The Effects of Uric Acid on Endothelial Function and Dysfunction

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Ph.D., The University of Edinburgh, 2006

Declaration

I hereby declare that the work described in this thesis was performed entirely by myself, and that it has not been accepted in any previous application for a degree.

Dedicated to John J. Marshall

Acknowledgments

There are many people who have helped me throughout the duration of my research and in the next few paragraphs I will attempt to thank them all.

Firstly, I wish to thank my Mum and Dad, who provided constant support, encouragement and advice throughout my research. The same degree of thanks must also go to my wife, Ali, who helped me through some difficult times and inspired me to strive on. I must also thank my brother, John, and his wife, Elaine, for their continual interest and encouragement. Similarly, I wish to express my gratitude to the Clarks, the Kennedys, and all other friends and family for their unending support.

Thanks also go to Fiona Gulliver-Sloan and Dr. Lynn Greig who welcomed me into the lab and made it a thoroughly enjoyable place to work. My appreciation also goes Dr. Ian Megson and Neil Johnston for their expert technical advice and extensive assistance. Likewise, I also wish to thank the people with whom I shared an office or lab, such as Dr. Alistair Thompson, Dr. Stephen Waring, Dr. Mark Miller and all the others at the CPU and George Square.

I also wish to express my great appreciation to Professor David Webb for his supervision, help and guidance throughout my research studentship. Appreciation also goes to the Medical Research Council for providing the funding which allowed me to conduct this research.

Finally, and most importantly, I wish to offer my sincerest thanks to my supervisor, Dr. Simon Maxwell, for his supervision, guidance and support.

To all these people and anybody I have forgotten to mention, I cannot thank you enough!

Abstract

The association between elevated uric acid (UA) concentrations and cardiovascular disease is well established in epidemiology, but the possibility that UA plays a specific role in the pathophysiology of cardiovascular disease remains a matter of debate. Although there are putative mechanisms by which UA could injure the cardiovascular system, it also has a number of properties that might be considered as protective. Most notably, its role as a radical-scavenging antioxidant might be expected to mitigate the effects of increased oxidative stress, which is characteristic of most risk factors for cardiovascular disease and is an important precipitant of endothelial dysfunction. The aim of the studies described in this thesis was to examine the effects of UA on endothelial function and investigate whether UA has the potential to reverse endothelial dysfunction induced by low-density lipoprotein (LDL).

Mesenteric arteries were isolated from Wistar-Kyoto rats and the responses to a vasoconstrictor (PE, phenylephrine) and endothelium-dependent (ACh, acetylcholine) and –independent (SNP, sodium nitroprusside) vasodilators examined using perfusion myography. This model was considered advantageous because it enabled the measurement of pharmacological responses in the presence of different luminal solutions in an experimental environment that most closely mimics the conditions found *in vivo*. Luminal perfusion with L-NAME and/or indomethacin demonstrated that nitric oxide synthase (NOS) –derived nitric oxide (NO) was the major vasodilator released by the endothelium in response to ACh in this experimental model.

Exposure of the vascular lumen to increasing concentrations of UA (200, 400, 600μM) or vehicle solution had no effect upon the responses to PE, ACh or SNP. This implied that, in this model, acute exposure to elevated UA does not impair endothelial function. In contrast, when the lumen was perfused with increasing concentrations of LDL (250, 500 and 1000μg/ml), maximal vasodilatation towards resting diameter in response to ACh was reduced to 42.6, 33.6 and 21.7% respectively. The failure of the NOS inhibitor L-NAME to further impair vasodilatation implied that major effect of LDL was to abolish endothelium-dependent NO-mediated vasodilatation. Supplementing the perfusing LDL

solution with 1mM L-arginine restored endothelium-dependent responses, implying that the LDL-induced endothelial dysfunction was in part explained by a disruption of L-arginine metabolism. Supplementation with the extracellular $O_2^{\cdot-}$ scavenger, superoxide dismutase (SOD), did not prevent the deleterious action of LDL.

Supplementation of 250μg/ml LDL with increasing concentrations of UA (200, 400, 600μM) partially reversed the inhibition of maximal vasodilatation towards resting diameter in response to ACh to 62.2, 69.5 and 74.4% respectively. No such improvement could be achieved in the presence of L-NAME. The beneficial effect of 400μM UA upon LDL-induced endothelial dysfunction contrasted with the lack of effect of two other water-soluble antioxidants, ascorbic acid (AA) and glutathione (GSH), at the same concentration.

The experiments then focused on investigating the potential mechanism by which UA prevented LDL-induced endothelial dysfunction. Isolated rings of thoracic aorta from Wistar-Kyoto rats were mounted in a wire myograph and exposed to ACh in the presence of varying concentrations of UA and 250μg/ml LDL. The superfusate was then transferred to endothelium-denuded rings and caused significant vasodilation in previously unresponsive ring segments. The extent of the vasodilatation in response to the transferred solution was dependent on the concentration of UA and ox-Hb sensitive. The decay in vasodilator response if the exposure of the denuded ring was delayed had a half-life of 29 minutes. These results implied that the stimulation of an endothelium-intact vessel by ACh in the presence of both UA and LDL results in the formation of an endothelium-independent vasodilator that releases NO and may be a derivative of UA.

In summary, the results of these experiments suggest that acute exposure to UA in physiological concentrations does not impair either endothelium-dependent or –independent vascular responses. Conversely, UA reverses the impairment of ACh-induced vasodilatation caused by LDL and does so more effectively than other high concentration hydrophilic antioxidants. Furthermore, UA appears to enable the formation of an NO-releasing compound when it is present with LDL and endothelial cells

stimulated by ACh. The presence and nature of a possible NO-donor compound formed in these circumstances requires further investigation. Taken together, this work implies that UA exposure is not directly injurious to vascular function and may protect against the effects of LDL on the vascular endothelium. This might offer a physiological role for a compound which is found in much higher concentration in the extracellular fluids of humans than almost any other species.

Table of Contents

List of Abbreviations

4-HNE	4-hydroxynonenal
AA	Ascorbic acid
ACh	Acetylcholine
AC	Adenylate cyclase
ADMA	Asymmetrical N^G,N^G dimethyl-arginine
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BH_4	Tetrahydriobiopterin
BSA	Bovine serum albumin
cAMP	Cyclic 3',5'-adenosine monophosphate
cGMP	Cyclic 3',5'-guanosine monophosphate
COX	Cyclo-oxygenase
DECTA	Diethyldithiocarbamic acid
DNA	Deoxyribonucleic acid
DOC	Deoxycholate
EC	Endothelial cells
EDCF	Endothelium-derived constricting factor
EDHF	Endothelium-derived hyperpolarising factor
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylene-diamine-tetraacetic acid
FAD	Flavin adenine dinucleotide
FCPR	Folin & Ciocalteu's phenol reagent
FMN	Flavin mononucleotide
GPX	Glutathione peroxidase
GSH	Glutathione
GSNO	S-nitrosoglutathione
GTN	Glyceryl trinitrate
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide

Hb	Haemoglobin
HLDL	High-density lipoprotein
HOCl	Hypochlorous acid
HUVEC	Human umbilical vein endothelial cell
HX	Hypoxanthine
IP_3	inositol 1,4,5-triphosphate
KBr	Potassium bromide
KCl	Potassium chloride
LDL	Low-density lipoprotein
Li_2CO_3	Lithium chloride
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
$LOO^{\cdot}$	Lipid peroxyl radicals
M	Molar
Mb	Methylene blue
MDA	Malondialdehyde
MLCK	Myosin light chain kinase
μM	Micromolar
mM	Millimolar
mmHg	Millimetres mercury
NaCl	Sodium chloride
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
NANC	Non-adrenergic, non-cholinergic
n-LDL	Native low-density lipoprotein
nM	Nanomolar
NOS	Nitric oxide synthase
NO	Nitric oxide
NO^+	Nitrosium ion
NO_2	Nitrogen dioxide
NO_2^-	Nitrite
NO_3^-	Nitrate

$O_2^{\cdot-}$	Superoxide
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
OH^-	Hydroxyl
$ONOO^-$	Peroxynitrite
ONOOH	Peroxynitrous acid
Ox-Hb	Oxyhaemoglobin
Ox-LDL	Oxidised low-density lipoprotein
PBS	Phosphate buffered saline
PE	Phenylephrine
PGI_2	Prostacyclin
PIP_2	Phosphatidylinositol
PKA	Protein kinase A
PKG	Protein kinase G
PLA	Phospholipase A
PLC	Phospholipase C
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
RSH	Thiol
RSNO	S-nitrosothiol
RSSR	Disulphide
S.E.M.	Standard error mean
sGC	Soluble guanyl cyclase
SNOC	S-nitrosocysteine
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
UA	Uric acid
VSMC	Vascular smooth muscle cells
w/v	Weight by volume

WKY	Wistar Kyoto
VLDL	Very low-density lipoprotein
XO	Xanthine oxidase
XDH	Xanthine dehydrogenase

CHAPTER 1

GENERAL INTRODUCTION

1. INTRODUCTION

1.1 The Vascular System

The vascular system is the set of structures in the body that are concerned with circulating the blood. The blood vessels are a major part of this system and form the route by which blood is carried away from the heart to the tissues of the body and then back to the heart. The structure and size of these vessels vary greatly and comprise a complex network of circulation and regulation. The arteries are the vessels which carry blood away from the heart to supply O_2 and nutrients to the various tissues in the body. The large arteries, such as the aorta and carotid or iliac artery, are known as the elastic arteries and transfer blood from the heart to the muscular or medium-sized arteries. These arteries then distribute the blood around the body by branching into smaller arteries, know as the arterioles. These arterioles connect with a network of microscopic vessels called capillaries, which are distributed near to all the body cells. The thin wall of these vessels facilitates the cellular exchange of O_2, nutrients and waste tissue with the blood.

The vascular wall of the arteries may be divided into three layers: the adventitia, the tunica media and the intima, as shown in Figure 1.1 (Rhodin, 1980). The adventitia consists mainly of connective tissue fibres. These attach the vessel to the surrounding tissue and also form a sheath over the external elastic lamina which covers the tunica media. The tunica media is formed by a layer of circumferential vascular smooth muscle cells (VSMC), responsible for vascular contraction and relaxation, and variable amounts of connective tissue. The intima is the inner layer of the blood vessel and consists of an elastic basement membrane, the internal elastic lamina, and a monolayer of endothelial cells (EC), which form the vascular endothelium.

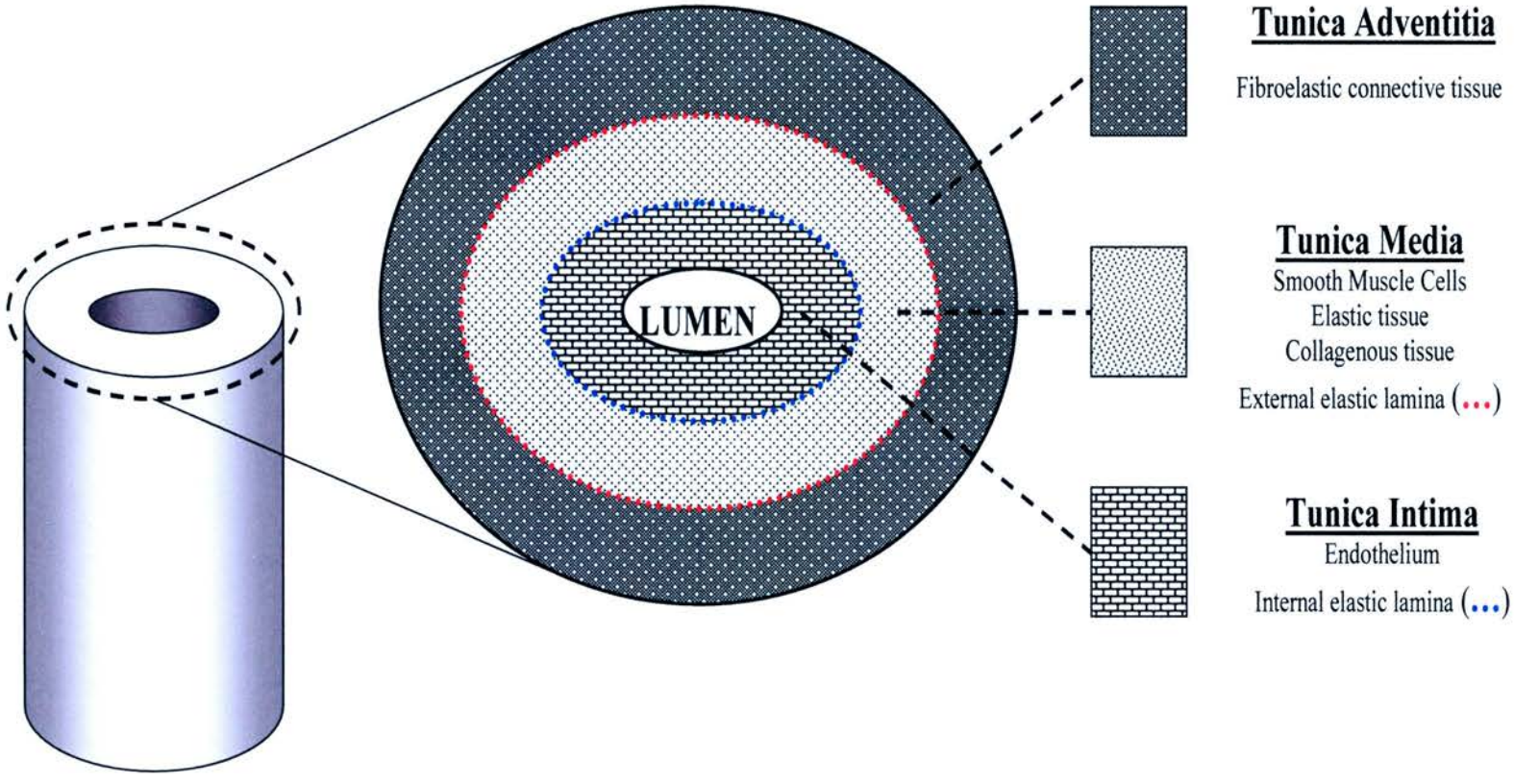


Figure 1.1. Diagram of the layer and tissue types that comprise the internal structure of the arterial wall.

1.2 The Endothelium

The endothelium lines the inner layer of the vascular wall and acts as a protective barrier to prevent extensive blood clotting and thrombus formation. EC release chemical inhibitors of platelet activation, cell proliferation and migration, to prevent unwanted thrombus formation. The endothelium also plays a crucial role in the regulation of vascular tone and homeostasis via the release of endothelial-derived vasoactive substances to the VSMC (Rubanyi, 1993). It releases a range of substances which induce vasodilatation, such as nitric oxide (NO) (Moncada *et al,* 1986; Furchgott *et al,* 1987; Palmer *et al,* 1987), prostacyclin (Moncada *et al,* 1976) and endothelium-derived hyperpolarising factor (EDHF) (Mombouli *et al*, 1992), and potent vasoconstrictors, such as endothelin-1 (ET-1), thromboxane A_2 and prostaglandin H_2 (Luscher & Tanner, 1993).

Neurotransmitters, hormones and platelet-derived substances may stimulate EC to influence vascular tone. Blood flow through the vascular lumen also exerts a "tonic" influence upon the endothelium. The nature of the substances released by the endothelium is dependent upon the shear stress exerted by the flowing blood upon the EC. For example, high shear stress induces the release of endothelium-derived relaxing factors (EDRF), such as NO and prostacyclin, to the underlying smooth muscle. Conversely, if shear stress is low, endothelium-derived contracting factors (EDCF), such as ET-1 and thromboxane A_2, are released to narrow the vessel diameter and ensure that good perfusion pressure is maintained. Consequently, damage to these cells results in the loss of both the control of vascular tone and the antithrombotic capacity provided by the endothelium. Pathophysiological conditions that damage the arterial wall and inhibit the endothelial control of vascular function may promote the onset of cardiovascular disease and the clinical complications associated with this disease state (Rubanyi, 1993; Shimokawa & Takeshita, 1995).

1.3 Nitric Oxide

1.3.1 Identification of NO as EDRF

The release of EDRF from EC was established when it was demonstrated that endothelial removal inhibited responses to ACh in rabbit aortic rings or strips, and stimulation of endothelium-intact aortic strips could induce vasodilatation in adjacent denuded strips (Furchgott & Zawadski, 1980). Other studies also reported that endothelium-intact tissue could be used as a donor to dilate endothelium-denuded VSMC (Griffith *et al,* 1984; Cocks *et al*, 1985; Gryglewski *et al*, 1986). Two independent research groups suggested that EDRF was the free radical, NO (Ignarro *et al,* 1986; Furchgott *et al,* 1987; Ignarro *et al,* 1988). Pharmacological comparison of EDRF and NO demonstrated that both could mediate vasodilatation via the generation of cGMP in VSMC, and was inhibited by Hb or $O_2^{\cdot-}$ (Palmer *et al,* 1987). Furthermore, infusion of L-NMMA, a nitric oxide synthase (NOS) inhibitor, into hand veins mimicked the effects of endothelial denudation and could be reversed by l-arginine supplementation, suggesting that EDRF was NO (Moncada *et al,* 1986). Several differences have also been reported (Cocks *et al,* 1985; Long *et al*, 1987; Moncada *et al,* 1991), but it is currently believed that the EDRF released from the endothelium to produce vasodilatation is NO.

1.3.2 Synthesis of NO

The vascular endothelium has receptors for a variety of circulatory and local hormones, such as ACh, bradykinin, substance P, noradrenaline, and adrenaline, which activate receptor-mediated mechanisms to stimulate the generation and release of NO from the EC. The endothelium also has ion channels that respond to increases in blood pressure, stretch or shear stress to enhance NO production (Calver *et al,* 1993). Following

agonist binding or ion channel opening, an elevation in the intracellular Ca^{2+} concentration occurs to activate NOS. This enzyme catalyses the oxidation of the guanidine nitrogen of l-arginine (Palmer *et al,* 1988; Sessa *et al,* 1994), for which it has a low substrate requirement (K_m=1-10μM) (Vallance, 1998; Bult *et al,* 1999; Hobbs *et al,* 1999), to produce NO and l-citrulline with a 1:1 stoichiometry (Stuehr & Griffith, 1992). The reaction pathway for this is shown in Figure 1.2 (Stuehr & Griffith, 1992). The reaction is Ca^{2+}-dependent and requires O_2, NADPH as a reducing cofactor, and other cofactors such as tetrahydrobiopterin (BH_4), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and haem (Sessa *et al,* 1994; Marletta, 1994). The terminal guanidine nitrogen of l-arginine undergoes a five-electron oxidation, followed by cleavage to form NO and l-citrulline. Ca^{2+}/calmodullin binds to NOS and the enzyme receives electrons from NADPH at the reductase domain. These are then transported by the flavin moiety to the oxidase domain where they reduce O_2 to provide a source for oxidising arginine.

1.3.3 NO Synthase

NOS exists in three isoforms in the human body – neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). The genes which code for these are located on chromosomes 7, 12 and 14 respectively (Moncada & Higgs, 1995). nNOS produces NO which acts as a neurotransmitter in non-adrenergic non-cholinergic (NANC) nerves (Toda & Okamura, 1990; Rajfer *et al,* 1992). iNOS is Ca^{2+}-independent isoform of this enzyme, and is active in VSMC and macrophages exposed to inflammatory cytokines, as part of the immunological response (Moncada, 1992; Kanno *et al,* 1994). eNOS-derived NO plays a crucial role in the regulation of blood pressure, vascular tone and platelet aggregation in the cardiovascular system (Radomski *et al,* 1990; Radomski *et al,* 1993; Moncada *et al,* 1991). eNOS and nNOS are expressed constitutively in cells and may be grouped together as constitutive NOS (cNOS). The enzymatic activity of these isoforms is regulated by intracellular Ca^{2+} concentrations and is fully active at

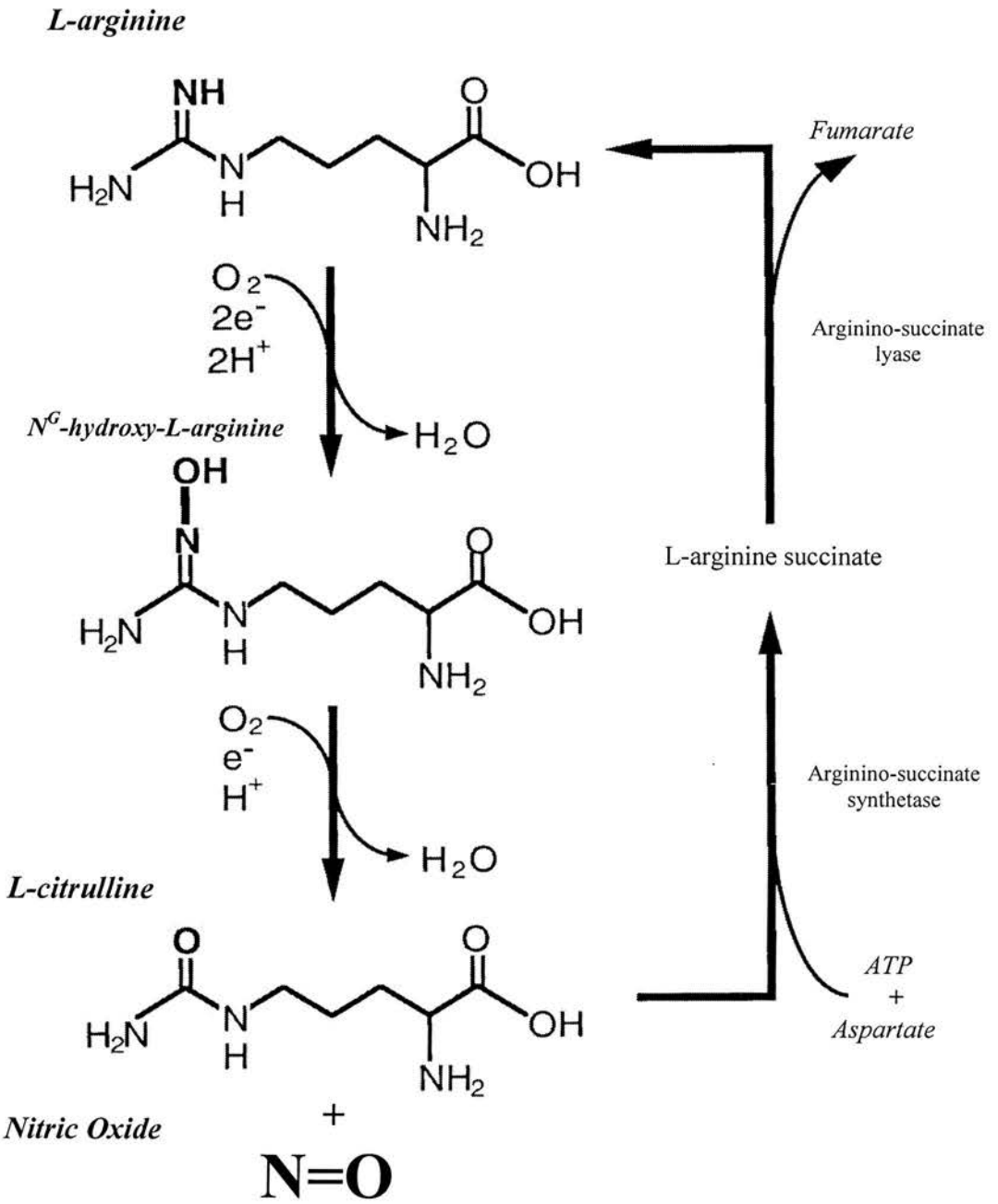


Figure 1.2. The synthesis of NO and l-citrulline from l-arginine by eNOS, and subsequent recycling of l-citrulline to l-arginine.

concentrations greater than 400nM (Ashley *et al*, 1984). In the absence of intracellular Ca^{2+}, NOS is localised within the caveolae and inhibited by caveolin. Increasing intracellular Ca^{2+} removes this inhibition (Michel *et al,* 1997). Under conditions in which there is a deficiency in co-factors such as BH_4 or substrate such as l-arginine, an imbalance in NOS activity may occur resulting in $O_2^{\cdot-}$ generation.

L-arginine analogues such as asymmetrical N^G,N^G-dimethyl-L-arginine (ADMA), N^G-monomethyl-L-arginine (L-NMMA), and N^G-nitro-L-arginine methyl ester (L-NAME) act as competitive reversible inhibitors of NOS to prevent NO generation (Rees *et al,* 1988). This can cause vasoconstriction of arteries and arterioles, and increases resting blood pressure (Rees *et al,* 1989; Vallance *et al,* 1989; Stamler *et al,* 1994). ADMA levels are increased in certain disease states, such as hypercholesterolaemia (Yu *et al,* 1994; Bode-Boger *et al,* 1996) and atherosclerosis (Bode-Boger *et al,* 1996), and may contribute to the pathophysiology of these conditions. NO may also inhibit NOS by binding to the haem iron in the enzyme to exert a negative feedback mechanism (Buga *et al,* 1993; Abu-Soud *et al,* 1995).

1.3.4 The properties of endothelial NO

NO has a diverse range of biological effects that may prevent the pathogenesis of cardiovascular disease, as demonstrated in Figure 1.3. (Beckman *et al,* 1990; Cooke *et al,* 1992; Rubbo *et al,* 1994; Rubbo *et al,* 1996; Hogg & Kalyanaraman *et al*, 1999; Patel *et al,* 2000). Basal NO prevents excessive vasoconstriction to regulate vascular tone (Vallance *et al,* 1989) and blood pressure (Haynes *et al,* 1993). It inhibits platelet aggregation and platelet adhesion to the endothelium (Radomski *et al,* 1987; Radomski & Moncada, 1993; Cooke & Tsao, 1994), as well as the activation and adhesion of leukocytes to the endothelium (Lefer, 1997). NO inhibits the activation of the redox-sensitive transcription factors, such as NF-κβ, in response to pro-inflammatory

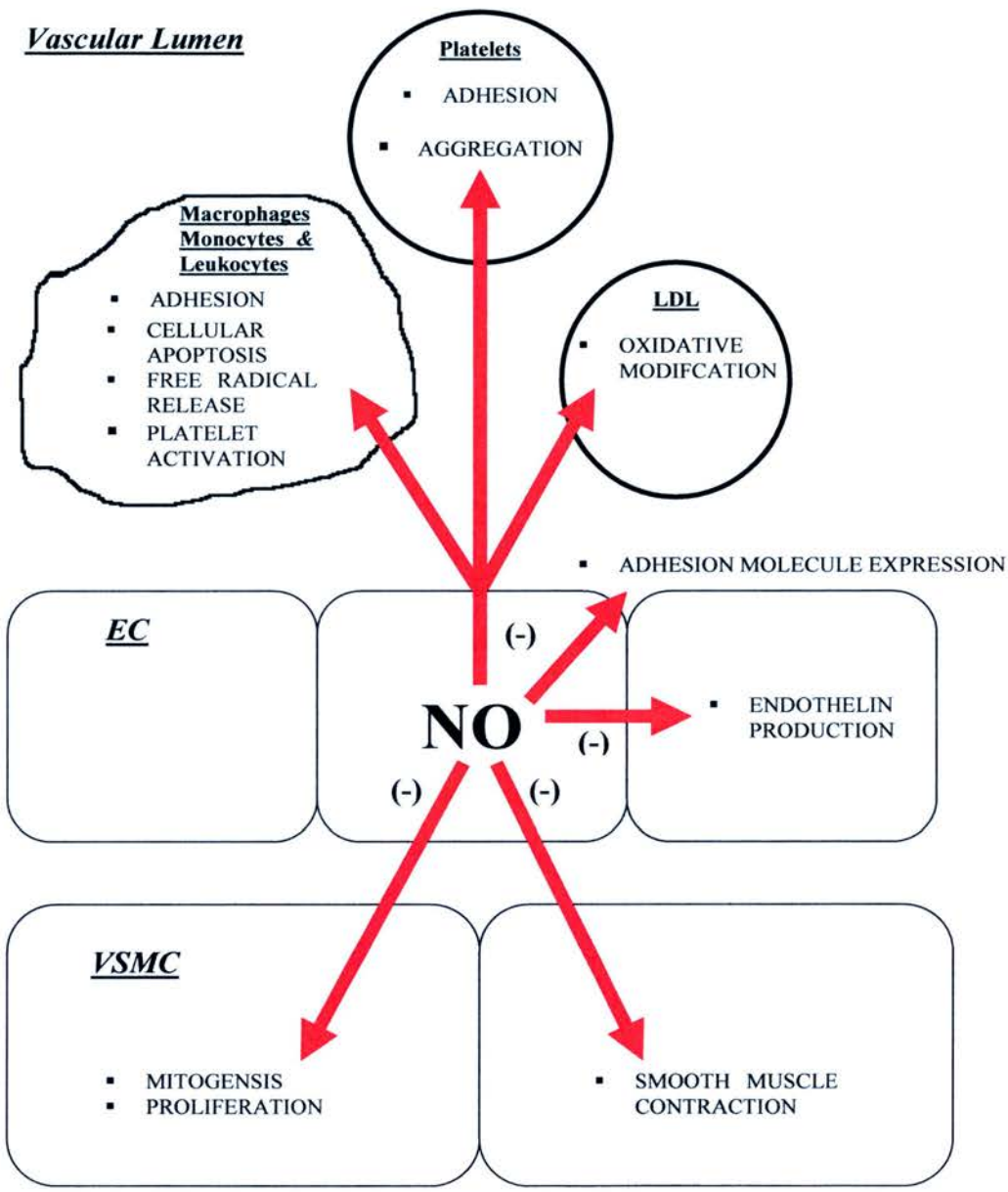


Figure 1.3. The major sites where endothelium-derived NO acts to prevent vascular injury and cardiovascular disease.

cytokines (Peng *et al,* 1995), which in turn prevents the transcription of chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and cellular adhesion molecules (Peng *et al,* 1995; Zeiher *et al,* 1995). NO also inhibits the mitogenesis and proliferation of VSMC (Garg & Hassid, 1989). NO relaxes contractile elements in EC to maintain tight junctions between the EC and preserve endothelial impermeability (Kanwar & Kubes, 1995; MacAllister, 1998). Platelets may also synthesise NO via NOS (Radomski *et al,* 1990a; Radomski *et al,* 1990b) and may store NO in vesicles as *S*-nitrosothiols (Hirayama *et al,* 1999). This is released when the platelets are activated, as a form of negative feedback to prevent platelet aggregation.

NO may also act as an antioxidant in human physiology to scavenge free radicals, protect against increased oxidative stress, and prevent vascular injury. It scavenges lipid peroxyl radicals ($LOO^{\cdot}$) in a termination reaction (Padmaja & Huie, 1993; O'Donnell *et al,* 1997), to inhibit lipid peroxidation (Rubbo *et al,* 1994; O'Donnell *et al,* 1997; Hogg & Kalyanaraman, 1999). Termination reactions are the likely point for the suppression of lipid peroxidation by NO in the extracellular environment. NO reacts with $LOO^{\cdot}$ in a two-step reaction, firstly it scavenges $LOO^{\cdot}$ to form a non-radical species that has similar characteristics to peroxynitrite ($ONOO^{-}$) (Padmaja & Huie, 1993). This unstable intermediate then reacts with a further NO molecule to form a stable nitrate (Patel *et al,* 2000). NO scavenges $LOO^{\cdot}$ approximately 10,000 times faster than α-tocopherol, the major hydrophobic antioxidant found in lipid phases (Liebler, 1993; O'Donnell *et al,* 1997). As a result, a lower concentration of NO (30nM) is required to scavenge $LOO^{\cdot}$, compared to α -tocopherol (20μM) (O'Donnell *et al,* 1997). Furthermore, the availability of NO is not limited by transport of a hydrophobic moiety and is freely diffusible between the aqueous and lipid phases, unlike α–tocopherol (Liu *et al,* 1998). NO rapidly scavenges $O_2^{\cdot -}$ (Huie & Padmaja, 1993), which prevents $O_2^{\cdot -}$-dependent cytotoxicity but produces $ONOO^{-}$, a potent pro-oxidant and cytotoxic species (Radi, 1998).

1.3.5 Vasoactive properties of NO

Endothelial NO also decreases intracellular Ca^{2+} within the VSMC to reduce contractility and induce vasodilatation. Once synthesised, NO diffuses from the EC to the underlying VSMC, where it stimulates soluble guanyl cyclase (sGC). NO has a high affinity for sGC and low concentrations of NO (10-100nM) activate sGC in a rapid rate reaction (10^7-10^8 $M^{-1}s^{-1}$) (Kharitonov *et al,* 1997; Stone & Marletta, 1996). sGC is a heterodimeric protein consisting of an α and β subunit, which may be divided into three sections, cGMP-binding domain, the haem-binding domain, and the dimerisation domain. sGC has some basal activity but is increased up to 500-fold by the interaction of the ferrous iron (Fe^{2+}) of the haem moiety with NO (Stone *et al,* 1996). This binding of NO to the haem moiety draws the Fe^{2+} from the plane of the porphyrin ring, breaking the his105 bond to produce a conformational change in the catalytic domain and stimulating the production of cGMP from GTP (Gerzer, 1981; Rapoport & Murad, 1983).

cGMP then activates protein kinase G (PKG) to oppose agonist-mediated increases in intracellular Ca^{2+} concentrations. Contractile agonists activate phospholipase C (PLC) to generate inositol 1,4,5,-triphosphate (IP_3). This releases Ca^{2+} from the sacroplasmic reticulum, which then binds to calmodulin to activate myosin light chain kinase (MLCK) and induce smooth muscle contraction. However, PKG inhibits this by phosphorylating PLC and IP_3, or by phosphorylating and down regulating the activity of the IP_3 receptor to prevent the increase in cystolic Ca^{2+}. PKG also promotes the cellular efflux of Ca^{2+} and uptake of cystolic Ca^{2+} into intracellular stores (Furkawa *et al,* 1988). PKG also decreases the sensitivity of the muscular contractile apparatus to cystolic Ca^{2+} (Carvajal *et al,* 2000).

1.4 Other vasoactive factors released by the endothelium

1.4.1 Prostacyclin

The EC also release prostacyclin (Moncada *et al,* 1976), a potent vasodilator and platelet anti-aggregator. Increases in endothelial intracellular Ca^{2+} also activate phospholipase A (PLA). This liberates arachidonic acid from membrane phospholipids, which may then metabolised by cyclo-oxygenase (COX) to eicosanoids, one of which is prostacyclin. Prostacyclin produces vasodilatation by activating adenylate cyclase (AC) and increasing the production of cyclic 3',5'-adenosine monophosphate (cAMP) within the VSMC. This acts via protein kinase A (PKA) to inhibit MLCK and vasoconstriction. Prostacyclin also increases cAMP within platelets to inhibit stimulation and aggregation. The anti-platelet properties of prostacyclin have a greater physiological role than its contribution to endothelium-dependent vasodilatation (Radomski *et al,* 1987; Luscher & Barton, 1997).

1.4.2 Endothelium-derived hyperpolarising factor

EDHF is a putative vasodilator released by the endothelium and has been identified in various vascular preparations (Garland & McPherson, 1992; Plane & Garland, 1992; Rand & Garland, 1992; Garland *et al*, 1995). The role of EDHF in agonist-stimulated endothelium-dependent vasodilatation varies between large and small blood vessels, in which NO and EDHF predominate respectively. The release of EDHF by EC is controlled by the intracellular Ca^{2+} concentration and is inhibited by calmodulin antagonists. EDHF is thought to exert is effect upon vascular smooth muscle by opening K^{+}-ion channels (Edwards *et al*, 1998).

Several possible suggestions have been proposed for the identity of EDHF. K^+, in low concentrations (<14 mM), induces hyperpolarisation and vasodilatation via the activation of NA^+/K^+-ATPase (Edwards *et al,* 1998; Edwards *et al,* 1999). However, other studies have concluded that K^+ is not EDHF (Doughty *et al,* 2000; Drummond *et al,* 2000; Lacy *et al,* 2000). Epoxyeicosatrienoic acids are metabolites of arachidonic acid, produced by EC through the cytochrome P450 mono-oxygenase pathway, and may function as EDHF (Rosolowsky & Campbell, 1993; Campbell *et al,* 1996; Rosolowsky & Campbell, 1996; Fisslthaler *et al,* 1999; Halcox *et al,* 2001). These acids can open Ca^{2+}-activated K^+ channels to cause vasodilatation. Substance P could also be an EDHF, as it promotes the endothelium-dependent hyperpolarisation of the VSMC in studies in guinea pig submucosal arterioles (Coleman *et al,* 2001). Hydrogen Peroxide (H_2O_2) also increases K^+ channel activity and hyperpolarises VSMC (Barlow *et al,* 2000) to induce vasodilatation (Beny & von der Weid, 1991; Matoba *et al,* 2000). Estrogen may also contribute to EDHF-mediated vasodilatation, as Wu *et al* (Wu *et al,* 2001) and Golding *et al* (Golding *et al,* 2001) demonstrated the varying contribution of EDHF to vasodilatation in females and males. An alternative explanation for the identification of EDHF, is the direct intercellular communication via gap junctions, as it has been demonstrated that these are involved in EDHF-mediated vasodilatation (Edwards *et al,* 1999; Harris *et al,* 2000; Goto *et al,* 2002; Chaytor *et al*, 2003; Karagiannis *et al,* 2004). The formation and release of cAMP from the EC may also contribute to the gap junction-dependent vasodilatation attributed to EDHF (Griffith & Taylor, 1999).

1.4.3 Endothelium-Derived Constracting Factors

The endothelium may also mediate vasoconstriction by releasing endothelium-derived constriction factors, such as ET-1, thromboxane A_2, prostaglandin H_2, and components of the renin-angiotensin system, such as angiotensin II (Ang-II) (Luscher & Barton, 1997). Ang-II activates angiotensin receptors on the endothelium to stimulate the release of ET-1, which may cause marked and sustained contractions (Yanagisawa *et al,* 1988; Ohnaka *et al*, 1993). Agonists such as arachidonic acid, serotonin, histamine and ACh may evoke the COX-mediated release of thromboxane A_2 and prostaglandin H_2 (Kato *et al,* 1990; Luscher & Barton, 1997), which activate thromboxane receptors in VSMC and platelets to counteract the effects of NO and prostacyclin.

1.5 Endothelial Dysfunction

The vascular endothelium has a fundamental role in the regulation of cardiovascular function. Consequently, damage to the EC and the resultant endothelial dysfunction, have been implicated in the pathophysiology of numerous cardiovascular diseases, and is regarded as an initial key event in the aetiology of these disease states (Rubanyi, 1993; Shimokawa & Takeshita, 1995; Luscher & Barton, 1997).). Endothelial dysfunction can be characterised as the loss of endothelium-dependent vasodilatation, the reduced release of relaxing factors or the enhanced release of vasoconstrictors from the endothelium (Yanagisawa *et al,* 1988; Vallance & Collier; 1994). However, the term 'endothelial dysfunction' commonly refers to an impairment of the NO-system alone, due to the major consequences this may have. The impairment of the NO-system may arise due to the inhibition of NO synthesis, reduced NO-availability, or as a result of the diminished effects of NO. NO contributes to the maintenance of basal vascular tone (Vallance *et al,* 1989) and blood pressure (Haynes *et al,* 1993). Therefore, a reduced efficacy of endogenous NO would cause impaired vasodilatation and increased

peripheral vascular resistance, resulting in the development of hypertension (Peart, 1980; Vallance & Collier; 1994; Ferro & Webb, 1997).

Impairment of the vascular NO system is present in hypertension (Panza *et al,* 1990), heart failure (Katz *et al,* 1993), hypercholesterolaemia (Creager *et al,* 1992), diabetes mellitus (Calver *et al,* 1992), cigarette smokers (Zeiher *et al,* 1995), and homocysteinamia (Mayer *et al,* 1996). All of these conditions are associated with the increased risk of vascular and cardiovascular disease. However, the role of endothelial dysfunction in these conditions may vary, as it may be a cause or an effect of the associated condition. For example, endothelial dysfunction may be a contributing factor to the development of hypertension (Panza *et al,* 1990), thus having a causative role in the development of this condition. However, in hypercholesterolaemia, the lipids present may directly damage the endothelium (Creager *et al,* 1992; Dart & Chin-Dusting, 1999), therefore endothelial dysfunction may occur as a result of the disease state.

1.6 Hypercholesterolaemia

Hypercholesterolaemia is a disease condition in which there is an excess concentration of fat, or lipids, in the blood. This condition is a risk factor for cardiovascular disease, as the association between elevated cholesterol in the blood and this pathophysiological condition is well established (Goldstein & Brown, 1977; Steinberg, 1983; Martin *et al,* 1986). Hypercholesterolaemia can occur as a consequence of a genetic malfunction in the physiological handling of cholesterol or due to the saturation of the liver via the high dietary intake of cholesterol and lipids, resulting in increased accumulation in plasma. This condition precipitates the development of cardiovascular disease via increased expression of endothelial surface adhesion molecules (Hwang *et al,* 1997; Abe *et al*, 1998), the recruitment and adhesion of monocytes and leukocytes (Tsao *et al,*

1994; Gauthier *et al,* 1995; Scalia *et al,* 1998), impaired fibrinolysis and excessive thrombosis (Puccetti *et al,* 2001; Puccetti *et al,* 2002), or impaired vascular function (Verbeuren *et al,* 1986; Zeiher *et al,* 1993; Anderson *et al,* 1995)

1.6.1 Hypercholesterolaemia and endothelial dysfunction

In vitro and *in vivo* data from both hypercholesterolaemic animal (Verbeuren *et al,* 1986; Anderson *et al,* 1995) and human studies (Zeiher *et al,* 1991) have demonstrated the impairment of endothelial function in hypercholesterolaemia. Human studies have shown that endothelial dysfunction is widespread in hypercholesterolaemia, and is not restricted to the coronary circulation (Chowienczyk *et al,* 1992). Impaired endothelium-dependent vasodilatation to ACh has been demonstrated in coronary blood vessels (Quyymi *et al,* 1995; Treasure *et al,* 1995; Mullen *et al,* 1997) and the peripheral small arteries (Vallance & Moncada, 1994; Goode *et al,* 1997) of hypercholesterolaemic human patients. This endothelial dysfunction may precede clinical evidence of vascular disease in both coronary (Drexler *et al,* 1991) and resistance vessels (Chowienczyk *et al,* 1992) in hypercholesterolaemic patients. Endothelial dysfunction has also been demonstrated in children with familial hypercholesterolaemia prior to the development of clinically evident vascular disease (Clarkson *et al,* 1997). Lipid lowering in hypercholesterolaemia improves coronary endothelial function (Leung *et al,* 1993; Chin & Dart, 1994; O'Driscoll *et al,* 1997; Tamai *et al,* 1997; Guven *et al,* 2006). This endothelial dysfunction is unlikely to result form an inability of VSMC to respond to NO, as vasodilatation to exogenous NO-donors such as glyceryl trinitrate (GTN) or sodium-nitroprusside (SNP) is generally not impaired in hypercholesterolaemia (Flavahan, 1992; Labinjoh & Webb, 1997; Wilkinson & Cockroft, 1998; Dart & Chin-Dusting, 1999). Therefore, the endothelial dysfunction that occurs in this pathophysiological state may reflect a reduction in NO-availability.

This reduced NO availability could occur due to decreased substrate availability, reduced NOS expression, reductions in the presence of NOS co-factors, or increased inactivation of NO by $O_2^{\cdot-}$. In hypercholesterolaemic patients, l-arginine restores endothelium-dependent vasodilatation to ACh (Creager *et al,* 1990; Creager *et al,* 1992; Chowienczyk *et al,* 1992), suggesting the reduced NO availability may be a result of impaired l-arginine metabolism. In hypercholesterolaemic rabbit aortic vessels, there is an increase rather than a reduction in NO generation (Minor *et al,* 1990). However, $O_2^{\cdot-}$ production is three times higher in these vessels than normal vessels, and is correlated with a significant impairment in endothelium-dependent relaxation to ACh (Minor *et al,* 1990). Ohara *et al.* also reported that hypercholesterolaemic vessels generated increased concentrations of endothelial $O_2^{\cdot-}$, which was related to the loss of endothelial function (Ohara *et al,* 1993). Furthermore, Ohara *et al.* demonstrated that the dietary correction of hypercholesterolemia in the rabbits prevented endothelial $O_2^{\cdot-}$ generation (Ohara *et al,* 1995). Similarly, the treatment of hypercholesterolaemic rabbits with SOD, which dimutates $O_2^{\cdot-}$, partially restores impaired endothelium-dependent vasodilatation (Mugge *et al,* 1991). Therefore, hypercholesterolaemia-induced endothelial dysfunction could be attributed to lipoprotein-induced increases in oxidative stress within the EC (Tanner *et al,* 1991).

1.7 Lipoproteins

1.7.1 Lipids and lipoproteins

Lipids are insoluble in plasma so are bound to a suitable transport vehicle. These lipid transport systems are known as 'lipoproteins' and are essential to life. The important components of this system are; chylomicrons, high-density lipoproteins (HDL), low-density lipoproteins (LDL) and very-low density lipoproteins (VLDL)

LDL is the major carrier of cholesterol in plasma and the main source of lipids found to accumulate in the blood. LDL consists of a cholesterol ester core surrounded by phospholipids and lipophilic antioxidants, such as α-tocopherol and β-carotene. LDL circulates in the blood and is recognised by LDL receptors on the peripheral cells that require cholesterol for cellular metabolism. Each LDL particle contains one apo-B_{100} protein that serves as a ligand for this LDL receptor. This facilitates the binding and internalisation of LDL by receptor-mediated endocytosis. The amount of LDL present in plasma is proportional to the amount of cholesterol in the plasma. Consequently, LDL may be the primary mediator of the deleterious actions of hypercholesterolaemia on endothelial and vascular function.

The endothelium is continuously exposed two forms of LDL, native (n-) and oxidised (ox-), and both may have direct effects on vascular function (Jacobs *et al*, 1990; Fontana *et al*, 1999). They may impair endothelium-dependent vasodilatation by inactivating or having a deleterious effect upon NO (Galle *et al,* 1991; Vergnani *et al,* 2000). In atherosclerotic patients, impaired vascular function and endothelial injury have been demonstrated at the site of the arterial plaque, formed by the accumulation of LDL within the vascular wall (Ludmer *et al*, 1986; Gordon *et al*, 1989; Helaly, 1990). As previously mentioned, there is extensive evidence to suggest that elevated plasma LDL concentrations are associated with impaired NO-mediated endothelium-dependent

vasodilatation and that reducing these can restore vasodilatation (Leung *et al,* 1993; Chin & Dart, 1994; O'Driscoll *et al,* 1997; Tamai *et al,* 1997; Matsuoka, 2001; Guven *et al,* 2006).

1.7.2 LDL and endothelial dysfunction

The oxidative modification of LDL causes profound changes in the physiochemical properties of the LDL particle and alters both the lipid and protein components (Cox & Cohen, 1996). It has been suggested that ox-LDL may be the most important single factor in producing endothelial dysfunction (Chin *et al,* 1992; Cox & Cohen, 1996; Steinberg, 1997; Rosendorff, 2002). Ox-LDL is a key component of endothelial injury (Ross, 1993; Dart & Chin-Dusting), and is known to impair endothelium-dependent vasodilatation by reducing NO availability (Galle *et al,* 1991; Plane *et al,* 1993; Myers *et al,* 1994; Galle *et al,* 1995; Lewis *et al,* 1997; Hein & Kuo, 1998; Fontana *et al,* 1999; Chan *et al,* 2003). This inhibition of vasodilatation by ox-LDL is irreversible (Jacobs *et al*, 1990; Kugiyama *et al*, 1990; Yokoyama *et al*, 1990; Simon *et al*, 1990). Ox-LDL may also impair vasodilatation to endothelium-independent vasodilators, such as SNP or GTN (Jacobs *et al,* 1990; Tanner *et al*, 1991), as it can impair cGMP-mediated vasodilatation (Ezaki *et al,* 1994; Pohl *et al,* 1995). Lysophosphatidylcholine or lysolecithin, produced upon the oxidative modification of LDL, may be responsible for the diminished vascular response to endothelial NO (Cox & Cohen, 1996). Lysolecithin derived from ox-LDL inhibits endothelium-dependent vasodilatation in rabbit aorta (Kugiyama *et al*, 1990; Yokoyama *et al*, 1990), whereas the remaining lipid fractions had no effect (Yokoyama *et al*, 1990).

Ox-LDL is also directly injurious to the endothelium and enhances EC permeability to macromolecules, acts as a chemotactic agent for monocytes, stimulates expression of adhesion molecules on the endothelium, and may cause EC apoptosis (Hessler *et al*,

1979; Frostegard *et al*, 1991; Frostegard *et al*, 1992; Labinjoh & Webb, 1997). Furthermore, ox-LDL can induce the generation and release of ET-1 from EC to promote vasoconstriction and prevent vasodilatation (Boulanger *et al,* 1992).

The role of n-LDL in lipoprotein-induced endothelial dysfunction remains one of great debate. Several *in vitro* studies using various isolated tissue preparation have reported that n-LDL can inhibit endothelium-dependent vasodilatation (Andrews *et al*, 1987; Jacobs *et al*, 1990; Tomita *et al*, 1990; Galle *et al*, 1991; Fontana *et al*, 1999). However, unlike the deleterious effects of ox-LDL, this occurs due to a rapid and reversible mechanism (Jacobs *et al,* 1990; Tomita *et al,* 1990; Galle *et al,* 1991). In contrast, several *in vitro* studies have failed to demonstrate any effect of n-LDL on or NO-availability or endothelium-dependent vasodilatation (Kugiyama *et al*, 1990; Simon *et al*, 1990; Yokoyama *et al*, 1990; Galle *et al,* 1994; Galle *et al,* 1995; Chan *et al,* 2003).

1.7.3 The mechanism of LDL-induced endothelial dysfunction

The deleterious effects of n- and ox-LDL on endothelial function may be mediated via impaired NO synthesis or reduced NO availability, as a consequence of increased oxidative stress within the vascular wall (Harrison, 1997). Both n-LDL and ox-LDL enhance eNOS activity (Galle *et al,* 1990; Schaefer *et al,* 1993; Fries *et al,* 1995; Allen *et al*, 1998), demonstrating that reduced NO availability is not a result of impaired enzymatic activity. The inhibitory action of human LDL may be prevented by supplementation with antioxidants, such as ascorbic acid (AA) and probucol (Plane *et al,* 1993; Brown & Goodman, 1998; Fontana *et al,* 1999; Chan *et al,* 2003), demonstrating that the inhibition of vasodilatation may be caused by increased oxidative stress. This may occur due to eNOS-mediated generation of $O_2^{\cdot-}$ (Galle *et al*, 1991; Ohara *et al*, 1993; Vergnani *et al*, 2000; Fleming *et al,* 2005). The generation of

this radical species increases intracellular oxidative stress and reduces NO availability, as $O_2^{\cdot-}$ radicals readily inactivate NO (Gryglewski *et al*, 1986; Rubanyi & Vanhoutte, 1986).

Prolonged endothelial exposure to n- or ox-LDL causes the enhanced generation of $O_2^{\cdot-}$ (Ohara *et al,* 1993; Pritchard *et al*, 1995; Vergnani *et al*, 2000; Fleming *et al,* 2005). This may be prevented by NOS inhibition (Huk *et al,* 1997). The incubation of isolated carotid arteries with ox-LDL or n-LDL stimulates an 8- and 4-fold increase in the generation of $O_2^{\cdot-}$ by eNOS, respectively (Stepp *et al,* 2002). This may be caused a lipoprotein-induced decrease in l-arginine availability (Huk *et al,* 1997; Vergnani *et al,* 2000), as the *in vitro* supplementation of cultured EC with high concentrations of l-arginine restores NO-availability (Pritchard *et al*, 1995; Huk *et al,* 1997; Vergnani *et al,* 2000). This is in accord with studies that demonstrated that administration of l-arginine restored vasodilatation in hypercholesterolaemia (Cooke *et al,* 1991; Drexler *et al*, 1991; Chowienczyk *et al*, 1994; Clarkson *et al,* 1996; Wolf *et al,* 1997; Yin *et al,* 2005). Therefore, LDL may cause endothelial dysfunction by uncoupling the l-arginine/NO-pathway, resulting in the eNOS-mediated generation of $O_2^{\cdot-}$ and reduced NO-availability. Consequently, enhanced oxidative stress has a prominent role in the development of LDL-induced vascular dysfunction and its clinical manifestations.

1.8 Atherosclerosis

Hypercholesterolaemia is an established risk factor for atherogenesis (Goldstein & Brown, 1977; Steinberg, 1983; Martin *et al,* 1986), as this is the major clinical manifestation of elevated plasma LDL concentrations (Cox & Cohen, 1996; Dart & Dusting, 1999). Atherosclerosis occurs due to the narrowing of the arteries by the deposition of lipids in the subendothelial layer. This can result in acute cardiovascular events such as myocardial infarction, stroke, peripheral vascular disease, renal failure

and other clinical syndromes, depending on the anatomical location of the atherosclerotic lesions.

Endothelial dysfunction is well established in atherosclerosis and is regarded as a major precursor of the disease (Harrison *et al*, 1987). LDL-induced endothelial dysfunction could have a prominent role in atherogenesis by increasing oxidative stress within the vascular wall, thus preventing the anti-atherogenic actions of NO and promoting the oxidative modification of LDL (Cox & Cohen, 1996; Dart & Dusting, 1999). The potential role of LDL in endothelial dysfunction and atherogenesis is demonstrated in Figure 1.4. The EC bind n-LDL (Baker *et al*, 1984) and generate free radicals that oxidise the attached LDL to form ox-LDL (Steinbrecher, 1987). Oxidative modification of LDL causes the destruction of the 'classic' LDL receptor-binding ligand to prevent n-LDL clearance and metabolism (Steinbrecher *et al*, 1988). Ox-LDL also promotes monocyte recruitment, monocyte adhesion to the vascular wall, and enhanced VSMC proliferation (Quinn *et* al, 1987; Quinn *et al,* 1988; Ross, 1993). The monocytes permeate the vascular wall were they differentiate into macrophages and accumulate ox-LDL (Steinbrecher *et al*, 1984) via their 'scavenger' receptors (Goldstein *et al,* 1979; Steinberg *et al,* 1989; Witzum & Steinberg, 1991). These cells become lipid-laden and form foam cells (Steinberg *et al*, 1989; Steinbrecher *et al*, 1990; Esterbauer *et al*, 1992), which grows into an advanced atherosclerotic lesion and develop into a fibrous plaque that impedes arterial blood flow (Ross, 1993). This is prone to rupture and may result in arterial occlusion (Berliner *et al*, 1995).

1.9 Oxidative Stress

Oxidative stress is an important aetiological factor in many pathophysiological states and may be a major cause of endothelial dysfunction in cardiovascular disease (Harrison, 1997; Cai & Harrison, 2000; Zalba *et al,* 2000; Schulz *et al,* 2004).

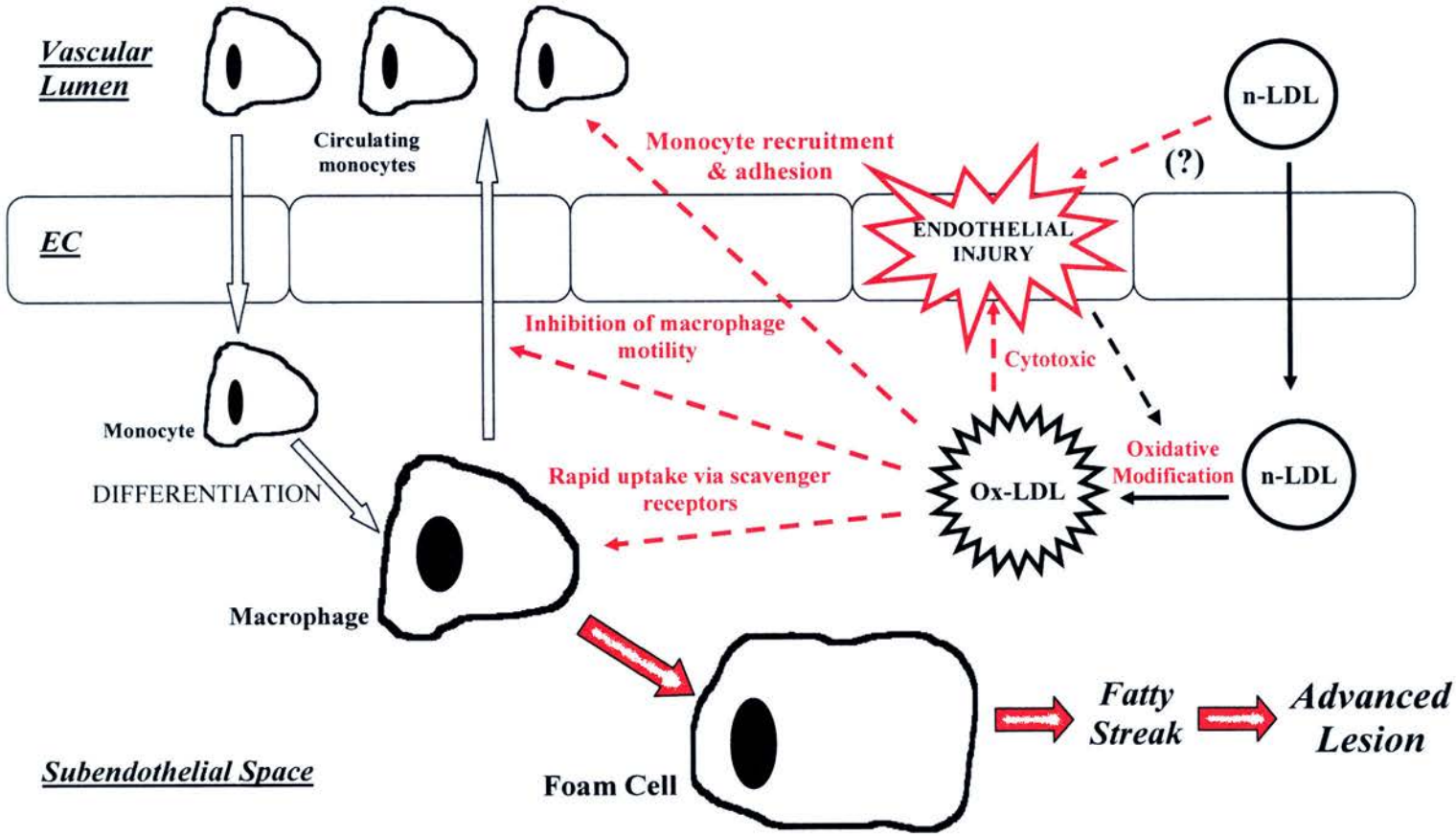


Figure 1.4. The role of n-LDL and ox-LDL in endothelial dysfunction and atherogenesis.

Oxidative stress is a situation in which cells are exposed to excessive levels of reactive oxygen species (ROS) due to an imbalance between pro-oxidant and antioxidant forces. A free radical is a highly reactive pro-oxidant species that contains one or more unpaired electron, and is capable of independent existence (Halliwell & Gutteridge, 1988). These radicals attack other molecules to produce an electron pair and a stable species. Consequently, the target molecule is oxidised and contains an unpaired electron, becoming a free radical which may further propagate a free radical chain reaction (Halliwell & Gutteridge, 1988). Oxygen radicals and other ROS initiate the chain reaction of lipid peroxidation that induces the oxidative modification of LDL and produces additional reactive intermediates, such as hydroperoxides, aldehydes and epoxides.

These in turn cause further damage to cells and tissues by affecting nucleic acids, DNA, RNA, cellular proteins and membrane constituents (Kukreja & Hess, 1992). Oxidative stress within the vascular wall may be caused by the release of ROS from the cells present. Several pathways and enzymes have been implicated in the generation of these free radicals, such as NADH/NADPH oxidase, XO, lipoxygenases, myeloperoxidase and NOS (Keaney & Vita, 1995; Harrison, 1997; Cai & Harrison, 2000; Zalba *et al,* 2000). The key role of excess ROS generation in the development of LDL-induced endothelial dysfunction has been demonstrated by the ability of antioxidants to restore endothelium-dependent vasodilatation in patients with risk factors for cardiovascular disease, such as hypercholesterolaemia (Anderson *et al,* 1995; Ting *et al,* 1996a; Ting *et al,* 1996b; Levine *et al,* 1996; Engler *et al,* 2003).

1.9.1 Superoxide

Superoxide ($O_2^{\cdot -}$) is the major free radical in biological systems and has a half-life of less than milliseconds (Fridovich, 1978). It is a potent free radical that may cause

oxidative damage within biological systems, as shown in Figure 1.5. The charged nature of $O_2^{\cdot-}$ prevents dissolution in the hydrophobic bilayer of cellular membranes and could prevent its diffusion into cells. However, whilst *in vitro* studies have suggested that $O_2^{\cdot-}$ cannot penetrate lipid bilayers (Dix & Aikens, 1993; Frimmer *et al,* 1996), the free radical may permeate biological membranes (Mao & Poznansky, 1992) via anion channels (Fridovich, 1978; Lynch & Fridovich, 1978; Gus'kova *et al,* 1984).

Under physiological conditions, $O_2^{\cdot-}$ is produced by all tissues via mitochondrial oxidative metabolism during aerobic respiration. $O_2^{\cdot-}$ reacts readily with NO to maintain low physiological concentrations of $O_2^{\cdot-}$ within the EC (Gryglewski *et al,* 1986; Rubanyi & Vanhoutte, 1986). Various enzymatic processes generate $O_2^{\cdot-}$ via the oxidation of NADH or NADPH by NAD(P)H oxidase (Loschen *et al,* 1974), which is constitutively active in smooth muscle cells. EC also contain several oxidase enzymes which are capable generating $O_2^{\cdot-}$ (Rubanyi & Vanhoutte, 1986; Katusic & Vanhoutte, 1989), such as NAD(P)H oxidase, XO (Engerson *et al,* 1987; Ohara *et al,* 1993), COX (Mugge *et al,* 1991), and cytochrome P_{450} (Sligar *et al,* 1974). In pathophysiological conditions, the generation of this potent free radical is may be increased. The deleterious action of LDL upon vascular function is attributed to the increased eNOS-mediated generation of $O_2^{\cdot-}$ (Ohara *et al,*1993). Furthermore, $O_2^{\cdot-}$ can cause vasoconstriction either by a direct action on smooth muscle cells or by an indirect action via the inactivation of basal endothelium-derived NO (Rubanyi & Vanhoutte, 1986; Gryglewski *et al,* 1986; Auch-Schwelk *et al*, 1989; Katusic & Vanhoutte, 1989). Endothelial removal decreases $O_2^{\cdot-}$ production in hypercholesterolaemic vessels, but increases production in normal vessels (Ohara *et al,* 1993). This suggests that, while the endothelium may be a source of $O_2^{\cdot-}$, it protects against the harmful effects of this radical via the production of basal NO to scavenge $O_2^{\cdot-}$. This also suggests that under physiological conditions, NO production is greater than basal $O_2^{\cdot-}$ production. Importantly, NO production is similar in normal and hypercholesterolaemic vessels (Ohara *et al,* 1993). The prominence of $O_2^{\cdot-}$ in pathophysiology, and its interaction and

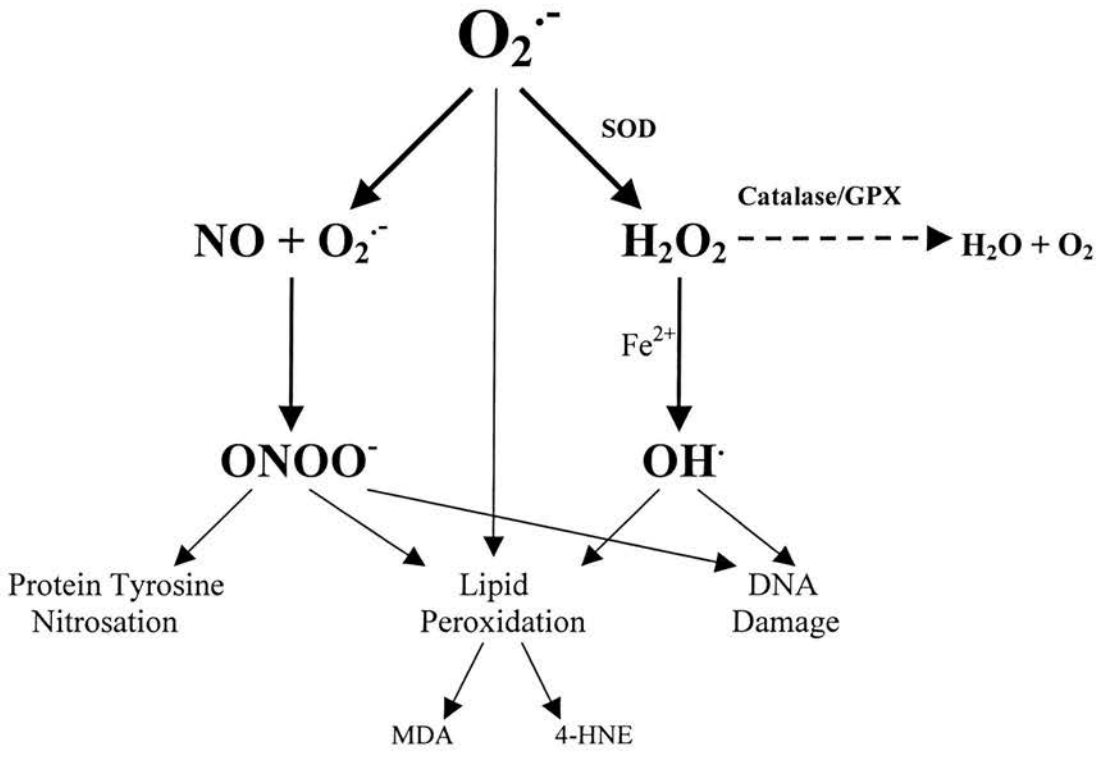


Figure 1.5. The major reactions of $O_2^{\cdot -}$. $O_2^{\cdot -}$ reacts rapidly with NO to form the highly reactive and cytotoxic radical $ONOO^-$. Dismutation with SOD converts $O_2^{\cdot -}$ to H_2O_2, which is then decomposed by catalase or GPX. An overview of the deleterious effects of the formation of these potentially-damaging radical species is also shown.

relationship with EC and NO, suggest it has a vital role in the aetiology of endothelial dysfunction and the disease states associated with this condition.

