UNIVERSITY OF LEICESTER

Remote Ischaemic Conditioning and its Effect on Maladaptive Cardiac Remodelling Following Myocardial Infarction

---

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

Dr Andrew Peter Vanezis

BSc (Hons.), MBChB, MRCP (UK)

Department of Cardiovascular Sciences

University of Leicester

2016

Remote Ischaemic Conditioning and its Effect on Maladaptive Cardiac Remodelling Following Myocardial Infarction

Andrew Peter Vanezis

Remote ischaemic conditioning (rIC) has shown benefit in protecting the myocardium from ischemia/reperfusion injury. However it's potential to attenuate maladaptive remodelling post myocardial infarction (MI) associated with the development of heart failure is less well investigated.

Using cellular models of endothelin-1 (ET-1) driven hypertrophy and fibrosis, we investigated the role that serum taken from healthy volunteers undergoing rIC (rIC-serum) had on various markers of both hypertrophy and fibrosis as well as attempting to elucidate some of the mechanisms involved. Furthermore we established a clinical trial to assess the degree to which repeated rIC post-MI can positively influence heart failure outcomes.

Hypertrophy was assessed by measuring H9c2 cardiomyoblast cell size using immunofluorescence, protein to DNA ratio and by abrogation of the expression of the pro-hypertrophic foetal genes BNP, βMHC, α-ACT and MS-1. This anti-hypertrophic mechanism was shown include the activation of the PKCε/AMPKα/eNOS/cGMP pathway and up-regulation of the anti-hypertrophic microRNAs miR-1 and miR-133.

RIC-serum was also shown to attenuate ET-1 induced markers of fibrosis including differentiation of fibroblasts to pathological myofibroblasts and proliferation of fibroblasts in a neonatal rat cardiac fibroblast model. Both unconditioned serum and rIC-serum attenuated ET-1 induced expression of α-SMA and increased the expression of MMP-2 and TIMP-1 but neither had any effect on ET-1 induced MMP-9.

Unconditioned serum also proved to have weak anti-hypertrophic properties which were greatly augmented by rIC. Increasing age diminished the innate protection afforded by unconditioned-serum and individuals with high levels of physical activity displayed higher levels of innate protection from hypertrophy akin to rIC.

Finally we describe the design and management of the DREAM study (Daily Remote Ischaemic Conditioning Following Acute Myocardial Infarction), a phase 2 randomised control trial established to evaluate the role of repeated rIC post-MI in modulating maladaptive cardiac remodelling with the presentation of some preliminary secondary outcome data.

Acknowledgements

I would like to thank Dr Glenn Rodrigo for his supervision, encouragement and wise words throughout my PhD and write-up. I would also like to thank Professor Sir Nilesh Samani for his support in setting up the DREAM study as well as overall supervision of my PhD. I am also grateful to Professor Iain Squire for his supervision.

I am grateful to many individuals within Dr Rodrigo's lab including Dr Sadat Edroos, Dr Helen Turrell, Dr Hayley Crumbie, Dr Chokanan Thaitirarot and Dr Madiha Butt. I am also indebted to the help of Mr Matthew Denniff, Mrs Martha Hardy, Dr Chris Nelson and Mrs Tina James for help with all aspects of my lab and trial work.

Pertaining to the DREAM study I would like to thanks all members of the Leicester Cardiovascular BRU who have helped me in some capacity but I give special mention to Mrs Jay Gracey, Mrs Sue Parish, Mrs Ellie Clarke, Ms Anna-Marie Marsh, Mr John McAdam, Dr Gerry McCann, Dr Jamal Khan and Dr Sheraz Nazir.

I would especially like to thank all the volunteers and all the patients who agreed to take part in either donating serum or participating in the DREAM study.

Finally I would like to thank my family for all their love and support and my long suffering wife Natalia who had to spent many a weekend and evening peering round the office door asking if it's finished yet.

This organ deserves to be styled the starting point of life and the sun of our microcosm just as much as the sun deserves to be styled the heart of the world. For it is by the heart's vigorous beat that the blood is moved, perfected, activated, and protected from injury and coagulation. The heart is the tutelary deity of the body, the basis of life, the source of all things, carrying out its function of nourishing, warming, and activating body as a whole.

William Harvey

Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus (An Anatomical Exercise on the Motion of the Heart and Blood in Living Beings), 1628

(translation by Kenneth J. Franklin, 1957)

Table of Contents

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

LIST OF TABLES

Chapter 4

Chapter 7

ABBREVIATIONS

AAR	Area At Risk
ACEi	Angiotensin Converting Enzyme Inhibitor
ARB	Angiotensin Receptor Blocker
α-act	Alpha Skeletal Muscle Actin
α-sma	Alpha Smooth Muscle Actin
AKT	also known as PKB, Protein Kinase B
ALD2	Aldehyde Dehydrogenase
AlRed	Aldose Reductase
AMI	Acute Myocardial Infarction
Ang-II	Angiotensin-2
ANOVA	Analysis of Variance
ANP	Atrial Natriuretic Peptide
ARB	Angiotensin Receptor Blocker
ARVM	Adult Rat Ventricular Myocyte
AT1	Angiotensin II Type 1 Receptor
ATP	Adenosine Triphosphate
β-ME	Beta Mercaptoethanol
βMHC	Beta Myosin Heavy Chain
Bad	BCL2 associated agonist of cell death
Bax	BCL2-associated X protein
Bcl-2	B-cell Lymphoma 2
Bcl-xL	B-cell Lymphoma Extra Large
BMI	Body Mass Index
BNP	Brain Natriuretic Peptide
BSA	Bovine Serum Albumin
C/EBPβ	CCAAT/enhancer binding protein beta
CABG	Coronary Artery Bypass Grafting surgery
CsM	$Ca2^{+}$-calmodulin
cAMP	Cyclic Adenosine Monophosphate

CamKII	Ca^{2+}/calmodulin-dependent protein kinase II
CDC42	Cell division control protein 42
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic Guanosine Monophosphate
CGRP	Calcitonin Gene Related Peptide
CK	Creatine Kinase
CNN2	Connective Tissue Growth Factor
CoQ	Coenzyme Q
COX-2	Cyclooxygenase-2
CRT (D/P)	Cardiac Resynchronization Therapy (Device/Pacemaker)
CTGF	Connective Tissue Growth Factor
C_T	Threshold cycle
CXCR4	Chemokine Receptor Type 4
CVA	Cerebral Vascular Accident
Cx43	Myocardial Connexin 43
DAF-FM	4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DMVN	Dorsal Motor Vagal Nucleus
dNTPs	Deoxynucleotide Triphosphates
DREAM	Daily Remote Ischaemic Conditioning Following Acute Myocardial Infarction
DTT	Dithiothreitol
eNOS	Endothelial Nitric Oxide Synthase
ECE	Endothelin Converting Enzyme
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
eGFR	Estimated Glomerular Filtration Rate
ELISA	Enzyme-Linked Immunosorbent Assay
EPO	Erythropoietin
ER	Endoplasmic Reticulum

ERK	Extracellular signal Regulated Kinase (a MAPK)
FBC	Full Blood Count
FBS	Foetal Bovine Serum
FGF2	Fibroblast Growth Factor 2
FITC	Fluorescein Isothiocyanate
FOV	Field of View
Gαq	Gq alpha subunit
GATA4	Transcription Factor for GATA4 gene
GLP1	Glucagon-Like Peptide 1
GLUT4	Glucose Transporter Type 4
GP130	Glycoprotein 130 receptor
GPCR	G-Protein Coupled Receptor
GSK3β	Glycogen Synthase Kinase-3β
H&E	Haematoxylin and Eosin
HDAC	Histone Deacetylases
Hem Oxy	Heme Oxygenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF-PEF	Heart Failure with Preserved Ejection Fraction
HF-REF	Heart Failure with Reduce Ejection Fraction
HIF1	Hypoxia Inducible Factor 1
HSP	Heat Shock Protein
I/R	Ischaemia/Reperfusion
ICAM-1	Intracellular adhesion molecule 1
IGF1	Insulin–like Growth Factor 1
IHD	Ischaemic heart disease
IkB	Inhibitor of Kappa
IL6	Interleukin-6
Inpp5F	Inositol Polyphosphate-5-phosphatase f
IP3	Inositol-1,4,5-triphosphate
IPAQ-SF	International Physical Activity Questionnaire Short Form
IPC	Ischaemic Preconditioning

IPostC	Ischaemic Postconditioning
Isl1	Islet 1 Transcription Factor
JAK	Janus Kinase
JNKs	Jun N-terminal Kinase (a MAPK)
KCCQ	Kansas City Cardiomyopathy Questionnaire
K_{ATP}	ATP Sensitive Potassium Channel
KLF4	Kruppel-like factor 4
LAD	Left Anterior Descending coronary artery
LCx	Left Circumflex coronary artery
LGE	Late Gadolinium Enhancement
L-NAME	L-N^G-Nitroarginine Methyl Ester
L-NNA	N^ω-Nitro-L-arginine
LTCC	L-Type Calcium Channel
LV	Left Ventricle
LVEDI	Left Ventricular End Diastolic Index
LVEDP	Left Ventricular End Diastolic Pressure
LVEDV	Left Ventricular End Diastolic Volume
LVEF	Left Ventricular Ejection Fraction
LVESI	Left Ventricular End Systolic Index
LVESV	Left Ventricular End Systolic Volume
LVH	Left Ventricular Hypertrophy
LVSD	Left Ventricular Systolic Dysfunction
MACCE	Major Adverse Cardiac and Cerebral Event(s)
MAPK	p38 Mitogen-Activated Protein Kinase
McL-1	Myeloid Cell Leukemia 1
MEF2	Myocyte Enhancer Factor 2
MEK1/2	Mitogen Activated Protein Kinase kinase 1/2
METS	Metabolic Equivalent of Task
MHC	Myosin Heavy Chain
MI	Myocardial Infarction
miR	Micro RNA

MLC2a	Myosin light chain 2 atrial form
MLC2v	Myosin light chain 2 ventricular form
MLP	Muscle LIM-protein
MMPs	Matrix Metalloproteinases
MOLLI	Modified Look Locker Inversion Recovery
MnSOD	Mitochondrial Antioxidant Manganese Superoxide Dismutase
mPTP	Mitochondrial Permeability Transition Pore
MS-1	Myocyte Stress-1 gene
mTOR	Mechanistic Target of Rapamycin
MVO	Microvascular Obstruction
NAD	Nicotinamide adenine dinucleotide
NADPD	Nicotinamide Adenine Dinucleotide Phosphate
NCX	Sodium/Calcium Exchanger
NELF-A	Negative Elongation Factor Complex Member A
NFAT	Nuclear Factor of Activated T cells
NFκB	Nuclear Factor kappa light polypeptide gene enhancer in B cells transcription factor
NHE	Sodium/Hydrogen Exchanger
Nix	Nip3-like protein X
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NPR	Natriuretic Peptide Receptor
NSTEMI	Non ST Elevation MI
NT-proBNP	N-Terminal Pro-Brain Natriuretic Peptide
NYHA	New York Heart Association
OMT	Optimal Medical Therapy
OPN	Osteopontin
P	Phosphorylation.
P70s6k	P70 Ribosomal S6 kinase
PI3K	Phosphoinositide 3-kinase
PBS	Phosphate Buffered Saline

PCI	Percutaneous Coronary Intervention
PDE5	Phosphodiesterase type 5
PDGF	Platelet-Derived Growth Factor
PFA	Paraformaldehyde
PGC1α	Peroxisome Proliferator-activated Receptor gamma, coactivator 1 alpha
PI3K	Phosphoinositide 3-Kinase
PLC	Phospholipase C
PKC	Protein Kinase
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PPARα	Peroxisome Proliferator-activated Receptor alpha
PPCI	Primary Percutaneous Coronary Intervention
PTEN	Phosphatase and Tensin Homolog
PVDF	Polyvinylidene Fluoride
RACK	Receptors for Activated C Kinase
RAAS	Renin Angiotensin/Aldosterone System
REC	Research Ethics Committee
RhoA	Ras homolog gene A
RIC	Remote Ischaemic Conditioning
RIP3	Receptor Interacting Protein 3
RIPC	Remote Ischaemic Preconditioning
RISK	Reperfusion Injury Salvage Kinase
ROS	Reactive Oxygen Species
RPostC	Remote Ischaemic Postconditioning
RTK	Receptor Tyrosine Kinase
RT-PCR	Real-time Quantitative PCR
RyR2	Ryanodine Receptor
S1P	Sphingosine 1 Phosphate
SAFE	Survivor Activating Factor Enhancement
SD	Standard Deviation
SDF-1α	Stromal cell Derived Factor 1 alpha

S.E.M	Standard Error of the Mean
SERCA	Sarco (Endoplasmic) Reticulum Ca^{2+}-ATPase
SGC	Soluble Guanylate-Cyclase
SNAP	(S)-Nitroso-N-acetylpenicillamine
SOCE	Store-Operated Ca^{2+} Entry
SOD	Superoxide Dismutase
SPK	Sphingosine Kinase
SR	Sarcoplasmic Reticulum
SRF	Serum Response Factor
ST	Slice Thickness
STAT	Signal Transducer and Activator of Transcription
STEMI	ST Elevation MI
SWOP	Second Window of Protection
T1	Longitudinal Relaxation Time
TBST	Tris Buffered Saline Tween-20
TGF-β	Transforming Growth Factor Beta
TIMI flow	**'**Thrombolysis In Myocardial Infarction' Coronary Flow Score
TIMPs	Tissue Inhibitor of Metalloproteinases
TLR	Toll Like Receptor
TnC	Troponin C
TnI	Troponin I
TnT	Troponin T
TNFα	Tumour Necrosis Factor alpha
TNFr	Tumour Necrosis Factor receptor
TR	Repetition Time
TRPC	Transient Receptor Potential Cation channel
U+E	Urea and Electrolytes
Ube	Ubiquitin conjugating enzymes
VEGF	Vascular Endothelial Growth Factor
VSMC	Vascular Smooth Muscle Cell

1. INTRODUCTION

1.1 Heart Failure Post Myocardial Infarction

The last few decades have witnessed a succession of landmarks advances in the fields of public health medicine, cardiovascular pharmacology and coronary artery intervention, which together have transformed acute myocardial infarction (MI) from a life threatening and frequently fatal insult into a manageable and quantifiable one, a fact that is reflected by the steady decline in mortality rates seen worldwide over this time period.[1-4] This success however has led to a large cohort of survivors of MI experiencing increased levels of morbidity as a consequence, often compounded by further ischaemic events and significant co-morbidities such as diabetes, hypertension, hypercholesterolaemia and continued negative lifestyle choices such as cigarette smoking. One major sequelae that can develop post-MI, due to a combination of infarcted myocardium and the acute post-MI remodelling process, is heart failure. Indeed in the UK coronary artery disease (CAD) as a whole accounts for around half of all heart failure cases.[5]

Heart failure is an umbrella term for a collection of clinical signs and symptoms which includes dyspnoea (during or after exertion or if severe at rest), fluid overload both peripherally and with accumulation in the pleural spaces or acutely in the lung parenchyma, low mood and depression and most commonly excessive fatigue. In essence it is caused by the failure of the heart to meet the metabolic demands of body tissues. It is nowadays commonly sub-classified as heart failure with reduced ejection fraction (HF-REF) or heart failure with preserved ejection fraction (HF-PEF). HF-REF is sometimes referred to as systolic heart failure and is predominantly a failure of the

ventricular pump mechanism. HF-PEF was previously referred to as diastolic heart failure and is predominantly a failure of adequate filling of the ventricles, usually due to increased wall stiffness often as a consequence of advancing age, diabetes or hypertension.[6] However, in reality heart failure is best defined on a spectrum between these two entities.[7]

Heart failure is a major cause of long-term mortality and morbidity following MI. The advances in myocardial salvage in the form of thrombolytic therapy and more recently primary percutaneous coronary intervention (PPCI), had led to concerns that those surviving their MI would fuel a wave of heart failure cases and see the overall prevalence of heart failure increase. Analysis of registries and of large clinical trials across the western world, conducted in the era of acute revascularisation, have reported incidence rates of post-MI heart failure ranging from 10% to 50%. This massive discrepancy is reflected by the complexity of accurately collecting and reporting such data and is dependent on a number of factors including the degree and location of infarcted myocardium, how MI and heart failure were defined, whether there was pre-existing heart failure, the treatment modalities used and the characteristics of the populations analysed.[8, 9]

A retrospective analysis of the Framingham Heart Study participants, demonstrated an increase in the incidence of post-MI heart failure from the 1970s to the 1990s, closely linked to a decrease in mortality in acute MI.[10] In tandem with this finding, the overall rate of hospitalisations for all types of heart failure in the US has increased by between 70-100% over the last few decades when analysing Medicare data.[11] In stark contrast, in the Olmsted County study where over 1500 patients who

were hospitalised with MI were recruited between 1979 to 1994, the overall incidence of heart failure declined by 2% per year.[12] Indeed, more contemporary data seems to suggest that heart failure rates may have plateaued or even have begun to fall from the mid-1990s onwards. Large epidemiological sampling from the US between 1998 and 2010 of almost 3 million patients of all ages enrolled in the Medicare system, showed a slight decrease in 1 year heart failure hospitalisation rates after MI down from to 16.1 to 14.2 per 1000 patient-years over this time. However, of those hospitalised, there was a non-significant mortality increase from 44.4% in 1998 to 45.5% in 2010.[13] Very recent data from the CVDNOR (Patients From the Cardiovascular Disease in Norway) Project, which analysed the total population of AMI patients hospitalized between 2001 and 2009 in Norway (86,771 individuals), showed that 12.6% were hospitalised with or died because of heart failure during a median follow-up time of 3.2 years. Follow-up heart failure incidence rates per 1000 person-years were 31 (95% CI, 30–32) for men and 46 (95% CI, 44–47) for women ($p < 0.01$). Furthermore the prevalence of heart failure increased with age, predictably being greatest in the over 75s age group (25.6% in men and 27.1% in women).[14]

The widespread establishment of acute revascularisation coupled with the more sophisticated management of heart failure as a whole may be in part responsible for the reduced incidence of heart failure in more recent years in the western world.[15, 16] However despite these relative successes, heart failure remains a common sequelae of MI with significant implications of mortality and morbidity compounded by an ageing population. We remain reliant on novel and emerging therapies if there are to be significant advance in the management of post-MI heart failure in years to come.

1.1.1 Myocardial Infarction

Despite overall declining mortality rates in the Western world, MI and ischaemic heart disease (IHD) as a whole, still accounts for 600,000 deaths per year in Europe and 1.9 million deaths per year in the US.[17, 18] MI is caused by the partial or total occlusion of one or more coronary arteries, usually caused by thrombus formation over an unstable or ruptured coronary plaque. Prolonged occlusion interrupts oxygen rich blood to the myocardium, which leads to cell death at first in the subendocardial layers and if allowed to continue, in time extends to the epicardial layer in a 'wavefront' phenomenon.[19] The degree of damage incurred is predominantly dependent on the length of time coronary blood flow is occluded but is also influenced by the territory size of myocardium the occluded vessel supplies and the metabolic demands of the myocardium at the time of the infarction.[20] Indeed limiting the infarct size incurred by reducing occlusion time by revascularisation has been at the thrust of the major advancements in modern MI therapies and underlies the modern interventional cardiologist's obsession with 'call/door to balloon time' i.e. the time it takes from the onset of cardiac pain signalling the onset of ischaemia to the time it takes to open the occluded coronary artery with initial balloon inflation.[21, 22] The use of thrombolytic agents or PPCI to bring about prompt revascularisation has been shown in numerous studies to reduce the final infarct size of the area at risk (AAR) i.e. the territory that was vulnerable to cell death, in around half of all cases.[23-25] In the acute phase of infarction, the majority of myocyte death is caused by coagulation necrosis characterised by an early influx of eosinophils and neutrophils and subsequent activation of apoptosis pathways. Figure 1.1 demonstrates

a typical microscopic appearance of myocardium stained with haematoxylin and eosin (H&E) stain in the first few hours following an MI.

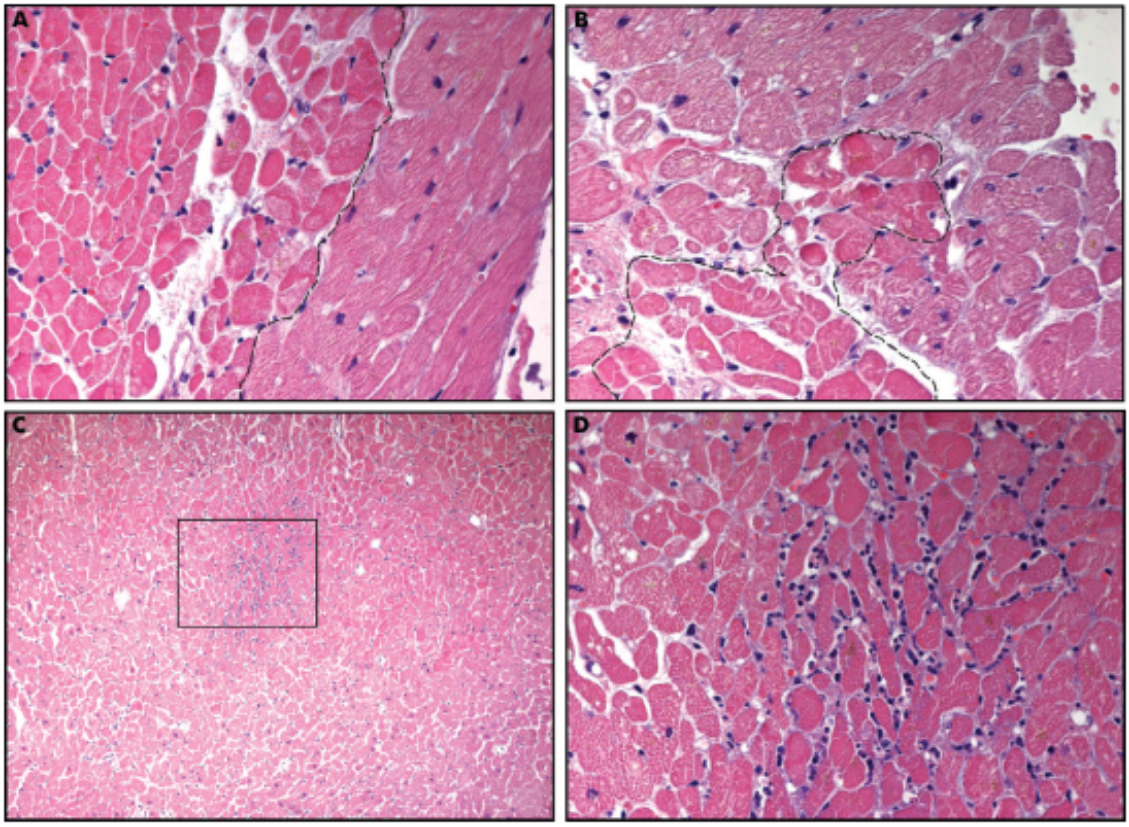


Figure 1.1. Microscopic changes of ischaemic myocardium shown with H&E staining. Ischaemic cardiomyocytes are demonstrated in the left of panel in A and lower part of panel B. Panel C shows an area of coagulation necrosis which is further magnified in panel D and characterised by hyper-eosinophilia. Reproduced from Passotti *et al.,* (2006).[26]

1.1.2 Reperfusion Injury

The sudden restoration of blood flow brought about by PCI or thrombolytic therapy can paradoxically cause a significant degree of damage, so called *reperfusion injury*.[27] Lethal reperfusion injury refers specifically to cardiomyocyte death and is thought to contribute up to 30-50% of the final infarct size in the setting of re-vascularised myocardium after a period of infarction.[28] Figure 1.2 demonstrates the degree to which ischaemia and reperfusion are thought to influence the final infarct size.

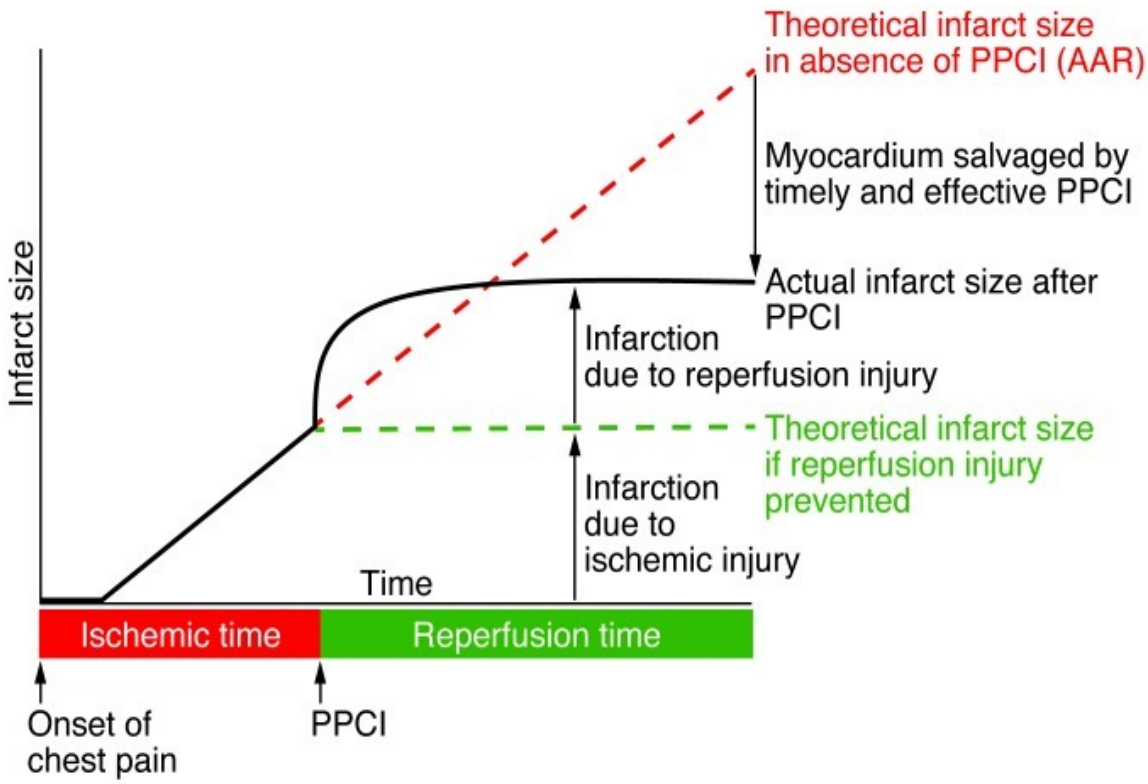


Figure 1.2. The relative contributions of ischaemia and reperfusion injury towards final infarct size. Even with successful reperfusion with PPCI, reperfusion can still go on to contribute a significant proportion of the final infarct size. Reproduced from Hausenloy *et al.,* (2013).[29]

The mechanism of reperfusion injury involves a number of interlinking processes. The first process of injury is as a direct result of oxidative stress. The sudden flood of oxygen to the myocardium after reperfusion leads to a decrease in circulating nitric oxide (NO) levels, a well-established cardioprotective molecule which leads to an increase in coronary blood flow via its effect on coronary endothelium, a reduction in inflammatory cell chemotaxis, inhibition of pro-inflammatory cytokines, the reduction in superoxide radical downstream pathways as well as a direct inhibitor of apoptosis.[30] Coupled to the direct effects of oxidative stress, the ensuing re-energisation of the mitochondria, which up until reperfusion had been depleted of substrate and molecular oxygen, triggers downstream pathways which ultimately converge and lead to cell necrosis and apoptosis.

The revitalised mitochondria generates a number of reactive oxygen species (ROS), in addition to those generated by ischaemia. This occurs mainly by the action of sustained electron flux at complex I (Nicotinamide adenine dinucleotide (NAD) dehydrogenase) and complex III (coenzyme Q (CoQ) and cytochrome C oxidoreductase) on the mitochondria that leads to increased generation and expulsion of ROS.[31, 32] A second later source of ROS is generated from nicotinamide adenine dinucleotide phosphate (NADPD) oxidase originating predominantly from the incoming neutrophils and supplemented by ROS generated by the conversion of xanthine dehydrogenase to xanthine oxidase after periods of prolonged ischaemia.[33, 34] The re-energisation of mitochondria during reperfusion also generates an excess of ATP. This occurs on a background of significant Ca^{2+}-loading during ischemia which is a product of the sodium/calcium exchanger (NHE) and sodium/calcium exchanger (NCX) coupled

exchanger mechanism.[35, 36] In this setting ROS and excess calcium levels in the cell activates the opening of the mitochondrial permeability transition pore (mPTP) which leads to cell death by the uncoupling of oxidative phosphorylation and a subsequent lack of ATP production, vital for cell viability.[37-39]

Widespread contraction band necrosis associated with reperfusion injury can also be caused by hyper-contracture of the myofibrils independently of the mPTP pathway. The reactivation of Sarcoplasmic Reticulum (SR) driven contraction due to oscillations in Ca^{2+} induces significant mechanical injury. This calcium loading is exacerbated by sarcolemmal membrane damage that occurs during ischaemia/reperfusion (I/R) injury and causes cellular and mitochondrial swelling and subsequent rupture of the mitochondria with the release of cytochrome-C, involved in apoptosis. This form of necrosis is commonly induced by the sudden release of contractile fibres which are held in contraction due to a lack of ATP during ischaemia but during reperfusion are rapidly released due to the expulsion of excess sarcoplasmic calcium initially accumulated during ischaemia. Opening of the mPTP can lead to similar pathological sequelae in more localised areas. Figure 1.3 highlights the typical microscopic findings of myocardium subjected to I/R injury.

Another downstream consequence of calcium loading is the activation of several degredative enzymes such as phospholipase A2 and calpains which generate a chemoattractive pull on neutrophils and other polymorphonuclear leukocytes.[40] As mentioned the influx of neutrophils adds to the circulating ROS pool. This process is already triggered by ischaemia but is exacerbated by reperfusion with the up-regulation of adhesion molecules such as CD11, CD 18, P-selectin and intracellular adhesion

molecule 1 (ICAM-1). As well as generating ROS, neutrophils release a number of enzymes which are tissue degraders such as proteases as well as causing vascular plugging.

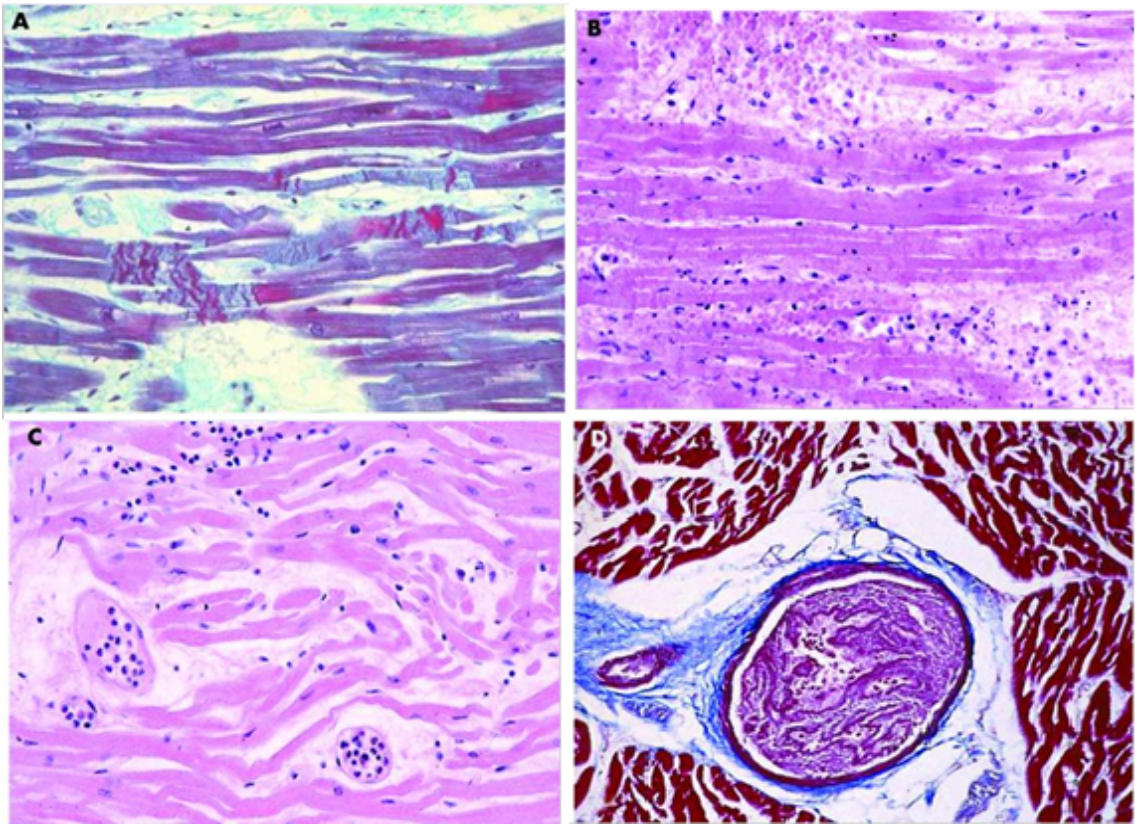


Figure 1.3. Microscopic changes of myocardium subjected to I/R injury shown with H&E staining (panels B and C) and trichrome Heindenhain staining (panels A and D). A. Contraction band necrosis. B. Interstitial haemorrhage. C. Neutrophil infiltration causing vascular plugging. D. Microembolisation of platelet/fibrin material. Reproduced from Basso *et al.,* (2006).[41]

Reperfusion also acts to rapidly correct the acidic environment generated by ischaemia within the cell. This is predominantly achieved by washing out lactic acid via the NHE and the sodium–bicarbonate symporter pathways. Paradoxically this sudden restoration to a physiological pH also activates mPTP opening as the correction of the extracellular acidosis means that the NHE can now operate effectively to remove H^+ from the cell in exchange for Na^+ leading to further sodium loading and calcium accumulation via the NCX which further contributes towards cell death.

A number of cellular, animal and clinical trials have attempted to target individual or groups of mediators at all stages of the reperfusion cascade by pharmacological means with largely minimal success. Some of the more prominent studies include the use of antioxidants to reduce the number of circulating ROS, the inhibition of calcium influx by targeting calcium channel transporters and symporters, the use of anti-inflammatory agents and the use of NO donors to increased circulating NO.[42-44] More recently there has been a growing interest in targeting microRNAs (miRs) which may modulate injury in reperfusion but the clinical translatability of these studies remains to be seen.[45-51] The shortcomings of these studies are discussed succinctly by Yellon *et al.,* (2007) in a review of this topic.[52] As they point out, there are numerous pathways thought to be involved in lethal reperfusion injury and therefore greater benefit may be seen by targeting a number of these pathways simultaneously. One way of targeting multiple pathways is by ischaemic conditioning, a powerful endogenous cardioprotective tool that will be discussed in detail in *section 1.2*. Traditional ischaemic conditioning in the context of I/R acts predominantly by inhibiting opening of the mPTP pore and hence a number of the downstream pathways that lead to cell death.

1.1.3 Cardiac Remodelling

Cardiac Remodelling encompasses the process of changes that occur following a substantial insult to the heart. Our traditional understanding of remodelling has largely come from animal models of MI and IHD but other insults can lead to remodelling such as pressure or volume overload states, heart muscle diseases such as cardiomyopathies, valvulopathies, diabetes, obesity and cardiac arrhythmias. However there are distinct changes that occur in the heart after an MI which this section will focus upon in particular, rather than discussing cardiac remodelling from all aetiologies as a whole.

Post-MI remodelling can be crudely divided into adaptive and maladaptive and the transition from the prior to the latter is broadly defined by a loss of the initial compensatory mechanism afforded by remodelling that leads to a decrease in cardiac output and heart failure.[53] In other words the point at which remodelling becomes maladaptive is the point at which the changes that have taken place post-MI no longer serve to maintain an adequate cardiac output but instead serve only to fuel a process of downward spiralling decline.

The properties of the final scar that is formed as an outcome of the remodelling process is dependent on a timely resolution of the process to ensure maximal tensile strength and minimal scar size. The scar itself is made up of predominantly of collagenous fibrous tissue. The greater the distribution and size of the scar, the greater the degree of myocardial stiffening and increased likelihood of ventricular arrhythmias. If remodelling is allowed to progress for too long (i.e. for more than a few days), a number of maladaptive pathways are activated which may extend the infarct zone and lead to a thin, poorly contractile scar with little tensile strength. This may then

degenerate into a downward spiral of further maladaptive processes which can ultimately lead to heart failure over the course of a few weeks to months after the initial insult. The *International Forum on Cardiac Remodelling* drew up a consensus paper in 2000 and defined maladaptive remodelling in clinical terms stating it as either an increase in end-diastolic volume (EDV) of >20% or end systolic volume (ESV) of > 15% at follow-up compared to baseline imaging assessment.[54]

The outcome of remodelling is very much dependent on the size and thickness of the infarcted area as well as the duration of the ischaemic event prior to adequate reperfusion.[55] However, independent of final infarct size, remodelling is also heavily influenced by concomitant microvascular obstruction (MVO), lethal reperfusion injury in the era of acute revascularisation and the evolving theory that scar is not inert but active in the remodelling process.[56-58] Broadly speaking there are three distinct phases to the process of post-MI remodelling: cell death and inflammation, proliferation and finally maturation. Overall the process can last anything up to 6 months but is typically complete at 1-2 months.[59, 60]

The process of remodelling is triggered by the initial I/R insult, which sets in to motion a chain of events. In the initial stages the changes in the left ventricle are predominantly caused by the effects of infarct expansion leading to cardiomyocyte necrosis, apoptosis and autophagy which ultimately leads to myocardial wall dilatation. Despite the presence of a small pool of local stem cells that are now thought to reside in the heart, these are not thought to play any substantial role in ameliorating this process.[61, 62] Following cell death, the process of infarct healing is partly driven by the substantial inflammatory process that ensues (and overlaps with the inflammatory

process seen in I/R post-MI described in *section 1.1.2*). This process involves complement activation, the production of ROS which induces an increase in cytokine and chemokine production followed by an influx of leukocytes, firstly neutrophils and other polymorphonuclear leukocytes and then later monocytes and macrophages, which act to remove dead cells and debris to clear the way for scar formation. This process is then followed by an infiltration of fibroblast which differentiate into myofibroblasts and secrete an array of matrix chemicals that leads to substantial extracellular matrix (ECM) turnover in border areas. This can lead to cell slippage and further dilatation.

Following the acute inflammatory and fibrotic process, the later stages of remodelling involves hypertrophy of cardiomyocytes that have been unaffected by I/R and reorganisation of the ECM with further deposition of scar tissue as the fibrotic process continues. From a whole-organ perspective, these changes impact on cardiac dimensions and function with an initial maintenance of adequate cardiac output in the face of a loss of functioning myocardium and in response to pressure and volume overloading, so called adaptive cardiac remodelling.[63] However, over time remodelling can become maladaptive with a drop away of the cardiac output that leads to the syndrome of heart failure. Indeed, the extent and the nature of remodelling and its progression is a powerful predictor for both heart failure and death following MI, as well as having prognostic implications for further MI, stroke and cardiac arrest.[64, 65] Furthermore a host of other adaptive responses including a change in calcium handling by cardiomyocytes, desensitisation of contractile units to beta-adrenergic stimulation and the expression of a number of foetal genes and miRs all combine to shift the balance

from adaptive to maladaptive remodelling. Figure 1.4 provides a general overview of the pathophysiological processes that occur in this process and the proceeding sections provide more details about the individual elements of remodelling.

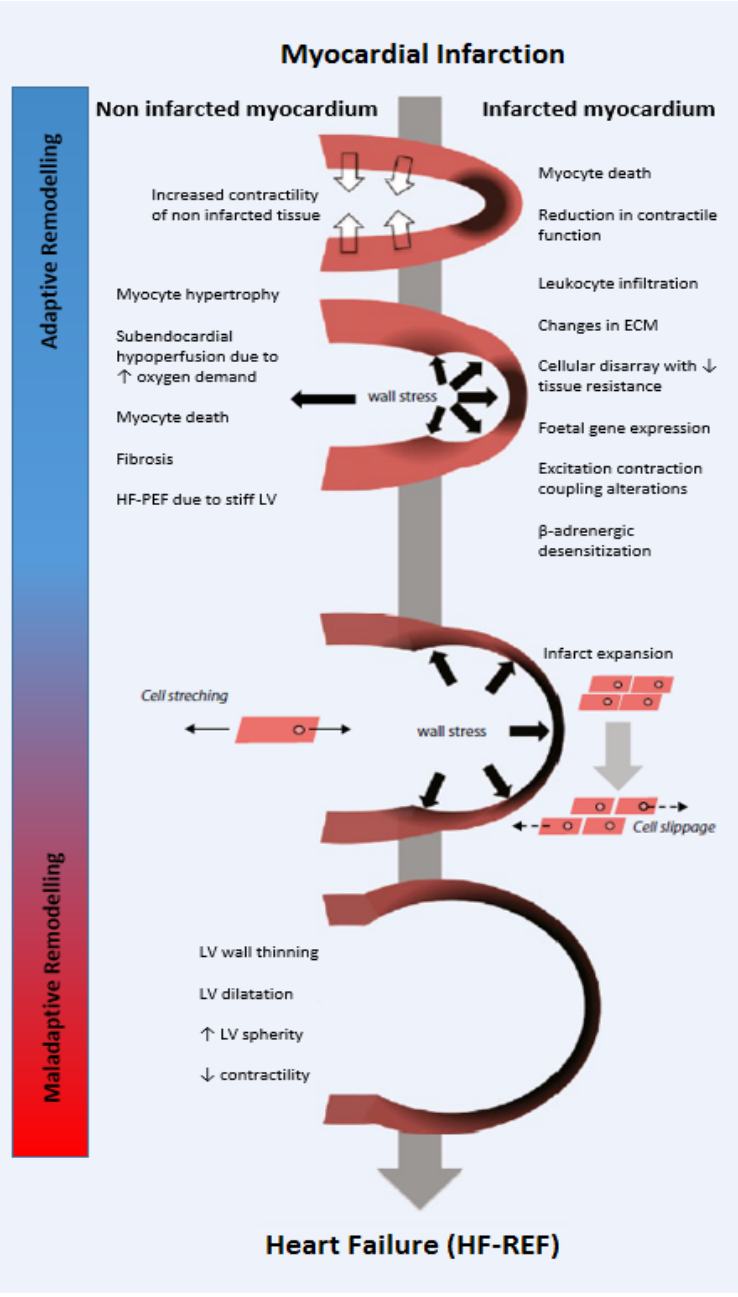


Figure 1.4. Schematic representation of remodelling after myocardial infarction. *LV* = left ventricular, *ECM* = extra cellular matrix, *HF-PEF* = heart failure with preserved ejection fraction, *HF-REF* = heart failure with reduced ejection fraction. Adapted from Brenner *et al.,* (2012).[66]

1.1.3.1 Cardiomyocyte Death

There are thought to be three broad mechanisms of cell death that occur during the remodelling process: apoptosis, programmed necrosis/oncosis and autophagy. A simplified schematic of these processes is shown in Figure 1.5.

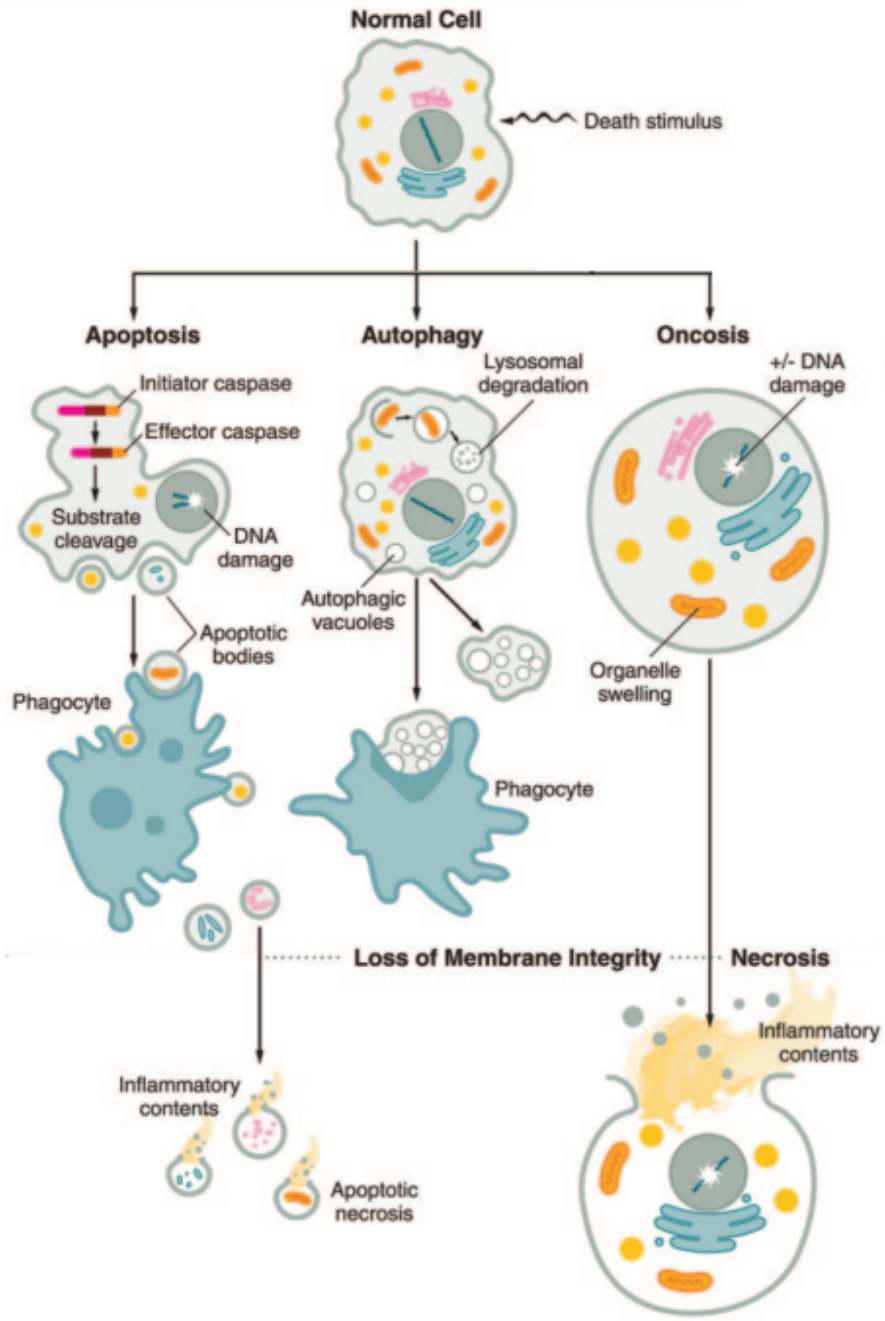


Figure 1.5. Schematic of the broad mechanisms of cell death which are universal across all human cell types. Adapted from Fink *et al*., (2005).[67]

1.1.3.1.1 Apoptosis and Programmed Necrosis

After the initial I/R insult, apoptosis and programmed necrosis are thought to predominate. Whilst both these terms refer to form of programmed cell death, apoptosis implies a genetically preserved process that is ATP dependent and important for the normal function of cells whereas programmed necrosis is triggered by environmental factors that are pro-inflammatory and act mainly via mPTP channel opening.[68]

Kajstura *et al.,* (1996) showed that MI induced in rats by coronary ligation produced on average 2.8 million apoptotic cardiomyocytes compared to 90,000 necrotic cardiomyocytes in the first 2 hours due to ischaemic injury.[69] Compare this to healthy myocardium where there are only 1 in 10,000-100,000 cells that are apoptotic at any given time.[70] Similar evidence of an ongoing apoptotic process has been demonstrated in a canine model of heart failure.[71] In cardiac remodelling apoptosis is regulated by two main pathways, the *extrinsic* and *intrinsic* pathways, otherwise known as the death receptor and mitochondrial pathways respectively. The *extrinsic* pathway is well described and involves activation of capsase enzymes culminating in the production of downstream capsases such as capsase 3 and 8. These downstream capsases are stimulated by extrinsic factors that act predominantly on tumour necrosis and Fas receptors on the myocyte membrane and culminate in the destruction of intracellular protein and DNA degradation.[72, 73] The *intrinsic* pathway is modulated by the expression of pro-apoptotic gene, in particular the Bcl-2 group such as Nip3-like protein X (Nix), that encourage permeabilisation of mitochondrial outer membranes.[74] More recent work suggests that these Nip proteins are acting on the mitochondrion to exacerbate

opening of mPTP caused by I/R, which leads to cellular death by programmed necrosis. They do this by exacerbating calcium loading which promotes mPTP opening.[75,76,77] It is likely that the environmental imbalance brought about at the time of I/R injury means that programmed necrosis predominates as the ultimate mode of cell death at this early time point but as remodelling ensues, apoptotic cell death that occurs independent of mPTP opening, may take over.

1.1.3.1.2 Oncosis

Microscopic passive necrosis, as opposed to the macroscopic destruction of cellular architecture, is the mechanism of cell death at a cellular level that is better termed oncosis or ischaemic cell death.[78] Oncosis is characterised by cell swelling, followed by vacuolisation and blebbing with increased permeability and finally necrosis with coagulation, shrinkage and karyolysis. Oncosis can occur as a by-product of hypertrophy as well occurring as a direct consequence of I/R. Furthermore oncosis can come about as a result of the concept of the 'ischaemic core' which dictates that as the myocardium hypertrophies, the cross sectional area exceeds the ability of oxygen to adequate diffuse into cells in the centre of the core.[79] This results in a decrease in ATP production by the mitochondria of these cells and predisposes the cell to necrosis. Furthermore this process is exacerbated by the fact that capillary angiogenesis struggles to keep up with the hypertrophy and further decreases the oxygen delivery to the core of the myocardium.[80] Over time, rates of cell death are thought to be low but as the remodelling process is a chronic one, the loss of cardiomyocyte numbers is thought to

play a major part in the process. This theory remains contentious although some evidence is provided by a murine model of hypertrophy.[81]

1.1.3.1.3 Autophagy

Finally, autophagy has been shown to play a role in exacerbating hypertrophy in the post-MI setting. Autophagy describes the process of cell recycling. During the process, the autophagosome isolates key components of the cytoplasm, such as the mitochondria, and degrades them by fusion with lysosomes.[82] In the context of remodelling, autophagy is a double edged sword. Whilst playing a role in maintaining normal cardiac function by restricting changes to the heart size and structure, it can in certain situations be deleterious. This is thought to be due to the greatly accelerated turnover of proteins that occur during the initial hypertrophic phase of remodelling which can disturb the normal balance of autophagy and lead to an increase in cell death from this process.[83]

1.1.3.2 The Inflammatory Response

There is considerable overlap between the inflammatory response, triggered by both ischaemia and then reperfusion, and the inflammatory response seen during the remodelling process. Conceptually it may be better to define the inflammatory process as an ongoing one that evolves through I/R and then into the acute early and then late phases of remodelling. As previously described, during I/R and in the early stages of remodelling, there is an influx of neutrophils into the infarcted and border areas with a subsequent influx of monocytes and macrophages which act to remove dead cells and

debris and pave the way for the foundation of scar material. This response is triggered by the dispersion of cell contents from damaged cells which activate toll like receptors (TLRs), in particular the TLR 2 and 4 pathways as well as activation of the complement, ROS pathways and activation of capsase-1 and the conversion of pro-interleukin (IL)-1β to mature IL-1β which act in tandem to create a chemoattractive pull to cells of the innate immune system and augment the inflammatory response.[84-86] As the remodelling process continues, the emphasis on infiltrating lymphocytes increases, in particular CD8 T lymphocytes which release pro-inflammatory mediators and a population of CD4 T lymphocytes which act to regulate the inflammatory process.[87] Furthermore in later remodelling there is switch of monocytes from class M1 (pro-inflammatory) to M2 (angiogenic and fibrotic).[88] Figure 1.6 summarises the main immunological cells involved in mediating and effecting inflammation during I/R and remodelling.

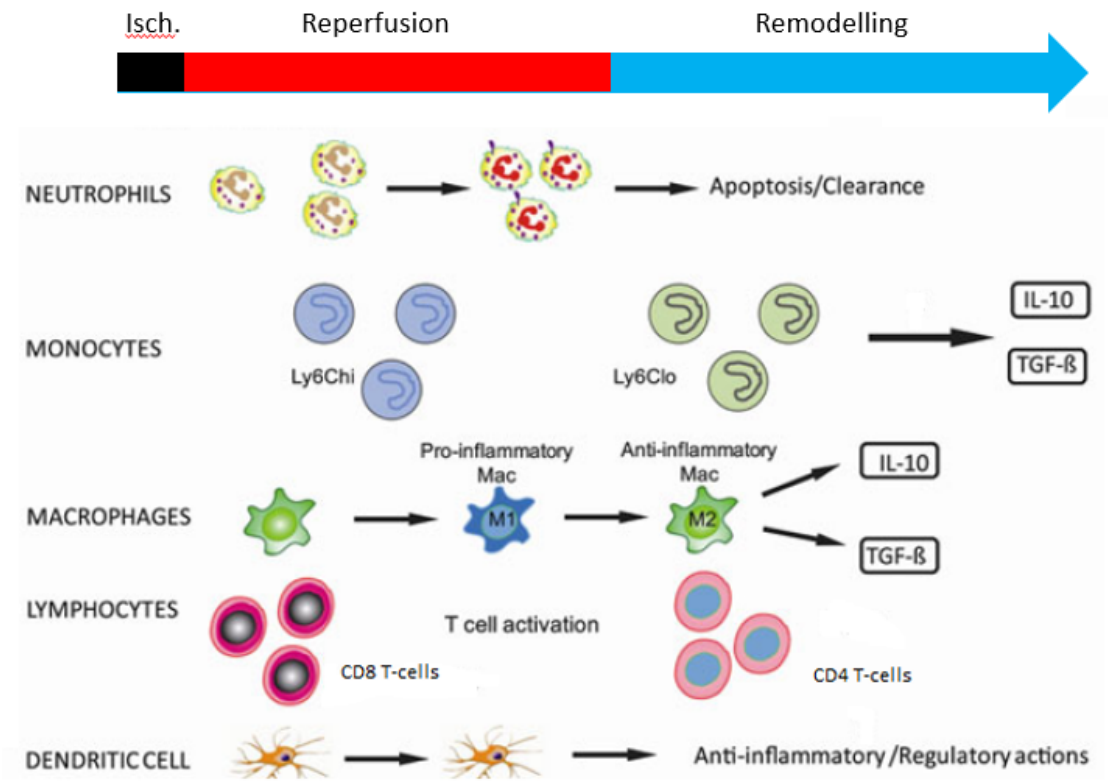


Figure 1.6. Predominant immunological cells that mediate inflammation during reperfusion and remodelling. Adaptive remodelling heralds the phenotypic change of most immunological cells displayed to ones that secrete anti-inflammatory mediators. However in maladaptive remodelling, the initial pro-inflammatory phenotype may predominate. Ly6Chi = pro-inflammatory monocytes, Ly6Clo = anti-inflammatory monocytes. Adapted from Jugdutt *et al.,* (2013).[89]

1.1.3.3 Fibroblasts and the Extra Cellular Matrix

The ECM is a dynamic and crucial component to maintaining cardiac integrity. As well as acting as a cardiac scaffold it is vital in signalling for cell migratory patterns, cardiomyocyte dimensions, growth and differentiation, proliferation and survival.[90] In addition, it acts as a transmitter of contractile force to provide adequate cardiac output and orchestrates cardiomyocyte geometry.[91] Two distinct phases are demonstrated in post-MI remodelling with regards to the heart's cytoskeleton: the initial enzymatic breakdown of the ECM followed by collagen production and scar formation. Although

the changes brought about act to stabilise the heart by reparative fibrosis, uncontrolled ECM turnover and collagen formation can lead initially to a stiff heart with impaired diastolic function resulting in (HF-PEF) and if allowed to progress ultimately the deterioration to systolic cardiac failure (HF-REF).

In health the ECM is produced and maintained by fibroblasts through their production of various proteins including structural collagens, elastins, adhesive proteins and integrins. Fibroblasts also produce a myriad of enzymes that keep a constant turnover of the ECM, namely matrix metalloproteinases (MMPs), a heterogeneous group of collagenase, gelatinses and other protein degradation enzymes, and tissue inhibitors of metalloproteinases (TIMPs) which act to nullify the effects of MMPs. Initial studies suggested that fibroblasts are more numerous than cardiomyocytes in the heart, making up around two thirds of the total cell number, however they are considerably smaller than cardiomyocytes and make up a much smaller percentage of the total heart mass.[92-94] More recent data by Banerjee *et al.,* (2007) has shown that a neonatal adult murine heart is made up of ~27% fibroblasts.[95] They play a major role in pathological LV remodelling as they are responsible for the fibrotic changes and ultimately scar formation as well as a majority of the gross structural changes that affect the haemodynamics of the heart. The fibroblast phenotype that is present in the normal, healthy heart is replaced by a *myo*fibroblast phenotype in the hypertrophic heart. Connective tissue growth factor (CTGF) and transforming growth factor β1 (TGFβ1), as well as a number of other neurohormonal factors implicated in cardiac hypertrophy as well as mechanical sheer stress act to induce myofibroblast differentiation from

fibroblasts.[96] In addition, myofibroblasts can be generated by induction of circulating bone marrow haematopoetic precursor cells during times of stress on the heart.[97]

Even before the discovery of MMPs, Judd and Wexler demonstrated over thirty years ago, a net loss of collagen production in the first few days post-MI in rats. However, after around 6 days, this changes to a net accumulation of collagen and the development of myocardial stiffening.[98] The first stage is now understood to be due to the action of MMPs released soon after MI causing collagen breakdown and the disruption of structural protein cross-links which are vital for cardiac integrity. Spinale *et al.,* (1998) demonstrated a fluctuation in circulating MMP levels after MI and showed a correlation to ventricular function.[99] Squire *et al.,* (2004) have shown that MMP-9 reached peak serum level in the six weeks after an MI. This peak showed a high degree of correlation to the degree of cardiac dysfunction (in particular left ventricular), as measured by echocardiography.[100] Further work by the same group using cardiac MRI parameters of heart failure suggests a more complicated role for MMP-9, where it is shown to have a negative correlation with brain natriuretic peptide (BNP) and may have some cardioprotective properties at lower circulating levels by increasing collagen deposition and hence reducing LV stretch as well as cardiomyocyte hypertrophy.[101] Reinhart *et al.,* (2002) extracted serum from patients with heart failure of both ischaemic and non-ischaemic aetiologies, and demonstrated up-regulation of MMP-9 in all causes of heart failure. Interestingly, in this study MMP-2 was not raised in heart failure of ischaemic aetiology.[102] However, numerous studies have demonstrated a positive correlation with levels of MMP-2 in post-MI remodelling as well as showing a negative correlation with MMP-1 and TIMP-1.[100, 103, 104]

Another integral component to the orchestration of remodelling of the ECM post-MI is osteopontin (OPN), a cytokine released by various tissues including fibroblasts, osteoblasts, osteocytes and dendritic cells.[105] The significance of OPN in remodelling was first demonstrated in mice where those knockout mice lacking the OPN gene demonstrated increased LV dilatation, greatly increased LV end-diastolic volume and reduced collagen deposition 28 days after MI when compared to the wild type mice.[106] OPN is up-regulated by the intracellular signal-regulated kinases (ERK)1/2 and JNK pathways and acts via various integrins to modulate the cytokine stimulated release (namely IL-1β) of various MMPs.[107]

1.1.3.4 Cardiac Hypertrophy

Cardiomyocyte hypertrophy is one of the major transformations that occur during remodelling and can occur without an overall increase in mass of the heart as the process technically refers to the changes that occur to the individual cardiomyocytes. *Pathological* hypertrophy is distinct from the *physiological* hypertrophy that occurs in athletes or pregnant women with distinct pathways. Whereas in physiological hypertrophy there is an organised re-alignment of myocardial component to accommodate hypertrophy and enhance cardiac output in response to increased tissue demands, in pathological hypertrophy there is often disorganised alignment of myocardial components with increased levels of fibrosis that often does not lead to an increase in cardiac output.[108] Pathological hypertrophy, accompanied by remodelling of the calcium regulated excitation-contraction coupling mechanism resulting in defective contraction, is the first stage of remodelling and ultimately leads to heart failure and

malignant arrhythmias. Many of our current treatments for heart failure work on the principle of slowing this hypertrophic process, a good example being ACEi which inhibits the action of angiotensin II, one of the main triggers of cardiac hypertrophy.

It was initially thought that cardiomyocytes are terminally differentiated and cannot proliferate. This dogma has been challenged in recent years with the discovery of a number of distinct populations of cardiac progenitor cells that are thought to reside within the heart. They are classified by their surface marker and include cardiosphere-derived cells, Islet 1 transcription factor (Isl1) positive stem cells, Sca-1 positive cells, and c-kit positive stem cells.[109-112] However, the clinical translatability of harnessing cardiac progenitor cells during post-MI remodelling has been challenging.[113] (See *section 1.1.3.9* for a more in-depth discussion of the therapeutic potential of cardiac progenitor cells in post-MI remodelling). Cardiomyocytes in general, therefore, can only increase in size in response to growth signals. After an MI, infarcted tissue stretches because of early degradation of the collagen scaffold that causes myofibril slippage and an increase in LV chamber size which over time leads to both pressure and volume overload. This triggers both lengthening and widening of cardiomyocytes. This initial compensatory hypertrophy by the heart occurs to maintain cardiac output and adequate haemodynamics, but over time the process become deleterious as haemodynamic stress develops on the heart and along with other ongoing process such as cell apoptosis (see *section 1.1.3.1*) there is a net increase in left ventricular EDV and a fall in left ventricular ejection fraction (LVEF).

Cardiac hypertrophy is triggered mainly by mechanical wall stress coupled with neurohormonal signals. The mechanism by which the mechanical stress placed on the

heart is transmitted as a messenger to hypertrophic induction for cardiomyocytes is thought to lay in a series of integrins that link the internal cytoskeleton of the cell to the extracellular matrix (ECM). These include melusin and the muscle LIM-protein (MLP).[114, 115] Other receptors involved in transducing mechanical stress include the angiotensin II type 1 receptor (AT1) which can be activated by mechanical stress independent of angiotensin II.[116] Furthermore, the transient receptor potential cation channel, subfamily C (TRPC) 6, an ion channel that lies within the cell membrane, has been shown to be up-regulated in mice subjected to pressure overload as well as in human hearts in the late stages of failure.[117]

Coupled to mechanical stretch transduction, neurohormonal signalling plays an important role in hypertrophy. Three main classes of molecules are important in neurohormonal transduction of hypertrophy: angiotensin II, epinephrine/phenylephrine, and endothelins. They act by binding to a G-protein coupled receptor, which possess a series of seven receptor components that span the cell membrane known as the Gαq/α11 receptors[118] and are coupled to phospholipase C (PLC) which leads to the production of diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). DAG then leads to activation protein kinase C (PKC). IP3 acts to free Ca^{2+} stores within the sarcoplasmic reticulum, a process also fundamental to muscle contraction in all muscle types. The IP3 pathway also plays an important role in hypertrophy by acting on various local transcription factors including the pro-hypertrophic transcription factor Nuclear Factor of Activated T cells (NFAT).[119] In addition IP3 acts on fibroblasts where maintenance of calcium levels is key to normal cell oscillation and functioning.[120] This calcium liberation in cardiomyocytes mediates

hypertrophy via calcineurin to activate NFAT transcription factors. PKC also activates histone deacetylases (HDAC), post-translational modifying enzymes, specifically HDAC class II (4,5,7 and 9) which inhibit myocyte enhancer factor 2 (MEF2) transcription and hence hypertrophy.[121-123] To counterbalance this process a pro-hypertrophic class of HDACs, class I (specifically HDAC 2) are also activated. These class I HDACs act to decrease the expression of anti-hypertrophic mediators including KLF4 and Inpp5f.[124] They are chiefly regulated by Heat Shock Protein 70 (HSP70) which are thought to react to ischaemia and other hypertrophic stimulators by increasing the anti-apoptotic pathways such as AKT with downstream up regulation of class I HDACs as well as the foetal gene ANP.[125]

Gαq/α11 receptors activation also up-regulates p38 mitogen activated protein kinase (MAPK) (predominantly the c-Jun N-terminal kinases (JNKs) and ERKs pathways) and increases transcription factor production for hypertrophy. The PI3K-AKT pathway is also implicated in hypertrophy. This pathway was initially thought to trigger hypertrophy with preserved cardiac function (i.e. physiological hypertrophy) but various studies have implicated it in pathological hypertrophy.[126, 127] It is activated by various neurohormonal molecules such as insulin–like growth factor 1 (IGF-1), epidermal growth factor (EGF), adenosine, bradykinin and opioids. Most of these downstream intracellular pathways are reliant on ROS to facilitate signalling.

Some of the main transcription factors that are targeted by these pathways include nuclear factor-κB (NFκB) which has been shown to increase cardiac hypertrophy in various mice and rat studies,[128] GATA4 which is primary responsible for cell differentiation but is also recruited in hypertrophic cardiac disease where over-

expression leads to hypertrophy,[129] MEF2 and NFAT as already mentioned and finally serum response factor (SRF), which induced a hypertrophic cardiomyopathy in transgenic mice over-expressing for SRF.[130] Negative feedback to hypertrophic signalling is provided by natriuretic peptides via the natriuretic peptide receptor (NPR)[131] and the IL-6 like proteins and TNFα activated JAK-STAT pathway, which has been shown in a series of knock-out mice experiments to prevent excessive remodelling, including hypertrophy, by their action on the regulation of foetal gene expression.[132] Figure 1.7 gives a detailed overview of the most clearly understood pathways involved in cardiomyocyte hypertrophy.

One common downstream pathway is the expression of panel of genes known as the foetal gene panel. Changes in these genes bring about a state similar to that seen during foetal cardiac development. Some of the most notable genes in this process are those that encode for atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), α and β-myosin heavy chain (MHC), TNFα, IL6, TGFβ, cardiac and alpha skeletal muscle actin (α-act) and proto-oncogenes c-myc and c-fos. These genes expressed act at various levels to modulate transcription, post-transcription and epigenetic regulation ultimately bringing about a phenotypic change in the heart. Despite its evolutionary role as a compensatory mechanism, foetal gene expression is considered to be maladaptive and after MI is a key contributor to the remodelling process.[133] Indeed studies have shown that patient with heart failure started on standard heart failure pharmacological therapy showed a reduction in typically expressed foetal genes.[134]

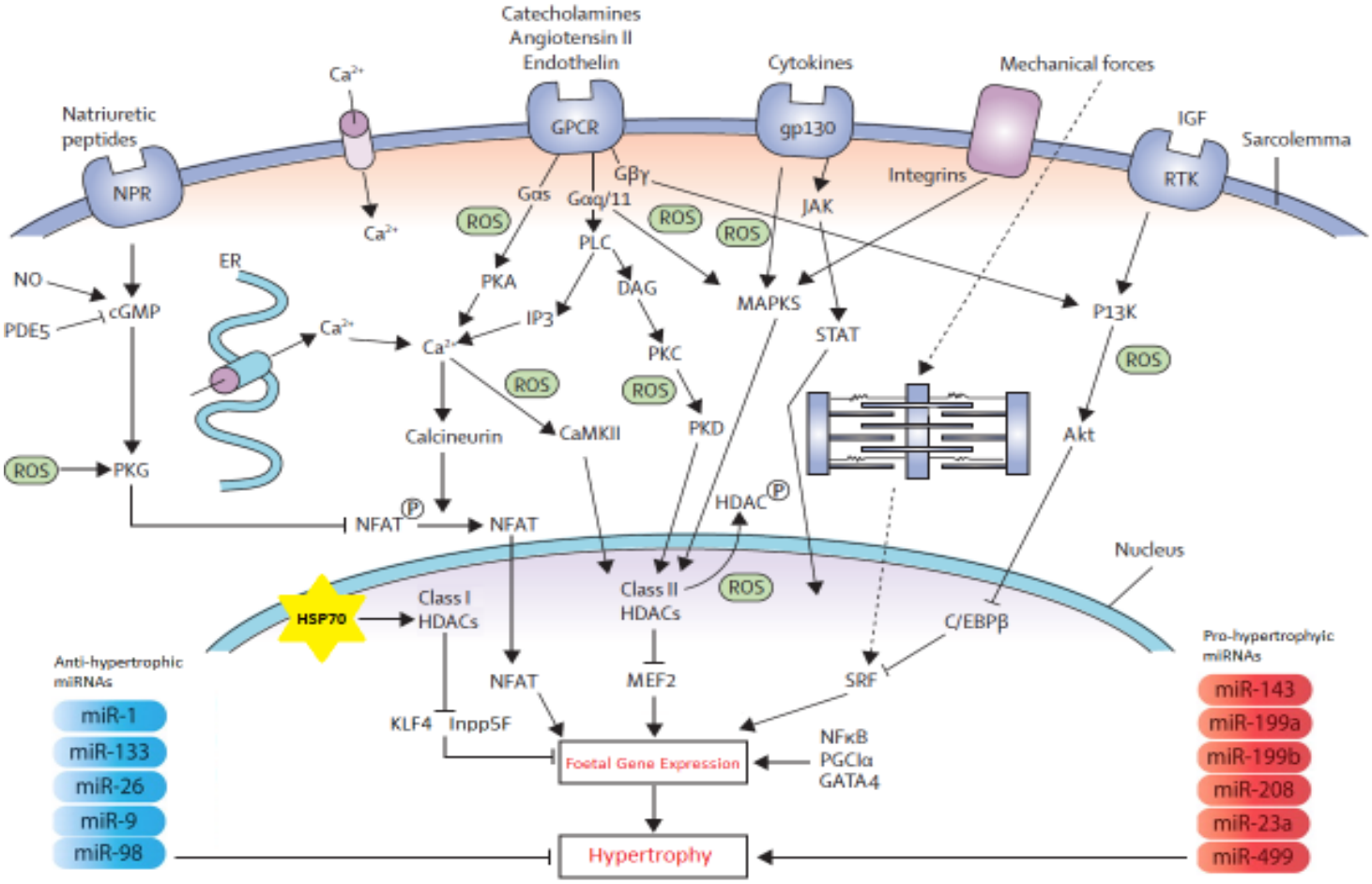


Figure 1.7. Schematic of the main signalling pathways in cardiomyocyte hypertrophy. Neurohormonal and mechanical factors act via cell surface receptors to initiate intracellular pathways which alter gene expression and ultimately the cell phenotype. Pro and anti-hypertrophic miRs influence all stages of signalling and are discussed in more detail in the text. For a full explanations of the abbreviations see the *Abbreviations* section. Adapted from Shah *et al.,* (2011), Da Costa Martins *et al.,* (2012) and Kee *et al.,* (2011).[135-137]

1.1.3.5 Excitation-Contraction

In the healthy heart excitation-contraction is controlled by close modulation of calcium handling. Coupling of excitation-contraction involves influx of Ca^{2+} via sarcolemmal L-type channels (LTCC), this influx of Ca^{2+} acts as a "trigger" for a large release of Ca^{2+} from the SR via ryanodine receptors (RyR), and this large rise in systolic Ca^{2+} leads to myofilament binding which initiates contraction. Cytosolic Ca^{2+} is then reduced as it is sequestered back to the SR via SR Ca^{2+} ATPase 2a channels (SERCA 2a) and effluxed from the cell via NCX on the sarcolemma, to bring about relaxation and replenish the SR Ca^{2+} stores for the whole process to begin afresh. For a more detailed review of this topic, please refer to Bers *et al*., (2002).[138]

In patients with heart failure, various abnormalities have been demonstrated in the excitation-contraction process including a prolonged action-potential, a decreased force of contraction generated by the myofibrils and a reduced capacity for relaxation after contraction. Abnormalities in the calcium handling process play a large part in this process. In heart failure there is a smaller intracellular rise in calcium in response to an action potential, the rate of rise of intracellular calcium is slowed and after contraction during relaxation, there is slower rate of calcium removal. These changes in calcium handling are caused by a number of molecular alteration to various structures involved in the process including decreased activity of the LTCCs on the cell membrane, as well as decreased SERCA activity with reduced SR calcium stores and phosholamban phosphorylation (responsible for normal SR functioning). There is also increased expression of the activity of the membrane NCX which encourages removal of calcium from the cell and is pro-arrhythmogenic. Yamamoto *et al.,* (1999) showed that RyR2

becomes hyper-phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) which appears to increase to compensate for the decreased calcium handling but eventually the calcium stores within the SR become depleted despite this compensation.[139] Indeed a body of evidence exists to suggest that phosphorylation of RyR2, brought about by the excessive β-adrenergic stimulation that occurs in the early stages of remodelling, can in fact lead to a worsening of cardiac function.[140] Disruption of the T-tubules has also been described in heart failure. This has a significant effect on calcium handling as it disrupts the usually close association of RyR2 and LTCCs.[141] Furthermore structural changes also occur in remodelling to the contractile proteins including the myosin heavy and light chains which revert back to foetal form, as does troponin T.

1.1.3.6 Beta-adrenergic Desensitization

Down-regulation of the β1-adrenergic receptor occurs during the remodelling process. This has been demonstrated in human ventricles removed at transplant procedures where there is significant reduction in β-adrenergic receptor numbers.[142] This is thought to be due to the excess levels of noradrenaline that exist locally around the receptors during the remodelling process, as well as increased expression of the beta-adrenergic receptor kinase pathways (βARK), which act to increase the net internalization of β-adrenergic receptors. Desensitization has mixed effects in that the reduced LV contractility that ensues is clearly damaging, however there is an overall reduction in energy required compared to an otherwise adrenergically sensitised heart. Recent evidence suggests β3 receptors may also play an important role in remodelling as they

have been shown to be up-regulated in heart failure and they have a mildly negatively inotropic effect when stimulated in the ventricle.[143] For a more in depth review of this topic, please refer to Triposkiadis *et* al., (2009).[144]

1.1.3.7 MicroRNAs

Post-transcriptional changes are also being recognised as important influences on remodelling. Much interest has been generate by the role of miRs over the last few years by their action on genes that are involved in all parts of the remodelling process including excitation-contraction, cell death, cardiomyocyte hypertrophy and fibrosis. MiRs are non-coding RNA segments that act as silencers of gene activity. The discovery of their involvement in remodelling has led to new avenues to potential therapy by controlling specific miR levels.

Particular focus has been paid to the role miRs play in hypertrophy (see Figure 1.7). Thum *et al.,* (2007) first showed that the foetal gene panel expression is driven in part by changes in the profile of miR expression.[145] The sheer number and variable targets of miR make the relevance of single miRs in health and disease difficult to interpret and for this reason miRs are often considered in clusters. An important cluster in remodelling and heart failure are the so-called *myo*miRs which include miR-1 and 133 (which are co-expressed) and to a lesser degree miR-206 and miR-208.[146] The net effect of these miRs is to inhibit hypertrophy and apoptosis via their action on a number of target. Other key anti-hypertrophic miRs include miR-9, 29 and 98. Conversely a number of miRs have targets with the net effect of increasing hypertrophy, namely miR-18b, 21, 23, 199a and b, 208 and 499.[147] In addition to its pro-hypertrophic properties, miR-199a

disrupts the normal cell architecture by down-regulated the Ubiquitin conjugating enzymes Ube2i and Ub2g1 which lead to disruption of the sarcomere and has knock on effects for its ability to perform ubiquitination. The STAT3 pathway, an important pathway for maintaining cardiac integrity and a key component of the second window ischaemic conditioning (see *section 1.2.3*) acts to down-regulate miR-199a.[148]

MiRs also play a key role in modulating the fibrotic response in remodelling. Heavily implicated miRs in this process are miR-21 and miR-29. MiR-21 is predominantly found in fibroblasts and regulates the ERK-MAPK pathway and increases interstitial fibrosis and fibroblast to myofibroblast differentiation.[149] Interestingly miR-21 seems to have a number of other function including up regulation of Sprouty-2 (Sprouty homolog 2) leading to hypertrophy.[150] MiR-29 up-regulates fibrosis by targeting a number of mRNA encoding for fibrotic proteins such as collagen, elastin and fibrillin.[151]

1.1.3.8 Reverse Remodelling: Current Therapeutic Strategies

Although traditionally thought of as a progressive, irreversible process, there is some evidence that the post-MI remodelling process can be partially reversed with certain therapeutic interventions. However, unlike cardiac failure caused by non-ischaemic aetiologies such as peripartum cardiomyopathy, takotsubo cardiomyopathy or viral myocarditis, where significant spontaneous remodelling is anticipated, even with therapeutic interventions, the degree of reverse remodelling in post-MI heart failure is modest at best.[152-154]

The most significant reverse remodelling is seen in post-MI heart failure patients who have received a left ventricular assist device (LVAD). LVADs are used in end-stage

heart failure and are electromechanical devices that help augment the circulatory actions of the heart. Traditionally they have been used as a bridge to transplant but they are now become an end destination procedure in themselves.[155, 156] Studies have shown a reduction in LV volume, cardiomyocyte size and overall cardiac mass.[157-159] This has been shown to occur by a reduction in the mean size of cardiomyocytes and improved calcium handling and β-adrenergic sensitivity.[160-162] The haemodynamic off-loading of the heart after LVAD implantation, reinforces the theory that heart failure is a consequence of continuous increased levels of myocardial wall stress and continuous activation of neurohormonal pathways.

Substantial reverse remodelling has also been demonstrated in cardiac resynchronisation therapy (CRT). CRT is a pacemaker with a left ventricular lead (actually placed in the coronary sinus via the venous circulation) to supplement the traditional right ventricular lead. The goal of the treatment is to allow a synchronous contraction between the left and the right side of the heart in patients with proven dyssynchrony to improve overall cardiac function.[163] The largest trial to date MADIT-CRT, which enrolled a total of 1820 participants in New York Heart Association (NYHA) class 1-2, demonstrated an increase in LVEF of 11%, a decrease in left ventricular end diastolic volume (LVEDV) by 21% and a decrease in left ventricular end systolic volume (LVESV) by 35% at 2.4 years in the treatment group compared to the control group.[164] Similar encouraging findings have been in seen in other smaller trials of similar design, often in patients in NYHA 3 or 4 classes.[165-167]

More modest reverse remodelling is seen with pharmacological therapies. Beta-adrenergic blockers have a wealth of data to show improved survival benefits[168] but

some trials have also demonstrated reverse remodelling. The cMRI sub-study of the MERIT-HF trial showed that the use of metoprolol for 6 months decreased left ventricular end diastolic index (LVEDI) by a mean of 16% and left ventricular end systolic index (LVESI) by a mean of 24%.[169] A number of similar trials have shown the reverse remodelling benefits of metoprolol as well as carvedilol in both ischaemic and non-ischaemic heart failure. The mechanisms for beta-blockade to encourage reverse remodelling have been demonstrated in animal studies and explanted human heart tissue and show improved handling of calcium in the sarcolemma as well as improved cardiomyocyte contractility with better contractile reserve.[170-172] With regards to blocking the renin angiotensin/aldosterone system (RAAS) antagonism with either angiotensin receptor inhibitors (ACEi) or angiotensin receptor blockers (ARBs), the evidence is less compelling.[173, 174] A more substantial reversal of remodelling is seen when more than one element of the RAAS system is blocked i.e. using a combination of ACEi, ARB and/or an aldosterone antagonist such as spironolactone.[175, 176] The novel combination medication of the ARB/neprilysin inhibitor valsartan/sacubitril (Entresto) was shown in the landmark PARADIGM-HF trial to be superior to angiotensin receptor inhibition alone, in the form or ramipril monotherapy, in reducing mortality and hospitalisation for patients with HF-REF.[177] The neprilysin inhibition afforded by the drug may augment the anti-remodelling properties of RAAS inhibition, by accelerating the breakdown of BNP, ANP, bradykinin and other vasoactive peptides implicated in acute maladaptive remodelling.[178, 179]

Another fairly novel pharmacological agent, Ivabradine, works to reduce the heart rate via its action on the “funny” channel (I*f*) in the sinoatrial node, has shown

promise in facilitating reverse remodelling in established heart failure. The SHI*F*T trial randomised patients with severe HF-REF (LVEF < 35%) with resting heart rates of greater than 70 bpm in sinus rhythm, to either optimal medical therapy (OMT) or OMT plus the addition of Ivabradine. The treatment groups had reduced LVESVI and reduced LVEDVI and improved LVEF compared to the control group at 8 months follow-up.[180] Similar 1 year results were demonstrated in the BEAUTIFUL echo sub-study trial in a population with stable CAD and HF-REF.[181] The exact mechanism of action has not been clearly demonstrated but animal studies have pointed towards reduction in fibrosis, improvement in endothelial function and stabilisation of calcium handling in the SR.[182-184]

1.1.3.9 Future Therapeutic Strategies to Combat Maladaptive Remodelling

As highlighted in the previous section, our ability to actively reverse established maladaptive cardiac remodelling after MI remains limited. Once established, maladaptive remodelling is much more challenging to combat compared to when it is evolving in the first few weeks after the ischaemic insult. One of the future goals of the healthcare professional must be to implement appropriate therapies in a timely manner to target acute remodelling.

With regards to novel pharmacological agents, the potential to target both acute and chronic remodelling post-MI is vast. Indeed a number of drugs are currently either in development or are being tested in clinical trials. These include istaroxime, a Na+ /K+ -ATPase inhibitor, which leads to improvements in sarcomeric contraction as well as displaying lusitropic properties.[185] Another encouraging chemically agent is serelaxin.

The large, multicentre RELAX-AHF2 phase IIIb trial currently recruiting, aims to investigate this new agent in acute heart failure patients (https://clinicaltrials.gov/ct2/show/NCT01870778). Seralaxin is a recombinant form of human relaxin-2 that targets the relaxin receptor, which leads to an increase in circulating NO and has shown symptom relief and clinical outcome benefit in the first RELAX-AHF trial.[186]

Other avenues of explorations in the future include gene targeted therapies. With regards to cardiac remodelling, the well-publicised CUPID phase 2 trial used an adeno-associated virus vector to administer SERCA2a cDNA into cardiomyocytes. The study population were participants with severe heart failure as measured by LVEF and VO_2max. The high dose gene therapy group showed subjective and objective improvements in measures of heart failure at 6 and 12 months however longer term data showed this therapy did not improve clinical outcomes in patients with HF-REF.[187] With regards to remodelling, there were improvements in LVEF, LVEDV and LVESV.[188] MiR and long noncoding (lnc) RNAs are also gene targets that are currently generating much interest and the likelihood is that a number of phase I and II trials in this area are on the horizon.

Another fascinating potential is that of regenerative medicine. The potential for novel targets falls into a three broad categories. The first of these categories includes those therapies that will harness and up-regulate progenitor cells already resident in the heart. One example is the CADUCEUS trial which enrolled patients up to 4 weeks after MI with LVEF ranging from 25-45% and took endomyocardial biopsies from which they cultured large population of cardiosphere-derived cells which we then infused back into

the patient's coronary circulation. Although the procedure was safe, there was no improvement in the treatment group in changes in LVEDV, LVESV or LVEF at 6 months as measured by cMRI.[189] The second category includes therapies that will encourage the migration of pluripotent bone marrow cells to migrate to the heart and differentiate. Once such agent is POL6326, a chemokine receptor type 4 (CXCR4) antagonist which stimulates the mobilisation of hematopoietic stem cells.[190] It is currently under investigation in the phase II CATCH-AMI which is testing its efficacy in post-ST elevation MI (STEMI) acute heart failure participants and is due to report its findings in late 2016 (https://clinicaltrials.gov/show/NCT01905475). Another approach may be the direct administration of autologous cardiopoietic stem cells either into the heart or into the blood stream with the feasibility of such an approach already established.[191] The SCIPIO trial has already shown an improvement in cardiac function with autologous cardiac derived progenitor cells in ischaemic cardiomyopathy[192] and further trials are currently underway to further elucidate this issue including two UK trials assessing the therapy in both chronic heart failure (REGENERATE-IHD, https://clinicaltrials.gov/ct2/show/NCT00747708) and acute heart failure post-MI (REGENERATE-AMI, https://clinicaltrials.gov/ct2/show/NCT00765453).

Perhaps the most exciting and all-encompassing of all of the novel and emerging therapies in the battle against post-MI heart failure is ischaemic conditioning, namely an intermittent and short-lived ischaemic stimulus at and around the time of an ischaemic insult that targets a plethora of downstream targets. In the author's opinion this therapy holds the greatest potential for providing new therapeutic benefits as it has the potential to target a number of important regulators and pathways in the context of

both I/R and acute cardiac remodelling post-MI. The remainder of this chapter will discuss our current understanding of this therapy as well as outlining future perspectives.

1.2 Ischaemic Conditioning

1.2.1 Preconditioning

Murry *et al.,* (1986) were the first to describe the phenomenon of ischaemic preconditioning (IPC). They demonstrated that by occluding the left circumflex coronary artery (LCx) of one group of dogs for 5 minutes followed by 5 minutes of reperfusion, repeated 4 times in succession prior to 40 minutes of sustained occlusion, the final infarct size that developed after 4 days following reperfusion was 25% smaller that the control group subjected to 40 minutes of LCx occlusion alone.[193] This work has since been replicated by numerous different research groups in various species. The first in-vivo study in humans assessing the effect of pre-conditioning was performed by Deutsch *et al.,* (1990). In a small group of patients undergoing elective PCI for an obstructed left anterior descending artery (LAD). They showed a reduction in electrographic, metabolic and clinical markers of ischaemia following the second cycle of balloon inflation compared to the first.[194]

Subsequent translational studies demonstrated the effectiveness of IPC in humans in a clinical context, specifically prior to Coronary Artery Bypass Grafting surgery (CABG),[195] although the larger ERICCA phase III clinical trial recently showed remote ischaemic conditioning (rIC) failed to improve long term outcomes after CABG.[196] Interestingly studies investigating patients who suffered angina prior to MI and

subsequent reperfusion showed they had better outcomes than patients without pre-infarction angina, hinting that these patients are in effect undergoing auto-IPC in a process sometimes termed 'warm-up angina'.[197, 198] Furthermore IPC has also been shown to be protective against post-MI arrhythmias and the development of acute cardiac failure within the first few days after the infarct.[199, 200]

The protection afforded by conditioning is time dependent i.e. the conditioning itself must be implemented at a certain time for a certain duration and then the effectiveness of conditioning wanes after a few hours. The length of conditioning required to induce a net benefit varies greatly in different animal models. In a rabbit model, whereas 2 minutes of IPC provided no benefit, 3-5 minutes of IPC significantly reduced the final infarct size.[201] The other major drawback of IPC as a new therapeutic tool is the fact that its use is limited to situations where sustained periods of ischaemia can be anticipated i.e. prior to major cardiac surgery or coronary angiography.

