Regulation of the Inducible L-arginine-Nitric Oxide Pathway by Oxidative Stress and Statins

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PhD

2009

Regulation of the Inducible L-arginine-Nitric Oxide Pathway by Oxidative Stress and Statins

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Submitted to the University of Hertfordshire in partial fulfilment of the requirements of the degree of PhD

June 2009

Abstract

Oxidative stress (OS) plays a critical role in the pathogenesis of atherosclerosis potentially through interaction with nitric oxide (NO) generated by the inducible nitric oxide synthase (iNOS) pathway. Although considerable literature supports a pro-atherogenic role for iNOS-induced NO, recent evidence suggest an anti-atherogenic property for this enzyme where iNOS-induced NO attenuates atherosclerotic lesions after immune injury, enhancing endothelial integrity, survival, protecting against OS-induced apoptosis and necrosis. We therefore hypothesize that iNOS may have a cardio-protective role in the atherosclerotic vessel and that under conditions of OS, expression and function of this enzyme may be impaired, thus contributing to the deleterious consequences of OS. Experiments have therefore been conducted to establish whether pro-oxidants regulate iNOS expression/function in rat cultured aortic smooth muscle cells (RASMCs). These cells were induced for 24 hours with LPS and IFN-γ to mimic inflammatory conditions. Oxidative stress inducers may modulate iNOS-induced NO production through alteration of the expression and/or function of the inducible L-arginine-NO pathway. We examined the effects of hydrogen peroxide (H_2O_2), antimycin A and diethyl maleate (DEM) on this pathway in vascular smooth muscle cells. H_2O_2 had little effect on NO production or L-arginine transport while antimycin A and DEM independently caused a concentration dependent inhibition of both processes. Only DEM induced heme-oxygenase-1 (HO-1) expression, monitored by western blotting as a marker of OS. The effects of statins on NO synthesis and L-arginine transport in the presence and absence of OS were also investigated. The benefits of statins therapy in cardiovascular medicine are ascribed in part to their lipid-lowering effect by inhibiting

3-hydroxy-3-methoxyglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme for cholesterol synthesis. However, statins may possess anti-inflammatory properties and are able to improve endothelial function, stabilize atherosclerotic plaque, and inhibit platelet aggregation, vascular smooth muscle cells proliferation and vessel wall inflammation. These effects may be exerted through novel actions of statins that include interaction with specific signalling pathways in cells which may be associated with the induction of iNOS and/or cationic amino acid transporters (CATs). Thus, we have extended our investigations to include an examination of the effects of statins on both iNOS and CAT function and expression under control conditions and following exposure of cells to OS. Atorvastatin caused a bell shaped response on NO production and iNOS expression and also enhanced L-arginine transport but in a non-concentration dependent manner. Simvastatin only affected NO synthesis without altering transporter activity. Pravastatin was without effect on either system. Further studies demonstrated that that atorvastatin was able to reverse the effects of antimycin A and DEM but only on NO production.

These findings confirm that the inducible L-arginine-NO pathway can be down-regulated by pro-oxidants. This mechanism may therefore contribute to the deleterious effects observed in disease states associated with OS. Moreover, statins (in particular atorvastatin) appear to be effective in reversing the inhibition of NO production caused by inducers of OS. This, together with the fact that atorvastatin and simvastatin can potentiate iNOS-induced NO production and indeed L-arginine transport (with atorvastatin), highlights a potential novel mechanism through which the cardio-protective actions of these compounds could be mediated.

Acknowledgements

I would here like to express my thanks to the people who have been very helpful to me during the time it took me to complete my PhD.

It's with great honour and joy that I thank my supervisors, I have been extremely fortunate with them. This thesis would not have been possible without the guidance, support, enthusiasm and patience of my principal supervisor Professor Anwar R. Baydoun, not to mention his advice and unsurpassed knowledge. The good advice, support and friendship of my second supervisor, Dr. Shori Thakur, has been invaluable on both an academic and personal levels, for which I am extremely grateful. I would also like to thank the valuable insight of my supervisor, Professor John Walker. Above all, I couldn't wish better to develop as a scientist and as a person.

To my parents and brother that gave me their unequivocal support throughout, even though we miss each other immensely, for which my mere expression of thanks likewise does not suffice. To my dear husband Luís, that supported me unconditionally at all times on my dream of pursuing a science career. His personal support and great patience made my path graceful and almost effortless.

I thank my all my friends for their support and encouragement throughout. I thank all my colleagues in the laboratory Marzieh Zamani, Peter Humphrey, Edmund Garr, Yogesh Kamra and James Crutchley.

I thank you all and will always keep you in my heart and in my life.

Abbreviations

Akt – Protein Kinase B

Ang-II – angiotensin II

AP-1 – Activator protein-1

ARE – Antioxidant responsive element

ATP – Adenosine 5'-triphosphate

BCA – Bicinchoninic Acid

BCRP - breast cancer resistance protein

BH_4 – Tetrahydrobiopterin

BSA – Bovine Serum Albumin

CAM – Cellular Adhesion Molecule

CAT – Cationic Amino acid Transporter

Cdc42 - cell division cycle 42 protein

CD68 – glycoprotein which binds to low density lipoprotein

cDNA – Complementary Deoxyribonucleic Acid

cGMP – cyclic adenosine monophosphate

CNC – cap-n-collar

CO – Carbon Monoxide

CRP – C-reactive protein

CYP3A4 – Cytochrome P450 3A4

DDW – Double Distilled Water

DEM – Diethyl maleate

Dil-Ac-LDL – Dil labelled acetylated low density lipoprotein

DMEM – Dulbecco's Modified Eagle's Medium

DNA – Deoxyribonucleic acid

DPM – Disintegrations Per Minute

EDRF – endothelium-derived relaxation factor

ECL – Enhanced chemiluminescence

ECM – Extra Cellular Matrix

EDTA – Ethylene Diamine Tetra Acetic Acid

EGF – epidermal growth factor

eNOS – endothelial Nitric Oxide Synthase

ERK – Extracellular signal Regulated Kinases

ESRD – End-Stage Renal Disease

FAD – Flavin Adenin Nucleotide

FBS – Foetal Bovine Serum

FGF – fibroblast growth factor

FITC – Fluorescein isothiocyanate

FMN – Flavin Mononucleotide

GO-1 – heterotrimeric G protein

GTP – Guanosine 5'-Triphosphate

gp91phox – transmembranar protein component of NAD(P)H oxidase

GPx – catalases and glutathione peroxidases family

GSH – Glutathione

H_2O_2 – hydrogen peroxide

HO^- – hydroxyl radical

HASMC – Human Aortic Smooth Muscle Cells

HDL – High Density Lipoprotein

HEPES – 4-(2-hydroxyethy)-1-peperazine ethanesulphonic acid

HMG-CoA – Hydroxymethyl Glutaryl - coenzyme A reductase

HO-1 – Heme Oxygenase-1

HO-2 – Heme Oxygenase-2

HO-3 – Heme Oxygenase-3

HRP – Horse Readish Peroxidase

HUVEC – Human Umbilical Vein Endothelial Cells

ICAM – Intercellular Adhesion Molecule-1

IFN-γ – Interferon-gamma

IgG – Immunoglobulin

IK-BK – inhibitory protein-κB

IKK-1 and 2 – Cytokine-activated IKappa B Kinases

IL-1 – Interleukin-1

IL-18 – Interleukin-18

IL-1β – Interleukin-1beta

iNOS – inducible Nitric Oxide Synthase

IR – Ischemia Reperfusion

IRP1 and 2/IRE – posttranscriptional cytoplasmic Iron Regulatory Proteins

JAK2 – Janus Kinase-2

JNK – c-Jun N-terminal Kinases

kDA – Kilodaltons

Km – Affinity constant (half maximal saturation constant)

LDL – Low Density Protein

LOX-1 – Lectin-like receptor-1

LPS – Lipopolysaccharide

mA – milliamp

MAPK – Mitogen Activated Protein Kinases

MCP-1 – monocyte chemotactic protein-1

MIF – macrophage inhibitory factor

MMP-2 – matrix metalloproteinase 2

MMP-9 – matrix metalloproteinase 9

MMPs – matrix metalloproteinases

MRE – metal responsive element

mRNA – messenger Ribonucleic Acid

MRP2 – multidrug resistance associated protein

MTF-1 – metal response element-binding transcription factor 1

MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

NF-κB – Nuclear Factor-κappa B

nNOS – neuronal Nitric Oxide Synthase

NO – Nitric Oxide

NO_2^- – nitrite

NOS – Nitric Oxide Synthase

Nox – NADPH oxidase subunit complex

Nrf2 – nuclear factor-erythroid 2-related factor 2

NTCP – sodium dependent taurocholate co-transporting polypeptide

OATP – organic anion transporting polypeptide

O_2 – Dioxygen

O_2^- – Superoxide anion

$ONOO^-$ – Peroxynitrite

OS – Oxidative Stress

oxLDL – oxidised Low Density Lipoprotein

PAECs – Porcine Aortic Endothelial Cells

PAK – P21-Activated Kinase

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PDGF – platelet-derived growth factor

PI3K – Phosphoinositide-3 Kinase

PKB – Protein Kinase B

PKC – Protein Kinase C

PMSF - phenyl methyl sulfonyl fluoride

PS – Penicillin/Streptomycin

PTPs – protein tyrosine phosphates

PVDF – Polyvinyldene difluoride

p38 MAPK – p38 Mitogen Activated Protein Kinase

RASMC – Rat Aortic Smooth Muscle Cells

rac1 – Ras-related C3 botulinum toxin substrate 1

Ras – family of genes encoding small GTPases

rBAT – Broad Scope Amino acid Transporter

Rho – Ras homolog gene family

RhoA – Ras homolog gene family, member A

RNA – Ribonucleic Acid

RNS – Reactive Nitrogen Species

ROS – Reactive Oxygen Species

S.E.M. – Standard Error Mean

SDS – Sodium Dodecyl Sulphate

SDS-PAGE – Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis

sGC – soluble Guanylyl Cyclase

SHR – spontaneously hypertensive rats

SMC – Smooth Muscle Cells

SOD – Superoxide Dismutase

Sp. – species

TEMED – NNN'N'-Tetramethylethylenediamine

TF – tissue factor

TGF-β – transforming growth factor β

TNF-α – Tumor Necrosis Factor-alpha

TRIS Base – Tris(hydroxymethyl)aminoethane

U.V. – Ultra Violet

VCAM – Vascular Adhesion Molecule

VCAM-1 – Vascular Adhesion Molecule-1

VEGFR-2 – vascular endothelial growth factor-2

VSMC – Vascular Smooth Muscle Cells

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1 . Introduction

1.1 . Oxidative stress

Oxidative stress (OS) occurs when the generation of reactive oxygen species (ROS) in a biological system exceeds the ability of the system to neutralize and eliminate these pro-oxidants. The imbalance can result from overabundance of ROS due to an environmental or behavioural stressor, or a lack of antioxidant capacity caused by disturbance in production and/or distribution of the antioxidant agents in the system (Sies 1991).

Reactive oxygen species may be generated in biological systems through various sources including certain drugs and various environmental stressors such as tobacco smoke (Noakes *et al.* 2007; Yeh *et al.* 2007), pollution (Diouf *et al.* 2006; Peretz *et al.* 2007), radiation and ultra violet (U.V.) light (Voss *et al.* 2007), pesticides (Lopez *et al.* 2007) and ozone (Calderon Guzman *et al.* 2006; Morrison *et al.* 2006; Chuang *et al.* 2007). Within cells, free radicals may be produced by different processes that may include mitochondria (Risom *et al.* 2005; Ago and Sadoshima 2006; Coughlan *et al.* 2007; Seddon *et al.* 2007; Zhang and Shah 2007), phagocytes (Sheeran and Pepe 2006), peroxisomes, xanthine oxidase (Ago and Sadoshima 2006; Seddon *et al.* 2007; Zhang and Shah 2007), myeloperoxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, uncoupled nitric oxide synthase (NOS) (Madamanchi *et al.* 2006; Sheeran and Pepe 2006; Seddon *et al.* 2007; Zhang and Shah 2007), auto-oxidation of glucose (Tan *et al.* 2007) or reactions involving iron and other transition metals (Risom *et al.* 2005).

Free radicals have a highly reactive molecular structure due to the presence of unpaired electrons in one or more of the atomic or molecular orbitals (Valko *et al.* 2007). This makes these oxygen species highly reactive in biological systems and

often cytotoxic. Some of these cytotoxic actions may however be physiologically relevant in terms of host defence mechanisms (Valko *et al.* 2007). During the inflammatory response, ROS and reactive nitrogen species (RNS) modulate phagocytosis, secretion, gene expression and apoptosis (Fialkow *et al.* 2007). Besides their potential toxic effects, ROS also play an important role as signalling molecules (Voeikov 2006; D'Autreaux and Toledano 2007; Sugino 2007). This is perhaps aided by the fact that ROS molecules are small, diffusible and ubiquitous in the body (Fialkow *et al.* 2007). As signalling molecules, ROS modulate contractile activity in skeletal muscle (Ji 2007; Niess and Simon 2007), regulate the redox sensitive transcription pathway of gene expression (Fialkow *et al.* 2007; Niess and Simon 2007), play a role in mitosis by redox sensitive processes (Chiarugi and Fiaschi 2007), are necessary for memory formation and synaptic plasticity (Kishida and Klann 2007), regulate cardiomyogenesis and vasculogenesis (Sauer and Wartenberg 2005), regulate apoptosis (Bickers and Athar 2006; Shen and Liu 2006), mediate signalling involved in vascular endothelial growth factor-2 (VEGFR-2) linked to endothelial cells migration and proliferation (Ushio-Fukai 2007), oxidise protein kinase C (PKC) and protein tyrosine phosphates (PTPs) and regulate mitogen activated protein kinase (MAPK) and P21-activated kinase (PAK) (Wu 2006; Fialkow *et al.* 2007; Zhang and Gutterman 2007).

1.2 . Oxidative stress and endogenous antioxidant systems

To control the redox state, cells possess systems that comprise of intracellular antioxidants such as ascorbic acid, glutathione (GSH), α-tocopherol, and several

ROS scavenging enzymes including superoxide dismutase (SOD), catalase, glutathione peroxidase and heme-oxygenase.

Mammals possess three types of SODs: Cu,Zn-SOD, Mn-SOD and an extracellular, (EC)-SOD. Their action is to convert superoxide ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) (Leopold and Loscalzo 2005; Sugino 2007). Cu,Zn-SOD is present in cytosol, lysosomes, peroxisomes, nucleus and mitochondrion while Mn-SOD is also present in mitochondria (Sugino 2007). Vascular smooth muscle cells (VSMCs) synthesise EC-SOD that binds extracellular matrix, and minimizes superoxide-dependent inactivation of endothelial derived nitric oxide (NO) (Leopold and Loscalzo 2005).

Catalase is composed of four monomers, each with one heme-active group. This enzyme acts by reducing H_2O_2 to water. Glutathione peroxidases on the other hand reduce hydrogen and lipid peroxides to water and alcohols, respectively. The glutathione enzymes have diverse functions: glutathione reductase reduces glutathione disulfide to reduced glutathione. In parallel, glutathione-S-transferase detoxifies oxidants by glutathiolation while the thiol-disulfide oxireductases and peroxiredoxins maintain redox state of protein thiol (Leopold and Loscalzo 2005). Catalases and glutathione peroxidases are part of the GPx family characterised by the unique amino acid selenocysteine within the active sites that use low molecular weight thiols such as glutathione (GSH) to reduce hydrogen and lipid peroxides to the correspondent alcohols. In the endothelium, the major superoxide metabolizing enzymes are Cu,Zn-SOD and Mn-SOD (Harrison 1997).

Heme-oxygenase is responsible for heme catabolism that is cytoprotective against oxidative injury and maintains tissue homeostasis (Maines 1988; Maines 1997; Dennery 2004; Maines and Gibbs 2005; Calabrese *et al.* 2006). There are

currently three isoforms referred to as: heme-oxygenase-1 (HO-1); heme-oxygenase-2 (HO-2) and heme-oxygenase-3 (HO-3). Heme-oxygenase-1 is a 32 kilodaltons (kDa) heat shock protein that is encoded by the *Hmox1* gene and is inducible but has low levels of expression under normal physiological conditions. In contrast, HO-2 and HO-3 are constitutive proteins with HO-2, a 36 kDa protein, being highly expressed in the brain (Motterlini *et al.* 2002; Dennery 2004). Expressed in the spleen, liver, thymus, prostate, heart, kidney, brain and testis, HO-3 is a 33 kDa protein lacking in catalytic activity and exhibits a 90 % homology with HO-2 (McCoubrey *et al.* 1997; Farombi and Surh 2006).

Induction of HO-1 can be initiated by several stress conditions including exposure to hyperthermia, U.V. irradiation, H_2O_2 and heavy metals. Moreover, HO-1 is also induced under inflammatory conditions and diseases such atherosclerosis. In the latter, expression of HO-1 may be coupled to iNOS-induced NO production (Kitamura *et al.* 1998) indicating that NO (*i.e.* iNOS-induced NO), acting through induction of HO-1, may protect against OS, thus preserving cellular homeostasis (Dennery 2004). Expression of HO-1 has been associated with an anti-inflammatory role and this is supported by the fact that mice lacking HO-1 develop a progressive inflammatory state while humans lacking the ability to express HO-1 may potentially die from a condition referred to as inflammatory syndrome (Otterbein *et al.* 2003). The anti-inflammatory and cytoprotective actions of HO-1 may be mediated through catabolism of heme, as shown in Figure 1.1, to carbon monoxide (CO), biliverdin (rapidly converted to bilirubin) and free iron (which leads to the induction of ferritin, an iron-binding protein). These metabolites can act as anti-inflammatory, anti-atherogenic and cytoprotective molecules. For instance, bilirubin has direct antioxidant effects while CO may exert tissue protective actions primarily through its

vasodilator and anti-platelet effects (Motterlini *et al.* 2002; Otterbein *et al.* 2003). Moreover, in rodent models of ischemia-reperfusion, allograft and xenograft survival, intimal hyperplasia following balloon injury or in chronic graft, the administration of CO, biliverdin, bilirubin or iron-binding compounds is reported to exert protective actions (Kitamura *et al.* 1998).

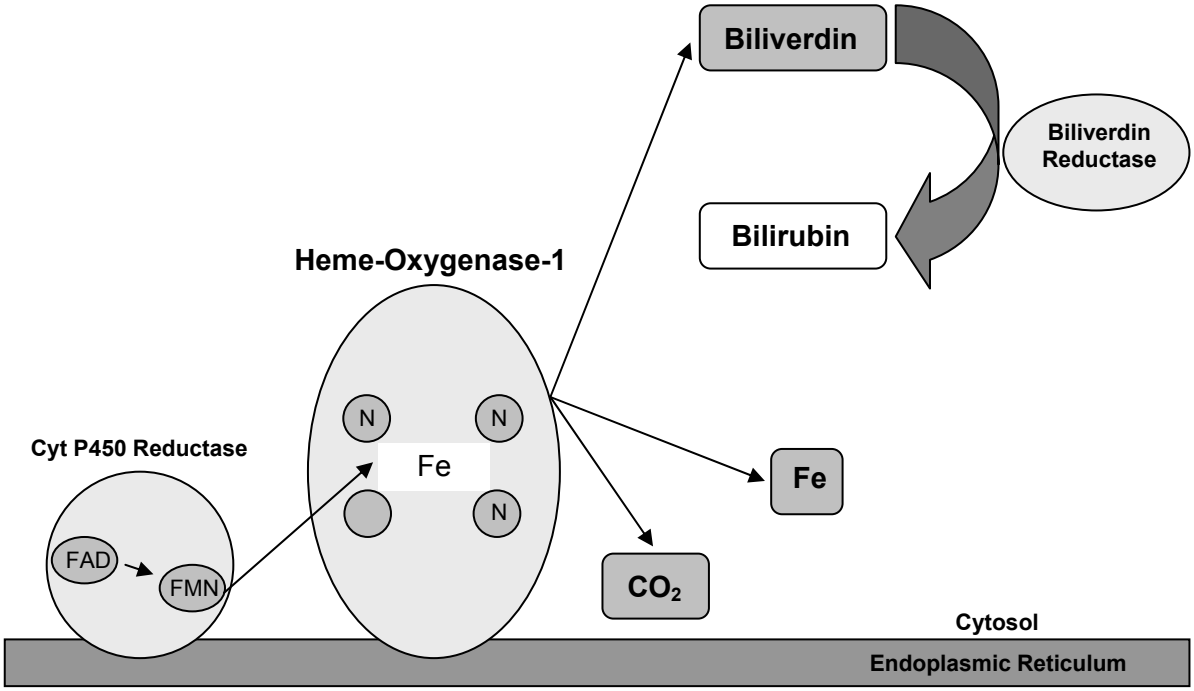


Figure 1.1. Catabolism of heme by heme-oxygenase-1 to carbon monoxide (CO), biliverdin and free iron.

Recent reports have suggested that there may be cross-talk between the NO synthase and CO systems (Kitamura *et al.* 1998; Otterbein *et al.* 2003). Both molecules have similar properties and cause smooth muscle cell relaxation (Dennery 2004). Like NO, HO-derived CO activates soluble guanylate cyclase (sGC) and elevates cyclic guanosine monophosphate (cGMP) production which serve to regulate both blood pressure and vascular contractility (Motterlini *et al.* 2002; Dennery 2004). It is worth noting however that CO has a much lower affinity for sGC than NO (Siow *et al.* 1999).

In addition to the above systems, several genes are also induced and regulated in the presence of oxidants which may activate factors such as metal response element-binding transcription factor 1 (MTF-1); the antioxidant response element (ARE); the MAF and the cap-n-collar (CNC-bZIP) transcription factors (Dalton *et al.* 1999). The activation of these elements contributes to the enhanced anti-oxidant response that may occur as a consequence of the oxidative stress. Moreover, the genes encoding for HO-1; γ-glutamylcysteine synthethase; thioredoxin reductase; glutathione-S-transferase and NADPH:quinone oxidoreductase may also be induced through the cis-acting transcriptional regulatory element ARE (Mann *et al.* 2007) resulting in the expression of their respective anti-oxidant proteins and these genes could potentially be targets for therapeutic interventions in the prevention of cardiovascular disease (Dalton *et al.* 1999; Mann *et al.* 2007; Siow *et al.* 2007).

1.3 . Oxidative stress and disease

Oxidative stress, linked to ROS and RNS, has been widely reported in several disease states including cancer (Valko *et al.* 2006), atherosclerosis (Lee and Hirani 2006), heart failure (Valko *et al.* 2006), hypertension, ischemia/reperfusion injury, diabetes mellitus, rheumatoid arthritis (Griffiths 2005), neurodegenerative diseases such as Alzheimer's (Adam-Vizi 2005; Moreira *et al.* 2007) and Parkinson's diseases (Adam-Vizi 2005), or even ageing (Davies 1995; Yu and Chung 2001). These disorders can be exacerbated, perhaps even initiated, by numerous environmental pro-oxidants and/or pro-oxidant drugs and foods (Davies 1995), including those highlighted above. In view of the focus of this thesis, the discussion will now concentrate on the role of OS in atherosclerosis.

1.3.1 . Atherosclerotic disease

Atherosclerosis is one of the most prevalent and major cardiovascular diseases, accounting for a high percentage of mortality rates in industrialised countries (Hansson and Libby 2006). Associated with blood vessels, atherosclerosis is characterized by the formation of atherosclerotic plaques in the vessel wall in response to chronic multifactorial injury. The development of this disease is however complex and may start as early as childhood and progress during adolescence and young adulthood (McMahan *et al.* 2006). Although asymptomatic, atherosclerosis was unveiled in a *post mortem* study by Tuzco in 17 % of children aged 13 to 19

years old. Moreover, in people aged over 50 the prevalence of coronary artery disease is about 85 % (Tuzcu *et al.* 2001).

Various stages of atherosclerosis show evidence of endothelial and smooth muscle dysfunction and metabolic abnormalities of the vessel wall which include: inflammation, OS and alterations of hormonal balance. Low-density lipoprotein (LDL) also accumulates in the vessel wall in the disease state and is modified through oxidation. Oxidised LDL (oxLDL) is subsequently accumulated in macrophages through scavenger receptors, resulting in the formation of foam cells (Linares *et al.* 2006). Macrophages, together with agents such as interferon-γ (IFN-γ) contribute to ROS formation (Schroecksnadel *et al.* 2006) which may then induce lipid peroxidation and oxidation of several substrates leading to vascular damage, signalling alterations and impairment of vascular tone (Chen *et al.* 2006). The precise mechanisms associated with the latter have been extensively studied but without clear consensus on the actual cellular events that lead to the observed changes in vascular tone. Part of the reason for this is that there may be multiple events which all culminate in the impairment of the vascular tone. One potential target is the disruption of NO synthesis and/or actions by ROS and/or other atherogenic agents. A multifactorial disease like atherosclerosis, may also be attributed to an increased anthropological pressure on the human genome by environmental stimuli (Lamon and Hajjar 2008).

1.3.2 . Formation of the atherosclerotic plaque

The advanced disease state is associated with the formation of a plaque which is composed of a cholesterol-rich lipid core, $CD4^+$T cells, macrophages and foam cells, covered by the injured endothelial cell layer (Ross 1993; Ross 1995; Pulido *et al.* 2004; Antohe 2006; Crouse 2006; Saam *et al.* 2007). Smooth muscle cells (SMCs) migrate to the intima, proliferate and produce abundant extracellular matrix during the early stages of the disease.

The progress of the atherosclerotic plaque starts with a combination of factors that induce endothelium imbalance, secretion of adhesion molecules, adhesion and infiltration of monocytes, release of more pro-inflammatory and adhesion molecules, SMCs proliferation, migration and finally the formation of an unstable plaque with a necrotic lipid core. Activated cells within the lesion, namely macrophages, release growth factors such as platelet-derived growth factor (PDGF); epidermal growth factor (EGF); fibroblast growth factor (FGF); transforming growth factor (TGF) which boost inflammation and SMCs proliferation (Lamon and Hajjar 2008). In addition, the proliferation of macrophages and their subsequent morphological alteration through accumulation of oxLDL generates foam cells (Schleicher and Friess 2007) which exist as fatty streaks and also release inflammatory cytokines such as TNF-α and interleukin-1β (IL-1β). The latter contribute to the subsequent inflammatory response (discussed below, Section 1.3.3), and are linked to proliferation of SMCs as well as their differentiation and subsequent apoptosis (Popa *et al.* 2007; Lamon and Hajjar 2008).

Smooth muscle cells recruited into the intima proliferate and dedifferentiate from the adult to the foetal phenotype becoming secretory cells, releasing growth factors and pro-inflammatory molecules (Bentzon *et al.* 2006; Doran *et al.* 2008). Smooth muscle cells also produce large amounts of collagen and extracellular matrix proteins (Doran *et al.* 2008), thereby facilitating the accumulation of sub-endothelial LDL to the negatively charged proteoglycans in the extracellular matrix (Schleicher and Friess 2007). Thus, the changes that SMCs undergo are not simply a consequence of the disease but in fact contribute to the pathogenesis of atherosclerosis. Moreover, the proliferation of SMCs together with fibres associated with the cells, cover the lipid necrotic core and contribute to plaque stability (Lamon and Hajjar 2008). Paradoxically, the lack of SMCs augments risk of thrombosis in patients with advanced plaque and there is a close relation between plaque instability caused by platelets and thrombus formation.

1.3.3 . Atherosclerosis as an inflammatory disease

The inflammatory aspects of atherosclerosis are associated with the release of PDGF, transforming growth factor β (TGF-β), macrophage inhibitory factor (MIF), cytokines and monocyte chemotactic protein-1 (MCP-1) (Doran *et al.* 2008). In the progression stages of the plaque, platelets become major players in releasing pro-inflammatory mediators and stimulating adhesion molecule expression in other cells (e.g. endothelium), modulating vascular inflammation, which may lead to vascular wall homeostatic imbalance (Langer and Gawaz 2008).

It is possible that inflammation together with hyperlipidemia act concomitantly in progressing atherosclerosis; and hyperlipidemia alone may not be as critical as initially thought. This suggestion is based on the fact that cardiovascular events associated with atherosclerosis may also occur in patients in whom plasma lipids concentrations are considered normal (Ross 1993; Lamon and Hajjar 2008). In the context of inflammation, the release of inflammatory cytokines such as TNF-α and IL-1β may, as already stated above, contribute in the subsequent inflammatory response that may cause further endothelial impairment.

A breach in endothelium homeostasis has been established as being the trigger for atherosclerosis. The homeostatic properties of the endothelium in healthy blood vessels provide a physical barrier to circulating blood constituents and also maintains the patency of the vessels (Lamon and Hajjar 2008). In response to endothelial damage, there seems to be an innate immune response (Lamon and Hajjar 2008) which is sustained by oxLDL, and this is believed to strongly amplify the release of cytokines causing the adherence and infiltration of leukocytes into the intima (Granger *et al.* 2004; Cook-Mills 2006).

In the inflammatory response, the endothelium is primarily defended by macrophages, which begin to digest oxLDL stimulating other cells of the immune system into action. In response, phagocytes migrate to the affected site and are activated to take up and destroy the modified LDL. Once activated, they also produce other cytokines that further activate other cells of the immune system. In a sense, the initial immune reaction leads to a cascade of other immune events. The inflammatory response is programmed to end with the phagocytic cells eliminating the foreign elements and this may subsequently result in apoptosis of these cells. Should the process fail, phagocytic cells which are unable to digest and thus

eliminate cholesterol may become irreparably damaged and thus undergo necrotic death. If the inflammatory stimulus is continuous, the helper T cells and the immune cells receive the “stay-alive” signal developing chronic inflammation within the lesion.

As a response to an inflammatory injury, the plasma levels of C-reactive protein (CRP) increase by up to a 1000 fold (Westhuyzen and Healy 2000). This protein is usually synthesised in hepatocytes at low rates and retained by the endoplasmic reticulum, but released into the blood stream following an inflammatory stimulus (Westhuyzen and Healy 2000). The build up of CRP in circulation is associated with, and used as a marker of, an increased risk of cardiovascular disease even though its mechanism of action is still not fully understood (Amezcua-Guerra *et al.* 2007; Pereira and Borba 2008). This protein exists in atherosclerotic plaque within foam cells (Vigushin *et al.* 1993; Westhuyzen and Healy 2000) and is believed to bind to damaged lipoproteins, microbial polysaccharides, phosphatidylcholine and also activates phagocytes and the complement cascade. These actions result in direct effects on vascular cells, including the induction of cytokines and pro-thrombotic factors (Westhuyzen and Healy 2000; Amezcua-Guerra *et al.* 2007).

One other consequence of the inflammatory process is the generation of ROS which may be produced from several sources including NADPH oxidase (Yokoyama *et al.* 2000; Sorescu *et al.* 2001; Cave *et al.* 2006; Lambeth 2007; Brandes and Schroder 2008; San Jose *et al.* 2008). The NADPH oxidase enzyme is composed of six subunits (see Table 1.1) including the GTPase Rho guanosine triphosphatase (usually Rac1 or Rac2) and five phagocytic oxidases (phox): p22phox, p40phox; p47phox; p67phoxand gp91 (Cross and Segal 2004; Quinn and Gauss 2004). In

VSMCs, gp91 is believed to be replaced by its homologous proteins nox1 and nox4 together with nox2 (Wassmann *et al.* 2002; Higashi *et al.* 2003; Ushio-Fukai 2006).

The NADPH oxidase enzyme is primarily described in phagocytises and fundamental in host defence generation of ROS to eliminate invading microorganisms. However, when deregulated, NADPH oxidase may contribute to the pathogenesis of diseases like atherosclerosis, hypertension and ischemia/ reperfusion injury (Aldieri *et al.* 2008). Its expression in vascular smooth muscle cells, adventitia or endothelium acts as a prominent source of ROS in the vascular wall even in the absence of inflammatory cells (Lambeth 2007; Brandes and Schroder 2008). Moreover, the generation of superoxide anions, by univalent reduction of oxygen, provides an environment of OS with the superoxide radicals readily reacting with NO forming the highly reactive peroxynitrite species with detrimental consequences (Forstermann 2006; Aldieri *et al.* 2008). Furthermore, the overproduction of NADPH, a co-factor for NADPH oxidase (and indeed NOS), by glucose-6-phosphate dehydrogenase activity induces OS by increasing the production of $O_2^{\cdot -}$ enhancing endothelial and vascular dysfunction (Gupte 2008).

The implication of the NADPH oxidase in atherosclerosis is supported by the fact that its expression is specially activated in endothelial cells at arterial bifurcations or branching points within the vascular bed where atherosclerotic lesions are most likely to occur (Wyatt *et al.* 2004; Rouhanizadeh *et al.* 2008). The increase in ROS generation could contribute toward endothelial dysfunction, a key precipitating factor in the development of atherosclerosis (Ross 1995). In addition ROS generation could lead to oxidation of LDL generating oxLDL which can play a crucial role in the onset and progression of atherosclerosis, mediating its effects in a two phase process. In the early phase the monocytes recruited into the vessel wall

will differentiate into macrophages and accumulate oxLDL through scavenger receptors such as SR-A, CD36 and lectin-like receptor-1 (LOX-1) becoming foam cells (Hofnagel *et al.* 2006; Lee *et al.* 2008).

Table 1.1. NADPH oxidase homologues.

Adapted from (Gao and Mann 2009).

Noxs	Nox1	Nox2	Nox3	Nox4	Nox5	Duox1 & 2
Detected in these tissues	**Vasculature,** kidney, retina, colon, uterus, prostate	**Vasculature,** phagocytes, heart, lung	Inner ear, kidney, liver, lung, spleen	**Vasculature,** kidney, heart, bone, ovary, eye, placenta, skeletal muscle	**Vasculature,** lymphoid tissue, testis, prostate, breast, brain	Thyroid, lung, salivary glands, gastrointestinal tract
Regulation of enzyme activity	p22phox, NoxO1, NoxA1, Rac1	p22phox, p47phox, Rac, p67phox, NoxO1	p22phox, NoxO1, Rac1	p22phox		Associated with p22phox but no effect on activity
Regulation of expression	Upregulated by INF-γ	Basal activity?, but upregulated by INF-γ		Basal activity. Upregulated by AngII, TGF-β, TNF-α, and inhibited by BMP-4		Upregulated by IL-1, IL-3, IL-4 and INF-γ
Cellular distribution	Membrane, associated with caveolin	Membrane	Associated with vinculin	Intracellular compartments		Membrane
References	(Ray and Shah 2005; Brandes and Schroder 2008)	(Ray and Shah 2005; Brandes and Schroder 2008)	(Ray and Shah 2005)	(Ray and Shah 2005; Brandes and Schroder 2008)	(Ray and Shah 2005; Brandes and Schroder 2008)	(Geiszt 2006)

In a second phase, oxLDL together with other unknown local factors may negatively regulate anti-inflammatory and antioxidant response (Maziere and Maziere 2008; Watanabe 2008). These effects, together, could enhance pro-inflammatory events leading to further damage to the endothelium and hence help progress the disease. Inhibition of ROS generation in the disease state may therefore help protect against some of the deleterious effects of the disease. In this context, studies using antioxidants have claimed to significantly inhibit cardiovascular events associated with ROS generation. However, the majority of other reports have failed to show any beneficial effects or any significant alteration of the atherosclerotic process following treatment with antioxidants (Jialal and Devaraj 2003). As examples of these contradictory findings, studies in patients on hemodialysis or suffering from diabetes with increased levels of ROS appear to have reduced incidences of myocardial infarction when administered with vitamin E (Boaz *et al.* 2000). In contrast, vitamin E was ineffective in reducing cardiovascular events in animal and human models of atherosclerosis. Interestingly, two other antioxidants, probucol and succinobucol, significantly reduced macrophage accumulation, VSMCs proliferation and stimulated endothelial repair (Wu *et al.* 2006; Tardif *et al.* 2008). Such discrepancies question the role of OS and ROS in the pathogenesis of diseases such as atherosclerosis and it has been suggested that OS may in fact not be a cause but rather aggravates the disease (Stocker and Keaney 2005; Forstermann 2008). It is also possible that these variations may reflect differences in the actions of antioxidants or even the complexity of *in vivo* redox reactions (Schleicher and Friess 2007).

An additional component to the inflammatory process may involve the induction of the enzyme NOS which generates nitric oxide. This molecule (discussed

below, Section 1.4.1), although physiologically relevant in regulating vascular tone and homeostasis, could contribute to the inflammatory process in atherosclerosis through its interaction with ROS to generate peroxynitrite ($ONOO^-$) (Pacher *et al.* 2007). The latter, in turn, can cause major alterations in cells that impair normal cellular function, thus consequences of OS (Upmacis *et al.* 2007).

1.4 . Nitric oxide pathways in the vessel wall

1.4.1 . Nitric oxide molecule

Nitrogen monoxide or NO is a natural gaseous signalling molecule that crosses biological membranes with ease. It has an unpaired electron, making NO an extremely reactive molecule which does not need a receptor to cross membranes (Stamler *et al.* 1992). Initially described as endothelium-derived relaxation factor (EDRF) in the eighties by Furchgott (Furchgott and Zawadzki 1980), NO has been extensively studied since its discovery and some excellent reviews summarising its biological properties and actions have been published (Moncada *et al.* 1988; Furchgott and Vanhoutte 1989; Moncada *et al.* 1991; Furchgott 1993; Kelly and Smith 1996; Fleming and Busse 1999; Ignarro 2002). The NO molecule has a short half life of between 4 and 50 seconds and due to its high reactivity forms intermediate molecules such as nitrogen dioxide (NO_2); nitrite (NO_2^-) and nitrate (NO_3^-) (Beckman *et al.* 1990).

1.4.2 . Nitric oxide synthases

The NOS family of enzymes is composed of three isozymes which are the constitutive endothelial NOS (eNOS; Type III, NOS-III and NOS-3), and neuronal NOS (nNOS; Type I, NOS-I and NOS-1) and the inducible NOS (iNOS; Type II, NOS-II and NOS-2). Both iNOS and nNOS are cytosolic but eNOS is specifically localized in the plasmalemmal membrane caveolae (Alderton *et al.* 2001; Wyatt *et al.* 2004). The human isoforms of these enzymes show 51 to 57 % homology and are products of three different genes (Alderton *et al.* 2001). Constitutive nNOS in the human genome is located on chromosome 12 and the protein is composed of 1434 amino acids with a 161 kDa weight. eNOS, a 131 kDa protein, is on chromosome 17 and composed of 1153 amino acids, while the 133 kDa inducible enzyme is composed of 1203 amino acids and located on chromosome 7 (Alderton *et al.* 2001). All the NOS enzymes share a common bi-domain structure where the N-terminal oxygenase domain binds haem and tetrahydrobiopterin (BH_4). The C-terminal reductase domain contains flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and NADPH binding sites with that for L-arginine linked by a calmodulin recognition site by this C-terminal (Richards and Marletta 1994; Ghosh and Stuehr 1995; McMillan and Masters 1995; Alderton *et al.* 2001). The constitutive isoforms require Ca^{2+}/calmodulin for activation while iNOS appears to be Ca^{2+} insensitive as it already has calmodulin tightly bound to its structure (Alderton *et al.* 2001). The primary structure of iNOS and the constitutive nNOS and eNOS differ in 40 to 50 amino acids in the FMN sub domain that is referred to as the auto-inhibitory loop of iNOS (Salerno *et al.* 1997; Alderton *et al.* 2001). Endothelial NOS can be activated by various agents including acetylcholine which binds to G protein-coupled

muscarinic receptors, activating phospholipase C, leading to a Ca^{2+} cytosolic increase. The increase in cytosolic Ca^{2+} in turn, results in activation of calmodulin and subsequently eNOS (Fleming and Busse 1999). Once activated, eNOS catalyses the formation of NO from oxygen and arginine, utilising various co-factors including NADPH, FMN, FAD and BH_4 (Figure 1.2.) (Ignarro 1990). Synthesis of NO via this enzymatic pathway is now widely accepted to perform as a physiological regulator of vascular tone, acting through the activation of sGC and generation of cGMP to induce smooth muscle relaxation (Silver 1985; Nakatsu and Diamond 1989; Luscher 1991). Guanylate cyclase is a heme-protein with a high affinity for NO (Stone and Marletta 1994; Koesling *et al.* 2004). Any changes within the vessel wall that impair eNOS activity would result not only in reduced NO synthesis but also in impaired vascular relaxation.

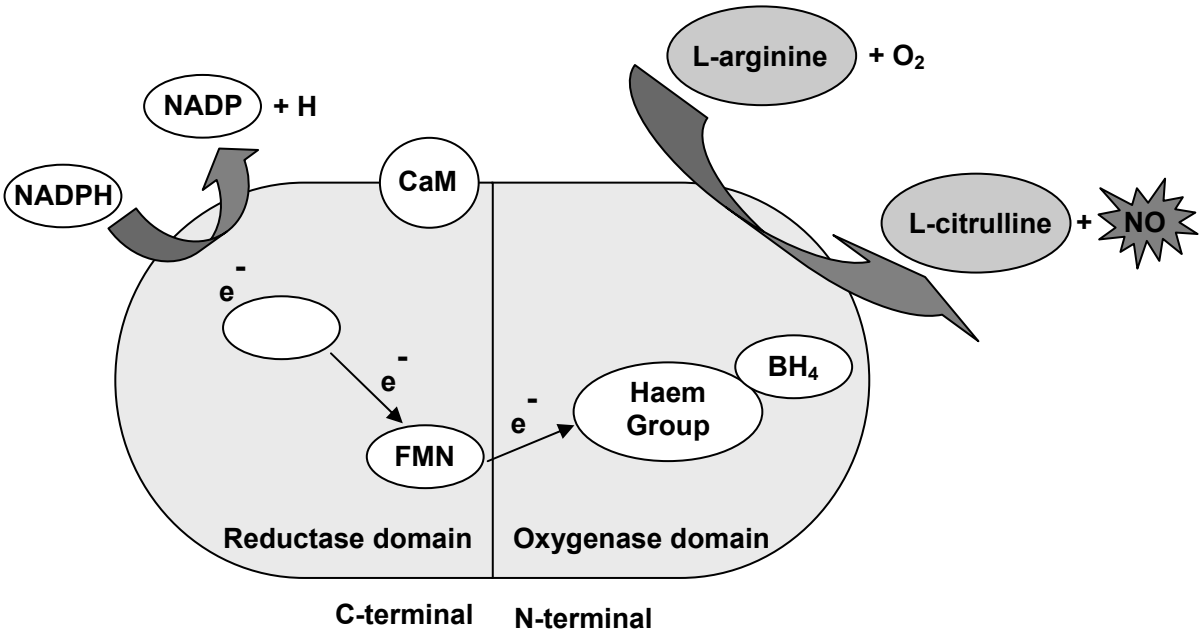


Figure 1.2. Synthesis of NO from L-arginine and Oxygen.

In contrast to eNOS, nNOS is expressed mostly in neuronal tissue and thus plays a less significant role in regulating vascular tone directly. By comparison, iNOS is often induced in the vessel wall following exposure to pro-inflammatory mediators such as bacterial lipopolysaccharide (LPS) and cytokines (Ravalli *et al.* 1998). This induction peaks after 18 hours of induction and generates nanomolar amounts of NO which are significantly higher than the low picomolar amounts produced by the constitutive isoforms (Ignarro 1999). The large amounts of NO produced by iNOS could have detrimental consequences, contributing to the pathophysiology of diseases such as atherosclerosis, where expression of iNOS may contribute to vascular damage through generation of $ONOO^-$. The latter is formed by the reaction between NO and $O_2^{\cdot -}$ thereby reducing NO bioavailability (Buttery *et al.* 1996; Cooke and Davidge 2002). More importantly, $ONOO^-$ can induce oxidation of BH_4 and nitration of proteins resulting in OS and impaired cellular function (Vasquez-Vivar *et al.* 1998; Kohnen *et al.* 2001; Kuzkaya *et al.* 2003; Alp and Channon 2004; Forstermann 2006; Forstermann and Munzel 2006; Upmacis *et al.* 2007). Peroxynitrite may also induce iNOS through nuclear factor kappa B (NF-κB) activation in endothelial cells resulting in sustained overproduction of NO and thus further vascular damage (Cooke and Davidge 2002).

1.4.3 . Nitric oxide, oxidative stress and atherosclerosis

As already indicated, atherosclerosis is considered to be a chronic inflammatory disease characterized by enhanced expression of pro-inflammatory cytokines, chemokines, and adhesion molecules. The cross-talk between cytokines,

chemokines and infiltrating immune cells amplifies the inflammatory cascade in the vessel wall, resulting in atherogenesis (Chandrasekar *et al.* 2006) and potentially the induction of iNOS. Immunostaining and *in situ* hybridization have indeed confirmed the presence of iNOS in atherosclerotic vessels, specifically localized to CD68-positive macrophages, foam cells and VSMCs (Buttery *et al.* 1996; Hemmrich *et al.* 2003). More importantly, the overexpression of iNOS may potentially contribute to the inflammatory response and further influence the pathologic features associated with the disease through, for instance, induction of nitrosative stress (Upmacis *et al.* 2007). A pathological role for iNOS-induced NO in atherosclerosis is supported by reports that iNOS knockout mice show lower plasma levels of lipoperoxides, often used as an index for OS (Chen *et al.* 2003).