1.9.2 The interaction of $O_2^{\cdot-}$ and NO

In various pathophysiological conditions, $O_2^{\cdot-}$ may form more reactive and damaging agents (Freeman & Crapo, 1982). The role of NO is different in the presence of $O_2^{\cdot-}$ compared with the absence (Rubbo *et al,* 1994). $O_2^{\cdot-}$ can directly decrease NO availability and impair NO-mediated dilatation (Beckham & Koppenol, 1996). However, more significantly, when produced in close proximity, NO and $O_2^{\cdot-}$ react rapidly to form the potent cytotoxic oxidant, peroxynitrite ($ONOO^-$) (Pryor & Squadrito, 1995; Beckman & Koppenol, 1996; Ma *et al*, 2000; Radi *et al*, 2001).

$$NO + O_2^{\cdot-} \longleftrightarrow ONOO^-$$

$ONOO^-$ is formed *in situ* when a vascular imbalance in the production of NO and $O_2^{\cdot-}$ occurs. These react rapidly to produce $ONOO^-$ at an almost diffusion-limited rate of 6.7 x $10^9 M^{-1} s^{-1}$ (Huie & Padmaja, 1993). This reaction is limited by the radical precursor produced at lower rates, but occurs optimally when $O_2^{\cdot-}$ and NO are present in an equimolar basis (Miles *et al,* 1996). $ONOO^-$ may be formed in either the aqueous or the hydrophobic phase of the cell. NO is more soluble in the hydrophobic phase (Khan & Wilson, 1995), and the reaction between NO and $O_2^{\cdot-}$ occurs approximately 300 times more rapidly within membranes than in the surrounding aqueous medium (Liu *et al,* 1998). NO has a biological half-life of seconds and freely diffuses across membranes (Denicola *et al*, 1996), however $O_2^{\cdot-}$ lasts less than milliseconds and may only permeate membranes via anion channels (Fridovich, 1978). Therefore, $ONOO^-$ formation will predominantly occur nearer to the site of $O_2^{\cdot-}$ generation.

1.9.3 Peroxynitrite

$ONOO^-$ is a highly reactive cytotoxic compound that may yield ROS and reactive nitrogen species (RNS) (Beckman *et* al, 1990; Koppenol *et al,* 1992; Koppenol, 1998; Radi *et al*, 2001). It is relatively stable anion, due to the delocalisation of the negative charge over the entire molecule. However, upon protonation to peroxynitrous acid (ONOOH) (p*K*a 6.8) it decays to nitrate. Under biological conditions, the ratio of $ONOO^-$ to ONOOH is dependent upon the pH. Under physiological conditions (pH 7.4), 80% of $ONOO^-$ is present in the anionic form and decomposes after only a few seconds (Beckman *et al,* 1990; Koppenol *et al,* 1992; Pryor & Squadrito, 1995). This generates further free radicals, such as $OH^{\cdot}$ or $NO_2^{\cdot}$ (White *et al*, 1994; Beckman & Koppenol, 1996; Squadrito & Pryor, 1998; Patel *et al*, 2000; Ma *et al*, 2000), which cause further radical reactions.

$ONOO^-$ is able to freely permeate membranes via anion channels ($ONOO^-$) and passive diffusion (ONOOH) (Marla *et al,* 1997; Denicola *et al,* 1998). It has an estimated half-life of less than 100ms (Radi *et al,* 1998; Romero *et al,* 1999), which is sufficient for $ONOO^-$ to potentially travel 5-20μm across extracellular and/or intracellular compartments. As a result, $ONOO^-$ has ready access to hydrophobic compartments within cells, and to hydrophobic structures such as atherosclerotic plaques, were it may induce membrane lipid and lipoprotein oxidation reactions (Radi, 1998).

$ONOO^-$ may react with a variety of biomolecules such as lipids, proteins, carbohydrates and DNA (Moreno & Pryor, 1992; Rubbo *et al,* 1994; Pryor & Squadrito, 1995; Salgo *et al,* 1995; Evans *et al,* 1996), and is implicated in the aetiology of numerous disease processes, such as cardiovascular disease, atherosclerosis and severe inflammatory conditions (Radi *et al,* 2001). The potential pathophysiological effects include inactivation of mitochondrial manganese-SOD (Ischiropoulos *et al,* 1992) and glutamine synthase (Berlett *et al,* 1998), oxidative modification of LDL (Steinberg *et*

al, 1989), lipid peroxidation (Radi *et al,* 1991), alteration of the lipid aggregatory properties of surfactant protein A (Berlett *et al,* 1998), inactivation of sodium transport, inactivation of α1-antiproteinase (Moreno & Pryor, 1992), modification of tyrosine phosphorylation (Gow *et al,* 1996; Kong *et al,* 1996). It may also cause one- or two-electron oxidations of sulphydryls, resulting in the formation of thiyl radicals, radical chain reactions and the depletion of thiols (Quijano *et al,* 1997). $ONOO^-$ also attacks and decreases plasma antioxidant levels, thus reducing the natural physiological defence mechanism and allowing oxidative damage to tissues and organs (van der Vliet *et al,* 1994; Pryor & Squadrito, 1995).

$ONOO^-$ reacts readily with phenolic compounds, such as tyrosine, to form nitrated and dimerised products, such 3-nitrotyrosine (Ischiropoulos, 1998) and dityrosine (van der Vliet *et al,* 1994). Protein tyrosine residues are key-targets for $ONOO^-$-mediated nitrations and modify protein and enzyme function (Ischiropoulos, 1998). The presence of 3-nitrotyrosine in proteins may represent modifications induced by the biological formation of $ONOO^-$ (Ischiropoulos, 1998). Endogenous $ONOO^-$ formation causes the nitration of certain cellular proteins *in vivo*, such as Mn-SOD (Hooper *et al,* 1997) and prostacyclin synthase (Haddad *et al,* 1996; MacMillan–Crow *et al,* 1998). $ONOO^-$ may also reduce NO-availability, by irreversibly inhibiting NOS via the oxidation of the haem thiolate bond in the catalytic site of the enzyme (Pasquet *et al,* 1996).

$ONOO^-$ may have contrasting effects upon the vascular system depending on the concentrations present. When present in low concentrations, $ONOO^-$ may form a vasoactive compound that can restore and maintain NO-mediated vascular function (Liu *et al,* 1994; Moro *et al,* 1995; van der Vliet *et al*, 1998). However, in greater concentrations (>30μM) the deleterious properties of $ONOO^-$ on the vascular system are predominant (Ma *et al,* 1997; Ma *et al,* 2000; Radi *et al*, 2001).

1.10 Antioxidants

The generation of harmful free radicals, such as $O_2^{\cdot -}$ and $ONOO^-$, can have disastrous effects upon human physiology. Consequently, an extensive antioxidant defence system is present within the human body. Three requirements must be fulfilled before a substance can be regarded as a physiological antioxidant. The potential antioxidant must interact with and scavenge biologically relevant oxidants and free radicals. This interaction must lead to the chemical modification of the antioxidant which generates a product that is physiologically less harmful than the initial oxidant or radical. Finally, the antioxidant should be present in adequate physiological concentrations to ensure a sufficient reaction.

The antioxidant defence system within the human body may be separated into three main classes:

- Antioxidant enzymes. This includes enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase which prevent the deleterious effects of ROS (Carlsson *et al,* 1995; Straitlin *et al,* 1995). These enzymes are mainly part of the intracellular antioxidant defence system.

- Preventative antioxidants. These are proteins, such as albumin, transferrin and caeruloplasmin, which bind transition metal ions to prevent the oxidation of LDL (Heinecke *et al,* 1984; Steinbrecher *et al,* 1984).

- Chain-breaking antioxidants. These are low molecular weight compounds that scavenge free radicals to prevent damage to important target molecules. They can be divided into lipid-soluble antioxidants, such as tocopherols, ubiquinol-10, and carotenoids, and water-soluble antioxidants, such as uric acid (UA), ascorbic acid (AA) and glutathione (GSH). Lipid-soluble antioxidants exist within cellular membranes and

lipoproteins, and have a major role in preventing the oxidative modification of LDL. LDL oxidation *in vitro* is preceded by the depletion of lipid-soluble antioxidants (Schlotte *et al,* 1998). Water-soluble, or hydrophilic, antioxidants prevent the lipid peroxidation induced by transition metal ions, peroxyl radicals and a number of different cell types (Frei *et al*, 1989). In addition to their radical scavenging ability, hydrophilic antioxidants such as AA and UA can sequester transition metal ions or modify the metal-binding sites of LDL to prevent metal-ion dependent oxidation (Retsky *et al,* 1993).

The various classes act synergistically to provide an efficient antioxidant defense system. For example, hydrophilic antioxidants may act as sacrificial antioxidants to prevent the consumption of those with in the lipid phase. Interaction also allows the regeneration of other antioxidants, for example, AA may regenerate the α-tocopherol radical, and UA may protect and stabilise AA (Abuja, 1999).

1.11 Uric Acid

UA is a weakly acidic (pKa=5.8), non-enzymatic compound distributed throughout the extracellular fluid in the body as urate, a monoanion salt. This is removed from plasma by glomerular filtration. 10% is filtered and secreted, but the remaining 90% is reabsorbed from the proximal renal tubule and returned to the blood. The concentration of UA in plasma varies widely between individuals and is determined by the rate of purine metabolism and renal clearance (Steele, 1971; Tykarski *et al,* 1991). However the typical reference range for physiological plasma concentration is 100-400μmol/l (Alderman *et al,* 1999; Fang & Alderman, 2000).

UA is derived exclusively from the breakdown of adenine and guanine-based compounds. These purine compounds are essential to all animal cells and subject to

continuous metabolic turnover. Through a series of enzymatic steps they are degraded to hypoxanthine (HX) then xanthine. UA is then formed by the further oxidation of xanthine (Figure 1.6) (Becker, 1993; Waring *et al*, 2000). This is exclusively catalysed by XDH (xanthine dehydrogenase), using NAD^+ as an electron acceptor, or by xanthine oxidase (XO), which transfers the reducing equivalents direct to O_2 to form ROS, such as $O_2^{\cdot-}$ and H_2O_2 (Parks & Granger, 1986; Rubanyi & Vanhoutte, 1986; Ohara *et al,* 1993). During normal metabolism UA is produced by XDH (Parks & Granger, 1986). However, under ischaemic conditions or oxidative stress, endothelial XDH is converted to XO (Rubanyi & Vanhoutte, 1986). XO activity is inhibited by NO and $ONOO^-$ (Ichimori *et al,* 1999; Lee *et al*, 2000). Therefore, UA production may be increased under conditions in which NO availability is reduced by the formation of $ONOO^-$. The major site of UA production in the cardiovascular system is the EC, as XO is primarily located within these cells (Jarasach *et al,* 1981; Nees *et al,* 1985; Jarasach *et al,* 1986). These cells release UA into the vascular lumen and abluminal compartment (Becker, 1993; Leyva *et al*, 1998).

1.11.1 UA solubility

UA is generally thought to be biologically unreactive in humans. However, it is poorly soluble and enhanced production and retention may be clinically manifest as crystal formation. This predisposes to the development of pathophysiological conditions such as gout or nephrolithiasis. UA accumulates in physiological fluids and tissues by several pathways, such as passive transudation from plasma, active transport as occurs in renal tubular cells, local generation by XO, and intracellular production from purine precursors. Although sparingly soluble in aqueous media, UA is insoluble in hydrophobic media, such as biological membranes and lipid phases. Therefore, UA is restricted to aqueous environments (Emerson, 1996). It is generally accepted that plasma UA saturation occurs at approximately 415μmol/l and that the concentration of

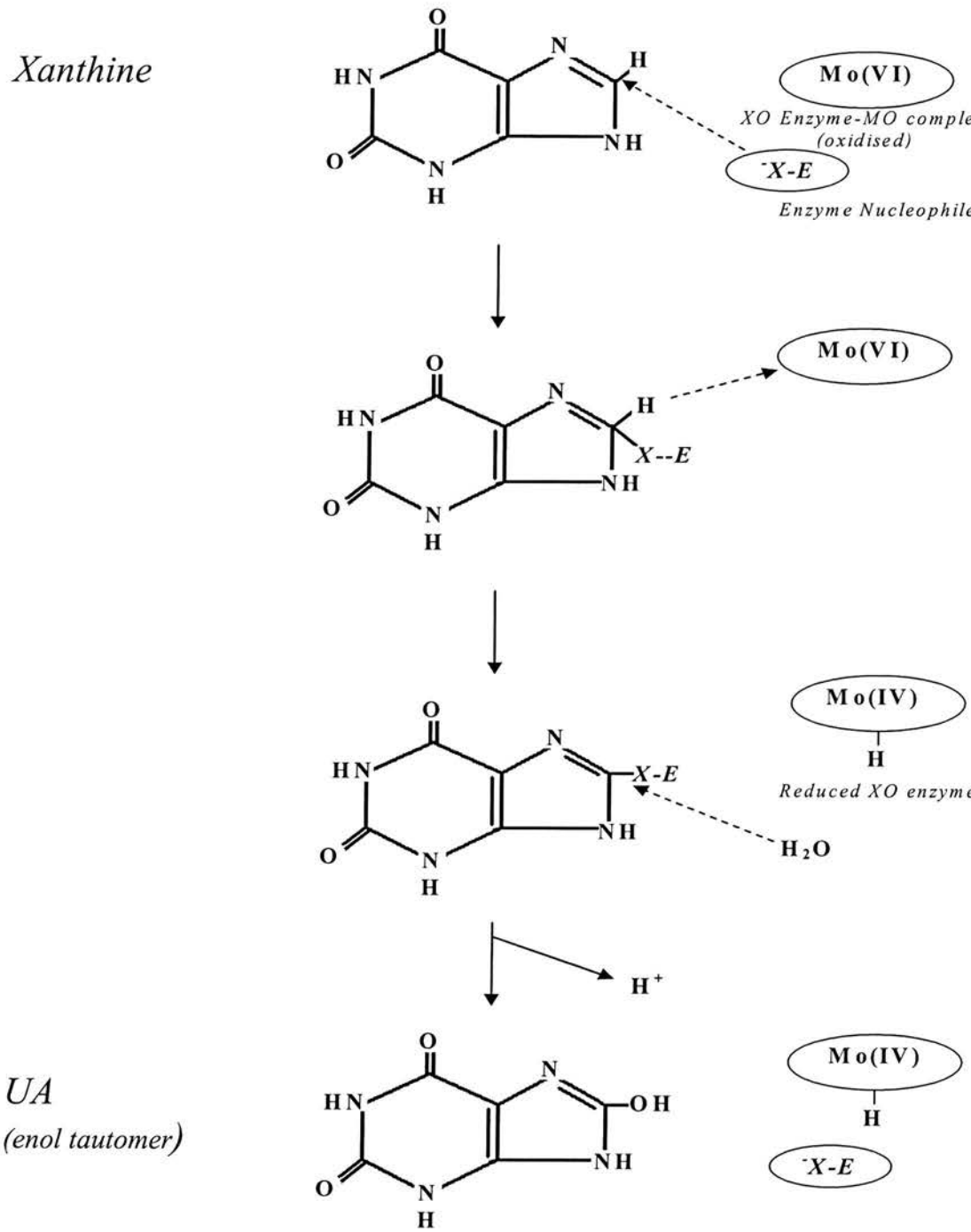


Figure 1.6. The formation of UA from Xanthine by XO. The reduced enzyme is subsequently re-oxidised by O_2 to yield H_2O_2.

UA in male blood approaches maximum solubility. As a result, humans with elevated concentrations are susceptible to crystal deposition and the resultant pathophysiological conditions (Emerson, 1996).

1.11.2 Comparative physiology

As previously mentioned UA is produced in human physiology as the end product of purine metabolism and undergoes renal excretion. UA is also the end-product of purine degradation and excreted in the urine of higher primates. The excess amino acid-nitrogen of terrestrial reptiles, birds and insects are also catabolised to UA. However, in other animals UA is further processed to more soluble products before excretion, as shown in Figure. 1.7. (Becker, 1993; Waring *et al*, 2000). Mammals, other than humans and higher primates, oxidise UA to their excretory product, allantoin, and fish and amphibians degrade this further prior to excretion. This occurs as these species express urate oxidase, the enzyme responsible for the oxidative degradation of UA. Consequently, the plasma levels of UA found in these species are less than 10% of those found in man. The concentration in human blood, 400μmol/l, is close to the maximum solubility. However, most mammals, such as rats or prosimians have markedly lower levels, less than 40μmol/l (Roch-Rammel & Peters, 1978).

In humans, the gene that codes for urate oxidase is located on chromosome 1. However, as the result of two non-sense mutations this is not expressed (Wu *et al,* 1992). This evolutionary process could suggest that elevated serum UA concentrations have an important role in human physiology (Ames *et al,* 1981). The increase in human UA levels that has occurred during the course of approximately 60 million years of evolution has been accompanied by a significant increase in life-span. It is possible that UA may be responsible for this. An interspecies comparison in mammals gives a positive correlation between plasma UA concentrations and species life-span. By way

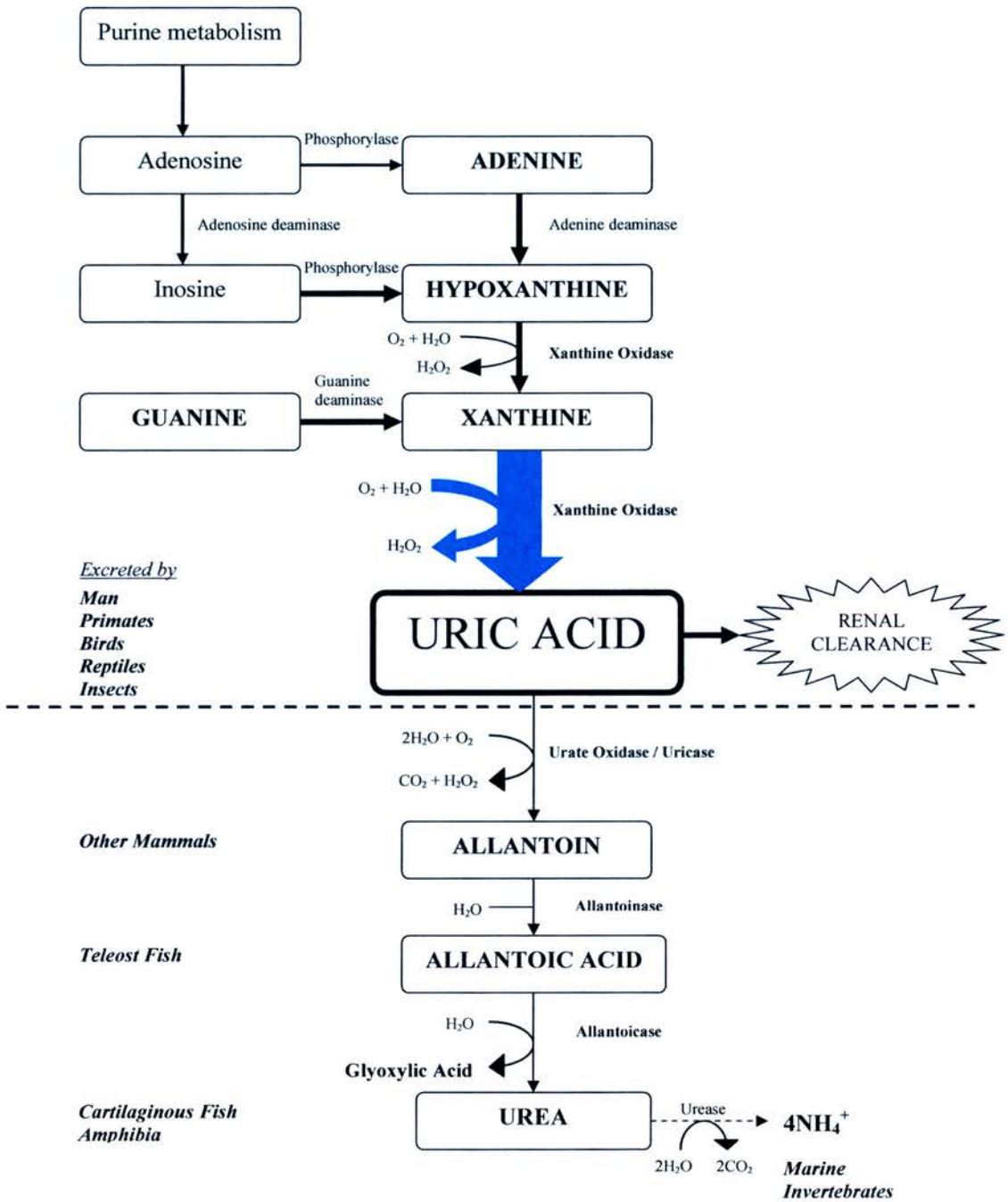


Figure 1.7. UA is produced in human physiology as the end product of purine metabolism and undergoes renal excretion. Other species that express uric oxidase further oxidise UA to produce more soluble excretory products.

of example, monkeys have higher plasma UA concentrations than prosimians, but less than longer-lived apes (Roch-Rammel & Peters, 1978). Oxidants and free radicals have a prominent role in the development of cellular aging and terminal diseases, such as cancer and cardiovascular disease, thus, the positive association could be attributed to the antioxidant ability of UA (Ames *et al,* 1981).

1.11.3 Is UA treated as a 'waste product' in human physiology?

In human physiology, UA may regarded as a waste product of purine metabolism. Purines such as adenine, guanine, HX, adenosine and inosine are readily removed from the blood and recycled into cellular purine pools. However, no such process occurs to recycle and reincorporate UA or xanthine (Manfredi & Holmes, 1985). Therefore, purines that are degraded to UA must be replaced by synthesis or by scavenging precursors, which results in the considerable loss of energy. However, despite the requirement to replenish the purine balance and the considerable loss of energy that occurs, dietary-ingested purines are not reabsorbed within the gastrointestinal tract but are converted exclusively to UA by mucosal XDH. This has been demonstrated as only UA appears in systemic circulation following purine consumption (Becker, 1993). Furthermore, as previously mentioned, 90% of filtered UA is reabsorbed from the proximal renal tubule. This physiological treatment of UA suggests that the human body does not treat it as a 'waste product' and may imply that it has a very important physiological role.

1.12 The association of UA with cardiovascular disease

Hyperuricaemia is usually defined as a serum UA concentration greater than 415μM in males and 357μM in females (Alderman *et al,* 1999; Fang & Alderman, 2000). These concentrations of UA are present in cardiovascular disease (Torun *et al,* 1998; Johnson

et al, 1999). Various epidemiological and clinical studies have linked these elevated UA concentrations to an increased risk of cardiovascular disease and it has been suggested that UA has a prominent role in the pathogenesis of this state (Fessel, 1980; Brand *et al*, 1985; Bonora *et al*, 1996; Persky *et al*, 1997; Bengtsson *et al,* 1998; Lehto *et al*, 1998; Ward, 1998; Liese *et al* 1999; Fang & Alderman, 2000; Johnson *et al,* 2003).

In contrast, several studies have suggested that the association between UA and cardiovascular disease is due to a non-causal relationship with pathophysiological conditions, in which cardiovascular risk is mediated by other risk factors. For example, under conditions of ischaemia and tissue hypoxia, vascular adenosine synthesis and release are upregulated to relax VSMC (Fredholm & Sollevi, 1986; Raatikainen *et al*, 1994). This adenosine may be rapidly degraded to UA by the EC (Kroll *et al,* 1992), as XO activity is increased under ischaemic conditions (Clark *et al,* 1990; Idstrom *et al,* 1990). Increased UA concentrations are also associated with insulin resistance syndromes, which result in the inhibition of insulin-mediated glucose metabolism and cause a significant increase in cardiovascular risk (Cleland *et al,* 1998). In this disease state, the effect of insulin upon glucose metabolism is impaired but the sensitivity of the renal tubule to insulin is not (Muscelli *et al,* 1996). Consequently, this condition could cause the accumulation of UA (Muscelli *et al,* 1996). Therefore, the link between UA and cardiovascular disease may arise from a non-causal relationship with conditions such as ischaemia, diabetes mellitus (Perry *et al,* 1995) and hypertension (Selby *et al,* 1990).

Despite the established clinical association between elevated concentrations of UA and cardiovascular disease, no causative mechanism has been proposed. Several, epidemiological studies have negated a predictive link between plasma UA levels and cardiovascular disease, suggesting that the relationship does not exist after accounting for co-existing risk factors (Reunanen *et al,* 1982; Iribarren *et al*, 1996; Wannamethee

et al, 1997; Culleton *et al,* 1999; Alderman, 2001; Alderman, 2002). Studies have associated elevated serum UA to hypertension, hypercholesterolaemia, reduced HDL cholesterol, hyperinsulinaemia, reduced physical activity, increased body mass index, and increased alcohol intake - all of which have a causal role in the development of cardiovascular disease (Patten *et al*, 1980; Tykarski, 1990; Agamagh *et al*, 1991; Lee *et al*, 1995; Alderman *et al*, 1999). Therefore, whilst elevated serum UA concentrations are *associated* with increased cardiovascular risk, it remains unclear whether UA is an *independent risk factor* or merely a predictor of cardiovascular disease and mortality, and whether it has a causative or protective role in cardiovascular disease.

1.13 The causative role of UA in cardiovascular disease

1.13.1 Evidence for the involvement of UA in the development of cardiovascular disease

UA has the potential to exert an antioxidant action and scavenges harmful radicals in the body. However, in conjunction with genetic or environmental factors, UA may be associated with vascular disease. Therapeutic interventions that lower serum UA concentrations have been reported to reduce the development of degenerative vascular disease in man (Emerson, 1979). UA has been found to enhance platelet and leukocyte adhesion to the vascular wall (Newland, 1975; Emerson, 1979; Boogaerts *et al,* 1983), and cause increased platelet lysis (Ginsberg *et al*, 1977), thus enhancing thrombus formation. UA can stimulate endothelial granulocyte adherence (Boogaerts *et al,* 1983) and the release of peroxide and $O_2^{\cdot-}$ from these cells (Boogaerts *et al,* 1983; Falasca *et al* 1993). UA may also enhance VSMC proliferation to contribute to arterial injury (Rao *et al,* 1991) and is strongly associated with the chronic inflammation that occurs in cardiovascular disease (Leyva *et al,* 1998). It may also traverse dysfunctional EC

and accumulate as crystals within atherosclerotic plaques (Kroll *et al,* 1992; Patetsios *et al,* 1996; Kanellis *et al,* 2003). Plaque samples have been found to contain increased concentrations of UA compared to control arteries (Suarna *et al,* 1995). Crystals consistent with urate have also been identified in diseased blood vessels (Lehto *et al,* 1998) and cardiac valves (Johnson *et al*, 1999). UA crystals are proinflammatory and stimulate neutrophils to release ROS, stimulate macrophages, and activate platelets and coagulation (Johnson *et al*, 1999). Therefore, UA could potentially have a role in the development of cardiovascular disease via its interaction with the cells present in the blood. This may indirectly enhance the pathophysiology of cardiovascular disease by increasing oxidative stress and exerting a further deleterious effect upon vascular function.

1.13.2 The pro-oxidant action of UA

The vital task of preventing the oxidative modification of LDL oxidation has been attributed to the major low molecular weight antioxidants within plasma, such as UA. However, under certain *in vitro* conditions UA may potentiate this (Sanguinetti *et al,* 2004). Many aqueous antioxidants have been found to promote metal-catalysed oxidation of LDL when the major endogenous antioxidant, α-tocopherol, is consumed (Abuja, 1999). UA shares this ability and can exert a pro-oxidant action when added after the partial depletion of α-tocopherol (Abuja, 1999; Bagnati *et al,* 1999). The pro-oxidant potential of UA is high compared to other conditional pro-oxidants, as it only requires the partial depletion of α-tocopherol, where as others only become pro-oxidant after most or all has been consumed (Abuja, 1999; Bagnati *et al,* 1999). Clinical data has also suggested that elevated UA production causes increased free radical formation (Anker *et al*, 1997; Leyva *et al*, 1997) and oxidative stress (Vasquez-Vivar *et al,* 1996). The oxidation of UA can also result in the generation of free radical metabolites (Maples & Mason, 1988). Furthermore, UA may react with the generated $ONOO^-$ to

produce a potentially damaging aminocarbonyl radical with the ability to cause further oxidative damage (Vasquez-Vivar *et al,* 1996; Santos *et al,* 1999). This is supported by *in vitro* experiments that demonstrated UA could potentiate $ONOO^-$-induced oxidation of LDL (Bowry & Stocker, 1993; Thomas *et al,* 1998). Therefore, UA has the potential to enhance the development of cardiovascular disease by potentiating the oxidative damage caused by transition metal ions (Smith *et al,* 1992; Garner *et al,* 1997) and $ONOO^-$ (Beckman & Koppenol, 1996).

1.13.3 UA and endothelial dysfunction

Evidence regarding the causal role of UA in endothelial dysfunction remains inconsistent (Alderman, 2002). Vascular EC are strong candidates for involvement in the pathological role of UA in vascular disease, as a result of their various functions and localisation. Chronic exposure of the endothelium to blood containing hyperuricaemic concentrations of UA, may trigger initial damage within the intimal layer of the vessel wall, and therefore, may represent a vital factor among the metabolic abnormalities responsible for pathologic damage and the of development vascular disease (Rathmann *et al,* 1993; Persky *et al*, 1997). *In vitro* and *in vivo* findings suggest that UA may contribute to endothelial dysfunction by inducing antiproliferative effects on endothelium and impairing NO production (Kanellis & Kang, 2005). Hyperuricaemic rats have impaired NO generation and decreased NO availability which can be reversed by lowering UA levels or by supplementation with l-arginine (Khosla *et al*, 2005). This suggests that UA can inhibit NOS and NO production to directly induce endothelial dysfunction (Khosla *et al*, 2005), and may provide a pathogenic mechanism by which UA can induce hypertension and cardiovascular disease.

In hyperuricaemic patients, endothelium-dependent vasodilatation is impaired when compared to control patients with normal UA levels but an elevated risk of cardiovascular disease (Mercuro *et al*, 2004). This is improved following a three-month therapeutic reduction of UA concentrations with allopurinol, which inhibits XO (Mercuro *et al*, 2004). Chronic heart failure is associated with reduced endothelial function, impaired peripheral blood flow, and is accompanied by hyperuricaemia. Treatment with allopurinol may improve endothelial function in this disease state (Farquharson *et al,* 1999; Farquharson *et al,* 2002). However, this may occur by preventing XO-mediated $O_2^{\cdot-}$ generation (Doehner *et al,* 2002). UA could also induce vascular dysfunction via an indirect deleterious action of UA upon the endothelium. For example, UA stimulates leukocyte adherence to the vascular endothelium (Boogaerts *et al,* 1983; Falasca *et al,* 1993), which may result in the inhibition of endothelial function. UA may also contribute to vascular injury by accumulating in the vascular wall to cause further damage (Kroll *et al,* 1992; Patetsios *et al,* 1996; Kanellis *et al,* 2003), and enhance the development of cardiovascular disease. However, a recent clinical study examined the direct effect of local or systemic administration of UA on vascular function in healthy adults (Waring *et al,* 2004). This study reported that UA administration caused a twofold increase in serum concentrations but did not acutely alter haemodynamic variables, basal forearm blood flow, or NO-dependent endothelial function (Waring *et al,* 2004).

1.14 The beneficial role of UA in cardiovascular disease

1.14.1 The antioxidant properties of UA

Although the role of elevated UA in endothelial dysfunction is uncertain, the well-established relationship between UA, cardiovascular disease and the associated risk factors may be misleading. Despite this association, UA is regarded as a major hydrophilic antioxidant in human physiology (Proctor, 1970; Ames *et al,* 1981; Becker, 1993; Nyyssonen *et al,* 1997; Glantzounis *et al,* 2005). Endogenous antioxidants constitute the first line of defence against oxidative stress and the damage caused by this. Therefore, hyperuricaemia may be a compensatory mechanism to counteract oxidative damage related to cardiovascular disease in humans.

Clinical studies have reported increased UA levels account for the compensatory increase in total oxidant-trapping capacity in patients with carotid atherosclerosis, (Nieto *et al*, 2000). This implies that UA may indeed have a cardioprotective role by increasing protection against mediated by oxidative stress. In ischaemic skeletal muscle HX and xanthine progressively accumulate to levels 15 times greater than normal (Idstrom *et al,* 1990). These are not converted to UA until reperfusion occurs. This occurs to combat the increased generation of ROS following reperfusion (Idstrom *et al,* 1990). Studies in perfused rat hindlimbs have proposed a similar mechanism in smooth muscle (Clark *et al,* 1990). Ischaemia and reperfusion also results in the local generation of large amounts of UA by both VSMC XO and EC XO (Parks & Granger, 1986; Castelli *et al,* 1995). This causes substantial increases in UA formation and the potential antioxidant capacity within the vessel wall (Rao *et al,* 1991). UA may prevent the impairment of endothelium-dependent vasodilatation caused by $O_2^{\cdot-}$ in the coronary system (Becker *et al,* 1991).

UA has the ability to exert a chain-breaking antioxidant action and is found in higher concentrations in the body than other potential non-enzymatic antioxidant compounds, such as AA (20-60μM), α-tocopherol (20-40μM) and GSH (1-10μM). UA contributes approximately 60% of the peroxyl-radical scavenging potential of the human serum (Wayner *et al,* 1987; Frei *et al,* 1988; Maxwell *et al*, 1997), and interacts with $ONOO^-$ to produce a stable, partially characterized, nitrate derivative and prevent any further damage to the vascular system (Skinner *et al,* 1998). UA is a major inhibitor of ozone-induced oxidation in plasma (Cross *et al,* 1992), scavenges singlet oxygen (Ames *et al,* 1981), and provides at least 15-20% of $OH^·$ radical scavenging activity (Thomas, 1992). At physiological concentrations, UA may prevent haem- and peroxide-catalysed peroxidation of erythrocyte membranes (Ames *et al,* 1981; Smith & Lawing, 1983). UA also chelates transition metal ions to prevent metal catalysed oxidation reactions (Davies *et al,* 1986).

UA also attenuates the oxidative modification of plasma lipids, lipoproteins and unsaturated fatty acids (Esterbauer *et al,* 1989; Bagnati *el,* 1999; Kopprasch *et a*l, 2000). UA can prevent the *in vitro* oxidative modification of n-LDL by preventing the consumption of endogenous LDL antioxidants. 100μM UA reduces the rate of α-tocopherol degradation by 50% and prevents PUFA oxidation (Schlotte *et al,* 1998). This indicates that UA is able to spare the oxidation of endogenous LDL antioxidants. UA can also act as a protective or sacrificial antioxidant for other hydrophilic antioxidants, such as AA (Sevanian *et al*, 1993; Lam *et al,* 1984). Therefore, UA has the potential to protect against the oxidative damage caused by oxidative stress and exert a cardioprotective effect in the pathophysiology of cardiovascular disease.

1.14.2 Mechanism of antioxidant action of UA

1.14.2.1 Metal Chelator

Transition metals are involved in the redox reactions that occur within the cells of the vascular wall (Garner *et al,* 1997), such as the metal-induced oxidative modification of LDL, and catalyse the generation of ROS (Aust *et al,* 1985). A potential role for UA in cardiovascular disease may be to protect against this by sequestering and inactivating transition metals, such as Fe^{2+} and Cu^{2+}, thus preventing reactions involving these redox-active metals. UA has metal-binding abilities and is able to form stable co-ordination complexes with transition metal ions, making them less redox active with regard to the initiation of oxidative reactions (Davies *et al,* 1986; Sevanian *et al*, 1991). On an equimolar basis, UA is approximately 10 times more effective than AA in preventing the Cu^{2+}-catalysed oxidative modification of LDL (Esterbauer *et al,* 1989). The metal-chelating abilities of UA also allow it to act as a sacrificial antioxidant to prevent the metal-catalysed oxidation of AA (Abuja *et al,* 1999). Furthermore, UA can regenerate AA that been oxidised (Lam *et al,* 1984; Sevanian *et al*, 1991) to and maintain the distinct antioxidant ability of this compound (Sevanian *et al*, 1985; Sevanian *et al,* 1991). Therefore, in view of the various mechanisms underlying cardiovascular disease, UA could have a beneficial role by chelating redox-active transition metal ions, thus inhibiting the initiation of metal-catalysed reactions, the oxidative modification of lipoproteins, and maintaining endogenous antioxidant levels.

1.14.2.2 Radical Scavenger

The radical scavenging ability of UA could confer a degree of protection against cardiovascular disease and the associated risk factors, such as hypercholesterolaemia, by preventing the deleterious actions of ROS. Under physiological conditions, 99% of

UA is present as urate (Simic & Jovanovic, 1989). Consequently, the antioxidant activity of UA is attributed the ability of this monoanion to scavenge free radicals via a one-electron redox process. Substitution of the hydroxy-group at the 8-position, with a halogen or phenyl group, results in the loss of the antioxidant ability of UA (Schlotte *et al,* 1998). Consequently, the 8-hydroxy-group may be essential for the stabilisation of the urate monoanion and is an important functional moiety for the antioxidant activity of UA (Schlotte *et al,* 1998). The one-electron oxidation of UA produces a free radical metabolite that contains an unpaired electron, located primarily on the five-membered ring of the purine structure (Maples & Mason, 1988). This free radical is a relatively stable compound due to the marked delocalisation of the unpaired electrons over the heterocyclic purine ring structure (redox potential = 0.59V, OH = 2.00V). The one-electron oxidation of UA by strong oxidants including hydroxyl ($OH^{\cdot}$), peroxyl, nitrite, and guanyl radicals produces a UA radical in neutral solution (Maples & Mason, 1988; Simic & Jovanovic, 1989; Vasquez-Vivar *et al,* 1996; Santos *et al,* 1999). UA reacts with hydroxyl radicals at a rate constant reaction of $7x10^9$ $M^{-1}s^{-1}$ (Becker, 1993) and significantly prevents the light emission evoked from luminol in the presence of various OH-generating systems in luminol-enhanced chemiluminescence (Howell & Wyngaarden, 1960; Becker *et al*, 1989; Radi *et al,* 1990). However, whilst UA may be regarded as a potent antioxidant for oxidants such as $OH^{\cdot}$ and $ONOO^{-}$ (Radi, *et al,* 2001) it does not react with weaker oxidants such as H_2O_2 or $O_2^{\cdot -}$ (Becker *et al*, 1989; Kaur & Halliwell, 1989). UA does not attenuate the reduction of cytochrome C (Becker *et al,* 1989) or nitrotetrazolium blue by $O_2^{\cdot -}$ radicals (Goshima *et al,* 1986). UA may only be oxidised in the presence of $O_2^{\cdot -}$ if the experimental conditions favour the secondary generation of $OH^{\cdot}$, for example, if H_2O_2 to was added to, or formed from, $O_2^{\cdot -}$ (Becker *et al,* 1989).

The UA-derived radical formed upon radical scavenging may further react with other antioxidants to regenerate UA (Abuja, 1999) and produce weaker oxidants, such as the ascorbate radical (redox potential = 0.28V) (Maples & Mason, 1988; Simic &

Jovanovic, 1989; Sevanian *et al,* 1991). However, in the absence of redox-partners to regenerate UA, the UA-derived radical may be further oxidised via two-electron transfer by oxidants such as H_2O_2 or HOCl. This forms allantoin and other products, such as allantoate, parabanic, oxaluric and oxalic acid (Kaur & Halliwell, 1989; Santos *et al,* 1999). Therefore, UA could potentially prevent the development of endothelial dysfunction and cardiovascular disease by scavenging free radicals to reduce oxidative stress, maintain NO-availability and prevent further oxidative damage to the vascular system.

1.14.3 The site of action of UA

Although the EC are a major site of UA production in the cardiovascular system (Becker, 1993), all tissue compartments, with the exception of the lipid phases, utilise UA as a replenishable antioxidant (Becker, 1993). UA has access to all extracellular fluid compartments, such as plasma, lymphatic, cerebrospinal, interstitial, synovial, intraocular, respiratory tract lining and amniotic fluid (Fleming *et al,* 1988; Hasegawa & Kuroda, 1989; Peden *et al,* 1990; Becker, 1993). Therefore, UA is not as strictly compartmentalised as enzymatic free radical scavengers, such as SOD and catalase. The transport of UA across cellular membranes may occur by non-ionic or anionic modes, although both forms are slow (Lucas-Heron & Fontenaille, 1979; Becker *et al,* 1989). Nevertheless, UA is avidly taken up into red blood cells, hepatocytes and cardiomyocytes, implying that it may act as an intracellular scavenger in cells other than those where it is produced. Therefore, in cardiovascular disease, other cells may take up the elevated concentrations of UA from the plasma to use as an intracellular antioxidant and protect against the oxidative stress and damage that are prominent this disease state. Further investigation is required to provide an increased understanding of the effect of UA upon vascular function. This would allow a clearer interpretation of

the potential role of the increased serum UA concentrations in endothelial dysfunction and cardiovascular disease.

1.15 Methodological approaches to assessing endothelial function

The study of isolated blood vessels eliminates the influence of surrounding tissue cells. This provides an understanding of the influence of the pharmacological properties of the vascular system upon blood flow. Comparing the reactivity of the blood vessels with and without endothelium is the only method that can demonstrate the involvement of EC in vascular function. For ethical reasons, the only technique that may be used to truly demonstrate endothelium-dependency in humans is the use isolated human blood vessels. However, the ability of EC to control the tone of vascular smooth muscle rapidly diminishes after death (Vedernikov *et al,* 1990), meaning that arteries must be isolated as early as possible and used immediately. This makes experiments difficult to plan and very restrictive. Therefore, the majority of isolated vessel studies are performed on animal models such as rabbits or rodents.

1.15.1 Perfusion myography

Resistance arteries within the microcirculation are chiefly responsible for the regulation of blood flow and capillary pressure (Furness & Marshall, 1974). Small arteries and arterioles are major determinants of peripheral vascular resistance, blood pressure and blood flow. Blood vessel control in the microcirculation also determines perfusion of the surrounding tissue. It has previously been suggested that alterations in the vascular structure and function of these vessels may have a prominent role in various

pathological conditions such as hypertension and congestive heart failure (Dunn & Gardiner, 1995). Therefore, an understanding of the physiological factors involved in the control of vascular tone in resistance arteries is essential. As a result of the causative or consequential implications of these arteries in vascular disease, the *in vitro* study of isolated resistance blood vessels is vital in investigating the aetiology of pathological conditions such as hyperuricaemia, hypertension and cardiovascular disease. However, due to their small size, the techniques used to perform *in vitro* studies on isolated resistance arteries are not as straightforward as the techniques used to study larger vessels such as the aorta.

Due to the difficulty in accurately measuring the biochemical generation of NO by the endothelium, a functional approach has been adopted. This is performed by assessing changes in vasomotor function in response to endothelium-dependent vasodilators, such as ACh, or by inhibiting NO synthesis. The methods most commonly used for examining the physiological and pharmacological properties of isolated resistance arteries are wire myography and perfusion, or pressurised, myography. Mulvany and Halpern developed the wire myography technique for the study of isolated resistance arteries. This involved the mounting of arterial ring preparations in an organ bath chamber by passing two wires through the lumen. Vasoactive responses were measured by recording the isometric force exerted by the vessels upon the wires (Mulvany & Halpern, 1976). This technique has since been modified to produce a method that better replicates physiological conditions for the study of isolated resistance vessels (Mulvany & Halpern, 1977; Duling *et al*, 1981; Sipkema & Westerhof, 1989; Halpern & Kelley, 1991; Hoogerwarf *et al*, 1992). In 1990 Van Bavel *et al* developed the technique of perfusion or pressurised myography (Van Bavel *et al*, 1990). This technique differs from wire myography as the vessel is mounted on size-matched cannula, perfused with an intraluminal solution and pressurised. The vasoactive responses are then measured as changes in intraluminal diameter.

1.15.2 The advantages of perfusion myography

Perfusion myograph may be more appropriate than techniques such as wire myography for the study of vascular and endothelial function in resistance arteries. The *in vitro* conditions under which experiments are performed alter the ability of agonists to induce vasoactive responses in isolated vessel preparation. The use of perfusion myography allows studies to be conducted in an experimental environment that mimics those found *in vivo*. Perfusion myography allows proportionately longer intact vessel segments to be mounted when compared to the ring segments commonly used in wire myography. This technique also allows longitudinal stretching of the mounted arterial segment to restore it to its physiological *in vivo* length, as the arteries undergo marked physical retraction upon dissection (Coats & Hillier, 1999). Longitudinal stretching of the arteries also increases the media surface area, thus increasing the availability and access of vasoactive drugs to the VSMC (Buss *et al*, 1994; Coats & Hillier, 1999).

Furthermore, this technique allows the arteries to be pressurised to passive or 'normal' *in vivo* conditions, which for the Wistar Kyoto rat (WKY) is 60mmHg (Halpern & Kelley, 1991). Pressurisation allows the arteries to assume a natural cylindrical shape as found *in vivo*. The pressurisation also allows the arteries under examination to more closely replicate the vasoactive responses produced *in vivo*, as pressure elevation significantly increases the sensitivity of WKY vessel concentration-responses to PE (Halpern & Kelley, 1991). It has previously been demonstrated in rat small mesenteric arteries that maximal sensitivity to methoxamine, an α-adrenoceptor agonist, occurs when transmural pressure is approximately 60mmHg or above (McPherson, 1992). This suggests that transmural pressure is a major influence on arterial function. However, in the wire myograph system the vessels may not be pressurised, but rather are stretched between two wires to place them under isometric tension instead. This results in the 'flattening' of the arterial ring segments and the loss of the natural cylindrical shape. Therefore, as a result of the influence exerted by transmural

pressure upon vasoactive responses, the technique used in these studies arguably provides a better model for assessing vascular function. Another advantage of this technique is that the cannulation of isolated vessels does not impose mechanical deformation upon the endothelium and vascular wall, and the endothelium is untouched upon cannulation. Therefore, there is less danger of endothelial disruption during mounting. This may occur with wire myography, as the wires on which the ring is mounted may damage the vascular wall. Endothelial damage would enhance vasoconstriction, impair endothelium-dependent vasodilatation, and have a significant influence upon any results produced

The use of perfusion myography also allows agents to be perfused through the vascular lumen and/or superfused abluminally through the organ bath. This may offer a more physiological method of study and may prevent the agents from interacting directly with the VSMC. Moreover, it may also prevent any interaction or inactivation of the vasoactive agents with any test solution, such as UA solution or LDL. Perfusion myography also prevents the contamination of the perfusate by the buffers or vasoactive agents, thus allowing the uncontaminated perfusate to be collected for analysis.

Finally, examining alterations in luminal or vascular diameter of the cannulated arteries may directly assess vascular function, rather than interpreting changes in the isometric force placed upon the wires on which an arterial ring segment is mounted. Therefore, assessment of the results produced using perfusion myography may be easier and of greater physiological relevance (Halpern & Osol, 1985).

1.16 Aims of Thesis

The role of UA in cardiovascular disease is still one of great debate. Several large population studies have identified the value of UA in predicting the risk of cardiovascular events. As a result, research has been conducted to determine possible mechanisms by which UA may have a direct or indirect effect upon the cardiovascular system. The specific role of UA has been difficult to identify because of its association with established cardiovascular risk factors. Currently, it is unclear whether UA has a protective or damaging effect in cardiovascular disease. Increased understanding of the effect of UA on the vascular system and the association of UA with elevated LDL concentrations may allow a clearer interpretation of the importance of increased serum UA concentrations in cardiovascular disease and the associated risk factors. This thesis aims to determine the role of UA in cardiovascular disease by investigating the direct action of UA upon endothelial and vascular function and examining the association between elevated UA and LDL in relation to their effects on the endothelium. In this way, this thesis will seek to investigate whether UA may exert a beneficial or harmful effect upon vascular function in isolated arteries exposed to conditions representative of those that predispose to the development of cardiovascular disease *in vivo*.

The general hypotheses of this thesis are:

- Direct exposure to elevated concentrations of UA is injurious to the endothelium and directly induces endothelial dysfunction, implying that UA has a direct causal role in development of cardiovascular disease and may be an independent risk factor in man.

- In conditions such as hypercholesterolaemia, which predispose to the development of cardiovascular disease, elevated UA concentrations prevent the development of vascular dysfunction. Thus, the association between UA and other cardiovascular risk

factors may represent a compensatory defence mechanism that serves to prevent and protect against the onset of cardiovascular disease.

The succeeding chapters aim to investigate these, and further elucidate the potential role of UA on endothelial function and dysfunction, by demonstrating that:

- The direct luminal exposure to increasing concentrations of UA impairs ACh-mediated vasodilatation in isolated rat mesenteric arteries.

- LDL will impair endothelial function in the perfusion myograph model using isolated rat mesenteric arteries.

- UA will mitigate the deleterious effects of LDL on endothelial function and will be able to do so as efficiently, on a molar basis, as other physiological radical-scavenging antioxidants.

- The beneficial effects of UA on LDL-induced endothelial dysfunction relate not only to its antioxidant effects but specifically to its potential to form an NO-dependent vasoactive species.

CHAPTER 2

METHODOLOGY

2. METHODS

2.1 Low-Density Lipoprotein solutions

2.1.1 Isolation of LDL

Venous blood samples were collected from healthy, non-smoking males following an overnight fast. Plasma was separated in heparinised tubes (Li Heparin 500 U/10 ml) by centrifugation at 1000 g for 10 minutes at 4°C, within 20 minutes of venepuncture. Lipoprotein sub-fractions were then isolated by a double spin rapid ultracentrifugation procedure similar to the methods described by other studies (Chung *et al,* 1980; Jacobs *et al,* 1990; MacDowell *et al,* 1995; Fontana *et al,* 1999; Woodburn *et al,* 1999). The density of the plasma was adjusted to 1.25g/ml by the addition of 2.823g of KBr to 7.65ml plasma. This was added to an 8ml Beckman polyallomer ultracentrifugation tube and over-layered with 0.35ml of 0.9% w/v NaCl solution (density=1.006g/ml). Ultracentrifugation was then performed in a Beckman Optima Ultracentrifuge (TL-100) at 420,000 g for 90 minutes at 5°C, using a Beckman 26° fixed angle rotor (MLA-80). The yellow lipoprotein layer was then removed by aspiration. This was added to a fresh 8ml Beckman ultracentrifugation tube and layered with an equal volume of KBr solution (density=1.21g/ml, 301mg KBr per ml lipoprotein). The tube was filled with 0.9% w/v NaCl and sealed. Ultracentrifugation was then performed, using the same equipment as before, at 420,000 g for 240 minutes at 5°C (Woodburn *et al,* 1999). The LDL sub-fractions were extracted by aspiration, placed on ice and purified immediately.

Isolation of the lipoprotein sub-fractions was carried out in the presence of a control tube containing Sudan Black, which stained the lipids to allow visualisation of the lipoprotein bands (Chapman *et al,* 1981; Gehrisch *et al,* 1989). This ensured that the LDL was extracted from the correct area of the ultracentrifuge tube. The separation of lipoprotein subfractions following ultracentrifugation is shown in Figure 2.1.

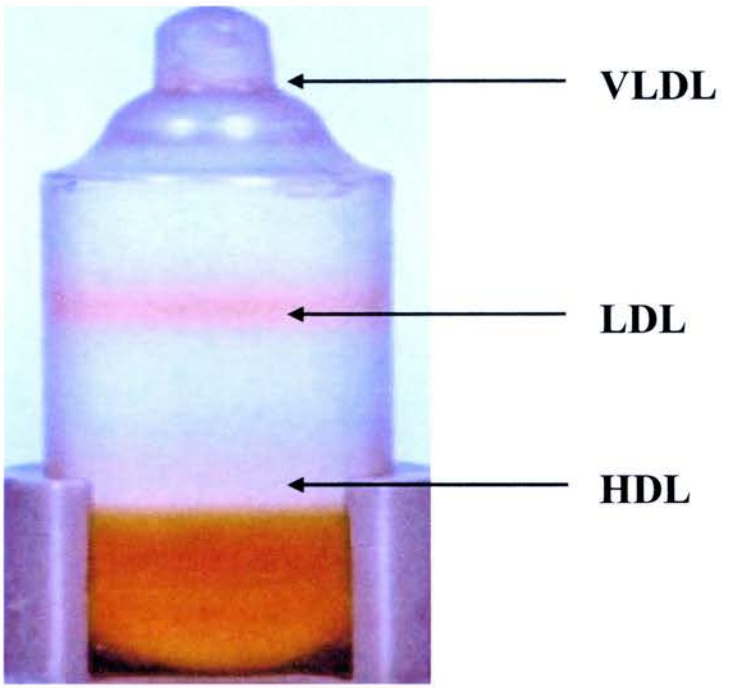


Figure 2.1. The separation of lipoprotein subfractions following density-gradient ultracentrifugation.

2.1.2 Purification of LDL

Small molecules, such as KBr, and water soluble antioxidants, such as AA and UA, were removed from the crude LDL sub-fractions by size exclusion chromatography using a Sephadex PD-10 column (Pharmacia Biotech, Bucks., UK) primed with phosphate-buffered solution (PBS, 0.01M, pH 7.4). LDL sub-fractions (1ml) were added to the column, followed by 1.5ml PBS and the elutant discarded. A further 2ml of PBS was added, and the elutant containing the purified LDL sub-fraction was collected. The purified LDL sub-fractions were then immediately placed on ice and the protein concentration assessed, or stored at –80°C as stock solutions for later use.

2.1.3 Determination of LDL protein concentration

The protein concentrations of the purified LDL samples were determined using a commercial kit a based on the Lowry precipitation assay (Sigma Chemical Company, Dorset, U.K.), which involved a spectrophotometric method with bovine serum albumin (BSA) as a standard (Lowry *et al*, 1951). This assay has been widely used in other studies to assess isolated lipoprotein concentrations (Frostegard *et al,* 1991; Lewis *et al*, 1997; Schlotte *et al,* 1998; Fontana *et al,* 1999; Santos *et al,* 1999).

2.1.3.1 Preparation of solutions

The Lowry reagent solution was prepared by adding 40ml of deionised water to a bottle of modified Lowry Reagent provided with the commercial kit. Folin & Ciocalteau's Phenol Reagent (FCPR) working solution was prepared by transferring 18ml of reagent solution to an amber glass bottle. The original reagent bottle was then rinsed with 80ml of deionised water and the rinsing added to give the working solution. The BSA protein standard solutions were prepared by adding 4.94ml deionised water to the vials

provided, giving a standard concentration of 400μg/ml. This solution was then refrigerated until required.

2.1.3.2 Procedure

Protein standards were prepared by diluting the protein standard solution with deionised water to a volume of 0.5ml in appropriately labelled eppendorf tubes, to give the following concentrations:

Protein Standard Solution (ml)	*Water (ml)*	*Protein Concentration (μg/ml)*
0.0	0.5	0
0.0625	0.4375	50
0.125	0.375	100
0.250	0.250	200
0.375	0.125	300
0.5	0.0	400

Samples of LDL were added to appropriately labelled tubes and diluted to a volume of 0.5ml:

LDL Solution (ml)	*Water (ml)*	*Dilution Factor*
0.0625	0.4375	1:8
0.03125	0.46875	1:16
0.015625	0.484375	1:32

50μl deoxycholate solution (DOC) was added to each standard and sample. After 10 minutes, 50μl trichloroacetic acid solution (TCA) was added to the standard and sample solutions. These were centrifuged for 10 minutes at 16,000 g to pellet the precipitates.

These pellets were dissolved with 0.5ml Lowry Reagent Solution and transferred to fresh tubes. The original tubes were rinsed with 0.5ml of de-ionised water and the rinsing added to the respective fresh tube. The solutions were allowed to stand at room temperature for 20 minutes, before 0.25ml of FCPR working solution was added to each tube. The tubes were then left to stand at room temperature for 30 minutes to allow the colour to develop. The absorbance of the standards was then measured vs. the blank (0μg/ml protein) at a wavelength of 595nm and a calibration curve constructed. The absorbance of the LDL samples was measured and the protein concentration determined using the calibration curve.

2.2 Functional studies using perfused isolated rat mesenteric arteries

2.2.1 Preparation of the rat mesenteric artery

Adult male WKY rats (250-300g) were obtained from the Biomedical Research Facility, Western General Hospital, Edinburgh, where they were maintained on standard chow and water. The animals were killed by cervical dislocation and the mesenteric bed immediately removed and pinned out in a silicone coated Petri dish (Sylgard, Dow-Corning, UK) containing cold Krebs-Ringer solution (Appendix 1).

Third order vessels of the superior mesenteric artery are found after the third arterial branch, a section of this artery (5-6mm in length, 250-350μm in diameter) (McPherson, 1992) was carefully dissected and cleaned of adhering perivascular fatty tissue under a dissection microscope (Zeiss, UK). To prevent damage to the artery, the adhering fat was carefully pulled away from the vessel to expose the connective membrane; this was cut to allow the removal of all adhering fat from the artery. The artery was then excised.

Care was taken to ensure that it was possible to differentiate between the two ends of the artery upon removal. This enabled the artery to be mounted in the correct orientation in the myograph, allowing any flow through the lumen to occur in the same direction as that of blood *in vivo* i.e. proximal to distal.

2.2.2 Mounting the isolated artery

The dissected artery was carefully transferred to a 10ml arteriograph chamber (Living Systems Instrumentation, Burlington, Vermont, USA) (Figure 2.2) containing oxygenated (95% O_2/ 5% CO_2) Krebs-Ringer solution. The arteriograph chamber contained two glass microcannulae, one of which was fixed (the efferent cannula) and another that was mounted on a manipulator (the afferent cannula) to facilitate positioning of the artery. The proximal end of the artery was mounted on the afferent cannula and secured using three knots of single strands of 4.0 silk suture that had previously been looped onto the cannula. The afferent cannula then attached to a miniature peristaltic pump connected to a pressure servo unit (P/S200, Living Systems Instrumentation, Burlington, Vermont USA) to facilitate the delivery and flow of the intraluminal solution through the arterial lumen (Figure 2.3). The artery was perfused slowly with Krebs-Ringer solution to remove any residual blood, ensuring that the intra-luminal pressure did not rise above 4-5mmHg to prevent endothelial damage. Once the arterial lumen was cleared of blood, the distal end was attached to the efferent cannula by the same means as before. If the dissected artery was not cannulated within 10 minutes a new vessel was dissected.

A stopcock was attached to the distal cannula and closed to allow the vessel to be pressurised. An intraluminal pressure of 60mmHg was achieved by slowly introducing Krebs-Ringer solution to the vascular lumen using the peristaltic pump. This was monitored using the pressure transducer attached to the afferent cannula. This pressure

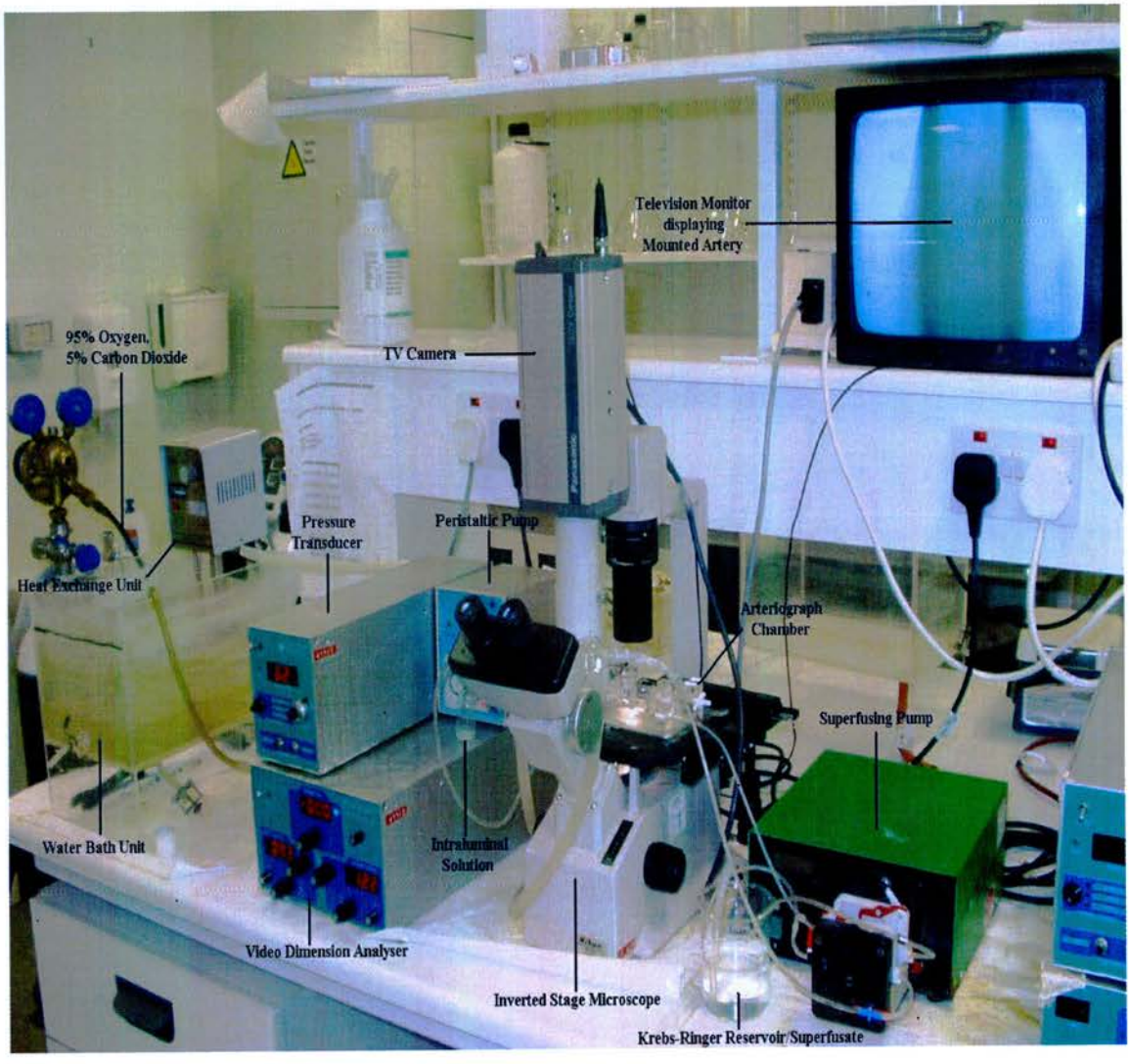


Figure 2.2. The arteriograph set-up used to perform perfusion myography.

