Investigations into the cardioprotective properties of resveratrol

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By

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"These are my principles, and if you don't like them... well, I have others."

-Groucho Marx

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TORYN POOLMAN

INVESTIGATIONS INTO THE CARDIOPROTECTIVE PROPERTIES OF RESVERATROL

ABSTRACT

Monocyte/macrophages play a pivotal role in the pathogenesis of atherosclerosis. Modulation of their effector functions is a potential therapeutic target and moreover, a good target for a chemopreventive agent such as resveratrol, a naturally occurring polyphenolic compound. In this study the ability of resveratrol to inhibit the activation of the monocyte respiratory burst was investigated.

Differentiated U937 (dU937) cells were pre-treated with resveratrol before stimulation with f-met-leu-phe (fMLP), phorbol 12-myristate-13-acetate (PMA) or arachidonic acid (AA). The production of reactive oxygen species was measured by luminol chemiluminescence, 2',7'-dichlorofluorescein, dihydrorhodamine and lucigenin. Resveratrol was found to inhibit ROS production induced by all three stimuli. Resveratrol was found to be a potent inhibitor of peroxidase-dependent ROS measuring principles, moreover, it was oxidised by the horseradish peroxidase/hydrogen peroxide system.

PMA was found to induce a rapid necrosis in dU937 cells, which was inhibited by resveratrol and partially inhibited by antioxidants and heat shock. PMA was not consistently found to alter the levels of heat shock protein 70, nor was resveratrol found to induce the expression of this protein. Free malondialdehyde (MDA) levels or the MDA DNA adduct (M_1G) were not altered in dU937 treated with PMA.

The cell signal transduction pathways activated by fMLP, PMA and AA were investigated. Only fMLP was found to activate phosphatidylinositol-3-kinase (PI3K) and Akt, using specific inhibitors of both kinases. Resveratrol inhibited PI3K activity and fMLP-induced Akt phosphorylation.

In conclusion, the inhibitory effect of resveratrol on ROS production can, in some cases, be explained by its oxidation by peroxidases that are required for the detection of cellular ROS. However, this property may also have further significance, due to the pro-atherogenic effects of peroxidases, such as myeloperoxidase, which could be modulated by resveratrol. The ability of resveratrol to inhibit the PI3K-Akt pathway represents an important anti-inflammatory pathway and further adds to its potential cardioprotective properties.

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ABBREVIATIONS:

AA - Arachidonic acid.

ABTS - 2,2¢-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid

ACE inhibitor - Angiotensin converting enzyme inhibitor

AD - Alzheimers disease

ADP - Adenosine diphosphate

AEBSF - 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride

AGE - Advanced glycation endproducts

mmAGE – Minimally modified AGE.

AKT – Protein kinase B

AMPK - AMP-activated protein kinase

ANOVA - One-way analysis of variance

ANP - Atrial natriuretic factor

APS - Ammonium persulphate

ATP - Adenosine triphosphate

Aβ - Amyliod β

BHT - Butylated hydroxyltoluene

BNP - Brain natriuretic factor

BSS - Balanced salt solution

CAD - Cardiovascular disease

CAMKK - Calcium/calmodulin-dependent protein kinase

CDK - Cyclin-dependent protein kinase

CHK - Checkpoint kinase

CK2 - Casein kinase 2

CL - Chemiluminescence

COX - Cylcooxygenase

CSK - C-terminal Src kinase

CXCL8 - Interleukin 8

DA - Deoxyadenosine

DAG - Diacylglycerol

DC - Deoxycytosine

DCF - 2',7'-Dichlorofluorescein

DEAE - Diethylaminoethyl

dG - Deoxyguanine

DHP-lysine - Dihydropyridine-type adduct

DHR - Dihydrorhodamine 123

DMBA - 7,12-dimethylbenzanthracene

DMSO - Dimethylsulphoxide

DPI - Diphenylene iodonium

DTT - Dithiothreitol

Duox1 and 2 - Dual oxidase 1 and 2

DYRK - Dual-specificity, tyrosine-phosphorylated and regulated kinase;

ECL - Enhanced-CL

EGF - Epidermal growth factor

eNOS - Endothelial nitric oxide synthase (eNOS)

EPC - Endothelial progenitor cells

ERK1/2 - Extra-cellular signal regulated protein kinases

FACS - Fluorescence Activated Cell Sorter

FDP-lysine - 3-formyl-3,4-dehydropiperidino adduct

FL - Fluorescence

fMLP - f-met-leu-phe

FOX - Ferrous oxidation in xylenol orange

FPR - Formyl receptor

FPRL1 - Formyl receptor 1

GCSF - Granulocyte colony stimulating factor

GEF - Guanine exchange factors

GFP - Green fluorescent protein

GMCSF - Granulocyte-macrophage granulocyte

GSK3 - glycogen synthase kinase 3

GTP - Guanosine triphosphate

HDL - High-density lipoprotein

HEPES - N-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic acid

4-HNE - 4-Hydroxynonenal

HR1 - Homologous region 1

HR2 - Homologous region 2

HR3 - Homologous region 3

HRP - Horse radish peroxidase

HSP70 - Heat shock protein 70

HSP90 - Heat shock protein 90

HUVECs - Human umbilical vein endothelial cells

ICAM-1 - Intercellular adhesion molecule 1

IL1 - interleukin-1

INFγ - Interferon-gamma

iNOS - Inducible nitric oxide synthase

Ins - Inositol

Ins1P - D-myo-inositol-1-phosphate

IκB - Cytosolic inhibitor of NFκB

JNK, c-Jun N-terminal kinase;

K_{ATP} - Potassium channels

LCK - Lymphocyte kinase;

LDL - Low-density lipoprotein

L-NAME - NG-nitro-L-arginine methylester

LPL - lipoprotein lipase

LPS - Lipopolysaccharide

M_1A - Deoxyadenosine MDA adduct

M_1C - Deoxycytosine MDA adduct

M_1G - Deoxyguanine MDA adduct

MAPK - Mitogen activated protein kinase

MAPKAP-K1 - MAPK-activated protein kinase-1 (or p90RSK2);

MAPKAP-K2 - MAPK-activated protein kinase 2;

MCP-1 - Monocyte chemoattractant protein-1

M-CSF - Macrophage colony stimulating factor

MDA - Malondialdehyde

MKK - MAPK kinase (or MEK)

MKKK - MAPK kinase kinase

MMP - Matrix metalloproteinases

MONICA - Monitoring trends and determinants in cardiovascular disease

MOPS - 4-morpholinepropanesulphonic acid

MPO - Myleoperxidase

MSK1 - Mitogen- and stress-activated protein kinase 1

NAC - N-acetylcysteine

NADPH - Nicotinamide adenine dinucleotide phosphate

Ncf - Neutrophil cytosolic factor

NFκB - nuclear factor κ B

NHE 2 Sodium/hydrogen exhanger

NOX1 - mitogen activated oxidase 1

NOX2 - Flavocytochrome b_{558} or glycoprotein 91

NOX3 - mitogen activated oxidase 3

NOX4 - Renox

OxLDL - Oxidised LDL

8-oxo-G - 8-oxo-deoxyguanosine

P38 - Stress-activated protein kinase 2a (SAPK2a)

P38β2 - Stress-activated protein kinase 2b (SAPK2b)

P38γ - Stress-activated protein kinase 3 (SAPK3)

P38δ - Stress-activated protein kinase 4 (SAPK4)

PAF - Platelet activating factor

PAK - p21-activated protein kinase

PBS - Phosphate buffered saline

PBST - Phosphate-buffered saline with Tween-20

PDGF - Platelet derived growth factor

PDK1 - PtdIns(3,4,5)P_3 dependent kinase 1

PECAM-1 - Platelet endothelial cell adhesion molecule 1

PGG_2 - Prostaglandin endoperoxide

PH domain - Pleckstrin homology domain

PHK - Phosphorylase kinase

PHOX - Phagocyte oxidase

PI - Propidium iodide

PI3K - Phosphaditylinositide-3-kinase

PIF - PDK1-interacting fragment

PIs - Phosphoinositides

PKA - cAMP-dependent protein kinase

PKB - Protein kinase B (or Akt)

PKC - Protein kinase C

PLA2 - Phospholipase A2

PLC - Phospholipase C

PLD - Phospholipase D

PMA - Phorbol 12-myristate-13-acetate

PRAK - p38-regulated/activated kinase

P-Rex1 - PtdIns(3,4,5)P_3-dependent Rac exchanger

PRK2 - PKC-related kinase 2

PS - Phosphatidylserine

PtdIns - Phosphatidylinositol

PtdIns(3)P_3 - Phosphatidylinositol(3) phosphate

PtdIns(3,4)P_2 - Phosphatidylinositol(3,4) phosphate

PtdIns(3,4,5)P_3 - Phosphatidylinositol(3,4,5) phosphate

PtdIns(3,5)P_2 - Phosphatidylinositol(3,5) phosphate

PtdIns(4)P - Phosphatidylinositol(4) phosphate

PtdIns(4,5)P_2 -Phosphatidylinositol(4,5) phosphate

PtdIns(5)P - Phosphatidylinositol(5) phosphate

PTEN - Phosphatase and tensin homolog on chromosome ten

PUFAs - Polyunsaturated fatty acids

PX domain - Phox homology

PYK2 Proline-rich tyrosine kinase

RA – Retinoic acid

ROCK-II - Rho-dependent protein kinase II

RPMI - Roswell Park Memorial Institute

S6K1 - p70 ribosomal protein S6 kinase

SAA - Serum amyliod A

SDF1 - Stromal cell derived factor-1

SDS - Sodium dodecylsulphate

SGK - Serum- and glucocorticoid-induced kinase

SH2 domains - Src homology domain 2

SH3 domains - Src-Homology domain 3

SHIP-1 - SH2-containing inositol-5-phosphatase

SMC - Smooth muscle cell

SOD - Superoxide dismutase

Sos - Son of sevenless

SR-A - Scavenger receptors

TBST - Tris-buffered saline with Tween-20

TEMED - N, N, N',N' –tetramethylethylenediamine

TGFβ - Transforming growth factor β

TNF - Tumour necrosis factor

TnI - Tropomyosoin-binding subunit

TRALI - Recurrent transfusion related acute lung injury

TnT - Troponin T

TXA_2 - Thromboxane A2

VCAM-1 - Vascular endothelial adhesion molecule 1

ViTD – Vitamin D_3

VEGF - Vascular endothelial growth factor

vLDL - Very low density lipoproteins

CHAPTER ONE:

INTRODUCTION

HEART DISEASE AND THE FRENCH PARADOX

1.1 GENERAL OVERVIEW

During the last century the diseases that cause the greatest number of deaths per year in the western world have changed considerably. The heavy use of antibiotics and better public health has massively reduced the death from infectious disease. However, this means we live longer and now chronic diseases (and mostly age-related) top the mortality lists (Cohen 2000). Some historians question if this is a fair swap "Death by infectious disease was quick and public" (Nikiforuk 1993).

Heart disease and Cancer are the greatest causes of death in the developed world. Cardiovascular disease accounts for 50% of deaths in the developed world and 15% in developing countries. However, since 80% of the world's death occurs in developing countries, the total number of deaths from heart disease is approximately equal between developed and developing countries (Betteridge and Morrell 1997). In the UK, 26% of the total death is due to cardiovascular disease (62% of this due to myocardial infarction). In 1994 this cost the NHS £1.42 billion, most of which was spent on medicines and hospital costs, with only 1% of the money used for health promotion. Fifty-three million working days are lost due to cardiovascular disease, £4 million in invalidity benefit and £3 billion in lost production (Betteridge and Morrell 1997).

There is a near perfect correlation between death from cardiovascular disease and serum cholesterol levels of different countries, but there are a few exceptions, namely France and Crete. The Seven Countries Study revealed that the level of cholesterol for Crete did not correlate with the incidence of cardiovascular disease (Keys et al., 1986). The World Health Organisation's MONICA study (**mon**itoring trends and determinants **in** **car**diovascular disease) revealed that Scotland and France have similar mean serum cholesterol and high-density lipoprotein (HDL) levels, and systolic pressure. There are slightly fewer smokers in France, but the greatest differences are in the diets of the two populations. The French consume more vegetables, high amounts of monounsaturated fats and wine. France leads the world in wine consumption and has the highest per capita overall alcohol intake. The question is what component(s) of their diet is responsible for reducing the levels of heart disease?

It has been suggested that ethanol is the active component in protection against cardiovascular disease. However, much attention has focused on polyphenols found in fruit and vegetables. These compounds have been at the heart of herbal medicine for centuries. The Ayruvedic herbal medicine, Darakchasava, used as a heart tonic contains polyphenols (Paul et al., 1999). If the polyphenols are cardioprotective, then how do they produce this protective effect? Plants have an array of antioxidant defence mechanisms, such as the tocopherols, flavonoids and other phenolic compounds, carotenoids and high levels of ascorbic acid (Halliwell 1996). Plants need them to prevent oxidative damage and these compounds could have a similar effect in humans (Halliwell 1996). Much attention has been paid to the involvement of free radicals contributing to the pathogenesis of disease. There is a large body of evidence that suggests oxidation plays a very important role in the pathogenesis of cardiovascular disease. This combined with epidemiological evidence provides a solid basis for the role of dietary antioxidants in the prevention of cardiovascular disease (reviewed by Witztum and Steinberg, 2001).

1.2 ANTIOXIDANTS AND HEART DISEASE

There is good evidence to suggest that oxidative damage plays an important role in the pathogenesis of atherosclerosis and ischaemic-reperfusion injury. Diets high in fruits, vegetables and possibly some alcoholic beverages (such as wine) are correlated with a lower incidence of coronary heart disease. Fruits, teas, grains, vegetables and wines are rich sources of antioxidants and therefore it is suggested that antioxidants should be cardioprotective. Using animal models of atherosclerosis that were given various

antioxidant preparations, e.g. Vitamin E and probucol, has resulted in highly significant results. A meeting in 1991 of the National Heart, Lung and Blood Institute led to the first clinical trials that were carried out with vitamin E, β-carotene and vitamin C. As pointed out by Witztum and Steinberg (2001) at the time there were surprisingly few positive results in animal models. Also, antioxidants such as vitamin E and β-carotene were originally grouped together because it was thought that they shared similar pharmacological properties. Although they both inhibit oxidation, they are mechanistically distinct (see section 1.8.1) and they would have vastly different pharmacokinetics (Witztum and Steinberg 2001).

The majority of antioxidant intervention trials for cardiovascular disease have produced results that are far from conclusive, some showing dramatic cardioprotective effects and others producing negative results. The ATBC trial was initially carried out to detect a decrease in lung cancer among heavy smokers and gave a daily dose of vitamin E and β-carotene (n=29133). This did not produce significant results for cardiovascular disease (ATBC group 1994), but possibly for prostate cancer (Heinonen et al., 1998). The dose of vitamin E was very low and β-carotene has been shown to have no effect on low-density lipoprotein oxidation (LDL). The CHAOS study (n=2002) gave much higher doses of vitamin E and resulted in a 47% reduction in cardiovascular death and non-fatal infarction, although there was no decrease in total cardiovascular mortality (Stephens et al., 1996). The HOPE trial (n=9541) gave combinations of vitamin E and the angiotensin converting enzyme inhibitor (ACE inhibitor) ramipril. The ACE inhibitor gave protection against stroke, non-fatal infarction and death from cardiovascular disease, whereas vitamin E had no effect (Yusuf et al., 2000). The GISSI trial (n=11324) gave vitamin E to patients that had an infraction within three months. They were then followed over three years with no effect on the primary outcome (GISSI 1999). The SPACE trial (n=146) gave vitamin E to patients thought to be at risk from oxidative stress and cardiovascular disease, due to their end-stage renal disease and haemodialysis. This resulted in 46% reduction in cardiovascular events (Boaz et al., 2001). However, this trial was criticised for its small patient numbers and short duration of follow up (Kritharides and Stocker 2002).

There are a number of suggestions to account for the failure of antioxidant therapy in humans, the most obvious being the dose used. Some of the studies have used low doses of vitamin E and some of the studies have not used biomarkers to determine the effect of vitamin E on oxidative stress (Halliwell 2000).

The animal models used for the validation of antioxidant therapy measured the ability of antioxidants to inhibit the development of the disease. The human studies administered vitamin E to patients that are in a different stage of the disease and it could be possible that antioxidant therapy has no use in end stage disease? Most of the studies use vitamin E and it may not be the correct antioxidant for humans, although the oxidative modification hypothesis suggests that vitamin E will prevent the oxidation of low density lipoprotein (LDL) and not cardiovascular disease (Witztum and Steinberg 2001). Other antioxidants may prove to be more effective and since the most impressive epidemiological evidence for the prevention of cardiovascular disease is wine consumption, there is the possibility that compounds found in wine will serve this purpose.

In trying to interpret beneficial observations of epidemiological studies, part of the problem arises from not knowing exactly which dietary components exert a cardioprotective effect or whether it is due to the diet as a whole? If the effect is just due to a few components then what is the mechanism, is it purely due to an antioxidant mechanism or is some other biological activity involved (Halliwell 2000).

1.3 WINE CONSUMPTION AND CORONARY HEART DISEASE

As previously mentioned, despite their high risk factors, Mediterranean countries have the lowest levels of coronary heart disease in the western world. The reasons for the "French Paradox" are not well understood. Is this anomaly due to higher consumption of vegetables, wine or some other factor? The relationship between vegetable foods and coronary heart disease is not as convincing as wine intake. If the calorific intake due to vegetable foods of France, Spain, Italy and Greece is compared, there is no correlation with the mortality rate from coronary heart disease. If the UK, France and Finland are compared there is a correlation between vegetable foods and coronary heart disease (Artaud-Wild et al., 1993). However, the Mediterranean countries all have high daily intake of wine. The two food types that are correlated with coronary heart disease are wine (-) and dairy products (+). Wine drinkers have been shown to have a 30-40% lower risk of cardiovascular death when compared to those who drink other alcoholic beverages (Klatsky et al., 1992).

THE PATHOGENESIS OF ATHEROSCLEROSIS

1.3.1. FATTY STREAKS AND THE DEVELOPMENT OF ATHEROSCLEROSIS

The pathology of atherosclerosis is complex and can take years to develop. There are a number of different theories as to the disease process, which have been amalgamated to form a combined hypothesis of events. Like most of the modern scourges, atherosclerosis has similarities to other diseases such as cancer (atherosclerotic plaques can be viewed as benign tumours) and other inflammatory disorders e.g. asthma and arthritis.

The disease could have its beginnings in early childhood or before birth, through the development of fatty streaks. Lipids (in the form of low density lipoprotein) can infiltrate the subendothelial space and be taken up by macrophages, (to form foam cells). Fatty streaks, of an inflammatory nature, could be sites of atheromatous plaques in later life. These fatty streaks occur at specific arterial sites such as branch points, bifurcations and curvatures, places where there are characteristic alterations in blood flow. The link between fatty streaks and atherosclerosis is a controversial issue (Ross 1998).

1.3.2 THE INITIAL LESION

It is very difficult to determine the precise nature of the initial lesion that results in the progression of atherosclerosis. One of the first steps in the formation of the atheromatous plaque is endothelial dysfunction, which is caused by some kind of damage e.g. high cholesterol and sheer stress, which is the basis for the response to injury hypothesis, first proposed by Ross and Harker (1976). Sheer stress is a typical example and changes in flow can alter the expression of genes that have elements in their promoters that respond to stress e.g. intercellular adhesion molecule 1 (ICAM-1) (Lusis 2000). Most of the traditional disease risk factors can induce some form of dysfunction. The modern term "dysfunction" does not just refer to loss of function due to injury, but decreased bioavailability of nitric oxide (the importance of nitric oxide will be discussed later).

The intimal cell mass theory suggests that there are focal accumulations of smooth mucle cells (SMC) (intimal cell masses) at branch points in certain vessels (particularly the coronary arteries). They could be focal points for lipid accumulation and injury. The reasons and mechanisms mediating lipid accumulation are not clear. However, local accumulations of specific glycosaminoglycans may mediate this process. These intimal cell masses could have a monoclonal origin i.e. they arose from one cell. This idea was generated from the

observation that SMCs in fibrous plaques are monoclonal or arose from very few cells (Schwartz and Murray 1998).

The initial injury to the endothelium stimulates it to produce a number of proinflammatory mediators, such as macrophage colony stimulating factor (M-CSF). The activated endothelium expresses the adhesion molecules E-and P-selectin, ICAM-1 and vascular endothelial adhesion molecule 1 (VCAM-1). Monocytes and T lymphocytes that are activated through the interaction between the selectins and VCAM-1 with their carbohydrate ligands e.g. glyCAM-1, can tether and begin rolling on the endothelial surface (Johnson-Leger et al., 2000).

The interactions mediated though leukocyte L-selectin or P-selectin glycoprotein ligand-1 (PSGL-1), allows rolling and increasing leukocyte recruitment to the site of inflammation (Patel et al., 1995). The slower velocity of rolling allows the leukocytes to favour an encounter with chemokines (see below), which co-operate with the adhesion molecule signals to activate the leukocyte. The leukocyte β2-integrins bind with ICAM-1 and platelet endothelial cell adhesion molecule 1 (PECAM-1) on the endothelial cell surface. The leukocyte can now transmigrate into the sub-endothelial space (Muller and Wang 1992).

Platelets also bind to the dysfunctional endothelium. Unlike leukocytes they bind directly to the site of inflammation or to areas under high shear stress. Once bound they release their granules, which contain cytokines, growth factors e.g. platelet derived growth factor (PDGF), free arachidonic acid and thromboxane A2 (TXA_2) (Lusis 2000).

The injured endothelium has increased permeability to lipoproteins, which is mediated by agents such as angiotensin II, endothelin and PDGF. Seventy percent of plasma lipoproteins are in the form of LDL, which consists of a hydrophobic core surrounded by phospholipids and apoproteins (B_{100}) that stabilise and act as ligands, for the complex (reviewed Betteridge and Morrell 1997). Once in the sub-endothelial space, LDL, can be oxidised (OxLDL) (see below) and taken up by macrophages through scavenger receptors i.e. SR-A and CD36 to form foam cells. This receptor is unregulated and macrophages are therefore able to take up massive amounts of LDL (Suzuki et al., 1997).

1.3.3. FIBROUS PLAQUES

A growing mass of extra-cellular lipid, the presence of SMCs in the intima and SMC-derived extra-cellular matrix e.g. collagen are the characteristics of a fibrous plaque (Lusis 2000). There is a plethora of growth factors released from the platelets, monocytes and the endothelium that are potent mitogens and chemokines for smooth muscle cells e.g. PDGF, angiotensin II, transforming growth factor β (TGFβ) and endothelin (Ross 1998). OxLDL also stimulates SMCs by inducing proliferation and migration to the intima (Adams et al., 2000). This is also mediated by the engagement of CD40-CD154 (CD40 ligand) on T-cells (also found on monocytes, SMCs and endothelial cells) and interferon (IFNγ) (Schonbeck et al., 2000). A discontinous endothelium also induces SMC proliferation and these cells produce a fibrous cap over the endothelium (Dalager-Pedersen et al., 1998).

1.3.4. ADVANCED LESIONS

The fibrous cap of this lesion harbours a necrotic core of lipid-laden cells and the composition of this plaque is the principle factor in determining its fate. A vulnerable plaque (one which could contribute to thrombosis-mediated acute coronary events) generally has a thin fibrous cap and increased numbers of inflammatory cells. T-cells produce interferon-gamma (INFγ) that can inhibit SMC extra-cellular matrix production and stimulate monocytes to produce proteases that would result in a thin fibrous cap. Plaque stability is also determined by the extent of neovascularisation and calcification. The intima can be calcified by pericytes that produce a matrix scaffold which is subsequently calcified. The process of angiogenesis or new blood vessel formation is a characteristic of advanced lesions, and vascularised lesions are easily accessed by inflammatory cells (Lusis, 2000 and Dalager-Pedersen et al., 1998).

1.3.5. ISCHAEMIC HEART DISEASE AND ACUTE CORONARY EVENTS

As previously suggested, atherosclerosis is a slowly developing disease and is also relatively benign. The growth of the lesion is discontinuous and appears to undergo episodic growth spurts, which are triggered by physical disruption and subsequent thrombosis. Stable angina pectoris often results from a coronary artery atheroma that can result in a reduced blood supply to the heart, which results in periods of chronic ischaemia. However, the disease can erupt into a severe life threatening acute myocardial ischemia. This is clinically presented as

acute coronary syndromes of unstable angina pectoris, acute myocardial infarction and sudden coronary death. Various reports have shown that the key event that produces this change arises from the formation of a coronary thrombus superimposed on an atherosclerotic plaque. This leads to occlusion of the artery and sometimes, peripheral embolisation (Dalager-Pedersen et al., 1998).

There are three types of physical disruption to the atheromatous lesion (reviewed by Libby 2002). Superficial erosions occur when small areas of endothelium are lost, possibly by apoptosis, lysis by T-cells, or matrix metalloproteinases (MMPs) stimulated by the presence OxLDL that removes their basement membrane. This results in the exposure of subendothelial collagen and subsequent platelet aggregation. This is a common form of disruption and is often asymptomatic, but accounts for a quarter of fatal coronary thrombosis. Neoangiogensis within the plaque as a result of numerous growth factors released from inflammatory cells e.g. vascular endothelial growth factor (VEGF), results in the formation of microvascular channels, these vessels are prone to haemorrhage. Intra-plague haemorrhaging could result in the growth of the plaque and progression of the disease through the production of thrombin. Thrombin has been shown to trigger the release of growth factors from platelets that could lead to the proliferation and migration of SMCs. The most common form of plaque disruption is fracture of the fiborous cap, although this depends on how thick the cap is. SMCs can produce extra-cellular matrix that stabilises the plaque, if the production of collagen is compromised the internal strength of the plaque is reduced. The inflammatory cells present within the plaque are able to reduce the production of collagen by SMCs through the action of IFN and the overexpression of MMPs (Virmani et al., 2002)

1.3.6. THE ROLE OF THE MACROPHAGE IN THE PATHOGENESIS OF ATHEROSCLEROSIS

It can be seen that the macrophage plays a vital role in the pathogenesis of atherosclerosis and is central in the oxidative modification hypothesis (see below). The importance of this cell type to the disease process has been demonstrated using ApoE-deficient mice and crossing them with animals that lack various receptors involved in macrophage activation (see below) or the cells themselves (Smith et al., 1995). Therefore, macrophage activation is a potential therapeutic target and downgrading their responses with antioxidants might be an effective mechanism to inhibit the progression of chronic inflammatory conditions (Gordon 2003). The role of the macrophage and the signal transduction mechanisms controlling its activation is discussed in the sections below.

Macrophage activation begins with the distribution of monocytes in the blood stream. They are able to enter all tissue compartments in the body. Their exit from the blood stream through the endothelium, interstitium, epithelium and entry into the target tissue is controlled by adhesion molecules. They are able to adapt to their local environment and depending on the tissue type form Kupffer cells (liver), alveolar macrophages (lung) and microglial cells (central nervous system). Monocytes can also remain within the endothelium and become endothelium resident macrophages. Tissue resident macrophages undergo local activation in response to inflammatory and immune responses and enhanced recruitment of monocytes results in the accumulation of tissue macrophages that have an altered phenotype. This altered phenotype is the result of activation, which is classically defined as the antigen non-specific response to a foreign body (innate immune response). There is also the existence of an alternative activation pathway that uses antigen specific immune responses (acquired immune response).

Figure 1.1 summaries the key role the macrophage plays in the progression of atherosclerosis. Once in the subendothelial space, the pattern of differentiation increases the levels of the scavenger receptors (SR-A and CD36), which normally recognise pathogens or apoptotic cells, but also molecular determinants of OxLDL. LDL can be further oxidised by the macrophage by various enzymes, such as NADPH oxidase. This leads to foam cell formation. Very low density lipoproteins (VLDL) are modified by lipoprotein lipase (LPL) and the resulting remnant (cholesterol-ester rich remnants) is oxidatively modified and engulfed by macrophages. T cells in the plaque mediate macrophage activation by the action of IFN, which results in the release of proinflammatory mediators e.g. tumour necrosis factor (TNF), interleukin-1 and 8 (IL1 and 8). This results in further recruitment of monocytes, stimulates the production of MMPs and increases the uptake of OxLDL that promotes cell death and formation of a necrotic core. These processes promote lesion progression and increase the susceptibility for plaque rupture (Li and Glass 2002).

OXIDATIVE STRESS AND ATHEROSCLEROSIS

1.4.1 THE OXIDATIVE MODIFICATION HYPOTHESIS

The oxidative modification hypothesis was put forward by two groups in the late seventies and early eighties. In Cleveland, Chislom and his colleagues showed that LDL could injure cells in culture and that the injury was dependent upon the modification of LDL. Meanwhile Steinberg in California showed that LDL could not induce foam cell formation unless it was modified and the modified LDL was recognised by a scavenger receptor. This group then showed that the modification was due to oxidation. Since then the role of oxidation in atherosclerosis has attracted considerable interest (reviewed by Chisolm and Steinberg 2000).

There is considerable evidence to suggest that the biological activities of OxLDL are pro-atherogenic. The small dense LDL particles are particularly susceptible to oxidation. However, there are defence mechanisms that prevent oxidation e.g. α-tocopherol and coenzyme-Q. When these defences have been consumed, a number of modifications can occur within LDL particles. The covalent modifications that occur on the apoprotein B_{100} prevent LDL from binding to its normal receptors, which results in highly negatively charged particles that are directly toxic to the endothelium (Steinberg 1991).

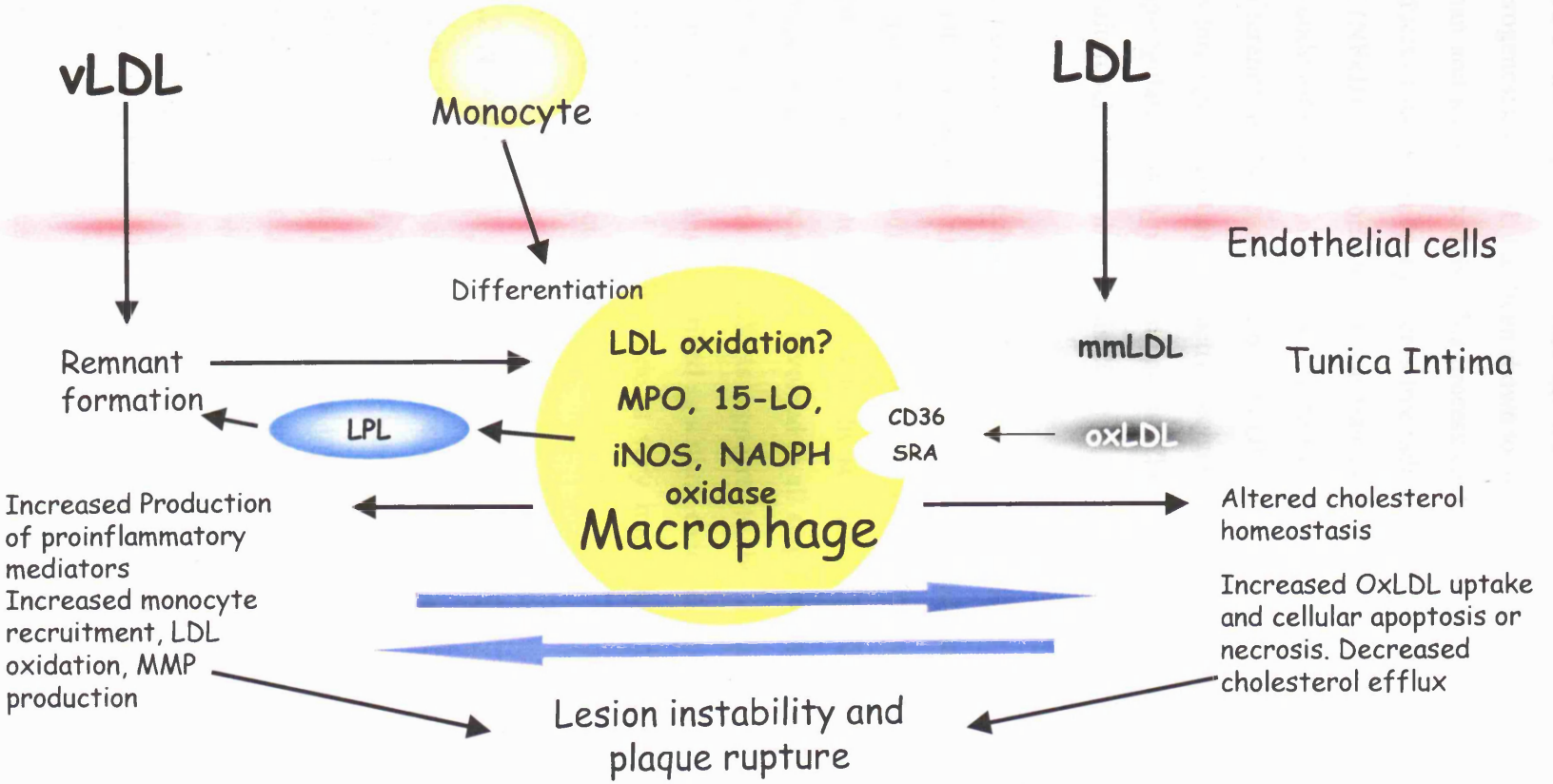

Figure 1.1. The formation of the foam cell. Monocyte recruitment into the artery wall is in response to inflammatory signals. After transmigration of the endothelial layer, locally produced factors induce monocyte differentiation, and up-regulate CD36 and the scavenger receptor A (SRA). Trapped low density lipoprotein (LDL) particles are subjected to oxidation by various enzymes, to form minimally modified LDL (mmLDL) and oxidised LDL (OxLDL). Very low density lipoprotein (vLDL) is modified by lipoprotein lipase (LPL) to cholesterol rich remnants. Oxidised LDL (OxLDL) induces further monocyte recruitment through the release of pro-inflammatory cytokines, this induces the production of matrix metalloproteinases (MMP) and promotes cell death and the formation of a necrotic core. These processes stimulate lesion growth and increase its potential for rupture. Modified from Li and Glass (2002).

OxLDL has a profound effect on the inflammatory process that is apparent during atherogenesis. OxLDL has been shown to increase monocyte adhesion to endothelial cells in human and animal models. This process could be directly mediated by OxLDL, or through products of its oxidation by excess free radicals ($O_2^{\bullet-}$ and OH^-) and subsequent nuclear factor κ B (NFκB) activation and nuclear translocation, which results in the expression of genes that code for chemokines, growth factors and adhesion molecules (Adams et al., 2000). Cell proliferation is also controlled by OxLDL. This species can induce apoptosis in endothelial cells through production of reactive oxygen intermediates. However, it has also been shown to up-regulate growth promoting genes. This leads to proliferation of SMCs and monocytes, and ultimately thickening (Lusis 2000).

The homeostatic mechanisms that control coagulation are also seriously disturbed by OxLDL, through its effect on the endothelium and platelets. OxLDL promotes platelet aggregation and thrombin generation, as well as altering the balance of prostacylin and endothelin to promote aggregation. In summary OxLDL is highly atherogenic and its biological effects appear to be involved in all stages of the disease. The mechanism through which OxLDL exerts its effects appears to be through antagonism of nitric oxide. Atherosclerosis could be described as a nitric oxide deficiency and the relationship between oxidative species and nitric oxide is very important in the pathogenesis of the disease (reviewed by Adams et al., 2000).

1.4.2 NITRIC OXIDE

In 1980 Furchgott and Zawadzki showed that endothelial cells were required for acetylcholine-induced relaxation of rabbit aortic preparations. They also showed that endothelial-dependent relaxation was inhibited by anoxia and under the same conditions restored by agents such as sodium nitrite and glyceryl trinitrite (Furchgott and Zawadzki 1980). The endothelium-dependent relaxing factor that they described was then shown to be nitric oxide (Palmer et al., 1987). Nitric oxide is a very important mediator of vascular tone and local blood flow.

Nitric oxide is produced by endothelial cells by the reaction of L-arginine with oxygen and catalysed by nitric oxide synthase (NOS). Nitric oxide is continuously produced and levels may be further increased by stimuli such as acetylcholine, bradykinin, or shear stress. Nitric oxide increases guanylate cyclase activity that brings about a reduction in intracellular calcium and then smooth muscle relaxation. Reduced bioavailability has been suggested to

be an early characteristic of endothelial dysfunction. Reduced nitric oxide levels can be easily replaced. However, continuous insults result in a permanent loss. Reduced nitric oxide levels results in loss of physiologic dilation and inappropriate constriction (Ludmer et al., 1986). Other activities of nitric oxide also include inhibition of platelet aggregation, inhibiting monocyte adhesion and transmigration into the endothelial space and foam cell formation (reviewed in Lloyd-Jones and Bloch 1996).

OxLDL has been shown to be an all round inhibitor of nitric oxide. Aortic rings from normal animals that are exposed to LDL show reduced endothelial-dependent relaxation, as do aortic rings from the hypercholesterolemic rabbit (Jayakody et al., 1987). LDL can reduce nitric oxide levels by a number of mechanisms. LDL metabolism produces superoxide (Ohara et al., 1993) that can react with nitric oxide to produce the peroxynitrite anion (Beckman et al., 1990), or it can prevent nitric oxide production. OxLDL can decrease DNA transcription of nitric oxide synthase though superoxide production (and subsequent NFκB translocation) (De Caterina et al., 1995) and protein kinase C (PKC) activation (Li and Cathcart, 1994). G-protein coupled receptors are also inhibited, leading to disturbed signal transduction within the endothelial cell. L-arginine uptake is also inhibited by OxLDL. Therefore, it is vital that the endothelium does not suffer a reduction in nitric oxide bioavailability.

1.4.3 REACTIVE OXYGEN SPECIES (ROS)

The bulk of oxygen that we breathe is used in oxidative phosphorylation, some is used for oxidases and very small amounts end up as free radicals. Superoxide is made accidentally (e.g. auto-oxidation reactions) and deliberately for phagocyte killing mechanisms and signalling pathways (Halliwell 1996).

Superoxide is a fairly inert radical, which is quite selective in what it reacts with. It does not react at significant rates with lipids, proteins or DNA. The damaging effect of superoxide is through conversion to other, far more reactive species e.g. hydroxyl and peroxynitrite species.

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2 \quad [1a]$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^- \quad [1b]$$

Superoxide can be converted to hydrogen peroxide through superoxide dismutase [1a] (SOD). Hydrogen peroxide can then form the highly reactive hydroxyl radical through a Fenton reaction (Fenton 1894). Fenton chemistry uses transition metals e.g. iron, copper and chromium as intermediates in the production of hydroxyl radicals (reviewed by Halliwell and Gutteridge 1990). Fenton chemistry and the formation of the hydroxyl radical [1b] has been a long standing contentious issue. The question is whether the radical is formed during the reaction or *in vivo*? (Halliwell 1998).

1.4.4 LIPID PEROXIDATION

Much attention has been paid to determining the role of lipid peroxidation in the pathogenesis of various diseases. As previously suggested, oxidised lipids are extremely atherogenic and they directly antagonise nitric oxide. Therefore, inhibiting lipid peroxidation could be used as a strategy for the prevention of atherosclerosis. There are many products of lipid peroxidation that can be toxic to cells, through modification of proteins, DNA, propagation of lipid peroxidation and the formation of ROS.

Polyunsaturated fatty acids (PUFAs) contain one or more methylene groups that are positioned between *cis* double bonds. These groups are the targets for oxidising agents (such as hydroxyl radicals), which can remove their hydrogen atoms to produce carbon-centred radicals. These radicals react with oxygen to form peroxyl radicals (the initial product of lipid peroxidation).

The next step depends upon the position of the peroxyl radical in the carbon chain of the fatty acid. If the radical appears at the end of one of the double bonds, it is reduced to a hydroperoxide, which is stable in the absence of metal ions. However, metal ion complexes are numerous in cells and they readily reduce hydroperoxides to alkoxyl radicals, which can form an array of other products (Marnett 1999, Dix 1993).

In cellular systems the reduction of hydroperoxides to alkoxyl radicals is carried out by another fatty acid that forms another carbon-centred radical that propagates the reaction. Large numbers of fatty acid molecules can be oxidised by one initiating event. If a molecule of vitamin E reduces the hydroperoxide the reaction is slowed. However, vitamin E can reduce hydroperoxides to alkoxyl radicals in low oxidant conditions (Barclay 1985).

Peroxyl radicals can also occur at internal positions of the carbon chain of fatty acids. Rather than be reduced to a hydroperoxide they form a cyclisation product with an adjacent double bond. This product can couple with oxygen to form a peroxyl radical which is then reduced to a hydroperoxide or another cyclisation reaction can occur to form a bicyclic peroxide. After further coupling to oxygen, a prostaglandin endoperoxide (PGG_2) analogue is produced, which through chemical conversion forms the common precursor for isoprostanes (Awad et al., 1993) and malondialdehyde (MDA) (Pryor and Stanley 1975). All of the hydroperoxide intermediates described above and their stereoisomers and regioisomers can be reduced by metal ion to alkoxyl radicals that decompose to a range of products such as hexanal, 4-hydroxynonenal (4-HNE) and ketones (Janero, 1990).

1.4.5. REACTIVE ALDEHYDES

There are a number of reactive aldehydes that are produced from the oxidation of PUFAs and glycation reactions, which are ideal "toxic second messengers". Compared to free radicals they are stable and can diffuse within the cell and attack targets far from their origin. They are also capable of inducing further oxidative stress, through hydrogen peroxide production or lipid peroxidation. Some of these aldehydes can modulate cellular signal transduction pathways and alter gene expression. Damage caused by these aldehydes has been implicated in the pathogenesis of atherosclerosis and cancer (Uchida 2000).

In most cases MDA is the most abundant individual aldehyde that results from lipid peroxidation (Uchida 2000). In 1983, Basu and Marnett showed that a highly purified preparation of MDA was mutagenic (Basu and Marnett 1983; reviewed in Marnett 1999) and the carcinogenic activity of MDA was determined by a two-year rodent bioassay that showed MDA was carcinogenic in rats, but not mice (Spalding 1988). The basis for the mutagenic properties of MDA is its reaction with nucleic acids. The carbonyl groups of MDA react with N-1 and N-2 of deoxyguanine (dG), with the loss of two water molecules to form pyrimidopurinone (Seto et al., 1983). This is a planar aromatic compound, which is unlike the condensation products formed with deoxyadenosine (dA) and deoxycytosine (dC). Only one carbonyl group of MDA reacts with exocyclic amino groups of dC and dA, which do not form cyclic adducts (Nair et al., 1984).

The monomeric MDA adduct (M_1G) is the major adduct formed, about five times greater than the deoxyadenosine adduct (M_1A), while the deoxycytosine adduct (M_1C) is only

formed in trace amounts. MDA can form dimers and trimers that react with DNA. The dimer reacts with dG to form a cyclo[3:3:1]nonene derivative and trimer only reacts with dA and dC. However, the formation of dimers or trimers at physiological pH is slow and the major adducts are monomeric (Marnett 1999).

The ability of MDA to induce base pair substitutions at dG, dA and dC residues corresponds with its ability to form adducts (Benamira et al., 1995). MDA does not form detectable adducts with deoxythymidine. The repair of M_1G is carried out by nucleotide excision repair, as determined by transforming M_1G-containing genomes in to *E. coli* strains that are deficient in DNA repair (Fink et al., 1997). The occurrence of M_1G in human DNA has been measured by various techniques for a range of different tissues (reviewed in Marnett 1999). The levels of M_1G adducts in normal breast tissue (determined by ^{32}P-post labelling) were 0.08 adducts per 10^7 nucleotides. The levels of M_1G in tissue from breast cancer patients were 0.2 adducts per 10^7 nucleotides (Wang 1995) and increases in M_1G have also been shown in leukocytes of women who were given a high fat diet (sunflower oil) compared to controls (Fang et al., 1996).

MDA can also react with proteins to form stable biomarkers. There are a number of protein adducts formed, especially to lysine residues of proteins or amine head groups of phospholipids. MDA reacts with primary amines to form for example, N-(2-propenal)lysine or the fluorescent dihydropyridine-type adduct (DHP-lysine) (Uchida 2000). The importance of MDA-lysine adducts becomes apparent in atherosclerosis and MDA can modify lysine residues of LDL apoprotein B_{100} producing the atherogenic OxLDL. The use of monoclonal antibodies raised against MDA-lysine showed this species to be present (along with other aldehydes) in atherosclerotic lesions (Uchida et al., 1997).

The lipid peroxidation product acrolein, is the strongest electrophile of the 2-alkenals and reacts with nucleophilic sites of proteins and DNA. This results in chromosome aberrations and point mutations. Acrolein has been described as an ubiquitous environmental pollutant. Acrolein forms a number of protein adducts with histidine and lysine such as the β-propenal adduct and 3-formyl-3,4-dehydropiperidino adduct (FDP-lysine) (Esterbauer et al., 1991).

Of the 4-hydroxyl-2-alkenals, 4-HNE is the most prominent aldehyde formed during lipid peroxidation that can accumulate in membranes at concentrations around 5mM. 4-HNE has a wide range of biological activities, such as growth inhibition, alterations in glutathione

levels, inhibition of enzymes, chemotactic activity towards neutrophils and inhibition of protein synthesis. This aldehyde is a potent alkylating agent and has been shown to be carcinogenic. 4-HNE is potent nucleophile that generally reacts with cysteine, histidine and lysine residues. The primary adducts formed with amino acids through a Michael reaction have a hemiacetal structure. However, the reaction with lysine residues forms both the pyrrole and the fluorescent cross-links. These lysine adducts have recently been identified in human atherosclerotic lesions (Salomon et al., 2000).

1.4.6 OXIDATIVE DNA DAMAGE

Oxygen radicals can induce DNA damage both indirectly i.e. through lipid peroxidation and via direct oxidation of DNA bases. The discovery of background DNA damage that changed with diet, tissue, gender, diurnal cycle and species (Randerath et al., 1990), combined with reports of the production of oxidised DNA bases that corresponded to metabolic rate (Adelman et al., 1988), focused carcinogenic research on endogenous compounds as a major damaging source. However, some of the analytical data produced by HPLC and GC/MS methods came under scrutiny, due to the wide range of values generated for human tissue e.g. for 8-oxo-deoxyguanosine (8-oxo-G) the levels ranged between one adduct in 10^3 and 10^7 nucleotides (Cadet 1999) (reviewed by Marnett 2000).

Oxidised DNA bases are thought to be formed by the generation of hydroxyl radicals, which can add or remove hydrogen atoms. However, the hydroxyl radical is highly reactive and does not diffuse far in the cell before it reacts with other molecules (Pryor 1986). Hydrogen peroxide can freely diffuse through the cell and in the presence of metal irons and can form hydroxyl radicals.

Peroxynitrite is also a highly reactive molecule and is capable of producing a wide range of oxidation products (Burney 1999). Peroxynitrite is more reactive towards 8-oxo-G than unmodified DNA bases, as the levels of 8-oxo-G increase they can compete with unmodified bases (Burney 1999). Although peroxynitrite can diffuse freely within the cell (Radi 1998), it could still react with glutathione and not reach the nucleus. Reduced glutathione levels have been correlated to increased susceptibility to DNA damage (Lenton et al., 1999).

The most extensively studied oxidised base adduct is 8-oxo-G (Marnett 2000), which has been shown to be mutagenic and to cause guanine to thymine transversions (Hussain and Harris 1998). The mutagenic potential of other oxidised bases has also been investigated: 8-

oxo-adenine, thymine glycol, 5-hydroxyuracil and uracil glycol are all mutagenic, the last two being highly so (Wang et al., 1998).

A study by Collins and his colleagues in 1998 showed an interesting correlation between 8-oxo-G and coronary heart disease. They looked at the differences in oxidative DNA damage in men and women from different European countries. By examining 8-oxo-G levels in lymphocytes they showed that in men there was a significant correlation between European countries with high coronary heart disease rates and high levels of 8-oxo-G (Collins et al., 1998). However, the high levels of oxidative DNA damage in men from Northern Europe did not correlate with low mean plasma vitamin C, E, or carotenoid levels. This study used the mean values for population groups, whereas other studies have used individual values of DNA damage and shown negative correlations between 8-oxo-G levels and carotenoid concentrations (Collins et al., 1998).

NADPH OXIDASE AND ATHEROSCLEROSIS

1.5.1 GENERAL OVERVIEW

The first line of defence against invading pathogens is through the respiratory burst and is marked by the rapid consumption of molecular oxygen. This process was first described in 1935 by Baldridge and Gerard. However, the reason for this increase in oxygen was not clear until the early 1960s when Lyer et al., (1961) continued the work of Sbarra and Karnovsky (1959) and showed that hydrogen peroxide was released during the respiratory burst. Babior and his co-workers showed that superoxide was the primary species released from phagocytes (Babior et al., 1973). The importance of NADPH oxidase is demonstrated by chronic granulomatous disease, a rare inherited condition and where NADPH oxidase in these patients is defective. These patients are highly susceptible to infections caused by bacteria and fungi, although treatment with prophylactic antibiotics has increased the life expectancy of these patients from middle childhood to adulthood (Babior 2000).

Much attention has been paid to the significance of oxidants in the pathology of many disorders and raises the question can this enzyme or its regulation by cell signalling pathways become a viable therapeutic target? Described below is the activation process of NADPH oxidase and the role it plays in the pathogenesis of atherosclerosis and related coronary syndromes.

1.5.2 NADPH OXIDASE SUBUNITS

$$2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+ \quad [2]$$

Equation [2] shows the reaction that is catalysed by a group of plasma membrane associated enzymes found in a variety of cells of mesodermal origin. The most thoroughly studied is the leukocyte NADPH oxidase, which is found in professional phagocytes and B cells, and produces superoxide by using NADPH as the electron donor. The core enzyme is composed of five subunits $p40^{PHOX}$, $p47^{PHOX}$, $p67^{PHOX}$, $p22^{PHOX}$ and gp91 (PHOX = **ph**agocyte **ox**idase).

In the resting cell, three of these subunits (p40, p47 and p67) exist as a complex in the cytosol while p22 and gp91 are found in membrane vesicles and specific granules. The subunits p22 and gp91 occur as a heterodimeric flavohaemoprotein known as cytochrome b_{558} (Babior 1999).

In order for activation to take place two low molecular weight guanine nucleotide binding proteins are required, Rac (in neutrophils Rac2 and monocytes Rac1) and Rap1A. Rac can be found in the cytoplasm in complex with Rho GDI (guanine nucleotide dissociation inhibitor) and Rap1A is found with cytochrome b_{558}. The activation of Rac induces the binding of guanosine triphosphate (GTP) and the complex then migrates along with the other cytosolic components to the cell surface. Rac translocation is independent of the other cytosolic subunits (Babior 1999).

1.5.3 NADPH OXIDASE ACTIVATION

MODEL FOR ACTIVATION. When the cell is activated phosphorylation of p47 disrupts the inter-SH3 domains (**S**rc-**H**omology **3**) between p40 and p67. p47 then translocates to the membrane and binds to p22 (p22 phosphorylation may also aid this process). The p47/p22 interaction forms the basis for the p67 binding site and the binding of Rac to gp91/p22 also forms a binding site for p67. The oxidase is now assembled and electrons are transferred from the substrate (NADPH) to molecular oxygen, by its electron carrying prosthetic groups (from flavin to haem groups) (Babior 1999). The activation of this enzyme requires two signals, one to initiate the translocation of p47-p40-p67 and the other to activate Rac. These

two components translocate independently of each other and interact with gp91 and p22 at the membrane (McPhail et al., 1999).

ROLE OF PROTEIN PHOSPHORYLATION. Activation of this enzyme can be discussed in terms of phosphorylation i.e. through activation of signalling pathways or subunit activation or through the organisation of various binding proteins. The activation of this enzyme provides a useful insight into the coordination of various binding proteins.

The most widely studied regulatory point in the activation of NADPH oxidase is phosphorylation. The subunits of the cytosolic trimer (p40, p47 and p67) have all been shown to be phosphorylated on multiple residues. p47 is extensively phosphorylated, on eight or nine serine residues in the terminal quarter of the molecule (S303-V390). Site directed mutagenesis of single serines revealed that S379 was essential for activity. However, it is not clear whether this serine is phosphorylated. Following mutagenesis of serines S303-S304 to alanines in EBV-transformed B cells, NADPH oxidase activity was found to be reduced by 20%. If the serines S359-S370 were changed to alanines, phosphorylation and translocation in whole cells and cell free systems was abolished (Babior 2002).

Several serine kinases have been implicated in the activation of NADPH oxidase. The PKC family of kinases (see section 1.75) has been the most extensively studied. However, there is also a role for protein kinase A and recently a role for Akt has been shown (see section 1.7.4).

p40 is phosphorylated in the resting cell and then gains an additional phosphate on activation. However, it is not so clear if p67 is phosphorylated. There are distinct signal transduction pathways controlling p40 and p47 phosphorylation that will be discussed below (Babior 1999). The role of p40 in the activation of the enzyme is not clear, although it does play a role in binding the lipid products of phosphaditylinositide-3-kinase (PI3K) (Ellson et al., 2001). p22 phosphorylation has been demonstrated in response to f-met-leu-phe (fMLP) and phorbol 12-myristate-13-acetate (PMA), with former being dependent on phospholipase D (PLD) (Regier et al., 2000).

ROLE OF PROTEIN-PROTEIN INTERACTIONS. Cysteine residues in p47 also play a role in its activation. Site directed mutagenesis studies in EBV-transformed B cells revealed that a C196A mutation increased activity and C111A/C378A mutations affected the rate of superoxide production. It has been suggested that this demonstrates the possibility of redox interactions between these cysteines that modulate the activity of the enzyme (Babior 2002).

The cytosolic subunits contain a number of functional domains that are vital for the activation of the oxidase. SH3 domains are the most widespread protein recognition module and they all share a highly conserved fold that is formed by two three-stranded β-sheets. They are able to bind proline rich sequences (Pro-x-x-Pro) independently of post-translational modifications. Eight classes of SH3 domains have been identified according to the similarities of the ligands that they bind (Cesareni et al., 2002). p47 contains tandem SH3 (a and b) domains located near the centre of the molecule and these can interact with an arginine/lysine rich region in the C-terminus of p47 (Huang and Klienberg, 1999). Phosphorylation of p47 disrupts this interaction, exposing the SH3 domain and allows binding to the proline rich domain of p22 (de Mendez et al., 1997). p67 also has tandem SH3 domains, SH3a is near the centre, while SH3b is at the C-terminal and binds to a proline rich sequence in the C-terminal of p47. p67 also contains four tetratricodecapeptide repeats (TPR) each one is a region of 34 degenerate amino acids. This region is required for activation and its disruption eliminates the binding of p67 to Rac, but may also serve as the arachidonic acid binding site (Ponting 1996). Amino acids 199-210 of p67 make up the cytochrome activation domain. Point mutations in this domain eliminate the activating role of p67 but do not affect the interaction with p40-p47 (Han et al., 1998). It is possible that this region of p67 regulates the activation of gp91, although there is no evidence that this region actually binds to gp91. However, it has been suggested that the binding of the other subunits contributes most of the binding energy and therefore the affinity between this domain and gp91 would not have to be high (Lambeth 2000).

The Phox homology (PX) domain is a phosphoinositide binding domain, which has recently been shown to be of great importance to NADPH oxidase regulation. The binding of PX domains in p40 and p47 to phosphatidylinositol (3)-phosphate (PtdIns(3)P_3) and phosphatidylinositol (3,4)-phosphate (PtdIns(3,4)P_2) has recently been shown to be essential for the function of NADPH oxidase. Phosphorylation of p47 could expose its PX domain and target this protein to the membrane through the binding of PtdIns(3,4)P_2 that is formed by the action of the 5-phosphatase, SHIP-1 (SH2-containing inositol-5-phosphatase), on

phosphatidylinositol (3,4,5)-phosphate (PtdIns(3,4,5)P_3). P40 could be activated through the interaction of p40 and PtdIns(3)P that could be formed through the action of a PtdIns(3,4)P_2-4 phosphatase (Ellson et al., 2001) (see figure 1.2). Phosphoinositide binding domains will be further discussed below.

The activation of NADPH oxidase requires interaction with the cytoskeleton, which must be intact (Granfeldt and Dahlgren 2001). Use of cytochalasin B, an inhibitor of actin polymerisation, demonstrated that actin polymerisation was required for sustained oxidase activity. Translocation of p47 and PKC has been shown in response to oxidase activation, not only in membrane preparations but also in detergent insoluble fractions (the cytoskeletal fraction) (Nixon and McPhail 1999). p47 contains a PKC binding site and is able to regulate the activity of the latter by promoting the targeting to coronin (an actin binding protein) (Reeves et al., 1999). The activation of Rac also modulates the re-organisation of the cytoskeleton, through the activation of p21-activated protein kinase (PAK) (Daniels and Bokoch, 1999). Upon stimulation the cytosolic subunits bind to actin filaments, where they (and the cytoskeleton) are phosphorylated by kinases, such as PKC and the subsequent binding of these cytosolic subunits to the membrane-associated subunits is dependent upon de-phosphorylation of the cytoskeleton.

CALCIUM AND ION TRANSPORT. The activation of NADPH oxidase has been shown to be both calcium-dependent and -independent, depending on the agonist used. Physiological stimuli, such as fMLP are calcium-dependent. The fMLP-induced respiratory burst is determined by increases in intra-cellular calcium. Two distinct calcium signals have been identified after stimulation with fMLP. The first is a result of the signalling cascade and is released from intracellular stores. The second is kinetically distinct and results from the opening of calcium channels from the cell surface. Interestingly, if cells are stimulated with calcium-free medium no response is observed and when calcium is added back the response occurs. The second calcium signal does not activate the respiratory burst *per se*, but acts in synergy with the first signal. If the cells are activated by fMLP without extra-cellular calcium, its appears that the cell remains static and waits for the second calcium signal (for at least 15 minutes) (Foyouzi-Youssefi et al., 1997).

Recent studies using CD38 knockout mice, showed that neutrophils from these animals displayed decreased bacterial clearance. CD38 is a transmembrane glycoprotein that can catalyse the production of cyclic ADP-ribose, which can induce the release of intracellular

calcium from ryanodine receptor regulated stores. CD38-/- neutrophils have a defective fMLP response and do not accumulate at the sites of infection. They also showed diminished intra-cellular calcium release and extra-cellular influx. Responses to interleukin-8 were not affected by the CD38 knockout (Partida-Sanchez et al., 2001)

The reaction in equation [2] results in the release of hydrogen ions in the cytoplasm, as does the generation of NADPH (equation [3]) by the hexose monophosphate shunt. NADPH is produced by the oxidation of glucose-6-phosphate that results in the production of hydrogen ions (Jankowski and Grinstein 2002).

$$\text{Glucose-6-phosphate} + 12NADP^{+} \rightarrow 12NADPH + Pi + 12H^{+} + 6CO_2 + 6H_2O \quad [3]$$

Carbon dioxide produced from this reaction can become hydrated and result in the formation of hydrogen carbonate. This can form more hydrogen ions, because carbon dioxide can diffuse through the cell and hydrogen ions will form in both the intra and extra-cellular media. It has been calculated that if the oxidase was active for 5 minutes the intracellular pH would drop by 0.33-0.66 pH units (Grinstein and Furuya 1986).

The acidification of cytosol is not mimicked in the phagosome. Hydrogen ions are used up in the dismutation of superoxide that generates hydrogen peroxide, therefore, the phagosome undergoes alkalinisation. There is a biphasic pH change and a small acidification is preceded by a large alkalinisation. However, the global pH change in the cytoplasm is of net alkalinisation. There are three processes that mediate the alkalinisation: 1) the activity of the sodium/hydrogen exchanger (NHE), 2) the activity of a vacuolar ATPase (Grinstein et al., 1986) and 3) the possibility that NADPH oxidase mediates a proton conductance pathway. It is postulated that the gp91 subunit serves this function, although this remains a contentious issue (Babior 1999).

The phagosome has been reported to undergo alkalinisation followed by a secondary acidification. This initial alkalinisation results from the dismutation of superoxide and the secondary acidification from the activity of the V-ATPase. It is important to note that these observations are found in neutrophils. In the macrophage, the pH of the phagosome is more acidic, the reason for this is not clear. It could be due to the lower NADPH oxidase activity

in macrophages and therefore a reduced conductance of hydrogen ions through the oxidase (Hackham et al., 1997 and Segal et al., 1981).

The role of potassium ions in phagosome pH regulation has been recently demonstrated. In neutrophils the initial alkalinisation is compensated for by the entry of hydrogen ions and potassium ions. The influx of hydrogen ions alone is not sufficient to compensate the pH change because proton compensation of electrons results in a neutral pH. The influx of potassium ions serves to increase the pH and raise the tonicity of the phagosome. The most important function of the potassium ions is to activate the proteases that initiate anti-microbial activity. Another important point raised in this study was the inability of hydrogen peroxide to kill bacteria (used at 100 mM with only 40% cell death) and the ability of myeloperoxidase to inhibit this effect. Hypochlorous acid was originally thought to be anti-microbial. The authors suggest that myeloperoxidase has a protective function in preventing the inactivation of anti-microbial enzymes (Reeves et al., 2002) and it is interesting to note that myeloperoxidase-deficient mice have an accelerated atherosclerosis (Brennan et al., 2001).

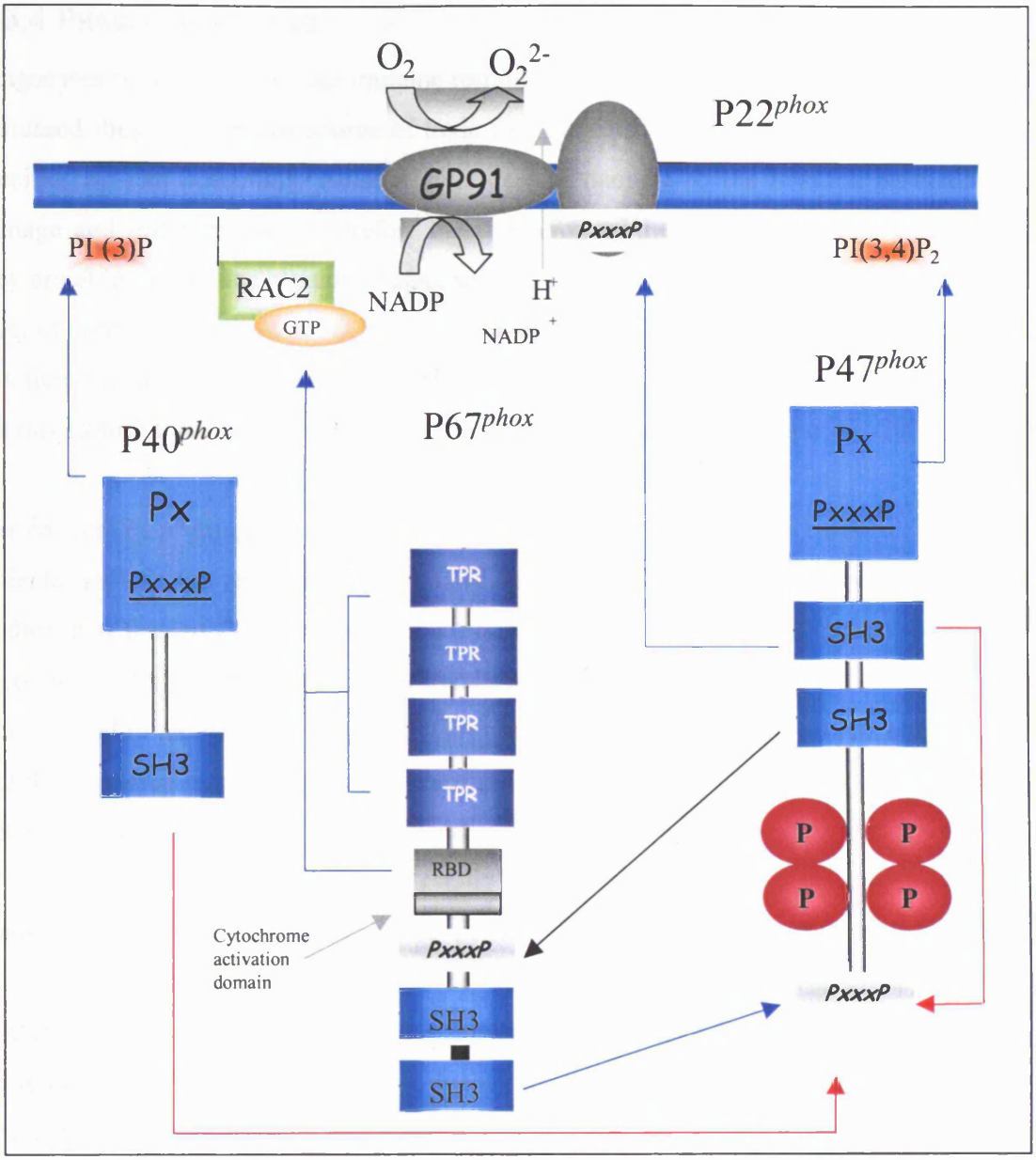

Figure 1.2. Phosphoinositide and SH3:PxxxP interactions in the membrane-targeted activation of NADPH oxidase. Model for the activation of NADPH oxidase (see text for details). PX domain - phox homology domain, SH3 - src homology domain 3, PxxxP - proline rich motif, TPR - tetratricopeptide repeat, P - phosphorylation site and RBD - RAC binding domain. Blue arrows represent interactions that are found in the activated complex and red arrows indicate an inhibitory interaction. Black arrows represent unknown interactions. Taken from Wishart et al., (2001).

1.5.4 PHAGOCYTE PRIMING

Phagocytes make up the innate immune response against invading pathogens. As previously discussed they are a major source of toxic free radicals. Their weapons of choice are non-specific and can potentially cause a great deal of damage to the host, in particular tissue damage and inflammation. Therefore the activation of these cells is tightly controlled and they are able to produce a graded response that is appropriate to the stimulus provided. This level of control is brought about by the action of priming agents. The majority of research in this field has focused on neutrophils. However, monocytes/macrophages are also primed by various stimuli that may also be relevant to several pathologies (Swain et al., 2002).

The concept of priming comes from the observations that neutrophils isolated from infected patients had greater responses to normally sub-stimulatory doses of activator. Subsequent studies then formed the basis for the neutrophil priming theory, in which neutrophils would encounter a stimulus that would not directly activate the cell, but prime it for another event that resulted in an increased response (Guthrie et al., 1984). However, it has been suggested that this two-step hypothesis is an over-simplification and that neutrophils can exist in many states from resting to highly active (Swain et al., 2002).

Phagocyte priming plays a vital role in host defence and in some cases the level of neutrophil priming has been related to the severity of the disease (Wakefield et al., 1993). However, in recent years the focus has turned to the role priming plays in the pathology of many disorders, such as diabetes, hypertension, atherosclerosis, pre-eclampsia, lung injury, recurrent transfusion-related acute lung injury (TRALI) (Win et al., 2001) and sepsis (Strassheim et al., 2002). Sepsis results from bacteria and bacterial products in the blood stream and is a result of the systemic response to infection. It is not due to an inadequate immune response, but rather to an overactive one and excessive inflammation results in damage to the kidneys, liver, lungs and cardiovascular system that leads to multiple organ failure. Inhibition of neutrophil and macrophage priming mechanisms might lead to a reduced response and an improved survival rate (Strassheim et al., 2002).

Neutrophils isolated from patients with type 2 diabetes displayed increased superoxide production in response to PMA, but a reduced response to zymosan particles, which is somewhat paradoxical, but does support the observation that diabetics are at a greater risk from bacterial infections (Shurtz-Swirski et al., 2001). AGE-treated neutrophils have been

shown to display enhanced ROS production in response to chemical or mechanical stimulus (Wong et al., 2002).

Priming is an important point of control for the activation of a macrophage. Under normal conditions the macrophage ROS production is relatively low when compared to neutrophils or eosinophils. Priming is the mechanism by which macrophage ROS production is up-regulated and during the pathogenesis of atherosclerosis M-CSF is thought to prime macrophages (Sakai et al., 2000). Many agents have been shown to prime neutrophils and macrophages, some of which are shown in table 1.1.

Priming agent	*Example*
Cytokines	Interleukin-1,3,6,8, interferon, granulocyte and granulocyte-macrophage colony stimulating factor (GCSF, GMCSF), TNF
Lipid mediators	Platelet activating factor (PAF), PMA, leukotriene B_4
Hormones and growth factors	Brain natriuretic factor (BNP), atrial natriuretic factor (ANP)
Oxidants	Hydrogen peroxide and peroxynitrite

Table 1.1 Some examples of agents known to prime phagocytes (modified from Swain et al., 2002).

The mechanism by which these agents mediate their effects is not well understood. Some of them act by activating signal transduction pathways that alter the phosphorylation status of NADPH oxidase subunits. Granulocyte-macrophage colony stimulating (GM-CSF) factor has been shown to prime neutrophils in a PI3K-dependent fashion that also involved protein kinase A (PKA) and the extra-cellular signal regulated protein kinases (ERK1/2). Priming by GM-CSF resulted in the increased production of PtdIns(3,4,5)P_3 and partial phosphorylation of p47 (Kodama et al.,1999). TNF, PAF and GM-CSF has been shown to prime neutrophils through the sustained accumulation of PtdIns(3,4,5)P_3, which was dependent on the activity of the class 1b PI3K isoform. It can be seen from the examples given in Table 1 that most of the receptors/signalling pathways that the agents activate are linked to PI3K, which makes this molecule an important control point for NADPH oxidase activation. The role of calcium is an integral part of NADPH oxidase activation and its mobilisation has also been suggested to play a role in phagocyte priming.

Monocyte NADPH subunit levels can be increased, through a chronic treatment with lipopolysaccharide (LPS) or IFN. Pre-treated of neutrophils with LPS, increase fMLP-induced ROS production 10-fold and is a result of enhanced assembly and redistribution (not phosphorylation) of NADPH oxidase subunits (DeLeo et al., 1998). In some cases the levels of NADPH oxidase are permanently upregulated. Increased levels of p22 have been shown in EBV-transformed B-lymphoblasts isolated from subjects with hypertension (Pettit et al., 2002). These cells showed increased ROS production in response to arachidonic acid and phorbol esters. Interestingly, EBV-transformed lymphoblasts from patients with pre-eclampsia showed increased ROS production with chemical stimulation (Lee et al., 2003) that was not due to an increase in NADPH oxidase subunits.

The ability of oxidants to act as priming agents has not received a great deal of attention. Peroxynitrite has been shown to prime neutrophils and hydrogen peroxide has been shown to prime macrophages through the phosphorylation of p47. Hydrogen peroxide has also been shown to activate alveolar macrophages and increase superoxide production in response to adenosine diphosphate (ADP) stimulation. Oxidants may represent an important control mechanism for NADPH oxidase. They could function through a feed forward mechanism that might result in ROS-induced ROS production (Giron-Calle and Foreman 2000). If oxidants were the major *in vivo* priming agents then there would be great potential for antioxidants to inhibit priming.

1.5.5 THE ROLE OF NADPH OXIDASE IN ATHEROSCLEROSIS

The balance of factors that leads to a pro-oxidant environment is very important in the development of atherosclerosis. High levels of LDL have been shown to lead to the activation of NADPH oxidase. The binding of LDL to endothelial receptors could activate signal transduction pathways that can activate NADPH oxidase, such as the phospholipase A_2 (PLA_2)-dependent release of arachidonic acid, a direct stimulator of NADPH oxidase. LDL metabolism in the endothelial cell may also lead to the production of arachidonic acid. The production of superoxide can generate a positive feedback mechanism through further activation of PLA_2 and production of arachidonic acid (Dana et al., 1998).

Macrophage oxidation of LDL has been shown to be dependent upon NADPH oxidase activity (Cathcart et al., 1989) and once LDL is oxidised, it can further activate NADPH oxidase. It has also been proposed that endothelial NADPH oxidase plays a role in oxidising LDL as it traverses the endothelial layer of the blood vessel. LDL that is taken up through

endocytosis could come into contact with superoxide and other factors for LDL oxidation e.g. transition metals (Meyer and Schmitt 2000).

Excessive superoxide production by endothelial cells could have other deleterious effects. Superoxide has been implicated as second messenger in cellular signalling pathways. Activation and nuclear translocation of NFκB leads to initiation of the inflammatory response, such as up-regulation of adhesion molecules and monocyte recruitment. The proliferation of SMCs has also been shown to take place in response to superoxide. By reducing nitric oxide bioavailability, superoxide can cause inappropriate smooth muscle constriction (Meyer and Schmitt 2000).

Only 50% of patients that present with atherosclerosis have hyperlipidemia (Ross 1998), and high levels of LDL are not the only activators of NADPH oxidase. Angiotensin II and shear stress have been shown to activate the endothelial oxidase, as have high levels of homocysteine (although this is a contentious issue) (Ross 1998).

Much has been learned about the role NADPH oxidase plays in atherosclerosis through the use of knockout mice. Mice lacking gp91 have been crossed with the ApoE deficient mouse that develops an atherosclerotic-like disorder. Surprisingly the development of the atherosclerotic-like lesions was unaffected (Kirk et al., 2000). However, mice lacking p47 were also crossed with the ApoE-deficient mouse and this resulted in reduced atherosclerotic lesions (Barry-Lane et al., 2001). The suggested reason for this difference is that the gp91 knockouts would still have other gp91 isoforms (such as NOX1) in endothelial cells that could be capable of promoting the disease (see below). The p47 knockout has also been used to demonstrate the role of NADPH oxidase in the development of hypertension and these mice do not display an increased blood pressure in response to angiotensin II infusion (Landmesser et al., 2002). Similar studies in the gp91 knockout would also be useful in determining the role of phagocyte NADPH oxidase in hypertension.

There have been several studies to determine the role of mutations in NADPH oxidase subunits in cardiovascular disorders. The C242T mutation in the p22 subunit was proposed in one study to be associated with a greater risk of cardiovascular disease (CAD) (Cahilly et al., 2000). However, population studies of this polymorphism revealed that Japanese (CAD) subjects did not have this mutation, but it was more prevalent in the control subjects. Similar studies have been carried out with American, European and Australian subjects, which also

produced negative results. It would appear that there is some confusion as to the relationship between these p22 mutations and CAD. The C242T mutation has also been studied in pre-eclamptic patients where it was not related to the outcome (Raijmarkers et al., 2002). The functional consequence of this mutation, is reduced superoxide production and it has been suggested that this may still augment the development of atherosclerosis. It has been suggested that increased superoxide production could augment the natural defence mechanism (Guzik et al., 2000). A similar situation has been identified in the pathogenesis of rheumatoid arthritis (RA). The use of rat models of arthritis has identified a polymorphism in the *ncf1* gene that encodes p47 and was responsible for an increased severity of the disease. Interestingly, this mutation leads to reduced superoxide in neutrophils isolated from these animals. The authors suggest that higher levels of ROS can reduce the numbers of arthritogenic T cells and activators of NADPH oxidase reduced the severity of disease (Olofsson et al., 2003). This may also explain why PKC inhibitors have failed to inhibit the early stages of adjuvant-induced arthritis (Birchall et al., 1994), which is thought to be neutrophil and macrophage driven. This highlights a potential problem of antioxidant therapy for similar conditions.

