MODULATION OF ENDOTHELIUM-DEPENDENT ARTERIAL RELAXATION BY INORGANIC ARSENIC AND GLUCOSE

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SUMMARY

Inorganic arsenic and elevated glucose concentrations increase endothelial production of superoxide, which impairs endothelium-derived nitric oxide bioavailability and associated nitric oxide-dependent arterial relaxations. However, there is now evidence in animal models of diabetes mellitus that relaxations attributed to endothelium-dependent smooth muscle hyperpolarization or endothelium-derived hyperpolarizing factor (EDHF) may be augmented and serve to compensate for the loss of nitric oxide, thereby maintaining arterial responsiveness. The effects of arsenite and elevated glucose concentrations on "EDHF-type" relaxations were thus investigated in isolated rabbit iliac artery rings using the G-protein-coupled agonist acetylcholine and the sarcoendoplasmic reticulum Ca^{2+} ATPase inhibitor cyclopiazonic acid. Arsenite and elevated glucose both potentiated EDHF-type relaxations evoked by cyclopiazonic acid. Differential effects of arsenite and glucose against EDHF-type responses to acetylcholine were identified in that arsenite attenuated relaxation, whereas glucose potentiated relaxation. Further experiments showed that the arsenite- and glucose-augmented components of relaxation were reversed to control levels by the hydrogen peroxide scavenger catalase and the NADPH oxidase inhibitor apocynin. Arsenite-augmented responses were also reversed by the cell-permeable superoxide dismutase/catalase mimetic manganese porphyrin. It follows that hydrogen peroxide derived from NADPH oxidase may augment EDHF-type relaxations in diabetes mellitus and arsenic toxicity, thus maintaining endothelial control of arterial tone when nitric oxide bioavailability is impaired by oxidative stress. These results are consistent with the demonstrations that hydrogen peroxide augments EDHF-type relaxations in the rabbit iliac artery by promoting endothelial Ca^{2+} mobilization and enhancing the opening of endothelial Ca^{2+}-activated K^+ channels, and that the increased activity of these channels underpins augmented EDHF-type arterial relaxations in animal models of diabetes.

ABBREVIATIONS

ACE	angiotensin converting enzyme
ACh	acetylcholine
ApoE	apolipoprotein E
ATP	adenosine triphosphate
BH_4	tetrahydrobiopterin
Bk	bradykinin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COX	cyclooxygenase
CPA	cyclopiazonic acid
DEA	diethylamine
(p)EC_{50}	(negative log) half maximal effective concentration
EDCF	endothelium-derived contracting factor
EDH	endothelium-dependent hyperpolarization
EDHF	endothelium-derived hyperpolarizing factor
EDR	endothelium-dependent relaxation
EDRF	endothelium-derived relaxing factor
EET	epoxyeicosatrienoic acid
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ESR	electron spin resonance
GK	Goto-Kakizaki
H_2O_2	hydrogen peroxide
HG	high glucose
(p)IC_{50}	(negative log) half maximal inhibitory concentration
$InsP_3(R)$	inositol 1,4,5-trisphosphate (receptor)
K_{ATP}	ATP-activated K^+ channel
(S, I, B)K_{Ca}	(small, intermediate, large-conductance) Ca^{2+}-activated K^+ channel
K_V	voltage-gated K^+ channel
LDL	low density lipoprotein
L-NAME	L^{ω}-nitro-arginine methyl ester
MAHMA NONOate	NOC-9,6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-hexanamine
MCP-1	monocyte chemoattractant protein 1
MEGJ	myoendothelial gap junction
MnTMPyP	Mn(III)-tetrakis-(1-methyl-4-pyridyl-porphyrin)tetratosylate hydroxide (manganese porphyrin)

mPTP	mitochondrial permeability transition pore
MR	muscarinic receptor
NADPH	nicotinamide adenine dinucleotide phosphate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOX	nicotinamide adenine dinucleotide phosphate oxidase
$O_2^{\bullet-}$	superoxide radical
ODQ	1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one
$OH^{\cdot}$	hydroxyl radical
OLETF	Otsuka Long-Evans Tokushima fatty rat
oxLDL	oxidized low density lipoprotein
OZFR	obese Zucker fatty rat
PDE3	phosphodiesterase 3
PE	phenylephrine
phox	phagocytic oxidase
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PMCA	plasma membrane Ca^{2+}-ATPase
PMVD	peripheral microvascular disease
RIA	rabbit iliac artery
R_{max}	maximal response
ROS	reactive oxygen species
SEM	standard error of the mean
SERCA	sarcoendoplasmic reticulum Ca^{2+}-ATPase
sGC	soluble guanylyl cyclase
SHR	spontaneously hypertensive rat
SIN-1	5-amino-3-(4-morphonilyl)-1,2,3-oxadiazolium chloride
SNAP	S-nitroso-N-acetyl penicillamine
SNP	sodium nitroprusside
SOC	store-operated Ca^{2+} channel
SOCE	store-operated Ca^{2+} entry
SOD	superoxide dismutase
STIM1	stromal interaction protein 1
STZ	streptozotocin
TEA	tetraethylammonium
TRPC	transient receptor potential canonical type channels
TRPV4	transient receptor vallinoid type 4 channels
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
WHO	World Health Organisation

CONTENTS

CHAPTER 5

THE ROLE OF HYDROGEN PEROXIDE IN EDHF PHENOMENA AUGMENTED BY ARSENITE AND D-GLUCOSE

CHAPTER 6

THE ROLE OF NADPH OXIDASE IN EDHF PHENOMENA AUGMENTED BY ARSENITE AND D-GLUCOSE

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Environmental arsenic exposure and diabetes mellitus are major health concerns in numerous countries across the world. The impact of arsenic toxicity and diabetes mellitus is fully illustrated on the World Health Organisation (WHO) website with alarming statistics. While the dangers of arsenic are now fully appreciated, the implementation of preventative measures is an extremely slow process. Diabetes mellitus, including its associated risk of cardiovascular complications, is a condition that is rapidly becoming an epidemic with increasing neglect of diet and physical activity.

1.1.1 Arsenic – "The Largest Poisoning of a Population in History"

Excessive quantities of inorganic arsenic species in drinking water has been correlated with an increased risk of a number of diseases including cancer, atherosclerosis and peripheral microvascular disease (PMVD) (States *et al.*, 2009). In addition, arsenic is now used clinically in the treatment of acute promyelocytic leukaemia, where its pro-apoptopic properties can lead to the destruction of cancerous stem cells (Tallman, 2008). Arsenic is plentiful in the earth's crust and poor drinking water is now recognised as the highest risk source. This has become a major public health concern in the United States of America, Taiwan, Chile, Mexico, China, Argentina, India and Bangladesh. An estimated 140 million people worldwide are at serious risk of exposure (www.who.int/topics/arsenic/en/). However, described as "the largest poisoning of a population in history", the current situation in Bangladesh is unprecedented and has been of increasing concern since the early 1990s (Smith *et al.*,

2000). There, ironically, drinking water was originally obtained from rivers and ponds that contained little or no arsenic, but were contaminated with infectious water-borne diseases including cholera, hepatitis and typhoid (Smith *et al.*, 2000). Attempts to install new and "safe" sources of drinking water unveiled arsenic as a new health concern. The tubewells, first constructed in Bangladesh in the 1940s as a safeguard against water-borne infection, obtained water from around 200 metres below ground. However, at this time, arsenic in drinking water was not recognised as a health risk and national water testing procedures did not include an appropriate protocol (www.unicef.org/arsenic, 1999). The alarm was first raised in neighbouring West Bengal, India, in 1983 when medical staff correlated cases of skin lesions with arsenic-contaminated tubewell water supplying a local population of 1.5 million (Mandal *et al.*, 1998). Despite similar cases being reported in Bangladesh, it was not until 1993 that contamination of water from the tubewells was confirmed (Khan *et al.*, 1997). The epidemiologist Allan Smith (of the University of California and World Health Organisation) published findings based on visits to Bangladesh between 1997 and 1998. These predicted that between 35 and 77 million of Bangladesh's 125 million population were at serious risk from drinking water contaminated with arsenic and it was urged that a public health emergency be declared (Smith *et al.*, 2000). Subsequent studies in an emerging field have shown that exposure to arsenic increases the risk of cancers of the bladder (Simeonova *et al.*, 2000; Steinmaus *et al.*, 2006), kidney (Parrish *et al.*, 1999; Yang *et al.*, 2004), lung (Posey *et al.*, 2008) and skin (Yeh *et al.*, 1968; Tseng *et al.*, 1968; Germolec *et al.*, 1998) as well as cardiovascular disease (Chang *et al.*, 2004; Yang *et al.*, 2006; Chiu *et al.*, 2007) and diabetes mellitus (Diaz-Villasenor *et al.*, 2006; Coronado-Gonzalez *et al.*, 2007). These diseases may manifest over a number of years or even decades.

While a long-term supply of fresh uncontaminated water appears to be the most effective preventative measure for arsenic-associated disorders, evident from the decline in cases of Blackfoot disease in Taiwan (www.who.int/topics/arsenic/en/), the task and cost involved in testing samples from all 1.3 million of Bangladesh's tubewells is enormous and still ongoing.

1.1.2 Arsenic and the Blackfoot Disease Epidemic

Blackfoot disease is a unique condition endemic to the south-western coast of Taiwan. It is characterized as a severe form of PMVD affecting the lower limbs which leads to gangrene ("black feet"). Drinking water in this area was previously supplied by artesian wells which contained high levels of arsenic. These wells, like the tubewells in Bangladesh, drew water from around 200 metres below ground where arsenic-rich rocks prevailed. This was later correlated with increased risk of PMVD with only a 50% survival rate 16 years after the onset of the disease (Tseng, 1961; 1977). Preclinical PMVD in these cases was found to be related to arsenic exposure in a dose-dependent manner with symptoms including impaired subcutaneous microcirculation (Tseng, 1977; Tseng *et al.*, 1995; Chiou *et al.*, 1997). Prevalence of the disease in the endemic villages was 7-19 per 1000 individuals (Tseng, 2002). Arsenic-accelerated atherogenesis has also been demonstrated experimentally in the widely-employed apolipoprotein $E^{(-/-)}$ mouse model of atherosclerosis (Simeonova *et al.*, 2003; Bunderson *et al.*, 2004; Srivastava *et al.*, 2007).

1.1.3 Diabetes Mellitus and Cardiovascular Disease

Diabetes mellitus, like chronic arsenic exposure, dramatically increases the risk of cardiovascular disease. It is a chronic disease that occurs when either the pancreas does not synthesize and secrete insulin (type I diabetes mellitus; affecting 10% of sufferers) or when normal insulin levels are offset by the body's insensitivity to the

hormone (type II diabetes mellitus; affecting 90% of sufferers). The result is elevated serum glucose levels which poses major health complications if uncontrolled. With approximately 180 million people worldwide affected by diabetes, this figure is expected to double within just 20 years (www.who.int/topics/diabetes-mellitus/en/). In 2005 1.1 million people worldwide died from a diabetes-related complication and this annual figure is expected to increase by 50% within the next 10 years (www.who.int/topics/diabetes-mellitus/en/). Furthermore, the figure for deaths by a diabetes-related cause is likely to be significantly higher than that projected since deaths that are attributed to cardiovascular diseases are often not correctly correlated with the condition. Diabetic retinopathy is one of the world's leading causes of blindness with 2% of diabetics suffering complete visual impairment after 15 years from the initial onset of the disease and 10-20% affected by some degree of visual impairment (www.who.int/topics/diabetes-mellitus/en/; Gariano & Gardner, 2005). Diabetes-associated neuropathy affects 50% of sufferers with symptoms including tingling, pain, numbness or weakness in the feet and hands (www.who.int/topics/diabetes-mellitus/en/; Casellini & Vinik, 2007). Diabetes is also among the leading causes of kidney failure, claiming the lives of 20-40% of sufferers (Radbill *et al.*, 2008). However, the most detrimental clinical manifestation of diabetes is cardiovascular disease which claims the lives of more than 50% of sufferers (www.who.int/topics/diabetes-mellitus/en/; Danaei *et al.*, 2006). Chronic elevated blood glucose levels are correlated with an accelerated onset of atherosclerosis and microvascular disease (Massi-Benedetti & Federici, 1999; Cardoso *et al.*, 2008), a phenomenon that has, like arsenic, been extensively demonstrated in animal models (for review see: Wu *et al.*, 2007).

1.2 Atherosclerosis

Atherosclerosis is a chronic and progressive inflammatory and occlusive disease characterized by the accumulation of lipid deposits in the walls of medium and large arteries. In regions of vessel curvature or branching, where the flow of blood is disturbed, the endothelium is more permeable to low density lipoprotein (LDL) and thus it is these sites that are particularly susceptible to atherosclerotic lesion formation (for review see: Lusis, 2000). Accumulating LDL in the vessel wall undergoes oxidative modifications by reactive oxygen species (ROS) originating from the surrounding vascular cells resulting in the formation of oxidized low density lipoproteins (oxLDL), which trigger an inflammatory response. Here, circulating monocytes migrate paracellularly from the lumen to the vessel wall where they rapidly absorb oxLDL (but not native LDL). The resulting "foam cells" then rupture, thereby promoting further monocyte migration via a positive feedback effect (for review see: Stocker & Keaney, 2004). Atherosclerosis is the principal causative factor of morbidity and mortality in the Western world, claiming more lives every year than all types of cancer combined, and is the underlying cause of most cardiovascular complications including stroke, coronary artery disease including myocardial infarction, and PMVD.

Although a major problem in developed nations, the World Health Organisation predicts that, with increasing economic prosperity in the Third World and the subsequent tendency to adopt a Western lifestyle, atherosclerosis will inevitably also become an epidemic in developing countries. While its onset is correlated with increasing age (beginning in the second or third decade of life), risk is accelerated by a number of factors. These include hypertension, diabetes mellitus, obesity, cigarette smoking, high serum LDL levels and, as previously outlined, chronic arsenic

exposure (see 1.1.1 and 1.1.2). Despite a multitude of distinct risk factors, endothelial oxidative stress and dysfunction is now appreciated to be a unifying preceding event and has provided an important focus for research. Oxidative stress is known to promote atherogenic signalling events in endothelial cells (for review see: Harrison *et al.*, 2003). Elucidation of the ROS-generating systems and redox signalling events which drive this phenomenon could potentially identify novel targets for pharmacological intervention. The recently identified pleiotropic antioxidant properties of statins in humans and animals have heightened this interest (Karatzis *et al.*, 2005; Mastalerz-Migas *et al.*, 2007; Verreth *et al.*, 2007; Schupp *et al.*, 2008). Thus, arsenic and hyperglycaemia have both been postulated to promote atherogenesis via pro-oxidative mechanisms, that stimulate platelet activation and vascular cell proliferation as well as endothelial production of vascular cell-adhesion molecule-1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1) and vascular endothelial growth factor (VEGF) (Lee *et al.*, 2002; Simeonova *et al.*, 2003; Lee *et al.*, 2005; Nuntharatanapong *et al.*, 2005; Tsou *et al.*, 2005; Pereira *et al.*, 2007; Scalia *et al.*, 2007; Piga *et al.*, 2007; Haubner *et al.*, 2007; Luppi *et al.*, 2008; Undas *et al.*, 2008; Al-Shabrawey *et al.*, 2008).

1.3 Oxidative Stress: a Definition

While the role of ROS in disease is now appreciated, the emerging field of "redox signalling" has lead to a revised definition of "oxidative stress" (Sies, 1991). Previously described as an "imbalance in the pro-oxidant/antioxidant equilibrium in favour of the pro-oxidants", the same author later modified this definition to "an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage" (Sies, 1997). The latter definition appreciates that increased

cellular levels of ROS do not necessarily evoke "oxidative damage" and can, in fact, mediate physiological responses (for review see: Touyz, 2005). In terms of vascular health and disease, oxidative stress may be reflected as a shift in endothelial redox status in favour of pro-oxidants leading to physiological signalling events or potentially, if uncontrolled, to cellular damage and dysfunction. Indeed, research in recent years has uncovered the important role of hydrogen peroxide (H_2O_2) as an endogenous mediator of arterial tone under normal conditions (Matoba *et al.*, 2000; 2002; Chaytor *et al.*, 2003; Larsen *et al.*, 2008; 2009; Edwards *et al.*, 2008).

1.4 The Endothelium

The entire circulation, including the heart, is lined by an endothelial monolayer on its luminal surface. The enormous surface area this generates, approximately 4000 - 7000 m^2 in a human adult, enables the endothelium to interact efficiently and optimally with all tissues of the body including cardiac and smooth muscle cells (Sumpio *et al.*, 2002). In capillaries, which are devoid of smooth muscle cells, the endothelium regulates delivery of essential nutrients to the target tissue. Initially thought to be merely a selective barrier between blood and tissue, the importance of the endothelium in the control of vascular tone has come to the fore since pioneering work in the 1970s and 1980s (see 1.4.2). Even to the present day, researchers are still deciphering the complexity of the interdependent mechanisms by which this monolayer is able to regulate vasomotor tone and hence provide efficient and optimal tissue perfusion.

1.4.1 Role as a Selective Barrier

The function of the endothelium as a selective barrier is determined by intercellular junctions, a negatively-charged glycocalyx surface layer and a vesicular system. Such regulation facilitates the efficient transfer of signalling molecules and nutrients, either by direct uptake via the plasma membrane or by paracellular transport, whilst restricting the entry of large plasma proteins thereby maintaining blood osmolarity, volume and viscosity (Pries & Keubler, 2006). Endothelial cells contain actin and myosin filaments that are components of intercellular junctions and stress fibres that "resist" the shearing effect of blood flow (Levick, 2000). Additionally, actin remodelling controls the integrity of interendothelial adhesive junctions (both tight and adherens junctions), thereby regulating the permeability of the endothelial barrier (for review see: Vandenbroucke *et al.*, 2008). Endothelial dysfunction and abnormal increases in endothelial permeability are associated with the accelerated onset of atherosclerosis (see 1.2). Correspondingly, the endothelium synthesizes and releases anti- and pro-thrombotic mediators that regulate platelet aggregation (nitric oxide (NO) and von Willebrand factor, respectively, for example) in addition to mediators of inflammatory cell adhesion, such as VCAM-1 and MCP-1 (Poredos, 2001).

1.4.2 Endothelial Control of Arterial Tone

In 1976 Vane and colleagues reported the ability of blood vessels to produce an unstable compound from prostaglandin endoperoxides that potently inhibited platelet aggregation and adhesion to endothelial cells (Moncada *et al.*, 1976). This compound, later identified as 6-keto-$PGF_{1\alpha}$ (prostacyclin), was subsequently reported to relax vascular smooth muscle (Whittaker *et al.*, 1976; Dusting *et al.*, 1977). In 1982, Vane was awarded the Nobel Prize for Physiology or Medicine jointly with Bergstrom and

Samuelsson for their earlier work on the isolation and molecular structure of prostaglandins (Bergstrom & Samuelsson, 1962; Nugteren *et al.*, 1966).

Subsequently, a seminal report by Furchgott and Zawadzki (1980) demonstrated the obligatory role of endothelial cells in the relaxation of vascular smooth muscle in response to acetylcholine, and thus the existence of an endothelium-derived relaxing factor (EDRF). Following this, the identity of EDRF was variously proposed as a product of lipooxygenase (De Mey *et al.*, 1982; Furchgott, 1983; Rapoport *et al.*, 1984; Forstermann & Neufang, 1985) or a short-lived carbonyl-containing compound with a biological half-life of ~6 seconds in oxygenated buffer (Griffith *et al.*, 1984). It was also reported that the bioavailability of EDRF was attenuated by superoxide anions ($O_2^{\bullet-}$) and potentiated by superoxide dismutase (SOD), which catalyses the single electron reduction of $O_2^{\bullet-}$ to form H_2O_2 (Rubanyi & Vanhoutte, 1986; Gryglewski *et al.*, 1986; Moncada *et al.*, 1986; de Nucci *et al.*, 1988), observations that would later prove fundamental to the study of endothelial dysfunction and cardiovascular disease (also see 1.2).

The obligatory role of cyclic guanosine monophosphate (cGMP) in the EDRF-type response was then established and followed, in 1986, by the independent proposals by Ignarro and Furchgott that EDRF was NO (Rapoport & Murad, 1983; Griffith *et al.*, 1985; Furchgott, 1988; Ignarro *et al.*, 1988). The close resemblance of EDRF to NO was confirmed in the following year (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; for review see: Moncada & Palmer, 1988). Notably, the smooth muscle relaxant effects of nitrodilators, such as sodium nitroprusside (SNP), and the involvement of cGMP in these responses had been reported during the previous decade (Schultz *et al.*, 1977; Murad *et al.*, 1978; Kukovetz *et al.*, 1979). In 1998, the Nobel Prize for Physiology or

Medicine was awarded jointly to Furchgott, Ignarro and Murad for their discovery of NO production by the vascular endothelium and its effects on smooth muscle tone.

As described in detail in Section 1.5, the endothelium is also able to mediate smooth muscle relaxation by promoting smooth muscle hyperpolarization, a phenomenon that was first described in the 1980s and appears to be quite distinct from its ability to synthesize NO and prostanoids (Bolton *et al.*, 1984; Feletou & Vanhoutte, 1988; Beny & Brunet, 1988; Chen *et al.*, 1988).

1.4.3 The Importance of Ca^{2+} Signalling

The divalent cation Ca^{2+} controls numerous endothelial signalling mechanisms in response to chemical messengers (Tran & Watanabe, 2006). Specifically, agonist-induced release of endothelium-derived vasodilators, such as NO and prostanoids, is critically reliant on increases in $[Ca^{2+}]_i$ (Griffith *et al.*, 1986; Gryglewski *et al.*, 2002; Simonsen *et al.*, 2009), whereas the role of endothelial Ca^{2+} mobilization in mechanical responses evoked by physical forces such as shear stress (flow) is controversial. For example, some authors claim that flow-induced endothelial NO synthesis is Ca^{2+}-independent, and mediated by Akt/protein kinase B (PKB)-dependent phosphorylation of serine 1177 of endothelial nitric oxide synthase (eNOS), whereas others suggest that extracellular Ca^{2+} influx via plasma membrane-bound mechanosensitive transient receptor potential vallinoid type 4 (TRPV4) channels is critical for shear stress-evoked NO-dependent dilatation (Dimmeler *et al.*, 1999; Hartmannsgruber *et al.*, 2004; Kohler *et al.*, 2006; Perez *et al.*, 2009).

Endothelial Ca^{2+} mobilization in response to agonists is mediated by receptor-operated Ca^{2+} channels and an inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive intracellular Ca^{2+} store. Depletion of the $InsP_3$-sensitive intracellular store may subsequently trigger a small and sustained influx of Ca^{2+} from the extracellular space

via store-operated Ca^{2+} entry (SOCE) (Griffith, 2004). Correspondingly, it is now established that following depletion of the endoplasmic reticulum (ER) Ca^{2+} store the ER Ca^{2+} sensor stromal interaction protein 1 (STIM1) and the store-operated Ca^{2+} channel protein Orai1 redistribute from the ER to the plasma membrane where the highly conserved 107 amino acid activation domain of STIM1 directly binds to the N and C termini of Orai1 causing clustering of individual Orai1 subunits and opening of the assembled channel. Refilling of the ER stores via the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) causes dissociation of the STIM1-Orai1 complex and disassembly of the channel (Park *et al.*, 2009; Sampieri *et al.*, 2009). The STIM1-Orai interaction has been reported to be important for SOCE in endothelial cells (Abdullaev *et al.*, 2008; Fernandez-Rodriguez *et al.*, 2009). Furthermore, it has also been reported that canonical-type TRP channels (TRPC) may contribute to SOCE by associating with Orai1 to form heteromeric channel complexes (Liao *et al.*, 2009).

Ca^{2+} efflux to the extracellular space is facilitated by the plasma membrane Ca^{2+}-ATPase (PMCA) and the Na^{+}/Ca^{2+} exchanger, the latter of which utilizes the electrochemical gradient as a driving force for Na^{+} entry and hence Ca^{2+} efflux (Szewczyk *et al.*, 2007). It has recently been reported that PMCA may be localised at caveolae, invaginations of the plasma membrane (Zhang *et al.*, 2009).

1.5 The EDHF Phenomenon and its Characterization

The residual component of endothelium-dependent relaxations observed in the presence of inhibitors of eNOS (e.g. N^{ω}-Nitro-L-arginine methyl ester, L-NAME) and COX (e.g. indomethacin) is now attributed to endothelium-dependent smooth muscle hyperpolarization (Griffith, 2004; Feletou & Vanhoutte, 2006). Such responses are

critical to the regulation of tissue perfusion due to their predominance in small vessels and the resistance vasculature (Shimokawa *et al.*, 1996; Berman *et al.*, 2002).

1.5.1 Endothelium-Dependent Smooth Muscle Hyperpolarization

The phenomenon of endothelium-dependent smooth muscle hyperpolarization was first described in guinea pig mesenteric arteries, rat aorta and main pulmonary artery, canine coronary small arteries and rabbit aorta (Bolton *et al.*, 1984; Feletou & Vanhoutte, 1988; Bény & Brunet, 1988; Chen *et al.*, 1988). One possibility is that agonist-stimulated endothelial cells release a freely diffusible endothelium-derived hyperpolarizing factor (EDHF) that causes hyperpolarization and relaxation by promoting smooth muscle K^+ efflux. Various factors, including H_2O_2, K^+ ions, epoxyeicosatrienoic acid (EET) metabolites of arachidonic acid and C-type natriuretic peptide, have been demonstrated to act as EDHFs in specific arteries (Campbell *et al.*, 1996; Edwards *et al.*, 1998; Matoba *et al.*, 2000; 2002; Chauhan *et al.*, 2003). However, evidence implicating a single factor as a universal EDHF has failed to emerge. The apparent species- and vessel-specific actions of each proposed mediator has thus divided opinions on the nature of the EDHF phenomenon. Indeed, an alternative hypothesis is that the phenomenon is mediated by electrotonic signalling, whereby agonist-stimulated endothelial hyperpolarization is transmitted through the arterial media via myoendothelial and homocellular smooth muscle gap junctions that provide electrical continuity between the different layers of the vessel wall (for reviews see: Griffith, 2004; Griffith *et al.*, 2004).

