Differential actions of the methylarginines in the rat aorta

A thesis submitted for the degree of Doctor of Philosophy in the College of Medical, Veterinary and Life Sciences at the University of Glasgow

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Summary

1. The main aim of this study was to determine whether $N^{G}N^{G}$- asymmetric dimethylarginine (ADMA), like N^{G}-monomethyl-L-arginine (L-NMMA), blocks basal but not acetylcholine-induced nitric oxide activity in female rat aorta, and if so, to investigate the possible mechanism(s) behind these seemingly differential actions.

2. In the rat aorta, ADMA, like L-NMMA, blocked basal nitric oxide activity, as assessed by its ability to enhance sub-maximal phenylephrine-induced tone, and to inhibit the endothelium-dependent, nitric oxide-mediated relaxation induced by superoxide dismutase or the PDE5 inhibitor, T0156.

3. In the rat aorta, ADMA had little effect on endothelium-dependent, nitric oxide-mediated relaxation induced by acetylcholine or the calcium ionophore, A23187, when studied at the same level of tone used in control tissues. It did, however, significantly block relaxation induced by calcitonin gene-related peptide-1, an agonist that had a significantly lower maximum response than acetylcholine or A23187.

4. ADMA did, however, produce potent, apparent blockade of acetylcholine-induced relaxation in tissues where the level of tone, due to blockade of basal nitric oxide activity, was permitted to rise above that of control tissues. This apparent blockade by ADMA was due to physiological antagonism rather than blockade of nitric oxide synthase, because control tissues taken to equivalent levels of tone were similarly inhibited.

5. $N^{G}N'^{G}$-symmetric dimethylarginine (SDMA), the inert analogue of ADMA, had no effect on basal or acetylcholine-induced activity of nitric oxide in rat aorta.

6. ADMA and L-NMMA behaved similarly to the endogenous substrate, L-arginine, in protecting acetylcholine-induced relaxation against blockade by L-NAME.

7. With the use of calmidazolium and wortmannin, respectively, the calcium-calmodulin and the phosphatidylinositol 3-kinase transduction mechanisms were found to play only a minor role in the stimulation of basal nitric oxide production, as assessed by the enhancement of phenylephrine tone or by blocking relaxation to superoxide dismutase or T0156 in rat aorta. In contrast, nitric oxide activity induced by acetylcholine, A23187 or calcitonin gene-related peptide-1 seems to be mediated mainly by the calcium-calmodulin transduction mechanism.

8. The M_3 partial agonist, butyrylcholine, elicited concentration-dependent relaxation in rat endothelium-containing aortic rings with a maximal relaxant response lower than that elicited by the full agonist, acetylcholine. Moreover, unlike with acetylcholine, this relaxation was significantly blocked by ADMA.

9. When rat endothelium-containing aortic rings were treated for 30 minutes with the irreversible receptor alkylating agent, phenoxybenzamine (3 μM), followed by washout and then submaximally contracted with endothelin-1, maximal relaxation to acetylcholine was blocked by ~50%, while that to butyrylcholine was almost abolished.

10. Under conditions where the apparent efficacy of acetylcholine was reduced to that of a partial agonist using phenoxybenzamine, ADMA produced powerful concentration-dependent blockade of the relaxation.

11. The results of this study strongly suggest that the seemingly differential abilities of L-NMMA and ADMA to block basal, but not acetylcholine-induced relaxation in rat aorta may be explained by the blocking action being critically determined by the efficacy of the relaxant stimulus i.e. low efficacy basal activity in comparison to the high efficacy acetylcholine-stimulated activity.

Contents

List of Figures

Chapter One

Chapter Two

Chapter Three

Chapter Four

Chapter Five

List of Tables

Chapter Two

Chapter Four

Abbreviations

5-HT	5-hydroxytryptamine
ACE	angiotensin-converting enzyme
ACh	acetylcholine
AD	adenylate cyclase
ADMA	N^GN^G- asymmetric dimethylarginine
ADP	adenosine diphosphate
ARDS	adult respiratory distress syndrome
AT_1	angiotensin receptor type 1
ATP	adenosine triphosphate
BCh	butyrylcholine
BH4	tetrahydrobiopterin
BK	bradykinin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CAT	catalase
CMZ	calmidazolium
COX1	cycloxygenase-1
DPI	diphenylene iodonium
ECE	endothelin-converting enzyme
EDCF	endothelium-derived constricting factor
EDHF	endothelium-derived hyperpolarising factor
EDRF	endothelium-derived relaxing factor
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
H_2O_2	hydrogen peroxide

HMGCoA	3-hydroxy-3-methylglutaryl coenzyme A
iNOS	inducible nitric oxide synthase
IP3	inositol trisphosphate
K_{ATP}	ATP-dependent potassium channels
L-NAME	N^G –nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
L-NOARG	N^G-nitro-L-arginine
NF-κB	nuclear factor- κB
NO	nitric oxide
NOHLA	N-hydroxy-L-arginine
NOS	nitric oxide synthase
O_2	molecular oxygen
O^-_2	superoxide anion
ODQ	1H- [1, 2, 4] oxadiazolo [4, 3,-a] quinoxalin-1-one
PAH	pulmonary arterial hypertension
PBZ	phenoxybenzamine
PDE5	phosphodiesterase type 5
PE	phenylephrine
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostaglandin I_2
PI 3-K	phosphatidylinositol 3-kinase
PPAR γ	peroxisome proliferator-activated receptor γ
PRMT	protein arginine methyltransferase
ROS	reactive oxygen species
SDMA	$N^GN'^G$-symmetric dimethylarginine

SP	substance P
sGC	soluble guanylate cyclase
SOD	superoxide dismutase
T0157	2-(2-methylpyridin-4-yl) methyl-4-(3,4,5-trimethoxyphenyl)-8-(pyrimidin-2-yl)methoxy-1,2-dihydro-1-oxo-2,7-naphthyridine-3-carboxylic acid methyl ester hydrochloride
TXA2	thromboxane A2
VEGF	vascular endothelium growth factor
Wtm	wortmannin

List of Publications

1. AL-Zobaidy MJ, Craig J, Brown K, Pettifor G, Martin W (2011). Stimulus-specific blockade of nitric oxide-mediated dilatation by asymmetric dimethylarginine (ADMA) and monomethyl-arginine (L-NMMA) in rat aorta and carotid artery. *Eur J Pharmacol* **673:** 78–84.

2. AL-Zobaidy MJ, Craig J, Martin W (2010). Differential sensitivity of basal and acetylcholine-induced activity of nitric oxide to blockade by asymmetric dimethylarginine (ADMA) in the rat aorta. *Br J Pharmacol* **160:** 1476–1483.

3. AL- Zobaidy M, Martin W (2011). The ability of asymmetric dimethylarginine (ADMA) to block endothelium-dependent, nitric oxide-mediated relaxation in rat aorta is determined by the efficacy of the relaxant agonist. Presented at the winter meeting of the British Pharmacological Society in London.

4. AL- Zobaidy M, Martin W (2010). Asymmetric dimethylarginine (ADMA) inhibits endothelium-dependent relaxation induced by superoxide dismutase or the PDE5 inhibitor, T0156, but not that induced by calcium ionophore A23187 in rat aorta. Presented at the winter meeting of the British Pharmacological Society in London.

5. AL- Zobaidy Mohammed, Craig John, Martin William (2009). Asymmetric dimethylarginine (ADMA) inhibits basal but not acetylcholine-induced activity of nitric oxide in rat aorta. Presented at the winter meeting of the British Pharmacological Society in London.

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Declaration

I declare that this thesis has been composed by myself and is a record of my work performed by myself. It has not been previously submitted for a higher degree.

The research was carried out in the College of Medical, Veterinary and Life Sciences, the School of Life Sciences, University of Glasgow, under the supervision of Professor William Martin.

Dr Mohammed Al-Zobaidy

2012

Chapter One

Introduction

Physiology of the vascular endothelium

General functions of the vascular endothelium

The lining of the blood vessels and chambers of the heart consists of a monolayer of cells termed the endothelium (Celemajer, 1997; Sader and Celemajer, 2002; Galley and Webster, 2004; Flammer and Lüscher, 2010). The vascular endothelium was classically regarded as a simple, passive barrier that separates the vascular wall from the blood. We now know, however, that the endothelium is a highly dynamic structure whose functional integrity is vital for many important physiological processes (Vanhoutte, 1989; Celemajer, 1997; Sader and Celemajer, 2002; Galley and Webster, 2004; Flammer and Lüscher, 2010).

Figure 1.1, adopted from Galley and Webster (2004) illustrates many of these diverse functions. For example, although the endothelium is a barrier to the diffusion of large molecules, it is under dynamic regulation in immune and inflammatory processes. Specifically, immune mediators (such as histamine, leukotrienes C_4 and D_4) induce endothelial contraction at the post-capillary venules, leading to the vascular leakage (local oedema) that characterises the inflammatory response. Moreover, endothelial damage leading to barrier dysfunction permits entry into the vascular wall of large, normally restricted molecules, such as cholesterol-rich low density lipoproteins, thus contributing to atherosclerosis. The endothelium also regulates haemostasis vs thrombosis through the balance between the release of a variety of procoagulant (such as von Willebrand factor, thromboxane A_2 and plasminogen activator inhibitor) and antithrombotic factors (such as prostacyclin, plasminogen activator inhibitor and antithrombin). Moreover, platelets adhesion and subsequent aggregation and activation is prevented by the synergistic actions of nitric oxide and the arachidonic acid derivative, prostacyclin. In addition, the

endothelium is an important site for the enzymatic activation of some mediators (e.g. angiotensin II) and the destruction of others (e.g. bradykinin).

Also, vascular endothelial cells express receptors for some endogenous hormones, such as oestrogen, progesterone, androgens and insulin, which may suggest a role for these receptors in regulating endothelial dysfunction (Vanhoutte, 1989; Sader and Celemajer, 2002, Flammer and Lüscher, 2010).

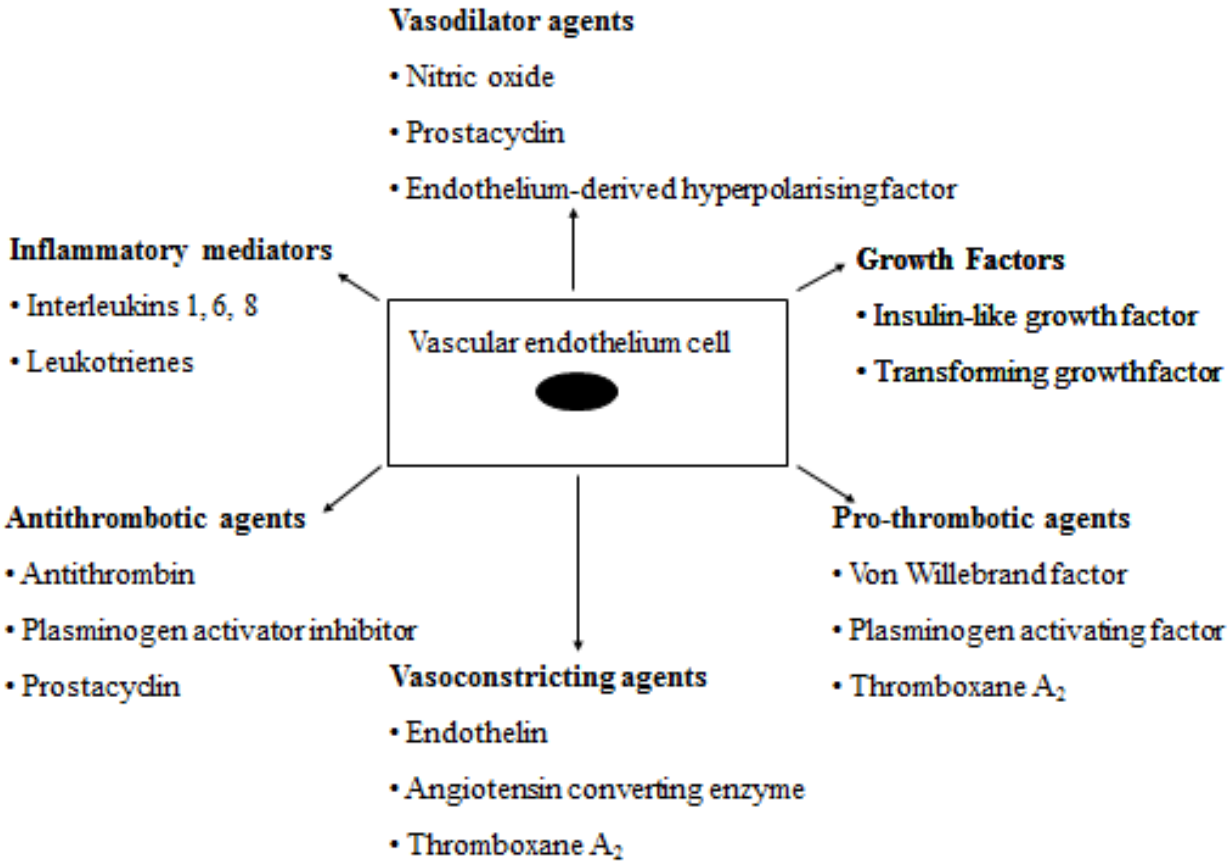

Figure 1.1. Functions of the vascular endothelium (Modified from Galley and Webster, 2004).

Regulation of vascular tone

The main focus of this study is to investigate the role the vascular endothelium plays in the regulation of blood vessel tone.

The vascular endothelium has an important function in the control of blood vessel contractility by the manufacture and secretion of a variety of endothelium-derived relaxing factors (EDRF) and endothelium-derived constricting factors (EDCF) that can alter the tone of the vascular wall (Furchgott and Zawadzki, 1980; Vanhoutte, 1989; Rubanyi, 1991; Celemajer, 1997; Féléton and Vanhoutte, 2000; Widlansky *et al*., 2003; Galley and Webster, 2004; Kozłowska *et al*., 2007; Flammer and Lüscher, 2010; Thorin and Clozel, 2010). The most important endothelium-derived relaxing factors are probably nitric oxide, which induces vasorelaxation via activation of the enzyme, soluble guanylate cyclase (sGC), in the vascular smooth muscle cells, and the endothelial-derived hyperpolarizing factor (EDHF) whose identity is still a matter of debate. Other important relaxing substances produced by the vascular endothelium include prostacyclin (Vane, 1971). Among the vasoconstricting substances released by the vascular endothelium is the peptide endothelin (ET) (Yanagisawa *et al*., 1988).

Nitric oxide

The vascular endothelial cells manufacture nitric oxide from the amino acid, L-arginine, through the chemical reaction that is catalysed by the enzyme nitric oxide synthase (Palmer *et al*., 1988). While the production of nitric oxide is inhibited by L-arginine analogues, such as N^G-monomethyl-L-arginine (L-NMMA), its formation is stimulated by the shearing force produced by flowing blood or by agonists such as acetylcholine, bradykinin or thrombin, that act on their own receptors expressed by the vascular endothelial cells (Pohl *et al*., 1986; Flammer and Lüscher, 2010). Nitric oxide induces vasorelaxation by

inducing the formation of its second messenger cyclic guanosine monophosphate (cGMP) via activation of the enzyme, soluble guanylate cyclase (sGC), in the vascular smooth muscle cells (Rapoport and Murad, 1983).

Endothelium-derived hyperpolarising factor (EDHF)

Stimulation of the vascular endothelium with shear stress or receptor-dependent agonists such as acetylcholine, bradykinin and substance P can induce vasorelaxation by mechanisms other than the release of NO such as hyperpolarisation of the underlying vascular smooth muscle cells. This vasorelaxation was found to be resistant to inhibitors of cycloxygenase, such indomethacin, or to agents that are known to block the synthesis and/or the actions of NO, such as L-NMMA, methylene blue and haemoglobin (Chen *et al*., 1988; Féléton and Vanhoutte 1988; Plane *et al*., 1995). Moreover, the resultant vasorelaxation does not correlate with increases in intracellular concentrations of second messengers, cGMP and cAMP, in the vascular smooth muscle cells. This hyperpolarisation was thought to be mediated by endothelium-derived hyperpolarisation factor(s) (EDHF) whose exact character is still under investigation. The vasorelaxation due to endothelium-dependent hyperpolarisation has been reported in the human coronary circulation as well as in animal vascular beds such as rat arteries, rabbit saphenous and ear arteries, and the canine coronary arteries (Komori and Suzuki, 1987a; Suzuki, 1988; Chen *et al*., 1988; Feletou and Vanhoutte, 1988; Chen and Suzuki, 1989; Celemajer, 1997; Kozłowska *et al*., 2007).

The exact character of the EDHF is still uncertain and there is a spectrum of molecules that are proposed to function as endothelium-derived hyperpolarisation factors depending on animal species and the vascular bed used (Matoba *et al*., 2000; Kozłowska *et al*., 2007; Griffith, 2004; Flammer and Lüscher, 2010). These include potassium ions (K^+) (Edwards

et al., 1998; Féléton and Vanhoutte, 2000; Griffith, 2004; Kozłowska *et al*., 2007), metabolic products of cytochrome P-450 epoxygenases like epoxyeicosatrienoic acids (Rosolowsky and Campbell, 1993; Fisslthaler *et al*., 1999), hydrogen peroxide (H_2O_2) (Matoba *et al.,* 2000; Féléton and Vanhoutte, 2000; Griffith, 2004; Shimokawa and Matoba, 2004; Kozłowska *et al*., 2007), a cannabinoid (Randall *et al*., 1996), C-type natriuretic peptide (Wei *et al*., 1994; Flammer and Lüscher, 2010). Alternatively, the EDHF process may function via electronic spread of endothelial hyperpolarisation to the underlying vascular smooth muscle cells through myoendothelial gap junctions that resulting in the closure of voltage-operated calcium channels with subsequent reduction in calcium influx leading to smooth muscle relaxation (Griffith, 2004; Griffith *et al*., 2004).

Prostacyclin (Prostaglandin I_2, PGI_2)

This endothelium-derived mediator is synthesised by the vascular endothelial cells from arachidonic acid by the cycloxygenase-1 (COX-1) enzyme, but it has a short half-life (~2-3 minutes) as it is rapidly transformed into its stable endproduct, 6-oxo-$PGF_{1\alpha}$ (Moncada *et al*., 1976; Moncada, 1982; Féléton and Vanhoutte, 2000; Flammer and Lüscher, 2010). The relaxant actions of PGI_2 are thought to be mediated by stimulating adenylate cyclase with the subsequent increase in intracellular concentration of the second messenger, cAMP (Gorman *et al*., 1977; Tateson *et al*., 1977). In some tissues such as the rabbit coronary arteries relaxation is accompanied by hyperpolarisation of the vascular smooth muscle cells as a result of opening of ATP-dependent potassium channels (K_{ATP}), as these effects are blocked by glibenclamide (Jackson *et al*., 1993; Féléton and Vanhoutte, 2000; Flammer and Lüscher, 2010). PGI_2 cooperates with NO in preventing platelet aggregation and subsequent thrombus formation, however, it differs from nitric oxide in that the latter is released continuously from the vascular endothelium while the former is produced transiently and does not therefore play a role in sustaining the resting vascular tone in

major arteries (Moncada, 1982). Furthermore, the release of PGI_2 is provoked by a range of stimuli such as shear stress, bradykinin, angiotensin II, thrombin, calcium ionophore A23187, adenine nucleotides (ADP and ATP) and certain anti-hypertensive drugs such as bendroflumethiazide and nitroglycerine (Moncada, 1982; Jackson *et al*., 1993; Celemajer, 1997; Flammer and Lüscher, 2010).

Endothelin (ET)

Although there are three isoforms of endothelin (ET-1, ET-2 and ET-3), only ET-1 is known to be produced by the vascular endothelium. The biosynthesis of ET-1 consists of two stages; initially the polypeptide proET-1 is sliced by a furin-like endopeptidase to yield another polypeptide molecule, big ET-1 (38 amino acids), then the endothelin-converting enzymes (ECEs) cleave the latter molecule to produce a 21-amino acid peptide, ET-1 (Yanagisawa et al., 1988; Kirkby *et al*., 2008; Barton and Yanagisawa, 2008; Thorin and Clozel, 2010).

ET-1 is the most potent spasmogens recognised so far and its pharmacological effects are proposed to be mediated through direct action on its own receptors, ET_A and ET_B (Masaki, 1989; Boulanger and Lüscher, 1990; Lüscher, 1990; Celemajer, 1997; Flammer and Lüscher, 2010).

ET_A receptors expressed by vascular smooth muscle cells and cardiomyocytes and mediate ET-1-induced contraction, and ET_B receptors expressed by the vascular endothelial cells and facilitate vasodilatation, ET-1 uptake and regulation of ET-1 release (Kirkby *et al*., 2008; Barton and Yanagisawa, 2008; Thorin and Clozel, 2010). However, an additional minor component of ET-1-induced vasoconstriction is also thought to be mediated by stimulating the release of renin, secondary to renal vasoconstriction, and/or

enhancing sympathetic output as a result of its effects on the central nervous system (Masaki, 1989; Rubanyi, 1991).

The release of ET-1 can be stimulated by a range of stimuli such as adrenaline, shear stress, thrombin, angiotensin II, calcium ionophore A23187, hypoxia and oxidised low density lipoproteins (Yoshizumi *et al*., 1989; Kohno *et al*., 1989; Kourembanas *et al*., 1991; Lüscher, 1990; Boulanger and Lüscher, 1990; Boulanger *et al*., 1992; Celemajer, 1997; Flammer and Lüscher, 2010). Moreover, vasospasm induced by ET-1 can be antagonised by calcium antagonist, nitric oxide and parenteral nitrovasodilators.

Within physiological concentrations, ET-1 acts to maintain basal vascular tone whereas higher concentrations are reported in pathophysiological conditions such as pulmonary arterial hypertension (PAH), coronary heart disease and cardiac failure (Boulanger and Lüscher, 1990; Celemajer, 1997; Flammer and Lüscher, 2010). Pharmacological antagonists of ET-1 such as the selective ET_A antagonists, sitaxsentan and ambrisentan, and the non-selective antagonist, bosentan, are currently prescribed for the treatment of PAH (Kirkby *et al*., 2008; Thorin and Clozel, 2010).

Further focus on the role played by nitric oxide in the regulation of vascular tone.

Synthesis of nitric oxide

In 1980 the vascular endothelial cells were first recognised to synthesise a powerful vasodilating agent, endothelium-derived relaxing factor (EDRF), when Furchgott and Zawadzki (1980) noticed that rabbit aortic rings relaxed in response to acetylcholine only when they were endothelium-intact. Many subsequent studies showed that the physical, chemical and pharmacological properties of EDRF were identical to those of nitric oxide (Palmer *et al*., 1987). The major problem at that time, however, was that no system existed

that could detect nitric oxide at the nanomolar concentrations that promote vasodilatation. The breakthrough occurred in 1987, however, when a highly sensitive chemiluminescence detection system for nitric oxide was developed (Palmer *et al*., 1987). The use of this system demonstrated not only that endothelial cells produced NO, but also that they did so in amounts that could fully account for the vasodilator action of EDRF.

Cells other than the vascular endothelial cells, such as neurons in the peripheral and central nervous systems (Garthwaite *et al*., 1988; Gillespie *et al*., 1989) are also able to produce nitric oxide as a physiological mediator. In addition, it was found that immunologically-activated macrophages synthesise nitric oxide from the amino acid L-arginine, (Marletta *et al*., 1987), and subsequent work showed that L-arginine was the substrate for the synthesis of nitric oxide in endothelial cells as well (Palmer *et al*., 1988).

Nitric oxide synthase (NOS), the key enzyme responsible for the production of NO, exists in three isoforms (Förstermann *et al*., 1991, Sader and Celemajer, 2002): NOS I or neuronal NOS (nNOS) is a soluble enzyme in the cytoplasm of the neurons of the central and peripheral nervous systems (Knowles *et al*., 1989; Toda and Toda, 2011); NOS III or endothelial NOS (eNOS) which is mainly a particulate enzyme in the caveolae of the endothelial cell membrane (Förstermann *et al*., 1991). These two isforms are normally found in the cells in the inactive state and require formation of the calcium-calmodulin complex for their activation. They produce small amounts of NO for signalling purposes. On the other hand, NOS II or inducible NOS (iNOS) is not normally expressed, but is synthesised by the macrophages, the vascular smooth muscle cells and other cell types after induction by cytokines, such as interferon-γ, or by bacterial endotoxins (Knowles *et al*., 1989; Buss & Mulsch, 1990; Gray *et al*., 1991; Toda and Toda, 2011), but does not require activation by the calcium-calmodulin complex. Tight calmodulin binding at low

concentrations of intracellular calcium ensures that iNOS is maximally active (assuming adequate supply of substrate, L-arginine) and makes massive amounts of NO that exert cytotoxic and cytostatic actions on invading microorganisms. This action represents an important component of the immune response. Thus, all three NOS isoforms catalyse the manufacture of nitric oxide from the cationic amino acid, L-arginine.

The reaction that yields nitric oxide requires the presence of oxygen as a co-substrate and results in the formation of the by-product, L-citrulline, in equimolar proportions (Bush *et al*., 1992; Toda and Toda, 2011). The reaction also requires 5 electrons supplied by the co-factor NADPH, and these flow through FAD, FMN and tetrahydrobiopterin (BH_4) to complete the synthesis of nitric oxide. This reaction is thought to be a two-stage reaction (Wallace & Fukuto, 1991; Pufahl *et al*., 1992). The first stage involves the N^G-hydroxylation of L-arginine to produce N^G-hydroxy-L-arginine (NOHLA) followed by the subsequent formation of nitric oxide and L-citrulline (Figure 1.2).

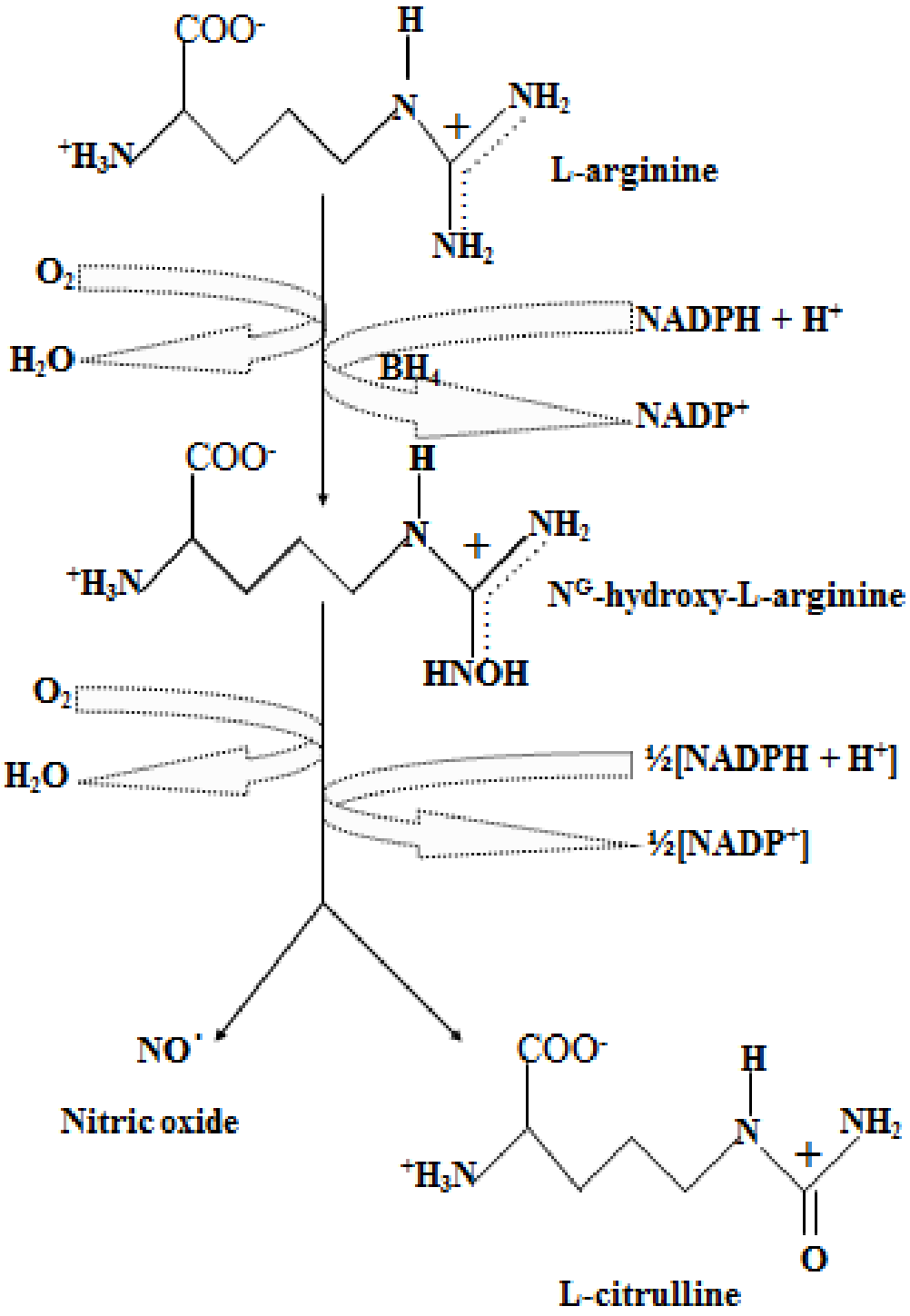

Figure 1.2. Nitric oxide synthase catalyses the oxidation of one of the guanidino nitrogens of L-arginine to produce nitric oxide (Modified from Knowles and Moncada, 1994).

Basal NO activity

It has been reported that the vascular endothelium generates nitric oxide in the absence of external stimuli, i.e. under basal conditions, and that this basal activity of nitric oxide exerts a tonic vasodilator effect that suppresses the actions of vasoconstrictor agents (Martin *et al*., 1986b; Rees *et al*., 1989; Moore *et al*., 1990; Mian & Martin, 1995). These findings are supported by the observation that removal of the endothelium from rat aortic rings results in an augmentation of contraction induced by vasoconstricting agents in comparison to those induced in control rings (Allan *et al*., 1983; Eglème *et al*., 1984).

A question raised is whether nitric oxide can satisfactorily account for the actions of both basal and agonist-stimulated EDRF activity. Kelm and co-workers (1998) found in cultured endothelial cells that bradykinin- and ATP-stimulation, produces nitric oxide as a free radical and this nitric oxide completely accounts for vasodilatation produced by EDRF. However, Mian and Martin (1995) found that superoxide generators, such as hypoxanthine/xanthine oxidase or pyrogallol, inhibit basal but not agonist-stimulated EDRF activity in rat aortic rings. Furthermore, they reported that the superoxide scavenger, superoxide dismutase (SOD) significantly enhanced basal EDRF activity, but it did not alter the acetylcholine-elicited EDRF activity.

The researchers who first documented the presence of basal nitric activity in rat aorta (Martin *et al*., 1986b; Rees *et al*., 1989; Moore *et al*., 1990) proposed that it was produced spontaneously by the vascular endothelium without the need for an external stimulus; this potentially excludes the existence of an intracellular signalling mechanism to control the synthesis of basal nitric oxide. This proposal was supported by the observations made by Rapoport and Murad (1983) that the resting levels of cGMP in entirely unstimulated

endothelium-containing rat aortic rings were 2-3-fold higher than those in endothelium-denuded rings. Their findings too suggest that the basal nitric oxide activity in endothelium-containing rings occurs in the absence of any stimulus.

On the other hand, other researchers have proposed the existence of triggering stimuli that operate within the vascular endothelium to generate basal nitric oxide activity. Such stimuli may include calcium ions that pass from the contracting vascular smooth muscle cells through myo-endothelial gap junctions to the adjacent endothelial cells (Dora *et al*., 2000; Jackson *et al*., 2008) where they (calcium ions) provoke the release of nitric oxide presumably via the calcium-calmodulin-mediated, calmidazolium-sensitive activation of endothelial nitric oxide synthase. Alternatively, Fleming *et al* (1999) proposed that isometric contraction may generate a mechanical stress on the endothelial cytoskeleton that triggers the release of nitric oxide via a phosphatidylinositol 3-kinase(PI 3-kinase)-mediated, wortmannin-sensitive phosphorylation and activation of endothelial nitric oxide synthase, similar to that induced by shear stress generated by blood flow.

Agonist-stimulated nitric oxide activity

Receptor-dependent agonists, such as acetylcholine, bradykinin, ATP and substance P, or receptor-independent agonists, such as calcium ionophore A23187, react with the vascular endothelium to promote the production and release of nitric oxide by stimulating eNOS via calcium-dependent mechanism that is proposed to induce a conformational change that permits dissociation of the enzyme from its inhibitory anchor protein, caveolin-1 (Michel *et al*., 1997; Moncada *et al*., 1991; Schini and Vanhoutte, 1992; Dudzinski and Michel, 2007). Moreover, the release of nitric oxide by this mechanism is inhibited by the calmodulin-antagonist, calmidazolium.

On the other hand, the shearing force of flowing blood induces the generation of nitric oxide via a wortmannin-sensitive, calcium-independent phosphorylation of Ser1177/1179 of eNOS through phosphatidylinositol 3-kinase and the downstream serine/threonine protein kinase Akt, resulting in enhanced NO formation (Fulton *et al*., 1999; Gallis *et al*., 1999; Dimmeler *et al*., 1999; McCabe *et al*., 2000; Galley et al., 2004). Other sites on eNOS are also targets for phosphorylation by protein kinases. For example, threonine 495 can be phosphorylated by AMP-activated kinase and protein kinase C (PKC) with the resultant suppression of eNOS enzymatic activity (Chen *et al*., 1999), and shear stress can result in phosphorylation of eNOS at Ser 116 (Gallis *et al*., 1999), however, the responsible protein kinase(s) and the functional implications of eNOS phosphorylation at this site are uncertain. Furthermore, the catalytic activity of eNOS can be reduced by phosphorylation of its tyrosine residues (Garćia-Gardeña *et al*., 1995) although the exact site(s) and the protein kinase(s) involved are not known. Certain agonists, including vascular endothelium growth factor (VEGF) and insulin, also stimulate nitric oxide production using this pathway (Zeng and Quon, 1996; Papapetropoulos *et al*., 1997; Fulton *et al*., 1999; Fleming *et al*., 2001).

Moreover, agonist-induced EDRF is more resistant to destruction by superoxide anion (O^-_2) than the basal EDRF (Mian and Martin, 1995). Further evidence that agonist-induced EDRF activity is resistant to destruction by superoxide anion has been reported in the rabbit aorta (Furchgott *et al*., 1994). These workers proposed that the identity of EDRF changed during stimulation with acetylcholine, such that it was resistant to destruction by superoxide anion immediately after activation, but became sensitive to destruction after 3-4 minutes of stimulation.

Mechanisms of action of nitric oxide

The majority of the signalling actions of nitric oxide are produced by stimulation of the soluble isoform of guanylate cyclase (sGC) (Hobbs *et al*., 1999). The combination between nitric oxide and the enzyme's heme subunit results in structural changes that enhance the action of this enzyme about 400-fold and leads to the formation of intracellular second messenger, cGMP (Figure 1.3). The latter catalyse the activation of protein kinase G and results in a series of phosphorylation steps (Ignarro and Kadowitz, 1985; Waldman and Murad, 1987). Myosin light chain kinase is phosphorylated, thus preventing its activation by the calcium-calmodulin complex. Furthermore, calcium mobilisation in the smooth muscle cells is blocked through inhibition of calcium release by the inositol trisphosphate (IP_3) mechanism, together with inhibition of calcium influx through the voltage-gated (L-type) and store-operated calcium channels. As a result, phosphorylation of myosin light chains is inhibited, diminishing smooth muscle tone and causing dilatation of blood vessels.

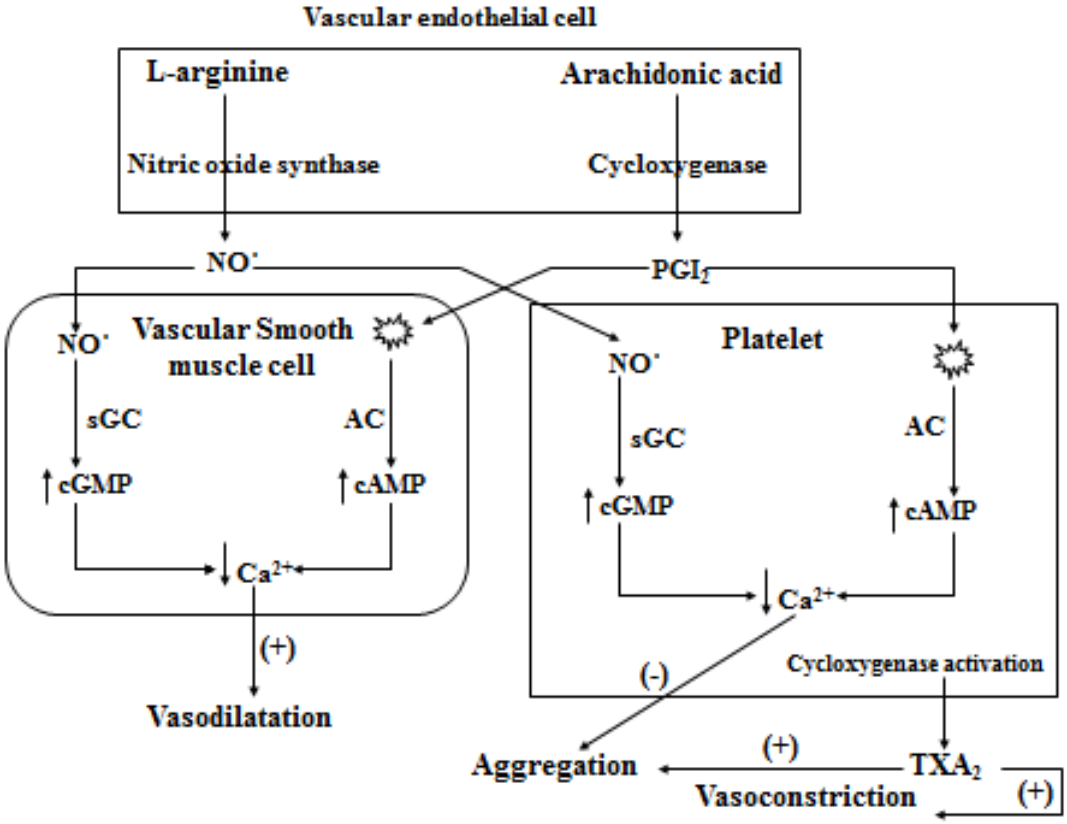

Figure 1.3. Mechanisms of action of nitric oxide and prostacyclin in vascular smooth muscle and blood platelets (Modified from Jerca *et al*., 2002).

In contrast to the effects of low concentrations of nitric oxide used as a signalling agent, high concentrations produced following induction of iNOS have different cellular targets that result in cytotoxic and cytostatic actions. In high concentrations, nitric oxide can result in constant suppression of the actions of certain enzymes such as complex I and complex II of the mitochondrial electron transport chain which is considered as a mechanism of macrophage-derived cytotoxicity (Nathan & Hibbs, 1991; Davis *et al*., 2001). Also, nitric oxide can slow down the action of ribonucleotide reductase, which is a regulatory enzyme in DNA synthesis. These actions of high concentrations cause nitric oxide to be cytotoxic or cytostatic to rapidly dividing cells or to invading microorganism (Figure 1.4). In addition, nitric oxide interacts with superoxide anion to yield the potent oxidant, peroxynitrite (Davis *et al*., 2001; Pacher *et al*., 2007). This agent can cause structural and, as a consequence, physiological injuries by nitrating and oxidising biological molecules such as proteins, lipids and nucleic acids.

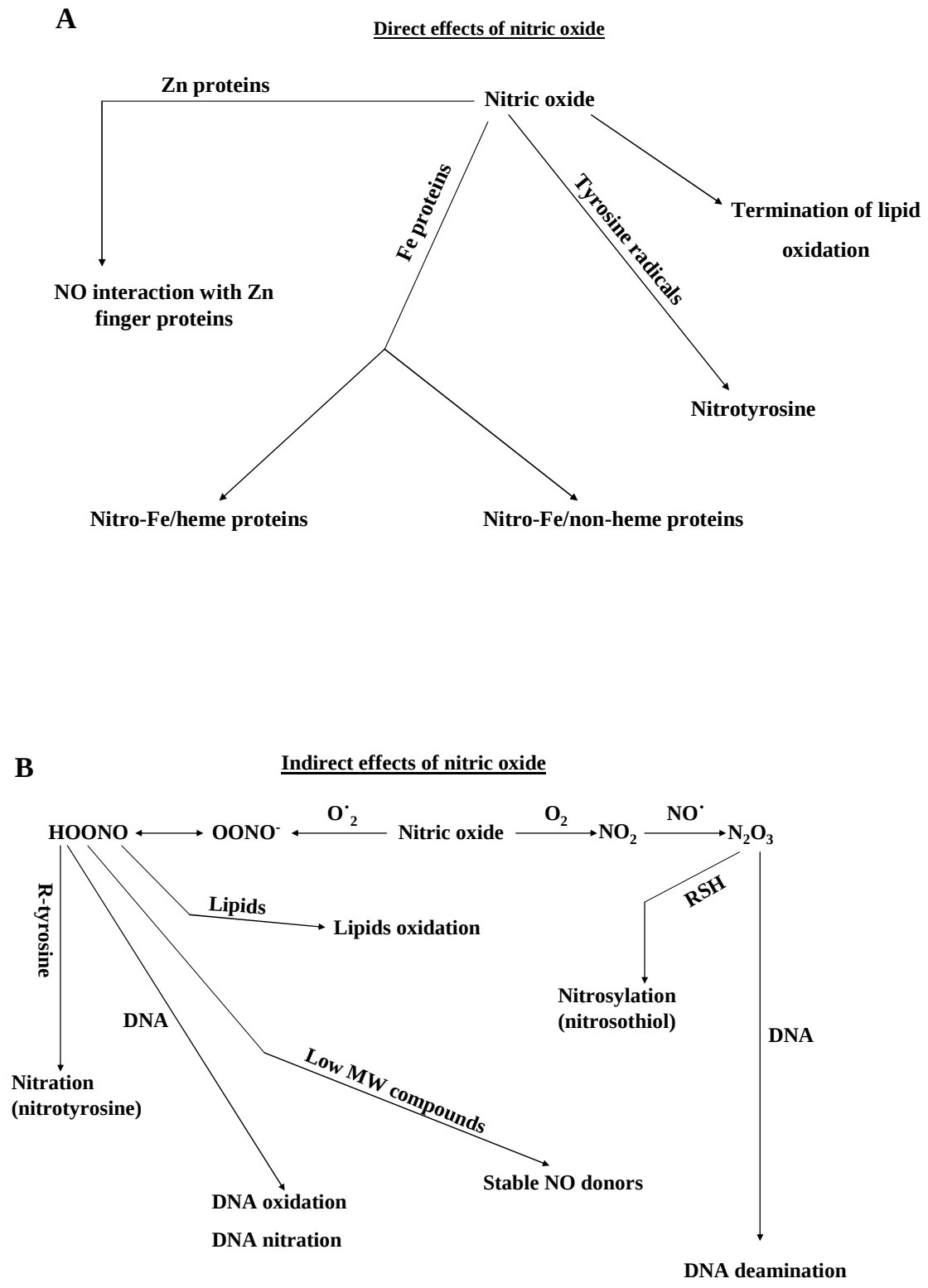

Figure 1.4. Summary of the targets of nitric oxide, the direct effects of nitric oxide (A) are frequently produced at low concentrations of nitric oxide, whereas the indirect effects (B) are produced at high concentrations of nitric oxide (Modified from Davis *et al.*, 2001).