1.5.6 NON-PHAGOCYTIC NADPH OXIDASE

NADPH oxidase subunits have been found in other cell types, however, the catalytic subunit appears to be genetically and structurally distinct. p47, p22, gp91 (NOX2) and p67 have all been found in human umbilical vein endothelial cells (HUVECs) (Meyer et al., 1999) and SMCs (Patterson et al., 1999). However, gp91 homologues have also been described (reviewed by Van Heerebeek et al., 2002).

NOX1 (or MOX1 as it was originally called), which has 82% homology to gp91, can generate reactive oxygen species in some non-phagocytic cells. It has been shown that over-expression of NOX1 in human fibroblasts leads to the production of superoxide. The proliferation of smooth muscle cells in response to PDGF may also be regulated through NOX1. Patients with chronic granulomatous disease, which resulted from defective gp91, had fibroblasts that were capable of normal superoxide production. It has been suggested that NOX1 may have a role in disorders such as cancer and atherosclerosis (Suh et al., 1999).

NOX3 is expressed in the foetal kidney (and the HepG2 cell line), but little is known about this analogue (Kikuchi et al., 2000). NOX4 (or RENOX) is also expressed in the kidney and

has been found in the renal cortex (in the proximal convoluted tubule). Over-expression of NOX4 in fibroblasts also displayed increased superoxide production. However, in contrast to NOX1-contining cells, these cells showed reduced cell growth (Shiose et al., 2001). NOX5 is also expressed in the kidney, although little is known about this analogue (Van Heerebeek et al., 2002). Duox1 and 2 (dual oxidase 1 and 2) are exclusively expressed in the thyroid gland and are believed to play a role in thyroxine production (De Dekan et al., 2001).

The function of NADPH oxidase in endothelial cells is not completely understood. Since the level of ROS produced from these cells is far less than in phagocytes, it is believed that they are involved in several important processes of vascular functioning. PMA is an established activator of ROS in phagocytes and has been shown to increase ROS production in aortic rings. Cytokines, such as TNF, that usually prime phagocytes, have a more direct effect on endothelial cells by inducing ROS production and increasing p22 levels (De Keulenaer et al., 1998). LPS also induces ROS production in aortic rings and increases p67 levels (Brandes et al., 1999). Angiotensin II is an important mediator in the development of hypertension and also been shown to be an activator of endothelial NADPH oxidase (Zafari et al., 1999). Increased ROS production in endothelial cells results in the depletion of NO and possibly the formation of peroxynitrite. Sheer stress also increases the production of ROS in endothelial cells and together with the other factors may alter the redox balance that contributes to the pathogenesis of hypertension and atherosclerosis (Bouloumie et al., 1997).

1.5.7 MYELOPEROXIDASE

Myleoperoxidase (MPO) is a tetrameric, heavily glycosylated, basic haem protein (150 kDa). The enzyme consists of two identical disulphide linked protomers. Each half contains a protoporphyrin containing a 59-64 kDa heavy subunit and a 14 kDa light subunit. MPO is abundant in neutrophils and monocytes, and accounts for 5 and 2% of their dry weight (Nauseef and Malech 1986).

$$Cl^- + H_2O_2 + H^+ \rightarrow HOCl + H_2O \quad [4]$$

This enzyme is found in primary azurophilic granules of leukocytes and when the respiratory burst is activated the protein is released into the phagosomal compartment and is followed by an extracellular burst of superoxide. The oxidising potential of hydrogen peroxide can be increased by MPO to form a variety of other radical species (reviewed in Podrez et al., 2000). Hydrogen peroxide oxidises the haem group of MPO to form a reactive intermediate

and then in the presence of halides the enzyme is reduced. Chloride ions are at the highest concentrations in the plasma and therefore they are the preferred substrate. The enzyme is restored to a ground state and hypochlorous acid (HOCL) (equation 4) is formed, which is an extremely efficient chlorinating agent. MPO is the only mechanism for producing chlorinated oxidants and products under physiological conditions (reviewed in Podrez et al., 2000).

CELLULAR SIGNALLING PATHWAYS THAT ACTIVATE NADPH OXIDASE

1.6.1 GENERAL OVERVIEW

The signalling mechanisms that activate NADPH oxidase are not well defined, with multiple pathways involved. Activators can be roughly split into two classes, soluble and insoluble. PMA and fMLP are classical soluble stimuli and the mechanisms by which they activate NADPH oxidase will be discussed below.

Over the past 20-25 years the mechanisms of fMLP- and PMA-induced leukocyte activation have been extensively studied and continues to develop into a vast field of research. The classical view of leukocyte signalling developed from the discovery of the importance of PKC in neutrophil activation and the fact that chemoattractant receptors were sensitive to pertussis toxin. The involvement of G-protein coupled receptors became paramount in chemoattractant-induced responses (Bokoch 1995). The study of chemoattractant signalling pathways has developed from the study of bacterial agents to that of endogenous chemoattractant cytokines (the chemokines) and important cell signalling mediators, such as PI3K that is also essential for the activation of NADPH oxidase. Inhibition of these pathways has great therapeutic potential not only for inflammatory conditions, such as atherosclerosis, but also for cancer, through the inhibition of cell growth and metastasis.

Presented below is a description of the major pathways and their components involved in the activation of NADPH oxidase, which attempts to encompass the main mediators that activate this enzyme. The contribution each pathway makes to inflammation and its relevance to atherosclerosis and other coronary syndromes is also discussed.

1.6.2 THE PI3K FAMILY

Phosphatidylinositol (PtdIns) is the basic building block for inositol lipids in eukaryotic cells and is made up of D-myo-inositol-1-phosphate (Ins1P) linked via its phosphate group to diacylglycerol. There are five hydroxyl groups on the inositol head of PtdIns, which can be phosphorylated (to form phosphoinositides or PIs) and these have been found in various combinations. They are membrane bound, where they are the targets for kinases, phosphatases and lipases (Vanhaesebroeck et al., 2001).

Eight PIs have been found in eukaryotic cells. The most abundant inositol lipid in mammalian cells under basal conditions is PtdIns, which is present at 10-20 times higher than the most abundant PIs (PtdIns(4)P and PtdIns(4,5)P_2). The latter are present in roughly equal amounts. Under basal conditions 90-96% of PIs have their inositol head groups phosphorylated once, in the form of PtdIns(4)P or PtdIns(3)P, while PtdIns(5)P only contributes 2-5%. The most abundant PtdIns with a double phoshorylated head group is PtdIns(4,5P)$_2$ and accounts for more than 99%, with PtdIns(3,4)P_2 and PtdIns(3,5)P_2 each making up about 0.2%. The levels of PtdIns(3,4,5)P_3 vary between cell types, although under basal conditions they are comparable to PtdIns(3,4)P_2 and PtdIns(3,5)P_2 (Rameh et al., 1997).

There are a number of pathways that allow PIs to be interconverted through the action of various kinases and phosphatases. PIs and their regulation have attracted considerable attention as therapeutic targets, although the main focus here will be the biological activities of PI3K and how it serves a pivotal role in inflammatory conditions such as atherosclerosis.

PI3K phosphorylates the 3-hydroxyl group of the inositol ring to form the following products: PtdIns(3)P, PtdIns(3,4)P_2, PtdIns(3,4,5)P_3 and PtdIns(3,5)P_2. PtdIns(5)P is formed by a PI 5-kinase called P235. The basal level of 3-phosphoinostides (3-PIs) is very low and only significant levels of PtdIns(3)P can be detected. However, upon receptor stimulation the levels increase rapidly, but remain relatively low when compared to the other PIs. Interestingly, the level of PtdIns(3)P remains constant during stimulation (Vanhaesebroeck et al., 2001).

PI3K was originally purified as a heterodimic complex that consisted of a 110 kDa and an 85kDa subunit, which now corresponds to the 110 kDa catalytic and 85 kDa regulatory subunits. There are currently eight mammalian PI3Ks that are divided into three classes based on sequence homology and substrate specificity (see table 1.2). The class 1 PI3K are the main focus here and will be discussed in detail. All PI3K share a homologous region that

is made up of a catalytic core domain HR1 (homologous region 1), which is linked to HR2 and a C2 domain (HR3) (Stein and Waterfield 2000).

CLASS I KINASES. There are four class 1 kinases that are divided into two sub-groups 1a and 1b, which signal downstream of tyrosine kinases and heterotrimeric G-protein coupled receptors respectively.

Class 1a PI3K heterodimer consists of a p110 kDa catalytic subunit of which there are three isoforms α, β and δ. The regulatory subunit is one of seven adaptor proteins that are generated from alternative splicing of three different genes (p85α, β, p55γ). All of these adaptor proteins contain two SH2 domains (Src homology domain 2), which consist of a modular domain of 100 amino acids that binds phosphorylated tyrosine residues. These domains allow the class 1a isoforms to associate with activated receptor tyrosine kinases or other phosphotyrosine containing proteins. The majority of tyrosine kinase coupled receptors are potent stimulators of class 1a isoforms e.g. PDGF- and insulin-receptors, with the exception of the epidermal growth factor receptor (EGFR), which is thought to be an inconsistent stimulus (Stein and Waterfield 2000). PI3K also has protein kinase activity, although only one substrate has been unequivocally demonstrated. p110α can phosphorylate the serine residue of the inter-SH2 domain region of p85α and β (Dhand et al., 1994), as can p110δ (serine 1039 of the C-teriminus). They are thought to be points of negative regulation (Vanhaesebroeck et al., 1999).

The p110γ subunit has a restricted distribution, only being found in white blood cells and is activated by an interaction through with the G-protein $\beta\gamma$-subunits (Leopoldt et al., 1998). In some cells types, G-protein coupled receptors have been shown to activate class 1a PI 3-kinases. However, the restricted distribution of the p110γ subunit may explain why G-protein coupled receptors do not always activate PI3K. G-protein coupled receptors have been shown to activate class 1a PI3K activity through a tyrosine kinase. However, there appears to be some discrepancy in the activation of these isoforms in response to chemoattractants. Ptasznik et al., (1996) and Coffer and Geijsen et al., (1998) showed that a tyrosine kinase signalling pathway accounts for most of the PtdIns(3,4,5)P_3 produced in response to fMLP, while others report the opposite (Cadwaller et al., 2002). The study by Cadwaller et al., (2002) suggests that different antibodies used in the PI3K assays in these reports may account for some of the discrepancies, although the study by Ptasznik et al.,

(1996) measures total phospholipids. Lectin-induced ROS production in THP1 cells has been shown to be dependent on both class 1a and b isoforms (Matsuo et al., 1996), while immunoglobulin induced PtdIns(3,4,5)P_3 production has also been shown to be dependent on both isoforms. In this case the initial rise in PtdIns(3,4,5)P_3 was due to the class 1b isoform with a secondary phase dependent on the class 1a isoform (Melendez et al., 1998). The p110β subunit of the class 1a PI 3-kinase can be directly activated by the G-protein Gβγ subunits. However, leukocytes from p110γ-null mice do not have G-protein-dependent PI3K activity despite the presence of p110β (Hirsch et al., 2000). The regulation of PI3K by G-protein receptors still requires further research to resolve these discrepancies.

CLASS II PI3K. There is much less known about class II PI3Ks, they have distinct substrate specificity and a C-terminal C2 domain that allows phospholipid binding in a calcium independent manner. Apart from a Ras-binding domain, the N-terminal regions show no homology to any other known protein (Vanhaesebroeck et al., 2001).

The molecular interactions by which extra-cellular stimuli are linked to class II enzymes is not well understood. *In vitro* kinase assays have shown that EGF, monocyte chemoattractant protein-1 (MCP-1) and insulin can all increase the lipid kinase activity of class II enzymes. However, their contribution to increased levels of PtdIns(3,4,5)P_3 in response to these stimuli *in vivo* remains unclear (Turner et al., 1998).

PI 3-kinase type	*Catalytic subunit*	*Regulatory subunit*	*Distribution*	*Lipid product*
Class 1a	P110αβδ	P55-P85	Broad	PtdIns(3,4,5)P_3
Class 1b	P110γ	P101	Mainly leukocytes	PtdIns(3,4,5)P_3
Class 2	PI3KCαβγ	-	Broad	PtdIns(3)P
Class 3	Human VSP34p	P150	Broad	PtdIns(3)P

Table 1.2. The phosphaditylinositol-3-kinase family (PI3K). Taken from Stephens et al., (2002).

PROTEIN TARGETS FOR PHOSPHOINOSITIDES. Numerous proteins have been shown to interact with PI-containing membranes and some of these actions have become recognised protein-lipid interactions. Several PI binding domains have been identified such as the pleckstrin homology domain (PH), the FYVE domain and the PX domain (discussed in 1.6.2).

The FYVE domain was named after the first five proteins in which it was discovered. It is a region of 60-80 amino acids with eight conserved cysteines that form two zinc coordination centres. The FYVE domain can act as a PtdIns(3)P binding module (Vanhaesebroeck et al., 2001).

The PH domain is widely distributed and consists of a seven-stranded β sandwich closed at one end with a C-terminal helix. These binding domains display dramatic electrostatic polarisation, with the C-terminal helix having a negative charge and the β-sheets having a positive charge. The function of these binding domains is to bind PIs or their inositol head groups and serve as regulated membrane anchoring sites. Group 1 mainly binds PtdIns(4,5)P_2 and inositol (Ins)(1,4,5)P_3 e.g. Sos1 and phospholipase (PLCδ). Group 2 binds PtdIns(3,4,5)P_3 and Ins(1,3,4,5)P_3 with high specificity and selectivity e.g. Bruton's tyrosine kinase. Group 3 binds PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, e.g. Akt, while group 4 displays very little PI binding e.g. Lbc proto-oncogene. Proteins with groups 2 or 3 PH-domains would be targets for PI3K (Maffucci and Falasca 2001).

It is through these binding domains that the lipid products of PI3K are able to mediate biochemical signals and there are now numerous downstream targets of PI3K, making it a central mediator for cell growth and apoptosis. The role PI3K plays in the orchestration of the immune response and inflammation is discussed here, with particular reference to leukocyte activation and the role of the Akt kinase.

PI3K INHIBITORS. There are two commonly used PI3K inhibitors, wortmannin and LY294002, both of which are potent inhibitors of all PI3Ks, although wortmannin is considerably less potent at inhibiting the class 1b isoform. Wortmannin is of fungal origin and acts as an irreversible inhibitor of PI3K (Powis et al., 1994) by binding covalently to the ATP binding site and inducing a conformational change, whereas LY294002 (Vlahos et al., 1994) is a competitive inhibitor (Walker et al., 2000). These compounds have no therapeutic use, since wortmannin is very unstable in solution and LY294002 is very insoluble (Stein and Waterfield 2000).

1.6.3 PI3K, CHEMOATTRACTANTS AND ATHEROSCLEROSIS

PI3K is a central biochemical mediator of a number of important phagocyte functions, such as phagocytosis and chemotaxis. As previously described, phosphoinositides play a vital role

in the assembly of NADPH oxidase subunits during its activation. They also play an important role as the main signal transduction mechanism for a number of pro-inflammatory mediators, in particular the family of **chemo**attractant cyto**kine**s (the chemokines) that have received a great deal of attention as new therapeutic targets.

There are currently 40 or more chemokines, which are classified according to the configuration of cysteine residues near the amino terminus and divided into four major families: CC, CXC, C and CX_3C. There are at least 20 or more chemokine receptors. Interleukin 8 (CXCL8) was the first cytokine found to be a chemoattractant and its receptor was cloned in 1991 (Holmes et al., 1991; reviewed by Rossi and Zlotnik 2000). For the previous 30 years, bacterial peptides were the more commonly studied chemoattractants, such as fMLP (via the FRP receptors) and products of the complement system. The fMLP receptor was defined in 1976 (Showell et al., 1976) and FRP-knockout mice display resistance to infection by *Listeria monocytogenes* (Gao et al., 1999). Chemokine and FRP receptors are closely related, as are neuropeptide receptors. It is possible that in terms of evolution the chemokine system goes as far back as *Drosophila*, where a CXCR4-like receptor (or stromal cell derived factor-1 SDF1) occurs (Proudfoot et al., 2000).

As described above, atherosclerosis is a multifactorial inflammatory disorder and chemoattractants play a very important role in the inflammatory response. There are four main functions of chemoattractants: 1) They provide the initial signal for a leukocyte to migrate towards the site of infection or dysfunction. This is achieved through the formation of a chemoattractant gradient; 2) the tethering of leukocytes to the endothelial wall, through intergrin signalling mediated, by the engagement of chemoattractants, 3) as leukocytes move towards the higher concentrations of chemoattractants, degranulation occurs releasing myleoperoxidase, elastases, histamine and other pro-inflammatory mediators and 4) the respiratory burst is also activated and follows the release of toxic radical species.

Some chemokines have been shown to induce angiogenesis, whereas others can inhibit this (angiostasis). Therefore, they might have a role in tumour rejection, through leukocyte recruitment and tumour destruction and by inducing angiostasis (Proudfoot et al., 2000).

The importance of chemotaxis to atherosclerosis has been demonstrated using the ApoE mouse model of atherosclerosis and blocking the function of MCP-1. Anti-MCP-1 gene therapy limits the disease progression in these animals (Inoue et al., 2002). Moreover, bone

marrow from MCP-1 over-expressing mice transplanted into irradiated apoE-deficient mice resulted in accelerated disease. Lipid staining in these animals was increased, the level of oxidised LDL was increased three times and the number of infiltrated macrophages was also increased (Aiello et al., 1999). The major biochemical mediator of MCP-1 signal transduction is PI3K, although inhibition of MCP-1 by a small molecule antagonist is a more attractive pharmaceutical strategy than PI3K inhibition, due to its inherent specificity. However, MCP-1 signal transduction is mediated through the class 1b PI3K isoform (and then the class1a) and has a restricted distribution to leukocytes (Stoyanov et al., 1995). PI3Kγ-deficient mice have been generated and have been shown to have defective neutrophil and macrophages responses e.g. respiratory burst and chemotaxis. There were no differences between the blood levels or differentiation of leukocytes and the cells were still able to respond to PI3K-independent stimuli, such as PMA (Hirsch et al., 2000). Interestingly PI3Kγ-deficient mice have also been shown to have resistance to thrombosis. Platelets from these mice have been shown to display diminished aggregation in response to ADP and thrombin, which was thought to be dependent on Akt signal transduction (Hirsch et al., 2001).

There has been renewed interest in formyl receptors, as chemokine receptors as they orchestrate the inflammatory response. The FPR receptor family is unusual, since it appears that of three known receptors, only the FPR receptor itself binds fMLP at a physiological concentration. FPRL1 is the low affinity receptor and FPRL2 does not actually bind fMLP. Phagocytes are not the only cell type to express these receptors, although the significance and function of FPR expression is not clear. Another feature that makes the FPR receptors, unique among chemoattractant receptors is that they have multiple pro-and anti-inflammatory agonists and antagonists from endogenous and exogenous sources (Le et al., 2002). Use of these agonists has indicated that these receptors might play a role in the pathogenesis of Alzheimers disease (AD) and HIV. In particular the FPRL1 has been shown to be a high affinity receptor for the serum amyloid A (SAA) and amyloid β (Aβ) proteins (Le et al., 2002). The amyloid precursor protein (APP) and Aβ have been shown to be present in atherosclerotic plaques (De Meyer et al., 2002). The FPRL1 receptor mediates Aβ-induced ROS and TNF production in monocytes, which is thought to play a key role in the pathogenesis of AD (Yazawa et al., 2001). During the progression of atherosclerosis it has been suggested that platelets entering the atheromatous plaque can be activated by collagen and release APP, which can then be hydrolysed to Aβ that activates macrophages

via the FRPL1. It is also suggested that platelets can be phagocytosed by macrophages and aid foam cell formation (Tedgui and Mallat 2002).

NADPH OXIDASE AND PI3K. The role of PI3K in the activation of NADPH oxidase (partly described in section 1.5.2) can be through the generation of PtdIns(3)P and its role in binding to PX domains required for oxidase activity. However, PI3K also activates a number of proteins that are involved in the NADPH oxidase activation process, such as Akt (see figure 1.3 and below), the MAPK pathway, PKC, PLD and PLC. The small nucleotide binding protein Rac is also under the control of PI3K (see figure 1.3).

Rac, a member of the Rho family of small GTPases, is essential for the activation of NADPH oxidase, phagocytosis and chemotaxis. Members of this family form a molecular switch between an inactive GDP bound form and an active GTP-bound form. Activation is achieved through the action of guanine exchange factors (GEF), the nature of which is not clear. Welch et al., (2002) recently identified a novel Rac activator, P-Rex1 (**P**tdIns(3,4,5)P_3-dependent **Rac** **ex**changer), which was identified by the observation that **P**tdIns(3,4,5)P_3 stimulates ROS production in neutrophil lysates. It was then discovered that the G-protein $\beta\gamma$-subunits and PtdIns(3,4,5)P_3 act synergistically to stimulate P-Rex1, which was found to be the major GEF in neutrophils. G$\beta\gamma$ and PtdIns(3,4,5)P_3 are thought to bind to different receptors on P-Rex1, possibly through a DEP domain and PH domain. P-Rex1 forms an important control point for the respiratory burst, as it appears to be designed to respond to both the G$\beta\gamma$ and PtdIns(3,4,5)P_3 and ensures that class 1a PI3K isoforms do not activate Rac. The activation of Rac in neutrophils is extremely rapid and occurs within 10 seconds, and P-Rex1 is found with the class 1b PI3K at the level of the membrane which avoids the requirement for translocation (Welch et al., 2002) (reviewed by Weiner 2002).

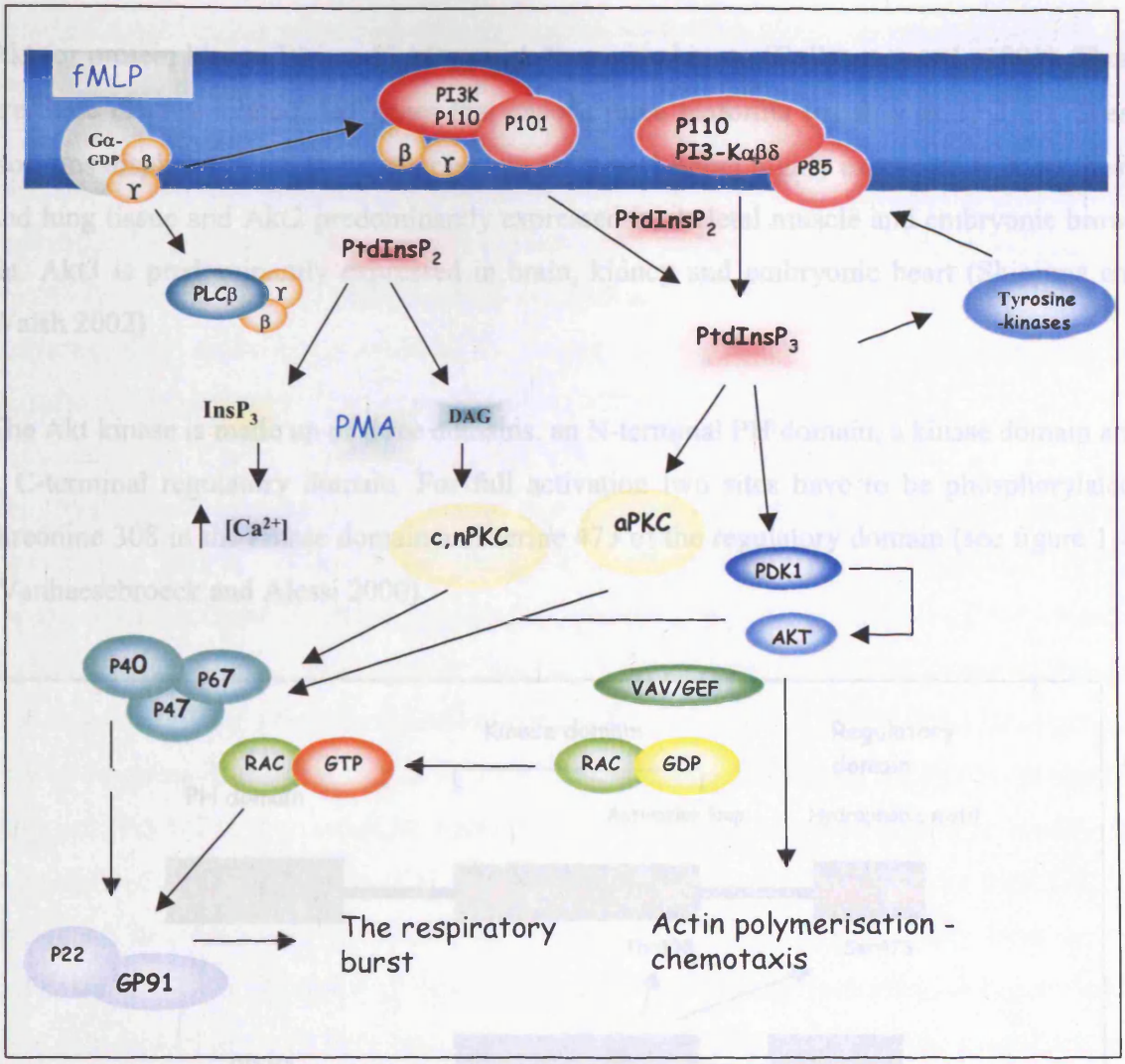

Figure 1.3 An overview of the role of phosphaditylinositide-3-kinase (PI3K) signalling pathways in the activation of NADPH oxidase. PI3K plays a pivotal role in f-met-leu-phe (fMLP)- (and many other chemoattractants and chemokines) stimulated signalling cascades. Upon receptor engagement, the Gβγ G-protein subunits directly activate the class 1b PI3K (that consists of a p110 catalytic and p101 regulatory subunits). The lipid products of PI3K (using PtdIns(4,5)P_2), PtdIns (3,4,5)P_3 and PtdIns(3,4)P_2) can then activate a number of downstream kinases through pleckstrin homology domains (PH) (see text for details). PtdIns(3,4,5)P_3 can also activate tyrosine kinases, e.g. Src, that result in phosphorylation of the p85 regulatory subunit of class 1a PI3K and therefore results in further production of PtdIns(3,4,5)P_3. PI3K also mediates the activation of the small GTP-binding protein Rac, phospholipase C (PLC), Akt and protein kinase C (PKC isoforms). Phorbol 12-myristate-13-acetate (PMA) acts as a diacylglyerol (DAG) analogue and activates classical and novel PKC isoforms. Modified from Wymann et al., (2000) and Rickert et al., (2000).

1.6.4 AKT (PROTEIN KINASE B) AND HEART DISEASE

Akt (or protein kinase B) is a 57 kDa serine/threonine kinase (Bellacosa et al., 1991). There are three closely related Akt genes that encode three isoforms (α, β, γ or 1, 2, 3). These isoforms display a broad tissue distribution, with Akt1 being highly expressed in brain, heart and lung tissue and Akt2 predominantly expressed in skeletal muscle and embryonic brown fat. Akt3 is predominantly expressed in brain, kidney and embryonic heart (Shiojima and Walsh 2002)

The Akt kinase is made up of three domains, an N-terminal PH domain, a kinase domain and a C-terminal regulatory domain. For full activation two sites have to be phosphorylated, threonine 308 in the kinase domain and serine 473 of the regulatory domain (see figure 1.4) (Vanhaesebroeck and Alessi 2000).

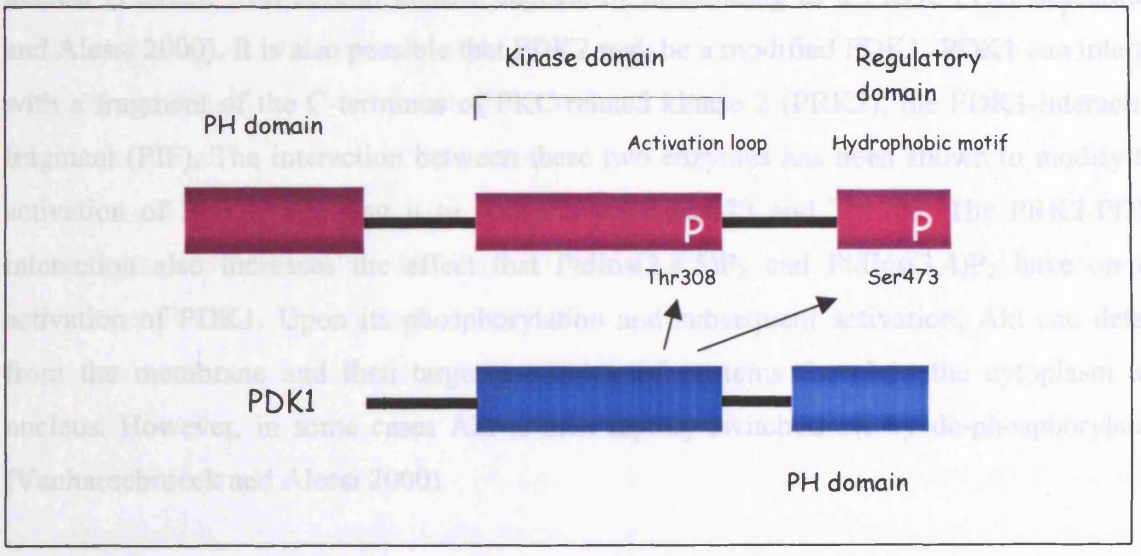

Figure 1.4. The structural layout of Akt and its phosphorylation by PDK1/2. Akt consists of three domains an N-terminal pleckstrin homology domain (PH), a kinase domain and a C-terminal regulatory domain. Two sites must be phosphorylated, Thr308 by PtdIns(3,4,5)P_3 dependent kinase 1 (PDK1) and Ser473 through the action of PtdIns(3,4,5)P_3 dependent kinase 2 (PDK2) (possibly a modified PDK1). Taken from Vanhaesebroeck and Alessi (2000).

Inactive Akt is found in the cytoplasm. The activation of PI3K and subsequent generation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 at the plasma membrane, initiates the translocation of Akt by interacting with its PH domain. Initial studies demonstrated that PtdIns(3)P (Franke et al.,

1995) and PtdIns(3,4,5)P3 (Franke et al., 1997) were responsible for the activation of Akt, although these studies were then found to be incorrect. The focus then turned towards Akt phosphorylation, which occurs on the T-loop or activation loop at Thr308 and in the hydrophobic motif on Ser473 (Alessi et al., 1997). Phosphoinositides have a direct effect on Akt activation, the presence of low level PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 enhances PtdIns(3,4,5)P_3 dependent kinase 1 (PDK1) phosphorylation at Thr308 (Alessi and James et al., 1997). The binding of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 to the PH-domain promotes Akt multimerisation and there is evidence to suggest that Akt forms dimers or trimers that are required for its regulation (Coffer and Jin et al., 1998).

The binding of phosphorylated PIs to the PH domain of Akt results in membrane translocation and a conformational change that allows PDK1 to phosphorylate Thr308. Phoshorylation of Ser473 occurs after Thr308. However, the precise identity of this kinase is unclear (PDK2), with several kinases capable of functioning as a PKD2 (Vanhaesebroeck and Alessi 2000). It is also possible that PDK2 may be a modified PDK1. PDK1 can interact with a fragment of the C-terminus of PKC-related kinase 2 (PRK2), the PDK1-interacting fragment (PIF). The interaction between these two enzymes has been shown to modify the activation of PDK1, allowing it to phosphorylate Ser473 and Thr308. The PRK2-PDK1 interaction also increases the effect that PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 have on the activation of PDK1. Upon its phosphorylation and subsequent activation, Akt can detach from the membrane and then target a number of proteins found in the cytoplasm and nucleus. However, in some cases Akt is then rapidly switched off by de-phosphorylation (Vanhaesebroeck and Alessi 2000).

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The PH domain of Akt is also a site of negative regulation and deletion of this domain leads to a higher basal Akt activity that is still regulated by PI3K (Alessi and James et al., 1997). The main method of Akt regulation is by phosphorylation at these sites, an event which has a relatively short half-life. Phosphatase 2A is thought to be a key enzyme that controls Akt de-phosphorylation (Andjelkovic et al., 1999), although most of the focus has been on PTEN and SHIP as negative regulators of Akt. PTEN can de-phosphorylate Akt at both Ser473 and Thr308 (Li and Yen et al 1997) (Myers et al., 1998). The major function of PTEN is a lipid phosphatase, which de-phosphorylates PtdIns(3,4,5)P_3 at the 3'-position. By reducing PtdIns(3,4,5)P_3, this enzyme diminishes the activity of PI3K (Ogg and Ruvkun 1998). The inositol 5'-phosphatase, SHIP, which converts PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2, has also

been shown to regulate the activity of Akt. Over-expression of SHIP results in reduced Akt activity and SHIP-null cells have prolonged Akt activity upon stimulation (Liu et al., 1998).

The regulation of Akt by PI3K is very well studied, which is not the case for PI3K-independent activation mechanisms, such as local increases in cellular calcium and cAMP or heat shock. Where calcium is involved the action of the calcium/calmodulin-dependent protein kinase (CAMKK) has been suggested (Yano et al., 1998).

In recent years the regulatory properties that Akt displays towards cell growth and death have received considerable attention. Indeed, the downstream targets of Akt are numerous and this makes it an attractive pharmacological target. The minimum sequence motif required for efficient phosphorylation of small peptide substrates by Akt is RXRXXS/T♣ (where X is any amino acid and ♣ is a bulky hydrophobic residue). The biological activities of Akt that have been most extensively studied are concerned with its role in apoptosis, exerting anti-apoptic signals through the phosphorylation of Bad, caspase-9, glycogen synthase 3 (GSK3), cytosolic inhibitor of NFκB (IκB) and forkhead transcription factors. The activities of Akt involve modulation of proteins involved in cell cycle regulation, protein synthesis and glucose metabolism (Vanhaesebroeck and Alessi 2000). However, the role Akt plays in the development of inflammation and cardiovascular disorders has received less attention and pharmacological intervention is already available by way of statin compounds e.g. Atorvastatin (Llevadot et al., 2001).

The role PI3K plays in chemotaxis, phagocytosis and the activation of NADPH oxidase is well established. Akt is a major down-stream mediator of PI3K and therefore is expected to be an important mediator of leukocyte functions. The first evidence for the involvement of Akt in cell movement comes from studies carried out in the slime-mould, *Dictyostelium* (Meili et al., 1999), and then in neutrophils by way of some very impressive research using GFP (green fluorescent protein)-tagged Akt. GFP-Akt was shown to migrate and polarise at the cell membrane (Servant et al., 2000). PAK is a downstream target of Akt that has been shown to be involved in phagocytosis (Chung et al., 2001). The family of small GTP-binding proteins are believed to be the major regulators of actin reorganisation that is required for cell movement and phagocytosis. Rho, Rac and Cdc42 are the most extensively studied and it has been suggested that Akt is a positive regulator of Rac (Genot et al., 2000). Most authors report that Rac and Cdc42 are up-stream regulators of Akt, and some suggest that they are activated in parallel (Welch et al., 1998).

In the pathogenesis of atherosclerosis, the activity of Akt and subsequent pharmacological intervention would depend upon which stage of the disease was targeted. The first stage where Akt would be involved is the attachment of monocytes to the endothelium associated with endothelial dysfunction. The importance of monocyte chemotaxis/adhesion was demonstrated by using MCP1-knockout mice that are resistant to the disease (see section 1.7.3).

Inhibition of Akt would also inhibit the activation of NADPH oxidase and subsequent ROS production. In phagocytes Akt activation would be acute. However, acute and chronic activation in the endothelium and cardiac myocytes would have different consequences. Overexpression of Akt in the U937-GM1 cell line results in the phosphorylation of the NADPH oxidase subunit p47 (Didichenko et al., 1996). Akt has also been shown to directly phosphorylate p47, using an *in vitro* kinase assay (Hoyal et al., 2003), and a specific inhibitor of Akt activation was found to inhibit fMLP-induced ROS production (see Chapter 6).

Growth factors, such as VEGF and many other endothelial stimuli mediate their effects through the PI3K-Akt pathway, promoting endothelial survival. Atherosclerotic plaques make up a highly active environment, which evolves from a simple fatty streak to a complex fibrous plaque. Apoptosis plays an important role in this development. However, the survival of endothelial cells is important for plaque stability. The actions of VEGF through Akt also promote endothelial cell migration and angiogenesis, which would be detrimental to plaque stability (Shiojima and Walsh 2002). The acute effect of Akt activation on the cardiomyocyte is inhibition of apoptosis, which has been shown to dramatically reduce infarction and cardiac dysfunction 24 hours after transient ischaemia. The chronic effect of Akt activation has been demonstrated using mice that have a cardiac specific expression of constitutive Akt, which resulted in cardiac hypertrophy. However, the results suggested that chronic Akt activation induced a wide range of phenotypes from moderate cardiac hypertrophy to massive cardiac dilation and sudden death (Cook et al., 2002; Matsui et al., 2002).

Endothelial nitric oxide synthase (eNOS) is also a major downstream target of Akt and VEGF has been shown to release NO from cultured cells, a process that can be inhibited by LY294002 (Papapetropoulos et al., 1997). Akt increases the activity of eNOS by

phosphorylation of Ser1177, using heat shock protein 90 (Hsp90) as scaffold protein (Fulton et al., 1999). The most intriguing aspect of the pharmacology of statin compounds is the effect they have on eNOS. Statins have been shown to activate Akt, resulting in eNOS activity (Kureishi et al., 2000). Lower doses of statins promote endothelial cell survival and capillary formation, whereas higher doses have been shown to inhibit angiogenesis (Urbich et al., 2002). An increase in endothelial progenitor cells (EPCs) from bone marrow and promotion of angiogenesis has also been shown to be dependent on PI3K-Akt (Dimmeler et al., 2001). The mechanism by which statins activate Akt in endothelial cells is not clear. Moreover, this phenomenon is exclusive to endothelial cells and not observed in smooth muscle cells or cardiac tissue (Kureishi et al., 2000). There have been reports that statin compounds can activate endothelial Ras and promote Akt and subsequent eNOS activation (Urbich et al., 2002).

Akt is a multifunctional protein kinase that plays an important role in inflammation and the development of cardiovascular disorders. However, its activation or inhibition would have different consequences depending upon which cell type or stage of the disease is targeted. Statins have been shown to be effective in primary and secondary prevention of heart disease and they may exert their effects through Akt activation in endothelial cells. They may also indirectly antagonise Akt-dependent activation of NADPH oxidase by inhibiting Rac (Bokoch and Prossnitz 1992). Given the multifunctional nature of Akt, therapeutic strategies involving the modulation of its activity could potentially result in both negative and positive consequences.

1.6.5 PROTEIN KINASE C (PKC)

There are at least 12 PKC isoforms, which comprise of a group of serine/threonine kinases that play a major role in signal transduction in response to a variety of stimuli. PKCs fall into three distinct groups. Conventional PKCs (cPKC) (α, βI, βII and γ) are dependent upon calcium and activated by both phosphatidylserine (PS) and DAG. The second messenger DAG is formed by the action of phospholipase C on PtdnIns(4,5)P_2. Novel PKCs (nPKC) (δ, ϵ, η, θ) are not dependent on calcium, but are still regulated by PS and DAG. Atypical PKC (ζ, ι and λ) isoforms are dependent on PS, but not calcium or DAG (reviewed by Way et al., 2000). The tissue distribution of PKC isoforms varies widely, as does their intracellular distribution in response to stimulation (De Vente et al., 1995).

PKC is a single polypeptide with an N-terminal regulatory region and a C terminal catalytic region. The polypeptide structure of PKC is made up of four conserved regions (C1-C4) and five variable regions (V1-V5). The basis of isoform identity is found within these latter regions. The N-terminal C1 and C2 domains are responsible for membrane targeting and interact with PS, calcium, DAG and phorbol esters. The cPKCs contain all four C-regions, whereas nPKCs have a C2-like domain that does not bind calcium. The PKC C-terminal contains the C3 and C4 regions, which make up the ATP and substrate binding domains. The C4-region is where many commonly used PKC inhibitors act, by competing with ATP for its binding domain (see figure 1.5) (Way et al., 2000).

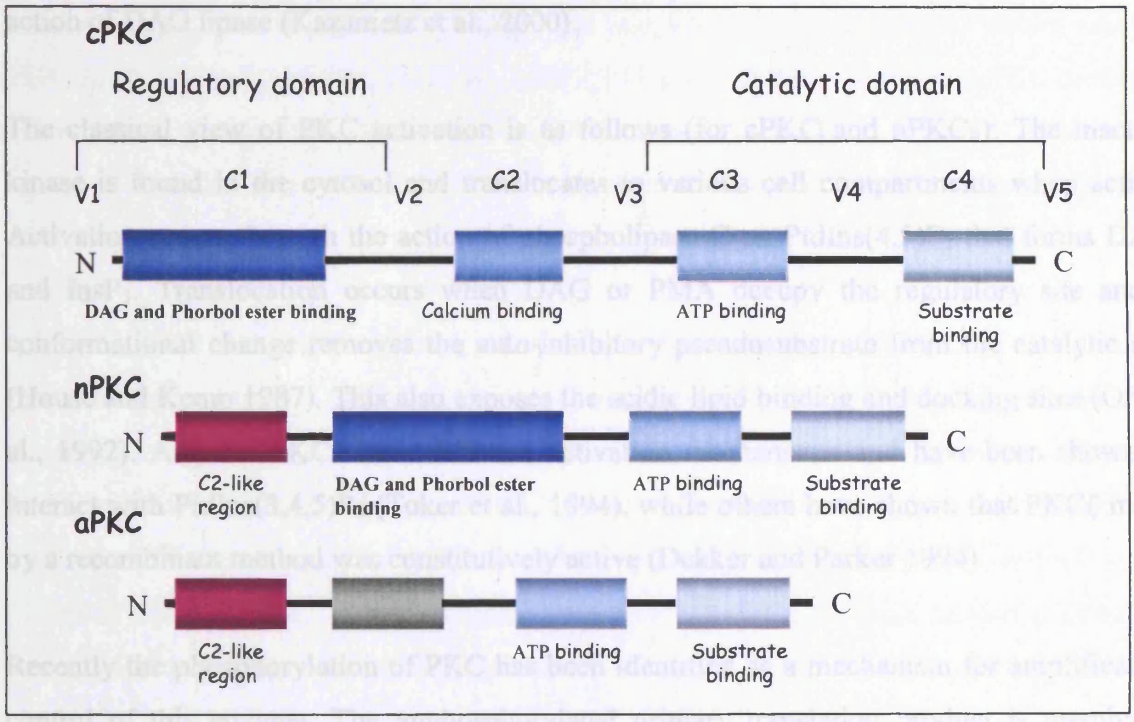

Figure 1.5. The structural organisation of the protein kinase C (PKC) family. The polypeptide structure of PKC consists of 4 conserved regions and 5 variable regions. The C1-C2 domains are responsible for membrane targeting and interacting with diacylglycerol (DAG), phosphatidylserine (PS) and phorbol esters. The C3-C4 domains contain the ATP and substrate binding domains. Classical PKC isoforms (cPKC) contain all 4 domains, whereas the novel PKC isoforms have C2 domain that does not bind calcium. Atypical PKCs do not bind calcium or DAG. Taken from Way et al., (2000).

The mode of activation of PKC depends on which isoform is being activated and the stimulus used. The most common form of PKC activator is the cell permeable phorbol ester, PMA, which activates cPKC and nPKC. Upon stimulation the cellular distribution of these isoforms is dramatically altered (Kiley and Parker 1995). PMA, a plant-derived compound, is an analogue of DAG, although unlike DAG, the effect of PMA is unregulated, whereas the formation of DAG from $PtdInsP_2$ is controlled at multiple levels and in particular by the action of DAG lipase (Kazanietz et al., 2000).

The classical view of PKC activation is as follows (for cPKC and nPKCs): The inactive kinase is found in the cytosol and translocates to various cell compartments when active. Activation occurs through the action of phospholipase C on $PtdIns(4,5)P_2$ that forms DAG and $InsP_3$. Translocation occurs when DAG or PMA occupy the regulatory site and a conformational change removes the auto-inhibitory pseudosubstrate from the catalytic site (House and Kemp 1987). This also exposes the acidic lipid binding and docking sites (Orr et al., 1992). Atypical PKCs have different activation mechanisms and have been shown to interact with $PtdIns(3,4,5)P_3$ (Toker et al., 1994), while others have shown that PKCξ made by a recombinant method was constitutively active (Dekker and Parker 1994).

Recently the phosphorylation of PKC has been identified as a mechanism for amplification control of this enzyme. The unphosphorylated primary translation product is membrane targeted where it becomes the substrate for various kinases acting on the activation loop (T497) and hydrophobic C-terminal sites (S657). Autophosphorylation (T638 for PKCα) renders the kinase in a closed conformation, but in a latent form. In this state the enzyme is stable and protected from phosphatases and can detach from the membrane. The enzyme can be reactivated by DAG, which completes the 3 stage priming hypothesis for PKC activation. All PKC isoforms have been shown to have a phosphorylation site in their activation loops or C-terminal hydrophobic domains. The kinases involved in the phosphorylation of PKC, include components of the PI3K-PDK1 pathway and other members of the PKC superfamily, which also have an important role in the activation of nPKCs (Parekh et al., 1999 and 2000).

PKC also has another point of regulation, since it has some unique structural features that makes it susceptible to oxidation. This results in its activation and therefore some antioxidants are able to negatively regulate PKC. The DAG/phorbol ester binding site of PKC contains zinc-fingers, which each contain 6 cysteine residues. The high concentration cysteine residues makes PKC a target for oxidation, which eliminates the zinc-finger conformation and removes autoinhibition that results in cofactor-independent activation (Gopalakrishna and Jaken 2000).

PMA is a classical activator of NADPH oxidase (DeChatelet et al., 1976). The addition of PMA to phagocytic cells results in a sustained release of superoxide and phagocytosis. The neutrophil p47 subunit of NADPH oxidase has been shown to have phosphorylation sites for PKC α, βII, δ, and ζ (Fontayne et al., 2002). PMA also induces some non-PKC mediated effects such as activation of the small GTP binding protein, Rac, although in general it would appear that most authors use PMA without considering these effects (Kazanietz et al., 2000).

Like Akt, PKC is a multifunctional mediator that has many targets including regulators of calcium homeostasis (Na/Ca exchanger), multiple membrane receptors, G-proteins, mitochondrial proteins, the K_{ATP} channel and nuclear proteins such as inhibitory κ B (IκB). PKC is also a mediator of normal myocardial contraction. The inhibitory subunit of troponin I (TnI), the tropomyosoin-binding subunit, troponin T (TnT) and Na/H antiporter are phosphorylated by PKC. The precise mechanism by which PKC affects cardiac contractility is not completely understood (Cain et al., 1999).

PKC INHIBITORS. Staurosporine was discovered over 20 years ago (Omura et al., 1977) and is now regarded as a general kinase inhibitor. However, it formed the basis for the development of new compounds such as the indolocarbazole and bisindolylmaleimide PKC inhibitors. The addition of a hydroxyl group to the C-7 carbon of the lactam ring produced UCN01 (7-hydroxy-staurosporine) and benzoylation of the amine within the glycoside ring produced PKC412 (Fabbro et al., 2000). While these inhibitors show some selectivity for PKC isoforms, they both inhibit other kinases. UCN01 has been shown to inhibit a wide range of kinases (at 1 μM) (Davies et al., 2000) and to inhibit PDK1 but not PI3K (Sato et al., 2002). PKC412 has been shown to be a potent inhibitor of Akt phosphorylation in Rat1a-fibroblasts (Tenzer et al., 2001), which may aid its use as an anticancer agent but not as a pharmacological tool.

The staurosporine-indolocarbazole structure has also been the means to produce the bisindolylmaleimide PKC inhibitors, and although they are less effective than staurosporine they are much more selective e.g. GF109203X. Table 1.2 shows the IC_{50} values for the PKC inhibitors used in this project.

	PKC isoform								
	α	β	βI	βII	γ	δ	E	ζ	η
GF109203X	8.4	ND	18	ND	ND	210	132	5800	ND
PKC412	24	-	17	32	18	360	4500	$>10^6$	60
UCN01	29	-	34	-	30	590	530	-	ND

Table 1.2 The IC_{50} values (nM) for three commonly used PKC inhibitors, (ND) not determined, (-) no effect (taken from Way et al., 2000)

Other methods to inhibit PKC offer greater selectivity, such as antisense oligonucleotide expression. This method has been employed to demonstrate the importance of PKCζ in the activation of NADPH oxidase in response to fMLP (Dang et al., 2001). Ribozyme technology, by which catalytic RNAs (ribozymes) bind to complementary mRNA and block the encoding protein by cleaving its mRNA, is also a promising method for selective inhibition (Way et al., 2000).

1.6.6 PKC AND HEART DISEASE

As mentioned briefly above, PKC might play a role in normal cardiac functioning, although more attention has been paid to the role it plays in cardiovascular disorders and inflammation. Cancer prevention studies focus on its inhibition because of the role it plays in tumour promotion. However, as described below PKC has a dual role in the development of cardiovascular disorders.

Acute coronary events as a result of decreased myocardial blood flow result in a region of contractile dysfunction. Rapid reperfusion allows the tissue to recover, but in a slow fashion and is referred to as "stunning" (Bolli and Marban 1999). Ischaemia can continue at a reduced level, and in response the myocardium functions at a reduced energy level, known as "hibernation" (Heusch and Schulz 1998). Prolonged ischaemia results in cell death, which

occurs through the reduction in ATP levels, leading to the inhibition of the Na^+/H^+ exchanger. Activation of the Ca^{2+}/Na^+ antiporter results in increased levels of intracellular calcium, which leads to cell death via mitochondrial uptake of calcium and inhibition of mitochondrial ATP production (Rang et al., 1995).

Murry and colleagues (1986) observed that a brief period of ischaemia resulted in protection against prolonged periods of ischaemia. It was also observed that patients had an attack of angina 48 hours prior to myocardial infarction had an improved prognosis. ADP, noradrenaline and calcium have all been shown to induce protection in response to ischaemia and it has been shown that these stimuli act through PKC. The mechanism by which PKC mediates its protective effects is not well understood and it has been suggested that activation of potassium channels (K_{ATP}) is involved (Hu et al., 1996). However, there is great potential for other kinases to be involved.

PKC plays a different role as an inflammatory mediator. LPS-induced TNF production in macrophages is dependent on PKC (Shapira et al., 1997) and TNF receptor signalling has also been shown to translocate PKC isoforms (Wyatt et al., 1997). Other cytokines such as IL-1 and 6 are dependent on PKC signal transduction (Meldrum et al., 1998), as are some chemokines such as IL-8 (Chabot-Fletcher et al., 1994). TNF is a myocardial depressant and can induce preconditioning. It has also been shown to be elevated during ischaemia and heart failure. It is clear that PKC is an important mediator for normal and pathological myocardial functioning (reviewed by Cain et al., 1999).

OxLDL has been shown to activate PKC in macrophages, which results in the release of M-CSF that serves to prime these cells for further stimulation and proliferation. Although macrophages are terminally differentiated and the tissue population is maintained by the influx of monocytes from the blood stream, these cells have been demonstrated to be proliferate in atherosclerotic plaques. OxLDL has been shown to induce this effect through the autocrine action of M-CSF (Sakai et al., 2000).

1.6.7 MITOGEN ACTIVATED PROTEIN KINASE

The MAPK family represents an important mechanism by which cells respond to extra-cellular stimuli. The MAPK signalling cascades are made up of three control levels (that make up a module), a MAPK kinase kinase (MKKK), which activates a MAPK kinase (MKK) which then activates MAPK. To date 14 MKKK, 7 MKK and 12 MAPK have been

identified. MAPK require both tyrosine and threonine phosphorylation to become active. A serine/threonine kinase, MKKK, phosphorylates a dual specificity MKK that phosphorylates a Thr-x-Tyr motif in the activation loop of a MAPK. This is an over-simplified scheme because there is great scope for cross-talk with other pathways.

A wide variety of stimuli have been shown to activate the MAPK signalling pathways. They range from receptor-dependent activation to receptor-independent pathways with agents such as hydrogen peroxide, PMA and mechanical stretch. Tyrosine kinase-linked receptors are shown to activate MAPK cascades. Cytokine and G-protein coupled receptors are also linked to these pathways and on the whole it can be seen that they are ubiquitous cell signalling intermediates (English et al., 1999).

The first three mammalian MAPK to be cloned were ERK1, 2 and 3 (Boulton et al., 1990). They represent some of the most commonly activated protein kinases in signal transduction. They have been widely linked to cell proliferation, but are multifunctional. The next group of MAPK, JNK/SAPK (stress activated protein kinase), are activated by environmental stresses e.g. UV light and cytokines and to a lesser extent growth factors (Hibi et al., 1993). The p38 subgroup was discovered as LPS signal transduction mediators and they are primarily also activated by environmental stresses (Han et al., 1994).

The activation of ERK was first deciphered for growth factor-dependent activation. Activation of receptor tyrosine kinase results in receptor autophosphorylation and subsequent binding of adapter molecules (through phosphotyrosine binding domains, SH2-domains) e.g. Shc and Grb2 that link the receptor to the guanine nucleotide exchange protein, son of sevenless (Sos). Sos activates Ras, through the catalysis of GDP release and the binding of GTP to Ras. Active Ras then binds to Raf, which then starts the first activation sequence of a MAPK module (reviewed in English et al., 1999).

G-protein coupled receptors also activate the MAPK pathways. All G-protein coupled receptors are linked to MAPK pathway, although the activation mechanism varies with cell type and stimulus. G-protein ($\beta\gamma$ subunits)-mediated ERK activation can be dependent or independent of Ras activation. Ras-dependent mechanisms may occur through the activation of non-receptor tyrosine kinases e.g. proline-rich tyrosine kinase (PYK2) and possibly through a PKC-dependent Ras-independent mechanism (reviewed by English et al., 1999). The FPR and chemokine receptors are all linked to MAPK signal transduction. However,

there are differences in the mode of activation and the involvement of PI3K. It has been suggested that there are four possible mechanisms for the activation of ERK by a chemoattractant G-protein receptor. First the $\beta\gamma$ G-protein subunits could activate either the class 1a (with the aid of a tyrosine kinase) or 1b PI3K isoforms, which then activate Ras. Conversely, the $\beta\gamma$ subunits could activate Ras, which then activates either the class 1a or 1b isoforms that then activate Raf. Finally the G-protein $\beta\gamma$ subunits could activate the class 1b isoforms, which then activate MEK or a PKC isoform through their serine kinase activity (Sotsios and Ward 2000).

The MAPK pathway has been extensively studied in neutrophils and has been shown to be vital for the activation of NADPH oxidase through the use of specific inhibitors. ERK, P38 and JNK have been shown to be activated by fMLP, although only the MEK inhibitors have been shown to consistently inhibit ROS production (Dewas et al., 2000). In some cases the p38 inhibitor, SB380530, has also been shown to have an inhibitory effect (Lal et al., 1999 and El Benna et al., 1996). It is unclear exactly how MAPKs activate NADPH oxidase as they could participate in NADPH oxidase subunit phosphorylation directly, or act indirectly via the involvement of other protein kinases. Their activation is required for neutrophil responsiveness. These pathways are important regulators of the inflammatory response and their blockage could inhibit cytokine production and the release of reactive oxygen species (Downey et al., 1998). The role these kinases have in chemotaxis is not well understood. Although they have been shown to be activated in response to many chemoattractants, inhibition of these pathways has been shown to have no effect on chemotaxis (Thelen et al., 1995).

It is often the case that a single stimulus will activate multiple MAPKs and these may then cross-regulate other family members. For example, over-expression of p38 enhances ERK activation in arsenite-stimulated kidney cells (Ludwig et al., 1998). However, in rat fibroblasts transfected with α-adrenoreceptors, this resulted in p38 activation, while basal and stimulated ERK phosphorylation was reduced (Alexandrov et al., 1998). Cross-talk has also been demonstrated with the PI3K pathway, where active Akt inhibits Raf and subsequent ERK phosphorylation. However, this was dependent on the dose of agonist used with lower doses not having this effect (Moelling et al., 2002). LPS signal transduction in monocytes and subsequent ERK, P38 and JNK activation was increased on inhibition of PI3K (Guha and Mackman et al., 2002).

MAPK INHIBITORS. There are several frequently used small molecule kinase inhibitors for the MAPK kinase pathways. SB203580 is an inhibitor of p38 (IC_{50} 48 nM), which displays selectivity for p38α and β. The crystal structure of the inhibitor-enzyme structure revealed that the Thr106 is critical for its inhibitory effect and the members of the ERK, JNK or other p38 kinases have amino acids that are not favourable for inhibition at this position (Eyers et al., 1998). Two MEK1/2 inhibitors are also commonly used, U0126 (IC_{50} 40 nM) and PD98059 (IC_{50} 4 μM). These inhibitors have an unusual inhibitory mechanism, as they do not compete for the ATP binding site, but rather inhibit through an allosteric interaction (Favata et al., 1998 and Alessi et al., 1995).

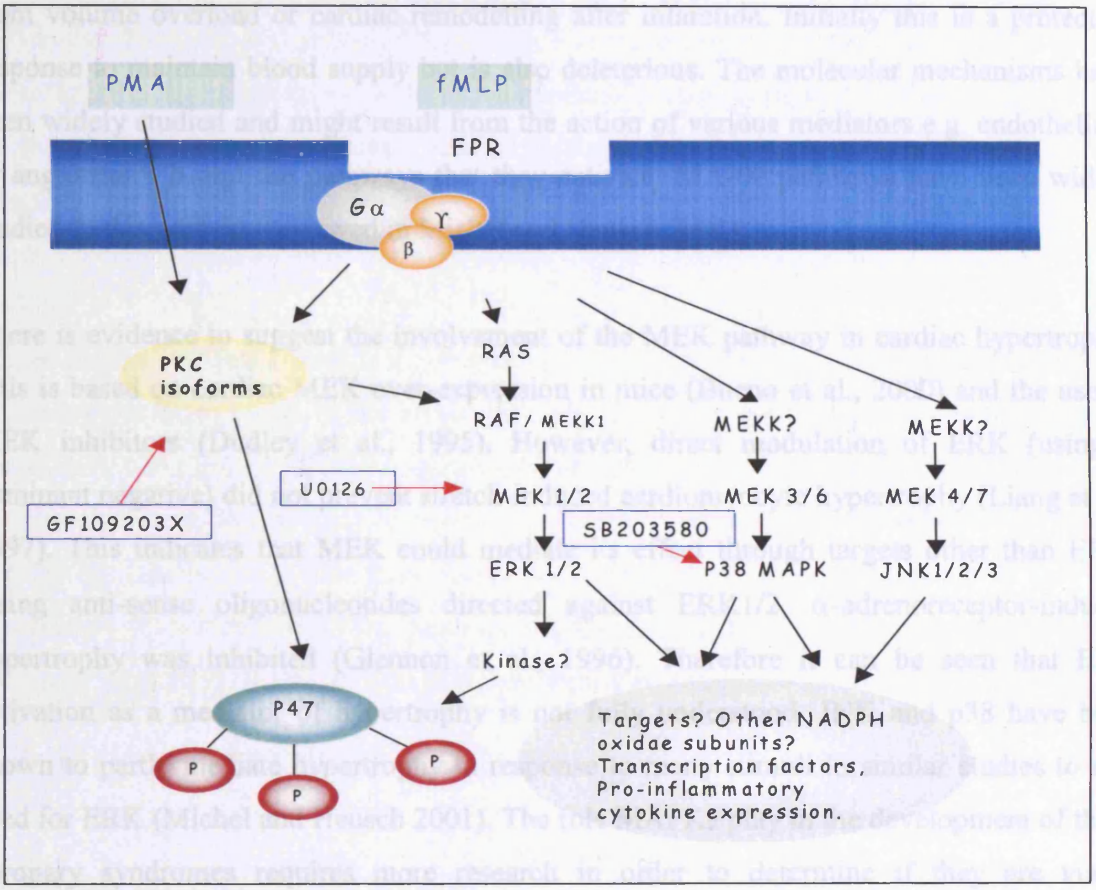

Figure 1.6. Hypothetical scheme for the mitogen activated protein kinase (MAP kinase) pathway during in the phosphorylation of p47 and activation of NADPH oxidase. Phorbol 12-myristate-13-acetate (PMA) can directly activate a number of protein kinase C (PKC) isoforms that lead to the phosphorylation of p47. These PKC isoforms can also activate the Raf/Erk1/2 pathway, although in some cases, this has been shown to be a minor pathway for p47 phosphorylation. f-met-leu-phe (fMLP) activates a PKC and Erk1/2 (and other MAPK pathways). The PKC inhibitor GF109203X has been shown to only partially inhibit fMLP-induced p47 phosphorylation, which suggests that multiple PKC isoforms are involved. P38 and JNK are also activated by fMLP, although in neutrophils these kinases have been shown not effect p47 phosphorylation. Taken from Dewas et al., (2000).

1.6.8 MAPK AND HEART DISEASE

The role these pathways play in ischaemia-reperfusion injury, ischaemic preconditioning and cardiac hypertrophy have been extensively studied. Activation of ERK, JNK and p38 have all been shown in response to prolonged ischaemia and preconditioning, However, there are discrepancies in their function and it is unclear if they are having a positive or negative effect on these situations. At present the MAPK pathway would not be a robust therapeutic target for the treatment of ischaemic heart disease or as a mediator of preconditioning (reviewed in Michel and Heusch 2001).

Cardiac hypertrophy results from increases in the stress on the wall of the heart that results from volume overload or cardiac remodelling after infarction. Initially this is a protective response to maintain blood supply but is also deleterious. The molecular mechanisms have been widely studied and might result from the action of various mediators e.g. endothelin-1 or angiotensin II and the pathways that they activate. MAPK pathways have been widely studied in this context (reviewed in Michel and Heusch 2001),

There is evidence to suggest the involvement of the MEK pathway in cardiac hypertrophy. This is based on cardiac MEK over-expression in mice (Bueno et al., 2000) and the use of MEK inhibitors (Dudley et al., 1995). However, direct modulation of ERK (using a dominant negative) did not prevent stretch-induced cardiomyocyte hypertrophy (Liang et al., 1997). This indicates that MEK could mediate its effect through targets other than ERK. Using anti-sense oligonucleotides directed against ERK1/2, α-adrenoreceptor-induced hypertrophy was inhibited (Glennon et al., 1996). Therefore it can be seen that ERK activation as a mediator of hypertrophy is not fully understood. JNK and p38 have been shown to partly mediate hypertrophy in response to many stimuli in similar studies to that used for ERK (Michel and Heusch 2001). The role MAPKs play in the development of these coronary syndromes requires more research in order to determine if they are viable therapeutic targets.

THE PHARMACOLOGICAL PROFILE OF RESVERATROL

1.7.1 ANTIOXIDANT PROPERTIES

Grapes contain a wide range of polyphenols, some of which are produced by plants in response to infection. There is a wide range of phenols in wine in the form of flavonoids (compounds with two phenols joined by an oxygen-containing carbon ring structure) and non-flavonoids. Most of the attention has been paid to the non-flavonoid stilbenes, which make up the bulk of anti-fungal phytoalexins that are only synthesised in response to injury. Resveratrol (3,4,5-trihydroxystilbene, see figure 1.7) was identified in 1977 from grapevines by Langcake and Pryce, although its presence in wine was not reported until 1992. It was then suggested that resveratrol could be a biologically active component of wine, after which its properties have been extensively studied. Bertelli and his colleagues (Bertelli et al., 1998) suggested that the average wine drinker can, in the long term, absorb enough resveratrol to explain the beneficial effect to human health.

The occurrence of resveratrol is much greater in red wine than white, the Merlot and Pinot Noir varieties being some of the richest sources (up to 5 mg/L). There are two isomers of resveratrol, trans and cis, which occur in a 60:40 ratio. Due to commercial availability more attention has been paid to trans-resveratrol, although cis-resveratrol can be obtained through ultra-violet irradiation of the trans form (N.B. from now on resveratrol refers to trans-resveratrol) (Fremont 2000). The pharmacological actions of resveratrol are diverse and it has many properties that make it an ideal cardioprotective agent.

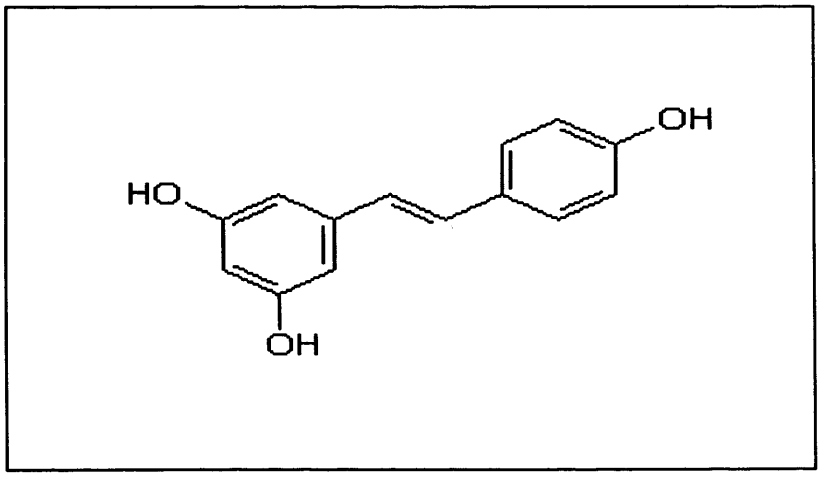

Figure 1.7 The structure of 3,4',5-Trihydroxy-trans-stilbene (resveratrol)

1.7.2 ANTIOXIDANT PROPERTIES.

Resveratrol has been shown to be, in some cases, a potent antioxidant that can inhibit both iron-dependent and -independent lipid peroxidation with IC_{50} values of 4.8 and 3.9 μM and the antioxidant effect was largely due to its free radical scavenging ability. However, the superoxide radical scavenging ability of resveratrol was only 6% that of trolox (a vitamin E analogue used as the standard scavenger in spin-trapping). It has been suggested that the inhibitory effect on lipid peroxidation was due to its peroxyl radical scavenging ability (Miura et al., 2000). The superoxide scavenging ability of flavanoids and other polyphenolic compounds can be quantified according to the bond dissociation energy of their hydroxyl groups and this determined that resveratrol is not a potent superoxide scavenger (Wright et al., 2001).

Resveratrol has been shown to prevent copper-induced peroxidation of LDL (Zou et al., 1999) and therefore has a potent ability to chelate copper (Belguendouz et al., 1997). It has also been shown to have a pro-oxidant effect on DNA through an interaction with ADP-Fe^{3+} and hydrogen peroxide (Miura et al., 2000). The antioxidant properties of resveratrol have been demonstrated in isolated rat heart models of ischaemia-reperfusion injury. Resveratrol (at 10 μM) was found to prevent MDA production, increase aortic flow and reduce the size of the subsequent myocardial infarction. The cardioprotective effect of resveratrol in this case could be mediated through increased production of nitric oxide and by its antioxidant capabilities (Hang et al., 2000).

1.7.3 INHIBITION OF PKC.

A number of protein kinases have been shown to be inhibited by resveratrol and one of the most extensively studied is protein kinase C (PKC), although the mechanism of inhibition is not clear. Resveratrol has been shown to inhibit PKC translocation at 15 μM (Subbaramaiah et al., 1998), However, results from PKC kinase assays show that the dose required is much greater (100 μM). High doses of resveratrol have been shown to inhibit all PKC isoforms (approximately 50%), which raises the possibility that resveratrol is an indirect inhibitor of this enzyme (Stewart et al., 1999). However, it has recently been shown that resveratrol inhibits PKC by binding to the DAG binding site (C1) and is independent of the concentration of calcium or PS (Slater et al., 2003). The effect of resveratrol on basal PKC activity induced by calcium and PS is very weak. Stewart et al., (1999) have suggested that membrane associated PKC activity is inhibited more effectively due to competition for DAG binding sites. However, PKC is also modulated by ROS and therefore, in some cases

resveratrol could be modulating PKC through an antioxidant mechanism e.g. in response to UV light (Yu et al., 2001). There are several other key enzymes that resveratrol is thought to modulate, PLD (Tou and Urbizo, 2001), protein kinase D (Stewart et al., 2000) and PI3K (Haider et al., 2002) have been suggested. However, evidence for direct inhibition of PI3K and PLD is currently lacking. Its ability to inhibit kinases and oxidation overlap, since many cell signalling pathways have been shown to be positively regulated by ROS. Thus antioxidants, such as resveratrol, could be potent indirect regulators of cell signalling pathways. Several important leukocyte functions that could be linked to atherosclerosis such as production of ROS, expression of adhesion molecules and inhibition of chemoattractant signalling events have been shown to be inhibited by resveratrol, although the mechanisms are unclear (Rotondo et al., 1998).

1.7.4 INHIBITION OF NFκB.

This pleiotropic transcription factor is an important mediator of the cell stress response, inflammation, cell proliferation and death. Resveratrol has been shown to inhibit LPS-induced expression of iNOS by suppressing the inactivation of the NFκB suppressor Iκ B. Resveratrol has been shown to inhibit NFκB activation in response to various cell stresses e.g. hydrogen peroxide, LPS, TNF, and PMA, demonstrated by Manna et al., (2000). However, their results should be viewed with caution because they claim that resveratrol is highly water soluble and all their experiments use resveratrol dissolved in water. This statement is incorrect because resveratrol is not soluble at the concentration at which their stock solution is made (1.14 mg/mL in Manna et al., (2000); resveratrol solubility 3 mg/100 mL with warming! Sigma-Aldrich product information).

1.7.5 INHIBITION OF CYLCO-OXYGENASES.

Prostanoids are formed from arachidonic acid and have been shown to be involved in a wide range of physiological processes, such as inflammation and also the promotion of tumour growth e.g. they promote tumour proliferation, angiogenesis, and suppress immune surveillance. Arachidonic acid is formed by the action of various phospholipases and is then metabolised by several pathways through the action of cylco-oxygenases (COX), to form numerous prostanoids (Rang et al., 1995). Resveratrol has been shown to inhibit the constitutive form of COX (COX1), while having no direct effect on the inducible form COX2 (Jang et al., 1997). However, in some cases resveratrol has been shown to antagonise COX2 activity through modulating its expression (Subbaramaiah et al., 1998).

1.7.6 MODULATION OF NOS.

NOS has also been suggested to be a target for resveratrol and expression of the inducible form of NOS (iNOS) has been shown to be down-regulated by resveratrol during classical macrophage activation. Conversely, chronic treatment with resveratrol of endothelial cells has been shown to result in an increase in the expression of endothelial NOS (eNOS). Resveratrol has also been shown to have a vaso-relaxing activity. Chen and Pace-Asciak (1996) showed that resveratrol could produce a nitric oxide mediated relaxation of the pre-conditioned (endothelium intact) rat aorta, in addition to a nitric oxide independent relaxation of denuded aorta.

1.7.7 INHIBITION OF PLATELET AGGREGATION.

Platelets are very important in the pathogenesis of atherosclerosis. Resveratrol has been shown *in vitro* to inhibit thrombin- and collagen-induced platelet aggregation, as well as platelet MDA production (a marker for the production of the vaso-constricting thromboxane A2) (Olas et al., 1999). Platelet aggregation is one of the few biological activities of resveratrol where the cis isomer is more active (Bertelli 1996). The mechanism by which resveratrol can inhibit platelet aggregation is currently unknown.

1.7.8 THE EFFECT OF RESVERATROL ON CELL GROWTH.

One of the most potent effects of resveratrol is on the enzyme, ribonucleotide reductase (Fontecave et al., 1998). However, its efficacy is related to its ability to scavenge tyrosyl radicals. In addition resveratrol has also been shown to inhibit DNA polymerase (Stivala et al., 2001).

It has been suggested that resveratrol displays some selectivity towards cells that are tumourigenic and in some cases has been shown to induce differentiation. In general the direct effect of resveratrol on cell growth is cell specific and in some cases can be anti-apoptotic e.g. preventing hydrogen peroxide-induced apoptosis. Resveratrol has been shown to induce differentiation in leukaemia cells and it has been suggested that it could be a candidate for *ex vivo* bone purging (Tsan et al., 2002).

1.7.9 THE EFFECT OF RESVERATROL ON CARCINOGENESIS.

Resveratrol has been shown to inhibit tumour formation in a mouse skin cancer model, which is induced by 7,12-dimethylbenzanthracene (DMBA) and uses PMA as a tumour promoter. This effect could be due to its ability to antagonise PKC or its ability to bind to the aryl hydrocarbon receptor and compete with DMBA. Treatment of rats with the kidney specific carcinogen $KBrO_3$ resulted in DNA damage that was inhibited by resveratrol (Cadenas and Barja 1999). The transcriptional regulation and activity of the cytochrome P450, CYP1A1, induced by the aryl hydrocarbon receptor activation has also been shown to be regulated by resveratrol (Ciolino et al., 1998).

PROJECT AIMS:

1. Using differentiated U937 cells (dU937) as the primary model in order to investigate a) the nature of the monocyte respiratory burst and b) and the ability of various priming agents to augment the respiratory burst.

2. To assess the ability of resveratrol to inhibit the production of ROS in dU937 cells. This is to be investigated using several different ROS measuring principles.

3. To investigate the effect that activation of the respiratory burst has on cell viability or oxidative stress status, such as lipid peroxidation and DNA damage. Determine the nature of any cellular insult and the effect of resveratrol.

4. Investigate the mechanism by which the respiratory burst is activated using various kinase inhibitors.

5. Investigate the ability of resveratrol to inhibit cell signalling pathways that are in involved in the activation of NADPH oxidase.

CHAPTER TWO:

MATERIALS AND METHODS

2.1 CHEMICALS

Unless otherwise stated all general chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK).

2.2 STOCK SOLUTIONS AND BUFFERS

ABTS ASSAY SOLUTION

2,2¢-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was prepared fresh for each experiment as a 100 mM solution in 100 mM sodium phosphate buffer (pH 7.4).

ACRYLAMIDE

Purchased as a 30% solution (Amersham Biosciences, UK).

AMMONIUM PERSULPHATE (APS)

10% (100 mg/mL) solution in water.

ANNEXIN BUFFER

10 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid, free acid (HEPES), pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$.

CELL LYSIS BUFFER 1 (C1 BUFFER)

1.28M sucrose, 40 mM Tris, 20 mM magnesium chloride and 4% Triton-X-100 at pH 7.5.

CELL LYSIS BUFFER 2 (C2 BUFFER).

137 mM sodium chloride, 2.7 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, 10% glycerol 1 mM EDTA and EGTA, 20 mM Tris, pH 8 at 4°C (see gel buffer). Before use 1% NP-40, 10 mM sodium fluoride, protease inhibitor cocktail (100x dilution) and 0.5 mM sodium orthovanadate were added (Hawkins et al., 1992).

EDTA AND EGTA SOLUTIONS.

A 250 mM solution was prepared in ultra-pure grade water, pH 3-3.5. Since EDTA is more soluble in the salt form, the suspension was dissolved by the gradual addition of concentrated sodium hydroxide (the pH needs to be greater than 8, Sigma-Aldrich product information). EGTA was prepared in a similar fashion.