1.5.2 Role of Gap Junctional Communication

Gap junctions play a pivotal role in direct chemical and electrical intercellular communication and are formed by the docking of two membrane-associated hemi-channels (connexons) at points of cell-to-cell contact, creating an aqueous pore, which

confers electrical and chemical (up to 1 kDa) continuity. Whole gap junction units aggregate, often in many hundreds, in plaque-like structures of a hexagonal array to increase intercellular communication (Griffith, 2004). Plaques have been visualised by electron microscopy at points of hetero- and homocellular contact between endothelial and smooth muscle cells (Spagnoli *et al.*, 1982; Sandow & Hill, 2000), with a correlation between plaque size and electrical coupling and dye transfer being evident (Bukauskas *et al.*, 2000). In addition, an increased frequency of gap junction plaques at points of cell-cell contact between endothelial and smooth muscle cells with decreasing vessel diameter (Aydin *et al.*, 1991; Kristek & Gerova, 1992; Sandow & Hill, 2000) may underpin the widely acknowledged dominant role of endothelium-dependent smooth muscle hyperpolarization in small vessels, as noted previously (see 1.4.2).

The mammalian vasculature expresses connexins 37, 40, 43 and, in certain vessel types, connexin 45 (each classified according to its molecular mass in kDa) which can form hexamers (hemi-channels/connexons) containing various combinations of connexin proteins. Immunohistochemical studies in our laboratory have confirmed the presence of connexins 37, 40 and 43 in the rabbit iliac artery. It was found that connexins 37 and 40 were predominantly expressed in homocellular interendothelial gap junction plaques, with connexin 43 predominantly expressed in homocellular smooth muscle gap junction plaques in the media (Chaytor *et al.*, 2003). The composition of myoendothelial gap junction plaques remains unknown.

In addition to "classical" pharmacological uncouplers of gap junctional communication, such as 18α-glycyrrhetinic acid and carbenoxolone, the recent development of novel "connexin-mimetic peptides" has enabled researchers to dissociate the relative contribution of individual connexin subtypes to gap junctional

communication. The peptides ^{37,43}Gap 27, ^{40}Gap27 and ^{43}Gap26 are short amino acid sequences homologous to connexin-specific variations in the Gap26 and Gap27 domains (1st and 2nd extracellular loops, respectively) of these proteins. Our laboratory has previously demonstrated the role of gap junctional communication in endothelial-dependent smooth muscle hyperpolarization and relaxation in rabbit arteries, with "EDHF-type" relaxations evoked by ACh being essentially abolished by a triple combination of the above peptides (Chaytor *et al.*, 2003). In addition, EDHF-type responses to cyclopiazonic acid (CPA), a compound that promotes SOCE by inhibiting Ca^{2+} reuptake into the ER via SERCA thus depleting the intracellular store, are abolished by ^{43}Gap26 in the rabbit iliac artery (Edwards *et al.*, 2007; 2008; Garry *et al.*, 2009). Correspondingly, ^{37,43}Gap27 causes retention of the dye calcein within the intima following intraluminal perfusion and preferential endothelial loading with its cell-permeant precursor calcein-AM by inhibiting the radial diffusion of calcein, formed by cleavage of the AM moiety, into the media via myoendothelial gap junctions (Griffith *et al.*, 2002).

While the molecular mechanism of action of these peptides is unclear, they are likely to interrupt channel gating, rather than junction and plaque stability, as wash-out of the peptide restores electrical coupling and dye transfer (Chaytor *et al.*, 1998; 2001; Martin *et al.*, 2005). Any possible non-junctional effects of these peptides have been discounted on the basis that agonist-evoked endothelial hyperpolarizations are not affected (Griffith *et al.*, 2005) without alteration in smooth muscle responsiveness to NO donors, K^+ channel openers and vasoconstrictor agonists (Chaytor *et al.*, 1997; 1998; Dora *et al.*, 1999; Richards *et al.*, 2001; Chaytor *et al.*, 2001; Sandow *et al.*, 2002; Ujiie *et al.*, 2002). Furthermore, non-specific effects of these peptides on endothelial NO biosynthesis in rabbit arteries can also dismissed as sandwich

bioassay experiments revealed that ACh induces an NO-dependent/^{37,43}Gap27-insensitive relaxation of a juxtaposed, but electrically uncoupled, smooth muscle strip indicating that NO transfer from donor endothelium to recipient smooth muscle is unaffected by the uncoupling of myoendothelial gap junctional communication (Chaytor *et al.*, 1998). Other studies, using different mammalian vessels and relaxant agonists, have also demonstrated a dominant role of gap junctional communication in EDHF-type relaxant responses (Edwards *et al.*, 1999; 2000; Doughty *et al.*, 2000; Allen *et al.*, 2002; Sandow *et al.*, 2002; Xu *et al.*, 2002).

1.5.3 The Integrated Role of K_{Ca} Channels

The activation of endothelial calcium-activated K^+ channels (K_{Ca}) is pivotal to endothelium-dependent smooth muscle hyperpolarization and relaxation mediated via gap junctions (Kagota *et al.*, 1999; Brandes *et al.*, 2000; Hill *et al.*, 2000; Taylor *et al.*, 2001; Chaytor *et al.*, 2001; 2002; Ujiie *et al.*, 2002; Ungvari *et al.*, 2002; Dora *et al.*, 2003; Sandow *et al.*, 2003; 2004; Griffith, 2004; Edwards *et al.*, 2008). The vascular endothelium expresses small- and intermediate-conductance K_{Ca} channels (SK_{Ca} and IK_{Ca}, respectively) that are activated by rises in endothelial $[Ca^{2+}]_i$ in response to agonists or shear stress (Dube & Canty, 2001; Miura *et al.*, 2001; Griffith, 2004). The sustained opening of these channels is now accepted to be dependent on SOCE from the extracellular space following intracellular store depletion (Marchenko & Sage, 1993; Sedova *et al.*, 2000; Nilius & Droogmans, 2001). While large-conductance BK_{Ca} channels predominate in smooth muscle cells they are variably expressed in the endothelium in some artery types (Kagota *et al.*, 1999; Ungvari *et al.*, 2002; Brakemeier *et al.*, 2003; Hilgers *et al.*, 2006). The individual and integrated role of these different K_{Ca} channel subtypes in EDHF-type responses appears to be vessel- and species-specific with different artery types displaying variable susceptibilities to

individual and combined K_{Ca} channel blockade, which may be agonist-specific (Freiden *et al.*, 1999; Marrelli *et al.*, 2003; Eichler *et al.*, 2003; Gluais *et al.*, 2005). Our laboratory has recently demonstrated an integrated role of these channels in gap junction-dependent EDHF-type relaxant responses in the rabbit iliac artery (Edwards *et al.*, 2008). Specific blockers of SK_{Ca}, IK_{Ca} and BK_{Ca} channels (apamin, TRAM-34 and iberiotoxin, respectively) revealed interdependence of these channels in the EDHF-type relaxant response following store depletion by CPA on the basis that apamin or TRAM-34 alone was ineffective, whereas their combination or iberiotoxin alone attenuated relaxation from ~86% to ~60% of induced tone. Only a triple combination of these blockers markedly reduced relaxation to ~35% with residual relaxation further attenuated to ~20% in the presence of catalase, an enzyme that destroys H_2O_2. This effect on CPA-evoked EDHF-type relaxation was indistinguishable from parallel experiments where gap junctional communication was interrupted with ^{43}Gap26, thereby implicating a crucial role of K_{Ca} channels in gap junction-dependent EDHF-type relaxant responses in this vessel. Furthermore, the additional attenuation of residual apamin+TRAM+iberiotoxin-insensitive relaxation by catalase was not due to H_2O_2 functioning as a freely diffusible EDHF because authentic H_2O_2 causes minimal smooth muscle hyperpolarization in the rabbit iliac artery (Chaytor *et al.*, 2003).

1.6 Hydrogen Peroxide and the EDHF Phenomenon

H_2O_2 has been proposed as a contributing mediator to vascular responses as diverse as coronary autoregulation, the myogenic response, flow-induced dilatation and reactive hyperaemia (Wolin *et al.*, 1990; Nowicki *et al.*, 2001; Koller & Bagi, 2004; Liu *et al.*,

2006; Yada *et al.*, 2006; 2008). Furthermore, H_2O_2 has also been demonstrated to act as an EDHF and an EDRF (Matoba *et al.*, 2000; 2002; Chaytor *et al.*, 2003).

H_2O_2 was first implicated as an EDHF in mouse and human mesenteric arteries on the basis that EDHF-type arterial relaxations and smooth muscle hyperpolarizations to ACh were sensitive to catalase (Matoba *et al.*, 2000; 2002). In these artery types, relaxation evoked by ACh was attributed to the endothelial production of H_2O_2 following stimulation by the agonist. Subsequent studies have shown inconsistency with a role of H_2O_2 as a freely diffusible EDHF. For example, our laboratory has shown that in the rabbit iliac artery, EDHF-type relaxation to A23187 (a Ca^{2+} ionophore), but not endothelium-dependent smooth muscle hyperpolarization, is sensitive to catalase. In the presence of catalase, endothelium-dependent smooth muscle hyperpolarization and the residual EDHF-type relaxation to A23187 was, however, abolished by connexin mimetic peptides. In addition, ACh itself only weakly stimulated H_2O_2 release from the endothelium (Chaytor *et al.*, 2003).

A further difficulty in implicating H_2O_2 as an EDHF is the observation that in a wide variety of human, rat, mouse, rabbit and pig arteries exogenous H_2O_2 only evokes smooth muscle hyperpolarizations and relaxations at "supraphysiological" concentrations, relative to the extracellular accumulation of H_2O_2 following agonist stimulation (Bény & von der Weid, 1991; Karasu *et al.*, 1999; Matoba *et al.*, 2000; Fujimoto *et al.*, 2001; Hattori *et al.*, 2003; Chaytor *et al.*, 2003; Gao *et al.*, 2003; Miura *et al.*, 2003). Indeed, measurements of extracellular H_2O_2 of the rat aorta (following stimulation with A23187) and human coronary arterioles (in response to flow) have revealed concentrations of just 10 – 100 nM and 200 nM in organ bath experiments, respectively (Cosentino *et al.*, 1998; Liu *et al.*, 2003). Conversely, while H_2O_2 is membrane diffusible, it has been estimated that only 1 – 15% of H_2O_2 applied

extracellularly is likely to be biologically available inside the cell (Schroder & Eaton, 2008). Additionally, intracellular H_2O_2 production and subsequent "redox-signalling" may speculatively involve microdomains of this ROS that are enzymatically-generated in proximity to their site of action. Correspondingly, accumulating evidence suggests that the specific subcellular localization of ROS-generating enzymes may be intricately linked to their function. For example, NADPH oxidase 4, which produces mostly H_2O_2 rather than $O_2^{\bullet-}$, is predominantly expressed in the ER membrane of endothelial and smooth muscle cells, and is critical for the oxidative regulation of the ER resident protein tyrosine phosphatase 1B (Hilenski *et al.*, 2004; Petry *et al.*, 2006; Chen *et al.*, 2008). The intracellular concentrations of H_2O_2 achieved following agonist or shear stress stimulation in the endothelium remain to be established.

H_2O_2 has previously been shown to mobilize endothelial Ca^{2+} via mechanisms that are variably dependent (Volk *et al.*, 1997) or independent (Hu *et al.*, 1998; 2000; Zheng & Shen, 2005) of phospholipase C activity. However, until recently, H_2O_2-evoked endothelial Ca^{2+} mobilization had not been investigated in the context of the EDHF phenomenon. Indeed, there is a growing appreciation of the dependency of EDHF-type relaxation responses on H_2O_2-generating enzymes such as cytosolic SOD (Cu/Zn-SOD) with attenuated EDHF-type relaxant responses having been reported in humans and animals either following pharmacological blockade of the enzyme or in Cu/Zn-SOD$^{(-/-)}$ mice (Cooke & Davidge, 2003; Morikawa *et al.*, 2003; 2004). In addition, the SOD-mimetic tempol restores EDHF-type relaxant responses blunted by enhanced $O_2^{\bullet-}$ production in hypertensive rats (Adeagbo *et al.*, 2003; Ozawa *et al.*, 2004; Christensen *et al.*, 2007).

Our group has shown that H_2O_2 enhances electrotonically-mediated EDHF-type relaxations evoked by CPA and ACh in the rabbit iliac artery (Edwards *et al.*, 2008;

Garry *et al.*, 2009). Here, measurements of endothelial $[Ca^{2+}]_i$ and ER store Ca^{2+} concentration ($[Ca^{2+}]_{ER}$) revealed that H_2O_2 enhanced endothelial Ca^{2+} mobilization evoked by CPA. Administration of 100 μM H_2O_2 alone affected neither $[Ca^{2+}]_i$ nor $[Ca^{2+}]_{ER}$. However, dual administration of 100 μM H_2O_2 and 10 μM CPA evoked an elevation of $[Ca^{2+}]_i$ and reduction of $[Ca^{2+}]_{ER}$ to levels indistinguishable from those observed when maximal responses were evoked by 30 μM CPA alone. This synergism may reflect oxidation and sensitization of the $InsP_3$ receptor by H_2O_2 on the basis that (i) elevations in $[Ca^{2+}]_i$ correlated with store depletion, (ii) the endothelium-specific isoform of the SERCA pump (SERCA3) is insensitive to H_2O_2 at concentrations <500 μM (Liu *et al.*, 1997; Grover *et al.*, 1997), and (iii) the thiol reagent thimerosal closely mimicked the effect of H_2O_2. Thimerosal has been shown to oxidize $InsP_3$ receptors and promote ER Ca^{2+} depletion (Bootman *et al.*, 1992; Parekh & Penner, 1995; Montero *et al.*, 2001; Bultynck *et al.*, 2004). In addition, although cumulative concentration-relaxation responses to H_2O_2 were indistinguishable between intact and endothelium-denuded rings, a potentiation was observed in intact rings in the presence of 10 μM CPA. This was partially reversed by blockade of BK_{Ca} channels (with iberiotoxin) and abolished after dual blockade of SK_{Ca} and IK_{Ca} (with apamin and TRAM-34, respectively), indicating downstream activation of endothelial K_{Ca} channels and subsequent hyperpolarization. Therefore, in the rabbit iliac artery, H_2O_2 potentiates EDHF-type vasorelaxation by promoting endothelial Ca^{2+} mobilization, probably by sensitizing the $InsP_3$ receptor, with downstream hyperpolarization and relaxation of smooth muscle mediated by K_{Ca} channels and electrotonic signalling via gap junctions (Edwards *et al.*, 2008).

1.7 Direct Smooth Muscle Actions of Hydrogen Peroxide

Distinct from the endothelial actions of H_2O_2 recently described in the rabbit iliac artery (Edwards *et al.*, 2008), this ROS is also able to modulate arterial smooth muscle tone directly. Indeed, the enhancing effect of exogenous H_2O_2 on EDHF-type relaxation in the rabbit iliac artery is superimposed on a direct smooth muscle relaxant action (Edwards *et al.*, 2008). However, smooth muscle responses to exogenous H_2O_2 in mammalian arteries display marked heterogeneity. For example, in contrast to the rabbit iliac artery, the responses of rat arteries to H_2O_2 are biphasic with low concentrations evoking constriction and high concentrations evoking relaxation (Gao *et al.*, 2003; Cseko *et al.*, 2004; Samora *et al.*, 2008; Garcia-Redondo *et al.*, 2009). The constrictor effects of H_2O_2 in the rat superior mesenteric artery are endothelium-independent (Gao *et al.*, 2003), but in 3rd order mesenteric arteries and skeletal muscle arterioles constriction is attenuated (not abolished) by removal of the endothelium (Cseko *et al.*, 2004; Samora *et al.*, 2008; Garcia-Redondo *et al.*, 2009). Endothelium-independent constriction to H_2O_2 in all of these artery types is abolished by inhibitors of COX or thromboxane A_2 synthesis (Gao *et al.*, 2003; Cseko *et al.*, 2004; Samora *et al.*, 2008; Garcia-Redondo *et al.*, 2009). H_2O_2 relaxations in the rat superior mesenteric artery are also endothelium-independent and are abolished by high $[K^+]_o$ or attenuated by tetraethylammonium (TEA), consistent with a direct action of H_2O_2 on smooth muscle K_{Ca} channels (Gao *et al.*, 2003). Similarly, endothelium-independent relaxation to H_2O_2 in skeletal muscle arterioles is also mediated, in part, by K_{Ca} channels although, in contrast to the superior mesenteric artery, this is superimposed on an endothelium-dependent component that involves the production of NO (Cseko *et al.*, 2004; Samora *et al.*, 2008).

Relaxation of pig coronary arterial smooth muscle by H_2O_2 is partly mediated by BK_{Ca} channels, although this is via a secondary mechanism which involves activation of phopholipase A_2 and the production of lipooxygenase-derived arachidonate metabolites (Barlow *et al.*, 1998; 2000). The above observations contrast with corresponding responses of the rabbit iliac artery which do not appear to involve any of the three K_{Ca} channels subtypes (Chaytor *et al.*, 2003; Edwards *et al.*, 2008). Correspondingly, 1 mM H_2O_2 only weakly hyperpolarizes (~7.5 mV) rabbit iliac arterial smooth muscle (Chaytor *et al.*, 2003), whereas the same treatment hyperpolarizes rat superior mesenteric arterial smooth muscle by ~15 mV (Gao *et al.*, 2003). Furthermore, while H_2O_2 induces Ca^{2+} mobilization and augmented K_{Ca} channel current in isolated endothelial cells, it has been reported that this ROS directly blocks BK_{Ca} at millimolar concentrations, probably via oxidation of cysteine 911 in the Ca^{2+} binding region of the α subunit of the channel thus impairing the binding of Ca^{2+} (Doan *et al.*, 1994; Hu *et al.*, 1998; Bychkov *et al.*, 1999; Brakemeier *et al.*, 2003; Tang *et al.*, 2004; Zheng *et al.*, 2005; Mergler *et al.*, 2005; Lu *et al.*, 2006).

While the studies of Gao *et al* (2003), Cseko *et al* (2004), Samora *et al* (2008) and Garcia-Redondo *et al* (2009) have highlighted heterogeneity in the responses of rat arteries to H_2O_2, this may be particularly evident in the human circulation. For example, in coronary arterioles H_2O_2–evoked relaxation is endothelium-independent and abolished by either high $[K^+]_o$ or apamin in combination with the non-specific IK_{Ca} and BK_{Ca} blocker charybdotoxin (Miura *et al.*, 2003). Furthermore, H_2O_2 evokes hyperpolarization of human coronary arteriolar smooth muscle (Matoba *et al.*, 2003). In contrast, H_2O_2–evoked relaxations of gut submucosal microvessels are endothelium-dependent with only a constrictor response observed in endothelium-

denuded preparations, despite the fact that EDHF-type relaxations in this vessel type are, like those of the mesenteric artery and coronary arterioles, sensitive to catalase (Matoba *et al.*, 2002; Hatoum *et al.*, 2005; Larsen *et al.*, 2008). While H_2O_2 has been postulated to be an EDHF in the human mesenteric artery and coronary arterioles (Matoba *et al.*, 2002; Miura *et al.*, 2003; Larsen *et al.*, 2008), the study of Hatoum and co-workers suggests that, in gut submucosal microvessels, H_2O_2 may be involved in the release of a chemically-distinct EDHF (Hatoum *et al.*, 2005). A further example of intraspecies heterogeneity is the dynamic range of vascular responsiveness to exogenous H_2O_2. For example, in mice, H_2O_2 relaxation in cerebral arteries occurs in the nanomolar range whereas micromolar concentrations are required in mesenteric arteries (Matoba *et al.*, 2000; Drouin *et al.*, 2007).

H_2O_2-evoked relaxations in rabbits are endothelium-independent, as demonstrated in the iliac and the superior and small mesenteric arteries (Fujimoto *et al.*, 2001; Edwards *et al.*, 2008). Furthermore, relaxations to H_2O_2 in these arteries may be explained by a decreased sensitivity of the smooth muscle contractile apparatus to Ca^{2+}, as has been reported in the rabbit aorta (Iesaki *et al.*, 1996). Furthermore, recent evidence has uncovered another mechanism by which H_2O_2 is able to mediate direct smooth muscle relaxation. Burgoyne and colleagues have identified a novel cysteine redox sensor (Cys42) of protein kinase G (PKG) that, upon application of exogenous H_2O_2, forms disulphide bridges between PKG monomers to form dimers, thereby increasing the affinity of this enzyme for its target substrates. Correspondingly, exogenous H_2O_2 triggered a relaxation of the rat thoracic aorta that was abolished by inhibition of PKG with Rp-8-Br-cGMP, but not by inhibition of soluble guanylyl cyclase (sGC) with 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) (Burgoyne *et al.*, 2007). This finding is in stark contrast to observations in mouse cerebral and

porcine coronary arteries, where relaxation to H_2O_2, though endothelium-independent, is attenuated by ODQ, thus suggesting a mechanism involving the activation of sGC rather than a direct downstream action on PKG (Hayabuchi *et al.*, 1998; Drouin *et al.*, 2007).

Taken together, it is apparent from the evidence discussed that H_2O_2 cannot be described unambiguously as an EDHF, and that the mechanisms through which it mediates direct smooth muscle relaxation require further clarification.

1.8 Arsenic, Glucose and the EDHF Phenomenon

Arsenic and hyperglycaemia both induce endothelial oxidative stress and impaired NO bioavailability. However, modulation of the EDHF phenomenon by arsenic has not yet been investigated, whilst the effect of diabetes on EDHF-type responses, despite fifteen years of investigation, remains controversial. Improving EDHF-type responses has recently been proposed as a novel potential drug target for the treatment of diabetes-associated impairments in tissue perfusion (Feletou, 2009). Therefore, elucidation of the mechanisms through which the EDHF phenomenon may compensate for impaired NO bioavailability, as has been demonstrated in diabetic models, may provide an important focus for pharmaceutical effort (Thomsen *et al.*, 2001; Timar-Peregrin & Guy, 2002; Shen *et al.*, 2003; Yousif *et al.*, 2005; Shi *et al.*, 2006; Malakul *et al.*, 2008; MacKenzie *et al.*, 2008; Patericza *et al.*, 2008).

1.8.1 Arsenic- and Hyperglycaemia-Evoked ROS Production in Endothelial Cells: Implications for Modulation of the EDHF Phenomenon

As previously highlighted (see 1.2), arsenic and hyperglycaemia have been implicated in the accelerated onset of atherosclerosis, and several studies have suggested that enhanced ROS production and subsequent endothelial dysfunction contribute to their

pro-atherogenic action (Nakagami *et al.*, 2005; Jindal *et al.*, 2008). However, the ROS-generating systems involved in arsenic- and glucose-evoked changes in endothelial redox status remain to be fully established.

Endothelial cells are capable of generating ROS via a number of systems including NADPH oxidase, uncoupled eNOS, xanthine oxidase, COX, cytochrome P_{450} and the mitochondrial electron transport chain (Wolin, 2000). While some authors suggest NADPH oxidase is principal source of ROS in the healthy endothelium (Griendling *et al.*, 2000; Ushio-Fukai *et al.*, 2002), it remains to be elucidated whether this enzyme is the dominant system involved in arsenic- and glucose-mediated ROS production. Indeed, arsenic has been shown to activate NADPH oxidase via a mechanism involving the translocation of Rac1, the GTPase essential for activation of the enzyme complex, to membrane fractions in endothelial cells (Smith *et al.*, 2001). Glucose is thought to activate this enzyme via a protein kinase C (PKC)-dependent mechanism, possibly involving phosphorylation of Rac1 (Cosentino *et al.*, 2003; Inoguchi *et al.*, 2003; Cave *et al.*, 2006; Ceolotto *et al.*, 2007). Glucose has also been reported to evoke $O_2^{\bullet-}$ release from the mitochondrial electron transport chain as a by product of oxidative metabolism (Nishikawa *et al.*, 2000; Quijano *et al.*, 2007). Arsenic may also facilitate ROS release from isolated mitochondria via direct interaction with vicinal thiols of the mitochondrial permeability transition pore (mPTP), although this has not yet been studied in endothelial cells (Larochette *et al.*, 1999; Tian *et al.*, 2005).

The production of ROS in endothelial cells under hyperglycaemic conditions and in the presence of arsenic has implications for modulation of the EDHF phenomenon. For example, glucose increases $O_2^{\bullet-}$ levels in endothelial cells in a concentration-dependent manner which correlates with rises in $[Ca^{2+}]_i$ (Wascher *et al.*, 1994; Graier *et al.*, 1996; 1997; Wu *et al.*, 1999). In addition, both arsenic and elevated glucose

promote endothelial production of H_2O_2 (Barchowsky *et al.*, 1999; Guo *et al.*, 2000; Lee *et al.*, 2008). As previously discussed (see 1.6), our laboratory has recently shown that H_2O_2 promotes endothelial Ca^{2+} mobilization and enhances EDHF-type relaxation in the rabbit iliac artery (Edwards *et al.*, 2008). While arsenite elevates $[Ca^{2+}]_i$ in multiple cell types, this has not yet been demonstrated in endothelial cells (Shen *et al.*, 2002; Mehta *et al.*, 2006; Sandoval *et al.*, 2007; Vahidnia *et al.*, 2008; Li *et al.*, 2009). Shen *et al* reported that low micromolar levels of arsenic mobilized intracellular Ca^{2+} stores of an oesophageal epithelial cell line, with peaks consistently reached after just 15 minutes (Shen *et al.*, 2002). Furthermore, Li and colleagues have recently demonstrated that arsenic-evoked vascular smooth muscle Ca^{2+} mobilization is mediated by a mechanism involving ROS (Li *et al.*, 2009).

Taken together, the acute effects of arsenic and elevated glucose on endothelial H_2O_2 production suggest that they might be capable of modulating EDHF-type relaxation in vessel types where H_2O_2 is known to participate in this phenomenon.

1.8.2 Arsenic, Hyperglycaemia and Endothelium-Dependent Arterial Relaxation

The effects of arsenic on endothelium-dependent arterial relaxation remain poorly understood. Indeed, to the author's knowledge, there are only four published articles investigating the effects of arsenic. Furthermore, despite over twenty years of investigation, the effects of hyperglycaemia remain controversial.