Peroxynitrite and other RNS are responsible for the *in vivo* toxic damage resulting from ROS reaction with NO (Cooke and Davidge 2002; Nanetti *et al.* 2007; Cooper and Magwere 2008). Oxidative stress also uncouples the NOS enzymes by oxidising BH_4 to BH_3 (Landmesser *et al.* 2003; Alp and Channon 2004) and administration of BH_4 or its precursor to animal models could restore NO bioactivity (Vasquez-Vivar *et al.* 1998; Laursen *et al.* 2001). The ROS generated by NADPH oxidase may directly cause vascular injury by oxidizing cellular constituents such as contraction proteins and depletion of NO either by formation of peroxynitrite or modification of NO binding sites (Abu-Soud *et al.* 1996; Elahi *et al.* 2007).

Although an increase of NO in cardiovascular disease has been argued to be detrimental, not only because of the actions described above but also because of NO-mediated depression of inotropy, mitochondrial respiration and β–adrenergic responsiveness, new lines of evidence have indicated that reduced NO bioavailability may impair vasodilation contributing to vascular dysfunction (Elahi *et*

al. 2007). Additionally, iNOS-induced NO production has been shown to have a protective role in the cell-mediated oxidative modification of LDL (Buttery *et al.* 1996; Suschek *et al.* 2003) and in an *in vivo* model, iNOS attenuated atherosclerotic lesions after immune injury and increased endothelial integrity and survival, protecting against OS-induced apoptotic or necrotic cell death (Rikitake *et al.* 1998; Hemmrich *et al.* 2003). These beneficial properties of iNOS may be associated with the NO generated by this enzyme. Furthermore, under inflammatory conditions, hepatocytes have been reported to increase NO generation through iNOS as a protection mechanism (Kuo *et al.* 1997). Moreover, macrophages challenged with pro-inflammatory mediators generate NO that appears to inhibit oxLDL formation (Yates *et al.* 1992). The beneficial effects of antioxidants have also been shown to be related to an increase in iNOS activity and decreasing ROS (Bernatova *et al.* 2002). These findings indicate a beneficial rather than a deleterious role of iNOS-induced NO. Such a notion, although gaining some credence, is however still controversial in light of the plethora of information saying otherwise. Detailed studies investigating the beneficial anti-atherosclerotic properties of iNOS are therefore essential. Moreover, a better understanding of the mechanisms that regulate iNOS expression in atherosclerosis may prove beneficial in developing novel strategies in controlling the disease state. This may be particularly relevant with regards to understanding how certain drugs used by atherosclerotic patients regulate iNOS expression and/or function. This also applies to the parallel regulation of the L-arginine cationic amino acid transporters (CATs) which, as discussed below (Section 1.4.4), clearly influence the activity of iNOS and could potentially be involved in the pathogenesis of atherosclerosis and/or OS.

1.4.4 . L-arginine transport in vascular cells

In inflammatory diseases, substrate supply to iNOS as well as expression and activity of the transporter(s) responsible for L-arginine influx into the cell (Durante *et al.* 1995; Simmons *et al.* 1996; Durante 2001; Kagemann *et al.* 2007) may be critical for sustained NO synthesis and thus the pathogenesis of the disease. Once induced, the ability of iNOS to generate NO may be critically regulated by L-arginine transport into cells (Baydoun *et al.* 1990; Bogle *et al.* 1992; Wileman *et al.* 1995).

The availability and transport of L-arginine may be critical for NO synthesis within the vasculature and could have a significant impact in atherosclerosis. To date, at least four systems (y^+, y^+L, $b^{o,+}$ and $B^{o,+}$) that mediate the uptake of cationic amino acids have been described. These are characterised according to their affinity for cationic amino acids and by their dependence on sodium (Deves and Boyd 1998; Palacin *et al.* 1998). The y^+L system has at least two distinct transporters that have in common the heavy chain 4F2hc glycoprotein and the light chains y^+LAT-1 or y^+LAT-2 (Torrents *et al.* 1998; Pfeiffer *et al.* 1999; Broer *et al.* 2000). This heterodimeric protein family characteristically transport neutral and basic amino acids via Na^+-dependent and independent mechanisms respectively. While y^+LAT-1 has a higher affinity for neutral amino acids (Km≈20-30 mM vs Km≈340 mM for L-arginine (Pfeiffer *et al.* 1999), y^+LAT-2 seems more specific for cationic amino acids (L-arginine Km≈120 mM vs leucine Km≈236 or glutamine ≈295) (Broer *et al.* 2000). The expression of these two proteins vary in different tissues with y^+LAT-1 being expressed preferentially in intestine, kidney, lung and leukocytes (Torrents *et al.* 1998; Pfeiffer *et al.* 1999) while y^+LAT-2 appears predominant in the brain, heart, testis, small intestine and parotids (Broer *et al.* 2000).

Systems $b^{o,+}$ and $B^{o,+}$ are broad-scope carriers, able to accept both neutral and cationic amino acids (Van Winkle *et al.* 1985; Bauch *et al.* 2003). The heteromeric amino acid transporter $b^{o,+}$ is Na^+ independent unlike $B^{o,+}$ which is Na^+ dependent (Bauch *et al.* 2003). $b^{o,+}$ however seems to be crucial as an exchanger at normal membrane potential in epithelial cells, showing higher affinity for L-cysteine than for cationic or large neutral amino acids (Bauch *et al.* 2003). This transporter is a transmembranar protein with a light chain catalytic subunit $b^{0,+}$ (glycoprotein associated) and the covalently associated heavy chain, type II glycoprotein r-broad scope amino acid transporter (rBAT) (Bauch *et al.* 2003). Although accepting a broad range of amino acids, $B^{o,+}$ shows selectivity for branched chained or benzonoid amino acids like leucine, isoleucine, tryptophan and phenylalanine (Van Winkle *et al.* 2006). First described in mouse blastocysts, $B^{o,+}$ is suggested to be functionally important in blastocyst implantation and adulthood complications such as obesity in humans (Van Winkle *et al.* 2006).

Of the transporters listed above, systems y^+ is perhaps the most diverse and widely studied. It is described as a widespread classical system, with high affinity (K_m in the micro-molar range) for cationic amino acids and is sodium independent (White *et al.* 1982; Closs and Mann 1999). In addition, this carrier is modulated by changes in membrane potential, with depolarization distinctly reducing L-arginine influx after stimulation with agonists such as bradykinin (Sobrevia *et al.* 1995; Bogle *et al.* 1996).

System y^+ is now known to comprise of a family of different cationic amino acid transport (CAT) proteins referred to as CAT-1 (Closs *et al.* 1993; Closs *et al.* 1993), CAT-2A (Closs *et al.* 1993), CAT-2B (Closs *et al.* 1993) and CAT-3 (Hosokawa *et al.* 1997; Ito and Groudine 1997). A more recent member of this family, hCAT-4, has been described and is expressed predominantly in brain, testis and placenta. This

carrier is encoded by the *SLC7A4* human gene which has a high homology with the *SLC7A1* and *SLC7A2* CAT genes that encode for CAT-1 and CAT-2 (Sperandeo *et al.* 1998; Dall'Asta *et al.* 2000). There is however some controversy as to whether the CAT-4 protein is in fact an actual transporter since it does not seem to exhibit the classical function of CATs (Sperandeo *et al.* 1998; Wolf *et al.* 2002; Closs *et al.* 2006). Expression of hCAT-4 complementary deoxyribonucleic acid (cDNA) in *Xenopus laevis* oocytes or human testis teratocarcinoma cell line NT2 did not result in the expression of a functional CAT protein that could transport neutral, anionic or cationic amino acids (Wolf *et al.* 2002). This therefore questions whether hCAT-4 is indeed a CAT. It is possible however that the protein may need additional factors to be functional (Wolf *et al.* 2002).

Of the CATs, CAT-1, a high-affinity carrier, was the first to be characterised at a molecular level with the deduced amino acid sequence of the cloned cDNA revealing a 622 amino acid glycoprotein of about 67 kDa with 12 to 14 transmembrane spanning domains (Closs *et al.* 1993; Closs *et al.* 1993). A truncated *Cat-2* gene was cloned shortly after *Cat-1* and initially named Tea (T-cell early activation receptor) because of its induction early in the response of normal T cells to mitogens. The full length cDNA which encodes a 658 amino acid protein (CAT-2B) was subsequently isolated and shown to have a 61 % homology with CAT-1 and 98 % homology with CAT-2A (Closs *et al.* 1993). Like CAT-1, CAT-2B has a high affinity for L-arginine (Km: 0.04-0.3 mM) despite its high sequence identity with CAT-2A. However, unlike CAT-1 and CAT-2A, CAT-2B is believed to be an inducible protein and the product of a differentially spliced *CAT-2* gene that also encodes for CAT-2A (Closs *et al.* 1993; Kakuda *et al.* 1999). These two products (CAT-2A and CAT-2B) differ by only 20 amino acids within a stretch of 41 amino acids in an alternatively

spliced region in the predicted fourth extracellular loop (Closs *et al.* 1993). This is however sufficient to render CAT-2B a high affinity carrier with CAT-2A now described as a high capacity but low affinity transporter of L-arginine. Moreover, this protein, unlike CAT-1 and CAT-2B is expressed more abundantly in the liver where it serves to remove surplus cationic amino acids from the portal circulation (Closs *et al.* 2006).

1.4.5 . L-arginine transport and NO synthesis

Concentrations of L-arginine in human plasma are reported to be between 80 to 120 μM (Mendes Ribeiro *et al.* 2001) or up to a normal maximum of 210 μM (Chin-Dusting *et al.* 2007). By comparison, concentrations in cells range between 0.1 to 0.8 mM (Baydoun *et al.* 1990; Mitchell *et al.* 1990). Despite the high intracellular concentrations, NO production by iNOS in cultured cell systems appears to be dependent not only on the presence of extracellular arginine (Granger *et al.* 1990; Mitchell *et al.* 1990; Assreuy and Moncada 1992; Bogle *et al.* 1992), but also directly related to the rate of transport of exogenous arginine (Assreuy and Moncada 1992; Bogle *et al.* 1992), and is inhibited following blockade of L-arginine entry into cells (Bogle *et al.* 1992). Removal of exogenous L-arginine or direct inhibition of L-arginine transport through these carriers also attenuates NO synthesis in blood vessels obtained from endotoxemic rats (Schott *et al.* 1993) and in SMCs exposed to endotoxin and inflammatory cytokines (Hattori *et al.* 1999; Wileman *et al.* 2003). Moreover, in knockout studies, suppression of L-arginine transport through abolition of CAT-2B expression results in a marked attenuation in NO production (Nicholson

et al. 2001). These findings demonstrate the important functional link between L-arginine transport and iNOS mediated NO synthesis. The close coupling between the two processes may be of particular clinical relevance as induction of iNOS generally coincides with the induction of transporter activity. This phenomenon, demonstrated *in vitro* in cultured cell systems, including SMCs (Durante *et al.* 1995; Wileman *et al.* 1995; Gill *et al.* 1996; Hattori *et al.* 1999), in experimental models of endotoxin-induced shock *in vivo* (Hattori *et al.* 1999; Schwartz *et al.* 2003; Huang *et al.* 2004; Chu *et al.* 2005; Yang *et al.* 2005) and, more importantly, in patients with septic shock (Reade *et al.* 2002) provides a mechanism for sustained substrate supply during enhanced NO synthesis.

Of the three CATs, there are strong indications that expression of the high affinity CAT-1 and/or CAT-2B may play an important regulatory role in determining the rate of NO synthesis within the vasculature. These carriers may be the critical targets for the regulation of NO synthesis and crucial because of their induction by inflammatory mediators which could provide a mechanism for substrate delivery critical for sustained NO production (Baydoun and Mann 1994; Rotoli *et al.* 2005; Baydoun *et al.* 2006).

In atherosclerosis, it is still not clear whether alterations in CATs activity contribute to the impaired vascular responses seen in the disease state. Moreover, there are conflicting reports on the effects of OS on L-arginine transport, some studies indicate that OS inhibits transport by system y^+, reducing L-arginine availability in endothelial cells, leading to eNOS uncoupling and OS (Zhang *et al.* 2006; Jin *et al.* 2007). Other report suggests that despite decrease of L-arginine availability, the transporters were not affected, instead, this has been linked to over expression of arginase-II which may deplete intracellular L-arginine pools by

breaking it in ornithine and urea thus increasing $ONOO^-$ concentration (Noris *et al.* 2004). In human aortic endothelial cells oxLDL induced an increase of arginase activity in a time and concentration dependent manner, contributing to the uncoupling of NOS and an increase of RNS. Arginase may therefore play a central role, contributing to the pathophysiology of lipid mediated endothelial damage (Ryoo *et al.* 2006; Ryoo *et al.* 2008). Furthermore, since arginase regulates cell proliferation, its inhibition may halt the progression of atherosclerosis (Hayashi *et al.* 2006).

The reasons for the discrepancies above are unclear and need careful investigation, paying particular attention to the systems used in the studies and to the conditions under which OS is being induced. It is not unreasonable to speculate however that part of the consequences of OS in atherosclerosis involves altered CATs expression and/or function. Such events would limit substrate supply to the NOS enzymes which in endothelium would attenuate eNOS-mediated NO and thus relaxation of vascular tone. By comparison, suppression of L-arginine supply to iNOS would attenuate iNOS-mediated NO production which should prove beneficial in the disease state because of the proposed potential pathological role of iNOS in atherosclerosis. More recent findings would however refute this notion and argue that the deleterious effects of OS may, in part, be associated with the suppression of iNOS-induced NO production through as yet undefined mechanisms that could potentially involve an action on the enzyme itself or on the CATs.

1.5 . Drug therapy for atherosclerosis

In view of the role of OS in the pathophysiology of atherosclerosis, one approach in treating the disease may be to diminish the status of OS within the vasculature. This could be achieved directly using pharmacological tools such as ascorbic acid, α-tocopherol, β-carotene or indirectly through enhancing the body's natural antioxidant defence mechanisms including up-regulating intracellular antioxidant defence mechanisms such as SOD, catalase and/or GPx (Inagi 2006). However, large trials of treatment with antioxidants in humans have failed when comparing with the successes in *in vitro* or animal studies. These differences may be due to the fact that gene regulation through long term and adequate dose of antioxidants may be more important than reducing ROS directly (Mann *et al.* 2007).

In addition to antioxidants, the fact that high levels of LDL directly correlate with cardiovascular complications, make targeting the lowering of plasma cholesterol levels critical for therapy. This could be achieved using well established lipid lowering drugs including anion-exchange resins, fibrates, nicotinic acid, and statins (Knopp *et al.* 2008). Of these, the statins have become the most effective and widely used drugs which act by reversibly and competitively inhibiting 3-hydroxy-3-methoxyglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme for cholesterol synthesis (Goldstein and Brown 1990; Chong *et al.* 2001). These drugs are discussed in further detail below (Section 1.5.1).

1.5.1 . Statins: 3-hydroxy-3-methoxyglutaryl coenzyme A (HMG-CoA) reductase inhibitors

Statins were discovered over 30 years ago and demonstrated to have a high capacity for reducing cholesterol levels (Endo *et al.* 1976; Endo *et al.* 1977). As mentioned above, these drugs are now increasingly prescribed for patients with coronary heart disease due to their efficiency in lowering cholesterol and protecting patients from cardiovascular events (Kronmann *et al.* 2007; Bartholomeeusen *et al.* 2008). In addition to lowering cholesterol, statins such as atorvastatin may reduce TNF-α-induced hypoxia-inducible factor-1α activation, indicating that these compounds may exert anti-inflammatory properties (Chandrasekar *et al.* 2006). Moreover, statins may also exert antioxidant effects which may be associated with their ability to inhibit $O_2^{\cdot-}$ production by NADPH oxidase, as demonstrated in a monocytes cell line (Delbosc *et al.* 2002a) and prevented angiotensin-II (Ang-II)-NADPH stimulation (Delbosc *et al.* 2002b). These actions may improve endothelial function, stabilize atherosclerotic plaque, and inhibit platelet aggregation, vascular SMC proliferation and vessel wall inflammation (Chandrasekar *et al.* 2006).

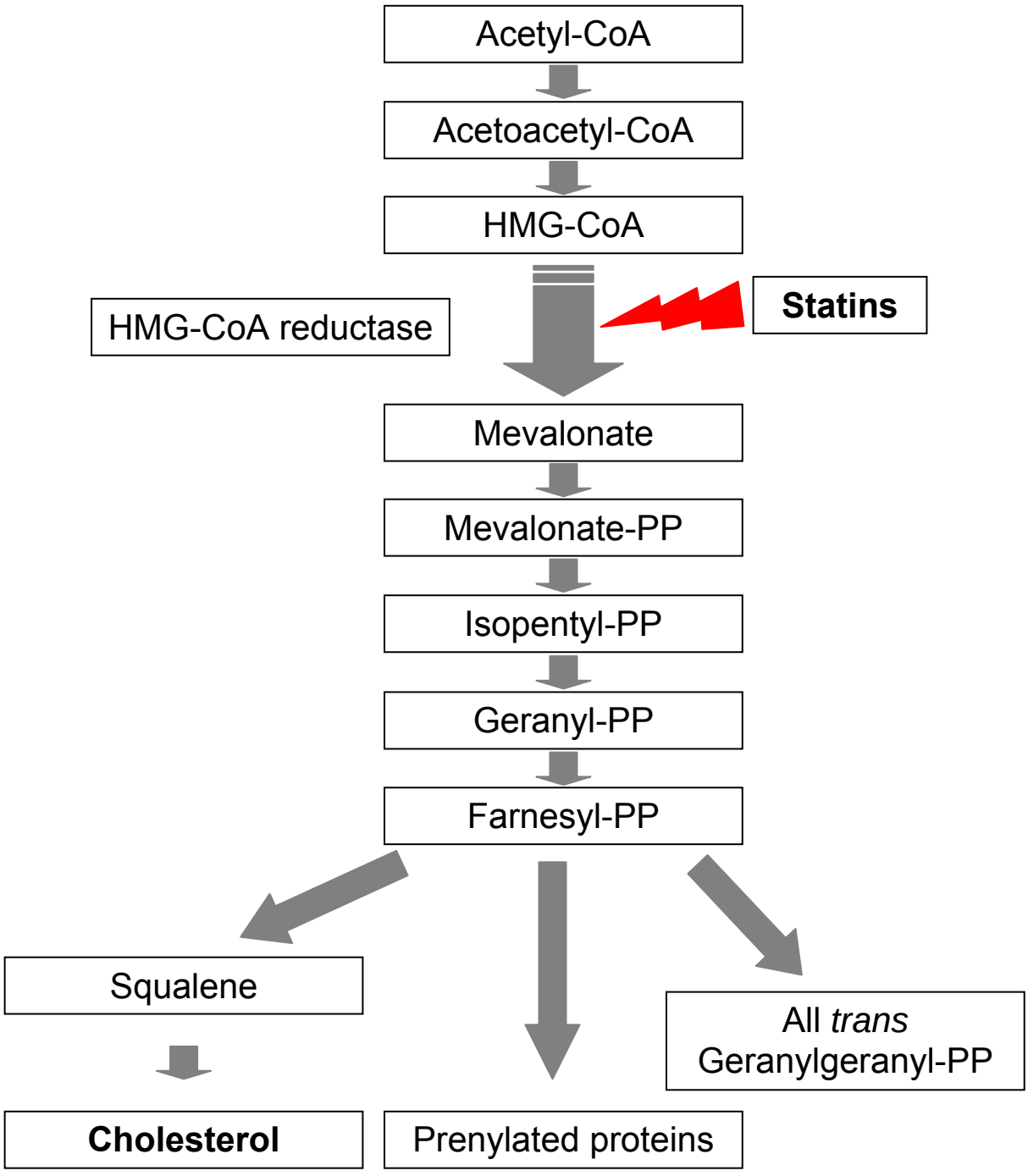
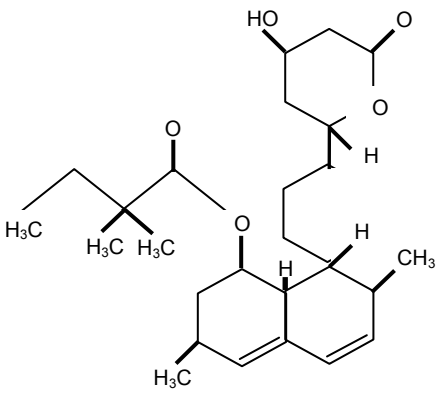


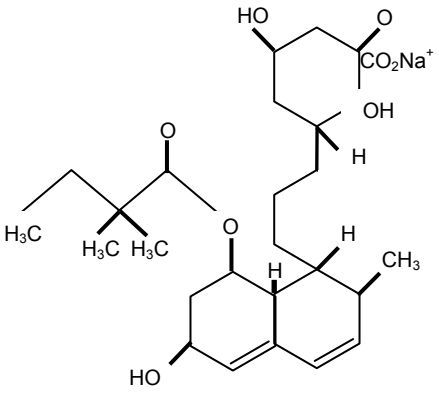
Figure 1.3. The mammalian mevalonate pathway.

It is worth noting that although all statins inhibit HMG-CoA reductase, this characteristic does not reflect a homology of structure and origin. Statins can be natural, synthetic or semi-synthetic and their molecular structure may vary (Manzoni and Rollini 2002) (Figure 1.4.). The derivate pravastatin can be obtained by the biotransformation of mevastatin obtained from *Penicillium citrinum*. Lovastatin on the other hand is obtained from the *Aspergillus terreus* strain whereas the semi-synthetic simvastatin is synthesised from lovastatin (Manzoni and Rollini 2002). The statins of natural origin share a characteristic hydroxy-hexahydro naphthalene ring system within the polyketide portion and differ on the linked varied side groups (Manzoni and Rollini 2002).

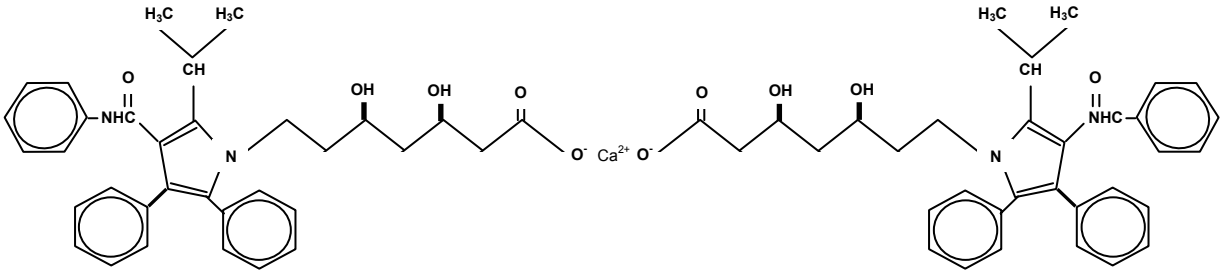
The molecular structure of statins is formed by three main groups: the HMG-CoA analogue; an hydrophobic ring that links the HMG-CoA analogue and a complex hydrophobic ring structure and finally the side groups linked to the rings (Schachter 2005). These structures confer the specific molecular properties of each statin. Synthetic atorvastatin and fluvastatin are derived from mevalonate and pyridine, respectively. Another insight is that statins can be lipophilic and hydrophilic, leading to different actions. Rosuvastatin although synthetic and hydrophilic is retained in circulation for 19 hours (Schachter 2005). Pravastatin is the most hydrophilic and simvastatin the most lipophilic, but the clinical relevance of the differences in solubility among the statins is not completely clear. Lipophilic statins may penetrate cells by passive diffusion, differing from pravastatin and rosuvastatin, both hydrophilic molecules that require a transporter system for entry into cells (Turner *et al.* 2007).



Simvastatin



Pravastatin



Atorvastatin

Figure 1.4. Chemical structure of HMG-CoA reductases.

1.5.2 . Statins pharmacology

In general, the pleiotropic effects of statins have been described in endothelial, other vascular cells and in platelets. Some of these effects include anti-inflammatory actions, vascular tone relaxation, plaque stabilization and anti-thrombotic effects (Boyle 2005; Mangat *et al.* 2007). Other actions include decrease of oxLDL (Hogue *et al.* 2008), ROS and NADPH oxidase activity (Rueckschloss *et al.* 2001; Wassmann *et al.* 2002; Yu *et al.* 2005; Forstermann 2008); the promotion of eNOS expression (Laufs *et al.* 1998) and activity by protein kinase B (Akt) phosphorylation (Nakata *et al.* 2007); down-regulation of caveolin expression that in excess binds eNOS reducing NO bioavailability (Feron *et al.* 2001); the inhibition of the farnesylation and geranylgeranylation of protein Ras homolog gene family, member A (RhoA) as well as RacC and cell division cycle 42 (Cdc42) in the mevalonate pathway resulting in the suppression of expression of eNOS (Ruperez *et al.* 2007). Vascular relaxation may also be improved by the diminished expression of endothelin-I, Ang-II receptor as well as the improvement in eNOS expression and hyperpolarization of calcium activated potassium channels (Hernandez-Perera *et al.* 1998; Mangat *et al.* 2007). In addition, vascular inflammation may be ameliorated through the inhibition of (i) LDL oxidation, (ii) leukocyte-endothelial cell adhesion, (iii) NF-κB activation, (iv) CRP and (v) homocysteine levels (Mangat *et al.* 2007) and (vi) release of pro-inflammatory cytokines. In human umbilical vein endothelial cells (HUVECs) simvastatin can induce neovascularization suggesting a mechanism of salvaging tissue from critical ischemia (Kureishi *et al.* 2000). In plaque stabilization statins may inhibit accumulation of cholesterol in macrophages, matrix metalloproteinases (MMPs) production and infiltration of inflammatory cells, which

together with the inhibition of platelets and tissue factor (TF) expression would have anti-thrombotic effects and could prove beneficial in atherosclerosis in preventing plaque rupture (Saini *et al.* 2005; Rubba 2007; Nakamura *et al.* 2008).

Treatment with statins reduces LDL levels which significantly correlates with plaque volume reduction, regression and a decline in arterial remodelling (Paciaroni *et al.* 2007). Interestingly, lipophilic but not hydrophilic statins apparently reduce neointimal formation and SMCs proliferation (Arnaboldi *et al.* 2007; Turner *et al.* 2007). The lipophilicity of statins may indeed reflect the more powerful pleiotropic effects of atorvastatin, simvastatin, fluvastatin and lovastatin.

Clinically, the dosage of statins given to patients appears to depend on the statin being used and varies between 20 and 80 mg/day. Their plasma half-life is normally between 2 to 4 hours, with the exception of atorvastatin which has a longer half-life of 14 to 20 hours (Chong *et al.* 2001; Manzoni and Rollini 2002). The plasma level of the drugs also depends on the statin used, with simvastatin for instance, reaching concentrations of 0.1 μM to 10 μM (Lin *et al.* 2005). After a 40 mg single therapeutic administration of lovastatin, peak serum concentrations vary between 0.1–0.3 μM (Pentikainen *et al.* 1992). In rats, plasma levels of atorvastatin reached 1 μM just after administration of 2 mg/ml, decreasing to a minimum of 0.1 μM, 2 to 4 hours after administration of this drug (Lau *et al.* 2006). Whereas simvastatin and lovastatin are available for patient use as lipophilic lactone pro-drugs, the other statins are given in the acidic form (Neuvonen *et al.* 2008). The pharmacokinetic differences between the statins may be crucial in clinical situations. The bioavailability of statins taken orally may vary accordingly with the activity of cytochrome P450 3A4 (CYP3A4) transporter proteins, the contents and pH of the gastrointestinal tract and is greater for fluvastatin (24 %), pravastatin (17 %) and

atorvastatin (12 %) but low for lovastatin and simvastatin (5 %) (Chong *et al.* 2001; Neuvonen *et al.* 2008). Different satins at varied concentrations show higher or lower potency in blocking LDL synthesis (see **Error! Reference source not found.**). Atorvastatin 80 mg has the most efficient lowering rate, decreasing cholesterol by 54 %, and the minimum dose (10 mg) reduces cholesterol by 38 %. Lovastatin at 80 mg reduces cholesterol levels by up to 48 %; while simvastatin at the same concentration reduces by up to 46 %. Similarly, at 40 mg, pravastatin reduced cholesterol by 34 %, and this was more efficient than fluvastatin which at the same concentration only inhibited by 23 % (Jones *et al.* 1998).

A vast majority of studies distinguish statins according to their lipophilicity which correlates with their pleiotropic effects. Lipophilic statins can enter cells by passive diffusion in contrast to hydrophilic molecules which are unable to penetrate the plasma membrane and therefore require a transport mechanism for entry into the cells. This involves the Na^+-independent organic anion transporting polypeptide (OATP) family (Hsiang *et al.* 1999; Ohtawa *et al.* 1999) expressed in several tissues: intestine, heart, liver, brain, kidney or testis (Hagenbuch and Meier 2003; Konig *et al.* 2006). Where these transporters are not expressed, it is possible that statins dependent on transport for entry into cells may not be effective. One such statin is pravastatin which comprises of a polar hydroxyl group and rosuvastatin, a methane sulphonamide group, conferring this molecule its hydrophilicity (McTavish and Sorkin 1991; McTaggart *et al.* 2001). These hydrophilic statins target, specially, hepatic cells and their lack of effect in other cell types, including smooth muscle cells, have been associated with their incapacity to cross cellular membranes (Rosenson and Tangney 1998; Schachter 2005). The lipophilic statins such as atorvastatin,

simvastatin or fluvastatin are less selective to hepatocytes as they are able to transpose membranes of peripheral cells (Schachter 2005).

Table 1.2. Percentage of reduction of LDL levels by statins.

Adapted from (Jones et al. 1998).

Statins/LDL reduction (%)					
Dose (mg)	**Atorvastatin**	**Pravastatin**	**Simvastatin**	**Fluvastatin**	**Lovastatin**
10	38	19	28	No data	No data
20	46	24	35	17	29
40	51	34	41	23	32
80	54	No data	46	36	48
Solubility	Lipophilic	Hydrophilic	Lipophilic	Lipophilic	Lipophilic

From the OATP family: OATP1B1, OATP2B1, OATP1A2 and sodium dependent taurocholate co-transporting polypeptide (NTCP) are responsible for the uptake of statins into hepatocytes (Kivisto and Niemi 2007; Neuvonen *et al.* 2008). Once in the cells, statins are oxidatively modified (hydroxylation) by the cytochrome P450 enzymes to a more water-soluble form for renal excretion (Aviram *et al.* 1998; Davidson 2000). Of the 50 CYP450 enzymes, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A5 metabolise 90 % of the drugs in humans (Lynch and Price 2007). Lovastatin, simvastatin and atorvastatin, like steroid drugs, are processed by CYP 3A4 (Davidson 2000).

Simvastatin and lovastatin lactones are oxidized by CYP3A4 (and CYP3A5) into their acidic forms in the intestinal wall and liver (Neuvonen *et al*. 2008). Pravastatin, unlike the other statins, is partially degraded in the stomach (Neuvonen *et al*. 2008). Finally statins are eliminated by their transport from the portal circulation to bile through multi-drug resistance associated protein-2 (MRP2), breast cancer resistance protein (BCRP) and bile salt export pump (Neuvonen *et al*. 2008).

Although the common belief is that statins are well tolerated and don't have dramatic side effects, these drugs may have hepatic and skeletal muscle toxicity with increased transaminase levels and may also cause myopathy (weakness and muscle pain), that could lead to rhabdomyolysis and renal failure (Chong *et al*. 2001). These effects may become more prevalent in the presence of CYP3A4 inhibitors (Davidson 2000) such as cyclosporin (immunosuppressant), erythromycin (arrhythmias medicine), ketoconazole and itraconazole (antifungals), nefazodone (antidepressant), mibefradil (calcium channel blocker) and grapefruit juice (Davidson 2000; Chong *et al.* 2001). Fluvastatin and pravastatin are less likely to exhibit drug-

drug interaction, thus making them the safest over the counter statins but they are definitely not the most effective at lowering cholesterol (Neuvonen et al. 2008).

1.5.3 . Statins and oxidative stress

The antioxidant properties of statins are not fully understood. However in macrophages and endothelial cells, involved in the onset and progression of atherosclerosis, simvastatin and cerivastatin (withdrawn from the market in 2001) blocked the production of free radicals (Terblanche *et al.* 2007). Statins also induce the stress protein HO-1 in rat and human aortic smooth muscle cells (HASMCs) and this could contribute in regulating antioxidant and anti-inflammatory events in atherosclerosis (Lee *et al.* 2004). These beneficial effects would have direct implications in clinical coronary interventions in patients with high levels of CRP, potentially being capable of reducing mortality.

The induction of HO-1 and subsequent production of CO by HO-1 provides protection against oxidative injury, cell death and inhibits cell proliferation (Terblanche *et al.* 2007) and could do this through bilirubin, a potent scavenger of free radicals. Moreover, atorvastatin metabolites inhibit lipoprotein oxidation (Aviram *et al.* 1998) and in human mesanglial cells, pitavastatin attenuates high glucose induced and Ang-II-induced activation of p42/44 MAP kinase and NADPH oxidase activity (Yu *et al.* 2005). Similarly, statin inhibition of Ang-II-induced stimulation of NADPH oxidase derived $O_2^{\cdot -}$ production was demonstrated in a monocytes cell line (Delbosc *et al.* 2002; Delbosc *et al.* 2002). Atorvastatin and pravastatin may also

reduce ras-related C3 botulinum toxin substrate 1 (rac1)-GTPase activity and thus NADPH oxidase induced ROS production which is dependent on rac1 (Korantzopoulos *et al.* 2006). In animal and *in vitro* experiments, inhibition of rac1 by statins inhibits NADPH oxidase related ROS production in VSMCs and cardiac myocytes leading to a reduction of cardiac hypertrophy (Maack *et al.* 2003). Furthermore, simvastatin blocked the translocation of p21 rac2 and p67phox, and subsequently NADPH oxidase activation, suggesting that this enzyme depends on geranylgeranylation for activation (Delbosc *et al.* 2002). All these effects would again be cardio-protective, providing additional beneficial effects beyond simply lowering blood cholesterol.

In endothelial cells, lovastatin and simvastatin induced NO production by increasing messenger ribonucleic acid (mRNA) stability (Laufs and Liao 1998). Atorvastatin seems to improve the endothelium homeostasis by blunting the p21-Rac mediated NADPH oxidase assembly, reducing therefore the production of superoxide and increasing NO bioavailability (Wagner *et al.* 2000). Additionally, this statin depresses the expression of NADPH oxidase 1 (Nox1) and p22phox in SMCs and endothelial cells respectively, thus highlighting another beneficial action as Nox1 and p22phox are NADPH subunits that contribute to the production of superoxide in the vessel wall (Wassmann *et al.* 2002). In the same study by Wassmann *et al*. an increase of catalase was detected, further contributing to the antioxidant effects of statins (Wassmann *et al.* 2002). In the same way, simvastatin lessen the oxidation of LDL and high density lipoprotein (HDL), the production of pro-inflammatory and cytotoxic aldehydes analysed from patients plasma as well as the oxidation of LDL in macrophages (Girona *et al.* 1999). Fluvastatin acts directly as a scavenger of $O_2^{\cdot-}$ and $^{\cdot}OH$ when tested in rat liver microsomes, inhibiting lipid peroxidation (Yamamoto

et al. 1998). A controversial study from Parker *at al.* suggested that lipophilic statins could in some conditions actually impair NO production through eNOS localization and promote OS (Parker *et al.* 2003), where other studies have shown the opposite (Ortego *et al.* 2005; Hofnagel *et al.* 2007; Adam and Laufs 2008; Singh *et al.* 2008). Whether any of these mechanisms contribute to the regulation of NO production and/or CATs is an interesting notion that is to be investigated.

1.6 . Aims and objectives

The iNOS-induced NO production may play a complex role in the pathogenesis of atherosclerosis. However, the putative role of iNOS, and indeed CATs, in the development and clinical expression of atherosclerosis is not yet clear. To further intricate the issue, it is even more confusing as to whether iNOS activity does in fact cause the observed oxidative damage seen in the disease state and whether iNOS and/or CATs expression and/or function may be regulated under OS.

This thesis was therefore aimed at unravelling how iNOS and CATs expression and function may be modified under conditions of OS by examining the effects of OS inducers on these proteins in rat cultured aortic smooth muscle cells. Smooth muscle cells were chosen for the studies due to their critical physiological role in regulating vascular tone, and the fact that some of the deleterious effects of OS are targeted at these cells.

Because of the central role of the endothelium in atherosclerosis it was intended that parallel studies would be carried out using endothelial cells isolated form porcine aorta. However, because of the restriction in tissue supply it was impossible to continue studies with this cell type. The limited data generated with the small samples of aortae obtained is presented but unfortunately has not been extended.

Cultured rat aortic smooth muscle cells were used to study the effects of oxidative stress inducers: H_2O_2 (from 0.01 to 1000 mM), antimycin A (25 to 150 μM) and DEM (1 to 150 μM). These cells were induced with LPS (100 μg/ml) and (IFN-γ 100 U/ml) for 24 hours to mimic inflammatory conditions. The induction and subsequent production of NO was measured using the Griess assay and the iNOS

protein expression detected by western blotting. The levels of L-arginine transport into RASMCs were detected using [^{3}H]L-arginine.

Additionally, studies examining the effects of atorvastatin, simvastatin and pravastatin (1 to 100 μM) on both iNOS and CATs function and on iNOS expression were also carried out to determine whether the cardio-protective effects of these compounds extend to regulating these proteins. Studies were performed over short incubation periods, (5 minutes to 3 hours) or over long incubation periods (24 hours incubation) when there is full induction of iNOS and the CAT transporters. Considering the anti-inflammatory and antioxidant properties of statins, the following stage was to co-incubate statins and oxidative stress inducers in our system to observe if there was any reversible effect.

2 . Methods

2.1.1 . Isolation and culture of rat aortic smooth muscle cells

Rat aortic smooth muscle cells (RASMC) were freshly isolated from the aortae of male Wistar rats (approximately 250 g) and collected in complete culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin, as initially described by Campbell and Campbell (1993). Each aorta was transferred into a Class II tissue culture hood, cleaned of connective and other tissue and cut open to expose the lumen which was gently scraped with a scalpel blade to remove the layer of endothelial cells. The aorta was then cut into approximately 2 mm sections and placed in a T25 flask containing 5 ml of complete culture medium. The flasks were initially incubated vertically in a tissue culture incubator (37ºC and 5 % CO_2) to allow aortic explants to attach following an overnight incubation. Flasks were then placed horizontally, ensuring that explants were covered in medium. An extra 5 ml of media was added to the explants and incubated for a period of approximately 5 days. During this period, cells migrated from the explants and proliferated in the flask. These cells were subsequently trypsinized (see Section 2.1.4), transferred to a T75 flask and allowed to grow to confluency.

2.1.2 . Maintenance of cells in culture

Cell cultures were maintained in complete culture medium throughout with the medium replenished on alternate days. When required, cells were used for experimentation from passage 2 to 6.

2.1.3 . Isolation and culture of porcine aortic endothelial cells

Porcine aortae were collected and maintained in ice-cold DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 mg/ml gentamycin and 200 µg/ml fungizone. Each aorta was flushed with 1x phosphorous buffered saline (PBS) to remove blood and other residues in the lumen before filling with 0.5 mg/ml collagenase made up in PBS (1x). Each vessel was incubated for 10 minutes at 37ºC in a tissue culture incubator. Cell suspension in collagenase was collected and centrifuged at 4ºC for 5 minutes at 2000 rpm. The supernatant was discarded and the cell pellet resuspended in complete culture medium supplemented with gentamycin (0.25 mg/ml) and fungizone (0.02 µg/ml). Cultures were incubated over night at 37ºC (5 % CO_2) before replacing the medium with the standard complete culture medium. This procedure was based on protocols described previously by Bogle *at al*. (1991; 1996).

2.1.4 . Trypsinization of cells

Prior to each study, confluent monolayers of cells were trypsinized by standard procedure. The spent culture medium was removed and the cell monolayers washed twice with PBS (1x) before incubating with 3 ml of 1 % trypsin-EDTA for 5 minutes, at 37ºC. Each flask was observed at regular intervals over this period. Detached cells were resuspended in 5 ml of complete media, further diluted as required and then subdivided in cell culture flasks or plated for experimentation. Plated monolayers were allowed to grow to confluency before being used in various studies.

2.2 . Biochemical characterisation of rat aortic smooth muscle cells for α-actin

The RASMCs isolated were identified using monoclonal anti-α smooth muscle actin antibody and anti-mouse IgG FITC conjugated secondary antibody as described by (Skalli *et al.* 1986). Cells were plated in Lab-Tek® wells at sub-confluent density and allowed to grow to 80 – 100 % confluency. The cells were then washed twice with PBS (1x) to remove media and serum before being fixed with ice-cold methanol (99.8 %) for 45 seconds and rinsed once with ice-cold PBS (1x). Fixed cells were blocked for 20 minutes with a solution containing 5 % bovine serum albumin (BSA). A 1:50 dilution of the anti-α actin antibody was made up in blocking buffer (10 mM TRIS, 100 mM NaCl, 0.1 % Tween 20 and 5 % fat free milk) and

incubated with cells for 1 hour. Following the primary antibody incubation, cells were washed four times with PBS (1x), changing every 5 minutes and incubated for one hour with an anti-mouse IgG-FITC conjugated secondary antibody diluted 1:50 in blocking buffer. Cells were subsequently treated for 15 seconds with 30 % glycerol. This procedure was repeated using 50 % and 80 % glycerol solution respectively. Finally the cells were mounted in 2 to 3 drops of glycerol (100 %) and a cover slip was placed over the cells. A seal was made using nail varnish and visualised under the U.V. microscope.

2.3 . Experimental procedures

2.3.1 . Induction of nitric oxide synthesis in rat aortic smooth muscle cells

Cells were routinely activated with LPS 100 µg/ml and IFN–γ 100 U/ml by replacing spent media with fresh complete media containing LPS and IFN–γ. Induction of NO synthesis was determined 24 hours after induction by the Griess assay (Section 2.4).

2.3.2 . Treatment of cells with oxidative stress inducers

2.3.2.1 . Short-term incubations

Confluent monolayers of cells in 96 well plates were either incubated in culture medium alone or in medium containing LPS (100 µg/ml) and IFN-γ (100 U/ml) for 21 to 23.5 hours prior to exposure to H_2O_2 (1 to 1000 µM), diethyl maleate (DEM) (5 µM) or antimycin A (100 µM) for periods of 5 to 180 minutes. Activation of cells prior to treatment with OS inducers was confirmed by the detection of nitrite production using the Griess assay.

For studies involving H_2O_2, a 20 mM stock solution was initially prepared in autoclaved double distilled water (DDW) and then diluted serially in complete or in serum-free culture media. In order to determine whether phenol red present in DMEM would mask any pro-oxidant action of H_2O_2 additional dilutions were prepared in media lacking phenol red but supplemented with 10 % FBS and 100 U/ml penicillin, 100 µg/ml streptomycin.

2.3.2.2 . Long-term incubation

Confluent monolayers of cells in 6 or 96 well plates were washed twice with PBS (1x) at 37ºC and pre-incubated with either H_2O_2 (1 to 1000 µM), DEM (1 to 150 µM) or antimycin A (25 to 150 µM) for 30 minutes before incubating in complete culture medium alone or in medium containing LPS (100 µg/ml) and IFN–γ (100 U/ml) for a further 24 hours. Nitrite production was determined by the Griess assay.

2.3.3 . Treatment of cells with statins

Confluent monolayers of cells in 6 or 96 well plates were washed twice with PBS (1x) at 37ºC and then incubated with complete media containing atorvastatin, pravastatin or simvastatin over the concentration range of 1 - 100 µM for 30 minutes. After this incubation period, the cells were activated with LPS (100 µg/ml) and IFN–γ (100 U/ml) co-incubated with a select statin for 24 hours.

Atorvastatin was prepared directly in DMEM at the concentration of 0.4 mM, and then serially diluted down to the concentration range of 1 µM to 100 µM. Simvastatin was prepared in 75 % ethanol and this solution used to prepare the final concentration solutions from 1 µM to 100 µM in complete medium. Pravastatin was diluted in sterile DDW at the concentration of 55 mM and in complete DMEM subsequently at the required concentrations.

2.3.4 . Treatment of cells with statins and oxidative stress inducers

Confluent monolayers of cells in 96 well plates were washed twice with PBS (1x) at 37ºC and then pre-incubated for 30 minutes with complete media containing atorvastatin, pravastatin or simvastatin over the concentration range of 1 - 100 µM. Cells were subsequently exposed to DEM (5 and 100 µM) or antimycin A (100 µM) for a further 30 minutes prior to activating with LPS (100 µg/ml) and IFN–γ (100 U/ml) and co-incubated with the statins for 24 hours.

