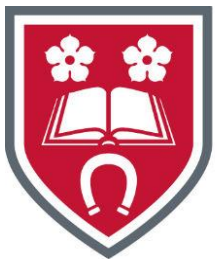

UNIVERSITY OF LEICESTER

Effects of Urotensin II on Excitation-Contraction Coupling in Hypertrophic Heart Failure

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled "Effects of Urotensin II on Excitation-Contraction Coupling in Hypertrophic Heart Failure" is based on work conducted by the author at the University of Leicester mainly during the period between July 2014 and June 2018.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other university.

Signed: Date:

Effects of Urotensin II on Excitation-Contraction Coupling in Hypertrophic Heart Failure

Hadeel Al Ali

Urotensin II (UII) is a potent vasoconstrictor endogenous hormone that binds to cognate G protein coupled receptors (GPCR) UT receptor. It has multiple actions on the cardiovascular system, and both the hormone and its receptor are upregulated in heart failure and may be linked to adverse remodeling. There is a marked elevation in circulating UII levels in patients suffering from heart failure, and this may lead to perturbations of Ca^{2+} homeostasis and adversely affect excitation-contraction coupling (E-C coupling), further contributing to the pathogenesis of heart failure.

The aims of this thesis were to determine the cellular effects of UII on hypertrophy and E-C coupling in ventricular cardiomyocytes and the cellular mechanism responsible, adopting in-vitro, ex-vivo and in-vivo animal models.

UII caused a significant reduction in left ventricular developed pressure in ex-vivo rat hearts and this is also observed in isolated rat ventricular cardiomyocytes, where the reduction in contraction strength is accompanied by a reduction in systolic $[Ca^{2+}]_i$. The reduction in systolic $[Ca^{2+}]_i$, appears to result from a combination of a reduction in L-type Ca^{2+}-current density and the SR Ca^{2+}-content. Moreover, the reduction in systolic $[Ca^{2+}]_i$ was reflected by a reduction in action potential duration ($APD_{30/50}$).

This study also showed that chronic exposure of isolated rat ventricular cardiomyocytes in culture to UII, induced hypertrophy after 24 hours. The study also showed this involved the UT receptor resulting in the activation of extracellular-regulated kinases (ERK1/2), p38 mitogen-activated protein kinases and calmodulin-dependent protein kinase II (CaMKII) signalling pathways and which were shown to be involved in the induction of hypertrophy. The UT receptor was not upregulated in-vitro study, neither in a mouse model of angiotensin II induced left ventricular hypertrophy (LV-hypertrophy) and LV-dysfunction. However, there was upregulation in atria tissue.

Increase in circulating UII may contribute to the development of LV-hypertrophy. The most obvious finding to emerge from this study is that the reduction in contraction will compound any LV-dysfunction in heart failure (HF). Pharmacological inhibition of the UII/UT system may prove beneficial in reducing adverse remodelling and alleviating contractile-dysfunction following heart disease.

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I would like to thank my supervisory Dr. Glenn Rodrigo for all the support, guidance, patience, encouragement, knowledge, and concern to achieve a desirable work that helped me to finish this study. I really appreciate the fact that his door was always open for any problem and that he would drop whatever he was doing when I need to talk to him in hard times. Working under his supervision has been very enjoyable and I feel fortunate to have given the opportunity to learn from his expertise as a scientist and human. I would like to thank my co-supervisor Professor David Lambert for all the guidance, advice and assistance in his laboratory. His guidance helped me in all the time of research and writing of this thesis.

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Conference abstracts

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Investigation role of Urotensin II on electrical activity of ventricular myocytes

Hadeel Al Ali, Dr. Glenn C Rodrigo, Professor David G Lambert

Frontiers in Cardiovascular Biology 2018, Vienna, Austria, 20th– 22nd April 2018

The effect of Urotensin II on calcium regulation during excitation-Contraction coupling

Hadeel Al Ali, Dr. Glenn C Rodrigo, Professor David G Lambert

Joint British Society for Cardiovascular Research and British Atherosclerosis Society Spring meeting 2017, Manchester, UK, 5th– 6th June 2017

Urotensin II induces cardiomyocyte hypertrophy via activation of the MAPK and CaMKII signalling pathways

Hadeel Al Ali, Dr. Glenn C Rodrigo, Professor David G Lambert

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AAA	Abdominal aortic aneurysm
AC	Adenylyl cyclase
ACE	Angiotensin converting enzyme
ACTβ	β-actin
ADP	Adenosine diphosphate
AdUT-IIR	Adenovirus expressing the urotensin receptor
Ag/AgCl	Silver/silver chloride
AKAP	A-kinase anchoring protein
Ang II	Angiotensin II
ANOVA	Analysis of variance
AP	Action potential
APD	Action potential duration
AT	Angiotensin receptors
AT_1	Angiotensin II receptor type 1
AT_2	Angiotensin II receptor type 2
AT_3	Angiotensin II receptor type 3
AT_4	Angiotensin II receptor type 4
ATP	Adenosine triphosphate
β2M	β-2-microglobulin
β-ADR	β-adrenergic receptors
βARK1	β-adrenergic receptors kinase 1
BDM	2,3-butanedione monoxime
BMK	Big map kinase
BSA	Bovine serum albumin
$[Ca^{2+}]_i$	Intracellular calcium ion
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CAD	Coronary artery disease
CaMK	Calcium/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
CAP	Cell attached patch

cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
CHF	Congestive heart failure
CHO_{hUT}	Chinese Hamster ovary cells expression human UT
CICR	Calcium induced calcium release
Cl^-	Chloride ion
Cs	Caesium
CSK	C-terminal Src kinase
C_t	Cycle threshold
ΔC_t	Delta cycle threshold
$\Delta\Delta C_t$	Delta delta cycle threshold
$CuSO_4$	Copper sulfate
Cys^5	Cysteine 5
DAD	Delayed after-depolarisation
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
E	Epinephrine
E-C coupling	Excitation-contraction coupling
ECM	Extracellular matrix
EF	Ejection fraction
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
E_{NCX}	Reversal potential of the sodium/calcium exchanger
EPAC	Guanine nucleotide exchange protein
ERK	Extracellular-regulated kinase
ET_A	Endothelin receptor type A
ET_B	Endothelin receptor type B
ET-1	Endothelin-1
F	Fluorescence
F0	Diastolic fluorescence between pulses
FAK	Focal adhesion kinase
FBS	Foetal bovine serum

FGF	Fibroblast growth factor
Fluo-3, AM	Fluo-3, AM acetoxymethyl ester
FRET	Fluorescence Resonance Energy Transfer
FS	Fractional shortening
Fura-2, AM	Fura-2, AM acetoxymethyl ester
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	Genomic DNA
G_i	Inhibitory GTP binding protein
GIO	Gene of interest
GPCR	G protein coupled receptor
G_q	Heterotrimeric G protein subunit
G_s	Stimulatory GTP binding protein
GSK-3	Glycogen synthase kinase 3
GUSβ	β-glucuronidase
HDAC	Histone deacetylases
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid)
HF	Heart failure
HPRT	Hypoxanthine guanine phosphoribosyl transferase
hUII	Human urotensin II
hUII prepro-mRNA	Human urotensin II prepro-mRNA
I_{Ca}	Calcium current
IC50	Half maximal inhibitory concentration
IGF	Insulin-like growth factor
I_{K1}	Inward rectifier K^+ current
I_{Kr}	Delayed outward rectifier K+ current, rapid
I_{Ks}	Delayed outward rectifier K+ current, slow
I_{Kur}	Delayed outward rectifier K+ current, ultra-rapid
I_{Na}	Inward sodium current
I_{NCX}	Reversible sodium/calcium current
InsP3R	Inositol triphosphate receptor
IOP	Inside-out patch
IP_3	Inositol triphosphate
ISO	Isoproterenol

I_{to}	Transient outward K^+ current
JAK/STAT	Janus kinase/signal transducers and activators of transcription
JNK	c-Jun N-terminal kinases
K^+	Potassium ion
KCl	Potassium chloride
KO	Knock out
LTCC	L-type calcium channel
LV-hypertrophy	Left ventricular hypertrophy
LVDP	Left ventricular developed pressure
LVEDV	Left ventricle end-diastolic volume
LVEF	Left ventricular ejection fraction
LVESV	Left ventricle end-systolic volume
MAPK	Mitogen-activated protein kinase
MCU	Mitochondrial uniporter
MEF2	Myocyte enhancer factor-2
$MgCl_2$	Magnesium chloride
MIQE	Minimum information qPCR experiments guidelines
miRNA	microRNA
MLC	Myosin light chain
MLCP	Myosin light chain phosphatase
mRNA	Messenger RNA
Na^+	Sodium ion
NaCl	Sodium chloride
NaHCO3	Sodium bicarbonate
NaH_2PO_4	Sodium phosphate monobasic
Na^+ K^+ tartrate	Potassium sodium tartrate tetrahydrate
NaOH	Sodium hydroxide
Na-pyruvate	Sodium pyruvate
NCX	Sodium/calcium exchanger
NE	Norepinephrine
NFAT	Nuclear factor of activated T cell
NMDG	N-methyl-D-glucamine
NO	Nitric oxide

NT	Normal Tyrode
OOP	Outside-out patch
p38	p38 mitogen-activated protein kinases
PAs	Phosphatidic acids
PDE	Cyclic nucleotide phosphodiesterase
PGI_2	Prostacyclin
PHE	Phenylephrine
PIP2	Phosphatidylinositol 4,5-biphospate
PKA	Protein kinase A
PKC	Protein kinase C
PLB	Phospholamban
PLC	Phospholipase C
PLCβ	Phospholipase C β
PMCA	Sarcolemmal Ca^{2+}-ATPase pump
POP4	Processing of precursor 4
PPIA	Peptidylprolyl isomerase A
qPCR	quantitative polymerase chain reaction
RG	Reference gene
RMP	Resting membrane potential
RNA	Ribonucleic acid
ROCK	Rho-associated kinase
RQ	Relative quantity
RT-PCR	Reverse transcription polymerase chain reaction
rUII	Rat UII
RyR2	Ryanodine receptor channel (Cardiac isoform)
%S	Percentage cell shortening
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SENR	Sensory epithelial neuropeptide-like receptor
Ser	Serine
SERCA2a	Sarcoplasmic/endoplasmic reticulum Ca^{2+}/ATPase (Cardiac isoform)

SR	Sarcoplasmic reticulum
6-TAMRA	6-carboxy-terta-methyl-rhodamine dye
TBS-T	Tris-buffered saline with tween 20
TC	Tissue culture
TEMED	Tetramethylethylenediamine
TGFβ	Transforming growth factor β
Thr	Threonine
TM	Tropomyosin
TN-C	Troponin C
TN-I	Troponin I
TN-T	Troponin T
TTCC	T-type calcium channel
Tyr	Tyrosine
UII	Urotensin II
UT	Urotensin receptor
WC	Whole cell
YWHAZ	5-monooxygenase activation protein zeta

Chapter 1: Introduction

1.1 Heart failure

Heart failure (HF) is a clinical syndrome, which results because the heart is unable to pump sufficient blood to maintain the tissues of the body (Sosin 2006). Heart failure can occur in either the right, left side or both sides of the heart. In right-sided HF, the pumping force of right ventricle is diminished and the output of the right ventricle is decreased, reducing the ability of lungs to oxygenate the blood. Right-side heart failure may be associated with breathlessness and is often associated with oedema in the lower limbs and ascites (Jackson 1993; Casey 2013). Left-sided heart failure occurs when the efficiency of the left ventricle fails resulting in inadequate pumping the blood into the systemic circulation, so that the body receives less oxygenated blood than is required. Left-sided heart failure is often associated with pulmonary congestion and it characterized by pressure elevation inside jugular vein as well as fatigue (Korczyk *et al.* 2012). People with left-side heart failure usually go on to develop right-side heart failure (Casey 2013). Patients with heart failure face difficulties for achievement of physical activities and lack of ability to do exercise as a result of decreased cardiac output (Clark *et al.* 1996).

1.1.1 Epidemiologic studies of heart failure

Heart failure represents a significant public health issue in the world. It affects a large section of the population worldwide. Over 23 million adults in the world are living with heart failure. There are several studies which estimate the prevalence of heart failure in different countries. In the western world, heart failure prevalence is 1% to 2% (Mendez and Cowie 2001). Around 5.8 million people have heart failure in the USA. According to the National Health and Nutrition Examination Survey the prevalence of heart failure disease in 2006 was 2.6% of population of USA (Lloyd-Jones *et al.* 2010). According to British Heart Foundation there are nearly 900,000 people living with heart failure in the UK and 60, 000 people have tendency to develop this condition per annum (Mehta *et al.* 2009).

In the Middle East, there is a lack of any comprehensive studies into the prevalence of this disease but there are a few scattered studies. For example, in Saudi Arabia the rate of mortality is 7.5% for 1090 cases at thirty days after release from the hospital (AlHabib *et al.* 2011). Another study in Oman state shows the prevalence of heart failure is 5.17 per 1000 persons (Agarwal *et al.* 2001).

Heart failure incidence rises with age and males have a greater tendency to present with this syndrome than females. According to data from the Framingham study, the incidence of heart failure in males is 5.64 per 1000 individual per annum while in females about 3.27 per 1000 population per annum (Levy *et al.* 2002). Similarly, based on Olmsted cohort study it was found that the incidence of disease in men was 3.78 per 1000 people and in women was 2.89 per 1000 population per year (Roger *et al.* 2004). In the USA, Bui and colleagues point out the incidence of heart failure is 2-5/1000 population per year (Bui *et al.* 2011). The rate of heart failure disease increases with progress in age. Data from the Framingham study show that the incidence of heart failure increases to 10 cases per 1000 individuals at ages ≥65 and this supported by Lloyd-Jones *et al*., which showed people in the fourth decade of age have tendency to develop heart failure, is about one from five (Lloyd-Jones *et al.* 2002).

In developed countries, such as European countries it was found that HF hospitalizations increase more than 1 million cases (Gheorghiade *et al.* 2005; Nieminen *et al.* 2005). Similarly, in the USA the hospitalizations rate >1 million patients with initial diagnosis of heart failure (Korczyk *et al.* 2012).

1.1.2 Impact of heart failure

Heart failure has a negative effect on life quality and is a major cause of mortality. The survival rate for heart failure has been estimated and compared with other diseases such as cancer and stroke. It has been found that the survival rate in patients with heart failure for five years was 26-52% while for cancer and stroke were 43% and 40-68%, respectively (Askoxylakis *et al.* 2010). Heart failure mortality is significantly increased with the duration of the disease, according to data obtained from Framingham study, one month fatality and one year fatality was 10% and 20-30%, respectively but this increased to 45-60% after five years. compare with (Levy *et al.* 2002). The annual cost to the economy of the USA relating to HF is rising, it reaches to $37 billion in year because

increase in prevalence associated with advance in age in spite development of medical technology and availability of medical care (Lloyd-Jones *et al.* 2009). The overall cost of hospital care for heart failure patients has been estimated by British Heart Foundation, it reached to £905 million in 2000. In USA and European countries such as Netherlands and Sweden, the rate of admission to hospital and old age wards was multiple. However, more recently this increase has plateaued and has become stable. The admission rate reaches to 5% in British hospitals (Davis *et al.* 2007). The rate of heart failure rehospitalisation is around 50% within 3-6 months from the first hospital discharge (Korczyk *et al.* 2012). Patient rehospitalisation has been reduced as a result of development in the diagnosis of disease and remedy (Al-Shamiri 2013). Use of angiotensin converting enzyme (ACE) inhibitors as a treatment for heart failure patients which can lead to reduced total cost of therapy (Davis *et al.* 2007). However, more targeted treatment strategies are still required, to both prevent and alleviate developed HF.

1.1.3 Types of heart failure

1.1.3.1 Systolic heart failure

Systolic heart failure results from an inability of the heart to contract during systole (*Systolic Dysfunction*) and therefore to pump blood into the large vessels in turn to all parts of the body during ventricular contraction (systole). Systolic heart failure is preceded by ventricular hypertrophy of the individual myocytes, the cardiac myocytes become elongated and mass of left ventricle is increased as a result of hypertrophy (Rubin and Reisner 2014). If ejection fraction of the left ventricle is less than 40%, it is a significant feature for diagnosis of HF (Korczyk *et al.* 2012). Systolic heart failure can be attributable to a reduction in cardiac contractility, in part due to the myofilaments becoming desensitised for Ca^{2+} or altered intracellular signal transduction mechanism which regulates excitation-contraction coupling (E-C coupling) in cardiac myocytes. Excitation-contraction coupling is impaired in systolic heart failure due to abnormality in intracellular Ca^{2+} haemostasis (Gwathmey *et al.* 1993). Another cause of systolic heart failure is myocardial fibrosis which either results from activation and proliferation of bone marrow circulating cells which convert to myofibroblasts and fibroblasts after entering the heart, or hyperactivity and rapidly proliferation of fibroblasts to induce fibrosis (Fan *et al.* 2012).

There are several structural changes in the extracellular matrix of cardiomyocytes associated with heart failure. The cardiac extracellular matrix is mainly composed of elastic fibers, collagen, glycoprotein, proteoglycans and laminin which represent the structural backbone of myocardium (Eghbali 1992; Berk *et al.* 2007). One of the main functions of extracellular matrix (ECM) is providing structural network for the heart in addition to transferring the extracellular signals to the myocardial cells (Weber *et al.* 1994). Excess accumulation of ventricular collagen has been linked with systolic dysfunction (Díez *et al.* 2002). Thus, in case of systolic dysfunction, the collagen fibres become degraded and disorganised in cardiomyocytes (Aurigemma *et al.* 2006). Abnormality in the metabolism of collagen might be implicated because elevated level of procollagen in the plasma have been observed (Rossi *et al.* 2004). Production of collagen degradation are collagen types I and III which consider as a hallmark of myocardial collagen degradation (Zannad *et al.* 2010).

1.1.3.2 Diastolic heart failure

Diastolic heart failure results from impairment of heart muscle to relax during diastole (*Diastolic Dysfunction*)**,** which adversely affects filling the ventricle with adequate amount of blood because stiffness the left ventricle wall, as a result decrease cardiac output (Jackson 1993). Abnormalities structure of ventricle could lead to diastolic heart failure which impedes flow of blood. It is likely to be these abnormalities either intramyocadial like diffuse myocardial fibrosis or extramyocardial like constrictive pericarditis (Gwathmey *et al.* 1993). In diastolic heart failure, there are essential alterations in the components of the extracellular matrix. Myocardial fibrosis is characterized by an increase in collagen types I and III which constitute about 90% of the total myocardium collagen (Weber *et al.* 1994). The main causes of diastolic heart failure are increased blood pressure and valvular heart disease (Mandinov *et al.* 2000).

Abnormality of intracellular Ca^{2+} handling in patients with heart failure can leads to reduced contractile function of ventricles (systolic dysfunction) as well as diastolic dysfunction (Morgan *et al.* 1990).

1.1.4 Causes of heart failure

Heart failure is a serious pathological state which tends to affect elderly people and it is more common in males than females. Many causes can develop heart failure indifferent ways. Usually, there are 5 common reasons of heart failure including, hypertension, coronary artery disease, heart valve diseases, cardiomyopathies and arrhythmias. There are additional causes leading to heart failure such as congenital heart disease, myocarditis, severe lung disease, alcohol, thyroid disease and severe anaemia (Korczyk *et al.* 2012).

1.1.4.1 Hypertension

Development of heart failure can be associated with chronic increase in systolic and diastolic blood pressure. Several studies have shown that arterial hypertension is a major cause of heart failure. With a sustained increase in blood pressure, the heart muscle is required to work harder to overcome the increased arterial pressure and maintain adequate flow of the blood to the systemic circulation. Over time, the cardiac muscle becomes stiffer or weaker and undergoes hypertrophy. It has been proposed that excess accumulation of myocardial collagen can lead to stiffness of myocardium during hypertrophy stage. Fibrosis is considered the main factor which is responsible for myocardium stiffness in hypertensive patients (Weber 1997). Serum concentration of procollagen type I C-terminal propeptide reflects synthesis of collagen type I which is correlated with myocardial fibrosis (Querejeta *et al.* 2000; López *et al.* 2001).

Pulmonary hypertension is a serious clinical condition. It is characterised by increased blood pressure inside the pulmonary arteries which supply the lungs (Kovacs *et al.* 2009). It leads to damage of the right ventricle resulting in the development of heart failure because weakness of the heart and decreases its ability to pump sufficient amount of blood to the lungs (Timmis and Davies 1992; Lam *et al.* 2009). In case of elevation of pulmonary arterial pressure, there are substantial alterations at the vascular level such as migration of elastic fibers and reticular fibrils, thickness of basement membrane as well as enlargement of endothelium of pulmonary vessels. These alterations in the composition of vascular tissue influences pulmonary vascular resistance and may cause elevation in and reduced pulmonary vascular permeability furthermore leading to right sided ventricular heart failure (Huang *et al.* 2001).

Levels of circulating endothelin-1 (ET-1) has been increased in patients with pulmonary hypertension (Stewart *et al.* 1991), endothelin-1 has been identified as an inducer of heart hypertrophy and development of heart failure. This finding shows a strong association between pulmonary hypertension and heart failure (Cody *et al.* 1992). It has been found that ET system is upregulated in hypertensive patients (Schiffrin 2001). Stimulation of endothelin receptor by ET-1 can induce contraction of vascular smooth muscle in addition to its proliferative and inflammatory effects on the vascular wall, these detrimental effects of ET-1 via activation of mitogen-activated protein kinase (MAPK) (Schiffrin 2005). Release of epinephrine (E) or norepinephrine (NE) from the adrenal gland can lead to raise of blood pressure and generate paroxysmal hypertension (Ito *et al.* 1992), paroxysmal hypertension is a dramatic episodes and volatiles of blood pressure rising (Mann 2015).

Findings from both animal experiments and clinical studies, have conclusively shown that activation of the renin-angiotensin system induces myocardial growth because high levels of angiotensin II (Ang II) in the plasma increases of blood pressure and this later leads to cardiac hypertrophy (Baker *et al.* 1992). Administration of angiotensin II to rats for a fortnight resulted in elevation of blood pressure as well as hypertrophy of vascular smooth muscle cells (Lombardi *et al.* 1999).

Although the correlation between UII and essential hypertension is poorly studied in human beings, limited published studies addressed the topic. A significant association between increased levels of circulating UII and hypertension have been observed in hypertensive patients in comparison to normotensive controls (Cheung *et al.* 2004; Peng *et al.* 2013). However, in another study with limited sample size, there was no association between level of UII in the circulating or cerebrospinal fluid and essential hypertension in hypertensive patients receiving long-term therapy for hypertension compared to normotensive controls, a positive association between mean arterial pressure and cerebrospinal fluid level of UII was demonstrated (Thompson *et al.* 2003).

1.1.4.2 Coronary artery disease (CAD)

Ischemic heart injury resulting from coronary artery disease (CAD), is the most common cause of heart failure. Coronary artery disease results from the accumulation of plaque inside the wall of these arteries. Over many years, plaque either becomes hardened or

ruptures. Hardened atherosclerosis plaque resulting in stenosis of the lumen of coronary arteries and reduces blood supply to the myocardium (Korczyk *et al.* 2012). Rupture of plaque causing embolism of coronary arteries due to formation of thrombosis, can result in complete or partial occlusion leading to development of myocardial infarction (Falk *et al.* 1995; Davies 2000; Korczyk *et al.* 2012).

Levels of circulating ET-1 increase in patients with coronary artery disease which may contribute to induced hypertrophy and heart failure (Chai *et al.* 2010). High levels of circulating catecholamines can produce cardiac dysfunction as a result to overload of intracellular Ca^{2+} in cardiac myocytes. The catecholamines are oxidized under stressful conditions resulting in formation of oxidative catecholamines. Cardiac dysfunction, coronary artery spasm and arrhythmias are produced due to the effects of these oxidation products, by altering of intracellular Ca^{2+} haemostasis in both sarcoplasmic reticulum (SR) and sarcolemmal (Adameova *et al.* 2009).

1.1.4.3 Heart valve diseases

Heart valves play an important role through controlling the blood flow that leaves the heart. Valve faults include: failure of these valves to close completely causing leakage of blood to the heart or narrowing of these valves lead to back-up of blood. Defects in the valves may result from infection of the heart like rheumatic fever or congenital (birth defect). Valve diseases (mitral regurgitation, aortic regurgitation and aortic stenosis) remain significant factors for development and progression of heart failure because of increased workload on the heart (Sutherland 2010). Stenosis of aorta can lead to left ventricular pressure overload (increase preload) while aortic and mitral regurgitation can result in volume overload (increase afterload) (Davis *et al.* 2007).

1.1.4.4 Cardiomyopathies

The term cardiomyopathy is defined as a disease of heart muscle and can be either genetic or acquired. Cardiomyopathies can be classified into three categories based on phenotype including: hypertrophic cardiomyopathy, dilated cardiomyopathy and restrictive cardiomyopathy (Korczyk *et al.* 2012), restrictive cardiomyopathy is a rare disorder of the heart muscle which is characterized by diastolic dysfunction and restrictive ventricular filling pattern, while systolic function is predominantly normal (Muchtar *et*

al. 2017). Hypertrophic cardiomyopathy involves thickness of the left ventricles and dysfunction in the contraction process, it may be this is related to a hereditary disorder. The pumping force of cardiomyocytes decreases as a result of power loss (Davis *et al.* 2007; Korczyk *et al.* 2012). Dilated cardiomyopathy has been linked with genetic factors, however, the main cause is unknown. In this condition, the cardiac muscle may become enlarged and the muscle becomes thin and the pumping action of myocardium decreases (Davis *et al.* 2007). The heart becomes weak and cannot pump adequate amounts of the blood and heart failure develops (Korczyk *et al.* 2012).

1.1.4.5 Arrhythmias

Arrhythmia may result in heart failure and it has a prominent role in acute and chronic heart failure. In this condition, the process of filling and pumping functions alter. In addition, the cardiac output decreases when the rhythmicity of the heart is slow, resulting in heart failure (Davis *et al.* 2007). For example, stroke volume decreases in response to atrial arrhythmias because reduction of functional capacity.

1.2 Excitation-contraction coupling in cardiac muscle

Excitation-contraction coupling refers to the cellular events whereby the cardiac action potential (AP) triggers a cardiomyocyte to produce a contraction, resulting from a transient rise in intracellular free calcium, subsequently followed by relaxation of the cardiomyocyte and return to the initial state of length and load (Bers 2002).

1.2.1 The Ventricular Cardiac Action Potential

An action potential is the starting point of E-C coupling and is responsible for initiating contraction of the heart, which is generated by the movement of ions (potassium ion (K^+), sodium ion (Na^+) and calcium ion (Ca^{2+})) through specific ion channels.

The ventricular action potential shown in Figure 1.1, is well described by five different phases numbered from 0 to 4. Phase 0 refers to rapid depolarization, phase 1 refers to partial repolarization, phase 2 is explained by plateau, phase 3 clearly explained the rapid, final repolarization and finally phase 4 refers to the final and maximum potential of repolarisation which known as resting membrane potential (RMP). Each phase will be discussed in more detail below.

1.2.1.1 Phase 4: Resting membrane potential

This phase refers to the resting membrane potential with the cardiomyocyte is at rest, in a period known as cardiac diastole. Differences in concentrations of Na^+, K^+, chloride ion (Cl^-) and Ca^{2+} cardiomyocytes membrane lead to generation of RMP. The RMP is generated principally by the movement of K^+, Na^+ and Cl^- through ion channels that regulate the membrane permeability of the cell to these ions. The resting potential of ventricular cardiomyocyte is between -70 and -80 (Bers 2001). The RMP can be calculated using the Goldman-Hodgkin-Katz equation (equation 1) (Hille 2001).

$$V_m = \frac{RT}{F} log_{10} pK \frac{[K^+]_O}{[K^+]_i} + pNa \frac{[Na^+]_o}{[Na^+]_i} + pCl \frac{[Cl^-]_i}{[Cl^-]_o} \text{ ..... (1)}$$

R = Gas constant

T = Absolute temperature

F = Faradays constant (9.65 x 104 mol-1)

pNa = Relative membrane permeability for sodium

pK = Relative membrane permeability for potassium

pCl = Relative membrane permeability for chloride

V_m = Membrane potential

$[K^+]_o$ = Extracellular potassium concentration

$[K^+]_i$ = Intracellular potassium concentration

$[Na^+]_o$ = Extracellular sodium concentration

$[Na^+]_i$ = Intracellular sodium concentration

$[Cl^-]_o$ = Extracellular chloride concentration

$[Cl^-]_i$ = Intracellular chloride concentration

1.2.1.2 Phase 0: The rapid upstroke of the cardiac action potential

Fast Na^+ channels (Nav1.5) open when membrane potential is more positive (-55mV) (Nerbonne and Kass 2005). This lead to increase the conductance which increases the Na^+ permeability (Levick 2013). These changes lead to upstroke the action potential of the heart and an overshoot as the membrane potential is driven towards the Na^+ equilibrium potential (E_{Na}) of +70mV. However, as the Na^+ channels rapidly inactivate and remain open for ~1 millisecond (Grant 2009) the overshoot reaches +70mV (Nerbonne and Kass 2005). The end of Phase 0 starts with the inactivation of Na^+ channels and peaks of membrane potential between +30mV and +50mV (Bers 2001). In

this time the main current is K^+ and here the next phase begins when closure of Na^+ channel gates (Bers 2001).

1.2.1.3 Phase 1: The early repolarisation phase

Inactivation of Na^+ channels is followed by activation of a K^+ (I_{to}) outward current, which can lead to incomplete and rapid repolarisation. I_{to} rapidly activates at potential -30mV and consists of two types of currents; I_{to},fast (the active channels are Kv4.2 and Kv4.3) and I_{to},slow (the active channels are Kv1.4) (Nerbonne and Kass 2005; Grant 2009). A Ca^{2+} activated Cl^- current (I_{to2}) could be a part of the current in addition to K^+ current (I_{to1}) (Grant 2009).

I_{to} in rat is strong (Varró *et al.* 1993) as Kv4.2 channels is heavily expressed in a high number (Schram *et al.* 2002), which results in a plateau phase that is relatively more negative than other species (Nerbonne and Kass 2005). I_{to} current and Kv4.2/Kv4.3 channels are reduced in left ventricular hypertrophy (LV- hypertrophy) and heart failure which associated with prolongation of action potential duration (APD) and increased arrhythmic risk (Kääb *et al.* 1998; Näbauer and Kääb 1998).

1.2.1.4 Phase 2: The plateau phase

During the plateau phase the cardiac action potential is prolonged and the membrane potential changes little compared with skeletal muscle cells (Grant 2009). Repolarisation is delayed, resulting in prolongation of the action potential duration (APD) which helps in preventing the re-excitation of cardiac muscle before the complete relaxation without happening of tetanisation (Bers 2001).

The early plateau is initiated when Ca^{2+} ions influx through voltage-gated L-type Ca^{2+}-channels (LTCC/Cav1.2 channels) down the electrochemical gradient leading to the generation of a Ca^{2+} current. The LTCC are rapidly activated and opened by depolarisation of the membrane at potentials positive to -40mV, and are slowly inactivated resulting in a long lasting of LTCC current (Grant 2009). Ca^{2+}-influx through LTCC during the plateau phase is the main key element that triggers further release of Ca^{2+} from SR via ryanodine receptor channels (RyR2) in a process termed Ca^{2+}-induced Ca^{2+}-release (CICR) (Bers 2002). Ca^{2+} also influx during the action potential can occur through "*reverse-mode*" Na^+/Ca^{2+} exchanger (NCX), however Ca^{2+} entry through LTCC

is the major source of Ca^{2+}-influx. NCX is thought to play a role during action potential, contributing to Ca^{2+}-influx early in the AP because the reversal potential of the exchanger (E_{NCX}) value is more negative than the upstroke of the AP. On the contrary, NCX extrudes accumulated Ca^{2+} during the second and third phases of action potential (Bers 2008). During systole, Ca^{2+} enters early through NCX in the early stage of AP (Bers 2001).

Outward delayed rectifier K^+ currents are responsible for balancing this inward I_{Ca}, and is due to rapid (I_{Kr}) and slow (I_{Ks}) delayed rectifier K^+ channels (Bers 2001), which are activated slowly at a membrane potential of ~-30mV during the plateau phase.

1.2.1.5 Phase 3: The late/rapid repolarisation phase

This phase is characterised by the repolarisation of the action potential and results from the closing of LTCC as they inactive and an increase in K^+ conductance resulting in repolarisation of the action potential from the plateau to RMP. The inward I_{Ca} decreases during this phase due to LTCC inactivation resulting in the decline in Ca^{2+} movement. The membrane potential is gradually driven towards K^+ equilibrium potential (E_K) because the conductance of the K^+ ion channels increase (Bers 2001). During this phase, the delayed rectifier K^+ current remains active because inactivation of the I_{Kr} and I_{Ks} channels is slow (Grant 2009), the membrane potential decrease towards resting levels leading to the gradual contribution of the inward rectifier current (I_{K1}), which does not contribute very much current at potentials positive to -20mV, because of blockage the channels by intracellular Mg^+ (Schmitt *et al.* 2014).

Phase 3 may also be enhanced by activity of the NCX (Sher *et al.* 2008). From above, two factors can effect on I_{NCX} direction and amplitude, membrane potential and the transmembrane Na^+ and Ca^{2+} concentration gradients. The "*forward-mode*" NCX can be stimulated by the high levels of $[Ca^{2+}]_i$ during systole, leading to efflux of cytosolic Ca^{2+}, which result in an increase in outward I_{NCX} during this phase (Bers 2001).

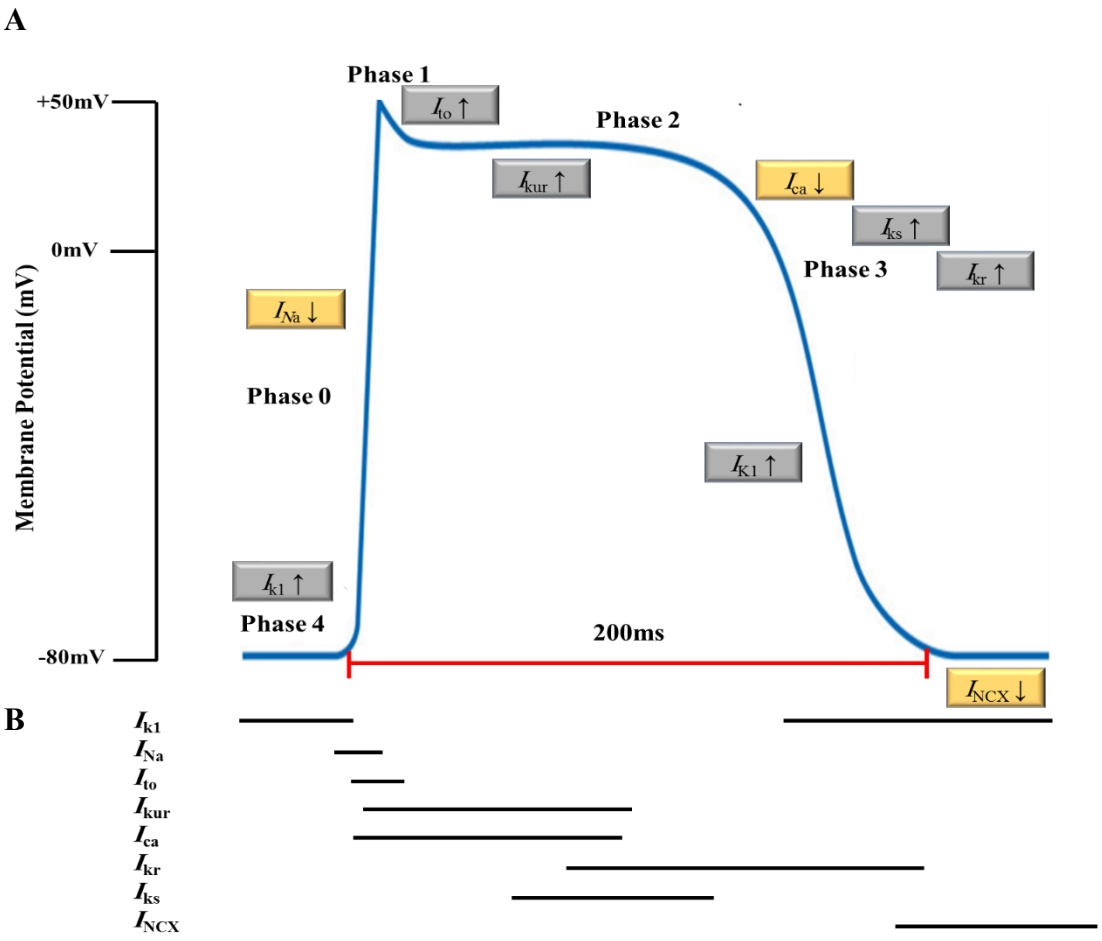


Figure 1.1: The ventricular cardiac action potential.
(A) A schematic of the ventricular action potential, the action potential has 5 separate phases. These phases are governed by movements of ions across the cardiac membrane.
(B) The figure shows the essential ionic currents contributing to the cardiac ventricular action potential. This figure was adapted from (Grant 2009).

1.2.2 Cardiac contraction

In the cardiac myocyte, the Ca^{2+}-transient that results from the action potential and which gives rise to contraction, results from a close interplay of Ca^{2+}-influx from outside the cell and the release of Ca^{2+} from intracellular organelles to increase Ca^{2+} followed by removal of this Ca^{2+} resulting in relaxation. Influx of Ca^{2+} into the cell occurs through voltage gated Ca^{2+}-channels and the NCX (Figure 1.2).

Myocardial contraction is initiated principally by interaction between contractile proteins and Ca^{2+}. As the intracellular concentration of Ca^{2+} increases inside the cardiac myocyte, it binds to troponin C (TN-C) and initiates the contraction process. Troponin I (TN-I) is one of a major regulatory proteins which modifies contraction process (England 1976). Conformational changes occur in the troponin I as a result binding of cytosolic Ca^{2+} to troponin C causing uncover of binding site of myosin on actin filament. At rest, troponin T (TN-T)/tropomyosin (TM) complex prevents interaction of myosin with actin. When the cardiac muscle is stimulated, Ca^{2+} binds to troponin C which results in a conformational change in tropomyosin protein, which allows myosin filament to interact with actin and form cross-bridges. Before starting the process of contraction, myosin heads bind to adenosine triphosphate (ATP) molecules, ATP is hydrolysed to ADP for supplying the contraction process by energy. Bending of myosin heads pulls actin filament over myosin filament resulting in shortening of the sarcomere and contraction is initiated (Bers 2001).

There are two types of voltage-dependent calcium channels in cardiac cells, through which Ca^{2+}-influx can occur to initiate the contractile cycle, the first channel is L-type Ca^{2+}-channel (LTCC) and the second is T-type Ca^{2+}-channel (TTCC), The LTCC is involved in the activation of contractile activity in cardiac cells (Bers 2008). During depolarization of cardiac myocyte, LTCC open in response to depolarisation and Ca^{2+}-influx occurs through these channels down their electrochemical gradient resulting in the generation of an inward Ca^{2+} current, which is responsible for maintaining the plateau of the action potential (Bers 2012) and causes a larger release of Ca^{2+} from the SR via ryanodine receptor channels, through a mechanism of Ca^{2+}-induced Ca^{2+}-release (Fabiato 1985).

In mammalian, the sodium/calcium exchanger (NCX) family is composed of three isoforms: NCX1 is expressed in the heart, in addition to the brain and kidney, NCX2 and NXC3 (Wanichawan *et al.* 2014). The NCX is a carrier for Ca^{2+} in cardiac tissues, which couples the movement of Na^{+} and Ca^{2+} in the opposing directions, with a coupling ratio of 3 Na^{+}: 1 Ca^{2+} (Kimura *et al.* 1986; Hinata *et al.* 2002). As a result the NCX is electrogenic and the direction of movement of Ca^{2+} and Na^{+} depends on the chemical gradient of the driving force of these ions as well as the membrane potential (electrical gradient) (Blaustein and Lederer 1999). The essential mechanism of Ca^{2+}-efflux in cardiac myocytes is NCX (Reeves and Hale 1984). During diastole NCX is responsible for regulating a low intracellular Ca^{2+} levels by removing the increase in Ca^{2+} during systole (Bers and Bridge 1989). Elevation of Ca^{2+} concentration leads to efflux of Ca^{2+} (resulting in an inward I_{NCX}), this activity of NCX is called *"forward-mode",* while high Na^{+} concentration and positive membrane potential favour entry of Ca^{2+} (resulting in an outward I_{NCX}) and is called *"reverse-mode"* (Peskoff and Langer 1998; Zahradnikova *et al.* 1999). It is thought that the NCX acting in reverse-mode can contribute to Ca^{2+}-influx during the early phase of the action potential (Bers 2001).

Sarcoplasmic reticulum is the primary store for intracellular Ca^{2+}. Calcium liberation from SR occurs through ryanodine receptor Ca^{2+}-release channels (RyR2), and the magnitude of release depends on the Ca^{2+}-content of SR as well as RyR2 activity (Laver 2007). SR content of calcium is based on the balance between calcium release via RyR2 and Ca^{2+} uptake which mediated by sarcoplasmic reticulum calcium ATPase pump (SERCA2a) (Diaz *et al.* 2005).

Ryanodine receptors or SR Ca^{2+}-release channels are located on the membrane of SR and the main function of RyR2 is release Ca^{2+} from the SR to increase cytosolic Ca^{2+} in response to a Ca^{2+}-signal from the LTCC and NCX that can be used for contraction of cardiac myocytes (Wagenknecht and Radermacher 1997; Williams *et al.* 2001). Calcium enters the myocyte in response to opening large number of LTCCs in the dyadic cleft of cardiomyocytes in response to the action potential, which in turn causes the ryanodine receptor channels to open. The concentration of local calcium at the RyR2 reaches 10-20μM. Couplon is a functional clusters of L-type Ca^{2+}-channels and ryanodine receptors in addition to junctional proteins of SR which has role during E-C coupling (Stern *et al.* 1997).

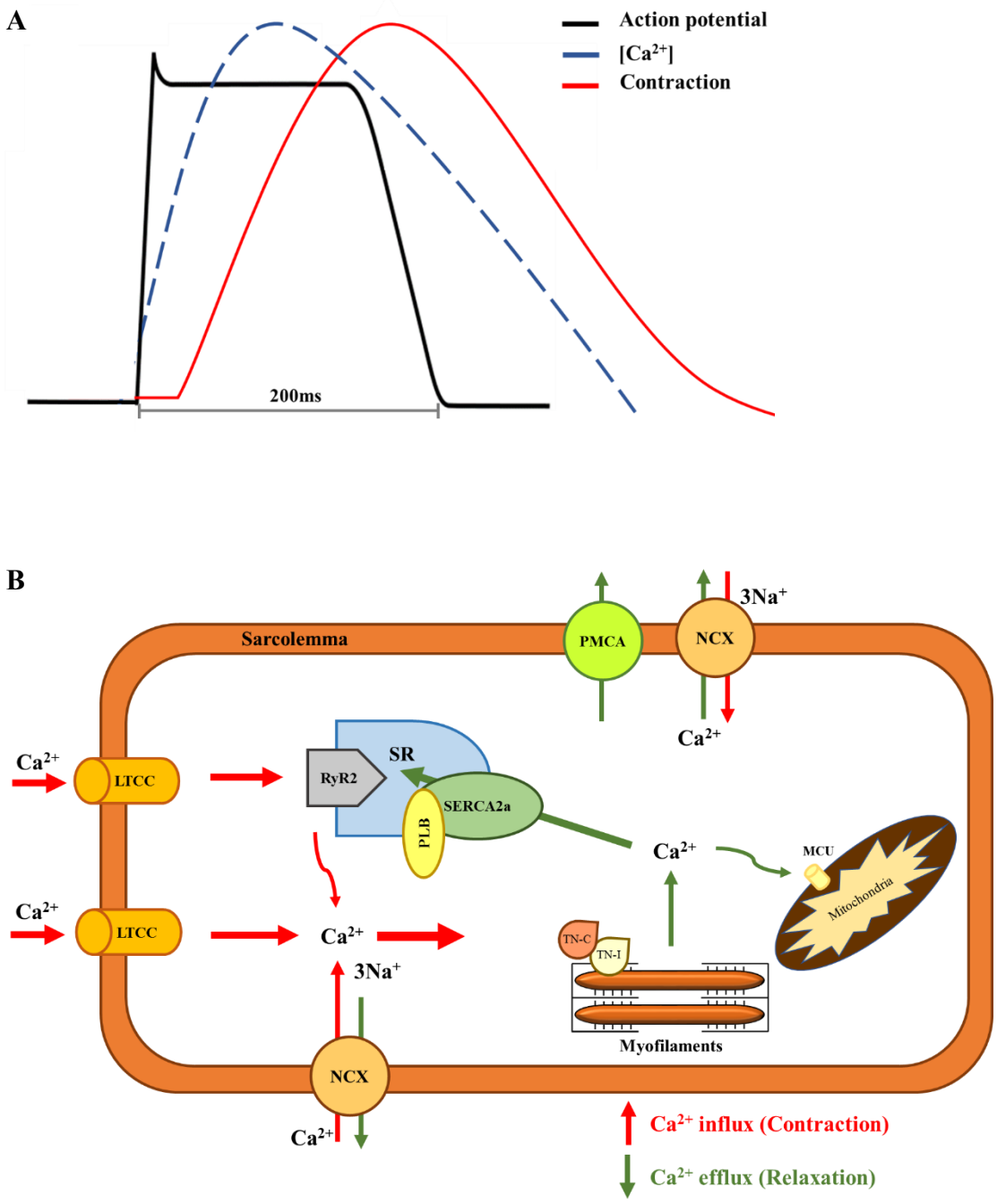


Figure 1.2: Cardiac excitation-contraction coupling mechanism.
(A) A schematic showing cardiac action potential, intracellular calcium $[Ca^{2+}]_i$ transient and relationship to the contractility of the heart.
(B) A schematic diagram showing of influx and efflux of Ca^{2+} in E-C coupling in a single ventricular cardiomyocyte. Phospholamban (PLB), Na^+/Ca^{2+} exchanger (NCX), sarcoplasmic reticulum (SR), ryanodine receptor (RyR2), troponin C (TN-C), troponin I (TN-I), L-type calcium channels (LTCC), sarcoplasmic reticulum calcium ATPase pump (SERCA2a), the sarcolemmal ATPase pump (PMCA) and the mitochondrial uniporter (MCU). This figure was adapted from (Bers 2002).

1.2.3 Cardiac relaxation

Following contraction, relaxation must occur to allow efficient refilling of ventricles during diastole. Relaxation of cardiomyocytes is initiated by a decrease in the level of intracellular Ca^{2+}, resulting in dissociation of Ca^{2+} from troponin C. Relaxation process depends on the inactivation of LTCC which in turn prevent further influx of Ca^{2+} into the cardiomyocyte and stop release of Ca^{2+} from sarcoplasmic reticulum (Sham *et al.* 1998; Bers 2002). Termination of release of Ca^{2+} from the SR is predominantly a result of ryanodine receptor (RyR2) closing due to a fall in SR luminal Ca^{2+} and its interaction with calsequestrin (Herzog *et al.* 2000). Ca^{2+} is transported out the cardiac myocyte via NCX, in addition, SR Ca^{2+} stores are replenished with Ca^{2+} through activation of SERCA2a, an energy dependent pump which pumps Ca^{2+} back to the lumen of the SR (Bassani *et al.* 1994).

Calcium is removed from the cytosol whereby different routes; SERCA2a, NCX, the mitochondrial Ca^{2+} uniporter (MCU) and sarcolemmal ATPase pump (PMCA), the quantity of Ca^{2+} that release from SR via RyR2 is balanced by SERCA2a (Bers 2002).To maintain a constant level of Ca^{2+} inside the cardiac myocyte, the amount of Ca^{2+} entry through the LTCC and NCX during systole should equal the amount of Ca^{2+} extrusion by NCX during the relaxation process and Ca^{2+} reuptake by SR should equal SR Ca^{2+}-release by RyR2. In this state, E-C coupling is maintained at steady state to avoid damage of the cardiac myocytes and the generation of arrhythmias.

SERCA2a exists in cardiac and skeletal muscle which is responsible for transport of cytosolic Ca^{2+} to SR by sequestering Ca^{2+} ions which pumps two molecules of Ca^{2+} ions to the SR lumen using one molecule ATP hydrolysis to power the transport process. Ca^{2+} enters the lumen of SR when a pore is created as a result of binding of Ca^{2+} to the transmembrane of SERCA2a causing addition of phosphate molecule to prevent Ca^{2+} return toward the opposite direction (Bers 2001). Phospholamban (PLB) is an endogenous inhibitor of SERCA2a when in its unphosphorylated state, leading to decrease the removal cytoplasmic Ca^{2+}. However, during the phosphorylation state of PLB, SERCA2a inhibition effect of PLB is reduced resulting in elevating the affinity of Ca^{2+} thus more felling down in $[Ca^{2+}]_i$ levels due to SERCA2a activation (Bers 2002; Bonow *et al.* 2011). Although, the contribution of Ca^{2+} removal by SERCA2a is species dependent, this mechanism is the main and fast Ca^{2+} removal system and extrusion in

mammals (Bers 2002; Bers 2008). The research show 90-95% of Ca^{2+} removal in cardiac tissues of rat is due to SERCA2a as it has greater density while 70% of the Ca^{2+} decline in humans is related to SERCA2a (Bers 2008).

In addition, removal of Ca^{2+} can be done by NCX, which results in efflux Ca^{2+} from the cardiomyocyte. This mechanism is responsible of extruding 5-8% of Ca^{2+} in rat but more in larger mammals and humans (Bers 2008). Calcium is immediately extruded after contraction process from the cytosol by NCX "*forward-mode*" due to repolarisation of membrane and increased levels of intracellular Ca^{2+} (Bers 2001). In most species, 98-99% of Ca^{2+} is removed from the cytosol as a result to a combination activity of both NCX and SERCA2a (Bers 2002; Bers 2008). Furthermore, 1-2% of cytosolic Ca^{2+} is removed by the slow systems such as MCU and PMCA.

1.2.4 Effect of β-adrenergic stimulation on contraction and relaxation process

The main function of sympathetic nervous system on the heart is to augment contractility of the cardiac muscle cell (positive inotropy) through an increase in $[Ca^{2+}]_i$ and heart rate (positive chronotropy) and expedite the relaxation process (positive lusitropy), through an increase in the rate of fall of Ca^{2+} (Katz 2010). This occurs by action of catecholamines on β-adrenergic receptors (β-ADR) via signalling cascade involving G protein coupled receptors (GPCR) and protein kinase A (PKA) (Bers 2002). Troponin I is phosphorylated in response to β-adrenergic stimulation (England 1976), which assists the relaxation process by rising cross-bridges cycling kinetics and reduces affinity of Ca^{2+} for cardiac TN-C (Katz 2010).

Many proteins that contribute to the E-C coupling process (such as RyR2, troponin I, LTCC and phospholamban) are phosphorylation targets for PKA, which is activated by cyclic adenosine monophosphate (cAMP). Binding of the neurotransmitter epinephrine or norepinephrine to β_1-ADR receptors, results in the activation of GTP-binding protein (G_s) which then stimulates adenylyl cyclase (AC) to form cAMP from ATP (Figure 1.3) (Bers 2002). There are several subtypes of β-adrenoceptors in myocardium (β_1, β_2 and β_3) which are belong to 7-transmembrane domain receptor family coupling with G-binding protein (Wallukat 2002). β_1 and β_2-ADR have the similar action in myocardium (positive inotropic and lusitropic impact) (Bers 2001).

Positive inotropic and lusitropic impacts occur through the classical β-ADR/cAMP/PKA pathway through stimulation of the β_1-ADR. The later consists of G_α, G_β and G_γ subunits therefore, it could consider as coupled stimulation for G_s. the β_1-ADR can be activated by binding of catecholamines which leads to adenylate cyclase (AC) activation. AC converts ATP into cAMP that then leads to the activation of PKA which in the ventricular myocyte, has a specific role in phosphorylating specific serine and threonine residues, including LTCC, PLB, RyR2 and TN-I (Bers 2001).

Activity of LTCC is enhanced by β-adrenergic receptors, which depend on two factors: phosphorylation of LTCC at sites Ser^{478} and Ser^{479} increases open probability and the channels remain open for a longer duration which promotes an increase in inward calcium current (Tsien *et al.* 1986; Bünemann *et al.* 1999). The resulting increase influx of Ca^{2+}, increases the Ca^{2+} concentration at the mouth of the RyR2 and enhances liberation of Ca^{2+} from RyR2 (Bers 2002). Consequently, increases the amplitude of the Ca^{2+}-transient which enhances contraction of cardiomyocyte (Tsien *et al.* 1986; Bers 2001).

Phospholamban is a key regulator for cardiomyocyte contraction. It is phosphorylated by PKA at Ser^{16} residue in response to rise in cAMP concentration during activation of cardiac β-adrenergic receptors. PLB has a negative action on the activity of SERCA2a, it decreases the activity of SERCA2a by interacting with SERCA2a and changing its affinity for Ca^{2+} (Simmerman and Jones 1998). Phosphorylation of PLB at Ser^{16}, leads to a conformational change that inhibits the interaction with SERCA2a, which relieves inhibition of SERCA2a thereby increase SERCA2a activity (Koss and Kranias 1996). This phosphorylation therefore increases pump activity of SERCA2a, in turn SERCA2a re-uptake of Ca^{2+} by the SR increases, which both accelerates the rate of relaxation and increase the competition for Ca^{2+} between SERCA2a and NCX (Bers 2001), with over time, increases the SR Ca^{2+}-content and hence subsequent Ca^{2+}-release (Katz 1990; Li *et al.* 2000). SR Ca^{2+} uptake is modified by PKA and calmodulin-dependent protein kinase II **(**CaMKII) through phosphorylation of the protein phospholamban, which inhibits the activity of SERCA2a under basal conditions (Bers 2008). Xiao and colleagues have demonstrated that phosphorylation of RyR2 by PKA occurs at two sites, Ser^{2808} and Ser^{2030}, it increases open probability of RyR2 and the duration of opening (Xiao *et al.* 2006). In addition, it changes the sensitivity of the RyR2 to Ca^{2+} (Marx *et al.* 2000; Bers 2001).

The total effect comes from all LTCC, RyR2 and PLB phosphorylation enhances the positive inotropic effect of β_1-ADR stimulation because the trigger of CICR can be increased with increasing the I_{Ca} and SR Ca^{2+}. The RyR2 can release greater Ca^{2+} due to the high level of RyR2 sensitivity to Ca^{2+} (Bers 2002).

Activation of β_3-adrenergic receptors result in negative inotropic impact on cells of the heart (Varghese *et al.* 2000). β_2 and β_3-ADR are coupled through G-protein (G_i) for production of cyclic guanosine monophosphate (cGMP). It is regulated by nitric oxide (NO) that is produced by endothelial nitric oxide synthesis (Bers 2001).

The changes of inotropism by the β_2-ADR stimulation appear to be dependent on the species. One clear example in humans, shows PKA phosphorylation of distant targets (PLB and RyR2) and an increase in activity during β_2-ADR (Kaumann and Molenaar 1997). However, only very small changes in PKA activity or phosphorylation of the distant target are observed in canine and mouse hearts (Altshuld *et al.* 1995; Sabri *et al.* 2000). While these differences tend to be moderate in rat as cAMP and phosphorylation of LTCC increase, without any changes in PKA-mediated phosphorylation of PLB or TN-I (Xiao 2001). These variations could be attributed to the activity of phosphodiesterase (PDE), PDE3 and PDE4 as PDE4 can mediate 10% of cAMP degradation in human and 60% in rat (Mika *et al.* 2012).

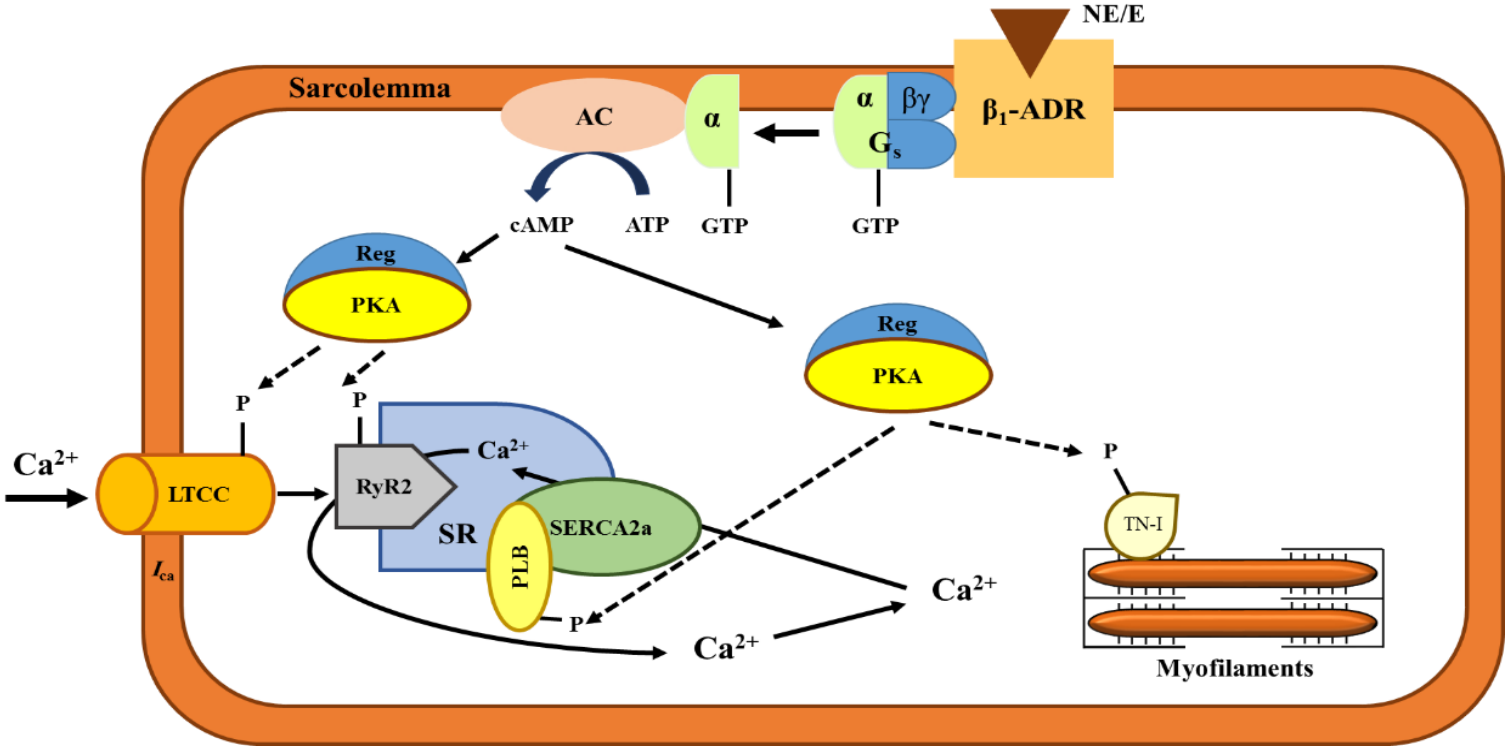


Figure 1.3: Effect of β-adrenergic stimulation on contraction and relaxation process in cardiac muscle.
Phosphorylation targets for protein Kinase A during β-ADR stimulation. Phospholamban (PLB), L-type calcium channel (LTCC) and ryanodine receptor (RyR2), sarcoplasmic reticulum calcium ATPase pump (SERCA2a), protein kinase A (PKA), adenylate cyclase (AC), cyclic adenosine monophosphate (cAMP), norepinephrine/epinephrine (NE/E), stimulatory GTP binding protein (G_s). This figure was adapted from (Bers 2002).

1.2.5 Role of CaMKII in regulation of heart Ca^{2+} homeostasis

Calcium/calmodulin-dependent protein kinase II (CaMKII) is one of multifunctional kinase family (CaMKs) which phosphorylates multiple protein targets that contain threonine or serine (Anderson 2005). Among the regulators of signal transduction of Ca^{2+}, the CaMKII appears to play pivotal role as well as other effectors of CaMKinases family such as CaMKI and CaMKIV (Braun and Schulman 1995). It is likely that CaMKII increases opening of L-type calcium channels, therefore influx of Ca^{2+} increases via these channels as well as mobilization of Ca^{2+} ion from sarcoplasmic reticulum via ryanodine receptor and by uptake of SR Ca^{2+} through SERCA2a (Anderson 2005). It can modulate many biological functions such as membrane excitability, regulation of gene expression in addition to Ca^{2+} handling (Lorca *et al.* 1993; Knott *et al.* 2006). The predominant isoform of CaMKII in the myocardium is CaMKIIδ (Hudmon and Schulman 2002). CaMKII is activated by numerous signalling pathways such as α and β-adrenergic stimulation and by an increase duration of action potential.

The hyperactivity of CaMKII is apparent in pathological and physiological events (De Koninck and Schulman 1998; Grueter 2006). CaMKII phosphorylates numerous substrates such as LTCC (the LTCC is phosphorylated by CaMKII when it binds with β_{2a} at site Thr^{498}) (Grueter 2006), RyR2 (Ser^{2814}) (Huke and Bers 2008) and PLB (Thr^{17}) (Sag *et al.* 2007) which are implicated in E-C coupling. It has been involved in distortion of normal E-C coupling, apoptosis and gene transcription (Grueter *et al.* 2007; McKinsey 2007). In-vitro study on the human cardiac tissue, it has been suggested that expression of CaMKII is upregulated in case of structurally diseases of heart (Hoch *et al.* 1999).