1.2.2 Postconditioning

The potential of ischaemic conditioning was soon realised in the MI setting where conditioning after the onset of MI and around the time of revascularization by PCI or thrombolysis was shown to be protective, so called postconditioning (IPostC). The reason for this seemingly paradoxical ability to condition after ischaemia is that conditioning is now known to protect predominantly from the harmful effects of reperfusion injury rather than ischaemia. Zhao *et al.,* (2003) first demonstrated the effectiveness of IPostC.[202] His group occluded the LADs of dogs for 60 minutes followed by a 3 hour reperfusion period. One group of dogs were preconditioned by occluding

the LAD for 5 minutes with 10 minutes of reperfusion prior to the 60 minutes of ischaemia and the other group were post-conditioned by staggering reperfusion after the 60 minutes of ischaemia with 3 cycles of 30 seconds occlusion/reperfusion. Interestingly they showed that IPostC was almost as effective as IPC in reducing the final infarct size, level of tissue oedema and various other markers of cardiac damage.

The effectiveness of IPostC was later demonstrated in humans. Laskey *et al.,* (2005) demonstrated that by staggering reperfusion during PCI by repetitively inflating and deflating an angioplasty balloon for short periods of time, final electrocardiographic ST segment elevation size was reduced and distal myocardial perfusion was increased.[203] Similarly Staat *et al.,* (2005) using a similar technique at the time of PCI, showed a significant reduction in creatine kinase (CK) biomarker release and an increase in myocardial reperfusion in the conditioned group.[204] In a similar vein but with slightly longer term implications, Thibault *et al.,* (2008) demonstrated that the effects of IPostC are not only protective with regard to acute infarct size, but also have a positive influence on more chronic markers such a final infarct size and myocardial contractility after 1 year; specifically they demonstrated a 7% increase in LVEF compared with the control group.[205]

1.2.3 Windows of Protection

It has become clear that there are two 'windows of protection' around the time of I/R in which ischaemic conditioning can be effectively utilised. The first is the classical window beginning immediately following MI and lasts up to 4 hours. Protection within this time period is mainly induced through post-translational protein changes. A delayed

or second window of protection (SWOP) then starts as early as 12 hours after the infarct and lasts up to 72 hours.[206-208] The SWOP is thought to induce cardioprotection mainly through gene transcription and it is to this second window that much recent attention has been focused as it supports the potential for a more prolonged and sustained conditioning technique. Figure 1.8 illustrates the temporal relationship of the each window of conditioning in relation to infarct sparing.

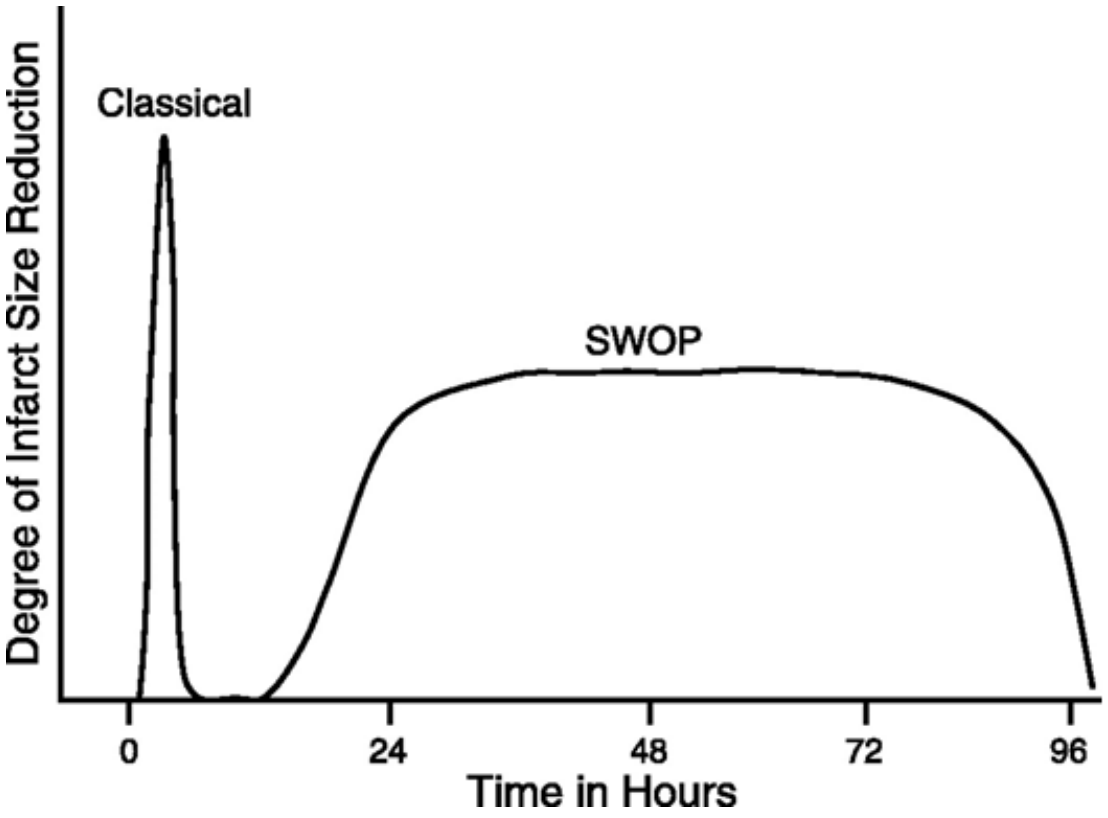


Figure 1.8. Schematic representation of the temporal nature of each window of conditioning with relative infarct sparing effects. Reproduced from Yellon *et al.,* (2003).[209]

1.2.4 Pathways of Ischaemic Postconditioning (Local and Remote)

There is significant overlap of the final intracellular signalling pathways involved in IPC and IPostC. Indeed despite the mystery that still surrounds the transmission of the signal from the distant muscle or tissue bed to the heart, the intracellular pathways of rIC share most of these final common pathways, with a few subtle differences.

One difference between the conditioning types is that IPostC only modulates the damage from reperfusion injury whereas IPC can influence ischaemia independent of its role in reperfusion, mainly by acting to slow the breakdown of ATP.[210] Furthermore, IPostC as well as activating similar pathways to IPC as described in following sections, also acts to prolong time to opening of the mPTP and hence reduce cell death e.g. by maintaining a low pH within the mitochondria during reperfusion as well as reducing the mitochondrial membrane potential and modulating mitochondrial calcium.[211] In addition Connexin 43 (Cx43), the main protein that forms gap junctions and hemi-channels in ventricular myocardium, is a key part of the signalling transduction cascade for IPC but not IPostC.[212]

A common theme of the intracellular signalling cascades is the central role the mitochondria play in inducing cardioprotection along with other vital components such as NO, PKC, the (Reperfusion Injury Salvage Kinase) RISK and Survivor Activating Factor Enhancement (SAFE) pathways; both in the setting of I/R as well as in the setting of acute remodelling. Figure 1.9 gives an overview of the extracellular triggers and intracellular signalling that infer cardioprotection from all forms of conditioning seen in early and late conditioning and the details of individual components of the intracellular signalling cascade are summarised in the following sections.

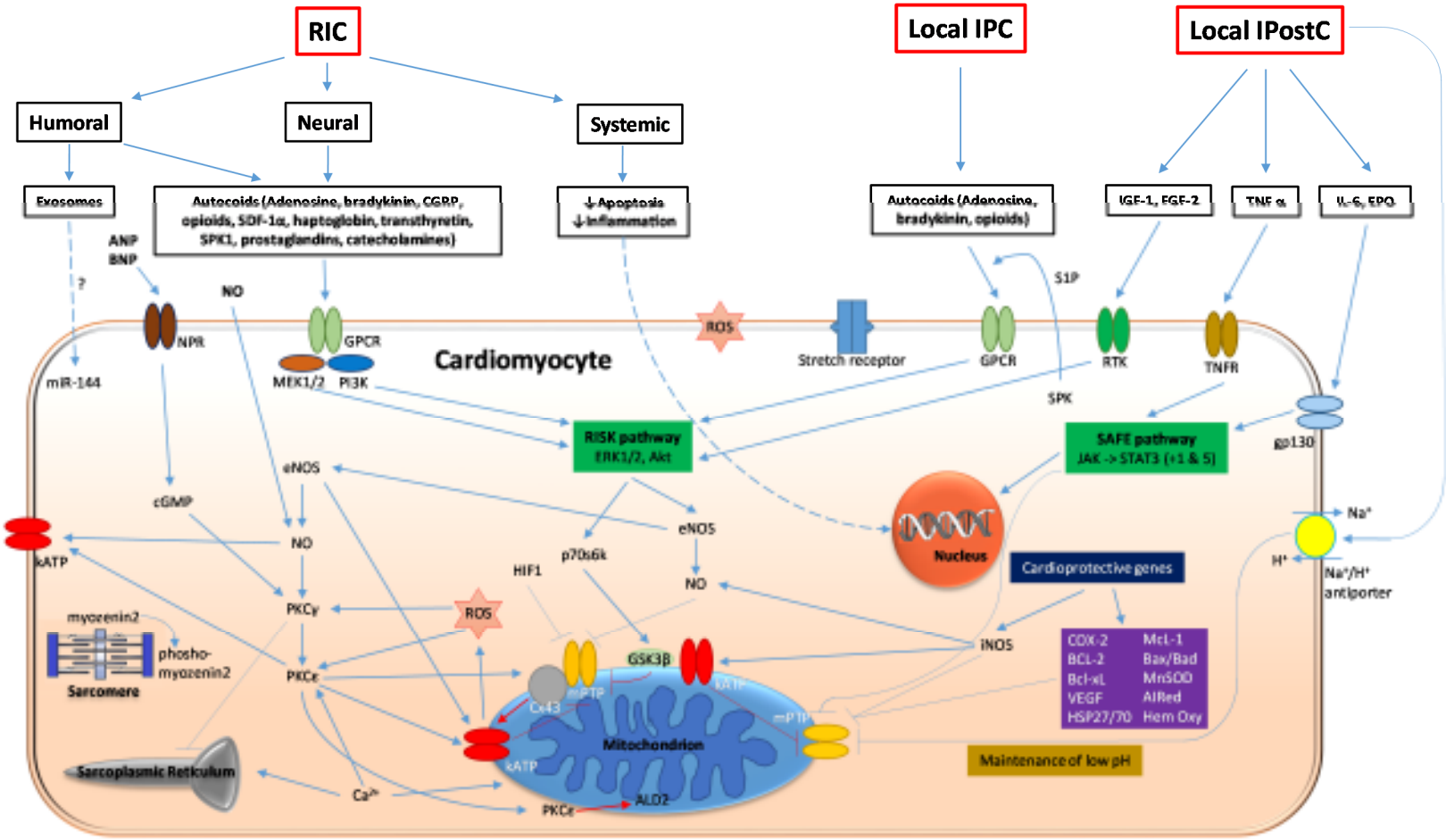


Figure 1.9. Schematic representation of the extra and intracellular pathways of ischaemic conditioning encompassing both windows of conditioning. For a full explanations of the abbreviations see the *Abbreviations* section at this beginning of the text.

1.2.4.1 RISK pathway

The Reperfusion Injury Salvage Kinase (RISK) pathway describes the p42/p44 (ERK 1/2) mitogen activated protein kinase (MAPK)-dependent axis, as well as encompassing the PI3K-AKT and GSK3β pathways, and in a broader sense includes the related JNK and p38MAPK pathways. The concept was introduced in 1999 by Yellon's group and its importance in ischaemic conditioning was first highlighted by Schulman *et al.,* (2002)[213] who along with other groups, demonstrated that activation of this axis in the post-MI reperfusion period reduced infarct size by up to 50%.[214] Further studies have also heavily implicated this pathway in the remodelling process. Yamazaki *et al.,* (2010) induced the RISK pathway in rats subjected to coronary artery occlusion by injecting the flavanol epicatechin. At 3 weeks post event there was a minimal change in the left ventricular end diastolic pressure (LVEDP) in the treated group but there was a significant increase in LVEDP in the control group. Furthermore there was a 33% decrease in infarct size in the treated group.[215] In this setting activation of and protection by the RISK pathway occurred after epicatechin induced ischaemic injury had been sustained.

The RISK pathway is activated by numerous triggers including bradykinin, adenosine and opioids acting via G-Protein Coupled Receptors (GPCR) and insulin and epidermal growth factors acting via tyrosine receptor kinase pathways as previously described. The pathway ultimately acts to inhibit mPTP opening and therefore reperfusion injury. More recent clinical trials have utilised various pharmacological agents such as ANP, GLP1, adenosine and erythropoietin to activate the RISK pathway during MI. Results vary but most agents trialled have shown some benefit, be it an improvement in LV systolic function, reduction in infarct size or improved clinical

outcome.[216-218] Although activation of the RISK pathway is clearly an important element of IPostC, it is not exclusive and indeed in some animals provides little or no protection at all, the most notable example being the study by Shyschally *et al.,* (2009) who failed to show any role for the RISK pathway in IPostC in pigs.[219]

1.2.4.2 Protein Kinase C pathways

PKC pathways are also heavily implicated in ischaemic conditioning and their role have been the subject of much debate. Initial studies placed PKC high up the cascade of intracellular signalling but more recent developments have shown it to be downstream of a number of intracellular signalling factors. Indeed the RISK hypothesis as previously described, places PKC downstream of the RISK pathway.[220] Adenosine receptors however, are thought to work directly to activate PKC.

The specific isoform(s) of PKC involved in conditioning are a subject of ongoing debate, however, PKCε has been heavily implicated. Gray *et al.,* (1997) abolished IPC by the application of the selective PKCε V1-2 peptide inhibitor in a rat cell culture model.[221] More recently Saurin *et al.,* (2002) showed that PKCε knock-out mice, PKCε -/-, were resistant to the infarct sparing effects of IPC perfusate compared to sibling heterozygous PKCε +/- controls.[222] One mechanism of action may be the mobilisation of PKCε to the mitochondria and phosphorylation of elements of the mPTP which prevents opening.[223] In a second proposed mechanism of protection derived from a rabbit model, Pain *et al.,* (2000) proposed that IPC acts via GPCR leading to the opening of mitochondrial K_{ATP} channels. This generates a small pool of ROS which then activates PKCε.[224] Recent work

by our group has also highlighted a role for PKCδ, which was shown in a rat model to work via sarcolemmal K_{ATP} channels to induce IPC against I/R.[225]

With regards to IPostC, Penna *et al.,* (2006) showed that the administration of a non-specific PKC inhibitor in rat hearts abolished the protective effects of IPost in limiting infarct size.[226] Similarly PKC has been shown to play a role in remote ischaemic preconditioning (rIPC). Wolfrum *et al.,* (2002) applied rIC to rats via mesenteric artery occlusion and demonstrated translocation of PKCε from the cytosol to the particulate compartment within the heart. This translocation was blocked by HOE140 (a bradykinin antagonist) as well as by a non-selective PKC blocker, chelerythrine.[227] This work has been mirrored by Weinbrenner *et al.,* (2002) who again abolished rIC in a rat model of I/R using chelerythrine.[228] With regards to a role for PKC in remote ischaemic postconditioning (rPostC), there is currently a scarcity of data. However a number of studies have shown the translocation of PKC isoforms in adenosine mediated cardioprotection (adenosine being a key component of rPostC).[229, 230]

1.2.4.3 SAFE pathway

The SAFE pathway encompasses the JAK-STAT pathway.[231] This pathway has yet to be fully elucidated but can act independently of the RISK pathway and is thought to be involved in the second window of protection. It involves activation by TNFα and IL-6 like peptides by binding to TNFr and gp130 respectively. This leads to the activation of JAK and the subsequent phosphorylation of the transcription factors STAT3 and STAT5. Phosphorylated STAT3/5 is then thought to act in two ways; firstly by translocation to the nucleus where it up-regulates various transcription factors involved in cell survival

such as the apoptotic transcription factors *Bcl-2*, *Bcl-xL* and vascular endothelial growth factor (VEGF) and down-regulates apoptotic genes such as *Bax*. Secondly, it is thought to act by its action on the mitochondrion by phosphorylating hence inactivating GSK3β, thereby inhibiting mPTP and conferring other mitochondrial cardioprotective benefits.[232] STAT3 has been shown to be more important to the SAFE pathway in rodent species whereas STAT5 seems to playing a greater part in humans.[233] The degree to which this pathway is utilised in repeated IPostC has yet to be established.[234] It is with great surprise that the role of SAFE pathway has yet to be investigated in the context of rIC in a clinical scenario.

1.2.4.4 CGMP/NO/PKG pathway

NO is well established as an important signalling molecule that mediates ischaemic conditioning, especially as part of the RISK pathway with increased levels in response to NOS activation, predominantly eNOS as previously described.[235, 236] Furthermore NO plays a fundamental role in attenuating pathological remodelling drivers post-MI. A number of studies have shown a direct inverse correlation between the levels of NO bioavailability in the heart and the degree of ensuing heart failure.[237-239] It is thought that the predominant protective properties of low levels of NO, released by Ca^{2+} dependent NOS such as eNOS, are its anti-hypertrophic and pro-apoptotic downstream effects, including the modulation of fibroblast growth factor (FGF), VEGF and TGF-β.[240, 241] Furthermore, NO has positive benefits in established heart failure by reducing myocardial oxygen consumption by competitive inhibition of oxygen of cytochrome oxidase within the mitochondria.[242, 243] NO also leads to increased myocardial

contractility by its effect on excitation-contraction by means of modulating calcium influx through sarcolemmal LTCC as well as increasing the release and re-uptake of calcium by the SR.[244, 245] Finally NO acts to increase coronary blood flow by increasing vasodilation and decreasing vascular resistance, mainly by its action on cGMP in vascular smooth muscle cells.[246, 247]

NO is thought to acts through two main downstream pathways during acute remodelling to attenuate ventricular hypertrophy.[248] Firstly it can act via the reversible *S*-nitrosylation of protein.[249, 250] S-nitroslyation is thought to inhibit or slow the progression of hypertrophy through inhibition of the pro-hypertrophic NF-κB and Inhibitor of Kappa (IκB).[251, 252] Secondly it activates the sGC/cGMP/PKG pathway resulting in phosphorylation of target proteins acting mainly via inhibition of the calcineurin-NFAT pathway.[253, 254] It does this by reducing Ca^{2+} loading by inhibiting LTCC and TRPC channels.[254-256] PKG is also thought to be crucial by interacting with PKC and the Akt to mediate the opening of mitochondrial K_{ATP} channels and subsequently the mPTP.[257] Furthermore increased PKG activity causes phosphorylation and activation of the GTPase regulator of G-protein signalling (RGS2/4). This inhibits GPCR-mediated PLCβ activity and attenuates hypertrophy.[258, 259] More recent work by Wang *et al.,* (2015), using human embryonic stem cell-derived cardiomyocytes, has identified a novel downstream target of PKG in modulating hypertrophy: Orai1, a pore-forming subunit of store-operated Ca^{2+} entry (SOCE).[260] In this model phosphorylation of Orai1-Ser-34 inhibited hypertrophy.

1.2.4.5 Other Key Pathways

Numerous other pathways have been implicated in ischaemic conditioning. One such pathway involves the lipid sphingosine kinase (SPK), which has been postulated to be an upstream regulator of the RISK pathway via the generation of sphingosine 1 phosphate (S1P). Jin *et al.,* (2008) showed that hearts from mice that lacked SPK1 were resistant to IPostC, sustained larger infarcts and did not activate the Akt or ERK1/2 pathways.[261]

Another important pathway involves hypoxia induced factor 1 alpha (HIF-1α), a gene transcription factor whose role is well established in traditional IPC. Cai *et al.,* (2008) highlighted the importance of HIF-1α in a heterozygous HIF-1α deficient mouse model where IPC against I/R was lost compared to wild type mice.[262] The same group also showed that HIF-1α is important in the SWOP by increasing levels of intracellular erythropoietin (EPO).[263] However, its role in rIC remains controversial. Although levels of HIF-1α are shown to increase in a mouse model of rIC, there has been conflicting data as to whether these increased levels actually translate into effective cardioprotection.[264, 265]

In data presented at the 8th Biennial Hatter Cardiovascular Institute Workshop 2015, two new mediators of rIC were presented which are implicated in rIC afforded cardioprotection, aldehyde dehydrogenase-2 (ALDH-2) and phospho-myozenin-2.[266] Firstly, ALDH-2 was shown by Kharbanda's group to play a key role in both a rabbit MI model and in healthy human volunteers. In the animal study, administration of an ALDH-2 inhibitor abrogated rIC induced cardioprotection. In the human study, volunteers with a Glu504Lys polymorphism in ALDH-2 showed high levels of resistance to rIC induced protection against ischaemia induced endothelial dysfunction.[267] Secondly, Suleiman's

group have shown that increased phosphorylation of phospho-myozenin-2 (a sarcomeric protein found within cardiomyocytes) as well as increased levels of numerous other phosphoproteins in the Z-disk within sarcomere were observed in a mouse model of rIC induced cardioprotection against I/R.[268]

1.2.5 Remote Ischaemic Conditioning

RIC allows for the ischaemic conditioning stimulus to be applied to a distant tissue or muscle bed, commonly by inflating a blood pressure cuff on the arm or leg. Przyklenk *et al.,* (1993) was the first to demonstrate rIC in dogs. They preconditioned the heart by occluding the LCx for brief periods and then induced prolonged ischaemia by completely occluding the LAD for an hour, followed by a prolonged period of reperfusion. By applying I/R to the LCx, they were able to confer protection to naive "remote" myocardium supplied by the LAD.[269] Kerendi *et al.,* (2005) later demonstrated the cardioprotective effects of rIC in the post-MI setting. After 30 minutes of coronary artery occlusion in rat hearts they remotely conditioned the kidneys then re-perfused the heart and showed a 50% decrease in infarct size compared to the control.[270] Similar results have been demonstrated using a pig model.[271] A summary of the timing and locality of conditioning in the context of an ischaemic event is given in Figure 1.10.

The technique of inducing ischaemia using a blood pressure cuff to the forearm in humans was first used by Kharbanda *et al.,* (2002).[272] More recently Bøtker *et al.,* (2010) looked at the effects of rIC in STEMI patients by applying 4x5-minute cycles of blood pressure cuff inflation and deflation to the forearm whilst patient were in the ambulance en route to receiving PPCI.[273] Conditioned patients with large anterior

infarcts had a significantly better myocardial salvage index than the control group. A smaller study by the same group was undertaken in STEMI patients where rIC was applied just after PCI using 4 bouts of 4-minute inflations of a forearm blood pressure cuff. With the addition of morphine there was a significant reduction of troponin T levels (a biomarker of cardiac damage) in the conditioned group compared to the control group.[274]

The timing of rPostC is critical with regards to modulating I/R after MI. Roubille *et al.,* (2011) described the damaged incurred by reperfusion as a 'wavefront' and showed that rPostC can be effective up to 30 minutes post-MI.[275] However, there has been so far only a tentative foray by the scientific community in examining the effects of rPostC on remodelling after MI once I/R damage has been established. Munk *et al.,* (2010) showed that in MI patients with an Area At Risk (AAR) of over 35%, those who received rIC after PPCI had a significant improvement of LVEF after 30 days compared to the control group: 51 ± 11% versus 46 ± 9% respectively.[276] However, the degree to which the difference in LVEF is due to remodelling as opposed to infarct size is difficult to ascertain.

1.2.5.1 Remote Ischaemic Conditioning Extracellular Signalling Pathways

Several current theories exist as to the mechanisms of rIC extracellular signalling to convey protection from a distant muscle or tissue bed to the heart to infer cardioprotection. These are summarised in Figure 1.11 (see also Figure 1.9).

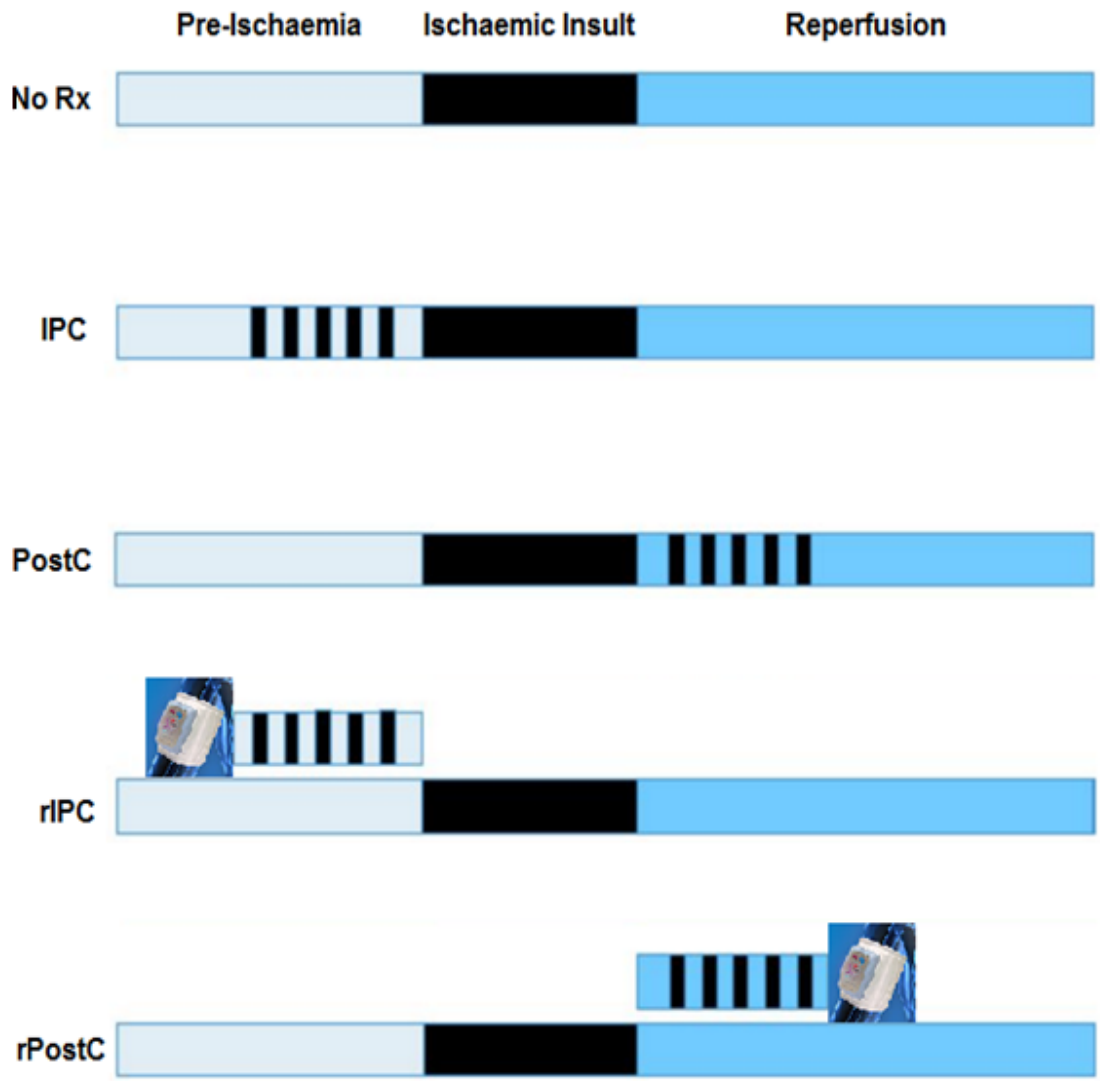


Figure 1.10. Schematic representation of the different conditioning techniques. Black bar = prolonged ischaemia, black lines = intermittent non-lethal ischaemia, light blue bar = pre-ischaemia, darker blue bar = reperfusion. *No Rx* = No conditioning, *IPC* = Ischaemic Preconditioning, *IPostC* = Ischaemic Postconditioning, *rIPC* = Remote Ischaemic Preconditioning, *rIPostC* = Remote Ischaemic Postconditioning. Adapted from McCafferty *et al.,* (2014).[277]

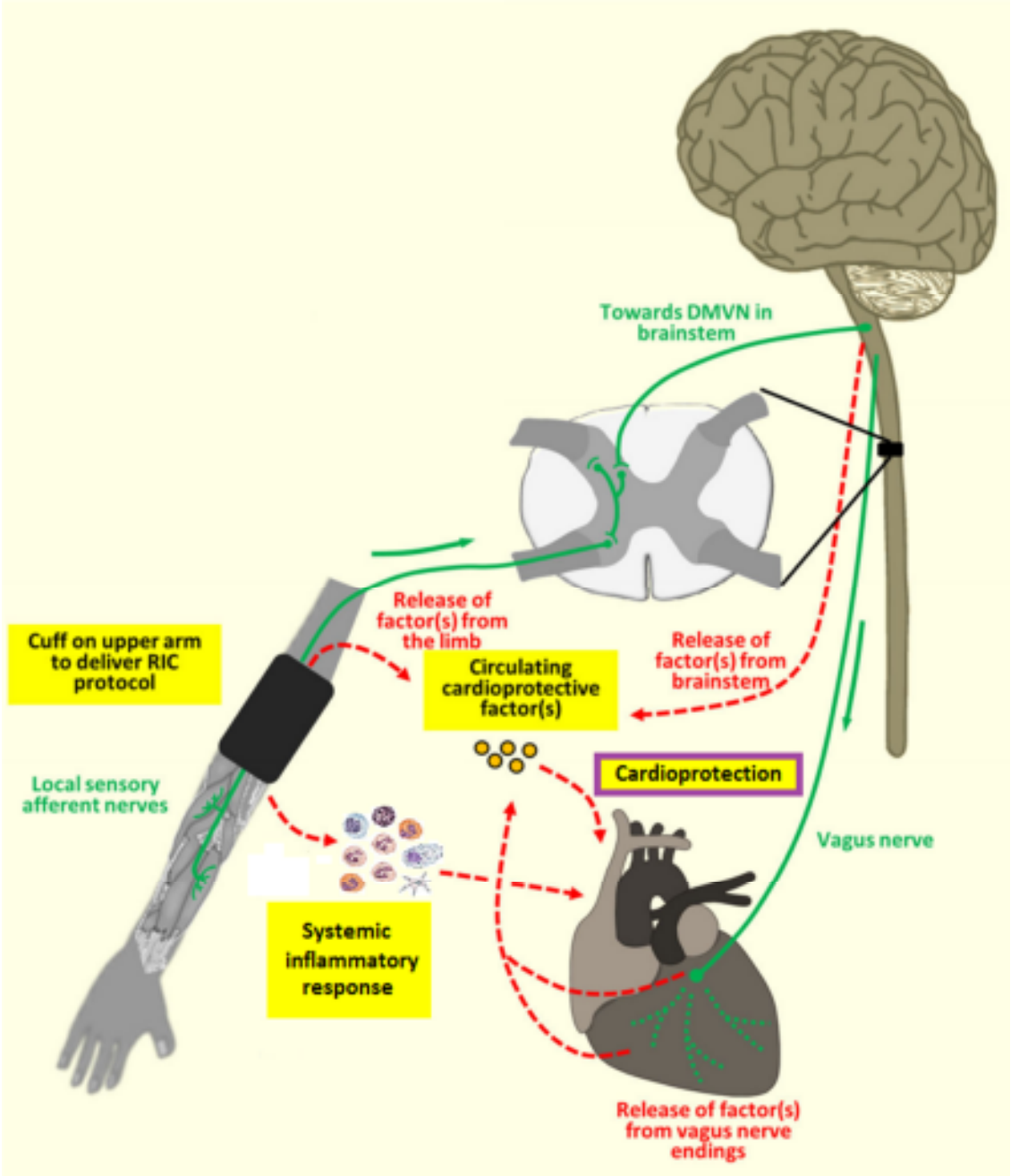


Figure 1.11. Schematic of the postulated remote ischaemic conditioning extracellular signalling pathways. The main signalling pathways are thought to be humoral and neural, with a significant interplay between the two. Modulation of the systemic inflammatory response is also fundamental, especially in the context of repeated rIC. *DMVN* = dorsal motor vagal nucleus. Adapted from Pickard *et al.,* (2015).[266]

1.2.5.1.1 Humoral Signalling

Humoral signalling is thought to play a prominent role in rIPC. This was succinctly demonstrated by Konstantinov *et al.,* (2005) who induced limb rIPC in a pig with a donor heart (which therefore had no autonomic innervation) and reduced the infarct size after MI, strongly indicating a humoral messaging system.[278] Other groups have pointed to the importance of humoral signalling by isolating animal hearts and treating them with perfusate from different rIPC animals and demonstrating a reduction in MI infarct size, classically by Dickson *et al.,* (1999) using rabbits.[279] Various humoral factors have been implicated including adenosine, bradykinin, NO, opioid peptides, natriuretic factors, endocannabinoids, angiotensin I and calcitonin gene related peptide (CGRP).

Current understanding points to a molecule or group of molecules that falls between the ranges of 3.5-8kDa in size and are hydrophobic.[280-282] This potentially rules out some molecules thought to be implicated such as adenosine, bradykinin and opioids which are all smaller than 3kDa. More recent candidates for the responsible humoral messenger include stromal cell-derived factor-1 (SDF-1a) which recruits stem cells via its action on CXCR4 and is activated by hypoxia[283] and a panel of anti-inflammatory proteins including haptoglobin and transthyretin.[284]

MiR-144 is the first miR to be identified as a possible humoral factor. Levels of circulating miR-144 were shown to be increased in mouse models of rIC induced protection against I/R with the effects of rIC completely negated after the administration of the antagomir to miR-144.[285, 286] Levels of this miR have also been shown to be increased in human volunteers undergoing standard rIC protocols.[287] Studies looking at the interaction of miR-144 and exosomes (circulating extracellular vesicles) highlighted

the importance of these exosomes to facilitate the carriage and release of miR-144 (or its precursor) to facilitate cardioprotection.[288] Exosomes are also heavily implicated in the transfer of other miRs as well as protein and mRNAs in the context of conferring cardioprotection from a rIC stimulus.[288, 289]

1.2.5.1.2 Neural Signalling

The first evidence for the involvement of neural signalling in rIC was provided by Gho *et al.,* (1996). By administering intravenous hexamethonium (a ganglion blocker), they abolished the protection afforded by rIC of anterior mesenteric artery or renal artery, against sustained MI.[290] Strong evidence for involvement of neural signalling in rIC was subsequently given by Ding *et al.,* (2001) who showed that they could abolish the cardioprotective effect of renal ischaemia by cutting the renal nerve in rabbits.[291] Similar results were seen in hind limb rIC in rats where the femoral nerve supply was cut.[292] Some studies have advocated C-fibres as the responsible sensory neural mechanism for rIC as application of the C-fibre inhibitor capsaicin prior to rIC can reduce the effective of cardioprotection seen.[293, 294] However, Weinbrenner *et al.,* (2004) could not block rIC induced cardioprotection in rats using hexamethonium[295] and subsequent work by Wolfrum *et al.,* (2005) points to a significant interspecies variability when it comes to the role of the afferent nervous system in rIC.[296] More recent work by Basalay *et al.,* (2012) using a rat model of ischaemia, suggested that rIC in the pre, per and immediate post-MI period was heavily dependent on parasympathetic messaging whereas delayed rPostC (>10 minutes after the event) appeared not to rely so heavily on this neural signalling, suggesting a greater importance of humoral signalling in this setting.[297]

Modulation of the autonomic nervous system has been recently shown by number group to play a significant role in rIC. Mastitskaya *et al.,* (2012) have shown that activation of the dorsal motor nucleus of the vagus nerve (DMNV) induced a similar level of cardioprotection as rIC and that this effect was blocked by atropine.[298] Earlier work have also pointed towards the importance of stimulation of the vagus nerve for inducing protection by attenuating the effect of rIC by performing bilateral vagotomy in a rat model.[299, 300] This theory is challenged by Buchholz *et al.,* (2012) who actually demonstrated an increase in final infarct size by inducing vagal nerve stimulation prior to an ischaemic insult in a rabbit model.[301] Furthermore Jones *et al.,* (2009), using a murine model of MI, showed that cardioprotection induced by rIC was not attenuated by effective spinal cord transection at level C7.[302]

1.2.5.1.3 Humoral and Neural Interplay Signalling

The two isolationist views on whether the rIC signal relayed to the heart is predominantly a humoral or neural mediated one is being gradually replaced by a more holistic model, which integrates the two mechanisms. The first such theory of an integrated mechanism of action was put forward proposing an adenosine mediated neural pathway. Ding *et al.,* (2001) demonstrated that in tandem with the importance of neural signalling in a rabbit model of rIC, a release of a small pool of adenosine. On its own this adenosine release was insignificant enough to act as a humoral signal molecule in isolation, but rather was thought to act as a neural trigger.[291] Other implicated humoral triggers of neural pathways in rIC include bradykinin[303] and CGRP.[304]

More recent work has demonstrated the humoral signalling pathway to lie downstream of a neural pathway. This is highlighted elegantly by Jensen *et al.,* (2012) who analysed the plasma dialysate of diabetics with peripheral sensory neuropathy and showed that they were unable to generate an effective humoral signal compared to their non-diabetic counterparts.[305] Redington's group have applied a number of neural stimulators and blockers in experimental models to further reinforce this theory. These include the application of capsaicin and the use of direct nerve stimulation,[306] elector-acupuncture[307] and electrical nerve stimulation.[308]

1.2.5.1.4 Signalling via Modulation of the Systemic Inflammatory Response

A final hypothesised mechanism of rIC signalling involves the modulation of the inflammatory response that is known to be important in initiating and controlling wound healing. After an MI, neutrophils migrate to the myocardium within a few hours and are subsequently followed by monophages, which become the dominant cellular infiltrate in the first 2 weeks after an MI (see *section 1.1.3.2* for more details). Cheung *et al.,* (2006) demonstrated that patients given an rIC stimulus prior to undergoing open-heart surgery had a reduced systemic inflammatory response and reduced levels of cardiac damage.[309] It is thought that rIC induces changes in gene transcription, specifically of polymorphonuclear leukocytes. Indeed Shimizu *et al.,* (2010) showed that repetitive rIC significantly altered the behaviour of neutrophils after MI with reduced levels of adhesion as well as a reduction in phagocytosis and a change in the prolife of cytokine release.[310] Li *et al.,* (2004) highlighted the importance of gene transcription factors by demonstrating a blunted cardioprotective response in mice deficient in NFκB subjected

to rIC.[311] NFκB plays a key role in modulating the immune response and is thought to be fundamental in the post-MI inflammatory process where it is predominantly pro-inflammatory.[312] The importance of NFκB was underlined by Wei *et al.,* (2001) in a rat model of repeated rIC and MI, where they demonstrated a significant reduction in phosphorylation of the NFκB subunit p65 and its inhibitory protein IκBα. In addition, this study showed a reduction in the infiltration of macrophages and neutrophils into the infarcted tissue in the rIC groups as well as a reduction in monocyte chemotactic protein 1 (MCP-1) in the border zone of infarcted tissue.[313] More recently Cai *et al.,* (2012) have shown up-regulation of expression of interleukin-10 (a potent anti-inflammatory cytokine) in a mouse model of rIC which lead to a reduction in MI size and improved cardiac contractility.[314] The role monocytes play in the remodelling process is complex and Nahrendorf *et al.,* (2010) suggests that there is a biphasic response in the monocyte population between the initial inflammatory surge of CD16-/14+ (Ly6Chi) cells and the subsequent reparative surge of CD16+/14- (Ly6Clo) cells.[315] There has been no attempt so far to show if rIC can significantly influence the populations of monocytes post-MI and whether such a change would positively influence the remodelling process.

1.2.5.2 Repeated Remote Conditioning

So far, most studies have focused on the effect of a one-off conditioning stimulus. However in many studies, one-off rIC at or around the time of MI has pointed towards the potential for this technique to reduce the incidence of chronic heart failure by attenuating the acute injury of reperfusion. However, the degree to which the difference in LVEF and other markers of heart failure are due to remodelling, as opposed to

attenuation of infarct size around the time of the acute event is difficult to ascertain. There is tentative evidence to suggest that a repeated conditioning stimulus may have long-term beneficially effects, especially in the context of cardiac remodelling post-MI, with the potential to target acute remodelling per se and not just act via its initial infarct size sparing benefit.

Animal studies by Reddington's group has hinted that the progression to heart failure can be strongly attenuated, in a "dose-dependent manner", by serial bouts of rIC soon after an ischaemic event. In a rat model of acute MI, Wei *et al.,* (2011) demonstrated the greatest improvement in LV chamber size, LV function and haemodynamic changes post-MI in the group that received repeated remote conditioning every day for 28 days compared to a control group and two groups receiving one-off applications of rIC either before or during ischemia.[313] This was brought about by positive modulation of key remodelling processes such as a reduction in oxidative stress, attenuation of the expression of genes associated with fibrosis and hypertrophy, and blunting of the inflammatory response with reduced levels of neutrophil and macrophage infiltration in the myocardium and reduced cytokine signalling. Interestingly, there was minimal effect in the rats who received conditioning every 3rd day instead of every day. The same group also showed that repeated rIC led to a reduction in oxidative stress as demonstrated by a reduction in NF-κB phosphorylation. Furthermore there was a reduction in genes associated with fibrosis (TGFβ1) and hypertrophy (ANP and βMHC) post-MI. They also showed an attenuated inflammatory response with repeated rIC, with reduced levels of neutrophil and macrophage

infiltration in the myocardium and reduced cytokine signalling, all of which are relevant to remodelling.[313]

Previously, the same group had demonstrated that repetitive rIC significantly altered the behaviour of neutrophils after MI with reduced levels of adhesion at day 1 and 10 as well as a reduction in phagocytosis at day 10, apoptosis at day 1 and 10 and an overall change in the prolife of cytokine release.[310] More recent work from this group has suggested the existence of separate and very distinct mechanisms by which 'one-off' traditional rIC and repeated rIC infer protection. Whilst traditional rIC acts through the pathways described previously, repeated rIC was shown in this study to increased production of the autophagosome proteins LC3-II, cathepsin-L and Atg5.[316] Yamaguchi *et al.,* (2015) reinforced the power of repeated rIC post-MI and implicated exosomes as the mediators for signalling in rIC, possibly by their action of transferring anti-fibrotic miRs such as miR29a as well as IGF-1, which is known to be protective in the context of remodelling.[317]

1.2.6 Future Perspectives in the Clinical Translation of Remote Ischaemic Conditioning

Multiple studies are underway to assess the impact of one-off rIC protocols at the time of MI on various heart failure related outcome. Following on from the first CONDI study,[318] CONDI2 (Effect of RIC on Clinical Outcomes in STEMI Patients Undergoing PPCI) is well underway. This study aims to recruit 2300 participants over a 36 months period from a number of sites across Europe (http://www.clinicaltrials.gov/ct2/show/NCT01857414) with the primary outcome of

assessing cardiovascular mortality and hospitalisation for heart failure at one year. Completion of the study is expected in late 2016. Running in collaboration with the CONDI-2 trial is the ERIC-PPCI (Effect of Remote Ischaemic Conditioning on clinical outcomes in ST-segment elevation myocardial infarction patients undergoing Primary Percutaneous Coronary Intervention) trial. This trial has recently started recruitment and aims to recruit 2000 participants in total across multiple sites to assess whether rIC at the time of PPCI for STEMI can reduce the combined primary outcome of cardiac death and hospitalisation for heart failure at 12 months (https://clinicaltrials.gov/ct2/show/NCT02342522).

DANAMI-3 (DANish Study of Optimal Acute Treatment of Patients with ST-elevation Myocardial Infarction) aims to assess the effect of local ischaemic conditioning on heart failure rates up to 3 years following PPCI for STEMI (http://clinicaltrials.gov/show/NCT01435408). The study has completed recruitment of over 2000 participants and preliminary results pertaining to acute outcomes have previously been presented.[319] RECOND (Reduction in Infarct Size by Remote Per-IPostC in Patients With ST-elevation Myocardial Infarction), a Swedish led study, aims to recruit 120 participants and apply remote *per*-conditioning during PPCI for STEMI. One of the aims of the study is to compare cMRI assessed remodelling parameters after 180 days between the conditioned and sham groups (https://clinicaltrials.gov/ct2/show/NCT02021760). Finally the RIC-STEMI trial (Remote Ischemic Conditioning in ST-elevation Myocardial Infarction as Adjuvant to Primary Angioplasty) is a Portuguese led study aiming to recruit 492 participants. Similarly this study will recruit from patients suffering STEMI and undergoing PPCI with a 1:1

randomisation to rIC approximately 10 minutes prior to first angiographic balloon inflation or sham conditioning. Rather than cMRI based outcomes, the primary endpoint in this study will be death or hospitalisation from heart failure at a minimum of one year (https://clinicaltrials.gov/ct2/show/NCT02313961).

Two phase II trials are underway with the hypothesis that repeated rIC use in the post STEMI period can positively influence cardiac remodelling and reduce the incidence of and progression to heart failure: DREAM (Daily REmote Conditioning in Acute Myocardial Infarction) (http://clinicaltrials.gov/show/NCT01664611) and CRIC (Chronic Remote Ischemic Conditioning to Modify Post-MI Remodelling) (http://clinicaltrials.gov/show/NCT01817114). The DREAM study was conceived as a clinical trial to accompany the basic science set out in this thesis and was conceived at the University of Leicester. Chapter 7 of this thesis lays out the hypothesis of the trial and the trial design. To summarise briefly in this section, DREAM is a UK based, multi-centre randomised control trial recruiting individuals who have suffered a STEMI and have had successful PPCI. Inclusion criteria includes post STEMI LVEF <45% on transthoracic echocardiography with no prior history of MI. The study aims to recruit 72 patients and is powered to detect a 5% increase in LVEF above natural recovery. Primary outcome data are obtained from baseline and 4 month cMRI to assess LVEF, LVEDV, LVESV, infarct size and oedema. An important facet of this trial is the intention to try and elucidate further our understanding of how much rIC in this context acts independently on remodelling when influences on the initial infarct size and MVO attenuation are reduced. This is done by beginning rIC 3 days after the acute event to avoid influencing the size of the infarct. RIC will continue for 4 weeks, performed daily

by the participant. The study will randomise participants 50:50 in the intervention or the control group. The intervention group will receive a device that inflates to 200mmHg in 4x5 minute cycles of inflation and deflation. The control group will receive identical looking devices that cycle as the intervention group but only inflate to a maximum of 10mmHg.

In a similar vein, The CRIC study is a multi-centre randomised controlled trial recruiting from a STEMI/PPCI population in Canada with a recruitment aim of 82. CRIC differs from DREAM in that the investigators will recruit LAD territory infarcts only and will exclude diabetic individuals. The reasons for focusing on non-diabetic patients who have suffered large anterior STEMIs in the CRIC study is based on prior work suggesting this group are most likely to respond to rIC and hence gain greater impact from the intervention. Furthermore rIC will start just prior to PPCI and continue for 4 weeks, therefore rIC in this context will likely have an influence on infarct size and MVO as well as subsequent remodelling. Primary outcome will be obtained by comparing cMRI at baseline and 28 days, primarily to compare LVEDV. Both the DREAM and CRIC trials are nearing completion and it is hoped that once these trials are completed we will be in a better position to assess the role of repeated rIC in remodelling and whether this technique merits investigation with larger phase III randomised control trials.

1.3 Summary

RIC is a powerful protective tool against I/R. It's potential for targeting the detrimental effects of maladaptive acute remodelling after MI is currently being realised. Furthermore the degree to which the underlying mechanisms of rIC overlap with I/R

have yet to be established and it is postulated that a number of novel and unique mechanism of cardioprotection come into play in the context of remodelling. We aim to elucidate some of the mechanisms by which rIC affords cardioprotection in acute cardiac remodelling in a rodent cell culture models of hypertrophy and fibrosis. Furthermore we aim to translate the postulated benefits of repeated rIC in a phase II post STEMI/PPCI clinical trial and assess its effect on subjective and objective heart failure outcomes.

1.4 Hypotheses

1. ***<u>General hypothesis:</u>*** **RIC leads to the release of chemicals into the bloodstream that can prevent post-MI adverse remodelling by modulating hypertrophy and fibrosis.**

2. ***<u>Hypertrophy hypothesis:</u>*** **The pro-hypertrophic phenotype brought about by ET-1 in a cellular model can be inhibited by rIC-serum from humans and rIC-perfusate from isolated rat hearts.**

   Means to test hypothesis:

   - Develop a cellular model of hypertrophy using H9C2 rat myocardioblasts.
   - Establish whether perfusate from conditioned isolated rat hearts could inhibit hypertrophy in this model.
   - Establish whether serum from healthy human volunteers undergoing rIC could inhibit hypertrophy in this model.
   - Assess the effects of age and levels of physical activity on influencing the anti-hypertrophic properties of rIC in this hypertrophic model.
   - Elucidate some of the mechanisms of action of rIC to inhibit hypertrophy in this model.

3. ***<u>Fibrosis hypothesis:</u>*** **The pro-fibrotic phenotype brought about by ET-1 in a cellular model can be inhibited by rIC-serum from humans and rIC-perfusate from isolated rat hearts.**

   Means to test hypothesis:

   - Develop a cellular model of fibrosis using neonatal rat fibroblasts.
   - Establish whether serum from healthy human volunteers undergoing rIC could inhibit fibrosis in this model.
   - Elucidate some of the mechanisms of action of rIC to inhibit fibrosis in this model.

4. ***<u>Clinical Translation:</u>*** **Daily serial rIC applied for 4 weeks following STEMI treated with PPCI, reduces the incidence and progression of heart failure by positively influencing cardiac remodelling, independent of its effect on initial infarct sparing.**

   Means to test hypothesis:

   - Establish a phase II clinical trial to assess the role of repeated rIC in the context of post-MI acute remodelling and heart failure.

Results are detailed in chapters 3-7 and the discussion is outlined in chapter 8.

2. MATERIALS AND METHODS

This chapter will outline the materials and general methods used. Furthermore the techniques of cell culture, DNA, RNA, miR and protein measurement, and statistical analysis techniques will be discussed. The relevant results chapters will provide a more detailed methodology of particular experiments along with the corresponding results.

2. 1 Materials and Equipment

This section will cover the chemicals and solution used for the individual experimental techniques used.

2.1.1 General Reagents and Chemicals

Recombinant Endothelin-1 (ET-1), all-trans retinoic acid, Phorbol 12-myristate 13-acetate (PMA), 6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine (compound-C), 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 2-[4-(4-Chlorobenzoyl)phenoxy]-2-methylpropanoic acid isopropyl ester (fenofibrate) and protease and phosphatase inhibitor cocktails (Sigma-Aldrich, UK). L-N^G-Nitroarginine methyl ester (L-NAME) (Cayman Chemicals, US), (S)-Nitroso-N-acetylpenicillamine (SNAP) (Tocris, US). All other products and laboratory compounds were obtained from Sigma-Aldrich or Fisher Scientific unless otherwise specified.

2.1.2 Experimental Solutions

Normal Tyrode (in mM) contained 135 NaCl, 5 KCl, 0.33 NaH_2PO_4, 5 sodium pyruvate, 10 glucose, 1 $MgCl_2$, 2 $CaCl_2$, and 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic

acid), titrated to pH 7.4 with NaOH. Calcium free Tyrode contained no $CaCl_2$. Ventricular myocycte isolation enzymatic solution contained collagenase type I (1 mg/ml), protease type XIV (0.67 mg/ml), bovine serum albumin (1.67 mg/ml) in 30 ml of Ca^{2+}-free Tyrode solution.

2.1.4 Immunofluorescent Staining

Fluorescein isothiocyanate (FITC)-labelled phalloidin bound to F-actin: excitation wavelength 495nm, emission wavelength 520nm. Hoechst 33342 nuclear stain: excitation wavelength 353-365nm, emission wavelength 460nm (Sigma-Aldrich, UK). Alpha smooth muscle actin (α-SMA)-Cy3™ antibody, excitation wavelength 552nm, emission wavelength 570nm (Abcore, US).

2.1.5 Immunofluorescent Cell Imaging and Capture

The Advanced Microscopy Group (AMG) EVOS f1 digital inverted fluorescence microscope (AMG, US) was used for image capture. The following excitation and emission filter cubes and mirrors were used: DAPI: 357 nm excitation 447 nm emission, GFP: 470 nm excitation 525 nm emission, RFP: 531 nm excitation 593 nm emission, Texas Red: 585 nm excitation 624 nm emission, Cy5: 628 nm excitation 692 nm emission. Images were taken at a magnification of x10 and analysed using the Volocity software programme (PerkinElmer, version 6.0) for analysis and measurements. Downstream data was exported to Prism software (GraphPad, version 6.01, US).

2.1.6 Cell culture

The embryonic rat ventricular cardiomyoblasts, H9c2,[320] were obtained from the European Collection of Cell Cultures (ECACC) at passage 13. Neonatal rat cardiac fibroblasts (Cell Applications Inc, US) were obtained at passage 2. Dulbecco's Modified Eagle's Medium (DMEM), M199 media, foetal bovine serum (FBS), penicillin and streptomycin (Invitrogen, US). Laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane, trypan blue solution, 1-Phenyl-1,2,3,4-tetrahydro-4-hydroxypyrrolo[2.3-b]-7-methylquinolin-4-one (blebbistatin) and all-trans-Retinoic acid (Sigma-Aldrich, UK). Corning T75 and T25 (75 and 25ml respectively) culture flasks (Fisher Scientific) and 8-well glass slides (BD Falcon Biosciences, US).

2.1.7 RNA and MicroRNA

RNeasy and QIAshredder kits (Qiagen, Germany). Reverse transcription kits (Invitrogen, US). The rat Taqman probes ACTB (house-keeper reference probe), BNP, βMHC, α-act and MS-1 (Life Technologies, US). The miR extraction kit mirVANA (Invitrogen, US), TaqMan miRs Reverse Transcription Kit and miR Taqman probes 1, 133a and U6 (Life technologies, US).

2.1.8 Western Blotting

Enhanced chemiluminesence (ECL) prime and Polyvinylidene Fluoride (PVDF) membranes (GE Healthcare, UK). Anti-phospho Ser-1177 eNOS rabbit polyclonal antibody, anti-total eNOS rabbit polyclonal antibody, anti-phosho Thr-172 AMPKα rabbit polyclonal antibody and anti-total AMPKα rabbit polyclonal antibody (Cell Signalling

Technology, New England Biolabs, UK). Anti-PKC-ε rabbit polyclonical antibody (Santa Cruz Biotechnology, US). Anti-α-tubulin mouse monoclonal antibody and goat anti-rabbit IgG and goat anti-mouse IgG horseradish peroxidase conjugated antibody (Sigma-Aldrich, UK).

A non-commercial detergent free lysis buffer (in mM) was made in our lab and contained 10 β-glycerophosphate, 10 Tris-HCl, 2 EGTA, 2 EDTA, 1 sodium orthovanadate, 1 benzamidine, 1 dithiothreitol (DTT) and 1 phenylmethylsulfonyl fluoride (PMSF) with 10 µl of both protease and phosphatase inhibitor cocktails (Sigma-Aldrich, UK) added to each ml of the buffer. Triton-lysis, non-detergent buffer contained 1% Triton X-100. 3 x SDS-PAGE loading buffer (in mM) contained 187.5 Tris HCl pH 6.8, 6% SDS, 30% glycerol, 150 DTT and bromophenol blue. Running buffer (in mM) contained 25 Tris, 192 glycine and 0.1% SDS. Transfer buffer contained (in mM) 25 Tris, 192 glycine, 0.01% SDS and 10% methanol. Tris Buffered Saline Tween-20 (TBST) (in mM) contained 50 Tris, 150 NaCl and 0.1% Tween-20.

2.1.9 Proprietary Assays

Cyclic GMP ELISA kit (Cayman Chemical, US). MMP2 ELISA kit and MMP9 ELISA kit (R&D Systems, US). TIMP1 ELISA kit (AbCam, US). DAF FM diacetate probe (Thermo-Scientific, US). DC BioRad colorimetric Protein Assay kit (BioRad, US). Biorad Fluorescent DNA Quantitation Kit (BioRad, US).

2.1.10 Clinical Imaging Tools

Screening echocardiograms for the DREAM study were performed using either the Philips iE33 scanner or the Phillips CX50 portable scanner. CMRI was performed on a Phillips Ingenia 3.0 Tesla scanner with the use of Dotarem (Gadoteric Acid) solution at 0.5 mmol/mL as a contrast agent.

2.1.11 Remote Ischaemic Conditioning

Figure 2.1 illustrates the protocol for inducing rIC in humans involving 4 inflation/deflation cycles cycling between 200mmHg and complete deflation for 5 minutes at a time. Conditioning in humans was performed either manually by the inflation of the Accoson Greenlight 300 mercury-free sphygmomanometer[321] (A C Cossor & Son) or automatically using the autoRIC™ device (CellAegis Devices Inc.). See Figures 2.2 and 2.3 for an examples of each device.

Figure 2.1. Protocol used in healthy human volunteers to induce rIC. RIC can be induced manually or by the automated CellAegis autoRIC™ device.

Figure 2.2. The Accoson Greenlight 300. A mercury-free manual sphygmomanometer.

Figure 2.3. The CellAegis autoRIC™ device. From left to right is the cuff which fits around the participant's arm, the device which slots into the cuff and the charging dock in which the device is placed when not in use.

2.2 Methods

2.2.1 Cell Culture

All cell culture work was carried out in a grade II laminar flow hood under aseptic conditions to avoid contamination. In all experiments (unless otherwise stated), cells were cultured as a monolayer in T75 flasks in 20ml of high glucose DMEM supplemented with 10% FBS, 4mM glutamine, 100units/ml penicillin and 100µg/ml streptomysin at a temperature of 37°C in a 5% CO_2 incubator. Passage was performed by removing the incubation media, briefly washing the cells in warmed PBS then incubating the cells with 5ml of 0.025% Trypsin for 3-5 minutes to ensure complete detachment of cells from the flasks and then cells were subjected to centrifugation at 13,000 x g for 5 minutes at 20°C to separate the cells from the Trypsin. The pellet was reconstituted in 10ml DMEM and an estimation of cell number was obtained using a haemocytometer. The desired number of cells were then added to each flask with 20ml DMEM supplemented as described.

Once the desired number of flasks of cells were obtained for experiments, the cells were allowed to reach the desired confluency of approximately 70-80%. For immunofluorescence experiments, cells were seeded onto glass slides attached to 8-well plastic incubation chambers (BD Falcon) to allow for direct visualisation without the need to transfer cells after treatment. The technique of incubating cells in this way is described in more detail in chapter 3. Cells were then washed with warmed PBS and serum starved for 48 hours by incubating the cells in 20ml of high glucose DMEM as previous but with no FBS added.[322] Serum starving prior to treatment serves a number of purposes. It synchronises the cell cycles of all starved cells with cells entering the

quiescent G_0/G_1 phase as well as reducing components of the serum which may degrade enzymes and inhibitors used in experimentation.[322]

After 48 hours, the cells were then washed again with warmed PBS and were then ready for experimentation. For most experiments, cells were then treated for 30 minutes with rIC-serum ± selective inhibitors of certain enzymes, unconditioned serum, rat perfusate or warmed PBS vehicle control. This treatment was then removed and cells were either processed immediately for downstream experimentation or incubated for 48 hours in serum-free media supplemented with 100nM ET-1, after which cells were processed immediately for downstream experimentation. A visual representation of the general cell culture techniques used are shown in Figure 2.4. The specific downstream culture of adult cardiomyocytes and H9c2 cells is described in chapter 3. The specific downstream culture of fibroblast cells is described in chapter 6.

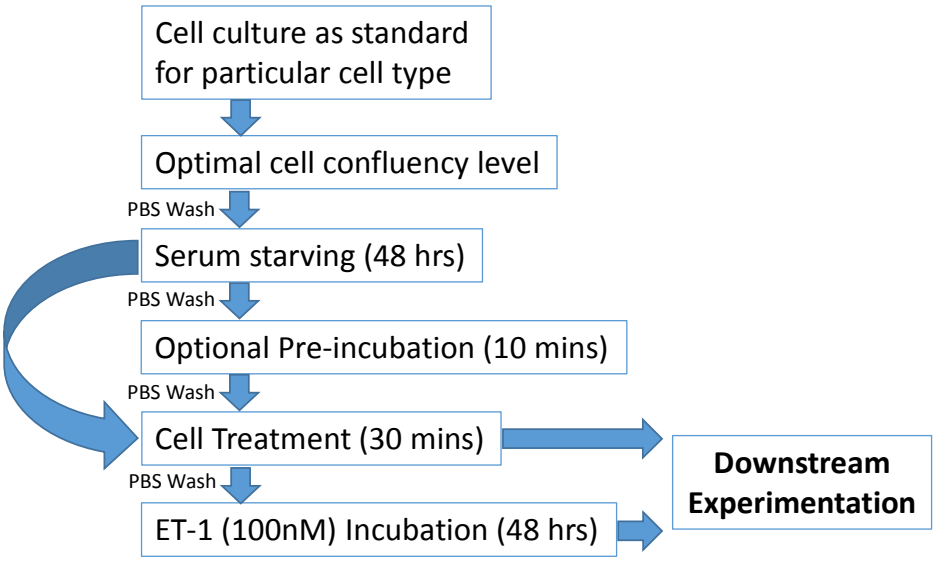


Figure 2.4 Schematic of the cell culture techniques used to prepare cells for experimentation. Certain inhibitors (such as Compound-C) require 10 minutes pre-incubation prior to incubation with serum to ensure adequate cell saturation.

2.2.2 Immunofluorescence

2.2.2.1 H9c2 Immunofluorescence

Immunofluorescence studies were performed to determine levels of hypertrophy in H9c2 cell subjected to hypertrophic stimuli. This was necessary as unstained H9c2 cells had ill-defined borders which made accurate surface area assessment challenging. Furthermore, staining facilitated determination of the phenotype of the cell (differentiation between blasts, myocytes and skeletal phenotypes) as demonstrated in Figure 2.5.

For immunofluorescence experiments, H9c2 cells were cultured on 8-well glass slides. 5000 cells were seeded per 0.7cm2 well and allowed to reach 70-80% confluency. Cells were then serum-starved for 48 hours and then subjected to treatment as described in *section 2.2.1* and outlined in Figure 2.4. The media was then removed from the wells and the cells were fixed for 20 minutes in 4% paraformaldehyde (PFA) in x1 strength PBS at room temperature. Fixation not only kills the cells but acts to terminate any ongoing biochemical reactions, allowing a preparation of tissue that is very close to the natural state at the time of fixation. The cells were washed 3 times in PBS and then permeabilised with 1% Triton X-100 (a detergent) in PBS for 5 minutes. Permeabilisation allows molecules, such as fluorescence labelled antibodies, to more easily pass through the cell wall membrane. The cells were washed twice in PBS and then 0.5% bovine serum albumin (BSA) in PBS was added for 30 minutes which acted as a blocking agent preventing nonspecific binding of the antibodies. FITC-Phalloidin (green) at a concentration of 5µg/ml in PBS was added to the cells and they were left to incubate at room temperature in the dark for one hour. The cells were then washed 3 times in PBS

and a Hoechst 33342 nuclear stain (blue) was added at a concentration of 1.0µg/ml in PBS and again the cells were left to incubate at room temperature in the dark for 15 minutes. The cells were washed a further 3 times in PBS and visualized using the fluorescence microscope.

For the analysis of cell morphology, 100 cells were counted from across 10 fields. Areas for analysis were chosen from those ranging from either a very low confluency (up to 30%), a moderate confluency (30-70%) to a maximal confluency of ≤70% in every experiment. The same number of cells were counted from fields of these three densities. Counting was performed after blinding the obtained images to cell treatment. Cells were initially analysed for the levels of differentiation to skeletal muscle cells. The skeletal phenotype was determined morphologically as evidenced by thin, stretched cells with central nuclei and myotube formation (the fusion of cells to form a multinucleated syncytia). Figure 2.5 shows H9c2 phenotype cells stained only with nuclear stain, cardiomyoblast H9c2 phenotype cells stained with both skeletal muscle and nuclear stain and skeletal muscle phenotype H9c2 cells stained with both skeletal muscle and nuclear stain. Skeletal cells were not included when assessing degree of hypertrophy induced. Each experiment was performed in triplicate and a mean was calculated from the pooled 300 cells from the three experiments. Cell areas were calculated by using the region of interest (ROI) tool in Volocity to draw a 2D outline of the cell as shown in Figure 2.6. The area obtained was doubled to take into account the area of the cell attached to the bottom of the flask. To avoid cell selection bias, all cells were counted in a field, including cells overlapping one another.

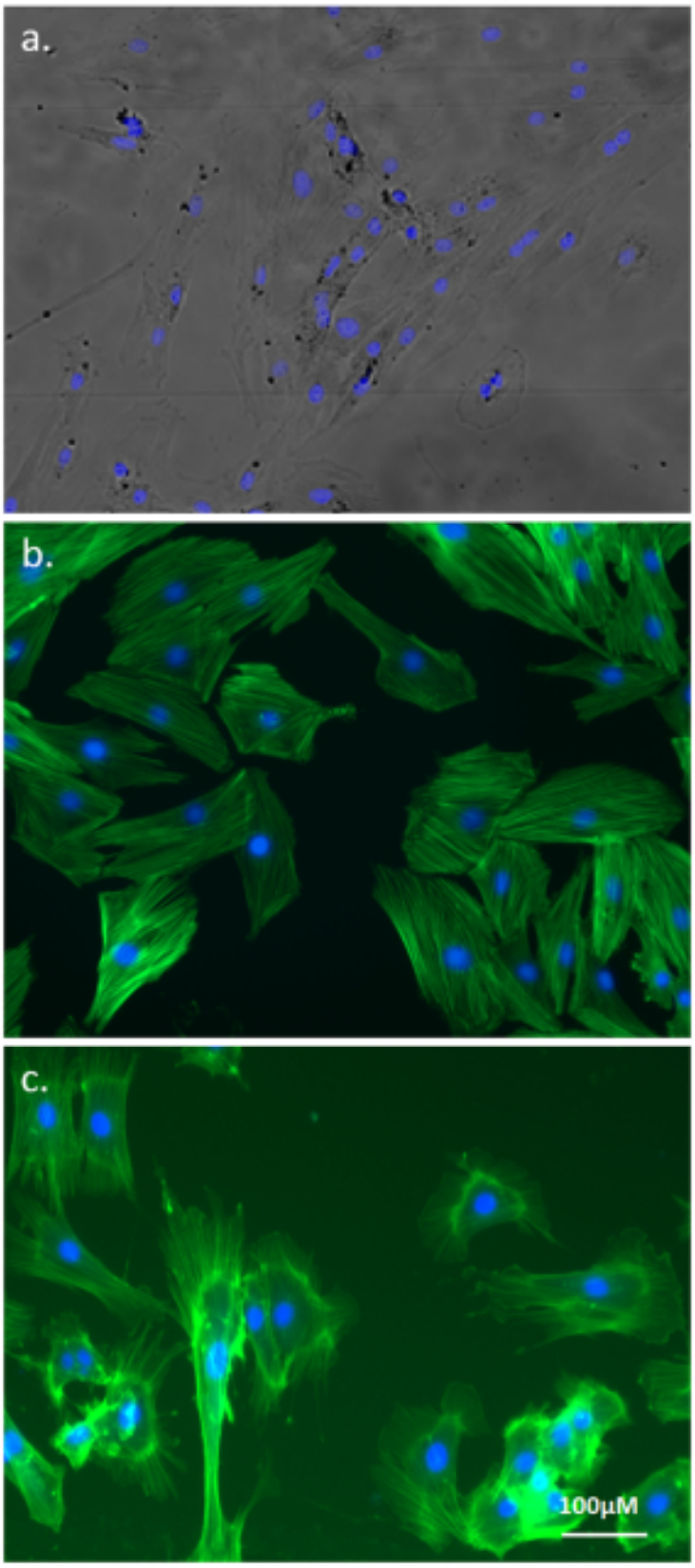


Figure 2.5. Immunofluorescence of H9c2 cells: a. nuclear stain (blue) b. F-actin stain (green) and nuclear stain (blue) displaying a myoblast phenotype c. F-actin stain (green) and nuclear stain (blue) displaying a skeletal phenotype. X10 magnification.

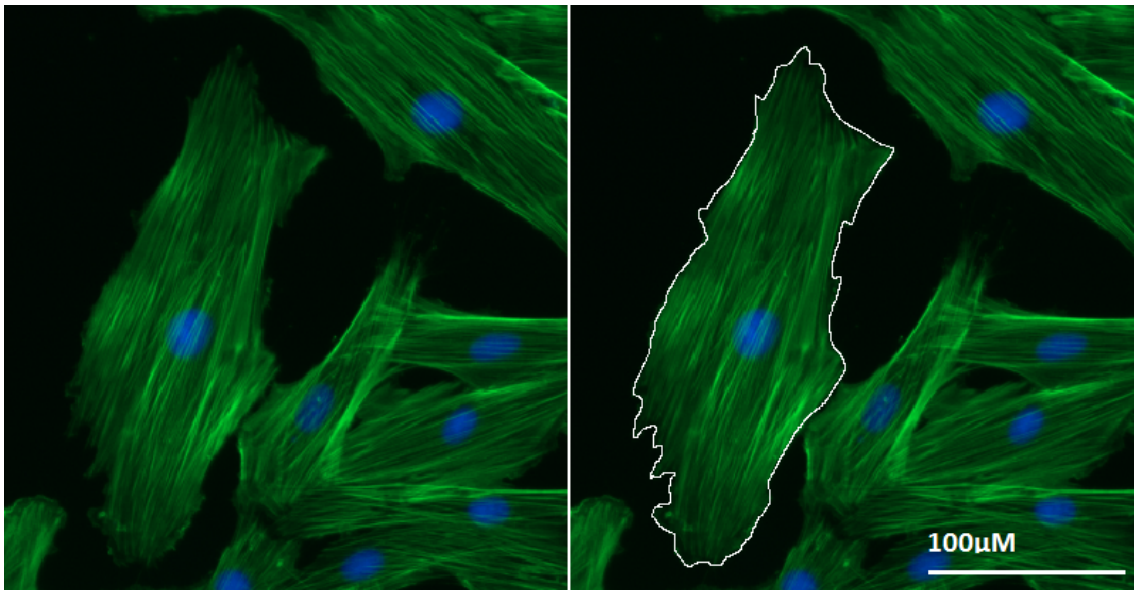


Figure 2.6. Measurement of cell surface area of H9c2 cells using Volocity software. The ROI tool allows tracing of the cell perimeter. X10 magnification.

2.2.2.2 Fibroblast Immunofluorescence

The purpose of immunofluorescence in the setting of fibroblasts experimentation was twofold: to allow better visualisation of the cells and assess proliferation and to quantify levels of fibroblast differentiation to myofibroblasts. Assessing proliferation was done by cell counting, which was performed manually, after blinding to cell treatment, using the Volocity software programme as described.

When assessing differentiation, surface marker identification of α-SMA antibody is a common technique employed as it is only expressed in myofibroblast populations.[323, 324] Fibroblast cells were prepared much the same ways as H9c2 cells in the initial stages (see *section 2.2.2.1*) however whilst incubating with the FITC-Phalloidin stain, the CY3- α-SMA antibody stain (red) at concentration of 5µg/ml in x1 PBS was also added. Images were analysed as per the H9c2 experiments using the same filter sets. As

seen in Figure 2.7 undifferentiated fibroblasts stained green with blue nuclei and myofibroblasts stained red with blue nuclei. It is known that fibroblasts are rich in F-actin bundles that helps generate wound contraction. With increased levels of α-SMA staining as fibroblasts transform into myofibroblasts, co-staining with F-acting becomes less apparent. Furthermore some authors has suggested that myofibroblasts have reduced levels of F-actin bundles as they are regarded as a transition cell between fibroblast to smooth muscle cells.[325-327] This remains controversial however. In a small population of cell, both red and green staining was seen in addition to blue nuclear staining. This was thought to represent a subpopulation of cells that were in transition from fibroblasts to myofiboblasts and hence which expressed both F-actin as well as α-SMA antibodies.[328]

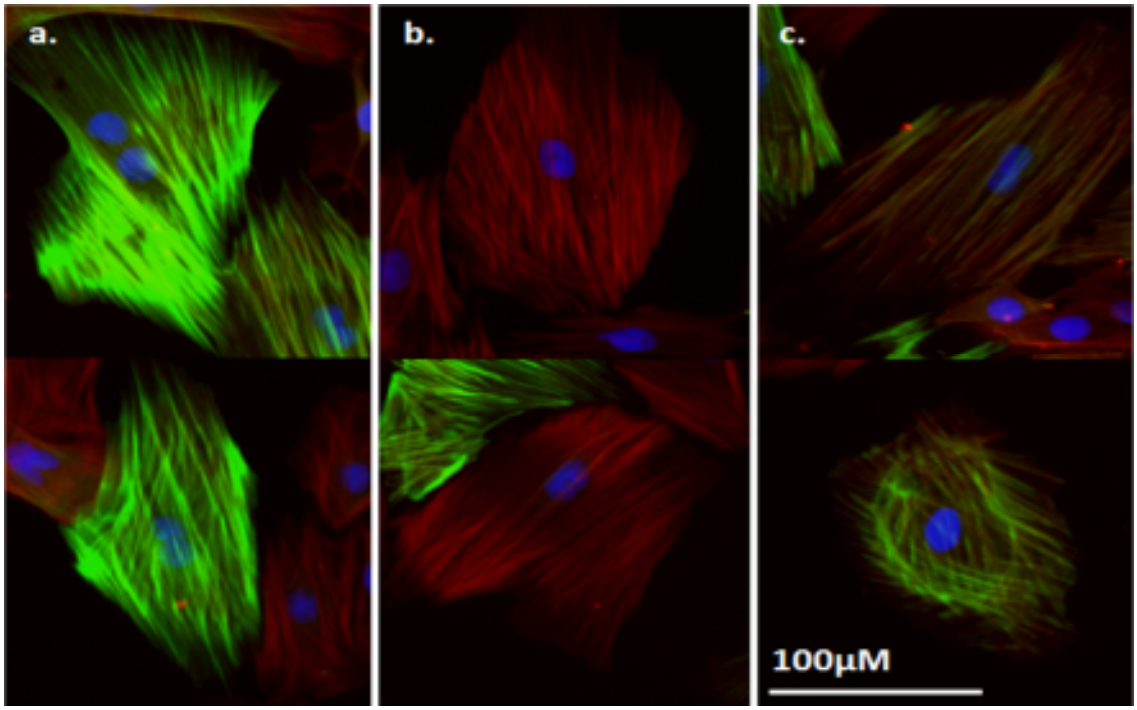


Figure 2.7. Immunofluorescence of neonatal rat fibroblasts: a. Fibroblasts staining with Phalloidin-FITC (green) and nuclear stain (blue) b. Myofibroblasts staining with CY3- α-SMA (red) and nuclear stain (blue) c. Fibroblast to myofibroblast transition cells with Phalloidin-FITC (green), CY3- α-SMA (red) and nuclear stain (blue). X10 magnification.

2.2.3 Real Time PCR of Messenger and Micro RNA

2.2.3.1 Sample Preparation

Cells were allowed to grow to ~70-80% confluence in the T75 flasks in cell culture before treatment by exposure to human serum or rat perfusate for 48 hours. After treatment, cells were trypsinized, the total number of cells was estimated using a haemocytometer to ensure a minimum of $0.5x10^6$ cells per flask to allow for adequate yields when performing RNA extraction. The media and cells were then transferred to 15ml centrifuge tubes and centrifuged for 5 minutes at 10,000 x *g* to obtain a pellet. The supernatant was then discarded and 5ml of ice cold PBS was added to the tubes, which were then centrifuged again. The PBS was then discarded using a pasteur pipette, being careful not to disrupt the pellet. The tubes were then inverted so as to keep the cell pellet separate from any residual PBS and placed in -80°C for later use or placed on dry ice if RNA extraction was to be performed immediately. If RNA was performed later, samples were frozen at -80°C for later use.

2.2.3.2 RNA Extraction

2.2.3.2.1 Messenger RNA Extraction

RNA extraction of H9c2 and neonatal rat fibroblasts cells in cell culture was performed using the Qiagen RNeasy Mini and Qiashredder kits (Qiagen, Germany). 350µl of buffer RLT (a guanidine isothiocycanate based solution that helps bind RNA to the silica membranes in the columns) pre-mixed with 10µl/ml 14.3M β-mercaptoethanol (β-ME) was added to the cell pellets obtained from culture as described in the previous sections. The pellets were disrupted by vigorous pipetting with cold lysis buffer to completely

dissolve the pellet. The solution was then pipetted into a Qiashredder homogeniser column placed on a 2ml collection tube and centrifuge for 2 minutes at 12,000 x *g* using an Eppendorf™ MiniSpin™ centrifuge. 350µl of 70% ethanol was then added to the homogenised lysate and mixed well. The 700µl solution was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 30 seconds at 7,000 x *g*. The follow-through was discarded and 700µl of buffer RW1 (a wash solution) was added to the RNeasy column and centrifuged for 30 seconds at 7,000 x *g*. The follow-through was discarded and 500µl of buffer RPE (a wash solution) pre-mixed with 4 volumes of pure ethanol was then added to the RNeasy column and centrifuged for 30 seconds at 7,000 x *g*. The follow-through was discarded and a further 500µl of buffer RPE was added to the column and centrifuged for 2 minutes at 7,000 x *g*. This longer centrifugation ensured that no ethanol was carried over during RNA elution which can interfere with some experiments downstream. A dry centrifugation of the column was carried out and subsequently the RNeasy column was then placed on a 1.5ml Eppendorf tube and 30µl of RNase-free water was added to the column and centrifuged for 1 minute at 7,000 x *g*. The RNA elute in the tube was then placed on ice and quantified immediately (see *section 2.2.3*). Figure 2.8 summarises the RNA extraction process.

Figure 2.8. Summary of the RNA extraction process using the Qiagen RNeasy column method. Adapted from the Qiagen RNeasy Mini Handbook.[329]

2.2.3.2.2 MicroRNA Extraction

MiR extraction of H9c2 cells from cell culture was performed using the mirVana extraction kit (Life technologies, US), a glass fibre based purification kit. 500µl Lysis/Binding solution was added to the pellets (containing up to up to 10^7 cells) obtained from culture (as previously described for RNA extraction) and disrupted by vigorous pipetting and vortexing. This Lysis/Binding solution acts to stabilise RNA and inactivates RNases. The solution was then pipetted into a Qiashredder homogeniser column placed on a 2ml collection tube and centrifuge for 2 minutes at 10,000 x *g*. 50µl of miR homogenate additive was added to the solution obtained from homogenisation and left on ice for 10 minutes. This homogenate additive was composed of the acid-phenol chloroform, which acts to remove most of the other cellular components and increase the purity of the sample. The mixture was then centrifuged for 5 minutes at 10,000 x *g* and the upper aqueous layer was removed (making sure to avoid contamination with chloroform) and pipetted into a separated Eppendorf tube, noting the volume. 165µl 100% ethanol was added to this top layer and vortexed. The mixture was placed on a filter cartridge on a collection tube and spun for 30 seconds at 7,000 x *g*. The follow-through was collected and 443µl of 100% ethanol was added and vortexed. The mixture was passed through a second filter cartridge and spun for 30 seconds at 7,000 x *g*. The filter was kept and the follow-through discarded. Following 2 wash steps, the filter cartridge was transferred onto a fresh tube and 30-100µl of the pre-heated elution solution was added and the mixture centrifuged for 30 seconds at 10,000 x *g*. The miR elute in the tube was then placed on ice and quantified immediately (see *section 2.2.3*). If after quantification the purity of the sample was deemed to be poor by

nanodrop analysis (see next section), a re-precipitation step was employed to improve the purity. 10% volume 3 M sodium acetate and 100% cold ethanol were added. The mixture was left at -80°C for 1 hour and then centrifuged for 20 minutes at 10,000 x *g* at 4°C. The supernatant was then re-suspend in 1 volume of water/elute solution and re-quantified. Figure 2.9 gives a summary of the miR extraction process.

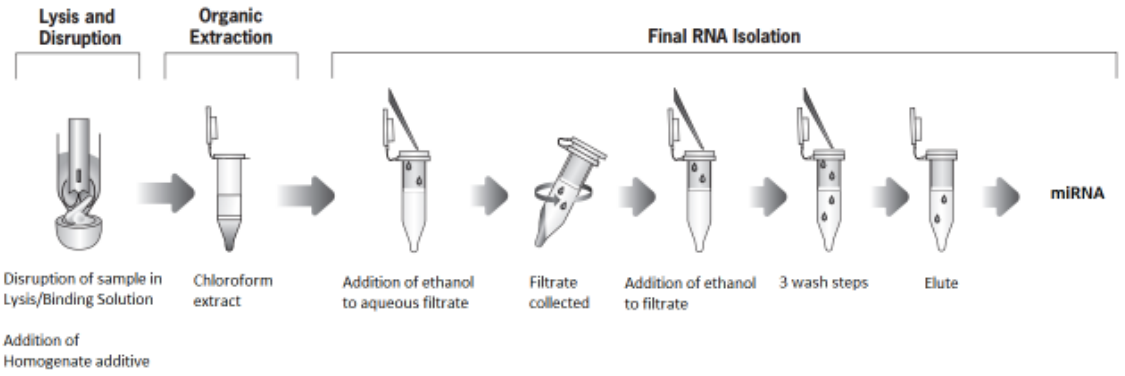


Figure 2.9. Summary of the miR extraction process using the mirVana extraction kit. Adapted from the mirVana miR Isolation Kit instruction protocol.[330]

2.2.3.3 RNA Quantification

Quantification of both mRNA and miR was performed using the Nanodrop 8000 v2 UV absorption spectrophotometer to obtain RNA yield (quantity of RNA), 260nm/280nm wavelength ratio (purity of RNA) and 260nm/230nm wavelength ratios (integrity of RNA). 1.5µl of RNA was added to the Nanodrop reader. Samples were discarded if the 260nm/280nm wavelength ratio was below 1.8 (2.1 indicates pure RNA) and likewise for the 260nm/230nm wavelength ratio. Samples with very poor yields i.e. below 50ng/µl

were also discarded. After quantification, RNA samples were frozen in -80°C or kept on ice if they were to be used immediately.

2.2.3.4 Reverse Transcription of RNA to cDNA

2.2.3.4.1 Messenger RNA Reverse Transcription

RNA was converted to cDNA using Taqman Reverse Transcription reagents (Invitrogen, US). RNA was diluted in 'MilliQ' deionised ultrapure water to allow for a maximum of 200ng RNA per 20µl reaction. The volume of water required to dilute 1ml of RNA elution was calculated by the following formula:

Volume water = (Concentration of sample x 8.6/200) – 1

The RNA/water mixture was placed on ice. The master mix was then made up in a 1.5ml Eppendorf tube on ice. The following quantities in the specified order of each reagent were added for one reaction:

- 4µl Buffer
- 2µl Magnesium Cloride
- 2µl Deoxynucleotide Triphosphates (dNTPs)
- 2µl (100µM) Dithiothreitol (DTT)
- 0.3µl Oligo dT16 primer
- 0.3µl Random Hexamers
- 0.5µl RNase Inhibitor
- 0.3µl MultiScribe RTase

The master mix was then mixed vigorously by pipetting and 11.4µl was transferred to a 0.5ml Eppendorf tubes. 8.6µl of the RNA/water mix was added to create a 20µl reaction. The 0.5µl Eppendorf was then placed into a Perkin Elmer Cetus DNA thermal cycler PCR machine and run using the following programme:

1. Hybridisation at 25°C for 10 minutes
2. Reverse Transcription at 42°C for 12 minutes
3. Enzyme Denaturation at 85°C for 5 minutes

The cDNA was then stored at -20°C until required.

2.2.3.4.2 MicroRNA Reverse Transcription

The TaqMan miRs Reverse Transcription kit was used to convert miR to cDNA. In contrast to the amplification of mRNA as described in *section 2.2.3.4.1*, miR reverse transcription utilises the stem–loop–specific reverse transcription Taqman pre-designed primers to amplify only the target miRs to these primers.[331] A total of 10 ng of miR was reverse transcribed for each miR primer per experiment, achieved by mixing with MilliQ water to a total of 5µl per tube. A master mix was made of the following constituents with volumes per reaction:

- 0.5µl 10x RT buffer
- 0.05µl dNTPs
- 0.03µl RNase inhibitor
- 100 nM dNTPs
- 1.42µl Nuclease free water
- 0.33µl Multiscribe reverse transcriptase

This master mix was added in each well along with 1 µL of miR-specific primer and 1.67 µL of miR. The RNA was denatured by heating to 65°C for 5 minutes then immediately chilled on ice to allow the primer to anneal to the RNA. Reverse transcription was carried out using the same following programme:

1. Hybridisation at 16°C for 30 minutes
2. Reverse Transcription at 42°C for 30 minutes
3. Enzyme Denaturation at 85°C for 5 minutes

The miR specific cDNA was then stored at -20°C until required.

2.2.3.5 Real-time Quantitative PCR

Real-time quantitative PCR was performed using the TaqMan® Gene Expression Assays (Invitrogen, US). Housekeeping genes were selected based on their stable expression profile in neonatal cardiomyoblasts and cardiomyocytes as described in the literature.[332-334] 1µl cDNA obtained from reverse transcription was diluted in 6.6µl of MilliQ water. Multiple reactions were performed in a master mix solution. Per reaction each well of a 96 well plate contained a total of 20µl solution made up of:

- 10.4µl TaqMan® Universal PCR Master Mix
- 1µl of the FAM™ labelled Taqman fluorescent probe:
    - For cDNA derived from H9c2 cells mRNA:
        - Brain Natriuretic Peptide - BNP (RefSeq NM_031545.1)
        - Myocyte Stress 1 - MS-1 (RefSeq NM_175844.2)
        - Alpha skeletal muscle actin - α-ACT (RefSeq NM_009606.2)
        - Beta Myosin Heavy Chain - βMHC (RefSeq NM_001017468.2)

- For cDNA derived from H9c2 cells miR:
  - miR-1 (miRBase ID rno-miR-1-3p)
  - miR-133a (miRBase ID rno-miR-133a-3p)
- For cDNA derived from neonatal fibroblasts cells:
  - Alpha smooth muscle actin - Acta-2 (RefSeq NM_031004.2)

- 1µl of the VIC® labelled Taqman fluorescent probe acting as the control housekeeper gene:
  - ACTB (RefSeq NM_112406.1) for mRNA derived cDNA
  - U6 (NCBI Accession # NR_004394) for miR derived cDNA
- 7.6µl cDNA/water mix

The following thermal profile was performed on the samples using the Applied Biosciences 9700 sequence detection system:

1. Initial denaturation at 50°C for 2 minutes (degrades the uracil-containing products)
2. Second denaturation step at 95°C for 10 minutes
3. 45 cycles of denaturation at 95°C for 15 seconds
4. Annealing and elongation at 60°C for 1 minute.