When used, H_2O_2 was prepared initially in sterile MilliQ water at a concentration of 100 µM and dilution in DMEM supplemented with FBS at concentrations ranging from 0.01 µM to 1000 µM. DEM was prepared initially in ethanol 50 % at the concentration of 50 mM and then serially diluted in complete medium to obtain concentrations of 5 µM to 150 µM. Antimycin A was diluted in ethanol 75% and then in complete DMEM to obtain concentrations ranging from 25 µM to 150 µM.

2.3.5 . Treatment of cells with statins and mevalonate

To determine whether the effects of statins could be reversed by mevalonate, confluent monolayers of cells in 96 well plates were washed twice with PBS (1x) at 37ºC and then pre-incubated for 30 minutes with complete media containing mevalonate (10 – 100 µM). A stock concentration of 0.8 mM mevalonate was

prepared in 75 % ethanol and subsequently diluted in complete DMEM. The cells were then exposed to atorvastatin, pravastatin or simvastatin (each at 10 μM) for a further 30 minutes before subsequent activation with LPS (100 μg/ml) and IFN–γ (100 U/ml) for 24 hours.

2.4 . Measurement of nitric oxide production

Nitric oxide production was measured by the standard Griess assay (Green *et al.* 1982) as described by Wileman *et al*. (1995). This assay relies on a diazotization reaction that was originally described by Griess in 1879 and detects NO_2^- in solution as shown in Figure 2.1.. In our studies, a 100 μl of media was removed from each well and transferred to the inner wells of a new 96 well plate. For studies carried out in 6 well plates, 100 μl of media was removed from each well in triplicates and transferred to a new 96 well plate. Sodium nitrite standards (1 to 10 pmoles/well) made up in complete culture medium were transferred to the outer wells and all samples were incubated at room temperature for 15 minutes with 100 μl of Griess reagent (2 % sulfanilamideamine and 0.2 % napthylethylenediamine plus 10 % phosphoric acid). The absorbance of each well was read at 540 nm on a microplate Multiskan Ascent (Labsystems) reader and the levels of nitrite in the media from cells determined using the standard curve constructed. A representative sodium nitrite standard curve is shown in Figure 2.2.

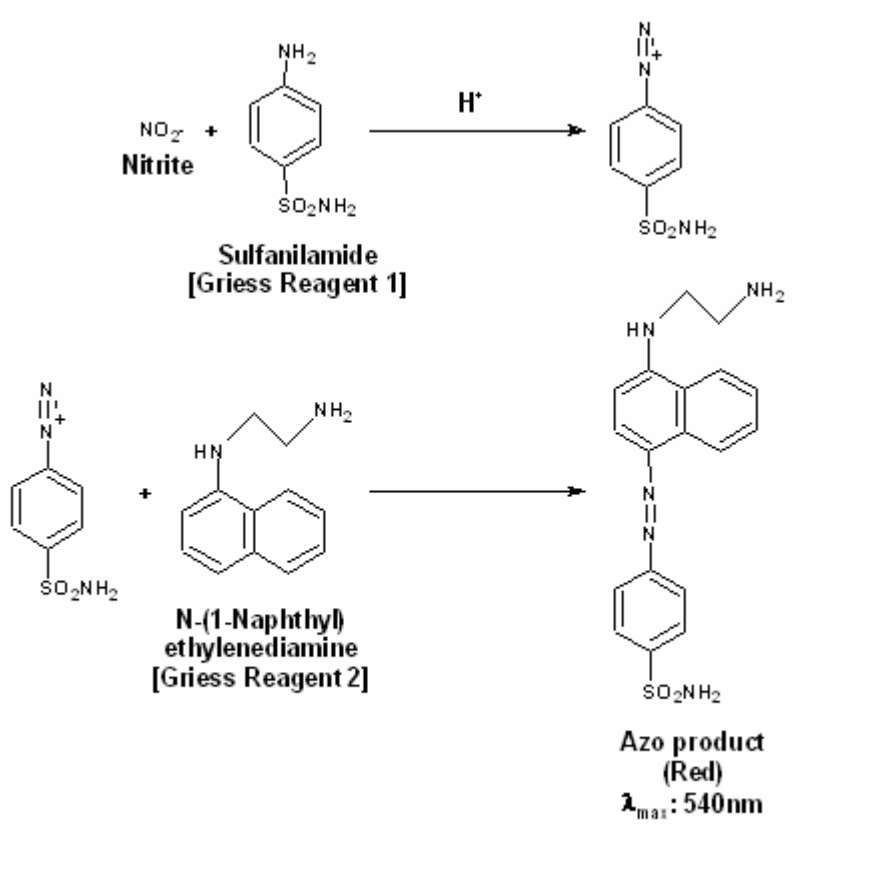


Figure 2.1. Detection of NO_2^- in solution by the Griess assay.

Nitrite is diazotized in sulfanilic acid and the product is then azo coupled with 1-naphthylamine to produce a coloured chromophore.

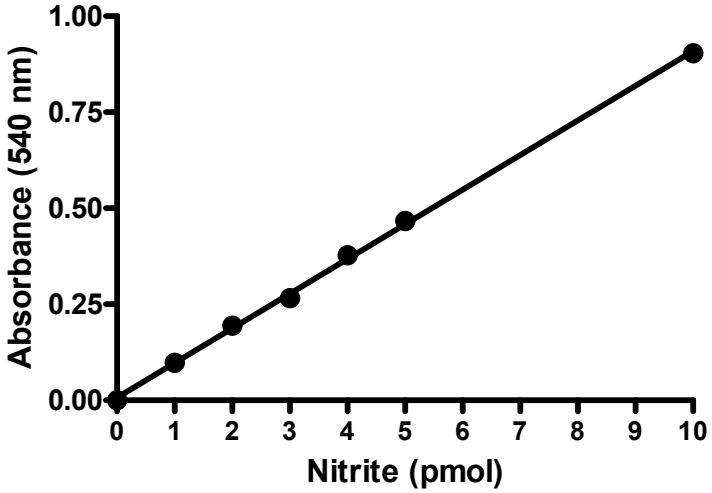


Figure 2.2. A representative nitrite standard curve.

Standards ranging from 1 to 10 pmoles per well were prepared in complete culture medium. A 100 µl aliquot from each standard was added in triplicate to the outer wells of a 96 well plate and incubated for 15 minutes with an equal volume of the Griess reagent. Absorbance values were taken at 540 nm on a Multiskan Ascent (Labsystems) plate reader. The data shown is the mean of three replicates for each concentration.

2.5 . Protein quantification using bicinchoninic acid

The bicinchoninic acid assay (BCA) is based on the Biuret reaction and chelation of a cuprous cation as shown in Figure 2.3.. The method combines the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the chelation of Cu^{+1} by the BCA reagent to form a purple-coloured product.

For the determination of total cell protein in our studies, 50 µl of DDW was added to each well containing cells. Protein standards (1 to 30 µg/ml) were made from a 1 mg/ml bovine serum albumin (BSA) stock solution in DDW and 50 µl of each standard was added in triplicates to the outer wells of the plate. The BCA reagent was prepared as instructed by the manufacturers by mixing BCA reagent B plus BCA reagent A at a ratio of 1/20 (v/v). A 100 µl aliquot of the reagent was added to each well and the plate incubated with mild shaking for 40 minutes and read at 620 nm on a Multiskan Ascent (Labsystems) plate reader. The absorbance values of the samples were converted to protein using the BSA standard curve constructed (Figure 2.4.). For the western blot protein quantification, 5 µl aliquot of each sample was taken and added to 50 µl of double distilled water in the inner wells of a 96 well plate. A 5 µl aliquot of lysis buffer was also added to each protein standard in the outer wells prior to adding 100 µl of the BCA reagent. Absorbance readings were read on a plate reader after 40 minutes at 620 nm.

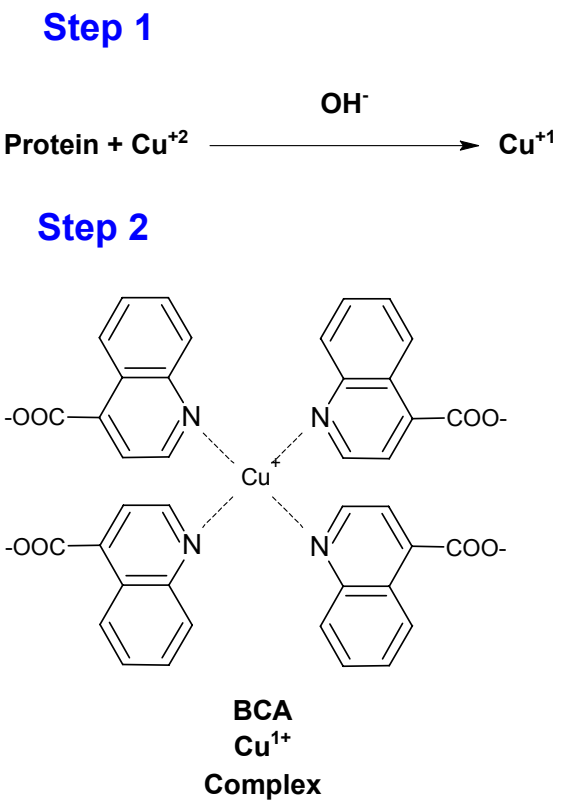


Figure 2.3. Protein detection method by the BCA method.

The peptides bind and reduce Cu^{2+} ions from the cupric sulfate to Cu^{1+}. The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. Two molecules of bicinchoninic acid chelate with each Cu^{1+} ion, forming a purple-coloured product that strongly absorbs light at a wavelength of 620 nm.

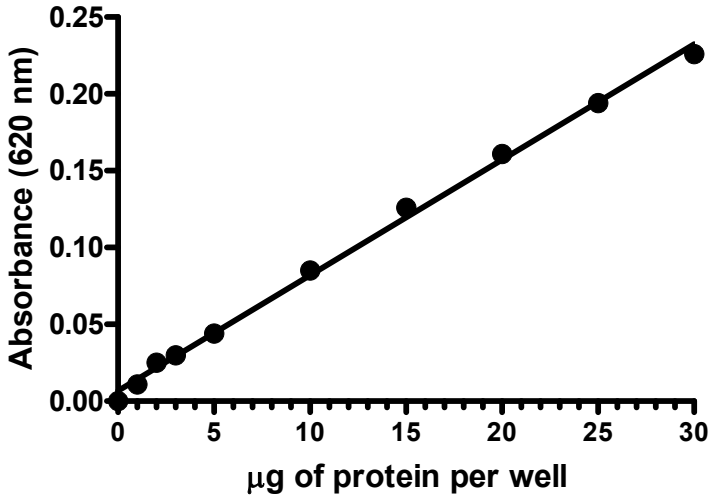


Figure 2.4. A representative protein standard curve.

Standards of bovine serum albumin (BSA) ranging from 1 to 30 μg per well were prepared in DDW. A 50 μl aliquot from each standard was added in triplicate to the outer wells of a 96 well plate and incubated for 45 minutes with 100 μl of the BCA reagent. Absorbance values were taken at 620 nm on a Multiskan Ascent (Labsystems) micro plate reader. The data shown is the mean of three replicates for each concentration.

2.6 . Measurement of [^{3}H]L-arginine transport into cells

Transport of [^{3}H]L-arginine was performed as described by Wileman *et al*. (1995). Briefly, plates were washed twice with 200 µl Krebs buffer (131 mM NaCl, 5.6 mM KCl, 25 mM $NaHCO_3$, 1 mM NaH_2PO_4, 5.5 mM D-glucose, 20 mM HEPES, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$) at 37ºC. Transport was initiated by the addition of 50 µl of transport buffer (Krebs buffer containing 1 µCi/ml [^{3}H]L-arginine plus 100 µM unlabelled L-arginine) to the cells at 37^{o}C.

In time-course studies, cells were incubated with the transport buffer for varying periods ranging from 30 seconds to 60 minutes. All other experiments were carried out over 2 minutes. The uptake of L-arginine is linear at this time point, allowing for the analysis of transport rates under unidirectional flux. At the end of the incubation period, transport was terminated by placing the plates on ice and washing 3x with ice-cold Krebs buffer containing 10 mM L-arginine. The plates were kept on ice until analysed for protein content.

The protein quantification was performed as described above (section 2.5) and the content of each well was transferred into a scintillation vial. Scintillation liquid (4 ml) was added to each vial, mixed on a vortex and counted on a Beckman Coulter^{TM} LS6500 β-scintillation counter. Blank disintegrations per minute (DPMs) were subtracted from each sample and the remaining DPMs converted to pmoles of L-arginine/µg protein/minute. The DPM value obtained from each sample was divided by the corresponding µg of protein (detected previously as described in section 2.5) and by the duration of incubation.

2.7 . Western blot analysis of inducible nitric oxide synthase and heme oxygenase-1 expression

Rat cultured smooth muscle cells were plated in 6 well plates and allowed to grow to confluency prior to treatment with drugs. At the end of the treatment period, wells were washed with ice-cold PBS and the cells lysed with 200 µl of lysis buffer (10 mM TRIS, 10 % sodium dodecyl sulphate (SDS), pH 7.4) containing 200 µM sodium orthovanadate (Na_3VO_4), 200 µM phenyl methyl sulfonyl fluoride (PMSF) and 1 mM sodium fluoride (NaF). The cells were scraped and transferred into an eppendorf tube. The samples were heated at 95ºC for 5 minutes and sonicated three times for 30 seconds. The protein quantification was performed as described above (Section 2.5). Samples were subsequently diluted with a 1:1 volume of loading buffer (250 µM TRIS, 4 % SDS, 10 % glycerol, 2 % β-mercaptoethanol, 0.006 % bromophenol blue, pH 6.8).

Samples were loaded onto a mini gel (stacking gel: 0.5 M TRIS, 4 % acrylamide, 10 % SDS, 10 % APS, 6.6 mM TEMED, pH 6.8; resolving gel: 1.5 M TRIS, 12 or 8 % acrylamide, 10 % SDS, 10 % ammonium persulphate (APS), 4 mM TEMED, pH 8.8) and run at 100 V for approximately 60 minutes. The proteins in the gel were then transferred to a polyvinylidene difluoride (PVDF) membrane that was pre-soaked in methanol (99.8+%) for 15 seconds prior to blotting. The membrane was placed in the transfer cells on a blotting filter paper followed by the gel and a second filter paper (see Figure 2.5.). The proteins were transferred for 90 to 150 minutes at 140 mA per cm^2 of membrane. The gel was stained in Coomassie brilliant blue reagent (0.1 % (w/v) Coomassie blue R350, 20 % (v/v) methanol, and 10 % (v/v) acetic acid) to check the efficiency of protein transferred to the membrane. The

gel was then incubated in destaining solution (50 % (v/v) methanol in water with 10 % (v/v) acetic acid). The membrane itself was blocked in blocking buffer (10 mM TRIS, 100 mM NaCl, 0.1 % Tween 20 and 5 % fat free milk) for a minimum of 60 minutes at room temperature with mild shaking, to prevent non-specific binding of antibodies to the membrane. An anti-iNOS monoclonal antibody was diluted in blocking buffer 1:2500 and incubated with the membrane over night. For detection of HO-1, a mouse monoclonal anti HO-1 antibody was diluted in blocking buffer 1:250 and incubated with the membrane for 48 hours at 4 ºC. An anti-β actin monoclonal antibody diluted 1:5000 was also added to the membrane to detect β-actin, except for experiments detecting HO-1. The latter was used to determine the equal loading of samples into the wells. The membrane was washed for 30 minutes, changing washing buffer (10 mM TRIS, 100 mM NaCl, pH 7.5) every 5 minutes. This was followed by further incubation of the membrane with the secondary antibody (goat polyclonal to mouse IgG) diluted 1:5000 dilution in blocking buffer. A horse radish peroxidase (HRP)-conjugate secondary antibody to the molecular weight marker diluted 1:500 was also used. These antibodies were incubated with membranes for 60 minutes. At the end of this incubation period, the membrane was again washed for 30 minutes changing washing buffer every 5 minutes. Protein bands were detected using the enhanced chemiluminescence (ECL) detection method.

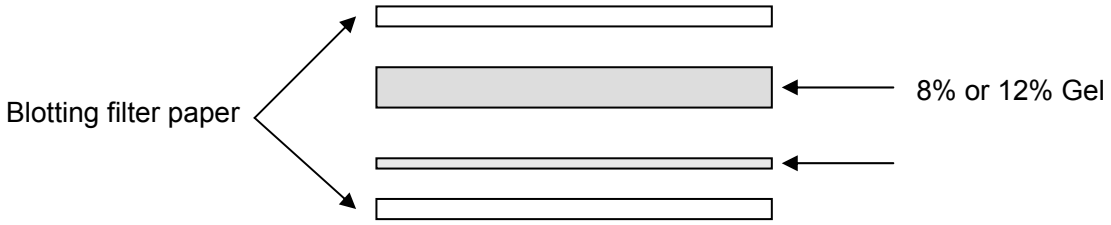


Figure 2.5. Transfer to PVDF membrane.

Scheme of protein transfer from the gel to the PVDF membrane in the semi-dry transfer cell.

2.8 . Determination of cell viability by the MTT assay

Cell viability was routinely determined by monitoring the metabolism of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan by viable cells (See Figure 2.6.). This reduction takes place only when mitochondrial reductase enzymes are active, and thus conversion is directly related to the number of viable cells. The production of purple formazan in cells treated with an agent is measured relative to the production in control cells.

In our experiments, confluent monolayers of cells were treated according to the designated experimental protocol and then exposed to MTT. A 20 µl aliquot of 0.5 mg/ml stock solution of MTT was added to cells and the plate incubated for 4 hour at 37ºC in a tissue culture incubator. Isopropanol (100 µl) was subsequently added to each well and incubated for a further 10 minutes. The plate was read at 540 nm, after mixing on the plate shaker.

2.9 . Statistical analysis

The data is normally the mean$\pm$ standard error mean (S.E.M.) of at least three independent experiments with 3 or 6 replicates per experiments unless otherwise stated. Where indicated, statistical analysis was carried out using the Mann-Whitney test, a one way ANOVA and a two way ANOVA test.

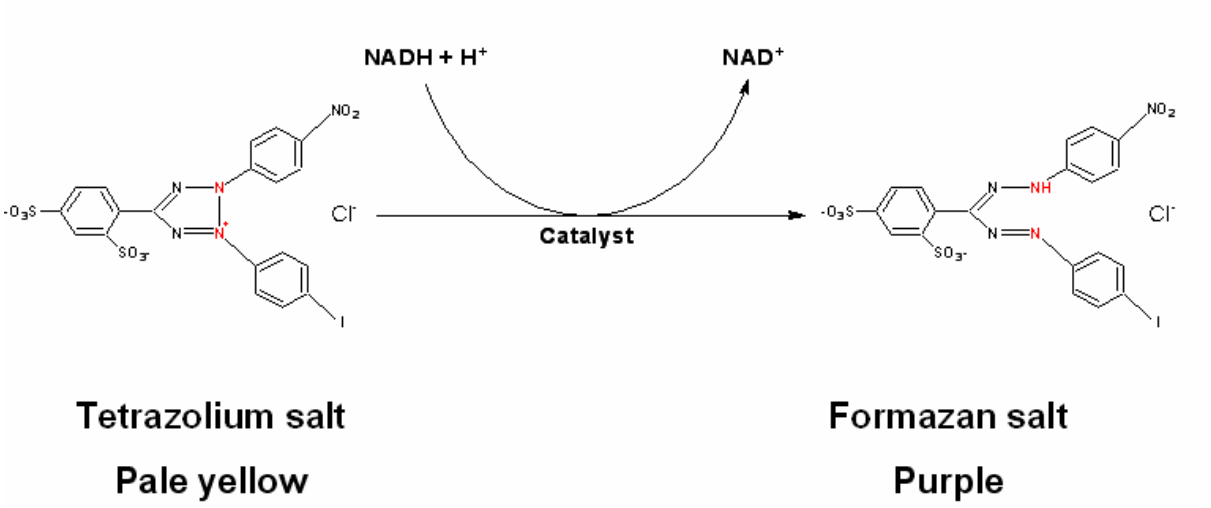


Figure 2.6. Metabolism of MTT to formazan by cells in culture.

Yellow MTT is reduced to purple formazan in living cells by common coenzymes NADH and NADPH that absorb U.V. light in their reduced forms, but do not in their oxidised forms. An ethanol solution, is added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this coloured solution can be quantified by measuring at a certain wavelength (540 nm). The metabolism by mitochondrial reductase into purple formazan was described by (Mosmann 1983).

3 . Results

3.1 . Biochemical characterization of rat cultured aortic smooth muscle cells

The characteristics of the cells isolated from rat or porcine aorta were determined morphologically. Rat aortic smooth muscle cells (RASMCs) were further biochemically characterized to ensure that studies were carried out on the required cell type. As described in the methods, SMCs were identified by staining for α-actin. As shown in Figure 3.1A, the confluent smooth muscle layer appear epithelioid in morphology consistent with a population of SMCs described in previous reports (Groves *et al.* 2005; Orlandi *et al.* 2005). The porcine aortic endothelial cells (PAECs) appeared cobbled-stoned but with the increase of passage number turned more elongated (Figure 3.1B).

Further biochemical characterisation revealed the positive α-actin staining of the SMCs (Figure 3.2). Moreover, cross staining of these cells with dil-labelled-acetylated-low density lipoprotein (Dil-Ac-LDL) (Voyta *et al.* 1984) did not show any positive staining for endothelial cells suggesting pure populations of smooth muscle cultures with little or no cross contamination.

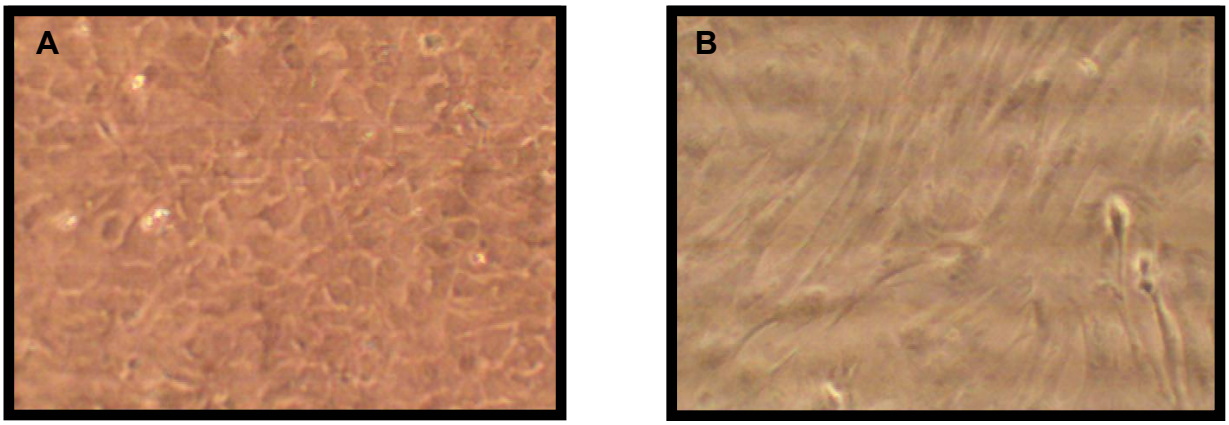


Figure 3.1. Morphology of rat cultured aortic smooth muscle cells (A), and porcine aortic endothelial cells (B).

Freshly isolated cells were cultured to confluency in a T-75 tissue culture flask as described in methods and then viewed under an inverted light microscope at 100X magnification.

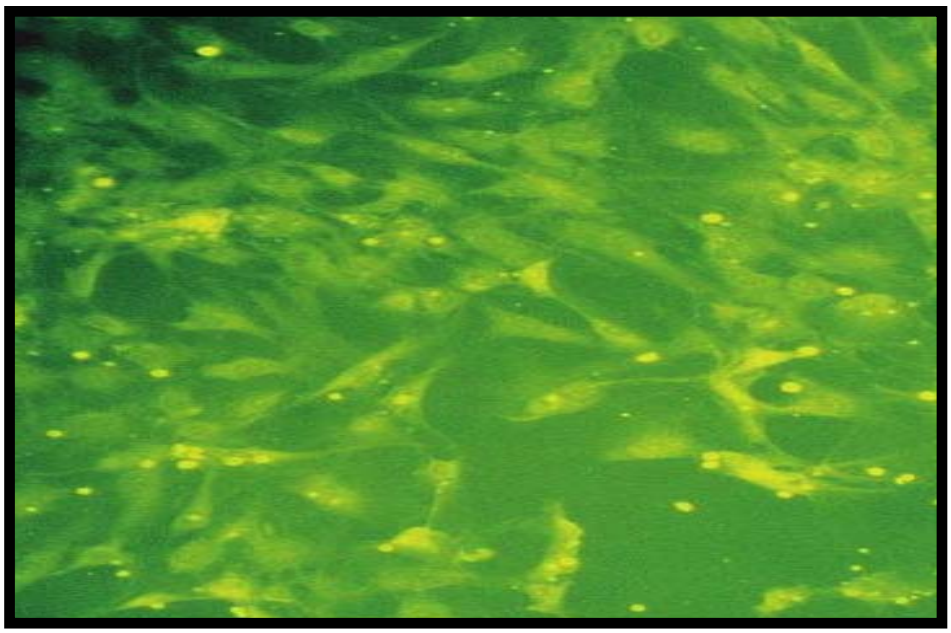


Figure 3.2. α-actin staining of rat cultured aortic smooth muscle cells.

Confluent cultures of rat aortic smooth muscle cells in Lab-Tek® wells were treated for α-actin staining as described in the Methods (Section 2.2) and viewed under U.V. microscope using a FITC filter at 400X magnification. The above picture is representative of at least three separate experiments carried out.

3.2 . Characterisation of L-arginine transport in porcine cultured aortic endothelial cells

Studies were carried out to determine the characteristics of the transporter(s) responsible for L-arginine uptake in these cells. This information is currently not available in the literature and is required in order to have a better understanding of the precise transporter potentially regulated by OS in cardiovascular disease states. Thus, experiments were aimed at determining the time-course and kinetic of uptake. Although cross-inhibition studies were planned to identify the transporters associated with L-arginine uptake, these experiments were not carried out because of the lack of subsequent aortae supply.

Represented in Figure 3.3 is the time-course of uptake of [^{3}H]L-arginine into PAECs. As shown in the graph, L-arginine was transported in a time-dependent manner, saturating at 60 minutes. This is consistent with studies in other cell types including RASMCs (Wileman *et al.* 1995). Kinetic analysis revealed that L-arginine uptake is not saturable even when substrate concentrations of up to 5 mM were used (Figure 3.4). This would indicate the expression of a high capacity carrier but further studies are required to conclusively demonstrate it.

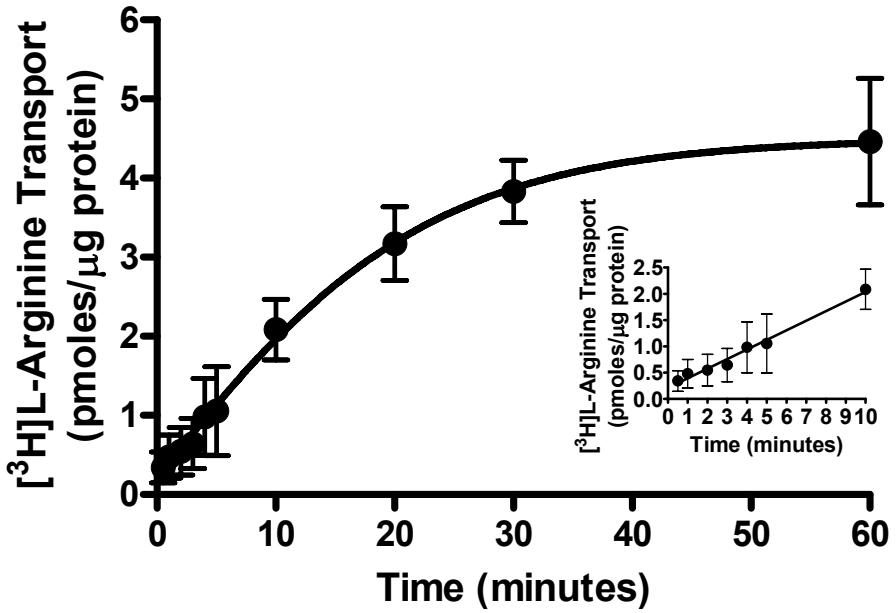


Figure 3.3. Time-course of L-arginine transport in PAECs.

Confluent monolayers of PAECs in 96 well plates were incubated with 100 µM L-arginine plus 1 µCi/ml [^{3}H]L-arginine for the time periods shown on the graph. Incubations were terminated after each time point and the cells processed as described in the Methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

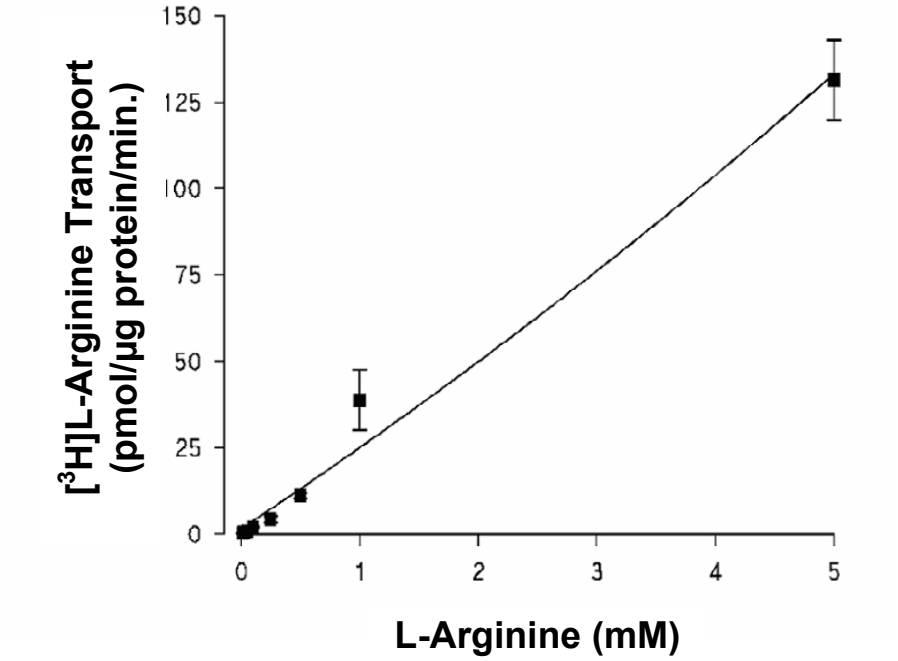


Figure 3.4. Kinetics of L-arginine transport in PAECs.

Confluent monolayers of PAECs in 96 well plates were incubated with transport buffer containing the concentrations of L-arginine indicated on the graph plus 1 µCi/ml [^{3}H]L-arginine. Incubations were terminated after 2 minutes and the cells processed as described in the Methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

3.3 . Effect of H_2O_2 on L-arginine transport in porcine cultured aortic endothelial cells

Cells were pre-exposed in an initial study to 0.01 – 100 μM of H_2O_2 but as these concentrations were found to have little or no effect (Figure 3.5), further experiments were carried out using H_2O_2 at 10 – 1000 μM and in the presence and absence of 10 % serum for periods ranging from 5 minutes to 180 minutes prior to initiating transport (Figure 3.6). The data represent single experiments and shows some degree of variability between conditions. Thus, it is difficult to draw any definite conclusions at this stage without increasing the n values. However, the indication from the preliminary data is that H_2O_2 may suppress L-arginine uptake when incubated with cells for 180 minutes at 1 mM. Preliminary cytotoxicity assays revealed that H_2O_2 may be cytotoxic at this concentration (Figure 3.7) and the effects observed may therefore be non-specific.

Figure 3.5. Time and concentration dependent effects of H_2O_2 on L-arginine transport in PAECs.

Confluent monolayers of PAECs in 96 well plates were incubated in complete culture medium and exposed to increasing concentrations of H_2O_2 for periods of 5 (Panel A), 15 (Panel B), 30 (Panel C), 60 (Panel D) and 180 (Panel E) minutes. L-arginine transport was carried out as described in the Methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

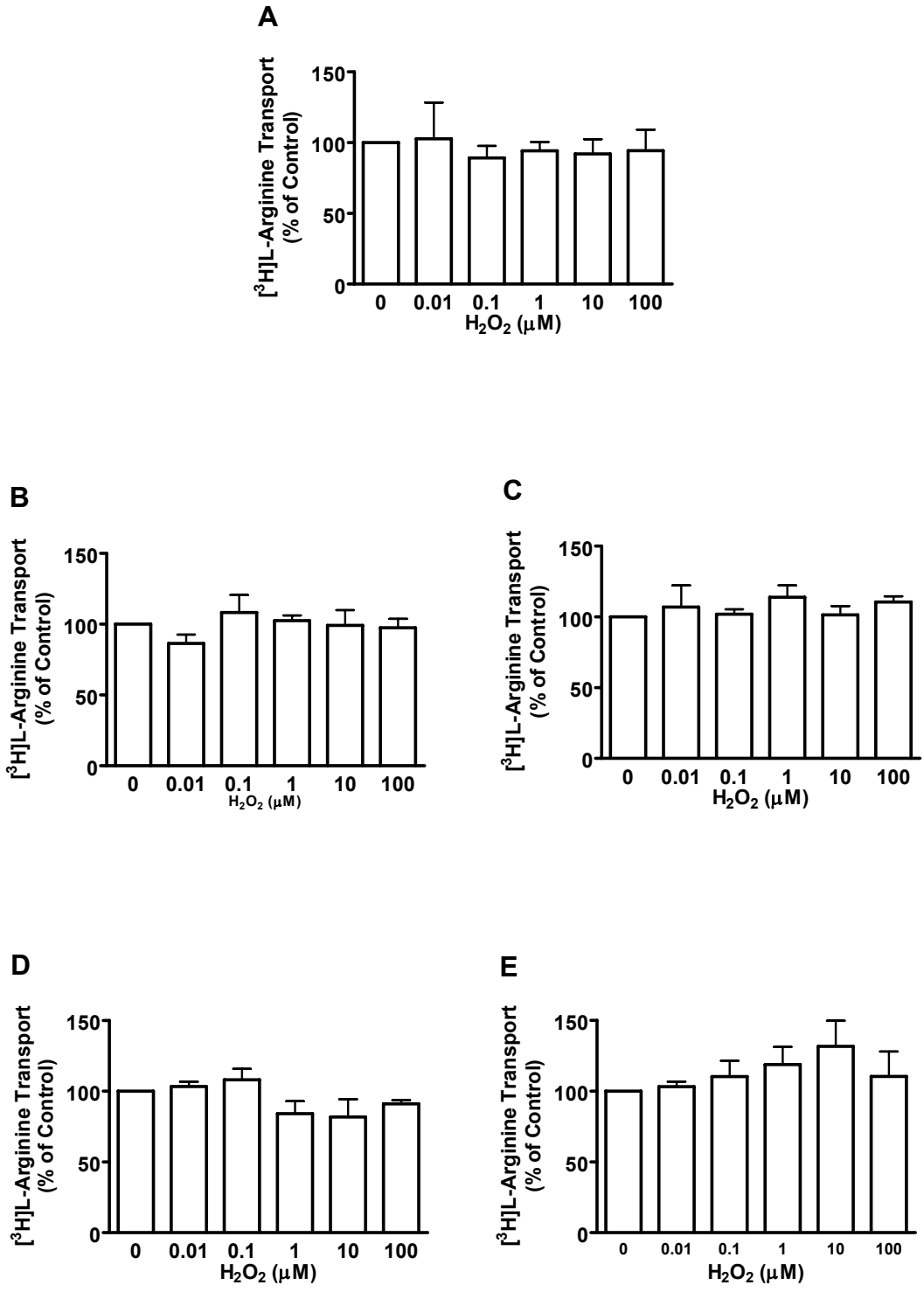

A
[3H]L-Arginine Transport (% of Control)
150
100
50
0
0 0.01 0.1 1 10 100
H2O2 (μM)
B
[3H]L-Arginine Transport (% of Control)
150
100
50
0
0 0.01 0.1 1 10 100
H2O2 (μM)
C
[3H]L-Arginine Transport (% of Control)
150
100
50
0
0 0.01 0.1 1 10 100
H2O2 (μM)
D
[3H]L-Arginine Transport (% of Control)
150
100
50
0
0 0.01 0.1 1 10 100
H2O2 (μM)
E
[3H]L-Arginine Transport (% of Control)
150
100
50
0
0 0.01 0.1 1 10 100
H2O2 (μM)

Figure 3.6. Time and concentration dependent effects of H_2O_2 on L-arginine transport in control and activated PAECs in complete media or without FBS.

Confluent monolayers of PAECs in 96 well plates were either incubated in complete culture medium (Controls) or culture medium with 0 % FBS. Exposure to increasing concentrations of H_2O_2 was performed for periods of 5 (Panel A), 15 (Panel B), 30 (Panel C), 60 (Panel D) and 180 (Panel E) minutes. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.D. of one single experiment with at least 3 replicates in each condition.

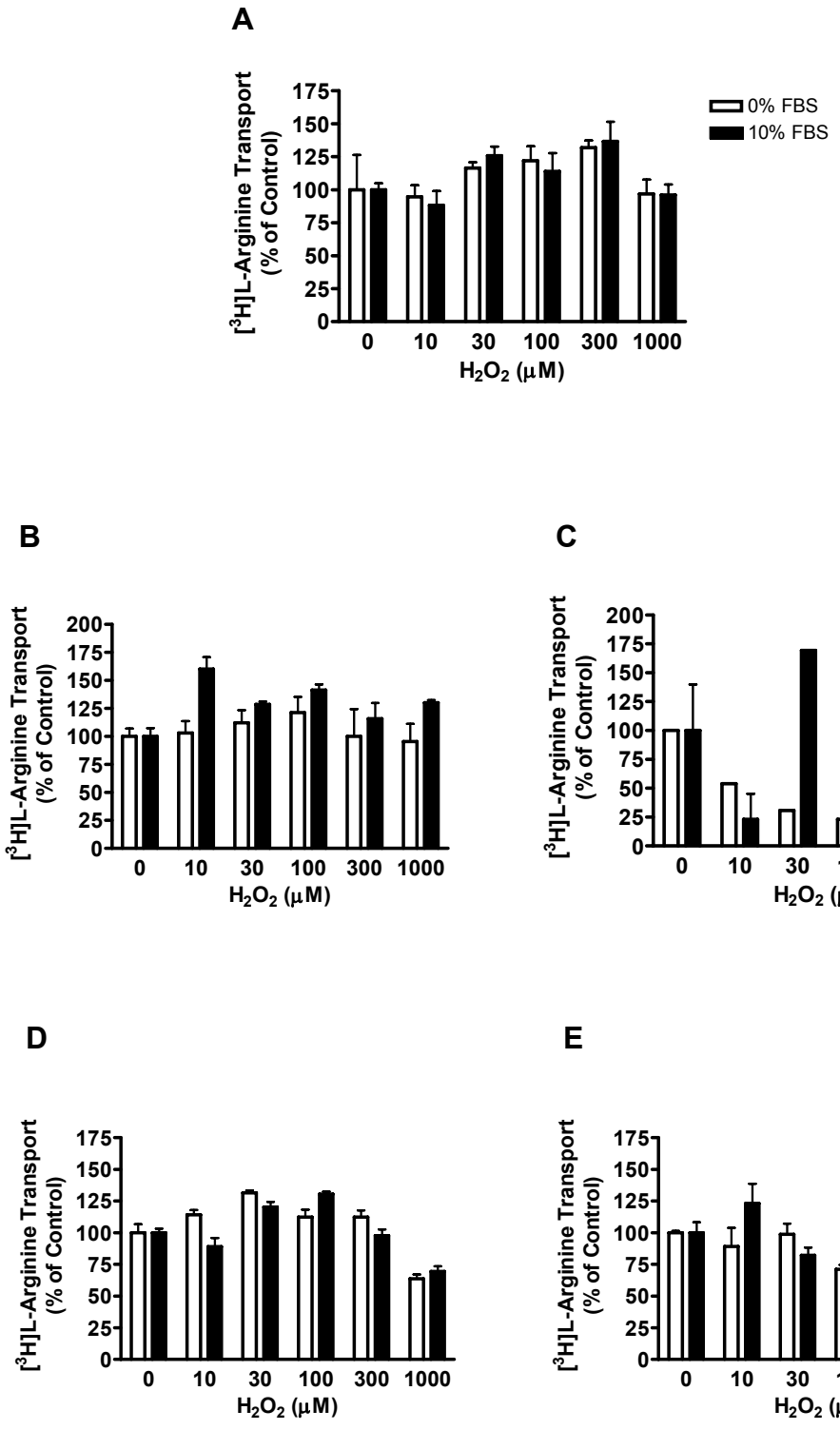
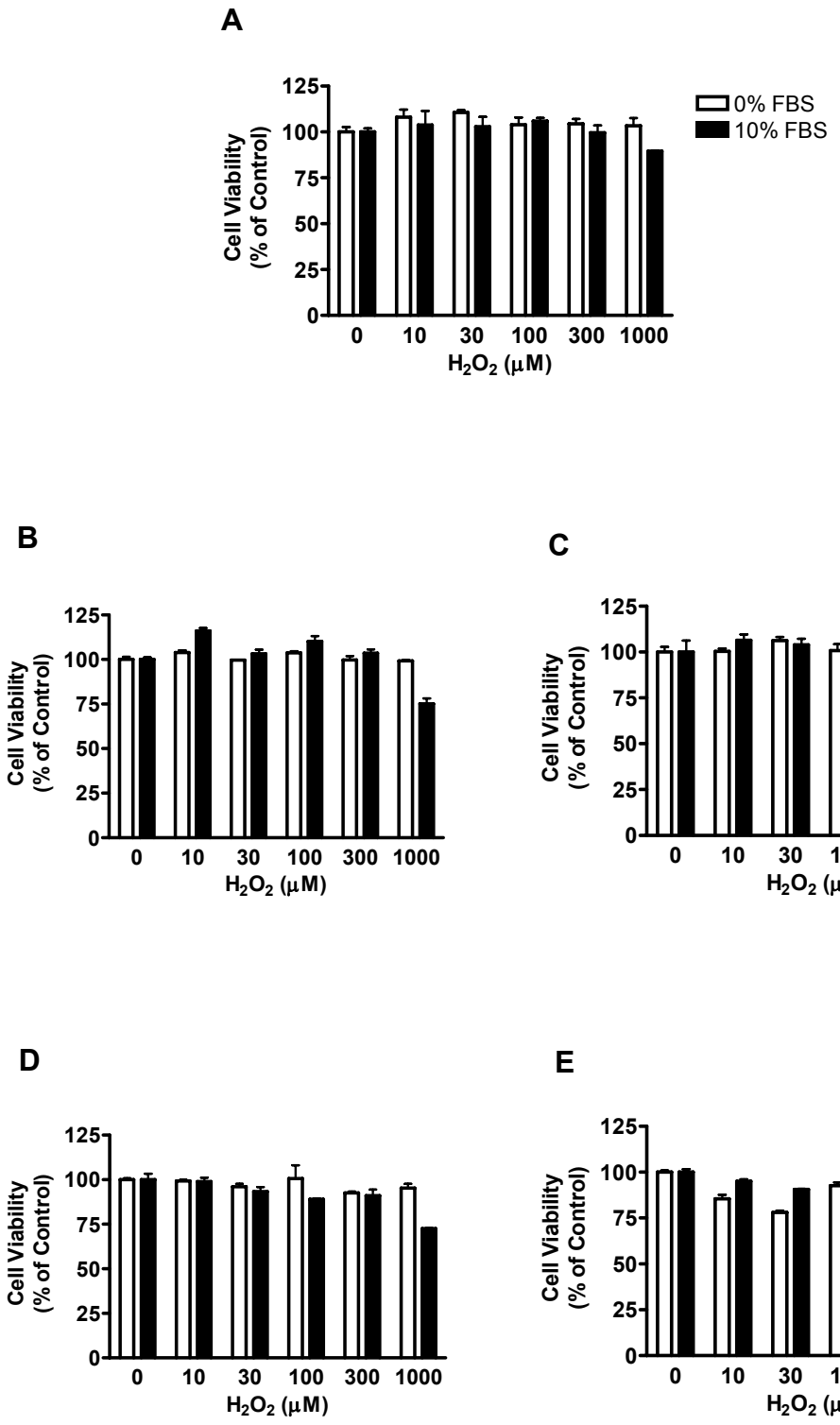

A
[3H]L-Arginine Transport (% of Control)
175
150
125
100
75
50
25
0
0% FBS
10% FBS
0 10 30 100 300 1000
H2O2 (μM)
B
[3H]L-Arginine Transport (% of Control)
200
175
150
125
100
75
50
25
0
0 10 30 100 300 1000
H2O2 (μM)
C
[3H]L-Arginine Transport (% of Control)
200
175
150
125
100
75
50
25
0
0 10 30 100 300 1000
H2O2 (μM)
D
[3H]L-Arginine Transport (% of Control)
175
150
125
100
75
50
25
0
0 10 30 100 300 1000
H2O2 (μM)
E
[3H]L-Arginine Transport (% of Control)
175
150
125
100
75
50
25
0
0 10 30 100 300 1000
H2O2 (μM)

Figure 3.7. Effects of H_2O_2 on viability of PAECs.