Previous studies have shown that alterations in CaMKII isoforms are associated with hypertension which induces hypertrophy and can result in heart failure (Hagemann *et al.* 2001; Yu *et al.* 2011). It is possible that these changes in CaMKII influence on intracellular Ca^{2+} handling which may cause abnormality in ventricular contractility (Hagemann *et al.* 2001).

1.2.6 Excitation-contraction coupling in heart failure

Urotensin II has been shown to impact on the cardiovascular system. It may result in a negative effect on E-C coupling and contributes to the etiology of the heart failure. Prejudicial changes in E-C coupling that reduce E-C coupling fidelity, contribute in the pathogenesis of heart failure. Accumulation of evidence from clinical studies on patients with heart failure shows that reduction of contractile function in heart failure is associated with changes in Ca^{2+}-transients or alterations in the mechanism of contraction (Gwathmey *et al.* 1987; Morgan *et al.* 1990; Houser *et al.* 2000). The main reason of dysfunction of the E-C coupling in failing heart is impairment in Ca^{2+}-handling by the SR Ca^{2+} during diastole due to upregulation of NCX and decrease the activity of the SERCA2a, which has effects of systolic function. There is also a change to NCX activity that compound changes to SERCA2a and RyR2 activity (Bers 2001). The outcome is that the cardiomyocytes are unable to release adequate Ca^{2+} for the next depolarization (Hasenfuss *et al.* 1994; Hasenfuss 1998; Lindner *et al.* 1998). Abnormalities occur on the cellular level in heart failure which influences Ca^{2+} handling, such as decreased Ca^{2+} transport through SERCA2a pump is linked with augment extrusion of Ca^{2+} by sarcolemmal NCX due to increase activity of this exchanger (Sossalla *et al.* 2010b) or leakage of SR Ca^{2+} as a result opening of RyR2 for long time (Maier *et al.* 2003; Ai *et al.* 2005) (Figure 1.4).

1.2.7 Alteration of β-adrenergic receptor signalling in heart failure

There have been several studies in the literature reporting that changes of β-adrenergic signalling pathway in heart failure (Bristow *et al.* 1982; Brodde 1993; Kiuchi *et al.* 1993). In normal mammalian cardiac tissues, β_1-receptor constitutes about 70-80% of the total β-adrenoceptor (Wallukat 2002). There is a reduction of β_1-receptor on the surface of cell membrane up to 50%. $G_{\alpha 1}$ levels are significant elevated up to 200% as well as increase efficacy of β-ADR kinase 1 (βADRK1) which consider the main cause of β-adrenergic desensitisation (Freedman and Lefkowitz 2004). All these changes are likely to contribute to the dysfunction of β-receptor signaling in heart failure. It is thought that the increase in circulating catecholamine in heart failure is the main cause of these changes in β-adrenergic signaling pathway, which results in impaired contractile function of the heart (Lohse *et al.* 2003). Long term activation of β-adrenergic receptor effects levels of G protein and adenylyl cyclase. Studies have found that G_i levels are upregulated in heart

failure in turn this change causing reduction of G_s:G_i ratio (Neumann *et al.* 1988; Ping and Hammond 1994). Moreover, adenylyl cyclase isoforms 5 and 6 expression at protein levels and messenger RNA (mRNA) are reduced (Ishikawa *et al.* 1994). Therefore, production of cAMP is reduced as a result of these alterations in signaling pathway. Successively, cAMP reduced activation of the second messenger protein kinase A (Brum *et al.* 2006).

Administration of isoproterenol to rats (for 7 days) resulted in stimulation of β-adrenergic receptors, causing myocardial hypertrophy linked with arrhythmias, fibrosis and development of heart failure (Szabo *et al.* 1975; Campos *et al.* 2006). Recently, it has been demonstrated that development of cardiac hypertrophy in response to isoproterenol treatment, involves an increase in cardiac protein expression, with levels of myosin light chain-2 and myosin light chain-3 reduced in rat treated with isoproterenol (Chowdhury *et al.* 2013), which is linked with myocardial hypertrophy (Wu *et al.* 2008).

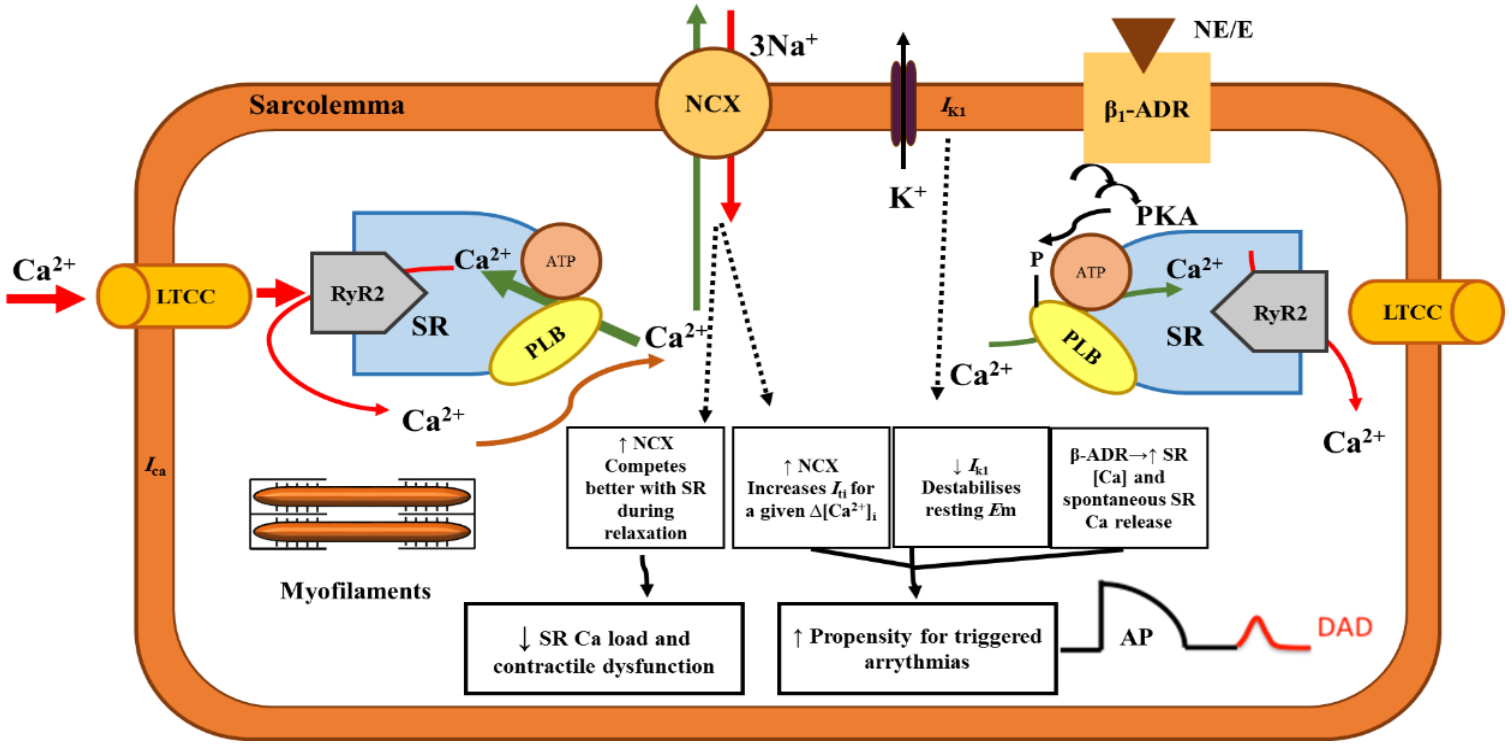


Figure 1.4: Schematic diagram shows change of excitation-contraction coupling in heart failure.
Phospholamban (PLB), Na^+/Ca^{2+} exchanger (NCX), sarcoplasmic reticulum (SR), ryanodine receptor (RyR2), L-type calcium channels (LTCC), protein kinase A (PKA), norepinephrine/epinephrine (NE/E), action potential (AP), delayed after-depolarisation (DAD). This figure was adapted from (Bers 2002).

1.3 Cardiac hypertrophy

Cardiac hypertrophy is a compensatory response in which the heart adapts to the changes such as genetic mutations or exposure to cardiac growth factors or sustained pressure overload on the wall of the heart (Sandler and Dodge 1963; Hood *et al.* 1968; Grossman *et al.* 1975). It often develops due to increased workload, while hypertrophy due to genetic mutations or growth factors is less common (Abel and Doenst 2011). Ischemic heart disease is one causes of pathological cardiac hypertrophy (Barry *et al.* 2008).

Cardiac hypertrophy is generally classified into two types: physiological cardiac hypertrophy and pathological hypertrophy (Abel and Doenst 2011). Physiological hypertrophy occurs in response to physiological stimuli; for example frequent exercise training. The morphology of the heart remains normal and the contractile function of the left ventricle is normal (McMullen and Jennings 2007). Cardiac pathological hypertrophy is commonly linked with development of myocardial dysfunction and increased cardiac fibrosis resulting in heart failure (Levy *et al.* 1990; Gunasinghe and Spinale 2004). Pathological hypertrophy is characterized by reduction in myosin ATPase effectiveness and weakness of left ventricle contractile function. However, physiological hypertrophy is associated with increment or regular activity of myosin ATPase enzyme (Wikman-Coffelt *et al.* 1979). Pathological cardiac hypertrophy is associated with dysregulation of Ca^{2+} cycling and abnormalities of electrical activity (Wickenden *et al.* 1998).

Pathological and physiological cardiac hypertrophy may be classified on the basis of alterations in the morphology of the heart into eccentric and concentric hypertrophy (Grossman *et al.* 1975; Pluim *et al.* 2000). Pathological hypertrophy can occur as a result of volume overload resulting from conditions such as arteriovenous fistulas or valve disease, which induces eccentric hypertrophy, the heart is characterized by thin wall and dilated chambers. Whereas pressure overload resulting from aortic stenosis or hypertension, which cause an increase the thickness of left ventricle leading to induced concentric hypertrophy, the morphology of heart is characterized by thickness of the wall of left ventricle and increment of left ventricle mass (Grossman *et al.* 1975).

1.3.1 Factors involved in left ventricular hypertrophy

Pathological cardiac hypertrophy occurs in response to many factors, such as mechanical stress or neuro-endocrine hormones (Sadoshima and Izumo 1997; Komuro 2001).

1.3.1.1 Mechanical stretch

Mechanical stretch is a central factor in development of left ventricular hypertrophy. Accumulating evidence has emerged that mechanical stretch is the main factor which causes left ventricular hypertrophy without accompanying of neurohormonal factors in response to hemodynamic load (Ruwhof and van der Laarse 2000). There are several changes occurring in the myocardial structure and function during development of hypertrophy in response to mechanical load, which include increased synthesis of cellular proteins and alterations in cardiac gene transcription (Komuro *et al.* 1990; Sadoshima *et al.* 1992; Kira *et al.* 1994). Previous research has shown that stretch of cardiomyocytes cultured in serum-free culture media results in an increase protein contents of cardiomyocytes because rise of protein synthesis rate as well as activation of immediate early genes and fetal genes induction (Komuro *et al.* 1990; Sadoshima *et al.* 1993). However, hormonal factors are released in response to mechanical stretch stimulation such as angiotensin II (Sadoshima *et al.* 1993), endothelin-1 (Yamazaki *et al.* 1996), in addition to other growth-promoting factors such as fibroblast growth factor (FGF), transforming growth factor β (TGFβ) (Villarreal and Dillmann 1992), and insulin-like growth factor (IGF)(Calderone *et al.* 1995; Neri Serneri *et al.* 1999).

Mechanical stretch induced hypertrophy generally is initiated by activation of several ionic channels in the cell membrane of heart such as Na^+ channels and L-type calcium channels as well as NCX (Sasaki *et al.* 1992; Sigurdson *et al.* 1992; Yamazaki *et al.* 1998a). Phospholipases C, D and A2 are activated in cardiac myocytes in response to mechanical stretch which lead to production of intracellular second messengers including arachidonic acid, inositol triphosphate (IP_3), diacylglycerol (DAG), and phosphatidic acids (PAs) (Sadoshima and Izumo 1997; Sussman *et al.* 2002). The second messenger IP_3 binds to inositol triphosphate receptor (InsP3R) on the surface of sarcoplasmic reticulum causing release and elevation concentration of intracellular Ca^{2+} (Figure 1.5). While DAG give rise to activation of protein kinase C (PKC) which cause rapid induction of immediate early gene such as c-fos and Egr-1 genes (Komuro 2001).

Integrins are transmembrane receptors which are capable to link the ECM with contractile protein (actin) at site focal adhesion (Hynes 1992; Juliano and Haskill 1993; Schwartz *et al.* 1995). Functional integrins are composed from α and β subunits which mainly are expressed in the heart (Hynes 1992). Integrins have widespread biological effects including cell growth, organizing adhesion of cell and migration. The functional role of integrins is act as a transmission for mechanical signals from the external cellular environment to the cellular cytoskeleton (Ingber 1991; Chen *et al.* 1997).

It has been observed that involvement of integrins in the development of cardiac hypertrophy in rat cardiomyocytes which is associated with overexpression of integrin receptors (Ross *et al.* 1998). Pathological cardiac hypertrophy may occur in response to mechanical stimuli via activation of integrins (Kuppuswamy *et al.* 1997). Furthermore, the stretching process leads to stimulate ERKs and c-Jun N-terminal kinases (JNK) transduction pathways via β integrin and extracellular matrix (MacKenna *et al.* 1998). Integrin induces phosphorylation both focal adhesion kinase (FAK) and C-terminal Src kinase, these kinases contribute in the development of hypertrophy in the myocardium of rat as a consequence of stimulation by mechanical stretch (Franchini *et al.* 2000; Laser *et al.* 2000).

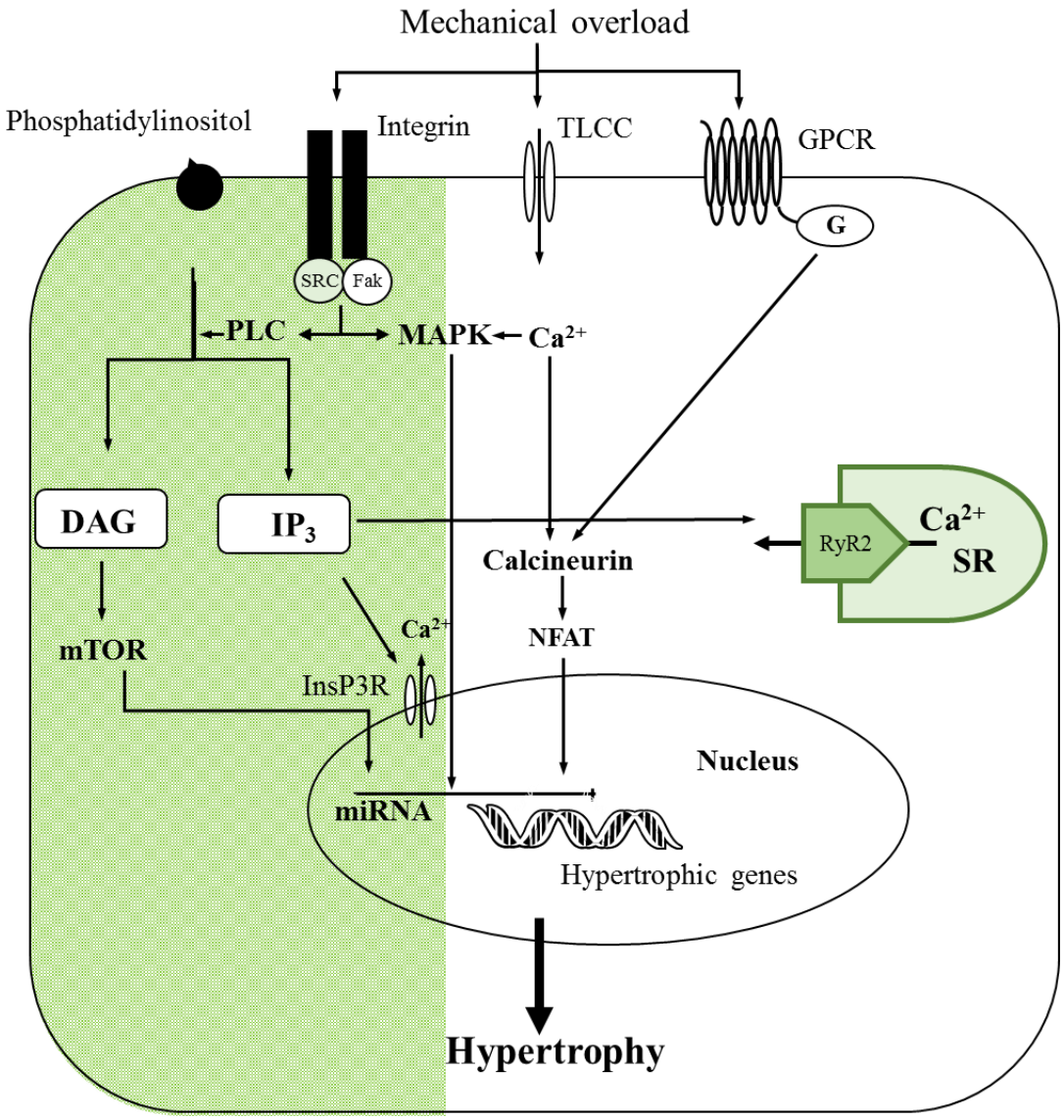


Figure 1.5: Cellular signaling pathways of cardiac hypertrophy induced by mechanical overload.

G protein coupled receptor (GPCR), type L calcium channel (TLCC), diacylglycerol (DAG), inositol 3,4,5-trisphosphate (IP_3), inositol triphosphate receptor (InsP3R), phospholipase C (PLC), nuclear factor of activated T cell (NFAT), mitogenic activated protein kinase (MAPK), mechanistic target of rapamycin (mTOR), microRNA (miRNA). This figure was adapted from (Garcia and Incerpi 2008).

1.3.1.2 Angiotensin II (Ang II)

Renin-angiotensin system plays a prominent role in regulating functions of heart and blood vessels. Angiotensin II (Ang II) is one of the most important neurohormones which can induce growth, proliferation and has apoptotic activity (Dzau 2001). Angiotensin II originally is produced by cardiac tissues, myofibroblasts and fibroblasts (Lindpaintner and Ganten 1991). Four angiotensin II receptor subtypes have been isolated and identified, AT_1, AT_2, AT_3 and AT_4 (Unger *et al.* 1996). Physiological effects of Ang II are mediated by AT_1, including, vasoconstriction, increased cardiac contractility, stimulation of aldosterone release, retention of salt and water and growth stimulation. While, it is thought that AT_2 has opposite actions to AT_1, for instance, vasodilatation, apoptosis, hypotension and antihypertrophic effects (Dasgupta and Zhang 2011).

Renin-angiotensin system components such as angiotensin converting enzyme, angiotensinogen and receptors of angiotensin (AT_1 and AT_2) are present in the cardiac myocytes, during growth response the levels of these components are elevated and overexpressed (Schunkert *et al.* 1990; Suzuki *et al.* 1993). Based on numerous studies on cultured cardiomyocytes conducted in the past two decades, addition of angiotensin II has been shown to mediate the process of hypertrophy. It is capable of inducing cardiac hypertrophy independently of mechanical factors which result from pressure overload (Baker and Aceto 1990; Schunkert *et al.* 1995). It is able to activate a number of signal transduction pathways involved in cardiac hypertrophy by binding to its receptor AT_1 including tyrosine kinases which activate a series of signalling cascades. Furthermore, it activates mitogen-activated protein kinases which in turn can activate some transcription factors (Sadoshima and Izumo 1993; Bernstein *et al.* 1998).

1.3.1.3 Endothelin-1 (ET-1)

Endothelin-1 is a vasoconstrictor neuropeptide which is produced by endothelial cells of the blood vessels and cardiomyocytes, in response to mechanical stretch (Battistini *et al.* 1993; Miyauchi and Masaki 1999). In addition to stretch, expression levels of preproendothelin-1 are also modulated by additional factors such as ANG II, have demonstrated that angiotensin II causes an increase in preproendothelin-1 mRNA expression in cardiomyocytes (Ito *et al.* 1993). It is probable therefore, that the production of ET-1 in the myocardium occurs in response to numerous inducers (Sakai *et al.* 1996b).

Levels of ET-1 are raised in the blood of heart failure patients (Sosin 2006). Hemodynamic actions have been observed in animals after infusion of exogenous ET-1 intravenously resulting in increased plasma levels of ET-1 to mimic those seen in pathological diseases (Lerman *et al.* 1991). Importantly, studies have demonstrated that in experimental animal models of heart failure, treatment with endothelin blockers can improve the clinical symptoms (Sakai *et al.* 1996a; Borgeson *et al.* 1998; Iwanaga *et al.* 1998).

Two subtypes of endothelin receptors had been identified in mammals (ET_A and $ET_{B)}$ which can mediate the biological action of ET-1 (Sakurai *et al.* 1990). This peptide acts on smooth muscles of arteries through its receptor A and leads to vasoconstriction of these muscles when stimulated. However, stimulation of ET_A in the heart can promote hypertrophy. An in-vivo animal experimental model was used by Yorikane and co-workers to research the concentration of ET-1 in rats with ventricular hypertrophy. Their analysis revealed that the concentration of ET-1 is significantly increased in pathological cardiac hypertrophy due to hemodynamic overload (Yorikane *et al.* 1993). ET-1 exerts mitogenic effects by inducing growth and proliferation of cardiomyocytes and vasculature smooth muscle cells (Miyauchi and Masaki 1999).

1.3.1.4 Norepinephrine/Epinephrine (NE/E)

There are two major types of adrenergic receptors, α-adrenergic receptors and β-adrenergic receptors, which are present in the heart (Yamazaki *et al.* 1998b). Cardiac sympathetic innervation has been involved in hypertrophic response. Binding of norepinephrine to β-receptor prompts conformational changes causing coupling to G-protein coupled receptor can result in activation of adenylyl cyclase. Levels of the myocardial cyclic AMP are increased causing activation of protein kinase A by triggering a series of intracellular pathway as well as activation of p38 mitogen-activated protein kinases (Dash *et al.* 2003).

It was recognized that myocardial hypertrophy is associated with elevation of concentration of circulating catecholamines (norepinephrine/epinephrine) and concentration of catecholamines are increased in patients with heart failure. Actually, the β-adrenergic receptors become desensitisation and the signals of β-adrenergic receptors are weaker in patients with heart failure (Sabbah 2004).

1.3.2 Cellular signalling pathways of ventricular hypertrophy

Many neurohormonal chemicals and growth factors can produce hypertrophy as a result of haemodynamic overload. These neurohormonal chemicals (such as Ang II, ET-1 and catecholamine) are released when cardiomyocytes experience for mechanical stretch (Bernardo *et al.* 2010). The mechanism action of these neurohormonal chemicals is started by binding of them with their receptors leading to stimulate G protein coupled receptors ($G_{\alpha q}$). In turn G protein activates phospholipase C β (PLCβ) causing hydrolysis of phosphatidylinositol 4,5-biphospate (PIP2) to diacylglycerol and cytosolic Inositol 1,4,5-trisphosphate (Rockman *et al.* 2002). Activation of cytosolic IP_3 leads to increased levels of intracellular Ca^{2+} by releasing it from SR, whilst diacylglycerol in the end activates protein kinase C. It has been shown that the ventricular hypertrophic signalling is mediated by increasing the level of liberated Ca^{2+} via activation of either CaMK or intracellular phosphatase calcineurin (Wilkins and Molkentin 2004; Wu *et al.* 2006) (Figure 1.6).

Multiple intracellular signalling pathways are activated to induce cardiac hypertrophy in response to various hypertrophic stimuli, including, activation of MAPKs signalling cascades such as ERKs and p38, Rac and Ras small GTPases, protein kinase C and the G_q family of heterotrimeric G proteins (Kyriakis and Avruch 1996). MAPK signalling cascades are successive sequence of acting kinases that eventually lead to phosphorylation and activation of three main groups, ERKs p38 and JNKs (Garrington and Johnson 1999). In cardiac myocytes, the MAPK signaling pathway is initiated by different stimuli, by stretch, GPCR (Ang II, adrenergic receptors and ET-1), cardiotrophin-1 (gp130 receptors) and receptor serine/threonine kinases (transforming growth factor-β (TGFβ)) (Sugden and Clerk 1998). A broad array of intracellular myocardial targets are phosphorylated, comprising transcription factors leading to activation and alteration of cardiac gene expression (Nishimoto and Nishida 2006).

The main signalling pathways of cardiac hypertrophy is MAPKs and CaMKII and UII may have similar signalling pathways to induce hypertrophy.

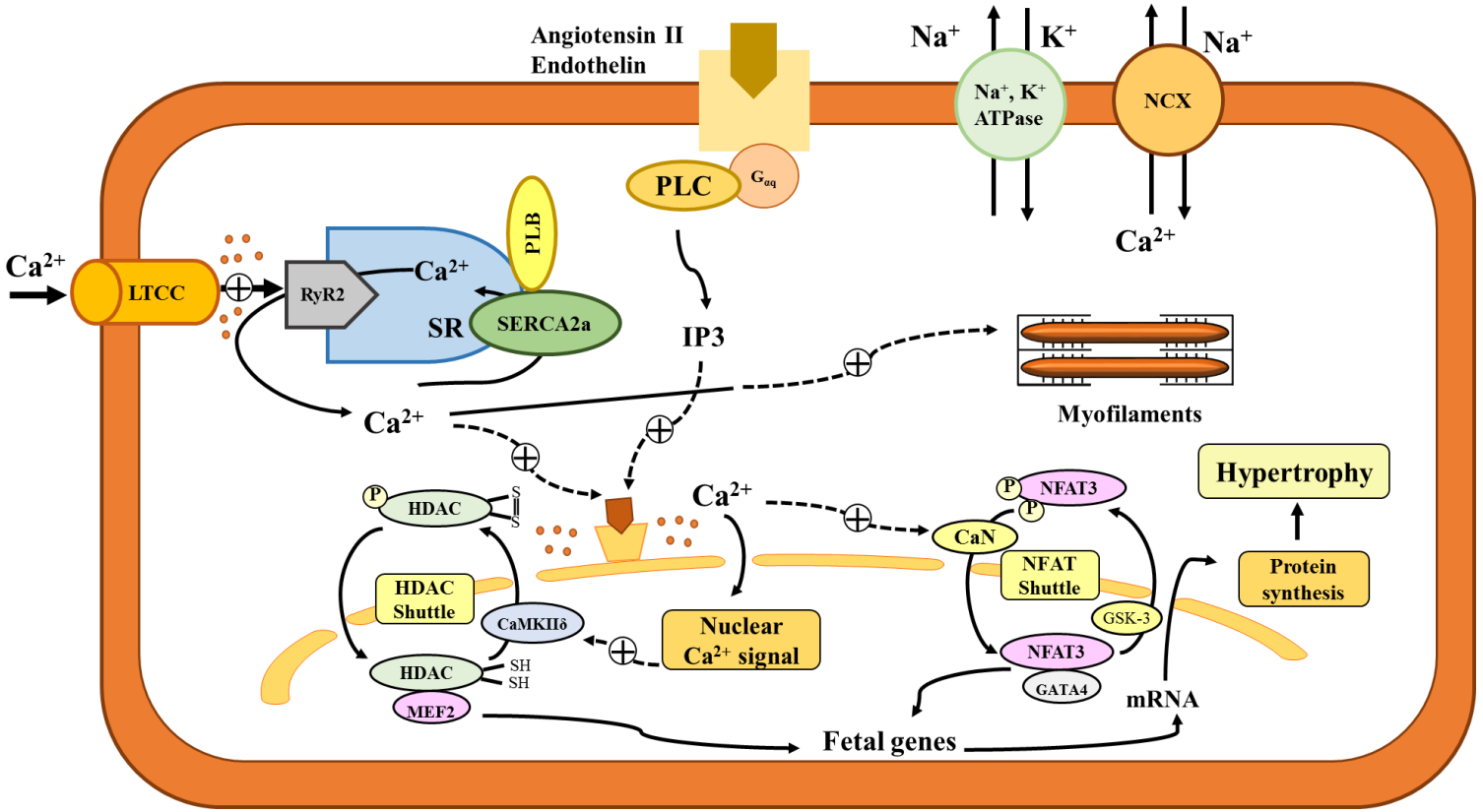


Figure 1.6: Intracellular effector pathways of cardiac hypertrophy induced by different neurohormonal chemicals.
G protein coupled receptor (GPCR), inositol 3,4,5-trisphosphate (IP_3), phospholipase C (PLC), nuclear factor of activated T cell (NFAT), phospholamban (PLB), Na^+/Ca^{2+} exchanger (NCX), sarcoplasmic reticulum (SR), ryanodine receptor (RyR2), histone deacetylases (HDAC), myocyte enhancer factor-2 (MEF2), glycogen synthase kinase 3 (GSK-3). This figure was adapted from (Berridge 2016).

1.4 Urotensin and Hypertrophic Heart Failure

Human urotensin II (hUII) is a vasoconstrictor hormone, and hUII prepro-mRNA is expressed in different tissue within the cardiovascular system. Urotensin II (UII) binds to cognate UT receptor and act as a system (UII/UT system). The (patho) physiological and pharmacological action of UII is mediated by its receptor. Previous studies have reported that the UII/UT system is likely to play a prominent role in regulation of physiological functions of the cardiovascular system as well as a role in certain disease states. In cardiovascular disease, levels of UII are elevated in patients with heart failure and this is suggested to play a role in its development, as it may be involved in cardiac hypertrophy and in adverse E-C coupling. The purpose of this study is to determine the involvement of the UII/UT system (UII and its receptor) in hypertrophy and/excitation-contraction coupling. Urotensin II (UII) has arrhythmogenic effects on right atrial trabeculae isolated from patients subjected to coronary artery bypass graft (Russell *et al.* 2001), the arrhythmogenic effect of UII is lesser than ET-1 (Burrell *et al.* 2000).

1.4.1 Urotensin II structure

Urotensin II is a vasoconstrictor neuropeptide which was initially described and isolated from the spinal cord of teleost fish (Pearson *et al.* 1980). It has since been found to be widely expressed in mammal species such as human, monkeys, rats and mice (Vaudry *et al.* 2010). UII has been postulated to have a role in the regulation of the function of the cardiovascular system (Kobayashi *et al.* 1986; Russell 2008) (Table 1.1). The normal physiological function of UII in human appears to be in the regulation of tone of the peripheral vessels (Douglas and Ohlstein 2000). However, its actions have been shown to vary between species and vascular beds (Ames *et al.* 1999).

Human urotensin II consists of 11 amino acid residues (H-Glu-Thr-Pro-Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH). There is a disulfide bridge which connects between amino acid Cys^{5} and amino acid Cys^{10} (Douglas and Ohlstein 2000). The amino acids of C-terminal arrange in cyclic group that may play an important role in biological activity, this active site of UII is fully preserved in all species. The N-terminal region of UII is different between species in the amino acids sequence and length (Kinney *et al.* 2002; Vaudry *et al.* 2010; Sun *et al.* 2014). For instance, rat UII (rUII) is different from human

UII in a number of amino acid residues, it is composed of 14 amino acid residues (Coulouarn *et al.* 1999) (Figure 1.7).

Tissue	**References**
<u>Vascular tissue response</u>	
Vasoconstriction	(Maguire *et al.* 2000)
Vasorelaxation	(Stirrat *et al.* 2001)
Mitogen of vascular smooth muscle cells	(Watanabe *et al.* 2001a; Watanabe *et al.* 2001b)
Mitogenic effect on endothelial cells	(Shi *et al.* 2006)
<u>Cardiac muscle response</u>	
Inotropic effect ↑ Force of contraction (positive inotropic)	(Russell *et al.* 2001)
↓ Force of contraction (negative inotropic)	(Morimoto *et al.* 2002; Quaile *et al.* 2009)
↑ Collagen synthesis in fibroblasts	(Tzanidis *et al.* 2003)
Hypertrophy	(Tzanidis *et al.* 2003)

Table 1.1: Effects of UII on cardiovascular system.

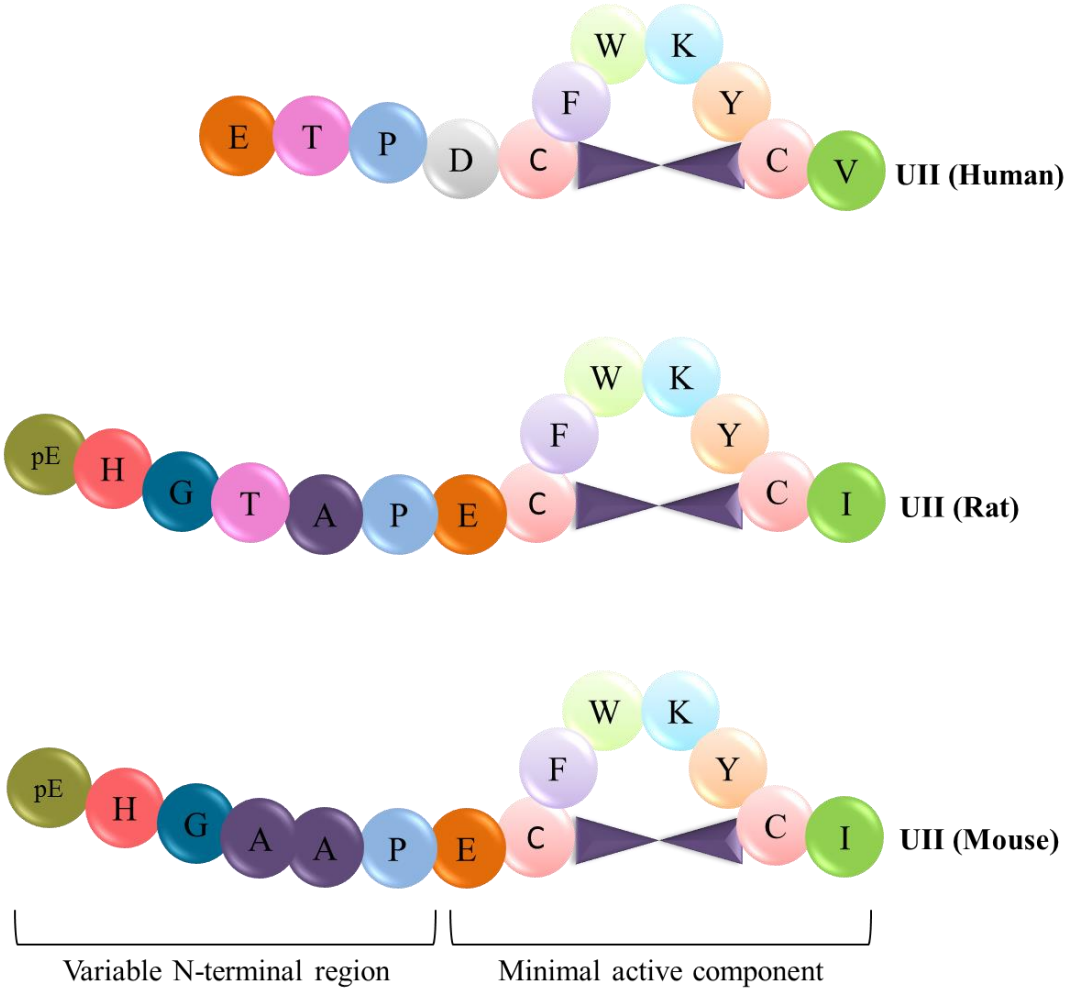


Figure 1.7: A comparison of urotensin II peptide sequences isolated from various mammalian species (human, rat and mouse).
The biological active site sequence of C-terminus is fully conserved across all species, while the N-terminus is various in the sequence and length. This figure was adapted from (Onan *et al.* 2004a).

1.4.2 Urotensin receptor

The UT receptor that binds UII is a G protein coupled receptor, is also called sensory epithelial neuropeptide-like receptor (SENR) because it has been recognized in sensory neurons and nervous tissues (Tal *et al.* 1995; Douglas and Ohlstein 2000). Human urotensin receptor consists of 389 amino acids (Ong *et al.* 2005) (Figure 1.8). UII and its receptor are widely expressed in the different organs of the body such as myocardium, pancreas, brain and kidneys in addition to some glands like (thyroid, adrenal and pituitary) (Matsushita *et al.* 2001; Totsune *et al.* 2001; Sugo *et al.* 2003; Totsune *et al.* 2003; Bousette *et al.* 2004).

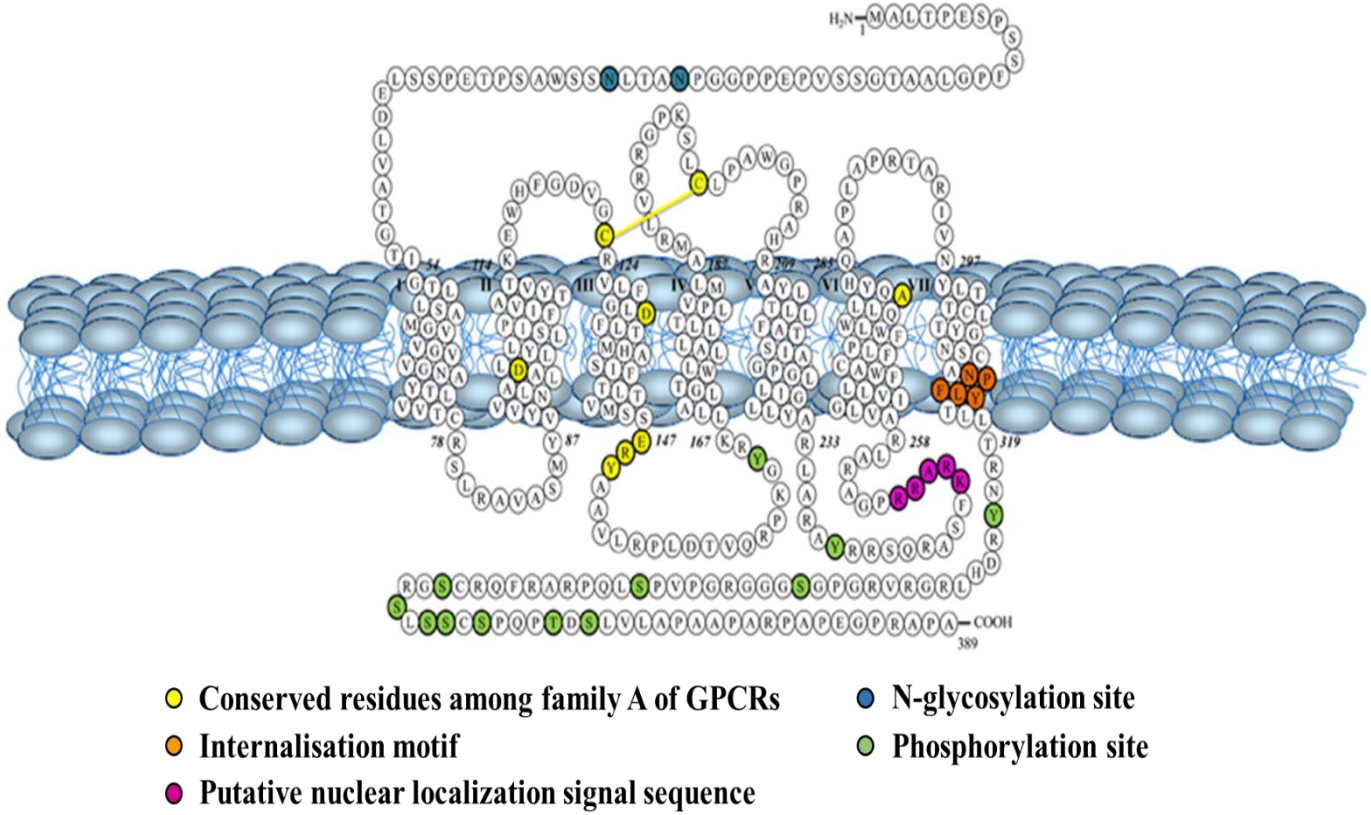


Figure 1.8: Structure of human urotensin receptor.
G protein coupled receptors (GPCRs). This figure was taken from (Douglas *et al.* 2000).

1.4.3 Role of UII in heart failure

Heart failure is a significant healthcare issue which contributes to increased mortality worldwide. Left ventricular remodeling progressively occurs during the progression of heart failure (Miner and Miller 2006).

Many neurohormonal systems are activated in heart failure, one of these is UII/UT-receptor system. The UT receptor is upregulated in cardiac myocytes, smooth muscle and endothelial cells of patients with heart failure (Douglas *et al.* 2002), and early studies have shown an increase in the level of circulating UII in some cardiovascular disorders, ischemic heart disease, hypertension, congestive heart failure, renal diseases and diabetes mellitus (Totsune *et al.* 2001; Totsune *et al.* 2003; Cheung *et al.* 2004; Heringlake *et al.* 2004; Khan *et al.* 2007). Ng and colleagues found that the normal concentration of circulating UII in healthy humans is 9.16pg/ml while plasma UII concentration is 30.6pg/ml in patients with heart failure (Ng *et al.* 2002). In another study, Richards and colleagues have demonstrated that the level of UII is 5.4pg/ml in patients with chronic heart failure in compare with healthy group is 2.6pg/ml (Richards *et al.* 2002).

UII has been elevated in heart failure and it has been recognized UII peptide can depress contractility of ventricular function in patients with heart failure (Douglas *et al.* 2002). Furthermore, the relationship between ejection fraction and levels of UII in patients with heart failure has been investigated, and reveals an inverse correlation between them (Lapp *et al.* 2004). As a result, it has been suggested that UII could be contribute to the reduced contractile function of the heart in this group of patients.

In spite of UII acting to increase contractility of the heart which may consider useful in the short term, long term activation is likely to lead to ventricular remodeling (Yamazaki *et al.* 1999; He *et al.* 2004). According to these events, UII could be involved in cardiac dysfunction and/or hypertrophy.

1.4.4 Role of UII in cardiac muscle

UII has been identified as a strong vasoactive peptide, its action exceeds other vasoconstrictor peptides such as endothelin-1, noradrenaline and serotonin (Russell *et al.* 2001). UII is also a powerful inotropic agent in human pathological conditions such as coronary artery disease (Russell and Molenaar 2004). The inotropic effect of UII has been

investigated by many researchers, however, the inotropic actions of UII are variable ranging from positive to negative inotropic effects. Positive inotropism has been found in cardiac tissues from right ventricle and right atrium of human patients, where the myocardial contractility is increased by endogenously applied UII (Russell *et al.* 2001). The positive inotropic effect of UII has also been shown in normal animal tissues. The research study by Gong and colleagues also found UII has a similar inotropic activity on strips of myocardial isolated from healthy rat tissues (Gong *et al.* 2004). Human UII has a positive inotropic effect on human heart, which results from stimulation of $G_{\alpha q}$-coupled receptor (UT) (Russell *et al.* 2001). However, it was found that UII has a negative inotropic effect on ventricular tissue isolated from left ventricle of canine (Morimoto *et al.* 2002). The research study by Quaile and colleagues also found that UII has a negative inotropic action on the myocardial activity in failing human heart (Quaile *et al.* 2009).

Increased force of cardiac contraction as action of hUII has been shown in the human right atrium through activation of a protein kinase C (PKC) dependent pathway (Russell and Molenaar 2004). It is possible that a PKC dependent pathway contributes to the inotropic effects of hUII by activation of Na^+/H^+ exchanger, resulting in an alkalinisation of intracellular pH, increasing the sensitivity of the myofilaments to Ca^{2+}. The pH of cardiac myocytes becomes alkaline due to action of Na^+/H^+ exchanger as well as these myofilaments become sensitised to Ca^{2+} ions (Allen and Orchard 1983; Krämer *et al.* 1991; Wang *et al.* 2000).

In cultured cardiomyocytes from neonatal rats, it was found that UII has the ability to induce hypertrophy (Tzanidis *et al.* 2003). Hypertrophy is induced in neonatal cardiomyocytes by UII which is mediated by MAPK signaling pathway, p38 mitogen-activated protein kinases and extracellular-regulated kinases (ERK1/2), this process is associated with trans-activation of epidermal growth factor receptor (EGFR) (Onan *et al.* 2004b).

1.4.4.1 Additional effect of UII on the vascular smooth muscle cells

Binding of hUII peptide with UII receptor on vascular smooth muscle cells produces a strong vasoconstriction effect (Ames *et al.* 1999; Giebing *et al.* 2005). Binding of hUII to the UT receptor leads to activation of G_q protein this later activates PKC, PLC and calmodulin (Opgaard *et al.* 2000; Rossowski *et al.* 2002; Tasaki *et al.* 2004) (Figure 1.9). Phospholipase C gives rise to the production of second messengers including DAG and IP_3 which causing release of intracellular Ca^{2+} from SR by binding of IP_3 to InsP3R on the surface of sarcoplasmic reticulum (Sauzeau *et al.* 2001; Proulx *et al.* 2008; Parys and De Smedt 2012). Successively these messengers can provoke influx of extracellular Ca^{2+} ions can result in constriction of vascular smooth muscles. Furthermore, the vasoconstrictor impacts are mediated by various pathway systems like Rho-associated kinase (RhoA-ROCK) and ERK. UII stimulates influx of Ca^{2+} depending on the type of cell which occurs via different types of voltage-gated Ca^{2+} channels. For example, in smooth muscle cells of arteries, UII stimulates influx of Ca^{2+} through LTCC (Sauzeau *et al.* 2001).

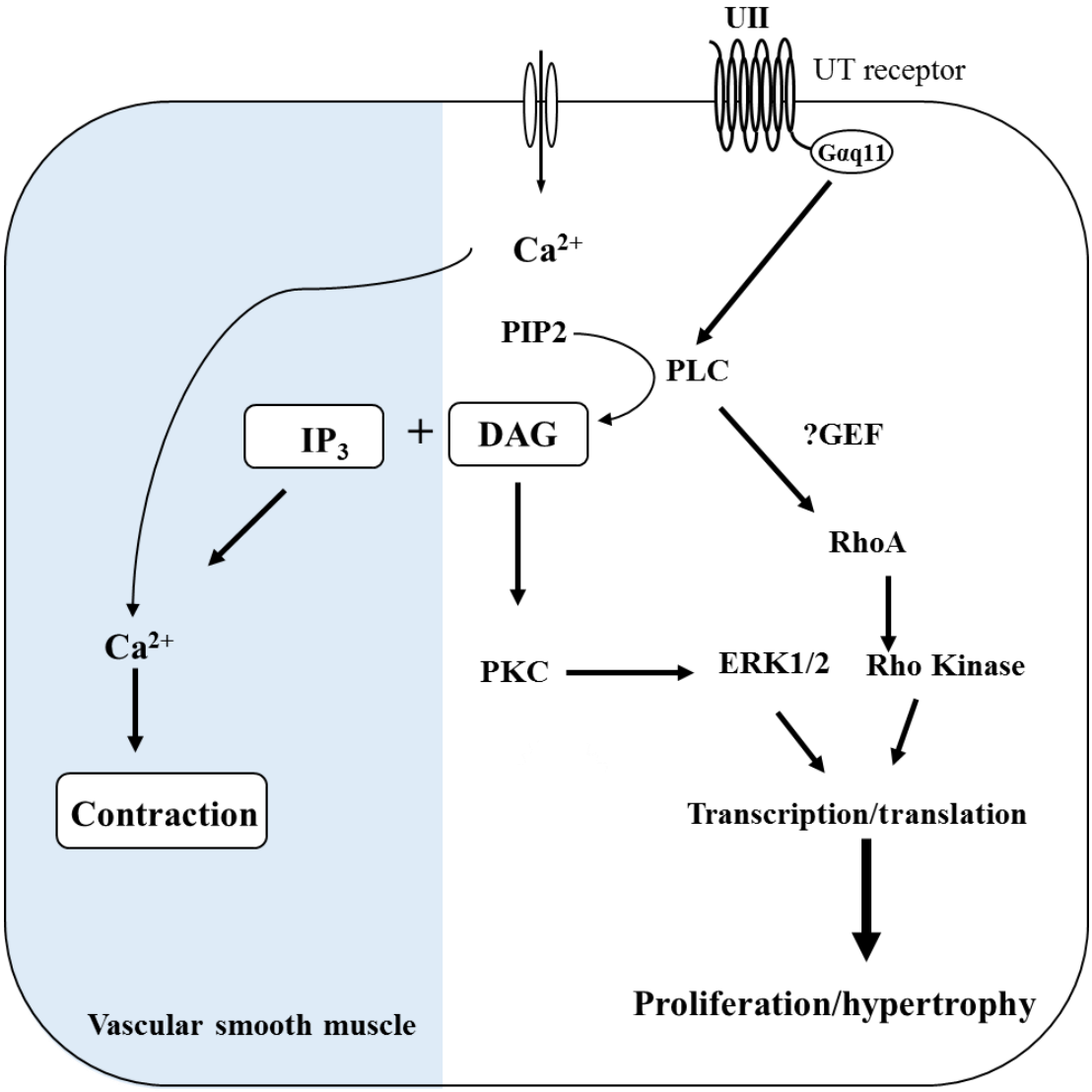


Figure 1.9: Schematic representation of UII signal pathways in vascular smooth muscle cell. Phosphatidylinositol 4,5-biphospate (PIP2), diacylglycerol (DAG), inositol 3,4,5-trisphosphate (IP3), phospholipase C (PLC), extracellular regulated kinase (ERK), protein kinase C (PKC). This figure was adapted from (Ong *et al.* 2005).

1.5 Aims and objectives

There have been several studies in the literature reporting elevation of circulating UII in failing heart (Ng *et al.* 2002; Richards *et al.* 2002). UII increases strength of the contraction of ventricular tissues. However, its action alters in diseases such as heart failure (Douglas *et al.* 2002). The hypothesis of this study is that UII has negative inotropic effects by altering Ca^{2+}-regulation during E-C coupling. UII also triggers hypertrophic effects in the heart which further influences Ca^{2+} and contractile regulation during E-C coupling. These effects involve UII binding to its receptor and triggering signaling pathways.

The main objectives of this study are to determine whether UII is involved in hypertrophy and heart failure and what signalling molecules are involved? The current study involves an investigation of the effects of UII on cardiac tissues, whether the increased levels of circulating UII may act as a driver of hypertrophy and whether there is a negative or positive inotropic effect. This study set out to determine:

1. **What is the impact of UII on excitation-contraction coupling of the ventricular muscle?**

The aim of the current study was to investigate effect of UII on excitation-contraction coupling and Ca^{2+}-regulation in normal ventricular cardiomyocytes and cultured ventricular cardiomyocytes, determine whether UII changes calcium regulation, contraction and electrical activity (action potential configuration, ion channel activity). Does it result in changes to E-C coupling that resemble heart failure (changes in Ca^{2+}-regulation that results in contractile dysfunction and arrhythmia).

2. **Can UII induce hypertrophy in adult ventricular muscle, and what are the signaling pathways implicated in hypertrophy in response to UII?**

The second aim of this project was to look at UII-induced hypertrophy in adult tissue, and to determine the signaling pathways involved. In order to facilitate this, a tissue culture model of hypertrophic heart failure was developed.

3. Does UT receptor upregulate in hypertrophy?

As UT receptor has been suggested to upregulate in heart failure, the third aim was to show the involvement of UT receptors in the response of ventricular cardiomyocytes to UII. This part of the study used gene expression assays, to detect expression of UT mRNA in freshly isolated cardiomyocytes and hypertrophic ventricular cardiomyocytes and in an animal model of hypertrophic heart failure.

Chapter 2: Materials and methods

2.1 Experimental animals and isolation of ventricular cardiomyocytes

2.1.1 Animals

Adult male Wistar rats (body weight 200-300g) were bred and held in specific environmentally controlled conditions at Preclinical Research Facility, University of Leicester. Animals were fed (ad libitum) and exposed to light for 12 hours from 6.00 to 18.00. The animals were at least 8-weeks old at the time of experimentation. The rat was euthanized by a sharp blow to the head followed by cervical dislocation (in compliance with schedule 1 of the Animals (Scientific Procedures Act, 1986)).

2.1.2 Ventricular cardiomyocytes isolation

Single cardiomyocytes were isolated from rat ventricles using a method previously described (Lawrence and Rodrigo 1999) and adapted from the original method (Mitra and Morad 1985). Animals were sacrificed and the heart exposed through an opening in the chest cavity, excised and transferred to cold Tyrode Ca^{2+}-free solution. The heart is then cannulated at the aorta and perfused using a Langendorff setup (Figure 2.1), with Ca^{2+}-free solution for 6 minutes, after which the heart was perfused with enzyme solution containing type I Collagenase, type XIV Protease and Bovine serum albumin (BSA) fraction V dissolved in Ca^{2+}-free Tyrode for 8 minutes. Following perfusion with enzyme solution, the heart was perfused with normal Tyrode (NT) for 3 minutes to wash off the enzyme. The solution used was continuously bubbled with 100% oxygen and maintained at 35 ± 1°C during the isolation process. The atria were dissected and the ventricles were cut in half, placed into 50ml conical flask containing 10ml normal Tyrode and shaken in a water bath (Stuart, SBS40) at a speed of 175 strokes/minute at 37˚C to release single cardiomyocytes. The cardiomyocytes suspension was filtered using a stainless steel sieve (Sigma) with a pore size of 200µm and the cell suspension centrifuged, speed 18g, and finally resuspended in fresh normal Tyrode, this process was repeated to wash the cells. Freshly isolated ventricular cardiomyocytes were stored in Petri dishes and maintained at room temperature until use within 8 hours (Figure 2.2).

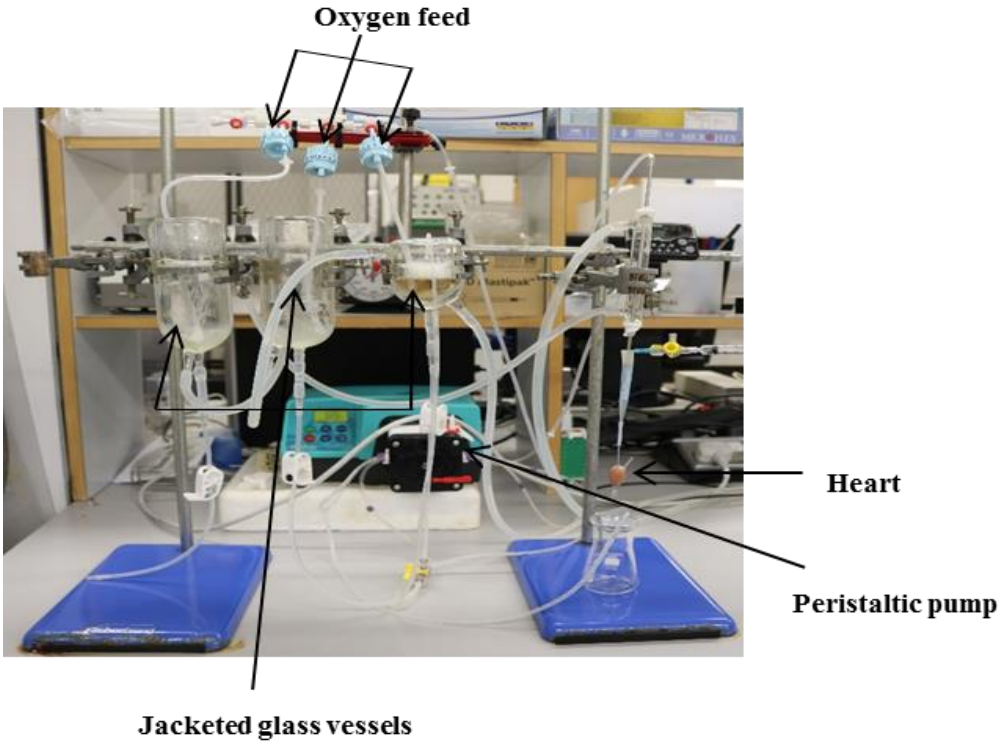


Figure 2.1: Langendorff isolated cardiomyocytes perfusion system.
This figure shows the Langendorff apparatus used for cardiomyocytes isolation. The system consists of three glass vessels with a constant flow provided by a peristaltic pump.

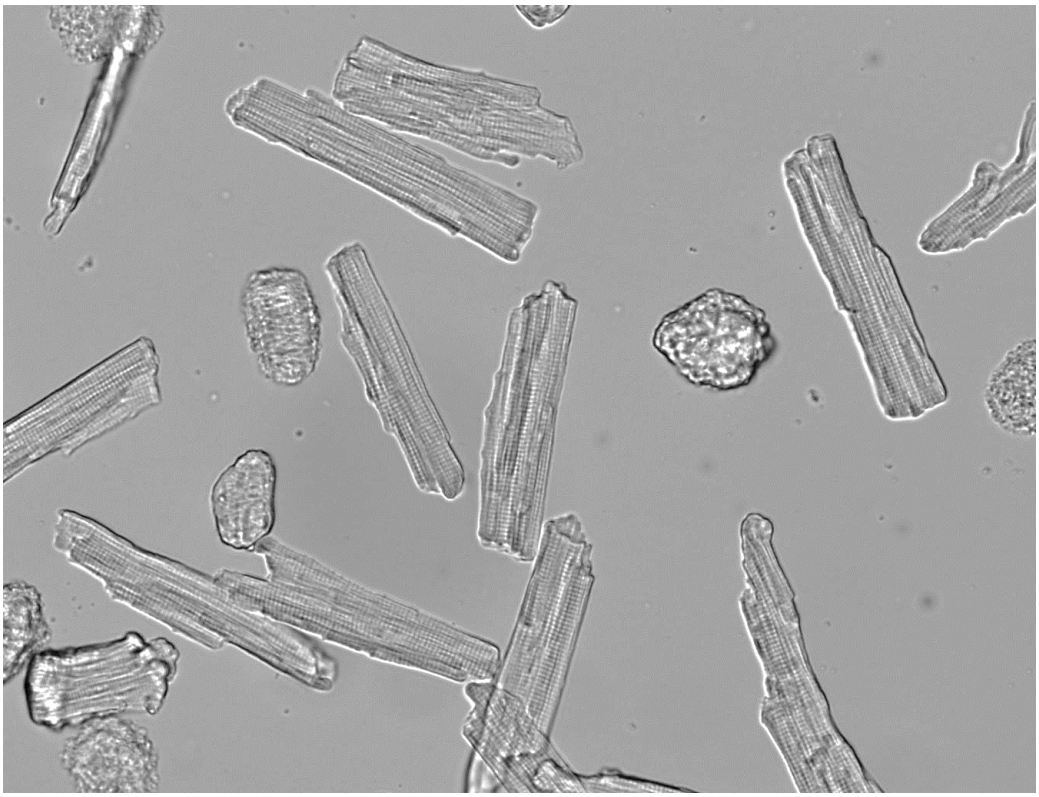


Figure 2.2: Isolated ventricular cardiomyocytes from Wistar rat in normal Tyrode, magnification (200X).

2.1.3 Mouse model of aortic aneurism and left ventricular hypertrophy

A mouse model of abdominal aortic aneurysm (AAA) was developed in which 6.129P2-ApoEtm1unc/J (ApoE KO) mice, were implanted with a mini-osmotic pump (alzet® 2004, Durect™; CA, USA) containing angiotensin II in a separate study undertaken by Dr. Wadhad Mahbuba and Professor David G Lambert. The aortic diameter of these mice plus sham and other control groups were checked after 28 days of angiotensin II exposure by non-invasive ultrasound scanning at different times which were 0, 7, 14, 21, and 28 days. At the day 28th, these mice were euthanized for the gross morphological and other assessments. The mice were grouped into controls (not expose to any treatment), Sham controls (treated with normal saline only for 28 days) and positive controls (angiotensin II 28 days). In these animals left ventricular hypertrophy was also seen to develop and was identified by ultrasound.

Firstly, the angiotensin II was diluted with normal saline to the desired dose under sterile conditions and injected into the mini-osmotic pump with a concentration of 1000ng/kg/minute. In the control sham animal, the pump was filled with normal saline only. These pumps were incubated at 37˚C for 24 hours.

Male mice were kept in cages with well-ventilated environment at 21˚C, 50% humidity and the light-dark cycles were 12/12 hours. These animals were provided by University of Leicester (Dr Emma Stringer) weighing 24.9± 0.7g and aged 72.8 ± 0.8 days. Surgical operations were done under complete veterinary supervision at the surgical unit in the Central Research Facility at University of Leicester. All in-vivo experiments were carried out under regulations of the Home Office (Scientific Procedure Act 1986, UK) and ethical approval of the institution (PPL 70/8740, PIL I2D933AF0). The protocols for these experiments were taken from (Choke *et al.* 2010).

Anaesthesia of the mice was initiated by applying inhalation of oxygen and 5% isoflurane and then maintained at 2% concentration. Then the surgery was started by clipping fur and wiping with 70% alcohol swab. After that, analgesic (10mg/kg Carprofen (Carprieve®, 50mg/ml), 0.1mg/kg) was subcutaneously injected. 20µl of bupivacaine hydrochloride (Marcaine®, 2.5mg/ml) diluted as 1:19 was locally subcutaneously injected between the shoulders with the animals in the prone position on a heated mat at the site of the proposed incision. Then a transverse incision was made on the skin to about

5mm following that creation of a subcutaneous pocket by bluntly dissecting on the back side which has to be fitted to the pump. Finally, insertion the pump to the pocket was done pushing it a bit away from the incision opening allowing to close the incision by two layers sutures.

The mice were moved to the holding area after its recovery from anaesthesia with gradual introduction of some soft food. The mouse was monitored for the general appearances of satisfactory recovery, looking at different features:

1- Starry coat, hunched, piloerection
2- Mouse grimace scale
3- Pain assessment by using a 0-2 facial expression-based scoring system. This system mainly focused on five categories (orbital tightening, cheek bulge, ear position, nose bulge and whisker change).

Two other doses of Carprofen could be given as 1 dose per day for pain relief.