1.8.2.1 Impaired NO-Dependent Relaxation Following Arsenic Exposure

Attenuation by arsenic of endothelium-dependent relaxation to ACh in the rat aorta has been variously attributed to reduced eNOS expression and reduced NO bioavailability mediated by oxidative stress (Lee *et al.*, 2003; Jindal *et al.*, 2008). Srivastava and colleagues (2007) also reported that arsenic impaired NO-dependent relaxations evoked by ACh in the mouse aorta, but did not investigate the nature of

the mechanisms involved. By contrast, Bilszta and co-workers (2006) reported that arsenic failed to alter relaxations of rat aorta and renal artery to ACh despite constrictor responses to phenylephrine being potentiated due reduced basal NO bioavailability.

1.8.2.2 Impaired NO-Dependent Relaxation in Diabetes

Impaired NO bioavailability due to oxidative stress is the most consistently reported phenomenon in studies of the modulation of endothelial function in diabetes, although several reports suggest that in the early stages of diabetes NO bioavailability may actually be increased, possibly via oxidant-induced Ca^{2+} mobilization (Wascher *et al.*, 1994; Graier *et al.*, 1996; 1997a; 1997b; 1998; Pieper *et al.*, 1999; Sercombe *et al.*, 2004). A number of studies have suggested that ROS derived from NADPH oxidase impair NO bioavailability in arteries from diabetic animals (Kim *et al.*, 2002; Coppey *et al.*, 2003; Sercombe *et al.*, 2004; Hayashi *et al.*, 2005; Bitar *et al.*, 2005; Tawfik *et al.*, 2006; Matsumoto *et al.*, 2007; Su *et al.*, 2008; Shukla *et al.*, 2008; Lopez-Lopez *et al.*, 2008), although the mitochondrial electron transport chain (Bagi *et al.*, 2004; Rosen and Wiernsperger, 2006) and cytochrome P_{450} (Elmi *et al.*, 2008) may be functionally significant sources in certain vascular beds. Furthermore, a number of studies have revealed the potential of SOD and its cell-permeable mimetic, tempol, to restore relaxant responses (Hattori *et al.*, 1991; Nassar *et al.*, 2002). It is also apparent that the effect of diabetes on NO-dependent relaxation may differ depending on the mode of endothelial stimulation (White & Carrier, 1986; Kamata & Hosokawa, 1997).

1.8.2.3 Altered Prostanoid Production in Diabetes

It was apparent from early studies of vascular function in diabetes that the condition may cause alterations in the production of dilator and/or constrictor prostanoids that may contribute to altered vascular responsiveness. For example, Mayhan and

colleagues reported that impaired endothelium-dependent relaxation of streptozotocin (an agent that induces type I diabetes by causing pancreatic ß cell necrosis) (STZ)-induced diabetic rat cerebral arterioles was mediated, at least in part, by the increased production of the endothelium-derived contracting factor (EDCF) thromboxane A_2, as indicated by the restoration of relaxant responses by the COX inhibitor indomethacin or a thromboxane A_2 receptor antagonist (Mayhan *et al.*, 1991). It was subsequently suggested that hyperglycaemia-evoked increases in endothelial thromboxane A_2 production may be mediated by a mechanism involving elevated ROS production (Kobayashi & Kamata, 2002; Cosentino *et al.*, 2003; Erdei *et al.*, 2007). By contrast, Alabadi *et al* (2001) reported that an increased production of dilator prostanoids in rabbit renal arteries during acute exposure to elevated glucose potentiated relaxant responses to ACh. However, studies of other vessel types have not provided evidence for functionally significant effect of COX inhibitors on endothelium-dependent relaxations in diabetes (Hattori *et al.*, 1991; Shi *et al.*, 2006).

1.8.2.4 Attenuation of the EDHF Phenomenon in Diabetes

A number of mechanisms have been postulated to contribute to impaired EDHF-type relaxation in diabetes. Fukao and colleagues (1997) reported impaired EDHF-type relaxations and endothelium-dependent smooth muscle hyperpolarizations to ACh in the mesenteric artery of STZ-diabetic rats, an effect that did not appear to involve ROS because responses were unaffected by SOD (Fukao *et al.*, 1997). By contrast, it was reported in the same artery type that either the individual application of SOD or the combined administration of SOD and catalase restored EDHF-type relaxations to ACh blunted by acute exposure to high glucose (Ozkan & Uma, 2005).

In small mesenteric arteries from STZ-treated apolipoprotein E null mice impaired EDHF-type relaxation to ACh involves, in part, decreased expression of SK_{Ca}

channels (Ding *et al.*, 2005). However, it was subsequently reported that impaired EDHF-type responses in the obese type II diabetic rat mesenteric artery were associated with the decreased activity, but not expression, of SK_{Ca} (Burnham *et al.*, 2006a). Additionally, these authors demonstrated that Ca^{2+}-dependent activation of BK_{Ca} channels located on the smooth muscle cells of these arteries was impaired in the same animal model of type II diabetes, and suggested that increased production of H_2O_2 may have accounted for the blockade of BK_{Ca} channels via the mechanism described by Tang and colleagues, whereby oxidation by H_2O_2 of the cysteine911 residue located in the Ca^{2+} bowl region of the α subunit virtually eliminated physiological activation of the channel (Tang *et al.*, 2004; Burnham *et al.*, 2006b). Alternatively, it has been proposed that the reduced Ca^{2+} sensitivity of BK_{Ca} channels of STZ-diabetic rat retinal arteriolar smooth muscle is mediated by the reduced expression of the $ß_1$ subunit (McGahon *et al.*, 2007).

A number of studies have also demonstrated that gap junctional communication may be impaired in diabetes. For example, the reduced expression of connexin 40 mediates impaired EDHF-type relaxations in the STZ-diabetic mouse coronary and type II diabetic rat mesenteric arteries (Makino *et al.*, 2008; Young *et al.*, 2008). Furthermore, the reduced expression of connexin 37 accounts, in part, for impaired EDHF-type relaxation in STZ-diabetic mouse small mesenteric arteries (Ding *et al.*, 2005). It has also been reported that elevated glucose evokes PKC-dependent phosphorylation of connexin 43 and impairs gap junction permeability in isolated aortic endothelial and smooth muscle cells (Inoguchi *et al.*, 1995; Kuroki *et al.*, 1998). Additionally, Matsumoto and colleagues have reported that impaired gap junction-dependent EDHF-type relaxations to ACh in STZ-diabetic rat mesenteric arteries are mediated by the reduced accumulation of cAMP resulting from the increased

expression of phosphodiesterase 3 and/or the reduced expression of adenylyl cyclase (Matsumoto *et al.*, 2003; 2004; 2005). Indeed, cAMP is known to play a permissive role in EDHF-type relaxations that involve gap junctional communication by modulating connexin functionality (Taylor *et al.*, 2001; Griffith *et al.*, 2002; Chaytor *et al.*, 2002; Griffith *et al.*, 2005).

1.8.2.5 Augmentation of the EDHF Phenomenon in Diabetes

Endo and colleagues were the first to report that EDHF-type relaxations may be resistant to diabetes and thus did not account for impaired endothelium-dependent relaxation in the STZ-diabetic rat aorta (Endo *et al.*, 1995). It was subsequently reported that in STZ-diabetic rat cremaster arterioles and the db/db (leptin receptor knockout) type II diabetic mouse small mesenteric artery EDHF-type relaxations were augmented and served to compensate for impaired NO bioavailability (Timar-Peregrin & Guy, 2001; Pannirselvam *et al.*, 2002). Following this, the studies of Thomsen and colleagues (2002) and Shen and colleagues (2003) provided evidence that augmentation of the EDHF phenomenon in diabetes was associated with an enhanced contribution of K_{Ca} channels, as demonstrated in STZ-diabetic rat sciatic nerve arterioles and the STZ-diabetic mouse aorta, respectively. These findings were later substantiated by Shi and co-workers (2006), who demonstrated augmented apamin+charybdotoxin-sensitive EDHF-type relaxations in the mesenteric and femoral arteries of STZ-diabetic rats. The reasons for the differential modulation of EDHF-type relaxation observed by the studies of Fukao *et al* and Shi *et al* are unclear as both groups analysed responses in the same artery of the same diabetic rat model after a similar duration of disease.

Malakul and co-workers (2008) later reported the existence of an EDHF-type relaxant response, mediated by apamin+charybdotoxin-sensitive K_{Ca} channels, in the aorta of

the STZ-diabetic rat, with such responses being absent from healthy animals. In addition, these authors reported that hypercholesterolaemia also revealed the contribution of an apamin+charybdotoxin-sensitive EDHF-type relaxant response, with the combination of diabetes and hypercholesterolaemia synergistically increasing NADPH oxidase activity and additively augmenting the EDHF phenomenon. These observations appear inconsistent with the previously mentioned findings of Endo and colleagues (1995), where EDHF-type responses were evident in healthy animals, and were unaffected by diabetes. Indeed, other authors have variously demonstrated the existence or absence of EDHF-type relaxations in the rat aorta under normal conditions (Chen *et al.*, 1988; Wu *et al.*, 1993; Iranami *et al.*, 1997).

MacKenzie and colleagues also reported augmented EDHF-type relaxations to bradykinin in human mesenteric arteries acutely exposed to high glucose, an artery type where H_2O_2 contributes to the EDHF phenomenon under control conditions (Matoba *et al.*, 2002; MacKenzie *et al.*, 2008). Pataricza and colleagues also reported an enhanced contribution of K_{Ca} channels to endothelium-dependent relaxation (in the absence of eNOS and COX inhibitors) to ACh in coronary arteries of alloxan-diabetic dogs that served to fully preserve vascular function (Pataricza *et al.*, 2008). Furthermore, Park and co-workers have recently reported that EDHF-type relaxations are abolished by catalase or apamin+charybdotoxin in coronary arterioles from type II diabetic mice, whereas these treatments only partially attenuate such responses in healthy mice (Park *et al.*, 2008). This observation appears to be consistent with the report of Edwards and colleagues (2008) that endogenous H_2O_2 may modulate concerted K_{Ca} channel activity and thus contribute to the EDHF phenomenon in the rabbit iliac artery.

Several reports have demonstrated an increased functional role of H_2O_2 in diabetic vessels. For example, H_2O_2 compensates for impaired NO bioavailability during ACh-evoked relaxant responses in aortae of STZ-diabetic rats (Karasu, 2000). However, it was not determined whether this was mediated via an EDHF-type mechanism. Furthermore, it has also been reported that exogenous H_2O_2 evokes more potent relaxation in the STZ-diabetic rat aorta, although the mechanism remains to be elucidated (Pieper & Gross, 1988; Karasu, 1999).

1.9 Cross-Talk Between the EDHF Phenomenon and NO

Several groups have suggested the existence of a reciprocal relation between NO-dependent and EDHF-type responses. Such "cross-talk" may facilitate a dynamic augmentation of the EDHF phenomenon when NO bioavailability is reduced. Thus, in canine coronary arterioles EDHF-type relaxations can completely offset the NO component (Nishikawa *et al.*, 2000b), whereas EDHF-type responses may be blunted by exogenous NO or under conditions where an excess of NO is released from the endothelium e.g. during sepsis (Beach *et al.*, 2001; Griffith *et al.*, 2005). NO has been reported to promote Ca^{2+} sequestration via S-glutathiolation of the SERCA pump while, downstream from NO, PKG may impair SOCE either via a direct mechanism involving phosphorylation of SOC or indirectly via phosphorylation of phospholamban leading to enhanced store refilling via the SERCA pump (Trepakova *et al.*, 1999; Mundina-Weilenmann *et al.*, 2000; Dora *et al.*, 2001; Adachi *et al.*, 2002). Therefore, reductions in NO bioavailability in diabetes could in theory augment the EDHF phenomenon directly.

1.10 Summary and Aims

From the evidence discussed above, it is apparent that NO bioavailability may be impaired by oxidative stress during diabetes and arsenic toxicity. Correspondingly, compelling evidence suggests that the increased endothelial production of ROS in diabetes and arsenic toxicity originates from NADPH oxidase. While the effect of arsenic on the EDHF phenomenon remains to be investigated, the modulation of EDHF-type relaxation during diabetes remains controversial despite extensive study. At the time of the last review of the EDHF phenomenon in diabetes in 2005 it was thought that this phenomenon was predominantly impaired in mammalian arteries, with the possible exception of mice (Fitzgerald *et al.*, 2005). However, studies subsequent to this review have provided evidence for augmented EDHF-type relaxations during diabetes in animals other than mice, including humans, rats and dogs (Shi *et al.*, 2006; MacKenzie *et al.*, 2008; Pataricza *et al.*, 2008; Malakul *et al.*, 2008). Furthermore, the recent study of Park and colleagues suggests that the contribution of H_2O_2 to EDHF-type relaxations may become increasingly significant in diabetes (Park *et al.*, 2008). Correspondingly, our group has demonstrated that authentic H_2O_2 potentiates the EDHF phenomenon by promoting endothelial Ca^{2+} mobilization in the rabbit iliac artery (Edwards *et al.*, 2008). However, it remains to be determined whether H_2O_2 plays a direct role in the augmentation of EDHF-type relaxations when NO-dependent responses are blunted by oxidative stress in diabetes and arsenic toxicity.

Considering these observations, it was thus hypothesized that arsenic and glucose might potentiate the EDHF phenomenon in the rabbit iliac artery via production of H_2O_2. The aim of the current study was to compare the effects of acute exposure to arsenic and elevated glucose on NO-dependent and EDHF-type relaxation responses

to receptor-dependent and receptor-independent endothelial stimulation. Further investigations were undertaken to elucidate the role of ROS, particularly H_2O_2, and NADPH oxidase in these responses.

CHAPTER 2

GENERAL METHODS

2.1 Iliac Artery Dissection

Male New Zealand White rabbits (2–2.5 kg) were sacrificed with sodium pentobarbitone (Euthatal; 150 mg kg^{-1}) administered intravenously via a marginal ear vein and inspected for nervous reflexes (in keeping with the Animal and Scientific Procedures Act, 1986). The tissue containing the iliac arteries, including surrounding skeletal muscle and adipose tissue (see Figure 2.1), was dissected and placed in ice cool Holman's buffer (composition in mM: 120 NaCl, 5 KCl, 2.5 CaCl, 1.3 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose and 10 sucrose) for transit back to the laboratory. External iliac arteries were subsequently dissected from the tissue section and any excess adipose tissue adjacent to the adventitial surface was carefully removed.

2.2 Ring Segment Preparation and Tension Recording Procedure

The iliac artery was cut into ring segments (2–3 mm wide) and mounted in a Mulvany type Myograph (Model 400, Danish Myotechnology, Aarhus, Denmark) containing oxygenated (95% O_2, 5% CO_2) Holman's buffer and maintained at 37°C (see Figure 2.2). Recording of tension was then commenced using Myodaq software (Danish Myotechnology, Aarhus, Denmark). The arterial segment diameter was gradually (in a step-wise fashion) increased over an equilibration period of 1 hour until a resting tension of 1 mN was established. The buffer in each channel was then replaced and the tension readjusted following stress relaxation. To study EDHF-type relaxations preparations were then incubated for 30 minutes with the cyclooxygenase inhibitor indomethacin (10 μM) and a concentration of the eNOS inhibitor L-NAME (300 μM)

that has previously been shown to abolish endothelium-dependent accumulation of cGMP in rabbit iliac arteries (Taylor *et al.*, 2001). Some preparations, where appropriate, were incubated with one of these inhibitors individually.

Subsequent incubation with other pharmacological agents is specified in each Chapter. Tone was induced with the α_1 adrenoreceptor agonist phenylephrine (PE, 1 μM) and the preparations then allowed to reach a stable plateau over approximately 15 minutes. PE was specifically selected as the α_1 adrenoreceptor is expressed in smooth muscle but not in the endothelium, thereby preventing direct activation of endothelial cells prior to the addition of relaxants (Dora *et al.*, 2000). Endothelium-dependent relaxations were then studied by constructing cumulative concentration-response curves for ACh (1 nM – 10 μM) or CPA (100 nM – 100 μM). Endothelium-independent responses to MAHMA NONOate (1 nM – 10 μM) were studied in iliac artery preparations that had been denuded of their endothelium.

This was achieved by rubbing the luminal surface with a wooden rod. Rings were then mounted and prepared for isometric analyses as described for endothelium-intact preparations (see above). Firstly, tone was induced by PE and successful removal of the endothelium confirmed by the absence of the relaxant response to 1 μM ACh (normally ~90% in intact preparations; see Chapters 3 and 4 for appropriate examples). Preparations were then washed repeatedly with fresh Holman's buffer (every 10 minutes) for 1 hour to remove PE and ACh. Tone was then readjusted to maintain a resting tension of 1 mN in preparation for further study.

2.3 Data Collection

The Myodaq-recorded experiment file was then analysed using Myodata software. Tension values were collected for the baseline, for the addition of PE (plateau) and for

each concentration of the cumulative concentration-response curves to ACh, CPA or MAHMA NONOate. Data were entered into Excel software and the PE-induced increase in tone calculated (tension at PE plateau *minus* baseline tension). Each data point on the cumulative concentration-response curves for ACh, CPA and MAHMA NONOate was then subtracted from the baseline tension and calculated as a percentage reversal of PE-induced tone (% relaxation). This data was then entered into Graphpad Prism Version 4.0 software for curve construction and statistical analysis.

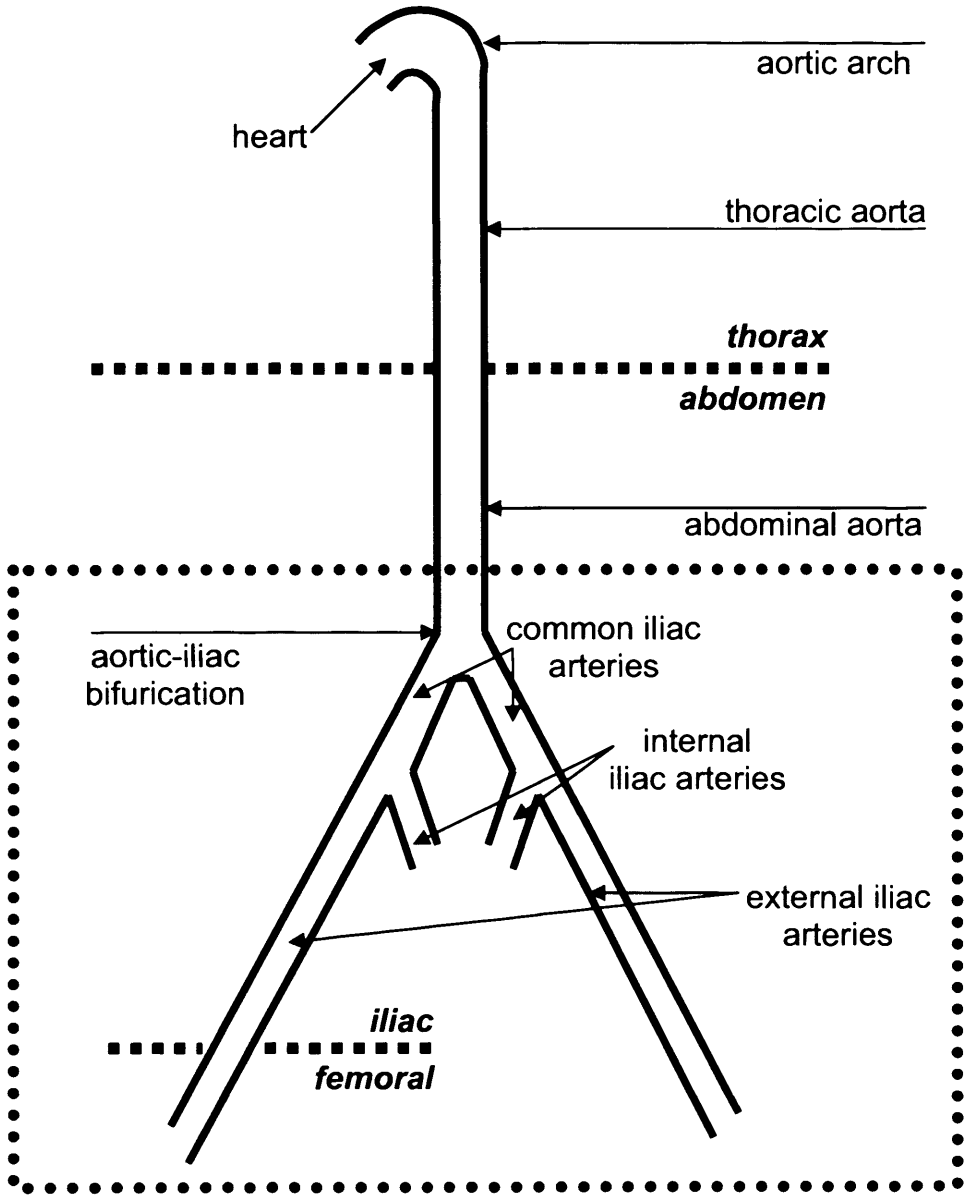
2.4 Statistical Analysis.

A small constrictor response evoked by low concentrations of CPA prior to the onset of EDHF-type relaxations meant that sigmoidal curves could not be plotted for these data. Therefore, the concentration of this agent giving 50% reversal of PE-induced tone (IC_{50}) was determined as an alternative to 50% maximal relaxation (as determined for ACh and MAHMA NONOate; EC_{50}). In addition, PE-induced tone and its maximal reversal (R_{max}) were determined and average PE-induced tone, R_{max} and pIC_{50}/EC_{50} (negative log molar IC_{50}/EC_{50}) were calculated as means±SEM. Normality was confirmed using the D'Agostino and Pearson omnibus test. Results were then compared using the Student's t-test for paired data or one-way ANOVA with Bonferroni multi-comparison post-tests as appropriate. Further comparisons of individual data points of concentration-response curves were obtained using the Student's t-test or two-way ANOVA with Bonferroni multi-comparison where appropriate. Variation was considered significant at $P<0.05$.

2.5 Reagent Information

All constituents of Holman's buffer were purchased from Fischer Scientific. Details of all other reagents studied in this thesis are provided in Table 2.1.

Figure 2.1 Schematic of the initial target dissection area. Initially, tissue containing the iliac arteries, including surrounding skeletal muscle and adipose tissue (indicated by dashed red box), was dissected for transit back to the laboratory before the arteries were finely-dissected and prepared for the experiment. Note that the initial tissue segment dissected includes the lower abdominal aorta and upper femoral artery to minimize damage to the iliac artery.


aortic arch
heart
thoracic aorta
thorax
abdomen
abdominal aorta
aortic-iliac
bifurication
common iliac
arteries
internal
iliac arteries
external iliac
arteries
iliac
femoral

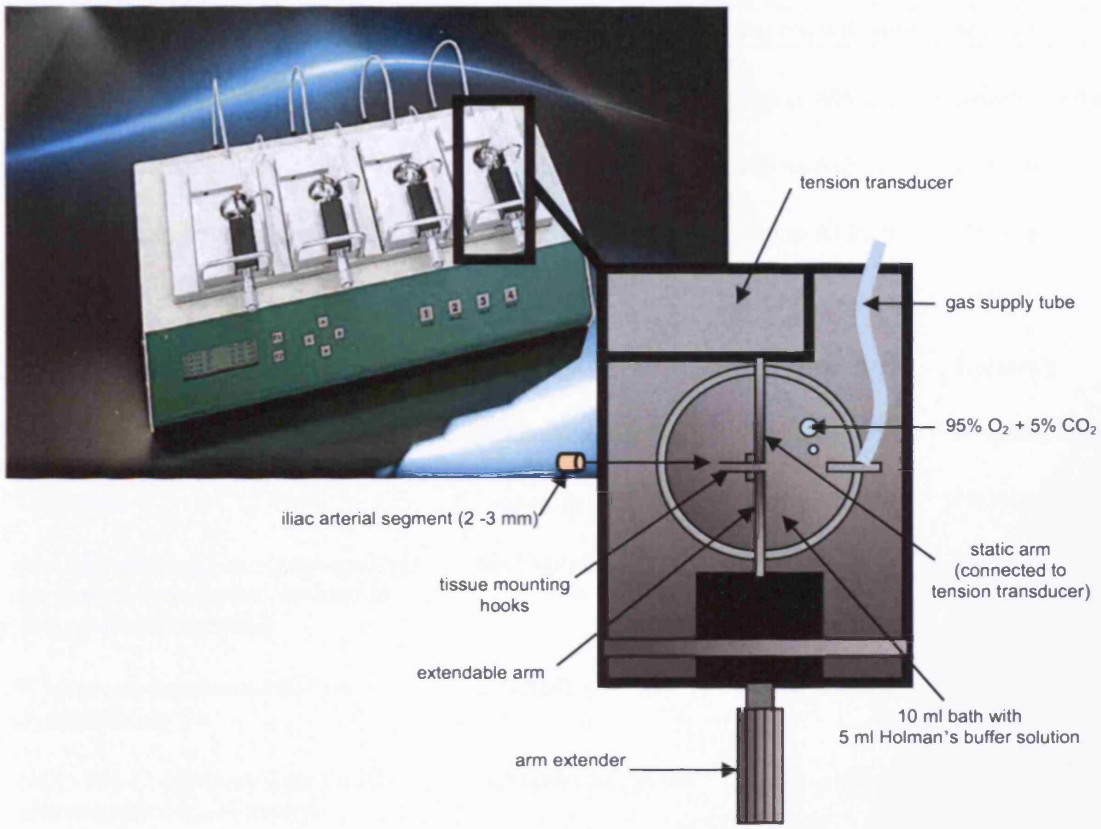


Figure 2.2 Schematic of a single channel of the Mulvany Myograph (original diagram). Photograph obtained from Danish Myotechnology, Aarhus, Denmark.