Confluent monolayers of PAECs in 96 well plates were either incubated in complete culture medium (Controls) or culture medium with 0 % FBS. Exposure to increasing concentrations of H_2O_2 was performed for periods of 5 (Panel A), 15 (Panel B), 30 (Panel C), 60 (Panel D) and 180 (Panel E) minutes. Cell viability was monitored as described in the methods (Section 2.8). The data represents the mean ± S.D. of one single experiment with at least 3 replicates in each condition.


A
125
100
75
50
25
0
Cell Viability
(% of Control)
0% FBS
10% FBS
0
10
30
100
300
1000
H2O2 (μM)
B
C
D
E

3.4 . Induction of nitric oxide synthesis and L-arginine transport in rat cultured aortic smooth muscle cells

Confluent monolayers of RASMCs in 96 well plates were incubated with either complete culture medium alone or with medium containing LPS (100 µg/ml) and IFN-γ (100 U/ml) for 24 hours. As expected, exposure of cells to LPS and IFN-γ resulted in the induction of NO synthesis. Consistent with many other studies, control RASMCs produced very little detectable nitrite (approximately 0.01 pmol/µg protein/24 hours) but the latter was significantly induced with average levels of 0.1 pmol/µg protein/24 hours being detected following exposure to LPS and IFN-γ.

In parallel with the induction of iNOS, L-arginine transport was also significantly increased in cells exposed to LPS and IFN-γ with rates increasing from 1 pmoles/µg protein/minute in controls to 2 pmoles/µg protein/minute in activated cells.

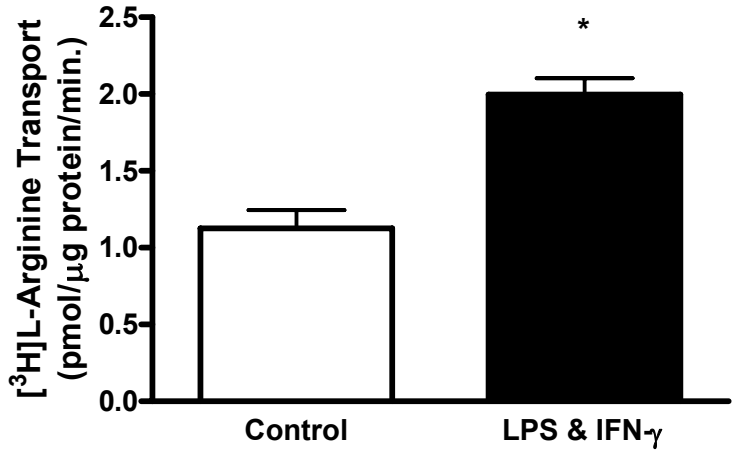


Figure 3.8. Effects of LPS and IFN-γ on transport in RASMCs.

Confluent monolayers of RASMCs in 96 well plates were either incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for at least 21 hours prior to transport studies. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). Values are expressed as the mean ± S.E.M. of 3 separate experiments with at least 3 replicates in each. *denotes $p<0.0001$ when compared to the control.

3.5 . Effects of oxidative stress inducers on L-arginine transport and NO production in rat cultured aortic smooth muscle cells

3.5.1 . Effects of acute exposure to oxidative stress inducers

In order to determine the effects of H_2O_2 on both control and induced transporter activity, cells were exposed to LPS and IFN-γ for at least 21 hours before exposure to H_2O_2 at concentrations of 10 to 1000 μM for periods of 5, 15, 30, 60 and 180 minutes prior to carrying out transport studies. Induction of cells was confirmed by determining nitrite levels in the culture medium 21 hours after activation. Activation of cells was successfully achieved in the experiments presented.

H_2O_2 was able to inhibit L-arginine transport in both control and activated cells but only when applied at a concentration of 1 mM and for 180 minutes prior to initiating transport. Under these conditions transport rates were inhibited by 66$\pm$23 % in control and 54$\pm$5 % in activated cells respectively (Figure 3.9). H_2O_2 was however without effect at any of the other conditions or concentrations used (Figure 3.9 A-D). Moreover, the inhibitions caused by H_2O_2 (1 mM; 180 minutes pre-incubation) may reflect a non selective cytotoxic effect as this was associated with a marked decrease in viability in both control and activated cells (Figure 3.10 E).

Phenol red is known to possess antioxidant properties (Grzelak *et al.* 2001; Briante *et al.* 2003; Lewinska *et al.* 2007), which may mask the effects of H_2O_2. Similarly, FBS may quench free radicals, thereby preventing any actions of oxidative stress inducers (Grzelak *et al.* 2001; Lewinska *et al.* 2007). Thus parallel

experiments were performed in media lacking FBS (Figure 3.11), phenol red (Figure 3.13) or both (Figure 3.14). As shown in Figure 3.11 and 21, removal of either component from the culture medium did not significantly alter the effects of H_2O_2 on L-arginine transport. Elimination of both FBS and phenol red did not show inhibition of transport when either control or activated cells were subsequently exposed to H_2O_2 (Figure 3.14). Omitting FBS from the culture medium did not cause any significant cytotoxicity to the cells (Figure 3.12).

Figure 3.9. Time and concentration dependent effects of H_2O_2 on L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were either incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for at least 21 hours prior to exposure to increasing concentrations of H_2O_2 for periods of 5 (Panel A), 15 (Panel B), 30 (Panel C), 60 (Panel D) and 180 (Panel E) minutes. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

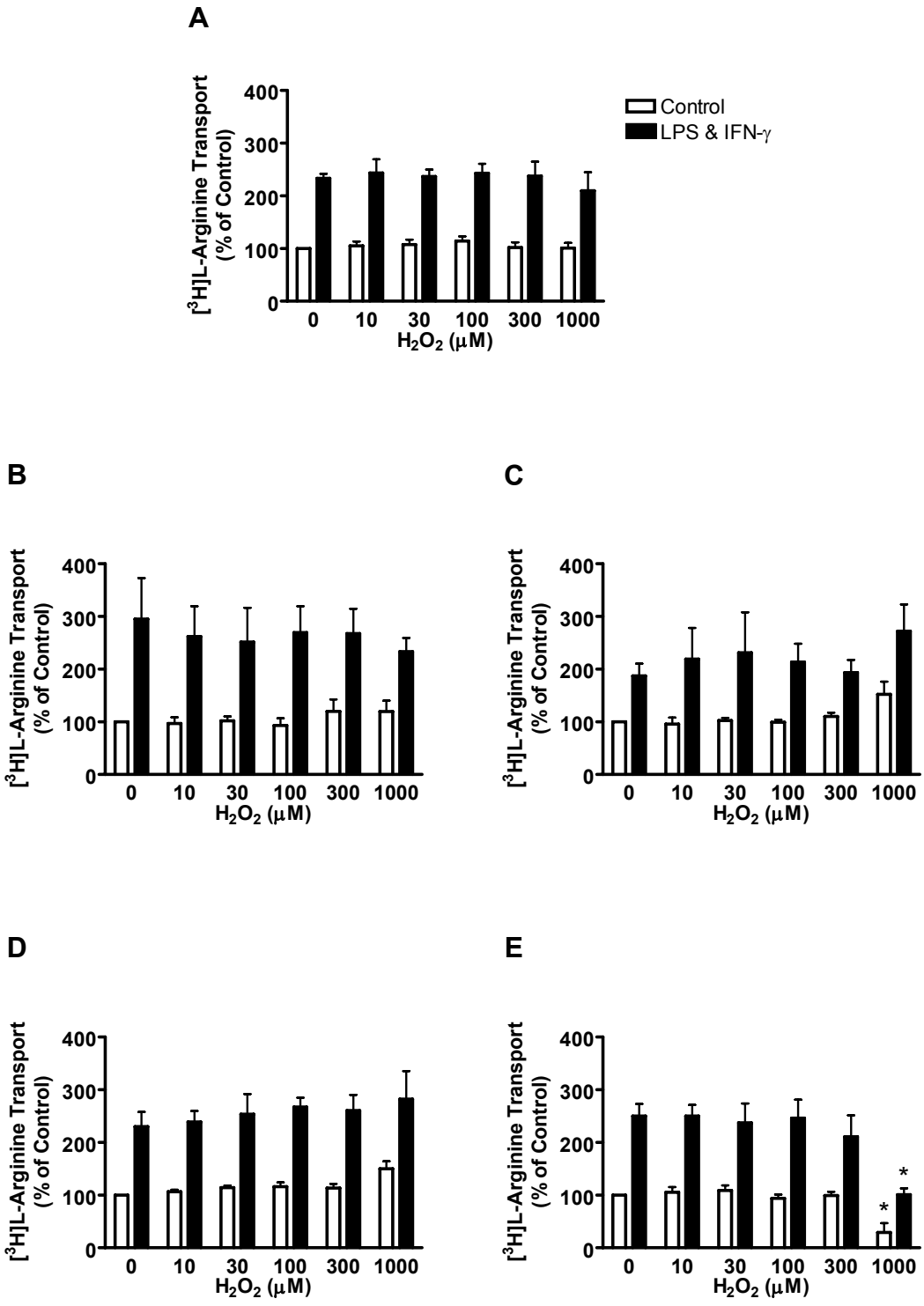

A
[3H]L-Arginine Transport (% of Control)
400
300
200
100
0
0 10 30 100 300 1000
H2O2 (μM)
Control
LPS & IFN-γ
B
C
D
E
*
*

Figure 3.10. Time and concentration dependent effects of H_2O_2 on viability of RASMCs.

Confluent monolayers of RASMCs in 96 well plates were either incubated in complete culture medium alone (Controls) or in medium containing LPS (100 µg) and IFN-γ (100 U/ml) for at least 21 hours prior to exposure to increasing concentrations of H_2O_2 for periods of 5 (Panel a), 15 (Panel B), 30 (Panel C), 60 (Panel D) and 180 (Panel E) minutes. Cell viability was monitored as described in the methods (Section 2.8). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

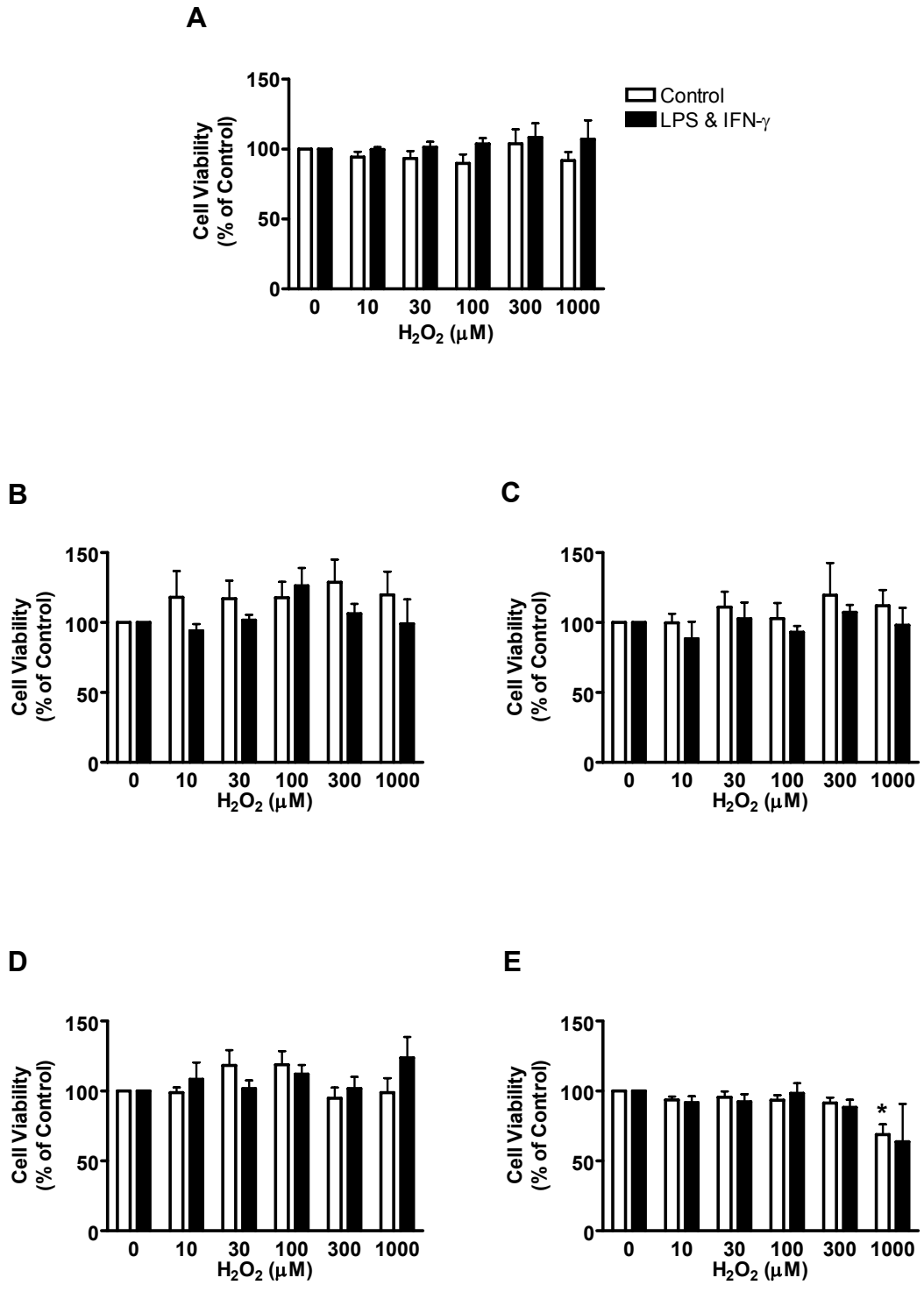

A
Control
LPS & IFN-γ
Cell Viability (% of Control)
150
100
50
0
0 10 30 100 300 1000
H2O2 (μM)
B
C
D
E
*

Figure 3.11. Time and concentration dependent effects of H_2O_2 on L-arginine transport in control and activated RASMCs in media without FBS.

Confluent monolayers of RASMCs in 96 well plates were incubated in culture medium in the absence (Controls) and presence of LPS (100 µg) and IFN-γ (100 U/ml) for at least 21 hours prior to exposure to FBS-free culture medium containing increasing concentrations of H_2O_2 for periods of 5 (Panel A), 15 (Panel B), 30 (Panel C), 60 (Panel D) and 180 (Panel E) minutes. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

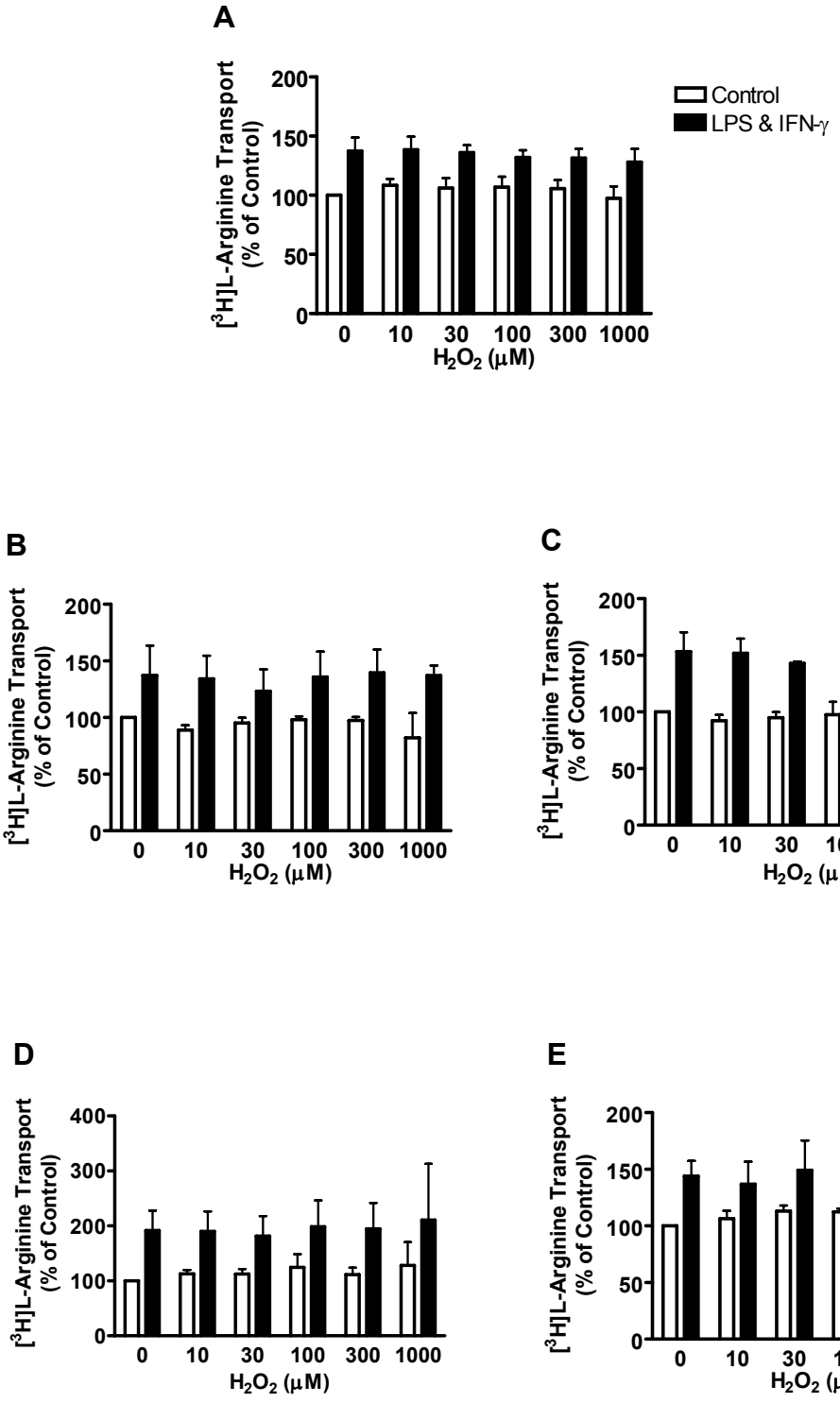

A
[3H]L-Arginine Transport (% of Control)
200
150
100
50
0
0 10 30 100 300 1000
H2O2 (μM)
Control
LPS & IFN-γ
B
[3H]L-Arginine Transport (% of Control)
200
150
100
50
0
0 10 30 100 300 1000
H2O2 (μM)
C
[3H]L-Arginine Transport (% of Control)
200
150
100
50
0
0 10 30 100 300 1000
H2O2 (μM)
D
[3H]L-Arginine Transport (% of Control)
400
300
200
100
0
0 10 30 100 300 1000
H2O2 (μM)
E
[3H]L-Arginine Transport (% of Control)
200
150
100
50
0
0 10 30 100 300 1000
H2O2 (μM)

Figure 3.12. Time and concentration dependent effects of H_2O_2 on viability of RASMCs in media without FBS.

Confluent monolayers of RASMCs in 96 well plates were incubated culture medium in the absence (Control) and presence of LPS (100 µg) and IFN-γ (100 U/ml) for at least 21 hours prior to exposure to FBS-free culture medium containing increasing concentrations of H_2O_2 for periods of 5 (Panel a), 15 (Panel B), 30 (Panel C), 60 (Panel D) and 180 (Panel E) minutes. Cell viability was monitored as described in the methods (Section 2.8). The data represents one single experiment with at least 3 replicates.

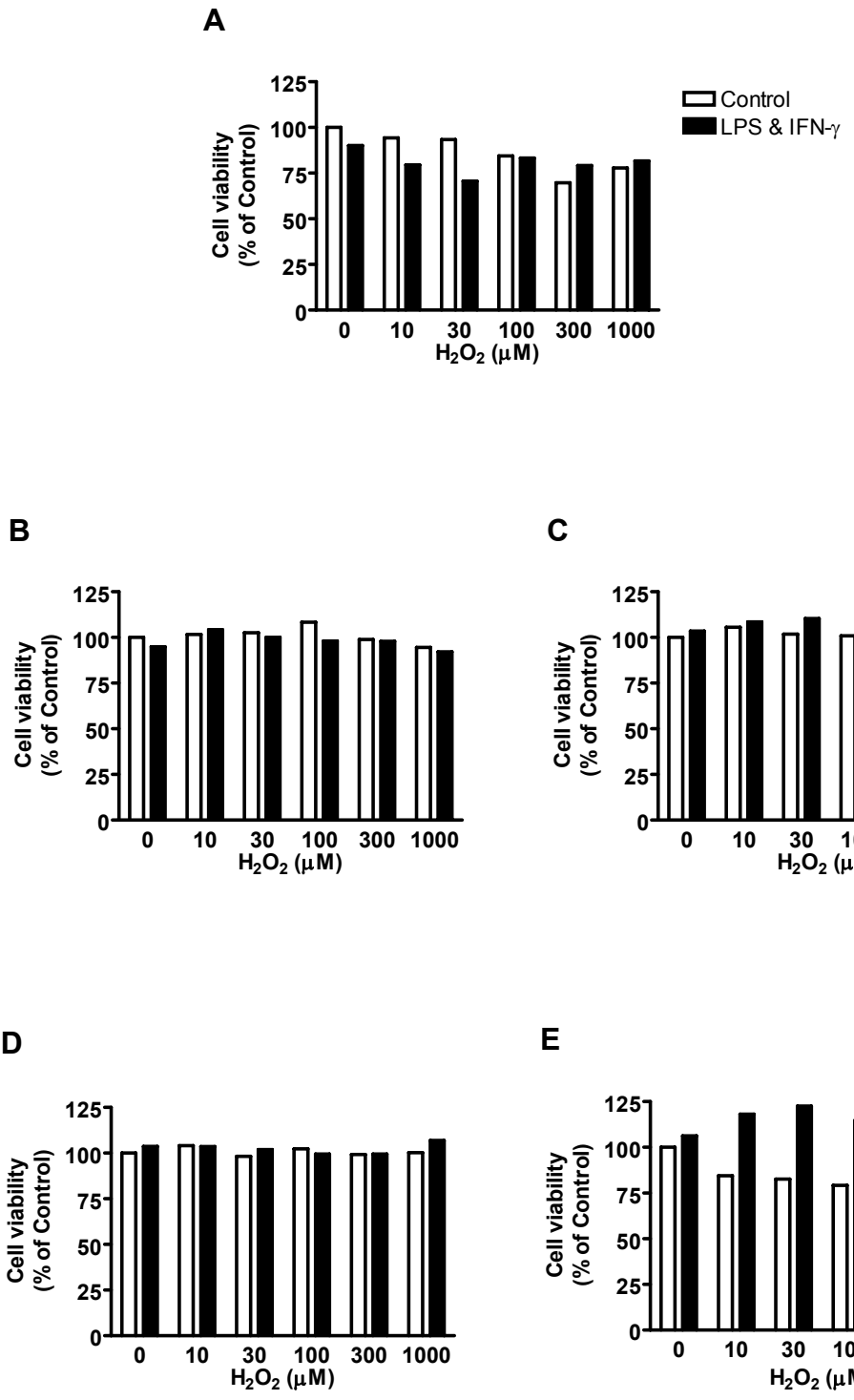

A
Control
LPS & IFN-γ
Cell viability (% of Control)
125
100
75
50
25
0
0 10 30 100 300 1000
H2O2 (μM)
B
C
D
E

Figure 3.13. Time and concentration dependent effects of H_2O_2 on L-arginine transport in control and activated RASMCs in media without phenol red.

Confluent monolayers of RASMCs in 96 well plates were incubated in culture medium in the absence (Controls) and presence of LPS (100 µg) and IFN-γ (100 U/ml) for at least 21 hours prior to exposure to phenol red-free culture medium containing increasing concentrations of H_2O_2 for periods of 5 (Panel A), 15 (Panel B), 30 (Panel C), 60 (Panel D) and 180 (Panel E) minutes. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

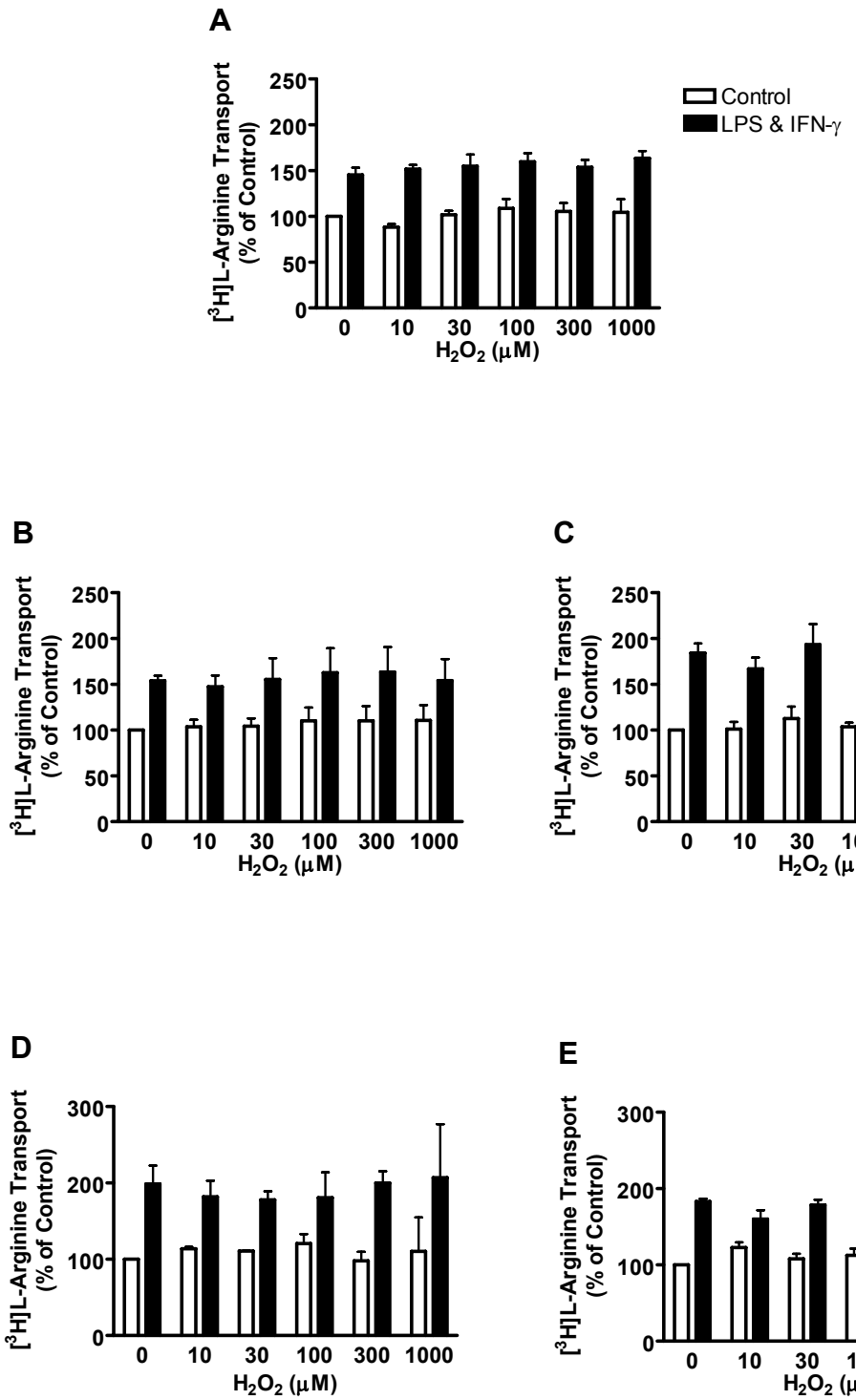
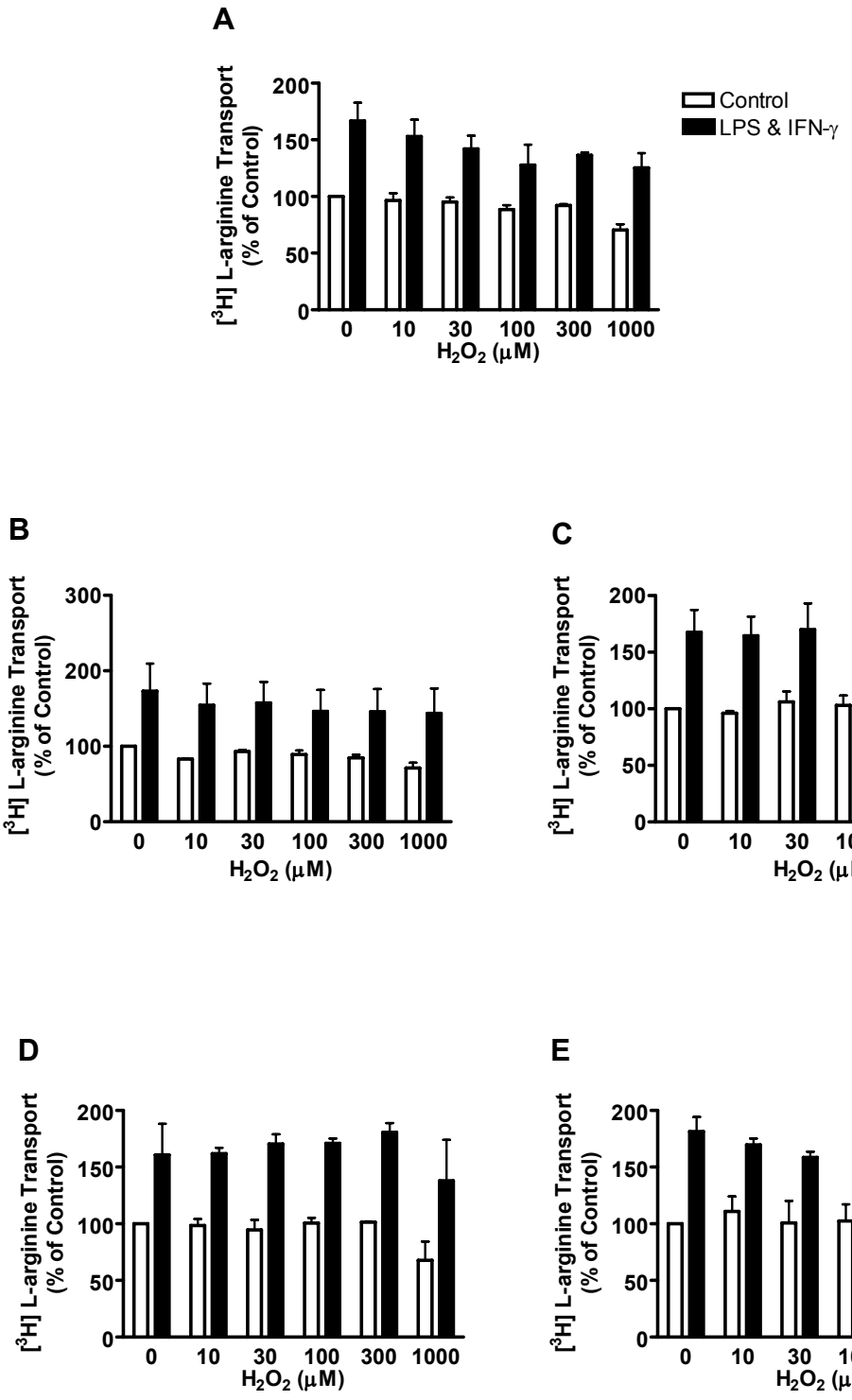

A
[3H]L-Arginine Transport (% of Control)
250
200
150
100
50
0
0 10 30 100 300 1000
H2O2 (μM)
Control
LPS & IFN-γ
B
[3H]L-Arginine Transport (% of Control)
250
200
150
100
50
0
0 10 30 100 300 1000
H2O2 (μM)
C
[3H]L-Arginine Transport (% of Control)
250
200
150
100
50
0
0 10 30 100 300 1000
H2O2 (μM)
D
[3H]L-Arginine Transport (% of Control)
300
200
100
0
0 10 30 100 300 1000
H2O2 (μM)
E
[3H]L-Arginine Transport (% of Control)
300
200
100
0
0 10 30 100 300 1000
H2O2 (μM)

Figure 3.14. Time and concentration dependent effects of H_2O_2 on L-arginine transport in control and activated RASMCs in media without FBS or phenol red.

Confluent monolayers of RASMCs in 96 well plates were incubated in culture medium in the absence (Controls) and presence of LPS (100 µg) and IFN-γ (100 U/ml) for at least 21 hours prior to exposure to increasing concentrations of H_2O_2 for periods of 5 (Panel A), 15 (Panel B), 30 (Panel C), 60 (Panel D) and 180 (Panel E) minutes, in media without FBS and phenol red. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.


A
[3H] L-arginine Transport (% of Control)
200
150
100
50
0
0 10 30 100 300 1000
H2O2 (μM)
Control
LPS & IFN-γ
B
[3H] L-arginine Transport (% of Control)
300
200
100
0
0 10 30 100 300 1000
H2O2 (μM)
C
[3H] L-arginine Transport (% of Control)
200
150
100
50
0
0 10 30 100 300 1000
H2O2 (μM)
D
[3H] L-arginine Transport (% of Control)
200
150
100
50
0
0 10 30 100 300 1000
H2O2 (μM)
E
[3H] L-arginine Transport (% of Control)
200
150
100
50
0
0 10 30 100 300 1000
H2O2 (μM)

The marginal effects with H_2O_2 would indicate that neither NO production nor L-arginine transport may be strongly regulated by OS. To confirm this, further studies were carried inducing OS using either antimycin A, an antibiotic that blocks electron transfer flow in Complex III in the mitochondrial respiratory chain (Izzo *et al.* 1978; Pietrobon *et al.* 1981), or DEM, an electrophile that alkylates the sulfhydryl group of glutathione, thereby blocking its function (Miccadei *et al.* 1988).

As in the previous experiments, cells were induced with LPS (100 µg) and IFN-γ (100 U/ml) for at least 21 hours prior to the incubation with either antimycin A (100 µM) or DEM (5 µM) for periods ranging from 5 minutes to 180 minutes. Transport of L-arginine was subsequently performed as described on section 2.6. Under these conditions neither antimycin A nor DEM caused any significant changes on L-arginine transport in control and activated cells (Figure 3.15 and Figure 3.16).

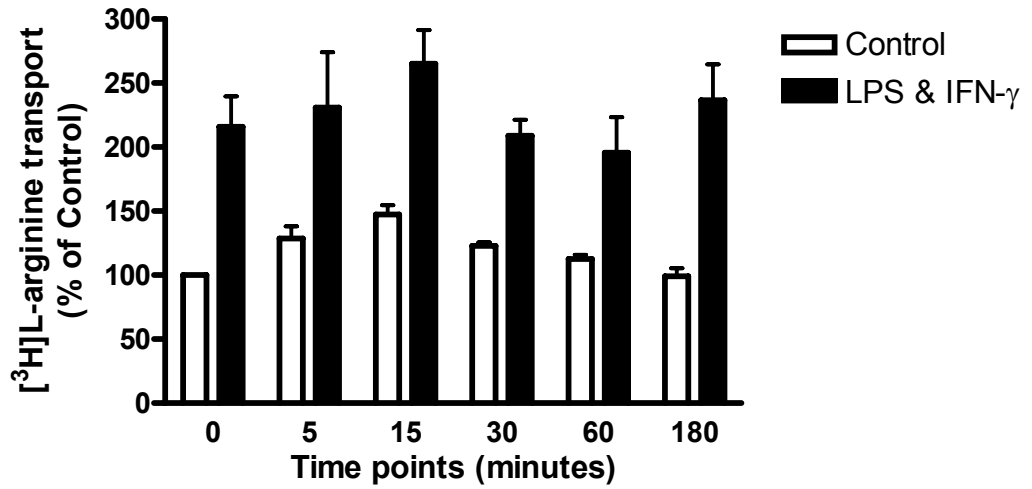


Figure 3.15. Effect of antimycin A on L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were incubated in culture medium in the absence (Controls) and presence of LPS (100 µg) and IFN-γ (100 U/ml) for at least 21 hours prior to exposure to antimycin A (100 µM) for the time points shown on the graph. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

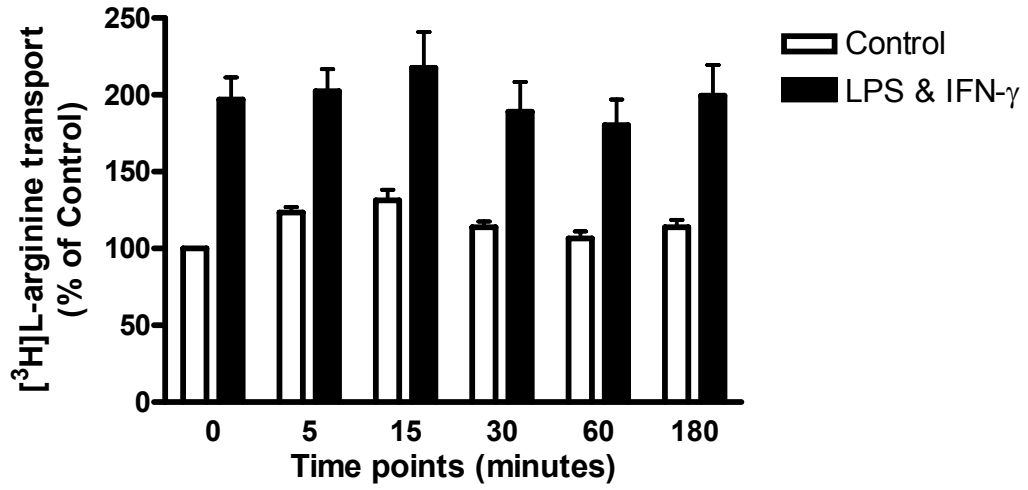


Figure 3.16. Effect of DEM on L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were either incubated in culture medium in the absence (Controls) and presence of LPS (100 µg) and IFN-γ (100 U/ml) for at least 21 hours prior to exposure to DEM 5 µM during the time points (5, 15, 30, 60 and 180 minutes). Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

3.5.2 . Effects of chronic exposure to oxidative stress inducers

In parallel with the above studies, additional experiments were carried out examining whether prolonged pre-exposure to H_2O_2, antimycin A or DEM would alter transporter activity. For these studies, H_2O_2 was used at a concentration of 300 μM which may regulate L-arginine transport without causing marked cytotoxicity (Figure 3.19), and was pre-incubated with RASMCs for 30 minutes prior to incubation with complete medium alone or with medium containing LPS (100 μg/ml) and IFN-γ (100 U/ml) for 24 hours. Under these conditions H_2O_2 caused a slight decrease in accumulated nitrite levels (Figure 3.17) and only marginally reduced induced L-arginine transport rates (Figure 3.18). A lower concentration of 100 μM was without effect (data not shown). Experiments with higher concentrations were not carried out due to cytotoxicity cause at 1 mM and above. In parallel with H_2O_2, pre-incubation of cells with antimycin A (25 μM -150 μM) or DEM (1 μM - 25 μM) caused a concentration dependent inhibition of induced NO synthesis which was virtually abolished with 150 μM antimycin A (Figure 3.20) or 25 μM DEM (Figure 3.22). Both compounds also concentration-dependently inhibited induced transporter activity, reducing the latter back to the control non-activated levels at ≥100 μM. Basal transport rates remained virtually unaltered (Figure 3.21 and Figure 3.23) and neither compound showed any cytotoxicity even at the highest concentrations used (Figure 3.24 and Figure 3.25). It is worth noting that the inhibition of induced L-arginine transport by DEM occurred at concentrations much higher than those required to inhibit NO synthesis (Figure 3.23). This would suggest that induced transporter activity may be less sensitive to DEM. Alternatively, DEM may be mediating these

effects (*i.e.* inhibition of NO synthesis and induced transporter activity) via different mechanisms which are yet to be determined.