Ultrasound scanning of the mice was performed under general anaesthesia, at day 0 and at days 7, 14, 21, and 28. Long and short axis B-mode was used to check aortic arch as well as the abdominal aorta while the M-mode was used to exam the wall thickness and contractility of the ventricular muscle.

In order to collect tissue (aorta, heart) for further in-vitro analysis, animals were humanly killed under general anaesthesia followed by a transverse incision to anterior upper abdomen. Finally, harvesting the heart was carefully done after cleaning it from any fascia. The heart was cut into three parts (atria, right ventricle and left ventricle) and kept in RNAlater™ till use.

2.2 Contraction studies and Calcium measurement

2.2.1 Superfusion of ventricular cardiomyocytes

Contraction and intracellular Ca^{2+}-transients were measured from single cardiomyocytes subjected to electrical field stimulation at a frequency 1Hz, by platinum electrodes placed in the bath (Figure 2.3), and connected to an electrical stimulator (Research stimulator, 6002, Harvard). Cardiomyocytes were placed into a superfusion chamber mounted on the stage of an inverted light microscope (Nikon Diaphot) and left for 5 minutes to settle on the bottom of the chamber. The cardiomyocytes were then perfused with normal Tyrode solution at a constant flow rate of 4mL/minute at 35 ± 1°C (Single Inline Solution Heater, SH-27B, Warner Instruments). All cells not contracting synchronously in response to field stimulation were excluded from the experiment.

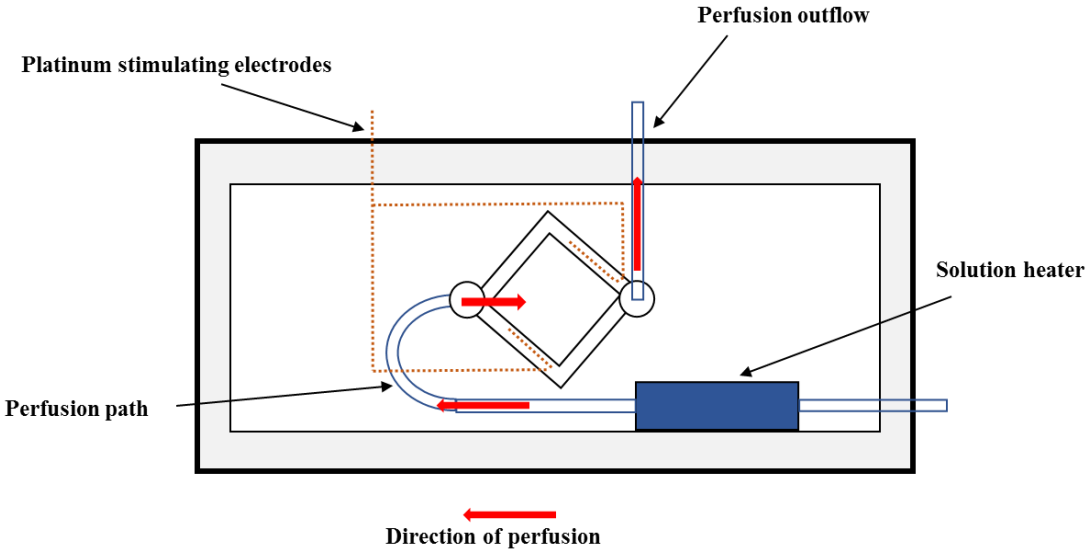


Figure 2.3: A schematic of the cardiomyocytes superfusion chamber.
This figure shows a diamond superfusion chamber used in all contraction and fluorescence microscopy experiments. The two platinum electrodes enabled electrical field stimulation of the ventricular cardiomyocytes in the superfusion chamber.

2.2.1.1 Cell length and contraction

Cell shortening was recorded from single ventricular cardiomyocytes as an indicator of the strength of contraction. A Nikon Diaphot inverted microscope was used to observe the cardiomyocytes, which linked to a black and white charge-coupled device camera (WATEC monochrome camera 1/2" Wat-902B). The edges of the cell were monitored using a video-edge detections system (Crescent Electronics Video Edge Detector VED-103). The camera rapidly transmitted an image of the cardiomyocyte to the cell motion detector and screen of the video monitor (Panasonic WV-5340). Any changes in cell length during shortening and re-lengthening were viewed as a video image using a x40 objective lens. The video edge detection amplifier produced an output that was proportional to cell length of the ventricular cardiomyocyte. Any changes in cell length were then converted to the percentage of cardiomyocyte shortening (%S) (Figure 2.4).

To determine the effect of UII on the contraction of ventricular cardiomyocytes, cells placed in a superfusion chamber were superfused with normal Tyrode and stimulated electrically at 1Hz and the cell shortening recorded and were then superfused with Tyrode containing UII (200nM) for 10 minutes. The force of contraction is expressed as percent cell shortening; data were collected using Clampfit software (version 10.4). The percentage of ventricular cardiomyocyte shortening (%S) was calculated according to the equation:

$$\%\ \textbf{cell shorten} = \left(\frac{\textbf{Diastolic cell length} - \textbf{Systolic cell length}}{\textbf{Diastolic cell length}}\right) \times \mathbf{100} \text{ ..... (2)}$$

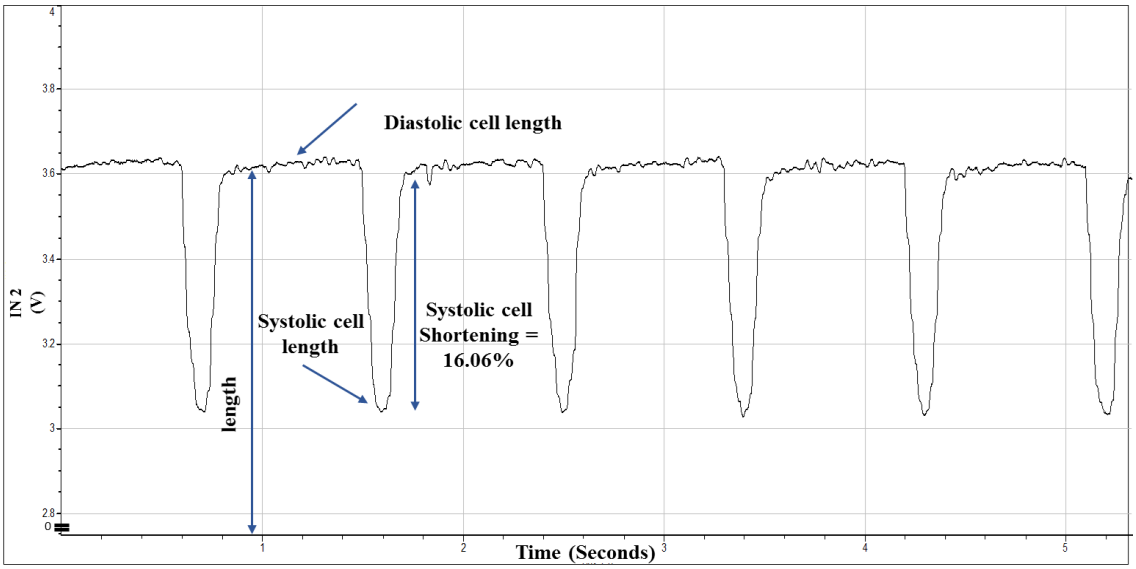


Figure 2.4: An example measuring of cell length contraction in ventricular cardiomyocyte. Figure shows a typical trace of cell length which represent cell contraction.

2.2.2 Fluorescence microscopy measurement of calcium

2.2.2.1 Theory

Fura-2 loaded cells were excited with illumination delivered from delta Ram X monochromator (PTI's model 101-M) alternately at 340 and 380nm with a frequency of 60Hz; light was reflected to the excited cell through an excitation dichroic mirror (Figure 2.5). Emitted light from excited cells at a wavelength of >510nm passed through a prism and was then directed to an emission filter for filtration. The emitted light from an excited single ventricular cardiomyocyte was delivered to the photon-counting photomultiplier tube detection system (PTI Felix 32 product guide) used for detection of the emitted light from excited ventricular cardiomyocyte. The 340/380 ratio was determined as a measure of calcium (Figure 2.6). Calcium transient data were collected, and analysis of fluorescence data was performed with PTI Felix 32 software.

2.2.2.1.1 Measurement of intracellular calcium

Intracellular Ca^{2+} was measured in single ventricular cardiomyocyte loaded with Fura-2-AM (acetoxymethyl ester). Fura-2-AM is a ratiometric dye and can cross the plasma membrane because ester groups render the Fura-2 lipid soluble. Once inside the cytoplasm of cell, the acetoxymethyl ester group is dissociated from the Fura-2 by the action of the endogenous esterase enzymes and the fluorochrome becomes lipid insoluble and is trapped in the cell. Fluorescence characteristics of Fura-2 change when bound to Ca^{2+}. The loaded cell is excited at 340nm (Ca^{2+}-bound) and 380nm (Ca^{2+}-free), respectively and the fluorescence intensity is recorded at 510nm. When Fura-2 binds Ca^{2+}, the intensity of the emitted light from 340nm increases and that of 380nm decreases and the ratio of the emitted light is directly proportional to the intracellular Ca^{2+} concentration (Figure 2.6) and enables measurement of the concentration of intracellular Ca^{2+} accurately. (Martínez *et al.* 2017).

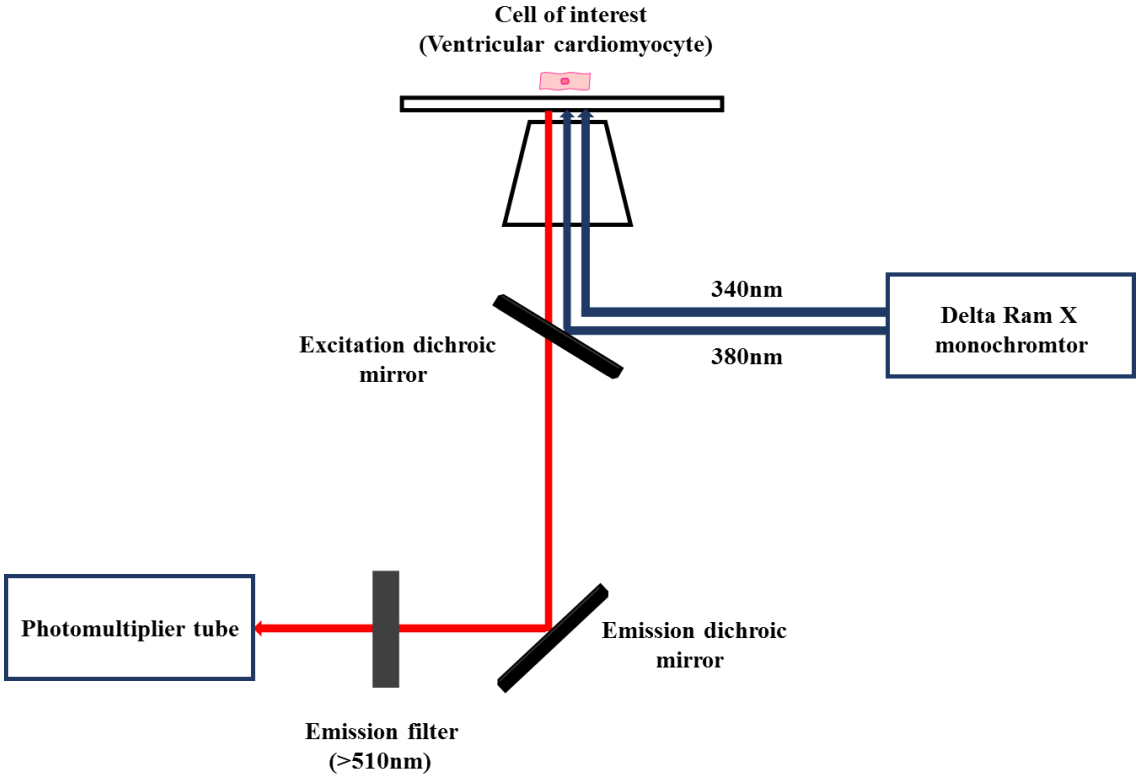


Figure 2.5: A schematic diagram of light pathway in a fluorescence microscope.
Figure illustrates the light pathway for a dual wavelength excitation/emission fluorescent dye Fura-2. Fura-2 loaded ventricular cardiomyocyte was excited reciprocally at 340 and 380nm, emitted light was collected from excited cell at >510nm.

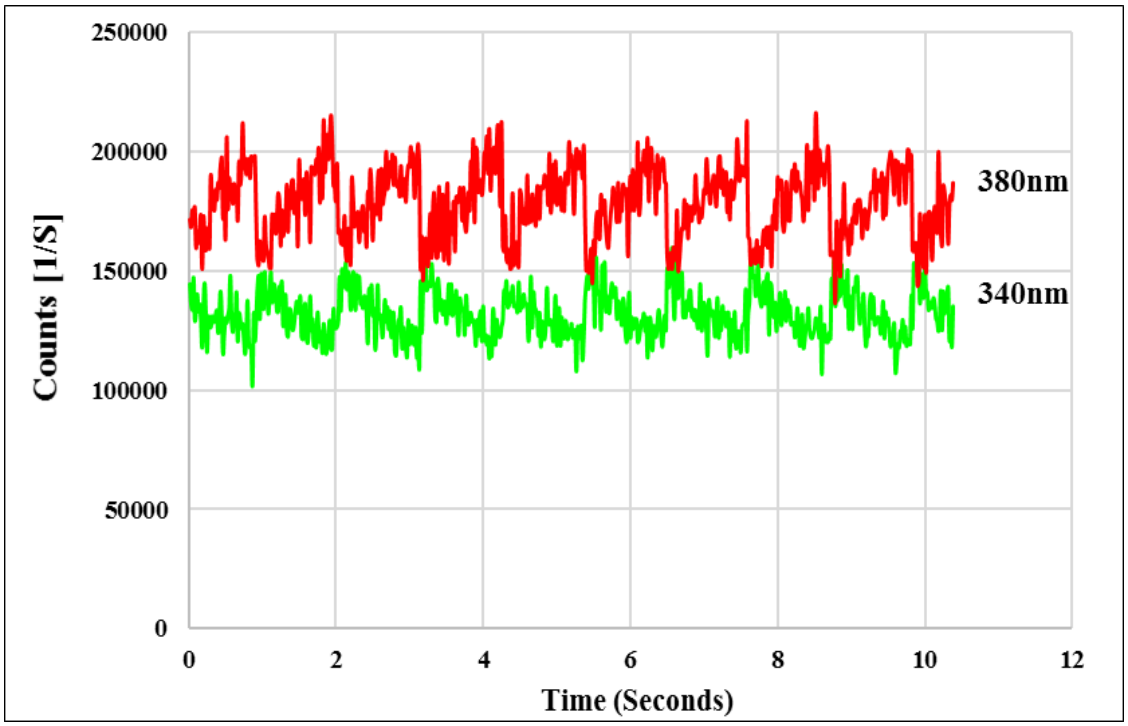


Figure 2.6: Example of fluorescence intensity at 340nm and 380nm emitted from single ventricular cardiomyocyte during excitation.

2.2.2.1.2 In-vivo calibration of $[Ca^{2+}]_i$ dependent Fura-2 fluorescence

Calibration of $[Ca^{2+}]_i$ was carried out as described previously (Hunt and Lambert 2013), using the equation of Grynkiewicz (equation 3), which requires the input of several constants. A Ca^{2+} dissociation constant (K_d) of 285nM at 37˚C (Groden *et al.* 1991), and the fluorescence ratio (R= F340/F380) determined as R_{min} (minimal Ca^{2+}-bound) and R_{max} (maximal Ca^{2+}-bound) and F_{380max}/F_{380min} (The ratio of the bound to unbound Ca^{2+} determined at 380nm). R_{min} was determined from single ventricular cardiomyocyte loaded with 2mM calcium chelator BAPTA-AM to buffer Ca^{2+} to very low levels, and R_{max} was achieved by using a microelectrode to mechanically disrupt the cell membrane of the same cell, allowing extracellular Ca^{2+} to enter the cell. The ratio of the bound to unbound Ca^{2+} determined at 380nm (F_{380max}/F_{380min}), was also obtained from these cells. $R_{min,}$ R_{max} and F_{380max}/F_{380min} ratios were determined by researchers in the lab in 41 cardiomyocytes isolated from 6 Wistar rats at different time points. $[Ca^{2+}]_i$ was calculated using the following equation below:

$$\mathbf{Ca^{+2} = K_d \left(\frac{R - R_{min}}{R_{max} - R}\right) \times \left(\frac{F_{380\ max}}{F_{380\ min}}\right)} \text{ ..... (3)}$$

R = Measured Fura-2 ratio

K_d = 285nM

R_{min} = 0.40

R_{max} = 4.96

F_{380max}/F_{380min} = 6.39

2.2.2.2 Methods

2.2.2.2.1 Measurement of Ca^{2+}-sequestration by the sarcoplasmic reticulum: activity of SERCA2a

The ventricular cardiomyocytes suspension was loaded with 5µM Fura-2, AM for 20 minutes in the dark room and then rinsed twice with normal Tyrode to remove excess extracellular Fura-2. The cells were left at room temperature for 30 minutes to allow de-esterification before measuring the intracellular Ca^{2+} in a single cardiomyocyte.

During diastole and relaxation, the increase in systolic Ca^{2+} is reduced by the actions of SERCA2a and the NCX as well as the sarcolemmal Ca^{2+}-ATPase (PMCA). In the rat cardiomyocyte the dominant mechanism is SERCA2a pump which actively pumps Ca^{2+} into the SR and accounts for >90% (Bers 2001). The function of SERCA2a can therefore be estimated in rat ventricular myocytes by calculating the exponential time constant of the decay of the electrically-provoked Ca^{2+}-transient. To calculate the exponential time constant for relaxation of the electrically-provoked Ca^{2+}-transient, the relaxation phase of the Ca^{2+}-transient was fitted with a single exponential decay curve using Excel (Figure 2.7).

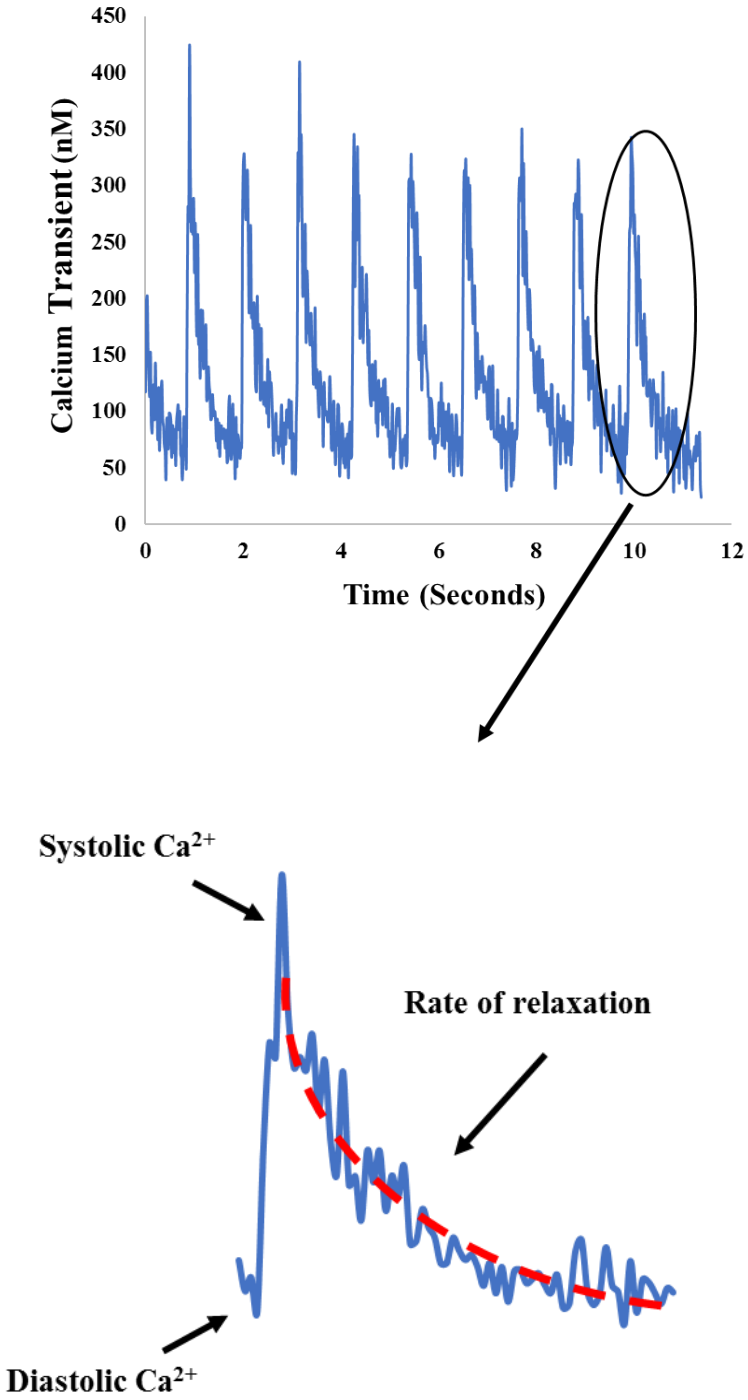


Figure 2.7: An example of record of intracellular Ca^{2+} from a single ventricular cardiomyocyte.

The ventricular cardiomyocytes were superfused with normal Tyrode and the Ca^{2+}-transient was recorded for 10 seconds. Figure illustrates measurements of systolic and diastolic calcium levels.

2.2.2.2.2 Measurement of sarcoplasmic reticulum calcium content

Caffeine at high concentrations opens the RyR2 and releases Ca^{2+} stored in the SR and can be used to estimate the Ca^{2+}-content of the SR (O'neill and Eisner 1990). To measure SR Ca^{2+}-load, the electrical stimulator was first switched off and 10mM of caffeine in normal Tyrode solution rapidly applied to the cell for 5 seconds, and the Ca^{2+}-releases from the SR lumen was clearly visible on the trace as a large $[Ca^{2+}]_i$ transient peak (Figure 2.8). The peak caffeine $[Ca^{2+}]_i$ transient was calculated and considered as an index of the SR Ca^{2+}. The resulted released Ca^{2+} peak of caffeine-induced Ca^{2+}-transients can refer to Ca^{2+} levels released during this time which found in SR (Bers 2001).

To calculate peak Ca^{2+}-release and exponential time constant of the caffeine-provoked Ca^{2+}-transient, the relaxation phase of the Ca^{2+}-transient was fitted with a single exponential decay curve using Microsoft Excel. The rate of decline of the caffeine-induced Ca^{2+}-transient is due to the function of the Na/Ca exchanger (NCX) (Bers 2001), and can be determined by calculating the exponential time constant of decay of the caffeine-induced Ca^{2+}-transients.

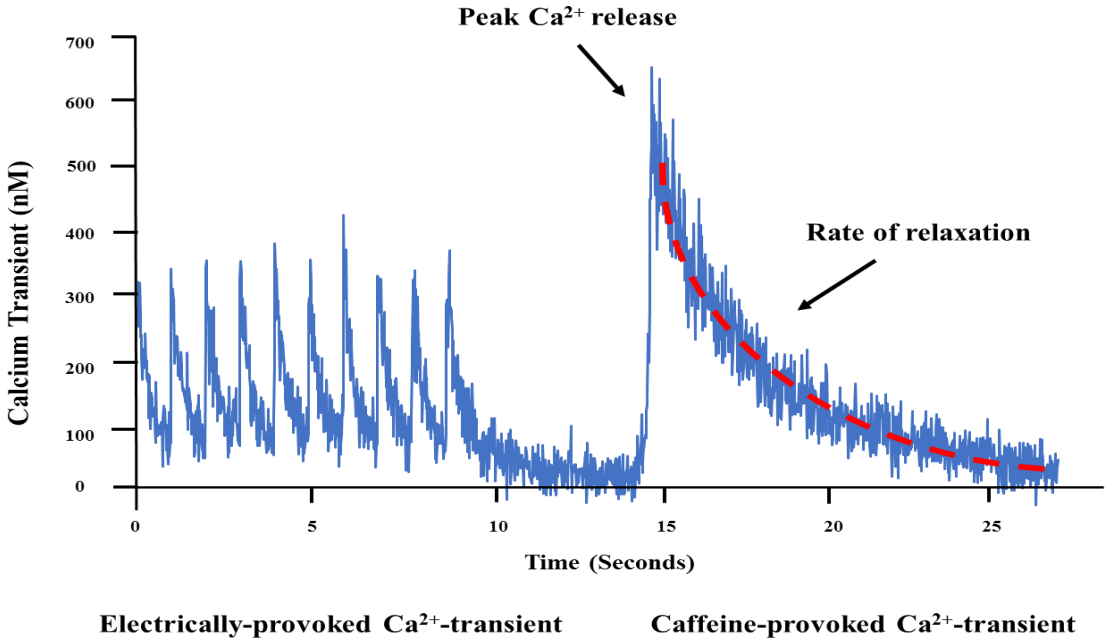


Figure 2.8: Example recording of electrically-provoked and caffeine-provoked $[Ca^{2+}]_i$ transient recorded from a ventricular cardiomyocyte in NT.

2.2.2.2.3 Sarcoplasmic reticulum Ca^{2+}-leak experiment

Fluo-3 is a non-ratiometric Ca^{2+}-sensitive dye, which provides a signal with a very low signal-to-noise ratio and was used to determine SR Ca^{2+}-leak. A single wavelength fluo-3 is absorbed at maximum of 506nm and emitted maximally at a wavelength of 525nm (Kao *et al.* 1989). Through using particular optical filter sets, the green-fluorescent emitted light of Ca^{2+}-bound fluo-3 can be detected. Fluorescence intensity of Fluo-3 is increased more than 100-fold upon Ca^{2+} binding (Harkins *et al.* 1993). 2ml of ventricular cardiomyocytes suspension were loaded with 10µM fluo-3 for 40 minutes in the dark to allow de-esterification. When cells are excited at a wavelength of 506nm the emitted light was at ~525nm. Cells were washed twice with normal Tyrode to remove excess of fluo-3.

Following perfusion of cells with normal Tyrode for 5 minutes and to obtain a steady state Ca^{2+}-transient, the ventricular cardiomyocytes were stimulated at 1Hz and $[Ca^{2+}]_i$ was recorded for 1 minute. Then, the electrical stimulator was switched off and the cells were superfused with Ca^{2+}/Na^{+}-free Tyrode (10mmol EGTA), for 90 seconds. The ventricular cardiomyocytes were superfused with Ca^{2+}/Na^{+}-free Tyrode in order to block fluxes of calcium across the cell membrane, at this point diastolic calcium is regulated by sarcoplasmic reticulum (Ca^{2+}-uptake and leak). To determine Ca^{2+}-leak, diastolic Ca^{2+} was measured in the absence and presence of 1mM tetracaine, a local anaesthetic that blocks the release of Ca^{2+} from SR through RyR2 (Shannon *et al.* 2002) (Figure 2.9).

The diastolic fluorescence (F0) between pulses was amassed, $[Ca]_d$ was measured for the cardiomyocytes at the beginning of experiment using fura-2. $K_{d(Ca)}$ represents the affinity of the fluorescence indictor for calcium and was measured to be 1100nM. Subsequently, according to the protocol fluorescence (F) was calibrated utilizing a pseudoratio below:

$$[\mathbf{Ca}]_\mathbf{i} = \frac{\mathbf{F/F_0}\left(\mathbf{K_{d(Ca)}}\right)}{\left(\left(\frac{\mathbf{K_{d(Ca)}}}{\mathbf{[Ca]_d}}\right) - \frac{\mathbf{F}}{\mathbf{F_0}} + \mathbf{1}\right)} \quad \text{..... (4)}$$

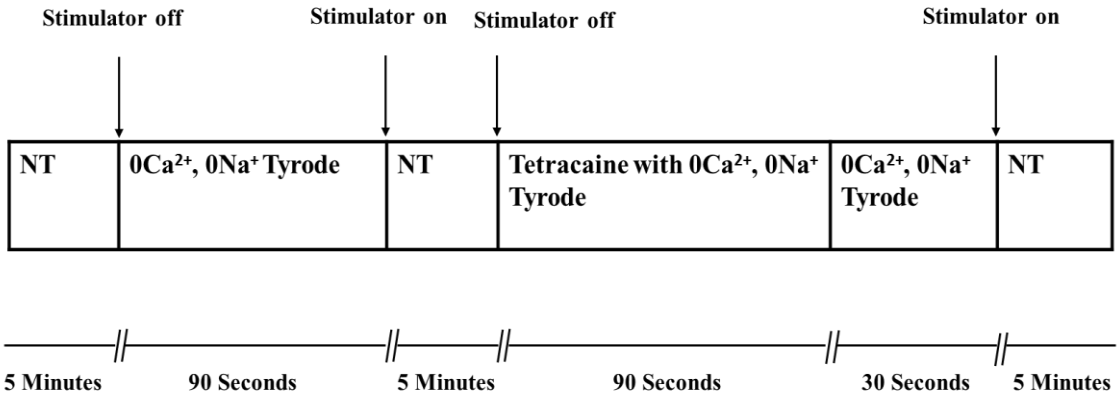


Figure 2.9: A schematic diagram of SR Ca^{2+}-leak experiment.

2.3 Cell culture

2.3.1 Primary culture of adult rat ventricular cardiomyocytes

To study hypertrophy and to see whether the short time tissue culture had an effect on UII receptor activation, adult rat ventricular cardiomyocytes were cultured. Freshly isolated ventricular cardiomyocytes were spun down at 18g and resuspended with prewarmed media199 HEPES containing supplements (2mgl/ml Insulin-Transferrin-Selenium, 2mM Carnitine, 5mM Creatine, 5mM Taurine and 10mM sodium pyruvate, 2ml of antibiotic 10000 units/ml penicillin/ 10mg/ml streptomycin) in addition 10mM 2,3-butanedione monoxime (BDM). The cells were washed twice with media to remove the normal Tyrode and dead cells. The cellular suspension was gently mixed by pipetting up and down till the suspension became homogenous. The ventricular cardiomyocytes were cultured in six-well plates. 2ml of cellular suspension was added to each well and the cells incubated for up to 48 hours at 37˚C with 5% CO_2.

Cultured ventricular cardiomyocytes were treated with 200nM UII (rUII or hUII) or 10µM phenylephrine was used as positive control to induce hypertrophy (Gan *et al.* 2005). Untreated cells were used as controls and media was replaced after 24 hours.

2.3.2 Measurement of ventricular cardiomyocyte hypertrophy

The hypertrophic effect of UII was studied. In order to do this, effect of UII was compared to phenylephrine in cultured adult rat ventricular cardiomyocytes. Sarcomeres are usually added in parallel in case of pathological hypertrophic growth (Dorn *et al.* 2003), and cells become wider. Therefore, length/width ratio was measured. The morphology and dimensions of single ventricular cardiomyocyte were quantified from light image after treating cells with UII or phenylephrine. The dimensions of length and width for ventricular cardiomyocytes were measured with straight line ImageJ software. The length width ratio was calculated, which was seen to get smaller with pathophysiological hypertrophy, as the width increases.

2.3.3 Cultivation of Chinese Hamster Ovary Cells expression human UT (CHOhUT)

Ham's nutrient mixture F-12 media was used to cultivate CHO cells stably expressing the human UT receptor (CHO_{hUT}) (University of Ferrara, Italy). Frozen cells were thawed quickly by immersion of a vial of cells at 37˚C in a water bath, 9ml of the growth media was added and the tube spun down at 125g for 10 minutes. The supernatant was discarded and the pellet resuspended with media in T-75 culture flask. When the culture reached 80-90% confluent the media was removed, cells were washed with phosphate buffered saline (1x) (PBS). Pre-warmed trypsin EDTA was added and incubated at 37˚C for 1 minute. 10ml of normal media were added to stop digestion of cells and the cells centrifuged at 1,200g for 3 minutes. The supernatant was discarded and the pellet was resuspended with 10ml growth media. The suspended cells were sub-cultured in growth media as ratio (1:3) in 6 well plates, at 37˚C in 5% CO_2 in an incubator. The cells were used experimentally when they reach to 90% confluency to study signal pathway of UII to induce hypertrophy.

2.4 Molecular biology

2.4.1 Reverse transcription polymerase chain reaction (RT-PCR)

2.4.1.1 Theory

Fluorescence-based real-time reverse transcription is an excellent technique used for the quantification of ribonucleic acid (RNA). The main methods commonly used in molecular biological studies for extraction RNA are, solid phase RNA extraction, the sample is degraded using a particular lysis buffer which is applied to a glass fiber filter column. RNA is absorbed to the column at a particular pH (pH $\geq$7). Washing buffer is used to remove undesired compounds such as protein, and the RNA eluted from the column in a purified state using water or Tris-EDTA buffer. The other method which was used in this study is the Guanidinium thiocyanate phenol-chloroform, Total RNA is isolated from cells and tissues. Genomic DNA (gDNA) is eliminated from using DNase enzymes to provide "clean" RNA free from gDNA contamination.

Particular primers are used for synthesis of complementary DNA (cDNA) from extracted RNA by reverse transcription, which is required as template for RT-PCR assays. The first

step of RT-PCR reaction is converting of mRNA to cDNA by using specific primers (Bustin 2000). RT-PCR reaction can be carried out in single-step (one-step) or a two-step approach, the two-step approach was utilised here. In single step qPCR reaction, reverse transcription and subsequent qPCR reaction are likely to perform in the same well. Advantage of this method is reduced risk of contamination but the cDNA synthesis cannot be stored for further uses, to study a wide range of genes of interest instead of one gene. The two-step approach includes firstly creating cDNA in separate tube (separate reverse transcription). Then a portion of the cDNA is transferred into separate well and used in qPCR assays. The main advantage of this method is that it enables the cDNA to be stored for later use. Additionally, using the two-step method the study of several genes of interest from a single sample (Bustin 2000). To ensure no contamination with genomic DNA, RT^- samples, in which a mock reverse transcription containing all reverse transcription reagents except reverse transcriptase enzyme. Any amplification in RT^- control indicate of genomic DNA contamination.

Amplification of cDNA is carried out at different temperatures which can largely be divided into three steps. In brief, melting of the double-stranded DNA result in two single stranded DNA templates. The next step is annealing of primers, primers bind specifically to the template DNA at low temperature. Complimentary strand is produced to the single stranded DNA by exploiting DNA polymerase enzyme, it binds to target DNA sequence. DNA polymerase adds deoxyribonucleotide 5'-triphosphate to the 3' hydroxyl group (Figure 2.10). The relative quantity of DNA is detected at the end of each RT-PCR cycle using a fluorometric probe, and the amount of emitted florescence is directly proportional to amount of products generated in each cycle.

A threshold quantity of florescence is determined for each reaction (cycle threshold (C_t)), and this value is inversely related to the amount of starting template. The C_t value of the gene of interest (GIO) is normalised to a reference gene (RG), to generate a delta C_t (ΔC_t) value which represents the difference in cycle thresholds between GIO and RG (Figure 2.11). Reference gene is an internal reaction control used in qPCR assays, which is used to compare the expression of gene of interest to that reference gene. The reference gene can be connected covalently to a different fluorescence molecule (fluorophore) so that the fluorescence molecule can emit a fluorescence at different wavelengths to that of the fluorescence molecule on the probe for the gene of interest thereby the detection system

of the PCR thermal cycler can differentiate between the two genes. Expression of reference gene should be unaffected by experimental treatment.

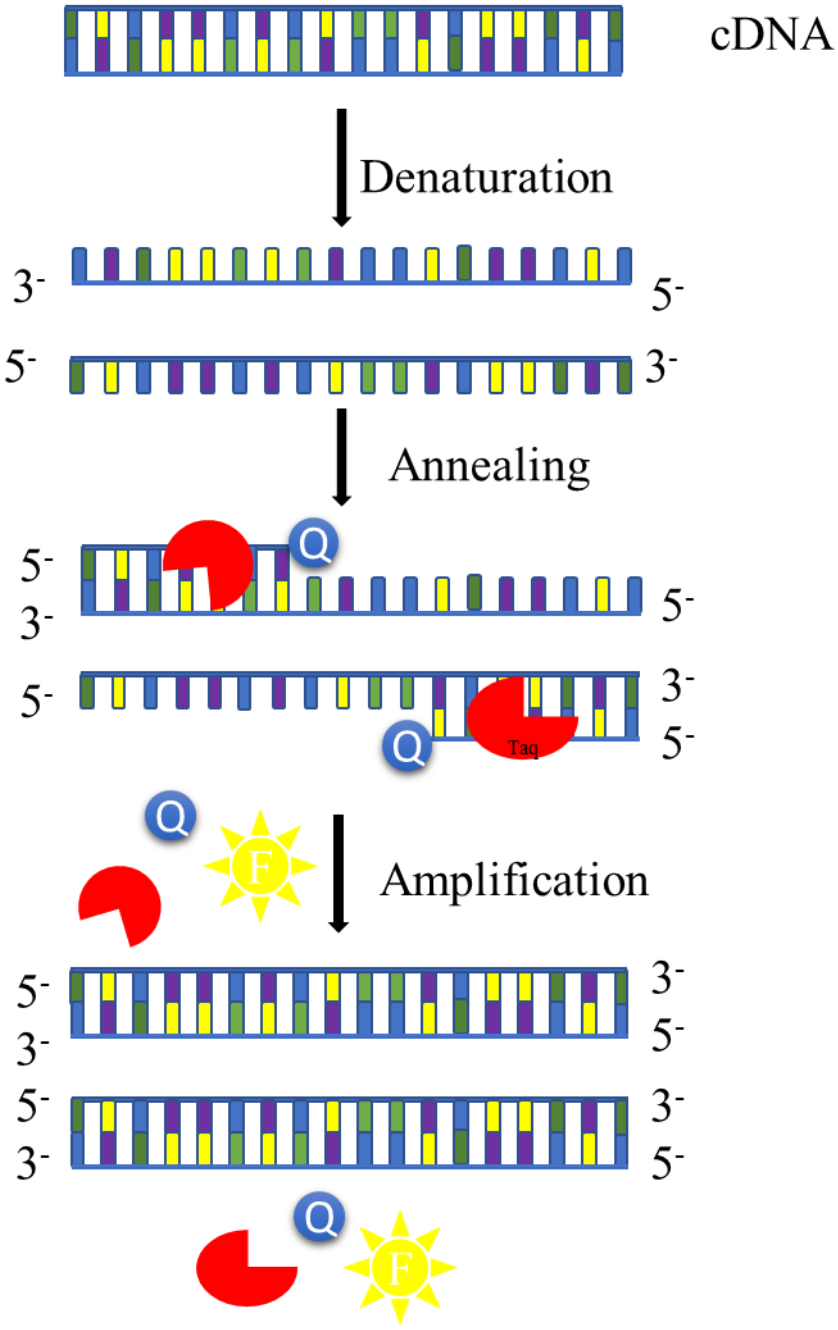


Figure 2.10: A schematic representation of RT-PCR.
RT-PCR product accumulating is detected, using a Taqman probe. Primers and specific probe are able to bind to its target sequence. Template strand is replicated by DNA polymerase. Cleavage of the probe causes release of quencher and fluorophore due to activity of polymerase, resulting in a measurable fluorescent signal its intensity directly proportion with amount of RT-PCR products.

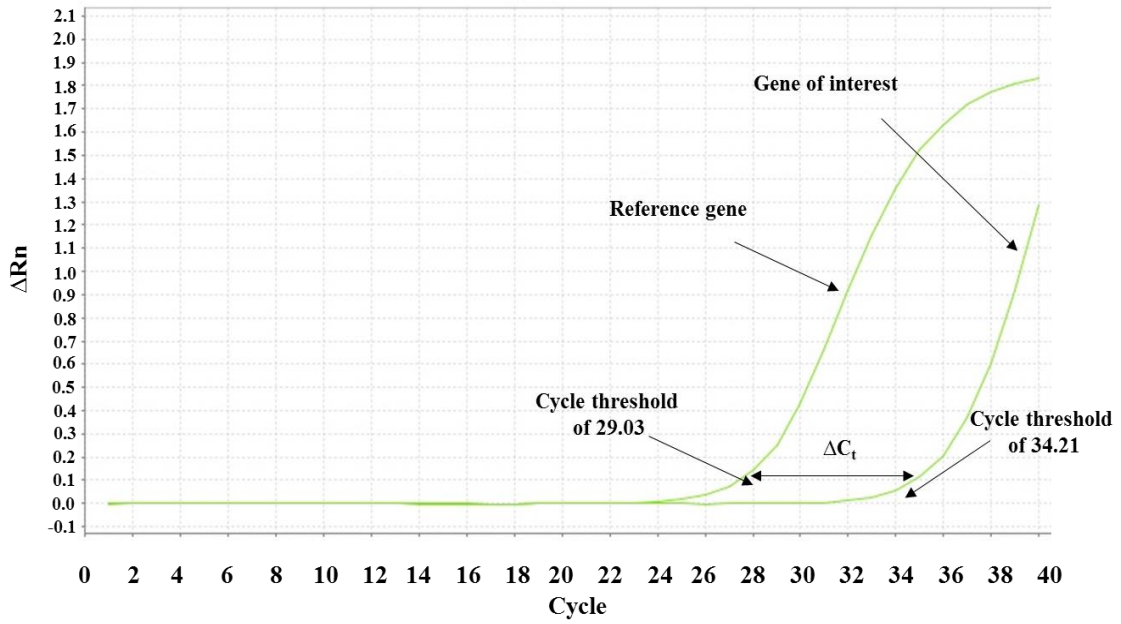


Figure 2.11: The internal amplification RT-PCR curves.

Curves were obtained from reactions of the RT-PCR assay for control and treated rat ventricular cardiomyocytes included in this study. RT-PCR graph showing cycle threshold (C_t) for gene of interest and reference gene. Delta Rn (ΔRn). The ΔC_t resulted from the difference between these two values, in this case 34.21 - 29.03 = 5.18.

2.4.1.2 TaqMan™ probes

TaqMan™ probes are specific hydrolysis probes that are designed to bind to a target region of interest and generate a fluorescent signal, used in RT-qPCR assays to increase specificity during experiments. The TaqMan™ probe contains a fluorophore covalently linked to the 5'-end of the nucleotide probe sequence and a non-fluorescent quencher molecule (minor groove binder) at the 3'-end of the probe (Kutyavin *et al.* 2000). There are several types of fluorophores available such as 6-carboxyfluorescein FAM and VIC 2′-chloro-phenyl-1,4-dichloro-6-carboxyfluorescein dyes and a popular non-fluorescent quencher is 6-carboxy-terta-methyl-rhodamine (TAMRA) dye. The quencher is often close to the fluorophore so that the role of quencher is to suppress the fluorescence emission from the fluorophore. The quencher molecule can quench the fluorescence intensity that is emitted by the fluorophore when excited by the qPCR cycler's light source through Fluorescence Resonance Energy Transfer (FRET). In qPCR assays the activity of *Taq* polymerase the elongation stage of the reaction cleaves the quencher molecule from the probe leading to an increase in the intensity of the fluorescence signal. Each target sequence is detected by specific TaqMan™ probes, specific for the genes sequence (Marras 2006).

2.4.1.3 Methods

2.4.1.3.1 Total RNA extraction

2.4.1.3.1.1 Mouse cardiac tissue

Phenol/chloroform method is one of several protocols used for the extraction of RNA. Cardiac tissue (atria, right and left ventricles) taken from mice were stored in 1ml RNAlater at 4˚C till use. 1ml Trizol (Tri reagent solution) was added per 50-100 mg tissue and TissueRuptor (Qiagen) was used for homogenizing the tissues.

2.4.1.3.1.2 Rat ventricular cardiomyocytes

The isolated rat ventricular cardiomyocytes were centrifuged at 1,000g and the resulting pellets lysed by adding 1ml Trizol per 5-10 million cells, to solubilize the cardiomyocytes. The sample was triturated using a pipette till the sample became homogenous. The lysed cells or tissues were frozen at -80˚C until RNA extraction.

Lysed cells or tissues were thawed at room temperature. 200µL of chloroform was added. Then the sample was mixed by a vortex (vortex-2 Genes, Scientific Industries) for 2 minutes until the sample converted to milky solution and incubated for 3 minutes at room temperature, then centrifuged at 16,000g for 15 minutes at 4˚C. The first layer (upper layer) was removed to a new microcentrifuge tube, this aqueous layer contains RNA, followed immediately by adding 500µL isopropanol and the sample was vortexed, it was left to incubate for 10 minutes at room temperature then centrifuged for 10 minutes at 4˚C. The supernatant was discarded and the pellet was resuspended by adding 70% ethanol at a volume 1ml. The sample was centrifuged at 16,000g for 5 minutes at 4˚C then the supernatant was removed and the pellet allowed to dry for few minutes at room temperature. The pellet was resuspended by adding 50µL of PCR grade water and vortexing (Figure 2.12).

RNA extraction was immediately followed by assessment of the quality of total RNA using Nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific). The spectrophotometer was set up and blanked using 3µL of RNase free water. Concentration of RNA was measured by pipetting 3µL of sample. Generally, the ratio A260/280 and A260/230 are used to determine contamination level of samples with proteins or solvents such as phenol. The A260/280 ratio is >1.8 and A260/230 is around 2 which indicate adequate purity of the sample. All samples were stored at -80˚C until required.

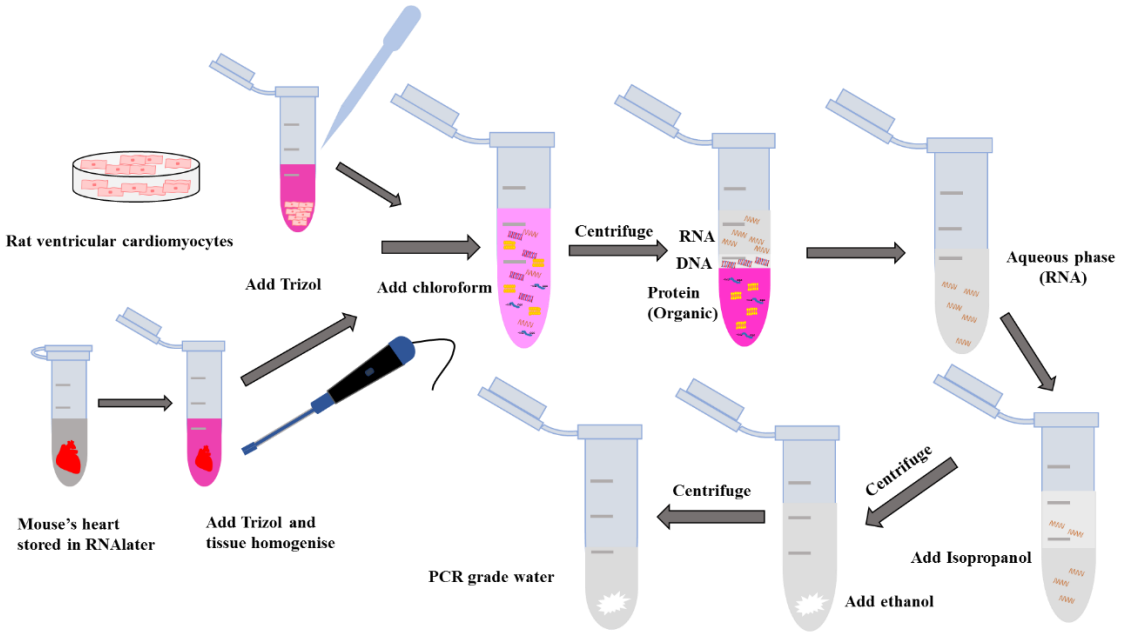


Figure 2.12: Schematic diagram of total RNA extraction.
Figure summarises steps of RNA extraction using phenol-chloroform extraction protocol.

2.4.1.3.2 RNA cleaning up

The total RNA samples isolated from rat ventricular cardiomyocytes or mouse cardiac tissues were processed to remove possible genomic DNA. Briefly, the frozen sample of total RNA and RNA kit were thawed on ice. The mixture was prepared according to equation 5. 5µg RNA sample was cleaned up and volume of sample should be added was calculated by using this below:

$$\textbf{Volume of sample} = \textbf{Weight/Concentration} \ldots\ldots\ldots \textbf{(5)}$$

The calculated volume of RNA sample (5µg) was added to 0.2ml tube then 5µL of DNase buffer and 1µL of DNase enzyme were added respectively. The volume of mixture was made up to a final volume of 50µL by adding water. The mixture was vortexed and centrifuged at 10,000g for 1 minute. The sample was then placed in a Mastercycler thermocycler (Eppendorf) at 37˚C for 30 minutes, after which the reaction was stopped by addition 5µL of inactivation agent and mixed by vortex. The supernatant was removed to a clean tube and stored at -80˚C to be ready for using for reverse transcription.

2.4.1.3.3 Reverse Transcription

Total RNA sample was reverse transcribed by using reverse transcription kit in a total volume 20µL. The reverse transcription mixture was prepared as follows; 1µL Multiscribe Reverse Transcription, 1µL RNase inhibitor, 0.8µL 25X dNTP mix (100nM), 2µL 10X Reverse transcription buffer, 2µL 10x Random Primers and 3.2µL PCR grade water. The negative control mixture was made by substituting the Multiscribe Reverse Transcription with PCR grade water. The mixture was vortexed and 10µL of total clean RNA samples was added into each positive or negative Eppendorf tube and the contents were mixed twice by pipetting the contents up and down. The positive and negative tubes were spun down using a mini centrifuge (Technico Mini) to eliminate air bubbles. The tubes were placed in a Mastercycler thermal cycler, programmed for cycles at 25˚C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and then held at 4°C. Then, the cDNA was stored in the freezer at -20˚C prior qPCR assay or proceed to qPCR (two-step approach).

2.4.1.3.4 qPCR

TaqMan® probes were used to assess gene expression using stepone plus software (Applied Biosystems). TaqMan gene expression probes and cDNA samples were thawed on ice and vortexed once defrosted prior to centrifugation for 30 seconds. 10µL of TaqMan gene expression Master mix was placed into PCR strip tube (Applied Biosystems, Life Technologies), 1µL of gene of interest (Uts2r (Rn00571932_s1) or Uts2r (Mm00520770_s1)), 1µL of reference gene and 6µL of PCR grade water. Then 2µL of cDNA sample was added to the mixture and spun by mini centrifuge to eliminate all air bubbles and to spin down the contents at the bottom of reaction tube. All samples were run in duplicate and non-template control for each sample was tested on single well. Reactions were run under the following cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds and 60°C for 1 minute, as incubated in accordance with the manufactures protocol.

Normalisation of target gene expression levels was performed for multiple reference genes (Table 2.1). Geometric mean value resulted from analysis data was used to evaluate the expression stability of candidate housekeeper genes using NormFinder (Latham 2010). The candidate reference genes used in rat experiment or mouse experiment were based on previously published data on the expression stability of these genes across these tissues.

The effect of UII (200nM) or phenylephrine (10µM) on UT mRNA was determined by normalising expression level of a specific target gene using $\Delta\Delta C_t$ according to this equation below:

$$\Delta\Delta C_t = \Delta C_t \text{ (treated cells)} - \Delta C_t \text{ (untreated cells)} \quad \text{..... (6)}$$

The data are expressed as a fold change expression $2^{-(\Delta\Delta C_t)}$.

A

Rat reference gene	Gene symbol	Applied biosystems assay I.D.	Amplicon length
β-actin	ACTβ	Rn00667869_m1	91
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Rn99999916_s1	87
β-2-microglobulin	β2M	Rn00560865_m1	58
Hypoxanthine phosphoribosyltransferase 1	HPRT1	Rn01527840_m1	64
Peptidylprolyl isomerase A	PPIA	Rn00690933_m1	149
Processing of precursor 4	POP4	Rn02347225_m1	93

B

Mouse reference gene	Gene symbol	Applied Biosystems assay I.D.	Amplicon length
β-actin	ACTβ	Mm00607939_s1	115
β-2-microglobulin	β2M	Mm00437762_m1	77
β-glucuronidase	GUSβ	Mm00446954_g1	91
Hypoxanthine guanine phosphoribosyl transferase	HPRT	Mm00446968_m1	65
Processing of precursor 4	POP4	Mm00481282_m1	94
Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta	YWHAZ	Mm03950126_s1	88

Table 2.1: Selected candidate TaqMan gene expression probes used in PCR.
(A) Table shows general information about housekeeping genes used in this investigation as reference genes for normalization to assess the UT mRNA expression in rat ventricular cardiomyocytes.
(B) Table shows general information about housekeeping genes used in this investigation as reference genes for normalization to assess the UT mRNA expression in mouse hearts.

2.4.2 Protein determination

Protein concentration of cell lysates were determined using a Lowry assay, a simple and common method for estimation of total protein. Bovine serum albumin was used as a protein standard. 1mg/ml of BSA stock solution was made up in 0.1M NaOH and different concentration of protein standards (0, 50, 100, 150, 200 and 250µg/ml) were prepared by mixing stock solution and 0.1M NaOH. 500µl of a set of diluted protein standards was pipetted out into different test tubes. Samples were also diluted with 500µl of NaOH, these unknown protein samples were replicate in dilution ratios 1:50 and 1:100. 2.5ml of color reagent mixture which consisted of: A ($NaHCO_3$ in 0.1M NaOH), B (1% $CuSO_4$) and C (2% Na^+ K^+ tartrate) made to the ratio 100:1:1, was added to all samples and standards and mixed well by vortex. The mixtures were incubated for 10 minutes at room temperature, followed by the addition of 250µL of Folin's reagent to all tubes (standards and samples) and vortexed. Then, the standards and samples were incubated for 30 minutes at room temperature to allow development of color. The absorbance was measured at 750nm after zeroing on the blank 0.1M NaOH (zero protein standard) using a spectrophotometer (Eppendorf). A standard curve was established from absorbance values for known concentration of BSA protein standard (Figure 2.13). The unknown sample protein concentration was assessed by comparing the absorbance to that of a serial dilution series of BSA standard.

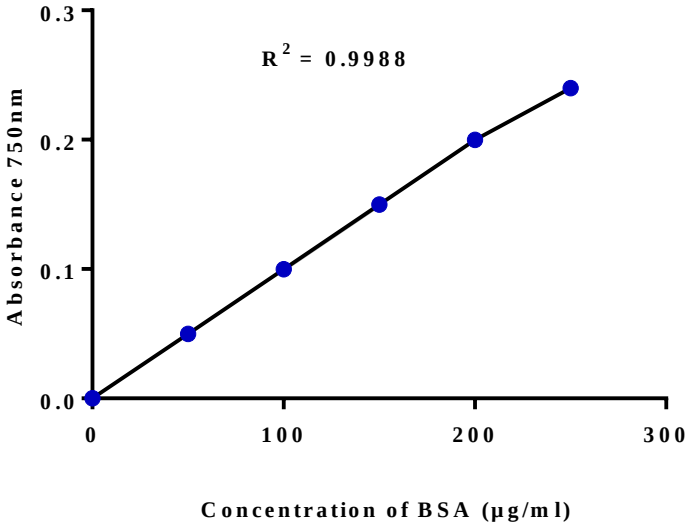


Figure 2.13: Lowry protein assay standard curve.
The curve was generated using bovine serum albumin (BSA) at 0, 50, 100, 150, 200 and 250µg/ml. A calibration was before every time protein was estimated.

2.4.3 Western Blotting

2.4.3.1 Theory

MAPK signalling pathways involve a sequence of protein kinase cascades which control among other things cell proliferation, and be measured using Western blotting, a ubiquitous fundamental technique to detect proteins from mixtures typically extracted from tissue samples. Cells were subjected to foetal bovine serum (FBS) starvation for 24 hours. Basal levels of MAPK phosphorylation are minimized due to remove of FBS, which allows the cellular mechanism of this pathway and its activation by agonists to be studied.

Cell lysate is mainly used in western blotting through lysis of cells with detergents and enzymes to ensure collecting of all the cytosolic proteins. Lysis buffer contains protease inhibitors which inactivate cellular proteolytic enzymes in order to prevent degradation of extracted proteins. The protein sample is further diluted with loading buffer which contains the following: 2M dithiothreitol (DTT) and sodium dodecyl sulphate (SDS) loading buffer, mixed to the ratio 100:900. Bromophenol blue is used to monitor progress of the sample moving through the gel while SDS has a detergent property can induce denaturation of protein. Extracted protein samples are heated to a high temperature in order to allow denaturing of the higher-order structure of protein. This denaturation process facilities movement of charged proteins through acrylamide gel (Figure 2.14) when driven by an electrical field (Mahmood and Yang 2012).

The Western blot gel is composed from two layers, each layer has a different function. The top layer is referred to as the stacking gel and the bottom layer is the resolving gel. The stacking gel has a low concentration of acrylamide and a pH of 6.8 and resolving gel is slightly basic (pH 8.8), this ensures the proteins are separated based on their molecular weight, the resolving gel has a high concentration of acrylamide (10%) it makes the pores of gel narrow.

Following this electrophoretic separation proteins are transferred from the gel to a nitrocellulose membrane by the application of an electrical field directly across the acrylamide gel (Figure 2.15). In order to obtain clear image, the acrylamide gel and blotting membrane should be in close contact each other. Both wet transfer system and semi-dry transfer system are often used.

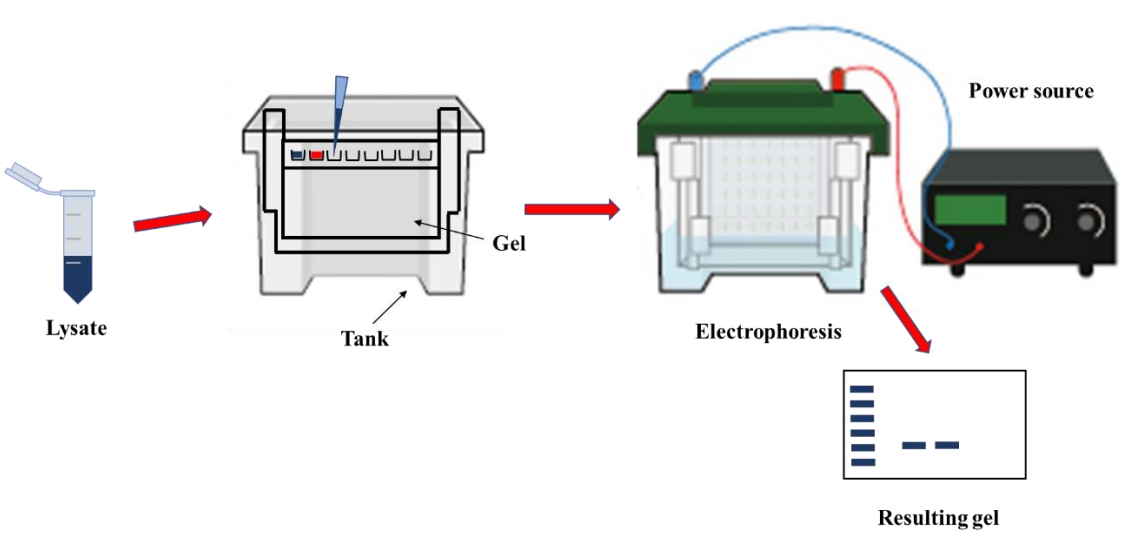


Figure 2.14: A schematic representation of a SDS-PAGE gel electrophoresis.
Figure shows step of electrophoresis of proteins through the gel according molecular weight.

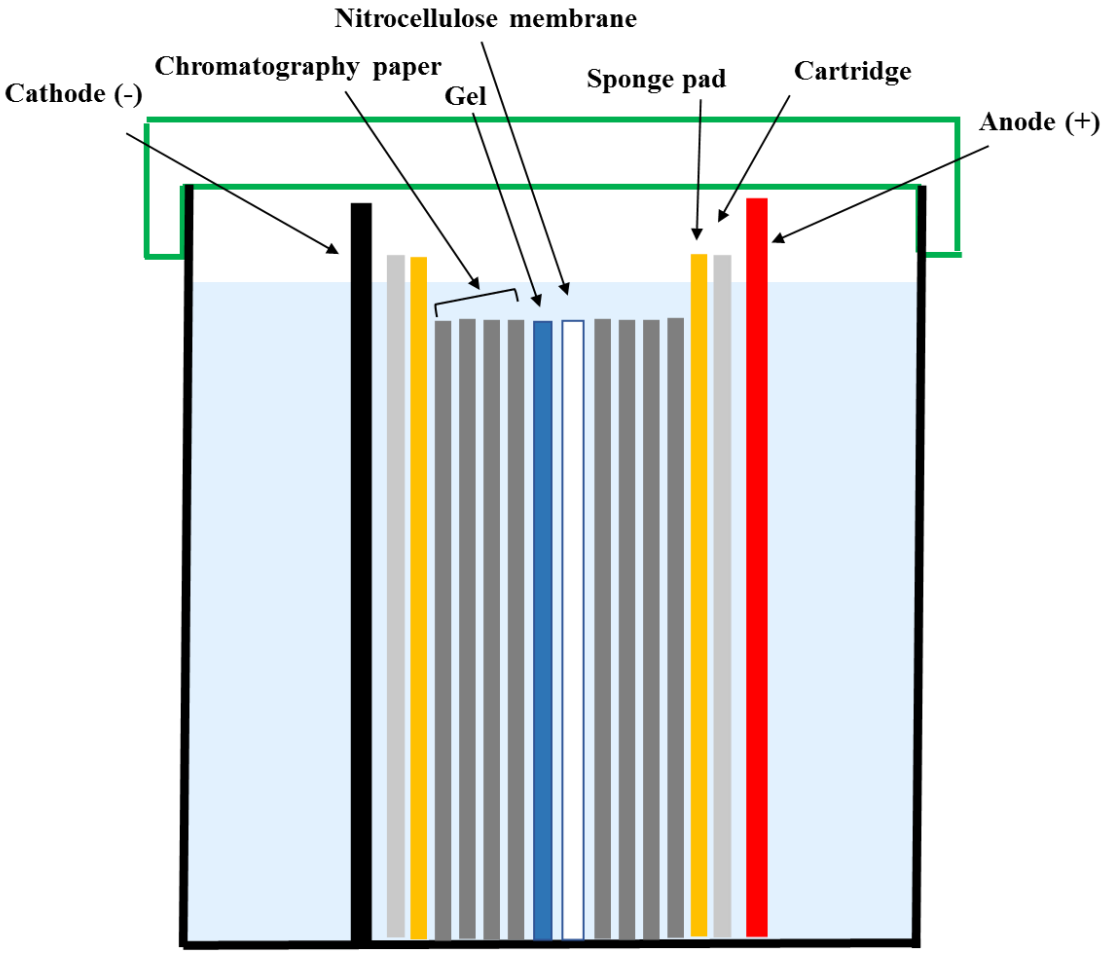


Figure 2.15: The setup of the sandwich cassette in the electrotransfer tank.
Proteins migrate from the gel to the blotting membrane, wet transfer method used in this study.