The vascular endothelium and cardiovascular disease

The normal, healthy vascular endothelium plays a critical role in maintaining vascular homeostasis. Generally, this is achieved, as stated above, through maintaining the equilibrium between the productions of endothelium-derived relaxing and contracting factors, and between the coagulation and fibrinolytic systems in addition to regulating inflammatory reactions (Vanhoutte, 1989; Celemajer, 1997; Sader and Celemajer, 2002; Widlansky *et al*., 2003; Galley, *et al*. 2004; Flammer and Lüscher, 2010).

However, in the presence of cardiovascular risk factors, whether the conventional ones such as age, cigarette smoking, dyslipidaemia, hypertension and hyperglycaemia, or the more recently recognized ones such as obesity, reduced physical activity and chronic systemic infections, a long-lasting inflammatory process starts in the vascular endothelium that eventually disturbs vascular homeostasis (Sorensen *et al*., 1994; Steinberg *et al*., 1996; Libby *et al.,* 2002; Prasad *et al*., 2002; Widlansky *et al*., 2003; Flammer and Lüscher, 2010). As a result, the endothelium-dependent release of vasoconstricting and pro-coagulant factors exceeds that of relaxing and anti-thrombotic ones. In addition, there is an abnormal increase in the expression of adhesion molecules and release of chemotactic factors and inflammatory mediators that promote adhesion of inflammatory cells, such as monocytes and lymphocytes, to endothelial cells and their subsequent migration into the vessel wall, the formation of foam cells (lipid-laden macrophages) and proliferation as well as migration of vascular smooth muscle cells. Finally, these events lead to the development of an atherosclerotic plaque. In addition, oxidative stress due to increased formation of reactive oxygen species (ROS) such as superoxide anion (O^{-}_{2}) or hydroxyl radical ($HO^{\cdot}$) play a major role in the development of endothelial dysfunction; the former by destroying, and thus reducing the bioavailability of nitric oxide, and/or by the formation of

peroxynitrite ($ONOO^-$) that oxidatively damages the intracellular macromolecules such as DNA or enzymes responsible for maintaining cellular physiology. Moreover, pathological conditions that may be caused by, or result from, impaired function of the vascular endothelium, such as renal dysfunction, systemic hypertension, atherosclerosis and pregnancy-induced hypertension, are associated with increased production of ROS (Winquist *et al*., 1984; Oyama *et al*., 1986; Lockette *et al.*, 1986; Durante *et al*., 1988; Cai and Harrison, 2000). Furthermore, inactivation of nitric oxide can also be induced by lipid radicals that are formed in the process of lipid oxidation and formation of oxidised low density lipoproteins (Tanner *et al*., 1991; Freeman *et al*., 1997; Cai and Harrison, 2000).

In addition, it was found that circulating concentrations of the endogenous inhibitor of endothelial nitric oxide synthase, asymmetric dimethylarginine (ADMA), increase in patients with pathological conditions that may be caused by, or result from, endothelial dysfunction such as renal dysfunction, systemic hypertension, atherosclerosis and pregnancy-induced hypertension (Vallance *et al*., 1992; Böger *et al*., 1998; Miyazaki *et al*., 1999; Kielstein *et al*., 1999; Böger 2003; Leiper and Vallance, 2006; Siroen *et al*., 2006; Andersshon *et al* 2010). These high concentrations may be due to increased production, diminished catabolism or impaired excretion of this inhibitor secondary to vascular endothelial dysfunction. Moreover, these high concentrations of ADMA further inhibit the bioavailability of nitric oxide and add to the burden created by the dysfunctional endothelium.

Modulation of nitric oxide bioactivity

Modulation of nitric oxide activity in the body underlies the ability of many drugs to provide clinical benefit in a number of pathologies including inflammation, sexual dysfunction and cardiovascular disease (Napoli and Ignarro, 2003). Indeed, a number of

traditionally used and recently introduced medicines act wholly or partly by modifying the nitric oxide signalling pathway. Such agents include:

Agents that enhance nitric oxide bioactivity

Superoxide dismutase

The main source of intracellular superoxide anions is the mitochondria through the respiratory chain enzymes (Yurkov *et al*., 2003). This generation process is enhanced when the cellular levels of nitric oxide are sufficient to inactivate mitochondrial enzymes such as cytochrome oxidase and NADH cytochrome c reductase (Poderoso *et al*., 1996). However, there are other generators of superoxide anion such as aldehyde oxidase, peroxidases, catecholamines and xanthine oxidase (Kerr *et al*., 1999). More recently, NADPH oxidase present on both endothelial and smooth muscle membranes has been implicated in the superoxide-mediated destruction of nitric oxide in a number of cardiovascular pathologies (Rajagopalan *et al*., 1996).

Interestingly, it has been demonstrated that when the cells are deprived of L-arginine, the substrate for NOS, or the cofactor BH_4, NOS shifts from production of nitric oxide to superoxide anion (Pou *et al*., 1992; Xia *et al*., 1996; Pou *et al*., 1999; Rosen *et al*., 2002). Under such conditions, NOS becomes uncoupled such that the electrons supplied by NADPH are now used to reduce the co-substrate O_2 to form superoxide anion (O^-_2).

One of the important pharmacological tools used so far to combat the destructive effects of O^-_2 against NO is the enzyme superoxide dismutase (SOD) (Gryglewski *et al*., 1986; Rubanyi and Vanhoutte, 1986; Martin *et al*., 1994; Mian and Martin, 1995; MacKenzie *et al*., 1999) and this protective effect of SOD extends to nitric oxide produced by the vascular endothelial cells and nitrergic nerves i.e. nitric oxide synthesised by eNOS and

nNOS, respectively. The proposed mechanism behind the protective action of SOD involves an oxidation-reduction (with the consumption of hydrogen ions) reaction that converts O^{-}_{2} into hydrogen peroxide (H_2O_2) with the liberation of molecular oxygen (Fridovich, 1983).

Excessive formation of superoxide anions diminishes the bioavailability of NO and results in dysfunctional vascular endothelium. The latter is associated with a spectrum of pathological conditions such as diabetes mellitus, hypertension, ischaemic heart disease and atherosclerosis (Mohan and Das, 1997; Laight *et al*., 1998; MacKenzie *et al*., 1999). As a consequence, by scavenging superoxide anions, SOD represents a potential therapeutic intervention in the management of such pathophysiological conditions.

In addition to its usefulness in situations of oxidative stress, SOD induces endothelium-dependent, nitric oxide-mediated relaxation in healthy blood vessels; an effect that is attributed to its ability to protect basal nitric oxide from destruction by superoxide anions produced by vascular endothelial cells under physiological circumstances (Gryglewski *et al*., 1986; Rubanyi and Vanhoutte, 1986; Ohlstein and Nichols, 1989; Mian and Martin 1995; MacKenzie *et al*., 1999). However, in light of the mechanism of action of SOD, relaxation induced by SOD seems to have two distinct components; the first through protection of basal NO activity from destruction by superoxide anions. This pathway is sensitive to blockade by nitric oxide synthase inhibitors, such as L-NAME and L-NMMA (Palmer *et al*., 1988; Rees *et al*., 1989; Aisaka *et al*., 1989; Rees *et al.,* 1990; Moore *et al*., 1990; Hobbs *et al*., 1999; Masuda *et al*., 2002). The second mechanism is through production of hydrogen peroxide as a result of dismutating superoxide anion. Hydrogen peroxide induces both endothelium-dependent and endothelium-independent relaxations

and this pathway is inhibited by catalase (Rubanyi and Vanhoutte, 1986; Furchgott *et al*., 1994; Yang *et al*., 1999; Iesaki *et al*., 1999; Itoh *et al*., 2003).

Phosphodiesterase (PDE) inhibitors

There are eleven isoforms of PDE enzyme (Beavo, 1995) whose main intracellular action is to hydrolyse and thereby curtail the actions of the second messengers, cyclic AMP (cAMP) and cyclic GMP (cGMP). PDE 5 and 6 selectively degrade cGMP (Beavo *et al*., 1994); PDE 3 and 4 are responsible for the decomposition of cAMP, while PDE 1 and 2 can degrade both cyclic nucleotides.

Zaprinast was the first agent developed with PDE 5 inhibitory activity, but it lacked the selectivity and potency to reach the clinic (Ballard *et al*., 1998). Another example is sildenafil (Viagra) which is currently used for the treatment of erectile dysfunction. It is a very effective and selective inhibitor of PDE 5 that is highly expressed in human penile smooth muscle. Thus, it potentiates the actions of nitric oxide by preventing the degradation of its second messenger cGMP (Sakuma *et al*., 2002; Rossoni *et al*., 2007).

Tadalafil and vardenafil are two more recent examples of PDE 5 inhibitors used to treat erectile dysfunction (Porst, 2002; Pryor, 2002; Kindirci *et al*., 2004). These have a longer biological half-life than sildenafil, and may therefore have improved therapeutic usefulness. There has been renewed interest in the use of PDE inhibitors to treat cardiovascular diseases resulting from impaired nitric oxide activity. Indeed, PDE 5 inhibitors may have utility in the treatment of pulmonary hypertension (Michelakis *et al*., 2002; Guazzi *et al*., 2004).

Angiotensin-converting enzyme (ACE) inhibitors

Pharmacological compounds that inhibit the rennin-angiotensin-aldosterone axis can indirectly influence the actions of nitric oxide in more than one way. Since ACE is the principle enzyme responsible for the catabolism of bradykinin, ACE inhibitors can enhance the nitric oxide -releasing actions of the peptide (Taylor-McCabe *et al*., 2001). Moreover, by reducing angiotensin II-mediated formation of superoxide anion by NADPH oxidase, ACE inhibitors indirectly prolong the half-life and actions of NO (Rajagopalan *et al*., 1996; Mancini *et al*., 1996).

Statins

The main indication of the 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase inhibiting agents (statins) is to lower the blood cholesterol levels (Shepherd *et al*., 1995). However, it has been shown that these agents can increase the expression of NOS in the endothelial cells and exhibit anti-oxidant effects, even without altering the level of cholesterol in the blood. In addition, they can inhibit the increase in vascular tone due to reduced expression of eNOS in response to hypoxia (Laufs *et al*., 1997; Wilson *et al*., 2001; Filusch *et al*., 2008; Rossoni *et al*., 2008). Furthermore, randomised clinical trials showed that the administration of statins to patients with clinically evident atherosclerotic coronary artery disease produced an improvement of coronary arterial tone (Anderson *et al*., 1995; Treasure *et al*., 1995).

Nitric oxide donors

These are pharmacologically effective agents that are able to donate nitric oxide *in vivo* or *in vitro* (Napoli and Ignarro, 2003).These agents are sub-categorised into:

Nitric oxide itself

Nitric oxide gas has a role in aiding respiration in premature infants and in adults with respiratory distress syndrome (ARDS). However, the use of NO gas has been restricted to the inhalational route of administration due to its limited half-life and quick interaction with molecular oxygen (Ignarro *et al*., 2002).

Nitric oxide donors requiring metabolism

These agents need to be biotransformed by body enzymes to donate nitric oxide. They include nitroglycerine, amyl nitrite, isosorbide dinitrate, isosorbide mononitrate and nicorandil. These agents represent the classical type of nitric oxide substitution treatment and are commonly employed for the treatment of angina pectoris. Although highly effective, this category of drugs has limiting side effects such as the development of tolerance and haemodynamic adverse actions such as headache (Ignarro *et al*., 2002). On the other hand, sodium nitroprusside, a different class of nitric oxide donor, is indicated for the management of acute hypertensive crisis. The advantages of this medication are narrowed by its drawbacks of being active only parenterally and, following continuous therapy, the possibility of thiocyanate poisoning and the development of tolerance (Gruetter *et al*., 1981; Ignarro *et al*., 2002).

Hybrid NO donors

These drugs are generated by adding functional NO-releasing groups, namely, nitrate esters and *S*-nitrosothiols groups to pharmacological compounds that are already in use without altering the therapeutic effectiveness of the principal drug (Ignarro *et al*., 2002). Nitrosocaptopril is an S-nitrosylated analogue of captopril, a vasodilator, angiotensin-converting enzyme inhibitor and anti-thrombotic drug (Loscalzo *et al*., 1989). The blood

pressure-lowering actions of the ACE inhibitor are augmented by the vasodilatory actions of nitric oxide.

Nitric oxide -releasing non-steroidal anti-inflammatory drugs (NONSAIDs) such as nitric oxide-aspirin represent another class of hybrid nitric oxide donors. These agents have the same anti-inflammatory actions of traditional NSAIDs, but the nitric oxide releasing group reduces the incidence of gastric ulceration (Wallace *et al*., 1997).

Agents that inhibit nitric oxide bioactivity

Inhibitors of soluble guanylate cyclase

The majority of signalling actions of nitric oxide are mediated by its combination with the heme group of the soluble guanylate cyclase enzyme (Hobbs *et al*., 1999). This was confirmed by use of methylene blue (Gruetter *et al*., 1981; Martin *et al*., 1985), or the chemical agent 1H- [1, 2, 4] oxadiazolo [4, 3,-a] quinoxalin-1-one (ODQ) (Moro *et al*., 1996; Toda and Toda, 2011), both of which inhibit the enzyme by oxidising its critical ferrous (Fe^{2+}) haem group to the ferric (Fe^{3+}) form.

Agents that destroy nitric oxide e.g. superoxide anion

The different routes of superoxide anion (O^{-}_{2}) formation are discussed above. This free radical reacts with nitric oxide at a rate ($7*10^{9}\ M^{-1}\ s^{-1}$) that is somewhat faster than the rate ($2*10^{9}\ M^{-1}\ s^{-1}$) of its reaction with superoxide dismutase (Pryor & Squadrito, 1995; MacKenzie *et al*., 1999; Toda and Toda, 2011). This rapid, favourable reaction leads to the loss of biological activity of nitric oxide and the production of the damaging oxidant, peroxynitrite.

Inhibitors of nitric oxide synthesis

The flavoprotein inhibitors

These agents, such as diphenylene iodonium (DPI), inhibit the NADPH-dependent flavoprotein (FAD) which is one of the important cofactors for the synthesis of nitric oxide (Stuehr *et al.*, 1991). The inhibition of NOS by these agents is irreversible in a way that neither NADPH nor FAD is able to restore the action of NOS.

The calmodulin inhibitors

These agents, such as calmidazolium and W 70, have the ability to inhibit the activity of constitutive NOS in the endothelial and neuronal cells but not the inducible NOS (Archer and Cowan, 1991; Schini and Vanhoutte, 1992). They therefore exert a degree of isoform selectivity.

Isoform selective inhibitors

Isoform selective inhibitors are useful tools for exploring the role of the different isoforms in different physiological or pathological functions. For example, in cerebral ischaemia (stroke) there is an enhanced stimulation of nNOS as a result of glutamate release, which results in the formation of high amounts of nitric oxide (Zhang & Snyder, 1995). This neuronal nitric oxide seems to augment neurotoxicity. Thus the use of nNOS selective inhibitors like 7-nitroindazole or N^G-propyl-L-arginine have the potential to block this neurotoxicity, whereas non-selective inhibitors would have inhibited eNOS and nNOS and led to exacerbation of cerebral damage by further reducing blood supply to the brain (Dawson *et al*., 1991; Zhang & Snyder, 1995; Toda and Okamura, 2003; Toda *et al*., 2009).

Moreover, selective inhibitors of iNOS, such as 1-amino-2-hydroxy-guanidine (Wu *et al*., 1995), have been shown to modulate the haemodynamic changes encountered in animal models of endotoxaemia. This indicates that these agents may have usefulness in the management of septic shock.

Inhibitors of NOS expression e.g. corticosteroids

It is well established that the vascular endothelial cells, smooth muscle cells, immune cells or other cell types express the inducible isoform of nitric oxide synthase (iNOS) when these cells are exposed for several hours to inflammatory mediators such as interferon-γ (INF-γ), or the bacterial cell wall component, lipopolysaccharide (LPS), whether in experimental settings or *in vivo*, e.g. in inflammatory bowel diseases or septicaemia (Radomski *et al*., 1990; McCall *et al*., 1991; Saura *et al*., 1995; Simmons *et al*., 1996; Linehan *et al*., 2004). In addition, when this enzyme is induced, it is capable of producing copious amounts of nitric oxide hence the precipitous fall in blood pressure seen in septic shock. One of the therapeutic strategies employed in septic shock and inflammatory bowel diseases is the administration of anti-inflammatory glucocorticoids such as hydrocortisone and dexamethasone (Radomski *et al*., 1990; McCall *et al*., 1991; Saura *et al*., 1995; Simmons *et al*., 1996). These therapeutic agents are known to block the synthesis of proteins whose expression is induced in response to inflammatory mediators, such as iNOS. However, the precise molecular mechanism behind the effects of these drugs may be more complex, as more than one mechanism is implicated. For example, some researchers concluded that the glucocorticoids, hydrocortisone and dexamethasone, inhibited in a receptor-mediated mechanism(s) the expression of iNOS, but they did not exert a direct blocking action on functionality of the enzyme (Radomski *et al*., 1990). This blocking action of glucocorticoids is inhibited in a concentration-dependent manner by

cortexolone, a partial agonist of glucocorticoid receptors. Other researchers proposed that dexamethasone might have blocked the expression of iNOS directly or, indirectly, by inhibiting the production and/or action of the inflammatory cytokines implicated in inducing its expression (McCall *et al*., 1991). In agreement with this finding, Linehan and co-workers (2004) concluded that the therapeutic effectiveness of the corticosteroids, prednisolone and budesonide, in the treatment of inflammatory bowel diseases such as colitis, is attributed to their ability to inhibit the formation of inflammatory mediators, such as IL-1, IL-12, TNF-α and prostaglandins, that are implicated in the induction of iNOS expression. However, another group of researchers reported that dexamethasone directly blocks both of the expression and functionality of the enzyme as a result of inhibition of the nuclear transcription factor, NF-κB, which controls the expression of iNOS (Saura *et al*., 1995). Moreover, a reduction in the accessibility of L-arginine and the cofactor, BH_4 for iNOS has been proposed to be the mechanism by which dexamethasone inhibits the activity of the enzyme in cytokine-induced endothelial cells of the cardiac microvasculature (Simmons *et al*., 1996). Steroids have no effect on expression or activity of eNOS or nNOS (Radomski *et al*., 1990).

The guanidino (N^G)-substituted analogues of L-arginine

In view of the finding that it is one of the equivalent guanidino nitrogens of L-arginine that is incorporated into nitric oxide (Yokoi *et al*., 1994), the earliest inhibitors of NOS were the guanidino **(**N^G)-substituted analogues of L-arginine (Rees *et al*., 1989, 1990; Moore *et al*., 1990; Hobbs *et al*., 1999; Toda and Toda, 2011):

N^G-monomethyl-L-arginine (L-NMMA)

N^G-nitro-L-arginine (L-NOARG)

N^G –nitro-L-arginine methyl ester (L-NAME)

These agents (Figure 1.5) are generally accepted to act as conventional competitive, reversible inhibitors of nitric oxide synthase, as it is possible to prevent or reverse their inhibitory effects by adding increasing concentrations of the endogenous substrate for nitric oxide synthase, L-arginine (Palmer *et al*., 1988). However, the use of these inhibitors has been almost entirely restricted to laboratory work where they are employed to investigate the importance of the L-arginine-nitric oxide pathway in the living body. This is probably because these agents indiscriminately block all the three isoforms of nitric oxide synthase which is perhaps not ideal for a potential therapeutic agent (Moncada *et al*., 1991; Nathan, 1992). These agents are also stereo-selective, i.e. the D-isomer, for example D-NMMA is inactive and the effects of the active L-isomer inhibitors can be prevented or reversed by L-arginine but not D-arginine (Palmer *et al*., 1988). However, the ability of L-arginine to reverse the blocking actions of these nitric oxide synthase inhibitors seems to be governed by the animal species, the vascular bed of interest and/or the inhibitor in question (Randall and Griffith, 1991). For example, in the rat aorta L-arginine was able to reverse the blockade of endothelium-dependent, acetylcholine-induced relaxation produced by L-NORG, 30 µM, but not that produced by 100 µM of the same inhibitor in the same tissue (Moore *et al*., 1990; Unmans, 1990). Moreover, L-arginine was only able to partially reverse the vasopressor effects induced by L-NOARG in the anaesthetised guinea pig, whereas those induced by L-NMMA were fully reversed (Steinberg *et al*., 1990). Furthermore, in the rabbit ear vascular bed, L-NAME produced a powerful blockade of basal as well as acetylcholine-induced nitric oxide activity, however, L-arginine was only able to reverse blockade of the former (Randall and Griffith, 1991). The authors attributed these findings to the possible mechanism behind nitric oxide production under basal and agonist-stimulated circumstances.

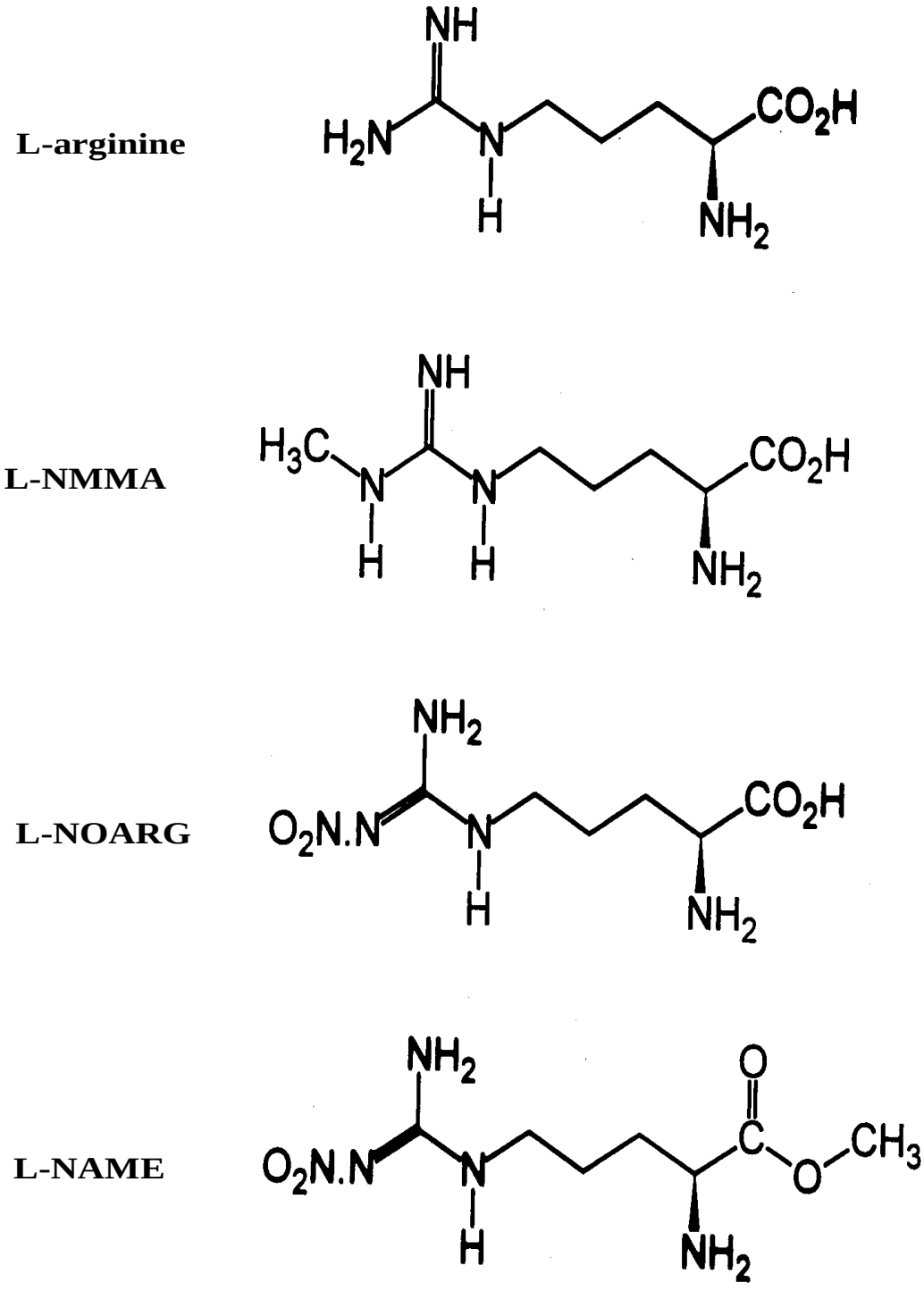

Figure 1.5. Structures of L-arginine and its guanidino (N^G)-substituted analogues used as inhibitors of nitric oxide synthase (Modified from Rees *et al.*, 1990).

Endogenous inhibitors of NOS (Endogenous guanidino (N^G)-substituted analogues of L-arginine)

Figure 1.6 shows the three examples of endogenous guanidino (N^G)-substituted analogues of L-arginine: N^G-monomethylarginine (L-NMMA), N^GN^G- asymmetric dimethylarginine (ADMA) and $N^GN'^G$-symmetric dimethylarginine (SDMA). These compounds are produced by adding methyl groups from methionine to the arginine guanidino nitrogens in proteins. This posttranslational adjustment, which takes place in the nucleus, is regulated by enzymes known as protein arginine methyltransferases (PRMT) (McBride & Silver, 2001; Vallance *et al*., 1992; Siroen *et al*., 2006; Leiper and Vallance, 2006). While ADMA and SDMA are synthesised by PRMT type 1 and type 2, respectively, L-NMMA can be synthesised by either type. The quantity of methylarginines produced is proportional to the level of arginine methylation in protein as well as to the rate of proteolysis, because these inhibitors are secreted into the plasma after proteolysis (Siroen *et al*., 2006).

ADMA is considered to be the major endogenous inhibitor of nitric oxide synthase (Vallance and Leiper, 2004; Siroen *et al*., 2006; Colonna *et al*., 2007; Anderssohn *et al*., 2010**),** because under normal conditions the circulating concentration of ADMA (0.68 µM) is higher than that of L-NMMA (0.11 µM) and because SDMA does not appear to be a competitive inhibitor of nitric oxide synthase (Vallance *et al*., 1992; Sydow and Münzel, 2003; Linz *et al*., 2012). Furthermore, it was found that circulating concentrations of ADMA increase in patients with renal dysfunction (Vallance *et al*., 1992; Kielstein *et al*., 1999) and in other pathological conditions that may be caused by, or result from, impaired function of the vascular endothelium, such as systemic hypertension, atherosclerosis and pregnancy-induced hypertension (Böger *et al*., 1998; Miyazaki *et al*., 1999; Böger 2003;

Leiper and Vallance, 2006; Siroen *et al*., 2006; Andersshon *et al* 2010). In addition, high plasma levels of ADMA were found to be positively correlated with the manifestations of insulin resistance syndrome, and drugs that recover insulin sensitivity, e.g. the PPARγ agonist, rosiglitazone, also restored plasma levels of ADMA (Stühlinger *et al*., 2002).

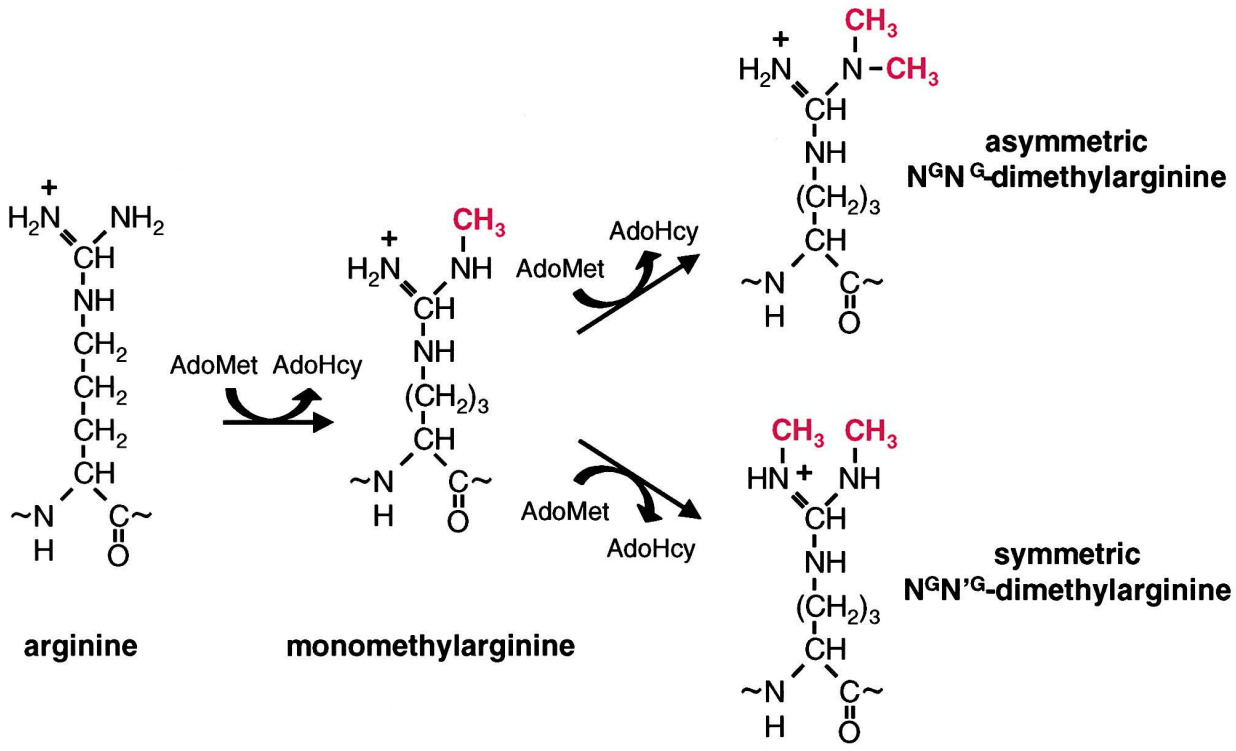

Figure 1.6. Methylation of arginine residues within the context of a protein requires the methyl donor, S-adenosyl methionine (AdoMet), which is converted, into S-adenosyl homocysteine (AdoHcy) (McBride & Silver, 2001).

Differential actions of L-NMMA on basal and agonist-induced activity of NO

Although generally regarded as a classical competitive inhibitor of nitric oxide synthase (Rees *et al*., 1989, 1990; Hobbs *et al*., 1999; Moore *et al*., 1990; Vallance *et al*., 1992; Leiper and Vallance, 2006), some findings with L-NMMA appear to be inconsistent with this view. For example, L-NMMA has been found to act as a "mechanism-based" insurmountable inhibitor of, and an alternative substrate for iNOS in murine macrophages; it is converted to produce N-methyl-N-hydroxy-L-arginine, which tends to irreversibly inhibit the enzyme (Olken, 1991; Feldman *et al*., 1993; Olken and Marletta, 1993). Moreover, in a different study, L-NMMA stimulated the production of superoxide anion by nNOS (Pou *et al*., 1999). This study showed that this effect of L-NMMA seems to be not due to metabolism of L-NMMA, but due to competitive interaction between L-NMMA and L-arginine that prevents the latter from inhibiting superoxide anion production.

Other studies have reported that L-NMMA does not inhibit nitrergic nerve-mediated relaxation in the bovine retractor penis muscle (Martin *et al*., 1993), or in the bovine penile artery (Liu *et al*., 1991) or ciliary artery (Overend and Martin, 2007), while both L-NAME and L-NOARG are effective inhibitors. Quite surprisingly in these tissues, L-NMMA seemed to be a better substrate for the formation of nitric oxide, because it was more potent than the endogenous substrate, L-arginine, at protecting nitrergic transmission from inhibition by L-NAME and L-NOARG. In addition, Cellek and Moncada (1997) concluded that, like L-arginine, L-NMMA is able to both prevent and reverse the blockade of nitrergic nerve-mediated relaxation induced by L-NORAG in the rabbit anococcygeus muscle.

Similar differential actions of L-NMMA have also been reported in blood vessels from other species. For example, while L-NMMA blocked acetylcholine-induced relaxation in rabbit aorta (Rees *et al*., 1989), it did not do so in rat aorta, although it did inhibit the basal activity of nitric oxide which exerts a powerful tonic vasodilator influence that opposes vasoconstriction in this tissue (Frew *et al*., 1993). In that study the inhibition of basal nitric oxide activity by L-NMMA was prevented by L-arginine. In contrast, L-NMMA behaved similarly to L-arginine in protecting acetylcholine- and ATP-induced relaxation from blockade by L-NOARG. Moreover, in rat aorta and pulmonary artery, L-NMMA behaved similarly to L-arginine, where both agents augmented nitric oxide production, assessed using a chemiluminescence assay, whereas L-NOARG produced the anticipated blockade of nitric oxide synthesis (Archer and Hampl, 1992). Thus, in rat aorta, L-NMMA has the curious effect of blocking basal nitric oxide activity in an L-arginine-reversible manner yet acts like L-arginine in preventing blockade of agonist-stimulated activity of nitric oxide by L-NOARG.

Differential actions of ADMA on basal and agonist-induced activity of NO

The short-term actions of ADMA are thought to be achieved by blocking the enzymatic activity of NOS and, in turn, inhibiting nitric oxide production by the vascular endothelium. Such effects were restored by L-arginine, but not D-arginine (Vallance *et al*., 1992; Cooke *et al*., 1997; Moncada & Higgs, 2002).

However, the correlation between elevated plasma levels of ADMA seen in various clinical conditions (Kielstein *et al*., 1999; Zoccali *et al*., 2002; Böger 2003) suggests that there are mechanisms other than simple inhibition of NOS that underlie the pharmacological actions of ADMA.

For example, Suda and co-workers (2004) suggested that some of the long-term vascular effects of ADMA might be mediated via AT_1 receptor activation, as they demonstrated that long-term administration of ADMA resulted in up-regulation of vascular ACE and enhanced the formation of superoxide anion in both wild-type and eNOS-knockout mice. Furthermore, they found that formation of both vascular and systemic nitric oxide was not affected by administration of ADMA and these effects of ADMA were not blocked by L-arginine. In addition, blockade of ACE or the AT_1 receptor abolished superoxide anion synthesis and the associated vascular actions of ADMA.

It is well-known that chronic administration of angiotensin II enhances the activity of vascular NADPH oxidases (Griendling *et al*., 1994) and most of the superoxide anion produced in blood vessels is through this pathway. These effects of angiotensin II are blocked by the AT_1 blocker losartan. More recently, Veresh and co-workers (2008) found that ADMA decreased basal arteriolar diameter and blocked the arteriolar dilatation in response to flow in rat skeletal muscle arterioles. These actions of ADMA were found to be inhibited by the ACE inhibitor, quinalapril, the AT_1 receptor blocker, losartan, the superoxide scavenger, superoxide dismutase, and by the inhibitor of NADPH oxidase, apocynin. As a result, the authors concluded that high concentrations of ADMA stimulate the RAS in the vascular wall, which in turn enhances the formation of angiotensin II and the latter stimulates the NAD(P)H oxidase pathway. The resultant production of superoxide anion reduces the biological activity of nitric oxide (Figure 1.7).

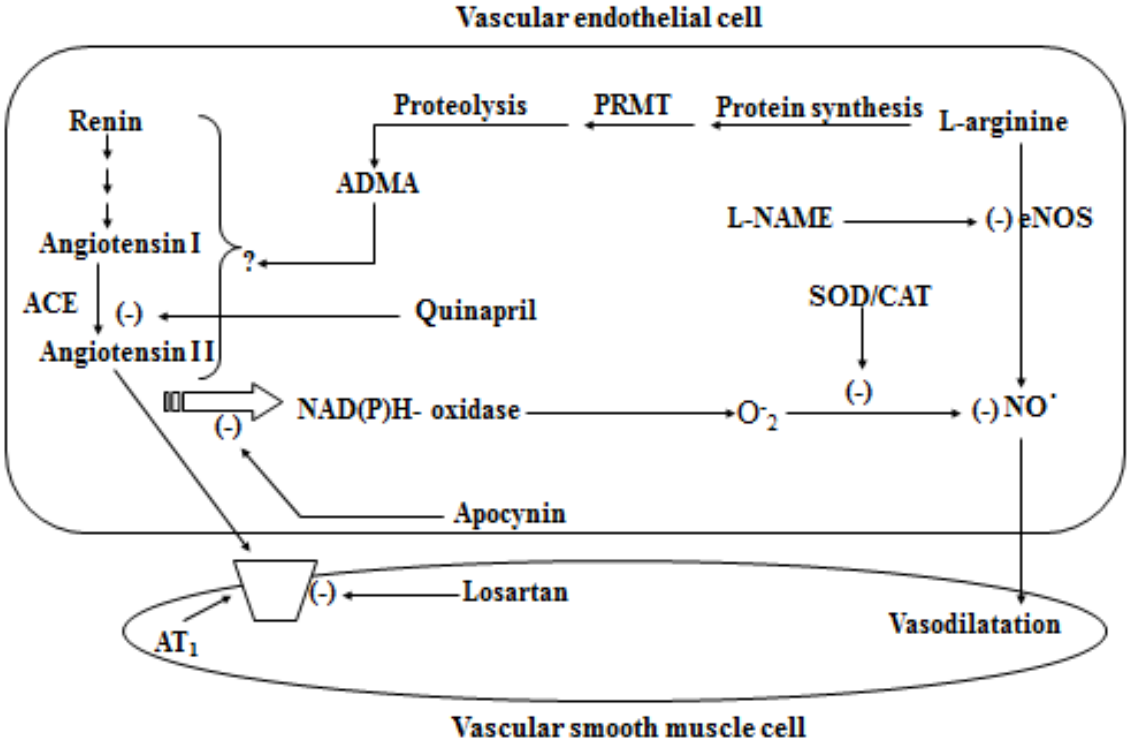

Figure 1.7. Proposed mechanisms by which ADMA induces oxidative stress and vasomotor dysfunction (Modified from Veresh *et al*., 2008).

The structural similarities between ADMA and L-NMMA may suggest that these two NOS inhibitors share similar actions mediated by the same mechanisms. Both these substituted L-arginine analogues are asymmetric in terms of the distribution of methyl group(s) around nitrogen atoms such that the single methyl group of L-NMMA and the two groups of ADMA are attached to one of the two guanidino nitrogen atoms of L-arginine (Leiper and Vallance, 1999; Kielstein *et al*., 2007). As a result, and in view of the finding that it is one of the equivalent guanidino nitrogens of L-arginine that is incorporated into nitric oxide (Yokoi *et al*., 1994), ADMA might act as an alternative substrate for, and/or a "mechanism-based" irreversible inhibitor of, nitric oxide synthase in a way similar to that proposed for L-NMMA (Olken, 1991; Feldman *et al*., 1993; Olken and Marletta, 1993). Moreover, similar to L-NMMA (Frew *et al*., 1993), ADMA may be able to block basal but not agonist-stimulated activity of nitric oxide in rat aorta and to protect acetylcholine-induced relaxation in rat aorta from blockade by L-NAME.

Thus, both L-NMMA and ADMA, two methylated analogues of L-arginine, exhibit anomalous actions that cannot be explained by a simple inhibition of NOS. Some of the pharmacology of L-NMMA and ADMA may arise from superoxide formation and some may arise from blockade of NOS. This dual pharmacology may explain some of the anomalous actions of these two agents.

Aim of the study

The aim of this study is to seek an explanation for some of the above differential actions of L-NMMA and ADMA. In particular, an attempt will be made to determine if a common mechanism is responsible for these differential actions of L-NMMA and ADMA.

The specific aims of the study are to:

1. Determine if previous experiments demonstrating that L-NMMA blocks basal but not agonist-stimulated nitric oxide activity in rat aorta (Frew *et al*., 1993) can be confirmed.

2. Determine if ADMA, like L-NMMA, blocks basal but not acetylcholine-induced nitric oxide activity in rat aorta, and if so, investigate the possible mechanism(s) behind these differential abilities.

It was hoped that these studies would provide an explanation for the seemingly differential actions of L-NMMA and ADMA. Those arising from the actions of ADMA may be clinically relevant, because this agent is known to accumulate in a growing number of pathological conditions.

Chapter Two

Introduction

Three decades have now elapsed since Furchgott and Zawadzki (1980) first discovered that the vascular endothelial cells produce a significant vasodilating agent, endothelium-derived relaxing factor (EDRF), which subsequent work identified as nitric oxide (Palmer *et al*., 1987).

The bioactivity of nitric oxide is liable to inhibition by different classes of agents, and those of particular interest to this study are the guanidino (N^G)-substituted analogues of L-arginine that act by inhibiting nitric oxide synthase (Rees *et al*., 1989, 1990; Moore *et al*., 1990; Hobbs *et al*., 1999). The initial representatives of this class were N^G- monomethyl-L-arginine (L-NMMA), N^G- nitro-L-arginine (L-NOARG) and N^G- nitro-L-arginine methyl ester (L-NAME). These agents are generally accepted to act as conventional competitive, reversible inhibitors of nitric oxide synthase, as it is possible to prevent or reverse their inhibitory effects by adding increasing concentrations of the endogenous substrate for nitric oxide synthase, L-arginine (Palmer *et al*., 1988). However, the use of these inhibitors has been almost entirely restricted to laboratory work where they are employed to investigate the importance of the L-arginine-nitric oxide pathway in the living body. This is probably because these agents indiscriminately block all the three isoforms of nitric oxide synthase which is perhaps not ideal for a potential therapeutic agent (Moncada *et al*., 1991; Nathan, 1992).

The interest in the properties of L-NMMA has increased when it was found that it was produced endogenously together with two other methylarginines: N^GN^G- asymmetric dimethylarginine (ADMA) and $N^GN'^G$-symmetric dimethylarginine (SDMA). These three methylarginines are formed following the breakdown of proteins that had been

posttransationally methylated by the enzyme protein arginine methyl transferase (Vallence *et al*., 1992; Siroen *et al*., 2006; Leiper and Vallence, 2006). In addition, it has been reported that the plasma levels of ADMA are raised in pathophysiological conditions that are caused by, or result from, vascular endothelial dysfunction (Vallance *et al*., 1992; Böger *et al*., 1998; Kielstein *et al*., 1999; Miyazaki *et al*., 1999; Böger 2003; Leiper and Vallence, 2006; Siroen *et al*., 2006; Andersshon *et al* 2010).

Thus, because of the current interest in ADMA as a potential contributor to human cardiovascular and other diseases, it seemed important to determine if this methylarginine showed some of the paradoxical actions of its closely related analogue, L-NMMA. Specifically, the aims of this part of the study were:

- To determine if ADMA, like L-NMMA, blocks basal but not agonist-induced activity of nitric oxide in the rat aorta.

- To determine if the blockade of basal activity of nitric oxide induced by ADMA is sensitive to reversal by L-arginine, the endogenous substrate for nitric oxide synthase.

- To determine if the availability of L-arginine is critical for basal as well as for acetylcholine-stimulated release of nitric oxide in the rat aorta.

- To determine if the level of phenylephrine-induced tone has an influence on acetylcholine-induced relaxation in control and ADMA-treated rat aortic rings.

- To determine if the choice of the contractile agent used (phenylephrine vs 5-hydroxytryptamine or $PGF_{2\alpha}$) has an influence on acetylcholine-induced relaxation in control and ADMA-treated rat aortic rings.

- To determine if ADMA, similarly to L-arginine, protects acetylcholine-induced relaxation against blockade by L-NAME in the rat aorta.