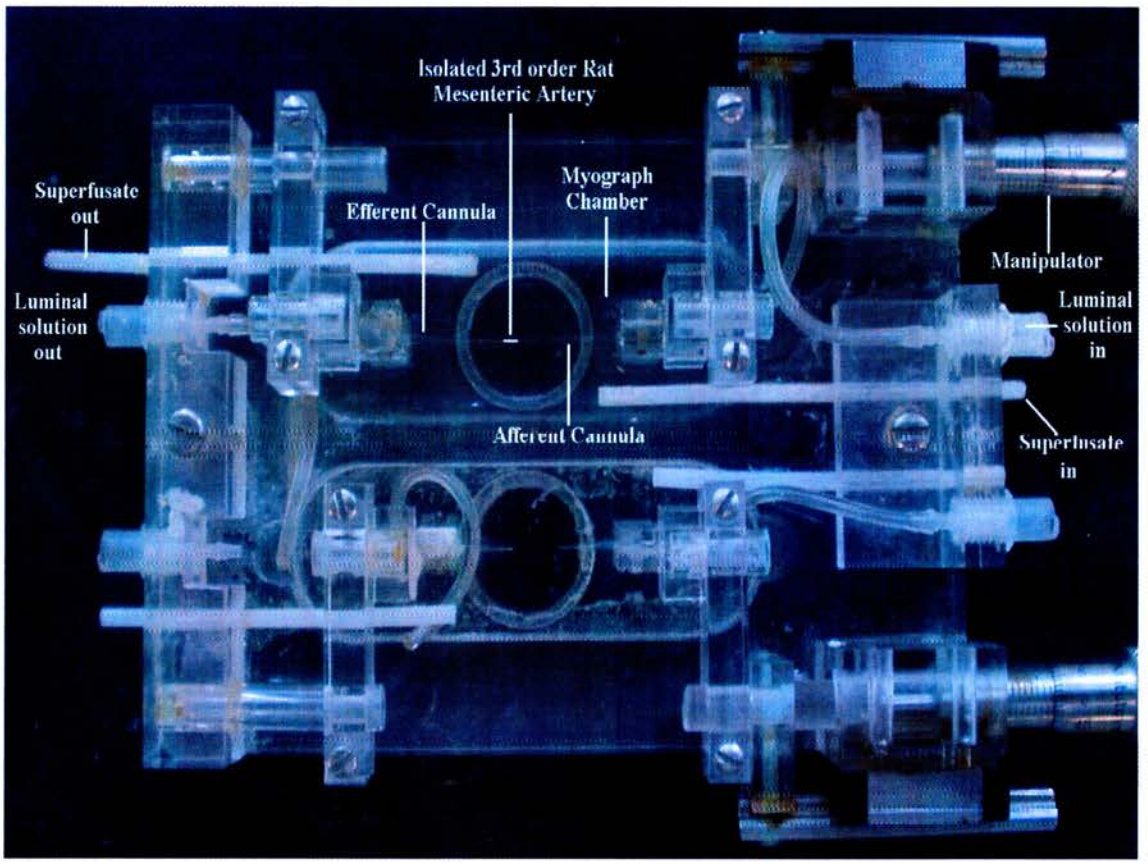


Figure 2.3. Arteriograph chamber containing isolated mesenteric artery.

is the physiological *in vivo* pressure in the animal model used (WKY) (Halpern & Kelley, 1991). Any buckling that developed in the vessel upon pressurisation was carefully removed by gently stretching the vessel using the micrometer attached to the proximal cannula. For the purpose of these experiments, the mounted arteries were stretched to 120% of their resting length to provide optimal experimental conditions (Coats & Hillier, 1999). Once pressurised, the artery was checked for leaks by changing the pressure servo from automatic to manual mode. If the pressure remained constant the mounted artery had no leaks. If the pressure fell, there was a leak in the vessel and perfusion circuit. If this occurred, the ties were further tightened or the artery removed, discarded and another vessel mounted.

The arteriograph chamber was then placed on an inverted stage microscope (Nikon TMS-F, Japan) with a monochrome TV camera (Burle, USA) attached to a viewing tube (Halpern & Osol, 1985), to allow the mounted arteries to be visualised on a television monitor (Burle, USA). The arteriograph chamber was connected to an oxygenated Krebs-Ringer reservoir. The mounted artery was superfused with this solution at a flow rate of $10ml/min^{-1}$ (Watson Marlow 505S; Falmouth UK) with a circulating volume of 30ml. The conditions within the arteriograph chamber were kept constant to maintain the vessel environment at $37^{o}C \pm 0.5$ and pH 7.4 ± 0.5. The temperature of the superfusate was maintained by passing the solution through a glass heat exchanger, which was warmed with circulating water from a water bath ($40^{o}C$) and a Peltier heat-exchange unit (MHE-1, Moor Instruments, Millwey, Axminter, Devon, UK). The temperature was measured using a digital thermometer held in close proximity to the mounted vessel and the pH was monitored using a basic pH meter (Dewar Instrument Company, USA).

2.2.3 General experimental protocol

For the purposes of this thesis, the term 'luminal perfusion' means that the vascular lumen was filled with a solution, pressurised and maintained in a non-flow state. This

was performed by opening the stopcock on the distal cannula and perfusing the arterial lumen with the intraluminal solution. The stopcock was then closed, luminal perfusion halted and the artery maintained in a **non-flow** state at an intraluminal pressure of 60 mmHg throughout the experiments. The vessels were allowed a 60 minute equilibration period before being contracted with high K^+ Krebs-Ringer solution (NaCl: 4.7mM; KCl 118mM) (Appendix 1), added extraluminally to the arteriograph via the Krebs-Ringer reservoir. The chamber was then washed for 10 minutes with Krebs-Ringer buffer to remove the high K^+ Krebs-Ringer solution and dilate the artery to its resting diameter. This process was repeated at 15 minute intervals until successive contractions remained constant, to ensure the vessel was responsive and maximal constriction was produced.

Following contraction with high K^+ Krebs-Ringer solution, the artery was washed with Krebs-Ringer solution and allowed to relax for 30 minutes. Concentration-response curves to phenylephrine (PE) ($1x10^{-9}$–$5x10^{-4}$M) were then performed. The drug was added cumulatively to the Krebs-Ringer reservoir and the arteries superfused with the modified solution. The arteries were exposed to each concentration of drug for 10 minutes and luminal diameter was measured immediately prior to the next drug addition. Contractions were then expressed as a 'percentage of maximal constriction to high K^+ Krebs-Ringer solution', and concentration-response curves constructed.

After washout and a 30 minute equilibration period, the arteries were preconstricted using EC_{80} PE. The vessels were allowed to equilibrate for 15 minutes and concentration-response curves to ACh ($1x10^{-9}$–$5x10^{-4}$M) and SNP ($1x10^{-9}$–$5x10^{-4}$M) performed, in a random order, by extraluminal cumulative addition, as previously described. Changes in intraluminal diameter were measured and expressed as 'percentage return towards resting luminal diameter' following preconstriction to PE. All concentration-response curves and 'best-fit' lines were produced using a non-linear regression analysis package (Biograph Version 1.0, Strathclyde University, UK).

Cumulative concentration-response curves were performed using a continuous reperfusion circuit. These experiments could be performed by switching off the superfusate and adding drugs directly to the arteriograph chamber. However, this method has several disadvantages; the O_2 within the chamber is consumed, resulting in anaerobic conditions, and a rapid and sustained decline in the chamber temperature occurs, both of which affect the vascular response to the vasoactive agents. Therefore, a continuous reperfusion circuit was used to maintain optimal conditions with in the vessel chamber.

2.2.4 Endothelial denudation

The most common approach to removing the endothelium in isolated blood vessels is to rub the inner surface with a small wire or hair (Osol *et al*, 1989; Megson *et al,* 1997). Resistance arteries are small and fragile making this technique difficult to apply. Chemical or enzymatic methods are difficult to control and may damage the adjacent VSMC, thus it cannot be determined if any alteration in vascular response is exclusively due the removal of the EC or the result of smooth muscle damage. An alternative method used for denuding the EC of resistance arteries is to perfuse the lumen with an air bubble (Ralevic *et al*, 1989; Bjorling *et al*, 1992; Falloon *et al,* 1993). For the purpose of this thesis, endothelial denudation of the mesenteric arteries was achieved using the 'air bubble technique' as described by Falloon *et al* (1993).

To do this, the pressure within the vessel was slowly reduced and the stopcock on the distal cannula opened. An air bubble of about one inch in length was introduced into the tubing attached to the proximal cannula. This was slowly moved down the tubing at a flow rate that produced an intraluminal pressure of 30-35mmHg. A series of 10-15 air bubbles were passed through the lumen of the vessel. To ensure any endothelial debris was removed, the flow of Krebs-Ringer solution through the lumen was continued for 2-3 minutes after all the air had passed. The stopcock was then closed and the pressure

within the vessel restored to 60mmHg. Endothelial denudation was confirmed by the failure of sub-maximally constricted vessel to respond to ACh (10 μM).

2.3 Functional studies using isolated rat aortic segments

2.3.1 Preparation of aortic ring segments

Adult male WKY rats (250-300g) were obtained from the Biomedical Research Facility, Western General Hospital, Edinburgh, where they were maintained on standard chow and water. The animals were killed by cervical dislocation. The aorta was immediately removed and pinned out in a silicone coated Petri dish (Sylgard, Dow Corning, UK) containing cold Krebs-Ringer solution. The isolated aorta was carefully cleaned of all adhering connective tissue and divided into 2 ring segments (2-3mm), one of which was then denuded.

The vessel rings were suspended between two intraluminal wires in a 10ml organ bath (Multi Tissue Bath System 700MO, Danish Myo Technology A/S, Copenhagen, Denmark) to record alterations in isometric tension. The aortic rings were bathed in oxygenated (95% O_2/ 5% CO_2) Krebs-Ringer solution at 37°C and placed under a resting tension of 1.5g by increasing the tension applied to the vessels in stepwise increments. The vessels were allowed to equilibrate for 60 minutes.

2.3.2 General experimental protocol

The aortic rings were contracted three times with high K^+ Krebs-Ringer solution (NaCl: 4.7mM; KCl: 118mM) to obtain maximum contraction. Following washout and an equilibration period, the rings were exposed to increasing concentrations of PE ($1x10^{-8}$–$1x10^{-5}$M). The drug was added cumulatively to the organ bath and the aortic rings were

exposed to each concentration of drug for 5 minutes. A concentration-response curve was constructed and a suitable concentration chosen to produce 80% contraction (EC_{80}).

The vessels were washed with Krebs-Ringer solution and allowed to equilibrate before being preconstricted with EC_{80} PE. Concentration-response curves to ACh ($1x10^{-8}$–$1x10^{-5}$M) were performed by cumulative addition as previously described. Changes in tension were measured and expressed as 'percentage return towards resting tension' prior to preconstriction with PE. Following washout, the aortic rings were allowed to relax. Test solutions were added to the organ bath and, following a 20 minute equilibration period, the rings were preconstricted with EC_{80} PE and vasodilatation to $1x10^{-5}$M ACh examined. Alterations in tension from the myography were processed by a MacLab/4e analogue digital converter and displayed through Chart™ software.

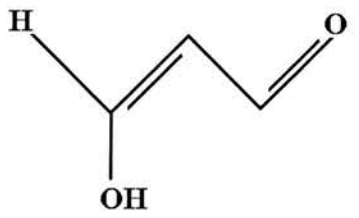
2.3.3 Endothelial Denudation

When required, aortic ring segments were denuded of their endothelium prior to mounting in the wire myograph system. Following dissection, the luminal surface of the aortic ring segment was rubbed to remove the endothelium (Megson *et al,* 1997). Endothelial denudation was confirmed by the failure of sub-maximally constricted vessels to respond to ACh (10 μM).

2.4 Determination of the oxidative status of LDL

The lipoprotein samples used in these studies are typical of the LDL found in human circulation (See Chapter 5). This should be unoxidised LDL as it is isolated from fresh human plasma and immediately used or stored at –80°C. To prevent auto-oxidation, 0.3mM EDTA was added to the solutions (Jacobs *et al*, 1990; Hayashi *et al,* 1994). However, to ensure that oxidative modification did not occur during the course of the

experiments, the oxidative status of the LDL samples before and after luminal perfusion was measured. This was performed using a commercial colourimetric assay kit (CN Biosciences, Nottingham, U.K.) that used a spectrophotometric method to measure 4-Hydroxynonenal (4-HNE) and malondialdehyde (MDA) as indices of lipid peroxidation (Esterbauer & Cheeseman, 1990). These have previously been used as indices of lipid peroxidation in several other studies examining the oxidative status of LDL *in vitro* (Jacobs *et al,* 1990; Janero, 1990; Frostegard *et al,* 1991; Galle *et al,* 1995; Pritchard *et al,* 1995; Schlotte *et al,* 1998; Vergnani *et al,* 2000).



MDA

4-HNE

Figure 2.4. The molecular structure of the aldehydes MDA and 4-HNE, which are used as an index to assess the oxidative status of the LDL samples in these experiments.

2.4.1 Preparation of solutions

18ml of 10.3mM N-methyl-2-phenylindole in acetonitrile was diluted by the addition of 6ml of 100% methanol. This was stored at 4°C. 200µl LDL samples were collected before and after perfusion through the vascular lumen, and immediately stored at –80°C. When performing the assay, samples were placed on ice and stored at 4°C.

2.4.2 Procedure

A working solution of 4-HNE was prepared by adding 1ml of 10mM 4-HNE solution to 99ml of Krebs-Ringer buffer, to give a final concentration of 100μM. Standards were then formed by adding 0-50μl of the working solution to Krebs-Ringer buffer to give a final volume of 200μl. This process was repeated using 10mM MDA solution to prepare MDA standards.

650μl of diluted N-methyl-2-phenylindole solution was added to a clean glass test-tube. 200μl of the standard were added to this solution and mixed by vortexing for 3-4 seconds. 150μl of methanesulphonic acid (15.4M) was added to the tube, the tube sealed tightly and the solution mixed. This solution was incubated in a water bath maintained at 45°C ± 1°C for 40 minutes. The standard samples were placed on ice and allowed to cool. The absorbance of the standards was then measured at 586nm and plotted against the corresponding aldehyde concentration to produce a calibration curve.

This experimental procedure was repeated using the LDL samples and the absorbency measured. This was compared to the calibration curve to determine the 4-HNE and MDA concentration in the LDL samples. This concentration was then multiplied by 5 to account for dilution during the assay. The concentration of MDA and 4-HNE present in the LDL samples before and after addition to the isolated vessels were compared and the percentage change in lipid peroxidation products calculated.

2.5 Analysis of Results

Techniques used for the analysis of the results for the individual studies are given in the 'Methods' section of the corresponding chapter. For the studies of mesenteric vessels all results are given as mean ± SEM of 8 independent experiments. For the studies of aortic

rings 6 independent experiments were performed. Statistical differences in concentration-response curves were analysed using a 2-factor ANOVA with replication and Bonferroni correction. The best fit concentration-response curves, and the concentration of agonist causing 50% (EC_{50}) responses for vasoconstriction and dilatation, were calculated using a non-linear regression analysis package (Biograph Version 1.0, Strathclyde University, UK). Estimated EC_{50} data were analysed using one-way ANOVA with Bonferroni correction. All other results were analysed using paired Student's *t*-test. The *P*-values quoted in the chapters are accepted as statistically significant when $P<0.05$. The statistical significance is indicated in the relevant figures, and the symbols used to represent points of significance are given in the corresponding figure legend.

2.5 Drug and Reagents

Key

Dilution

H_2O:	Diluted in water
H_2O/Kr.:	Diluted in water, then Krebs-Ringer buffer
Kr.:	Diluted in Krebs-Ringer buffer
Li_2CO_3 vehicle:	Diluted in Li_2CO_3 vehicle solution
Meth.:	Diluted in 100% methanol
Sal.:	Diluted in saline
Solution:	Supplied as a solution

Storage

day:	Prepared on the day of use
week:	Stored at 4°C and used within 1 week of preparation
month:	Stored at 4°C and used within 1 month of preparation
3 months:	Used within 3 months of preparation as advised
6 months:	Used within 6 months of preparation as advised

Drug	Dilution	Storage	Source
ACh	Kr	day	Sigma
AA	H_2O /Kr	day	Sigma
BSA	H_2O	day	Sigma
DOC	solution	3 months	Sigma
FCPR	H_2O	3 months	Sigma
GSH	H_2O /Kr	day	Sigma
Indomethacin	sal.	month	Sigma
Krebs buffer salts	H_2O	day	BDH
Li_2CO_3	H_2O/Dextrose solution	month	Sigma
Li_2CO_3 vehicle	Kr	day	-
Lowry reagent	H_2O	6 months	Sigma
L-NAME	Kr	week	Sigma
MDA	solution	day	CN Biosciences
Methanesulphonic acid	solution	month	CN Biosciences
N-methyl-2-phenylindole			
In acetonitrile	Meth.	day	CN Biosciences
PBS	H_2O	day	Pharmacia Biotech
PE	Kr	day	Sigma
SOD	Kr	day	Sigma
SNP	Kr	day	Sigma
TCA	solution	6 months	Sigma
UA	Li_2CO_3 vehicle	month	Sigma
UA solution	Kr	day	-
4-HNE	solution	day	CN Biosciences

A detailed description of the preparation of the various LDL, antioxidant and vehicle solutions is provided in the 'Methods' section of the relevant chapters.

All compressed gases were supplied by Linde.

Company Locations

BDH	-	Merck Ltd., Lutterworth, U.K.
CN Biosciences	-	Nottingham, U.K.
Linde	-	Linde Gas UK Ltd., West Bromich, U.K.
Sigma	-	Poole, U.K.
Pharmacia Biotech	-	Amersham Biosciences, Bucks., U.K.

CHAPTER 3

EXAMINATION OF THE ROLE OF NO IN THE VASCULAR FUNCTION OF ISOLATED RAT MESENTERIC ARTERIES

3.1 INTRODUCTION

It is well established that NO has a central role in ACh-induced endothelium-dependent vasodilatation in large vessels (Bellan *et al,* 1993; Shimokawa *et al,* 1996; Cohen *et al,* 1997). For example, chronic treatment with L-NAME caused a 10-fold reduction in cGMP and prevented ACh-induced vasodilatation in isolated rat aorta (Arnal *et al,* 1992; Henrion *et al,* 1996). However, the role of NO in vasodilatation to ACh in isolated rat mesenteric arteries is more controversial. In these arteries, muscarinic agonists such as ACh can stimulate the release of an uncharacterised EDHF that is neither prostacyclin nor NO (Chen *et al*, 1989; Taylor & Weston, 1988; Garland & MacPherson, 1992; Garland *et al*, 1995). Consequently, endothelium-dependent vasodilatation of isolated resistance arteries can be mediated by both NO and EDHF (Henrion *et al,* 1997). NO may not be the primary mediator of endothelium-dependent vasodilatation in isolated rodent arterioles, as L-NAME reduces vasodilatation to ACh by less than 50% (Dora *et al*, 1997). Similarly, this NOS inhibitor only caused a slight and non-significant reduction in the concentration-dependent vasodilatation to ACh isolated rat mesenteric arteries (Garland & MacPherson, 1992). Similarly, the COX inhibitor, indomethacin, which prevents the generation of prostacyclin, did not impair ACh-mediated vasodilatation (Garland & MacPherson, 1992; Henrion *et al,* 1997), although it significantly reduced dilatation in chronic L-NAME-treated vessels (Henrion *et al,* 1997). It has previously been demonstrated that agonist-mediated vasodilatation in rat mesenteric arteries is primarily due to the release of EDHF and the resultant hyperpolarisation of the VSMC (Dora *et al,* 1997; Edwards *et al*, 1998). It has been suggested that K^+ is the EDHF, and primary EDRF, released in rat mesenteric arteries following agonist stimulation with ACh (Wu *et al,* 1993; Edwards *et al*, 1998).

Therefore, whilst the use of perfusion myography arguably provides the most favourable model for assessing vascular function, the role of NO in ACh-induced vasodilatation in resistance arteries is uncertain. For that reason, the role of NO in the vascular function

of this experimental model must be investigated. This chapter aims to clarify the role of NO, prostacyclin, and subsequently, EDHF in isolated rat mesenteric arteries, by examining the effect of arterial perfusion with L-NAME and indomethacin on the vasoactive responses to PE, ACh and SNP. This chapter also intends to validate the use of perfusion myography in future studies examining the effect of various substances on NO-mediated vascular function. This will be achieved by demonstrating that:

1). NO is the major vasodilator released by the endothelium in response to ACh, demonstrated by the abolition of the vascular response to ACh by L-NAME, an eNOS inhibitor.

2). Inhibition of endothelial prostacyclin generation by indomethacin, will not have any significant effect upon vascular function in the isolated arteries.

3.) Perfusion myography using third order rat mesenteric arteries stimulated/activated with ACh is a suitable technique to examine NO-mediated vascular function.

3.2 METHODS

3.2.1 Preparation

Adult male Wistar Kyoto rats (250-300g) were killed, the mesenteric beds removed, and a segment of a third order arterial branch mounted in the perfusion myograph chamber as described in Chapter 2 (Section 2.2).

3.2.2 Experimental protocol

Concentration-response curves to PE, ACh and SNP were performed, as described in Chapter 2 (Section 2.2), when perfused with 100μM L-NAME, 10μM indomethacin, 100μM L-NAME + 10μM indomethacin, or control Krebs-Ringer buffer. This procedure was also repeated on endothelial denuded vessels perfused with Krebs-Ringer solution.

3.2.3 Intraluminal Solutions

1mM indomethacin stock solution was prepared by dissolving 0.179g in 500ml saline. Working solution (10μM) was prepared by diluting 1ml of the stock solution in 99ml Krebs-Ringer buffer on the day of use. 100μM L-NAME solution was prepared daily by dissolving 0.135g L-NAME in 500ml Krebs-Ringer solution to give a 1mM solution, then adding 1ml of this to a further 9ml of Krebs Ringer.

3.2.4 Statistical Analysis

Statistical analysis was performed to determine any significant difference in the results obtained for the various intraluminal solutions when compared to the physiological Krebs-Ringer solution.

3.3 RESULTS

3.3.1 Vasoconstrictor response to PE in the presence of L-NAME and/or indomethacin

The maximal response obtained when the intact lumen was perfused with Krebs-Ringer solution was 101.2 $\pm$ 2.5%, and 101.3 $\pm$ 2.1% when the endothelium was removed (Fig 3.1). Perfusion with 10μM indomethacin, 100μM L-NAME, or100μM L-NAME + 10μM indomethacin produced maximal responses to PE of 96.6 $\pm$ 1.2%, 99.4 $\pm$ 3.5% and 98.6 $\pm$ 3.3%, respectively. Vasoconstriction was significantly greater when all responses to $5x10^{-8}$ ($P<0.05$) and $1x10^{-7}$ PE ($P<0.01$) were compared to the physiological control.

3.3.2 Endothelium-dependent vasodilatation in the presence of L-NAME and/or indomethacin

ACh produced a maximal vasodilatation of 100.5 $\pm$ 2.1% in endothelium-intact arteries perfused with physiological Krebs-Ringer solution (Fig 3.2). Vasodilatation when perfused with 100μM L-NAME (maximal = 42.3 $\pm$ 3.2%) or 100μM L-NAME + 10μM indomethacin (maximal = 36.3 $\pm$ 3.6%) were similar, but significantly lower than the responses when perfused the physiological control or 10μM indomethacin only ($1x10^{-8}$-$5x10^{-4}$, $P<0.01$). Vasodilatation to ACh when perfused with 10μM indomethacin was similar to the physiological control. All responses produced by endothelium-intact arteries were significantly greater than those obtained following endothelial denudation ($P<0.01$), as this abolished vasodilatation to give a maximal response of 4.3 $\pm$ 5.5%.

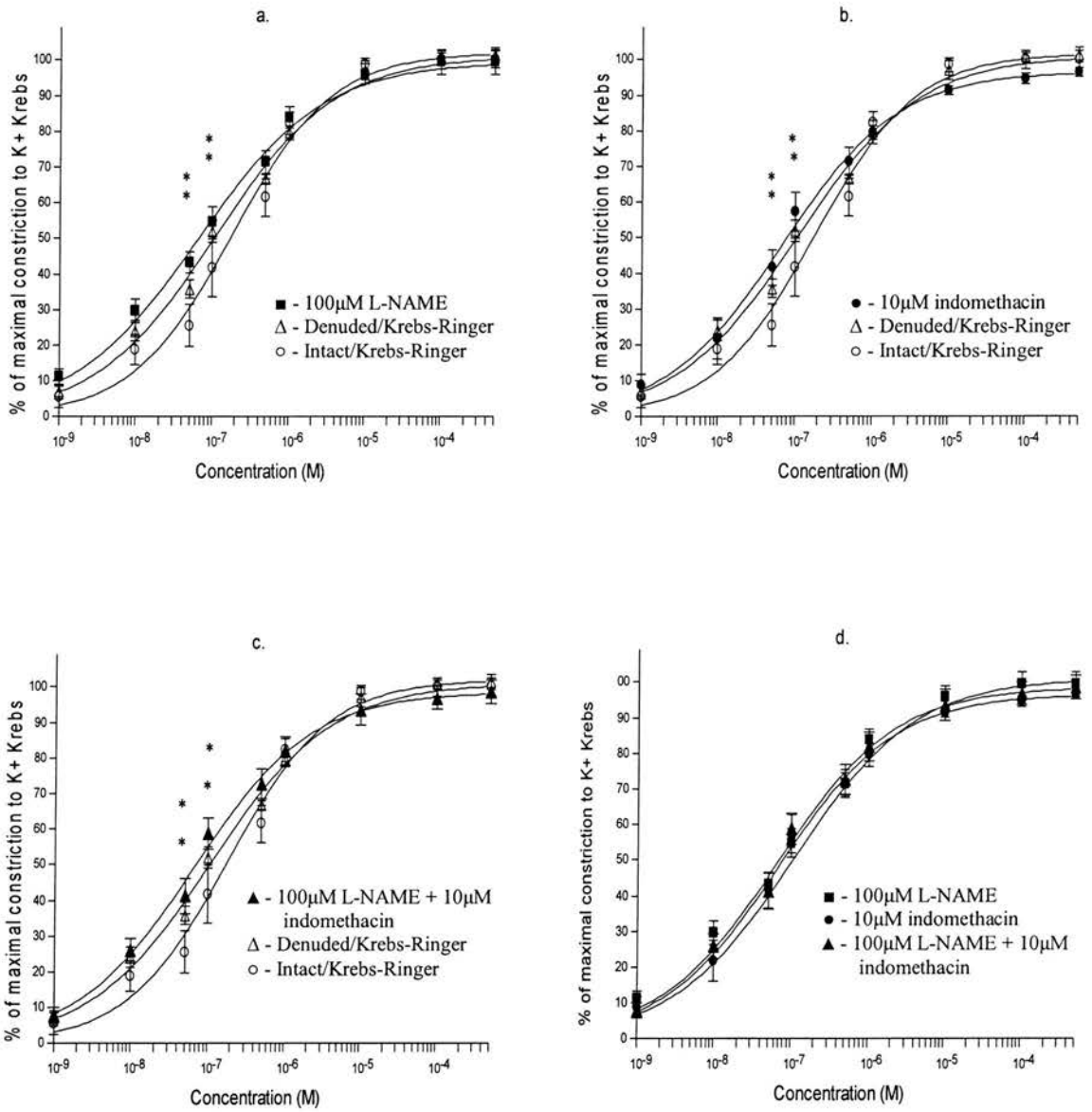


Figure 3.1. The effect of luminal perfusion with L-NAME and/or indomethacin on vasoconstriction to PE in isolated rat mesenteric arteries: a.) 100μM L-NAME, b.) 10μM indomethacin, and c.) 100μM L-NAME + 10μM indomethacin compared to intact or denuded vessels perfused with Krebs-Ringer solution. *$p<0.05$ vs. intact vessels (2-factor ANOVA with replication and Bonferroni correction, n=8). d.) compares the effect of L-NAME, indomethacin, and L-NAME + indomethacin. All values are the mean $\pm$ SEM (n=8).

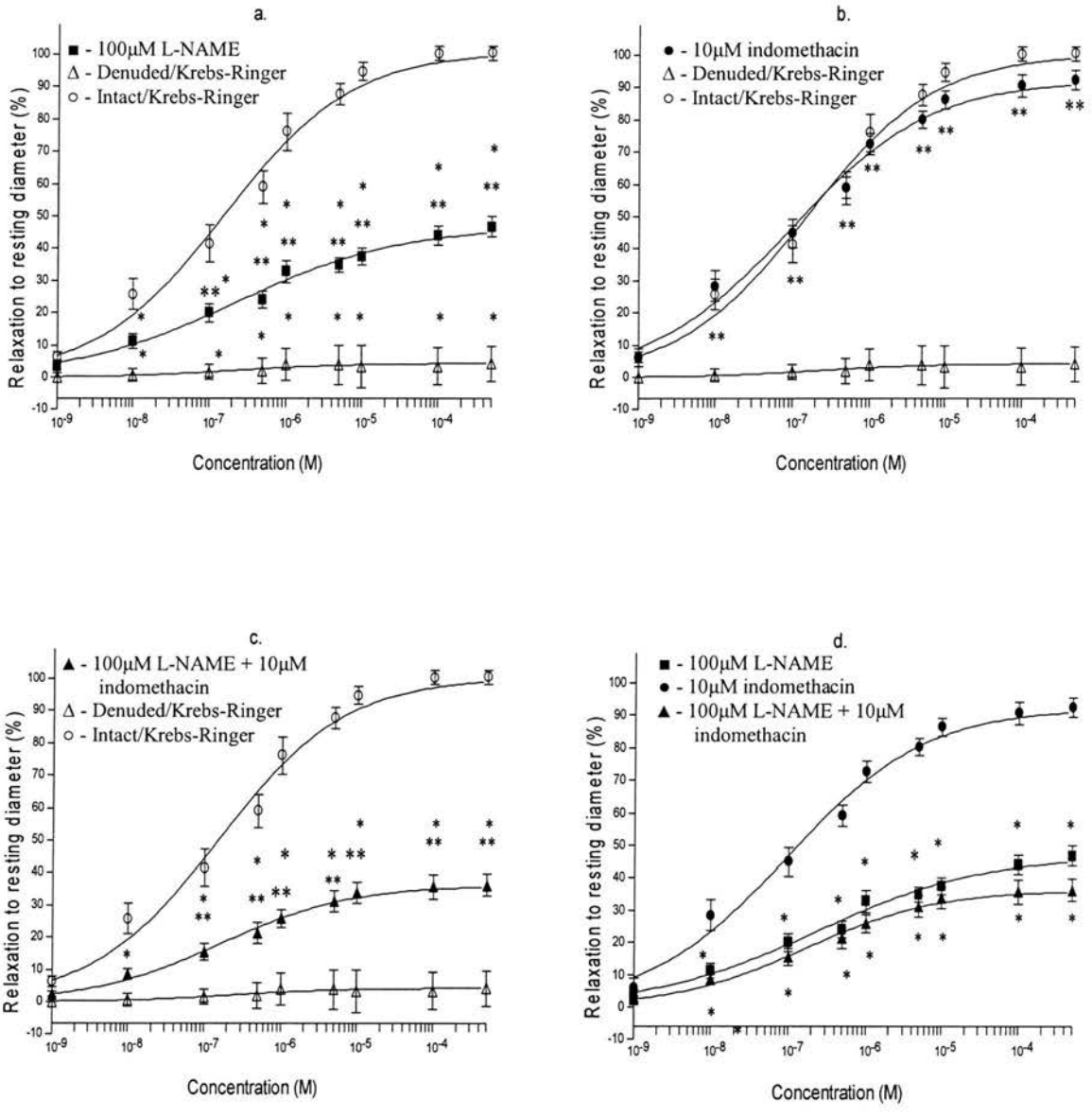


Figure 3.2. The effect of luminal perfusion with L-NAME and/or indomethacin on vasodilatation to ACh in isolated rat mesenteric arteries: a.) 100μM L-NAME, b.) 10μM indomethacin, and c.) 100μM L-NAME + 10μM indomethacin compared to intact or denuded vessels perfused with Krebs-Ringer solution. *$p<0.01$ vs. intact vessels, **$p<0.01$ vs. denuded vessels (2-factor ANOVA with replication and Bonferroni correction, n=8). d.) compares the effect of L-NAME, indomethacin, and L-NAME + indomethacin. *$p<0.01$ vs. indomethacin (2-factor ANOVA with replication and Bonferroni correction, n=8). All values are the mean ± SEM.

3.3.3 Endothelium-independent vasodilatation to SNP in the presence of L-NAME and/or indomethacin

All responses to SNP were similar when the vessels were perfused with Krebs-Ringer solution, 10μM indomethacin, 100μM L-NAME, 100μM L-NAME + 10μM indomethacin or following endothelial denudation (Fig 3.3).

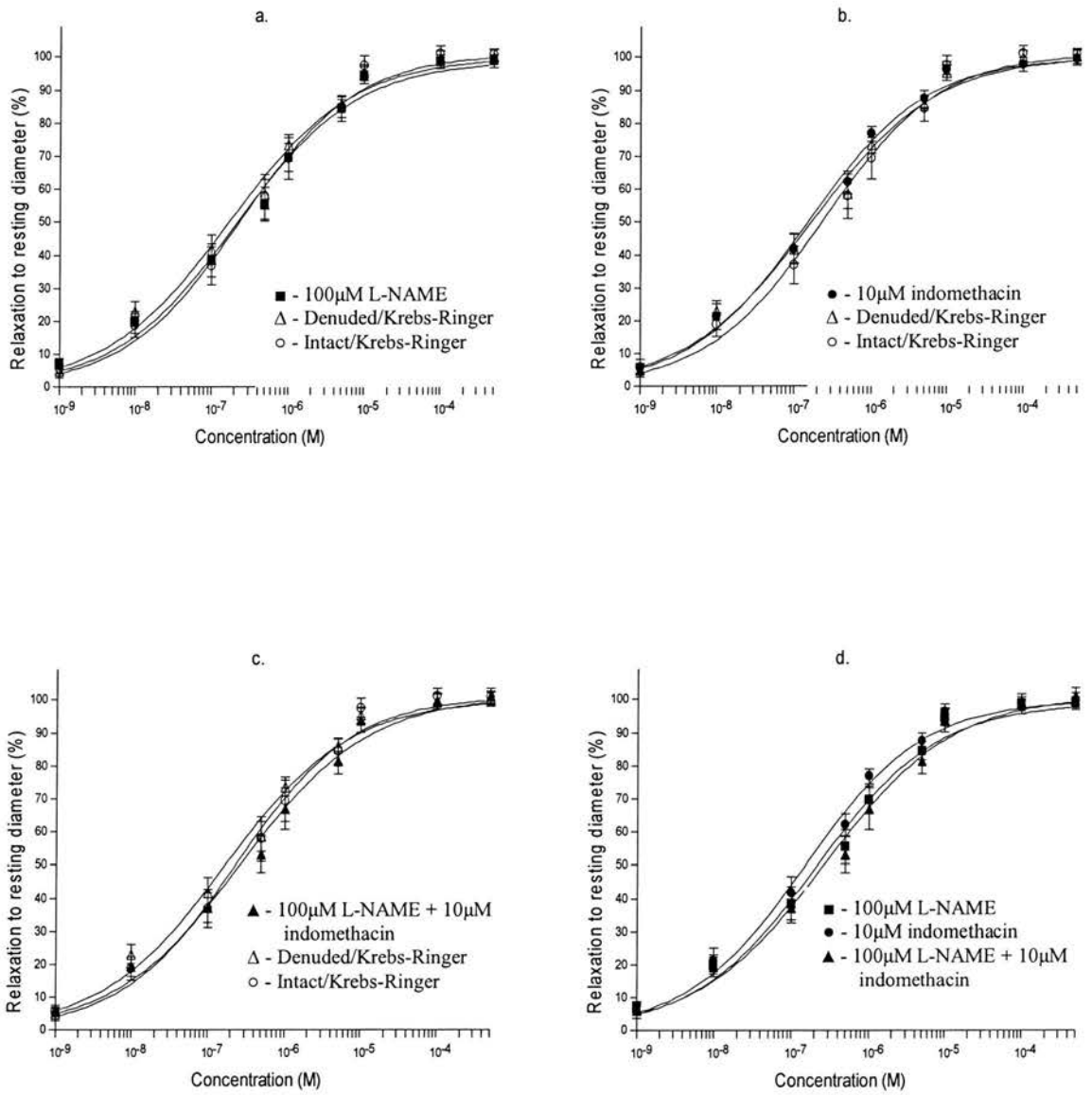


Figure 3.3. The effect of luminal perfusion with L-NAME and/or indomethacin on vasodilatation to SNP in isolated rat mesenteric arteries: a.) 100μM L-NAME, b.) 10μM indomethacin, and c.) 100μM L-NAME + 10μM indomethacin compared to intact or denuded vessels perfused with Krebs-Ringer solution. d.) compares the effect of L-NAME, indomethacin, and L-NAME + indomethacin. All values are the mean ± SEM (n=8).

3.4 DISCUSSION

Luminal perfusion with L-NAME (100μM) and/or indomethacin (10μM) only enhanced vasoconstriction to PE at lower concentrations. Perfusion with L-NAME significantly inhibited vasodilatation to ACh in isolated rat mesenteric arteries. Indomethacin did not significantly alter the response to ACh. The inhibition of ACh-induced vasodilatation following luminal perfusion with L-NAME + indomethacin was not significantly different from the inhibition produced when perfused with L-NAME only. Vasodilatation to SNP was unaffected by luminal perfusion with L-NAME and/or indomethacin.

3.4.1 The effect of L-NAME and/or indomethacin on vasoconstriction to PE

PE is a sympathomimetic amine that causes vasoconstriction by acting directly upon VSMC. PE-mediated vasoconstriction occurs via the activation of the α_1-adrenoceptors, although blood vessels also possess α_2-receptors that may cause vasoconstriction. These G-protein-coupled receptors are coupled to PLC, which converts PIP_2 to IP_3, causing the release of stored intracellular Ca^{2+}. This combines with calmodulin to activate MLCK, which phosphorylates myosin allowing it to interact with actin and initiate the contractile process. Activation of these receptors constricts arteries, arterioles, and veins resulting in increased peripheral resistance, reduced venous capacity, and consequently, increased arterial blood pressure

Vasoconstriction to PE was largely unaffected by perfusion with the various solutions in the current studies. The constriction produced was calculated and expressed as a percentage of the maximum achieved with KCl. However, it should be noted that the

vasoconstriction to KCl was also performed in the presence of the various test solutions. Consequently, this could mask any changes that the various solutions had on the maximal responses produced. Nevertheless, at lower concentrations, endothelial denudation or luminal perfusion with L-NAME and/or indomethacin significantly enhanced vasoconstriction (Fig 3.1). It has previously been reported that the suppression of NO by L-NAME can enhance agonist-induced vasoconstriction (Moncada *et al*, 1991) and augment vasoconstriction to PE in rat mesenteric arteries (Dora *et al,* 2000). The results produced following endothelial denudation or the inhibition of NOS activity suggest that the endothelium and NO synthesis confer a degree of protection against agonist-induced vasoconstriction. This may occur as a compensatory defence mechanism to prevent exaggerated vasoconstriction and disorders such as vasospasm (Dora *et al,* 1997).

3.4.2 The role of NO in endothelium-dependent vasodilatation to ACh

Due to difficulties in assessing the biochemical generation of NO, research into the role of the endothelium and NO in the vasculature is conducted using a functional approach. Studies using larger vessels such as the aorta are commonly performed using wire myography. In such vessels, vasodilatation and repolarisation to ACh can be fully accounted for by NO, with little contribution from EDHF (Cohen *et al,* 1997). Perfusion myography is technically demanding but may better reflect the *in vivo* properties of arteries when assessing the impact of various conditions on endothelial function *in vitro* (Halpern & Kelley, 1991; Buss *et al*, 1994; Coats & Hillier, 1999). However, studies performed by this method use smaller resistance arteries and the role of NO in ACh- and agonist-induced vasodilatation in these arteries is questionable. Therefore, before this experimental model could be regarded as a suitable technique for the assessment NO-mediated vascular function, it had to be demonstrated that NO was the primary vasodilator released in response to ACh. The interpretation of the vascular

responses to ACh is complicated (Chowienczyk *et al,* 1993; Chowienczyk *et al,* 1995). ACh acts on the G-protein-coupled M_2 or M_3 muscarinic receptors on the EC to stimulate the release of EDRF, and can induce endothelium-dependent smooth muscle relaxation that is associated with membrane hyperpolarisation (Bolton *et al,* 1984; Chen *et al*, 1989; Feletou & Vanhoutte, 1988; Brayden 1990; McPherson & Angus, 1991; Garland & McPherson, 1992; Rand & Garland, 1992). However, in the rat aorta and pulmonary artery, oxyhaemoglobin (ox-Hb) or methylene blue impair vasodilatation but do not block this hyperpolarisation. This indicates that a distinct EDHF is released from the EC at the same time as NO, although the latter is the cause of the endothelium-dependent vasodilatation (Chen *et al,* 1989; Taylor & Weston, 1988; Garland & McPherson, 1992).

In rabbit and rat aortic rings, ACh-induced vasodilatation is almost exclusively mediated by the endothelial release of NO (Bellan *et al,* 1993; Shimokawa *et al,* 1996). However, in smaller vessels, such as rat mesenteric arteries, vasodilatation to ACh may be produced by NO-independent EDHF-mediated increases in VSMC membrane potential (Waldron & Garland, 1994; Shimokawa *et al,* 1996; Dora *et al,* 1997; Dora *et al,* 2000). This could account for more than 75% of the vasodilatation induced (Waldron & Garland, 1994). In canine mesenteric arteries, ACh induces cell membrane hyperpolarisation in VSMC by the endothelial release of a diffusible substance. This is NO-independent and has been was attributed to the release of EDHF (Komori *et al,* 1988). It has previously been reported that NO had little or no involvement in ACh-induced dilatation in rat mesenteric arteries of a similar size and breed to those used in the current study (WKY), as treatment with L-NAME only caused a slight decrease in vasodilatation (Garland & McPherson, 1992).

However, the results of this study contradict these findings. The current study demonstrates that luminal perfusion of the isolated rat mesenteric artery with 100μM L-NAME solution produces a maximal ACh-mediated vasodilatation 42.3%. This is a

reduction of 57.7% when compared to the physiological control. L-NAME is an l-arginine analogue that binds within the active site of eNOS to make the haem iron unreactive to oxygen and prevent the oxidation l-arginine and the formation of NO (Abu-Soud *et al,* 1994). The current results indicate that approximately 60% of vasodilatation to ACh occurred via the generation and release of NO. These results imply that NO is the primary EDRF released to this agonist in the experimental model used. This is supported by previous studies that demonstrated a significant impairment of ACh-induced vasodilatation in isolated rat mesenteric arteries following acute and chronic L-NAME (Plane *et al,* 1992; Dowell *et al,* 1996). Several other studies have also demonstrated that the inhibition NO synthesis markedly impairs, but does not abolish ACh-induced vasodilatation in rat mesenteric arteries (Moore *et al,* 1990; Khan *et al,* 1992; Le Marquer-Domagala & Finet, 1997; Kimura & Nishio, 1999). The removal of NO by haemoglobin (Hb) and methylene blue also markedly attenuated vasodilatation to ACh in this experimental preparation (Furchgott *et al,* 1987, Randall & Hilley, 1988). The contradictory results produced between this study and those previous could be due to the experimental size of isolated vessels used (Dora *et al,* 2000), as the relative contributions of NO to *in vitro* vasodilatation by ACh may vary in vessels of different sizes from the rat mesenteric vascular bed (Hwa *et al,* 1994).

Nevertheless, the results of the current study suggest that NO is the primary EDRF released following agonist stimulation with ACh in preconstricted rat mesenteric arteries. These results support the use of perfusion myography, with this isolated vessel preparation, to examine NO-mediated vascular function. This experimental model allows isolated vessel studies to be conducted under conditions that are more reflective of those found *in vivo* when compared to the use of isometric ring or strip preparations. The experimental advantages that this technique offers for assessing vascular function have previously been cited in Chapter 1 (Section 1.14.2).

3.4.3 The role of prostacyclin in endothelium-dependent vasodilatation to ACh

Activation of EC by ACh increases intracellular Ca^{2+} and activates PLA. This liberates arachadonic acid from membrane phospholipids, which can then be metabolised to prostacyclin via the enzyme COX-1, a constitutive enzyme found in most cells. Indomethacin is a COX inhibitor that is relatively selective for COX-1. This prevents the conversion of arachadonic acid to cyclic endoperoxides, thus preventing the biosynthesis of vasoactive prostaglandins or thromboxanes. The inability of indomethacin (10μM) to inhibit dilatation demonstrates that COX-derived mediators such as prostacyclin do not make a significant contribution to ACh-mediated vasodilatation in the current study. This is in agreement with other studies that have reported the failure of indomethacin to inhibit vasodilatation to ACh (Chen *et al,* 1989; Feletou & Vanhoutte, 1998). ACh-induced vasodilatation in rabbit aortic ring segments (Andrews *et al,* 1987) and rat small mesenteric arteries were unaffected by 10μM indomethacin (Garland & MacPherson, 1992). However, whilst acute indomethacin (10μM) did not significantly alter the responses in this experiment, it caused a significant reduction in flow-induced dilatation in chronic L-NAME-treated isolated rat mesenteric arteries (Henrion *et al,* 1997). Therefore, COX-prostacyclin may have a greater role in endothelium-dependent vasodilatation following the inhibition or abolition of NO. Nevertheless, the anti-platelet properties of prostacyclin are thought to have a greater contribution to its role in human physiology than its vasoactive capabilities (Radomski *et al,* 1987; Luscher & Barton, 1997).

3.4.4 Implication for the role of EDHF in endothelium-dependent vasodilatation to ACh

Perfusion of the arteries with L-NAME + indomethacin inhibits the synthesis of both NO and COX-derived mediators. Therefore, the results produced indirectly imply that EDHF accounts for approximately 36% of ACh-induced vasodilatation in this experimental model. It has been demonstrated that ACh stimulates vasodilatation via endothelium-dependent increases in VSMC K^+ conductance and raising external K^+ concentrations will reduce dilatation to ACh by 50% (Chen *et al* 1989). However, the current study suggests that ACh-mediated vasodilatation of the rat mesenteric artery primarily depends on the stimulated release of NO by NO synthase. Nevertheless, while the results demonstrate that EDHF is not the primary EDRF released in this experimental model, they do imply that it makes a significant contribution to ACh-induced vasodilatation in the current experimental model. This EDHF may be released to ACh to act synergistically with NO and produce vasodilatation via different mechanisms in isolated rat mesenteric arteries (Waldron & Garland, 1994).

3.4.5 Endothelium-independent vasodilatation to SNP

Denudation or perfusion of the isolated arteries with L-NAME and/or indomethacin had no significant effect upon vasodilatation when compared to the physiological control. SNP is an exogenous NO-donor and acts directly upon the VSMC to produce vasodilatation by mimicking the action of endogenous NO (Ignarro *et al,* 1981). SNP releases NO that enters directly into the VSMC where it may either form a reactive nitrosothiol intermediate or act alone to activate intracellular sGC and increase in cGMP levels. This decreases intracellular Ca^{2+} and activates PKG to prevent VSMC constriction and induce vasodilatation. There was no significant alteration in the

responses to SNP compared to the physiological control, demonstrating that, as expected, eNOS- and COX-inhibitors had no significant effect upon dilatation to this agent.

3.4.6 Summary

ACh caused concentration-dependent vasodilatation in isolated rat small mesenteric arteries previously constricted with PE. This was totally abolished by endothelial denudation. The significant reduction of the vascular response (>50%) to ACh by L-NAME (100μM), an eNOS inhibitor, demonstrates that NO is the major vasodilator released by the endothelium to ACh in rat mesenteric arteries. The contribution of prostacyclin to vasodilatation in this experimental model is minimal, as the response to ACh is not inhibited following luminal perfusion with indomethacin (10μM). The remainder of the endothelium-dependent vasodilatation most likely occurs via hyperpolarisation to EDHF, based on the residual dilatation produced to ACh following luminal perfusion with L-NAME + indomethacin. Vasodilatation to SNP was unaffected by prior exposure to L-NAME and/or indomethacin. The results of this study establish NO as the major vasodilator released by the endothelium in response to ACh (Fig 3.4). This in turn demonstrates that the experimental model and technique employed is suitable for the examination of NO-mediated vascular function.

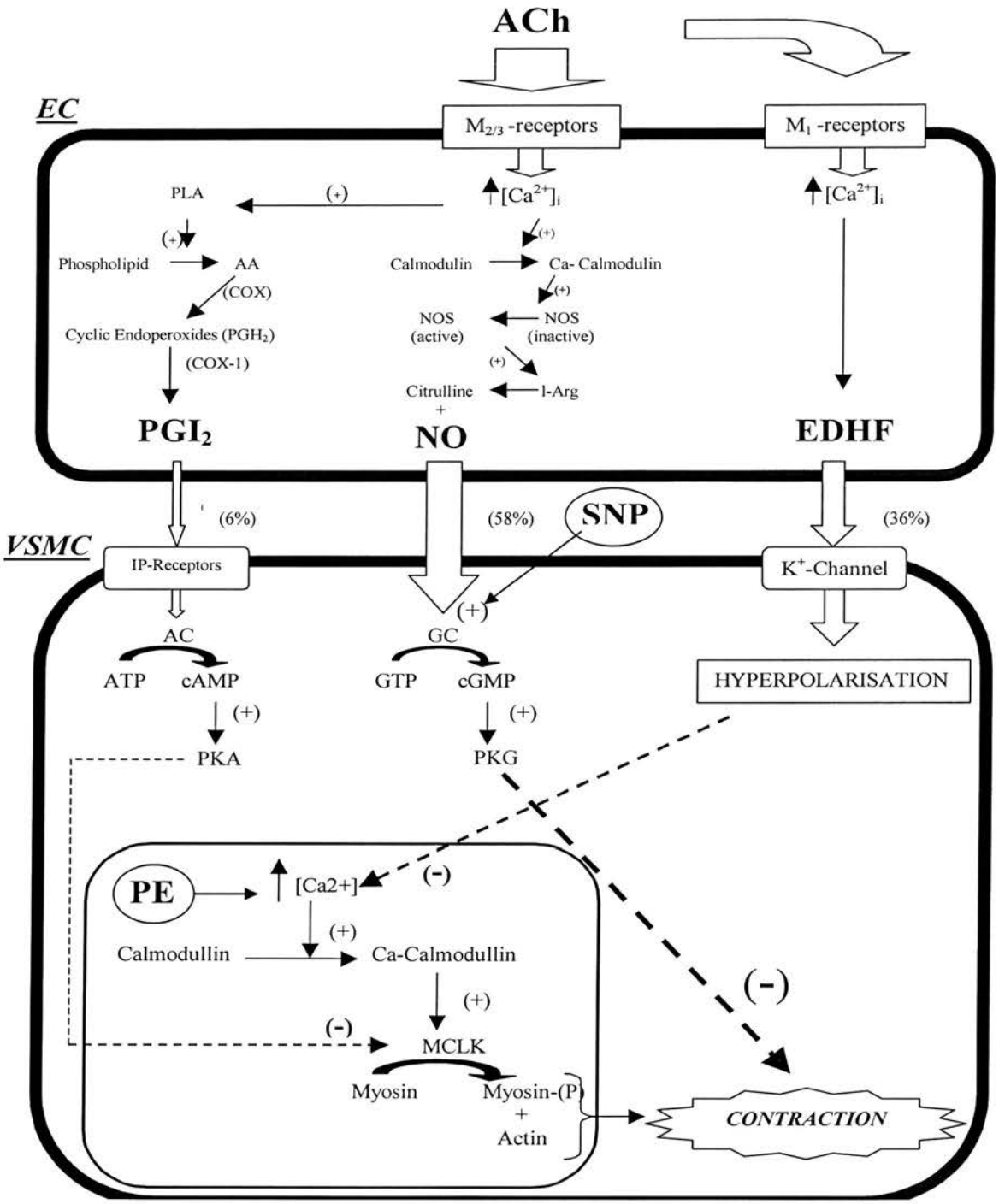


Figure 3.4. Relaxation of VSMC to EDRF released from rat mesenteric EC in response to ACh. NO increases cGMP to activate PKG and oppose agonist-induced increases in $[Ca^{2+}]i$. EDHF causes hyperpolarisation, thus preventing voltage-gated Ca^{2+}-channels from opening and inhibiting the intracellular concentration increase. PGI_2 increases cAMP to activate PKA and inhibit MCLK to inhibit contraction and induce relaxation.

CHAPTER 4

THE EFFECT OF LUMINAL PERFUSION WITH URIC ACID ON ENDOTHELIAL AND VASCULAR FUNCTION

4.1 INTRODUCTION

Having demonstrated the role of NO in mesenteric vascular function in the previous chapter, the relationship between UA and endothelial dysfunction can now be investigated. Endothelial dysfunction can promote the onset of cardiovascular disease by many mechanisms and is characteristically found in the blood vessels of patients with cardiovascular risk factors such as hypertension, type II diabetes, hypercholesterolaemia and atherosclerosis (D'Angelo *et al,* 1978; Ross, 1993; Blann *et al,* 1995; Celermajer, 1997; Wilkinson & Cockroft, 1998).

These disease states are frequently accompanied by an elevation in the serum UA level (Lehto *et al,* 1998; Ward, 1998; Fang & Alderman, 2000; Alderman, 2002). However, the role of this elevated UA in cardiovascular disease has yet to be established. Several studies suggest that UA is an independent risk factor for the development of cardiovascular disease (Bengtsson *et al,* 1998; Brand *et al*, 1985; Bonora *et al*, 1996; Fang & Alderman, 2000; Fessel, 1980; Lehto *et al*, 1998; Liese *et al*, 1999; Persky *et al*, 1997; Ward, 1998; Johnson *et al,* 2003), whilst others have concluded that this relationship is secondary to the association between UA and other cardiovascular risk factors such as obesity, hypertension, hypercholesterolaemia and increased triglyceride levels (Reunanen *et al,* 1982; Iribarren *et al*, 1996; Wannamethee *et al,* 1997; Culleton *et al,* 1999; Alderman, 2001; Alderman, 2002).

Evidence regarding the causal role of UA in endothelial dysfunction remains inconsistent (Alderman, 2002). The functional impairment of the endothelium provides a potential means by which UA could directly contribute to the pathogenesis of cardiovascular disease. Elevated serum UA may constitute a novel risk factor for endothelial dysfunction. Impaired endothelial-dependent vasodilatation is present in hyperuricaemic patients in the absence of any overt cardiovascular disease (Kato *et al,* 2005) and increasing serum UA concentrations, even in the physiological range, reduce

flow-mediated dilatation in healthy human subjects (Edrogan *et al,* 2005). Clinical studies performed using angiography (Crouse *et al,* 1987) or carotid ultrasound (Schneidau *et al,* 1989; Vigna *et al,* 1992) demonstrated a linear relationship between elevated UA concentrations and cardiovascular disease. The infusion of UA into the brachial artery of healthy volunteers impairs endothelium-dependent vasodilatation (Waring *et al,* 2000), and reducing serum UA concentrations has been reported to prevent the development of degenerative vascular disease (Emerson, 1979). This may also restore endothelium-dependent vasodilatation in hyperuricaemic patients (Farquharson *et al,* 1999; Mercuro *et al,* 2004; Baldus *et al,* 2005). Furthermore, *in vitro* and *in vivo* studies have demonstrated that UA can impair endothelial NO production, thus inhibiting endothelium-dependent vasodilatation (Kanellis & Kang, 2005; Khosla *et al,* 2005). In pre-eclampsia, a disease characterised by endothelial injury, the plasma UA concentration correlates with the increased markers for endothelial damage such as thrombomodullin and endothelin-1 (Clark *et al,* 1992; Hsu *et al,* 1993; Garrone & Broso, 1997; Leyva *et al*, 1998). Therefore, UA could be directly injurious to the endothelial lining of the vascular system and, as a result, could have a direct role in the pathogenesis of cardiovascular disease.

This chapter investigates whether acute UA elevation has a deleterious action on the endothelium by examining the direct effect of acute luminal exposure to various concentrations of UA upon vascular function. The hypotheses for this study are:

1). UA is an independent risk factor with a direct role in the pathogenesis of cardiovascular disease, hence the reported correlation between elevated UA concentrations and cardiovascular mortality.

2). UA is injurious to the endothelium and directly induces endothelial dysfunction, thereby inhibiting the vascular response to endothelial-dependent vasodilatory agents, such as ACh, to cause severe functional impairment.

3). The extent of endothelial injury is related to the concentration of UA to which the vessel is exposed. This effect will be concentration-dependent and manifest at physiological concentrations seen in human extracellular fluids.

4). Although UA is sparingly soluble in aqueous solution, it can be delivered directly into the arterial lumen after being dissolved in Li_2CO_3, which will not influence vascular function.

4.2 METHODS

4.2.1 Preparation

Adult male WKY rats (250-300g) were killed, the mesenteric beds removed, and a segment of a third order arterial branch mounted in the perfusion myograph chamber as described in Chapter 2 (Section 2.2).

4.2.2 Experimental protocol

Concentration-response curves to PE, ACh and SNP were performed, as described in Chapter 2 (Section 2.2), when perfused with 200μM, 400μM or 600μM UA solution, control Krebs-Ringer buffer, or the vehicle Li_2CO_3 solution.

4.2.3 Intraluminal Solutions

UA stock was made using Li_2CO_3 solution as the vehicle. The vehicle Li_2CO_3 stock was made by dissolving 1.6mg/ml Li_2CO_3 in a mixture of distilled water: 5% dextrose solution (1:4). 2g/ml of UA were then dissolved in the vehicle solution to give a stock solution of 11.9mM. 16.6ml/l, 33.3ml/l and 49.9ml of stock were then added to Krebs-Ringer solution to give 200μM, 400μM and 600μM UA solution respectively. For the purpose of the control vehicle solution, 49.9ml/l of Li_2CO_3 stock was added to Krebs-Ringer solution. This was used as it was the concentration of Li_2CO_3 present in the 600μM UA solution (216μM).

4.2.4 Statistical Analysis

Statistical analysis was performed to determine any significant concentration-dependent difference between the results obtained for the various UA solutions, and any significant difference when compared to the physiological control and vehicle Li_2CO_3 solutions. To determine the effect of the vehicle solution on vascular function, the results for the Li_2CO_3 solution and the physiological Krebs-Ringer buffer were compared.

4.3 RESULTS

4.3.1 The effect of UA on vascular contractility to PE

All responses to PE were similar when the vessels were perfused with 200μM, 400μM or 600μM UA, physiological control or Li_2CO_3 vehicle solution (Fig. 4.1).

4.3.2 The effect of UA on endothelium-dependent responses to ACh

The concentration-dependent responses to ACh were similar when the arterial lumen was perfused with 200μM, 400μM or 600μM UA solution (Fig. 4.2). The responses were also similar to those obtained when the arteries were perfused with the vehicle Li_2CO_3 solution. The ACh-induced vasodilatation when perfused with the various concentrations of UA appeared to be reduced when compared with the physiological control. However, only the reduced response to $1x10^{-6}$M ACh when perfused with 200μM UA was found to be statistically significant ($P<0.03$). There was no statistically significant difference in the calculated EC_{50} values (Table 4.1).

4.3.3 The effect of UA on endothelium-independent vasodilatation to SNP

Concentration-dependent vasodilatation to SNP was similar when the arteries were perfused with the physiological, vehicle or UA solutions (Fig. 4.3).

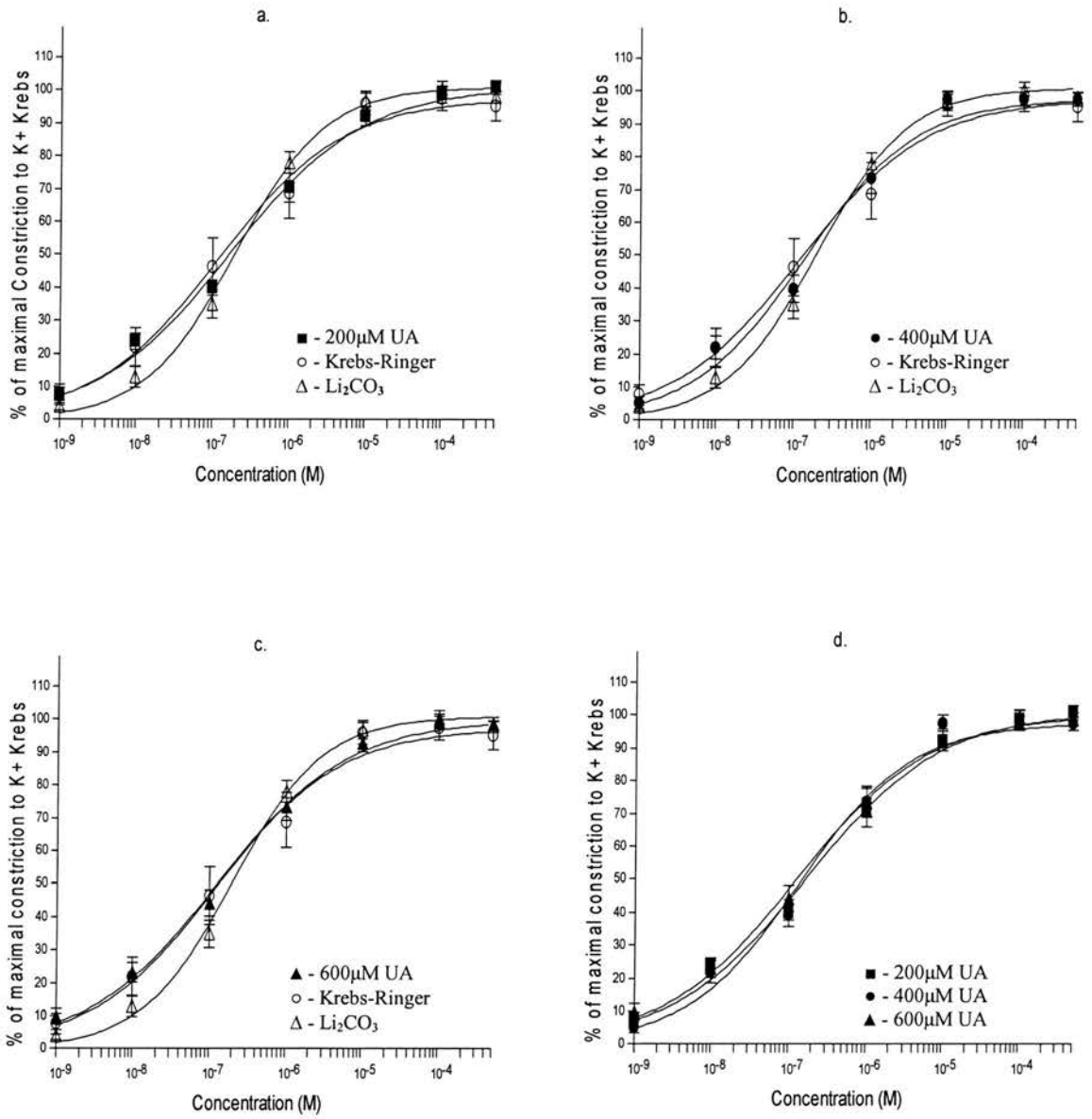


Figure 4.1. The effect of luminal perfusion with UA on vasoconstriction to PE in isolated rat mesenteric arteries: a.) 200μM UA, b.) 400μM UA, and c.) 600μM UA compared to Krebs-Ringer or Li_2CO_3 solution. d.) compares the effect of each concentration of UA. All values are the mean ± SEM (n=8).