Full name	Short-name (in text)	Purchased from	Solvent
Acetovanillone	Apocynin	Sigma-Aldrich	Absolute Ethanol
Acetylcholine chloride	ACh	Sigma-Aldrich	Holman's
Catalase (from bovine liver)	Catalase	Sigma-Aldrich	Holman's
Cyclopiazonic Acid	CPA	Sigma-Aldrich	DMSO
D-glucose	D-glucose	Fisher Scientific	Holman's
Indomethacin	Indomethacin/Indo	Sigma-Aldrich	Absolute Ethanol
L-glucose	L-glucose	Sigma-Aldrich	Holman's
Mn(III)-tetrakis-(1-methyl-4-pyridyl) porphyrin, tetratosylate, hydroxide (Manganese Porphyrin)	MnTMPyP	Calbiochem	Holman's
N^{ω}-Nitro-L-arginine methyl ester hydrochloride	L-NAME	Sigma-Aldrich	Holman's
NOC-9. 6-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-hexanamine	MAHMA NONOate	Sigma-Aldrich	dH_2O
Phenylephrine hydrochloride	PE	Sigma-Aldrich	Holman's
Sodium-meta-Arsenite	Arsenite	Sigma-Aldrich	Holman's

Table 2.1 Reagent Information

CHAPTER 3

MODULATION OF ENDOTHELIUM-DEPENDENT ARTERIAL RELAXATION BY ARSENITE

3.1 Introduction

Arsenic is well recognised as an environmental toxin, whose atherosclerosis-accelerating properties have been confirmed in animal models (Simeonova *et al.*, 2003; Bunderson *et al.*, 2004; Srivastava *et al.*, 2007). Endothelial "dysfunction" resulting from chronic oxidative stress is associated with a number of cardiovascular risk factors, including arsenic exposure, hypertension, hyperlipidaemia and diabetes, and is now widely thought to underpin the early onset of atherosclerosis (for reviews see: Stocker & Keaney, 2004; States *et al.*, 2009). Correspondingly, the trivalent arsenical, arsenite (the predominant species in arsenic-contaminated drinking water), has been reported to activate the $O_2^{\bullet-}$-generating enzyme NADPH oxidase in endothelial and vascular smooth muscle cells leading to increased ROS production (Barchowsky *et al.*, 1999; Lynn *et al.*, 2000; Smith *et al.*, 2001; Qian *et al.*, 2005; Straub *et al.*, 2008). $O_2^{\bullet-}$ is known to inactivate NO via a reaction that results in the production of the species peroxynitrite, which is directly toxic to endothelial cells. Furthermore, the associated reduction in NO bioavailability impairs endothelium-dependent relaxation and promotes prothrombotic mechanisms (for review see: Schafer & Bauersachs, 2008).

Despite comprehensive study and characterization of arsenite-mediated ROS production in endothelial cells, its effects on endothelium-dependent arterial relaxation remain poorly understood. Indeed, it appears that only four published studies have addressed this issue. Furthermore, none of these studies have addressed

the effect of arsenite on endothelium-dependent relaxations that are independent of the activation of G-protein coupled receptors, e.g. mediated by SERCA inhibition. The studies of Jindal *et al* (2008) and Lee *et al* (2003) demonstrated impaired relaxant responses of rat aorta to ACh that were attributed to impaired NO bioavailability mediated by oxidative stress and reduced eNOS expression, respectively. In addition, Srivastava *et al* (2007) demonstrated that arsenite impaired NO-dependent relaxation to ACh in the mouse aorta. However, only a single study has investigated the effect of arsenite on endothelium-dependent relaxations that are independent of the action of NO. Bilszta *et al* (2006) analysed the reactivity of isolated rat aorta and renal artery to PE and ACh after intravenous infusion of arsenite (6 mg kg^{-1} for 4 hours). These authors found that relaxation to ACh was unaffected by arsenite, either in the absence or presence of L-NAME, despite PE-induced tone being potentiated as a consequence of reduced basal levels of NO (Bilszta *et al.*, 2006). It should be noted that these authors did not employ a COX inhibitor in combination with L-NAME in their experiments, and as a result these responses cannot, by definition, be described as EDHF-type relaxations.

The effect of arsenite on EDHF-type arterial relaxation in organ bath experiments has not previously been investigated. One study examined the effect of chronic administration of inorganic arsenic on the formation of vascular gap junctions, and demonstrated reduced expression of connexins 37, 40 and 43 in endothelial cells (Chou *et al.*, 2007), and a single study has investigated the effects of arsenite on K_{Ca} channel activity, demonstrating augmented TEA-sensitive currents in HeLa cells (Dong *et al.*, 2005). However, increases in $[Ca^{2+}]_i$ in response to arsenite have been reported in a variety of cell types, albeit not in endothelial cells (Shen *et al.*, 2002; Mehta & Shaha, 2006; Sandoval *et al.*, 2007; Vahidnia *et al.*, 2008). For example, Li

and colleagues have recently reported that arsenite enhances vascular smooth muscle $[Ca^{2+}]_i$ which correlated with increased ROS production (Li *et al.*, 2009). Indeed, arsenite is widely acknowledged to increase endothelial and smooth muscle cell production of H_2O_2, which in this context is probably the end-product of the dismutation of $O_2^{\bullet-}$ derived from NADPH oxidase (Barchowsky *et al.*, 1999; Lynn *et al.*, 2000; Smith *et al.*, 2001; Tsai *et al.*, 2001; Yeh *et al.*, 2002). The fact that endogenous H_2O_2 contributes to the EDHF phenomenon in a number of artery types and, when applied exogenously, potentiates EDHF-type relaxation in the rabbit iliac artery, therefore justifies analysis of the effect of arsenite on the EDHF phenomenon (Matoba *et al.*, 2000; 2002; Kimura *et al.*, 2002; Lacza *et al.*, 2002; Larsen *et al.*, 2008; Edwards *et al.*, 2008; Garry *et al.*, 2009).

The aim of the current Chapter was therefore to characterize the acute effects of arsenite on endothelium-dependent arterial relaxation in the rabbit iliac artery. Time- and concentration-dependent effects on the relaxing actions of ACh (receptor-dependent) and CPA (receptor-independent) were investigated in the absence and presence of inhibitors of eNOS and COX (L-NAME and indomethacin, respectively). This study was thus directed at dissociating the effects of arsenite on NO/prostanoid-dependent and EDHF-type relaxant responses.

3.2 Methods

Male New Zealand White rabbits were sacrificed and arterial rings studied isometrically as described in 2.1 and 2.2. The rings were maintained at a resting tension of 1 mN over a 1 hour equilibration period and the buffer repeatedly replaced, with any subsequent alterations in baseline tension due to stress relaxation corrected. Preparations were then incubated for 30 minutes in the absence or presence of L-

NAME (300 μM) and indomethacin (10 μM) to compare the effects of arsenite on EDHF-type relaxation. Arsenite (as 30 or 100 μM sodium arsenite dissolved in Holman's) was added for 30, 90 or 180 minutes prior to constriction with PE (1 μM). Once PE-induced tone had reached a stable plateau (after approximately 15 minutes) relaxant responses were studied by constructing cumulative concentration-response curves to ACh (1 nM – 10 μM) or CPA (100 nM – 100 μM). Data was collected and analysed as described in 2.3 and 2.4.

3.3 Results

3.3.1 Effects of arsenite on responses to CPA in the absence of L-NAME/indomethacin

Incubation with 100 μM arsenite for 30, 90 or 180 minutes did not affect relaxant responses to CPA (n=4-5; Figure 3.1A, B, C; Table 3.1). Time-matched control data was obtained with preparations incubated for similar periods in the absence of arsenite. Neither maximal relaxation (R_{max}) to CPA (Table 3.1) nor PE-induced tone (Table 3.2) was affected by incubation with arsenite.

3.3.2 Effects of arsenite on EDHF-type responses to CPA

In the presence of L-NAME and indomethacin, 30 minutes incubation with 30 μM arsenite did not affect EDHF-type relaxations evoked by CPA (n=5; Figure 3.2A; Table 3.1), whereas 100 μM arsenite potentiated these responses, and shifted the concentration-response curve to the left (pIC_{50}: from 4.79±0.04 to 5.07±0.05; $P<0.001$; n=45; Figure 3.2A; Table 3.1). 90 minutes incubation with either 30 or 100 μM arsenite potentiated EDHF-type relaxations, and shifted the concentration–

response curve to the left in each case (pIC_{50}: from 4.85±0.04 to 5.19±0.05 and from 4.89±0.10 to 5.31±0.12; $P<0.01$ and $P<0.05$, respectively; Figure 3.2B; Table 3.1). 180 minutes incubation with 100 μM arsenite also potentiated EDHF-type relaxations, and shifted the concentration-response curve to the left relative to time-matched control data (pIC_{50}: from 4.79±0.09 to 5.18±0.04; $P<0.001$; Figure 3.2C; Table 3.1). R_{max} to CPA was not affected by incubation with arsenite (see Table 3.1). PE-induced tone was unaffected by incubation with L-NAME+indomethacin and/or arsenite (Table 3.2).

3.3.3 Effects of arsenite on responses to ACh in the absence of L-NAME/indomethacin

30 minutes incubation with 100 μM arsenite did not affect relaxations to ACh (n=6; Figure 3.3A; Table 3.3), with 90 minutes incubation with either 30 or 100 μM arsenite also being ineffective (n=7 and 6, respectively; Figure 3.3B; Table 3.3). While 180 minutes incubation with 30 μM arsenite did not affect responses after 180 minutes incubation (n=5; Figure 3.3C; Table 3.3), 100 μM arsenite attenuated relaxation at this time point, reducing R_{max} relative to the time-matched control data (from 75.0±2.5 to 37.6±3.6%; $P<0.001$; n=7; Figure 3.3C; Table 3.3). Neither pEC_{50} (Table 3.3) nor PE-induced tone (Table 3.2) was affected by incubation with arsenite.

3.3.4 Effects of arsenite on EDHF-type responses to ACh

In the presence of L-NAME and indomethacin, 30 minutes incubation with 100 μM arsenite did not affect EDHF-type relaxations evoked by ACh (n=6; Figure 3.4A). 90 minutes incubation with 100 μM arsenite attenuated maximal EDHF-type relaxations to ACh (R_{max}: from 68.6±3.4 to 43.9±2.5%; $P<0.001$; n=6; Figure 3.4B; Table 3.3),

whereas 30 μM arsenite did not affect responses at this time point (n=4; Figure 3.4B; Table 3.3). 180 minutes incubation with 30 or 100 μM arsenite attenuated relaxation to ACh, reducing R_{max} from 60.1±3.4 to 36.9±3.0% and from 59.2±3.7 to 23.4±2.0% (P<0.001 for each; n=5 for each; Figure 3.4C; Table 3.3), respectively. pEC_{50} was not affected by incubation with arsenite (Table 3.3). PE-induced tone was not affected by incubation with L-NAME+indomethacin and/or arsenite (Table 3.2).

Figure 3.1 Effect of arsenite on relaxations evoked by CPA in the absence of L-NAME and indomethacin. Concentration-response curves were unaffected by 100 μM arsenite after (A) 30, (B) 90 or (C) 180 minutes incubation. n denotes the number of animals studied.

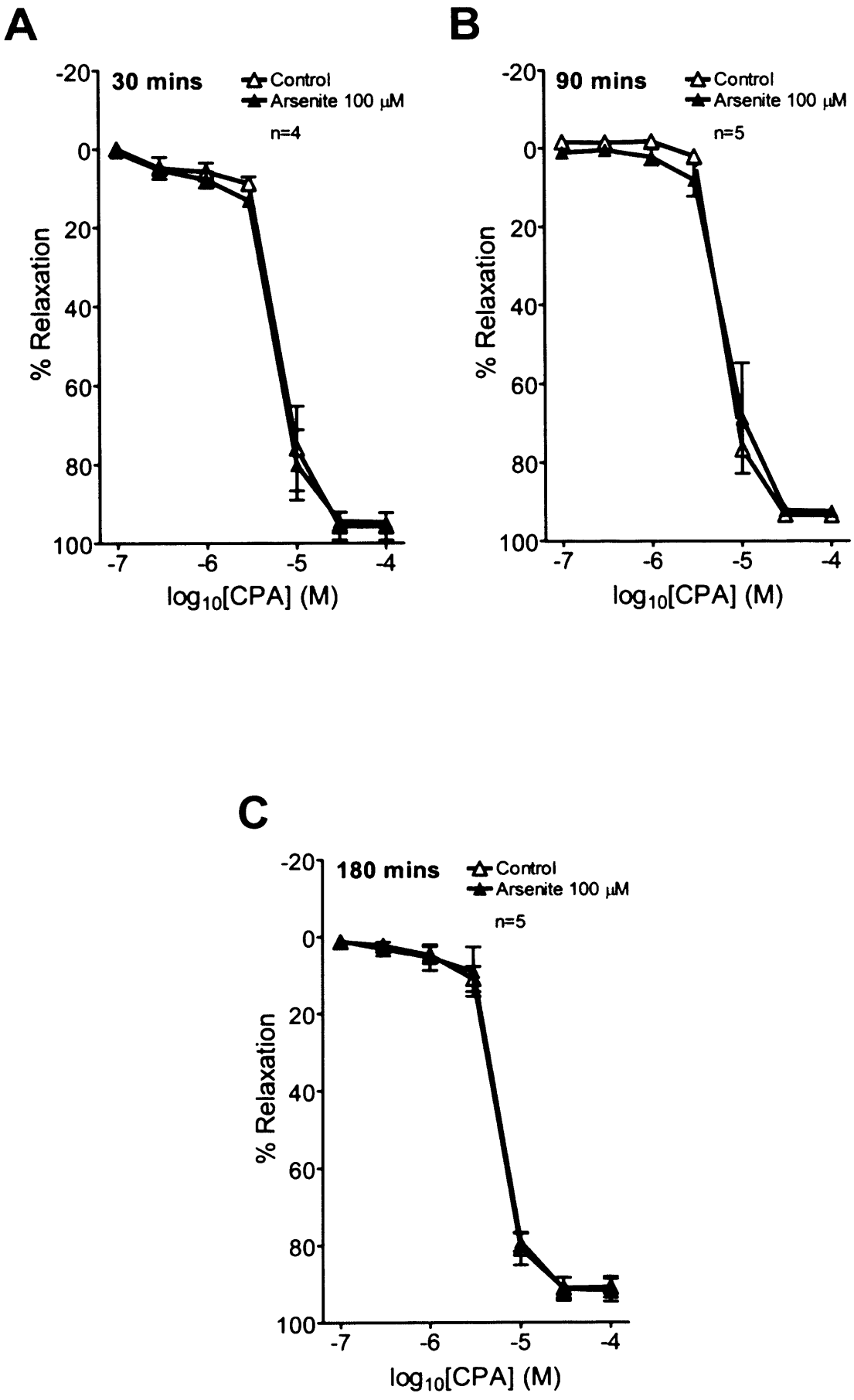

A
30 mins
Control
Arsenite 100 μM
n=4
% Relaxation
log10[CPA] (M)
B
90 mins
Control
Arsenite 100 μM
n=5
% Relaxation
log10[CPA] (M)
C
180 mins
Control
Arsenite 100 μM
n=5
% Relaxation
log10[CPA] (M)

Figure 3.2 Effects of arsenite on relaxations evoked by CPA in the presence of L-NAME and indomethacin. (A) Concentration-response curves revealed that arsenite at 100 μM, but not 30 μM, augmented EDHF-type relaxations after 30 minutes incubation. (B) EDHF-type relaxations were augmented by both 30 and 100 μM arsenite after 90 minutes incubation. (C) Potentiation by 100 μM arsenite was still evident after 180 minutes incubation. * and *** denote $P<0.05$ and $P<0.001$ compared with time-matched preparations incubated in the absence of arsenite. n denotes the number of animals studied.

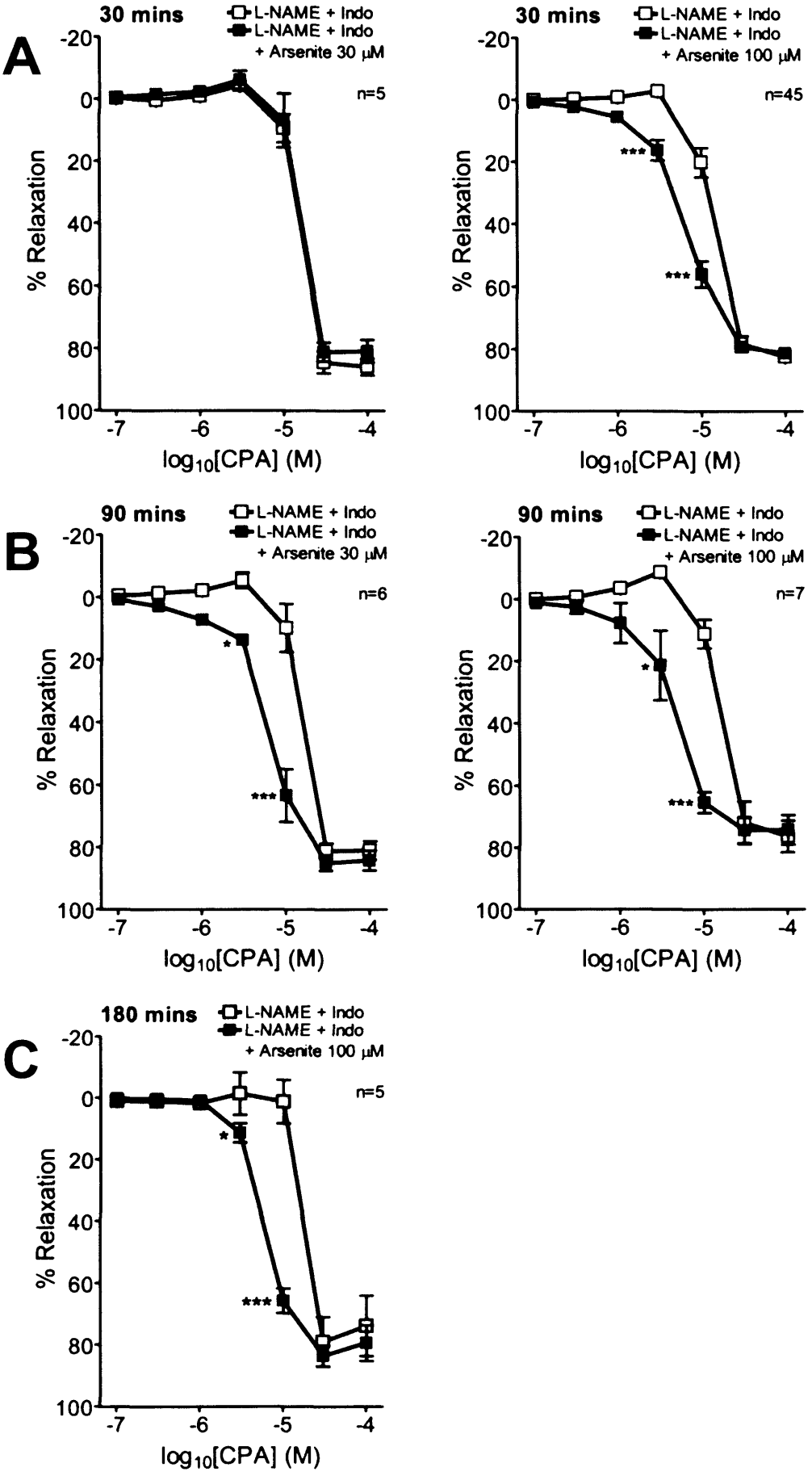
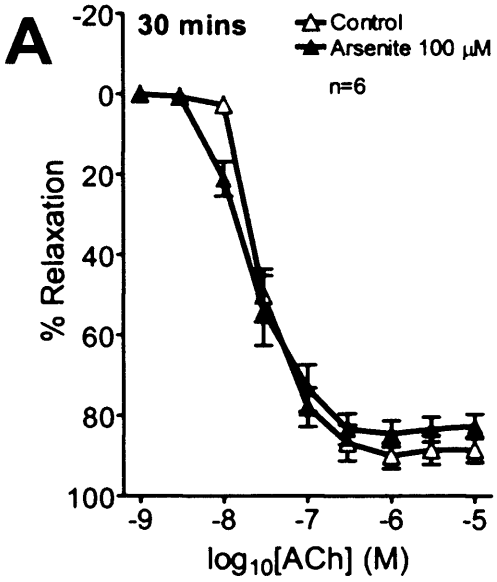
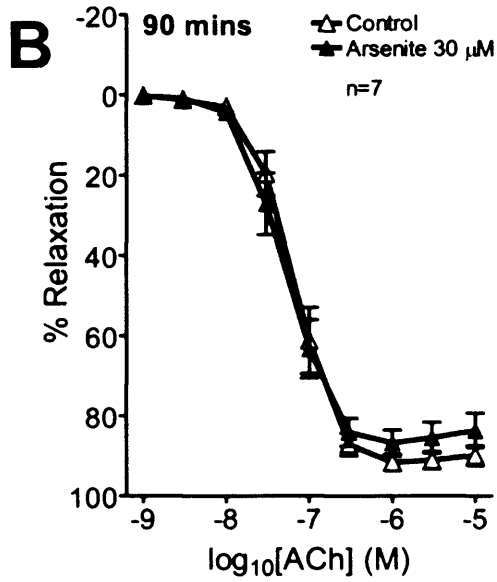
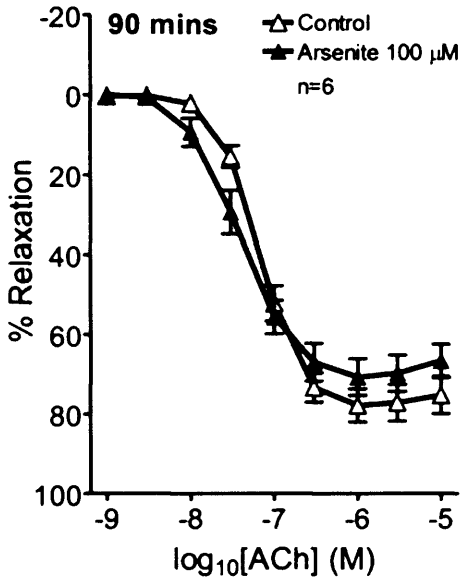
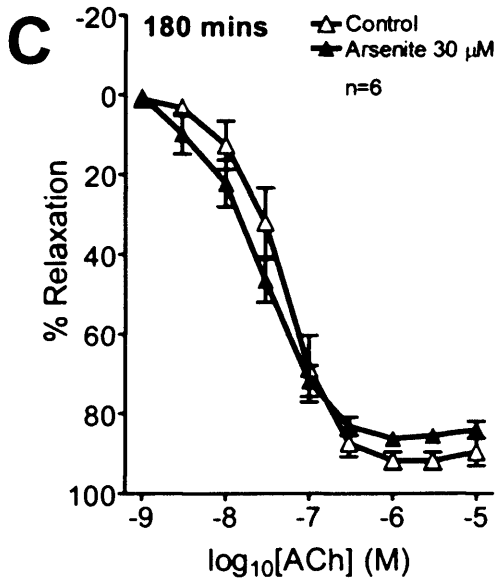

A
30 mins
L-NAME + Indo
L-NAME + Indo
+ Arsenite 30 µM
n=5
% Relaxation
log10[CPA] (M)
30 mins
L-NAME + Indo
L-NAME + Indo
+ Arsenite 100 µM
n=45
***
***
% Relaxation
log10[CPA] (M)
B
90 mins
L-NAME + Indo
L-NAME + Indo
+ Arsenite 30 µM
n=6
*
***
% Relaxation
log10[CPA] (M)
90 mins
L-NAME + Indo
L-NAME + Indo
+ Arsenite 100 µM
n=7
*
***
% Relaxation
log10[CPA] (M)
C
180 mins
L-NAME + Indo
L-NAME + Indo
+ Arsenite 100 µM
n=5
*
***
% Relaxation
log10[CPA] (M)

Figure 3.3 Effects of arsenite on relaxations evoked by ACh in the absence of L-NAME and indomethacin. (A) Concentration-response curves were unaffected by 100 μM arsenite after 30 minutes incubation. (B) Relaxation was unaffected by either 30 or 100 μM arsenite after 90 minutes incubation. (C) Relaxation was attenuated by 100 μM, but not 30 μM, arsenite after 180 minutes incubation. * and *** denote $P<0.05$ and $P<0.001$ compared with time-matched preparations incubated in the absence of arsenite. n denotes the number of animals studied.


A
30 mins
Control
Arsenite 100 μM
n=6
% Relaxation
log10[ACh] (M)


B
90 mins
Control
Arsenite 30 μM
n=7
% Relaxation
log10[ACh] (M)


90 mins
Control
Arsenite 100 μM
n=6
% Relaxation
log10[ACh] (M)


C
180 mins
Control
Arsenite 30 μM
n=6
% Relaxation
log10[ACh] (M)

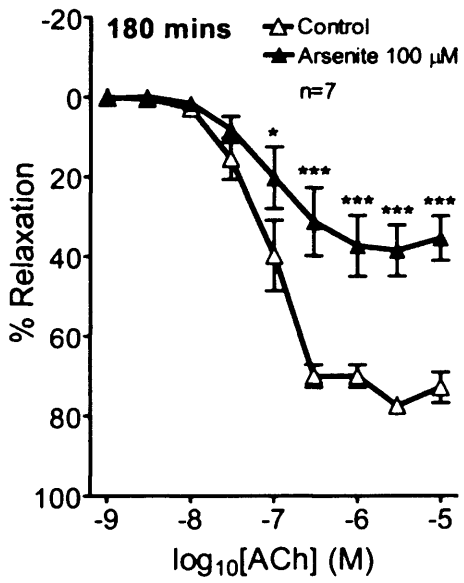

180 mins
Control
Arsenite 100 μM
n=7
*
***
***
***
***
% Relaxation
log10[ACh] (M)

Figure 3.4 Effects of arsenite on relaxations evoked by ACh in the presence of L-NAME and indomethacin. (A) Concentration-response curves were unaffected by 100 μM arsenite after 30 minutes incubation. (B) EDHF-type relaxations were attenuated by 100 μM but not 30 μM arsenite after 90 minutes incubation. (C) EDHF-type relaxations were attenuated by both 30 and 100 μM arsenite after 180 minutes incubation. *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$ compared with time-matched preparations incubated in the absence of arsenite. n denotes the number of animals studied.