Material and Methods

Preparation of aortic rings

The preparation of aortic rings was essentially similar to that previously discussed (Al-Zobaidy *et al*., 2010). Specifically, female Wistar rats weighing 150-200 g were killed by stunning and exsanguination. The descending thoracic aorta was exposed by removing the heart and lungs, and excised from just above the diaphragm to just below the aortic arch. The vessel was cleared of adhering fat and connective tissue, placed in a Petri dish containing Krebs' solution, and cut transversely into 2.5 mm wide rings using a device with parallel razor blades. The vessel was handled carefully to avoid unintentional damage to the intimal surface. In some experiments, the endothelium was removed from some rings by gently rubbing the intimal surface for 30 s with a wooden stick dampened in Krebs' solution. Care was taken not to over-stretch the rings during rubbing in order to avoid damaging their muscular layer. Endothelial denudation was considered successful if the endothelium-dependent vasodilator, acetylcholine (1 μM), failed to produce relaxation.

Tension recording

The aortic rings were mounted under 10 mN resting tension on two stainless steel hooks in 10 ml organ baths at 37 ºC. The upper hook was connected with thread to a tension transducer and the lower was connected to a rigid bar. The bathing solution was Krebs solution composed of (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11, and continuously gassed with 95% O_2 and 5% CO_2. The temperature of the solution within the baths was maintained using a water jacket heated by a thermostatically controlled pump (Techne, Circulator C-85D). Contractions were recorded isometrically with Grass FT03C transducers and responses displayed and recorded on a PowerLab (AD Instruments, Hastings, UK). Tissues were allowed to

equilibrate for approximately 90 min, before executing experimental protocols, during which time the resting tension was re-adjusted to 10 mN when required.

Cumulative concentration-effect curves to phenylephrine and the effects of the vascular endothelium on phenylephrine-induced contraction

The aims, from performing cumulative concentration-effect curves to phenylephrine in female rat endothelium-containing and endothelium-denuded aortic rings, were to determine if the contractions produced were concentration-dependent, to obtain the maximal response (E_{max}) and -log EC_{50} (pEC_{50}), and to assess the vasodepressant effect of basal nitric oxide generated by the vascular endothelium on phenylephrine-induced contractions.

In these experiments, endothelium-containing or -denuded aortic rings were allowed to equilibrate for 90 min and the resting stretch was re-adjusted to 10 mN, if required. Phenylephrine was then added in increasing concentrations to the tissues in the organ baths using the sequence 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 μM, 3 μM and 10 μM, without washing out after each concentration. Each successive concentration of phenylephrine was added after stabilisation of tone produced by the preceding concentration. When the point was reached that the next concentration of phenylephrine failed to produce a further increment in tone, it was concluded that the maximal response to phenylephrine had been achieved. The drug was then either washed out to conduct another concentration-response curve, or papaverine (300 μM) was added to produce full relaxation of the tissues.

Cumulative concentration-effect curves to acetylcholine and the effects of the vascular endothelium on acetylcholine-induced relaxation

The aims, from performing cumulative concentration-effects curves to acetylcholine in female rat endothelium-containing and endothelium-denuded aortic rings, were to determine if relaxations were concentration-dependent, to obtain the maximal response (E_{max}) and -log EC_{50} (pEC_{50}), and to assess the endothelium-dependence of the relaxations produced.

In these experiments, endothelium-containing or -denuded aortic rings were allowed to equilibrate for 90 min and the resting stretch re-adjusted to 10 mN, if required. Tissues were precontracted to ~50% of maximal tone using phenylephrine (100-300 nM). After stabilisation of phenylephrine-induced tone, increasing concentrations of acetylcholine (1 nM-10 μM) were added to the tissues in the organ baths using the sequence 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 μM, 3 μM and 10 μM, without washing out after each concentration. Each successive concentration of acetylcholine was added after relaxation to the preceding concentration had maximised. When the point was reached that the next concentration of acetylcholine did not produce further relaxation, it was concluded that the maximal response to acetylcholine had been achieved. The drug was then either washed out to conduct another concentration-response curve, or papaverine (300 μM) was added to produce full relaxation of the tissues.

The effect of L-arginine on basal nitric oxide activity

The rationale for conducting these experiments was to assess if the availability of L-arginine, the endogenous substrate for nitric oxide synthase (Palmer *et al*., 1988; Schmidt *et al*., 1988), was limiting for production of the basal nitric oxide activity that suppresses

vasoconstrictor tone (Martin *et al*., 1986b; Moore *et al*., 1990; Rees *et al*., 1989; Mian & Martin, 1995). If the availability of L-arginine was limiting, it was expected that its addition would result in a fall in phenylephrine-induced tone.

The approach employed in these experiments involved induction of intermediate (10.7 ± 1.0 mN) phenylephrine (50-70 nM) tone in female rat endothelium-containing aortic rings. After stabilisation of tone, L-arginine (10 mM, 1 h) was added to the tissues in the organ baths and its effects on tone assessed. Time-matched controls were treated with the solvent (0.9% normal saline solution) used in the preparation of L-arginine.

The effect of L-arginine on acetylcholine-induced relaxation

The rationale for conducting these experiments was to assess if the availability of L-arginine, the endogenous substrate for nitric oxide synthase, is limiting for the production of nitric oxide stimulated by acetylcholine. If the availability of L-arginine was limiting, it was expected that its addition would potentiate acetylcholine-induced relaxation.

In these experiments, control female rat endothelium-containing aortic rings and rings treated with L-arginine (10 mM, 1 h) were precontracted to ~50% of maximal phenylephrine tone. After stabilisation of tone, a full cumulative concentration-response curve to acetylcholine (1 nM-10 μM) was conducted as indicated previously. When the maximal response to acetylcholine had been achieved, the drug was then either washed out to conduct another concentration-response curve, or papaverine (300 μM) was added to produce full relaxation of tissues.

Effects of nitric oxide synthase inhibitors on basal nitric oxide activity

Basal activity of nitric oxide generated by the vascular endothelium exerts a tonic vasodilator effect that suppresses the actions of vasoconstrictor drugs (Martin *et al*., 1986b; Moore *et al*., 1990; Rees *et al*., 1989; Mian & Martin, 1995). As a result, agents that reduce the synthesis or actions of nitric oxide produce an enhancement of vasoconstrictor-induced tone by removing this endothelium-dependent suppression of vasoconstriction. Therefore, the aim of these experiments was to investigate the effects of nitric oxide synthase inhibitors on basal nitric oxide activity by assessing their ability to enhance phenylephrine-induced contraction in female rat endothelium-containing aortic rings.

In these experiments two strategies were employed. In the first strategy, blockade of basal nitric oxide activity was assessed by measuring the enhancement of phenylephrine-induced tone observed following the addition of nitric oxide synthase inhibitors to female rat endothelium-containing aortic rings. In these experiment, after the tissues had equilibrated and the resting stretch was re-adjusted to 10 mN, a low-level (~2–5 mN) tone was induced with phenylephrine (60 nM). After the tone had stabilised, the tissues were treated with L-NMMA, L-NAME, ADMA (all at 100 μM) or SDMA (1 mM) with the resulting enhancement of tone measured after 1 h.

In another experiment in this series, the ability of a range of concentrations of ADMA (0.3-300 μM) to enhance low-level phenylephrine-induced tone was assessed. The threshold concentration of ADMA, its pEC_{50} and the concentration producing maximal enhancement of phenylephrine-induced tone were determined.

In the second strategy, blockade of basal nitric oxide activity was evaluated by measuring the augmentation of phenylephrine-induced contractions occurring in female rat endothelium-containing aortic rings that had been pre-treated with the nitric oxide synthase inhibitors 1 h prior to constructing a cumulative concentration-effect curve to phenylephrine (1 nM-10 µM), when compared with time-matched controls.

Using this same approach, the concentration-dependent ability of ADMA to block basal nitric oxide activity in female rat aorta was also investigated. In these experiments, ADMA was added at three different concentrations (100, 300 and 1000 µM) for 1 h before performing a full concentration-response curve to phenylephrine (1 nM-10 µM).

This protocol was also employed to investigate the endothelium-dependent ability of ADMA to block basal nitric oxide activity in female rat aorta. In these experiments, endothelium-containing and endothelium-denuded rat aortic rings were treated with ADMA (100 µM, 1 h) prior to conducting a full concentration-response curve to phenylephrine (1 nM-10 µM) and the resulting contractions were compared in the two groups.

The ability of L- or D-arginine to reverse and/or prevent the blockade of basal nitric oxide activity induced by nitric oxide synthase inhibitors

As will be seen in the Results, nitric oxide synthase inhibitors enhance phenylephrine-induced tone by blocking basal nitric oxide activity in female rat endothelium-containing aortic rings. Further experiments were aimed to investigate whether this blockade can be reversed and/ or prevented by the substrate of nitric oxide synthase, L-arginine, or its optical isomer, D-arginine.

In the experiments designed to investigate the ability of L- or D-arginine to reverse the blockade of basal nitric oxide activity, a low-level (~2–5 mN) of phenylephrine (60 nM) tone was induced and after the tone had stabilised, the tissues were treated with L-NAME, L-NMMA or ADMA (all at 100 µM) for 1 h or until the enhanced tone had stabilised. L- or D-arginine (10 mM) was then added to the tissues and the effects on tone assessed after 1 h.

In the experiments designed to investigate the ability of L- or D-arginine to prevent the blockade of basal nitric oxide activity, L- or D-arginine (10 mM) was added to the tissues for 1 h prior to the induction of a low-level (~2–5 mN) of phenylephrine (60 nM) tone. After the tone had stabilised, the nitric oxide synthase inhibitor was added and the effects on tone assessed after 1 h.

The effect of ADMA on the contractile response to 5-hydroxytryptamine or prostaglandin $F_{2\alpha}$

These experiments were conducted to determine if the ability of ADMA (100 µM) to augment vasoconstrictor tone through blockade of basal nitric oxide activity in female rat aorta is independent of the nature of the contractile agent.

In these experiments, cumulative concentration-response curves were conducted to 5-hydroxytryptamine (1 nM-100 µM) or prostaglandin $F_{2\alpha}$ (1 nM-30 µM) in control and in ADMA (100 µM; 1 h)-treated female rat endothelium-containing aortic rings. Comparisons were then made of the contractile actions observed in control and ADMA-treated rings.

The effects of nitric oxide synthase inhibitors on acetylcholine-induced nitric oxide activity

The previous experiments in this Chapter investigated the effects of nitric oxide synthase inhibitors on basal nitric oxide activity in female rat aorta. The aim of these experiments was to investigate the effects of these inhibitors on acetylcholine-induced nitric oxide activity.

In these experiments, female rat endothelium-containing aortic rings were allowed to equilibrate for 90 min and resting stretch was re-adjusted to 10 mN. The tissues were then treated with L-NAME (100 µM or 1 mM for 30 min), L-NMMA (100 µM or 1 mM for 45 min), ADMA (100 µM or 1 mM for 1 h) or SDMA (1 mM for 1 h). After this time the treated tissues and their time-matched controls were pre-contracted to ~50% of maximal phenylephrine tone. Care was taken to induce comparable levels of tone both in control and treated tissues by using lower concentrations of phenylephrine in the latter. After stabilisation of tone, a full concentration-response curve to acetylcholine (1 nM-10 µM) was conducted. In some experiments involving L-NMMA at 100 µM or ADMA at 1 mM, this was followed by a second and a third concentration-response curve to acetylcholine where the tissues were washed out in between, and treated again with the same concentration of L-NMMA or ADMA and pre-contracted to ~50% of maximal phenylephrine tone . The aim here was to determine if the sensitivity of control tissues to acetylcholine or the blocking actions of L-NMMA or ADMA changed with time.

Effect of the level of tone on acetylcholine-induced relaxation in control and ADMA-treated tissues

The aims of these experiments were to determine if the level of phenylephrine-induced tone affected the magnitude of acetylcholine-induced relaxation in control tissues or the ability of ADMA to block this relaxation in female rat endothelium-containing aortic rings.

In these experiments, endothelium-containing aortic rings were contracted to ~50% of maximal phenylephrine tone using phenylephrine (100-300 nM) before conducting a full concentration-response curve to acetylcholine (1 nM-10 µM). The tissues were then washed out and treated with ADMA (100 µM, for 1 h) before being contracted using the same concentration of phenylephrine (100-300 nM), which this time produced a higher level of tone, due to blockade of basal nitric oxide activity. A full concentration-response curve to acetylcholine (1 nM-10 µM) was then conducted at the highest level of tone. The tissues were then washed out, treated again with ADMA (100 µM, for 1 h) and this time contracted to the original ~50% of maximal phenylephrine tone using a lower concentration of phenylephrine before performing a full concentration-response curve to acetylcholine (1 nM-10 µM). The ability of acetylcholine to produce relaxation at each level of tone was then compared.

In another set of experiments, control endothelium-containing aortic rings and rings treated with ADMA (100 µM, for 1 h) were contracted using phenylephrine to different levels of tone ranging from low, maximal to supra-maximal, before full concentration-response curves to acetylcholine (1 nM-10 µM) were conducted. The ability of acetylcholine to produce relaxation at these different levels of tone in control and ADMA-treated tissues was then compared.

The effect of L-arginine, L-NMMA, ADMA and SDMA on blockade of acetylcholine-induced relaxation by L-NAME

As will be seen in the Results, that when experiments were conducted at similar levels of sub-maximal tone that the inhibitor of nitric oxide synthase, L-NAME, produced powerful blockade of acetylcholine-induced relaxation in female rat endothelium-containing aortic rings, but L-NMMA and ADMA had little effect. Therefore, the aim of the current experiments was to investigate if L-NMMA or ADMA could interfere with the ability of L-NAME to produce blockade of acetylcholine-induced relaxation. The possibility that the substrate, L-arginine, or the inactive analogue, SDMA, could interfere with the ability of L-NAME to produce blockade of acetylcholine-induced relaxation was also investigated.

In these experiments two approaches were employed. In the first, to determine if blockade produced by L-NAME (30 µM) could be prevented by L-arginine, L-NMMA, ADMA or SDMA (all at 1 mM), the latter were added to the tissues in the organ baths 1 h prior to the addition of L-NAME. Matched intermediate tone was then induced with phenylephrine both in control and treated tissues to be followed by a full concentration-response curve to acetylcholine (1 nM-10 µM). The ability of acetylcholine to produce relaxation in the different conditions was then compared

In the second approach, to determine if the L-NAME-induced blockade could be reversed by L-arginine, L-NMMA, ADMA or SDMA, the tissues were treated with L-NAME (30 µM for 1 h) to establish the blockade then treated with L-arginine, L-NMMA, ADMA or SDMA (all at 1 mM) for 1 h prior to induction of matched intermediate phenylephrine-induced tone. After stabilisation of phenylephrine-induced tone, a full

concentration-response curve to acetylcholine (1 nM-10 μM) was conducted. The ability of acetylcholine to produce relaxation in the different conditions was then compared.

Drugs and chemicals

Acetylcholine chloride (ACh), N^G , N^G –dimethyl-L-arginine dihydrochloride (ADMA), L-arginine hydrochloride, 5-hydroxytryptamine (5-HT), N^G –nitro-L-arginine methyl ester (L-NAME), N^G –monomethyl-L-arginine acetate (L-NMMA), papaverine hydrochloride, phenylepherine hydrochloride (PE) and prostaglandin $F_{2\alpha}$ were all obtained from Sigma, Poole, UK. N^G , N'^G –dimethyl-L-arginine dihydrochloride (SDMA) was obtained from Enzo Life Sciences, Exeter, UK. All drugs were dissolved and diluted in 0.9% saline.

Data analysis

Contractions were measured in milliNewtons. Papaverine (300 μM) was added at the end of each experiment to produce full relaxation of tissues and the relaxant responses to acetylcholine were expressed as percentage of this relaxation. Data are expressed as the mean ± S.E.M of n separate observations. Concentration-effect curves were analysed and drawn by fitting the data to following formula (Randall and Griffith, 1991):

$$R = \frac{R_{max} \times A^{n_H}}{EC_{50}^{n_H} + A^{n_H}}$$

Where **R** is the percentage tissue response to agonist, **A** the concentration of agonist, $\mathbf{R_{max}}$ the maximum response induced by agonist, $\mathbf{n_H}$ is the slope and $\mathbf{EC_{50}}$ is the concentration of agonist that induces 50% of the maximal response.

Statistical analysis was done by one-way analysis of variance followed by Bonferroni's post-test or by Student's *t* test, as appropriate, with the aid of a computer-based program (Graph Pad, San Diego, USA). Values were considered to be statistically different when P was ≤ 0.05.

Results

Effects of the vascular endothelium and N^G–nitro-L-arginine methyl ester on phenylephrine-induced contraction

A full concentration-effect curve to phenylephrine (1 nM-10 nM) in female rat endothelium-containing aortic rings resulted in an E_{max} of 14.4 ± 1.5 mN and a pEC_{50} of 6.75 ± 0.07 (Figure 2.1). Removal of the endothelium or pretreatment with N^G –nitro-L-arginine methyl ester (L-NAME, 100 µM) significantly increased the tissue sensitivity to phenylephrine (pEC_{50} 8.25 ± 0.14 and 7.80 ± 0.12, $P< 0.001$, respectively), but had no effect on the E_{max} (18.2 ± 1.0 mN and 18.2 ± 1.6 mN, $P> 0.05$, respectively), consistent with the presence of basal nitric oxide activity suppressing vasoconstrictor tone in this tissue.

Effects of the vascular endothelium and L-NAME on acetylcholine-induced relaxation

Following sub-maximal (7.8 ± 0.6 mN, n= 7) contraction with phenylephrine (100-300 nM), acetylcholine (1 nM-10 µM) produced concentration-dependent relaxation in endothelium-containing rings of female rat aorta (E_{max} 91.8 ± 1.6% and pEC_{50} of 7.70 ± 0.02) (Figure 2.2). In endothelium-denuded (EC-) rings taken to the same sub-maximal level of tone as control tissues, acetylcholine failed to produce relaxation. In endothelium-containing rings treated with the nitric oxide synthase (NOS) inhibitor, L-NAME (100 µM), and taken to the same sub-maximal level of tone, acetylcholine-induced relaxation was significantly blocked (E_{max} 19.8 ± 1.6% and pEC_{50} of 6.35 ± 0.06).

Effects of L-arginine on basal nitric oxide activity

Following induction of intermediate tone (10.7 ± 1.0 mN) in rat endothelium-containing aortic rings using phenylepherine (50-70 nM), subsequent addition of the NOS substrate, L-arginine (10 mM, 1 h), did not significantly affect the level of tone (Figure 2.3). It is

therefore unlikely that the availability of L-arginine is limiting for the production of basal nitric oxide activity that suppresses vasoconstrictor tone.

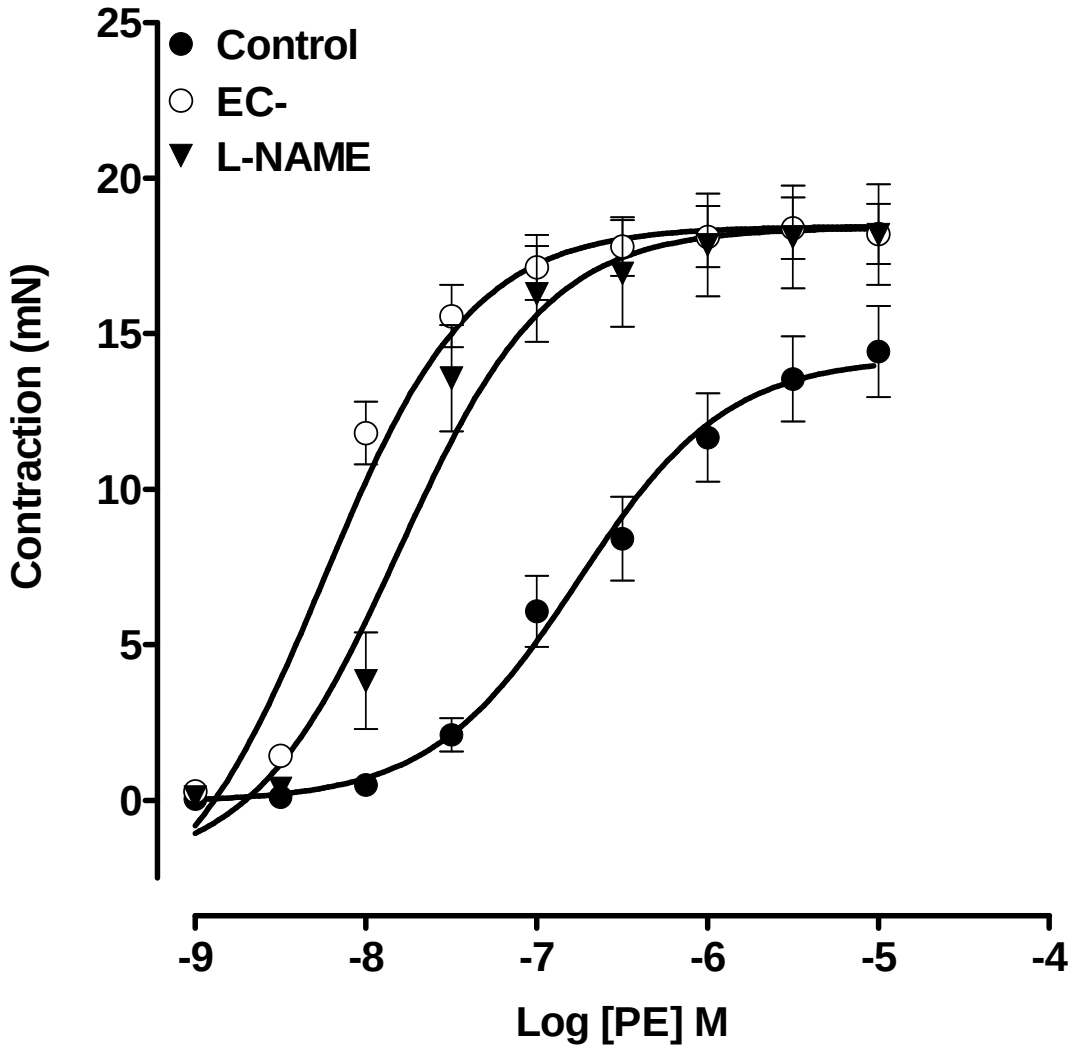

Figure 2.1. Cumulative concentration-effect curves showing contractile responses to PE in female rat aortic rings. Responses are shown for tissues in the presence (control) or in the absence (EC-) of endothelium, or tissues with endothelium but treated with L-NAME (100 µM). The sensitivity of tissues was enhanced by both endothelial denudation and L-NAME treatment. Each point represents the mean ± SEM of 6-15 observations.

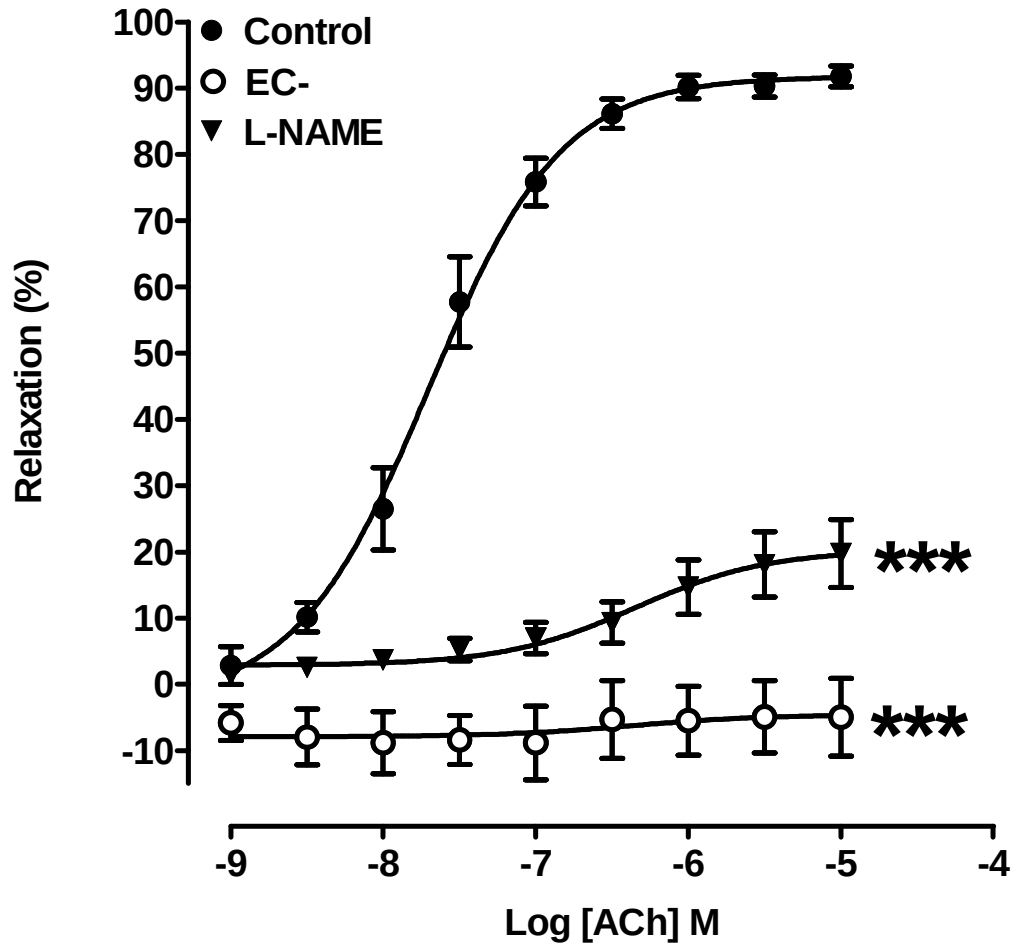

Figure 2.2. Cumulative concentration-effect curves showing relaxation to acetylcholine in female rat aortic rings submaximally contracted with PE. Responses are shown for tissues in the presence (control) or the absence (EC-) of endothelium, or tissues with endothelium treated with L-NAME (100 µM). Acetylcholine-induced relaxation was powerfully blocked by both endothelial denudation and L-NAME treatment. Each point represents the mean ± SEM of 6-7 observations. *** $P < 0.001$ indicates a significant difference from control.

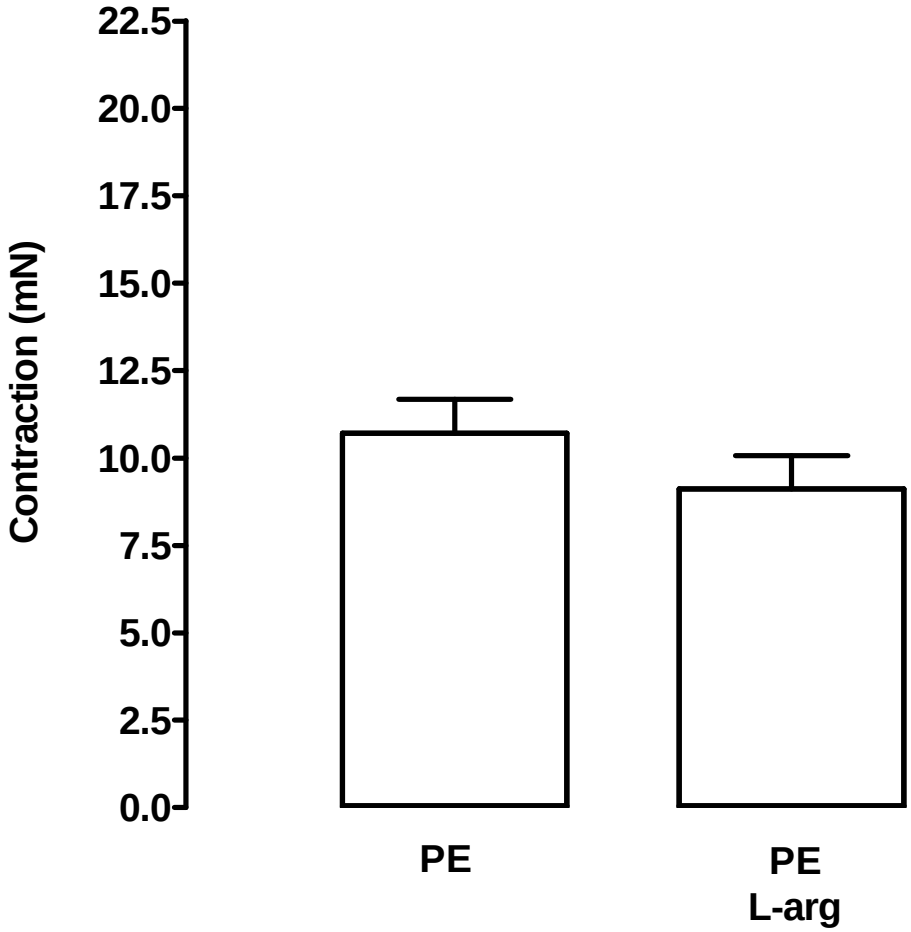

Figure 2.3. Histogram showing the contractile response to PE (50-70 nM) in female rat endothelium-containing aortic rings in the absence and presence of L-arginine (10 mM for 1 h). L-arginine did not significantly affect PE-induced contraction. Data represent the mean ± SEM of 7 observations.

Effects of L-arginine on acetylcholine-induced relaxation

Following treatment with L-arginine (10 mM, 1h) and contraction to ~ 50% of maximal phenylephrine tone, acetylcholine-induced relaxation (E_{max} 75.4 ± 4.1% and pEC_{50} of 7.59 ± 0.02) was similar to that of control tissues (E_{max} 82.7 ± 2.7% and pEC_{50} of 7.63 ± 0.03) (Figure 2.4). It is therefore unlikely that the availability of L-arginine is limiting for the production of nitric oxide stimulated by acetylcholine.

Effects of L-NAME on basal nitric oxide activity

Following induction of low tone (4.3 ± 0.6 mN) in female rat endothelium-containing aortic rings using phenylepherine (30-60 nM), subsequent addition of L-NAME (100 µM, 1h) significantly potentiated the tone, consistent with blockade of basal nitric oxide activity (Figure 2.5). L-arginine (at 10 mM, 1 h), given as a post-treatment or pre-treatment, significantly reversed and prevented, respectively, the potentiation of phenylephrine-induced tone by L-NAME.

Effects of N^G –monomethyl-L-arginine on basal nitric oxide activity

Following induction of low tone (4.0 ± 0.4 mN) in female rat endothelium-containing aortic rings using phenylephrine (30-60 nM), subsequent addition of N^G –monomethyl-L-arginine (L-NMMA, 100 µM for 1 h) significantly potentiated the tone, consistent with blockade of basal nitric oxide activity (Figure 2.6). L-arginine (at 10 mM, 1 h), given as a post-treatment or pre-treatment, significantly reversed and prevented, respectively, the potentiation of phenylephrine-induced tone by L-NMMA.

Effects of N^G, N^G –dimethyl-L-arginine and of N^G, N'^G–dimethyl-L-arginine on basal nitric oxide activity

Following induction of low tone (3.5 ± 0.5 mN) in female rat endothelium-containing aortic rings using phenylephrine (30-60 nM), subsequent addition of N^G , N^G –dimethyl-L-

arginine (ADMA, 100 µM for 1 h) significantly enhanced the tone (18.3 ± 2.2 mN, P< 0.001) consistent with blockade of basal nitric oxide activity (Figures 2.7 and 2.8). L-arginine (10 mM, 1 h), given as a post-treatment or pre-treatment, significantly reversed and prevented, respectively, the potentiation of phenylephrine-induced tone by ADMA. In contrast, D-arginine, the other optical isomer of L-arginine, neither reversed nor prevented the potentiation of phenylephrine-induced tone by ADMA.

In contrast to ADMA, N^G, $N^{,G}$ –dimethyl-L-arginine (SDMA, 1 mM for 1 h) did not affect phenylephrine-induced tone in rat endothelium-containing aortic rings (Figure 2.9).

Concentration-dependence and endothelium-dependence of the ability of ADMA to block basal nitric oxide activity

Following induction of low tone (3.1 ± 0.6 mN) in rat endothelium-containing aortic rings using phenylepherine (30-60 nM), subsequent addition of ADMA (0.3-300 µM; the response to each concentration was measured after 1 h), resulted in a concentration-dependent enhancement of the tone (Figure 2.10). The threshold concentration of ADMA enhancing tone was 0.3 µM, the pEC_{50} was 4.78 ± 0.06 and the maximum was obtained at 100 µM.

A concentration-effect curve to phenylepherine (1 nM-10 µM) produced a maximal contraction of 14.4 ± 1.5 mN and a pEC_{50} of 6.75 ± 0.07 in rat endothelium-containing aortic rings (Figure 2.11). Pretreatment with ADMA (100 µM, 1 h) significantly enhanced both of the tissue maximal response (E_{max} 22.2 ± 0.8 mN) and sensitivity (pEC_{50} 7.88 ± 0.04) to phenylepherine. Increasing the concentration of ADMA to 300 or 1000 µM produced a very little further enhancement.

In contrast to the effect on endothelium-containing rings, ADMA did not enhance phenylephrine-induced tone in the absence of the endothelium (Figure 2.12).

Effects of ADMA on the contractile responses to 5-hydroxytryptamine

A full concentration-effect curve to 5-hydroxytryptamine (1 nM-100 µM) in rat endothelium-containing aortic rings resulted in an E_{max} of 17.7 ± 2.1 mN and a pEC_{50} of 5.33 ± 0.13 (Figure 2.13). Pretreatment with ADMA (100 µM, 1 h) significantly enhanced both the tissue maximal response (E_{max} 24.3 ± 0.8 mN) and sensitivity (pEC_{50} 5.83 ± 0.07) to 5-hydroxytryptamine, consistent with blockade of basal nitric oxide activity.

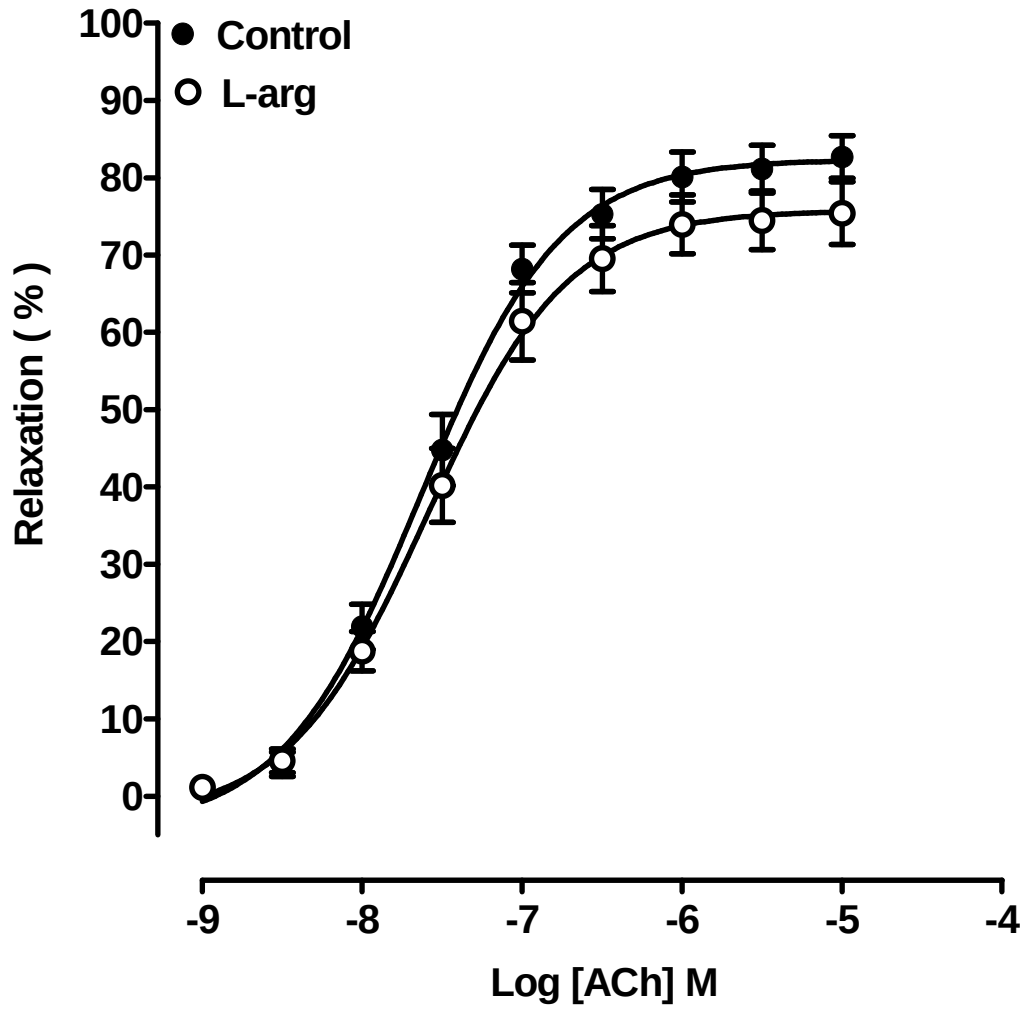

Figure 2.4. Cumulative concentration-effect curves showing relaxation to acetylcholine in female rat endothelium-containing aortic rings submaximally contracted with PE in the absence and presence of L-arginine (10 mM). L-arginine had no effect on acetylcholine–induced relaxation. Each point represents the mean ± SEM of 6 observations.

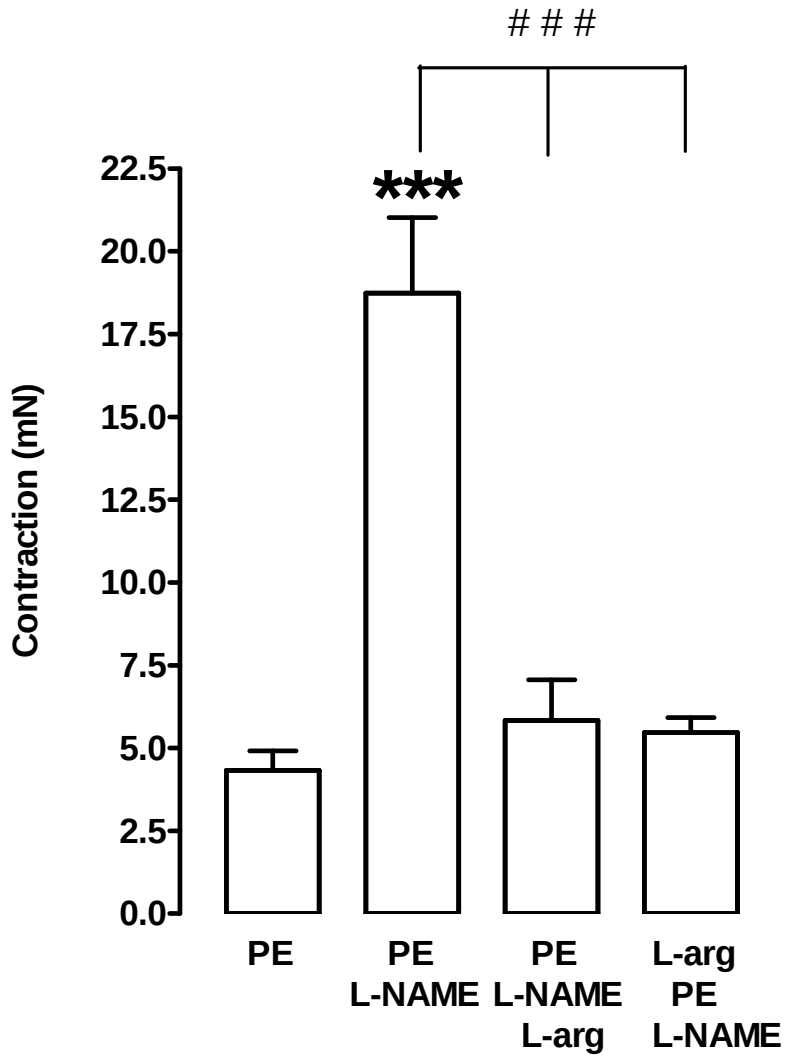

Fig.2.5. Histogram showing the contractile response to PE (30-60 nM) in female rat endothelium-containing aortic rings. Responses are shown for control PE-contracted tissues, PE-contracted tissues treated with L-NAME (100 µM for 1 h), PE-contracted tissues treated with L-NAME then with L-arginine (10 mM for 1 h), or tissues treated with L-arginine (10 mM for 1 h) before contraction with PE then treatment with L-NAME (100 µM for 1 h). L-arginine, the substrate for nitric oxide synthase, both reversed and prevented the potentiation of PE-induced tone by L-NAME. Data represent the mean ± SEM of 4-9 observations. *** $P < 0.001$ indicates a significant difference from PE; ### $P < 0.001$ indicates significant differences from tissues treated with L-NAME alone.

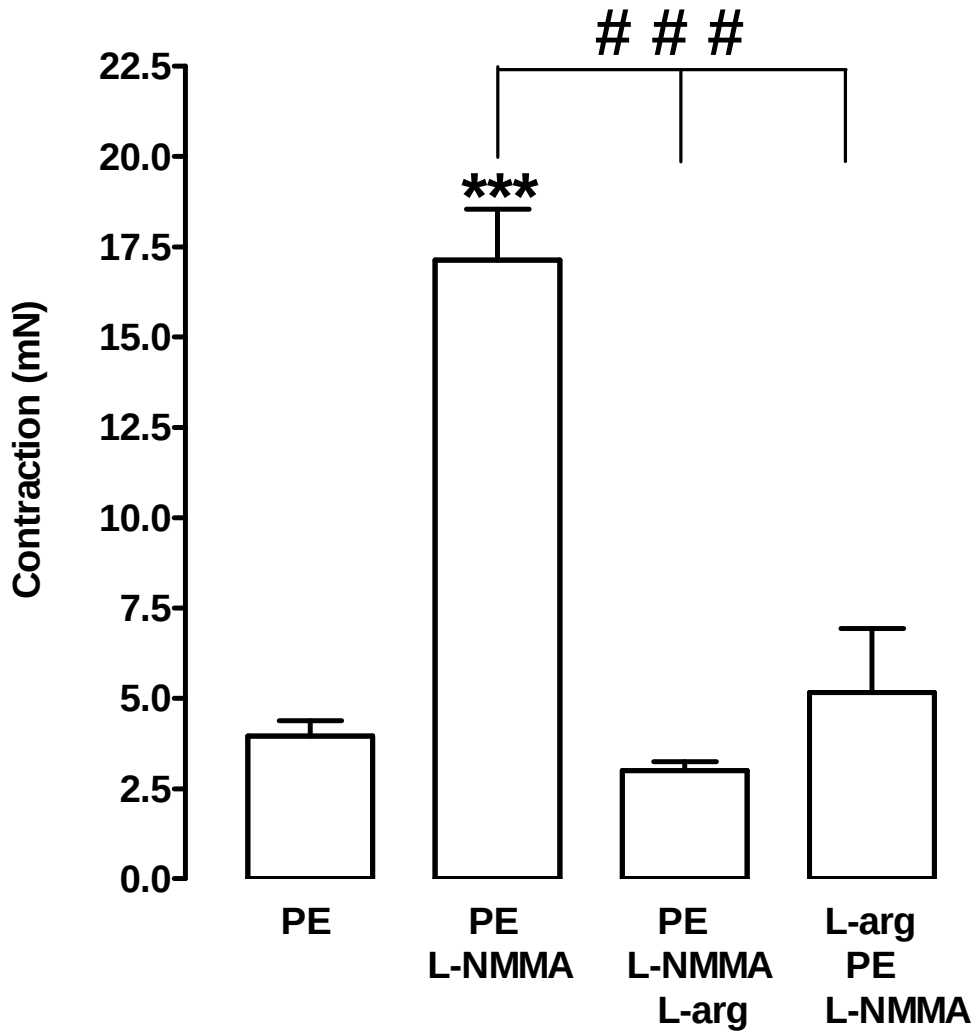

Figure 2.6. Histogram showing the contractile response to PE (30-60 nM) in female rat endothelium-containing aortic rings. Responses are shown for control PE-contracted tissues, PE-contracted tissues treated with L-NMMA (100 µM for 1 h), PE-contracted tissues treated with L-NMMA then with L-arginine (10 mM for 1 h), or tissues treated with L-arginine (10 mM for 1 h) before contraction with PE then treatment with L-NMMA (100 µM for 1 h). L-arginine, the substrate for nitric oxide synthase, both reversed and prevented the potentiation of PE-induced tone by L-NMMA. Data represent the mean ± SEM of 4-9 observations. *** $P < 0.001$ indicates a significant difference from PE; ### $P < 0.001$ indicates significant differences from tissues treated with L-NAME alone.