FOX REAGENT 1 (F1)

The F1 stock solution contained 2.5 mM ammonium ferrous sulphate, 2.5 M sulphuric acid (diluted from a 18 M stock solution of concentrated sulphuric acid). Stored at 4°C.

FOX REAGENT 2 (F2)

The F2 stock solution contained 125 μM xylenol orange and 4 mM BHT in methanol. Stored at 4°C.

GEL BUFFER

1.5 M Tris at pH 8.8. The pH of Tris is temperature-dependent and decreases approximately 0.03 pH units for each 1°C increase.

GENERAL LYSIS BUFFER 1 (G1 BUFFER)

800 mM guanidine-HCl, 30 mM Triss, 30 mM EDTA, 0.5% Tween-20 and 0.5% Triton-X-100 at pH 8.

HEPES WASH BUFFER (H-BUFFER)

20 mM HEPES, 5 mM magnesium chloride, 1 mM DTT, added fresh from 1 M stock, pH 7.6 at 4°C.

KP1 BUFFER

10 mM dipotassium hydrogen orthophosphate and the pH was adjusted to 7 using orthophosphoric acid.

KP2 BUFFER

100 mM dipotassium hydrogen orthophosphate and the pH adjusted to 7 using orthophosphoric acid.

LITHIUM CHLORIDE WASH BUFFER (LC-BUFFER)

0.5 M lithium chloride, 0.1 M Tris, pH 8 at 4°C.

PHOSPHATE BUFFERED SALINE (PBS)

Made with 1 phosphate buffered saline tablet (Oxoid, Dulbecco A) per 100 mL of ultra-pure grade water.

PI3K-BUFFER

20 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 30 mM sodium chloride, 1 mM DTT added fresh from a 1 M stock, pH 7.2 at 4°C.

PBS WITH TWEEN-20 (PBST)

PBS with 0.1% Tween-20.

PROTEASE INHIBITOR COCKTAIL

This was purchased as a 100X stock solution in dimethylsulphoxide (DMSO) and contained: 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) 104 mM, aprotinin 0.08 mM, leupeptin 2 mM, bestatin 4 mM, pepstatin A 1.5 mM, E-64 1.4 mM (Sigma-Aldrich product information).

QBT BUFFER.

750 mM sodium chloride, 50 mM 4-morpholinepropanesulphonic acid (MOPS), 15% isopropanol and 0.15% Triton-X-100 at pH 7.

QC BUFFER

1 M sodium chloride, 50 mM MOPS and 15% isopropanol at pH 7.

QF BUFFER

1.5M sodium chloride, 50 mM Tris and 15% isopropanol at pH 8.5.

RELAXATION BUFFER

100 mM potassium chloride, 3 mM sodium chloride, 3.5 mM magnesium chloride, 1.25 mM EGTA, protease inhibitors (100x dilution) and 0.5 mM sodium orthovanadate, pH 7.3.

R1 SOLUTION.

6 mL of 10.3 mM N-methyl-2-phenylindole in acetonitrile mixed with 12 mL of methanol.

SAMPLE BUFER (3X CONCENTRATION)

0.187 M Tris pH 6.8, 6% sodium dodecylsulphate (SDS), 30% glycerol, 300 mM DDT, bromophenol blue 0.03% and stored at –20°C for up to 6 months.

SDS SOLUTION

10% (100 mg/mL) solution in water.

SODIUM CHLORIDE WASH BUFFER (SC-BUFFER).

0.15 M sodium chloride, 10 mM Tris, 1 mM EDTA, pH 7.6 at 4°C.

SODIUM ORTHOVANADATE

Sodium orthovanadate was prepared using the method described by Gordon (1991). Two hundred mM solution of sodium orthovanadate (in Ultra-pure grade water), pH adjusted to 10 using either 10 M sodium hydroxide or 12 N hydrochloric acid. At pH 10.0 the solution was yellow and was then boiled until it turned clear, after which it was cooled on ice. The pH was then readjusted back to 10 and cycle repeated until the solution remained at pH 10 after the boiling step. After the solution was adjusted back to its original volume with water, 0.5 mL aliquots were stored at -20°C.

STACKING BUFFER

0.5 M Tris at pH 6.8.

STRIPPING BUFFER

62.5 mM Tris, pH 6.7; 2% SDS, 100 mM β-mercaptoethanol.

TANK BUFFER

Made up as a 10x stock solution containing 0.025 M Tris, 0.192 M glycine and 0.1% SDS.

TRIS-BUFFERED SALINE WITH TWEEN-20 (TBST)

Made up as a 10x stock soltion containing 0.05 M Tris pH 7.5, 0.15 M sodium chloride and 0.1% Tween-20

TRANSFER BUFFER.

Made up as 10 L containing 0.0462 M Tris, 0.038 M glycine, 0.0037% SDS and 20% methanol (Fischer-Scientific, UK).

U937 BALANCED SALT SOLUTION (BSS)

140 mM sodium chloride, 5 mM potassium chloride, 2.8 mM sodium hydrogen carbonate, 1 mM magnesium chloride, 1.5 mM calcium chloride (Calbiochem, Merck Biosciences, Nottingham, UK), 15 mM HEPES, 0.06 mM and magnesium sulphate (Calbiochem), adjusted to pH 7.3 with 10 M sodium hydroxide. This solution was made as a 5x stock solution and filtered before use, using acridisk (0.2μ filter) (Walters et al., 1996).

2.3 AGONISTS AND INHIBITORS

AGONISTS. PMA (Calbiochem) was reconstituted in DMSO and stored at -20°C as 5 mM aliquots and diluted in DMSO before use. fMLP (Calbiochem) was reconstituted in DMSO and stored at -20°C as 20 mM aliquots. Arachidonic acid (purchased as 10 mg of oil) was made up in ethanol as 30 mM stock and stored at -20°C. ATP was dissolved in BSS at 50 mM and diluted to 5 mM before use. Insulin was dissolved in water at pH 4 and then the pH adjusted to 7.2 with 50 mM HEPES (sodium salt) and stored at -20°C as 200 mM aliquots. TNFα was reconstituted in a 1% bovine serum albumin (BSA) solution (1% BSA in PBS and stored at -20°C as 10 µg/mL aliquots). AGE was a gift from Professor L. Ng, University of Leicester and was prepared by heating glucose with human serum albumin (HSA) or BSA for 6 weeks and dialysed against PBS before storing at 20°C as 2 mg/mL solution. Hydrogen peroxide was diluted from an 8.8 M stock in BSS.

INHIBITORS. The following inhibitors were all reconstituted in DMSO, aliquoted and stored at -20°C (all from Calbiochem unless otherwise stated). Resveratrol (100 mM); GF109203X

(2.4 mM); UCN01 (10 mM) and PKC412 (1 mM) were a gift from Professor A. Gescher, University of Leicester. LY294002 (50 mM); Wortmannin (5 mM); U0126 (10 mM, this compound is only stable for 1 week in solution). SB203580 (10 mM); L-NAME (182 mM); trolox (100 mM); Akt inhibitor (1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate) (10 mM); zVAD-fMK (10 mM, was a gift from Dr. M. Macfarlane, Universty of Leicester); deferioxamine (desferal) (Sigma-Aldrich) (made fresh each time as 100 mM in BSS). SOD was purchased as a 5 units/μL solution. Catalase was made as a 23.1 K-units/mL in BSS salt solution. N-acetylcysteine (NAC) (Sigma-Aldrich) was made as a 1 M stock in BSS.

2.4 ANTIBODIES

The following antibodies were used (working concentration and source). Anti-p47 was a gift from Professor L. Ng (1 μg/mL, rabbit); phospho-specific Akt (Ser473) antibody (Biosource, 1:1000, rabbit); phospho-specific (Thr308) Akt antibody; anti-Akt1 (Santa Cruz biotechnology, 1:1000, goat); anti-PKCα (1:1000, mouse) antibody was a gift from Professor A. Gescher; anti-BCL-2 (Dako A/S, Denmark, 1:1000, mouse); phospho-specific ERKs 1 and 2 (Tyr 204) antibody (Santa Cruz biotechnology, 1:1000, mouse); anti-ERK1 (which also reacts with ERK2) (Santa Cruz biotechnology, 1:1000, rabbit); anti-tubulin (Oncogene, 1:1000, mouse); anti-p85α (Upstate Biotechnology, mouse and Santa Cruz Biotechnology, mouse). M_1G antibody (mouse) was a gift from Dr. L. Marnett, with stock solutions (0.33 mg/mL) diluted 1:6 (33.3 μL antibody +166.7 μL PBS) and then 1:8000 before use. Anti-Heat shock protein 70 (inducible form) (1:1000, Bioquote, UK mouse); phospho-tyrosine (PY99) antibody (Santa Cruz biotechnogy, 1:1000 or 10 μg for immunoprecipitation). With one exception antibodies were used in TBST containing 5% milk (non-fat milk powder, Marvel, Nestle Ltd) for western blotting. The M_1G antibody was used in PBST, containing 0.5% milk.

2.5 CELL CULTURE

U937 monocytes were obtained from the European Tissue Culture Collection (CAMR, Salisbury, UK). One mL containing $3x10^6$ U937 cells, was rapidly thawed from liquid nitrogen in a water bath at 37°C. The cells were transferred to a 15 mL centrifuge tube and added to a further 5 mL of medium (RPMI supplemented with 10% foetal calf serum and 5%

glutamine). The cells were spun at 200x g for 3 minutes and resuspended in 6 mL of medium. The cells were grown in a small flask for 48 hours or until the medium had turned yellow, after which they were washed in medium and resuspended in 6 mL of fresh medium. A 50 µl sample was mixed with an equal volume of tryphan blue (0.4%) and 50 µl of this solution was placed under the cover slip of the haemocytometer. Cells were counted in 32 squares, the average of 16 taken and using a conversion factor of 10^4, converted to number of cells per mL. The cells were then seeded at a density of $2x10^5$ cell per mL in a medium sized flask. After this the cells were split 1:10 every 3 days and once every week they were spun and resuspended in fresh medium.

B-lymphoblasts (transformed with Epstein–Barr virus by Professor L. Ng, University of Leicester) were resurrected from liquid nitrogen in a identical fashion to the U937 monocytes, although they were grown in a smaller volume (5 mL) for several days before being transferred to a medium sized tissue culture flask. These cells grow at a much slower rate and are easily seeded at a density that is unfavourable for their growth. Before being passaged, they were dispersed with a pipette and every 3 days they were split 1:3.

For long-term storage, $3x10^7$ cells (B-lymphocytes or U937) were resuspended in 10 mL of freezing medium which contained 4 mL of serum free medium, 1 mL of DMSO and 5 mL of foetal calf serum. 1 mL aliquots were frozen at -80°C for 1 week and then transferred to liquid nitrogen.

2.6 U937 CELL DIFFERENTIATION AND ANALYSIS OF GROWTH.

DIFFERENTIATION PROTOCOL. U937 cells were allowed to grow for 2 days before being differentiated. They were split 1:10 in a large tissue culture flask, after which they were treated with 10 nM dihydroxy-vitamin D_3 and 1 µM all-trans retinoic acid (Calbiochem) for 4 days. Twenty mL fresh medium was added after 2 days. Retinoic acid was reconstituted as a 50 mM solution, aliquoted and stored in liquid nitrogen. Dihydroxy-vitamin D_3 was reconstituted as a 500 µM solution, aliquoted and stored in liquid nitrogen. Fifty mL of U937 cells were treated with 1 µL of each compound.

GROWTH CURVES. U937 ($2x10^5$) cells were added to 24-well plate and treated with 1 μM retinoic acid and 10 nM vitamin D_3. Cell numbers were estimated at 24, 48, 72 and 96 hour intervals, by collecting all the cells in each well, including two washes with medium from each well and centrifuging at 200x g. Cells were resuspended in 200 μL of PBS solution, which was then added to 9.8 mL of isoton solution. Cells were counted using a Coulter Counter (Beckton-Dickinson).

2.7 MEASUREMENT OF REACTIVE OXYGEN SPECIES (ROS)

Five different methods were employed to determine ROS production from dU937 monocytes. Three chemiluminescence (CL) methods were used (luminol, isoluminol and lucigenin-CL) and two fluorescence (FL) detection systems, dihydrorhodamine 123 (Molecular Probes) (DHR) and 2',7'-dichlorofluorescein (Molecular Probes) (DCF) (NB. Older literature refers to this compound as 2',7'-dichlorofluorescin and the oxidised form as 2',7'-dichlorofluorescein). CL- measurements were carried out using a FluoStar plate reader and cellular FL was determined using a flow cytometer and the FluorStar plate reader.

Isoluminol and luminol (50 mM stock) were dissolved in 0.1 M sodium hydroxide and stored as 100 μL aliquots at -20°C. Lucigenin was freshly prepared for each experiment by dissolving in water as a 1 mM solution. Horse radish peroxidase (HRP) is required for luminol/islominol-CL and was dissolved in water at 5000 units per mL. Aliquots of 8 μL (40 units) were stored at –20°C. DCF was dissolved in DMSO at 20 mM, aliquoted and stored at –20°C. DHR was purchased as a 5 mM solution (stabilised in DMSO). Both dyes were stored under nitrogen to prevent oxidation in air.

Two resveratrol treatment conditions were used in this study: treatment A consisted of pre-treatment of dU937 cells with resveratrol for 1 hour, followed by washing (one wash in balanced salt solution then re-suspension in balanced salt solution (containing the FL- or CL-probe of interest) and treatment B, where cells were re-suspended in balanced salt solution containing FL- or CL-probe of interest, incubated with resveratrol for 5 minutes and then stimulated.

LUCIGENIN AND LUMINOL/ISOLUMINOL-CL PROTOCOL. The luminol/isoluminol method has been modified from Lundqvist and Dahlgren (1996) and lucigenin method modified from Li et al.,

(1998). Differentiated U937 monocytes were harvested in 50 mL centrifuge tubes, spun at 200 x g and resuspended in 10 mL of fresh medium. The cell suspension was adjusted to $1x10^6$ cells per mL using a haematocrit. One mL aliquots were treated with inhibitors for the indicated times. They were then spun at 200 x g and washed once in BSS. They were then resuspended in 1 mL of CL-buffer, which consisted of BSS supplemented with 50 μM luminol or isoluminol and 4 units per mL of HRP. Lucigenin was used at 50 μM without HRP. A bioluminescence plate was pre-warmed to 37°C and 100 μL of cell suspension was used per well. Before stimulation the plate was allowed to warm to 37°C in the FluorStar plate reader. Cells were stimulated with PMA and AA, using a multi-channel pipette to add 50 μL of 3x PMA and AA (100 μL cells + 50 μL agonist). The FluorStar plate reader has in internal injection system that allows rapid cellular events to be measured. fMLP measurements were carried out in this fashion. A base line was obtained for each well (for 45 seconds) and then 50 μL of 3x fMLP was injected to each well.

CL-measurements were quantified according to three different parameters (Alexandrova et al., 2001). The velocity of phagocyte activation (T_{max}) was represented as the time to peak response after agonist addition. The maximum phagocytic response (I_{max}) was represented as the maximum CL-response. Total oxidative phagocyte capacity (S) was proportional to parameter S, the area under the kinetic curve (AUC). The AUC was calculated for 0-5 min for fMLP and 0-40 min for PMA or AA.

DCF AND DHR PROTOCOL 1. These methods have been modified from Bass et al., (1983) and Myhre et al., (2000). Differentiated U937 monocytes were harvested in 50 mL centrifuge tubes, spun at 200 x g and resuspended in 10 mL of fresh medium. The cell suspension was adjusted to $1x10^6$ cells per mL using a haematocrit. One mL aliquots were treated with inhibitors for the indicated times, after which, they were spun at 200 x g and washed in 1 mL of BSS and then resuspended in 1 mL of BSS. A Nunc F98 plate was pre-warmed to 37°C and 100 μL of cell suspension was used per well. The cells were allowed to warm to 37°C, after which, 50 μL of DCF or DHR solution was added to all wells. This solution consisted of 6 μM of either dye in 5 mL BSS. The cells were incubated for 5 minutes and then using a multi-channel pipette, 50 μL of agonist was added (4x concentration, 150 μL cells + 50 μL agonist). 50 μL of BSS was added to the control cells in order to ensure the dye concentration was the same for all cells.

FLUOSTAR OPTIMA PLATE READER PROTOCOLS. The FluorStar Optima can measure luminescence and FL through the use of interchangeable reading heads. The main consideration before experimentation is the gain setting, which allows the sensitivity to be adjusted in order to remain within the measurement scale. For luminescence experiments the gain range is 0-4095 and 4000 was used for all experiments. This allowed the fMLP response to be measured consistently and produced a large response with PMA and AA.

DCF AND DHR PROTOCOL 2. The method was modified from Bass et al., (1983). Differentiated U937 monocytes were harvested in 50 mL centrifuge tubes, spun at 200 x g and resuspended in 10 mL of fresh medium. The cell suspension was adjusted to $1x10^6$ cells per mL using a haematocrit. 1 mL aliquots were treated with inhibitors for the indicated times, after which, they were then spun at 200 x g and washed in 1 mL of BSS and then resuspended in 1 mL of BSS. Five hundred μL of this suspension was added flow cytometer tubes and warmed to 37°C in a covered water bath. DCF or DHR solution (250 μL) (prepared in the same fashion to protocol 1) was added to all cells and incubated in the water bath for 5 minutes. Agonist (250 μL) was added to stimulate the cells and 250 μL of BSS to the control cells. The cells were incubated in the dark for 30 or 60 minutes, after which the tubes were placed on ice but still kept in the dark.

Flow cytometry was carried out using a Beckton-Dickenson flow cytometer. A dot-plot was used to find the live cell population. Forward scatter (FSC) (which measures cell size) vs side scatter (SSC) (which measures granularity) was used to identify live cells and exclude smaller dead cells and debris. Cell aggregates were also excluded in this fashion because they have a higher FL. To measure cellular-FL a histogram was used. The FL1 filter was used for DCF/DHR measurements and 10 000 cells were counted for each sample.

DCF PROTOCOL 3. The cell free oxidation of DCF was carried out using de-esterified DCF, according to the method by Cathcart et al., (1983) One mM DCF was dissolved in 0.5 mL of methanol, to which 2 mLs of 0.01 M sodium hydroxide was added. The mixture was incubated in the dark for 30 minutes. The solution was neutralised by the addition of 10 mL of 100 mM sodium phosphate buffer. The solution was stored in the dark at 4° C until use and a fresh solution was prepared for each experiment. DCF oxidation was carried in a 96-well plate. One μM HRP was dissolved in 100 μL sodium phosphate buffer (100 mM), then 2 μM DCF and 250 μM hydrogen peroxide was added. In some cases, various concentrations

of resveratrol (0.1-50 μM) were added, which were pre-diluted in sodium phosphate buffer. DCF oxidation was measured as described in DCF protocol 1.

2.8 DNA MODIFICATION AND EXTRACTION

DNA was extracted, using a Qiagen genomic DNA extraction kit (Qiagen LTD, UK) from both undifferentiated and differentiated U937 cells. Qiagen genomic DNA-tips consist of an anion exchange resin (consisting of positively charged diethylaminoethyl (DEAE) groups) that bind the negatively charged phosphate groups of DNA. Only buffers with a high salt concentration will extract DNA from the column and therefore lower (<1.5 M) salt concentrations are used to remove impurities.

No more than $1x10^7$ monocytes were used per treatment group to yield 45 μg of DNA ($2x10^7$ is the maximum number of cells for this tip size).

The cells were treated with the appropriate inhibitor and stimulus for the indicated times and since longer treatment times were used the cells were kept in complete medium. After stimulation the cells were harvested in 50 mL tubes and spun at 200x g for 3 minutes. When PMA was the stimulus, a cell scraper was used to dislodge cells that had become adherent. The medium was discarded and the cells were washed twice in cold PBS (4°C). The cells were resuspended in 2 mL of cold PBS, to which 6 mL of cold water (4°C ultra-pure grade) and 2 mL of cold C1 buffer were added. The suspension was mixed by inverting and incubated on ice for 10 minutes, after which the lysed cells were centrifuged at 1300x g for 15 minutes (4°C). The supernatant was discarded and 1 mL of C1 buffer and 3 mL of water were added. The pellet was resuspended by vortexing and then centrifuged at 1300x g for 15 minutes.

The supernatant was then discarded and 5 mL of G1 buffer added, the pellet is vortexed for 10-30 seconds in order to completely resuspend the pellet. Proteinase K (95 μL, Qiagen) and 162.5 μL of RNAase A were added and the suspension was incubated at 37°C for 2 hours. If necessary the incubation period can be extended if there is still soluble matter. Otherwise, the suspension can be centrifuged to clear the solution. However, this appeared to reduce the DNA yield.

A 100/G genomic Qiagen tip was placed in a 50 mL centrifuge tube using an adapter that is supplied with the kit. Five mL of QBT buffer was used to equilibrate the tip. The sample was briefly vortexed and loaded into the tip. The flow rate should be roughly 10-20 drops per minute. If the flow rate is slow, the sample can be diluted with QBT buffer or a positive pressure can be applied using a piece of Nesco film (Fisons Scientific Apparatus, UK). Once the sample had passed through the tip it was washed with 2x 7.5 mL QC buffer. The DNA was eluted with 5 mL of QF, which was warmed to 50 °C to increase the flow rate.

The DNA was precipitated by the addition of 5 mL of isopropanol and mixed by inverting the tubes 10-20 times. To increase the yield the tubes were placed at –20°C and left overnight. The tubes were then centrifuged at 5000x g (4°C) for 10 minutes and once the supernatant had been discarded, 2 mL of cold 70% ethanol was added. The solution was centrifuged at 5000x g (4°C) and the supernatant removed. The precipitate was allowed to air dry for about 10 minutes, after which 200 μL of water (ultra-pure grade) was added and the DNA allowed to dissolve.

The concentration of DNA was determined by measuring the absorbance at 260 and 280 nm. The concentration was calculated according to the formula: 260nm reading x dilution factor x 50 = μg/mL. Five μL of DNA sample was diluted in 95 μL of water (Ultra-pure) to obtain the readings.

DNA MODIFICATION. Calf thymus DNA (3.5 mg) was dissolved overnight in 10 mL of 100 mM KP2 buffer. 1 mL aliquots of this solution were treated with various concentrations of sodium MDA (a gift from Dr E. Grieche, University of Leicester). One mL of isopropanol was used to precipitate the DNA, which was then treated as described above. The DNA was dissolved in 250 μL of water (ultra-pure water) and 3.5 μg was prepared for M_1G analysis as described below.

2.9 DETERMINATION OF M_1G LEVELS BY THE IMMUNOSLOT BLOT METHOD

The immunoslot blot is a sensitive method to measure the M_1G DNA adduct (Leuratti et al., 1999). This method involves transferring dilutions of calf thymus DNA containing known amounts of adducts, along with sample DNA to nitrocellulose. The nitrocellulose is then probed with an antibody specific for M_1G and the amount of adduct quantified using a CL-detection system.

PREPARATION OF THE CALF-THYMUS DNA STANDARD CURVE. Calf thymus DNA that had been treated with MDA (from Dr. R. Singh, University of Leicester) was diluted with control calf thymus DNA to give a series of standards ranging from 0.4-10 fmol of adducts per sample.

Thirty five μL of each standard was added to a microtube along with 65 μL of KP1 buffer and 150 μL of PBS. The standards were then vortexed and centrifuged at 14000x g for 1 minute, then sonicated for 20 minutes, to break up the DNA into ~100 base pair strands. To produce single-stranded DNA, the standards were heated (100°C) for 5 minutes and then rapidly cooled on ice for at least 10 minutes. They were then centrifuged for 2 minutes at 14000x g and 250 μL of 2 M ammonium acetate was added before vortexing and centrifuging as before.

PREPARATION OF SAMPLE DNA. Sample DNA (3.5 μg) was made up to 100 μL with KP1 buffer. The samples were then treated in exactly the same fashion as the standards.

IMMUNOSLOT BLOT PROTOCOL. Two pieces of gel blotting papers (Schleicher and Schuell, Anderman and Company Ltd, Kingston Upon Thames, Surrey, UK) were soaked in 1 M ammonium acetate and then placed in one half of the immunoslot blot apparatus. A piece of nitrocellulose filter (Schleicher and Schuell) was cut to size and soaked first in water (ultra-pure) and then in 1 M ammonium acetate. The nitrocellulose filter was then placed on top of the blotting paper and the immunoslot blot apparatus assembled. The apparatus was then connected to an aspirator.

One μg of DNA was added to each well and allowed to run dry, after which 200 μL of 1 M ammonium acetate was added and again allowed to run dry for 20 minutes. The apparatus was dismantled and the first and last wells were marked (on the underside of the filter). The nitrocellulose filter was placed in a filter paper tent and baked in a vacuum oven at 80°C for 1.5 hours. The filter was then placed in a tray containing a PBST solution with 5% milk and allowed to gently rock for 1 hour after which the filter was washed twice with 50 mL of PBST and then incubated with the M_1G antibody. The filter was incubated with the primary antibody for 2 hours at room temperature followed by an overnight incubation at 4°C.

The filter was then washed in PBST for 3x 5 minute washes and then incubated with the a HRP-conjugated anti-mouse secondary antibody (Dako A/S, Denmark). For this stage 8 μL

of antibody was diluted in 32 mL of PBST containing 0.5% milk to cover the filter, which was left rocking for 2 hours at room temperature. The filter was then washed with 3x 50 mL of PBST. Following the last wash, 8 mL of Supersignal Ultra (PerBio) solution was prepared by mixing 4 mL of the luminol enhancer solution with peroxidase solution, both solutions having been allowed to warm to room temperature before use. The filter was bathed in the solution for 5 minutes before being wrapped in saran film and then exposed to ECL-hyperfilm (Amersham Biosciences). To quantify the level of adduct in each sample, the CL-signal for each sample was quantified using a Biorad Fluor S Multi-imager.

PROPIDIUM IODIDE STAINING. Following the detection of DNA adducts, the amount of DNA on the filter was quantified using propidium iodide (PI) staining. The nitrocellulose filter was first washed overnight in PBS and then incubated with 50 mL of PBS-PI (50 mL PBS with 50 μL of PI solution (5 mg/mL)) in a covered plastic box. The filter was then washed for 1 hour in PBS and then for 2 further 30 minute washes. PI staining was quantified using a Biorad Fluor S multi-imager.

CALCULATION OF ADDUCT LEVELS. The optical density (adduct level) and PI (total DNA) were obtained for all standards (see Figure 2.1) and samples. The average PI value for the standard curve was used to correct for DNA binding. A corrected curve standard (adjusted for DNA binding) was used to quantify the adduct level in an unknown sample (see Figure 4.8).

2.10 MEASUREMENT OF LIPID PEROXIDATION

Lipid peroxidation was measured using a method that is based on a kit supplied from Calbiochem (Erdelmeier et al., 1998) that only measures free intracellular MDA and 4-HNE. The method uses N-methyl-2-phenylindole, which reacts with MDA and 4HNE at 45°C to produce a stable chromophore. The absorbance of this chromophore was measured at 586 nm using a spectrophotometer.

A standard curve for this assay was produced by using an MDA solution provided by Calbiochem or a solution of sodium MDA. The Calbiochem MDA solution (10 mM), was diluted 1:100 to make a series of standards from 0-50 (final volume 200 μL). The standards were then diluted 5-fold in the reaction mixture (see below). Sodium MDA was stored as a 110 mM stock solution that was diluted in the same way.

U937 monocytes ($3x10^6$ per mL, differentiated and undifferentiated) were treated for the indicated times, after which they were centrifuged at 200x g for 3 minutes and then washed in PBS.

Water (250 μL)(Ultra-pure) was added to the cell pellet, which was freeze-thawed using dry ice and a water bath set at 45°C for 6 cycles. Fifty μL of the resulting lysate was used for a protein assay (see section 2.11) and the remaining 200 μL assayed for MDA levels. R1 solution (650 μL) was added to 200 μL of cell sample and mixed in a glass vial by vortexing. The standards were also treated in the same fashion. Twelve N hydrochloric acid (150 μM) was added and mixed well. The samples and standards were then incubated at 45°C for 45 minutes. The vials are then cooled on ice for 10 minutes to stop the reaction. Pure MDA reacts with N-methyl-2-phenylindole to yield a blue colour. However, the samples turn pink due to the formation of other chromophores that have an absorbance of 505 nm. The presence of these chromophores did not interfere with the absorbance of the MDA chromophore. The absorbance of standards and samples was read at 586 nm. The samples were quantified using the following formula: MDA concentration = (absorbance of sample x 5 (dilution factor) - absorbance of the blank)/molecular extinction coefficient (obtained from slope of the standard curve).

Following protein estimation (see section 2.11) the concentration of MDA was expressed as μM and all samples were corrected to the equivalent of 1 mg protein.

2.11 PREPARATION OF CELL LYSATES, FRACTIONATION AND IMMUNOPRECIPITATION FOR WESTERN BLOTTING AND KINASE ASSAYS.

Three large flasks of dU937 monocytes were harvested and resuspended (as previously described) at a cell density between $0.5\text{-}1x10^6$ per mL for kinase assays and $2x10^6$ for western blotting. One mL aliquots were treated with inhibitors for the indicated times (at 37°C) and then centrifuged for 3 minutes at 200x g and resuspended in 500 μL of BSS. Cell stimulation was carried out at 37°C by the addition of 250μL agonist at 3x concentration (for PMA, AA, fMLP and ATP). Insulin was added to the inside of the microtube lid, which was then closed and the tube inverted.

Once the cells have been stimulated for the indicated time they were rapidly centrifuged (14000x g for 10 seconds) and the supernatant aspirated. Cold (4°C) cell lysis buffer was added (200 μL for western blotting and 300 μL for immunoprecipitation) and the samples and vortexed. After a 15 minute incubation on ice, the samples were centrifuged at 14000x g (4°C) to clear the lysate and 10 μL of each sample was then used for a protein assay (see section 2.12).

IMMUNOPRECIPITATION. The immunoprecipitation protocol was modified from Hawkins et al., (1992) and the Amersham Biosciences technical literature (Amersham Biosciences Technical Tips 156). The cell lysate (500 μg) was diluted with cold cell lysis buffer, without NP40. Some antibodies bind more efficiently if the detergent concentration is below 1%. Each sample was incubated with 5 μg of antibody at 4°C overnight. Protein A sepharose (200 μL) was washed in 1 mL of lysis buffer (centrifuged at 14000x g), equilibrated for 1 hour at 4°C and resuspended in 200 μL of lysis buffer (making a 1:1 protein A/ buffer mix). Forty μL of this suspension was then added to the 1 mL lysates and incubated for 1 hour while rotating at 4°C. The protein A pellets were collected by centrifugation (14000x g) and then washed, according to which assay was to be performed), lipid kinase (see section 2.13) or western blotting (see section 2.12).

PREPARATION OF CELL MEMBRANES. Cell membrane fractionation was carried out, with modifications according to Clark et al., (1990). dU937 cells ($1x10^7$) were treated as described above and after stimulation were rapidly centrifuged and resuspended in 0.5 mL relaxation buffer. The cells were then sonicated for 2x 15 second blasts on ice using a probe sonicator and then centrifuged at 500x g (4°C) to remove the nuclei and unbroken cells. The supernatant was then centrifuged for 30 minutes at 100000x g (4°C), after which the soluble fraction was referred to as the cytosolic fraction and the pellet as the membrane fraction. Crude membranes were resuspended in relaxation buffer containing 1% NP-40.

2.12 PROTEIN DETERMINATION

The Bradford protein assay (Bradford 1976) was used according to the manufacturer's instructions (BioRad). The lower limit of detection of this assay is 1 μg and a standard curve ranging from 2-20 μg was prepared. However, at higher concentrations (often less than 20 μg) the curve flattens out as the reaction saturates.

Four μL of sample was made up to 1 mL with water (Ultra-pure) and 800 μL of this was added to 200 μL of Bradford reagent (BioRad). The solutions were thoroughly mixed and left for 15 minutes, to allow the reaction to stabilise. The absorbance was then read at 595 nm, a mixture of water and Bradford reagent serving as a blank. The protein concentration was calculated using the following formula: (sample absorbance-blank)/slope of standard curve = amount of protein (μg).

2.13 WESTERN BLOTTING

The BioRad minigel apparatus was assembled according to the manufacture's instructions to produce 1.5 mm thick gels. The separating and stacking gels were prepared according to Table 2.1.

	Final gel concentration				
Resolving gel	5%	7.5%	10%	12.5%	15%
Acrylamide solution (mL)	3.3	5	6.7	8.3	10
Gel buffer (mL)	5	5	5	5	5
10% SDS (mL)	0.2	0.2	0.2	0.2	0.2
H_2O (mL)	11.4	9.7	8	6.4	4.7
APS (μL)	150	150	150	150	150
TEMED (μL)	10	10	10	10	10
Stacking gel					
Acrylamide solution (mL)	0.88				
Stacking buffer (mL)	1.66				
10% SDS (μL)	66				
H_2O (mL)	4.0				
APS (μL)	100				
TEMED (μL)	5				

Table 2.1. Gel recipes for 5-15% acrylamide gels

Ammonium persulphate (APS) and N, N, N',N' –tetramethylethylenediamine (TEMED) were added to initiate the polymerisation. After pouring the resolving gel, water was placed on top to give a level finish. When set, the water was removed and the stacking gel was then added along with the appropriate well comb. After the removal of the comb, the wells were washed out with tank buffer.

Protein sample was mixed 3:1 with sample buffer and then heated to 100°C for 5 minutes. After centrifugation for 10 seconds at 14000x g, a volume equal to 35 μg of protein was loaded into each well. The gel was run at 110 volts until the dye front reached the bottom of the gel.

One piece of nitrocellulose (Hybond nitrocellulose, Amersham Biosciences, UK) and two pieces of filter paper were prepared for each gel. A transfer case was prepared by soaking 2 sponges, the filter paper and the nitrocellulose in transfer buffer. The gel was removed from the glass plates, soaked in transfer buffer (for 10 minutes) and assembled according to Figure 2.1. It was important to make sure that there were no air bubbles on the nitrocellulose. The transfer tank was filled with transfer buffer and the transfer cases were inserted into the tank in the correct orientation. Transfers were carried out at 100 volts for 2 hours (on ice) or 30 volts overnight.

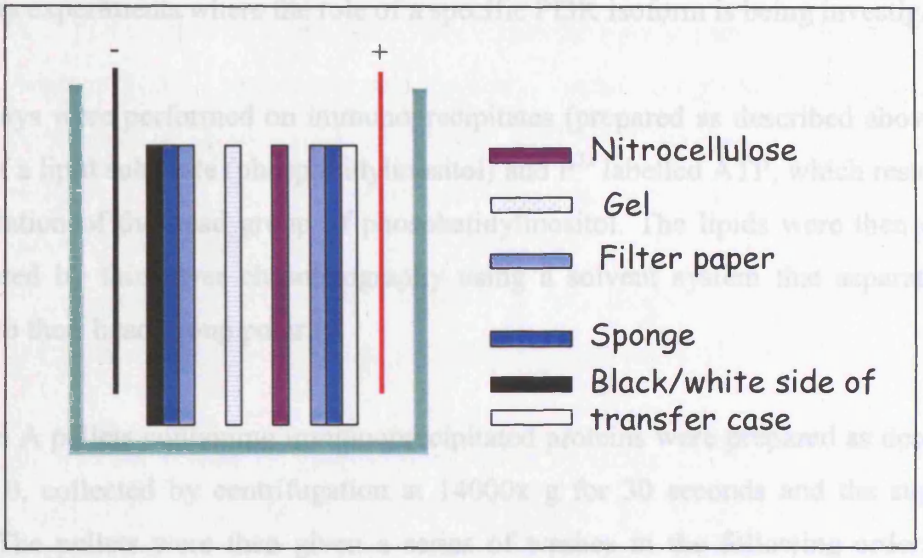

Figure 2.1 A schematic representation of the electrophoretic blotting system used for polyacrylamide gels.

Following transfer, the nitrocellulose filter was blocked in TBST with 5% milk for 1 hour after which it was incubated with the primary antibody (see section 2.3) for 2 hours (room temperature) or overnight (4°C). The filter was washed with 3 x 10 mL of TBST and then incubated with the secondary antibody for 1 hour. After washing as before, it was incubated for 1 min with 1 mL of ECL reagent (made by adding equal quantities of the luminol and

enchancer solutions (Amersham Biosciences). The filter was wrapped in saran film and exposed to ECL film (Amersham Biosciences).

The exposure times varied between different antibodies, the maximum time being 20 minutes, which is the duration of the signal for ECL reagent. Hyperfilms were quantified by densitometry using a Syngene Chemigenius (Synoptics UK) or BioRad densitometer (BioRad Lab Technologies, UK).

2.14 LIPID KINASE ACTIVITY ASSAY

This method is based on that published by Hawkins et al., (1998). The assay involved immunoprecipitation of PI3K using an antibody against one of the PI3K subunits or phosphotyrosine. The lack of commercially available high affinity antibodies against the catalytic subunit of PI3K (personal communication, Dr L. Stephens, Cambridge University, UK) hinders experiments where the role of a specific PI3K isoform is being investigated.

Kinase assays were performed on immunoprecipitates (prepared as described above) in the presence of a lipid substrate (phosphitidylinositol) and P^{32} labelled ATP, which results in the phosphorylation of the head group of phosphatidylinositol. The lipids were then extracted and separated by thin layer chromatography using a solvent system that separates lipids according to their head group polarity.

The protein A pellets containing immunoprecipitated proteins were prepared as described in section 2.10, collected by centrifugation at 14000x g for 30 seconds and the supernatant removed. The pellets were then given a series of washes in the following order: 1x C2-buffer, 2x LC-buffer, 1x SC-buffer, 1x H-buffer, 1x PI3K-buffer.

The protein A pellets were finally resuspended in 40 μL of kinase buffer. Phosphatidylinositol (5 mg in chloroform) was dried in speed vac. This was carried out on a low setting to avoid over-heating and subsequent degradation. Five mg of phosphatidylserine was suspended in 1.6 mL of PI3K buffer containing 1% cholate, which was then added to the dried phosphatidylinositol and sonicated for 5 minutes, using a water bath sonicator. It was imperative that the lipids were not over-sonicated because this would result in oxidation. Once they were dissolved (forming micelles), 20 μL of this mixture was added to the protein

A pellets and incubated at 37°C for 5 minutes. A solution of 3 μM ATP and 7.5 mM magnesium chloride was prepared, 390 μL of which was added to 10 μL of 250 μM Ci [γP ^{32}] ATP. Forty μL of this solution was added to the protein A pellets and incubated at 37°C for 15 minutes.

The reaction was terminated by the addition of 450 μL of chloroform/methanol (1:2 ratio) and mixed by vortexing to yield a homogenous extraction phase. Hydrochloric acid (150 μL of 0.1 M) and chloroform (150 μL) were added, vortexed and centrifuged for 10 minutes at 6000 x g. The lower organic phase was removed using a pipette (pre-equilibrated with chloroform). Synthetic upper phase (600 μL, methanol/HCl 1:1) was added to the organic phase, which was then vortexed and centrifuged as before. The lower phase was extracted as before and the remaining organic phase was dried in a speed vac (on a low setting). The lipids were then resuspended in a solution of chloroform/methanol/HCl (25 μL of 200:100:1).

Silica gel TLC plates (Merck Kieselgel, Merck Eurolab) were prepared by heating in an oven for 1 hour at 100°C, to remove any moisture. Some authors suggest that the plates should be impregnated with potassium oxalate and EDTA (1% oxalate, 1 mM EDTA in 40% methanol) before drying, to retard any calcium that would otherwise prevent poly-phosphoinositides from moving up the plate. Most plates are low in residual calcium, apart from silica G (gypsum). In this case, PtIns(3)P is less likely to be effected by calcium retardation (personal communication, Dr. L. Stephens, University of Cambridge, UK).

The TLC tank solvent system of methanol/chloroform/water/ammonia (300:210:75:45) was allowed to equilibrate. Once the TLC plate had cooled, the lipids were spotted in 5 μL aliquots 2 cm apart (1.5 cm from the bottom of the plate). The plate was run until the solvent front was 2 cm from the top. The plate was then air dried and exposed to autoradiography film or visualised using a phosphoimager screen. To determine which spot was the one of interest and the Rf value for PtdIns(3)P, a non-radioactive standard was used. PtdIns(4)P (Calbiochem) (50 μg) (which has the same Rf value as PtdIns(3)P) was run against a PI/PS mixture (60 μg of each) as described above. The lipids were visualised using iodine vapour. The Rf value of PI(4)P was found to be approximately 0.9.

LIPID KINASE ASSAY METHOD FOR KINASE SCREEN (as described by Kurosu rt al., 1997). The assays were conducted in a final volume of 50 μl containing 0.1% bovine serum albumin,

2 mM EGTA, 0.2 mM EDTA, 10 mM $MgCl_2$, 120 mM NaCl, 40 mM HEPES, pH 7.4, 1 mM dithiothreitol, 1 mM β-glycerophosphate. Thirty µL of lipid vesicles (225 µM phosphatidylserine and 75 µM phosphatidylinositol) were mixed with either Gβγ complexes or their vehicle and incubated on ice for 8 minutes. Thereafter the enzyme fraction (1-10 ng) was added and the mixture was incubated for further 10 minutes at 4 °C in a final volume of 40 µl. The assay was then started by adding 50 µM ATP (1 µCi of [γ-^{32}P]ATP in 10 µl of the above assay buffer (30 °C). After 15 minutes the reaction was stopped with ice-cold 150 µl of 1 N HCl and placing the tubes on ice. The lipids were extracted by vortexing samples with 450 µl of chloroform/methanol (1:1). After centrifugation and removing of the aqueous phase, the organic phase was washed twice with 200 µl of 1 N HCl. Subsequently, 40 µl of the organic phase were resolved on potassium oxalate-pretreated TLC plates with 35 ml of 2 N acetic acid and 65 ml of 1-propanol as the mobile phase. Dried TLC plates were analysed using a phosphoimager.

2.15 KINASE ACTIVITY SCREEN

These assays were carried out in the laboratory of Dr P. Cohen (University of Dundee, UK) and these methods taken from that described by Davies et al., (2000). Unless stated otherwise, all protein kinases were of human origin and were expressed either as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* or as hexahistidine (His_6)-tagged proteins in insect Sf9 or Sf21 cells. GST fusion proteins were purified by affinity chromatography on glutathione–Sepharose, and His_6-tagged proteins were purified on nickel/nitrilotriacetate–agarose.

Expressed in E. coli: The following kinases were expressed in *E. coli*: MKK3, MKK4, MKK6, MKK7, rabbit MKK1, murine ERK2 and human JNK 1, SAPK2a/p38, SAPK2b/p382, SAPK3/p38, SAPK4/p38, MAPK-activated protein kinase 2 (MAPKAP-K2), checkpoint kinase 1 (CHK1) and CHK2. CHK2 contained an additional six histidine residues at its C-terminus to aid purification of the full-length protein.

Expressed in Sf9 cells: The following kinases were expressed in Sf9 cells: protein kinase B (PKB also known as Akt), SGK (serum- and glucocorticoid-induced kinase), p38-regulated/activated kinase (PRAK), rat ROCK-II-(1–543) (where ROCK is Rho-dependent protein kinase), mitogen- and stress-activated protein kinase 1 (MSK1), casein kinase 2 (CK2), lymphocyte kinase (LCK) and the p110 catalytic subunit of bovine PI3K. *Expressed*

in Sf21 cells: The following kinases were expressed in Sf21 cells: 3-phosphoinositide-dependent protein PDK1, GSK3, S6K1 (p70 ribosomal protein S6 kinase). *Expressed in human embryonic kidney 293 cells.* GST–PRK2-(501–984) and purified on glutathione–Sepharose. *Tissue purified*: Phosphorylase kinase (PHK), MAPKAP-K1b (also known as RSK2) and skeletal muscle myosin light chain kinase (SkMLCK) were purified from rabbit skeletal muscle, PKA was purified from bovine heart and AMP-activated protein kinase (AMPK) was purified from rat liver. MLCK was purified from chicken gizzard smooth muscle (SmMLCK). Protein kinase C and CAM-KII were purchased commercially.

Kinase assays: All protein kinase activities were performed either manually for 10 min at 30 °C in 50 μl incubations using [γ-^{32}P]ATP, or with a Biomek 2000 Laboratory Automation Workstation in a 96-well format for 40 min at ambient temperature in 25 μl incubations using [^{33}P]ATP. The concentrations of ATP and magnesium acetate were 0.1 mM and 10 mM respectively, unless stated otherwise. Manual assays were terminated by spotting aliquots of each incubation on to phosphocellulose paper, followed by immersion in 50 mM phosphoric acid. Robotic assays were terminated by the addition of 5 μl of 0.5 M phosphoric acid before spotting aliquots on to P30 filter mats. All papers were then washed four times in 50 mM phosphoric acid to remove ATP, once in acetone (manual incubations) or methanol (robotic incubations), and then dried and counted for radioactivity.

GSK3, S6K1, MAPKAP-K1b/RSK2, PKA, CHK1, CHK2, MSK1 and SGK were assayed in 8 mM MOPS, pH 7.0, containing 0.2 mM EDTA. Substrate peptides were: GSK3, YRRAAVPPSPSLSRHSSPHQS(PO_4)EDEEE (20 μM); S6K1, KKRNRTLTV (100 μM); MAPKAP-K1b/RSK2, KKKNRTLSVA (30 μM); PKA, LRRASLG (30 μM); CHK1 and CHK2, KKKVSRSGLYRSPSMPENLNRPR (200 μM); MSK1 and SGK, GRPRTSSFAEG (30 μM). PKB was also assayed with GRPRTSSFAEG (30 μM) in 50 mM Tris/HCl, pH 7.5, containing 0.05% 2-mercaptoethanol. ERK2, SAPK2a/p38, SAPK2b/p382, SAPK3/p38 and SAPK4/p38 were assayed in 25 mM Tris/HCl, pH 7.5, containing 0.1 mM EGTA, with myelin basic protein (0.33 mg/ml) as substrate. MAPKAP-K2 and PRAK were assayed in 50 mM sodium β-glycerophosphate, pH 7.5, containing 0.1 mM EGTA, with the peptides KKLNRTLSVA (30 μM) and KKLRRTLSVA (30 μM) respectively as substrate. CAM-KII was assayed in 50 mM Hepes, pH 7.4, containing 5 mM $CaCl_2$ and 0.03 mg/ml calmodulin, with the peptide KKLNRTLSVA (60 μM) as substrate. JNK1, ROCK-II and PRK2 were assayed in 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 0.1% 2-mercaptoethanol with the following peptides: JNK1, 3 μM GST–ATF2-(19–96) (where ATF2 is activating transcription factor 2); PRK2, 30 μM AKRRRLSSLRA; ROCK-II,

KEAKEKRQEQIAKRRRLSSLRASTSKSGGSQK. MLCK was assayed as for JNK1, ROCK-II and PRK2, except that EGTA was replaced by 0.1 mM $CaCl_2$, calmodulin (0.1 μM) was included and the substrate was skeletal muscle heavy meromyosin (0.5 mg/ml) (SkMLCK) or 100 μM KKRAARATSNVFA (SmMLCK). AMPK was assayed in 50 mM HEPES, pH 7.4, 1 mM dithiothreitol, 0.02% Brij-35 and 0.2 mM AMP, with HMRSAMSGLHLVKRR (0.2 mM) as substrate. PKCα was assayed in 20 mM Hepes, pH 7.4, 0.03% Triton X-100, 0.1 mM $CaCl_2$, 0.1 mg/ml phosphatidylserine and 10 μg/ml 1,2-dioleoyl-*sn*-glycerol, with 0.1 mg/ml histone H1 as substrate. PKC was assayed in an identical manner to PKC, except that $CaCl_2$ was replaced with 0.1 mM EGTA. CK2 was assayed in 20 mM Hepes, pH 7.6, 150 mM NaCl, 0.1 mM EDTA, 5 mM dithiothreitol and 0.1% Triton X-100, with RRRDDDSDDD (165 μM) as substrate. LCK was assayed in 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 0.1 mM Na_3VO_4, using KVEKIGEGTYGVVYK (250 μM) as substrate. PHK was assayed in 50 mM Tris, 50 mM sodium β-glycerophosphate, pH 8.6, and 0.04 mM $CaCl_2$, using phosphorylase b (0.5 mg/ml) as substrate. MKK1 was assayed via its ability to activate ERK2 (0.07 mg/ml) in incubations containing 25 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 0.01% Brij-35 and MgATP. After incubation for 15 minutes at 30 °C, activated ERK2 was assayed as described above. PDK1 was assayed in 50 mM Tris/HCl, pH 7.5, containing 0.1% 2-mercaptoethanol, using the peptide KTFCGTPEYLAPEVRREPRILSEEEQEMFRDFDYIADWC (PDKtide) as substrate. PI 3-kinase was assayed as described above (see 2.14).

2.16 ABTS ASSAY

The activity of HRP was determined using the ABTS chromogen (as described by Nunez-Delicado et al., 2003). The peroxidase catalysed reduction of hydrogen peroxide to water is coupled to the 1 electron oxidation of ABTS, which forms a stable cation. This cation makes a bright green/blue solution, the absorbance of which can be measured at 405 nm. One nM of HRP and 1 mM of ABTS (final concentration) were incubated in 100 μL sodium phosphate buffer (pH 7.4). The reaction was initiated by the addition of 250 μM hydrogen peroxide (final concentration). Reactions were carried out in a 96-well plate and the absorbance continuously measured at 405 nm for 6 minutes. In some cases various concentrations of resveratrol were added. Resveratrol was diluted in 100 mM sodium phosphate buffer before addition to the reaction mixture.

2.17 FLOW CYTOMETRIC DETERMINATION OF CELL DEATH

U937 monocytes (differentiated and undifferentiated) were treated and stimulated for the indicated times in 24 well plates. The cells were harvested in 1 mL microtubes, making sure that all the cells were removed from the plate. After being spun at 500x g they were washed in BSS and finally resuspended in 500 μL of annexin-V binding buffer. The cells were transferred to FACS tubes and then incubated with FITC (fluorescein-isothiocyanate)-conjugated annexin-V (100 ng/mL) for 10 minutes, after which 12.5 μL of PI (of 50 μg per mL solution) was added. The cells were then immediately analysed by flow cytometry.

The method was similar to that described in section 2.6. However, in this case 2 dot plots were obtained. The first was FCS against SSC (as in section 2.6) and the second was green-FL (FL1) against red-FL (FL2). The FL spectra measured by FL2 overlaps with FL1 fluorescence and to ensure both were being read independently of each other, compensation was used.

2.18 FOX ASSAY

The FOX (ferrous oxidation in xylenol orange) assay measures peroxide down to a concentration of 1 μM. The basis of this assay is the complexing of ferric ion (Fe^{+2}) by H_2O_2 in the presence of Xylenol Orange. Peroxides in the sample oxidise Fe^{+2} to Fe^{+3}. Fe^{+3} will form a coloured complex with xylenol orange that can be read at 560 nm. Several modifications have been published in order to increase the sensitivity of the FOX assay (using sorbitol; Gay and Gebicki, 2000) or use it to measure both lipid and protein hydroperoxides (Gay and Gebicki, 2003). A hydrogen peroxide standard curve was prepared (1-100 μM). A working solution of hydrogen peroxide was made from a 30% solution (8.8M). A working FOX reagent was prepared by mixing 1 volume of FOX1 solution with 100 of FOX2. The working FOX solution is stable for at least 12 hours. One volume of test sample or hydrogen peroxide standard was mixed with 10 volumes of working FOX reagent. The reaction was allowed to proceed at room temperature for 15-20 minutes, centrifuged for 1 min at 10 000x g and read at 560 nm. The concentration of hydrogen peroxide in a test sample was determined using the slope of the hydrogen peroxide standard cure.

2.19 ANALYSIS OF RESVERATROL OXIDATION

One μM HRP was added to 100 μL sodium phosphate buffer and mixed in a quartz cuvette. Using an U2000 spectrophotometer an absorbance spectrum was generated from 450 to 350 nm. In its resting state HRP produces a characteristic absorbance pattern with a peak in the 405 region. Fifty µM of hydrogen peroxide was added and the changes in the HRP spectrum measured by recording a series of scans. Fifty μM resveratrol was added (before and after the addition of hydrogen peroxide). The oxidation of resveratrol was measured by recoding a series of scans in the presence of 1 μM HRP/50 μM hydrogen peroxide. The absorbance of resveratrol is 305/317 nm and the phenoxyl radical form 253 nm.

2.20. DATA ANALYSIS

Data was analysed using an one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. A P value of <0.05 was considered significant (both 0.5* and 0.01** significance levels were used). The area under the kinetic curve was calculated using the trapezoidal rule, based on method shown below (Le Floch et al., 1990). IC_{50} values were estimated by using a method that is based on the logit-log plot (or pseudo-Hill plot) (Noh et al., 1999). The percentage of activity remaining at each inhibitor concentration is defined as p and I represents the concentration of inhibitor. The value calculated for, log $[p/(100-p)]$ was determined for each concentration of inhibitor $[I]$ used. This value was then added to log $[I]$. The mean of these values was used as an estimation of the IC_{50} value.

$$log\,[p/(100-p)] = n\,log\,[I] + n\,log\,IC^{50}$$

	B	D	
	Time (min)	Values	AREA
11	0	0	
12	5	0	((D11+D12)*(B12-B11))/2
12	10	0	((D12+D13)*(B13-B12))/2
14	15	0	0.000
15	20	0	0.000
16	25	0	0.000
17	30	0	0.000

AUC (0-X) 0.000 µg/ml.min

AUC (X-∞) = Conc last / λ2 (Kel)

= 0 / 0

= 0.000

AUC (0-X) = 0.000

AUC (X-∞) = 0

TOTAL AUC (0-∞) 0

CHAPTER THREE:

THE EFFECT OF RESVERATROL ON THE RESPIRATORY BURST OF U937 MONOCYTES

INTRODUCTION

ROS produced from phagocytes are thought to contribute towards the progression of many pathologies. The initial source of these oxidants arises from the activity of NADPH oxidase. This enzyme is found throughout the immune system and a number of non-phagocytic sources, such as vascular smooth muscle cells (Babior 1999). The main focus here is the activation of the respiratory burst in monocytes. In recent years antioxidant strategies for the prevention of atherosclerosis have attracted considerable attention. The monocyte/macrophage plays a key role in the development of atherosclerosis and therefore it is a potential pharmacological target for antioxidants, such as resveratrol. This study is concerned with the ability of resveratrol to modulate an important monocyte/macrophage function, the respiratory burst, the activation of which, contributes significantly to the oxidative burden in the development of atherosclerosis.

3.1 DIFFERENTIATION OF MONOCYTIC CELL LINES

In recent years the monocyte/macrophage has received much attention, due to the pivotal role it plays in the pathogenesis of atherosclerosis. They are difficult cells to study, as they make up less than 1% of the circulating white blood cells and using primary cells is not always practical. Moreover, when primary monocytes are cultivated they rapidly loose their capacity to produce ROS. Mouse macrophage cell lines (e.g. RAW 264) have provided a useful model and have been widely used. However, several important discoveries have been made using human monocytic cell lines that can be chemically differentiated. Several cell lines, such as U937, THP1 and HL60, have been extensively used. In their native form they are rapidly growing, easy to maintain and provide large numbers of cells. Upon differentiation with an appropriate stimulus (chemical or cytokine) the cells progress towards a mature monocyte/macrophage phenotype. During this process the cells become phagocytic, various receptor functions are gained and the subunits of NADPH oxidase are up-regulated. This corresponds with the ability to produce ROS by a respiratory burst.

Differentiation is controlled by the actions of various transcription factors, the balance between them and the signals that they receive deciding the fate of a developing cell. The haematopoietic stem cell (HSC) can differentiate into a common myeloid precursor (CMP), which is the starting point for red blood cells, megakaryocytes, monocytes, neutrophils and eosinophils (see figure 3.1). The U937 cell is a pre-monocyte, committed to the monocyte lineage, therefore differentiation results in a monocyte. However, the HL60 cell line is a pre-myeloid cell and depending on the differentiating agent used, can progress towards a neutrophil or a monocyte-like cell (Zhu and Emerson 2002).

U937 monocytes can be differentiated with several agents, including those that are polar and planar, such as DMSO (at 1.3% v/v) and dcAMP. 1,25-α-Dihydroxy-vitamin D_3 has also been shown to be an important regulator of cellular differentiation. The nuclear vitamin D receptors can be found in complexes with retinoic acid receptors (RXR and RAR). Differentiation by VitD has been shown to be enhanced by all-trans and 9-cis RA, which led to the use of retinoids as potential chemotherapeutic agents.

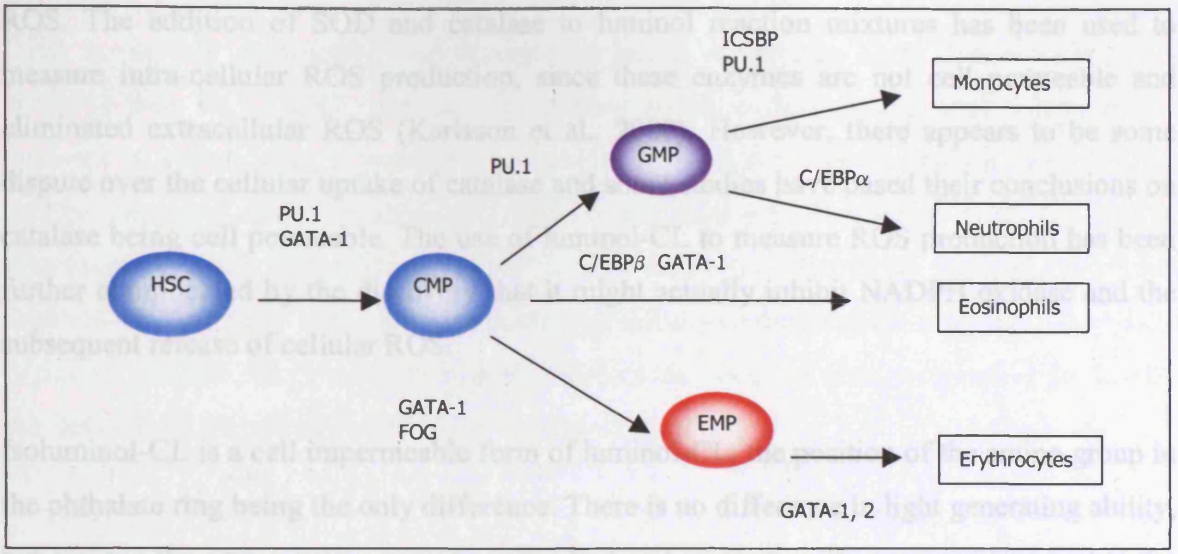

Figure 3.1 The transcriptional regulation of the common myeloid precursor (CMP) commitment. The haematopoietic stem cell (HSC) can be driven to the common myeloid precursor (CMP) through the dual expression of PU.1 and GATA-1. This is then the precursor for either a granulocytic-monocytic lineage (GMP) or an erythroid-megakaryocyte lineage (EMP). PU.1 expression is restricted to the GMP lineage, whereas the sole expression of GATA-1 leads to the EMP lineage. There is also a possible route to an eosinophil through the expression of C/EBPβ and GATA-1. Taken from Zhu and Emerson (2002).

3.1.2 MEASUREMENT OF CELLULAR ROS PRODUCTION

Receptor activation in phagocytic cells (or in some cases receptor-independent mechanisms) leads to the subsequent one or two step reduction of molecular oxygen to form a superoxide anion or hydrogen peroxide. The electron donor NADPH is formed from the hexose monophosphate shunt. Many techniques have been developed to measure the respiratory burst, although none provide the perfect method for measuring the production of ROS from phagocytes. Dahlgren and Karlsson (1999) have set criteria for a good measuring principle, the main requirements of which are: 1) high specificity for a particular reactive oxygen metabolite, 2) high sensitivity 3), no interference with cellular processes and 4) ability to distinguish where the ROS are generated. A probe that satisfies most of these criteria is luminol-CL.

Luminol is excited by ROS (in the presence of peroxidase) and the resultant energy is released in the form of light. Luminol-CL is an activity amplifier and the quantum yield of the molecule is very high, making the technique very sensitive. Luminol passes freely across cellular membranes, and therefore, measures both intra- and extracellular release of cellular

ROS. The addition of SOD and catalase to luminol reaction mixtures has been used to measure intra-cellular ROS production, since these enzymes are not cell permeable and eliminated extracellular ROS (Karlsson et al., 2000). However, there appears to be some dispute over the cellular uptake of catalase and some studies have based their conclusions on catalase being cell permeable. The use of luminol-CL to measure ROS production has been further complicated by the discovery that it might actually inhibit NADPH oxidase and the subsequent release of cellular ROS.

Isoluminol-CL is a cell impermeable form of luminol-CL, the position of the amino group in the phthalate ring being the only difference. There is no difference in light generating ability, but moving the amino group away from the first carbon atom in the aromatic ring makes the molecule more hydrophilic. Therefore, isoluminol-CL provides a highly sensitive method for measuring extracellular release of ROS. Precise kinetic measurements can be made and the probe does not affect the cellular production of ROS, which makes isoluminol-CL an excellent choice of probe (Dahlgren and Karlsson 1999).

Lucigenin is another commonly used probe to determine extracellular superoxide. The ability of lucigenin to measure superoxide is not dependent on peroxidases. However, under certain conditions it has been shown to redox cycle and form superoxide (Li et al., 1998).

The measurement of intracellular ROS production is slightly more problematical (as already discussed with luminol-CL). The most common method is with fluorescent dyes, such as 2,7-dichlorofluorescein diacetate (DCF) and dihydrorhodamine 123 (DHR). DCF, the most extensively studied fluorescent probe, rapidly diffuses across cellular membranes, where it is de-acetylated by the activity of intra-cellular esterases. De-acetylated DCF is non-fluorescent, but upon oxidation the molecule becomes highly fluorescent and can be measured using a fluorescence plate reader or fluorescence activated cell sorting (FACS). Like most probes, DCF is non-specific, however, as other non-oxidant DCF oxidations have been documented. DCF can be directly oxidised by cytochrome c (Burkitt and Wardman 2001) and changes in the glutathione content of cells can also lead to the oxidation of DCF (Jakubowski and Bartosz 2000). It can also leak from cells, bringing into question the ability to measure only intra-cellular ROS production. There are DCF analogues that have better cellular retention. These problems have brought the use of DCF into question, but despite these technical difficulties, it still remains a popular probe for measuring the cellular redox status.

DHR is another commonly used fluorescent probe, which has been shown to be more sensitive than DCF. DHR also rapidly diffuses across cellular membranes, where in the presence of peroxidases, it reacts with peroxides to yield a highly fluorescent molecule that can be measured using a fluorescence plate reader or a FACS machine. DHR has similar drawbacks to DCF, although its oxidation has not been extensively studied. The main problem of using DCF and DHR to measure the activity of the intra-cellular respiratory burst is due to kinetics. The time of maximal fluorescence does not always represent the kinetic window where the activity of NADPH oxidase is maximal.

RESULTS

3.2 DIFFERENTIATION OF U937 MONOCYTES BY RETINOIC ACID AND VITAMIN D_3

As previously discussed monocytes/macrophages are difficult cells to study and therefore suitable substitute cell models have to be used. The U937 cell line was chosen as the main cell model in this study. There are many agents that can be used to differentiate these cells, RA and VitD were also chosen for their consistency to induce an oxidative capacity. After treatment with these agents the cell population remains viable (see below) and unlike other differentiating agents e.g. low dose PMA, the cells remain in suspension with only minimal adherence.

Figure 3.2A shows the effect that RA and VitD have on the growth of U937 cells, where it can be seen that there is a slight alteration in the kinetics in the growth of differentiating cells, but the total cell number after 4 days is similar to the untreated cells. The expression of the p47 subunit of NADPH oxidase differs greatly between differentiated and undifferentiated cells (Figure 3.2B). The combination of RA and VitD is thought to result in a greater degree of differentiation than the sole use of either compound. However, the use of RA alone is capable of inducing up-regulation of p47.

Treating U937 cells with a combination of 1 μM retinoic acid (RA) and 10 nM vitamin D_3 (VitD) over a period of 4 days was found to up-regulate the p47 NADPH oxidase subunit. Several authors have investigated the efficiency of RA and VitD to induce differentiation in U937 cells. Chateau et al., (1996) suggested using equal doses of RA and VitD (at 0.1 μM); however, here the use of 1 μM RA and 10 nM VitD has produced satisfactory results.

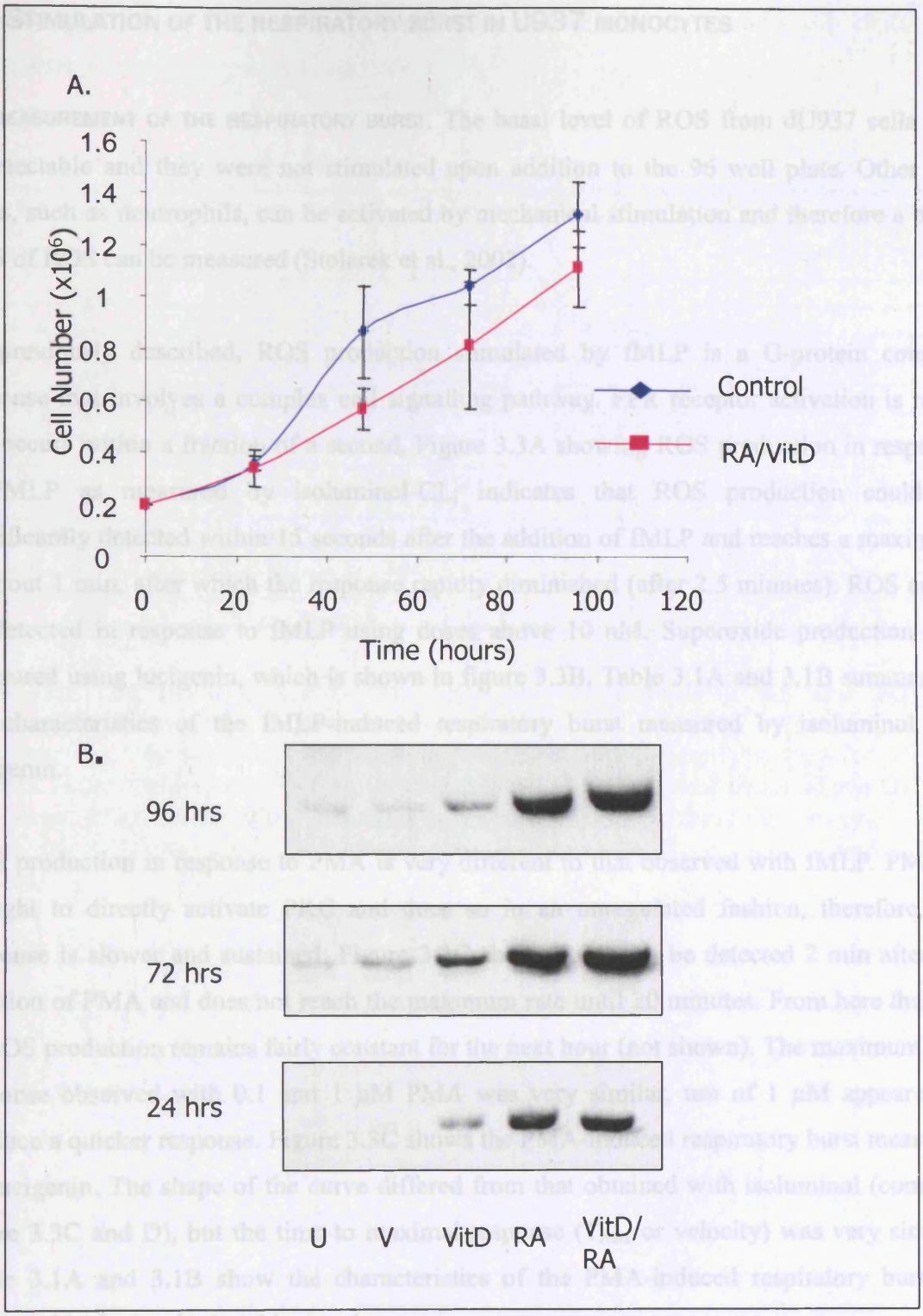

Figure 3.2 Differentiation up-regulates NADPH oxidase sub-units in U937 monocytes. A) The effect of 1 μM retinoic acid (RA) and 10 nM vitamin D_3 (VitD) on the growth of U937 monocytes. Results shown are means (±S.D) of three experiments. B) The effect of RA (1 μM) and VitD (10 nM) combinations on p47 expression in U937 monocytes as measured by western blotting. U, untreated; V, vehicle (DMSO). Representative of three experiments.

3.3 STIMULATION OF THE RESPIRATORY BURST IN U937 MONOCYTES

CL-MEASUREMENT OF THE RESPIRATORY BURST. The basal level of ROS from dU937 cells was undetectable and they were not stimulated upon addition to the 96 well plate. Other cell types, such as neutrophils, can be activated by mechanical stimulation and therefore a basal level of ROS can be measured (Stolarek et al., 2002).

As previously described, ROS production stimulated by fMLP is a G-protein coupled response that involves a complex cell signalling pathway. FPR receptor activation is rapid and occurs within a fraction of a second. Figure 3.3A showing ROS production in response to fMLP as measured by isoluminol-CL, indicates that ROS production could be significantly detected within 15 seconds after the addition of fMLP and reaches a maximum at about 1 min, after which the response rapidly diminished (after 2.5 minutes). ROS could be detected in response to fMLP using doses above 10 nM. Superoxide production was measured using lucigenin, which is shown in figure 3.3B. Table 3.1A and 3.1B summarises the characteristics of the fMLP-induced respiratory burst measured by isoluminol and lucigenin.

ROS production in response to PMA is very different to that observed with fMLP. PMA is thought to directly activate PKC and does so in an unregulated fashion, therefore, the response is slower and sustained. Figure 3.3C shows ROS can be detected 2 min after the addition of PMA and does not reach the maximum rate until 20 minutes. From here the rate of ROS production remains fairly constant for the next hour (not shown). The maximum CL-response observed with 0.1 and 1 μM PMA was very similar, use of 1 μM appeared to produce a quicker response. Figure 3.3C shows the PMA-induced respiratory burst measured by lucigenin. The shape of the curve differed from that obtained with isoluminol (compare figure 3.3C and D), but the time to maximal response (T_{max} or velocity) was very similar. Table 3.1A and 3.1B show the characteristics of the PMA-induced respiratory burst as measured by isoluminol and lucigenin.

Extracellular ROS production induced by AA (measured by isoluminol), was found to be rapid and reached a maximum within 1 minute (figure 3.3E). The dose required to induce ROS production was found to be greater than 10 μM. Although AA induced the rapid production of ROS, the response was sustained over the entire measuring period.

Interestingly, AA did not induce a measurable response when extracellular superoxide production was being measured (see table 3.1B).

A. Isoluminol-CL	Dose (μM)	T_{max} (min)	I_{max} (RLU/10^3)	Phagocytic capacity (RLU/min 0-X) 10^3
fMLP	0.01	1	0.1	0.4
	0.1	0.9	3.2± 0.1	3.4± 0.4
	1	0.9	6.6± 0.2	8.7± 0.5
PMA	0.01	36.7± 3.2	4± 1	63.7± 20.7
	0.1	34± 3.5	7.4± 0.3	148.1± 31.4
	1	32± 4	8.6± 0.5	231.4± 10.6
AA	10	19.5± 2	5.7± 0.1	148.5± 6.5
	25	3.2± 0.3	13.8± 1.9	215.1± 7.5
	50	2.2± 0.3	16.2± 2.5	250.4± 22.5
B. Lucigenin-CL				
fMLP	1	0.83± 0.14	2.2± 0.8	3.8± 1.9
PMA	0.1	43	4.12± 0.9	65.6± 12.1
AA	50	-	-	-

Table 3.1 The characteristics of the isoluminol (extracellular ROS)- and lucigenin (superoxide)-CL responses induced by fMLP, PMA and AA. I_{max} refers to the maximum CL response, T_{max} represents the time to the maximum response. Phagocytic capacity refers to the area under the kinetic curve and was calculated 0-5 min for fMLP and 0-40 min (relative light units, RLU) for PMA and AA. Results are means (±SD) of three experiments. Results shown for fMLP, measured by isoluminol are means (±SD) of two experiments. (-), not detected.

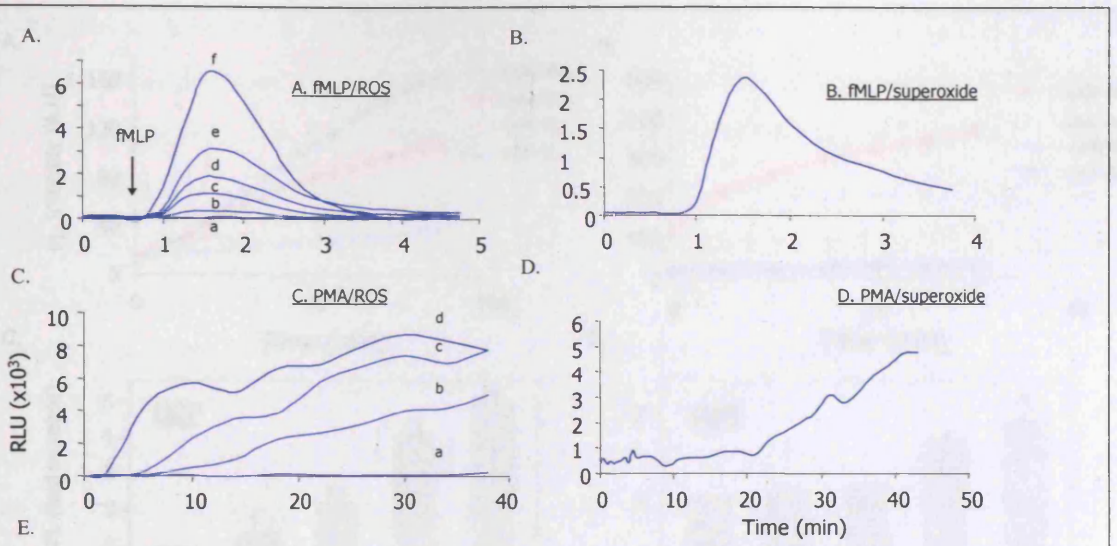

Figure 3.3 Activation of the respiratory burst in dU937 cells. **A)** dU937 cells were stimulated with fMLP at 45 seconds a) 0.01 μM, b) 0.04 μM, c) 0.06 μM, d) 0.08 μM, e) 0.1 μM, f) 1 μM measured by isoluminol. **B)** Cells were stimulated with 1 μM fMLP (at 45 seconds) measured by lucigenin. **C)** Cells were stimulated at time zero with PMA a) 0.001 μM, b) 0.01 μM, c) 0.1 μM, d) 1 μM, measured by isluminol. **D)** Cells stimulated with 100 nM PMA at time zero measured by lucigenin. **E)** Cells were stimulated with AA at time zero a) 10 μM, b) 25 μM, c) 50 μM, measured by isoluminol. AA did not induce a lucigenin-CL response (not shown).