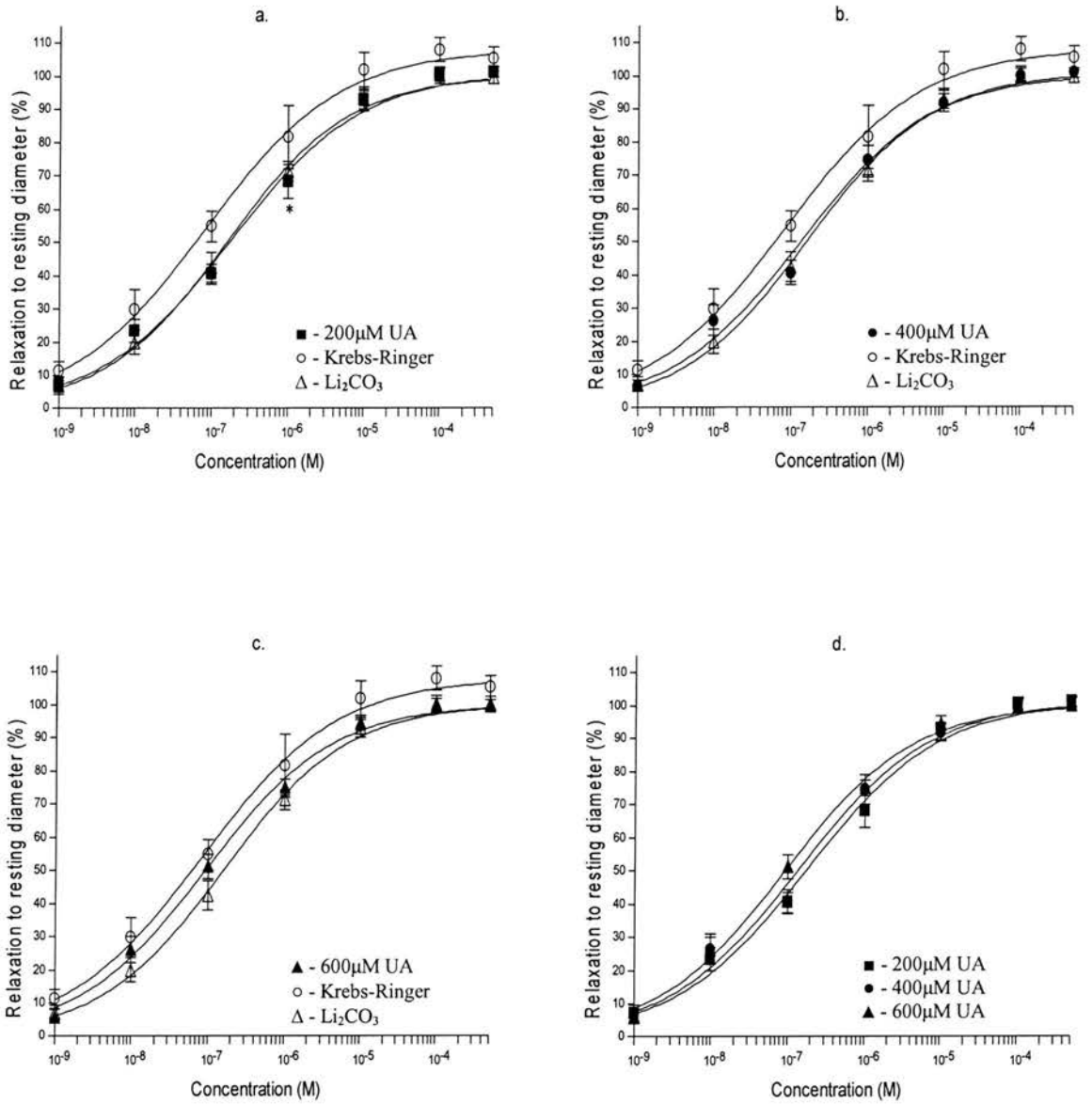


Figure 4.2. The effect of luminal perfusion with UA on vasodilatation to ACh in isolated rat mesenteric arteries: a.) 200μM UA, b.) 400μM UA, and c.) 600μM UA compared to Krebs-Ringer or Li_2CO_3 solution. d.) compares the effect of increasing concentrations of UA. All values are the mean $\pm$ SEM (n=8). *$p < 0.03$ vs. Krebs-Ringer solution (2-factor ANOVA with replication and Bonferroni correction).

Intraluminal Solution	EC_{50}	Maximal Dilatation
Krebs-Ringer	107.4 ± 30.2nM	105.9 ± 3.6%
Li_2CO_3	197.5 ± 44.8nM	100.3 ± 2.5%
200μM UA	155.5 ± 30nM	101.4 ± 1.6%
400μmM UA	178 ± 58.2nM	100.7 ± 1.4%
600μM UA	125.6 ± 37.6nM	100.3 ± 2.3%

Table 4.1. The effect of luminal perfusion with UA on vasodilatation to ACh in isolated rat mesenteric arteries: Calculated mean EC_{50} and maximal response values, ± SEM, for concentration-dependent vasodilatation to ACh in isolated rat mesenteric arteries when perfused with the above intraluminal solutions. There was no statistically significant difference in the mean EC_{50} values ($P>0.05$; One-way ANOVA with Bonferroni correction, n=8).

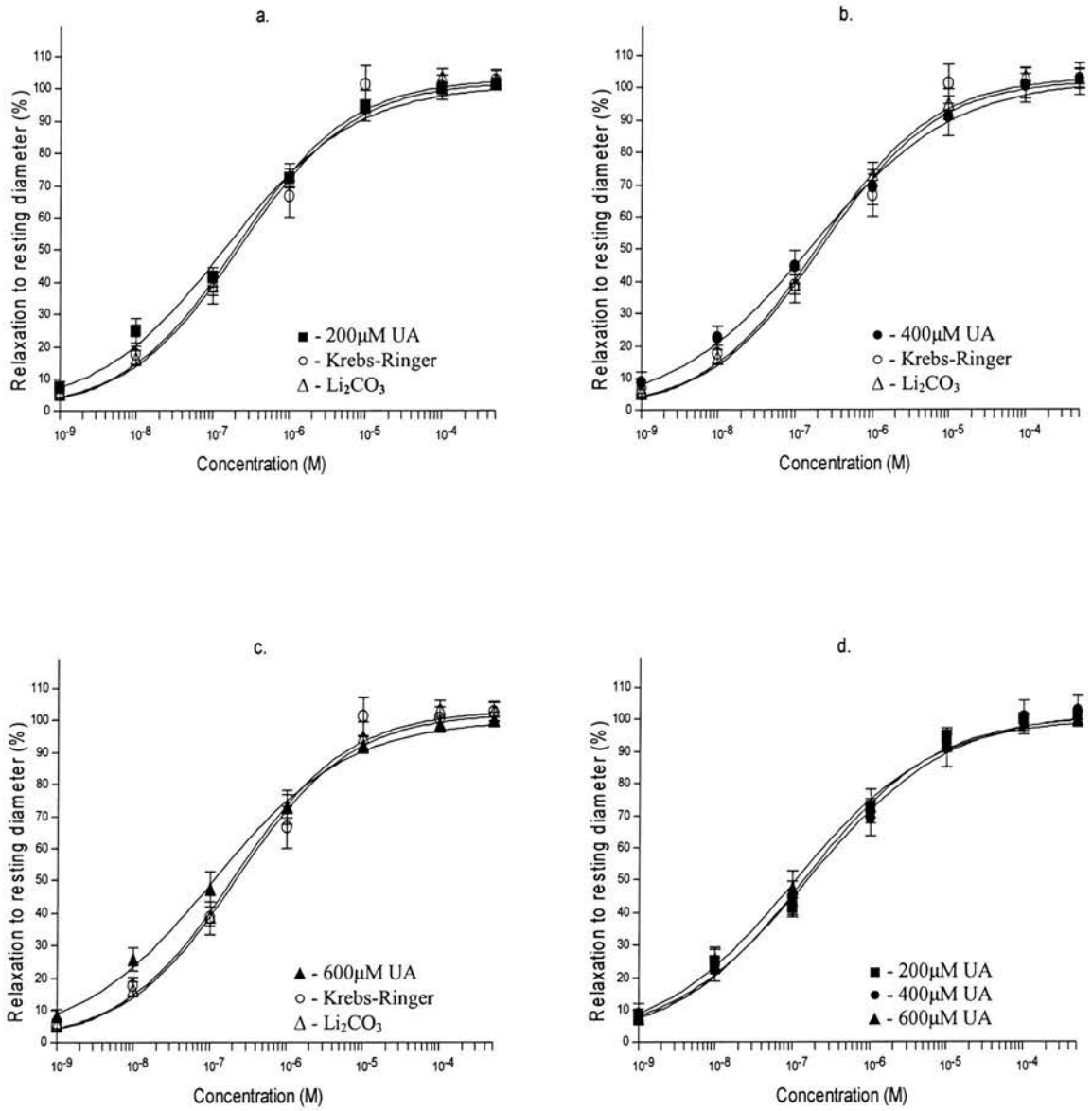


Figure 4.3. The effect of luminal perfusion with UA on vasodilatation to SNP in isolated rat mesenteric arteries: a.) 200μM UA, b.) 400μM UA, and c.) 600μM UA compared to Krebs-Ringer or Li_2CO_3 solution. d.) compares the effect of increasing concentrations of UA. All values are the mean ± SEM (n=8).

4.4 DISCUSSION

Perfusion of the arterial lumen with increasing concentrations of UA (200μM, 400μM and 600μM) had no effect upon the vasoconstriction to increasing concentrations of PE, endothelium-dependent vasodilatation to ACh, or endothelium-independent vasodilatation to SNP, when compared to the physiological control or vehicle control responses.

4.4.1 The effect of UA on vascular function

Many cardiovascular risk factors are accompanied by endothelial dysfunction, which may be characterised by excessive vasoconstriction or vasospasm (Galle *et al,* 1990; Cox & Cohen 1996). However, acute luminal exposure to UA at physiological and elevated concentrations does not directly potentiate vasoconstriction to PE. This suggests that UA does not impair vascular dysfunction and may provide limited evidence that increased concentrations of UA are not injurious to the vascular system.

It was hypothesised that UA is injurious to the endothelium and directly impairs endothelium-dependent vasodilatation. However, in the current study, vasodilatation to ACh was similar when the isolated arteries were perfused with increasing concentrations of UA, the vehicle Li_2CO_3 solution, or physiological Krebs-Ringer solution (Fig. 4.2). This demonstrates that acute luminal perfusion with increasing concentrations UA, to a level that is greater than that found in human plasma, does not attenuate endothelium-dependent vasodilatation to ACh, or induce vasoconstriction to this agonist, as may occur in endothelial dysfunction (Ludmer *et al,* 1986; Zeiher *et al,* 1991; Zeiher *et al,* 1993). These results provide evidence that acute endothelial exposure to increasing concentrations of UA does not directly impair vascular function. The failure of UA to induce vascular dysfunction is further demonstrated by the

vasodilatation produced to SNP when the arteries were perfused UA solution or physiological Krebs-Ringer solution. All the concentration-dependent responses were similar, confirming that UA does not exert a direct inhibitory effect upon the relaxation of VSMC. These findings negate the hypotheses that UA directly induces the functional impairment of the endothelium and demonstrate that UA is not cytotoxic or directly injurious to the endothelium. Consequently, they do not support a causal link between elevated UA concentrations and endothelial or vascular dysfunction. This is in accord with a recent clinical study which reported that acute exposure to high concentrations of UA did not impair forearm vascular function in healthy men (Waring *et al,* 2004).

The current results conflict with several studies which concluded that elevated UA concentrations caused endothelial dysfunction (Farquharson *et al,* 1999; Mercuro *et al,* 2004; Baldus *et al,* 2005; Edrogan *et al,* 2005; Kanellis & Kang, 2005; Kato *et al,* 2005; Khosla *et al,* 2005). However, these studies did not examine the direct effect of UA on vascular function and the results produced may be misleading. Kato *et al* and Edrogan *et al* both demonstrated the impairment of endothelial-dependent vasodilatation in hyperuricaemic patients and concluded that UA causes this endothelial dysfunction (Edrogan *et al,* 2005; Kato *et al,* 2005). This conclusion may be speculative, as whilst these studies demonstrate a correlation between hyperuricaemia and endothelial dysfunction, it cannot be concluded if UA is the direct cause of this dysfunction. Similarly, a number of the studies based their conclusions upon the restoration of endothelial function following the lowering of *in vivo* UA concentrations in hyperuricaemic patients (Farquharson *et al,* 1999; Mercuro *et al,* 2004; Baldus *et al,* 2005; Khosla *et al,* 2005). This restoration was achieved by the therapeutic reduction of UA levels with allopurinol or oxypurinol, which inhibit XO activity. These studies did not examine the direct action of UA on vascular function, but rather studied the effects of inhibiting XO-mediated UA generation. XO is a prominent source of ROS (Engerson *et al,* 1987; Ohara *et al,* 1993; Anker *et al,* 1997; Leyva *et al,* 1997; Culleton *et al,* 1999) and the stoichiometry of the XO reaction produces an excess of free radicals over UA (Becker *et al,* 1991). These free radicals reduce NO availability and impair

NO-mediated vasodilatation (Huie & Padmaja, 1993; Beckman & Koppenol, 1996). Consequently, interpretation of the results produced is not straightforward, as the restoration of endothelial function by XO-inhibitors could be attributed to the inhibition of excess generation of XO-mediated free radicals (Farquharson *et al,* 1999; Doehner *et al,* 2002; Farquharson *et al,* 2002). The current study used exogenous UA to examine the direct effects on vascular function, thus the results produced were not complicated by XO-mediated free radical generation. This could potentially explain the conflicting results produced in this *in vitro* study when compared to previous *in vivo* studies in hyperuricaemic patients.

4.4.2 Li_2CO_3

UA is insoluble in hydrophobic media and sparingly soluble in aqueous media. Physiological concentrations in human blood are near saturation (415μM). Consequently, poor solubility has impaired the *in vivo* and *in vitro* study of UA on vascular function, however this has recently been overcome (Waring *et al,* 2000). In the present study, concentrations of UA near to and above the level of saturation were required. Dissolution of UA in Li_2CO_3 solution allowed the preparation of such concentrations (Waring *et al*, 2000). To minimise the Li_2CO_3 present in the perfusing solutions, and any influence this could have upon vascular function, high concentrations of UA were dissolved in the vehicle solution and then further diluted with physiological Krebs-Ringer solution.

The results obtained in this study demonstrate no significant influence of any of the interventions, or vehicle on endothelium-dependent dilatation to ACh. Therefore, it seems unlikely that the Li_2CO_3 vehicle was an important influence on the effects of UA in this study. This is supported by the calculated mean EC_{50} values, which are similar when the vessels are perfused with the vehicle solution compared to the physiological control. This is in agreement with a clinical study that demonstrated that Li_2CO_3

provided a safe and suitable vehicle for the *in vitro* administration of UA in humans (Waring *et al,* 2002).

However, the results produced do not agree with previous studies that have demonstrated the variable impact of lithium and Li_2CO_3 on vascular function in isolated vessel preparations. However, such studies have used higher concentrations of Li_2CO_3 and longer durations of exposure than this study. Li_2CO_3 could potentially impair vasodilatation to ACh by inhibiting the formation of IP_3 (Drummond, 1987), which is responsible for the release of intracellular Ca^{2+} from the endoplasmic reticulum in EC. Similarly, lithium could also reduce vasodilatation by inhibiting cGMP synthesis (Kanba *et al,* 1991). In contrast, it has been reported that lithium can enhance eNOS activity and NO-mediated vasodilatation via the endothelial accumulation of cystolic Ca^{2+} (Marsden *et al,* 1990), or by inducing a transient increase in IP_3 (Berridge *et al,* 1982). Dehpour *et al* demonstrated that the alteration of endothelial function by Li_2CO_3 was dependent upon the chronicity of exposure, with prolonged exposure to lithium enhancing ACh-mediated vasodilatation (Dehpour *et al,* 2000). However, in the current study, the EC of the isolated vessels were only acutely exposed to the perfusing Li_2CO_3 solution. Dehpour *et al.* also reported that the effect of lithium was concentration-dependent, as 2mM lithium significantly increased ACh-induced dilatation in isolated rat aorta, while 500μM significantly reduced vasodilatation (Dehpour *et al*, 1995). However, incubation of the aorta with 500μM lithium did not alter the dilatory response to less than 10^{-6}M ACh (Dehpour *et al*, 1995). The current study is in partial agreement with this as luminal perfusion with the Li_2CO_3 vehicle solution had no effect upon vasodilatation to ACh at any concentration studied. This may be because the concentration of lithium in the vascular lumen in our studies was low (216μM). Similarly, the inhibitory action of lithium upon IP_3 and cGMP synthesis only occurs at concentrations (1.5mM) far greater than those used in the present study (Kanba *et al,* 1991).

4.4.3 The association of UA with cardiovascular disease

An epidemiological link between elevated UA and cardiovascular events has been established for several years (Becker, 1993; Waring *et al,* 2000). Evidence regarding the association between elevated plasma UA concentrations and the development of cardiovascular disease is inconsistent (Jossa *et al,* 1994). Therefore, it remains unclear whether UA has a causative or protective role in this condition. Several epidemiological studies have reported that UA is an independent risk factor for this disease state (Fessel, 1980; Brand *et al*, 1985; Bonora *et al*,1996; Persky *et al*, 1997; Bengtsson *et al,* 1998; Lehto *et al*,1998; Ward, 1998; Liese *et al*, 1999; Fang & Alderman, 2000;), whilst others have reported that the link does not persist after correcting for other risk factors (Reunanen *et al*, 1982; Iribarren *et al*, 1996; Wannamethee *et al,* 1997; Culleton *et al,* 1999; Wannamethee, 2005). The latter findings are in agreement with the results produced in the present study, as UA does not have a direct effect upon vascular and endothelial dysfunction following acute exposure in isolated vessels. This does not support the hypothesis that elevated UA directly contributes to the pathogenesis of cardiovascular disease via the impairment of vascular function. Rather, it suggests that the epidemiological association of elevated UA with increased cardiovascular risk may be mediated by other means. The strong association between UA and cardiovascular risk may be misleading, as elevated UA may be present to combat the pathogenesis of this disease or may simply be a non-causal marker for existing damage or other cardiovascular risk factors.

4.4.3.1 The potential indirect causative role of UA in cardiovascular disease

The present study has demonstrated that short-term exposure to elevated UA is not directly injurious to the endothelium and does not induce endothelial dysfunction; however an indirect causative role for UA in this condition cannot be discounted. There

is extensive clinical and experimental evidence to suggest that UA can indirectly impair endothelial function by other means. Biochemical and *in vitro* data indicate that UA can react to form radical species and induce various pro-oxidant effects in the vascular wall which could impair endothelial function (Vasquez-Vivar *et al,* 1996; Abuja, 1999; Bagnati *et al,* 1999; Santos *et al,* 1999). This could not be examined in the present study as the vessels were not placed under oxidative stress. *In vitro* and *in vivo* findings suggest that UA may contribute to endothelial dysfunction by inducing antiproliferative effects on endothelium and impairing NO production (Kanellis & Kang, 2005). Elevated concentrations of UA may cause chronic inflammation through the activation of various blood components, such as leukocytes (Duff *et al,* 1983; Hutton *et al*, 1985; Leyva *et al,* 1998; Mazzali *et al,* 2002; Kanellis *et al,* 2003), which can have a deleterious effect upon the endothelium. UA can enhance platelet adhesion, aggregation, (Mustard *et al,* 1963; Newland, 1975; Ginsberg *et al,* 1977) and lysis (Ginsberg *et al,* 1977). Platelet and leukocyte activation promotes peroxide and $O_2^{\cdot-}$ radical liberation from these cells (Boogaerts *et al,* 1983; Falasca *et al,* 1993). This can increase oxidative stress within the vascular wall and reduces NO availability. Therefore, UA may indirectly impair NO-mediated vascular function by the activation of various cells in the blood. These more chronic effects *in vivo* could not be addressed by the current study as there were no blood components present in the vascular lumen.

4.4.3.2 UA as marker for other risk factors or existing damage

The association between cardiovascular disease and elevated UA could occur as a non-specific marker for other cardiovascular risk, such as hypercholesterolaemia, hypertension, disordered glucose metabolism, obesity, or as a marker for existing damage (Reunanen *et al,* 1982; Iribarren *et al*, 1996; Wannamethee *et al,* 1997; Culleton *et al,* 1999; Alderman, 2001; Alderman, 2002). For example, the link between elevated serum UA and cardiovascular disease may arise through a non-causal relationship with insulin resistance syndromes, in which cardiovascular risk is caused by other factors. In this state, insulin-mediated glucose metabolism is impaired but the renal effects remain

(Perry *et al,* 1995; Muscelli *et al,* 1996). As insulin concentrations are greater in this condition, there is enhanced renal insulin activity, and as such, reduced UA clearance resulting in increased serum UA concentrations (Perry *et al,* 1995; Muscelli *et al,* 1996). The association could also occur as elevated serum UA may be a marker for tissue ischaemia. Under conditions of tissue ischaemia and hypoxia, there is a significant increase in the synthesis and circulating concentration of adenosine to relax the VSMC (Fredholm & Sollevi, 1986; Raatikainen *et al,* 1994). However, the adenosine synthesised locally by the VSMC is rapidly degraded by the EC to UA (Nees *et al,* 1985; Jarasch *et al,* 1986; Becker, 1993). Therefore, elevated serum UA may be a marker of underlying ischaemia and or hypoxia. Similarly, the association between elevated serum UA and increased cardiovascular risk may be secondary to the increased XO activity that occurs under increased oxidative stress (Castelli *et al,* 1995). NO exerts a potent dose-dependent inhibition on XO (Fukahori *et al*, 1994; Rinaldo *et al,* 1994; Hassoun *et al,* 1995). This inhibition is removed in oxidative stress and endothelial dysfunction and XO activity is enhanced (Sobey *et al,* 1992; Sobey *et al,* 1993), resulting in increased UA generation.

4.4.3.3 Association of UA with cardiovascular disease as protective role

The inability of UA to impair vascular function in the current study suggests that UA does not have a direct harmful effect upon endothelial function. Alternatively, the association between elevated UA, endothelial dysfunction and cardiovascular disease could occur as UA has a variety of potential protective effects, as outlined in Chapter 1. UA is an established antioxidant and the elevated concentrations present in cardiovascular disease may occur as a compensatory mechanism in the face of chronic risk factors or established vascular disease. For example, elevated UA levels are frequently associated with hypercholesterolaemia (Wannamethee *et al,* 1997), in which there is an increased generation of $O_2^{\cdot-}$ and ROS within the vascular wall (Ohara *et al,* 1993). UA may be present in greater concentrations in this disease state to prevent the oxidative damage caused by these free radicals. This potentially beneficial role for

elevated UA in conditions of cardiovascular risk could explain why humans have greater concentrations of UA compared to other species, as a positive correlation exists between plasma UA and species life-span (Ames *et al,* 1981; Becker, 1993). It has previously been suggested that the high levels of UA present in human physiology are not harmful to the body but are the result of an evolutionary process to provide an effective protective mechanism against radical-induced oxidative damage (Ames *et al,* 1981). As the current study has demonstrated that UA does not directly impair vascular function, further studies should focus upon investigating the potentially protective effects of UA upon endothelial and vascular function.

4.4.4 Study Limitations

Possible limitations of this investigation include the duration of arterial exposure to UA. The isolated vessels were only acutely exposed to the UA solutions (approximately 7 hours). However, patients at risk of developing cardiovascular disease, or those with hyperuricaemia, may be exposed to elevated concentrations of UA in the plasma for extensive periods of time. Therefore, it is possible that the onset of vascular or endothelial dysfunction by UA may take longer the time scale for this study would allow. Indeed, it has been suggested that chronic exposure to blood containing hyperuricaemic concentrations of UA may trigger initial damage within the intimal layer of the vessel wall (Rathmann *et al,* 1993; Persky *et al*, 1997). Furthermore, *in vitro* studies examining the effect of incubation with elevated UA concentrations upon the structural and functional integrity of HUVEC in culture reported that the extent of endothelial injury was correlated to UA concentration and the duration of incubation (Kuhne *et al*, 1999). Another possible limitation of this study is the presence of Li_2CO_3 as the vehicle solution. However, as previously mentioned, the concentration within the arterial lumen in these studies was relatively low (216μM) and exposure to the vehicle solution had no significant effect on the vascular responses. Finally, any alterations in

the maximum vasoconstrictor responses may have been hidden, as the data was calculated in relation to the maximum response to KCl, which was also performed in the presence of the various test solutions.

4.4.5 Summary

This study demonstrated that perfusing the lumen of isolated rat mesenteric arteries with increasing concentrations of UA did not potentiate vasoconstriction or reduce endothelial-dependent vasodilatation. This demonstrates that acute exposure of the endothelium to UA does not induce severe functional impairment and is not directly cytotoxic or injurious to the EC. On the basis of this study, it is hypothesised that the UA does not have a direct causal role in endothelial dysfunction. Therefore, the correlation between elevated UA concentrations and the pathogenesis of cardiovascular disease does not occur by the direct impairment of endothelial function. However, the relationship could be due to either the indirect actions of UA upon the endothelium, the production of free radicals by XO, or increased generation of UA as a compensatory mechanism to combat conditions of increased oxidative stress. As a result, it is believed that examining the effect of UA on endothelial function secondary to other established risk factors, such as high concentrations of LDL, will help to further elucidate the role of elevated UA in cardiovascular disease.

CHAPTER 5

THE EFFECT OF LUMINAL EXPOSURE TO LDL ON ENDOTHELIAL AND VASCULAR FUNCTION

5.1 INTRODUCTION

The previous chapter demonstrated that elevated concentrations of UA do not directly impair vascular function. This elevation in serum UA concentrations frequently accompanies hypercholesterolaemia (Wannamethee *et al,* 1997), an established cardiovascular risk factor (Goldstein & Brown, 1977; Steinberg, 1983; Martin *et al,* 1986), in which endothelial dysfunction is prevalent (Chowienczyk *et al,* 1992). This dysfunction is attributed to LDL-induced damage to the endothelium. However, evidence regarding the role of LDL in the development of endothelial dysfunction remains inconsistent, and must be clarified before the relationship between UA, LDL and vascular function can be examined.

The oxidative modification of LDL to ox-LDL was previously regarded as a key process in the development of endothelial dysfunction (Steinberg *et al,* 1989; Esterbauer *et al,* 1992; Cox & Cohen, 1996). The harmful properties of LDL are commonly attributed to the action of ox-LDL (Cox & Cohen, 1996; Dart & Chin-Dusting, 1999; Rosendorff, 2002) and the deleterious effects of ox-LDL on NO-mediated vascular function are widely established (Hein & Kuo, 1998; Vergnani *et al,* 2000; Mukherjee *et al,* 2001; Chan *et al,* 2003; Fleming *et al,* 2005). However, the role of unmodified LDL in vascular and endothelial dysfunction is less clear, as *in vitro* studies performed to elucidate the direct effects of LDL on endothelial function have produced conflicting results. Several studies have reported that LDL does not impair vascular function in isolated vessel preparations (Kugiyama *et al*, 1990; Yokoyama *et al*, 1990; Simon *et al*, 1990; Plane *et al,* 1992; Galle *et al,* 1994; Galle *et al,* 1995; Chan *et al,* 2003), while several have demonstrated that LDL significantly reduces NO-availability and impairs vasodilatation (Andrews *et al*, 1987; Jacobs *et al*, 1990; Tomita *et al,* 1990; Galle *et al*, 1991; Hein & Kuo, 1998; Fontana *et al*, 1999; Vergnani *et al,* 2000).

The mechanism by which LDL could impair endothelium-dependent vasodilatation also remains uncertain, but is likely arise due to the decreased release of, or the increased inactivation of, endothelium-derived NO. These may occur due to LDL-induced increases in ROS and oxidative stress within the vascular wall (Gryglewski *et al*, 1986; Ohara *et al*, 1993). It has been reported that a three-fold increase in ROS occurs in hypercholesterolemia, which coincides with a decrease in vascular relaxation (Ohara *et al*, 1993; Ohara *et al*, 1995). This may be prevented by SOD, the major tissue defence against $O_2^{\cdot-}$, which restores NO-mediated vascular function in *in vitro* models of hypercholesterolaemia (Mugge *et al*, 1991; White *et al*, 1994; Hien & Kuo, 1998). This restoration implies a central role for $O_2^{\cdot-}$ in the deleterious actions of LDL on endothelial function. The endothelial generation of $O_2^{\cdot-}$ may be enhanced by LDL via the direct impairment of endothelial l-arginine metabolism and the resultant effects this has upon eNOS activity (Pritchard *et al*, 1995; Pritchard *et al,* 2002; Stepp *et al,* 2002). Consequently, LDL could have a causative role in cardiovascular disease by increasing oxidative stress within the EC, which in turn reduces NO availability, thus inducing endothelial dysfunction.

This chapter aims to clarify the contradictory literature and investigate the role of LDL in endothelial dysfunction. This chapter will use perfusion myography to examine the effect of acute luminal exposure to LDL upon vascular function in isolated rat mesenteric arteries. This chapter will also examine the effect of supplementation of the LDL solutions with L-NAME, l-arginine and SOD. This chapter aims to establish the effect of LDL on vascular function, and the possible mechanisms of action, by demonstrating that:

1.) Luminal exposure to LDL in a perfusion myograph model induces vascular dysfunction in a concentration-dependent manner.

2.) LDL induces vascular dysfunction by impairing NO-dependent vasodilatation in this experimental model, which is not augmented by the prevention of NOS activity with L-NAME.

3.) The inhibition of vascular function is mediated by impaired l-arginine metabolism and increased oxidative stress within the vascular wall. This can be reversed by supplementation with l-arginine or the dismutation of $O_2^{\cdot -}$ by SOD.

4.) The inhibitory effects of LDL upon endothelium-dependent vasodilatation are fully reversible.

5.) LDL does not require oxidative modification to ox-LDL to induce endothelial dysfunction in this experimental model.

5.2 METHODS

5.2.1 Lipoprotein Isolation

LDL was isolated, purified, and the protein concentration determined using a commercial kit based on the Lowry precipitation assay (Sigma Chemical Company, Dorset, U.K.), as previously described (Chapter 2).

5.2.2 The effect of LDL on vascular function

Segment of third order branches of the rat mesenteric artery were excised, mounted and concentration-responses curves to PE, ACh and SNP performed when the lumen was perfused with LDL solution (250, 500 or 1000μg/ml) or control Krebs-Ringer solution. This was also repeated on endothelial denuded vessels perfused with Krebs-Ringer solution.

5.2.3 The reversibility of the inhibitory action of LDL on endothelium-dependent vasodilatation

The arteries were prepared as before and the lumen perfused with Krebs-Ringer solution. Following constriction with high K^+ Krebs solution, cumulative concentration-response curves to PE were performed. The arteries were washed, allowed to relax, and then preconstricted with EC_{80} PE (1-5μM). ACh concentration-response curves ($1x10^{-7}$–$1x10^{-5}$M) were then performed. The stopcock attached to the distal cannula was then opened to allow the arterial lumen to be slowly perfused with LDL solution (250, 500 or 1000μg/ml). The stop-cock was closed and the vessel re-

pressurised to 60mmHg. The vessels were allowed a 60-minute equilibration period, preconstricted with PE, and ACh concentration-response curves repeated. The lumen was then perfused slowly with Krebs-Ringer solution for 10 minutes to remove all LDL solution and ensure the lumen was completely perfused with physiological buffer. The arteries were re-pressurised to 60mmHg and, allowed a 60-minute equilibration period, preconstricted, and ACh concentration-response curves repeated.

5.2.4 The effect of L-NAME upon the action of LDL on vascular function

Arteries were prepared and mounted as before. The lumen was perfused with 250μg/ml LDL solution, 100μM L-NAME solution, 250μg/ml LDL + 100μM L-NAME solution, or Krebs-Ringer buffer, and concentration-response curves to PE, ACh and SNP performed.

5.2.5 The effect of SOD upon the action of LDL on vascular function

The experimental preparation and protocol (5.2.2) was repeated when the arterial lumen was perfused with 250μg/ml LDL solution, 250μg/ml LDL + 150U/ml SOD solution or Krebs-Ringer buffer.

5.2.6 The effect of l-arginine supplementation upon the action of LDL on vascular function

The experimental preparation and protocol (5.2.2) were repeated when the arterial lumen were perfused with 250μg/ml LDL solution, 250μg/ml LDL + 1mM l-arginine solution or Krebs-Ringer buffer.

5.2.7 Intraluminal Solutions

LDL solutions were prepared daily from stock by dilution with Krebs-Ringer solution to give a final intraluminal lipoprotein concentrations of 250, 500 and 1000μg/ml. To prevent the oxidative modification of LDL, 0.3mM EDTA was added to the solutions. 1mM L-NAME stock solution was prepared by dissolving in Krebs-Ringer solution. Working solutions were prepared daily by dilution with Krebs-Ringer to give a final concentration of 100μM. L-NAME-supplemented LDL solutions were prepared daily by diluting stock LDL solution with Krebs-Ringer buffer and stock L-NAME solution to give a final intraluminal lipoprotein concentration of 250μg/ml and L-NAME concentration of 100μM. 10mM l-arginine solution was prepared daily by dissolving 1.74mg/ml l-arginine in Krebs-Ringer buffer. This was then used to dilute the stock LDL solution, along with Krebs-Ringer buffer, to give an l-arginine concentration of 1mM and lipoprotein concentration of 250μg/ml. SOD protein was dissolved daily in 250μg/ml LDL solution to give a final SOD concentration of 150U/ml.

5.2.8 Lipid Peroxidation

Samples of the intraluminal LDL and supplemented solutions were collected before and after luminal perfusion to measure the extent of LDL lipid peroxidation and examine any changes in oxidative status. The hydroxynonenal (4-HNE) and malondialdehyde (MDA) concentrations of the intraluminal solutions were determined using a commercial colorimetric assay kit (CN Biosciences, Nottingham, U.K.).

5.2.9 Statistical Analysis

The statistical significance of the impairment of vascular function by LDL was determined by comparing the results obtained for the various concentrations of LDL with the physiological control responses. These were also compared with each other to demonstrate that the inhibitory effects of LDL were concentration-dependent. The reversibility of the effect of LDL upon endothelial function was analysed by determining any statistical significance in endothelium-dependent vasodilatation before, during and after luminal perfusion with LDL solution. Statistical analysis was performed to determine the effect of LDL supplementation with L-NAME, SOD or l-arginine on vascular function when compared to LDL or the physiological control. The effect of the LDL supplementation with L-NAME was also compared to the vascular responses when perfused with L-NAME only. Statistical analysis was performed using a paired Student's t-test to compare the oxidative status of the intraluminal LDL solution before and after luminal perfusion.

5.3 RESULTS

5.3.1 The effect of LDL on vascular function

5.3.1.1 The effect of LDL on vascular contractility

PE produced concentration-dependent vasoconstriction in mesenteric arteries perfused with Krebs-Ringer solution, with a maximal response of 96.6 $\pm$ 3.5% (Fig 5.1). All maximal responses were similar when perfused with the various intraluminal solutions. All concentrations of LDL produced similar results. However, 250μg/ml ($5x10^{-7}$-$1x10^{-6}$, $P<0.01$), 500μg/ml ($5x10^{-7}$-$1x10^{-6}$, $P<0.01$) and 1000μg/ml LDL ($5x10^{-8}$-$1x10^{-6}$, $P<0.01$) significantly enhanced vasoconstriction when compared to the control and denuded artery responses. Nevertheless, there was no statistically significant difference in the calculated EC_{50} values (Table 5.1).

5.3.1.2 The effect of LDL on endothelium-dependent vasodilatation

ACh produced concentration-dependent vasodilatation with a maximal response of 106.1 $\pm$ 2.9% in arteries perfused with Krebs-Ringer solution (Fig 5.2). Endothelial denudation abolished the dilatory responses to ACh ($1x10^{-9}$-$5x10^{-4}$, $P<0.001$). 250μg/ml LDL ($1x10^{-9}$-$5x10^{-4}$, $P<0.01$), 500μg/ml LDL ($1x10^{-9}$-$5x10^{-4}$, $P<0.03$), and 1000μg/ml LDL ($1x10^{-9}$-$5x10^{-4}$, $P<0.01$) significantly reduced vasodilatation when compared to the physiological control, producing maximal responses of 42.6 $\pm$ 2.1%, 33.6 $\pm$ 3.7% and 21.7 $\pm$ 2.3%, respectively. However, these responses were significantly greater than the responses in endothelial denuded arteries (250μg/ml: $1x10^{-8}$-$5x10^{-4}$, $P<0.05$; 500μg/ml: $1x10^{-7}$-$5x10^{-4}$, $P<0.01$; 1000μg/ml: $5x10^{-7}$-$5x10^{-4}$, $P<0.04$). The inhibition of vasodilatation by LDL was concentration-dependent.

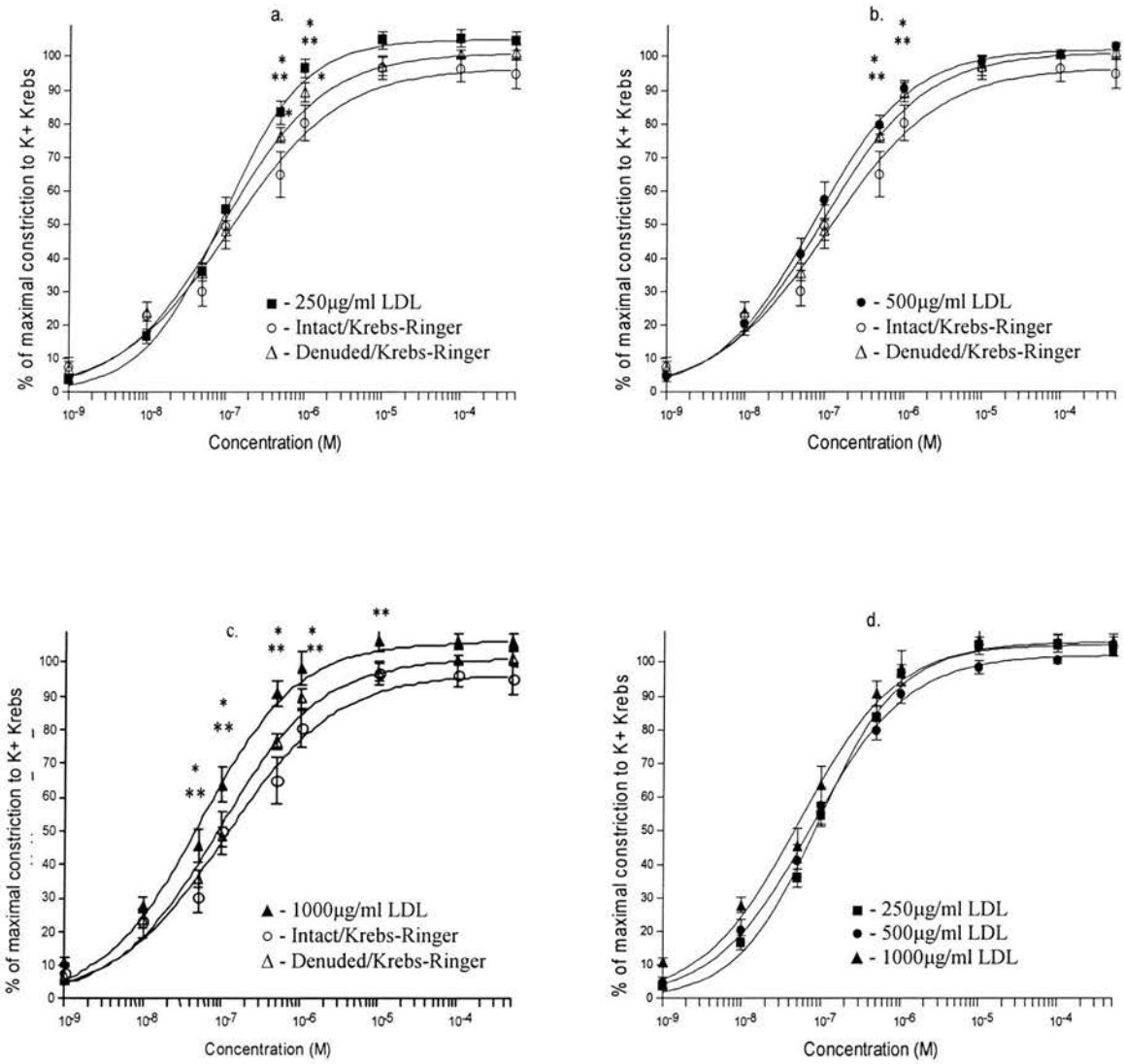


Figure 5.1. The effect of luminal perfusion with LDL on vasoconstriction to PE in isolated rat mesenteric arteries: a.) 250µg/ml LDL, b.) 500µg/ml LDL, and c.) 1000µg/ml LDL compared to intact or denuded vessels perfused with Krebs-Ringer solution. *$p<0.05$ vs. intact vessels, **$p<0.05$ vs. denuded vessels (2-factor ANOVA with replication and Bonferroni correction, n=8). d.) compares the effect of the various concentrations of LDL. All values are the mean $\pm$ SEM (n=8).

Intraluminal Solution	EC_{50}	Maximal Response (% of vasoconstriction to KCl)
Intact / Krebs-Ringer	103.7 + 36.4nM	96.6 + 3.5%
Denuded / Krebs-Ringer	124.2 + 14.3nM	101.3 + 2.1%
250μg/ml LDL	100.29 + 11.4nM	105.3 + 2.6%
500μg/ml LDL	99.4 + 23.4nM	102.8 + 1.2%
1000μg/ml LDL	71.3 + 18.1nM	106.4 + 2.9%

Table 5.1. The effect of luminal perfusion with LDL on vasoconstriction to PE in isolated rat mesenteric arteries: Calculated mean EC_{50} and maximal response values, $\pm$ SEM, for concentration-dependent vasoconstriction to PE when perfused with the above intraluminal solutions. There was no statistically significant difference in the mean EC_{50} values ($P>0.05$; One-way ANOVA with Bonferroni correction, n=8).

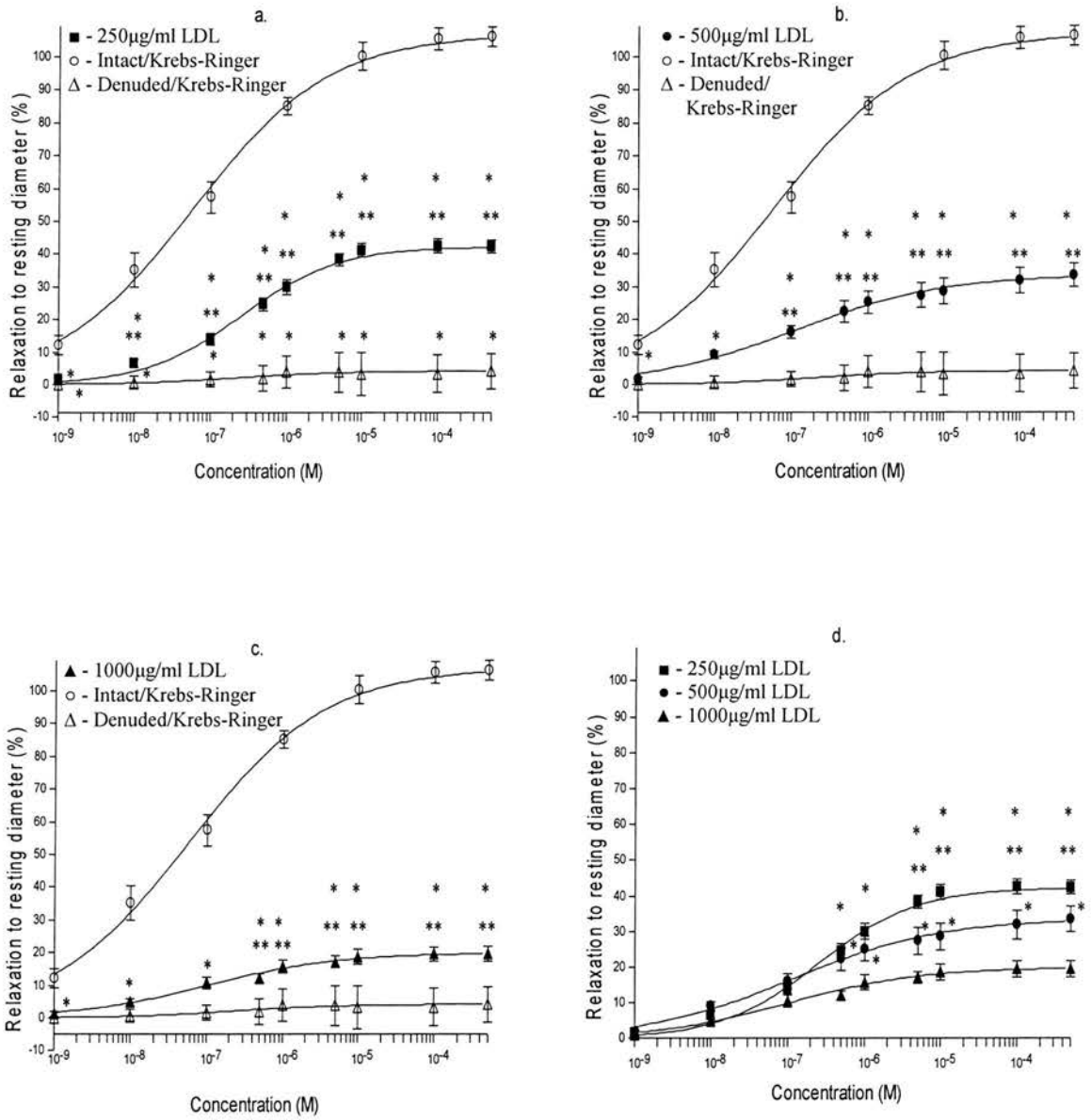


Figure 5.2. The effect of luminal perfusion with LDL on vasodilatation to ACh in isolated rat mesenteric arteries: a.) 250µg/ml LDL, b.) 500µg/ml LDL, and c.) 1000µg/ml LDL compared to intact or denuded vessels perfused with Krebs-Ringer solution. *$p<0.03$ vs. intact vessels, **$p<0.04$ vs. denuded vessels (2-factor ANOVA with replication and Bonferroni correction, n=8). d.) compares the effect of the various concentrations of LDL. *$p<0.01$ vs. 1000µg/ml LDL, **$p<0.01$ vs. 500µg/ml LDL (2-factor ANOVA with replication and Bonferroni correction, n=8). All values are the mean $\pm$ SEM (n=8).

5.3.1.3 The effect of LDL on endothelium-independent vasodilatation to SNP

Vasodilatation to SNP was similar following luminal perfusion with Krebs-Ringer solution, 250, 500 or 1000μg/ml LDL (Fig. 5.3), producing maximal dilatory responses of 100.9 ± 1.3%, 99.7 ± 1.5%, 99.7 ± 1.8% and 96.9 ± 4.4%, respectively. The vasodilatation in endothelium denuded arteries was also largely similar and produced a maximal dilatation of 100 ± 2.1%. However, 250μg/ml ($1x10^{-7}$–$1x10^{-6}$, $P<0.02$), 500μg/ml ($1x10^{-6}$, $P<0.01$) or 1000μg/ml LDL ($1x10^{-6}$, $P<0.01$) did reduce dilatation to SNP when compared to vasodilatation in denuded arteries.

5.3.2 The reversibility of the inhibitory action of LDL on endothelium-dependent vasodilatation

Luminal perfusion with 250, 500 or 1000μg/ml LDL significantly attenuated concentration-dependent vasodilatation to ACh when compared to the dilatation prior to perfusion with LDL (Fig. 5.4). This was completely reversible, as vasodilatation to ACh was restored following LDL washout and reperfusion with Krebs-Ringer solution.

5.3.3 The effect of L-NAME upon the inhibitory action of LDL on endothelium-dependent vasodilatation

Perfusion with 100μM L-NAME significantly reduced vasodilatation to ACh when compared to Krebs-Ringer buffer ($1x10^{-8}$-$5x10^{-4}$, $P<0.02$; Fig 5.5). The maximal dilatation was 44.9 ± 3.2%. Perfusion with 250μg/ml LDL + 100μM L-NAME produced a maximal dilatation of 38.6 ± 3.5%. These dilatations to ACh were largely similar to the responses for 250μg/ml LDL ($5x10^{-6}$, $P<0.02$) or 100μM L-NAME

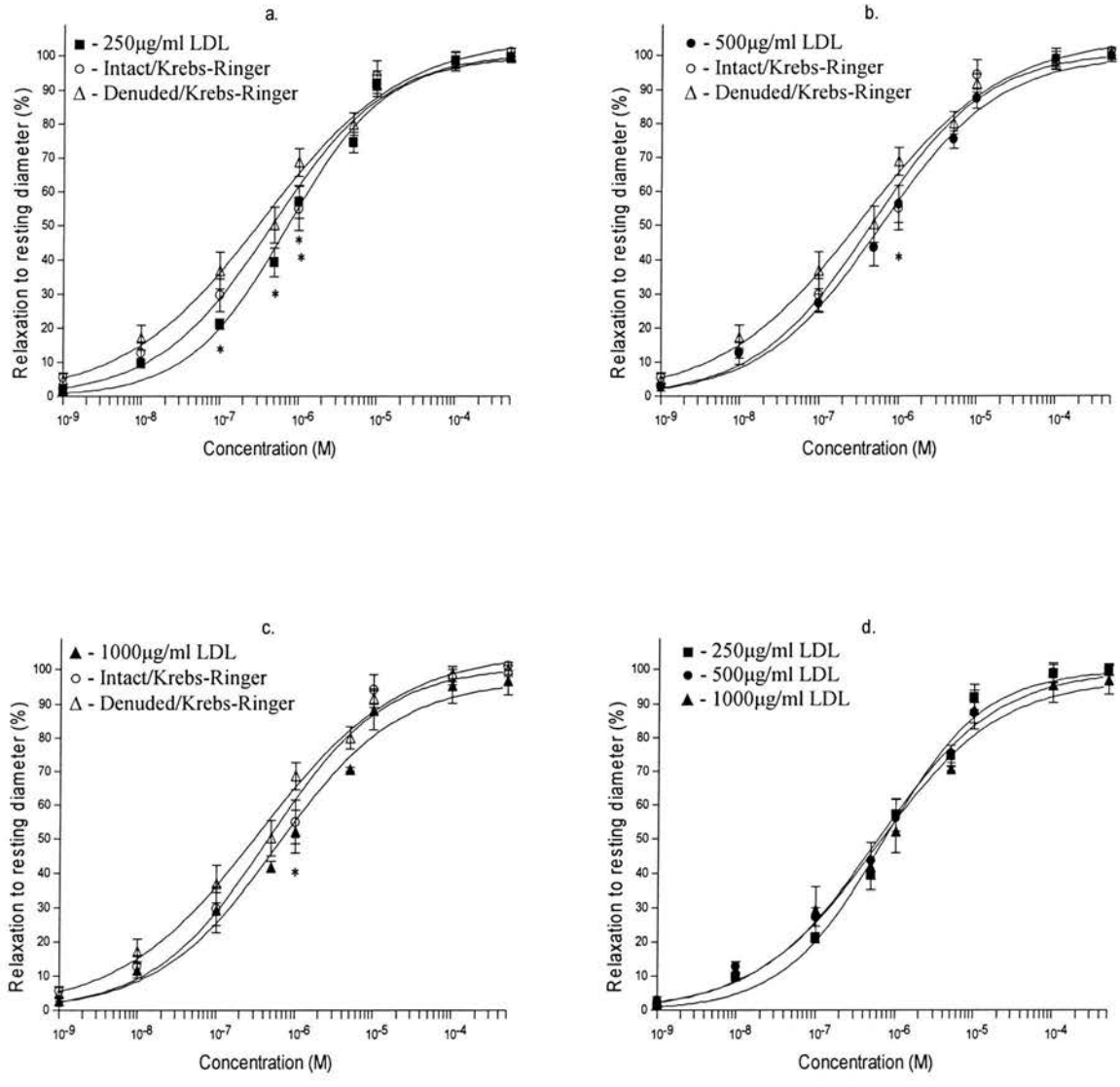


Figure 5.3. The effect of luminal perfusion with LDL on vasodilatation to SNP in isolated rat mesenteric arteries: a.) 250µg/ml LDL, b.) 500µg/ml LDL, and c.) 1000µg/ml LDL compared to intact or denuded vessels perfused with Krebs-Ringer solution. *$p<0.02$ vs. denuded vessels (2-factor ANOVA with replication and Bonferroni correction, n=8). d.) compares the effect of the various concentrations of LDL. All values are the mean $\pm$ SEM (n=8).

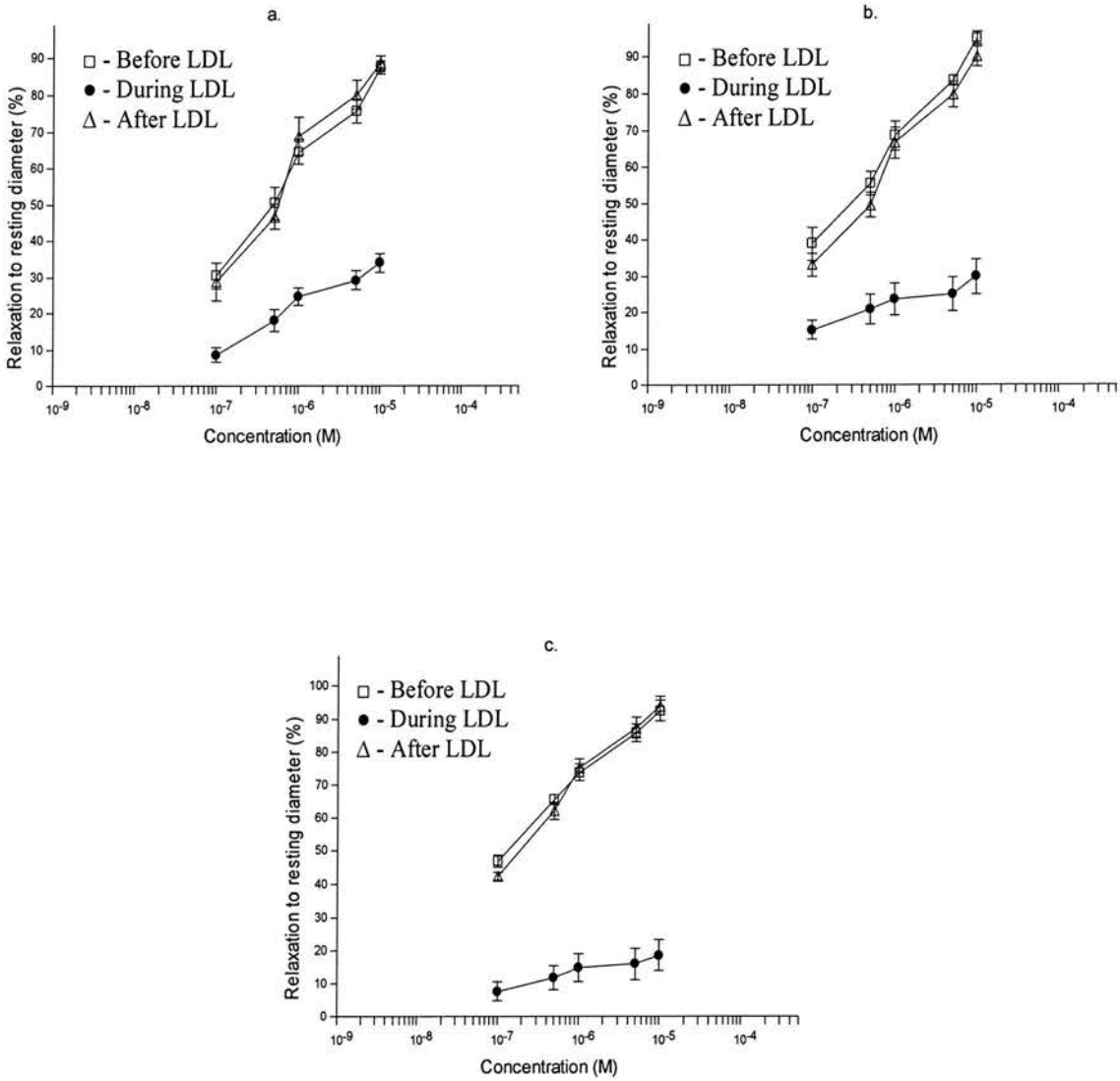


Figure 5.4. The reversibility of the impaired endothelium-dependent vasodilatation to ACh in isolated rat mesenteric arteries perfused with LDL: before, during, and after luminal perfusion with a.) 250μg/ml LDL, b.) 500μg/ml LDL, and c.) 1000μg/ml LDL. All values are the mean ± SEM (n=8).

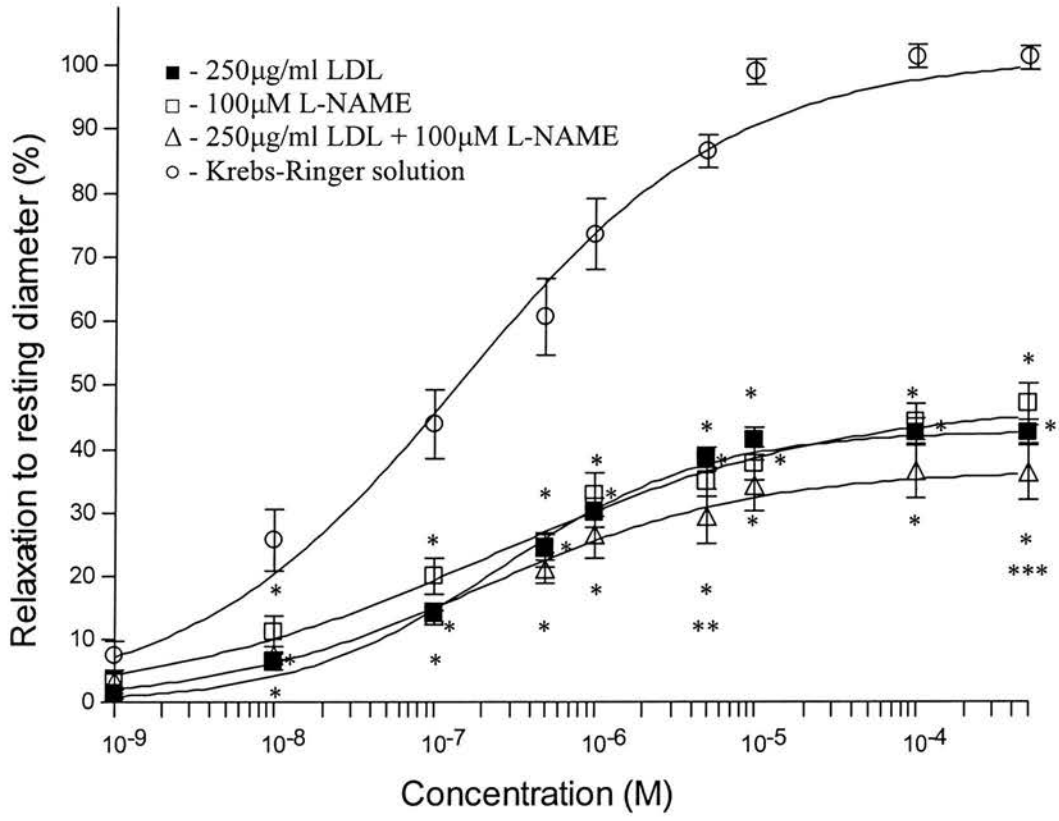


Figure 5.5. The effect of L-NAME on vasodilatation to ACh in isolated rat mesenteric arteries perfused with LDL: 250μg/ml LDL, Krebs-Ringer solution, 100μM L-NAME and 250μg/ml LDL + 100μM L-NAME. *$p<0.01$ vs. Krebs-Ringer solution, **$p<0.02$ vs. 250μg/ml LDL, ***$p<0.02$ vs. 100μM L-NAME (2-factor ANOVA with replication and Bonferroni correction, n=8). All values are the mean ± SEM (n=8).

($5x10^{-4}$, $P<0.02$) but significantly reduced when compared to the physiological control ($1x10^{-8}$-$5x10^{-4}$, $P<0.01$).

5.3.4 The effect of SOD upon the action of LDL on endothelium-dependent vasodilatation

250μg/ml LDL + 150U/ml SOD significantly attenuated concentration-dependent vasodilatation to ACh when compared to the physiological responses ($1x10^{-8}$-$5x10^{-4}$, $P<0.01$; Fig 5.6), and the maximal vasodilatation was 46 ± 3.6%. Luminal perfusion with 250μg/ml LDL + 150U/ml SOD did not significantly alter the vasodilatation to ACh when compared to arterial perfusion with 250μg/ml LDL only.

5.3.5 The effect of l-arginine supplementation upon the action of LDL on vascular function

Perfusion with 250μg/ml LDL + 1mM l-arginine significantly increased ACh concentration-dependent vasodilatation when compared to LDL only ($1x10^{-8}$-$5x10^{-4}$, $P<0.01$; Fig 5.7), producing a maximal vasodilatation of 84.2 ± 4.8%. However, this vasodilatation was still significantly lower than the physiological control responses ($5x10^{-7}$-$5x10^{-4}$, $P<0.04$).

5.3.6 Lipid peroxidation

There was no significant alteration in MDA and 4-HNE concentrations solutions following luminal perfusion (Fig. 5.8).

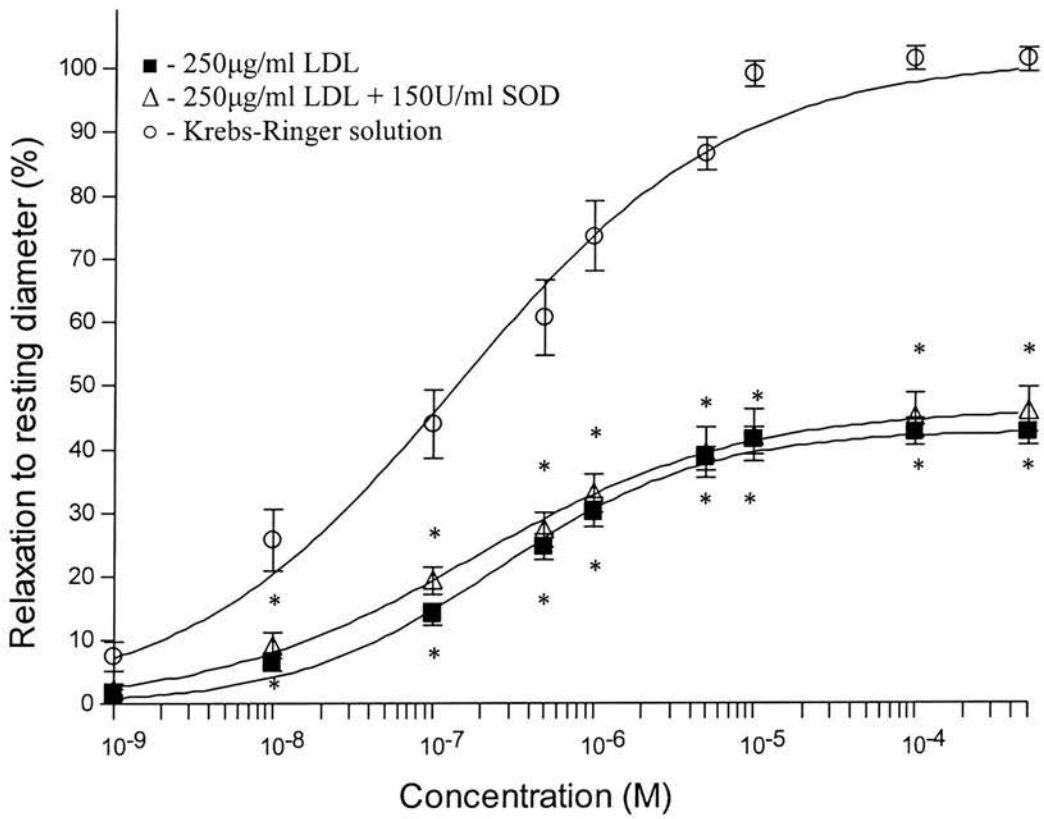


Figure 5.6. The effect of SOD on vasodilatation to ACh in isolated rat mesenteric arteries perfused with LDL: 250μg/ml LDL, Krebs-Ringer solution, 250μg/ml LDL + 150U/ml SOD. *$p<0.01$ vs. Krebs-Ringer solution (2-factor ANOVA with replication and Bonferroni correction, n=8). All values are the mean ± SEM (n=8).