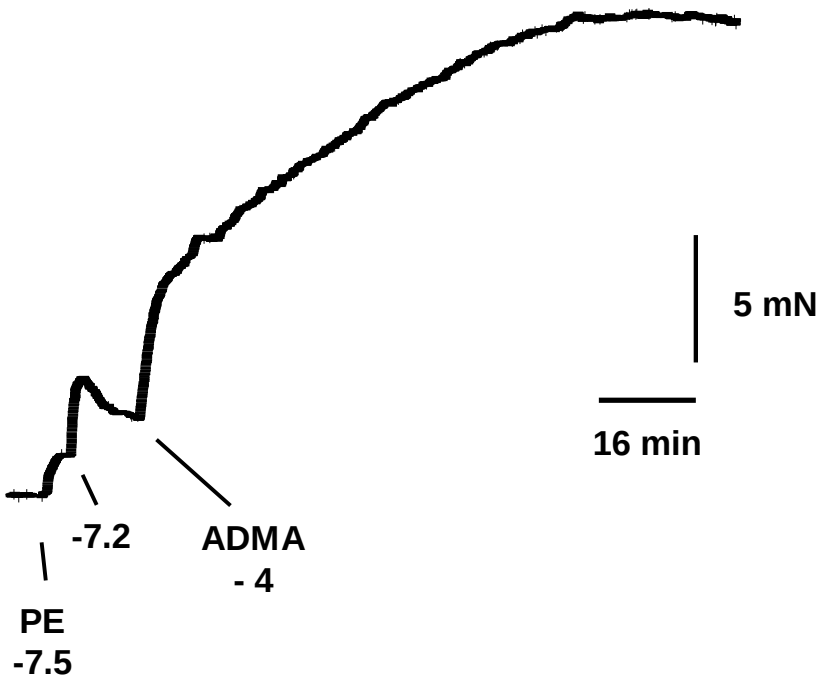

Figure 2.7. Trace showing a female rat endothelium-containing aortic ring contracted with PE to a low level of tone then treated with ADMA (for 1 h). ADMA powerfully potentiated PE-induced tone. Concentrations of PE and ADMA are given as log molar units.

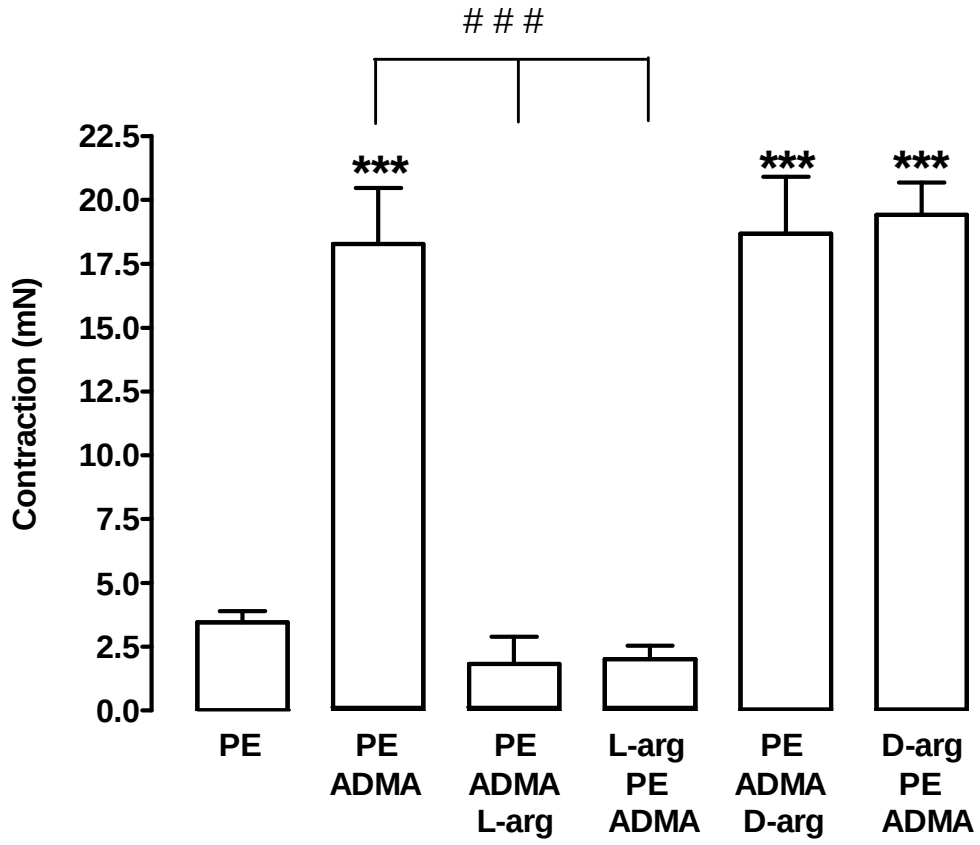

Figure 2.8. Histogram showing the contractile response to PE (30-60 nM) in female rat endothelium-containing aortic rings. Responses are shown for PE-contracted tissues, PE-contracted tissues treated with ADMA (100 µM for 1 h), PE-contracted tissues treated with ADMA then with L-arginine or D-arginine (both at 10 mM for 1 h), or PE-contracted tissues treated with L-arginine or D-arginine (both at 10 mM for 1 h) before contraction with PE then treatment with ADMA (100 µM for 1 h). L-arginine, the substrate for nitric oxide synthase, but not D-arginine, both reversed and prevented the potentiation of PE-induced tone by ADMA. Data represent the mean ± SEM of 6 observations. *** $P < 0.001$ indicates a significant difference from PE; ### $P < 0.001$ indicates significant differences from tissues treated with ADMA alone.

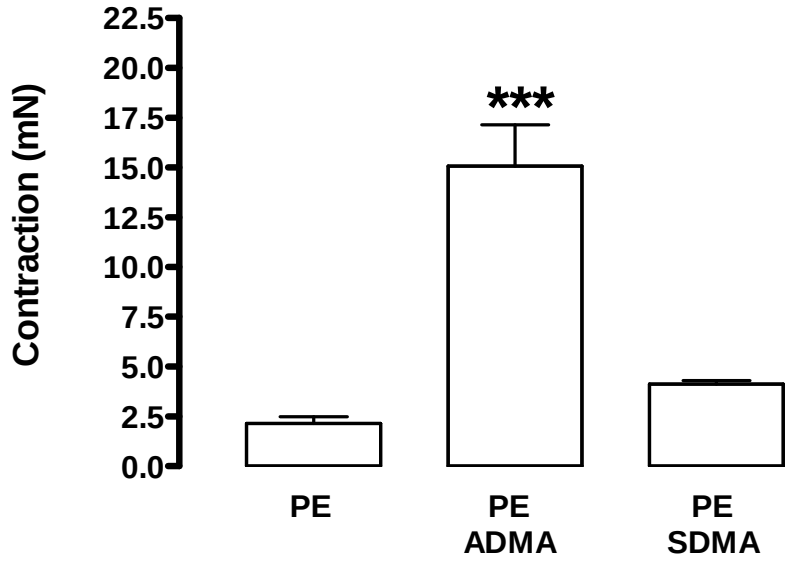

Figure 2.9. Histogram showing the contractile response to PE (30-60 nM) in female rat endothelium-containing aortic rings. Responses are shown for PE-contracted tissues or PE-contracted tissues treated with the L-arginine analogues, ADMA (100 µM for 1 h) or SDMA (1 mM for 1 h). PE-induced contraction was significantly enhanced by ADMA, but not by SDMA. Data represent the mean ± SEM of 6 observations. *** $P < 0.001$ indicates a significant difference from PE.

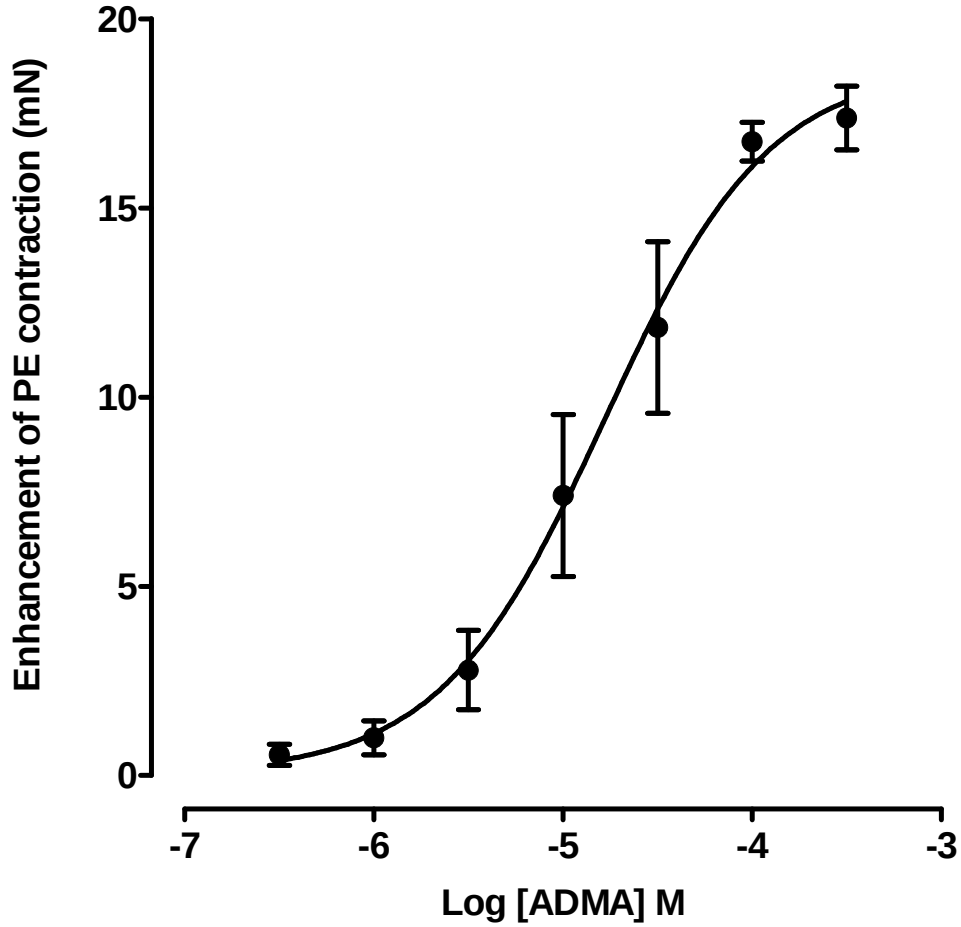

Figure 2.10. Concentration-effect curve showing the enhancement of low level PE (30-60 nM)-induced contraction by ADMA in female rat endothelium-containing aortic rings. The response to each concentration of ADMA was measured after 1 h. Each point represents the mean ± SEM of 4-5 observations.

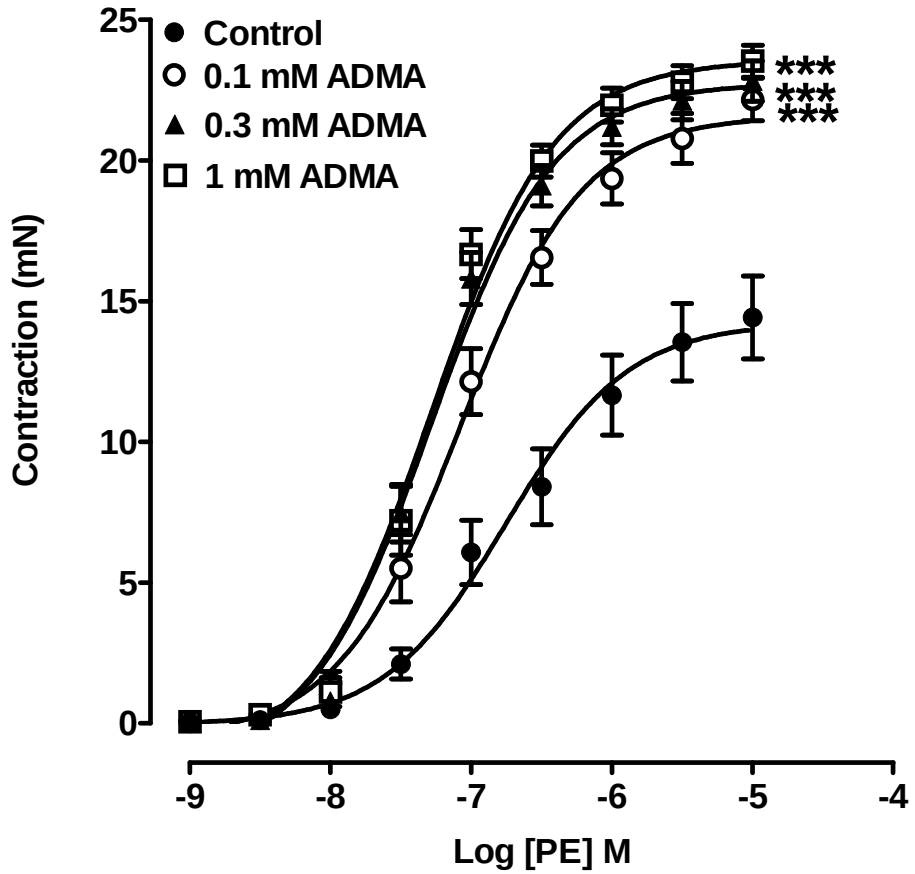

Figure 2.11. Cumulative concentration-effect curves showing contractile responses to PE in female rat endothelium-containing aortic rings in the absence and presence of ADMA (0.1 mM, 0.3 mM or 1 mM, all for 1 h). Contractions were almost maximally enhanced by 0.1 mM ADMA; higher concentrations produced little additional enhancement. Each point represents the mean ± SEM of 8-15 observations. *** $P < 0.001$ indicates a significant difference from control.

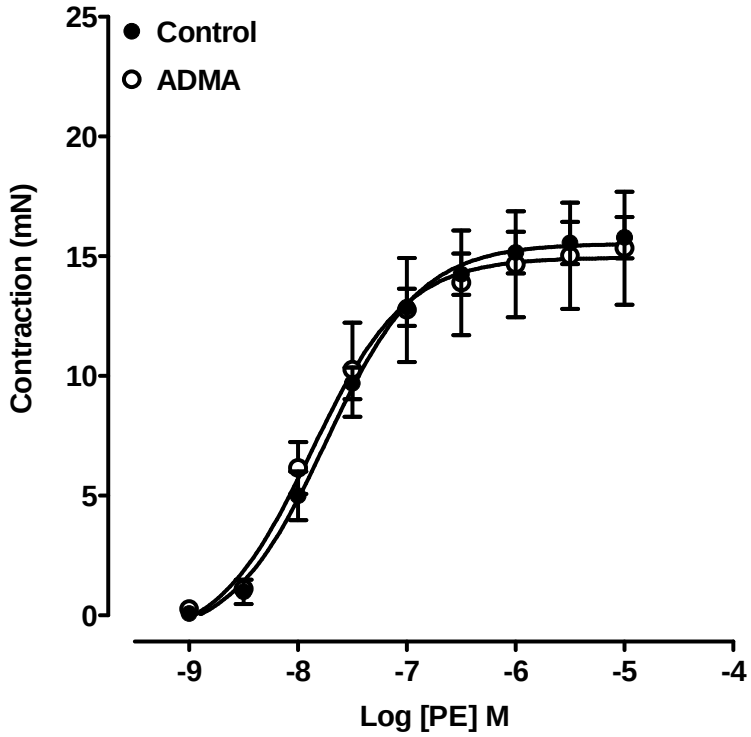

Figure 2.12. Cumulative concentration-effect curves show contractile responses to PE in female rat endothelium-denuded aortic rings in the absence and presence of ADMA (100 µM for 1 h). ADMA did not enhance PE-induced contractions in the absence of the endothelium. Each point represents the mean ± SEM of 6 observations.

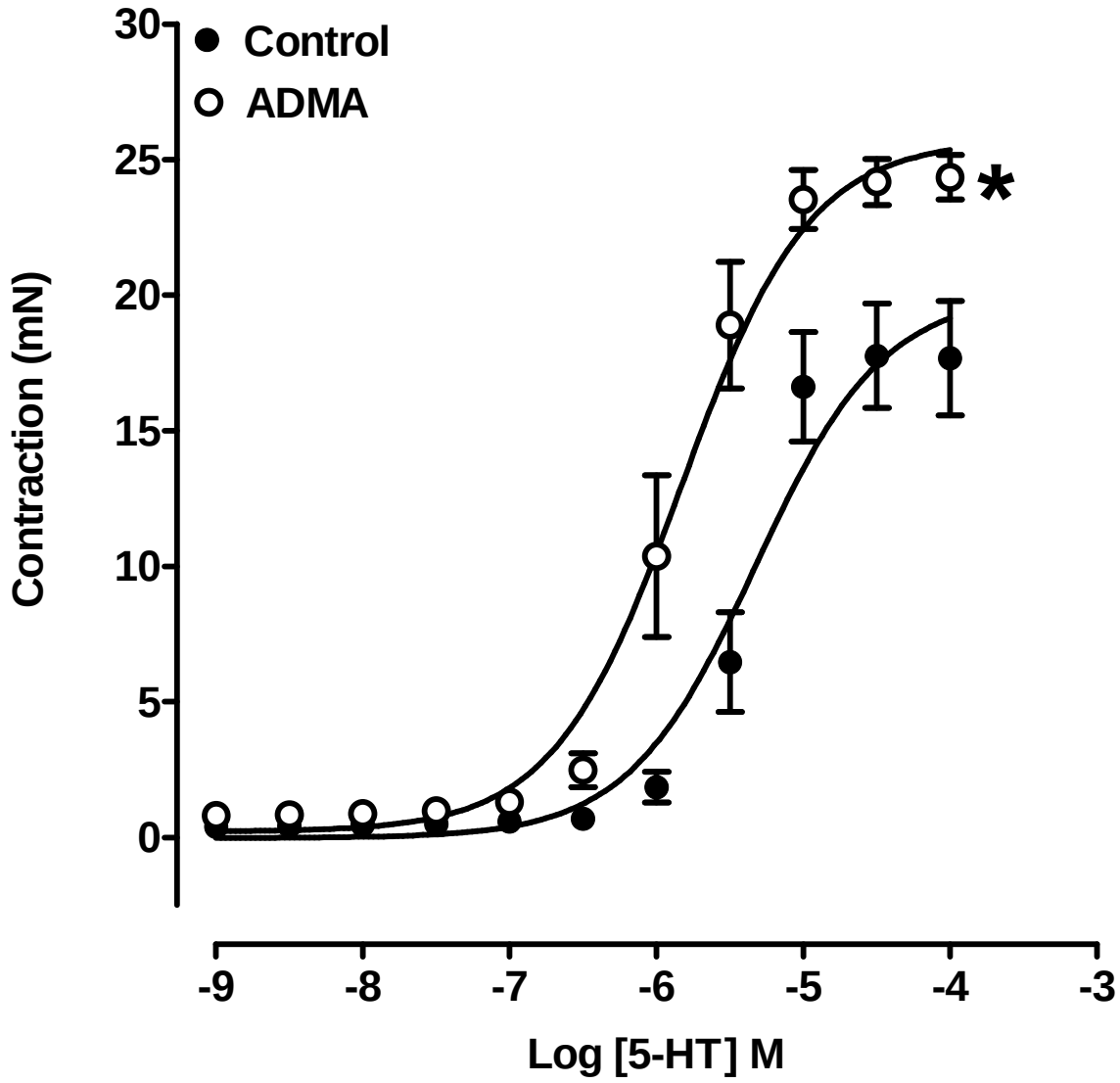

Figure 2.13. Cumulative concentration-effect curves showing contractile responses to 5-hydroxytryptamine (5-HT) in female rat endothelium-containing aortic rings in the absence and presence of ADMA (100 µM for 1 h). The sensitivity and maximal contraction to 5-HT were enhanced in the presence of ADMA. Each point represents the mean ± SEM of 6 observations. * $P < 0.05$ indicates a significant difference from control.

Effects of ADMA on the contractile responses to the prostaglandin $F_{2\alpha}$

A full concentration-effect curve to prostaglandin $F_{2\alpha}$ (1 nM-30 µM) in rat endothelium-containing aortic rings resulted in an E_{max} of 19.4 ± 0.7 mN and a pEC_{50} of 4.96 ± 0.19 (Figure 2.14). Pre-treatment with ADMA (100 µM, 1 h) significantly enhanced both the tissue maximal response (E_{max} 23.8 ± 0.8 mN) and sensitivity (pEC_{50} 5.67 ± 0.13) to prostaglandin $F_{2\alpha}$, consistent with blockade of basal nitric oxide activity.

Effects of L-NAME and L-NMMA on acetylcholine-induced relaxation

Following induction of ~ 50% of maximal phenylepherine-induced tone in rat endothelium-containing aortic rings in the presence of the NOS inhibitor, L-NAME (100 or 1000 µM for 30 min), acetylcholine (1 nM-10 µM)-induced relaxation was powerfully blocked when compared with control tissues set at the same level of tone (Figure 2.15; Table 2.1).

In contrast to L-NAME, and as previously shown by Frew *et al*. (1993), L-NMMA (100 or 1000 µM for 45 min) had little effect on acetylcholine-induced relaxation when experiments were conducted at ~ 50% of maximal phenylepherine-induced tone (Figure 1.15; Table 1.1). When tissues were tested after 2 h and 3 h, there was a small progressive reduction in tissue sensitivity to the relaxant action of acetylcholine both in control and L-NMMA (100 µM)-treated tissues (Figure 2.16). The maximal response of L-NMMA-treated tissues to acetylcholine was not significantly different from that seen in time-matched controls.

Effect of ADMA and SDMA on acetylcholine-induced relaxation

Like L-NMMA, but unlike L-NAME, ADMA (100 or 1000 µM for 1 h) produced a small but significant decrease in tissue sensitivity to acetylcholine without affecting the

E_{max} when experiments were conducted at ~ 50% of maximal phenylepherine-induced tone (Figure 2.17; Table 2.1). When tissues were tested after 3 and 4.5 h, there was a small progressive reduction in tissue sensitivity to the relaxant action of acetylcholine both in control and ADMA (1 mM)-treated tissues, but with a greater reduction in the latter (Figure 2.18). However, the maximal response of ADMA-treated tissues to acetylcholine was not significantly different from that seen in time-matched controls.

On the other hand, following induction of ~ 50% of maximal phenylepherine-induced tone in female rat endothelium-containing aortic rings pre-treated with the inactive analogue, SDMA (1 mM, 1h), acetylcholine (1 nM-10 µM)--induced relaxation was similar to that of control tissues (Figure 2.19; Table 2.1).

Effect of the level of tone on the apparent ability of ADMA to inhibit acetylcholine - induced relaxation

When rat endothelium-containing aortic rings were contracted with phenylepherine (100-300 nM) to an intermediate level of tone (11.9 ± 2.1 mN), acetylcholine (1 nM-10 µM) produced concentration-dependent relaxation with an E_{max} of 77.5 ± 7.8% and a pEC_{50} of 7.30 ± 0.03 (Figures 2.20A and 2.21). When the tissues were washed and ADMA (100 µM) added for 1 h, the same concentration of phenylepherine (100-300 nM) now produced a higher level of tone (18.8 ± 1.5 mN), consistent with blockade of basal activity of nitric oxide, and acetylcholine-induced relaxation appeared to be significantly blocked (E_{max} 22.1 ± 3.1%; pEC_{50} 6.91 ± 0.04). However, when these same tissues were washed and constricted with a lower concentration of phenylepherine (10-30 nM) in the presence of ADMA to give an intermediate level of tone similar to control vessels, the maximal response to acetylcholine was no longer blocked (E_{max} 77.6 ± 4.0%), but there was a decrease in tissue sensitivity to acetylcholine by ~ 1.7 fold (pEC_{50} 7.06 ± 0.04). It was

therefore possible that the apparently potent blockade of acetylcholine-induced relaxation seen with ADMA at a higher level of tone than control tissues might be due to physiological antagonism, rather than to blockade of NOS.

The effect of the level of tone on control acetylcholine-induced relaxation

Having established that the level of tone was critical in determining whether or not ADMA appeared to block acetylcholine-induced relaxation, it became important to determine how the level of tone affected the magnitude of acetylcholine-induced relaxation in control tissues.

In control, rat endothelium-containing aortic rings precontracted with phenylephrine to two different but submaximal levels of tone (3.7 ± 0.4 and 10.3 ± 0.5 mN), acetylcholine (1 nM-10 µM) produced similar relaxant responses (Figure 2.22a). However, relaxation was significantly depressed when the level of phenylepherine-induced tone was maximal for endothelium-containing tissues (14.1 ± 0.5 mN). When tissues constricted with phenylephrine to sub-maximal levels of tone of 4.1 ± 0.6 or 10.2 ± 0.5 mN in the presence of ADMA (100 µM), the magnitude of acetylcholine-induced relaxation was similar to that in control tissues precontracted with phenylephrine to comparable levels of tone (Figure 2.22b). When ADMA-treated tissues were constricted with phenylephrine to 15.7 ± 0.5 mN tone, acetylcholine produced the same depressed maximal relaxation to that in control tissues precontracted to the same level of phenylephrine tone (Figures 2.22a, b). At levels of phenylepherine tone of 20.3 ± 0.7 mN and 24.7 ± 0.6 mN, which cannot be induced in control tissues, acetylcholine-induced relaxation was further depressed.

It was therefore clear that the level of tone is critical in determining the magnitude of acetylcholine-induced relaxation in both control and ADMA-treated tissues, and that little

blockade by the latter was seen when the level of tone was matched with that of control tissues.

The effect of L-NMMA and ADMA on the blockade of acetylcholine-induced relaxation by L-NAME

Having established that L-NMMA and ADMA had little effect on acetylcholine–induced relaxation when tone was matched with that of control tissues, it was important to determine if they could interfere with the blocking action of L-NAME.

In the presence of L-NAME (30 µM, 1h) and following induction of ~50% of maximal phenylephrine-induced tone in rat endothelium-containing aortic rings, acetylcholine (1 nM-10 µM)-induced relaxation was reduced to a maximum of ~ 40% (Figure 2.23). This blockade by L-NAME was significantly prevented as well as reversed following pre-treatment or post-treatment, respectively, with the endogenous substrate of NOS, L-arginine (1mM, Figure 2.23a). L-NMMA (1 mM) alone decreased tissue sensitivity to the relaxant action of acetylcholine by ~2.6-fold without reducing the maximal response to acetylcholine (Figure 2.23b). Moreover, like with L-arginine, treatment with L-NMMA (1 mM) both prevented and reversed the blockade of acetylcholine-induced relaxation by L-NAME. ADMA (1 mM) too on its own produced a 3-fold reduction in sensitivity to acetylcholine without reducing its maximal relaxation (Figure 2.23c). It too largely prevented the blockade of acetylcholine-induced relaxation by L-NAME, but did not reverse it (Figure 2.23c). SDMA (1 mM) alone had no effect on acetylcholine-induced relaxation and it had no effect on blockade of acetylcholine-induced relaxation by L-NAME (Figure 2.23d).

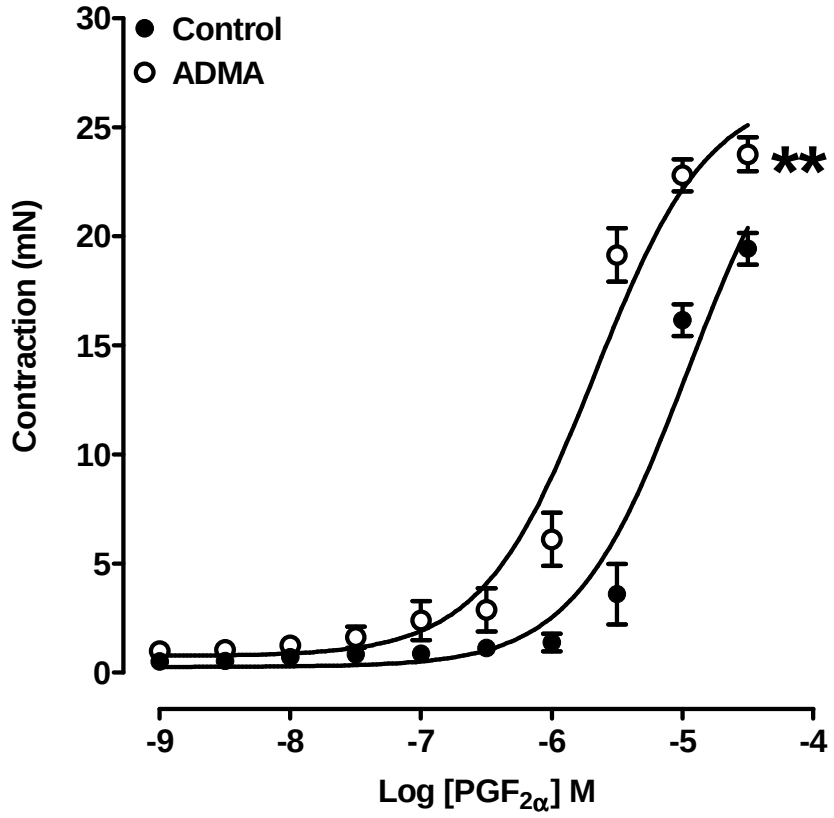

Figure 2.14. Cumulative concentration-effect curves show contractile responses to prostaglandin $F_{2\alpha}$ in female rat endothelium-containing aortic rings in the absence and presence of ADMA (100 µM for 1 h). Contractions were significantly enhanced in the presence of ADMA. Each point represents the mean ± SEM of 6 observations. ** P< 0.01 indicates a significant difference from control.

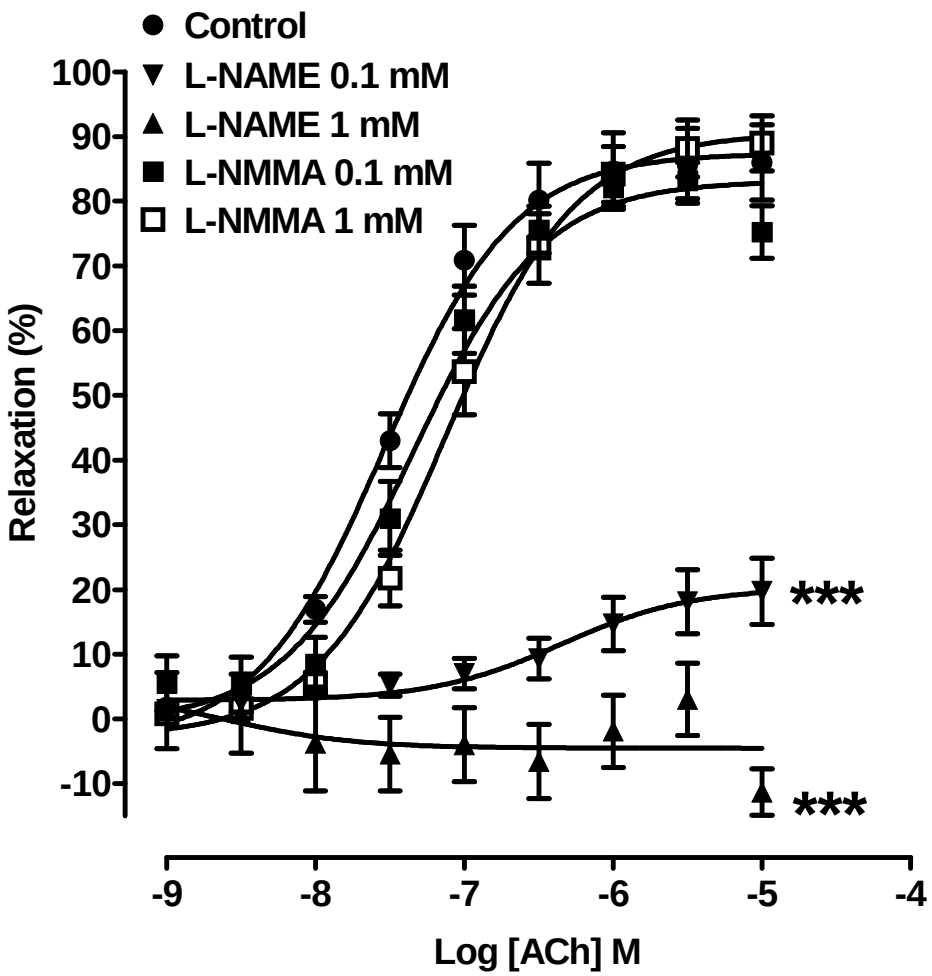

Figure 2.15. Cumulative concentration-effect curves showing relaxation to acetylcholine in female rat endothelium-containing aortic rings submaximally contracted with PE in the absence and presence of L-NAME or L-NMMA (both at 0.1 and 1mM). Maximal relaxation was powerfully blocked by L-NAME at both concentrations, but not affected by either concentration of L-NMMA. Each point represents the mean ± SEM of 6-9 observations. *** $P < 0.01$ indicates a significant difference from control.

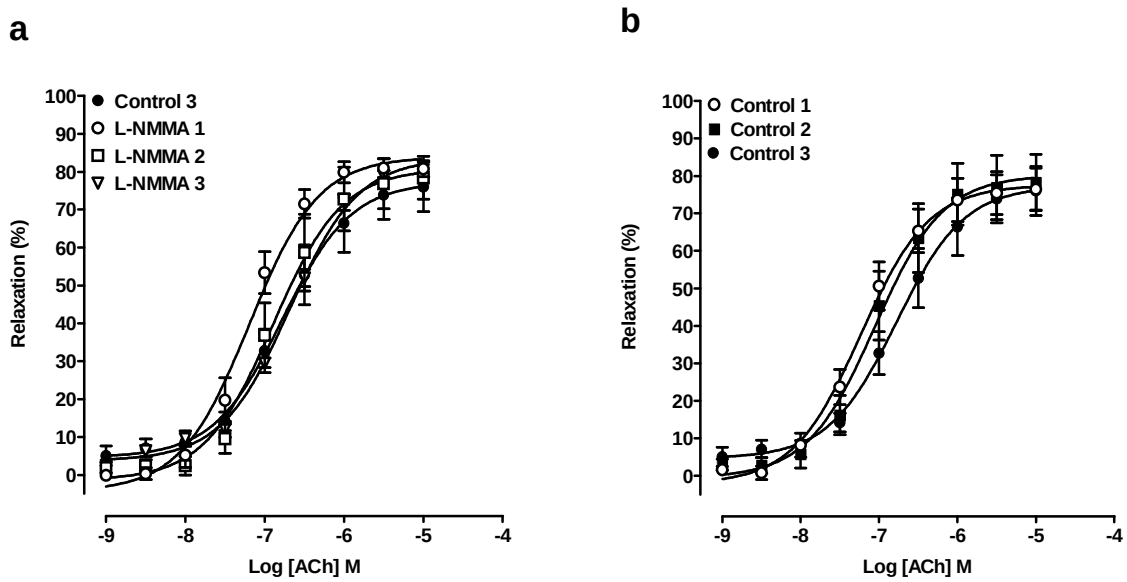

Figure 2.16. Concentration–effect curves showing relaxation to acetylcholine in female rat endothelium-containing aortic rings submaximally contracted with PE. Responses are shown for the same tissues treated with L-NMMA at 0.1 mM (a) for 45 minutes (L-NMMA 1), 2 h (L-NMMA 2) or 3 h (L-NMMA 3), and for their time-matched controls (b); control 1, control 2 and control 3, respectively. There is no significant time-dependent inhibition of maximal acetylcholine-induced relaxation by L-NMMA. Each point is the mean ± SEM of 6 observations.

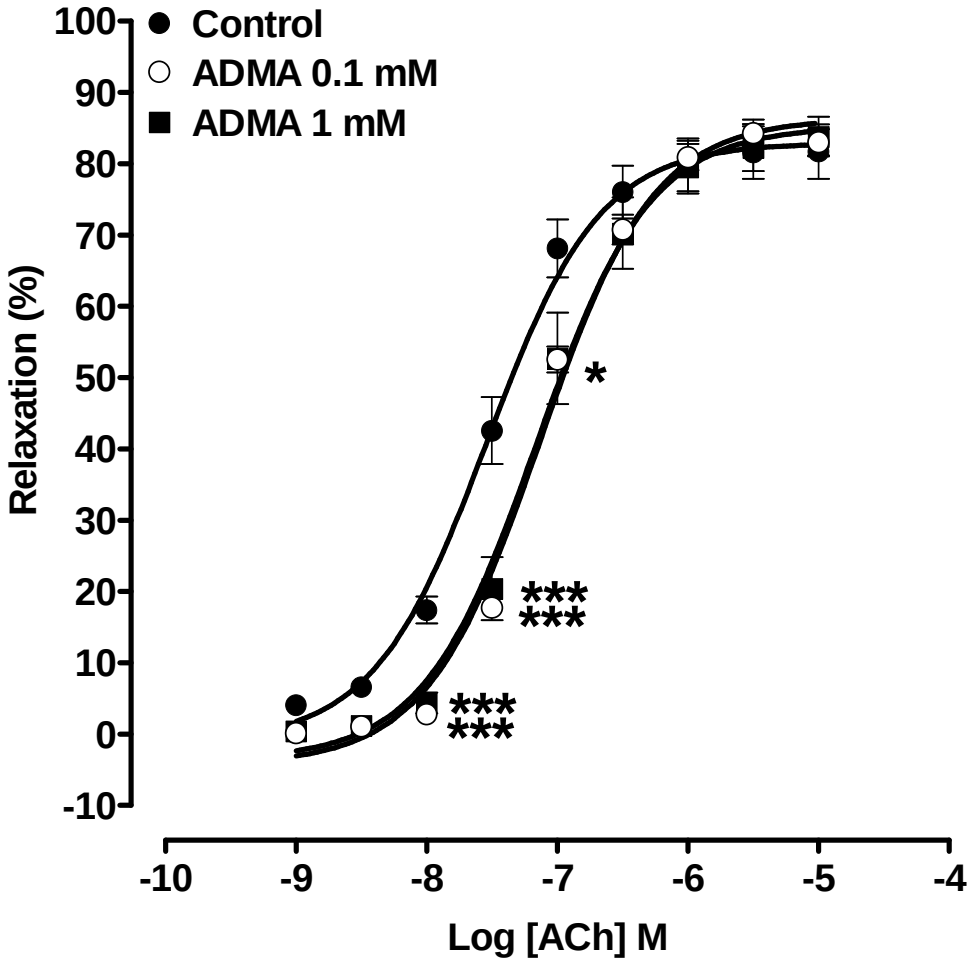

Figure 2.17. Cumulative concentration-effect curves showing relaxation to acetylcholine in female rat endothelium-containing aortic rings submaximally contracted with PE in the absence and presence of ADMA (0.1 and 1 mM, 1h). ADMA produced a small but significant decrease in tissue sensitivity to acetylcholine without affecting the maximal relaxation. Each point represents the mean ± SEM of 5-9 observations. * $P < 0.05$ and *** $P < 0.001$ indicate significant differences from control.

Table 2.1 The effects of different NOS inhibitors on acetylcholine-induced relaxation in rat endothelium-containing aortic rings submaximally contracted with PE, together with their time-matched controls.

Treatment	**Concentration**	**pEC_{50}**	**E_{max} ± SEM (%)**
Control	----------	7.54 ± 0.05	81.7 ± 3.8
ADMA	0.1 mM (1 h)	7.13 ± 0.07 **	84.3 ± 1.9
ADMA	1 mM (1 h)	7.16 ± 0.06 **	83.8 ± 2.8
Control	----------	7.54 ± 0.05	86.0 ± 5.8
L-NMMA	0.1 mM (30 min)	7.34 ± 0.11	83.2 ± 2.8
L-NMMA	1 mM (30 min)	7.12 ± 0.04 *	89.0 ± 4.3
L-NAME	0.1 mM (30 min)	6.35 ± 0.10 ***	19.8 ± 5.1 ***
L-NAME	1 mM (30 min)	ND	3.0 ± 5.6 ***
Control	----------	7.51 ± 0.03	93.3 ± 2.8
SDMA	1 mM (1 h)	7.52 ± 0.04	91.2 ± 4.4

Data are the mean ± SEM of 5-9 observations. * P< 0.05, ** P< 0.01 and *** P< 0.001 indicate significant differences from relevant control.

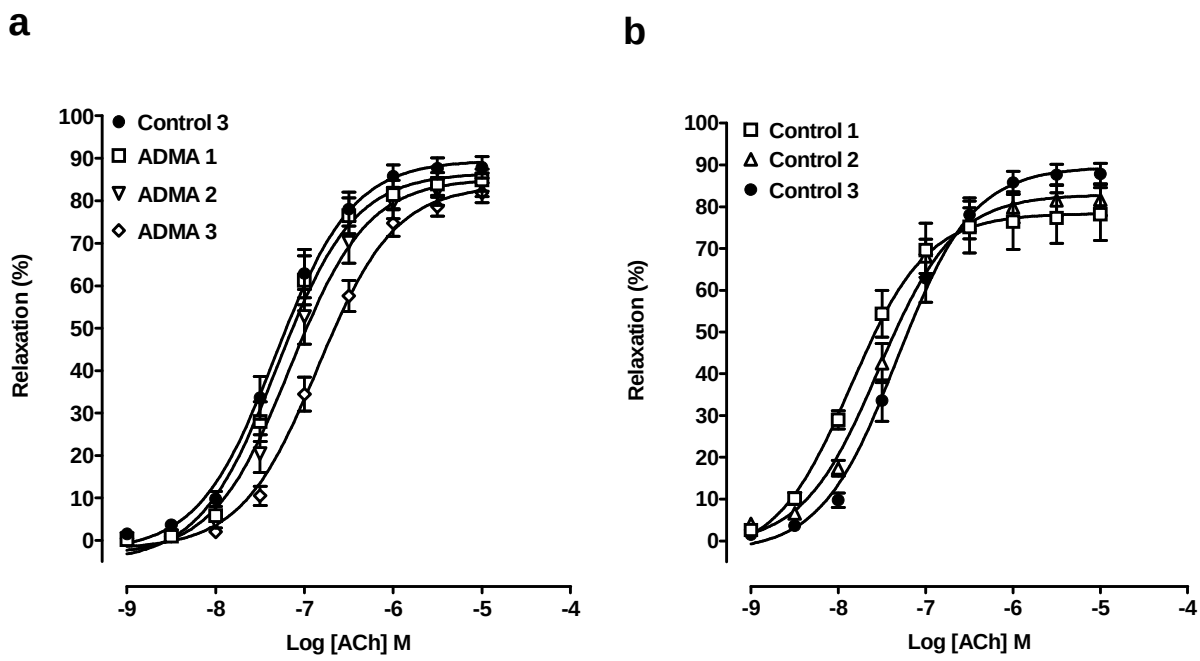

Figure 2.18. Concentration–effect curves showing relaxation to acetylcholine in female rat endothelium-containing aortic rings submaximally contracted with PE. Responses are shown for the same tissues treated with ADMA at 1 mM (a) for 1 h (ADMA 1), 3 h (ADMA 2) or 4.5 h (ADMA 3), and for their time-matched controls (b); control 1, control 2 and control 3, respectively. There was no significant time-dependent inhibition of maximal acetylcholine-induced relaxation by ADMA. Each point is the mean ± SEM of 5-6 observations.