FLUORESCENT MEASUREMENT OF THE RESPIRATORY BURST. Using a microplate method to measure fluorescence (bulk or total fluorescence), DCF was found to be an effective probe for the measurement of PMA and AA-induced ROS production, but not when using fMLP (not shown). The response measured using PMA (figure 3.4A, dose response in figure 3.4C) and AA (figure 3.4B) were found to be kinetically distinct, with AA producing a much faster and greater response. Using DHR as the probe to measure the respiratory burst produced similar results to that found with DCF. DHR was not able to measure fMLP-induced ROS production (not shown), but produced kinetically distinct responses when PMA (figure 3.4A, dose response in figure 3.4D) or AA (figure 3.4B) were used as the stimuli. DCF was found to be the superior fluorescent probe, since it produced a greater response than DHR, while still having low baseline values.

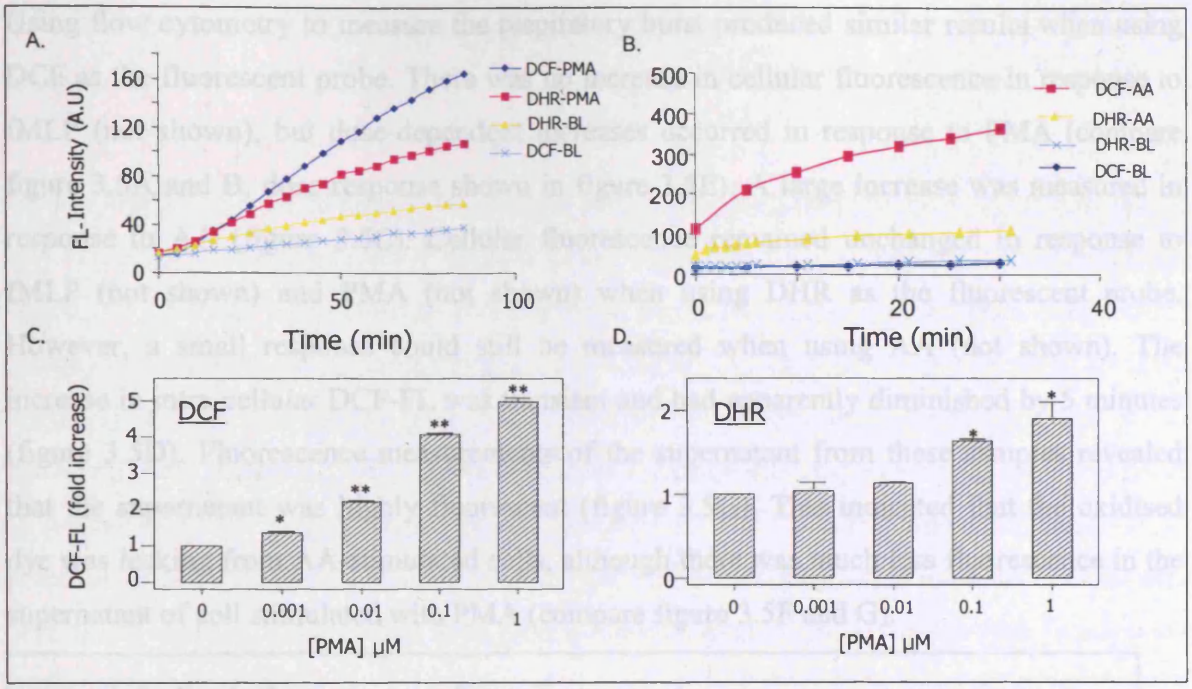

Figure 3.4 The effect of PMA and AA on DCF and DHR-FL (total ROS). dU937 cells were loaded with 2 µM DCF or DHR for 5 min before stimulation with 50 µM AA or the indicated dose of PMA. A) The typical response to PMA (1 µM) as measured by DCF or DHR. B) The typical response to AA (50 µM) as measured by DCF and DHR. dU937 cells were loaded with (C) DCF or (D) DHR (as described above) before stimulation with PMA (0.001-1 µM) for 85 minutes. Results shown are represented as fold increase from base line (BL). Results are means (±SD) of three experiments. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the unstimulated cells as determined by a one way ANOVA.

Using flow cytometry to measure the respiratory burst produced similar results when using DCF as the fluorescent probe. There was no increase in cellular fluorescence in response to fMLP (not shown), but dose-dependent increases occurred in response to PMA (compare figure 3.5A and B, dose response shown in figure 3.5E). A large increase was measured in response to AA (figure 3.5C). Cellular fluorescence remained unchanged in response to fMLP (not shown) and PMA (not shown) when using DHR as the fluorescent probe. However, a small response could still be measured when using AA (not shown). The increase in intra-cellular DCF-FL was transient and had apparently diminished by 5 minutes (figure 3.5D). Fluorescence measurements of the supernatant from these samples revealed that the supernatant was highly fluorescent (figure 3.5G). This indicated that the oxidised dye was leaking from AA-stimulated cells, although there was much less fluorescence in the supernatant of cell stimulated with PMA (compare figure 3.5F and G).

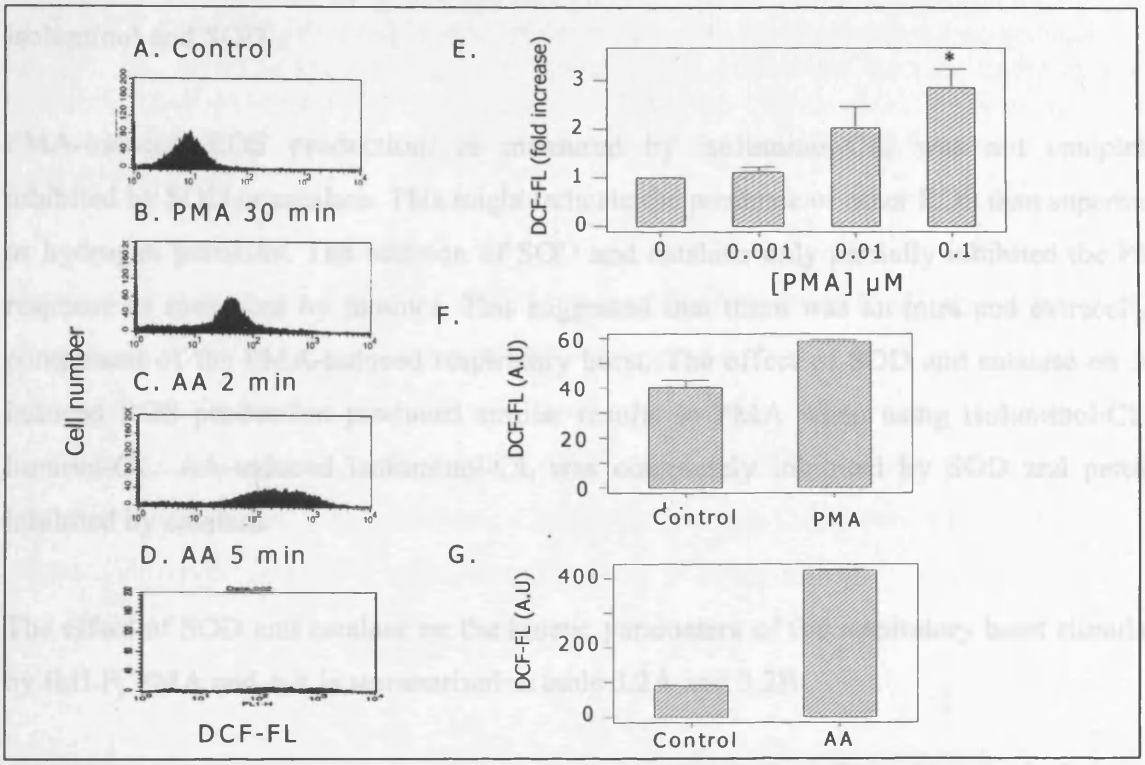

Figure 3.5 The effect of PMA and AA on DCF-FL measured by flow cytometry (cellular ROS). dU937 monocytes were pre-loaded with 2 µM DCF for 5 min before stimulation with 0.1 µM PMA or 50 µM AA. A and B are histograms (cell number against DCF-FL) showing the changes in intracellular fluorescence for control and PMA stimulated cells. The change in DCF-FL after a 2 min (C) or 5 min (D) AA stimulation is also shown as a histogram. E) PMA (µM) dose-response measured by DCF. Results shown are means (±SD) of three experiments, statistical significance of control vs PMA stimulated cells measured by a one way ANOVA, $p<0.05$*. F) Cells were stimulated with 0.1 µM PMA for 30 minutes in the presence of DCF (as previously described) the cells were centrifuged and the fluorescence of the supernatant was then measured (control vs stimulated). G) As F except that 50 µM AA was the stimulus and cells were stimulated for 5 minutes before the supernatant was measured.

3.4 THE EFFECT OF SOD AND CATALASE ON THE U937 MONOCYTE RESPIRATORY BURST.

CHEMILUMINESCENCE MEASUREMENTS. Catalase and SOD were used to investigate the nature and the location of the reactive species being measured by the luminenscent/fluorescent probes. It is well established that isoluminol measures extra-cellular ROS production, although the nature of the reactive species is unclear. Pre-incubating cells with SOD (200 units/mL) before stimulation with fMLP reduced the isoluminol response by over 90%. Catalase (1000 units/mL) had a much less of an effect on this response. If luminol was used as the probe, where both intracellular and extracellular ROS is being measured, an identical response was produced in response to fMLP (not shown). Using a combination of SOD and catalase to inhibit this response produced a similar level of inhibition to that found with isoluminol and SOD.

PMA-induced ROS production, as measured by isoluminol-CL, was not completely inhibited by SOD or catalase. This might indicate the presence of other ROS than superoxide or hydrogen peroxide. The addition of SOD and catalase only partially inhibited the PMA response as measured by luminol. This suggested that there was an intra and extracellular component of the PMA-induced respiratory burst. The effect of SOD and catalase on AA-induced ROS production produced similar results to PMA when using isoluminol-CL or luminol-CL. AA-induced isoluminol-CL was completely inhibited by SOD and partially inhibited by catalase.

The effect of SOD and catalase on the kinetic parameters of the respiratory burst stimulated by fMLP, PMA and AA is summarised in table 3.2A and 3.2B.

A. Luminol-CL	T_{max}(min)	I_{max} (%)	Phagocytic capacity (%)
fMLP	0.75	100	100
fMLP+SOD/CAT	1.25	6.3± 0.3	10.1± 1
PMA	39	100	100
PMA+SOD/CAT	34.3± 5	23.8± 1.5	25.8± 4.1
AA	2.2± 0.8	100	100
AA+SOD/CAT	2.8± 1	35.10± 7.17	37.5± 6.9
B. Isoluminol-CL			
fMLP	1	100	100
fMLP+SOD	1	5.2± 4.5	8.8± 3.6
fMLP+CAT	1.17± 0.29	57.6± 11.4	51.8± 10.9
PMA	3.8± 0.3	100	100
PMA+SOD	35.7± 4.2	16.3± 3.8	12.1± 1.8
PMA+CAT	41.7± 0.3	33.1± 5.2	20.9± 3.3
AA	6± 1.5	100	100
AA+SOD	16.6± 21	2.2± 0.7	2.3± 1.2
AA+CAT	7± 2	36.2± 3.1	31.1± 2.4

Table 3.2 The effect of SOD and catalase (1000 units/mL) on fMLP (0.1 μM), PMA (0.1 μM) and AA (50 μM)-induced luminol (total ROS) – (A) and isoluminol (extracellular ROS) CL (B). I_{max} refers to the maximum CL response, T_{max} represents the time to the maximum response. Phagocytic capacity refers to the area under the kinetic curve and was calculated 0-5 min for fMLP and 0-40 min for PMA and AA. Results shown are means (±SD) of three determinations.

FL-MEASUREMENTS. Using DCF or DHR to measure PMA-induced ROS production the effect of extracellular SOD or catalase appeared to be minimal. There was no effect of either SOD or catalase on PMA-induced DCF or DHR-FL measured by flow cytometry or fluorimetry (not shown). However, if the level of DCF-FL was measured in the supernatant of PMA-stimulated cells treated with catalase, a slight decrease in fluorescence was measured (not shown). However, this effect of catalase was found to be inconsistent.

3.5 THE EFFECT OF PRIMING AGENTS ON THE U937 MONOCYTE RESPIRATORY BURST

U937 cells can be stimulated to produce ROS in response to a variety of stimuli. However, in order to further investigate the activation mechanism in these cells, their ability to be primed in a manner described for neutrophils was investigated (see Chapter 1.5.4). Phagocytes do not usually produce high levels of ROS upon exposure to a priming agent, but ROS production can be greatly enhanced when the cells are subsequently stimulated. The effect of resveratrol would then be determined on any observed priming affect.

The results for the effect of priming agents on dU937 monocytes are summarised in table 3.3. ROS production could not be detected upon exposure to these priming agents (under the conditions described).

AGE. AGE has been shown to prime neutrophils under the conditions used in this study (Wong et al., 2002). Pre-treatment with AGE-BSA or minimally-modified AGE (mmAGE) (for 2 hours) had no effect on either PMA- or fMLP-induced ROS production in dU937 monocytes. There was no effect of AGE on I_{max} or the total phagocytic capacity. The T_{max} was also unaffected.

TNF AND IFN. TNF has been used as the classical priming agent by many investigators. The conditions used in this study were similar to those used by Cadwallader et al., 2002. Pre-treatment with TNFα (10 μg/mL for 30 min) had no effect on AA or PMA-induced ROS. There was no effect on I_{max} or the total phagocytic capacity. TNFα had a small effect on fMLP-induced ROS, with changes in I_{max} and phagocytic capacity, but not T_{max}. IFNγ (100 units/mL for 30 minutes) did not affect fMLP, AA or PMA-induced ROS, with no effect on the kinetics of NADPH oxidase activation or total phagocytic capacity.

HYDROGEN PEROXIDE. Some authors have identified redox sensitive signalling pathways that have been shown to prime macrophages (see Forman and Torres, 2002 and references therein). Pre-treating dU937 monocytes with hydrogen peroxide (100 μM 30 minutes) did not affect PMA or AA-induced ROS production, either total phagocytic capacity or T_{max}. However, fMLP-induced ROS was marginally affected by hydrogen peroxide, with changes in I_{max} and phagocytic capacity.

AA. Low doses of AA have been shown to induce the membrane translocation of PKC, which raises the possibility that pre-treatment with a low dose of AA would be able to enhance the subsequent stimulatory effects of fMLP or AA. Pre-treating dU937 monocytes with a low dose of AA (5 μM, 2 minutes, not sufficient to activate ROS) did not affect PMA- or AA–induced (50 μM) ROS production, with no changes in I_{max}, phagocytic capacity, T_{max} or rate. However, fMLP-induced ROS production was reduced by pre-treatment with AA.

Stimulus	Priming Agent	T_{max} (%)	I_{max} (%)	Phagocytic capacity (%)
fMLP	AGE	100	87.2± 15.6	95.2± 8.4
PMA*	AGE	110.8± 15.3	98.2± 10.5	104.2± 20.2
fMLP	mmAGE	100	96.2± 0.9	86.3± 25.6
PMA*	mmAGE	95.6± 6.8	104.8± 6.3	94.3± 8.6
fMLP	TNF	88.3± 17.2	154.8± 19.2	156.4± 28.2
PMA	TNF	115.2± 10.4	95.6± 24.2	96.7± 15.2
AA	TNF	120.2± 15.6	109.5± 6	110.6± 7.8
fMLP	AA	110.5± 8.9	13.9± 14.2	14.4± 14.5
PMA	AA	99.6± 10.1	106.7± 15.2	111.2± 15.2
AA	AA	105.6± 5.5	89.3± 17.2	92.3± 13
fMLP	H_2O_2	100	115.3± 1.9	118.7± 2.8
PMA	H_2O_2	105.2± 15.8	114.2± 8.8	109.7± 0.7
AA	H_2O_2	120.8± 19.2	121.5± 27.2	118.4± 5.6
fMLP	IFN	100	98.1± 15.3	102.2± 15.3
PMA	IFN	95.6± 30.2	110.2± 13.2	109.8± 14.7
AA	IFN	115.3± 18.3	98.3± 5.3	103.6 ±6

Table 3.3. The effect of priming agents on the dU937 respiratory burst. dU937 monocytes were pre-treated with AGE, 200 μg/mL; minimum-AGE (mmAGE), 200 μg/mL for 2 hours; TNFα 10 ng/mL, for 30 minutes; AA 5 μM, for 2 minutes; hydrogen peroxide, 100 μM for 30 minutes and IFNγ, 100 units/mL for 30 minutes. Cells were then stimulated with 1 μM fMLP, 100 nM PMA or 50 μM AA. ROS production was measured using isoluminol-CL, with the exception of those marked *, where luminol was used. I_{max} and phagocytic capacity was expressed as % stimulated control for TNF, IFN and hydrogen peroxide. AA pre-treated cells were expressed as a percentage of the cells stimulated in the presence of ethanol and AGE in the presence of BSA. Results shown are means (±SD) of three experiments.

3.6 OXIDATION OF POLYPHENOLS IN CELL CULTURE MEDIA

Recently the use of some polyphenolic compounds has come into question because of the possibility that they can generate hydrogen peroxide when applied to commonly used cell culture media. Therefore, before the antioxidant efficiency of a polyphenol can be determined, any possible interaction with media has to be determined. Incubating resveratrol in RPMI medium for 1 hour did not produce hydrogen peroxide. However, epigallocatechin-3-gallate (EGCG) did produce large quantities of hydrogen peroxide when incubated in RPMI (figure 3.7).

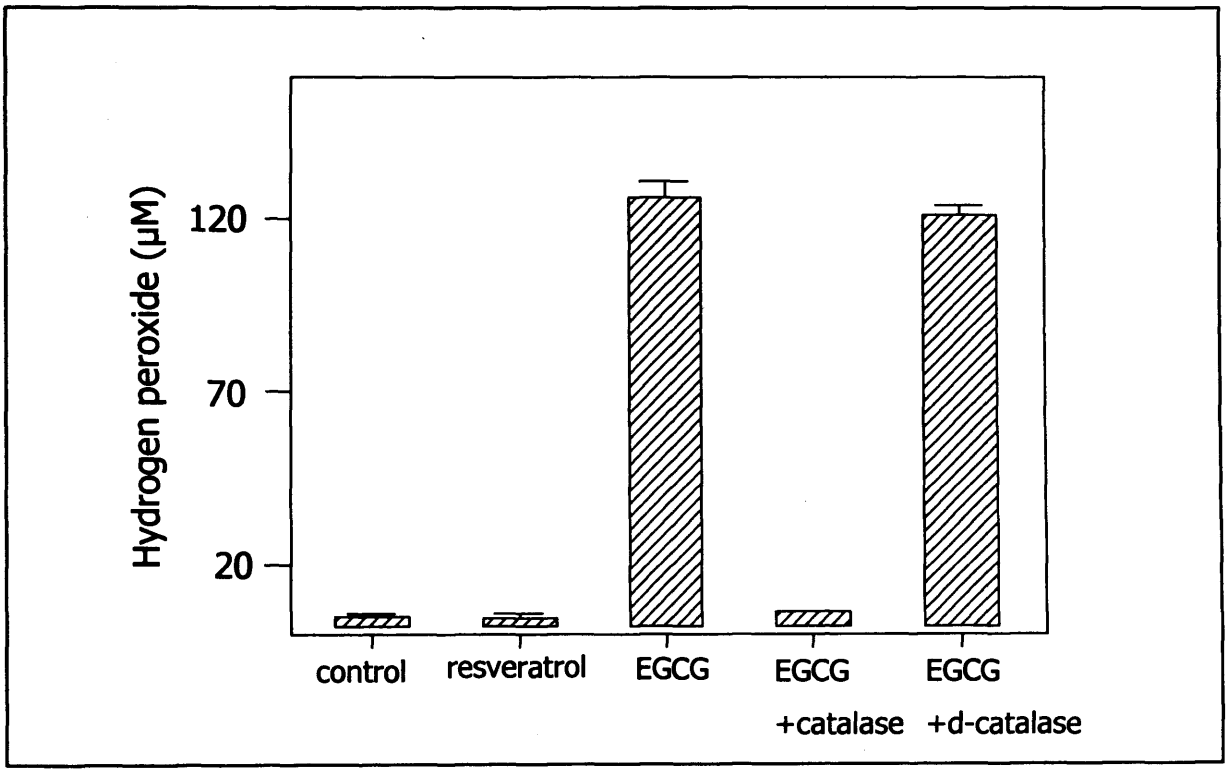

Figure 3.6 Oxidation of polyphenolic compounds in RPMI cell culture medium. Resveratrol (50 µM) did not generate significant amounts of hydrogen peroxide in RPMI. EGCG (100 µM) produced large amounts of hydrogen peroxide. This was confirmed using catalase and denatured catalase (d-catalase). Polyphenols were incubated in RPMI for 1 hour. Hydrogen peroxide was measured by ferrous oxidation in xylenol orange (FOX assay). Results shown are means (±SD) of three experiments.

3.7 THE EFFECT OF RESVERATROL ON THE U937 MONOCYTE RESPIRATORY BURST

CL-MEASUREMENTS. Two resveratrol treatment conditions were used in this study: treatment A consisted of pre-treatment of dU937 cells with resveratrol for 1 hour, followed by washing (one wash in balanced salt solution then re-suspension in balanced salt solution containing CL- or FL-probe of interest) and treatment B, where cells were re-suspended in balanced salt solution containing CL- or FL-probe of interest, incubated with resveratrol for 5 minutes and then stimulated. Treatment A resulted in a dose-dependent inhibition of fMLP-induced (1 μM) extracellular ROS production measured by isoluminol-CL. Resveratrol was found to be a potent inhibitor of this response with a dose-dependent decrease in I_{max} (figure 3.7A) and phagocytic capacity (IC_{50} 2.5 μM) (table 4.5A). There was no significant change in T_{max}. Moreover, the efficacy of resveratrol could be increased under the conditions of treatment B (IC_{50} 0.24 μM) (figure 3.7B). The T_{max} values for direct inhibition by resveratrol was also not significant. Similar results were obtained for luminol-CL (figure 3.7C, D and table 3.4B), with the IC_{50} values for treatment A and B estimated to be 1.5 vs 0.18 μM respectively.

The ability of resveratrol to inhibit PMA-induced (100 nM) ROS production measured by isoluminol-CL was also investigated. Treatment A (figure 3.8A) resulted in a dose-dependent decrease in I_{max} and phagocytic capacity (IC_{50} 5.6 μM). There was no significant change in T_{max}. Greater inhibition was achieved under the conditions of treatment B (inhibition of phagocytic capacity IC_{50} 1.4 μM) (figure 3.8B), but there was no significant change in T_{max}. Using luminol there was less inhibition for treatment A (inhibition of phagocytic capacity IC_{50} 23.8 μM) (figure 3.8C). Treatment B increased the inhibition (inhibition of phagocytic capacity IC_{50} 14.1 μM, figure 3.8D) and resulted in a significant change in T_{max} (see table 3.5A and B). Significantly less inhibition was measured if the dose of PMA was increased to 1 μM. This was apparent for both luminol and isoluminol-CL (table 3.5A and B). Lower doses of resveratrol appeared to increase the level of ROS stimulated by 1 μM PMA.

Resveratrol was found to dose-dependently inhibit AA-induced ROS production. Treatment A inhibited AA-induced isoluminol-CL (figure 3.9A and table 3.6A). I_{max} and the phagocytic capacity (IC_{50} 5.6 μM) were reduced, although T_{max} was not significantly affected (see table 3.6). The effect of resveratrol could be increased under the conditions of treatment B (figure

3.9B). Both I_{max} and phagocytic capacity (IC_{50} 0.5 μM) were inhibited and the T_{max} significantly decreased.

A. Isoluminol-CL	Dose (μM)	T_{max} (min)	I_{max} (%)	Phagocytic capacity (%)
fMLP Treatment A	0.1	100	83.3± 16.6	81.4± 3.5
	1	100	67.9± 0.5**	65.6± 1.1*
	10	100	27.7± 1.9**	27± 1.1**
	25	100	10.5± 4.4**	17.7± 9.4**
	50	83.3± 23.6	2.8± 0.9**	10.6± 8.2**
fMLP Treatment B	0.1	122.2±50.9	73.4± 13.4	79.9± 20.8
	1	88.9± 19.2	37.2± 27.8*	30.4± 23.2**
	10	66.7	1± 0.5**	1.1± 0.4**
	25	77.8± 19.2	0.3± 0.3**	0.7± 0.6**
B. Luminol-CL				
fMLP Treatment A	0.1	100	93.5± 11.6	83.7± 4.4*
	1	100	57± 14.4**	46.3± 6.6**
	10	107.1±10.1	13.1± 9.5**	3.2± 3.8**
	25	100± 9.1	21.1± 7.3**	22.7± 4.8**
	50	110.5	9.5± 4.9**	11.5± 5.2**
fMLP Treatment B	0.1	94.7	82.8± 16.7*	72.2± 15.9*
	1	105.3± 9.1	46.8± 1**	41.1± 3.9**
	10	105.3± 9.1	2.7± 0.6**	0.8± 0.2**
	25	110.5	0.6± 0.2**	0.3**

Table 3.4. A) The effect of resveratrol on fMLP-induced isoluminol (extracellular ROS) and (B) luminol (total ROS) -CL. dU937 monocytes were pre-treated with resveratrol for one hour (followed by washing, treatment A) or five minutes, before stimulation (treatment B) with 1 μM fMLP. I_{max} and phagocytic capacity are expressed as % RLU/min of cells stimulated in the presence of DMSO. Results shown are means (±SD) of three experiments (except for one-hour resveratrol measured by isoluminol, mean of four experiments). **($p<0.001$) and * ($p<0.05$) indicate significant differences from the DMSO (100%) control cells as determined by a one way ANOVA. B) as A) carried out with luminol.

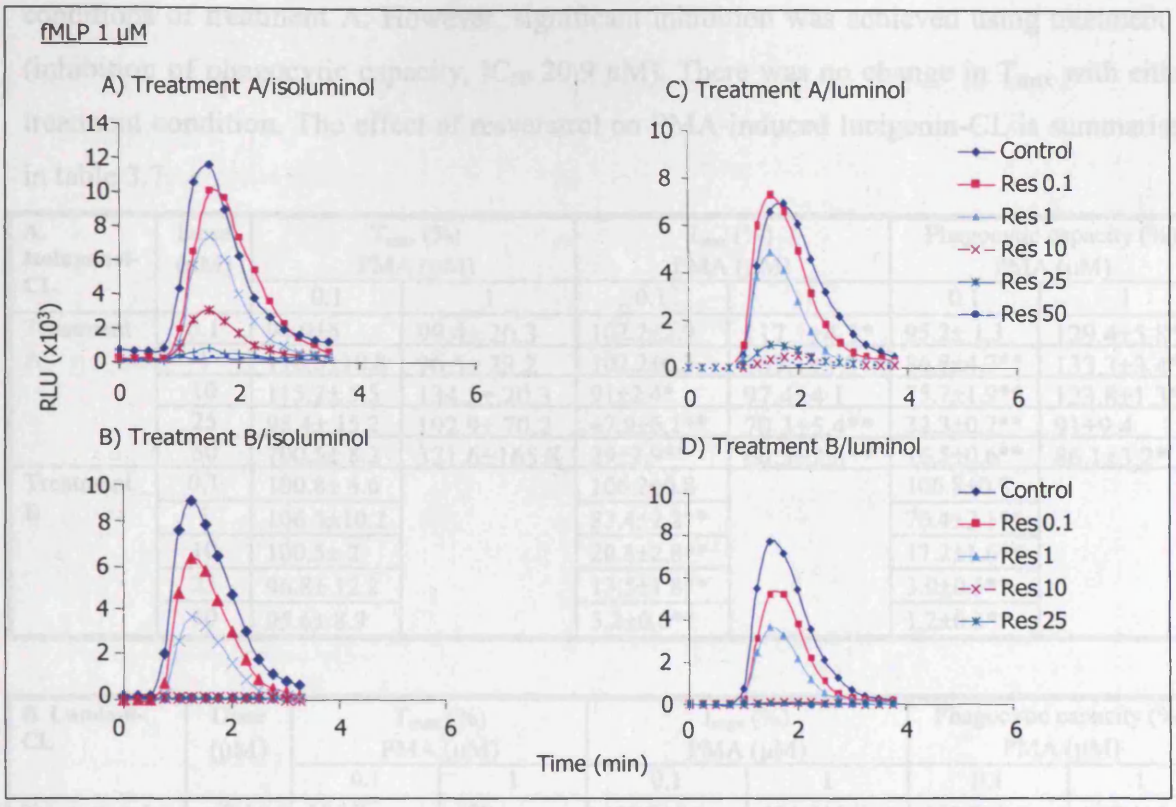

Figure 3.7 The effect of resveratrol on fMLP-induced ROS production in dU937 monocytes. Cells were pre-treated with resveratrol for 1 hour followed by washing (treatment A) (A and C) or added directly into the reaction mixture and incubated for 5 minutes (treatment B) (B and D). A and B were measured using isoluminol and C and D with luminol. Control represents fMLP-stimulated cells in the presence of DMSO. The CL-response for unstimulated cells or those treated with resveratrol alone is not shown, since it was found to be less than 0.05×10^3 RLU/min.

Using luminol-CL, resveratrol was found to be a less effective inhibitor under the conditions of treatment A (inhibition of phagocytic capacity, IC_{50} 54.1 μM) (figure 3.9C and table 3.6A). However, treatment B resulted in increased inhibition, similar to that found with isoluminol (inhibition of phagocytic capacity, IC_{50} 0.8 μM) (see figure 3.9D and table 3.6B). There was also a significant increase in T_{max} with 50 µM resveratrol.

The use of lucigenin-CL to measure the effect of resveratrol on the respiratory burst is shown in table 3.7. Resveratrol was found to inhibit fMLP-induced lucigenin-CL (inhibition of phagocytic capacity, IC_{50} 15.1 μM) (figure 3.10A and table 3.7). However, treatment B resulted in reduced inhibition (inhibition of phagocytic capacity, IC_{50} 45.3 μM) (figure 3.10B and table 3.7). There were no significant changes in T_{max} with either treatment condition. PMA-induced ROS was not significantly inhibited by resveratrol under the

conditions of treatment A. However, significant inhibition was achieved using treatment B (inhibition of phagocytic capacity, IC_{50} 20.9 μM). There was no change in T_{max} with either treatment condition. The effect of resveratrol on PMA-induced lucigenin-CL is summarised in table 3.7.

A. Isoluminol-CL	Dose (μM)	T_{max} (%) PMA (μM)		I_{max} (%) PMA (μM)		Phagocytic capacity (%) PMA (μM)	
		0.1	1	0.1	1	0.1	1
Treatment A	0.1	94.9±5	99.4± 20.3	102.2±2.9	117.1±4.3*	95.2± 1.3	129.4±5.8*
	1	110.5±10.8	96.5± 23.2	102.2±0.3	107.1±1.7	86.8±4.7**	133.3±3.4**
	10	115.2± 5.5	134.5± 20.3	91±2.4*	97.4±4.1	75.7±1.9**	123.8±1.3**
	25	98.4± 15.2	192.9± 70.2	47.9±6.1**	70.3±5.4**	32.3±0.7**	91±9.4
	50	100.5± 8.2	321.6±165.8	29±2.9**	60.5±2.8**	16.5±0.6**	86.1±3.2*
Treatment B	0.1	100.8± 4.6		106.2±0.8		106.8±0.8	
	1	106.5±10.2		83.4±2.2**		70.4±2.1**	
	10	100.5± 2		29.8±2.8**		17.2±1.6**	
	25	96.8± 12.2		13.5±1.8**		3.9±0.5**	
	50	95.6± 8.9		3.2±0.6**		1.2±0.3**	

B. Luminol-CL	Dose (μM)	T_{max} (%) PMA (μM)		I_{max} (%) PMA (μM)		Phagocytic capacity (%) PMA (μM)	
		0.1	1	0.1	1	0.1	1
Treatment A	0.1	101.7	100	103.3±3.8	131.8±8.6**	100.8±8.3	107.4±6.5
	1	100± 3	90.5±16.5	100.6±1.7	118.6±3.6	87.3±4.5	102.1±3.3
	10	98.3± 3	100	99.7±2.4	112.6±3.8	87.2±6.7	96.9±3.6
	25	98.3± 6	138.1±43.6	59.9±9.6**	113±4.3	42.1±8.6**	101±2.7
	50	96.5± 9	147.6±16.5	65±2.9**	78.3±3.6*	43.2±5**	73.4±2.1**
Treatment B	0.1	92.4±14.4		120.3±5.6		122.1±10.1	
	1	111.4±11.4		117.3±2.9		99.9±5.5	
	10	121±3.3		72.3±11.6*		38.6±10.2**	
	25	121±3.3		29.4±11.8**		10.1±6.5**	
	50	122.9*		13.6±12.5**		4.5±4**	

Table 3.5. A) The effect of resveratrol on PMA-induced isoluminol (extracellular ROS) and (B) luminol (total ROS) -CL. dU937 monocytes were pre-treated resveratrol for one hour (followed by washing, treatment A) or five minutes, before stimulation (treatment B) with 0.1 or 1 μM PMA. I_{max} and phagocytic capacity are expressed as % RLU/min of cells stimulated in the presence of DMSO. Results shown are means (±SD) of three. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (100%) control cells as determined by a one way ANOVA. B) as (A) except that luminol was used.

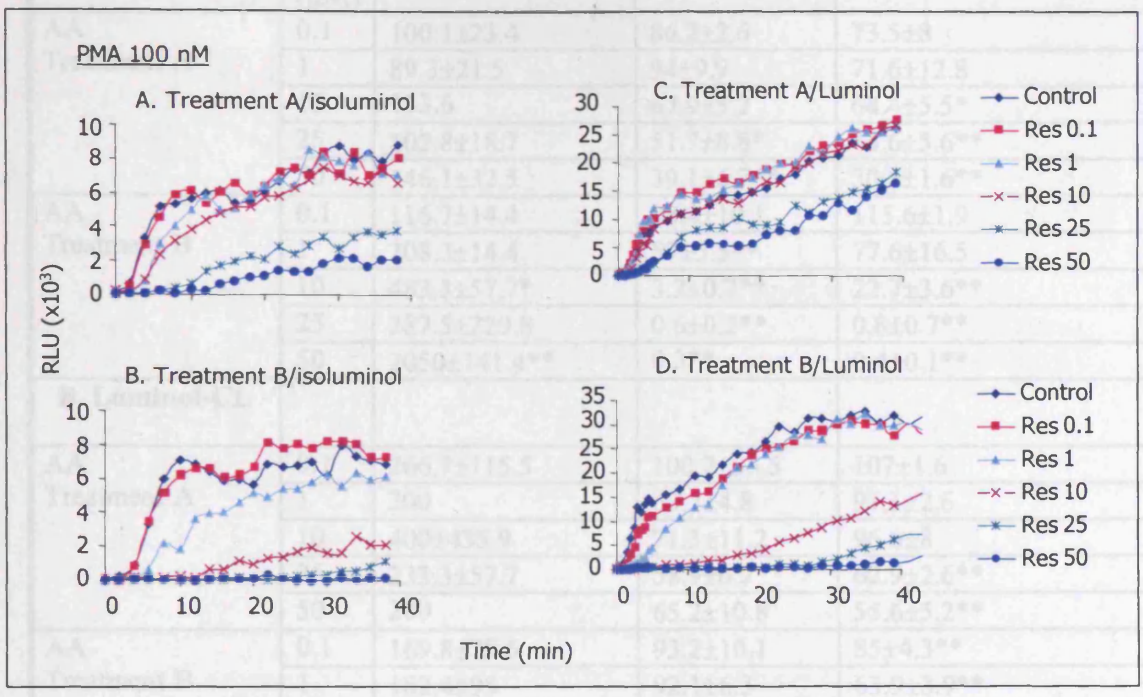

Figure 3.8 The effect of resveratrol on PMA-induced ROS production in dU937 monocytes. Cells were pre-treated with resveratrol for 1 hour followed by washing (treatment A) (A and C) or resveratrol was added directly into the reaction mixture and incubated for 5 minutes (treatment B) (B and D). A and B were measured using isoluminol and C and D made with luminol. Control represents PMA-stimulated cells in the presence of DMSO. The CL-response for unstimulated cells or those treated with resveratrol alone is not shown, since it was found to be less than $0.05x10^3$ RLU/min.

A. Isoluminol-CL	Dose (μM)	T_{max} (%)	I_{max} (%)	Phagocytic capacity (%)
AA Treatment A	0.1	100.1±23.4	86.2±2.6	73.5±8
	1	89.3±21.5	94±9.9	71.6±12.8
	10	113.6	67.9±5.2	64.4±5.5*
	25	102.8±18.7	51.7±8.8*	44.6±5.6**
	50	146.1±32.5	39.1±6.7**	30.5±1.6**
AA Treatment B	0.1	116.7±14.4	98.4±10.1	115.6±1.9
	1	208.3±14.4	57±5.5**	77.6±16.5
	10	483.3±57.7*	3.2±0.7**	22.7±3.6**
	25	287.5±229.8	0.6±0.2**	0.8±0.7**
	50	2050±141.4**	0.3**	0.4±0.1**
B. Luminol-CL				
AA Treatment A	0.1	266.7±115.5	100.2±33.5	107±1.6
	1	200	63.7±4.8	91.1±2.6
	10	400±435.9	71.3±11.2	96.6±8
	25	233.3±57.7	58.9±6.7	62.9±2.6**
	50	200	65.2±10.8	55.6±5.2**
AA Treatment B	0.1	169.8±75.5	93.2±10.1	85±4.3**
	1	182.4±95	92.1±6.3	63.9±3.9**
	10	358.5±196.1	12.1±10.4**	9.7±0.8**
	25	471.7±196.1	1.5±0.2**	0.8**
	50	628.9±106.7*	0.8±0.2**	0.4±0.1**

Table 3.6 A) The effect of resveratrol on AA-induced isoluminol (extracellular ROS) and (B) luminol (total ROS) -CL. dU937 monocytes were pre-treated with resveratrol for one hour (followed by washing, treatment A) or five minutes, before stimulation (treatment B) with 50 μM AA. I_{max} and phagocytic capacity are expressed as % RLU/min of cells stimulated in the presence of DMSO. Results shown are means (±SD) of three. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (100%) control cells as determined by a one way ANOVA. B) as (A) accept that luminol (total ROS production measured) was used.

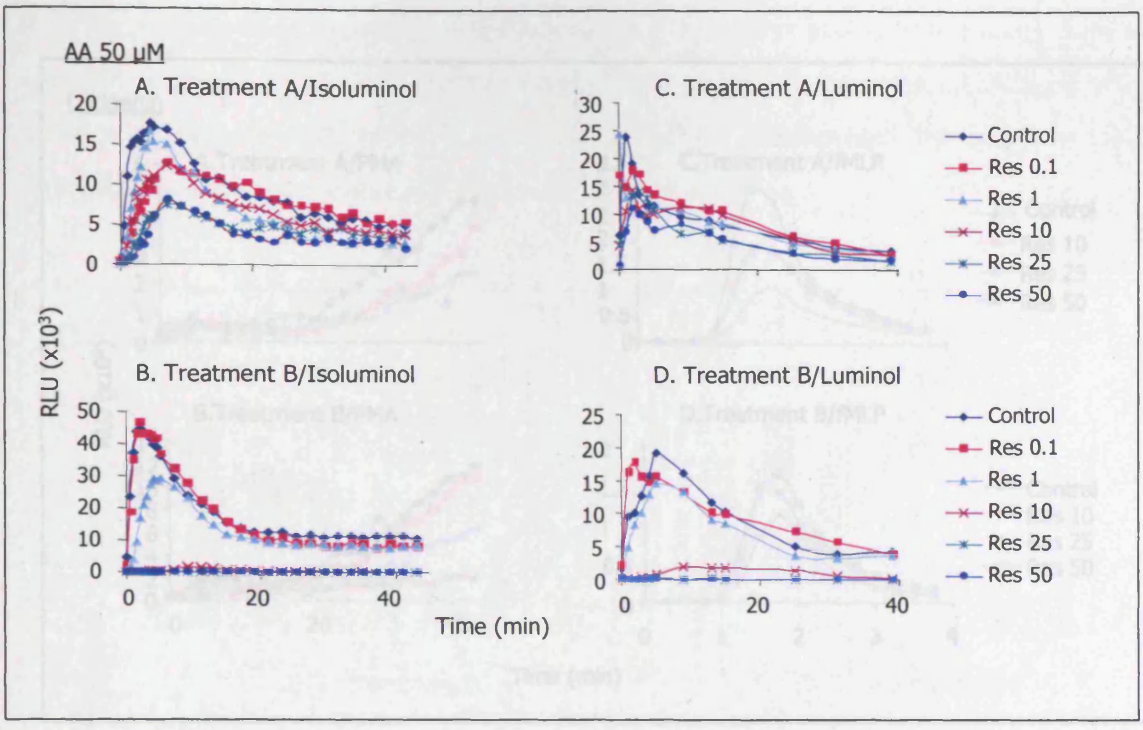

Figure 3.9 The effect of resveratrol on AA-induced ROS production in dU937 monocytes. Cells were pre-treated with resveratrol for 1 hour followed by washing (treatment A) (A and C) or added directly into the reaction mixture and incubated for 5 minutes (treatment B) (B and D). A and B were measured using isoluminol (extracellular ROS) and C and D using luminol (total ROS). Control represents AA-stimulated cells in the presence of DMSO. The CL-response for unstimulated cells or those treated with resveratrol alone is not shown, since it was found to be less than $0.05x10^3$ RLU/min.

	Dose (µM)	T_{max} (%)	I_{max} (%)	Phagocytic capacity (%)
fMLP 1µM Treatment A	10	100	78.7± 13.2*	51.8± 5.1**
	25	100.7±1.5	64.6± 6.5**	46± 2.6**
	50	101.3±1.8	52.5± 11.7 **	25.2± 7.8**
fMLP Treatment B	10	100	103.1± 27.8	80± 4.7*
	25	111.1±9.6	93± 25.4	64.9± 10.5**
	50	106.3±17.8	56.3± 19.6	50.2± 6.3**
PMA 100 nM Treatment A	10	100	83.2± 32.9	72.2± 21.2
	25	100	65.9± 4.6	69± 18.7
	50	100	71.6± 14.7	70.7± 12.2
PMA 100 nM Treatment B	10	100	87.2± 7.9*	77.7± 4.8**
	25	100	50.8± 2.5**	47.6± 0.8**
	50	100	14± 1.6**	18.8± 3.6**

Table 3.7. The effect of resveratrol on fMLP and PMA-induced lucigenin (superoxide)-CL. dU937 monocytes were pre-treated with resveratrol for one hour (followed by washing, treatment A) or five minutes, before stimulation (treatment B) with 1 µM fMLP or 100 nM PMA. I_{max} and phagocytic capacity are expressed as % RLU/min of cells stimulated in the presence of DMSO. Results shown are means (±SD) of three experiments. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (100%) control cells as determined by a one way ANOVA.

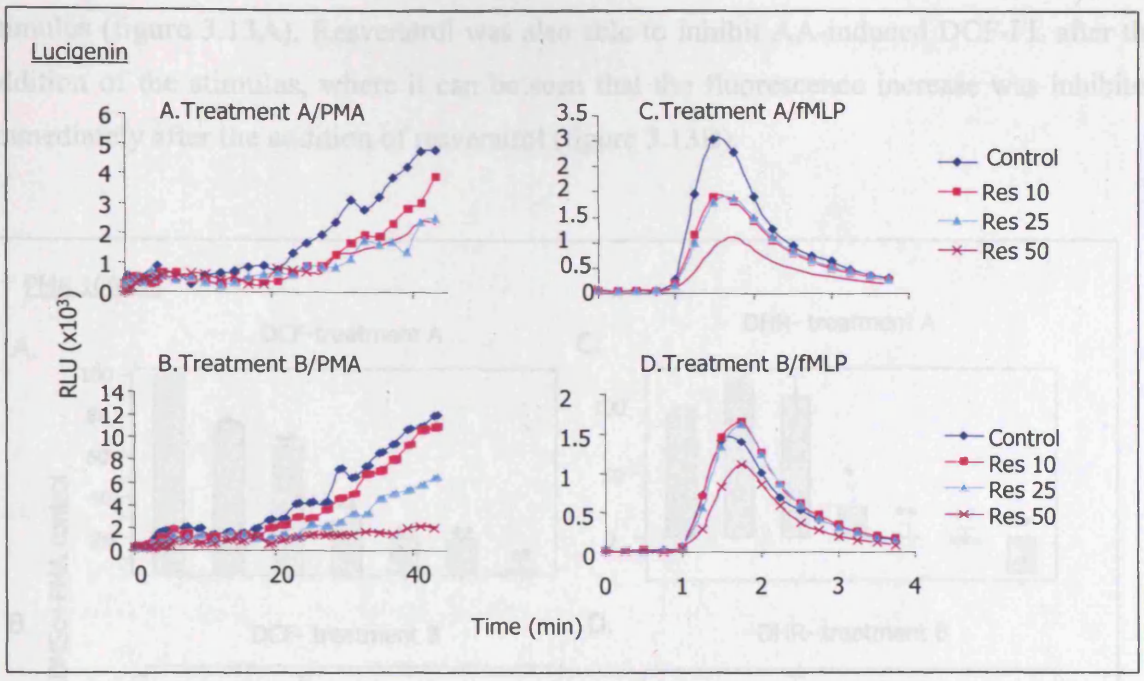

Figure 3.10 The effect of resveratrol on fMLP- and PMA-induced superoxide production in dU937 monocytes. Cells were pre-treated with resveratrol for 1 hour followed by washing (treatment A) (A and C) or resveratrol added directly into the reaction mixture and incubated for 5 minutes (treatment B) (B and D). A and B were stimulated with 100 nM PMA and C and D with 1 μM fMLP. Control represents fMLP- or PMA-stimulated cells in the presence of DMSO. The CL-response for unstimulated cells or those treated with resveratrol alone is not shown, since it was found to be less than 0.01×10^3 RLU/min.

FL-MEASUREMENTS. Using a fluorimeter to measure PMA- and AA-induced DCF- or DHR-FL it was found that resveratrol caused a dose-dependent inhibition. Treatment A resulted in potent inhibition of PMA-induced DCF-FL (IC_{50} 2.8 μM) (figure 3.11A). Treatment B did not result in a large increase in inhibition (IC_{50} 1.8 μM), which was seen with luminol or isoluminol (figure 3.11B). PMA-induced DHR-FL was also inhibited under the conditions of treatment A (IC_{50} 4.4 μM, figure 3.11C). Treatment B resulted in a less inhibition (IC_{50} 10.1 μM figure 3.11D). AA-induced DCF-FL was also inhibited by resveratrol. There was also less difference between the two treatment conditions for AA-induced DCF-FL (treatment A, IC50 2.7 μM and treatment B, 4.5μM) than seen in the previous section, using luminol or isoluminol. AA-induced DHR-FL was found to be dose-dependently inhibited by resveratrol using treatment condition A (IC_{50} 3.9 μM, figure 3.12C). Greater inhibition was achieved by adding resveratrol directly into the reaction mixture (IC_{50} 0.12 μM figure 3.12D).

Resveratrol was able to inhibit AA-induced DCF-FL when added at the same time as the stimulus (figure 3.13A). Resveratrol was also able to inhibit AA-induced DCF-FL after the addition of the stimulus, where it can be seen that the fluorescence increase was inhibited immediately after the addition of resveratrol (figure 3.13B).

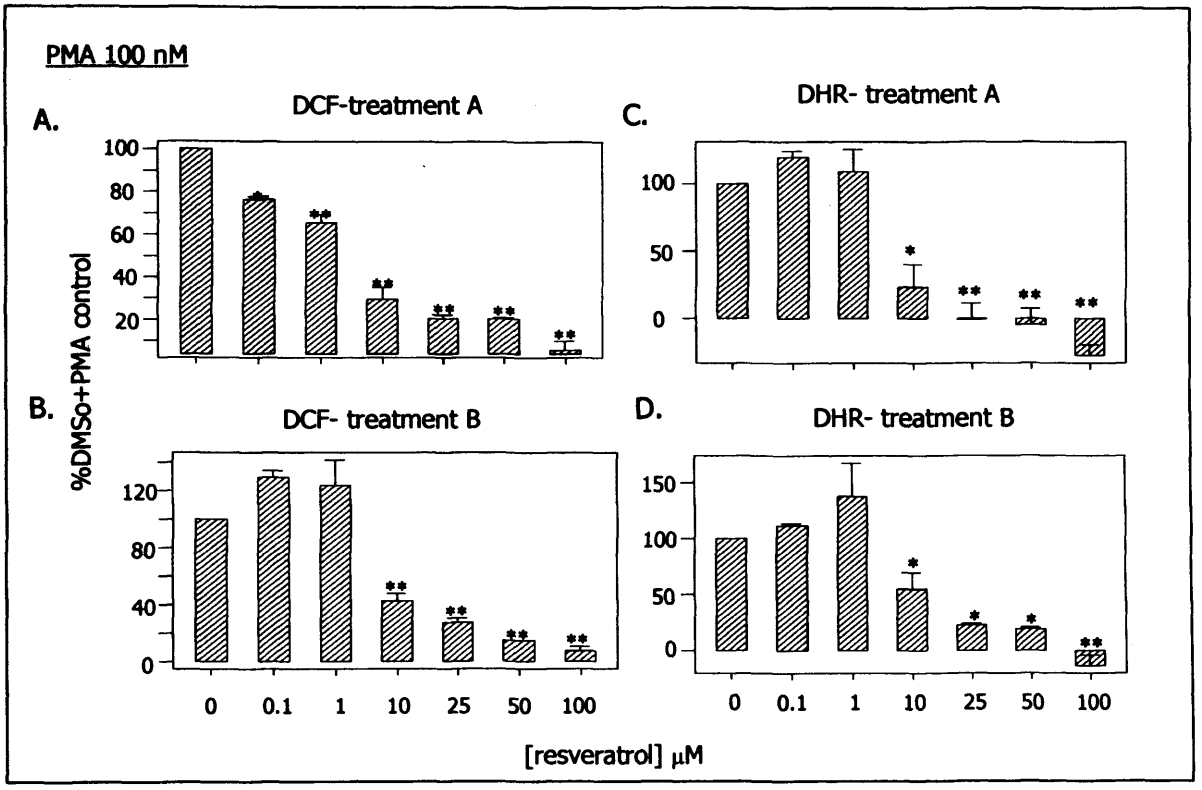

Figure 3.11 The effect of resveratrol on PMA-induced DCF- and DHR-FL (total ROS) in dU937 monocytes. Cells were pre-treated with resveratrol for 1 hour followed by washing (treatment A) (A and C) or resveratrol was added directly into the reaction mixture and incubated for 5 minutes (treatment B) (B and D). Cells were then stimulated with 100 nM PMA. Changes in total fluorescence were then measured for 85 minutes using a microplate reader. Results are expressed as % PMA-stimulated cells in the presence of DMSO (shown as 0). **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (100%) control cells as determined by a one way ANOVA.

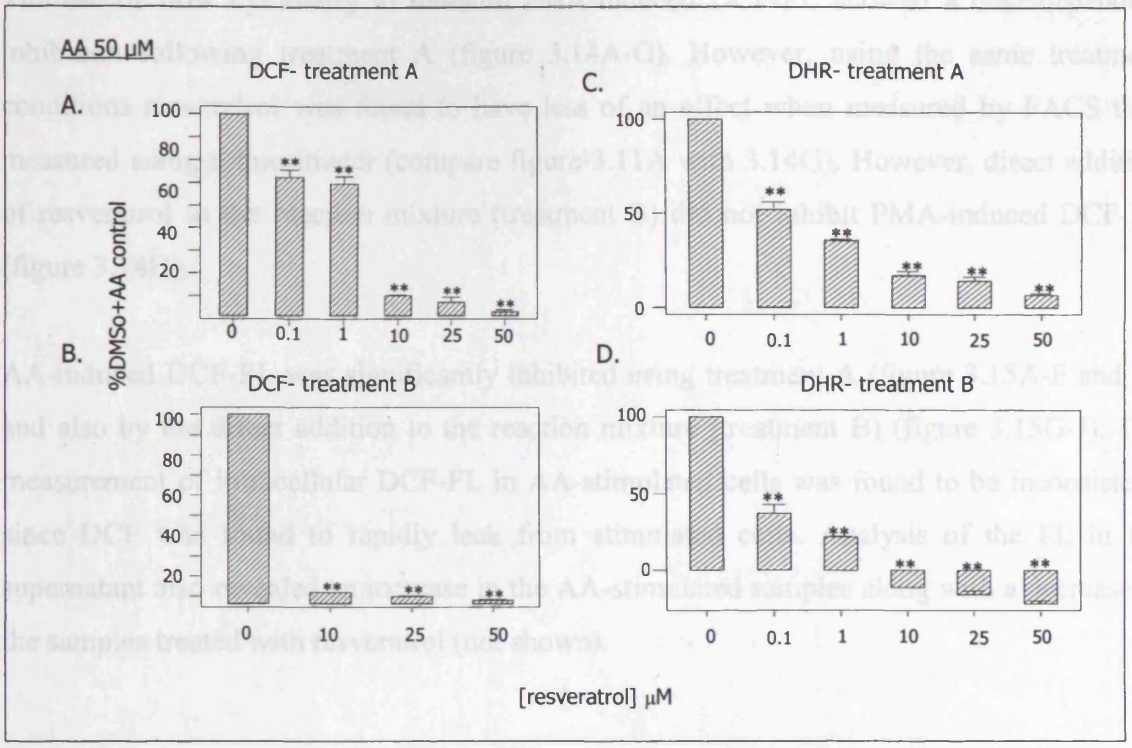

Figure 3.12 The effect of resveratrol on AA-induced DCF- and DHR-FL (total ROS) in dU937 monocytes. Cells were pre-treated with resveratrol for 1 hour followed by washing (treatment A) (A and C) or added directly into the reaction mixture and incubated for 5 minutes (treatment B) (B and D). Cells were then stimulated with 50 µM AA. Changes in total fluorescence were then measured for 30 minutes using a microplate reader. Results are expressed as % AA-stimulated cells in the presence of DMSO (shown as 0). **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (100%) control cells as determined by a one way ANOVA.

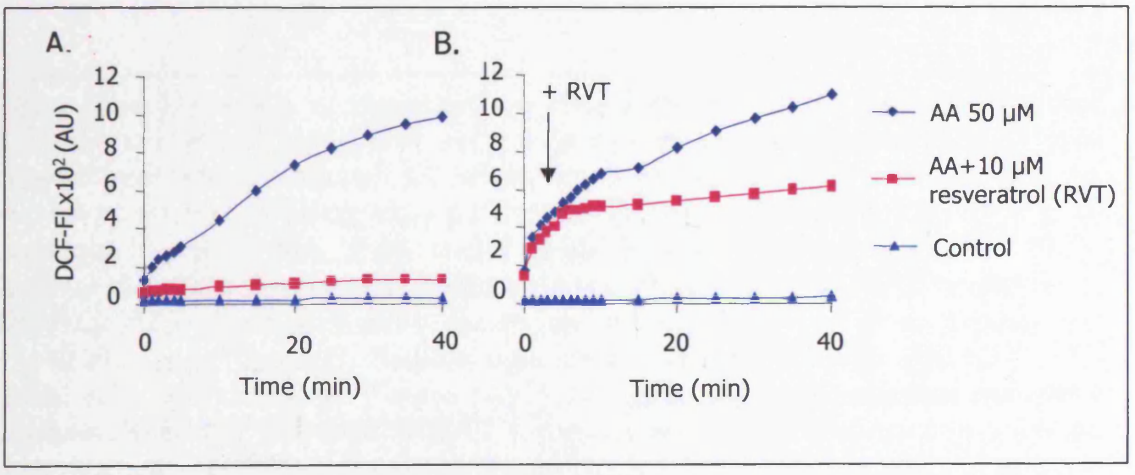

Figure 3.13 AA-induced DCF-FL (total ROS) can be inhibited when resveratrol is added with or after the stimulus. A) dU937 cells were loaded with 2 μM DCF for 5 minutes and stimulated with 50 μM AA or a mixture of AA and 10 μM resveratrol. B) as A except that cells were stimulated with 50 μM AA for 5 minutes before the addition of BSS containing 0.1% DMSO or 10 μM resveratrol (indicated by the arrow). Results shown are representative of two experiments.

The use of flow cytometry to measure PMA-induced DCF-FL showed a dose-dependent inhibition following treatment A (figure 3.14A-G). However, using the same treatment conditions resveratrol was found to have less of an effect when measured by FACS than measured using a fluorimeter (compare figure 3.11A with 3.14G). However, direct addition of resveratrol to the reaction mixture (treatment B) did not inhibit PMA-induced DCF-FL (figure 3.14H).

AA-induced DCF-FL was significantly inhibited using treatment A (figure 3.15A-F and K) and also by the direct addition to the reaction mixture (treatment B) (figure 3.15G-J). The measurement of intracellular DCF-FL in AA-stimulated cells was found to be inconsistent, since DCF was found to rapidly leak from stimulated cells. Analysis of the FL in the supernatant also revealed an increase in the AA-stimulated samples along with a decrease in the samples treated with resveratrol (not shown).

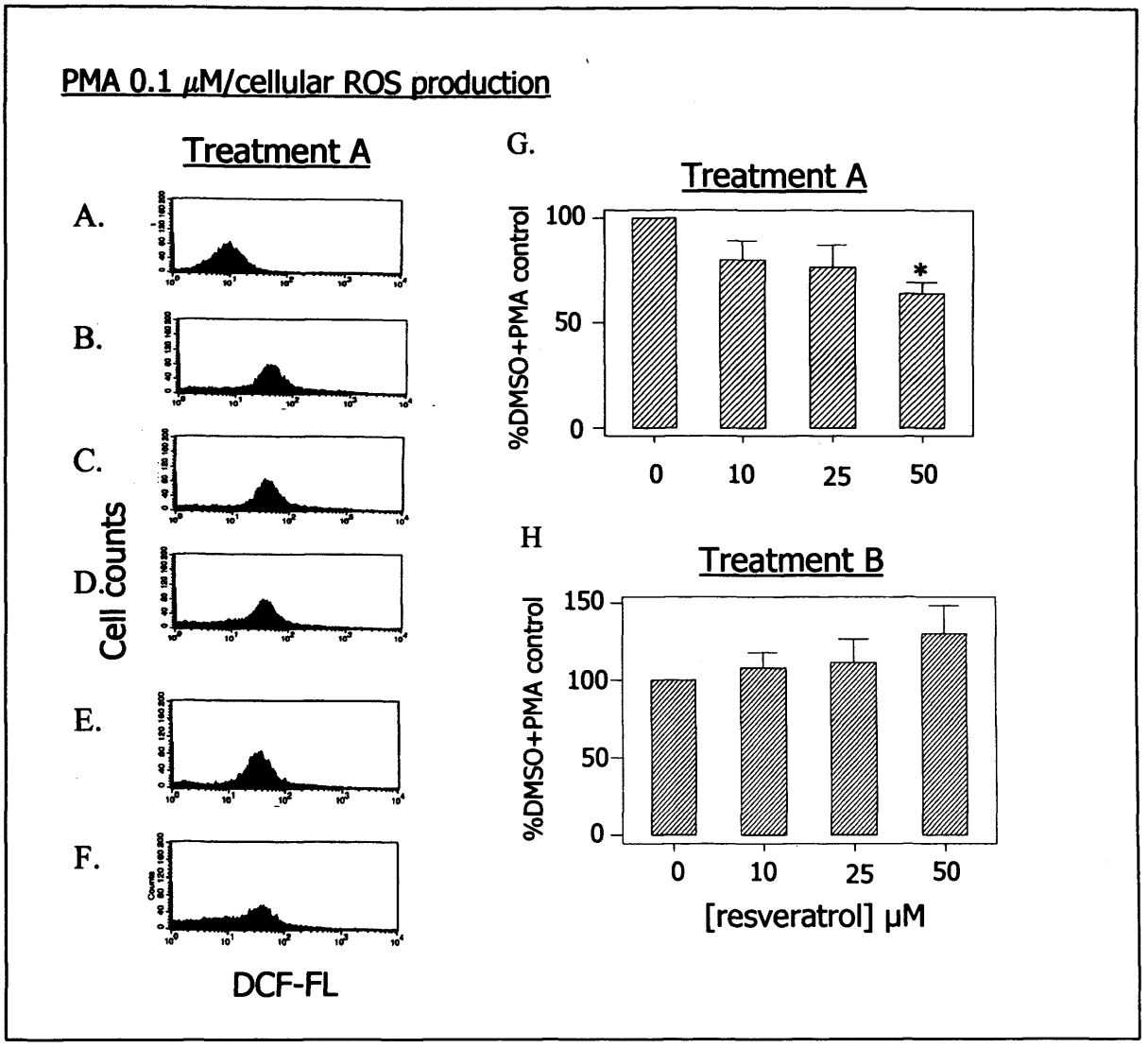

Figure 3.14 The effect of resveratrol on PMA-induced DCF-FL measured by flow cytometry (cellular ROS). dU937 cells were pre-treated with resveratrol for 1 hour followed by washing (treatment A), before they were stimulated with 100 nM PMA for 30 minutes (shown as histograms) A) Control, B) PMA, C) DMSO 0.1% +PMA, D) resveratrol 10 μM + PMA, E) resveratrol 25 μM +PMA, F) resveratrol 50 μM + PMA. G) shows the effect of resveratrol (treatment A) on PMA–induced DCF-FL (expressed as %DMSO+PMA control). Results shown are means (±S.D) of three experiments. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (100%) control cells as determined by a one way ANOVA. H) shows the effect of resveratrol (treatment B) on PMA–induced DCF-FL (expressed as %DMSO+PMA control). Results shown are means (±S.D) of three experiments.

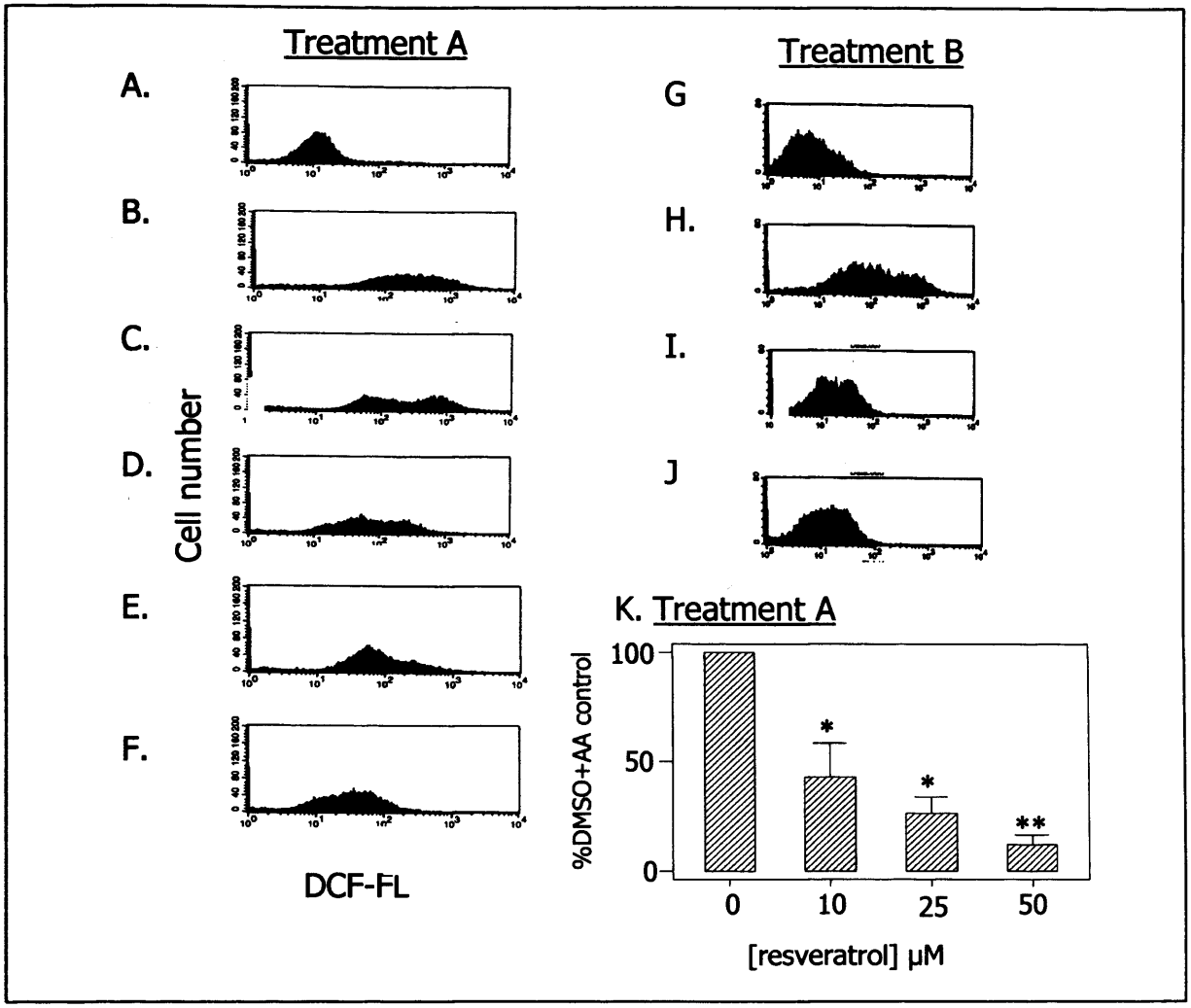

Figure 3.15 The effect of resveratrol on AA-induced DCF-FL measured by flow cytometry (cellular ROS). dU937 cells were pre-treated with resveratrol for 1 hour followed by washing, (treatment A), before they were stimulated with 50 μM AA for 2 minutes (shown as histograms) A) Control, B) AA, C) DMSO 0.1% +AA, D) resveratrol 10 μM + AA, E) resveratrol 25 μM +AA, F) resveratrol 50 μM + AA. Histograms G-J show the effect of resveratrol on AA-induced DCF-FL using treatment B (added directly into the reaction mixture). G) control, H) 50 μM AA, I) AA+ 25 μM resveratrol, J) AA + 50 μM resveratrol. Results shown in (K) is the effect of resveratrol (treatment A) on AA-induced DCF-FL (expressed as %DMSO+AA control). Results shown are means (±S.D) of three experiments. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (100%) control cells as determined by a one way ANOVA.

The effect of resveratrol on the basal oxidation of DCF of DHR in dU937 cells was also investigated. However, inconsistent results were obtained. In some cases resveratrol was found to inhibit basal DCF oxidation whereas on other occasions no effect was measured. The reasons for this are not clear, however, it appears that negative values for DHR-FL (samples that had less fluorescence than the untreated control) were only consistently measured in cells that were stimulated with PMA or AA.

3.8 THE EFFECT OF TROLOX ON THE U937 MONOCYTE RESPIRATORY BURST

Trolox was found to be a potent inhibitor of fMLP, PMA-induced luminol and isoluminol-CL (not shown). Trolox was originally used to compare its antioxidant ability with resveratrol, however, it has been shown that trolox can quench luminol radicals and is also a substrate for HRP (see discussion). Using flow cytomertry, trolox was found to inhibit PMA-induced DCF-FL, a 1 hour pre-treatment of trolox (treatment A) resulted in more inhibition than the direct addition to the reaction mixture (treatment B) (see figure 3.16A and B).

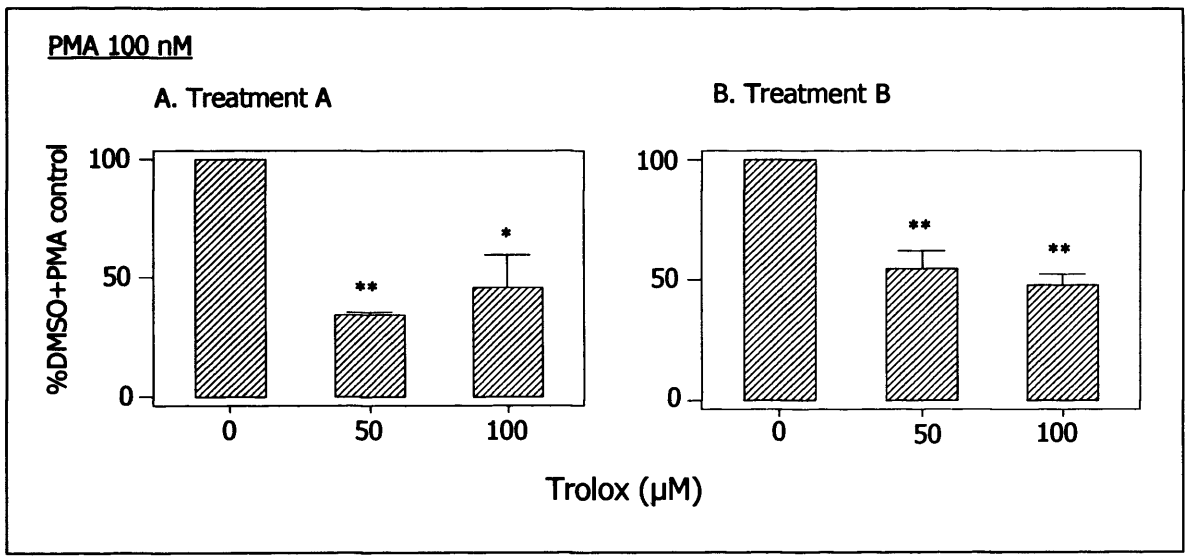

Figure 3.16 The effect of trolox on DCF-FL as measured by flow cytometry (cellular ROS). A) dU937 cells were pre-treated with trolox (μM) for 1 hour followed by washing (treatment A) before stimulated with PMA (100 nM) for 30 minutes. B) As (A) except that trolox was added after the addition of DCF (treatment B). Results shown are means (±SD) of three experiments. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (100%) control cells as determined by a one way ANOVA.

3.9 THE EFFECT OF RESVERATROL ON PMA-INDUCED ROS PRODUCTION IN EBV-TRANSFORMED B-CELLS

EBV-transformed B lymphoblasts were used as a comparison to the U937 cells. The B cells were isolated from healthy subjects as shown by Petitt et al., (2001). These cells do not have to be differentiated in order to induce a respiratory burst. B cells produce a kinetically distinct response compared to dU937 monocytes. In general they produce a smaller and slower response to PMA. Very little was measured when using AA.

Resveratrol was found to inhibit PMA-induced ROS after a 1 hour pre-treatment (followed by washing). Resveratrol was found to reduce all three measured parameters of the respiratory burst (see table 3.8).

Isoluminol-CL	Resveratrol (μM)	T_{max} (%)	I_{max} (%)	Oxidative capacity (%)
PMA 1μM	0.1	100.4(6.1)	105.5(9.5)	103.7(5.5)
	1	98.5(8.8)	102.1(2.9)	100.7(2.8)
	10	108.7(15.4)	94.6(9.6)	88.3(10.5)
	25	137.8(12.9)	82.2(6.4)	64.9(5.1)
	50	155.4(18.2)	69.2(7.1)	52.2(7.2)

Table 3.8 The effect of resveratrol on PMA-induced ROS production in EBV-transformed B cells as measured by isoluminol (extracellular ROS)-CL. B cells were pre-treated with resveratrol for 1 hour followed by washing. T_{max} refers to the time taken to reach the maxiumum CL-value. I_{max} represents the maximum CL-value and oxidative capacity represents the area under the kinetic curve. Results are means (±SD) of six experiments and expressed as %DMSO+PMA control.

3.10 RESVERATROL IS OXIDISED BY THE HRP/H_2O_2 SYSTEM

Resveratrol was found to be potent inhibitor of ROS production (by all three stimuli used) when measured by a peroxidase-dependent redox probe. Isoluminol, luminol, DCF and DHR all use peroxidases to detect ROS. Lucigenin is the only probe used that has consistently been shown to be directly oxidised by ROS e.g. superoxide. Resveratrol was found be less effective at inhibiting lucigenin measured ROS, which could be due to its antioxidant properties. However, if resveratrol can be oxidised by peroxidase/H_2O_2 then it would be able to compete with the redox probe for the peroxidase. This is an important consideration, particularly if the ability of an antioxidant to scavenge H_2O_2 has been overlooked by many authors who have investigated the antioxidant properties of resveratrol. All peroxidases have common mechanisms, therefore if a compound is oxidised by HRP it is likely to be oxidised by many other peroxidases e.g. myeloperoxidase.

HRP produces a characteristic UV-spectrum (shown in figure 3.17A) that, upon addition of hydrogen peroxide, shows changes in absorbance to form HRP-compound I and II. The addition of resveratrol to the HRP-hydrogen peroxide system resulted in the conversion of HRP back to its native form. The addition of resveratrol to HRP did not change its UV-spectrum. However, the addition of hydrogen peroxide to this mixture resulted in changes to the HRP-spectrum that were more apparent in the absence of resveratrol. Resveratrol has absorbance at 305 and 317 nm (see figure 3.17B). The large peak with absorbance in the 220 nm region was due to DMSO. The HRP-hydrogen peroxide system oxidises resveratrol to a phenoxyl radical that has absorbance at 253 nm, which can clearly be seen, despite the DMSO peak. There was no change in absorbance when resveratrol was incubated with HRP alone, and hydrogen peroxide did not oxidise resveratrol in the absence of HRP.

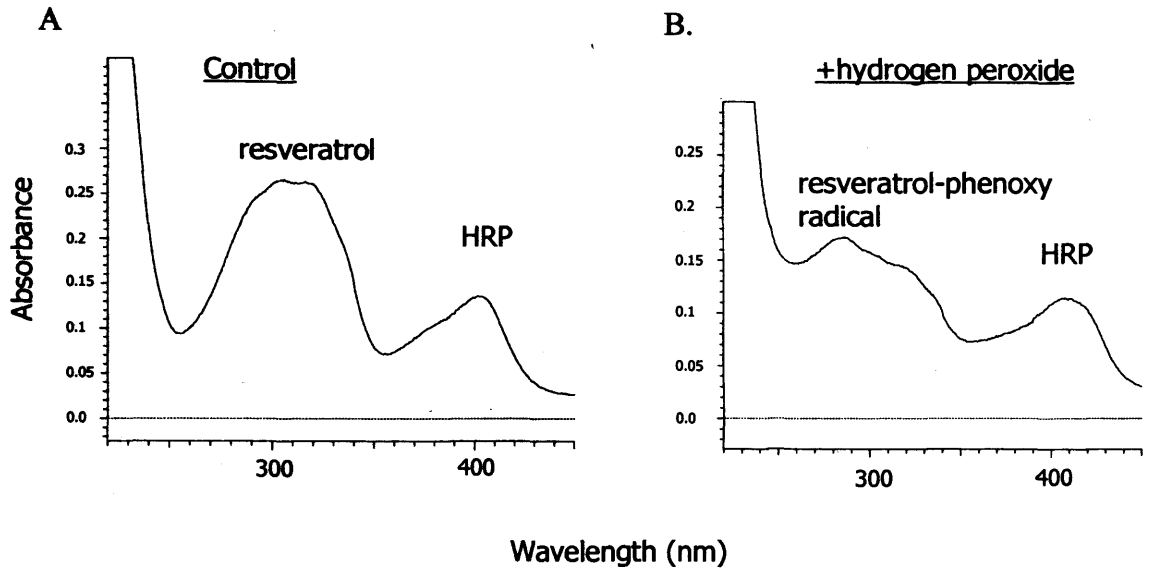

Figure 3.17 The oxidation of resveratrol by the HRP/hydrogen peroxide system. 1 μM HRP was dissolved in 25 mM sodium phosphate buffer. UV-spectral analysis was performed in the presence of 10 μM resveratrol and 50 μM hydrogen peroxide. A) HRP (405 nm) was incubated with 10 μM resveratrol (305 and 317 nm). B) 50 μM hydrogen peroxide was added to a mixture of resveratrol and HRP, which resulted in the formation of the phenoxy-radical of resveratrol (253 nm). Representative of 6 experiments.