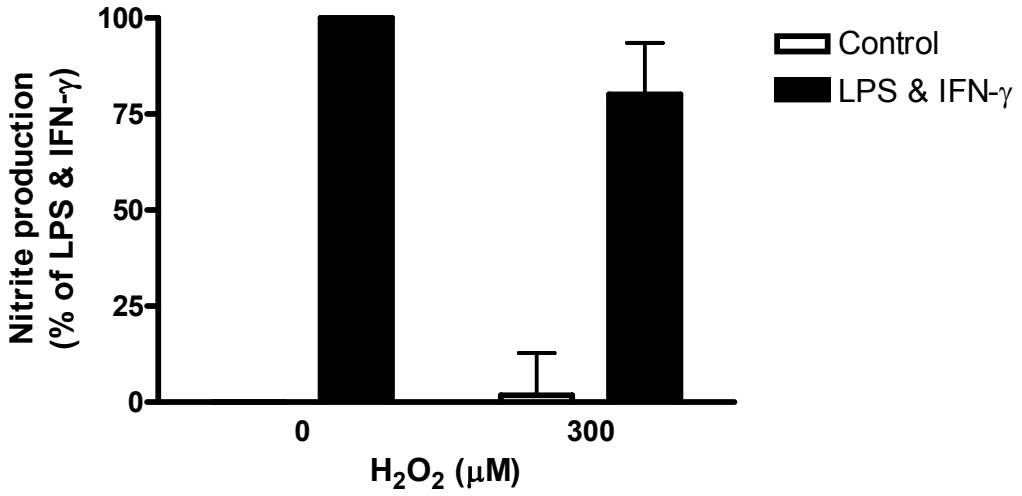


Figure 3.17. Effect of H_2O_2 on nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with H_2O_2 at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for a further 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

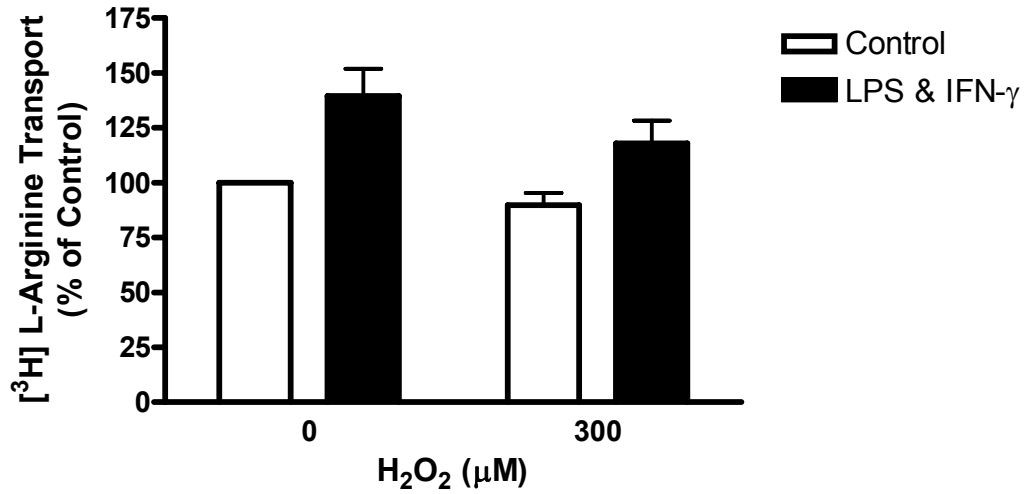


Figure 3.18. Effect of H_2O_2 on L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with H_2O_2 at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for a further 24 hours. Transport of [^{3}H]L-arginine was determined in the cell monolayer as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

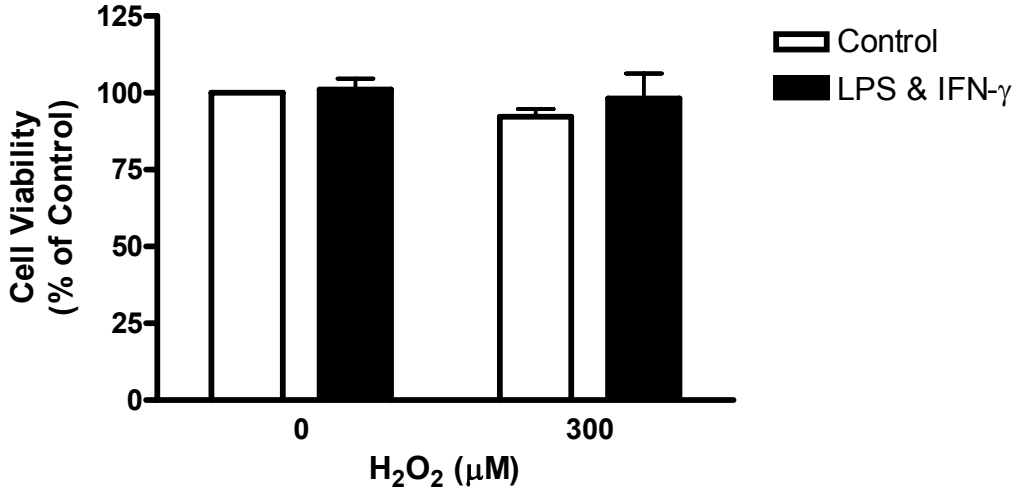


Figure 3.19. Effect of H_2O_2 on viability in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with H_2O_2 at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 µg) and IFN-γ (100 U/ml) for a further 24 hours. Cell viability was monitored as described in the methods (Section 2.8). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

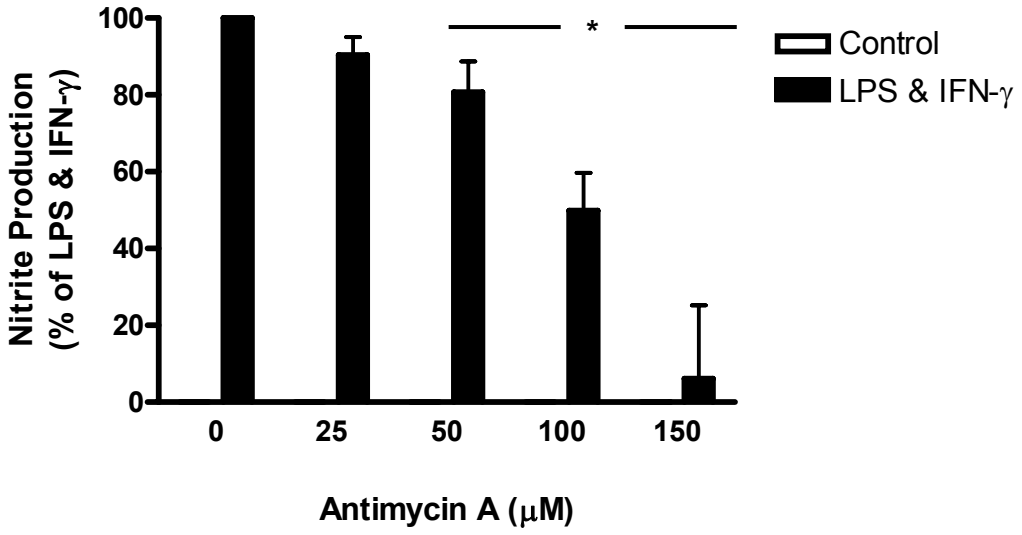


Figure 3.20. Effect of antimycin A on nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with antimycin A at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for a further 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

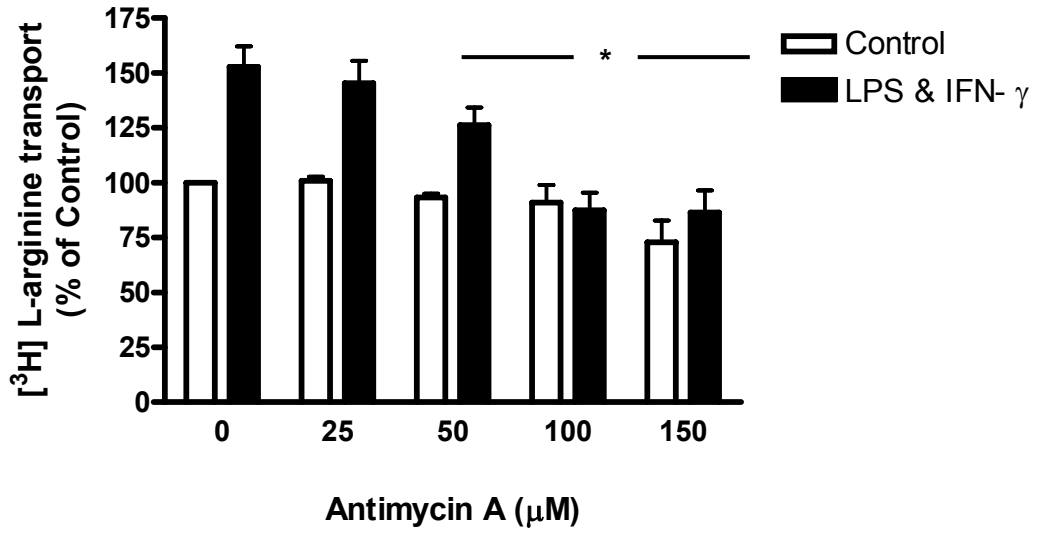


Figure 3.21. Effect of antimycin A on L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with antimycin A at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for a further 24 hours Transport of [^{3}H]L-arginine was determined in the cell monolayer as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

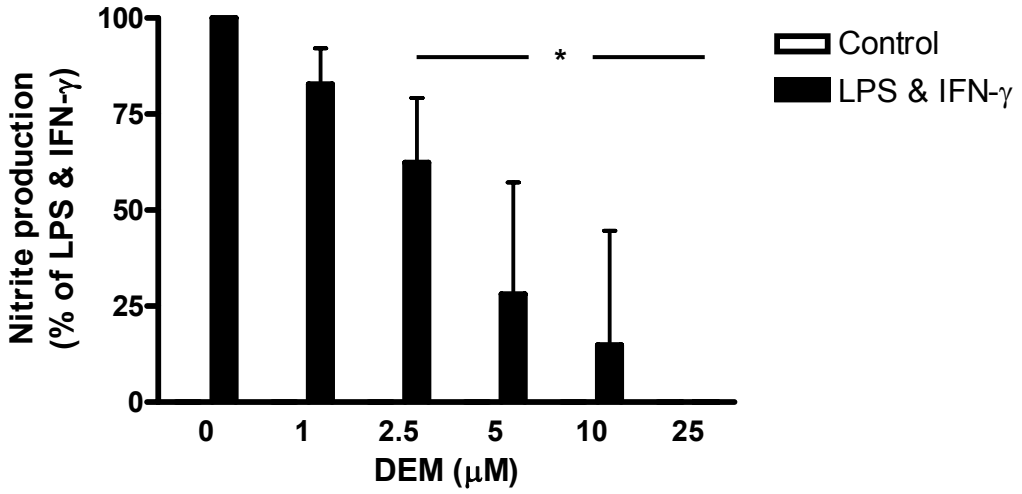


Figure 3.22. Effect of DEM on nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with DEM at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for a further 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

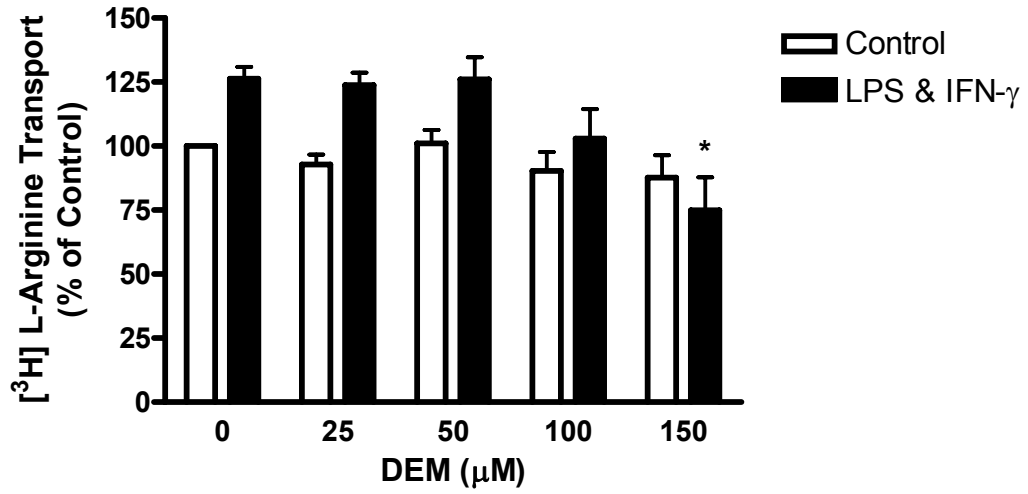


Figure 3.23. Effect of DEM on L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with DEM at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for a further 24 hours. Transport of [^{3}H]L-arginine was determined in the cell monolayer as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

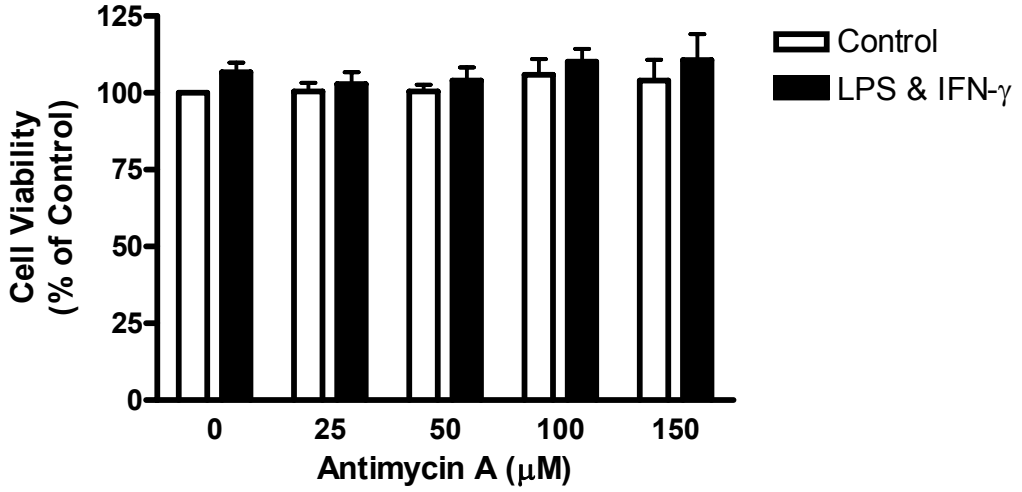


Figure 3.24. Effect of antimycin A on viability in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with antimycin A at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for a further 24 hours. Cell viability was monitored as described in the methods (Section 2.8). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

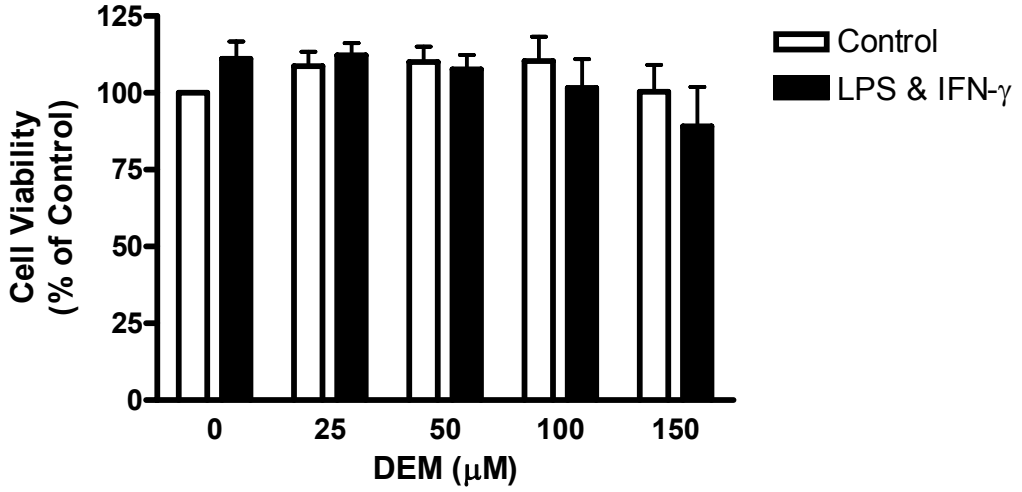


Figure 3.25. Effect of DEM on viability in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with DEM at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for a further 24 hours. Cell viability was monitored as described in the methods (Section 2.8). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

3.5.3 . Does the regulation of NO synthesis and/or L-arginine transport by antimycin A or DEM occur at the functional or expression levels?

To determine whether the effects described above were mediated via direct inhibition or through regulation of the induction of either NOS and/or transporter activity, additional experiments were carried out exposing cells to antimycin A (150 μM) or DEM (150 μM) either 30 minutes prior to, or at different time points (3, 6, 9 and 18 hours) after activation with LPS (100 μg) and IFN-γ (100 U/ml). Changes in NO production and in L-arginine transport were assessed 24 hours after activation. As shown in Figure 3.26, addition of antimycin A for 30 minutes prior to or for up to 3 hours after activation of cells resulted in a marked inhibition of NO synthesis. In contrast, when added at 6 hours or later, antimycin A failed to cause any statistically significant change in NO production (Figure 3.26). By comparison, L-arginine transport remained suppressed unless when antimycin A was added 18 hours after exposure of cells to LPS (100 μg) and IFN-γ (100 U/ml) (Figure 3.27). Similarly, addition of DEM 18 hours after activation of cells failed to inhibit NO synthesis (Figure 3.28) or L-arginine transport (Figure 3.29) but caused marked inhibition of both processes when added at the earlier time points where the iNOS and CAT genes may not have been fully induced. Nitric oxide production after exposure to DEM was affected by concentrations as low as 1 μM (Figure 3.22) but L-arginine transport was less susceptible and only affected by concentrations higher than 100 μM in induced cells (Figure 3.23). Initially, NO production was also examined using DEM at 25 μM to 150 μM (data not shown) and was completely inhibited even at the

25 μM concentration. The expression of iNOS on the other hand decreased in a concentration dependent manner from 25 μM and was completely abolished at 150 μM (Figure 3.32).

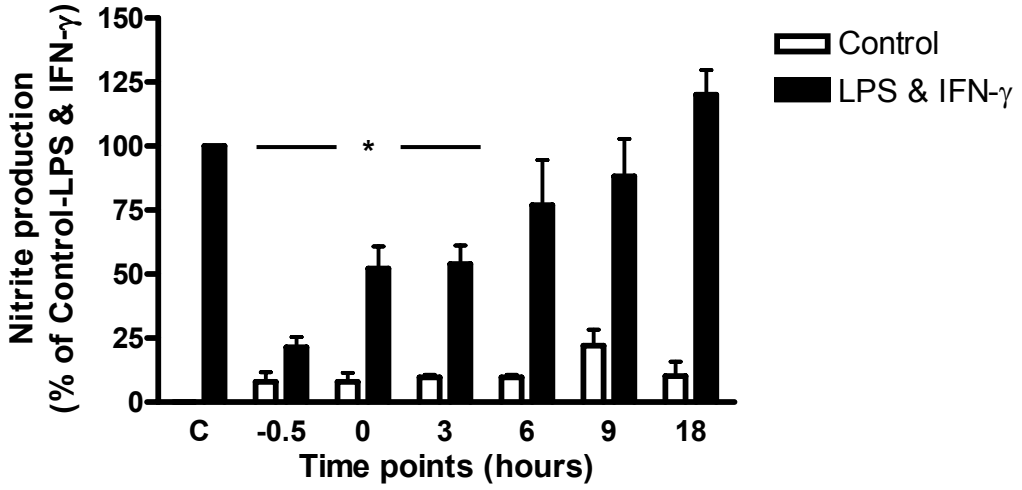


Figure 3.26. Time dependent effects of antimycin A on nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were incubated for 24 hours either in culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml). When applied, antimycin A (150 μM) was added at the time points shown on the graph. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

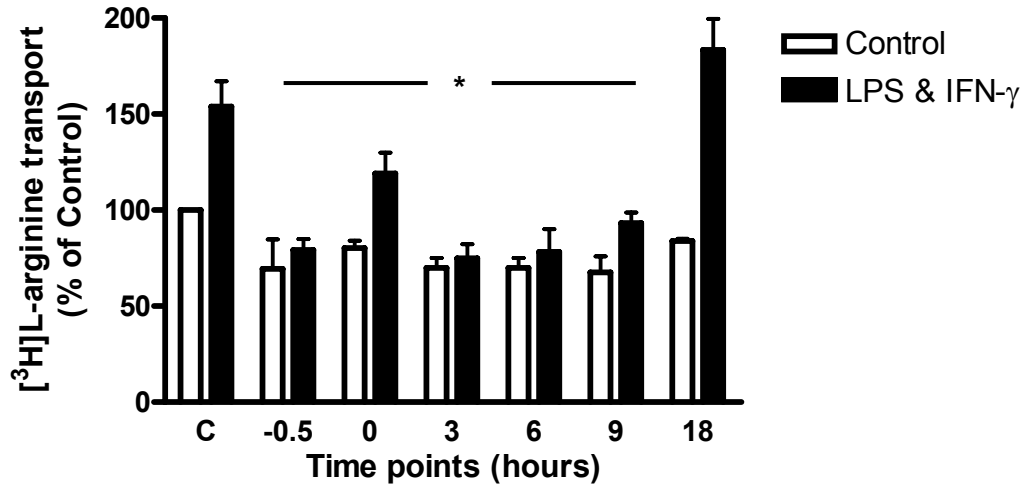


Figure 3.27. Time dependent effects of antimycin A on L-arginine transport in control and activated RASMC.

Confluent monolayers of RASMCs in 96 well plates were incubated for 24 hours either in culture medium alone (Controls) or in medium containing LPS (100 µg) and IFN-γ (100 U/ml). When applied, antimycin A (150 µM) was added at the time points shown on the graph. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

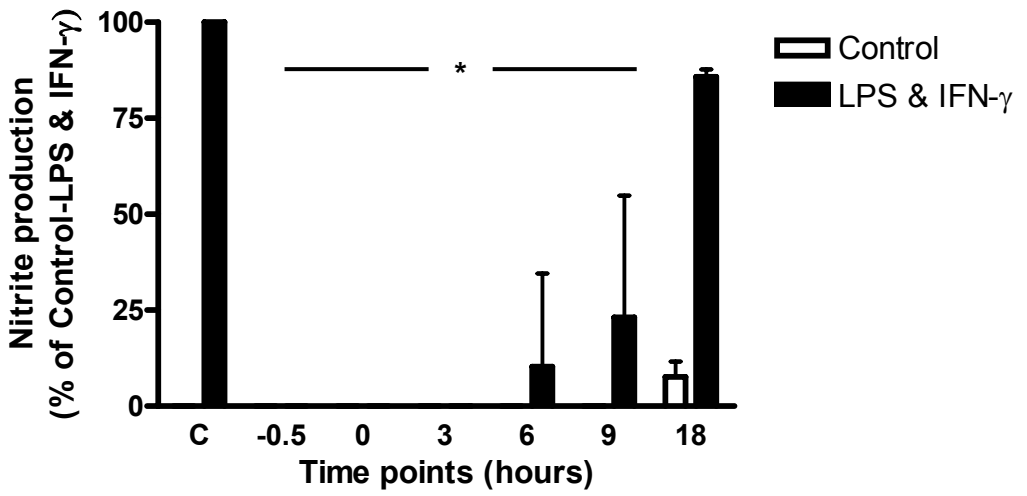


Figure 3.28. Time dependent effects of DEM on nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were incubated for 24 hours either in culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml). When applied, DEM (150 μM) was added at the time points shown on the graph. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

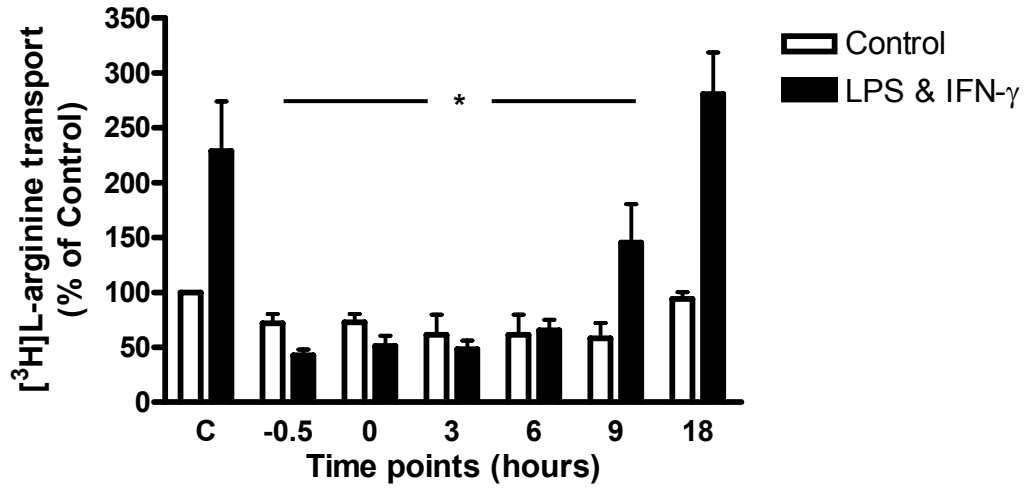


Figure 3.29. Time dependent effects of DEM on L-arginine transport in control and activated RASMC.

Confluent monolayers of RASMCs in 96 well plates were incubated for 24 hours either in culture medium alone (Controls) or in medium containing LPS (100 µg) and IFN-γ (100 U/ml). When applied, DEM (150 µM) was added at the time points shown on the graph. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

3.6 . Effects of oxidative stress inducers on iNOS protein

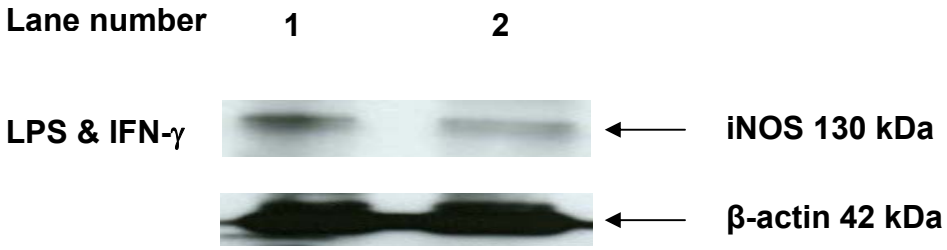
As a follow on to the time-course studies, experiments were carried out investigating changes in iNOS protein expression in order to confirm that the actions described for the oxidative stress inducers were in fact mediated via suppression of the expression of this enzyme. Studies looking at the CATs were not carried out as specific antibodies against these proteins are currently not commercially available.

There was no detectable expression of iNOS in control non-activated cells. Exposure to LPS and IFN-γ did however cause a marked increase in the expression of iNOS which was inhibited by about 50 % in the presence of H_2O_2 at a concentration of 300 μM (Figure 3.30). This contrasts with the effects of H_2O_2 on NO production where only marginal inhibitions were observed. Antimycin A also suppressed iNOS protein expression but this was only significant at 150 μM. Although partial inhibitions were seen with the lower concentrations, these were not concentration dependent nor significantly different from the control activated samples (Figure 3.31). These results do not correlate well with the concentration dependent decrease in NO production reported for the functional studies above (Figure 3.20). Exposure of cells to DEM concentration-dependently suppressed iNOS expression but, unlike the changes seen with NO production (Figure 3.22), iNOS expression was not abolished except at concentrations $\geq$100 μM (Figure 3.32). The reasons for the discrepancies between the effects of these compounds on NO production and iNOS expression are currently not known but it is likely that the action of some compounds on NO production may not only reside in blocking the induction of iNOS but potentially also in regulating the function of the expressed enzyme.

Figure 3.30. Effect of H_2O_2 on expression of inducible NOS in control and activated RASMCs.

Confluent monolayers of RASMCs in 6 well plates were pre-treated for 30 minutes with H_2O_2 at the concentrations shown on the graph prior to activation with LPS (100 µg) and IFN-γ (100 U/ml) for 24 hours. Lysates were generated and analysed by western blotting as described in the Methods (Section 2.7). The lanes represent: 1: control and 2: H_2O_2 300 µM. Panel A is one representative blot from three separate experiments and Panel B is the densitometric analysis of the blots ± S.E.M. *denotes $p<0.05$.

A


Lane number
1
2
LPS & IFN-γ
iNOS 130 kDa
β-actin 42 kDa

B

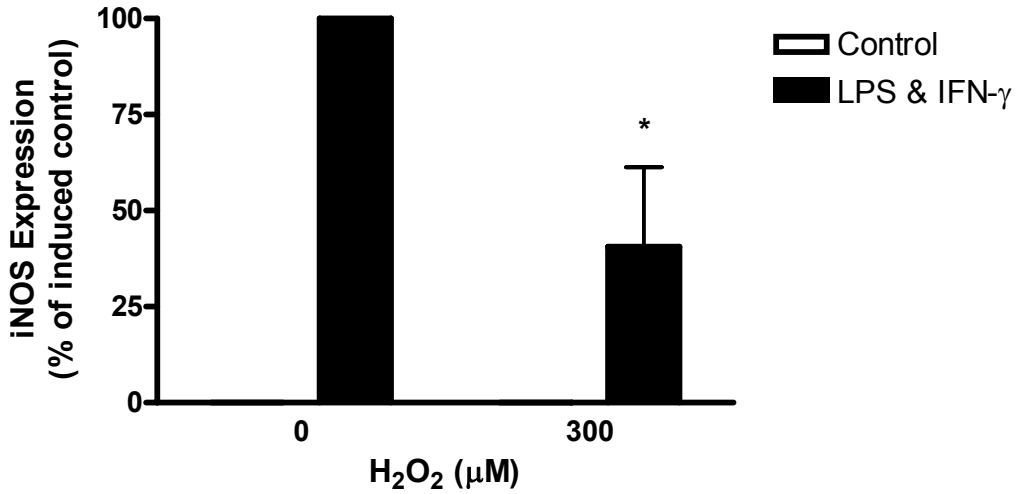
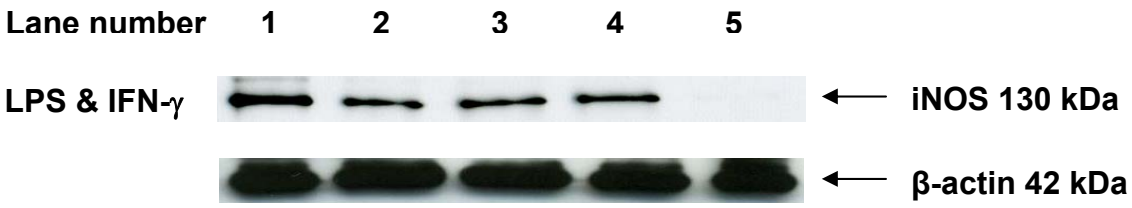

iNOS Expression
(% of induced control)
100
75
50
25
0
*
0
300
H2O2 (μM)
Control
LPS & IFN-γ

Figure 3.31. Effect of antimycin A on expression of inducible NOS in control and activated RASMCs.

Confluent monolayers of RASMCs in 6 well plates were treated with antimycin A at the concentrations shown for 30 minutes prior to activation with LPS (100 μg) and IFN-γ (100 U/ml) for 24 hours. Lysates were generated and analysed by western blotting as described in the Methods (Section 2.7). The lanes represent: 1: control; 2: antimycin A at 25 μM; 3: antimycin A at 50 μM; 4: antimycin A at 100 μM and 5: antimycin A at 150 μM. Panel A is one representative blot from 3 separate experiments and Panel B is the densitometric analysis of the 3 blots represented as the mean ± S.E.M. * denotes $p<0.05$.

A


Lane number 1 2 3 4 5
LPS & IFN-γ
iNOS 130 kDa
β-actin 42 kDa

B

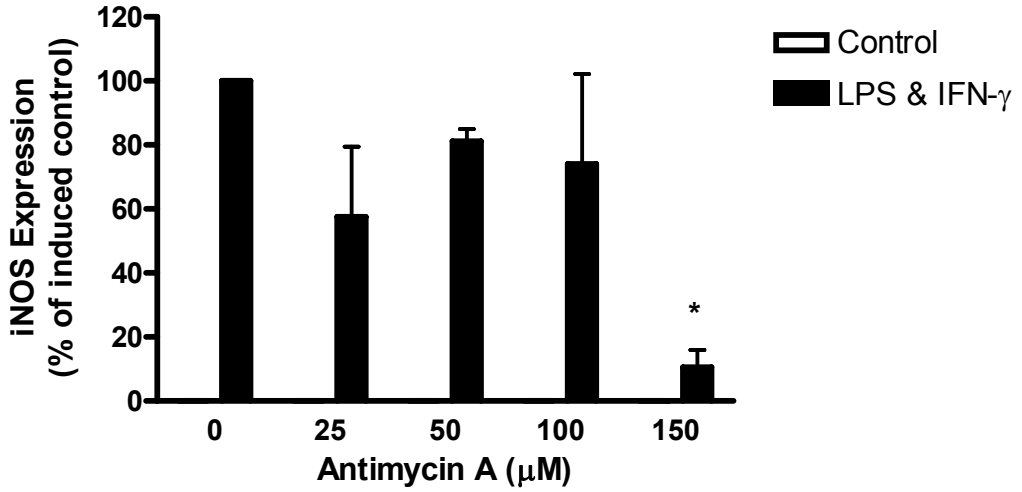
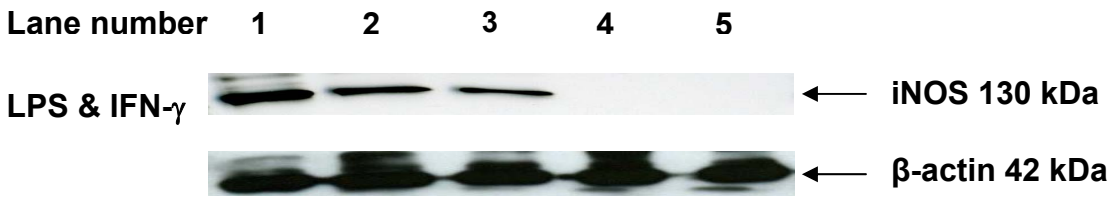

iNOS Expression
(% of induced control)
120
100
80
60
40
20
0
0
25
50
100
150
Antimycin A (μM)
*
Control
LPS & IFN-γ

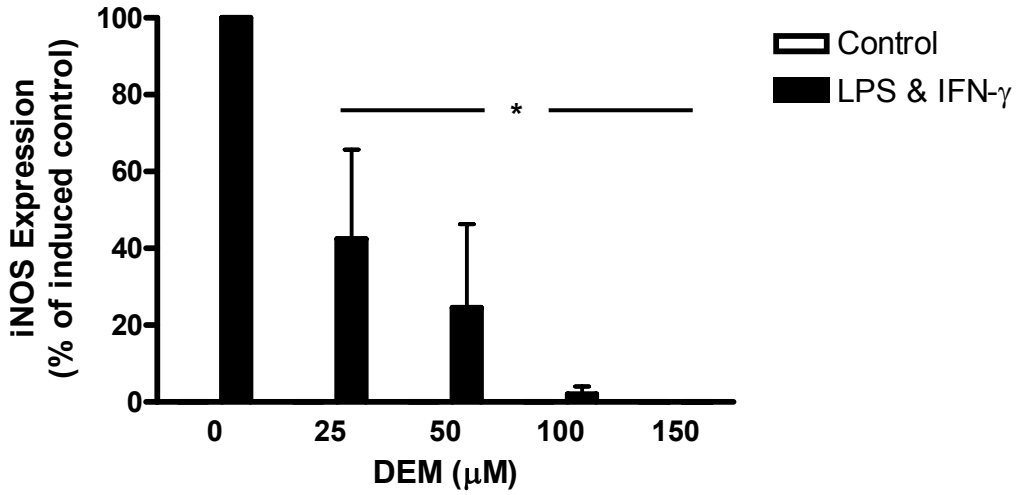
Figure 3.32. Effect of DEM on expression of inducible NOS in control and activated RASMCs.

Confluent monolayers of RASMCs in 6 well plates were treated with DEM at the concentrations shown for 30 minutes prior to activation with LPS (100 μg) and IFN-γ (100 U/ml) for 24 hours. Lysates were generated and analysed by western blotting as described in the Methods (Section 2.7). The lanes represent: 1: control; 2: DEM 25 μM; 3: DEM 50 μM; 4: DEM 100 μM and 5: DEM 150 μM. Panel A is one representative blot from 3 separate experiments and Panel B is the densitometric analysis of the 3 blots represented as the mean ± S.E.M. * denotes $p<0.05$.

A


Lane number 1 2 3 4 5
LPS & IFN-γ
iNOS 130 kDa
β-actin 42 kDa

B


iNOS Expression
(% of induced control)
100
80
60
40
20
0
*
0
25
50
100
150
DEM (μM)
Control
LPS & IFN-γ

3.7 . Effect of oxidative stress inducers on Heme-Oxygenase-1 expression in rat cultured aortic smooth muscle cells

The HO-1 protein is a stress protein, expressed in response to oxidative stress. In these studies, cells were pre-treated with each compound individually prior to exposure to either medium alone (Control) or medium containing LPS (100 μg) and IFN-γ (100 U/ml) for 24 hours. The western blots of lysates generated did not show any detectable or significant expression of HO-1 in controls or in cells treated with H_2O_2 (Figure 3.33). Antimycin A on the other hand induced a 32 kDa protein that was detectable with an anti-HO-1 specific antibody. The expression was however identified only at 150 μM in induced cells (Figure 3.34). Interestingly, in the series of experiments with DEM, HO-1 was detected in lysates from LPS (100 μg) and IFN-γ (100 U/ml) treated cells and this was increased in a concentration-dependent manner by DEM, reaching a maximum at 150 μM (Figure 3.35). The reason for the induction of HO-1 by LPS (100 μg) and IFN-γ (100 U/ml) in this series of experiments but not in the others is unclear. It is possible however that this reflects the degree of stress that may have been induced on the cells at any given time. Should this be higher in the experiments carried out with DEM then this would perhaps account for the detectable levels of HO-1.

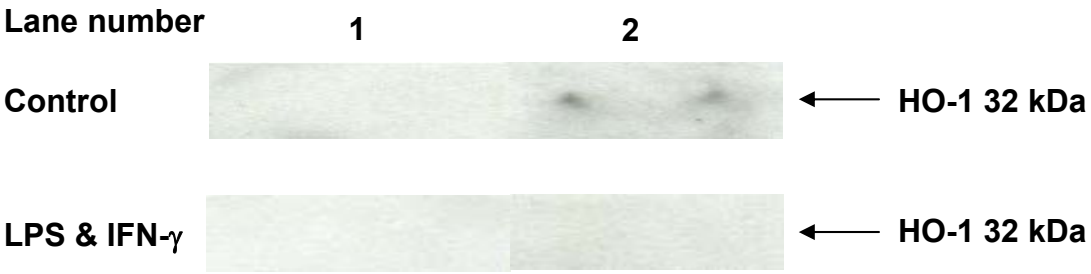


Figure 3.33. Effect of H_2O_2 on expression of heme-oxygenase-1 in control and activated RASMCs.

Confluent monolayers of RASMCs in 6 well plates were treated with H_2O_2 at the concentrations shown for 30 minutes prior to activation with LPS (100 µg) and IFN-γ (100 U/ml) or incubated in medium alone without LPS and IFN-γ (Control) for 24 hours. Lysates were generated and analysed by western blotting as described in the Methods (Section 2.7). The lanes represent: 1: control; 2: H_2O at 300 µM. One representative blot from 3 separate experiments.

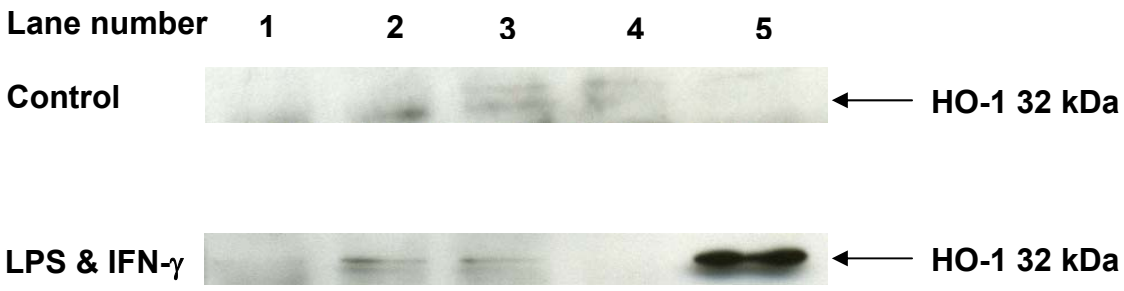


Figure 3.34. Effect of antimycin A on expression of heme-oxygenase-1 in control and activated RASMCs.

Confluent monolayers of RASMCs in 6 well plates were treated with antimycin A at the concentrations shown for 30 minutes prior to activation with LPS (100 µg) and IFN-γ (100 U/ml) or incubated in medium alone without LPS and IFN-γ (Control) for 24 hours. Lysates were generated and analysed by western blotting as described in the Methods (Section 2.7). The lanes represent: 1: control; 2: antimycin A 25 µM; 3: antimycin A 50 µM; 4: antimycin A 100 µM and 5: antimycin A150 µM. One representative blot from 3 separate experiments.

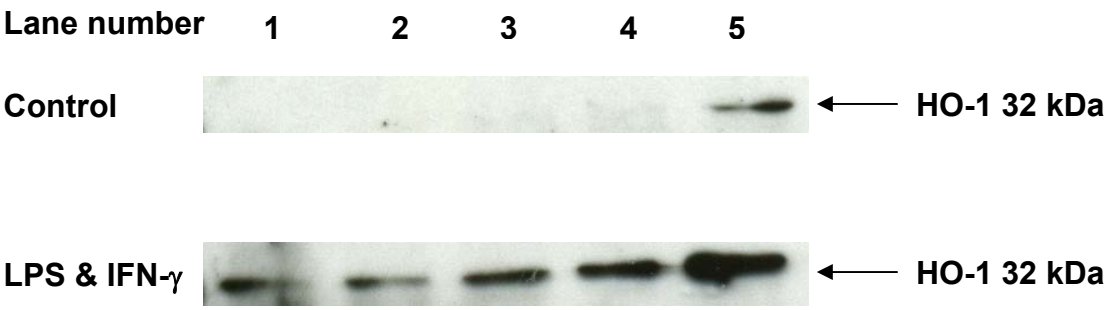


Figure 3.35. Effect of DEM on expression of heme-oxygenase-1 in control and activated RASMCs.

Confluent monolayers of RASMCs in 6 well plates were treated with DEM at the concentrations shown for 30 minutes prior to activation with LPS (100 µg) and IFN-γ (100 U/ml) or incubated in medium alone without LPS and IFN-γ (Control) for 24 hours. Lysates were generated and analysed by western blotting as described in the Methods (Section 2.7). The lanes represent: 1: control; 2: DEM 25 µM; 3: DEM 50 µM; 4: DEM 100 µM and 5: DEM 150 µM. One representative blot from 3 separate experiments.

3.8 . Effect of statins on nitrite production and L-arginine transport in rat cultured aortic smooth muscle cells

As highlighted in the introduction chapter, statins are now known to exert a diverse range of actions which are not simply restricted to cholesterol lowering. We have therefore initiated studies to determine the effects of statins on the expression and function of the L-arginine-NO pathway in RASMCs. Studies have been carried out using atorvastatin, simvastatin and pravastatin, which are widely administered to patients with coronary artery disease to lower their blood cholesterol levels (Bauersachs *et al.* 2006; Chapidze *et al.* 2006). In our experiments, cells were pre-incubated with increasing concentrations of statins (1-100 μM) for 30 minutes prior to activation with LPS (100 μg/ml) and IFN-γ (100 U/ml) for 24 hours. Changes in nitrite production and L-arginine transport were subsequently determined.

Atorvastatin caused a bell-shaped response on LPS and IFN-γ induced NO synthesis, initially increasing this in a concentration-dependent manner up to 10 μM and decreasing thereafter. Even at a 100 μM atorvastatin, the amount of nitrite detected, although lower than that measured with 10 μM, was still higher than the levels detected with LPS and IFN-γ alone (Figure 3.36). Basal nitrite levels remained undetectable and unaffected by the drug (Figure 3.36). As with atorvastatin, simvastatin also produced a bell-shaped response curve on NO production which was enhanced at 3 μM to 10 μM, declining to just below those detected with LPS and IFN-γ at 100 μM (Figure 3.37). In contrast to the previous two statins, pravastatin failed to cause any significant change in NO production even at concentrations of up

to 100 μM (Figure 3.38). None of the drugs showed any cytotoxicity at any of the concentrations used (Figure 3.39, Figure 3.40 and Figure 3.41).

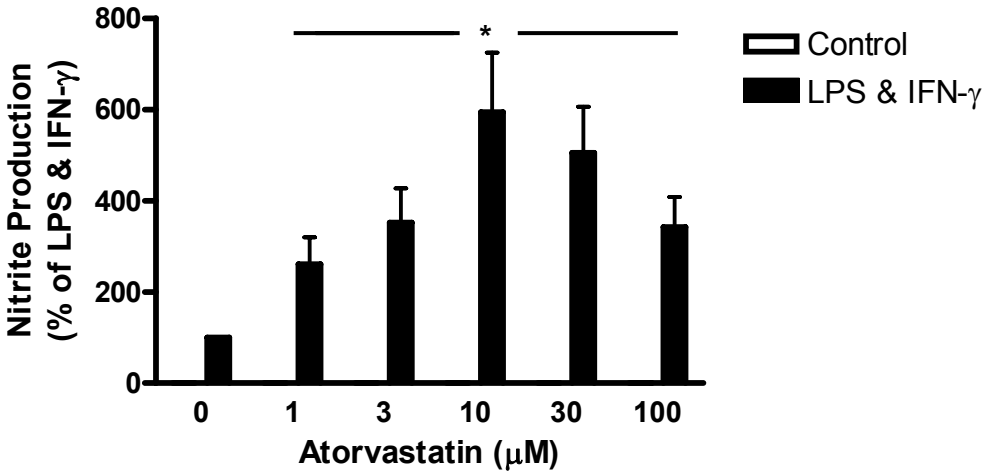


Figure 3.36. Concentration dependent effect of atorvastatin on nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in complete culture medium for 30 minutes with atorvastatin at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for a further 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

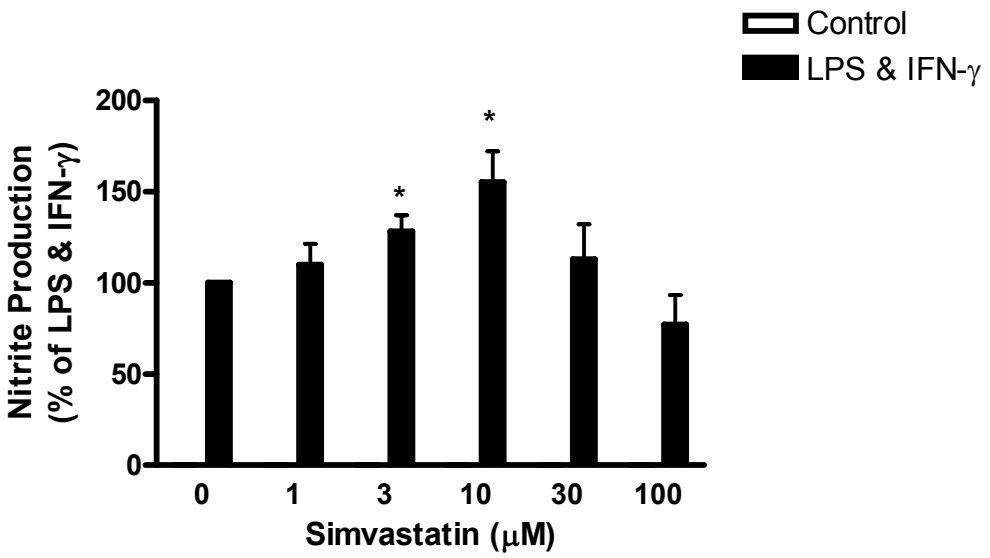


Figure 3.37. Concentration dependent effect of simvastatin on nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with simvastatin at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for a further 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

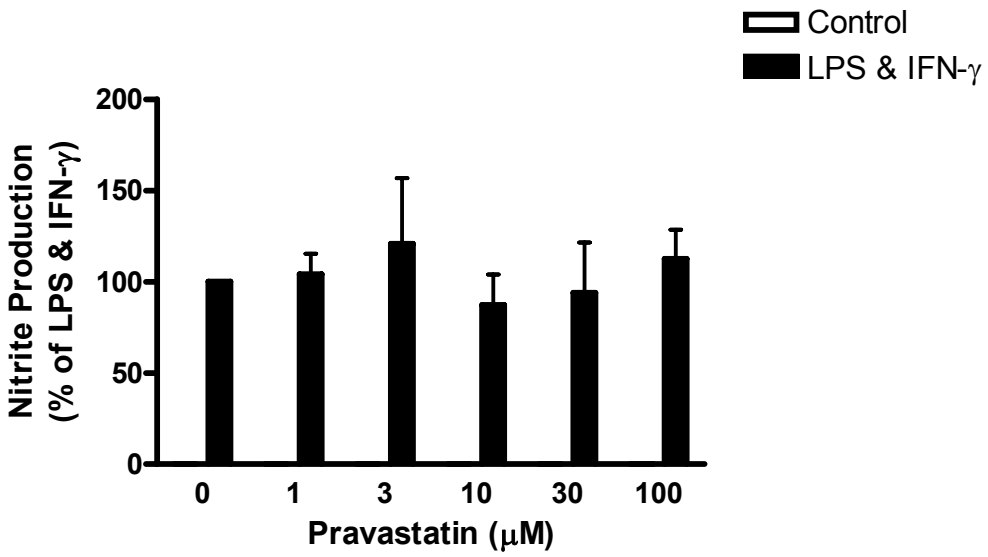


Figure 3.38. Concentration dependent effect of pravastatin on nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with pravastatin at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for a further 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

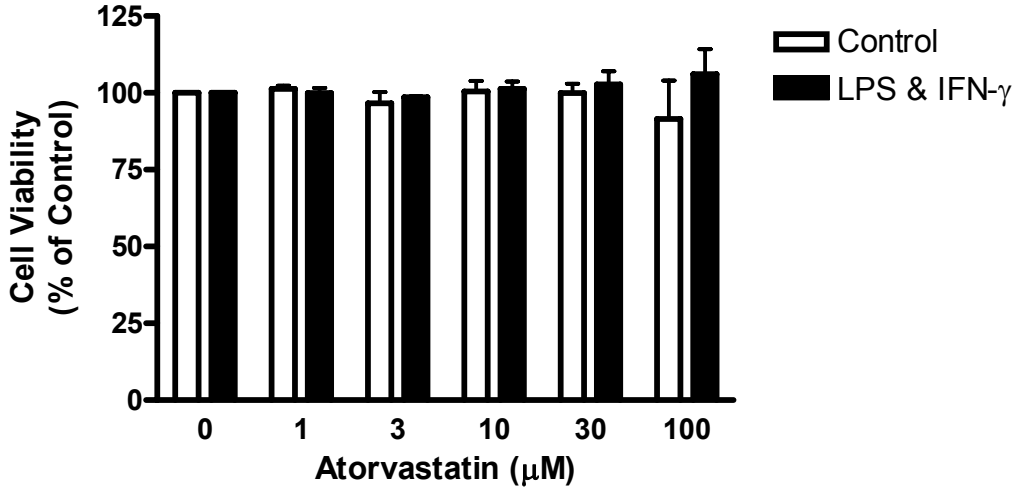


Figure 3.39. Effect of atorvastatin on viability in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with atorvastatin at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for a further 24 hours. Cell viability was monitored as described in the methods (Section 2.8). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

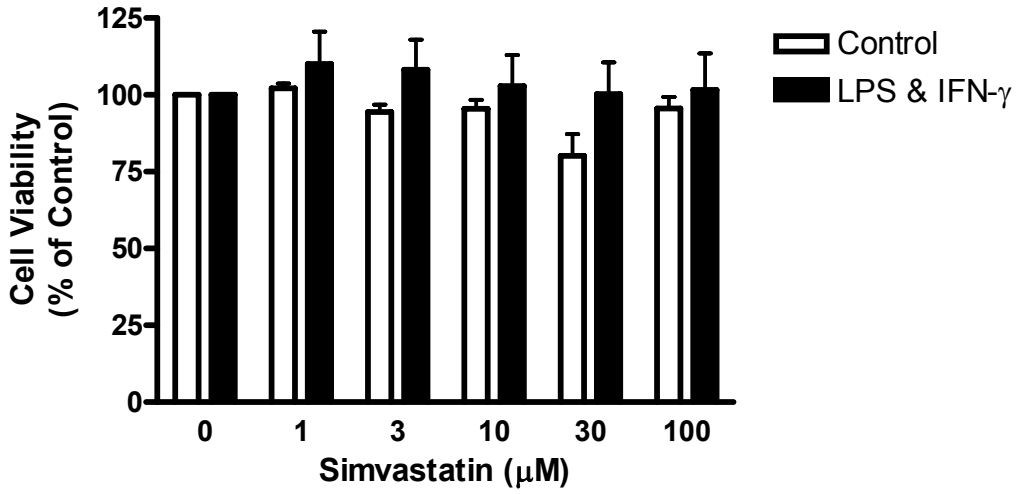


Figure 3.40. Effect of simvastatin on viability in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with simvastatin at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for a further 24 hours. Cell viability was monitored as described in the methods (Section 2.8). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

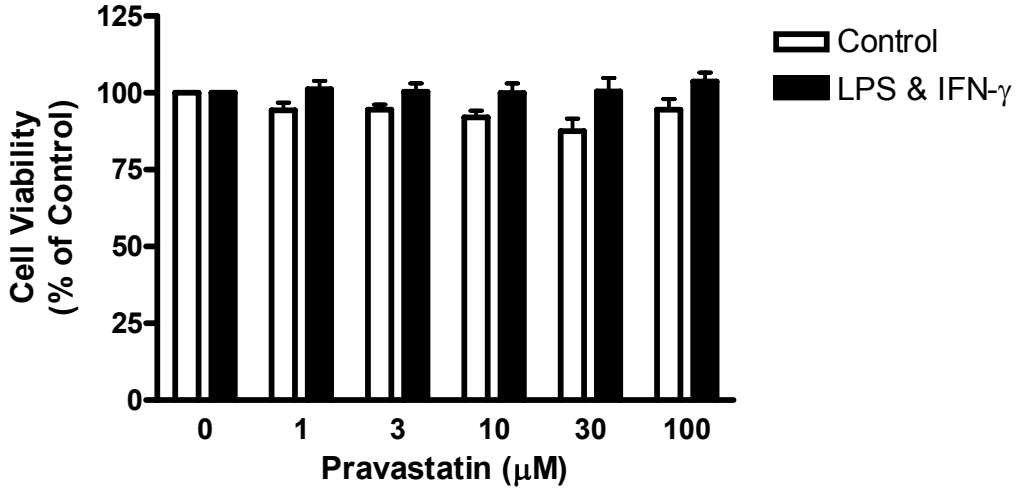


Figure 3.41. Effect of pravastatin on viability in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with pravastatin at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 µg) and IFN-γ (100 U/ml) for a further 24 hours. Cell viability was monitored as described in the methods (Section 2.8). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

Consistent with its effects on induced NO synthesis, atorvastatin also enhanced L-arginine transport. However, this did not appear to be concentration dependent in that the increases observed were virtually comparable at all concentrations used except for 100 μM which inhibited transport rates, albeit marginally, in both control and activated cells (Figure 3.42). In contrast to atorvastatin, simvastatin did not alter L-arginine transport (Figure 3.43), contrasting with its effects on NO production. Pravastatin also failed to modify transporter activity in either control or activated cells (Figure 3.44) which is consistent with its lack of effects on induced NO synthesis.