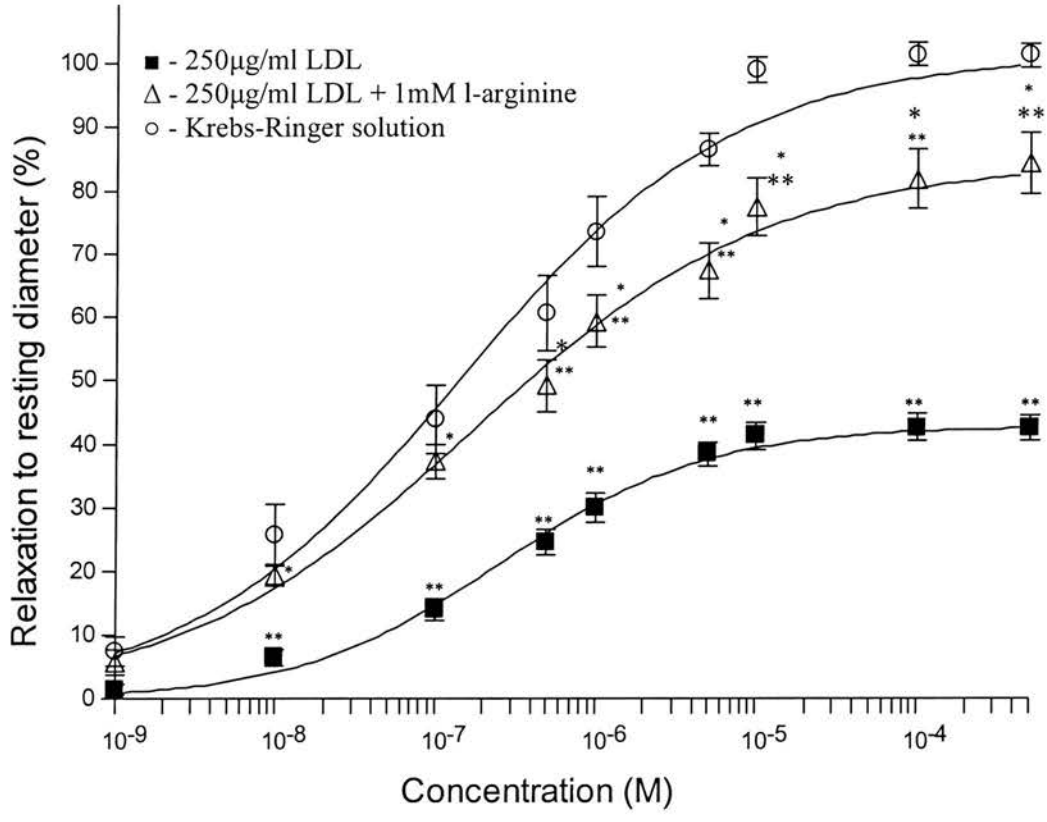


Figure 5.7. The effect of l-arginine on the action of LDL on vasodilatation to ACh in isolated rat mesenteric arteries: 250μg/ml LDL, Krebs-Ringer solution, 250μg/ml LDL + 1mM l-arginine. *$p<0.01$ vs. 250μg/ml LDL, **$p<0.04$ vs. Krebs-Ringer solution (2-factor ANOVA with replication and Bonferroni correction, n=8). All values are the mean $\pm$ SEM (n=8).

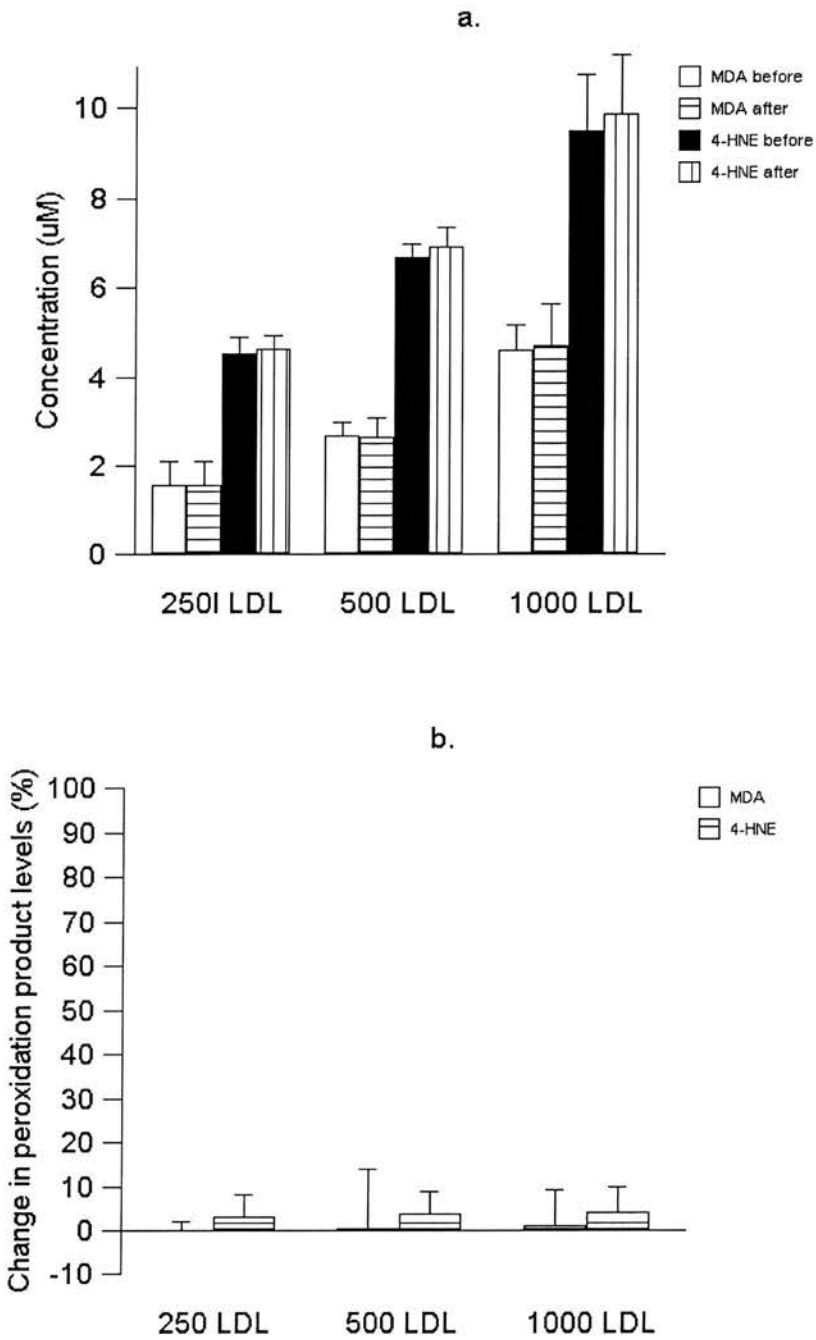


Figure 5.8. Alterations in lipid peroxidation product levels: a.) Concentration of MDA and 4-HNE present in 250μg/ml, 500μg/ml and 1000μg/ml LDL solutions before and after luminal perfusion. b.) Mean percentage change in the concentration of MDA and 4-HNE following luminal perfusion. All values are mean ± SEM (n=8).

5.4 DISCUSSION

The results show that luminal perfusion with LDL inhibits vascular function in isolated rat mesenteric arteries, resulting in the marked attenuation of endothelium-dependent vasodilatation to increasing concentrations of ACh. Vasodilatation to SNP was not significantly affected. The effects of LDL were similar to those of L-NAME, and supplementation of LDL with L-NAME did not augment vasodilatation LDL-induced impairment of vasodilatation to ACh. This impairment was reversible upon washout, and could be largely reversed by l-arginine supplementation, but was not affected by SOD.

5.4.1 The effect of LDL on vascular function

Protein and cholesterol constitute approximately 20% and 33% of the weight LDL, respectively (Lewis *et al*, 1997; Abuja, 1999). Therefore, 1mg/ml LDL contains 200μg LDL protein/ml and 330μg LDL cholesterol/ml (Esterbauer *et al,* 1991; Lewis *et al*, 1997; Bagnati *et al,* 1999). Consequently, the LDL samples used in this study (250, 500 and 1000μg/ml protein) equate to 1.25, 2.5 and 5mg/ml LDL, and should contain approximately 412.5, 825, and 1650μg/ml, or 1.65mg/ml, LDL cholesterol respectively. Hypercholesterolaemic patients have a total cholesterol concentration greater than 2.4mg/ml (6mmol/l) (Chowienczyk *et al,* 1992; Ting *et al,* 1997; Wilkinson & Cockroft, 1998), and LDL cholesterol greater than 1.6mg/ml (Ting *et al,* 1997; Hornig *et al,* 1998). Similarly, hypercholesterolaemic patients have a plasma LDL protein concentration of approximately 2mg/ml or greater (Jacobs *et al,* 1990), while the physiological concentration is approximately 1mg/ml (Lees *et al,* 1970; Schonfeld *et al,* 1974; Beghin *et al,* 2000). As a result, the concentrations of LDL used in the present study are relevant to human physiology and pathophysiological conditions.

Luminal perfusion with LDL enhanced vasoconstriction to PE in a concentration-dependent manner when compared to the physiological control. However, the maximal response was only increased by approximately 10% at the highest concentration of LDL used, whilst there was no significant difference in any of the calculated EC_{50} values. It has previously been demonstrated that LDL has little direct constrictor effect upon isolated blood vessels in the absence of preconstriction, but may constrict vessels that have been pretreated with a contractile agonist (Galle *et al,* 1990; Simon *et al,* 1990; Murohara *et al*, 1994). This is likely to be mediated by the inhibition of basal NO release from the EC, which occurs to prevent excessive vasoconstriction (Bullock *et al,* 1986; Moncada *et al*, 1991; Dora *et al*, 1997; Dora *et al*, 2000).

ACh-induced endothelium-dependent vasodilatation was impaired by LDL. This inhibition was LDL concentration-dependent, with 1000μg/ml causing approximately 78% inhibition (Fig. 5.2). The failure of L-NAME to significantly enhance the inhibition of vasodilatation when added to the 250μg/ml LDL solution demonstrated that the prevention of NOS activity did not augment the inhibition of endothelium-dependent vasodilatation by LDL (Fig 5.5). This suggests that all endothelial NO-dependent vasodilatation was impaired, and implies that the deleterious action of LDL is largely caused by the impairment of endothelial NO production and may be mediated via an inhibitory action upon eNOS. Conversely, the results produced may also suggest that LDL impairs endothelium-mediated NO-independent vasodilatation. It has previously been demonstrated (Chapter 3) that NO accounts 58% of vasodilatation in this experimental model. However, luminal exposure to higher concentrations of LDL (500 and 1000μg/ml) caused a 66% and 78% reduction in vasodilatation to ACh, respectively, suggesting that LDL may also impair vasodilatation to other EDRF's. This is supported by the failure of 1mM l-arginine to fully restore vasodilatation to ACh, demonstrating that there is a component of the LDL-induced inhibition of endothelium-mediated vasodilatation that is independent of the NO system. It has been demonstrated that the role of prostacyclin as an EDRF in this experimental model is

minimal (Chapter 3). Therefore, the NO-independent inhibition is likely to be caused by the impairment of EDHF-mediated vasodilatation.

There was no significant reduction in vasodilatation to SNP when the arterial lumen was perfused with LDL compared to the physiological control (Fig 5.3). This is in agreement with numerous studies that have demonstrated that LDL does not alter vasodilatation to exogenous nitrosovasodilators, such as SNP and GTN (Andrews *et al*, 1987; Jacobs *et al,* 1990; Lewis *et al,* 1997; Hein & Kuo, 1998; Fontana *et al,* 1999; Jiang *et al,* 2001; Ji *et al,* 2003). However, LDL reduced dilatation to SNP when compared to vasodilatation in denuded arteries (Fig 5.3, Table 5.3). This may initially appear surprising, but could occur as the inhibitory effects of LDL are mediated via the endothelium. For example, LDL may promote the excess endothelial generation of $O_2^{\cdot -}$ which could potentially impair endothelium-independent vasodilatation to SNP by inactivating the NO released. Endothelial denudation would remove this and enhance the endothelium-independent vasodilatation produced.

5.4.2 Mechanism of action of LDL on vascular function

The results of the current study have demonstrated that LDL impairs endothelial NO-dependent vasodilatation. It is unlikely that the effects of LDL are mediated by the endocytosis of LDL, as LDL inhibits ACh-mediated vasodilatation in aortic rings from Watanabe hereditary hyperlipidaemic rabbits (Bruckdorfer *et al*, 1988) which lack the functional high affinity receptors required for the endocytosis and degradation of LDL (Baker *et al*, 1984). The current study supports this, as these receptors are fully saturated at LDL concentrations of 100μg/ml in rabbits and rodents (Baker *et al*, 1984; Jacobs *et al,* 1990). However, increasing the concentration of LDL from 250μg/ml, at which the receptors should already be saturated in the rodent model used, to 500μg/ml and 1000μg/ml increased the level of inhibition induced.

The inhibitory action of all concentrations of LDL upon endothelium-dependent vasodilatation was reversible upon washout and reperfusion with physiological Krebs-Ringer solution (Fig 5.4). These results support the hypothesis that the inhibitory effect of LDL occurs via a direct impairment of NO-availability rather than a cytotoxic or injurious effect upon the EC. This may endorse an *in vivo* study that reported an improvement in impaired vasodilatation to ACh in hypercholesterolaemic patients following LDL apheresis (Tamai *et al*, 1997). The reversible nature of the inhibition also implies that the deleterious action of LDL is not mediated by the internalisation of LDL in the EC or accumulation in subendothelial space (Brown & Goldstein, 1979; Baker *et al,* 1984), as this LDL would not be washed out and the inhibition would not be reversed.

The relatively rapid nature of the inhibition also suggests that the LDL does not exert its inhibitory action by undergoing oxidation to ox-LDL as this requires prolonged exposure to transition metals or EC (Jacobs *et al*, 1990). Examination of MDA and 4-HNE in the lipoprotein samples before and after luminal perfusion implies that the LDL did not undergo further oxidative modification during the course of these experiments, and should therefore have been representative of unmodified LDL.

The four sites were LDL is most likely to exert an effect are shown in Fig. 5.9. It is unlikely that the inhibitory action of LDL is mediated by a kinetic phenomenon or effect, for example the binding of the LDL to ACh, as the technique used would not allow the LDL and ACh to come into contact and interact (Fig 5.9 **(1)**). The failure of LDL to impair vasodilatation to SNP establishes that LDL does not inactivate NO within the extracellular space (Fig 5.9 **(3)**) or alter VSMC function (Fig 5.9 **(4)**). This suggests that the inhibitory action of LDL is endothelium-specific (Ezaki *et al,* 1994). Therefore, it is highly likely that LDL inhibits NO-mediated vasodilatation by altering physiological eNOS activity (Fig 5.9 **(2)**).

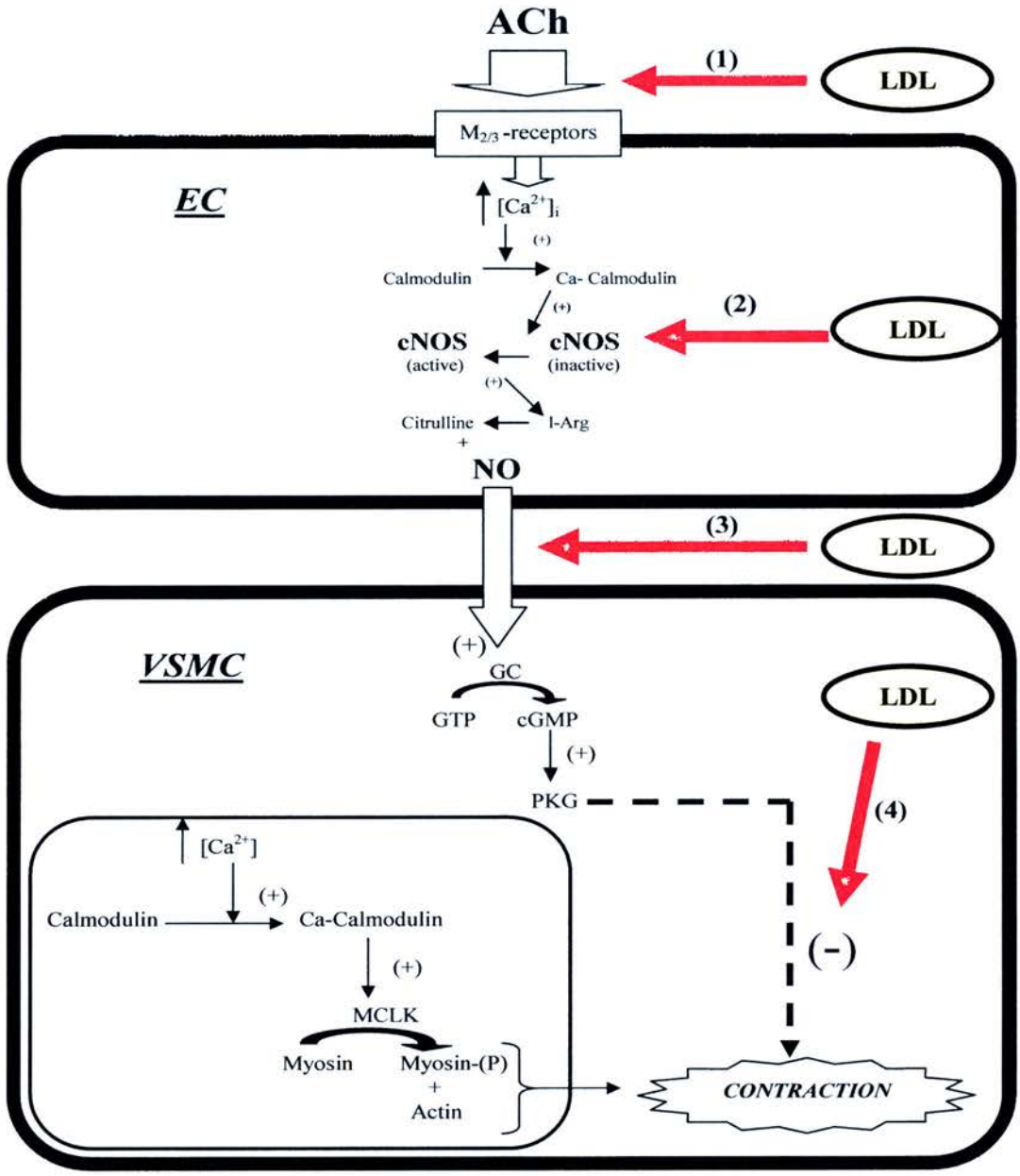


Figure 5.9. Potential sites where LDL may impair endothelium-dependent vasodilatation to ACh: 1) LDL may prevent the interaction between ACh and the $M_{2/3}$-receptor. 2) LDL may alter eNOS activity. 3) LDL may promote the inactivation of NO following the generation and release from EC. 4) LDL may inhibit the mechanism by which NO opposes agonist-induced increases in $[Ca^{2+}]i$ to inhibit contraction and induce relaxation.

5.4.3 The role of $O_2^{\cdot-}$ in LDL-induced vascular dysfunction

The current study has demonstrated that acute luminal exposure LDL impairs NO-mediated vasodilatation. However, numerous studies have demonstrated that eNOS activity and NO production are in fact enhanced in hypercholesterolaemic models or by exposure to LDL (Minor *et al*, 1990; Galle *et al*, 1991; Schaefer *et al,* 1993; Liao *et al,* 1995; Pritchard *et al*, 1995; Allen *et al,* 1998; Vidal *et al,* 1998). Under these conditions, endothelial NO may undergo enhanced oxidative degradation (Ohara *et al*, 1993; Ohara *et al*, 1995; Pritchard *et al*, 1995; Vergnani *et al*, 2000). This is commonly attributed to a LDL-induced imbalance in eNOS activity that increases eNOS-mediated $O_2^{\cdot-}$ generation. Hypercholesterolaemic rabbits generate 3 to 5 fold more $O_2^{\cdot-}$ than control animals (Ohara *et al*, 1993; Ohara *et al*, 1995). When produced in close proximity, $O_2^{\cdot-}$ reacts readily with NO to decrease NO availability. It was therefore hypothesised that the inhibition of vasodilatation by LDL is a result of increased oxidative degradation of NO due to $O_2^{\cdot-}$ generation within the vascular wall.

The current study examined the role of $O_2^{\cdot-}$ in LDL induced vascular dysfunction by supplementing 250μg/ml LDL solution with exogenous SOD. SOD is the major tissue defence against the harmful effects of $O_2^{\cdot-}$. Endogenous SOD and SOD mimetics facilitate the dismutation of $O_2^{\cdot-}$(Kasten *et al*, 1994; Kasten *et al*, 1995; Mian & Martin, 1995) at a rate of $2x10^9M^{-1}s^{-1}$ (Huie & Padmaja, 1993). The potential restoration of vasodilatation by the addition of SOD to the LDL solution would demonstrate the role of $O_2^{\cdot-}$ in the impairment of vascular function. However, supplementation with SOD had no significant effect upon the response to ACh in the current study.

This could suggest that $O_2^{\cdot-}$ generation is not involved in the inhibitory action of LDL. However, there are several other possible explanations for this finding. NO is rapidly inactivated by $O_2^{\cdot-}$ at a diffusion-limited rate (Huie & Padmaja, 1993), and although the reaction rates are similar, the reaction between of $O_2^{\cdot-}$ and NO is over 3 times faster

than the dismutation of $O_2^{\cdot-}$ by SOD (Ducroq, 1999). As a result, the reaction between excessive concentrations of $O_2^{\cdot-}$ and NO is likely to occur before the reaction with SOD. Furthermore, exogenous SOD is unable to permeate cellular membranes and dismute intracellular sources of $O_2^{\cdot-}$ (Laight *et al*, 1998). SOD can reverse the inhibition of ACh-mediated vasodilatation caused by the extracellular generation of $O_2^{\cdot-}$ in both rat (Mian & Martin, 1995) and rabbit aorta (Abrahamsson *et al*, 1992; MacKenzie & Martin, 1998), but cannot prevent the inhibition caused by DECTA (Mian & Martin, 1995; Laight *et al*, 1998), which inhibits endogenous Cu-Zn SOD to generate intracellular oxidative stress (Heikkila *et al,* 1976). Therefore, the exogenous SOD used to supplement the LDL solution would only be able to exert an extracellular action, which could explain the negative results produced. These agree with a previous study that demonstrated the inability of exogenous SOD to restore endothelial function following acute exposure to LDL *in vitro* (Plane *et al*, 1993).

It has previously been concluded, using exogenous SOD *in vivo,* that endothelial dysfunction in hypercholesterolaemic patients is not due to increased extracellular NO breakdown by $O_2^{\cdot-}$ (Garcia *et al*, 1995). The failure of exogenous SOD to restore vasodilatation may indirectly suggest that LDL-induced endothelial dysfunction is caused by the intracellular inactivation of NO. This has previously been demonstrated by the cell permeable SOD mimetic, manganese (III) tetra is (1-methyl-4-pyridyl) porphyry (MnTMPyP), which can react with $O_2^{\cdot-}$ at a rate constant of $1x10^9 M^{-1}s^{-1}$ (Faulkner *et al*, 1994), and restores vasodilatation in aortic rings (Fontana *et al*, 1999) or isolated conduit arteries exposed to LDL (Hein & Kuo, 1998).

Nevertheless, the current study does not support the hypothesis that exogenous SOD can restore endothelial function following luminal exposure to LDL. However, this does not discount the hypothesis that LDL-induced vascular dysfunction is due to increased oxidative stress within the vascular wall, as the ability of exogenous SOD to combat this may have impaired by an inability to gain endothelial intracellular access.

Consequently, it is suggested that LDL-induced vascular dysfunction is mediated by the intracellular generation of $O_2^{\cdot-}$ and resultant inactivation of NO.

The generation of $O_2^{\cdot-}$ may be attributed an imbalance in eNOS activity caused by the impairment in l-arginine metabolism (Pritchard *et al,* 2002; Stepp *et al,* 2002). In an arginine-depleted environment, eNOS still accepts electrons from NADPH and becomes electron rich. Under these conditions eNOS activity uncouples and utilises molecular O_2 as a principal substrate to produce $O_2^{\cdot-}$ (Mayer *et al,* 1991; Klatt *et al,* 1993; Griffith & Steuhr, 1995; Pritchard *et al,* 1995), making eNOS a further source of oxidative stress (Shinozaki *et al,* 1999). Huk *et al* previously proposed that LDL reduced l-arginine availability, resulting in a conformational change in eNOS that promoted the generation of $O_2^{\cdot-}$ from O_2 (Huk *et al*, 1997). Previous studies have demonstrated that l-arginine supplementation can prevent LDL-induced increases in endothelial $O_2^{\cdot-}$ generation and enhance NO-availability (Pritchard *et al*, 1995; Huk *et al,* 1997; Vergnani *et al*, 2000). This is in accord with the current study, which demonstrates that the inhibitory action of LDL can be reversed by supplementation with 1mM l-arginine. This implies that LDL-induced endothelial dysfunction could occur, in part, via the inhibition of the utilisation of l-arginine by eNOS.

The restorative action of l-arginine supplementation may be specific for LDL-induced endothelial dysfunction, as this improves vascular function in hypercholesterolaemia (Creager *et al,* 1992; Clarkson *et al,* 1996; Wolf *et al,* 1997; Yin *et al,* 2005), but not in hypertension or insulin-dependent diabetes mellitus (MacAllister *et al,* 1995). The *Km* of eNOS for l-arginine is in the order of 1-10μM (Vallance, 1998; Bult *et al,* 1999; Hobbs *et al,* 1999) and the cellular l-arginine concentration ranges from 40μM to 1mM, respectively (MacAllister *et al,* 1995). Therefore, the supply of cellular l-arginine is excessive and should not be rate-limiting for the formation of NO. However, the restoration of NO-mediated vasodilatation in the present study could occur as supplementation increases the intracellular l-arginine concentration and shifts the

equilibrium towards the removal of the excess l-arginine, resulting in increased NO formation and availability.

There are several possible means by which LDL could impair l-arginine metabolism. The inhibition may be due to the formation of ADMA, an endogenous eNOS inhibitor, which accumulates at high levels in the plasma of hypercholesterolemic animals (Yu *et al*, 1994; Bode-Boger *et al*, 1996). This competes with l-arginine as a substrate for eNOS to prevent NO formation. Supplementation with l-arginine may increase the intracellular concentration to sufficient levels to compete with this, restore eNOS activity and normalise NO production (Boger *et al*, 1997). Alternatively, LDL could increase EC membrane rigidity through the donation of cholesterol (Pritchard *et al*, 1991). This would alter the lipid dynamics of the membrane, which may impede the binding, internalisation, and availability of l-arginine to eNOS. The resultant generation of $O_2^{\cdot-}$ could further impede the internalisation of l-arginine, as the primary means of l-arginine transport into EC, the system y^+ transporter (Ogonowski *et al*, 2000), is impaired by oxidants and oxidative stress (Patel *et al,* 1996; Ogonowski *et al*, 2000). Nevertheless, the demonstrated restoration of endothelial function by l-arginine suggests that LDL impairs intracellular l-arginine availability. This is likely to cause eNOS uncoupling and the endothelial generation of $O_2^{\cdot-}$, which in turn increases oxidative stress and reduces NO availability (Fig 5.10).

5.4.4 Tissue preparation

Contradictory results have been reported with regards to the effect of LDL upon isolated vessel preparations. This may be due to the techniques applied, as the action of LDL upon vascular function *in vitro* could be dependent upon the experimental preparation used. Galle *et al*. reported that incubation with LDL did not impair

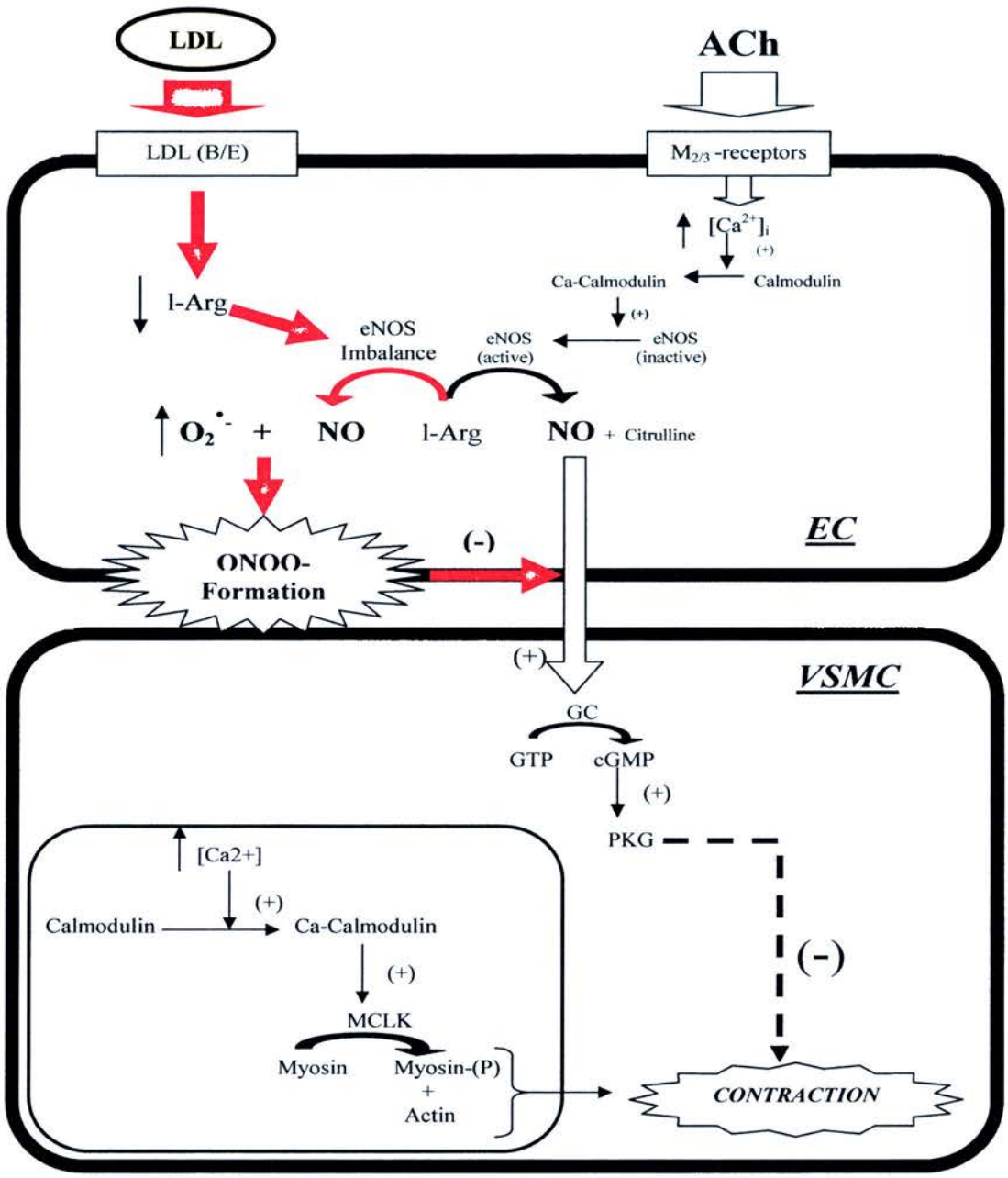


Figure 5.10. The proposed mechanism of LDL-induced endothelial dysfunction. From the experiments performed in this chapter, it is hypothesised that LDL impairs l-arginine metabolism to cause an imbalance in eNOS activity. This inturn increases eNOS-generated $O_2^{\cdot-}$ which then inactivates endothelial NO to reduce NO bioavailability and impair NO-mediated vasodilatation.

endothelium-dependent vasodilatation in intact rabbit femoral artery segments, but did significantly inhibit the responses in arterial ring preparations (Galle *et al*, 1991). Similarly, several other *in vitro* studies have demonstrated the impairment of endothelium-dependent vasodilatation using ring or strip preparations (Andrews *et al*, 1987; Tomita *et al*, 1990; Takahashi *et al,* 1990; Jacobs *et al,* 1990; Plane *et al,* 1993; Lewis *et al*, 1997; Fontana *et al,* 1999). The immersion of ring or strip preparations in the LDL solution allows the lipoprotein to act directly upon the smooth muscle layers, allowing rapid infiltration into the vessel wall. Consequently, the impaired vasodilatation may not be exclusively attributable to the action of LDL upon the endothelium, but could result from an action of the LDL directly upon the VSMC.

Up to this point, the majority of *in vitro* vessel studies examining LDL have immersed ring preparations mounted under isometric conditions (See above). Perfusion myography is more technically demanding and time-consuming than wire myography, but may provide the optimal technique for assessing the impact of LDL on endothelial function. This technique allows the vessels to be exposed to LDL exclusively via the endothelial surface under physiological pressures, thus removing any influence produced by the direct interaction of LDL with VSMC. As a result, the inhibition of endothelium-dependent vasodilatation observed in this study may be directly attributed to an action upon the endothelium and mitigates any direct contractile effect of LDL on VSMC. Furthermore, this technique also prevents any interaction or inactivation of the vasoactive agents, such as PE or ACh, with the LDL solution. Therefore, the conditions used in this study may better replicate the *in vivo* interaction between LDL, EC and vascular function when compared to experimental models used by other studies.

5.4.5 The Oxidative Status of LDL

It is has previously been demonstrated that LDL does not require oxidative modification to induce endothelial dysfunction (Chowienczyk *et* al, 1992; Drexler & Zeiher, 1991; Zeiher *et al*, 1991). The current results appear to support this, but in order to attribute the impairment of vascular function to the action of unoxidised LDL it must be ensured that there was no oxidative modification of the LDL. Such a change could alter the effect upon vascular function (Liao & Clark, 1995) and eNOS expression (Liao *et al*, 1995; Hirata *et al*, 1995). The LDL used in these experiments may have undergone minor degrees of oxidation *in vitro* or prior to the experiments being performed, due to variations in the initial oxidative state, the antioxidant content of the LDL samples, or spontaneous oxidation. This could explain any observed variation in the responses to the vasoactive agents in the individual experiments. However, throughout the experiments special care was taken to avoid any oxidative modification. The lipoprotein should have been representative of unmodified LDL as it was collected from fresh blood samples, and there is little direct evidence that ox-LDL exists within circulation (Frei *et al*, 1988; Bowry *et al*, 1992; Bowry & Stocker 1993). Furthermore, the LDL subfractions were immediately frozen at –80°C following isolation and purification.

It is also unlikely that the oxidative modification of LDL would have occurred *in vitro* during these experiments, as this requires prolonged exposure to transition metals or EC within the vascular wall (Jacobs *et al,* 1990; Cox & Cohen, 1996). 0.3mM EDTA was also added to the LDL to prevent any oxidation *ex vivo* and *in vitro*. This is an established technique that prevents oxidative modification (Jacobs *et al*, 1990; Hayashi *et al,* 1994), without altering vascular function to endothelium-dependent or independent vasodilators (Green *et al,* 1999). Supplementation with EDTA is extensively used in studies examining the effect of LDL in isolated vessels and has no effect upon the vascular responses produced (Andrews *et al,* 1987; Jacobs *et al*, 1990;

Galle *et al,* 1991; Plane *et al,* 1993; Hayashi *et al,* 1994; Lewis *et al,* 1997; Fontana *et al,* 1999; Vergnani *et al*, 2000). The reversibility of the induced endothelial dysfunction also suggests that the lipoprotein used was not oxidised, as ox-LDL produces an inhibition that is partially or completely irreversible (Jacobs *et al*, 1990; Simon *et al*, 1990; Plane *et al*, 1992). However, without adequate detection or analysis of the oxidative state of the LDL, the observed effects cannot be attributed to LDL. Therefore, MDA and 4-HNE concentrations were measured as indices of lipid peroxidation (Esterbauer & Cheeseman, 1990). There was no significant alteration in the concentration of these before and after the experiments were performed, demonstrating that modification did not occur.

5.4.6 Study Limitations

As previously, the maximum constriction to KCl was performed in the presence of the various test solutions rather than Krebs-Ringer only. This could have masked any effect the test solutions had on the maximal vasoconstriction to PE. Another limitation of the current study is that only one endothelium-dependent vasodilator, ACh, was used. The inhibitory effect of LDL may be concentration-dependent, as lower concentrations selectively inhibit ACh-mediated dilatation but at higher concentrations this selectivity is less apparent (Flavahan, 1992). For example, at concentrations of 200μg/ml or greater, LDL may inhibit vasodilatation to several agonists (Lewis *et al*, 1997). Therefore, while it has been demonstrated that acute luminal perfusion with LDL inhibits endothelial function in isolated mesenteric arteries, it cannot be determined if the effects are selective for ACh or generalisable to other agonists. Another limitation is the addition of human LDL to rodent tissue. Previous studies have used a similar method to examine the effects of LDL, although the arteries used were from a different species of rat (Sprague-Dawley) (Lewis *et al*, 1997). The concentrations of LDL used to perfuse the isolated arteries in this study were relevant

to the rodent model used, as the plasma LDL concentration in WKY rat is approximately 240μg/ml (Kitts *et al,* 1998). Finally, this study can only speculate that the action of LDL occurs via the intracellular inactivation of NO by $O_2^{\cdot -}$ as exogenous SOD, which exerts an extracellular action, failed to restore vasodilatation. The experiments should be repeated using a SOD-mimetic, such as MnTMPyP, before a definitive conclusion can be made regarding the role and site of action of $O_2^{\cdot -}$ in LDL induced endothelial dysfunction.

5.4.7 Summary

The current study demonstrates that luminal exposure to various concentrations of LDL in a perfusion myograph model induces endothelial dysfunction in a concentration-dependent manner. This is caused by the inhibition of NO-mediated vascular function, as demonstrated by the experiments using L-NAME. The restoration of vascular function by l-arginine supplementation implies that this inhibition is due to the impairment of l-arginine availability following acute endothelial exposure to LDL. This is likely to result in an uncoupling in eNOS activity, which causes eNOS to generate $O_2^{\cdot -}$, and in turn, reduce NO availability and inhibit NO-mediated vasodilatation. The inhibition of vasodilatation by LDL is reversible and demonstrates that LDL is not cytotoxic or directly injurious to EC. These results suggest a causal role for LDL in cardiovascular disease and demonstrate that it does not require oxidative modification to induce endothelial dysfunction. This deleterious action of LDL has been demonstrated using perfusion myography and intact arterial segments, rather than immersed strips or ring preparations. Use of this technique removed any direct influence that the addition of LDL may have had upon the VSMC, to better replicate the *in vivo* interaction of LDL with endothelial and vascular function.

CHAPTER 6

THE EFFECT OF URIC ACID ON VASCULAR DYSFUNCTION INDUCED BY LDL

6.1 INTRODUCTION

Hypercholesterolaemia is strongly associated with vascular dysfunction (Zeiher *et al,* 1993; Anderson *et al,* 1995; Wilkinson & Cockroft, 1998). The mechanism that produces this is attributed to lipoprotein-induced damage of the endothelial lining of the blood vessels. The capacity of LDL to induce vascular dysfunction was established in the previous chapter, using isolated rat mesenteric arteries mounted in a perfusion myograph. The inhibition of vascular function was attributed to the onset of endothelial dysfunction, mediated by the impairment of l-arginine availability (Chapter 5). This increases eNOS-generated free radical production and oxidative stress within the vascular wall (Pritchard *et al*, 1995; Shinozaki *et al,* 1999), which reduces NO availability and prevents NO-mediated vasodilatation (Huie & Padmaja, 1993). This increased oxidative stress also potentiates the oxidative modification of LDL to further enhance the development of endothelial dysfunction and cardiovascular disease (Steinberg *et al,* 1989; Esterbauer *et al,* 1992).

The human body has an extensive antioxidant defence system to protect against this. Several of these antioxidants may protect against the development of endothelial dysfunction (Plane *et al,* 1993; Brown & Goodman, 1998; Fontana *et al,* 1999). UA is an established hydrophilic antioxidant in human physiology (Ames *et al,* 1981; Cross *et al,* 1992; Becker, 1993; Maxwell *et al*, 1997), and the potent radical scavenging ability (Maxwell *et al*, 1997; Nyyssonen *et al,* 1997; Nieto *et al*, 2000) of this compound could confer several advantages in conditions of increased oxidative stress, such as LDL-induced endothelial dysfunction. UA could potentially prevent the deleterious actions of LDL upon vascular function by scavenging the free radicals generated within the vascular wall, to maintain NO-availability and protect endothelium-dependent vasodilatation. In point of fact, elevated UA levels are frequently associated with hypercholesterolaemia (Wannamethee *et al,* 1997).

However, the potential role of increased serum UA concentrations in this pathophysiological condition remains unclear. For example, under certain conditions UA can prevent (Davies *et al,* 1986; Schlotte *et al*, 1998; Abuja, 1999; Bagnati *et al,* 1999), or promote (Schlotte *et al*, 1998; Abuja, 1999; Bagnati *et al*, 1999; Patterson *et al,* 2003) the oxidative modification of LDL and enhance oxidative-damage (Vasquez-Vivar *et al*, 1996; Santos *et al,* 1999). Several epidemiological studies have shown that elevated UA, rather than conferring a physiological advantage, is associated with the development of cardiovascular disease (Fessel, 1980; Brand *et al*, 1985; Bonora *et al*, 1996; Persky *et al*, 1997; Bengtsson *et al,* 1998; Ward, 1998; Liese *et al* 1999; Fang & Alderman, 2000).

Nevertheless, it has been demonstrated in this thesis (Chapter 4) that physiological and supraphysiological concentrations of UA do not directly impair vascular function. This suggests that UA does not directly contribute to the development of cardiovascular disease, or pathophysiological conditions that predispose to this, by the induction of endothelial dysfunction. Therefore, it is logical to investigate a potential protective role for UA in cardiovascular disease, by examining the ability of UA to mitigate the harmful effect of LDL upon vascular function.

The aim of this chapter is to examine the effect of UA supplementation upon LDL-induced vascular dysfunction, following acute luminal exposure, in isolated rat mesenteric arteries. The following hypotheses are examined in this chapter:

1). UA is a powerful chain-breaking antioxidant that protects against the inhibitory effects of LDL on endothelial function by scavenging free radicals to reduce oxidative stress and increase NO-availability within the vascular wall.

2.) The ability of UA to prevent the inhibitory action of LDL is concentration-dependent, as increasing concentrations of UA confer greater protection upon vasodilatation.

3.) The effect of UA on LDL-induced endothelial dysfunction results from its ability to increase the availability of endothelium-derived NO and will not be apparent in the absence of NO synthase activity.

6.2 METHODS

6.2.1 Lipoprotein Isolation

LDL was isolated, purified and the protein concentration determined using a commercial kit based on the Lowry precipitation assay (Sigma Chemical Company, Dorset, U.K.), as previously described (Chapter 2).

6.2.2 The effect of UA upon the action of LDL on vascular function

Adult male Wistar Kyoto rats (250-300g) were killed, the mesenteric beds removed, and a segment of a 3rd order branch of the mesenteric arteries mounted in the perfusion myograph chamber as described in Chapter 2. Concentration-response curves to PE, ACh and SNP were performed when perfused with Krebs-Ringer buffer, 250μg/ml LDL solution, UA-supplemented 250μg/ml LDL solution (200μM, 400μM or 600μM UA), or 250μg/ml LDL solution supplemented with the vehicle Li_2CO_3 solution.

6.2.3 The effect of L-NAME upon the reversal of LDL-induced vascular dysfunction by UA

Arteries were mounted and perfused with 250μg/ml LDL solution, 250μg/ml LDL + 400μM UA, 250μg/ml LDL + 100μM L-NAME, 250μg/ml LDL + 400μM UA + 100μM L-NAME, or Krebs-Ringer buffer. The concentration-response curves to PE, ACh and SNP were then repeated.

6.2.4 Intraluminal Solutions

LDL solutions were prepared daily from stock by dilution with supplemented Krebs-Ringer solution to give an intraluminal concentration of 250μg/ml LDL. UA stock was prepared as previously described. The stock solution was diluted with Krebs-Ringer solution and used to supplement the LDL solutions to give a final intraluminal LDL concentration of 250μg/ml and UA concentrations of 200μM, 400μM or 600μM. For the purpose of the vehicle solution control, Li_2CO_3 stock was used to supplement the 250μg/ml LDL solutions to give final intraluminal concentration of 216μM. 1mM L-NAME stock solution was prepared as previously described in Chapter 3. L-NAME supplemented solutions were prepared daily by diluting stock LDL solution with Krebs-Ringer buffer, UA solution and stock L-NAME solution to give final intraluminal concentrations of 250μg/ml LDL, 400μM UA and 100μM L-NAME.

6.2.5 Lipid Peroxidation

Samples of the intraluminal LDL and UA-supplemented LDL solutions were collected before and after the concentration-response curves to the vasoactive drugs were performed to measure the extent of LDL lipid peroxidation and examine any changes in oxidative status. The MDA and 4-HNE concentrations of the intraluminal solutions were determined using a commercial colorimetric assay kit (CN Biosciences, Nottingham, U.K.).

6.2.6 Statistical Analysis

The ability of UA to prevent the effect of LDL upon vascular function was determined by comparing the results obtained for the various concentrations of UA with each other,

with LDL only, with the physiological control responses. The effect of the Li_2CO_3 vehicle solution on LDL was analysed by comparing the vascular responses to those obtained when the vessels were perfused with LDL only. The significance of L-NAME upon the reversal of LDL induced vascular dysfunction by UA was analysed by comparing the vascular responses when perfused with LDL + UA + L-NAME to the responses when perfused with LDL + UA, LDL + L-NAME, LDL only or the physiological control. Statistical analysis was also performed using paired Student's *t*-tests to compare the oxidative status of the intraluminal LDL solutions before and after luminal perfusion.

6.3 RESULTS

6.3.1 The effect of UA upon the action of LDL on vascular function

6.3.1.1 The effect of UA upon the action of LDL on vascular contractility to PE

PE produced concentration-dependent vasoconstriction in arteries perfused with physiological Krebs-Ringer solution, giving a maximal response of 99.1 $\pm$ 2.3% (Fig 6.1). 250μg/ml LDL enhanced vasoconstriction to PE at concentrations from $1x10^{-7}$ to $1x10^{-6}$ ($P<0.01$). The concentration-dependent responses to PE were similar when perfused with the physiological solution or the UA-supplemented LDL solutions. However, supplementation of LDL with 200μM ($1x10^{-7}$-$1x10^{-5}$, $P<0.01$), 400μM ($1x10^{-8}$-$1x10^{-5}$, $P<0.03$) and 600μM UA ($1x10^{-8}$-$1x10^{-5}$, $P<0.03$) significantly reduced vasoconstriction when compared to LDL alone. Supplementation with Li_2CO_3 had no effect upon vasoconstriction when compared to LDL, but significantly increased vasoconstriction when compared to the physiological control ($5x10^{-8}$-$1x10^{-6}$, $P<0.04$).

6.3.1.2 The effect of UA upon LDL-induced endothelial dysfunction

ACh produced concentration-dependent vasodilatation with a maximal response of 101.5 + 2.3% in the physiological control. Perfusion with LDL markedly impaired this ($P<0.01$; Fig 6.2) and produced a maximal dilatation of 41.1 + 4.3%. Supplementation of the perfusing LDL solution with 200μM, 400μM or 600μM UA significantly increased the concentration-dependent responses to ACh, when compared to LDL only ($1x10^{-7}$-$5x10^{-4}$, $P<0.01$), and produced maximal vasodilatations of 62.2 + 4.8%, 69.5 + 1.3% and 74.4 + 3.4%, respectively. However, the vasodilatation was still significantly lower than the physiological control ($1x10^{-8}$-$5x10^{-4}$, $P<0.01$). Supplementation with 600μM UA produced greater restoration of vasodilatation than 400μM, which produced

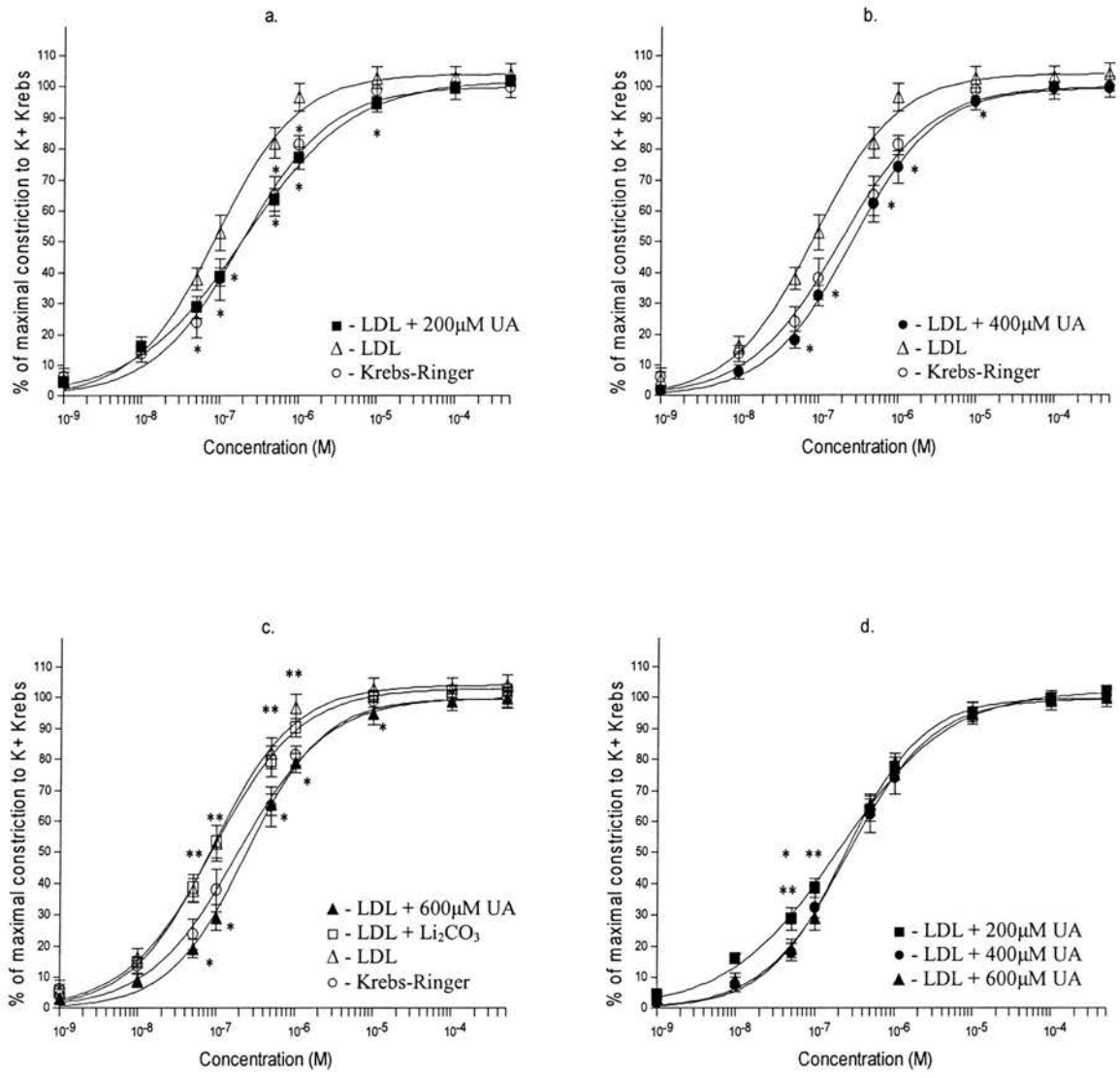


Figure 6.1. The effect of UA on vasoconstriction to PE in isolated rat mesenteric arteries perfused with LDL: a.) 200μM UA, b.) 400μM UA, and c.) 600μM UA or Li_2CO_3 solution compared to Krebs-Ringer solution or 250μg/ml LDL. *$p<0.03$ vs. 250μg/ml LDL, **$p<0.04$ vs. Krebs-Ringer solution (2-factor ANOVA with replication and Bonferroni correction, n=8). d.) compares the effect of each concentration of UA. *$p<0.01$ vs. LDL + 400μM UA, **$p<0.02$ vs. LDL + 600μM UA (2-factor ANOVA with replication and Bonferroni correction, n=8). All values are mean ± SEM (n=8).

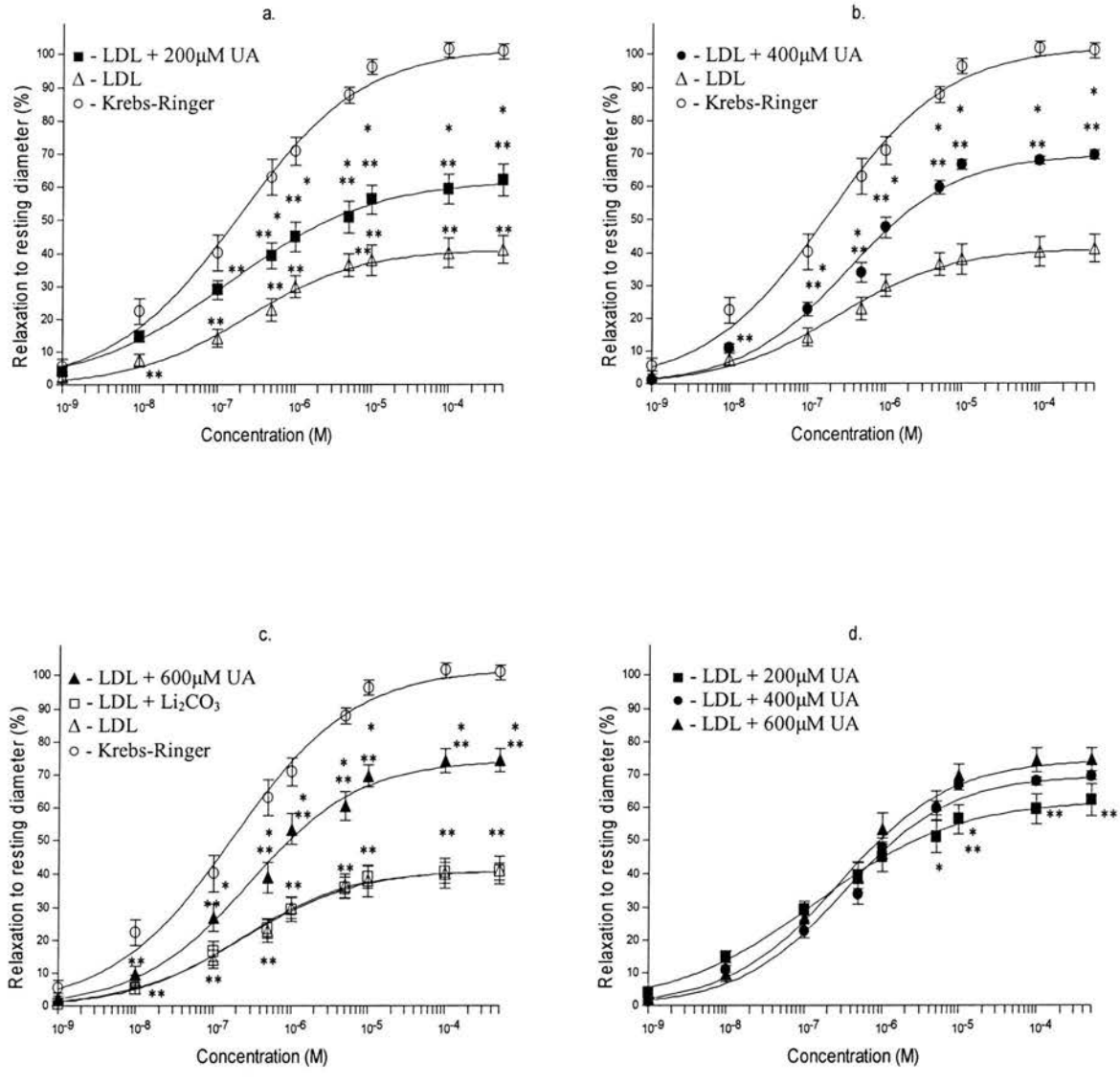


Figure 6.2. The effect of UA on vasodilatation to ACh in isolated rat mesenteric arteries perfused with LDL: a.) 200μM UA, b.) 400μM UA, and c.) 600μM UA or Li_2CO_3 solution compared to Krebs-Ringer solution or 250μg/ml LDL. *$p<0.01$ vs. 250μg/ml LDL, **$p<0.01$ vs. Krebs-Ringer solution (2-factor ANOVA with replication and Bonferroni correction, n=8). d.) compares the effect of each concentration of UA. *$p<0.05$ vs. LDL + 400μM UA, **$p<0.02$ vs. LDL + 600μM UA (2-factor ANOVA with replication and Bonferroni correction, n=8). All values are mean ± SEM (n=8).

greater vasodilatation than 200μM UA ($5x10^{-6}$-$1x10^{-5}$, $P<0.05$). Supplementation of LDL with Li_2CO_3 had no effect upon the inhibitory action of LDL, and vasodilatation was significantly reduced when compared to the physiological control ($1x10^{-8}$ -$5x10^{-4}$, $P<0.01$).

6.3.1.3 The effect of UA upon the action of LDL on endothelium-independent vasodilatation to SNP

Concentration-dependent vasodilatation to SNP was similar when the arteries were perfused with the physiological, LDL, or supplemented LDL solutions (Fig 6.3). However, the supplementation of LDL with 200 and 400μM UA significantly enhanced vasodilatation to $1x10^{-7}$ ($P<0.03$), and $1x10^{-7}$ and $5x10^{-7}$ SNP ($P<0.03$) respectively, when compared the LDL only,.

6.3.2 The effect of L-NAME upon the reversal of LDL-induced endothelial dysfunction by UA

Arterial perfusion with LDL + 400μM UA + 100μM L-NAME significantly impaired the concentration-dependent vasodilatation to ACh when compared to the physiological control ($1x10^{-8}$-$5x10^{-4}$, $P<0.01$; Fig 6.4) or LDL + 400μM UA ($5x10^{-7}$-$5x10^{-4}$, $P<0.01$), producing a maximal dilation of 39.7 $\pm$ 5.5%. However, the vasodilatation produced was similar to that obtained in the presence of LDL + 100μM L-NAME or LDL only. .

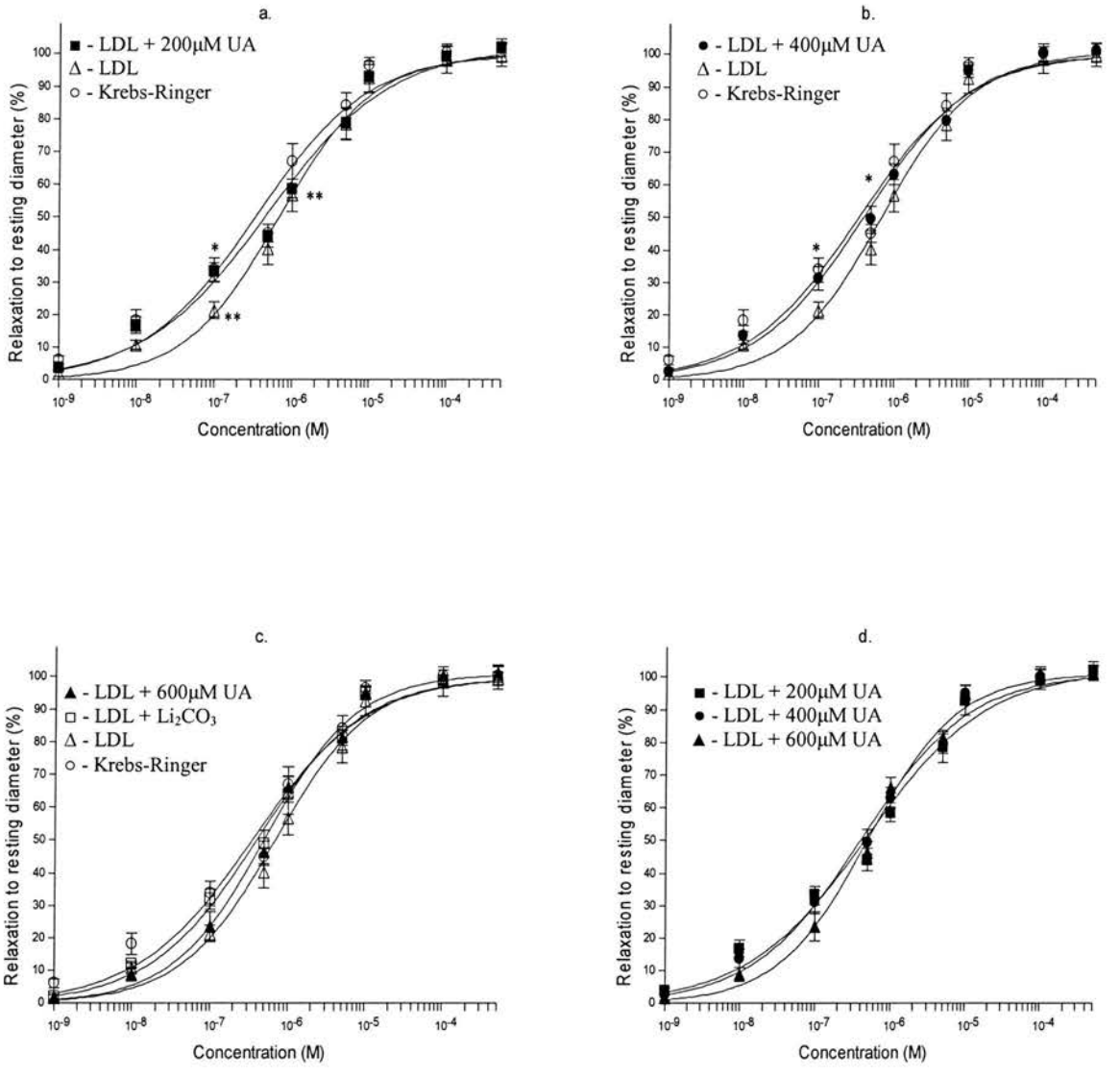


Figure 6.3. The effect of UA on vasodilatation to SNP in isolated rat mesenteric arteries perfused with LDL: a.) 200μM UA, b.) 400μM UA, and c.) 600μM UA or Li_2CO_3 solution compared to Krebs-Ringer solution or 250μg/ml LDL. *$p<0.03$ vs. 250μg/ml LDL, **$p<0.03$ vs. Krebs-Ringer solution (2-factor ANOVA with replication and Bonferroni correction, n=8). d.) compares the effect of each concentration of UA. All values are the mean $\pm$ SEM (n=8).