Use of a peroxidase assay, where ABTS is the substrate for HRP, resulted in the formation of an insoluble green product. Although it can be seen that resveratrol inhibits the formation of the ABTS product in a dose-dependent fashion, the results were not statistically significant (using a two-way ANOVA for dosage against time). The effect of resveratrol was determined using different doses of ABTS. Although greater inhibition was seen using lower doses, the assay had diminished sensitivity. These results were also statistically insignificant (see figure 3.18A and B).

The incubation of HRP and de-esterified DCF results in an increase in DCF-FL. The addition of hydrogen peroxide to this mixture results in further increases in the FL. The addition of 100 nM resveratrol to HRP inhibits the DCF-FL (figure 3.19A). The addition of an excess of hydrogen peroxide to this mixture results in reduced DCF-FL (Figure 3.19B). The ability of resveratrol to inhibit HRP-induced oxidation was dependent on the addition order of the reactants. Greater inhibition was observed if resveratrol was added with HRP, before the addition of DCF and hydrogen peroxide.

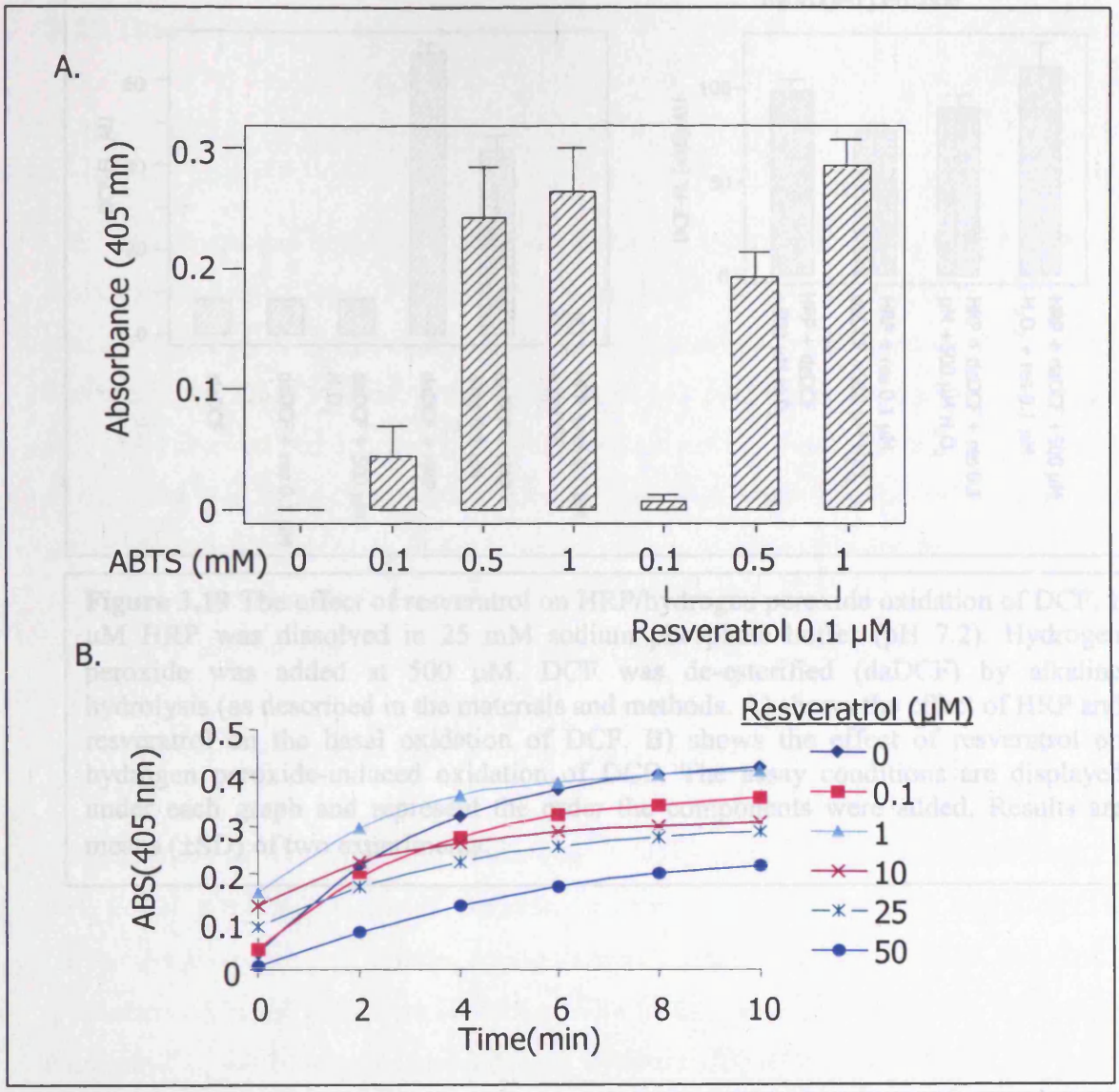

Figure 3.18 The effect of resveratrol on the ABTS-peroxidase assay. A) 0.1 µM HRP was dissolved in 25 mM sodium phosphate buffer (pH 7.2) with 0, 0.1, 0.5 or 1 mM ABTS. Resveratrol (0.1 µM) was also included in some experiments. The reaction was started with addition of 50 µM hydrogen peroxide. The change in absorbance was measured at 405 nm for 10 minutes. Results are means (±S.D) of three experiments. The presence of resveratrol did not significantly effect ABTS absorbance determined by a one way ANOVA. B) 0.1 µM HRP, 1 mM ABTS and the indicated concentration of resveratrol (µM). The reaction was started after the addition of 50 µM hydrogen peroxide. The change in absorbance was measured at 405 nm for 10 minutes. Results are means of three experiments.

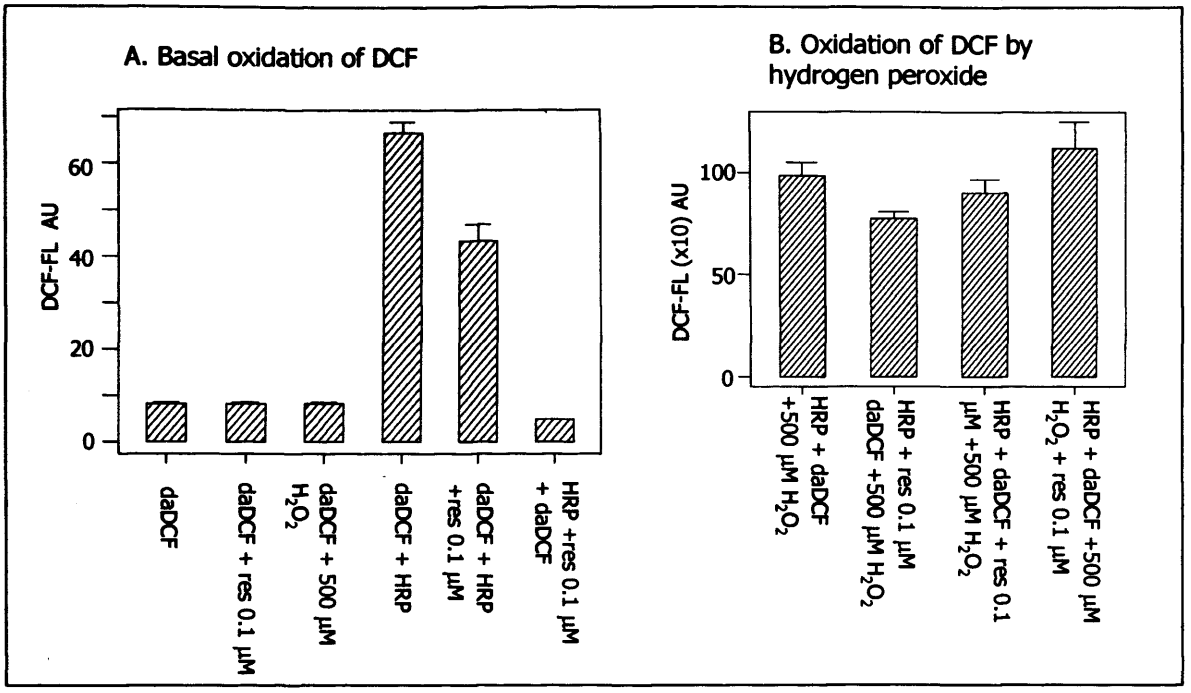

Figure 3.19 The effect of resveratrol on HRP/hydrogen peroxide oxidation of DCF. 1 µM HRP was dissolved in 25 mM sodium phosphate buffer (pH 7.2). Hydrogen peroxide was added at 500 µM. DCF was de-esterified (daDCF) by alkaline hydrolysis (as described in the materials and methods. A) shows the effect of HRP and resveratrol on the basal oxidation of DCF. B) shows the effect of resveratrol on hydrogen peroxide-induced oxidation of DCF. The assay conditions are displayed under each graph and represent the order the components were added. Results are means (±SD) of two experiments.

3.11 DISCUSSION

DIFFERENTIATION OF U937 MONOCYTES

U937 monocytes can be differentiated with numerous agents. This study uses RA and VitD and it can be seen in figure 3.2 that over a 4 day period the p47 subunit of NADPH oxidase was up-regulated. A combination of RA and VitD resulted in enhanced expression of p47. The use of RA alone resulted in up-regulation of p47 over either agent alone. Obermeier et al., (1995) showed that RA treatment alone was not able to induce the up-regulation of any of the other NADPH oxidase subunits, or produce ROS upon stimulation. Analysis of cell growth during differentiation showed that the cells were still viable and proliferating. Other studies have used higher doses of ViTD3 (1 μM; Chateau et al., 1996), but the conditions used here (1 μM RA and 100 nM VitD3) produced satisfactory results.

ACTIVATION OF THE RESPIRATORY BURST IN DU937 MONOCYTES

Three distinct stimuli were used to stimulate NADPH oxidase in dU937 monocytes. PMA and fMLP produced a graded response, whereas AA only induced significant ROS production above 10 μM. Similar responses were measured using lucigenin-, luminol- and isoluminol-CL when cells were stimulated with fMLP, but not PMA or AA. PMA-induced lucigenin-CL and luminol/isoluminol have different shaped curves. AA did not induce a lucigenin-CL. Lucigenin has been shown to be a fairly specific probe for superoxide, whereas luminol/isoluminol have been shown to measure a variety of oxidants (Hasegawa et al., 1997). Lucigenin is thought to only measure extracellular superoxide (Dieter et al., 1995), which suggests that AA does not induce the extracellular formation of superoxide. Superoxide does not cross cellular membranes and requires a transport process, whereas hydrogen peroxide and hypochlorous acid can leak from the cell (Chateau et al., 1996). PMA and fMLP both induced extracellular superoxide, but the PMA-induced lucigenin response was kinetically distinct from that found with luminol or isoluminol. Superoxide production induced by PMA is released in the later stages of the respiratory burst. These results have important consequences for measuring the respiratory burst, since lucigenin-CL does not always measure the initial activation of the respiratory burst.

Using redox sensitive dyes (DCF and DHR) to measure the respiratory burst produced a different pattern of ROS production compared to the CL-probes. Although these dyes do not provide reliable kinetic data, there was a significant difference in ROS production stimulated with fMLP, PMA and AA, as measured by DCF or DHR. fMLP-induced ROS could not be measured by DCF or DHR. This suggested that fMLP-induced ROS was exclusively extra-cellular or these techniques do not have the sensitivity to measure this response. PMA-induced ROS was measured by DCF and DHR-FL using fluorimetry, but only DCF could measure PMA-induced ROS when using flow cytometry. The reasons for this are not clear. However, it could be due to the sensitivity of the two dyes. DCF measured the greater response to PMA using fluorimetry (Vowells et al., 1995). Although it has been suggested that DHR offers greater sensitivity, it has been shown that DHR is not capable of measuring PMA-induced ROS in dU937 cells (Chateau et al., 1996). Using fluorimetry to measure the oxidation of these dyes is thought to represent both intra-cellular and extracellular ROS, whereas flow cytometry only measures intracellular-FL. When DCF-FL was measured in the supernatant of PMA-stimulated cells, only a slight increase was apparent.

DCF and DHR were both able to detect AA-induced oxidants when measured by both fluorimetry and flow cytometry. A large, rapid increase in DCF-FL was measured, whereas DHR-FL increases were much smaller. DHR was found to be an inconsistent probe for the flow cytometric measurement of AA-induced ROS production. Interestingly, increase in DCF-FL as measured by flow cytometry was found to be transient. After 5 minutes the cells appeared to lose their DCF-FL. Analysis of the supernatant revealed a large increase in DCF-FL, which suggests that oxidised DCF had leaked from the cell. This may be due to the high dose of AA used, which has been described as membrane damaging (Brosnan 2002). The use of flow cytometry to measure DCF-FL has been criticised, since oxidised DCF can leak from cells and be taken up by non-responding cells (Vowells et al., 1995). However, when DCF had leaked from AA-stimulated cells all the fluorescence was in the supernatant and could not be measured by flow cytometry.

There is also a possibility that the changes in DCF-FL could be due to a mechanism independent of ROS formation. Since the oxidation of DCF is dependent on peroxidases, e.g. the activity of lipoxygenase (Hempel et al., 1997) or prostaglandin H synthase (or COX) (Larsen et al., 1996) induced by AA have been shown to lead to the oxidation of DCF.

THE EFFECT OF SOD AND CATALASE ON THE ACTIVATION OF THE RESPIRATORY BURST

To determine the cellular location of the U937 monocyte respiratory burst the luminol/isoluminol-CL system described by Karlsson et al., (2000) was used, which examines the difference between the respiratory burst measured by luminol-CL and isoluminol-CL in the presence or absence of SOD and catalase. However, this method assumes that SOD and catalase are cell impermeable and extracellular ROS have no role in propagating the respiratory burst. When cells are stimulated in the presence of SOD and catalase, luminol-CL measurements are thought to represent intra-cellular ROS production. When dU937 monocytes were stimulated with fMLP under these conditions the majority of the CL-response was inhibited. This further suggested that the majority of fMLP-induced ROS were extracellular. In contrast, when cells were stimulated with PMA and AA under these conditions a luminol-CL response was still measured. This suggested that the respiratory burst stimulated by PMA and AA have both intra and extracellular components. These results are in direct opposition to those published by Chateau et al., (1996), where it was found that PMA-induced ROS in dU937 monocytes measured by luminol-CL was completely inhibited by a combination of SOD and catalase.

PMA, fMLP and AA-induced isoluminol-CL(extracellular ROS) responses were completely inhibited by the addition of SOD, which suggests that superoxide is the main reactive species being measured. However, catalase also had a partial inhibitory effect. This result might appear to be paradoxical, since if isoluminol measures superoxide and hydrogen peroxide then SOD would have no effect on isoluminol-CL, some authors have suggested that luminol does not measure hydrogen peroxide, but rather hypochlorous acid (in the absence of HRP) (Stolarek et al., 2002). Other studies suggest that superoxide is the main oxidant being measured. The precise nature of the oxidant being measured is not completely clear (Lundqvist and Dahlgren 1996).

The scheme below shows the light generating mechanism of luminol (as proposed by Thorpe and Kricka 1986). This scheme suggests that hydrogen peroxide is indirectly converted to superoxide, which should enhance the CL-reaction. However, since SOD was used in excess it would expected to inhibit the peroxidase-dependent measurement of hydrogen peroxide.

$$HRP + H_2O_2 \rightarrow HRP(\text{compound-I}) + H_2O$$

$$HRP(\text{compound-I}) + \text{luminol}^- \rightarrow HRP(\text{compound-II}) + \text{luminol}^{\bullet -} + H_2O$$

$$\text{luminol}^{\bullet -} + O_2^{\bullet -}$$

$$\text{luminol}^{-\bullet} + O_2^{\bullet -} \rightarrow \text{luminol}\ O_2^{2-}$$

$$\text{luminol}\ O_2^{2-} \rightarrow N_2 + {}^*AP^{2-}$$

$${}^*AP^{2-} \rightarrow \text{light} + AP^{2-}$$

A similar scheme has been suggested in the measurement of lipid hydroperoxides, which are used with a cytochrome c fragment (microperoxidase) in place of HRP (proposed by Yamamoto et al., 1987 and Hirayama et al., 1997).

SOD and catalase were found to have no effect on PMA or AA-induced DCF or DHR-FL, measured by fluorimetry or flow cytometry. The peroxidase activity of SOD has been shown to result in the oxidation of DCF (Liochev and Fridovich, 2001) and therefore it is not surprising that DCF-FL is not affected. However, assuming that SOD and catalase do not enter the cell, these results initially suggested that extracellular oxidants play no part in the response measured by these fluorescent probes. Although the supernatant from AA stimulated cells (measured by DCF) was highly fluorescent, the oxidation of DCF could still have occurred inside the cell. The decomposition of hydrogen peroxide by catalase proceeds by the formation of catalase-compound-I, which is a ferryl type structure with a porphyrin π-cation radical. These catalase-iron complexes are thought to be able to oxidise DCF (La Bel et al., 1990). These results add to the complexity of using DCF to measure oxidative stress, and even more so when antioxidant efficacy is being determined.

CELLULAR PRIMING OF DU937 MONOCYTES

Phagocyte "priming" is the control mechanism by which the respiratory burst is regulated in order to mount an appropriate inflammatory response. Phagocyte priming is thought to play a key role in the development of numerous inflammatory disorders, which makes these mechanisms potential therapeutic targets. The precise mechanism by which a priming agent increases ROS production upon stimulation is not well defined. There are several mechanisms proposed, which depend on the stimuli. Some *in vitro* cell models use chronic treatments of IFN and LPS to achieve macrophage priming, which is commonly used for rodent macrophage cell lines (Wang and Mazza 2002). In this study, the interest was focused

on the ability of an acute pre-treatment of a priming agent to augment the production of ROS from dU937 monocytes via the modulation of a cell signalling pathway. These priming mechanisms have been extensively studied in neutrophils, with very little attention given to monocytes.

AGE has been shown to prime neurtrophils to give a 2-fold increase in ROS production (Wong et al., 2002). However, pre-treatment with AGE of dU937 monocytes did not affect the subsequent production of ROS upon stimulation with fMLP or PMA. Using the less glycated, mmAGE, also had no effect on fMLP-induced ROS production. AGE is thought to act via a cell surface receptor for advanced glycation subunits (RAGE). U937 cells have been shown to express this receptor but it appeared to have no direct effect on priming or ROS production in dU937 monocytes (Ichikawa et al., 1998).

TNFα has been shown to be a potent neutrophil priming agent in response to a variety of stimuli, such as fMLP (Cadwallader et al., 2002) and AA (Li et al., 1996). TNF has also been shown to have a small direct effect on ROS production (Cadwallader et al., 2002). Pre-treatment with TNF was found to have a marginal effect on ROS production stimulated by fMLP and no effect on AA or PMA-induced ROS. TNF has been shown to activate PI3K (and possibly a tyrosine kinase) and lead to the accumulation of PtIns(3,4,5)P_3 (Cadwallader et al., 2002). Monocytes have been found to release TNFα during their activation and any effect of TNFα on dU937 monocytes could have represented an autocrine regulatory mechanism (Wang et al., 1997).

IFN plays a key role in a process termed "classical macrophage activation" and during the pathogenesis of atherosclerosis it is thought to be released from T_H1 cells (Gordon 2003). IFN is thought to promote macrophage-mediated LDL oxidation (Folcik et al., 1998) and compromise atherosclerotic plaque stability through the release of MMPs (Nold et al., 2003). Although in this study an acute treatment of IFN was found to have no effect of fMLP, PMA or AA-induced ROS production, longer (days) pre-treatments of IFN have been shown to differentiate U937 monocytes when used in conjunction with RA or VitD3 (Obermeier et al., 1995). This suggests that IFN can only affect monocyte ROS production through increases in the expression of NADPH oxidase subunits.

Several oxidants have been found to regulate the respiratory burst of phagocytes. Peroxynitrite has been shown to prime neutrophils (Rohn et al., 1999) and hydrogen

peroxide has been shown prime alveolar macrophages (Giron-Calle and Forman 2000). In this study hydrogen peroxide was used as a priming agent in the same manner as described by Forman et al., (2002). It was found to have no effect on PMA- or AA-induced ROS production, but had a marginal effect on fMLP-induced ROS production. It is still possible that other oxidants could prime dU937 monocytes, or that hydrogen peroxide released from these cells can activate cell signalling pathways that might further affect the release of ROS. The study of oxidants as priming agents is a relatively new area of research and it is not clear how they mediate their effect.

High doses of AA are a potent stimulus of NADPH oxidase, although the precise mechanism is not completely clear. It has been suggested that AA directly activates the translocation of NADPH oxidase subunits (Shiose and Sumimoto, 2000). Some authors have reported that low doses of AA can induce the translocation of PKC (O'Flaherty et al., 2001) and activate Akt via PI3K-dependent and MAPK by PI3K-independent mechanisms (Hii et al., 2001). Therefore, the effect of low dose AA (5 μM) on subsequent fMLP, PMA and high dose (50 μM) AA-induced ROS was determined. Surprisingly, low dose AA had no effect on PMA or AA (high dose)-induced ROS, but inhibited ROS production when cells were stimulated by fMLP. This might represent an important feed-back mechanism, since activated U937 monocytes have been shown to mobilise AA stores (Withnall et al., 1995). Other authors have shown that the majority of fatty acids are capable of priming neutrophils, with the exception of PGE_2 that was found to significantly inhibit fMLP-induced ROS in neutrophils (Li et al., 1996). Since some authors have shown that AA can activate the PI3K and PKC pathways, it is possible that they are desensitising the fMLP receptor or signalling pathway. Pre-treating cells with PMA has been shown to desensitise fMLP-induced responses (Kong et al., 1993). It has been reported that a combination of PMA and AA (high dose) stimulates more ROS from U937 monocytes than either stimulus alone. This is an example of synergy and not priming, since both these stimuli are potent activators of NADPH oxidase (Sellmeyer et al., 1996).

It can be concluded that dU937 cells do not readily prime in a manner compared to neutrophils or rodent monocyte/macrophage cell lines. However, the negative results in this study could be due to the priming agents used, since there are many other priming agents that might activate these cells. It has been suggested that dU937 cells function in a manner akin to a monocyte/macrophage already in a primed state (Johnston et al., 1978). It was determined that dU937 monocytes release superoxide upon stimulation with fMLP, PMA

and AA (see section 3.3). Monocytes have been shown to only release superoxide in a primed state, whereas they would only release hydrogen peroxide by diffusion from the phagosome.

The classical view of cellular priming has been recently been questioned. The notion that the action of one agent is solely responsible for a change in cellular activity is probably over-simplified. Priming is likely to result from phenotypic changes that are induced by a number of factors, such as cellular interactions (Kroegel et al., 2000). Eosinophils isolated from asthmatics have a distinctive phenotype (termed hypodense or low density) that is reversible (Kroegel et al., 1994). It is possible that monocytes/macrophages also undergo distinctive phenotypic changes, which are a reflection of their environment, in disorders such as atherosclerosis.

THE EFFECT OF RESVERATROL ON FMLP-INDUCED ROS

Pre-treating dU937 monocytes with resveratrol (1 hour followed by one wash) was found to be a potent inhibitor of fMLP-induced ROS production measured by isoluminol or luminol-CL. There was a dose-dependent decrease in I_{max} and phagocytic capacity, although there was no change in T_{max}. The direct addition of resveratrol to the reaction mixture increased the potency of resveratrol. However, it is unlikely that this increased potency is due to an antioxidant property of resveratrol, but as a co-reducing substrate for HRP (see below). Use of lucigenin to measure fMLP-induced ROS production produced a different pattern of results. The 1 hour pre-treatment conditions produced greater inhibition than addition of resveratrol to the reaction mixture. This suggested that resveratrol might be antagonising the release of ROS from dU937 monocytes. As previously discussed lucigenin and luminol measure different oxidants and it is possible that resveratrol is not an effective antioxidant of lucigenin-measured oxidants. Since the latter measures superoxide, this agrees with data where resveratrol has been shown to be a relatively weak superoxide scavenger (Miura et al., 2000). In contrast to lucigenin, luminol and isoluminol were used in the presence of a peroxidase. Given the promiscuous nature of the luminol and isoluminol measurement systems, it is very difficult to determine the antioxidant ability of resveratrol. Luminol-CL can be inhibited by classical scavenging mechanisms. There are also a number of indirect mechanisms, such as quenching of luminol intermediates (Lissi et al., 1994) or providing an alternative co-reducing substrate for the peroxidase (HRP) (Halliwell 1995).

THE EFFECT OF RESVERATROL ON PMA-INDUCED ROS

Pre-treating dU937 monocytes with resveratrol inhibited subsequent PMA-stimulated ROS measured by both luminol and isoluminol-CL. A dose dependent decrease in I_{max} and phagocytic capacity was measured. Greater inhibition was achieved when isoluminol was used as the CL substrate. Since luminol measures both intra and extracellular ROS, it is possible that resveratrol is less effective at inhibiting the intracellular component of the PMA-induced respiratory burst. The later stages of the PMA-induced ROS that are measured by luminol might represent intracellular ROS production. Direct addition of resveratrol to the reaction mixture increases the potency of resveratrol to inhibit the PMA-induced respiratory burst, measured by both luminol and isoluminol-CL. This either suggests that resveratrol can directly scavenge PMA-induced ROS or compete with luminol as the substrate for HRP. Using the 1 hour resveratrol treatment protocol followed by stimulation with 1 μM PMA, reduces the inhibitory effect of resveratrol. The response seen with 0.1 μM PMA is very similar to that with 1 μM PMA. Resveratrol has recently been shown to bind to the C1-regulatory domain of cPKCs and therefore is a phorbol ester/DAG antagonist. Increasing the dose of PMA could potentially reverse this inhibition through competitive inhibition (Slater et al., 2003).

The activation of the respiratory burst in EBV-transformed B cells was also shown to be inhibited by resveratrol and the inhibition was similar to that found in dU937 monocytes using 1 μM PMA. The respiratory burst measured from B cells was found to be smaller than that measured from the monocytes, which reflects the specialised nature of these cells. However, it has been shown that the respiratory burst of both B cells and monocytes is much smaller than that of an eosinophil or neutrophil (Giron-Calle and Forman 2000).

When using DCF or DHR to measure the respiratory burst stimulated by PMA, resveratrol was able to inhibit oxidation of FL-probes. Pretreatment with resveratrol or direct addition resulted in a similar pattern of inhibition with both probes, as measured by fluorimetry. Using flow cytometry it was found that a 1 hour pre-treatment with resveratrol was able to inhibit PMA–induced DCF-FL. However, higher doses were required to inhibit DCF-FL measured by flow cytometry when compared to fluorimetry. When resveratrol was added 5 minutes before or after the addition of DCF, there was no change in PMA-induced DCF-FL. Moreover, higher doses of resveratrol appeared to increase DCF-FL. Interestingly, when the supernatant of these samples was measured there was less DCF-FL in the samples treated

with resveratrol. This also raises the possibility that resveratrol is less effective at inhibiting intracellular ROS production. However, it is difficult to determine how the changes in fluorescence measured by fluorimetry or flow cytometry can be directly compared. It assumes that when DCF-FL is measured by fluorimetry the changes in total-FL are measured and flow cytometry only measures changes in cellular-FL.

THE EFFECT OF RESVERATROL ON AA-INDUCED ROS PRODUCTION

Resveratrol was found to inhibit AA-induced luminol and isoluminol chemilulinescence, although significant inhibition was only achieved for luminol when resveratrol as added directly into the reaction mixture. Since AA is thought to activate the respiratory burst in a direct fashion, these results might suggest that resveratrol can directly inhibit NADPH oxidase. If resveratrol was a potent inhibitor of NADPH oxidase then its inhibitory effects would not be dependent on the stimulus or measuring principle e.g. shown with DPI. This raises the possibility that the results from this study suggest the inhibitory effects of resveratrol differ between stimulus and measuring principle.

AA-induced ROS was measured by flow cytometry using DCF and DHR. However DHR-FL was found to be inconsistent. Pretreating the cells for 1 hour or directly adding resveratrol into the reaction mixture resulted in dose-dependent inhibition of DCF-FL. DCF-FL of the supernatant was also measured after AA stimulation and resulted in a dose-dependent decrease by resveratrol. The increase in DCF-FL after AA stimulation was far greater than that found with PMA. Resveratrol was found to be a potent inhibitor of the AA-induced response. The nature of the ROS measured by DCF in response to AA is not clear. It is possible that the oxidants are different to those formed by PMA. This raises the possibility that resveratrol is more effective at inhibiting these reactive species than the oxidants measured by the CL-techniques. It has been shown that resveratrol is potent scavenger of peroxyl radicals and possesses some transition metal chelating ability (Mirua et al., 2000), which might explain its potency for inhibiting DCF and DHR-FL. However, there could still be an intra-cellular target that resveratrol can inhibit in the PMA and AA-induced signal transduction pathways.

As previously described, the activities of lipoxygenase and prostaglandin H synthase have been shown to oxidise DCF independently of ROS production. Resveratrol has been shown to be a potent inhibitor of these enzymes. These results cast further doubt on the specificity

of DCF as a redox sensitive probe. Table 3.9 shows a summary of the IC_{50} values for the inhibitory effects of resveratrol on the respiratory burst.

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	fMLP (1 μM)				
Treatment condition	Isoluminol Extracellular Ros	Luminol Total ROS	Lucigenin Superoxide	DCF Total ROS?	DHR Total ROS?
A	2.5	1.5	15.6	-	-
B	0.24	0.18	45.3	-	-
	PMA (0.1 μM)				
A	8.6	23.8	>50	2.8	4.2
B	1.3	14.2	20.9	1.5	10.1
	AA (50 μM)				
A	5.6	52.4	-	2.7	3.9
B	0.8	0.5	-	4.5	0.12

Table 3.9 The estimated IC_{50} values for the inhibitory effects of resveratrol on the dU937 respiratory burst. Treatment A) consisted of a 1 hour pre-treatment followed by washing and B) where resveratrol was added to the cells for 5 minutes before stimulation. (-) indicates that no response could be measured with this technique.

THE ROLE OF PEROXIDASES IN THE MEASUREMENT OF THE RESPIRATORY BURST.

The use of DCF to measure oxidative stress in cells is very popular and has been proposed as a convenient method to study the efficacy of antioxidants, despite numerous publications highlighting potential pitfalls. The HRP-mediated oxidation of DCF has been proposed to result in the formation of superoxide and hydrogen peroxide through the formation of a DCF-phenoxy radical, which makes the system auto-catalytic. De-esterified DCF can be oxidised by HRP in the presence of hydrogen peroxide to form a DCF phenoxyl radical. This intermediate can then, in the presence of a reducing agent e.g. GSH or NADH, be converted to DCF with the release of superoxide (forming GS• and NAD+, see figure 3.20). These results make increases in DCF-FL in cells undergoing oxidative stress difficult to interpret (Rota et al., 1999). The use of this system for measuring the efficacy of antioxidants is not

straightforward, due to the formation of free radicals in the oxidation process and therefore it is difficult to determine whether the antioxidant is reacting with the initiator compound or radicals formed during its oxidation.

$$\text{Peroxidase} + \text{ROOH} \rightarrow \text{compound I} + \text{ROH}$$

$$\text{Compound I} + \text{XOH(DCF/luminol/resveratrol/DHR)} \rightarrow \text{compound II} + \text{XO}^{\bullet}$$

$$\text{Compound II} + \text{XOH} \rightarrow \text{XO}^{\bullet} + \text{H}_2\text{O}$$

As previously mentioned, peroxidases are required for the ability of DCF, DHR, luminol and isoluminol to measure oxidative stress (see scheme above). In some cases DCF and DHR can be directly oxidised e.g. peroxynitrite or hyperchlorous acid (Crow 1997). Lucigenin-CL does not require peroxidases in order to measure ROS. The ability of the HRP-hydrogen peroxide system to oxidise resveratrol was documented by Calderon et al., (1990) and has been further described by Galati et al., (2002). The peroxidase-dependent oxidation of resveratrol appears to be identical to that of DCF. Moreover, the effect of many other phenolic compounds have also been shown to inhibit or enhance chemiluminescence reactions (Diaz et al., 1998). Therefore the effect of resveratrol on HRP-dependent oxidation was determined. Only high doses of resveratrol were able to inhibit the oxidation of ABTS. This suggested that resveratrol was not a potent direct inhibitor of HRP, but could still be acting as a reducing co-substrate. If the concentration of ABTS (below 1 mM) was lowered, the sensitivity of the assay was reduced. Resveratrol was found to have greater inhibition if the dose of ABTS was lowered to 0.5 and 1 mM. ABTS has to be used at high concentrations (1 mM) far higher than the dose of resveratrol (50 μM) used in this study. However, the CL- or FL-probes are all used at lower doses (2 μM for DCF and DHR with luminol and isoluminol used at 50 μM) and were comparable to resveratrol. Therefore, it could compete with the probe for HRP as an alternative substrate.

De-esterified DCF was incubated with HRP, which produced an increase in DCF-FL. Resveratrol was able to inhibit HRP-induced DCF-FL at 100 nM, with the concentration of DCF used 20x higher than resveratrol. When dU937 cells were incubated with resveratrol, the basal levels of DCF and DHR were not consistently reduced, which is surprising given the effect of resveratrol on HRP-induced DCF-FL. However, when dU937 cells were stimulated with PMA or AA, resveratrol was found to reduce the fluorescence values below that of the unstimulated controls. The reasons for this were not clear, but could be due to the basal oxidation of DCF or DHR in dU937 cells occurring at a low level.

It is possible that some of the inhibitory effects of resveratrol in HRP-dependent detection systems are due to competition with the probe for HRP, since they are oxidised in a very similar fashion. This might be particularly relevant when resveratrol is added to the cells before stimulation. Different results are obtained when the cells are pre-treated for 1 hour and then washed. However, under these conditions (treatment A) there is also the potential that resveratrol could be inhibiting a cell signalling intermediate.

THE EFFECT OF TROLOX ON THE DU937 RESPIRATORY BURST

A well established antioxidant was used to inhibit the dU937 monocyte respiratory burst, in order to provide a comparison to resveratrol. Trolox, an extremely effective antioxidant, was chosen, because it has been compared to resveratrol in cell free systems and does not inhibit PKC directly. However, trolox has been shown to quench luminol intermediates (Lissi et al., 1994) and is a substrate for HRP (Nakumura 1990). This makes its use in luminol-CL reactions problematical. The effect of trolox on PMA-induced DCF-FL (measured by flow cytometry) differed to that found with resveratrol, in that trolox was more effective when directly added to the reaction mixture and after a 1 hour pre-treatment followed by washing. Trolox was found to be more effective at inhibiting PMA-induced DCF-FL (measured by flow cytometry) than resveratrol.

HOW CAN RESVERATROL INHIBIT THE RESPIRATORY BURST?

Three possible scenarios are proposed that explain the inhibitory effect of resveratrol on the respiratory burst:

Resveratrol can directly inhibit NADPH oxidase. Resveratrol has been shown to inhibit various enzymes including COX1 and 2, 5-lipoxygenase and cytochrome P450 enzymes. If resveratrol was a potent direct inhibitor of NADPH oxidase then it might be expected to behave like other NADPH oxidase inhibitors, such as diphenylene iodonium (DPI). DPI has been shown to inhibit NADPH oxidase-dependent ROS production induced by a variety of stimuli and measuring principles (Myhre et al., 2000). The results in this study show that the inhibitory effects of resveratrol are dependent on the stimulus and its measuring principle.

Resveratrol can directly scavenge ROS produced by the respiratory burst. It is difficult to make a precise conclusion, since with the exception of lucigenin, the redox probes used

here do not offer solid evidence of the existence of any particular oxidant. Resveratrol was a potent inhibitor of luminol- and isoluminol-CL, however. Its inhibitory effects were greater if the redox probe was dependent on peroxidases. Resveratrol is a peroxidase substrate and could be inhibiting these reactions by acting as an alternative substrate. Indeed, resveratrol appears to be oxidised in an identical fashion to the DCF fluorescent probe. The most abundant oxidants produced by dU937 cells are most likely to be superoxide and hydrogen peroxide. Resveratrol does not directly react with hydrogen peroxide and its ability to remove superoxide (determined with lucigenin-CL) was found to be weak. However, resveratrol was found to be a potent inhibitor of fMLP-induced ROS, regardless of the measuring principle. Although fMLP was found to be the weakest activator of ROS production, there is still the possibility that resveratrol can directly antagonise the activation pathway induced by this stimulus.

Resveratrol can inhibit cell signalling pathways that activate the respiratory burst. The ability of resveratrol to inhibit fMLP-induced ROS was found to be greater after a 1 hour pre-treatment than following direct addition to the reaction mixture. Interestingly, the effect of resveratrol on PMA-induced lucigenin-CL produced results that were opposite to that found with fMLP. The signal transduction pathways that active NADPH oxidase after stimulation with fMLP or PMA have been shown (in neutrophils) to be distinct. Therefore, the inhibitory effects of resveratrol on fMLP-induced lucigenin-CL could be due to inhibition of a cellular signalling intermediate. Resveratrol has been shown to inhibit PKC, which supports some of the inhibitory effects of resveratrol on PMA-induced ROS production. The activation of the respiratory burst by fMLP is not well understood and may involve multiple cell signalling intermediates, some of which could be potential targets for resveratrol.

Figure 3.20 summarises the inhibitory effects of resveratrol on NADPH oxidase and a possible pro-oxidant pathway via the formation of a resveratrol phenoxyl radical.

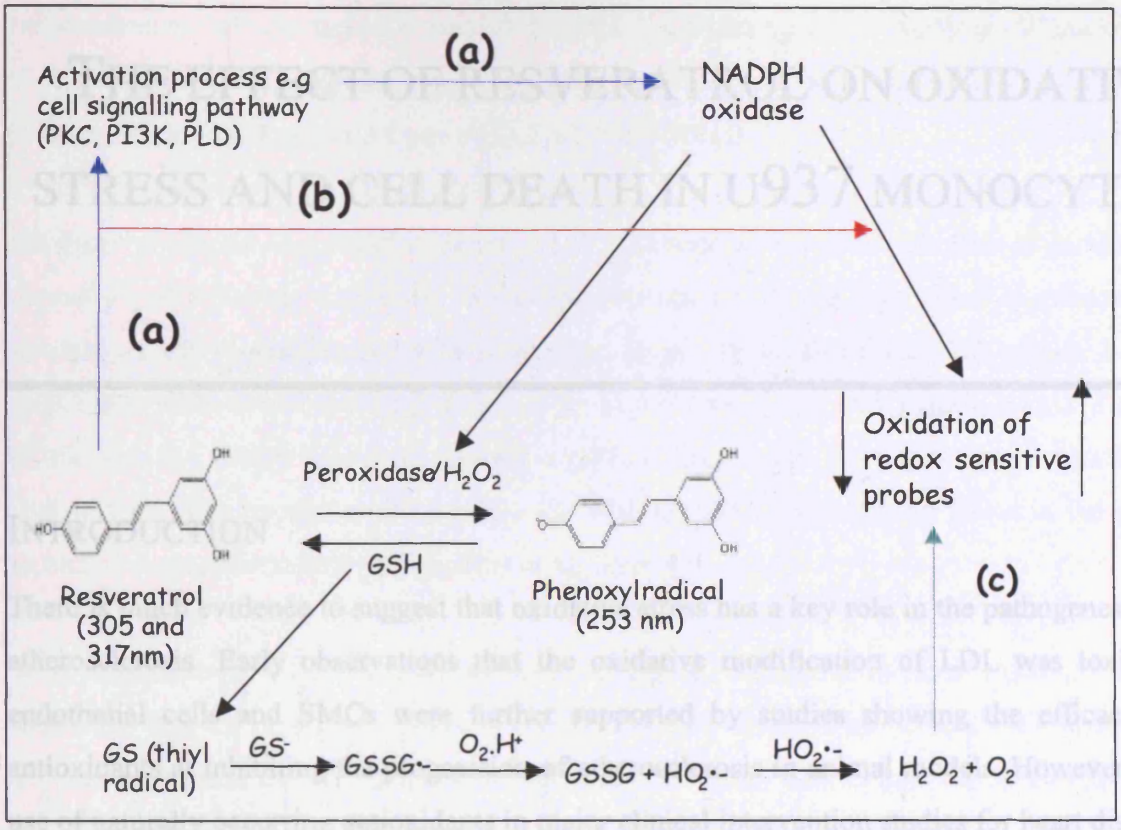


Figure 3.20 How can resveratrol inhibit or increase NADPH oxidase dependent oxidative stress? A) Pre-treatment of dU937 cells with resveratrol can potentially lead to the inhibition of cell signalling pathways involving protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K) and phospholipase D (PLD) that activate NADPH oxidase. B) Resveratrol could also directly scavenge intra- or extracellular oxidants generated by NADPH oxidase or interfere with the detection method. C) If resveratrol was added directly before the activation of NADPH oxidase an increase in intra-cellular oxidants could be detected. This might be due to the oxidation of resveratrol by the action of a peroxidase, which has been shown to lead to the formation of a phenoxyl-radical. In the presence of a reducing agent such as glutathione (GSH), this intermediate is converted back to resveratrol with the formation of superoxide (modified from Galati et al., 2002)

CHAPTER FOUR:

THE EFFECT OF RESVERATROL ON OXIDATIVE STRESS AND CELL DEATH IN U937 MONOCYTES

INTRODUCTION

There is much evidence to suggest that oxidative stress has a key role in the pathogenesis of atherosclerosis. Early observations that the oxidative modification of LDL was toxic to endothelial cells and SMCs were further supported by studies showing the efficacy of antioxidants at inhibiting the progression of atherosclerosis in animal models. However, the use of naturally occurring antioxidants in major clinical intervention studies for heart disease has been largely ineffective. The reasons for this failure are not completely clear, although it is most likely due to the dose of antioxidant used, which has been generally very low. Secondly, few of these trials have used oxidative biomarkers to determine the efficacy of the antioxidant. Recently it has been shown that vitamin E only reduces the plasma levels of F_2-isoprostanes when administered at doses of 800IU or above (Roberts et al., 2002)

The main problem of these clinical trials is that they represent antioxidant chemotherapy, rather than the chemoprevention that has been demonstrated by the animal models. Atherosclerosis takes years to develop in humans and perhaps when antioxidants, such as vitamin E, are used in clinical trials it is too late for successful intervention (FitzGerald and Witztum, 2001). There is great interest in the use of flavonoids and other related polyphenolic compounds, e.g. resveratrol and EGCG, which potentially have a broad range of anti-inflammatory actions. Their antioxidant properties have been widely studied, which in some cases may not result from a reaction with the oxidant itself but from a secondary effect e.g. inhibition of a cell signalling pathway or enzyme that generates ROS. The secondary effects of increased ROS production are also prevented by the action of antioxidants. Reactive aldehydes formed from the oxidation of lipids or sugars are long

lived toxic second messengers that can cause further stress away from the site of initial oxidation. Some of these modifications, such as the formation of AGE, can alter the responsiveness of the immune system through the priming of neutrophils (Wong et al., (2001). They are also capable of activating cell signalling pathways that would result in pro-inflammatory gene expression e.g. 4-HNE activates NFκB.

Oxidants produced as a result of stress, or their secondary products, are able to induce the formation of a variety of protein and DNA adducts, which are convenient biomarkers of oxidative stress and have a potential prognostic value. The levels of the DNA adduct, 8-oxo-guanine in leukocytes have been shown to be higher in subjects with cardiovascular disease (Collins et al., 1998). Moreover there is a positive correlation for populations in countries that suffer a high incidence of heart disease with the levels of 8-oxo-G, found in the DNA extracted from their leukocytes (Collins et al., 1998).

When investigating the antioxidant properties of a given compound *in vitro,* it is important to remember that the conditions of the system that confer "antioxidant" efficacy. For example, a given compound can act as an antioxidant in one system and as a prooxidant in another. The importance of an antioxidant *in vivo* depends on which ROS is produced, how it is generated and the type of damage measured. A good example of this is the effect of vitamin C on gas-phase cigarette smoke-treated plasma. Vitamin C is able to inhibit lipid peroxidation in this plasma, but not protein carbonyl formation. Human plasma contains a variety of antioxidants that all have different properties. Lipid peroxidation in plasma is inhibited by transferrin and caeruloplasmin, whereas oxidative damage induced by nitrogen dioxide (found in polluted air) is inhibited by uric acid. Uric acid does not inhibit oxidative damage that is induced by hypochlorous acid. Therefore, it is important that antioxidants are thoroughly characterised. Some investigators use methods that claim to measure total antioxidant capacity. A given compound is incubated with human plasma and then a water soluble azo-initiator 2,2'-azobis(2-amidinopropane hydrochloride) (AAPH) yields carbon-centred radicals that react with oxygen to give peroxyl radicals. These assays only detect the peroxyl scavenging efficacy of plasma. Water soluble antioxidants, such as vitamin C, will excel at this. Different results would be obtained if lipid-soluble radicals or hydrophobic antioxidants were used (reviewed by Halliwell 1995).

The aim here is to determine the consequence of a biologically relevant source of ROS (from the respiratory burst in U937 monocytes). This process is thought to contribute to the

oxidative burden during the pathogenesis of atherosclerosis. The consequences of activating NADPH oxidase depend on the cell type. Neutrophils are capable of producing vast quantities of oxidants. They have a short life span, and after activation they undergo apoptosis and are cleared by cells such as macrophages (Arroyo et al., 2002). Very little is known about the life span of a macrophage, which is much longer than a neutrophil (possibly years). Therefore they do not undergo apoptosis as readily as neutrophils. Some authors have suggested that Akt1 is an important survival factor for macrophages (Liu et al., 2001). In the case of atherosclerosis, the fate of the macrophage could affect the outcome of the disease. It is believed that macrophages are subjected to oxidative stress and it has been shown that iNOS expression, nitrotyrosine and lipid peroxidation are prevalent in plaque macrophages (Cromheeke et al., 1999). High levels of DNA synthesis/repair have also been demonstrated in this cell type and this is thought to be in response to DNA damage. p53 and p21 expression have been demonstrated in plaque macrophages (Ihling et al., 1997) and therefore, it is suggested that p53 induces cell cycle arrest in response to DNA damage. However, DNA damage induces the activation of poly (ADP-ribose) polymerase (PARP), which binds to strand breaks. Excessive PARP activation can lead to the depletion of NAD^+ and attempts to re-synthesize NAD^+ result in ATP depletion, energy deprivation and a necrotic cell death (see figure 4.1). Caspase activation usually leads to the cleavage and subsequent inhibition of PARP. However, caspases can be oxidatively modified or nitrosylated, which leads to their inactivation (Szabo et al., 1996). Macrophages that are apoptotic/necrotic need to be cleared because necrosis will further the inflammatory response and lead to the formation of a necrotic core, which is a prominent feature of a mature atheroma. At present it is not clear if apoptosis is beneficial or detrimental in the mature atheroma. Reduced macrophage numbers would promote plaque stability through reduced MMP levels and subsequent collagen breakdown. This would also lead to reduced clearance of apoptotic bodies, the formation of a necrotic core and the activation of thrombin (Kockx and Knaapen 2000) (see figure 4.1).

These processes were investigated using two biomarkers, the formation of MDA and the levels of the M_1G DNA adduct. Any subsequent cell death was also studied as was the effect of several commonly used antioxidants e.g. trolox, NAC and desferal. The effect of resveratrol on oxidative stress in these cells was then determined, in order to further characterise the antioxidant or prooxidant properties of resveratrol.

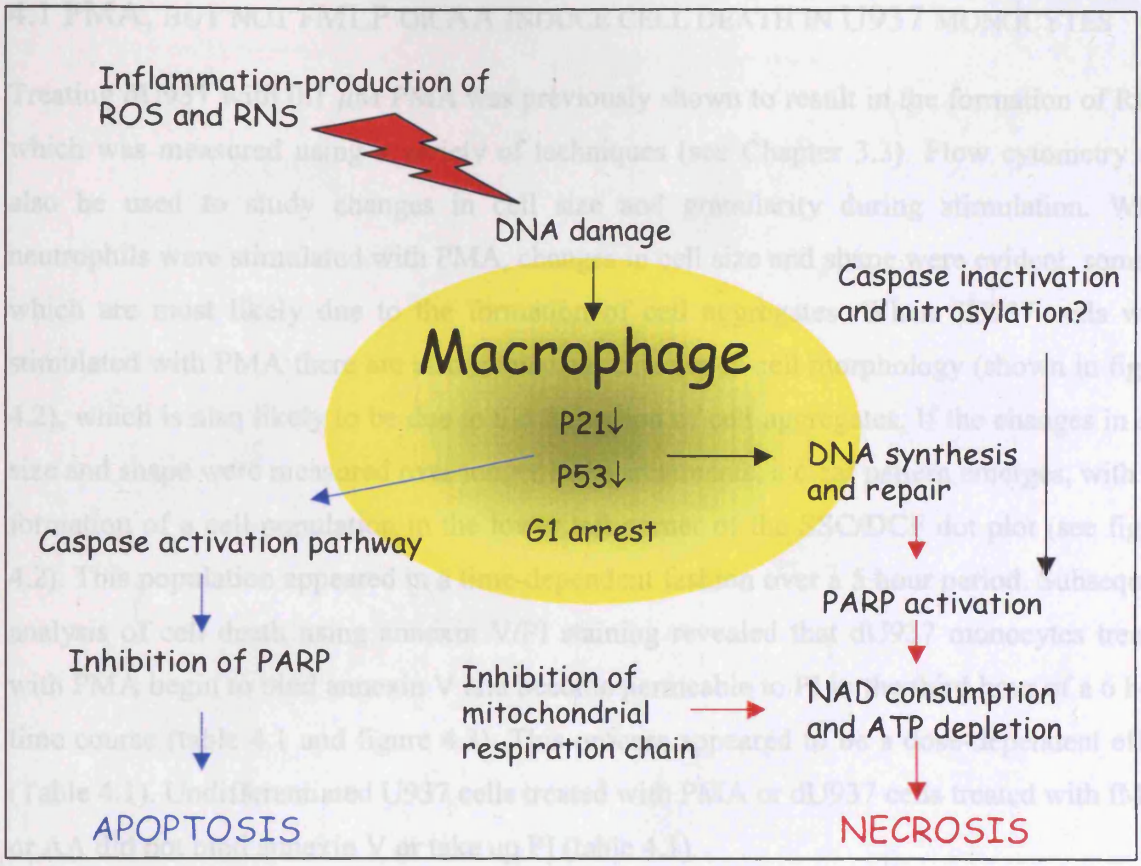


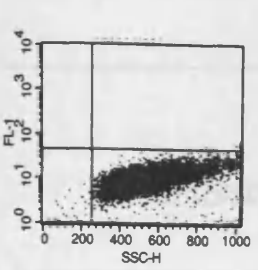
Figure 4.1. Macrophage apoptosis and necrosis in atherosclerosis. During the pathogenesis of atherosclerosis, macrophages show high levels of DNA synthesis and repair. Some macrophages undergo classical apoptosis, while others inactivate their caspases e.g. by nitrosylation and are resistant to nitric oxide-induced apoptosis. DNA damage and subsequent repair and synthesis could lead to excessive PARP activation that would result in NAD^+ consumption, ATP depletion and necrosis (taken from Kockx and Knaapen 2000).

4.1 PMA, BUT NOT FMLP OR AA INDUCE CELL DEATH IN U937 MONOCYTES

Treating dU937 with 0.1 μM PMA was previously shown to result in the formation of ROS, which was measured using a variety of techniques (see Chapter 3.3). Flow cytometry can also be used to study changes in cell size and granularity during stimulation. When neutrophils were stimulated with PMA, changes in cell size and shape were evident, some of which are most likely due to the formation of cell aggregates. When dU937 cells were stimulated with PMA there are also immediate changes in cell morphology (shown in figure 4.2), which is also likely to be due to the formation of cell aggregates. If the changes in cell size and shape were measured over longer PMA treatments, a clear pattern emerges, with the formation of a cell population in the lower left corner of the SSC/DCF dot plot (see figure 4.2). This population appeared in a time-dependent fashion over a 5 hour period. Subsequent analysis of cell death using annexin V/PI staining revealed that dU937 monocytes treated with PMA begin to bind annexin V and become permeable to PI in the third hour of a 6 hour time course (table 4.1 and figure 4.3). This process appeared to be a dose-dependent effect (Table 4.1). Undifferentiated U937 cells treated with PMA or dU937 cells treated with fMLP or AA did not bind annexin V or take up PI (table 4.1).

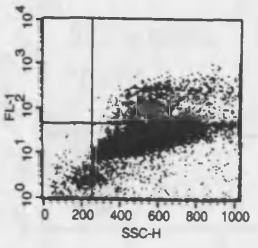
	Live	Apoptotic	Necrotic
Control	96.2	1.3	1.5
PMA 100 nM 30 min	94.8	1.9	2
"60	95.4	1.5	1.4
"120	90.2	2.6	4.9
"180	53.5(10.8)	19.7(7.8)	25.3(4.9)
"360	53.5	18.6	26.1
PMA 1 nM (for 3 hours)	97.6(0.4)	1.1(0.5)	1.8
10 nM	88.1(1)	3.8(0.4)	7.2(0.4)
100 nM	53.5(10.8)	19.2(7.2)	25.3(4.9)
fMLP 1 μM	95.3	1.4	1.7
AA 50 μM	94.6	1.1	1.8
Undifferentiated U937	97.3	1.6	1
" stimulated with 100 nM PMA	94.2	2.7	2.6

Table 4.1 The effect of fMLP, PMA and AA on cell death in dU937 monocytes. dU937 cells were differentiated as previously described. Cells were stimulated with fMLP (1 μM for 3 hours) or PMA (indicated time and dose) in complete medium. AA (50 μM 3 hours) cell stimulation was carried out in serum free medium. Cells were then harvested and washed in PBS before being incubated with annexin/PI and analysed by flow cytometry. Five thousand cells were counted for each sample. Results shown are expressed as percentage of the total counted (±SD n=3).



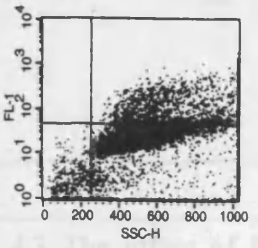
Control

Quad	Events	% Total
UL	0	0.00
UR	28	0.28
LL	176	1.76
LR	9796	97.96



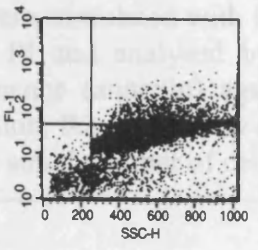
30 min

Quad	Events	% Total
UL	1	0.01
UR	1720	17.20
LL	398	3.98
LR	7881	78.81



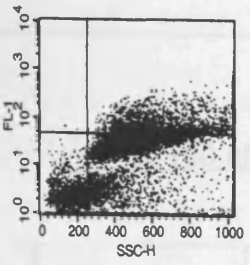
1-hour

Quad	Events	% Total
UL	0	0.00
UR	2263	22.63
LL	489	4.89
LR	7248	72.48



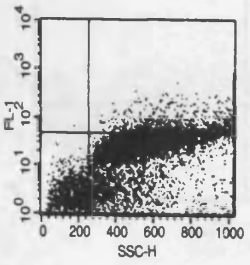
2-hour

Quad	Events	% Total
UL	0	0.00
UR	2026	20.26
LL	658	6.58
LR	7316	73.16



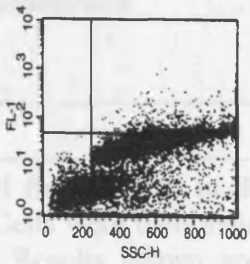
3-hour

Quad	Events	% Total
UL	3	0.03
UR	2133	21.33
LL	1252	12.52
LR	6612	66.12



4-hour

Quad	Events	% Total
UL	1	0.01
UR	2200	22.00
LL	1138	11.38
LR	6661	66.61



5-hour

Quad	Events	% Total
UL	0	0.00
UR	1395	13.95
LL	1829	18.29
LR	6776	67.76

Figure 4.2. Time course of PMA activation in dU937 monocytes. dU937 cells were stimulated with 100 nM PMA for the indicated time before being loaded with 2 μM DCF. Dot plots represent FL-1 fluorescence (DCF) against side scatter (SSCH). Side scatter represents cell surface irregularity.

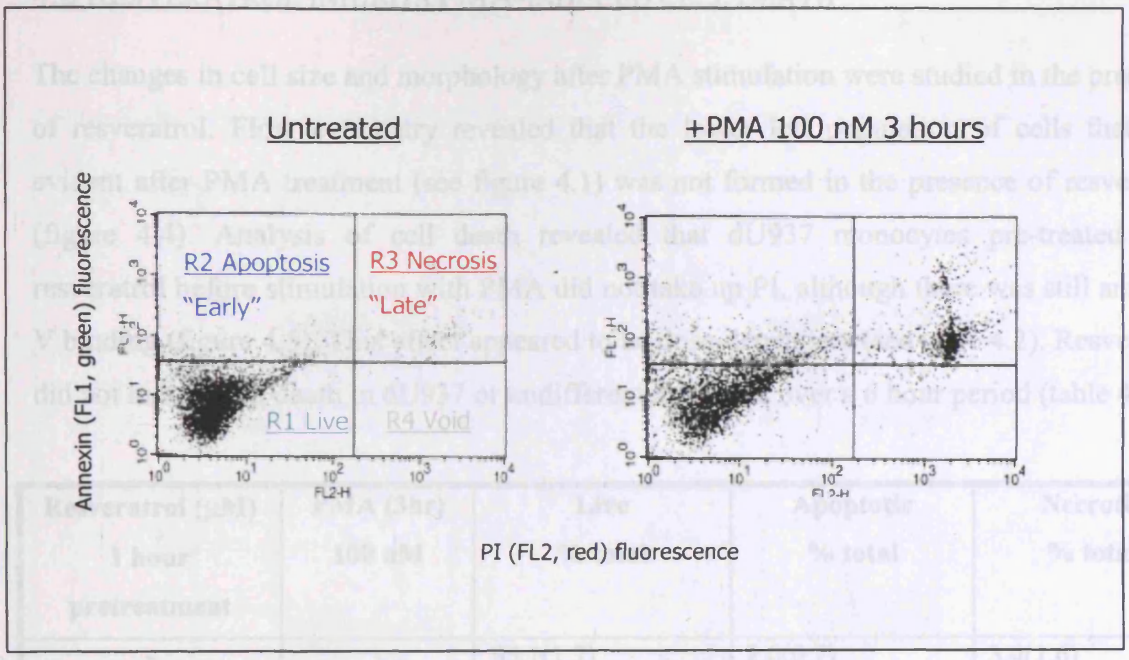


Figure 4.3 The effect of PMA on annexin/PI fluorescence in dU937 cells. dU937 cells were stimulated with PMA for 3 hours. Cells were then incubated with annexin V and PI and analysed by flow cytometry. Results shown are dot-plots of FL1 fluorescence (annexin) against FL2 fluorescence (PI). R1 represents the live cell population, R2 is the early apoptotic phase, R3 is the late apoptotic or necrotic phase and the small number of cells that appear in R4 are excluded from the analysis.

4.2 RESVERATROL INHIBITS PMA-INDUCED CELL DEATH

The changes in cell size and morphology after PMA stimulation were studied in the presence of resveratrol. Flow cytometry revealed that the lower left population of cells that was evident after PMA treatment (see figure 4.1) was not formed in the presence of resveratrol (figure 4.4). Analysis of cell death revealed that dU937 monocytes pre-treated with resveratrol before stimulation with PMA did not take up PI, although there was still annexin V binding (figure 4.5). This effect appeared to be dose-dependent (see table 4.2). Resveratrol did not induce cell death in dU937 or undifferentiated cells over a 6 hour period (table 4.2).

Resveratrol (μM) 1 hour pretreatment	**PMA (3hr) 100 nM**	**Live % total**	**Apoptotic % total**	**Necrotic % total**
-	-	95.1(1.7)	2.0(0.7)	3.4(1.6)
-	+	53.5(10.8)	19.2(7.2)	25.3(4.9)
DMSO	+	51.3(10.8)	17.5(8)	29.0(5.7)
0.1	+	46.0(12.9)	23.5(9.3)	27.3(7.5)
5	+	66.9(16.6)	15.8(8.3)	15.9(7.9)
10	+	72.7(8.7)	14.6(6.5)	11.4(4.1)
20	+	81.3(5.1)	14.9(4.3)	3.5(1.5)
25 μM resveratrol added with PMA	+	80.2(10.2)	9.6(7.2)	7.2(3.7)
25 μM resveratrol added 1 hour after PMA	+	95.4	2.0(0.4)	2.2(0.7)
25	-	95.1	1.9	2.5
-	+ (6 hr)	53.5	18.6	26.1
25	+ (6 hr)	83.4	9.4	6.44
25 (6 hr)	-	93.6	0.9	5.7

Table 4.2 The effect of resveratrol on PMA-induced cell death in U937 cells. U937 cells were incubated with resveratrol (μM) for 1 hour, after which they were stimulated with 100 nM PMA. Cells were then incubated with annexin/PI and analysed by flow cytometry. Five thousand cells were counted for each sample. Results shown are expressed as percentage of the total counted (means of 6 ±SD).

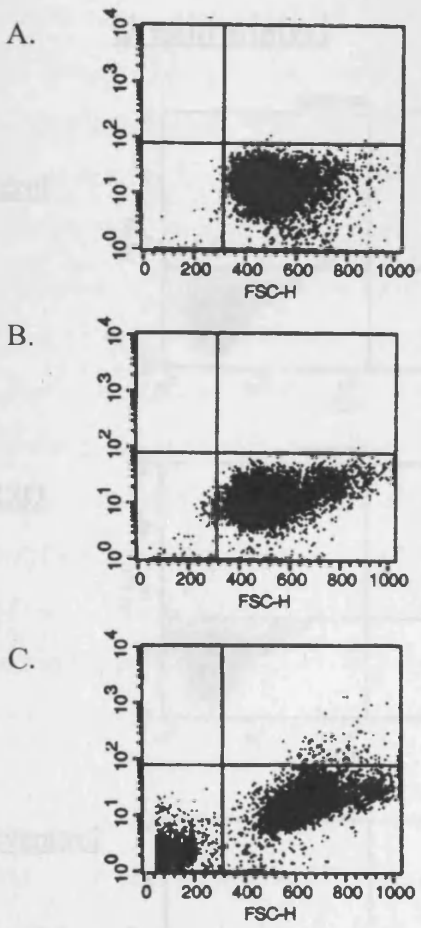


Figure 4.4. Resveratrol inhibits the formation of a low fluorescence population in dU937 cells. dU937 cells were pretreated with resveratrol (25 μM) for 1 hour before stimulation with 100 nM PMA for 3 hours. Cells were then loaded with 2 μM DCF and analysed by flow cytometry. Graphs shown are DCF fluorescence against cell size (FSCH). A-control, B-resveratrol, C-PMA, D-resveratrol+PMA, E-DMSO+PMA

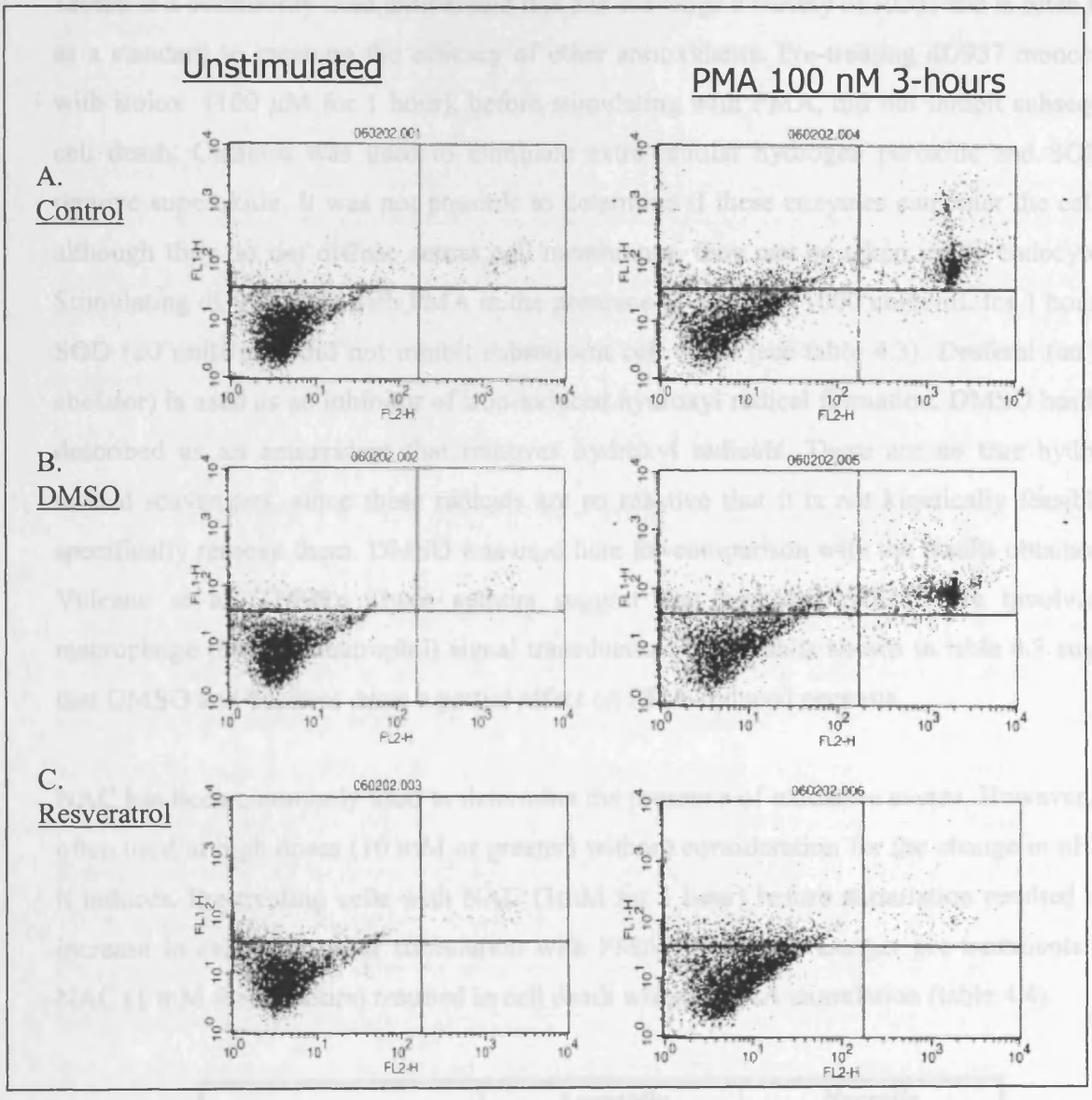


Figure 4.5 The effect of resveratrol on PMA-induced cell death in dU937 cells. U937 cells were incubated with resveratrol (μM) for 1 hour, after which they were stimulated with 100 nM PMA. Cells were then incubated with annexin/PI and analysed by flow cytometry. Results shown are dot-plots of FL1 fluorescence (annexin) against FL2 fluorescence (PI).

4.3 PMA-INDUCED CELL DEATH IS NOT COMPLETELY INHIBITED BY CLASSICAL ANTIOXIDANTS

Trolox is a commonly used antioxidant that can scavenge a variety of ROS, and is often used as a standard to measure the efficacy of other antioxidants. Pre-treating dU937 monocytes with trolox (100 μM for 1 hour), before stimulating with PMA, did not inhibit subsequent cell death. Catalase was used to eliminate extra-cellular hydrogen peroxide and SOD to remove superoxide. It was not possible to determine if these enzymes can enter the cell, as although they do not diffuse across cell membranes, they can be taken up by endocytosis. Stimulating dU937 cells with PMA in the presence of catalase (1000 units/mL for 1 hour) or SOD (20 units/mL) did not inhibit subsequent cell death (see table 4.3). Desferal (an iron chelator) is used as an inhibitor of iron-induced hydroxyl radical formation. DMSO has been described as an antioxidant that removes hydroxyl radicals. There are no true hydroxyl radical scavengers, since these radicals are so reactive that it is not kinetically feasible to specifically remove them. DMSO was used here for comparison with the results obtained by Vulcano et al., (1998). These authors suggest that hydroxyl radicals are involved in macrophage (but not neutrophil) signal transduction. The results shown in table 4.3 suggest that DMSO and desferal have a partial effect on PMA-induced necrosis.

NAC has been commonly used to determine the presence of oxidative events. However, it is often used at high doses (10 mM or greater) without consideration for the change in pH that it induces. Pre-treating cells with NAC (1mM for 1 hour) before stimulation resulted in an increase in cell death after stimulation with PMA (table 4.3). Longer pre-treatments with NAC (1 mM for 24 hours) resulted in cell death without PMA stimulation (table 4.4).

	Apoptotic % increase(+) or decrease(-)	**Necrotic % increase (+) or decrease (-)**
Trolox	66.9 ± 34.4	-2.2 ± 15.1
DMSO 1%	-44.3 ± 6.6	-40 ± 21
Desferal	-32.1± 34.4	-42.2 ± 4.9
SOD	5.3	8.3
Catalase	1.4	-4.2
NAC	-21.3± 34	55 ± 87.9

Table 4.3 The effect of antioxidants on PMA-induced cell death in dU937 cells. dU937 cells were pretreated with trolox (100 μM), DMSO (1%), desferal (1 mM), catalase (1000 units/mL), SOD (20 units/mL), NAC (1mM) for 1 hour before stimulation with 100 nM

PMA. Cells were then incubated with annexin/PI and analysed by flow cytometry. Results are expressed as percentage change in apoptosis or necrosis (means of 3 experiments ±SD).

	PMA	Live	Apoptotic	Necrotic
Control	-	93.38	0.8	4.1
	+	73.3	0.4	14.5
NAC (1 mM)	-	92.8	1.2	4.7
	+	71.1	2.5	15.6
NAC (10 mM)	-	7.6	0.2	90.7
	+	5.3	0.1	96.7

Table 4.4 The effect of NAC on PMA-induced cell death. dU937 cells were pretreated with NAC (1 or 10 mM) for 16-hours before stimulation with 100 nM PMA for 3 hours. Cells were then incubated with annexin/PI and analysed by flow cytometry. Results shown are expressed as percentage of the total counted.

4.4 THE EFFECT OF MODULATORS OF APOPTOSIS ON PMA-INDUCED CELL DEATH IN DU937 CELLS

Caspases are redox-sensitive enzymes that have a critical role in the execution of apoptosis. Using a caspase inhibitor such as zVAD-fmk, it has been determined that neutrophils undergo a caspase-dependent cell death after the respiratory burst. Moreover, if a strong stimulus is used e.g. PMA, the caspase inhibitor increases cell death. Some authors have shown that zVAD-fmk can inhibit the neutrophil respiratory burst. Pre-treating dU937 monocytes with the caspase inhibitor zVAD-fmk (10 μM for 30 minutes) did not affect PMA-induced ROS production, measured by DCF-FL (not shown) or cell death (table 4.5). Caspases are thought to be redox-regulated, which renders them inactive. This is thought to take place in the atheromatous plaque. If these enzymes are inactivated, oxidative damage that would normally induce apoptosis would not take place. Excessive oxidative DNA damage and inactivated caspases result in excessive PARP activation. Cellular necrosis occurs because of an attempt to regenerate NAD+, which results in ATP depletion. The PARP inhibitor, 3-aminobenzamide (3AB) (1 mM for 1 hour) did not have any effect on PMA-induced cell death (table 4.5).

Since these reactions were carried out in complete medium, there would be the potential for the activation of nitric oxide synthase. In theory this would be unlikely, since dU937 cells are not primed and most investigators have to use large doses of LPS before the activity of

nitric oxide synthase can be measured. The competitive inhibitor of NOS, L-NAME (1 mM), did not have any effect on PMA-induced cell death (table 4.5).

Some compounds with antioxidant or cytoprotective properties have been shown to induce the expression of heat shock protein 70 as a protective mechanism to oxidative stress. Heat shock proteins are molecular chaperones that have been shown to prevent cell death from a number of cell stresses (Beere 2001). Moreover, heat shock has been shown to inhibit PMA-induced ROS production in macrophages. Incubating dU937 monocytes at 42°C for 30 minutes with a recovery period at 37°C, resulted in inhibition of PMA-induced cell death (table 4.5). Heat shock was found to inhibit PMA-induced isoluminol-CL by 25% (of the phagocytic capacity) (not shown). The expression of HSP70 in dU937 monocytes was determined by western blotting (figure 4.6). U937 cells have high levels of HSP70 and pre-treatment does not significantly alter the levels of this protein. Initial results suggested that HSP70 was down-regulated during PMA stimulation and this decrease was inhibited by resveratrol. The levels of BCL2 were also investigated after PMA treatment, since over expression of this protein has been shown to prevent oxidative cell death. The levels of BCL2 remained unchanged during PMA stimulation (see figure 4.6).

	Apoptotic % increase(+) or decrease(-)	**Necrotic % increase (+) or decrease (-)**
3-Aminobenzamide	-4.4 ± 15.6	-3.7 ± 8.7
L-NAME	6.4 ± 2.9	10.6 ± 15.2
Heat shock	-65.1 ± 8.6	-70.6 ± 5.2
zVAD-fmk	-17.7 ± 6.9	-19.5 ± 9.2

Figure 4.5 The effect of modulators of apoptosis on PMA-induced cell death. dU937 cells were pretreated with L-NAME (1 mM) and 3-aminobenzamide (1 mM) for 1 hour, or zVAD-fmk (10 μM) for 30 minutes. Heat shock was performed by incubating cells at 42°C for 20 minutes, followed by a 2 hour recovery period at 37°C. Cells were then stimulated with 100 nM PMA for 3 hours and incubated with annexin/PI for analysis by flow cytometry. Results shown are expressed as percentage of the PMA control (means of 3 experiments ±SD).