Following the transfer of the protein, the blotting nitrocellulose membrane is incubated in a blocking solution (tris-buffered saline with tween 20 (TBS-T) and 10% dry milk), to reduce non-specific binding of antibodies. The primary antibody used in western blot to detect the protein of interest (phospho-p38, phospho-ERK1/2, phosphor-JNK and phosphor-CaMKII, total p38, total ERK1/2, total JNK and total CaMKII) are diluted to working concentrations in 5% BSA-TBST and incubated with the blotting membrane at (4˚C) overnight. The nitrocellulose membrane was then washed with TBS-T to remove any unbound antibody, as a result reduces background interference.

Following washing the blotting membrane is incubated with horseradish peroxidase (HRP) conjugated anti-rabbit IgG secondary antibody which is targeted to the primary antibody. Combination of conjugated secondary antibody with an appropriate substrate can generate a detectable signal (Figure 2.16).

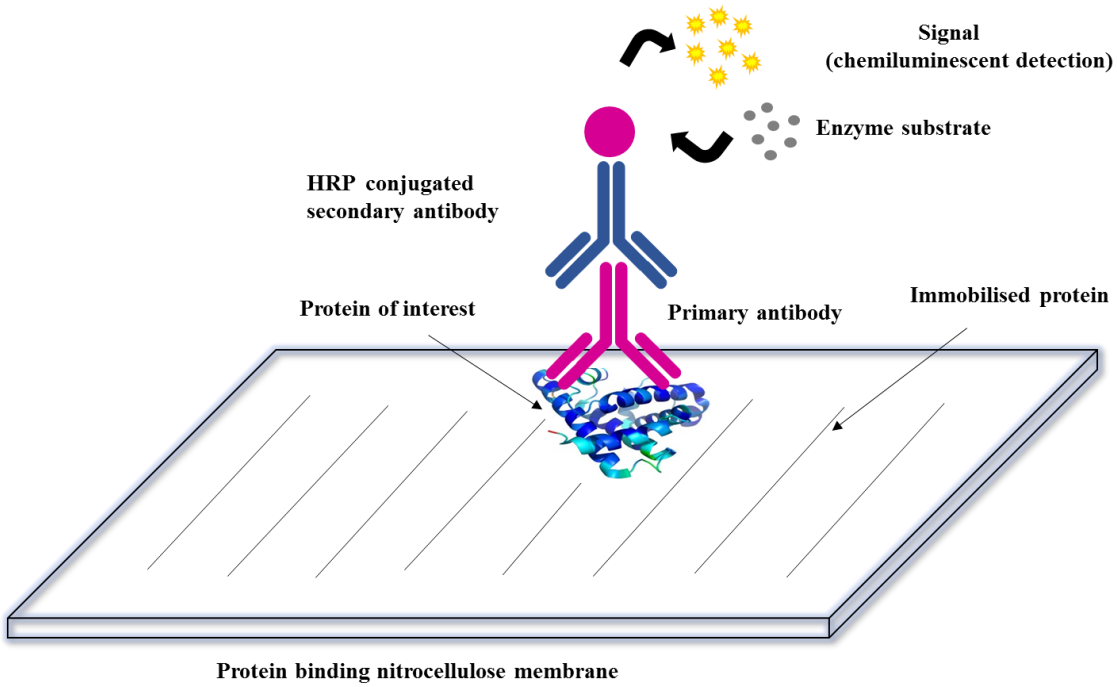


Figure 2.16: A primary antibody binds to target protein on the blotting membrane. Secondary antibody which is targeted to the primary antibody, a chemiluminescence signal will be produced when the secondary antibody conjugate is exposed to the enzyme substrate.

2.4.3.2 Methods

In this study, the signalling pathway mediated by UII was studied using two type of cells, CHO_{hUT} cells and ventricular cardiomyocytes.

CHO_{hUT} were cultured in 6 well plates and allowed to adhere and grow to 90% confluence. Cells were starved for 24 hours with serum free media before stimulation, cells were subjected to foetal bovine serum starvation. Media were then replaced by pre-warmed Krebs buffer (2ml each well) and incubated for 15 minutes at 37˚C in 5% CO_2. The cells were incubated with UII (200nM) for various time points. Basal levels of MAPK were measured in the absence of UII. Reactions were terminated by aspiration of the Krebs buffer and addition of 200µL ice cold RIPA buffer, for 2 minutes on ice.

Isolated ventricular cardiomyocytes were cultured in 6 well plates using media 199 plus HEPES. After 24 hours, the cultured cardiomyocytes were stimulated with 200nM UII for different durations to obtain a time course for activation of signaling proteins. Cells were centrifuged at 1,000g for 1 minute using a mini-centrifuge (Eppendorf). Media was aspirated and the cell pellet resuspended in 100µL of ice cold RIPA buffer. Lysates were incubated for 30 minutes on ice, then centrifuged at 13,300g for 10 minutes at 4˚C. Supernatants were then transferred into new Eppendorf tubes. Protein concentration of lysates was measured and samples were diluted with loading buffer to a concentration of 60µg/µL. Following this, the diluted samples were denatured at 95˚C for 5 minutes. Samples can be used immediately and loaded into SDS acrylamide gel or stored at -20˚C until required.

To identify the target protein, 40µL of denatured protein alongside 3µLof blue protein ladder and biotin ladder run through 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) mini-gels. A tank was filled with running buffer and gels were loaded with samples and run at a constant voltage 150V for 1.5 hours. Immediately after electrophoresis of the protein samples, the gels were transferred onto a nitrocellulose membrane (Fisher Scientific) via an overnight wet transfer at 30V at room temperature. After overnight transfer, the nitrocellulose membranes were blocked in a 10% skimmed milk powder and TBS-T solution for 2 hours. The membranes were washed with TBS-T (2-3) times. Then, the membranes were incubated overnight with the primary antibody solution of interest, such as phosphorylated-ERK1/2 antibody (p-ERK1/2) (1µl in 6ml

TBS-T), phosphorylated-p38 (p-p38) (1µl in 3ml TBS-T), phosphorylated-JNK (p-JNK) (1µl in 3ml TBS-T) and phosphorylated CaMKII (p-CaMKII) (1µl in 3ml TBS-T), maintaining overnight at 4˚C with gentle agitation. Following incubation process with the antibody of interest, the membranes were washed for 30 minutes with TBS-T to remove unbound antibody, the washing process being repeated six times each 5 minutes. The nitrocellulose membranes were afterwards incubated in the appropriate secondary antibody, anti-rabbit IgG secondary antibody with horseradish peroxidase for 1 hr diluted in TBS-T (10µL in 10ml) containing 5% skimmed milk powder and 10µL of anti-biotin secondary antibody. Incubation was at room temperature with gentle agitation. Whereupon the membranes were subsequently washed with TBS-T six times for 30 minutes.

The density of immunoreactive bands was visualised and detected using a Biorad ChemiDoc^{TM} MP imager (BIO-Rad), utilising a chemiluminescence technique. Enhanced chemiluminescence (ECL) reagent applied and coated the nitrocellulose membrane for 1 minute. Excess ECL reagent was drained off.

Following detection of the phosphorylated protein, the excess ECL reagent was removed by washing the nitrocellulose membrane in TBS-T three times for 15 minutes. The membranes were then incubated with stripping buffer for 15 minutes to remove the antibody. The membranes were again washed in TBS-T three times for 15 minutes and reblocked in 10% skimmed milk TBS-T solution for 1 hour at room temperature before incubation with antibody. Following several washings in TBS-T buffer, the membranes were incubated overnight at 4˚C with appropriate new antibody specific to the total protein of interest (1µl in 3ml TBS-T). The process was again repeated, which as mentioned above and levels of total protein were quantified and used for normalisation of the target phosphorylated protein.

2.5 Ex-vivo isolated Langendorff heart

2.5.1 Theory

The Langendorff perfused mammalian heart assay permits the simultaneous determination of cardiac inotropism, chronotropism and coronary flow. The aorta is cannulated and the heart is retrograde perfusion with oxygenated perfusate. The perfusate is delivered to the heart down the aorta in the opposite direction to normal blood flow

causing closure of leaflets of aortic valve, shutting of the aortic valve lead to flowing of the perfusion solution via the coronary ostia (right and left) located along the circumference of the aortic root. Then, the perfusate flows through vascular beds and drains to the coronary sinus in the right atrium through the coronary veins. During the procedure, the ventricle chambers are entirely empty and without any fluid filling them.

2.5.2 Method

Isolated hearts were immersed in $0Ca^{2+}$ Tyrode solution (4˚C) in order to arrest beating and to minimise any risk of ischemic injury. The hearts were then immediately mounted onto a cannula by the aorta and held in place with a small artery clip (Figure 2.17). The cannula should be situated above the coronary ostia to avoid rupture of the aortic valve leaflets. A suture was tied tightly around the aorta and the artery clip was removed. It is necessary to ensure that both the cannula with perfusate glass vessels are filled with perfusate and completely free from air bubbles.

In order to monitor the heart rate (HR) and left ventricular developed pressure (LVDP), a deflated plastic wrap balloon size 3-4mm, connected to a pressure transducer by a catheter, which was calibrated to mmHg using a conventional sphygmomanometer (previously done by members of our group). The balloon catheter was inserted gently through the opened left atrial appendage and mitral valve into the left ventricle. The balloon was inflated with perfusate solution using a syringe by opening a stopcock and forcing fluid into the balloon to gradually increase the preload left ventricular end diastolic pressure between 6-8mmHg and to give a maximum systolic pressure of 80mmHg. The rhythmic force of contraction exerted by the heart on the balloon was transferred through the pressure transducer and consequently recorded continuously throughout the experiment and visualised on a computer monitor and this readout was used to calculate heart rate. To maintain a normothermia, hearts are maintained in a warm water jacketed organ bath filled with perfusate solution.

Hearts were perfused at a constant flow of 10ml/minute with oxygenated normal Tyrode for 15-20 minutes to reach steady-state. After stabilisation of the heart rate and left ventricular developed pressure were measured for 5 minutes (Figure 2.18). Subsequently, the hearts were switched to UII (50nM) for 10 minutes and the contractile parameters

were recorded then the hearts were washed out with normal Tyrode for 30 minutes followed by reperfusion again with 50nM UII for 10 minutes.

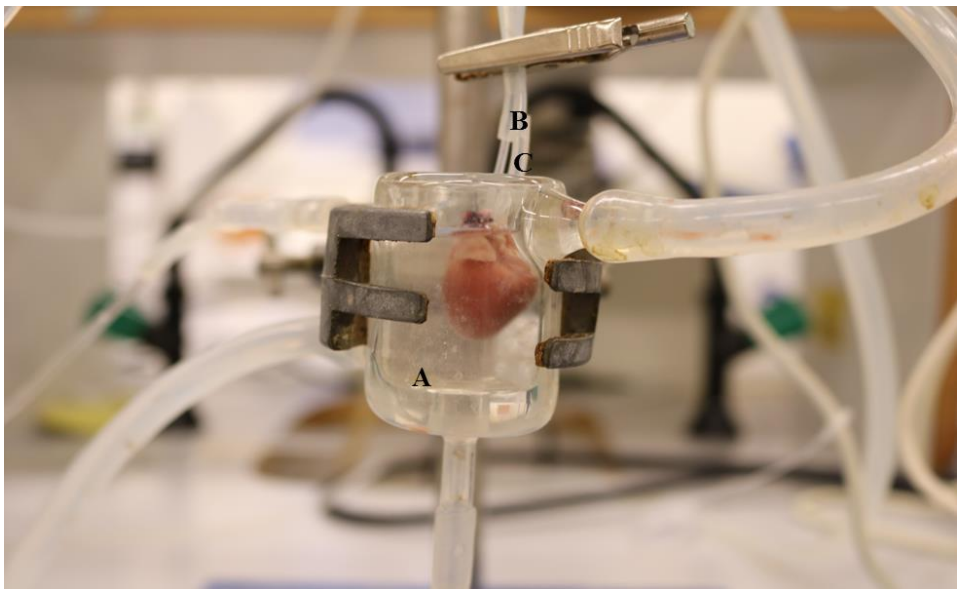


Figure 2.17: Isolated rat heart perfused in the Langendorff mode.
(A) The heart was immersed in an organ bath.
(B) The aorta was cannulated.
(C) Intraventricular balloon was inserted in the left ventricle.

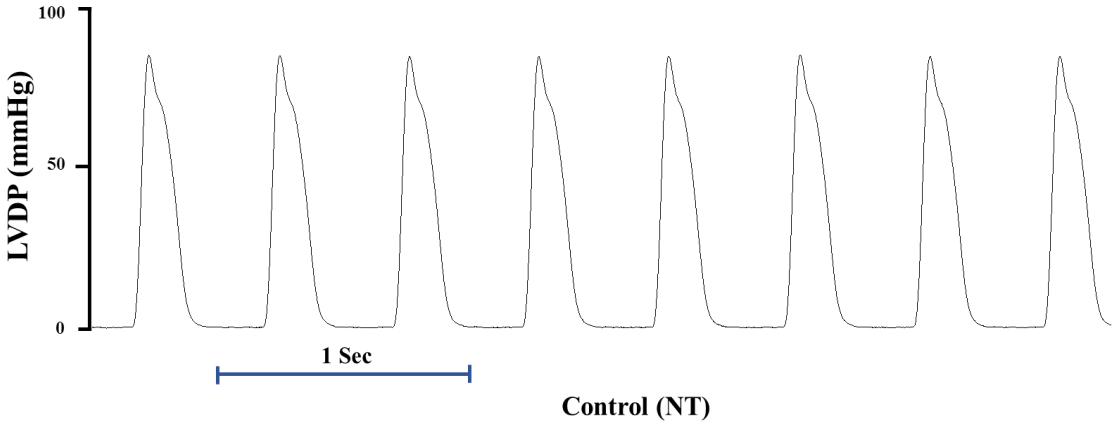


Figure 2.18: Representative pressure trace from rat ventricle (normal Tyrode).
Pressure recorded by the LV balloon upon ventricular contraction.

2.6 Electrophysiological recordings

2.6.1 Theory

In 1976, Neher and Sakmann developed a patch clamp recording technique which uses a glass microelectrode to study single or multiple ion channel current activity in mammalian cells. Different patch clamp measurement configurations are available and can be used depending on topic of interest including cell attached patch (CAP), excised patch (inside-out patch (IOP) and outside-out patch (OOP)) and whole cell (WC). Whole cell was used in this research to record the action potential duration and (APD) and single currents (voltage dependent L-type calcium (I_{Ca})) in ventricular cardiomyocytes.

2.6.1.1 Electrophysiology setup

Ventricular cardiomyocytes were placed in a diamond superfusion chamber and mounted on the stage of inverted microscope (Nikon eclipse, TE200) and bathed with normal Tyrode. Cells were left 5-10 minutes to allow them to settle and then perfused with normal Tyrode at rate of 2-4 ml/minute using a gravity fed perfusion system. Perfusion solutions in the chamber were maintained at 35 ± 1˚C with the aid of a preheater system (Single Inline Solution Heater, SH-27B, Warner Instruments). The microscope was installed on a steady anti-vibration platform and covered with a Faraday cage in order to minimise external electrical nose. The patch electrode was connected to a pre-amplifier (headstage) (Axon CNS, HS-9A x0.1) moved in a straight line along three axes over the sample chamber by a micromanipulator (Scientifica, ACCi UI). A silver wire coated with silver chloride (Ag/AgCl) was used to make contact between patch glass microelectrode filling solution and pre-amplifier and to act as an earth (zero potential) for the bath solution. Therefore, all voltage and current clamp recordings were made in respect to earth potential (zero mV).

2.6.1.2 Glass pipette

In the patch clamp technique, glass microelectrodes were made from filamented capillary glass. Borosilicate glass capillary tubes (1.5mm O.D., Harvard apparatus) were used and pulled using a two-step microelectrode puller (Narishige PB-7). To obtain low electrode capacitance, thick-walled glass was used because capacitance is inversely proportional to a thickness. Typical patch pipette resistances of 12-15MΩ were used for recording action potentials and restricted dialysis of the cell content. However, low-resistance patch

pipettes of 2-4MΩ were used to measure ion currents during voltage clamp. It is necessary to use low resistance pipettes which allow rapid clamping of the membrane potential. Accordingly, errors in measurement of voltage and current are minimised during the experiment via decreased the size and time constant in series resistance errors (Ogden 1994).

2.6.2 Methods

2.6.2.1 Current Clamp; Action potential recording

Electrical recordings of membrane potentials under current clamp in the whole cell configuration, were measured using an Axon integrated Patch clamp instrument (AXOPATCH 200B) and high-resolution, low-noise data acquisition system (DIGIDATA 1322A). Collection and analysis of data were made using Clampfit 10.4 software. The patch pipettes were filled with K^+ electrode solution (see section 2.8.1) and had a high resistance of 12-15MΩ. Super threshold pulses were applied to obtain a consistent firing of action potential (1.5 times threshold for 5ms). Action potentials were elicited at 1Hz by continuous stimulation.

For action potential recordings, cardiomyocytes were perfused with normal Tyrode for 10 minutes. Following forming the whole cell configuration, the initial data were immediately collected once a steady state was reached. The cells were superfused with 200nM of UII for 10 minutes, sufficient to reach a maximal response and steady state. During experiments all action potentials were continuously monitored on computer screen. Action potentials were recorded for analysis at the end of perfusion for each solution. 10 consecutive action potential sweeps were averaged and analysed by Clampfit 10.4. Action potential durations were measured at 30% (APD_{30}), 50% (APD_{50}) and 90% (APD_{90}) repolarisation (Figure 2.19). Action potential duration was estimated as the duration from the amplitude of action potential (50mV) to different percentages of repolarization (30: APD_{30}, 50: APD_{50} and 90: APD_{90}).

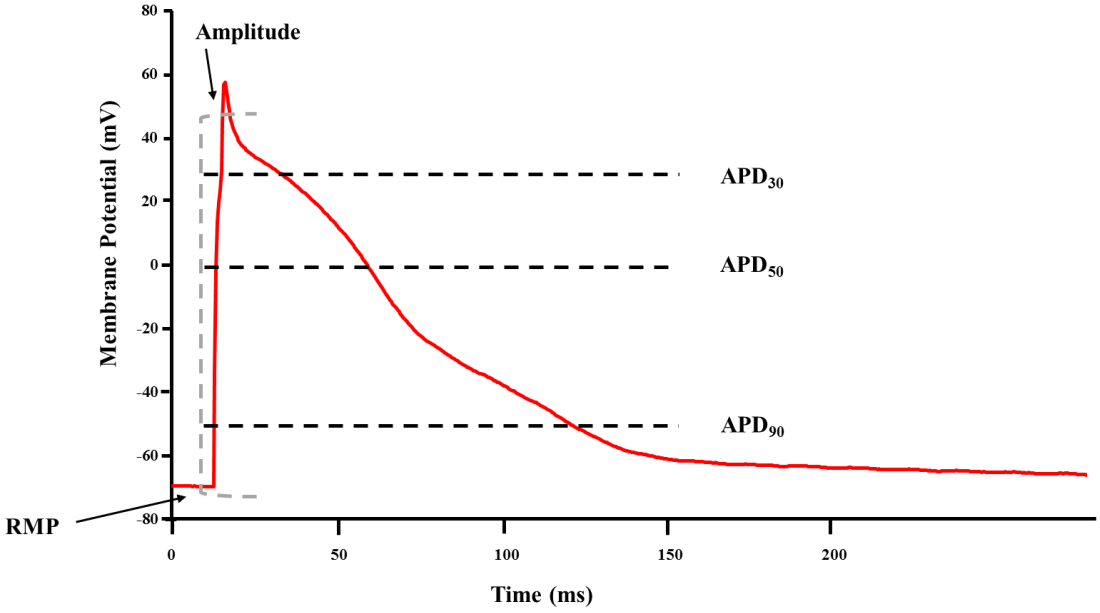


Figure 2.19: An example recording of an action potential in ventricular cardiomyocytes. Action potential recorded from ventricular cardiomyocytes in normal Tyrode showing measurement of resting membrane potential (RMP) and the action potential duration at 30% (APD_{30}), 50% (APD_{50}) and 90% (APD_{90}) repolarization.

2.6.2.2 Whole-cell patch-clamping

A tight seal is created between the microelectrode and cell membrane, by the application of negative pressure; termed a gigaseal (1GΩ). Following forming of gigaseal, fast capacity transient was compensated using neutralisation of microelectrode capacitance facility of the clamping amplifier.

Whole-cell recording was achieved by application of further suction to rupture a patch of cell membrane allowing electrical access between the cell interior and the glass microelectrode filled with an appropriate microelectrode solution (caesium (Cs) based solution). The membrane capacitance was determined and series resistance compensated at 80%.

2.6.2.2.1 Voltage-clamp; Ionic current recording

Voltage clamp protocols were used to record the L-type Ca^{2+}-current in isolated cardiomyocytes using an Axon Axoclamp Microelectrode Amplifier (AXOPATCH 200B) and data acquisition system (DIGIDATA 1322A). Electrophysiology software Clampfit 10.4 was used for collection and analysis of data. Low resistance microelectrode was used in this experiment with a rate of (2-4MΩ). The microelectrodes were filled with a Cs-electrode solution. The membrane capacitance (Cm) was determined using the "Membrane Test" function of pClamp and electrode resistance and membrane capacitance compensated at 80%.

In this study, current of interest was isolated for recording in voltage clamp mode. Caesium in the electrode diffuses into the cell and blocks outward potassium currents. The cardiomyocytes were clamped at a holding potential -70mV then followed by depolarisation pre-pulse (50ms) to-40mV to inactivate voltage-dependent sodium currents (I_{Na}). The membrane potential was subsequently stepped from -40mV to +70mV +10mV increments for duration of 300ms. Recordings were sampled at 10KHz and filtered at 5KHz using a low pass Bessel filter, the data were analysed using Clampfit version 10.4 software. The currents recorded were normalised to cell capacitance (pA/pF).

2.7 Statistical analysis

In-vitro data are shown as number of hearts/animals; number of cells. All data from experiments were arranged and tabulated in Excel program. Results were expressed as mean ± S.E.M. Statistical analysis was performed using one-way and two-way ANOVA and t-test in GraphPad Prism 7.0 software. Experimental groups were analysed by one-way or two-way ANOVA which show significant differences by multiple comparisons with Sadik, Tukey or Dunnett *post hoc* analysis. P-value <0.05 was considered as significant.

2.8 Experimental drugs and solutions

2.8.1 Experimental solutions

All experimental solutions were made using Milli-Q de-ionised water

Normal Tyrode solution contained the following (in mM): NaCl 135, $MgCl_2$ 1, KCl 6, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10, glucose 10, sodium pyruvate 5, NaH_2PO_4 0.33 and $CaCl_2$ 2. 1M NaOH or 1M HC1 was used to titrate the pH to 7.4.

Calcium free Tyrode solution contained all the materials mentioned above except $CaCl_2$ was excluded.

Ca^{2+} and Na^+ free Tyrode contained the following (in mM): N-methyl-D-glucamine (NMDG) 1, NaH_2PO_4 1, Glucose 10, HEPES 10. The solution was then titrated by stepwise addition of 1M of HCl. 1mM of EGTA was added and the pH was adjusted by addition 1M KOH till the pH reached to 7.4. Following titration of the pH with KOH, the K concentration was made up to 6mM.

Digestion enzymes solution used in isolation of cardiomyocytes was prepared by dissolving collagenase type I (1mg/ml), Bovine serum albumin (1.66mg/ml) and protease type XIV (0.67g/ml) in zero Ca^{2+} Tyrode.

K^+ electrode solution (normal K^+ electrode solution) contained the following (in mM) KCl 130, Na_2ATP 3, MgCl2 5, HEPES 10. 1M KOH was added to adjust the pH of the solution to 7.2. Then the solution was filtered using a falcon filter and frozen at -20°C.

Caesium electrode solution contained the following (in mM): CsCl 130, $CaCl_2$ 0.4, $MgCl_2$ 5, Na-ATP 3, EGTA 10, HEPES 10. 1M of CsOH was used to adjust the pH of solution, which was then filtered and stored at -20°C.

Folin Ciocalteu's phenol reagent was diluted at a ratio 1:4 in distilled water.

Krebs-HEPES buffer contained the following (in mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 1.3, Glucose 11.9, HEPES 10. The pH of solution was adjusted to 7.2 by filtering with 1M NaOH then 1.3M $CaCl_2$ was added.

Tris-buffered saline with tween 20 (TBS-T) contained the following (in mM): NaCl 5, Tris-HCl 1. The pH of solution was titrated to 7.5 by addition of 1M NaOH. 0.5ml of Tween-20 was added and volume made up to 1L.

Running buffer 10x Tris/Glycine/SDS was diluted with deionized water.

Transfer buffer contained the following (in mM): Tris base 48, glycine 39, SDS 0.037% W/V. The solids were dissolved first and 200ml Methanol (20% W/V) was added at the end in a total volume of 1L.

Ponceau S solution

Ponceau stain is used for the staining of protein bands immobilized on nitrocellulose membranes. 1% W/V Ponceau S in 1% acetic acid.

Running gel was prepared by addition of Acrylamide 3.35ml, 1.5M Tris-HCl (PH 8.8) 2.5ml, 10% SDS 100µL, distilled water 3.95ml, 10% AMPS 100µL and TEMED 4µL.

Stacking gel was prepared by addition of Acrylamide 0.85ml, 1.5M Tris-HCl (PH 6.8) 0.625µL, 10% SDS 50µL, distilled water 3.6ml, 10% AMPS 50µL and TEMED 5µL.

SDS loading buffer

Blue loading buffer contained the following Tris-HCl (pH 6.8) (100mM), SDS 2%, glycerol 10% and bromophenol blue sodium salt 0.1%.

Colour reagents

Protein A, A stock solution of protein A contained the following 2% Na_2CO_3 in 0.1M NaOH.

Protein B, A stock solution of protein B contained the following 1% (W/V) copper sulphate ($CuSO_4$).

Protein C, A stock solution of protein C contained the following 2% (W/V) sodium potassium tartrate ($NaK_4H_4O_6.4H_2O$).

Assay proteins ABC mix Colour reagents (A, B and C) were mixed in a ratio 100:1:1.

2.8.2 Drugs

Antagonist SB706375 was made up as 1mM stock solution dimethyl sulfoxide (DMSO). 1µM SB706375 was prepared on the day of experiment by addition of SB706375 to normal Tyrode.

Caffeine: 10mM stock solution was made up to assess release of Ca^{2+} from stores inside SR.

Isoproterenol (ISO): 10mM stock solution of isoproterenol hydrochloride was prepared by dissolving 12.35mg in 5ml absolute alcohol (ethanol). 5nM isoproterenol was made up from 10mM stock solution prior carry out the experiment and flask was wrapped with foil paper.

KN-93 is a CaMKII inhibitor. A 5mM stock solution was made in Milli-Q water and stored at -20˚C.

PD184352 is an ERK1/2 inhibitor. A 5mM stock solution was made in DMSO and stored at -20˚C.

Phenylephrine: A 0.1M stock solution of phenylephrine was made in Milli-Q water, stored at 4˚C.

SB202190 is a p38 inhibitor. A 10mM stock solution was made in DMSO and stored at -20˚C.

Tetracaine: A 1mM tetracaine was prepared on the day of experiment by addition of tetracaine to Ca^{2+} and Na^{+} free Tyrode (10mM EGTA).

Urotensin II

Human urotensin II was made by Professor R Guerrini (University of Ferrara, Italy), rUII was purchased from Sigma or made by Dr A Jamieson (Leicester Department of Chemistry). Stock solution of hUII was made by dissolving in normal Tyrode at 2mM and stored at -20˚C till use, rUII in normal Tyrode at 0.5mM. Working solutions were made in the day of experimentation by diluting stock solution in normal Tyrode.

2.8.3 Growth media

Culture media for ventricular cardiomyocytes

Tissue culture media was prepared by addition of the following supplements to 100ml Media 199 HEPES (2mgl/ml Insulin-Transferrin-Selenium, 2mM Carnitine, 5mM Creatine, 5mM Taurine and 10mM sodium pyruvate, 10mM 2,3-butanedione monoxime (BDM)) as well 2ml of antibiotic 10000 units/ml penicillin/ 10mg/ml streptomycin a sterile flask. All supplements and media were mixed and filtered using a sterile syringe-driven filter (Millex) and stored in falcon conical tubes at 4˚C to be ready for using as a growth medium within 5 days.

Culture media for culture CHO_{hUT} cells

Dulbecco's modified Eagle medium: Hams nutrient mixture F-12 media 50/50 mix (DMEM/F12 (50/50)) for culture CHO_{hUT} cells supplemented with 10% FBS, 2.5µg/ml fungizone, 100IU/ml penicillin, 100µg/ml streptomycin and 400µg/ml Geneticin (G418).

2.8.4 Fluorescent dyes

Fura-2

To prepare stock solution of 2mM of Fura-2 (the vial contained 50µg of the fura-2), the dye was dissolved by adding 25µL of DMSO which containing 5% of Pluronic acid. To avoid lysis of dye, it should be wrapped by foil paper and it was kept in a freezer.

Fluo-3

Fluo-3 AM is a fluorescence calcium indicator; the vial contained 50µg of the fluo-3. Stock (2mM) was made by addition of 22µl of 5% Pluronic acid in DMSO to a 50µg vial and wrapped in a foil. The labelled vial was frozen at -20˚C.

Chapter 3: UII and excitation-contraction coupling in ventricular Cardiomyocytes

3.1 Introduction

As indicated in the introduction UII has been implicated in E-C coupling dysfunction. In this chapter, it is suggested that E-C coupling and Ca^{2+}-regulation are affected by UII levels, as UII is elevated in heart failure patients and, therefore, may affect E-C coupling. During a myocardial action potential, a sequence of events is triggered within the cardiac myocyte resulting in contraction of the myocardium. Calcium ions play a crucial role in regulating function of cardiomyocytes contractility. The contractile machinery of the cardiac muscle is fuelled by calcium influx from the extracellular milieu and from intracellular stores (sarcoplasmic reticulum). This process involves the sarcolemmal bound L-type Ca^{2+} channels and NCX, and ryanodine receptors (RyR2) and Ca^{2+} pumps (see section1.2.2).

Detrimental changes to Ca^{2+} homeostasis adversely affects E-C coupling and contributes to the pathogenesis of heart failure. In cardiac hypertrophy, gross physiological alterations in the E-C coupling occur. These abnormalities in E-C coupling are accompanied by changes in the $[Ca^{2+}]_i$ transient, notably a raise in $[Ca^{2+}]_i$ transient amplitude. Cardiac hypertrophy can progress to heart failure where myocardial contractility and relaxation are impaired, and a reduction of $[Ca^{2+}]_i$ transient amplitude occur. A growing body evidence has suggested that abnormal expression and function Ca^{2+} handling proteins are a hallmark which contribute to cardiac dysfunction. Defects in E-C coupling manifest in alterations in $[Ca^{2+}]_i$ transient, reduction $[Ca^{2+}]_i$ transient amplitude and increase duration (see section 1.2.6).

Plasma levels of UII are elevated in patients with heart failure (see introduction, section 1.4.3). However, it is not currently known whether this elevation in UII is a result of heart failure or if UII may be involved in the initial development of heart failure. A number of studies have shown that UII contributes to the development of pathogenesis and progress of heart failure disease (Ng *et al.* 2002; Russell *et al.* 2003; Lapp *et al.* 2004). In addition, the development of heart failure has been attributed to altered calcium handling in cardiomyocytes, (Meyer *et al.* 1995; Hasenfuss and Pieske 2002; Houser and Margulies

2003), suggesting that elevated level of UII may contribute to altered E-C function and therefore the progression to heart failure.

There is a conflict in the literature about the action of UII on myocardial contractility. Urotensin II peptide has been reported to exert a positive inotropic action on isolated normal heart tissues (Gong *et al.* 2004; Quaile *et al.* 2009) and diseased heart tissues (Russell *et al.* 2001; Russell and Molenaar 2004). In human heart tissue, UII decreased contractility of failing heart tissue but potently increased myocardial contractility in non-failing human heart, concomitant with increment phosphorylation of PLB and increase of intracellular Ca^{2+} mobilization, causing increases in SR content (Quaile *et al.* 2009). On the other hand, the study by Fontes-Sousa and colleagues has shown that UII has negative inotropic effect in isolated rabbit papillary muscles (Fontes-Sousa *et al.* 2009). These effects may also be drivers of heart failure, as a decrease in activity may result in impairment of LV-function leading to stress.

The hypothesis of this study is that UII diminishes contraction amplitude of ventricular cardiomyocytes which is associated with changes in Ca^{2+}-transients during E-C coupling and causes alterations in the mechanism of contraction in heart failure.

Therefore, the initial aim of project was to investigate the influence of UII on excitation-contraction coupling and calcium regulation in single normal healthy ventricular cardiomyocytes.

3.2 Results

3.2.1 The effects of UII on contraction

UII has been reported to have an inotropic effect but its action is variable between tissues and species. There have been several studies in the literature reporting that UII has positive inotropic effect while other studies show that UII has negative inotropic properties in cardiac tissues (see section1.4.4).

3.2.1.1 Effect of UII on Langendorff rat heart preparation

Effects of UII on intact whole heart tissue was investigated, to determine whether the isolation of single cells using enzymes alters the receptor kinetics.

The cannulated heart was perfused with normal Tyrode and LVDP was measured (Figure 3.1A). Exposure of rat heart to 50nM UII caused a reduction in LVDP from a control in NT of (1.79 ± 0.17mmHg) to (1.42 ± 0.12mmHg), which was a reduction of (19.7 ± 2.5%) (8 hearts, $p<0.01$) (Figure 3.1B). However, there was no significant difference in heart rate when the heart was perfused with NT (154 ± 12.82) or after being subjected to UII (157.1 ± 8.07) ($p>0.05$) (Figure 3.1C).

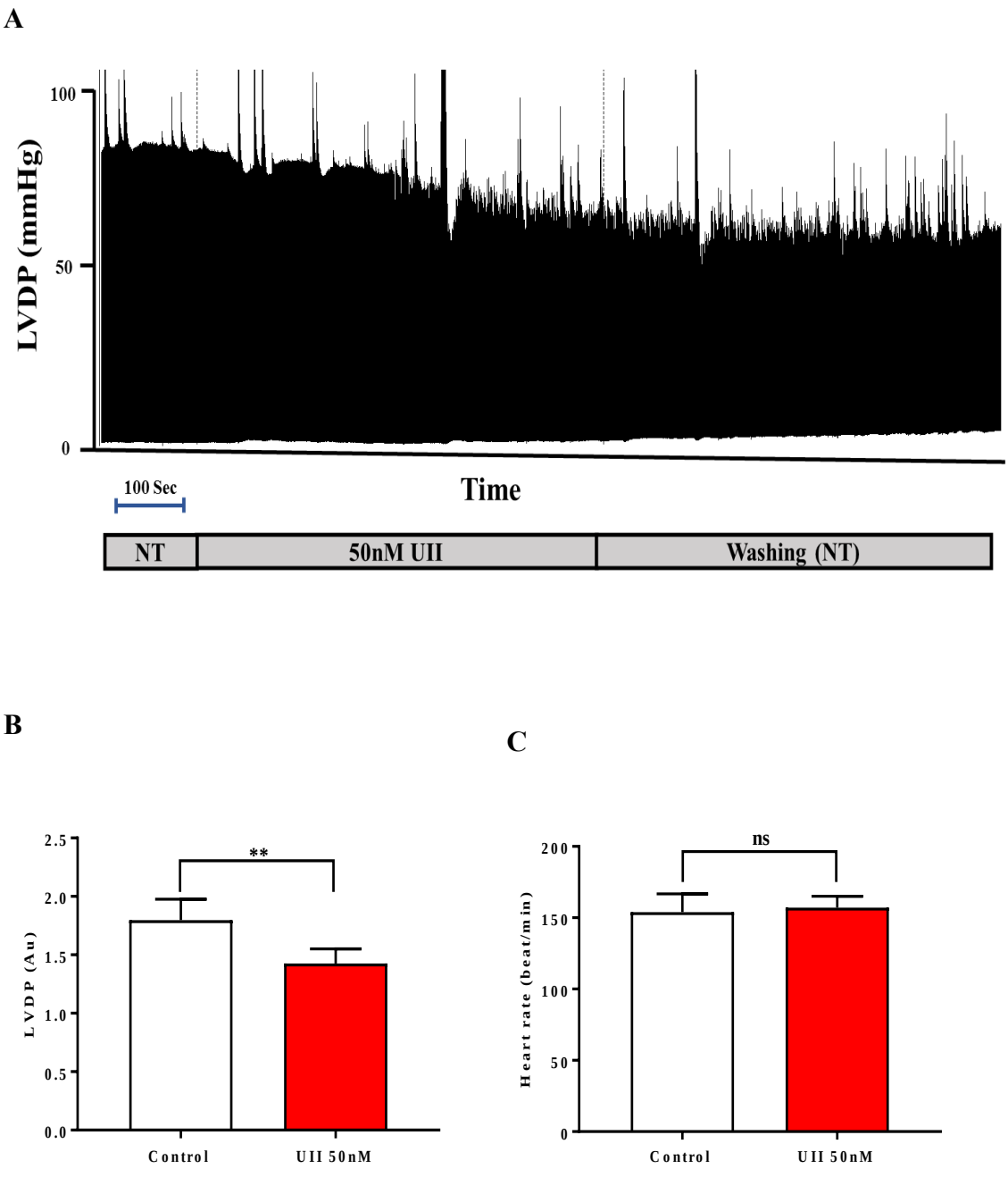


Figure 3.1: Ex-vivo isolated Langendorff rat heart and effect of UII (50nM).
(A) Representative pressure trace from adult rat ventricle, contractile parameters were recorded after perfusion of the heart with NT then 50nM UII, respectively.
(B) Bar chart shows effect of 50nM of UII on LVDP recorded from rat heart. N= 8 hearts.
(C) Bar chart shows heart rate after 50nM of UII.
The results are expressed as mean ± S.E.M. **p<0.01. Paired t-test.

3.2.1.2 Impact of temperature on ventricular cardiomyocytes contractility

The effects of UII on myocardial contractility in physiological and non-physiological conditions were examined. Recently, researchers have shown an increased interest in the influence of UII on myocardial contractility in ventricular cardiomyocytes from Sprague Dawley rats. They found that the amplitude of sarcomere shortening increased after application of 100nM of UII. However, these experiments were conducted at room temperature of 24˚C (Zhang *et al.* 2015). Therefore, the acute actions of UII on % cell shortening were recorded at room temperature and compared these to 37ºC, to see whether temperature modifies the response or ventricular cardiomyocytes to UII.

Perfusion of ventricular cardiomyocytes with 100nM UII at room temperature (24˚C) caused a significant reduction in % cell shortening from 12.6 ± 0.6% in NT to 11.3 ± 0.6% in 100nM UII (4 hearts; 24 cells $p<0.01$) (Figure 3.2A). However, this reduction was less pronounced than that recorded at 37˚C, where the reduction was from 11.5 ± 0.5% in NT to (9.5 ± 0.5%) in 100nM UII (4 hearts; 29 cells, $p<0.0001$) (Figure 3.2B). The percentage reduction in contraction strength (% cell shortening) in response to UII (100nM) for ventricular cardiomyocytes at room temperature (24˚C) (7.53 ± 3.78%) or 37˚C (18.15 ± 2.14%) changed significantly ($p<0.05$) (Figure 3.2C).

Collectively these data suggest that the amplitude of cell shortening decreased in physiological and non-physiological conditions after UII (100nM) treatment and the % change of cell length was higher at 37˚C than room temperature. Although, a higher negative inotropic response of cells at 37˚C than at room temperature. Data presented here contradict those of Zhang and colleagues and show this is not due to the different temperature of the experimental conditions (Zhang *et al.* 2015).

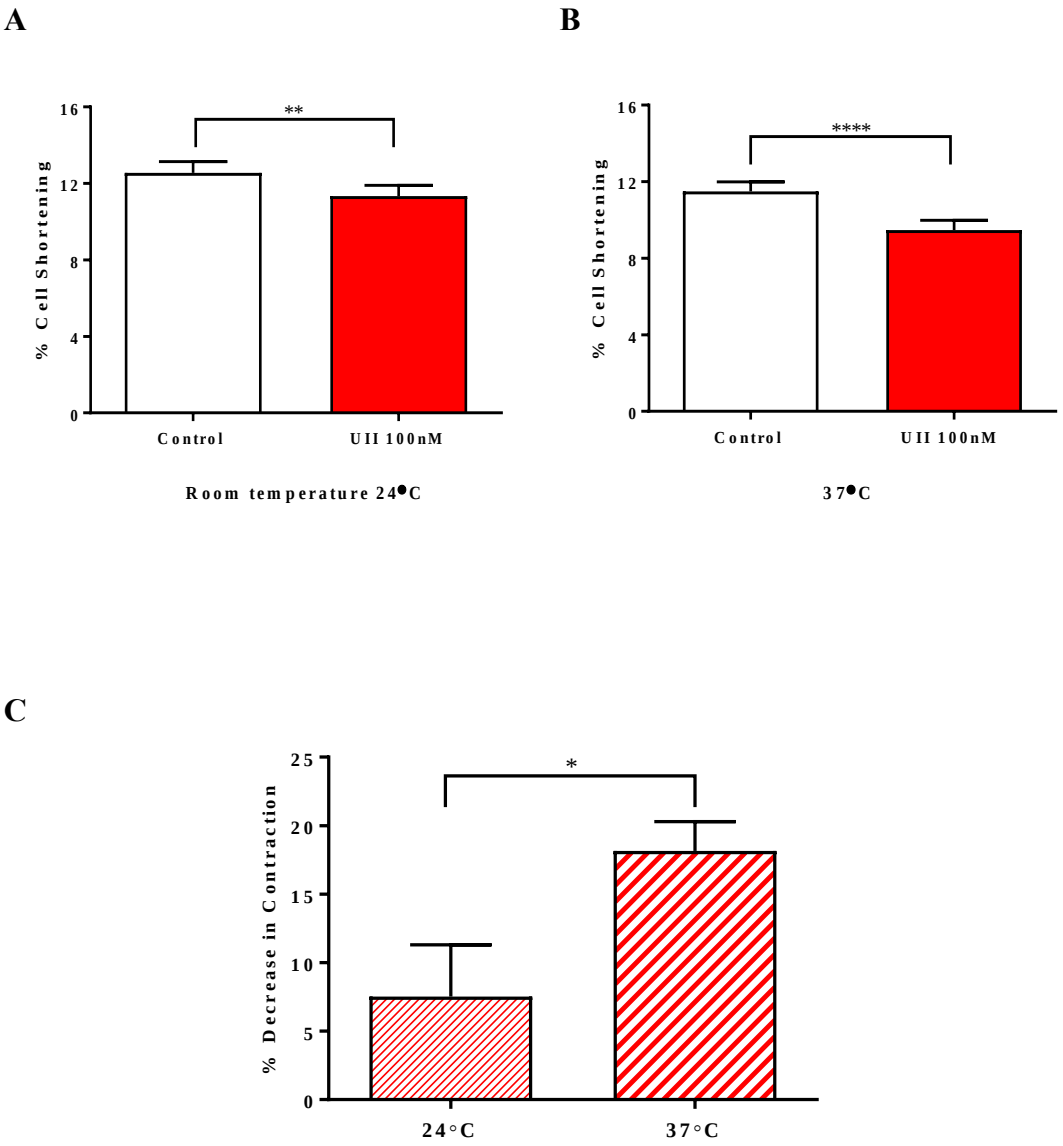
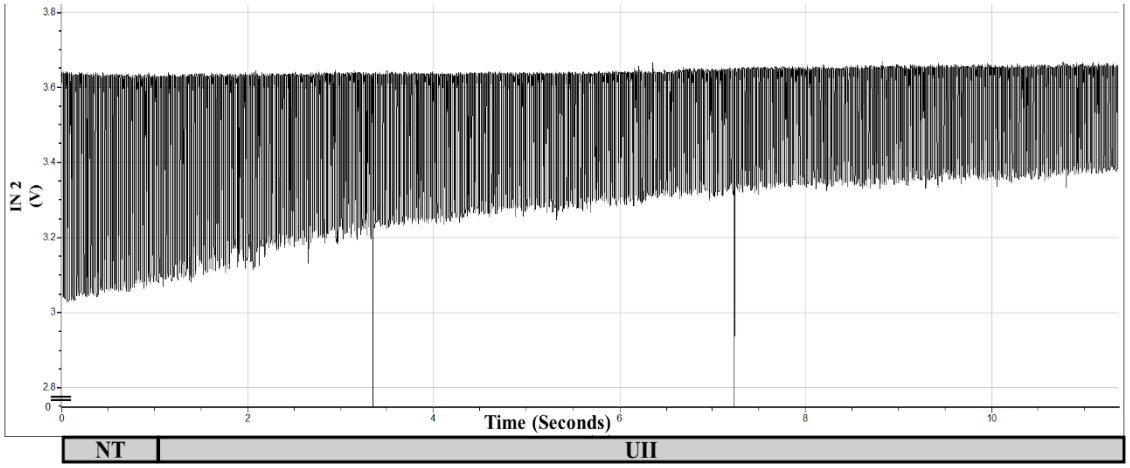


Figure 3.2: Measurement of cell length in cardiomyocytes treated with UII 100nM.
(A) Effect of UII at room temperature (24˚C) on contraction of freshly isolated ventricular cardiomyocytes. N= 4 hearts; 29 cells. The results are expressed as mean ± S.E.M. **p<0.01. Paired t-test.
(B) Effect of UII at 37˚C on contraction of freshly isolated cardiomyocytes. N= 4 hearts; 24 cells. The results are expressed as mean ± S.E.M. ****p<0.0001. Paired t-test.
(C) % change of cell shortening at room temperature and 37˚C. N= 8 hearts; (29 & 24) cells. The results are expressed as mean ± S.E.M. *p<0.05. Unpaired t-test.

3.2.1.3 Effects of UII on contraction strength of isolated rat ventricular cardiomyocytes

To obtain a measure of contraction strength, changes in cell length were measured in electrically stimulated ventricular cardiomyocytes (1Hz) in response to UII (Figure 3.3A). Figure 3.3B shows the mean data of percentage cell shortening for ventricular cardiomyocytes under control conditions when superfused with normal Tyrode and after superfusion with Tyrode containing UII (200nM). The strength of contraction as determined by change in cell length was reduced in the presence of 200nM UII. There was highly significant reduction in percentage cell shortening from (12.6 ± 0.5%) control to (10.9 ± 0.5%) (12 hearts; 71 cells, $p<0.0001$) in UII-treated cardiomyocytes. These data show that UII caused a negative inotropic effect in both ex-vivo whole hearts and isolated ventricular cardiomyocytes.

A



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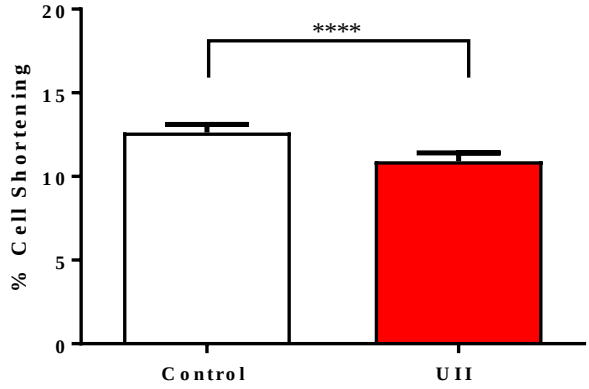


Figure 3.3: Cell length in control and 200nM UII-treated cardiomyocytes.
(A) Example trace of cell length recorded from a single cardiomyocyte in normal Tyrode and superfused with 200nM UII as indicated on the trace.
(B) Bar chart of percentage cell shortening recorded from freshly isolated ventricular cardiomyocytes in NT and after treated with UII. N= 12 hearts; 71 cells. The results are expressed as mean ± S.E.M. **** $p<0.0001$. Paired t-test.

3.2.1.4 Effects of long-term tissue culture on response of ventricular cardiomyocytes to isoproterenol and UII

Urotensin II is the endogenous agonist for the UT receptor, which is a G_q protein coupled receptor, and all physiological and pathological actions of UII are thought to be mediated by its receptor (Ames *et al.* 1999). Urotensin II has high binding affinity for the UT receptor, and upon binding of the peptide to the receptor phospholipase C activity is increased leading to the production of the second messenger IP_3 followed by increased level of intracellular Ca^{2+} (Opgaard *et al.* 2000). Several mechanisms are responsible for the regulatory functions of GPCRs signaling, including internalisation, desensitization, recycle and down-regulation. Prolonged exposure of GPCRs ligand(s) results in receptor desensitization (GPCRs lose their response to the ligands), which physiologically acts to prevent the GPCR from being overstimulated.

Stimulation of UT receptor with UII can promote internalisation of the receptor, with UT internalisation machinery destination driven by to the involvement of specific clusters of serine in the C-terminal tail of UT which efficiently facilities this process (Proulx *et al.* 2005). This may result in the loss of receptors and reduce or alter the response to exogenous applied agonist and, consequently, the study set out to determine whether endogenous UII levels in the rat had resulted in loss of receptors in the isolated ventricular cardiomyocytes. To facilitate this, cells were cultured in media to promote resynthesis of receptors and removal of endogenous agonists.

3.2.1.4.1 Effects of UII on contraction of ventricular cardiomyocytes after 24 and 48 hours tissue culture

The main purpose of the initial investigation was to see whether the effects of UII on contraction were altered following long-term tissue culture (48 hours). Preliminary experiments were conducted to determine whether long-term tissue culture altered the contractile properties of the ventricular cardiomyocytes (Figure 3.4A). 2,3-Butanedione monoxime was used in culture media, which prevents spontaneous contraction and preserves the contractile properties of ventricular cardiomyocytes in tissue culture over multiple days (Chung *et al.* 2015). However, BDM inhibits myosin ATPase in cardiomyocytes and inhibits contraction (Louch *et al.* 2011). Therefore, the cultured cells

were removed from the media and washed in NT and then left at room temperature for at least 30 minutes prior to use, to facilitate complete washout of the BDM.

Figure 3.4B shows the mean data of percent cell shortening for freshly isolated cells and cultured cells (24 and 48 hours), percentage cell shortening was measured for all cells in NT. There was significant difference in contraction recorded after 24 and 48 hours in tissue culture, with percentage cell shortening of freshly isolated cells of (13.9 ± 0.8%) (7 hearts; n= 47 cells), reduced to (9.9 ± 0.1%) after 24 hours tissue culture (8 hearts; n= 46 cells) ($p<0.0001$) and after 48 hours (10.1 ± 0.6%) (3 hearts; n= 18 cells) ($p<0.01$) in percent cell shortening. The drop in contraction strength happens after 24 hours, following which, there is no further decline.

Decline in contraction may result from altered SR function, and to see whether long term culture affected SR function, an exponential time constant was determined, which reflected the rate of decline in $[Ca^{2+}]_i$ and therefore SERCA2a activity (see section 1.2.3). Figure 3.4C is the bar chart of the time constant for relaxation for fresh and cells cultured for 24 hours. The rate of relaxation of fresh cells was significantly reduced following 24 hours in tissue culture, with the exponential time constant of 45.2 ± 2.4ms in freshly isolated cells compared to 59.9 ± 4.0ms in cells after 24 hours of tissue culture ($p<0.01$).

These data show that tissue culture results in a reduction in strength of contraction (percentage cell shortening) and that this may reflect impairment of SERCA2a activity.

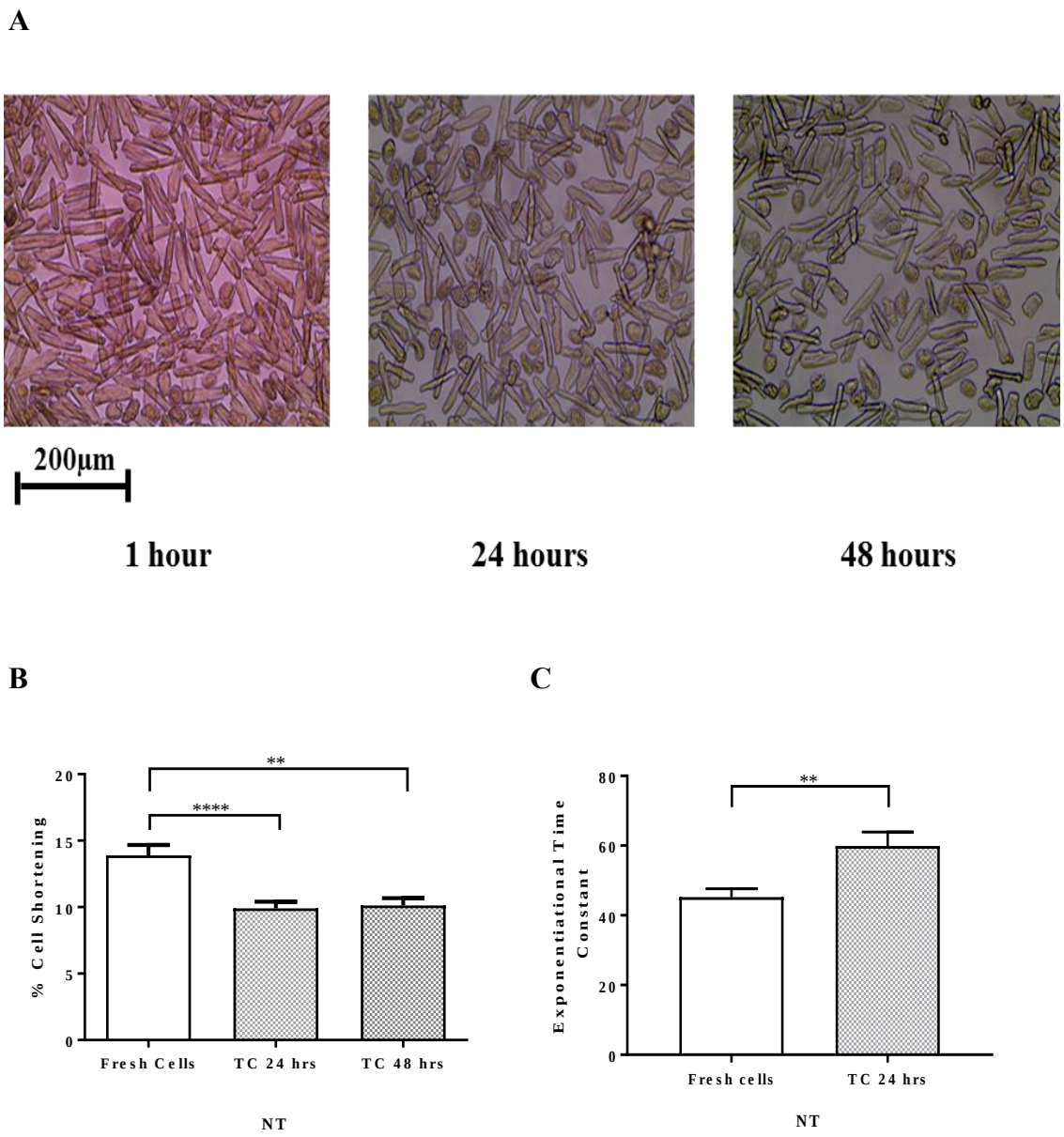


Figure 3.4: Measurement of cell length and exponential time constant in fresh and tissue culture ventricular cardiomyocytes.

(A) Isolated ventricular cardiomyocytes images in culture media (1, 24 and 48) hours, the ventricular cardiomyocytes were cultured in Media 199 HEPES (with supplements and 10mM BDM) and incubated in 5% CO_2 incubator at 37˚C.

(B) Bar chart of percentage cell shortening recorded from freshly isolated ventricular cardiomyocytes, and following 24 hours and 48 hours in tissue culture. N= 7 hearts; 47 cells for fresh cell experiment, n= 8 hearts; 46 cells for 24 hours cultured cells experiment, n= 3 hearts; 18 cells for 48 hours cultured cells experiment. The results are expressed as mean ± S.E.M. **p<0.01, ****p<0.0001. One-way ANOVA followed by a Sidak's post hoc test.

(C) Bar chart of exponential time constant of relaxation recorded from freshly isolated ventricular cardiomyocytes and after 24 hours in tissue culture. N= 7 hearts; 47 cells for fresh cell experiment, n= 8 hearts; 46 cells for 24 hours cultured cells experiment. The results are expressed as mean ± S.E.M. **p<0.01. Unpaired t-test.

3.2.1.4.2 The effects of tissue culture on the response of ventricular cardiomyocytes contraction to β-ADR stimulation with ISO

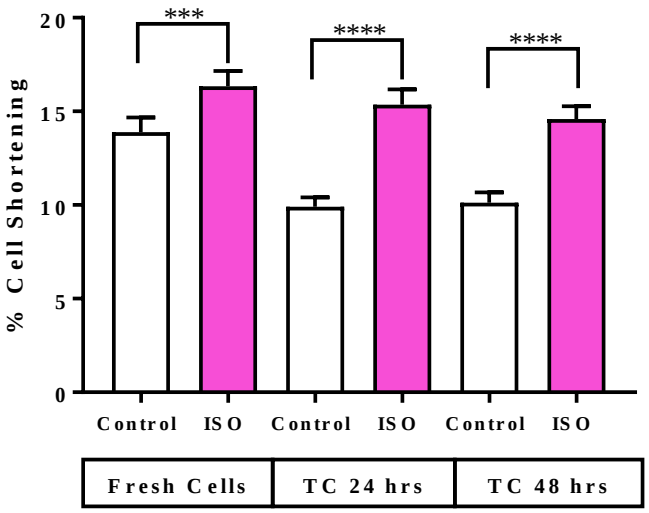
Ventricular cardiomyocytes from hypertrophic hearts, show reduced response to β_1-ADR stimulation (Engelhardt *et al.* 1999; Lohse *et al.* 2003). Therefore, the effect of the β-ADR agonist isoproterenol (5nM) on cell shortening was examined to determine potency of ISO on myocardial contractility in ventricular cardiomyocytes in tissue culture as a control to experiments where the cells would be subject to a hypertrophic stimulus by UII, prior to β-ADR stimulation with ISO. The ventricular cardiomyocytes following tissue culture for 24 and 48 hours, were superfused with normal Tyrode and % cell shortening recorded (control) and then perfused with ISO and cell shortening recorded again.

Figure 3.5A shows the effects of ISO on percent cell shortening in control cells (freshly isolated) and cells in culture for 24 and 48 hours. The increase in strength of contraction recorded from freshly isolated cells in response to ISO (5nM) was from (13.9 ± 0.8%) in NT to (16.3 ± 0.8%) in ISO (7 hearts; 47 cells, $p<0.001$). Cells after 24 and 48 hours of tissue culture had a reduced strength of contraction as previously shown (see section 3.2.1.4.1). However, superfusion with 5nM ISO still increases the strength of contraction from (9.9 ± 0.5%) in NT to (15.35 ± 0.8%) in ISO (8 hearts; 46 cells, $p<0.0001$) in cells cultured for 24 hours and from (10.1 ± 0.55%) in NT to (14.6 ± 0.7%) in ISO (3 hearts; 18 cells, $p<0.0001$) in cells cultured for 48 hours.

Figure 3.5B shows exponential time constant for freshly isolated ventricular cardiomyocytes and 24 and 48 hours cultured cells. The results show no reduction in relaxation time in freshly isolated cells in response to 5nM ISO control (45.2 ± 2.4ms) versus ISO-treated cells (44.4 ± 3.3ms) (7 hearts; 47 cells, $p>0.05$). Cells after 24 hours of tissue culture had also no any reduction in the relaxation time control (59.9 ± 3.97ms) versus ISO-treated cells (51.97 ± 3.66ms) (8 hearts; 46 cells, $p = 0.0528$), no change in the rate of relaxation has also been shown after 48 hours tissue culture between control (44.7 ± 3.2ms) and ISO-treated cells (36.9 ± 2.8ms) (3 hearts; 18 cells, $p>0.05$).

Based on the results, ISO increases the strength of contraction for both freshly isolated and cultured cells. It has positive inotropic effect on contraction of ventricular tissues. While there was no change in the rate of relaxation in each group of cells.

A



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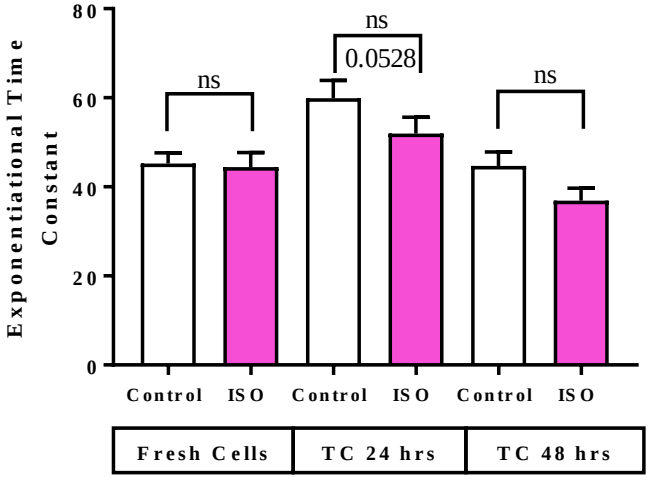


Figure 3.5: Measurement of cell length and rate of relaxation in fresh and tissue culture ventricular cardiomyocytes in response to 5nM ISO.