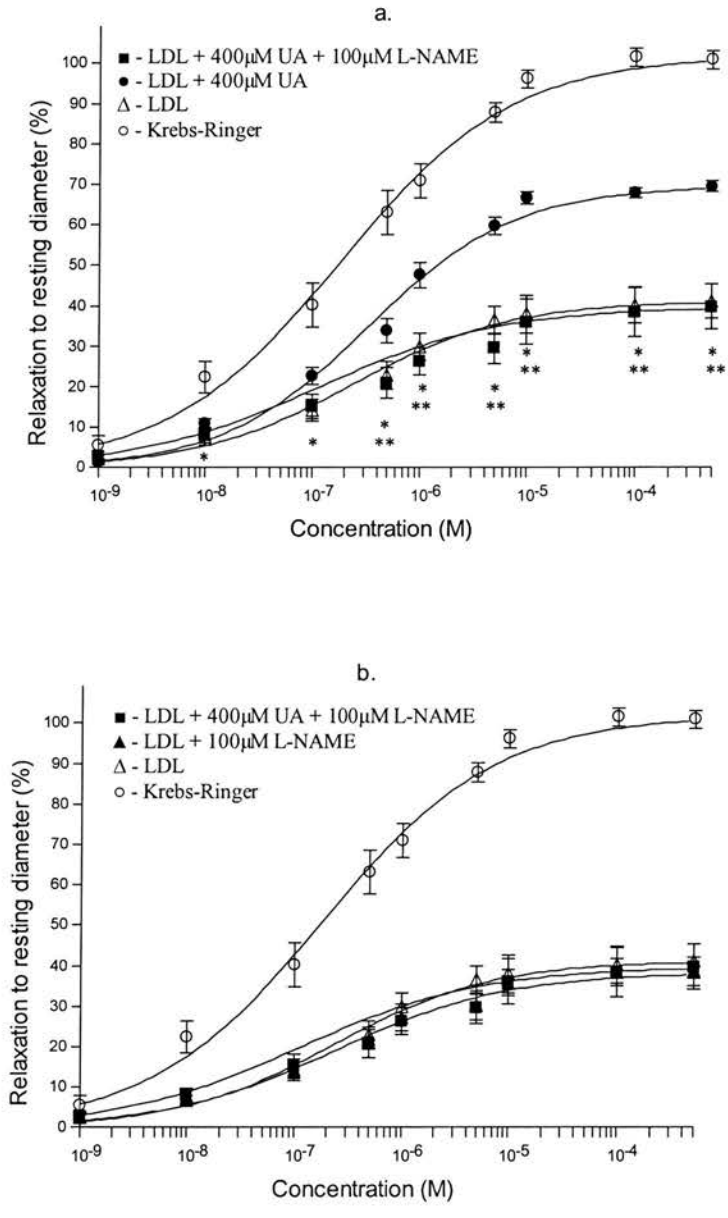


Figure 6.4. The effect of L-NAME on vasodilatation to ACh in isolated rat mesenteric arteries when perfused with LDL and UA: a.) The effect of LDL + 400μM UA + 100μM L-NAME is compared to LDL + 400μM UA, 250μg/ml LDL or Krebs-Ringer solution. *$p<0.01$ vs. Krebs-Ringer solution, **$p<0.01$ vs. LDL + 400μM UA (2-factor ANOVA with replication and Bonferroni correction, n=8). b.) The effect of LDL + 400μM UA + 100μM L-NAME is compared to LDL + 100μM L-NAME, All values are the mean ± SEM (n=8).

6.3.3 Lipid peroxidation

There was no significant alteration in the MDA and 4-HNE concentrations of the LDL and supplemented LDL solutions following luminal perfusion. This suggests that there was no change in the oxidative status of the LDL during the experiments (Fig 6.5).

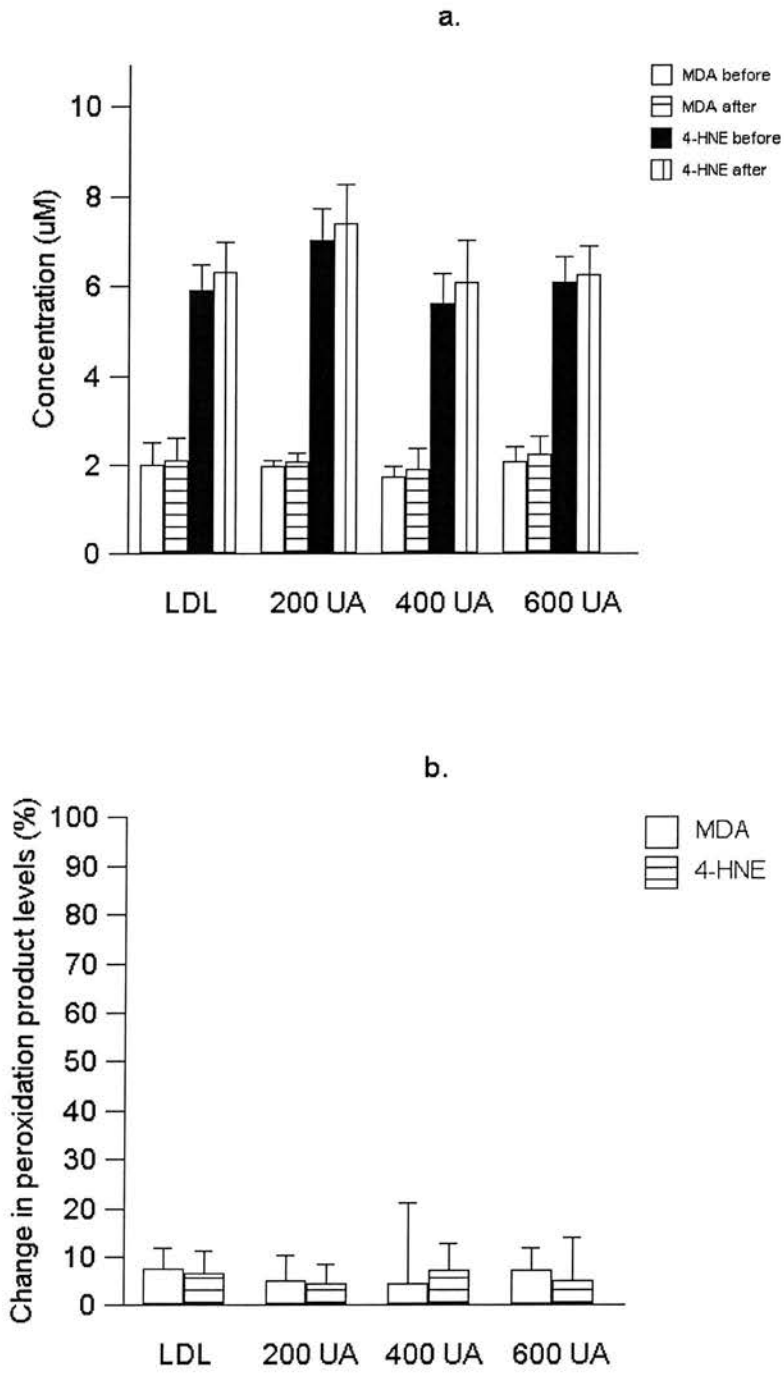


Figure 6.5. Alterations in lipid peroxidation product levels: a.) Concentration of MDA and 4-HNE present in 250μg/ml LDL, LDL + 200μM UA, LDL + 400μM UA and LDL + 600μM UA solutions before and after luminal perfusion. b.) Mean percentage change in the concentration of MDA and 4-HNE following luminal perfusion. All values are mean ± SEM (n=8).

6.4 DISCUSSION

In the previous chapter it was demonstrated that ACh-mediated vasodilatation is reduced in rat mesenteric arteries when they are exposed to 250μg/ml LDL. The results produced in this series of experiments demonstrate that supplementation of the LDL solution with increasing concentrations of UA restores endothelium-dependent vasodilatation to ACh in a concentration-dependent manner and prevents the potentiation and attenuation of the vasoactive responses to PE and SNP respectively. Furthermore, the restoration of endothelial function by UA can be prevented by L-NAME.

6.4.1 Mechanism of action of UA upon LDL-induced vascular dysfunction

The previous chapter demonstrated that luminal perfusion with LDL impaired NO-mediated vasodilatation to ACh in rat mesenteric arteries. This is thought to occur due to the stimulation of $O_2^{\cdot-}$ production in the vascular wall (Ohara *et al,* 1993; Vergnani *et al,* 2000). This reacts rapidly to inactivate NO and can also directly impair eNOS activity (Peterson *et al,* 1999), thus preventing the vasoactive actions of NO. Various pathophysiological conditions associated with elevated lipoproteins levels are accompanied by increased UA concentrations (Wannamethee *et al*, 1997).

In this study, UA improved the deleterious effects of LDL on endothelial function in a concentration-dependent manner. This was NO-dependent and could be prevented by the inhibition of eNOS by L-NAME. The significance of these results is that the concentrations used are equivalent to those that LDL would be exposed to in plasma in various *in vivo* conditions (200-600μM). This suggests that elevated UA *in vivo* could provide greater protection against the inhibitory effects of LDL on vascular function and

prevent the subsequent development of cardiovascular disease. This may be a potential reason for the continual generation and release of this antioxidant from the vascular endothelium, and the excessive concentrations found in pathophysiological conditions such as hypercholesterolaemia (Wannamethee *et al*, 1997).

The oxidation of LDL is known to enhance its deleterious effects on endothelial NO production (Vergnani *et al*, 2000), however, there was no evidence of any significant oxidation of the luminal LDL during our control experiments. This does not rule out mild degrees of oxidation, particularly in particles entering the vessel wall, but argues against the effect of UA being due to an antioxidant action causing a significant retardation of the oxidation of LDL. Instead, it may suggest that UA acts to attenuate the direct action of LDL upon NO-mediated vascular function. There are four possible sites of action were UA may impair the inhibitory action of LDL. UA could act independent of its antioxidant ability to directly prevent the uptake of LDL into the EC. Moreover, it could prevent the deleterious action of LDL upon l-arginine uptake and metabolism which causes the imbalance in NOS enzymatic activity and resultant generation of excess O_2^-. However, this seems highly unlikely, as there is no evidence of an interaction between UA and LDL uptake or the impairment of l-arginine availability, in the literature. Therefore, it is more probable that the restoration is a direct result of the antioxidant properties of UA. The two most probable sites were UA can prevent the LDL-induced inhibition of NO-mediated endothelial-dependent vasodilatation are shown in Figure 6.6. UA could prevent LDL-induced endothelial dysfunction by scavenging eNOS-generated $O_2^{\cdot-}$ to impair the inactivation of NO, thus restoring vascular function (Fig 6.6). Similarly, it is possible that UA could scavenge $ONOO^-$, produced by the rapid reaction between NO and $O_2^{\cdot-}$ within the EC (Fig 6.6). However, while the latter would prevent further oxidative damage to the endothelium, it may not necessarily restore NO-mediated vasodilatation.

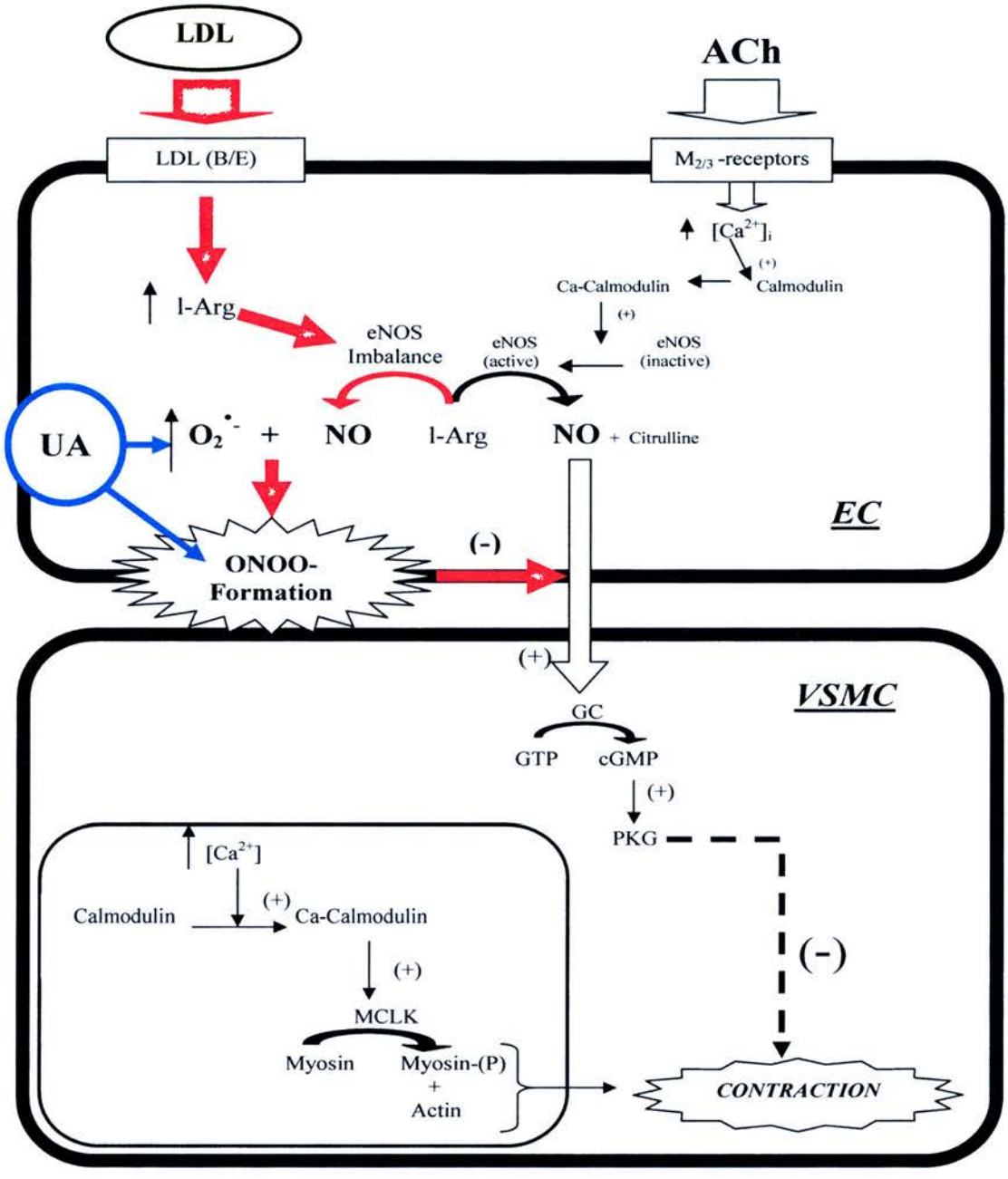


Figure 6.6. The proposed sites where UA may prevent LDL-induced inhibition of NO-mediated endothelial-dependent vasodilatation. It is hypothesised that UA scavenges either $O_2^{\bullet-}$ or $ONOO^-$ to restore endothelial NO-bioavailability.

6.4.2 UA and $O_2^{\cdot-}$

UA is a potent antioxidant and may be capable of reacting with and inactivating $O_2^{\cdot-}$ (Ames *et al,* 1981), although this has been disputed (Becker *et al,* 1989; Becker, 1993). The lack of restoration of vasodilatation when L-NAME was added to the UA and LDL solution demonstrates that the beneficial effects of UA are dependent upon eNOS activity. It is tempting to speculate that the restoration of vascular function by UA occurs via a simple antioxidant mechanism that removes $O_2^{\cdot-}$, generated by uncoupled eNOS, and improves the availability of NO. In the previous chapter (Chapter 5), the failure of SOD to prevent the deleterious action of LDL demonstrated that the inactivation of NO by $O_2^{\cdot-}$ was unlikely to occur in the extracellular space. NO is likely to be inactivated within the EC in the current experimental model, as the simultaneous generation of NO and $O_2^{\cdot-}$ within close proximity results in the rapid inactivation of NO at the site of production (Radi, 1998).

The major site of UA production in the cardiovascular system is the EC. The UA is then released to plasma and extracellular fluid compartments, such as lymphatic, interstitial and cerebrospinal (Becker, 1993). UA may readily permeate cellular membranes (Becker *et al,* 1989), via ionic and non-ionic means (Lucas-Heron & Fontenaille, 1979), and is not compartmentalised, as may occur with other radical scavengers. UA is able to cross cellular membranes (Kroll *et al,* 1992) and has the potential to act as an intracellular antioxidant in cells other than those where it is produced. Therefore, exogenous UA may be able to enter EC and prevent the inactivation of NO by scavenging $O_2^{\cdot-}$, thus preventing the inhibition of endothelial NO-mediated vasodilatation. However, this is unlikely as although it has been reported that UA may react with $O_2^{\cdot-}$ react at a rate constant of $8x10^8M^{-1}s^{-1}$ (Santus *et al,* 2001), NO is a far more likely reactant for $O_2^{\cdot-}$, in rapid diffusion-limited reaction with a rate of $6.9x10^9M^{-1}s^{-1}$. Therefore, the ability of UA to directly scavenge $O_2^{\cdot-}$ is debatable. Previous studies have demonstrated that UA does not attenuate the reduction of ferricytochrome C by

$O_2^{\cdot-}$, generated from allopurinol and XO (Becker *et al,* 1989), or the reduction of nitrotetrazolium blue by $O_2^{\cdot-}$ (Goshima *et al,* 1986). It has been demonstrated that the decomposition of UA to allantoin, a product of UA oxidation, only occurs in the presence of $O_2^{\cdot-}$ if the reaction conditions favour the secondary generation of $OH^{\cdot}$ i.e. if H_2O_2 is added to $O_2^{\cdot-}$, or, allowed to form from $O_2^{\cdot-}$ (Becker *et al,* 1989). As a result, it has been suggested that UA does not react directly with $O_2^{\cdot-}$ but rather with secondary formed intermediates (Becker *et al,* 1989; Becker, 1993). Therefore, UA is unlikely to be able to prevent the inactivation of NO by $O_2^{\cdot-}$. This may suggest that the enhanced NO-mediated vasodilatation that occurs in the current study is produced via a restorative, rather than preventative, action of UA upon NO availability.

6.4.3 UA and $ONOO^-$

The rapid inactivation of NO by $O_2^{\cdot-}$ leads to the formation of $ONOO^-$. This is a potent oxidant that causes cytotoxicity and cellular damage. It reacts with a variety of biomolecules such as lipids, proteins, and carbohydrates (Moreno & Pryor, 1992; Rubbo *et al,* 1994; Pryor & Squadrito, 1995; Salgo *et al,* 1995; Evans *et al,* 1996) and is implicated in the aetiology of numerous disease processes such as atherosclerosis (Radi, 1998). The half-life of this oxidant is estimated to be less than 100ms (Radi, 1998; Romero *et al,* 1999), which allows it to potentially travel 5-20μm across extracellular and/or intracellular compartments. It can freely permeate membranes (Finklestein & Cass, 1967), via anion channels ($ONOO^-$) and passive diffusion (ONOOH) (Marla *et al,* 1997; Denicola *et al,* 1998), and readily accesses hydrophobic compartments within cells and hydrophobic structures. $ONOO^-$ can accumulate in atherosclerotic plaques and may play an important role in membrane and lipoprotein lipid oxidation reactions. Furthermore, the life-span of $ONOO^-$ may be prolonged in hydrophobic environments, such as atherosclerotic lesions, than in the aqueous media. This could imply that

$ONOO^-$ undergoes a rapid degradation or inactivation in aqueous media which contains hydrophilic antioxidants.

UA is a major hydrophilic antioxidant and an endogenous scavenger of $ONOO^-$ (Hooper *et al,* 1997; Hooper *et al,* 1998). It is possible that the benefits of UA on vasodilatation relate to this well-recognised ability to scavenge $ONOO^-$ (Squadrito *et al,* 2000). This could prevent potential damage to the endothelium and eNOS (Milstien & Katusic, 1999). The reaction between UA and $ONOO^-$ has only been partially characterised but occurs at a rate of $5x10^2M^{-1}s^{-1}$ at pH 7.4 and 37°C (Santos *et al*, 1999). It has been demonstrated that UA can be readily oxidised by $ONOO^-$ to form intermediates that may be further oxidised, by $ONOO^-$, to metabolites such as allantoin, parabanic acid, urazole and oxonic acid (Skinner *et al*, 1998; Santos *et al,* 1999). Excessive production of $ONOO^-$ decreases UA levels in the environment of the site of $ONOO^-$ production and results in a reduced antioxidant capacity. In the presence of high UA concentrations, tyrosine nitration by $ONOO^-$ is largely inhibited (Sawa *et al*, 2000). Peroxidation of plasma lipids (Wayner *et al,* 1985; Frei *et al,* 1988) and erythrocyte membrane fatty acids (Ames *et al,* 1981; Smith & Lawing, 1983; Miki *et al,* 1989), and lipid peroxidation in isolated rabbit kidneys (Green *et al,* 1986) can also be prevented or inhibited by UA. However, as with the reaction between UA and $O_2^{\cdot-}$, it has been suggested that UA does not react rapidly or directly with $ONOO^-$. When present in relatively high concentrations, as found in the hyperuricaemic conditions associated with hypercholesterolaemia, UA reacts with secondary intermediates of $ONOO^-$ reactions (Skinner *et al,* 1998). Furthermore, the scavenging of $ONOO^-$ by UA may also form a UA-derived free radical that can propagate $ONOO^-$-mediated damage (Vasquez-Vivar *et al*, 1996) and enhance the oxidative modification of LDL (Santos *et al,* 1999).

In relation to the current study, the simple scavenging of $ONOO^-$ and subsequent oxidation of UA would not restore NO-mediated vasodilatation. This would not prevent

the inactivation of NO or necessarily restore NO-availability, although it is biologically plausible that this reaction could form a UA-based NO donor (Skinner *et al,* 1998). UA could also restore endothelium-dependent vasodilatation in the current study by preventing the inhibition of eNOS by $ONOO^-$ (Cosentino & Katusic, 1995; Pasquet *et al,* 1996; Milstien & Katusic, 1999). L-NAME prevents the beneficial action of UA and demonstrates that the restoration of vasodilatation is mediated via an eNOS-dependent process. The inhibition of eNOS by $ONOO^-$ is irreversible (Pasquet *et al,* 1996) and is mediated by the oxidation of the haem thiolate bond in the catalytic site of the enzyme (Pasquet *et al,* 1996), or via the oxidation of BH_4 (Cosentino & Katusic, 1995; Milstien & Katusic, 1999). However, the restorative action of UA demonstrates that eNOS activity is not impaired following endothelial exposure to LDL and could suggest that UA prevents the deleterious action of $ONOO^-$ on eNOS. Therefore, the $ONOO^-$-scavenging ability of UA has the potential to be involved in the restoration of vascular function following luminal exposure to LDL. However, further assessment is required as it remains to be determined if the restoration of vascular function is specific for UA in this experimental model.

6.4.4 Study Limitations

A limitation of the present study is that the LDL solution is supplemented with exogenous UA, and as such, the increase in UA concentration is not accompanied by the radical production that occurs during enzymatic UA production in pathophysiological conditions. Therefore, this study may not represent the entire *in vivo* conditions. Similarly, the vessels did not undergo chronic exposure to UA so there was no crystallisation or accumulation in the tissue, as occurs *in vivo* (Boogaerts *et al,* 1983). Furthermore, the vessels were not perfused with red blood cells and as such the pro-inflammatory effects of UA (Boogaerts *et al,* 1983; Duff *et al,* 1983; Hutton *et al*, 1985; Leyva *et al,* 1998; Mazzali *et al,* 2001; Mazzali *et al,* 2002; Kanellis *et al,* 2003) were

not demonstrated. These properties may damage the vascular wall and impair vascular function, thus enhancing the development of endothelial dysfunction and vascular disease. The results produced also only relate to vascular dysfunction produced using unmodified LDL. UA may prevent the oxidation of LDL but may induce the further oxidation of mildly oxidised LDL *in vitro*. Consequently, it may potentiate the deleterious action of oxidised LDL on vascular function by enhancing the oxidative modification of lipoproteins. Therefore, whilst the present study has demonstrated that UA inhibits endothelial dysfunction to LDL, it could propagate the inhibitory action of LDL via properties that were not examined in the present study. Another limitation may be the presence of $LiCO_3$ in all the UA solutions. However, it has previously been established that this vehicle solution does not directly alter vascular function (Chapter 4), and the current study demonstrated that supplementation LDL with Li_2CO_3 did not prevent the inhibitory action of LDL. This demonstrates that the restorative action of UA is not influenced by the presence of Li_2CO_3, and defends the use of this solution as a vehicle for UA in the current study. Finally, the maximum constriction to KCl was performed in the presence of the various test solutions rather than Krebs-Ringer only. Consequently, this could have masked the effect the test solutions had on the maximal vasoconstriction to PE.

6.4.5 Summary

This study demonstrated that supplementation of LDL solution with UA significantly restored NO-dependent vasodilatation to ACh in a concentration-dependent manner. This suggests that elevated UA in plasma may provide greater protection against the inhibitory effect of LDL upon endothelial function, a key-early event in the subsequent development of cardiovascular disease. The results of this study may lend further support to the hypothesis that an evolutionary loss of urate oxidase expression occurred as increased UA confers a physiological advantage in humans and may be related to

their prolonged life-span (Ames *et al,* 1981). UA could confer this physiological advantage by protecting or restoring endothelium-dependent vasodilatation following arterial exposure to LDL. However, additional research is required to clarify the mechanism by which vascular function is restored. Further research should investigate if the restoration is mediated via UA-specific means or via a generalised action that can be replicated with other endogenous hydrophilic antioxidants.

CHAPTER 7

THE EFFECT OF URIC ACID AND OTHER ENDOGENOUS ANTIOXIDANTS ON LDL-INDUCED VASCULAR DYSFUNCTION

7.1 INTRODUCTION

In the previous chapter (Chapter 6), it was demonstrated that UA can partially restore NO-mediated vascular function, in a concentration-dependent manner, following luminal perfusion with LDL. This is thought to occur due to the antioxidant properties of UA increasing NO-availability. However, it remains to be determined if the observed restoration is mediated via a UA-specific mechanism or if it can be achieved with other endogenous hydrophilic antioxidants, such as ascorbic acid (AA) or glutathione (GSH).

Several large epidemiological studies have suggested that dietary intake of AA and plasma AA concentrations are inversely associated with oxidative stress and the development of vascular disease (Gey *et al,* 1987; Riemersma *et al,* 1989; Singh *et al,* 1994; Singh *et al*, 1995; Khaw *et al,* 2001; Knekt *et al,* 2004). This may mediated by the ability of AA to effectively scavenge $O_2^{\cdot-}$ and other ROS (Som *et al*, 1983; Halliwell & Gutteridge, 1988; Gotoh & Niki, 1992), as AA is a powerful antioxidant *in vitro* and *in vivo* (Frei *et al,* 1988; Frei *et al,* 1989; Frei, 1994). It has previously been demonstrated that AA can restore endothelium-dependent vasodilatation in endothelium-intact isolated rabbit aortic rings exposed to LDL (Plane *et al,* 1993; Fontana *et al*, 1999). This antioxidant can also increases the resistance of isolated LDL to oxidative modification and decreases the rate of oxidation *ex vivo,* thus preventing any deleterious effects that may caused by the conversion from LDL to ox-LDL (Esterbauer *et al*, 1991). Furthermore, AA can also spare other endogenous antioxidants from consumption, thus providing further protection against the deleterious action of LDL and increased oxidative stress (Frei *et al,* 1988; Frei *et al,* 1989; Retsky *et al,* 1993). Therefore, the antioxidant ability of AA could potentially prevent the deleterious action of LDL upon NO availability in the isolated rat mesenteric arteries (Chapter 5).

GSH is also a major endogenous antioxidant in human physiology (Fahey & Sundquist, 1991). It is found in varying concentrations in the body, with high concentrations in VSMC, EC, platelets and red blood cells (0.5-5mM), but lower circulating levels in human plasma (2-20μM) (Mills & Lang, 1996; Jones *et al,* 2000). Intracellular GSH concentrations are important to maintain the intracellular redox balance and provide protection against the physiological and pathophysiological formation of ROS (Meister, 1994; Griffith, 1999). Consequently, GSH may maintain NO availability by preventing inactivation by ROS. It has previously been suggested that increased intracellular GSH concentrations may be an adaptive mechanism to LDL-induced oxidative stress in EC (Cho *et al,* 1999; Moellering *et al,* 2002; Bea *et al,* 2003). This implies that increased GSH concentrations protect against the deleterious action of LDL, and may suggest that GSH supplementation can prevent LDL-induced vascular dysfunction.

However, although AA and GSH are arguably two of the most recognisable antioxidants in human physiology, UA is the strongest determinant of plasma antioxidant capacity (Nyyssonen *et al*, 1997). The concentration of UA in human plasma (200-500μM) is 5-10 times greater than the concentration of AA (20-60μM) and 100 times greater than GSH (>2μM). The concentration of UA is also significantly greater in humans than in other species (Becker, 1993). When taken in conjunction, these may suggest that the elevated UA concentrations found in human plasma confer a greater physiological advantage over the other endogenous antioxidants present (Ames *et al*, 1981; Becker, 1993).

This chapter aims to examine and compare the effect of supplementation with stoichiometric equivalent concentrations of UA, AA and GSH on the responses to vasoactive drugs when isolated rat mesenteric arteries are perfused with 250μg/ml LDL solution. This will investigate whether UA confers greater protection than the other major hydrophilic antioxidants on vascular function in the presence of LDL. This chapter also aims to determine if the restorative action demonstrated in Chapter 6 is unique for UA. The hypotheses examined are:

1.) The beneficial effect of UA on LDL-induced endothelial dysfunction is mediated by antioxidant potential, and is replicated by elevated concentrations of other major hydrophilic antioxidants.

2.) At stoichiometric equivalent concentrations UA exerts a greater inhibitory effect than other endogenous antioxidants on LDL-induced endothelial dysfunction, and physiological concentrations of AA and GSH will provide little or no protection.

3.) At lower concentrations, similar to those found in animals that express urate oxidase, UA has little effect against the deleterious action of LDL upon endothelial function.

7.2 METHODS

7.2.1 Lipoprotein Isolation

LDL was isolated, purified and the protein concentration determined using a commercial kit based on the Lowry precipitation assay (Sigma Chemical Company, Dorset, U.K.), as previously described (Chapter 2).

7.2.2 Comparison of the effect of UA and AA

A segment of a third order branch of a mesenteric artery was mounted in the perfusion myograph chamber. Concentration-response curves to PE, ACh and SNP were performed when arteries were perfused with Krebs-Ringer buffer, 250μg/ml LDL solution, UA-supplemented LDL solution (40μM or 400μM), or AA-supplemented LDL solution (40μM or 400μM).

7.2.3 Comparison of the effect of UA and GSH

The above experimental protocol was repeated for arteries perfused with 250μg/ml LDL + 40μM or 400μM GSH solution, and the results compared to those previously obtained for UA-supplemented LDL solution, 250μg/ml LDL only, and physiological Krebs-Ringer solution .

7.2.4 Intraluminal Solutions

LDL solutions and UA stock were prepared as previous. The UA stock solution was diluted with Krebs-Ringer buffer and used to supplement the 250μg/ml LDL solutions daily, to give final intraluminal UA concentrations of 40μM and 400μM. AA stock solution was made by dissolving 0.704mg/ml in distilled water to give a stock solution of 4mM. The stock solution was diluted with Krebs-Ringer buffer and used to supplement the 250μg/ml LDL solutions daily, to give final intraluminal AA concentrations of 40μM and 400μM. GSH stock solution was made by dissolving 1.23mg/ml in distilled water to give a stock solution of 4mM. The stock solution was diluted with Krebs-Ringer buffer and used to supplement the 250μg/ml LDL solutions daily, to give final intraluminal GSH concentrations of 40μM and 400μM.

7.2.5 Lipid Peroxidation

Samples of the intraluminal LDL and antioxidant-supplemented solutions were collected before and after the concentration-response curves to the vasoactive drugs were performed, and the MDA and 4-HNE concentrations determined using a commercial colorimetric assay kit (CN Biosciences, Nottingham, U.K.).

7.2.6 Statistical Analysis

The results achieved when LDL was supplemented with UA were compared to the results produced when LDL was supplemented with stoichiometric equivalent concentrations of AA or GSH. These results were also compared to the physiological control and the results for LDL solution only. Analysis was also performed to determine if supplementation with 40μM or 400μM antioxidant had a significant

concentration-dependent effect. Statistical analysis was performed using paired Student's *t*-tests to compare the oxidative status of the intraluminal LDL solutions before and after luminal perfusion.

7.3 RESULTS

7.3.1 Comparison of the effect of UA and AA upon LDL-induced vascular dysfunction

7.3.1.1 Comparison of the effect upon vasoconstriction to PE

Supplementation with UA or AA did not have a significant effect on the vasoconstriction to PE when compared to LDL only (Fig 7.1), although the responses to $1x10^{-6}$ PE were reduced ($P<0.01$). Supplementation of the LDL with the antioxidant solutions produced largely similar results, however, vasoconstriction was reduced when supplemented with 400μM UA compared to 400μM AA ($5x10^{-8}$ - $1x10^{-7}$, $P<0.04$). There was no statistically significant difference in the calculated EC_{50} values, as shown in Table 7.1.

7.3.1.2 Comparison of the effect upon LDL-induced endothelial dysfunction

Perfusion with LDL or LDL supplemented with 40μM UA, 40μM AA or 400μM AA produced similar concentration-dependent vasodilatation to ACh (Fig 7.2). The maximal dilatations produced were 44 ± 2.8%, 45.6 ± 4.2%, 44.7 ± 8.3%, and 44.7 ± 6.2%, respectively. The supplementation of LDL with 400μM UA produced a maximal dilatation of 66.7 ± 1.9% and significantly enhanced vasodilatation when compared to LDL only ($1x10^{-7}$-$5x10^{-4}$, $P<0.01$), or LDL supplemented with 400μM AA ($1x10^{-6}$-$5x10^{-4}$, $P<0.01$) or 40μM UA ($5x10^{-7}$-$5x10^{-4}$, $P<0.01$). However, this was still significantly lower than the physiological control ($1x10^{-7}$-$5x10^{-4}$, $P<0.05$).

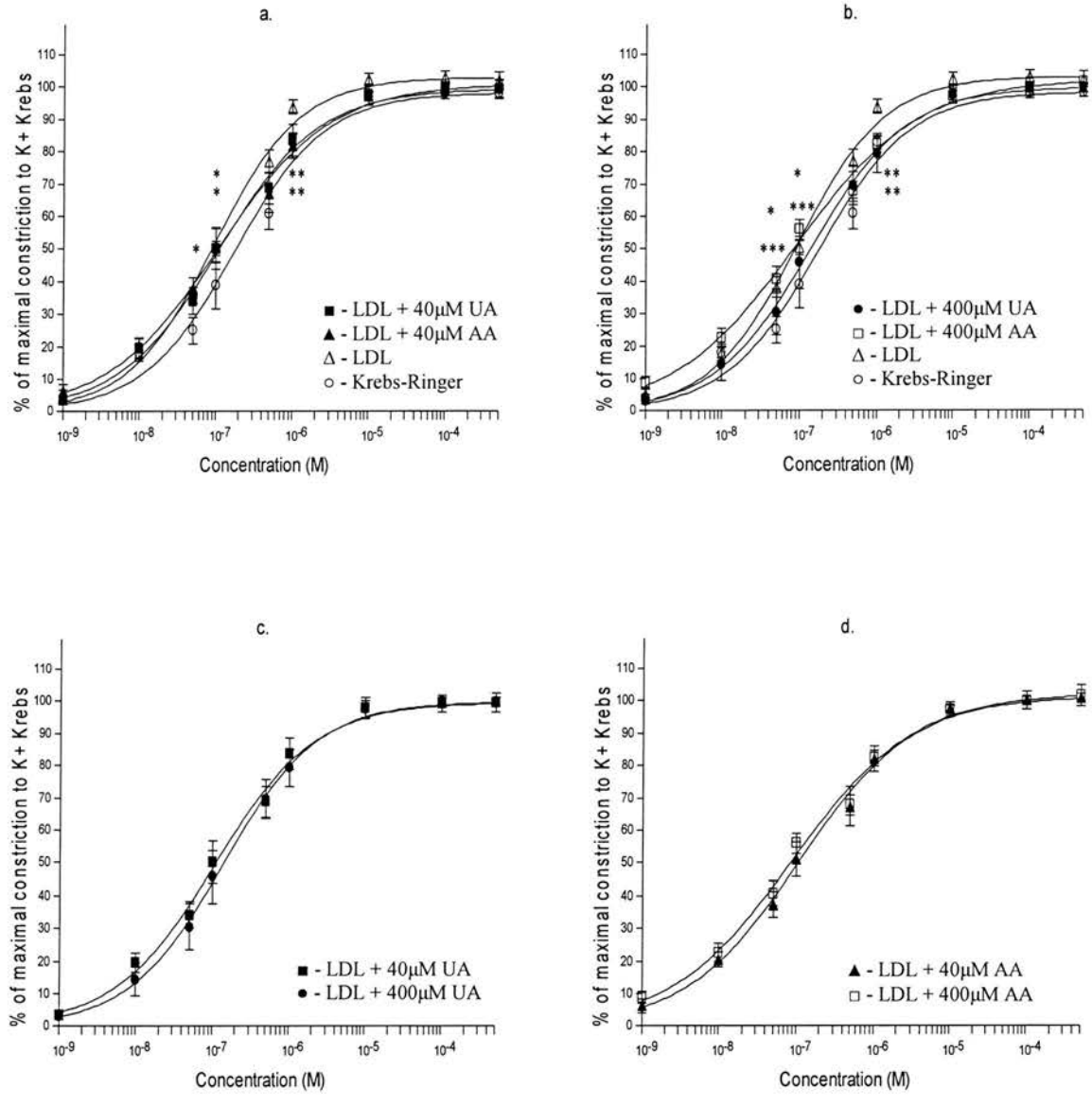


Figure 7.1. Comparison of the effect of UA and AA on vasoconstriction to PE in isolated rat mesenteric arteries are perfused with LDL: **a.)** Comparison of LDL + 40μM UA with LDL + 40μM AA. *$p<0.03$ vs. Krebs-Ringer. **$p<0.01$ vs. 250μg/ml LDL (2-factor ANOVA with replication and Bonferroni correction, n=8). **b.)** Comparison of LDL + 400μM UA, LDL + 400μM AA. *$p<0.01$ vs. Krebs-Ringer, **$p<0.01$ vs. 250μg/ml LDL, ***$p<0.04$ vs. LDL + 400μM UA (2-factor ANOVA with replication and Bonferroni correction, n=8). **c.)** Comparison of LDL + 40μM or 400μM UA. **d.)** Comparison of LDL + 40μM or 400μM AA. All values are the mean ± SEM (n=8).

Intraluminal Solution	EC_{50}	Maximal Response (% of vasoconstriction to KCl)
Krebs-Ringer	240.4 ± 60.9nM	98.3 ± 1.7%
250μg/ml LDL	95.4 ± 11nM	102.9 ± 2.2%
250μg/ml LDL + 40μM UA	106.4 ± 27.1nM	99.5 ± 2.9%
250μg/ml LDL + 40μM AA	100.8 ± 20.1nM	100.9 ± 1.2%
250μg/ml LDL + 400μM UA	230.1 ± 61.7nM	99.7 ± 1.5%
250μg/ml LDL + 400μM AA	145.6 ± 34.5nM	101.5 ± 3.3%

Table 7.1. Comparison of the effect of UA and AA on vasoconstriction to PE when isolated rat mesenteric arteries are perfused with LDL: Mean EC_{50} and maximal response values, ± SEM, for concentration-dependent vasoconstriction to PE when perfused with the above intraluminal solutions. There is no statistically significant difference in the calculated EC_{50} values ($P>0.05$; One-way ANOVA with Bonferroni correction, n=8).

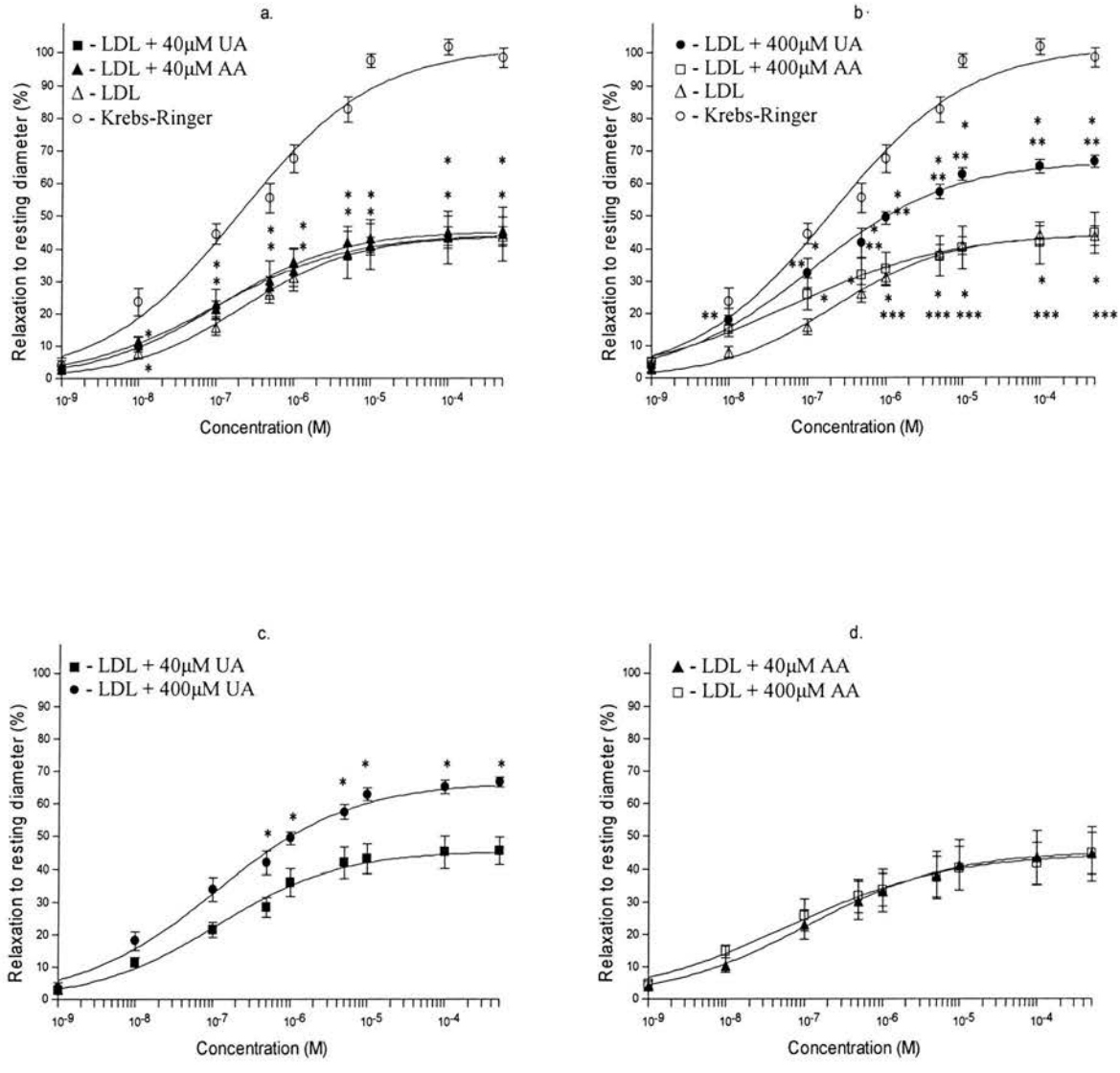


Figure 7.2. Comparison of the effect of UA and AA on vasodilatation to ACh in isolated rat mesenteric arteries perfused with LDL: **a.)** Comparison of LDL + 40μM UA with LDL + 40μM AA. *$p<0.01$ vs. Krebs-Ringer (2-factor ANOVA with replication and Bonferroni correction, n=8). **b.)** Comparison of LDL + 400μM UA with LDL + 400μM AA. *$p<0.01$ vs. Krebs-Ringer, **$p<0.01$ vs. 250μg/ml LDL, ***$p<0.01$ vs. LDL + 400μM UA (2-factor ANOVA with replication and Bonferroni correction, n=8). **c.)** Comparison of LDL + 40μM or 400μM UA. *$p<0.01$ vs. LDL + 40μM UA (2-factor ANOVA with replication and Bonferroni correction, n=8). **d.)** Comparison of LDL + 40μM or 400μM AA. All values are the mean ± SEM (n=8).

7.3.1.3 Comparison of the effect upon the action of LDL on endothelium-independent vasodilatation to SNP

Perfusion with Krebs-Ringer solution or LDL supplemented with 40μM UA or AA, or 400μM UA or AA produced comparable concentration-dependent vasodilatation to SNP (Fig 7.3). Supplementation with 400μM UA or AA significantly enhanced vasodilatation when compared to LDL only (400μM UA: $1x10^{-8}$-$1x10^{-6}$, $P<0.02$; 400μM AA: $1x10^{-7}$-$1x10^{-6}$, $P<0.01$).

7.3.2 Comparison of the effect of UA and GSH upon LDL-induced vascular dysfunction

7.3.2.1 Comparison of the effect upon vasoconstriction to PE

Vasoconstriction was similar when the arteries were perfused with LDL solution supplemented with 40μM or 400μM GSH (Fig 7.4). This vasoconstriction was similar to the responses produced when perfused with LDL and LDL supplemented with UA, but greater than the physiological control ($5x10^{-8}$-$1x10^{-7}$; 40μM GSH: $P<0.02$, 400μM GSH: $P<0.04$).

7.3.2.2 Comparison of the effect upon LDL-induced endothelial dysfunction

The concentration-dependent vasodilatation to ACh was comparable when the arteries were perfused with LDL and LDL supplemented with 40μM or 400μM GSH (Fig 7.5). Vasodilatation was similar when perfused with 40μM GSH- or UA–supplemented LDL solution. Supplementation with 400μM GSH markedly reduced vasodilatation when compared to 400μM UA ($1x10^{-7}$-$5x10^{-4}$, $P<0.01$).

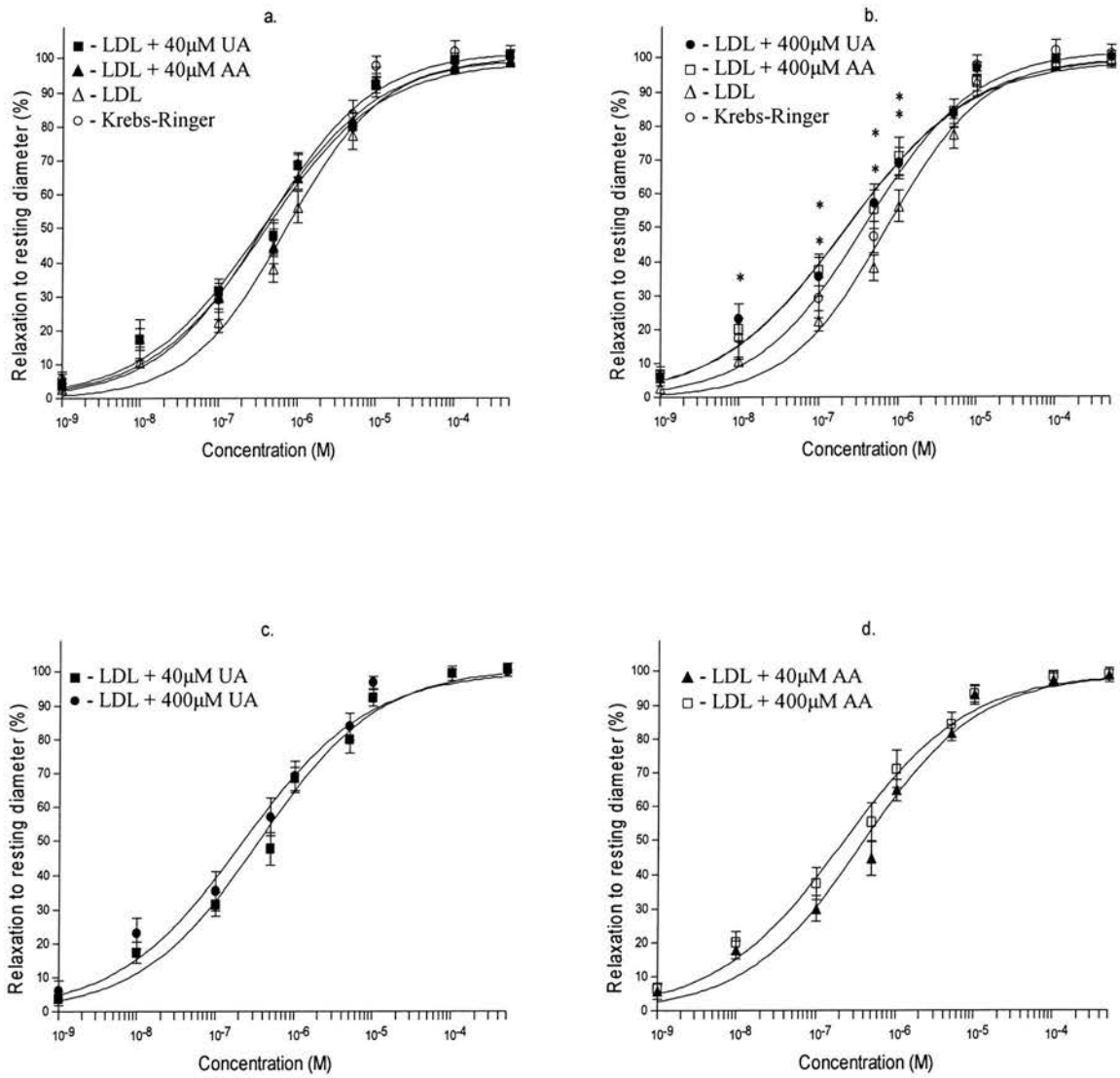


Figure 7.3. Comparison of the effect of UA and AA on vasodilatation to SNP in isolated rat mesenteric arteries perfused with LDL: **a.)** Comparison of LDL + 40μM UA with LDL + 40μM AA. **b.)** Comparison of LDL + 400μM UA, LDL + 400μM AA. *$p<0.02$ vs. 250μg/ml LDL (2-factor ANOVA with replication and Bonferroni correction, n=8). **c.)** Comparison of LDL + 40μM or 400μM UA. **d.)** Comparison of LDL + 40μM or 400μM AA. All values are the mean ± SEM (n=8).

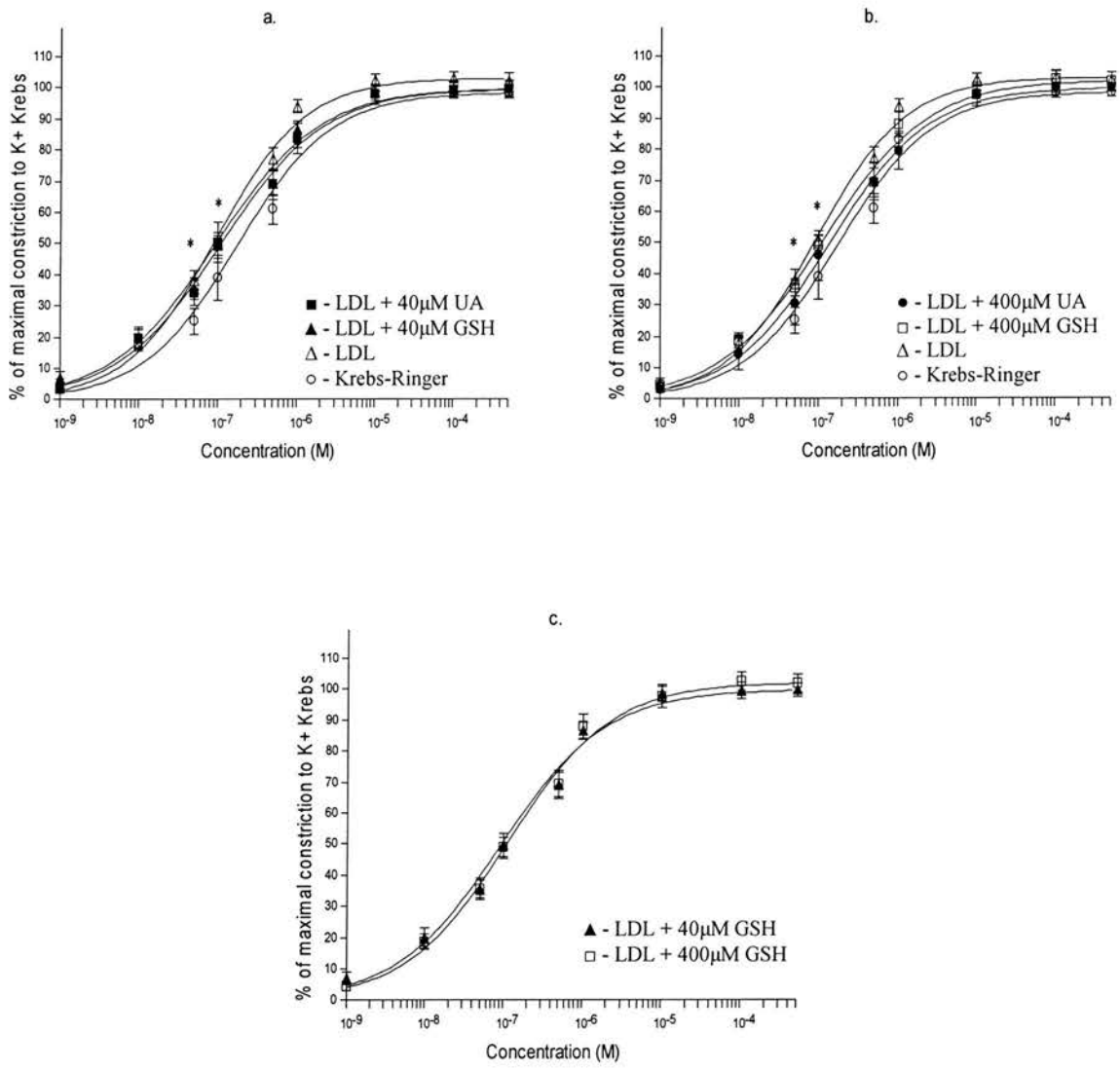


Figure 7.4. Comparison of the effect of UA and GSH on vasoconstriction to PE in isolated rat mesenteric arteries are perfused with LDL: **a.)** Comparison of LDL + 40μM UA with LDL + 40μM GSH. *$p<0.02$ vs. Krebs-Ringer (2-factor ANOVA with replication and Bonferroni correction, n=8). **b.)** Comparison of LDL + 400μM UA with LDL + 400μM GSH. *$p<0.04$ vs. Krebs-Ringer (2-factor ANOVA with replication and Bonferroni correction, n=8). **c.)** Comparison of LDL + 40μM or 400μM GSH. All values are the mean $\pm$ SEM (n=8).

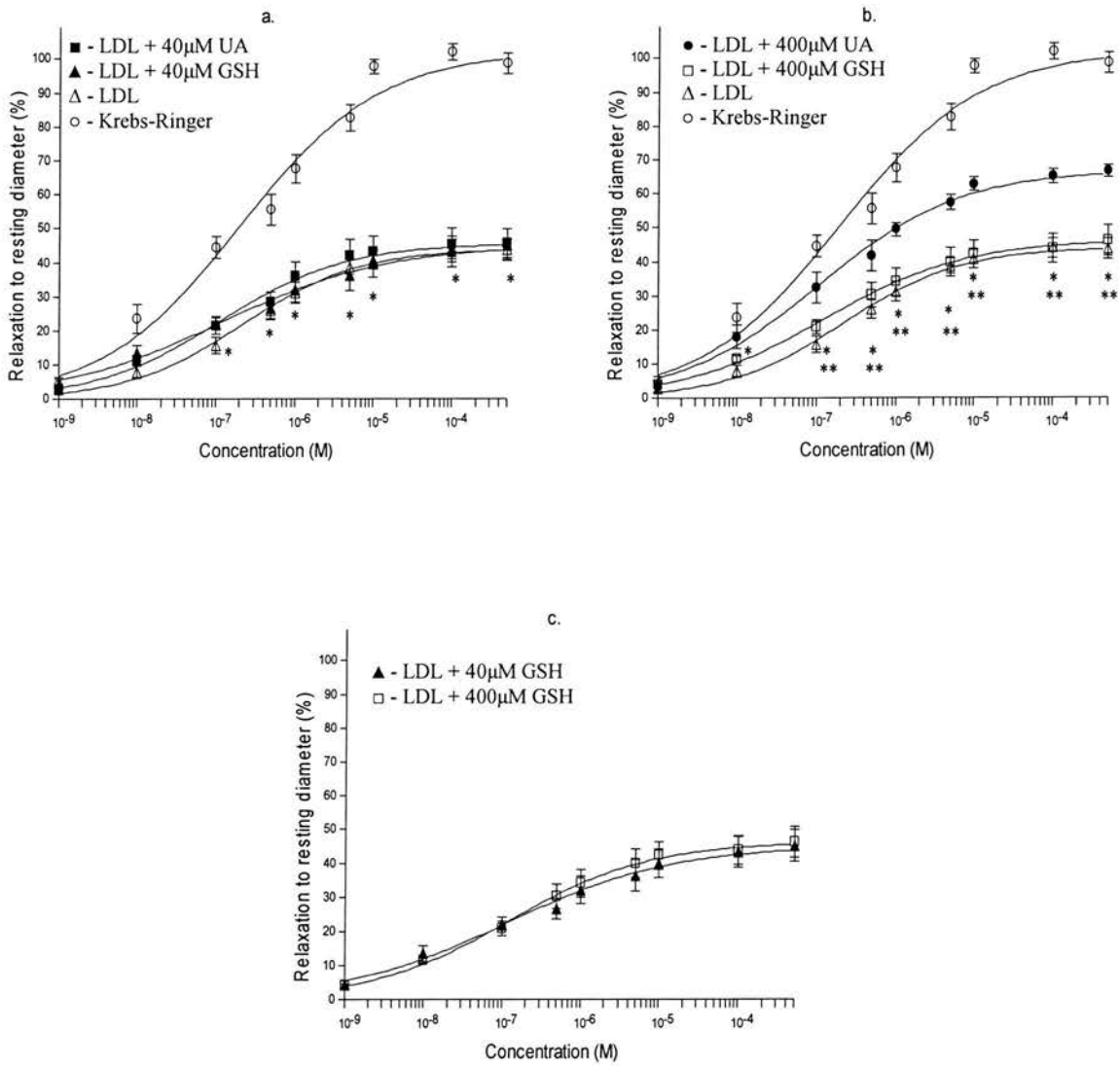


Figure 7.5. Comparison of the effect of UA and GSH on vasodilatation to ACh in isolated rat mesenteric arteries are perfused with LDL: **a.)** Comparison of LDL + 40μM UA with LDL + 40μM GSH. *$p<0.01$ vs. Krebs-Ringer (2-factor ANOVA with replication and Bonferroni correction, n=8). **b.)** Comparison of LDL + 400μM UA with LDL + 400μM GSH. *$p<0.01$ vs. Krebs-Ringer, **$p<0.01$ vs. LDL + 400μM UA (2-factor ANOVA with replication and Bonferroni correction, n=8). **c.)** Comparison of LDL + 40μM or 400μM GSH. All values are the mean ± SEM (n=8).

7.3.2.3 Comparison of the effect upon the action of LDL on endothelium-independent vasodilatation to SNP

Perfusion with 40μM or 400μM GSH-supplemented LDL solution produced similar vasodilatation to SNP. This vasodilatation was comparable to the results produced when perfused with Krebs-Ringer solution, LDL only, or UA-supplemented LDL solutions (Fig 7.6).

7.3.3 Lipid Peroxidation

No significant alteration in MDA and 4-HNE concentrations occurred in the LDL and supplemented LDL solutions following luminal perfusion (Fig 7.7 and 7.8). This demonstrates that the oxidative status of the LDL was unchanged during the experiments.

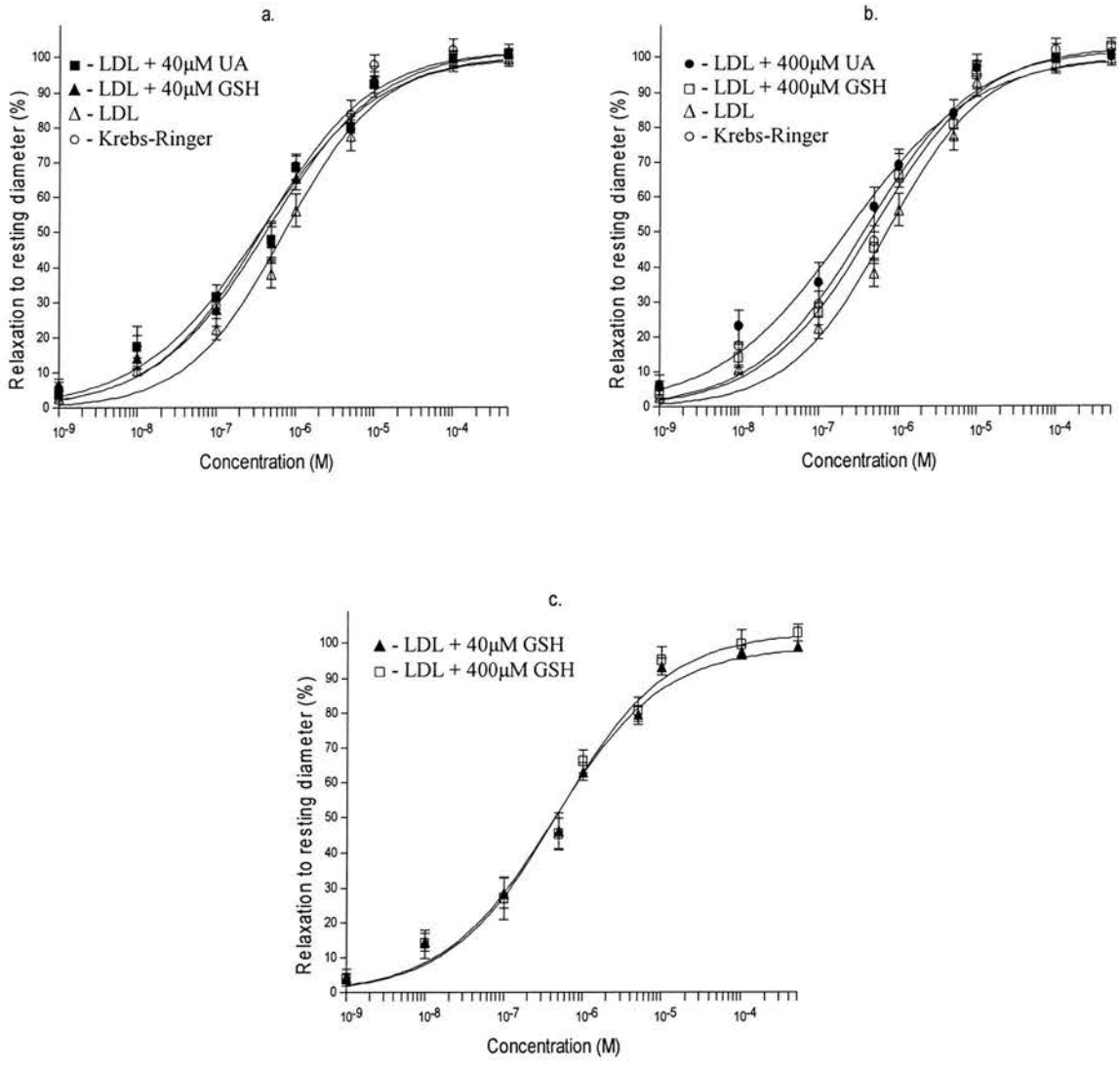


Figure 7.6. Comparison of the effect of UA and GSH on vasodilatation to SNP in isolated rat mesenteric arteries are perfused with LDL: **a.)** Comparison of LDL + 40μM UA with LDL + 40μM GSH. **b.)** Comparison of LDL + 400μM UA with LDL + 400μM GSH. **c.)** Comparison of LDL + 40μM or 400μM GSH. All values are the mean ± SEM (n=8).