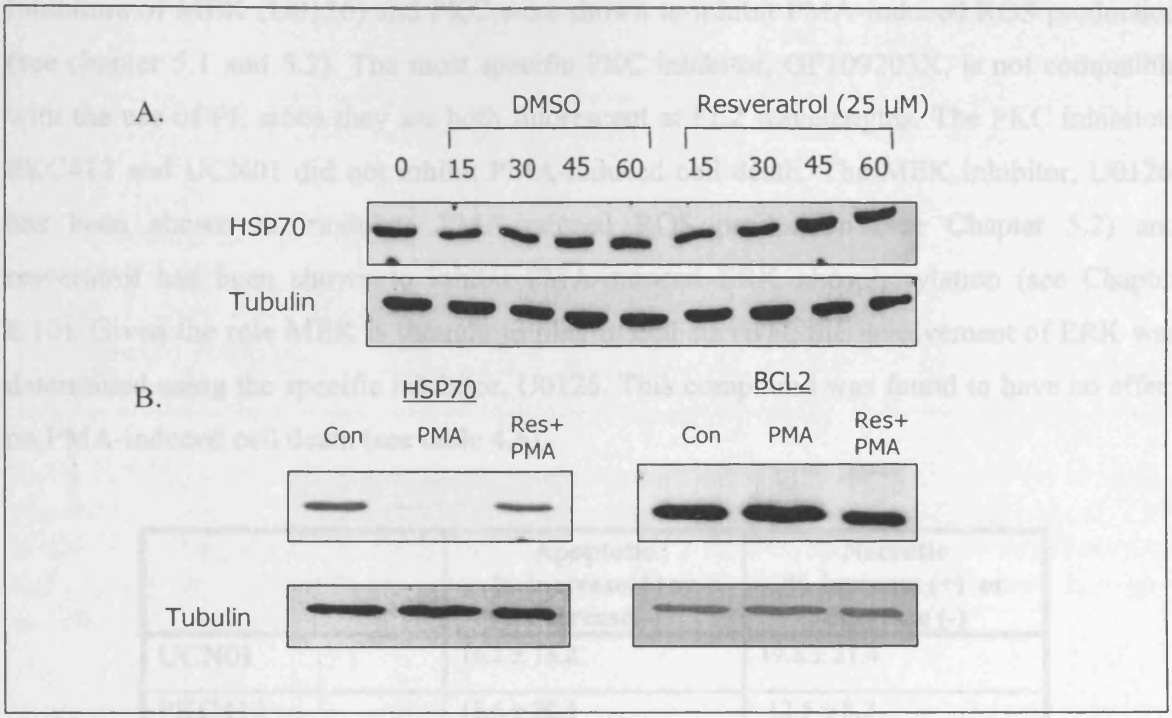


Figure 4.6 The effect of resveratrol on HSP70 and BCL2 expression in dU937 monocytes. A) dU937 cells were incubated with resveratrol (25 μM) or DMSO (0.1%) for the indicated time. Cell lysates were prepared and analysed by Western blotting as described in the materials and methods. Subsequent blots were probed for HSP70. B) dU937 cells were incubated for 1 hour with resveratrol before being stimulated with 100 nM PMA for 3 hours. Cell were prepared and analysed as described above. Subsequent blots were probed for HSP70 or BCL2. A) Representative of two experiments and B) of a single experiment.

4.5 THE EFFECT OF MEK AND PKC INHIBITORS ON PMA-INDUCED CELL DEATH

Inhibitors of MEK (U0126) and PKC were shown to inhibit PMA-induced ROS production (see chapter 5.1 and 5.2). The most specific PKC inhibitor, GF109203X, is not compatible with the use of PI, since they are both fluorescent at FL2 wavelengths. The PKC inhibitors PKC412 and UCN01 did not inhibit PMA-induced cell death. The MEK inhibitor, U0126, has been shown to modulate PMA-induced ROS production (see Chapter 5.2) and resveratrol has been shown to inhibit PMA-induced ERK phosphorylation (see Chapter 6.10). Given the role MEK is thought to play in cell survival, the involvement of ERK was determined using the specific inhibitor, U0126. This compound was found to have no effect on PMA-induced cell death (see table 4.6).

	Apoptotic % increase(+) or decrease(-)	**Necrotic % increase (+) or decrease (-)**
UCN01	16.2 ± 18.2	19.8 ± 21.4
PKC412	15.6 ± 20.4	-12.5 ± 8.2
U0126	5.6	1.4

Table 4.6 The effect of PKC and MEK-inhibitors on PMA-induced cell death in dU937 cells. dU937 cells were pretreated with UCN01 (1 μM) and PKC412 (1 μM) for 15 minutes and U0126 (10 μM) for 1 hour. Cells were then stimulated with 100 nM PMA and then incubated with annexin/PI and analysed by flow cytometry. Results shown are expressed as percentage PMA control (means of 3 experiments ±SD).

4.6 THE EFFECT OF RESVERATROL ON LIPID PEROXIDATION IN U937 MONOCYTES

Lipid peroxidation was measured using a colorimetric assay that is thought to be specific for the formation of MDA. dU937 cells treated with vitamin C and iron (III) chloride (100 μM of each for 15 minutes), which did not show increased MDA levels. Pre-treating U937 monocytes with 1 mM sodium MDA resulted in a large increase in the intra-cellular levels of MDA (table 4.7). Interestingly, pre-treating U937 cells with sodium MDA resulted in an increase in DCF-FL that was comparable to 100 μM H_2O_2 (figure 4.7). However, pre-treating dU937 cells with PMA or fMLP did not result in a significant increase in MDA over a 3 hour period. Resveratrol did not alter the levels of MDA in the absence or presence of PMA (table 4.7). TNF has been shown to increase MDA levels in numerous cell lines. However, the results shown here do not support this fact (table 4.7).

	MDA (μM)
Control	2.0±1.2 n=28
Sodium MDA (1 mM)	16.3±6.2 n=3
TNF (1 nM)	2.4±1.3 n=20
fMLP (1μM)	3.0±1.2 n=6
PMA 40minutes (undifferentiated U937, 100 nM)	1.8±1.2 n=4
PMA 3 hours (100 nM)	1.9±0.6 n=3
Resveratrol (50 μM)	1.5±0.4 n=3
PMA+resveratrol	2.3±0.8 n=3

Table 4.7 The effect of resveratrol on lipid peroxidation in dU937 cells. Lipid peroxidation was induced in U937 cells by incubating cells with sodium MDA (1 mM, for 1 hour); TNF (1 nM or 175 μg/ml, for 1 hour); fMLP (1 μM, for 1 hour); PMA (100 nM for 40 minutes and 1 hour); resveratrol (50 μM pre-treatment for 1 hour). MDA levels (±SD) were measured as described in the material and methods.

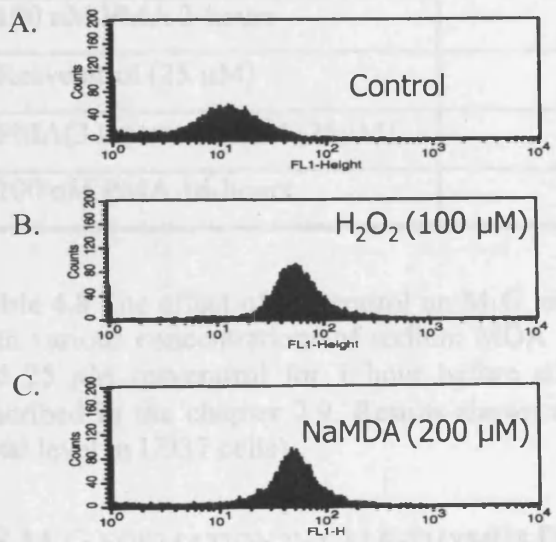


Figure 4.7. The effect of sodium MDA on DCF-FL in dU937 cells. dU937 cells were suspended in BSS and loaded with 2 μM DCF for 5 minutes before stimulation with 100 μM hydrogen peroxide for 30 minutes or 200 μM sodium MDA for 1 hour. Cells were then analysed by flow cytometry. Results shown are histograms of cell counts against DCF fluorescence. A) untreated cells, B) hydrogen peroxide, C) sodium MDA.

4.7 THE EFFECT OF RESVERATROL ON M_1G FORMATION IN U937 MONOCYTES

The M_1G DNA adduct can be used as a marker for DNA damage that has occurred via lipid peroxidation and direct radical oxidation (through base propenal formation, see below) (Plastaras et al., 2000). The immunoslot blot method was used to measure the levels of M_1G in dU937 cells and revealed that these cells have high levels of this adduct. Following stimulation with PMA for 4 or 16 hours there was no significant increase in M_1G levels. Resveratrol was found to have no effect on M_1G levels over a 3 hour period (basal or stimulated). Treating dU937 cells with sodium MDA (0.5, 1 2 mM) did not result in any significant changes in M_1G levels.

	M_1G (adducts per 10^8 nucleotides)
Calf-thymus DNA standard	6.1(1.02)
Untreated U937	96.7(48.5)
Sodium MDA 0.5 mM	61.5(29)
"1 mM	66.1(36.3)
"2 mM	91.6(25.1)
100 nM PMA 3-hours	111.8(47.5)
Resveratrol (25 μM)	117.3(63.6
PMA(3 hr)+resveratrol (25μM)	115.4(82.3)
100 nM PMA 16-hours	45(12.9)

Table 4.8 The effect of resveratrol on M_1G levels in U937 cells. U937 cells were incubated with various concentrations of sodium MDA for 16 hours, 100 nM PMA for 3 or 16 hour and 25 μM resveratrol for 1 hour before stimulation with PMA. M_1G was analysed as described in the chapter 2.9. Results shown are means (±SD) of 3 experiments (6 for the basal level in U937 cells).

4.8 M_1G FORMATION IN CALF-THYMUS DNA

Since attempts to increase the levels of M_1G in U937 cells all failed, the immuno-slot blot method was tested using calf-thymus DNA. In order to examine the reaction between MDA and DNA, calf-thymus DNA was treated with sodium MDA (1,10, 20 mM) for 96 hours (see figure 4.8 and 4.9). This resulted in significant increases in M_1G at 10 and 20 mM. A time course of MDA treatment over 96 hours using 1,5,10, 20 mM sodium MDA resulted in

significant changes in M_1G levels at 24, 72 and 96 hours for the 10 and 20 mM treatments. M_1G formation by sodium MDA was found to be accelerated when calf-thymus DNA was incubated with sodium MDA at a lower pH (pH 4.5 compared to 7.4) resulting in an approximately 8-fold increase in M_1G formation.

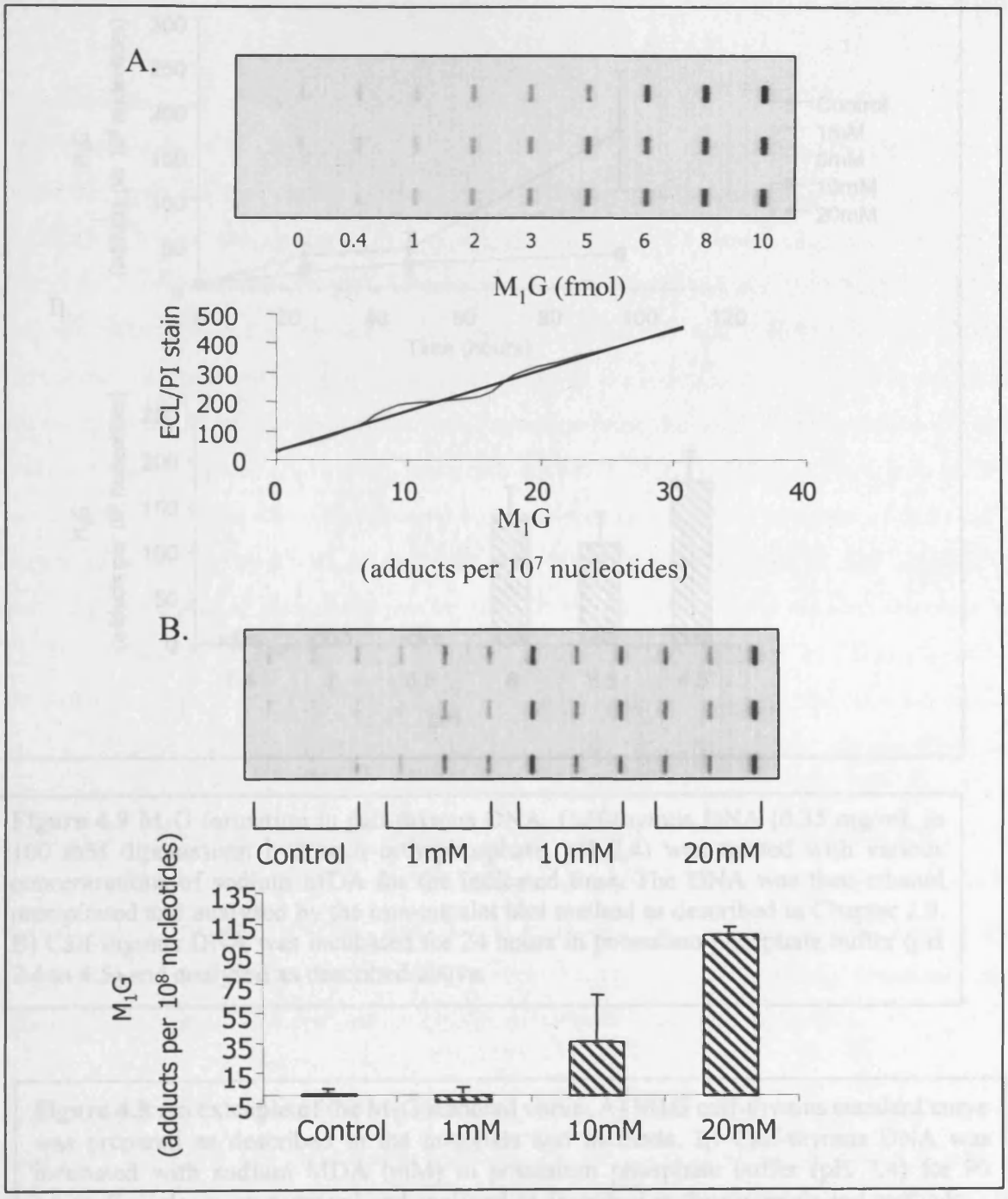


Figure 4.8 An example of the M_1G standard curve. A) M_1G calf-thymus DNA standard curve was prepared as described in Chapter 2.9. B) Calf-thymus DNA was incubated with sodium MDA (mM) in potassium phosphate buffer (pH 7.4) for 96 hours. Samples were prepared and analysed as described in Chapter 2.9. Results shown are means (±SD) obtained from a single experiment.

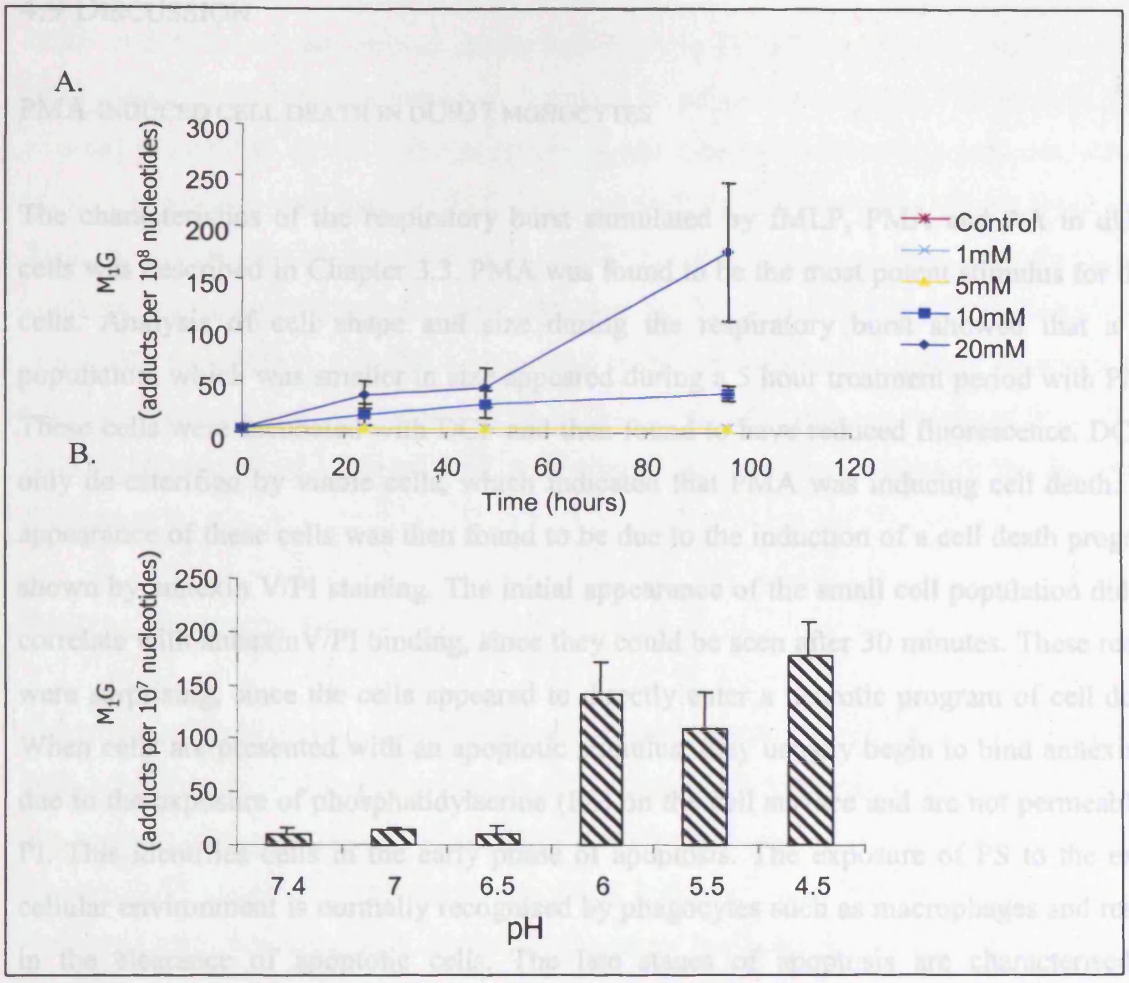


Figure 4.9 M_1G formation in calf-thymus DNA. Calf-thymus DNA (0.35 mg/mL in 100 mM dipotassium hydrogen orthophosphate, pH 7.4) was treated with various concentrations of sodium MDA for the indicated time. The DNA was then ethanol precipitated and analysed by the immunoslot blot method as described in Chapter 2.9. B) Calf-thymus DNA was incubated for 24 hours in potassium phosphate buffer (pH 7.4 to 4.5) and analysed as described above.

Figure 4.8 An example of the M_1G standard curve. A) M1G calf-thymus standard curve was prepared as described in the materials and methods. B) Calf-thymus DNA was incubated with sodium MDA (mM) in potassium phosphate buffer (pH 7.4) for 96 hours. Samples were prepared and analysed as described in the materials and methods

4.9 DISCUSSION

PMA-INDUCED CELL DEATH IN DU937 MONOCYTES

The characteristics of the respiratory burst stimulated by fMLP, PMA and AA in dU937 cells was described in Chapter 3.3. PMA was found to be the most potent stimulus for these cells. Analysis of cell shape and size during the respiratory burst showed that a cell population, which was smaller in size appeared during a 5 hour treatment period with PMA. These cells were incubated with DCF and then found to have reduced fluorescence. DCF is only de-esterified by viable cells, which indicated that PMA was inducing cell death. The appearance of these cells was then found to be due to the induction of a cell death program, shown by annexin V/PI staining. The initial appearance of the small cell population did not correlate with annexinV/PI binding, since they could be seen after 30 minutes. These results were surprising, since the cells appeared to directly enter a necrotic program of cell death. When cells are presented with an apoptotic stimulus, they usually begin to bind annexin V, due to the exposure of phosphatidylserine (PS) on the cell surface and are not permeable to PI. This identifies cells in the early phase of apoptosis. The exposure of PS to the extra-cellular environment is normally recognised by phagocytes such as macrophages and results in the clearance of apoptotic cells. The late stages of apoptosis are characterised by membrane permeability to PI. Under normal conditions apoptotic cells are cleared by phagocytes, before they become necrotic. If this process is compromised, apoptotic cells then become necrotic, which acts as a pro-inflammatory stimulus. In general, necrosis is viewed as a passive process that lacks a coordinated mechanism. However, the fragmentation of DNA during necrosis has been shown to result in orderly fragments and some authors suggest that cell death has more than the two classical forms (Kok et al., 2002).

In the case of PMA-treated dU937 monocytes, the cells remain intact until at least 2 hours after PMA treatment and then become necrotic. Oxidative insults have been shown to induce apoptosis in a variety of cell types (Simon et al., 2000), whereas excessive oxidative insults have been shown to directly induce necrosis (Gardner et al., 1997). PMA was found to induce necrosis in a dose-dependent fashion. However, doses of PMA less than 100 nM induced much less necrosis. PMA did not induce cell death in undifferentiated U937 cells

over a 3 hour period. However, some authors have shown that PMA induces ROS production and cell death in undifferentiated U937 cells (Datta et al., 2000).

fMLP and AA were not able to induce cell death in dU937 monocytes. Neutrophils have been shown to undergo apoptosis in response to physiological stimuli as well as PMA, whereas monocytes do not undergo apoptosis under normal activating conditions. AA was show to produce a large burst of ROS (see Chapter 3.3). However, this did not result in cell death. AA is thought to directly activate NADPH oxidase which could suggest that it is not involved in the induction of cell death. However, the kinetics of AA and PMA-induced ROS production are distinct, with PMA-inducing a sustained response. Although the results from Chapter 3 suggest that AA-induces large intra-cellular responses (as measured by DCF and DHR), the activity of LOX or COX may contribute significantly towards the increase in fluorescence.

RESVERATROL INHIBITS PMA-INDUCED CELL DEATH

Pre-treating dU937 monocytes with resveratrol was found to dose dependently inhibit PMA-induced cell death. The low fluorescence cell population formed during PMA stimulation did not appear when cells were pre-treated with resveratrol (shown in figure 4.2). Analysis of annexin V/PI staining revealed that resveratrol inhibited PMA-induced necrosis, however, there was still some annexin V binding. Resveratrol has been shown to induce apoptosis in several cell types, including U937 monocytes. In this case resveratrol did not induce cell death in dU937 cells over a 6 hour period. Annexin V staining is only a semi-quantitative method for measuring apoptosis. Cells that are permeable to PI normally display a 1000-fold increase in FL2 fluorescence, therefore it is easy to distinguish between necrotic and live cells (see figure 4.5). Annexin V binding does not result in large changes in fluorescence, therefore it is often difficult to make a clear distinction between cells that are not binding annexin V. There is also the added complication that cell aggregates appear to have a higher fluorescence, therefore, in some cases small increases in annexin V fluorescence should be viewed with caution. The small increase in annexin V staining that remains after PMA stimulation in the presence of resveratrol, might not be significant if these factors are taken into account, especially considering that PMA has been shown to induce the formation of cell aggregates (in the presence of calcium) (Van Pelt et al., 1996).

THE ROLE OF OXIDANTS IN PMA-INDUCED CELL DEATH

Studies using differentiated HL60 cells, which are thought to resemble a neutrophil-like cell revealed that PMA stimulation results in the rapid formation of apoptotic cells (Arroyo et al., 2002). These authors showed that the addition of SOD and catalase inhibited this process. The addition of catalase to dU937 cells before PMA stimulation did not affect the resulting necrosis. As discussed in Chapter 3, there is some debate as to whether catalase can affect intracellular oxidation, since it is thought to be cell impermeable. Some cell types can take up catalase by endocytosis. In order to exclude these problems, trolox was used in an attempt to inhibit PMA-induced necrosis. Trolox has been shown to be a highly effective antioxidant that can react with most oxidising species, although it does not inhibit PKC, which vitamin E has been shown to inhibit along with p47 translocation (Cachia et al., 1998). Trolox was found to have no inhibitory effect on PMA-induced necrosis, but rather caused an increase.

NAC has been used in many studies as an antioxidant and as a method of increasing intracellular glutathione levels. Pre-treatment of 1 mM NAC for 1 hour did not inhibit PMA-induced necrosis, but rather increased it. Longer pre-treatments of 24 hours, did not inhibit PMA-induced necrosis and higher doses of NAC (10 mM) induced cell death in the absence PMA stimulation. High doses of NAC (1 mM or above) resulted in a reduced pH of the cell solution, in fact 1 mM NAC was able to lower the pH to below 5. This is often an overlooked aspect of NAC and the majority of studies use this compound at 1 mM or above. Interestingly, the study by Datta et al (2000), which demonstrates that undifferentiated U937 cells can produce superoxide, uses 30 mM NAC.

Desferal is an iron chelator that is thought to prevent the formation of hydroxyl radicals. Pre-treatment of dU937 monocytes with 1 mM desferal partially inhibited PMA-induced ROS production, although this compound was less effective than resveratrol. DMSO can be used as a crude antioxidant and is a potential scavenger of hydroxyl radicals. However, as mentioned above, it is not a true antioxidant. DMSO also had a partial effect on PMA-induced necrosis. These results tentatively suggest a role for transition metal catalysed hydroxyl radical formation in this response. However, this was not supported by the negative results obtained using trolox. Resveratrol has been shown to have some ability to prevent transition metal mediated-hydroxyl radical formation and can also prevent subsequent

glutathione oxidation. It is unlikely that these properties of resveratrol are solely responsible for its inhibitory effect on PMA-induced cell death, since in that case desferal would be more effective.

The inability of some highly effective and commonly used compounds to inhibit PMA–induced necrosis, suggested that oxidants were not involved in the cell death process, which was in complete contrast to other studies involving neutrophils or HL60 cells. This also suggested that the antioxidant properties of resveratrol were not responsible for its inhibition of PMA-induced necrosis. Although it is assumed that the antioxidant ability of trolox is far greater than that of resveratrol, there is the possibility that resveratrol can directly inhibit the activation of the respiratory burst or some other process that accounts for the appearance of necrotic cells.

THE ROLE OF CASPASES, PARP AND NOS IN OXIDATIVE CELL DEATH

Caspases are redox sensitive enzymes, which can be inactivated by an oxidative insult. It has been shown in HL60 cells that the pan-caspase inhibitor, zVAD-fmk, can inhibit the production of ROS (Arroyo et al., 2002). In the present study 10 μM zVAD-fmk had no effect on ROS production or cell death induced by PMA in dU937 monocytes. If the oxidative insult is excessive, caspases are thought to be inactivated and therefore the subsequent cell death has a necrotic phenotype (Borutaite et al., 2001). It has been suggested that macrophages can inactivate their caspsases. This may occur in conditions such as atherosclerosis, possibly through nitrosylation or oxidation. Although antioxidants had no effect this did not completely discard nitrosylation (Kockx and Knaapen 2000). However, the inhibitor of nitric oxide, L-NAME, also had no effect. During the apoptotic process caspases represent the primary enzymatic effectors, which are activated through mitochondrial makers of apoptosis e.g. cytochrome c or through receptor-mediated effectors e.g. Fas-ligand (Zimmermann et al., 2001). After caspases have been activated, PARP is cleaved and inactivated (Chang and Yang 2000). PARP is a nuclear protein that aids the DNA repair process (Benjamin and Gill 1980). Following large oxidative insults, e.g. 1 mM hydrogen peroxide, PARP inhibitors have been shown to prevent subsequent necrosis (Gardner et al., 1997). PARP facilitates the repair process, which is induced by oxidative damage. However, if the insult is large the caspases (e.g. caspase 3) that would normally inhibit over-activation of process are deactivated. This results in excessive PARP activation which results in the excessive consumption of the enzyme cofactor NAD+. The consumption

of NAD+ leads to ATP depletion and results in necrosis. To determine the involvement of PARP and a possible inhibitory effect of resveratrol on this enzyme, the PARP-inhibitor 3-aminobezamide was used. At 1 mM it had no effect on PMA-induced necrosis, so the involvement of PARP was excluded.

THE ROLE OF HSP IN PMA-INDUCED CELL DEATH

Heat shock proteins are the molecular chaperones of the cell. They function to maintain protein function during cell stress such as heat shock and oxidative stress. They have more recently been associated with various cell signalling pathways and kinases, e.g. Akt and are believed to play a role in apoptosis (Beere 2001). Heat shock (Chen et al., 1999) and agents shown to induce the expression of heat shock proteins (Lu et al., 2002) have been shown to protect against oxidative insults. As previously described by Jacquier-Sarlin et al (1995), exposing cells to higher temperatures (44°C) for 20 minutes followed by a recovery period of two hours, resulted in complete inhibition of ROS production upon PMA stimulation. The results in this study confirm this, however the inhibition was much less. Heat shock was, with the exception of resveratrol, the only treatment found to inhibit PMA induced necrosis. Analysis of HSP70 after resveratrol pre-treatment revealed that dU937 cells have high levels of this protein. Resveratrol induced a slight increase in the expression of HSP70 over a 1 hour period. Initial results suggested that PMA stimulated dU937 cells display reduced levels of HSP70, an effect which was inhibited by resveratrol. Subsequent results did not support this hypothesis, since PMA failed to reduce the levels of HSP70 to the same dramatic effect.

BCL2 is an onocoprotein that has been shown to protect cells from oxidative insults (Kane et al., 1993 and Steinman 1995), which is suggested to be responsible for its anti-apoptotic properties (Antonsson and Martinou 2000). Some authors have over expressed BCL2 and the resulting cells have been found to be more resistant to oxidative insults. Moreover, heat shock has been shown to increase the expression of BCL2 in U937 cells, which inhibited hydrogen peroxide-induced cell death (Polla et al., 1996). Pre-treatment with resveratrol or PMA stimulation had no effect on the protein levels of BCL2 over a 3 hour period in dU937 cells. Changes in BCL2 levels are generally shown to occur over longer time periods than used in this study.

THE ROLE OF PKC IN PMA-INDUCED CELL DEATH

The results so far demonstrate that PMA-induced necrosis only occurred in *differentiated* U937 cells (over a 3 hour period) and could be inhibited by resveratrol and heat shock. Resveratrol can potentially inhibit the production of ROS and heat shock has been demonstrated to do this by other authors (Jacquier-Sarlin et al., 1995). Therefore, to test whether it was the activation of the respiratory burst *per se* rather than the oxidants produced that were responsible for the necrosis, inhibitors of PKC and MEK, were used. Surprisingly, the PKC inhibitors UCN01 and PKC412 did not affect PMA-induced necrosis, although both these compounds significantly reduced PMA-induced ROS production, as measured by DCF-FL, luminol-CL, isoluminol-CL and lucigenin-CL.

The MEK inhibitor, U0126, did not affect PMA-induced necrosis. U0126 was found to partially inhibit PMA-induced ROS (chapter 5.2) and resveratrol was found to inhibit PMA-induced ERK phosphorylation (chapter 6.10). Much attention has been paid to the activation of ERK during oxidative stress-induced cell death. It has been suggested that ERK activation has a dual role in cell survival in response to an oxidative insult. In some cases inhibition of ERK activition results in enhanced cell death following an oxidative insult, while others have reported that ERK activity can contribute to cell death. ERK activity and the consequences for stress-induced cell death are largely cell type-dependent. However, its pro or anti-apoptotic functions have been classified according to the kinetics of its activation. Rapid and transient ERK activation is thought to mediate anti-apoptotic signals, whereas, delayed and sustained activation results in apoptotic signals (reviewed by Martindale and Holbrook, 2002). In this study, PMA-induced ERK activity was found to be rapid and transient (see Chapter 6.9), although in this case inhibition of ERK activity did not result in increased cell death.

The inability of PKC inhibitors and classical antioxidants to inhibit PMA-induced cell death suggests that the ability of PMA to induce ROS production was not responsible for cellular necrosis. This leads towards the possibility that some other activity of PMA was inducing the cell death, which could be PKC-independent. Resveratrol has been shown to inhibit PKC. However, it has a different inhibitory mechanism to the other PKC inhibitors used in this study. PMA binds to the C1-domain of PKC and resveratrol has also been shown to bind to this domain, therefore resveratrol is thought to be a phorbol ester antagonist. The C1-

domain occurs in many proteins, other than PKC that are also involved in cell survival, which is often overlooked. PMA should not be viewed as a specific PKC activator and there are many other cellular effects of PMA that are PKC-independent. Calcium channels are activated by PMA and this could be an important regulator of cell death. However, PMA-induced necrosis only occurred in dU937 cells and not undifferentiated cells. In other studies, PMA has been shown to induce cell death in undifferentiated U937 cells, which the authors suggest is as a result of oxidative stress (Datta et al 2000). In this study PMA-induced ROS could not be measured in undifferentiated U937 cells and therefore, the initial hypothesis was that the cell death was dependent on NADPH oxidase derived ROS. It would appear that this was not case and some other effect of PMA was responsible for cell death in dU937 monocytes. Although heat shock was found to inhibit ROS production and subsequent cell death, it is difficult to interpret these results, since with the exception of resveratrol, it was the only treatment found to inhibit PMA-induced necrosis.

LIPID PEROXIDATION

The ability of PMA to induce oxidative stress was further investigated with the use of biomarkers for lipid peroxidation and DNA damage. The formation of MDA is a well established biomarker for oxidative stress, since it is the most abundant reactive aldehyde formed as a result of lipid peroxidation. Although the commonly used assay for MDA, such as the thiobarteric reactive substances (TBARS) assay, are often criticised for their lack of specificity, they still provide useful information in isolated cell systems. In this study, the MDA assay used was much more specific than the TBARS assay, since assay conditions favour greater specificity for MDA and the heating step is at a lower temperature and avoids artificial generation of MDA.

Stimulating dU937 cells with PMA did not result in significant increases in intracellular MDA levels. Moreover, resveratrol did not affect basal or PMA stimulated MDA levels. It was subsequently shown by Gieseg et al., (2001) that exposure of U937 cells to high levels of sustained oxidative stress (up to 21 hours) did not result in lipid peroxidation. However, protein oxidation was evident and was measured by the formation of protein hydroperoxides using the FOX assay in precipitated proteins (devoid of lipids). Their method involved measuring protein hydroperoxides in a large number of cells, but the resulting levels of protein hydroperoxides appeared to be low.

M_1G FORMATION

The M_1G adduct is thought to be formed through the reaction of MDA with guanine residues, although it became clear during this study that the reaction between MDA and DNA was very slow. High levels of sodium MDA had to be used in order to measure significant levels of M_1G in calf thymus DNA using the immuno-slot blot method. This method is a sensitive and efficient means of measuring M_1G, allowing 24 sample analysis per assay. The formation of M_1G was accelerated by reacting sodium MDA with calf-thymus DNA at a lower pH. It is difficult to envisage where such conditions occur physiologically, although the activation of the respiratory burst does result in significant pH changes, although they are efficiently buffered.

It was subsequently shown by Palstaras et al (2000) that there are two pathways that lead to M_1G formation. The modification efficiency of MDA can be predicted from the results in figure 4.9, by calculation from the slope of the curve after 24 hour sodium MDA treatment. This was estimated to be 1.6 adducts per 10^8 bases per mM of sodium MDA. Palstaras et al., (2000) calculated it to be 1.3. Using the immuno-slot blot method they also show that M_1G formation by MDA is very slow, but M_1G formation is greatly enhanced from base propenals. Base propenals are formed through the action of ROS on DNA bases, such as adenine. No changes were measured in M_1G levels during the stimulation of the respiratory burst by PMA. There was no change over 3 hour stimulation, where cell death was measured with annexin/PI staining. Longer PMA pre-treatments also had no effect on the levels of M_1G.

CONCLUSIONS -RESVERATROL AND THE C1 DOMAIN

The initial theory tested here was that resveratrol was inhibiting the respiratory burst, either through direct radical scavenging or inhibition of the activation of NADPH oxidase. The chronic activation of the respiratory burst by PMA led to an unusual cellular necrosis that could be dose-dependently inhibited by resveratrol. An attempt was made to characterise PMA-induced necrosis, however, this was largely unsuccessful. A striking feature of this mode of cell death was that at no point during the time course did the apopotic cells out number the necrotic cells. Large oxidative insults, such as 1 mM hydrogen peroxide, usually induce this type of response. However, since PKC inhibitors or other antioxidants did not

inhibit this response (albeit a partial effect), oxidative damage was all but ruled out as the causative factor. This suggested that PMA-induced necrosis was not dependent on the respiratory burst and some other property of PMA was inducing this response. PMA binds to the C1 domain of PKC, which is also the DAG binding site. Resveratrol has been shown to compete for PMA for this binding site and thus reduce PMA-induced PKC activity. The effect of resveratrol on PKC appears to be dependent on the method used to measure PKC activity. Resveratrol has been shown in some cases to strongly inhibit membrane associated PKC activity and is a weak competitor of ATP for its binding site.

The ability of PMA to induce non-PKC mediated effects does not receive a great deal of attention. There are a number of non-PKC effects of PMA, which are thought to be mediated through the C1 binding domain. Resveratrol is a potential inhibitor of these PMA induced effects. Calcium channels have been shown to be activated by DAG and it is possible that PMA-induced cell death is calcium mediated and is perhaps a result of calcium overload. Calcium channels are thought to be a viable therapeutic target for leukocyte activation (Li and Westwick et al., 2002). Therefore the effect of resveratrol on C1 domain binding requires further investigation and might add to its chemopreventive properties.

CHAPTER FIVE: CELLULAR SIGNALLING PATHWAYS THAT ACTIVATE NADPH OXIDASE IN U937 MONOCYTES

INTRODUCTION

In order to further understand the mechanism by which resveratrol can inhibit the respiratory burst the kinases involved in the activation process need to be identified. The stimuli used in this study (fMLP, PMA and AA) are all likely to activate different cell signalling pathways. Many kinases have been shown to be involved in the activation of NADPH oxidase and the majority of this research has focused on the activation process in neutrophils. In this chapter the activation process was studied in monocytes, which has received much less attention, but is still highly relevant to a variety of pathologies. Using specific inhibitors of protein kinases the activation of NADPH oxidase can be determined.

The activation of NADPH oxidase requires the coordination of multiple cell signalling pathways. The range of pathways involved depends on the stimuli. However, they all share common features, in that a single stimulus results in two separately amplified signals. One initiates the translocation of the small GTP-binding protein, Rac, and the other for the cytosolic NADPH oxidase subunits.

The current literature describes many kinases that are involved in the activation of NADPH oxidase. The review by Bokoch (1995) is one of the few examples where an attempt has been made to summarise chemoattractant signalling pathways, seven in all and the activation of Rac. To add to this complexity there is also the possibility that the activation of NADPH oxidase may involve biphasic activation of various kinases and there may also be different pathways controlling intra- and extra-cellular release of ROS.

Protein kinase mediated signalling cascades are the primary mechanism by which a cell is able to respond to an environmental stimulus e.g. growth factors (Reith 2001). They also mediate signals induced by noxious stimuli e.g. bacterial, viral and parasitic infections, non-digestible particles and chemical irritants. Chronic inflammation caused by biological and chemical irritants is thought to accelerate the carcinogenic processes in some organ sites. Much attention has been paid to the role of arachidonic acid metabolism and prostaglandin synthesis, which has now been linked with many human cancers. Inhibition of prostaglandin synthesis has been identified as a major chemopreventive strategy e.g. inhibition of COX and LOX (Steele et al., 2003).

Phagocytes are major mediators of the inflammatory process, which serves to combat invading pathogens. In some cases their activation is believed to contribute towards a number of pathologies. These cells produce a number of non-specific mediators, such as ROS and RNS through the activities of NADPH oxidase and iNOS. The activity of these cells is mediated by multiple protein kinase signalling pathways, which allows them to respond to environmental stimuli in an appropriate fashion.

Most of the current research involving the activation of NADPH oxidase has been carried out in neutrophils or neutrophil-like cell lines e.g. HL60 and there is much less known about the activation process in monocytes/macrophages. However, it is important to determine the differences between these cell types, given that in some pathologies, such as atherosclerosis, the initiation of the disease is mediated by macrophages. This chapter attempts to address these issues for the activation of NADPH oxidase in monocytes. Using several different kinase inhibitors, their effect on both intra- and extra-cellular ROS production is determined. This is carried out to assess their potential as therapeutic targets to compare their effects to those of resveratrol and to identify potential therapeutic targets. The kinases that activate NADPH oxidase might provide potential chemopreventive targets as they are involved in cellular priming mechanisms, which orchestrate an appropriate immune response and in some cases, result in pathology.

5.1 PMA AND FMLP, BUT NOT AA-INDUCED ROS PRODUCTION ARE SENSITIVE TO PKC INHIBITORS

Three different PKC inhibitors were used to examine the role of PKC in the activation of NADPH oxidase in response to PMA, fMLP and AA. PKC has been shown to phosphorylate multiple sites on the p47 subunit of NADPH oxidase (Dang 2001). Their efficacy towards

various PKC isoforms is described in Chapter 1.7.5 (table 1.3). PKC has been shown to be inhibited be resveratrol, therefore it is important to determine which of the stimuli activates PKC-dependent signalling pathways.

Figure 5.1A-C shows the effect of GF109203X, UCN-01 and PKC412 on PMA-induced ROS production measured by DCF-FL using a plate reader, which determines the change in total fluorescence. Negative values were measured for the highest doses of GF109203X and UCN-01. The use of flow cytometry to measure ROS production (figure 5.1D- F) resulted in a similar, though less prominent, pattern of inhibition. Figure 5.2 shows the effect of these inhibitors on isoluminol and lucigenin-CL. UNC-01 produced the greatest inhibition of the three inhibitors. Analysis of the effect of these inhibitors on the kinetic parameters of the isoluminol and lucigenin responses revealed a dose-dependent decrease in phagocytic capacity and I_{max} and only PKC412 produced a significant change in the T_{max} (table 5.1A). UCN01 and PKC412 produced significant changes in T_{max} when lucigenin was used to measure PMA-induced ROS (table 5.1B)

Figure 5.3A shows the effect of GF109203X on fMLP-induced ROS production, while B and C show the effect of PKC412 and UCN-01 respectively. All of these compounds were effective inhibitors of this response, displaying similar efficacies. GF109203X was also found to inhibit fMLP-induced lucigenin-CL and is shown in figure 5.3D. One μM GF109203X reduced the response to 67.6% ± 25.6 of the control, compared to 26.5% ±5.2 for isoluminol-CL. The effect of these PKC inhibitors on isoluminol- and lucigenin-CL response (Table 5.1) revealed a dose-dependent decrease in phagocytic capacity and no change in T_{max}.

Figure 5.4 shows the effect of the PKC inhibitors on AA-induced ROS production and no significant effect was measured using DCF (A) or isoluminol (B) chemiluminescence. Kinetic analysis also revealed no change in any of the parameters (table 5.1).

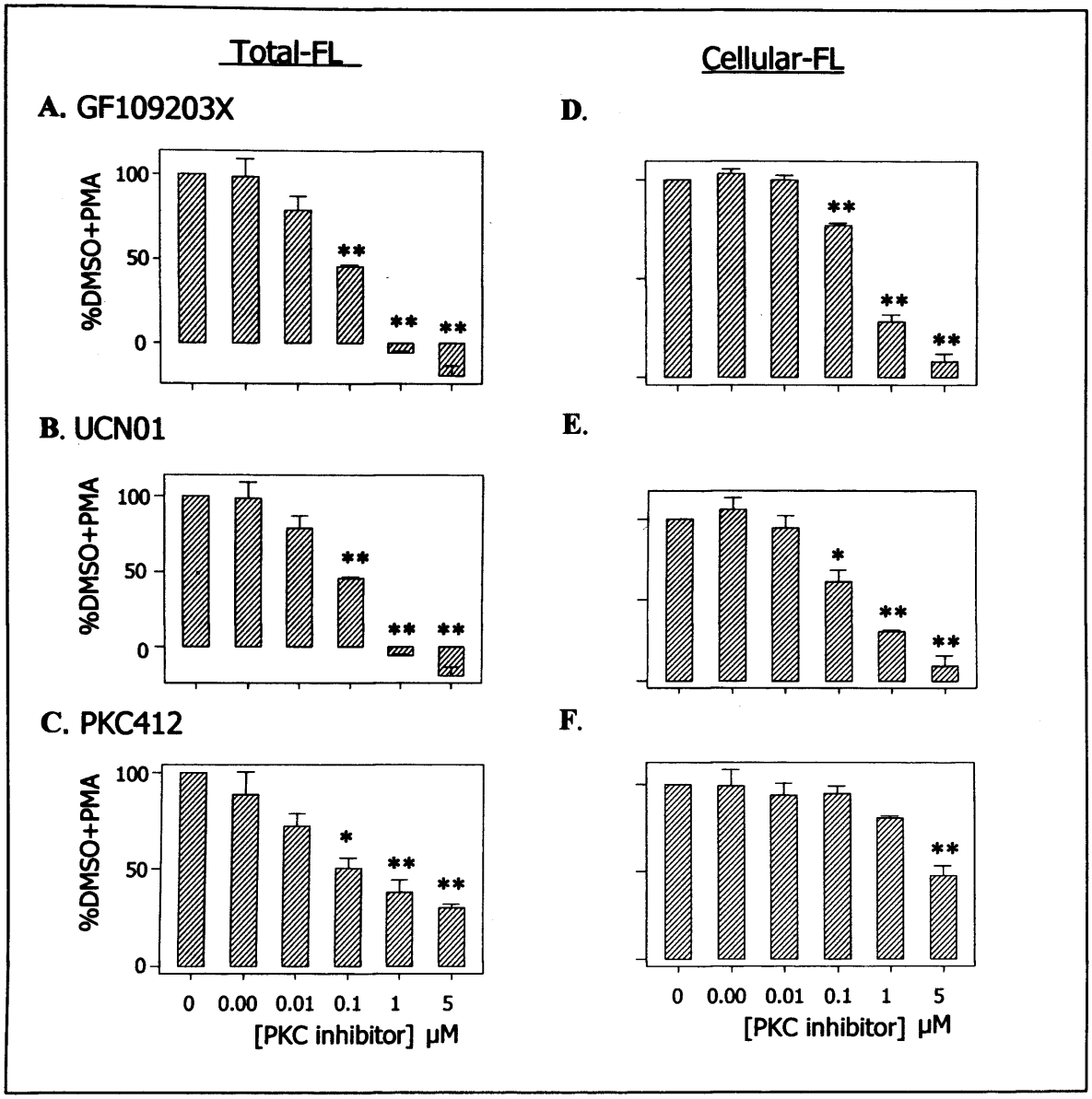


Figure 5.1 The effect of PKC inhibitors on PMA-induced DCF-FL. dU937 cells were pre-treated with the indicated PKC inhibitor for 15 minutes. Cells were pre-loaded with 2 µM DCF for 5 minutes before stimulation with 100 nM PMA for 75 minutes (A-C) and 30 minutes (D-F). The effect of the inhibitors was measured using a fluorescence plate reader (A-C) or flow cytometry (D-F). GF109203X (A and D), UCN01 (B and E) and PKC412 (C and F). Results shown are expressed as %DMSO+PMA control and show means (±S.D) of three experiments. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (0) control cells as determined by a one way ANOVA.

A. Isoluminol	Stimulus	T_{max}	I_{max}	Phagocytic capacity
GF109203X (1 μM)	fMLP	160± 87.1	23.8± 4.5**	26.5± 5.2**
	PMA	99.8± 7.4	24.5± 3.3**	19.5± 2.5**
	AA	100.7± 20.4	110.6± 8.8	98.7± 5
UCN01 (1 μM)	fMLP	104.4± 7.6	5.9± 9.2**	15.7± 18.1**
	PMA	87.1± 54.5	0.5± 0.54**	3± 3.8**
	AA	115.6± 10.5	95.6± 20.5	90.7± 4.8
PKC412 (1 μM)	fMLP	102.2± 3.8	39.7± 22.5*	46.1± 25.8*
	PMA	133.33± 14.4*	63.3± 3.7**	78.9± 14.1*
B.Lucigenin-CL				
GF109203X (1μM)	fMLP	100	68.2± 23.9	67.6± 25.6
	PMA	79.8± 14.2	17.7± 2.8**	17.6± 1.6**
UCN01 (1μM)	PMA	9.6± 10.3**	9.1± 1.75**	5.94± 1.62**
PKC412 (1μM)	PMA	10.8± 0.6**	24.6± 4.4**	22.2± 0.7**

Table 5.1 The effect of PKC inhibitors on isoluminol (extracellular ROS)- and lucigenin (superoxide)-CL. dU937 cells were treated with the indicated PKC inhibitor for 15 minutes (followed by washing) before stimulation with 1 μM fMLP, 100 nM PMA or 50 μM AA. Results shown in (A) are measured with isoluminol and (B) lucigenin-CL. Results are expressed as a % of cells stimulated in the presence of DMSO obtained from means (±SD) of three experiments. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (100%) control cells as determined by a one way ANOVA.

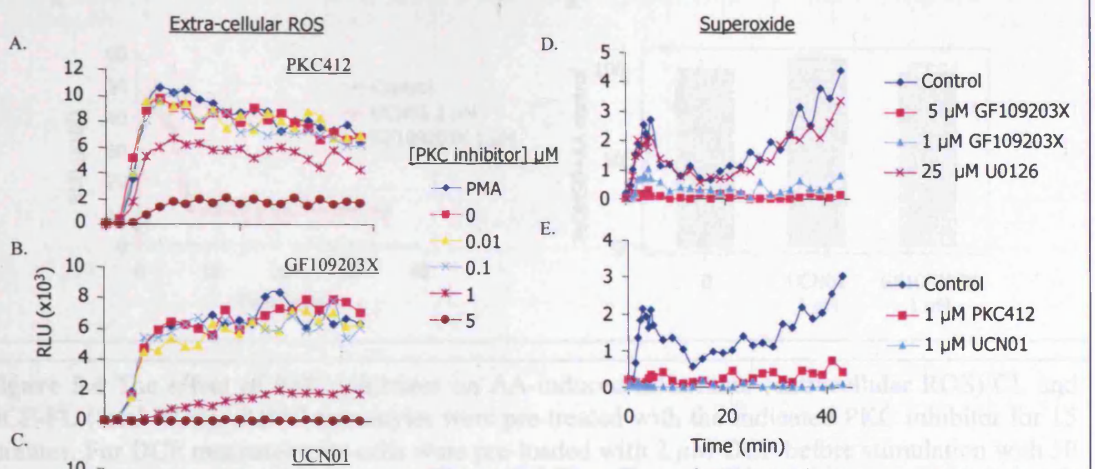


Figure 5.2 The effect of PKC inhibitors on PMA-induced isoluminol- (extra-cellular ROS) and lucigenin (superoxide)-CL. dU937 monocytes were pre-treated with the indicated PKC inhibitor for 15 minutes. Cells were stimulated with PMA (100 nM) at time zero as measured by isoluminol-CL (A-C) or lucigenin-CL (D-E). Control represents cells stimulated in the presence of DMSO (0). Results shown are representative of three experiments.

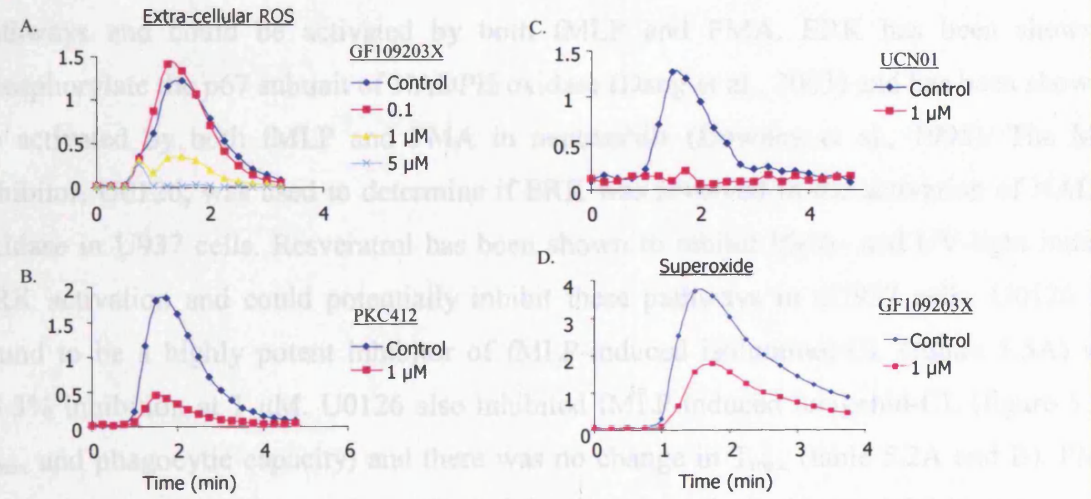


Figure 5.3 The effect of PKC inhibitors on fMLP-induced isoluminol-(extra-cellular ROS) and lucigenin (superoxide)-CL. dU937 monocytes were pre-treated with the indicated PKC inhibitor for 15 minutes. Cells were stimulated with fMLP (1 μM) at 45 seconds and measured by isoluminol-CL (A-C) or lucigenin-CL (D). GF109203X (A and D), PKC412 (B) and UCN01 (C). Control represents cells stimulated in the presence of DMSO. Results shown are representative of three experiments.

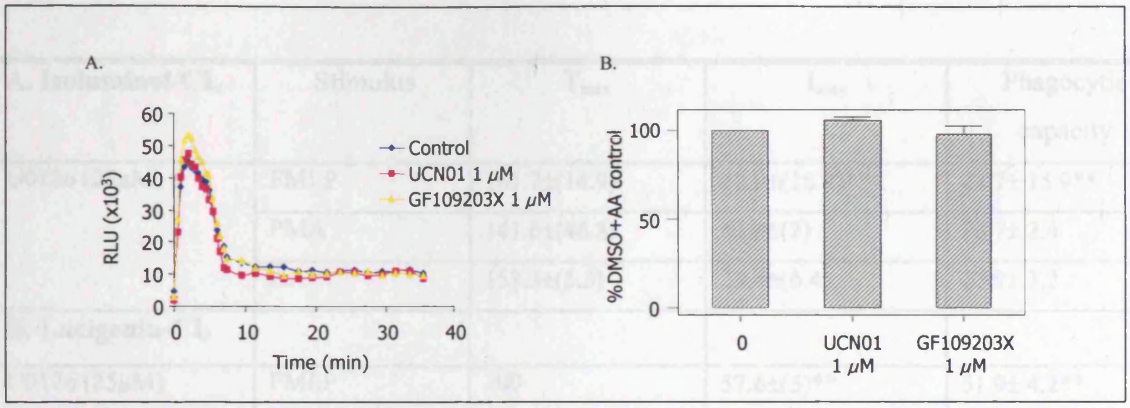


Figure 5.4 The effect of PKC inhibitors on AA-induced isoluminol (extracellular ROS)-CL and DCF-FL (total ROS). dU937 monocytes were pre-treated with the indicated PKC inhibitor for 15 minutes. For DCF measurements cells were pre-loaded with 2 μM DCF before stimulation with 50 μM AA for 30 minutes (B) and measured using a fluorescence plate reader. Results shown in (A) are representative of three experiments and in (B) are expressed as %DMSO+AA control and means (±SD) of three experiments. For isoluminol measurements AA was added at time zero (A).

5.2 THE MEK INHIBITOR, U0126, INHIBITS PMA, FMLP AND AA-INDUCED ROS PRODUCTION

The MAPK pathway represents a potential convergence point for numerous cell signalling pathways and could be activated by both fMLP and PMA. ERK has been shown to phosphorylate the p67 subunit of NADPH oxidase (Dang et al., 2003) and has been shown to be activated by both fMLP and PMA in neutrophils (Downey et al., 1998). The MEK inhibitor, U0126, was used to determine if ERK was involved in the activation of NADPH oxidase in U937 cells. Resveratrol has been shown to inhibit PMA- and UV-light induced ERK activation and could potentially inhibit these pathways in dU937 cells. U0126 was found to be a highly potent inhibitor of fMLP-induced isoluminol-CL (figure 5.5A) with 53.3% inhibition at 1 μM. U0126 also inhibited fMLP-induced lucigenin-CL (figure 5.5B) (I_{max} and phagocytic capacity) and there was no change in $T_{max.}$ (table 5.2A and B). PMA-induced isoluminol-CL was less affected by U0126 and significant inhibition was only measured at 10 and 25 μM (figure 5.5C). U0126 produced no change in T_{max} and a dose-dependent decrease in phagocytic capacity and I_{max} (table 5.2). PMA-induced lucigenin-CL was significantly affected by U0126 (figure 5.5D). AA-induced ROS production was only significantly affected by U0126 at the highest dose used (25 μM) producing a significant decrease in phagocytic capacity, but not T_{max} (figure 5.5E and table 5.2).

U0126 did not significantly inhibit PMA- (figure 5.6A) or AA-induced (figure 5.6B) ROS when measured by DCF-FL.

A. Isoluminol-CL	Stimulus	T_{max}	I_{max}	Phagocytic capacity
U0126 (25μM)	FMLP	101.7±(14.9)	22.9±(18.2)**	23.7± 15.9**
	PMA	141.6±(46.8)	62.8±(2)	23.7± 2.4
	AA	153.3±(5.3)	23.4±(6.4)	23.9± 3.2
B. Lucigenin-CL				
U0126 (25μM)	FMLP	100	57.6±(5)**	51.9± 4.2**
	PMA	96.8±(2.6)	72.6±(14.2)*	80.7± 9.1*

Table 5.2 The effect of U0126 on isoluminol (extracellular ROS)- and lucigenin (superoxide)-CL. dU937 were treated with the indicated dose of U0126 for 60 minutes (followed by washing) before stimulation with 1 μM fMLP, 100 nM PMA or 50 μM AA. Results shown in (A) are measured with isoluminol and (B) lucigenin-CL. Results are expressed as a % of cells stimulated in the presence of DMSO obtained from three experiments. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (100%) control cells as determined by a one way ANOVA.

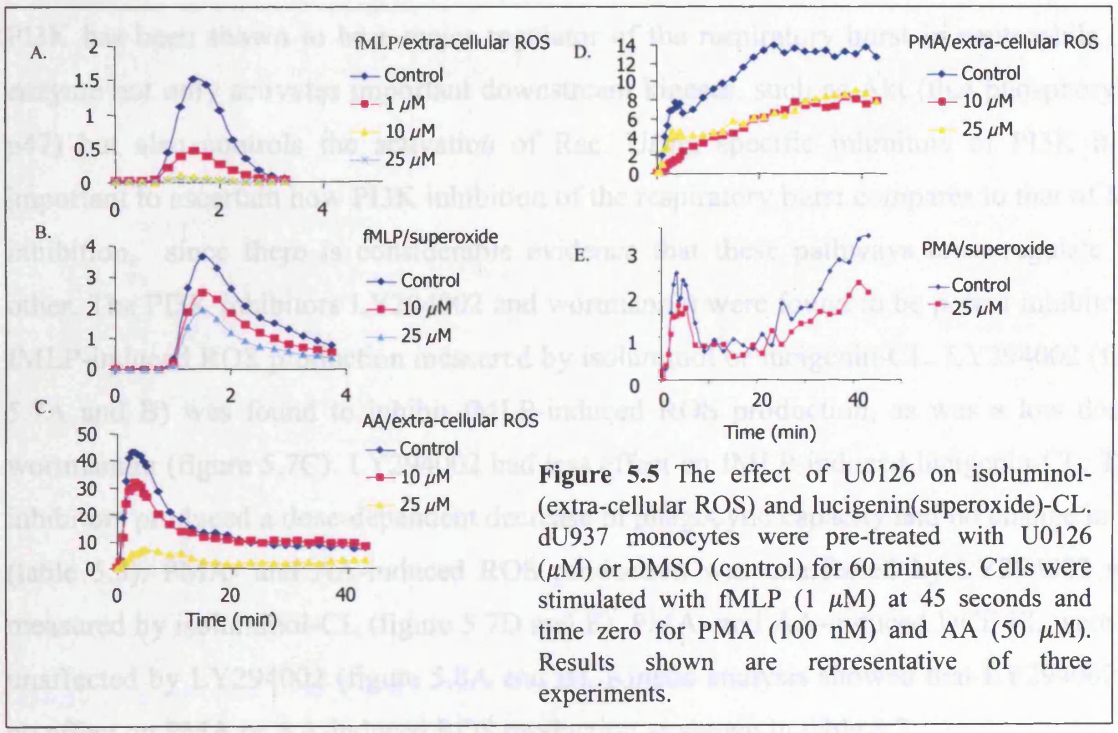


Figure 5.5 The effect of U0126 on isoluminol-(extra-cellular ROS) and lucigenin(superoxide)-CL. dU937 monocytes were pre-treated with U0126 (μM) or DMSO (control) for 60 minutes. Cells were stimulated with fMLP (1 μM) at 45 seconds and time zero for PMA (100 nM) and AA (50 μM). Results shown are representative of three experiments.

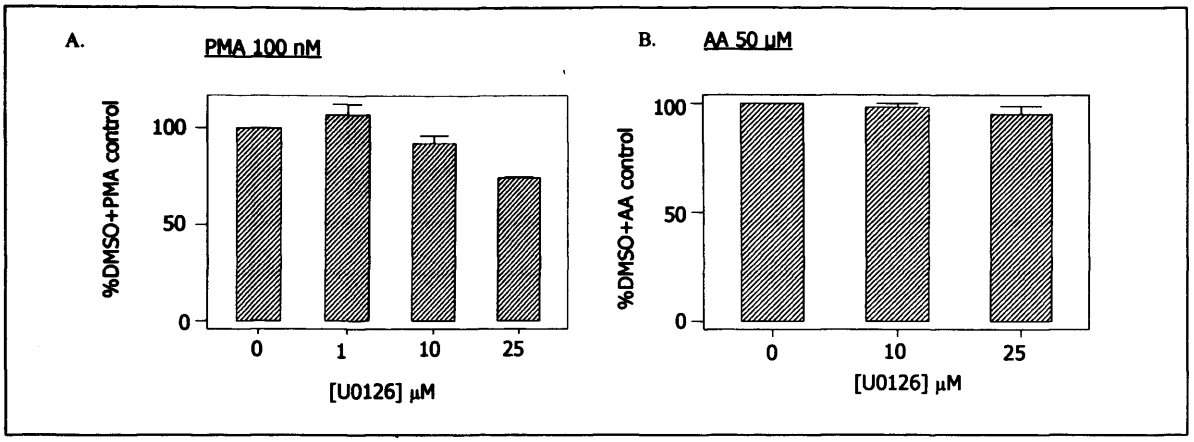


Figure 5.6 The effect of U0126 on DCF measured ROS production (total ROS). The effect of U0126 on PMA-and AA-induced DCF-FL. dU937 cells were pre-treated with U0126 for 60 minutes. Cells were pre-loaded with 2 μM DCF for 5 minutes before stimulation with (A) PMA for 75 minutes and (B) AA for 30 minutes. The effect of these inhibitors was measured using a fluorescence plate reader. Results shown are expressed as %DMSO+PMA control and means (± S.D) of three experiments.

5.3 fMLP-INDUCED ROS PRODUCTION IS SENSITIVE TO PI3K INHIBITORS

PI3K has been shown to be a major regulator of the respiratory burst in neutrophils. This enzyme not only activates important downstream kinases, such as Akt (that phosphorylates p47) but also controls the activation of Rac. Using specific inhibitors of PI3K it was important to ascertain how PI3K inhibition of the respiratory burst compares to that of MEK inhibition, since there is considerable evidence that these pathways cross-regulate each other. The PI3K inhibitors LY294002 and wortmannin were found to be potent inhibitors of fMLP-induced ROS production measured by isoluminol or lucigenin-CL. LY294002 (figure 5.7A and B) was found to inhibit fMLP-induced ROS production, as was a low dose of wortmannin (figure 5.7C). LY294002 had less effect on fMLP-induced lucigenin-CL. These inhibitors produced a dose-dependent decrease in phagocytic capacity and no change in T_{max} (table 5.3). PMA- and AA-induced ROS production was unaffected by LY294002 when measured by isoluminol-CL (figure 5.7D and E). PMA- and AA-induced DCF-FL were also unaffected by LY294002 (figure 5.8A and B). Kinetic analysis showed that LY294002 had no effect on PMA or AA-induced ROS production as shown in table 5.3.

A. Isoluminol-CL	Stimulus	T_{max}	I_{max}	Phagocytic capacity
LY294002 (50μM)	fMLP	133.3± 33.3	5.4± 4.7**	17.09± 2.9**
	PMA	84.9± 14.3	123.2± 2.2**	118.5± 21.4
	AA	95.6± 20.6	110.6± 15.8	105.8± 10.6
Wortmannin (100 nM)	fMLP	79.2± 15.9*	7.9± 10.8**	7.2± 7.8**
B. Lucigenin-CL				
LY294002 (50 μM)	fMLP	108.3± 11.7	69.9± 8.2	60.8± 2
	PMA	102.5± 16.8	110.7± 14.6	107.8± 13.6

Table 5.3 The effect of PI3K inhibitors on isoluminol (extracellular ROS)- and lucigenin (superoxide)-CL. dU937 were treated with the indicated dose of LY294002 or wortmannin for 60 minutes (followed by washing) before stimulation with 1 μM fMLP, 100 nM PMA or 50 μM AA. Results shown in (A) are measured with isoluminol and (B) lucigenin-CL. Results are expressed as a % of cells stimulated in the presence of DMSO obtained from three experiments. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (100%) control cells as determined by a one way ANOVA.

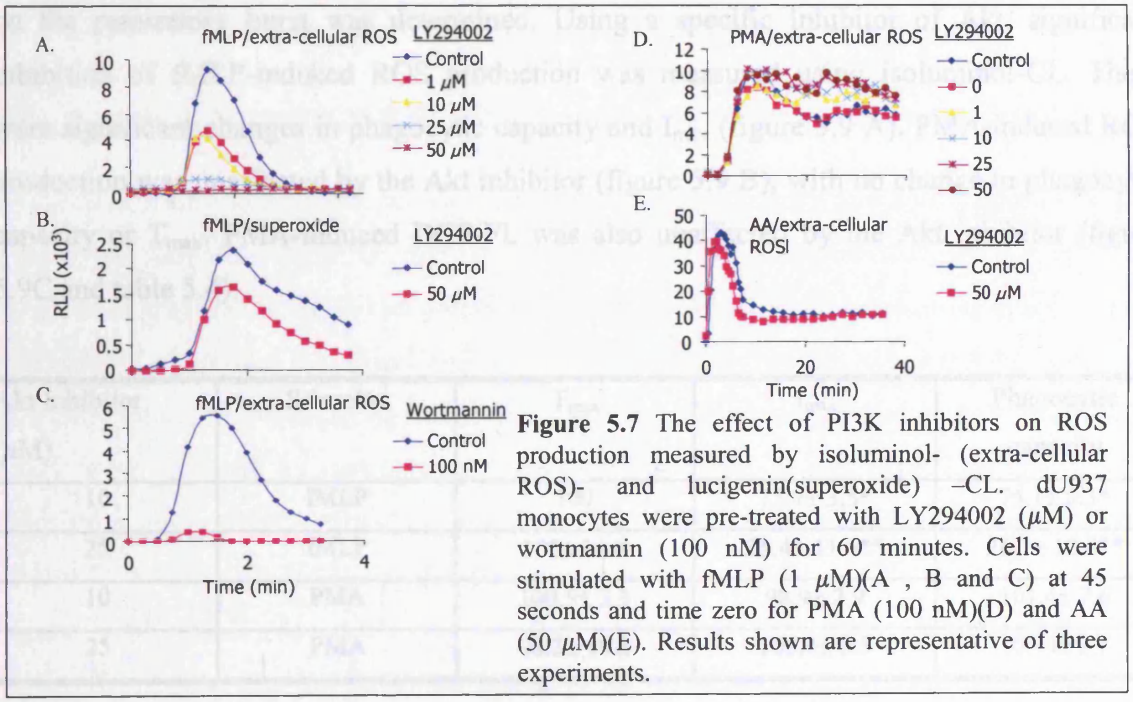


Figure 5.7 The effect of PI3K inhibitors on ROS production measured by isoluminol- (extra-cellular ROS)- and lucigenin(superoxide) –CL. dU937 monocytes were pre-treated with LY294002 (μM) or wortmannin (100 nM) for 60 minutes. Cells were stimulated with fMLP (1 μM)(A , B and C) at 45 seconds and time zero for PMA (100 nM)(D) and AA (50 μM)(E). Results shown are representative of three experiments.

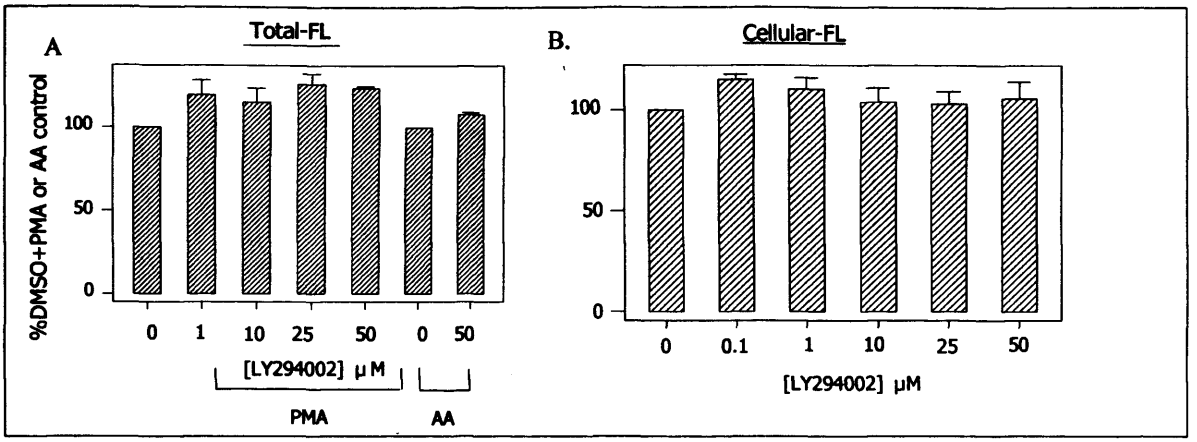


Figure 5.8 The effect of LY294002 on PMA- and AA-induced DCF-FL. dU937 cells were pre-treated with LY294002 (μM) for 1 hour. Cells were pre-loaded with 2 μM DCF for 5 minutes before stimulation with A) 100 nM PMA for 75 minutes or 50 μM AA for 30 minutes. Total DCF-FL (ROS) was measured by fluorimetry. B) dU937 cells were pre-treated as in (A) except that they stimulated for 30 minutes and measured by flow cytometry (cellular ROS). Results shown are means (±SD) of three experiments

5.4 A SPECIFIC INHIBITOR OF AKT ACTIVATION INHIBITS FMLP-INDUCED ROS PRODUCTION

Given its role in the phosphorylation of neutrophil p47, the activation of Akt and its effect on the respiratory burst was determined. Using a specific inhibitor of Akt, significant inhibition of fMLP-induced ROS production was measured using isoluminol-CL. There were significant changes in phagocytic capacity and I_{max} (figure 5.9 A). PMA-induced ROS production was unaffected by the Akt inhibitor (figure 5.9 B), with no change in phagocytic capacity or T_{max}. PMA-induced DCF-FL was also unaffected by the Akt inhibitor (figure 5.9C and table 5.4).

Akt Inhibitor (μM)	Stimulus	T_{max}	I_{max}	Phagocytic capacity
10	fMLP	100	75.7± 3.5*	75.1± 5.3*
25	fMLP	100± 16.6	25.4± 11.6**	26.2± 12.8**
10	PMA	100.5± 3.3	98.9± 2.9	101.4± 7.6
25	PMA	98.3± 10.2	103.4± 3.5	105.4± 2.7

Table 5.4 The effect of an Akt-inhibitor on isoluminol (extracellular ROS)-CL. dU937 were treated with the indicated dose of Akt inhibitor for 2 hours (followed by washing) before stimulation with 1 μM fMLP or 100 nM PMA. Results are expressed as a % cells stimulated in the presence of DMSO obtained from three experiments for PMA and four for fMLP. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (100%) control cells as determined by a one way ANOVA.

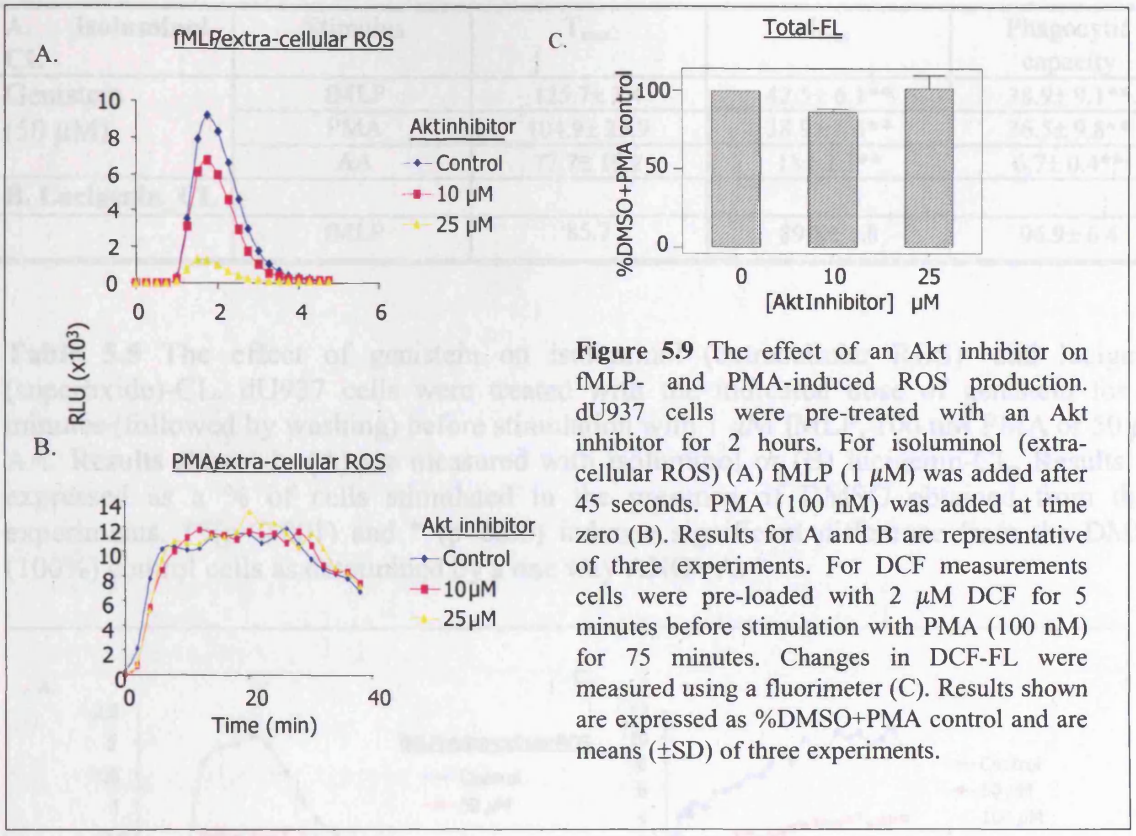


Figure 5.9 The effect of an Akt inhibitor on fMLP- and PMA-induced ROS production. dU937 cells were pre-treated with an Akt inhibitor for 2 hours. For isoluminol (extra-cellular ROS) (A) fMLP (1 μM) was added after 45 seconds. PMA (100 nM) was added at time zero (B). Results for A and B are representative of three experiments. For DCF measurements cells were pre-loaded with 2 μM DCF for 5 minutes before stimulation with PMA (100 nM) for 75 minutes. Changes in DCF-FL were measured using a fluorimeter (C). Results shown are expressed as %DMSO+PMA control and are means (±SD) of three experiments.