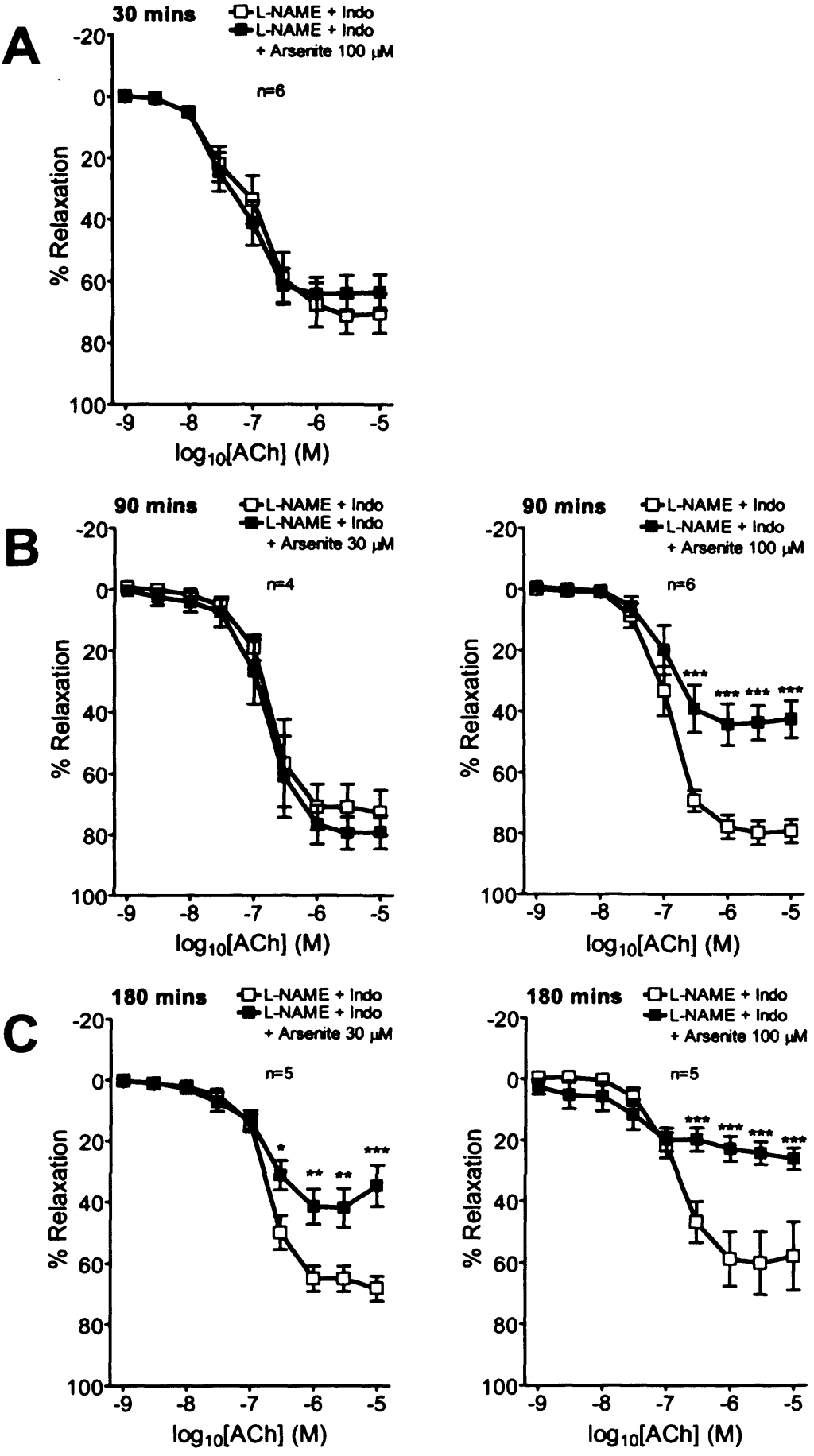

A
30 mins
L-NAME + Indo
L-NAME + Indo
+ Arsenite 100 µM
n=6
% Relaxation
log10[ACh] (M)
B
90 mins
L-NAME + Indo
L-NAME + Indo
+ Arsenite 30 µM
n=4
90 mins
L-NAME + Indo
L-NAME + Indo
+ Arsenite 100 µM
n=6
C
180 mins
L-NAME + Indo
L-NAME + Indo
+ Arsenite 30 µM
n=5
180 mins
L-NAME + Indo
L-NAME + Indo
+ Arsenite 100 µM
n=5

Table 3.1 Time- and concentration-dependent effects of arsenite on endothelium-dependent arterial relaxation evoked by CPA. Experiments were performed in the absence and presence of L-NAME (300 μM) and indomethacin (10 μM) and the effect of arsenite (30 or 100 μM) was subsequently determined. Potency (negative $logIC_{50}$) and maximal responses (R_{max}) are given as means±SEM. *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$ compared with time-matched preparations incubated in the absence of arsenite. n denotes the number of animals studied.

Intervention	n	pIC_{50}	R_{max} %
30 minutes Arsenite incubation			
Control	4	5.17±0.07	95.8±3.5
Arsenite 100 μM		5.22±0.06	94.7±2.4
L-NAME + Indo	5	4.85±0.04	85.9±2.7
L-NAME + Indo + Arsenite 30 μM		4.82±0.03	81.7±3.2
L-NAME + Indo	45	4.79±0.04	82.8±1.6
L-NAME + Indo + Arsenite 100 μM		5.07±0.05***	82.2±1.7
90 minutes Arsenite incubation			
Control	5	5.16±0.06	93.6±1.7
Arsenite 100 μM		5.16±0.09	92.4±1.7
L-NAME + Indo	6	4.85±0.04	81.8±2.6
L-NAME + Indo + Arsenite 30 μM		5.19±0.05**	85.6±2.7
L-NAME + Indo	7	4.89±0.10	77.8±4.6
L-NAME + Indo + Arsenite 100 μM		5.31±0.12*	76.6±3.5
180 minutes incubation			
Control	5	5.22±0.05	91.3±2.7
Arsenite 100 μM		5.20±0.05	91.8±2.9
L-NAME + Indo	5	4.79±0.09	81.0±6.5
L-NAME + Indo + Arsenite 100 μM		5.18±0.04***	83.6±3.3

Table 3.2 Effects of L-NAME/indomethacin and arsenite on rabbit iliac arterial tone induced by PE. It was determined that L-NAME/indomethacin and/or arsenite did not alter PE-induced tone compared to time-matched preparations incubated for similar periods without these treatments. Data given as means±SEM. n denotes the number of animals studied (data pooled from Tables 3.1 and 3.3).

Intervention	n	PE-induced tone (mN)
30 minutes Arsenite incubation		
Control	10	37.2±3.2
Arsenite 100 μM	10	34.8±4.9
L-NAME+Indo	56	39.9±1.9
L-NAME+Indo + Arsenite 30 μM	5	36.1±5.9
L-NAME+Indo + Arsenite 100 μM	51	39.1±1.5
90 minutes Arsenite incubation		
Control	18	38.2±3.3
Arsenite 30 μM	7	32.9±5.2
Arsenite 100 μM	11	37.6±2.8
L-NAME+Indo	23	36.2±2.4
L-NAME+Indo + Arsenite 30 μM	10	34.2±6.1
L-NAME+Indo + Arsenite 100 μM	13	31.4±4.8
180 minutes Arsenite incubation		
Control	18	39.7±3.1
Arsenite 30 μM	6	34.6±5.3
Arsenite 100 μM	12	30.6±6.7
L-NAME+Indo	15	35.7±2.7
L-NAME+Indo + Arsenite 30 μM	5	31.8±6.9
L-NAME+Indo + Arsenite 100 μM	10	29.9±5.3

Table 3.3 Time- and concentration-dependent effects of arsenite on endothelium-dependent arterial relaxation evoked by ACh. Experiments were performed in the absence and presence of L-NAME (300 μM) and indomethacin (10 μM) and the effect of arsenite (30 and 100 μM) was subsequently determined. Potency (negative $logEC_{50}$) and maximal responses (R_{max}) are given as means±SEM. *** denotes $P<0.001$ compared with time-matched preparations incubated in the absence of arsenite. n denotes the number of animals studied.

Intervention	n	pEC_{50}	R_{max} %
30 minutes Arsenite incubation			
Control	6	7.56±0.03	87.5±1.8
Arsenite 100 μM		7.74±0.07	83.9±2.4
L-NAME + Indo	6	7.06±0.13	72.8±4.6
L-NAME + Indo + Arsenite 100 μM		7.30±0.11	65.0±3.1
90 minutes Arsenite incubation			
Control	7	7.18±0.04	91.5±2.0
Arsenite 30 μM		7.30±0.06	86.0±2.4
Control	6	7.18±0.04	77.1±1.8
Arsenite 100 μM		6.88±0.07	69.4±2.2
L-NAME + Indo	4	6.79±0.08	72.2±3.0
L-NAME + Indo + Arsenite 30 μM		6.81±0.09	79.7±4.3
L-NAME + Indo	6	7.44±0.08	68.6±3.4
L-NAME + Indo + Arsenite 100 μM		7.05±0.10	43.9±2.5***
180 minutes Arsenite incubation			
Control	6	7.33±0.06	91.9±2.8
Arsenite 30 μM		7.60±0.07	86.2±2.2
Control	7	7.08±0.06	75.0±2.5
Arsenite 100 μM		7.07±0.19	37.6±3.6***
L-NAME + Indo	5	6.75±0.09	60.1±3.4
L-NAME + Indo + Arsenite 30 μM		6.87±0.14	36.9±3.0***
L-NAME + Indo	5	6.87±0.11	59.2±3.7
L-NAME + Indo + Arsenite 100 μM		7.38±0.36	23.4±2.0***

3.4 Discussion

Investigations into modulation of endothelium-dependent relaxation by arsenite in the rabbit iliac artery unveiled differential effects between two pharmacologically distinct modes of endothelial stimulation. In the absence of L-NAME and indomethacin, arsenite (at 100 μM) was without effect on relaxations to CPA over the 180 minute incubation period studied. However, when the EDHF-type component was isolated by the addition of L-NAME and indomethacin, the same concentration of arsenite increased the potency of CPA after just 30 minutes, with subsequent experiments confirming this augmentatory effect was sustained at incubation intervals of 90 and 180 minutes. A time- and concentration-dependent augmentation was confirmed by experiments showing that 30 μM arsenite was without effect after 30 minutes but potentiated EDHF-type relaxations by CPA after 90 mins.

By contrast, in the absence of L-NAME and indomethacin, though 30 μM arsenite was ineffective after 180 minutes incubation, 100 μM arsenite attenuated maximal relaxations to ACh (without influencing its potency) after 180 minutes. Further experiments demonstrated that 30 μM arsenite attenuated EDHF-type relaxations to ACh after 180 minutes incubation (to ~60% of that observed in time-matched controls), whereas 100 μM arsenite attenuated relaxations after just 90 minutes incubation (to ~55% of that observed in time-matched controls).

While both ACh and CPA evoke rises in endothelial Ca^{2+}, increases in $[Ca^{2+}]_i$ in response to ACh are mediated principally by $InsP_3$-induced Ca^{2+} release from the ER store, whereas CPA evokes SOCE secondary to depletion of the ER Ca^{2+} store following SERCA inhibition (Griffith, 2004; Edwards *et al.*, 2008; Garry *et al.*, 2009). Therefore, the results of this Chapter suggest that arsenite may promote depletion of the ER Ca^{2+} store and/or evoke an antagonistic effect on the ACh receptor and/or its

downstream signalling mechanism. Indeed, arsenite has been shown to inhibit muscarinic receptors directly via interaction with critical thiols on the basis that (i) the affinity of the receptor for the antagonist (3H)quinuclidinyl benzilate was impaired (IC_{50} increased), and (ii) this effect of arsenite was only apparent during co-administration of the thiol reductant dithiothreitol (Fonseca *et al.*, 1991). Investigations to determine whether arsenite interferes with the coupling of muscarinic receptors to phospholipase C via G-proteins, which underpins the synthesis of $InsP_3$ following the binding of ACh, have not yet been undertaken.

The effects of arsenite on vascular function are largely unexplored. Indeed, it was not until 2003 that this issue was first investigated, when Lee *et al* (2003) demonstrated that arsenite impaired NO-dependent relaxation to ACh in the rat aorta, which they attributed to reduced bioavailability of NO. Jindal *et al* (2008) also observed impaired relaxation to ACh, though impaired NO bioavailability in their study was attributed to elevated intracellular levels of $O_2^{\bullet-}$, whereas Lee *et al* found that this was mediated by reduced expression and/or activity of eNOS, as indicated by the reduced conversion of L-arginine to L-citrulline (Lee *et al.*, 2003; Jindal *et al.*, 2008). However, the only investigation into the effect of arsenite on endothelium-dependent relaxation that was independent of eNOS activity (although, by definition, these were not EDHF-type responses as COX activity had not been inhibited) concluded that there was no effect in the aorta and renal artery of the rat (Bilszta *et al.*, 2006).

In studies of isolated endothelial and vascular smooth muscle cells, a number of authors have proposed that arsenite activates NADPH oxidase leading to increased endothelial $O_2^{\bullet-}$ production, thereby (i) impairing NO bioavailability and increasing peroxynitrite formation (measured as nitration of tyrosine residues) and (ii) increasing endothelial production of H_2O_2 subsequent to the dismutation of NADPH oxidase-

derived $O_2^{\bullet-}$ (Wang *et al.*, 1996; Barchowsky *et al.*, 1996; Wang *et al.*, 1997; Barchowsky *et al.*, 1999; Liu & Jan, 2000; Smith *et al.*, 2001; Bunderson *et al.*, 2002; Yeh *et al.*, 2002; Hirano *et al.*, 2003; Bunderson *et al.*, 2006; Straub *et al.*, 2008). While such alterations in endothelial redox status play a pathological role in impairing NO function, subsequent parallel elevations of H_2O_2 may represent a novel mechanism by which vascular function can be preserved during oxidative stress.

The results of the current Chapter have demonstrated that arsenite may augment EDHF-type relaxations, and this effect only becomes apparent during a mode of endothelial stimulation that bypasses membrane receptor and second messenger mechanisms involving $InsP_3$ by directly inhibiting the SERCA pump. From the experiments with CPA it was apparent that arsenite potentiated EDHF-type relaxation while having no net effect on endothelial function in the absence of L-NAME and indomethacin. This suggests that the augmented EDHF-type component may offset an impaired NO/prostanoid component, thereby preserving endothelium-dependent vascular function. It should be stressed, however, that while it has been demonstrated that the EDHF phenomenon may acutely preserve arterial responsiveness in arsenic toxicity, it is uncertain whether such mechanisms compensate for the vasculoprotective effects of NO that normally limit atheroma formation, such as its ability to reduce endothelial permeability, thrombogenesis and inflammatory cell adhesion and migration.

Accumulating reports of arsenite-evoked endothelial oxidative stress and recent observations demonstrating the ability of exogenous H_2O_2 to potentiate the EDHF phenomenon in the rabbit iliac artery (Edwards *et al.*, 2008) warrant further investigation into the role of this ROS in responses modulated by arsenite. Investigations in models of hyperglycaemia and diabetes have also demonstrated

oxidant stress-mediated impairment of NO bioavailability and associated NO-dependent relaxations (Coppey *et al.*, 2003; Sercombe *et al.*, 2004; Bitar *et al.*, 2005; Hayashi *et al.*, 2005; Tawfik *et al.*, 2006; Reyes-Toso *et al.*, 2007; Matsumoto *et al.*, 2007; 2008; Lopez-Lopez *et al.*, 2008; Shukla *et al.*, 2008; Su *et al.*, 2008; Elmi *et al.*, 2008). Additionally, a number of studies have postulated a compensatory role of the EDHF phenomenon in diabetes that may serve to offset impaired NO function (Thomsen *et al.*, 2001; Timar-Peregrin & Guy, 2002; Shen *et al.*, 2003; Yousif *et al.*, 2005; Shi *et al.*, 2006; Pataricza *et al.*, 2008; MacKenzie *et al.*, 2008; Malakul *et al.*, 2008). However, it remains to be determined whether arsenite and elevated D-glucose modulate vascular function, in particular the EDHF phenomenon, via similar mechanisms. Investigation of the effects of glucose on vascular function is the subject of the following Chapter and will form the basis of comparative analyses in subsequent Chapters.

CHAPTER 4

MODULATION OF ENDOTHELIUM-DEPENDENT ARTERIAL RELAXATION BY D-GLUCOSE

4.1 Introduction

Diabetes mellitus affects around 200 million people worldwide and is responsible for approximately 5% of all deaths each year. Diabetes-associated fatalities are likely to double in the next 10 years with growing neglect of diet and physical inactivity (www.who.int/topics/diabetes-mellitus/en/). High plasma glucose concentrations (hyperglycaemia) are, like arsenic, associated with an increased risk of atherosclerosis (for review see: Schaefer & Bauersachs, 2008). Furthermore, hyperglycaemia-accelerated atheroma formation, like arsenic, is now appreciated to be the result of chronic endothelial oxidative stress and cell dysfunction. Endothelial oxidative stress results in impaired NO bioavailability and associated NO-dependent arterial relaxations (Schaefer & Bauersachs, 2008). In contrast to the lack of studies with arsenic, the influence of diabetes on endothelium-dependent arterial relaxation has been extensively investigated.

Early studies of the modulation of NO-dependent relaxation in diabetes provided evidence for impaired responses in the aortae of biobreeding rats, which spontaneously develop type I diabetes (Durante *et al.*, 1988; Kappagoda *et al.*, 1989). A study by Pieper demonstrated that NO-dependent relaxations in the aorta of STZ-diabetic rats may be increased during the early stages of STZ-induced diabetes, with responses progressively decreasing with disease duration (Pieper, 1999). The involvement of $O_2^{\bullet-}$ in impaired responses was first evidenced by the studies of Pieper

et al (1988) and Hattori *et al* (1991) in aortae from STZ-diabetic rats, the latter of which demonstrated that these responses could be recovered by incubation with SOD. Correspondingly, there is now compelling evidence that in chronic diabetes NADPH oxidase-derived $O_2^{\bullet-}$ production is increased and contributes to impaired NO-dependent relaxations in animals (Kim *et al.*, 2002; Kanie & Kamata, 2002; Coppey *et al.*, 2003; Sercombe *et al.*, 2004; Hayashi *et al.*, 2005; Bitar *et al.*, 2005; Ling *et al.*, 2005; Tawfik *et al.*, 2006; Matsumoto *et al.*, 2007; Lopez-Lopez *et al.*, 2008; Shukla *et al.*, 2008; Su *et al.*, 2008) and humans (Guzik *et al.*, 2000; 2002).

Modulation of EDHF-type relaxation in diabetes nevertheless remains controversial, as evidenced by observations of enhanced, resistant and impaired responses. Confounding opinions were evident from the earliest studies, the first of which demonstrated that the EDHF phenomenon was not impaired in the STZ-diabetic rat aorta, and thus did not contribute to depressed endothelium-dependent relaxations (Endo *et al.*, 1995). By contrast, it was demonstrated that EDHF-type relaxations and endothelium-dependent smooth muscle hyperpolarizations to ACh were impaired in the mesenteric artery of STZ-diabetic rats, although this has since been contested by another group (Fukao *et al.*, 1997; Shi *et al.*, 2006). Furthermore, studies of isolated endothelial cells conducted by the group of Graier provided evidence that hyperglycaemia-mediated oxidative stress may actually promote the mobilization of intracellular Ca^{2+} stores in response to agonists and SERCA inhibitors (Wascher *et al.*, 1994; Graier *et al.*, 1996; 1997), with similar findings having since been reported by other authors (Wu *et al.*, 1999; Dragonmir *et al.*, 2004; Tamareille *et al.*, 2006). Correspondingly, a number of groups have now provided evidence that the EDHF phenomenon may be augmented during diabetes and serve to offset impaired NO bioavailability and preserve relaxation (Timar-Peregrin & Guy; 2001; Thomsen *et al.*,

2002; Yousif *et al.*, 2002; Pannirselvam *et al.*, 2002; Shen *et al.*, 2003; Shi *et al.*, 2005; Malakul *et al.*, 2008; MacKenzie *et al.*, 2008; Pataricza *et al.*, 2008). The promotion of endothelial Ca^{2+} mobilization by ROS during diabetes could in theory potentiate the EDHF phenomenon, with our group having shown that exogenous H_2O_2 potentiates EDHF-type relaxation in the rabbit iliac artery via a mechanism involving enhanced Ca^{2+} mobilization (Edwards *et al.*, 2008).

Diabetes has also been demonstrated to alter prostanoid production by COX, with evidence of increases in both vasodilator (Alabadi *et al.*, 2001) and constrictor products, such as thromboxane A_2 (Mayhan *et al.*, 1991; Taylor and Poston, 1994; Kamata *et al.*, 2004; Akamine *et al.*, 2006; Cheng *et al.*, 2007; Csanyi *et al.*, 2007). Taken together, such diversity may be indicative of interactions between competing or complementary EDRF, EDHF-type and EDCF activities that superimpose to modulate vascular function. Furthermore, a disease duration-dependent effect of diabetes on vascular function was observed in the aorta of STZ- and alloxan-treated rats. The study of Pieper (1999) observed a triphasic effect of the diabetic state, with NO-dependent relaxations increased 24 hours after STZ injection, unaltered after 1–2 weeks and impaired after 8 weeks. This appears to be consistent with studies where acute exposure of endothelial cells to elevated glucose increased the activity of the Ca^{2+}/NO pathway (Graier *et al.*, 1993; Wascher *et al.*, 1994; Graier *et al.*, 1996; 1997).

The aim of the current Chapter was to characterize acute time- and concentration-dependent effects of D-glucose on endothelium-dependent relaxation in the rabbit iliac artery in a similar fashion to the experiments described for arsenite in Chapter 3. Furthermore, in addition to experiments with or without the combination of L-NAME and indomethacin conducted according to the protocols described in Chapter 3, these

inhibitors were administered individually in order to dissociate potential alterations in prostanoid synthesis that have previously been demonstrated to modulate relaxation in animal models of diabetes.

4.2 Methods

Male New Zealand White rabbits were sacrificed and arterial rings studied isometrically as described in 2.1 and 2.2. The rings were maintained at a resting tension of 1 mN over a 1 hour equilibration period, after which the buffer was replaced and any subsequent alteration in baseline tension due to stress relaxation corrected. Preparations were then incubated for 30 minutes in the absence or presence of L-NAME (300 μM) and/or indomethacin (10 μM). Additional D-Glucose was then added (22 or 33 mM to give total buffer concentrations of 33 or 44 mM, respectively) for 30, 180 or 360 minutes prior to constriction with PE (1 μM). Once PE-induced tone had reached a stable plateau (after approximately 15 minutes) relaxant responses were studied by constructing cumulative concentration-response curves to ACh (1 nM – 10 μM) or CPA (100 nM – 100 μM). Data was collected and analysed as described in 2.3 and 2.4.

4.3 Results

4.3.1 Effects of D-glucose on responses to CPA in the absence of L-NAME

Incubation with 44 mM D-glucose for 30, 180 or 360 minutes, either in the absence or presence of indomethacin, did not affect relaxant responses to CPA (n=4-10; Figure 4.1A, B, C; Table 4.1). Control data was obtained from preparations incubated for similar periods in standard Holman's buffer containing 11 mM D-glucose. Neither the

R_{max} for CPA (Table 4.1) nor PE-induced tone (Table 4.2) was affected by incubation with indomethacin and/or D-glucose.

4.3.2 Effects of D-glucose on responses to CPA in the presence of L-NAME

In the presence of L-NAME, 30 minutes incubation with 44 mM D-glucose potentiated relaxations evoked by CPA, either in the absence or presence of indomethacin, and shifted the concentration-response curve to the left in each case (pIC_{50}: from 4.87±0.05 to 5.11±0.05 and from 4.85±0.04 to 5.10±0.04, respectively; $P<0.001$ for each; Figure 4.2A; Table 4.1). 30 minutes incubation with 33 mM D-glucose did not affect EDHF-type relaxations (n=16; Figure 4.2A; Table 4.1). 180 minutes incubation with 44 mM D-glucose, either in the absence or presence of indomethacin, potentiated relaxations evoked by CPA, and shifted the concentration-response curve to the left in each case (pIC_{50}: from 4.84±0.03 to 5.10±0.04 and from 4.83±0.02 to 5.23±0.07, respectively; $P<0.001$ for each; Figure 4.2B; Table 4.1). 180 minutes incubation with 33 mM D-glucose also potentiated EDHF-type relaxations evoked by CPA, and shifted the concentration-response curve to the left (pIC_{50}: from 4.79±0.06 to 5.12±0.07; $P<0.05$; Figure 4.2B; Table 4.1). 360 minutes incubation with 44 mM D-glucose, either in the absence or presence of indomethacin, potentiated relaxation evoked by CPA, and shifted the concentration-response curve to the left in each case (pIC_{50}: from 4.85±0.06 to 5.30±0.11 and from 4.78±0.06 to 5.33±0.08, respectively; $P<0.001$ for each; Figure 4.2C; Table 4.1). Neither the R_{max} for CPA (Table 4.1) nor PE-induced tone (Table 4.2) was affected by incubation with indomethacin and/or D-glucose. L-NAME did not affect PE-induced tone relative to time-matched preparations incubated in the absence of L-NAME (Table 4.2).

4.3.3 Effects of D-glucose on responses to ACh in the absence of L-NAME

Incubation with 44 mM D-glucose for 30, 180 or 360 minutes, either in the absence or presence of indomethacin, did not affect relaxation to ACh (n=5 for each; Figure 4.3A, B, C; Table 4.3). Time-matched control data was obtained with preparations incubated for similar periods in standard Holman's buffer containing 11 mM D-glucose. Neither the R_{max} for ACh (Table 4.3) nor PE-induced tone (Table 4.2) was affected by incubation with indomethacin and/or D-glucose. Concentration-response curves, constructed either in the absence or presence of 44 mM D-glucose, were unaffected by indomethacin relative to equivalent preparations incubated for similar periods in the absence of indomethacin (Table 4.3).