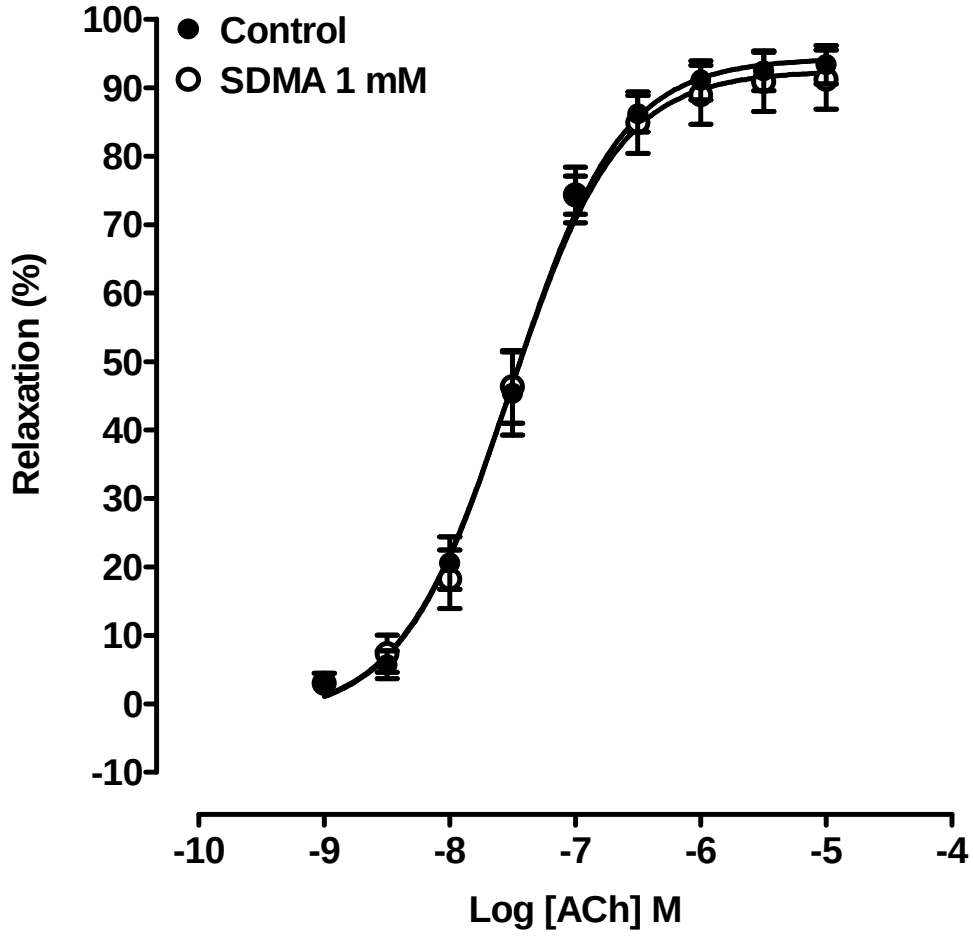

Figure 2.19. Cumulative concentration-effect curves showing relaxation to acetylcholine in female rat endothelium-containing aortic rings submaximally contracted with PE in the absence and presence of SDMA (1 mM, 1h). SDMA had no effect on acetylcholine-induced relaxation. Each point represents the mean ± SEM of 6 observations.

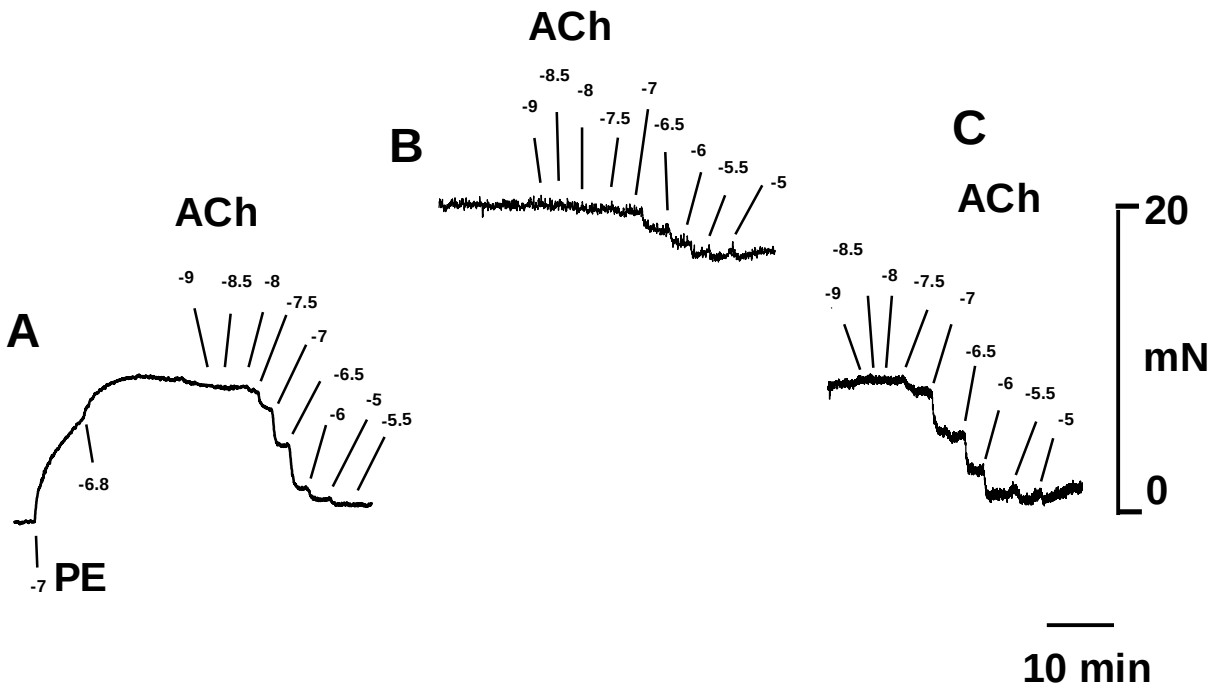

Fig. 2.20. Traces show acetylcholine-induced relaxation in a control (trace A) endothelium-containing rat aortic ring submaximally contracted with PE (160 nM). When the same tissue was washed and treated with ADMA (100 µM for 1 h) and contracted with the same concentration of PE, the level of tone was higher (trace B) and acetylcholine-induced relaxation seems to be blocked. However, when the same tissue was washed again, treated with ADMA and contracted to the same level as the control by using a lower concentration (25 nM) of PE (trace C), the blockade of acetylcholine-induced relaxation was almost entirely abolished. Concentrations of PE and ACh are given in log molar units.

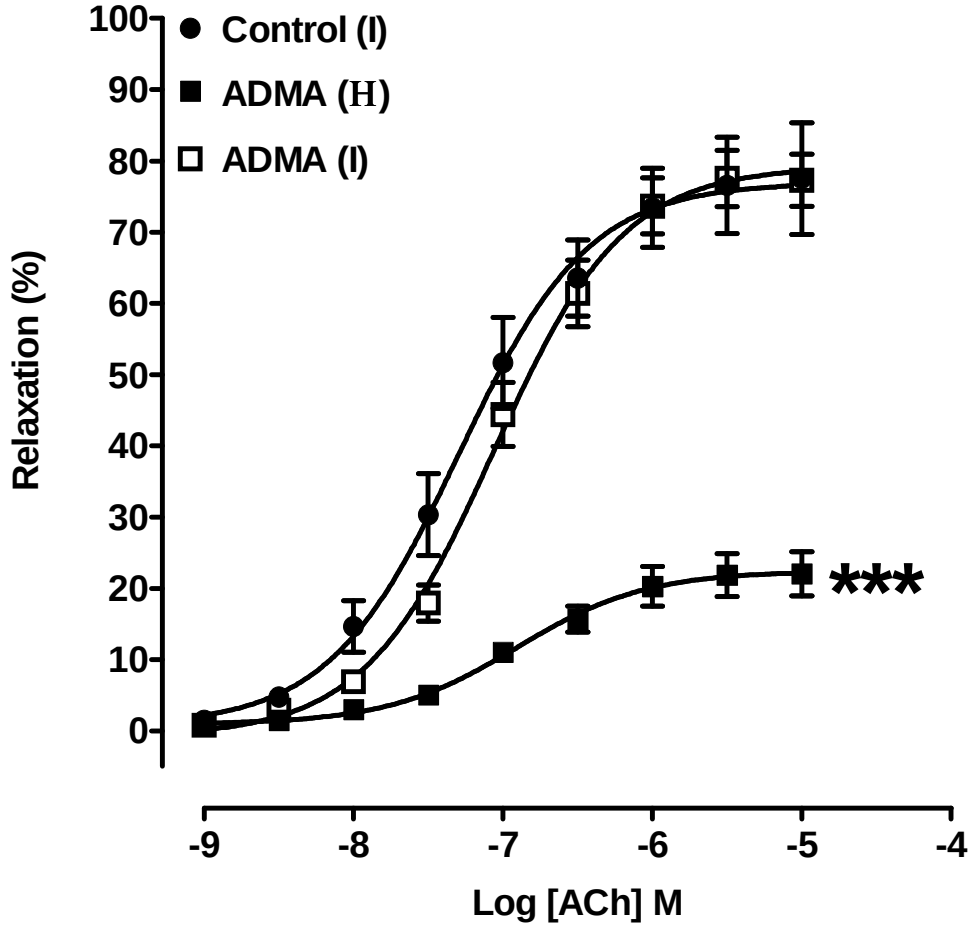

Figure 2.21. Cumulative concentration-effect curves showing acetylcholine-induced relaxation in female rat endothelium-containing aortic rings contracted to an intermediate (I) level of tone with PE (100-300 nM). When ADMA (100 µM, 1 h) enhanced PE (100-300)-induced tone to a high (H) level, acetylcholine-induced relaxation appeared powerfully inhibited. However, when these same tissues were washed, with intermediate PE (10–30 nM)-induced tone (I) re-established in the presence of ADMA, the blockade of acetylcholine-induced relaxation was almost entirely abolished. Each point is the mean ± SEM of six observations. *** P< 0.001 indicates a significant difference from control.

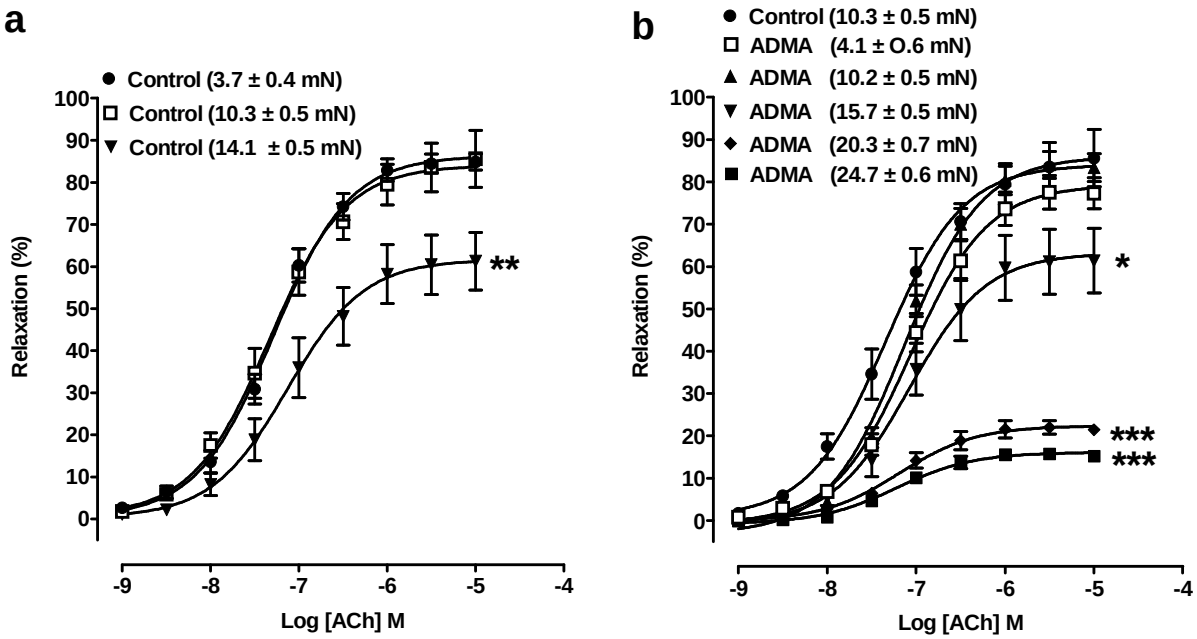

Figure 2.22. Cumulative concentration-effect curves showing relaxation to acetylcholine in female rat endothelium-containing aortic rings precontracted with PE to different levels of tone (shown in mN). Responses are shown for (a) control tissues and (b) ADMA-treated tissues (0.1 mM for 1 h). Acetylcholine-induced relaxation was significantly inhibited both in control and ADMA-treated tissues precontracted to high but not low or intermediate levels of PE-induced tone. Each point represents the mean ± SEM of 6-9 observations. * P< 0.05, ** P< 0.01 and *** P< 0.001 indicate significant differences from control.

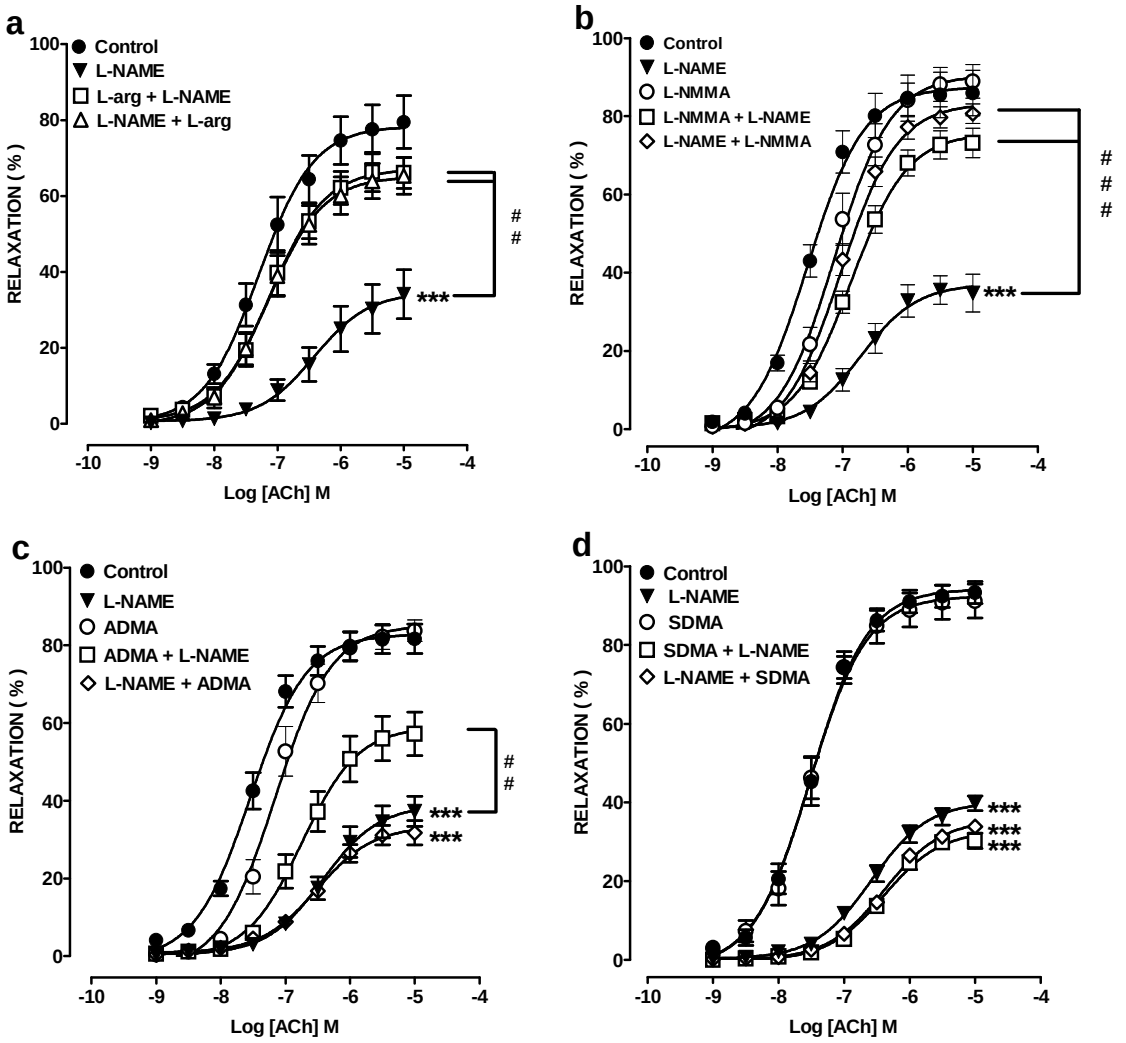

Figure 2.23. Concentration–response curves showing acetylcholine-induced relaxation in female rat endothelium-containing aortic rings submaximally contracted with PE and blockade of this relaxation with L-NAME (30 µM, 1 h). The ability of (a) L-arginine, (b) L-NMMA, (c) ADMA and (d) SDMA (all at 1 mM) to either protect against or reverse blockade by L-NAME is also shown. Note that the order of listing of drugs reflects their order of addition to tissues. Each point is the mean ± SEM of 6-9 observations. *** P< 0.001 indicates significant blockade by L-NAME; ## P< 0.01 and ### P< 0.001 indicate significant protection against or reversal of blockade induced by L-NAME.

Discussion

Basal nitric oxide activity in female rat aorta is significantly blocked by L-NAME, L-NMMA and ADMA, but not affected by SDMA or L-arginine

It has been reported that the vascular endothelium generates nitric oxide in the absence of external stimuli, i.e. under basal conditions, and that this basal activity of nitric oxide exerts a tonic vasodilator effect that suppresses the actions of vasoconstrictor drugs (Martin *et al*., 1986b; Rees *et al*., 1989; Moore *et al*., 1990; Mian & Martin, 1995). These findings are supported by the observation that removal of the endothelium from rat aortic rings results in an augmentation of contraction induced by vasoconstricting agents in comparison to those induced in control rings (Allan *et al*., 1983; Eglème *et al*., 1984). As a consequence of these findings, it is generally accepted that agents that inhibit nitric oxide synthesis or block the actions of nitric oxide are expected to produce an enhancement of vasoconstrictor-induced tone by removing this endothelium-dependent suppression of vasoconstriction.

In agreement with the concept that basal activity of nitric oxide exerts a tonic vasodilator influence on blood vessels, the results of this study showed that endothelial denudation or treatment of endothelium-containing female rat aortic rings with the nitric oxide synthase inhibitors, L-NAME, L-NMMA or ADMA, resulted in augmentation of low level phenylephrine-induced tone. This augmentation of tone was both prevented and reversed by adding a high concentration of the endogenous substrate for nitric oxide synthase, L-arginine, but D-arginine was ineffective. This suggests that L-arginine produced its effects in a stereospecific way (Eddahibi *et al*., 1992) and that the inhibition of basal nitric oxide activity by L-NAME, L-NMMA or ADMA takes place through classical surmountable

antagonism (Palmer *et al*., 1988; Rees *et al*., 1989; Aisaka *et al*., 1989; Rees *et al.*, 1990; Moore *et al*., 1990; Hobbs *et al*., 1999; Masuda *et al*., 2002).

An additional important observation in this study is that L-arginine itself had no effect on phenylephrine-induced tone in endothelium-containing rings of rat aorta. This suggests that the availability of L-arginine is not limiting for the production of the basal nitric oxide activity that suppresses vasoconstrictor tone. This finding is in agreement with previous reports (Cooke *et al*., 1991; Moritoki *et al*., 1991) which showed that pre-treatment with L-arginine did not affect the contractile responses to norepinephrine or phenylephrine in rabbit and rat aortic rings, respectively. Moreover, this finding is consistent with that reported by Jun and Wennmalm (1994) that intravenous infusion of L-arginine did not change mean arterial blood pressure in normotensive rats. Thus, the availability of L-arginine does not appear to be limiting for basal production of nitric oxide either *in vitro* or *in vivo*.

A likely explanation for the inability of exogenous L-arginine to promote further basal nitric oxide synthesis may be found when we compare the endogenous level of L-arginine (100-300 µM) reportedly found in endothelial cells with estimates of its K_m for nitric oxide synthase (2-3 µM). Thus, it would appear that endogenous levels are such saturated that the enzyme would normally be fully saturated (Sydow and Münzel, 2003; Teerlink *et al.*, 2009; Zhang *et al*., 2011). Moreover, within the context of the control experiments conducted in this study, there is no inhibitor of nitric oxide synthase such as ADMA presents, which would compete with L-arginine and thus necessitate additional substrate to overcome this surmountable inhibition. *In vivo* in humans, however, where evidence for the presence of ADMA has been presented (Böger, 2004; Bode-Böger *et al*., 2007;

Teerlink *et al*., 2009), the addition of L-arginine was, in contrast, seen to increase nitric oxide activity. This ability of exogenously administered L-arginine to enhance nitric oxide activity *in vivo* has been termed the "arginine paradox" and is believed to result from reversal of blockade by endogenous ADMA, rather than from an abnormally low endogenous level of L-arginine.

In addition, the results of the present study showed that SDMA did not affect phenylephrine-induced tone in rat endothelium-containing aortic rings, and so had no effect on basal nitric oxide activity, which is in agreement with previous data indicating that SDMA is not an inhibitor of nitric oxide synthase (Masuda *et al*., 2002; Siroen *et al*., 2006).

The blockade of basal nitric oxide activity by ADMA is both endothelium- and concentration-dependent

The results of this study showed that the threshold concentration of ADMA to enhance submaximal phenylephrine-induced contraction through blockade of basal nitric oxide activity in rat aorta, was 0.3-1 µM, which is consistent with the concentrations of ADMA reported in the plasma of patient with pathological conditions associated with renal dysfunction or vascular endothelial dysfunction (Vallance *et al*., 1992; Siroen *et al*., 2006). Moreover, this effect of ADMA seemed to be concentration dependent with an IC_{50} of 17 µM and treatment with 100 µM of ADMA was almost enough to abolish basal activity of nitric oxide, as increasing the concentration to 1000 µM produced very little further effect.

It is worth noting that ADMA augmented submaximal contractions induced not just by phenylephrine, but by a range of different vasoconstrictors, such as 5-hydroxytryptamine

and prostaglandin $F_{2\alpha}$, in female rat endothelium-containing aortic rings. Thus, basal nitric oxide activity, suppresses tone induced by a range of vasoconstrictors in rat aorta.

In endothelium-denuded rat aortic rings, ADMA, like the other nitric oxide synthase inhibitors, L-NMMA and L-NAME, failed to augment phenylephrine-induced contractions. These findings were as expected, because endothelium-denuded vessels are unable to produce basal nitric oxide activity.

Acetylcholine-induced relaxation in female rat aorta is endothelium-dependent and nitric oxide-mediated

Data from this study showed that the concentration-dependent relaxation induced by acetylcholine in rat aorta was abolished by endothelial denudation or treatment with the standard nitric oxide synthase inhibitor, L-NAME. These findings are consistent with previous reports (e.g. Furchgott & Zawadzki, 1980) that the lining endothelium of blood vessels plays a vital role in facilitating vasodilator responses to relaxant agents such as acetylcholine, substance P and the calcium ionophore A23187, due to the production of an endothelium-derived relaxing factor (EDRF), later shown to be nitric oxide (Palmer *et al*., 1987).

Acetylcholine-induced relaxation in female rat aorta is not affected by L-NMMA, ADMA or SDMA

Probably the most important finding of this study is that despite producing potent blockade of basal nitric oxide activity in rat aorta, ADMA at 100 µM produced only a very modest effect on acetylcholine-induced relaxation; there was a slight (2-3-fold) decrease in tissue sensitivity without affecting the maximal response to acetylcholine. Even increasing

the concentration of ADMA to 1000 μM and prolonging the duration of treatment to 3 h failed to produce further blockade. Moreover, the ineffectiveness of ADMA against acetylcholine-induced relaxation was seen in rat aorta regardless of the agent used to induce contraction (phenylephrine, 5-hydroxytryptamine or $PGF_{2\alpha}$). Most experiments were conducted using submaximal phenylephrine-induced tone matched to that of control tissues, but very little blockade was seen.

These effects of ADMA in blocking basal but not acetylcholine-induced activity of nitric oxide are similar to those of L-NMMA reported by Frew and co-workers (1993) in the same tissue.

The level of tone has an influence on acetylcholine-induced relaxation in control and L-NMMA- or ADMA-treated female rat aorta rings

The results from this study showed that when phenylephrine-induced tone in the presence of ADMA or L-NMMA was held at intermediate levels, virtually no blockade of acetylcholine-induced relaxation was seen; there was a modest reduction in tissue sensitivity without any effect on the maximal relaxation. However, under conditions where ADMA had potentiated phenylephrine-induced tone to near maximal levels, it appeared to produce blockade of acetylcholine-induced relaxation. This latter effect of ADMA was almost certainly due to physiological antagonism rather than blockade of nitric oxide synthase, since matching this increased tone in control tissues with additional phenylephrine produced a similar degree of apparent blockade.

The findings of this study are clearly in conflict with previous studies on rat aorta which reported blockade of acetylcholine-induced relaxation by ADMA (Vallance *et al*., 1992; Jin and D'Alecy, 1996; Feng *et al*., 1998). Since these authors made no mention of

matching the tone in control and ADMA-treated tissues, it is likely that the “blockade of acetylcholine-induced relaxation” they reported resulted from physiological antagonism, due to over-contraction, rather than to blockade of nitric oxide synthase.

Furthermore, the results of this study showed that SDMA, the endogenously produced inert analogue of ADMA, had no effect on tissue sensitivity, or the maximal response, to acetylcholine, consistent with previous reports that SDMA has no effect on nitric oxide activity (Masuda *et al*., 2002; Siroen *et al*., 2006).

Although all of the results presented in this study relate to the anomalous abilities of L-NMMA and ADMA to block basal but not agonist–stimulated activity of nitric oxide in female rat aorta, such actions seem not to be gender- or site-specific. Evidence for this has separately been reported in male rat aorta and in female rat carotid artery (AL-Zobaidy *et al*., 2011), where L-NMMA and ADMA each enhanced sub-maximal phenylephrine-induced tone, consistent with blockade of basal nitric oxide activity, but they had almost no effect on acetylcholine-induced relaxation, whereas L-NAME uniformly inhibited both basal and agonist-stimulated activity .

L-arginine availability for acetylcholine-stimulated nitric oxide activity

The results of this study showed that endothelium-dependent, nitric oxide-mediated relaxation to acetylcholine in female rat aortic rings, precontracted with phenylephrine, is not affected by treatment with L-arginine. This suggests that availability of the substrate for nitric oxide synthase is not limiting for the production of nitric oxide in response to acetylcholine in this tissue. These findings are in agreement with those reported by Moritoki and co-workers (1991) who found that endothelium-dependent acetylcholine-induced relaxation in male rat aortic rings was not affected by pre-treatment with L-

arginine although the substrate on its own induced a slowly developing, concentration-dependent relaxation in both endothelium-containing and denuded rings. The authors attributed these relaxations to an endothelium-independent, but nitric oxide-mediated mechanism, as relaxation was blocked by the nitric oxide synthase inhibitors L-NMMA and L-NOARG. Moreover, Cooke and co-workers (1991) found that endothelium-dependent, acetylcholine-induced relaxation in isolated aortic rings obtained from normal rabbits infused *in vivo* with L-arginine, was similar to that of untreated rabbits. These authors attributed their findings to the existence of a high intracellular concentration of L-arginine which saturated endothelial nitric oxide synthase.

In contrast to the situation in normal animals, L-arginine supplementation does appear to improve endothelial function in animals with dysfunctional vascular endothelium resulting from hypercholesterolaemia (Böger *et al*., 1995; Candipan *et al*., 1996; Böger and Bode-Böger, 2001). Similar findings were reported in humans with pathological conditions associated with vascular dysfunction (Rector *et al*., 1996; Blum *et al*., 1999; Watanabe *et al*., 2000; Böger and Bode-Böger, 2001). However, little or no effect of L-arginine has been reported in normal healthy volunteers. The vascular effects of L-arginine observed in pathological conditions caused by vascular endothelial dysfunction has been attributed to the higher circulating concentrations of ADMA in association with these conditions (Vallance *et al*., 1992; Böger *et al*., 1998; Kielstein *et al*., 1999; Miyazaki *et al*., 1999; Böger 2003; Leiper and Vallence, 2006; Siroen *et al*., 2006; Andersshon *et al* 2010). In addition, it has been reported that endothelium-independent mechanism(s) such as stimulating the release of histamine or vasodilator hormones such as insulin might contribute to the vascular actions of infused L-arginine (Smulders *et al*., 1994; Mehta *et al*., 1996; Guigliano *et al*., 1997).

The possible explanations for the lack of vascular effect of L-arginine in rat isolated aortic rings demonstrated by the results of the present study include the lack of availability of vasodilator hormones that could potentially contribute to *in vivo*. Moreover, the aortic rings used in the present study were obtained from normal, healthy animals where it is known rats and it is well-known that the intracellular L-arginine concentrations (100-300 µM) are high enough to saturate endothelial nitric oxide synthase (K_m 2-3 µM) (Sydow and Münzel, 2003; Teerlink *et al.,* 2009; Zhang *et al*., 2011).

ADMA and L-NMMA, like L-arginine, protect acetylcholine-induced relaxation against blockade by L-NAME

The results of this study showed that the acetylcholine-induced, endothelium-dependent, nitric oxide-mediated, relaxation in female rat aorta was powerfully blocked by the standard nitric oxide synthase inhibitor, L-NAME. In addition, although the early findings of this study showed that L-NMMA, another inhibitor of nitric oxide synthase, blocked basal but not acetylcholine-induced relaxation, the subsequent results of this study showed that L-NMMA acted similarly to L-arginine, the endogenous substrate for nitric oxide synthase by both preventing and reversing the blockade of acetylcholine-induced relaxation produced by L-NAME. These findings are in agreement with those reported by Frew and co-workers (1993) who hypothesised that L-NMMA may function as an alternative substrate for nitric oxide synthase when it is stimulated with agonists like acetylcholine or ATP. Moreover, chemiluminescence detection has shown that L-NMMA, in common with L-arginine, increases the synthesis of nitric oxide in rat aorta and pulmonary artery, whereas L-NOARG inhibits it (Archer and Hampl, 1992).

Therefore, in light of the similarities between ADMA and L-NMMA at blocking basal but not acetylcholine-stimulated activity of nitric oxide in rat aorta, it was interesting to investigate whether ADMA can protect acetylcholine-induced relaxation against blockade by L-NAME. Indeed, the results of this study showed that ADMA behaves similarly to L-arginine; it protects acetylcholine-induced relaxation against blockade by L-NAME, however, it failed to reverse that blockade after an hour of treatment, perhaps due to a lower potency of ADMA than L-arginine or L-NMMA. Whether or not longer treatments on higher concentrations ADMA would have reversed the blockade by L-NAME was not investigated.

In light of the similarities between L-NMMA and ADMA found in this study so far, ADMA; like L-NMMA and L-arginine might have behaved as a substrate for acetylcholine-stimulated nitric oxide synthase so as to prevent the blockade by L-NAME.

It is unlikely that a demethylation reaction to form L-arginine explains the abilities of ADMA and L-NMMA to behave as alternative substrates for agonist-stimulated endothelial nitric oxide synthase, because the blockade they produce of basal nitric oxide activity is sustained, suggestive of on-going stability of these agents.

Possible explanations for the anomalous abilities of L-NMMA and ADMA to block basal but not agonist–stimulated activity of nitric oxide

One theory that may explain these anomalous actions of ADMA and L-NMMA is the existence, within the vascular endothelial cells, of more than one isoform of nitric oxide synthase; one that catalyses the synthesis of nitric oxide under basal conditions, and another that catalyses agonist-stimulated nitric oxide production. Other workers, using

aorta from endothelial and neuronal nitric oxide synthase enzymes knockout mice, or studying the effects in intact rats or human volunteers of S-methyl-L-thiocitrulline, a presumably selective blocker of neuronal nitric oxide synthase, have postulated that the latter enzyme catalyses the synthesis of nitric oxide under basal conditions, and the endothelial nitric oxide synthase catalyses agonist-stimulated nitric oxide production (Wakefield et al., 2003; Nangle et al., 2004; Melikian et al., 2009; Seddon et al., 2009).

Another possible hypothesis behind the anomalous actions of ADMA and L-NMMA, is that the different signal transduction mechanisms triggered by different stimuli may result in structural or conformational changes in endothelial nitric oxide synthase that alter its requirement for substrates and inhibitors. Such changes could perhaps result in nitric oxide synthase recognising ADMA or L-NMMA as substrates rather than as inhibitors. For example, nitric oxide production induced by agonists that signal through calcium-calmodulin pathway, such as acetylcholine (Schini and Vanhoutte, 1992), is little affected by ADMA, whereas flow-mediated production of nitric oxide, believed to be mediated through phosphorylation of nitric oxide synthase by the phosphatidylinositol 3-kinase/Akt pathway (Fulton *et al*., 1999; Gallis *et al*., 1999), is inhibited by ADMA in humans (Boger *et al*., 1998; Vladimirova-Kitova *et al*., 2008). Further studies would be required, however, to compare the effects of ADMA and L-NMMA against relaxant stimuli that bring about their effects through calcium-calmodulin or the phosphatidylinositol 3-kinase/Akt pathway, using same tissue.

One other potential explanation for the anomalous abilities of ADMA and L-NMMA to block basal but not agonist-stimulated activity of nitric oxide may relate to the degree to which endothelial nitric oxide synthase is stimulated. Specifically, the low level activation

of the enzyme which underpins basal activity of nitric oxide may be easier to block than the powerful enzyme activity resulting from agonist stimulation. Indeed, this is supported by previous work which suggests that the efficacy of the relaxant agonist is critical in determining the ability of nitric oxide synthase inhibitors to block endothelium-dependent relaxation (Martin *et al*., 1992). The results of testing this hypothesis will be presented in Chapter Four.

In conclusion, the findings so far demonstrate that in female rat aorta, the basal nitric oxide activity that suppresses vasoconstrictor tone is blocked, in an L-arginine-preventable and reversible manner, by the nitric oxide synthase inhibitors, L-NAME, L-NMMA and ADMA. In sharp contrast, however, acetylcholine-stimulated nitric oxide activity is little affected by L-NMMA or ADMA alone, but these agents like L-arginine oppose the blocking action of L-NAME.

A summary of the effects of L-arginine, L-NAME, L-NMMA, ADMA and SDMA on basal and acetylcholine-stimulated activity of nitric oxide in the rat aorta is shown below (Figure 2.24).

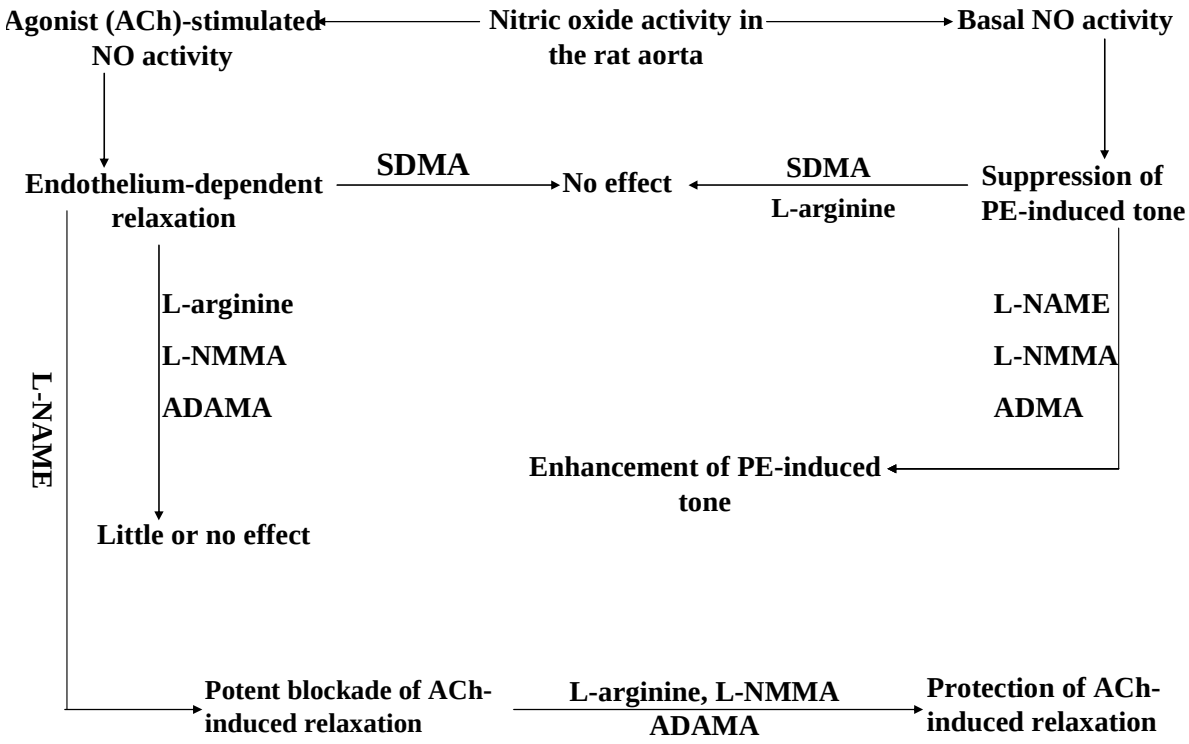

Figure 2.24. Summary of the effects of L-arginine and methylarginines on basal and acetylcholine-stimulated activity of nitric oxide in the rat aorta.

In the next Chapter the anomalous abilities of L-NMMA and ADMA to block basal but not agonist–stimulated activity of nitric oxide will be further explored by using different experimental approaches. For example, the effects of L-NMMA and ADMA on endothelium-dependent relaxation induced by superoxide dismutase or the phosphodiesterase isoform 5 inhibitor, T-0156, will be examined as these two agents are known to induce endothelium-dependent relaxation by potentiating basal nitric oxide activity (MacKenzie *et al*., 1999; Mochida *et al*., 2002; Mochida *et al*., 2004). Moreover, the effects of L-NMMA and ADMA will be examined on nitric oxide activity stimulated by a range of different agonists that are known to produce endothelium-dependent, nitric oxide-mediated relaxations in rat aorta, such as the calcium ionophore A23187 (Weinheimer and Osswald, 1986; Schini and Vanhoutte, 1992), and calcitonin gene-related peptide-1 (Gray and Marshall, 1992; Wisskiechoen *et al*., 1999).

In addition, the transduction mechanism(s) that may play a role in the release of nitric oxide, under basal and stimulated conditions, will be explored using the calcium-calmodulin antagonist, calmidazolium (Schini and Vanhoutte, 1992), and the phosphatidylinositol 3-kinase/Akt pathway inhibitor, wortmannin (Michell *et al*., 1999; Fulton *et al*., 1999; Dimmeler *et al*., 1999).

Chapter Three

Introduction

The findings of the first part of this study showed that L-NMMA and ADMA exhibit anomalous abilities to block basal but not agonist (acetylcholine)-stimulated activity of nitric oxide in female rat aorta. In addition, the powerful blockade of basal nitric oxide activity produced by these two inhibitors of nitric oxide synthase is surmountable, i.e. it can both be prevented and reversed by adding a higher concentration of L-arginine, the endogenous substrate for nitric oxide synthase. Although, L-NMMA and ADMA had little effect on acetylcholine-stimulated activity of nitric oxide, they displayed properties similar to L-arginine, i.e. they prevented the ability of L-NAME to block acetylcholine-induced relaxation.

The above effects of L-NMMA or ADMA on basal and agonist-stimulated activity of nitric oxide were examined by assessing their effects on phenylephrine-induced tone and on acetylcholine-induced relaxation in rat aortic rings, respectively. The purpose of this part of the study was to explore further these differential abilities of L-NMMA and ADMA to block basal but not agonist–stimulated activity of nitric oxide, using different experimental approaches. Specifically, superoxide dismutase is known to produce endothelium-dependent relaxation by protecting basal nitric oxide against inactivation by superoxide anions (Ohlstein and Nichols, 1989; Mian and Martin, 1995; MacKenzie *et al*., 1999). In addition, phosphodiesterase isoform 5 inhibitors, including T-0156, are known to elicit endothelium-dependent relaxation because they potentiate basal nitric oxide activity by extending the life span of the nitric oxide-dependent second messenger, cyclic guanosine monophosphate (cGMP), by preventing its degradation in the vascular smooth muscle cells (Martin *et al*., 1986a; Mochida *et al*., 2002; Mochida *et al*., 2004). As a consequence, the effects of L-NMMA and ADMA on basal nitric oxide activity will be

examined by assessing the influence they exert on endothelium-dependent, nitric oxide-mediated relaxation induced by superoxide dismutase and T0156 in female rat aorta.

On the other face of the coin, agonists such as calcitonin gene-related peptide-1 and the calcium ionophore A23187 are known to produce endothelium-dependent, nitric oxide-mediated relaxation by receptor- and non-receptor-mediated actions, respectively (Weinheimer and Osswald, 1986; Gray and Marshall, 1992a; Schini and Vanhoutte, 1992; Wisskirchen *et al*., 1999). Thus, the effects of L-NMMA and ADMA on the activity of nitric oxide stimulated by these agonists in female rat aorta will be compared with their actions on that induced by acetylcholine.

In addition, the differential abilities of L-NMMA and ADMA to block basal but not agonist-stimulated activity of nitric oxide in rat aorta may be related to differences in the transduction mechanisms that control the generation of nitric oxide by nitric oxide synthase under basal and agonist-stimulated conditions. This hypothesis will be examined by assessing the influence of the calcium-calmodulin inhibitor, calmidazolium (Archer and Cowan, 1991; Schini and Vanhoutte, 1992), and the phosphatidylinositol 3-kinase inhibitor, wortmannin (Michell *et al*., 1999; Fulton *et al*., 1999; Dimmeler *et al*., 1999), on basal and agonist-stimulated nitric oxide activity. this will be done by comparing the impact they have on the endothelium-dependent, nitric oxide-mediated relaxation induced by superoxide dismutase and T0156, on the one hand, and on that induced by acetylcholine, A23187 and calcitonin gene-related peptide-1, on the other hand.

Materials and Methods

Preparation of aortic rings for tension recording

The preparation of aortic rings for tension recording was essentially similar to that described in the previous Chapter except that the animals were killed by CO_2 overdose.

Role of the vascular endothelium in relaxation induced by superoxide dismutase or the PDE 5 inhibitor, T-0156

In the previous Chapter, basal nitric oxide activity was assessed by examining the enhancement of phenylephrine-induced tone seen upon the addition of nitric oxide synthase inhibitors. In this Chapter an alternative means of assessing this was sought by producing endothelium-dependent relaxation through potentiation of basal nitric oxide activity.