All reactions were performed in triplicate. Fold-change of the gene of interest was calculated using the comparative C_T (threshold cycle) method using the formula:

$$2^{(-\Delta\Delta CT)}$$

$\Delta\Delta C_T$ = (C_T target - C_T control) of calibrator - (C_T target - C_T sample of interest) of sample

This formula is based on the assumption that both the gene of interest and the housekeeper gene have an identical efficiency with regards to the doubling of product with every cycle[335] therefore validation analysis were performed for each probe to ensure this assumption held true.

2.2.4 Western Blot Protein Analysis

2.2.4.1 Sample Preparation

For western blot protein analysis, cells were treated as per mRNA and miR experiments to generate cell pellets. Thereafter the frozen pellets when needed were re-suspended in the appropriate lysis buffer for the particular experiment.

For downstream analysis of AMPK or eNOS levels, a detergent based lysis buffer (Triton) was used containing added protease and phosphatase inhibitors. The frozen pellet was re-suspended in 100µl of cold Triton lysis buffer and then homogenised in 1.5ml Eppendorf tubes using homogenising beads and mechanical force. Briefly this involved the addition of approximately 10 x 0.1mm homogenizer beads (Cayman Medical) to each tube. The tubes when then vortexed at top speed using the vortex machine (Stuart Vortex SA8) for 1 minute at a time. The lysate was then left on ice for 30 minutes to allow for complete lysis. A final centrifugation stage at 13,000 x *g* for 10 minutes ensured complete removal of any remaining debris. The protein rich supernatant was retained.

In the case of downstream analysis of PKCε fractions, 100 µl of cold non-detergent bases lysis was used (see materials section) initially to re-suspend the pellet and then homogenised in 1.5ml Eppendorf tubes using homogenising beads and

mechanical force. The lysate was then left on ice for 30 minutes to allow for complete lysis. The cell lysate was the place in an ultracentrifuge and spun at 50,000 x *g* for 30 minutes at 4°C. This supernatant, which contained the cytosolic protein fraction, was retained. For a summary of this process see Figure 2.10. Protein concentrations were analysed using a protein assay that was compatible with detergent (DC BioRad Protein Assay) and sample were diluted in Triton lysis buffer to fall within a pre-determined bovine serum albumin (BSA) derived linear standard curve (~7.5µg/µl).

2.2.4.2 Gel Electrophoresis and Transfer

Proteins were separated on 7-15% SDS-PAGE gels (depending on the size of the protein). Gels were pre-made using glass spacer plates in casting stands (BioRad, US). 20µg of protein was loaded into each well and run at a constant voltage of 100 V for 15 minutes followed by 120 V for a further 70 minutes within an enclosed ice-bath to avoid over-heating. A semi-dry transfer technique to polyvinylidene fluoride (PVDF) membrane was performed at 100 V for 70 minutes.

Figure 2.10. Protocol for separating out the cytosolic fraction of H9c2 cells for downstream processing. The cytosolic protein fraction is used in western blot analysis of PKCε translocation (see *section 5.2.3*). Adapted from Turrell., (2009).[336]

2.2.4.3 Immunodetection

The PVDF membrane was rinsed in TBST washing buffer and blocked in 5% non-fat dried milk for 1 hour at room temperature with constant agitation on a stirring machine. The membrane was further rinsed in TBST and then primary antibodies diluted in either 5% non-fat dried milk or 5% BSA (according to manufacturer's protocols) for a minimum of 2 hours at room temperature with constant agitation. After further TBST rinsing steps, the PVDF membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies in 5% non-fat dried milk for 1 hour at room temperature with constant agitation. After final TBST rinsing, protein bands were visualised by incubation with Enhanced Chemiluminescence (ECL) reagents (GE Healthcare, US). A digital imaging system (ImageQuant LAS 4000) was used to detect the bands. To perform semi-quantitative analysis of the bands, the GeneGenus bio-imaging system software (Syngene, US) was used. Band densities where assessed with GeneSnap and GeneTools software (Syngene, US) to ascertain relative protein expressions for each sample and were normalised by division of the corresponding housekeeping protein (α-tubulin) density for the each sample.

2.2.5 MMP, TIMP and cGMP ELISAs

All enzyme-linked immunosorbent assay (ELISAs) were performed according to the manufacturers' protocols. Initial steps included the generation of a standard curve using standard solution provided. Sample plates were processed in a Novostar luminescence plate reader (BMG Labtech, Germany) and data interpreted against the standard curved to yield final the results.

2.2.6 Nitric Oxide Levels Detected by DAF-FM Diacetate

DAF-FM diacetate is non-fluorescent molecule until it reacts with NO, after which it begins to fluoresce.[337] It permeates cell membranes easily and is it is deacetylated by intracellular esterases to its active form DAF-FM.

H9c2 cells were prepared in culture on standard 96 well plates. After treatment, PBS containing 10μM of DAF-FM diacetate was added to each well and incubated for 20 minutes at room temperature. Wells were washed in PBS and then cell media was used to incubate the cells for an additional 15 minutes to allow complete de-esterification of the intracellular diacetates. The Novostar luminescence plate reader was then used, set to an excitation wavelength of 494nm and emission wavelength of 515nm. Measurements were taken every 1 minute for an hour.

2.3 Statistical Analysis

Statistical analysis was performed using Prism software (GraphPad, version 6.01, US). For all wet-lab work, statistical analyses were performed using either two-tailed, unpaired Student *t*-test for comparison of data with only 2 groups or one-way ANOVA analyses with either Tukey's or Dunnett's post-*hoc* analysis modification for data requiring multiple comparison.[338, 339] The Tukey's tests compares every mean with every other mean. The Dunnett's test compares every mean to a control mean. In the majority of cases, Tukey's test was used however Dunnett's was used in the experiments of control group design (specifically experiments laid out in *section 4.3.2*) as it is more powerful than Tukey's at discovering small but significant differences among combinations of groups.[340]

In this setting, data is presented as means ± standard error of the mean (S.E.M). Data is presented as n = repetition of experiment / samples from separate individuals or animal per experiment / total cell number used for each experiment when assessing hypertrophy, for example 3/8/500 = 8 experimental samples performed in triplicate with 500 cells analysed in each experiment. With regards to non-hypertrophy experiments, the meaning of the numbers is explained in the figure legend.

For analysis of demographic, screening and secondary outcome data from the DREAM study, two-tailed Fisher's exact testing was used to analyse categorical variables. Continuous variables were compared using either two-tailed, unpaired Student T-test for parametric data or the Wilcoxon rank-sum test for non-parametric data. In this setting continuous variable data is presented as means ± standard deviation (SD). *P*-values were deemed statistically significant at $p<0.05$. As displayed in figures, * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$.

3. Development of Cell Culture Based Models of Hypertrophy and Fibrosis

3.1 Chapter Introduction

As outlined in *section 1.2.5.1.1*, humoral signalling plays a key role in the transmission of rIC protection to the heart in I/R, and may also play a key role in modifying acute remodelling following I/R. This chapter describes the development of a murine cellular models of hypertrophy and fibrosis that are robust and reproducible. To these models we test the effects of perfusate derived from Langendorff explanted rat hearts that have undergone ischaemic conditioning protocols as well as serum from healthy male volunteers who have undergone rIC. The focus of these experiments is to test the functional effects of rIC perfusate and serum on hypertrophy and fibrosis and the results are laid out in this chapter.

3.2 Cell Type

3.2.1 Adult Rat Ventricular Myocytes (ARVMs)

The use of ARVMs in cell culture has grown in importance over the last few years, fuelled by the evolution of gene transfection studies, and the technique is now well established.[341] Indeed, ARVMs are deemed to reflect a more physiological state than neonatal myoblasts when replicating the heart in both structural and functional experiments. A number of studies have used this model to assess hypertrophy including analysis of cell size over time, the re-organisation of contractile elements within the cell and the up-regulation of foetal genes. However, significant challenges remain in isolating large numbers of healthy cardiomyocytes and maintaining them for long

periods of time in cell culture. This can ultimately lead to the sacrifice of a large number of animals. Studies have shown that ARVMs can be maintained for up to 2 weeks, however, there is approximately a 50-70% death rate after the first week.[342, 343] Furthermore cell morphology begins to change in the first few days of culture with rounding of the myocyte ends and the internalisation of intercalated discs.[344] There is also evidence from some studies that changes occur to the electrical and contractile properties of the cell.[345]

3.2.1.1 Collection of Adult Rat Ventricular Myocytes from Explanted Rat Hearts

Ventricular myocytes and rIC-perfusate was obtained from Langendorff explanted heart preparation as described by Turrell *et al.,* (2014).[39] All experiments in this context were performed in accordance with the University of Leicester Biomedical Services Ethical Review and Animal Welfare Committee guidelines and in compliance with the United Kingdom Home Office Animals Scientific Procedures Act of 1986.

The Langendorff perfusion system has been described extensively.[346, 347] After euthanizing the rat, the heart is quickly isolated via thoracotomy and perfused retrogradely by direct cannulation of the aorta which provides constant flow through the coronary arteries with the physiological isotonic Tyrode's solution. This is brought about due to the fact that the aortic valve remains closed due to the constant retrograde flow. After a period of stable perfusion with calcium free Tyrode's solution for a minimum of 6 minutes, the heart was perfused with calcium free Tyrode's solution containing collagenase type I (1mg/ml), protease type XIV (0.67mg/ml) and bovine serum albumin fraction V (1.67mg/ml). This was perfused through the heart for 6

minutes and led to interstitial digestion will sloughing of the cardiac material, including ventricular myocytes into the perfusate. The first 2 minutes of perfusate run-off was discarded and then the enzyme products were recycled back through the system. Following digestion of the heart, the enzyme was then washed out with 6 minutes of perfusion with normal Tyrode's solution. After this process the ventricle was dissected with scissors and the tissue pieces placed in normal Tyrode's solution (containing calcium) and placed in a shaking water bath at 37°C. ARVMs were then decanted and filtered through dissociation sieves (Sigma-Aldrich, UK) and washed in normal Tyrode's solution through 2 cycles of centrifugation at 20 x *g* for a minute at a time. In most fractions, at least 60% of cells were viable (i.e. rods). Figure 3.1 shows a typical fraction of ARVMs after isolation.

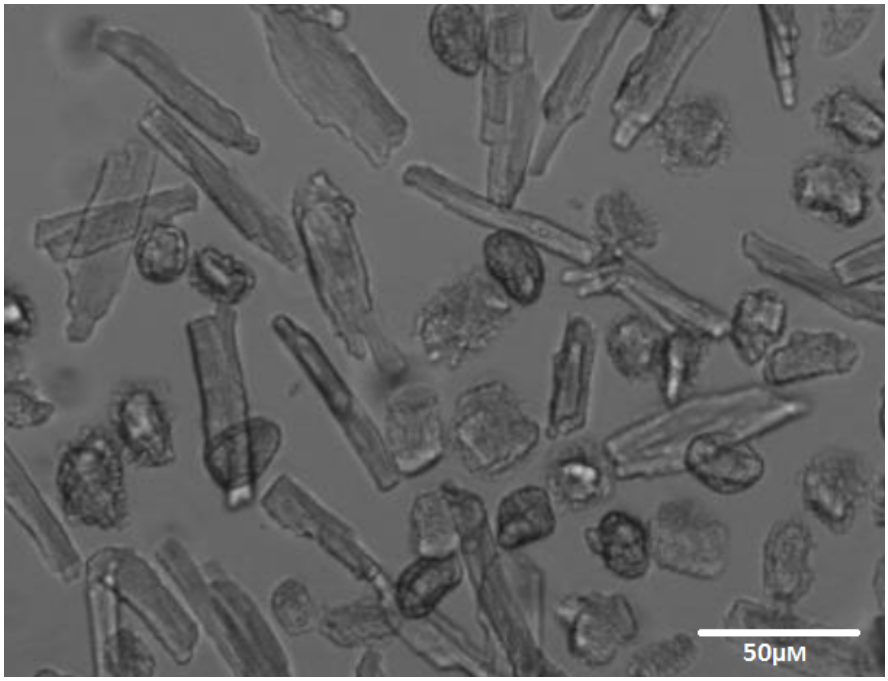


Figure 3.1. Assessment of ARVM viability after isolation. Microscope bright field view displaying both viable cells (rods) and non-viable cells (circular). x40 magnification.

3.2.1.2 ARVM Culture

ARVMs were re-suspended in M199 media with 100units/ml penicillin and 100µg/ml streptomyosin added. Cells were counted using a haemocytometer and a 0.5ml sample was tested with Trypan blue stain to assess viability. This stain is derived from the organic solvent toluene and it is unable to pass through intact cell membrane therefore only dead cells will be stained.[348] Adequate media was then added to the cells to make a suspension of 10,000 cells per ml.

8-well glass slides were treated with laminin to allow myocyte adhesion. Enough 10µg/ml laminin in phosphate buffered saline (PBS) solution was added to each slide so it was completely covered and left for an hour at room temperature. The laminin was then removed and ½ ml (approximately 5000 cells) of the media cell mixture was added to each 0.7cm^2 well and left to incubate at 37°C for 2 hours. The media was then removed gently taking with it dead, unattached cells, and replaced with either M199 media with antimicrobials only or M199 media with antimicrobials and 25µM of the myosin II selective inhibitor blebbistatin, which prevents the beating of individual myocytes.[349] For prolonged experiments, media was replaced every 48 hours to remove waste products and replenish the nutrients. Cells were seeded into multiple wells and divided into 8 groups. To determine the ability to maintain ARVMs in culture over 8 days, cell viability was determined every 24 hours of the culture period. For each day, a sample of the culture ARVMs were stained with 0.1% Trypan blue stain in PBS for 5 minutes. Cell morphology and staining was assessed at x10 magnification under a light microscope. Five different fields from three wells were used therefore a mean was obtained from 15 fields. Cell we classed as alive if they had the distinctive rod shape and did not stain blue

or dead if they appeared rounded and stained blue. Any cell with a rod like appearance which stained blue was classed as dead. The percentage of viable myocytes was calculated by using the formula:

% viable myocytes = [unstained rods / (unstained rods + stained rounded cells + stained rods)] x 100

Due to bacterial contamination of 3 groups of cell wells in culture, analysis was not possible on 3 out of 8 days and therefore there is no data for days 2, 5 and 7. Furthermore it proved challenging to maintain a healthy population of ARVMs past 6 days, not long enough to maintain cells in culture for rIC-serum experimentation. As per the reported literature, there was a significant death rate over time as shown in Figure 3.2. This was quite significantly attenuated by the addition of blebbistatin. Survival of ARVMs had dropped to 6.4 ± 3.7% by day 4 and was zero by day 6. In contrast, 58.4 ± 3.6% of myocytes treated with blebbistatin were still alive by day 8 ($p < 0.005$ versus control). The use of blebbistatin in this setting did pose the question as to whether it had any unknown effects hypertrophy in this model, potentially via its action on myofilament Ca^{2+} desensitization.[350, 351] Furthermore fluorescent staining of ARVMs proved unsuccessful despite various well established techniques and variations of the protocol used successfully in H9c2 cells (see *sections 3.2.2.1 and 3.2.2.2*).[352] The use of ARVMs was therefore abandoned in favour of H9c2 rat cardiomyoblasts.

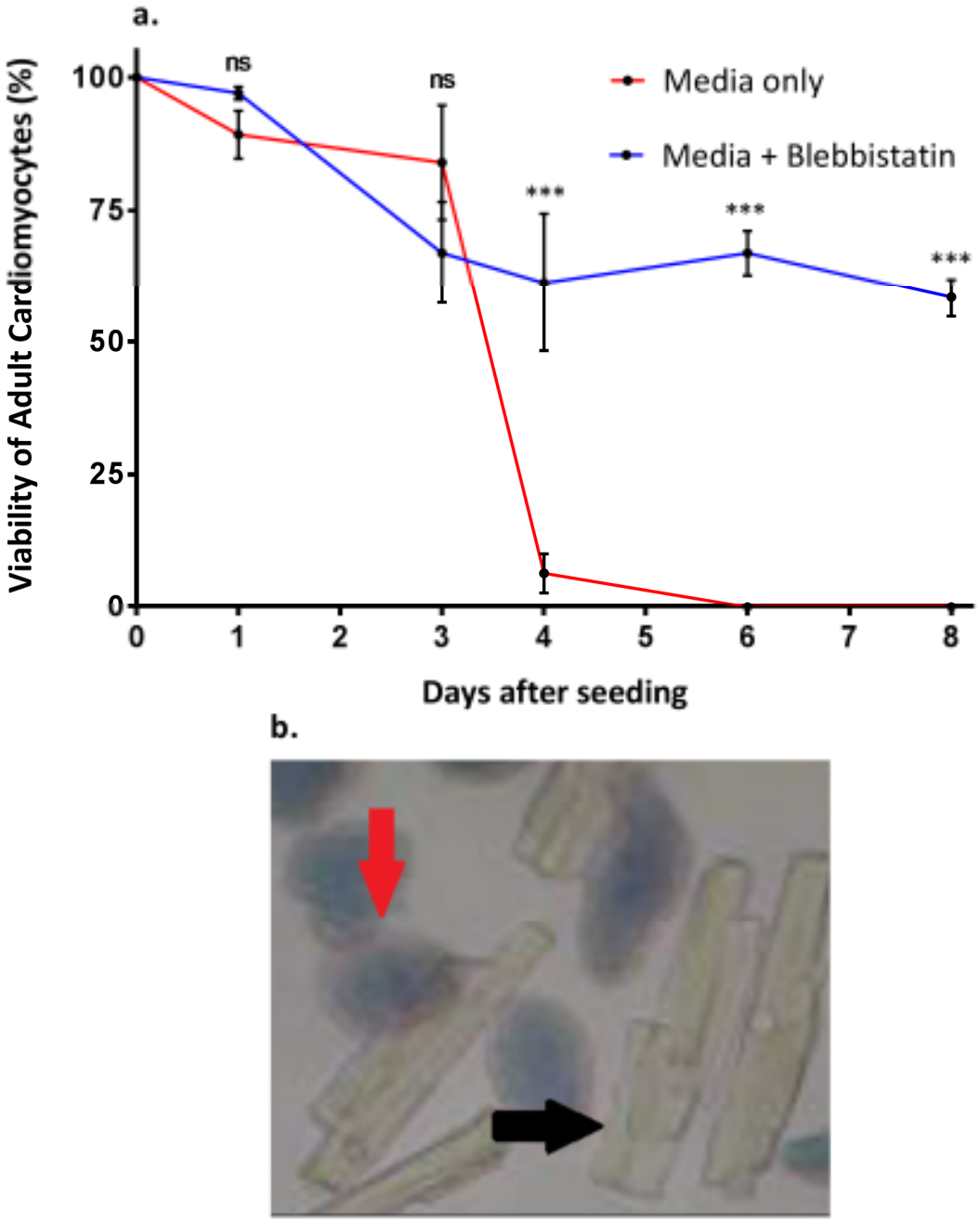


Figure 3.2. Viability of ARVMs over time *a.* Viability with and without the use of blebbistatin assessed by morphology and trypan blue exclusion. In the media only group, viability had dropped to almost 0 by day 4. The blebbistatin stained myocytes showed similar survival to the untreated group in the first 3 days but maintained their survival to above 50% up until day 8. Means with S.E.M, n=4/3/200 for each group, ns = non-significant, ***p<0.001. *b.* Representative image with black arrow showing a viable, unstained rod and the red arrow showing a stained and non-viable rounded myocyte. x40 magnification.

3.2.2 H9c2 Rat Cardiomyoblasts

The embryonic rat ventricular cardiomyoblasts H9c2 is a subclone of an original clone derived from BDIX rat heart tissue (a leukaemia rat model), and was first described in 1976 by Kimes and Brandt as a potential rat cell culture model for various cardiac disease states.[320, 353] They are an immortalised mononucleated myoblast derived directly from serial passages of ventricular tissue. It is one of only a handful of established commercially available cardiomyocyte cell clone lines along with the HL-1 cells derived from mouse atrial tumours.[354] H9c2 cell have been shown to be useful in models of cell death[355-362] as well as in the development of hypertrophic models.[363-372] They have also been used as a model for cardiac toxicity.[373-375] Although H9c2 are not full differentiated, they can be considered pre-designated with the appearance of a number cardiomyocyte specific markers. They do not actively beat and unlike ARVMs lack gap junctions, caveolae and T-tubule. In common with ARVMs however, they share similar GPCR expression profiles, a common morphology of most membrane components as well having similar electrophysiological properties including preservation of an LTCC current influenced by cGMP and cAMP pathways and increased expression of genes related to calcium transporters (SERCA, phospholamban, sarcolipin and ryanodine receptor) in the presence of all-trans-retinoic acid.[353, 376, 377]

H9c2 myoblasts have also been shown to respond to similar hypertrophic signalling pathways as ARVMs including ET-1, Ang-II and noradrenaline.[378] Initial criticism for the use of H9c2 in hypertrophied models has been levelled as the cells are capable of proliferation and differentiation from their myoblast stage into either cardiac or skeletal phenotypes and certain stresses can cause a change in phenotype. As

myoblasts they express both myosin light chain 2 atrial form (MLC2a) and myosin light chain 2 ventricular form (MLC2v) membrane markers, which are also seen in cardiomyocytes from neonatal rats grown in cell culture.[379, 380] When the cells reach confluence they form multinucleated tubular structures and adopt skeletal muscle features with the expression of nicotinic receptors, therefore care must be taken in cell culture to avoid confluency and hence skeletal cell muscle differentiation. A potential mechanism of ensuring cardiac rather than skeletal cell differentiation is by the addition of all-trans retinoic acid to a low serum media. This has been shown to induce a predominantly cardiac phenotype with expression of the alpha-1 subunit of LTCCs.[377, 381] The mechanism by which all-trans retinoic acid acts to encourage a cardiac phenotype over a skeletal phenotype is not clearly understood but is thought to involve activation of MAPK pathways including p38, ERK and JNK 1 and 2 as well as decreased expression of myogenin and MyoD genes, which play a role in skeletal cell differentiation.[377, 382, 383]

3.2.2.1 General H9c2 Rat Myoblast Culture

H9c2 cells were obtained from the European Collection of Cell Cultures (ECACC) at passage 13 and were maintained for experiments until passage 25. They were cultured as described in *section 2.1.* Cells were allowed to reach a maximum of 70-80% confluency to avoid differentiation to skeletal cells (indicated by the formation of myotube), usually after 2-3 days and then split 2-4 ways depending on number of flasks required. Cells were seeded at a density of ~$0.5x10^6$cells per T75 flask. During

maintenance in culture, but not during experimentation, H9c2 culture media was supplemented with 10nM all-trans retinoic acid to maintain a myocyte phenotype.

3.2.2.2 Determination of Optimal Serum Concentration for H9c2 Cell Culture

As previously alluded to, an important consideration for the cell culture of H9c2 cells is the concentration of FBS used during incubation and experimentation. Pagano *et al.,* (2004) has previously demonstrated that H9c2 cells are more likely to differentiate into skeletal cells at a lower serum concentration.[384] This is thought to be due to the reduced levels of cyclic adenosine monophosphate (cAMP) levels and reduced adenylate cyclase activity with the cells in such conditions. Therefore, experiments were performed with various foetal bovine serum (FBS) concentrations to determine a concentration that allowed for minimal background hypertrophy without a hypertrophic stimulus but maintained a cardiac rather than a skeletal cell phenotype. H9c2 cells were incubated in serum free, 0.1%, 1% and 10% serum in T75 flasks over a 5 day period in multiple flasks. After every 24 hours, the cells were lysed and analysed per flask to obtain a mean cell surface area (see *section 2.2.1.2* for how this is performed). The results are shown in Figure 3.3. As well as looking for signs of skeletal cell differentiation, signs of cell stress in the serum free and low serum media flasks were monitored by assessing the degree of cell blebbing, cell shrinkage and nuclear morphological changes.

There was a natural tendency towards an increase in cell size over time in all serum concentration groups. After a slight increase in size over the initial 24 hours in the serum free media incubated cells, there was a stabilisation in size. This stabilisation occurred after 48 hours in the 0.1 and 10% serum groups but no clear stabilisation was

seen in the 1% group. The most hypertrophy was seen in the 1% and 10% serum treated cells. The 1% serum treated cells increased by 2.3 ± 1.6 fold by day 5 ($p < 0.001$ versus serum free media control) and the 10% serum treated cells increased by 2.1 ± 1.4 fold ($p < 0.05$ versus serum free media control). The serum-free treated cells increased by 1.6 ± 1.0 fold by day 5. There was no significant difference between this increase and the increase seen in 0.1% serum treated cells by day 5 of 1.7 ± 1.0 fold ($p = 0.29$ versus serum free media control). It was decided that for experiments with H9c2, a serum free environment would be utilised to minimise the effect of serum on cellular hypertrophy. Such experiments however would be limited in time as prolonged serum free exposure would lead to skeletal cell differentiation, even in the presence of all-trans retinoic acid.

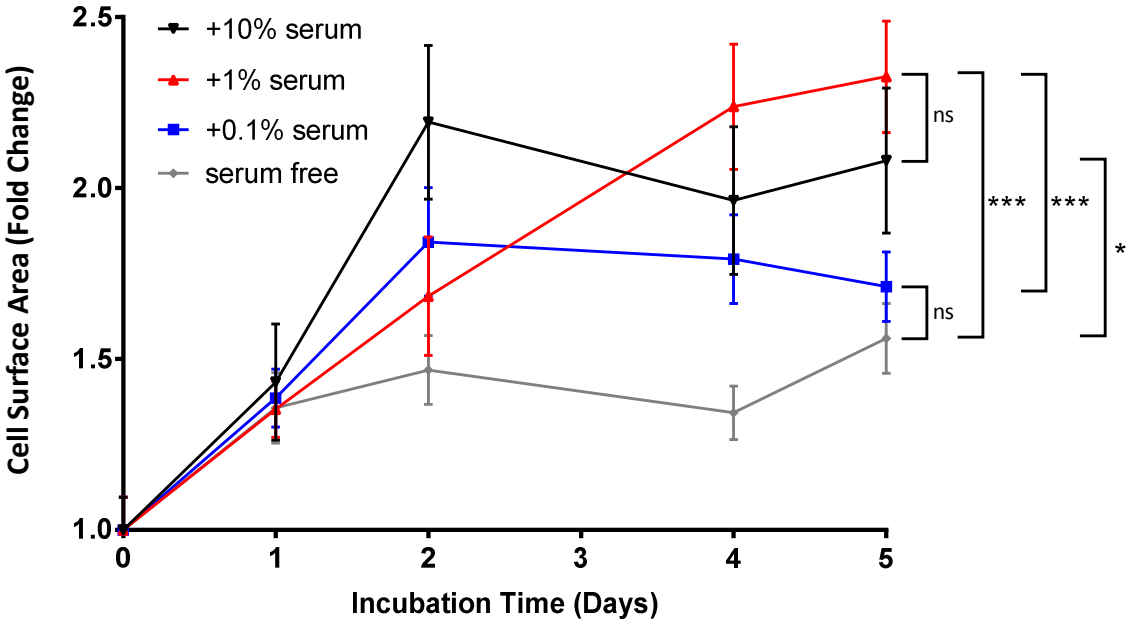


Figure 3.3. Line graph of the effects of various concentrations of FBS on H9c2 cell size. The greatest increase in cell size was seen in the higher serum concentration groups of 1 and 10%. In all groups the maximal growth was seen in the first 48 hours except in the 1% serum group which showed an almost linear increase in growth over the 5 day period. Means with S.E.M, n=4/3/200 for each group, *ns* = non-significant, *$p < 0.05$, ***$p < 0.001$.

3.2.3 Neonatal Rat Cardiac Fibroblast

Neonatal cardiac fibroblasts are well established in modelling fibrosis and exhibit only a slight delay in their conversion to myofibroblasts in response to fibrotic triggers, compared with their adult counterparts.[385] They were preferred to adult cardiac fibroblasts because the reported average passage number in literature before they displayed spontaneous phenotype change and senescence was longer in the neonatal cells.[386]

3.2.3.1 Neonatal Rat Cardiac Fibroblast Culture

Neonatal rat cardiac fibroblasts were obtained from Cell Applications Inc (US). The cells are derived from neonatal Sprague Dawley rat heart tissue harvested at 2 days old and were received at passage 2 and were maintained for experiments until passage 10. They were cultured in a monolayer in T75 flasks in 20ml of specific rat fibroblast growth medium 2 (PromoCell), which contained low levels of serum (2%). The serum was supplemented with 100units/ml penicillin and 100µg/ml streptomysin and the cells were kept at a temperature of 37°C in a 5% CO_2 incubator. Cells were allowed to reach confluency at which point they were either harvested for experiments or split 2-4 ways depending on number of flasks required. Cells were seeded at a density of ~$0.5x10^6$cells per T75 flask.

3.3 Selecting a Hypertrophic and Fibrotic Stimulating Agent

3.3.1 Endothelin-1

Endothelin-1 (ET-1) is a peptide made up of 21 amino acids expressed by vascular endothelial cells and cardiomyocytes. It is formed by cleavage of a precursor form "big endothelin" by endothelial cell derived endothelin converting enzyme (ECE). It is a key paracrine and autocrine factor that plays a central role in many pathophysiological process in the heart.[387] There is increased production of ET-1 in response to hypoxia and it plays a vital role in post-MI injury and recovery. Indeed some studies have shown improved survival rates with ET-1 treated cardiomyocytes susceptible to apoptotic stressors.[388] In vitro studies have also confirmed it's positively inotropic properties in heart failure.[389]

ET-1 acts on 2 receptor types: ET_A and ET_B. Both of these receptors are coupled to a GPCR and predominantly found on endothelial and VSMCs, however they are also present on cardiomyocytes where ET-1 act via these receptors to trigger PKC and ERK1/2 to induce hypertrophy.[390] A major pathway in ET-1 induced hypertrophy is calcium signalling via calcineurin, which leads to up-regulation of pro-hypertrophic genes such as NFAT as shown in Figure 3.4.[391] ET-1 also up-regulates gene expression that leads to the increase action of the hypertrophic agents Ang-II, phenylephrine and as well as further ET-1.[392] Indeed Ito *et al.,* (1993) showed that ARVMs in culture exposed to ET-1, released Ang-II in an autocrine and paracrine fashion, which augmented hypertrophy.[393] See *section 1.1.3.4* for more details of the downstream effects of ET-1 in modulating cardiomyocyte hypertrophy.

ET-1 also stimulates fibroblast proliferation, differentiation into myofibroblasts and increased deposition of fibrotic materials via a number of mechanisms including PKC activated pathways, increased levels of TGFβ and up-regulation of connective tissue growth factor (CNN2).[394-397] Figure 3.4 highlights the main pathways in ET-1 induced hypertrophy in cardiomyocytes and fibrosis via its action on fibroblasts as well as summarising its other key downstream effects in endothelial and VSMCs. For these reason ET-1 was tested as a hypertrophic and fibrosis stimulating agent to be used in the cell culture models (see *section 3.3.1.1*).

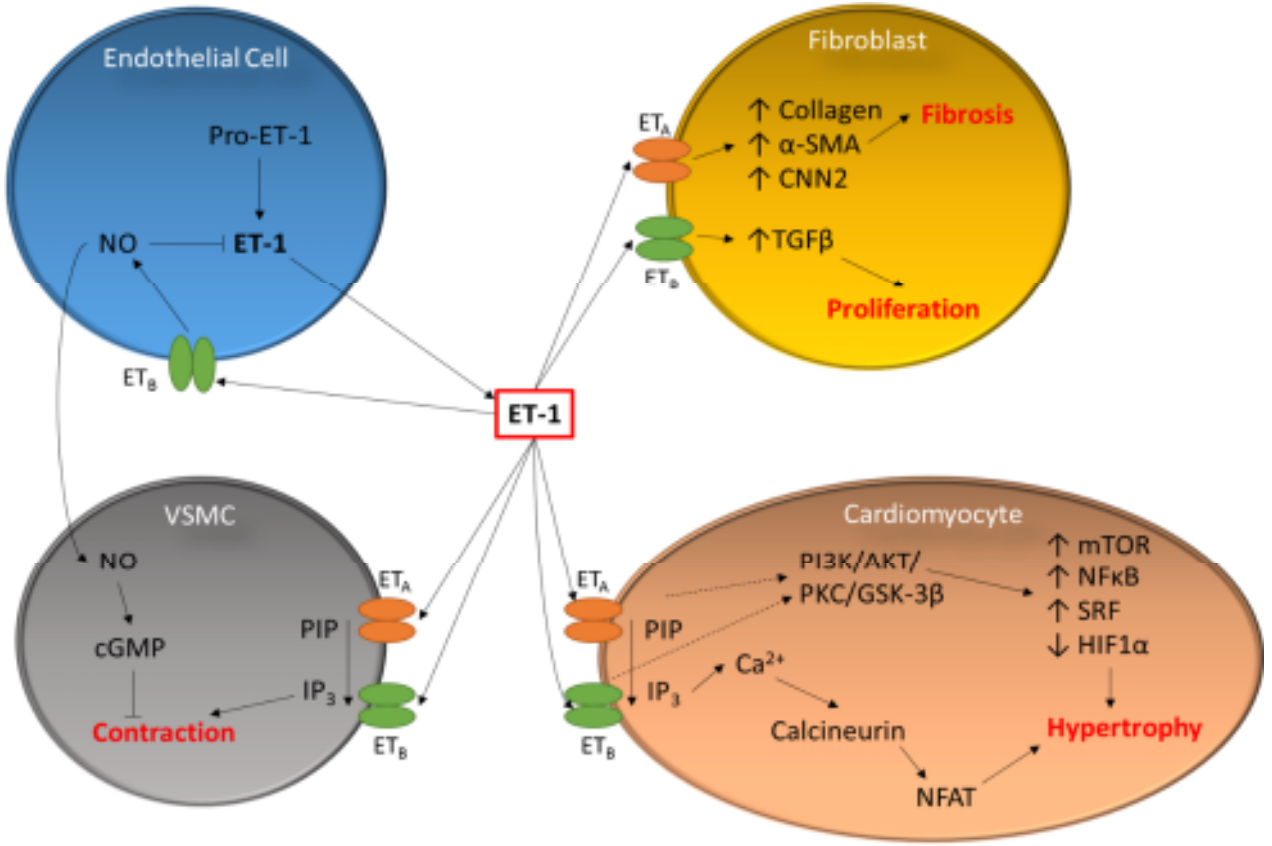


Figure 3.4. Effect of ET-1 on cardiomyocytes, fibroblasts, endothelial cells and VSMCs. ET-1 acts via ETA/B GPCR on a variety of cells. In cardiomyocytes ET-1 activates a number of downstream pathways which lead to hypertrophy. In fibroblasts ET-1 increases the production of collagen and triggers differentiation to a myofibroblast phenotype with proliferation. In VSMCs ET-1 leads to an increase in contraction. Negative feedback loops exist, mediated by NO in endothelial cells and VSMCs. In scenarios where the balance of ET-1 to NO is disrupted (e.g. endothelial dysfunction), the downstream effects of ET-1 are exaggerated.[398] For a full explanations of the abbreviations see the *Abbreviations* section.

3.3.1.1 Endothelin-1 Induced Hypertrophy Assessed by Immunofluorescence

As previously demonstrated in *section 3.2.2.2*, there is a natural increase in H9c2 cell size in serum free media over time with the greatest increase in the first 24 hours after passage and a plateau phase from approximately 48 hours onwards. For this reason experimentation was only carried out once this plateau stage had been reached. To assess the effects of ET-1 on H9c2 cells in culture over time, 5 different ET-1 concentrations were added to serum-free media and cell size was measure every day for 4 days. As shown in Figures 3.5 and 3.6, there was a modest increase in size over and above the natural increase seen with ET-1 at concentrations of between 0.1, 1 and 10nm; 2.3 ± 1.4 fold, 2.5 ± 2.2 fold and 2.7 ± 1.9 fold respectively. There was a much larger increase in size with a steeper rate of growth over the first 48 hours with ET-1 at 100nM and 250nM. The most significant increase in cell size over time was seen after 48 hours of ET-1 treatment, with an increase in relative cell surface increase compared to baseline of 3.5 ± 1.9 fold in the 100nM ET-1 group and 3.4 ± 2.2 fold in the 250nM ET-1 group (in both cases $p < 0.001$ compared to serum free media control at same time point). Relative fold increase in cell size plateaued after 2 day in the 100nM and 250nM groups. There remained a significant difference between these higher treatment groups and the other treatment groups at day 4, with an overall relative fold increase of 3.3 ± 1.8 fold in the 100nM ET-1 group and 3.3 ± 2.1 fold in the 250nM ET-1 group (in both cases $p < 0.001$ compared to serum free media control at same time point). From these experiments it was elected to use 100nM of ET-1 as the optimal concentration to induce hypertrophy in H9c2 cells and to incubate the cells for 48 hours, after which no significant incremental hypertrophy was achieved in this model.

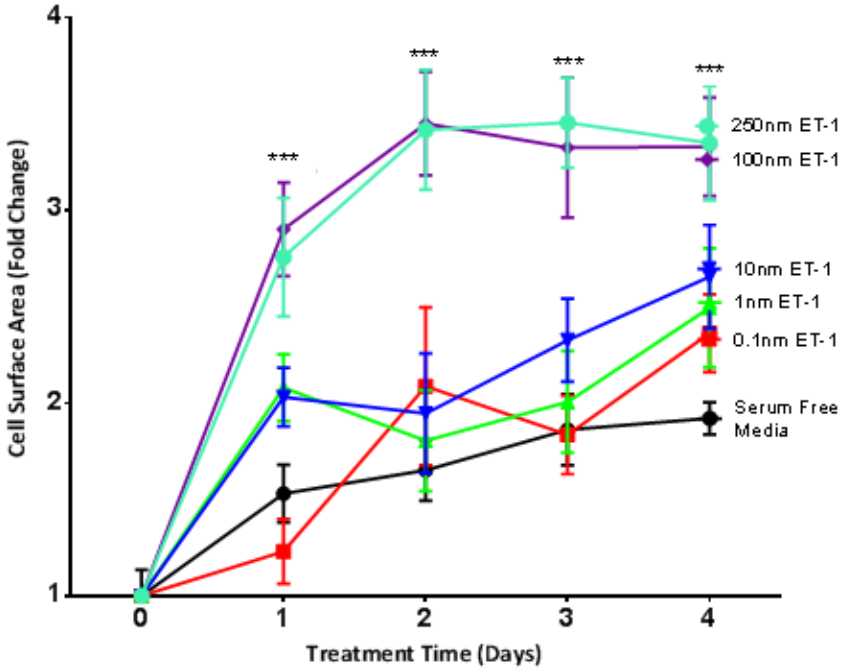


Figure 3.5. Line graph of the effects of various concentrations of ET-1 on H9c2 cell size in adherent culture over time. 100nM ET-1 (purple) and 250nM ET-1 (light blue) induced the greatest hypertrophy after 2 days. Significance above each day compares 100nM ET-1 to serum free media for that day. Means with S.E.M, n=4/3/200 for each group, *ns* = non-significant, ***p < 0.001**.**

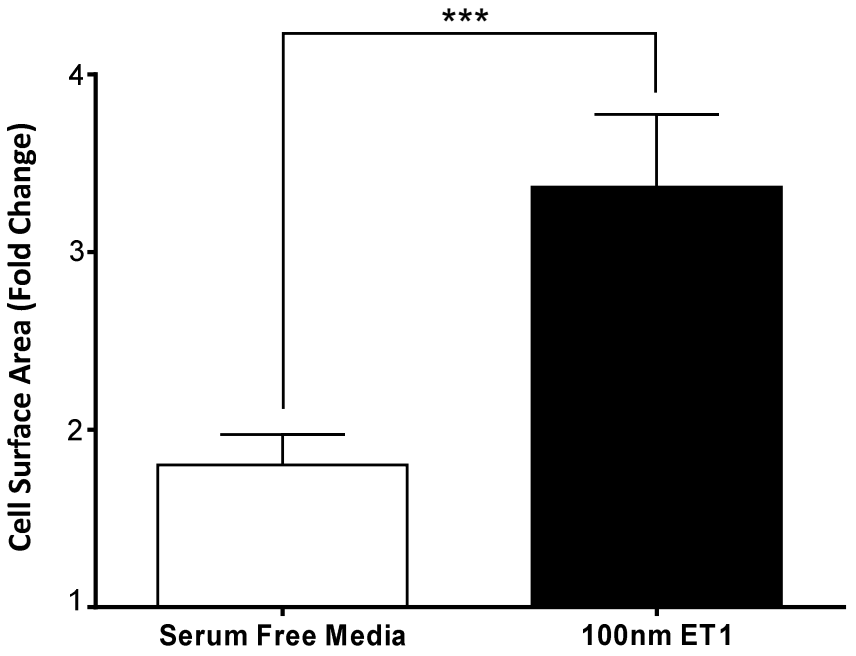


Figure 3.6. Bar chart of the effects of 100nM of ET-1 on H9c2 cell size in adherent culture after 48 hours. The greatest incremental change in cell size was seen in the 100nM ET-1 group after 48hrs compared to cell at the same time point kept in serum free media. Means with S.E.M, n=4/3/200 for each group, ***p<0.001.

3.3.2 Hypoxia

An attempt was made to induce hypertrophy in H9c2 cells using hypoxia. This was based on numerous studies in the literature that used this technique to induce hypertrophy in ARVMs[399, 400] as well as in H9c2 myoblasts.[401, 402] The earliest work utilising hypoxia induce hypertrophy in a cell culture model was by Webster *et al*., (1993), implicating sustained increased levels of the proto-oncogenes *c-fos* and *c-jun* in this model for hypertrophy.[403] Interestingly, Ito *et al.,* (1996) showed that one of the major mechanisms by which hypoxia induces cardiomyocyte hypertrophy, is through the release of the ET-1 precursor preproET-1 (ppET-1) with increased local mRNA levels and an increase of ET-1 production within cardiomyocytes. Furthermore, hypoxia induced hypertrophy was attenuated by blocking ET_A receptors.[399] More recent work by Chen *et al.,* (2007) has shown that in an *in vivo* murine model, intermittent hypoxia induced hypertrophy via a number of mechanisms and was dependent on the length of time of hypoxia. They found TNFα and IGF-II to be important upstream mediators in both short and long periods of hypoxia, with increased levels of p38, STAT-1 and STAT-3 in rats exposed to short periods of hypoxia and increased levels of IL-6, MAPK-5 and ERK5 increased in the longer exposure groups.[404] Hypoxia was therefore tested as an alternative model for hypertrophy in *in vitro* H9c2 cells (see *section 3.3.2.1*).

3.3.2.1 Hypoxia Induced Hypertrophy Assessed by Immunofluorescence

A protocol was developed using a sterile humidified hypoxic chamber (Galaxy 48R incubator; New Brunswick Eppendorf, Germany). H9c2 cells in 10% FBS were incubated in the hypoxic chamber using nitrogen to displace oxygen forming an inert environment.

95% nitrogen and 5% carbon dioxide insufflation was used reducing oxygen levels to 0.1% following protocols described for both ARVMs and H9c2 cells in the literature with incubation times of 8-10 hours.[405-407] Despite numerous modifications to the protocol, only modest levels of hypertrophy could be induced before a rapid cell death was seen after approximately 8 hours. The use of serum free media as an incubating media led to complete cell death and detachment from the flask within 30 minutes. Figure 3.7 demonstrates the modest changes in cell size over time seen for H9c2 cells in serum-rich media. At 8 hours there was a 1.4 ± 0.8 fold increase in the hypoxic group compared to a 1.3 ± 0.6 fold increase in the normoxic group (p = 0.59). This method of inducing hypertrophy was therefore abandoned in favour of the use of ET-1 which produce more marked results (see *section 3.4.1.1*).

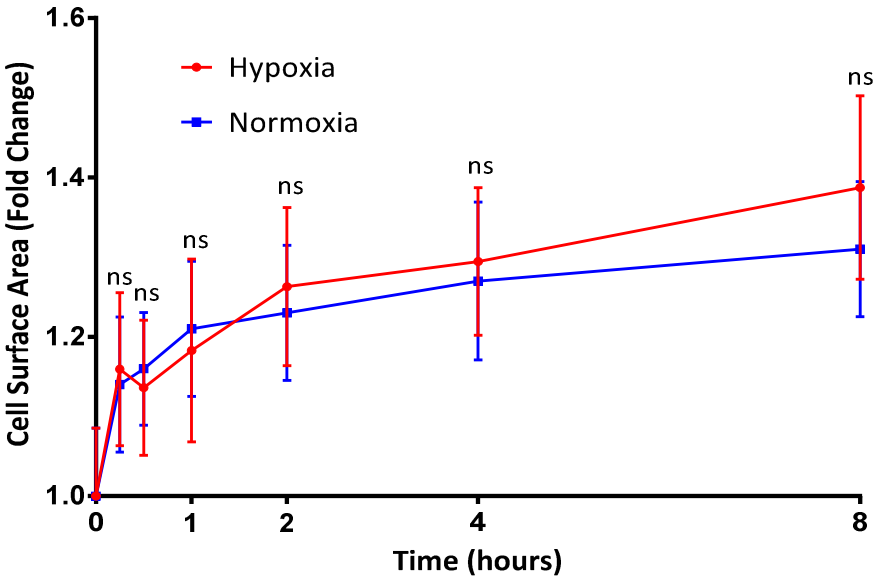


Figure 3.7. Line graph of hypoxia induced hypertrophy of H9c2 cells over time. Only modest hypertrophy is seen in the hypoxic group (red) compared to the normoxic group (blue). Data is not shown after 8hrs as most cells were not viable after this time-point in the hypoxia group. Means with S.E.M, n=3/3/200 for each group, *ns* = non-significant.

3.6 Endothelin-1 Induced Fibroblast Proliferation and Differentiation

As discussed in *section 3.4.1* and shown in Figure 3.4, ET-1 is known to act on cardiac fibroblasts via ET_A and ET_B to induce fibrosis, myofibroblast differentiation and proliferation. ET-1 was tested on neonatal rat cardiac fibroblasts to assess the degree of proliferation and myofibroblast differentiation induced and whether this could be used as a model to later test the effects of rIC-rat perfusate and rIC-human serum. ET-1 at 100nM was used as it was showed to induce maximally hypertrophy in H9c2 cells and reports from the literature showed it be effective at this dose in the context of inducing a pathological phenotype in fibroblast.[395, 408, 409]

3.6.1 Endothelin-1 Induced Fibroblast Proliferation

Care was taken to seed an equal number of cells in each well. A balance was struck between not over seeding wells to allow adequate proliferation analysis over time and under seeding wells as low cell density seeding is known to accelerate the differentiation to myofibroblasts.[410] Cells were grown to approximately 25% confluency in 10% FBS rich media in T25 flask at the start of the experiment. Serum was then withdrawn and replaced with serum free media for 48 hours. After 48 hours the media was replaced with serum free media with or without 100nM ET-1. Thereafter 3 flasks from each treatment group was selected after 2 days and after 4 days, the cells trypsinised and cell number was measured by use of a haemocytometer and the brightfield mode of the AMG EVOS f1 digital inverted fluorescence microscope. 10 x 10µl fields were counted per sample and the total cells per flask calculated. The cell number obtained at the start

of the experiment (i.e. the seeding number) was set at 0% and changes in cell number were told in terms of percentage change from this baseline control measurement. Results from these experiments are shown in Figure 3.8. After 2 days there was increase in proliferation of fibroblasts in the ET-1 group at 8.5 ± 2.1% compared to the untreated group at 3.1 ± 1.9% with a trend towards significance ($p = 0.573$). After 4 days there was a significance difference between the ET-1 treated group at 19.9 ± 3.3 and the control group at 7.6 ± 4.6 ($p < 0.05$).

3.6.2 Endothelin-1 Induced Fibroblast Differentiation

Fibroblast cells were grown in 8-well glass well chambers and stained as described in *section 2.2.2* for α-SMA (CY3) and F-actin (Phalloidin). Fibroblasts stained with Phalloidin attached F-actin (green) and myofibroblasts with α-SMA attached CY-3 (red). Cells from both the serum-free and ET-1 treated groups were stained after days 2 and 4 post-treatment. For each treatment on each day, 3 different wells were assessed and a total of 200 cells were analysed. Results from these experiments are shown in Figure 3.9. After 2 days there was a large transformation in the ET-1 treated cells with 78.2 ± 19.7% of the total cell population staining positive for α-SMA, indicative of myofibroblast differentiation. This is compared to 5.4 ± 2.3% in the untreated group after 2 days ($p < 0.001$). There was minimal further transformation in the ET-1 after day 4 at 81.3 ± 15.9% compared to 11.9 ± 4.9% in the untreated group ($p < 0.01$).

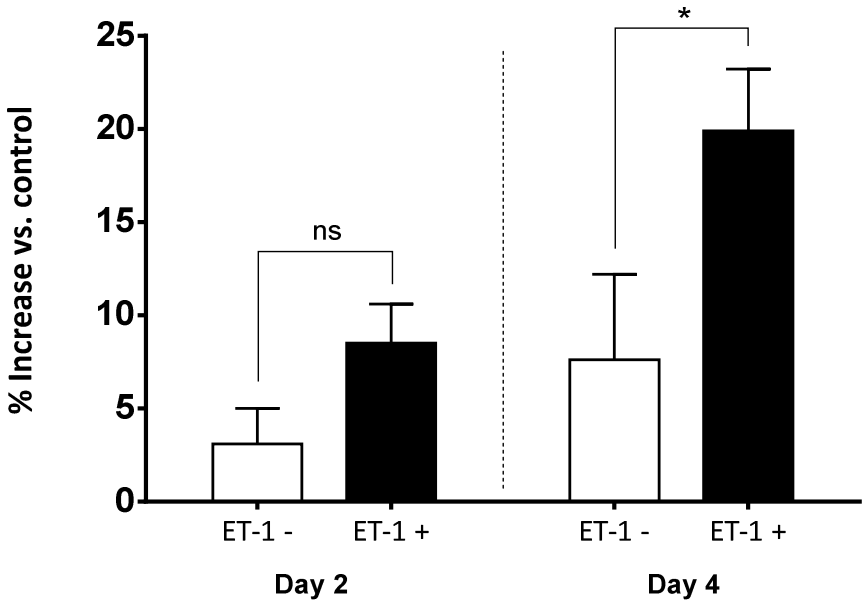


Figure 3.8. Bar chart of the effect of ET-1 on cardiac fibroblast proliferation. Exposure of cardiac fibroblasts to 100nM of ET-1 in serum free media induced a significant increase after 4 days (black) compared to time matched control (white). Means with S.E.M, n=3/200 for each group, *ns* = non-significant, *p < 0.05.

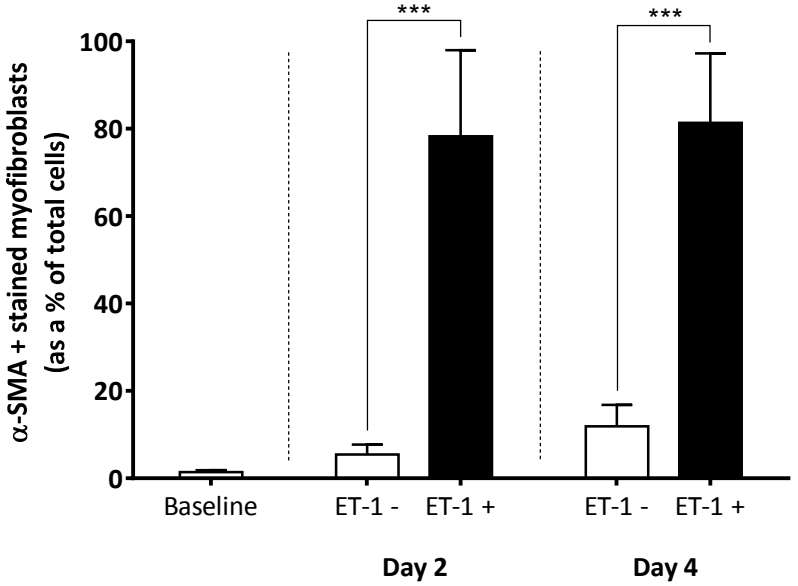


Figure 3.9. Bar chart of the effect of ET-1 on cardiac fibroblast differentiation. Exposure of cardiac fibroblasts to 100nM of ET-1 in serum free media induced a transformation of the majority of fibroblasts to myofibroblasts after day 2 which was maintained at day 4 (black). This is compared to a very minimal degree of differentiation in the untreated groups (white). Means with S.E.M, n=3/200 for each group, ***p < 0.001.

3.7 Summary

ET-1 induced hypertrophy of H9c2 cardiomyoblasts in culture proved a reliable and reproducible model as assessed by immunofluorescence. This cell line has been used by a number of other groups using a range of hypertrophic triggers including ET-1 as in this study,[378, 411] Ang-II[412, 413] and phenylephrine[414] and have also been shown to undergo the same hypertrophic growth and induction of foetal gene expression in response to ET-1 and Ang-II, as ARVMs.[378] The advantage of this model was its ability to introduce rIC-treated rat perfusate and rIC-human serum to assess their effects on hypertrophy by assessing not only overall cell size via immunofluorescence but also changes in the expression of foetal gene pro-hypertrophic gene panel, changes in the levels of anti-hypertrophic miR expression and changes in the levels of intra and extracellular proteins implicated in the hypertrophic pathway (see chapter 4-5).

In a similar manner, ET-1 proved to be a robust trigger in inducing neonatal rat cardiac fibroblasts to differentiate into myofibroblasts. This is one of the first key stages in the fibrotic process of remodelling. Again this model provided the ability to introduce rIC treated rat perfusate or human serum to assess their effects on not only differentiation via immunofluorescence but also markers of fibrosis by assessing changes in the expression of pro-fibrotic genes such as α-SMA and changes in the levels of pro and anti-fibrotic MMP/TIMPs (see chapter 6 for more details).

4. Functional Effects of Remote Ischaemic Conditioning on ET-1 Induced Hypertrophy in a Rat Cardiomyoblast Model

4.1 Chapter Introduction

Taking the H9c2 cardiomyoblast cell line model as described in chapter 3, this chapter deals with the functional effects of the introduction of both rIC perfusate from isolated explant adult rats hearts and serum from healthy male volunteers undergoing a standard rIC protocol into this model to assess whether the humoral molecular signalling generated by rIC can inhibit ET-1 induced hypertrophy. The expression of a panel of foetal genes namely BNP, α-ACT, βMHC and MS-1, which are surrogates for cardiomyocyte hypertrophy, were also assessed as a separate model of the effect of rIC-perfusate and rIC-serum on ET-1 induced hypertrophy.

4.2 Generation of Remote Ischaemic Conditioned Signals

As detailed in *section 1.2.5.1.1,* humoral signalling is a fundamental mechanism by which cardioprotection is inferred from a distant tissue bed or muscle to protect the heart from I/R injury. In this chapter we aim to test the anti-hypertrophic actions of rIC. To this end we collected perfusate from explanted Langendorff adult rat hearts which had undergone a traditional IPC protocol. In addition we collected blood from healthy male volunteers, undergoing a standard rIC protocol, to process into serum. The rat perfusate and human serum were then used in a series of experiments based on a cell culture model of hypertrophy to the test the hypothesis that rIC acts via humoral signalling to infer ant-hypertrophic effects on the heart by blocking the pro-hypertrophic ET-1

signalling pathway, one of the key pathways that induces hypertrophy in acute remodelling post-MI.

4.2.1 Induction and Collection of IPC Perfusate from Explanted Rat Hearts

Adult male Wistar rats were humanely sacrificed by cervical dislocation. The heart was then removed and isolated using the standard Langendorff preparation as described (see *section 2.2.1.1*). The heart was first perfused with pyruvate free Tyrode's solution[415] using a constant flow generated by a multi-roller peristaltic pump at a rate of approximately 10 ml/min per estimated gram of heart at temperature 37°C. After 15 minutes of stable perfusion, ischaemic conditioning was instigated by subjecting the heart to 5 minutes of global no-flow ischaemia by pausing the peristaltic pump. Flow was then allowed to resume for a further 5 minutes. This was performed a total of 3 times and 3ml of the perfusate was collected at the start of each perfusion cycle. The myocytes were screened to ensure successful IPC of the heart by subjecting them to metabolic inhibition and re-energization injury. Hearts were also discarded if they did not reach above 180 beats per minute. Perfusate from successfully preconditioned hearts was stored at -20°C or kept on ice if being used immediately.

4.2.2 Collection of rIC-serum from Healthy Human Volunteers

Ethical approval was granted by the Leicester NHS research Ethics Committee on 18th May 2012 for the taking of, use and storage of blood from volunteers within NHS property, in this instance the Leicester Cardiovascular Biomedical Research Unit. All

human samples were collected and stored in accordance with Good Clinical Practice and the Declaration of Helsinki 1964. Samples were taken from 19 healthy male volunteers between the ages of 22-70 after obtaining written informed consent (see appendix for ethical approval form and related documents). The blood provided by the volunteers provided serum which was used in a number of experiments. Serum rather than plasma was used as serum lacks anticoagulants which may affect protein interactions. Furthermore plasma may retain certain elements of blood such as erythrocytes and platelets which can remain metabolically active and may influence any downstream experiments. Finally studies have shown that plasma is less stable when stored for long periods, event at -80°C compared to serum, predominantly due to the retention of cellular debris.[416, 417]

Human blood was obtained from volunteers from 2 separate age cohorts: under the age of 40 and over the age of 50. Information was collected on the volunteers including age, ethnicity, medical history, blood pressure, smoking status, drug history and level of physical activity. Measure of physical activity was collated based on a simple but well validated questionnaire of levels of activity in healthy adults, the International Physical Activity Questionnaire Short Form (IPAQ-SF) (a template of which can be found in the appendix).[418, 419] Volunteers were grouped in low, moderate or high levels of activity based on their answers to the questionnaire. Table 4.1 shows the demographics for the volunteers, divided into the 2 age cohorts with the mean age and standard error of the mean (S.E.M) displayed below.

Blood was collected both prior to conditioning (unconditioned serum) and after conditioning (rIC-serum). RIC was induced by inflating a standard blood pressure cuff on

the non-dominant arm to 200mmHg for 4 cycles of 5 minutes at a time with 5 minute full deflations in between in cycle or via the use of the CellAegis autoRIC™ device which used an identical but automated protocol (see Figures 2.1-2.3). Some unconditioned blood was sent to the Glenfield hospital pathology lab for full blood count, urea and electrolytes, glucose and non-fasting lipid profile testing to ensure the volunteers had no undiagnosed comorbidities such as diabetes, pre-diabetes or hypercholesterolaemia. Blood for processing was collected in SSTII advance vacutainer blood tubes (BD Sciences, US), which contain clot activator and gel for serum separation. The tubes were gently inverted a minimum of eight times and then left undisturbed for 1 hour at room temperature before further processing to ensure clotting of the blood. The tubes were centrifuged at 500 x *g* for 20 minutes at 4°C. The serum was then transferred carefully to 1.0ml barcoded cryo tubes (Thermo Scientific, US), labelled and if not being used immediately, placed in -80°C for later use. Serum was used for experimentation within 3 months of storage. Figure 4.1 shows the process of collecting and processing blood samples to obtain unconditioned and rIC-serum.

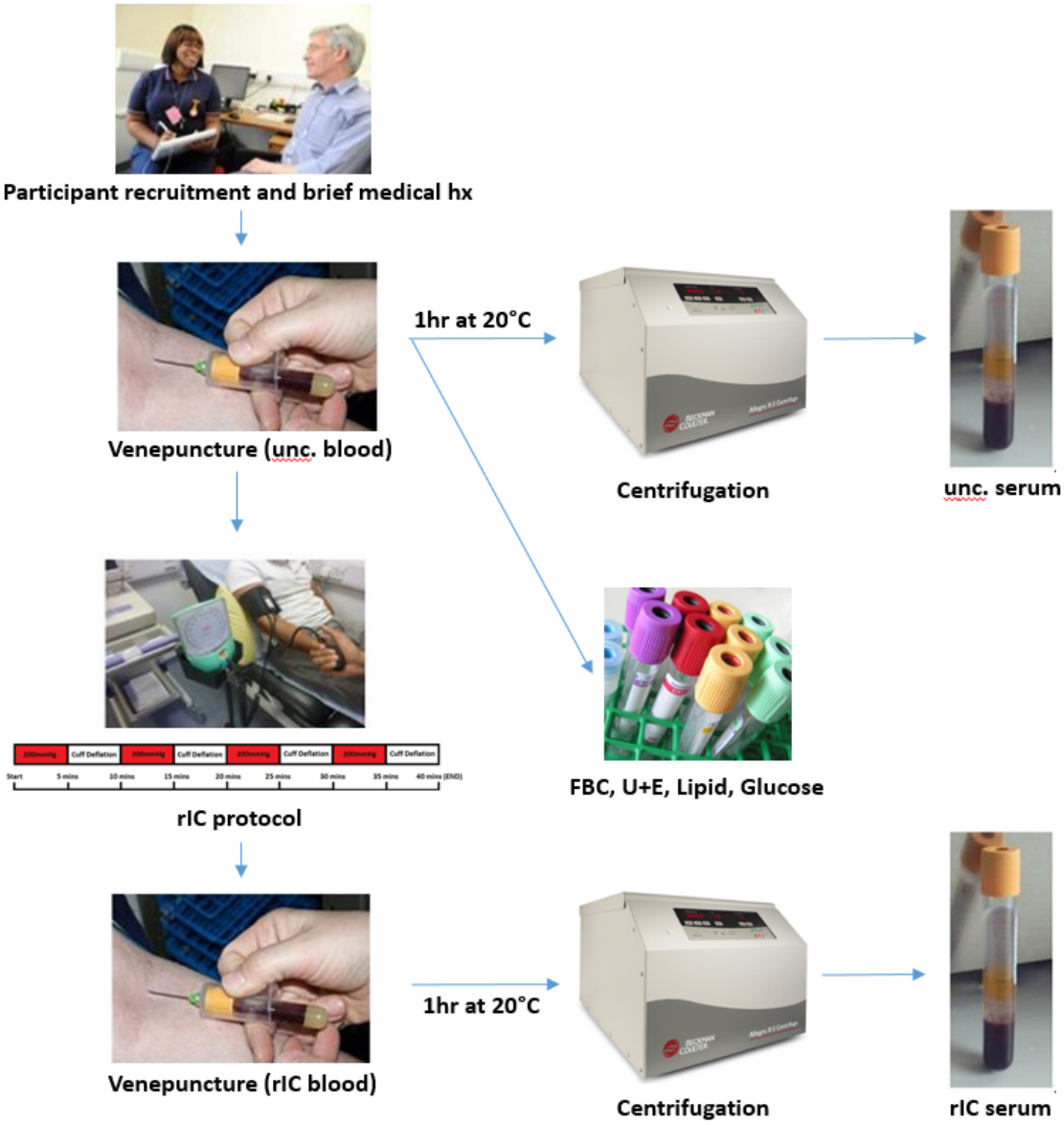


Figure 4.1. Flowchart detailing the collection and processing of unconditioned and conditioned blood from healthy male volunteers. *FBC* = full blood count, *U+E* = urea and electrolytes, *unc.* = unconditioned, *rIC* = remote ischaemic conditioned/ing.

	#	Age	Medical History	Blood screen	Blood Pressure	Smoker	Medications	Ethnicity	Activity level
<40 years of age	1	26	None	Normal	Normal	No	None	White European	Moderate
	2	22	None	Normal	Normal	Yes	None	White European	Moderate
	3	28	None	Normal	Normal	No	None	South Asian	High
	4	33	None	Normal	Normal	No	None	South Asian	High
	5	22	None	Normal	Normal	No	None	South Asian	Low
	6	36	None	Normal	Normal	No	None	White European	Moderate
	7	25	None	Normal	Normal	No	None	White European	Moderate
	8	28	None	Normal	Normal	No	None	White European	Moderate
	9	23	None	Normal	Normal	No	None	East Asian	High
	10	23	None	Normal	Normal	No	None	South Asian	Moderate
	11	23	None	Normal	Normal	No	None	White European	Moderate
	12	38	None	Normal	Normal	No	None	South Asian	Moderate
	13	28	None	Normal	Normal	No	None	South Asian	High
	14	24	None	Normal	Normal	No	None	White European	Moderate
	15	23	None	Normal	Normal	No	None	White European	High
	16	26	None	Normal	Normal	Yes	None	White European	Moderate
>50 years of age	17	55	None	Normal	Normal	No	None	White European	Low
	18	66	Asthma	Normal	Mildly ↑	No	Salbutamol inhaler	White European	Low
	19	56	None	Normal	Normal	No	None	East Asian	Moderate
	20	55	Asthma	Normal	Mildly ↑	No	None	White European	Low
	21	54	None	Normal	Normal	No	None	South Asian	Low
	22	52	None	Normal	Normal	No	None	South Asian	Low
	23	52	None	↑ cholesterol	Normal	No	None	White European	Low
	24	55	None	Normal	Normal	Yes	None	White European	Moderate
	25	70	None	Normal	Mildly ↑	No	None	South Asian	Low

Table 4.1. Demographic table detailing medical history, co-morbidities and activity levels of the healthy male volunteer group. Mean age + S.E.M of younger cohort = 26.8 ± 1.2. Mean age + S.E.M of older cohort = 57.2 ± 2.1. Combined mean age + S.E.M = 37.7 ± 3.6.

4.3 The Effects of RIC on ET-1 Induced Hypertrophy

4.3.1 The Optimum Treatment Time to Confer Anti-Hypertrophic Action

To determine the optimum treatment time required with rIC perfusate (collected from IPC conditioned explanted rat hearts) and human serum (collected after rIC induction on the upper arm) to infer maximal cardioprotection, the H9c2 model was used to assess the final cell surface area as measure by immunofluorescence. Cells were seeded onto pre-treated 8-well glass slides at a density of 10^4 per 0.7cm^2 well in 0.5ml of serum-rich media and allowed to attach for 24 hours. The serum-rich media was then removed and the cells were washed twice with serum-free media and then incubated for a further 48 hours in serum free media. The cells were washed again after this period had elapsed and then treated with rIC perfusate or human serum for 10, 20 or 30 minutes (in these initial experiments, all human serum used for experimentation came from healthy male volunteers, under the age of 40 with variable levels of physical activity). One groups of cells were incubated in PBS only in the same fashion as the treated cells, serving as the negative control. After treatment cells were washed once more and then incubated in serum free media with 100nM ET-1 for 48 hours. Cells were maintained in a 37°C, 5% CO_2 incubator throughout. Numerous studies have demonstrated that a number of cell lines including H9c2 cells can be maintained in serum free media for long periods of time if good cell culture practice is maintained.[420] After 48 hours the cells were washed and stained and measured as described in *section 2.2.1.1*. The results are shown in Figure 4.2.

PBS caused a small attenuation in final cell size but this was non-significant, 30 minutes PBS treatment final cell size = 2.4 ± 0.9x10^4 μm^2 versus ET-1 treated only = 2.5

± 1.3x10^4 µm^2 ($p = 0.07$). RIC-perfusate treatment for 10, 20 and 30 minutes significantly attenuated final cell size in response to ET-1 treatment with a non-significant difference between the 3 time points. 30 minutes rIC-perfusate treatment final cell size = 1.6 ± 0.6x10^4 µm^2 versus ET-1 treated only ($p < 0.001$). RIC-serum treatment for 10, 20 and 30 minutes also significantly attenuated final cell size. However there was a significant difference between the effect after 10 minutes and 30 minutes on final cell size, 1.7 ± 0.8x10^4 µm^2 and 1.3 ± 0.5x10^4 µm^2 respectively ($p < 0.05$). From these experiments it was clear that a maximal attenuation of final cell size was seen with 30 minutes of treatment with both rIC-perfusate and rIC-serum therefore all experiments from here on in, unless otherwise stated, were performed using 30 minute treatment time with rIC-perfusate or rIC-serum.

Subsequent experiments revealed that the human serum could be diluted 1:5 in PBS without a loss of its anti-hypertrophic properties. All subsequent experiment were therefore performed using human serum with a 1:5 dilution of serum in PBS to allow for a greater number of experiments to be performed.

4.3.2 Establishing Positive and Negative Controls

Three negative and two positive control were used to allow a better interpretation of the final results. The first negative control involved simply treating and incubating the cells in serum free media only. The second negative control involved treatment with serum free media the incubation with 100nM of ET-1. The third control involved pre-treating the cells for 30 minutes in warmed PBS and then incubating for 48 hours in ET-1. To directly inhibit the effects of ET1, the fourth control used fenofibrate as a

treatment. Fenofibrate is a PPARα inhibitor which has been shown to reduce ET-1-induced cardiomyocyte hypertrophy and protein synthesis culture.[421, 422] PPARα has been shown to act in a number of ways to inhibit hypertrophy and aid LV remodelling in both cell culture and animal models. It acts to inhibit GATA-4 binding to NFATc leading to decreased levels of its transactivation and reduced levels of hypertrophy and also acts by blocking JNK.[421, 423] PPARα is also known to inhibit NF-κB DNA-binding activity which leads to decreased levels of the pro-inflammatory cytokines IL-6, TNF-α and COX-2 implicated in hypertrophy.[424] In this setting fenofibrate was used as a positive control at a concentration of 10 μM. Finally unconditioned-serum derived from a matched blood sample taken from healthy human volunteers immediately prior to the induction of the rIC protocol, was used as a second positive control. Table 4.2 summarises the treatment and incubation protocols. Results shown in Figure 4.3.

Both rIC-perfusate and rIC-serum treatment brought about a significant attenuation of the increase in cell size after ET-1 treatment for 48 hours when compared to the ET-1 control group of $2.4 \pm 0.7 \times 10^4$ μm^2 reduced to $1.6 \pm 0.4 \times 10^4$ μm^2 ($n = 400$, $p < 0.001$) in response to conditioning with rIC-perfusate and $1.3 \pm 0.3 \times 10^4$ μm^2 ($n = 400$, $p < 0.001$) for rIC-serum. As expected, fenofibrate significantly attenuated the effect of ET-1 hypertrophy with a final cell size of $1.7 \pm 0.4 \times 10^4$ μm^2 ($n = 400$, $p < 0.001$). Compared to fenofibrate treatment, there was a significant different between the rIC-serum group ($n = 400$, $p < 0.001$) but there was no significant difference between the rIC-perfusate group ($n = 400$, $p = 0.062$). Unconditioned human serum also caused a significant attenuation of ET-1 induced hypertrophy with a final cell size of $1.8 \pm 0.5 \times 10^4$ μm^2 ($n = 400$, $p < 0.001$). However the effect of rIC-serum augmented the evident anti-

hypertrophic effects of unconditioned-serum alone in this model with a significant difference between the 2 serum types ($p < 0.001$). Of note the rIC pre-treated cells displayed a final cell size smaller than the untreated cells over 48 hours suggesting that not only did rIC inhibit ET-1 induce hypertrophy but also had some effect on the natural growth in H9c2 size when grown in culture over time (see Figure 3.5).

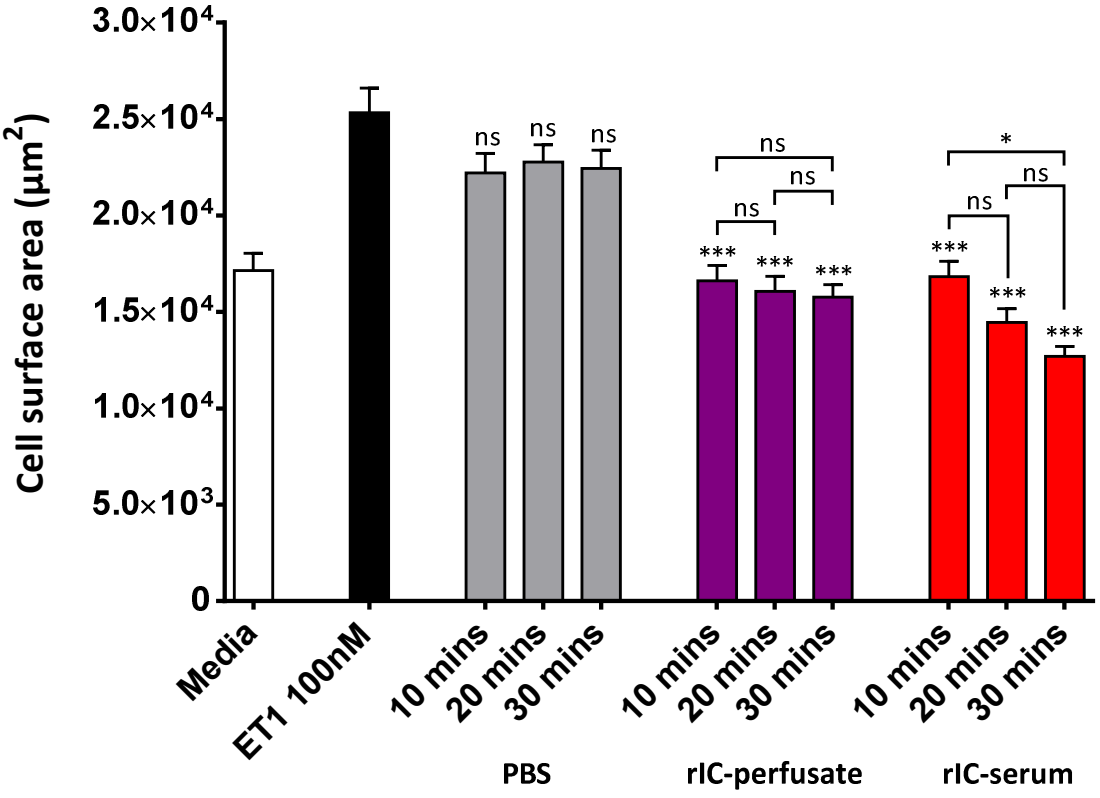


Figure 4.2. Bar chart of H9c2 cell size after 48 hours ET-1 with 10, 20 and 30 minutes rIC-perfusate and serum treatment pre-treatment. Media only (white), media only then ET-1 (black), PBS then ET-1 (grey), rIC-perfusate then ET-1 (purple) and rIC-serum then ET-1 (red). Statistical significance above bars = comparison to ET-1 column. Means with S.E.M, n=3/4/200 for each group, *ns* = non-significant, *$p < 0.05$, ***$p < 0.001$.

	Treatment (30 mins)	Incubation (48 hrs)	Graph Colour
Controls	SFM (-)	SFM	
	SFM (-)	100nM ET-1 in SFM	
	Warmed PBS (-)	100nM ET-1 in SFM	
	10 µM fenofibrate (+)	100nM ET-1 in SFM + 10 µM fenofibrate	
	Human unc.-serum (+)	100nM ET-1 in SFM	
rIC	Rat rIC-perfusate	100nM ET-1 in SFM	
	Human rIC-serum	100nM ET-1 in SFM	

Table 4.2. Table of upstream treatment arms of cultured H9c2s for immunofluorescence experiments. Cells were treated for 30 minutes (as shown) and then placed back in the incubator for 48 hours of ET-1 treatment before downstream processing took place. Each treatment and incubation protocol combination colour coded and will be for that treatment/incubation in all figures hereafter. *PBS* = phosphate buffered saline, *ET-1* = endothelin-1, *rIC* = remote ischaemic conditioned, unc. = unconditioned, *SFM* = serum free media, (-) = negative control, (+) = positive control.

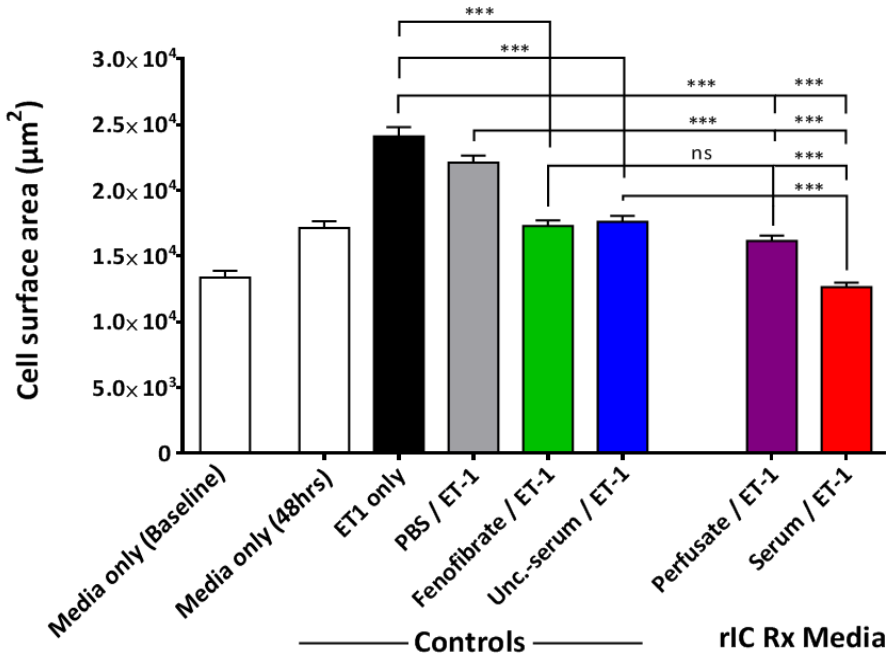


Figure 4.3. Bar chart of H9c2 cell size after 48 hours ET-1 with 30 minutes rIC-perfusate/serum pre-treatment and controls. The control groups are shown on the left and the rat perfusate pre-treatment group (purple) and rIC-serum pre-treatment group (red) are shown on the right. Values given are those for pre-treatment as shown followed by 48 hours ET-1 incubation, except for the media only control (white). Means with S.E.M, n=3/4/400 for each group, *ns* = non-significant, *p < 0.05, ***p < 0.001.

4.3.3 Human rIC-serum Anti-Hypertrophic Properties Validation

To ensure the reproducibility and validity of the anti-hypertrophic effects seen in the initial phase of experiments, two further experiments were performed. The first experiment entailed the recruitment of a larger separate cohort of healthy volunteers so that matched unconditioned and rIC-serum could be tested again to ensure reproducibility. To ensure the validity of this model of hypertrophy in which cell surface area was measured, protein and DNA quantification was performed to provide a parallel index of hypertrophy.

4.3.3.1 Larger Separate Cohort of Healthy Volunteers

Matched serum samples (unconditioned and rIC) from a total of 16 healthy male volunteers were tested for their ability to attenuate ET-1 induced hypertrophy in the H9c2 model. 1600 cells were measured on 3 separate occasions for each of the 16 individuals. The means for each treatment groups were combined to give overall means. The results are shown in Figure 4.4. The data supported results of the first preliminary phase of experiments. RIC-serum attenuated the effects of ET-1 induced hypertrophy from $2.0 \pm 1.0 \times 10^4$ μm^2 (ET-1 control cells) to $1.2 \pm 0.3 \times 10^4$ μm^2 (rIC-serum treated cells) ($n = 1600$, $p < 0.001$). However in contrast to the initial phase of experiments, unconditioned-serum no longer afforded significant protection against hypertrophy with final mean measurements of $1.8 \pm 0.4 \times 10^4$ μm^2 ($n = 1600$, $p = 0.68$).

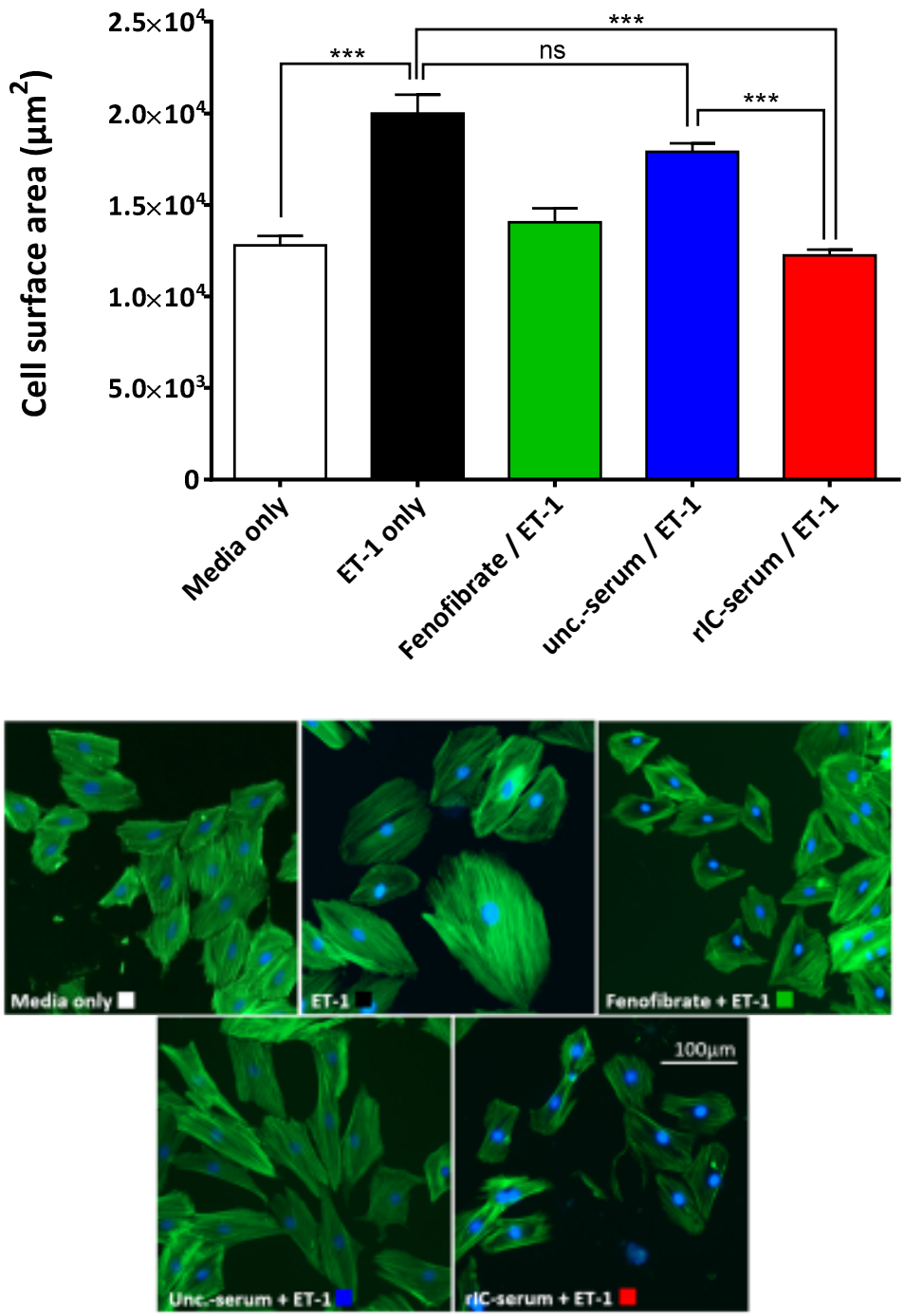


Figure 4.4 H9c2 cell size after 48 hours ET-1 with 30 minutes rIC-perfusate/serum treatment, n = 16. a.) Bar chart showing the effects of unconditioned-serum (blue) and rIC-serum (red) pre-treatment on subsequent ET-1 induced H9c2 hypertrophy b.) Representative immunofluorescence images of H9c2 cardiomyoblasts for each treatment group, x10 magnification. Means with S.E.M, n=3/16/1600, ns = non-significant, ***p < 0.001.

4.3.3.2 Protein/DNA Ratio Analysis

A possible limitation with this model of hypertrophy in cultured H9c2 cardiomyoblasts, is the ability to accurately interpret hypertrophy, which is a three dimensional phenomenon using essentially a two dimensional measuring technique, where hypertrophy is determined from a measure of cell size. As previously discussed, all measurements were doubled to take into account both the visualised cell surface area and the area attached to the chamber slide. The technique of 2D cell surface measuring is used widely and is well established as a simple means of establishing cell size in culture.[425] Indeed this technique has been shown to correlate well with measurements obtained from 3D volume measurements using confocal microscopy.[426] Yet despite this, concern remained that rather than hypertrophy, ET-1 in this setting may in fact just be acting instead to increase cell attachment to the growing membrane, in essence causing the cell to be spread thin and appear larger. If this was the case, rIC-serum may be acting to disrupt these attachments and hence appear to make the cell looks smaller when measured. Another situation that may mimic true hypertrophy is that of disturbed water balance leading to intracellular oedema and increased cell size in culture. Such confounding factors would clearly make interpretation of these results from the immunofluorescence experiments previously described more challenging.

Another marker of hypertrophy which is less susceptible to such confounding factors is the measurement of overall protein levels in relation to DNA. In true hypertrophy, protein levels will increase whilst DNA levels will remain relatively unchanged for any given cell. For this reason, total protein and DNA levels were measured to provide a ratio and attempt to validate the results seen with

immunofluorescence. H9c2 cells were grown to 70-80% confluency in T25 flasks and treated as described in Table 4.2 and previously in the text. After the 48 hours incubation period, cells were lysed and the total cell count estimated by use of a haemocytometer. Each flask was then split into two and the fractions centrifuged to produce a pellet. One pellet was used to assess protein levels using the Biorad DC Colorimetric protein assay (a modified Lowry assay)[427] and the other was used to assess DNA levels using the Biorad Fluorescent DNA Quantitation Kit, which utilises the fluorochrome Hoechst 33258, following manufacturer's protocol. This provided a total concentration of protein and DNA per sample which was subsequently adjusted according to the number of cells per sample and then standardised to the control (media only) set at 100%. The results are shown in Figure 4.5.

The protein to DNA ratio increased by 148 ± 19% in the ET-1 group compared to the media only control group ($p < 0.001$). This increased ratio was significantly reduced by both unconditioned and rIC-serum, 81 ± 10% ($p < 0.05$ compared to ET-1 group) and 120 ± 8% ($p < 0.001$ compared to ET-1 group) respectively. The reduction was more marked and the effects of rIC-serum treatment group were significantly greater than the unconditioned-serum treatment group. Furthermore there was a significance difference between these 2 serum groups ($p < 0.05$). These results correlate well to the data obtained with the immunofluorescence assessment of cell size area and provides further proof that rIC-serum significantly attenuates ET-1 induced hypertrophy.