(A) Bar chart shows effect of ISO on percentage cell shortening recorded from fresh ventricular cardiomyocytes, 24 and 48 hours cultured cells. N= 7 hearts; 47 cells for fresh cell experiment, n= 8 hearts; 46 cells for 24 hours cultured cells experiment, n= 3 hearts; 18 cells for 48 hours cultured cells experiment.

(B) Bar chart of rate of relaxation recorded from fresh ventricular cardiomyocytes and 24 and 48 hours cultured cells before and after treated with ISO. N= 7 hearts; 47 cells for fresh cell experiment, n= 8 hearts; 46 cells for 24 hours cultured cells experiment, n= 3 hearts; 18 cells for 48 hours cultured cells experiment.

The results are expressed as mean ± S.E.M. ***p<0.001, ****p<0.0001. Two-way ANOVA followed by a Sidak's *post hoc* test.

3.2.1.4.3 The influence of long-term tissue culture on the acute effects of UII on ventricular cardiomyocytes

Ventricular cardiomyocytes were cultured for 24 and 48 hours, and the effect of acute application of UII on contraction strength (% cell shortening) determined and compared to freshly isolated ventricular cardiomyocytes. The main purpose of this initial investigation was to demonstrate if there was any change in the effects of UII on strength of contraction between freshly isolated ventricular cardiomyocytes and cultured cells (24 and 48 hours in culture).

Figure 3.6 shows the mean data of the percentage cell shortening for freshly isolated ventricular cardiomyocytes and cells cultured for 24 and 48 hours, superfused with normal Tyrode and in the presence of 200nM UII. UII caused a significant reduction in cell shortening in freshly isolated cells, from (12.6 ± 0.5%) in normal Tyrode to (10.9 ± 0.5%) (12 hearts; 71 cells, $p<0.0001$) in UII. UII also significantly reduced contraction in 24 hours cultured cells from (10.8 ± 0.4% in NT) to (8.5 ± 0.4%) in UII (12 hearts; 67 cells, $p<0.0001$). However, in ventricular cardiomyocytes cultured for 48 hours, there was no significant difference between control (9.7 ± 0.8%) and UII-treated cells (8.8 ± 0.8%) (2 hearts; 9 cells, $p>0.05$) in percentage cell shortening.

The data show that UII decreases contraction strength in freshly isolated ventricular cardiomyocytes and following 24 hours of tissue culture.

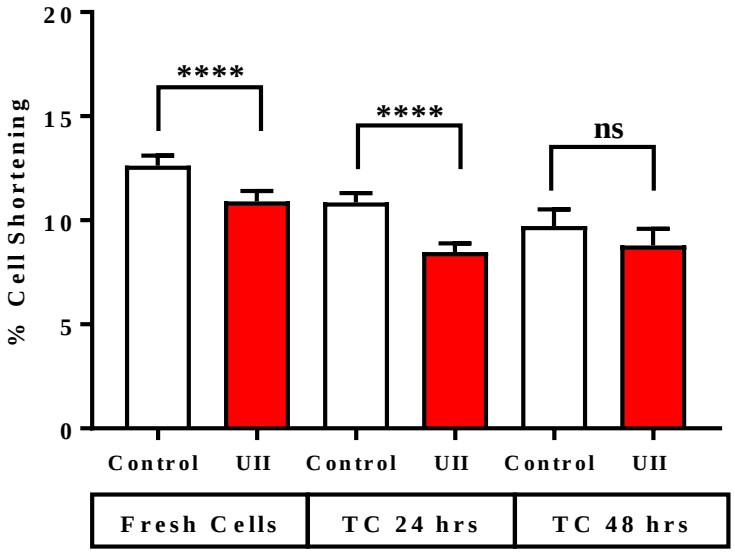


Figure 3.6: Effect of UII on strength of contraction in freshly isolated and cultured ventricular cardiomyocytes.

Bar chart of percentage cell shortening recorded from fresh ventricular cardiomyocytes and cultured cells in normal Tyrode and after treated with 200nM UII. N= 12 hearts; 71 cells for fresh cell experiment, n= 12 hearts; 67 cells for 24 hours cultured cells experiment, n= 2 hearts; 9 cells for 48 hours cultured cells experiment. The results are expressed as mean ± S.E.M. ****p<0.0001. Two-way ANOVA followed by a Sidak's *post hoc* test.

3.2.2 Concentration and time-dependent effects of UII on intracellular calcium

Cellular Ca^{2+} is the major factor for E-C coupling process. Urotensin II affects myocardial activity and has a cardiac inotropic effect (see section 1.4.4). Any change that occurs in the modulating factors of intracellular Ca^{2+}dynamics which affects and modifies SR functional properties (Kockskämper *et al.* 2008). Ca^{2+}-release has an obvious role during Ca^{2+} signaling in cardiac muscle tissue, as it leads to activation of RyR2 and elicits CICR in cardiomyocytes (MacMillan *et al.* 2005). It has been shown that activation of G_q protein coupled receptors prompts entry of Ca^{2+} leading to increases in the concentration of Ca^{2+} which binds with calmodulin (Rothman *et al.* 1992; Cobb *et al.* 1994). Binding of Ca^{2+} to calmodulin can activate CaMKII, resulting in phosphorylation of LTCC and RyR2 causing increased open probability of LTCC and influx of Ca^{2+} via LTCC and mobilization of Ca^{2+} from SR, which increase the level Ca^{2+}-transient (Anderson 2005).

UII binds to G_q protein coupled receptors, causing activation of phospholipase C, which in turn results in hydrolysis of phosphatidylinositol 4,5-bisphosphate into inositol trisphosphate and diacylglycerol (Matsushita *et al.* 2001; Papadopoulos *et al.* 2008). Inositol trisphosphate gives rise to release of intracellular Ca^{2+} from the SR (Hidalgo and Núñez 2007). Intracellular Ca^{2+} rises in the cytosol when IP_3 freely diffuses to the cytoplasm triggering IP_3 channels (InsP3R) and subsequently provokes the opening of neighbouring channels (RyR2) (Foskett *et al.* 2007). The function of the SR is based on interaction between InsP3R and RyR2 which mediate Ca^{2+}-release to the cytosol and then back flux to SR thorough the SERCA2a.

3.2.2.1 Dose-response to human and rat UII

In order to look at the effects of UII on E-C coupling, a dose/response curve was conducted to determine the effective concentration of UII, which could then be related to the UII serum concentration and used in subsequent in-vitro and ex-vivo experiments.

Figure 3.7A is a record of $[Ca^{2+}]_i$ from a single freshly isolated ventricular cardiomyocyte perfused with UII (100nM). Figure 3.7B is a record of $[Ca^{2+}]_i$ from a single cell perfused with UII 200nM. The cells were superfused with normal Tyrode for 5 minutes, and control Ca^{2+}-transient was recorded. The ventricular cardiomyocytes were then superfused with either rat (rUII) or human urotensin II (hUII) at various concentrations

(100, 200 and 500nM). Each ventricular cardiomyocyte was exposed to only one concentration of UII.

Figure 3.7C is the dose response curve of effects of hUII and rUII on systolic $[Ca^{2+}]_i$. The data show the IC_{50} is 89nM for hUII and 158nM for rUII.

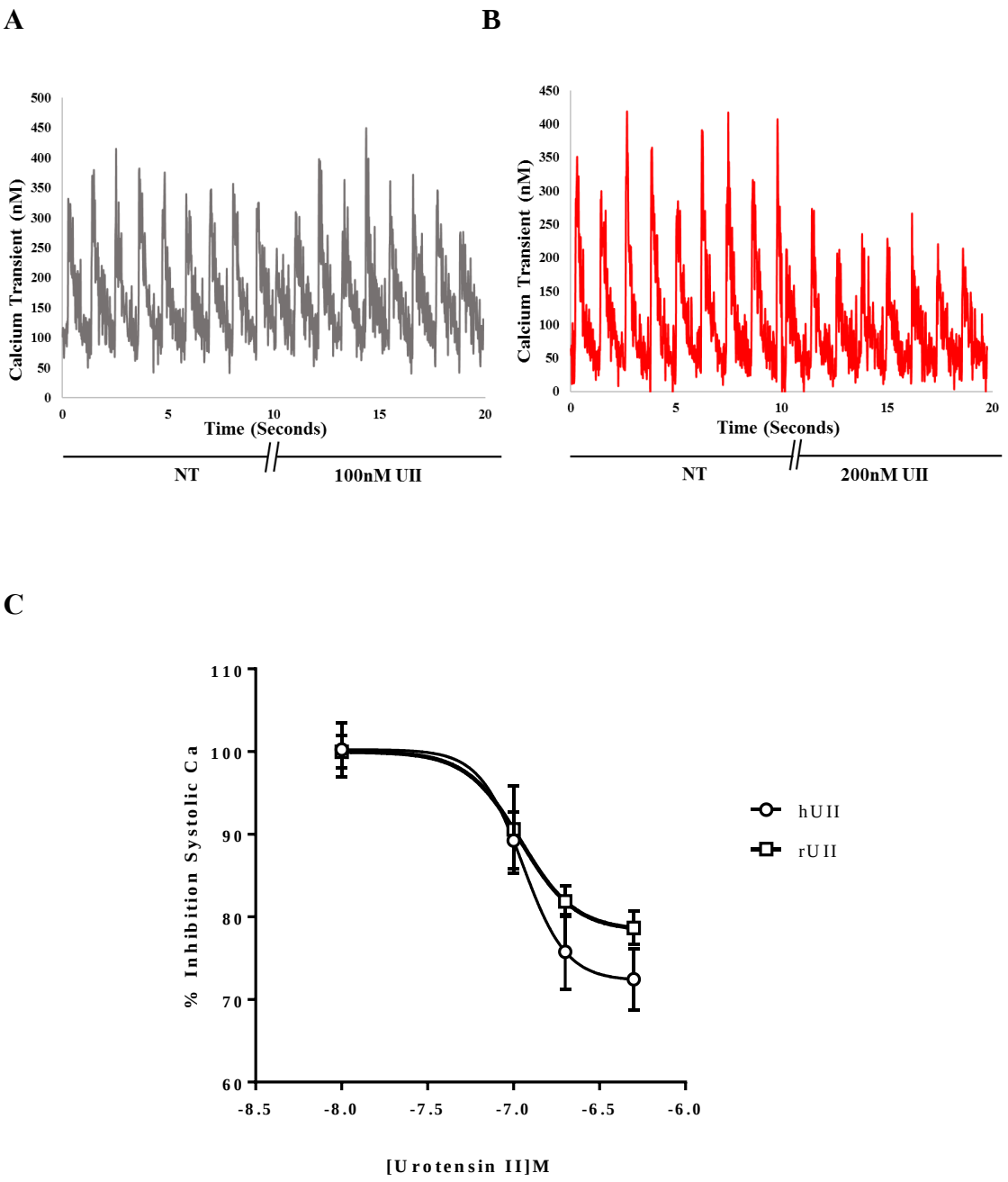


Figure 3.7: Effect of different concentrations of hUII (100, 200, and 500nM) and rUII (100, 200, and 500nM) on systolic $[Ca^{2+}]_i$ in ventricular cardiomyocytes.

(A) An example records of intracellular calcium from a single ventricular cardiomyocyte in response to hUII 100nM. The first 10 seconds of trace in the Ca^{2+}-transient recorded in normal Tyrode. The ventricular cardiomyocytes were superfused with hUII for 10 minutes and the Ca^{2+}-transient was recorded for 10 second.

(B) An example recording of intracellular calcium from a single ventricular cardiomyocyte in response to hUII 200nM.

(C) Concentration-response curve (% inhibition) of systolic $[Ca^{2+}]_i$ to hUII and rUII, n= (7-10) hearts; (55-79) cells for hUII and n= (6-7) hearts; (44-72) cells for rUII. Note: Y-axis is rescaled for clarity.

3.2.2.2 Time-dependent effects of UII

The effects of UII may result from the binding of UII to its receptor triggering a signalling pathway but may also result from direct interaction of UII with LTCC channels. Therefore, the purpose of these experiments was to determine the optimal time for this response. The effect of UII on intracellular Ca^{2+}-transient recorded from electrically stimulated ventricular cardiomyocytes was measured at different times (5, 10, 15) minutes to determine the time dependence of the response to UII. Figure 3.8A shows a trace of Ca^{2+}-transient recorded from single ventricular cardiomyocyte. To protect the Fura-2 loaded cell from photo-bleaching, records of Ca^{2+} -transients were made for short periods (10 seconds) at 5, 10 and 15 minutes after start of superfusion with UII at 200nM. Figure 3.8B is a bar chart of the mean ± S.E.M. showing diastolic $[Ca^{2+}]_i$ in normal Tyrode (control) and 200nM UII at 5, 10 and 15 minutes. The results also show that there were no significant differences between control (53.7 ± 4.9nM) and treated cells with UII after 5 minutes (55.1 ± 4.1nM) and after 10 minutes (49.3 ± 3.4nM) or after 15 minutes (55.6 ± 9.6nM) ($p>0.05$) (4 hearts; 16 cells) in diastolic $[Ca^{2+}]_i$. Figure 3.8C shows systolic $[Ca^{2+}]_i$ measured from single ventricular cardiomyocytes after superfusion with UII at 5, 10 and 15 minutes. Significant differences were found in systolic $[Ca^{2+}]_i$ between control (480.8 ± 61.6nM) and UII after 5 minutes (360.1 ± 51.7nM) ($p<0.05$) and after 10 minutes (310.7 ± 46.3nM) ($p<0.01$) or after 15 minutes (301.1 ± 43.9nM) ($p<0.05$) (4 hearts; 16 cells). There was no difference in systolic calcium recorded at 10 minutes (310.7 ± 46.3nM) versus 15 minutes (301.1 ± 43.9nM) ($p>0.05$) (4 hearts; 16 cells), it could be reach steady-state after 10 minutes and the effect seemed to be saturated. Therefore, the ventricular cardiomyocytes were perfused with UII for 10 minutes.

The data show a time-dependent reduction in systolic $[Ca^{2+}]_i$ due to UII in adult rat ventricular cardiomyocytes.

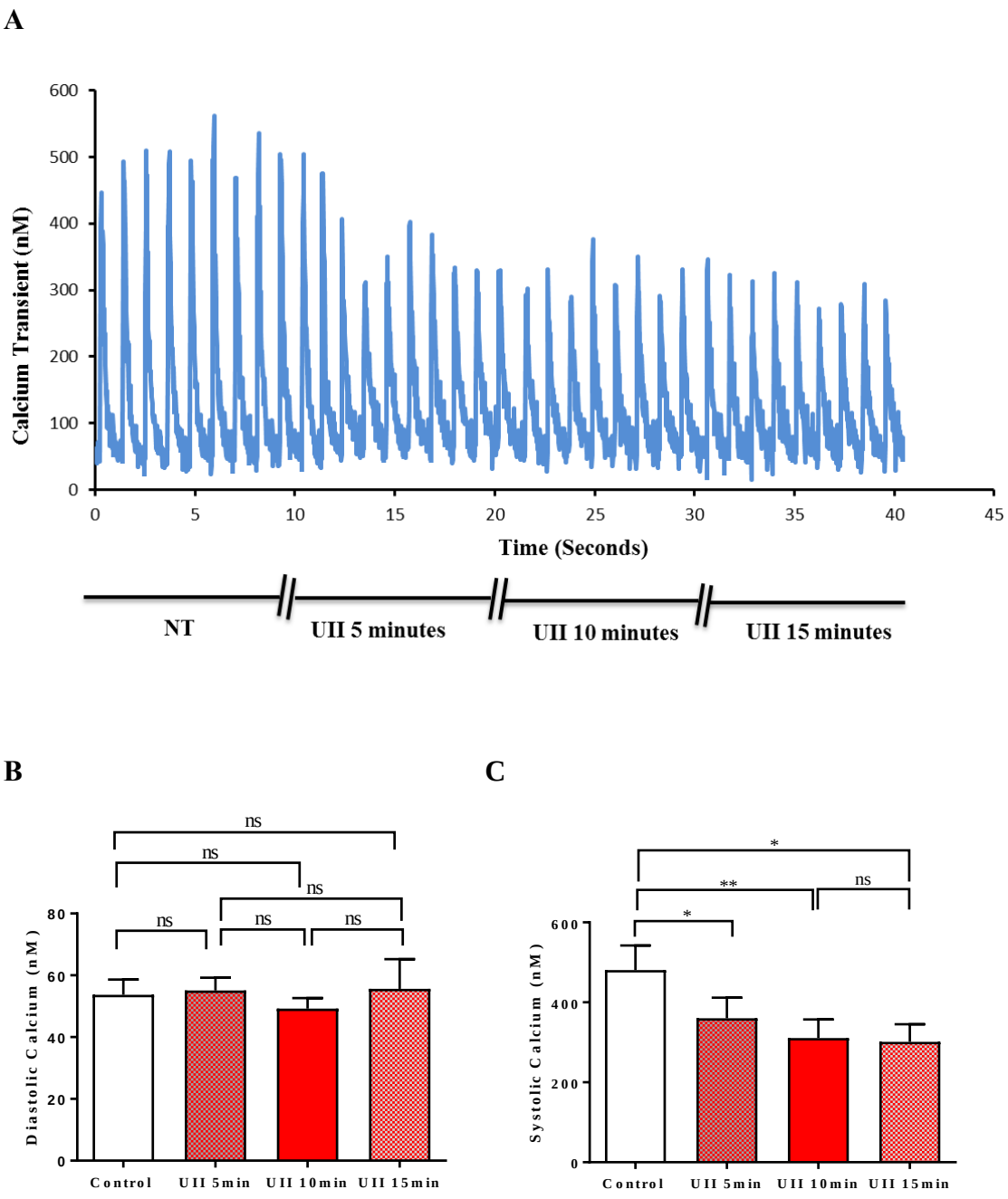


Figure 3.8: Measurement of intracellular Ca^{2+}-transient in ventricular cardiomyocytes.
(A) Example of recording of intracellular Ca^{2+} from a single ventricular cardiomyocyte. The ventricular cardiomyocytes were superfused with 200nM UII and the Ca^{2+}-transient was recorded for 10 seconds at 5, 10 and 15 minutes.
(B) Bar chart of diastolic $[Ca^{2+}]_i$ recorded from ventricular cardiomyocytes in normal Tyrode and at 5, 10 and 15 minutes after perfusion with 200nM UII. N= 4 hearts; 16 cells.
(C) Bar chart of systolic $[Ca^{2+}]_i$ recorded from ventricular cardiomyocytes in normal Tyrode and at 5, 10 and 15 minutes after superfusion with 200nM UII. N= 4 hearts; 16 cells.
Data are the mean ± S.E.M. *p<0.05, **p<0.01. One-way ANOVA followed by a Sidak's *post hoc* test.

3.2.2.3 The effects of urotensin II on Ca^{2+}-regulation during excitation

In the next set of experiments, we set out to investigate mechanisms for the UII-induced negative inotropism and in particular the cellular mechanism for the decline in systolic Ca^{2+} Figure 3.9A. The previous experiment, comparing the effect of UII on contraction between freshly isolated ventricular cardiomyocytes and those in long-term tissue culture (see section 3.2.1.4.3) shows no significant difference between their response to UII, however, there was a reduction in contraction strength in tissue cultured cells. Therefore, freshly isolated cells were used in this study.

Figure 3.9B shows the mean data of diastolic $[Ca^{2+}]_i$ for freshly isolated ventricular cardiomyocytes before (normal Tyrode) and after treatment with UII (200nM). The data showed no significant difference in the effect of UII on diastolic $[Ca^{2+}]_i$ between control (53.97 ± 5.25nM) and treated cells with UII (55.85 ± 3.85nM) (7 hearts; 73 cells, $p>0.05$).

Figure 3.9C showing the mean data of systolic $[Ca^{2+}]_i$ for freshly isolated ventricular cardiomyocytes. The data show significant differences between control (372.8 ± 18.86nM) and UII (306.1 ± 11.58nM) (7 hearts; 73 cells, $p<0.0001$).

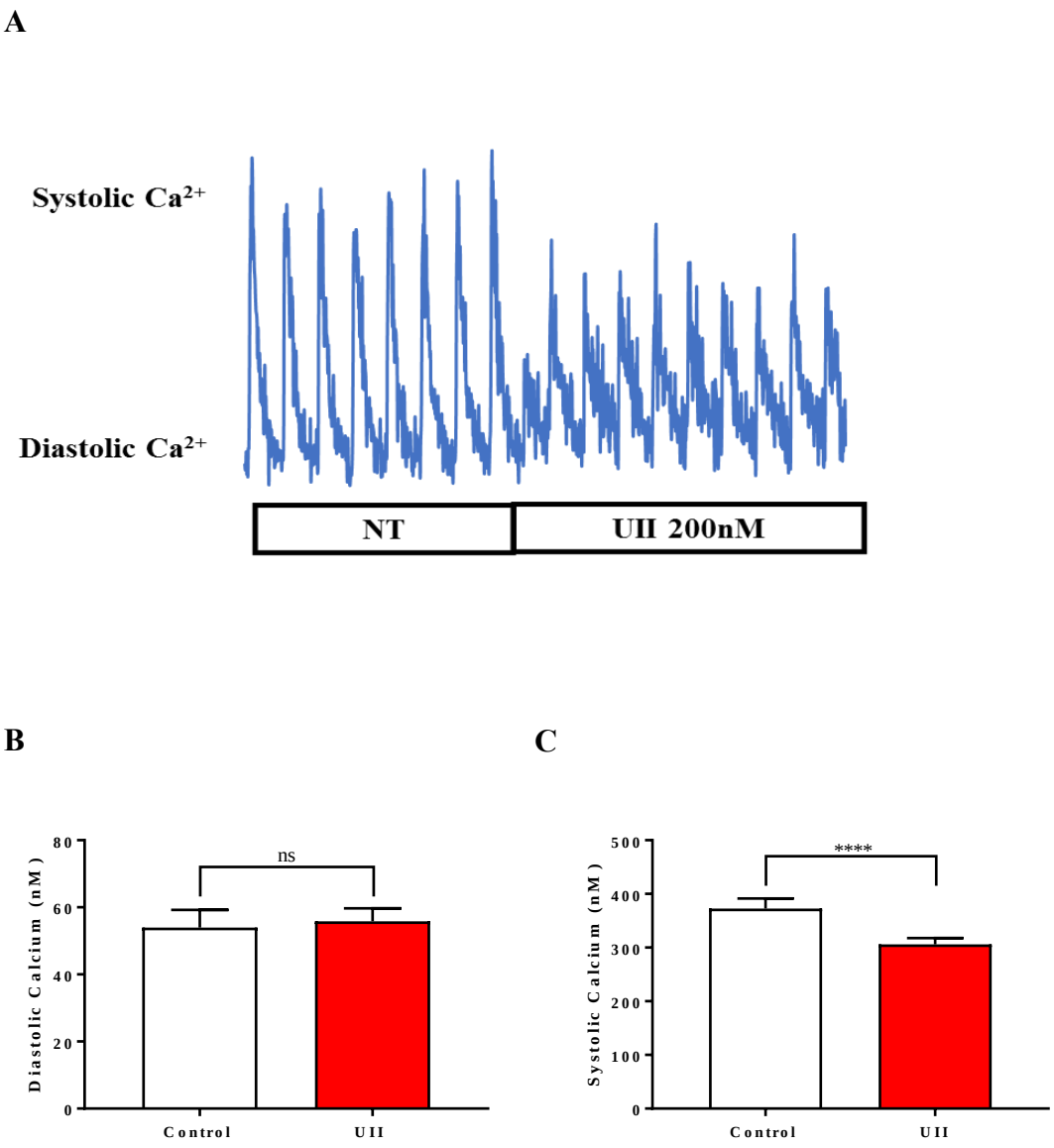


Figure 3.9: Measurement of intracellular Ca^{2+}-transient in freshly isolated ventricular cardiomyocytes.

(A) An example of record of intracellular Ca^{2+} from a single ventricular cardiomyocyte in NT and presence of 200nM UII.

(B) Bar chart of diastolic $[Ca^{2+}]_i$ recorded from freshly isolated ventricular cardiomyocytes in normal Tyrode and after treatment with 200nM UII. N= 7 hearts, 73 cells.

(C) Bar chart of systolic $[Ca^{2+}]_i$ recorded from freshly isolated ventricular cardiomyocytes before and after treatment with 200nM UII. N= 7 hearts; 73 cells.

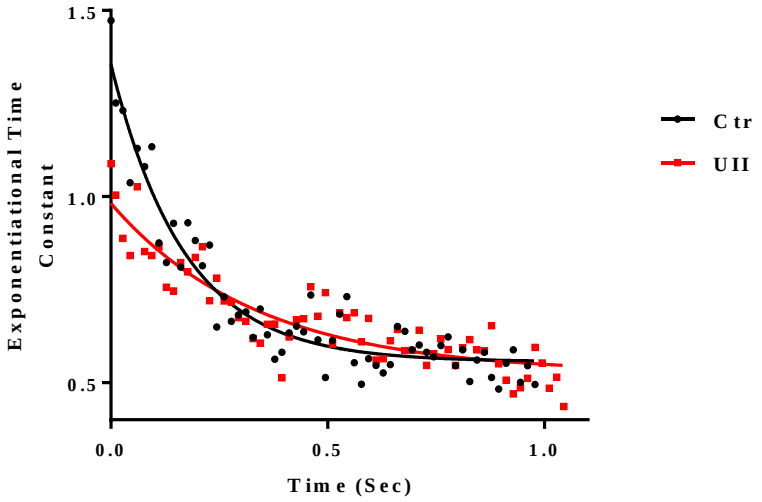
The results are expressed as mean ± S.E.M. ****p<0.0001. Paired t-test.

3.2.2.4 Effect of UII on ventricular cardiomyocyte relaxation

Myocardial relaxation is initiated when intracellular Ca^{2+}-transient level is decreased in the cardiac muscle and in rat ventricular cardiomyocytes, this is mainly attributed to the activity of SERCA2a (Stammers *et al.* 2015).

Figure 3.10A shows a single exponential curve fitted to show the exponential decay of Ca^{2+}-transient under control conditions (NT) and then in the presence of UII (200nM). The mean exponential time constant of the decay of the electrically-provoke Ca^{2+}-transient recorded in ventricular cardiomyocytes is shown in Figure 3.10B. The data show no significant difference in relaxation time following UII stimulation (147.5 ± 3.4ms) compare to control (145.2 ± 3.2ms) (6 hearts; 36 cells, $p>0.05$).

A



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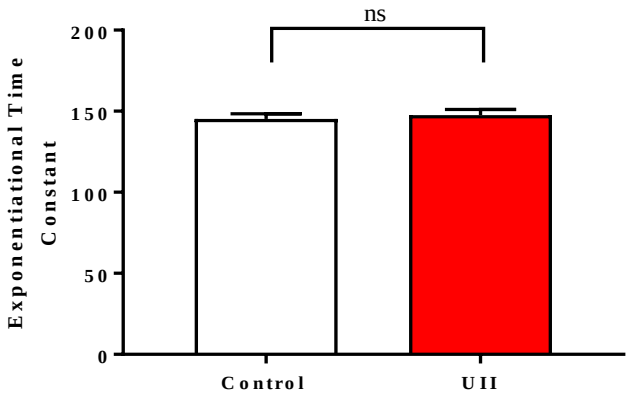


Figure 3.10: Exponential time constant for relaxation of the electrically-provoked Ca^{2+}-transients for ventricular cardiomyocytes.
(A) Fit curve shows time constants Tau for both control and stimulated cells with 200nM UII**.**
(B) Bar chart showing exponential time constant for control and treated cells with 200nM UII. N= 6 hearts; 36 cells. The results are expressed as mean ± S.E.M. Paired t-test.

3.2.2.5 Effects of UII on sarcoplasmic reticulum calcium content

In the rat ventricular cardiomyocyte, the Ca^{2+}-released from the SR contributes around 80-90% of the Ca^{2+} to systolic $[Ca^{2+}]_i$ (Wier *et al.* 1994). Therefore the SR Ca^{2+}-content was investigated, to determine whether this was responsible for the reduction in systolic $[Ca^{2+}]_i$ by UII. To determine the effect of UII on SR Ca^{2+}-content, the rapid pulse of caffeine (10mM for 5 seconds) was used, which opens the RyR2 and causes the release of Ca^{2+} from SR, and this was then measured as an indicator of total SR Ca^{2+}-content. The amplitude of this caffeine-induced Ca^{2+}-transient represents Ca^{2+}-content of the SR (Bers 2001).

Figure 3.11A shows a trace of intracellular Ca^{2+} in response to the application of caffeine. Ca^{2+}-content was measured in this way in normal Tyrode as a control and then after superfusion with UII. After the caffeine-provoked Ca^{2+}-transient declined, the electrical stimulation was then resumed. The cardiomyocytes were then perfused with 200nM UII for 10 minutes and subjected to 1Hz electrical field stimulation. Once $[Ca^{2+}]_i$ had reached a steady state level, electrically provoked $[Ca^{2+}]_i$ transients and caffeine provoked $[Ca^{2+}]_i$ transients were recorded again as mentioned above.

Figure 3.11B shows a record of $[Ca^{2+}]_i$ from single ventricular cardiomyocytes. Electrically-stimulated Ca^{2+}-transients were recorded and at steady-state the stimulator was switched off and the cell superfused with caffeine (10mM) to liberate the SR Ca^{2+}-content and the peak of caffeine-induced Ca^{2+}-release measured. The peak caffeine-induced Ca^{2+}-transient was reduced from 440.8 ± 13.9nM in normal Tyrode to 412.1 ± 14.7nM after superfusion with 200nM UII for 10 minutes (n= 11 hearts; 85 cells, $p<0.01$).

The exponential decay of the caffeine-provoke Ca^{2+}-transient is an indication of the activity of sarcolemmal bound Ca^{2+}-extrusion mechanisms (NCX and PMCA), and this data is shown in Figure 3.11C for the control (NT) and UII. The data show that a reduction in relaxation time in freshly isolated ventricular cardiomyocytes from 151.6 ± 4.4ms in NT to 138.9 ± 3.8ms in UII (n= 11 hearts; 69 cells, $p<0.0001$).

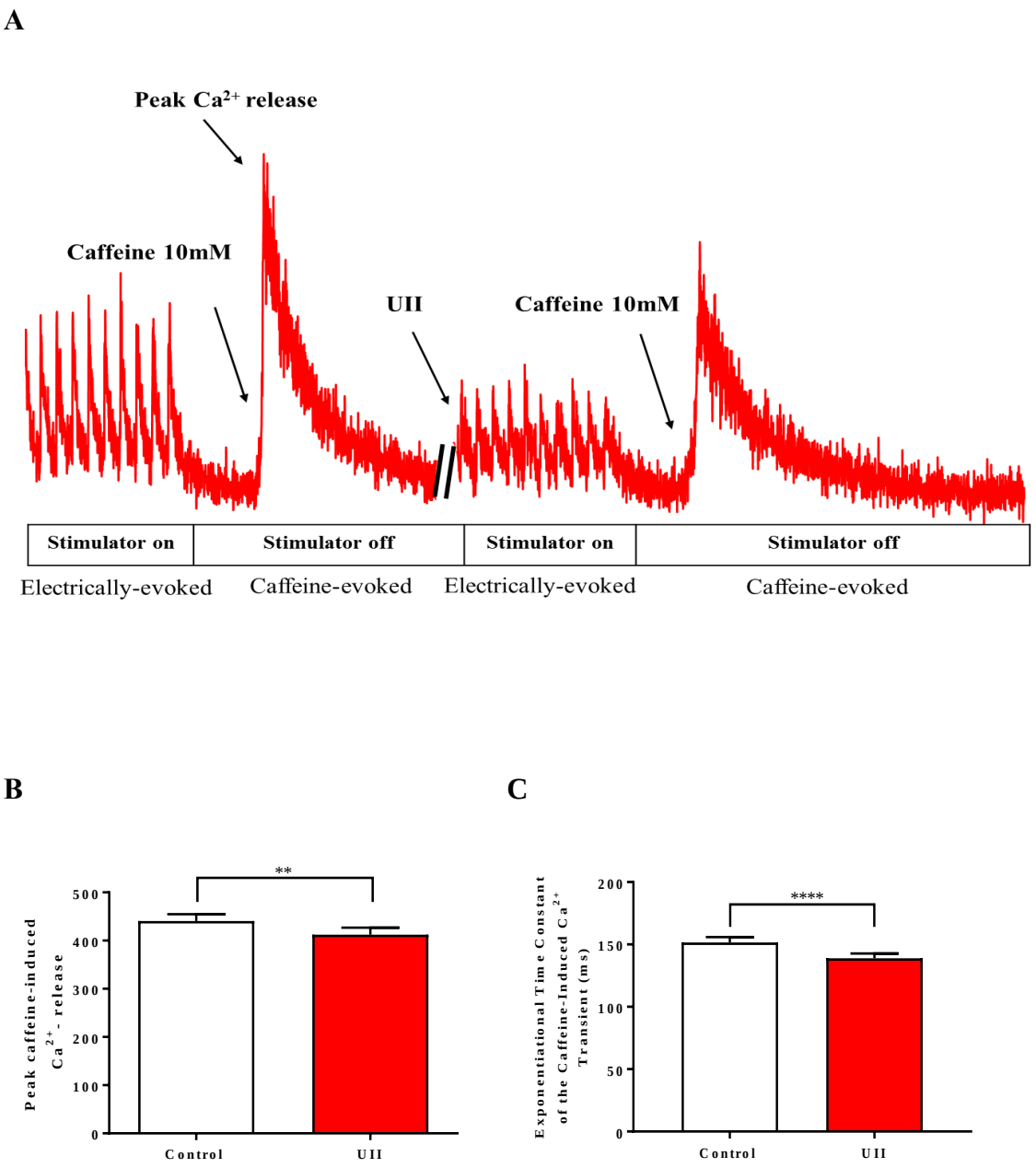


Figure 3.11: Measurement of SR Ca^{2+}-content after induced the ventricular cardiomyocytes by 10mM caffeine before and after treatment with 200nM UII.

(A) Example trace of $[Ca^{2+}]_i$ recorded from a single ventricular cardiomyocyte, showing the response to caffeine (10mM) in normal Tyrode (control) and in the presence of UII (200nM).

(B) Bar chart shows the peak caffeine-induced Ca^{2+}-transient recorded from ventricular cardiomyocytes before (normal Tyrode) and after 10 minutes superfusion with UII (200nM). N= 11 hearts; 85 cells.

(C) Exponential time constant of the decay of the caffeine-provoked transient recorded from ventricular cardiomyocytes in NT and after stimulation with 200nM UII. N= 11 hearts; 69 cells. The results are expressed as mean ± S.E.M. **p<0.01, ****p<0.000. Paired t-test.

3.2.2.6 Effect of antagonist SB657510 on Ca^{2+}-regulation

To determine whether negative inotropic effects of rUII were a result of a decline in systolic $[Ca^{2+}]_i$, experiments were conducted in ventricular cardiomyocytes loaded with Fura-2 to measure $[Ca^{2+}]_i$ (see methods, section 2.2.2.2.1). The effects of SB657510 UT receptor antagonist on intracellular Ca^{2+}-transient recorded from electrically stimulated ventricular cardiomyocytes were studied in presence or absence of rUII 200nM. At the beginning of experiment, diastolic $[Ca^{2+}]_i$ and systolic $[Ca^{2+}]_i$ were examined in the ventricular cardiomyocytes in presence of 200nM UII (Figure 3.12A & B).

There was no significant difference between control (59.47 ± 1.67nM) and superfused ventricular cardiomyocytes with SB657510 (1µM) alone for 5 minutes (61.87 ± 1.75nM) ($p>0.05$) in diastolic $[Ca^{2+}]_i$. Diastolic calcium markedly elevated after superfusion of the ventricular cardiomyocytes with UII 200nM and 1µM of SB657510 together (66.4 ± 1.8nM) ($p<0.001$). Additionally, there was significant difference between SB657510 group (61.8 ± 1.75nM) and UII+SB657510 group (66.4 ± 1.8nM) ($p<0.05$) (n= 7 hearts; 78 cells) (Figure 3.12C).

However, there was no significant difference in systolic $[Ca^{2+}]_i$ between control (normal Tyrode) (332.4 ± 6.3nM) and superfused ventricular cardiomyocytes with antagonist SB657510 alone (324.9 ± 5.7nM) ($p>0.05$) or after superfusion of the cardiomyocytes with UII+SB657510 (331.6 ± 5.8nM) ($p>0.05$). Also, there was no significant change in the reduction of systolic $[Ca^{2+}]_i$ between SB657510 group (324.9 ± 5.7nM) and UII+SB657510 group (331.6 ± 5.8nM) ($p>0.05$) (n= 7 hearts; 78 cells) (Figure 3.12D).

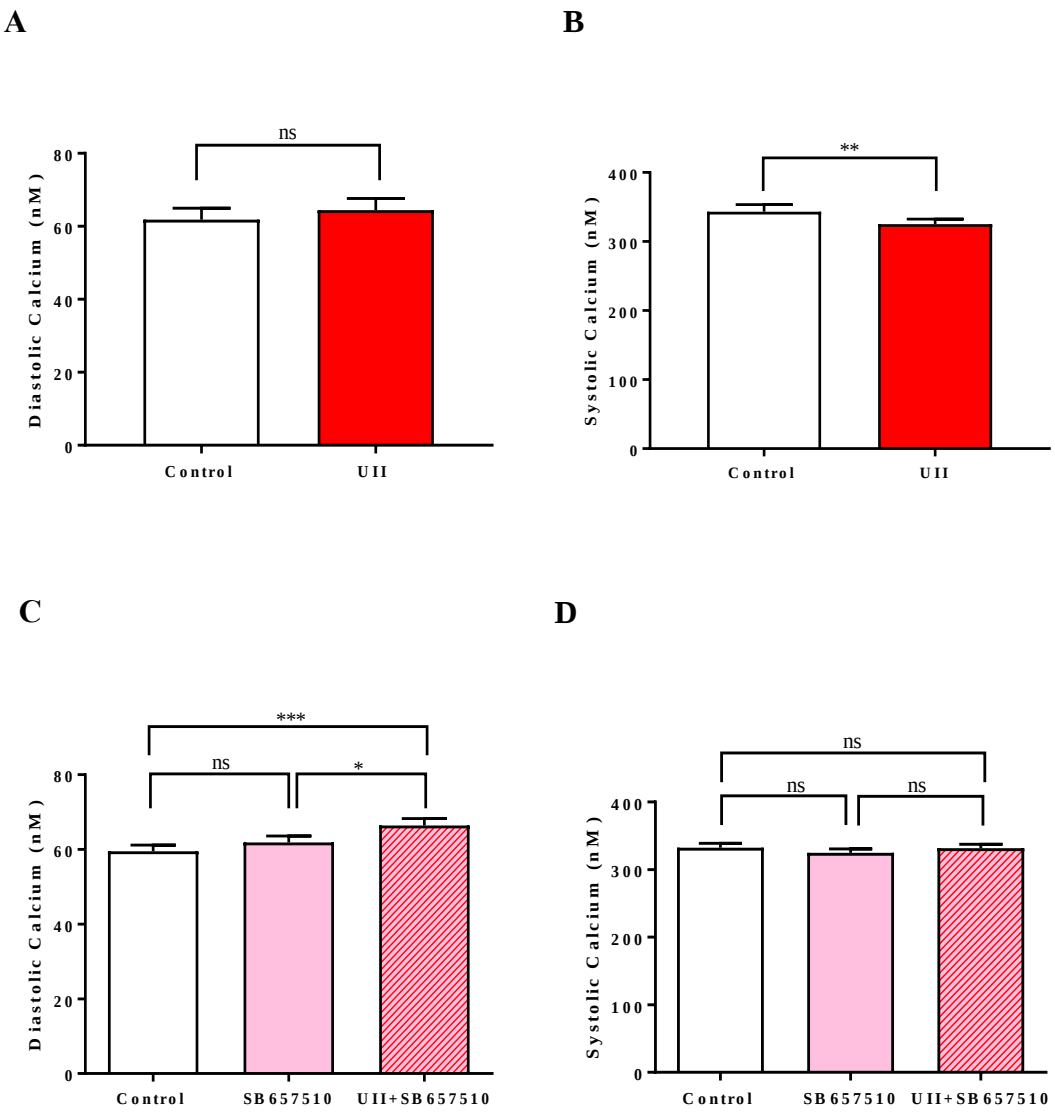


Figure 3.12: Effect of SB657510 on intracellular Ca^{2+}-transient of ventricular cardiomyocytes.

(A) Bar chart of diastolic $[Ca^{2+}]_i$ recorded from ventricular cardiomyocytes in normal Tyrode and after treated with 200nM UII. N= 7 hearts, 27 cells. The results are expressed as mean ± S.E.M. Paired t-test.

(B) Bar chart of systolic $[Ca^{2+}]_i$ recorded from ventricular cardiomyocytes before and after treated with 200nM UII. N= 7 hearts; 27 cells. The results are expressed as mean ± S.E.M. **p<0.01. Paired t-test.

(C) Measurement of diastolic $[Ca^{2+}]_i$ after superfusion of cells with SB657510 (1µM) alone or SB657510 (1µM)+UII 200nM. N= 7 hearts; 78 cells. The results are expressed as mean ± S.E.M. *p<0.05, ***p<0.001. One-way ANOVA followed by Sidak's *post hoc* test.

(D) Measurement of systolic $[Ca^{2+}]_i$ after superfusion of cells with SB657510 (1µM) alone or SB657510 (1µM)+UII 200nM. N= 7 hearts; 78 cells. The results are expressed as mean ± S.E.M. One-way ANOVA followed by Sidak's *post hoc* test.

3.2.3 The effect of UII stimulation on ventricular cardiomyocytes action potential

To determine the effects of UII on electrical activity, the effects of UII on the configuration of the action potential was investigated (Figure 3.13A), in particular the study looked at percentage change in action potential duration ($APD_{30/50/90}$) in response to UII (200nM) in freshly isolated ventricular cardiomyocytes. The data show APD_{30} was significantly reduced from (9.5 ± 1.4ms) in NT to (5.3 ± 0.9ms) in UII ($p<0.01$) and APD_{50} from (19.5 ± 2.1ms) in NT to (12.1 ± 1.6ms) in UII ($p<0.001$). However, there was no significant change in APD_{90} between UII (50.9 ± 6.0ms) compared to NT (57.2 ± 5.2ms) ($p>0.05$) (n= 6 hearts; 41 cells) (Figure 3.13B).

Figure 3.13C shows action potentials recorded from cultured ventricular cardiomyocytes for 24 hours using the whole cell patch clamp. The percentage change in action potential duration ($APD_{30/50/90}$) was calculated in response to UII (200nM). The data show APD_{30} was significantly reduced from (14.4 ± 2.2ms) in NT to (9.6 ± 1.2ms) in UII ($p<0.05$) and APD_{50} from (29.9 ± 2.81ms) in NT to (22.7 ± 2.66ms) in UII ($p<0.01$). However, there was no significant change in APD_{90} between UII (111.9 ± 11.0ms) compared to NT (121.6 ± 9.9ms) ($p>0.05$) (n= 6 hearts; 44 cells).

Figure 3.14A shows the mean data of action potential duration for freshly isolated cells and 24 hours cultured cells. The percentage change in action potential duration ($APD_{30/50/90}$) was calculated for freshly isolated and 24 hours cultured ventricular cardiomyocytes after superfusion of these cells with normal Tyrode. The data show, there was no significant change in APD_{30} between freshly isolated cells (9.5 ± 1.4ms) and cultured cells (14.4 ± 2.2ms) ($p>0.05$) and APD_{50} in freshly isolated cells (19.5 ± 2.1ms) and cultured cells (29.9 ± 2.81ms) ($p>0.05$). While, there was a significant difference in APD_{90} between freshly isolated cells (57.2 ± 5.2ms) and cultured cells (121.6 ± 9.9ms) ($p<0.0001$) (Figure 3.14B).

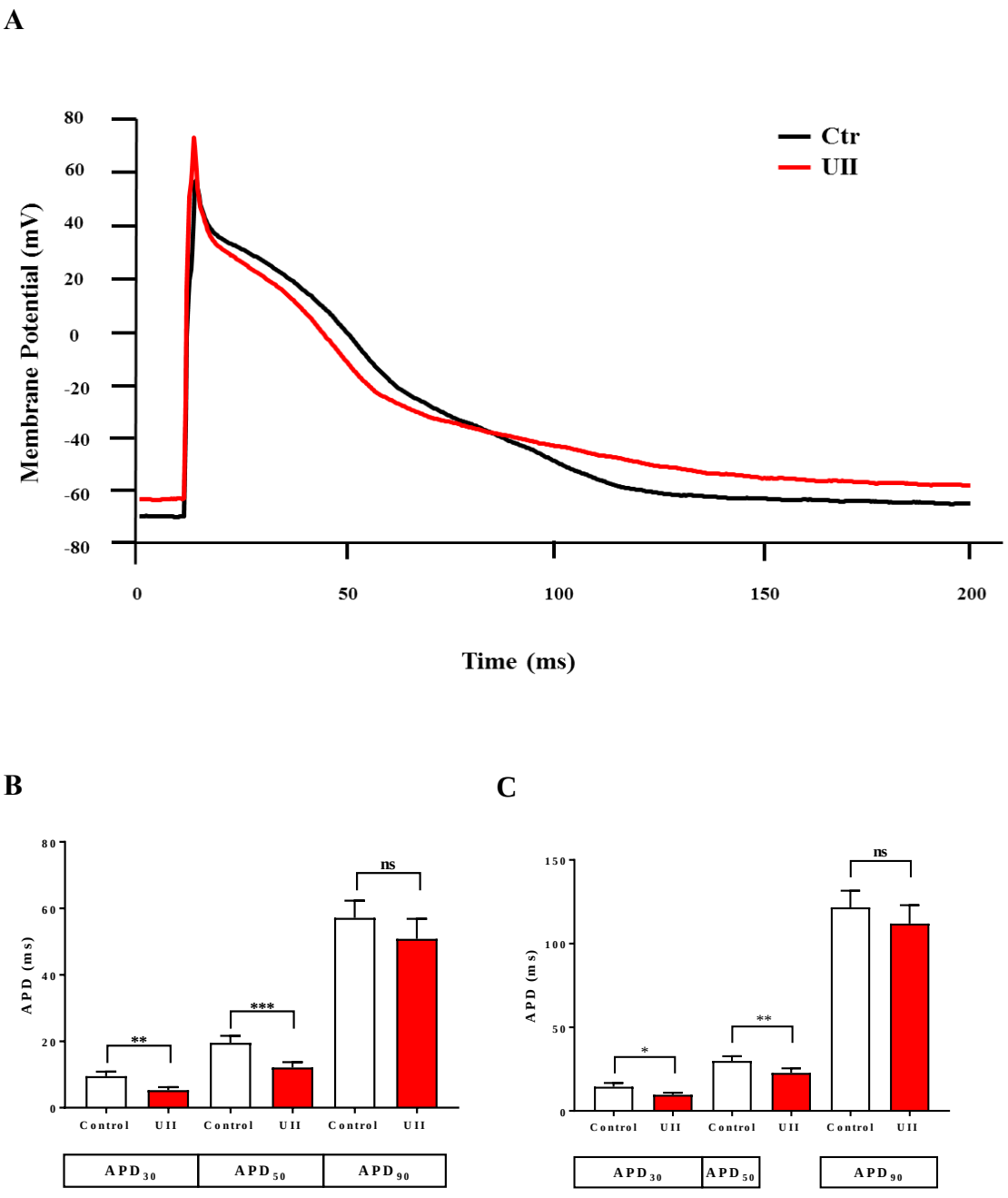
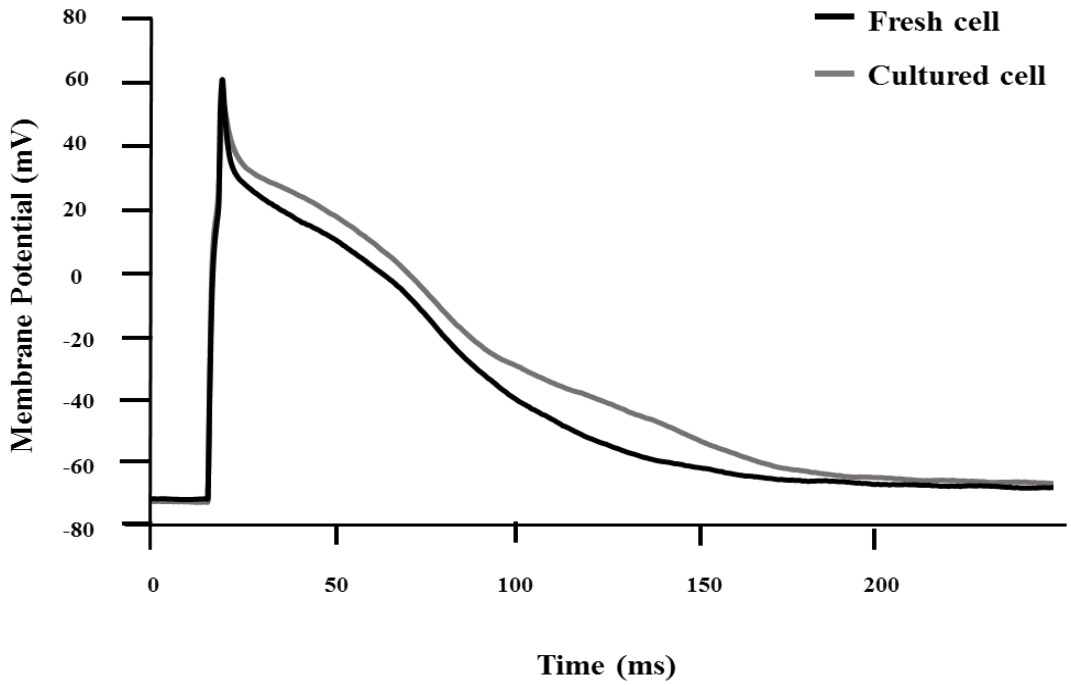


Figure 3.13: Effect of UII (200nM) on ventricular cardiomyocytes action potential.
(A) Representative action potential recordings from a single ventricular cardiomyocyte superfused with normal Tyrode and 200nM UII.
(B) Bar chart showing; APD_{30}, APD_{50} and APD_{90} recorded from freshly isolated ventricular cardiomyocytes superfused with NT followed by 200nM UII. N= 6 hearts; 41 cells.
(C) Bar chart shows APD_{30}, APD_{50} and APD_{90} recorded from cultured ventricular cardiomyocytes. N= 6 hearts; 44 cells.
Data are mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001. One-way ANOVA, Sidak's *post hoc* test.

A



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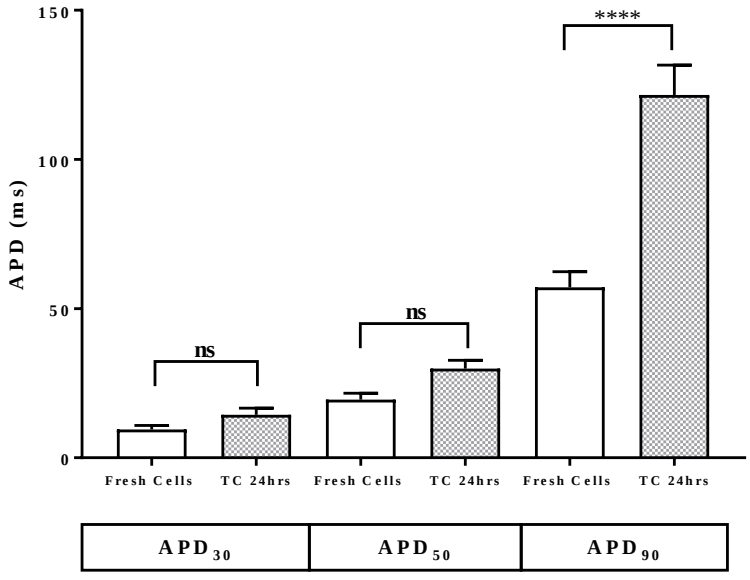


Figure 3.14: Comparison of action potential duration recorded from freshly isolated and cultured ventricular cardiomyocytes.

(A) Representative action potential recordings from freshly and cultured ventricular cardiomyocytes superfused with normal Tyrode.

(B) Bar chart shows APD_{30}, APD_{50} and APD_{90} recorded from freshly isolated and cultured ventricular cardiomyocytes for 24 hours. N= 6 hearts; 41 cells for fresh cell experiment, n= 6 hearts; 44 cells for 24 hours cultured cells experiment. The results are expressed as mean ± S.E.M. ****$p<0.0001$. One-way ANOVA followed by a Sidak's post hoc test.

3.2.4 Effects of UII on the L-type Ca^{2+}-current density

In the previous study by Zhang *et al.*, UII resulted in an increase in the L-type Ca^{2+}-current (Zhang *et al.* 2015). The result showed that the APD_{50} is reduced by UII (200nM), which suggests a reduction in the LTCC. Therefore, the effects of acute UII (200nM) for 10 minutes on the LTCC density was examined. The data show that UII (200nM) decreased the LTCC recorded from ventricular cardiomyocytes at -10mV from 14.7 ± 0.6 pA/pF in NT to 12.6 ± 0.7 pA/pF (n= 1 heart; 4, cells $p<0.001$) (Figure 3.15).

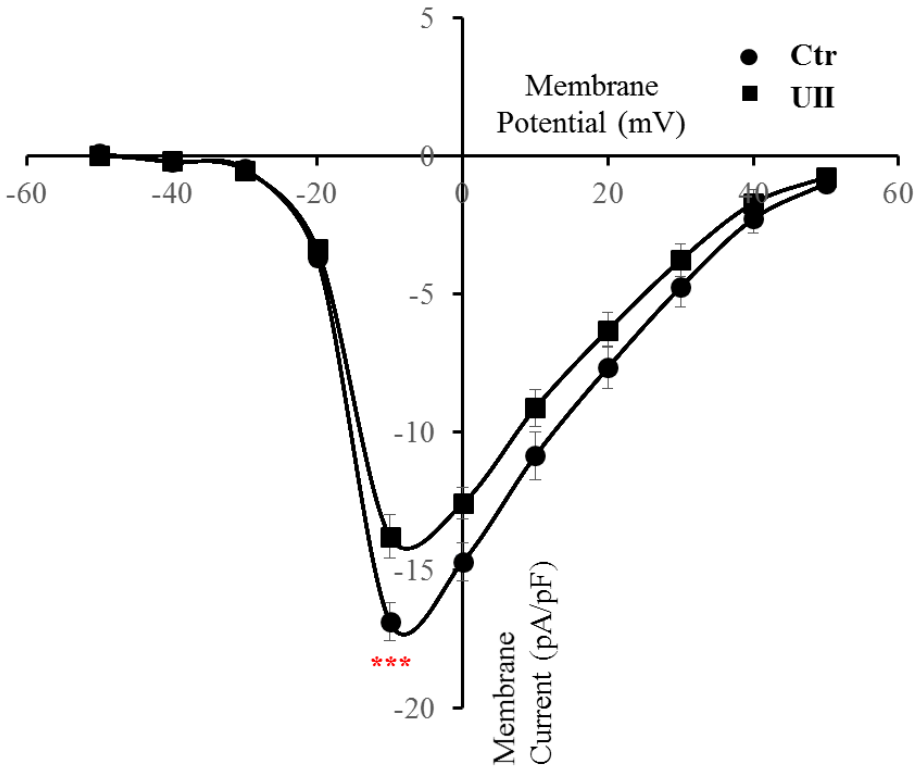


Figure 3.15: The effect of UII (200nM) on the L-type calcium current (LTCC).
Current-voltage relationship of the LTCC of freshly isolated ventricular cardiomyocytes, recorded in NT and in response to UII. N= 1 heart; 4 cells. Data are mean ± S.E.M. ***p<0.001. One-way ANOVA. Sidak's *post hoc* test.

3.3 Discussion

This study is an experimental investigation to explore the role of UII on E-C coupling in healthy ventricular cardiomyocytes.

1- The data show that acute application of UII caused a reduction in LVDP recorded from ex-vivo rat hearts with no effect on heart rate.

2- Urotensin II decreases cardiac contractility. It caused a negative inotropic effect determined as a reduction in % cell shortening in isolated adult rat ventricular cardiomyocytes.

3- Urotensin II causes a reduction in systolic $[Ca^{2+}]_i$. This reduction in systolic $[Ca^{2+}]_i$ may result from the decline in SR Ca^{2+}-content and a decrease in L-type Ca^{2+}-current.

4- The results also show that ventricular cardiomyocytes cultured for 48 hours, exhibit a similar level of a reduction in contractile function but still respond to UII in a similar fashion to freshly isolated ventricular cardiomyocytes.

3.3.1 Acute application of UII causes a decrease in myocardial contractility

The present study demonstrates that acute application of UII-induced a negative inotropic effect in whole hearts and isolated cells, which reflects the reduction in systolic Ca^{2+}. This finding is consistent with finding of past studies by Fontes-Sousa and colleagues, which shows a decrease in myocardial contractility of isolated papillary muscles of rabbit induced by UII, which is mediated by neurohormonal factors such as ET-1 and Ang II (Fontes-Sousa *et al.* 2009). The negative inotropic effect of UII is linked with a reduction in peak systolic $[Ca^{2+}]_i$. Normally, cardiac muscle contraction occurs as a result of an increase in concentration of intracellular Ca^{2+} in the cardiomyocytes, which in the rat is predominantly from the SR (90%) (Wier *et al.* 1994).

As discussed in the introduction (see section 1.4.3), UII levels in patients with heart failure are elevated and this may impact on E-C coupling in a negative way. A significant reduction in systolic $[Ca^{2+}]_i$ transients has been observed in ventricular cardiomyocytes isolated from heart failure patients (Beuckelmann *et al.* 1992). However, other studies have shown no changes in the peak of systolic $[Ca^{2+}]_i$ transients between end-stage failing human heart and control in isolated isometric contracting ventricular muscle and $[Ca^{2+}]_i$

was recorded using aequorin as probe for Ca^{2+} (Gwathmey *et al.* 1990; Morgan 1991). On the other hand, systolic $[Ca^{2+}]_i$ transients become shorter in isometrically contracting strip preparations taken from terminally failing human hearts when stimulation frequencies are increased, suggesting these changes in cardiac muscle loaded with aequorin can be observed at higher stimulation frequencies (Pieske *et al.* 1995).

The data shows that the reduction in strength of contraction on the ventricular cardiomyocytes was mirrored by decrease in systolic Ca^{2+}, which relies upon both influx through the L type Ca^{2+}channel and NCX, and efflux from the SR. It is known that the reduction in cytosolic Ca^{2+} could be either due to less LTCC current or less Ca^{2+}-induced Ca^{2+}-release and this can be modulated by the action potential duration. Therefore, the impairment of systolic $[Ca^{2+}]_i$ in ventricular cardiomyocytes in response to UII may reflect alterations in either the activity or expression of Ca^{2+} handling proteins such as SERCA2a, PLB, RyR2 or LTCC or it could be alteration in action potential configuration. Each of these possibilities will be discussed in greater detail below.

The observed difference in systolic $[Ca^{2+}]_i$ between control and ventricular cardiomyocytes superfused with UII may result from decrease in SR Ca^{2+}-content, which may account for the reduction in systolic $[Ca^{2+}]_i$. The results confirmed this as using caffeine revealed a reduction in SR Ca^{2+}-content. Normally, calcium uptake during diastole and therefore SR Ca^{2+}-content over time, relies on SERCA2a pump activity. Therefore, the reduction in SR Ca^{2+}-content could result from a reduction in the activity of SERCA2a and thereby reduced Ca^{2+}-content of the SR. The decrease in SERCA2a activity could be as a consequence of decreased expression of SERCA2a or upregulation of PLB mRNA expression or dephosphorylation of PLB. It is unfortunate that the study did not include determination of expression of SERCA2a or PLB and their protein levels. When SERCA2a activity is reduced, as there exists a competition between SERCA2a which sequesters Ca^{2+} to SR and NCX which drives Ca^{2+}-efflux, this will subsequently lead to decrease of SR contents. In this case, it is likely that NCX will be dominant and more Ca^{2+} extrusion during diastole to create a balance and reach to steady-state otherwise cytosolic Ca^{2+} gradually will be accumulated. In cardiac cells, several factors control the amount of Ca^{2+} that is released to the cytosol from the SR which mediated by RyR2 including, adequate concentrations of SR Ca^{2+} (Bers 2002), functional activity of RyR2 and SR regulatory proteins (Györke and Terentyev 2007; Bers 2008), as well as

activity of LTCC to trigger CICR (Bassani *et al.* 1995). Interestingly, the data of current study showed no effect of UII on the rate of relaxation of the electrically-evoked Ca^{2+}-transient, suggesting little effect on SERCA2a activity, which dominates removal of systolic Ca^{2+} during diastole in the rat myocardium (Wier *et al.* 1994). However, there was an increase in the rate of relaxation of the caffeine-induced Ca^{2+}-transient, which suggests an increase in either the activity of the NCX and/or the plasma membrane Ca^{2+}-ATPase.