In these experiments, female rat endothelium-containing or endothelium-denuded aortic rings were allowed to equilibrate for 90 min and the resting stretch was re-adjusted to 10 mN, if required. Some tissues were then treated with catalase (3600 u ml^{-1} for 30 min to remove any H_2O_2 present), L-NAME (100 µM for 30 min) or the combination of catalase and L-NAME. After this time the treated tissues and their time-matched controls were pre-contracted to ~50% of maximal phenylephrine tone. Lower concentrations of phenylephrine were required to induce the same level of tone in endothelium-denuded and L-NAME treated tissues as in control tissues because of removal of the vasodepressant action of basal nitric oxide. After stabilisation of tone, increasing concentrations of SOD (0.1-300 u ml^{-1}) were added cumulatively to the tissues in the organ baths using the sequence 0.1 u ml^{-1}, 0.3 u ml^{-1}, 1 u ml^{-1}, 3 u ml^{-1}, 10 u ml^{-1}, 30 u ml^{-1}, 100 u ml^{-1} and 300 u ml^{-1} and relaxation assessed. The drug was then either washed out to conduct another

concentration-response curve, or papaverine (300 μM) was added to produce full relaxation of the tissues.

As will be seen in the Results, SOD-induced relaxation appeared to comprise separate components due to nitric oxide and H_2O_2. As a consequence, all further experiments with SOD were conducted in the presence of catalase (3600 u ml^{-1}) to ensure any relaxation produced was solely due to nitric oxide.

In another set of experiments, female rat endothelium-containing or endothelium-denuded aortic rings were allowed to equilibrate for 90 min and the resting stretch was re-adjusted to 10 mN, if required. The tissues were then pre-contracted to ~50% of maximal tone using phenylephrine. After stabilisation of phenylephrine-induced tone, increasing concentrations of SOD or the PDE 5 inhibitor, T-0156, were added cumulatively to the tissues in the organ baths and relaxations assessed. The drug was then either washed out to conduct another concentration-response curve, or papaverine (300 μM) was added to produce full relaxation of the tissues.

Effects of nitric oxide synthase inhibitors on relaxation induced by superoxide dismutase or T-0156

In these experiments, female rat endothelium-containing aortic rings were allowed to equilibrate for 90 min and resting stretch was re-adjusted to 10 mN, if required. The tissues were then treated with L-NAME (100 μM for 30 min), L-NMMA (100 μM 45 min) or ADMA (100 μM for 1 h). After this time the treated tissues and their time-matched controls were pre-contracted to ~50% of maximal phenylephrine tone. Care was taken to induce comparable levels of tone both in control and treated tissues by using lower

concentrations of phenylephrine in the latter. After stabilisation of tone, a full concentration-response curve to superoxide dismutase (0.1-300 u ml^{-1}) or T-0156 (1-300 nM) was conducted. Thereafter, papaverine (300 µM) was added to produce full relaxation of the tissues.

Effects of calmidazolium and wortmannin on relaxation induced by superoxide dismutase or T-0156

The aim of these experiments was to determine the role of endothelial calcium and the phosphatidylinositol 3-kinase (PI 3-kinase) pathway in relaxation induced by SOD or T-0156. This was investigated using the calcium-calmodulin antagonist, calmidazolium (Illiano *et al*., 1992; Schini and Vanhoutte, 1992), and the PI3 kinase inhibitor, wortmannin (Michell *et al.*, 1999; Fulton *et al*., 1999; Dimmeler *et al*., 1999), respectively.

In these experiments, female rat endothelium-containing aortic rings were allowed to equilibrate for 90 min and resting stretch was re-adjusted to 10 mN, if required. The tissues were then treated with calmidazolium (10 µM) alone, wortmannin (100 nM) alone, or with a combination of the two agents, for 1 h. After this time the treated tissues and their time-matched controls were pre-contracted to ~50% of maximal phenylephrine tone. After stabilisation of tone, a full concentration-response curve to SOD (0.1-300 u ml^{-1}) or T-0156 (1-300 nM) was conducted and relaxation assessed. Thereafter, papaverine (300 µM) was added to produce full relaxation of the tissues.

Effects of calmidazolium and wortmannin, compared with L-NAME, on basal nitric oxide activity assessed by their effects on phenylephrine-induced contraction

The previous experiments in this Chapter investigated the effects of L-NAME,

calmidazolium and wortmannin on basal nitric oxide activity assessed by their ability to affect relaxation induced by SOD or T-0156 in female rat endothelium-containing aortic rings. The current experiments were conducted to investigate and compare the effects of these three agents, alone and in combination, on basal nitric oxide activity as assessed by their ability to enhance the contractile effects of phenylephrine.

In these experiments, female rat endothelium-containing aortic rings were allowed to equilibrate for 90 min and the resting stretch was re-adjusted to 10 mN, if required. The tissues were then treated with calmidazolium (10 μM) alone, wortmannin (100 nM) alone, L-NAME (100 μM) alone, calmidazolium and L-NAME, wortmannin and L-NAME, calmidazolium and wortmannin, or a combination of the three agents, before performing a full concentration-response curve to phenylephrine (1 nM-10 μM). The differential ability of these treatments to enhance phenylephrine-induced contraction through inhibition of the depressant action of basal nitric oxide activity was then assessed.

Effects of nitric oxide synthase inhibitors on relaxation induced by acetylcholine, calcium ionophore A23187 or calcitonin gene-related peptide-1

In the previous Chapter it was found that L-NMMA and ADMA had little effect on endothelium-dependent relaxation of rat aorta induced by acetylcholine, despite powerfully inhibiting basal nitric oxide activity. The aim of these experiments was to investigate the effects of L-NMMA and ADMA on the endothelium-dependent relaxation induced by a range of agents, i.e. acetylcholine, calcium ionophore A23187 or calcitonin gene-related peptide-1 (CGRP-1) in female in female rat endothelium-containing aortic rings.

In these experiments, female rat endothelium-containing aortic rings were allowed to equilibrate for 90 min and resting stretch was re-adjusted to 10 mN, if required. The tissues

were then treated with L-NAME (100 µM, 30 min), L-NMMA (100 µM, 45 min) or ADMA (100 µM, 1 h). After this time, the treated tissues and their time-matched controls were pre-contracted to ~50% of maximal phenylephrine tone. Care was taken to induce comparable levels of tone both in control and treated tissues by using lower concentrations of phenylephrine in the latter. After stabilisation of tone, a full concentration-response curve to acetylcholine (1 nM-10 µM), A23187 (1 nM-1 µM) or CGRP-1 (0.1 nM-1 µM) was conducted. Thereafter, papaverine (300 µM) was added to produce full relaxation of the tissues.

Effects of calmidazolium and wortmannin on relaxation induced by acetylcholine, calcium ionophore A23187 or calcitonin gene-related peptide-1

The aim of these experiments was to determine the role of endothelial calcium and the PI 3-kinase pathway in relaxation induced by acetylcholine, A23187 or CGRP-1. This was investigated using the calcium-calmodulin antagonist, calmidazolium (Illiano *et al*., 1992; Schini and Vanhoutte, 1992), and the PI3 kinase inhibitor, wortmannin (Michell *et al*., 1999; Fulton *et al*., 1999; Dimmeler *et al*., 1999), respectively.

In these experiments, female rat endothelium-containing aortic rings were allowed to equilibrate for 90 min and resting stretch was re-adjusted to 10 mN, if required. The tissues were then treated with calmidazolium (10 µM) or wortmannin (100 nM) for 1 h. After this time the treated tissues and their time-matched controls were pre-contracted to ~50% of maximal phenylephrine tone. After stabilisation of tone, a full concentration-response curve to acetylcholine (1 nM-10 µM), A23187 (1 nM-1 µM) or CGRP-1 (0.1 nM-1 µM) was conducted and relaxation assessed. Thereafter, papaverine (300 µM) was added to produce full relaxation of the tissues.

Effects of L-NAME on relaxation induced by isoprenaline or forskolin

The rationale for conducting these experiments was to determine if isoprenaline or forskolin induce a nitric oxide-dependent component of relaxation that is susceptible to blockade by the nitric oxide synthase inhibitor, L-NAME, in female rat endothelium-containing aortic ring. If so, this would give an opportunity to examine the effects of L-NMMA and ADMA on agents that produce a component of relaxation via nitric oxide release stimulated by agents that elevate cAMP levels (Boo and Jo, 2003).

In these experiments, female rat endothelium-containing aortic rings were allowed to equilibrate for 90 min and resting stretch was re-adjusted to 10 mN, if required. The tissues were then treated with L-NAME (100 µM, 30 min). After this time the treated tissues and their time-matched controls were pre-contracted to ~50% of maximal phenylephrine tone. Care was taken to induce comparable levels of tone both in control and treated tissues by using lower concentrations of phenylephrine in the latter. After stabilisation of tone, a full concentration-response curve to isoprenaline (1 nM-10 µM) or forskolin (1-300 nM) was conducted and relaxation assessed. Thereafter, papaverine (300 µM) was added to produce full relaxation of the tissues.

Drugs and chemicals

Acetylcholine chloride (ACh), asymmetric N^G, N^G-dimethyl-L-arginine dihydrochloride (ADMA), calmidazolium chloride, N^G-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine acetate (L-NMMA), papaverine hydrochloride, phenylephrine hydrochloride (PE), superoxide dismutase (SOD; from bovine erythrocytes), isoprenaline hydrochloride and forskolin hydrochloride were all obtained from Sigma, UK. Calcium ionophore A23187 and wortmannin were obtained from Enzo Life Sciences, UK. Catalase (bovine liver) and calcitonin gene-related peptide-1 (human) were obtained from

Calbiochem, UK. T-0156 (2-(2-methylpyridin-4-yl) methyl-4-(3,4,5-trimethoxyphenyl)-8-(pyrimidin-2-yl)methoxy-1,2-dihydro-1-oxo-2,7-naphthyridine-3-carboxylic acid methyl ester hydrochloride) was obtained from Tocris, UK. All drugs were dissolved and diluted in 0.9% saline, with the exceptions of calmidazolium, T-0156, calcium ionophore A23187 and wortmannin, which were dissolved in 100% DMSO, and calcitonin gene-related peptide-1 which was dissolved in 5% acetic acid (all 10 mM stocks).

Data analysis

Contractions were measured in milliNewtons. Papaverine (300 μM) was added at the end of each experiment to produce full relaxation of tissues and the relaxant responses to agonists were expressed as a percentage of the full relaxation induced by this agent. Data are expressed as the mean ± S.E.M of n separate observations. Concentration-effect curves were analysed and drawn by fitting the formula mentioned on page 56, and statistical analysis was performed using one-way analysis of variance followed by Bonferroni's post-test or by Student's *t* test, as appropriate, with the aid of a computer-based program (Graph Pad, San Diego, USA). Values were considered to be statistically different when P was $\leq$ 0.05.

Results

Superoxide dismutase-induced relaxation

Following induction of ~ 50% of maximal phenylephrine tone in female rat endothelium-containing aortic rings, superoxide dismutase (SOD, 0.1-300 u ml^{-1}) induced concentration-dependent relaxation (maximal relaxation 63.3 ± 6.0% of initial tone; Figure 3.1). This relaxation was not significantly affected by 30 min pretreatment with catalase at 3600 u ml^{-1} , but it was partially blocked following a 30-min pretreatment with the NOS inhibitor, L-NAME, at 100 µM. However, in the presence of catalase, SOD-induced relaxation was almost completely abolished by L-NAME (Figure 3.1), or after endothelial denudation (Figure 3.2). Since SOD-induced relaxation appeared to comprise separate components due to nitric oxide and H_2O_2, all further experiments with this agent were conducted in the presence of catalase to allow further study of the former component only.

Two other NOS inhibitors, ADMA and L-NMMA (both at 100 µM) in the presence of catalase, significantly blocked SOD-induced relaxation in endothelium-containing vessels (E_{max} 15.9 ± 4.2 and 21.5 ± 3.6 for ADMA and L-NMMA, respectively; Figure 3.3).

SOD-induced relaxation in endothelium-containing rat aortic rings was not changed by either the PI 3-kinase inhibitor, wortmannin (100 nM) alone, or the calmodulin antagonist, calmidazolium (10 µM) alone, but the combination of the two agents resulted in a slight but significant inhibition of relaxation (Figure 3.4).

It is therefore likely that in the presence of catalase, SOD-induced relaxation is mediated by protecting basal nitric oxide activity from destruction by superoxide anions, and that this explains its blockade by endothelial denudation or pretreatment with NOS inhibitors.

The PI 3-kinase and calcium-calmodulin pathways are likely to play only a minor role in basal nitric oxide production.

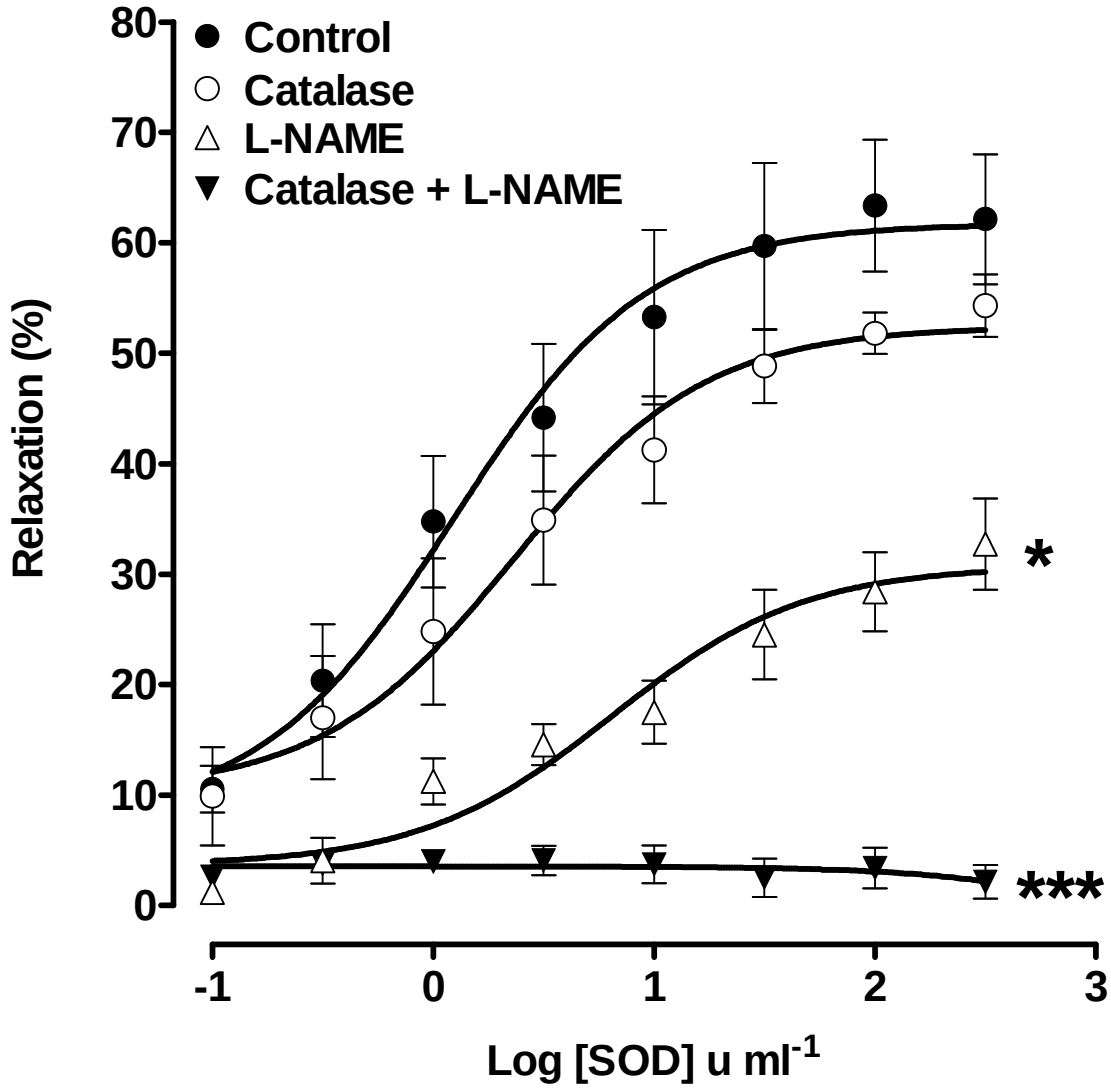

Figure 3.1. Cumulative concentration-effect curves showing relaxation to superoxide dismutase (SOD) in rat endothelium-containing aortic rings sub-maximally contracted with PE. Responses are shown for control tissues, tissues in the presence of (3600 u ml^{-1}) of catalase alone, or L-NAME (100 µM) alone, or tissues treated with both catalase and L-NAME. Although SOD-induced relaxation was significantly depressed in the presence of L-NAME, it was completely abolished by L-NAME in the presence of catalase. Each point represents the mean ± SEM of 5-6 observations. * $P< 0.05$ and *** $P< 0.001$ indicate significant differences from control.

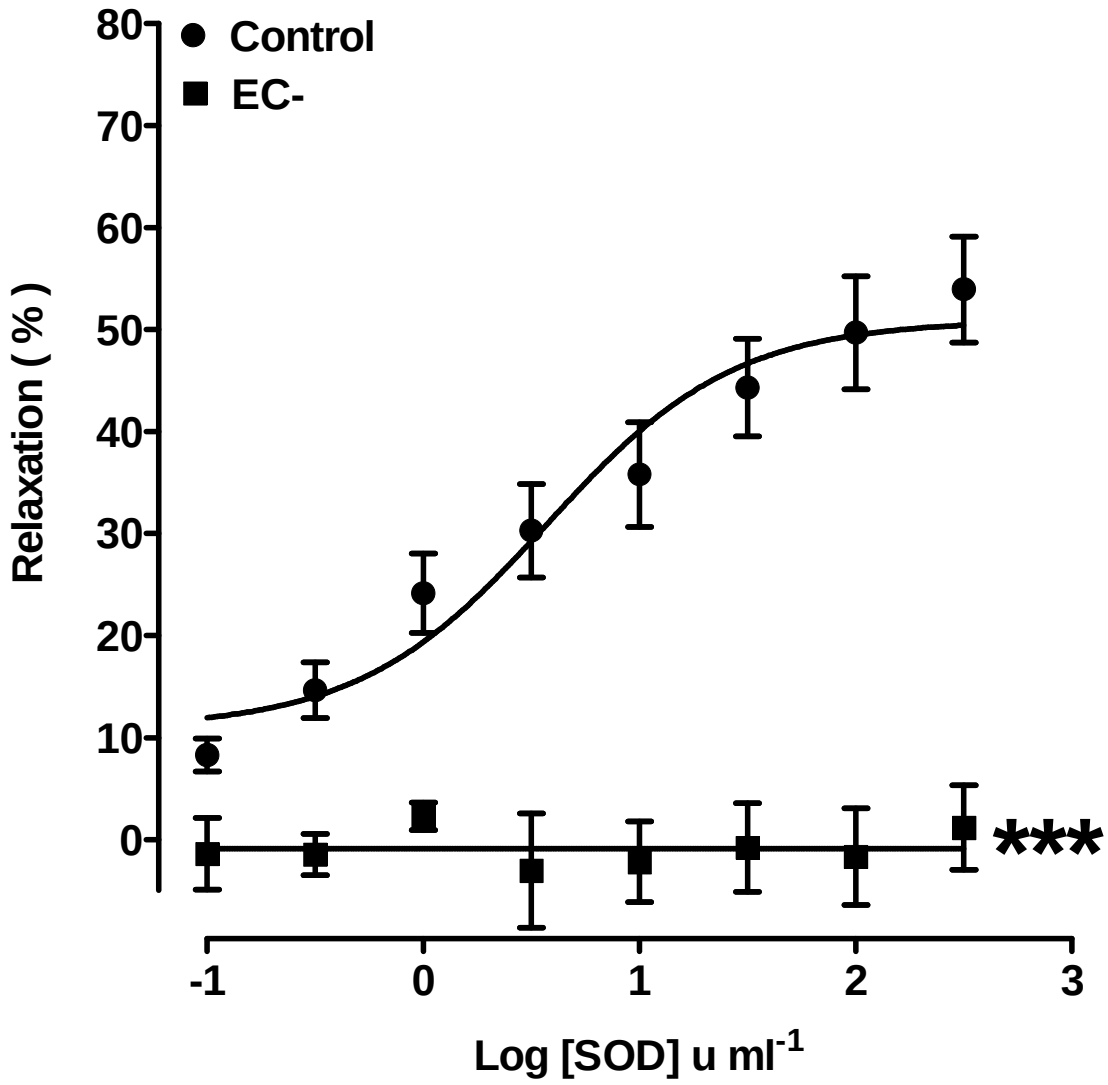

Figure 3.2. Cumulative concentration-effect curves showing relaxation to SOD in rat aortic rings submaximally contracted with PE in the presence (control) and absence (EC-) of endothelium. SOD-induced relaxation was completely abolished by endothelial denudation. All experiments were conducted in the presence of catalase (3600 u $ml^{-1)}$ to remove the influence of any H_2O_2 produced. Each point represents the mean ± SEM of 6 observations. *** P< 0.001 indicates a significant difference from control.

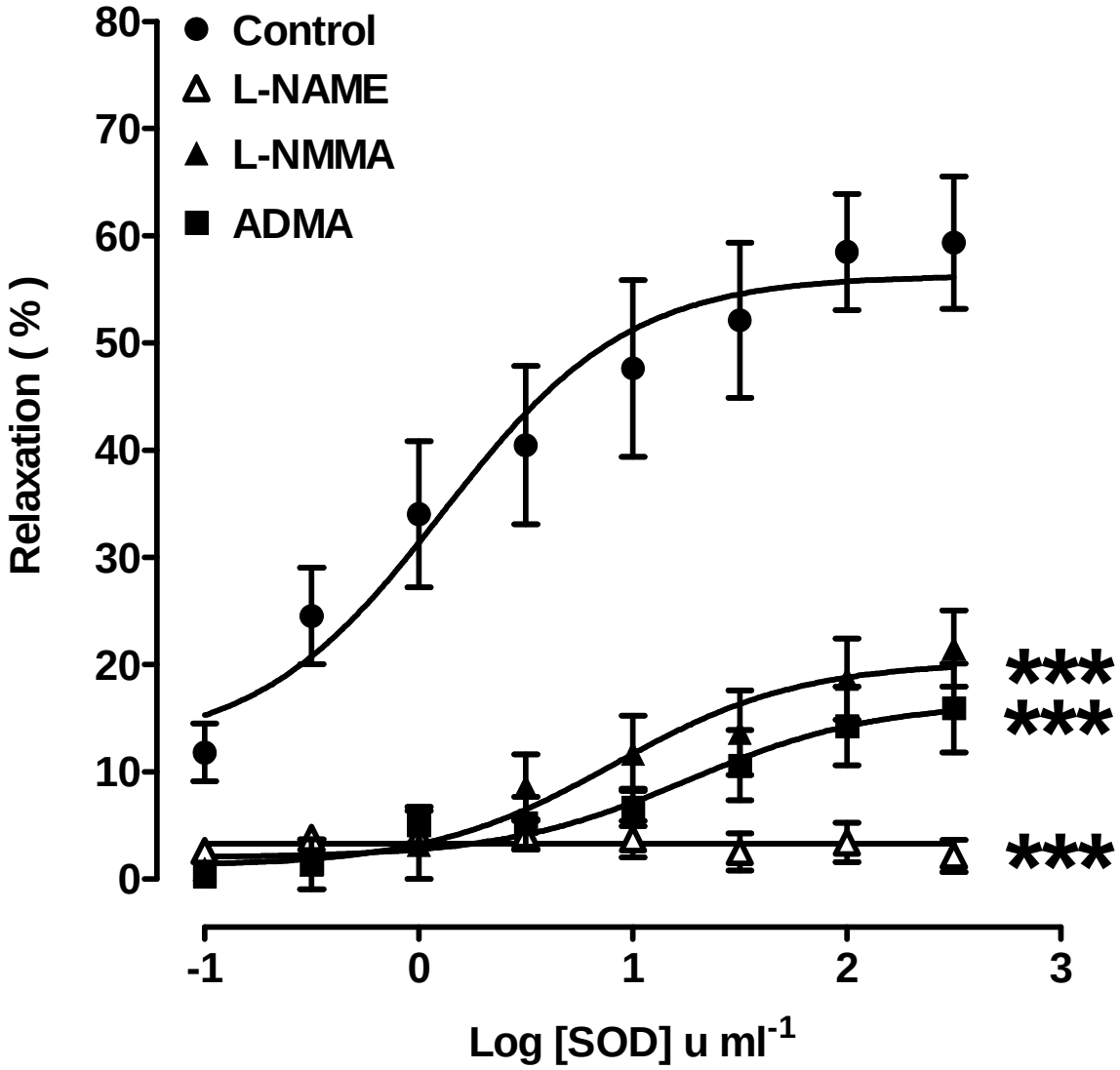

Figure 3.3. Cumulative concentration-effect curves showing relaxation to SOD in rat endothelium-containing aortic rings submaximally contracted with PE in the absence and presence of L-NAME, L-NMMA or ADMA, all at 100 µM. SOD-induced relaxation was powerfully blocked by each of the three nitric oxide synthase inhibitors. All experiments were conducted in the presence of catalase (3600 u ml^{-1}) to remove the influence of any H_2O_2 produced Each point represents the mean ± SEM of 5-6 observations. *** P< 0.001 indicates a significant difference from control.

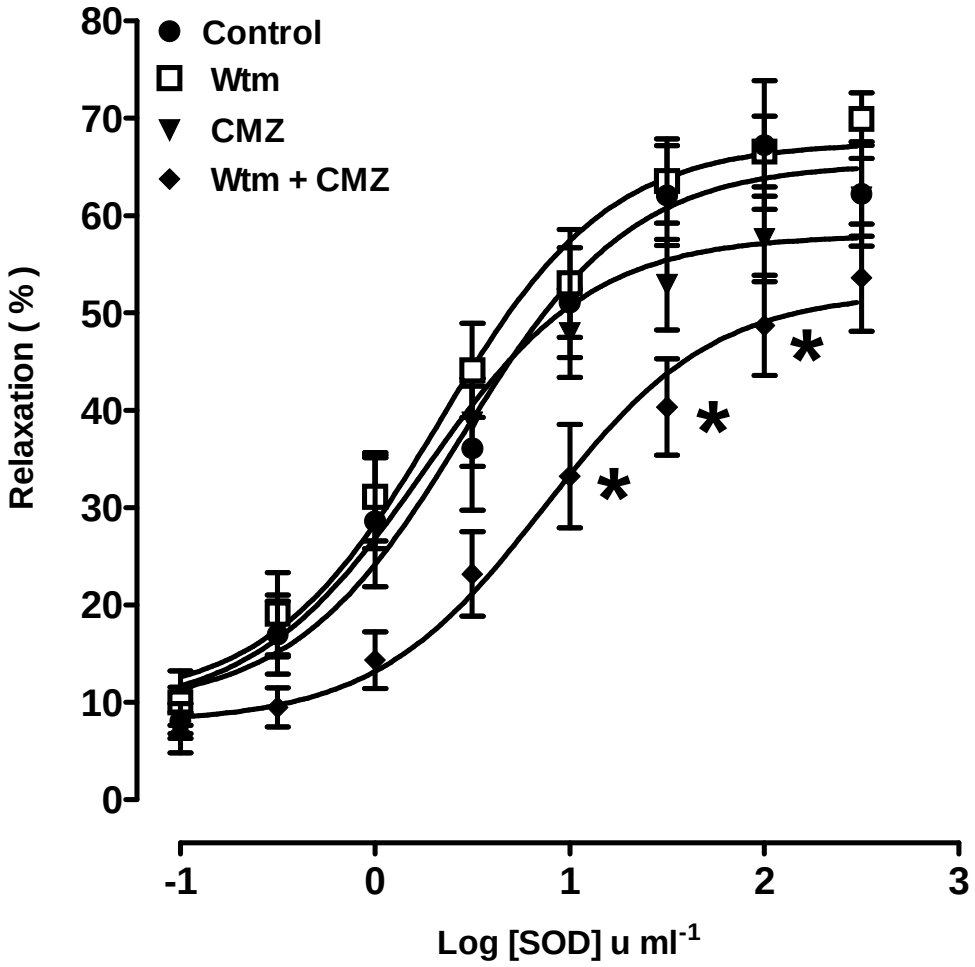

Figure 3.4. Cumulative concentration-effect curves showing relaxation to SOD in rat endothelium-containing aortic rings submaximally contracted with PE in the absence and presence of wortmannin (Wtm) at 100 nM, calmidazolium (CMZ) at 10 µM, or both. Neither wortmannin nor calmidazolium alone had an effect on SOD-induced relaxation, however, the combination of the two drugs produced significant blockade. All experiments were conducted in the presence of catalase (3600 u $ml^{-1)}$ to remove the influence of any H_2O_2 produced. Each point represents the mean ± SEM of 5-7 observations. * $P< 0.05$ indicates a significant difference from control.

Relaxation induced by the PDE 5 inhibitor, T-0156

The PDE 5 inhibitor, T-0156 (1-300 nM), produced more powerful relaxation in endothelium-containing than in endothelium-denuded rings of rat aorta (E_{max} 89.9 ± 3.2% and 53.5 ± 3.4%, respectively; Figure 3.5). The endothelium-dependent component of T-0156-induced relaxation was significantly blocked by each of the NOS inhibitors, L-NAME, L-NMMA or ADMA, all at 100 µM (Figure 3.6).

Relaxation induced by T-0156 in endothelium-containing aortic rings was not affected by either wortmannin (100 nM) alone, or calmidazolium (10 µM) alone, but the combination of the two agents resulted in a slight but significant inhibition of relaxation (Figure 3.7).

The endothelium-dependent component of T-0156-induced relaxation is likely to occur through potentiation of basal nitric oxide activity and this explains its blockade by the NOS inhibitors. These experiments also suggest that the PI 3-kinase and calcium-calmodulin pathways are likely to play only a minor role in basal nitric oxide production.

Effects of wortmannin and calmidazolium on basal nitric oxide activity assessed by their effects on PE-induced contractions

The maximal response as well as the sensitivity of rat endothelium-containing aortic rings to phenylephrine (1nM-10 µM) was significantly enhanced by pretreatment with L-NAME (100 µM), consistent with blockade of basal nitric oxide activity (Figure 3.8). Wortmannin (100 nM) alone, calmidazolium (10 µM) alone, and the combination of the two also led to an enhanced sensitivity and maximal response to phenylephrine, but the magnitude of these was much lower than for L-NAME. Moreover, the ability of

wortmannin or calmidazolium, either alone or combined, to enhance PE-induced contraction was abolished when the tissues were pre-treated with L-NAME.

Therefore, basal nitric oxide activity which suppresses vasoconstrictor tone is greatly blocked by pre-treatment with the NOS inhibitor, L-NAME. These experiments suggest that the PI 3-kinase and calcium-calmodulin pathways play a role in basal nitric oxide production, but only to a minor extent.

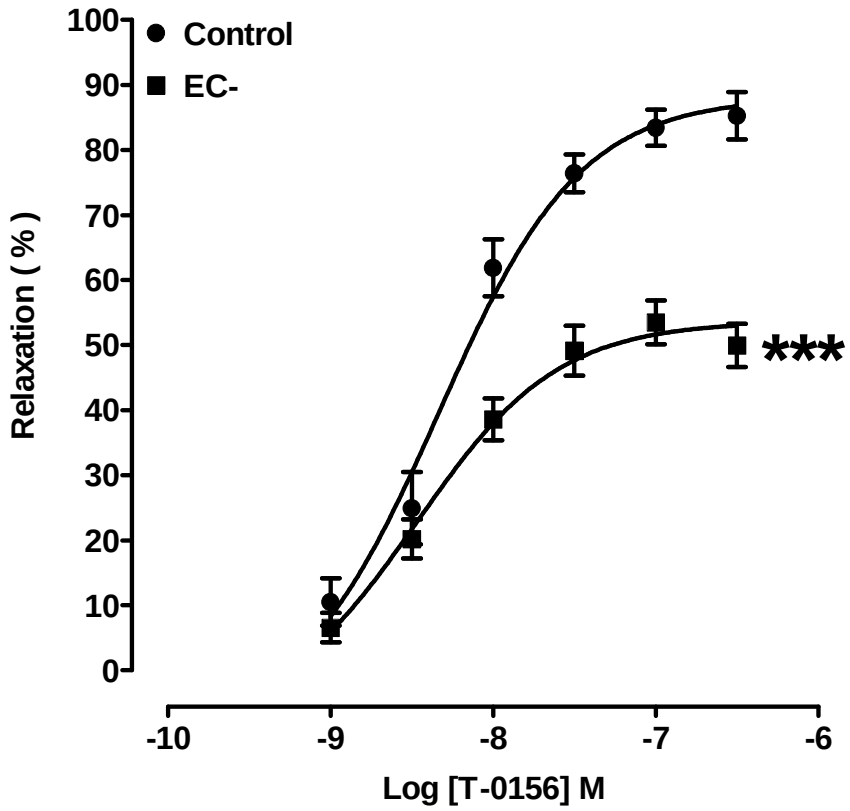

Figure 3.5. Cumulative concentration-effect curves showing relaxation to the PDE 5 inhibitor, T-0156, in rat aortic rings submaximally contracted with PE in the presence (control) and absence (EC-) of endothelium. T-0156 has both endothelium-dependent and endothelium-independent components of relaxation. Each point represents the mean ± SEM of 6 observations. *** P< 0.001 indicates a significant difference from control.

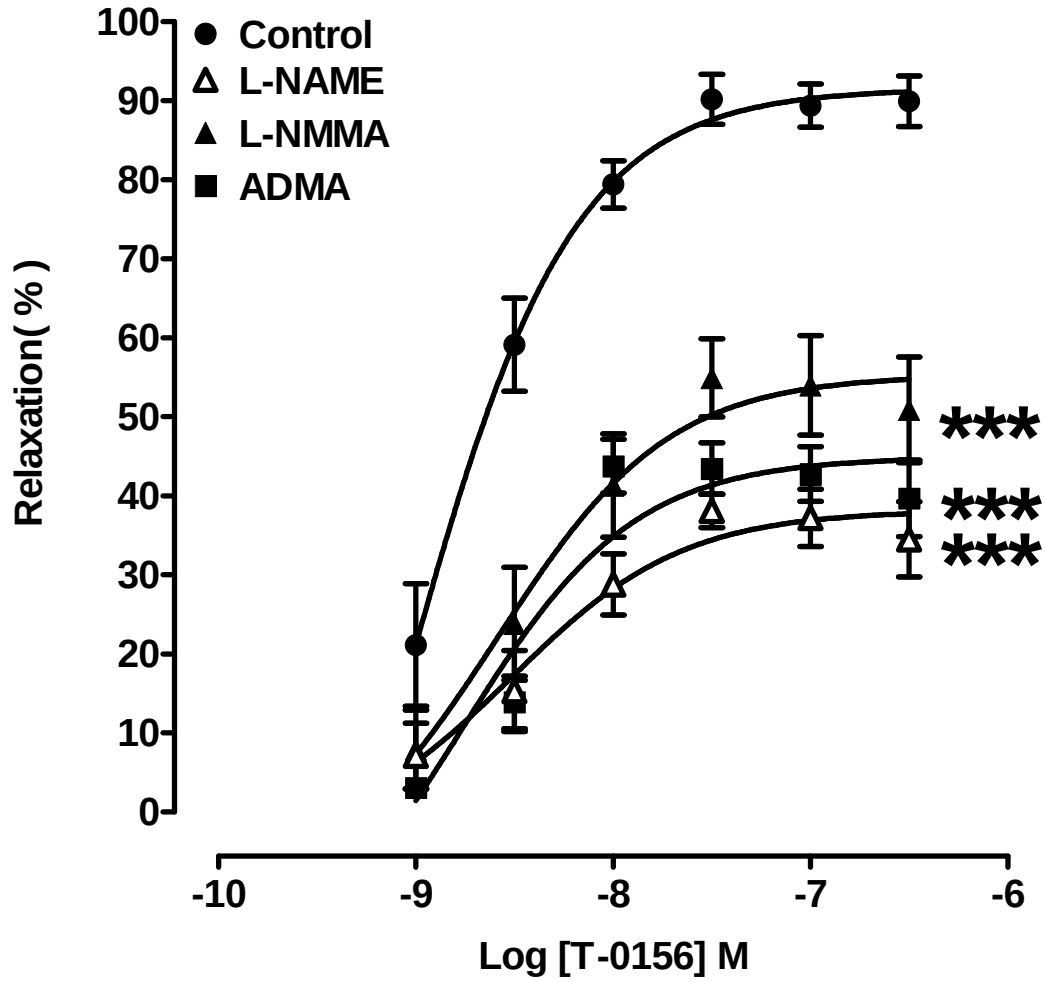

Figure 3.6. Cumulative concentration-effect curves showing relaxation to T-0156 in rat endothelium-containing aortic rings submaximally contracted with PE in the absence and presence of L-NAME, L-NMMA or ADMA, all at 100 µM . The endothelium-dependent component of T-0156-induced relaxation was significantly blocked by each of the three nitric oxide synthase inhibitors. Each point represents the mean ± SEM of 6 observations. *** P< 0.001 indicates a significant difference from control.

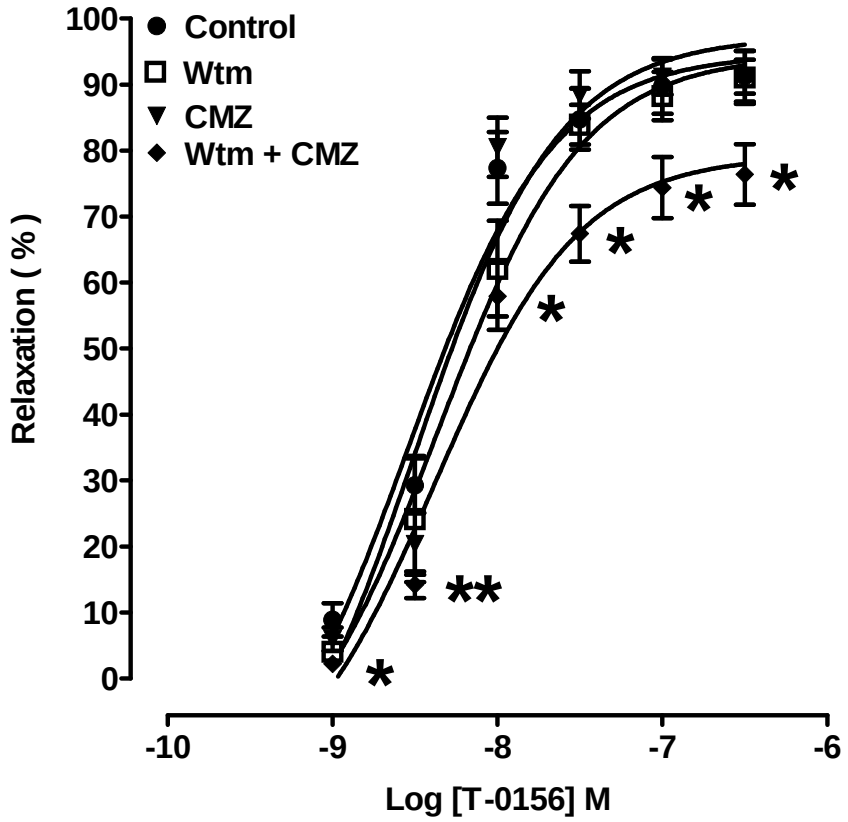

Figure 3.7. Cumulative concentration-effect curves showing relaxation to T-0156 in rat endothelium-containing aortic rings submaximally contracted with PE in the absence and presence of wortmannin (Wtm) at 100 nM, calmidazolium (CMZ) at 10 µM, or both. Neither wortmannin nor calmidazolium alone had an effect on T-0156-induced relaxation; however, the combination of the two drugs produced a slight but significant blockade. Each point represents the mean ± SEM of 5-7 observations. * $P < 0.05$ and ** $P < 0.01$ indicate significant differences from control.

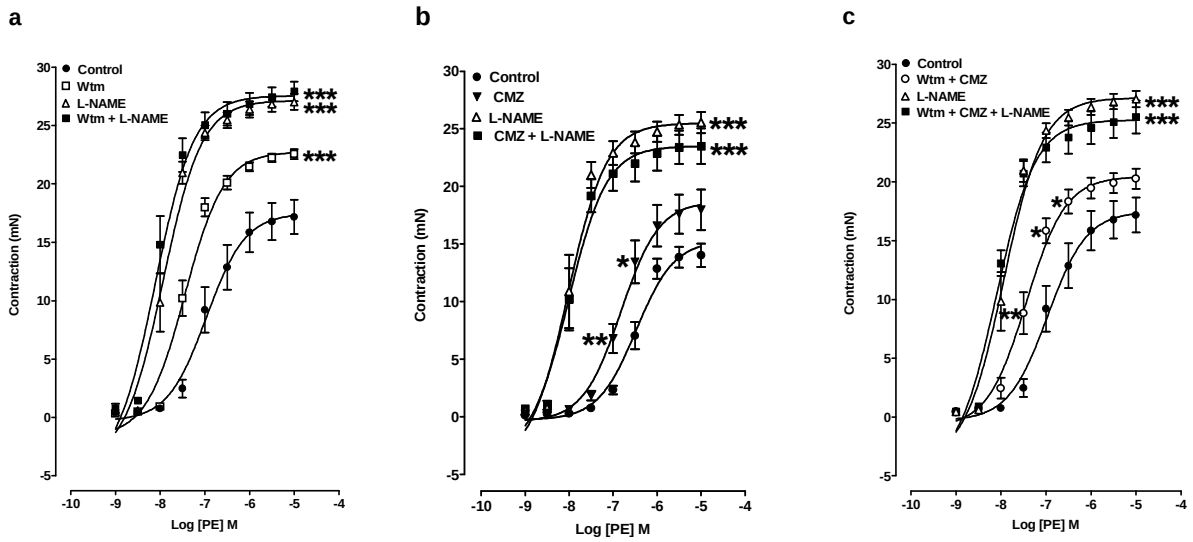

Figure 3.8. Cumulative concentration-effect curves showing contractile response to PE in rat endothelium-containing aortic rings in the absence or presence of (a) wortmannin (Wtm; 100 nM), L-NAME (100 µM) or both, (b) calmidazolium (CMZ; 10 µM), L-NAME or both, and (c) wortmannin and calmidazolium, L-NAME or all the three agents. PE-induced contraction was significantly enhanced by L-NAME or by wortmannin or calmidazolium alone and combined. However, the magnitude of the enhancement of PE-induced contraction produced by wortmannin and calmidazolium, either alone or in combination was lower than that produced by L-NAME. Each point represents the mean ± SEM of 5-7 observations. * $P< 0.05$, ** $P< 0.01$ and *** $P< 0.001$ indicate significant differences from control.

Acetylcholine-induced relaxation

Having established the effects of NOS inhibitors, wortmannin or calmidazolium on basal nitric oxide activity, it became important to investigate the effects of these agents on agonist-stimulated nitric oxide activity.

Following induction of ~ 50% of maximal phenylephrine tone in rat endothelium-containing aortic rings, acetylcholine (1 nM-10 µM) produced concentration-dependent relaxation that was significantly blocked by L-NAME (100 µM), but little affected by L-NMMA or ADMA (both at 100 µM; Figure 3.9a), in keeping with findings in the previous Chapter.

Acetylcholine-induced relaxation was significantly blocked by calmidazolium (10 µM), but unaffected by wortmannin (100 nM; Figure 3.9b).

Thus, acetylcholine-induced relaxation appears to involve calcium-calmodulin but not the PI 3-kinase pathway.