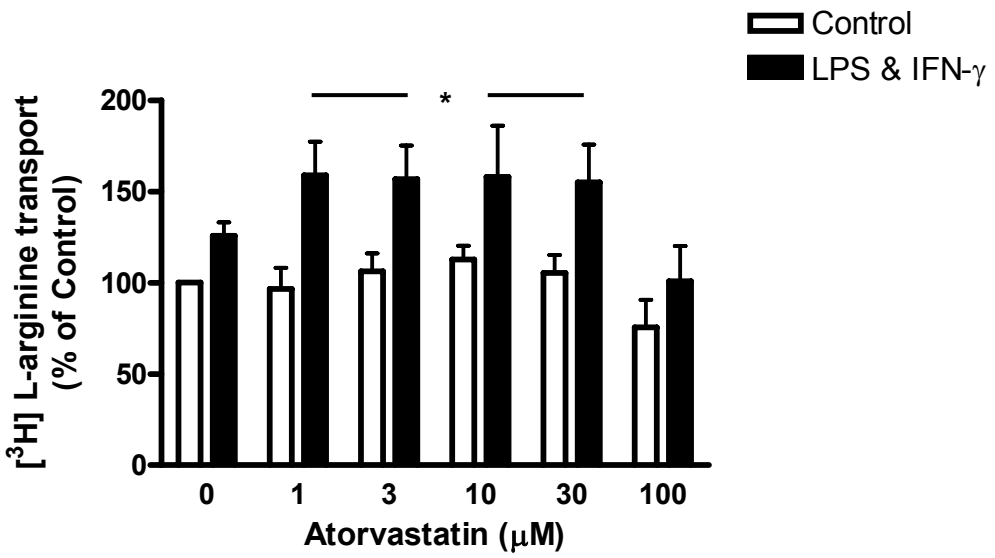


Figure 3.42. Effect of atorvastatin on L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with atorvastatin at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml) for a further 24 hours Transport of [^{3}H]L-arginine was determined in the cell monolayer as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

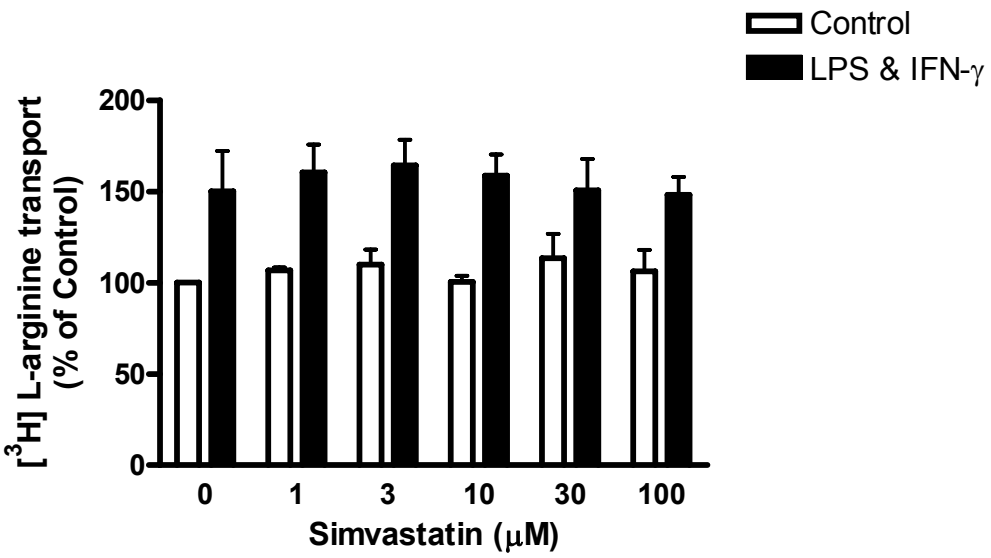


Figure 3.43. Effect of simvastatin on L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with simvastatin at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 µg) and IFN-γ (100 U/ml) for a further 24 hours Transport of [^{3}H]L-arginine was determined in the cell monolayer as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

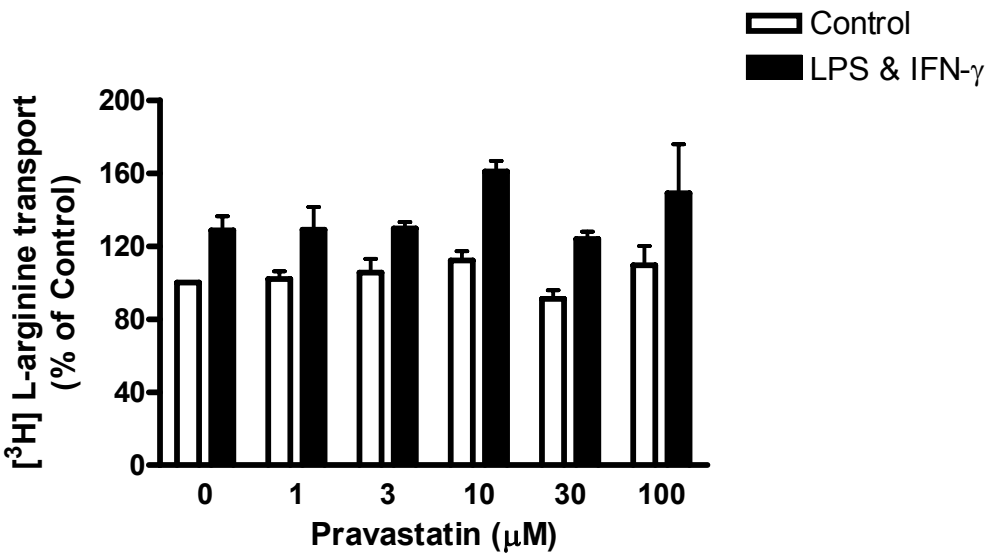


Figure 3.44. Effect of pravastatin on L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-treated in normal culture medium for 30 minutes with pravastatin at the concentrations shown on the graph. Cells were subsequently incubated in complete culture medium alone (Controls) or in medium containing LPS (100 µg) and IFN-γ (100 U/ml) for a further 24 hours Transport of [^{3}H]L-arginine was determined in the cell monolayer as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

3.9 . Effects of statins on iNOS protein expression

In order to determine whether the changes induced by atorvastatin and simvastatin on nitrite production were associated with changes in the induction of iNOS, western blot analysis of the enzyme was carried out under identical conditions to those described above. In these studies, atorvastatin clearly enhanced iNOS expression in activated cells. More importantly, the enhancements correlated well with the changes seen in NO production (Figure 3.36) in that 10 μM and 30 μM atorvastatin produced the maximum increase in enzyme protein expression with 100 μM decreasing the latter (Figure 3.45). Simvastatin also appeared to enhance iNOS expression (Figure 3.46) over the concentration range that increased NO production (Figure 3.37) while pravastatin failed to cause any significant change in iNOS induction (Figure 3.47).

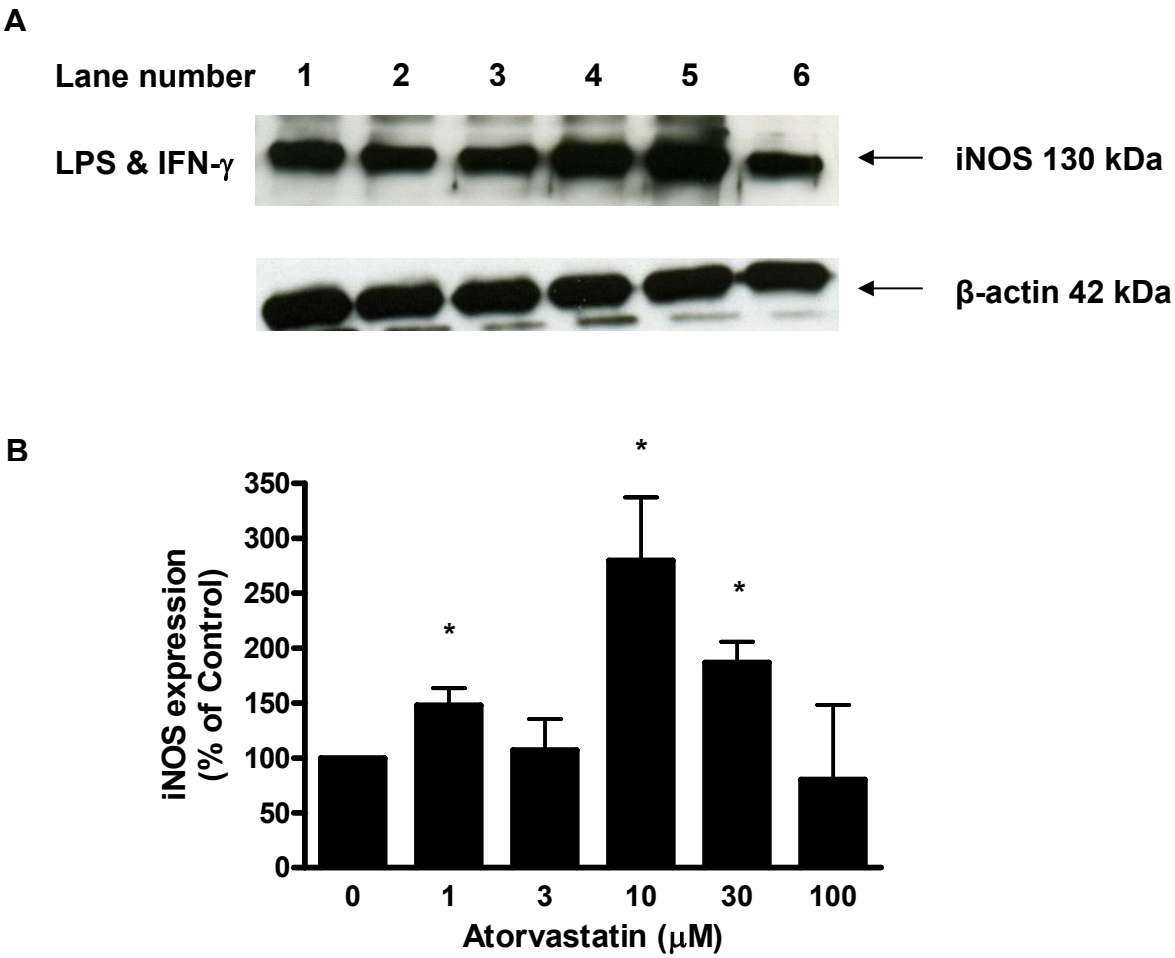


Figure 3.45. Effect of atorvastatin on expression of inducible NOS in control and activated RASMCs.

Confluent monolayers of RASMCs in 6 well plates were treated with atorvastatin at the concentrations shown for 30 minutes prior to activation with LPS (100 μg) and IFN-γ (100 U/ml) for 24 hours. Lysates were generated and analysed by western blotting as described in the Methods (Section 2.7). The lanes represent: 1: control; 2: atorvastatin 1 μM; 3: atorvastatin 3 μM; 4: atorvastatin 10 μM, 5: atorvastatin 30 μM and 6: atorvastatin 100 μM. Panel A is one representative blot from 3 separate experiments and Panel B the densitometry of ± S.E.M. of at least 3 separate experiments.

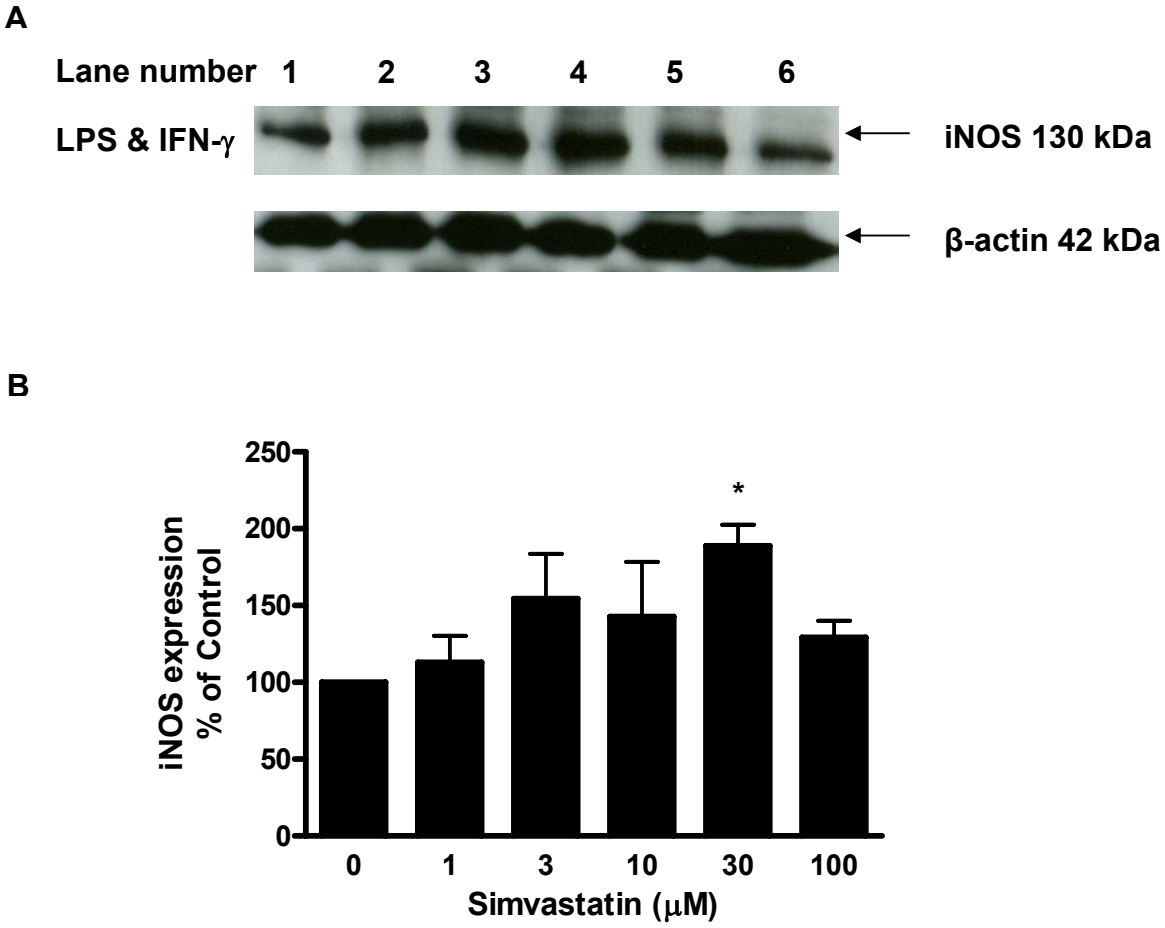


Figure 3.46. Effect of simvastatin on expression of inducible NOS in control and activated RASMCs.

Confluent monolayers of RASMCs in 6 well plates were treated with simvastatin at the concentrations shown for 30 minutes prior to activation with LPS (100 μg) and IFN-γ (100 U/ml) for 24 hours. Lysates were generated and analysed by western blotting as described in the Methods (Section 2.7). The lanes represent: 1: control; 2: simvastatin 1 μM; 3: simvastatin 3 μM; 4: simvastatin 10 μM, 5: simvastatin 30 μM and 6: simvastatin 100 μM. Panel A is one representative blot from 3 separate experiments and Panel B the densitometry of ± S.E.M. of at least 3 separate experiments.

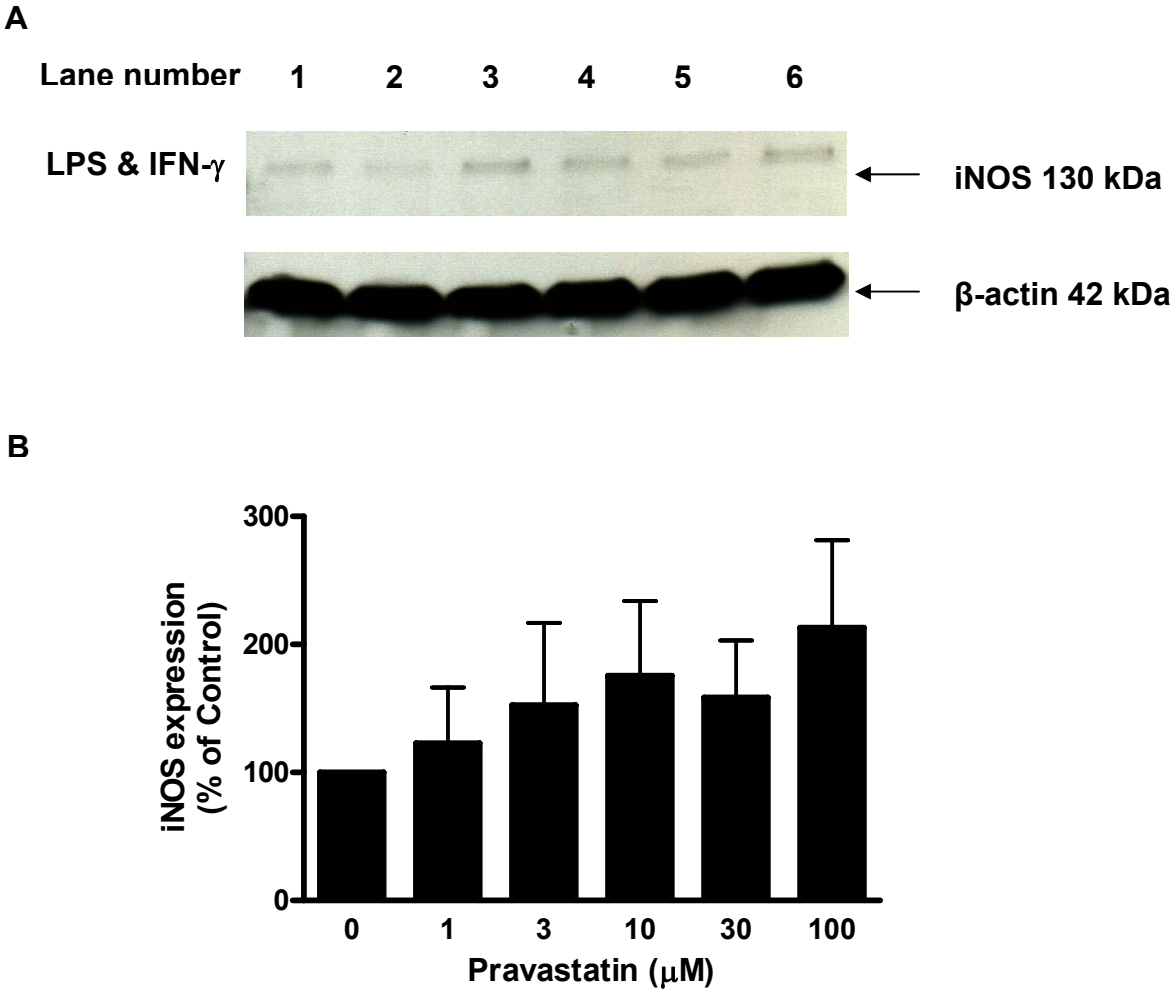


Figure 3.47. Effect of pravastatin on expression of inducible NOS in control and activated RASMCs.

Confluent monolayers of RASMCs in 6 well plates were treated with pravastatin at the concentrations shown for 30 minutes prior to activation with LPS (100 μg) and IFN-γ (100 U/ml) for 24 hours. Lysates were generated and analysed by western blotting as described in the Methods (Section 2.7). The lanes represent: 1: control; 2: pravastatin 1 μM; 3: pravastatin 3 μM; 4: pravastatin 10 μM, 5: pravastatin 30 μM and 6: pravastatin 100 μM. Panel A is one representative blot from 3 separate experiments and Panel B the densitometry of ± S.E.M. of at least 3 separate experiments.

3.10. Effect of statins on oxidative stress induced changes in NO synthesis and L-arginine transport in rat cultured aortic smooth muscle cells

In view of the effects described above for atorvastatin and simvastatin, studies with these compounds were extended to determine whether they could reverse or counteract the actions of the OS inducers described earlier (Section 3.5). In these studies experiments were performed by incubating cultured RASMCs with atorvastatin, simvastatin or pravastatin at the concentration ranging from 1 to 100 μM 30 minutes prior to the co-incubation with antimycin A (100 μM) or DEM (5 or 100 μM) for a further 30 minutes before activation with LPS (100 μg) and IFN-γ (100 U/ml) for 24 hours. Controls were incubated in culture medium alone and another control was used incubating cells with the oxidative stress inducer only.

Consistent with the original observations, antimycin A at 100 μM significantly inhibited NO production as shown in Figure 3.20. More importantly, pre-incubating cells with atorvastatin concentration-dependently reversed these inhibitions with 1 μM virtually restoring levels to those seen with LPS and IFN-γ alone. Moreover, higher concentrations of up to 30 μM caused further potentiation of accumulated nitrite levels which is consistent with the effects observed previously with the statin alone. At 100 μM, atorvastatin suppressed NO production back to the activated control values (Figure 3.48). In contrast, simvastatin (Figure 3.49) and pravastatin (Figure 3.50) modestly recovered the inhibitions of NO production caused by antimycin A but these effects did not appear to be statistically different to the inhibited values. Moreover, neither compound enhanced NO production beyond the

induced values. Furthermore, none of the statins altered the blockage of L-arginine transport seen with antimycin A (Figure 3.51, Figure 3.52 and Figure 3.53).

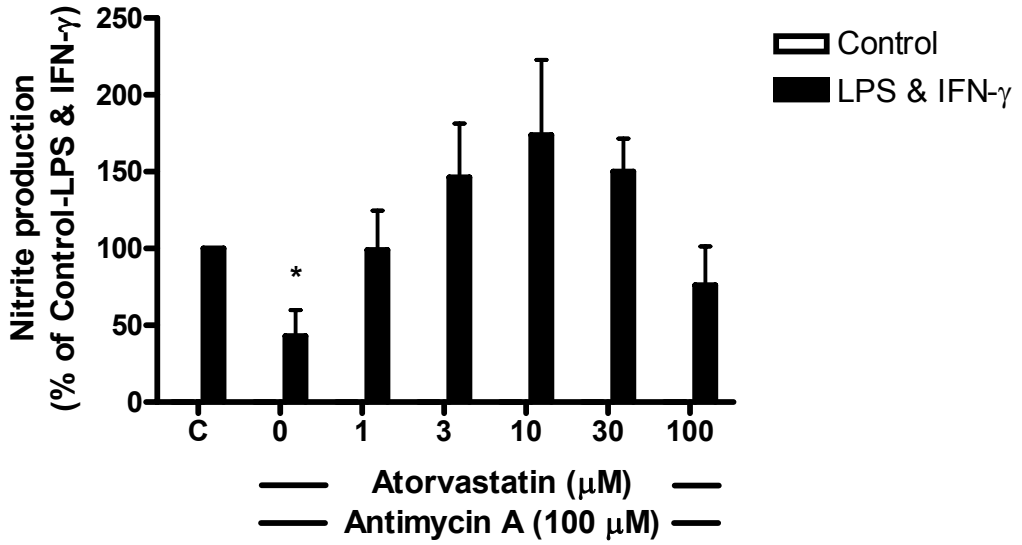


Figure 3.48. Concentration dependent effect of atorvastatin on antimycin A-induced suppression of nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of atorvastatin for 30 minutes before co-incubation with antimycin A (100 μM) for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to all the other conditions.

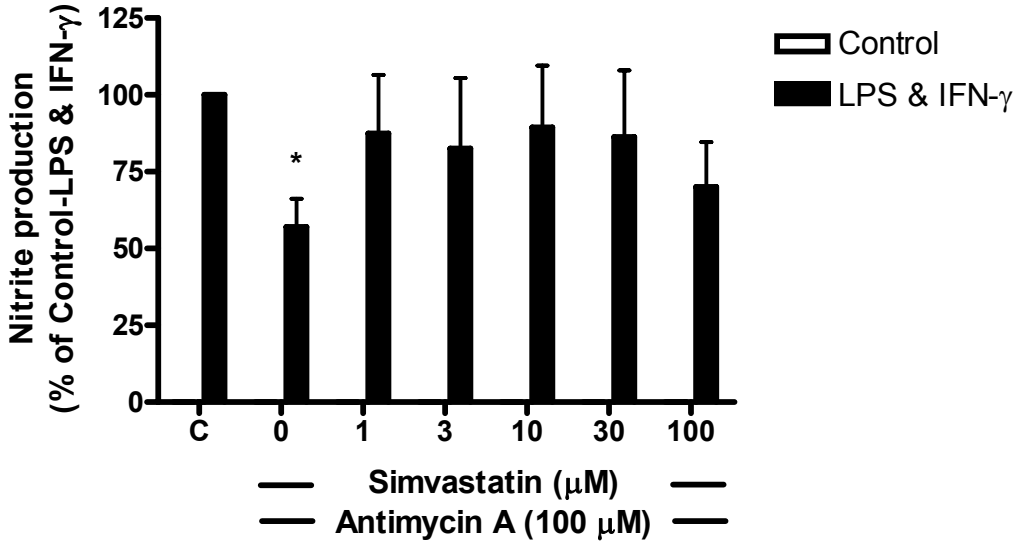


Figure 3.49. Concentration dependent effect of simvastatin on antimycin A-induced suppression of nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of simvastatin for 30 minutes before co-incubation with antimycin A (100 μM) for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. * denotes $p<0.05$ when compared with control.

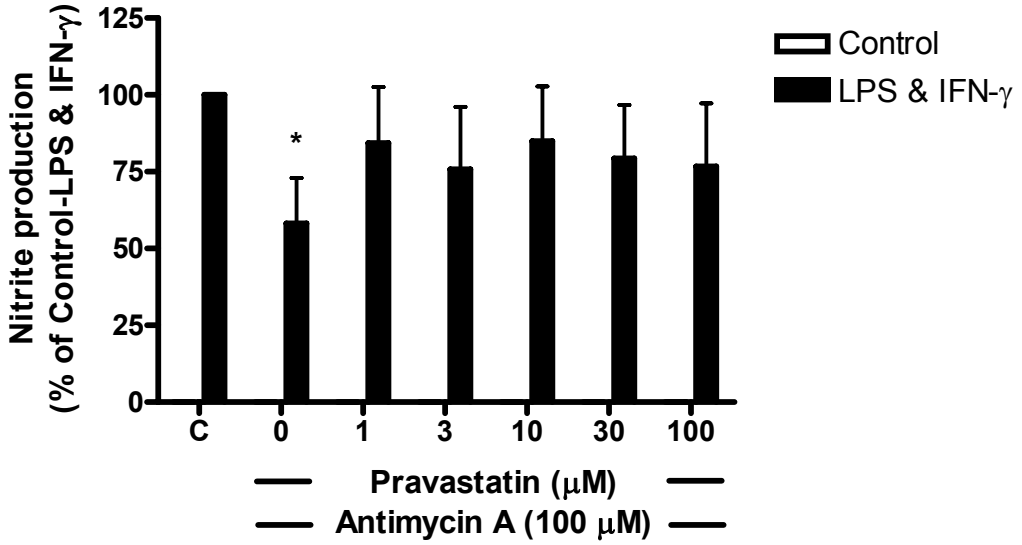


Figure 3.50. Concentration dependent effect of pravastatin on antimycin A-induced suppression of nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of pravastatin for 30 minutes before co-incubation with antimycin A (100 μM) for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. * denotes $p<0.05$ when compared with control.

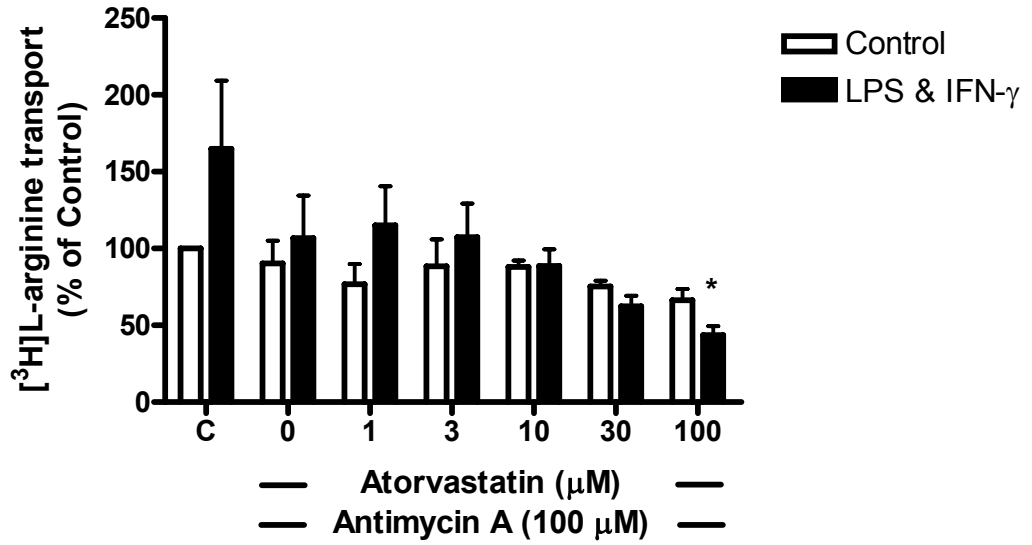


Figure 3.51. Concentration dependent effect of atorvastatin on antimycin A-induced suppression of L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of atorvastatin for 30 minutes before co-incubation with antimycin A 100 μM for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when the values in activated cells (black bars) are compared to the activated control.

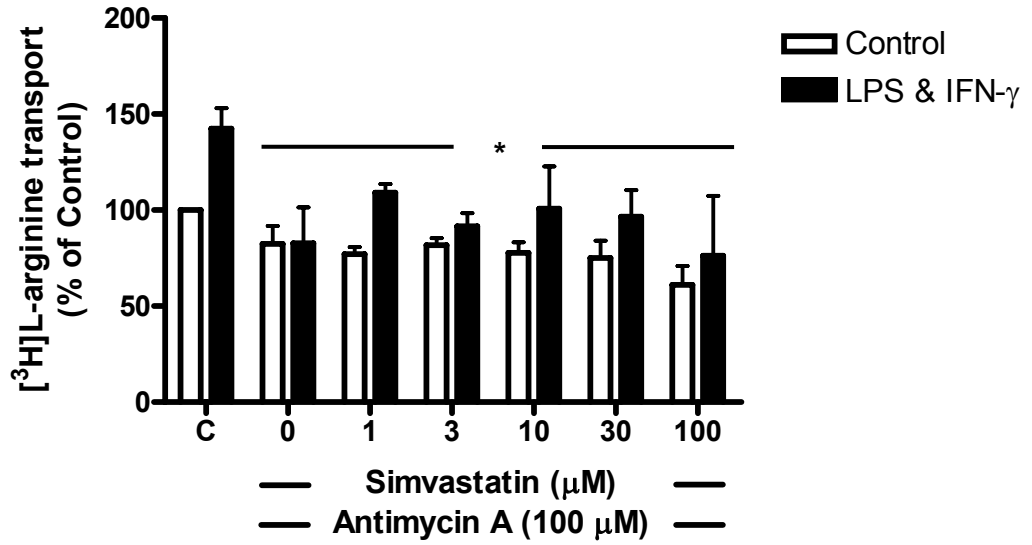


Figure 3.52. Concentration dependent effect of simvastatin on antimycin A-induced suppression of L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to simvastatin increasing concentrations for 30 minutes before co-incubation with antimycin A 100 μM for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when the values in activated cells (black bars) are compared to the activated control.

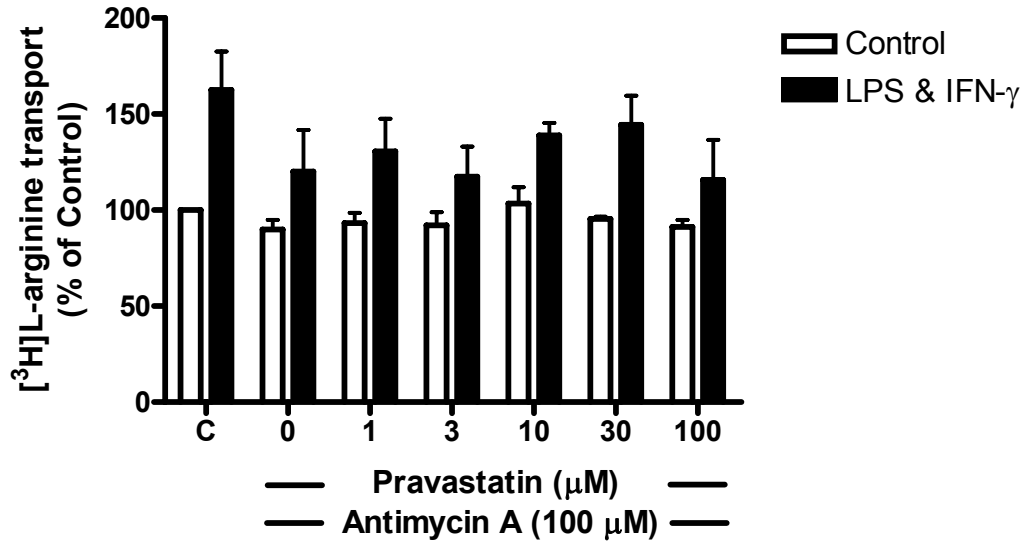


Figure 3.53. Concentration dependent effect of pravastatin on antimycin A-induced suppression of L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to pravastatin increasing concentrations for 30 minutes before co-incubation with antimycin A 100 μM for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

3.11. Effect of statins on DEM-induced changes in NO synthesis and L-arginine transport in rat cultured aortic smooth muscle cells

As with antimycin A, studies were carried out investigating the effects of statins on DEM-induced changes in both NO synthesis and L-arginine transport. Two concentrations of DEM were selected for these experiments, one at 100 μM, and the other at 5 μM. The higher concentration was selected to ensure changes in L-arginine transport could be observed and at this concentration, DEM completely abolished nitrite accumulation and inhibited induced L-arginine transport back to the basal control levels. Higher concentrations of atorvastatin (≥30 μM) together with the 100 μM DEM appear to suppress transport rates in both control and activated cells below the basal values (Figure 3.57). At 5 μM, DEM selectively but only partially, inhibited NO production with no significant effects on transport. When applied, atorvastatin was able to reverse the inhibition of NO synthesis in the presence of 5 μM DEM (Figure 3.60). Simvastatin (Figure 3.61) and pravastatin (Figure 3.62) did not cause any significant changes in the inhibitions produced by 5 μM DEM and all three statins failed to reverse the abrogation of NO synthesis in the presence of DEM 100 μM (Figure 3.54, Figure 3.55 and Figure 3.56 respectively for atorvastatin, simvastatin and pravastatin).

The inhibitions of L-arginine transport induced by DEM at 100 μM were not altered by any of the statins (Figure 3.57, Figure 3.58 and Figure 3.59).

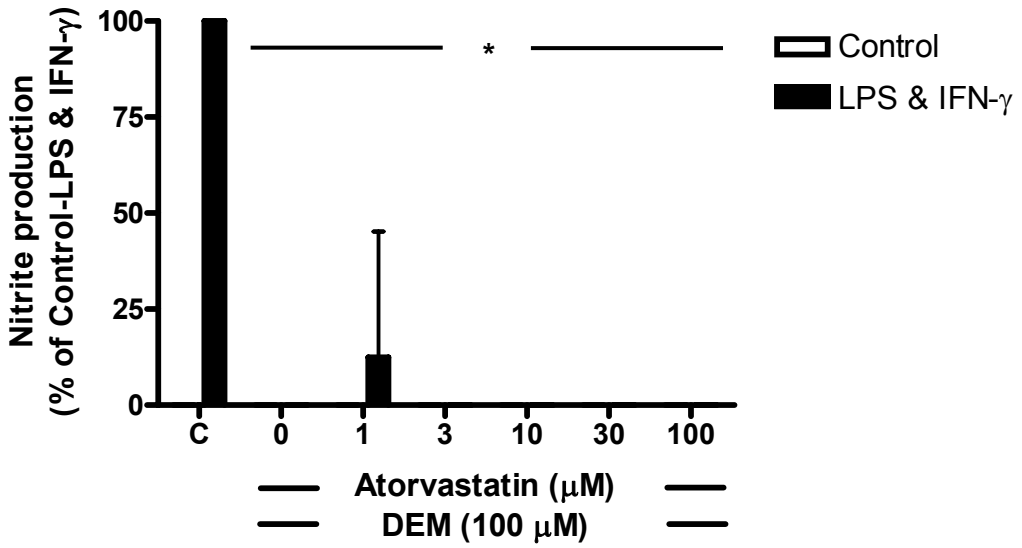


Figure 3.54. Concentration dependent effect of atorvastatin on DEM-induced suppression of nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of atorvastatin for 30 minutes before co-incubation with DEM (100 μM) for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.005$ when the values in activated cells (black bars) are compared to the activated control.

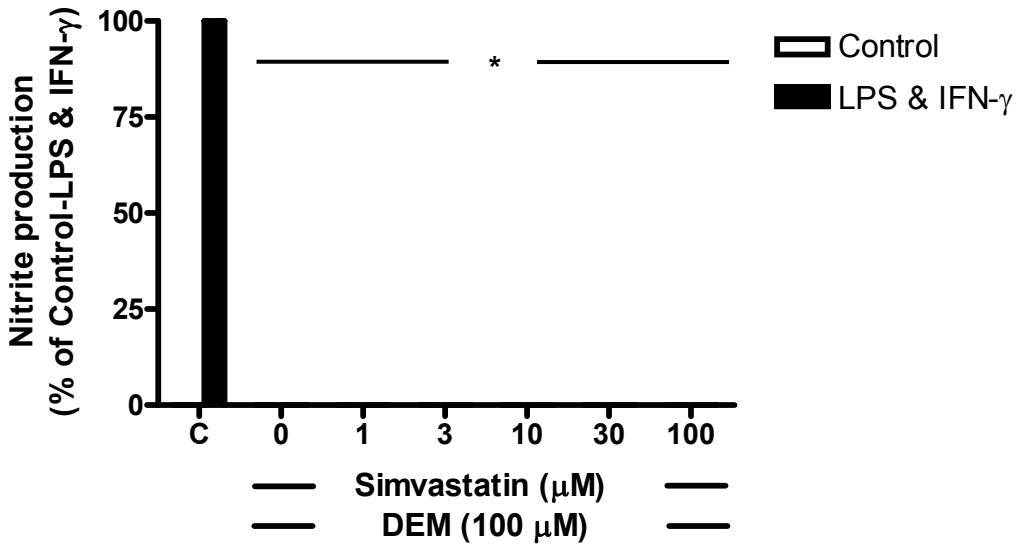


Figure 3.55. Concentration dependent effect of simvastatin on DEM-induced suppression of nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of simvastatin for 30 minutes before co-incubation with DEM (100 μM) for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.005$ when the values in activated cells (black bars) are compared to the activated control.

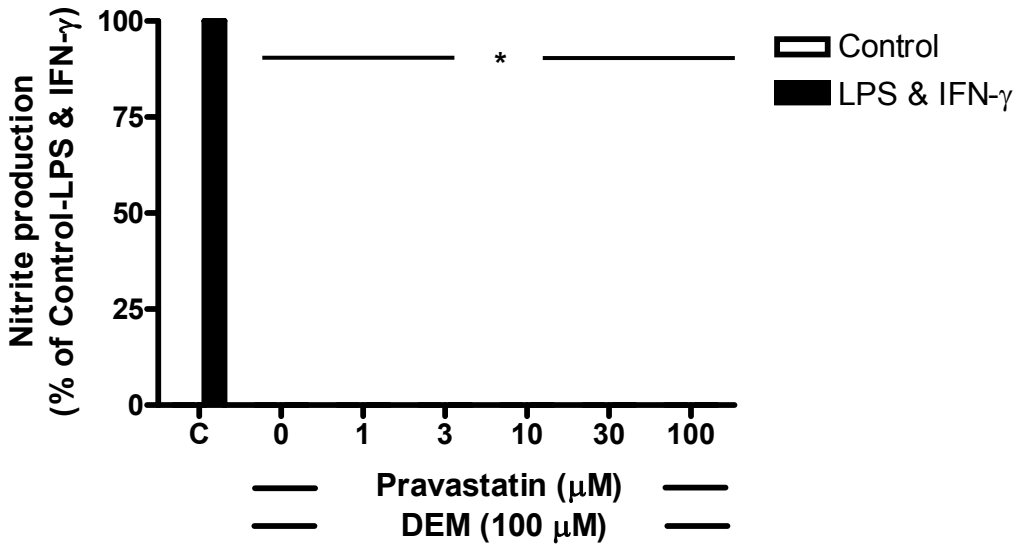


Figure 3.56. Concentration dependent effect of pravastatin on DEM-induced suppression of nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of pravastatin for 30 minutes before co-incubation with DEM (100 μM) for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.005$ when the values in activated cells (black bars) are compared to the activated control.