Another possibility for the reduction of systolic $[Ca^{2+}]_i$ transients, could be that UII has a direct action on RyR2 causing diastolic leak thereby indirectly affecting accumulation of Ca^{2+} and the SR will become depleted. The decrease in the systolic $[Ca^{2+}]_i$ transients observed in ventricular cardiomyocytes after subjecting these cells to UII may be related to changes in the activity of RyR2. In the past few years, the regulatory mechanisms of RyR2 have been subjected to intensive investigations inasmuch to their pathophysiological relevance with heart diseases. Ca^{2+}-release from SR to the cytosol by RyR2 is essential for contraction. SR is refilled with Ca^{2+} and primed for the next contraction, therefore RyR2 should be remain closed during diastole thereby permitting SR Ca^{2+}-content to increase and refill the SR for subsequent contractions (Bers 2002). Hyperphosphorylation of RyR2 occurs at Ser^{2808} residue by PKA and may result from persistent stimulation of sympathetic system (increase activation of β-adrenergic system). It has been shown that PKA-phosphorylation induced dissociation of FKBP12.6 from RyR2. Eventually, an outcome is leakage of Ca^{2+} from SR and increased diastolic Ca^{2+} (Hain *et al.* 1995; Marx *et al.* 2000). It has been observed that the amount of both protein phosphatase 1 and 2A which associate to RyR2, are reduced in failing human heart. It is likely exacerbate phosphorylation of RyR2 by PKA, in turn FKBP12.6 (Marx *et al.* 2000). Urotensin II through IP_3 can lead to activation of CAMKII which phosphorylates the RyR2 and can increase open probability and may be responsible for an increase in SR Ca^{2+}-leak.

Another possible cause for the observed reduction in the amplitude of systolic $[Ca^{2+}]_i$ is the LTCC and a decreased influx of Ca^{2+}, resulting in less activation of RyR2. A decrease in LTCC may directly contribute to reduction of systolic $[Ca^{2+}]_i$ via a smaller signal for CICR or indirectly by shortening of the APD. When the cardiomyocyte is depolarized, a small amount of Ca^{2+}-influx occurs via LTCC, triggering calcium release from the

intracellular store (CICR) via RyR2 to the cytosol (Bers 2002). The plateau repolarization phase is predominantly due to entry of calcium to the cytosol via LTCC and both a reduction in APD50 and in the LTCC density were observed. Relaxation is initiated by closure of LTCC result in stop influx of Ca^{2+}, followed by extrusion of Ca^{2+} out of the cell by NCX, sequestration of Ca^{2+} through pumping it back into SR by SERCA2a (Bers 2008).

There was also an apparent increase in activity of NCX, as suggested by the increase in the rate of relaxation of the caffeine-induced Ca^{2+}-transient in freshly isolated ventricular cardiomyocytes treated with UII. In the steady-state of E-C coupling, the balance between influx of Ca^{2+} via LTCC and efflux across NCX is required for maintaining stable Ca^{2+} homeostasis (Bers 2002; Eisner *et al.* 2009). Increase in NCX activity could shift the balance of Ca^{2+}-fluxes causing a reduction in SR Ca^{2+}-content, NCX competes with SERCA2a to remove cytosolic Ca^{2+} when the its activity increased and may yield a reduction of SR Ca^{2+} store.

Although any effect on NCX activity seen in these experiments in response to acute exposure of ventricular cardiomyocytes to UII are likely to result from post-translational modification on the NCX proteins, numerous studies have found that increase of NCX mRNA and their protein levels in both animal models of heart failure (Bers 2001; Rodriguez *et al.* 2014) and cases of human heart failure (Studer *et al.* 1994; Flesch *et al.* 1996b; Hasenfuss and Pieske 2002) but some results which were obtained from studies in human heart failure are less consistent with results of animal models. Furthermore, some studies have observed NCX functional capacity to efflux of Ca^{2+} is increased in terminally heart failure (Flesch *et al.* 1996a; Reinecke *et al.* 1996). In heart failure, increased functional activity of NCX could be as compensatory mechanism for the reduction in SERCA2a pump level. The increased in activity of NCX can aid in relaxation of the heart although decreasing amounts of Ca^{2+} that are released from SR during cardiac systole (Pieske *et al.* 1995; Hasenfuss *et al.* 1997). Interestingly, increased NCX activity is sequentially associated with upregulation of NCX expression in hypertrophied hearts stimulated with pressure overload (Studer *et al.* 1997). Therefore, against a background of increased NCX protein levels in heart failure combined with increased levels of circulating UII would both work to increase NCX activity and hence alter SR Ca^{2+}-content.

Ligand binding leads to GPCR kinase activation, producing multisite phosphorylation of the GPCR (Pitcher *et al.* 1998), which successively acts to promote recruitment and binding of β-arrestins. Ultimately, β-arrestins binding leads to uncoupling of G-proteins from receptors thus precluding further signalling via the G-protein and promoting receptor internalisation into the cell by endocytosis, rapid down-regulation of the receptor's target due to a decrease in receptor number on the cell surface (Oakley et al. 2001). β-arrestin acts as an adapter protein which can intimately control the function of the GPCR. It binds to clathrin which facilitates entry of receptors into clathrin-coated pits and mediates the endocytosis process (Claing *et al.* 2002). Following internalisation, the receptor is trafficked either to lysosomes for proteolysis (Martin *et al.* 2003) or recycled back toward the plasma membrane (Tanowitz and von Zastrow 2002).

As the UT receptor binds UII with high affinity we decided to determine whether UT receptor was either occupied by the presence of endogenous UII with the heart in-vivo prior to excision and isolation of cells and/or damage to receptors during the cell isolation process. The study looked at whether the sensitivity of the ventricular cardiomyocytes to UII changed by culturing the cells (>24 hours). The percentage cell shortening was decreased in normal cultured cells after 24 and 48 hours, however, this reduction was complete after 24 hours with no difference between 24 and 48 hours. In spite of this reduction in contraction strength of ventricular cardiomyocytes in tissue culture they still respond to UII and ISO. The present study showed that the reduction in percentage cell shortening by UII was similar between freshly isolated ventricular cardiomyocytes and cultured cells after 24 hours but this effect of UII was not significant after 48 hours. However, the data set are limited (48 hours).

Because the results of the project have shown that UII has a negative inotropic effect, the effect of SB657510 antagonist was investigated to detect whether the negative inotropic effects of rUII was a result of a reduction in systolic $[Ca^{2+}]_i$ in ventricular cardiomyocytes. There was no significant difference in the systolic $[Ca^{2+}]_i$ and the effects of UII were reversed by the selective antagonist. The negative inotropic effects of rUII may due to a decline in systolic $[Ca^{2+}]_i$.

As contraction strength can also reflect changes in electrical activity, the effects of acute UII on the configuration of the action potential was determined. APD was measured in this investigation, the study was shown for the first time a reduction in APD. Acute

application of UII decreased APD_{30} and APD_{50} in both freshly isolated ventricular cardiomyocytes and cultured cells.

This marked reduction in APD may be indicative of a decrease in the LTCC currents, which through a reduction in Ca^{2+}-influx would decrease Ca^{2+}-release from the SR. In preliminary experiments, a significant reduction in LTCC density by UII (200nM) was shown. However, a reduction in APD could also have an indirect effect on contraction strength by modulating NCX activity during diastole. As stated previously, the removal of intracellular Ca^{2+} during diastole, results from a competition between SR sequestration by SERCA2a and Ca^{2+}-efflux through the NCX. With a shorter APD, the NCX favours Ca^{2+}-efflux (forward-mode) and this will, over time, reduce SR Ca^{2+} load. The reduction in APD_{30} could may reflect a reduction in the transient outward current (I_{to}), however, the transient outward current was not investigated in this study.

3.3.2 Study limitations

In this chapter effect of exogenous UII has shown on E-C coupling. The initial aim was to investigate the influence of UII on excitation-contraction coupling and calcium regulation in single healthy ventricular cardiomyocytes. The major limitation of this study is that the model used was isolated ventricular cardiomyocytes from healthy animals. During the isolation, the heart muscle is expose to variations in $[Ca^{2+}]_i$ as well as short periods of ischemia and actions of protease which may target the UT receptors. However, a similar reduction in contraction strength and in APD was seen in cells cultured for 24 and 48 hours. In further studies we would suggest looking at cardiomyocytes isolated from animals with heart failure, where the normal E-C coupling processes are impacted by the disease and therefore more closely reflects the clinical state.

Effect of UII on strength of contraction was measured in only 9 cultured cardiomyocytes after 48 hours, compared with 71 freshly isolated cells and 67 after 24 hours in tissue culture. The number of cells was limited in tissue cultured cells because the cells become fragile and difficult to study. In addition, the cardiomyocytes appeared to lose their ability to contract efficiently after long-term culture.

Only preliminary experiments in which LTCC currents were recorded from 4 cardiomyocytes, show a reduction in current density. However, more experiments need

to be conducted and in addition, the effects of UII on the transient outward current should be included. We would also look at RyR leak from freshly isolated cardiomyocytes.

3.3.3 Conclusion

The present study was designed to determine the effect of UII on myocardial contractility and its role in healthy ventricular tissues. The following conclusion can be drawn UII reduces the myocardial contractile activity of healthy isolated fresh and cultured rat ventricular cardiomyocytes. This may be due to a decreased SR Ca^{2+}-load as a result of decrease SERCA2a activity or increase RyR2 open causing leakage of $Ca^{2+.}$ The acute negative inotropic effects of UII and reduction in systolic $[Ca^{2+}]_i$ could be associated with action potential duration shortening and LTCC current density. The second major finding was that although cultured cardiomyocytes exhibit a significant reduction in normal E-C coupling parameters in cultured cells, they still show a significant response to UII or ISO.

Chapter 4: Cellular and molecular events mediated by UII

4.1 Introduction

Cardiac hypertrophy has been identified as one of the greatest independent risk factors for cardiac morbid events (Richey and Brown 1998). Much effort has been concentrated around characterizing the intracellular signaling pathways involved in, and the neurohormonal stimuli that are associated with ventricular cardiomyocyte hypertrophy. Ventricular cardiomyocytes hypertrophy develops as an adaptive response when the cells are stimulated with a wide array of growth stimuli. Nevertheless, sustained hypertrophy is often significantly associated with an increase in the risk of the progression to heart failure. Cardiovascular regulating factors that participate in cardiac hypertrophy; exemplified by Ang II, ET-1 and catecholamines (α-adrenergic), bind to G protein coupled receptors (GPCR) causing conformational changes and dissociation of $G_{\alpha q}$ resulting in activation of downstream signaling proteins (Molkentin and Dorn II 2001).

Myocardial hypertrophy is accompanied by changes in specific patterns of gene expression and cellular and molecular events. It is initiated as a result of different stimulating factors and signals which contributed to the development of disease, presumably reflective of these events at the level of cardiomyocyte (Clerk *et al.* 2007). The MAPK family has been reported to be important for induction of cardiac hypertrophy and have been widely investigated (Wang *et al.* 1998; Zhang *et al.* 2003b), including ERKs, p38 and JNK (Wang *et al.* 1998; Zou *et al.* 2001a; Onan *et al.* 2004b).

UII is a neurohormonal peptide; both peptide and its receptor are ubiquitously expressed in cardiac tissue of patients with end stage heart failure, it is mainly increased in cardiomyocytes but to a lesser extent also in endothelial cells and vascular smooth muscle cells (Douglas *et al.* 2002). The relationship between UII levels and cardiac dysfunction have been widely investigated in patients with cardiac disease (Ng *et al.* 2002; Richards *et al.* 2002; Russell *et al.* 2003; Simpson *et al.* 2006; Khan *et al.* 2007). However, there are some studies that have shown no correlation between UII and disease state (Dschietzig *et al.* 2002; Jołda-Mydłowska *et al.* 2006). Potentially the differences between these studies could be related to difference in sample populations collected from patients (different tissues were collected or age of patients) or in assays that have been used (Bousette and Giaid 2006).

Evidence directly implicating UII in modulation of cardiac dysfunction and pathogenicity has been accumulated (Tzanidis *et al.* 2003; Onan *et al.* 2004b). In-vitro and in-vivo experimental studies have been undertaken to identify the role of UII in cardiac and vascular remodeling which contributes to the progression of cardiovascular disease. These studies generally have included monitoring the effect of UII either on morphological changes and protein synthesis via treatment of cells in culture with UII or determination of structural and functional changes to the cardiovascular system following administration of the peptide directly to animals. For instance, treatment of cultured neonatal cardiac fibroblasts with UII leads to profibrogenic responses and overexpression of fibronectin, increased level of procollagen mRNA type I and III and stimulation of collagen production (Tzanidis *et al.* 2003). It was suggested that overexpression of UT receptor alone does not trigger cell growth (Tzanidis *et al.* 2003; Onan *et al.* 2004b). In neonatal cardiomyocytes transfected with recombinant adenovirus UT receptors, to produce high levels of expression, there was no hypertrophy in the absence of UII stimulation.

The hypothesis of this study is that UII promotes strong phenotypic changes and as plasma levels of UII are elevated in heart failure, they may be involved in the development of pathological cardiac hypertrophy. The initial objective of the research project was to determine whether UII is involved in hypertrophy. Therefore, it has been sought to induce hypertrophy in cultured adult rat ventricular cardiomyocytes in response to UII and to identify the signalling pathways of hypertrophy induced by UII.

4.2 Results

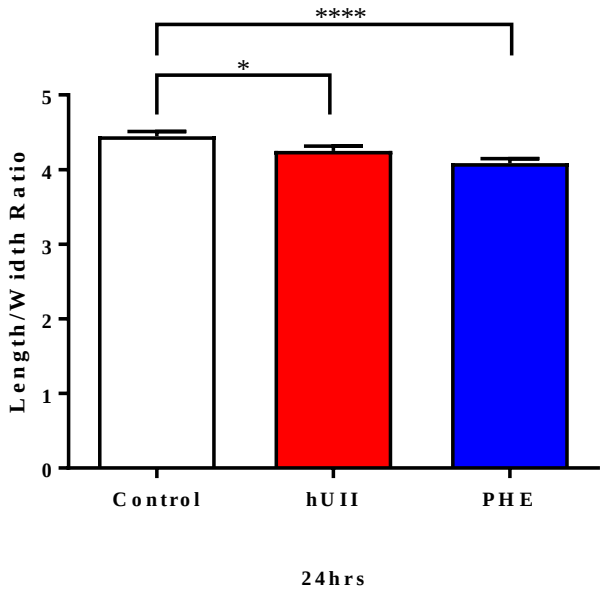
4.2.1 UII drives hypertrophy of adult ventricular cardiomyocytes in primary culture

In order to induce hypertrophy in adult rat ventricular cardiomyocytes, tissue culture was established and optimized. The purpose of this experiment was to detect whether UII is involved in the regulation of cardiomyocyte hypertrophic growth. Ventricular cardiomyocytes were treated with 200nM UII or 10µM phenylephrine as a positive control for 24 and 48 hours and cells cultured in normal medium for 24 and 48 hours used as the negative control. Change of myocardial morphology was measured as outlined in the methods section (see methods, section 2.3.2).

Ventricular cardiomyocytes demonstrated characteristic phenotypic changes associated with pathophysiological hypertrophy, in response to either phenylephrine or UII, as indicated by significant decrease in length/width ratio after 24 and 48 hours incubation in culture. Phenylephrine caused a significant reduction in length/width ratio after 24 hours from 4.45 ± 0.06 (n=335) to 4.09 ± 0.06 (n=338) ($p<0.0001$) (Figure 4.1A) and after 48 hours from 4.536 ± 0.10 (n=126) to 3.77 ± 0.08 (n=141) ($p<0.0001$) (Figure 4.1B). These data demonstrate the ability of our tissue culture system to show a significant level of hypertrophy in response to the well documented hypertrophic stimulus of phenylephrine (Gan *et al.* 2005).

Similarly, treatment of ventricular cardiomyocytes with UII (200nM) lead to alteration in the morphology of cardiomyocytes, with a significantly reduced length/width ratio after incubation with hUII for 24 hours from 4.45 ± 0.06 (n=335) to 4.25 ± 0.06 (n=362) ($p<0.05$) (Figure 4.1A). After 48 hours treatment from 4.53 ± 0.10 (126) to 3.99 ± 0.06 (n=209); ($p<0.0001$) (Figure 4.1B). Ventricular cardiomyocytes stimulated with hUII (200nM) became hypertrophied at a level equivalent to that produced by phenylephrine (10µM).

A



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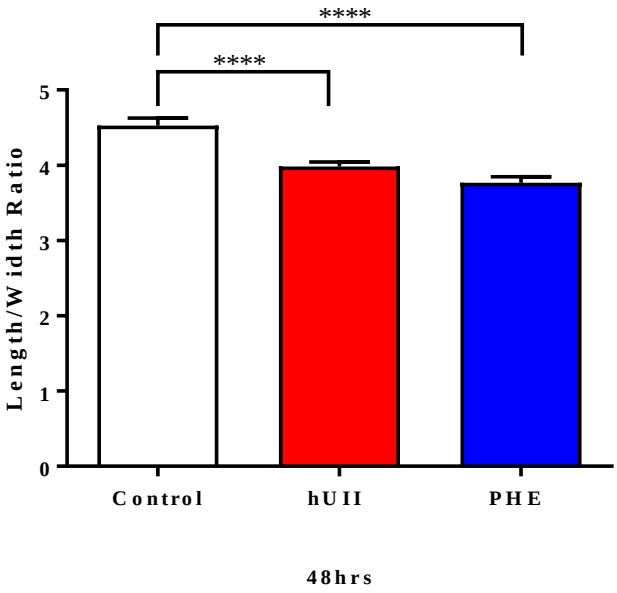


Figure 4.1: Length/width ratio of ventricular cardiomyocytes in response to hUII and phenylephrine.

(A) Measurement of length/width ratio after 24 hours treatment with hUII (200nM) or phenylephrine (10µM). N= 6 hearts; 335, 362 & 338 cells, respectively.

(B) Measurement of length/width ratio after 48 hours treatment with hUII (200nM) or phenylephrine (10µM). N= 6 hearts; 126, 209 & 141 cells, respectively.

The results are expressed as mean ± S.E.M. *p<0.05, ****p<0.0001 One-way ANOVA followed by Sidak's *post hoc* test.

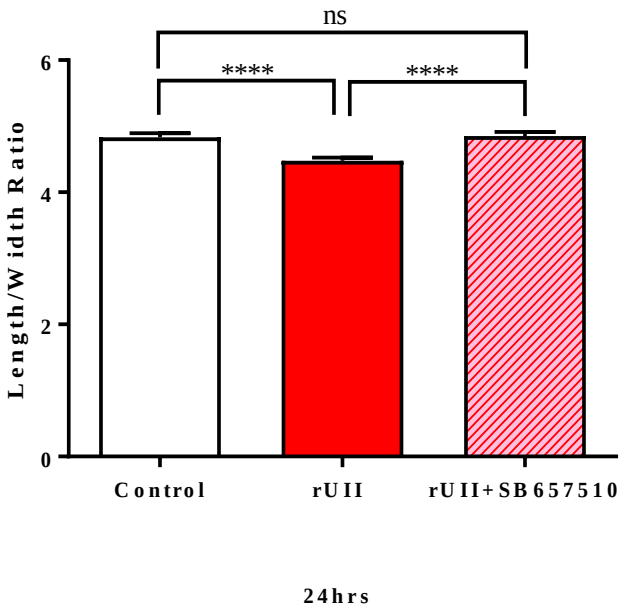
4.2.1.1 UT receptor involvement; experiments with the UT antagonist SB657510

To show that the hypertrophic effect of UII on ventricular cardiomyocytes involved binding of UII to the UT receptor, the cells were stimulated with rUII (200nM) in primary culture for 24 hours and 48 hours in the presence or absence of the UT receptor antagonist SB657510. Length/width ratio following incubation with rUII significantly decreased (4.48 ± 0.05, n= 519) compared with control (4.83 ± 0.0.59, n= 420) after 24 hours ($p<0.0001$) (Figure 4.2A) and to (4.37 ± 0.06, n= 380) after 48 hours in comparison with untreated cells (4.62 ± 0.06, n= 328) ($p<0.01$) (Figure 4.2B), these differences measured now with rUII were consistent with the previous hUII data.

Cultured ventricular cardiomyocytes were pretreated with 1µM antagonist SB657510 (Park *et al.* 2013) for 15 minutes before addition/and incubation with rUII 200nM. The effects of UII on the reduction in length/width ratio (hypertrophy) were blocked by the selective receptor antagonist. The length/width ratio after 24 hours of (4.85 ± 0.06, n= 441) in response to rUII+SB657510 compared to (4.48 ± 0.05, n= 519) in rUII alone ($p<0.0001$) (Figure 4.2A), and after 48 hours (4.78 ± 0.07, n= 304) in response to rUII+SB657510 compared with (4.37 ± 0.06, n= 380) in rUII alone ($p<0.0001$) (Figure 4.2B).

There was no significant difference between rUII+SB657510 group and control group after incubation for 24 hours (4.85 ± 0.06, n= 441 vs. 4.83 ± 0.06, n= 420) ($p>0.05$) (Figure 4.2A) or after 48 hours (4.78 ± 0.07, n= 304 vs. 4.62 ± 0.06, n= 328) ($p>0.05$) (Figure 4.2B). The ability of the specific UII receptor antagonist SB657510 to block the hypertrophic response to UII, shows the direct involvement of UII receptor in these responses.

A



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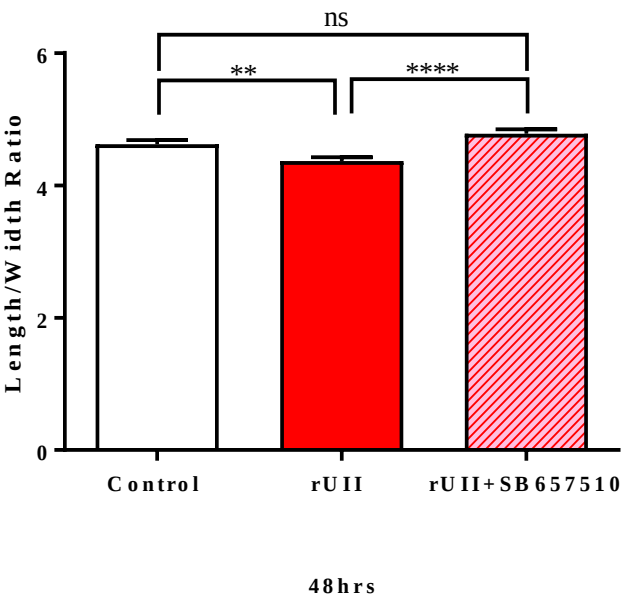


Figure 4.2: Effect of UT receptor antagonist on rUII induced hypertrophy in ventricular cardiomyocytes.

(A) Measurement of length/width ratio after rUII (200nM) or cells pretreated with SB657510 (1µM) before exposure to rUII for 24 hours. N= 6 hearts; 420, 519 & 441 cells, respectively.

(B) Measurement of length/width ratio after rUII (200nM) or pretreated the cells with SB657510 (1µM) at 48 hours. N= 6 hearts 328, 380 & 304 cells, respectively.

The results are expressed as mean ± S.E.M. **p<0.01 & ****p<0.0001. One-way ANOVA followed by Sidak's *post hoc* test.

4.2.1.2 Effect of UII on the diastolic Ca^{2+} of ventricular cardiomyocytes in culture

In the previous chapter looking at the acute effects of UII, it was noted that the diastolic Ca^{2+} increased and this may impact on both contractile function and contribute to the hypertrophic response to UII. Consequently, the effects of chronic exposure of ventricular cardiomyocytes to 200nM UII for 24 hours on Ca^{2+}-regulation of the ventricular cardiomyocytes were investigated. The cells were placed into tissue culture in the presence of 200nM UII for 24 hours. Prior to measurement of $[Ca^{2+}]_i$, the cells were washed with NT and loaded with fura-2 in order to measure a Ca^{2+}-transient (see methods, section 2.2.2.2.1). Diastolic $[Ca^{2+}]_i$ was increased from to 43.5 ± 1.4nM in control cells cultured in the absence of UII to 51.3 ± 1.6nM in cells cultured for 24 hours in the presence of UII (200nM) (n= 8 hearts; 94 cells, $p<0.0001$) (Figure 4.3).

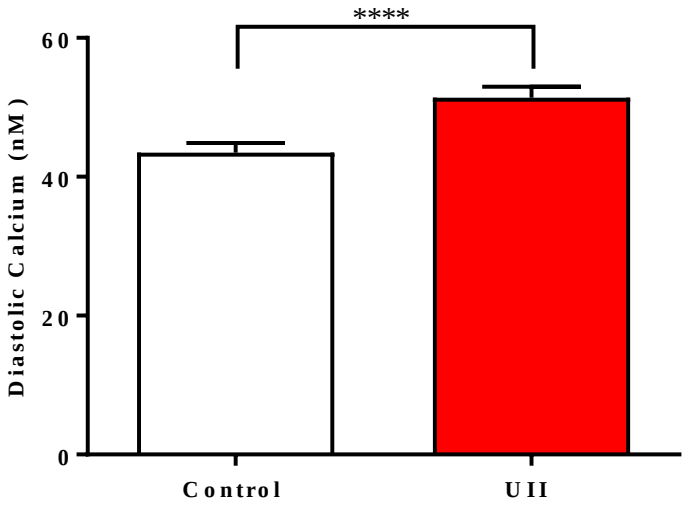


Figure 4.3: Measurement of intracellular diastolic Ca^{2+}-transient in cultured ventricular cardiomyocytes.

Diastolic $[Ca^{2+}]_i$ recorded from ventricular cardiomyocytes after 24 hours tissue culture in the absence (control) and presence of 200nM UII. N= 8 hearts; 94 cells.

The results are expressed as mean ± S.E.M. ****$p<0.0001$. Paired t-test.

4.2.1.3 Effect of UII on sarcoplasmic reticulum Ca^{2+}-leak

Several studies that employed experimental animal models of ventricular hypertrophy and human models of heart failure have consistently showed diastolic SR Ca^{2+}-leak; which is mediated through RyR2 (Shannon *et al.* 2003; Bers 2012). This effect has been attributed to an increase in RyR2 open probability, which has the effect of increasing the leakiness of the SR and increasing diastolic Ca^{2+}, which then depletes SR Ca^{2+}-content and increases diastolic Ca^{2+}, which is negatively inotropic and pro-hypertrophic (Lipskaia *et al.* 2007; Kawase and Hajjar 2008).

Because of the increase in diastolic $[Ca^{2+}]_i$ it is hypothesised that treatment of ventricular cardiomyocytes with UII could enhance the diastolic SR Ca^{2+}-leak. To determine whether UII had increased SR Ca^{2+}-leak, tetracaine was used to block RyR2 leak whilst measuring diastolic Ca^{2+} in the absence of sarcolemmal Ca^{2+}-fluxes (Shannon *et al.* 2002).

To determine the effects of UII on SR Ca^{2+}-leak, ventricular cardiomyocytes cultured for 24 hours in the absence or presence of UII (200nM), were washed with NT and loaded with Fluo-3 to measure $[Ca^{2+}]_i$. Cells were perfused in NT and stimulated at 1Hz until steady-state, Ca^{2+}-transients were recorded and then the stimulator switched off and cells were superfused with Ca^{2+} and Na^+-free Tyrode (10mmol EGTA) (for detail description see methods, section 2.2.2.2.3), to determine diastolic $[Ca^{2+}]_i$ in the absence of Ca^{2+}-flux across the cell membrane (LTCC and NCX). Following this the cells were superfused with NT and electrically stimulated at 1Hz until normal Ca^{2+}-transient was recorded. The cells were then superfused with 1mM tetracaine in EGTA solution ($0Ca^{2+}$, Na^+) for 90 seconds (Figure 4.4). Tetracaine blocks RyR2 and prevents Ca^{2+}-leak from the SR and in the absence of Ca^{2+}-flux across the cell membrane diastolic $[Ca^{2+}]_i$ declines reflecting the absence of Ca^{2+}-leak though RyR2. The difference between the diastolic $[Ca^{2+}]_i$ was measured in the presence and absence of tetracaine which blocks RyR2, reflecting SR Ca^{2+}-leak through RyR2 (Shannon *et al.* 2002). The data show that UII had no significant effect on SR Ca^{2+}-leak, with a fall of diastolic $[Ca^{2+}]_i$ of 15.3 ± 2.6nM in control cells cultured for 24 hours (n=19 cells) compared to 17.1 ± 2nM in ventricular cardiomyocytes cultured for 24 hours in the presence of UII (n = 25 cells) ($p>0.05$) (Figure 4.5).

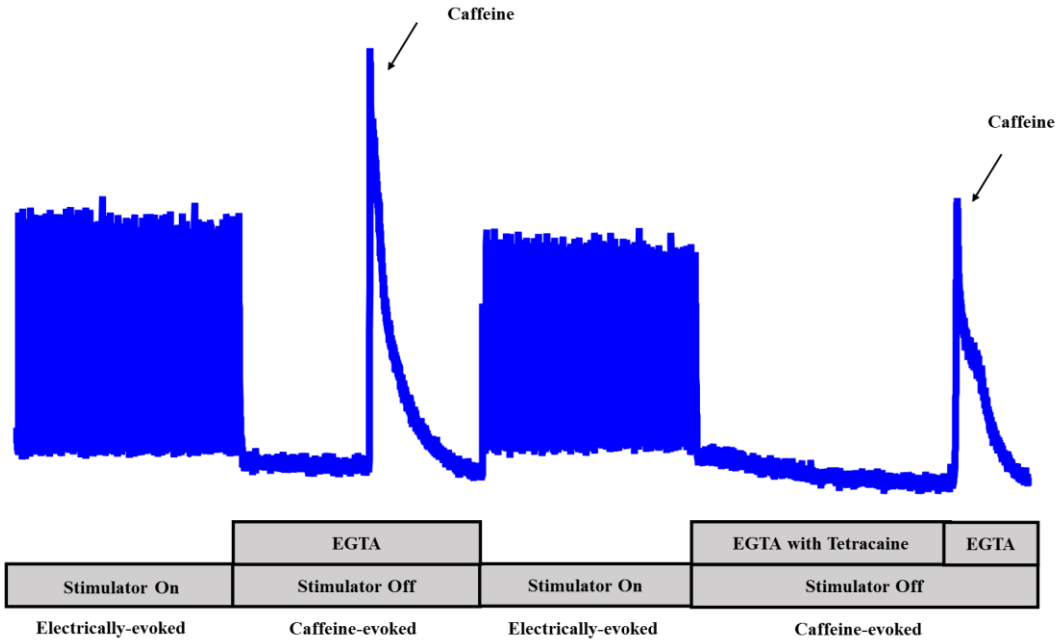


Figure 4.4: An example recording of showing the protocol and $[Ca^{2+}]_i$ to determine SR Ca^{2+}-leak.

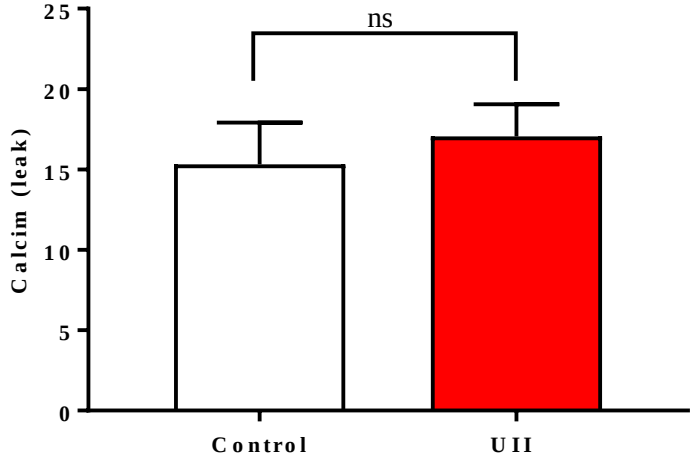


Figure 4.5: Diastolic SR Ca^{2+}-leak in cultured ventricular cardiomyocytes.
Cells cultured for 24 hours in the absence (control) and presence of UII (200nM) for 24 hours. The difference in the diastolic $[Ca^{2+}]_i$ was measured in the presence and absence of 1mM tetracaine for both control and treated cell. . N= 6 hearts; 19 control cells & 25 treated cells. The results are expressed as mean ± S.E.M. Unpaired t-test.

4.2.2 Signalling pathway involved in the UII-induced hypertrophy of ventricular cardiomyocytes

Cardiac hypertrophy induced by UII may be attributed to activation of MAPKs signaling molecules. MAPKs are a messenger system, involved in many signal transduction pathways that control a variety of cellular physiological processes such as differentiation, cell proliferation, cell growth, stress response, motility and apoptosis (Nishida and Gotoh 1993; Chang and Karin 2001; Pearson *et al.* 2001). MAPKs comprise four subfamilies, including ERK1/2, p38, JNK and big map kinase (BMK or ERK5) (Pearson *et al.* 2001). Onan and co-workers observed a pronounced activation of ERK1/2 and p38 in adenovirus expressing the urotensin receptor (AdUT-IIR) infected neonatal ventricular cardiomyocytes after stimulation with100nM UII in time-dependent manner (Onan *et al.* 2004b).

The aim of this current series of experiments was to seek to elucidate the signaling pathway(s) implicated in hypertrophy as a consequence of exposure to UII.

4.2.2.1 UII activates ERK in ventricular cardiomyocytes and CHO_{hUT}

In all of the Western blot experiments on ventricular cardiomyocytes, cells were cultured in normal media for 24 hours prior to treatment with UII. In order to confirm the role of MAPKs signalling in the hypertrophic response to UII, Western blot analysis of protein phosphorylation was performed. ERK1/2 can be activated by phosphorylation at residues Thr^{202} and Tyr^{204}. Because phosphorylation of MAPKs increases in CHO_{hUT} cells, these cells were used as positive control to the ventricular cardiomyocytes. The result revealed that the ventricular cardiomyocytes activate MAPKs in response to UII.

To assess the activation of ERK1/2 by UII. Western blots were carried out and densitometry to determine the ratio of phosphorylated ERK1/2 to total protein. CHO_{hUT} cells (n= 6), were treated with UII (200nM) and samples taken at various time points. UII led to a significant time-dependent increase in the phosphorylation of ERK1/2 (0.72 ± 0.25) after 5 minutes, (0.90 ± 0.22) at time 7.5 minutes and (0.91 ± 0.19) at 10 minutes when compared to basal (0.07 ± 0.03) unstimulated cells. The statistic results demonstrated a significant increase over basal activity (Figure 4.6).

Then, it has been determined whether the exogenous UII (200nM) could induce phosphorylation of ERK1/2 in ventricular cardiomyocytes. The ventricular cardiomyocytes from 6 rat hearts were used in this experiment and were cultured for 24 hours prior to stimulate with UII (200nM). The results revealed that in the cell treated with 200nM UII, the phosphorylation of ERK increased (0.44 ± 0.05) after 5 minutes, and reached a plateau at time 7.5 minutes (0.46 ± 0.08) and 10 minutes (0.44 ± 0.08), which was then was sustained for up 15 minutes (0.28 ± 0.02) compared to the basal cellular protein level in untreated cells (0.17 ± 0.03) as illustrated in Figure 4.7.

ERK1/2 phosphorylation level increased in both CHO_{hUT} cells and ventricular cardiomyocytes in response UII in a time-dependent manner. Response of ventricular cardiomyocytes and CHO_{hUT} for ERK1/2 after normalization to the basal is illustrated in Figure 4.8.

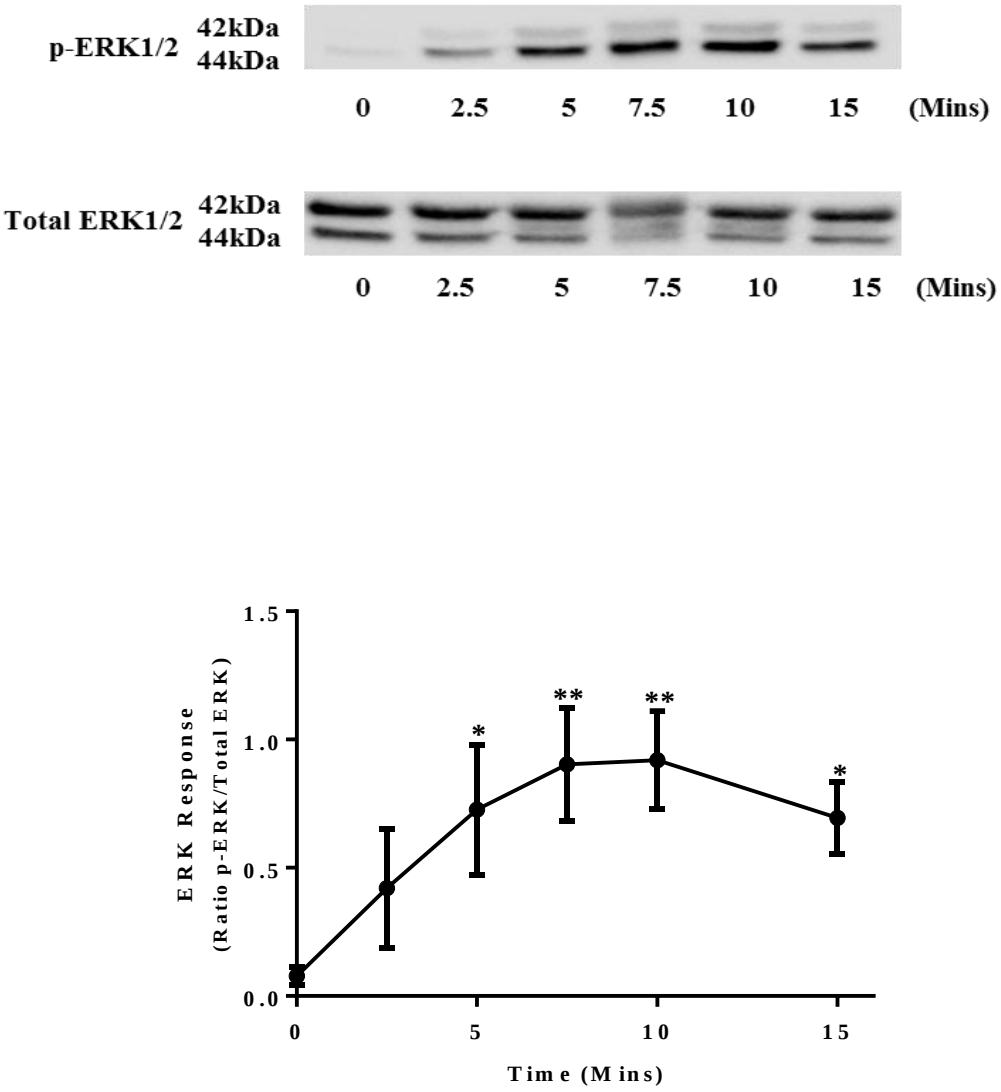


Figure 4.6: Phosphorylation time course of ERK in CHO_{hUT} by 200nM UII.
Phosphorylation of ERK and total ERK was determined by Western blot analysis. N= 6 hearts. The results are expressed as mean ± S.E.M. *p<0.05 & **p<0.01. One-way ANOVA followed by Dunn's *post hoc* test.

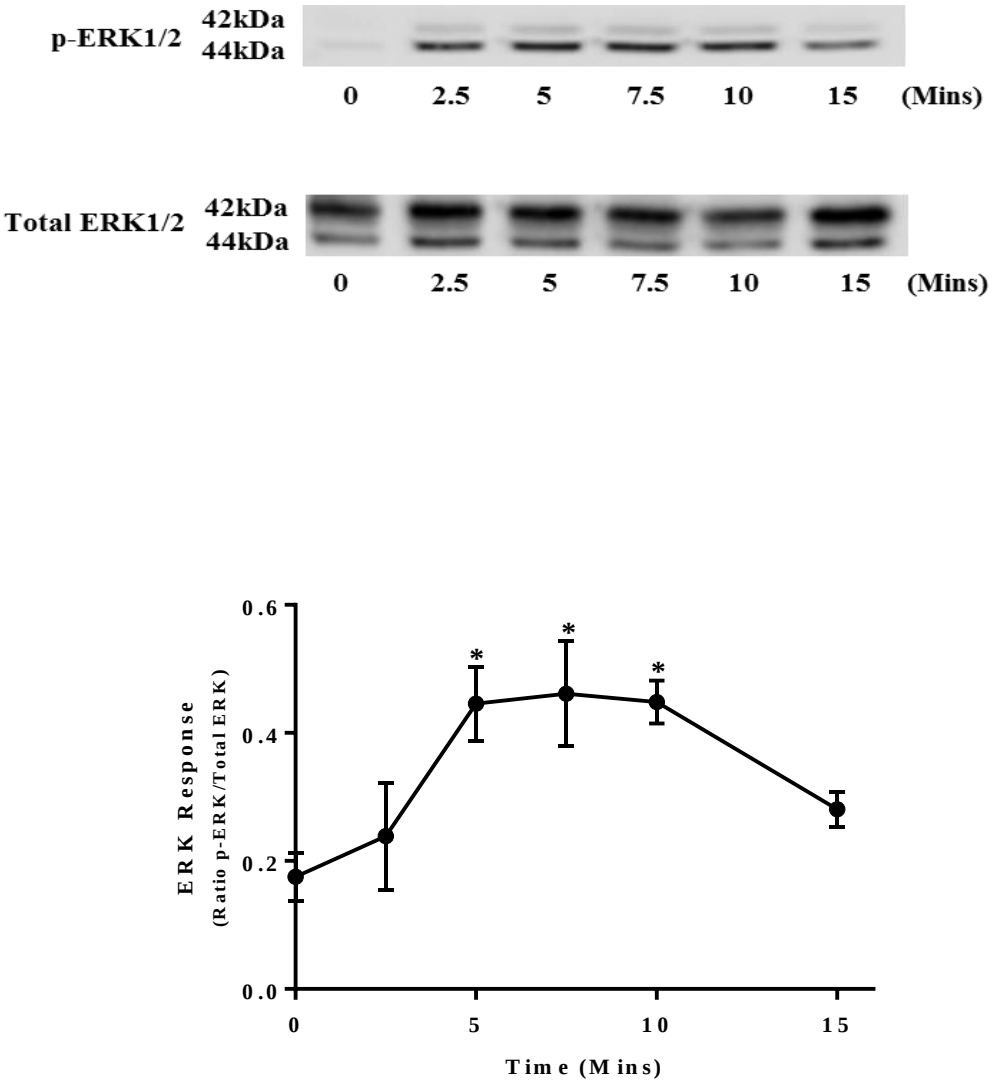


Figure 4.7: Phosphorylation time course of ERK in ventricular cardiomyocytes by 200nM UII.

Phosphorylation of ERK and total ERK was detected by Western blot analysis. N= 6 hearts. The results are expressed as mean ± S.E.M. *p<0.05. One-way ANOVA followed by Dunn's *post hoc* test.

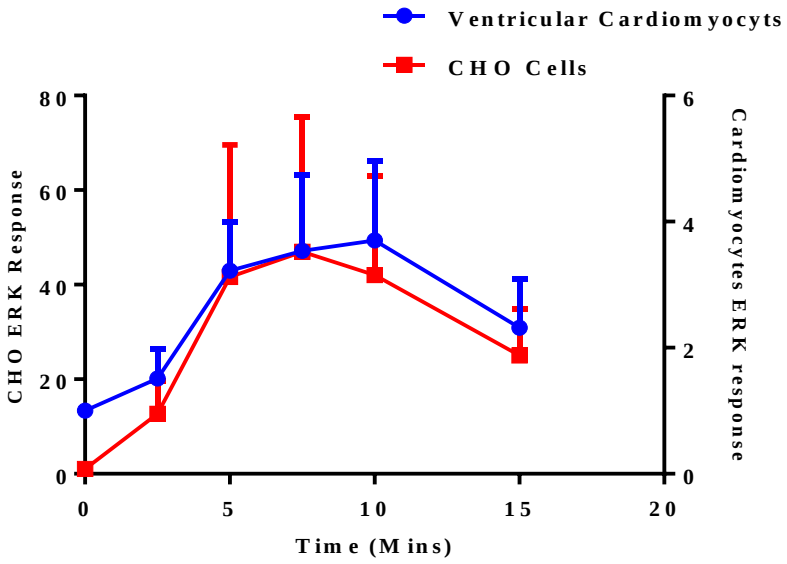


Figure 4.8: Comparison of UII time courses in ventricular cardiomyocytes and CHO_{hUT} for ERK.

4.2.2.2 UII activates p38 signalling pathway in ventricular cardiomyocytes and CHO_{hUT}

Western blotting was applied to identify the content of phosphorylation and total p38 in both types of cells CHO_{hUT} and ventricular cardiomyocytes. The cells were incubated with UII at various time points, as per the ERK1/2 experiments. The results showed a significant increase over basal activity (0.35 ± 0.07) in CHO_{hUT} cells (n= 6) after 10 minutes (0.89 ± 0.11) ($p<0.05$) or after 15 minutes (1.13 ± 0.15) ($p<0.01$) (Figure 4.9). In a separate set of experiments, the activation of p38 was determined over a longer duration of 0-60 minutes and the data show that p38 phosphorylation reached a peak after 15 minutes which remained elevate for at least 60 minutes. Western blot analysis showed that the phosphorylation levels of p38 were increased after 15 minutes (1.33 ± 0.10) stimulation of cells with UII (n= 7 hearts) compare to the basal level activity (0.67 ± 0.09) ($p<0.01$), providing evidence that the activation of p38 dependent signalling pathways is associated with the UII-induced hypertrophy (Figure 4.10). Response of ventricular cardiomyocytes and CHO_{hUT} for p38 after normalization to the basal is illustrated in Figure 4.11.

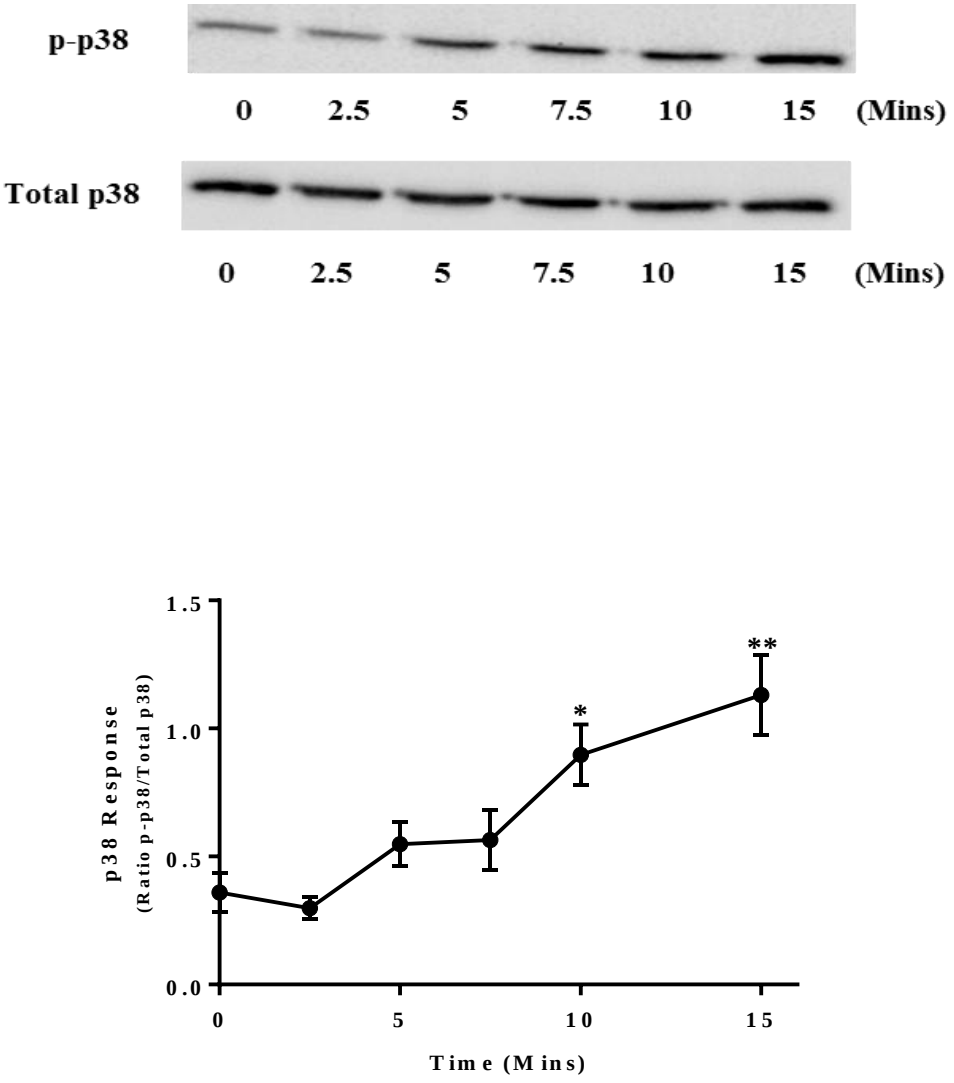
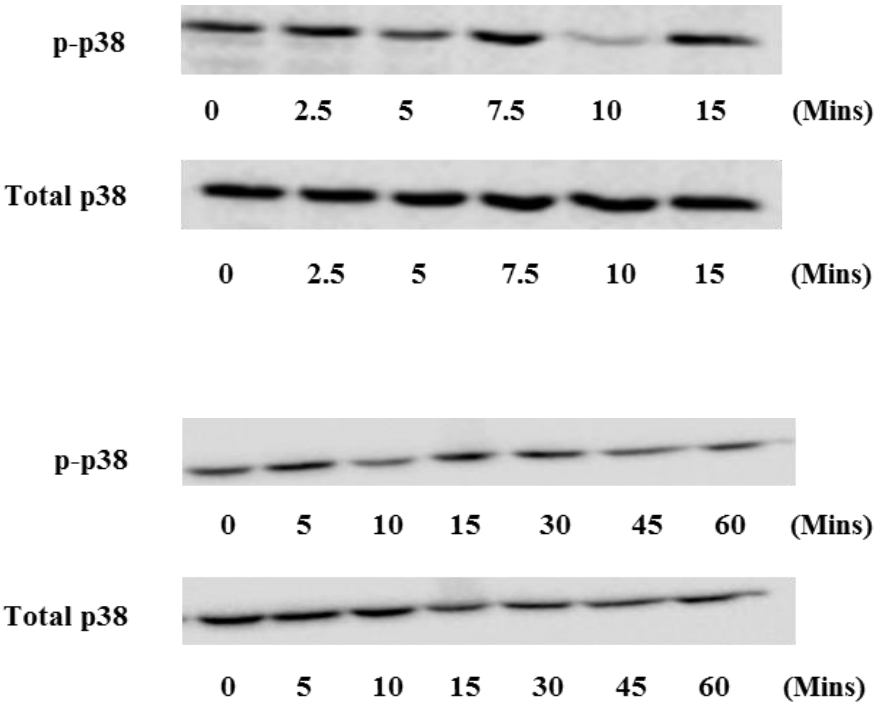


Figure 4.9: UII (200nM) induced p38 phosphorylation time course in CHO_{hUT} cells.
Phosphorylation of p38 and total p38 was detected by Western blot analysis. N= 6 hearts. The results are expressed as mean ± S.E.M. *p<0.05, **p<0.01. One-way ANOVA followed by Dunn's *post hoc* test.



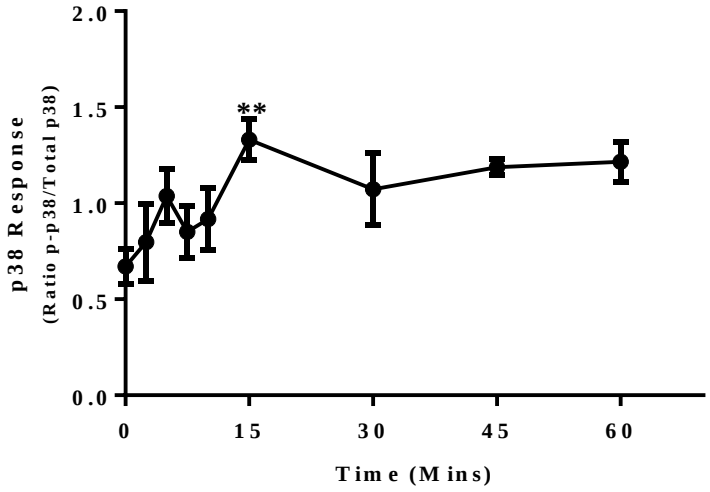


Figure 4.10: UII (200nM) induced p38 phosphorylation time course in ventricular cardiomyocytes.

Ventricular cardiomyocyte p38 response was detected by Western blot analysis. N= 7 hearts. The results are expressed as mean ± S.E.M. **p<0.01. One-way ANOVA followed by Dunn's *post hoc* test.

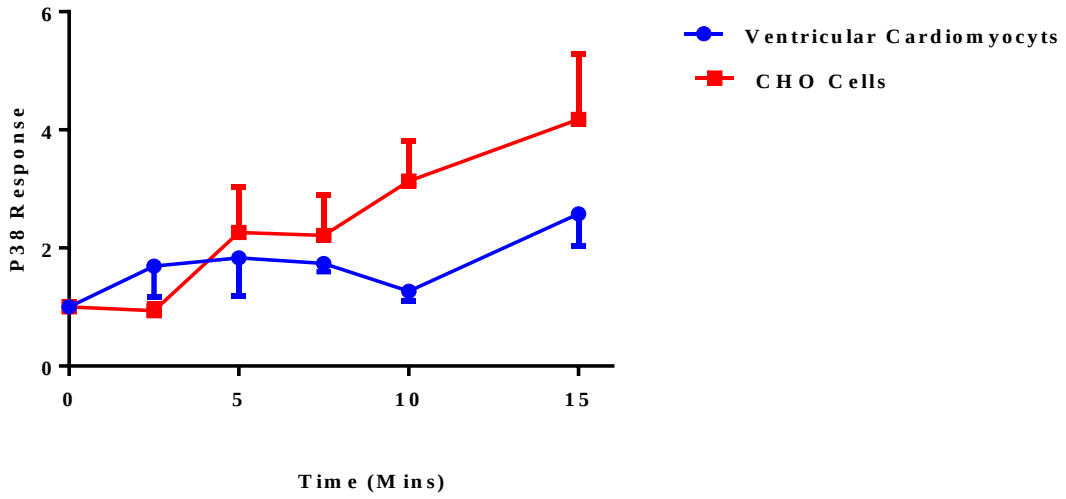


Figure 4.11: Response of ventricular cardiomyocytes and CHO_{hUT} cells with respect to p38.

4.2.2.3 UII induces phosphorylation of CaMKII in ventricular cardiomyocytes

CaMKII is a multifunctional CaM kinase act as transducer for Ca^{2+} signalling, which regulates multiple cellular process and phosphorylates a broad extent of substrate proteins. The second messenger Ca^{2+} can bind to calmodulin through the Ca^{2+} receptor CaM when the intracellular Ca^{2+} concentration increase causing phosphorylation of CaMKII. Indeed, these alterations in the concentration of Ca^{2+} have acute and chronic impacts on the function of cardiac muscle (Zhang and Brown 2004). CaMKIIδc isoform is predominantly expressed in the heart and the pathological role of this enzyme is reaffirmed by overexpression of this kinase which induced cardiac hypertrophy (Zhang *et al.* 2003a).

As we noticed an increase in diastolic Ca^{2+} levels in response to chronic exposure to UII, the study looked at the ability of UII to induce phosphorylation of CaMKII in ventricular cardiomyocytes. The ventricular cardiomyocytes (n= 5 hearts) stimulated with UII (5, 10, 15, 30, 45, 60 & 90 minutes). The results demonstrated statistically a significant increase in phosphorylation of CaMKII over basal cellular protein level activity (1.54 ± 0.25) after 90 minutes (3.34 ± 0.5) ($p<0.05$) (Figure 4.12).

During these experiments, no bands were appeared when CaMKII antibody from Cell Signaling was used however, when the same antibody from Abcam was added, the bands were clearly showed.

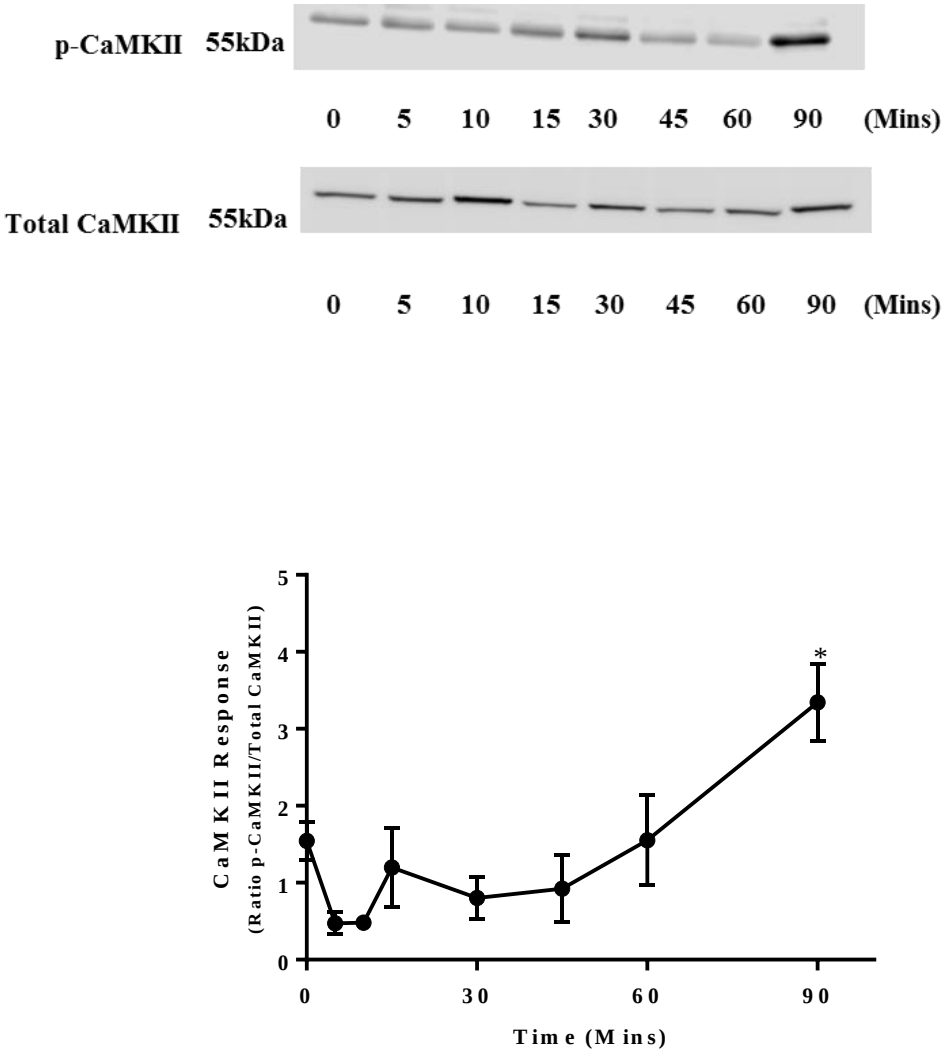


Figure 4.12: Time course for UII-induced phosphorylation of CaMKII in ventricular cardiomyocytes after treatment with 200nM UII.

Phosphorylation of CaMKII and total CaMKII was detected by Western blot analysis. N= 5 hearts. The results are expressed as mean ± S.E.M. *p<0.05. One-way ANOVA followed by Dunn's *post hoc* test.

4.2.2.4 Does UII induce phosphorylation of JNK in ventricular cardiomyocytes?

c-Jun N-terminal kinase signalling pathway is considered one of the major intracellular signalling cassettes of MAPK family, which is implicated in the MAPK signalling and has an active role in the development of the cardiac hypertrophy. Because UII-induced hypertrophy in cultured ventricular cardiomyocytes it possible that JNK signalling pathway is activated in response to UII. Therefore, phosphorylation of JNK in cultured ventricular cardiomyocytes in response to UII was measured using Western blotting. UII was incubated with cultured ventricular cardiomyocytes for different time points (5, 10, 15, 30 and 45 minutes), the phosphorylation of JNK was not affected by UII treatment at any time point examined (Figure 4.13).

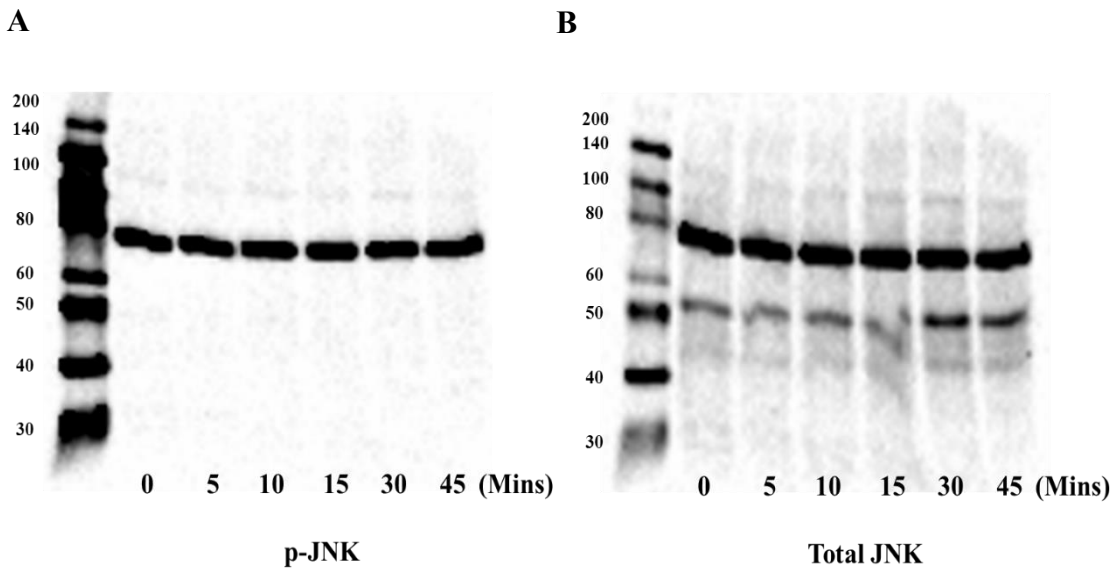


Figure 4.13: Representative blots indicating that UII did not phosphorylate JNK in ventricular cardiomyocytes, n= 3.