Relaxation induced by the calcium ionophore A23187

After induction of submaximal phenylephrine-induced tone in rat endothelium-containing aortic rings, the calcium ionophore A23187 (1 nM-1 µM), produced concentration-dependent relaxation (E_{max} 96.2 ± 1.2%) that was significantly blocked by pre-treatment with 100 µM L-NAME (E_{max} 36.4 ± 1.6%); Figure 3.10a). A23187-induced relaxation was powerfully blocked by pre-treatment with 10 µM calmidazolium (E_{max} 56.7 ± 2.7%), but unaffected by wortmannin (100 nM; Figure 3.10b).

Therefore, relaxation produced A23187, like that to acetylcholine, seems to be susceptible to blockade by L-NAME, but not L-NMMA or ADMA, and occurs via the calcium-calmodulin but not the PI 3-kinase pathway.

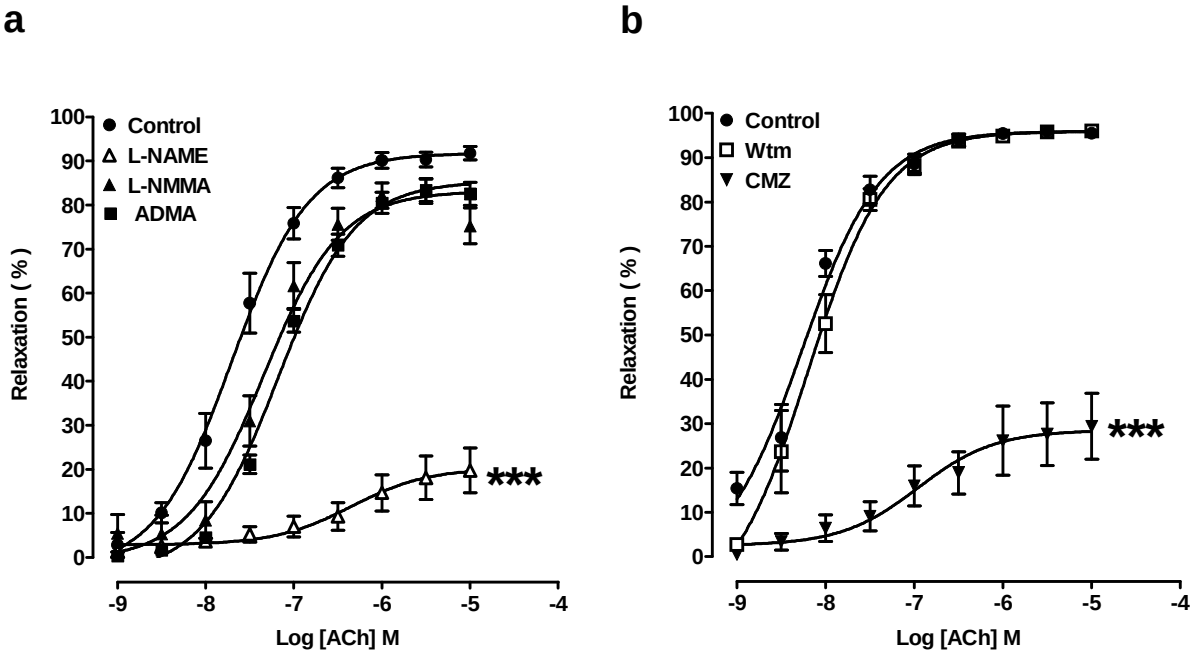

Figure 3.9. Cumulative concentration-effect curves showing relaxation to acetylcholine in rat endothelium-containing aortic rings submaximally contracted with PE in the absence and presence of (a) L-NAME, L-NMMA or ADMA (all at 100 µM), or (b) wortmannin (Wtm) at 100 nM or calmidazolium (CMZ) at 10 µM. Relaxation was significantly blocked by L-NAME or calmidazolium, but not by L-NMMA, ADMA or wortmannin. Each point represents the mean ± SEM of 6-15 observations. *** $P < 0.001$ indicates a significant difference from control.

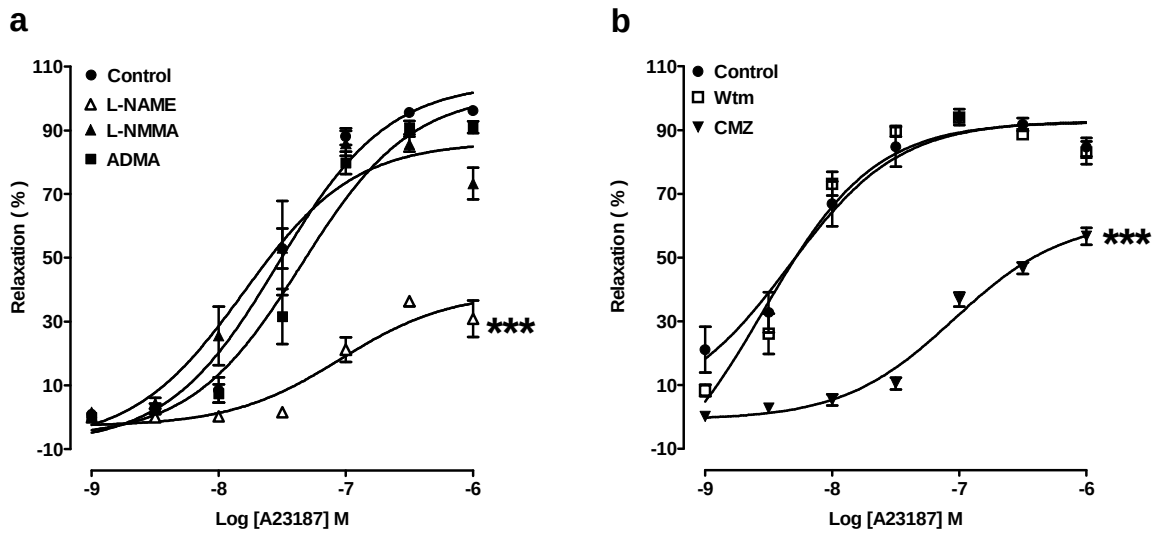

Figure 3.10. Cumulative concentration-effect curves showing relaxation to calcium ionophore A23187 in rat endothelium-containing aortic rings submaximally contracted with PE in the absence and presence of (a) L-NAME, L-NMMA or ADMA (all at 100 µM), or (b) wortmannin (Wtm) at 100 nM or calmidazolium (CMZ) at 10 µM. Relaxation was significantly blocked by L-NAME or calmidazolium, but not by L-NMMA, ADMA or wortmannin. Each point represents the mean ± SEM of 5-7 observations. *** $P < 0.001$ indicates a significant difference from control.

Relaxation induced by calcitonin gene-related peptide-1

After induction of submaximal phenylephrine -induced tone in rat endothelium-containing aortic rings, calcitonin gene-related peptide-1 (CGRP-1; 0.1 nM-1 μM) elicited concentration-dependent relaxation (E_{max} 57.6 ± 3.2%) that was significantly blocked by L-NAME (100 μM; Figure 3.11a). Unlike with acetylcholine and A23187, however, CGRP-1-induced relaxation was also blocked by L-NMMA and ADMA (both 100 μM).

CGRP-1-induced relaxation was also powerfully blocked by calmidazolium (10 μM), but not by wortmannin (100 nM; Figure 3.11b).

Thus, surprisingly, despite appearing to operate by the same calcium-calmodulin-dependent, PI 3-kinase-independent pathway used by acetylcholine and A23187, CGRP-1-induced relaxation is blocked by all three NOS inhibitors and not just L-NAME. Why CGRP-1 behaves so differently from acetylcholine and A23187 in this regard requires further investigation.

Relaxation induced by isoprenaline and forskolin

After induction of ~ 50% of maximal phenylephrine tone in female rat endothelium-containing aortic rings, isoprenaline (1 nM-10 μM) and forskolin (1 nM- 300 nM) each produced relaxation that was only slightly depressed by L-NAME (100 μM; Figure 3.12a and b). Since there was no substantial nitric oxide-dependent component to relaxation by these agents, the effects of L-NMMA, ADMA, wortmannin and calmidazolium were not examined.

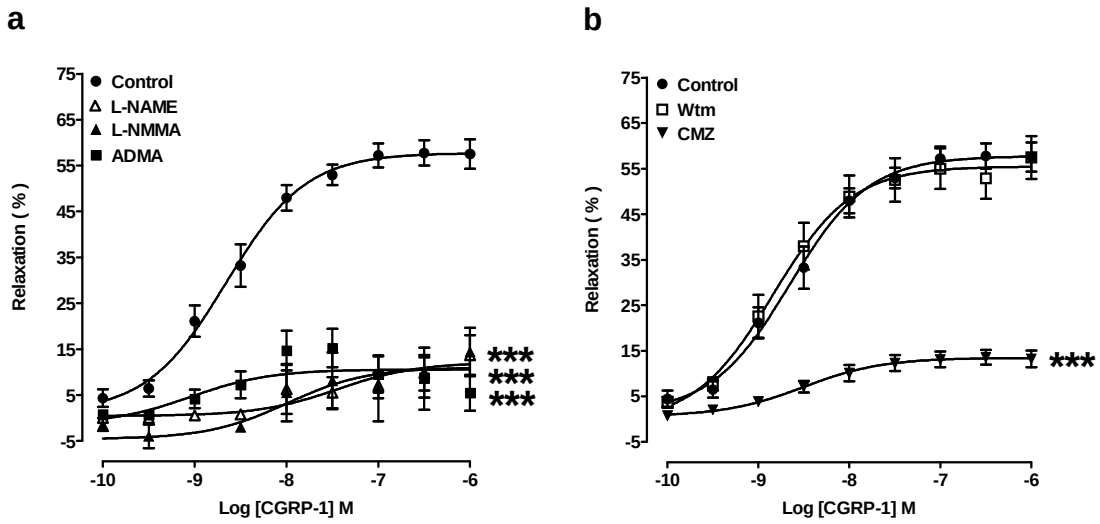

Figure 3.11. Cumulative concentration-effect curves showing relaxation to calcitonin gene-related peptide-1 (CGRP-1) in rat endothelium-containing aortic rings submaximally contracted with PE in the absence and presence of (a) L-NAME, L-NMMA or ADMA (all at 100 µM), or (b) wortmannin (Wtm) at 100 nM or calmidazolium (CMZ) at 10 µM. Relaxation was significantly blocked by L-NAME, L-NMMA, ADMA and calmidazolium, but not by wortmannin. Each point represents the mean ± SEM of 5-6 observations. *** P< 0.001 indicates a significant difference from control.

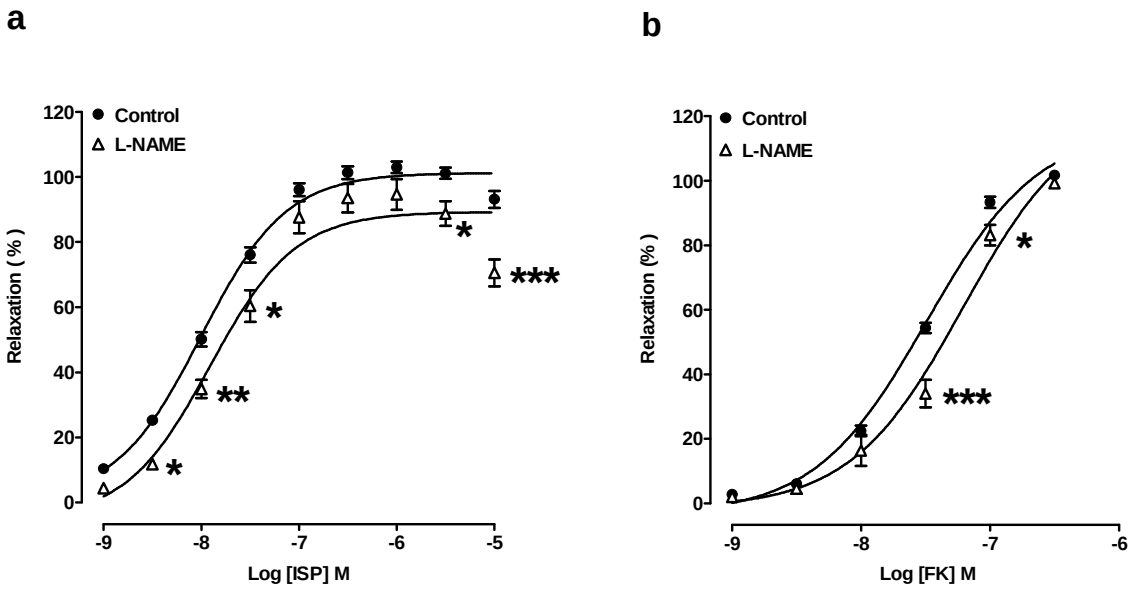

Figure 3.12. Cumulative concentration-effect curves showing relaxation to (a) isoprenaline (ISP) or (b) forskolin (FK) in rat endothelium-containing aortic rings submaximally contracted with PE in the absence or presence of L-NAME (100 µM). Relaxations were only slightly depressed in the presence of L-NAME. Each point represents the mean ± SEM of 6 observations. * P< 0.05, ** P< 0.01 and *** P< 0.001 indicate significant differences from control.

Discussion

Superoxide dismutase-induced relaxation in the presence of catalase in female rat aorta occurs through potentiation of basal nitric oxide activity and is blocked by L-NAME, L-NMMA and ADMA

The findings of this part of the study showed that superoxide dismutase produced a concentration-dependent relaxation in endothelium-containing rings of rat aorta that was partially blocked by pre-treatment with L-NAME. However, in the presence of the hydrogen peroxide scavenger, catalase, superoxide dismutase-induced relaxation was virtually abolished by L-NAME or endothelial denudation. These findings are consistent with previous work showing that the concentration-dependent, endothelium-mediated relaxation induced by superoxide dismutase is attributed to protection of basal nitric oxide from destruction by superoxide anion ($^{\bullet}O^{-}_{2}$) (Gryglewski *et al*., 1986; Rubanyi and Vanhoutte, 1986; Ohlstein and Nichols, 1989; Mian and Martin 1995; MacKenzie *et al*., 1999). However, the partial blockade of superoxide dismutase-induced relaxation by L-NAME in the absence of catalase indicates that this relaxation has at least two components; an L-NAME-sensitive nitric oxide-mediated component and a catalase-sensitive hydrogen peroxide-mediated component, as relaxation was almost completely blocked by co-treatment with L-NAME and catalase (Rubanyi and Vanhoutte, 1986; Furchgott *et al*., 1994; Yang *et al*., 1999; Iesaki *et al*., 1999; Itoh *et al*., 2003).

Therefore, in the presence of catalase, superoxide dismutase-induced relaxation is likely to be mediated solely through protecting basal nitric oxide activity from breakdown by superoxide anion. L-NMMA and ADMA, like L-NAME, were also able to significantly block superoxide dismutase-induced relaxation suggesting that they too block the synthesis of basal nitric oxide. The outcome of these experiments is therefore consistent with those

in the previous Chapter where L-NMMA and ADMA were deemed to block basal nitric oxide activity as assessed by their ability to enhance phenylephrine-induced tone in endothelium-containing rings.

The endothelium-dependent component of relaxation to the PDE isoform 5 inhibitor, T-0156, occurs through potentiation of basal nitric oxide activity and is abolished by L-NAME, L-NMMA and ADMA

Previous reports showed that the phosphodiesterase isoform 5 inhibitors, zaprinast (M&B 22948), T1032 and T0156, exhibit endothelium-dependent and endothelium-independent components of relaxation (Martin *et al*., 1986a; Hobbs *et al*., 1999; Mochida *et al*., 2002; Kimura *et al*., 2003; Mochida *et al*., 2004; Galley *et al*., 2004; Bauer and Sotnikova, 2010). The endothelium-dependent component has been attributed to potentiation of basal nitric oxide activity generated by the vascular endothelium, as the activation induced by these agents is associated with elevation of the nitric oxide-dependent second messenger, cyclic guanosine phosphate (cGMP), within the vascular smooth muscle cells. These previous results provide an explanation for findings from this study which showed that T0156 produced more powerful relaxation in endothelium-containing rat aortic rings than in endothelium-denuded. In addition, the nitric oxide synthase inhibitors, L-NAME, L-NMMA and ADMA, each produced a blockade of T0156-induced relaxation that was as powerful as that produced by endothelial denudation. These findings, like those with superoxide dismutase, are therefore consistent with the ability of L-NAME, L-NMMA and ADMA to block basal nitric oxide activity in rat aorta.

Calcium-calmodulin and the phosphatidylinositol 3-kinase pathways have a minor role in basal nitric oxide production in female rat aorta

A difference in the transduction mechanisms that govern basal and agonist-stimulated synthesis of nitric oxide could potentially explain the ability of L-NMMA and ADMA to block the former but not the latter in rat aorta. However, the researchers who first documented the presence of basal nitric oxide activity in rat aorta (Martin *et al*., 1986b; Rees *et al*., 1989; Moore *et al*., 1990) proposed that it was produced spontaneously by the vascular endothelium without the need for an external stimulus; this potentially excludes the existence of an intracellular signalling mechanism to control the synthesis of basal nitric oxide. This proposal was supported by the observations made by Rapoport and Murad (1983) that the resting levels of cGMP in entirely unstimulated endothelium-containing rat aortic rings were 2-3-fold higher than those in endothelium-denuded rings. Their findings too suggest that the basal nitric oxide activity in endothelium-containing rings occurred in the absence of any stimulus.

On the other hand, other researchers have proposed the existence of triggering stimuli that operate within the vascular endothelium to generate basal nitric oxide activity. Such stimuli may include calcium ions that pass from the contracting vascular smooth muscle cells through myo-endothelial gap junctions to the underlying endothelial cells (Dora *et al*., 2000; Jackson *et al*., 2008) where they (calcium ions) provoke the release of nitric oxide presumably via a calcium-calmodulin-mediated, calmidazolium-sensitive activation of endothelial nitric oxide synthase. Alternatively, Fleming *et al* (1999) proposed that isometric contraction may generate a mechanical stress on the endothelial cytoskeleton that triggers the release of nitric oxide via a phosphatidylinositol 3-kinase-mediated,

wortmannin-sensitive phosphorylation and activation of endothelial nitric oxide synthase, similar to that induced by shear stress generated by blood flow.

The findings of this study showed that calmidazolium and wortmannin each produced a statistically significant augmentation of phenylephrine-induced contraction which may suggest an inhibitory effect of these agents on basal nitric oxide production in rat aorta. However, the extent to which calmidazolium or wortmannin enhanced phenylephrine-induced contraction by blocking basal nitric oxide synthesis was substantially smaller than that produced by L-NAME. Moreover, neither of these agents blocked the endothelium-dependent nitric oxide mediated relaxation induced by superoxide dismutase or T0156 to a significant extent, although a combination of the two did produce a small degree blockade. Taken together, these data indicate that calcium-calmodulin-dependent and phosphatidylinositol 3-kinase-mediated activation of endothelial nitric oxide synthase play only a minor role in basal nitric oxide production in female rat aorta. The major component of basal nitric oxide production appears to occur, however, through spontaneous, unstimulated activity of the enzyme.

Relaxation induced by calcitonin gene-related peptide-1 and calcium ionophore A23187 in female rat aorta is differentially sensitive to blockade by L-NMMA and ADMA

One of the main findings from this study was that although L-NAME powerfully blocked acetylcholine-induced relaxation, L-NMMA and ADMA had a small inhibitory effect on tissue sensitivity to acetylcholine (2-3-fold) but did not affect the maximal response to this agent; this finding with L-NMMA supports the earlier observations of Frew and co-workers (1993). The aim of this part of the study was to determine if L-NMMA and

ADMA were poor inhibitors of relaxations induced by other agents, namely calcitonin gene-related peptide-1 and calcium ionophore A23187.

The data from this study showed that calcitonin gene-related peptide-1 induced endothelium-dependent, nitric oxide-mediated relaxation in rat aorta, as relaxation is powerfully blocked by the nitric oxide synthase inhibitor, L-NAME. Surprisingly, however, and in contrast with the findings with acetylcholine, both L-NMMA and ADMA powerfully blocked relaxation to calcitonin gene-related peptide-1. These data are in agreement with previous reports (Gray and Marshall, 1992; Abdelrahman *et al*., 1992, de Hoon *et al*., 2003; Thakor and Giussani, 2005) which showed that the endothelium-dependent relaxation induced by gene-related peptide-1 is mediated by nitric oxide.

In addition, the findings of this study showed that relaxation induced by calcium ionophore A23187, which acts to increase endothelial calcium levels by a receptor-independent mechanism, is powerfully blocked by L-NAME. This strongly suggests that this relaxation is endothelium-dependent and nitric oxide-mediated, which is consistent with previous reports (Weinheimer and Osswald, 1986; Schini and Vanhoutte, 1992; Taniguchi *et al*., 1999; Shi *et al*., 2007). As with acetylcholine, but unlike with calcitonin gene-related peptide-1, L-NMMA and ADMA, had little effect on relaxation induced by calcium ionophore A23187. It was clear, therefore, that an explanation was required for the ability of L-NMMA and ADMA to block the endothelium-dependent, nitric oxide-mediated relaxation induced by calcitonin gene-related peptide-1, but not by acetylcholine or A23187.

One potential explanation for these differential effects of L-NMMA and ADMA is the possibility that the endothelium-dependent relaxation induced by calcitonin gene-related

peptide-1 in the female rat aorta is mediated by relaxing factor(s) other than nitric oxide as has been proposed in other tissues such as the male rat aorta, cultured human umbilical vein endothelial cells or rabbit cutaneous microvasculature (Grace *et al*., 1987; Crossman *et al*., 1987; Hughes and Brain, 1994). This explanation can be ruled out, however, because in keeping with previous reports (Gray and Marshall, 1992a; Abdelrahman *et al*., 1992, Hoon *et al*., 2003; Thakor and Giussani, 2005), the standard nitric oxide synthase inhibitor, L-NAME, virtually abolished relaxation to calcitonin gene-related peptide-1, showing it is almost certainly mediated by nitric oxide.

Another potential explanation for the differential effects of L-NMM and ADMA on relaxation induced by calcitonin gene-related peptide-1, acetylcholine and A23187 is that these agonists might stimulate the release of nitric oxide by nitric oxide synthase through the activation of different transduction pathways, and this is explored in the next section.

The endothelium-dependent, nitric oxide-mediated relaxation induced by acetylcholine, calcium ionophore A23187 and calcitonin gene-related peptide-1 are all mediated by the calcium-calmodulin pathway

Previous work has shown that the shearing force of flowing blood induces the generation of nitric oxide via the wortmannin-sensitive, phosphatidylinositol 3-kinase/Akt pathway which activates endothelial nitric oxide synthase through a calcium-independent phosphorylation of serine 1177 (Dimmeler *et al*., 1999; Fulton *et al*., 1999; McCabe *et al*., 2000). Certain agonists, including VEGF and insulin, also stimulate nitric oxide production using this pathway (Zeng and Quon, 1996; Papapetropoulos *et al*., 1997; Fulton *et al*., 1999; Fleming *et al*., 2001). In contrast, the generation of nitric oxide by other agonists such as acetylcholine, A23187 or substance P is stimulated via the calmidazolium-

sensitive, direct activation of nitric oxide synthase by calcium-calmodulin (Weinheimer and Osswald, 1986; Archer and Crowan, 1991; Schini and Vanhoutte, 1992; Taniguchi *et al*., 1999; Sunagawa *et al*., 2000; Schneider *et al*., 2003). In agreement with this latter conclusion, data from the present study showed that calmidazolium powerfully blocked the endothelium-dependent, nitric oxide-mediated relaxation induced by acetylcholine, A23187 or calcitonin gene-related peptide-1, while the phosphatidylinositol 3-kinase inhibitor, wortmannin, had no effect. These findings strongly suggest that these three distinct agonists signal through a seemingly similar calcium-calmodulin-dependent transduction mechanism to stimulate the release of nitric oxide in rat aorta. It is therefore clear that the ability of L-NMMA and ADMA to block relaxation to calcitonin gene-related peptide-1, but not to acetylcholine or A23187, cannot be explained by these agonists acting via different transduction pathways.

Isoprenaline and forskolin possess endothelium-dependent and endothelium-independent components of relaxation in female rat aorta

The non-selective β-adrenoceptor agonist, isoprenaline, and the adenylate cyclase activator, forskolin, have been reported to relax rat aorta by two distinct mechanisms: an endothelium-dependent, nitric oxide-mediated component, and a component due to direct effect on the vascular smooth muscle cells (Kamata *et al*., 1989; Gray and Marshall, 1992b; Karasu *et al*., 1997).

The endothelium-dependent component of relaxation to isoprenaline and forskolin is believed to be due to the calcium-independent activation of endothelial nitric oxide synthase through phosphorylation of serine 1177 by cyclic AMP-dependent protein kinase A (PKA). A potential opportunity thus arose to compare the effects of L-NMMA and

ADMA with those of L-NAME on relaxation induced through activation of endothelial nitric oxide synthase by PKA-mediated phosphorylation.

The data from this study showed that isoprenaline and forskolin each induced potent concentration-dependent relaxation of endothelium-containing rings. However, in the presence of L-NAME, the relaxations obtained to both agents were not substantially different from those in control tissues. Why this study appeared to generate a smaller component due to nitric oxide than previous studies (Gray and Marshall, 1992b; Mikio *et al*., 1997) is unclear. One possibility relates to the level of tone employed in the different studies. In this study, the levels of tone were carefully matched in control and L-NAME-treated preparations. The previous reports (Gray and Marshall, 1992b; Mikio *et al*., 1997) did not indicate the level of tone used, so it is possible that in these the level of tone was substantially higher in treated tissues than in controls due to blockade of basal nitric oxide activity. If so, the substantially higher levels of blockade reported might have resulted from physiological antagonism rather than blockade of agonist-stimulated production of nitric oxide.

Thus, because no substantial endothelium-dependent, nitric oxide-mediated component of relaxation could be observed in response to isoprenaline or forskolin it was, therefore, impossible to conduct the planned experiments to determine the effects of L-NMMA and ADMA on this component.

The ability of L-NMMA and ADMA to block endothelium-dependent, nitric oxide-mediated relaxation in female rat might be determined by the efficacy of the relaxant agent

Previous studies (Grace *et al*., 1987; Gray and Marshall, 1992a) showed that calcitonin gene-related peptide-1 induced concentration-dependent relaxation in endothelium-containing rat aortic rings, and the maximal response elicited by this agonist was lower than that induced by acetylcholine in the same tissue. Moreover, these studies showed that calcitonin gene-related peptide-1-induced relaxation was powerfully blocked by L-NMMA and L-NOARG.

The results of the present study also showed that the maximum relaxation calcitonin gene-related peptide-1 produced in female rat aorta is lower than that elicited by acetylcholine or A23187. Also, the results of this study showed that this relaxation, in contrast to that induced by acetylcholine or A23187, was powerfully blocked by L-NMMA and ADMA.

It is possible therefore that the ability of L-NMMA and ADMA to block relaxation to calcitonin gene-related peptide-1 but not to acetylcholine or A23187 might somehow related to the maximum relaxation these agents can elicit. In light of this possibility, a question emerged of whether the lower efficacy of calcitonin gene-related peptide-1, in comparison with that of acetylcholine or A23187, was the reason why relaxation induced by this agent was susceptible to blockade by L-NMMA or ADMA. This point will be further investigated in the next Chapter by use of the M_3 receptor partial agonist, butyrylcholine, and by lowering the apparent efficacy of acetylcholine by using the irreversible alkylating agent, phenoxybenzamine (Martin *et al*., 1992).

Chapter Four

Introduction

The findings of the present study so far showed that, in the female rat aorta, the inhibitors of nitric oxide synthase, L-NMMA and ADMA, have seemingly differential abilities to block basal but not agonist (acetylcholine or A23187)-stimulated activity of nitric oxide. However, the findings also showed that these inhibitors significantly blocked the endothelium-dependent, nitric oxide-mediated relaxation induced by calcitonin gene-related peptide-1 in the same tissue.

It was clear that the ability of L-NMMA and ADMA to block relaxation to calcitonin gene-related peptide-1 but not to acetylcholine or A23187 was not related to differences in transduction mechanisms used by these agents to produce relaxation, since all these agents seemed to stimulate endothelial nitric oxide production via the same calcium-calmodulin-dependent, calmidazolium-sensitive pathway.

One other aspect that also appeared to differentiate calcitonin gene-related peptide-1 from acetylcholine and A23187 was its lower maximal relaxant capacity. It is therefore possible that the differential effects of L-NMMA and ADMA might be related to the efficacy of the relaxant agonist.

This possibility is supported by a previous report showing that the ability of L-NAME to block endothelium-dependent, nitric oxide-mediated relaxation in the rabbit jugular vein is determined by the efficacy of the relaxant agent (Martin *et al*., 1992). In this latter study, the researchers found in rat aorta that L-NAME produced powerful blockade of acetylcholine-induced, endothelium-dependent, nitric oxide-mediated relaxation, whereas in rabbit jugular vein L-NAME had little effect on same relaxation. However, in the latter

tissue L-NAME powerfully blocked endothelium-dependent relaxation induced by α-methyl-5-hydroxytryptamine, a partial agonist at endothelial 5-HT_2 receptors (Leff *et al*., 1987), or relaxation induced by the M_3 muscarinic receptor partial agonists, pilocarpine and butyrylcholine. The researchers concluded that the differential effects of L-NAME were not due to different receptor subtypes or to different endothelium-derived relaxing factors, but were potentially attributed to the effectiveness of receptor-effector coupling. To test this hypothesis, the researchers employed the irreversible alkylating agent, phenoxybenzamine, to reduce the apparent efficacy of acetylcholine in the rabbit jugular vein to the extent that acetylcholine behaved similar to a partial agonist. Under these conditions they showed that L-NAME now produced powerful blockade of acetylcholine-induced relaxation in rabbit jugular vein. Taking these observations together, Martin and co-workers (1992) concluded that the ability of L-NAME to block endothelium-dependent, nitric oxide-mediated relaxation in the rabbit jugular vein is determined by the efficacy of the relaxant agent.

With the finding of Martin and co-workers (1992) in mind, the aim of the experiments in this part of the study was to determine whether the ability of L-NMMA or ADMA to block relaxation induced by calcitonin gene-related peptide-1 but not acetylcholine or A23187 is attributed to the lower maximal response elicited by this agonist.

This possibility was investigated using the M_3 receptor partial agonist, butyrylcholine, and by lowering the apparent efficacy of acetylcholine using the irreversible alkylating agent, phenoxybenzamine (Martin *et al*., 1992).

Material and Methods

Preparation of aortic rings for tension recording

The preparation of aortic rings for tension recording was essentially similar to that described in the previous Chapter except that the animals were killed by CO_2 overdose.

The effects of L-NMMA and ADMA on acetylcholine- and butyrylcholine-induced relaxation

Experiments from the previous Chapters investigated and compared the effects of the nitric oxide synthase inhibitors, L-NMMA and ADMA, on relaxation induced by acetylcholine, A23187 or calcitonin gene-related peptide-1 in female rat endothelium-containing aortic rings.

The aims of these experiments were to investigate if the limited ability of L-NMMA and ADMA to block relaxation induced by acetylcholine or A23187, on the one hand, but significantly block relaxation induced by CGRP-1, on the other hand, is related to the efficacy of the relaxant agonist. This was examined by comparing the effects of L-NMMA and ADMA on relaxation induced by the full agonist, acetylcholine, with those on relaxation to the M_3 partial agonist, butyrylcholine (Martin *et al*., 1992).

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In experiments involving the muscarinic partial agonist, butyrylcholine, female rat endothelium-containing aortic rings were allowed to equilibrate for 90 min with the resting stretch re-adjusted to 10 mN, if required. The tissues were then treated with L-NMMA (100 µM or 1 mM for 30 min) or ADMA (100 µM or 1 mM for 1 h). After this time the treated tissues and their time-matched controls were pre-contracted to ~50% of maximal phenylephrine tone. Care was taken to induce comparable levels of tone in control and

treated tissues by using lower concentrations of phenylephrine in the latter. After stabilisation of tone, a full concentration-response curve to butyrylcholine (100 nM-300 µM) was conducted. Thereafter, papaverine (300 µM) was added to produce full relaxation of the tissues.

Effects of the contractile agent on relaxation induced by butyrylcholine or acetylcholine

The aim of these experiments was to determine if the ability of acetylcholine or butyrylcholine to induce endothelium-dependent relaxation in rat aorta differed depending on the contractile agent used to raise tone. These preliminary experiments were required because, as will be seen later, experiments were conducted to lower the apparent efficacy of acetylcholine using the irreversible alkylating agent, phenoxybenzamine. Since this agent also inhibits the contractile effects of phenylephrine, a suitable alternative contractile agent needed to be identified.

In these experiments, female rat endothelium-containing aortic rings were allowed to equilibrate for 90 min and the resting stretch re-adjusted to 10 mN, if required. The tissues were then pre-contracted to a submaximal level of phenylephrine- or endothelin-1 (20-40 nM)-induced tone. After stabilisation of tone, a full concentration-response curve to acetylcholine (1 nM-10 µM) or to butyrylcholine (100 nM-300 µM) was conducted. Thereafter, papaverine (300 µM) was added to produce full relaxation of the tissues.

Effects of L-NMMA or ADMA on acetylcholine-induced relaxation in tissues pre-contracted with endothelin-1

The aim of these experiments was to investigate if the limited ability of L-NMMA or ADMA to block the endothelium-dependent relaxation induced by acetylcholine in rat aorta is also seen when endothelin-1 is used as the contractile agent.

In these experiments, female rat endothelium-containing aortic rings were allowed to equilibrate for 90 min with the resting stretch re-adjusted to 10 mN, if required. The tissues were then treated with L-NMMA (100 µM or 1 mM for 30 min) or ADMA (100 µM or 1 mM for 1 h). After this time the treated tissues and their time-matched controls were pre-contracted to a submaximal level of endothelin-1-induced tone. Care was taken to induce comparable levels of tone in control and treated tissues by using lower concentrations of endothelin-1 in the latter. After stabilisation of tone, a full concentration-response curve to acetylcholine (1 nM-10 µM) was conducted. Thereafter, papaverine (300 µM) was added to produce full relaxation of the tissues.

Effects of phenoxybenzamine on relaxation induced by acetylcholine or butyrylcholine

The aim of these experiments was to determine the extent to which the irreversible alkylating agent, phenoxybenzamine (Martin *et al*., 1992), reduced the efficacy of the full agonist, acetylcholine, and the partial agonist, butyrylcholine, in rat aorta.

In these experiments, female rat endothelium-containing aortic rings were allowed to equilibrate for 90 min with the resting stretch re-adjusted to 10 mN, if required. Some tissues were then treated with phenoxybenzamine (3 µM for 30 min followed by washout)

and others were left as controls. The tissues were then pre-contracted to a submaximal level of endothelin-1-induced tone before conducting a full concentration-response curve to acetylcholine (1 nM-10 µM) or butyrylcholine (10 µM-300 µM). Thereafter, papaverine (300 µM) was added to produce full relaxation of the tissues.

Effects of L-NMMA and ADMA on acetylcholine-induced relaxation after treatment with phenoxybenzamine

The aim of these experiments was to determine if the limited ability of L-NMMA and ADMA to block acetylcholine-induced relaxation in female rat endothelium-containing aortic rings was influenced by lowering the apparent efficacy of acetylcholine using the irreversible alkylating agent, phenoxybenzamine.

In these experiments, female rat endothelium-containing aortic rings were allowed to equilibrate for 90 min with the resting stretch re-adjusted to 10 mN, if required. Some tissues were then treated with phenoxybenzamine (1 µM or 3 µM, for 30 min followed by washout) and others were left as controls. Some of the phenoxybenzamine-treated tissues were then treated with L-NMMA (100 µM or 1 mM, 30 min) or ADMA (100 µM or 1 mM, 1 h), respectively. After this time the treated tissues and their time-matched controls were pre-contracted to a submaximal level of endothelin-1-induced tone. Care was taken to induce comparable levels of tone both in control and nitric oxide synthase inhibitor-treated tissues by using lower concentrations of endothelin-1 in the latter. After stabilisation of tone, a full concentration-response curve to acetylcholine (1 nM-10 µM) was conducted. Thereafter, papaverine (300 µM) was added to produce full relaxation of the tissues.

Drugs and chemicals

Acetylcholine chloride (ACh), asymmetric N^G, N^G-dimethyl-L-arginine dihydrochloride (ADMA), N^G-monomethyl-L-arginine acetate (L-NMMA), papaverine hydrochloride, phenylephrine hydrochloride (PE), endothelin-1 (ET-1; human), butyrylcholine chloride (BCh) and phenoxybenzamine hydrochloride were all obtained from Sigma, UK. All drugs were dissolved and diluted in 0.9% saline except phenoxybenzamine which was dissolved in 100% ethanol.

Data analysis

Papaverine (300 μM) was added at the end of each experiment to produce full relaxation of tissues and the relaxant responses to agonists were expressed as a percentage of the full relaxation induced by this agent. Data are expressed as the mean ± S.E.M of n separate observations. Concentration-effect curves were analysed and drawn by fitting the data to formula detailed on page 56, and statistical analysis was performed by using one-way analysis of variance followed by Bonferroni's post-test or by Student's *t* test, as appropriate, with the aid of a computer-based program (Graph Pad, San Diego, USA). Values were considered to be statistically different when P was ≤ 0.05.

Results

Butyrylcholine-induced relaxation

The muscarinic partial agonist, butyrylcholine (100 nM-300 μM), elicited concentration-dependent relaxation in female rat endothelium-containing aortic rings precontracted to ~50% of maximal PE tone (Figure 4.1a and b; Table 4.1). Consistent with its action as a partial agonist, butyrylcholine had a significantly lower E_{max} of 72.6 ± 5.4% than that seen with the full agonist, acetylcholine (E_{max} 91.8 ± 1.5%; $P < 0.01$; Figure 4.2). Unlike with acetylcholine (Chapter Two), relaxation to butyrylcholine was significantly blocked in a concentration-dependent manner by L-NMMA and ADMA (both at 0.1 or 1 mM).

Thus, L-NMMA or ADMA appears to block relaxation induced by the partial agonist, butyrylcholine, but not to the full agonist, acetylcholine (Chapter Two). The possibility that the efficacy of the relaxant agonist determines whether or not relaxation will be blocked by L-NMMA or ADMA seems worthy of further investigation.

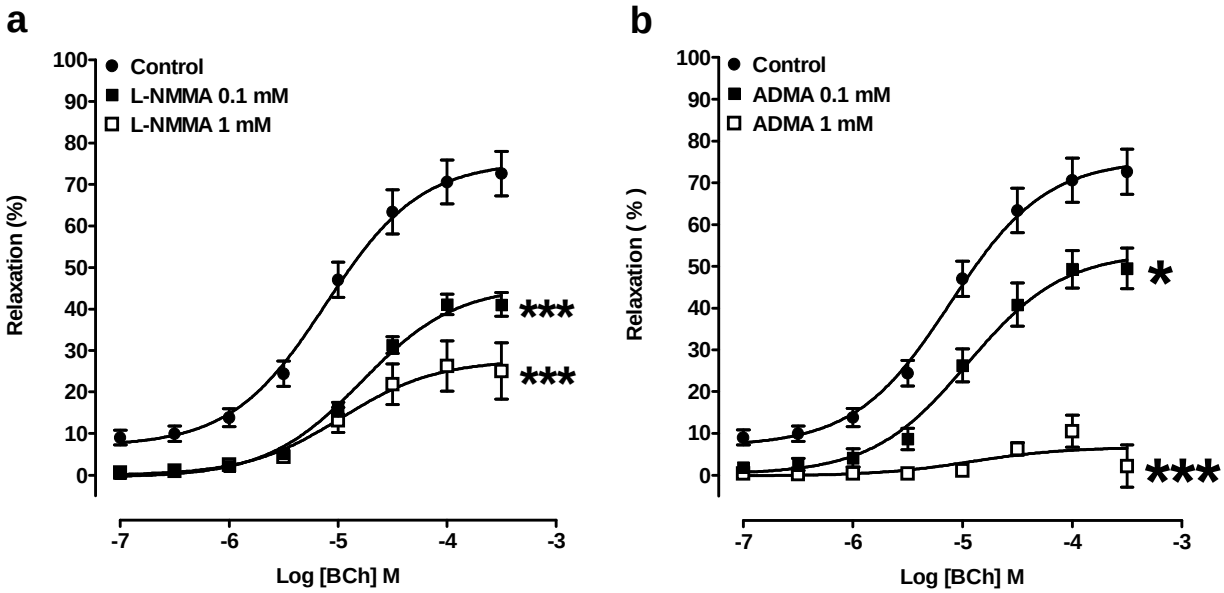

Figure 4.1 Cumulative concentration-effect curves showing relaxation to butyrylcholine (BCh) in female rat endothelium-containing aortic rings submaximally contracted with PE in the absence and presence of (a) L-NMMA for 30 minutes or (b) ADMA for 1 h (both at 0.1 and 1 mM). Both L-NMMA- and ADMA-treated tissues show concentration-dependent blockade of relaxation. Each point represents the mean ± SEM of 6-12 observations. * P< 0.05 and *** P< 0.001 indicate significant differences from control.

Table 4.1 The effects of ADMA (for 1 h) or L-NMMA (for 30 min) on butyrylcholine-induced relaxation in female rat endothelium-containing aortic rings submaximally contracted with PE.

Treatment	**pEC_{50}**	**E_{max} ± SEM (%)**
Control	5.11 ± 0.05	72.6 ± 5.4
ADMA 0.1 mM	4.95 ± 0.07	49.5 ± 4.9 *
ADMA 1 mM	4.92 ± 0.83	10.5 ± 3.9 ***
L-NMMA 0.1 mM	4.78 ± 0.08 **	41.1 ± 2.9 ***
L-NMMA 1 mM	4.97 ± 0.1	26.3 ± 6.0 ***

Data are the mean ± SEM of 6-12 observations. * P< 0.05, ** P< 0.01 and *** P< 0.001 indicate a significant difference from control.

Effect of the contractile agent on relaxation induced by butyrylcholine or acetylcholine

Butyrylcholine (100 nM-300 µM) produced concentration-dependent relaxation in female rat endothelium-containing aortic rings precontracted to ~50% of maximal PE tone (E_{max} 72.6 ± 5.4%; pEC_{50} 5.11 ± 0.05) (Figure 4.2a), however, the tissue sensitivity as well as its maximal relaxation to butyrylcholine were decreased when it was precontracted to a similar level with endothelin-1, achieved using (20-40 nM).

When acetylcholine (1 nM-10 µM) was the relaxant agent, it induced concentration-dependent relaxation (E_{max} 91.8 ± 1.5%; pEC_{50} 7.70 ± 0.03) in tissues submaximally contracted with PE (Figure 4.2b). The tissue sensitivity was reduced (pEC_{50} 7.29 ± 0.03, $P< 0.001$), but its maximal response to acetylcholine was not significantly affected when endothelin-1 was used to induce submaximal tone.

In the forthcoming experiments when the tissues are to be treated with the irreversible alkylating agent, phenoxybenzamine, in order to reduce the efficacy of acetylcholine, and contraction to PE is no longer possible, endothelin-1 will be used to induce submaximal tone.