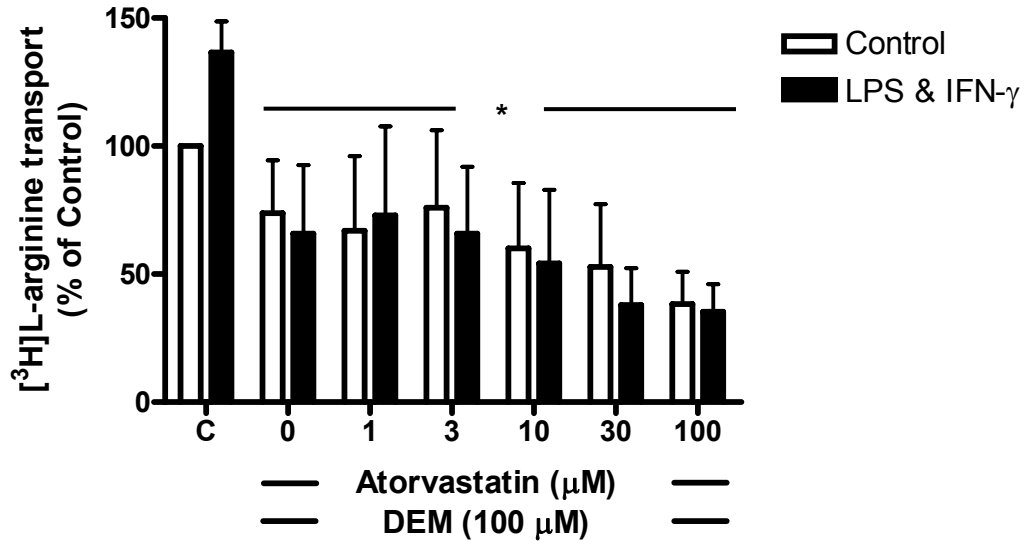


Figure 3.57. Concentration dependent effect of atorvastatin on DEM-induced suppression of L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of atorvastatin for 30 minutes before co-incubation with DEM (100 μM) for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when the values in activated cells (black bars) are compared to the activated control.

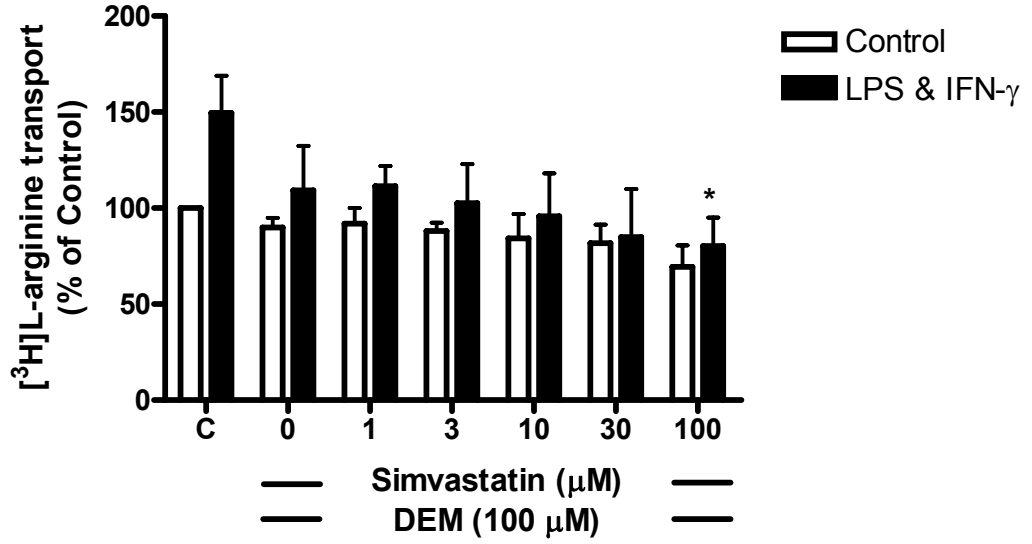


Figure 3.58. Concentration dependent effect of simvastatin on DEM-induced suppression of L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of simvastatin for 30 minutes before co-incubation with DEM (100 μM) for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

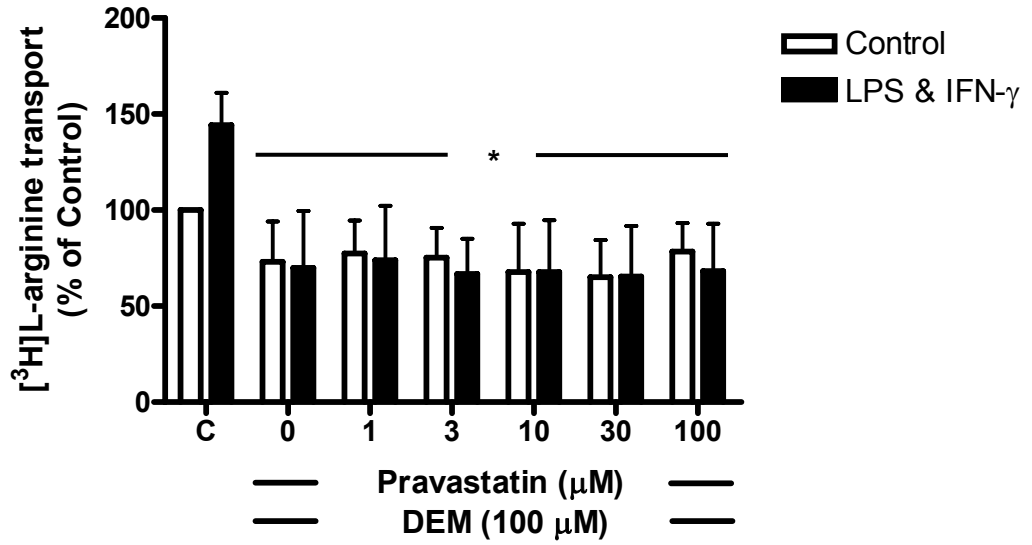


Figure 3.59. Concentration dependent effect of pravastatin on DEM-induced suppression of L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of pravastatin for 30 minutes before co-incubation with DEM (100 μM) for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

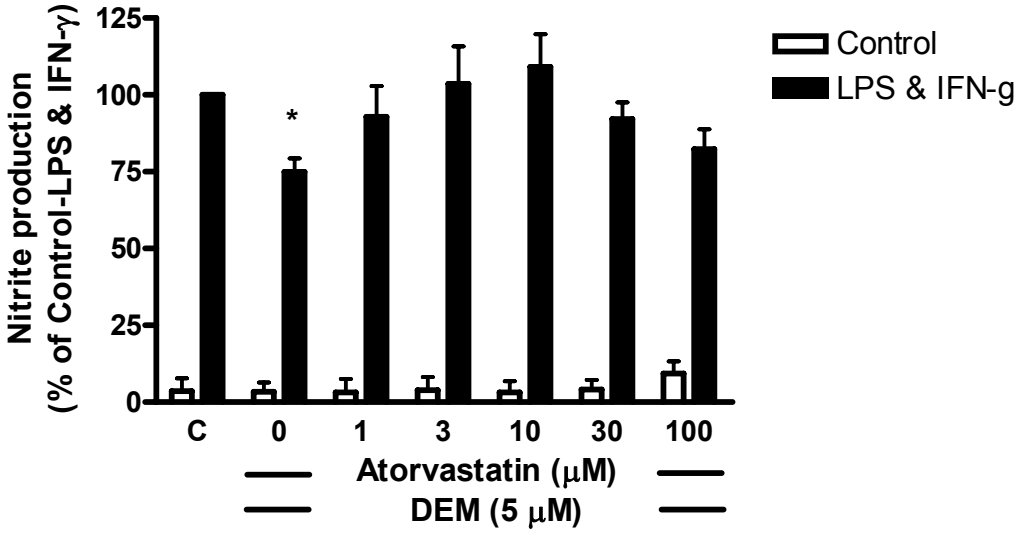


Figure 3.60. Concentration dependent effect of atorvastatin on DEM-induced suppression of nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of atorvastatin for 30 minutes before co-incubation with DEM (5 μM) for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. * denotes $p<0.05$ when compared with the activated control.

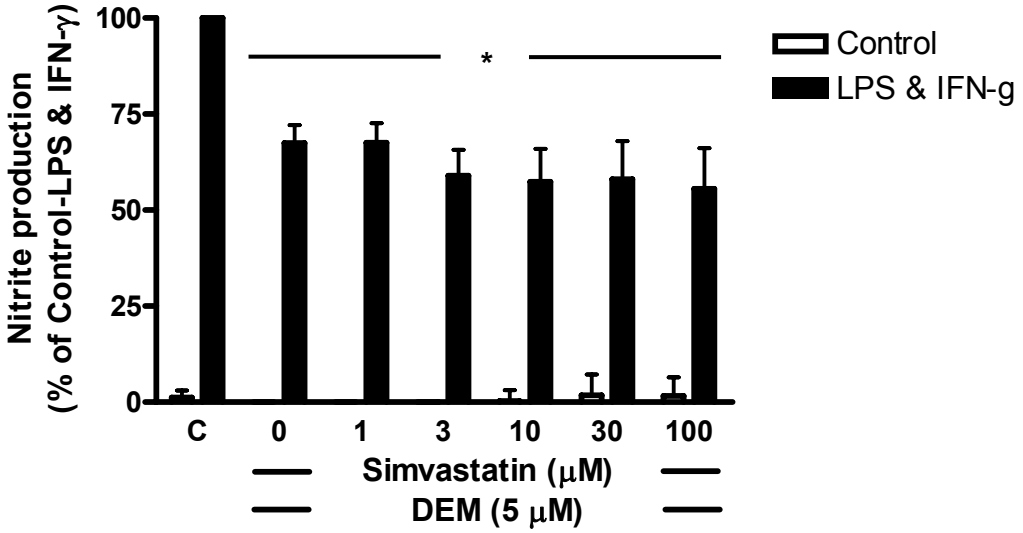


Figure 3.61. Concentration dependent effect of simvastatin on DEM-induced suppression of nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of simvastatin for 30 minutes before co-incubation with DEM (5 μM) for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

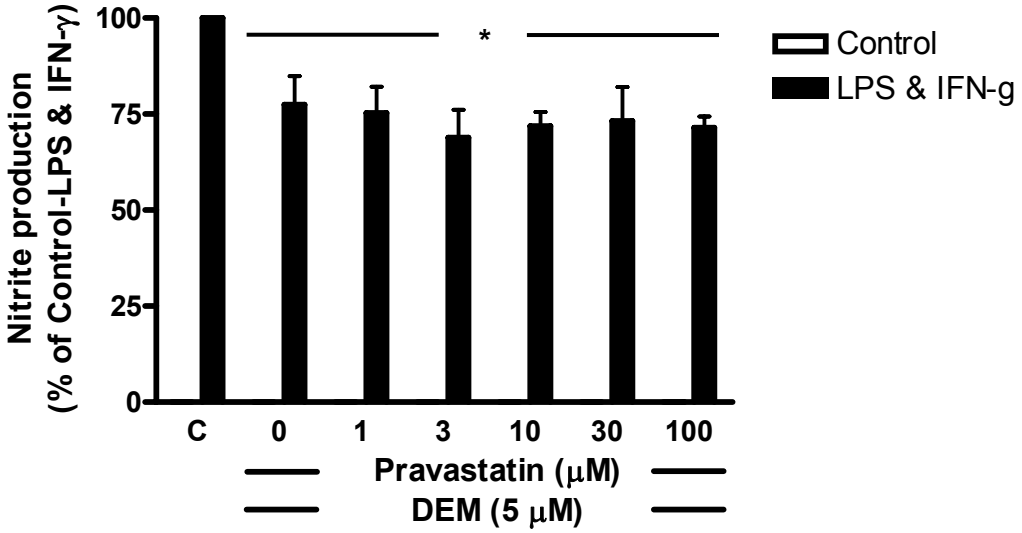


Figure 3.62. Concentration dependent effect of pravastatin on DEM-induced suppression of nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of pravastatin for 30 minutes before co-incubation with DEM (5 μM) for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

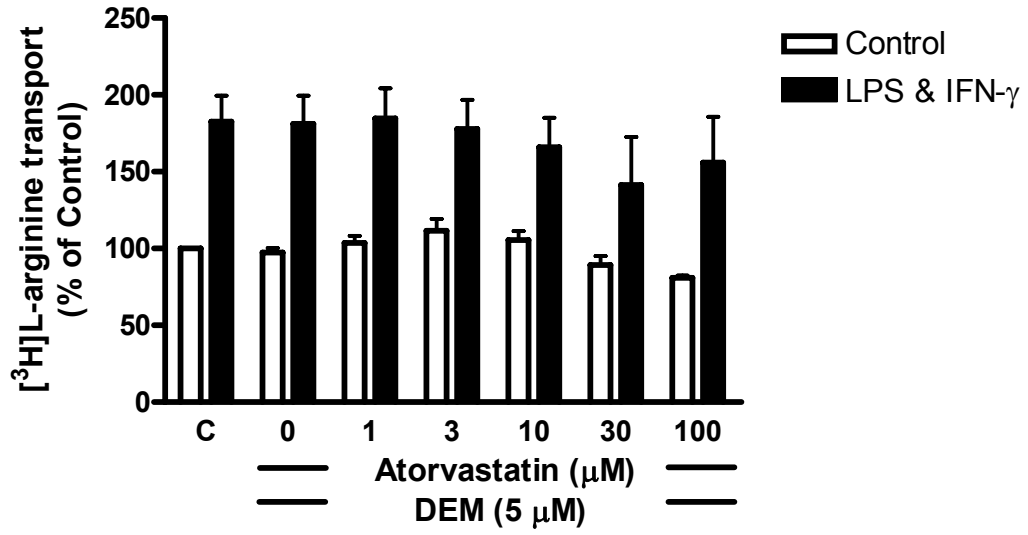


Figure 3.63. Concentration dependent effect of atorvastatin on DEM-induced suppression of L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of atorvastatin for 30 minutes before co-incubation with DEM (5 μM) for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

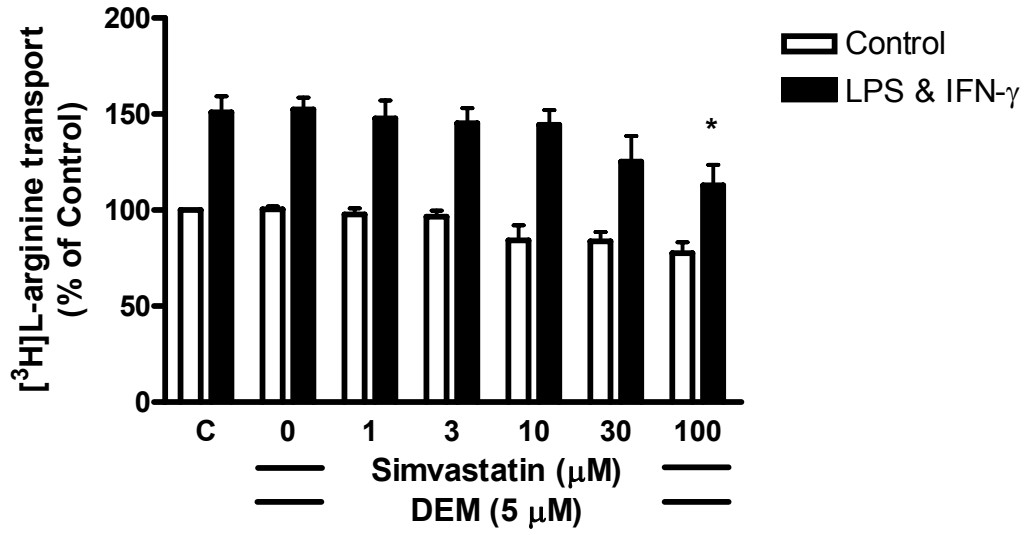


Figure 3.64. Concentration dependent effect of simvastatin on DEM-induced suppression of L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of simvastatin for 30 minutes before co-incubation with DEM (5 μM) for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each. *denotes $p<0.05$ when compared to the activated control.

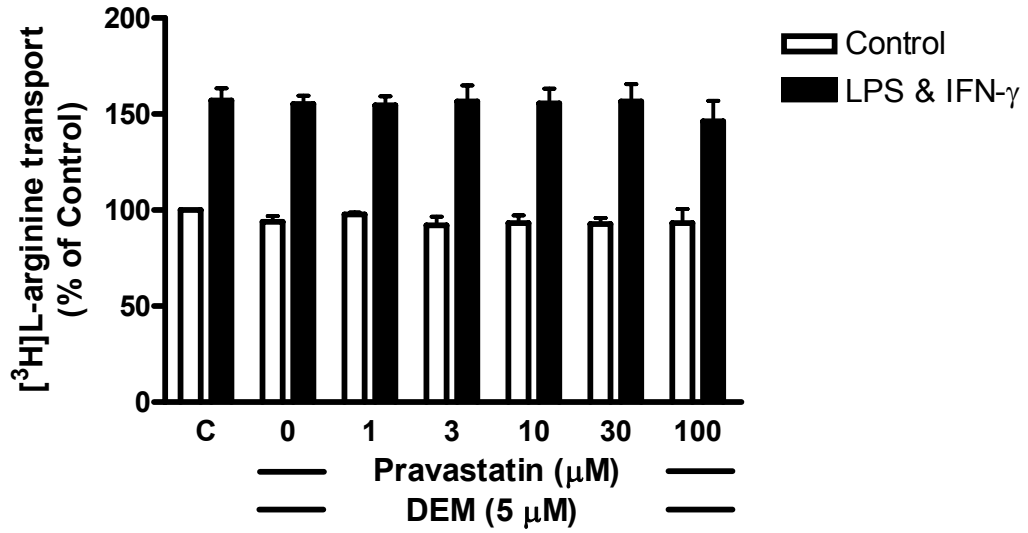


Figure 3.65. Concentration dependent effect of pravastatin on DEM-induced suppression of L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of pravastatin for 30 minutes before co-incubation with DEM (5 μM) for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

3.12. Effect of mevalonate on statins induced changes in NO synthesis and/or L-arginine transport in rat cultured rat aortic smooth muscle cells

Statins inhibit the HMG-CoA reductase enzymes and may deplete cells of mevalonic acid as a consequence. Mevalonate is the product of the reduction of HMG-CoA by the HMG-CoA reductase enzyme and inhibition of its production would ultimately inhibit cholesterol synthesis. To determine whether the effects observed in our studies with the statins are directly related to HMG-CoA inhibition, experiments were carried out supplementing the culture medium with mevalonate at 50 μM and 100 μM. Mevalonate was administered to cultured cells prior to addition of each statin, following induction with LPS and IFN-γ as described previously. Consistent with our earlier observations, induction of NO synthesis was enhanced by atorvastatin at a concentration of 10 μM and by simvastatin at the same concentration. When present, mevalonate appeared to modify the effects of the both atorvastatin and simvastatin (Figure 3.66 and Figure 3.67), reducing at least the simvastatin enhancement of nitrite production back to the LPS and IFN-γ stimulated levels. It is worth noting however that simvastatin did not cause a very marked enhancement in this series of experiments. Interestingly, the responses observed for transport appear to show differences with the statins. Atorvastatin induced transporter activity albeit marginally, and these effects were inhibited by mevalonate (Figure 3.68). When co-incubated simvastatin and mevalonate, the transporter activity was unaffected (Figure 3.69). When applied alone, mevalonate had no distinct effects on either nitrite levels (Figure 3.70) or L-arginine transport (Figure

3.71). Cell viability assays for mevalonate suggest that non cytotoxic concentrations were used (Figure 3.72).

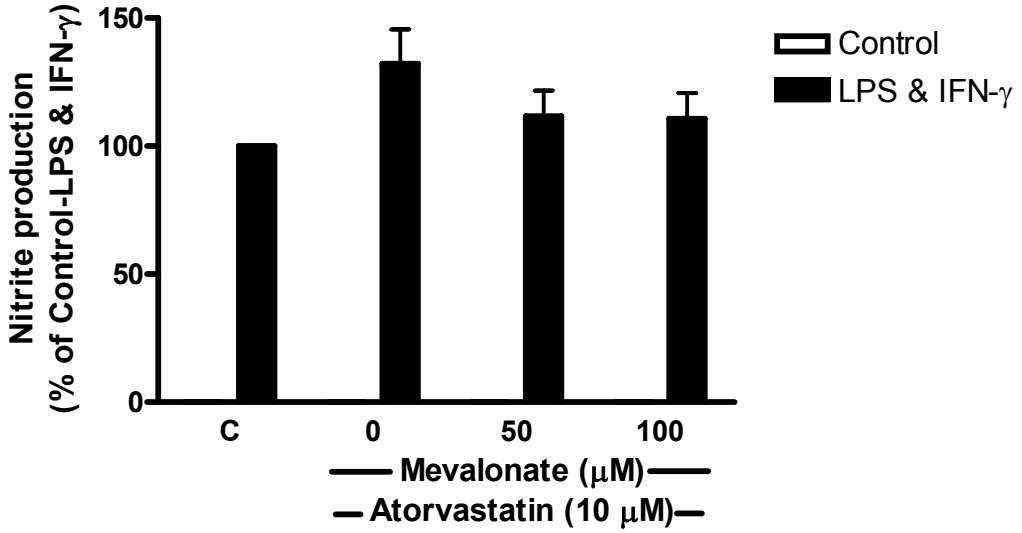


Figure 3.66. Concentration dependent effect of mevalonate on atorvastatin-induced enhancement of nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of mevalonate for 30 minutes before co-incubation with 10 μM atorvastatin for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

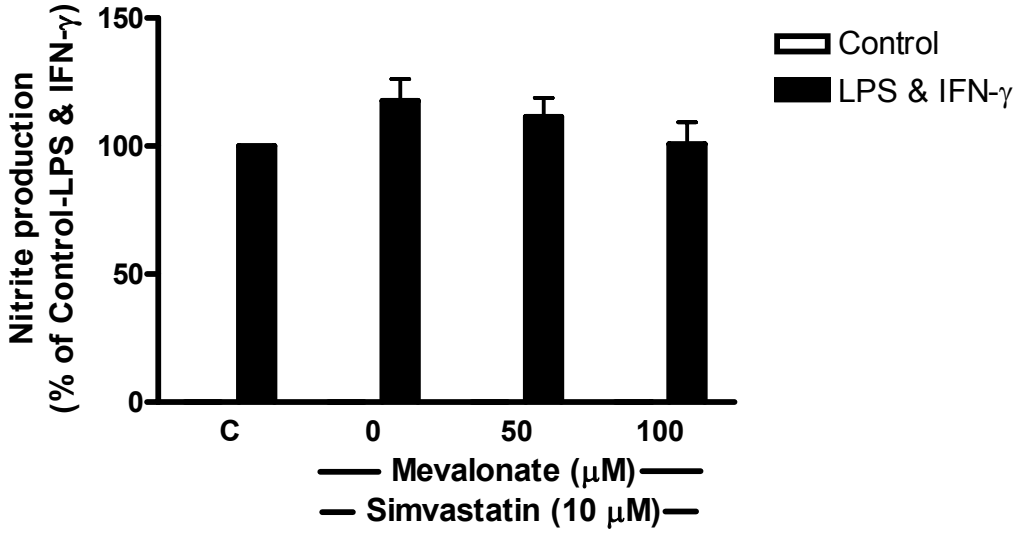


Figure 3.67. Concentration dependent effect of mevalonate on simvastatin-induced enhancement of nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of mevalonate for 30 minutes before co-incubation with 10 μM simvastatin for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

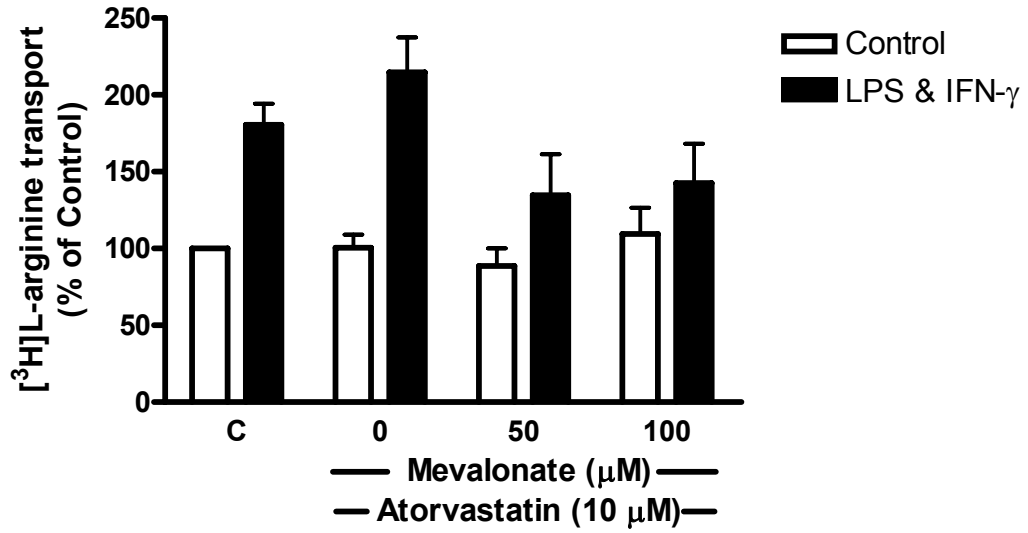


Figure 3.68. Concentration dependent effect of mevalonate on atorvastatin-induced enhancement of L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of mevalonate for 30 minutes before co-incubation with 10 μM atorvastatin for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

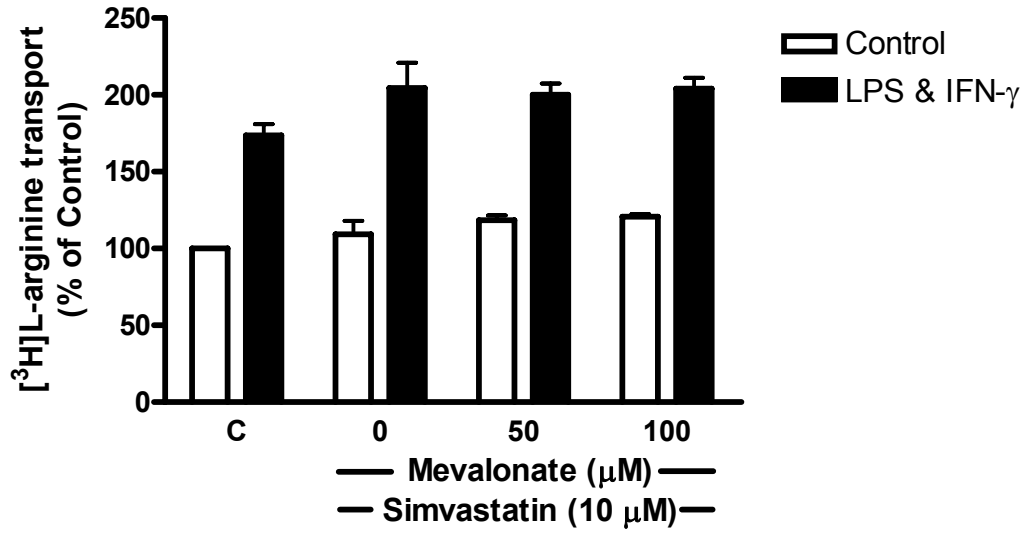


Figure 3.69. Concentration dependent effect of mevalonate on simvastatin induced of L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of mevalonate for 30 minutes before co-incubation with 10 μM simvastatin for a further 30 minutes. Cells were subsequently incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

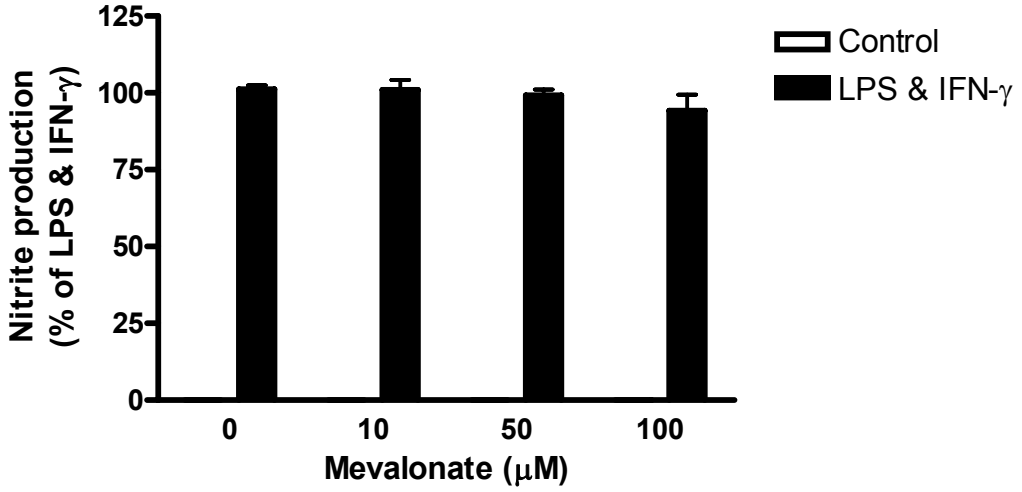


Figure 3.70. Concentration dependent effect of mevalonate on nitrite production in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of mevalonate for 30 minutes before cells were incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Nitrite production was monitored as described in the methods (Section 2.4). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

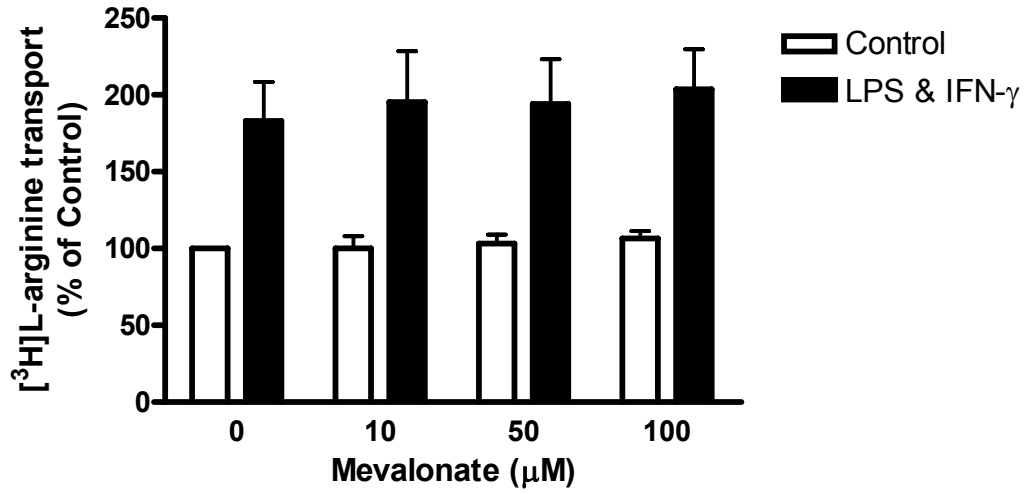


Figure 3.71. Concentration dependent effect of mevalonate on L-arginine transport in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of mevalonate for 30 minutes before cells were incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Transport of [^{3}H]L-arginine was initiated and monitored as described in the methods (Section 2.6). The data represents the mean ± S.E.M. of at least 3 separate experiments with 3 replicates in each.

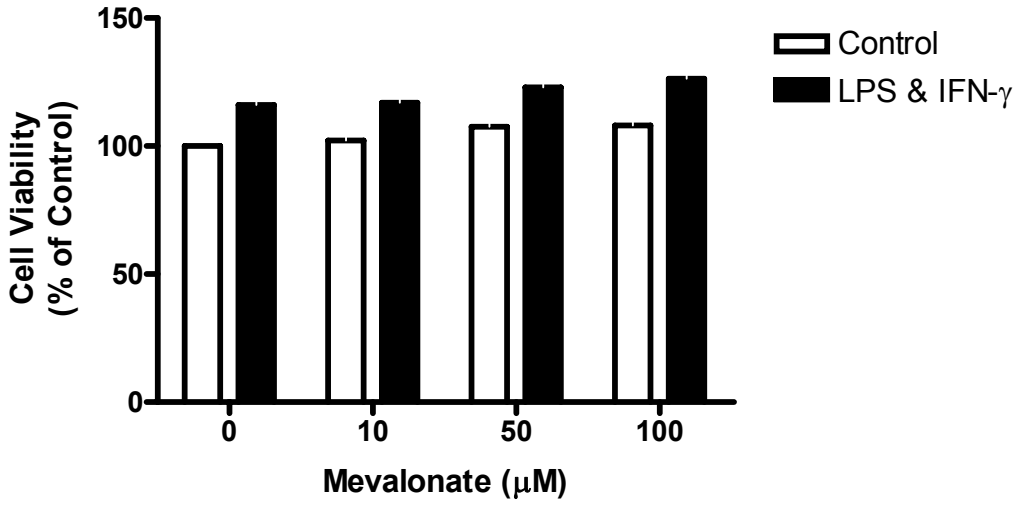


Figure 3.72. Effect of mevalonate on viability in control and activated RASMCs.

Confluent monolayers of RASMCs in 96 well plates were pre-exposed to increasing concentrations of mevalonate for 30 minutes before cells were incubated either in complete culture medium alone (Controls) or in medium containing LPS (100 μg) and IFN-γ (100 U/ml), for 24 hours. Cell viability was monitored as described in the methods (Section 2.8). The data represents the mean of 2 separate experiments with 3 replicates in each.

4 . Discussion

The studies outlined in this thesis were carried out to determine whether NOS and CATs expression and function may be regulated under conditions of oxidative stress (OS). The studies were initially planned for endothelial cells, where the constitutive NOS pathway exists; and for smooth muscle cells where the expression of the inducible enzyme can be initiated. These two cell types play a critical role in regulating vascular tone under normal physiological conditions and are targets for OS induced impairment of vascular function. Disruption of NO synthesis in endothelial cells is characteristic of OS-induced impairment of vascular relaxation, and is a process that may involve an inhibition in eNOS expression, function or even sequestration of eNOS derived NO by ROS generated during an OS insult. Although conclusive evidence is lacking, the latter may also alter L-arginine transport into cells, thereby restricting NO synthesis.

This however requires vital proof and studies aimed at addressing this issue were originally planned in endothelial cells cultured from porcine aorta. Moreover, as with the investigations in SMCs, the effects of statins were also going to be examined in PAECs in order to establish if this group of lipid lowering drugs protect L-arginine transport and NO synthesis and bioavailability under conditions of OS. Such an effect would highlight an additional mechanism of action for these compounds which may contribute towards their beneficial therapeutic actions in man. However difficulties encountered with tissue supply (see discussion below) meant the experiments in endothelial cells could not be carried out.

For the studies in smooth muscle cells, the aim was to determine what role iNOS and CATs play in diseases associated with OS and further determine if the actions of OS on these proteins could be modulated by statins. These studies are

timely and pertinent to the focus of this thesis, especially because of the existing controversy over the role of iNOS in diseases, such atherosclerosis, where controversial data suggest both deleterious and protective actions for this enzyme. iNOS-derived NO has been implicated in the ensuing inflammatory response and has been suggested to influence the pathologic features of the disease through various actions, including the induction of nitrosative stress and enhancing levels of lipoperoxides (Beckman *et al.* 1990; Graham *et al.* 1993; Hogg *et al.* 1993; Huang *et al.* 1999). In contrast to these reports, iNOS-induced NO has been shown to reduce oxidative modification of LDL (Jessup and Dean 1993; Malo-Ranta *et al.* 1994; Rikitake *et al.* 1998; Hemmrich *et al.* 2003) and attenuate atherosclerotic lesions after immune injury in *in vivo* models of the disease (Russell *et al.* 1995; Koglin *et al.* 1998; Koglin 2000). Thus it is clear that iNOS and the NO derived from it can, under certain conditions, be beneficial. We hypothesised in this thesis that the deleterious actions of OS may be mediated, in part, through suppression of the expression and/or activity of iNOS, and potentially L-arginine transport which mediates substrate supply to the enzyme. Such effects would abolish the protective actions of the inducible L-arginine-NO pathway, contributing to the pathogenesis of disease states. This is a rather controversial notion but with emerging evidence in support; and with a fascinating potential of generating new data which may lead to novel ideas for treatment of OS-mediated pathologies such as atherosclerosis. The latter, as already highlighted in the introduction, is a progressive disease which can be initiated as early as in the first decade of life with the identification of “fatty streaks” in vessels such as the coronary artery and abdominal aorta (McMahan *et al.* 2006). The disease develops at different stages and by the second decade of life, the “fatty streak lesions” may become evident in coronary artery spreading to the cerebral

arteries by the third and fourth decade of life (Bonomini *et al.* 2008). The early formation of atherosclerotic plaque starts with injury caused to the endothelium (Chester *et al.* 1990; Creager *et al.* 1990; Braddock *et al.* 1998). This in turn results in structural, functional and biochemical changes in the affected vessel with accumulation of macrophage and lipid formed foam cells, smooth muscle cell, cholesterol crystals and other cell debris accumulating in what is referred to as a fibrous plaque (Pulido *et al.* 2004; Antohe 2006; Crouse 2006; Saam *et al.* 2007). One of the other fundamental changes that occur is the increased production of ROS and initiation of an inflammatory response which contribute to the progression of the disease state.

In the vessel wall, the major sources of ROS are NADPH oxidase, xanthine oxidase, enzymes from the mitochondrial respiratory chain and dysfunctional NOS (Figure 4.1). (Hattori *et al.* 2004; Inagi 2006; Elahi *et al.* 2007; Seddon *et al.* 2007; Forstermann 2008). Of these, the NADPH oxidase system appears to contribute the largest amount of ROS in smooth muscle, endothelial cells and fibroblast (Bengtsson *et al.* 2003; Herkert *et al.* 2004; Ushio-Fukai and Alexander 2004; Escobales and Crespo 2005; Rouhanizadeh *et al.* 2008; Selemidis *et al.* 2008). The NADPH oxidase enzyme is composed of six subunits including a GTPase (Rho guanosine triphosphatase: usually Rac1 or Rac2 - Rac stands for Rho-related C3 botulinum toxin substrate); p40phox; p47phox; p67phox; p22phox and gp91. The latter is barely detectable in VSMCs, and is believed to be replaced by its homologous proteins nox1 and nox4 together with nox2 (Wassmann *et al.* 2002; Higashi *et al.* 2003; Ushio-Fukai 2006).

Nox proteins are prominent sources of vascular reactive oxygen species. These proteins are differentially expressed depending on the cell type and may have

diverse functions such as mediating cellular activation, differentiation, proliferation and angiogenesis, as well as contributing to signal transduction and gene expression (Lambeth 2007). Excessive formation of nox-dependent reactive oxygen species may be associated with pathologies such as atherosclerosis where 60 % of the ROS generated are from nox enzymes (Lambeth 2007) (Figure 4.1). In other conditions such as hypertension, hyperlipidemia or diabetes, nox-derived ROS may also contribute to vascular dysfunction resulting in defective angiogenesis and activation of the inflammatory process (Maytin *et al.* 1999; Cave *et al.* 2006; Selemidis *et al.* 2008). Inhibition of nox proteins may therefore provide a novel approach in preventing and treating cardiovascular diseases (Cave *et al.* 2006; Brandes and Schroder 2008).

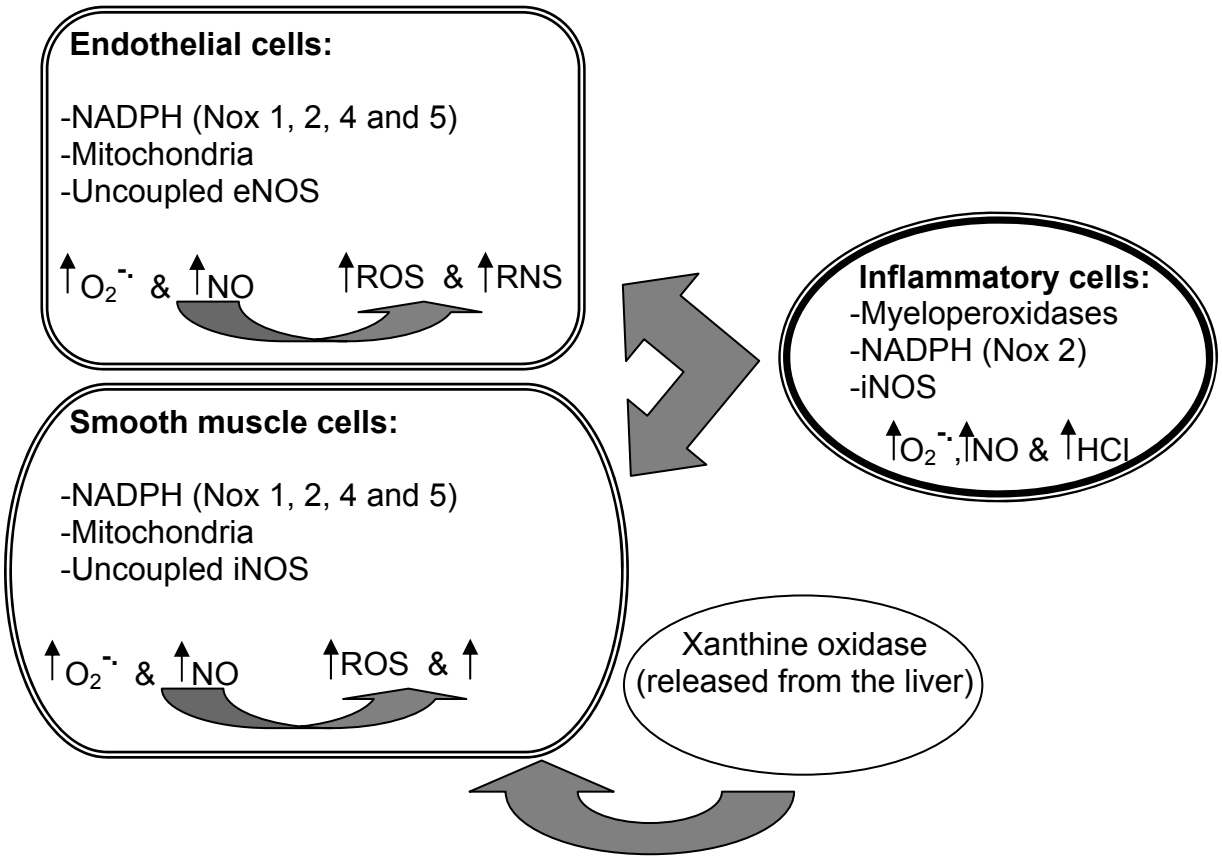


Figure 4.1. Sources of ROS in the vasculature.

Adapted from (Seddon et al. 2007; Forstermann 2008).

The initial studies conducted for this thesis were aimed at identifying and characterising the transport system(s) for L-arginine in aortic endothelial cells with a view to understanding how these carriers may be regulated by OS and further exploring what the consequence would be in modulating NO production. However, only limited experiments were carried out because of the disruption in supply of porcine aortae. The latter were obtained from a link with GlaxoSmithKline as there is no abattoir close to the University that supplied porcine aorta. The supply by GlaxoSmithKline was restricted to limited periods when their studies were being conducted and this eventually ceased when their studies were concluded. Thus, the further experiments planed with PAECs had to be abandoned. However, in the limited studies carried out, it was demonstrated that uptake of L-arginine into these cells was linear for up to 10 minutes, saturating thereafter reaching a plateau at 60 minutes. In addition, the limited kinetics experiments showed transport did not obey saturable Michaelis-Menten kinetics. The later would suggest the expression of a high capacity but low affinity carrier. This however requires further investigation by PCR analysis. Furthermore, cross-inhibition studies with select substrate amino acids are also required to check the selectivity of the transporter(s) expressed.

It is worth noting that previous investigations have shown that L-arginine transport into endothelial cells may be mediated through a high affinity carrier which, under normal physiological conditions, may be CAT-1. In other studies in porcine pulmonary aortic endothelial cells, it appears that L-arginine may be transported through a Na^+-independent system that resembles system y^+ (around 70 % of the uptake) (Greene *et al.* 1993; Bogle *et al.* 1996) and a Na^+-dependent system that is characteristic of $B^{0,+}$ (Greene *et al.* 1993). Thus, it is likely that several transporters

may be associated with uptake of L-arginine into the endothelium, but with system y^+ constituting the predominant carrier system.