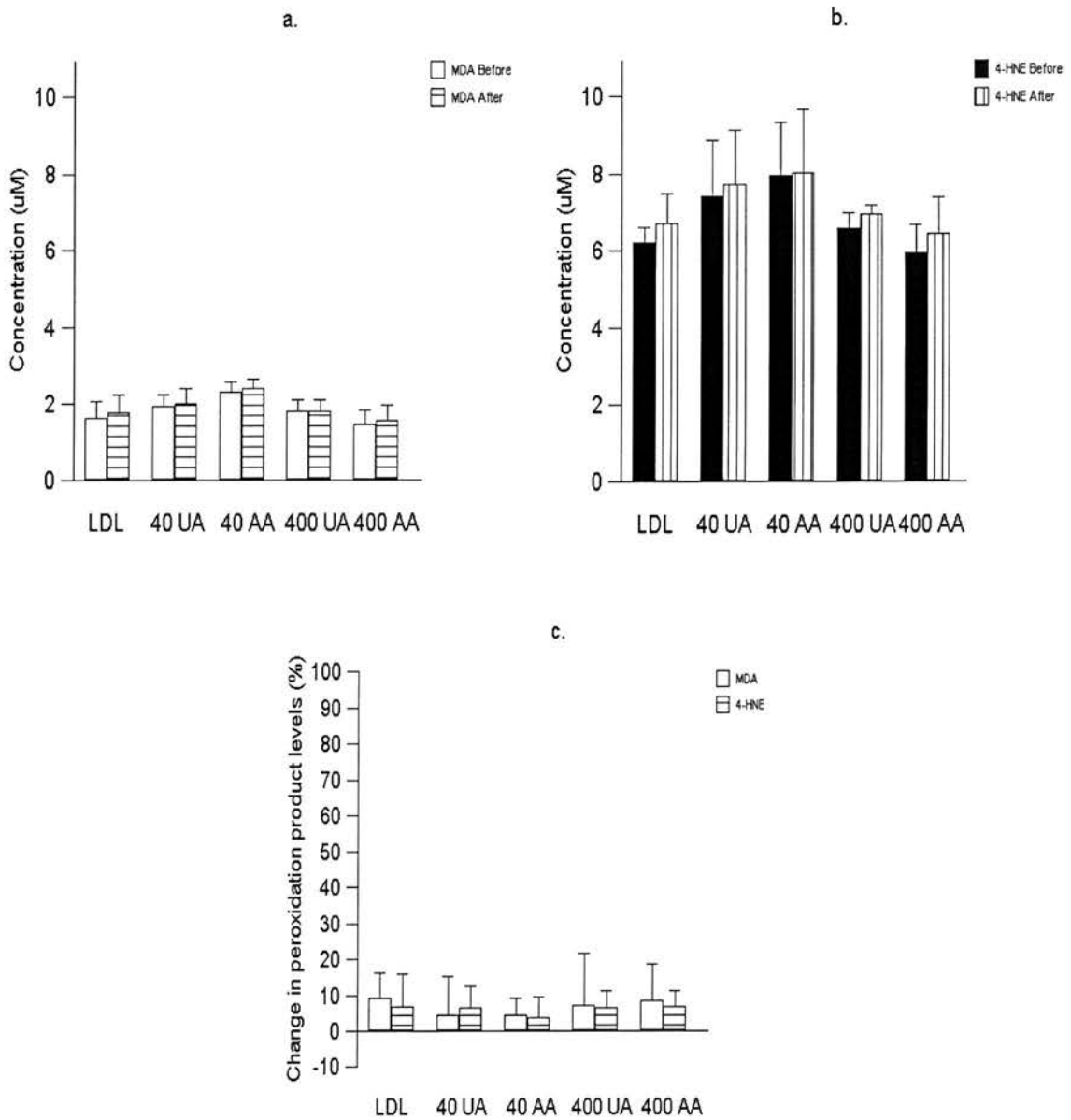


Figure 7.7. Alterations in lipid peroxidation product levels: a.) Concentration of MDA present in 250μg/ml LDL and LDL solution supplemented with 40μM UA, 40μM AA, 400μM UA and 400μM AA, before and after luminal perfusion. b.) Concentration of 4-HNE present in 250μg/ml LDL and LDL solution supplemented with 40μM UA, 40μM AA, 400μM UA and 400μM AA, before and after luminal perfusion. c.) Mean percentage change in the concentration of MDA and 4-HNE following luminal perfusion. All values are mean ± SEM (n=8).

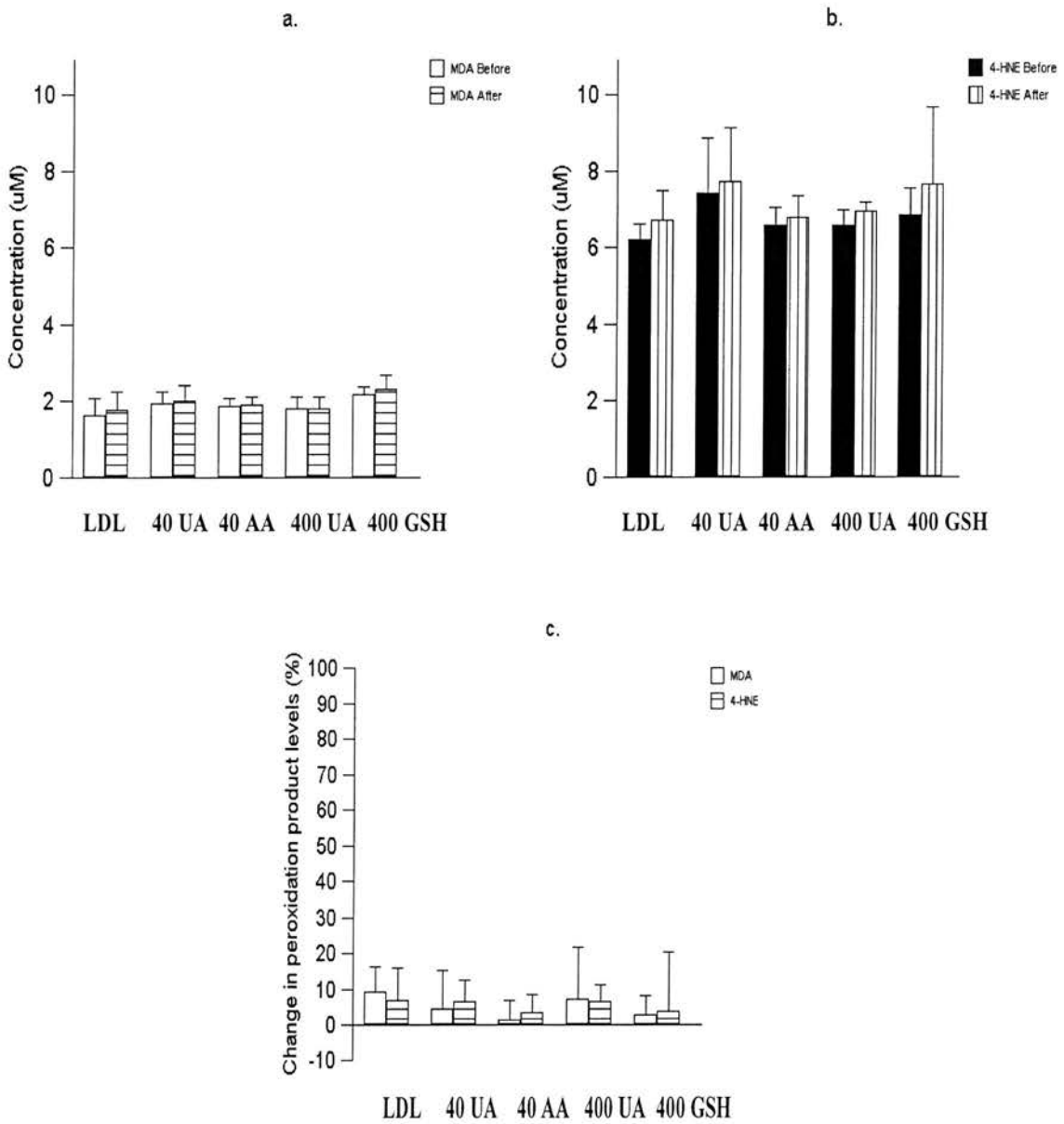


Figure 7.8. Alterations in lipid peroxidation product levels: a.) Concentration of MDA present in 250μg/ml LDL and LDL solution supplemented with 40μM UA, 40μM GSH, 400μM UA and 400μM GSH, before and after luminal perfusion. b.) Concentration of 4-HNE present in 250μg/ml LDL and LDL solution supplemented with 40μM UA, 40μM GSH, 400μM UA and 400μM GSH, before and after luminal perfusion. c.) Mean percentage change in the concentration of MDA and 4-HNE following luminal perfusion. All values are mean ± SEM (n=8).

7.4 DISCUSSION

400μM UA prevented the slight enhancement of vasoconstriction to PE by LDL, but AA and GSH had no effect. 400μM UA was also the only antioxidant solution to significantly improve LDL-induced endothelial dysfunction in this model. Supplementation of LDL with 400μM UA or AA also enhanced vasodilatation to SNP.

7.4.1 The effect of AA upon LDL-induced vascular dysfunction

7.4.1.1 AA and $O_2^{\cdot-}$

The deleterious effect of LDL upon endothelium-dependent vasodilatation has been widely attributed to the inactivation of NO by $O_2^{\cdot-}$ within the EC (Galle *et al,* 1991; Pritchard *et al,* 1995; Dart & Chin-Dusting, 1999; Vergnani *et al*, 2000). AA is a major water-soluble antioxidant found in plasma (Frei *et al*, 1988; Frei *et al*, 1989; Retsky *et al,* 1998) and effectively scavenges $O_2^{\cdot-}$ (Som *et al,* 1983; Bendich *et al,* 1986; Halliwell & Gutteridge, 1988). The molecular structure of this antioxidant is shown in Figure 7.9. However, the luminal perfusion with AA at physiological and elevated plasma concentrations (40 and 400μM, respectively) had no significant effect upon the deleterious action of LDL on vascular function. This is surprising as it has previously been demonstrated that intra-arterial infusion of AA in hypercholesterolaemic patients improves NO-mediated vasodilatation (Ting *et al,* 1997), although negative results have also been reported (Gilligan *et al,* 1994). Similarly, dietary supplementation with AA improves endothelial function in hyperlipidaemic children (Engler *et al,* 2003) and patients with coronary artery disease (Gokce *et al,* 1999; Tousoulis *et al,* 2005). It has also been demonstrated *in vitro* that high dose AA (1mM) can partially restore acute lipoprotein induced endothelial dysfunction in rabbit aortic rings immersed in 150μg/ml LDL (Fontana *et al*, 1999). Antioxidant therapy with AA may also improve endothelium-dependent vasodilatation in other conditions associated with increased

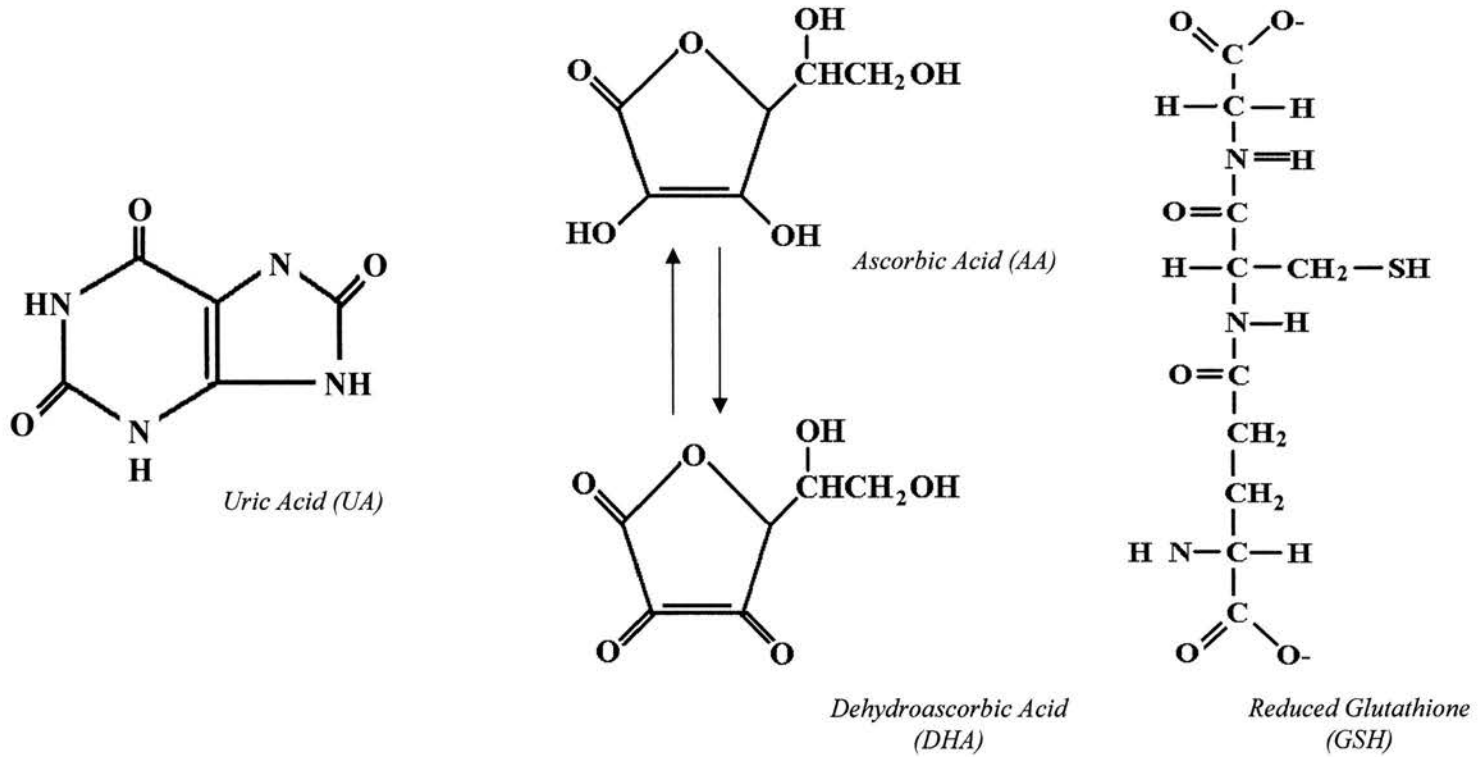


Figure 7.9. The structure of the hydrophilic antioxidants used to supplement LDL in the present study, and DHA which may be rapidly taken up by cells and reduced intracellularly to enhance AA concentrations.

oxidative stress, such as hypertension, type II diabetes and smoking (Heitzer *et al,* 1996; Levine *et al,* 1996; Solzbach *et al,* 1997; Hornig *et al,* 1998; Raitakari *et al*, 2000), although contradictory results have also been reported (Duffy *et al,* 2001; Darko *et al,* 2002; Pellegrini *et al,* 2004; Kinlay *et al,* 2004; Widlansky *et al,* 2004; Chen *et al,* 2006).

The beneficial effects of AA have been attributed to its ability to scavenge $O_2^{\cdot-}$, as demonstrated by the ability of AA to restore NO-mediated vasodilatation following the extracellular generation of $O_2^{\cdot-}$ by HX/XO or pyrogallol (Dudgeon *et al*, 1998; Jackson *et al*, 1998). However, the ability of exogenous AA to restore NO-dependent vasodilatation under conditions of intracellular oxidant stress is less certain. Exogenous AA, at concentrations exceeding the physiological range, was ineffective at protecting NO-dependent vasodilatation following the inhibition of Cu-Zn SOD by DECTA (Jackson *et al*, 1998). This implies that exogenous AA does not exert an intracellular action. This could explain the ineffective nature of AA supplementation in the current study. The cellular uptake of exogenous AA across membranes occurs at a slow rate (Winkler *et al,* 1994). The intracellular penetration of exogenous AA is limited as it must undergo extracellular oxidation to DHA, the major transport form of AA, before intracellular accumulation can occur (Mann & Newton, 1975; Washko *et al,* 1993). Once transported into cells, DHA will undergo intracellular reduction to regenerate AA (Washko *et al,* 1993). Therefore, the failure of exogenous AA to restore vasodilatation could result from the inability of AA to access the EC.

Nevertheless, the ability of exogenous AA to scavenge intracellular endothelial $O_2^{\cdot-}$ in the current study should not be discounted. However, the rate of this reaction is $3x10^5$ $M^{-1}s^{-1}$ (Nishikimi, 1975; Gotoh & Niki, 1992; Tsujimoto *et al,* 1993), which is 10^4 times slower than the reaction rate of $ONOO^-$ formation. As a result, the kinetics and reaction rates favour the reaction between $O_2^{\cdot-}$ and NO and it is highly unlikely that AA could scavenge $O_2^{\cdot-}$ within the stimulated EC. Therefore, while the failure of AA to restore NO-mediated vascular function is initially surprising, the negative results could arise

from the inability of AA to access the intracellular compartment in sufficient concentrations to compete with the inactivation of NO by $O_2^{\cdot-}$. The failure of AA to prevent LDL-induced vascular dysfunction does not discount the role of $O_2^{\cdot-}$, but may further support the hypothesis that the inactivation of NO in this experimental model is intracellular. This is in agreement with a previous study that examined the effects of LDL upon vascular function (Fontana *et al*, 1999)

7.4.1.2 AA and $ONOO^-$

It is unlikely that supplementation with exogenous AA would be able to scavenge any $ONOO^-$ formed in this experimental model. Physiological concentrations of AA are insufficient to scavenge $ONOO^-$ *in vivo* due to the small rate constant for the reaction. ($1x10^2M^{-1}s^{-1}$) (Bartlett *et al,* 1995). The estimated concentrations of AA in human tissue (90μM in heart) are typically higher than those in plasma (Bergsten *et al,* 1990), but are still unlikely to be sufficient to effectively scavenge $ONOO^-$ (Bartlett *et al,* 1995). However, Squadrito *et al* suggested that supraphysiological concentrations of AA (5.5mM) could effectively scavenge $ONOO^-$ (Squadrito *et al*, 1995). This concentration of AA is still significantly greater than the highest concentration used in the current study. Therefore, it is unlikely that the levels of exogenous AA present in the arterial lumen would be sufficient to scavenge and protect against $ONOO^-$.

7.4.1.3 AA and endothelium-independent vasodilatation

Supplementation of the LDL solutions with 400μM AA produced enhanced endothelium-independent vasodilatation to SNP when compared to UA, despite the inability of AA to restore ACh-mediated vasodilatation. This may be related to ability of AA to potentiate the release of NO from NO-donors such as SNP or *S*-nitroso-N-acetylpenicillamine (SNAP), which act through S-nitrosothiols (RSNO) within the VSMC (Singh *et al,* 1996). The ability of AA to stimulate the release of NO from RSNO may account for the direct vasodilator action of this antioxidant in some isolated

preparations (Dudgeon *et al,* 1998) and could explain the disagreement between studies that have demonstrated the restoration of endothelium-dependent vasodilatation by AA (Ting *et al,* 1997; Levine *et al,* 1995; Heitzer *et al,* 1996; Solzbach *et al,* 1997; Hornig *et al,* 1998; Fontana *et al,* 1999) and the negative results produced in the current study.

7.4.2 The effect of GSH on LDL-induced vascular dysfunction

Thiols are critical endogenous intracellular antioxidants against ROS and $ONOO^-$, and GSH is the main intracellular thiol (Fahey & Sundquist, 1991; Boesgaard *et al,* 1993). The molecular structure of this established antioxidant is shown in Figure 7.9. Increased intracellular concentrations are a common response to oxidant stress and prevent oxidative damage by enhancing the capacity to scavenge radical species and repair oxidised protein thiols and lipid peroxides (Padgett & Whorton, 1997; Griffith, 1999; Lu, 1999; Rahman & MacNee, 2000). GSH is also a reducing cofactor in the production of NO and is required for full NOS enzymatic activity (Stuehr *et al,* 1990; Hofmann & Schmidt, 1995; Harbrecht *et al,* 1997). Therefore, altering GSH levels could potentially protect against the deleterious action of LDL on vascular function by preventing the ROS-mediated inactivation of NO or increasing NO synthesis. The depletion of intracellular GSH may be related to the oxidative injury that occurs in hypercholesterolaemia (Ma *et al,* 1997), and increased intracellular GSH may prevent LDL-induced endothelial ROS generation (Kuzuya *et al,* 1989). This close interaction between GSH and NO availability in the vascular wall made this an interesting antioxidant to examine and compare with UA. However, unlike UA, the current did study did not report any reversal of LDL-induced endothelial dysfunction when the LDL solution was supplemented with exogenous GSH.

The inhibitory action of LDL on vascular function is attributed increased oxidative stress and the inactivation of NO by $O_2^{\cdot-}$ (Chapter 5). The negative results of the current

study suggest that supplementation of the LDL solution with GSH could not prevent this inactivation. It is highly unlikely that GSH could directly protect against NO inactivation due to the rapid rate of the reaction between $O_2^{\cdot-}$ and NO. However, a recent study demonstrated that GSH can protect NO from inactivation by ROS (Ford *et al,* 2006). It has also been widely demonstrated that GSH can reverse endothelial dysfunction and improve NO-availability under other conditions of increased oxidative stress (Cheung & Schultz, 1997; Kevelatis *et al,* 1997; Kugiyama *et al,* 1998; Vita *et al,* 1998; Prasad *et al,* 1999; Cheung *et al,* 2000; Nascimento *et al,* 2003; Schauer *et al,* 2004). This has been attributed to the antioxidant ability of GSH preventing the inactivation of NO by ROS. This is supported by the induction of acute oxidative stress, reduced NO-availability and alterations in vascular function in experimental models following intracellular GSH depletion (Ghigo *et al,* 1993; Laursen *et al,* 2001; Ganafa *et al,* 2002; Ford *et al,* 2006).

Therefore, the failure of GSH supplementation to restore endothelium-dependent vasodilatation in the current study is initially surprising. However, the beneficial effects of GSH in the previous studies have been attributed to the intracellular action of the antioxidant. In the current study, this may have been impeded by the tendency for exogenous GSH to undergo extracellular breakdown by γ-glutamyl transpeptidase (Chang *et al,* 1992; Ghigo *et al,* 1993). As a result, exogenous GSH is unlikely to penetrate the EC and exert a direct intracellular action in the current experimental model (Ghigo *et al,* 1993). Consequently, GSH supplementation in the current study may only provide an alternative approach to enhancing the *extracellular* antioxidant status. It has previously been demonstrated that increasing the extracellular GSH concentrations protects against the extracellular generation of ROS (Boesgaard *et al,* 1993; Bilzer *et al,* 1999). However, in the current experimental model the generation of $O_2^{\cdot-}$ and the subsequent inactivation of NO are likely to occur within the EC, as both are generated in the same cell. The failure of exogenous GSH to restore vascular function supports the hypothesis.

The extracellular breakdown of GSH does provide a source of substrate for intracellular GSH synthesis (Tsan *et al,* 1989; Hiraishi *et al,* 1994; Griffith, 1999; Sies, 1999), which could potentially protect EC against intracellular oxidative injury (Chang *et al,* 1992). However, this reconstitution is time-dependent and the failure of GSH to restore vasodilatation may be due to the relatively short time-scale of the current experiment when compared to the previous studies. The disparity in the results could also be due to concentrations of GSH used as it has been demonstrated that supplementation of rat cells with less than 1mM GSH is insufficient to produce a sustained increase in intracellular concentrations (Visarius et al, 1996). Several of the *in vitro* studies to demonstrate a beneficial action of GSH in oxidative stress used greater concentrations than the current study (>1mM) (Ghigo *et al,*1993; Visarius et al, 1996; Cheung & Schultz, 1997; Kevelatis *et al,* 1997; Bilzer *et al,* 1999).

The beneficial effects of GSH demonstrated in these studies may arise from its ability to scavenge $ONOO^-$ (Wu *et al,* 1994; Balazy *et al,* 1998; Radi, 1998; Radi *et al,* 2001), rather than $O_2^{\cdot-}$. This reaction occurs with a second-order rate constant of $2\text{-}5x10^2M^{-1}s^{-1}$ (Quijano *et al,* 1997; Arteel *et al,* 1999). Increased GSH concentration may be an adaptive response to $ONOO^-$ (Buckley & Whorton, 2000) and cellular GSH depletion increases the detection and biological effects of $ONOO^-$ (Ma *et al,* 1997; Radi, 1998; Radi *et al,* 2001). GSH can convert this potent free radical into potential NO donors such as *S*-nitrosoglutathione (GSNO) and *S*-nitroglutathione (Wu *et al,* 1994; Mayer *et al,* 1995; Mayer *et al,* 1998; White *et al,* 1997). These exhibit physiological effects similar to those of NO and can induce vasodilatation (Nossuli *et al,* 1998), which may account for the restorative action of GSH demonstrated in several studies (Cheung & Schulz, 1997; Cheung *et al*, 2000). However, this reaction is inefficient and yields maximally 1% GSNO (Moro *et al,* 1994; Mayer *et al,* 1995). There was no evidence of such an effect in the current study.

7.4.3. Comparison of supplementation with UA, AA and GSH

The previous chapter demonstrated that UA improved LDL-induced endothelial dysfunction at physiological concentrations. To determine if this ability was unique for UA or could be replicated by other major hydrophilic antioxidants, the beneficial effects were compared with AA and GSH at equivalent molar concentrations (40 and 400 μM each). These concentrations were chosen as they approximate the concentrations of AA and UA in human plasma. GSH is found in lower concentrations in plasma (2-20μM), although greater concentrations are present within tissue. The results produced imply that the restorative action of UA supplementation on LDL-induced endothelial dysfunction is distinct from its antioxidant ability, as both AA and GSH are potent antioxidants but have no effect. However, it must be noted that whilst antioxidants have similar antioxidant potential, the primary mechanisms of action are distinct to each compound and cannot be extrapolated to other antioxidants. The results support the hypothesis that UA confers greater protection against the deleterious action of LDL-induced endothelial dysfunction at stoichiometric equivalent concentrations when compared to AA and GSH. The failure of UA to prevent LDL-induced endothelial dysfunction when present in concentrations similar to those of other endogenous plasma antioxidants (40μM), and those found in animals that express urate oxidase, could have further implications regarding the increased concentrations of UA in human physiology and may indirectly imply that elevated UA confers a physiological advantage.

The current study was also performed to help elucidate the mechanism of action by which UA restores vascular function following acute luminal exposure to LDL. However, it should also be acknowledged that this study may only have examined the *extracellular* action of the exogenous antioxidants, given the question marks over their ability to readily permeate the cell membrane and access the intracellular compartments. The proposed sites at which the exogenous antioxidants could prevent LDL-induced endothelial dysfunction are demonstrated in Figure 7.10. The superiority of 400μM UA

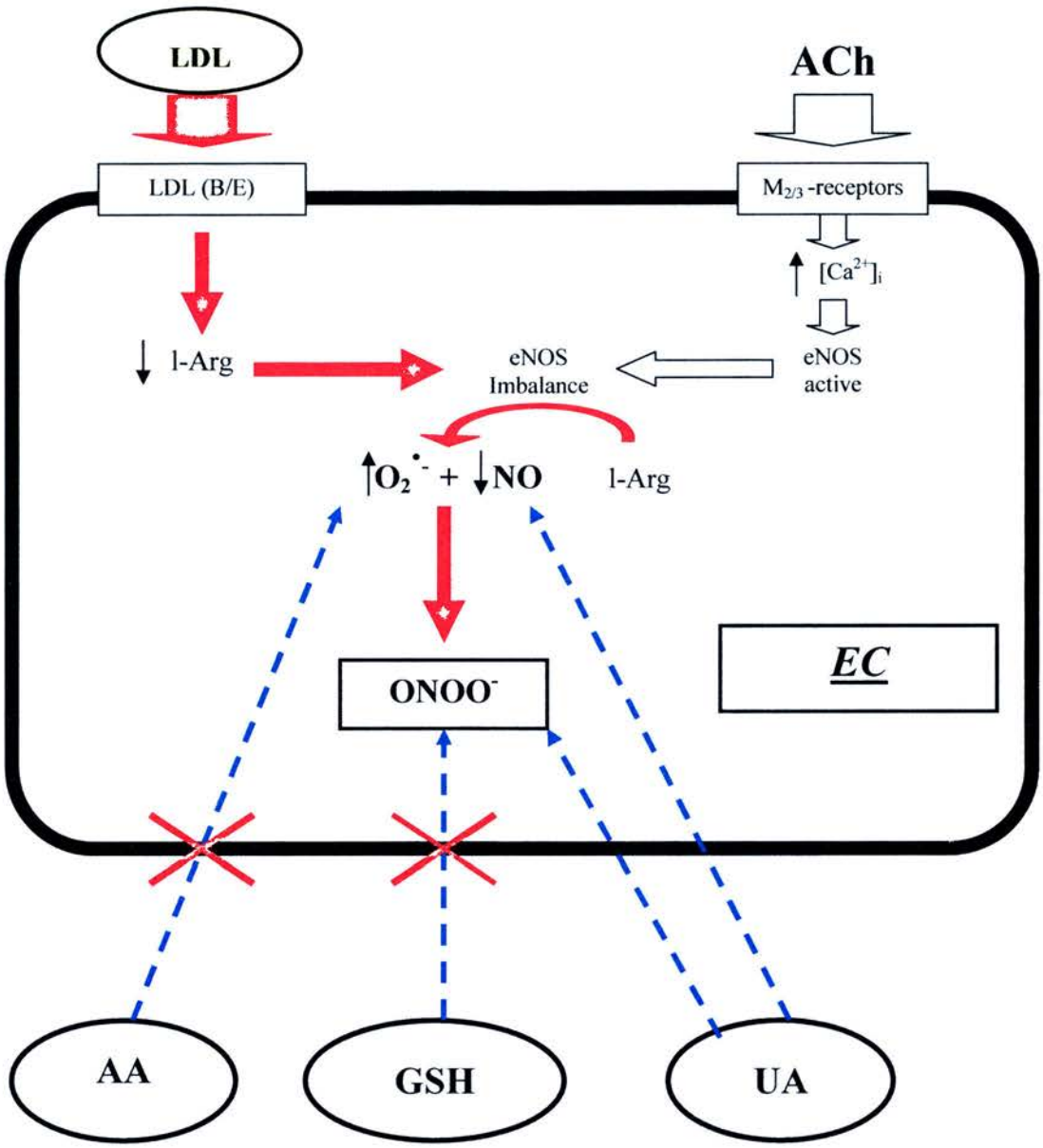


Figure 7.10. The proposed sites where the exogenous antioxidants may prevent LDL-endothelial dysfunction. AA and GSH are potent $O_2^{\cdot-}$ and $ONOO^-$ scavengers, respectively, but may be impeded extracellular breakdown. The superiority of UA over the other antioxidants could occur as only UA is able to gain access to the intracellular environment in sufficient concentrations to restore NO-mediated vasodilatation, or because UA has a unique biochemical action that is not shared by the other antioxidants.

over the other antioxidants suggests a unique ability for UA in the current experimental model. This could occur as only UA is able to gain access to the intracellular environment in sufficient concentrations to restore NO-mediated vasodilatation, or because UA has a unique biochemical action that is not shared by the other antioxidants. Unlike GSH and AA which undergo extracellular breakdown (Mann & Newton, 1975; Chang *et al,* 1992; Ghigo *et al,* 1993; Washko *et al,* 1993), UA may readily permeate cellular membranes (Becker, 1989) and exert an intracellular action (Becker, 1993). The rapid reaction between $O_2^{\cdot-}$ and NO within the EC all but negates the ability of UA to restore NO-mediated vasodilatation by preventing the intracellular inactivation of NO. Consequently, supplementation with UA would not prevent the intracellular formation of $ONOO^-$. However, UA is able to scavenge $ONOO^-$ (Kooy *et al,* 1994; Whiteman & Halliwell, 1996; Kuzkaya *et al,* 2005). This could mediate the restoration of vascular function in the current experiments by preventing $ONOO^-$ from eNOS (Pasquet *et al,* 1996) or by forming a nitrated UA derivative with the potential to induce vasodilatation (Skinner *et al,* 1998). The formation of such a vasoactive species may be the unique biochemical ability demonstrated by UA. However, further investigation is required before the restorative action of UA can be attributed to the formation of a UA-derived vasodilator species.

7.4.4 Study Limitations

The current study has several limitations. The major limitation of the current study is that the acute endothelial exposure to LDL supplemented with exogenous GSH or AA may only allow the extracellular action of these antioxidants to be examined, as mentioned above. To demonstrate the intracellular action of AA upon O_2^- and the formation of $ONOO^-$, this series of experiments could have been repeated using DHA instead of AA. AA is taken up at a slow rate by EC. However, these cells may rapidly take up DHA under similar conditions and readily converted to AA, which may

scavenge intracellular $O_2^{\cdot-}$ to restore vascular function. Furthermore, it is worth noting that in the present study the action of AA and GSH may have been impaired by the presence of substances in the physiological buffer. Bicarbonate may partially inhibit $ONOO^-$-dependent oxidation of GSH and is present in the Krebs-Ringer solution, thus, in the current study it may have prevented GSH from scavenging $ONOO^-$ to form GSNO. Similarly, during the current experiments EDTA was added to the intraluminal LDL solution to prevent the oxidative modification by transition metals. EDTA may be used as a transition metal ion chelator to chelate Cu^{2+}. It is possible that this may mask the formation of an NO-donor by GSH in the present study, by preventing the Cu^{2+}-catalysed release of NO from GSNO. It should also be noted that, as previous, the maximum constriction to KCl was performed in the presence of the various test solutions rather than Krebs-Ringer only, which may have hidden any effect the test solutions had on the maximal vasoconstriction to PE.

7.4.5 Summary

This study demonstrates the ability of UA, but not the other water-soluble antioxidants, AA or GSH, to ameliorate endothelial dysfunction induced by luminal perfusion with LDL. The concentrations of antioxidant used to supplement the LDL solutions in the current study are typical of those found in plasma for AA (40μM) and UA (400μM). The superiority of UA over the other major aqueous antioxidants in restoring vascular function implies that the action of UA may be distinct from its antioxidant ability, as both AA and GSH are potent free radical scavengers. It is hypothesised that the ability of UA to restore vascular is not due to the direct inhibition of $O_2^{\cdot-}$- mediated NO inactivation but rather to alternative mechanisms. One possibility is that UA mediates the formation of a vasoactive NO-donor species, via the scavenging $ONOO^-$ (Skinner *et al,* 1998). However, further investigation is required before the demonstrated restorative action of UA can be attributed to this.

CHAPTER 8

INVESTIGATION OF THE POTENTIAL FORMATION OF A URIC ACID-DERIVED VASODILATOR

8.1 INTRODUCTION

The mechanism by which supplementation of LDL solution with UA partially restores vascular function (Chapter 6) has still to be determined. However, the apparent superiority of UA over the other major aqueous antioxidants in restoring vascular function (Chapter 7) suggests that the action of UA may be distinct from its antioxidant ability.

The inhibitory action of LDL is thought to occur due to an imbalance in NOS activity, resulting in enhanced $O_2^{\cdot-}$ generation. This inactivates NO in a rapid diffusion-controlled reaction to yield $ONOO^-$ (Pryor & Squadrito, 1995; Beckman & Koppenol, 1996; Kissner *et al,* 1997). This reduces NO availability and, consequently, inhibits NO-mediated vasodilatation. There are several potential sites where UA may act to prevent this inhibition (Chapter 6), but the restoration of vascular function is most likely to occur via the free radical scavenging ability of UA. UA could prevent the deleterious effect of LDL by scavenging $O_2^{\cdot-}$ to directly reduce oxidative stress and prevent the inactivation of NO. However, the ability of UA to directly react with $O_2^{\cdot-}$ is debatable, as positive (Santus *et al,* 2001) and negative results (Becker *et al,* 1989; Becker *et al,* 1993) have been reported. It is unlikely that UA could prevent the inactivation of NO by $O_2^{\cdot-}$, due to the rapid rate of this reaction.

For that reason, it is suggested that the restorative action of UA does not occur prior to the inactivation of NO but after the formation of $ONOO^-$. UA is an efficient scavenger of $ONOO^-$ (Kooy *et al,* 1994; Whiteman & Halliwell, 1996; Kuzkaya *et al,* 2005), but the reaction has only been partially characterised (Uppu *et al,* 1996; Vasquez-Vivar *et al,* 1996; Skinner *et al,* 1998; Tretyakova *et al,* 1999). The oxidation of UA by $ONOO^-$ can produce two partially characterised products, an aminocarbonyl free radical (Vasquez-Vivar *et al,* 1996; Santos *et al,* 1999; Kuzkaya *et al,* 2005) and a nitrate derivative that is able to release NO (Skinner *et al,* 1998). These products have been detected in human plasma treated with $ONOO^-$ (Skinner *et al,* 1998; Vasquez-Vivar *et*

al, 1996), suggesting that they can be produced *in vivo* and may be involved in the physiological role of UA. However, the formation of a UA-derived vasoactive species has yet to be demonstrated *in vivo* or in intact biological systems *in vitro*, such as isolated tissue preparations.

The aim of this study is to demonstrate the formation of a potential UA-derived vasodilator in the presence of LDL in an intact biological system. This is investigated by examining the restoration of endothelium-dependent vasodilatation to ACh, following the addition of UA to rat endothelium-intact aortic ring segments exposed to LDL in a wire myograph model. The solution from these organ baths will then be transferred to chambers containing endothelium-denuded ring segments, and the potential to induce vasodilatation examined. Furthermore, the ability of ox-Hb, an exogenous NO-scavenger, to reverse any vasodilatation produced in the denuded aortic rings will also be examined.

To this point, perfusion myography has been used as an experimental model for assessing vascular function in isolated tissue preparations. However, while this technique offers several advantages for the *in vitro* study of vascular function, it does have disadvantages. For example, the size of the vessels and the manner in which they are perfused make the collection and transfer of the intraluminal solution difficult. This is due to dilution of the intraluminal solution upon collection and complications upon retransfer into the lumen of other arteries. Therefore, wire myography and aortic ring segments will be used in this study to facilitate the ease of transfer of solutions from the organ baths containing endothelium-intact to endothelium-denuded vessels. This will also allow the generation of greater volumes of any UA-derived product that may be formed under the experimental conditions used.

The hypotheses tested in this study are:

1). UA restores LDL-induced endothelial dysfunction because of its potential to form an aqueous endothelium-independent vasodilator following stimulation of healthy endothelium by ACh in the presence of LDL.

2). This UA-derived vasodilator is an NO donor compound, formed following a reaction with $ONOO^-$, and will be inactivated by the presence of ox-Hb.

3). The extent of the vasodilator formation will be dependent on the concentration of UA.

8.2 METHODS

8.2.1 Lipoprotein Isolation

LDL was isolated, purified and the protein concentration determined using a commercial kit based on the Lowry precipitation assay (Sigma Chemical Company, Dorset, U.K.), as previously described (Chapter 2).

8.2.2 Examination of vasodilator formation

The aorta was removed from adult male WKY rats, prepared as ring segments and mounted in a wire myograph, as described in Chapter 2 (Section 2.3). Endothelium-intact and -denuded aortic ring segments were bathed in Krebs-Ringer solution and concentration-response curves to PE performed. Concentration-response curves to ACh were then performed to ensure all denuded vessels were unresponsive and all endothelium-intact rings produced similar responses. After washout, the ring segments were bathed in 250μg/ml LDL + 200, 400 or 600μM UA. The vessels were preconstricted with EC_{80} PE, and ACh (10μM) added to the ring segments to examine endothelium-dependent vasodilatation.

5 minutes after the addition of ACh, the organ bath containing the endothelium-denuded ring was drained, the solution present in the endothelium-intact vessel chamber immediately transferred to the denuded chamber, and any vasodilatation recorded. Following 5 minutes exposure to the transferred solution, 10μM ox-Hb was added to the denuded ring segments and the alterations in tension recorded. For control purposes, the above experiment was repeated when aortic rings were bathed in Krebs-Ringer solution, 250μg/ml LDL solution, or 200, 400 and 600μM UA solution only, following completion of the concentration-response curves to PE and ACh.

8.2.3 Vasodilator stability

Aortic ring segments were prepared as previous and concentration-response curves to PE and ACh performed. 250μg/ml LDL + 400μM UA were added to the aortic ring segments, which were then preconstricted with PE (1-3μM) and ACh (10μM) added. After 5 minutes exposure to ACh, the intact aortic ring segment was removed from the myograph chamber. At various time points after removal (0, 3, 7, 15, 30, 60 and 120 minutes), the solution from the intact chamber was transferred to the chamber containing the denuded ring and vasodilatation measured. The half-life of the UA-derived dilatory product was calculated using the equation: $y = R_{Max}/ (1 + (x/Ec50)*P)$ to fit the formed curve (Biograph Version 1.0, Strathclyde University, UK).

8.2.4 Solutions

LDL solutions were prepared as previously described and diluted using Krebs-Ringer solution to give a final organ bath concentration of 250μg/ml. UA solution was prepared as previously described and added to the Krebs-Ringer solution or 250μg/ml LDL solutions to give a final organ bath concentration of 200, 400 or 600μM UA.

8.2.5 Lipid Peroxidation

Samples of the LDL and UA-supplemented solutions were collected before and after the experiments were performed. The MDA and 4-HNE concentrations of the solutions were determined using a commercial colorimetric assay kit (CN Biosciences, Nottingham, U.K.), as described in Chapter 2.

8.2.6 Statistical Analysis

The results shown are the mean values ± SEM of 6 independent experiments. The statistical significance of the results were analysed using paired Student's *t*-tests. Statistical analysis was performed to determine any significant difference in the vasodilatation produced by the transfer of the various organ bath solutions from the endothelium-intact aortic ring to the denuded ring segment. Student's *t*-tests were also used to compare the oxidative status of the test LDL solutions before and after exposure to the aortic ring segments.

8.3 RESULTS

8.3.1 Examination of vasodilator formation

8.3.1.1 Assessment of aortic contractility to PE and endothelium-dependent vasodilatation to ACh

PE ($1x10^{-8}$–$1x10^{-5}$M) produced concentration-dependent vasoconstriction in endothelium-intact aortic ring segments, bathed in physiological Krebs-Ringer solution. This vasoconstriction was significantly greater in endothelial denuded ring segments at concentrations of $1x10^{-8}$-$3x10^{-7}$ PE ($p<0.05$). Similarly, the EC_{50} of the denuded ring segments (45.8 $\pm$ 6.2nM) was significantly lower than the intact segments (67.1 $\pm$ 4.8nM) ($P<0.01$; Paired Student's *t*-test, n=48). However, the vasoconstriction to greater concentrations of PE ($7x10^{-7}$ to $1x10^{-5}$) was similar (Fig. 8.1.).

ACh ($1x10^{-8}$–$1x10^{-5}$M) produced concentration-dependent vasodilatation in endothelium-intact vessels, with a maximal dilatation of approximately 98% towards resting diameter. All endothelial-denuded vessels were unresponsive to ACh (Fig 8.2.).

8.3.1.2 Endothelium-dependent vasodilatation to ACh in intact aortic ring segments

In the physiological control, ACh (10μM) produced vasodilatation of 79.8 $\pm$ 4.6%. Dilatation to ACh in the presence of 250μg/ml LDL was significantly reduced (24.3 $\pm$ 2.7%) ($P<0.01$). Following the supplementation of LDL with 200μM, 400μM or 600μM UA, ACh produced a vasodilatation of 33.1 $\pm$ 5.9%, 42 $\pm$ 2.1% and 48.5 $\pm$ 4.7%, respectively. These were significantly greater than the dilatation in LDL only ($P<0.01$), but markedly attenuated when compared to the physiological or UA control ($P<0.01$).

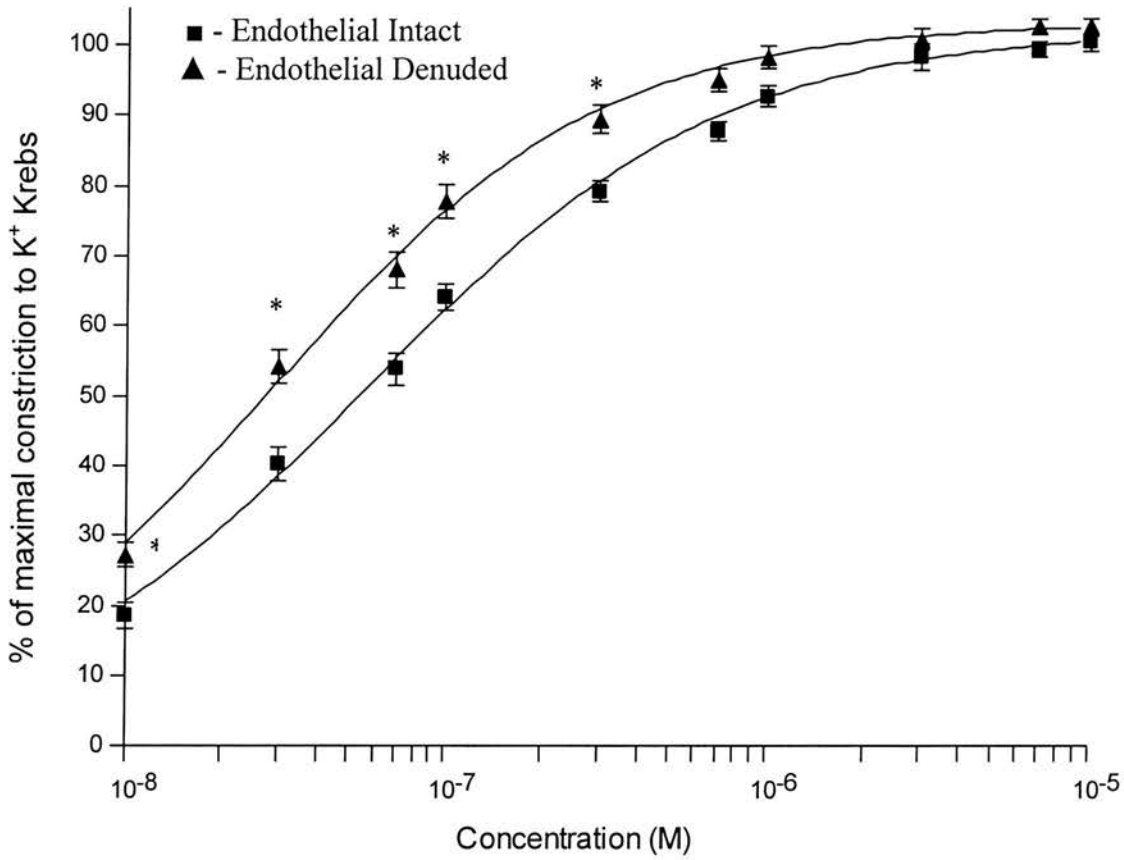


Figure 8.1. The concentration-dependent vasoconstriction to PE in endothelial-intact and –denuded rat aortic ring segments, prior to the addition of the various LDL and UA solutions. *$p<0.05$ vs. intact vessels (2-factor ANOVA with replication and Bonferroni correction, n=48). All values are the mean $\pm$ SEM.

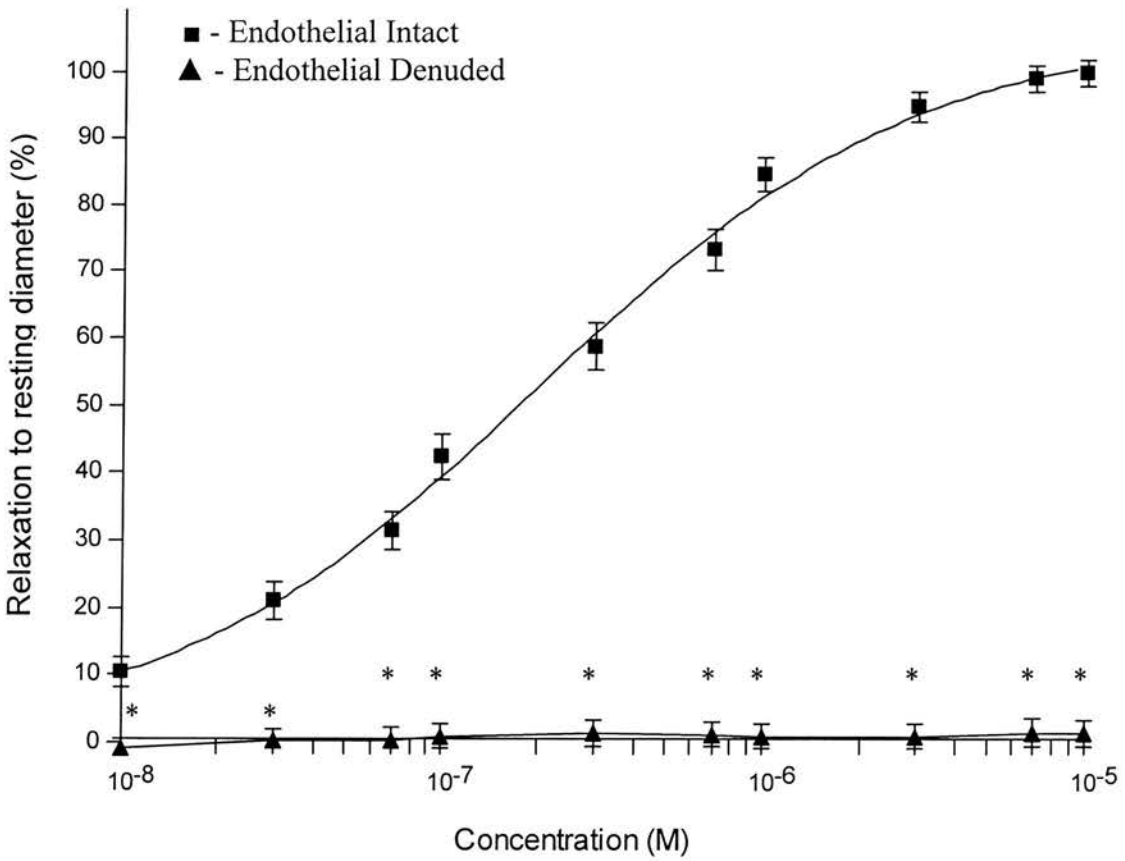


Figure 8.2. The concentration-dependent vasodilatation to ACh in endothelial-intact and –denuded rat aortic ring segments, prior to the addition of the various LDL and UA solutions. *$p<0.02$ vs. intact vessels (2-factor ANOVA with replication and Bonferroni correction, n=48). All values are the mean $\pm$ SEM.

8.3.1.3 Endothelium-dependent vasodilatation to ACh in denuded aortic ring segments

There was no significant vasodilatation to 10μM ACh in the denuded aortic rings, regardless of whether the segments were bathed in Krebs-Ringer, UA, LDL, or UA supplemented LDL solutions (Fig 8.3).

8.3.1.4 Investigation into vasodilator formation by examining vasodilatation in denuded aortic rings

Transferring Krebs-Ringer or 250μg/ml LDL solution from the intact ring segments to the denuded rings produced relaxation towards resting tension of 0.9 ± 1.4% and 3.2 ± 4.9%, respectively. Transferring 200μM, 400μM or 600μM UA solution produced vasodilatation of -0.5 ± 2.2%, 2.7 ± 2.5% and 1.3 ± 2%. The transfer of the of the LDL solution supplemented with 200μM (Fig 8.4), 400μM (Fig 8.5) or 600μM UA (Fig 8.6) produced vasodilatation of 21.2 ± 1.9%, 33.1 ± 6% and 49.2 ± 6.6%, respectively. These were significantly greater than the vasodilatation induced by the transfer of physiological Krebs-Ringer solution ($P<0.01$), LDL ($P<0.01$), or UA only solution ($P<0.01$). The vasodilatation produced by the UA-supplemented LDL solutions was significantly greater when LDL was supplemented with increased UA concentrations ($P<0.01$; Fig 8.7).

8.3.1.5 The role of NO in the vasodilatation produced by the solution transfer

10μM Ox-Hb reversed the vasodilatation induced by the transfer of the LDL solution supplemented with 200, 400 or 600μM UA. Ox-Hb constricted the vessels to 103.1 ± 3.1%, 103.9 ± 5.9% and 96.2 ± 4.8% of preconstriction with PE, respectively (Fig 8.8).

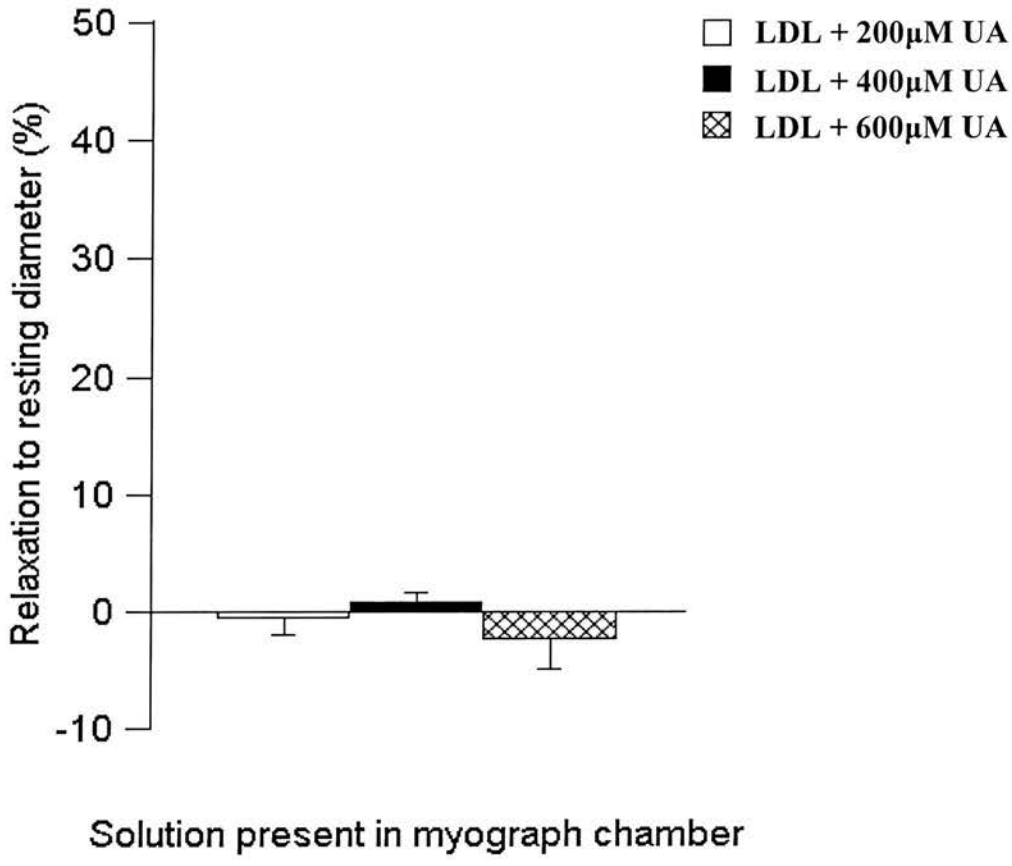


Figure 8.3. Comparison of vasodilatation to ACh in the presence of 250µg/ml LDL + 200µM, 400µM, or 600µM UA in denuded aortic ring segments. All values are the mean ± SEM (n=6).

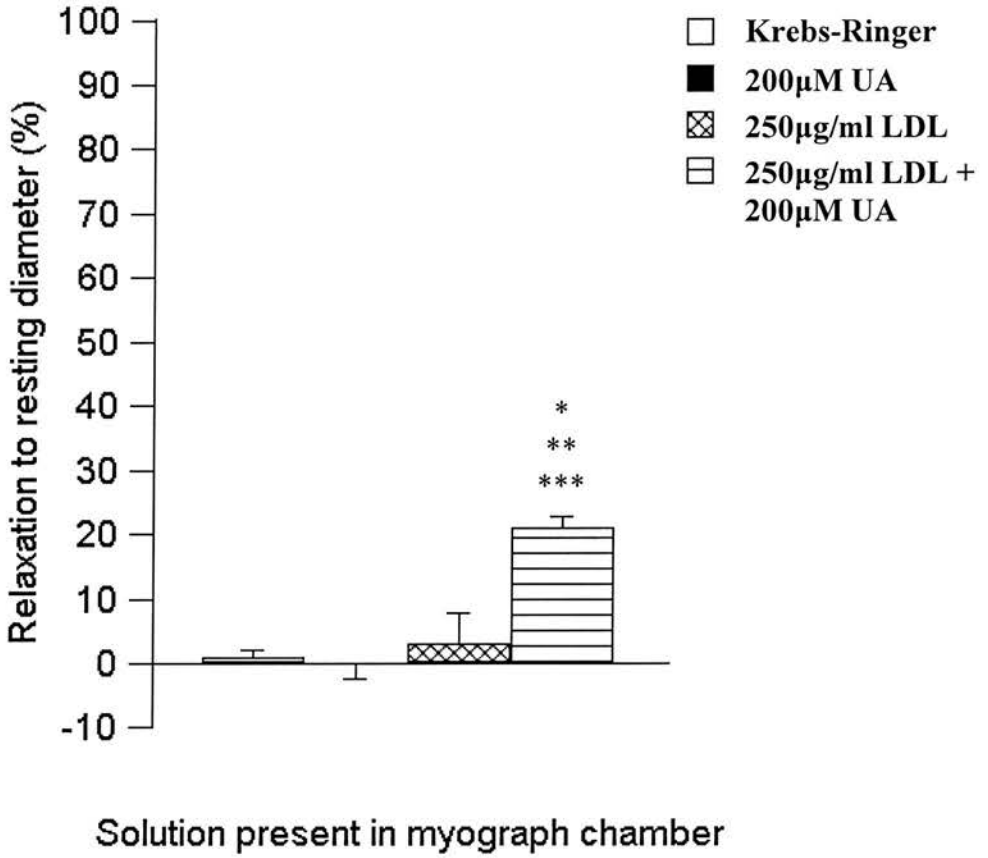


Figure 8.4. Investigation into donor formation by examining vasodilatation in denuded aortic rings: Comparison of vasodilatation when transferring Krebs-Ringer, 200µM UA, 250µg/ml LDL, or 250µg/ml LDL + 200µM UA acid from intact aortic rings to denuded rings. *$p<0.01$ vs. Krebs-Ringer (paired Student's t-test, n=6), **$p<0.01$ vs. 200µM UA (paired Student's t-test, n=6), ***$p<0.01$ vs. 250µg/ml LDL (paired Students t-test, n=6). All values are the mean $\pm$ SEM (n=6).

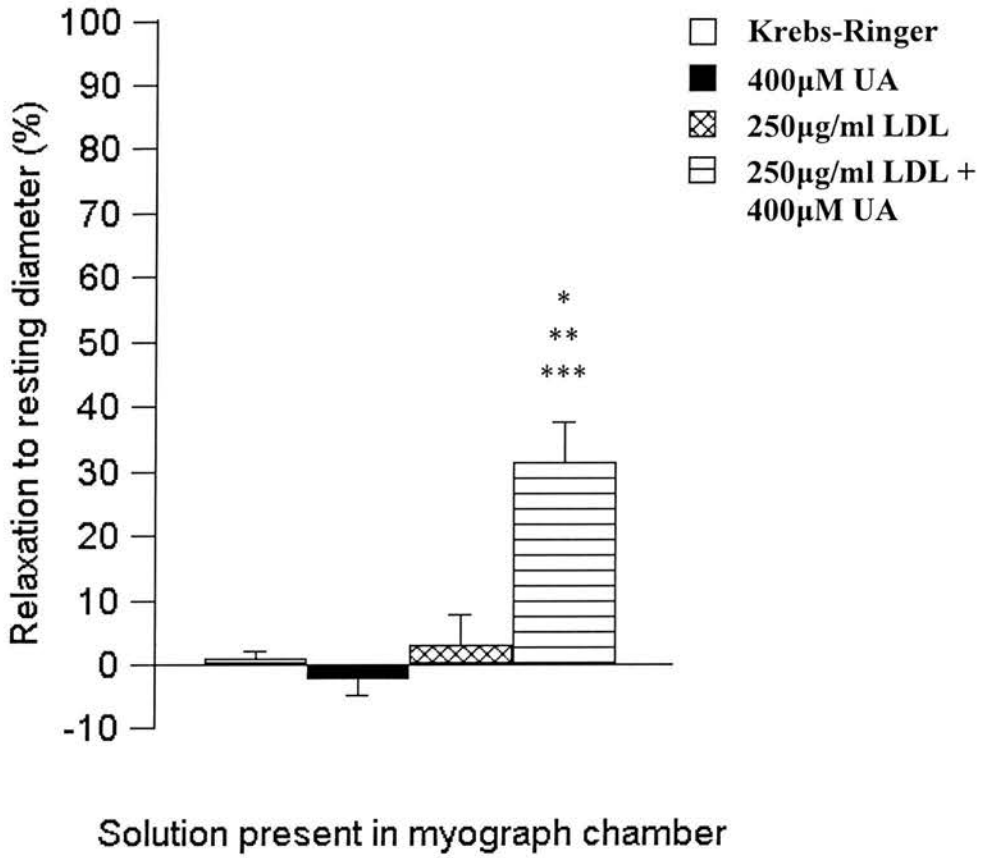


Figure 8.5. Investigation into donor formation by examining vasodilatation in denuded aortic rings: Comparison of vasodilatation when transferring Krebs-Ringer, 400µM UA, 250µg/ml LDL, or 250µg/ml LDL + 400µM UA from intact aortic rings to denuded rings. *$p<0.01$ vs. Krebs-Ringer (paired Student's t-test, n=6), **$p<0.01$ vs. 400µM UA (paired Student's t-test, n=6), ***$p<0.01$ vs. 250µg/ml LDL (paired Students t-test, n=6). All values are the mean $\pm$ SEM (n=6).

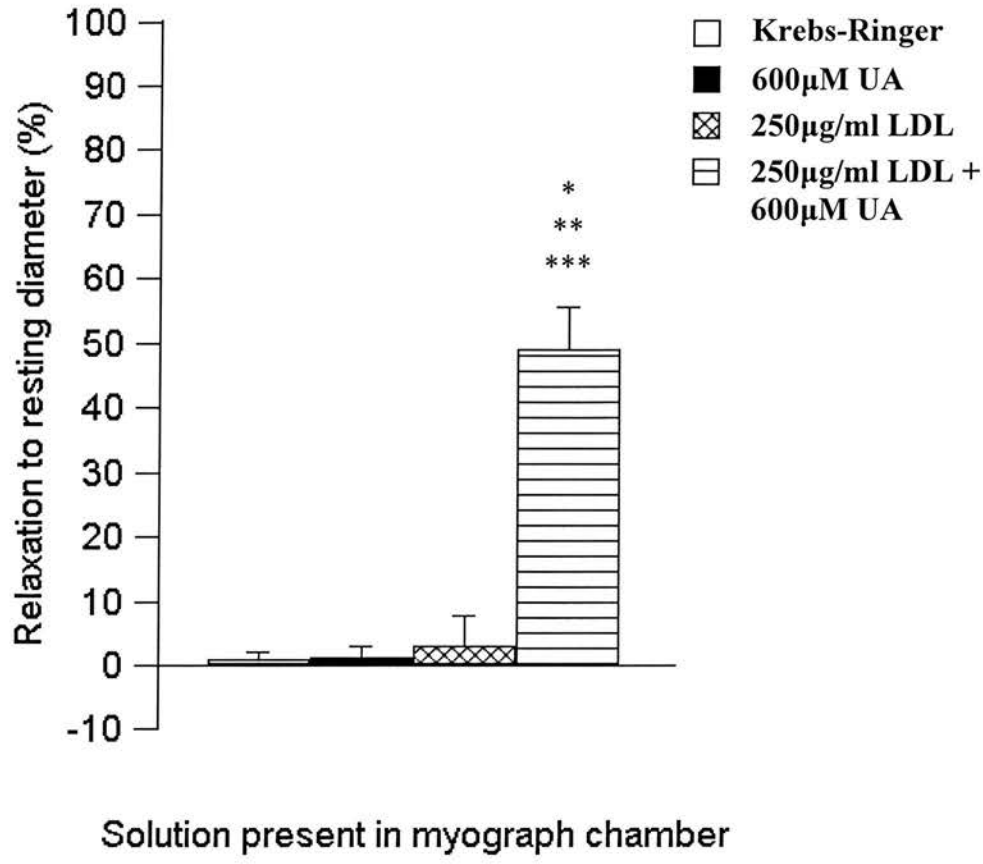


Figure 8.6. Investigation into donor formation by examining vasodilatation in denuded aortic rings: Comparison of vasodilatation when transferring Krebs-Ringer, 600µM UA, 250µg/ml LDL, or 250µg/ml LDL + 600µM UA from intact aortic rings to denuded rings. *$p<0.01$ vs. Krebs-Ringer (paired Student's t-test, n=6), **$p<0.01$ vs. 600µM UA (paired Student's t-test, n=6), ***$p<0.01$ vs. 250µg/ml LDL (paired Students t-test, n=6). All values are the mean $\pm$ SEM (n=6).