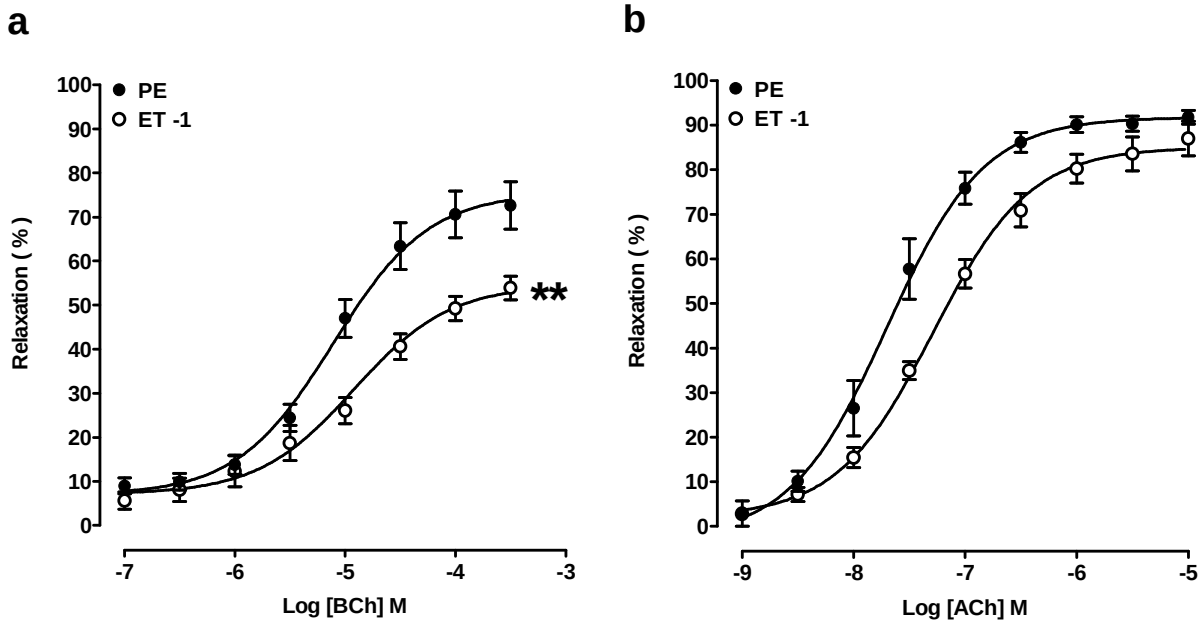

Figure 4.2. Cumulative concentration-effect curves showing relaxation to (a) butyrylcholine and (b) acetylcholine in female rat endothelium-containing aortic rings submaximally contracted with PE or endothelin-1 (ET-1). Tissues precontracted with ET-1 show a decrease in sensitivity to the two relaxants than those precontracted with PE, with the greater reduction occurring with the partial agonist, butyrylcholine. Each point represents the mean ± SEM of 6-13 observations. ** $P < 0.01$ indicates a significant difference from PE.

Effect of L-NMMA or ADMA on acetylcholine-induced relaxation in tissues precontracted with endothelin-1

Acetylcholine (1 nM-10 µM) induced concentration-dependent relaxation in female rat endothelium-containing aortic rings precontracted with endothelin-1 (Figure 4.3). This relaxation was unaffected by treatment with L-NMMA at 0.1 or 1 mM or by ADMA at 0.1 mM. However, ADMA at 1 mM produced a very slight depression of maximal relaxation to acetylcholine.

Therefore, regardless of whether tissues are contracted with PE or with endothelin-1, acetylcholine-induced relaxation appears to be largely resistant to blockade by L-NMMA or ADMA.

Effects of phenoxybenzamine on relaxation induced by butyrylcholine or acetylcholine

When female rat endothelium-containing aortic rings were treated with the irreversible alkylating antagonist, phenoxybenzamine (3 µM for 30 min), followed by washout and subsequent constriction with ET-1, dilatation to butyrylcholine (100 nM-300 µM) or acetylcholine (1 nM-10 µM) was significantly blocked in comparison to their time-matched control tissues (Figure 4.4a and b, respectively). Relaxation to the partial agonist, butyrylcholine, was almost abolished (E_{max} 53.9 ± 2.7 and 8.5 ± 1.9 for control and treated tissues, respectively), while that to acetylcholine was reduced but to a lesser degree (E_{max} 87.9 ± 2.6 and 63.3 ± 3.5 for control and treated tissues, respectively).

Moreover, following treatment with phenoxybenzamine, acetylcholine (1 nM-10 µM)-induced relaxation was inhibited by both L-NMMA and ADMA (both at 0.1 or 1 mM) (Figure 4.5a and b; Tables 4.2 and 4.3, respectively).

Therefore, although L-NMMA and ADMA have little effect on acetylcholine-induced relaxation in control tissues, they produce powerful blockade when the efficacy of acetylcholine is reduced to that of a partial agonist, by reducing the number of functional muscarinic receptors through the use of the irreversible alkylating agent, phenoxybenzamine.

Thus, these experiments, when taken together with those using the partial agonist, butyrylcholine, suggest that the ability of L-NMMA or ADMA to block agonist-induced relaxation seems to be determined by the efficacy of the relaxant agonist.

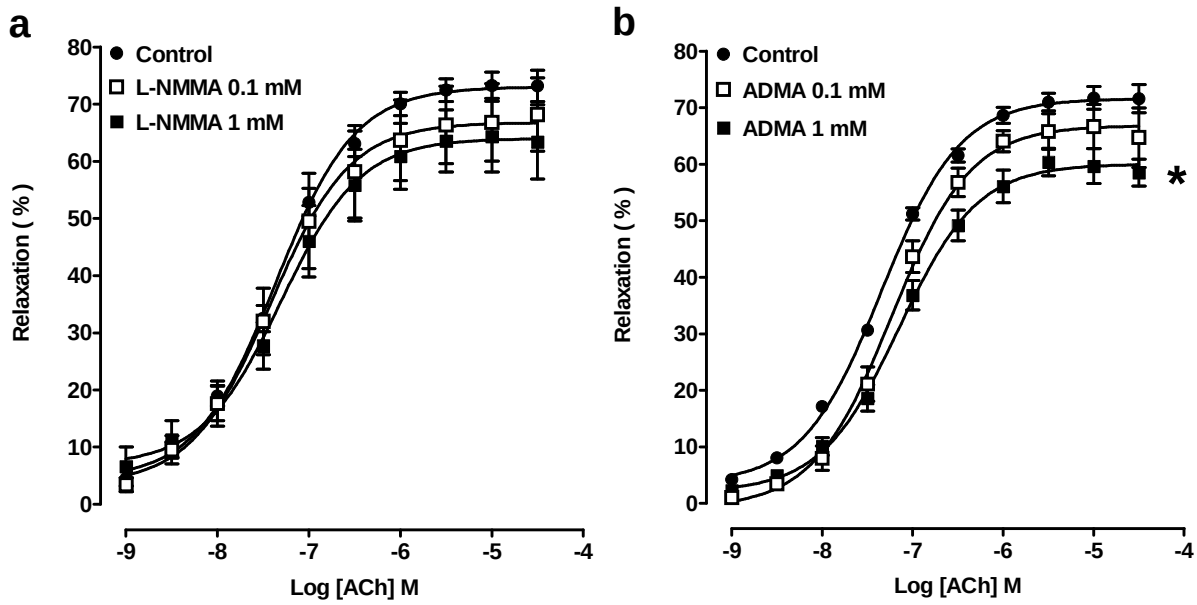

Figure 4.3. Cumulative concentration-effect curves showing relaxation to acetylcholine in rat endothelium-containing aortic rings submaximally contracted with endothelin-1 in the absence and presence of (a) L-NMMA for 30 minutes or (b) ADMA for 1 h (both at 0.1 and 1 mM). Relaxation was very slightly depressed in the presence of 1 mM ADMA, but was unaffected by L-NMMA. Each point represents the mean ± SEM of 5-11 observations. * $P < 0.05$ indicates a significant difference from control.

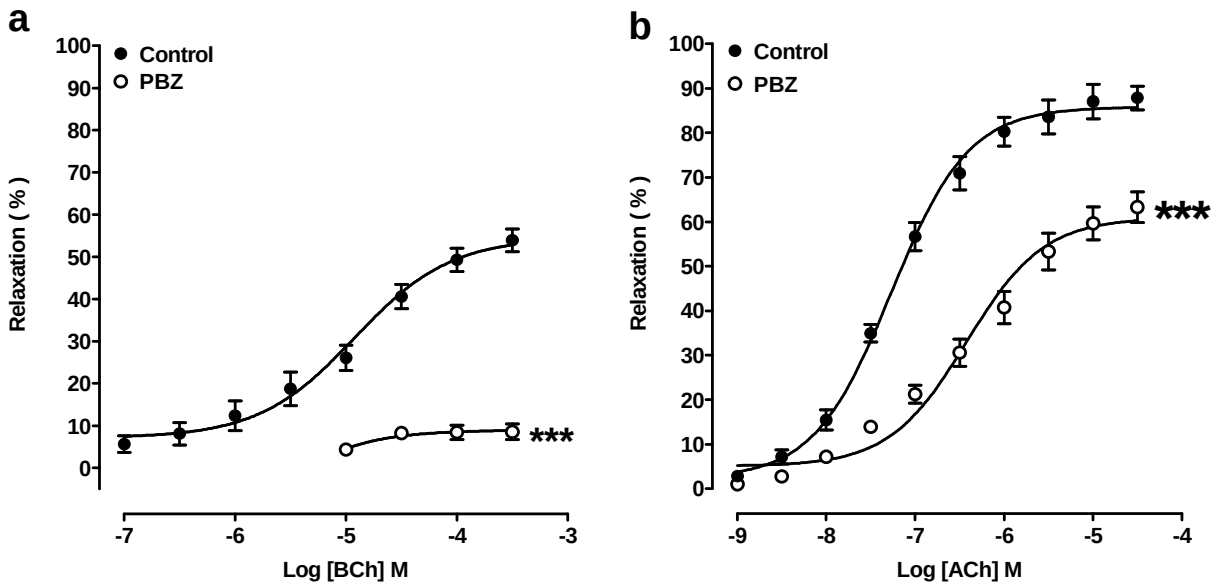

Figure 4.4. Cumulative concentration-effect curves showing relaxation to (a) butyrylcholine and (b) acetylcholine in rat endothelium-containing aortic rings submaximally contracted with endothelin-1. Responses are shown for control tissues and for tissues treated with the irreversible alkylating agent, phenoxybenzamine (3 μM for 30 minutes followed by washout). Relaxations to both relaxants were significantly blocked by phenoxybenzamine; however, those in response to the partial agonist, butyrylcholine, were more powerfully blocked. Each point represents the mean ± SEM of 6-13 observations. *** $P < 0.001$ indicates a significant difference from control.

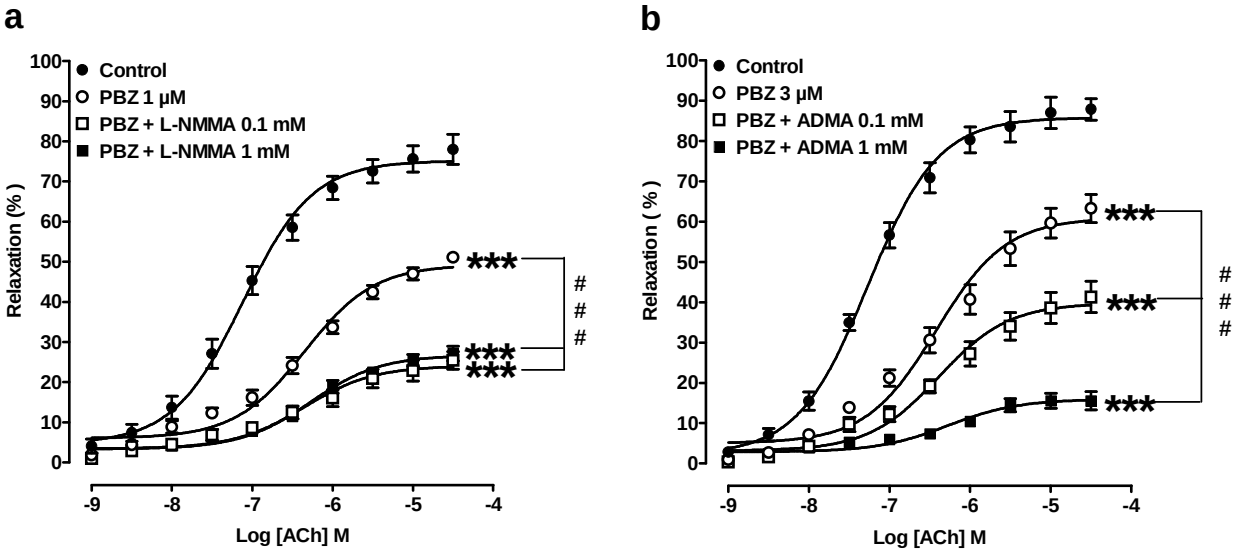

Figure 4.5. Cumulative concentration-effect curves showing relaxation to acetylcholine in rat endothelium-containing aortic rings precontracted with endothelin-1. Responses are shown for control tissues, tissues treated with phenoxybenzamine 1 µM (a) or 3 µM (b) for 30 minutes followed by washout, and tissues treated with phenoxybenzamine then with L-NMMA for 30 minutes (a) or ADMA for 1 h (b) (both at 0.1 and 1 mM). Following treatment with phenoxybenzamine, L-NMMA and ADMA produced potent blockade of relaxation. Each point represents the mean ± SEM of 5-10 observations. *** P< 0.001 indicates a significant difference from control; ### P< 0.001 indicates significant differences from phenoxybenzamine only-treated tissues.

Table 4.2 Effects of phenoxybenzamine (PBZ; 1 µM for 30 min followed by washing) alone or PBZ then L-NMMA (0.1 or 1 mM, for 30 min) on acetylcholine-induced relaxation in female rat endothelium-containing aortic rings submaximally contracted with endothelin-1.

Treatment	**pEC_{50}**	**E_{max} ± SEM (%)**
Control	7.12 ± 0.05	78.0 ± 3.7
PBZ	6.35 ± 0.11 ***	51.1 ± 1.2 ***
PBZ + L-NMMA (0.1 mM)	6.35 ± 0.13 ***	25.4 ± 2.2 *** ###
PBZ + L-NMMA (1 mM)	6.28 ± 0.09 ***	27.1 ± 1.8 *** ###

Data are the mean ± SEM of 5-10 observations. *** P< 0.001 indicates a significant difference from control; ### P< 0.001 indicates a significant difference from PBZ-treated tissues.

Table 4.3 Effects of phenoxybenzamine (PBZ; 3 µM for 30 min followed by washing) alone or PBZ then ADMA (0.1 or 1 mM, for 1 h) on acetylcholine-induced relaxation in rat endothelium-containing aortic rings submaximally contracted with endothelin-1.

Treatment	**pEC_{50}**	**E_{max} ± SEM (%)**
Control	7.27 ± 0.04	87.9 ± 2.7
PBZ	6.42 ± 0.11 ***	63.3 ± 3.5 ***
PBZ + ADMA (0.1 mM)	6.39 ± 0.11 ***	41.3 ± 3.9 *** ###
PBZ + ADMA (1 mM)	6.28 ± 0.16 ***	15.6 ± 2.3 *** ###

Data are the mean ± SEM of 6 observations. *** P< 0.001 indicates a significant difference from control; ### P< 0.001 indicates a significant difference from PBZ-treated tissues.

Discussion

Butyrylcholine-induced relaxation

The findings of this part of the study showed that the M_3 partial agonist, butyrylcholine (Martin *et al.*, 1992), induced concentration-dependent relaxation in female rat endothelium-containing aortic rings. As expected, the maximal relaxant response elicited by this relaxant agent was lower than that by the full agonist, acetylcholine, in the same tissue. Strikingly, the nitric oxide synthase inhibitors, L-NMMA and ADMA, which have relatively little effect on acetylcholine-induced relaxation, produced potent blockade of butyrylcholine-induced relaxation.

Previous results of this study showed that calcitonin gene-related peptide-1 induced concentration-dependent relaxation in female rat endothelium-containing aortic rings, but the maximal response elicited by this agonist was lower than that elicited by acetylcholine or A23187 in the same tissue (Chapter Three). Furthermore, the nitric oxide synthase inhibitors, L-NMMA and ADMA, almost abolished relaxation induced by calcitonin gene-related peptide-1, whereas they had little effect on relaxation induced by acetylcholine or A23187 in the same tissue.

Previous work by others has suggested that the efficacy of the relaxant agonist is critical in determining the ability of L-NAME to block endothelium-dependent, nitric oxide-mediated relaxation (Martin *et al*., 1992). Taken together, it seemed possible that the lower relaxant efficacy of calcitonin gene-related peptide-1 and butyrylcholine, in comparison with acetylcholine or A23187, was the reason why either relaxant effect was susceptible to blockade by L-NMMA or ADMA. This possibility was investigated further in this study by

lowering the apparent efficacy of acetylcholine using the irreversible alkylating agent, phenoxybenzamine (Martin *et al*., 1992).

Effect of the contractile agent on relaxation induced by butyrylcholine or acetylcholine

The aim of conducting these experiments was to determine whether the contractile agent used to induce tone has an influence on the ability of acetylcholine or butyrylcholine to elicit endothelium-dependent relaxation in rat aorta. These preliminary experiments were required because, as will be seen later, experiments were conducted to lower the apparent efficacy of acetylcholine using the irreversible alkylating agent, phenoxybenzamine. Since this agent also inhibits the contractile effects of α-adrenergic agonists such as phenylephrine (Kirpekar and Wakade, 1970; Nina and Guzzetta, 2007), a suitable alternative contractile agent needed to be identified.

Previous results from this study (Chapter Two) showed that acetylcholine-induced relaxation in female rat endothelium-containing aortic rings (E_{max} 81.7 ± 3.8%, pEC_{50} 7.54 ± 0.05) was relatively similar regardless of the contractile agent used to induce tone. However, neither 5-hydroxytryptamine nor prostaglandin $F_{2\alpha}$, proved to be suitable alternatives to phenylephrine. 5-hydroxytryptamine-induced contraction is also blocked by phenoxybenzamine (Trevor *et al*., 2008) and prostaglandin $F_{2\alpha}$-induced contraction was insufficiently stable in the presence of this blocking agent. Endothelin-1 was therefore tested as a replacement for phenylephrine.

The results showed that in female rat endothelium-containing aortic rings, the tissue sensitivity and maximal response to the relaxant action of the partial agonist, butyrylcholine, were significantly reduced when the tissues were precontracted with

endothelin-1 in comparison to their time-matched controls precontracted with phenylephrine to the same level of tone. However, when the tissues were precontracted with endothelin-1 instead of phenylephrine, and acetylcholine was the relaxant agent, the tissue sensitivity was reduced but its maximal response was not affected. In these experiments 100-300 nM phenylephrine was needed to induce an intermediate level of tone, whereas only 10-40 nM endothelin-1 was needed to induce a comparable level of tone.

The above outcomes could be potentially attributed to the greater vascular reactivity of endothelin-1 when compared phenylephrine (Kurcer *et al*., 2006), which resulted in a kind of physiological antagonism that opposed relaxation to the partial agonist, butyrylcholine, more powerfully than to the full agonist, acetylcholine.

As a consequence of the above, when the tissues were to be treated with phenoxybenzamine to lower the apparent efficacy of acetylcholine, new control concentration-response curves were required for acetylcholine in the presence of endothelin-1-induced submaximal tone.

The findings of this study showed that as with phenylephrine-induced tone, the nitric oxide synthase inhibitors, L-NMMA and ADMA, did not block acetylcholine-induced relaxation in female rat endothelium-containing aortic rings precontracted with endothelin-1, apart from a slight depression in the maximal response caused by ADMA at 1000 µM. These results are in general agreement with previous work (Frew *et al*., 1993) and in agreement with the results from Chapters two and three of this study which showed that acetylcholine-induced relaxation is largely resistant to blockade by L-NMMA or ADMA,

regardless of animal gender (female vs male), the blood vessel examined (rat aorta vs carotid artery) or the contractile agent used (phenylephrine, 5-hydroxytryptamine or prostaglandin $F_{2\alpha}$).

Effects of phenoxybenzamine on relaxation induced by acetylcholine or butyrylcholine in female rat aorta

The aim of conducting these experiments was to determine the extent to which the irreversible alkylating agent, phenoxybenzamine (Martin *et al*., 1992), reduced the relaxant efficacy of the full agonist, acetylcholine, and the partial agonist, butyrylcholine, in rat aorta.

The findings of this study showed that when female rat endothelium-containing aortic rings were treated for 30 minutes with phenoxybenzamine (3 μM), followed by washout and then submaximally contracted with endothelin-1, relaxation to acetylcholine or butyrylcholine was significantly blocked in comparison to control tissues, however, those in response to the partial agonist, butyrylcholine, were more powerfully blocked. Previous work showed that in rabbit jugular vein, similarly treated with phenoxybenzamine, acetylcholine now acted as a partial agonist and induced approximately 75% of its normal maximal response (Martin *et al*., 1992). However, in that study, butyrylcholine induced a maximal response in control tissues higher than that induced in the control rat aortic rings used in the present study. This difference may due to the presence of significantly more spare receptors in the rabbit jugular vein in comparison to rat aorta (Martin *et al*., 1992), as the maximal response elicited by a partial agonist is enhanced when the population of receptors is increased (Tan *et al*., 2003).

By definition, butyrylcholine as a partial agonist which even when occupies 100% of endothelial M_3 receptors will fail to produce the same maximum response as the full agonist, acetylcholine (Stephenson, 1956; Schild, 1957; Furchgott, 1966; Pliška, 1999; Foreman and Johansen, 2003; Kenakin, 2004). Moreover, the full agonist, acetylcholine, can elicit its maximal relaxant effect by occupying only a small percentage of the total endothelial M_3 receptor pool, i.e. there is a considerable receptor reserve. The population of functional receptors available to bind the agonist can be reduced using irreversible receptor antagonists (Stephenson, 1956; Furchgott, 1966; Foreman and Johansen, 2003). These are chemically reactive molecules that combine with the ligand binding subunit of the receptor. The resultant covalent bond between the receptor protein and the irreversible antagonist molecule results in irreversible blockade of the receptor and new receptor molecules need to be synthesised before binding agonist and responsiveness can resume. Moreover, in the presence of an irreversible antagonist the number of active drug-receptor complexes that can be formed, which determine the magnitude of agonist effect, is decreased, so that the maximal response elicited by the agonist is depressed and the resultant dose-response curve is shifted to the right. Thus, on the basis of receptor theory one would predict that phenoxybenzamine would reduce the maximal relaxant effect elicited by the partial agonist, butyrylcholine, to a far greater extent than to the full agonist, acetylcholine. Thus, the findings of this study in rat aorta are entirely consistent with receptor theory.

The most important finding of this study, however, was that when the apparent efficacy of acetylcholine was reduced to that of a partial agonist using phenoxybenzamine, both L-NMMA and ADMA, which normally have little effect, now produced potent concentration-dependent blockade of the relaxation. The degree of inhibition by L-NMMA

and ADMA was similar to their blocking effect on relaxation induced by butyrylcholine. Previous work showed in rabbit jugular vein that when the apparent efficacy of acetylcholine was reduced, using 3 μM phenoxybenzamine for 30 minutes, so that it attained approximately 75% of its normal maximum effect, L-NAME now produced powerful blockade of relaxation, similar to its blocking effect on relaxation induced by the partial agonist, α-methyl-5-hydroxytryptamine (Martin *et al*., 1992).

Thus, the ability of L-NMMA and ADMA to block relaxation to the partial agonist, butyrylcholine, and to acetylcholine when its efficacy is reduced is in striking contrast with their lack of effect when acetylcholine is acting as a full agonist.

Previous findings of this study (Chapter Three) showed that the endothelium-dependent, nitric oxide-mediated relaxation induced by the low efficacy agonist, calcitonin gene-related peptide-1, but not by the powerful agents, acetylcholine and A23187, was powerfully blocked by L-NMMA and ADMA in rat aorta. When taken together with the results of the present chapter, these findings strongly suggest that the efficacy of the relaxant agonist is critical in determining the ability of L-NMMA and ADMA to block endothelium-dependent, nitric oxide-mediated relaxation. This conclusion is supported by previous findings in rabbit jugular vein that efficacy of relaxant agonist is critical in determining the blocking ability of L-NAME (Martin *et al*., 1992).

Thus, the major conclusion of this study is that the seemingly differential abilities of L-NMMA and ADMA to block basal, but not acetylcholine-induced relaxation in rat aorta may be explained by the blocking action being critically determined by the efficacy of the relaxant stimulus (presumably low efficacy basal activity vs high efficacy acetylcholine-

induced stimulated activity). A summary of the effects of L-NMMA and ADMA on relaxation induced by acetylcholine or butyrylcholine in the rat aorta is shown below (Figure 4.6).

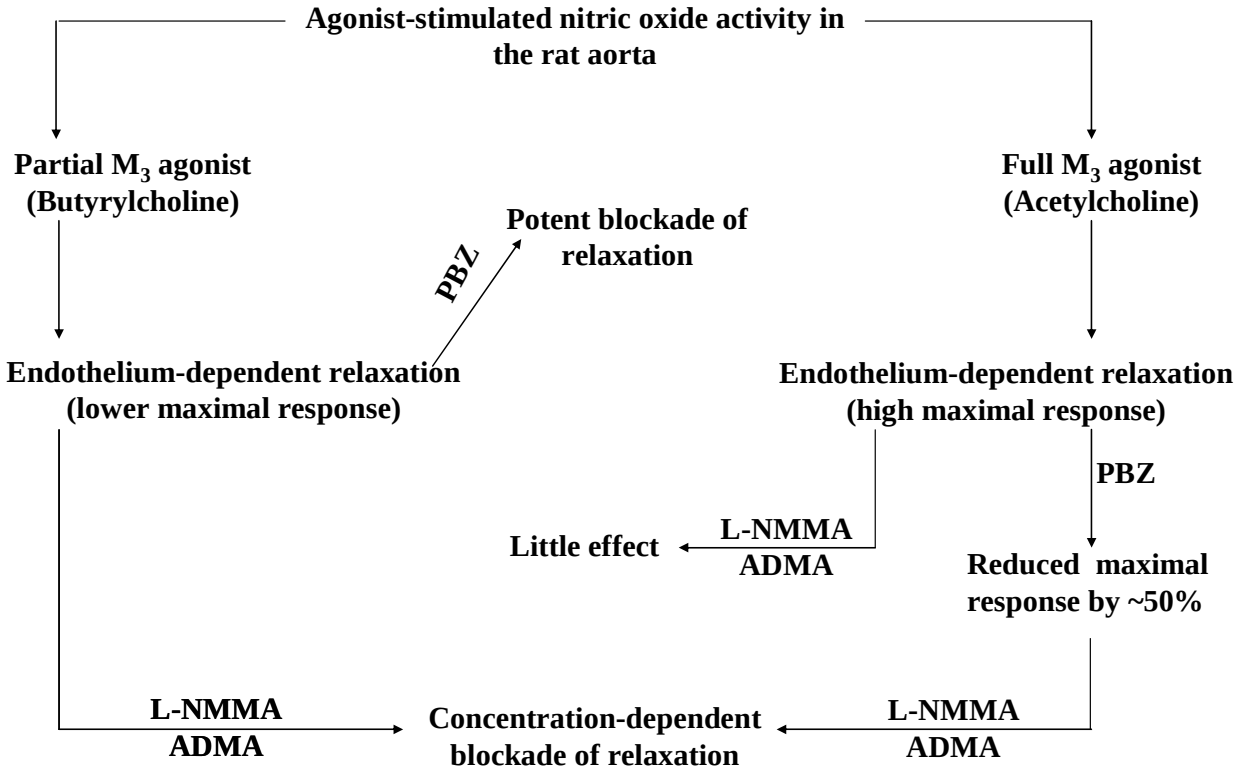

Figure 4.6. Summary of the effects of L-NMMA and ADMA on relaxation induced by acetylcholine or butyrylcholine in the rat aorta.

Chapter Five

General Discussion

The findings from the first part of this study showed that ADMA has differential abilities to block basal but not acetylcholine-stimulated activity of nitric oxide in rat aorta. These actions of ADMA are similar to those of L-NMMA in the same tissue (Frew *et al*., 1993), but in contrast to the actions of the standard nitric oxide synthase inhibitor, L-NAME, which similarly blocks basal as well as acetylcholine-stimulated nitric oxide activity in rat aorta.

It has been reported that the vascular endothelium generates nitric oxide in the absence of external stimuli, i.e. under basal conditions, and that this basal activity of nitric oxide exerts a tonic vasodilator effect that suppresses the actions of vasoconstrictor drugs (Martin *et al*., 1986b; Rees *et al*., 1989; Moore *et al*., 1990; Mian & Martin, 1995). Therefore, agents that inhibit nitric oxide synthesis or block the actions of nitric oxide are expected to produce an enhancement of vasoconstrictor-induced tone by removing this endothelium-dependent suppression of vasoconstriction.

In agreement with this concept, the results of this study showed that endothelial denudation or treatment of endothelium-containing female rat aortic rings with the nitric oxide synthase inhibitors, L-NAME, L-NMMA or ADMA, resulted in augmentation of low level phenylephrine-induced tone. This augmentation of tone was both prevented and reversed by adding a high concentration (10 mM) of the endogenous substrate for nitric oxide synthase, L-arginine. These findings suggest that all of these inhibitors of nitric oxide synthase act as classical competitive antagonists of the enzyme responsible for basal nitric oxide synthesis (Palmer *et al*., 1988; Rees *et al*., 1989; Aisaka *et al*., 1989; Rees *et al*., 1990; Moore *et al*., 1990; Hobbs *et al*., 1999; Masuda *et al*., 2002).

Additionally, the findings showed that the availability of L-arginine is not limiting for the production of the basal nitric oxide activity that suppresses vasoconstrictor tone. This latter finding is supported by previous *in vitro* and *in vivo* studies (Cooke *et al*., 1991; Moritoki *et al*., 1991; Jun and Wennmalm, 1994) and it may be due to the high endogenous level of L-arginine (100-300 µM) reportedly found in endothelial cells compared with estimates of the K_m for nitric oxide synthase (2-3 µM). Thus, under normal circumstances, the enzyme would normally be fully saturated with substrate (Sydow and Münzel, 2003; Teerlink *et al*., 2009; Zhang *et al*., 2011).

Also, the findings of this study showed that ADMA augmented the contractile responses to a range of spasmogens (phenylephrine, 5-hydroxytryptamine or prostaglandin $F_{2\alpha}$). While the threshold concentration of ADMA to block basal nitric oxide activity, as assessed by enhancement of phenylephrine-induced tone, in rat aorta was at 0.3–1 µM, it had almost completely blocked basal activity at a concentration of 100 µM. Furthermore, SDMA, the inert isomer of ADMA, had no effect on basal nitric oxide activity consistent with data indicating that SDMA is not an inhibitor of nitric oxide synthase (Masuda *et al*., 2002; Siroen *et al*., 2006).

Probably the most important finding of this study is that ADMA at a concentration of 100 µM, which almost completely blocks basal nitric oxide activity, has virtually no effect on acetylcholine-induced relaxation when the level of control and treated tissues was matched; there was a slight (2-3-fold) decrease in tissue sensitivity without affecting the maximal response to acetylcholine. Even increasing the concentration of ADMA to 1000 µM and prolonging the duration of treatment to 3 h failed to produce further blockade.

In contrast, under conditions where ADMA had been permitted to potentiate phenylephrine-induced tone as a result of blockade of basal nitric oxide activity, it appeared to produce blockade of acetylcholine-induced relaxation. This latter effect of ADMA was almost certainly due to physiological antagonism rather than blockade of nitric oxide synthase, since matching this increased tone in control tissues with additional phenylephrine produced a similar degree of apparent blockade. These findings are clearly in conflict with previous studies on rat aorta which reported blockade of acetylcholine-induced relaxation by ADMA (Vallance *et al*., 1992; Jin and D'Alecy, 1996; Feng *et al*., 1998). These authors made no mention of matching the tone in control and ADMA-treated tissues, so the observed "blockade of acetylcholine-induced relaxation" was likely to have been due to physiological antagonism rather than to blockade of nitric oxide synthase.

The results of this study also showed that ADMA did not inhibit acetylcholine-induced relaxation in rat aortic rings contracted submaximally with other agents, i.e. 5-hydroxytryptamine or prostaglandin $F_{2\alpha}$. Also, the inert isomer, SDMA, has no effect on acetylcholine-induced relaxation in rat aorta consistent with previous reports that this agent has no effect on nitric oxide synthase (Masuda *et al*., 2002; Siroen *et al*., 2006). In addition, the results showed that the availability of L-arginine is not limiting for the production of nitric oxide in response to acetylcholine in rat aorta. This finding could be attributed to the existence of a high intracellular concentration of L-arginine which saturates endothelial nitric oxide synthase (Cooke *et al*., 1991; Moritoki *et al*., 1991) although exogenous supplementation of L-arginine does appear to improve endothelium-dependent vasodilatation in humans and animals with pathological conditions associated with vascular dysfunction (Böger *et al*., 1995; Candipan *et al*., 1996; Rector *et al*., 1996; Blum *et al*., 1999; Watanabe *et al*., 2000; Böger and Bode-Böger, 2001).

In a further deviation from the classically expected outcome, the results of this study also showed that both of L-NMMA and ADMA behaved similarly to the endogenous substrate, L-arginine, by protecting acetylcholine-induced relaxation against blockade by the standard nitric oxide synthase inhibitor, L-NAME. L-arginine and L-NMMA both prevented and reversed blockade by L-NAME, however, ADMA failed to reverse that blockade after an hour of treatment, perhaps due to a lower potency of ADMA than L-arginine or L-NMMA. These findings are supported by previous reports suggesting that L-NMMA may act as an alternative substrate for nitric oxide synthase and thereby increase the synthesis of nitric oxide in rat aorta and pulmonary artery (Archer and Hampl, 1992; Frew *et al*., 1993). Thus, L-NMMA and ADMA behaved more like alternative substrates for acetylcholine-induced activity of nitric oxide in rat aorta despite acting as classical competitive antagonists of basal nitric oxide synthesis.

One theory that could potentially explain these anomalous actions of ADMA and L-NMMA to block basal but not acetylcholine-stimulated activity of nitric oxide is the existence, within the vascular endothelial cells, of more than one isoform of nitric oxide synthase; one that catalyses the synthesis of nitric oxide under basal conditions, and another for agonist-stimulated production.

Another possible hypothesis behind these anomalous actions of ADMA and L-NMMA, is that the different signal transduction mechanisms triggered by different stimuli may result in unique structural or conformational changes in endothelial nitric oxide synthase that alter its requirement for substrates and inhibitors.

One other potential explanation for these anomalous abilities of ADMA and L-NMMA may relate to the degree to which endothelial nitric oxide synthase is stimulated.

In the second section of this study the above possibilities were explored by employing a variety of different experimental approaches.

The results from the second section of this study showed that, in the presence of catalase (to remove any formed hydrogen peroxide), L-NMMA and ADMA, like L-NAME, were able to almost completely abolish relaxation induced by superoxide dismutase; this relaxation is attributed to protection of basal nitric oxide from destruction by superoxide anion ($^{\bullet}O^{-}_{2}$) (Gryglewski *et al*., 1986; Rubanyi and Vanhoutte, 1986; Ohlstein and Nichols, 1989; Mian and Martin 1995; MacKenzie *et al*., 1999). Also, the results showed that these nitric oxide synthase inhibitors each produced blockade of relaxation induced by the phosphodiesterase isoform 5 inhibitor, T0156, that was as powerful as that produced by endothelial denudation; this relaxation is attributed to potentiation of basal nitric oxide activity generated by the vascular endothelium due to slowed hydrolysis of its second messenger cGMP (Martin *et al*., 1986a; Hobbs *et al*., 1999; Mochida *et al*., 2002; Kimura *et al*., 2003; Mochida *et al*., 2004; Galley *et al*., 2004; Bauer and Sotnikova, 2010). Thus, the uniform outcome is that L-NMMA and ADMA block basal activity of nitric oxide irrespective of whether it is assessed via SOD- or T0156-induced relaxation or through enhancement of phenylephrine-induced tone.

As has been mentioned before, a difference in the transduction mechanisms that govern basal and agonist-stimulated synthesis of nitric oxide could potentially explain the ability of L-NMMA and ADMA to block the former but not the latter in rat aorta. However, the

use of calmidazolium or wortmannin indicated that calcium-calmodulin-dependent and phosphatidylinositol 3-kinase-mediated activation of endothelial nitric oxide synthase appears to play only a minor role in basal nitric oxide production in female rat aorta. The major component of basal nitric oxide production appears to occur, however, through spontaneous, unstimulated activity of the enzyme.

The finding of this study that L-NMMA and ADMA have a little effect on the receptor-dependent acetylcholine-induced relaxation in rat aorta seems to be not unique for this agonist, since these nitric oxide synthase inhibitors also have little effect on relaxation induced by calcium ionophore A23187 in the same tissue. This relaxant agent is known to produce endothelium-dependent, nitric oxide-mediated relaxation via a receptor-independent elevation of intracellular endothelial calcium levels (Weinheimer and Osswald, 1986; Schini and Vanhoutte, 1992; Taniguchi *et al*., 1999; Shi *et al*., 2007). In stark contrast, this study showed that both L-NMMA and ADMA powerfully blocked relaxation to calcitonin gene-related peptide-1, which induces endothelium-dependent, nitric oxide-mediated relaxation in rat aorta (Gray and Marshall, 1992; Abdelrahman *et al*., 1992, de Hoon *et al*., 2003; Thakor and Giussani, 2005). Another apparent difference among the three agents was that the maximal response elicited by calcitonin gene-related peptide-1 was lower than that elicited by acetylcholine or A23187 in the same tissue. In addition, the results from this section of the study showed that these three agonists (acetylcholine, A23187 and calcitonin gene-related peptide-1) signal through a seemingly similar calmidazolium-sensitive, calcium-calmodulin-dependent transduction mechanism to stimulate the release of nitric oxide in rat aorta. Taken together, it is clear that the ability of L-NMMA and ADMA to block relaxation to calcitonin gene-related peptide-1, but not to acetylcholine or A23187, cannot be explained by these agonists acting via different

transduction pathways. It is possible therefore that the differential effect L-NMMA and ADMA exert on relaxations induced by these three agonists might somehow related to the maximum relaxation these agents can elicit. This possibility is further supported by previous work suggesting that the efficacy of relaxant agonist is critical in determining the ability of L-NAME to block endothelium-dependent, nitric oxide-mediated relaxation (Martin *et al*., 1992). Thus, this possibility was investigated in the next section of the study using the muscarinic M_3 receptor partial agonist, butyrylcholine, and by lowering the apparent efficacy of acetylcholine by using the irreversible alkylating agent, phenoxybenzamine (Martin *et al*., 1992).

The results from this part of the study showed that the M_3 partial agonist, butyrylcholine (Martin *et al*., 1992), induced concentration-dependent relaxation in female rat endothelium-containing aortic rings. As expected, the maximal relaxant response elicited by this partial agonist was lower than that of the full agonist, acetylcholine, in the same tissue. Strikingly, the nitric oxide synthase inhibitors, L-NMMA and ADMA, which have relatively little effect on acetylcholine-induced relaxation, produced powerful blockade of butyrylcholine-induced relaxation.

Previous results of this study showed similar findings with the low efficacy agonist, calcitonin gene-related peptide-1.

Taken together, it seemed possible that the lower relaxant efficacy of calcitonin gene-related peptide-1 and butyrylcholine, in comparison with acetylcholine or A23187, might explain why these relaxant effects were susceptible to blockade by L-NMMA or ADMA. This possibility was investigated further in this study by lowering the apparent efficacy of

acetylcholine using the irreversible alkylating agent, phenoxybenzamine (Martin *et al*., 1992).

The results showed that when the apparent efficacy of acetylcholine was reduced (by ~50%) to that of a partial agonist using phenoxybenzamine, both of L-NMMA and ADMA produced concentration-dependent blockade of the relaxation. The degree of inhibition by L-NMMA and ADMA was similar to their blocking effect on relaxation induced by butyrylcholine.

In conclusion, the findings of this study strongly suggest, in common with previous findings with L-NAME (Martin *et al*., 1992), that the efficacy of the relaxant agonist is critical in determining the degree to which L-NMMA and ADMA can block endothelium-dependent, nitric oxide-mediated relaxation. An explanation for the ability of L-NMMA and ADMA to block acetylcholine-induced relaxation therefore rests on this conclusion.

A summary of the differential actions of L-NMMA and ADMA on basal and agonist-stimulated activity of nitric oxide in the rat aorta is shown below (Figure 5.1).

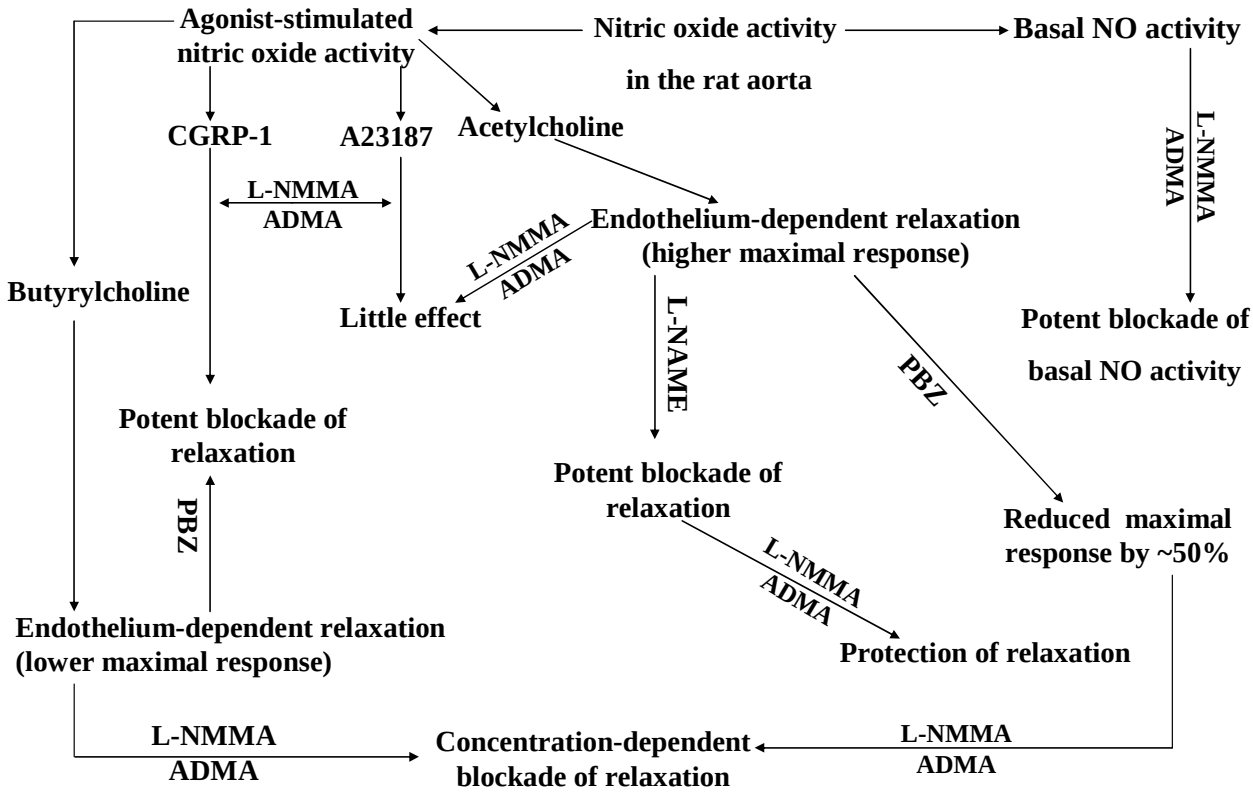

Figure 5.1. Summary of the differential actions of L-NMMA and ADMA on basal and agonist-stimulated activity of nitric oxide in the rat aorta

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