5.5 GENISTEIN INHIBITS FMLP, PMA AND AA-INDUCED ROS PRODUCTION

Tyrosine kinases have been shown to regulate the respiratory burst in neutrophils and monocytes. However, these kinases have not been shown to be substrates for the cytosolic NADPH oxidase subunits, but upstream regulators e.g. PI3K, Rac. Kinases such as Src have been shown to activate fMLP-induced Akt activation in neutrophils. Genistein was also used as a comparison to the effect of resveratrol, since it is also a polyphenolic compound that is likely to be oxidized by peroxidases. Genistein was found to inhibit fMLP-induced ROS production measured by isoluminol, but much less inhibition was measured by lucigenin-CL (figure 5.10 A and B). PMA- and AA-induced isoluminol-CL was also inhibited by genistein (figure 5.10C and D). Genistein was also found to inhibit PMA- and AA-induced DCF-FL (figure 5.11A and B). The effect of genistein on the kinetics of the respiratory burst are shown in table 5.4. There was inhibition of I_{max} and phagocytic capacity with all stimuli used, with no change in T_{max}.

A. Isoluminol-CL	Stimulus	T_{max}	I_{max}	Phagocytic capacity
Genistein (50 μM)	fMLP	125.7± 3.4	42.5± 6.1**	38.9± 9.1**
	PMA	104.9± 25.9	38.9± 6.5**	36.5± 9.8**
	AA	77.7± 19.2	18± 2.3**	6.7± 0.4**
B. Lucigenin_CL				
	fMLP	85.7	89.8± 1.8	96.9± 6.4

Table 5.5 The effect of genistein on isoluminol (extracellular ROS)- and lucigenin (superoxide)-CL. dU937 cells were treated with the indicated dose of genistein for 60 minutes (followed by washing) before stimulation with 1 μM fMLP, 100 nM PMA or 50 μM AA. Results shown in (A) are measured with isoluminol or (B) lucigenin-CL. Results are expressed as a % of cells stimulated in the presence of DMSO obtained from three experiments. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (100%) control cells as determined by a one way ANOVA.

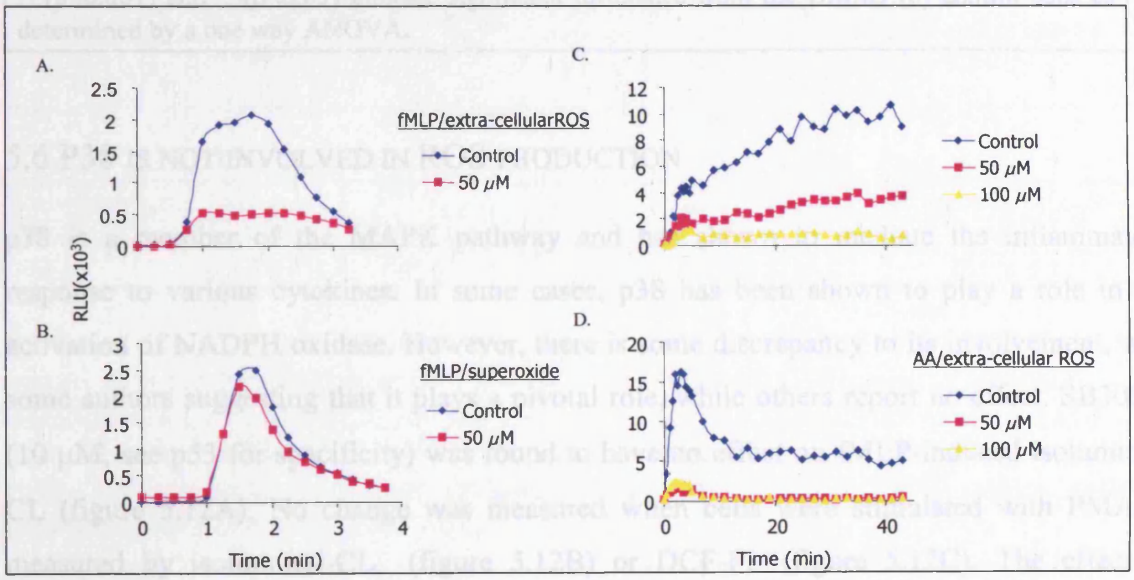


Figure 5.10 Figure 5.5 The effect of genistein on isoluminol(extra-cellular ROS)- and lucigenin (superoxide)-CL. dU937 monocytes were pre-treated with genistein (μM) for 60 minutes. Cells were stimulated with fMLP (1 μM)(A and B) at 45 seconds and time zero for PMA (100 nM)(C) and AA (50 μM)(D). Results shown are representative of three experiments.

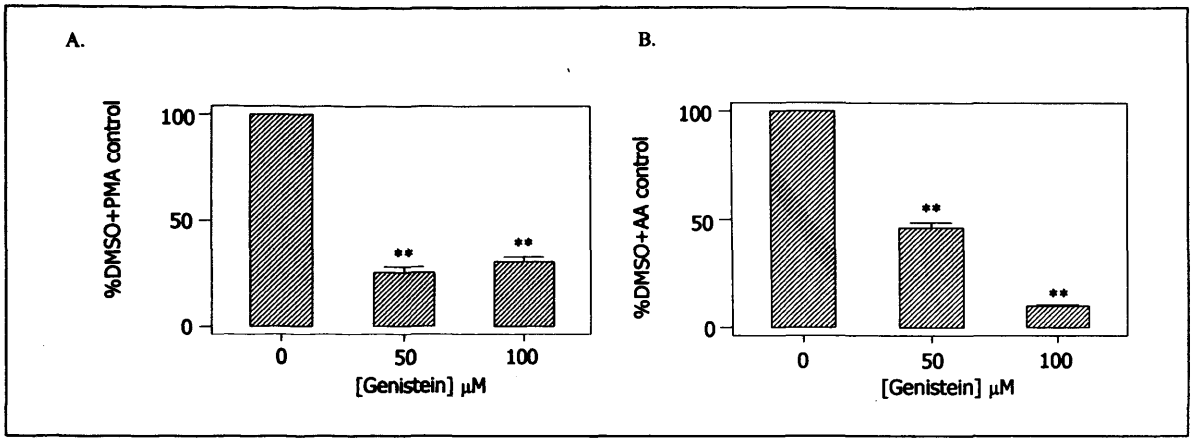


Figure 5.11 The effect of genistein on PMA- and AA-induced DCF-FL(total ROS). dU937 monocytes were pre-treated with genistein (μM) for 60 minutes. Cells were pre-loaded with 2 μM DCF for 5 minutes before stimulation with 100 nM PMA (A) for 75 minutes and AA for 30 minutes (B). The effect of these inhibitors was measured using a fluorescence plate reader. Results shown are expressed as %DMSO+PMA or AA control and means (± S.D) of three experiments. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (0) control cells as determined by a one way ANOVA.

5.6 P38 IS NOT INVOLVED IN ROS PRODUCTION

p38 is a member of the MAPK pathway and has shown to mediate the inflammatory response to various cytokines. In some cases, p38 has been shown to play a role in the activation of NADPH oxidase. However, there is some discrepancy to its involvement, with some authors suggesting that it plays a pivotal role, while others report no effect. SB30580 (10 μM, see p53 for specificity) was found to have no effect on fMLP-induced isoluminol-CL (figure 5.12A). No change was measured when cells were stimulated with PMA as measured by isoluminol-CL (figure 5.12B) or DCF-FL (figure 5.12C). The effect of SB203580 on the kinetics of the isoluminol-CL response is shown in table 5.6.

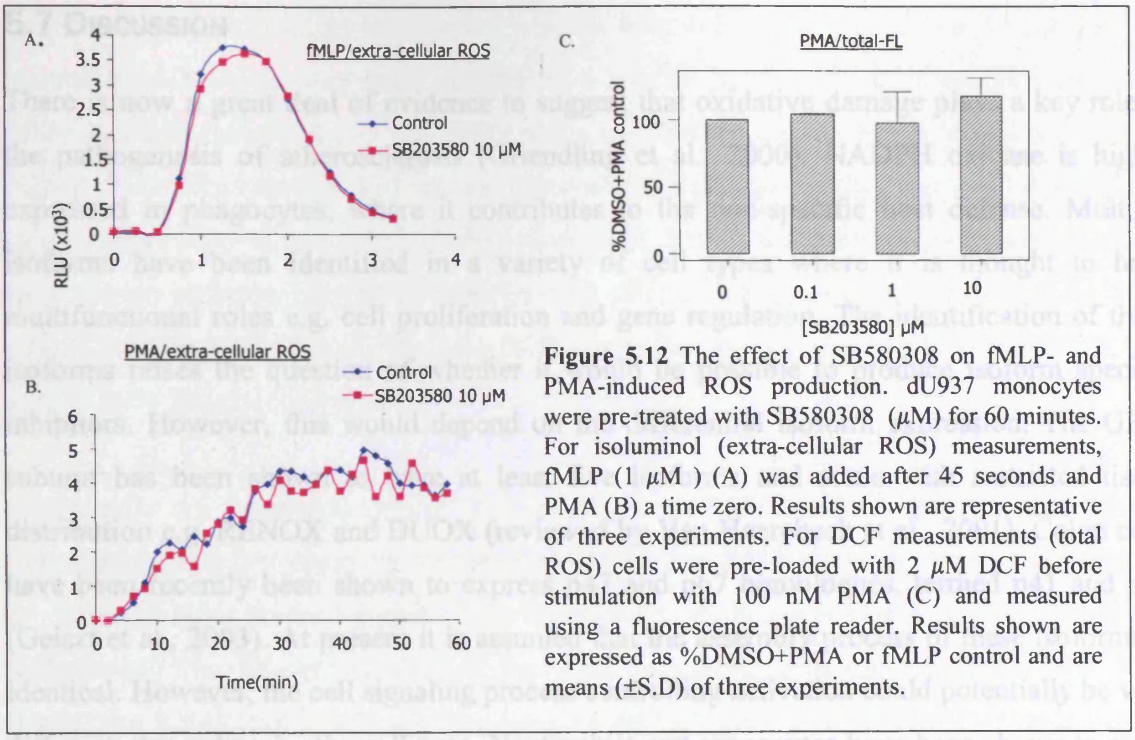


Figure 5.12 The effect of SB580308 on fMLP- and PMA-induced ROS production. dU937 monocytes were pre-treated with SB580308 (μM) for 60 minutes. For isoluminol (extra-cellular ROS) measurements, fMLP (1 μM) (A) was added after 45 seconds and PMA (B) a time zero. Results shown are representative of three experiments. For DCF measurements (total ROS) cells were pre-loaded with 2 μM DCF before stimulation with 100 nM PMA (C) and measured using a fluorescence plate reader. Results shown are expressed as %DMSO+PMA or fMLP control and are means (±S.D) of three experiments.

Isoluminol-CL	Stimulus	T_{max}	I_{max}	Phagocytic capacity
SB203580 (10 µM)	fMLP	120	80.4± 44.7	85.7± 48.1
	PMA	95.6± 8.8	100.4± 2.7)	106.5± 9.3

Table 5.6 The effect of SB203580 on isoluminol (extracellular ROS)-CL. dU937 cells were treated with the indicated dose of SB203580 for 60 minutes (followed by washing) before stimulation with 1 μM fMLP, 100 nM PMA or 50 μM AA. Results are expressed as a % of cells stimulated in the presence of DMSO obtained from three experiments. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (100%) control cells as determined by a one way ANOVA.

5.7 DISCUSSION

There is now a great deal of evidence to suggest that oxidative damage plays a key role in the pathogenesis of atherosclerosis (Griendling et al., 2000). NADPH oxidase is highly expressed in phagocytes, where it contributes to the non-specific host defense. Multiple isoforms have been identified in a variety of cell types where it is thought to have multifunctional roles e.g. cell proliferation and gene regulation. The identification of these isoforms raises the question of whether it would be possible to produce isoform specific inhibitors. However, this would depend on the differential isoform expression. The GP91 subunit has been shown to have at least five isoforms and some with restricted tissue distribution e.g. RENOX and DUOX (reviewed by Van Heerebeek et al., 2001). Colon cells have been recently been shown to express p47 and p67 homologues, termed p41 and p51 (Geiszt et al., 2003). At present it is assumed that the assembly process of these isoforms is identical. However, the cell signaling process controlling activation could potentially be very different, depending on the cell type. Neutrophils and monocytes have been shown to use a variety of cell signalling processes controlling their priming and activation, which makes these signal transduction pathways potential therapeutic targets. The PI3Kγ isoform has been shown to have a restricted distribution in that it is only expressed in phagocytes. Mice lacking this isoform are viable and have neutrophils that display reduced chemotaxis and ROS production upon stimulation. In addition these animals have been shown to display reduced platelet aggregation (Hirsch et al., 2001 and 2000). The cell signalling pathways controlling the activation of NADPH oxidase are of great importance in order to mount an appropriate immune response, while over-activation results in disastrous consequences as seen in sepsis (Shaked et al., 1994).

THE ROLE OF PKC

The first known regulator of NADPH oxidase was PKC. This was based on the observation that PMA is a potent stimulus for ROS production by neurophils (DeChatelet et al., 1975). In this study three different PKC inhibitors were used to inhibit ROS production stimulated by fMLP, PMA and AA. The use of staurosporine-analogue PKC inhibitors is heavily criticised due to their lack of specificity. The published IC_{50} values for these inhibitors against various PKC isoforms are much lower than the doses used in this study, which is due to their mechanism of action. These inhibitors compete with ATP and therefore the concentration of ATP *in vivo* is much higher than normally used in kinase assays (Davies et al., 2001). GF109203X and UCN01 were found to be potent inhibitors of PMA-induced ROS

production as measured by isoluminol, whereas PKC412 was less effective. Using DCF-FL to measure the respiratory burst resulted in a similar pattern of inhibition. However, less inhibition was measured and the higher doses of inhibitor did not result in negative values. Although DCF is thought to measure intracellular ROS, measuring the total fluorescence (with a plate reader) can potentially measure both. Some authors have suggested that there are different isoforms controlling the intra- and extra-cellular production of ROS. It has been shown in neutrophils that intracellular ROS production is less sensitive to GF109203X than extracellular (Karlsson et al., 2000)

Neutrophils are known to contain two pools of NADPH oxidase, granular and plasma membrane pools (reviewed by Karlsson and Dahlgren 2002). Monocytes/macrophages do not have granules so the purpose of multiple PKCs activating NADPH oxidase is not well understood. The CL-results showed that PKC inhibitors dose-dependently inhibit ROS production but do not alter T_{max}. The main target for PKC is the NADPH oxidase subunit p47, which has multiple phosphorylation sites (Fontayne et al., 2002). It could be that different PKC isoforms are involved in different phases of the respiratory burst. The initial phase of ROS production is thought to be extracellular, whereas later phases are intracellular. Given the promiscuous nature of PKC inhibitors and the pitfalls of using DCF-FL to measure intracellular ROS production, only limited conclusions can be drawn from these results.

GF109203X, PKC412 and UCN01 were also shown to inhibit fMLP-induced ROS production. UCN01 and PKC412 have both been shown to have an inhibitory effect on the PI3K/Akt signalling pathway. UCN01 has been shown to inhibit PDK1 (Sato et al., 2002) and PKC412 has been shown to inhibit Akt phosphorylation through an unknown mechanism (Tenzer et al., 2001). GF109203X displays a greater specificity, particularly towards PKCα, but was still found to inhibit fMLP-induced ROS production. Interestingly, the order of potency towards fMLP-induced ROS production was similar to that found with PMA. However, PKC412 was much more effective against fMLP.

PMA has been suggested to activate redundant cell signalling pathways that are not activated in a physiological setting. This was proposed from the observation that the PKC inhibitor did not inhibit the initial phases in the pathogenesis of arthritis (Birchall et al., 1994), which is thought to be dependent on ROS generated from phagocytes. However, the results shown here and elsewhere demonstrate that fMLP-induced ROS is dependent on PKC. It has also

been shown that immunoglobulin-induced ROS was also inhibited by GF109203X and to a lesser extent PKC412 (Larsen et al., 2000). fMLP has been shown to activate a variety of PKC isoforms, and the use anti-sense oligonucleotides to inhibit PKCζ resulted in complete inhibition of fMLP–induced ROS. Cell stimulation is assumed to activate multiple isoforms of PKC and multiple isoforms have been shown to phosphorylate p47. It is unclear which is the major PKC isoform required for activation, even though the selective inhibition of PKCζ results in the complete abolition of ROS. GF109203X does not inhibit PKCζ, but still completely inhibits ROS production (Way et al., 2001 and Davies et al., 2001).

AA-induced ROS was not inhibited by any of the PKC inhibitors used. This is a useful control for these inhibitors, since it can now be determined that they do not directly act upon NADPH oxidase. The inability of these compounds to inhibit AA-induced ROS, supports the hypothesis that its mechanism of action does not involve the phosphorylation of the cytosolic NADPH oxidase subunits e.g. p47. Lower doses of AA have been shown to activate PKC translocation and in some cases activate Akt by PI3K-dependent and -independent mechanisms (Gorin et al., 2001 and Hii et al., 2001).

THE ROLE OF THE MAPK PATHWAY

The MEK inhibitor, U0126, was shown to inhibit fMLP, PMA and AA-induced ROS production. The activation of the MAPK pathway is thought to play an important role in the activation of neutrophils (Rane et al., 1997). fMLP-induced ROS production was completely inhibited by U0126, whereas PMA and AA-induced ROS were much less affected. The characteristics of the PMA-induced respiratory burst was significantly altered by U0126, with a dose-dependent decrease in I_{max} and phagocytic capacity that did not result in complete inhibition. This suggested that ERK is not required for activation *per se* but might have some regulatory role. Higher doses of U0126 significantly altered the AA-induced respiratory burst, with much less effect on total ROS production (I_{max} and phagocytic capacity) but significant changes in T_{max}. Although 25 μM U0126 was required to measure this effect, this compound has a unique mechanism of action and has been shown to be fairly specific, however, it is far more efficacious *in vivo* than *in vitro* and could have other targets.

MEK represents an important convergence point in the activation of the respiratory burst. fMLP-induced ROS was completely inhibited by low doses of U0126, which suggests that MEK plays an important role in the direct activation of the enzyme. There are numerous reports that demonstrate that PI3K can activate MEK. Although it is not possible to

determine any cross-talk from initiation of ROS production alone, these interactions should not be discounted (LopezIlasaca et al., 1997). U0126 was able to partially inhibit PMA and AA-induced ROS. It could be possible that ROS produced by PMA and AA are capable of activating cell signalling pathways that can further activate NADPH oxidase e.g. ERK, which can be activated as a result of oxidative stress. It is possible that hydrogen peroxide formed in and around the cell could activate the MAPK pathway and act as a feed forward mechanism. Initial ROS induced by AA from its direct action on NADPH oxidase could then activate other pathways that could induce the further activation of the enzyme. This hypothesis was not supported by the results shown in Chapter 3.5, where pre-treating the cells with hydrogen peroxide does not affect subsequent ROS production. PMA-induced ROS might also be propagated in this fashion, since PKC is thought to be regulated by ROS (Meyer et al., 1993). The activation of ERK by fMLP could be dependent on a number of cellular signalling intermediates such as PKC or PI3K, therefore, inhibition of ERK could potentially inhibit the upstream signals from these kinases.

p38 has been shown to be activated in response to fMLP in neutrophils and monocytes (Yagisawa et al., 1999). In some cases p38 activation has been linked to ROS production and neutrophil de-granulation. The results here show that there is no relation between p38 activation and ROS production in response to fMLP or PMA. This was determined using SB203580 and does not rule out the involvement of p38 isoforms that are not inhibited by this compound (Eyers et al., 1998). Rane et al., (2001) suggested that MAPKAPK-2, a kinase downstream of p38, functions as a PDK2 for Akt in neutrophils. The interaction between these pathways cannot be ruled out. However, due to the inability of SB203580 to inhibit ROS production it is unlikely.

Formyl receptors have been shown to activate a broad range of MAPK family members (Rane et al., 1997), such as JNK. It is not clear as to their role in activation; the most likely influence would be on cell survival. Most of the studies regarding the regulation of NAPDH oxidase have been carried out in neutrophils. These cells undergo a rapid cell death after NADPH oxidase activation, whereas monocytes/macrophages have much longer life spans. The pathways controlling their survival under normal and pathological conditions are not well understood. The phagocyte priming process also effects cell survival, which has been shown in neutrophils and it is possible that the MAPK pathways are involved in these processes.

As expected fMLP-induced ROS was found to be dependent on PI3K as shown by the action of LY294002 and wortmannin. PMA- and AA-induced ROS were unaffected by LY294002, which confirms that this inhibitor has no direct effect on NADPH oxidase. It has been shown that PMA activates intra-cellular ROS that is dependent on wortmannin (Karlsson et al., 2000). This study suggested that in neutrophils PKC was activating PI3K, whereas in the present study intracellular ROS induced by PMA was found not to be dependent on PI3K. MEK inhibitors were also shown to inhibit PMA-induced intra-cellular ROS production. This further supports the notion that multiple cell signalling pathways act in co-operation in order to activate the respiratory burst. Low doses of wortmannin were found to completely inhibit fMLP-induced ROS. Actin polymerisation has been shown to be activated by insulin and ATP in U937 monocytes, and in this case ATP was shown to be eight times less sensitive to wortmannin than insulin (Walters et al., 1996). This is thought to be due to the different PI3K isoforms activated by ATP and insulin, where ATP only activates the class 1b isoform and insulin activates the class 1a (Melendez et al., 1998). The results in this study tentatively suggest the involvement of multiple PI3K isoforms, which has also been suggested for fMLP-induced ROS in monocytes (Pan et al., 2000). Most authors have reported that wortmannin does not display PI3K isoform selectivity, therefore further experimentation would be required to determine which isoforms are involved in fMLP-induced ROS production e.g. using dominant negatives.

PI3K has been shown to be a central mediator of a number of important phagocyte functions, such as chemotaxis and ROS production (Stephens et al., 2002). There are also a number of studies that have used PI3Kγ knockout mice to show the importance of PI3K in chemotaxis and ROS production (as previously discussed). The downstream mediators of PI3K are numerous, although much attention has been paid to the serine/theronine kinase, Akt. This kinase has been shown to be an important mediator of cardiovascular pathologies, where overexpression results in cardiac hypertrophy (Matsui et al., 2002). Akt has been shown to activate eNOS in endothelial cells (Dimmeler et al., 1999) and statins have been show to activate Akt through a mechanism that is exclusive to endothelial cells (Kureishi et al., 2000). Much less attention has been paid to its role in phagocyte functions, although early reports showed that constitutively active PI3K results in Akt and p47 phosphorylation (Didichenko et al., 1999), and Akt has been recently shown to directly phosphorylate p47 *in vitro* (Chen et al., 2003 and Hoyal et al., 2003). In this study a phosphoinositide ether

analogue was shown to inhibit fMLP-induced ROS production. This compound has been shown to selectively inhibit Akt activation by binding to PH-domains of Akt and preventing its interaction with PtdIns(4,5)P_2 (Hu et al., 2000). Subsequent membrane translocation and activation is then inhibited. Moreover, this compound does not inhibit PI3K (Hu et al., 2000). PMA-induced ROS production was not affected by this compound, which suggests that it does not directly inhibit NADPH oxidase or act as an antioxidant. These results further strengthen the role Akt plays in the activation of NADPH oxidase and also highlights Akt as a potential anti-inflammatory drug target. Akt has been shown to be key regulator of chemotaxis and phagocytosis, which raises the possibility that Akt could play a role in the macrophage-mediated oxidation of LDL. Akt1 has been shown to be an important macrophage survival factor (Goyal et al., 2002 and Liu et al., 2001), which suggests that Akt could potentially effect macrophage apoptosis, which might have some bearing on macrophage mediated disease processes such as atherosclerosis.

THE ROLE OF TYROSINE KINASES

Genistein has been used in numerous studies to demonstrate the role of tyrosine kinases in the activation of NADPH oxidase. Genistein was found to inhibit fMLP, PMA and AA-induced ROS production, although the specificity of this compound could be questioned, it has been shown that PKC and PI3K are not affected by genistein. Despite the possibility that genistein has antioxidant properties it is still used as an inhibitor of tyrosine kinases in phagocytes (Edashige et al., 1993). Genistein has been used to show that tyrosine kinases are responsible for the activation of PI3K in neutrophils by fMLP. The ability of genistein to inhibit fMLP-induced ROS was expected, although PMA-induced ROS was also inhibited. fMLP has been shown to activate tyrosine phosphorylation of a variety of proteins. However, PMA activates a different battery of kinases. PMA-induced ROS would be less likely to involve tyrosine kinases, since PKC is capable of directly activating NADPH oxidase. Some PKC isoforms are thought to be tyrosine phosphorylated. PKC phosphorylation is the mechanism by which the kinase is primed for activation and the nature of phosphorylation depends upon the PKC isoform. Classical PKCs undergo phosphorylation after translation of the native peptide, which is therefore constitutive, whereas phosphorylation of novel or atypical PKCs can occur upon cell stimulation.

The activation of ERK is dependent on the dual phoshorylation of tyrosine and threonine residues, which is inhibited by U0126. Genistein had a much greater effect on PMA-induced

ROS than U0126. This suggested that other kinases were involved or that genistein has some antioxidant activity.

AA-induced ROS was also inhibited by genistein. AA is thought to activate NADPH oxidase independently of phosphorylation events, despite the wide range of information regarding the effect of AA on kinases such as PI3K, PKC, P38 and Akt. Although the activation of PKC and PI3K were ruled out by the negative effects of UCN01, GF109203X and LY294002, AA is a highly bioactive mediator and could have other effects. Genistein was an effective inhibitor of AA-induced ROS production, but it is also likely to be a co-reducing substrate for the peroxidase/hydrogen peroxide system, which makes these results inconclusive. Interestingly, genistein had much less effect on fMLP-induced superoxide production, when measured using lucigenin (a peroxidase-independent measuring principle).

There are numerous tyrosine kinases that have been shown to regulate the activation of NADPH oxidase (Brumell et al., 1996 and Fialkow et al., 1993). In particular, the Src family of tyrosine kinases (lyn, fyn, hck, yes) have been shown to play some role in the activation of Akt (in neutrophils Nijhuis et al., 2001) and bacterial responsiveness of monocytes (Shahan et al., 2000).

ACTIVATION OF THE RESPIRATORY BURST IN U937 MONOCYTES

The activation of NADPH oxidase is coordinated by series of multiple protein kinases, which is dependent on the stimuli encountered. Superoxide produced from the activation of NADPH oxidase can be released into the extracellular space through anion channels, or is released into the phagosome during phagocytosis. Neutrophils contain two pools of NADPH oxidase that have been shown to display differential regulation by protein kinases. The intracellular production of ROS can be induced by PMA, but is still dependent on PI3K. PMA-induced extracellular ROS was shown to be independent of PI3K inhibitors. Monocytes are not thought to contain distinct pools of NADPH oxidase, although it appeared that intra-cellular ROS measured by DCF-FL was less sensitive to the inhibitors used. It is difficult to determine if this is an artefact of the measuring principle, since there are many problems using DCF-FL (as discussed in chapter 3). Monocytes have been suggested to release superoxide in response to soluble stimuli and release intracellular ROS in response to insoluble stimuli. The mechanism by which this is regulated is unclear. PMA

is a soluble stimulus that activates PKC isoforms that can also be activated by insoluble particles (Larsen et al., 2000).

Isoluminol and lucigenin both measure the extracellular production of ROS. However, lucigenin is thought to be relatively specific for superoxide. Most of the inhibitors tested in this study had less effect on lucigenin-CL induced by fMLP or PMA. The reasons behind this discrepancy are not clear. The use of lucigenin has been criticised, since it is known to redox cycle (and release superoxide during its excitation), which makes the use of the probe problematic (Li et al., 1998)

The results shown here suggest that the respiratory burst can be inhibited by a variety of protein kinase inhibitors. Agents inhibiting PKC, MEK and PI3K-Akt were all shown to be effective inhibitors of fMLP-induced ROS. The role of MEK, Akt and PI3K in fMLP-induced ROS was determined using reasonably specific inhibitors, U0126 and LY294002. Results using PKC inhibitors should be treated with more caution, where GF109203X is perhaps the most specific PKC inhibitor. MEK, PI3K and PKC could all be involved in fMLP-induced activation of NADPH oxidase. U0126 and LY294002 were both highly effective inhibitors of fMLP-induced ROS, with the Akt inhibitor having less of an effect. It is very difficult to determine cross-talk between these pathways by measuring ROS production alone. The results shown here demonstrate that PMA-induced ROS is predominantly dependent on PKC, but independent of the PI3K-Akt pathway. However, there was a degree of inhibition measured using U0126. In conclusion, fMLP is dependent on multiple cell signalling pathways that include PI3K-Akt, MEK and PKC. PMA-induced ROS was predominantly dependent on PKC, with a possible role of MEK. AA is thought to activate NADPH oxidase in through a direct interaction with the enzyme, while U0126 and genistein were able to inhibit the AA response it is not clear if this is a non-specific effect of the inhibitors.

The main problem of using protein kinase inhibitors to modulate the activity of NADPH oxidase activity is complicated by the concept of redundancy. Some of these activating pathways could be redundant in the physiological setting. fMLP is the major physiological stimulus used and shows some similarity to the chemokine family of chemoattractants. PI3K is a major mediator of this response and has been called "the gatekeeper of inflammation"

(Weaver and Ward 2001). Therefore, it remains an important therapeutic target and has a great potential as a chemoprentive mechanism for chronic inflammatory disorders.

CHAPTER SIX:

THE EFFECT OF RESVERATROL ON SIGNAL TRANSDUCTION PATHWAYS THAT ACTIVATE NADPH OXIDASE IN U937 MONOCYTES

INTRODUCTION

Glucocorticoid (GC) hormones have been employed as anti-inflammatory agents for over forty years. In theory they would be an effective treatment for many pathologies that are a result of chronic inflammatory responses. However, their use is restricted due to their serious side effects. Steroid drugs and GC bind to glucocorticoid receptors (GR), which are bound to heat shock proteins. On binding these drugs, the GR dimerises and interacts with the glucocorticoid response element (GRE) in gene promoters. The GC/GR complex can also interact with transcription factors AP-1 and Nf-κB, which are regulators of a number of pro-inflammatory processes. The interaction between GC hormones and these transcription factors is thought to be the mechanism by which these drugs mediate their anti-inflammatory effects. The interaction with GRE is thought to be responsible for their side effects (reviewed by Cato and Wade 1996). Therefore, an ideal anti-inflammatory agent would inhibit the transcription factors AP-1 and Nf-κB without interaction with GRE (Favata et al., 1998).

This concept was the rationale behind the development of the MEK inhibitor, U0126, which inhibits AP-1 activity without interacting with GRs (see figure 6.1). Inhibition of AP-1 in phagocytes would potentially lead to the inhibition of cytokine and chemokine production, expression of adhesion molecules and MMP production (Cato and Wade 1996). However, there are many other kinases involved in the activation of immune cells that are also

potential therapeutic targets, such as PKC and PI3K (see figure 6.1 modified from Duncia et al., 1998).

These kinases are excellent therapeutic targets for chemopreventive agents. As previously discussed resveratrol has antioxidant properties. However, it has many other potential anti-inflammatory properties. Oxidative events make up one of many contributing factors in the pathogenesis of atherosclerosis. Mice lacking the monocyte chemoattractant receptor have been crossed with the APO-E-deficient mouse (which develops an atherosclerotic-like lesion). This results in reduced development of the disease, which not only highlights the possibility of using MCP receptor antagonists, but also using a PI3K inhibitor (Gosling et al., 1999). PI3K is the central biochemical mediator of MCP and many other chemokines (Turner et al., 1998). The p47 subunit of NADPH oxidase also plays an important role. Knockout mice lacking this subunit have also been crossed with the APO-E-deficient mouse, which also leads to reduced lesion formation (Bary-Lane et al., 2001). PI3K plays a pivotal role in the activation of NADPH oxidase (as shown in Chapter 5.3). Therefore it makes an ideal therapeutic target, since its inhibition could potentially reduce the oxidative burden and activation of inflammatory processes that are associated with atherosclerosis.

In the previous chapter, several commonly used kinase inhibitors were used to investigate the signal transduction pathways that are activated by fMLP and PMA. The PI3K-Akt and MAPK pathways were found to be involved in the activation of fMLP-induced ROS. In the studies presented in this chapter, the ability of resveratrol to antagonise these pathways was determined, with a view to highlighting a potential anti-inflammatory mechanism.

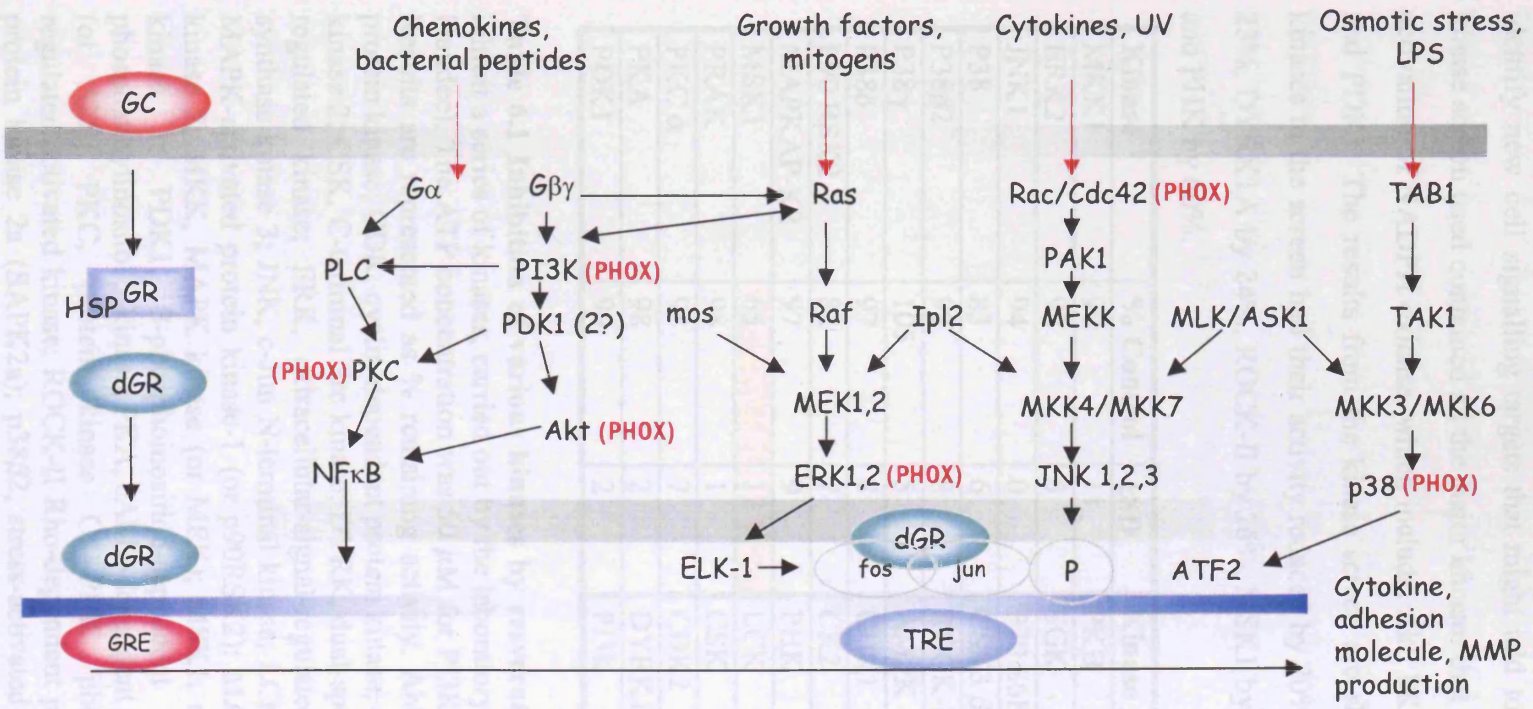


Figure 6.1. The mechanism of action of glucocorticoid hormones (GC) with the mitogen activated protein kinase (MAPK) and phosphotidylinostide-3-kinase (PI3K) signalling cascades. GC act via a glucocorticoid receptor that is found complexed to a heat shock protein (HSP). Upon dimerisation (dGR) this complex interacts with the glucocorticoid response element (GRE) and 12-O-tetraecanoylphorbol 13-acetate (TPA or PMA)-responsive elements (TRE). The kinases that have been shown to activate NADPH oxidase are marked with PHOX. Some of the interacting proteins and pathways are not shown. Gα,βγ, G-protein subunits; PLC, phospholipase C; PDK1/2, 3-phosphoinositide-dependent protein kinase 1/2; Akt, protein kinase B; NFκB, nuclear factor κ B; Mos, c-mos proto-oncogene product; tpl2, tumour progression locus; ERK, extracellular-signal-regulated protein kinase; PAK, p21-activated protein kinase; PKC, protein kinase C; P38, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; MLK, mixed lineage kinase; ASK, apoptosis signal-regulating kinase; TAB1; transforming growth factor activated protein kinase 1-binding protein-1; TAK1, transforming growth factor activated protein kinase; AP-1 complex is also known as c-fos/jun; MEK, mitogen-activated protein kinase; MEKK, mitogen activated protein kinase kinase; MMP, matrix metalloproteinase; ATF2, activating transcription factor 2; LPS, lipopolysaccharide; UV, ultraviolet light. Modified from Duncia et al., 1998.

6.1 THE EFFECT OF RESVERATROL IN A KINASE ACTIVITY SCREEN.

The inhibitory effect of resveratrol on a series of protein kinases was carried out in order to identify new cell signalling targets that might add to its cardioprotective properties. The kinase screen used contained all the major kinases that have been shown to play a role in the activation of NADPH oxidase, which include; Akt, PKC, MEK, ERK, p38, Src, PI3K, CK2 and PDK1. The results from the kinase screen are shown in Table 6.1. Seven of the 28 kinases in the screen had their activity reduced by 20% or more. P70 S6K was inhibited by 23%, DYRK1A by 24%, ROCK-II by 26%, MSK1 by 35%, AMPK by 41%, PI3K by 47% and PHK by 60%.

Kinase	% Control	±SD	Kinase	% Control	±SD
MKK1	90	11	PKB	97	7
ERK2	92	5	SGK	110	0
JNK1	94	0	P70 S6K	77	2
P38	83	6	GSK3 β	92	10
P38β2	92	8	ROCK-II	74	14
P38γ	105	5	AMPK	59	7
P38δ	97	8	CHK1	82	2
P90 RSK2	82	7	CK2	96	3
MAPKAP-K2	97	9	PHK	40	3
MSK1	65	12	LCK	81	12
PRAK	98	1	CSK	88	1
PKC α	98	7	CDK2	89	11
PKA	98	2	DYRK1A	76	13
PDK1	93	2	PI3K	53	2

Table 6.1 Inhibition of various kinases by resveratrol. Ten μM resveratrol was used to inhibit a series of kinases, carried out by the laboratory of Professor P. Cohen (University of Dundee). The ATP concentration was 50 μM for PI3K, all other assays contained 100 μM. Results are represented as % remaining activity. Abbreviations: AMPK, AMP-activated protein kinase; CDK, cyclin-dependent protein kinase; CHK, checkpoint kinase; CK2, casein kinase 2; CSK, C-terminal Src kinase; DYRK, dual-specificity, tyrosine-phosphorylated and regulated kinase; ERK, extracellular-signal-regulated protein kinase; GSK3, glycogen synthase kinase 3; JNK, c-Jun N-terminal kinase; LCK, lymphocyte kinase; MAPKAP-K1, MAPK-activated protein kinase-1 (or p90RSK2); MAPKAP-K2, MAPK-activated protein kinase 2; MKK, MAPK kinase (or MEK); MSK1, mitogen- and stress-activated protein kinase 1; PDK1, 3-phosphoinositide-dependent protein kinase 1; PI 3-kinase, phosphatidylinositol 3-kinase; PKA, cAMP-dependent protein kinase; PKB, protein kinase B (or Akt); PKC, protein kinase C; PHK, phosphorylase kinase; PRAK, p38-regulated/activated kinase; ROCK-II Rho-dependent protein kinase II; p38, stress-activated protein kinase 2a (SAPK2a); p38β2, stress-activated protein kinase 2b (SAPK2b); p38γ, stress-activated protein kinase 3 (SAPK3); p38δ, stress-activated protein kinase 4 (SAPK4); SGK, serum- and glucocorticoid-induced kinase; S6K1, p70 ribosomal protein S6 kinase.

6.2 THE EFFECT OF RESVERATROL ON PI3K ACTIVITY.

Increased lipid kinase activity was found in phosphotyrosine immunoprecipitates after a 1 minute stimulation with 1 μM fMLP (figure 6.2A). Lipid kinase activity was also found in insulin-stimulated immunoprecipitates (figure 6.2A). The presence of ^{32}P radiolabelled PI(3)P was confirmed using a cold standard, PI(4)P. Using a methanol/chloroform/ammonia/water solvent system poly-phosphoinositides are separated according to their head-group polarity, therefore they are separated according to the number of phosphates attached to the inositol head group. PI(4)P and PI(3)P have identical Rf values when separated by thin layer chromatography.

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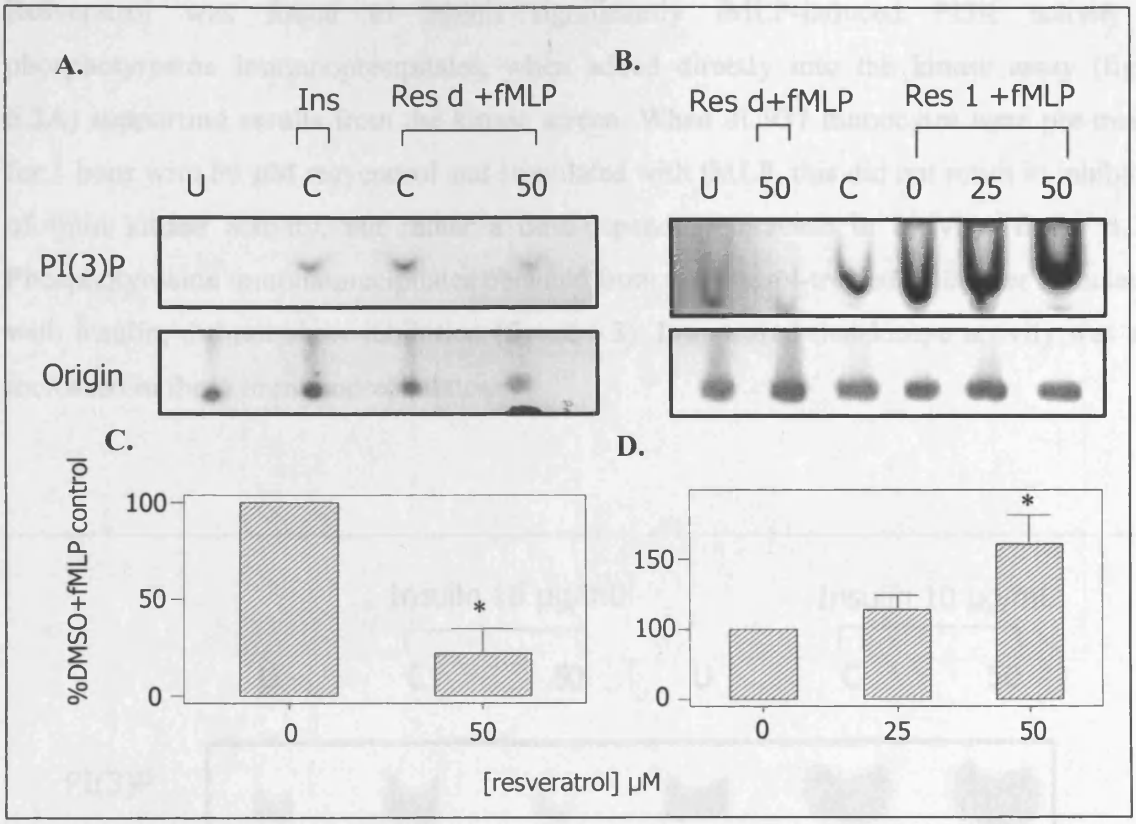

Figure 6.2 The effect of resveratrol on fMLP-induced PI3K activity. Immunoprecipitates were prepared from dU937 cells as described in the materials and methods. A) PI3K activity was measured in immunoprecipates from cells stimulated with insulin (10 μg/mL for 1 min) or fMLP (1 μM for 1 min). Immunoprecipitates from fMLP stimulated cells were also pre-incubated with resveratrol (50 μM) for 5 min before the addition of phosphatidylinositol/phosphatidylserine and ATP. PI3K activity was measured as described in the materials and methods. U, unstimulated control; C, stimulated control; Res d, immunoprecipitates pre-incubated with resveratrol. B) PI3K activity measured in immunoprecipitates from cells pretreated with resveratrol (25 and 50 μM) for 1 hour before stimulation with fMLP (1 μM for 1 min). U, unstimulated control; C, stimulated control; Res 1, immunoprecipitates prepared from cells pre-incubated with resveratrol (0 (DMSO), 25 and 50 μM) for 1 hour before stimulation with fMLP (1 μM for 1 min). C) Inhibition of PI3K activity by addition of resveratrol to immunoprecipitates from stimulated cells. D) The effect of resveratrol on PI3K activity in immunoprecipitates prepared from cells pre-treated with resveratrol for 1 hour before stimulation with fMLP (1 μM for 1 min). Autoradiography films were quantified by densiometry and results expressed as %DMSO+fMLP control. Results shown are means (± S.D) of three experiments. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (0) control cells as determined by a one way ANOVA.

Resveratrol was found to inhibit significantly fMLP-induced PI3K activity in phosphotyrosine immunoprecipitates, when added directly into the kinase assay (figure 6.2A) supporting results from the kinase screen. When dU937 monocytes were pre-treated for 1 hour with 50 μM resveratrol and stimulated with fMLP, this did not result in inhibition of lipid kinase activity, but rather a dose-dependent increase in activity (figure 6.2b). Phosphotyrosine immunoprecipitates obtained from resveratrol-treated cells after stimulation with insulin, did not show inhibition (figure 6.3). It appeared that kinase activity was also increased in these immunoprecipitates.

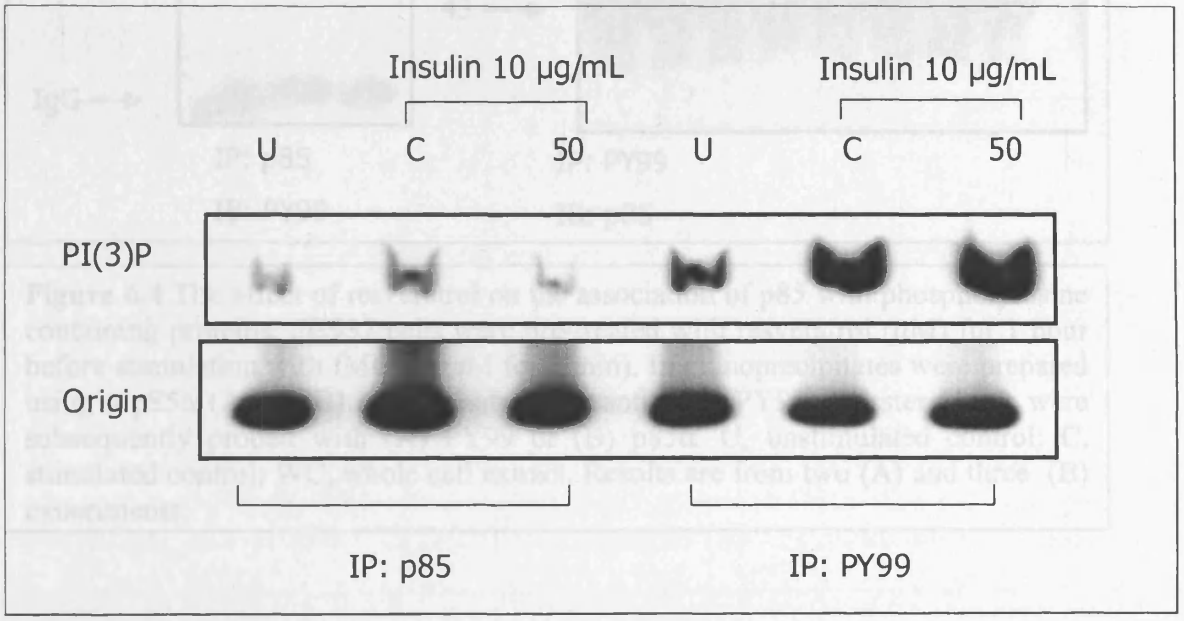

Figure 6.3 The effect of resveratrol on insulin-induced PI3K activity. dU937 cells were pre-treated with resveratrol (50 μM) for 1 hour and stimulated with insulin (10 μg/mL) for 1 minute. Immunoprecipitates (IP) were prepared using a p85α or a phosphotyrosine antibody (PY99) and PI3K activity was measured as described in the materials and methods. U, unstimulated control; C, stimulated control. Representative of a single experiment.

Immunoprecepitates of p85 from insulin-stimulated cells were found to have lipid kinase activity. Immunoprecipitates prepared from cells pre-treated with resveratrol before stimulation with insulin displayed reduced kinase activity (figure 6.3). Initial results suggested that p85 immunoprecipitates from fMLP-stimulated cells did not have kinase activity (data not shown). p85-immunoprecipitates were not found to have phosphotyrosine-containing proteins at the correct molecular weight. Reciprocal immunoprecipitations also yielded negative results (figure 6.4A and B).

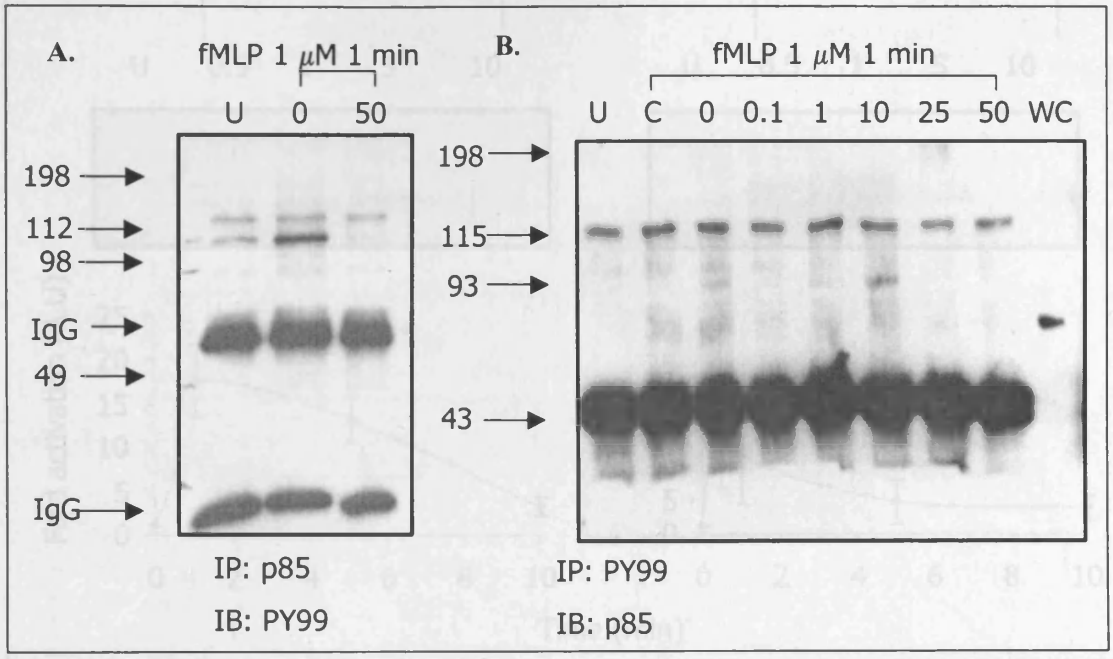

Figure 6.4 The effect of resveratrol on the association of p85 with phosphotyrosine containing proteins. dU937 cells were pre-treated with resveratrol (μM) for 1 hour before stimulation with fMLP (1 μM for 1 min). Immunoprecipitates were prepared using a p85α (A) or (B) a phosphotyrosine antibody (PY99). Western blots were subsequently probed with (A) PY99 or (B) p85α. U, unstimulated control; C, stimulated control; WC, whole cell extract. Results are from two (A) and three (B) experiments.

6.3 fMLP STIMULATES AKT AND ERK PHOSPHORYLATION.

One μM fMLP was found to rapidly induce the phosphorylation of Akt in dU937 monocytes. Akt phosphorylation was measured within 30 seconds of stimulation and was found to be diminished after 5 minutes (figure 6.5A). ERK was also found to be phosphorylated in response to fMLP and displayed similar kinetics to that of Akt phosphorylation (6.5B). However, Akt phosphorylation was sustained longer to that of ERK, with ERK phosphorylation being diminished by 5 minutes.

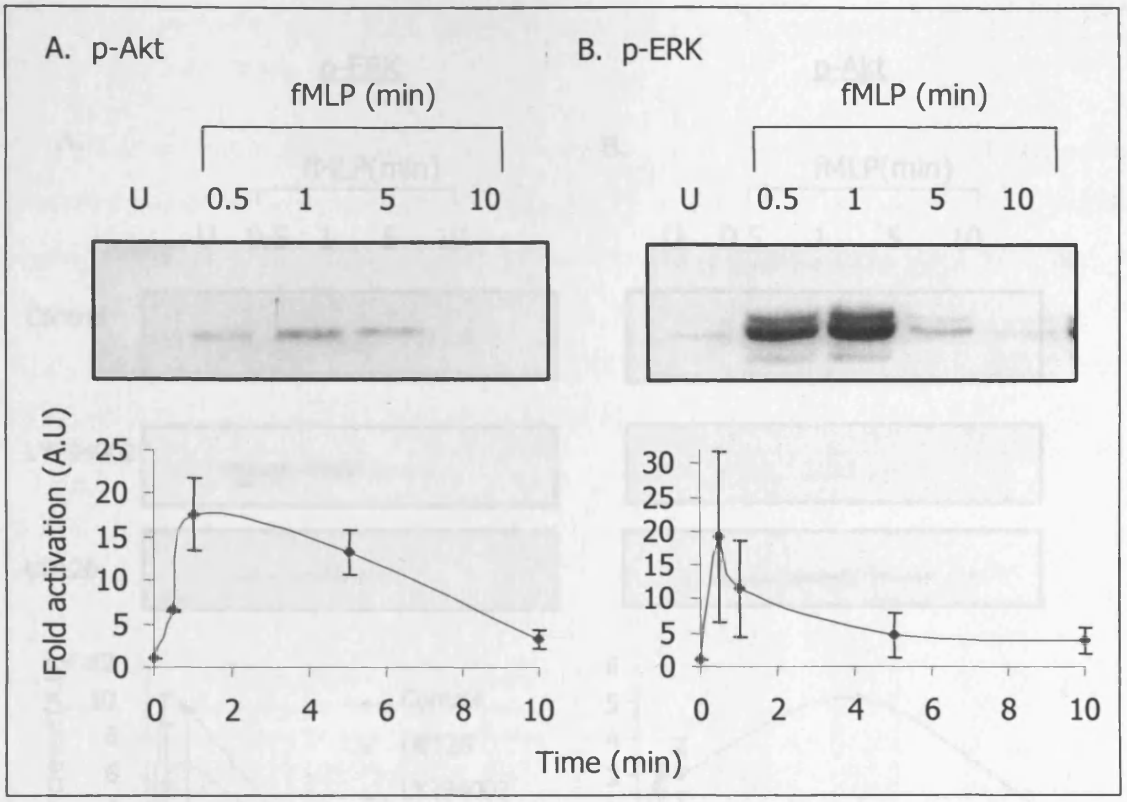

Figure 6.5 fMLP stimulates the time-dependent phosphorylation of Akt and ERK. dU937 cells were resuspended in BSS and then stimulated with 1 μM fMLP for the indicated time. Cell lysis and sample preparation was performed as described in the materials and methods. Thirty five μg of protein was used for Western blotting using phospho-specific Akt (ser473) and ERK antibodies. A) Akt phosphorylation in response to fMLP. B) ERK phosphorylation in response to fMLP. Results in graphs are means (±SD) of three (Akt) and four experiments (ERK) U, unstimulated control.

6.4 THE EFFECT OF U0126 AND LY294002 ON FMLP-INDUCED AKT AND ERK PHOSPHORYLATION.

Ten μM U0126 was found to reduce fMLP-induced ERK phosphorylation over a 10 minute time course (figure 6.6A). Ten μM U0126 did not completely inhibit early fMLP-induced Akt phosphorylation. However, the later stages of the response were completely inhibited (figure 6.6B).

Fifty μM LY294002 was found to completely inhibit fMLP-induced Akt phosphorylation over a 10 minute time course (figure 6.6B). fMLP-induced ERK phosphorylation was found to be much less affected by LY294002. Fifty μM LY294002 was found to reduce fMLP-induced ERK phosphorylation by 36% (figure 6.6A).

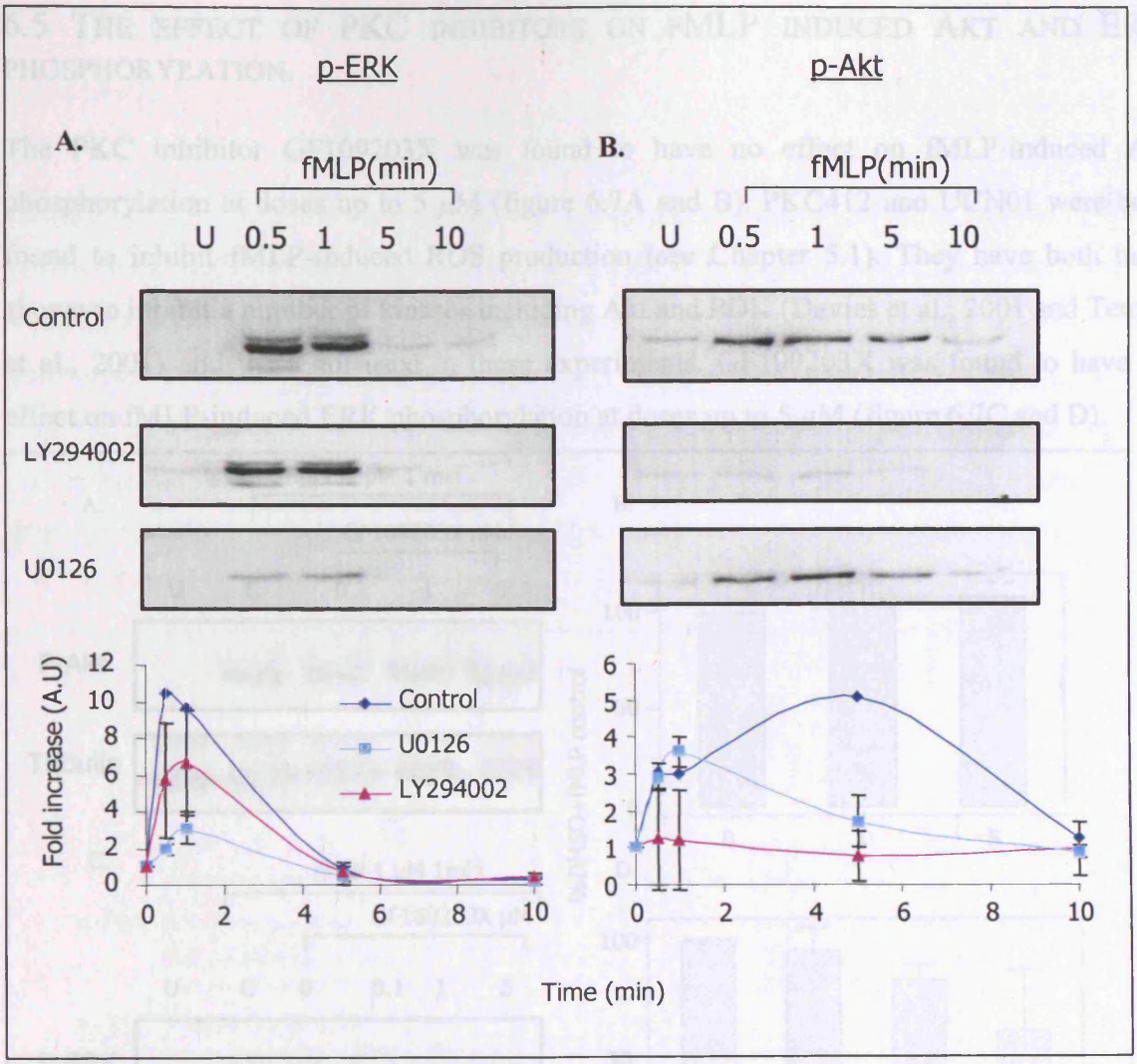

Figure 6.6 The effect of U0126 and LY294002 on fMLP-induced ERK and Akt phosphorylation. dU937 cells were treated with 50 μM LY294002 and 10 μM U0126 in complete medium for 1 hour. Cells were washed and resuspended in BSS. One μM fMLP was added for the indicated time. Thirty five μg of protein was used for western blotting using phospho-specific Akt (ser473) and ERK antibodies. A) ERK phosphorylation B) Akt phosphorylation. Results in graphs are means (±SD) of two experiments; U, unstimulated control.

6.5 THE EFFECT OF PKC INHIBITORS ON FMLP INDUCED AKT AND ERK PHOSPHORYLATION.

The PKC inhibitor GF109203X was found to have no effect on fMLP-induced Akt phosphorylation at doses up to 5 μM (figure 6.7A and B). PKC412 and UCN01 were both found to inhibit fMLP-induced ROS production (see Chapter 5.1). They have both been shown to inhibit a number of kinases including Akt and PDK (Davies et al., 2001 and Tenzer et al., 2001) and were not used in these experiments. GF109203X was found to have no effect on fMLP-induced ERK phosphorylation at doses up to 5 μM (figure 6.7C and D).

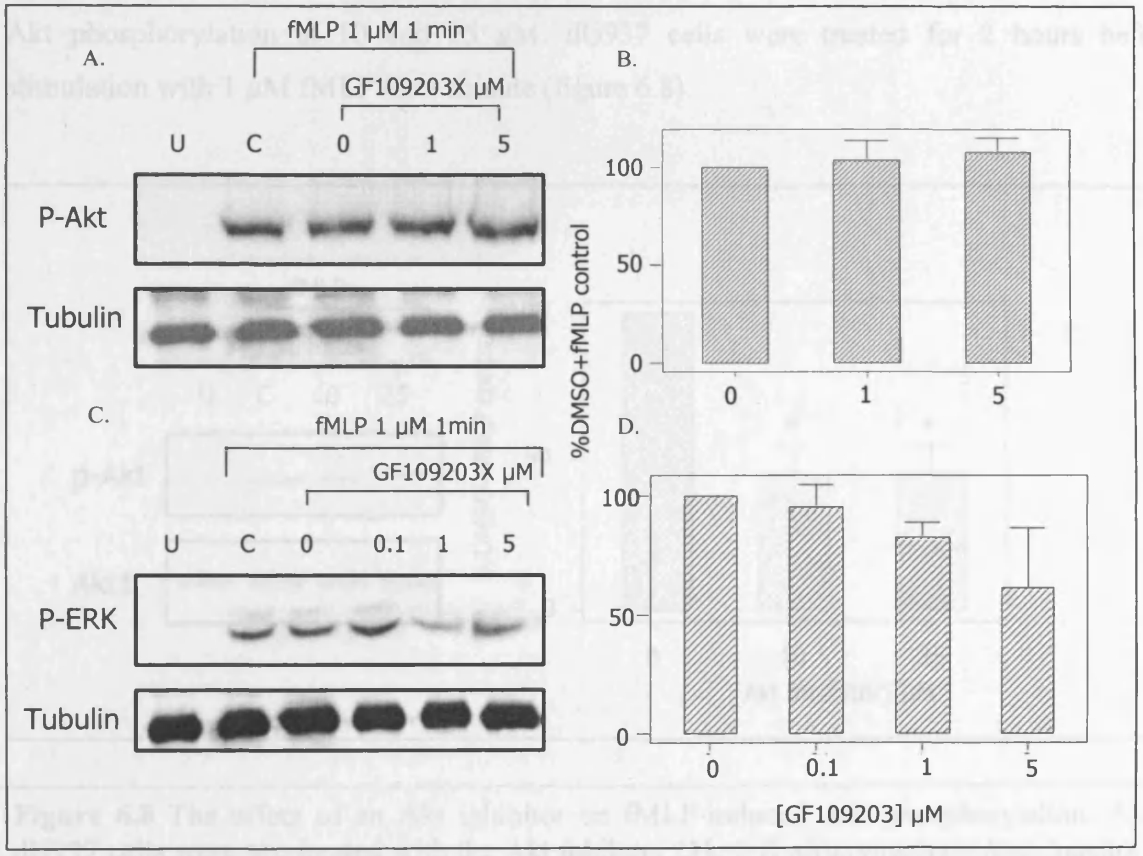

Figure 6.7 The effect of GF109203X on fMLP-induced ERK and Akt phosphorylation. dU937 cells were pre-treated with GF109203X (μM) for 15 minutes before stimulation with fMLP (1 μM for 1 min in BSS). Whole cell extracts were prepared and analysed by western blotting as described in the materials and methods. Thirty five μg of protein was used for western blotting using phospho-specific Akt (ser473) and ERK antibodies and reprobed using an anti-tubulin antibody. A) The effect of GF109203X on fMLP-induced Akt phosphorylation. B) Densitometry was carried out on autoradiography films and the results expressed as %DMSO+fMLP control. Results shown are means (±S.D) of two experiments. C) The effect of GF109203X on fMLP-induced ERK phosphorylation. D) Densitometry was carried out on autoradiography films and the results expressed as %DMSO+fMLP control. Results shown are means (±S.D) of two experiments. U, unstimulated control; C, stimulated control.

6.6 fMLP-INDUCED AKT PHOSPHORYLATION IS INHIBITED BY A PI-ETHER ANALOGUE.

Akt has recently been shown to phosphorylate the p47 subunit of NADPH oxidase (Chen et al., 2003). 1L-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate is a phosphoinositide ether analogue that binds to the PH domain of Akt. The membrane translocation of Akt is then inhibited (Hu et al., 2000). This compound is weak inhibitor of PI3K (IC_{50} <80 μM). The PI-ether analogue was found to significantly inhibit fMLP-induced Akt phosphorylation at 10 and 25 μM. dU937 cells were treated for 2 hours before stimulation with 1 μM fMLP for 1 minute (figure 6.8).

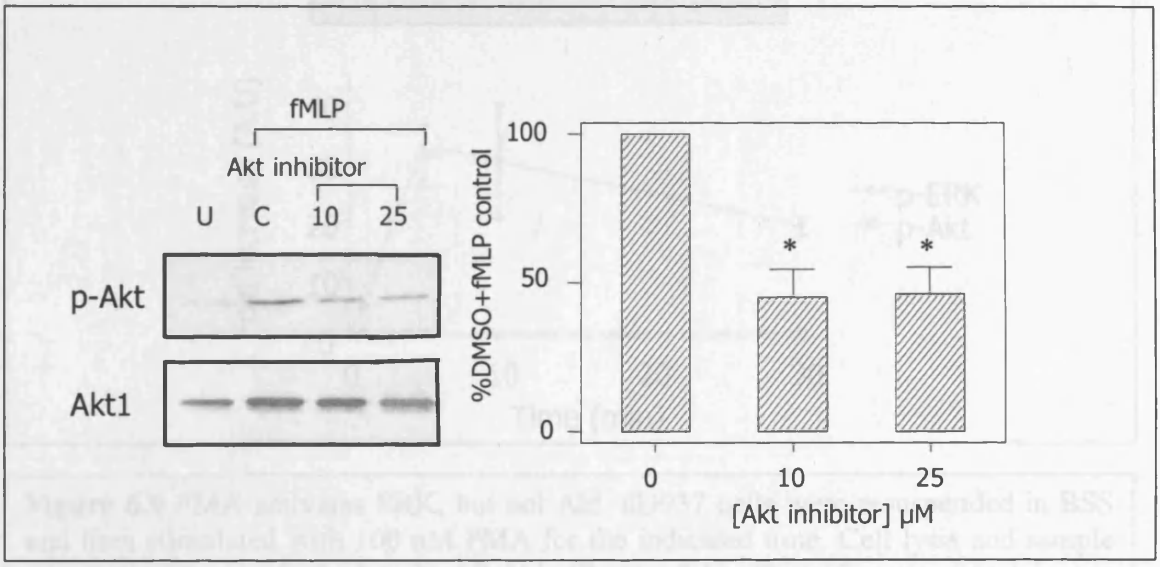

Figure 6.8 The effect of an Akt inhibitor on fMLP-induced Akt phosphorylation. A) dU937 cells were pre-treated with the Akt inhibitor (1L-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate, μM) for 2 hours before stimulation with fMLP (1 μM for 1 min in BSS). Whole cell extracts were prepared and analysed by western blotting as described in chapter 2.13. Thirty five μg of protein was used for western blotting using a phospho-specific Akt (ser473) antibody and reprobed using an Akt1 antibody. B) Densitometry was carried out on autoradiography films and the results expressed as %DMSO+fMLP control. Results shown are means (±SD) of four results (from two separate experiments). **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (0) control cells as determined by a one way ANOVA.

6.7 PMA STIMULATES ERK, BUT NOT AKT.

PMA (100 nM) was found to induce phosphorylation of ERK over a 30 minute time course. The response was slow, reaching a maximum at 10 minutes and was found to be diminished by 30 minutes. Akt phoshorylation was not found to be stimulated by 100 nM PMA over a 30 minute time course (not shown).

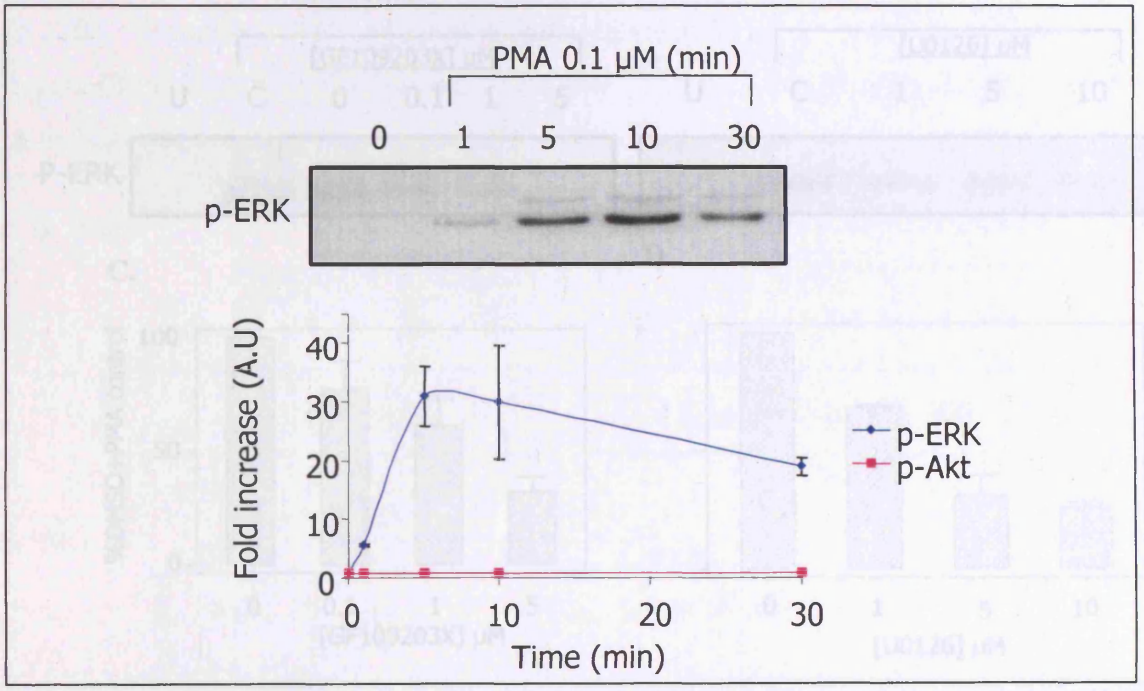

Figure 6.9 PMA activates ERK, but not Akt. dU937 cells were resuspended in BSS and then stimulated with 100 nM PMA for the indicated time. Cell lysis and sample preparation was performed as described in Chapter 2.13. Thirty five μg of protein was used for western blotting using phospho-specific Akt (ser473) and ERK antibodies (p-ERK). A) ERK phosphorylation in response to fMLP. Results in graphs are means (±SD) of two experiments; U, unstimulated control.

6.8 PMA STIMULATES PKC-DEPENDENT ERK PHOSPHORYLATION.

GF109203X was found to inhibit PMA-induced (100 nM for 10 minutes) ERK phosphorylation at doses from 1μM (figure 6.10A). U0126 was also found to dose-dependently inhibit PMA-induced (100 nM for 10 minutes) ERK phosphorylation (figure 6.10B).

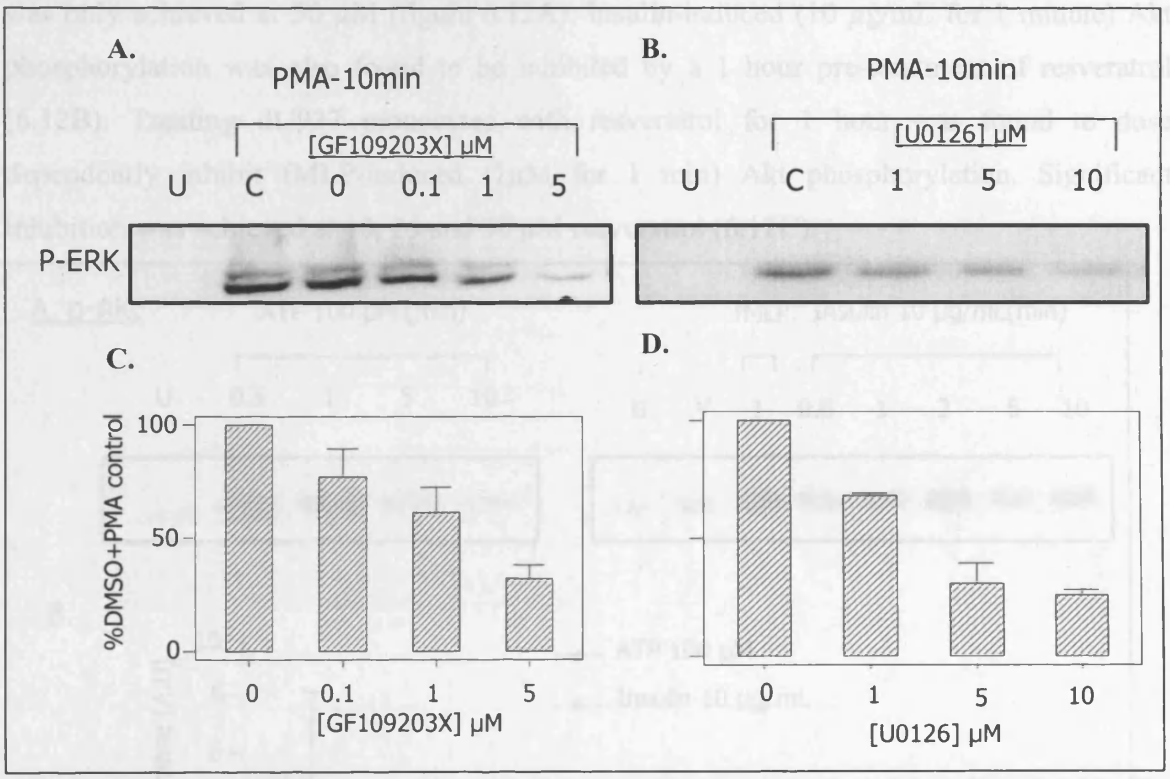

Figure 6.10 The effect of GF109203X and U0126 on PMA-induced ERK phosphorylation. A and C) dU937 cells were pre-treated with GF109203X (μM) for 15 minutes before stimulation with fMLP (1 μM for 1 min in BSS). Whole cell extracts were prepared and analysed by western blotting as described in Chapter 2.13. Thirty five μg of protein was used for western blotting using a phospho-specific ERK antibody. B and D) as (A) except that cells were pre-treated with U0126 for 1 hour before stimulation with PMA. C and D Densitometry was carried out on autoradiography films and the results expressed as %DMSO+fMLP control. Results shown are means (±S.D) of two experiments.