4.3.4 Effects of D-glucose on responses to ACh in the presence of L-NAME

In the presence of L-NAME, 30 minutes incubation with 44 mM D-glucose potentiated relaxations evoked by ACh either in the absence or presence of indomethacin (R_{max}: from 57.3±3.6 to 79.2±2.9% and from 51.5±2.1 to 70.5±2.0%; $P<0.01$ and $P<0.001$; n=6 and 13, respectively; Figure 4.4A; Table 4.3), without significantly altering pEC_{50} values (Table 4.3). This effect of 44 mM D-glucose was sustained following 180 minutes incubation (R_{max}: from 50.9±2.5 to 69.1±3.0% and from 52.5±3.7 to 69.0±2.6%; $P<0.05$ and $P<0.001$; n=5 and 15, respectively; Figure 4.4B; Table 4.3) and 360 minutes incubation (R_{max}: from 28.7±3.2 to 46.4±4.5% and from 20.0±1.7 to 48.4±4.8%, respectively; $P<0.01$, n=6 for each; Figure 4.4C; Table 4.3). Indomethacin did not alter L-NAME-insensitive control relaxations to ACh or their potentiation by 44 mM D-glucose (Table 4.3). It should be noted, however, that maximal NO-independent control relaxations to ACh in preparations incubated for 360 minutes, either in the absence or presence of indomethacin, were impaired

relative to equivalent preparations incubated for 30 minutes ($P<0.01$ for each; Table 4.3). As a result, relaxations in preparations incubated in the presence of 44 mM D-glucose for 360 minutes were potentiated to a level comparable with control preparations incubated for 30 minutes (Table 4.3). A time- and concentration-dependent potentiation of EDHF-type relaxation was confirmed in preparations incubated with 33 mM D-glucose, with responses being unaffected after 30 minutes but potentiated following 180 minutes incubation (R_{max}: from 58.3±3.0 to 76.2±3.2%; $P<0.05$; n=6; Figure 4.4A, B; Table 4.3). PE-induced tone was not altered by L-NAME and/or indomethacin and/or D-glucose relative to time-matched preparations incubated in the absence of L-NAME (Table 4.2).

4.3.5 Effects of low D-glucose concentrations on EDHF-type responses to ACh

EDHF-type relaxations evoked by ACh were unaffected by 30 minutes incubation with either 1.1 or 5.5 mM D-glucose relative to time-matched preparations incubated for similar periods in standard Holman's buffer containing 11 mM D-glucose (n=4; Figure 4.5; Table 4.4). PE-induced tone was unaffected by incubation with either 1.1 or 5.5 mM D-glucose (Table 4.2).

4.3.6 Effects of L-glucose on EDHF-type responses to ACh

Neither 30 nor 180 minutes incubation with 33 mM L-glucose (to give a total of 44 mM glucose as 11 mM D-glucose was present in the buffer at all times) affected EDHF-type relaxations evoked by ACh (n=5-15; Figure 4.6A, B; Table 4.4). PE-induced tone was not altered by incubation with L-glucose (Table 4.2).

Figure 4.1 Effects of D-glucose on relaxations evoked by CPA in the absence of L-NAME. Concentration-response curves were unaffected by 44 mM D-glucose, either in the absence or presence of indomethacin, after (A) 30, (B) 180 or (C) 360 minutes incubation. n denotes the number of animals studied.

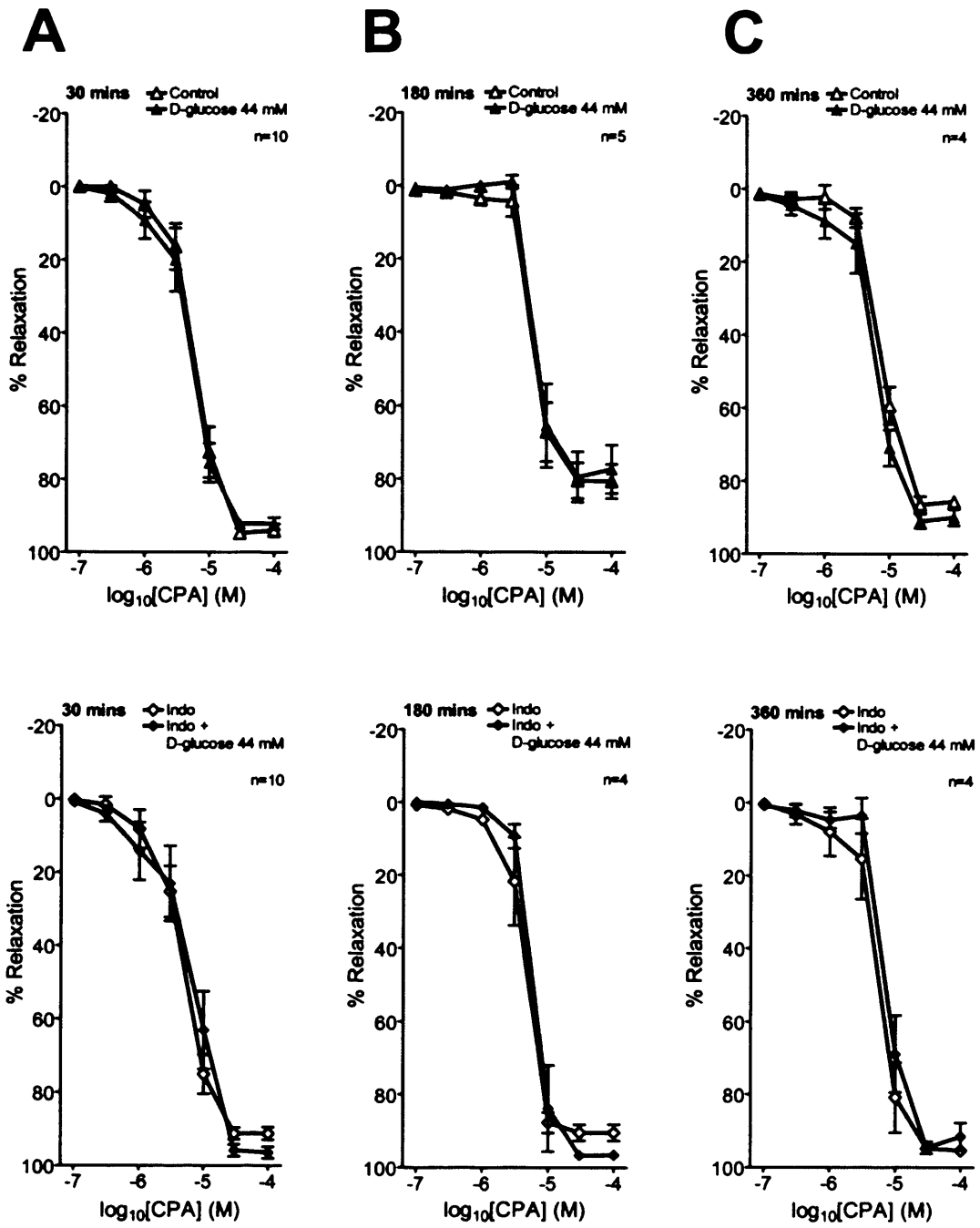

A
B
C
30 mins
180 mins
360 mins
Control
D-glucose 44 mM
n=10
n=5
n=4
Indo
Indo +
D-glucose 44 mM
% Relaxation
log10[CPA] (M)

Figure 4.2 Effects of D-glucose on relaxations evoked by CPA in the presence of L-NAME. Concentration-response curves revealed that 44 mM D-glucose augmented relaxation, either in the absence or presence of indomethacin after (A) 30, (B) 180 and (C) 360 minutes incubation. Additional experiments confirmed a time- and concentration-dependent augmentation of EDHF-type relaxation by D-glucose, with 33 mM being ineffective after 30 minutes (A) but potentiating responses after 180 minutes incubation (B). *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$ compared with time-matched control preparations incubated in standard Holman's buffer containing 11 mM D-glucose. n denotes the number of animals studied.

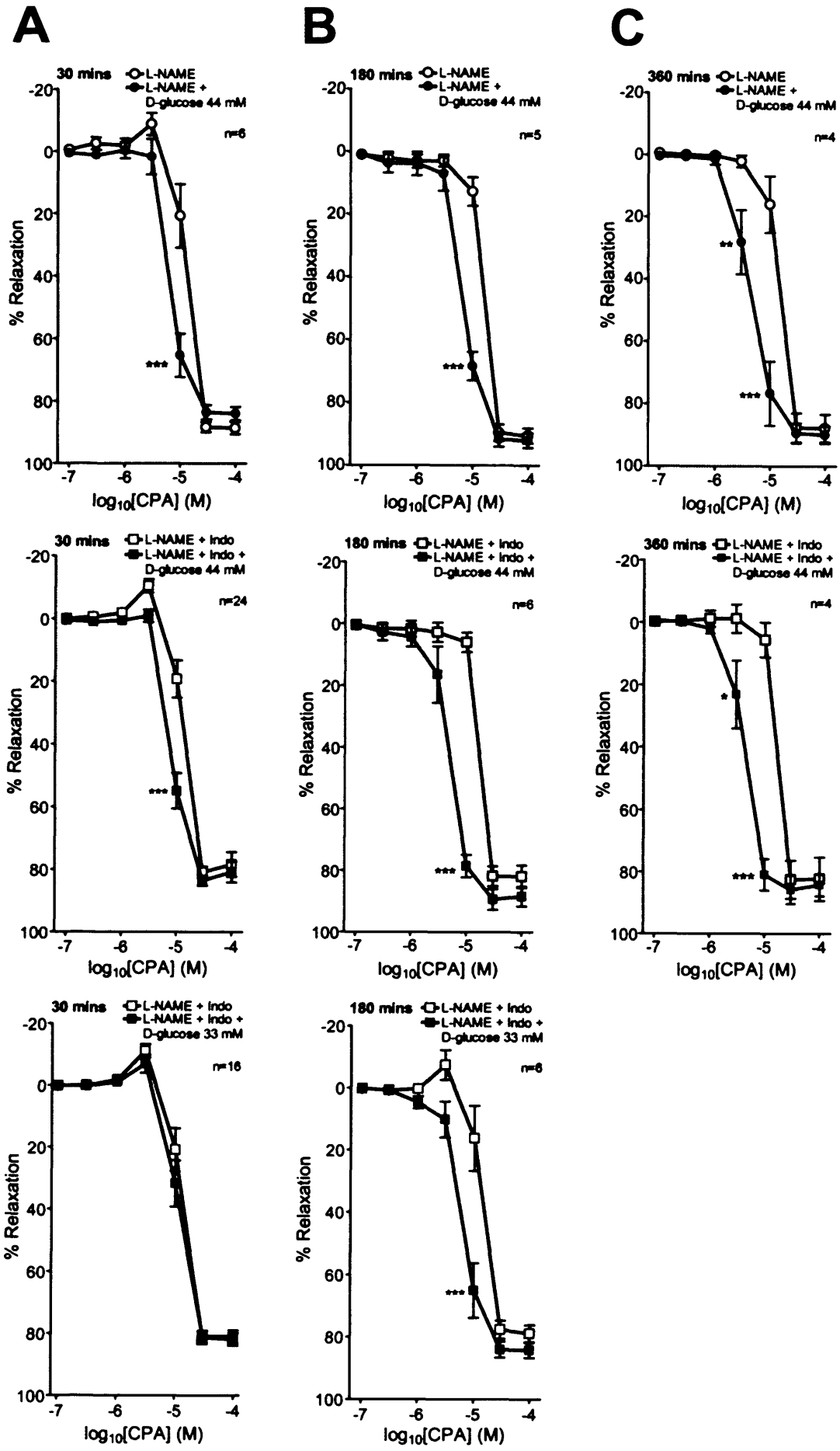

A
B
C
30 mins
180 mins
360 mins
L-NAME
L-NAME +
D-glucose 44 mM
L-NAME + Indo
L-NAME + Indo +
D-glucose 33 mM
n=6
n=5
n=4
n=24
n=16
% Relaxation
log10[CPA] (M)
-20
0
20
40
60
80
100
-7
-6
-5
-4
*
**
***

Figure 4.3 Effects of D-glucose on relaxations evoked by ACh in the absence of L-NAME. Concentration-response curves were unaffected by 44 mM D-glucose, either in the absence or presence of indomethacin, after (A) 30, (B) 180 or (C) 360 minutes incubation. n denotes the number of animals studied.

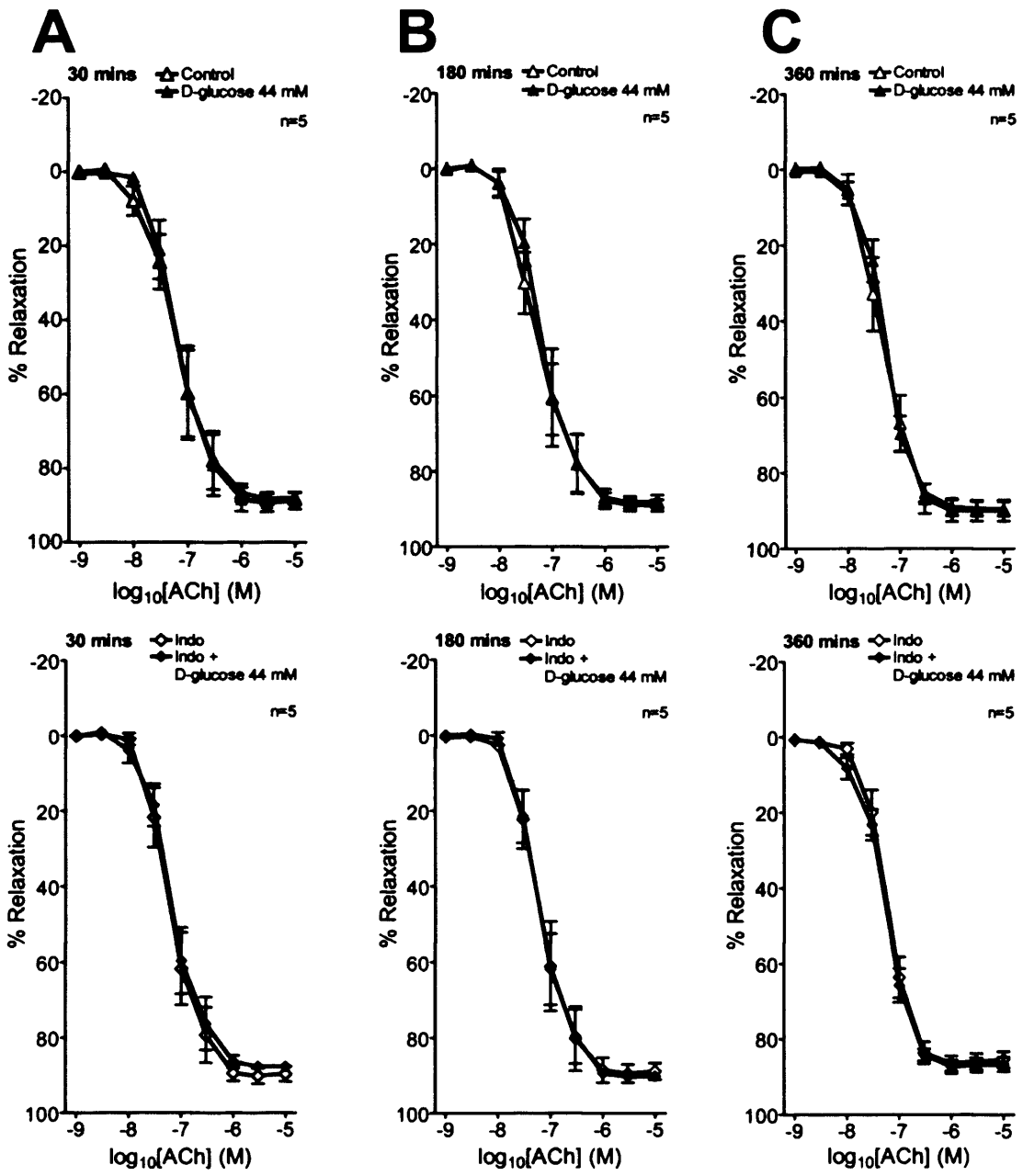

A
B
C
30 mins
180 mins
360 mins
Control
D-glucose 44 mM
Indo
Indo +
D-glucose 44 mM
n=5
% Relaxation
log10[ACh] (M)

Figure 4.4 Effects of D-glucose on relaxations evoked by ACh in the presence of L-NAME. Concentration-response curves revealed that 44 mM D-glucose augmented relaxation, either in the absence or presence of indomethacin, after (A) 30, (B) 180 and (B) 360 minutes incubation. Additional experiments confirmed a time- and concentration-dependent augmentation of EDHF-type relaxation by D-glucose, with 33 mM D-glucose being ineffective after (A) 30 minutes but potentiating responses after (B) 180 minutes incubation. *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$ compared with time-matched control preparations incubated in standard Holman's buffer containing 11 mM D-glucose. n denotes the number of animals studied.

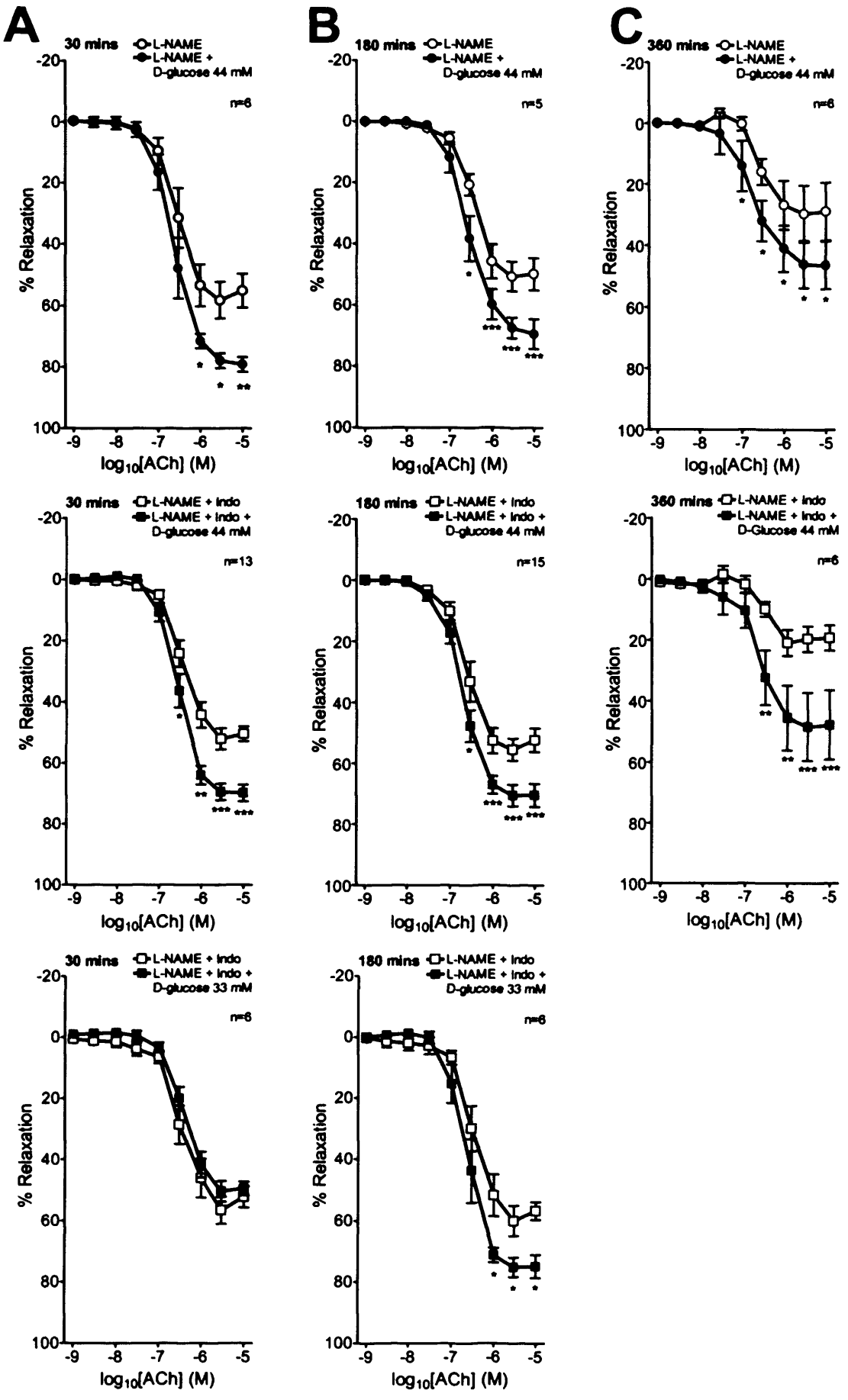

A
B
C
30 mins
180 mins
360 mins
L-NAME
L-NAME +
D-glucose 44 mM
n=6
n=5
L-NAME + Indo
L-NAME + Indo +
n=13
n=15
D-Glucose 44 mM
n=6
D-glucose 33 mM
n=6
% Relaxation
log10[ACh] (M)

Figure 4.5 Effects of low concentrations of D-glucose on EDHF-type relaxations evoked by ACh. Concentration-response curves were unaffected by 30 minutes incubation with either 1.1 or 5.5 mM D-glucose. n denotes the number of animals studied.

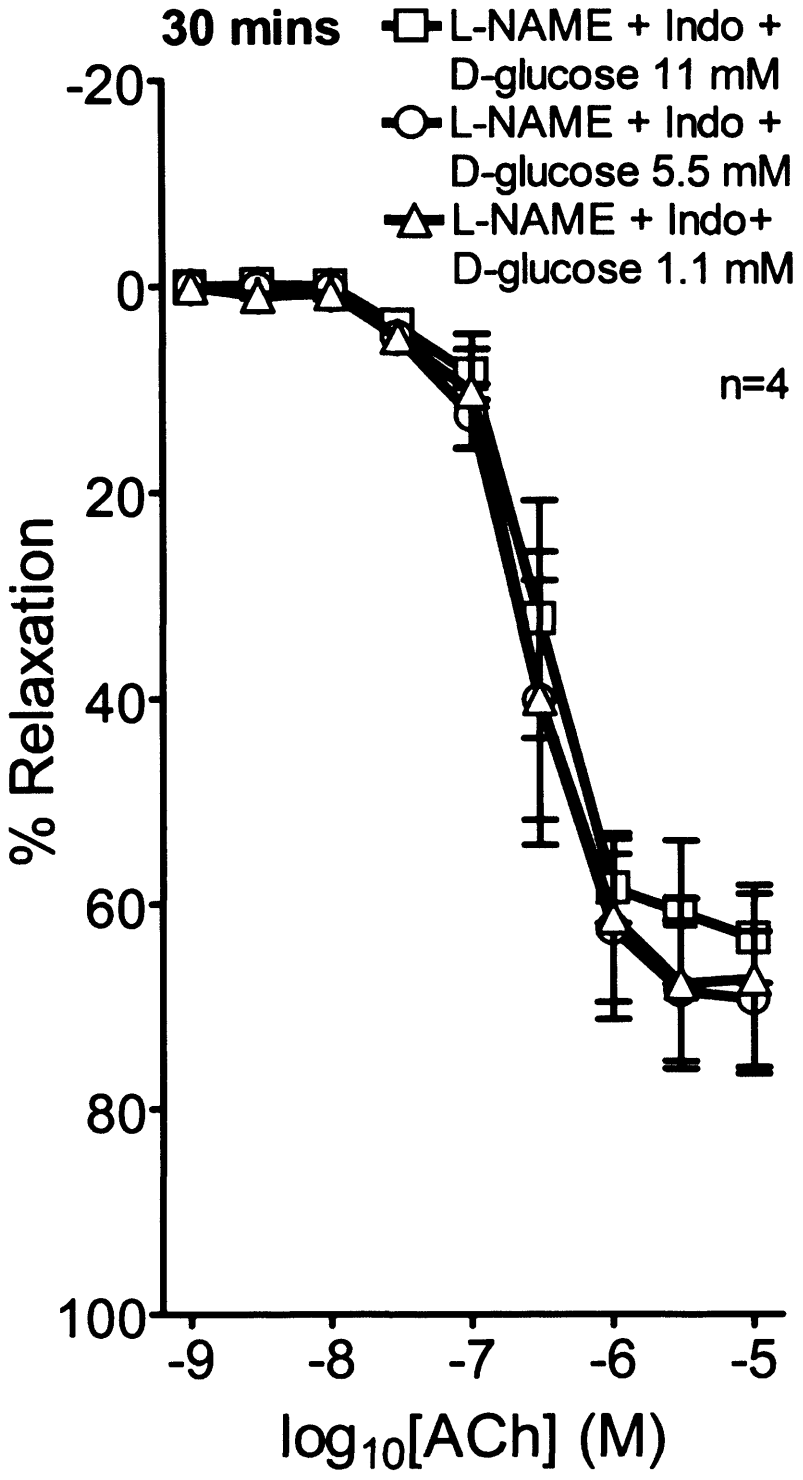
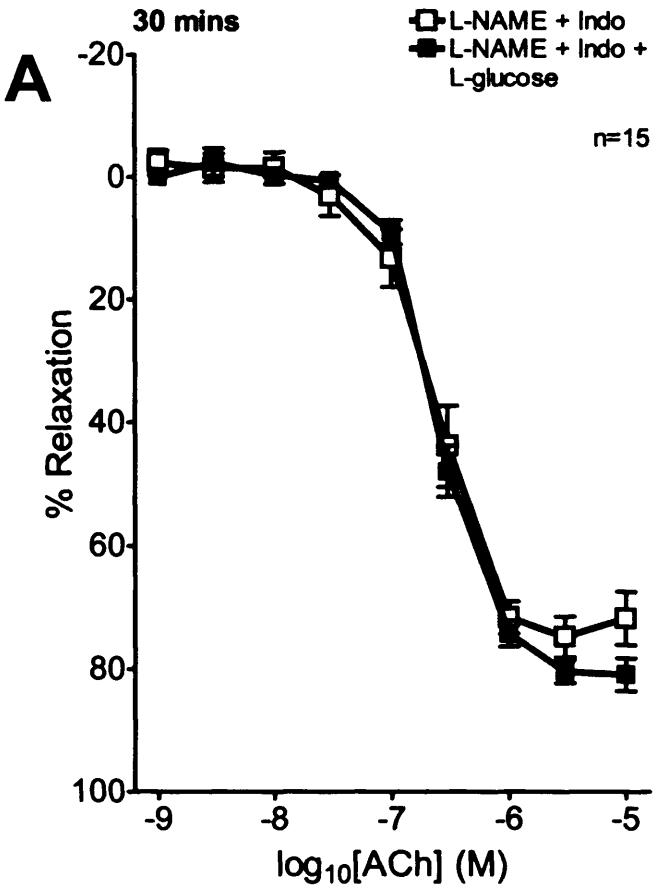

30 mins
L-NAME + Indo + D-glucose 11 mM
L-NAME + Indo + D-glucose 5.5 mM
L-NAME + Indo+ D-glucose 1.1 mM
n=4
% Relaxation
-20
0
20
40
60
80
100
-9
-8
-7
-6
-5
log10[ACh] (M)

Figure 4.6 Effects of L-glucose on EDHF-type relaxations evoked by ACh. Concentration-response curves were unaffected by 33 mM L-glucose (totalling 44 mM glucose with the 11 mM D-glucose normally present in the buffer) after (A) 30 minutes or (B) 180 minutes incubation. n denotes the number of animals studied.