4.2.2.5 Unexpected bands on a Western Blot

Unexpected bands were found at 80kDa in all the blots. Anti-rabbit IgG secondary antibody was used in western blot assays related to ventricular cardiomyocytes and incubated with blots. Because some endogenous proteins may bind non-specifically for some primary antibodies, the blot of ventricular cardiomyocytes was incubated just with anti-rabbit IgG peroxidases secondary antibody without addition of primary antibody. However, these bands were still evident. Then, the ventricular cardiomyocytes lysates were run at different time points with CHO_{hUT} in the same gel and incubated just with secondary antibody. Unexpected bands related to the ventricular cardiomyocytes were also seen in CHO_{hUT} (Figure 4.14). Therefore, two new different secondary antibodies were used, the first antibody is peroxidase-conjugated affinipure donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch) with two dilutions were used (1:1000 and 1:5000) (Figure 4.15A) and the other one is anti-rabbit IgG HPR-linked antibody (Cell Signalling) (Figure 4.15B) but the bands were still present.

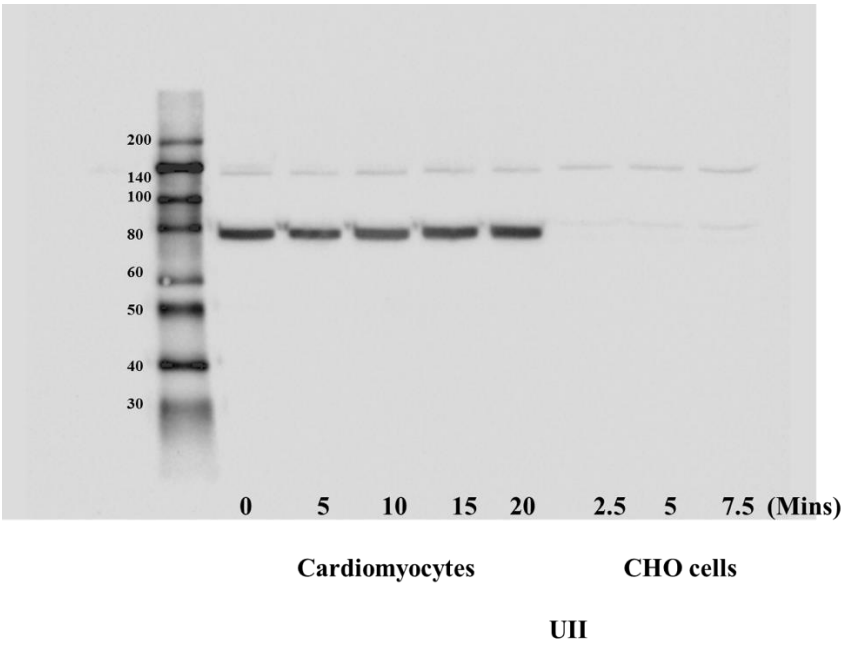


Figure 4.14: Non-specific binding at molecular weight 80kDa.
Unexpected bands were seen after run Western blot for ventricular cardiomyocytes. Incubation of blot just with anti-rabbit IgG peroxidases secondary antibody for ventricular cardiomyocytes and CHOhUT cells were used as positive control.

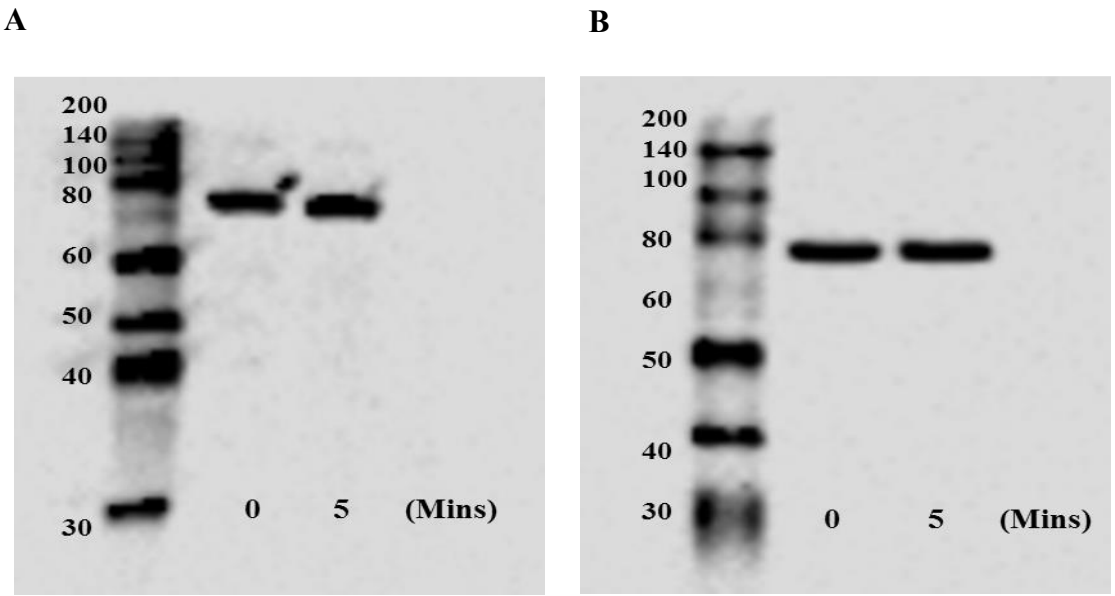


Figure 4.15: Unexpected bands on blots related to the ventricular cardiomyocytes when various types of secondary antibodies were used.
(A) Peroxidase-conjugated affinipure donkey anti-rabbit IgG secondary antibody, n= 2 hearts.
(B) Anti-rabbit IgG HPR-linked antibody, n= 2 hearts.

4.2.2.6 The involvement of ERK1/2 signalling pathway in the UII-induced hypertrophy of ventricular cardiomyocytes

Having shown that UII simulated many pro-hypertrophic signalling molecules (ERK1/2, p38 and CAMKII), we then determined to see whether these pathways were indeed involved in the hypertrophic response to UII. To study the role of ERK1/2 signalling in UII-induced hypertrophy, cultured ventricular cardiomyocytes were treated with 5μM of PD184352 (ERK1/2 inhibitor) for 30 minutes prior to stimulation with 200nM UII (Ley *et al.* 2003). The cells were incubated under tissue culture conditions with UII for 48 hours. The length/width ratio was decreased after 48 hours from (4.87 ± 0.06, n=358) under control condition to (3.87 ± 0.05, n=420) in UII group ($p<0.0001$). Inhibition of ERK1/2 signalling pathway completely blocked the UII-induced hypertrophy after 48 hours from (3.87 ± 0.05, n=420) in UII group to (4.64 ± 0.08, n=195) in UII+PD184352 group ($p<0.0001$). There was no significant difference between control group and PD184352 group after incubation for 48 hours (4.87 ± 0.06, n=358 vs 4.80 ± 0.07, n=253) ($p>0.05$). There were also no significant differences between control group and UII+PD184352 group after incubation for 48 hours (4.87 ± 0.06, n=358 vs. 4.64 ± 0.08, n=195) ($p>0.05$) (Figure 4.16).

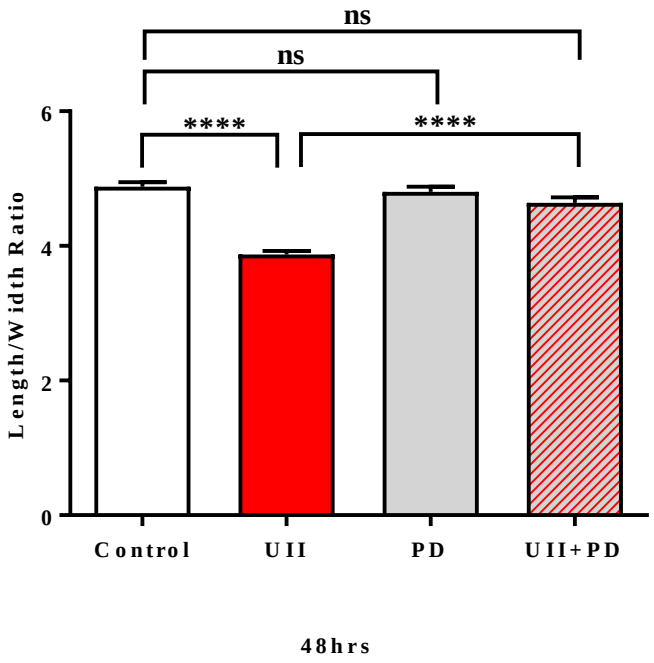


Figure 4.16: Effect of ERK1/2 inhibitor (PD184352) on UII-induced hypertrophy in cultured ventricular cardiomyocytes.
PD184352 blocked the UII-induced hypertrophy after 48 hours. N= 6 hearts; 358, 420, 253 & 195 cells, respectively. The results are expressed as mean ± S.E.M. ****p<0.0001. One-way ANOVA followed by Sidak's *post hoc* test.

4.2.2.7 The involvement of the p38 signalling pathway in UII-induced hypertrophy of ventricular cardiomyocytes

To study the role of p38 signalling in UII-induced hypertrophy, cultured ventricular cardiomyocytes were treated with 10µM antagonist SB202190 (P38 inhibitor) for 30 minutes prior to stimulation with 200nM UII (Chen *et al.* 2012). The cells were incubated under tissue culture conditions with UII for 48 hours. The length/width ratio was decreased after 48 hours from (4.87 ± 0.06, n=358) in control group to (3.87 ± 0.05, n=420) in UII group ($p<0.0001$). Inhibition of p38 signalling pathway completely blocked the UII-induced hypertrophy after 48 hours from (3.87 ± 0.05, n=420) in UII group to (4.85 ± 0.08, n=212) in UII+SB202190 group ($p<0.0001$). There was no significant difference between control group and SB202190 group after incubation for 48 hours (4.87 ± 0.06, n=358 vs 4.80 ± 0.07, n=278 ($p>0.05$). There were also no significant differences between control group and UII+SB202190 group after incubation for 48 hours (4.87 ± 0.06, n=358 vs. 4.85 ± 0.08, n=212) ($p>0.05$) (Figure 4.17).

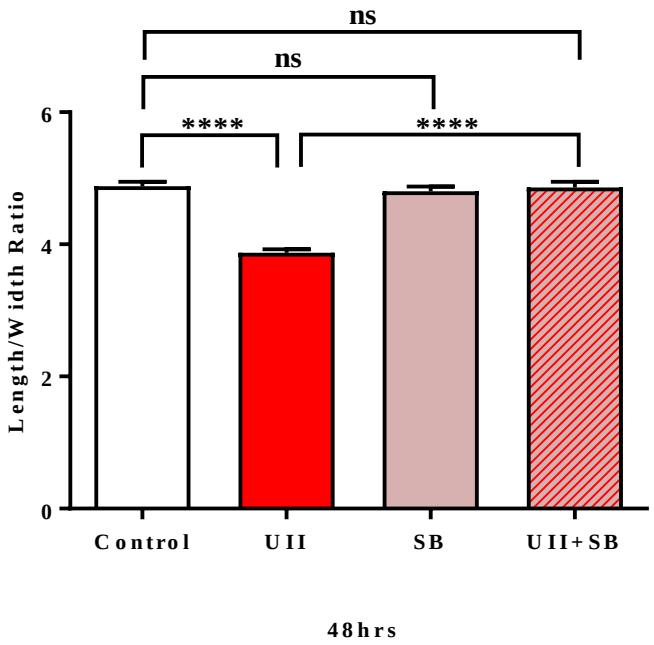


Figure 4.17: Effect of p38 inhibitor (SB202190) on UII-induced hypertrophy in cultured ventricular cardiomyocytes.

SB202190 blocked UII-induced hypertrophy after 48 hours. N= 6 hearts; 358, 420, 278 & 212 cells, respectively. Results are expressed as mean ± S.E.M. ****p<0.0001. One-way ANOVA followed by Sidak's *post hoc* test.

4.2.2.8 KN-93 inhibitor blocks CaMKII signalling pathway

An increase in diastolic calcium can induce hypertrophy via activation of the Ca^{2+}-dependent CaMKII enzyme. The role of CaMKII signalling in UII-induced hypertrophy was studied in cultured ventricular cardiomyocytes treated with 5µM antagonist KN-93 (CaMKII inhibitor) for 30 minutes prior to stimulation with 200nM UII (Shi *et al.* 2016). The cells were incubated under tissue culture conditions with UII for 48 hours. The length/width ratio was decreased after 48 hours from (4.14 ± 0.05, n=320) in control group to (3.39 ± 0.04, n=386) in UII group ($p<0.0001$). Inhibition of CaMKII signalling pathway completely blocked the UII-induced hypertrophy after 48 hours from (3.39 ± 0.04, n=386) in UII group to (4.14 ± 0.06, n=223) in UII+KN-93 group ($p<0.0001$). There was no significant difference between control group and KN-93 group after incubation for 48 hours (4.14 ± 0.05, n=320 vs 4.22 ± 0.06, n=282) ($p>0.05$). There were also no significant differences between control group and UII+KN-93 group after incubation for 48 hours (4.14 ± 0.05, n=320 vs. 4.14 ± 0.06, n=223) ($p>0.05$) (Figure 4.18).

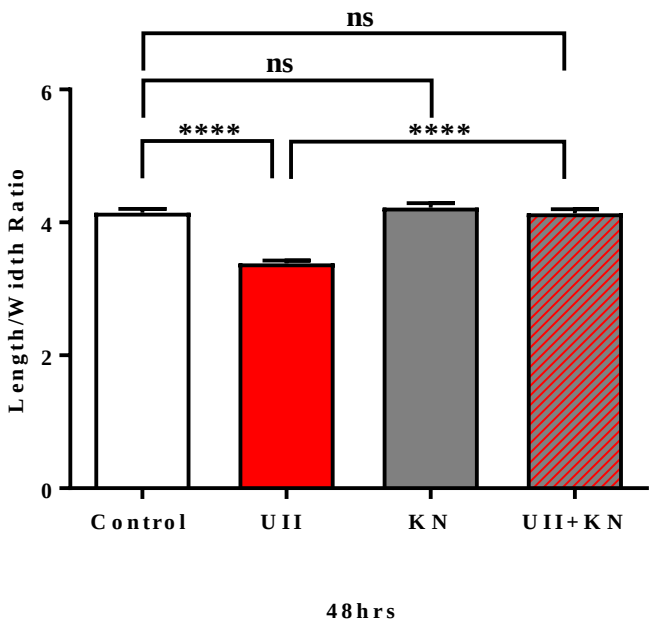


Figure 4.18: Effect of CaMKII inhibitor (KN-93) on UII-induced hypertrophy in cultured ventricular cardiomyocytes.

KN-93 blocked UII-induced hypertrophy after 48 hours. N= 6 hearts; 320, 386, 282 & 223 cells, respectively. Results are expressed as mean ± S.E.M. ****p<0.0001. One-way ANOVA followed by Sidak's *post hoc* test.

4.3 Discussion

The precise molecular mechanisms of UII have not been fully elucidated, and whether the increase in circulating UII in patients with HF is a cause or effect of LV-hypertrophy and heart failure. Therefore, the research has looked at whether endogenous applied UII is hypertrophic in in-vitro tissue culture based systems.

1- In this chapter it was demonstrated that UII (200nM) is able to induce hypertrophy in an in-vitro model of hypertrophy involving adult rat ventricular cardiomyocytes and this hypertrophic response involved the binding of UII to the UT receptor.

2- The present study then sought to elucidate the intracellular signaling pathways implicated in cardiac hypertrophy mediated by UII. The results demonstrated that UII led to the phosphorylation of ERK1/2, P38 and CaMKII,

3- Moreover, blocking intracellular signaling pathways implicated in hypertrophy mediated by UII with selective blockers significantly inhibited UII mediated cardiomyocytes hypertrophy. However, JNK pathway in our system was not activated by UII and so did not appear to be involved in UII mediated cardiac hypertrophy.

4- Diastolic $[Ca^{2+}]_i$ was increased in cultured ventricular cardiomyocytes after 24 hours treatment with UII, this may be due to SR Ca^{2+}-leak resulting from hypertrophy. SR Ca^{2+}-leak was measured and the data show UII had no effect on SR Ca^{2+}-leak.

4.3.1 Isolated cultured ventricular cardiomyocytes are appropriate model to study hypertrophy

To study the pharmacological role of UII in cardiac hypertrophy, cultured ventricular cardiomyocytes were used as an experimental model. Neither neonatal cardiomyocytes nor H9C2 were used in this study because both cells possess several disadvantages. H9C2 cells are immature non-contractile cardiomyoblasts, in addition to prevent de-differentiation, the culture media must be supplemented with a growth differentiation factor. The neonatal cardiomyocytes are characterized by immature phenotype (Peter *et al.* 2016). Although neonatal and H9C2 cardiomyocytes respond to hypertrophic factors and have been used as in-vitro model, ventricular cardiomyocytes were used as the best model to study hypertrophy because they are terminally differentiated adult cells and

more closely reflect the cells that are driven to hypertrophy and the morphology and contractile behavior of these cells are similar to those cardiomyocytes in intact tissue (Peter *et al.* 2016). Ventricular cardiomyocytes isolated from adult rat can be maintained in culture for short periods of time and are ideal for using in contractile and electrophysiological studies due to mature myofibril structure, the adult cardiomyocytes are a good model to study cellular and molecular changes in contrast to the neonatal or H9C2 cardiomyocytes which can be maintained in culture for long term studies but are non-contractile and abnormal Ca^{2+}-regulation and electrical activity when compared to adult heart tissue (Peter *et al.* 2016).

A tissue culture model of hypertrophy was developed in adult ventricular cardiomyocytes using 199 media HEPES provided with supplements to maintain as closely as the conditions in-vivo. Physiological and morphological properties of the ventricular cardiomyocytes are also maintained in culture (Mitcheson et al. 1997). The addition of selected supplements to the tissue culture medium such taurine, carnitine and creatine, has been shown to increase the half-life of rod shaped ventricular cardiomyocytes up to 14 days (Volz *et al.* 1991) and the presence of insulin and pyruvate to media is important to increase the energy content of the media and thereby ameliorate the metabolic properties of ventricular cardiomyocytes in tissue culture (Ellingsen *et al.* 1993; Berger *et al.* 1994). In addition, BDM was included to the medium which prevents contracture-induced injury and is shown to reduce spontaneous contractions in cardiomyocytes under culture.

Phenylephrine was used in this study as positive control to induce hypertrophy. Phenylephrine is a selective α_1-adrenergic receptor agonist, widely used to experimentally induce pathologic cardiac hypertrophy (Siddiqui *et al.* 2004; Peng *et al.* 2017; Romano *et al.* 2018). The results in this study regarding phenylephrine data confirmed those observed in earlier studies and supported the use of adult rat ventricular cardiomyocytes as a model for hypertrophy. UII promotes hypertrophy in ventricular cardiomyocytes in tissue culture after 24 hours and 48 hours. During pathological cardiac hypertrophy, the width of the cardiomyocytes increases while the length is constant (Dorn *et al.* 2003).

A growing body of literature has investigated the role of UII in cardiac tissue. In In-vivo and in-vitro animal model studies, many changes occur at a cellular level in UII-induced

hypertrophy, including increased cellular proteins, cell size, collagen synthesis and enhanced myofibrillar reorganization (Zou *et al.* 2001a; Zhang *et al.* 2007; Chao *et al.* 2014). However, Onan and co-workers demonstrated that UII does not cause hypertrophy in uninfected cultured cardiomyocytes with an adenovirus. It has been suggested that increased expression of UT receptor as a likely cause of pathological cardiac hypertrophy mediated by UII. Two branches of MAPKs are activated during this hypertrophy by involvement of epidermal growth factor receptor trans-activation, including ERK1/2 and p38. These findings are somewhat contradictory and, could be related to differences in culture technique (Onan *et al.* 2004b). Induction of hypertrophic response in the ventricular cardiomyocytes was monitored by measuring length/width ratio as an indicator. This ratio significantly decreased after 24 hour and 48 hours stimulation with UII, which is indicative of pathophysiological hypertrophy (Gerdes 2002). To induce hypertrophy, two isoforms of UII (human and rat) were used. Hypertrophic response induced by hUII was similar to that induced by rUII. This confirms that there was no species related differences and agree with those of other studies and suggesting equal activity of both isoforms. Potentially this is due to structure of the biological active C-terminal ring, which is fully preserved in all isoforms of UII in different species (Onan *et al.* 2004a). The data of the present study support previous findings in cultured neonatal cardiomyocytes; UII at a dose 100nM is capable of inducing maximal hypertrophy in cultured neonatal cardiomyocytes without any upregulation of UT receptor (Zou *et al.* 2001a). Conversely, Onan and co-workers found that the same concentration of UII (100nM) can induce hypertrophy in cultured neonatal cardiomyocytes accompanied by overexpression of UT receptor (Onan *et al.* 2004b). Similarly, Gruson and colleagues observed the same findings in adult rat cardiomyocytes when UII was incubated with these cells for 48 hours, which was associated with activation of GSK-3 (Gruson *et al.* 2010).

4.3.2 UT receptor involved in the UII-induced hypertrophy

The UT receptor is expressed (UT mRNA) in ventricular cardiomyocytes (see chapter 5, section 5.2.1.1). Therefore, the effects of a selective UT receptor antagonist SB657510 on the hypertrophic response induced by UII were investigated to confirm UT receptor involvement in hypertrophy. The effect of UII to induce hypertrophy was reversed by the

antagonist SB657510, confirming that that the hypertrophic response to UII did involve receptor binding.

4.3.3 Intracellular signalling pathways in cardiac hypertrophy

The UT receptor is a $G_{\alpha q}$-coupled GPCR. There are several hormones that can bind to GPCR on the cell membrane of cardiomyocytes. Coupling of theses agonist to G_q can give rise to generation of intracellular messengers, DAG and IP_3, the second messenger DAG can activate PKC and MAPK subfamilies (see section 1.3.2).

4.3.3.1 Involvement of MAPKs signalling pathways in UII-induced hypertrophy

UII stimulation increased phosphorylation of ERK1/2, p38 MAPKs in both CHO_{hUT} cells and ventricular cardiomyocytes. ERK1/2 were activated by UII in ventricular cardiomyocytes at (5-10) minutes and was sustained for up 15 minutes by UII at 200nM. The present findings seem to be consistent with other research which found the effect of UII on neonatal cardiomyocytes and activation of ERK1/2 (Zou *et al.* 2001a; Onan *et al.* 2004b) and extend these findings to adult cardiomyocytes and show that activation is involved in the hypertrophic response to UII, which was completely blocked by inhibition of ERK1/2 with PD184352.

Activation of p38 signalling pathway is associated with different pathological stresses in the heart which can response to stress stimuli. Most notably, activated p38 involves in a broad range of cardiac diseases such as hypertrophy, heart failure and myocardial infarction (Marber *et al.* 2011). A number of independent features are stimulated when the p38 signalling pathway is activated during hypertrophy including, an increase in cardiomyocyte surface area and sarcomere assembly as well as re-expression of specific cardiac hypertrophic fetal genes (Wang et al. 1998).

The signalling pathway of p38 was investigated in cultured ventricular cardiomyocytes in response to UII in time dependent manner which activates in hypertrophy. The data showed activation of p38 within 15 minutes and remained elevated for at least 60 minutes. Involvement of p38 signalling was initially probed for by using of selective antagonist SB202190 to block the UII-induced hypertrophic response, p38 was activated which linked to hypertrophic response to UII as it was completely blocked by the SB202190 inhibitor.

During hypertrophy ERKs and p38 are activated in cultured cardiomyocytes in response to UII via GPCR mediated by trans-activation of epidermal growth factor receptor (Onan *et al.* 2004b; Liu *et al.* 2009). Furthermore, Rho family proteins have been suggested that involvement in UII-induced activation of ERK1/2 in cardiomyocytes (Zou *et al.* 2001a), and progression of cardiac hypertrophy. Rho is one member of Rho family of GTPases and is thought to link to hypertrophy in a number of ways. Activation of Rho can lead to expression of atrial natriuretic factor gene and stimulation of c-fos in the cardiomyocytes (Aikawa *et al.* 1999; Kuwahara *et al.* 1999; Clerk and Sugden 2000). It has been shown that UII induces proliferation and contraction of arterial smooth muscle cells beside formation of actin stress fibre, and this requires activation of GTPase RhoA and Rho kinase pathway (Sauzeau *et al.* 2001). Cardiac hypertrophic stimuli activate subcellular signal pathways, in turn these signals immediately transduce to the nucleus and lead to activation of transcription factors. Yanazume and colleagues demonstrated that activation of Rho-ROCK pathway lead to activation of a GATA-4 factor. Cardiac GATA-4 factor is necessitated for activation of ERK1/2. Phosphorylation of GATA-4 factor at serine residue resulting in activation of ERK1/2 pathway. Accordingly, the study suggested there is a linkage between Rho and ERK1/2 pathways during cardiac hypertrophy (Yanazume *et al.* 2002). As far as downstream intracellular signalling pathways are concerned which are activated during hypertrophy, role of UII to induce hypertrophy was elucidated in this study (Figure 4.19).

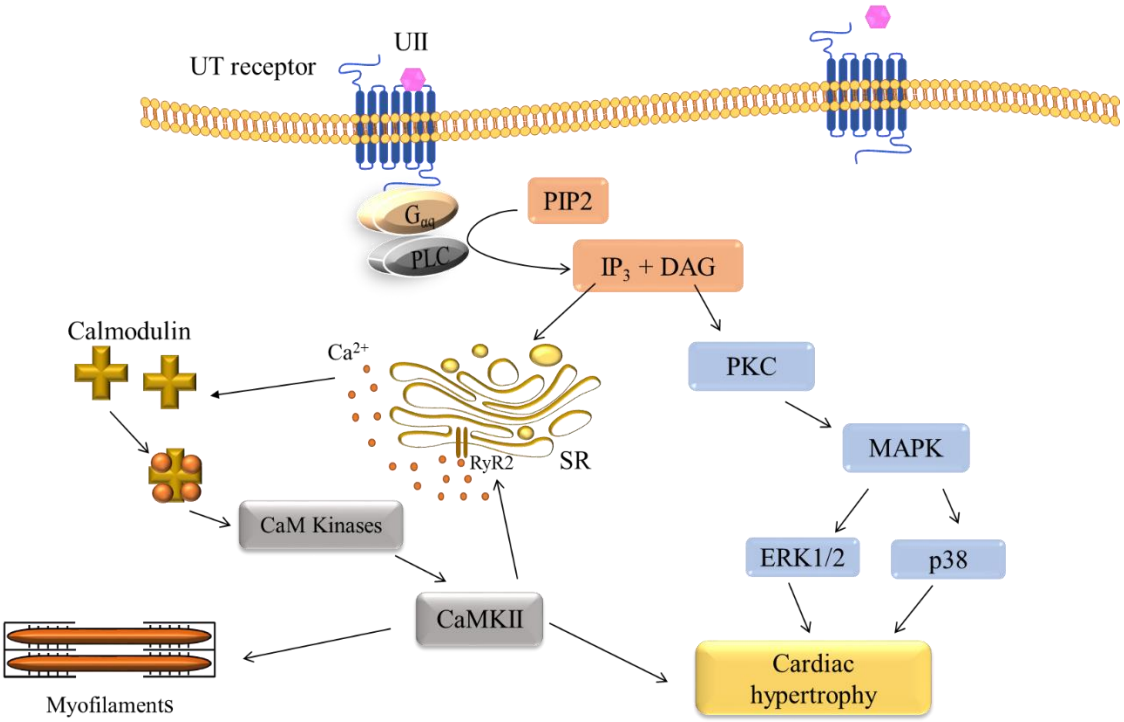


Figure 4.19: Schematic diagram of UII signalling pathways during hypertrophy.

4.3.3.2 Involvement of CaMKII signalling pathway in UII-induced hypertrophy

Previous studies have reported CaM kinases and calcineurin are profoundly implicated in the development of pathological cardiac hypertrophy in response to hypertrophic agents such as Ang II, ET-1 and catecholamines (Molkentin *et al.* 1998; Zhu *et al.* 2000; Zou *et al.* 2001b). It has been confirmed that expression and activity levels of CaMKII are upregulated in both animal and human studies of cardiac hypertrophy and heart failure (Kirchhefer *et al.* 1999; Kato *et al.* 2000; Maier and Bers 2002; Colomer *et al.* 2003). CaMKII is involved in the development of cardiac hypertrophy, it is activated re-expression of several hypertrophic fetal genes encompassing, atrial natriuretic peptide, brain natriuretic peptide, skeletal actin and β-myosin heavy chain (Colomer and Means 2000) .

We showed using Western blotting to identify the activation on CAMKII by UII as indicated by the level of phosphorylated and total of CaMKII. CaMKII was activated in the ventricular cardiomyocytes stimulated in a time dependent manner in response to UII, phosphorylation of CaMKII was increased significantly over basal activity at 90 minutes. Moreover, the presence of the inhibitor KN93 completely blocked the hypertrophic response to UII. This finding supports previous research into this cardiac hypertrophy area which links UII and signal pathway. Stimulation of neonatal cardiomyocytes with UII for 48 hours is capable to induce hypertrophy via phosphorylation of CaMKII and its downstream PLB signalling pathway (Shi *et al.* 2016). CaM kinase is required for induction of specific hypertrophic genes as a consequence of UII stimulation (Valencia and Thomas 2006). Calcium/calmodulin-dependent protein kinase II is a downstream target phosphorylated by CaM kinase and can promote development of heart failure and vascular disease (Erickson *et al.* 2011).

There are two ways for activation of CaMKII either directly via elevation of intracellular Ca^{2+}-transient concentration or alternatively via intracellular second messenger cAMP regulatory mechanism. Indirect activation of CaMKII could happen when adenylyl cyclase is activated, consecutively intracellular cAMP level is upregulated. As a result of the increased level of cAMP, a guanine nucleotide exchange protein (EPAC) is directly activated when binds to cAMP with high-affinity. The function of EPAC associated with cardiac hypertrophy is activate either phospholipase C or CaMKII resulting in increasing

intracellular Ca^{2+}-release during cardiac E-C coupling thus EPAC can induce the hypertrophic program (Morel *et al.* 2005; Pereira *et al.* 2007; Oestreich *et al.* 2009).

Selective KN-93 inhibitor was used to establish the global role of CaMKII in regulating ventricular cardiomyocytes hypertrophy. Inhibition of CaMKII signalling pathway completely blocked the UII-induced hypertrophy in ventricular cardiomyocytes. Notably, the minimum time was selected for incubation of these inhibitors with the cells and low concentration. It can thus be suggested that the hypertrophy was blocked completely by theses inhibitors. Effect of inhibitors to block was shown after 48 hours to be sure the response of maximum level, therefore, any inhibition will be seen. Co-incubation of KN-93 and UII with neonatal cardiomyocytes prevented UII-induced phosphorylation of CaMKII and hypertrophic phenotype response as well as increase of protein content level (Shi *et al.* 2016). The pharmacological companies are competing to develop CaMKII inhibitors in cardiovascular fields as effective therapies and with an acceptable safety profile. Inhibition can be achieved allosterically either directly by inhibition of kinase activity via small molecules which are capable blocking binding of ATP, Ca^{2+} and calmodulin or protein substrates or indirectly by blocking essential targeting sites on substrate or anchoring protein (Schulman and Anderson 2010). The KN-93 inhibitor wraps around the helical CaM target segment, the binding site becomes free from the catalytic kinase domain (Pellicena and Schulman 2014). Only a few protein kinases are influenced by the inhibitory effect of KN-93. Simultaneously, KN-93 has a direct effect on unrelated molecules to CaM kinase family (Ledoux *et al.* 1999; Gao *et al.* 2006).

4.3.3.3 Are the RyR channels and SR Ca^{2+}-leak involved in CaMKII activation?

Hyperphosphorylation of RyR2 at Ser^{2808} mediated by PKA has been shown in heart failure resulting from β-adrenergic stimulation, impaired FKBP12.6 binding to the channels and the final outcome leak of Ca^{2+} from SR (Marx *et al.* 2000; Ono *et al.* 2000). Role of CaMKII in heart failure has been investigated, which mediated phosphorylation of RyR2 and causing change the functional activity of channels (Chelu *et al.* 2009). Recently, CaMKII levels have been shown to be elevated in failing hearts, thereby increased phosphorylation of RyR2 at Ser^{2008} and Ser^{2814} (in some species Ser^{2815}) residues which is mediated by CaMKII (Dobrev and Wehrens 2014). Numerous studies have been shown that the pathological influence of CaMKII on RyR2 behaviour related to hyperactivity of this kinase in animal cardiac hypertrophy models and human disease

(Maier *et al.* 2003; Zhang *et al.* 2003a; Kohlhaas *et al.* 2006; Sag *et al.* 2009; Sossalla *et al.* 2010a; Toischer *et al.* 2010). RyR2 are not entirely closed and increased their open probability during diastole when these channels are hyperphosphorylated by CaMKII causing a substantial elimination of SR Ca^{2+}, leading to a local restriction SR Ca^{2+}-release events (Ca^{2+} sparks) (Maier *et al.* 2003; Zhang *et al.* 2003a). Spontaneous diastolic opening of single channel can lead to trigger the opening of neighbouring RyRs clusters through the process CICR (Endo 1977). In this manner, SR Ca^{2+}-content is depleted and eventually initiated diastolic SR Ca^{2+}-leak. Consequently, the contractile function of the heart is impaired on one hand and further arrhythmias are triggered on the other hand (Maier *et al.* 2003; Zhang *et al.* 2003a). Leaky Ca^{2+} is immediately extruded from the cytosol by NCX thus generates transient inward current and delayed afterdepolarizations (Bers 2002). Diastolic SR Ca^{2+}-leak can be blocked with RyR2 inhibitors. Interestingly, recent study has been shown that a selective CaMKII inhibitor (GS-680) can reduce diastolic SR Ca^{2+}leak in atrial cardiomyocytes from patient with hypertrophic obstructive cardiomyopathy which act on CaM and block ATP-binding site, confirmed involvement of CaMKII in SR Ca^{2+}-leak (Lebek *et al.* 2018).

SR Ca^{2+}-leak could attribute to excessive activation of CaMKII in hypertrophy, which mediates phosphorylation of RyR2 resulting in increased release of Ca^{2+} from SR and thereby SR Ca^{2+}-leak events related to activation of CaMKII downstream signalling processes. Effect of chronic exposure of ventricular cardiomyocytes to UII on Ca^{2+}-transient was investigated. The most interesting finding was that there was increase in diastolic in $[Ca^{2+}]_i$ after 24 hours UII treatment compared to control. It seems possible that these results are due to SR Ca^{2+}-leak resulting from hypertrophy. Surprisingly, no differences were found in diastolic $[Ca^{2+}]_i$ in the presence and absence of tetracaine. Two reasons may associate with that; first, UII could not cause a sustained leak and second the chronic effect of UII and the diastolic $[Ca^{2+}]_i$ which was measured after 24 hours. The measurement probably was late and the effect was not seen due to the hypertrophy which was happened because of the effect of UII within the first 2 hours. Thus, CaMKII can induce RyR2 stimulation which leads to diastolic Ca^{2+}-leak. Therefore, to study RyR2 leak should look at the effect over acute period between 1 and 2 hours.

E-C coupling is initiated in cardiac muscle by triggering Ca^{2+}. Calcium/calmodulin-dependent protein kinase II is a regulatory protein, responsible for regulation of

intracellular Ca^{2+}. CaMKIIδc isoform is ubiquitously expressed in the heart. Calcium/calmodulin-dependent protein kinase II phosphorylates PLB Thr^{17} in the cytoplasmic helix can relieve the inhibitory action of PLB on the SR, causing relaxation of muscle. Upregulation of CaMKII in cardiomyocytes result in altered of Ca^{2+} handling properties in cardiomyocytes that resulted in myocardial hypertrophy and heart failure (Huke *et al.* 2011). PLB, LTCCs and RyR2 are phosphorylated by CaMKII at specific sites, phosphorylation of LTCCs by CaMKII directly cause influx of Ca^{2+} into cardiomyocytes across these channels at high heart rate (DeSantiago *et al.* 2002). Action of SERCA2a is reversibly inhibited by dephosphorylation of PLB thereby reducing Ca^{2+} uptake into the SR by SERCA2a (Simmerman and Jones 1998). Phosphorylation of PLB by PKA at Ser^{16} and CaMKII at Thr^{17} alleviates, it makes SERCA2a more efficient and pumping Ca^{2+} into the SR which increases Ca^{2+} accumulation (Simmerman and Jones 1998; MacLennan and Kranias 2003).

4.3.4 Study limitations

In this chapter, it has been shown the hypertrophic response of ventricular cardiomyocytes mediated by UII and the signaling pathways of this peptide ligand to induce hypertrophy. The initial aim was to investigate the role of UII on cultured ventricular cardiomyocytes. A number of important limitations need to be considered. Isolated cultured adult ventricular cardiomyocytes were used for morphological cellular and molecular mechanisms assays. Ventricular cardiomyocytes cannot be maintained after isolation for long time in culture and the drawbacks were with replacement of culture media because the cells were suspended in culture.

Although the study has successfully demonstrated signal pathways of hypertrophy induced by UII it has certain limitations with Western blot investigations in terms of using of freshly isolated ventricular cardiomyocytes. In contrast to the phosphorylation of ERK, p38 and CaMKII in cultured cardiomyocytes, Urotensin II did not stimulate ERK, p38 and CaMKII phosphorylation in freshly isolated ventricular cardiomyocytes.

There were significant technical issues with CaMKII antibody (Cell Signalling). No bands were visible on the blots and CaMKII target protein was not detected. Therefore, another antibody (Abcam) was used in this study, it was effective for detection the CaMKII target protein.

c-Jun N-terminal kinase signalling pathway was investigated, Western blotting data suggested that JNK was not activated by UII, JNK signalling pathway may be not involved. However, JNK inhibitor was not used in this study.

Two experiments were performed at various time points to investigate action of UII to induce phosphorylation of p38 in ventricular cardiomyocytes; 0-15 and 0-60 minutes, the data have been shown from 7 experiments of which 4 had time course (0-15) extended to 60 minutes in a further three. Although the p38 MAPK was phosphorylated at 15 minutes, the results statistically were not significant. Therefore, the signalling pathway of p38 which was activated during hypertrophic ventricular cardiomyocytes in response to UII at time dependent manner (0-60) minutes was investigated. The p38 was activated in ventricular cardiomyocytes at 15 minutes.

Unexpected (additional) bands were seen on the blot around molecular weight 80kDa when the ventricular cardiomyocytes lysate was run, it is possible that these bands are non-specific for the secondary antibody. Therefore, the blots were incubated just with secondary antibody without addition of primary antibody and CHO_{hUT} cells were used as positive control for ventricular cardiomyocytes but the additional bands remained.

4.3.5 Conclusion

In the current study, the ability of exogenous UII to induce hypertrophy in cultured ventricular cardiomyocytes was examined. This study has shown involvement of UII in the development of hypertrophy in cardiac myocytes. As far as intracellular signal transduction pathways downstream are concerned, the signalling pathways responsible for hypertrophy in ventricular cardiomyocytes response to UII were shown. The results indicated increased activation of ERK1/2, p38 and CaMKII in hypertrophic responses. The involvement of these signaling pathways in the observed hypertrophy of these cells was confirmed in ventricular cardiomyocytes treated with selective inhibitors of the kinases; ERK1/2 (PD184352), p38 (SB202190) and CaMKII (KN-93), respectively. Addition of these inhibitors suppressed the morphological changes and blocked hypertrophy in ventricular cardiomyocytes. Diastolic $[Ca^{2+}]_i$ significantly increased in ventricular cardiomyocytes treated with UII for 24 hours however, any significant increase in SR Ca^{2+}-leak was not seen. These findings suggested that activation of

ERK1/2, p38 MAPKs and CaMKII pathways are involved in the hypertrophic response to UII.

Chapter 5: UT receptor gene expression in hypertrophy and heart failure

5.1 Introduction

UT receptor gene is extensively expressed in various region of the cardiovascular system, at the level of myocardium in both the atria and ventricles, at the vascular level including thoracic aorta, coronary artery, great saphenous vein as well as umbilical vein (Ames *et al.* 1999; Davenport and Maguire 2000; Matsushita *et al.* 2001; Zhang *et al.* 2007).

There is some evidence to suggest that UII system is upregulated in the failing heart (Johns *et al.* 2004). Exposure to chronic hypoxia lead to increased binding of UII to its receptor suggests that there is an increase in density of receptors (Zhang *et al.* 2002). Interestingly, Huang *et al*., have observed that upregulation of UT receptor mRNA in the myocardium of rats which were chronically subjected to hypoxia is accompanied by development of right ventricular hypertrophy (Huang *et al.* 2006). Expression of UII and together with its receptor has been demonstrated to be raised among congestive heart failure (CHF) patients mainly in cardiac muscle tissue, and this increased expression inversely correlates with physiological function of the heart and with a direct correlation to disease severity (Douglas *et al.* 2002).

In the previous chapter, the results showed that exogenous UII-induced hypertrophy in cultured ventricular cardiomyocytes. UII is a strong vasoconstrictor peptide, this could result in a sustained hypertension which could also drive LV-hypertrophy. There are other stimuli can induce cardiac hypertrophy such as Ang II, ET-1, phenylephrine and isoproterenol. Angiotensin II is an active octapeptide in the renin angiotensin aldosterone system.

We hypothesise that UII stimulates hypertrophy in ventricular cardiomyocytes in-vivo via upregulation of cardiac UT receptors. Therefore, a legitimate model in which to study LV-hypertrophy and heart failure.

The aim was to see whether the UII-induced hypertrophy of isolated rat ventricular cardiomyocytes in-vitro, was also associated with the upregulation of UT receptors. Gene expression assays were used to detect expression of UT mRNA in freshly isolated and

hypertrophic ventricular cardiomyocytes. Furthermore, in hypertrophy induced by chronic exposure to angiotensin II in-vivo, leads to UT receptor upregulation.

5.2 Results

UT receptor expression may be upregulated in response to hypertrophic stimuli or in response to chronic exposure to UII. In-vitro and in-vivo studies have been done to detect expression of UT mRNA in response to hypertrophy. RT-qPCR assays were performed to measure expression of UT receptor in adult ventricular cardiomyocytes and cardiac mouse tissue after treatment with hypertrophic stimuli. In order to normalise our data of UT receptor expression it is requisite to validate the reference genes to be sure the treatments we have adopted do not in themselves affect expression of reference genes. The expression levels of the candidate reference genes were evaluated in rat and mouse experiments.

5.2.1 Rat results

Overexpression of G_q signaling has been shown to induce maladaptive cardiac apoptosis (Althoefer *et al.* 1997; Adams *et al.* 1998; Adams *et al.* 2000) while moderate levels of G_q activation lead to adaptive hypertrophy (LaMorte *et al.* 1994; Milano *et al.* 1994; D'Angelo *et al.* 1997). A sequence of events is associated with cardiac hypertrophy when the heart is exposure to hormonal stimuli or a pressure overload stimulus, results in pathophysiological gene transcription and ventricular hypertrophy (Hilfiker-Kleiner *et al.* 2006). Binding of the UII to its receptor is thought to initiate a signalling process that drives hypertrophy (Liu *et al.* 2009).

5.2.1.1 UT expression in Wistar rat ventricular cardiomyocytes

Assessment of UT mRNA expression in rat ventricular cardiomyocytes was performed by RT-PCR assays. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference gene to determine quantitative UT mRNA expression in ventricular cardiomyocytes isolated freshly from adult rat from 9 hearts. The mean C_t value for GAPDH was 13.84 ± 2.20 while for UT was 35.0 ± 1.40 giving a ΔC_t value of 21.18 ± 3.12. Results showing expression of UT mRNA in rat ventricular cardiomyocytes are in Table 5.1.

Samples	C_t GAPDH	C_t UT	ΔC_t
Sample 1	15.62	36.84	21.22
Sample 2	11.76	34.69	22.92
Sample 3	12.39	34.59	22.20
Sample 4	13.66	35.69	22.04
Sample 5	13.29	36.27	22.98
Sample 6	12.27	34.67	22.40
Sample 7	13.29	35.67	22.38
Sample 8	13.39	34.82	21.43
Sample 9	18.91	31.93	13.08
Mean	13.84 ± 2.20	35.02 ± 1.40	21.18 ± 3.12

Table 5.1: UT mRNA expression in fresh rat ventricular cardiomyocytes measured by RT-PCR.

Cycle thresholds (C_t) for the housekeeper (GAPDH) and Gene of interest (UT) are presented along with the relative difference ΔC_t. Each cycle represents a doubling of starting material and high values indicate low expression. N= 9 hearts.

5.2.1.2 Validation of reference genes

In tissue expression experiments, selection of appropriate reference genes is indispensable for reliable analysis and avoiding misinterpretation of results (Huggett *et al.* 2005). To obtain a satisfactory normalisation of mRNA expression experiments and allow conclusions in biological gene expression studies, a list of endogenous reference gene is required.

Validation of standard reference genes (endogenous control) was performed either on rat ventricular cardiomyocytes that had incubated with 200nM UII or 10µM phenylephrine for 24 hours or an in-vivo experiment where mouse cardiac tissue was exposed to Ang II for 28 days. The stability of expression for selected reference genes was calculated by analysing the cycle threshold of each reference gene with NormFinder® (Andersen *et al.* 2004).

5.2.1.2.1 NormFinder® methodology

Appropriate endogenous reference gene selection is a crucial and essential aspect for interpretation of data generated from RT-PCR assays (Bustin *et al.* 2009). NormFinder® is an algorithm for determining the most stably expressed gene among candidate reference genes and the ideal number required of these genes for accurate RT-PCR normalisation (Jensen and Ørntoft 2004).

The input RT-PCR data must be on a linear scale to allow NormFinder® which is available as an Excel add-in, to process the relative gene expression data. Mean cycle threshold values of samples are transformed to relative quantities (RQ) according to the formula below:

$$RQ = 1 / (2^{\text{Ct value - minimum Ct value}}) \quad \ldots\ldots\ldots (7)$$

The following five steps were carried out to find the best reference gene for each tissue species.

Step 1- The mean cycle threshold values of the RT-PCR were input into an Excel sheet file. Table 5.2 shows RT-PCR data for 12 samples of rat ventricular cardiomyocytes (including the controls) sorted in columns and labelled (A-N) and 6 traditional candidate endogenous reference genes were measured, sorted in rows. To estimate the variances by the NormFinder® software each category of the group has to be identified for each sample. Numbers in the last row (1, 2 and 3) for each sample corresponding to a specific group category (control, UII or phenylephrine).

Sample	A	B	C	D	E	F	G	H	I	K	L	N
GAPDH	22.031	18.864	18.963	20.985	17.004	19.726	18.619	17.305	17.983	18.740	17.278	16.908
ACTβ	29.277	25.430	24.989	29.654	24.065	26.100	24.605	24.826	24.399	24.862	23.761	23.819
β2M	26.097	22.185	21.199	24.828	20.594	22.515	21.827	20.937	21.666	22.520	20.664	20.592
HPRT	28.660	23.862	23.536	27.619	22.724	24.940	24.437	23.011	23.379	23.960	23.193	22.705
PPIA	30.960	27.389	26.153	32.249	24.846	26.617	25.804	25.624	25.828	25.956	23.143	22.964
POP4	35.933	31.141	30.519	35.515	29.194	31.170	30.766	30.119	29.576	30.417	28.729	28.460
	1	1	1	1	2	2	2	2	3	3	3	3

Table 5.2: The first step in NormFinder® software. Spreadsheet of Ct values of samples for the six candidate reference genes.

Step 2- The lowest C_t value obtained during amplification for each of the endogenous reference gene in the row was subtracted from the C_t value of the other samples for that endogenous reference gene. For example, as illustrated in Table 5.3 the minimum C_t value is 20.592 for sample N for β2M gene.

β2M C_t Value of sample		**Minimum C_t Value**		**C_t Value - min C_t Value**
26.097	-	20.592	=	5.505
22.185	-	20.592	=	1.592
21.199	-	20.592	=	0.607
24.828	-	20.592	=	4.236
20.594	-	20.592	=	0.001
22.515	-	20.592	=	1.923
21.827	-	20.592	=	1.235
20.937	-	20.592	=	0.345
21.666	-	20.592	=	1.074
22.520	-	20.592	=	1.928
20.664	-	20.592	=	0.072
20.592	-	20.592	=	0.000

Table 5.3: The second step of NormFinder® software.
The table show the lowest C_t value for β2M reference gene subscribed from the C_t values of other samples for β2M reference gene.

Step 3- The resulting values from step 2 were entered into the calibration formula 1 / ($2^{\text{Ct value - minimum Ct value}}$) as illustrated in Table 5.4. The lowest C_t value for endogenous reference gene was replaced with the number 1.

		RQ Value
$1/(\mathbf{2^{5.505}})$	=	0.022
$1/(2^{\mathbf{1.592}})$	=	0.332
$1/(2^{\mathbf{0.606}})$	=	0.657
$1/(2^{\mathbf{4.235}})$	=	0.053
$1/(2^{\mathbf{0.001}})$	=	0.999
$1/(2^{\mathbf{1.922}})$	=	0.264
$1/(2^{\mathbf{1.235}})$	=	0.425
$1/(2^{\mathbf{0.344}})$	=	0.788
$1/(2^{\mathbf{1.073}})$	=	0.475
$1/(2^{\mathbf{1.927}})$	=	0.263
$1/(2^{\mathbf{0.071}})$	=	0.951
$1/(2^{\mathbf{0.000}})$	=	1.000

Table 5.4: The third step in NormFinder® software.
The table shows calculation of relative quantities (RQ) for each sample for β2M reference gene.

Step 4- A new table was created which contains RQ values for each sample for each reference gene rather than C_t values (Table 5.5).

Sample	A	B	C	D	E	F	G	H	I	K	L	N
GAPDH	0.029	0.258	0.241	0.059	0.936	0.142	0.305	0.760	0.475	0.281	0.774	1.000
ACTB	0.022	0.314	0.427	0.017	0.810	0.198	0.557	0.478	0.643	0.466	1.000	0.960
B2M	0.022	0.332	0.657	0.053	0.999	0.264	0.425	0.788	0.475	0.263	0.951	1.000
HPRT	0.016	0.448	0.562	0.033	0.987	0.212	0.301	0.809	0.627	0.419	0.713	1.000
PPIA	0.004	0.047	0.110	0.002	0.271	0.079	0.140	0.158	0.137	0.126	0.884	1.000
POP4	0.006	0.156	0.240	0.008	0.601	0.153	0.202	0.317	0.461	0.257	0.830	1.000
	1	1	1	1	2	2	2	2	3	3	3	3

Table 5.5: The fourth step of NormFinder® software, Ct values converted to a linear scale (RQ).

Step 5- To start the NormFinder® add-in, samples; arranged in table under different name categories, gene names and group identifiers, were highlighted and these categories were also selected on the program window box (Figure 5.1). Then the ‘Log transform data‘ box was ticked without selecting the ‘Simple output only‘ box and the NormFinder® algorithm was run by clicking ‘GO‘.

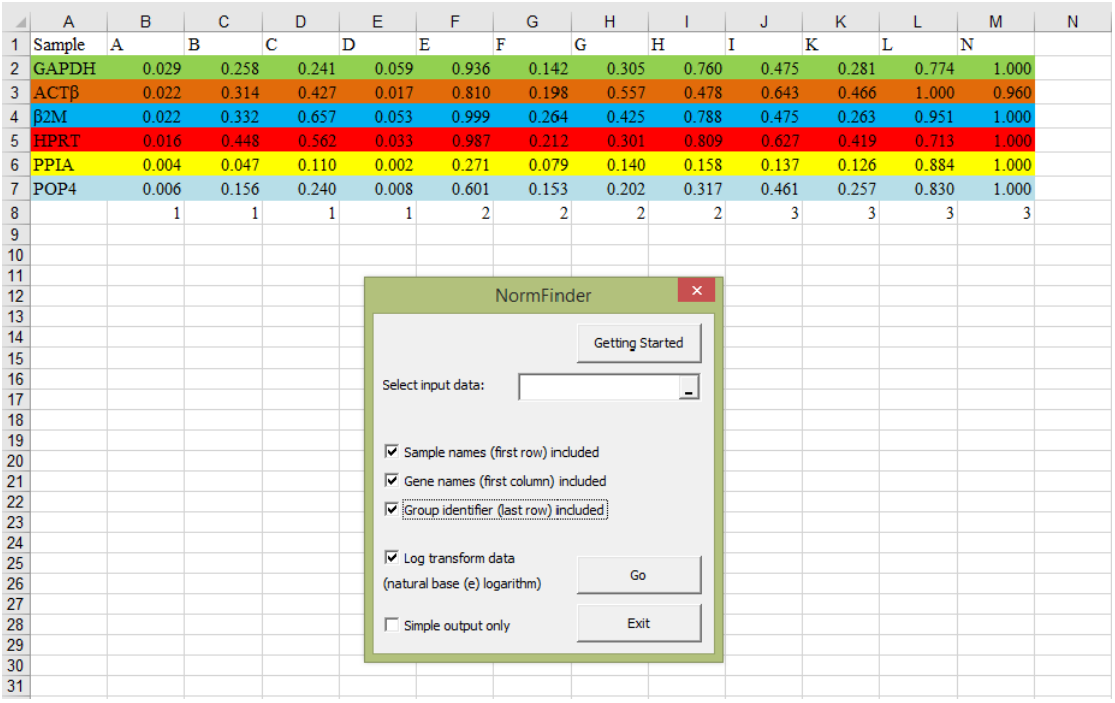


Figure 5.1: The fifth step in NormFinder® software.

A statistic sheet was presented by NormFinder® including stability values for all six endogenous reference genes (Figure 5.2).

	A	B	C	D	E	F	G	H
1								
2		**Gene name**	**Stability value**			**Best gene**	ACTβ	
3		GAPDH	0.348			**Stability value**	0.180	
4		ACTβ	0.180					
5		β2M	0.308			**Best combination of two genes**	β2M and POP4	
6		HPRT	0.291			**Stability value for best combination of two genes**	0.107	
7		PPIA	0.488					
8		POP4	0.280					
9								
10		**Intragroup variation**						
11		Group identifier	1	2	3			
12		GAPDH	0.628	0.094	0.001			
13		ACTβ	0.016	0.104	0.068			
14		β2M	0.098	0.008	0.001			
15		HPRT	0.034	0.051	0.102			
16		PPIA	0.438	0.037	0.484			
17		POP4	0.086	0.027	0.001			
18								
19		**Intergroup variation**						
20		Group identifier	1	2	3			
21		GAPDH	0.274	-0.059	-0.215			
22		ACTβ	0.035	-0.027	-0.008			
23		β2M	0.321	0.031	-0.353			
24		HPRT	0.223	-0.045	-0.178			
25		PPIA	-0.569	0.080	0.489			
26		POP4	-0.284	0.019	0.265			
27								
28								

Figure 5.2: Statistical analysis produced by NormFinder® software of stability values for candidate reference genes.

5.2.1.3 Measurement of gene stability and selection of reference genes for cultured ventricular cardiomyocytes

Six potential reference genes for ventricular cardiomyocytes were selected for control and cells treated with UII or phenylephrine in an in-vitro culture model. Data were analysed using NormFinder® program to identify the optimal normalisation gene. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTβ), β-2-microglobulin (β2M), hypoxanthine guanine phosphoribosyl transferase (HPRT), peptidylprolyl isomerase A (PPIA) and processing of precursor 4 (POP4) were used in this experiment. The expression levels of target gene in RT-PCR should be normalised to compensate intragroup variation and intergroup variation using reference genes (endogenous control), which expressed in control and treated samples. Reference gene analysis data are in Appendix 6A explains expression stability of the reference genes was verified using NormFinder® algorithm to calculate the most stable reference genes. Based on the results of NormFinder® analysis, the best single reference gene was ACTβ and the stability value was 0.180 while the best combination of two reference genes were β2M and POP4 with stability value of 0.107 (n= 4) (Figure 5.3). Appendix 6B & C show intergroup and intragroup variations for each reference gene as reported by NormFinder® software (variation between RT-PCR runs or between samples).

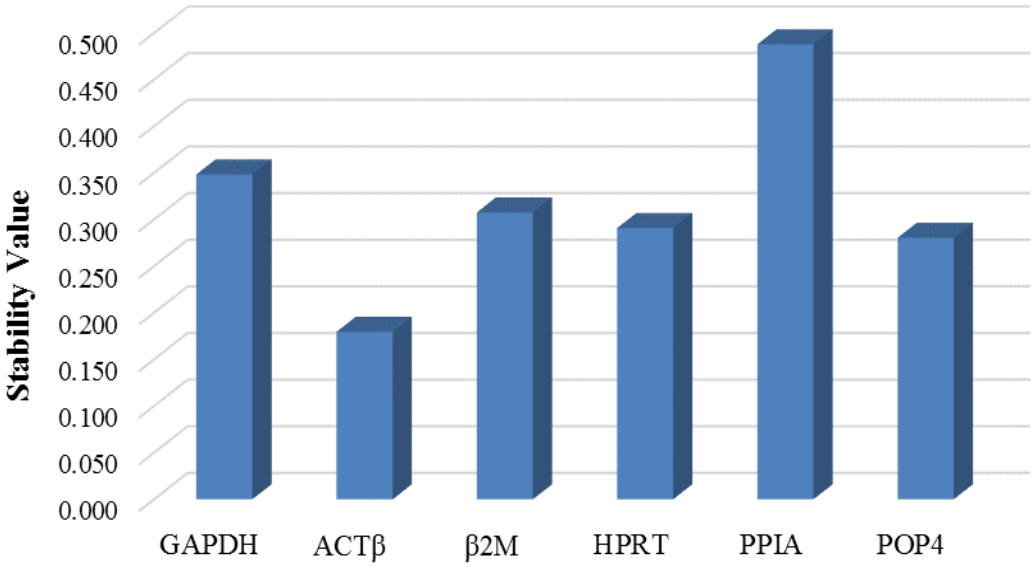


Figure 5.3: Stability value of different reference genes calculated by NormFinder® in cultured ventricular cardiomyocytes.
N= 4 hearts. The raw data in appendix 6.

5.2.1.4 UT mRNA gene expression in cultured ventricular cardiomyocytes

Relative quantification of gene expression for UT receptor was subsequently determined using two Taqman endogenous control probes (rat POP4 and rat β2M) in a duplex assay. Assay reactions of RT-PCR were run on a StepOne plus instrument. UT expression was detected in control (untreated samples).

UT receptor was expressed in freshly isolated ventricular cardiomyocytes therefore effect of treatment was investigated in cultured ventricular cardiomyocytes after incubation the cells with UII or phenylephrine for 24 hours to induce hypertrophy and see whether the UT receptor expression upregulates in response to either UII or phenylephrine induced hypertrophy, the experiment was carried out against two reference genes that were previously shown and compared to cells cultured for 24 hours in normal media. The effect of UII (200nM) or phenylephrine (10µM) on UT mRNA after 24 hours treatment was determined using $\Delta\Delta C_t$ (Livak and Schmittgen 2001), the phenylephrine was used in the experiment to see whether phenylephrine induced hypertrophy affects expression. The results showed that treatment of cardiomyocytes with UII or phenylephrine did not affect on the expression of UT mRNA (Table 5.6).

Investigation was carried out to determine the effects of chronic exposure to either UII or phenylephrine on expression of the UT receptor. The phenylephrine was used as positive control for a hypertrophic signal. There were no differences in ΔC_t between treated cells with UII (6.81 ± 0.59) or phenylephrine (7.10 ± 0.42) and untreated cardiomyocytes for the reference gene POP4 (6.45 ± 0.53) ($p>0.05$, $p>0.05$, respectively) (Figure 5.4A). There were also no significant differences in ΔC_t values for the reference gene β2M between treated cells with UII (15.99 ± 0.76) or phenylephrine (16.06 ± 0.73) in primary culture and control (16.27 ± 0.72) ($p>0.05$, $p>0.05$, respectively) (Figure 5.4B). Statistical analysis showed that there was also no significant change in ΔC_t (against geometric) between untreated cardiomyocytes (cells cultured for 24 hours in normal media) (11.89 ± 0.64) and treated cells with UII (11.87 ± 0.69) or phenylephrine (12.03 ± 0.63) ($p>0.05$, $p>0.05$, respectively) (Figure 5.4C).

Sample	C_t Mean POP4	C_t Mean β2M	Geometric Mean	C_t Mean UT	ΔC_t	$\Delta\Delta C_t$	Fold change
Control	28.32	18.49	22.87	34.76	11.89		
Treated cells with UII	27.94	18.76	22.89	34.76	11.87	-0.02	1.04
Treated cells with PHE	27.91	18.95	22.99	35.01	12.03	0.13	1.07

Table 5.6: Effect of UII (200nM) and phenylephrine (10μM) on UT receptor using endogenous control (POP4 and β2M).
N= 11 hearts. The raw data in appendices 7 and 8.