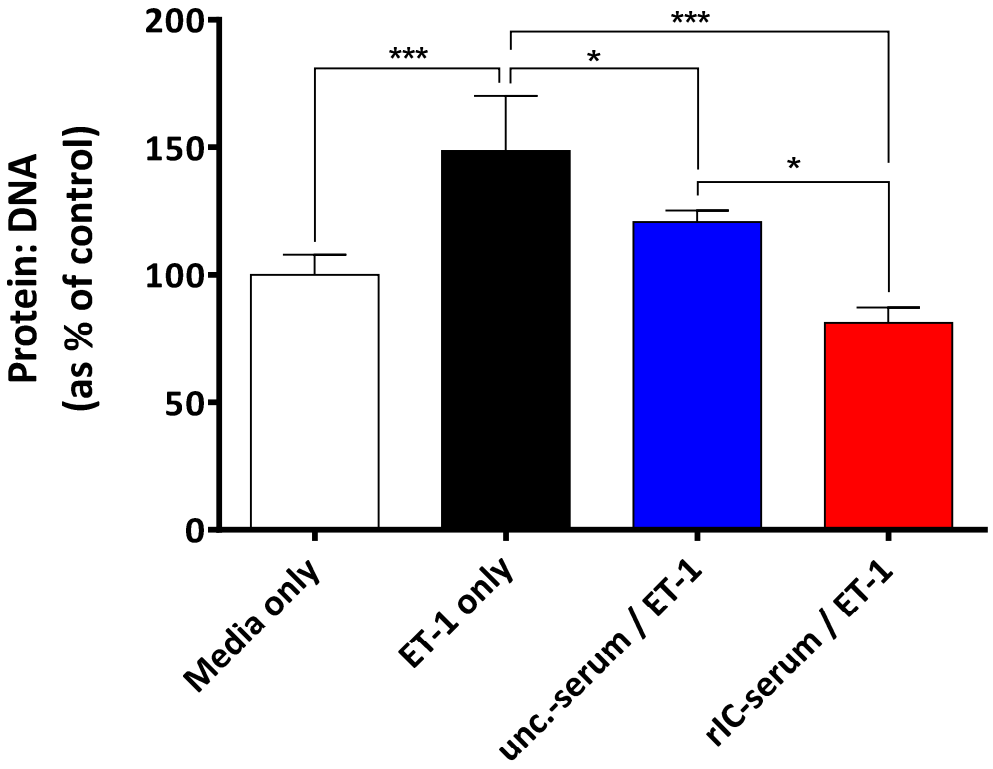


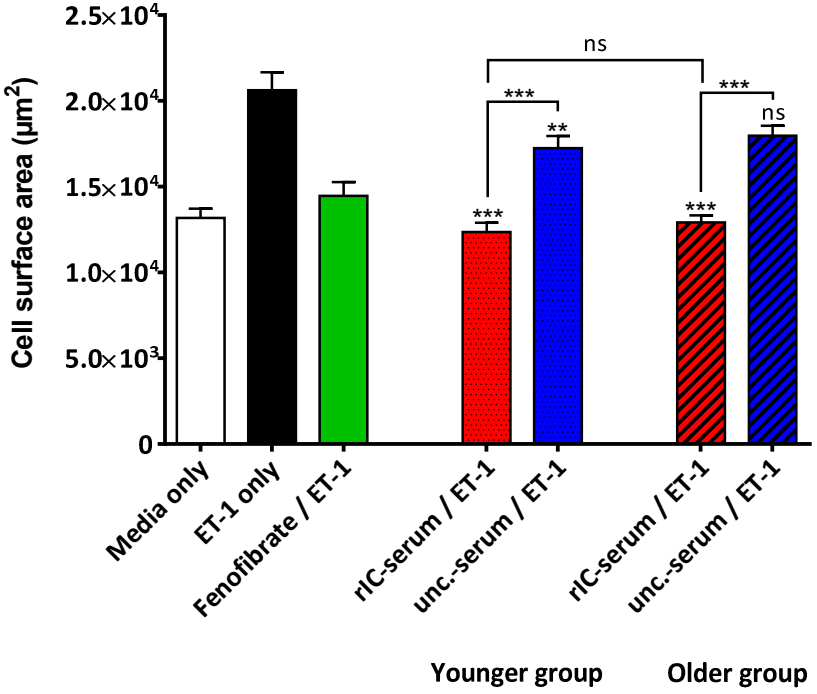
Figure 4.5 Protein to DNA ratio analysis of ET-1 treated H9c2 cells. Bar chart showing the effects of unconditioned-serum (blue) and rIC-serum (red) pre-treatment followed by 48 hours of ET-1 treatment on the total ratio of protein to DNA. Means with S.E.M, n=3/8/400, *<0.05, ***p<0.001.

4.3.4 The Effect of Age on RIC Inhibition of Hypertrophy

The cardioprotective effects of rIC are known to diminish with age. This has been demonstrated by a number of animal studies as well as clinical studies using elderly subjects. The mechanisms for reduction of rIC seen in the ageing heart has not been fully elucidated but is thought in part to involve changes in some of the downstream pathways that modulate rIC including down-regulation of PKC translocation and reduced activation and phosphorylation of proteins that are constituents of the RISK pathway of rIC including ERK1/2, Akt, GSK3b and p38.[428-430] Therefore the effect of age on the

effectiveness of rIC-serum to inhibit ET-1 hypertrophy was next tested. 8 healthy male volunteers from a younger cohort and 8 healthy male volunteers from an older cohort were recruited. Matched serum was used as described in previous experiments and the results are shown in Figure 4.6a and b.

The mean age of the younger group was 26.0 ± 1.7 years of age and the mean age of the older group was 57.5 ± 2.2 years of age. Interestingly the serum from both groups behaved very similarly. In both the younger and the older age groups rIC-serum treatment significantly attenuated hypertrophy compared to ET-1 treatment alone reducing the size from $2.06 \pm 0.1 \times 10^4$ μm^2 in ET-1 only treated group to $1.24 \pm 0.05 \times 10^4$ μm^2 in the younger group and $1.29 \pm 0.04 \times 10^4$ μm^2 in the older group (both groups $p < 0.001$). In the younger group, the unconditioned-serum also significantly reduced final cell size at $1.72 \pm 0.07 \times 10^4$ μm^2 ($p < 0.01$) although there remained a large and significant difference between the attenuation seen between rIC-serum and unconditioned-serum ($p < 0.005$). In the older groups, the unconditioned-serum showed a trend towards significance with regards to attenuating final cell size at $1.80 \pm 0.06 \times 10^4$ μm^2 ($p = 0.075$) and a significance difference was seen between the rIC and unconditioned groups ($p < 0.005$).



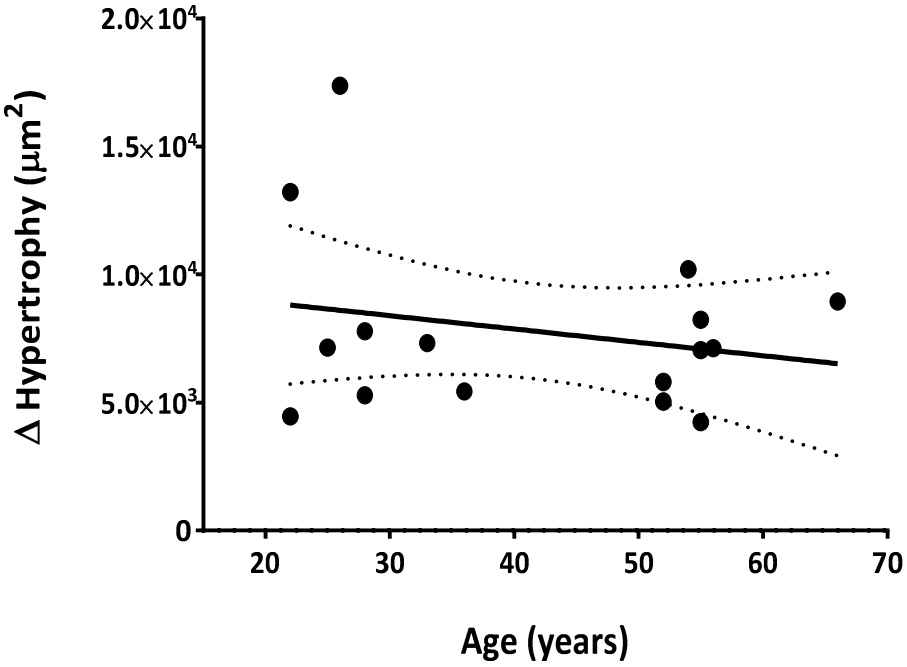


Figure 4.6.a.) Effect of age on the degree of hypertrophic attenuation afforded by rIC-serum on final H9c2 cell size. Bar chart of the effects of unconditioned-serum (blue) and rIC-serum (red) pre-treatment from a younger and older cohort, followed by 48-hours of ET-1 treatment. Statistical significance above bars = comparison to ET-1 column. Means with S.E.M, n=3/8/800 per age group, *ns* = non-significant, **<0.01, ***p<0.001.

Figure 4.6.b.) Linear regression plot of age against difference in hypertrophic attenuation between unconditioned and rIC-serum. Difference in hypertrophy between unconditioned-serum pre-treatment and rIC-serum pre-treatment (Δ Hypertrophy) with the younger cohort clustered on the left and the older cohort clustered on the right. Means with 95% confidence intervals, n=3/100 per plot, r^2 = 0.2862.

4.3.5 The Effect of Levels of Physical Activity on RIC Inhibition of Hypertrophy

The effect of levels of physical activity on the effectiveness of rIC and unconditioned-serum to inhibit ET-1 hypertrophy was next tested. All volunteers for this experiment were males under the age of 40. Volunteers were divided into 3 groups based on their responses to questions regarding their level of activity: low, moderate and high, derived from the IPAQ-SF questionnaire.[418] A total of 4 volunteers were recruited from each of these 3 groups. Matched serum (unconditioned versus rIC-serum) from the volunteers was used as described in previous experiments and the results are shown in Figure 4.7.

There was a significant attenuation of ET-1 induced hypertrophy using serum from volunteers with low levels of activity with a marked difference in effect between rIC-serum and unconditioned serum, $1.03 \pm 0.11 \times 10^4$ μm^2 and $2.02 \pm 0.15 \times 10^4$ μm^2 respectively ($p < 0.001$). As level of physical activity increased, there was a less marked difference between the effectiveness of rIC-serum over unconditioned-serum and indeed in the high level of activity group, there was no significant difference in the degree of attenuation of hypertrophy between the 2 serum groups, rIC-serum measuring at $1.33 \pm 0.67 \times 10^4$ μm^2 and unconditioned-serum measuring at $1.37 \pm 0.81 \times 10^4$ μm^2. In the low activity group, there is a non-significance difference between the ET-1 control cells and the unconditioned-serum treated cells, $2.03 \pm 0.53 \times 10^4$ μm^2 and $2.02 \pm 0.15 \times 10^4$ μm^2 respectively ($p = 0.94$). However the difference is significant for both the moderate activity group measuring at $1.69 \pm 0.88 \times 10^4$ μm^2 and high levels activity group measuring at $1.37 \pm 0.81 \times 10^4$ μm^2, when comparing unconditioned-serum and ET-1 control cells ($p < 0.01$ and $p < 0.001$ respectively).

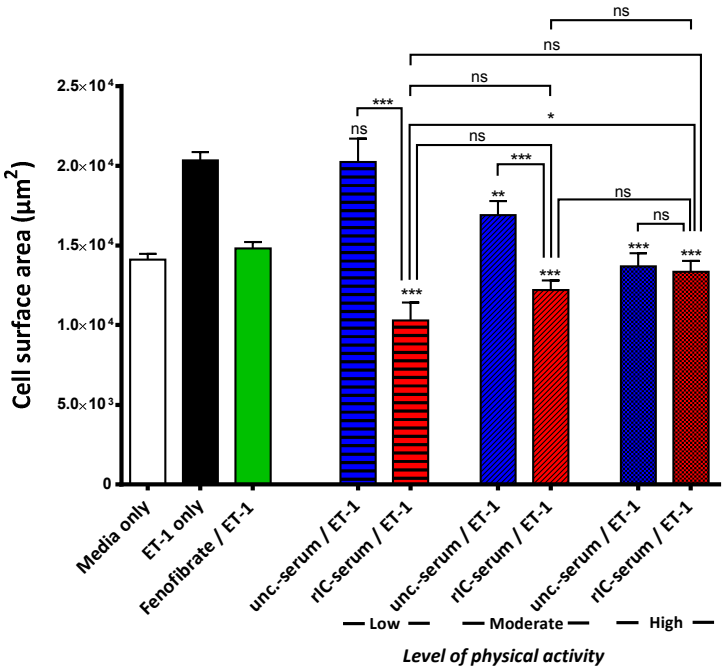


Figure 4.7.a.) Effect of levels of physical activity in young males on the degree of hypertrophic attenuation afforded by rIC-serum on final H9c2 cell size. Bar chart of the effects of unconditioned-serum (blue) and rIC-serum (red) pre-treatment from groups with low, moderate and high levels of physical activity, followed by 48-hours of ET-1 treatment. Statistical significance above bars = comparison to ET-1 column. Means with S.E.M, n=3/12/1200, 4 per activity group, *ns* = non-significant, *p<0.05, **p<0.01, ***p<0.001.

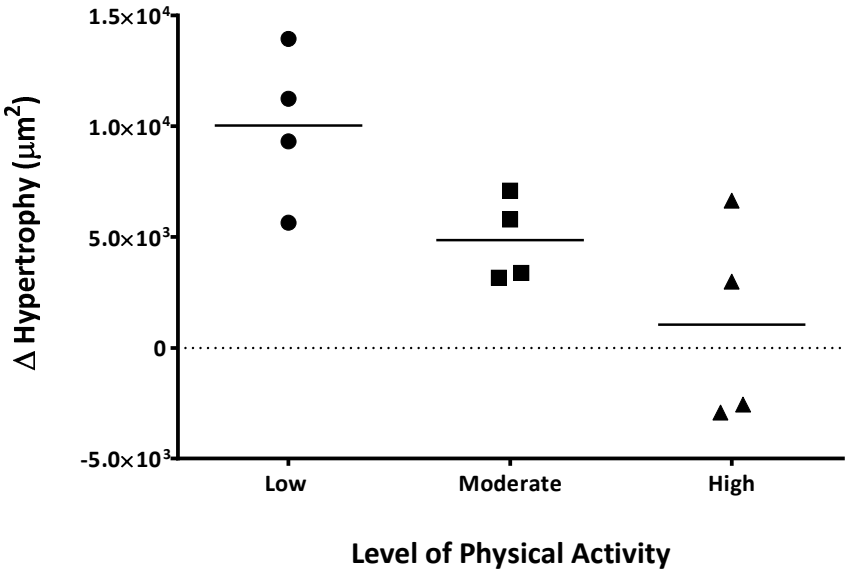


Figure 4.7.b.) Scatter plot of individual volunteer differences in hypertrophic attenuation between unconditioned and rIC-serum, grouped by physical activity level. Difference in hypertrophy between unconditioned-serum pre-treatment and rIC-serum pre-treatment (Δ Hypertrophy) with three levels of physical activity: low, moderate and high. Means, n=3/100 per individual.

4.5 Foetal Gene Panel Expression as a Surrogate for RIC-serum Attenuation of ET-1 Induced Hypertrophy

The *"foetal gene panel"* is the term given to the groups of genes that are up-regulated in post-MI hypertrophy and remodelling that mimic the patterns of gene expression seen in foetal cardiac development. Aberrant expression of these foetal genes has been shown to be maladaptive with regards to a number of processes including unregulated cardiac hypertrophy, abnormal calcium handling as well as effecting myocardial contractility.[431]

Four genes whose up-regulation is associated with maladaptive cardiac remodelling were selected for experimentation: Brain Natriuretic Peptide (BNP), Beta Myosin Heavy Chain (βMHC), Alpha Skeletal Muscle Actin (α-ACT) and Myocyte Stress 1 (MS-1). BNP is a small peptide hormone released by the cardiac ventricle in response to stretch as well as ET-1 in adults, often in the context of heart failure.[432] In this setting it acts to increase natriuresis and decrease systemic vascular resistance and venous pressure to decrease the afterload and preload on the heart. It also act to initially prevent cardiac hypertrophy via various pathways including cGMP,[433] however, its continually activation has been shown to lead to a pro-hypertrophic phenotype via downstream up-regulation of pro-hypertrophic transcription factors.[434] βMHC is a sub-unit of myosin filaments which has a lower level of contractility compared to αMHC.[435] ET-1 can trigger an increased ratio of βMHC to αMHC expression and is associated with cardiac hypertrophy.[436, 437] ET-1 also increases the expression of α-ACT[438], another cytoskeletal protein which is highly expressed in the foetal heart but not the adult heart in health. Up-regulation in adulthood is associated with cardiac hypertrophy and

dysfunction.[439] Finally MS-1 is a striated muscle-specific actin-binding protein that has been implicated in foetal development of the heart, although it is not classically considered part of the foetal gene programme. It acts to activate SRF-dependent transcription to bring about hypertrophy and cardiac dysfunction in adult rat and mouse models.[364, 440-442]

H9c2 cells were pre-treated with unconditioned rat perfusate or rIC rat perfusate for 30 minutes and then either trypsinized immediately or after 48 hours of ET-1 treatment as described in *section 3.2.2.1*. H9c2 cells were also pre-treated with unconditioned human serum or rIC human serum for 30 minutes and then either trypisinized immediately or after 48 hours of ET-1 treatment as described. Real-time quantitative PCR was performed as described in *section 2.2.* The results of the rat perfusate treated cells are shown in Figure 4.8. The results of the human serum treated cells are shown in Figure 4.9.

A significant relative increase in gene expression of βMHC, α-ACT and MS-1 was seen in ET-1 treated cells after 48 hours compared to untreated cells at baseline in the rat perfusate group of experiments (fold-change = 7.1 ± 0.4, 7.7 ± 0.4 and 36.1 ± 3.5 respectively, $p < 0.001$ in all 3 genes). A trend towards significance was seen in BNP expression in ET-1 treated cells after 48 hours (fold-change = 2.0 ± 0.1, $p = 0.062$). Expression levels of ET-1 induced BNP was attenuated by rIC perfusate (fold-change = 0.4 ± 0.1 versus 2.0 ± 0.1, $p < 0.01$) but not unconditioned perfusate (fold-change = 1.27 ± 0.3 versus 2.0 ± 0.1, $p = 0.19$). Expression levels of ET-1 induced βMHC was attenuated by rIC perfusate (fold-change = 2.5 ± 0.3 versus 7.1 ± 0.4, $p < 0.001$) but not unconditioned perfusate (fold-change = 6.5 ± 0.5 versus 7.1 ± 0.4, $p = 0.64$). Similarly

expression levels of ET-1 induced α-ACT was attenuated by rIC perfusate (fold-change = 1.7 ± 0.1 versus 7.7 ± 0.4, $p < 0.001$) but not unconditioned perfusate (fold-change = 8.0 ± 0.4 versus 7.7 ± 0.4, $p = 0.95$) and expression levels of ET-1 induced MS-1 was attenuated by rIC perfusate (fold-change = 13.0 ± 1.4 versus 36.1 ± 3.5, $p < 0.001$) but not unconditioned perfusate (fold-change = 31.6 ± 2.7 versus 36.1 ± 3.5, $p = 0.57$).

In the human serum group, a significant relative increase in gene expression of all four genes was seen after 48 hours compared to untreated cells at (BNP fold-change = 1.9 ± 0.1, βMHC fold-change = 10.7 ± 0.7, α-ACT fold-change = 7.7 ± 0.5 and MS-1 fold-change = 26.6 ± 2.4, $p < 0.001$ in all 4 genes). Increased expression levels induced by 48 hours ET-1 was significantly attenuated by both rIC and unconditioned-serum pre-treatment (p at least < 0.01 for groups). There was no significant difference in this attenuation between rIC and unconditioned-serum in BNP expression (fold-change of 0.4 ± 0.1 for rIC-serum versus 0.5 ± 0.1 in unconditioned-serum, $p = 0.94$). For βMHC a significantly greater attenuation was seen in rIC-serum than unconditioned-serum (fold-change of 3.8 ± 0.4 for rIC-serum versus 7.5 ± 0.7 in unconditioned-serum, $p < 0.001$). Interestingly unconditioned-serum led to a greater attenuation of ET-1 induced expression of α-ACT after 48 hours than rIC-serum (fold-change of 2.2 ± 0.2 for rIC-serum versus 0.9 ± 0.1 in unconditioned-serum, $p < 0.05$). Finally there was no significant difference in this attenuation between rIC and unconditioned-serum in MS-1 expression (fold-change of 7.0 ± 0.7 for rIC-serum versus 9.2 ± 1.7 in unconditioned-serum, $p = 0.8$).

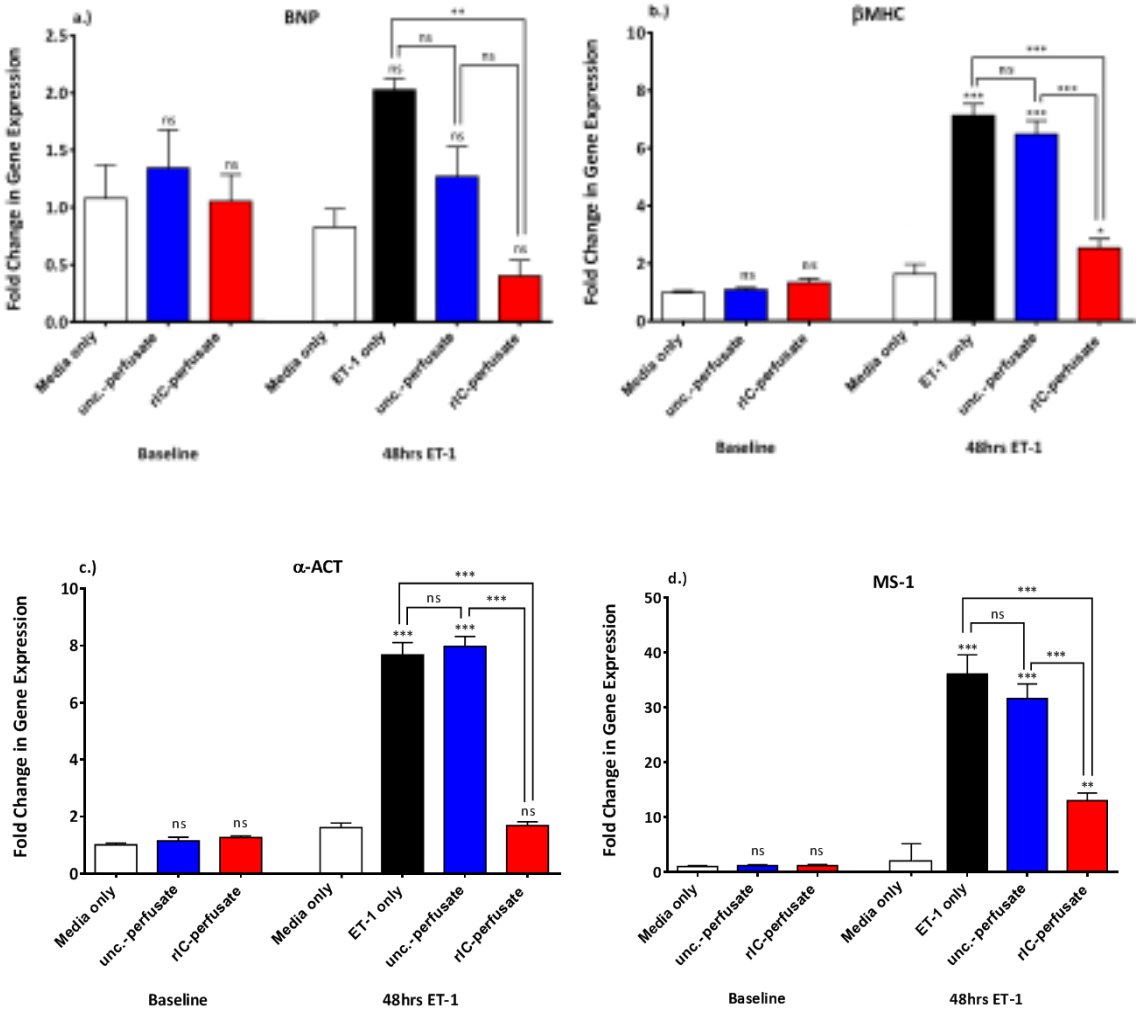


Figure 4.8 Bar charts of the effect of unconditioned and rIC rat perfusate on relative fold-expression of 4 genes expressed as part of the 'foetal panel' of gene expression in remodelling, taken at baseline and 48 hours: a.) BNP b.) c.) α-ACT d.) MS-1. Normalized to the housekeeper probe ACTB. Statistical significance above bars = comparison to media only column. Means with S.E.M, n=8/3, *ns* = non-significant, *p<0.05, **p<0.01, ***p<0.001.

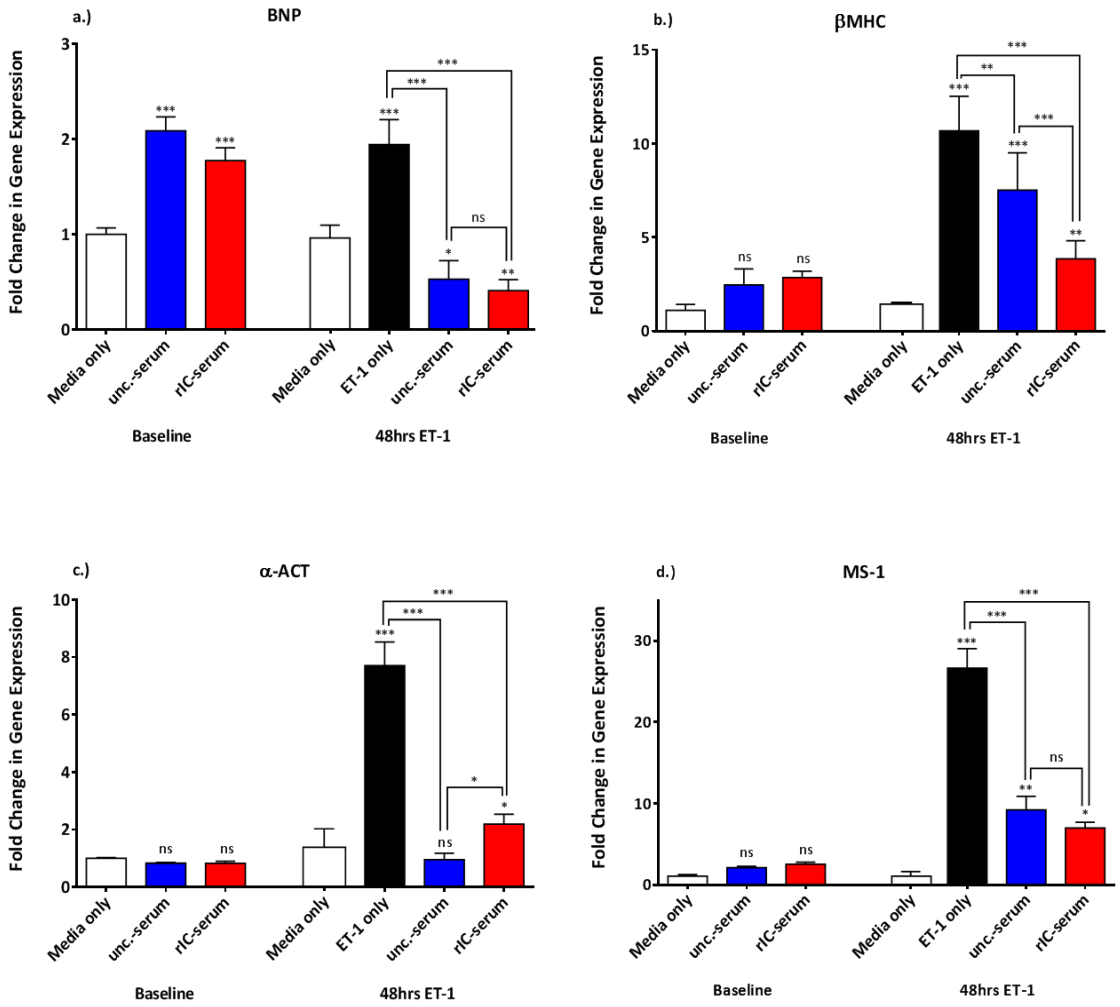


Figure 4.9 Bar charts of the effect of unconditioned and rIC human serum on relative fold-expression of 4 genes expressed as part of the 'foetal panel' of gene expression in remodelling, taken at baseline and 48 hours: a.) BNP b.) βMHC c.) α-ACT d.) MS-1. Normalized to the housekeeper probe ACTB. Statistical significance above bars = comparison to media only column. Means with S.E.M, n=8/3, *ns* = non-significant, *p<0.05, **p<0.01, ***p<0.001.

4.6 Summary

The results from this chapter demonstrate the ability of perfusate from isolated rat hearts that have undergone cycles of I/R and serum from healthy volunteers undergoing cycles of rIC to attenuate the hypertrophic effects of ET-1 in an H9c2 rat cardiomyoblast model. Furthermore, matched unconditioned-serum from healthy volunteers has a much less marked effect on blocking hypertrophy. This adds weight to the theory that rIC exerts many of its effect via a blood borne humoral agent, or more likely a panel of agent. Furthermore the technique of immunofluorescence staining and measuring cell surface area using computer software, was validated using protein: DNA ratios, which proved it to be an accurate measure of assessing real cellular hypertrophy, rather than a measure for intra-cellular swelling or cellular adhesion to the growing surface.

The effects of age and physical activity levels were also tested on this model. Age proved to have no significant effect on the degree of attenuation of hypertrophy from rIC-serum, however the innate, lower level protection afforded by unconditioned-serum in the younger group was lost in the older group. Interestingly, a greater degree of rIC-serum induced hypertrophic attenuation was seen in the low activity group, although all groups still induced significant ant-hypertrophic effects. This may be due to the high levels of circulating protective agents in the higher physical activity groups with a small spike seen with a rIC stimuli than is seen in the lower activity groups.

The results also showed that rIC-serum led to a down regulation of a number of pro-hypertrophic genes (part of the so-called 'foetal gene panel') including BNP, βMHC, α-ACT and MS-1. The next chapter deals with some of the potential mechanisms by which rIC-serum attenuates ET-1 induced hypertrophy in this model.

5. Mechanisms of RIC in ET-1 induced hypertrophy

5.1 Chapter Introduction

Chapter 4 has demonstrated the functional effects of rIC-serum on an H9c2 cardiomyoblast model of hypertrophy and remodelling. Following on from this, this chapter will attempt to elucidate some of the cellular mechanisms responsible for this anti-hypertrophic action of rIC-serum. This includes assessing the role of the signalling molecules AMPK, the activation of the endothelial NO synthase/soluble guanylate-cyclase (cGC) signalling pathway and the role of PKCε translocation. Furthermore this chapter will assess the effect of rIC-serum on the expression miR-1 and 133a, which are heavily implicated in cardiomyocyte hypertrophy.

5.2 The Role of PKCε Signalling as a Mechanism for RIC-serum Attenuation of ET-1 Induced Hypertrophy

5.2.1 The Effect of Endothelin-1 on PKCε Translocation in H9c2 Cardiomyoblasts

ET-1 has been shown to induce hypertrophy via a number of mechanisms, including rapid PKCε translocation (see *section 3.3.1* and Figure 3.3 for more details).[443, 444] We tested the effects of ET-1 on PKCε in our H9c2 model of hypertrophy using western blot techniques as described in *section 2.3.* PMA was used as a positive control. PMA is a potent activator of PKC with an analogous structure to DAG and acts by binding to the C1 domain inducing membrane translocation.[445] In the PMA groups, cells were pre-treated for 30 minutes in serum-free media with 1μM PMA co-incubation. The results of this experiment are shown in Figure 5.1.

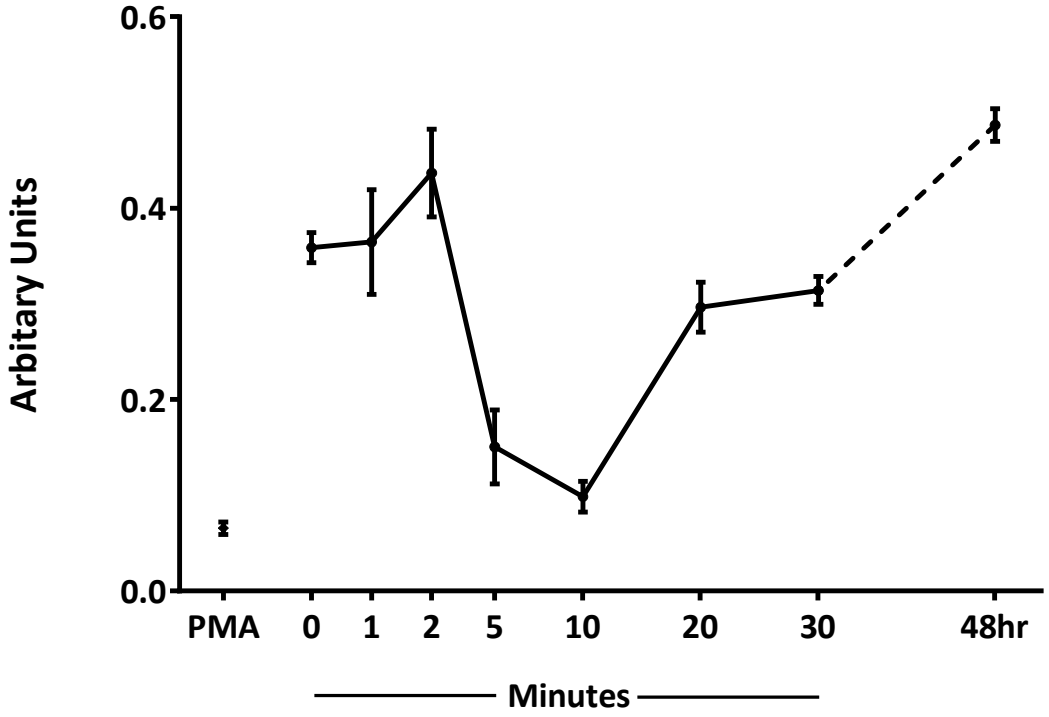


Figure 5.1 Line graph showing the level of the cytosolic component of PKCε over time after continuous treatment with ET-1. Levels of PMA treated cells at 1 minute shown as reference. Means with S.E.M, n=4/3/3.

In the context of IPC, Ping *et al.,* (1997) were the first group to show in a rabbit model that PKCε translocation from the cytosol to the membrane, rather than overall PKCε activation, is the key mechanism by which IPC acts.[446] Cytosolic PKCε levels therefore, act as a surrogate for activity in an inverse manner i.e. lower the levels of cytosolic PKCε and higher the membrane levels, are indicative of an increase in the overall PKCε activity. ET-1 incubation in this model caused a large degree of translocation by 5 minutes, reaching a peak at around 10 minutes. By 20 minutes most of the PKCε had relocated back to the cytosol therefore become inactive again. This implies that ET-1 has

a role in an acute phase response mechanism via PKCε which activated downstream pathways but does not sustain PKCε despite ongoing incubation for 48 hours.

5.2.2 The Effect of RIC Pre-treatment on Endothelin-1 Treated H9c2 Cardiomyoblast Levels of PKCε Translocation

The degree of PKCε translocation was next tested in cells pre-treated with either unconditioned or rIC human serum for 30 minutes followed by ET-1 incubation for either 30 minutes or 48 hours. Samples were lysed for protein analysis after the 30 minutes pre-treatment and 48 hours of ET-1 incubation. The results are shown in Figures 5.2 and 5.3. ET-1 caused activation of PKCε with a greatly decreased cytosolic fraction after 30 minutes from 0.44 ± 0.02 arb. units in the media only group to 0.30 ± 0.01 arb. units in the ET-1 treated group, indicating an increased membrane fraction. Pre-treatment with rIC-serum for 30 minutes followed by ET-1 incubation sustained the membrane fraction of PKCε at 30 minutes of ET-1 incubation compared to ET-1 incubation only, 0.09 ± 0.02 arb. units versus 0.30 ± 0.01 arb. units respectively ($p < 0.001$). This increased augmentation of cytosolic to membrane translocation was seen only with rIC-serum pre-treatment and not unconditioned-serum pre-treatment. However, this effect is lost by 48 hours where there is no significant difference between all treatment groups. This may suggest that any effects ET-1 or serum pre-treatment prior to ET-1 incubation occur acutely and these effects have dissipated when testing at 48 hours. A PKCε inhibitor was not used in the experiments due to concerns over non selectivity of available inhibitors of PKCε over other PKC isoforms.

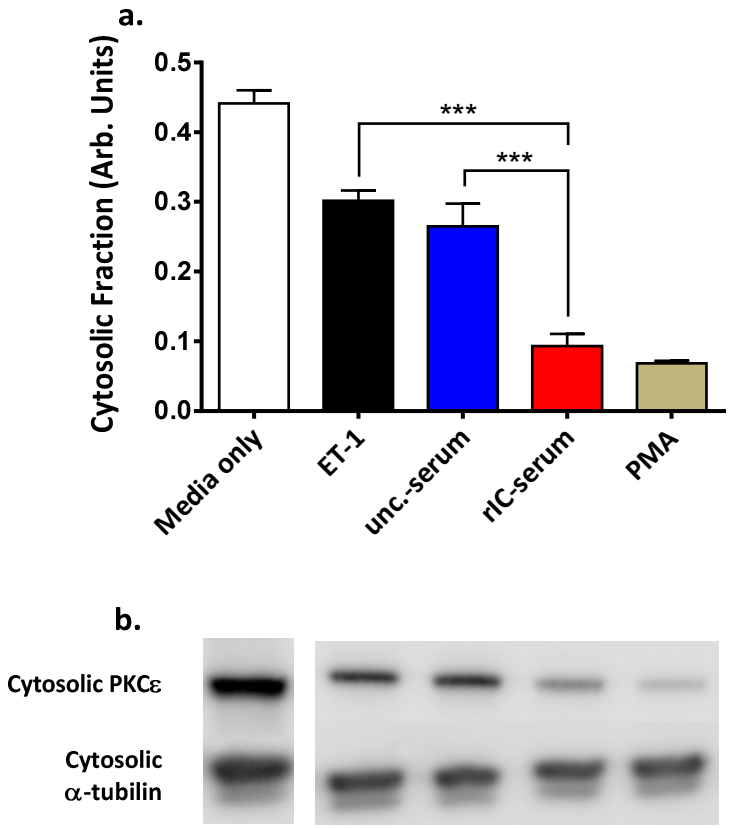


Figure 5.2.a.) Bar chart of the effect of rIC-serum pre-treatment prior to ET-1 incubation for 30 minutes on the level of the cytosolic component of PKCε. b.) Representative western blot images with control. Means with S.E.M, n=6/3/12, ***p<0.001.

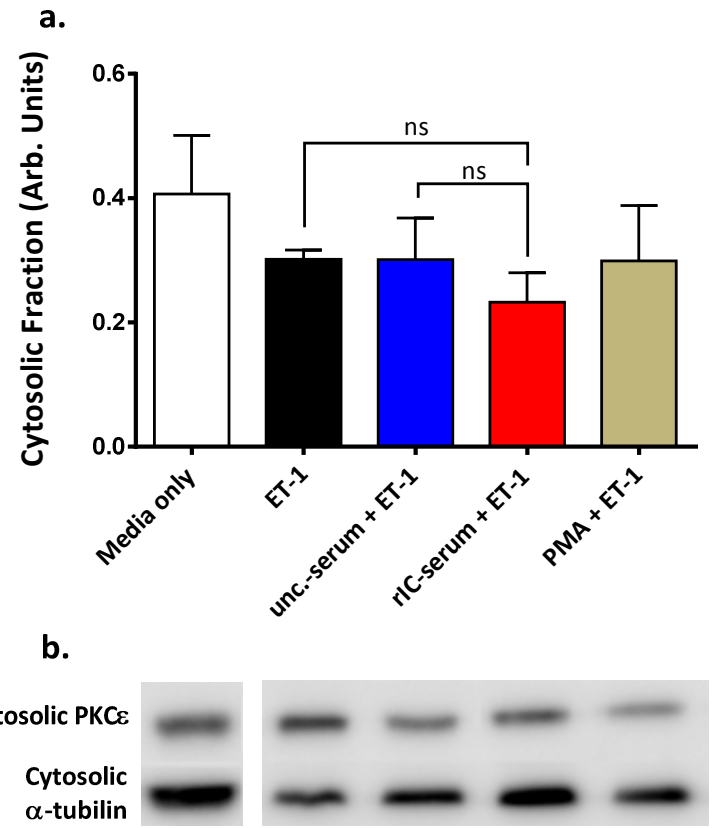


Figure 5.3.a.) Bar chart of the effect of rIC-serum pre-treatment prior to ET-1 incubation for 48 hours on the level of the cytosolic component of PKCε. b.) Representative western blot images with control. Means with S.E.M, n=6/3/12, *ns* = non-significant.

5.3 The Role of AMPK Signalling as a Mechanism for RIC-serum Attenuation of ET-1 Induced Hypertrophy

We investigated the role of AMPK in the anti-hypertrophic action of rIC-serum in the model of ET-1 induced hypertrophy of H9c2 cardiomyoblasts. The AMPK inhibitor compound C (dorsomorphin) was used to assess the role of AMPK in this model. Compound-C is a cell permeable, selective ATP-competitive inhibitor of AMPK.[447] 10μM of compound-C was dissolved in rIC-serum during H9c2 pre-treatment with rIC-serum prior to 48 hours of ET-1 incubation to assess levels of hypertrophy using immunofluorescence. H9c2 cells were also treated for 30 minutes with rIC-serum, unconditioned-serum or rIC-serum + 10μM compound-C. The results are shown in Figure 5.4 and 5.5. The anti-hypertrophic action of rIC-serum pre-treatment was significantly reduced in the presence of compound-C with a mean cell surface area measurement of $1.82 \pm 0.19 \times 10^4$ μm^2 in the ET-1 only group compared to $1.31 \pm 0.33 \times 10^4$ μm^2 in the rIC-serum pre-treatment groups versus a mean cell surface measurement of $1.64 \pm 0.47 \times 10^4$ μm^2 in rIC-serum + compound-c group ($p < 0.001$).

Furthermore levels of AMPK activation were assessed by measuring the levels of phosphorylated AMPKα and total AMPKα using western blotting, the results of which are shown in Figure 5.5b. RIC-serum caused a significant increase in phosphorylated AMPK levels of 2.5 ± 0.3 fold over unconditioned-serum ($p < 0.001$). This activation was completely blocked by compound-C ($p < 0.001$).

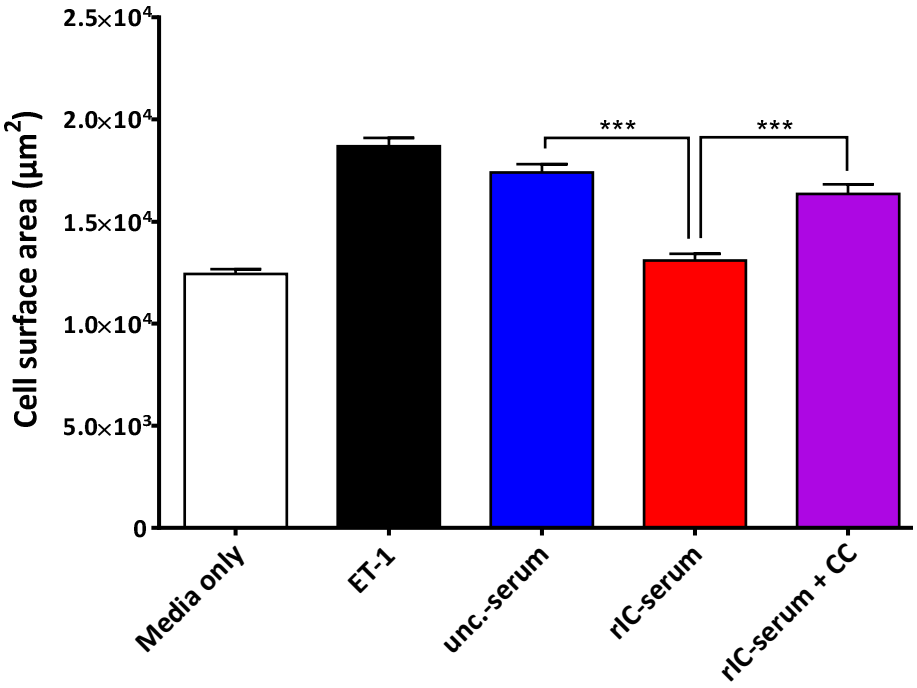


Figure 5.4 Bar chart showing the effect of AMPK inhibition by compound-c on rIC-serum attenuation of ET-1 induced hypertrophy of H9c2 cells. *CC* = compound-c. Means with S.E.M, n=8/3/500, ***p<0.001.

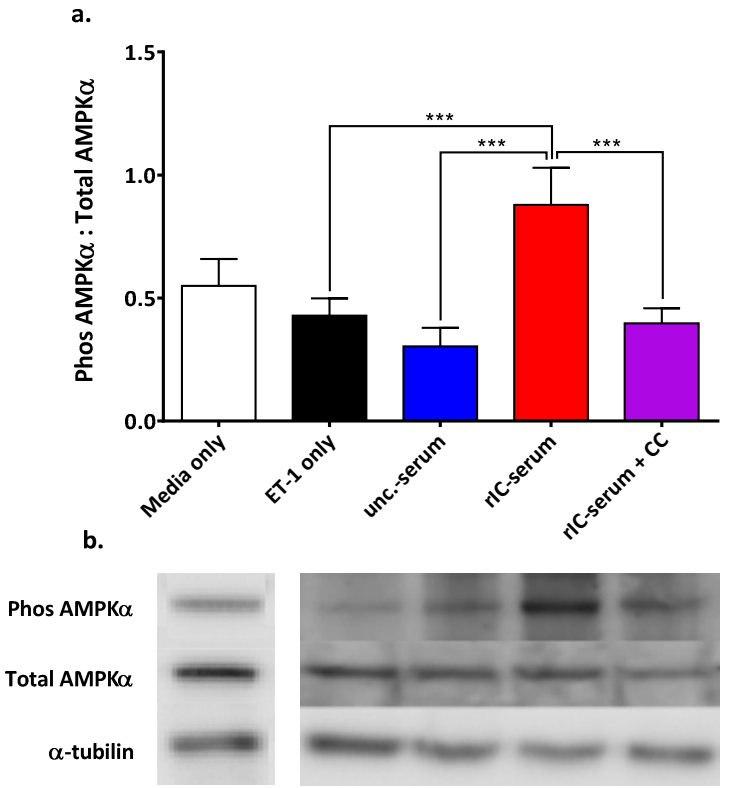


Figure 5.5.a.) Bar chart showing the effect of rIC-serum treatment for 30 minutes on the level of phosphorylated and total AMPK shown as a ratio. b.) Representative western blot images with control. *CC* = compound-c. Means with S.E.M, n=8/3/500, ***p<0.001.

5.4 The Role of eNOS/NO/SGC/CGMP Signalling as a Mechanism for RIC-serum Attenuation of ET-1 Induced Hypertrophy

5.4.1 The Role of eNOS as Mechanisms for RIC Attenuation of ET-1 Induced Hypertrophy

The non-specific NOS inhibitor L-NAME was used to determine the role of NOS (in particular eNOS) in the model of anti-hypertrophic actions of rIC-serum. 100µM of L-NAME was dissolved in rIC-serum during H9c2 pre-treatment prior to 48 hours of ET-1 incubation to assess levels of hypertrophy. Final cell size was assessed via immunofluorescence. H9c2 cells were also treated for 30 minutes with either ET-1, rIC-serum, unconditioned-serum or rIC-serum + L-NAME. The results, shown in Figure 5.6, demonstrate that the anti-hypertrophic actions of rIC-serum was completely blocked by L-NAME with a mean cell surface area measurement of $1.3 \pm 0.3x10^4$ μm^2 in the rIC-serum pre-treatment groups versus a mean cell surface measurement of $1.8 \pm 0.6x10^4$ μm^2 in rIC-serum + L-NAME group ($p < 0.001$), compared to $1.86 \pm 0.14x10^4$ μm^2 in the ET-1 only group.

In order to further show the involvement of eNOS in particular (as opposed to overall NOS involvement), levels of phosphorylated eNOS were assessed by western blotting. Cells were pre-treated with either ET-1, rIC-serum, unconditioned-serum or rIC-serum + 10µM compound-C for 30 minutes. The results of which are shown in figure 5.7. RIC-serum caused a significant increase in phosphorylated eNOS levels of 2.0 ± 0.05 fold over unconditioned-serum which caused a modest 1.0 ± 0.05 fold ($p < 0.001$). This activation was completely blocked by compound-C ($p < 0.001$).

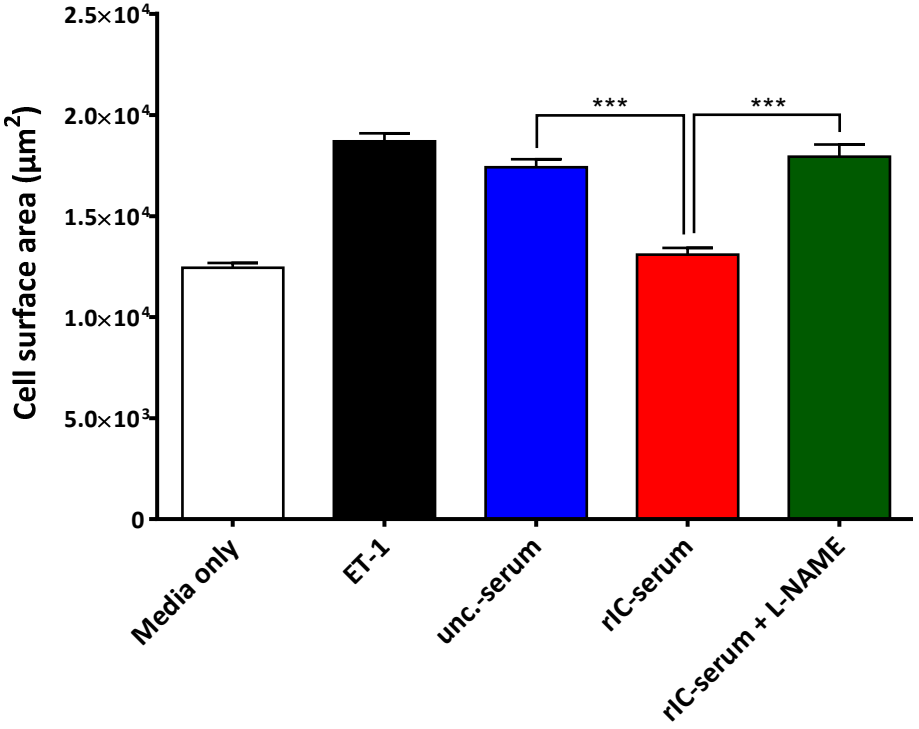
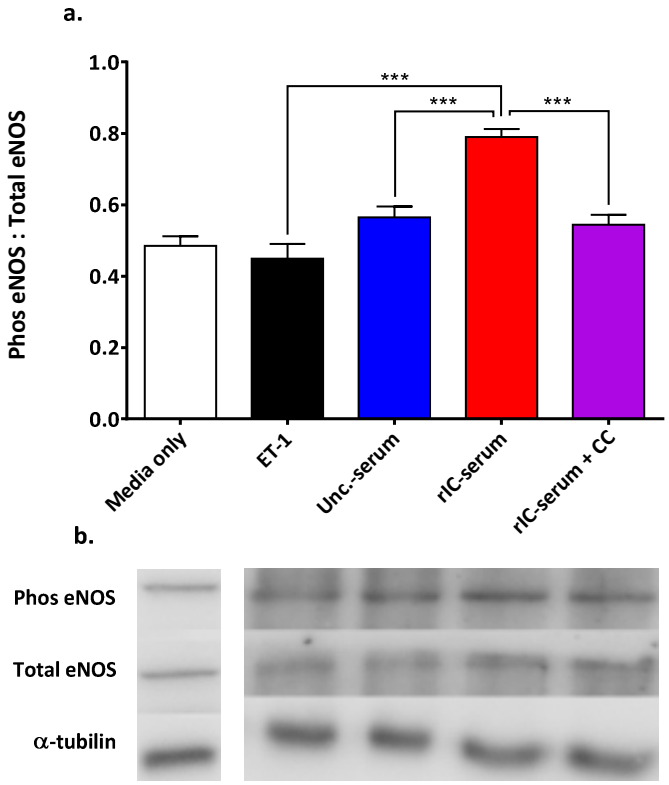


Figure 5.6 Bar chart showing the effect of eNOS inhibition by L-NAME on rIC-serum attenuation of ET-1 induced hypertrophy of H9c2 cells. Means with S.E.M, n=8/3/500, ***p<0.001.



238

Figure 5.7.a.) Bar chart showing the effect of rIC-serum treatment for 30 minutes on the level of phosphorylated and total eNOS shown as a ratio. b.) Representative western blot images with controls. *CC* = compound-c. Means with S.E.M, n=8/3/500, ***p<0.001.

5.4.2 The Role of NO as Mechanisms for RIC-serum Attenuation of ET-1 Induced Hypertrophy

We used DAF-FM fluorescence to detect levels of NO production from H9c2 cells subjected to rIC-serum. DAF-FM is a fluorescent benzotriazole which only fluoresces in the presence of NO and gives a direct indication of intracellular NO levels. The NO donor (S)-Nitroso-N-acetylpenicillamine (SNAP) was used as a positive control. SNAP is a synthetic S-nitrosothiol that under physiological conditions decays to release NO and a disulfide at a steady rate.[448, 449] H9c2 cells were treated for 10 minutes in DAF-FM alone to allow adequate cell entry. The cells were then left for 30 minutes to allow for de-esterification of the DAF to its benzotriazole derivative which is more fluorescent than DAF-FM. The cells were then treated for 20 minutes with either media only, unconditioned serum, rIC-serum, rIC-serum + compound-C (10μM) or media + SNAP (1mM) and levels of fluorescence were measured every minute. No groups with subsequent 48 hours of ET-1 treatment were tested as it was felt that this pathway was likely to be affected only for short period time, if at all, by rIC-serum and as such experiments were only performed out to 20 minutes. The results are shown in Figure 5.8a-c.

There was no significant difference in fluorescence between any of the groups at 5 minutes. SNAP induced a rise in fluorescence levels (and hence detectable NO) which was not seen at 5 minutes but clearly demonstrated at 20 minutes. There was no significant difference at 20 minutes between the fluorescence levels demonstrated in the SNAP treated cells at 1.07 ± 0.002 adjusted units, and the rIC-serum treated cells at 0.96 ± 0.01 adjusted units, $p = 0.13$. Although visually there appeared to be a greater

release of NO in the rIC treated cells compared to both the unconditioned-serum treated cells and the rIC + compound C treated cells at 20 minutes, these differences were non-significant, p = 0.47 and 0.21 respectively.

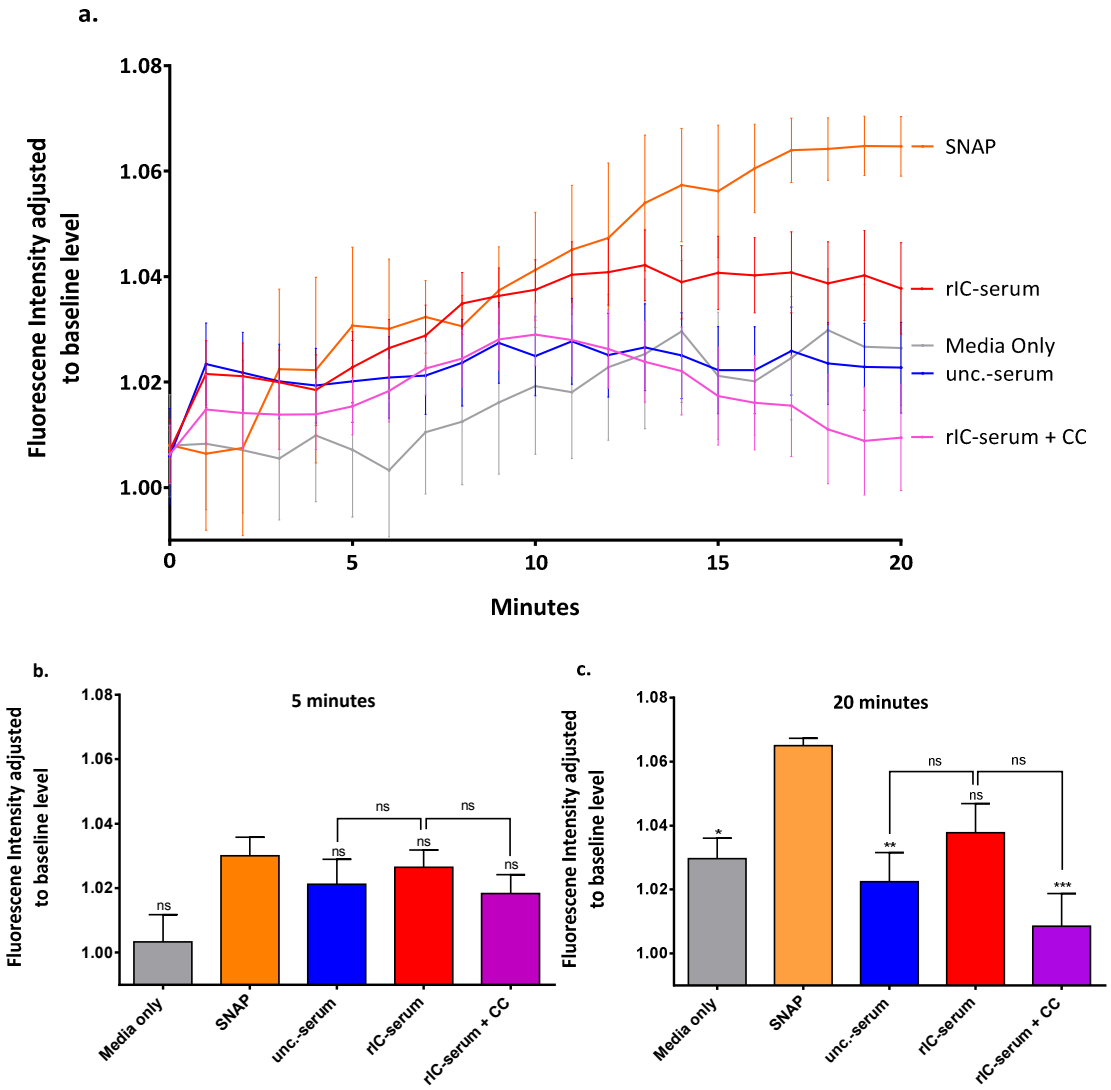


Figure 5.8. Increase in DAF fluorescence as a measure of NO production from H9c2 cardiomyoblasts adjusted to baseline levels. a). Plot of DAF fluorescence intensity recorded from H9c2 cardiomyoblasts in response to 30 minutes of treatment with either serum free media (grey), 1mM SNAP diluted in serum-free media (orange), unconditioned human serum (blue), rIC-serum (red) or rIC-serum with 10µM compound C added (purple). b). Data at 5 and 20 minutes after treatment. *CC* = Compound C. Means with S.E.M, n=12 subjects/3 experimental runs/3 wells. Statistical significance above bars = comparison to SNAP column. Ns = non-significant, *p<0.05, **p<0.01, ***p<0.001.

5.4.3 The Role of SGC and cGMP as Mechanisms for RIC-serum Attenuation of ET-1 Induced Hypertrophy

To determine the influence sGC/cGMP pathway in particular plays in the anti-hypertrophic actions of rIC in the ET-1 induced hypertrophy model, we incubated H9c2 cardiomyoblasts with 10µM of the inhibitor of sGC ODQ, present for 10 minutes prior to and during treatment with rIC-serum, followed by ET-1 medium for 48 hours to induce hypertrophy. The results are shown in Figure 5.9. The inhibition of sGC with ODQ reduced the anti-hypertrophic action of rIC-serum from $1.3 \pm 0.3x10^4$ μm^2 in the rIC-serum group to $1.7 \pm 0.6x10^4$ μm^2 in the rIC-serum + ODQ group ($p < 0.001$). There was no significant difference between the unconditioned-serum treated cells measuring at $1.74 \pm 0.40x10^4$ μm^2 and the ET-1 only treated cells measuring at $1.87 \pm 0.39x10^4$ μm^2 ($p = 0.077$).

Furthermore, we used a competitive ELISA cGMP assay kit to assess levels of intracellular cGMP in H9c2 cells after 30 minutes treatment with either rIC-serum, unconditioned serum, rIC-serum with 10µM ODQ or rIC-serum with 100µM of L-NAME. We also treated the cells for 30 minutes as described and then incubated them for 48 hours in serum free media. After each time period, samples were lysed from the cell culture flasks using 0.1M HCl and reconstituted in a pre-made ELISA buffer. Cell underwent an acetylation step to increase the sensitivity of the assay and downstream process took place as per the manufacturer's instructions. The results are shown in Figure 5.10. Both rIC and unconditioned-serum increased relative levels of cGMP compared to baseline. This appeared greater in the rIC-serum group but was not statistically significant (relative increased levels of 1.6 ± 0.2, $p < 0.001$ in the rIC-serum

group and 1.5 ± 0.1, $p < 0.01$ in the unconditioned-serum group, $p = 0.57$ between groups). The increased level caused by rIC-serum was significantly attenuated by the co-treatment with ODQ (1.1 ± 0.1, $p < 0.05$) but not with L-NAME (1.4 ± 0.1, $p = 0.56$). There was no significant difference between any of the treatment groups after 48 hours of ET-1 incubation.

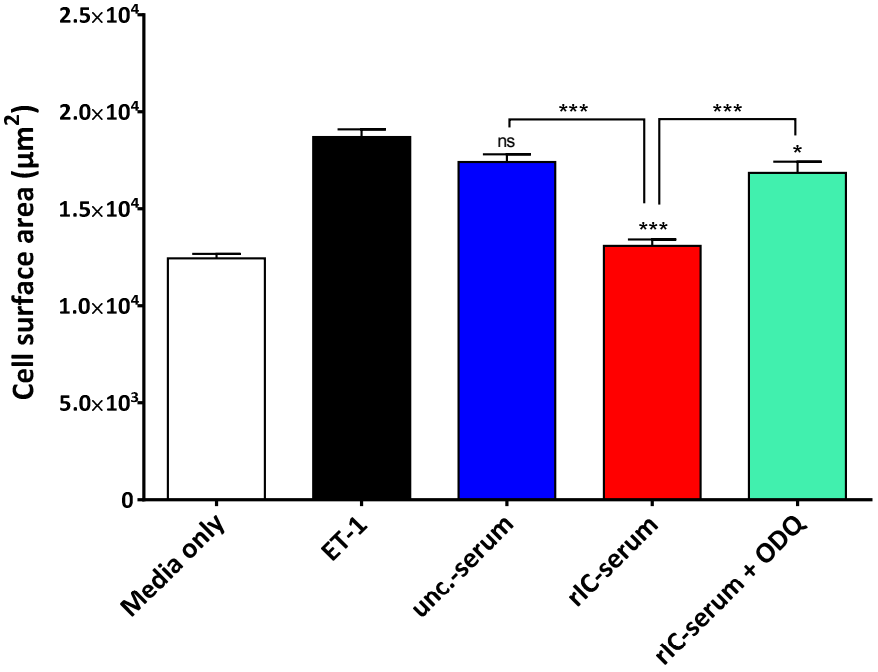


Figure 5.9 Bar chart of the effect of sGC inhibition by ODQ on rIC-serum attenuation of ET-1 induced hypertrophy of H9c2 cells. Statistical significance above bars = comparison to ET-1 only (black). Means with S.E.M, n=8/3/500, *ns* = non-significant, *p<0.05, ***p<0.001.

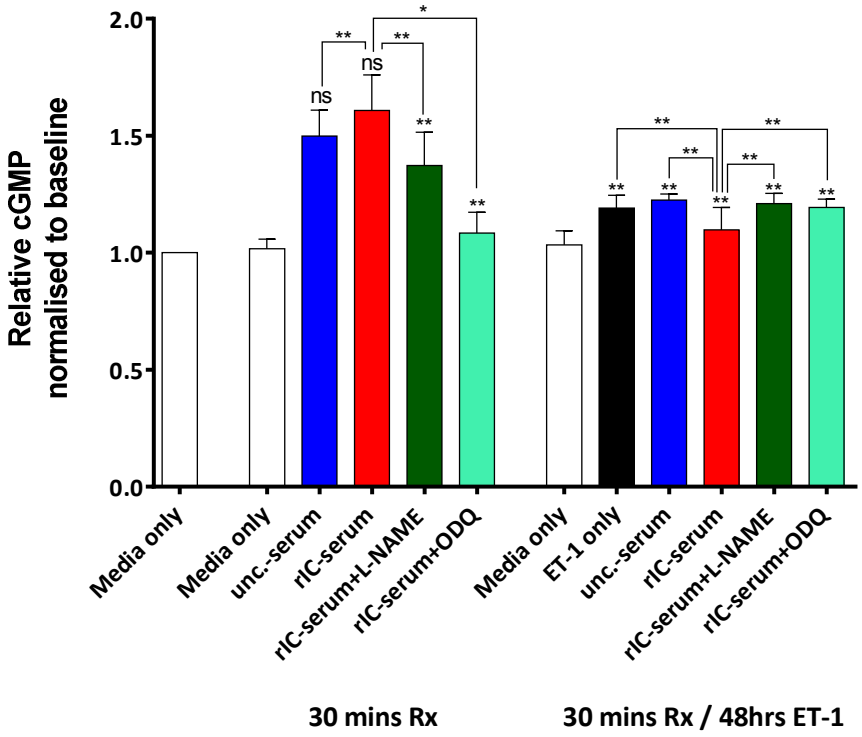


Figure 5.10 Bar chart of the effect of rIC-serum on cGMP levels after 30 minutes treatment and 30 minutes treatment followed by 48 hours ET-1 incubation. Statistical significance above bars = comparison to media only at baseline. Means with S.E.M, n=6/3, *ns* = non-significant, *p<0.05, **p<0.01, ***p<0.001.

5.5 The Role of Micro RNA 1 and 133a as a Mechanism for RIC-serum Attenuation of ET-1 Induced Hypertrophy

A number of miRs have been shown to attenuate cardiac hypertrophy and maladaptive remodelling, including the so called myo-miR (striated muscle-specific miRs), of which miR 1 and 133 are members (*see section 1.1.3.7*). However, as yet there have been no reports of a role for miRs in regulation of hypertrophy in the context of ischemic conditioning. We analysed the expression of miR-1 and miR-133a in H9c2 cells pre-treated with unconditioned human serum or rIC human serum and then subjected to 48 hours of ET-1 as previously described. In addition, to determine whether the AMPK and/or the eNOS-cGMP pathway, which was shown to be involved in the antihypertrophic action of rIC-serum, was involved in any changes in miR-1 and/or 133a, rIC-serum pre-treatment of cells was also performed in the presence of compound-C (10µmol/L), L-NAME (100µmol/L) or ODQ (10µmol/L). The results are shown in Figure 5.11.

ET-1 treatment alone did not significantly increase the expression of either miR. However 30 minutes of rIC-serum pre-treatment did significantly up-regulate miR-1 and 133a after 48 hours of ET-1 treatment (fold-change = 5.9 ± 2.0 for miR-1 and 4.3 ± 0.6 for miR-133a compared to untreated cells at baseline, both $p < 0.001$). This up-regulation by rIC-serum was completely inhibited by compound-C, L-NAME and ODQ co-treatment for both miR-1 and miR-133a (p at least < 0.01 for all). There was a significant difference between the effects of unconditioned-serum compared to rIC-serum. Unconditioned-serum pre-treatment only led a mild increase in miR-1 which was small but non-significant (fold-change = 1.3 ± 0.2, $p = 0.95$). A larger relative fold-change was

seen in miR-133a in unconditioned pre-treated cells (fold-change = 3.1 ± 0.7, $p < 0.05$). In both miR, this increased fold-change by unconditioned-serum pre-treatment was significantly smaller than that seen by rIC pre-treatment ($p < 0.001$ for both).

5.6 Summary

The results from this chapter demonstrate for the first time that a humoral agent present in the blood of healthy human volunteers, generated after 4 cycles of rIC, has strong anti-hypertrophic properties. The mechanism appears to involve short-term translocation of PKCε from the cytosolic to the membrane component, associated with increased activation of PKCε. Furthermore, activation of the AMPK/eNOS/NO/cGMP/PKG axis was implicated in this process as well as the up-regulation of the anti-hypertrophic miR-1 and 133a. Interestingly, unconditioned human serum collected before rIC of an arm appeared to have a mild anti-hypertrophic action, although this was not seen with unconditioned rat perfusate.

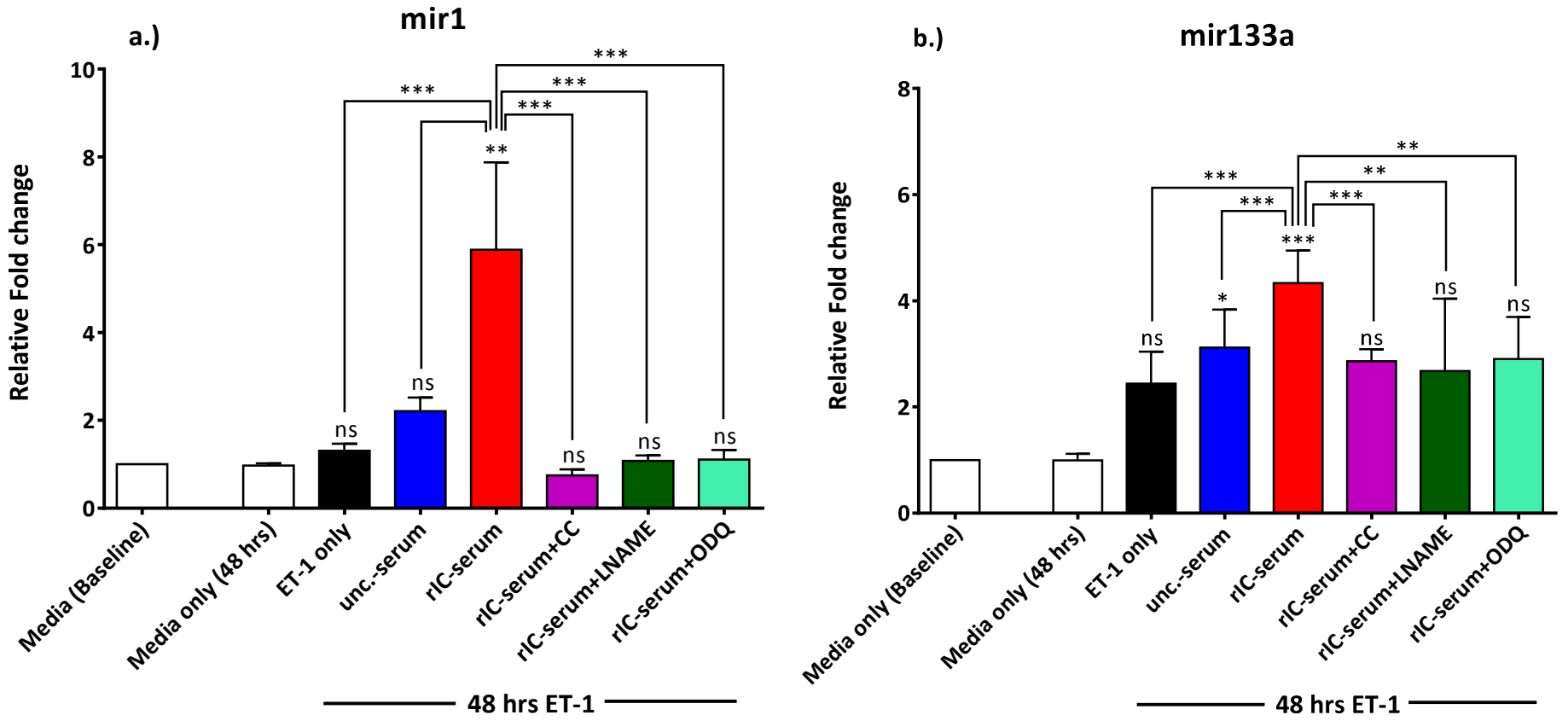


Figure 5.11a&b Bar charts of MiR-1 and miR-133a up-regulation by rIC-serum. Mean fold-change in a.) miR-1 and b.) miR-133a at 48 hours in response to ET-1 and after treatment with either unconditioned-serum or rIC-serum prior to exposure to ET-1, in the absence and presence of compound-C (10µmol/L), L-NAME (100µmol/L) and ODQ (10µmol/L), normalized to the housekeeper probe U6. *CC* = compound C. Statistical significance above bars = comparison to media only column. Means with S.E.M, n=4-8/3, *ns* = non-significant, *p<0.05, **p<0.01, ***p<0.001.

6. FUNCTIONAL EFFECTS OF REMOTE ISCHAEMIC CONDITIONING IN ET-1 INDUCED FIBROSIS IN A RAT FIBROBLAST MODEL

6.1 Chapter Introduction

Chapters 4 and 5 focused on a cellular model of ventricular hypertrophy, an important element of the initial remodelling process post-MI. Another component of remodelling, which differentiates physiological remodelling versus pathological remodelling seen post-MI, is the degree of fibrosis that takes place. In this chapter we assessed the response of neonatal rat cardiac fibroblasts in cell culture to ET-1 with regards to a number of markers of fibrosis including the production of the enzymes MMP-2, MMP-9 and TIMP-1, the expression of α-SMA gene and the degree of fibroblast to myofibroblast differentiation. ET-1 is a well-recognised trigger of cardiac fibroblast induced fibrosis[450, 451] and in preliminary experiments was shown to increase fibroblast proliferation and differentiation to myofibroblasts. In addition we analysed the effect rIC-serum had on the ET-1 triggered effects on cardiac fibroblasts.

6.2 The Effect of RIC on Fibroblast Proliferation

It was our intention to test the effect of rIC-serum on ET-1 induced fibroblast proliferation. As described in *section 3.6.1* and shown in Figure3.8, ET-1 increased fibroblast proliferation significantly from baseline both after 2 and 4 days.

Unfortunately due to bacterial contamination of the fibroblast grown in cell culture for this set of experiments, all results were abandoned as it is likely that the bacterial contamination will have significantly affected interpretation of these results.

6.3 The Effect of RIC on Fibroblast Differentiation

ET-1 induced marked differentiation from a fibroblast to a myofibroblast phenotype at day 2 compared to media only treated cells (81.3 ± 18.7% versus 6.5 ± 3.8% respectively, $p < 0.005$), as assessed by immunofluorescence staining of α-SMA, a cellular marker expressed only by myofibroblasts. This level of differentiation increased only slightly after a further 2 days of ET-1 treatment (88.3 ± 10.6% for ET-1 treatment versus 13.5 ± 5.6% for media only treatment, $p < 0.005$). 30 minutes of pre-treatment with rIC-serum prior to exposure to ET-1 significantly attenuated the degree of differentiation seen at day 2 compared to ET-1 treatment alone (29.9 ± 8.0% versus 81.3 ± 18.7% respectively, $p < 0.05$), however by day 4, differentiation levels were comparable in the rIC-serum pre-treated cells to the ET-1 only treated cells (79.3 ± 13.5% versus 88.3 ± 10.6% respectively, $p = 0.94$). 30 minutes of pre-treatment with unconditioned-serum had no effect on the degree of differentiation induced by ET-1 compared to ET-1 treatment alone at either day 2 (75.6 ± 15.2% versus 81.3 ± 18.7% respectively, $p = 0.99$) or day 4 (85.3 ± 13.4% versus 88.3 ± 10.6% respectively, $p = 0.99$). These results are shown in Figure 6.1.

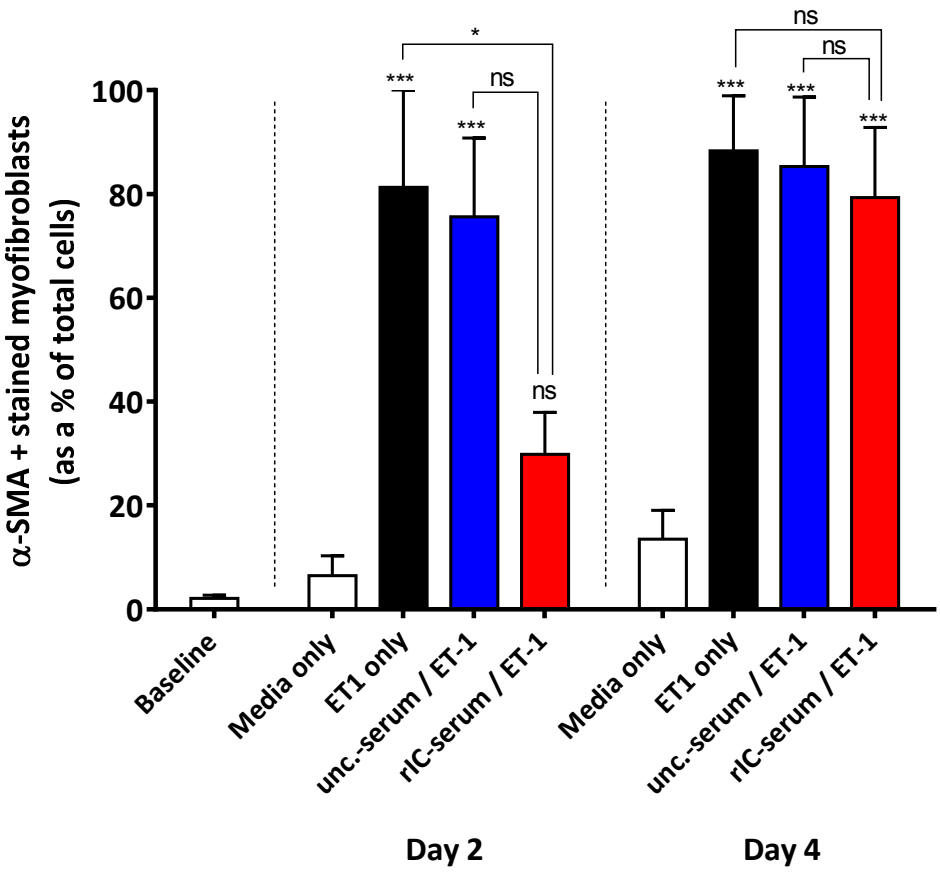


Figure 6.1. Bar chart of the effect of rIC-serum on ET-1 induced cardiac fibroblast differentiation. Percentage of myofibroblast seen in culture is shown after 2 and 4 days of ET-1 treatment. Statistical significance above bars = comparison to media only column for that day. Means with S.E.M, n=4/3/400, *ns* = non-significant, *p<0.05, ***p<0.001.

6.4 The Effect of RIC on MMP and TIMP Production by Fibroblasts

As discussed in *section 1.1.3.3*, the coordination and turnover of ECM after MI is a key component in modulating the remodelling response and the degree to which this becomes maladaptive. In this experiment we specifically focussed on 3 key enzymes that have been shown to play a significant role in this process: MMP-2 and MMP-9 whose up-regulation has been shown to positively correlate with increased levels of fibrosis[100, 101] and maladaptive remodelling and TIMP-1 whose up-regulation is known to negatively correlate with levels of fibrosis.[103]

In this experiment we analysed the effect of either ET-1 treatment alone for 4, 24 and 48 hours, 30 minutes of rIC-serum pre-treatment followed by ET-1 treatment for 4, 24 and 48 hours and 30 minutes of unconditioned-serum followed by ET-1 treatment for 4, 24 and 48 hours on the expression of MMP-2, MMP-9 and TIMP-1 by cardiac fibroblasts in culture. With regards to MMP-2 expression, there was no significant difference between the groups except between the ET-1 only treated cells and the rIC-serum pre-treated cells at 24 hours (0.9 ± 0.2ng/ml versus 2.3 ± 0.3ng/ml respectively, $p < 0.05$). With regards to MMP-9 expression, there was no significant difference between the groups at any time-points. RIC-serum pre-treatment increased MMP-9 levels at 48 hours compared to untreated cells (0.10 ± 0.04ng/ml versus 0.03 ± 0.01ng/ml respectively, $p < 0.05$) but this was not significant when compared to other treatment groups at that time-point. TIMP-1 expression was increased by rIC-serum pre-treatment when compared to ET-1 treatment alone at both 24 hours (4.1 ± 1.0ng/ml versus 1.4 ± 0.1ng/ml respectively, $p < 0.05$) and 48 hours (2.8 ± 0.4ng/ml versus 1.2 ± 0.2ng/ml respectively, $p < 0.05$). This increased level of TIMP-1 expression was mirrored by unconditioned-serum pre-treatment for both time-points. Bar charts of these results are shown in Figure 6.2a-c.

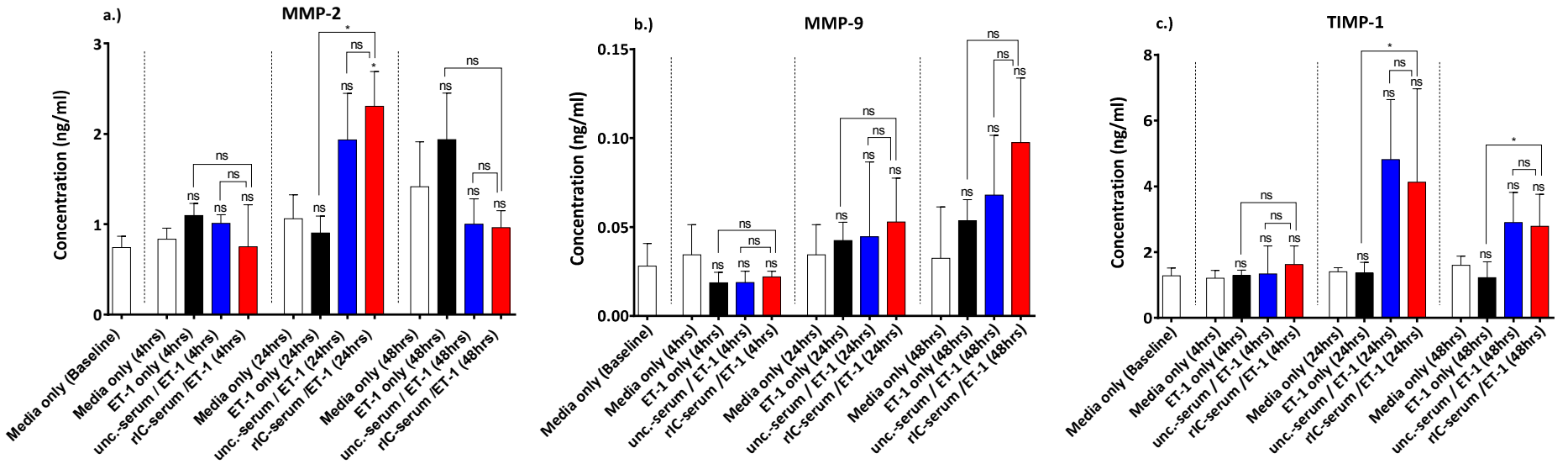


Figure 6.2.a-c. Bar charts of the effect of rIC-serum pre-treatment on concentrations of a.) MMP-2 b.) MMP-9 and c.) TIMP-1 in ET-1 treated H9c2 cells at 4, 24 and 48 hours. Statistical significance above bars = comparison to untreated cells. Means with S.E.M, n=4/3, *ns* = non-significant, *p<0.05, **p<0.01, ***p<0.001.

6.5 The Effect of RIC on Alpha Smooth Muscle Antibody Gene Expression

Differentiation of fibroblast to myofibroblasts can also be measured by the expression of α-SMA, expression of which is increased in myofibroblasts.[452, 453] In this experiment we assessed the effect of rIC-serum pre-treatment on ET-1 induced α-SMA expression by cardiac fibroblasts in culture. 48 hours of ET-1 treatment significantly increased α-ACT expression compared to media only treated cells (fold-change of 2.8 ± 0.4 for ET-1 treatment compared to media only treated cells, $p < 0.05$). Neither 30 minutes of rIC-serum nor unconditioned-serum pre-treatment attenuated the expression of α-SMA after 48 hours of ET-1 treatment. These results is shown in Figure 6.3.

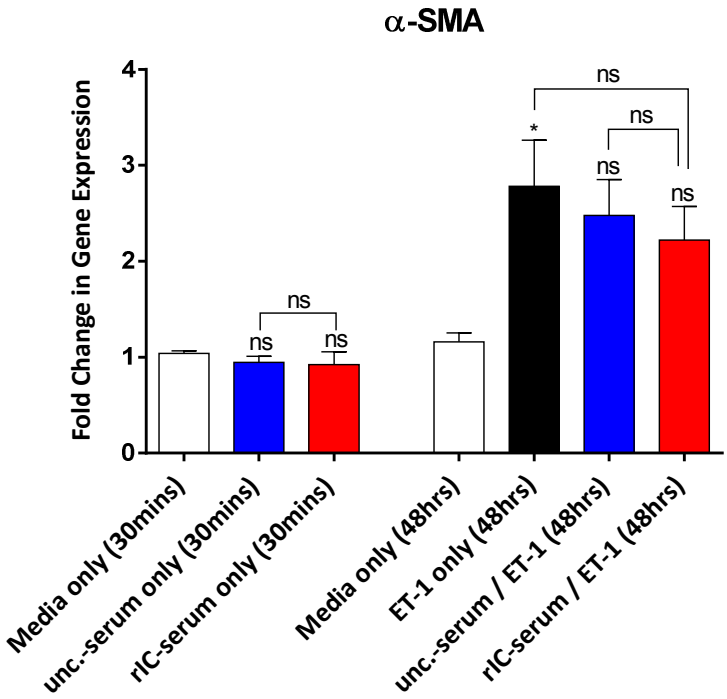


Figure 6.3 Bar chart of the effect of unconditioned and rIC-serum on relative fold-expression of α-SMA. Results are shown at baseline and after 48 hours ET-1 treatment in rat neonatal fibroblasts, normalized to the housekeeper probe ACTB. Statistical significance above bars = comparison to media only (30mins) column. Means with S.E.M, n=6/3, *ns* = non-significant, *p<0.05.