A
30 mins
L-NAME + Indo
L-NAME + Indo + L-glucose
n=15
% Relaxation
-20
0
20
40
60
80
100
-9
-8
-7
-6
-5
log10[ACh] (M)

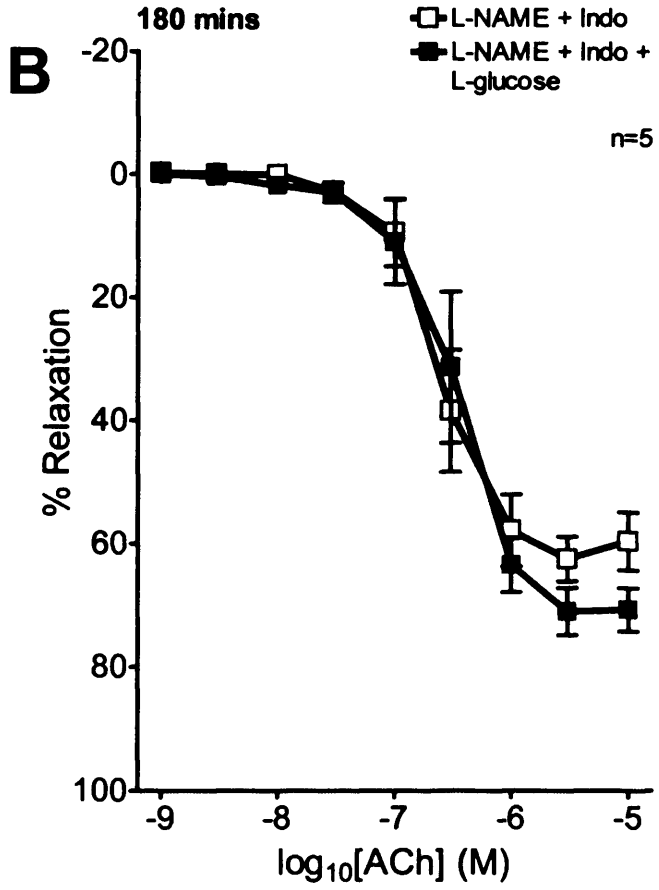

B
180 mins
L-NAME + Indo
L-NAME + Indo + L-glucose
n=5
% Relaxation
-20
0
20
40
60
80
100
-9
-8
-7
-6
-5
log10[ACh] (M)

Table 4.1 Time- and concentration-dependent effects of D-glucose on endothelium-dependent arterial relaxation evoked by CPA. Experiments were performed in the absence and presence of L-NAME (300 μM) and/or indomethacin (10 μM) and the effect of D-glucose (33 or 44 mM) was subsequently determined. Potency (negative $\log IC_{50}$) and maximal responses (R_{max}) are given as means±SEM. * and *** denote $P<0.05$ and $P<0.001$ compared with time-matched preparations incubated in standard Holman's buffer containing 11 mM D-glucose. n denotes the number of animals studied.

Intervention	n	pIC_{50}	R_{max} %
30 minutes D-glucose incubation			
Control	10	5.24±0.08	95.6±3.1
D-glucose 44 mM		5.27±0.10	93.2±3.5
Indo	10	5.31±0.09	92.6±3.3
Indo + D-glucose 44 mM		5.28±0.14	98.7±2.0
L-NAME	6	4.87±0.05	88.9±1.7
L-NAME + D-glucose 44 mM		5.11±0.05***	84.1±2.1
L-NAME + Indo	24	4.85±0.04	81.7±2.2
L-NAME + Indo + D-glucose 44 mM		5.10±0.04***	82.8±2.1
L-NAME + Indo	16	4.85±0.04	81.7±2.2
L-NAME + Indo + D-glucose 33 mM		4.96±0.04	82.8±2.2
180 minutes D-glucose incubation			
Control	5	5.11±0.05	81.3±4.8
D-glucose 44 mM		5.05±0.06	79.9±6.6
Indo	4	5.37±0.07	90.6±2.2
Indo + D-glucose 44 mM		5.25±0.08	96.7±0.8
L-NAME	5	4.84±0.03	90.6±2.4
L-NAME + D-glucose 44 mM		5.10±0.04***	92.1±2.3
L-NAME + Indo	6	4.83±0.02	82.5±3.3
L-NAME + Indo + D-glucose 44 mM		5.23±0.07***	89.6±3.4
L-NAME + Indo	6	4.79±0.06	79.4±2.7
L-NAME + Indo + D-glucose 33 mM		5.12±0.07*	84.4±2.5
360 minutes D-glucose incubation			
Control	4	5.07±0.04	87.3±2.0
D-glucose 44 mM		5.17±0.07	91.0±1.9
Indo	4	5.24±0.13	95.4±1.3
Indo + D-glucose 44 mM		5.13±0.06	94.8±1.4
L-NAME	4	4.85±0.06	88.9±4.3
L-NAME + D-glucose 44 mM		5.30±0.11***	90.2±3.0
L-NAME + Indo	4	4.78±0.06	83.1±6.6
L-NAME + Indo + D-glucose 44 mM		5.33±0.08***	86.1±4.5

Table 4.2 Effects of L-NAME, indomethacin and D-glucose on arterial tone induced by PE. It was determined that L-NAME and/or indomethacin and/or D-glucose or L-glucose did not alter PE-induced tone compared with time-matched preparations incubated for similar periods without these treatments. Data given as means±SEM. n denotes the number of animals studied (data pooled from Tables 4.1, 4.3 and 4.4).

Intervention	n	PE-induced tone (mN)
30 mins D-Glucose incubation		
Control	15	35.5±2.3
D-glucose 44 mM	15	34.8±3.1
Indo	15	36.2±2.9
Indo+ D-glucose 44 mM	15	35.6±4.0
L-NAME	12	37.8±4.1
L-NAME + D-glucose 44 mM	12	36.2±2.2
L-NAME+Indo	80	37.7±0.8
L-NAME+Indo + D-glucose 1.1 mM	4	36.9±5.9
L-NAME+Indo + D-glucose 5.5 mM	4	34.6±4.8
L-NAME+Indo + D-glucose 33 mM	24	36.4±2.7
L-NAME+Indo + D-glucose 44 mM	37	33.6±2.0
L-NAME+Indo + L-glucose 33 mM	15	37.2±3.3
180 mins D-Glucose incubation		
Control	10	38.2±3.7
D-glucose 44 mM	10	36.2±2.9
Indo	9	37.8±3.2
Indo+ D-glucose 44 mM	9	33.5±2.6
L-NAME	10	36.5±2.2
L-NAME + D-glucose 44 mM	10	31.5±3.6
L-NAME+Indo	37	35.4±1.3
L-NAME+Indo + D-glucose 33 mM	12	36.7±3.6
L-NAME+Indo + D-glucose 44 mM	21	33.4±2.5
L-NAME+Indo + L-glucose 33 mM	5	35.3±5.9
360 mins D-Glucose incubation		
Control	9	34.2±4.4
D-glucose 44 mM	9	33.2±5.0
Indo	9	35.3±3.0
Indo+ D-glucose 44 mM	9	33.9±2.9
L-NAME	10	36.4±1.9
L-NAME + D-glucose 44 mM	10	34.7±3.4
L-NAME+Indo	10	38.8±2.7
L-NAME+Indo + D-glucose 44 mM	10	36.1±1.1

Table 4.3 Time- and concentration-dependent effects of D-glucose on endothelium-dependent arterial relaxation evoked by ACh. Experiments were performed in the absence and presence of L-NAME (300 μM) and/or indomethacin (10 μM) and the effect of D-glucose (33 or 44 mM) was subsequently determined. Potency (negative $logEC_{50}$) and maximal responses (R_{max}) are given as means±SEM. *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$ compared with time-matched preparations incubated in standard Holman's containing 11 mM D-glucose. †† denotes $P<0.01$ compared to corresponding preparations incubated for 30 minutes. n denotes the number of animals studied.

Intervention	n	pEC_{50}	R_{max} %
30 minutes D-glucose incubation			
Control	5	7.23±0.08	88.3±3.5
D-glucose 44 mM		7.20±0.07	88.9±3.2
Indo	5	7.22±0.06	89.5±2.8
Indo + D-Glucose 44 mM		7.19±0.06	87.1±2.5
L-NAME	6	6.58±0.09	57.3±3.6
L-NAME + D-Glucose 44 mM		6.64±0.06	79.2±2.9**
L-NAME + Indo	13	6.48±0.05	51.5±2.1
L-NAME + Indo + D-glucose 44 mM		6.55±0.04	70.5±2.0***
L-NAME + Indo	8	6.52±0.08	54.3±2.9
L-NAME + Indo + D-glucose 33 mM		6.42±0.05	50.6±2.1
180 minutes D-glucose incubation			
Control	5	7.29±0.09	88.1±3.6
D-glucose 44 mM		7.20±0.06	88.4±2.7
Indo	5	7.22±0.07	89.0±3.2
Indo + D-Glucose 44 mM		7.21±0.06	89.7±2.6
L-NAME	5	6.44±0.06	50.9±2.5
L-NAME + D-Glucose 44 mM		6.58±0.06	69.1±3.0*
L-NAME + Indo	15	6.64±0.11	52.5±3.7
L-NAME + Indo + D-glucose 44 mM		6.82±0.06	69.0±2.6**
L-NAME + Indo	6	6.52±0.07	58.3±3.0
L-NAME + Indo + D-glucose 33 mM		6.63±0.06	76.2±3.2*
360 minutes D-glucose incubation			
Control	5	7.27±0.13	82.7±5.1
D-glucose 44 mM		7.18±0.07	83.4±3.0
Indo	5	7.18±0.03	84.5±1.4
Indo + D-glucose 44 mM		7.23±0.03	86.0±1.3
L-NAME	6	6.56±0.12	28.7±3.2††
L-NAME + D-glucose 44 mM		6.75±0.16	46.4±4.5**
L-NAME + Indo	6	6.51±0.12	20.0±1.7††
L-NAME + Indo + D-glucose 44 mM		6.67±0.16	48.4±4.8**

Table 4.4 Effects of L-glucose and low concentrations of D-glucose on EDHF-type relaxation evoked by ACh. All experiments were performed in the presence of L-NAME (300 μM) and indomethacin (10 μM) and the effects of L-glucose (33 mM; totalling 44 mM glucose) and D-glucose (1.1 or 5.5 mM) were subsequently determined. Potency (negative $logEC_{50}$) and maximal responses (R_{max}) are given as means±SEM. n denotes the number of animals studied.

Intervention	n	pEC_{50}	R_{max} %
30 minutes D/L-glucose incubation			
L-NAME + Indo + D-glucose 11 mM (control)	4	6.54±0.07	63.0±3.4
L-NAME + Indo + D-glucose 5.5 mM		6.60±0.09	69.6±4.2
L-NAME + Indo + D-glucose 1.1 mM		6.59±0.10	67.6±4.8
L-NAME + Indo	15	6.63±0.05	74.6±2.7
L-NAME + Indo + L-glucose 33 mM		6.60±0.02	80.4±1.5
180 minutes L-glucose incubation			
L-NAME + Indo	5	6.63±0.06	61.2±3.0
L-NAME + Indo + L-glucose 33 mM		6.47±0.07	72.3±4.1

4.4 Discussion

The principal finding of this Chapter was that 44 mM D-glucose potentiated EDHF-type relaxations to CPA and ACh in the rabbit iliac artery, while having no overall effect on endothelial function in the absence of L-NAME and indomethacin. In experiments with CPA, this effect of D-glucose was comparable with that of arsenite, whereas the potentiating effect of D-glucose on EDHF-type relaxations to ACh contrasts with the attenuating effect of arsenite on such responses demonstrated in Chapter 3. EDHF-type relaxations to both CPA and ACh were potentiated following 30 minutes exposure to 44 mM D-glucose, an effect that was sustained after 180 and 360 minutes. Time- and concentration-dependent effects were confirmed by the demonstration that 33 mM D-glucose potentiated relaxation after 180 minutes incubation but was ineffective after 30 minutes.

It was noted, however, that control responses to ACh observed in the presence of L-NAME (either individually or in combination with indomethacin) for 360 minutes were impaired relative to corresponding preparations incubated for 30 minutes, a phenomenon that was not evident in experiments with CPA. This is therefore likely to reflect an impairment in endothelial signalling mechanisms specific to ACh, which mobilizes endothelial Ca^{2+} principally via the receptor-coupled synthesis of $InsP_3$, whereas CPA evokes SOCE by depleting the ER Ca^{2+} store following inhibition of SERCA (Edwards *et al.*, 2008; Garry *et al.*, 2009). Additionally, it is unlikely that either the activity of K_{Ca} channels or gap junctional communication was impaired in preparations incubated for 360 minutes given that EDHF-type relaxations evoked by both ACh and CPA are electrotonic in nature in this artery (Taylor *et al.*, 1998; 2001; Griffith *et al.*, 2002; Chaytor *et al.*, 2002; 2003; 2005; Griffith *et al.*, 2005; Edwards *et al.*, 2007; 2008; Garry *et al.*, 2009).

Some authors have demonstrated that the production of constrictor (Pannirselvam *et al.*, 2005; Erdei *et al.*, 2007; Cheng *et al.*, 2007) or dilator (Alabadi *et al.*, 2001; Shen *et al.*, 2003) prostanoids may be increased in diabetes, whereas others have suggested that changes in prostanoid synthesis do not contribute to altered vascular responsiveness (Hattori *et al.*, 1991; Malakul *et al.*, 2008). In the presence of L-NAME only, control NO-independent relaxations to CPA and ACh, and their potentiation by 44 mM D-glucose were not significantly affected by indomethacin, thus excluding effects attributable to altered prostanoid synthesis in the rabbit iliac artery. In contrast to the effects of high glucose buffer, 30 minutes incubation with low concentrations of D-glucose (1.1 or 5.5 mM) did not affect EDHF-type relaxations evoked by ACh. Additionally, L-glucose did not affect EDHF-type relaxations to ACh over the 180 minute incubation period studied, thus indicating that the potentiating effects of D-glucose on the EDHF phenomenon are not evident with its metabolically inert stereoisomer. Other groups have also demonstrated that metabolically inert sugars have no effect on endothelium-dependent relaxations (Tesfamariam *et al.*, 1990; Dorigo *et al.*, 1997).

At the time that the effect of diabetes on the EDHF phenomenon was reviewed by Fitzgerald and colleagues (2005) there was evidence to suggest that EDHF-type relaxations were impaired by the condition, with the possible exception of murine arteries (Fitzgerald *et al.*, 2005). Subsequent investigations have also demonstrated augmented responses in mice, rats, dogs and humans (Shi *et al.*, 2006; Malakul *et al.*, 2008; Pataricza *et al.*, 2008; MacKenzie *et al.*, 2008). The present demonstration that elevated D-glucose increased maximal EDHF-type relaxations to ACh in the rabbit iliac artery without alterations in pharmacological potency is therefore consistent with findings in the STZ-diabetic rat mesenteric artery, in which an augmented maximal

EDHF-type relaxation, sensitive to the combination of apamin+charybdotoxin, was found to compensate for an impaired NO-dependent response (Shi *et al.*, 2006). While the increased activity of endothelial K_{Ca} channels is thought to play an important role in augmented EDHF-type relaxations in diabetes, the subcellular mechanisms that might be involved remain unidentified (Thomsen *et al.*, 2002; Shen *et al.*, 2003; Shi *et al.*, 2006; Malakul *et al.*, 2008; Pataricza *et al.*, 2008).

Several authors have postulated that increased endothelial production of H_2O_2 secondary to increased generation of $O_2^{\bullet-}$ during diabetes may compensate for NO via its ability to act as an EDHF, whereas others have proposed that augmentation of the EDHF phenomenon may be a direct result of alterations in NO bioavailability (Shi *et al.*, 2006; Malakul *et al.*, 2008; Park *et al.*, 2008). For example, Park and colleagues (2008) have demonstrated that EDHF-type relaxations in coronary arterioles of db/db mice are abolished by catalase or apamin+charybdotoxin, whereas these inhibitors only partially attenuate responses in healthy animals. It was therefore suggested that the role of H_2O_2 in modulating K_{Ca} channel activity and the EDHF phenomenon may become increasingly significant in diabetes, and thereby contribute to the maintenance of dilator function when NO bioavailability is impaired. These observations appear consistent with the demonstration that H_2O_2 may contribute to the EDHF phenomenon in the rabbit iliac artery by promoting the mobilization of endothelial Ca^{2+} stores with secondary activation of K_{Ca} channels (Edwards *et al.*, 2008). Furthermore, Shi and co-workers (2006; 2007) demonstrated that augmented EDHF-type relaxations in the STZ-diabetic rat femoral artery are associated with increased endothelial H_2O_2 production. Indeed, it has been suggested that H_2O_2 may fully compensate for the loss of NO in arterioles isolated from patients with coronary artery disease via an EDHF-type mechanism (Phillips *et al.*, 2007). Alternatively, it has been

suggested that because NO appears to attenuate the EDHF phenomenon under control conditions by suppressing agonist-evoked endothelial Ca^{2+} mobilization, impaired NO bioavailability in diabetes may unmask the EDHF phenomenon as a back-up mechanism (Bauersachs *et al.*, 1996; Shi *et al.*, 2006). Indeed, it has been demonstrated that the EDHF phenomenon may compensate for the absence of NO, either following chronic inhibition of eNOS or in eNOS knockout mice, and fully preserve endothelium-dependent relaxation (Nishikawa *et al.*, 2000b; Brandes *et al.*, 2000; also see 1.9)

By contrast, the impaired function or expression of endothelial SK_{Ca} channels has been proposed to contribute to impaired EDHF-type responses in small mesenteric arteries from STZ-diabetic mice (Ding *et al.*, 2005; Burnham *et al.*, 2006a). Additionally, impaired gap junctional communication may also contribute to attenuated EDHF-type relaxations in animal models of type I and type II diabetes, and has variously been suggested to involve the reduced expression of connexins 37 and 40 (Ding *et al.*, 2005; Makino *et al.*, 2008; Young *et al.*, 2008), and impaired agonist-evoked cAMP accumulation (Matsumoto *et al.*, 2003; 2004; 2005), which is known to play a permissive role in gap junction-dependent EDHF-type responses (Taylor *et al.*, 2001; Griffith *et al.*, 2002; Chaytor *et al.*, 2002; Griffith *et al.*, 2005). Additionally, it has been demonstrated that elevated glucose may evoke PKC-mediated hyperphosphorylation of connexin 43 in cultured vascular smooth muscle cells, leading to impaired intercellular communication via gap junctions (Kuroki *et al.*, 1998). Furthermore, acute exposure of rat mesenteric arteries to elevated glucose impairs EDHF-type relaxations via a mechanism directly involving oxidative stress, with the combination of superoxide dismutase plus catalase restoring responses to control levels (Ozkan & Uma, 2005).

Studies with isolated endothelial cells have also suggested that elevated glucose could in theory augment or impair the EDHF phenomenon. For example, the group of Graier have demonstrated that high concentrations of glucose may promote the mobilization of endothelial Ca^{2+} stores via a mechanism involving ROS on the basis that (i) elevated glucose potentiated increases in $[Ca^{2+}]_i$ in response to the SERCA inhibitor thapsigargin, and (ii) this was reversed to control levels by various antioxidants, including vitamins C and E and the thiol reductant glutathione (Graier *et al.*, 1997). Correspondingly, our group has demonstrated that glutathione monoethyl ester attenuates H_2O_2-promoted endothelial Ca^{2+} mobilization in response to CPA (Edwards *et al.*, 2008).

It was demonstrated in isolated bovine aortic endothelial cells that elevated glucose, via a mechanism involving OH^- radicals, attenuated the sustained increase in $[Ca^{2+}]_i$ (attributed to SOCE) in response to thapsigargin despite an increase in the rate of the initial increase in Ca^{2+} levels (attributed to depletion of the ER store) (Pieper & Dondlinger, 1997; Kimura *et al.*, 1998). Furthermore, elevated glucose has been shown to attenuate BK_{Ca} channel currents in HEK293 (a human embryonic kidney cell line) and vascular smooth muscle cells via a mechanism underpinned by H_2O_2, which was suggested to involve oxidation of cysteine 911 of the α subunit, a residue located near the Ca^{2+} bowl region of this channel (Tang *et al.*, 2004; Lu *et al.*, 2006). This mechanism was also postulated by Burnham and colleagues (2006b) to mediate the impaired sensitivity of BK_{Ca} channels to Ca^{2+} in mesenteric small arterial smooth muscle from Zucker fatty rats (a model of type II diabetes).

There is now compelling evidence that the increased production of ROS by endothelial cells in diabetes originates from NADPH oxidase, and that this may contribute to impaired NO bioavailability and associated NO-dependent relaxations in

animals (Kim *et al.*, 2002; Coppey *et al.*, 2003; Ling *et al.*, 2005; Bitar *et al.*, 2005; Hayashi *et al.*, 2005; Tawfik *et al.*, 2006; Su *et al.*, 2008; Matsumoto *et al.*, 2008; Shukla *et al.*, 2008; Lopez-Lopez *et al.*, 2008) and humans (Guzik *et al.*, 2000; 2002). However, it remains to be determined whether elevated endothelial NADPH oxidase-derived H_2O_2 production in diabetes may compensate for the loss of NO by augmenting the EDHF phenomenon.

The data from the current and previous Chapter have demonstrated that both D-glucose and arsenite augment EDHF-type relaxations to CPA in the rabbit iliac artery, and that these responses may offset impaired NO-dependent relaxations. However, as highlighted in Chapter 3 with arsenic, while it has been demonstrated that the EDHF phenomenon acutely maintains arterial responsiveness during exposure to high glucose, it is uncertain whether such mechanisms are able to provide vasculoprotection against atheroma formation that is facilitated by NO. Subsequent Chapters will investigate the role of oxidative stress, in particular H_2O_2, in the augmented EDHF-type components of relaxation evoked by both arsenite and D-glucose.

CHAPTER 5

THE ROLE OF HYDROGEN PEROXIDE IN EDHF PHENOMENA AUGMENTED BY ARSENITE AND D-GLUCOSE

5.1 Introduction

The first two experimental Chapters of this thesis characterized the time- and concentration-dependent effects of arsenite and D-glucose on endothelium-dependent relaxation in the rabbit iliac artery. It was found that EDHF-type relaxations evoked independently of $InsP_3$-mediated Ca^{2+} mobilization (with CPA) were acutely augmented (after 30 minutes) by both agents (Chapters 3 and 4, respectively). By contrast, arsenite impaired EDHF-type relaxations evoked by ACh (Chapter 3), whereas D-glucose potentiated these responses (Chapter 4). These differential effects of arsenite suggest that it may inhibit the ACh receptor and/or interfere with subsequent second messenger signalling. Indeed, arsenite has previously been reported to inhibit muscarinic receptors directly by interfering with critical thiol groups on the receptor itself (Fonseca *et al.* 1991).

Only four studies appear to have investigated the effects of arsenite on endothelium-dependent relaxation, and the only relaxant employed in these studies was ACh (Lee *et al.*, 2003; Bilszta *et al.*, 2006; Jindal *et al.*, 2007; Srivastava *et al.*, 2007). Furthermore, only one of these studies investigated the effects of arsenite following inhibition of eNOS (Bilszta *et al.*, 2006), while the effects of arsenite on endothelium-dependent relaxations following inhibition of eNOS *and* COX (i.e. EDHF-type) have not previously been explored.

By contrast, the effects of diabetes on endothelium-dependent arterial relaxation have been extensively studied. However, the high degree of variability reported in the literature has, thus far, obscured a clear understanding of the effects of diabetes on endothelial-dependent relaxation. While ROS are regarded as the major contributor to modulation of endothelial function in these models, particularly with reference to compromised NO bioavailability (Hattori *et al.*, 1991; Nassar *et al.*, 2002; Kim *et al.*, 2002; Lopez-Lopez *et al.*, 2008; Shukla *et al.*, 2008), the mechanisms underlying modulation of the EDHF phenomenon are unclear, as evidenced by observations of enhanced, unchanged and attenuated responses (see Table 5.1). However, as with the possible heterogenous nature of the EDHF phenomenon itself (for reviews see: Griffith, 2004; Feletou & Vanhoutte, 2006), it is apparent that the effect of diabetes on EDHF-type relaxation may be vessel- and species-specific. Furthermore, disease duration is also likely to be an important factor (MacKenzie *et al.*, 2008).