6.9 RESVERATROL INHIBITS INSULIN-, ATP- AND FMLP-INDUCED AKT PHOSPHORYLATION.

Insulin (10 μg/mL) and ATP were both found to induce a time-dependent phosphorylation of Akt (figure 6.11A and B). ATP-induced (100 μM for 1 minute) Akt phosphorylation was also found to be inhibited by a 1 hour pre-treatment of resveratrol, statistical significance was only achieved at 50 μM (figure 6.12A). Insulin-induced (10 μg/mL for 1 minute) Akt phosphorylation was also found to be inhibited by a 1 hour pre-treatment of resveratrol (6.12B). Treating dU937 monocytes with resveratrol for 1 hour was found to dose dependently inhibit fMLP-induced (1μM for 1 min) Akt phosphorylation. Significant inhibition was achieved at 10, 25 and 50 μM resveratrol (6.12C).

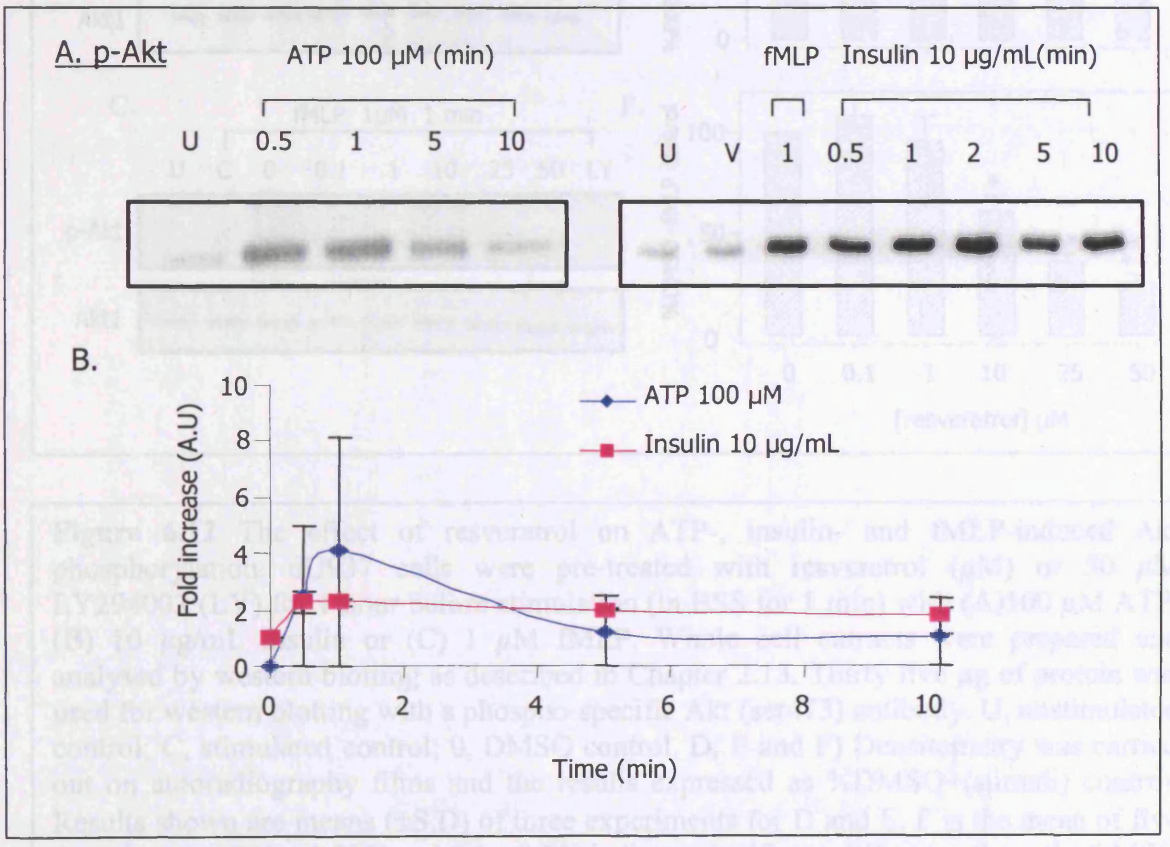

Figure 6.11 ATP and insulin stimulate the time-dependent phosphorylation of Akt. A) dU937 cells were resuspended in BSS and then stimulated with 100 μM ATP, 10 μg/mL insulin or 1 μM fMLP for the indicated time. Cell lysis and sample preparation was performed as described in Chapter 2.13. Thirty five μg of protein was used for western blotting using a phospho-specific Akt (ser473) antibody. B) Results are means (±SD) of two experiments U, unstimulated control; V, vehicle (HEPES).

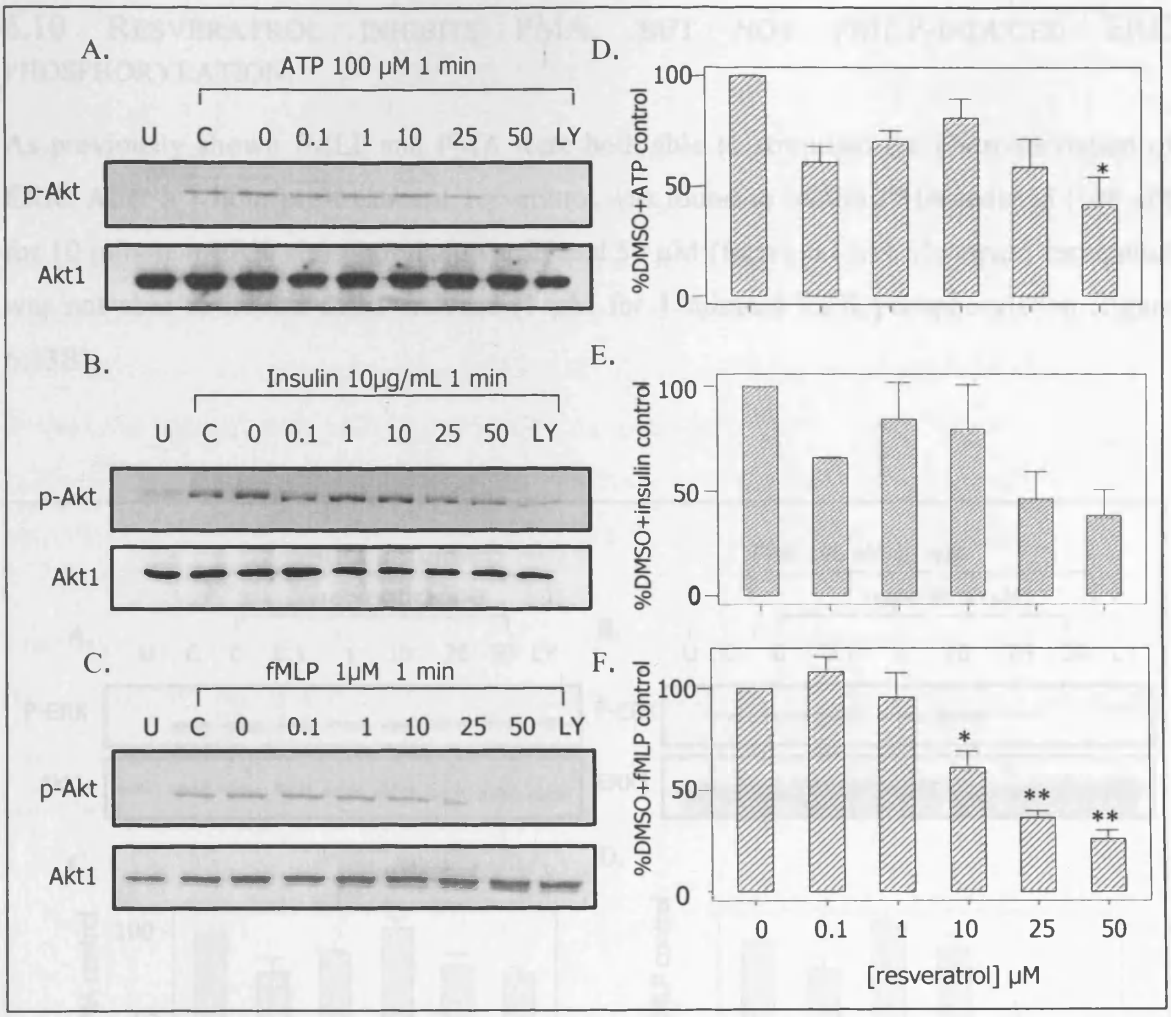

Figure 6.12 The effect of resveratrol on ATP-, insulin- and fMLP-induced Akt phosphorylation. dU937 cells were pre-treated with resveratrol (μM) or 50 μM LY294002 (LY) for 1 hour before stimulation (in BSS for 1 min) with (A)100 μM ATP, (B) 10 μg/mL insulin or (C) 1 μM fMLP. Whole cell extracts were prepared and analysed by western blotting as described in Chapter 2.13. Thirty five μg of protein was used for western blotting with a phospho-specific Akt (ser473) antibody. U, unstimulated control; C, stimulated control; 0, DMSO control. D, E and F) Densitometry was carried out on autoradiography films and the results expressed as %DMSO+(stimuli) control. Results shown are means (±S.D) of three experiments for D and E. F is the mean of five experiments. **($p<0.001$) and * ($p<0.05$) indicate significant difference from the DMSO (0) control cells as determined by a one way ANOVA

6.10 RESVERATROL INHIBITS PMA, BUT NOT FMLP-INDUCED ERK PHOSPHORYLATION.

As previously shown fMLP and PMA were both able to stimulate the phosphorylation of ERK. After a 1 hour pre-treatment, resveratrol was found to inhibit PMA-induced (100 nM for 10 minutes) ERK phosphorylation at 25 and 50 μM (figure 6.13A). However, resveratrol was not able to inhibit fMLP-induced (1 μM for 1 minute) ERK phosphorylation (figure 6.13B).

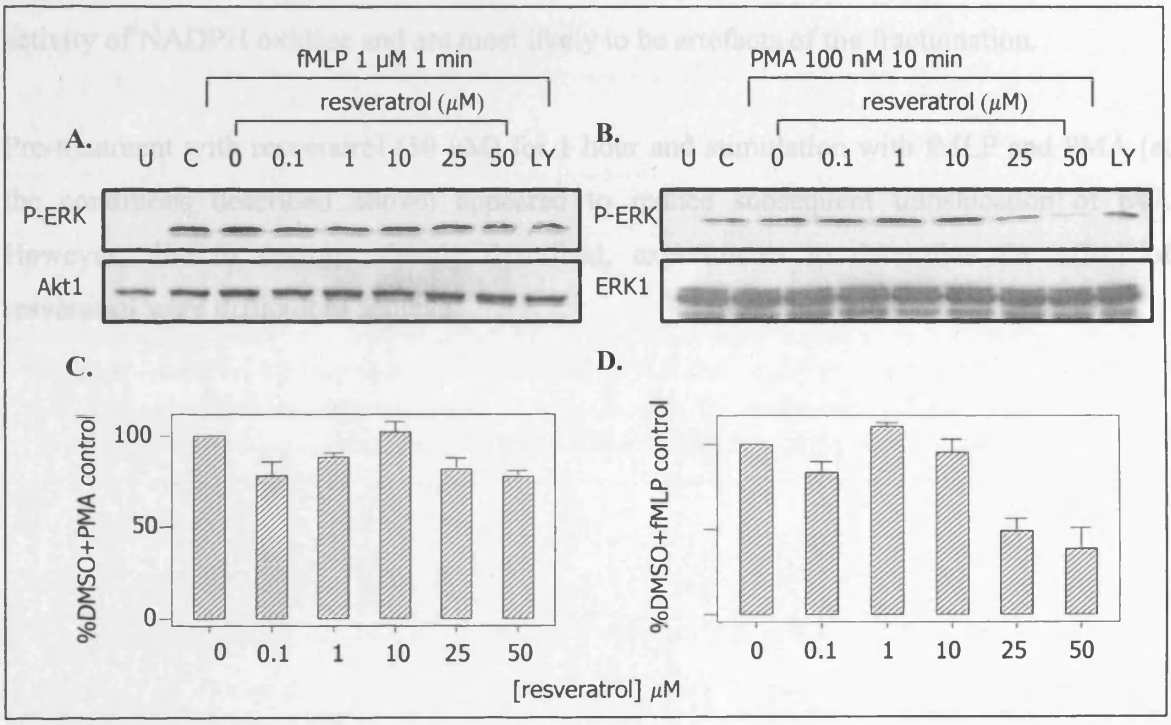

Figure 6.13 The effect of resveratrol on fMLP- and PMA-induced ERK phosphorylation. dU937 cells were pre-treated with resveratrol (μM) or 50 μM LY294002 (LY) for 1 hour before stimulation (in BSS) with (A) 1 μM fMLP for 1 min or (B) 100 nM PMA for 10 min. Whole cell extracts were prepared and analysed by western blotting as described in the materials and methods. Thirty five μg of protein was used for western blotting. Subsequent blots were probed with a phospho-ERK antibody and reprobed with (A) Akt1 and (B) ERK1 for equal loading. U, unstimulated control; C, stimulated control; 0, DMSO control. C and D) Densitometry was carried out on autoradiography films and the results expressed as %DMSO+PMA control. Results shown are means (±S.D) of three experiments for C and two for D.

6.11 THE EFFECT RESVERATROL ON FMLP- AND PMA-INDUCED P47 TRANSLOCATION AND PHOSPHORYLATION.

Both fMLP and PMA were able to induce the membrane translocation of the cytosolic NADPH oxidase subunit, p47 (figure 6.14). However, it should be noted that in general, the translocation of p47 in dU937 cells was inconsistent. This subunit is highly expressed in differentiated cells and it was difficult to obtain a membrane preparation that did not contain p47. PMA-induced membrane translocation of PKCα was also investigated and this was also found to be inconsistent, with high levels being found in the membrane fraction (not shown). High basal levels of p47 and PKCα in membrane preparations do not correlate with the activity of NADPH oxidase and are most likely to be artefacts of the fractionation.

Pre-treatment with resveratrol (50 μM) for 1 hour and stimulation with fMLP and PMA (at the conditions described above) appeared to reduce subsequent translocation of p47. However, due to reasons already described, experiments to determine the effect of resveratrol were difficult to achieve.

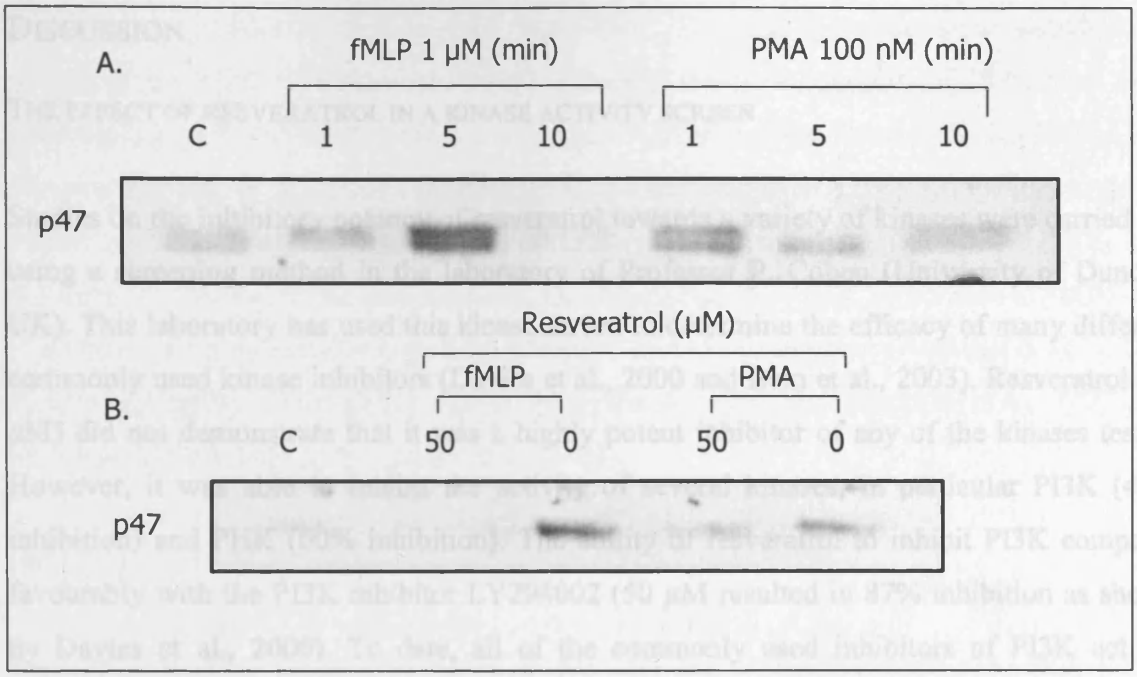

Figure 6.14 The effect of resveratrol on fMLP- and PMA-induced p47 membrane translocation. A) dU937 cells were stimulated in BSS for the indicated duration with 1μM fMLP and 100 nM PMA. B) dU937 cells were pre-treated with resveratrol (50 μM) for 1 hour before stimulation with 1 μM fMLP for 1 min or 100 nM PMA for 10 min. Cell membranes were prepared as described in the materials and methods. Fifty μg of membrane equivalents were used for western blotting using a p47 antibody.

DISCUSSION

THE EFFECT OF RESVERATROL IN A KINASE ACTIVITY SCREEN.

Studies on the inhibitory potency of resveratrol towards a variety of kinases were carried out using a screening method in the laboratory of Professor P. Cohen (University of Dundee, UK). This laboratory has used this kinase screen to determine the efficacy of many different commonly used kinase inhibitors (Davies et al., 2000 and Bain et al., 2003). Resveratrol (10 μM) did not demonstrate that it was a highly potent inhibitor of any of the kinases tested. However, it was able to inhibit the activity of several kinases, in particular PI3K (47% inhibition) and PHK (60% inhibition). The ability of resveratrol to inhibit PI3K compares favourably with the PI3K inhibitor LY294002 (50 μM resulted in 87% inhibition as shown by Davies et al., 2000). To date, all of the commonly used inhibitors of PI3K act via competition with ATP for the nucleotide binding pocket (Walker et al., 2001) and therefore, their activity in kinase screens is much higher than *in vivo* where ATP levels are much higher (mM) (Davies et al., 2001). However, some kinase inhibitors act through ATP-independent mechanisms. The MEK inhibitor, U0126, is a good example of this and is more effective *in vivo* than in kinase assay systems. The ability of U0126 to inhibit MEK was discovered through its ability to inhibit AP-1 transactivation in cell-based reporter gene assays (Favata et al., 1998). Using similar techniques it was shown that resveratrol can inhibit PMA-induced AP-1 activity (Subbaramaiah et al., 1998). These authors also showed that resveratrol is a potent inhibitor of membrane-associated PKC activity, while having very little effect on cytosolic PKC activity (at low doses 15 μM or below).

The results shown here suggest that resveratrol does not inhibit PKCα. This was due to the conditions used in the kinase assay. Resveratrol has been shown to be a weak direct inhibitor of multiple PKC isoforms when tested in kinase assays such as this. However, if the conditions of the assay are changed and membrane-associated kinase activity is measured then resveratrol has been shown to have greater efficacy (Slater et al., 2003; Stewert et al., 1999). It can be seen that caution should be taken before drawing conclusions from kinase screens, since negative results can be a product of the assay conditions.

PI3K was the only kinase shown to be inhibited by resveratrol in the kinase screen that is known to activate NADPH oxidase. PI3K represents one of the key control points in the activation of the NADPH oxidase. It is involved in the rapid signal transduction process of bacterial peptides, chemokines and immunoglobulins (Welch et al., 2001). Multiple cell

signalling intermediates, such as PKC, Akt, ERK, PLD and Rac have been shown to be controlled by PI3K (Welch et al., 2003). Recently the lipid products of PI3K have been shown to play pivotal roles in the assembly of NADPH oxidase through the binding of PX-domains (Ellson et al., 2001). Therefore, PI3K represents an ideal therapeutic target to inhibit a number of leukocyte processes such as chemotaxis, phagocytosis, ROS and cytokine production. In the case of resveratrol, inhibition of a PI3K might also explain some of its broad ranging activities, such as inhibition of ROS production (Poolman et al., 2002), platelet aggregation (Wang et al., 2002), tissue factor production (Pendurthi et al., 1998) and smooth muscle hypertrophy (Haider et al., 2002).

INHIBITION OF PI3K BY RESVERATROL IN DU937 MONOCYTES.

The class 1b PI3K isoform (p110γ subunit) was used in the kinase screen, which was originally discovered in the U937 cell line as was its regulatory subunit, the p101 adaptor protein. However, measuring the activity of PI3K induced in cells by fMLP was problematical. In order to reliably measure changes in the cellular pools of poly-phosphoinositides, cells need to be metabolically labelled with [γP^{32}] ATP. After stimulation the lipids are extracted from the cells, separated by TLC and quantified by HPLC. Due to the large amounts of radioactive waste produced by this technique it was not desirable to carry out these experiments. A lipid kinase assay was far more convenient to perform. This method requires that the enzyme or protein that it is associated with be immunoprecipitated. After incubation with the phosphoinositide substrate (and carrier lipids i.e. phosphatidylserine) and [γP^{32}] ATP, the radioactive lipids can be separated by TLC according to the head group polarity e.g. the more phosphate groups the lower the Rf value (originally described by Whittman et al., 1988).

The next difficulty is deciding on the immunoprecipitation antibody. In theory it would be practical to immunoprecipitate the p110 subunit. However, this is hindered by a lack of high affinity antibodies (personal communications from Dr L. Stephens, University of Cambridge). Some research groups have made their own antibodies and carried out successful lipid kinase assays (Cadwallader et al., 2002). Using class 1b, p110 antibodies in lipid kinase assays also highlights the observation that this isoform requires the constant association with the G-protein $\beta\gamma$ subunits (Stephens et al., 1994). The most common immunopreciptation protocols are targeted against the regulatory, p85 subunit. However, fMLP-induced PI3K activity in phagocytes has been shown to be independent of the class 1a

isoforms (Cadwallader et al., 2002), while others have shown that p85 is activated by fMLP (Pan et al., 2000). In this study, anti- phosphotyrosine immunoprecipitates were found to have PI3K lipid kinase activity. However, p85 from fMLP-stimulated cells was found not to be tyrosine phosphorylated or associated with tyrosine phosphorylated proteins. There is the possibility that the class 1a isoform activated by fMLP is not associated with the p85 regulatory subunit. Other adapter molecules might be involved e.g. p55. The class 1b isoform, PI3Kγ, is thought to be the initial PI3K isoform activated by fMLP. Secondary activation of class 1a PI3K isoforms has been proposed. PtIns(3,4,5)P_3 is thought to activate a tyrosine kinase that could activate the class 1a isoforms. This would increase the accumulation of PtIns(3,4,5)P_3 and aid signal amplification (reviewed by Weiner et al., 1998). This hypothesis has been supported by the use of cell permeable esters of PtIns(3,4,5)P_3 that elicit cell responses in a wortmannin sensitive fashion (Niggli et al., 2000).

Cadawaller et al., (2002) showed that TNF-activated lipid kinase activity in p110γ, but not p85 or 4G10 (phosphotyrosine) immoprecipitates. These authors suggest that some of the published discrepancies are due to the specificity of the phosphotyrosine antibodies used. The 4G10 antibody used by Cadawaller et al., (2002) might offer greater specificity than the PY99 used in this study. However, Ptasznik et al., (1996) show that fMLP-induces tyrosine kinase dependent PtIns(3,4,5)P_3 production, when measuring the total changes phosphoinositides. It is clear that *in situ* methods for determining the levels of PI3K lipid products need to be used e.g. confocal imaging. An ELISA microplate method has recently been developed that measures PtdIns(3,4,5)P_3 production from immnuoprecipiates. Although this would not overcome the difficulties observed with antibody specificities, it might be more reliable than the classical TLC methods. In general, the activity of PI3K is a very difficult to measure reliably. Most of the available methods have many potential pitfalls, even the composition of the kinase buffer is thought to affect kinase activity. Class 1a isoforms have been shown to perform better in a HEPES buffer, whereas, class 1b favour Tris-based buffers (personal communication from Dr. R Drees, Elchon Incorporated, Salt Lake City, USA).

Incubation of phosphotyrosine immunoprecipitates from fMLP-stimulated cells with resveratrol resulted in inhibition of PI3K activity. Pre-treatment of dU937 cells with resveratrol for 1 hour before stimulation and immunoprecipitation with phosphotyrosine antibodies does not result in inhibition of PI3K activity. These results initially suggest that

resveratrol does not inhibit PI3K *in vivo,* where ATP levels are much higher than commonly used in kinase assays. The PI3K protocol requires that immunoprecipitates are thoroughly washed and it could be possible that the interaction between resveratrol and the ATP-binding site is diminished. PI3K activity in immunoprecipitates from cells pretreated with resveratrol before fMLP stimulation had increased kinase activity. The reasons for this are unclear. Given that in some cases anti-phosphotyrosine antibodies can bind non-specifically, these results could be an artefact of the kinase assay. Initial results using insulin (a well known stimulus of PI3K) showed that a 1 hour incubation with resveratrol before stimulation with insulin inhibited lipid kinase activity in p85, but not in PY99 immunoprecipitates.

She et al., (2003) have shown that resveratrol does not inhibit EGF-induced PI3K, using 10 μM resveratrol with a 20 minute incubation. Haider et al., (2002) showed that resveratrol could inhibit the association of p85 with tyrosine phosphorylated proteins and Akt phosphorylation. The EGF receptor does not have a p85 binding consensus sequence and has been shown to be a relatively weak agonist of PI3K activity. The classical model for p85 recruitment to a receptor tyrosine kinase is thought to be dependent on multiple YXXM motifs that are phosphorylated by the receptor. This permits the binding of PI3K through its SH2-domains. The EGF receptor has been shown to associate with growth factor receptor bound-1 protein {Grb-1}-associated protein (Gab1), which permits it to activate the class 1a PI3K isoforms. Interestingly, Gab1 does not only activate PI3K, but is also a PI3K substrate and therefore completes a positive feed back mechanism (Rodrigues et al., 2000). Differences in the recruitment process might explain the inability of resveratrol to inhibit EGF-induced PI3K and Akt phosphorylation. These data suggest inhibition of PI3K *in vitro* might be due to an ATP-independent mechanism, such as inhibiting the recruitment of the PI3K to effector molecules. This would be similar to the mechanism by which resveratrol has been shown to inhibit PKC. In this case resveratrol was shown to inhibit membrane associated PKC activity through an interaction with its C1-domain.

THE EFFECT OF RESVERATROL ON AKT PHOSPHORYLATION.

Stimulation of dU937 cells with fMLP resulted in Akt phosphorylation, which could be detected within 30 seconds. The response peaked at 1 minute and was diminished after 5 minutes. The signal is similar to the response measured by fMLP-induced luminol/isoluminol/lucigenin-CL, although Akt phosphorylation was evident up to 5 minutes after stimulation. Insulin and ATP were also shown to induce Akt phosphorylation with

similar kinetics to fMLP, although insulin-induced Akt phosphorylation was found to produce a more sustained signal. The effect of insulin on the respiratory burst is not completely clear (Oldenborg 1999), although it has been shown to induce actin polymerisation in U937 cells (Walters et al., 1996). ATP has been shown to activate the purine, P2x receptor and also induce actin polymerisation (Walters et al., 1996). The effect of purines, such as ATP, have been shown to have a varied effects on the respiratory burst. In some cell types e.g. alveolar macrophages, ADP has been shown to be a potent activator of ROS production (Gozal et al., 2001). ATP has been shown to be much less potent. fMLP is also thought to induce actin polymerisation (Simchowitz and Cragoe 1990). However, of the three stimuli only fMLP is a consistent activator of NADPH oxidase.

Resveratrol was found to significantly inhibit fMLP-induced Akt phosphorylation at doses above 10 μM (after a 1 hour pre-treatment followed by washing). Insulin-induced Akt phosphorylation was also found to be inhibited with doses of resveratrol above 10 μM. ATP-induced Akt phosphorylation was also inhibited by resveratrol, although higher doses were required (above 25 μM). Some authors have shown that insulin and ATP activate distinct PI3K isoforms. Melendez et al., (1998) showed that insulin and ATP activate class 1a and 1b isoforms respectively. They also showed that the levels PtdIns(3,4,5)P_3 could not be increased in dU937 monocytes by insulin when cells were transfected with a p85 dominant negative, whereas ATP could still induce PtdIns(3,4,5)P_3 in the presence of the dominant negative.

Pre-treatment with PI3K inhibitors has been shown to inhibit agonist-induced and basal levels of Akt phosphorylation. Some authors have suggested that Akt phosphorylation is a good indicator for the cellular levels of PtIns(3,4,5)P_3, the major lipid product of PI3K. Other downstream targets of PI3K can still be activated even when Akt phosphosphorylation is diminished, which is thought to be due to the low affinity PH domain of Akt (which has equal affinity for PtIns(3,4,5)P_3 / PtIns(3,4,)P_3) (reviewed by Parker, 2001) . PDK has a high affinity PH domain, therefore low levels of PtIns(3,4,5)P_3 could still result in its activation (Stephens et al., 1998). This makes Akt a good therapeutic target, since a relatively small change in PtIns(3,4,5)P_3 / PtIns(3,4,)P_3 levels results in a large change in the activation status of Akt.

THE EFFECT OF RESVERATROL ON ERK PHOSPHORYLATION.

The phosphorylation of ERK provided a convenient point of convergence, being induced by fMLP and PMA. fMLP was shown to phosphorylate ERK and the kinetics of this activation was very similar to that measured with Akt. PMA was also shown to phosphorylate ERK in a much slower fashion and the initial activation was similar to that measured by PMA-induced luminol/isoluminol-CL. However, ERK phosphorylation was significantly diminished after 30 minutes, whereas PMA-induced ROS production was still evident.

Resveratrol was found to have no effect on fMLP-induced ERK phosphorylation (at doses up to 50 μM). This suggested that resveratrol was not antagonising the fMLP receptor and that the G-protein, $\beta\gamma$ subunits are able to elicit other signalling pathways. PMA-induced ERK phosphorylation was found to be inhibited by resveratrol at doses above 10 μM. Since PMA did not induce Akt phosphorylation it is unlikely that PMA is activating ERK through a PI3K-dependent mechanism.

ERK has been shown to be activated in a PI3K-dependent mechanism, for which there are several different activation mechanisms (as discussed in Chapter 1.6.7). One hypothesis was examined by constructing PI3Kγ hybrids that have differential lipid kinase activity. It was determined that the lipid kinase activity of PI3K was not required for the activation of the MAPK cell signalling cascade, whereas the protein kinase activity was essential for this function (Bondeva et al., 1998). The only reliable protein kinase substrate for PI3K is its own regulatory subunit (Stein and Waterfield 1998 and Vanhaesebroeck et al., 1999). There are also PI3K-independent mechanisms proposed for the activation of the MAPK pathway. The activated G-protein subunits ($\beta\gamma$) have been shown to activate Ras (Koch et al., 1994) and a number of MAPK signalling intermediates. Ras has also been shown to interact with PI3Kγ (Rubio et al., 1997). In this study there was limited evidence to suggest cross-talk between the MAPK and PI3K pathways. The PI3K inhibitor LY294002 was found to partially inhibit fMLP-induced ERK phosphorylation and U0126 had a minimal inhibitory effect on fMLP-induced Akt phosphorylation.

It could be possible that PMA- and fMLP-induced activation involve distinctive cell signalling pathways. This might explain why resveratrol does not inhibit fMLP-induced ERK phosphorylation (see below). The ability of resveratrol to antagonise PMA-induced responses has been well studied in animal models and many cell types. Resveratrol has been

shown to be a potent inhibitor of PMA-induced COX2 expression and AP-1 activity (Subbaramaiah et al., 1998), ERK and src activation (Yu et al., 2001). In this study it was shown that the MEK inhibitor, U0126, had some effect on ROS production. However, caution should be used when determining the physiological relevance of PMA-induced responses and some authors have described these pathways as "redundant". This concept is discussed below.

CELL SIGNAL TRANSDUCTION PATHWAYS THAT ACTIVATE NADPH OXIDASE.

One of the first receptor mediated activation processes, which was shown to be dependent on phosphoinositide production was the respiratory burst. In dU937 monocytes, fMLP-induced Akt phosphorylation was found to be inhibited by LY294002 and a specific inhibitor of Akt activation. Since the activation of Akt displays identical kinetics to fMLP-induced ROS production (see Chapter 5.4) and both PI3K and Akt inhibitors are effective against fMLP-induced ROS production. Therefore, it is proposed that the PI3K/Akt pathway is a major regulatory cell signalling pathway for the activation of the respiratory burst in dU937 monocytes. Resveratrol was found to inhibit Akt phosphorylation and thereby explaining its inhibitory effect on fMLP-induced ROS production. Akt has recently been shown to directly phosphorylate the p47 subunit (Cheng et al., 2003).

The PKC, inhibitor GF109203X was found to have no effect on fMLP-induced Akt phosphorylation. GF109203X is a relatively specific PKC inhibitor and its inability to inhibit fMLP-induced Akt phosphorylation suggests that PKC is not involved in this process. Neutrophils have been shown to have a granular pool of NADPH oxidase that is activated by a PKC-dependent pathway, which is sensitive to wortmannin (Karlsson et al., 2001). PMA does not activate Akt and PMA-induced ROS production is not inhibited by inhibitors of PI3K or Akt. Therefore, in dU937 cells PMA and fMLP activate distinctive pathways. In Chapter 5.1 it was shown that GF109203X can inhibit fMLP-induced ROS production. If fMLP does activate PKC isoforms, it is most likely that it is downstream of PI3K. The lipid products of PI3K have been shown to be involved in the activation process of some PKC isoforms, through their phosphorylation and possibly their direct activation. It is unlikely that resveratrol can affect PKC phosphorylation under the treatment conditions used in this study, since PKC phosphorylation occurs after translation and is then unaffected by PI3K inhibitors. Direct activation of PKC by PI3K has been postulated for some PKC isoforms, but is still not completely clear. The fMLP receptor could activate PLC, which could activate

PKC though DAG production (the classical activation pathway). There are some PLC isoforms that have been shown to be activated by the lipid products of PI3K (Piccolo et al., 2002) and some PLC isoforms can be directly activated through PI3K-independent mechanisms (Rhee et al., 2001).

Both fMLP- and PMA-induced ERK phosphorylation were shown to be inhibited by the MEK inhibitor, U0126. ERK phosphorylation was investigated since it is activated by fMLP and PMA. However, fMLP-induced ERK activation was found to be independent of PI3K and only partially inhibited PKC inhibitors. PMA-induced ERK phosphorylation was found to be dependent on PKC and independent of PI3K. These results might explain why resveratrol inhibits PMA-induced ERK, but not fMLP-induced ERK phosphorylation, since resveratrol is a well established inhibitor of PKC *in vivo*.

Several other cell signalling intermediates are activated in response to fMLP, which have not been investigated in this study. In particular, PLD has been shown to be activated and can been inhibited by resveratrol (Tou and Urbizo 2001). However, the dose required for inhibition was greater than that required to inhibit PI3K or Akt phosphorylation in this study. PI3K has been shown to be an upstream regulator of PLD through the activation of GEFs (Powner et al., 2002). PKC has also been demonstrated to activate PLD (Regier et al., 2000). However, it is unclear if PMA activates PLD in dU937 cells. There are other enzymes, such as PLC and Src that are also thought to play a role in the activation of NADPH oxidase that could also be inhibited by resveratrol. Resveratol has also been shown to inhibit Src activity (Yu et al., 2001) induced by PMA and UV-light.

The activation of NADPH oxidase also involves the activation of the small GTP binding protein, Rac (Rac1 in neutrophils and Rac2 in monocytes). It has recently been shown that Rac1 knockout mice display selective neutrophil defects (Glogauer et al., 2003). Statins have been shown to inhibit fMLP-induced ROS production by inhibiting the isoprenylation of Rac (Bokoch and Prossnitz 1992). Although, the effect of resveratrol on Rac has not been investigated it is unlikely that it can directly inhibit Rac. If this was the case then resveratrol would have an equal inhibitory effect on ROS production induced by fMLP and PMA.

It is clear that the activation of NADPH oxidase is tightly regulated and its activation depends on the simultaneous activation of multiple kinases. These enzymes bring about the phosphorylation of the cytosolic subunits that initiate activation. In chapter 5.2 and 5.3 it was

shown that inhibitors of PI3K and ERK were both able to inhibit ROS production and in this chapter it was shown that these pathways are largely activated independently of each other. Although it is possible that the two pathways are activated with different kinetics, with ERK being activated quicker than Akt. To date, Akt has been shown to phosphorylate the p47 subunit and ERK can phosphorylate the p67 subunit. The regulatory process involved in the activation of NADPH oxidase can be exploited, since it appears that only one pathway needs to be inhibited to attenuate the respiratory burst. Similar results have been found in neutrophils using various kinase inhibitors and with dominant negatives or antisense oglionucleotides. Resveratrol was shown to inhibit the translocation of p47 in response to both PMA and fMLP. This provides further evidence that resveratrol can directly antagonise the activation of NADPH oxidase. However, the mechanism is still unclear, and may be due to its ability to bind to lipid binding domains e.g. the C1 domain in the inhibition of PKC. It could be possible that resveratrol can bind to PH domains that are involved in the activation of PI3K and NADPH oxidase.

CHAPTER SEVEN:

GENERAL DISCUSSION

CELL SIGNAL TRANSDUCTION PATHWAYS THAT ACTIVATE NADPH OXIDASE AS POTENTIAL THERAPEUTIC TARGETS?

Studies using knockout mice have suggested that the monocyte and its activation state are pivotal to the pathogenesis of atherosclerosis. As previously mentioned, mice that have fewer macrophages (through deletion of M-CSF) can be crossed with the ApoE-deficient strain (that develops experimental atherosclerosis), resulting in reduced lesion formation in the double knockout (Smith et al., 1995). Moreover, if mice lacking the chemokine receptor for MCP1 are crossed with the ApoE-deficient strain, reduced disease progression is again observed in the double knockout (Inouse et al., 2002). Similar results have now been observed for the chemokine receptor for fractalkine (Combadiere et al., 2003). Chemokines, such as MCP1, offer the pharmaceutical industry an ideal chance to develop small molecule receptor antagonists that could be the next generation of anti-inflammatory agents. The signal transduction pathway that these receptors activate has attracted considerable attention. The PI3K family has been identified as the central mediator, with its activation involved in chemotaxis, cellular priming, the respiratory burst and phagocytosis. Therefore, inhibition of the PI3K signalling pathway also offers a viable therapeutic target. However, the pharmaceutical industry would argue that the inherent specificity that a receptor antagonist would offer is lost with a kinase inhibitor. In theory it would be relatively straightforward to develop a receptor antagonist. However, the chemokine system had additional problems. Chemokines are known to use multiple receptors, making it difficult to completely antagonise an individual chemokine.

BX471 is an orally active non-peptide antagonist of the CCR1 chemokine receptor (Liang et al., 2000), which binds the chemokine RANTES (Regulated upon activation normal T cell

expressed and secreted). BX471 has been used to inhibit heart transplant rejection in rats (Horuk et al., 2001), renal fibrosis (which leads to renal failure) (Anders et al., 2002) and CCL16 mediated angiogenesis (Strasly et al., 2003). This highlights the importance of these mediators for a variety of disorders, and more importantly PI3K is likely be the central signal transducer in these cases.

The main question is whether a kinase inhibitor can be developed that specifically inhibits a particular PI3K isoform? There are two commonly used PI3K inhibitors, LY294002 and wortmannin, but although they have been successfully used at deciphering cell signal transduction pathways, their physical properties make them unsuitable for *in vivo* application. The second generation PI3K inhibitors will hopefully further increase our knowledge of the role PI3K plays in inflammation. Neither wortmannin nor LY294002 display reliable PI3K isoform selectivity. Despite the evolutionary conservation of kinase ATP binding pockets (García-Echeverría et al., 2000), it has been possible to develop ATP competitive compounds that are specific e.g. IRESSA™ developed by AstraZeneca. Recently several compounds have been developed that have been shown to display PI3K isoform selectivity (reviewed by Ward and Finan 2003). A quinolone and a pyridopyrimidine compound show some selectivity for PI3Kα and β (100-fold) over PI3Kγ. The ICOS corporation has described several compounds that are apparently selective for PI3Kδ e.g. IC87114 (Sadhu et al., 2003). The PI3Kγ isoform, which displays a tissue distribution restricted to phagocytes, is an excellent target for an inhibitor of this nature. However, to date no such compound has been described. The crystal structures of PI3Kγ-inhibitor complexes have been described, which will aid the development of new inhibitors. The crystal structures of PI3Kγ complexed to quercetin, myricetin, staurosporine, LY294002 and wortmannin have all been investigated (Edward et al., 2000). Surprisingly, all these compounds bind to the ATP-binding site with different orientations, which explains why structurally unrelated compounds are able to inhibit PI3K.

The activation of NADPH oxidase has been shown to depend on the lipid products of PI3K, not only through the activation of downstream kinases, but also for membrane binding of the cytosolic phox subunits e.g. p47 binds to PtdIns(3,4)P_2 (Kanai et al., 2001). In this study the PI3K inhibitor, LY294002, was found to inhibit fMLP-, but not PMA- or AA-induced ROS production. This suggests that there are PI3K-independent pathways that activate NADPH oxidase. Upon cell stimulation ,the p47 subunit is phosphorylated and this exposes the its PX domain, which aids the membrane targeting of p47. If p47 is isolated from PMA-stimulated

cells, it is incapable of binding to membranes, which suggests that the differences between fMLP- and PMA-induced ROS production are not only due to different kinases but also to membrane targeting (Ago et al., 2003).

Inhibitors of MEK and PKC were also found to inhibit fMLP-induced ROS, which suggests that the coordination of multiple signalling pathways is required for activation. This makes inhibition of the respiratory burst an ideal target, since inhibition of any one pathway attenuates the response. However, there is always the possibility of redundancy in cell signalling pathways and the kinases studied involved *in vitro* might have less significance *in vivo*. Therefore, a robust kinase target is required that is likely to be activated *in vivo* by cellular mediators that contribute to disease states. PI3K has been shown to be activated by many inflammatory mediators (including all chemokines studied) (Sotsios and Ward 2000). Moreover, it has been shown to activate many down stream signalling intermediates. Indeed, PI3K has been described as the "Gatekeeper of inflammation" (Weaver and Ward 2001), which makes it an ideal therapeutic target.

A specific inhibitor of Akt was used to determine its role in fMLP-induced ROS production (see below), which is further strengthened by recent publications demonstrating that the p47 subunit of NADPH oxidase was a substrate for Akt (Chen et al., 2003). Although this does not prove that it will be the case *in vivo*, the use of a specific Akt inhibitor does further strengthen this hypothesis. Some authors have reported that the MAPK pathway is downstream of PI3K (Nomura et al., 2001) and moreover, ERK has been shown to phosphorylate the p67 subunit (Dang et al., 2003) of NADPH oxidase. It could be argued that ERK is of equal importance to Akt as a mediator of ROS production, since the MEK inhibitor (U0126) is a potent inhibitor of this response. However, Akt has been shown to play important roles in cell movement (Hannigan et al., 2002) and phagocytosis (Chung et al., 2001), whereas ERK has not been shown to be a robust target (reviewed by Sotsios and Ward 2000). The inhibition of Akt has some potential pitfalls, in that it is a mediator of nitric oxide synthase (Fulton et al., 1999), and the activation of eNOS endothelial cells is dependent on Akt. Statins have been shown to activate Akt in endothelial cells (Dimmeler et al., 2001), which would promote their survival and increase the levels of nitric oxide (Zeng et al., 2000). This would reduce the endothelial dysfunction that is associated with many cardiovascular disorders. However, the chemopreventive ideology is one of modulation. It is proposed that the increased activity of the PI3K-Akt pathway, which has in some cases been

shown to be responsible for the phagocyte priming phenomenon, could be reduced to a normal level by resveratrol.

PEROXIDASES AND RESVERATROL

Resveratrol has been shown to inhibit the oxidation of various redox sensitive probes, including DCF (Rota et al., 1999) and luminol (Nakamura and Nakamura 1998). These results prompted several authors to suggest that resveratrol was a potent inhibitor of cellular ROS production. The effect of resveratrol on ROS production from dU937 cells was investigated in this study, with a view to providing a mechanistic explanation. However, several studies that aimed to determine the antioxidant efficacy of resveratrol using cellular models (Rotondo et al., 1998; Jang et al., 1999; Leiro et al., 2002; Martinez and Moreno, 2000; DeSavia et al., 2002; Ignatowicz et al., 2003; Cheng et al., 2003) have not taken into account the ability of resveratrol to inhibit the oxidation of these probes through as an alternative co-reducing substrate. The majority of redox sensitive probes require peroxidases for their oxidation, which are added to the reaction conditions e.g. in the case of luminol or isoluminol-CL where HRP is added. Some probes use intracellular peroxidases, such as DCF or DHR and their oxidation is very rapid in the presence of a peroxidase.

Resveratrol has been shown to inhibit hydrogen peroxide induced DCF oxidation (DeSalvia et al., 2002). Hydrogen peroxide is relatively unreactive and resveratrol does not directly affect this oxidant. DCF can be oxidised by a mixture of iron and hydrogen peroxide. However, this method of oxidation is not as great as that seen with HRP and hydrogen peroxide (LeBel et al., 1992). Resveratrol, like most polyphenolic compounds, is a co-substrate for peroxidases and could compete with DCF for the peroxidase. It has recently been demonstrated that resveratrol and DCF are oxidised in a very similar fashion (Galati et al., 2002).

HRP reacts with hydrogen peroxide to form a product with a spectroscopically detectable higher oxidation state called Compound I, which is then reduced back to the native enzyme (through the formation of compound II). Both DCF and resveratrol form a phenoxy-radical intermediate that can be reduced back to the original compound (for example by GSH) with the generation of superoxide (Rota et al., 1999 and Galati et al., 2002). It has been suggested that this represents a pro-oxidant pathway for resveratrol and can make the measurement of cellular ROS using DCF problematical.

In some cases resveratrol has been shown to inhibit peroxidase activity in a non-competitive fashion that did not depend on its ability to act as a co-substrate. Resveratrol has been shown to inhibit the peroxidase activity of COX1 and 2 in a non-competitive manner (Johnson et al., 1998). In this study it was shown that resveratrol had a marginal effect on peroxidase activity when measured using ABTS as a substrate. This peroxidase assay requires high concentrations of ABTS (1 mM), therefore resveratrol does not directly inhibit HRP. The concentration of DCF used was 2 μM and the resveratrol concentration ranged from 0.1-50 μM. Therefore, resveratrol would be able to directly compete with DCF as a peroxidase substrate. When cellular oxidation of DCF is considered, where a mixture of ROS is produced, DCF is only likely to be oxidised by three different oxidants generated in this system. DCF does not detect superoxide and the presence of reactive nitrogen species is excluded by the absence of L-arginine. DCF is oxidised by hydrogen peroxide and hypochlorous acid (Crow et al., 1997), with the latter being dependent on myeloperoxidase. Hydrogen peroxide is likely to be the major oxidant measured. The activity of MPO has been shown to vary with the stimulus used (Stolarek et al., 2002) and it is difficult to determine the contribution of hypochlorous acid towards DCF oxidation.

Luminol and isoluminol are also oxidised by HRP is a similar fashion, however these compounds have been shown to measure a variety of ROS. Despite this, HRP is apparently required regardless of the ROS being measured. Some authors have suggested that superoxide plays a role in the detection of peroxides by isoluminol (see Chapter 3, Yamamoto et al., 1985). Therefore, it is important to use a variety of measuring principles, including methods that are independent of peroxidases. In this study, lucigenin was used as a probe that does not require peroxidases and is specific for superoxide. However, there are reports that lucigenin redox cycles and releases superoxide (Vasquez-Vivar et al., 1997) and then over-estimates superoxide production. The method has been heavily criticised (Liochev and Fridovich 1998) and then validated (Li et al., 1998), whereas others question if it does undergo redox cycling in the first place (Afanas'ev et al., 1999). Using lucigenin to measure the respiratory burst resulted in significant differences between the inhibitory effect of resveratrol measured with any other probe that was HRP-dependent. Given that resveratrol is oxidised by peroxidases, it is most likely that its inhibitory effects on peroxidase-dependent ROS measurement is due to its ability to act as a co-substrate. All of the current reports that show that resveratrol can inhibit the respiratory burst use a peroxidase-dependent ROS

measuring principle. Most authors have reported a low μM IC_{50} value (1.4 μM) to inhibit the neutrophil respiratory burst (Rotondo et al., 1998).

In this study two treatment conditions were used, a 1 hour pre-treatment followed by washing and the direct addition of resveratrol to the reaction. When cells were pre-treated with resveratrol, its inhibitory effects were greatly reduced and correlated with the inhibitory effect of resveratrol on various cell signalling pathways (see below). The inhibitory effects of genistein on the respiratory burst can also be questioned, since it is also likely to be a co-substrate for HRP. Its inhibitory effects should also be viewed with caution. The other inhibitors used in the study are unlikely to be co-substrates for peroxidases, since they were found to inhibit the respiratory burst in a stimulus-specific manner (see Chapter 5).

The oxidation of resveratrol by peroxidases does have some important implications for its cardioprotective properties. LDL has been shown to be oxidised by peroxidase/H_2O_2 systems, moreover, resveratrol has already been shown to inhibit this type of LDL oxidation. Tyrosine is also oxidised by peroxidase systems, which has been shown to have some important implications for lipid peroxidation and atherosclerosis. Tyrosine, is present in human plasma (40-80 μM) (Heinecke et al., 2002). The phenol group of tyrosine is oxidised by peroxidases in the same fashion as the one electron oxidation of resveratrol (to form the phenoxyl radical), but a tyrosyl radical is the resulting species (Heinecke, 2002). Phagocyte myeloperoxidase has been shown to oxidise free tyrosine under physiological conditions leading to the formation of the tyrosyl radical and o,o'-dityrosine (formed by the reaction of two tyrosyl radicals) (Stvenkova et al., 1994). The tyrosyl radical can then diffuse from the active site of the enzyme and react with another tyrosyl radical or protein tyrosyl residue (to form a protein tyrosyl radical). The protein tyrosyl radical could have several fates. It could react with free tyrosyl radical to form a tyrosylated protein or react with juxtaposed protein tyrosyl radical to form a protein cross link (reviewed by Heinecke, 2002). The reaction with tyrosyl radicals and PUFAs has been suggested to be a major physiological initiator of lipid peroxidation. Myeloperoxidase has been found in atherosclerotic plaques and is found to be localised with lipid laden macrophages (Daugherty et al., 1994).

LDL from human vascular tissue at autopsy contained 100 times more o,o'-dityrosine than circulating LDL. Fatty streaks have been shown to contain 11 times more o,o'-dityrosine and there has been shown to be a 6 fold increase in mature atherosclerotic aorta (Leeuwenburgh et al., 1997). Resveratrol has been shown to be a scavenger of tyrosyl

radicals (as shown in its inhibition of ribonuclease reductase (Fontecave et al., 1998). Moreover, resveratrol could potentially compete for tyrosine and therefore prevent its oxidation. It can be seen that the ability of resveratrol to inhibit tyrosine oxidation by myeloperoxidase can only be speculated upon. However, there is a great deal of similarity between the mechanism of resveratrol and tyrosine oxidation.

The phenoxy-radical species of resveratrol has been shown to be biologically active. Resveratrol has been shown to be an effective inhibitor of the dioxygenase activity of 5-lipoxygenase (Pinto et al., 1999). However, the oxidised form was found to be an equally effective inhibitor of this enzyme. The effect of resveratrol on peroxidase enzymes requires mechanistic studies in order to determine any possible contribution to its cardioprotective effects. The nature and properties of the oxidised form of resveratrol should also be characterised. This would be particularly relevant when resveratrol is added to cell culture systems that are used to study oxidative stress. The fate of a polyphenolic compound in these systems needs to be determined to avoid artificial results. This has been demonstrated by Long et al., (2000) who showed that a polyphenol found in green tea, EGCG, is oxidised in all commonly used cell culture media. Resveratrol is not oxidised in this fashion, but its fate in cell culture systems should be determined.

THE ANTIOXIDANT PROPERTIES OF RESVERATROL

The oxidative burden is thought to play a significant role in the pathogenesis of atherosclerosis and since the discovery that oxidised LDL is highly atherogenic, much attention has been paid to an antioxidative therapeutic strategy. To date, antioxidant therapy has been inconsistent e.g. with the use of vitamin E, where some trials showed no positive results, while others have been very successful (as discussed in Chapter 1.2). The reasons for this are not completely clear. However, one possibility is that antioxidant properties of the compounds have not been correctly considered. To describe a compound as an antioxidant does not convey any real information, since there are many different antioxidant mechanisms. Any given compound can be shown to have antioxidant properties if the correct experimental conditions are used. It is also important to consider the possibility that a compound can be an antioxidant in one system and acts as a pro-oxidant in another (Halliwell 1995). This has been shown for vitamin E, which can both inhibit and increase lipid peroxidation (Iwatsuki et al., 1995). Pro-oxidant effects of vitamin C have also been reported (Paolini et al., 1999). In some systems resveratrol has been shown to have pro-

oxidant properties. However, this has been discounted since the reaction conditions were found not to have physiological relevance (Burkitt and Duncan 2000).

Therefore, it is important not only to characterise the antioxidant properties of resveratrol, but determine if these will have any relevance *in vivo*. Such properties should also be determined using a biologically relevant source of ROS. The monocyte respiratory burst is thought to be a major factor in the development of atherosclerosis. Resveratrol was found to be a potent inhibitor of this response. However, it was difficult to determine the precise mechanism as to how this effect was achieved. Given the nature of the oxidants produced in this system, which mainly consists of superoxide, hydrogen peroxide and hypochlorous acid, how effective is resveratrol at scavenging these oxidants? Resveratrol has been shown to be a relatively weak inhibitor of superoxide (Wright et al., 2001) and has no direct effect on hydrogen peroxide (see chapter 3.10). The direct effect of resveratrol on hypochlorous acid has not been determined and the release of this oxidant from dU937 cells has not been confirmed.

There are many non-antioxidant mechanisms by which a compound can reduce oxidative stress e.g. reduced ROS production through the modulation of signalling pathways or alterations in the cellular antioxidant defence system. Compared to vitamin E, resveratrol is likely to be a poor antioxidant and moreover, the *in vivo* levels of vitamin E are far greater than could be realistically achieved with resveratrol. Therefore, which of the other biological activities of resveratrol can reduce the oxidative burden associated with atherosclerosis?

RESVERATROL AS A KINASE INHIBITOR

The primary function of protein kinase signalling pathways is to mediate the cellular response to environmental stimuli. They are also the major mediators of inappropriate and toxic stimuli, which induce cell death and inflammatory processes. Therefore, inhibition of these kinases has great therapeutic potential for a variety of pathologies (Reith, 2001). A number of natural products have been used as kinase inhibitors for many years (e.g. staurosporine) that act through competitive inhibition with ATP (Reith, 2001). The ATP binding site demonstrates considerable conservation and these early inhibitors were then found to inhibit a variety of kinases. Despite this it has been possible to develop inhibitors with a high degree of specificity, Moreover, some kinase inhibitors e.g. U0126 have been developed to act in a manner that is independent of ATP or protein kinase substrate (Duncia et al., 1998). Given the role kinases play in the inflammatory process they are an excellent

target for a chemopreventive agent, such as resveratrol. Protein kinases are the primary mediators that control the effector functions of all leukocyte cell types. This not only includes activation of NADPH oxidase, but the responsiveness of these cells through the modulation of the priming process. Therefore, one theory is that dietary resveratrol enters the blood stream (assuming that it is not completely metabolised) where it would come into contact with blood leukocytes, the majority of which would be neutrophils. Monocytes are crucial to the pathological process of atherosclerosis. However the question is can sufficient amounts of resveratrol reach these cells in order to modulate their activity? These cells are long lived and it could be possible that the levels of resveratrol can accumulate in these cells or in atheromatous plaques. This can only be speculated upon at present, however it is estimated that resveratrol is unlikely to exceed concentrations higher than 1 μM. There is also the possibility that resveratrol has pharmacologically active metabolites, which to date has not been thoroughly investigated.

In this study it was shown that resveratrol could inhibit the cell signalling pathways that were dependent on PI3K and PKC. Resveratrol was found to inhibit fMLP-induced Akt phosphorylation at 10 μM or above and PMA-induced ERK phosphorylation (not dependent on PI3K) at higher doses (25 μM). fMLP-induced ERK phosphorylation was not found to be dependent on PKC and was only slightly inhibited by LY294002 (a specific PI3K inhibitor). Resveratrol was found to have no effect on fMLP-induced ERK phosphorylation. The effect of resveratrol on PKC signalling pathways has been well studied and as previously mentioned, acts through an ATP-independent mechanism. Resveratrol inhibited PI3K dependent signalling pathways with greater efficacy, but the mechanism is not clear. In kinase screen resveratrol was found to inhibit PI3K by 47% at 10 μM (carried out by Professor P Cohen, University of Dundee). The majority of the other kinases tested were not greatly affected by resveratrol (with the exception of phosphorylase kinase). The method used (a TLC technique) was similar to that used in this study, only that a recombinant enzyme was used. The mechanism by which resveratrol achieves inhibition in these assays is most likely due to competition with ATP. The technique used to measure PI3K activity consists of an immunoprecipiated enzyme, a lipid substrate mixture (consisting of phosphatidylinositol and phosphatidylserine) and a magnesium-ATP complex. Phosphatidylserine is usually included in these assays, but is not required for the enzyme activity of PI3K when phosphatidylinositol is used as a substrate. Phosphatidylserine is only required if poly-phosphoinositides are used as substrates, e.g. if PtIns(4,5)P_2 is used as the

substrate. Therefore, the only variable in these experiments is the amount of substrate used and it is likely that resveratrol competes with ATP.

It is difficult to determine if the effect of resveratrol on fMLP-induced Akt phosphorylation is due to inhibition of PI3K by this mechanism. fMLP-induced Akt phosphorylation was found to be exclusively controlled by PI3K and resveratrol was found to have no effect on PDK (an upstream regulator of Akt). It is still possible that resveratrol can inhibit PI3K *in vivo* through inhibition of the ATP binding site. It has been suggested that Akt phosphorylation is not a quantitative measurement of PI3K activity (Stephens et al., 1998). The lipid products of PI3K are able to activate a wide variety of kinases. Upon activation of PI3K the cellular levels of PtIns(3,4,5)P_3 increase greatly. In order to control the subsequent activation of a variety of kinases, the binding sites for these lipids, the PH-domains, have variable affinities. The PH-domain of Akt that binds PtdIns(3,4)P_2 has less affinity than other kinases, such as PDK (Stephens et al., 1998). Therefore, it is possible that small changes in PI3K activity would have a great effect on Akt phosphorylation. There is also the possibility that resveratrol can inhibit PI3K in an ATP-independent fashion, such as competing with lipids for various binding domains involved in the activation of PI3K. The Akt inhibitor used in this study (1L-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate) binds to the PH domain of Akt and inhibits its translocation and subsequent phosphorylation. Resveratrol was found to have more inhibitory activity on fMLP-induced Akt phosphorylation than that induced with ATP or insulin, which suggests that it could be affecting some other kinase involved in the activation of Akt that was not investigated in this study e.g. tyrosine kinases such as Src. *In situ* methods of measuring PI3K activity would be highly beneficial to determine the effect of resveratrol on poly-phosphoinositide production.

THE USE OF PHORBOL ESTERS AS PHARMACOLOGICAL TOOLS

PMA has been widely used as an activator of PKC for many years. However, most investigators use it without considering its non-PKC mediated effects. There are a number of non-kinase receptors for PMA. The ability of PMA to induce ROS production in phagocytes depends on its ability to induce the activation of PKC, but also activation of the small-GTP binding protein Rac. The activation of Rac is poorly understood, but it might represent a non-PKC mediated effect of PMA. Phorbol esters and DAG have high affinity non-PKC receptors called chimaerins e.g. protein kinase D, Ras guanyl nucleotide-releasing protein

(RasGRP), Munc13s and diacylglycerol kinase γ (DAG kinase γ). PMA mimics the effect of DAG, but it remains in the cell for many hours, since it is not metabolised (whereas DAG is removed by DAG lipases). Protein kinase D has been found to be inhibited by resveratrol, albeit at high doses. Interestingly resveratrol inhibited *in vitro* kinase activity more effectively than *in vivo* activity (Haworth and Avkiran, 2001).

It was originally thought that all proteins that contained C1 domains would be activated by PMA and DAG. However, in some cases there is no concrete evidence and the situation is further complicated by the presence of C1 domains that are not responsive to PMA/DAG. The activation of the MAPK pathway by PMA has been well studied in numerous cell types and is largely attributed to a PKC-independent mechanism. In Chapter 6.8 it was shown that the PKC inhibitor, GF109203X, was able to inhibit PMA-induced ERK phosphorylation. This inhibitor has been used in this setting by several investigators who reached similar conclusions. Studies using RasGRP mutants have suggested that the activation of the MAPK pathway by PMA is not dependent on PKC, whereas most of the evidence against this hypothesis is reliant on PKC inhibitors. Although these inhibitors are often criticised for their inherent non-specificity, they are still of some use as pharmacological tools and potential anti-cancer drugs. It was also shown in Chapter 6.5 that fMLP-induced ERK phosphorylation was less affected by GF109203X and that Akt phosphorylation was completely unaffected.

In Chapter 4 it was shown that PMA induced a rapid form of cellular necrosis that was difficult to characterise, since the only conclusive modulator of this response was resveratrol. Initially it was thought that this response might be due to the large oxidative insult that is associated with the chronic activation of NADPH oxidase. However, no increases in lipid peroxidation or changes in the levels of M_1G DNA adducts. Moreover, classical antioxidants did not inhibit this response nor did inhibitors of PKC or MEK. This suggested that the necrosis was not correlated with the activity of NADPH oxidase or PKC. However, the only means used to inhibit PKC activity were the inhibitors, PKC412 and UCN01, which have been shown to inhibit many other kinases.

It is unclear as to the mechanism by which PMA induces cell death in dU937 monocytes or how resveratrol can inhibit this effect. Given that PMA is a DAG analogue it might be that PMA is opening receptor-activated calcium channels, which have been shown to be activated by DAG e.g. short transient receptor potential channel (STRPC) (Li and Westwick

et al., 2002). Calcium influx is a common feature during leukocyte activation and inhibitors of calcium influx are postulated to inhibit agonist-induced inflammatory responses. The long cellular life span of PMA might lead to some form of chronic activation and subsequent cell death. After many years of continuous use, the classical PKC inhibitors and activators should be replaced with more conclusive methods of modulating PKC cell signalling e.g. RNAi.

Inhibition of COX2 is an excellent chemopreventive strategy. Resveratrol has been found to inhibit PMA-induced COX2 expression (Subbaramaiah et al., 1998). The problem with using a non-physiological stimulus is that the pathway being activated might not be involved in the physiological setting. The activation of NADPH oxidase by PMA is thought to involve multiple PKC isoforms, some of which will not be activated by a physiological stimulus. This was highlighted in Chapter 6.10, where it was shown that resveratrol inhibited PMA-induced ERK phosphorylation, but not that induced by fMLP. Subbaramaiah et al., (1998) showed that resveratrol inhibited PM- induced AP-1 activity, which was due to its ability to inhibit PKC activity. In dU937 cells, fMLP-induced ERK phosphorylation was found to be largely independent of PKC, therefore it could be questioned if resveratrol would inhibit fMLP-induced AP-1 activity.

A MOLECULAR EXPLANATION OF THE FRENCH PARADOX.

An important question that is still to be answered is which of the reported properties of resveratrol is most likely to convey its cardioprotective effect. A recent study by Rosencrantz et al., (2001) suggested that the molecular explanation of the French Paradox is due to the ability of wine polyphenols to inhibit PDGF receptor signalling and subsequent smooth muscle hypertrophy. Given the multifactorial nature of atherosclerosis, several different molecular explanations can be postulated. Using the results obtained from this study a hypothesis can be generated that focuses on the role of the macrophage and the potential for resveratrol to modulate its activation.

In order to make a case for the dietary chemopreventive activity of a given compound suitable concentrations of the compound have to be achieved in vivo. The majority of research carried out with resveratrol has used concentrations between 1-100 μM. Although the pharmacokinetics of resveratrol have not been well studied, the amount of resveratrol obtained from a dietary source would unlikely to be greater than 1 μM. There are very few studies that have shown that resveratrol has biological activity at concentrations lower than 1

μM *in vitro*. If high doses of a compound are required to achieve an effect in cell based assays, it is unlikely to have greater potency *in vivo*. Sub-micromolar levels of resveratrol have been shown to inhibit the activity of lipoxygenase and ribonuclease reductase, with the latter being dependent on the scavenging of the tyrosyl radical (Fontecave et al., 1998).

Resveratrol was found to inhibit luminol or isoluminol-CL at doses lower than 1 μM irrespective of the stimulus used. This was thought to be due to the ability of resveratrol to act as a co-reducing substrate and compete with luminol or isoluminol for HRP oxidation. Resveratrol has been shown to inhibit HRP-dependent oxidation of LDL (Pietraforte et al., 2001) and as described above, myeloperoxide-mediated lipid peroxidation (through the formation of tyrosyl radicals) could also be potentially inhibited by resveratrol. This could be an important cardioprotective mechanism, since myeloperoxidase mediated lipid peroxidation is thought to be a major physiological pathway. The ability to modulate the activity of peroxidase enzymes requires further investigation in order to determine if this contributes towards its cardioprotective properties.

The ability of resveratrol to inhibit various kinases was determined and it was found that it was not a highly potent kinase inhibitor. However, resveratrol was still found to inhibit the phosphorylation of Akt at doses of 10 μM or above. It is still unclear which mechanism achieves this, e.g. binding to an ATP binding site or binding to a lipid binding domain and it is difficult to determine if the low doses of dietary resveratrol will modulate these pathways. The dU937 cell model used in this study is activated by supra-physiological doses of agonist e.g. the levels of fMLP found *in vivo* would range from pM to low nM levels and activation would be induced by multiple agonists. However, modulation of monocyte recruitment to the atherosclerotic lesion is of considerable significance as a chemopreventive mechanism, since this process is an early event in the disease process (Libby, 2002). If resveratrol was used as a food supplement, where higher doses could be achieved, the potential to modulate the PI3K pathway and effectively antagonise chemokine induced activation is much greater. Moreover, the PI3Kγ subunit has been recently implicated in platelet aggregation (Hirsch et al., 2001), which might explain the inhibitory effect of resveratrol on this process. Figure 7.1 shows a different hypothesis to that described by Rosencrantz et al., (2001). Although a different target cell type is shown, the molecular mechanism is similar (inhibition of PI3K), as is the overall outcome.

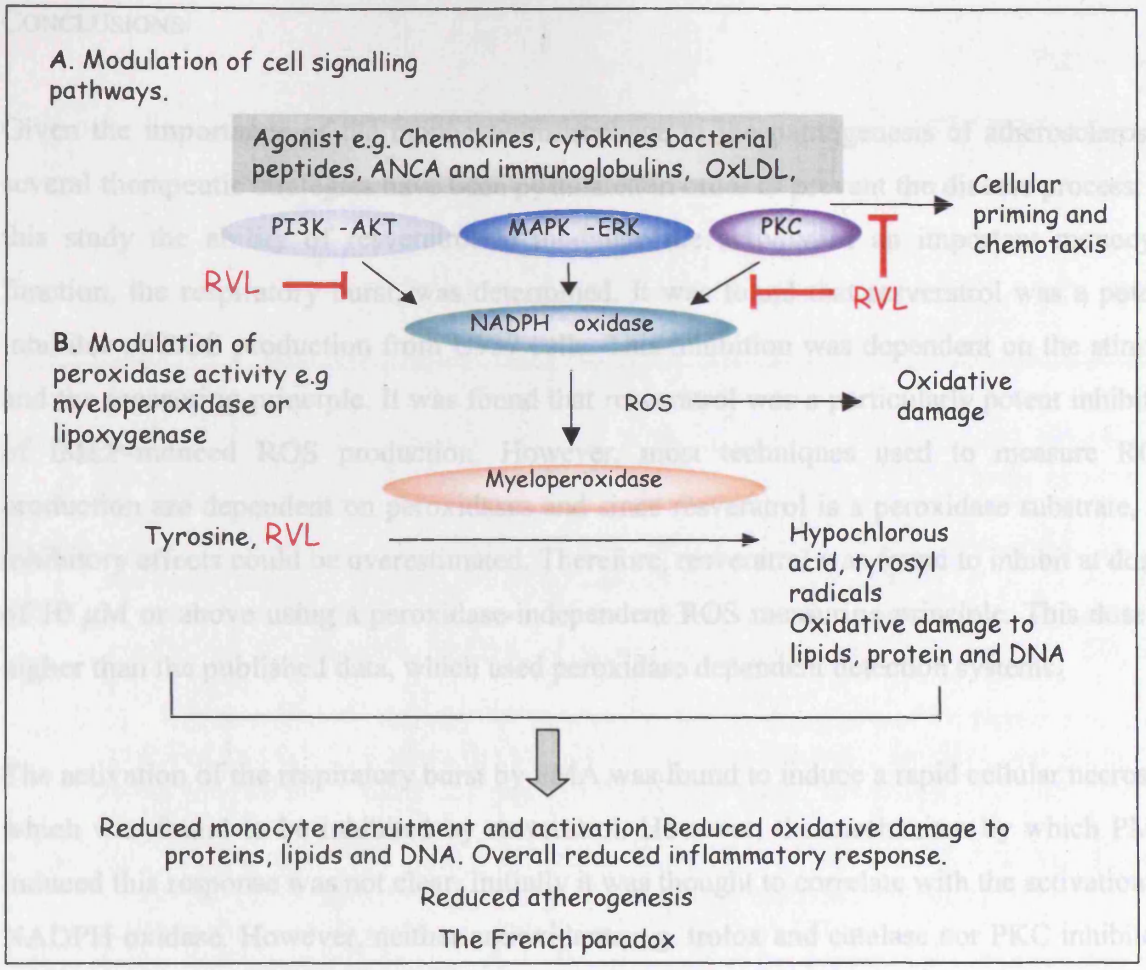

Figure 7.1 The cardioprotective properties of resveratrol-a hypothetical molecular explanation of the French Paradox. A) Inhibition of monocyte activation through kinase inhibition: Monocyte activation is an early event in atherogenesis. These cells are activated by a dysfunctional endothelium, through the release of cytokines and in particular chemoattractant cytokines (chemokines) e.g. fractalkine and monocyte chemoattractant protein 1. Other activators might include anti-neutrophilic cytosolic antibodies (ANCA) or bacterial peptides. These agonists activate common cell signalling pathways such as PI3K, PKC and MAPK, which mediate cell recruitment and activation e.g. the activation of NADPH oxidase. Attenuation of these processes would potentially reduce the recruitment of these cells to the atherosclerotic lesion and reduce the subsequent oxidative burden. B) The modulation of peroxidase activity: resveratrol has been shown to modulate the activity of lipoxygenase and cyclooxygenase enzymes. Resveratrol could act as a co-substrate for myeloperoxidase and thus support its peroxidase activity, but compete with endogenous co-substrates, such as tyrosine that have been shown to induce lipid peroxidation. The formation of tyrosyl radical is thought to be a major contributor towards lipid peroxidation that contributes towards the atherogenic process. The overall outcome is that inflammatory processes that mediate atherogenesis are attenuated, which could be a contributing factor towards the French Paradox.

CONCLUSIONS

Given the importance of the monocyte/macrophage to the pathogenesis of atherosclerosis, several therapeutic strategies have been postulated in order to prevent the disease process. In this study the ability of resveratrol to modulate the activity of an important monocyte function, the respiratory burst, was determined. It was found that resveratrol was a potent inhibitor of ROS production from U937 cells. This inhibition was dependent on the stimuli and the measuring principle. It was found that resveratrol was a particularly potent inhibitor of fMLP-induced ROS production. However, most techniques used to measure ROS production are dependent on peroxidases and since resveratrol is a peroxidase substrate, its inhibitory effects could be overestimated. Therefore, resveratrol was found to inhibit at doses of 10 μM or above using a peroxidase-independent ROS measuring principle. This dose is higher than the published data, which used peroxidase dependent detection systems.

The activation of the respiratory burst by PMA was found to induce a rapid cellular necrosis, which was found to be inhibited by resveratrol. However, the mechanism by which PMA induced this response was not clear. Initially it was thought to correlate with the activation of NADPH oxidase. However, neither antioxidants e.g. trolox and catalase nor PKC inhibitors were able to inhibit this response. This suggested that it could be due to a non-PKC mediated effect and the inhibitory action of resveratrol could be due to its ability to bind to the PMA binding site (the C1-domain that is found in many proteins).

Resveratrol was found to inhibit the PI3K signalling pathway. It was found to be an inhibitor of fMLP-induced PI3K activity and was also found to inhibit the phosphorylation of Akt (a downstream target of PI3K). The PI3K-Akt pathway was found to be a major mediator of fMLP-induced ROS production, which was determined using inhibitors of both enzymes. Inhibition of these cell signalling pathways correlated with the effect of resveratrol on fMLP-induced ROS production. This suggested that it could directly antagonise the release of ROS from U937 cells. Given the importance of these pathways in a number of disease states, the inhibitory effect of resveratrol on the PI3K cell signalling pathway might represent an important anti-inflammatory mechanism.

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