6.6 Summary

In preliminary experiments we showed that exposure of neonatal rat cardiac fibroblast in culture with ET-1 (100nM) significantly increased cellular proliferation after 2 and 4 days of treatment (see Figure 3.8). Furthermore in this chapter we have demonstrated that ET-1 induces phenotype differentiation from fibroblast to myofibroblast when assessed by levels of α-SMA cellular expression. This differentiation is partially attenuated by pre-treatment with rIC-serum followed by 24 hours of ET-1 treatment but not 48 hours of ET-1 treatment. ET-1 also increases the levels of α-SMA mRNA expression, also associated by fibroblast differentiation, however rIC-serum pre-treatment did not attenuate this effect. Furthermore both untreated and rIC-serum pre-treatment significantly increased the expression of MMP-2 compared to ET-1 treatment alone for 24 hours. Similarly both untreated and rIC-serum pre-treatment significantly increased the expression of TIMP-1 compared to ET-1 treatment alone for both 24 hours and 48 hours. Untreated and rIC-serum pre-treatment had no effect on ET-1 induced MMP-9 expression in this model.

7. DREAM – PHASE II RANDOMISED CONTROL TRIAL

7.1 Chapter Introduction

In conjunction with the basic science research set out in this thesis in the previous chapters, this chapter outlines the clinical research trial designed and coordinated in conjunction with the basic science work as already set out. The DREAM study (**D**aily **RE**mote Ischaemic Conditioning Following **A**cute **M**yocardial Infarction) is a multi-centre, first-in-man, phase II, double-blinded randomised control trial which at the time of writing is nearing the end of the active recruitment phase. The trial is a 4 centre NIHR portfolio adopted UK trial which is centrally coordinated from the Leicester Glenfield Hospital and is sponsored by the University of Leicester.

7.2 Hypothesis

Daily serial remote ischaemic conditioning applied for 4 weeks following STEMI treated with PPCI, reduces the incidence and progression of heart failure by positively influencing cardiac remodelling, independent of its effect on initial infarct sparing.

7.3 Endpoints

7.3.1 Primary Endpoint

The main primary endpoint of the DREAM study is:

The mean change in LVEF as assessed by cMRI immediately post-MI and at 4 months post-MI.

LVEF remains one the most commonly used measures of cardiac systolic function in both clinical and research scenarios. It is relatively easy to measure and has been shown to provide a surrogate of cardiac output.[454, 455] However care must be taken in situations that significantly raise cardiac afterload, such as arterial hypertension or aortic stenosis, as this can depress the LVEF. Furthermore caution is advised in the context of severe mitral regurgitation and other causes of raised preload where despite a normal LVEF, overall cardiac output is reduced due to increases in EDV.[456] In the context of MI, a reduced LVEF as assessed by echocardiography is associated with a poor outcome.[457]

In the DREAM study, screening echocardiography was used in the assessment of LVEF. Participants were only recruited if LVEF measured below 45% (at least moderately impaired systolic function). Echocardiographic assessment of LVEF was performed by measuring EDV and ESV in the apical 4 chamber and apical 2 chamber views to obtain the modified Simpson's Biplane LVEF.[458] Figure 7.1 shows an anonymised example 2D echo to illustrate this. Simpson's Biplane LVEF is calculated by the following formula:

Simpson's Biplane LVEF (%) = (Apical 4 chamber [(EDV - ESV) / EDV] x 100) + (Apical 2 chamber [(EDV - ESV) / EDV] x 100) / 2

For the primary endpoint measures the cMRI measurements of LVEF were made by QMass 7.1 (Medis, Leiden, Netherlands) software platform. Measurements of the LV endocardial and epicardial borders were made using a series of short axis cuts 10mm apart along the length of LV from the basal/mitral level to the apical level performed in diastole and systole to assess EDV and ESV.[459-461] Figure 7.2 shows an anonymised example short axis series of the LV. The same calculation as used for echocardiography

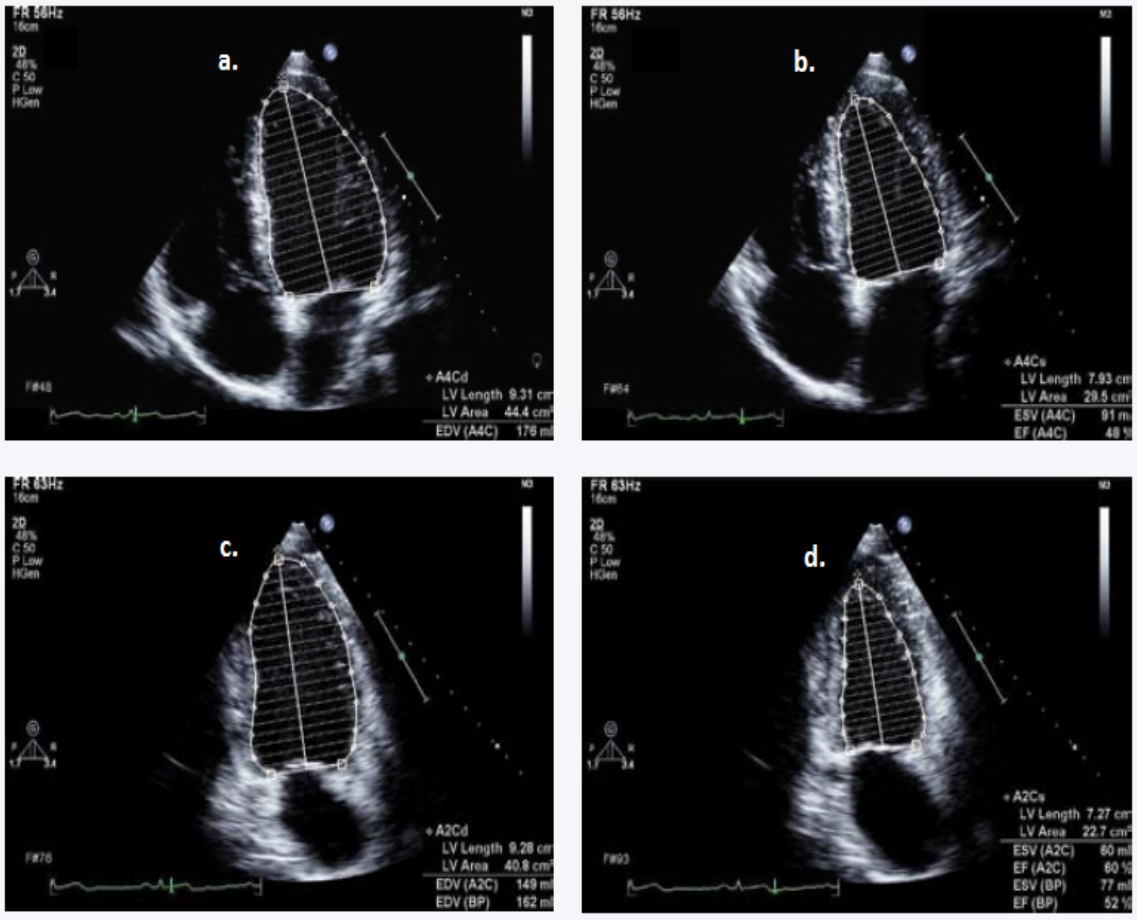


Figure 7.1 2D echocardiographic measurement to obtain LVEF by Simpson's Biplane technique. The endocardial border of LV is traced manually and computer software generates a series of parallel discs which are collated to assess 3D volume and allow calculation of LVEF. Views: a.) Apical 4 chamber EDV b.) Apical 4 chamber ESV c.) Apical 2 chamber EDV d.) Apical 2 chamber ESV. Adapted from Wharton *et al.,* (2015).[462]

was used for cMRI to calculate LVEF from EDV and ESV across all short axis slices. Compared to conventional 2D echocardiography, cMRI has been shown to have much greater specificity, less intra and inter-observer variability and higher inter-study reproducibility for the accurate assessment of LVEF and therefore this was chosen as the modality for analysis of the primary endpoint.[463-465] In addition, cMRI allows for the assessment of a more accurate assessment of infarct size, MVO and fibrosis, indices that were measured as secondary outcome endpoints.[466]

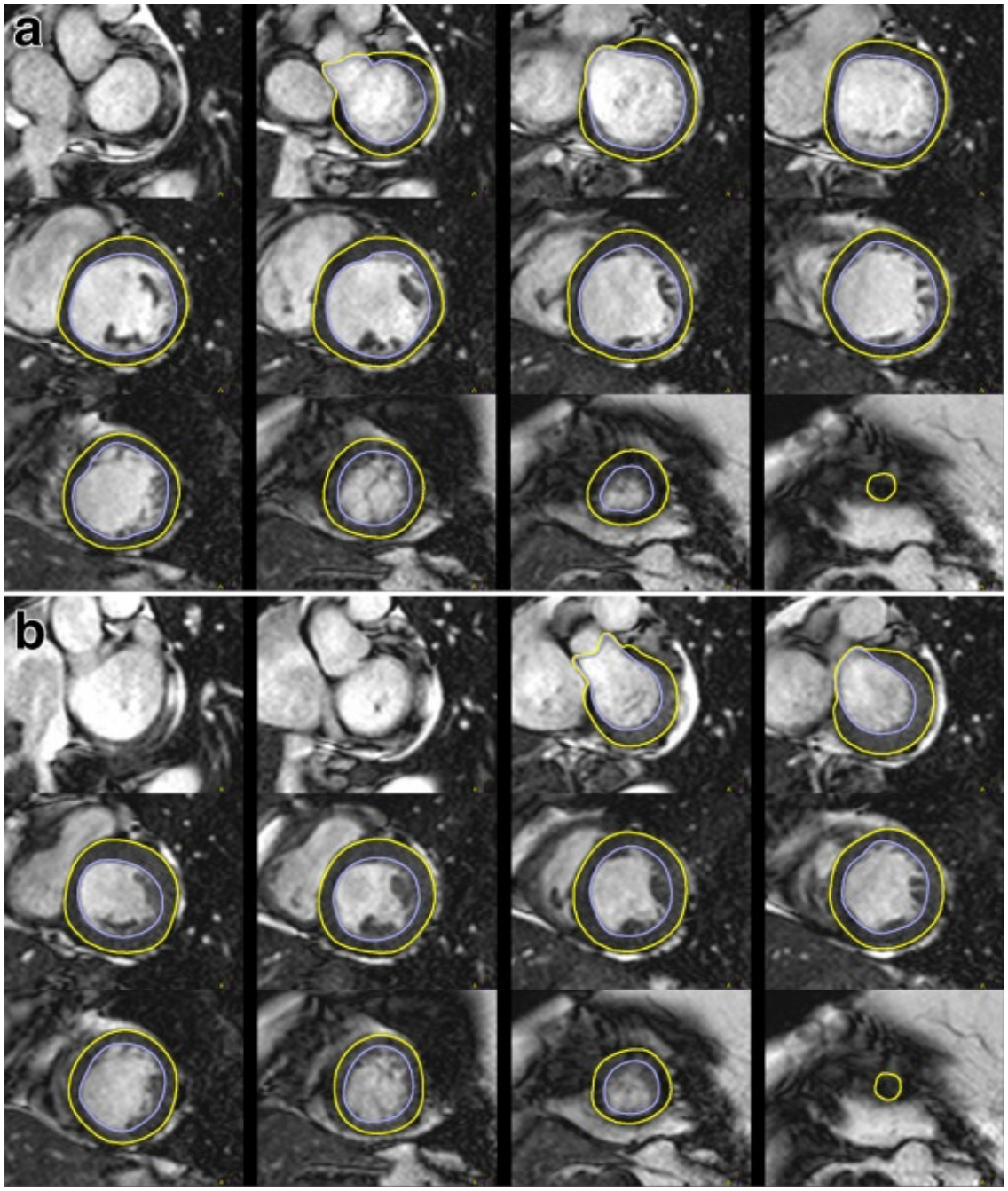


Figure 7.2 Cardiac MRI short axis LVEF assessment. 12 short-axis slices along the LV in a.) diastole and b.) systole. The images are read horizontally from LV base in the top left corner down to LV apex in the bottom right corner in each image. The epicardial delineation is shown in yellow and the endocardial delineation is shown in blue. Adapted from Schulz-Menger *et al.,* (2013).[467]

7.3.2 Secondary Endpoints

7.3.2.1 CMRI Secondary Endpoints

As well as assessing LVEF at baseline and 4 months, a number of other cMRI endpoints are used. The first of these are standard LV chamber dimensions and volumes including LVEDV, LVESV, LVEDD and LVESD, both raw and indexed to body surface area. These dimensions and volumes are obtained as described in *section 7.3.1* as they are used to calculate LVEF. In addition, T1 mapping techniques (see *section 7.6.1*) were used in combination with gadolinium contrast to assess levels of fibrosis, MVO and ascertain overall infarct size.

7.3.2.2 Blood Biomarker Secondary Endpoints

Blood biomarkers associated with heart failure and ventricular remodelling were measured from samples taken at baseline and 4 months. The first of these biomarker was N-terminal pro-hormone of brain natriuretic peptide (NT-proBNP). This is a well-established biomarker of heart failure in current clinical use typically used to evaluate treatment efficacy in patients with established heart failure. It has also shown to provide prognostic information in patients undergoing acute remodelling post-MI and hence was chosen for this study.[468] In addition, MMP-9 and TIMP-1 levels were also analysed from samples collected at baseline and 4 month follow-up. Both these enzymes are well-established biomarkers of fibrosis and inflammation in the context of cardiac remodelling and acute heart failure and are discussed in more detail in *section 1.1.3.3*.[469, 470]

7.3.2.3 Health Questionnaire Secondary Endpoint

The Kansas City Cardiomyopathy Questionnaire (KCCQ) score was used as a qualitatively assessment of the effects of heart failure on the participant. It was administered by a member of the research team at 1 month (after 28 days of conditioning) and 4 months. It is a well validated and commonly used 23 point questionnaire which has been shown to be valid both in the context of HF-REF and HF-PEF.[471, 472] It sets out to quantify participants' physical capabilities, the frequency, severity and onset of symptoms, how these symptoms impact on social interactions as well as addressing the participants' understanding of their condition and giving an overall measure of their quality of life. The full KCCQ is detailed in the appendix.

7.4 Study design

This first in man study aims to recruit a total of 72 STEMI patients who have had successful PPCI who have an ejection fraction of less than 45% on their screening echocardiogram at 48-72 hours post infarct. To incorporate a drop-out rate of 20%, the total recruitment number will be 90. Participants are recruited from one of 4 sites; the Leicester Glenfield Hospital, Kettering General Hospital, the Royal Free Hospital London and the Royal Derby Hospital. Once a patient is identified and deemed to pass the inclusion/exclusion criteria, they are approached by a member of the research team who explains the study to them, provides them with a participation information sheet and allows them adequate time to read the information sheet, ask questions and discuss with family members and/or friends. A full list of the inclusion and exclusion criteria are detailed in Table 7.3. After signing the trial consent form agreeing to participate in the

trial, participants are randomised into the treatment or control arms (see *section 7.5* for more details). Participants randomised to the treatment arm receive daily rIC for 28 days following MI and participants randomised to the control arm will receive sham conditioning for 28 days following MI, starting at day 3 post-MI. Conditioning is applied with the use of the 'autoRIC™ device', a CE marked licenced product developed by CellAegis Devices Inc. (see Figure 2.3) to administer an rIC stimulus or a placebo stimulus in the first instance with the option of using a standard electronic sphygmomanometer as a back-up manual device if required (see Figure 2.2). The cuff with the machine pod in situ is applied to the non-dominant arm of the participant. The rIC stimulus involves 4 cycles of 200mmHg systolic cuff inflation and deflation lasting a total of 40 minutes i.e. each period of inflation/deflation lasts 5 minutes as previously described in chapter 2 and shown in Figure 2.1. The placebo stimulus involves 4 cycles of ~10mmHg total cuff inflation and deflation for 40 minutes. After 28 days conditioning ceases and the participant returns the device to the study investigators. The participant returns at 4 months for a repeat cMRI, further blood tests (including FBC, U+E and heart failure biomarker NT-proBNP, MMP9 and TIMP1) and completion of the quality of life questionnaire (KCCQ). After this 4 month visit their involvement in the study ends. An overview of the participant journey in the study is given in Figure 7.1. Active participant recruitment started in November 2012 with an expected completion date for recruitment of October 2016 and estimated closure of the study with dissemination of findings by early 2017.

Inclusion criteria
LVEF < 45% on baseline echocardiogram
First STEMI
Successful revascularisation by PPCI (improved TIMI flow in culprit vessel)
Able to attend research centre for baseline and 4 month follow-up appointment
Competent to consent
Exclusion criteria
<18 years of age
ICD or CRTP/D in-situ
Prior history of heart failure (HF-REF or HF-PEF)
Haemoglobin < 11.5 g/dl
Creatinine > 200µmol/L and/or eGFR<30ml/min/m^2
Known malignancy or other comorbid condition which in the opinion of the investigator is likely to have significant negative influence on life expectancy
Significant complications or illness following MI including: • Severe cardiogenic shock • Ischaemic mitral regurgitation • Ventricular wall rupture • Severe pericarditis Recurrent malignant arrhythmias e.g. as VT/VF
cMRI contraindicated. Reasons include: • Permanent pacemaker or ICD • Brain Aneurysm Clip • Implanted neural stimulator • Cochlear implant (specific implant must be checked that it is MR safe) • Ocular foreign body (e.g. metal shavings) unless removed • Other implanted medical devices: (e.g. Swan Ganz catheter) • Insulin pump • Metal shrapnel or bullet • Renal dysfunction (eGFR <30ml/min/m^2) cMRI unobtainable due to claustrophobia or significantly raised BMI
Further planned coronary interventions
Enrolment in another clinical trial

Table 7.1. Inclusion and Exclusion criteria of the DREAM study.

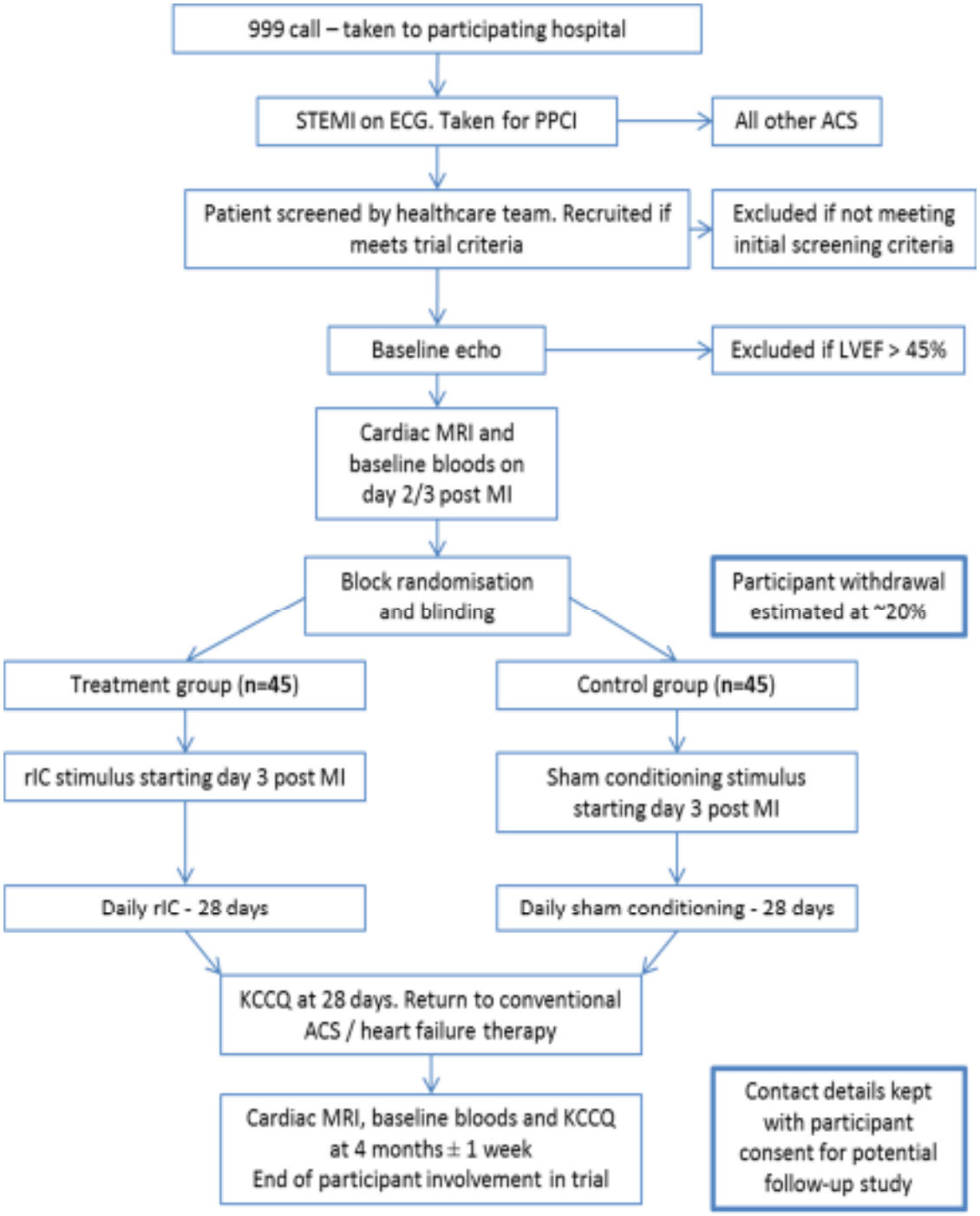


Figure 7.3. Flow diagram of participant journey within the DREAM study.

7.5 Randomisation

The participants are randomised to either the treatment or control arm using block randomisation to control for age (<45 or ≥45), gender (male or female) and infarct location (anterior infarct versus all other infarct locations). This is done using the online randomisation service 'Sealed Envelope' (http://www.sealedenvelope.com). The study is double-blinded in that the participants will not be aware which group they are in and the research team members tasked with analysing the primary outcome cMRI data will similarly be blinded to arm the participant whose cMRI they are analysing belongs to. However members of the research team involved in recruiting participants and overseeing the use of the autoRIC™ device will not be blinded to which arm the participant is receiving, because of the serial numbers on the machines and easy assessment of whether a device is inflating to 200mmHg or 10mmHg.

7.6 Assessment and Follow-up

The timelines for the study procedures are shown in the Table 7.2. Baseline denotes the first day of conditioning which is day 3 post-MI. Follow-up is at 4 months (± 2 weeks). Participants recruited at Glenfield Hospital, Kettering General Hospital and the Royal Derby Hospital attend the Glenfield Hospital for baseline and 4 month venepuncture and cMRI. All participant require a 20 guage cannula or larger for the cMRI. Participants recruited at the Royal Free Hospital have all procedures performed locally. Blood samples obtained at the Royal Free are processed locally and the serum and plasma is shipped on dry-ice to the Glenfield Hospital for storage and experimentation. CMRI scans performed at the Royal Free Hospital are anonymised and sent via secure post on

a compact-disc to Glenfield Hospital for analysis centrally. After undergoing the 4 month follow-up investigations, participants have completed the trial. However consent has been gained by all participants to retain their contact details in a secure and linked-anonymised fashion so that they may be contacted in the future if further studies are planned or follow-up data is required such as 5 or 10 year Major Adverse Cardiac and Cerebral Event (MACCE) outcome data.

Activity	**Time-point and Location**			
	Day 0+ Baseline *(Inpatient)*	**Day 3-31 *(Participant's home)***	**Day 31 (*Participant's home)***	**4 months ± 2 weeks *(Research facility)***
Randomisation	+			
rIC	+ (if still an inpatient on day 3)	+		
Collection of rIC device from participant			+	
Blood tests	+			+
Screening Echo	+			
Cardiac MRI	+			+
KCCQ questionnaire			+	+

Table 7.2. Timeline and location of study procedures. Clock starts at day 0 = day of MI, specifically at time of PPCI. RIC starts on day 3, which may be in hospital or in the participant's own home depending on the speed of discharge following the participant's MI. Baseline cMRI should ideally take place on day 2 or 3 but can take place anytime between day 1 to day 5 (from 24 up to 120 hours post PPCI).

7.6.1 Cardiac Magnetic Resonance Imaging

CMRI is performed at baseline following PPCI ideally 48-72 hours post procedure (usually on the day of discharge for uncomplicated cases) although where there is an anticipated delayed to obtain cMRI e.g. at the weekend or bank holidays or where the participant cannot have a cMRI e.g. if they have an intra-aortic balloon pump or temporary wire insitu, the participant are scanned at any time between 24 to 120 hours of PPCI. Any delay past this time leads to the participant being withdrawn from the study. We have ensured that no systematic bias between the treatment and placebo group exists as to the day post PPCI they undergo their baseline cMRI scan as this could influence the calculated infarct size.

The cMRIs performed at Leicester Glenfield hospital are performed on 3-Tesla platforms (4 Siemens Avanto, Erlangen, Germany, and 1 Philips Intera, Best, the Netherlands) and the cMRIs performed at the Royal Free hospital are performed on the 1.5-Tesla equivalent machine. Both machines utilise a 6-channel phased-array cardiac receiver coil and were supervised at the time of the scan by Cardiologists with a specialist interest in cMRI allowing for optimisation of image acquisition. An identical protocol is used for the baseline and follow-up cMRI scans as shown in Table 7.3. The participants are required to take breath holds during image acquisition. Retrospective ECG gating is used to time acquisitions and cine imaging to obtain functional information pertaining to LV and RV dimensions and function. Longitudinal relaxation time (T1) mapping is used and is defined as the time taken for longitudinal proton magnetization to recover approximately 63% of its equilibrium value.[473] T1 mapping utilising late gadolinium enhancement (LGE) allows for an assessment of myocardial fibrosis, oedema

and infarct size.[474] The Modified Look Locker Inversion Recovery (MOLLI) technique is used to reduce issues with participant movement and the prolonged acquisition time.[475] The gadolinium contrast agent Dotarem® (gadoterate meglumine) is used at a dose of 0.15mmol/kg and LGE images are obtained 10 minutes after contrast injection. Each scan takes approximately 50 minutes.

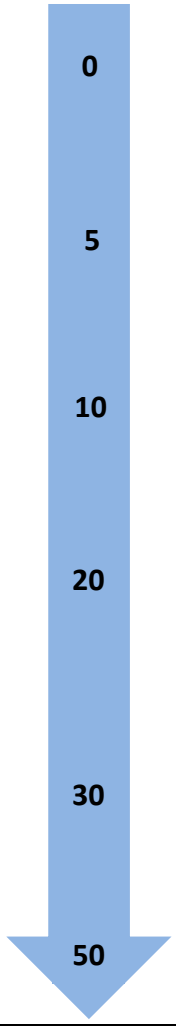
cMRI Acquisition Procedure	Time (mins)
Localisers and Pre-scan	
3 plane, low resolution slices to plot scan slices and align participant in the magnet iso-centre. Calibration involving shimming, coil tuning and matching, centre frequency calibration, transmitter and receiver gain adjustment and dummy cycle stimulation	
Cine imaging (4, 2 & 3 chamber long axis)	
8mm ST, 25mm gap, matrix 208x256, FOV optimized for participant **Assesses LV and RV function**	
Pre contrast T1 mapping (short and long axes)	
8mm ST, 2mm slice gap, MOLLI utilised	
0.15 mmol/kg Gadolinium (Dotarem) injection	
Cine imaging (short axis stacks)	
8mm ST, 25mm gap, matrix 208x256, FOV optimized for participant **Assesses LV dimension and function**	
Post contrast T1 mapping with LGE (4, 2 & 3 chamber long axis and short axis)	
10mm ST, 0mm slice gap, matrix 208x256, FOV optimized for participant, TR = R wave to R wave interval -100ms **Assesses Infarct Size and degree of MVO**	

Table 7.3. DREAM study cMRI Protocol. FOV = field of view, LGE = late gadolinium-enhanced, LV = left ventricular, MOLLI = modified look locker inversion recovery, MVO = microvascular obstruction, RV = right ventricular, ST = slice thickness, TR = repetition time.

7.7 Potential Risks, Benefits and Inconveniences

7.7.1 Potential Risks and Inconveniences

Possible risks of rIC include discomfort, paraesthesia and a mild rash on the arm on which the device is inflated. The protocol proposed may cause low-grade discomfort but is not considered dangerous. We have applied this manoeuvre locally in a previous study with no significant complications (REC reference 10/H0405/52). Other groups report no complications of note.[272, 273] To date there have been no reported adverse events (AEs) related directly to the inflation of the blood pressure cuff itself. There is minor discomfort associated with taking blood and a small risk of bruising. Bloods are taken by appropriately trained and experienced health practitioners, according to local policy. Up to 30ml of blood is taken on 2 separate occasions.

7.7.2 Potential Benefits

Although there is no human trial that indicates a direct clinical benefit to the participant of repeated rPostC, there may be a positive effect on the severity of heart failure as suggested by prior cellular and animal research as discussed in this thesis. Participants benefit from an echocardiogram and two cMRIs which are not routinely performed as part of clinical care.

7.8 Participant Withdrawal

Participants may leave the study at any time for any reason. The investigators can decide to withdraw a participant from the study if the participant is re-hospitalized with a further MI or serious condition that requires a protracted inpatient admission.

7.9 Safety Reporting

7.9.1 Adverse Events

An AE is defined as any undesirable experience occurring to a participant during a clinical trial. An adverse device event (ADE) is defined as any untoward and unintended response to a medical device. A serious adverse event (SAE) is an untoward medical event or effect that:

1. results in death (at the time of the event)
2. is life-threatening (at the time of the event)
3. requires inpatient hospitalization or prolongation of existing hospitalization
4. results in persistent or significant disability/incapacity
5. is a congenital anomaly/birth defect
6. requires intervention to prevent permanent impairment or damage

Medical judgement is exercised by the trial coordinators in deciding whether an AE is serious in other situations. Important AEs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above are also considered serious and reported as such.

7.9.2 Reporting Procedures

Participants are given contact details for the research team should any unforeseen events occur. Monitoring is performed in accordance with local guidance. All AEs are reported to the designated representative at the Leicester Cardiovascular BRU as well as to CellAegis Devices Inc. Any questions concerning AE reporting are directed to the

Chief Investigator in the first instance. Non serious AEs, whether expected or not, are not recorded. For SAEs, an SAE form is completed within 24 hours. However, relapse and death due to MI or its related sequelae and hospitalisations for elective treatment of a pre-existing condition are not reported as unexpected SAEs but are reported as expected SAEs.

SAEs are reported to the accredited ethics committee and sponsor (University of Leicester) according to the requirements of the ethics committee as well as to CellAegis Devices Inc. Reports of related and unexpected AEs are submitted within 2 days (if there is a risk of death or serious harm to the patient) or 7 days of the Chief Investigator becoming aware of the event. The Chief Investigator also notifies the Sponsor of all SAEs.

7.10 Statistics and Data Analysis

7.10.1 Sample size

Previous researchers from our group have charted the change in LVEF after MI using cMRI in 85 patients. Patients with a LVEF < 45% had an average LVEF of 36.5 ± 6.7% immediately after MI which increased to 44.8 ± 8.6% after 4 months (unpublished data). This data was comparable to previously published recovery rates of LVEF after MI and may in part reflect recovery from stunning in the immediate post-MI period.[476] This informed the power calculation to estimate the sample sizes needed to detect a 5% absolute difference in LVEF between treatment and control group for this study.

We based our power calculation on requiring an additional 5% absolute improvement in LVEF above what is to be expected by natural recovery. We assumed LVEF to be normally distributed. Power calculations were calculated using the

biomath.info online calculator with an alpha value of 0.05 and a power level of 0.8 using a one-sided test. The power calculation were checked by departmental statistician Dr Chris Nelson. We estimated a sample size of 36 in each group to achieve adequate power, giving a total study sample size of 72. To allow for a dropout rate of up to 20%, an estimated 90 participants will need to be recruited in total.

7.10.2 Data analysis

Data will be analysed to assess the effect of daily rIC both within groups and between groups for primary and secondary outcome data as well analysis of subgroups of cMRI, blood and questionnaire data. Data will be assessed to see if it is normally distributed using histogram analysis and the Kolmogorov-Smirnov test and if so, the principal statistical test used will be a one-way ANOVA to analyse both primary and secondary outcome data. Where ANOVA is used and is significant, subsequent p values will be corrected with Tukey correction for repeated measures.[338] Where data is not normally distributed, the data will be analysed using the Mann–Whitney U-test and expressed as medians (25%-75% interquartile range).

7.11 Roles and Responsibilities

Professor Sir Nilesh Samani is the Principle Investigator and head of the Cardiovascular BRU. I act as the research team coordinator and investigator and I am involved in participant recruitment and enrolment, monitoring and post recruitment analysis and dissemination of data. For a full list of all other individuals involved in the DREAM study including their defined roles and responsibilities, please refer to the appendix.

7.12 Regulatory Issues

7.12.1 Conduct and Confidentiality

The study is conducted according to Good Clinical Practice and the Declaration of Helsinki 1964. All protocol amendments have been approved by the research ethics committee (REC). Confidentiality is maintained at all times by labelling all paper and electronic records and laboratory specimens with a participant specific code. However each participant's name, study number, randomisation status, date of birth, address, contact telephone number and GP details is collected on the case report form to allow contact of participants during the course of the trial. This is done on the Redcap computer system (http://project-redcap.org/) which is a secure system kept on the University of Leicester central server with restricted access. Paper documents are stored in a locked filing cabinet in the Biomedical Research Unit, Clinical Research Facility offices in Glenfield Hospital, which require both swipe and keycode access to gain entry. Study data is also stored on University of Leicester computers in a drive accessible by the investigators in linked anonymous form. University computers are password protected. Clinical information is not released without written permission of the participant, except as necessary for monitoring by designated research regulatory authorities or the sponsor of the trial. Copies of the data are sent to the GP with the participants consent by post.

7.12.2 Sponsorship and Ethical Approval

The University of Leicester acts as the Sponsor for this study. The study has full approval from the local NHS Research Ethics Committee and Trust R&D approval (see appendix).

7.12.3 Consent

Consent to enter the study is sought from each participant only after a full explanation has been given, a patient information leaflet provided and time allowed for consideration. Signed participant consent is obtained (see appendix for participant information sheet and consent form templates). The right of the participant to refuse to participate without giving reasons is always respected. After the participant has entered the study the clinician remains free to give alternative treatment to that specified in the protocol at any stage if he/she feels it is in the participant's best interest, but the reasons for doing so is recorded. In these cases the participants remain within the study for the purposes of follow-up and data analysis. All participants are free to withdraw at any time from the protocol treatment without giving reasons and without prejudicing further treatment.

7.12.5 Indemnity

The University of Leicester holds negligent harm insurance policies which apply to the design and management of this study and the University Hospitals of Leicester NHS Trust holds standard NHS Hospital Indemnity and insurance cover with NHS Litigation Authority for NHS Trusts in England, which apply to the conduct of this study.

7.12.7 Funding

Part funding has been secured from the Masonic Samaritan Fund to support the work of the research nurse. The Leicester Cardiovascular Biomedical Research Unit meets all

additional costs that are required under the Cardiovascular Investment theme. Participants receive no incentive payment for taking part.

7.12.8 Audits

The study has been subjected to an inspection and audit process by the University of Leicester under their remit as sponsor on August 26th 2015 to ensure adherence to good clinical practice.

7.13 Study Management

Study data is entered on the Leicester Cardiovascular BRU administered RedCap database service. Participant communication data e.g. NHS number, address, telephone number, next of kin and GP details are kept on the Leicester Cardiovascular BRU CiviCRM system. Participant communication data collected from Kettering General Hospital, the Royal Free Hospital and the Royal Derby Hospital are paper-based and secure scans of these paper documents are relayed to the study team at Glenfield via nhs.net email accounts. Those sites without access to RedCap also send study data to Glenfield hospital via nhs.net email accounts. Tissue samples are processed using the CaTissue software[477] administered by the Leicester Cardiovascular BRU and tissue samples are stored in barcoded tubes in specially tracked and designated -80°C freezers. Data belongs to the University of Leicester and will be kept in accordance with GCP for 5 years once the trial is complete and 15 years with regards to data documented in the participant's medical records. The chief investigator will notify the ethics committee and sponsor at the end of the study within a period of 8 weeks. The end of the study is

defined as the last patient's last visit.

7.14 Publication Policy

The results of the trial will be published in the scientific literature. All active members of the study will co-author any publications. In addition an end of study report will be submitted to the accredited EC and sponsor within a year of the end of the study.

7.15 Demographic, Clinical Screening and Preliminary Secondary Outcome Data of DREAM Study Participants

At the time of writing, a total of 1159 participants have been screened across the four hospital sites, 87 of which have been recruited, of which 8 have been withdrawn or dropped out before completion of the study. A total of 64 participants have completed the study with a further 15 currently taking part. The estimated final completion date of active participant involvement is 31st August 2016. Table 7.4 outlines the demographic, screening, KCCQ and 4 month MACCE outcome data of the 79 participants that have completed their participation in the study or are currently taking part. The full results of the study including primary outcome cMRI and secondary outcome cMRI and blood data will not be analysed until the final participant has completed the study to avoid bias and therefore are not discussed in this thesis.

Participants in each treatment arm were well matched by demographic data and clinical history. Furthermore there was no significant differences in factors that may influence infarct size and acute remodelling such as pain to balloon time (median of 150 minutes in the treatment arm and 180 minutes in the placebo arm, p = 0.11), culprit

lesion (the majority of which were LAD lesions; 65.9% in the treatment arm and 78.9 in the placebo arm, p = 0.22) and LVEF on baseline screening echocardiogram (mean of 38.1 ± 5.3% in the treatment arm and 38.9 ± 5.3% in the placebo arm, p = 0.47). The only significant difference between groups was seen in the maximal high sensitivity troponin T level recorded which was significantly higher in the placebo group with a mean of 39.9 ± 2.7μg/l versus 26.8 ± 3.3μg/l, p = 0.011. However the level of Troponin T in both groups was significantly raised compared to the threshold common used for aiding in the diagnosis of ACS which is around 0.01-0.015μg/l.[478, 479] Furthermore the degree to which the absolute level of Troponin T correlates with infarct size or the degree of myocardial damage remains contentious.[480] It must also be noted that although the aim was for Troponin T to be recorded at 3 hours after pain, on many occasions the levels were often taken at any point before, during or post PPCI and hence significant sampling error exists. With regards to qualitative improvement in patient's symptoms and quality of life, there was no difference in the change in overall KCCQ score from 1 month to 4 months between the two groups with both groups showing a modest improvement rising by a mean of 3.5 ± 2.4 points in the treatment arm and 3.2 ± 1.9 points in the placebo arm, p = 0.55. Furthermore there was no difference in recorded MACCE outcomes at 4 months between the groups although the overall event numbers were low likely due to the short follow-up time.

Participant Characteristics	Total (n = 79)	Treatment arm (n = 41)	Placebo arm (n = 38)	P Value
Age*	64.0 ± 13.2	63.8 ± 13.2	64.2 ± 13.6	0.87 (ns)
Gender (%)				
Male	86.1	87.8	84.2	0.54 (ns)
Female	13.9	12.2	15.8	
Ethnicity (%)				
White British	89.5	85	91.9	0.64 (ns)
South Asian Origin	7.9	10	8.1	
Other White Origin	1.3	2.5	0	
Other Mixed Origin	1.3	2.5	0	
BMI				
Median	28	28	28.1	0.49 (ns)
Interquartile range	25.8-31	26-31.7	25.5-29.9	
Smoking status (%)				
Current / Ex	77.2	75.6	78.9	0.79 (ns)
Never	22.8	24.4	21.1	
Medical History (%)				
Hypertension	27.8	34.1	21.1	0.13 (ns)
Hyperlipidaemia	15.2	19.5	10.5	0.35 (ns)
NSTEMI/Unstable Angina	0	0	0	-
Stable Angina	1.3	0	2.6	0.48 (ns)
Stroke/TIA	2.5	4.9	0	0.49 (ns)
Diabete Mellitus	15.2	9.8	21.1	0.22 (ns)
Peripheral Vacsular Disease	2.5	4.9	0	0.49 (ns)
COPD	5.1	2.4	7.9	0.35 (ns)
Maximal Troponin T (µg/l)*	32.1 ± 2.0	26.8 ± 3.3	37.9 ± 2.7	0.011 (*)
Pain to Balloon Time (mins)				
Median	162	150	180	0.11 (ns)
Interquartile range	112.5-295	116-254	109.8-418.8	
Culprit Vessel (%)				
LAD	72.2	65.9	78.9	0.22 (ns)
Other	27.8	34.1	21.1	
LVEF on baseline echo (%)*	38.5 ± 5.3	38.1 ± 5.3	38.9 ± 5.3	0.47 (ns)
Inpatient stay duration (days)*	3.6 ± 2.7	3.9 ± 3.6	3.4 ± 1.0	0.35 (ns)
Δ KCCQ score (1 to 4 months)§	3.4 ± 2.5	3.5 ± 2.4	3.2 ± 1.9	0.55 (ns)
MACCE event at 4 months§				
ACS	2	1	1	1 (ns)
Stroke/TIA	1	1	0	1 (ns)
Cardiovascular Mortality	0	0	0	-
Heart failure hospitalisation	1	1	0	1 (ns)
Other cause hospitalisation	7	5	2	0.43 (ns)

* Mean ± SD, § n = 62 (completed study)

Table 7.4. Table of the demographic, screening and preliminary secondary outcome data of the participants enrolled in the DREAM study to date. Ns = non-significant, *p<0.05, **p<0.01, ***p<0.001.

7.16 DREAM Study Summary

RIC, in the form of 4 five minutes cycles of ischaemia and reperfusion administered on the forearm, now has proven benefits in reducing the final infract size associated with STEMI when used at or around the time of PPCI.[273, 274, 276, 318, 481-483] The DREAM study has been specifically designed to assess the effects of rIC, independent of its established protection afforded against infract size, by initiating 4 weeks of daily rIC starting 3 days after the ischaemic insult thereby specifically targeting acute remodelling and not infract size per se. The trial is nearing completion and will hopefully shed light on whether rIC is a clinically relevant tool in targeting acute remodelling post-MI.

8. DISCUSSION

8.1 Summary of Findings

The role of rIC in attenuating I/R both in preclinical and clinical studies is well established. However, the ability of rIC to attenuate acute cardiac remodelling that occurs post-MI has garnered little attention to date. Wei *et al.,* (2011) showed, for the first time, that adult rats undergoing repeated bouts of rIC around the time of MI had increased survival with reduced infarct size, attenuated LV dimensions and reduced markers of inflammation and fibrosis with a dose-dependent effect that altered remodelling and survival over and above the attenuation of reperfusion injury.[313]

The aims of the work set out in this thesis was to examine whether rIC-serum taken from human volunteers could attenuate the response of humoral agents known to be up-regulated and involved in acute cardiac remodelling post-MI using two cell culture models of remodelling. We specifically focused on two key processes that can tip the balance of acute remodelling in favour of a maladaptive process, namely cardiomyocyte hypertrophy and fibroblast induced fibrosis. Hypertrophy is the first herald of maladaptive remodelling and subsequent myocardial thinning and myocyte slippage. Post-MI the main triggers of hypertrophy are stretch, brought about by the changing haemodynamics in the heart and neurohormonal processes including activation of Ang II, adrenaline and endothelin receptors. Fibrosis is the process by which viable myocardium is replaced with scar tissues, which is stiff and has less compliance than normal myocardium and also predisposes individuals to the development of heart failure. In addition to demonstrating the functional effects of human rIC-serum on hypertrophy and fibrosis, we also investigated the role of some of the intracellular

signalling pathways known either to play a role in myocyte hypertrophy or rIC protection against I/R.

This study is the first to demonstrate that a humoral agent present in the blood of healthy human volunteers after 4 cycles of rIC, has strong anti-hypertrophic and anti-fibrotic properties. We have shown that rIC-serum taken from healthy human volunteers and perfusate collected from explanted rat hearts undergoing IPC, can attenuate ET-1 induced hypertrophy in a cell culture model. Unconditioned-serum from humans had some innate anti-hypertrophic properties but this was boosted by rIC. Increasing age was shown to diminish the innate protection afforded by unconditioned-serum to hypertrophy however older individuals were afforded the same protection to hypertrophy after undergoing rIC compared to their younger counterparts. Furthermore, we showed that increased levels of physical activity was able to mirror the protection against ET-1 induced hypertrophy as afforded by rIC, with individuals with high levels of activity reaching a threshold of protection in their serum that was not further augmented by rIC. In contrast individuals with lower levels of physical activity did not show this innate protection and required rIC for this protection to be seen. We have also demonstrated a number of intracellular signalling pathways to be involved in attenuating hypertrophy in this model including PKCε, AMPK, eNOS, NO, SGC/CGMP as well as showing that rIC-serum can attenuate gene expression associated with hypertrophy including the foetal gene panel genes of BNP, βMHC, α-ACT and MS-1 and up-regulate the anti-hypertrophic microRNAs miR-1 and miR-133. We have also shown that human rIC-serum can attenuate indices of ET-1 induced fibrosis in a cell culture model by limiting the differentiation of fibroblasts to pathological myofibroblasts

assessed by immunofluorescence and assessing expression of expression of α-SMA, as well as increasing MMP-2 and TIMP-1. Finally, we have described the design, and implementation of the DREAM study, a phase 2 randomised control trial aiming to evaluate the role of repeated rIC post-MI to attenuated maladaptive cardiac remodelling and report some preliminary secondary outcome data.

8.2 Is an In-vitro Tissue Culture Model an Appropriate Surrogate Model for In-vivo Clinical Studies?

We utilised a cell culture based system in which various aspects of remodelling reflected to allow for a reasonably hi-throughout of experiments as well as allow for the utilisation of human serum in experiments.

8.2.1 Cardiac Hypertrophy In-vitro Tissue Culture Model

We have developed a model of ET-1 induced hypertrophy in H9c2 cardiomyoblasts derived from ventricular tissue. Utilising this model we have shown that rIC-serum collected after rIC in healthy human volunteers and rat perfusate from explanted rat hearts that have undergone IPC on a Langendorff circuit can attenuate the hypertrophic response of ET-1 in H9c2 cardiomyoblasts. This attenuation of hypertrophy supports the hypothesis of a blood-borne humoral mechanism of rIC protection. However, there was a detectable anti-hypertrophic action of unconditioned-serum as well as rIC, although the protective effects of unconditioned-serum were augmented by rIC. RIC has been implicated with the release of a number of humoral agent[484] and these agent are likely to be present in serum at rest albeit at lower levels than those seen in rIC. The modest

depression of ET-1 induced hypertrophy by unconditioned-serum may therefore reflect basal levels of these agents.

The use of H9c2 cardiomyoblast cells as a model for ventricular hypertrophy remains controversial. As described in chapter 3, we were unable to culture ARVMs for any length of time to allow meaningful experimentation therefore H9c2 cells were used as the main model of hypertrophy. Watkins *et al.,* (2011) addressed the concerns of using H9c2 in the context of hypertrophy in an elegant series of experiments. Using cell culture techniques she compared H9c2 cells to primary rat neonatal cardiomyocytes in their response to the hypertrophic triggers ET-1 and angiotensin 2 (Ang2). Hypertrophy was determined by overall changes in cell size as well as rearrangement of cytoskeleton and induction of the foetal gene panel. Interestingly in almost every measure of hypertrophy, H9c2 cells showed very similar responses to those observed in primary cardiomyocytes.[378]

The use of ET-1 is described in detail in *section 3.3.1*. As outlined, ET-1 is a key trigger of cardiomyocyte hypertrophy as part of the neurohormal activation of hypertrophy post-MI. The use of ET-1 in H9c2 cell culture provoked a strong hypertrophic response in terms of overall visual cell size, protein synthesis and the increased expression of the foetal gene panel. Furthermore the addition of fenofibrate, which has been shown to be an inhibitor of LV hypertrophy in a rat model and ET-1 induced hypertrophy in ARVMs via activation of PPARα, also completely blocked ET-1 induced hypertrophy in H9c2 cells in this study.[421, 485] This alludes to the involvement of the calcineurin-dependent dephosphorylation of NFATc in hypertrophy of H9c2, a pathway central to the hypertrophy described *in vivo* in the post-MI setting.[423] However

this work requires confirmation in an animal model of either LAD-ligation[486] or transverse aorta constriction of hypertrophy.[487]

It is clear that cell culture modelling of hypertrophy has a number of limitations. One of the main limitations is the growth of these cells in isolation from other cell types, thereby denying them of the usual paracrine communication between cell types. Furthermore, a cell culture model of this type utilising rigid flask and slides, does not allow the application of stretch to the cells which is known to be important as a trigger for hypertrophy post-MI. Not-withstanding the use of animal modelling and clinical testing, 3D cell culture modelling techniques remain in their infancy but may provide an avenue for accurately replicating post-MI remodelling in the future that represent a reasonable surrogate.[488] However current 2D cell culture techniques remain a useful tool for early hypothesis generating and we felt in this case was powerful enough make inferences to the real-life clinical picture.

8.2.2 Cardiac Fibrosis In-vitro Tissue Culture Model

One common measure of assessing fibrosis in cell culture is the use of a collagen gel contraction assay which measures the degree of constriction, a process mediated exclusively by myofibroblasts.[489] Other common techniques include the use of gelatin zymography to assess the enzymatic activity of MMPs and TIMPs and the use of migration assays, as chemotactic pull is altered depending on the phenotype of the cell.[490] In this study we focussed on some of the more traditional and well established markers of fibrosis *in vitro*, namely the degree to which various conditions effect proliferation of cardiac fibroblasts as well as their differentiation to myofibroblasts

responsible for downstream fibrosis using immunofluorescence techniques, the production of pro-enzymatic factors MMP-2 and MMP-9 and anti-enzymatic factor TIMP-1 detected by western blot analysis and the expression of α-SMA by RT-PCR as a marker for phenotype change to myofibroblasts.

Neonatal rat cardiac fibroblasts were used for the reasons outlined in *section 3.2.3*. In summary they are a well-established *in vitro* model of cardiac fibrosis, are obtained at an earlier passage than their adult counterpart allowing more passage cycles before spontaneous differentiation to myofibroblasts occur, and are more resistant than their adult counterparts to oxidative stress.[491] However, similar issues remain as seen with the use of H9c2 cells for modelling hypertrophy including issues with growing fibroblasts in isolation from other cells devoid of paracrine communication and the 2D nature of current standard cell culture practices, which do not allow for the inclusion of ECM modelling as it is becoming more and more apparent that the ECM plays a crucial role in chemical as well as mechanical signalling.[492]

Again ET-1 was used as the triggering molecule in this model as it is well established that in the post-MI phase, increased levels of ET-1 lead to cardiac fibrosis via a number of mechanisms including PKC activation and synergistic effects with Ang-II, TGF-β and platelet-derived growth factor (PDGF).[394, 493] ET-1 proved to increase markers associated with fibrosis including levels of cardiac fibroblast proliferation, differentiation and α-SMA RNA expression and as such was thought to provide a good model in which to test the effects of human rIC-serum.

8.3 RIC-serum from humans and IPC rat perfusate attenuates ET-1 induced hypertrophy in H9c2 cells

We have clearly shown in this study that the application of human rIC-serum or rat IPC perfusate for 30 minutes prior to treatment with ET-1, significantly attenuates ET-1 induced hypertrophy in H9c2 cardiomyoblasts after 48 hours. The inhibition of ET-1 hypertrophy was more marked in the human rIC-serum pre-treated cells that rat IPC perfusate pre-treated cells. We validated the results obtained by immunofluorescence by using protein:DNA ratio measurements and showed similar outcomes in rIC-serum treated cells compared to ET-1 treated cells only, confirming that the changes seen were not due to a change in shape or stretch of the H9c2 cells in culture, but reflected true changes in cell volume.

8.3.1 What are the mechanisms for the anti-hypertrophic actions of rIC-serum?

8.3.1.1 PKCε Signalling

PKCε is one of a number of structurally similar serine-threonine protein kinases. PKC can be broadly divided into 3 separate groups:

1. The conventional PKC isoforms (α, βI, βII and γ) which are calcium and DAG sensitive.
2. The novel PKC isoforms (δ, ε, θ and η) which are insensitive to calcium but sensitive to DAG.
3. The atypical PKC isoforms (λ,μ,ι and ζ) which are insensitive to both calcium and DAG.[494]

Studies in the early to mid-1990s implicated PKC translocation from the cytosol to the membrane of cardiac sarcomeres as well as the membranes of various other cellular structures to a lesser extent (such as the nucleus, mitochondria and cytoskeletal structures) as a mechanism of protection against ischaemia. In particular isoforms α, ε and ι were most heavily implicated.[495-497] As described in *section 1.2.4.2*, the isoform PKCε (the most abundant of the novel PKC isoforms in rats[498]) has been shown to play a role in the protection afforded by ischaemic conditioning against ischaemia. However, the role PKCε plays in cardiac hypertrophy is complex and the intricacies of its role in particular to hypertrophy in the context of heart failure following myocardial infarction in particular remains controversial.

A number of studies have suggested a pro-hypertrophic role for PKCε. In a rat cell culture model of hypertrophy, treatment with PKCε antisense reduced myotrophin-induced stimulation of protein synthesis.[499] Takeishi *et al.,* (2000) showed in a transgenic mouse model of PKCε overexpression, that although this phenotype led to concentric hypertrophy compared to non-trangenic controls, there was no associated heart failure suggesting that this isoform of PKC, unlike other isoforms such as PKCβ, may play a role in compensatory hypertrophy but not hypertrophy associated with maladaptive remodelling and heart failure.[500]

Conversely some studies have failed to demonstrate the pro-hypertrophic effects of PKCε and indeed some have shown it to be anti-hypertrophic in certain situations. In a knock-out mouse model, mice expressing an inhibitor to PKCε (εV1) were more likely to develop a spontaneous dilated cardiomyopathy compared to wild-type controls.[501] Furthermore, Mochly-Rosen *et al.,* (2000) used a transgenic mouse model

which expressed the PKCε stabiliser pseudo Receptors for Activated C Kinase epsilon (ψεRACK) and showed, that although this mouse displayed greater concentric hypertrophy compared to the wild-type mouse, this was due predominantly to cardiomyocyte proliferation and not hypertrophy. In fact mean cardiomyocyte size was 10% smaller in the transgenic group compared to control. The authors suggest that PKCε is part of a compensatory signalling pathway that is pro-proliferative and not hypertrophic and that this may be an early post-natal feature.[501] This hypothesis is supported by work from Wu *et al.,* (2000) who used a double transgenic mouse of both PKCε and Gαq (a pro-hypertrophic signalling pathway linked to PKC) overexpression. This mouse showed a reduction in cardiac hypertrophy with improved cardiac function compared to single transgenic mice over-expression Gαq alone.[502] Furthermore Hamasaki *et al.,* (2000) showed that in a thyroid hormone-induced live *in vivo* rat model of cardiac hypertrophy, there was decreased mRNA expression of PKCε as well as decreased PKCε immunoreactivity in both the cytosolic and membrane fractions.[503] Mochly-Rosen' group showed that specific regulatory components of PKCε interact with RACKs to facilitate translocation.[504] PKCε translocation within cardiac myocytes occurs via binding to the cardiac Z-lines in a slow process with an approximate half-life of 8 minutes and the receptor binding site eV1 domain of PKCε in association with RACK2 plays a key role translocation kinetics.[505, 506]

In this study, our aim was to try and integrate our current understanding of PKCε in the context of ischaemic conditioning against direct ischaemic injury, and that of hypertrophy, using the ET-1 hypertrophic H9c2 model as described. In the experiments described in *section 5.2* we have demonstrated a link between a more sustained PKCε

activation (via its translocation to the membrane component of predominantly cardiac sarcomeres) with rIC-serum pre-treatment prior to ET-1 exposure compared to ET-1 exposure alone. Coupled with the anti-hypertrophic properties of rIC in this model, this suggests a link between this sustained translocation of PKCε and the attenuation of ET-1 induced hypertrophy.

It may be that in our cellular model of hypertrophy, a component of rIC-serum is inducing sustained DAG activation which is maintaining a prolonged PKCε translocation to the membrane component. This may occur via disruption of RACK and ψRACK PKC-binding sites which are responsible for the stabilisation of PKC in its membrane form.[504, 507] Alternatively, there may be a component of rIC-serum not seen in conditioned serum which acts like PMA i.e. is structurally analogous to DAG in its binding of C1 binding sites on PKCε but does not activate the DAG pathway directly. For a sustained effect, this component of rIC must remain in the intracellular space for at least 30 minutes and is presumably washed out, excreted or degraded by 48 hours where the effect is lost. If PKCε translocation is not sustained for 48 hours as suggested by the results, it may be that PKCε translocation acts as an upstream trigger of downstream pathways that lead to more sustained anti-hypertrophic effects. Finally as the experiments did not demonstrate translocation directly but rather inferred translocation by the decrease in the cytosolic fraction, it may be possible that rIC-serum is either changing the distribution of translocation from cardiac sarcomeres to other intracellular organelles or cytosolic PKCε degradation without any meaningful translocation to the membrane.

In the acute setting post I/R, sustained PKCε may be one mechanism by which ischaemic conditioning infers cardioprotection. In our model we failed to show a

sustained membrane population of PKCε at 48 hours, implying that any anti-hypertrophic effects seen in this model may be triggered by initially sustained PKCε membrane levels in the acute setting which then act on downstream pathways to attenuate hypertrophy. The most well documented of these downstream pathways in the context of ischaemic conditioning is AMPK signalling[225, 508, 509] and this is discussed in the next section.

8.3.1.2 AMPK Signalling

AMPK is a highly conserved group of kinases with 3 subunits: α, β and γ. In mammalian species it is a key regulator of intracellular fatty acid and glycogen homeostasis.[510] It is activated in scenarios where ATP levels are depleted such as ischaemia in relation to MI.[511] In addition to its key role in energy haemostasis within the heart, AMPK is known to regulate cardiomyocyte growth. Tian *et al.,* (2001) showed that AMPK levels increased in a chronic overload model of hypertrophy in rats as a compensatory mechanism to increase glycogen homeostasis.[512]

AMPK can be activated either allosterically to induce around a 10-fold activation or via phosphorylation of the α-subunit at Thr172 to induce up to a 1000-fold activation.[513, 514] As well as its role in energy homeostasis, AMPK has been shown to be involved in classical IPC[225] as well as in rIC via adenosine and opioid humoral signalling[227, 515] which act to increase phosphorylation of AMPK at Thr172.[516-518] Furthermore, activation of AMPK pathways by metformin or the AMPK activator AICAR has been shown to decrease levels of protein synthesis associated with hypertrophy in phenylephrine-induced cardiac myocyte hypertrophy as well pressure-overload induced

hypertrophy.[519, 520] Implicated downstream targets of AMPK activation include mechanistic target of rapamycin (mTOR), PPARα, p70S6 kinase, glucose transporter type 4 (GLUT4) and ERK1/2.[521-523] However the direct role rIC plays in modulating AMPK activation to attenuate hypertrophy remains unclear.

The data as outlined in *section 5.3* suggests that human rIC-serum attenuation of ET-1 induced hypertrophy in our model involves AMPK signalling by increasing the degree of AMPKα phosphorylation at threonine-172. A number of authors have shown AMPK activation by phosphorylation in the context of protection from I/R by IPC to be downstream of PKCε,[524-526] however there is a scarcity of data with regards to the role of PKCε activation of AMPK in the context of rIC or IPC protection against hypertrophy. Downstream to AMPK signalling is the eNOS/NO/SGC/CGMP axis and its role in this model of hypertrophy is discussed in the next section.

8.3.1.3 eNOS/NO/SGC/CGMP Signalling

AMPK activation has been shown to activate eNOS by phosphorylation of Ser-1177 in the presence of Ca^{2+}-calmodulin (CaM)[527], which leads to increased levels of NO and triggering of the soluble guanylate cyclase (SGC) and protein kinase G (PKG) pathways.

The data as outlined in *section 5.4* showed that rIC-serum increases eNOS phosphorylation and that inhibition with the non-selective NOS blocker L-NAME blocked the anti-hypertrophic action of ET-1. Taken together this data suggests that ET-1 induced hypertrophy involves eNOS signalling by increasing the degree of eNOS phosphorylation. Furthermore, the effect of compound-C on blocking e-NOS phosphorylation in this model places eNOS signalling downstream from AMPK signalling in rIC induced anti-

hypertrophic pathway. This is in agreement with Chen *et al.,* (1999) who in a rat model of ischaemia showed that AMPK phosphorylation phosphorylates Ser-1177 in the presence calmodulin to activate eNOS.[527] Phosphorylation of eNOS by AMPK in endothelial cells and myocytes provides a further regulatory link between metabolic stress and cardiovascular function. Work by our group has confirmed the work of others to place PKCε upstream of NOS.[528, 529] Specifically using IPC perfusate from rats (obtained as described in this study), NOS inhibition blocked the induced IPC protection against I/R but did not affect the IPC induced translocation of PKCε, placing PKCε upstream of NOS.[336]

With regards to the effects of rIC on the detectable intracellular levels of NO in the cells, we were unable to detect a significant difference in NO levels in the rIC treated cells compared to the controls at either 5 or 20 minutes, although there was a non-significant increase of NO levels associated with rIC-serum pre-treatment at 20 minutes. This is at odds with what we expected to see as we expected NO levels to rise in the rIC treated groups due to the increased levels of eNOS phosphorylation. It may be the absolute levels of NO change are so small that we were unable to detect using DAF-FM testing. Another possibility is that the DAF-FM method to detect changes in NO levels in this setting was unable to detect intracellular NO changes due to large background detections in the fluorescence, which may have affected the sensitivity of this assay. DAF-FM also binds to nitrites and nitrates and may therefore give falsely high readings in the context of purely NO detection.

Finally we analysed the role of the sGC/cGMP pathway in attenuating hypertrophy. The results of these two experiments suggest an important role for the

sGC/cGMP pathway in attenuating hypertrophy in this setting. RIC-serum pre-treatment attenuated H9c2 cell surface area after 48 hours of ET-1 incubation. This attenuation was negated by the co-treatment with the sGC inhibitor ODQ, implicating the sGC pathway in the anti-hypertrophic properties of rIC in this model. However, ODQ does not fully inhibit rIC inhibition of hypertrophy, suggesting alternative pathways at work that bypass the sGC, possibly by the S-nitroslyation of the pro-hypertrophic NF-κB and IκB. Furthermore, both unconditioned and rIC-serum increased the initial levels of cGMP in H9c2 cells, an effect again blocked by ODQ suggesting that human serum temporarily increases levels of cGMP via NO/sGC activation and that rIC-serum in particular augments the increased level of cGMP seen.

8.3.1.4 Foetal Gene Panel

The data as outlined in *section 4.5* suggest that in this model, rIC rat perfusate but not unconditioned perfusate attenuated ET-1 induced expression of 4 selected genes that make up part of the foetal gene panel. In contrast both unconditioned and rIC-human serum attenuated ET-1 induced gene expression to similar degrees (except in the case of βMHC) suggesting an innate ability of unconditioned human serum to inhibit the pro-hypertrophic foetal gene panel that is not significantly augmented by rIC. This would suggest that the anti-hypertrophic properties of human rIC-serum over unconditioned-serum seen in our model is not significantly regulated by an attenuation of the expression of BNP, α-ACT and MS-1, however βMHC may have a role to play. However IPC rat perfusate did significant attenuate the expression of all 4 genes compared to unconditioned rat perfusate. The differences between the 2 conditioning media in this

context may be related to species variation, differing conditioning techniques used for rIC in humans and IPC in isolated rat hearts or an innate conditioning response in our human volunteers meaning no significant difference in attenuation in gene expression was seen between the pre-treatment with unconditioned or rIC-serum.

A further difficulty in interpreting the significance of the effect of human serum, both unconditioned and rIC, on the foetal gene panel is understanding the role these genes playing in cardiac hypertrophy post-MI. Debate still exists as to whether these genes are the drivers of hypertrophy or just are downstream effectors of other hypertrophic triggers. BNP, βMHC and α-ACT have been commonly shown to be up regulated as part of the foetal gene programme but MS-1 is not classically grouped with these genes in this context, although it is thought to be activated by similar stressors and triggers hypertrophy in a similar way to the more traditional foetal genes. The more widely held view is that up-regulation of these genes are an adaptation to MI in the remodelling phase caused by metabolic variations within the cells of the heart such as increased levels of glycogenolysis and glucose transport as well as biomechanical stress caused by both pressure and volume overload that can occur post-MI.[431, 530] A number of transcription factors have linked to up-regulating the foetal genes that ultimately lead to hypertrophy including GATA4, NFAT, SRF, MEF2. Some of these transcription factors are regulated by HDACs class I and II. There are also a number of epigenetic influences and finally post-translational factors play a role, chief amongst them being the microRNAs (discussed in the following section).[431, 531] Clearly in this context examining foetal gene expression in isolation may be misleading. Instead assessing the activity and expression of a number of these influencing factors in tandem may be more revealing.

It is for this reason we examined the expression of key regulators of hypertrophy post-MI, miR-1 and 133a, discussed in the following section.

8.3.1.5 Micro RNA-1 and 133a

In a rat model of pressure-overload hypertrophy, up-regulation of miR-1 by adenoviral delivery has been shown to regress cardiac hypertrophic response and blunt adverse cardiac remodelling. This was linked to reduced activity of the pro-hypertrophic pathways that are induced by ET-1 including the MAPK phospho-p38 and phospho-ERK 1/2 pathways.[532] In a neonatal mouse cardiomyoblast model, overexpression of miR-1 and 133 reduced ET-1 and phenylephrine induced markers of hypertrophy.[533] MiR-1 and 133 have also been shown to play a major role in IPC.[534, 535] In rat hearts exposed to 4 cycles of rIC, miR-1 was significantly up-regulated from 6 hours onwards.[536] He e*t al*., (2011) in a rodent model of I/R demonstrated that miR-1 and miR-133a were up-regulated by IPostC compared to I/R alone. They showed a link between increased miR-133a levels and decreased protein expression levels of the pro-apoptoic capsase-9.[537] MiR-1 and 133 has also been showed to modulate hypertrophy via their effects on a number of key transcription factors including calmodullin, MEF2a and GATA4.[431] Some studies have also shown that over-expression of both miR-1 and 133 in murine models can inhibit expression of foetal genes associated with hypertrophy including ANP, α-ACT 1 and βMHC, as discussed in the previous section.[533, 538]

The data as outlined in *section 5.5* showed that rIC-serum pre-treatment significantly up-regulated miR-1 and 133a after 48 hours of ET-1 treatment and was completely inhibited by compound-C, L-NAME and ODQ co-treatment for both miR-1

and miR-133a. Unconditioned-serum did not up-regulate either miR to the same effect. These result point towards a mechanism by which rIC up-regulates the anti-hypertrophic miR-1 and 133a via activation of the AMPK/eNOS/NO/sGC pathway. Although there have been no reports of a role for miRs in regulation of hypertrophy in the context of ischemic conditioning to date, up-regulation of miR expression via the pathway described have been described by other groups in the past, including Yuhas *et al.,* (2014) who demonstrated increased expression on miR-155 via cGMP/PKG pathways in human epithelial cells and Hur *et al.,* (2015) who demonstrated decreased regulation of miR-451 in fatty acid-induced inflammation via AMPK pathways in mouse hepatocytes.[539, 540] Caution must be taken as inferences as to the role of rIC in miR-1 and miR-133a via these pathways remain speculative in nature. Further confirmation would be gained by developing molecular knock-down strategies of the identified miRs with antagomirs, to establish any causal effect as well as analyse the effects antagomirs have on foetal gene expression.

8.3.1.6 Summary

Based on the finding as laid out in chapter 5, we propose a mechanism of rIC-serum induced anti-hypertrophic signalling via PKCε and AMPK/eNOS/NO/cGMP/PKG signalling converging on the calcineurin-dependent dephosphorylation of NFATc activated by ET-1[253] as well as a number of other downstream targets that lead to a hypertrophic phenotype. Although a link is known to exist between miRs 1/133a and suppression of the foetal gene panel, we did not explicitly show this in this study. A schematic of this proposed pathway is shown in Figure 8.1.

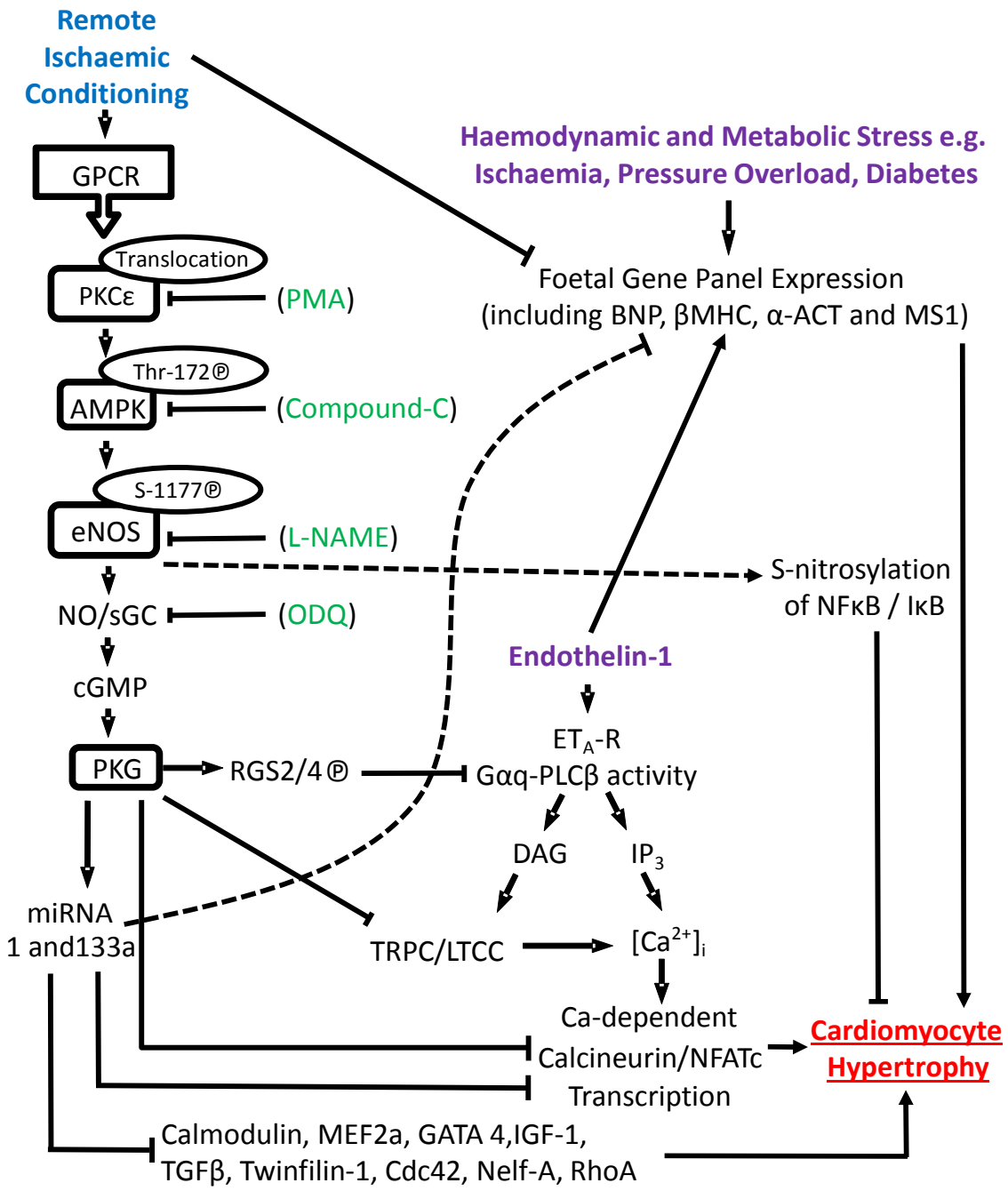


Figure 8.1 Proposed mechanism of rIC-serum induced anti-hypertrophic signalling converging on the ET-1 activated calcineurin-dependent de-phosphorylation of NFATc plus a number of other downstream targets that lead to a hypertrophic phenotype. Ⓟ = phosphorylation. For a full explanations of the abbreviations see the *Abbreviations* section at this beginning of the text.

8.3.2 What effects does age play on rIC attenuation of hypertrophy in this model?

The data suggests the capacity to infer protection against hypertrophy from rIC in the serum is not reduced with age. This is in contrast to a number of animal and clinical studies in the context of I/R that demonstrate decreasing protection from ischaemic condition with age.[541-544] However, work performed in our lab on a murine cellular model of rIC using healthy ARVMs affording protection against I/R, demonstrated no effect of age (i.e. the age of the human serum donor) on the degree of protection afforded.[545] The author did however present data from his model that reinforced the well-recognised phenomenon of reduced protection afforded from rIC in individuals with certain cardiovascular co-morbidities, in particular diabetes mellitus, and that it is these co-morbidities and not age per-se that dampens the benefits seen by rIC.[546]

One must be careful when analysing the effects of age as demonstrated in our study as two elements must be borne into consideration. First is the signal generated by rIC in the ischaemic arm which is an interplay of skeletal muscle, vascular smooth muscle and endothelium, the responses of which are influenced by the individuals' age. Second is the response to these signalling molecules in H9c2 cells which are an immortalised cell line and hence their lack normal cellular senescence makes interpretation of such data difficult. However keeping these limitations in mind, our data also suggests that the body's natural protection against hypertrophy, as evidence by the protection afforded by unconditioned-serum, diminishes slightly with age. The reason for this is unclear but one can speculate this is due in part to changes in the balance in circulating humoral factors that are anti-hypertrophic as one ages. Furthermore as both groups were made

up of non-diabetics with little cardiovascular co-morbidities, one reason for the observed difference may lie in the level of physical activity seen between the 2 groups. Out of the 8 individual in the younger group, none fell into the low activity group, 5 into the moderate activity group and 3 into the high activity group. This is compared to the old group where 5 fell into the lower activity group, 3 into the moderate group and 0 into the high activity group. This suggests that individuals with higher levels of activity have a degree of innate self-conditioning and is discussed in more detail in the next section.

8.3.3 What effects do levels of physical activity play on rIC attenuation of hypertrophy in this model?

It is well established that ischaemic conditioning can be induced by exercise. Separate animal models have shown episodes of brief exercise prior to I/R reduces final infarct size.[547, 548] Exercise has also been shown to mimic both early and late IPC.[548] This effect has also been demonstrated in humans in a number of clinical studies including Abete *et al.,* (2000) who showed that increased levels of physical activity in elderly patients protected against in-hospital mortality for myocardial infarction.[549] The same author has also previously shown that exercise boosts the protective benefits of ischaemic conditioning against I/R in the elderly.[550] Indeed the mechanistic nature by which exercise induced cardioprotection was discussed by Michelsen *et al.,* (2012) who demonstrated that vigorous exercise shared many of the mechanistic properties of rIC and that like rIC, humoral factors are likely to play a major role.[551]

A number of pathways have been implicated in so called 'exercise IPC' including heat shock protein 72 (HSP72), Ca^{2+} handling proteins such as SERCA, K_{ATP} channels (particularly sarcolemmal channels) and circulating antioxidants such as superoxide dismutase (SOD) and these share a number of the components described in the down-stream signalling of rIC.[225, 552] The close relationship between exercise and rIC may partly explain the importance of cardiac rehabilitation programmes after ischaemic events. Rock-Willoughby *et al.,* (2013) have recently shown that individuals with cardiac disease undergoing vigorous exercise (measured by VO_2max and METS) during cardiac rehabilitation programmes displayed improved LVEF, a reduction in their requirement for CABG surgery and overall fewer cardiovascular risk factors.[553] This study alluded to the protection afforded by exercise on cardiac remodelling as assessed by LVEF. Indeed, exercise training has also been shown to prevent hypertrophy caused by pathological sympathetic hyperactivity, an important pathway of post-MI hypertrophy. The mechanisms highlighted to be invoked in this anti-hypertrophic action included many associated with rIC such as modulation of pro-inflammatory cytokines, a reduction in foetal gene expression such as ANP and β-MHC and increased levels/activation of intra-cellular eNOS or AMPK.[554-556] This may in part explain the anti-hypertrophic properties of unconditioned-serum of the higher physical activity level group in this study.

The results in the experiments in *section 4.3.5* demonstrates unconditioned-serum contains a basal level of conditioning molecules which are additive as level of activity increases and reaches a threshold in the high activity group so that the degree of attenuation from unconditioned-serum and rIC-serum in this group is approximately equal. This is akin to the threshold reached with IPC cardioprotection which also displays

such a threshold. Interestingly in the rIC-serum group, an increased degree of rIC hypertrophy attenuation is seen in the low activity group compared to the high activity group. One possibility is that individuals with higher levels of activity maintained a steady state of circulating protective factors such that when a rIC signal is generated, the triggered peak in circulating factors which was ultimately collected in this experiment was not a high as the peak seen in the low activity group, as shown in Figure 4.7. This hypothesised higher level of circulating rIC like components in the higher activity groups may also explain the reason that as activity level increases, the effects of unconditioned-serum on attenuating ET-1 induced hypertrophy increases. It must be emphasised that this data is obtained from an entirely male subject group however there is also data to support the hypothesis of innate ischaemic conditioning in more active women as well as men.[550, 553, 557]

Another possible reason that serum from individual with higher levels of activity appears to be naturally protective against hypertrophy but with less dramatics peaks in protection compared to the lower activity group may be the way they deal with oxidative stress. Individuals with higher levels of activity tend to have a higher density of anaerobic glycolytic fibres and in addition are better are reducing ATP demand by metabolic economisation and reduced overall muscle force.[558]

Care must be taken in making sweeping analysis from this small cohort of quite heterogeneous individuals. One must also bear in mind that although levels of activity were assessed in broad terms, the intricacies of activity type were not ascertained including whether the activity can be classified as high intensity interval training (HIT) or not. This is pertinent as the mechanisms by which HIIT is thought infer cardioprotection

is somewhat different to that of conventional, low or medium level activity.[559] Furthermore high degrees of exercise can be detrimental in some case, associated with increased levels of ROS. This is thought to be especially true as the one gets older.[560]

8.4 RIC-serum from humans attenuates ET-1 induced markers of early fibrosis in neonatal rat cardiac fibroblasts

In chapter 6 we showed that the role rIC-serum plays in the modulating cardiac fibrosis in the post-MI setting is not straightforward. Whilst rIC-serum did reduce the degree of differentiation from fibroblast to myofibroblast at day 2, by day 4 the numbers of myofibroblasts was equal in all groups at day 4. Furthermore neither unconditioned-serum nor rIC-serum had any significant effect on attenuating ET-1 induced expression of α-SMA mRNA, also associated with fibroblast differentiation. Both untreated and rIC-serum pre-treatment did however significantly increase the expression of MMP-2 and TIMP-1 but neither had any effect on ET-1 induced MMP-9 release in this model.

This model of fibrosis, whilst providing some insights into the early influences of rIC and unconditioned-serum on ET-1 induced changes in neonatal cardiac fibroblasts in culture, ultimately was not powerful enough to allow an assessment on the role rIC plays in fibrosis as this is a complex and evolving process that relies on a multitude on changes not only pertaining to fibroblast but also in relation to other cell types, infiltrating inflammatory cells and ECM turnover. To model fibrosis over time following an MI and better simulate the *in vivo* conditions, cell culture remains limited. Ultimately, the ideal models would involve temporary LAD artery ligation in an animal model to induce I/R with these animals subjected to rIC and fibrosis detected mainly by echocardiographic

assessment in life and post-mortem morphological assessment of ECM deposition and fibrosis using collagen staining, biochemical analysis of fibrosis as well as mRNA and protein expression of MMPs and TIMPs.[561, 562]

8.5 The DREAM study

The primary outcome data and hence conclusions of the DREAM study have yet to be revealed. However some pertinent issues regarding the construction of the trial and overall methodology will be discussed in this section.

The basic hypothesis of the trial, namely that daily rIC from 4 weeks post-MI can modulate cardiac remodelling, is based mainly on the discoveries of one pre-clinical trial, Wei *et al.,* (2011) showing the attenuation of maladaptive remodelling when applying daily rIC in a rat model of I/R.[313] This concept of repeated rIC or more widely repeated IPC to infer cardioprotection has subsequently been repeated in more recent studies.[317, 563, 564] One criticism that may be levelled is the lack of animal data to corroborate these findings or phase 1 trial work to assess the effects of rIC in healthy volunteers before embarking on a phase 2 clinical trial. A counter argument is that for successful clinical translation, promising and breakthrough wet lab findings must be entered into the clinical forum at an early stage to maximise the possibilities of a potential new therapy.

In the study by Wei *et al.,* (2011), daily conditioning was significantly more effective at modulating remodelling that conditioning on every 3rd day. This is likely due to a degradation in the humoral signals that are generated and release by rIC over time. Indeed work by our lab in a ARVM cell culture model exposed to human rIC-serum obtained in the same way as described in this study, showed that there was degradation

of the protective signal against I/R after an hour of the conditioning stimulus, with no protection seen at all after 4 hours.[545] It is reasonable therefore to keep the interval period between conditioning cycles as short as practically possible to maintain as close to a steady state of circulating protective humoral mediators as possible.

With regards to participant selection, DREAM aims to look at the effects of rIC on naïve myocardium i.e. not previously subjected to significant ischaemia hence participants with previous MIs were excluded. Furthermore any existing chronic remodelling process that has led to heart failure out with the context of ischaemia was deemed to make interpretation of any new acute remodelling process post-MI difficult. Hence any participants with LVEF < 45% were excluded, however participant with potential HF-PEF were not and this fact was an unquantifiable factor in the trial that was not addressed. Furthermore participants with certain comorbidities known to reduce the efficacy of rIC such as Diabetes Mellitus were not excluded.[565, 566] To complicate matters a number of the diabetic participants were taking metformin or other diabetic medications at the time of their MI and subsequent rIC therapy. Metformin is thought to work via a number of pathways including the up-regulation of AMPK to augment IPC whereas other common diabetic agents such as sulphonyurea agents such as Glicazide attenuate IPC, partly via their action on K_{ATP} channels.[567]

Primary outcome data is to be revealed by cardiac MRI. LVEF was used as the index change by which to power the study i.e. to detect a 5% increase in LVEF above natural recovery over a 4 month period. The validity of this power equation is based on the assumptions that the natural recovery of LVEF seen in the small studies and local data analysed is representative of post-MI populations as a whole. Furthermore, LVEF

does not take into account changes such as overall volume change, pressure changes as seen in HF-PEF and actual cardiac output however it remains the gold standard for studies of this type for historical reasons and for the fact that it is an easy index to screen for on echo and measure by cMRI.

A potential criticism of the trial is the fact that rIC does not start until day 3 post-MI. This was the intention of the trial designers as we wanted to see the effects of rIC on acute remodelling alone, independent of the effects of infarct size. This is in contrast to the ongoing CRIC-RIC trial which starts rIC around the time of PPCI and hence will influence infarct size as well as remodelling.

A further possible criticism of the trial is the implementation and oversight of rIC on a daily basis for 4 weeks by the participant. Whilst the automated devices are simple and easy to use even for the frailest participants, in their current form they have no function to allow for interrogation to assess concordance. As such we relied on participant diaries, similar to ones used in drug trials, to encourage and evaluate concordance. Current research from drug trials suggests general concordance rates are approximately 70%. With direct oversight or a way or monitoring concordance, this rises to above 90%.[568]

8.6 Future Work

The DREAM study is on course to complete active recruitment of all participants by the end of August 2016. Thereafter all data collected will be analysed and disseminated. One area of future work which may prove illuminating is the use of collected serum to assess levels of a number of circulating factors implicating in rIC. One of the main focuses in

the area of rIC over the last few years has been to identify the responsible circulating humoral factor(s) responsible for protection in the context of I/R both in the heart as well as other organ, a holy grail which has so far has remained relatively elusive. It may be that rather than the release of a few key molecules, rIC shifts the balance of a slew of circulating molecules with the net effect being one of protection. Efforts to detect such changes in experimental models using proteomics may continue to prove challenging, especially when using animal models or healthy volunteers. Serum collected from DREAM study participants may provide a better real world understanding of the changes that occur in the bloodstream at various time-points in relation to MI and rIC and ethical approval is in place to perform detailed follow-up proteomic experiments from this participant group in the future.

Furthermore in our model we identified the anti-hypertrophic properties of rIC-serum when cells were exposed to rIC-serum on a one-off basis. To expand upon the idea of chronic rIC augmenting and providing continual protection, a natural progression of this work be to subject cells in culture to rIC-serum on a regular basis over a series of days before exposure cells to ET-1 i.e. continuous rIC. Furthermore one could use both one-off and continuous rPostC in this model i.e. treat cells with rIC-serum after exposure to ET-1.

Another key potential therapeutic target of remodelling post MI is the inflammatory process that occurs as described in *section 1.1.3.2* and *section 1.2.5.1.4*. In particular the migration of neutrophils and macrophages to the infarcted area and the border zones play a key role in remodelling therefore it would be possible to develop assays by which rIC-serum is added to a population of these cells in culture and their

migration tracked over time following an appropriate trigger, compared to a control group. Furthermore we are beginning to understand that platelets may play a role in the protection afforded by rIC (in particular microparticles release by platelets)[569, 570] and as such, all experiments described in this thesis could be repeated with serum or plasma containing platelets.

8.7 Conclusions

We present cellular models for both cardiac hypertrophy and fibrosis, important components of the acute remodelling process post-MI. We used these model to assess the effect of rIC-serum from healthy male volunteers and IPC perfusate from isolated rat hearts on both hypertrophy and fibrosis in these models. In addition we designed a phase 2 clinical trial to assess the effect of daily rIC post STEMI treated by PPCI on acute remodelling assessed by cMRI, blood biomarkers and participant qualitative indices.

We demonstrated that rIC-serum and IPC rat perfusate attenuated ET-1 induced hypertrophy. The attenuation afforded by rIC was not significantly diminished with age or physical activity, although individuals with higher levels of physical activity displayed innate conditioning properties as evidenced by hypertrophic attenuation by unconditioned serum. The attenuation caused by rIC-serum was shown to be mediated by a number of intracellular pathways. We demonstrated an increase in PKCε translocation to the membrane component leading to an increase in AMPK phosphorylation and the subsequent activation of the eNOS/sGC/cGMP/PKG signalling pathway. Furthermore we showed that rIC-serum and rat IPC perfusate attenuated the increase in a number of genes associated with the foetal gene programme (BNP, βMHC

and α-ACT) or cardiac hypertrophy (MS-1). In addition rIC-serum was shown in our model to increase the expression of the anti-hypertrophic miR-1 and miR-133a. We hypothesis that the anti-hypertrophic actions of rIC-serum and rat IPC perfusate are brought about by the activation or modulation of these various mechanism which act in large part via the calcineurin-dependant phosphorylation of NFATc.