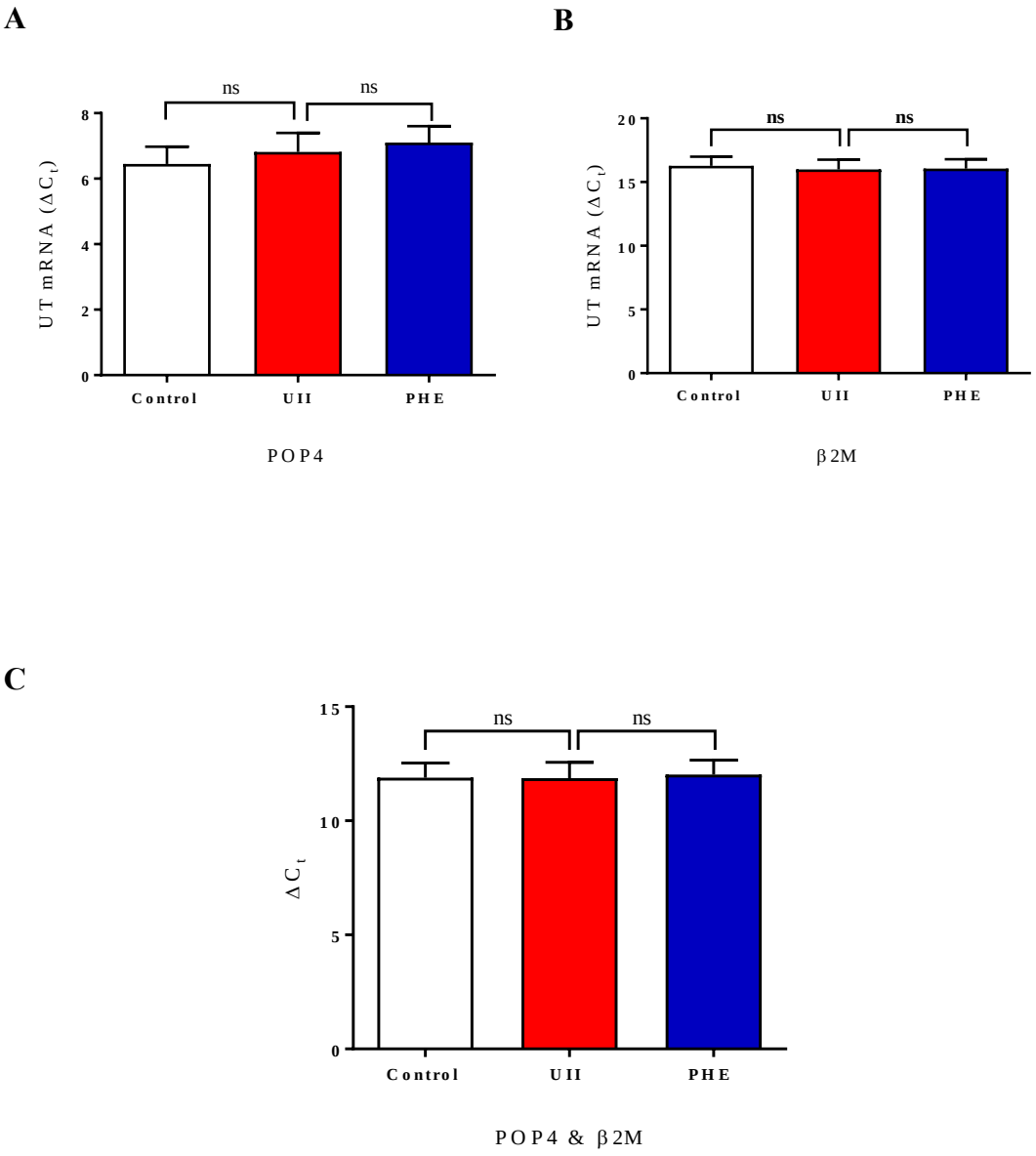


Figure 5.4: Expression of UT mRNA in treated ventricular cardiomyocytes with UII (200nM) or phenylephrine (10µM) and untreated cells using different reference genes.
(A) Determine the effects of chronic exposure to either UII or phenylephrine on expression of the UT receptor. Comparison of ΔC_t mean of treated cells with UII (200nM) or phenylephrine (10µM) and control using POP4 as reference gene. N= 11 hearts
(B) Determine the effects of chronic exposure to either UII or phenylephrine on expression of the UT receptor. Comparison of ΔC_t mean of treated cells with UII (200nM) or phenylephrine (10µM) and control using β2M as reference gene. N= 11 hearts.
(C) Comparison of ΔC_t of treated cells with UII (200nM) or phenylephrine (10µM) and control. Data are expressed as mean ± S.E.M. $p>0.05$. ANOVA followed by Sidak's *post hoc* test.

5.2.2 Mouse cardiac tissue results

Expression of UT increases in plasma and cardiac tissue of failing heart patients. Cardiac mass is increased due to hypertrophic of cardiomyocyte which is associated with myocardial injury. There are different neurohumoral stimuli can mediate hypertrophy exemplified by ET-1, catecholamines, Ang II as well as inflammatory cytokines. UII peptide and hypertrophic ligands (ET-1 and Ang II) are similar which share the same biological activity. Left ventricular hypertrophy was developed in ApoE KO mice by treatment with Ang II (1000ng/kg/minute), which induced by abdominal aortic aneurysm (Choke *et al.* 2010).

The aim of this experiment was to determine whether UT receptor will upregulate after induction of LV-hypertrophy in an in-vivo mouse heart model using another stimulus such as Ang II rather than UII.

5.2.2.1 Mouse left ventricular hypertrophy and cardiac function

Angiotensin II is involved in the pathophysiology of cardiovascular disorders, including endothelial dysfunction, hypertension, inflammation, atherosclerosis and heart failure. In addition to its physiological effects to mediate regulation of vasoconstriction and blood pressure (Mehta and Griendling 2007). Using in-vivo experiments in mice, Ang II induced left ventricular hypertrophy, as identified using echocardiography and later confirmed post-mortem measurements of the isolated heart (Figure 5.5), this model was performed at University of Leicester (Janus *et al.* 2018). To determine whether UT receptor will upregulate in mouse hearts after induction of LV-hypertrophy, ApoE KO mice were treated with Ang II and cardiac dysfunction/remodelling. At 1-2 weeks post, LV-function was depressed in ApoE mice (n=3) with an ejection fraction (EF) of 36.3% versus sham group (n=3) 57.2% ($p<0.05$) and fractional shortening of 17.16% versus 29.87% ($p<0.05$) (Table 5.7).

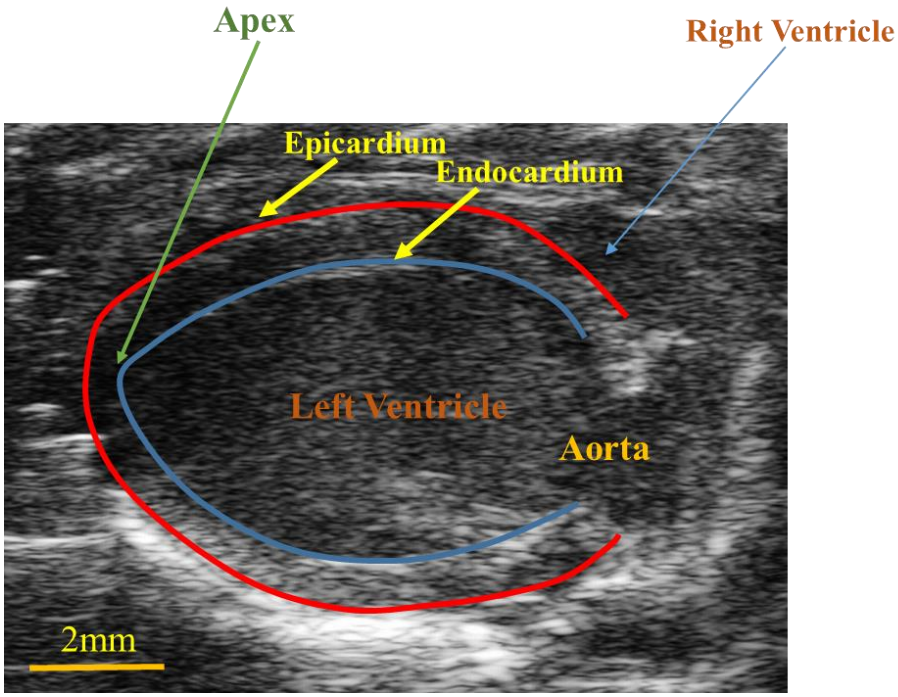


Figure 5.5: A representative echocardiography showing left ventricle from a control mouse. The image shows the left ventricle, the red outline indicates epicardium, the blue outline indicates endocardium and the aorta is situated on the right of the image.

Sample	EF (%)	FS diameter (%)	Corrected LV mass (mg)	LVEDV (µl)	LVESV (µl)
Sham	57.2 ± 0.92	29.87 ± 0.66	79.34 ± 6.04	89.56 ± 4.76	37.78 ± 0.88
Ang II Treatment	36.3 ± 5.01	17.16 ± 2.75	90.21 ± 10.49	60.72 ± 11.39	38.21 ± 6.40
p-value	p<0.05	p<0.05	p>0.05	p= 0.07	p>0.05

Table 5.7: Cardiac morphology and function of sham-operated and ApoE mice with left ventricular hypertrophy.
Ejection fraction (EF); fractional shortening (FS); left ventricle end-diastolic volume (LVEDV); left ventricle end-systolic volume (LVESV). N of sham group = 3, n of treated group = 3. The results are expressed as mean ± S.E.M. Unpaired t-test. The raw data in appendix 9.

5.2.2.2 Evaluation of suitable reference genes for UT mRNA gene expression in mouse cardiac tissue

To identify the ideal reference genes for accurate gene expression normalisation in mouse cardiac tissues, six potential reference genes were evaluated, β-glucuronidase (GUSβ), ACTβ, β2M, HPRT, Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) and POP4. In order to yield representative results to normalise RT-PCR data of hypertrophy experiments in mice, 5 treated animals with Ang II were used and 5 animals as control (Appendix 10A). NormFinder® analysis showed that two endogenous reference genes were found to be sufficient for reliable measurement and accurate normalisation of the RT-PCR data. The use of NormFinder® software resulted in the selection of POP4 and β2M as the most two stable genes across all tested samples of the study with stability value 0.216. However, the best single gene was β2M with the stability value 0.289 (Figure 5.6). Appendix 10B & C show intergroup and intragroup variations of the six candidate reference genes between samples according to NormFinder® software.

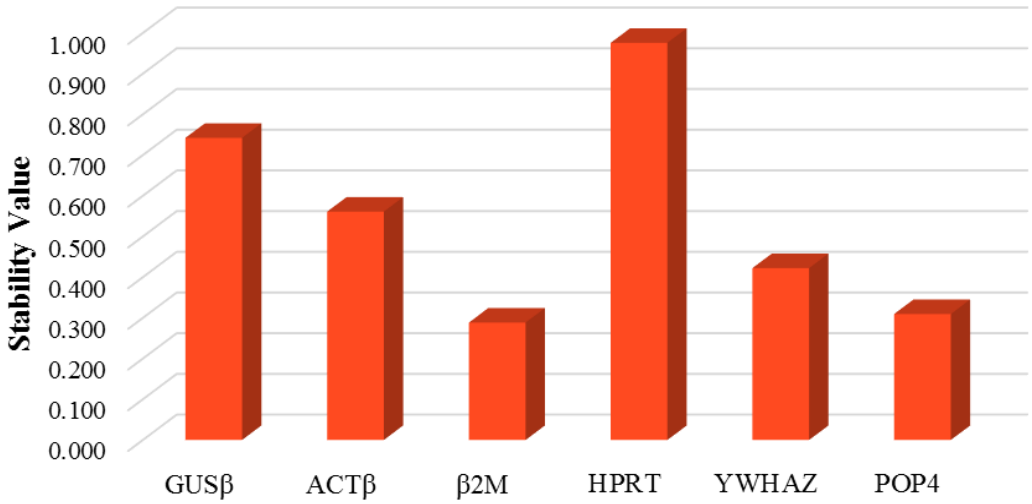


Figure 5.6: Expression of stability value of various endogenous reference genes calculated by NormFinder® in mouse cardiac tissue.
N= 5 hearts (left ventricle) for control and 5 hearts (left ventricle) treated with Ang II. The raw data in appendix 10.

5.2.2.3 UT mRNA gene expression in mouse cardiac tissue

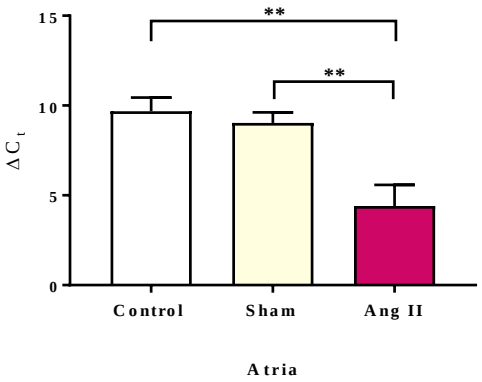
After identification of suitable reference genes, three independent RT-PCR assay reactions were performed on mRNA from cardiac tissue including, atria, right ventricle and left ventricle in order to compare the expression levels of UT. Five hearts were harvested from each group: 1. Control, 2. Ang II-treated and 3. Sham-operated. Geometric mean and ΔC_t were calculated for all cardiac tissues (Table 5.8). Using the stable reference genes POP4 and β2M, gene expression (UT mRNA) showed a significant increase in mRNA expression in the atria of ANG II-treated group, with a ΔCt of 4.4 ± 1.85 (Ang II-treated), versus 9.03 ± 0.59 (Sham-operated) and 9.67 ± 0.76 (control) ($p<0.01$), as shown in Figure 5.7A. However, there were no significant differences in ΔC_t values between right ventricle tissue treated with Ang II (5.92 ± 1.22) compared to the sham (5.87 ± 2.50) or control group (7.19 ± 1.13) ($p>0.05$) (Figure 5.7B). There was also no significant difference in levels of UT-receptor expression in left ventricular tissue collected from treated animals with Ang II with a ΔC_t value of (7.80 ± 1.41), versus in sham-operated group (6.07 ± 2.33) and control group (7.62 ± 0.90) ($p>0.05$) (Figure 5.7C).

Atria					
Sample	**C_t Mean POP4**	**C_t Mean β2M**	**Geometric Mean**	**C_t Mean UT**	**ΔC_t**
Control	28.63	20.64	24.28	33.96	9.68
Sham	26.58	21.81	24.07	33.10	9.03
Ang II Treatment	30.75	24.82	27.56	31.96	4.40
Right ventricle					
Sample	**C_t Mean POP4**	**C_t Mean β2M**	**Geometric Mean**	**C_t Mean UT**	**ΔC_t**
Control	27.61	20.90	24.00	31.19	7.19
Sham	28.77	25.09	26.79	32.67	5.87
Ang II Treatment	29.77	23.16	26.24	32.17	5.93
Left ventricle					
Sample	**C_t Mean POP4**	**C_t Mean β2M**	**Geometric Mean**	**C_t Mean UT**	**ΔC_t**
Control	28.09	20.44	23.92	31.55	7.62
Sham	27.88	24.26	25.95	32.02	6.07
Ang II Treatment	27.02	20.12	23.27	31.07	7.80

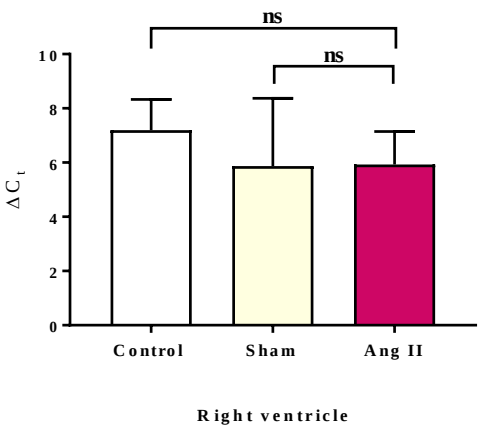
Table 5.8: Effect of Ang II on UT receptor expression in cardiac tissues using endogenous control (POP4 and β2M).

The table shows C_t value mean, geometric mean, ΔC_t mean for cardiac tissue samples. N of control = 5 hearts, n of sham = 5 hearts and n of treated animals with Ang II = 5 hearts. The raw data in appendices 11, 12 and 13.

A



B



C

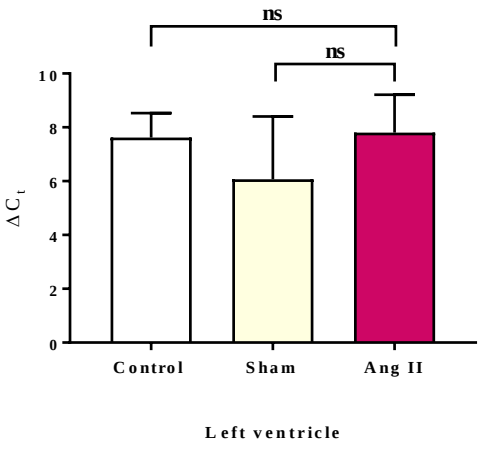


Figure 5.7: Expression of UT receptor in mouse cardiac tissues using endogenous control (POP4 and β2M).

(A) Expression of UT receptor in atria of mouse.

(B) Expression of UT receptor in right ventricle of mouse.

(C) Expression of UT receptor in left ventricle of mouse.

N of control = 5 hearts, n of sham = 5 hearts and n of Ang II treatment = 5 hearts.

The results are expressed as mean ± S.E.M. **p<0.01. One-way ANOVA followed by Sidak's *post hoc* test.

5.3 Discussion

In the present chapter, UT mRNA expression was investigated in an in-vitro and in-vivo model of cardiac hypertrophy. RT-PCR is still the most prevalent technique to quantify mRNA and study alterations in gene expression that are associated with diseases.

1- UT mRNA was detected in freshly isolated ventricular cardiomyocytes and GAPDH was individually used as reference gene which is most frequently used to normalise the results of RT-PCR analysis in cardiovascular research.

2- In order to use this technique to assess any quantitative treatment-related differences a formal assessment of reference gene stability was carried out as recommended in the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin *et al.* 2009). Various endogenous reference genes were subsequently evaluated and validated to select the best for use in cardiac hypertrophy research to measure UT mRNA expression.

3- Gene expression level of UT was not altered in isolated ventricular cardiomyocytes that developed hypertrophy following treatment with UII (200nM) or phenylephrine (10μM) for 24 hours.

4- In our in-vivo model, LV-hypertrophy and LV-dysfunction was induced in ApoE KO mice in-vivo treated with Ang II using mini-osmotic pump. The UT mRNA expression was corrected using the POP4 and β2M reference genes selected from NormFinder® software. Using both combinations of reference genes suggested by this software to normalise the data provided by RT-PCR. The results showed that animals treated with Ang II for 28 days, UT receptor expression was not affected in either left or right ventricles, however, levels of expression in the atria was upregulated, compared to the sham or control.

5.3.1 Relevance of selecting specific reference genes for the evaluation of UT receptor expression in hypertrophy

Although UII has been shown to induce cardiac hypertrophy (Zou *et al.* 2001a; Onan *et al.* 2004b), the molecular mechanisms underlying development of ventricular hypertrophy remain unclear until today. Indeed, several articles have extensively

reviewed the signalling cascades responsible for mediating physiological and pathological events and alteration of cellular mechanism within the heart in case of LV-hypertrophy and heart failure (Rothman *et al.* 1992; Hilfiker-Kleiner *et al.* 2006; Bernardo *et al.* 2010; van Berlo *et al.* 2013; Lorenz *et al.* 2014; Tham *et al.* 2015). Gene expression analysis is fundamental to gain a better understanding alteration of UT receptor expression level in cardiac hypertrophy. In the previous chapter, the results showed that UII-induced hypertrophy in ventricular cardiomyocytes and the MAPKs (ERK1/2 and p38) and CaMKII signalling pathways were activated in cultured ventricular cardiomyocytes in response to UII. However, in addition to an increase in UII levels associated with heart failure upregulation of UT-receptor has also been implicated (Douglas *et al.* 2002; Richards *et al.* 2002), and this may result in an increase in binding of UII and/or as an outcome of the hypertrophic response.

Biological variations correlate with variability of mRNA level across different tissues or cell culture conditions, while analytical variations correlate with disparities in RNA quality, PCR efficiency as well as extraction protocols (Caradec *et al.* 2010). Currently, reference genes are extensively used as internal group for normalisation of mRNA data and correction for the variations resulting from cellular and experimental procedures. Moreover, the accuracy of measurement can be increased when using multiple reference genes rather use single reference gene (Bustin et al. 2009). Rates of protein synthesis and protein accumulation are increased in cultured cells than freshly isolated cells, mRNA levels of many receptors or enzymes are dramatically increased after treatment of these cells or tissues. For example, subjecting of animals to ventricular tachypacing lead to induced heart failure and gene expression levels are changed in cardiac tissue. The results showed that changes in genes expression level which are upregulated in atrium more than ventricle tissue (Cardin et al. 2008). In this study, adult rat freshly and cultured ventricular cardiomyocytes were used to study expression of UT receptor, different mouse cardiac tissues (atria and ventricles) were also used. There are variations between samples therefore data should be normalised using reference genes.

Determination of the optimum number of reference genes for normalisation is therefore considered more accurate, compared to normalisation against single reference gene to avoid variant expression during RT-PCR assays (Bustin *et al.* 2009). This method identified a two reference genes POP4 and β2M that were suitable for use in either rat

tissue or mouse tissue, that could be used in further mechanistic studies in both in-vitro rat cultured cardiomyocytes or in-vivo mouse model. To control for biological and analytical variations during RT-PCR assays, normalisation of data is a principle component and it allows us to correct for these variations.

RT-PCR reference gene instability in congestive heart failure and myocardial infarction has been clearly described. For example, GAPDH expression level is reduced in comparison to 18S rRNA which significantly affected the results and analysis of mRNA expression levels (Brattelid et al. 2007). High expression levels of GAPDH, ACTβ, β2M or 18S rRNA have been observed in in-vitro study on fibroblasts, the expression levels of theses references genes affect by experimental conditions such as normalisation of reaction to contain exactly the same amount of RNA and the other condition that the reaction is not normalised RNA in qPCR assays (Schmittgen and Zakrajsek 2000). It has been found that expression level of GAPDH is invariant in rat neonatal cultured cardiomyocytes in response to various hypertrophic stimuli (Winer *et al.* 1999). Therefore, the outcomes of a study and conclusions of gene transcription drawn from data using PCR determination of mRNA expression, may be affected by inappropriate selection of reference genes for mRNA expression normalisation, therefore care needs to be taken to avoid reference genes upregulation or downregulation in myocardial infarction studies (Everaert *et al.* 2011). In the current study, UII was shown to induce hypertrophy through its receptor in cultured ventricular cardiomyocytes, however UT receptor gene expression was not found to have been altered in the current study. It is possible that difference in the results was seen in other studies due to use inappropriate reference genes.

5.3.2 UT mRNA expression in isolated rat ventricular cardiomyocytes in response to hypertrophy induced by UII

Using GAPDH as reference gene may not be appropriate to the study. According to NormFinder® analysis, POP4 and β2M were the best reference genes for normalisation of mRNA RT-PCR data. Results of tissue culture model of ventricular cardiomyocytes did not show any change in levels of UT receptor. The results in this study are better due to use of a combination of reference genes not affected by treatments. Several studies have examined UT expression in hypertrophy and heart failure. Zhang and co-workers demonstrated that UT mRNA upregulates in rat cardiomyocytes treated with

isoproterenol (Zhang *et al.* 2007). Harris and colleagues found administration of UII and volume overload together can increase UT receptor expression in aortic smooth muscle of rat, but in contrast, administration of UII alone is unable to change expression level of UT mRNA (Harris *et al.* 2010). In-vivo study of myocardial tissue in an animal model of CHF, in which rats underwent coronary artery ligation, both UII and its receptor are overexpressed in cardiac tissues. Furthermore, this study has also investigated the ability of the UT antagonist SB611812 to ameliorate of myocardial dysfunction. Treatment the animals with SB611812 for 8 weeks after ligation, causing improved in cardiac function including reduction of left ventricle end-diastolic pressure, decrease systolic pressure of right ventricle. In addition, blockade of the UT receptor can reduce about 50% of cardiomyocyte hypertrophy in a rat model of CHF (Bousette et al. 2006), the finding of this study was based on reports that showed increased UII levels in the plasma of patients with heart failure (Richards *et al.* 2002; Lapp *et al.* 2004).

The present study has produced results which corroborate the findings of a great deal of the previous work in this field. However, although the current study showed that UII was able to induce hypertrophy in ventricular cardiomyocytes (see section 4.2.1), UII did not significantly increase the UT receptor expression in this study. Furthermore, treatment of these cells with UT antagonist SB657510 lead to block cardiomyocytes hypertrophy. The data in this study are different from other studies may due to use of adult rat ventricular cardiomyocytes while other studies have used neonatal cardiomyocytes.

5.3.3 Upregulation of UT mRNA expression in mouse cardiac tissue in response to LV-hypertrophy and heart failure induced by Ang II

Angiotensin II mouse model is a good model of hypertrophy to look for UT receptor upregulation. A mouse model which develops LV-hypertrophy dependent on Ang II infusion was used (Matsumoto *et al.* 2013; Tsuruda *et al.* 2016). The animal model used in this study developed LV-hypertrophy after infusion of Ang II, myocardial hypertrophy was developed after 14-28 days. Although, this model was initially developed of aortic aneurysms, it has been shown that infusion of Ang II causes LV-hypertrophy and heart failure after 28 days (Regan *et al.* 2015; Tsuruda *et al.* 2016).

Remarkably, Ang II but not UII or ischemic heart disease was used to induce hypertrophy in mouse in the current study. Therefore, the results may differ from other studies.

Ang II binds to G_q-coupled AT_1 receptor in cardiac tissue triggering a wide range of biological activities affecting cardiovascular function. It is produced in various organs such as kidney, heart, and liver (Dzau 1987). The problems associated with chronically elevated Ang II include fibrosis, myocardial hypertrophy, remodeling and risk of developing pathological hypertrophy as intermediate steps to heart failure (Ruiz-Ortega *et al.* 2001; Mehta and Griendling 2007). AT_2 receptor has usually opposite action to AT_1, and effects of AT_1 receptor are counteracted by transient expression of AT_2 receptor, which promotes apoptosis and prevent cell growth during hypertrophy (Van Kesteren *et al.* 1997). In-vivo cardiac function studies in mouse models have shown that cardiac function is impaired following chronic exposure to Ang II (Huggins *et al.* 2003). Role of Ang II has also been shown in heart failure which contributes to impair cardiac contractile function and elicits an alteration of inotropic response causing decrease shortening both amplitude and velocity (Allen *et al.* 1988). The pronounced role of Ang II in hypertrophy is manifested through upregulation of its receptor, promoting classical features of hypertrophy, cardiac remodeling and the development of heart failure (Paradis *et al.* 2000). Four molecular mechanisms have been identified to explain how Ang II can contribute to the development of pathological cardiac hypertrophy: (1) activation of MAPKs signalling pathway, JNKs and Janus kinases (JAK) signal pathway via DAG and PKC. (2) Activation of MAPKs via IP_3. (3) Matrix metalloproteinases activations involved in hypertrophy through the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signal pathway. (4) G12/13 protein mediates cardiac hypertrophy induced by Ang II through activation of Rho-kinases (Balakumar and Jagadeesh 2010).

UII plays an important role in perturbation of the cardiovascular system causing pathological myocardial hypertrophy and the accompanied by upregulation of UT receptor (Onan *et al.* 2004b). In this regard, Douglas and co-workers showed that UII/UT system is overexpressed in diseased hearts of failing patients with end-stage of disease. Moreover, localisation of UII is detected in cardiomyocytes and both smooth muscle cells and endothelial cells of coronary artery, strong expression is noticed in subendocardial surviving myocardium of patients with ischemia. However, at early-stage of CHF, myocardial tissue displayed weak expression of UII (Douglas *et al.* 2002).

UT mRNA expression levels were evaluated in left and right ventricular and atrial tissues from hearts isolated from a mouse model of LV-hypertrophy and heart failure induced by Ang II for 28 days. Although, the UT mRNA did not upregulate in left or right ventricular tissue in ApoE mouse treated with Ang II compare to sham or control, there was an interesting finding in this study, UT mRNA had higher expression pattern in atria in ApoE mouse treated with Ang II for long term compare to sham or control. These results agree with the findings of another study, in which UT receptor expression has been reported to increase in atrial tissues but not ventricle tissues of rat with CHF (Nakayama *et al.* 2008). This is in contrast to a study by Leonard and co-workers (2009) who showed a decrease in UT-receptor expression in atria from patients with ischemia heart disease with reduced LVEF (<50%), (Leonard *et al.* 2009). To understanding the functional role of UII in heart diseases, Tzanidis and colleagues have demonstrated that a coronary artery ligation rat model of heart failure, stimulates expression of UII/UT system. In fact, upregulation of UII peptide and UT mRNA are both observed in infarct and noninfarct zones of the left ventricle. Interestingly, the study also showed that UII has a pronounced effect on cultured neonatal cardiomyocytes and can induce hypertrophy, in addition it has been shown that UII is involved in myocardial fibrosis due to increase collagen production (Tzanidis *et al.* 2003). Similarly, Johns and co-workers found that UII/UT system is significantly upregulated immediately after coronary ligation. Notably, the study also demonstrated that involved of UII in the development of pathological hypertrophy in H9C2 cardiomyocytes (Johns *et al.* 2004). The results of this study will now be compared to the findings of previous work, UII-induced hypertrophy in adult rat ventricular cardiomyocytes, however, the findings of the current study showed that UII expression levels did not change in ventricular cardiomyocytes.

In the current study, the mouse model of LV-hypertrophy was induced by using Ang II. It has been primarily focused on left ventricular systolic function and ejection fraction as an indicator of severity of LV-hypertrophy. After development of LV-hypertrophy, ejection fraction was significantly reduced, this reduction is more pronounced in Ang II treated group in compared to sham-operated. According to echocardiographic data, ejection fraction (≥30%) was reduced in ApoE mice treated with Ang II compared to sham group as well as reduction in fractional shortening was shown in this study. The study showed that myocardial (atria) UT mRNA expression was significantly higher in heart samples taken from mice treated with Ang II than sham or control, which was

associated with drop in ejection fraction, other parameters such as left ventricular mass did not show much of the observed variance. It is possible this related to the data of ultrasound that analysed only after 7 and 14 days. Accordingly, these findings are consistent with those of other studies and suggesting that Ang II induced LV-hypertrophy and UT receptor was upregulated in response to hypertrophy, UII may be play important role pathophysiology of heart failure. In-vivo cardiac mouse model, there was no idea about plasma levels of UII when these animals were treated with Ang II, UII/UT system could be elevated in-vivo.

There are contradictory results concerning circulating UII level in patients with heart failure. Several studies investigating UII/UT system have been carried out on heart failure patients, showing increased expression of UII and its receptor are supported by elevation of UII plasma level (Richards *et al.* 2002; Russell *et al.* 2003; Lapp *et al.* 2004). The UT receptor is present in both healthy individuals and diseased hearts in various area of cardiovascular system such as cardiomyocytes, vascular smooth muscle cells and endothelial cells, these cells exhibit greater binding sites for UII which significantly increased with severity of disease in group heart failure (Douglas *et al.* 2002). It is worth mentioning that atrial remodelling was observed in patients with symptomatic congestive heart failure with ejection fraction 25%, after subjecting the patients to cardiac electrophysiological tests, atria undergo both electrical and structural changes (Sanders *et al.* 2003). Together these studies provide important insights into the atria that are an alternate marker for cardiovascular disease. One possibility is that the presence of an increase in UII in addition to driving hypertrophy could also lead to an increase in expression of the UT-receptor. To see the effect of UII on atrial cardiomyocytes, it could be useful to culture these cells and look at the effects of UII.

Russell *et al.*, observed a marked concentration gradient of circulating hUII peptide between aortic root and pulmonary artery of CHF patients, suggesting that UII is produced by the heart (Russell *et al.* 2003). Some reports have shown no difference in circulating UII between healthy and CHF patients (Dschietzig *et al.* 2002; Krüger *et al.* 2005). However, other reports have shown a large increase in circulating UII and this is inversely proportional to disease status as measured by ejection fraction (Douglas *et al.* 2002; Gruson *et al.* 2006; Chen *et al.* 2009). On the other hand, there is an apparent variance in results with other studies, no difference was found either in circulating UII

levels or tissue levels between patients with heart failure and healthy individual (Dschietzig *et al.* 2002; Krüger *et al.* 2005). Together, these studies using various assays provide strong evidence of a correlation between UII levels and disease efficiency, but these studies cannot determine whether this elevation of peptide is cause or effect. Therefore, it is not entirely clear the functional role of elevated UII levels in the failing heart.

5.3.4 Study limitations

There are several limitations associated with the present study that should be emphasized. In this current study, UT mRNA expression in a mouse model of LV-hypertrophy was ` independently of circulating UII. Unfortunately, it was not possible to measure the level of UII in plasma. The current study has only examined UT receptor mRNA in ventricular cardiomyocytes and in-vivo mouse model treated with Ang II. Protein was not measured in this study because there is no good UT antibody. Plasma UII levels were not measured in ApoE mice after developed LV-hypertrophy to see whether the level of UII was changed or not.

5.3.5 Conclusion

A conclusion that can be drawn from these results is that UII in-vitro treatment does not alter UT mRNA expression in rat cultured ventricular cardiomyocytes. In the present study, in-vivo data obtained in a mouse model of LV-hypertrophy indicate upregulation of the UT receptor in atria but not ventricles, suggesting no link between LV-hypertrophy, induced by chronic exposure to Ang II, and UT receptor upregulation. The increase in the atria requires further study to look for a possible mechanistic link between UT receptors and atrial function in heart failure.

Chapter 6: Clinical implications of UII in E-C coupling and cardiac hypertrophy

Urotensin II is elevated in heart failure and has been implicated in both the hypertrophic remodelling of the ventricles and the resultant dysfunction of ventricular function, which may contribute to the development of heart failure (see section 1.4.3). The present study was designed to determine the effect of UII on both contractile function of ventricular cardiomyocytes and molecular mechanisms that are responsible for UII-induced hypertrophy.

6.1 Ventricular Hypertrophy

We have shown in this study, that UII can induce hypertrophy in ventricular cardiomyocytes after 24 hours of incubation. The study showed that MAPKs (ERK1/2 and p38) and CaMKII signalling pathways were activated in cultured ventricular cardiomyocytes in response to UII and responsible for the induction of hypertrophy. However, in the current study this was not linked to any upregulation in UT receptor, contrary to some previous studies (see section 5.3.2).

Precise understanding of the intracellular mechanisms that are responsible for induction of hypertrophy by UII will give new insights into targeting these signalling mechanisms and will be helpful to find novel therapy. The signalling pathways that was identified are common to the MAPKs pathways identified for many $G_{\alpha q}$-coupled GPCRs such as Ang II and ET-1 (see introduction, section 1.3.2). Therefore, many pharmacological treatments identified to target normal signalling associated with ischaemic heart disease and pressure-overload (hypertension and valve disease) are also likely to have a positive impact on UII-induced ventricular remodelling.

6.2 E-C coupling

The effect of UII on E-C coupling in the heart was demonstrated in both normal and disease conditions. However, whilst some studies have shown that UII has a positive inotropic action (Russell *et al.* 2001; Zhang *et al.* 2015), other studies have been observed that UII peptide has a negative inotropic action (Morimoto *et al.* 2002; Fontes-Sousa *et al.* 2009). In the present study, a significant negative inotropic action of UII was

demonstrated in both isolated whole rat hearts and isolated single rat ventricular cardiomyocytes. The reduction in strength of contraction was reflected by reduction is systolic Ca^{2+}, which was accompanied by a decrease in L-type Ca^{2+}-current density, SR Ca^{2+}-content and a shortening of the APD determined at 30 and 50% repolarization.

Expression of myocardial hUII is upregulated in patients with heart failure (Douglas *et al.* 2002). It negatively affects on contraction of cardiac muscle in LV-hypertrophy and failing myocardium. The results of current study agree with the findings of another study, in which UII negatively affects on contraction of cardiac muscle in LV-hypertrophy and patients with heart failure (Quaile *et al.* 2009). E-C coupling is impaired in heart failure patients, and various factors appear to contribute to this depression in contractility in heart failure, including decrease SR Ca^{2+}-content, decreased $[Ca^{2+}]_i$ transients or diastolic SR Ca^{2+}-leak (Piacentino *et al.* 2003). There is strong evidence that SR Ca^{2+}-content is reduced in heart failure due to impaired function of SERCA2a, which contributes to slow down rate of decay of the Ca^{2+}-transient, thence affects diastolic function (Schmidt *et al.* 1998; Jiang *et al.* 2002). Simultaneously, NCX function/expression is increased in heart failure and Ca^{2+} is extruded from the cytosol by "*forward- mode*". As a result of these changes, SR Ca^{2+} store became underfilled and depleted (Belevych *et al.* 2007). Abnormal Ca^{2+} homeostasis can be recognised in patients with heart failure due to the rendering of RyR2 which become more active in heart failure, these channels are hyperphosphorylated by CaMKII or PKA resulting in SR Ca^{2+}-leak and reduction in SR Ca^{2+}-content (Shannon *et al.* 2003; Kubalova *et al.* 2005).

An increase in circulating UII and in the UT receptors would result in a further reduction in contraction. Therefore, pharmacological interventions to inhibit the UII system in ventricular tissue might be a target for supporting the failing heart, in conditions where the UII system is upregulated.

6.3 Future work

The study looked at acute effect of UII on E-C coupling and chronic effect of UII on ventricular cardiomyocytes in tissue culture for 48 hours. Future research should therefore concentrate on the investigation the role of UII using in-vivo models to look at hypertrophy because the ventricular cardiomyocytes undergo morphological and functional alteration over time in culture media. It would be better preferable to now look at implication of UII in-vivo from 14-21 days and its action then look for its action on E-C coupling at 7, 14 and 21 days.

Another possible area of future research would be to extend the investigation into gene expression of UT after induced hypertrophy in-vivo, to include identification of protein levels and more importantly surface membrane levels of active receptors.

In-vivo

What about using mini-pump with UTII to drive hypertrophy and then looking at the signalling pathways that have identified in this study (inhibitors of the signalling pathways). Using this model, we could also see whether chronic exposure to UII affects the UT receptor expression and activity.

In-vitro signalling

To demonstrate possible interaction between ERK1/2, p38 and CaMKII signalling in the hypertrophic response to UII. We could treat cells with the inhibitors and look for an effect on phosphorylation and activation of the other two signalling pathways.

Appendix

Appendix 1

Materials

Chemical materials	Supplier
Acetic acid	Fisher Scientific
Acrylamide	Gene Flow
Adenosine 5'-triphosphate sodium salt (Na_2ATP)	Sigma
Ammonium persulfate (AMPS)	Sigma
Amphotericin B	Gibco
BAPTA tetrapotassium salt	Santa Cruz Bio Technology
Biotinylated ladder	Cell Signalling
Blot stripping buffer	Thermo Scientific
Bromophenol blue sodium salt	Sigma
Bovine serum albumin (BSA)	Sigma
2,3-Butanedione monoxime (BDM)	Sigma
Caffeine	VWR
Calcium chloride	Acros
Calcium chloride solution ($CaCl_2$)	Fluka
Chloroform	Fisher Scientific
Copper sulphate ($CuSO_4$)	Fisons analytical Reagents
Collagenase from clostridium histolyticum type I	Sigma
Creatine anhydrous	Sigma
D-glucose anhydrase	Fisher
Dimethyl Sulfoxide (DMSO)	Sigma
Dithiothreitol	Fisher Bioreagent
Ethanol	Fisher Scientific
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Ethylene glycol-bis(2-amino-ethylether) *N,N,N′,N′*-tetraacetic acid (EGTA)	Sigma
Fetal Bovine Serum (FBS)	Thermo Fisher Scientific
Fluo-3, AM Molecular Probes	Life Technologies

Folin Ciocalteu's phenol reagent	Sigma
Fungizone	Gibco®/ Thermo Fisher Scientific
Fura-2, AM Molecular Probes	Invitrogen/Thermo Fisher Scientific
Geneticin (G418)	Gibco®/ Thermo Fisher Scientific
Glucose	Sigma
Glycerol	Sigma
Glycine	Sigma
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich
Industrial denatured alcohol Industrial methylated sprit (IMS)	Genta Medical
Insulin-Transferrin-Selenium (ITS)	Corning
(-)-Isoproterenol hydrochloride (ISO)	Sigma
KN-93	Sigma
Magnesium chloride Solution ($MgCl_2$)	Fluka
Magnesium sulfate ($MgSO_4$)	Fisher
Methanol	Fisher scientific
N-methyl-D-glucamine (NMDG)	Fluka
PCR-grade Water	Roche
8-(p-sulfo-phenyl)theophylline hydrate	Sigma
PD184352	Sigma
Penicillin (100 IU/ml) and streptomycin (100μg/ml)	Gibco®/ Thermo Fisher Scientific
Potassium chloride (KCl)	Sigma-Aldrich
Potassium dihydrogen orthophosphate (KH_2PO_4)	Fisons
Potassium sodium tartrate	Fisons Analytical Reagents
Ponceau S	Sigma
Prestained ladder	Cell Signalling
2-Propanol (Isopropanol)	Sigma
Protease from Streptomyces griseus type XIV	Sigma
(R)-(-)-phenylephrine hydrochloride	Sigma
RNAlater	Sigma
SB202190	Synkinase
SB657510	Tocris

Skimmed milk	The Co-operative
Sodium carbonate (Na_2Co_3)	Sigma-Aldrich
Sodium chloride (NaCl)	Fisher
Sodium dodecyl sulphate (SDS)	Fisher
Sodium bicarbonate ($NaHCO_3$)	Sigma
Sodium carbonate anhydrase (Na_2CO_3)	Fisher
Sodium hydroxide (NaOH)	Sigma
Sodium orthovanadate	Sigma
Sodium phosphate dibasic (anhydrous)	Sigma
Sodium pyrophosphate	Sigma-Aldrich
Sodium Pyruvate	Sigma-Aldrich
Taqman gene expression Master mix	Applied Biosystems™/Thermo Fisher Scientific
Taurine	Sigma
Tetracaine hydrochloride	Sigma
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Sigma
Tris-HCL (Trizma®base)	Sigma
Tris/glycine/SDS Buffer (TGS) (10x)	Bio-Rad
Tri reagent solution	Invitrogen
Tween® 20	Sigma
Trypsin	Lonza
Urotensin II human	Professor R Guerrini
Urotensin II rat	Sigma-Aldrich/ Dr A Jamieson

Appendix 2

Antibodies

Antibody	Supplier
Anti-biotin secondary antibody	Cell Signalling
Anti-rabbit IgG HPR-linked antibody	Cell Signalling
Anti-rabbit IgG peroxidases secondary antibody	Cell Signalling
Peroxidase-conjugated affinipure donkey anti-rabbit IgG secondary antibody	Jackson ImmunoResearch
Phosphorylated CaMKII primary antibody	Abcam
Phosphorylated CaMKII primary antibody	Cell Signalling
Phosphorylated ERK 1/2 primary antibody	Cell Signalling
Phosphorylated JNK primary antibody	Cell Signalling
Phosphorylated p38 primary antibody	Cell Signalling
Total CaMKII primary antibody	Cell Signalling
Total ERK 1/2 primary antibody	Cell Signalling
Total JNK primary antibody	Cell Signalling
Total p38 primary antibody	Cell Signalling
vinculin antibody	Cell Signalling

Appendix 3

Kits

Kit	Supplier
Clarity™ western ECL substrate	Bio-Rad
cOmplete Lysis M	Roche
High-Capacity cDNA reverse transcription kit	Applied Biosystems™/Thermo Fisher Scientific
RNA kit	Ambion/Life Technology

Appendix 4

Tissue culture Media

Media	Supplier
Dulbecco's modified Eagle medium: Hams nutrient mixture F-12 media 50/50 mix (DMEM/F12 (50/50))	Corning
Medium 199 HEPES modification	Sigma

Appendix 5

TaqMan gene expression probes

Reference gene	Supplier
Beta-actin (β-actin) (Rat)	Applied Biosystems™/Thermo Fisher Scientific
Glyceraldehyde 3-phosphate dehydrogenase (Rat)	Applied Biosystems™/Thermo Fisher Scientific
Beta-2-microglobulin (Rat)	Applied Biosystems™/Thermo Fisher Scientific
Hypoxanthine phosphoribosyltransferase 1 (Rat)	Applied Biosystems™/Thermo Fisher Scientific
Peptidylprolyl isomerase A (Rat)	Applied Biosystems™/Thermo Fisher Scientific
Processing of precursor 4 (Rat)	Applied Biosystems™/Thermo Fisher Scientific
Urotensin 2 receptor (Rat)	Applied Biosystems™/Thermo Fisher Scientific
Beta-actin (β-actin) (Mouse)	Applied Biosystems™/Thermo Fisher Scientific
Beta-2-microglobulin (Mouse)	Applied Biosystems™/Thermo Fisher Scientific
Beta-glucuronidase (Mouse)	Applied Biosystems™/Thermo Fisher Scientific
Hypoxanthine guanine phosphoribosyl transferase (Mouse)	Applied Biosystems™/Thermo Fisher Scientific
Processing of precursor 4 (Mouse)	Applied Biosystems™/Thermo Fisher Scientific
Tyrosine3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta polypeptide (Mouse)	Applied Biosystems™/Thermo Fisher Scientific
Urotensin 2 receptor (Mouse)	Applied Biosystems™/Thermo Fisher Scientific

Appendix 6

A

Gene name	Stability value
GAPDH	0.348
ACTβ	0.180
β2M	0.308
HPRT	0.291
PPIA	0.488
POP4	0.280

B

Group identifier	1	2	3
GAPDH	0.274	-0.059	-0.215
ACTβ	0.035	-0.027	-0.008
β2M	0.321	0.031	-0.353
HPRT	0.223	-0.045	-0.178
PPIA	-0.569	0.080	0.489
POP4	-0.284	0.019	0.265

C

Group identifier	1	2	3
GAPDH	0.628	0.094	0.001
ACTβ	0.016	0.104	0.068
β2M	0.098	0.008	0.001
HPRT	0.034	0.051	0.102
PPIA	0.438	0.037	0.484
POP4	0.086	0.027	0.001

Appendix 6: Evaluation of expression stability for candidate reference genes in ventricular cardiomyocytes.
(A) Represent stability of six candidate reference genes in cultured cardiomyocytes. N= 4 hearts. The NormFinder® statistic algorithm showed that POP4 and β2M were the most stable genes with a stability value of 0.107.
(B) Intergroup RT-PCR variation estimated by NormFinder®. Group 1= control, group 2= treated cells with UII, group 3= treated cells with phenylephrine.
(C) Intragroup RT-PCR variation.

Appendix 7

A

Sample	Control			Treated cells with UII			Treated cells with Phenylephrine		
	C_t Mean POP4	**C_t Mean UT**	**ΔC_t Mean**	**C_t Mean POP4**	**C_t Mean UT**	**ΔC_t Mean**	**C_t Mean POP4**	**C_t Mean UT**	**ΔC_t Mean**
Sample 1	28.49	34.94	6.45	26.89	34.02	7.13	26.63	34.58	7.94
Sample 2	26.03	35.06	9.03	27.28	36.98	9.70	26.58	34.83	8.25
Sample 3	25.82	34.14	8.33	25.96	34.46	8.50	25.99	35.91	9.92
Sample 4	25.97	34.66	8.69	26.36	35.39	9.03	26.76	36.15	9.39
Sample 5	31.52	35.93	4.42	27.91	34.35	6.45	26.90	34.35	7.45
Sample 6	28.14	35.83	7.69	27.36	35.43	8.08	30.63	36.56	5.93
Sample 7	29.35	34.33	4.97	28.11	32.99	4.87	28.39	34.17	5.79
Sample 8	27.98	34.06	6.08	28.75	34.84	6.08	28.38	34.44	6.07
Sample 9	30.18	34.21	4.03	32.43	35.50	3.07	29.27	33.76	4.48
Sample 10	28.03	33.16	5.13	27.32	33.01	5.70	28.38	34.70	6.32
Sample 11	29.96	36.07	6.11	29.01	35.35	6.34	29.13	35.71	6.58
Mean	28.316	34.76	6.45	27.94	34.76	6.81	27.91	35.01	7.10

B

Sample	Control			Treated cells with UII			Treated cells with Phenylephrine		
	C_t Mean β2M	C_t Mean UT	ΔC_t Mean	C_t Mean β2M	C_t Mean UT	ΔC_t Mean	C_t Mean β2M	C_t Mean UT	ΔC_t Mean
Sample 1	18.77	34.94	16.16	17.43	34.02	16.59	17.62	34.58	16.95
Sample 2	15.60	35.06	19.46	17.24	36.98	19.74	16.76	34.83	18.08
Sample 3	15.41	34.14	18.73	15.49	34.46	18.97	16.29	35.91	19.63
Sample 4	15.53	34.66	19.14	16.50	35.39	18.89	16.56	36.15	19.59
Sample 5	19.84	35.93	16.09	19.99	34.35	14.36	19.62	34.35	14.73
Sample 6	18.46	35.83	17.37	18.40	35.43	17.03	20.03	36.56	16.53
Sample 7	20.32	34.33	14.01	19.88	32.99	13.11	20.50	34.17	13.67
Sample 8	19.50	34.06	14.57	20.57	34.84	14.26	20.70	34.44	13.75
Sample 9	21.27	34.21	12.94	22.57	35.50	12.93	20.75	33.76	13.01
Sample 10	20.10	33.16	13.06	19.78	33.01	13.23	20.93	34.70	13.77
Sample 11	18.61	36.07	17.46	18.52	35.35	16.82	18.74	35.71	16.97
Mean	18.49	34.76	16.27	18.76	34.76	15.99	18.95	35.01	16.06

Appendix 7: RT-PCR data of cultured ventricular cardiomyocytes for reference genes and target gene.

(A) C_t value for POP4 reference gene and UT.

(B) C_t value for β2M reference gene and UT.

Appendix 8

A

Sample	Control				
	C_t Mean POP4	**C_t Mean β2M**	**Geometric Mean**	**C_t Mean UT**	**ΔC_t**
Sample 1	28.49	18.77	23.13	34.94	11.81
Sample 2	26.03	15.60	20.15	35.06	14.91
Sample 3	25.82	15.41	19.95	34.14	14.19
Sample 4	25.97	15.53	20.08	34.66	14.58
Sample 5	31.52	19.84	25.01	35.93	10.93
sample 6	28.14	18.46	22.79	35.83	13.04
Sample 7	29.35	20.32	24.42	34.33	9.91
Sample 8	27.98	19.50	23.36	34.06	10.70
Sample 9	30.18	21.27	25.34	34.21	8.88
Sample 10	28.03	20.10	23.74	33.16	9.42
Sample 11	29.96	18.61	23.61	36.07	12.46
Mean	28.32	18.49	22.87	34.76	11.89

B

Sample	Treated cells with UII						
	C_t Mean POP4	**C_t Mean β2M**	**Geometric Mean**	**C_t Mean UT**	**ΔC_t**	**$\Delta\Delta C_t$**	**Fold change**
Sample 1	26.89	17.43	21.65	34.02	12.37	0.56	0.68
Sample 2	27.28	17.24	21.68	36.98	15.30	0.39	0.76
Sample 3	25.96	15.49	20.06	34.46	14.41	0.21	0.86
Sample 4	26.36	16.50	20.86	35.39	14.53	-0.05	1.04
Sample 5	27.91	19.99	23.62	34.35	10.73	-0.19	1.14
sample 6	27.36	18.40	22.44	35.43	12.99	-0.05	1.03
Sample 7	28.11	19.88	23.64	32.99	9.35	-0.56	1.47
Sample 8	28.75	20.57	24.32	34.84	10.51	-0.19	1.14
Sample 9	32.43	22.57	27.05	35.50	8.45	-0.43	1.34
Sample 10	27.32	19.78	23.24	33.01	9.77	0.35	0.79
Sample 11	29.01	18.52	23.18	35.35	12.17	-0.29	1.22
Mean	27.94	18.76	22.89	34.76	11.87	-0.02	1.04

C

Sample	Treated cells with Phenylephrine						
	C_t Mean POP4	**C_t Mean β2M**	**Geometric Mean**	**C_t Mean UT**	**ΔC_t**	**$\Delta\Delta C_t$**	**Fold change**
Sample 1	26.63	17.62	21.67	34.58	12.91	1.10	0.47
Sample 2	26.58	16.76	21.10	34.83	13.73	-1.18	2.26
Sample 3	25.99	16.29	20.58	35.91	15.34	1.14	0.45
Sample 4	26.76	16.56	21.05	36.15	15.10	0.52	0.70
Sample 5	26.90	19.62	22.97	34.35	11.38	0.45	0.73
sample 6	30.63	20.03	24.76	36.56	11.79	-1.25	2.38
Sample 7	28.39	20.50	24.12	34.17	10.05	0.15	0.90
Sample 8	28.38	20.70	24.23	34.44	10.21	-0.49	1.41
Sample 9	29.27	20.75	24.64	33.76	9.11	0.24	0.85
Sample 10	28.38	20.93	24.37	34.70	10.33	0.90	0.53
Sample 11	29.13	18.74	23.37	35.71	12.34	-0.11	1.08
Mean	27.91	18.95	22.99	35.01	12.03	0.13	1.07

Appendix 8: RT-PCR data obtained from cultured ventricular cardiomyocytes.

(A) Table shows C_t value, geometric mean, ΔC_t for 11 control samples.

(B) Table shows C_t value, geometric mean, ΔC_t, $\Delta\Delta C_t$, and fold change expression in 11 treated samples with UII.

(C) Table shows C_t value, geometric mean, ΔC_t, $\Delta\Delta C_t$, and fold change expression in 11 treated samples with phenylephrine.

Appendix 9

A

	Ang II pump	EF (%)	FS; diameter (%)	LV mass (corrected) (mg)	LV Vol; d (ul)	LV Vol; s (ul)
tp1	AE5625	36.26	16.769	81.355	39.926	25.448
tp2	AE5659	27.644	12.606	78.153	63.084	45.645
tp2	AE5671	45.005	22.114	111.115	79.158	43.533

B

	Sham controls	EF (%)	FS; diameter (%)	LV mass (corrected) (mg)	LV Vol; d (ul)	LV Vol; s (ul)
tp1	AE5666	57.394	29.946	81.442	85.69	36.509
tp1	AE5667	58.692	30.982	88.5889	99.037	39.471
tp1	AE5672	55.522	28.680	67.977	83.963	37.345

Appendix 9: Echocardiographic assessment obtained from sham-operated and ApoE mice with LV hypertrophy obtained.
EF, ejection fraction; FS, fractional shortening; LVEDV, left ventricle end-diastolic volume; LVESV, left ventricle end-systolic volume. B-mode long axis. tp1 = 7 days post-surgery, tp2 = 14 days post-surgery.
(A) Treated group with Ang II, n= 3 animals.
(B) Sham group, n= 3 animals.

Appendix 10

A

Gene name	Stability value
GUSβ	0.742
ACTβ	0.561
β2M	0.289
HPRT	0.976
YWHAZ	0.422
POP4	0.309

B

Group identifier	1	2
GUSβ	-0.384	0.384
ACTβ	0.007	-0.007
β2M	0.190	-0.190
HPRT	-0.409	0.409
YWHAZ	0.201	-0.201
POP4	0.396	-0.396

C

Group identifier	1	2
GUSβ	1.781	3.940
ACTβ	0.806	2.596
β2M	0.777	0.167
HPRT	10.642	1.214
YWHAZ	0.668	1.146
POP4	0.452	0.507

Appendix 10: Evaluation of expression stability for candidate reference genes in mouse cardiac tissue.
(A) Represent gene expression stability of six candidate reference genes in mouse cardiac tissue. N= 5 hearts for control and 5 treated with Ang II. The most real stable genes were POP4 and β2M with a stability value of 0.216 according to NormFinder® statistic algorithm.
(B) Intergroup RT-PCR variation estimated by NormFinder®. Group 1= control, group 2= ApoE mice treated with Ang II.
(C) Intragroup RT-PCR variation.

Appendix 11

A

Control					
Atria					
Sample	**C_t Mean POP4**	**C_t Mean β2M**	**Geometric Mean**	**C_t Mean UT**	**ΔC_t**
1- Ctr	29.22	19.09	23.62	32.61	8.99
2- Ctr	29.78	20.08	24.46	32.32	7.86
3- Ctr	29.62	19.52	24.04	32.56	8.52
4- Ctr	27.23	22.25	24.61	36.22	11.61
5- Ctr	27.32	22.27	24.67	36.08	11.41
Mean	28.63	20.64	24.28	33.96	9.68

B

Sham					
Atria					
Sample	**C_t Mean POP4**	**C_t Mean β2M**	**Geometric Mean**	**C_t Mean UT**	**ΔC_t**
1- Sham H1	26.20	22.92	24.51	33.13	8.62
2- Sham H2	24.01	20.69	22.29	31.67	9.38
3- Sham H3	31.25	25.09	28.00	35.46	7.46
4- Sham H4	25.42	20.22	22.67	33.73	11.06
5- Sham H5	26.01	20.11	22.87	31.50	8.62
Mean	26.58	21.81	24.07	33.10	9.03

C

Ang II Treatment					
Atria					
Sample	**C_t Mean POP4**	**C_t Mean β2M**	**Geometric Mean**	**C_t Mean UT**	**ΔC_t**
1- Sample 24	34.25	32.38	33.30	33.27	-0.03
2- Sample 25	24.28	21.72	22.96	29.95	6.98
3- Sample 58	34.56	28.64	31.46	36.46	5.00
4- Sample H59	29.96	20.47	24.76	29.21	4.45
5- Sample H71	30.70	20.89	25.33	30.92	5.60
Mean	30.75	24.82	27.56	31.96	4.40

Appendix 11: Results obtained from the RT-PCR assay.

Effect of Ang II on UT receptor expression in atria. The table shows mean C_t values for endogenous control (POP4 and β2M), geometric mean and ΔC_t for mouse cardiac tissue samples.
(A) Atria collected from 5 control animals.
(B) Atria collected from 5 sham animals.
(C) Atria collected from 5 treated animals with Ang II.

Appendix 12

A

Control					
Right ventricle					
Sample	**C_t Mean POP4**	**C_t Mean β2M**	**Geometric Mean**	**C_t Mean UT**	**ΔC_t**
1- Ctr	29.10	19.06	23.55	29.53	5.97
2- Ctr	29.56	20.90	24.86	30.95	6.09
3- Ctr	28.46	22.25	25.17	29.39	4.23
4- Ctr	25.87	21.59	23.63	33.82	10.19
5- Ctr	25.05	20.72	22.78	32.27	9.49
Mean	27.61	20.90	24.00	31.19	7.19

B

Sham					
Right ventricle					
Sample	**C_t Mean POP4**	**C_t Mean β2M**	**Geometric Mean**	**C_t Mean UT**	**ΔC_t**
1- Sham H1	25.24	19.63	22.26	31.90	9.64
2- Sham H2	29.84	24.38	26.97	36.65	9.67
3- Sham H3	28.82	23.33	25.93	29.53	3.60
4- Sham H4	33.12	36.88	34.95	31.94	-3.01
5- Sham H5	26.83	21.21	23.85	33.31	9.45
Mean	28.77	25.09	26.79	32.67	5.87

C

Ang II Treatment					
Right ventricle					
Sample	**C_t Mean POP4**	**C_t Mean β2M**	**Geometric Mean**	**C_t Mean UT**	**ΔC_t**
1- Sample 24	24.83	18.73	21.57	31.00	9.43
2- Sample 25	23.83	20.76	22.24	29.73	7.49
3- Sample 58	34.64	29.02	31.70	34.43	2.73
4- Sample H59	31.84	22.57	26.81	33.04	6.23
5- Sample H71	33.71	24.74	28.88	32.63	3.75
Mean	29.77	23.16	26.24	32.17	5.93

Appendix 12: Results obtained from the RT-PCR assay.
Effect of Ang II on UT receptor expression in right ventricle. The table shows mean C_t values for endogenous control (POP4 and β2M), geometric mean and ΔC_t for mouse cardiac tissue samples.
(A) Right ventricle collected from 5 control animals.
(B) Right ventricle collected from 5 sham animals.
(C) Right ventricle collected from 5 treated animals with Ang II.

Appendix 13

A

Control					
Left ventricle					
Sample	**C_t Mean POP4**	**C_t Mean β2M**	**Geometric Mean**	**C_t Mean UT**	**ΔC_t**
1- Ctr	28.56	18.60	23.05	29.42	6.37
2- Ctr	32.61	22.75	27.24	32.90	5.66
3- Ctr	27.69	17.87	22.24	28.79	6.54
4- Ctr	25.28	21.03	23.06	32.27	9.21
5- Ctr	26.32	21.94	24.03	34.35	10.32
Mean	28.09	20.44	23.92	31.55	7.62

B

Sham					
Left ventricle					
Sample	**C_t Mean POP4**	**C_t Mean β2M**	**Geometric Mean**	**C_t Mean UT**	**ΔC_t**
1- Sham H1	29.62	23.80	26.55	36.16	9.61
2- Sham H2	23.52	17.94	20.54	30.65	10.11
3- Sham H3	29.68	24.51	26.97	31.44	4.47
4- Sham H4	24.62	20.41	22.41	30.95	8.54
5- Sham H5	31.95	34.66	33.27	30.91	-2.36
Mean	27.88	24.26	25.95	32.02	6.07

C

Ang II Treatment					
Left ventricle					
Sample	**C_t Mean POP4**	**C_t Mean β2M**	**Geometric Mean**	**C_t Mean UT**	**ΔC_t**
1- Sample 24	26.31	18.85	22.27	33.86	11.60
2- Sample 25	25.90	21.12	23.39	33.69	10.30
3- Sample 58	27.33	23.95	25.59	29.47	3.88
4- Sample H59	27.11	19.25	22.84	28.76	5.92
5- Sample H71	28.42	17.45	22.27	29.58	7.31
Mean	27.02	20.12	23.27	31.07	7.80

Appendix 13: Results obtained from the RT-PCR assay.
Effect of Ang II on UT receptor expression in left ventricle. The table shows mean C_t values for endogenous control (POP4 and β2M), geometric mean and ΔC_t for mouse cardiac tissue samples.
(A) Left ventricle collected from 5 control animals.
(B) Left ventricle collected from 5 sham animals.
(C) Left ventricle collected from 5 treated animals with Ang II.

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