In addition to the time course and kinetic experiments, limited studies were also conducted investigating the effects of H_2O_2 on L-arginine transport. This molecule is believed to be produced under conditions of OS and to be one of the major reactive oxygen intermediates that can activate many of the pathways associated with stress. In our studies, H_2O_2 was without significant effect even when applied at different concentrations and in the absence of FBS which is believed to quench the pro-oxidant actions of H_2O_2. Where changes were observed (such as at 1 mM), this was often associated with cytotoxicity and therefore reflect a non-specific action of the compound. These findings indicate that L-arginine transport in PAECs is not susceptible to modification by OS. Alternatively, it is possible that H_2O_2 did not induce stress under the conditions used but this needs further clarification by, for instance, determining whether ROS production was in fact induced following H_2O_2 treatment. Changes in expression of stress proteins such as HO-1 could also be determined as performed to RASMCs (discussed below).

It is worth pointing out that H_2O_2 has indeed been shown to induce OS in several cell types including leukocytes (Arendt *et al.* 2005), chinese hamster ovary cells (Borradaile *et al.* 2006), H9c2 cells (Pesant *et al.* 2006), endothelial cells (Haendeler *et al.* 2005), hepatic cells (Itagaki *et al.* 2005), adipocytes (Kamigaki *et al.* 2006), HeLa epithelial-like cells and Human lens epithelial B-3 cells (Adachi *et al.* 2004; Narayan *et al.* 2005; Seomun *et al.* 2005), cardiomyocytes (Pesant *et al.* 2006) and SMCs (Adachi *et al.* 2004; Lee *et al.* 2004; Ryer *et al.* 2005). These were observed over the same concentration range used in this thesis. Thus, the

concentrations of H_2O_2 employed in our studies should have induced some degree of OS and the reasons why this was not the case is unclear.

In parallel with the PAECs, RASMCs were exploited as the second cellular model, and used specifically to determine how the inducible L-arginine-NO pathway may be regulated under conditions of OS. As pointed out in the introduction, iNOS-induced NO has been widely reported to exert detrimental effects in various inflammatory conditions, inducing deleterious effects largely through its interaction with ROS to generate highly reactive intermediate species such as $ONOO^-$.

The L-arginine-NO pathway in RASMCs had already been characterised previously by the group (Wileman *et al.* 1995) and the current studies conducted using these cells relied on the previous data already generated. For instance, the expression of iNOS and up-regulation of L-arginine transport rates were routinely induced by exposing cells to LPS (100 µg/ml) and IFN-γ (100U/ml). These agents have already been shown to cause consistent and sustained induction of these processes (Wileman *et al.* 1995). As in previous studies (MacNaul and Hutchinson 1993; Wileman *et al.* 1995; Wileman *et al.* 2003), this combination of LPS and IFN-γ up-regulated iNOS-induced NO production and enhanced L-arginine transport, increasing the latter above the basal rates seen in control non activated cells.

The effects of OS on both iNOS expression and enhanced L-arginine transport in RASMCs were investigated using two approaches in which cells were either activated and then exposed to the OS inducers or pre-exposed to these agents before activating with LPS and IFN-γ. In the former studies, exposure of RASMCs to OS inducers was preceded by induction with LPS and IFN-γ for 21 hours and activation was always confirmed by the Griess assay. These cells were subsequently exposed to H_2O_2 at different concentrations and time points prior to

monitoring L-arginine transport. Over these shorter incubation periods, H_2O_2 suppressed L-arginine transport but only when used at 1 mM and exposed to cells for periods of up to 180 minutes in complete culture medium. Lower incubation times and concentrations were without significant effect. This may reflect the amount of free radicals generated over this period and may indicate that high amounts of free radicals are required over prolonged periods to suppress L-arginine uptake. Alternatively, since there was some degree of cytotoxicity seen at the extreme conditions, it is likely that the inhibition of transport rates may in fact reflect cytotoxicity.

It was considered that the lack of significant effect with H_2O_2 in complete medium may be due to the presence of phenol red and FBS, which both have antioxidant potentials (Zheng *et al.* 2006; Lewinska *et al.* 2007). To eliminate this possibility, further experiments were carried out omitting these from the culture medium during incubation of cells with H_2O_2. In these studies, there were no significant changes in either basal or induced transport rates at any of the concentrations of H_2O_2 used or time points examined. These observations indicate clearly that the lack of effects seen with H_2O_2 in complete medium was in fact not due to any of the antioxidant actions of either phenol red of FBS but perhaps due to H_2O_2 not being able to modify transport, al least in acute incubations (<180 minutes). Similar to the findings with short term treatments, chronic exposure of RASMCs to H_2O_2 did not result in any marked changes in basal or induced transporter activities. Accordingly, induced NO synthesis was also not significantly affected by H_2O_2. Since H_2O_2 was added prior to inducing cells with LPS and IFN-γ, it is clear therefore that the induction process associated with enhanced L-arginine transport or NO production were not modified by H_2O_2. Taken together, the data in RASMCs is

consistent with the preliminary findings in PAECs in that neither the induction of iNOS-induced NO synthesis nor the up-regulation in L-arginine transport appeared to be susceptible to H_2O_2. As already discussed for PAECs, it is not clear whether this is because OS was not induced or whether these processes (iNOS-induced NO synthesis and L-arginine transport) may in fact be insensitive to the oxidant actions of H_2O_2. Unfortunately it was not possible to determine the production of ROS due to the facilities not being available in the laboratory. However, the effects of H_2O_2 on HO-1 expression were determined and this is discussed below.

In other studies where H_2O_2 has been employed, experiments were carried out in PBS or media with low FBS (0.1 %) (Adachi *et al.* 2004; Lee *et al.* 2004) and with higher concentrations of H_2O_2 (≥1 mM) to induce apoptosis (Borradaile *et al.* 2006). Moreover, these studies revealed the effects of H_2O_2 at 72 hours incubation in low FBS medium. Our experiments were however limited to a maximum of 24 hours incubation because of decreased cell viability over prolonged exposure time points. Our aim was not to induce apoptosis but rather to induce OS and maintain cell viability without any adverse apoptotic actions being evident. In addition, the focus of our studies was to determine how OS may regulate the function and/or induction of iNOS/L-arginine transport. This needed to be examined over the period where the induction takes place; and for iNOS this is reported to start at 3 hours reaching a peak at 18 hours (Boughton-Smith *et al.* 1993).

One other issue worth noting is the fact that there may be differences in the ability of various systems to cope with OS and this may reflect differences in their antioxidant capacities. For instance, it is reported that endothelial and VSMCs from humans contain low levels of catalase activity and as a result, these cells are more susceptible to the effects of H_2O_2 (Shingu *et al.* 1985; Lin *et al.* 2007). The fact that

the same is not true in RASMCs may additionally reflect, perhaps, species differences.

The protection of tissue and cells against OS is provided by anti-oxidative and phase 2 enzymes (Li *et al.* 2006). In RASMCs the antioxidant defence triggers a time and concentration dependent increase of cellular antioxidants and phase 2 enzymes including SOD, catalase, glutathione, glutathione reductase, glutathione peroxidase, glutathione *S*-transferase (GST), and NADPH:quinone oxidoreductase-1 (NOQ1) (Li *et al.* 2006). Studies in SMCs show that when exposed to oxidative stress or oxLDL, these cells respond by inducing the expression of stress proteins like HO-1 and MSP23, as a cytoprotective mechanism (Siow *et al.* 1995). Although the induction of HO-1 is important under OS, this protein has a crucial role in preventing atherosclerosis as described in a study where a HO-1 deficient child had severe atherosclerotic lesions (Kawashima et al. 2002). The transcription factor Nuclear Factor erythroid 2 (NF-E2)-related factor 2 (Nrf2) regulates phase 2 antioxidant response by binding to the antioxidant response elements (ARE) in the promoter region of genes like HO-1, GPx and GSH enzymes (Nguyen *et al.* 2003). In a study with spontaneously hypertensive rats (SHR) the GSH system was impaired in aortic SMCs further contributing to OS (Wu and Juurlink 2001).

The lack of effect with H_2O_2 in our systems is rather unexpected not least because of the discussion in the paragraph above but also because H_2O_2 has been shown to have many physiological functions and is reported to regulate cell proliferation, differentiation, and migration. It can also act as a second messenger modulating the growth of SMCs and fibroblasts and regulating the expression of inflammatory mediators (Schleicher and Friess 2007). Moreover, H_2O_2 may be able to activate many signalling pathways, inducing the phosphorylation and activation of

proteins such as c-Jun N-terminal Kinases (JNK), extracellular signal regulated kinases (ERK)1/2, protein kinase B (PKB) and p38 MAPK in vascular cells (Ushio-Fukai *et al.* 1998; Chevalier *et al.* 2000; Blanc *et al.* 2004). These proteins, and in particular the p38 MAPK, may play a significant role in regulating both induced NO synthesis and L-arginine transport (Baydoun *et al.* 1999).

Because of the moderate effects of H_2O_2 in RASMCs, our studies were extended to other inducers of OS in order to determine conclusively whether OS regulated the inducible L-arginine–NO pathway. The additional compounds used were antimycin A and DEM. Antimycin A is produced by *Streptomyces* sp. bacteria and exerts its effects by blocking the flow of electrons from semiquinone to ubiquinone in the Q-cycle of complex III in mitochondrial oxidative phosphorylation. By disrupting the proton gradient across the inner membrane, antimycin A prevents the production of adenosine 5'-triphosphate (ATP) as protons are unable to flow through the ATP synthase complex. The inhibition of Complex III by antimycin A results in the formation of large quantities of the toxic free radical ($O_2^{\cdot -}$) augmenting intracellular OS (Bundy *et al.* 2005). In cells such as the As4.1 from pigeons hearts, H_2O_2 may be one of the products generated by antimycin A (Boveris and Chance 1973; Boveris *et al.* 1976; Han *et al.* 2007). The effects of antimycin A on mitochondria may also contribute largely to the increases in peroxynitrite production which is promoted by the reaction between NO and superoxide from mitochondria (Packer *et al.* 1996).

As with H_2O_2, studies using either antimycin A or DEM examined both acute (5 minutes to 180 minutes) and chronic effects (24 hours) of these compounds on L-arginine transport in control and activated cells. Consistent with the H_2O_2 data, neither antimycin A nor DEM altered transport rates when exposed to cells acutely

and after activation with LPS and IFN-γ. It can be concluded therefore that OS, or at least the OS inducers used in the studies, do not regulate the function of the transporters for L-arginine in RASMCs. This would however contradict at least one other report in the literature which has suggested that L-arginine transport in porcine pulmonary aortic endothelial cells may be regulated under conditions of OS (Patel *et al.* 1996). This is thought to occur through S-nytrosilation of the transporters by sulfhydryl agents that modify protein thiol groups thereby reducing the function of the carriers and thus L-arginine transport (Patel *et al.* 1996).

In the 24 hours exposure studies, both antimycin A and DEM treatment resulted in a concentration dependent inhibition of both nitrite accumulation and L-arginine transport. Interestingly, the latter was affected only in activated cells with the enhanced transporter activity being reduced back to control levels but with basal rates remaining virtually unaltered. Moreover, the inhibitions on induced L-arginine transport were more apparent at lower concentrations of antimycin A when compared to suppression of NO synthesis where 150 μM of the drug was required to virtually abolish the response. By comparison, 100 μM antimycin A was sufficient to reverse induced L-arginine transport back to basal levels. This suggests that the transporter(s) may be more sensitive to regulation by OS or at least by antimycin A.

The precise mechanism via which these effects of antimycin A are mediated remain to be determined. However, the fact that both induced NO production and L-arginine transport were abolished without affecting controls suggests that antimycin A may be acting at the induction level, potentially inhibiting these processes. Since antimycin A can deplete ATP levels due to the disruption of oxidative phosphorylation, it would not be unreasonable to suggest that its ability to suppress both NO production and L-arginine transport may, in part, be through this

mechanism. Indeed, cellular models of inflammation are known to require higher energy production for gene transcription and *de novo* protein synthesis (Berg *et al.* 2003). Thus inhibition of ATP generation would inevitably disrupt these processes. Also of significance to transport is the fact that it is an energy dependent process which may be suppressed significantly if cells are depleted of ATP. This would be an effect that directly affects the functioning of the transporter rather than their induction. Such a mechanism would however be contradicted by the fact that antimycin A was without effect on basal transporter activity which should also be dependent on cellular ATP. Thus the more likely explanation for the actions of antimycin A may be that the compound is influencing cellular signalling pathways that are responsible for the induction of iNOS and the CATs. This in turn leads, potentially, to a suppression of gene transcription and translation and thus reduced protein expression which is reflected in a decrease in function. Unfortunately, because of the constraints of time, gene analysis studies could not be carried out and these studies are clearly needed. Expression of iNOS protein was however monitored and this is discussed further below. Changes in CAT proteins could not be determined because of lack of appropriate available antibodies.

As with antimycin A, prolonged incubation of cells with DEM also inhibited NO production and induced L-arginine transport. However, the latter appeared less susceptible to DEM when compared to NO production because transport remained virtually unaltered at concentrations of DEM (25 μM) that virtually abolished NO synthesis. This shows a clear differential regulation of transport and NO synthesis by DEM and may highlight differences in the mechanisms associated with the induction of these processes.

The inhibition of NO production by DEM in RASMCs is consistent with its reported effects in hepatocytes where it has been shown to decreases levels of nitrite and iNOS expression (Kang *et al.* 1999; Tirmenstein *et al.* 2000). Although we have not addressed the underlying mechanisms of action of DEM in our studies, it is possible that this may be linked to its ability to deplete glutathione levels which in turn induces OS (Boyland and Chasseaud 1967; Miccadei *et al.* 1988; Russo *et al.* 1995). DEM possesses an electrophilic core that interacts with the glutathione S-transferase system, modifying thiol groups, albeit non specifically, and reducing GSH levels to 50 % when used at concentrations ranging from 125 μM to 250 μM (Boyland and Chasseaud 1967; Yang *et al.* 2004). These reductions could have detrimental consequences and in a rat animal model, the depletion of GSH induced hypertension, decreased NO bioavailability and may contribute to the pathogenesis of atherosclerosis (Vaziri *et al.* 2000).

It has been demonstrated in other studies that administration of concentrations ≤100 μM of DEM in fibroblasts enhanced GSH content and induced transport of its precursor L-cysteine, but L-cystine transport declines when higher concentrations (up to 1 mM) are used (Bannai 1984). The increase in L-cysteine may be a cell protective response to OS against electrophiles (Ruiz *et al.* 2003) and interestingly, DEM has been used as a therapeutic agent to prevent organ damage in extreme inflammatory conditions such as septic shock (Jones *et al.* 1999). In such conditions, there is an overproduction of NO which could lead to marked hypotension and eventually end on organ failure. In such a state, DEM may conceivably reduce NO production and bioavailability, thereby reducing the chance of organs collapse. The underlying mechanism is likely to involve the generation of free radicals which uncouple NOS and blunt NO produced. In addition, signalling events that lead to the

induction of iNOS may potentially be inhibited leading to a decrease of iNOS expression and potentially L-arginine transport. Moreover, ROS may also oxidise BH_4 and uncouple NOS (Landmesser *et al.* 2003; Bevers *et al.* 2006; Tang *et al.* 2007; Sweazea and Walker 2008; Zhang *et al.* 2008). In the absence of sufficient BH_4, NOS changes its functional profile and instead of oxidising L-arginine, oxygen is reduced to superoxide. In these conditions, the flux of superoxide will be higher due to the increased activity of iNOS (Loscalzo 2000). Disruption of NADPH, associated with oxidation of L-arginine by iNOS, may lead to an interruption in NO production and further perpetuating superoxide production (Vasquez-Vivar *et al.* 1997; Vasquez-Vivar *et al.* 1997; Vasquez-Vivar *et al.* 1998; Panda *et al.* 2004). It is worth noting that should this process go unchecked it could result in detrimental consequences.

The different profiles of inhibition of iNOS-induced NO and L-arginine transport caused by antimycin A and DEM may well reflect their different actions in terms of the actual cellular targets regulated by these compounds or their products. However, since the effects are only visible following pre-treatment of cells prior to activation, it is likely that both compounds act through inhibition of the induction process rather than affecting the actual direct activity of either the transporters or iNOS. What has not been established in this thesis is whether the actions of antimycin A or DEM are indeed mediated through ROS generation, which these compounds are clearly well documented to do, or through other mechanisms independent of ROS generation. What is clear however is the fact that the literature supports that antimycin A and DEM (and indeed H_2O_2) induce OS at the concentrations used. This cannot therefore be ruled out as a potential factor in the effects reported in the thesis. Studies measuring changes in levels of ROS produced

are clearly required to further develop the studies and these should be part of the future work planned.

As an alternative to ROS generation, changes in the expression of the stress protein HO-1 was monitored as an index of the induction of OS. Heme oxygenase-1 is a stress protective protein induced under oxidative imbalance. The products of its catabolism are carbon monoxide (CO), biliverdin (which is rapidly converted to bilirubin) and free iron (which leads to the induction of ferritin, an iron-binding protein). The actions of these products could be valuable physiological and therapeutic agents. For instance, CO like NO modulates intracellular cGMP levels, platelet aggregation and smooth muscle relaxation, but has a much lower affinity for sGC than NO (Siow *et al.* 1999). Similarly, bilirubin acts as a potent cellular antioxidant by rapid interconversion to biliverdin, removing ROS at up to 30,000 times its concentration (Siow *et al.* 1999; Otterbein *et al.* 2003). Contrasting with these beneficial effects, it is reported that under certain conditions, CO may have detrimental effects if produced inappropriately. One example where this may be the case is in chronic hypoxia where generation of ROS and the production of CO by induced HO-1 is reported to lead to vasoconstriction of mesenteric arteries (Sweazea and Walker 2008).

In our studies, experiments were carried out exploring the changes in expression of HO-1 under control conditions and in cells exposed to the OS inducers. The data obtained to date show that HO-1, which is not expressed in controls, is induced following exposure to DEM. Surprisingly, antimycin A had a less pronounced effect which was only evident at the highest concentration used (150 μM) while H_2O_2 was virtually without effect. These studies suggest a difference in the mechanism of actions of the three compounds and indicate that effects of H_2O_2 and,

potentially, antimycin A may be independent of an action on HO-1 expression. It is possible that with DEM, the depletion of glutathione may account for the expression of HO-1 and also for its effects on both NO production and L-arginine transport which may all be linked to the induction of OS.

Oxidative stress can regulate several signalling events as already highlighted. One of its targets in cells is NF-κB (Sen and Packer 1996; Chakraborti and Chakraborti 1998) which is implicated in the induction of not only iNOS (Xie *et al.* 1994; Katsuyama *et al.* 1999; Matsumura *et al.* 2001; Zhang *et al.* 2001; Pingle *et al.* 2003) but also L-arginine transport (Hammermann *et al.* 2000; Visigalli *et al.* 2004). This transcription factor is normally maintained in the inactive state by the inhibitory protein IκB. Its activation and subsequent translocation to the nucleus is preceded by the phosphorylation of IκB kinases (IKK) which phosphorylate IκB, resulting in ubiquitination, dissociation and proteasomal degradation of NF-κB. The latter next migrates from the cytoplasm to the nucleus and stimulates gene transcription (Zapolska-Downar *et al.* 2004). The ability of DEM to regulate NF-κB by down-regulating its activation could therefore account for its effects in suppressing induced NO synthesis and L-arginine transport in RASMCs (Jones *et al.* 1999; Vos *et al.* 1999; Szaszi *et al.* 2005; Lou and Kaplowitz 2007).

To confirm that the inhibitions of NO synthesis were associated with a suppression of iNOS expression, western blotting was performed on lysates generated from cells treated chronically with either antimycin A or DEM. Studies with the maximum non-cytotoxic concentration (300 μM) of H_2O_2 were also performed for completion even though it was found not to have any significant effect on NO production. In these studies, antimycin A and DEM suppressed iNOS expression as did H_2O_2 which, interestingly, did not significantly alter NO synthesis. The reason for

this discrepancy with H_2O_2 is currently unclear. The inhibition was however only partial and it is possible that the expressed enzyme protein may be sufficient to sustain NO production. This is however only a hypothesis.

The inhibitions caused by antimycin A, unlike its effects on NO production, were not concentration dependent. In contrast, DEM caused a concentration-dependent reduction in iNOS protein expression but this was only evident at concentrations well above those required to completely block NO synthesis. The latter was seen with 25 μM DEM, a concentration which only partially suppressed iNOS expression. From these observations, it is feasible to suggest that DEM may be exerting a dual effect where both the function and expression of iNOS may be inhibited. As already indicated earlier, parallel studies examining changes in CAT protein expressions could not be conducted because of lack of appropriate antibodies to the carriers. Although analysis of gene expression was planned the restricted timescale towards the end of the project meant these studies could not be conducted either but are again essential in developing the project further.

In conjunction with the above, further experiments were carried out examining the time dependent inhibition of NO production and L-arginine transport by either antimycin A or DEM. In these studies, cells were either pre-exposed to the drugs for 30 minutes prior to, or added together with or at various time points from 3 hours to 18 hours after activation with LPS and IFN-γ. This is the time period over which the induction of both *iNOS* and *CATs* genes were expected to occur, reaching a peak at 18 hours. When added prior to activation, antimycin A and DEM inhibited both NO production and induced L-arginine transport rates. The inhibitions with antimycin A became less pronounced when the compound was added several hours after activation of cells. In fact there did not appear to be any significant difference

between the levels of nitrite detected in activated controls when compared to those where antimycin A was added ≥6 hours after activation. In contrast, L-arginine transport in activated cells was suppressed at all the time points except when antimycin A was added 18 hours after activation. Whether this reflects that *CAT* gene induction occur over a much delayed time period or whether their activity could be affected directly is not clear at present. However, since direct exposure of induced transporters to antimycin A did not alter transporter activity in our 'acute' exposure studies, it is likely that the inhibitions reflect an effect of antimycin A at the induction level. Quantitative PCR analysis of gene expression is again required to confirm this suggestion.

Similar experiments conducted with DEM were carried out using a concentration of 150 μM to ensure changes in L-arginine transport could also be determined. This concentration was however supra-maximal for inhibition of NO synthesis and as such the inhibitions observed in the time course studies were much more prolonged. No nitrite was detected in samples obtained from cells treated either before or for up to 3 hours after activation but was virtually restored in samples where DEM was added 18 hours after LPS and IFN-γ. Similarly, induced L-arginine transport was back to the control activated levels when cells were exposed to DEM 18 hours after activation but remained suppressed at all other time points. Taken together, these results again highlight that DEM, as with antimycin A, suppressed both processes by potentially inhibition the induction of either iNOS or CATs.

The second part of this thesis focused on establishing the effects of statins on both iNOS and L-arginine transport, and on the changes in these processes induced by antimycin A and DEM. Statins are amongst the drugs used for lowering cholesterol (Endo *et al.* 1976; Endo *et al.* 1977), and, as already stated, inhibit the

rate limiting HMG-CoA reductases in the cholesterol synthetic pathway (Goldstein and Brown 1990; Chong *et al.* 2001). These statins are either lipophilic or hydrophilic and can be naturally occurring or synthetic. A vast majority of studies distinguish between these compounds according to their lipophilicity which correlates with their pleiotropic effects. Lipophilic statins can enter cells by passive diffusion in contrast to hydrophilic molecules such as pravastatin that are unable to penetrate the plasma membrane of most cells (Turner *et al.* 2007). Certain cells such as hepatocytes have specific transporters to hydrophilic drugs and can therefore easily take up these compounds (Turner *et al.* 2007).

Statins are increasingly prescribed due to their efficiency in lowering cholesterol and in protecting patients from cardiovascular events (Kronmann *et al.* 2007; Bartholomeeusen *et al.* 2008). Some of these effects are however not exclusively related to their cholesterol lowering actions. Statins pleiotropic effects comprise anti-inflammatory properties, improvement of endothelial function, stabilizing atherosclerotic plaque, inhibiting platelet aggregation and vascular SMC proliferation (Nakagami *et al.* 2003; Chandrasekar *et al.* 2006; Greenwood and Mason 2007; Jasinska *et al.* 2007; Kronmann *et al.* 2007; Bersano *et al.* 2008; Shaw *et al.* 2009). The pharmacology of these drugs is also influential for patient's treatment; therefore the plasma concentration, half-life and lipophilicity are determinant to the outcomes both on lowering cholesterol and on their pleiotropic effects. The statins used in this study differ in these parameters. Pravastatin for instance is a selective hydrophilic inhibitor. Its oral absorption is 34 % and its bioavailability is 17 % (Pan 1991). In patients, the maximum plasma concentrations is proportional to the doses administered and this drug, although binding 45 % to plasma proteins, shows no evidence of drug accumulation (Pan 1991). Up to 95 % of

lipophilic statins such as simvastatin and atorvastatin may bind to plasma proteins (Neuvonen *et al.* 2008).

The mean plasma concentration of pravastatin may vary from 60 μM to 140 μM while simvastatin reaches 10 μM after administration of a single dose of 40 mg (Neuvonen *et al.* 1998; Neuvonen *et al.* 2008). Plasma concentrations of atorvastatin after the administration of single doses of 40 and 80 mg could reach concentration of up to 28.6 μM and 66.2 μM respectively (Lins *et al.* 2003). In our studies the concentrations used range was from 1 to 100 μM, correlating with the human plasma concentrations found in patients given statins. Lipophilic statins like simvastatin and atorvastatin are however thought to be accumulated in cells and tissues, further propagating their pleiotropic effects (Turner *et al.* 2007).

Statins demonstrate beneficial effects not only in patients with high cholesterol, but also in other pathologies. For instance, in patients with diabetes and normal LDL levels statins may improve vascular dysfunction by NADPH inhibition (Gao and Mann 2009).

They may act on specific signalling molecules, regulating their activity, and in doing so alter the cellular processes which the molecules regulate. Some of these processes include regulation of iNOS gene transcription and thus expression and subsequent NO synthesis which has a direct bearing to the thesis.

For our studies, three satins were used, including the synthetic compound atorvastatin which is commonly known as Zarator®. The other two are simvastatin or Zocor®, a synthetic derivate of a fermentation product of *Aspergillus terreus*, and pravastatin or Pravachol®, originally identified in a bacterium called *Nocardia autotrophica.* The data obtained with these compounds shows marked differences in their ability to regulate NO production and L-arginine transport. Atorvastatin, for

instance, produced a concentration-dependent bell shaped response on NO production with the maximal increase observed at 10 μM and represented a 6 fold increase when compared to the levels seen with LPS and IFN-γ alone. Even though these levels decreased thereafter, the amounts of nitrite detected in the presence of 30 μM and 100 μM were still higher than those in the activated controls. Atorvastatin also enhanced L-arginine transport but only in induced cells where the activated rates were further enhanced in the presence of the drug. In this case the increase at 1 μM to 30 μM appeared similar with no obvious concentration difference but the rate of transport declined back to the activated control levels with 100 μM. It is evident therefore that induction of NO synthesis was more susceptible than the transporters to regulation by atorvastatin. This trend was more evident with simvastatin which selectively enhanced induced NO synthesis without regulating L-arginine transport in either controls or activated cells. Moreover, pravastatin was without significant effect on either process. Although all three compounds inhibit HMG-CoA reductases and lower cholesterol, they do not appear to regulate the inducible L-arginine-NO pathway in a consistent similar manner, highlighting for the first time, differences in the alternative actions of these compounds. The results on NO production are corroborated by data from western blots in which iNOS protein expression was enhanced by both atorvastatin and simvastatin and over the concentration range that induced the increases in nitrite accumulation. Pravastatin again failed to cause any obvious significant change in iNOS expression. The reasons for the lack of effect with pravastatin are as yet undefined but as it is hydrophilic, it is possible that its penetration into RASMCs may be limited. There is however no data to indicate whether this may be the case or whether RASMCs actually lack the specific transporters required to internalising this compound.

In reviewing the literature, the following effects of statins on the NOS pathways were identified: simvastatin administered to endotoxic rat *in vivo* reduced iNOS expression in endothelial cells but not in SMCs (Giusti-Paiva *et al.* 2004; Alvarez de Sotomayor *et al.* 2008). Both eNOS and iNOS are up-regulated by atorvastatin in brain and aorta of hypertensive rats (Kishi *et al.* 2003). Simvastatin significantly increase eNOS but decrease iNOS mRNA and protein expression in rat hearts after ischemia-reperfusion (Di Napoli *et al.* 2001). Statins also consistently improve endothelium-dependent vasorelaxation in hyperlipidemic patients by increased NO bioavailability (Egashira *et al.* 1994; Treasure *et al.* 1995; Jiang *et al.* 2004; Landmesser *et al.* 2004). Interestingly, simvastatin has been shown to cause relaxation of contracted arteries in the absence of endothelium (Perez-Guerrero *et al.* 2005) suggesting that not all its actions are endothelium dependent. Statins such as cerivastatin and fluvastatin have been shown to have little effect on NO production when added after the induction of iNOS, suggesting that these compounds do not regulate the activity of the enzyme once induced (Yamamoto *et al.* 2003).

Currently, there is little or no information on the effects of statins on L-arginine transport and the data presented in this thesis, is to our knowledge, the only information currently available. What is important from the data is the clear discrimination between the statins in terms of their ability to regulate iNOS expression and the induced transporter activity and also the fact that the three statins selected for our studies demonstrated quite a completely different profile, when examined against NO production and L-arginine transport.

Differences in the profile of actions of statins have been highlighted from clinical data with atorvastatin being more effective in reducing circulating cholesterol

levels with patients requiring 10 mg/day to achieve this. By comparison, patients on simvastatin required 40 mg/day to achieve the same effectiveness (Doggrell 2006). Atorvastatin is also reported to be more efficient than simvastatin and pravastatin in lowering both LDL and CRP and TNF-α levels (Ridker *et al.* 2002; Ridker *et al.* 2005; Doggrell 2006). In contrast, HDL levels are apparently increased more effectively by simvastatin and pravastatin with atorvastatin causing a less prominent effect in patients with diabetes or metabolic syndrome, except when combined with fibrates (Doggrell 2006). A more potent drug, rosuvastatin, depletes LDL levels more drastically than atorvastatin with rosuvastatin at 10, 20 and 40 mg/day reducing LDL by 46, 52 and 55 % while atorvastatin at 20, 40 and 80 mg/day lowered LDL-cholesterol by 34, 45 and 51 % respectively. Atorvastatin at 20 mg/day is reported to reduce intimal thickness over 12 months and stabilised the process at lower concentrations. In contrast, simvastatin did not alter intimal thickening but the latter was reversed if patients were switched to atorvastatin (Doggrell 2006; Rubba 2007; Turner *et al.* 2007; Yu *et al.* 2007).

Further analysis of the actions of the hydrophilic pravastatin revealed that the incidences of cardiovascular events were consistently lower when using this statin in patients with a history of myocardial infarction, than when using atorvastatin or simvastatin, where the benefits were more modest (Paciaroni *et al.* 2007). Similarly, in a model of pulmonary hypertension, pravastatin but not atorvastatin, significantly reduced hypertension and right ventricular pressure even though both drugs were used at equal concentrations (Rakotoniaina *et al.* 2006). In this study, pravastatin was reported to also diminished apoptosis at the same time that it up-regulated eNOS. By comparison, atorvastatin caused only modest changes (Rakotoniaina *et al.* 2006). These selected examples highlight the differences in the actions and

effectiveness of statins and suggests exercising some caution in not generalising their expected actions either in man or in experimental models.

The mechanisms that regulate the effects of atorvastatin or simvastatin were not examined in this thesis because of lack of time but clearly warrant investigation. Speculating on potential mechanisms, it is likely that these compounds may act through cellular targets such as the small GTPase Rho. The latter is known to be negatively coupled to iNOS induction and its inhibition by statins, a common action widely reported (Chen *et al.* 2000; Muniyappa *et al.* 2000; Chen *et al.* 2001; Ikeda *et al.* 2001; Yamamoto *et al.* 2003; Madonna *et al.* 2005), may prevent the ability of Rho to repress *iNOS* gene expression, thereby resulting in a potentiation of iNOS protein and thus NO synthesis. Evidence in support of such actions was obtained with fluvastatin which apparently increased iNOS mRNA through the blockade of the Rho/Rho kinase pathway in rat VSMCs (Yamamoto *et al.* 2003). However, in embryonic cardiac myoblasts challenged with IL-1 or TNF-α, simvastatin inhibited iNOS expression and mRNA levels (Madonna *et al.* 2005). Whether this is a Rho mediated effect or simply reflecting differences in the cellular actions of statins is unclear. Interestingly, and of relevance to the findings in this thesis, pravastatin was unable to blunt Rho activity in SMCs (Yamanouchi *et al.* 2005) highlighting perhaps why it was ineffective in the RASMCs used in our studies.

Additional mechanisms of actions could potentially include regulation of NOS gene transcription and in this regard, the transcriptional activity of the iNOS promoter has been shown to be augmented by atorvastatin in human VSMCs (Kolyada *et al.* 2001). This statin is claimed to stabilise eNOS mRNA, increasing its half-life from 14 up to 27 hours and this was considered to be responsible for the increase in NO bioavailability (Laufs *et al.* 1998; Laufs and Liao 1998). Additional to the above

actions, lipophilic statins such as cerivastatin and fluvastatin increase the cellular content of BH_4 (Hattori *et al.* 2002) and this could also enhance the production of NO by coupled iNOS.

Some reports have indicate statins may negatively couple with NF-κB and simvastatin has been shown to inhibit TNF-α stimulated activation of NF-κB, as demonstrated by elevated levels of p65 in the cytosol of TNF-α-stimulated endothelial cells (Zapolska-Downar *et al.* 2004). Simvastatin has also been shown to reduce NF-κB activation in epithelial cells (Sakoda *et al.* 2006) while atorvastatin exerted the same effect in SMCs and monocytes stimulated with TNF-α (Ortego *et al.* 1999). Simvastatin and atorvastatin both inhibited the activation of NF-κB in response to oxidized LDL in human umbilical vein endothelial cells and rat ischemic model (Sironi *et al.* 2006; Singh *et al.* 2008). In a rabbit model of atherosclerosis, atorvastatin also reduced the activity of NF-κB in vascular macrophages and SMCs (Bustos *et al.* 1998). These reports clearly implicating NF-κB in the actions of statins provide another potential pathway through which these compounds could regulate not only the induction of iNOS but also L-arginine transport as these are also NF-κB dependent as already discussed earlier. However, the studies carried out in this thesis show clearly that atorvastatin and simvastatin enhance induction of iNOS and NO synthesis. The negative coupling of these statins to NF-κB would therefore be at odds with these findings. Thus it would be essential to established whether statins are able to regulate induced NO production and L-arginine transport through regulation of NF-κB activation in RASMCs.

Amongst the growing profile of actions exerted by statins, their ability to act as antioxidants has a direct influence in regulating OS induced modification of cellular processes. In a trial with diabetic patients, characterised by high ROS levels,

atorvastatin was able to prevent cardiovascular events (Collins *et al.* 2003) and in hyperglycaemic model it induced the re-coupling of eNOS through inhibition of NADPH oxidase expression (Wenzel *et al.* 2008). Furthermore, atorvastatin has been reported to induce several other antioxidant effects including: (i) increase catalase mRNA expression and activity (Wassmann *et al.* 2002), (ii) reducing ROS generation in a rat hypertensive model (Mondo *et al.* 2006) and (iii) down-regulating angiotensin II (AT_1) receptor expression, consequently decreasing p22phox and exerting an antioxidant effect in endothelial cells (Wassmann *et al.* 2001; Zhou *et al.* 2004). The antioxidant effects of statins have also been attributed to the inhibition of the Rho-kinase pathway and inhibition of the latter using the potent Rho-kinase inhibitor, farsudil, was able to deplete NADPH oxidase subunit (nox1, nox4, gp91phox and p22phox) expression in VSMCs thereby diminishing OS in these cells (Higashi *et al.* 2003).

In view of the potential antioxidant properties of statins, studies were extended to include an examination of the effects of these compounds on the responses induced by antimycin A and DEM. As shown in the results section, atorvastatin restored NO production when applied in the presence of either antimycin A or DEM, reversing the inhibitory actions of these compounds. Moreover, atorvastatin potentiated levels of accumulated nitrite beyond those seen with LPS and IFN-γ, showing a similar bell-shaped response to that seen when atorvastatin was applied alone. By comparison, simvastatin and pravastatin were much less effective in overcoming the effects of antimycin A or DEM when compared to atorvastatin. None of the statins recovered the inhibition of induced L-arginine transport, further indicating that these processes may be regulated differentially.

The first product blocked by statins in the mevalonate pathway is mevalonic acid which is produced by the rate limiting enzyme HMG-CoA reductase. Because of this, additional studies were conducted examining the effects of mevalonate on induced NO synthesis and L-arginine transport. Moreover, the ability of mevalonate to modulate the actions of statins was also investigated. On its own, mevalonate was without significant effect on either nitrite production or L-arginine transport but appeared to inhibit the increments in NO production caused by atorvastatin and simvastatin. In contrast, the effects of the latter on L-arginine transport remained unaffected while the enhancements caused by atorvastatin were blocked. This is a novel finding which has not been reported before and again indicate that atorvastatin and simvastatin may regulate these processes via different mechanisms which have not as yet been fully elucidated. It would appear however that the actions of both statins on NO production may be mediated via the Rho pathway and would be consistent with published literature as discussed earlier. It is also possible that the effects of atorvastatin on transport may potentially involve Rho because of its susceptibility to mevalonate but this needs to be confirmed and an alternative mechanism of action cannot be ruled out. In this regard, a study in neuronal cells and macrophages, where statins inhibited iNOS and pro-inflammatory cytokines, co-incubation with cholesterol or ubiquinone, the end products of the mevalonate pathway, did not reverse the actions of statins (Pahan *et al.* 1997).

4.1 . Summary and conclusions

The data generated in this thesis has highlighted some important differences in the regulation of the inducible L-arginine-NO pathway in cultured vascular smooth muscle cells by OS inducers and thus potentially OS. One of the key findings is the fact that H_2O_2, a key OS inducer and intermediate in the process of OS, was without effect while antimycin A and DEM both regulated induced NO synthesis and L-arginine transport. The data with the latter two confirm that the inducible L-arginine-NO pathway can be down-regulated by OS inducers and this may contribute to the deleterious effects observed in disease states associated with OS. However, antimycin A and DEM exhibited different profiles of action and only DEM induced HO-1 expression indicating perhaps that the effects of antimycin A were not related to the induction of OS.

In the second half of the studies, statins were found to regulate both induced NO production and L-arginine transport but the effects produced depended on the statin used and unlike their cholesterol lowering actions, not all statins may have the ability to regulate the inducible L-arginine-NO pathway. A good illustration of this is the fact that pravastatin was without effect in our system while atorvastatin and simvastatin both enhanced iNOS expression and NO synthesis. Only atorvastatin regulated L-arginine transport, elevating the latter in LPS and IFN-γ treated cells but not in controls. Moreover, atorvastatin reversed the inhibition of NO production caused by either antimycin A or DEM but the inhibitions of L-arginine transport caused by OS inducers were unaffected by statins.

Taken together, the data highlighting the effects of statins (especially atorvastatin) on NO production and L-arginine transport are encouraging if we

consider the original hypothesis proposed which is the deleterious effect of OS may be mediated in part through suppression of iNOS induced NO production whereby the protective and beneficial effects of the latter are lost, contributing to the pathogenesis of atherosclerosis. The ability of statins such as atorvastatin to restore NO production in the presence of OS inducers could contribute to the cardio-protective actions of these compounds and the data from this thesis may help further in understanding how these drugs act.

5 . Future work

The limited experiments with PAECs raise questions about which carrier would be involved on L-arginine transport under the conditions presented. Further studies on kinetics, amino acid cross-inhibitions studies to verify the transporter selectivity and ultimately investigation by PCR analysis are necessary.

There is need to confirm the presence of ROS and determine the levels released under the different experimental conditions especially when using pro-oxidants. Inflammation is also a source of ROS and their detection should be measured in the presence of LPS and IFN-γ alone or/and together with pro-oxidants (Javesghani *et al.* 2003). The presence and amount of ROS formed during the incubation periods would also allow us to infer more accurately if the effects are actually due to the free radicals generation or other mechanisms. The detection of ROS could be carried out by chemiluminescence using lucigenin (Javesghani *et al.* 2003).

Quantitative PCR analysis of iNOS and CATs induction or lack of it in the presence of antimycin A or DEM (and possibly H_2O_2) is required to confirm whether the changes some of the effects observed are related to a change in gene expression of either iNOS and/or CATs.

The effects observed with OS inducers and/or statins are most likely affecting signalling pathways and studies looking at key signalling molecules known to regulate iNOS expression and/or CATs should be conducted following treatment of

cells under the different conditions investigated. Some of the pathways could include Rho, the P38 MAPKs, PI3K and the transcription factor NF-kB.

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7 . Appendix

Table 7.1. Transfer buffer

Reagent	Concentration
Glycine	39 mM
TRIS	48 mM
SDS	0.0375 %

Table 7.2. Washing buffer

Reagent	Concentration
TRIS	10 mM
NaCl	100 mM

Table 7.3. Tank buffer

Reagent	Concentration
TRIS	250 mM
Glycine	2 M
SDS	1 %

8 . Materials

Absolute ethanol – Fluka

Acrylamide, ProtoGel – National Diagnostics

Agarose – Sigma

Ammonium Persulphate – AMRESCO

Anti-α monoclonal smooth muscle actin – Sigma

Anti-biotin, HRP-linked antibody, Detection Pack – Cell Signalling

Anti-Heme Oxygenase-1 mouse monoclonal antibody (HO-1-1) – Calbiochem

Anti-mouse IgG (whole molecule) FITC Conjugate developed in rabbit – Sigma

Anti-iNOS/NOS Type II monoclonal antibody – Transduction Laboratories

Anti-β actin monoclonal antibody – Sigma

Anti-mouse secondary antibody, Goat anti-mouse HRP – Transduction Labs

Antimycin A from *Streptomyces* sp. – Sigma

Atorvastatin Calcium – Pfizer

BCA, Bicinchoninic Acid protein assay regent kit – Pierce

Biotinylated Protein Ladder, Detection Pack – Cell Signalling

Bromophenol Blue – Sigma

BSA, Bovine Serum Albumin – Sigma

$CaCl_2$, Calcium Chloride – BDH

Chloroform $\geq$99.8 % – Sigma-Aldrich

Collagenase – Roche Diagnostics

Coomassie brilliant blue – Fluka

Diethyl maleate (DEM) – Sigma

Developer (Dektol) – Kodak

D-glucose – BDH

Dil-Ac-LDL, Dil labelled acetylated low density lipoprotein – Calbiochem

DMEM, Dulbecco's Modified Eagle's Medium – GIBCO

ECL detection reagents – Amersham Biosciences

Ethanol – BDH

Extra Thick Blot Paper – BIO-RAD

Ficoll – Sigma

FITC, Fluorescein isothiocyanate – Sigma

Fixer (Unifix) – Kodak

FBS, Foetal Bovine Serum – GIBCO

Formaldehyde- Sigma

Fungizone – GIBCO

Gentamycin – GIBCO

Acetic acid – Fisher Scientific

Glycerol – Sigma

Glycine – BDH

H_2O_2, hydrogen peroxide – Sigma

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) – Sigma

Hyperfilm (ECK & X-ray) – Amersham

Horseradish Peroxidase-linked Antibody – Cell Signalling, Detection Pack

[H^3]L-arginine (2 μ Ci/ml) – Amersham Biosciences

IMS, Industrial Methylated Spirit – BDH

Interferon-γ, rat, recombinant, *E. coli* – Calbiochem

Isopropanol – BDH

KCl, potassium chloride – BDH

L-(+)-Arginine Hydrochloride – Sigma

LPS, Lipopolysaccharide from *E. Coli* – Fluka

Lab-Tek® well plates – Nunc

Methanol – Fisher Scientific

Mevalonate, (±)-Mevalonolactone – Sigma

MOPS, 3-(N-Morpholino)propanesulfonic acid sodium salt – Sigma

$MgCl_2$, Magnesium Chloride – BDH

MTT (5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) – Sigma

Na_3VO_4, Sodium Orthovanadate – Sigma

NaCl, Sodium Chloride – BDH

NaF, Sodium Fluoride - Sigma

NaH_2PO_4, Sodium Dihydrogen Orthophosphate 1-hydrate – BDH

$NaHCO_3$, Sodium Bicarbonate – BDH

NaOH, Sodium hydroxide – Sigma

$NaNO_3$, Sodium Nitrate – Sigma

Napthylethylenediamine – Sigma-Aldrich

NO_2^-, Nitrite – Sigma

PBS, Phosphate Buffered Saline – GIBCO

Penicillin/Streptomycin – GIBCO

Phosphoric Acid – BDH

PMSF, phenyl methyl sulfonyl fluoride – Sigma

Pravastatin sodium salt – Calbiochem

PVDF membrane, Membrane Immobilon-P transfere – Millipore

Random hexamers – Promega

Scintillation Liquid (Liquiscint) – National Diagnostics

SDS, Sodium Dodecyl Sulphate – Sigma

Simvastatin sodium salt – Calbiochem

Skimmed milk – Marvel

Sulfanilamideamine – Sigma-Aldrich

TEMED, NNN'N'-Tetramethylethylenediamine – BDH

Trizma base (TRIS) – Sigma

Trypsin-EDTA – GIBCO

Tween 20 – Sigma

Unifix (fixer) – Kodak

Xylene cyanol – Sigma-Aldrich

β-mercaptoethanol – Sigma