We also demonstrated that rIC-serum reduced one marker of cardiac fibrosis: the degree of differentiation of fibroblast to myofibroblast. However some data was conflicting as both unconditioned and rIC-serum increased both MMP-2 and TIMP-1 and rIC-serum increasing MMP-9 after a delay as well as no effect of rIC-serum on attenuating the expression of ET-1 induced α-ACT.

Another interesting aspect of this work was the cross-species protection afforded by human rIC-serum in rat cell culture models. Such cross-species protection by rIC has been replicated by a number of other groups in the context of I/R[280, 305, 571] however to our knowledge this is the first time that this cross-species protective property of rIC has been shown in the context of hypertrophy and fibrosis.

Finally the phase II DREAM study is nearing completion and will help elucidate the potential therapeutic benefit of repeated daily rIC post-MI via its action on acute remodelling.

9. APPENDICES

9.1 Posters

1. The PKCε/AMPK/eNOS is implicated as a mechanism by which remote ischaemic conditioning attenuates ET-1 mediated cardiomyocyte hypertrophy (presented at the British Society of Cardiovascular Research Spring Meeting 2014)
2. The role of PKCε and AMPK signalling in attenuation of ET-1 induced cardiomyocyte hypertrophy by remote ischaemic conditioning (presented at the British Society of Cardiovascular Research 2013)
3. Remote ischaemic conditioning attenuates ET-1 induced hypertrophic response in rat cardiomyoblasts (presented at the Physiological Society's Cardiac Meeting 2012)
4. Remote ischaemic conditioning attenuates adverse cardiac remodelling in rat cardiomyoblasts (presented at the Postgraduate Day, University of Leicester 2012)

1. Poster presented at the British Society of Cardiovascular Research Spring Meeting 2014

THE PKCε/AMPKα/ENOS PATHWAY IS IMPLICATED AS A MECHANISM BY WHICH REMOTE ISCHAEMIC CONDITIONING ATTENUATES ENDOTHELIN-1 MEDIATED CARDIOMYOCYTE HYPERTROPHY

University of Leicester

Vanezis AP, Thaitirarot C, Butt M, Squire I, Samani NJ, Rodrigo GC

Department of Cardiovascular Sciences, University of Leicester, UK.

NHS National Institute for Health Research

Background and Purpose

- Remote Ischaemic Conditioning (RIC) protects the heart against ischaemia/reperfusion (I/R) injury by intermittent ischaemia to a distant limb.
- RIC may also attenuate hypertrophy associated with cardiac remodelling after a myocardial infarction.
- We have shown in a rat cell culture model that application of human RIC-serum attenuates endothelin-1 (ET-1) induced hypertrophy via PKCε translocation and AMPKα phosphorylation.
- Endothelial nitric oxide synthase (eNOS) is a key downstream enzyme in RIC protection against I/R.

Hypothesis

- RIC acts via a PKCε/AMPKα/eNOS axis to attenuate ET-1 induced cardiac hypertrophy in a rat cell culture model.

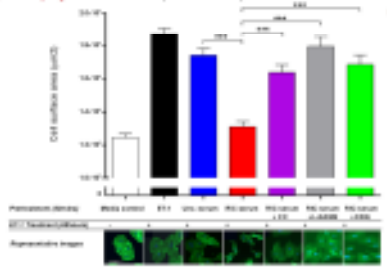
Methods

- Healthy male volunteers were bled before and after undergoing 4x supra-systolic BP cuff inflation/deflation on the arm to isolate serum.
- A rat cardiomyoblast model was used whereby cells were treated for 30 minutes with serum prior to ET-1 (100nM) treatment for 48 hours. Compound C (10μM) was used to inhibit AMP-activated protein kinase (AMPK), L-NAME (100μm) to inhibit NOS and ODQ (10μm) to inhibit soluble guanylate cyclase (sGC).
- FITC-labelled F-actin stain allowed for cell visualisation and measurements were made on Volocity 6.3 to calculate cell surface areas.
- Total/phosphorylated eNOS was assessed using western blots, normalised to α-tubulin. Densitometry was performed using Image J.
- cGMP levels were measured using a standard 96-well ELISA kit.

Statistical Analysis

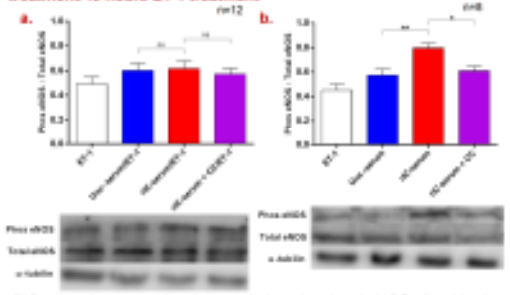
- Data was analysed in GraphPad Prism v6 using one-way ANOVA/Tukey's test. Histograms are displayed with SEM. * = p<0.05, ** = p<0.01, *** = p<0.001.

Figure 1. Effect of RIC-serum pre-treatment on ET-1 induced hypertrophy



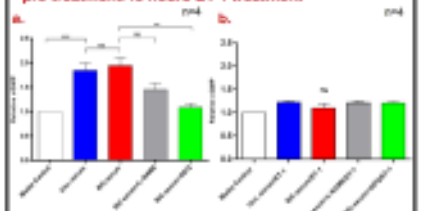
- ET-1 induced significant hypertrophy in rat cardiomyoblast in culture.
- Hypertrophy was significantly attenuated by pre-treatment with RIC-serum but not unconditioned serum.
- Blocking various stages of the proposed signalling pathway (figure 4) negated the anti-hypertrophic effect of RIC-serum.

Figure 2. Western blots of RIC-serum effect on eNOS phosphorylation after a. 30mins pre-treatment b. 30mins pre-treatment/48 hours ET-1 treatment



- RIC-serum increases the degree of phosphorylated eNOS after 30 mins.
- This increase is negated by the inhibition of AMPK by compound C.
- After 48 hours of ET-1 treatment, there was no significant difference in phosphorylation levels between groups.

Figure 3. Effect of RIC-serum on cGMP levels after a. 30mins pre-treatment alone b. 30mins pre-treatment/48 hours ET-1 treatment



- Both RIC and Unc.-serum increased cGMP acutely.
- L-NAME inhibition partially blocked this increase.
- After 48 hours of ET-1 treatment, there was no significant difference in cGMP concentrations between groups.

Figure 4. Proposed signalling pathway

Conclusions

- RIC-serum attenuates ET-1 induced hypertrophy in a rat cell culture model.
- Downstream eNOS phosphorylation and cGMP production are implicated as potential mechanisms in this process.

2. Poster presented at the British Society of Cardiovascular Research 2013

The Role of PKCε and AMPK Signalling in Attenuation of ET-1 Induced Cardiomyocyte Hypertrophy by Remote Ischaemic Conditioning

University of Leicester

Andrew Vanezis, Madiha Butt, Nilesh Samani, Glenn Rodrigo

Department of Cardiovascular Sciences, University of Leicester, UK.

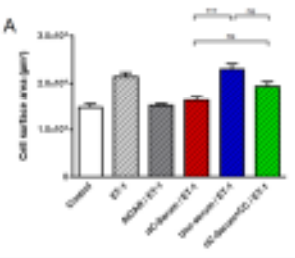
Background and Objectives

- ***Ischaemic Conditioning*** utilises the body's endogenous defence mechanism against sustained ischaemia/reperfusion (I/R) by inducing short, non-lethal episodes of I/R.
- Protection can be induced by performing conditioning in a distant muscle or tissue bed, so called ***Remote Ischaemic Conditioning (rIC)***.
- rIC is thought to work via a combination of humoral, neuronal and inflammatory mechanisms and may also attenuate hypertrophy associated with cardiac remodelling that takes place following a myocardial infarction and hence slow the progression to heart failure.
- Our lab has previously shown that in a rat cell culture model, the application of rIC-serum from healthy human volunteers attenuates cardiac hypertrophy induced by endothelin-1 (ET-1) both in cell size and in hypertrophy associated gene expression.
- AMPK and PKCε signalling pathways are known to be key to the protection afforded by rIC in the context of I/R. We hypothesis that both these pathways are also important in the attenuation of cardiac hypertrophy by rIC.

Methods

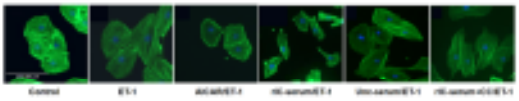
- ***Human RIC-serum:*** Healthy human volunteers underwent 3x supra-systolic BP cuff inflation/deflation on the upper arm. Blood was taken before/after and serum isolated.
- ***Cell Culture Model:*** ET-1 (100nM) to rat cardiomyoblast H9c2 cells for 48 hours to induce hypertrophy. Conditioning was induced by the application of rIC-serum for 30 minutes prior to ET-1 treatment. Unconditioned serum was used as a serum control. AICAR (1mM) was the positive control for AMPK and PMA (5μM) the positive control for PKCε. AMPK inhibition was achieved by co-incubating cells with Compound C (10μM)+rIC-serum.
- ***Immunofluorescence:*** FITC-labelled F-actin stain was used to image cells and obtain cell surface area. Measurements were made using Volocity software.
- ***Western blotting:*** Total/phosphorylated AMPKα and cytosolic/membrane PKCε were assessed using western blots, normalised to α-tubulin. Densitometry was performed using Image J software.
- ***Data analysis:*** GraphPad Prism v6 was used. Statistical significance was calculated using one-way ANOVA/Dunnett's test. * = p<0.05, ** = p<0.01 and *** = p<0.001.

Figure.1



A. Acute treatment with rIC-serum as well as AICAR inhibits subsequent ET-1 induced hypertrophy.

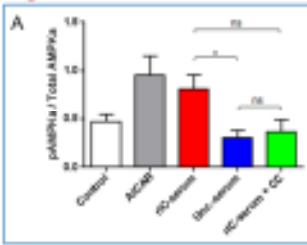
Histogram of cell surface area (mean+SEM) after 48 hours ET-1 incubation. Cells were pre-treated for 30 minutes with ET-1 (n = 8 & 663), AICAR (n = 8 & 1000), rIC-serum (n = 4, 2 & 169, unconditioned serum (n = 4, 2 & 642) or rIC-serum+CC (n = 4, 2 & 678). Control cells (n = 8 & 450) were incubated in serum free media throughout.



B. Representative fluorescence images of cells subjected to different treatments.

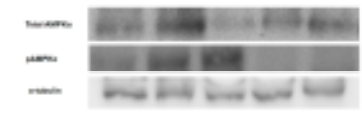
The data shows there is significant attenuation of ET-1 induced hypertrophy by rIC-serum (p<0.01). This effect is mimicked by AICAR. rIC + compound C does not inhibit this protective effect but there is a trend towards significance (NUM).

Figure.2



A. Acute treatment with rIC-serum increased the degree of AMPKα phosphorylation in H9c2 cardiomyoblasts.

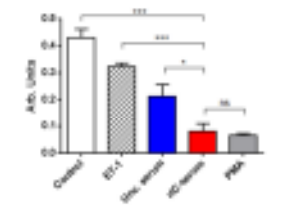
Histogram of western blot analysis (mean+SEM) of phosphorylated AMPKα/Total AMPKα normalised to α-tubulin control (n = 6, 12).



B. Representative blots

The data shows that there is a significantly increased level of AMPKα phosphorylation after acute treatment with rIC-serum. rIC + compound C does not show increased levels of phoshorylation but there is a trend towards significance (p=0.082).

Figure.3

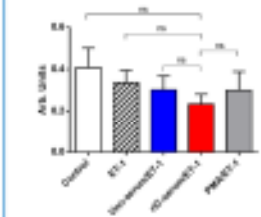


Acute treatment with rIC-serum decreased cytosolic PKCε and increased membrane PKCε indicating an increased level of translocation in H9c2 cardiomyoblasts.

Histogram of western blot analysis (mean+SEM) of cytosolic PKCε fraction normalised to α-tubulin control (n = 6, 12).

The data shows that rIC-serum increases translocation of PKCε from the cytosol to the membrane measured acutely, significantly more that unconditioned serum which cause only partial translocation.

Figure.4



Acute treatment with rIC-serum followed by 48hrs of ET-1 incubationhad no significant effect on PKCε translocation in H9c2 cardiomyoblasts.

Histogram of western blot analysis (mean+SEM) of cytosolic PKCε fraction normalised to α-tubulin control (n = 4, 8).

The data shows that rIC-serum does not increase translocation of PKCε from the cytosol to the membrane measured after 48 hours.

Conclusions

- Human rIC-serum reduces the hypertrophic effect of ET-1 on rat cardiomyoblasts.
- AMPK activation may play a role in the anti-hypertrophic property of rIC-serum.
- AMPK activation in this context may occur by increased AMPK phosphorylation.
- PKCε translocation is increased by rIC-serum treated cells both at baseline but not at 48 hours, suggesting acute but not chronic PKCε activation plays a role the rIC attenuation of hypertrophy.

References

Hausenloy DJ & Yellon DM. *Cardiovascular Research.* 2008. 79: 377-386.

Rodrigo GC & Samani NJ. *Am J Physiol Heart Circ Physiol.* 2008. 294(1): 524-31.

Wei M, Xin P, Li S et al. *Circ Res.* 2011;108:1220-1225

This work is funded by the NIHR Leicester Cardiovascular BRU

3. Poster presented at the Physiological Society's Cardiac Meeting 2012

Remote Ischaemic Conditioning Attenuates Endothelin-1 Induced Hypertrophic Response in Rat Cardiomyoblasts

University of Leicester

Andrew Vanezis, Sadat Edroos, Nilesh Samani, Glenn Rodrigo

Department of Cardiovascular Sciences, University of Leicester, UK.

The Physiological Society

Background

- Ischaemic Conditioning utilises the body's endogenous defence mechanism against sustained ischaemia/reperfusion (I/R) by inducing short, non-lethal episodes of I/R.
- Protection can be induced by performing conditioning ischaemia in a distant muscle or tissue bed, typically by 3-4 cycles of supra-systolic inflation and deflation of a blood pressure cuff in the upper arm or legs, so called *remote ischaemic conditioning (RIC)*.
- RIC has been attributed to humoral, neural and inflammatory signals. Our work focuses on humoral signalling. The exact molecule(s) responsible have yet to be indentified but candidates include bradykinin, adenosine, nitric oxide, endocannabinoids, calcitonin gene-related peptide and opioids.
- RIC may also attenuate adverse cardiac remodelling that takes place in the days to weeks post MI and hence slow the progression to heart failure.
- Using a cellular model of heart failure, we test the hypothesis that RIC can:
  1. reduce cellular hypertrophy
  2. modulate gene expression associated with adverse cardiac remodelling.

Abbreviations

α-actin	Alpha cardiac skeletal actin	**IR**	Ischaemic/Reperfusion
βMHC	Beta Myosin Heavy Chain	**MI**	Myocardial Infarction
BNP	Brain Natriuretic Peptide	**MS-1**	Myocardial Stress Gene 1
ET-1	Endothelin-1	**RIC**	Remote Ischaemic Conditioning

Methods

- ***Rat RIC-Superfusate:*** Langendorff perfused rat hearts were subjected to global ischaemia and superfusate was collected at the start of reperfusion.
- ***Human RIC-serum:*** Healthy human volunteers underwent 3 cycles of supra-systolic inflation/deflation of a blood pressure cuff on the upper arm. Blood was taken before/after conditioning and serum isolated by centrifugation.
- ***Cell Culture Model:*** Cardiac hypertrophy was induced by applying 100nM (248ng/ml) of endothelin-1 (ET-1) in cell culture media to rat cardiomyoblast H9c2 cells for 48 hours. Ischaemic conditioning was induced by application of either rat RIC-superfusate or human RIC-serum for 30 minutes prior to treatment with ET-1. Immunofluorescence staining was used to obtain cell area measured using computer software (**Figure 1**).
- ***Conditioning of Cardiomyoblasts:*** Cells were treated for 30 minutes with either the RIC-superfusate or serum. Phosphate Buffered Saline was used as a control for the rat superfusate and unconditioned human serum was the control for the RIC human serum.
- ***Gene Expression:*** Cells were conditioned and trypsinized at 0 and 48 hours, the RNA extracted and converted to cDNA by reverse transcription PCR. qRT-PCR was used to quantify the expression of 4 genes: BNP (a marker of hypertrophy), βMHC and α-actin (part of the foetal gene programme) and MS-1 (a cardiac stress gene).
- ***Data analysis and Statistics:*** Data was analysed using GraphPad Prism version 5. Statistical significance was calculated using one-way ANOVA and Tukey's test and *p* values <0.05 were considered significant; * = p<0.05, ** = p<0.01 and *** = p<0.001.

Results

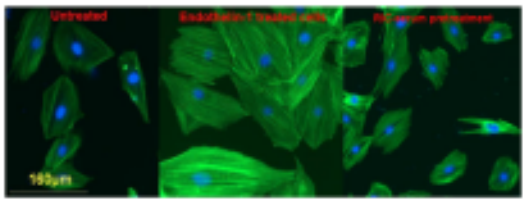


Figure 1. H9c2 cells stained with Phalloidin-FITC which binds F-actin (green) and the nuclear stain Hoechst (blue) after 48 hours.

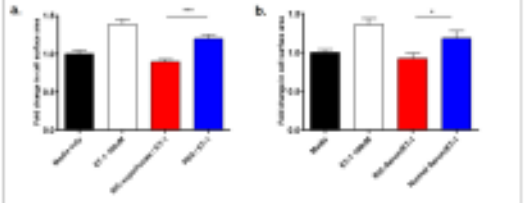


Figure 2. Impact of conditioning on hypertrophy of H9c2 cells in response to ET-1 *a.* rat RIC-superfusate (n=4, p<0.0001) *b.* human RIC-serum (n=4, p<0.05).

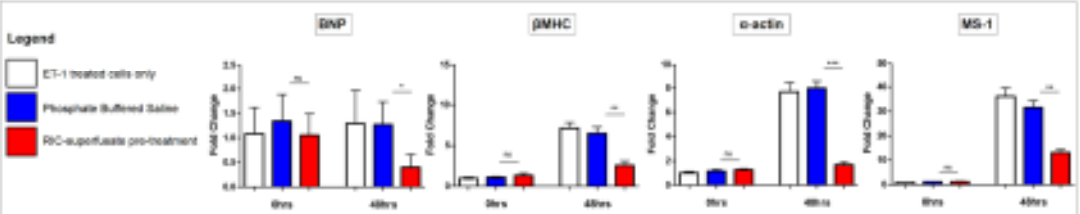


Figure 3. Effect of rat RIC-superfusate on gene expression after 48 hours ET-1 treatment. RIC-superfusate significantly reduced the expression of α-actin (p<0.01), βMHC (p<0.01), BNP (p<0.05) and ms-1 (p<0.01). N=4 for all experiments.

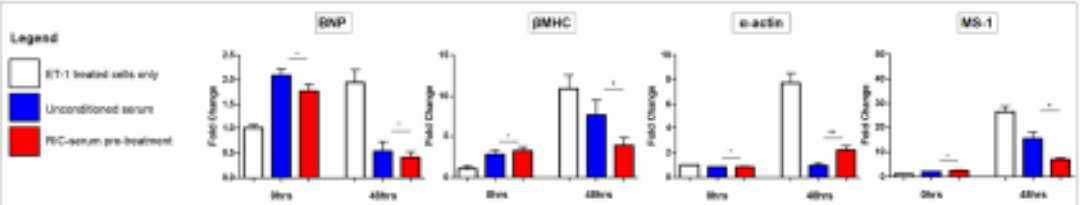


Figure 4. Effect of human RIC-serum on gene expression after 48 hours ET-1 treatment. RIC-serum significantly reduced the expression of BNP (p<0.05), βMHC (p<0.05) and ms-1 (p<0.05). N=4 for all experiments.

Conclusions

- Rat RIC-superfusate and human RIC-serum reduces the hypertrophic effect of ET-1 on rat cardiomyoblasts.
- Rat RIC-superfusate and human RIC-serum modulate the expression of 4 genes that are associated with adverse cardiac remodelling.
- This work reinforces the hypothesis that the humoral signalling initiated by RIC attenuates the deleterious process of cardiac remodelling and presents an exciting new development in the management of MI-induced heart failure.

References

Hausenloy DJ & Yellon DM. *Cardiovascular Research.* 2008. 79: 377-386.

Rodrigo GC & Samani NJ. *Am J Physiol Heart Circ Physiol.* 2008. 294(1): 524-31.

Wei M, Xin P, Li S et al. *Circ Res.* 2011;108:1220-1225.

Acknowledgements

This work is funded by the NIHR Leicester Cardiovascular BRU

Leicester Cardiovascular Biomedical Research Unit

4. Poster presented at the Postgraduate Day, University of Leicester 2012

Remote Ischaemic Conditioning Attenuates Adverse Cardiac Remodelling in Rat Cardiomyoblasts

Andrew Vanezis, Sadat Edroos, Helen Turrell, Glenn Rodrigo, Nilesh Samani

Department of Cardiovascular Sciences, University of Leicester, UK

Leicester Cardiovascular Biomedical Research Unit

University of Leicester

NHS National Institute for Health Research

Background

Ischaemic Conditioning is a technique that harnesses the body's own endogenous self defence mechanism against **sustained ischaemia/reperfusion (IIR)** by inducing short, non-lethal episodes of ischaemia/reperfusion. It can be employed prior to (**preconditioning**) or after (**postconditioning**) an ischaemic event, classically a myocardial infarction (MI).

Furthermore the protection can be induced away from the heart, so called **remote ischaemic conditioning (RIC)**, by inducing I/R in a distant muscle or tissue bed, typically by cycles of inflation and deflation of a blood pressure cuff in the upper arm or leg. This protection is afforded by a combination of humoral, neural and inflammatory signals.

New evidence suggests that RIC can not only reduce the short-term impact of MI induced I/R damage but can also attenuate **adverse cardiac remodelling** that takes place in the days to weeks post MI, which if left unchecked can ultimately lead to heart failure.

Using a cellular model of heart failure, we test the hypothesis that RIC can both reduce cellular hypertrophy and modulate foetal gene expression associated with remodelling.

Figure 1. Schematic of Pre and Post Ischaemic Conditioning protocols

Methods

Preconditioned Rat Superfusate

Langendorff perfused isolated rat hearts were preconditioned by inducing global ischaemia for 3x5 minutes interspersed with 3x5 minutes of reperfusion. Superfusate was collected from the heart at the start of each reperfusion period. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Remotely Conditioned Human Serum

Healthy human volunteers underwent 3 cycles of upper arm blood pressure cuff inflation 30mmHg above systolic pressure for 5 minutes interspersed with 5 minutes of reperfusion. Blood was taken before and after the conditioning cycles and serum isolated by centrifugation.

Cell Culture Model

Cardiac remodelling was simulated by applying 100ng of the key remodelling molecule endothelin-1 (ET-1) in cell culture media to the rat cardiomyoblast cell line H9C2 for 48 hours. The protection afforded by ischaemic conditioning was induced by application of either the preconditioned rat superfusate or RIC human serum for 30 minutes prior to the application of ET-1 in media.

Cell Hypertrophy

Cells were seeded onto glass 8-well chamber slides and allowed to adhere to the glass surface for 24 hours in serum rich media. The cells were then treated for 30 minutes with either the rat superfusate or the RIC human serum. Control groups were created by pre-treating cells with either 1. unconditioned human serum 2. culture media only 3. 5μM Fenofibrate (partial inhibitor of ET-1) in media 4. PBS. After 48 hour treatment with ET-1, cells were stained with Phalloidin-FITC and Hoechst and cell area measured using computer software analysis.

Foetal Gene Expression

Cells were treated in T75 culture flasks as described above. Cells were trypsinized at 0, 24 and 48 hours and the RNA extracted. RNA was converted to cDNA by RT-PCR and real time semi-quantitative PCR was used to quantify the expression of 4 genes associated with remodelling: ANP, BNP, βMHC and ACTA.

This work is funded by the NIHR: Leicester Cardiovascular BRU

Results

Figure 2. Staining of cardiomyoblasts with Phalloidin-FITC which binds F-actin (green) and the nuclear stain Hoechst (blue), before and after ET-1 treatment.

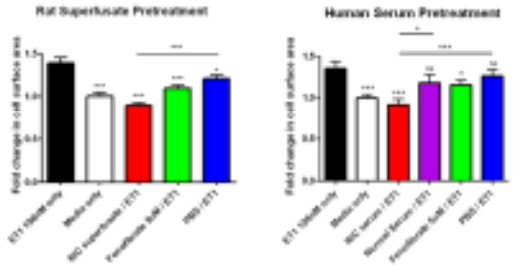


Figure 3. Bar charts of the degree of cell hypertrophy blunting with preconditioned rat superfusate pre ET-1 (16.4±0.7mm² vs. 22.6±1.1mm², p<0.0001) and human RIC serum pre ET-1 (16.1±1.2mm² vs. 23.6±1.5mm², p<0.0001).

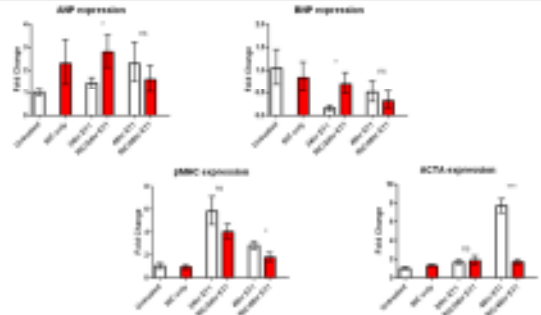


Figure 4. Bar charts of the fold expression of 4 genes that are expressed as part of the foetal panel of gene expression in remodelling. Preconditioned rat superfusate significantly increases expression of ANP (1.44±0.13 vs. 2.79±0.41, p<0.05) and BNP (0.15±0.03 vs. 0.7±0.14, p<0.05) at 24 hours but significantly decreases expression of βMHC (1.78±0.22 vs. 2.81±0.2, p<0.05) and ACTA (1.68±0.14 vs. 7.67±0.44, p<0.0005) at 48 hours. (White column = ET-1 treatment only, red column = superfusate treatment prior to ET-1).

Conclusions

- Both preconditioned rat superfusate and RIC human serum reduces the hypertrophic effect of ET-1 on rat cardiomyoblasts.
- Preconditioned rat superfusate also modulates the expression of 4 genes that are over-expressed in remodelling.
- This work reinforces the hypothesis that the humoral signalling pathways initiated by RIC attenuate the deleterious process of cardiac remodelling and presents an exciting new development in the management of MI-induced heart failure.

References

1. Rodrigo, G.C. and N.J. Standen, *Ischemic preconditioning of the whole heart confers protection on subsequently isolated ventricular myocytes.* Am J Physiol Heart Circ Physiol, 2005. 284(1): p. H324-31.
2. Murry CE, Reimer KA, Jennings RB. *Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium.* Circulation (New York, NY). 1990;74(5):1124-36.

9.2 Publications

- Remote Ischaemic Conditioning and Remodelling Following Myocardial Infarction: Current Evidence and Future Perspectives - Accepted for publication by *Heart Failure Reviews* and awaiting publication (DOI: 10.1007/s10741-016-9560-9)
- rIC: Beyond traditional acute cardioprotection - Published in *Hospital Healthcare Europe Bulletin* 2014

'Remote Ischaemic Conditioning and Remodelling Following Myocardial Infarction: Current Evidence and Future Perspectives' - Heart Fail Rev DOI 10.1007/s10741-016-9560-9.[572]

Heart Fail Rev
DOI 10.1007/s10741-016-9560-9

Remote ischaemic conditioning and remodelling following myocardial infarction: current evidence and future perspectives

A. P. Vanezis[1] · G. C. Rodrigo[1] · I. B. Squire[1] · N. J. Samani[1]

Abstract Remote ischaemic conditioning (rIC) has demonstrated its effectiveness as a powerful cardioprotective tool in number of preclinical and limited clinical settings. More recently, ischaemic *postconditioning* given after an ischaemic event such as a myocardial infarction (MI) has shown not only to reduce infarct size but also to have beneficial effects on acute remodelling post-MI and to reduce the burden of heart failure and other detrimental outcomes. Building on this platform, *repeated* rIC over a number of days has the potential to augment the protective process even further. This review considers the current evidence base from which the concept of rIC in the setting of post-MI remodelling has grown. It also discusses the ongoing and planned clinical trials which are attempting to elucidate whether the protection imparted by rIC in the preclinical setting can be translated to the clinic and become a realistic weapon in the clinician's armoury to tackle acute remodelling and heart failure post-MI.

Keywords Cardioprotection · Heart failure · Remodelling · Myocardial infarction · Remote ischaemic conditioning

Introduction

Remote ischaemic conditioning (rIC) is a non-invasive therapeutic technique whereby intermittent interruption of blood to an organ or muscle confers protection against ischaemia/reperfusion (I/R) injury to a distant organ. RIC can be implemented prior to an expected ischaemic insult (preconditioning), during the evolution of an ischaemic insult (per-conditioning) or soon after the completion of an ischaemic insult (postconditioning). For the purposes of this review, the term rIC will encompass all of these techniques.

The technique evolved from the phenomenon of local ischaemic conditioning of the heart and has been successfully used to reduce myocardial damage and improve cardiovascular outcomes in the context of primary percutaneous intervention (PPCI) for acute myocardial infarction (MI) [1, 2], elective coronary angioplasty [3–5], coronary artery bypass surgery [6], valve surgery [7] and paediatric cardiac surgery [8]. Beyond the well-established acute protective phase, early preclinical studies have hinted at an additional role for rIC, predominantly in positively influencing post-MI ventricular remodelling. In addition to directly affecting final infarct size, rIC may act to increase recruitment of stunned myocardium as well as modulating remodelling processes such as cell death with an increased emphasis on autophagy, cardiomyocyte hypertrophy, extracellular matrix (ECM) changes and the influx of pro-inflammatory cells to the damaged myocardium. This potential new role for rIC may have a profound effect in reducing the incidence and impact of post-MI heart failure.

Remodelling following myocardial infarction

Heart failure is a major cause of long-term mortality and morbidity after MI. Analysis of registries and of large clinical trials across the western world, conducted in the era of acute revascularisation, has reported incidence rates of post-MI heart failure ranging from 10 to 50 %, depending on a number of factors including the degree and

✉ A. P. Vanezis
andrew.vanezis@nhs.net

[1] Department of Cardiovascular Sciences, Glenfield General Hospital, University of Leicester, Leicester LE3 9QP, UK

Published online: 13 May 2016

location of infarcted myocardium, how MI and heart failure were defined, whether there was pre-existing heart failure, the treatment modalities used and the characteristics of the populations analysed [9]. A retrospective analysis of Framingham Heart Study participants demonstrated an increase in the incidence of post-MI heart failure from the 1970s to the 1990s, closely linked to a decrease in mortality in acute MI, likely due to advances in myocardial salvage over this time period [10].

The development of chronic heart failure following MI most commonly results from adverse remodelling of the left ventricle, a process of structural reorganisation which occurs within the first few weeks to months after the acute event. Such remodelling is directly related to the extent of myocardial damage (due to initial necrosis and secondary apoptosis) and is most likely to occur following transmural infarction, as well as being heavily influenced by concomitant microvascular obstruction and lethal reperfusion injury in the era of acute revascularisation [11, 12]. The process of remodelling is triggered by the initial ischaemia/reperfusion insult which sets into motion a number of events. In the initial stages, the changes in the left ventricle are predominantly due to the effects of infarct expansion causing cardiomyocyte necrosis and apoptosis which ultimately leads to myocardial wall dilatation via a number of mechanisms including changes in excitation–contraction coupling and an increased expression of foetal genes leading to an alteration in proteins produced. In the later stages, remodelling is largely fuelled by hypertrophy of surviving cardiomyocytes in response to pressure and volume changes and neurohumoral signalling, reorganisation of the ECM with deposition of scar tissue and an inflammatory-driven process whereby substantial ECM turnover in border areas leads to cell slippage and further dilatation. From a whole-organ perspective, these changes impact on cardiac dimensions and function. These initial changes act to maintain an adequate cardiac output in the face of a loss of functioning myocardium; however, over time remodelling becomes maladaptive. Indeed the extent and the nature of remodelling (both compensatory and subsequently maladaptive), and its progression is a powerful predictor for both heart failure and death following MI, as well as having prognostic implications for further MI, stroke and cardiac arrest [13, 14]. Preventing or modifying some or all of the drivers for remodelling may go some way to reducing major adverse cardiovascular events in this setting.

Local ischaemic conditioning

In 1986, Murry et al. [15] first described an endogenous cardioprotective mechanism in a canine model of MI termed ischaemic preconditioning (IPC), whereby intermittent, non-lethal occlusion and reperfusion of the left anterior descending artery (LAD) immediately prior to a period of sustained occlusion significantly reduced the final infarct size. The first in vivo study in humans assessing the effect of preconditioning was performed by Deutsch et al. [16]. In a small group of patients undergoing elective PCI for an obstructed (LAD), they showed a reduction in electrographic, metabolic and clinical markers of ischaemia during the second cycle of balloon inflation compared to the first. Yellon et al. [17] later utilised IPC prior to coronary artery bypass grafting surgery (CABG), demonstrating preserved levels of myocardial adenosine triphosphate during cardiopulmonary bypass. Over the intervening years, evolution of this technique has seen it applied to situations of unpredictable cardiac ischaemia (as opposed to anticipated ischaemia from elective surgery). Zhao et al. [18] in 2003 introduced the concept of ischaemic postconditioning (IPostC) whereby the conditioning stimulus is applied immediately or soon after the index ischaemic event by intermittent inflations and deflations of the intracoronary balloon to stagger reperfusion. Using a canine model, they demonstrated the effectiveness of IPostC in the context of acute MI with comparable levels in infract size reduction and levels of tissue oedema as well as a variety of markers of cardiac damage when compared to IPC. The possible clinical applicability of IPostC in the setting of acute coronary events was quickly realised. By staggering reperfusion during PCI by repetitively inflating and deflating the angioplasty balloon in the culprit vessel for short periods of time, Laskey et al. [19] showed a reduction in final electrocardiographic ST-segment elevation size and an increase in distal myocardial perfusion. Staat et al. [20], using a similar technique at the time of PCI, showed a significant reduction in creatine kinase release and an increase in myocardial reperfusion in the conditioned group.

Windows of protection and delayed conditioning

Two distinct phases of cardioprotection resulting from ischaemic preconditioning have been shown to exist and are commonly termed 'windows of protection' [21]. The first window begins immediately following the conditioning stimulus and lasts up to 4 h. Protection within this time period is mainly induced through posttranslational modification of proteins. The second or delayed window of protection occurs 12–72 h after the conditioning event and confers protection mainly through gene transcriptional changes [22–24].

In the context of protection against the long-term effects of I/R and subsequent remodelling, the timing of the conditioning stimulus is paramount. Early studies suggested that to impart meaningful protection, conditioning must be

implemented before, during or immediately after the clinical event as reperfusion injury is thought to occur within the first 15 min after the event. Dispelling this belief somewhat, Roubille et al. [25] described the damage associated with reperfusion as a 'wave front' and showed that rIC after I/R can be effective up to 30 min post-MI. Basalay et al. [26] also found a similar but more modest phenomenon in a rat model of I/R where rIC was effective in reducing injury when started up to 10 min into reperfusion time. The ability to impart protection, even after a significant time after the acute event, may prove clinically useful in the context of protection against adverse remodelling in post-MI in patients presenting late to hospital, as the remodelling process continues to evolve for several days after the initial insult.

Proposed mechanisms of remote ischaemic conditioning

RIC took the concept of IPC a step further, allowing the conditioning stimulus to be applied away from the heart in a distant tissue bed. Przyklenk et al. [27] were the first to demonstrate rIC in an animal model of ischaemia/reperfusion. By preconditioning the left circumflex coronary (LCx) artery in dogs, they were able to protect the remote myocardium supplied by the LAD following transient ligation to induce MI and reperfusion. Kerendi et al. [28] later demonstrated the cardioprotective effects of rIC in the post-MI setting. After 30 min of coronary artery occlusion in rat hearts, they remotely conditioned the kidneys, then reperfused the heart and showed a 50 % decrease in infarct size compared to the control.

In humans, the most practical application of rIC is by sequentially inflating a blood pressure cuff on the arm or leg, commonly using 3–4 cycles of inflation and deflation. This non-invasive technique affords protection not only to the heart but also to a number of other organs, most notably the brain and kidneys (for review, see Ref. [29]). Although the exact mechanisms of signal transduction from the tissue/organ undergoing rIC to the target organ have yet to be elucidated, various authors have highlighted the importance of humoral and neural signalling pathways as well as modulation of the systemic inflammatory response, perhaps working in an interdependent manner [30, 31].

The humoral signalling theory postulates that blood-borne factors are released locally by the tissue undergoing rIC and are then relayed in the blood to the target organ, where they bind to G-protein-coupled receptors triggering a number of intracellular signalling pathways. A number of research groups have illustrated the importance of humoral signalling by isolating naïve animal hearts and treating them with superfusate from rIC-treated animals or human donors and demonstrating cardioprotection [32, 33]. We have shown this in our laboratory using isolated adult rat cardiomyocytes [34]. Over the years, numerous humoral factors have been implicated including adenosine, bradykinin, nitrate/nitrites, opioid peptides, prostaglandins, natriuretic peptides, endocannabinoids, angiotensin I and calcitonin gene-related peptide. It is currently believed that the signalling factor(s) is between 3.5 and 15 kDa in size and is hydrophobic [35, 36]. More recent candidates for the responsible humoral messenger include stromal cell-derived factor-1 (SDF-1a) which recruits stem cells and is activated by hypoxia [37], circulating extracellular vesicles [38] and a panel of anti-inflammatory proteins including haptoglobin and transthyretin [39].

The first evidence for the involvement of neural signalling in rIC was given by Gho et al. [40]. By administering intravenous hexamethonium (a ganglion blocker), they abolished protection afforded by remote ischaemic *pre*conditioning of anterior mesenteric artery or renal artery against sustained MI. Subsequent experiments by Ding et al. [41] showed that by directly severing the renal nerve, one could abolish the cardioprotective effect of renal ischaemia rIC in rabbits. Mastitskaya et al. [42] proposed that rIC involves transmission via vagal preganglionic neurones, whilst further studies have advocated C-fibres as the sensory neural mechanism responsible for rIC [43]. Indeed there is some suggestion that a combined humoral/neural signalling relay exists where adenosine (or other candidate factors) acts via modulation of afferent neural pathway [44]. Jensen et al. [45] demonstrated that the dialysates from type 2 diabetic individuals with peripheral neuropathy did not afford protection against infarction in a rabbit model, whereas the dialysate from non-diabetics and diabetics without peripheral neuropathy did, implying a fundamental role for neuronal signalling in this process. Furthermore, Basalay et al. [26] suggested that rIC in the pre-, per- and immediate post-MI period is heavily dependent on sympathetic messaging, whereas delayed remote ischaemic postconditioning i.e. >10 min after the event, appears not to rely so heavily on this neural signalling. This suggests a greater level of importance for humoral signalling in late postconditioning as well as potentially for repeated rIC.

A final hypothesised mechanism of rIC signalling involves modulation of the inflammatory response, important in initiating and controlling wound healing. Cheung et al. [8] demonstrated that patients given a rIC stimulus prior to undergoing open-heart surgery had a reduced systemic inflammatory response and reduced levels of cardiac damage. Li et al. [46] also highlighted the importance of inflammation by demonstrating a blunted cardioprotective response in mice deficient in NFκB (a transcription factor involved in most inflammatory

processes) subjected to rIC. The importance of NFκB was underlined by Wei et al. [47] in a rat model of repeated rIC and MI where they demonstrated significantly reduction in phosphorylation of the NFκB subunit p65 and its inhibitory protein IκBα. In addition, this study showed a reduction in the infiltration of macrophages and neutrophils into the infarcted tissue in the rIC groups as well as a reduction in monocyte chemotactic protein 1 (MCP-1) in the border zone of infarcted tissue. More recently, Cai et al. [48] have shown up-regulation of expression of interleukin-10 (a potent anti-inflammatory cytokine) in a mouse model of rIC which leads to a reduction in myocardial infarct size and improved cardiac contractility.

Although some mystery still exists as to the mechanisms of rIC signalling, once the signal reaches the intended organ, the downstream intracellular pathways of rIC are thought to share much in common with local ischaemic conditioning. A number of intracellular pathways have been implicated including the reperfusion injury signalling kinase (RISK) pathway, involving ERK 1/2, p38 MAPK, PI3K-AKT and GSK3β, acting ultimately to prevent opening of the mitochondrial permeability transition pore (mPTP) at the time of reperfusion. Another important downstream pathway is the survivor activating factor enhancement (SAFE) pathway, involving activation of the JAK-STAT3/5 axis, a protective transcription factor in the context of acute ischaemia (for a detailed review, see Ref. [49]). The first window of protection is thought to depend heavily on the RISK pathway, nitric oxide (NOS), PKCε, PKCγ and reactive oxygen species. The second window of protection is more dependent on the SAFE pathway and inducible nitric oxide (iNOS) as well as retaining a significant overlap with some of the pathways implicated in the first window of protection [21, 50]. For a detailed discussion of our current understanding of the mechanisms of rIC, see the proceedings from the most recent Biennial Hatter Cardiovascular Institute Workshop [51].

Remote ischaemic conditioning and acute myocardial infarction

The simple and safe technique of inducing ischaemia by inflating a blood pressure cuff applied to the forearm to a level greater than the systolic blood pressure was first used in the setting of acute MI by Bøtker et al. [1] in the CONDI trial. In this landmark study 4 × 5-min cycles of blood pressure cuff inflation/deflation were applied to the forearm of a cohort of ST-segment elevation MI (STEMI) patients in the ambulance on-route to PPCI and showed that with large anterior MIs caused by total occlusion of the LAD, conditioned patients had a significantly better myocardial salvage index as assessed by gated single-photon emission CT (SPECT) than the control group. A smaller study by Rentoukas et al. [2] was undertaken in STEMI patients where rIC was applied just after PCI using 4 × 4-min cycles of a forearm blood pressure cuff inflation/deflation in combination with morphine. There was a significant reduction in troponin *T* levels in the conditioned group compared to the control group as well as ST-segment deviation resolution. More recent work by White and colleagues further demonstrated the benefits of rIC, implemented in this setting just prior to PPCI in the context of STEMI. They showed a reduction in myocardial oedema and infarct size as measured by cardiac magnetic resonance imaging (cMRI) as well as reduced levels of troponins in the conditioned group [52]. The excitement generated by these trials must be tempered by the difficulty in interpreting individual studies with small sample sizes and significant population heterogeneity which often assesses non-clinical outcome measures. Reassuringly, a recent comprehensive systematic review and meta-analysis of the available trial data by Le Page et al. [53] showed significant reductions in the hard end points of MACCE and all-cause mortality in conditioned groups compared to controls in this setting.

Remote ischaemic conditioning and remodelling postmyocardial infarction

Thibault et al. first hinted at the prospect that the effects of local IPostC after an MI may have a positive influence on myocardial contractility [54]. They demonstrated a 7 % greater left ventricular ejection fraction (LVEF) after 1 year compared with the control group ($p = 0.04$) [55]. Similarly, Munk et al. [54] in a sub-study of the CONDI trial showed that in MI patients with an area at risk (AAR) of over 35 %, those who received rIC immediately prior to PPCI had significant improvement in LVEF after 30 days compared to the control group (51 ± 11 vs. 46 ± 9 %, $p = 0.03$). Furthermore, Hoole et al. [5], as well as demonstrating reduced levels of Troponin *T* in patients undergoing elective PCI who received rIC compared to control, showed that at 6 months, the major adverse cardiac and cerebral event rate (MACCE) was lower in the rIC group (4 vs. 13 events, $p = 0.018$). More recent data published by the CONDI investigators underlined some of the long-term benefits of rIC [56]. They followed 256 patients who had suffered a STEMI to a median of 3.8 years, split equally between those who had received rIC at the time of PPCI and those who had received PPCI only. MACCE occurred in 13.5 % of the intervention group compared to 25.6 % of the control group (HR 0.49, CI 0.27–0.89, $p = 0.018$). However, due to the small sample size, no solid inferences could be made about a

number of secondary outcome measures, including the development of chronic heart failure.

In all these studies, one-off rIC at or around the time of MI has pointed towards the potential for this technique to reduce the incidence chronic heart failure. However, the degree to which the difference in LVEF and other markers of heart failure is due to remodelling, as opposed to attenuation of infarct size around the time of the acute event, is difficult to ascertain. Animal studies by Reddington's group have hinted that the progression to heart failure can be strongly attenuated, in a 'dose-dependent manner', by serial bouts of rIC soon after an ischaemic event. In a rat model of acute MI, Wei et al. [47] demonstrated the greatest improvement in LV chamber size, LV function and haemodynamic changes post-MI in the group that received repeated remote conditioning every day for 28 days compared to a control group and two groups receiving one-off applications of rIC either before or during ischaemia. The benefit appears to be in addition to the initial improvement seen due to reduction in infarct size and points towards novel mechanism of cardioprotection acting directly on remodelling. The study highlighted a variety of ways in which repeated rIC may work in this context including a reduction in oxidative stress, attenuation of the expression of genes associated with fibrosis and hypertrophy, and blunting of the inflammatory response with reduced levels of neutrophil and macrophage infiltration in the myocardium and reduced cytokine signalling. Previously, the same group had demonstrated that repetitive rIC significantly altered the behaviour of neutrophils after MI with reduced levels of adhesion at days 1 and 10 as well as a reduction in phagocytosis at day 10, apoptosis at days 1 and 10 and an overall change in the prolife of cytokine release [57]. More recent work from this group has suggested the existence of separate and very distinct mechanisms by which 'one-off' traditional rIC and repeated rIC infer protection. Whilst traditional rIC acts through the pathways described previously, repeated rIC was shown in this study to increased production of the autophagosome proteins LC3-II, cathepsin-L and Atg5 [58]. Yamaguchi et al. reinforced the power of repeated rIC post-MI and implicated exosomes as the mediators for signalling in rIC, possibly by their action of transferring anti-fibrotic microRNAs such as miR29a as well as IGF-1, which is known to be protective in the context of remodelling [59]. In addition, work by our laboratory showed that superfusate taken from ischaemic-conditioned Langendorff perfused rat hearts as well as serum taken from human volunteers immediately after undergoing rIC stimulation both independently inhibited endothelin-1-induced hypertrophy in a cellular model of hypertrophy alluding to a humoral mechanism of action [60].

Future perspectives

Multiple studies are underway to assess the impact of one-off rIC protocols at the time of MI on various heart failure-related outcome. Following on from the first CONDI study [56], CONDI-2 (Effect of RIC on Clinical Outcomes in STEMI Patients Undergoing PPCI) is well underway. This study aims to recruit 2300 participants over a 36-months period from a number of sites across Europe (http://www.clinicaltrials.gov/ct2/show/NCT01857414) with the primary outcome of assessing cardiovascular mortality and hospitalisation for heart failure at 1 year. Completion of the study is expected in late 2016. Running in collaboration with the CONDI-2 trial is the ERIC-PPCI (Effect of Remote Ischaemic Conditioning on clinical outcomes in ST-segment elevation myocardial infarction patients undergoing Primary Percutaneous Coronary Intervention) trial. This trial has recently started recruitment and aims to recruit 2000 participants in total across multiple sites to assess whether rIC at the time of PPCI for STEMI can reduce the combined primary outcome of cardiac death and hospitalisation for heart failure at 12 months (https://clinicaltrials.gov/ct2/show/NCT02342522).

DANAMI-3 (DANish Study of Optimal Acute Treatment of Patients with ST-elevation Myocardial Infarction) aims to assess the effect of local ischaemic conditioning on heart failure rates up to 3 years following PPCI for STEMI (http://clinicaltrials.gov/show/NCT01435408). The study has completed recruitment of over 2000 participants, and preliminary results pertaining to acute outcomes have previously been presented [61]. RECOND (Reduction in Infarct Size by Remote Per-postconditioning in Patients With ST-elevation Myocardial Infarction), a Swedish-led study, aims to recruit 120 participants and apply remote *per*-conditioning during PPCI for STEMI. One of the aims of the study is to compare cMRI-assessed remodelling parameters after 180 days between the conditioned and sham groups (https://clinicaltrials.gov/ct2/show/NCT02021760). Finally, the RIC-STEMI trial (Remote Ischaemic Conditioning in ST-elevation Myocardial Infarction as Adjuvant to Primary Angioplasty) is a Portuguese-led study aiming to recruit 492 participants. Similarly, this study will recruit from patients suffering STEMI and undergoing PPCI with a 1:1 randomisation to rIC approximately 10 min prior to first angiographic balloon inflation or sham conditioning. Rather than cMRI-based outcomes, the primary endpoint in this study will be death or hospitalisation from heart failure at a minimum of 1 year (https://clinicaltrials.gov/ct2/show/NCT02313961).

Two phase II trials are underway with the hypothesis that chronic, repeated rIC use in the post-STEMI period can positively influence cardiac remodelling and reduce

the incidence of and progression to heart failure: DREAM (Daily REmote Conditioning in Acute Myocardial Infarction) (http://clinicaltrials.gov/show/NCT01664611) and CRIC (Chronic Remote Ischaemic Conditioning to Modify Post-MI Remodelling) (http://clinicaltrials.gov/show/NCT01817114). The DREAM study is a UK-based, multi-centre randomised control trial recruiting individuals who have suffered a STEMI and have had successful PPCI. Inclusion criteria includes post-STEMI LVEF <45 % on transthoracic echocardiography with no prior history of MI. The study aims to recruit 72 patients and is powered to detect a 5 % increase in LVEF above natural recovery. Primary outcome data are obtained from baseline and 4-month cMRI to assess LVEF, left ventricular end diastolic volume and systolic volume, infarct size and oedema. An important facet of this trial is the intention to try and elucidate further our understanding of how much rIC in this context acts independently on remodelling when influences on the initial infarct size and MVO attenuation are reduced. This is done by beginning rIC 3 days after the acute event to avoid influencing the size of the infarct. RIC will continue for 4 weeks, performed daily by the participant. The study will randomise participants 50:50 in the intervention or the control group. The intervention group will receive a device that inflates to 200 mmHg in 4 × 5-min cycles of inflation and deflation. The control group will receive identical-looking devices that cycle as the intervention group but only inflate to a maximum of 10 mmHg. As conditioning commences on day 3 post-MI, a greater focus is on the modulation of the remodelling process rather than the infarct sparing properties of rIC.

In a similar vein, the CRIC study is a multi-centre randomised controlled trial recruiting from a STEMI/PPCI population in Canada with a recruitment aim of 82. CRIC differs from DREAM in that the investigators will recruit left anterior descending (LAD) territory infarcts only and will exclude diabetic individuals. The reasons for focusing on non-diabetic patients who have suffered large anterior STEMIs in the CRIC study are based on prior work, suggesting that this group is most likely to respond to rIC and hence gains greater impact from the intervention [1, 62, 63]. Furthermore, rIC will start just prior to PPCI and continue for 4 weeks; therefore, rIC in this context will likely have an influence on infarct size and MVO as well as subsequent remodelling. Primary outcome will be obtained by comparing cMRI at baseline and 28 days, primarily to compare LVEDV. Both the DREAM and CRIC trials are nearing completion, and it is hoped that once these trials are completed we will be in a better position to assess the role of chronic rIC in remodelling and whether this technique merits investigation with larger phase III randomised control trials.

Challenges of remote ischaemic conditioning

The recent high-profile ERICCA trial, which showed no clinical outcome benefit at 1 year when using rIC compared to sham conditioning during elective on-pump CABG surgery, has tempered the enthusiasm in some quarters for rIC as a potential new cardioprotective therapy [64]. Pertaining to cardioprotection in the context of MI and remodelling, a number of key obstacles remain in effectively translating the protection afforded by rIC in animal and early clinical trials into larger clinical trials and ultimately into routine clinical practice.

One major challenge is that of timing of rIC. Patients having an MI presenting late to centres that can administer rIC may have completed their infarct and as such will derive minimal benefit from the procedure with regard to limiting I/R injury, although they may derive benefits from remodelling [65]. Similarly, patients presenting with small infarcts or those receiving PPCI or thrombolysis very early may derive little benefit from rIC as the scope for additional cardioprotection in this setting is limited [66, 67].

Another significant challenge is that of the large comorbidities and polypharmacy that is often encountered in the MI patient population. In particular type 2 diabetes, hyperlipidaemia, obesity and hypertension have all been shown to increase the threshold required for effective rIC [68]. Conversely, a number of the medications used in the context of MI or commonly taken by this group of patients already provide a significant degree of cardioprotection, namely ace inhibitors, statins, opioids, insulin and a number of oral hypoglycaemic agents including metformin [69]. There are also a few medication that can inhibit the effects of rIC including sulfonylureas [70]. These issues muddy the waters and make trial design and subsequent clinical translation challenging.

Finally, from a practical perspective, because rIC involves the application of a device on the arm that requires a number of inflation and deflation cycles, even with the use of an automated device, this can pose logistical problems in the ambulance or the catheter laboratory during PPCI where time is of the essence and gaining arterial and venous access with the cuff in situ may pose an issue. Furthermore, in scenarios, where rIC must be administered on a regular basis by the patient to target remodelling post-MI, the authors foresee significant concordance issues which may limit the therapy in this context. The use of automated rIC devices that can be interrogated may go some way to overcomeing this issue.

Conclusions

RIC is only now beginning to reach its translational potential with regard to protection from ischaemic/reperfusion injury. Long-term outcome data for one-off rIC at

the time of MI are awaited from the CONDI-2, ERIC-PPCI, DANAMI-3, RECOND and RIC-STEMI trials to supplement promising results from smaller preliminary studies. It is yet to be established whether early preclinical data suggesting a clinically useful role for chronic, repeated rIC use in the context of post-MI remodelling will be borne out in the trial data, but it is hoped that results from both the DREAM and CRIC trials will go some way to answering this question and potentially open the door for larger clinical trials to follow.

Acknowledgments APV would like to acknowledge the generous funding received by the NIHR Leicester Cardiovascular Biomedical Research Unit.

Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests. In addition, the authors have no affiliations or financial involvement with any organisation or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

References

1. Bøtker HE, Kharbanda R, Schmidt MR, Bøttcher M, Kaltoft AK, Terkelsen CJ, Munk K, Andersen NH, Hansen TM, Trautner S, Lassen JF, Christiansen EH, Krusell LR, Kristensen SD, Thuesen L, Nielsen SS, Rehling M, Sørensen HT, Redington AN, Nielsen TT (2010) Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: a randomised trial. Lancet 375:727–734. doi:10.1016/s0140-6736(09)62001-8
2. Rentoukas I, Giannopoulos G, Kaoukis A, Kossyvakis C, Raisakis K, Driva M, Panagopoulou V, Tsarouchas K, Vavetsi S, Pyrgakis V, Deftereos S (2010) Cardioprotective role of remote ischemic periconditioning in primary percutaneous coronary intervention: enhancement by opioid action. J Am Coll Cardiol Interv 3:49–55. doi:10.1016/j.jcin.2009.10.015
3. Luo SJ, Zhou YJ, Shi DM, Ge HL, Wang JL, Liu RF (2013) Remote ischemic preconditioning reduces myocardial injury in patients undergoing coronary stent implantation. Can J Cardiol 29:1084–1089. doi:10.1016/j.cjca.2012.11.022
4. Ahmed RM, el Mohamed HA, Ashraf M, Maithili S, Nabil F, Rami R, Mohamed TI (2013) Effect of remote ischemic preconditioning on serum troponin T level following elective percutaneous coronary intervention. Catheter Cardiovasc Interv 82:18
5. Hoole SP, Heck PM, Sharples L, Khan SN, Duehmke R, Densem CG, Clarke SC, Shapiro LM, Schofield PM, O'Sullivan M, Dutka DP (2009) Cardiac remote ischemic preconditioning in coronary stenting (CRISP Stent) study: a prospective, randomized control trial. Circulation 119:820–827. doi:10.1161/circulationaha.108.809723
6. Thielmann M (2013) Cardioprotective and prognostic effects of remote ischaemic preconditioning in patients undergoing coronary artery bypass surgery: a single-centre randomised, double-blind, controlled trial. Lancet 382:597–604
7. Xie JJ, Liao XL, Chen WG, Huang DD, Chang FJ, Chen W, Luo ZL, Wang ZP, Ou JS (2012) Remote ischaemic preconditioning reduces myocardial injury in patients undergoing heart valve surgery: randomised controlled trial. Heart 98:384–388. doi:10.1136/heartjnl-2011-300860
8. Cheung MMH, Kharbanda RK, Konstantinov IE, Shimizu M, Frndova H, Li J, Holtby HM, Cox PN, Smallhorn JF, Van Arsdell GS, Redington AN (2006) Randomized controlled trial of the effects of remote ischemic preconditioning on children undergoing cardiac surgery: first clinical application in humans. J Am Coll Cardiol 47:2277–2282. doi:10.1016/j.jacc.2006.01.066
9. Weir RA, McMurray JJ, Velazquez EJ (2006) Epidemiology of heart failure and left ventricular systolic dysfunction after acute myocardial infarction: prevalence, clinical characteristics, and prognostic importance. Am J Cardiol 97(10A):13F–25F. doi:10.1016/j.amjcard.2006.03.005
10. Velagaleti RS, Pencina MJ, Murabito JM, Wang TJ, Parikh NI, D'Agostino RB, Levy D, Kannel WB, Vasan RS (2008) Long-term trends in the incidence of heart failure after myocardial infarction. Circulation 118:2057–2062. doi:10.1161/circulationaha.108.784215
11. Ndrepepa G, Mehilli J, Kastrati A, Schömig A, Schwaiger M, Martinoff S (2007) Evolution of left ventricular ejection fraction and its relationship to infarct size after acute myocardial infarction. J Am Coll Cardiol 50:149–156. doi:10.1016/j.jacc.2007.03.034
12. Garcia-Dorado D, Ruiz-Meana M, Piper HM (2009) Lethal reperfusion injury in acute myocardial infarction: facts and unresolved issues. Cardiovasc Res 83(2):165–168. doi:10.1093/cvr/cvp185
13. White H, Norris R, Brown M, Brandt P, Whitlock R, Wild C (1987) Left ventricular end-systolic volume as the major determinant of survival after recovery from myocardial infarction. Circulation 76:44–51. doi:10.1161/01.cir.76.1.44
14. Verma A, Meris A, Skali H, Ghali JK, Arnold JMO, Bourgoun M, Velazquez EJ, McMurray JJV, Kober L, Pfeffer MA, Califf RM, Solomon SD (2008) Prognostic implications of left ventricular mass and geometry following myocardial infarction: the VALIANT (VALsartan In Acute myocardial iNfarcTion) Echocardiographic Study. J Am Coll Cardiol Imaging 1:582–591. doi:10.1016/j.jcmg.2008.05.012
15. Murry CE, Reimer KA, Jennings RB (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 74:1124–1136
16. Deutsch E, Berger M, Kussmaul WG, Hirshfeld JW, Herrmann HC, Laskey WK (1990) Adaptation to ischemia during percutaneous transluminal coronary angioplasty. Clinical, hemodynamic, and metabolic features. Circulation 82(6):2044–2051. doi:10.1161/01.cir.82.6.2044
17. Yellon DM, Alkhulaifi AM, Pugsley WB (1993) Preconditioning the human myocardium. Lancet 342:276–277
18. Zhao Z-Q, Faraz K, Michael EH, Joel SC, Wang N-P, Guyton RA, Vinten-Johansen J (2003) Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol 54:H579
19. Laskey WK (2005) Brief repetitive balloon occlusions enhance reperfusion during percutaneous coronary intervention for acute myocardial infarction: a pilot study. Catheter Cardiovasc Interv 65:361–367. doi:10.1002/ccd.20397
20. Staat P, Ovize M, Bonnefoy E, André-Fouët X, Aupetit J-F, L'Huillier I, Cottin Y, Cung TT, Piot C, Rioufol G, Finet G

'rIC: Beyond Traditional Acute Cardioprotection' - Published in Hospital Healthcare Europe Bulletin 2014[573]

rIC: Beyond traditional acute cardioprotection

Automated remote ischaemic conditioning (rIC) aims to replicate preclinical data suggesting post-MI remodelling benefits from chronic rIC

Andrew Vanezis BSc MB ChB MRCP
Department of Cardiovascular Sciences, University of Leicester, UK
Nilesh Samani BSc MB ChB MD FRCP FACC FAHA F Med Sci
Head of Department of Cardiovascular Sciences; Director Leicester NIHR Biomedical Research Unit in Cardiovascular Disease, University of Leicester, UK

Traditional cardioprotection as a concept refers broadly to 'all mechanisms and means that contribute to the preservation of the heart by reducing or even preventing myocardial damage'.[1] More recently, this term has been associated with protection afforded against acute ischaemia–reperfusion injury at the time of myocardial infarction (MI) and subsequent re-vascularisation. In particular, the use of remote ischaemic conditioning (rIC), a form of cardioprotection that involves intermittent interruption of blood flow to an organ or muscle bed distant to the heart (usually the upper arm), has been used successfully in the context of acute MI,[2,3] as well as planned coronary angioplasty,[4–7] to reduce myocardial damage and improve cardiovascular outcomes. Recent preliminary work has hinted at an additional role for rIC in positively influencing cardiac remodelling that occurs soon after an MI,[8,9] which may consequently have a profound impact in reducing the incidence and impact of heart failure that develops in a large number of patients post-MI.

Heart failure and cardiac remodelling

Heart failure is a major cause of long-term mortality and morbidity after MI. In the US, 7% of men and 12% of women under 70 years and 22% of men and 25% of women 70 years and over will develop heart failure (usually left ventricular systolic dysfunction) within five years of their first MI.[10] Heart failure that develops following an MI is usually a combination of infarcted myocardium due to the initial insult and cardiac remodelling that occurs in the weeks and months after the event. Remodelling is the process of reorganisation of the remaining myocardium in order to compensate for that which has been lost and no longer contributes to cardiac output. The degree of remodelling that occurs is roughly proportional to the degree of necrotic muscle infarction[11] with the changes occurring at a cellular level, where cellular homeostasis and signalling is modified, and at a whole-organ level, where heart dimensions and haemodynamics are altered. This process is initially compensatory, but over time, this process ultimately becomes detrimental. The level of remodelling and its progression is a powerful predictor for both heart failure and death following MI, with prognostic implications for further MIs, cerebral vascular accidents (CVA) and cardiac arrest.[12,13]

Remote ischaemic conditioning

Murry et al first described an endogenous cardioprotective mechanism called ischaemic preconditioning in an animal model, whereby intermittent occlusion and reperfusion of a coronary artery territory prior to sustained occlusion significantly reduced final infarct size.[14] Evolution of this technique has seen it applied after an acute ischaemic event in the clinical setting (postconditioning)[15-16] and subsequently, remotely from the target organ (rIC) in a non-invasive fashion affording protection to the heart,[2,3,19] as well other organs such as the brain and kidney.[20-21]

Expanding the paradigm

Thibault et al demonstrated that the effects of post-conditioning post-MI also have a positive influence on more chronic markers such as final infarct size and myocardial contractility. They demonstrated a 7% increase in left ventricular ejection fraction after one year compared with the control group.[18] Similarly, Munk et al showed that in MI patients with an Area At Risk (AAR) of over 35%, those who received rIC after primary percutaneous coronary intervention (PPCI) had a significant improvement of left ventricular ejection fraction (LVEF) after 30 days compared with the control group: 51±11% versus 46±9%, respectively.[22] Furthermore, Hoole et al, as well as demonstrating reduced levels of troponin T (a blood biomarker of cardiac damage) in patients undergoing elective PCI who received rIC compared with control, showed that the major adverse cardiac and cerebral event rate was lower in the rIC group at six months (4 versus 13 events; $p = 0.018$).[5]

Kharbanda's group took this concept a step further in humans by applying rIC during an ongoing MI. They applied a rIC protocol using a blood pressure cuff to the forearm of ST segment elevation MI (STEMI) patients during their ambulance journey to receive PPCI. Conditioned patients with a large MI showed a significantly improved myocardial salvage index (MSI), a radiographic marker of cardiac damage, than unconditioned subjects.[2,19] A smaller study by the same group was undertaken in STEMI patients where rIC was applied to the forearm just

after PCI. With the addition of morphine, there was a significant reduction of troponin T levels in the conditioned group compared with the control group, although there was little effect of conditioning without the addition of morphine.[5]

In all the studies to date, the rIC stimulus was implemented on a one-off basis, either pre- or immediately post-MI. Tantalisingly, one rodent study recently hinted that the progression to heart failure can be averted (or at least delayed) by serial bouts of rIC soon after an ischaemic event. Wei et al demonstrated the greatest improvement in LV chamber size, LV function and haemodynamic changes post-MI in the group that received repeated remote conditioning every day for 28 days compared with a control group and two groups receiving one-off applications of rIC either before ischaemia (pre-conditioning) or during (peri-conditioning). The benefit appears to be in addition to the initial improvement seen due to reduction in scar size. The study highlighted a variety of ways in which repeated rIC may work, including a reduction in oxidative stress, attenuation of the expression of genes associated with fibrosis and hypertrophy, and blunting of the inflammatory response with reduced levels of neutrophil and macrophage infiltration in the myocardium and reduced cytokine signalling.[8] Complementary work by Shimzu et al also demonstrated the important role neutrophils play in chronic rIC.[9]

Automated rIC

The autoRIC™ Device, developed by CellAegis Devices Inc, automates the process of rIC. The device automatically inflates to 200mmHg for five minutes interspersed with five minutes of deflation for a total of four cycles. This device enables trial participants to automate rIC in their own home with little or no input from a healthcare professional after first use. Prior to the implementation of the autoRIC™ Device, the authors administered rIC for trial purposes using a manual sphygmomanometer. For some studies where daily rIC was needed after the participant was discharged from hospital, this required visits to participants' homes, which were time-consuming and incurred significant travel costs. The feedback received from trial participants to date is that the autoRIC™ Device is simple to use and gives a sense of empowerment as they are, to a degree, responsible for their own treatment.

Current trials

At the present time there are two trials underway with the hypothesis that

chronic rIC use in the post-STEMI period can positively influence cardiac remodelling and reduce the incidence of and progression to heart failure: DREAM (Daily REmote Conditioning in Acute Myocardial Infarction; clinicaltrials.gov/show/NCT01664611) and CRIC (Chronic Remote Ischemic Conditioning to Modify Post-MI Remodelling; clinicaltrials.gov/show/NCT01817114). Both trials are utilising the autoRIC™ Device.

The DREAM study is a UK based randomised control trial recruiting individuals who have suffered a STEMI and have had successful PPCI. Inclusion criteria includes having a post-STEMI LVEF on transthoracic echocardiography of <45% on a background of a previously documented normal myocardium. The study aims to recruit 72 patients. Primary outcome data are obtained from baseline and four-month cardiac magnetic resonance imaging (cMRI) to assess EF, left ventricular end diastolic volume (LVEDV) and systolic volume (LVESV), infarct size and oedema.

CRIC will recruit from a similar patient population in Canada with a recruitment aim of 82. CRIC differs from DREAM in that the investigators will recruit only left anterior descending (LAD) territory infarcts only, will not screen post-STEMI LVEF on echo and will exclude diabetic individuals. Furthermore, primary outcome will be obtained by comparing cMRI at baseline and 28 days to compare LVEDV. Both trials aim to complete recruitment by early 2015. It is hoped that once these trials are complete, we will be in a better position to assess the role of chronic rIC in remodelling and whether this technique merits investigation with larger Phase III, randomised, controlled trials (RCTs).

Conclusions

rIC is only now beginning to reach its translational potential with regards to protection from ischaemic/reperfusion injury. It is yet to be established whether early preclinical data suggesting that chronic rIC use in the context of post-MI remodelling will be borne out in the trial data, but it hoped that results of both the DREAM and CRIC trials will go some way to answering this question and potentially open the door for larger RCTs to follow. ✦

References

1. Kübler W, Haass M. Cardioprotection: definition, classification, and fundamental principles. Heart 1996;75:330–3.
2. Bøtker HE et al. Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: a randomised trial. Lancet 2010;375:727–34.
3. Rentoukas I et al. Cardioprotective role of remote ischemic periconditioning in primary percutaneous coronary intervention: Enhancement by opioid action. J Am Coll Cardiol Interv 2010;3:49–55.
4. Luo SJ et al. Remote ischemic preconditioning reduces myocardial injury in patients undergoing coronary stent implantation. Can J Cardiol 2013;29:1084–9.
5. Hoole SP et al. Cardiac remote ischemic preconditioning in coronary stenting (CRISP Stent) study: a prospective, randomized control trial. Circulation 2009;119:820–7.
6. Ghaemian A et al. Remote ischemic preconditioning in percutaneous coronary revascularization: a double-blind randomized controlled clinical trial. Asian Cardiovasc Thorac Ann 2013;20:548–54.
7. Ahmed RM et al. Effect of remote ischemic preconditioning on serum troponin T level following elective percutaneous coronary intervention. Catheter Cardiovasc Interv 2013;82:E647–53.
8. Wei M et al. Repeated remote ischemic postconditioning protects against adverse left ventricular remodeling and improves survival in a rat model of myocardial infarction. Circ Res 2011;108:1220–5.
9. Shimizu M et al. Remote ischemic preconditioning decreases adhesion and selectively modifies functional responses of human neutrophils. J Surg Res 2010;158:155–61.
10. Rosamond W et al. Heart disease and stroke statistics-2008 Update: A report from the American Heart Association statistics committee and stroke statistics subcommittee. Circulation 2008;117:e25–e46.
11. McKay R et al. Left ventricular remodeling after myocardial infarction: a corollary to infarct expansion. Circulation 1986;74:693–702.
12. White H et al. Left ventricular end-systolic volume as the major determinant of survival after recovery from myocardial infarction. Circulation 1987;76:44–51.
13. Verma A et al. Prognostic implications of Left Ventricular Mass and Geometry Following Myocardial Infarction: The VALIANT (VALsartan In Acute myocardial iNfarcTion) Echocardiographic Study. J Am Coll Cardiol Imag 2008;1:582–91.
14. Murry CE, Reimer KA, Jennings RB. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986;74:1124–36.
15. Laskey, W.K. Brief repetitive balloon occlusions enhance reperfusion during percutaneous coronary intervention for acute myocardial infarction: A pilot study. Catheter Cardiovasc Interv 2005;65:361–7.
16. Zhao Z-Q et al. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: Comparison with ischemic preconditioning. Am J Physiol 2003;54:H579.
17. Staat P et al. Postconditioning the human heart. Circulation 2005;112:2143–8.
18. Thibault H et al. Long-term benefit of postconditioning. Circulation 2008;117:1037–44.
19. Kharbanda R et al. Transient limb ischemia induces remote ischemic preconditioning in vivo. Circulation 2002;106:2881–3.
20. Hahn CD et al. Remote ischemic per-conditioning: a novel therapy for acute stroke? Stroke 2011;42:2960–2.
21. Candilio L, Malik A, Hausenloy DJ. Protection of organs other than the heart by remote ischemic conditioning. J Cardiovasc Med 2013;14:193–205.
22. Munk K et al. Remote ischemic conditioning in patients with myocardial infarction treated with primary angioplasty / clinical perspective. Circ Cardiovasc Imag 2010;3:656–62.

9.3 Awards

- East Midlands Engineering and Science Professional Prize Winner 2012 – Press Release

East Midlands Engineering and Science Professional Prize Winner 2012 Press Release

UNIVERSITY OF LEICESTER

Web Editor Log in

Search Site Search

University Home | University A-Z | Maps and Directions

Home / Academic Departments / Graduate School / Postgraduate Community at Leicester / Continued Success for Leicester Science PhDs

University of Leicester Triumphs Again at 2012 Regional Competition for Postgraduate Researchers

Andrew Vanezis Wins East Midlands Engineering and Science Professionals Prize

The University of Leicester has made it two years in a row with PhD candidate Dr Andrew Vanezis of the Department of Cardiovascular Sciences winning the 2012 East Midlands Engineering and Science Professionals Prize.

Andrew, who is supervised by Dr Glenn Rodrigo and Professor Nilesh Samani, beat off a strong field of postgraduates from other East Midlands universities to win the prize for his work into how temporarily blocking blood flow can actually help the heart become more resistant to damage caused during heart attacks. Andrew's success follows that of fellow Leicester researcher Will Nicholson who won in 2011.

Heart failure is a debilitating condition in which the heart is unable to supply sufficient blood and therefore oxygen and nutrients for the bodies needs, and is a major cause of morbidity and death in the UK. In spite of the introduction of strategies to unblock the coronary arteries of patients suffering a heart attack, many surviving individuals go on to develop heart failure, and it is estimated that around 2% of people in the UK over 45 years of age suffer from some degree of cardiac failure.

Andrew, who started his PhD in 2011, is conducting exciting research adopting a new angle on an old story. At Leicester Andrew and his group have been conducting research into the phenomenon of "conditioning" in which **the arm of a patient is "squeezed" using a pressure cuff for short periods resulting in protection of the heart against the acute injury of a heart attack**. They have shown that the arm responds to this transient stoppage in the blood by releasing substances into the blood which then travel to the heart where they bind to receptors on the surface of the cells and stimulate pro-survival pathways rendering the heart resistant to injury.

The process by which a damaged heart begins to fail involves a complex "remodelling" of the individual cells and the underlying architecture of the wall of the pump. What Andrew and his group have noticed is that many of the pathways triggered by the blood born agents released during "conditioning" are also known to prevent this remodelling process. Their results show that these agents in addition to protecting the heart cells possess a powerful ability to prevent the adverse enlargement of damaged cells, which is characteristic of the remodelling process. The group is currently investigating the molecular mechanisms responsible for this.

This work which utilizes a completely safe technique to recruit an endogenous protective mechanism of the body has the potential to not only reduce injury during a heart attack but also to prevent adverse changes to the heart of the recovering patient. Andrew and his group hope to be able to use the results of their research, which is supported by the NIHR Biomedical Research Unit in Cardiovascular Disease, to develop a treatment regime for patients during and after a heart attack.

Andrew, who received as his prize £500 and an engraved silver tankard, said "I was both surprised and honoured to have my research recognised in this way. The quality of the presentations on the day was very strong; ultimately I think the judges were impressed with the translational nature of my research and the potential of remote ischaemic conditioning to become an important clinical tool in the fight against heart failure post heart attack".

About East Midlands Engineering and Science Professionals

East Midlands Engineering and Science Professionals provides a voice on the common issues for the engineering and science profession in the East Midlands and works to make people more aware of the vital contribution that engineering and science make to the economy, prosperity, and quality of life in the region.

The EMESP Prize is an annual award given to a postgraduate researcher from one of the region's universities. In 2011 and 2012 the EMESP Prize has been won by the University of Leicester.

Learn More

- Department of Cardiovascular Sciences
- Leicester Cardiovascular Biomedical Research Unit
- University of Leicester Graduate School
- Our Research Degrees

GRADUATE SCHOOL

Share this page: Like 0 Tweet 0+1 0 Share

9.4 Healthy Male Volunteer Recruitment Study Documents

- NRES Ethical Approval Letter
- UHL R&D Approval Letter
- History and Blood Results template
- Physical Activity Questionnaire (IPAQ-SF)
- Advertisement for recruitment
- Participant Information Sheet
- Consent Form

NRES Committee East Midlands - Leicester
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 01158839440
Facsimile: 01159123300

18 May 2012

Dr Glenn Rodrigo
Lecturer in Cardiovascular Sciences
University of Leicester
Clinical Sciences Department
Glenfield Hospital
Groby Road Leicester
LE3 9QP

Dear Dr Rodrigo,

Study title:	**Remote Ischaemic Conditioning in Healthy Individuals to Assess the Cardioprotective Effects of Their Serum on a Cellular Model of Post Myocardial Infarction Ventricular Remodelling**
REC reference:	**12/EM/0145**
Protocol number:	**UNOLE0305**

Thank you for your letter of 24 May 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Advertisement	1.0	05 March 2012
Advertisement	1.0	05 March 2012
Covering Letter		09 March 2012
Evidence of insurance or indemnity		16 August 2011
GP/Consultant Information Sheets	1.0	05 March 2012
Investigator CV		21 June 2010
Letter of invitation to participant	1.0	05 March 2012
Other: Student CV: DR Andrew Vanezis		05 March 2012
Other: CV: Dr S A Edroos		05 March 2012
Other: GCP Certificate: Dr S A Edroos		29 November 2011
Other: GCP Certificate: Dr Andrew Peter Vanezis		29 November 2011
Other: Research Governance Certificate: Dr Glenn Rodrigo		01 July 2010
Other: CV: Mrs Mary Elizabeth Harrison (nee Oakley)		25 January 2012
Other: GCP Certificate: Mary Harrison		16 January 2012
Other: CV: Elaine Logtens		03 May 2011
Other: GCP Certificate: Elaine Logtens		15 February 2011
Other: CV: Robyn Lotto		02 November 2010
Other: GCP Certificate: Robyn lotto		28 October 2010
Other: Participant Process Flowchart	1.0	05 March 2012
Other: Signed D3 by Academic Supervisor		24 April 2012
Participant Consent Form	1.0	29 February 2012
Participant Consent Form	2.0	24 April 2012
Participant Information Sheet	1.0	29 February 2012
Participant Information Sheet	2.0	24 April 2012
Protocol	1.0	29 February 2012
REC application	99031/30476	16 March 2012

	3/1/584	
Referees or other scientific critique report		09 March 2012
Response to Request for Further Information		24 May 2012

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/EM/0145	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely,

Dr Carl Edwards
Chair

Email: Sam.Tuite@nottspct.nhs.uk

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Mrs Carolyn Maloney, University Hospitals of Leicester NHS Trust*

UHL R&D Approval Letter for Healthy Male Volunteer Recruitment Study Page 1

University Hospitals of Leicester NHS
NHS Trust

DIRECTORATE OF RESEARCH & DEVELOPMENT

Director: **Professor D Rowbotham**
Assistant Director: **Dr David Hetmanski**
R&D Manager: **Carolyn Maloney**

Research & Development Office
Leicester General Hospital
Gwendolen Road
Leicester
LE5 4PW

Direct Dial: (0116) 258 8351
Fax No: (0116) 258 4226

09th August 2012

Dr Glen Rodrigo
Clinical Lecturer in Cardiovascular Sciences,
University of Leicester,
Department of Cardiovascular Sciences,
Clinical Science Wing,
Glenfield Hospital,
Groby Road,
Leicester,
LE3 9QP

Dear Dr Rodrigo,

Ref: 99031
Title: **Remote Ischaemic Conditioning in Healthy Individuals to Assess the Cardioprotective Effects of Their Serum on a Cellular Model of Post Myocardial Infarction Ventricular Remodelling.**
Project Status: **Approved**
End Date: **31st November 2014**

I am pleased to confirm that with effect from the date of this letter, the above study has Trust Research & Development permission to commence at University Hospitals of Leicester NHS Trust. The research must be conducted in line with the Protocol and fulfil any contractual obligations agreed with the Sponsor. If you identify any issues during the course of your research that are likely to affect these obligations you must contact the R&D Office.

In order for the UHL Trust to comply with targets set by the Department of Health through the 'Plan for Growth', there is an expectation that the first patient will be recruited within 30 days of the date of this letter. If there is likely to be a problem achieving this target, please contact the office as soon as possible. You will be asked to provide the date of the first patient recruited in due course. In addition, the Title, REC Reference number, local target recruitment and actual recruitment for this study will be published on a quarterly basis on the UHL Trust external website.

All documents received by this office have been reviewed and form part of the approval. The documents received and approved are as follows:

Document Title	Version	Date	REC Approval
REC Favourable Opinion Letter	N/A	18/05/12	N/A
Protocol	1.0	29/02/12	18/05/12
Participant Information Sheet	2.0	24/04/12	18/05/12
Participant Consent Form	2.0	24/04/12	18/05/12
Letter of Invitation to Participant	1.0	05/03/12	18/05/12
GP/Consultant Information Sheet	1.0	05/03/12	18/05/12
Advertisement: Email	1.0	05/03/12	18/05/12
Advertisement: Poster	1.0	05/03/12	18/05/12
Participant Process Flowchart	1.0	05/03/12	18/05/12

Please be aware that any changes to these documents after approval may constitute an amendment. The process of approval for amendments should be followed. Failure to do so may invalidate the approval of the study at this trust.

Undertaking research in the NHS comes with a range of regulatory responsibilities. Please ensure that you and your research team are familiar with, and understand the roles and responsibilities both collectively and individually.

Documents listing the roles and responsibilities for all individuals involved in research can be found on the R&D pages of the Public Website. It is important that you familiarise yourself with the Standard Operating Procedures, Policies and all other relevant documents which can be located by visiting www.leicestershospitals.nhs.uk/aboutus/education-and-research

The R&D Office is keen to support and facilitate research where ever possible. If you have any questions regarding this or other research you wish to undertake in the Trust, please contact this office. Our contact details are provided on the attached sheet.

This study has been reviewed and processed by the Leicestershire, Northamptonshire & Rutland Comprehensive Local Research Network (LNR CLRN) using the Coordinated System for gaining Trust Permission (CSP). If you require any further information on the approval of this study please contact the LNR CLRN office on 0116 258 6185 making reference to the CSP number which is located at the top of this letter.

We wish you every success with your research.

Yours sincerely

Carolyn Maloney
R&D Manager

Encs: .R&D Office Contact Information

History and Blood Results Template Used in Healthy Male Volunteer Recruitment Study

University of Leicester

THIS DOCUMENT IS CONFIDENTIAL

Patient study number _____

Leicester Cardiovascular Biomedical Research Unit

Demographics

Name _____

Date of Birth _____

Assessment Date _____

Medical History

Drug History

Allergies? _____

Social History

Smoker? – Y/N/Ex (please circle). If Y/Ex, pack year hx _____

Levels of physical activity – low/moderate/high (please circle)

Vital Signs

Heart Rate _____

Blood Pressure _____

Fasting Blood Tests

Hb	Na+	Glu
MCV	K+	Trig
Platelets	U	LDL
WCC	CR	HDL

Physical Activity Questionnaire (IPAQ-SF) in Healthy Male Volunteer Recruitment Study Page 1

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

   _____ **days per week**

   ☐ No vigorous physical activities → ***Skip to question 3***

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

   _____ **hours per day**

   _____ **minutes per day**

   ☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

   _____ **days per week**

   ☐ No moderate physical activities → ***Skip to question 5***

SHORT LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised August 2002.

Physical Activity Questionnaire (IPAQ-SF) in Healthy Male Volunteer Recruitment Study Page 1

Physical Activity Questionnaire (IPAQ-SF) in Healthy Male Volunteer Recruitment Study Page 2

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ **days per week**

☐ No walking → ***Skip to question 7***

6. How much time did you usually spend **walking** on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

This is the end of the questionnaire, thank you for participating.

SHORT LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised August 2002.

Advertisement Poster Used in Healthy Male Volunteer Recruitment Study

University of Leicester

Leicester Cardiovascular Biomedical Research Unit

DID YOU KNOW THAT…

Most people who suffer a heart attack in the UK survive thanks to specialist medical treatment.

HOWEVER…

Many of the people who survive heart attacks develop **Heart Failure** with:

- A shortened life expectancy
- Breathing difficulties
- Body water retention
- Extreme tiredness

Do you want to help with Heart Failure research?

Do you want a free heart check-up at the same time?

The Biomedical Research Unit at Glenfield Hospital is looking for healthy people who are interested in taking part in a research study into protecting against heart failure following heart attacks. The study will take about an hour and involve having a blood pressure cuff inflated on an arm with blood tests either side of this. We will also carry out a physical examination and blood tests to check the blood count, kidney and liver function, cholesterol levels and look for diabetes at the same time, which if you agree will be sent on to your GP.

Interested?

Please contact Dr Andrew Vanezis on
0116 250 2650 / *apv4@le.ac.uk*

Participation Information Sheet Used in Healthy Male Volunteer Recruitment Study

Page 1

University of Leicester | Leicester Cardiovascular Biomedical Research Unit | NHS National Institute for Health Research

Dr Andrew Peter Vanezis
BSc (Hons), MB ChB, MRCP (UK)
Clinical Research Fellow
Department of Cardiovascular Sciences
Glenfield Hospital, Groby Road
LEICESTER LE3 9QP
Tel: +44 (0)116 250 2650
Email: *apv4@le.ac.uk*

Remote Ischaemic Conditioning and Ventricular Remodelling

Principal Investigator
Dr Glenn Rodrigo
Department of Cardiovascular Sciences
Glenfield Hospital

PARTICIPANT INFORMATION SHEET

We would like to invite you to take part in our research study.

Before you decide we would like you to understand why the research is being done and what it would involve for you. You have been sent this leaflet to read at your leisure and if you agree to participate, one of our team will go through the information sheet with you and answer any questions you may have. Please ask us if there is anything that is not clear or you would like more information about.

This study aims to study the effectiveness of a new technique that can potentially be used after people have suffered a heart attack to reduce the chance of those people developing heart failure. This is important as it has the potential to improve quality of life as well as prolonging life expectancy. This technique involves the use of a standard blood pressure machine.

Participation Information Sheet Used in Healthy Male Volunteer Recruitment Study

Page 2

Study Title

Remote Ischaemic Conditioning and Ventricular Remodelling.

Background information

Worldwide over 7 million people suffer a heart attack every year. Thankfully due to advances in the way we now treat heart attacks, the majority of people survive. However many of the survivors goes on to develop subsequent problems including shortness of breath, extreme tiredness, retention of water in the body, chest pain, irregular heart rhythms as well as having a significantly shortened life expectancy. This is collectively known as *heart failure* and is usually caused by ongoing changes to the heart that takes place many weeks and months after the heart attack has occurred.

What is the purpose of the study?

This study will test a new technique to help combat the changes that occur after a heart attack with the aim of improving peoples' quality of life and life expectancy. This technique is called *Remote Ischaemic Conditioning* and involves the use of a blood pressure cuff which inflates and deflates on your arm and generates chemical messages that protect the heart. By studying the chemical released in the blood in healthy volunteers, we aim to gain a better understanding of how this techniques works when applied to people who have suffered heart attacks.

What do I have to do if I agree to take part?

Your participation in this study is completely voluntary. If you agree to participate, you will be given this information sheet to keep and you will be asked to sign a consent form if the researchers feel you meet the criteria for inclusion. Please be aware that despite interest on your part, you may not be eligible for inclusion, for example if you suffer from a heart condition. If you do participate in the study, **you will be required to fast on the morning of the study.** This means no food from midnight the night before and on the morning of the appointment. You are allowed to drink water but nothing else e.g. tea, coffee, fruit/soft drinks are not allowed. This is necessary for some of the blood tests. You will be able to eat and drink freely after the procedure is complete. On attending the BRU at Leicester Glenfield hospital, a medical doctor will ask some questions on your general health and carry out a physical examination. At this stage you may be precluded from taking part in the study based

Participation Information Sheet Used in Healthy Male Volunteer Recruitment Study

Page 3

on information gained from the history and/or the clinical examination. If this is the case you will of course still be reimbursed your travel and parking expenses.