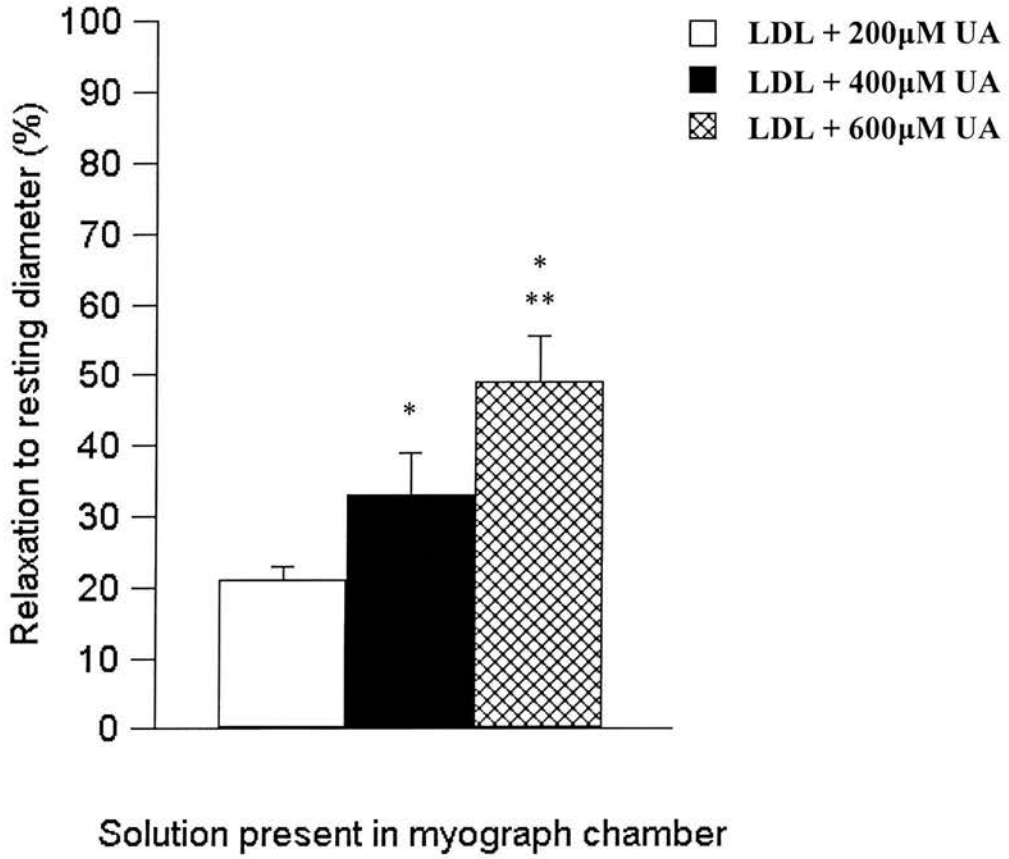


Figure 8.7. Comparison of vasodilatation when transferring 250µg/ml LDL + 200µM, 400µM, or 600µM UA from intact aortic rings to denuded rings. *$p<0.01$ vs. 250µg/ml LDL + 200µM UA (paired Student's t-test, n=6), **$p<0.01$ vs. 250µg/ml LDL + 400µM UA (paired Student's t-test, n=6). All values are the mean $\pm$ SEM (n=6).

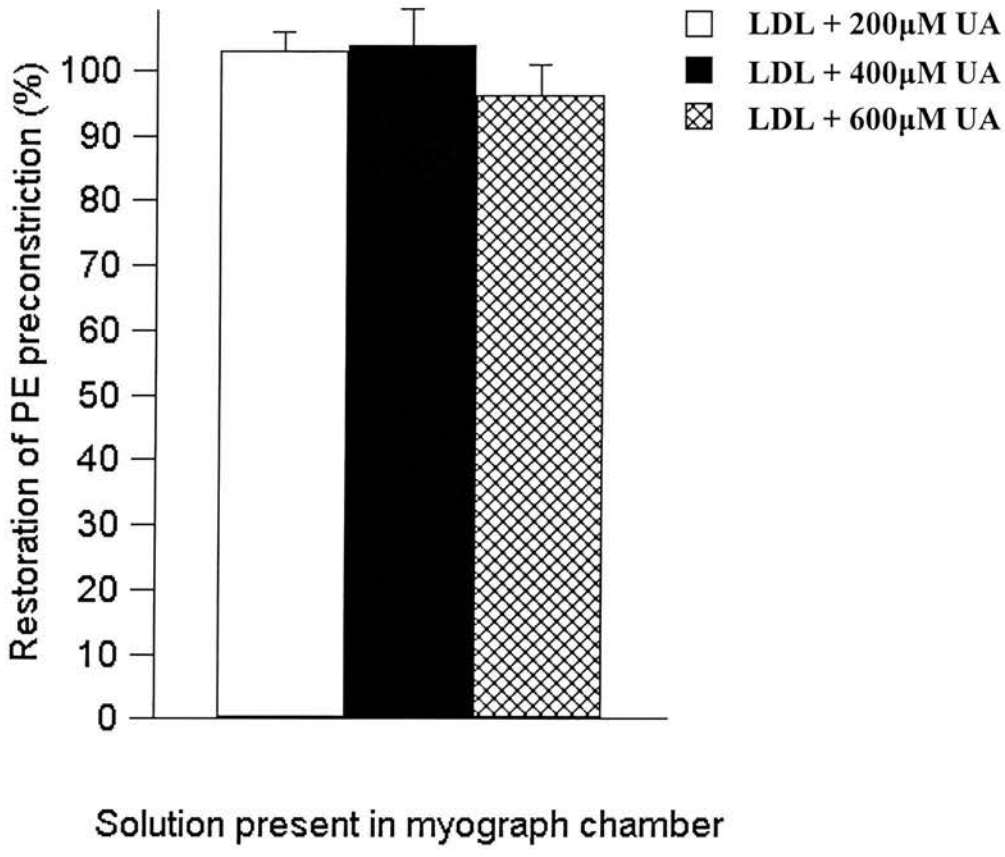


Figure 8.8. The role of NO in vasodilatation to the UA-derived vasodilator: Reversal of vasodilatation, following the transfer of 250μg/ml LDL + 200μM, 400μM, or 600μM UA solution from intact aortic rings to denuded rings, by Ox-Hb. All values are the mean ± SEM (n=6).

8.3.2 Donor stability

Solution transfer at 0 minutes after the removal of the intact aortic ring resulted in 33.1 $\pm$ 6.9% vasodilatation (Fig 8.9). After 15 minutes, the solution transfer produced 23 $\pm$ 3.9% vasodilatation. Transfer after 120 minutes resulted in 4.2 $\pm$ 2.3% vasodilatation (Fig 8.9). The decay of the NO-dependent product formed was exponential and the calculated half-life was 29.3 $\pm$ 3.1 minutes at 37°C.

8.3.3 Lipid peroxidation

There was no significant alteration in the MDA and 4-HNE concentrations of the LDL solutions, suggesting that no change in the oxidative status of the LDL occurred during the experiments (Fig 8.10).

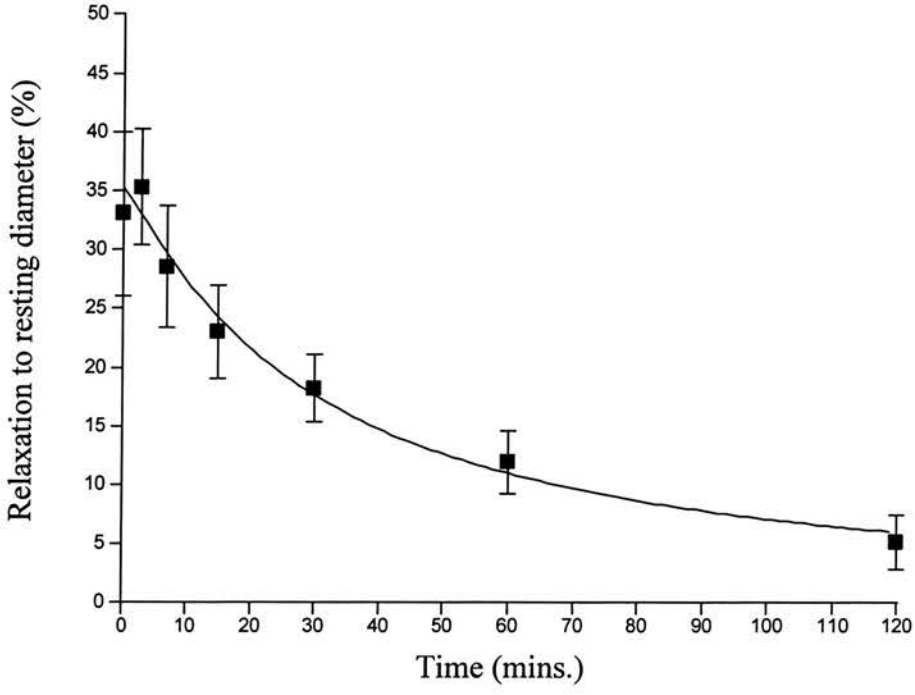


Figure 8.9. Examination of the stability of the UA-derived vasodilator: The effect of time from formation upon vasodilatation in denuded rings following the transfer of 250μg/ml LDL + 400μM UA solution from intact aortic rings. All values are the mean + SEM (n=6).

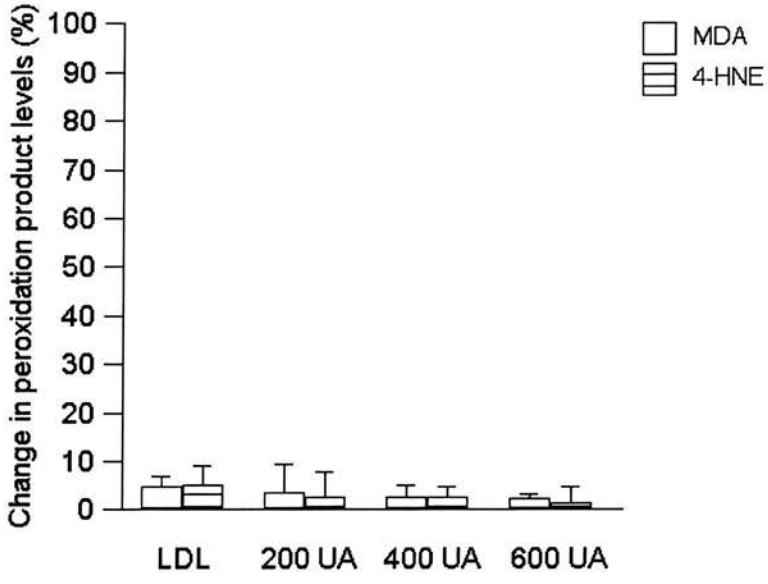


Figure 8.10. Alterations in lipid peroxidation product levels: Mean percentage change in the concentration of MDA and 4-HNE following addition to aortic rings. All values are mean $\pm$ SEM (n=6).

8.4 DISCUSSION

Superfusion of endothelium-intact aortic ring segments with LDL solution significantly impaired vasodilatation to ACh when compared to the physiological control. However, supplementation of the LDL solution with UA (200, 400, 600μM) significantly restored ACh-mediated dilatation in a concentration-dependent manner, as previously demonstrated in the isolated mesenteric arteries. Superfusion with UA only had no significant effect upon vasodilatation. Endothelial denudation abolished all vasodilatation to ACh in the aortic rings, regardless of the superfusing solution.

Following the transfer of the solution from intact vessels to denuded vessels, only the superfusing solution containing UA and LDL caused significant vasodilatation, all other solutions (Krebs, LDL and UA only) were ineffective. This vasodilatation was related to the concentration of UA and could be completely reversed by the addition of ox-Hb to the organ bath. The half-life of the formed dilatory product was 29.3 $\pm$ 3.1 minutes at physiological temperature and pH.

8.4.1 Restoration of Vascular Function by UA

In Chapter 6, 200 to 600μM UA improved the deleterious effects of LDL on endothelial function in a concentration-dependent manner. The dependency of the benefits of UA on the release and activity of NO was clarified by the lack of any improvement in vasodilator response in the presence of L-NAME, an eNOS inhibitor. It has previously been suggested that UA is an antioxidant capable of reacting with and inactivating $O_2^{\cdot -}$ (Ames *et al*, 1981; Santus *et al,* 2001), and it is tempting to speculate that UA restores vascular function via this simple antioxidant mechanism. This is unlikely, as $O_2^{\cdot -}$ is far more likely to react with NO than UA. Instead, it is more probable that the restoration of vasodilatation relates to the ability of UA to scavenge $ONOO^-$ (Squadrito *et al*,

2000), which has the potential to further damage the endothelium and eNOS (Milstien *et al*, 1999). The apparent superiority of UA over other aqueous antioxidants in the perfusion myograph model led to the hypothesis that stimulation of intact vessels with ACh in the presence of LDL and UA led to the formation of a vasodilator in solution.

This was demonstrated in the current study when transferring the superfusing solutions from the endothelial intact rings to the denuded rings, as only the solution containing both UA and LDL caused dilatation of the denuded vessels. The transfer of the Krebs-Ringer solution (with and without UA) may also have been expected to induce vasodilatation, as the technique used was similar to that used by Furchgott and Zawadski to establish the release of EDRF from EC (Furchgott & Zawadski, 1980). This group demonstrated that the stimulation of endothelium-intact aortic strips with ACh could induce vasodilatation in adjacent denuded strips (Furchgott & Zawadski, 1980). Therefore, following EC stimulation with ACh in the current study, the Krebs-Ringer solution from the intact vessel should contain NO, which could potentially dilate the denuded vessels upon transfer. However, the failure of this solution to induce vasodilatation may be due to the relatively short half-life of NO. NO has a half-life of only a few seconds (Beckman & Crow, 1993; Vallance & Collier, 1994; Denicola *et al*, 1996) but the solution was not transferred until 5 minutes after EC stimulation, thus any NO produced may have been consumed or decomposed prior to transfer.

The previous lack of effect of ACh in denuded rings confirmed the abolition of endothelium-dependent vasodilatation, and proved ACh was not responsible for the vasodilatation upon transfer. Consequently, vasodilatation of the denuded ring segments demonstrated the presence of an endothelium-independent vasodilator in the transferred solution. Therefore, it is concluded that the stimulation of the EC in the presence of both LDL and UA caused the formation of a vasoactive substance. However, although the vasodilatation produced is endothelium-independent, the formation of the dilatory product is endothelium-dependent, as it requires stimulation of the intact endothelium prior to transfer. This was as shown by the failure of UA to

restore dilatation in denuded vessels exposed to ACh and LDL. The transfer experiments also suggest an absolute requirement for UA with LDL, as neither UA nor LDL alone caused vasodilatation when transferred to the denuded segments. The dilatation was also related to the concentration of UA, as greater UA concentrations produced greater dilatation following transfer. This supports the hypothesis that the vasodilator produced is a derivative of UA.

8.4.2 Mechanism of action of vasodilating factor

The dilatation of the endothelial denuded aortic rings by the UA-derived vasodilator was reversed by ox-Hb, an exogenous NO-scavenger (Martin *et al,* 1985; Joshi *et al,* 2002). This suggests that the vasodilatation is produced by the release of NO, or a closely related molecule, in the extracellular environment. However, if NO is released from the UA-derived vasodilator in such a manner, then it remains unclear why it is not subjected to the same reduction in availability that apparently occurs in the presence of LDL alone, via the reaction with $O_2^{\cdot -}$. This may reflect the increased stability of the donor in the vessel wall or an altered site of NO release. For example, the NO may be released in close proximity to the VSMC, at a site that is removed from any endothelial $O_2^{\cdot -}$, allowing it to interact with VSMC before it can be inactivated.

The half-life of the UA-derived vasodilator formed in this study is 29.3 minutes $\pm$ 3.1 minutes at physiological temperature and pH. It has previously been demonstrated that $ONOO^-$ may react with biological molecules to form a vasodilator species that releases NO by a thiol and Cu^+-dependent mechanism (Moro *et al,* 1995; White *et al,* 1997). However, the release of NO from the UA-derived vasodilator in the current study is spontaneous. This is in agreement with the findings of Skinner *et al*, who demonstrated the release of NO was not accelerated by the addition of Cu^{2+} or thiols (Skinner *et al,* 1998).

Therefore, the results suggest that the UA-derived vasoactive substance formed is an NO-donating species that produces vasodilatation via the spontaneous release of NO in the extracellular environment. However, this awaits direct confirmation and can only be inferred at present. The proposed mechanism of action of the UA-derived vasodilator factor is demonstrated in Figure 8.11. It is likely that this NO-mediated vasodilatation occurs via a cGMP-dependent mechanism, but this requires further investigation and may be answered following the identification of the formed vasodilator.

8.4.3 Formation of the vasodilatory substance

The molecular nature of the vasodilator has not been characterised, but it may be a nitration product formed by the ability of UA to scavenge LDL-generated $ONOO^-$. $ONOO^-$ formation in the vascular wall occurs via the generation of both NO and $O_2^{\cdot-}$ by EC (Oury *et al,* 1993; Van der Vliet *et al*, 1994). In the current study, the generation of $O_2^{\cdot-}$ and NO occurs from exposure of aortic EC to LDL and ACh, to stimulate NO production. As both are produced in close proximity and react rapidly, $ONOO^-$ formation within the EC is likely to occur. UA is a potent endogenous scavenger of $ONOO^-$ (Hooper *et al,* 1997; Hooper *et al,* 1998) and their interaction has been well documented. UA may react with $ONOO^-$ to form a variety of derivatives (Santos *et al,* 1999). Oxidation of UA by $ONOO^-$ may form two partially characterised products, an aminocarbonyl free radical intermediate centred on an electron-deficient carbon atom, which may potentiate harmful effects of $ONOO^-$ (Vasquez-Vivar *et al,* 1996), and a nitrate derivative which may release NO (Skinner *et al,* 1998). Although these products have been detected in human plasma treated with $ONOO^-$, their production *in vivo* has still to be demonstrated and the reaction between UA and $ONOO^-$ remains poorly characterised (Uppu *et al,* 1996; Vasquez-Vivar *et al,* 1996; Skinner *et al,* 1998; Tretyakova *et al,* 1999). However, the formation of a UA-based NO donor is biologically plausible. The ability of UA to form an NO-mediated vasodilator gives further support to the hypothesis that UA is found in elevated concentrations in

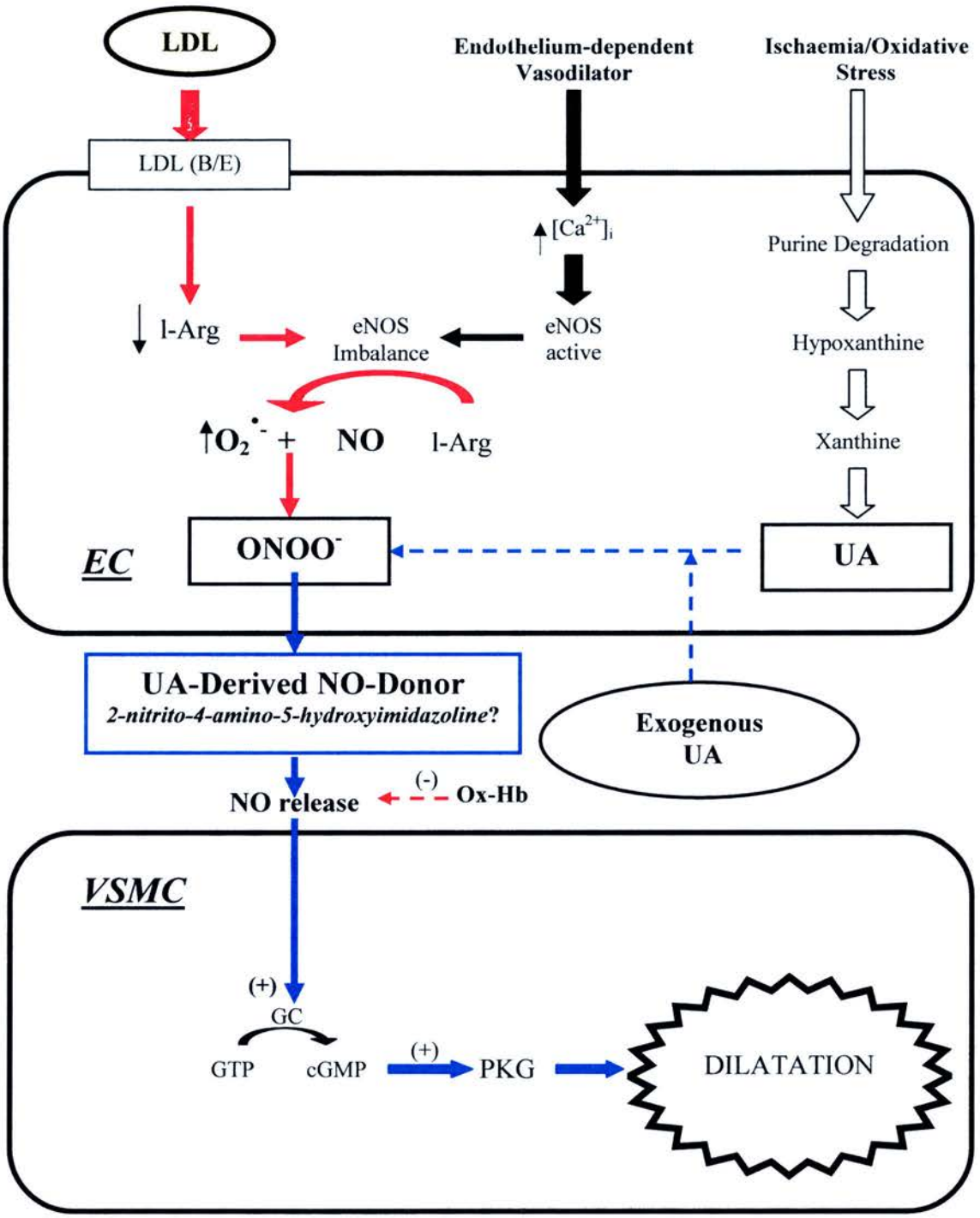


Figure 8.11. The proposed mechanism by which UA restores NO-dependent vasodilatation following endothelial exposure to LDL. It is hypothesised that UA scavenges the $ONOO^-$, formed within the EC, to produce a nitrated UA-derivative that spontaneously releases NO, thus restoring endothelial NO-dependent vasodilatation.

hypercholesterolaemia, and other pathophysiological conditions, as a defence system to minimise the deleterious effect of LDL on NO-availability. This may provide a previously undetermined role for UA in human physiology and the pathophysiology of cardiovascular disease.

8.4.4 Identification of the vasodilatory substance

The results produced in this series of experiments are similar to those of Skinner *et al*, who demonstrated that the addition of UA to human plasma or a physiological buffer containing $ONOO^-$ *in vitro* produced a nitrated UA product (Skinner *et al,* 1998). This nitrated UA product was identified as 2-nitrito-4-amino-5-hydroxyimidazoline. The proposed molecular structure of this dilatory species is shown in Figure 8.12 (Skinner *et al,* 1998). It was demonstrated that this could act as a vasoactive NO-donor and produce concentration-dependent relaxation of rat aortic ring segments. This vasodilatation was endothelium-independent and ox-Hb sensitive (Skinner *et al,* 1998). The vasoactive species produced in the current study also produced UA-concentration dependent vasodilatation of denuded rat aortic ring segments and, as has already been demonstrated, shared several of the characteristics of the UA-derived NO-donor produced by the Skinner group. However, a discrepancy exists between the results of the current study and the findings by Skinner *et al*. Electrochemical quantification confirmed that the UA/$ONOO^-$ reaction resulted in the release of NO from a nitrated UA-derivative with a decay half-life of 125 minutes (Skinner *et al,* 1998). The half-life of the vasoactive species formed in this study (29.3 minutes) was significantly less than the half-life of the nitrated-UA derivative formed by Skinner *et al.* However, the Skinner group performed their studies at 10°C, but the present study was performed at physiological temperature (37°C). This could account for the difference as a 10°C decrease in temperature will approximately double the half-life. Therefore, it is hypothesised that a similar nitrated UA product is responsible for the current results. However, while the Skinner study demonstrated the *ex vivo* formation of a vasoactive

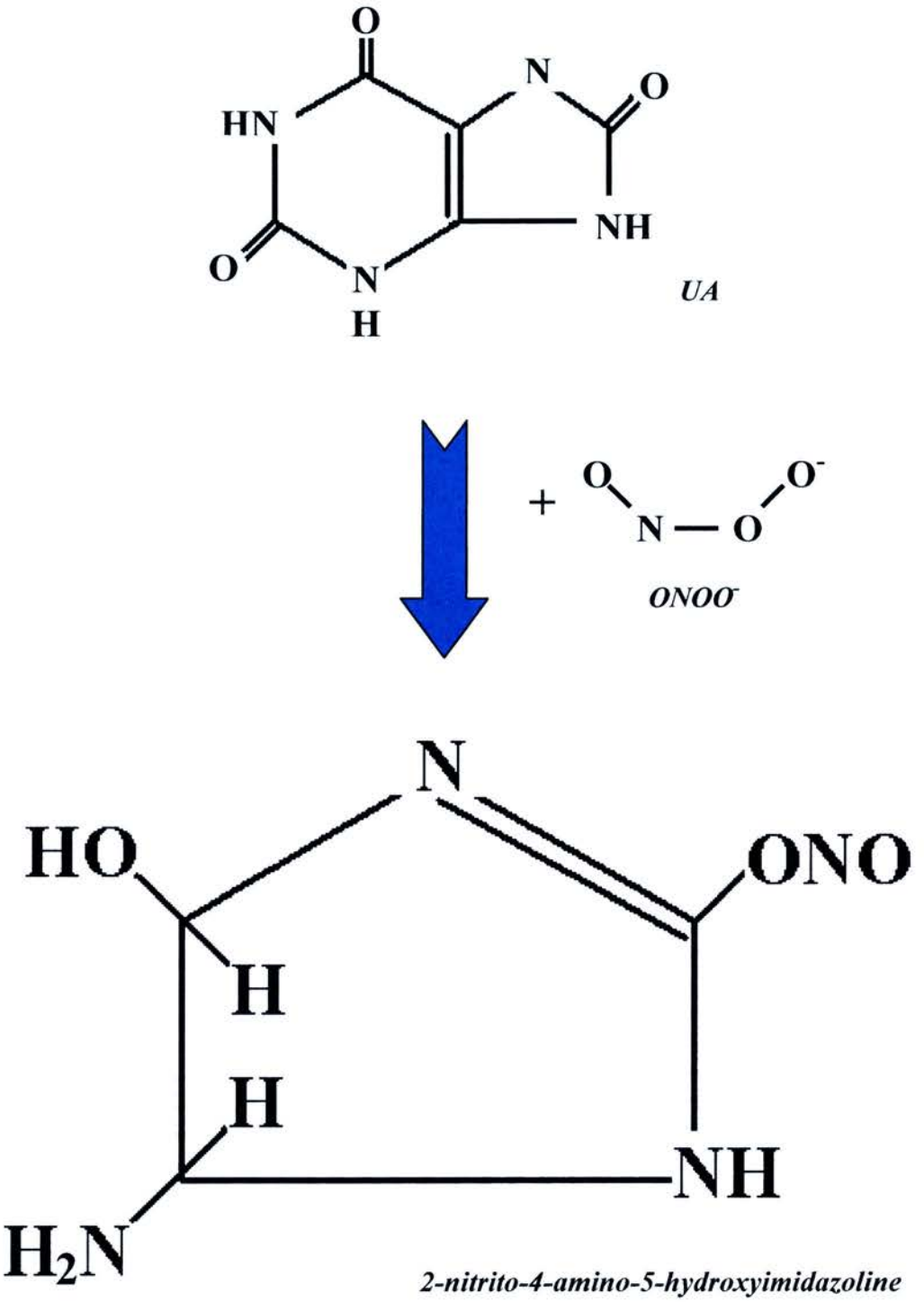


Figure 8.12. The proposed molecular structure of the UA-derived vasodilator formed by the reaction of UA with $ONOO^-$ (Skinner *et al*, 1998). It is proposed that this compound, 2-nitrito-4-amino-5-hydroxyimidazoline, acts as a NO-donating species (Skinner *et al*, 1998), and is responsible for the NO-mediated vasodilatation in the current study.

species prior to addition to isolated vessels, this study demonstrates the formation a UA-derived vasodilator in aortic ring preparations. These results, when taken in conjunction with those of previous chapters, could provide the first evidence of a UA-based NO-donor being formed in an intact biological system.

8.4.5 Physiological Relevance

The formation of a nitrated UA-derivative *in vivo* could have wide reaching implications for human physiology and the association between elevated UA concentrations and pathophysiological conditions predisposing to cardiovascular disease, such as hypercholesterolaemia. The observation that the UA-derived vasodilator releases NO in a spontaneous and continuous fashion suggests that it could play a significant role in the maintenance and restoration of vascular function in human physiology. The current results provide a mechanism by which the higher concentrations of UA present in plasma and extracellular fluids could offer an important biological advantage in human physiology and the pathophysiology of cardiovascular disease. This may in turn be responsible for the evolutionary loss of UA breakdown by urate oxidase, and the increased UA concentrations in human physiology, which are positively associated with an increase in human life-span (Ames *et al,* 1981).

8.4.6 Study Limitations

A limitation of this study is the speculation that the effects of LDL on vascular function resulted in the formation of $ONOO^-$, as the formation of $ONOO^-$ was not examined. In addition, whilst the formation of the vasodilator and subsequent dilatation was UA-concentration dependent, the concentration of $ONOO^-$ present was not established, and as such, the concentration-dependent effect of $ONOO^-$ could not be determined. Furthermore, the effects of LDL concentration upon the formation of the vasoactive

species were not examined as only one concentration of LDL was present. Similarly, only one concentration of ACh was used to stimulate the EC. Repeating the experiments using other concentrations of ACh could help to determine the role of endothelial stimulation and endothelial-derived NO upon the formation of a UA-derived vasodilator in isolated vessel preparations acutely exposed to LDL. The current study is also limited as there is no direct measurement of NO release; this was demonstrated by assessing changes in vascular function and the reversal of vasodilatation following solution transfer by ox-Hb. Finally, EDTA and Li_2CO_3 were present within the LDL and UA solutions respectively. However, it has previously been demonstrated that the presence of these would be unlikely influence the results produced (Chapter 4, 5 and 6).

8.4.7 Summary

The present study concluded that UA can partially restore LDL-induced endothelial dysfunction via the formation of a UA-derived vasoactive species. The formation of this vasodilator is UA-concentration-dependent, and occurs following the stimulation of healthy endothelium by ACh in the presence of LDL. This vasodilator exerts an endothelium-independent action and the vasodilatation produced is ox-Hb-sensitive, suggesting that the UA-derived vasodilator is likely to be an NO donor. It is hypothesised that this is formed by the reaction of UA with $ONOO^-$, generated as a result of EC exposure to LDL. The ability of UA to scavenge $ONOO^-$ is well recognised (Squadrito *et al,* 2000) and it has previously been demonstrated that the treatment of human plasma with $ONOO^-$ produces a UA derivative that causes vasodilatation similar to the current study (Skinner *et al,* 1998). This reference is widely cited, but the formation of this UA-derived NO-donor has never been demonstrated *in vivo*. However, the current study could provide the first evidence for the formation of such a species in an intact biological system. This UA-derived vasodilator could play a vital role in human physiology by restoring NO-mediated function following endothelial exposure to LDL.

CHAPTER 9

GENERAL DISCUSSION

9. GENERAL DISCUSSION

9.1 INTRODUCTION

UA is the major product of purine metabolism and is found in abundant concentrations in human extracellular fluid. It is a major hydrophilic antioxidant and is present in considerably higher concentrations in man than most other species, due to the loss of the ability to express the enzyme urate oxidase (Ames *et al*, 1981). This evolutionary process and the physiological treatment of UA suggest that the human body does not regard UA as a 'waste product', but may imply that it has a very important physiological role.

However, there has been substantial clinical and epidemiological evidence reporting a positive association between UA, cardiovascular disease and various cardiovascular events, such as stroke and myocardial infarction (Fessel, 1980; Brand *et al*, 1985; Bonora *et al*, 1996; Persky *et al*, 1997; Bengtsson *et al,* 1998; Lehto *et al*, 1998; Liese *et al*, 1999; Ward, 1998; Fang & Alderman, 2000). Vascular injury and endothelial dysfunction are key events in conditions such atherosclerosis, hypertension, diabetes mellitus and hypercholesterolaemia, which predispose to the development of cardiovascular disease (D'Angelo *et al,* 1978; Blann *et al,* 1995). Increased oxidative stress is a major factor in the development of this dysfunction, and although UA is an established antioxidant, these conditions are often accompanied by elevated UA concentrations (Wannamethee *et al,* 1997). Consequently, it has been suggested that UA is an independent risk factor for the development of cardiovascular disease. However, the specific role of UA in the pathogenesis of this condition has been difficult to ascertain, due to the association of UA with other established cardiovascular risk factors. Therefore, it remains to be determined if UA has a damaging or protective role under these circumstances. Increased understanding of the effect of UA in the vascular system and the relationship between UA and the associated risk factors for

cardiovascular disease would allow a clearer interpretation of the association between serum UA concentrations and cardiovascular disease.

This thesis aimed to determine the role of UA in cardiovascular disease by investigating the direct action of UA upon endothelial and vascular function and examining the association between elevated UA and LDL. This was performed using isolated rat mesenteric arteries in an *in vitro* perfusion myograph system. Perfusion myography allowed the UA and lipoprotein solutions to be perfused through the vascular lumen to mimic the endothelial exposure to these compounds that occurs in plasma *in vivo*. In addition, a wire myograph model was used to investigate the potential ability of UA to form a vasoactive species in isolated rat aortic ring segments exposed to ACh in the presence of LDL.

9.2 THE USE OF PERFUSION MYOGRAPHY TO ASSESS VASCULAR AND ENDOTHELIAL FUNCTION

The technique employed for the majority of these studies is more technically demanding and time consuming than alternative techniques, such as wire myography. However, perfusion myography is believed to be the optimal technique for assessing the impact of LDL upon endothelial function as it better replicates the *in vivo* conditions, proportionately longer vessel segments can be mounted, there is less danger of endothelial damage or disruption during mounting, and the vessels can be exposed to LDL via the endothelial surface under physiological pressures. This experimental technique was selected for this series of studies as luminal perfusion prevented the results from being influenced by any direct action of the LDL upon the VSMC. This technique also allowed the studies to be performed using isolated resistance arteries. These vessels are major determinants of peripheral vascular resistance, blood pressure, blood flow and perfusion of the surrounding tissue. The *in vitro* study of these arteries is vital to investigating the pathophysiology of cardiovascular disease, as alterations in

the structure and function of these blood vessels may have a prominent role in the various pathological conditions that predispose to this disease state (Dunn & Gardiner, 1995).

However, the role of NO in ACh-induced vasodilatation in isolated rat mesenteric arteries is uncertain. Therefore, the ability of this experimental model to examine NO-mediated vascular function was investigated in Chapter 3. These results demonstrated that perfusion with 100μM L-NAME reduced vasodilatation to ACh by approximately 58%. Perfusion with 10μM indomethacin only reduced vasodilatation by 8%. Following luminal perfusion with both 100μM L-NAME and 10μM indomethacin, the vasodilatation produced was only 36% of the physiological response. It is hypothesised that this residual vasodilatation was produced via hyperpolarisation to EDHF. It was therefore concluded that NO was the major EDRF released in response to ACh in this experimental model under physiological conditions, and as such, perfusion myography using rat mesenteric arteries was suitable for the examination of NO-mediated vascular function.

9.3 UA AND ENDOTHELIAL DYSFUNCTION

Chapter 4 examined the direct interaction of UA with vascular and endothelial function. It has previously been suggested that elevated UA concentrations may be directly injurious to the endothelial lining of the vascular system, thus precipitating a causal role in vascular and cardiovascular disease. However, luminal perfusion with UA at physiological, elevated physiological and supraphysiological concentrations did not affect vasoconstriction to PE, endothelium-dependent vasodilation to ACh, or endothelium-independent vasodilatation to SNP. These results demonstrated that acute endothelial exposure to UA is not cytotoxic or injurious to the EC in this model and does not induce severe functional impairment. This also mitigates a direct causal role for UA in the development of cardiovascular disease via the development of endothelial

dysfunction. Therefore, it is suggested that the correlation between elevated UA and the pathogenesis of cardiovascular disease may be due to either the indirect association of UA with vascular disease or its risk factors, or a compensatory response to protect against the development of this disease. For example, UA is a major hydrophilic antioxidant and serum concentrations could be elevated to combat the increased $O_2^{\cdot -}$ and oxidative stress that occurs in various conditions that predispose to the development to cardiovascular disease.

9.4 THE INTERACTION OF UA AND LDL ON ENDOTHELIAL FUNCTION

The role of UA in cardiovascular disease may be further determined by examining the interaction of UA with established cardiovascular risk factors. Elevated UA concentrations are commonly associated with hypercholesterolaemia (Wannamethee *et al,* 1997), a recognised risk factor for the development of cardiovascular disease (Goldstein & Brown, 1977; Steinberg, 1983; Martin *et al,* 1986). However, before the interaction of UA and LDL could be examined, the effects of LDL upon vascular function had to be clarified.

Chapter 5 focused on the inhibitory action of LDL on vascular function following perfusion into the arterial lumen, and demonstrated that NO-mediated ACh-induced vasodilatation was reduced in rat mesenteric arteries exposed to increasing concentrations of human LDL. This finding is in agreement with previous studies that have shown that both ox- and LDL can impair endothelium-dependent responses in isolated vessel preparations *in vitro* (Andrews *et al*, 1987; Jacobs *et al*, 1990; Plane *et al*, 1993; Fontana *et al*, 1999). This deleterious action is believed to occur via LDL-induced generation of $O_2^{\cdot -}$ by eNOS (Ohara *et al,* 1993; Keaney *et al,* 1995; Vergnani *et* al, 2000). The increased oxidative stress and generation of $O_2^{\cdot -}$ by eNOS could be caused by an impairment of l-arginine metabolism in the EC, as the uncoupling of l-

arginine oxidation and O_2 reduction by the oxygenase and reductase domains of eNOS respectively allows eNOS to be a source of $O_2^{\cdot-}$ generation (Klatt *et al*, 1993; Griffith & Steuhr, 1995; Pritchard *et al*, 1995; Shinozaki *et al,* 1999). This proposed mechanism is supported by the restoration of vascular function when the lipoprotein solution was supplemented with 1mM l-arginine in the current study. The central role of $O_2^{\cdot-}$ is supported by the observation that impaired endothelium-dependent vasodilatation in hypercholesterolaemic conditions is improved by the administration of SOD or SOD-mimetics (White *et al,* 1994; Fontana *et al,* 1999). This was not demonstrated in the current study, as supplementation of the LDL solution with SOD did not restore vasodilatation. However, this may be due by the failure of the exogenous SOD to access the EC and compete effectively with the rapid reaction between $O_2^{\cdot-}$ and NO.

Chapter 6 examined the effect of UA upon the deleterious action of LDL by supplementing the lipoprotein solution with various concentrations of UA prior to luminal perfusion. Vasodilatation to ACh was significantly restored when UA was added to the perfusing LDL solution at concentrations of 200, 400 or 600μM (62%, 70%, and 74% respectively), but was abolished by the addition of 100μM L-NAME. These results demonstrated that UA was able to restore NO-mediated vascular function in a concentration-dependent manner. There was no significant oxidation of luminal LDL during these experiments or the experiments with LDL only. This does not rule out mild degrees of oxidation but argues against the effect of UA being due to the antioxidant ability of UA causing significant retardation of the oxidative modification of LDL. It is more probable that UA improves NO-mediated vascular function by protecting NO from the inhibitory action of LDL or restoring NO availability following inactivation within the vascular wall. UA is a potent antioxidant that may be capable of reacting with and inactivating $O_2^{\cdot-}$ (Ames *et al*, 1981; Santus *et al* 2001), although this has been debated (Becker *et al,* 1989; Becker *et al,* 1991; Becker *et al,* 1993). It is tempting to speculate that the beneficial action of UA may be mediated via this simple antioxidant mechanism. However, this seems unlikely as $O_2^{\cdot-}$ reacts more rapidly with NO (Huie & Padmaja, 1993). Instead, it seems more likely that the benefits of UA on

vasodilatation are related to the established ability of UA to scavenge $ONOO^-$ (Skinner *et al,* 1998; Squadrito *et al,* 2000). Therefore, the protection of NO-mediated vasodilatation by UA seems more likely to occur due to an action that restores NO-availability following inactivation by $O_2^{\cdot-}$, rather than via a protective mechanism that prevents the inactivation of NO by LDL.

To determine if this protective effect was unique for UA, supplementation with two other major hydrophilic antioxidants was examined. UA was compared to AA and GSH at physiological concentrations (40 and 400μM), chosen as they approximate the concentrations of UA, AA and thiols in human plasma. Only 400μM UA was able to improve LDL induced vascular dysfunction. The results demonstrated that UA conferred greater protection on vascular function in the presence of LDL when compared to AA and GSH. These results also support the hypothesis that the action of UA was distinct from its antioxidant radical-scavenging ability, as both AA and GSH are potent free radical scavengers. The apparent superiority of UA over these antioxidants could occur because only UA is able to gain access to the intracellular environment in sufficient concentrations to restore NO-mediated vasodilatation (Becker, 1989; Becker, 1993). However, none of the antioxidants studied could be expected to compete effectively with the rapid reaction between $O_2^{\cdot-}$ and NO, to prevent the formation of $ONOO^-$. However, UA is an efficient scavenger of $ONOO^-$ in biological systems (Skinner *et al,* 1998; Squadrito *et al,* 2000). The restoration of vascular function by UA could be independent from its antioxidant potential, and due to the unique ability to scavenge $ONOO^-$ and form a UA derivative with the potential to induce vasodilatation (Skinner *et al,* 1998).

9.5 THE MECHANISM BY WHICH UA RESTORES NO-MEDIATED VASCULAR FUNCTION FOLLOWING IMPAIRMENT BY LDL

Chapter 8 investigated the mechanism by which UA could restore the impairment of NO-mediated vascular function by LDL. The chapter focused on the ability of UA to form an NO-donor. This was examined by transferring the superfusate from organ bath chambers containing endothelium-intact vessels to endothelium-denuded vessels, following the stimulation of intact vessels with ACh in the presence of LDL and UA. This experiment was performed using wire myography and thoracic aorta ring segments from the same rat species as used in the previous studies. The experimental model was changed to facilitate the ease of transfer. The transfer of physiological, UA or LDL solutions containing ACh did not cause vasodilatation of the denuded ring segments. However, exposure of stimulated intact aortic ring segments in the presence of both LDL and UA led to the formation of an endothelium-independent vasodilator in solution, with a half-life of approximately 29-30 minutes. The vasodilatation produced by this species was UA concentration-dependent. The formation of this species also required ACh-mediated stimulation of the intact vessels to be formed. The molecular nature of this UA-derived vasodilator was not characterised, but is believed to be a nitration product of the reaction between UA and $ONOO^-$. The role of $ONOO^-$ in the formation of this species is supported by the requirement for LDL, which increases the local oxidative stress and subsequently generates $ONOO^-$. The decay time of the vasodilator product is comparable to that of the UA-derived vasodilating species formed in the studies by Skinner *et al* (Skinner *et al*, 1998). The ox-Hb-sensitive nature of the vasodilatation suggests that the UA-derived vasodilator is a NO-donor which produces vasodilatation via the spontaneous release of NO in the extracellular environment. This is likely to induce vasodilatation via a cGMP-dependent mechanism but requires further investigation.

Nevertheless, these studies have demonstrated that UA can restore endothelial function in the presence of LDL most likely by forming a UA-derived vasoactive species that acts as an NO-donor to restore vascular function. This restoration is unique when compared to other major hydrophilic antioxidants. The NO-donor is produced as a result of the ability of UA to scavenge $ONOO^-$, generated following EC exposure to LDL and the resultant increase in $O_2^{\cdot-}$ generation. It is believed that this study provides the first evidence that a UA-based vasodilator can be formed in an intact biological system.

9.6 PHYSIOLOGICAL RELEVANCE

It is intriguing to speculate that the interaction between UA and LDL may be relevant to the function of human blood vessels. In human physiology, arteries are exposed to higher lipoprotein concentrations than most other species and this has the potential to have adverse effects on arterial structure and function. The mechanism demonstrated in this study suggests that UA could mitigate some of these effects, specifically by improving the availability of NO in the vascular wall. The high UA concentrations found in human plasma may be a factor that enables the vascular system to remain healthy and responsive when persistently exposed to LDL. The elevated UA concentrations present could occur due to LDL increasing oxidative stress in the vascular wall, which in turn activates XO (Sobey *et al,* 1992; Tesfamariam e*t al,* 1992; Sobey *et al,* 1993; Castelli *et al,* 1995), resulting in the enhanced generation and release of UA by the EC (Parks & Granger, 1986). This may also explain the correlation between elevated UA and other conditions associated with increased oxidative stress.

UA is the most abundant radical-scavenging antioxidant in human extracellular fluid (Stocker & Frei, 1991; Maxwell *et al,* 1997), and all aqueous tissue compartments contain UA as a replenishable antioxidant. Intravenous UA supplementation significantly increases plasma antioxidant activity in healthy individuals (Waring *et al,*

2001). The high concentrations of UA in human physiology, the evolutionary loss of urate oxidase and the manner in which the body re-absorbs and conserves UA suggest it may confer some physiological advantage in humans and could be associated with to the prolonged life-span when compared to other species (Ames *et al,* 1981). The epidemiological evidence that associates UA with cardiovascular disease does not necessarily contradict the present study. This may be explained by the close association of hyperuricaemia with established risk factors and the release of UA to combat increased oxidative stress. From the results of the current study, it may be hypothesised that the elevated UA concentrations associated with conditions such as hypercholesterolaemia occur as a compensatory mechanism to protect against the detrimental effects of LDL and prevent the development of cardiovascular disease. However, relating the findings of the current study to the *in vivo* situation may not be so simple.

Under physiological conditions, the generation of UA from xanthine is catalysed by XDH. This reaction is not accompanied by free radical production and the antioxidant ability of UA is prominent. However, under pathophysiological conditions, such as oxidative stress and hypercholesterolaemia, XO activity is increased and catalyses the generation of UA (Ohara *et al,* 1993). This could occur as a compensatory mechanism to generate greater concentrations of UA to combat the increased oxidative stress and reduced NO-availability. However, the stoichiometry of the XO system results in the excess generation of $O_2^{\cdot -}$ over UA (Becker *et al,* 1991; Ohara *et al,* 1993). The excess generation of $O_2^{\cdot -}$ could potentially negate the protection conferred by increased UA production *in vivo*. This must be taken into consideration when interpreting the results produced. Increased UA concentrations were achieved in the current studies by supplementation with exogenous UA. Therefore, the experimental conditions used were not complicated by the generation of $O_2^{\cdot -}$ that accompanies the increased UA production associated with hypercholesterolaemia. Consequently, the current study did not examine the potential effect of increased UA *generation*, and may only be used to

interpret the direct action of elevated UA *following* the generation and release from the EC to plasma.

The protection conferred by UA upon NO-mediated vascular function in the current studies has been attributed to the formation of a UA-derivative that spontaneously releases NO. The *in vivo* formation of this vasoactive species remains to be established, however, the generation of a UA-based vasodilator or NO-donor is biologically plausible. The formation of such a compound has previously been reported in human plasma *in vitro* (Skinner *et al,* 1998). The current study has provided supportive evidence for the formation of a UA-derived NO-donor in an intact biological system. This could have wide reaching implications for the association between elevated UA concentrations and pathophysiological conditions predisposing to cardiovascular disease in human physiology. The observation that the formed vasodilator releases NO in a spontaneous and continuous fashion could suggest that the product plays a significant role in the restoration of vascular function. The ability to form this vasoactive species *in vivo* may provide a beneficial reason for the evolutionary elevation in plasma UA concentrations, as this may provide enhanced protection against high levels of circulating LDL. The UA-derived vasodilator could play a vital role in human pathophysiology by releasing NO to maintain vascular function, blood flow and tissue perfusion (Kengatharan *et al,* 1996; Mellander *et al*, 1997). Furthermore, a nitrated UA product could also potentially exhibit the anti-inflammatory properties of NO by preventing platelet aggregation and adherence (Radomski *et al,* 1993; Radomski & Moncada, 1993; Cooke & Tsao, 1994) as well as inhibiting leukocyte adhesion to the endothelial surface (Granger & Kubes, 1994).

The experimental model used may affect the ability to relate the current results to human physiology, as the current studies were performed using rodent tissue. The use of isolated human blood vessels is difficult to plan and very restrictive. Therefore, the isolated vessel studies were performed using animal tissue. However, the lipoprotein samples were extracted from human plasma and the concentration used for the majority

of the studies (250μg/ml) was typical of that found in human and WKY rat plasma (Kitts *et al,* 1998). The impaired vascular function demonstrated in these studies is also in accord with previous human *in vivo* studies that have demonstrated impaired responses under hypercholesterolaemic conditions. Therefore, the inhibition and restoration of vasodilatation demonstrated in the *in vitro* model may be relevant with regards to human physiology.

9.7 EVOLUTIONARY IMPLICATIONS

The potential role of UA as a restorative mediator to protect against the deleterious action of increased LDL levels may have evolutionary implications. UA is present in the human plasma largely as the monoanion urate (Simic & Javanovic, 1989) and the concentration of UA in human blood (200-400μM) is almost saturating. This is 5-10 times greater than the concentrations of AA in plasma (20-60μM) and 100 times greater than GSH (>2μM). A major factor in lengthening human life span during the course of human evolution is the improved antioxidant defences against oxidative damage by free radicals. Several mutational events have resulted in the loss of urate oxidase activity, which oxidises UA to allantoin (Wu *et al,* 1992), and the development of an active kidney re-absorption system for UA. This has resulted in plasma UA concentrations in humans that are 10 times greater than those found in most other mammals (Roch-Rammel & Peters, 1978). Interspecies comparison gives a positive correlation between plasma UA concentrations and species life-span. This positive association may be attributed to the antioxidant ability of UA (Ames *et al,* 1981; Becker 1993). The current study demonstrated that a UA concentration comparable that those found in species expressing urate oxidase did not prevent LDL-induced endothelial dysfunction, however concentrations physiologically relevant to humans did. This supports the evolutionary hypothesis that the loss of urate oxidase expression occurred as elevated serum UA concentrations have an important role in human physiology, and may be an adaptive mechanism to combat the increasing role of lipoproteins in pathophysiology. This

could also explain the established association of hyperuricaemia and disease states such as hypercholesterolaemia (Wannamethee *et al,* 1997),

The evolutionary loss of enzymatic UA oxidation has also been accompanied by a loss of ascorbate synthetase activity during human evolution. This prevents the synthesis of AA, thus requiring *in vivo* concentrations to be replenished by dietary consumption. It has been proposed that the loss of urate oxidase occurred to compensate for the loss of ascorbate synthetase (Ames *et al,* 1981). As a result, humans and higher primates may have higher concentrations of serum UA to offset the decreased antioxidant potential caused by the reduction of circulating AA concentrations. This evolutionary process could have occurred as the ability of AA to act as an antioxidant may be inhibited by its tendency under certain conditions to auto-oxidise, resulting in the generation of oxygen radicals (Sevanian *et al,* 1991) and mutagens (Koh *et al,* 1980). However, the current results demonstrated the superiority of UA in preventing the deleterious action of LDL on vascular function when compared to AA and GSH. Therefore, it is hypothesised that the evolutionary elevation of UA concentrations is an adaptive mechanism to provide greater protection against the continual exposure of the human vascular system to increased concentrations of LDL. It is also hypothesised that this has occurred because UA is the major antioxidant for restoring vascular function following the impairment of NO availability by increased oxidative stress in the vascular wall. This is supported by the current studies, as the other major endogenous hydrophilic antioxidants were ineffective against the direct action of LDL upon vascular function.

9.8 CLINICAL AND THERAPEUTIC POTENTIAL

Although the current studies were performed to investigate the *in vivo* relationship between elevated UA levels and cardiovascular disease, the ability of UA to scavenge $ONOO^-$ and form a potential NO-donating species may be of significant therapeutic interest. Due to the occurrence of endothelial dysfunction in cardiovascular disease, the

delivery of exogenous NO to areas of reduced NO availability is a frequently used therapy and drugs that release NO have been clinically utilised for over a hundred years. NO-donors may induce vasodilatation and prevent oxidative stress-mediated EC injury in cardiovascular disease (Chang *et al,* 1996; Terada *et al,* 1996). They may also be used to treat and prevent tissue damage associated with other conditions such pulmonary hypertension, acute respiratory distress and various other disease states.

Organic nitrates, such as GTN, are the most widely used NO-donor drugs and rapidly dilate veins, coronary arteries and collateral vessels (Parker, 1987; Bauer *et al,* 1995). However, tolerance, defined as the loss of therapeutic effect with continued administration, is a major limitation of these compounds (Megson & Webb, 2002), and may involve the induction of haemodynamic compensatory mechanisms (Dupuis *et al,* 1990; Parker *et al,* 1991; Munzel *et al,* 1995) and oxidative stress (Munzel *et al,* 1995; Munzel *et al,* 1999). Increased oxidative stress may induce tolerance (Munzel *et al,* 1999), and studies have shown that tolerance to organic nitrates may be partially reversed by several antioxidants, including AA (Bassenge *et al*, 1998; Watanabe *et al,* 1998). It is possible that the use of a UA-derived NO donor could prevent the induction of tolerance by increased oxidative stress, as the UA-derived species could potentially exert a radical scavenging ability following the release of NO, to prevent any inactivation by ROS. Tolerance may also develop due to depletion of tissue thiols, previously thought to be essential to the metabolic process involved in the release of NO from organic nitrates (Needleman *et al,* 1973; Ignarro & Gruetter, 1980; Munzel *et al,* 1995). Thiols may be depleted under conditions of oxidative stress, such as hypercholesterolaemia, thus diminishing the therapeutic effect of these NO-donors (Hanspal *et al,* 2001). The development of new drugs that negate these limitations and have a wider range of applications is of great interest and potential benefit. The release of NO from the UA-derived compound formed is spontaneous and is not believed to be thiol-dependent. This is in agreement with the findings of Skinner *et al* that demonstrated the release of NO was not accelerated by the addition of thiols (Skinner *et al,* 1998). Therefore, the use of the nitrated UA-derivative as an NO-donor would not

be hampered by the depletion of intracellular thiols. This is a potential therapeutic advantage of the mechanism of action of the UA–derived NO donor formed in the present study.

Furthermore, while this species could act as a NO-donor, it could also exert other physiological properties. The NO-mediated dilating species formed in this study is thought to be a nitrosated UA-adduct (Skinner *et al,* 1998). Nitrosated-adducts may act as hybrid NO-donors; these are NO-donating species formed from compounds that are structurally modified to incorporate NO-groups. This class of NO-donors can release NO whilst retaining the pharmacological activity of the original compound. The development of a combined NO-donor/antioxidant compound would be of great therapeutic interest, as this may release NO to restore vascular function and also enhance the antioxidant potential. The compound formed in the present study could fulfill such criteria, given the established antioxidant and $ONOO^-$ scavenging potential of UA and the ability of the formed product to induce NO-mediated vasodilatation. It is interesting to speculate that such a species could be used to treat clinical conditions in which increased oxidative stress and diminished antioxidant potential contribute to the development of vascular dysfunction. The combined vasoactive and antioxidant properties of such a UA-derived NO-donor may also make it ideally suited for the treatment and prevention of vascular dysfunction and cardiovascular disease. This product could also be used to treat other clinical conditions associated with oxidative tissue damage, such as organ transplantation and multiple organ dysfunction. The release of NO from the UA-derived product could also minimise tissue damage following conditions such as ischaemia or reperfusion, which may be caused by increased oxidative stress.

However, before this may be regarded as a realistic therapeutic option several limitations must be considered. Therapeutic treatment using an UA-derived NO-donor may result in the accumulation of excessive UA concentrations in the plasma. UA is sparingly soluble in aqueous media and reaches saturation at approximately 415μM.

Persistent exposure to excessive serum concentrations predisposes to crystal depositions within soft tissues and the development of conditions such as gout (Emerson, 1996). Furthermore, UA may also stimulate the adherence of granulocytes and macrophages to the endothelium, which may have detrimental effects upon the vascular system (Duff *et al,* 1983; Hutton *et al*, 1985; Leyva *et al,* 1998; Mazzali *et al,* 2002; Kanellis *et al,* 2003). These could not be examined in the current study, as no blood cells were present within the organ bath, but must be investigated before the UA-derived vasodilator can be considered as a realistic therapeutic alternative.

9.9 FURTHER STUDY

The present studies demonstrated that the association of elevated UA and hypercholesterolaemia could occur due to a unique mechanism by which UA restores impaired NO-mediated vasodilatation. However, further investigation is required to fully clarify the mechanism by which this occurs and gain a better understanding of relationship between UA and the development of cardiovascular disease

The priority of any future study must be to identify the formed vasoactive species. It has previously been suggested by Skinner *et al.* that the scavenging of $ONOO^-$ by UA *in vitro* results in the formation of 2-nitrito-4-amino-5-hydroxyimidazoline, a nitrated UA-derivative that produces NO-mediated vasodilatation of rat aortic ring segments (Skinner *et al,* 1998). The findings of Skinner *et al* are widely cited with regards to the role of UA in human physiology and pathophysiology. However, to our knowledge this has never been expanded upon and the formation a UA-derived NO-donor in an intact biological system has never been demonstrated. Therefore, this study is believed to be the first to report the formation of such a compound in an intact biological system. Consequently, it is of the utmost importance that the vasodilatory species formed in the current experimental model is identified. This could be identified by repeating the experiments, collecting the intraluminal LDL solution and performing electron spin

resonance, HPLC and electrospray mass spectroscopic analysis to study the reaction of UA with $ONOO^-$ and identify the vasodilator produced. This could be supported by experiments examining the formation of a UA-derived compound in EC cultures incubated with various concentrations of LDL and UA. The solution present could then be added to isolated vessel preparations and vascular function examined. This solution would also be collected and analysed to identify any vasodilator formed.

The previous chapter demonstrated that UA did not cause vasodilatation in denuded ring segments bathed in LDL. These experiments should be repeated to examine the effects of solution transfer from denuded aortic rings to other denuded rings, as this was not performed in the current studies. This should demonstrate that the proposed formation of a UA-derived vasodilator has an absolute requirement on the presence of an intact endothelium as a source of NO. Further study is also required to determine the potential site of action of UA, prior to the formation of the UA-derived vasodilator. This could be achieved by examining the ability of UA to generate a vasodilatory species in models of intra- and extracellular oxidative stress. The experiments performed in Chapter 8 could be repeated and LDL replaced with pyrogallol, as a source of extracellular $O_2^{\cdot-}$, or DECTA to inhibit endogenous Cu-Zn SOD and increase intracellular $O_2^{\cdot-}$.

Investigation is also required to fully understand the mechanism of NO-dependent vasodilatation. The dilatation produced is likely to occur via the direct activation of VSMC cGMP by NO (Ignarro & Gruetter, 1980; Ignarro *et al,* 1986; Waldman & Murad, 1987). However, vasodilatation to NO is not exclusively mediated by cGMP (Trottier *et al,* 1998; Weisbrod *et al,* 1998) and several NO-donating species may act via cGMP-independent mechanism (Brunner *et al,* 1996; Plane *et al,* 1998; Homer & Wanstall, 2000). This may be influenced by the site of NO release. The UA-derived vasodilator produces ox-Hb-sensitive vasodilatation, implying that NO is released in the extracellular environment, and could produce dilatation by cGMP-independent mechanism. Therefore, the vessel studies should be repeated in the presence of 1H-[1,2,3]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), a selective inhibitor of sGC,

(Garthwaite *et al*, 1995; Hobbs *et al,* 1997), to examine if the vasodilatation produced is mediated through the NO/cGMP pathway. It would also be of significant interest to measure the concentration of NO released from this dilator species and compare to the physiological release of NO from EC in this experimental model. The role of NO was demonstrated in the present studies by assessing changes in vascular function. However, the direct generation of NO from the UA-derived vasodilator may be examined using an NO-electrode. The quantification of NO could be achieved by collecting organ bath samples, following endothelial activation, and measuring the NO released from the UA-derived vasodilator using an isolated NO-electrode in the presence and absence of vascular tissue, with and without endothelium. The NO release would be compared to calibration curves produced by the generation of known NO concentrations using authentic NO solution, formed in the laboratory. Such experiments would clarify the mechanism of action of the UA-derived vasodilator and may help to further elucidate the role of elevated UA concentrations in human physiology.

Following identification of the UA-derived vasodilator and the mechanism of action, future studies should examine the specificity of the restorative action, to ascertain if the restoration of vascular function also occurs in other cardiovascular risk factors with which increased UA is associated. Elevated UA concentrations are frequently associated with other pathophysiological conditions such as hypertension and type II diabetes (Lehto *et al,* 1998; Ward, 1998; Fang & Alderman, 2000; Alderman, 2002). Endothelial dysfunction is prevalent in these states and, as with hypercholesterolaemia, may be caused by increased oxidative stress and the inactivation of NO by ROS within the vascular wall. Consequently, UA could be expected to exert a similar restorative action. The demonstration of this would give further support to the potentially beneficial role of UA in human physiology and pathophysiology, and should be examined to further determine the relationship between, UA, cardiovascular disease and the associated risk factors.

9.10 CONCLUSION

The results of these studies mitigate any direct causal role for UA in endothelial dysfunction and cardiovascular disease, as it is demonstrated that UA is not directly injurious to the endothelium. On the contrary, the results highlight the importance of UA in protecting vascular function from the detrimental action of LDL. It is shown that LDL may have a prominent role in cardiovascular disease by increasing oxidative stress within the vascular wall, which in turn impairs NO-mediated endothelium-dependent vasodilatation. The present studies have demonstrated that UA is able to restore NO-mediated vascular function following endothelial exposure to LDL, and does so more effectively than other hydrophilic antioxidants. The ability of UA to prevent the inhibitory effect of LDL is thought to be due to a restorative action that occurs via the unique ability of UA to scavenge $ONOO^-$ and form UA-derived NO-dependent vasoactive species. It is believed that the current study provides the first evidence for the formation of such a UA-derived vasodilator in an intact biological system. These results have demonstrated a distinct physiological role for UA in the prevention of LDL-induced vascular dysfunction, and suggest a means by which the higher concentrations of UA in human plasma may confer an important biological advantage in human physiology. This could provide a beneficial reason for the loss of urate oxidase activity and the subsequent increases in UA concentrations that have occurred during the course of human evolution.

APPENDIX I

PREPARATION OF SOLUTIONS

10. Appendix I – Preparation of Solutions

Krebs-Ringer Buffer

Amounts stated are to make 1L Krebs solution (final concentration in mM).

	Stock	Normal Krebs		High K^+ Krebs	
NaCl	g	6.9g	(118)	0.28g	(4.7)
KCl	g	0.35g	(4.7)	8.8g	(118)
$MgSO_4$	g	1.45g	(1.2)	1.45g	(1.2)
$NaHCO_3$	g	2.1g	(25)	2.1g	(25)
KH_2PO_4	g	0.16g	(1.2)	0.16g	(1.2)
Glucose	g	2.0g	(11.1)	2.0g	(11.1)

Make up to 950ml with de-ionised water and bubble with 95% O_2/5% CO_2 for 10 minutes.

$CaCl_2$	g	2.8g	(2.5)	2.8g	(2.5)

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