The doctor will place a plastic tube (cannula) in the vein and take up to 60mls of blood. The cannula will be secured using clinical tape so that subsequent blood tests can be done from the cannula without having to reintroduce the needle. We will interrupt the blood flow to the opposite arm for 5 minutes using a blood pressure cuff. Following this flow is returned to normal. This procedure is repeated 3 times. This is a safe procedure. At the end another blood sample (up to 50ml) is taken from the cannula. Therefore in total we will take up to 110 ml of blood (about 7 tablespoons), which is about a quarter of what is taken when donating blood. The whole process will take around an hour of your time from start to finish.

As we are taking routine blood samples (blood count, kidney test, liver test, cholesterol test and sugar levels) and a physical examination, we are happy to send these test results on to your GP. We will not do this if you would prefer **unless we find any information that is clinically significant and may warrant action by your GP, this includes clinically relevant information discovered in your medical records.** It will be at your GP's discretion to inform you of any such findings.

Please be aware you are free to withdraw from the study at any time without giving a reason and this will not affect the way you are treated at this or any other hospital.

What are the possible benefits of taking part?

There will be no direct benefit to you by participating in this study however you will receive a screening medical examination and blood tests. Your involvement in the study will however will be helpful in our study into a major health condition.

What are the risks involved?

We will be taking blood at two separate time-points. This involves a needle puncture using a venous cannula. This is a piece of plastic that sits over a needle and it inserted into the vein. This is a safe procedure, carried out by experienced health professionals, but some people find it painful and occasionally there is a small amount of bruising after the procedure has been carried out. Some people are very sensitive to needles and the sight of their own blood and may feel lightheaded or faint when a blood sample is taken. This is not uncommon and can be reduced by lying down while the sample is taken. Occasionally more than one attempt may be required to locate a vein but no more than three unsuccessful attempts will be carried out before abandoning the procedure. The plastic cannula will remain in the arm for around

Participation Information Sheet Used in Healthy Male Volunteer Recruitment Study

Page 4

half an hour (the needle is removed once the cannula is in the vein) whilst the blood pressure cuff inflation and deflation occurs in the other arm. Some people can find the cannula uncomfortable or painful and if this is the case it will be removed. On removing the cannula, significant pressure must be applied to the wound site for at least 2 minutes and a plaster placed on the wound.

The inflation of the blood pressure cuff is uncomfortable, and may cause tingling of the fingers and a mild rash on the upper arm which will quickly fade, but will not cause any lasting problems in healthy people and has been used in previous trial at this centre. We are also carrying out screening blood tests and a physical check-up, and we sometimes find something unexpected that may lead to more investigations and treatments.

What about expenses and payments?

You will not receive payment for participating in this study. Travel expenses and hospital parking fees will be reimbursed at the time of your visit. A maximum claim of £30 per participant can be made and will be agreed between the participant and a member of the research team prior to the visit.

Who will see my records and know that I am taking part in the study?

If you agree to take part, the study team and where relevant, authorised responsible individuals from the University of Leicester NHS Trust, regulatory authorities or other organisations including staff at the Biomedical Research Unit, will have access to your medical records and information taken about you in the study. This information will remain strictly confidential.

What will happen to the samples I give?

Routine sampling of your blood will be sent to the pathology laboratory for analysis. In addition we will do some more complex tests that are associated with heart failure. Data gained from your participation may be retrospectively removed from the study based on laboratory blood findings for example a previously unknown raised cholesterol. Any blood that is not used initially will be confidentially labelled and stored in a University of Leicester freezer for up to 5 years.

Participation Information Sheet Used in Healthy Male Volunteer Recruitment Study

Page 5

What will happen if I don't want to carry on with the study?

Your participation in the study is voluntary and you are free to withdraw at any time without giving any reason and without my medical care or legal rights being affected.

Medication / health

We are studying normal responses in healthy individuals. If you have any known chronic disease, or if you have taken any medication, prescribed or otherwise, please discuss this in confidence with the doctor.

What will happen to the results of the study?

The results of the study will be presented at scientific meetings and conferences and published in the medical and scientific literature. Your identity will be not be divulged at any time. If you so wish, you will be able to obtain details of the results of any publications from the research team. If clinically relevant information is discovered, it will be revealed to your GP to act at his/her discretion.

Who is sponsoring and funding the research?

The study is sponsored by the University of Leicester and funded by the Leicester Cardiovascular Biomedical Research Unit.

Who has reviewed the study?

Research that involves NHS patients and information from NHS medical records must be given a Favourable Opinion by an NHS Research Ethics Committee before it starts. The committee comprises of an independent group of people who study the research protocol to protect your safety, rights, wellbeing and dignity. If a study is given a Favourable Opinion it means that the committee is satisfied that your rights will be respected, that risks have been minimised and that you have been given sufficient information to make an informed decision about taking part.

Participation Information Sheet Used in Healthy Male Volunteer Recruitment Study

Page 6

Leicestershire Partnership NHS Trust, Ground Floor, Bradgate Mental Health Unit, Groby Road, Glenfield General Hospital, Leicester, Leicestershire, LE3 9EJ.

Further information and contact details

You will be given a copy of this information sheet and a signed consent form to keep. If at any time you would like any further information about the study, please contact:

Dr Andrew Vanezis
Clinical Research Fellow
Department of Cardiovascular Sciences
Clinical Science Wing
Glenfield Hospital, Groby Road
Leicester. LE3 9QP
Tel: 0116 2502650
Email: apv4@le.ac.uk

We would like to take this opportunity to thank you for taking the time to consider participating in this study.

Consent Form Used in Healthy Male Volunteer Recruitment Study

University of Leicester

National Institute for Health Research
Leicester Cardiovascular
Biomedical Research Unit

NHS
National Institute for
Health Research

Remote Ischaemic Conditioning and Ventricular Remodelling

Researcher: Dr Andrew Vanezis

CONSENT FORM

Please initial each box:

1. I confirm that I have read the information sheet dated 24/04/2012 version 2.0 for the above study and I have understood it, including the risks and benefits involved. I have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason and without my medical care or legal rights being affected. ☐

3. I agree to my general practitioner or any other doctor treating me being notified of my participation in the study. ☐

4. I understand and agree that relevant sections of my medical notes and/or data may be looked at by the sponsor, regulatory authorities, or authorised individuals from the NHS Trust who oversee research where it is relevant and I give permission for these individuals to access my records. ☐

5. I agree to samples of my bodily tissue collected during the course of this study being used in future research. ☐

6. I agree to take part in this study. ☐

Participant name:	Signature:	Date:
_____	_____	_____
Investigator name and title:	Signature:	Date:
_____	_____	_____

9.5 DREAM Study Documents

- NRES Ethical Approval Letter
- UHL R&D Approval Letter
- Participant Information Sheet
- Consent Form
- Kansas City Cardiomyopathy Questionnaire (KCCQ)
- Roles and Responsibilities of Staff Within the Trial

NRES Ethical Approval Letter for DREAM Study Page 1

NHS
Health Research Authority

NRES Committee East Midlands - Northampton
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 0115 8839435
Facsimile: 0115 8839294

14 September 2012

Professor Nilesh Samani
British Heart Foundation Professor of Cardiology, Head of Department of Cardiovascular Sciences, Glenfield General Hospital, Director Leicester NIHR Biomedical Research Unit in Cardiovascular Disease
University of Leicester and Leicester Biomedical Research Unit
Department of Cardiovascular Sciences
Clinical Science Wing
Glenfield Hospital, Groby Road, Leicester
LE3 9QP

Dear Professor Samani

Study title:	**DREAM Study (Daily Remote Ischaemic Conditioning following Acute Myocardial Infarction)**
REC reference:	**12/EM/0304**
Protocol number:	**UNOLE 0306**

Thank you for your letter of 07 September 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Covering Letter		19 July 2012
Evidence of insurance or indemnity		16 August 2011
GP/Consultant Information Sheets	1.0	16 July 2012
Investigator CV		16 January 2012
Other: CV - Dr Vanezis		05 March 2012
Other: CV - Glen Rodrigo		28 June 2012
Other: CV - Dr Edroos		05 March 2012
Other: CV - Dr Marsh		12 July 2012
Other: CV - Robyn Lotto		02 November 2010
Other: CV - Mrs Harrison		25 January 2012
Other: CV - Elaine Logtens		03 May 2011
Other: Flow Diagram	1.0	16 July 2012
Other: Letter from the funder		23 May 2012
Other: GCP Certificate - Elaine Logtens		15 February 2011
Other: GCP Certificate - Mary Harrison		16 January 2012
Other: GCP Certificate - Robyn Lotto		28 October 2010
Other: GCP Certificate - Anna Marsh		02 February 2011
Other: GCP Certificate - Dr Edroos		29 November 2011
Other: GCP Certificate - Dr Vanezis		29 November 2011
Other: GCP Certificate - Glenn Rodrigo		18 July 2012

Other: GCP Certificate - Professor Samani		16 May 2011
Other: Linked anonymous data participants log	1	24 August 2012
Participant Consent Form	2.0	24 August 2012
Participant Information Sheet	3.0	07 September 2012
Protocol	1.0	18 July 2012
Questionnaire: The Kansas City Cardiomyopathy		
REC application	101254/3450 37/1/20	19 July 2012
Referees or other scientific critique report		20 March 2012
Referees or other scientific critique report		15 March 2012
Referees or other scientific critique report		13 March 2012
Response to Request for Further Information		24 August 2012
Response to Request for Further Information	3.0	07 September 2012

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/EM/0304	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

NRES Ethical Approval Letter for DREAM Study Page 4

Yours sincerely

Mr Paul hamilton
Chair

Email: georgia.copeland@nottspct.nhs.uk

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Mrs Wendy Gamble*
Mrs Carolyn Maloney, University Hospitals of Leicester NHS Trust

UHL R&D Approval Letter for DREAM Study Page 1

University Hospitals of Leicester NHS
NHS Trust

DIRECTORATE OF RESEARCH & DEVELOPMENT

Director: **Professor D Rowbotham**
Assistant Director: **Dr David Hetmanski**
R&D Manager: **Carolyn Maloney**

Research & Development Office
Leicester General Hospital
Gwendolen Road
Leicester
LE5 4PW

Direct Dial: (0116) 258 8351
Fax No: (0116) 258 4226

25 October 2012

Professor Nilesh Samani
Professor of Cardiology
Department of Cardiovascular Sciences
Clinical Sciences Wing
Glenfield Hospital
Groby Road,
Leicester,
LE3 9QP

Dear Professor Samani

Ref: **101254**
Title: **DREAM Study (Daily Remote Ischaemic Conditioning following Acute Myocardial Infarction)**
Project Status: **Approved**
End Date: **30 November 2015**

I am pleased to confirm that with effect from the date of this letter, the above study has Trust Research & Development permission to commence at University Hospitals of Leicester NHS Trust. The research must be conducted in line with the Protocol and fulfil any contractual obligations agreed with the Sponsor. If you identify any issues during the course of your research that are likely to affect these obligations you must contact the R&D Office.

In order for the UHL Trust to comply with targets set by the Department of Health through the 'Plan for Growth', there is an expectation that the first patient will be recruited within 30 days of the date of this letter. If there is likely to be a problem achieving this target, please contact the office as soon as possible. You will be asked to provide the date of the first patient recruited in due course. In addition, the Title, REC Reference number, local target recruitment and actual recruitment for this study will be published on a quarterly basis on the UHL Trust external website.

All documents received by this office have been reviewed and form part of the approval. The documents received and approved are as follows:

Document Title	Version	Date	REC Approval
REC Favourable Opinion Letter	N/A	14/09/12	N/A
Protocol	1.0	18/07/12	14/09/12
Other: Flow Diagram	1.0	16/07/12	14/09/12
Participant Information Sheet	3.0	07/09/12	14/09/12

Participant Consent Form	2.0	24/08/12	14/09/12
GP/Consultant Information Sheet	1.0	16/07/12	14/09/12
Questionnaire: The Kansas City Cardiomyopathy	N/A	N/A	14/09/12
Other: Linked anonymous data participants log	1	24/08/12	14/09/12

Please be aware that any changes to these documents after approval may constitute an amendment. The process of approval for amendments should be followed. Failure to do so may invalidate the approval of the study at this trust.

Undertaking research in the NHS comes with a range of regulatory responsibilities. Please ensure that you and your research team are familiar with, and understand the roles and responsibilities both collectively and individually.

Documents listing the roles and responsibilities for all individuals involved in research can be found on the R&D pages of the Public Website. It is important that you familiarise yourself with the Standard Operating Procedures, Policies and all other relevant documents which can be located by visiting www.leicestershospitals.nhs.uk/aboutus/education-and-research

The R&D Office is keen to support and facilitate research where ever possible. If you have any questions regarding this or other research you wish to undertake in the Trust, please contact this office. Our contact details are provided on the attached sheet.

This study has been reviewed and processed by the Leicestershire, Northamptonshire & Rutland Comprehensive Local Research Network (LNR CLRN) using the Coordinated System for gaining Trust Permission (CSP). If you require any further information on the approval of this study please contact the LNR CLRN office on 0116 258 6185 making reference to the CSP number which is located at the top of this letter.

We wish you every success with your research.

Yours sincerely

Dr David Hetmanski
Assistant Director of R&D

Encs: .R&D Office Contact Information

Version 11, 16/10/2012

Participation Information Sheet Used in DREAM Study Page 1

University of Leicester

NHS National Institute for Health Research

PARTICIPANT INFORMATION SHEET

The DREAM Study: Daily REmote Ischaemic Conditioning Post-Acute Myocardial Infarction

We would like to invite you to take part in our research project that is being run by the NIHR Leicester Cardiovascular Biomedical Research Unit based at Glenfield Hospital. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. We'd suggest this should take about 20 minutes. Talk to others about the study if you wish. Please ask us if anything is not clear from this leaflet.

What is the purpose of the study?

In a heart attack part of the heart muscle dies. As a consequence changes can occur in the heart that makes its pumping action less efficient. In turn this may cause symptoms such as breathlessness and fatigue. Recent research has shown that if a blood pressure cuff is applied to the arm and inflated intermittently for a short period, it releases chemicals which may reduce the adverse changes in the heart after a heart attack. This is termed *remote ischemic conditioning*. In this project we want to test whether this simple technique applied on a daily basis for 28 days after a heart attack can reduce the damage it sustains. Each participant will be randomised to either receiving a high or a low level of blood pressure cuff inflation treatment. Participants will not know which treatment they are receiving. Such clinical trials are essential to prove or disprove whether treatments like these are beneficial before their wider use.

Why have I been invited?

You have been invited because you have recently had a heart attack treated by coronary artery angioplasty (balloon, wire or stent therapy).

Do I have to take part?

It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

What will happen to me if I take part?

- You will be asked to sign a consent form.
- We will collect some information about your health and your heart attack from speaking to you and from your medical records.
- We will perform an initial bedside heart scan called an echocardiogram (ECHO) to assess your suitability for the study whilst you are still in hospital. If you do not meet the criteria for the study, unfortunately you can no longer take part and your details will be removed.
- Prior to or soon after discharge we will perform a cardiac magnetic resonance (MRI) heart scan, repeated again at 4 months. The reason for doing this scan is that it provides very accurate information about the functioning of the heart and will be used in our analyses. The scan takes around 30 minutes, is painless and involves no radiation. As part of the scan, we will insert a cannula into a vein. This can cause minor discomfort and may leave a small bruise. MRI scans are not routinely performed after heart attacks as part of NHS care unless specifically indicated.
- Prior to discharge you will be taught how to implement the blood pressure cuff inflation treatment using the autoRIC™ device. At this time you will be asked to sign a release form so that the treatment equipment can be loaned to you for use in your own home for 28 days.

- After discharge you be asked to implement the treatment using the loaned equipment at roughly the same time each day for 28 days. You will be asked to keep a diary to note down the time of treatments and whether any issues that were encountered.
- After discharge you will also be visited by a member of the research team (a nurse or doctor) intermittently to monitor the use of the equipment and resolve any issues. This will be at dates and times convenient to you. After 28 days of treatment a member of the research team will visit you at home and collect the equipment from you along with the treatment diary you have been asked to keep. This technique has been used before and has been shown to be safe.
- At 4 months when you attend for your MRI scan, we will also ask you to fill in a health questionnaire to see how you are functioning in daily activities.
- We will also take two blood samples of up to 30mls (2 tablespoons) at a time. This will be done whilst you are still in hospital and at your 4 month follow-up appointment. This is to allow us to measure chemicals in the blood which may change with treatment and which may in the future be used as a guide to its effect. Participation in the project will therefore last for four months.

Will my taking part in the study be kept confidential?

Yes. Your participation in this project will be confidential. With your permission we will inform your GP. You will be assigned a unique study number, which will be used to label your data and samples. When any researcher uses your information or your samples they will only know your study number. This will protect your identity.

Your data will be stored in a purpose built secure research database in the University of Leicester. MRI and echo data is stored in addition in the University Hospital of Leicester's PACS system which is a secure imaging system available only to authorised hospital staff.

What will happen to any samples I give?

Your samples will be stored in secure facilities in the Biomedical Research Unit. We perform a number of tests on the blood samples to assess for levels of specific chemicals in the blood. Stored samples will be kept for up to 5 years. You can be reassured that no confidential health information on you will be released, published or presented in a manner that can identify you. The staff managing the project will use your NHS number to source data from your medical records (now and in the future).

The research may be inspected or audited by the Research Sponsor or the UK Authorities to ensure that it is being conducted properly. The inspectors or auditors may access your records where it is relevant to the conduct of the study.

What are the possible benefits of taking part?

The purpose of the research is to determine whether the treatment being studied is effective or not. If this is the case then it may be clinically applied in the future. We do not know this yet and so you should not anticipate any direct benefit. Also, please note that in a randomised clinical trial like this, there is a 50:50 chance that you will be allocated to either the high or low level of treatment. You will not know which group you are in.

There is no payment for participating in the study. Travel expenses and hospital parking fees for study visits will however be reimbursed, if these do not coincide for a visit for your normal care.

What will happen to the results of the research study?

As you are randomly allocated to either the low or high level of treatment, we will not provide you with results of the findings from their scans or other tests on a routine basis. However at the end of the trial when the results have been analysed we plan to send out a letter which details the study summary results as well as holding a meeting to inform all participants of the results and answer any questions.

What are the possible disadvantages and risks of taking part?

The blood pressure cuff treatment that we will use has been used before both locally and by other research groups and is safe. Inflation of the cuff may cause transient discomfort but there should be no lasting ill effects. The blood samples that will be taken and the insertion of a small venous cannula may cause transient discomfort and a small bruise. The MRI machine is noisy and you will be give ear muffs for this. The dye we give for the scan may cause transient warmth when injected. Very rarely it may cause an allergic reaction but we have procedures in place for treating this.

While we do not anticipate any adverse effects from the treatment that we plan to test and as for any clinical trial, strict reporting procedure will be in place to monitor for any side effects that you report. These will be reviewed regularly by the Principal Investigator and any significant adverse effects reported immediately to the relevant authorities in the Hospital and the University.

What will happen if I don't want to carry on with the study?

You are free to withdraw from the research project at any time without any prejudice to the care you receive or your legal rights, by informing the NIHR Leicester Cardiovascular Biomedical Research Unit administrator at the address shown on the end of this leaflet. You can ask for the samples and data you have provided to be destroyed or allow the samples and data you have already donated to continue to be used but not to have any further involvement.

What if there is a problem?

If you have a concern about any aspect of this study, you can speak to the researchers by phoning 0116 250 2429. They will do their best to answer your questions. For independent advice you can contact University Hospitals of Leicester Patient Information and Liaison Service on 08081 788337.

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against the University of Leicester but you may have to pay your legal costs. The University of Leicester hold an indemnity policy with Newline Underwriting Management Limited. This project has been reviewed by a Research Ethics Committee to ensure that your rights and wellbeing are protected.

Who is organising and funding the research?

This project is managed by the NIHR Leicester Cardiovascular Biomedical Research Unit, which is funded by the National Institute for Health Research which is part of the Department of Health. The Unit is a partnership of the University Hospitals of Leicester and the University of Leicester.

Funding for this project has been provided by the Mason's Grand Charity and Samaritans' Fund and the Leicester Cardiovascular Biomedical Research Unit.

The Northamptonshire Research Ethics Committee has reviewed this project and given a favourable opinion of it. Research Ethics Committees are independent bodies which review all NHS research projects to ensure that your rights and wellbeing are upheld and risks are managed properly.

Further information and keeping in touch

Your donation of samples, and consent to access data will allow researchers to undertake important medical research. We would like to keep you informed about the research through our regular NIHR Leicester Cardiovascular BRU newsletter. If you want to receive the newsletter, please contact the administrator on 0116 250 2429. If you would like to discuss any of the information in this leaflet or your participation, please contact:

Leicester Cardiovascular Biomedical Research Unit.
Department of Cardiovascular Sciences, Clinical Science Wing.
Glenfield Hospital, Groby Road. Leicester. LE3 9QP. UK
Telephone Number: 0116 250 2429 // email: lcbru@le.ac.uk

We thank you for considering participation in the DREAM Study.

Consent Form Used in DREAM Study

University of Leicester

NHS National Institute for Health Research

Patient name, address, Date of Birth (or ID label)

CONSENT FORM

The DREAM Study: Daily REmote Ischaemic Conditioning Post-Acute Myocardial Infarction

Please initial each box to indicate you agree with the statement:

1.	I confirm that I have read the information sheet dated 7.9.12 version 3.0 for the above study and I have understood it, including what participation will involve and any risks and benefits. I have had the opportunity to ask questions.	
2.	I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason and without my medical care or legal rights being affected.	
3.	I agree to the research team accessing my medical records to obtain information necessary for the study. This includes an initial screen of my medical notes to ensure suitability for enrolment to the study.	
4.	I agree to my general practitioner or other health care professionals treating me being notified of my participation in the study.	
5.	I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the research team, the sponsor, regulatory authorities or from UHL NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records	
6.	I agree that the samples I provide can be stored and used for future research (please initial one box only).	Yes / No
7.	I agree to take part in this study.	

Participant name: _____ Signature: _____ Date: _____

Investigator name and title: _____ Signature: _____ Date: _____

White sheet: study file; pink sheet: hospital medical notes, yellow sheet: patient

Enquiries about the project can be made to:

Leicester Cardiovascular Biomedical Research Unit.
Department of Cardiovascular Sciences, Clinical Science Wing.
Glenfield Hospital, Groby Road. Leicester. LE3 9QP. UK
Telephone Number: 0116 250 2429 // email: lcbru@le.ac.uk

KCCQ Used in DREAM Study page 1

Cardiomyopathy Questionnaire (Kansas City)

The following questions refer to your **heart failure** and how it may affect your life. Please read and complete the following questions. There are no right or wrong answers. Please mark the answer that best applies to you.

1. **Heart failure** affects different people in different ways. Some may mainly feel shortness of breath while others mainly fatigue. Please indicate how limited you have been by **heart failure** (for example, shortness of breath or fatigue) in your ability to do the following activities over the past 2 weeks.

Please put an **X** in one box on each line

Activity	**Extremely** limited	**Quite a bit** limited	**Moderately** limited	**Slightly** limited	**Not at all** limited	**Limited for other reasons or did not do the activity**
Dressing yourself	☐	☐	☐	☐	☐	☐
Showering or having a bath	☐	☐	☐	☐	☐	☐
Walking 100 yards on level ground	☐	☐	☐	☐	☐	☐
Doing gardening, housework or carrying groceries	☐	☐	☐	☐	☐	☐
Climbing a flight of stairs without stopping	☐	☐	☐	☐	☐	☐
Jogging or hurrying (as if to catch a bus)	☐	☐	☐	☐	☐	☐

2. Compared with 2 weeks ago, have your symptoms of **heart failure** (for example, shortness of breath, fatigue, or ankle swelling) changed?

My symptoms of **heart failure** are now…

Much worse	Slightly worse	Not changed	Slightly better	Much better	I've had no symptoms over the last 2 weeks
☐	☐	☐	☐	☐	☐

KCCQ - UK/English

KCCQ Used in DREAM Study page 2

3. Over the past 2 weeks, how many times have you had **swelling** in your feet, ankles or legs when you woke up in the morning?

Every morning	3 or more times a week, but not every day	1-2 times a week	Less than once a week	Never over the past 2 weeks
☐	☐	☐	☐	☐

4. Over the past 2 weeks, how much has **swelling** in your feet, ankles or legs bothered you?

Extremely bothersome	**Quite a bit** bothersome	**Moderately** bothersome	**Slightly** bothersome	**Not at all** bothersome	I've had **no swelling**
☐	☐	☐	☐	☐	☐

5. Over the past 2 weeks, on average, how many times has **fatigue** limited your ability to do what you wanted?

All of the time	Several times a day	At least once a day	3 or more times a week but not every day	1-2 times a week	Less than once a week	Never over the past 2 weeks
☐	☐	☐	☐	☐	☐	☐

6. Over the past 2 weeks, how much has your **fatigue** bothered you?

Extremely bothersome	**Quite a bit** bothersome	**Moderately** bothersome	**Slightly** bothersome	**Not at all** bothersome	I've had **no fatigue**
☐	☐	☐	☐	☐	☐

7. Over the past 2 weeks, on average, how many times has **shortness of breath** limited your ability to do what you wanted?

All of the time	Several times a day	At least once a day	3 or more times a week but not every day	1-2 times a week	Less than once a week	Never over the past 2 weeks
☐	☐	☐	☐	☐	☐	☐

KCCQ - UK/English

KCCQ Used in DREAM Study page 3

8. Over the past 2 weeks, how much has your **shortness of breath** bothered you?

Extremely bothersome	**Quite a bit** bothersome	**Moderately** bothersome	**Slightly** bothersome	**Not at all** bothersome	I've had **no shortness of breath**
☐	☐	☐	☐	☐	☐

9. Over the past 2 weeks, on average, how many times have you been forced to sleep sitting up in a chair or with at least 3 pillows to prop you up because of **shortness of breath**?

Every night	3 or more times a week, but not every night	1-2 times a week	Less than once a week	Never over the past 2 weeks
☐	☐	☐	☐	☐

10. **Heart failure** symptoms can worsen for a number of reasons. How sure are you that you know what to do, or whom to call, if your **heart failure** gets worse?

Not at all sure	**Not very** sure	**Somewhat** sure	**Mostly** sure	**Completely** sure
☐	☐	☐	☐	☐

11. How well do you understand what things you are able to do to keep your **heart failure** symptoms from getting worse (for example, regularly weighing yourself, eating a low salt diet etc.)?

Do not understand at all	Do not understand very well	Somewhat understand	Mostly understand	Completely understand
☐	☐	☐	☐	☐

12. Over the past 2 weeks, how much has your **heart failure** limited your enjoyment of life?

It has **extremely** limited my enjoyment of life	It has limited my enjoyment of life **quite a bit**	It has **moderately** limited my enjoyment of life	It has **slightly** limited my enjoyment of life	It has **not limited** my enjoyment of life at all
☐	☐	☐	☐	☐

13. If you had to spend the rest of your life with your **heart failure** the way it is right now, how would you feel about this?

Completely dissatisfied	Mostly dissatisfied	Somewhat satisfied	Mostly satisfied	Completely satisfied
☐	☐	☐	☐	☐

14. Over the past 2 weeks, how often have you felt discouraged or down in the dumps because of your **heart failure**?

I have felt that way **all of the time**	I have felt that way **most of the time**	I have **occasionally** felt that way	I have **rarely** felt that way	I have **never** felt that way
☐	☐	☐	☐	☐

15. How much does your **heart failure** affect your lifestyle? Please indicate how your **heart failure** may have limited your participation in the following activities over the past 2 weeks.

Please put an **X** in one box on each line

Activity	**Extremely** limited	**Quite a bit** limited	**Moderately** limited	**Slightly** limited	**Not at all** limited	**Limited for other reasons or did not do the activity**
Hobbies, recreational activities	☐	☐	☐	☐	☐	☐
Working or doing household chores	☐	☐	☐	☐	☐	☐
Visiting family or friends	☐	☐	☐	☐	☐	☐
Intimate or sexual relationships	☐	☐	☐	☐	☐	☐

Roles and Responsibilities of Staff within the DREAM study

- *Professor Sir Nilesh Samani:* Principle Investigator, head of Leicester Cardiovascular BRU. PhD supervisor to AV. PhD supervisor to Dr Andrew Vanezis.
- *Dr Andrew Vanezis:* Research team coordinator and investigator involved in patient recruitment and enrolment, monitoring and post recruitment analysis and dissemination of data. Also assists research nurse in follow-up visits. Coordinates trial activity at other trial sites.
- *Dr Anna-Marie Marsh*: Research team investigator and Senior Cardiac Physiologist performing patient echocardiograms.
- *John McAdam*: Research team investigator and Cardiac Physiologist performing patient echocardiograms.
- *Dr Glenn Rodrigo*: Research team investigator involved in monitoring and post recruitment analysis and dissemination of data. PhD supervisor to Dr Andrew Vanezis.
- *Professor Iain Squire*: Research team investigator involved in monitoring and post recruitment analysis and dissemination of data. PhD supervisor to AV.
- *Dr Gerry McCann*: Responsible for designing cMRI protocol, involved in MRI scanning and reporting.
- *Dr Jamal Khan*: Responsible for participant cMRIs scanning and reporting.
- *Dr Sheraz Nazir*: Responsible for participant cMRIs scanning and reporting.
- *Dr Chris Nelson*: Statistician and analysis of data.
- *Jay Gracey:* Senior Research Nurse involved in all aspects of study running.

- *Sue Parish*: Research Nurse involved in all aspects of study running.
- *Ellie Clarke*: Research Nurse involved in all aspects of study running.
- *Leicester Cardiovascular BRU nursing staff*: BRU nursing staff assist in all aspects of the study based onsite i.e. within BRU premises.
- *Dr Simon Hetherington:* Chief Investigator at Kettering General Hospital.
- *Professor Gerry Coghlan:* Chief Investigator at Royal Free Hospital, London.
- *Dr Kamal Chitkara:* Chief Investigator at Royal Derby Hospital.

10. REFERENCES

1. Keeley EC. Primary angioplasty versus intravenous thrombolytic therapy for acute myocardial infarction: A quantitative review of 23 randomised trials. *Lancet*. 2003;361:13-20

2. Haw SJ, Gruer L. Changes in exposure of adult non-smokers to secondhand smoke after implementation of smoke-free legislation in scotland: National cross sectional survey. *BMJ*. 2007;335:549

3. Moran AE, Forouzanfar MH, Roth GA, Mensah GA, Ezzati M, Murray CJL, Naghavi M. Temporal trends in ischemic heart disease mortality in 21 world regions, 1980 to 2010: The global burden of disease 2010 study. *Circulation*. 2014;129:1483-1492

4. Setoguchi S, Glynn RJ, Avorn J, Mittleman MA, Levin R, Winkelmayer WC. Improvements in long-term mortality after myocardial infarction and increased use of cardiovascular drugs after discharge: A 10-year trend analysis. *J Am Coll Cardiol*. 2008;51:1247-1254

5. Fox KF CM, Wood DA et al. Coronary artery disease as the cause of incident heart failure in the population. *European Heart Journal*. 2001;22:228-236

6. Sanderson JE. Hfnef, hfpef, hf-pef, or dhf. What is in an acronym? *JACC: Heart Failure*. 2014;2:93-94

7. Paulus WJ, Tschöpe C, Sanderson JE, Rusconi C, Flachskampf FA, Rademakers FE, Marino P, Smiseth OA, De Keulenaer G, Leite-Moreira AF, Borbély A, Édes I, Handoko ML, Heymans S, Pezzali N, Pieske B, Dickstein K, Fraser AG, Brutsaert DL. How to diagnose diastolic heart failure: A consensus statement on the diagnosis of heart failure with normal left ventricular ejection fraction by the heart failure and echocardiography associations of the european society of cardiology. *European Heart Journal*. 2007;28:2539-2550

8. Weir RAP, McMurray JJV, Velazquez EJ. Epidemiology of heart failure and left ventricular systolic dysfunction after acute myocardial infarction: Prevalence, clinical characteristics, and prognostic importance. *Am J Cardiol* 2006;97:S13-25

9. Jhund PS, McMurray JJV. Heart failure after acute myocardial infarction: A lost battle in the war on heart failure? *Circulation*. 2008;118:2019-2021

10. Velagaleti RS, Pencina MJ, Murabito JM, Wang TJ, Parikh NI, D'Agostino RB, Levy D, Kannel WB, Vasan RS. Long-term trends in the incidence of heart failure after myocardial infarction. *Circulation*. 2008;118:2057-2062

11. Fang J, Mensah GA, Croft JB, Keenan NL. Heart failure-related hospitalization in the u.S., 1979 to 2004. *Journal of the American College of Cardiology*. 2008;52:428-434

12. Hellermann JP, Goraya TY, Jacobsen SJ, Weston SA, Reeder GS, Gersh BJ, Redfield MM, Rodeheffer RJ, Yawn BP, Roger VL. Incidence of heart failure after myocardial infarction: Is it changing over time? *American Journal of Epidemiology*. 2003;157:1101-1107

13. Chen J, Hsieh AF-C, Dharmarajan K, Masoudi FA, Krumholz HM. National trends in heart failure hospitalization after acute myocardial infarction for medicare beneficiaries: 1998–2010. *Circulation*. 2013;128:2577-2584

14. Sulo G, Igland J, Vollset SE, Nygård O, Ebbing M, Sulo E, Egeland GM, Tell GS. Heart failure complicating acute myocardial infarction; burden and timing of occurrence: A nation-wide analysis including 86 771 patients from the cardiovascular disease in norway (cvdnor) project. *Journal of the American Heart Association*. 2016;5

15. Stewart S, MacIntyre K, MacLeod MMC, Bailey AEM, Capewell S, McMurray JJV. Trends in hospitalization for heart failure in scotland, 1990–1996. An epidemic that has reached its peak? *European Heart Journal*. 2001;22:209-217

16. Schaufelberger M, Swedberg K, Köster M, Rosén M, Rosengren A. Decreasing one-year mortality and hospitalization rates for heart failure in sweden. *Data from the Swedish Hospital Discharge Registry 1988 to 2000*. 2004;25:300-307

17. Garcia-Dorado D, Ruiz-Meana M, Piper HM. Lethal reperfusion injury in acute myocardial infarction: Facts and unresolved issues. *Cardiovascular research*. 2009;83:165-168

18. Jones H, Hopkins N, Bailey TG, Green DJ, Cable NT, Thijssen DH. Seven-day remote ischemic preconditioning improves local and systemic endothelial function and microcirculation in healthy humans. *American journal of hypertension*. 2014

19. Reimer KA, Lowe JE, Rasmussen MM, Jennings RB. The wavefront phenomenon of ischemic cell death. 1. Myocardial infarct size vs duration of coronary occlusion in dogs. *Circulation*. 1977;56:786-794

20. Reimer KA, Jennings RB. The "wavefront phenomenon" of myocardial ischemic cell death. Ii. Transmural progression of necrosis within the framework of ischemic bed size (myocardium at risk) and collateral flow. *Lab Invest*. 1979;40:633-644

21. Menees DS, Peterson ED, Wang Y, Curtis JP, Messenger JC, Rumsfeld JS, Gurm HS. Door-to-balloon time and mortality among patients undergoing primary pci. *New England Journal of Medicine*. 2013;369:901-909

22. Cannon C, Gibson C, Lambrew C, Shoultz D, Levy D, French W, Gore J, Weaver W, Rogers W, Tiefenbrunn A. Relationship of symptom-onset-to-balloon time and door-to-balloon time with mortality in patients undergoing angioplasty for acute myocardial infarction. *JAMA*. 2000;283:2941-2947

23. Miura T, Miki T. Limitation of myocardial infarct size in the clinical setting: Current status and challenges in translating animal experiments into clinical therapy. *Basic Research in Cardiology*. 2008;103:501-513

24. Rathore SS, Curtis JP, Chen J, Wang Y, Nallamothu BK, Epstein AJ, Krumholz HM. Association of door-to-balloon time and mortality in patients admitted to hospital with st elevation myocardial infarction: National cohort study. *BMJ*. 2009;338

25. De Luca G, Suryapranata H, Ottervanger JP, Antman EM. Time delay to treatment and mortality in primary angioplasty for acute myocardial infarction: Every minute of delay counts. *Circulation*. 2004;109:1223-1225

26. Pasotti M, Prati F, Arbustini E. The pathology of myocardial infarction in the pre- and post-interventional era. *Heart*. 2006;92:1552-1556

27. Carden DL, Granger DN. Pathophysiology of ischaemia–reperfusion injury. *The Journal of Pathology*. 2000;190:255-266

28. Dirksen MT, Laarman GJ, Simoons ML, Duncker DJGM. Reperfusion injury in humans: A review of clinical trials on reperfusion injury inhibitory strategies. *Cardiovascular research*. 2007;74:343-355

29. Hausenloy DJ, Yellon DM. Myocardial ischemia-reperfusion injury: A neglected therapeutic target. *The Journal of clinical investigation*. 2013;123:92-100

30. Phillips L, Toledo A, Lopez-Neblina F, Anaya-Prado R, Toledo-Pereyra L. Nitric oxide mechanism of protection in ischemia and reperfusion injury. *J Invest Surg*. 2009;22:46-55

31. Braunersreuther V, Jaquet V. Reactive oxygen species in myocardial reperfusion injury: From physiopathology to therapeutic approaches. *Curr Pharm Biotechnol*. 2012;13:97-114

32. Kalogeris T, Bao Y, Korthuis RJ. Mitochondrial reactive oxygen species: A double edged sword in ischemia/reperfusion vs preconditioning. *Redox Biology*. 2014;2:702-714

33. Wright HL, Moots RJ, Bucknall RC, Edwards SW. Neutrophil function in inflammation and inflammatory diseases. *Rheumatology*. 2010;49:1618-1631

34. Zhang Z, Blake DR, Stevens CR, Kanczler JM, Winyard PG, Symons MC, Benboubetra M, Harrison R. A reappraisal of xanthine dehydrogenase and oxidase in hypoxic reperfusion injury: The role of nadh as an electron donor. *Free Radic Res*. 1998;28:151-164

35. Tani M, Neely JR. Role of intracellular na+ in ca2+ overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of h+-na+ and na+-ca2+ exchange. *Circ Res*. 1989;65:1045-1056

36. Allen DG, Xiao XH. Role of the cardiac na+/h+ exchanger during ischemia and reperfusion. *Cardiovascular research*. 2003;57:934-941

37. Hearse D. Reperfusion of the ischemic myocardium. *J Mol Cell Cardiol*. 1977;9:605-616

38. Rodrigo GC, Standen NB. Role of mitochondrial re-energization and ca2+ influx in reperfusion injury of metabolically inhibited cardiac myocytes. *Cardiovascular research*. 2005;67:291-300

39. Turrell HE, Thaitirarot C, Crumbie H, Rodrigo G. Remote ischemic preconditioning of cardiomyocytes inhibits the mitochondrial permeability transition pore independently of reduced calcium-loading or sarckatp channel activation. *Physiol Rep*. 2014;2

40. Halestrap AP. A pore way to die: The role of mitochondria in reperfusion injury and cardioprotection. *Biochem Soc Trans*. 2010;38:841-860

41. Basso C, Thiene G. The pathophysiology of myocardial reperfusion: A pathologist's perspective. *Heart*. 2006;92:1559-1562

42. Steffens S, Montecucco F, Mach F. The inflammatory response as a target to reduce myocardial ischaemia and reperfusion injury. *Thrombosis and Haemostasis*. 2009;102:240-247

43. Théroux P, Chaitman B, Danchin N, al. e. Inhibition of the sodium-hydrogen exchanger with cariporide to prevent myocardial infarction in high-risk ischemic situations: Main results of the guardian trial. *Circulation*. 2000;102:3032-3038

44. Downey JM. Free radicals and their involvement during long-term myocardial ischemia and reperfusion. *Annu Rev Physiol*. 1990;52:487-504

45. Yao L, Lv X, Wang X. Microrna 26a inhibits hmgb1 expression and attenuates cardiac ischemia-reperfusion injury. *Journal of Pharmacological Sciences*. 2015;1:In press

46. Wang JX, Zhang XJ, Li Q, Wang K, Wang Y, Jiao JQ, Feng C, Teng S, Zhou LY, Gong Y, Zhou ZX, Liu J, Wang JL, Li PF. Microrna-103/107 regulate programmed necrosis and myocardial ischemia/reperfusion injury through targeting fadd. *Circ Res*. 2015;117:352-363

47. Weiss J, Eisenhardt S, Stark G, Bode C, Moser M, Grundmann S. Microrna s in ischemia-reperfusion injury. *Am J Cardiovasc Dis*. 2012;2:237-247

48. Zeymer U, Suryapranata H, Monassier JP, Opolski G, Davies J, Rasmanis G, Linssen G, Tebbe U, Schröder R, Tiemann R, Machnig T, Neuhaus K-L. The na+/h+ exchange inhibitor eniporide as an adjunct to early reperfusion therapy for acute myocardial infarction1results of the evaluation of the safety and cardioprotective effects of eniporide in acute myocardial infarction (escami) trial. *Journal of the American College of Cardiology*. 2001;38:E1644-E1650

49. Armstrong P, Granger C, Adams P, Hamm C, Holmes D, O'Neill W, Todaro T, Vahanian A, Van de Werf F. Pexelizumab for acute st-elevation myocardial infarction in patients undergoing primary percutaneous coronary intervention: A randomized controlled trial. *JAMA*. 2007;297:43-51

50. Mehta SR, Yusuf S, Diaz R, Zhu J, Pais P, Xavier D, Paolasso E, Ahmed R, Xie C, Kazmi K, Tai J, Orlandini A, Pogue J, Liu L, Investigators C-ETG. Effect of glucose-insulin-

potassium infusion on mortality in patients with acute st-segment elevation myocardial infarction: The create-ecla randomized controlled trial. *JAMA*. 2005;293:437-446

51. Kitakaze M, Asakura M, Kim J, Shintani Y, Asanuma H, Hamasaki T, Seguchi O, Myoishi M, Minamino T, Ohara T, Nagai Y, Nanto S, Watanabe K, Fukuzawa S, Hirayama A, Nakamura N, Kimura K, Fujii K, Ishihara M, Saito Y, Tomoike H, Kitamura S, investigators J-W. Japan working groups of acute myocardial infarction for the reduction of necrotic damage (jwind) by anp or nicorandil), a large-scale trial using atrial natriuretic peptide or nicorandil as an adjunct to percutaneous coronary intervention for st-segment elevation acute myocardial infarction. *Clinical Cardiology*. 2007;30:99-99

52. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *The New England journal of medicine*. 2007;357:1121-1135

53. Gaudron P, Eilles C, Kugler I, Ertl G. Progressive left ventricular dysfunction and remodeling after myocardial infarction. Potential mechanisms and early predictors. *Circulation*. 1993;87:755-763

54. Cohn JN, Sharpe N, Ferrari R. Cardiac remodeling—concepts and clinical implications: A consensus paper from an international forum on cardiac remodeling. *Journal of the American College of Cardiology*. 2000;35:569-582

55. McKay R, Pfeffer M, Pasternak R, Markis J, Come P, Nakao S, Alderman J, Ferguson J, Safian R, Grossman W. Left ventricular remodeling after myocardial infarction: A corollary to infarct expansion. *Circulation*. 1986;74:693-702

56. Kloner RA, Ganote CE, Jennings RB. The "no-reflow" phenomenon after temporary coronary occlusion in the dog. *J Clin Invest*. 1974;54:1496-1508

57. Wu KC, Zerhouni EA, Judd RM, Lugo-Olivieri CH, Barouch LA, Schulman SP, Blumenthal RS, Lima JA. Prognostic significance of microvascular obstruction by magnetic resonance imaging in patients with acute myocardial infarction. *Circulation*. 1998;97:765-772

58. Kidambi A, Mather AN, Motwani M, Swoboda P, Uddin A, Greenwood JP, Plein S. The effect of microvascular obstruction and intramyocardial hemorrhage on contractile recovery in reperfused myocardial infarction: Insights from cardiovascular magnetic resonance. *J Cardiovasc Magn Reson*. 2013;15:58

59. Takemura G, Nakagawa M, Kanamori H, Minatoguchi S, Fujiwara H. Benefits of reperfusion beyond infarct size limitation. *Cardiovascular research*. 2009;83:269-276

60. Kanamori H, Takemura G, Goto K, Maruyama R, Tsujimoto A, Ogino A, Takeyama T, Kawaguchi T, Watanabe T, Fujiwara T, Fujiwara H, Seishima M, Minatoguchi S. The role of autophagy emerging in postinfarction cardiac remodelling. *Cardiovascular research*. 2011;91:330-339

61. Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, Wang M, Wu TD, Guerquin-Kern JL, Lechene CP, Lee RT. Mammalian heart renewal by pre-existing cardiomyocytes. *Nature*. 2013;493:433-436

62. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabé-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisén J. Evidence for cardiomyocyte renewal in humans. *Science*. 2009;324:98-102

63. Sabbah HN, Goldstein S. Ventricular remodelling: Consequences and therapy. *Eur Heart J*. 1993;14 Suppl C:24-29

64. Verma A, Meris A, Skali H, Ghali JK, Arnold JMO, Bourgoun M, Velazquez EJ, McMurray JJV, Kober L, Pfeffer MA, Califf RM, Solomon SD. Prognostic implications of left ventricular mass and geometry following myocardial infarction: The valiant (valsartan in acute myocardial infarction) echocardiographic study. *J Am Coll Cardiol Img*. 2008;1:582-591

65. White H, Norris R, Brown M, Brandt P, Whitlock R, Wild C. Left ventricular end-systolic volume as the major determinant of survival after recovery from myocardial infarction. *Circulation*. 1987;76:44-51

66. Brenner S, Ertl G. Remodelling and adverse remodelling in cad. *Herz*. 2012;37:590-597

67. Fink SL, Cookson BT. Apoptosis, pyroptosis, and necrosis: Mechanistic description of dead and dying eukaryotic cells. *Infection and Immunity*. 2005;73:1907-1916

68. Dorn GW. Molecular mechanisms that differentiate apoptosis from programmed necrosis. *Toxicologic Pathology*. 2013;41:227-234

69. Kajstura J, Cheng W, Reiss K, Clark W, Sonnenblick E, Krajewski S, Reed J, Olivetti G, Anversa P. Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. *Lab Invest*. 1996;74:86-107

70. Soonpaa MH, Field LJ. Survey of studies examining mammalian cardiomyocyte DNA synthesis. *Circulation research*. 1998;83:15-26

71. Sharov VG, Sabbah HN, Shimoyama H, Goussev AV, Lesch M, Goldstein S. Evidence of cardiocyte apoptosis in myocardium of dogs with chronic heart failure. *Am J Pathol*. 1996;148:141-149

72. MacLellan WR, Schneider MD. Death by design: Programmed cell death in cardiovascular biology and disease. *Circulation research*. 1997;81:137-144

73. Diwan A, Dorn GW, 2nd. Decompensation of cardiac hypertrophy: Cellular mechanisms and novel therapeutic targets. *Physiology (Bethesda)*. 2007;22:56-64

74. Latif N, Khan MA, Birks E, O'Farrell A, Westbrook J, Dunn MJ, Yacoub MH. Upregulation of the bcl-2 family of proteins in end stage heart failure. *Journal of the American College of Cardiology*. 2000;35:1769-1777

75. Abbate A, Narula J. Role of apoptosis in adverse ventricular remodeling. *Heart Fail Clin*. 2012;8:79-86

76. Kinnally KW, Peixoto PM, Ryu SY, Dejean LM. Is mptp the gatekeeper for necrosis, apoptosis, or both? *Biochim Biophys Acta*. 2011;1813:616-622

77. Dorn GW. Apoptotic and non-apoptotic programmed cardiomyocyte death in ventricular remodelling. *Cardiovascular research*. 2009;81:465-473

78. Zong WX, Thompson CB. Necrotic death as a cell fate. *Genes Dev*. 2006;20:1-15

79. Vatner SF. Reduced subendocardial myocardial perfusion as one mechanism for congestive heart failure. *Am J Cardiol*. 1988;62:94E-98E

80. Hudlicka O, Brown M, Egginton S. Angiogenesis in skeletal and cardiac muscle. *Physiol Rev*. 1992;72:369-417

81. Shiojima I, Sato K, Izumiya Y, Schiekofer S, Ito M, Liao R, Colucci WS, Walsh K. Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. *J Clin Invest*. 2005;115:2108-2118

82. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell*. 2008;132:27-42

83. Levine B, Yuan J. Autophagy in cell death: An innocent convict? *J Clin Invest*. 2005;115:2679-2688

84. Riad A, Jager S, Sobirey M, Escher F, Yaulema-Riss A, Westermann D, Karatas A, Heimesaat MM, Bereswill S, Dragun D, Pauschinger M, Schultheiss HP, Tschope C. Toll-like receptor-4 modulates survival by induction of left ventricular remodeling after myocardial infarction in mice. *J Immunol*. 2008;180:6954-6961

85. Arslan F, Smeets MB, O'Neill LA, Keogh B, McGuirk P, Timmers L, Tersteeg C, Hoefer IE, Doevendans PA, Pasterkamp G, de Kleijn DP. Myocardial ischemia/reperfusion injury is mediated by leukocytic toll-like receptor-2 and reduced by systemic administration of a novel anti-toll-like receptor-2 antibody. *Circulation*. 2010;121:80-90

86. Van Tassell BW, Toldo S, Mezzaroma E, Abbate A. Targeting interleukin-1 in heart disease. *Circulation*. 2013;128:1910-1923

87. Littman DR, Rudensky AY. Th17 and regulatory t cells in mediating and restraining inflammation. *Cell*. 2010;140:845-858

88. Nahrendorf M, Swirski FK. Monocyte and macrophage heterogeneity in the heart. *Circ Res*. 2013;112:1624-1633

89. Jugdutt B, Dhalla N. *Cardiac remodeling: Molecular mechanisms*. New York: Springer-Verlag 2013.

90. Lukashev M. Ecm signalling: Orchestrating cell behaviour and misbehaviour. *Trends in cell biology*. 1998;8:437-441

91. Weber KT, Pick R, Jalil JE, Janicki JS, Carroll EP. Patterns of myocardial fibrosis. *J Mol Cell Cardiol*. 1989;21 Suppl 5:121-131

92. Nag AC. Study of non-muscle cells of the adult mammalian heart - a fine-structural analysis and distribution. *Cytobios*. 1980;28:41-61

93. Zehr JE, Feigl EO. Suppression of renin-activity by hypothalamic stimulation. *Circulation research*. 1973;32:17-27

94. Baudino TA, Carver W, Giles W, Borg TK. Cardiac fibroblasts: Friend or foe? *American Journal of Physiology - Heart and Circulatory Physiology*. 2006;291:H1015-H1026

95. Banerjee I, Fuseler JW, Price RL, Borg TK, Baudino TA. Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse. *American Journal of Physiology - Heart and Circulatory Physiology*. 2007;293:H1883-H1891

96. Driesen RB, Nagaraju CK, Abi-Char J, Coenen T, Lijnen PJ, Fagard RH, Sipido KR, Petrov VV. Reversible and irreversible differentiation of cardiac fibroblasts. *Cardiovascular research*. 2014;101:411-422

97. Endo J, Sano M, Fujita J, Hayashida K, Yuasa S, Aoyama N, Takehara Y, Kato O, Makino S, Ogawa S, Fukuda K. Bone marrow–derived cells are involved in the pathogenesis of cardiac hypertrophy in response to pressure overload. *Circulation*. 2007;116:1176-1184

98. Judd J, Wexler B. Prolyl hydroxylase and collagen metabolism after experimental mycardial infarction. *American Journal of Physiology -- Legacy Content*. 1975;228:212-216

99. Spinale FG, Coker ML, Thomas CV, Walker JD, Mukherjee R, Hebbar L. Time-dependent changes in matrix metalloproteinase activity and expression during the progression of congestive heart failure: Relation to ventricular and myocyte function. *Circulation research*. 1998;82:482

100. Squire IB, Evans J, Ng LL, Loftus IM, Thompson MM. Plasma mmp-9 and mmp-2 following acute myocardial infarction in man: Correlation with echocardiographic and neurohumoral parameters of left ventricular dysfunction. *J Card Fail*. 2004;10:328-333

101. Orn S, Manhenke C, Squire IB, Ng L, Anand I, Dickstein K. Plasma mmp-2, mmp-9 and n-bnp in long-term survivors following complicated myocardial infarction: Relation to cardiac magnetic resonance imaging measures of left ventricular structure and function. *J Card Fail*. 2007;13:843-849

102. Reinhardt D, Sigusch HH, Hensse J, Tyagi SC, Korfer R, Figulla HR. Cardiac remodelling in end stage heart failure: Upregulation of matrix metalloproteinase (mmp) irrespective of the underlying disease, and evidence for a direct inhibitory effect of ace inhibitors on mmp. *Heart*. 2002;88:525-530

103. Matsunaga T, Abe N, Kameda K, Hagii J, Fujita N, Onodera H, Kamata T, Ishizaka H, Hanada H, Osanai T, Okumura K. Circulating level of gelatinase activity predicts

ventricular remodeling in patients with acute myocardial infarction. *Int J Cardiol*. 2005;105:203-208

104. Hirohata S, Kusachi S, Murakami M, Murakami T, Sano I, Watanabe T, Komatsubara I, Kondo J, Tsuji T. Time dependent alterations of serum matrix metalloproteinase-1 and metalloproteinase-1 tissue inhibitor after successful reperfusion of acute myocardial infarction. *Heart*. 1997;78:278-284

105. Ashizawa N, Graf K, Do YS, Nunohiro T, Giachelli CM, Meehan WP, Tuan TL, Hsueh WA. Osteopontin is produced by rat cardiac fibroblasts and mediates a(ii)-induced DNA synthesis and collagen gel contraction. *Journal of Clinical Investigation*. 1996;98:2218-2227

106. Trueblood NA, Xie Z, Communal C, Sam F, Ngoy S, Liaw L, Jenkins AW, Wang J, Sawyer DB, Bing OHL, Apstein CS, Colucci WS, Singh K. Exaggerated left ventricular dilation and reduced collagen deposition after myocardial infarction in mice lacking osteopontin. *Circulation research*. 2001;88:1080-1087

107. Singh M, Foster CR, Dalal S, Singh K. Osteopontin: Role in extracellular matrix deposition and myocardial remodeling post-mi. *J Mol Cell Cardiol*. 2010;48:538-543

108. McMullen JR, Jennings GL. Differences between pathological and physiological cardiac hypertrophy: Novel therapeutic strategies to treat heart failure. *Clinical and Experimental Pharmacology and Physiology*. 2007;34:255-262

109. Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, Henckaerts E, Bonham K, Abbott GW, Linden RM, Field LJ, Keller GM. Human cardiovascular progenitor cells develop from a kdr+ embryonic-stem-cell-derived population. *Nature*. 2008;453:524-528

110. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell*. 2003;114:763-776

111. van Vliet P, Roccio M, Smits AM, van Oorschot AAM, Metz CHG, van Veen TAB, Sluijter JPG, Doevendans PA, Goumans MJ. Progenitor cells isolated from the human heart: A potential cell source for regenerative therapy. *Netherlands Heart Journal*. 2008;16:163-169

112. Bollini S, Smart N, Riley P. Resident cardiac progenitor cells: At the heart of regeneration. *J Mol Cell Cardiol*. 2011;50:296-303

113. Zamilpa R, Navarro MM, Flores I, Griffey S. Stem cell mechanisms during left ventricular remodeling post-myocardial infarction: Repair and regeneration. *World Journal of Cardiology*. 2014;6:610-620

114. Brancaccio M, Fratta L, Notte A, Hirsch E, Poulet R, Guazzone S, Acetis MD, Vecchione C, Marino G, Altruda F, Silengo L, Tarone G, Lembo G. Melusin, a muscle-specific integrin [beta]1-interacting protein, is required to prevent cardiac failure in response to chronic pressure overload. *Nature medicine*. 2003;9:68-75

115. Knöll R, Hoshijima M, Hoffman HM, Person V, Lorenzen-Schmidt I, Bang M-L, Hayashi T, Shiga N, Yasukawa H, Schaper W, McKenna W, Yokoyama M, Schork NJ, Omens JH, McCulloch AD, Kimura A, Gregorio CC, Poller W, Schaper J, Schultheiss HP, Chien KR. The cardiac mechanical stretch sensor machinery involves a z disc complex that is defective in a subset of human dilated cardiomyopathy. *Cell (Cambridge)*. 2002;111:943-955

116. Zou Y, Akazawa H, Qin Y, Sano M, Takano H, Minamino T, Makita N, Iwanaga K, Zhu W, Kudoh S, Toko H, Tamura K, Kihara M, Nagai T, Fukamizu A, Umemura S, Iiri T, Fujita T, Komuro I. Mechanical stress activates angiotensin ii type 1 receptor without the involvement of angiotensin ii. *Nat Cell Biol*. 2004;6:499-506

117. Koichiro K, Yanggan W, John M, James AR, et al. Trpc6 fulfills a calcineurin signaling circuit during pathologic cardiac remodeling. *The Journal of clinical investigation*. 2006;116:3114

118. Rockman HA, Koch WJ, Lefkowitz RJ. Seven-transmembrane-spanning receptors and heart function. *Nature*. 2002;415:206-212

119. Cooling M, Hunter P, Crampin EJ. Modeling hypertrophic ip3 transients in the cardiac myocyte. *Biophysical Journal*. 2007;93:3421-3433

120. Harootunian AT, Kao JP, Paranjape S, Adams SR, Potter BV, Tsien RY. Cytosolic ca2+ oscillations in ref52 fibroblasts: Ca(2+)-stimulated ip3 production or voltage-dependent ca2+ channels as key positive feedback elements. *Cell Calcium*. 1991;12:153-164

121. Vega RB, Matsuda K, Oh J, Barbosa AC, Yang X, Meadows E, McAnally J, Pomajzl C, Shelton JM, Richardson JA, Karsenty G, Olson EN. Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. *Cell*. 2004;119:555-566

122. Chang S, McKinsey TA, Zhang CL, Richardson JA, Hill JA, Olson EN. Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. *Mol Cell Biol*. 2004;24:8467-8476

123. Zhang CL, McKinsey TA, Chang S, Antos CL, Hill JA, Olson EN. Class ii histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. *Cell*. 2002;110:479-488

124. Trivedi CM, Luo Y, Yin Z, Zhang M, Zhu W, Wang T, Floss T, Goettlicher M, Noppinger PR, Wurst W, Ferrari VA, Abrams CS, Gruber PJ, Epstein JA. Hdac2 regulates the cardiac hypertrophic response by modulating gsk3 beta activity. *Nat Med*. 2007;13:324-331

125. Kee HJ, Eom GH, Joung H, Shin S, Kim JR, Cho YK, Choe N, Sim BW, Jo D, Jeong MH, Kim KK, Seo JS, Kook H. Activation of histone deacetylase 2 by inducible heat shock protein 70 in cardiac hypertrophy. *Circ Res*. 2008;103:1259-1269

126. Matsui T, Li L, Wu JC, Cook SA, Nagoshi T, Picard MH, Liao R, Rosenzweig A. Phenotypic spectrum caused by transgenic overexpression of activated akt in the heart. *Journal of Biological Chemistry*. 2002;277:22896-22901

127. Condorelli G, Drusco A, Stassi G, Bellacosa A, Roncarati R, Iaccarino G, Russo MA, Gu Y, Dalton N, Chung C, Latronico MVG, Napoli C, Sadoshima J, Croce CM, Ross J. Akt induces enhanced myocardial contractility and cell size in vivo in transgenic mice. *Proceedings of the National Academy of Sciences*. 2002;99:12333-12338

128. Brasier A. The nf-κb regulatory network. *Cardiovascular Toxicology*. 2006;6:111-130

129. Liang Q, Molkentin JD. Divergent signaling pathways converge on gata4 to regulate cardiac hypertrophic gene expression. *J Mol Cell Cardiol*. 2002;34:611-611

130. Zhang X, Azhar G, Chai J, Sheridan P, et al. Cardiomyopathy in transgenic mice with cardiac-specific overexpression of serum response factor. *American journal of physiology. Heart and circulatory physiology*. 2001;49:H1782

131. Pagel-Langenickel I, Buttgereit J, Bader M, Langenickel T. Natriuretic peptide receptor b signaling in the cardiovascular system: Protection from cardiac hypertrophy. *Journal of Molecular Medicine*. 2007;85:797-810

132. Boengler K, Drexler H, Hilfiker-Kleiner D, Schulz R, Heusch G. The myocardial jak/stat pathway: From protection to failure. *Pharmacology & therapeutics (Oxford)*. 2008;120:172-185

133. Rajabi M, Kassiotis C, Razeghi P, Taegtmeyer H. Return to the fetal gene program protects the stressed heart: A strong hypothesis. *Heart failure reviews*. 2007;12:331-343

134. Dirkx E, da Costa Martins PA, De Windt LJ. Regulation of fetal gene expression in heart failure. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*. 2013;1832:2414-2424

135. Shah AM, Mann DL. In search of new therapeutic targets and strategies for heart failure: Recent advances in basic science. *Lancet*.378:704-712

136. Da Costa Martins PA, De Windt LJ. Micrornas in control of cardiac hypertrophy. *Cardiovascular research*. 2012;93:563-572

137. Kee HJ, Kook H. Roles and targets of class i and iia histone deacetylases in cardiac hypertrophy. *Journal of Biomedicine and Biotechnology*. 2011;2011:928326

138. Bers DM. Cardiac excitation-contraction coupling. *Nature*. 2002;415:198-205

139. Yamamoto T, Yano M, Kohno M, Hisaoka T, Ono K, Tanigawa T, Saiki Y, Hisamatsu Y, Ohkusa T, Matsuzaki M. Abnormal ca2+ release from cardiac sarcoplasmic reticulum in tachycardia-induced heart failure. *Cardiovascular research*. 1999;44:146-155

140. Phrommintikul A, Chattipakorn N. Roles of cardiac ryanodine receptor in heart failure and sudden cardiac death. *International Journal of Cardiology*. 2006;112:142-152

141. He J-Q, Conklin MW, Foell JD, Wolff MR, Haworth RA, Coronado R, Kamp TJ. Reduction in density of transverse tubules and l-type ca2+ channels in canine tachycardia-induced heart failure. *Cardiovascular research*. 2001;49:298-307

142. Bristow MR. ß-adrenergic receptor blockade in chronic heart failure. *Circulation*. 2000;101:558-569

143. Gauthier C, Rozec B, Manoury B, Balligand J-L. Beta-3 adrenoceptors as new therapeutic targets for cardiovascular pathologies. *Current Heart Failure Reports*. 2011;8:184-192

144. Triposkiadis F, Karayannis G, Giamouzis G, Skoularigis J, Louridas G, Butler J. The sympathetic nervous system in heart failure: Physiology, pathophysiology, and clinical implications. *Journal of the American College of Cardiology*. 2009;54:1747-1762

145. Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW, Doevendans PA, Mummery CL, Borlak J, Haverich A, Gross C, Engelhardt S, Ertl G, Bauersachs J. Micrornas

in the human heart: A clue to fetal gene reprogramming in heart failure. *Circulation*. 2007;116:258-267

146. Callis TE, Wang DZ. Taking micrornas to heart. *Trends Mol Med*. 2008;14:254-260

147. Gladka MM, da Costa Martins PA, De Windt LJ. Small changes can make a big difference — microrna regulation of cardiac hypertrophy. *J Mol Cell Cardiol*. 2012;52:74-82

148. Haghikia A, Missol-Kolka E, Tsikas D, Venturini L, Brundiers S, Castoldi M, Muckenthaler MU, Eder M, Stapel B, Thum T, Haghikia A, Petrasch-Parwez E, Drexler H, Hilfiker-Kleiner D, Scherr M. Signal transducer and activator of transcription 3-mediated regulation of mir-199a-5p links cardiomyocyte and endothelial cell function in the heart: A key role for ubiquitin-conjugating enzymes. *Eur Heart J*. 2011;32:1287-1297

149. Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, Galuppo P, Just S, Rottbauer W, Frantz S, Castoldi M, Soutschek J, Koteliansky V, Rosenwald A, Basson MA, Licht JD, Pena JT, Rouhanifard SH, Muckenthaler MU, Tuschl T, Martin GR, Bauersachs J, Engelhardt S. Microrna-21 contributes to myocardial disease by stimulating map kinase signalling in fibroblasts. *Nature*. 2008;456:980-984

150. Sayed D, Rane S, Lypowy J, He M, Chen IY, Vashistha H, Yan L, Malhotra A, Vatner D, Abdellatif M. Microrna-21 targets sprouty2 and promotes cellular outgrowths. *Mol Biol Cell*. 2008;19:3272-3282

151. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, Hill JA, Olson EN. Dysregulation of microrna after myocardial infarction reveals a role of mir-29 in cardiac fibrosis. *Proc Natl Acad Sci U S A*. 2008;105:13027-13032

152. McCarthy RE, 3rd, Boehmer JP, Hruban RH, Hutchins GM, Kasper EK, Hare JM, Baughman KL. Long-term outcome of fulminant myocarditis as compared with acute (nonfulminant) myocarditis. *N Engl J Med*. 2000;342:690-695

153. Chau EM, Chow WH, Chiu CS, Wang E. Treatment and outcome of biopsy-proven fulminant myocarditis in adults. *Int J Cardiol*. 2006;110:405-406

154. Templin C, Ghadri JR, Diekmann J, Napp LC, Bataiosu DR, Jaguszewski M, Cammann VL, Sarcon A, Geyer V, Neumann CA, Seifert B, Hellermann J, Schwyzer M, Eisenhardt K, Jenewein J, Franke J, Katus HA, Burgdorf C, Schunkert H, Moeller C, Thiele H, Bauersachs J, Tschope C, Schultheiss HP, Laney CA, Rajan L, Michels G, Pfister R, Ukena C, Bohm M, Erbel R, Cuneo A, Kuck KH, Jacobshagen C, Hasenfuss G, Karakas M, Koenig W, Rottbauer W, Said SM, Braun-Dullaeus RC, Cuculi F, Banning A, Fischer TA, Vasankari T, Airaksinen KE, Fijalkowski M, Rynkiewicz A, Pawlak M, Opolski G, Dworakowski R, MacCarthy P, Kaiser C, Osswald S, Galiuto L, Crea F, Dichtl W, Franz WM, Empen K, Felix SB, Delmas C, Lairez O, Erne P, Bax JJ, Ford I, Ruschitzka F, Prasad A, Luscher TF. Clinical features and outcomes of takotsubo (stress) cardiomyopathy. *N Engl J Med*. 2015;373:929-938

155. Birks EJ. A changing trend toward destination therapy: Are we treating the same patients differently? *Texas Heart Institute Journal*. 2011;38:552-554

156. Rose EA, Gelijns AC, Moskowitz AJ, Heitjan DF, Stevenson LW, Dembitsky W, Long JW, Ascheim DD, Tierney AR, Levitan RG, Watson JT, Meier P, Ronan NS, Shapiro PA, Lazar RM, Miller LW, Gupta L, Frazier OH, Desvigne-Nickens P, Oz MC, Poirier VL, Randomized Evaluation of Mechanical Assistance for the Treatment of Congestive Heart Failure Study G. Long-term use of a left ventricular assist device for end-stage heart failure. *N Engl J Med*. 2001;345:1435-1443

157. Zafeiridis A, Jeevanandam V, Houser SR, Margulies KB. Regression of cellular hypertrophy after left ventricular assist device support. *Circulation (New York, N.Y.)*. 1998;98:656

158. Fida N, Loebe M, Estep JD, Guha A. Predictors and management of right heart failure after left ventricular assist device implantation. *Methodist Debakey Cardiovasc J*. 2015;11:18-23

159. James KB, McCarthy PM, Thomas JD, Vargo R, Hobbs RE, Sapp S, Bravo E. Effect of the implantable left ventricular assist device on neuroendocrine activation in heart failure. *Circulation*. 1995;92:II191-195

160. Dipla K, Mattiello JA, Jeevanandam V, Houser SR, Margulies KB. Myocyte recovery after mechanical circulatory support in humans with end-stage heart failure. *Circulation*. 1998;97:2316-2322

161. Chaudhary KW, Rossman EI, Piacentino V, 3rd, Kenessey A, Weber C, Gaughan JP, Ojamaa K, Klein I, Bers DM, Houser SR, Margulies KB. Altered myocardial ca2+ cycling after left ventricular assist device support in the failing human heart. *J Am Coll Cardiol*. 2004;44:837-845

162. Klotz S, Barbone A, Reiken S, Holmes JW, Naka Y, Oz MC, Marks AR, Burkhoff D. Left ventricular assist device support normalizes left and right ventricular beta-adrenergic pathway properties. *J Am Coll Cardiol*. 2005;45:668-676

163. Lalani GG, Birgersdotter-Green U. Cardiac resynchronisation therapy in patients with chronic heart failure. *Heart*. 2015;101:1008-1014

164. Moss AJ, Hall WJ, Cannom DS, Klein H, Brown MW, Daubert JP, Estes NAM, Foster E, Greenberg H, Higgins SL, Pfeffer MA, Solomon SD, Wilber D, Zareba W. Cardiac-resynchronization therapy for the prevention of heart-failure events. *New England Journal of Medicine*. 2009;361:1329-1338

165. Duncan A, Wait D, Gibson D, Daubert JC, Trial M. Left ventricular remodelling and haemodynamic effects of multisite biventricular pacing in patients with left ventricular systolic dysfunction and activation disturbances in sinus rhythm: Sub-study of the mustic (multisite stimulationin cardiomyopathies) trial. *Eur Heart J*. 2003;24:430-441

166. Cleland JGF, Daubert J-C, Erdmann E, Freemantle N, Gras D, Kappenberger L, Tavazzi L. The effect of cardiac resynchronization on morbidity and mortality in heart failure. *New England Journal of Medicine*. 2005;352:1539-1549

167. St. John Sutton M, Ghio S, Plappert T, Tavazzi L, Scelsi L, Daubert C, Abraham WT, Gold MR, Hassager C, Herre JM, Linde C, Group oBotRrRiSlvdS. Cardiac resynchronization induces major structural and functional reverse remodeling in patients with new york heart association class i/ii heart failure. *Circulation*. 2009;120:1858-1865

168. Domanski MJ, Krause-Steinrauf H, Massie BM, Deedwania P, Follmann D, Kovar D, Murray D, Oren R, Rosenberg Y, Young J, Zile M, Eichhorn E, Investigators B. A comparative analysis of the results from 4 trials of beta-blocker therapy for heart failure: Best, cibis-ii, merit-hf, and copernicus. *J Card Fail*. 2003;9:354-363

169. MERIT-HF Study Group. Effect of metoprolol cr/xl in chronic heart failure: Metoprolol cr/xl randomised intervention trial in congestive heart failure (merit-hf). *Lancet*. 1999;353:2001-2007

170. Kubo H, Margulies KB, Piacentino V, 3rd, Gaughan JP, Houser SR. Patients with end-stage congestive heart failure treated with beta-adrenergic receptor antagonists have improved ventricular myocyte calcium regulatory protein abundance. *Circulation*. 2001;104:1012-1018

171. Wang R, Miura T, Harada N, Kametani R, Shibuya M, Fukagawa Y, Kawamura S, Ikeda Y, Hara M, Matsuzaki M. Pleiotropic effects of the beta-adrenoceptor blocker carvedilol on calcium regulation during oxidative stress-induced apoptosis in cardiomyocytes. *J Pharmacol Exp Ther*. 2006;318:45-52

172. Gwathmey JK, Kim CS, Hajjar RJ, Khan F, DiSalvo TG, Matsumori A, Bristow MR. Cellular and molecular remodeling in a heart failure model treated with the beta-blocker carteolol. *Am J Physiol*. 1999;276:H1678-1690

173. Greenberg B, Quinones MA, Koilpillai C, Limacher M, Shindler D, Benedict C, Shelton B. Effects of long-term enalapril therapy on cardiac structure and function in patients with left ventricular dysfunction. Results of the solvd echocardiography substudy. *Circulation*. 1995;91:2573-2581

174. Wong M, Staszewsky L, Latini R, Barlera S, Volpi A, Chiang YT, Benza RL, Gottlieb SO, Kleemann TD, Rosconi F, Vandervoort PM, Cohn JN, Val-He FTHFTI. Valsartan benefits left ventricular structure and function in heart failure: Val-heft echocardiographic study. *J Am Coll Cardiol*. 2002;40:970-975

175. Chan AK, Sanderson JE, Wang T, Lam W, Yip G, Wang M, Lam YY, Zhang Y, Yeung L, Wu EB, Chan WW, Wong JT, So N, Yu CM. Aldosterone receptor antagonism induces reverse remodeling when added to angiotensin receptor blockade in chronic heart failure. *J Am Coll Cardiol*. 2007;50:591-596

176. Granger CB, McMurray JJ, Yusuf S, Held P, Michelson EL, Olofsson B, Ostergren J, Pfeffer MA, Swedberg K, Investigators C, Committees. Effects of candesartan in patients with chronic heart failure and reduced left-ventricular systolic function intolerant to angiotensin-converting-enzyme inhibitors: The charm-alternative trial. *Lancet*. 2003;362:772-776

177. McMurray JJV, Packer M, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, Rouleau JL, Shi VC, Solomon SD, Swedberg K, Zile MR. Angiotensin–neprilysin inhibition versus enalapril in heart failure. *New England Journal of Medicine*. 2014;371:993-1004

178. Daniels LB, Maisel AS. Natriuretic peptides. *J Am Coll Cardiol*. 2007;50:2357-2368

179. Lu B, Gerard NP, Kolakowski LF, Jr., Bozza M, Zurakowski D, Finco O, Carroll MC, Gerard C. Neutral endopeptidase modulation of septic shock. *J Exp Med*. 1995;181:2271-2275

180. Tardif J-C, O'Meara E, Komajda M, Böhm M, Borer JS, Ford I, Tavazzi L, Swedberg K. Effects of selective heart rate reduction with ivabradine on left ventricular remodelling and function: Results from the shift echocardiography substudy. *European Heart Journal*. 2011;32:2507-2515

181. Ceconi C, Freedman SB, Tardif JC, Hildebrandt P, McDonagh T, Gueret P, Parrinello G, Robertson M, Steg PG, Tendera M, Ford I, Fox K, Ferrari R. Effect of heart

rate reduction by ivabradine on left ventricular remodeling in the echocardiographic substudy of beautiful. *International Journal of Cardiology*. 2011;146:408-414

182. Dedkov EI, Zheng W, Christensen LP, Weiss RM, Mahlberg-Gaudin F, Tomanek RJ. Preservation of coronary reserve by ivabradine-induced reduction in heart rate in infarcted rats is associated with decrease in perivascular collagen. *American Journal of Physiology - Heart and Circulatory Physiology*. 2007;293:H590-H598

183. Milliez P, Messaoudi S, Nehme J, Rodriguez C, Samuel J-L, Delcayre C. Beneficial effects of delayed ivabradine treatment on cardiac anatomical and electrical remodeling in rat severe chronic heart failure. *American Journal of Physiology - Heart and Circulatory Physiology*. 2009;296:H435-H441

184. Gupta RC, Wang M, Ilsar I, Sabbah MS, Dye K, Cavanagh A, Rastogi S, Sabbah HN. Heart rate reduction with ivabradine improves sarcoplasmic reticulum calcium cycling in left ventricular myocardium of dogs with moderate heart failure. *Journal of the American College of Cardiology*. 2011;57:E323

185. Gheorghiade M, Blair JE, Filippatos GS, Macarie C, Ruzyllo W, Korewicki J, Bubenek-Turconi SI, Ceracchi M, Bianchetti M, Carminati P, Kremastinos D, Valentini G, Sabbah HN, Investigators H-H. Hemodynamic, echocardiographic, and neurohormonal effects of istaroxime, a novel intravenous inotropic and lusitropic agent: A randomized controlled trial in patients hospitalized with heart failure. *J Am Coll Cardiol*. 2008;51:2276-2285

186. Teerlink JR, Cotter G, Davison BA, Felker GM, Filippatos G, Greenberg BH, Ponikowski P, Unemori E, Voors AA, Adams KF, Jr., Dorobantu MI, Grinfeld LR, Jondeau G, Marmor A, Masip J, Pang PS, Werdan K, Teichman SL, Trapani A, Bush CA, Saini R, Schumacher C, Severin TM, Metra M. Serelaxin, recombinant human relaxin-2, for treatment of acute heart failure (relax-ahf): A randomised, placebo-controlled trial. *The Lancet*. 2013;381:29-39

187. Greenberg B, Butler J, Felker GM, Ponikowski P, Voors AA, Desai AS, Barnard D, Bouchard A, Jaski B, Lyon AR, Pogoda JM, Rudy JJ, Zsebo KM. Calcium upregulation by percutaneous administration of gene therapy in patients with cardiac disease (cupid 2): A randomised, multinational, double-blind, placebo-controlled, phase 2b trial. *The Lancet*.387:1178-1186

188. Jessup M, Greenberg B, Mancini D, Cappola T, Pauly DF, Jaski B, Yaroshinsky A, Zsebo KM, Dittrich H, Hajjar RJ, Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease I. Calcium upregulation by percutaneous administration of gene therapy in cardiac disease (cupid): A phase 2 trial of intracoronary gene therapy of sarcoplasmic reticulum ca2+-atpase in patients with advanced heart failure. *Circulation*. 2011;124:304-313

189. Makkar RR, Smith RR, Cheng K, Malliaras K, Thomson LE, Berman D, Czer LS, Marban L, Mendizabal A, Johnston PV, Russell SD, Schuleri KH, Lardo AC, Gerstenblith G, Marban E. Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (caduceus): A prospective, randomised phase 1 trial. *Lancet*. 2012;379:895-904

190. Debnath B, Xu S, Grande F, Garofalo A, Neamati N. Small molecule inhibitors of cxcr4. *Theranostics*. 2013;3:47-75

191. Bartunek J, Behfar A, Dolatabadi D, Vanderheyden M, Ostojic M, Dens J, El Nakadi B, Banovic M, Beleslin B, Vrolix M, Legrand V, Vrints C, Vanoverschelde JL, Crespo-Diaz R, Homsy C, Tendera M, Waldman S, Wijns W, Terzic A. Cardiopoietic stem cell therapy in heart failure: The c-cure (cardiopoietic stem cell therapy in heart failure) multicenter randomized trial with lineage-specified biologics. *Journal of the American College of Cardiology*. 2013;61:2329-2338

192. Bolli R, Chugh AR, D'Amario D, Loughran JH, Stoddard MF, Ikram S, Beache GM, Wagner SG, Leri A, Hosoda T, Elmore JB, Goihberg P, Cappetta D, Solankhi NK, Fahsah I, Rokosh DG, Slaughter MS, Kajstura J, Anversa P. Effect of cardiac stem cells in patients

with ischemic cardiomyopathy: Initial results of the scipio trial. *Lancet*. 2011;378:1847-1857

193. Murry CE, Reimer KA, Jennings RB. Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. *Circulation*. 1986;74:1124-1136

194. Deutsch E, Berger M, Kussmaul WG, Hirshfeld JW, Herrmann HC, Laskey WK. Adaptation to ischemia during percutaneous transluminal coronary angioplasty. Clinical, hemodynamic, and metabolic features. *Circulation*. 1990;82:2044-2051

195. Yellon DM, Alkhulaifi AM, Pugsley WB. Preconditioning the human myocardium. *Lancet*. 1993;342:276-277

196. Hausenloy DJ, Candilio L, Evans R, Ariti C, Jenkins DP, Kolvekar S, Knight R, Kunst G, Laing C, Nicholas J, Pepper J, Robertson S, Xenou M, Clayton T, Yellon DM. Remote ischemic preconditioning and outcomes of cardiac surgery. *New England Journal of Medicine*. 2015;373:1408-1417

197. Kloner RA, Shook T, Przyklenk K, Davis VG, Junio L, Matthews RV, Burstein S, Gibson CM, Poole WK, Cannon CP, McCabe CH, Braunwald E. Previous angina alters in-hospital outcome in timi 4 : A clinical correlate to preconditioning? *Circulation*. 1995;91:37-45

198. Ishihara Md M, Sato Md H, Tateishi Md H, Kawagoe Md T, Shimatani Md Y, Kurisu Md S, Sakai Md K, Ueda Md K. Implications of prodromal angina pectoris in anterior wall acute myocardial infarction: Acute angiographic findings and long-term prognosis. *Journal of the American College of Cardiology*. 1997;30:970-975

199. Cave AC, Hearse DJ. Ischaemic preconditioning and contractile function: Studies with normothermic and hypothermic global ischaemia. *J Mol Cell Cardiol*. 1992;24:1113-1123

200. Shiki K, Hearse DJ. Preconditioning of ischemic myocardium: Reperfusion-induced arrhythmias. *American Journal of Physiology - Heart and Circulatory Physiology*. 1987;253:H1470-H1476

201. Miura T, Adachi T, Ogawa T, Iwamoto T, Tsuchida A, Iimur O. Myocardial infarct size—limiting effect of ischemic preconditioning: Its natural decay and the effect of repetitive preconditioning. *Cardiovascular Pathology*.1:147-154

202. Zhao Z-Q, Faraz K, Michael EH, Joel SC, Wang N-P, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: Comparison with ischemic preconditioning. *Am J Physiol*. 2003;54:H579

203. Laskey WK. Brief repetitive balloon occlusions enhance reperfusion during percutaneous coronary intervention for acute myocardial infarction: A pilot study. *Cathet Cardiovasc Int*. 2005;65:361-367

204. Staat P, Ovize M, Bonnefoy E, André-Fouët X, Aupetit J-F, L'Huillier I, Cottin Y, Cung TT, Piot C, Rioufol G, Finet G. Postconditioning the human heart. *Circulation*. 2005;112:2143-2148

205. Thibault H, Rossi R, Macia JC, Bonnefoy E, André-Fouët X, Bontemps L, Angoulvant D, Sportouch C, Raczka F, Kirkorian G, Cung TT, Piot C, Finet G, Itti R, Derumeaux G, Ovize M, Aupetit J-F, Staat P, Rioufol G. Long-term benefit of postconditioning. *Circulation*. 2008;117:1037

206. Marber M, Latchman D, Walker J, Yellon D. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation*. 1993;88:1264-1272

207. Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada M. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circulation research*. 1993;72:1293-1299

208. Ren C, Yan Z, Wei D, Gao X, Chen X, Zhao H. Limb remote ischemic postconditioning protects against focal ischemia in rats. *Brain Research*. 2009;1288:88-94

209. Yellon D, Downey J. Preconditioning the myocardium: From cellular physiology to clinical cardiology. *Physiological reviews*. 2003;83:1113-1151

210. Murphy E, Steenbergen C. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiological reviews*. 2008;88:581-609

211. Gateau-Roesch O, Argaud L, Ovize M. Mitochondrial permeability transition pore and postconditioning. *Cardiovascular research*. 2006;70:264-273

212. Schulz R, Boengler K, Totzeck A, Luo Y, Garcia-Dorado D, Heusch G. Connexin 43 in ischemic pre- and postconditioning. *Heart failure reviews*. 2007;12:261-266

213. Schulman D, Latchman DS, Yellon DM. Urocortin protects the heart from reperfusion injury via upregulation of p42/p44 mapk signaling pathway. *American Journal of Physiology - Heart and Circulatory Physiology*. 2002;283:H1481-H1488

214. Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia–reperfusion injury: Targeting the reperfusion injury salvage kinase (risk)-pathway. *Cardiovascular research*. 2004;61:448-460

215. Yamazaki KG, Taub PR, Barraza-Hidalgo M, Rivas MM, Zambon AC, Ceballos G, Villarreal FJ. Effects of (-)-epicatechin on myocardial infarct size and left ventricular remodeling after permanent coronary occlusion. *J Am Coll Cardiol*. 2010;55:2869-2876

216. Kloner RA, Forman MB, Gibbons RJ, Ross AM, Alexander RW, Stone GW. Impact of time to therapy and reperfusion modality on the efficacy of adenosine in acute myocardial infarction: The amistad-2 trial. *European Heart Journal*. 2006;27:2400-2405

217. Kitakaze M, Asakura M, Kim J, Shintani Y, Asanuma H, Hamasaki T, Seguchi O, Myoishi M, Minamino T, Ohara T, Nagai Y, Nanto S, Watanabe K, Fukuzawa S, Hirayama A, Nakamura N, Kimura K, Fujii K, Ishihara M, Saito Y, Tomoike H, Kitamura S. Human

atrial natriuretic peptide and nicorandil as adjuncts to reperfusion treatment for acute myocardial infarction (j-wind): Two randomised trials. *Lancet*.370:1483-1493

218. Nikolaidis LA, Mankad S, Sokos GG, Miske G, Shah A, Elahi D, Shannon RP. Effects of glucagon-like peptide-1 in patients with acute myocardial infarction and left ventricular dysfunction after successful reperfusion. *Circulation*. 2004;109:962-965

219. Skyschally A, van Caster P, Boengler K, Gres P, Musiolik J, Schilawa D, Schulz R, Heusch G. Ischemic postconditioning in pigs. *Circulation research*. 2009;104:15-18

220. Le Good JA, Ziegler WH, Parekh DB, Alessi DR, Cohen P, Parker PJ. Protein kinase c isotypes controlled by phosphoinositide 3-kinase through the protein kinase pdk1. *Science*. 1998;281:2042-2045

221. Gray MO, Karliner JS, Mochly-Rosen D. A selective epsilon-protein kinase c antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. *J Biol Chem*. 1997;272:30945-30951

222. Saurin AT, Pennington DJ, Raat NJH, Latchman DS, Owen MJ, Marber MS. Targeted disruption of the protein kinase c epsilon gene abolishes the infarct size reduction that follows ischaemic preconditioning of isolated buffer-perfused mouse hearts. *Cardiovascular research*. 2002;55:672-680

223. Mitchell MB, Meng X, Ao L, Brown JM, Harken AH, Banerjee A. Preconditioning of isolated rat heart is mediated by protein kinase c. *Circulation research*. 1995;76:73-81

224. Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV, Downey JM. Opening of mitochondrial k(atp) channels triggers the preconditioned state by generating free radicals. *Circ Res*. 2000;87:460-466

225. Rodrigo GC, Norman RI, Dickens M, Standen NB. Phenylephrine preconditioning involves modulation of cardiac sarcolemmal katp current by pkc delta, ampk and p38 mapk. *Journal of molecular and cellular cardiology*. 2011;51:370-380

226. Penna C, Cappello S, Mancardi D, Raimondo S, Rastaldo R, Gattullo D, Losano G, Pagliaro P. Post–conditioning reduces infarct size in the isolated rat heart: Role of coronary flow and pressure and the nitric oxide/cgmp pathway. *Basic Res Cardiol*. 2006;101:168-179

227. Wolfrum S, Schneider K, Heidbreder M, Nienstedt J, Dominiak P, Dendorfer A. Remote preconditioning protects the heart by activating myocardial pkcepsilon-isoform. *Cardiovascular research*. 2002;55:583-589

228. Weinbrenner C, Nelles M, Herzog N, Sarvary L, Strasser RH. Remote preconditioning by infrarenal occlusion of the aorta protects the heart from infarction: A newly identified non-neuronal but pkc-dependent pathway. *Cardiovascular research*. 2002;55:590-601

229. Downey JM, Davis AM, Cohen MV. Signaling pathways in ischemic preconditioning. *Heart Fail Rev*. 2007;12:181-188

230. Cohen MV, Downey JM. Adenosine: Trigger and mediator of cardioprotection. *Basic Res Cardiol*. 2008;103:203-215

231. Lecour S. Activation of the protective survivor activating factor enhancement (safe) pathway against reperfusion injury: Does it go beyond the risk pathway? *J Mol Cell Cardiol*. 2009;47:32-40

232. Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN, Sollott SJ. Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J Clin Invest*. 2004;113:1535-1549

233. Heusch G, Musiolik J, Kottenberg E, Peters J, Jakob H, Thielmann M. Stat5 activation and cardioprotection by remote ischemic preconditioning in humans: Short communication. *Circ Res*. 2012;110:111-115

234. Hilfiker-Kleiner D, Hilfiker A, Drexler H. Many good reasons to have stat3 in the heart. *Pharmacology & Therapeutics*. 2005;107:131-137

235. Cohen MV, Yang X-M, Downey JM. Nitric oxide is a preconditioning mimetic and cardioprotectant and is the basis of many available infarct-sparing strategies. *Cardiovascular research*. 2006;70:231-239

236. Jones SP, Bolli R. The ubiquitous role of nitric oxide in cardioprotection. *J Mol Cell Cardiol*. 2006;40:16-23

237. Recchia FA, McConnell PI, Bernstein RD, Vogel TR, Xu X, Hintze TH. Reduced nitric oxide production and altered myocardial metabolism during the decompensation of pacing-induced heart failure in the conscious dog. *Circ Res*. 1998;83:969-979

238. Mohri M, Egashira K, Tagawa T, Kuga T, Tagawa H, Harasawa Y, Shimokawa H, Takeshita A. Basal release of nitric oxide is decreased in the coronary circulation in patients with heart failure. *Hypertension*. 1997;30:50-56

239. Prabhu SD. Nitric oxide protects against pathological ventricular remodeling: Reconsideration of the role of no in the failing heart. *Circulation research*. 2004;94:1155-1157

240. Wollert KC, Drexler H. Regulation of cardiac remodeling by nitric oxide: Focus on cardiac myocyte hypertrophy and apoptosis. *Heart Fail Rev*. 2002;7:317-325

241. Eliseyeva MR, PhD, ScD. Endothelium: A long road from mystery to discovery. *International Journal of BioMedicine*. 2013

242. Cleeter MW, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AH. Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. *FEBS Lett*. 1994;345:50-54

243. Shen W, Hintze TH, Wolin MS. Nitric oxide. An important signaling mechanism between vascular endothelium and parenchymal cells in the regulation of oxygen consumption. *Circulation*. 1995;92:3505-3512

244. Campbell DL, Stamler JS, Strauss HC. Redox modulation of l-type calcium channels in ferret ventricular myocytes. Dual mechanism regulation by nitric oxide and s-nitrosothiols. *J Gen Physiol*. 1996;108:277-293

245. Xu L, Eu JP, Meissner G, Stamler JS. Activation of the cardiac calcium release channel (ryanodine receptor) by poly-s-nitrosylation. *Science*. 1998;279:234-237

246. Traverse JH, Chen Y, Hou M, Bache RJ. Inhibition of no production increases myocardial blood flow and oxygen consumption in congestive heart failure. *American Journal of Physiology - Heart and Circulatory Physiology*. 2002;282:H2278-H2283

247. Chen Y, Traverse JH, Du R, Hou M, Bache RJ. Nitric oxide modulates myocardial oxygen consumption in the failing heart. *Circulation*. 2002;106:273-279

248. Treuer AV, Gonzalez DR. Nitric oxide synthases, s-nitrosylation and cardiovascular health: From molecular mechanisms to therapeutic opportunities (review). *Molecular Medicine Reports*. 2015;11:1555-1565

249. Costa AD, Garlid KD, West IC, Lincoln TM, Downey JM, Cohen MV, Critz SD. Protein kinase g transmits the cardioprotective signal from cytosol to mitochondria. *Circ Res*. 2005;97:329-336

250. Sun J, Morgan M, Shen RF, Steenbergen C, Murphy E. Preconditioning results in s-nitrosylation of proteins involved in regulation of mitochondrial energetics and calcium transport. *Circ Res*. 2007;101:1155-1163

251. Marshall HE, Stamler JS. Inhibition of nf-kappa b by s-nitrosylation. *Biochemistry*. 2001;40:1688-1693

252. Reynaert NL, Ckless K, Korn SH, Vos N, Guala AS, Wouters EF, van der Vliet A, Janssen-Heininger YM. Nitric oxide represses inhibitory kappab kinase through s-nitrosylation. *Proc Natl Acad Sci U S A*. 2004;101:8945-8950

253. Rohini A, Agrawal N, Koyani CN, Singh R. Molecular targets and regulators of cardiac hypertrophy. *Pharmacol Res*. 2010;61:269-280

254. Fiedler B, Lohmann SM, Smolenski A, Linnemuller S, Pieske B, Schroder F, Molkentin JD, Drexler H, Wollert KC. Inhibition of calcineurin-nfat hypertrophy signaling by cgmp-dependent protein kinase type i in cardiac myocytes. *Proc Natl Acad Sci U S A*. 2002;99:11363-11368

255. Nishida M, Watanabe K, Sato Y, Nakaya M, Kitajima N, Ide T, Inoue R, Kurose H. Phosphorylation of trpc6 channels at thr69 is required for anti-hypertrophic effects of phosphodiesterase 5 inhibition. *J Biol Chem*. 2010;285:13244-13253

256. Koitabashi N, Aiba T, Hesketh GG, Rowell J, Zhang M, Takimoto E, Tomaselli GF, Kass DA. Cyclic gmp/pkg-dependent inhibition of trpc6 channel activity and expression negatively regulates cardiomyocyte nfat activation novel mechanism of cardiac stress modulation by pde5 inhibition. *J Mol Cell Cardiol*. 2010;48:713-724

257. Burley DS, Ferdinandy P, Baxter GF. Cyclic gmp and protein kinase-g in myocardial ischaemia-reperfusion: Opportunities and obstacles for survival signaling. *British Journal of Pharmacology*. 2007;152:855-869

258. Hao J, Michalek C, Zhang W, Zhu M, Xu X, Mende U. Regulation of cardiomyocyte signaling by rgs proteins: Differential selectivity towards g proteins and susceptibility to regulation. *J Mol Cell Cardiol*. 2006;41:51-61

259. Huang J, Zhou H, Mahavadi S, Sriwai W, Murthy KS. inhibition** of gα_q-dependent plc-β1 activity by pkg and pka is mediated by phosphorylation of rgs4 and grk2 . *American Journal of Physiology - Cell Physiology*. 2007;292:C200-C208

260. Wang Y, Li ZC, Zhang P, Poon E, Kong CW, Boheler KR, Huang Y, Li RA, Yao X. Nitric oxide-cgmp-pkg pathway acts on orai1 to inhibit the hypertrophy of human embryonic stem cell-derived cardiomyocytes. *Stem Cells*. 2015;33:2973-2984

261. Jin Z-Q, Karliner JS, Vessey DA. Ischaemic postconditioning protects isolated mouse hearts against ischaemia/reperfusion injury via sphingosine kinase isoform-1 activation. *Cardiovascular research*. 2008;79:134-140

262. Cai Z, Zhong H, Bosch-Marce M, Fox-Talbot K, Wang L, Wei C, Trush MA, Semenza GL. Complete loss of ischaemic preconditioning-induced cardioprotection in mice with partial deficiency of hif-1 alpha. *Cardiovascular research*. 2008;77:463-470

263. Cai Z, Manalo DJ, Wei G, Rodriguez ER, Fox-Talbot K, Lu H, Zweier JL, Semenza GL. Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury. *Circulation*. 2003;108:79-85

264. Cai Z, Luo W, Zhan H, Semenza GL. Hypoxia-inducible factor 1 is required for remote ischemic preconditioning of the heart. *Proc Natl Acad Sci U S A*. 2013;110:17462-17467

265. Kalakech H, Tamareille S, Pons S, Godin-Ribuot D, Carmeliet P, Furber A, Martin V, Berdeaux A, Ghaleh B, Prunier F. Role of hypoxia inducible factor-1alpha in remote limb ischemic preconditioning. *J Mol Cell Cardiol*. 2013;65:98-104

266. Pickard JM, Botker HE, Crimi G, Davidson B, Davidson SM, Dutka D, Ferdinandy P, Ganske R, Garcia-Dorado D, Giricz Z, Gourine AV, Heusch G, Kharbanda R, Kleinbongard P, MacAllister R, McIntyre C, Meybohm P, Prunier F, Redington A, Robertson NJ, Suleiman MS, Vanezis A, Walsh S, Yellon DM, Hausenloy DJ. Remote ischemic conditioning: From experimental observation to clinical application: Report from the 8th biennial hatter cardiovascular institute workshop. *Basic Res Cardiol*. 2015;110:453

267. Contractor H, Støttrup N, Cunnington C, Manlhiot C, Diesch J, Ormerod JM, Jensen R, Bøtker H, Redington A, Schmidt M, Ashrafian H, Kharbanda R. Aldehyde

dehydrogenase-2 inhibition blocks remote preconditioning in experimental and human models. *Basic research in cardiology*. 2013;108:1-10

268. Abdul-Ghani S, Heesom KJ, Angelini GD, Suleiman MS. Cardiac phosphoproteomics during remote ischemic preconditioning: A role for the sarcomeric z-disk proteins. *Biomed Res Int*. 2014;2014:767812

269. Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation (New York, N.Y.)*. 1993;87:893

270. Kerendi F, Kin H, Halkos ME, Jiang R, Zatta AJ, Zhao ZQ, Guyton RA, Vinten–Johansen J. Remote postconditioning. *Basic Res Cardiol*. 2005;100:404-412

271. Andreka G, Vertesaljai M, Szantho G, Font G, Piroth Z, Fontos G, Juhasz ED, Szekely L, Szelid Z, Turner MS, Ashrafian H, Frenneaux MP, Andreka P. Remote ischaemic postconditioning protects the heart during acute myocardial infarction in pigs. *Heart*. 2007;93:749-752

272. Kharbanda RK, Mortensen UM, White PA, Kristiansen SB, Schmidt MR, Hoschtitzky JA, Vogel M, Sorensen K, Redington AN, MacAllister R. Transient limb ischemia induces remote ischemic preconditioning in vivo. *Circulation*. 2002;106:2881-2883

273. Bøtker HE, Kharbanda R, Schmidt MR, Bøttcher M, Kaltoft AK, Terkelsen CJ, Munk K, Andersen NH, Hansen TM, Trautner S, Lassen JF, Christiansen EH, Krusell LR, Kristensen SD, Thuesen L, Nielsen SS, Rehling M, Sørensen HT, Redington AN, Nielsen TT. Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: A randomised trial. *Lancet*. 2010;375:727-734

274. Rentoukas I, Giannopoulos G, Kaoukis A, Kossyvakis C, Raisakis K, Driva M, Panagopoulou V, Tsarouchas K, Vavetsi S, Pyrgakis V, Deftereos S. Cardioprotective role

of remote ischemic periconditioning in primary percutaneous coronary intervention: Enhancement by opioid action. *J Am Coll Cardiol Intv*. 2010;3:49-55

275. Roubille F, Covinhes A, Nargeot J, Piot C, Franck-Miclo A, Redt-Clouet C, Lafont C, Combes S, Cransac F, Sportouch-Dukhan C, Vincent A, Barrère-Lemaire S, Fontanaud P. Delayed postconditioning in the mouse heart in vivo. *Circulation*. 2011;124:1330

276. Munk K, Andersen NH, Schmidt MR, Nielsen SS, Terkelsen CJ, Sloth E, Bøtker HE, Nielsen TT, Poulsen SH. Remote ischemic conditioning in patients with myocardial infarction treated with primary angioplasty / clinical perspective. *Circ Cardiovasc Imaging* 2010;3:656-662

277. McCafferty K, Forbes S, Thiemermann C, Yaqoob MM. The challenge of translating ischemic conditioning from animal models to humans: The role of comorbidities. *Dis Model Mech*. 2014;7:1321-1333

278. Konstantinov IE, Li J, Cheung MM, Shimizu M, Stokoe J, Kharbanda RK, Redington AN. Remote ischemic preconditioning of the recipient reduces myocardial ischemia-reperfusion injury of the denervated donor heart via a katp channel-dependent mechanism. *Transplantation*. 2005;79:1691-1695

279. Dickson EW, Lorbar M, Porcaro WA, Fenton RA, Reinhardt CP, Gysembergh A, Przyklenk K. Rabbit heart can be “preconditioned” via transfer of coronary effluent. *AJP-Heart*. 1999;277:H2451-H2457

280. Shimizu M, Tropak M, Diaz R, Suto F, Surendra H, Kuzmin E, Li J, Gross G, Wilson G, Callahan J, Redington A. Transient limb ischaemia remotely preconditions through a humoral mechanism acting directly on the myocardium: Evidence suggesting cross-species protection. *Clin Sci (Lond)*. 2009;117:191-200

281. Serejo FC, Rodrigues LFJ, da Silva Tavares KC, de Carvalho ACC, Nascimento JHM. Cardioprotective properties of humoral factors released from rat hearts subject to ischemic preconditioning. *J Cardiovasc Pharmacol* 2007;49:214-220

282. Lang S, Elsässer A, Scheler C, Vetter S, Tiefenbacher C, Kübler W, Katus H, Vogt A. Myocardial preconditioning and remote renal preconditioning. *Basic Res Cardiol*. 2006;101:149-158

283. Davidson S, Selvaraj P, He D, Boi-Doku C, Yellon R, Vicencio J, Yellon D. Remote ischaemic preconditioning involves signalling through the sdf-1α/cxcr4 signalling axis. *Basic Res Cardiol*. 2013;108:1-10

284. Hibert P, Prunier-Mirebeau D, Beseme O, Chwastyniak M, Tamareille S, Pinet F, Prunier F. Modifications in rat plasma proteome after remote ischemic preconditioning (ripc) stimulus: Identification by a seldi-tof-ms approach. *PLoS ONE*. 2014;9:13

285. Li J, Rohailla S, Gelber N, Rutka J, Sabah N, Gladstone RA, Wei C, Hu P, Kharbanda RK, Redington AN. Microrna-144 is a circulating effector of remote ischemic preconditioning. *Basic Res Cardiol*. 2014;109:423

286. Norata GD, Sala F, Catapano AL, Fernández-Hernando C. Micrornas and lipoproteins: A connection beyond atherosclerosis? *Atherosclerosis*. 2012;227:209-215

287. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF, Tewari M. Argonaute2 complexes carry a population of circulating micrornas independent of vesicles in human plasma. *Proceedings of the National Academy of Sciences*. 2011;108:5003-5008

288. Giricz Z, Varga ZV, Baranyai T, Sipos P, Paloczi K, Kittel A, Buzas E, Ferdinandy P. Cardioprotection by remote ischemic preconditioning of the rat heart is mediated by extracellular vesicles. *J Mol Cell Cardiol* 2014;68:75-78

289. Yellon DM, Davidson SM. Exosomes: Nanoparticles involved in cardioprotection? *Circulation research*. 2014;114:325-332

290. Gho BCG, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in noncardiac tissue. *Circulation*. 1996;94:2193-2200

291. Ding Y, Zhang M, He R. Role of renal nerve in cardioprotection provided by renal ischemic preconditioning in anesthetized rabbits. *Sheng Li Xue Bao*. 2001;53:7-12

292. Dong Y, Liu Y, Ji E, He R. [limb ischemic preconditioning reduces infarct size following myocardial ischemia-reperfusion in rats] article in chinese. *Sheng Li Xue Bao*. 2004;56:41-46

293. Tang Z-L, Dai W, Li Y-J, Deng H-W. Involvement of capsaicin-sensitive sensory nerves in early and delayed cardioprotection induced by a brief ischaemia of the small intestine. *Naunyn-Schmiedeberg's Archives of Pharmacology*. 1999;359:243-247

294. Brzozowski T, Konturek PC, Konturek SJ, Pajdo R, Kwiecien S, Pawlik M, Drozdowicz D, Sliwowski Z, Pawlik WW. Ischemic preconditioning of remote organs attenuates gastric ischemia–reperfusion injury through involvement of prostaglandins and sensory nerves. *Eur J Pharmacol* 2004;499:201-213

295. Weinbrenner C, Schulze F, Sárváry L, Strasser RH. Remote preconditioning by infrarenal aortic occlusion is operative via δ1-opioid receptors and free radicals in vivo in the rat heart. *Cardiovascular research*. 2004;61:591-599

296. Wolfrum S, Nienstedt J, Heidbreder M, Schneider K, Dominiak P, Dendorfer A. Calcitonin gene related peptide mediates cardioprotection by remote preconditioning. *Regulatory Peptides*. 2005;127:217-224

297. Basalay M, Barsukevich V, Mastitskaya S, Mrochek A, Pernow J, Sjöquist P-O, Ackland GL, Gourine AV, Gourine A. Remote ischaemic pre- and delayed postconditioning – similar degree of cardioprotection but distinct mechanisms. *Exp Physiol* 2012;97:908-917

298. Mastitskaya S. Cardioprotection evoked by remote ischaemic preconditioning is critically dependent on the activity of vagal pre-ganglionic neurones. *Cardiovasc. Res.* 2012;95:487-494

299. Katare RG, Ando M, Kakinuma Y, Arikawa M, Handa T, Yamasaki F, Sato T. Vagal nerve stimulation prevents reperfusion injury through inhibition of opening of mitochondrial permeability transition pore independent of the bradycardiac effect. *J Thorac Cardiovasc Surg*. 2009;137:223-231

300. Calvillo L, Vanoli E, Andreoli E, Besana A, Omodeo E, Gnecchi M, Zerbi P, Vago G, Busca G, Schwartz PJ. Vagal stimulation, through its nicotinic action, limits infarct size and the inflammatory response to myocardial ischemia and reperfusion. *J Cardiovasc Pharmacol*. 2011;58:500-507

301. Buchholz B, Donato M, Perez V, Ivalde FC, Hocht C, Buitrago E, Rodriguez M, Gelpi RJ. Preischemic efferent vagal stimulation increases the size of myocardial infarction in rabbits. Role of the sympathetic nervous system. *Int J Cardiol*. 2012;155:490-491

302. Jones WK, Fan GC, Liao S, Zhang JM, Wang Y, Weintraub NL, Kranias EG, Schultz JE, Lorenz J, Ren X. Peripheral nociception associated with surgical incision elicits remote nonischemic cardioprotection via neurogenic activation of protein kinase c signaling. *Circulation*. 2009;120:S1-9

303. Schoemaker RG, van Heijningen CL. Bradykinin mediates cardiac preconditioning at a distance. *Am J Physiol Heart Circ Physiol*. 2000;278:H1571-1576

304. Xiao L, Lu R, Hu CP, Deng HW, Li YJ. Delayed cardioprotection by intestinal preconditioning is mediated by calcitonin gene-related peptide. *Eur J Pharmacol*. 2001;427:131-135

305. Jensen R, Støttrup N, Kristiansen S, Bøtker H. Release of a humoral circulating cardioprotective factor by remote ischemic preconditioning is dependent on preserved neural pathways in diabetic patients. *Basic research in cardiology*. 2012;107:1-9

306. Redington K, Disenhouse T, Strantzas S, Gladstone R, Wei C, Tropak M, Dai X, Manlhiot C, Li J, Redington A. Remote cardioprotection by direct peripheral nerve stimulation and topical capsaicin is mediated by circulating humoral factors. *Basic research in cardiology*. 2012;107:1-10

307. Redington K, Disenhouse T, Li J, Wei C, Dai X, Gladstone R, Manlhiot C, Redington A. Electroacupuncture reduces myocardial infarct size and improves post-ischemic recovery by invoking release of humoral, dialyzable, cardioprotective factors. *The Journal of Physiological Sciences*. 2013;63:219-223

308. Merlocco A, Redington K, Disenhouse T, Strantzas S, Gladstone R, Wei C, Tropak M, Manlhiot C, Li J, Redington A. Transcutaneous electrical nerve stimulation as a novel method of remote preconditioning: In vitro validation in an animal model and first human observations. *Basic research in cardiology*. 2014;109:1-13

309. Cheung MMH, Kharbanda RK, Konstantinov IE, Shimizu M, Frndova H, Li J, Holtby HM, Cox PN, Smallhorn JF, Van Arsdell GS, Redington AN. Randomized controlled trial of the effects of remote ischemic preconditioning on children undergoing cardiac surgery: First clinical application in humans. *J Am Coll Cardiol* 2006;47:2277-2282

310. Shimizu M, Saxena P, Konstantinov IE, Cherepanov V, Cheung MMH, Wearden P, Zhangdong H, Schmidt M, Downey GP, Redington AN. Remote ischemic preconditioning decreases adhesion and selectively modifies functional responses of human neutrophils. *J Surg Res* 2010;158:155-161

311. Li G, Labruto F, Sirsjö A, Chen F, Vaage J, Valen G. Myocardial protection by remote preconditioning: The role of nuclear factor kappa-b p105 and inducible nitric oxide synthase. *Eur J Cardiothorac Surg* 2004;26:968-973

312. Valen G, Yan Z, Hansson GK. Nuclear factor kappa-b and the heart. *Journal of the American College of Cardiology*. 2001;38:307-314

313. Wei M, Redington AN, Liu M, Tao J, Xin P, Zhu W, Li J, Li J, Li Y, Li S. Repeated remote ischemic postconditioning protects against adverse left ventricular remodeling and improves survival in a rat model of myocardial infarction. *Circ Res*. 2011;108:1220-1225

314. Cai ZP, Parajuli N, Zheng X, Becker L. Remote ischemic preconditioning confers late protection against myocardial ischemia-reperfusion injury in mice by upregulating interleukin-10. *Basic Res Cardiol*. 2012;107:012-0277

315. Nahrendorf M, Pittet MJ, Swirski FK. Monocytes: Protagonists of infarct inflammation and repair after myocardial infarction. *Circulation*. 2010;121:2437-2445

316. Rohailla S, Clarizia N, Sourour M, Sourour W, Gelber N, Wei C, Li J, Redington AN. Acute, delayed and chronic remote ischemic conditioning is associated with downregulation of mtor and enhanced autophagy signaling. *PLoS ONE*. 2014;9:e111291

317. Yamaguchi T, Izumi Y, Nakamura Y, Yamazaki T, Shiota M, Sano S, Tanaka M, Osada-Oka M, Shimada K, Miura K, Yoshiyama M, Iwao H. Repeated remote ischemic conditioning attenuates left ventricular remodeling via exosome-mediated intercellular communication on chronic heart failure after myocardial infarction. *Int J Cardiol*. 2015;178:239-246

318. Sloth AD, Schmidt MR, Munk K, Kharbanda RK, Redington AN, Schmidt M, Pedersen L, Sørensen HT, Bøtker HE, Investigators C. Improved long-term clinical outcomes in patients with st-elevation myocardial infarction undergoing remote ischaemic conditioning as an adjunct to primary percutaneous coronary intervention. *Eur Heart J* 2013

319. Hofsten DE, Kelbaek H, Helqvist S, Klovgaard L, Holmvang L, Clemmensen P, Torp-Pedersen C, Tilsted HH, Botker HE, Jensen LO, Kober L, Engstrom T. The third danish study of optimal acute treatment of patients with st-segment elevation myocardial infarction: Ischemic postconditioning or deferred stent implantation versus conventional primary angioplasty and complete revascularization versus treatment of culprit lesion only: Rationale and design of the danami 3 trial program. *American heart journal*. 2015;169:613-621

320. Kimes BW, Brandt BL. Properties of a clonal muscle cell line from rat heart. *Experimental Cell Research*. 1976;98:367-381

321. Graves JW, Tibor M, Murtagh B, Klein L, Sheps SG. The accoson greenlight 300, the first non-automated mercury-free blood pressure measurement device to pass the international protocol for blood pressure measuring devices in adults. *Blood Press Monit*. 2004;9:13-17

322. Pirkmajer S, Chibalin AV. Serum starvation: Caveat emptor. *Am J Physiol Cell Physiol*. 2011;301:C272-279

323. Grinnell F. Fibroblasts, myofibroblasts, and wound contraction. *J Cell Biol*. 1994;124:401-404

324. Gabbiani G. The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol*. 2003;200:500-503

325. Swaney JS, Roth DM, Olson ER, Naugle JE, Meszaros JG, Insel PA. Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102:437-442

326. Welch MP, Odland GF, Clark RA. Temporal relationships of f-actin bundle formation, collagen and fibronectin matrix assembly, and fibronectin receptor expression to wound contraction. *J Cell Biol*. 1990;110:133-145

327. Na S, Meininger GA, Humphrey JD. A theoretical model for f-actin remodeling in vascular smooth muscle cells subjected to cyclic stretch. *J Theor Biol*. 2007;246:87-99

328. Langevin HM, Storch KN, Cipolla MJ, White SL, Buttolph TR, Taatjes DJ. Fibroblast spreading induced by connective tissue stretch involves intracellular redistribution of alpha- and beta-actin. *Histochem Cell Biol*. 2006;125:487-495

329. Qiagen. Rneasy® mini handbook. *https://**www.qiagen.com/gb/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en***. 2012

330. Tecnologies L. Mirvana™ mirna isolation kit. *https://**www.thermofisher.com/order/catalog/product/AM1560***. 2011

331. Kramer MF. Stem-loop rt-qpcr for mirnas. *Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.]*. 2011;CHAPTER:Unit15.10-Unit15.10

332. Galiveti CR, Rozhdestvensky TS, Brosius J, Lehrach H, Konthur Z. Application of housekeeping npcrnas for quantitative expression analysis of human transcriptome by real-time pcr. *RNA*. 2010;16:450-461

333. Pérez S, Royo LJ, Astudillo A, Escudero D, Álvarez F, Rodríguez A, Gómez E, Otero J. Identifying the most suitable endogenous control for determining gene expression in hearts from organ donors. *BMC Molecular Biology*. 2007;8:1-7

334. Tan SC, Carr CA, Yeoh KK, Schofield CJ, Davies KE, Clarke K. Identification of valid housekeeping genes for quantitative rt-pcr analysis of cardiosphere-derived cells preconditioned under hypoxia or with prolyl-4-hydroxylase inhibitors. *Mol Biol Rep*. 2012;39:4857-4867

335. Pfaffl MW. A new mathematical model for relative quantification in real-time rt-pcr. *Nucleic acids research*. 2001;29:e45

336. Turrell HE. Phenylephrine preconditioning of isolated ventricular myocytes. 2009;PhD

337. Räthel TR, Leikert JF, Vollmar AM, Dirsch VM. Application of 4,5-diaminofluorescein to reliably measure nitric oxide released from endothelial cells in vitro. *Biological Procedures Online*. 2003;5:136-142

338. Tukey JW. Comparing individual means in the analysis of variance. *Biometrics*. 1949;5:99-114

339. Cabral HJ. Multiple comparisons procedures. *Circulation*. 2008;117:698-701

340. McHugh ML. Multiple comparison analysis testing in anova. *Biochem Med (Zagreb)*. 2011;21:203-209

341. Louch WE, Sheehan KA, Wolska BM. Methods in cardiomyocyte isolation, culture, and gene transfer. *Journal of molecular and cellular cardiology*. 2011;51:288-298

342. Dubus I, Samuel JL, Marotte F, Delcayre C, Rappaport L. Beta-adrenergic agonists stimulate the synthesis of noncontractile but not contractile proteins in cultured myocytes isolated from adult rat heart. *Circ Res*. 1990;66:867-874

343. Schwarzfeld TA, Jacobson SL. Isolation and development in cell culture of myocardial cells of the adult rat. *J Mol Cell Cardiol*. 1981;13:563-575

344. Jacobson SL, Piper HM. Cell cultures of adult cardiomyocytes as models of the myocardium. *J Mol Cell Cardiol*. 1986;18:661-678

345. Mitcheson JS, Hancox JC, Levi AJ. Action potentials, ion channel currents and transverse tubule density in adult rabbit ventricular myocytes maintained for 6 days in cell culture. *Pflugers Arch*. 1996;431:814-827

346. Bell RM, Mocanu MM, Yellon DM. Retrograde heart perfusion: The langendorff technique of isolated heart perfusion. *J Mol Cell Cardiol*. 2011;50:940-950

347. Skrzypiec-Spring M, Grotthus B, Szelag A, Schulz R. Isolated heart perfusion according to langendorff-still viable in the new millennium. *Journal of Pharmacological and Toxicological Methods*. 2007;55:113-126

348. Strober W. Trypan blue exclusion test of cell viability. *Current protocols in immunology / edited by John E. Coligan ... [et al.]*. 2001;Appendix 3:Appendix 3B

349. Kabaeva Z, Zhao M, Michele DE. Blebbistatin extends culture life of adult mouse cardiac myocytes and allows efficient and stable transgene expression. *American Journal of Physiology - Heart and Circulatory Physiology*. 2008;294:H1667-H1674

350. Baudenbacher F, Schober T, Pinto JR, Sidorov VY, Hilliard F, Solaro RJ, Potter JD, Knollmann BC. Myofilament ca(2+) sensitization causes susceptibility to cardiac arrhythmia in mice. *The Journal of clinical investigation*. 2008;118:3893-3903

351. Kovács M, Tóth J, Hetényi C, Málnási-Csizmadia A, Sellers JR. Mechanism of blebbistatin inhibition of myosin ii. *Journal of Biological Chemistry*. 2004;279:35557-35563

352. Srinivasan S. A method to fix and permeabilize isolated adult mouse cardiomyocytes for immuno-staining and confocal imaging. 2011

353. Hescheler J, Meyer R, Plant S, Krautwurst D, Rosenthal W, Schultz G. Morphological, biochemical, and electrophysiological characterization of a clonal cell (h9c2) line from rat heart. *Circulation research*. 1991;69:1476-1486

354. Rao F, Deng CY, Wu SL, Xiao DZ, Yu XY, Kuang SJ, Lin QX, Shan ZX. Involvement of src in l-type ca2+ channel depression induced by macrophage migration inhibitory factor in atrial myocytes. *J Mol Cell Cardiol*. 2009;47:586-594

355. Bonavita F, Stefanelli C, Giordano E, Columbaro M, Facchini A, Bonafe F, Caldarera CM, Guarnieri C. H9c2 cardiac myoblasts undergo apoptosis in a model of ischemia consisting of serum deprivation and hypoxia: Inhibition by pma. *FEBS Lett*. 2003;536:85-91

356. Chen QM, Tu VC, Wu Y, Bahl JJ. Hydrogen peroxide dose dependent induction of cell death or hypertrophy in cardiomyocytes. *Arch Biochem Biophys*. 2000;373:242-248

357. Ekhterae D, Lin Z, Lundberg MS, Crow MT, Brosius FC, 3rd, Nunez G. Arc inhibits cytochrome c release from mitochondria and protects against hypoxia-induced apoptosis in heart-derived h9c2 cells. *Circ Res*. 1999;85:e70-77

358. Gustafsson AB, Tsai JG, Logue SE, Crow MT, Gottlieb RA. Apoptosis repressor with caspase recruitment domain protects against cell death by interfering with bax activation. *J Biol Chem*. 2004;279:21233-21238

359. Han H, Long H, Wang H, Wang J, Zhang Y, Wang Z. Progressive apoptotic cell death triggered by transient oxidative insult in h9c2 rat ventricular cells: A novel pattern of apoptosis and the mechanisms. *Am J Physiol Heart Circ Physiol*. 2004;286:H2169-2182

360. Pesant M, Sueur S, Dutartre P, Tallandier M, Grimaldi PA, Rochette L, Connat JL. Peroxisome proliferator-activated receptor delta (ppardelta) activation protects h9c2 cardiomyoblasts from oxidative stress-induced apoptosis. *Cardiovascular research*. 2006;69:440-449

361. Tanaka H, Sakurai K, Takahashi K, Fujimoto Y. Requirement of intracellular free thiols for hydrogen peroxide-induced hypertrophy in cardiomyocytes. *J Cell Biochem*. 2003;89:944-955

362. Turner NA, Xia F, Azhar G, Zhang X, Liu L, Wei JY. Oxidative stress induces DNA fragmentation and caspase activation via the c-jun nh2-terminal kinase pathway in h9c2 cardiac muscle cells. *J Mol Cell Cardiol*. 1998;30:1789-1801

363. Huang CY, Chueh PJ, Tseng CT, Liu KY, Tsai HY, Kuo WW, Chou MY, Yang JJ. Zak re-programs atrial natriuretic factor expression and induces hypertrophic growth in h9c2 cardiomyoblast cells. *Biochem Biophys Res Commun*. 2004;324:973-980

364. Koekemoer AL, Chong NW, Goodall AH, Samani NJ. Myocyte stress 1 plays an important role in cellular hypertrophy and protection against apoptosis. *FEBS Lett*. 2009;583:2964-2967

365. Zhou Y, Jiang Y, Kang YJ. Copper inhibition of hydrogen peroxideinduced hypertrophy in embryonic rat cardiac h9c2 cells. *Experimental Biology and Medicine*. 2007;232:385-389

366. Vindis C, D'Angelo R, Mucher E, Negre-Salvayre A, Parini A, Mialet-Perez J. Essential role of trpc1 channels in cardiomyoblasts hypertrophy mediated by 5-ht2a serotonin receptors. *Biochem Biophys Res Commun*. 2010;391:979-983

367. Brostrom MA, Reilly BA, Wilson FJ, Brostrom CO. Vasopressin-induced hypertrophy in h9c2 heart-derived myocytes. *The International Journal of Biochemistry & Cell Biology*. 2000;32:993-1006

368. Hwang GS, Oh KS, Koo HN, Seo HW, You KH, Lee BH. Effects of kr-31378, a novel atp-sensitive potassium channel activator, on hypertrophy of h9c2 cells and on cardiac dysfunction in rats with congestive heart failure. *Eur J Pharmacol*. 2006;540:131-138

369. Laufs U, Kilter H, Konkol C, Wassmann S, Bohm M, Nickenig G. Impact of hmg coa reductase inhibition on small gtpases in the heart. *Cardiovascular research*. 2002;53:911-920

370. Huang CY, Kuo WW, Chueh PJ, Tseng CT, Chou MY, Yang JJ. Transforming growth factor-beta induces the expression of anf and hypertrophic growth in cultured cardiomyoblast cells through zak. *Biochem Biophys Res Commun*. 2004;324:424-431

371. Liu CJ, Cheng YC, Lee KW, Hsu HH, Chu CH, Tsai FJ, Tsai CH, Chu CY, Liu JY, Kuo WW, Huang CY. Lipopolysaccharide induces cellular hypertrophy through calcineurin/nfat-3 signaling pathway in h9c2 myocardiac cells. *Mol Cell Biochem*. 2008;313:167-178

372. Kuo H-F, Lai Y-J, Wu J-C, Lee K-T, Chu C-S, Chen I-J, Wu J-R, Wu B-N. A xanthine-derivative k(+)-channel opener protects against serotonin-induced cardiomyocyte hypertrophy via the modulation of protein kinases. *International Journal of Biological Sciences*. 2014;10:64-72

373. Newman RA, Hacker MP, Krakoff IH. Amelioration of adriamycin and daunorubicin myocardial toxicity by adenosine. *Cancer Res*. 1981;41:3483-3488

374. Yancy SL, Shelden EA, Gilmont RR, Welsh MJ. Sodium arsenite exposure alters cell migration, focal adhesion localization and decreases tyrosine phosphorylation of focal adhesion kinase in h9c2 myoblasts. *Toxicol Sci*. 2005;84:278-286

375. Sardao VA, Oliveira PJ, Holy J, Oliveira CR, Wallace KB. Morphological alterations induced by doxorubicin on h9c2 myoblasts: Nuclear, mitochondrial, and cytoskeletal targets. *Cell Biol Toxicol*. 2009;25:227-243

376. Sipido KR, Marban E. L-type calcium channels, potassium channels, and novel nonspecific cation channels in a clonal muscle cell line derived from embryonic rat ventricle. *Circ Res*. 1991;69:1487-1499

377. Branco AF, Pereira SP, Gonzalez S, Gusev O, Rizvanov AA, Oliveira PJ. Gene expression profiling of h9c2 myoblast differentiation towards a cardiac-like phenotype. *PLoS ONE*. 2015;10:e0129303

378. Watkins S, Borthwick G, Arthur H. The h9c2 cell line and primary neonatal cardiomyocyte cells show similar hypertrophic responses in vitro. *In Vitro Cellular & Developmental Biology - Animal*. 2011;47:125-131

379. Ménard C, Pupier S, Mornet D, Kitzmann M, Nargeot J, Lory P. Modulation of l-type calcium channel expression during retinoic acid-induced differentiation of h9c2 cardiac cells. *Journal of Biological Chemistry*. 1999;274:29063-29070

380. Rybkin, II, Markham DW, Yan Z, Bassel-Duby R, Williams RS, Olson EN. Conditional expression of sv40 t-antigen in mouse cardiomyocytes facilitates an inducible switch from proliferation to differentiation. *J Biol Chem*. 2003;278:15927-15934

381. Menard C, Pupier S, Mornet D, Kitzmann M, Nargeot J, Lory P. Modulation of l-type calcium channel expression during retinoic acid-induced differentiation of h9c2 cardiac cells. *J Biol Chem*. 1999;274:29063-29070

382. Ren X, Li Y, Ma X, Zheng L, Xu Y, Wang J. Activation of p38/mef2c pathway by all-trans retinoic acid in cardiac myoblasts. *Life Sci*. 2007;81:89-96

383. Albagli-Curiel O, Carnac G, Vandromme M, Vincent S, Crepieux P, Bonnieu A. Serum-induced inhibition of myogenesis is differentially relieved by retinoic acid and triiodothyronine in c2 murine muscle cells. *Differentiation*. 1993;52:201-210

384. Pagano M, Naviglio S, Spina A, Chiosi E, Castoria G, Romano M, Sorrentino A, Illiano F, Illiano G. Differentiation of h9c2 cardiomyoblasts: The role of adenylate cyclase system. *Journal of Cellular Physiology*. 2004;198:408-416

385. Santiago J-J, Dangerfield AL, Rattan SG, Bathe KL, Cunnington RH, Raizman JE, Bedosky KM, Freed DH, Kardami E, Dixon IMC. Cardiac fibroblast to myofibroblast differentiation in vivo and in vitro: Expression of focal adhesion components in neonatal and adult rat ventricular myofibroblasts. *Developmental Dynamics*. 2010;239:1573-1584

386. Golden HB, Gollapudi D, Gerilechaogetu F, Li J, Cristales RJ, Peng X, Dostal DE. Isolation of cardiac myocytes and fibroblasts from neonatal rat pups. *Methods Mol Biol*. 2012;843:205-214

387. Drawnel FM, Archer CR, Roderick HL. The role of the paracrine/autocrine mediator endothelin-1 in regulation of cardiac contractility and growth. *Br J Pharmacol*. 2013;168:296-317

388. Araki M, Hasegawa K, Iwai-Kanai E, Fujita M, Sawamura T, Kakita T, Wada H, Morimoto T, Sasayama S. Endothelin-1 as a protective factor against beta-adrenergic agonist-induced apoptosis in cardiac myocytes. *J Am Coll Cardiol*. 2000;36:1411-1418

389. Bupha-Intr T, Haizlip KM, Janssen PML. Role of endothelin in the induction of cardiac hypertrophy in vitro. *PLoS ONE*. 2012;7:e43179

390. Agapitov AV, Haynes WG. Role of endothelin in cardiovascular disease. *Journal of Renin-Angiotensin-Aldosterone System*. 2002;3:1-15

391. Olson EN, Molkentin JD. Prevention of cardiac hypertrophy by calcineurin inhibition: Hope or hype? *Circulation research*. 1999;84:623-632

392. Miyauchi T, Masaki T. Pathophysiology of endothelin in the cardiovascular system. *Annu Rev Physiol*. 1999;61:391-415

393. Ito H, Hirata Y, Adachi S, Tanaka M, Tsujino M, Koike A, Nogami A, Murumo F, Hiroe M. Endothelin-1 is an autocrine/paracrine factor in the mechanism of angiotensin ii-induced hypertrophy in cultured rat cardiomyocytes. *J Clin Invest*. 1993;92:398-403

394. Piacentini L, Gray M, Honbo NY, Chentoufi J, Bergman M, Karliner JS. Endothelin-1 stimulates cardiac fibroblast proliferation through activation of protein kinase c. *J Mol Cell Cardiol*. 2000;32:565-576

395. Shi-Wen X, Chen Y, Denton CP, Eastwood M, Renzoni EA, Bou-Gharios G, Pearson JD, Dashwood M, du Bois RM, Black CM, Leask A, Abraham DJ. Endothelin-1 promotes myofibroblast induction through the eta receptor via a rac/phosphoinositide 3-kinase/akt-dependent pathway and is essential for the enhanced contractile phenotype of fibrotic fibroblasts. *Molecular Biology of the Cell*. 2004;15:2707-2719

396. Leask A. Tgfβ, cardiac fibroblasts, and the fibrotic response. *Cardiovascular research*. 2007;74:207-212

397. Gojniczek K, Jurzak M, Garncarczyk A. The role of connective tissue growth factor (ctgf) in fibroproliferative processes and tissues fibrosis. *Advances in Cell Biology*. 2008;-1:1

398. Böhm F, Pernow J. The importance of endothelin-1 for vascular dysfunction in cardiovascular disease. *Cardiovascular research*. 2007;76:8-18

399. Ito H, Adachi S, Tamamori M, Fujisaki H, Tanaka M, Lin M, Akimoto H, Marumo F, Hiroe M. Mild hypoxia induces hypertrophy of cultured neonatal rat cardiomyocytes: A possible endogenous endothelin-1-mediated mechanism. *J Mol Cell Cardiol*. 1996;28:1271-1277

400. Chu W, Wan L, Zhao D, Qu X, Cai F, Huo R, Wang N, Zhu J, Zhang C, Zheng F, Cai R, Dong D, Lu Y, Yang B. Mild hypoxia-induced cardiomyocyte hypertrophy via up-regulation of hif-1alpha-mediated trpc signalling. *J Cell Mol Med*. 2012;16:2022-2034

401. Nehra S, Bhardwaj V, Kalra N, Ganju L, Bansal A, Saxena S, Saraswat D. Nanocurcumin protects cardiomyoblasts h9c2 from hypoxia-induced hypertrophy and apoptosis by improving oxidative balance. *J Physiol Biochem*. 2015;71:239-251

402. Nehra S, Bhardwaj V, Saraswat D. Amlodipine protects rat ventricular cardiomyoblast h9c2 from hypoxia-induced apoptosis and restores oxidative balance by akt-1-dependent manner. *J Cardiovasc Pharmacol*. 2014;64:375-384

403. Webster KA, Discher DJ, Bishopric NH. Induction and nuclear accumulation of fos and jun proto-oncogenes in hypoxic cardiac myocytes. *J Biol Chem*. 1993;268:16852-16858

404. Chen L-M, Kuo W-W, Yang J-J, Wang S-GP, Yeh Y-L, Tsai F-J, Ho Y-J, Chang M-H, Huang C-Y, Lee S-D. Eccentric cardiac hypertrophy was induced by long-term intermittent hypoxia in rats. *Experimental Physiology*. 2007;92:409-416

405. Moon CH, Jung YS, Kim MH, Park RM, Lee SH, Baik EJ. Protein kinase c inhibitors attenuate protective effect of high glucose against hypoxic injury in h9c2 cardiac cells. *Jpn J Physiol*. 2000;50:645-649

406. Liu SX, Zhang Y, Wang YF, Li XC, Xiang MX, Bian C, Chen P. Upregulation of heme oxygenase-1 expression by hydroxysafflor yellow a conferring protection from anoxia/reoxygenation-induced apoptosis in h9c2 cardiomyocytes. *Int J Cardiol*. 2012;160:95-101

407. Sun HY, Wang NP, Halkos ME, Kerendi F, Kin H, Wang RX, Guyton RA, Zhao ZQ. Involvement of na+/h+ exchanger in hypoxia/re-oxygenation-induced neonatal rat cardiomyocyte apoptosis. *Eur J Pharmacol*. 2004;486:121-131

408. Shi-wen X, Denton CP, Dashwood MR, Holmes AM, Bou-Gharios G, Pearson JD, Black CM, Abraham DJ. Fibroblast matrix gene expression and connective tissue remodeling: Role of endothelin-1. 2001;116:417-425

409. Nishida M, Onohara N, Sato Y, Suda R, Ogushi M, Tanabe S, Inoue R, Mori Y, Kurose H. Galpha12/13-mediated up-regulation of trpc6 negatively regulates endothelin-1-induced cardiac myofibroblast formation and collagen synthesis through nuclear factor of activated t cells activation. *J Biol Chem*. 2007;282:23117-23128

410. Masur SK, Dewal HS, Dinh TT, Erenburg I, Petridou S. Myofibroblasts differentiate from fibroblasts when plated at low density. *Proceedings of the National Academy of Sciences*. 1996;93:4219-4223

411. Porchia F, Papucci M, Gargini C, Asta A, De Marco G, Agretti P, Tonacchera M, Mazzoni MR. Endothelin-1 up-regulates p115rhogef in embryonic rat cardiomyocytes during the hypertrophic response. *J Recept Signal Transduct Res*. 2008;28:265-283

412. Gu S, Zhang W, Chen J, Ma R, Xiao X, Ma X, Yao Z, Chen Y. Epc-derived microvesicles protect cardiomyocytes from ang ii-induced hypertrophy and apoptosis. *PLoS ONE*. 2014;9:e85396

413. Hernandez JS, Barreto-Torres G, Kuznetsov AV, Khuchua Z, Javadov S. Crosstalk between ampk activation and angiotensin ii-induced hypertrophy in cardiomyocytes: The role of mitochondria. *J Cell Mol Med*. 2014;18:709-720

414. Passariello CL, Zini M, Nassi PA, Pignatti C, Stefanelli C. Upregulation of sirt1 deacetylase in phenylephrine-treated cardiomyoblasts. *Biochem Biophys Res Commun*. 2011;407:512-516

415. Wang QD, Tokuno S, Valen G, Sjoquist PO, Thoren P. Cyclic fluctuations in the cardiac performance of the isolated langendorff-perfused mouse heart: Pyruvate abolishes the fluctuations and has an anti-ischaemic effect. *Acta Physiol Scand*. 2002;175:279-287

416. Oddoze C, Lombard E, Portugal H. Stability study of 81 analytes in human whole blood, in serum and in plasma. *Clin Biochem*. 2012;45:464-469

417. Bowen RA, Hortin GL, Csako G, Otanez OH, Remaley AT. Impact of blood collection devices on clinical chemistry assays. *Clin Biochem*. 2010;43:4-25

418. Lee PH, Macfarlane DJ, Lam TH, Stewart SM. Validity of the international physical activity questionnaire short form (ipaq-sf): A systematic review. *Int J Behav Nutr Phys Act*. 2011;8:115

419. Paffenbarger RS, Jr., Blair SN, Lee IM, Hyde RT. Measurement of physical activity to assess health effects in free-living populations. *Med Sci Sports Exerc*. 1993;25:60-70

420. van der Valk J, Brunner D, De Smet K, Fex Svenningsen A, Honegger P, Knudsen LE, Lindl T, Noraberg J, Price A, Scarino ML, Gstraunthaler G. Optimization of chemically defined cell culture media--replacing fetal bovine serum in mammalian in vitro methods. *Toxicol In Vitro*. 2010;24:1053-1063

421. Irukayama-Tomobe Y, Miyauchi T, Sakai S, Kasuya Y, Ogata T, Takanashi M, Iemitsu M, Sudo T, Goto K, Yamaguchi I. Endothelin-1–induced cardiac hypertrophy is inhibited by activation of peroxisome proliferator–activated receptor-α partly via blockade of c-jun nh2-terminal kinase pathway. *Circulation*. 2004;109:904-910

422. Liang F, Wang F, Zhang S, Gardner DG. Peroxisome proliferator activated receptor (ppar)alpha agonists inhibit hypertrophy of neonatal rat cardiac myocytes. *Endocrinology*. 2003;144:4187-4194

423. Le K, Li R, Xu S, Wu X, Huang H, Bao Y, Cai Y, Lan T, Moss J, Li C, Zou J, Shen X, Liu P. Pparalpha activation inhibits endothelin-1-induced cardiomyocyte hypertrophy by prevention of nfatc4 binding to gata-4. *Arch Biochem Biophys*. 2012;518:71-78

424. Diep QN, Benkirane K, Amiri F, Cohn JS, Endemann D, Schiffrin EL. Ppar alpha activator fenofibrate inhibits myocardial inflammation and fibrosis in angiotensin ii-infused rats. *J Mol Cell Cardiol*. 2004;36:295-304

425. Hirotani S, Otsu K, Nishida K, Higuchi Y, Morita T, Nakayama H, Yamaguchi O, Mano T, Matsumura Y, Ueno H, Tada M, Hori M. Involvement of nuclear factor-kappab and apoptosis signal-regulating kinase 1 in g-protein-coupled receptor agonist-induced cardiomyocyte hypertrophy. *Circulation*. 2002;105:509-515

426. Fredj S, Bescond J, Louault C, Potreau D. Interactions between cardiac cells enhance cardiomyocyte hypertrophy and increase fibroblast proliferation. *J Cell Physiol*. 2005;202:891-899

427. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*. 1951;193:265-275

428. Tani M, Honma Y, Hasegawa H, Tamaki K. Direct activation of mitochondrial k(atp) channels mimics preconditioning but protein kinase c activation is less effective in middle-aged rat hearts. *Cardiovascular research*. 2001;49:56-68

429. Przyklenk K, Li G, Simkhovich BZ, Kloner RA. Mechanisms of myocardial ischemic preconditioning are age related: Pkc-epsilon does not play a requisite role in old rabbits. *J Appl Physiol (1985)*. 2003;95:2563-2569

430. Peart JN, Gross ER, Headrick JP, Gross GJ. Impaired p38 mapk/hsp27 signaling underlies aging-related failure in opioid-mediated cardioprotection. *Journal of molecular and cellular cardiology*. 2007;42:972-980

431. Dirkx E, da Costa Martins PA, De Windt LJ. Regulation of fetal gene expression in heart failure. *Biochim Biophys Acta*. 2013;1832:2414-2424

432. Bruneau BG, Piazza LA, de Bold AJ. Bnp gene expression is specifically modulated by stretch and et-1 in a new model of isolated rat atria. *Am J Physiol*. 1997;273:H2678-2686

433. Rosenkranz AC, Hood SG, Woods RL, Dusting GJ, Ritchie RH. B-type natriuretic peptide prevents acute hypertrophic responses in the diabetic rat heart: Importance of cyclic gmp. *Diabetes*. 2003;52:2389-2395

434. Kuwahara K. Role of nrsf/rest in the regulation of cardiac gene expression and function. *Circ J*. 2013;77:2682-2686

435. Gustafson TA, Bahl JJ, Markham BE, Roeske WR, Morkin E. Hormonal regulation of myosin heavy chain and alpha-actin gene expression in cultured fetal rat heart myocytes. *J Biol Chem*. 1987;262:13316-13322

436. Hui HP, Li XY, Liu XH, Sun S, Lu XC, Liu T, Yang W. [adeno-associated viral gene transfer of serca2a improves heart function in chronic congestive heart failure rats]. *Zhonghua Xin Xue Guan Bing Za Zhi*. 2006;34:357-362

437. Krenz M, Robbins J. Impact of beta-myosin heavy chain expression on cardiac function during stress. *J Am Coll Cardiol*. 2004;44:2390-2397

438. Ito H, Hirata Y, Hiroe M, Tsujino M, Adachi S, Takamoto T, Nitta M, Taniguchi K, Marumo F. Endothelin-1 induces hypertrophy with enhanced expression of muscle-specific genes in cultured neonatal rat cardiomyocytes. *Circ Res*. 1991;69:209-215

439. Ren R, Oakley RH, Cruz-Topete D, Cidlowski JA. Dual role for glucocorticoids in cardiomyocyte hypertrophy and apoptosis. *Endocrinology*. 2012;153:5346-5360

440. Mahadeva H, Brooks G, Lodwick D, Chong NW, Samani NJ. Ms1, a novel stress-responsive, muscle-specific gene that is up-regulated in the early stages of pressure overload-induced left ventricular hypertrophy. *FEBS Lett*. 2002;521:100-104

441. Arai A, Spencer JA, Olson EN. Stars, a striated muscle activator of rho signaling and serum response factor-dependent transcription. *Journal of Biological Chemistry*. 2002;277:24453-24459

442. Ounzain S, Kobayashi S, Peterson RE, He A, Motterle A, Samani NJ, Menick DR, Pu WT, Liang Q, Chong NW. Cardiac expression of ms1/stars, a novel gene involved in cardiac development and disease, is regulated by gata4. *Molecular and Cellular Biology*. 2012;32:1830-1843

443. Clerk A, Bogoyevitch MA, Anderson MB, Sugden PH. Differential activation of protein kinase c isoforms by endothelin-1 and phenylephrine and subsequent stimulation of p42 and p44 mitogen-activated protein kinases in ventricular myocytes cultured from neonatal rat hearts. *Journal of Biological Chemistry*. 1994;269:32848-32857

444. Jiang T, Pak E, Zhang H, Kline RP, Steinberg SF. Endothelin-dependent actions in cultured at-1 cardiac myocytes: The role of the ε isoform of protein kinase c. *Circulation research*. 1996;78:724-736

445. Manger B, Hardy KJ, Weiss A, Stobo JD. Differential effect of cyclosporin a on activation signaling in human t cell lines. *J Clin Invest*. 1986;77:1501-1506

446. Ping P, Zhang J, Qiu Y, Tang X-L, Manchikalapudi S, Cao X, Bolli R. Ischemic preconditioning induces selective translocation of protein kinase c isoforms ε and η in the heart of conscious rabbits without subcellular redistribution of total protein kinase c activity. *Circulation research*. 1997;81:404-414

447. Liu X, Chhipa RR, Nakano I, Dasgupta B. The ampk inhibitor compound c is a potent ampk-independent antiglioma agent. *Molecular Cancer Therapeutics*. 2014;13:596-605

448. Konorev EA, Tarpey MM, Joseph J, Baker JE, Kalyanaraman B. S-nitrosoglutathione improves functional recovery in the isolated rat heart after cardioplegic ischemic arrest-evidence for a cardioprotective effect of nitric oxide. *J Pharmacol Exp Ther*. 1995;274:200-206

449. Ignarro LJ, Napoli C, Loscalzo J. Nitric oxide donors and cardiovascular agents modulating the bioactivity of nitric oxide: An overview. *Circ Res*. 2002;90:21-28

450. Seccia TM, Belloni AS, Kreutz R, Paul M, Nussdorfer GG, Pessina AC, Rossi GP. Cardiac fibrosis occurs early and involves endothelin and at-1 receptors in hypertension due to endogenous angiotensin ii. *J Am Coll Cardiol*. 2003;41:666-673

451. Leask A. Potential therapeutic targets for cardiac fibrosis: Tgfβ, angiotensin, endothelin, ccn2, and pdgf, partners in fibroblast activation. *Circulation research*. 2010;106:1675-1680

452. Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillessen D, Gabbiani G. A monoclonal antibody against alpha-smooth muscle actin: A new probe for smooth muscle differentiation. *J Cell Biol*. 1986;103:2787-2796

453. Ehrlich HP, Allison GM, Leggett M. The myofibroblast, cadherin, alpha smooth muscle actin and the collagen effect. *Cell Biochem Funct*. 2006;24:63-70

454. Carlsson M, Andersson R, Bloch KM, Steding-Ehrenborg K, Mosen H, Stahlberg F, Ekmehag B, Arheden H. Cardiac output and cardiac index measured with cardiovascular magnetic resonance in healthy subjects, elite athletes and patients with congestive heart failure. *J Cardiovasc Magn Reson*. 2012;14:51

455. Mangano DT, Van Dyke DC, Ellis RJ. The effect of increasing preload on ventricular output and ejection in man. Limitations of the frank-starling mechanism. *Circulation*. 1980;62:535-541

456. Cikes M, Solomon SD. Beyond ejection fraction: An integrative approach for assessment of cardiac structure and function in heart failure. *Eur Heart J*. 2015

457. de Waha S, Eitel I, Desch S, Fuernau G, Lurz P, Stiermaier T, Blazek S, Schuler G, Thiele H. Prognosis after st-elevation myocardial infarction: A study on cardiac magnetic resonance imaging versus clinical routine. *Trials*. 2014;15:249

458. Otterstad JE. Measuring left ventricular volume and ejection fraction with the biplane simpson's method. *Heart*. 2002;88:559-560

459. Sakuma H, Fujita N, Foo TK, Caputo GR, Nelson SJ, Hartiala J, Shimakawa A, Higgins CB. Evaluation of left ventricular volume and mass with breath-hold cine mr imaging. *Radiology*. 1993;188:377-380

460. Longmore DB, Klipstein RH, Underwood SR, Firmin DN, Hounsfield GN, Watanabe M, Bland C, Fox K, Poole-Wilson PA, Rees RS, et al. Dimensional accuracy of magnetic resonance in studies of the heart. *Lancet*. 1985;1:1360-1362

461. Nijveldt R, Beek AM, Hirsch A, Stoel MG, Hofman MB, Umans VA, Algra PR, Twisk JW, van Rossum AC. Functional recovery after acute myocardial infarction: Comparison between angiography, electrocardiography, and cardiovascular magnetic resonance measures of microvascular injury. *J Am Coll Cardiol*. 2008;52:181-189

462. Wharton G, Steeds R, Allen J, Phillips H, Jones R, Kanagala P, Lloyd G, Masani N, Mathew T, Oxborough D, Rana B, Sandoval J, Wheeler R, O'Gallagher K, Sharma V. A minimum dataset for a standard adult transthoracic echocardiogram: A guideline protocol from the british society of echocardiography. *Echo Res Pract*. 2015;2:G9-G24

463. Joshi SB, Connelly KA, Jimenez-Juan L, Hansen M, Kirpalani A, Dorian P, Mangat I, Al-Hesayen A, Crean AM, Wright GA, Yan AT, Leong-Poi H. Potential clinical impact of cardiovascular magnetic resonance assessment of ejection fraction on eligibility for cardioverter defibrillator implantation. *J Cardiovasc Magn Reson*. 2012;14:69

464. Grothues F, Smith GC, Moon JC, Bellenger NG, Collins P, Klein HU, Pennell DJ. Comparison of interstudy reproducibility of cardiovascular magnetic resonance with two-dimensional echocardiography in normal subjects and in patients with heart failure or left ventricular hypertrophy. *Am J Cardiol*. 2002;90:29-34

465. Bellenger NG, Burgess MI, Ray SG, Lahiri A, Coats AJ, Cleland JG, Pennell DJ. Comparison of left ventricular ejection fraction and volumes in heart failure by echocardiography, radionuclide ventriculography and cardiovascular magnetic resonance; are they interchangeable? *Eur Heart J*. 2000;21:1387-1396

466. Goenka AH, Wang H, Flamm SD. Cardiac magnetic resonance imaging for the investigation of cardiovascular disorders. Part 2: Emerging applications. *Texas Heart Institute Journal*. 2014;41:135-143

467. Schulz-Menger J, Bluemke DA, Bremerich J, Flamm SD, Fogel MA, Friedrich MG, Kim RJ, von Knobelsdorff-Brenkenhoff F, Kramer CM, Pennell DJ, Plein S, Nagel E. Standardized image interpretation and post processing in cardiovascular magnetic resonance: Society for cardiovascular magnetic resonance (scmr) board of trustees task force on standardized post processing. *J Cardiovasc Magn Reson*. 2013;15:35

468. Nilsson JC, Groenning BA, Nielsen G, Fritz-Hansen T, Trawinski J, Hildebrandt PR, Jensen GB, Larsson HB, Sondergaard L. Left ventricular remodeling in the first year after acute myocardial infarction and the predictive value of n-terminal pro brain natriuretic peptide. *Am Heart J*. 2002;143:696-702

469. Halade GV, Jin Y-F, Lindsey ML. Matrix metalloproteinase (mmp)-9: A proximal biomarker for cardiac remodeling and a distal biomarker for inflammation. *Pharmacology & Therapeutics*. 2013;139:32-40

470. Sundström J, Evans JC, Benjamin EJ, Levy D, Larson MG, Sawyer DB, Siwik DA, Colucci WS, Sutherland P, Wilson PWF, Vasan RS. Relations of plasma matrix metalloproteinase-9 to clinical cardiovascular risk factors and echocardiographic left ventricular measures: The framingham heart study. *Circulation*. 2004;109:2850-2856

471. Green CP, Porter CB, Bresnahan DR, Spertus JA. Development and evaluation of the kansas city cardiomyopathy questionnaire: A new health status measure for heart failure. *Journal of the American College of Cardiology*. 2000;35:1245-1255

472. Spertus J, Peterson E, Conard MW, Heidenreich PA, Krumholz HM, Jones P, McCullough PA, Pina I, Tooley J, Weintraub WS, Rumsfeld JS. Monitoring clinical changes in patients with heart failure: A comparison of methods. *Am Heart J*. 2005;150:707-715

473. Hwang SH, Choi BW. Advanced cardiac mr imaging for myocardial characterization and quantification: T1 mapping. *Korean Circ J*. 2013;43:1-6

474. Jellis CL, Kwon DH. Myocardial t1 mapping: Modalities and clinical applications. *Cardiovascular Diagnosis and Therapy*. 2014;4:126-137

475. Messroghli DR, Radjenovic A, Kozerke S, Higgins DM, Sivananthan MU, Ridgway JP. Modified look-locker inversion recovery (molli) for high-resolution t1 mapping of the heart. *Magn Reson Med*. 2004;52:141-146

476. Baks T, van Geuns R-J, Biagini E, Wielopolski P, Mollet NR, Cademartiri F, Boersma E, van der Giessen WJ, Krestin GP, Duncker DJ, Serruys PW, de Feyter PJ. Recovery of left ventricular function after primary angioplasty for acute myocardial infarction. *European Heart Journal*. 2005;26:1070-1077

477. McIntosh LD, Sharma MK, Mulvihill D, Gupta S, Juehne A, George B, Khot SB, Kaushal A, Watson MA, Nagarajan R. Catissue suite to openspecimen: Developing an extensible, open source, web-based biobanking management system. *Journal of Biomedical Informatics*.57:456-464

478. Shah ASV, Newby DE, Mills NL. High-sensitivity troponin assays and the early rule-out of acute myocardial infarction. *Heart*. 2013

479. Mahajan VS, Jarolim P. How to interpret elevated cardiac troponin levels. *Circulation*. 2011;124:2350-2354

480. Hallen J. Troponin for the estimation of infarct size: What have we learned? *Cardiology*. 2012;121:204-212

481. White SK, Frohlich GM, Sado DM, Maestrini V, Fontana M, Treibel TA, Tehrani S, Flett AS, Meier P, Ariti C, Davies JR, Moon JC, Yellon DM, Hausenloy DJ. Remote ischemic conditioning reduces myocardial infarct size and edema in patients with st-segment elevation myocardial infarction. *JACC Cardiovasc Interv*. 2015;8:178-188

482. Crimi G, Pica S, Raineri C, Bramucci E, De Ferrari GM, Klersy C, Ferlini M, Marinoni B, Repetto A, Romeo M, Rosti V, Massa M, Raisaro A, Leonardi S, Rubartelli P, Oltrona Visconti L, Ferrario M. Remote ischemic post-conditioning of the lower limb during primary percutaneous coronary intervention safely reduces enzymatic infarct size in anterior myocardial infarction: A randomized controlled trial. *JACC: Cardiovascular Interventions*. 2013;6:1055-1063

483. Prunier F, Angoulvant D, Saint Etienne C, Vermes E, Gilard M, Piot C, Roubille F, Elbaz M, Ovize M, Biere L, Jeanneteau J, Delepine S, Benard T, Abi-Khalil W, Furber A. The ripost-mi study, assessing remote ischemic perconditioning alone or in combination with local ischemic postconditioning in st-segment elevation myocardial infarction. *Basic Res Cardiol*. 2014;109:400

484. Sadat U. Signaling pathways of cardioprotective ischemic preconditioning. *International Journal of Surgery*. 2009;7:490-498

485. Li CB, Li XX, Chen YG, Zhang C, Zhang MX, Zhao XQ, Hao MX, Hou XY, Gong ML, Zhao YX, Bu PL, Zhang Y. Effects and mechanisms of pparalpha activator fenofibrate on myocardial remodelling in hypertension. *J Cell Mol Med*. 2009;13:4444-4452

486. Muthuramu I, Lox M, Jacobs F, De Geest B. Permanent ligation of the left anterior descending coronary artery in mice: A model of post-myocardial infarction remodelling and heart failure. *J Vis Exp*. 2014

487. Del Monte F, Butler K, Boecker W, Gwathmey JK, Hajjar RJ. Novel technique of aortic banding followed by gene transfer during hypertrophy and heart failure. *Physiol Genomics*. 2002;9:49-56

488. Mudera V, Smith AS, Brady MA, Lewis MP. The effect of cell density on the maturation and contractile ability of muscle derived cells in a 3d tissue-engineered skeletal muscle model and determination of the cellular and mechanical stimuli required for the synthesis of a postural phenotype. *J Cell Physiol*. 2010;225:646-653

489. Vernon RB, Gooden MD. An improved method for the collagen gel contraction assay. *In Vitro Cellular & Developmental Biology - Animal*. 2002;38:97-101

490. Xiao J, Sheng X, Zhang X, Guo M, Ji X. Curcumin protects against myocardial infarction-induced cardiac fibrosis via sirt1 activation in vivo and in vitro. *Drug Des Devel Ther*. 2016;10:1267-1277

491. Norris RA, Borg TK, Butcher JT, Baudino TA, Banerjee I, Markwald RR. Neonatal and adult cardiovascular pathophysiological remodeling and repair. *Annals of the New York Academy of Sciences*. 2008;1123:30-40

492. Rienks M, Papageorgiou A-P, Frangogiannis NG, Heymans S. Myocardial extracellular matrix: An ever-changing and diverse entity. *Circulation research*. 2014;114:872-888

493. Leask A. Getting to the heart of the matter: New insights into cardiac fibrosis. *Circulation research*. 2015;116:1269-1276

494. Armstrong SC. Protein kinase activation and myocardial ischemia/reperfusion injury. *Cardiovascular research*. 2004;61:427-436

495. Prasad MR, Jones RM. Enhanced membrane protein kinase c activity in myocardial ischemia. *Basic Res Cardiol*. 1992;87:19-26

496. Yoshida K, Hirata T, Akita Y, Mizukami Y, Yamaguchi K, Sorimachi Y, Ishihara T, Kawashiama S. Translocation of protein kinase c-alpha, delta and epsilon isoforms in ischemic rat heart. *Biochim Biophys Acta*. 1996;1317:36-44

497. Albert CJ, Ford DA. Protein kinase c translocation and pkc-dependent protein phosphorylation during myocardial ischemia. *American Journal of Physiology - Heart and Circulatory Physiology*. 1999;276:H642-H650

498. Bogoyevitch MA, Ketterman AJ, Sugden PH. Cellular stresses differentially activate c-jun n-terminal protein kinases and extracellular signal-regulated protein kinases in cultured ventricular myocytes. *Journal of Biological Chemistry*. 1995;270:29710-29717

499. Sil P, Kandaswamy V, Sen S. Increased protein kinase c activity in myotrophin-induced myocyte growth. *Circulation research*. 1998;82:1173-1188

500. Takeishi Y, Ping P, Bolli R, Kirkpatrick DL, Hoit BD, Walsh RA. Transgenic overexpression of constitutively active protein kinase c epsilon causes concentric cardiac hypertrophy. *Circ Res*. 2000;86:1218-1223

501. Mochly-Rosen D, Wu G, Hahn H, Osinska H, Liron T, Lorenz JN, Yatani A, Robbins J, Dorn GW. Cardiotrophic effects of protein kinase c ε: Analysis by in vivo modulation of pkcε translocation. *Circulation research*. 2000;86:1173-1179

502. Wu G, Toyokawa T, Hahn H, Dorn GW. E protein kinase c in pathological myocardial hypertrophy: Analysis by combined transgenic expression of translocation modifiers and gαq. *Journal of Biological Chemistry*. 2000;275:29927-29930

503. Hamasaki Y, Shinohara O, Ishida H, Hayashi Y, Nakazawa H. Decreased protein kinase c-epsilon expression in hypertrophied cardiac ventricles induced by triiodothyronine treatment in the rat. *Life Sci*. 2000;67:1859-1868

504. Mochly-Rosen D, Khaner H, Lopez J. Identification of intracellular receptor proteins for activated protein kinase c. *Proc Natl Acad Sci U S A*. 1991;88:3997-4000

505. Robia SL, Ghanta J, Robu VG, Walker JW. Localization and kinetics of protein kinase c-epsilon anchoring in cardiac myocytes. *Biophysical Journal*. 2001;80:2140-2151

506. Johnson JA, Gray MO, Chen C-H, Mochly-Rosen D. A protein kinase c translocation inhibitor as an isozyme-selective antagonist of cardiac function. *Journal of Biological Chemistry*. 1996;271:24962-24966

507. Schechtman D, Craske ML, Kheifets V, Meyer T, Schechtman J, Mochly-Rosen D. A critical intramolecular interaction for protein kinase cepsilon translocation. *J Biol Chem*. 2004;279:15831-15840

508. Blanco K. Mechanisms of mitochondrial regulation and ischemic neuroprotection by the pkcε pathway. *Open Access Dissertations. Paper 1224.* 2014;1224

509. Morris-Blanco KC, Cohan CH, Neumann JT, Sick TJ, Perez-Pinzon MA. Protein kinase c epsilon regulates mitochondrial pools of nampt and nad following resveratrol

and ischemic preconditioning in the rat cortex. *J Cereb Blood Flow Metab*. 2014;34:1024-1032

510. Hardie DG. The amp-activated protein kinase pathway--new players upstream and downstream. *J Cell Sci*. 2004;117:5479-5487

511. Baron SJ, Li J, Russell RR, 3rd, Neumann D, Miller EJ, Tuerk R, Wallimann T, Hurley RL, Witters LA, Young LH. Dual mechanisms regulating ampk kinase action in the ischemic heart. *Circ Res*. 2005;96:337-345

512. Tian R, Musi N, D'Agostino J, Hirshman MF, Goodyear LJ. Increased adenosine monophosphate-activated protein kinase activity in rat hearts with pressure-overload hypertrophy. *Circulation*. 2001;104:1664-1669

513. Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D, Hardie DG. Characterization of the amp-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates amp-activated protein kinase. *J Biol Chem*. 1996;271:27879-27887

514. Suter M, Riek U, Tuerk R, Schlattner U, Wallimann T, Neumann D. Dissecting the role of 5'-amp for allosteric stimulation, activation, and deactivation of amp-activated protein kinase. *J Biol Chem*. 2006;281:32207-32216

515. Surendra H, Diaz RJ, Harvey K, Tropak M, Callahan J, Hinek A, Hossain T, Redington A, Wilson GJ. Interaction of delta and kappa opioid receptors with adenosine a1 receptors mediates cardioprotection by remote ischemic preconditioning. *J Mol Cell Cardiol*. 2013;60:142-150

516. Li L, Zhang H, Li T, Zhang B. Involvement of adenosine monophosphate-activated protein kinase in morphine-induced cardioprotection. *J Surg Res*. 2011;169:179-187

517. Pang T, Rajapurohitam V, Cook MA, Karmazyn M. Differential ampk phosphorylation sites associated with phenylephrine vs. Antihypertrophic effects of

adenosine agonists in neonatal rat ventricular myocytes. *Am J Physiol Heart Circ Physiol*. 2010;298:H1382-1390

518. Sajan MP, Bandyopadhyay G, Miura A, Standaert ML, Nimal S, Longnus SL, Van Obberghen E, Hainault I, Foufelle F, Kahn R, Braun U, Leitges M, Farese RV. Aicar and metformin, but not exercise, increase muscle glucose transport through ampk-, erk-, and pdk1-dependent activation of atypical pkc. *Am J Physiol Endocrinol Metab*. 2010;298:E179-192

519. Chan AY, Soltys CL, Young ME, Proud CG, Dyck JR. Activation of amp-activated protein kinase inhibits protein synthesis associated with hypertrophy in the cardiac myocyte. *J Biol Chem*. 2004;279:32771-32779

520. Fu YN, Xiao H, Ma XW, Jiang SY, Xu M, Zhang YY. Metformin attenuates pressure overload-induced cardiac hypertrophy via ampk activation. *Acta Pharmacol Sin*. 2011;32:879-887

521. Proud CG. Ras, pi3-kinase and mtor signaling in cardiac hypertrophy. *Cardiovascular research*. 2004;63:403-413

522. Meng R, Pei Z, Zhang A, Zhou Y, Cai X, Chen B, Liu G, Mai W, Wei J, Dong Y. Ampk activation enhances pparalpha activity to inhibit cardiac hypertrophy via erk1/2 mapk signaling pathway. *Arch Biochem Biophys*. 2011;511:1-7

523. Sun D, Nguyen N, DeGrado TR, Schwaiger M, Brosius FC, 3rd. Ischemia induces translocation of the insulin-responsive glucose transporter glut4 to the plasma membrane of cardiac myocytes. *Circulation*. 1994;89:793-798

524. Xie Z, Dong Y, Scholz R, Neumann D, Zou MH. Phosphorylation of lkb1 at serine 428 by protein kinase c-zeta is required for metformin-enhanced activation of the amp-activated protein kinase in endothelial cells. *Circulation*. 2008;117:952-962

525. Nishino Y, Miura T, Miki T, Sakamoto J, Nakamura Y, Ikeda Y, Kobayashi H, Shimamoto K. Ischemic preconditioning activates ampk in a pkc-dependent manner and

induces glut4 up-regulation in the late phase of cardioprotection. *Cardiovascular research*. 2004;61:610-619

526. Heidrich F, Schotola H, Popov AF, Sohns C, Schuenemann J, Friedrich M, Coskun KO, von Lewinski D, Hinz J, Bauer M, Mokashi SA, Sossalla S, Schmitto JD. Ampk - activated protein kinase and its role in energy metabolism of the heart. *Current cardiology reviews*. 2010;6:337-342

527. Chen ZP, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR, Kemp BE. Amp-activated protein kinase phosphorylation of endothelial no synthase. *FEBS Lett*. 1999;443:285-289

528. Thaitirarot C, Crumbie H, Rodrigo G. 251 the role of nitric oxide and calcium regulation in cardioprotection from remote ischaemic preconditioning. *Heart*. 2013;99:A133

529. Halestrap AP, Clarke SJ, Khaliulin I. The role of mitochondria in protection of the heart by preconditioning. *Biochimica et Biophysica Acta*. 2007;1767:1007-1031

530. Taegtmeyer H, Sen S, Vela D. Return to the fetal gene program: A suggested metabolic link to gene expression in the heart. *Annals of the New York Academy of Sciences*. 2010;1188:191-198

531. Akazawa H, Komuro I. Roles of cardiac transcription factors in cardiac hypertrophy. *Circulation research*. 2003;92:1079-1088

532. Karakikes I, Chaanine AH, Kang S, Mukete BN, Jeong D, Zhang S, Hajjar RJ, Lebeche D. Therapeutic cardiac-targeted delivery of mir-1 reverses pressure overload-induced cardiac hypertrophy and attenuates pathological remodeling. *J Am Heart Assoc*. 2013;2:e000078

533. Care A, Catalucci D, Felicetti F, Bonci D, Addario A, Gallo P, Bang ML, Segnalini P, Gu Y, Dalton ND, Elia L, Latronico MV, Hoydal M, Autore C, Russo MA, Dorn GW, 2nd,

Ellingsen O, Ruiz-Lozano P, Peterson KL, Croce CM, Peschle C, Condorelli G. Microrna-133 controls cardiac hypertrophy. *Nat Med*. 2007;13:613-618

534. Kukreja RC, Yin C, Salloum FN. Microrna: New players in cardiac injury and protection. *Mol Pharmacol*. 2011;80:558-564

535. Yin C, Salloum FN, Kukreja RC. A novel role of microrna in late preconditioning: Upregulation of endothelial nitric oxide synthase and heat shock protein 70. *Circ Res*. 2009;104:572-575

536. Brandenburger T, Grievink H, Heinen N, Barthel F, Huhn R, Stachuletz F, Kohns M, Pannen B, Bauer I. Effects of remote ischemic preconditioning and myocardial ischemia on microrna-1 expression in the rat heart in vivo. *Shock*. 2014;42:234-238

537. He B, Xiao J, Ren AJ, Zhang YF, Zhang H, Chen M, Xie B, Gao XG, Wang YW. Role of mir-1 and mir-133a in myocardial ischemic postconditioning. *J Biomed Sci*. 2011;18:22

538. Sayed D, Hong C, Chen IY, Lypowy J, Abdellatif M. Microrna play an essential role in the development of cardiac hypertrophy. *Circ Res*. 2007;100:416-424

539. Yuhas Y, Berent E, Ashkenazi S. Effect of nitric oxide on microrna-155 expression in human hepatic epithelial cells. *Inflamm Res*. 2014;63:591-596

540. Hur W, Lee JH, Kim SW, Kim JH, Bae SH, Kim M, Hwang D, Kim YS, Park T, Um SJ, Song BJ, Yoon SK. Downregulation of microrna-451 in non-alcoholic steatohepatitis inhibits fatty acid-induced proinflammatory cytokine production through the ampk/akt pathway. *Int J Biochem Cell Biol*. 2015;64:265-276

541. Abete P, Testa G, Cacciatore F, Della-Morte D, Galizia G, Langellotto A, Rengo F. Ischemic preconditioning in the younger and aged heart. *Aging and Disease*. 2011;2:138-148

542. Ataka K, Chen D, Levitsky S, Jimenez E, Feinberg H. Effect of aging on intracellular ca2+, phi, and contractility during ischemia and reperfusion. *Circulation*. 1992;86:II371-376

543. Abete P, Cioppa A, Ferrara P, Caccese P, Ferrara N, Rengo F. Reduced aerobic metabolic efficiency in postischemic myocardium dysfunction in rats: Role of aging. *Gerontology*. 1995;41:187-194

544. Abete P, Cioppa A, Calabrese C, Pascucci I, Cacciatore F, Napoli C, Carnovale V, Ferrara N, Rengo F. Ischemic threshold and myocardial stunning in the aging heart. *Exp Gerontol*. 1999;34:875-884

545. Edroos SA. Myocardial ischaemia - reperfusion injury and its reduction by remote ischaemic preconditioning in health and diabetes mellitus *Cardiovascular Sciences*. 2014;PhD:259

546. Wider J, Przyklenk K. Ischemic conditioning: The challenge of protecting the diabetic heart. *Cardiovascular Diagnosis and Therapy*. 2014;4:383-396

547. Yamashita N, Hoshida S, Otsu K, Asahi M, Kuzuya T, Hori M. Exercise provides direct biphasic cardioprotection via manganese superoxide dismutase activation. *The Journal of Experimental Medicine*. 1999;189:1699-1706

548. Domenech R, Macho P, Schwarze H, Sánchez G. Exercise induces early and late myocardial preconditioning in dogs. *Cardiovascular research*. 2002;55:561-566

549. Abete P, Ferrara N, Cacciatore F, Sagnelli E, Manzi M, Carnovale V, Calabrese C, de Santis D, Testa G, Longobardi G, Napoli C, Rengo F. High level of physical activity preserves the cardioprotective effect of preinfarction angina in elderly patients. *Journal of the American College of Cardiology*. 2001;38:1357-1365

550. Abete P, Calabrese C, Ferrara N, Cioppa A, Pisanelli P, Cacciatore F, Longobardi G, Napoli C, Rengo F. Exercise training restores ischemic preconditioning in the aging heart. *J Am Coll Cardiol*. 2000;36:643-650

551. Michelsen MM, Stottrup NB, Schmidt MR, Lofgren B, Jensen RV, Tropak M, St-Michel EJ, Redington AN, Botker HE. Exercise-induced cardioprotection is mediated by a bloodborne, transferable factor. *Basic Res Cardiol*. 2012;107:260

552. Quindry JC, Hamilton KL. Exercise and cardiac preconditioning against ischemia reperfusion injury. *Current cardiology reviews*. 2013;9:220-229

553. Rock-Willoughby J, Boardley D, Badenhop D, Tinkel J. Vigorous exercise mimics remote ischemic preconditioning and provides benefit in cardiac rehabilitation patients. *J Clin Exp Cardiolog*. 2013;4

554. Silva JA, Santana ET, Manchini MT, Antônio EL, Bocalini DS, Krieger JE, Tucci PJF, Serra AJ. Exercise training can prevent cardiac hypertrophy induced by sympathetic hyperactivity with modulation of kallikrein-kinin pathway and angiogenesis. *PLoS ONE*. 2014;9:e91017

555. Serra AJ, Santos MH, Bocalini DS, Antonio EL, Levy RF, Santos AA, Higuchi ML, Silva JA, Magalhaes FC, Barauna VG, Krieger JE, Tucci PJ. Exercise training inhibits inflammatory cytokines and more than prevents myocardial dysfunction in rats with sustained beta-adrenergic hyperactivity. *J Physiol*. 2010;588:2431-2442

556. Solskov L, Magnusson NE, Kristiansen SB, Jessen N, Nielsen TT, Schmitz O, Botker HE, Lund S. Microarray expression analysis in delayed cardioprotection: The effect of exercise, aicar, or metformin and the possible role of amp-activated protein kinase (ampk). *Mol Cell Biochem*. 2012;360:353-362

557. Bogaty P, Kingma Jr JG, Robitaille NM, Plante S, Simard S, Charbonneau L, Dumesnil JG. Attenuation of myocardial ischemia with repeated exercise in subjects with chronic stable angina: Relation to myocardial contractility, intensity of exercise and the adenosine triphosphate–sensitive potassium channel. *Journal of the American College of Cardiology*. 1998;32:1665-1671

558. Lanza IR, Wigmore DM, Befroy DE, Kent-Braun JA. In vivo atp production during free-flow and ischaemic muscle contractions in humans. *The Journal of Physiology*. 2006;577:353-367

559. Gayda M, Ribeiro PA, Juneau M, Nigam A. Comparison of different forms of exercise training in patients with cardiac disease: Where does high-intensity interval training fit? *Can J Cardiol*. 2016;32:485-494

560. Polidori MC, Mecocci P, Cherubini A, Senin U. Physical activity and oxidative stress during aging. *Int J Sports Med*. 2000;21:154-157

561. Sun Y, Weber KT. Animal models of cardiac fibrosis. In: Varga J, Brenner DA, Phan SH, eds. *Fibrosis research: Methods and protocols*. Totowa, NJ: Humana Press; 2005:273-290.

562. Gay-Jordi G, Guash E, Benito B, Brugada J, Nattel S, Mont L, Serrano-Mollar A. Losartan prevents heart fibrosis induced by long-term intensive exercise in an animal model. *PLoS ONE*. 2013;8:e55427

563. Madias JE, Koulouridis I. Effect of repeat twice daily sessions of remote ischemic conditioning over the course of one week on blood pressure of a normotensive/prehypertensive subject. *Int J Cardiol*. 2014;176:1076-1077

564. Epps J, Dieberg G, Smart NA. Repeat remote ischaemic pre-conditioning for improved cardiovascular function in humans: A systematic review. *IJC Heart & Vasculature*. 2016;11:55-58

565. Edroos SA, Vanezis AP, Davies MJ, Samani NJ, Rodrigo GC. 108 remote ischaemic conditioning is impaired in diabetes. *Heart*. 2012;98:A61

566. Lejay A, Fang F, John R, Van JAD, Barr M, Thaveau F, Chakfe N, Geny B, Scholey JW. Ischemia reperfusion injury, ischemic conditioning and diabetes mellitus. *Journal of molecular and cellular cardiology*. 2016;91:11-22

567. Rahmi Garcia RM, Rezende PC, Hueb W. Impact of hypoglycemic agents on myocardial ischemic preconditioning. *World Journal of Diabetes*. 2014;5:258-266

568. Czobor P, Skolnick P. The secrets of a successful clinical trial: Compliance, compliance, and compliance. *Molecular Interventions*. 2011;11:107-110

569. Starlinger P, Gruenberger T. Role of platelets in systemic tissue protection after remote ischemic preconditioning. *Hepatology*. 2014;60:1136-1138

570. Shan LY, Li JZ, Zu LY, Niu CG, Ferro A, Zhang YD, Zheng LM, Ji Y. Platelet-derived microparticles are implicated in remote ischemia conditioning in a rat model of cerebral infarction. *CNS Neurosci Ther*. 2013;19:917-925

571. Skyschally A, Gent S, Amanakis G, Schulte C, Kleinbongard P, Heusch G. Across-species transfer of protection by remote ischemic preconditioning with species-specific myocardial signal transduction by reperfusion injury salvage kinase and survival activating factor enhancement pathways. *Circ Res*. 2015;117:279-288

572. Vanezis A, Rodrigo G, Squire I, Samani N. Remote ischaemic conditioning and remodelling following myocardial infarction: Current evidence and future perspectives. *Heart Fail Rev*. 2016

573. Vanezis A, Samani N. Ric: Beyond traditional acute protection. *Hospital Healthcare Europe Bulletin* 2014