Endothelial cells are capable of synthesizing ROS via a number of systems including NADPH oxidase, the mitochondrial electron transport chain, uncoupled eNOS, COX, cytochrome P_{450} and xanthine oxidase (Cai & Harrison, 2000; Matoba *et al.*, 2000; Fleming *et al.*, 2001; Matoba et al., 2002; Liu *et al.*, 2003; McNally *et al.*, 2003; Larsen *et al.*, 2009). There is now compelling evidence that the increased activity of NADPH oxidase contributes to impaired NO-dependent relaxations in diabetes, while studies of isolated endothelial cells suggest that arsenite may also activate the same enzyme leading to increased ROS production and impaired NO bioavailability (Barchowsky *et al.*, 1999; Smith *et al.*, 2001; Bunderson *et al.*, 2002; 2004; Lopez-Lopez *et al.*, 2008; Shukla *et al.*, 2008; Su *et al.*, 2008). Furthermore, a recent study using vascular smooth muscle cells suggests that the increased production of ROS by arsenite may also promote Ca^{2+} mobilization (Li *et al.*, 2009). Conversely, the end-

product of $O_2^{\bullet-}$ dismutation, H_2O_2, has attracted much interest in the last decade as an important regulator of vascular function under normal conditions, with both NADPH oxidase, neuronal NOS (nNOS), the mitochondrial electron transport chain and eNOS having been proposed as important sources (Matoba *et al.*, 2000; 2002; Liu *et al.*, 2003; Leung *et al.*, 2006; Capettini *et al.*, 2008; Larsen *et al.*, 2009). Originally thought to be one of the most toxic ROS, H_2O_2 has controversially emerged as a "classical" EDHF, i.e. as a freely transferable hyperpolarizing factor of vascular smooth muscle cells (Bény & von der Weid, 1991; Matoba *et al.*, 2000; 2002; Lacza *et al.*, 2002; Yada *et al.*, 2003; Matoba *et al.*, 2003; Larsen *et al.*, 2008), in addition to playing an important signalling role in coronary autoregulation, the myogenic response and reactive hyperaemia (Wolin *et al.*, 1990; Nowicki *et al.*, 2001; Koller & Bagi, 2004; Yada *et al.*, 2006; 2008). However, some authors have recently suggested that endothelial H_2O_2 may contribute to the EDHF phenomenon via autocrine actions, thus questioning its role as a freely diffusible EDHF (Hatoum *et al.*, 2005; Edwards *et al.*, 2008; Hercule *et al.*, 2009). For example, our laboratory has recently demonstrated that H_2O_2 promotes the mobilization of intracellular Ca^{2+} stores in rabbit endothelial cells and potentiate EDHF-type relaxation in the rabbit iliac artery (Edwards *et al.*, 2008). Rather than serving as a classical EDHF, H_2O_2 in this vessel promotes endothelial hyperpolarization by modulating integrated K_{Ca} channel activity secondary to increases in endothelial $[Ca^{2+}]_i$. This endothelial hyperpolarization may then be transmitted electrotonically to underlying smooth muscle cells via myoendothelial gap junctions (Edwards *et al.*, 2008). Indeed, gap junctional communication is integral to EDHF-type relaxation in this vessel and many other vascular beds (Taylor *et al.*, 1998; Harris *et al.*, 2000; Chaytor *et al.*, 2001; Griffith *et al.*, 2002; De Vriese *et al.*, 2002; Edwards *et al.*, 2007; 2008; Garry *et al.*, 2009). It is

therefore plausible that the EDHF phenomenon may serve to offset impaired NO bioavailability in this vessel under conditions of oxidative stress via a mechanism involving H_2O_2.

Correspondingly, a recent study has demonstrated that EDHF-type relaxations evoked by ACh in coronary arterioles of db/db mice are abolished by catalase or the combination of apamin and charybdotoxin, while these treatments only partially attenuate such responses in healthy animals (Park *et al.*, 2008). Additionally, the group of Gutterman demonstrated that EDHF-type relaxations evoked by ACh in adipose arterioles of patients with coronary artery disease were augmented via a mechanism involving H_2O_2 in such a manner that impaired NO bioavailability was offset and overall vascular function was preserved (Phillips *et al.*, 2007). Furthermore, the group of Vanhoutte proposed that the enhanced activity of K_{Ca} channels contributed to enhanced EDHF-type relaxation in diabetic rat mesenteric and femoral arteries (Shi *et al.*, 2006). However, although these authors later demonstrated that endothelial H_2O_2 production was increased in the femoral artery of this diabetic rat model, they did not elucidate whether this ROS was directly involved in the augmented EDHF-type response (Shi *et al.*, 2007). Several other groups have also reported an increased contribution of K_{Ca} channels to endothelium-dependent relaxation and the EDHF phenomenon in arteries from diabetic animals, although again it is unknown whether increased production of H_2O_2 contributes to these responses (Thomsen *et al.*, 2002; Shen *et al.*, 2003; Pataricza *et al.*, 2008; Malakul *et al.*, 2008; see Table 5.1).

The aim of the current Chapter was to elucidate the involvement of H_2O_2 in the augmented EDHF-type relaxations evoked by arsenite and D-glucose that were previously characterized in Chapters 3 and 4. H_2O_2 is hypothesized to be the most

likely candidate for these augmented responses on the basis that (i) reduced NO bioavailability associated with diabetes and arsenic toxicity is caused by endothelial oxidative stress (Barchowsky *et al.*, 1999; Smith *et al.*, 2001; Bunderson *et al.*, 2002; 2004; also see Table 5.1), (ii) augmented EDHF-type relaxations in diabetic models have been proposed to involve enhanced K_{Ca} channel activity, with the recent study of Park *et al* (2008) specifically implicating H_2O_2 in this modulation, and (iii) H_2O_2 facilitates integrated K_{Ca} channel activity in the rabbit iliac artery (Edwards *et al.*, 2008). Evidence for the involvement of H_2O_2 would provide evidence for a compensatory role of the EDHF phenomenon under conditions of oxidative stress that are known to impair NO bioavailability.

Table 5.1 Modulation of EDHF-type arterial relaxation in models of diabetes mellitus. Observations are approximately equally-weighted between enhanced, unaltered and reduced relaxation which is further complicated by controversy regarding the mechanism(s) involved. The most consistent mechanistic observation appears to be enhanced K_{Ca} channel activity in association with augmented responses. More specifically, the study of Park *et al* (2008) demonstrated this to be mediated by H_2O_2. Our group has recently confirmed the modulation of integrated K_{Ca} channel activity by endogenous and exogenous H_2O_2 in the RIA and the ability of exogenous H_2O_2 to potentiate the EDHF phenomenon by promoting the mobilization of endothelial ER Ca^{2+} stores (Edwards *et al.*, 2008). Streptozotocin evokes pancreatic ß cell necrosis, thereby mimicking type I diabetes; alloxan evokes ß cell necrosis and insulin resistance thereby mimicking type II diabetes; db/db mice and obese Zucker fatty rats (OZFR) are a genetic models of type II diabetes. EDH: endothelium-dependent smooth muscle hyperpolarization; HG: high glucose *in vitro*; n/i: not investigated; n/a: not applicable; PDE3: phosphodiesterase 3; PKA: protein kinase A. *assumed by authors to be EDHF-type relaxation on the basis that NO activity is functionally insignificant in this artery under control conditions.

Study	Species	Induction of diabetes	Artery	Relaxant	Effect on EDHF-type relaxation	Attributed to:
Park *et al.*, 2008	Mouse	streptozotocin	coronary arterioles	ACh	↑(relative to NO)	H_2O_2/↑K_{Ca} channel activity
Pataricza *et al.*, 2008	Dog	alloxan	carotid	ACh	↑	↑K_{Ca} channel activity
MacKenzie *et al.*, 2008*	Human	HG medium	mesenteric	ACh/Bk	↑	n/i
Malakul *et al.*, 2008	Rat	streptozotocin	aorta	ACh	↑	↑K_{Ca} channel activity
Shi *et al.*, 2006	Rat	streptozotocin	femoral, mesenteric	ACh	↑	↑K_{Ca} channel activity
Shen *et al.*, 2003	Mouse	streptozotocin	aorta	ACh	↑	↑K_{Ca} channel activity
Yousif *et al.*, 2002	Rat	streptozotocin	mesenteric	Histamine/A23187	↑	n/i
Thomsen *et al.*, 2002	Rat	streptozotocin	sciatic nerve arterioles	ACh	↑ (duration)	↑K_{Ca} channel activity
Pannirselvam *et al.*, 2002	Mouse	db/db	small mesenteric	ACh	↑	n/i
Timar-Peregrin & Guy, 2001	Rat	streptozotocin	cremaster arterioles	ACh	↑	n/i
Edgley *et al.*, 2008	Rat	streptozotocin	renal	ACh/Bk	↔	n/a
Hirabara, 2007	Rat	streptozotocin	mesenteric	ACh	↔	n/a
Pannirselvam *et al.*, 2006	Mouse	C57BL/KsJ db/db	small mesenteric	ACh/Bk	↔	n/a
Yousif, 2005	Rat	streptozotocin	renal	Histamine	↔	n/a
Yousif *et al.*, 2005	Rabbit	alloxan	renal	ACh	↔	n/a
Ozkan *et al.*, 2005	Rat	HG medium	mesenteric	A23187	↔	n/a
Endo et al., 1995	*Rat*	*streptozotocin*	*aorta*	*ACh*	↔	*n/a*
Makino *et al.*, 2008	Mouse	streptozotocin	coronary	ACh	↓	↓connexin 40 expression
Young *et al.*, 2008	Rat	IR OZFR	mesenteric	ACh	↓	↓connexin 40 expression
Burnham *et al.*, 2006a	Rat	zucker diabetic fatty	small mesenteric	ACh	↓(EDH)	↓SK_{Ca} channel activity
Ding *et al.*, 2005	Mouse	streptozotocin	small mesenteric	ACh	↓	↓connexin 37/SK_{Ca} expression
Ozkan & Uma, 2005	Rat	HG medium	mesenteric	ACh	↓	H_2O_2
Matsumoto *et al.*, 2005	Rat	streptozotocin	mesenteric	ACh/A23187/CPA	↓	↑PDE3 activity
Matsumoto *et al.*, 2004	Rat	streptozotocin	mesenteric	ACh	↓	↓PKA expression
Matsumoto *et al.*, 2003	Rat	streptozotocin	mesenteric	ACh	↓	↑PDE3 activity
Fukao *et al.*, 1997	Rat	streptozotocin	mesenteric	ACh	↓	n/i

5.2 Methods

Male New Zealand White rabbits were sacrificed arterial rings studied isometrically as described in 2.1 and 2.2. The rings were maintained at a resting tension of 1 mN over a 1 hour equilibration period and the buffer repeatedly replaced, with any subsequent alterations in baseline tension due to stress relaxation corrected. All preparations were then incubated with L-NAME (300 μM) and indomethacin (10 μM), and some preparations were also incubated with catalase (2000 units ml^{-1}; to scavenge H_2O_2) or manganese porphyrin (MnTMPyP 100 μM; to scavenge $O_2^{\bullet-}$ /H_2O_2), for 30 minutes. Following this, D-Glucose (total buffer concentration of 44 mM) or arsenite (100 μM) was added for 30 minutes prior to constriction with PE (1 μM). Once PE-induced tone had reached a stable plateau (after approximately 15 minutes) relaxant responses were studied by constructing cumulative concentration-response curves to ACh (1 nM – 10 μM) or CPA (100 nM – 100 μM). Data was collected and analysed as described in 2.3 and 2.4.

5.3 Results.

5.3.1 Effects of arsenite and D-glucose on EDHF-type responses to CPA

EDHF-type relaxations evoked by CPA were potentiated by 30 minutes prior incubation with either 100 μM arsenite or 44 mM D-glucose, shifting the concentration-response curve to the left in each case (pIC_{50}: from 4.92±0.05 to 5.21±0.07 and from 4.85±0.04 to 5.17±0.04; $P<0.001$ and $P<0.01$, n=9 and 11, Figure 5.1A and B, respectively; Table 5.2). Subanalyses revealed that arsenite potentiated relaxations in preparations more responsive to CPA (threshold 10 μM CPA; pIC_{50}: from 4.99±0.05 to 5.28±0.07; $P<0.01$; n=4; Figure 5.2A; Table 5.2), whereas D-glucose was ineffective in such preparations (n=4; Figure 5.2B; Table 5.2). In

preparations less responsive to CPA (threshold 30 μM) both arsenite and D-glucose potentiated relaxation, shifting the concentration-response curve to the left in each case (pIC_{50}: from 4.89±0.05 to 5.07±0.05 and from 4.78±0.04 to 5.19±0.09; $P<0.05$ and $P<0.001$, n=5 and 7, Figures 5.2A and B, respectively; Table 5.2). Additionally, 100 μM arsenite potentiated relaxation in the MnTMPyP group, shifting the concentration-response curve to the left (pIC_{50}: from 4.68±0.07 to 5.05±0.05; $P<0.05$; n=8; Figure 5.3; Table 5.3). Neither the R_{max} for CPA (Tables 5.2 and 5.3) nor PE-induced tone (Table 5.4) was affected by incubation with arsenite or D-glucose.

5.3.2 Effects of catalase on arsenite- and D-glucose-augmented EDHF-type responses to CPA

Catalase (2000 units ml^{-1}) did not affect relaxations evoked by CPA, in either the arsenite group or the D-glucose group, under control conditions in the combined data (n=9 and 11; Figure 5.1A and 5.1B, respectively) or in preparations responsive at 30 μM CPA (n=5 and 7; Figure 5.2A and 5.2B, respectively). However, catalase attenuated relaxation in preparations responsive at 10 μM CPA, shifting the concentration-response curve to the right in both the arsenite and D-glucose groups (pIC_{50}: from 4.99±0.05 to 4.78±0.03 and from 5.11±0.04 to 4.79±0.05; $P<0.01$ for each; Figure 5.2A and B, respectively; Table 5.2). Catalase also reversed the enhancing effects of both arsenite and D-glucose in the combined data (Figure 5.1A and 5.1B, respectively; Table 5.2) and in preparations responsive at 30 μM CPA (Figure 5.2A and 5.2B, respectively; Table 5.2). Additionally, in preparations responsive at 10 μM CPA, catalase reversed the enhancing effect of arsenite to a level comparable with preparations incubated with catalase individually (Figure 5.2A;

Table 5.2). Neither the R_{max} to CPA (Table 5.2) nor PE-induced tone (Table 5.4) was affected by catalase individually or in combination with arsenite or D-glucose.

5.3.3 Effects of MnTMPyP on arsenite-augmented EDHF-type responses to CPA

MnTMPyP (100 μM) attenuated relaxation at 10 μM CPA ($P<0.01$; n=8; Figure 5.3) but did not evoke a significant shift in the concentration-response curve (Table 5.3). MnTMPyP also reversed the enhancing effect of arsenite to a level comparable with time-matched control preparations (Figure 5.3), with a pIC_{50} value comparable with either time-matched control preparations or those incubated with MnTMPyP alone (Table 5.2). Neither the R_{max} to CPA (Table 5.3) nor PE-induced tone (Table 5.4) was affected by MnTMPyP alone or its combination with arsenite.

5.3.4 Effects of D-glucose on EDHF-type responses to ACh

Maximal EDHF-type relaxations evoked by ACh were potentiated by 30 minutes incubation 44 mM D-glucose (R_{max}: from 55.6±3.4 to 77.1±2.7%; $P<0.01$; n=6; Figure 5.4), whereas the pEC_{50} value was not altered (Table 5.5). PE-induced tone was not affected by D-glucose (Table 5.4).

5.3.5 Effects of catalase on D-glucose-augmented EDHF-type responses to ACh

EDHF-type relaxations evoked by ACh were unaffected by catalase (2000 units ml^{-1}) under control conditions (Figure 5.4; Table 5.5). Catalase reversed the enhancing effect of D-glucose to control levels (Figure 5.4; Table 5.5). PE-induced tone was unaffected by catalase alone or its combination with D-glucose (Table 5.4).

Figure 5.1 Effect of catalase on arsenite- and D-glucose-augmented EDHF-type relaxations evoked by CPA. Original recordings and concentration-response curves showing that catalase abolished the enhancing effects of both (A) arsenite and (B) D-glucose. *, ** and *** denote $P<0.05$, $P<0.01$, $P<0.001$ compared with time-matched controls. n denotes the number of animals studied.

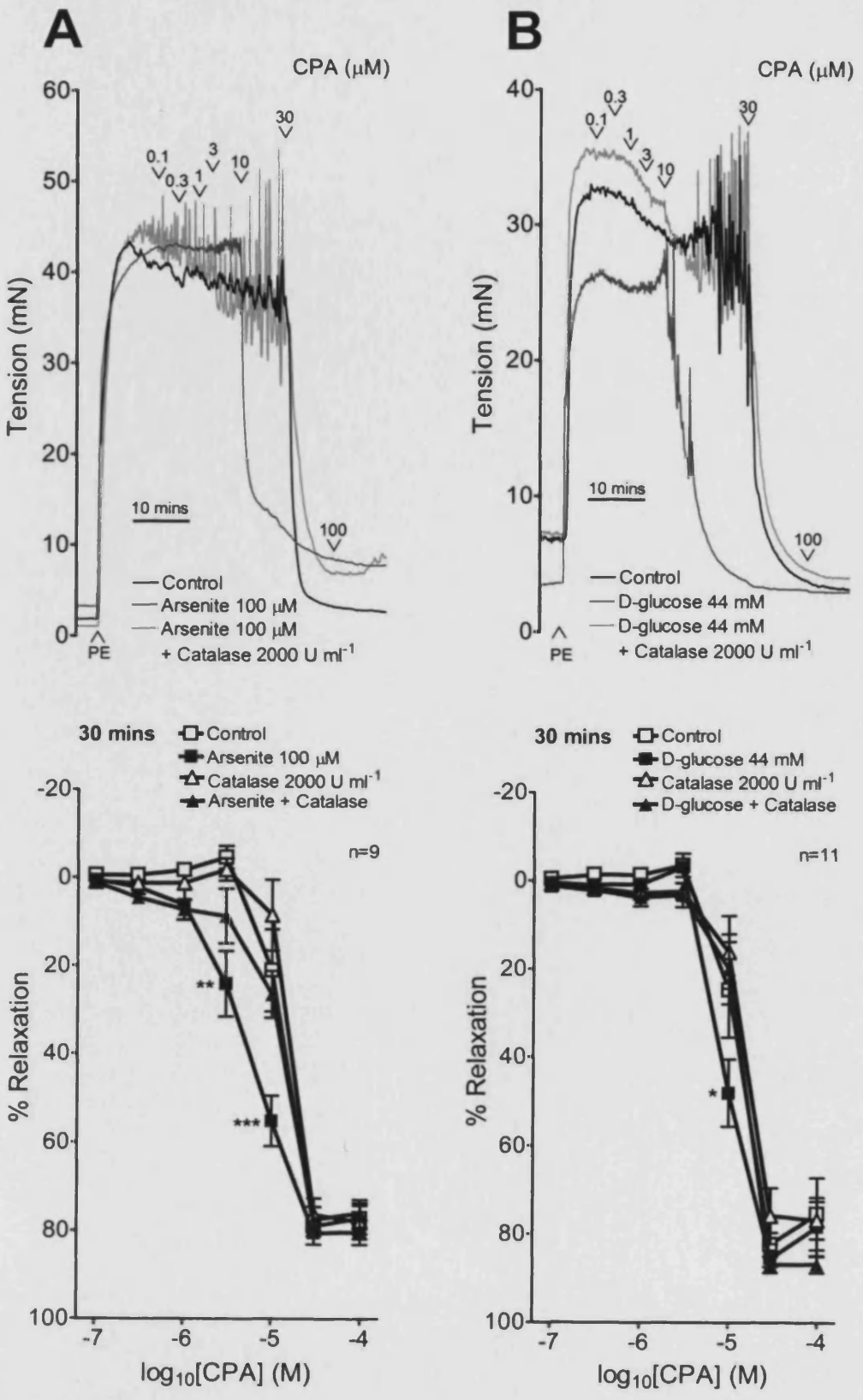

A
CPA (μM)
Tension (mN)
0.1
0.3
1
3
10
30
100
10 mins
Control
Arsenite 100 μM
Arsenite 100 μM
+ Catalase 2000 U ml-1
PE
B
CPA (μM)
Tension (mN)
10 mins
Control
D-glucose 44 mM
D-glucose 44 mM
+ Catalase 2000 U ml-1
PE
30 mins
Control
Arsenite 100 μM
Catalase 2000 U ml-1
Arsenite + Catalase
n=9
% Relaxation
log10[CPA] (M)
30 mins
Control
D-glucose 44 mM
Catalase 2000 U ml-1
D-glucose + Catalase
n=11
% Relaxation
log10[CPA] (M)

Figure 5.2 Subanalyses showing differential effects of arsenite, D-glucose and catalase on EDHF-type relaxations according to the threshold of the response to CPA. Where the threshold was 10 μM CPA, (A) arsenite, but not (B) D-glucose, augmented relaxation at 10 and 30 μM CPA. In these preparations, catalase attenuated relaxation at 10 μM CPA under control conditions (A and B) and also reversed the enhancing effect of arsenite to a level indistinguishable from preparations incubated with catalase individually (A). Catalase was ineffective in preparations responsive at 30 μM CPA under control conditions (A and B) but abolished the enhancing effect of both (A) arsenite and (B) D-glucose. *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$ compared with time-matched controls. n denotes the number of animals studied.

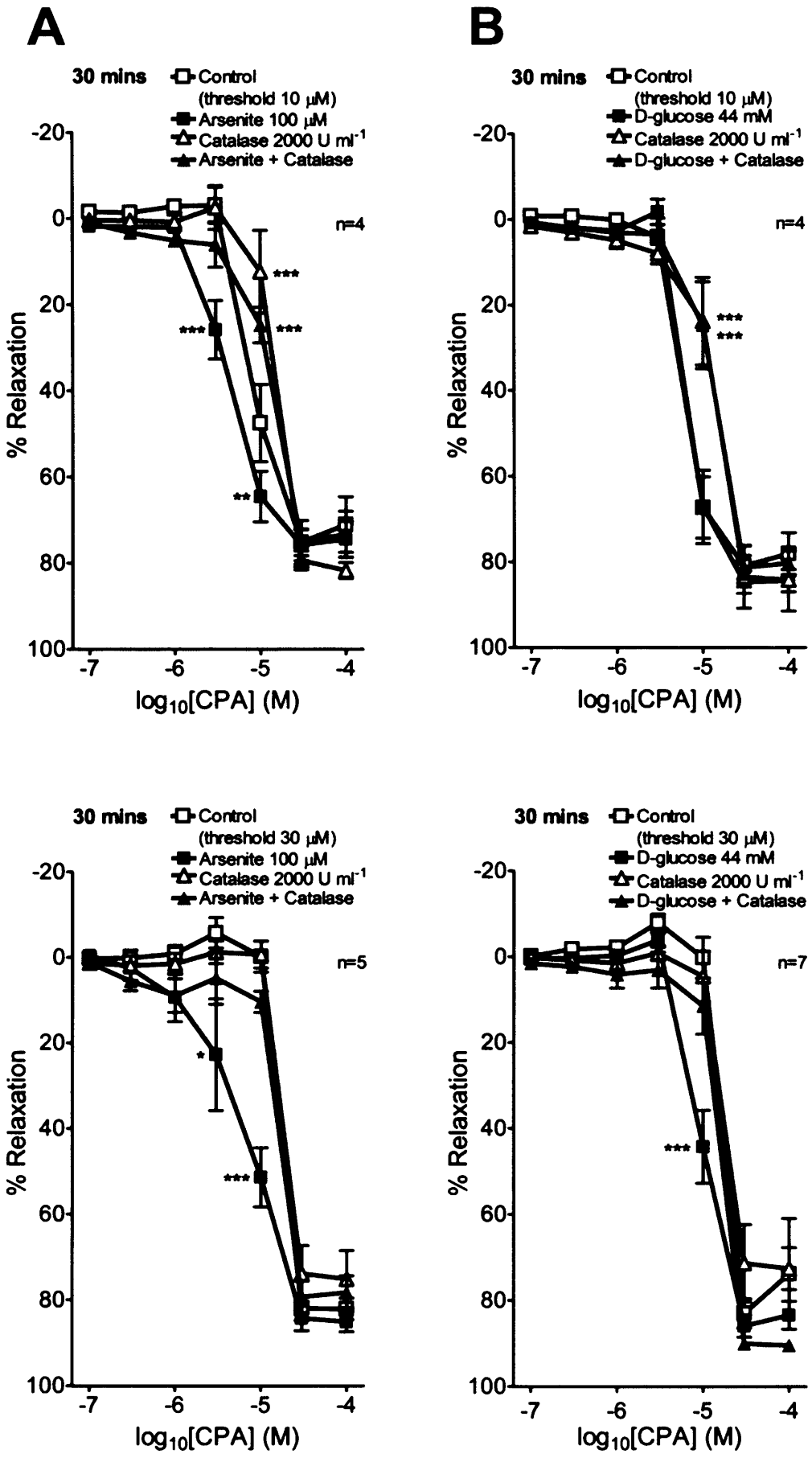
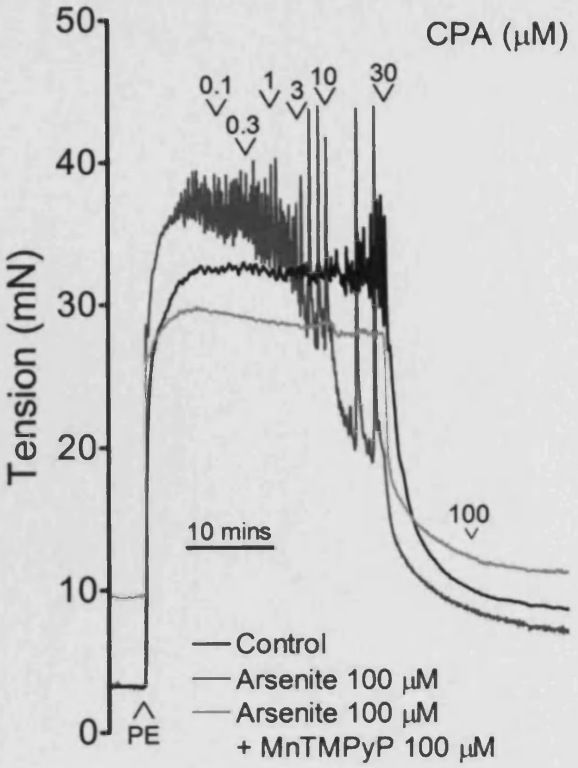

A
30 mins
Control
(threshold 10 μM)
Arsenite 100 μM
Catalase 2000 U ml-1
Arsenite + Catalase
n=4
% Relaxation
log10[CPA] (M)
B
30 mins
Control
(threshold 10 μM)
D-glucose 44 mM
Catalase 2000 U ml-1
D-glucose + Catalase
n=4
% Relaxation
log10[CPA] (M)
30 mins
Control
(threshold 30 μM)
Arsenite 100 μM
Catalase 2000 U ml-1
Arsenite + Catalase
n=5
% Relaxation
log10[CPA] (M)
30 mins
Control
(threshold 30 μM)
D-glucose 44 mM
Catalase 2000 U ml-1
D-glucose + Catalase
n=7
% Relaxation
log10[CPA] (M)

Figure 5.3 Effect of MnTMPyP on arsenite-augmented EDHF-type relaxations evoked by CPA. Original recording and concentration-response curves showing that MnTMPyP abolished the enhancing effect of arsenite. MnTMPyP attenuated relaxation at 10 μM CPA under control conditions. * and ** denote $P<0.05$ and $P<0.01$ compared with time-matched controls. n denotes the number of animals studied.


CPA (μM)
0.1
0.3
1
3
10
30
100
Tension (mN)
50
40
30
20
10
0
10 mins
PE
Control
Arsenite 100 μM
Arsenite 100 μM
+ MnTMPyP 100 μM

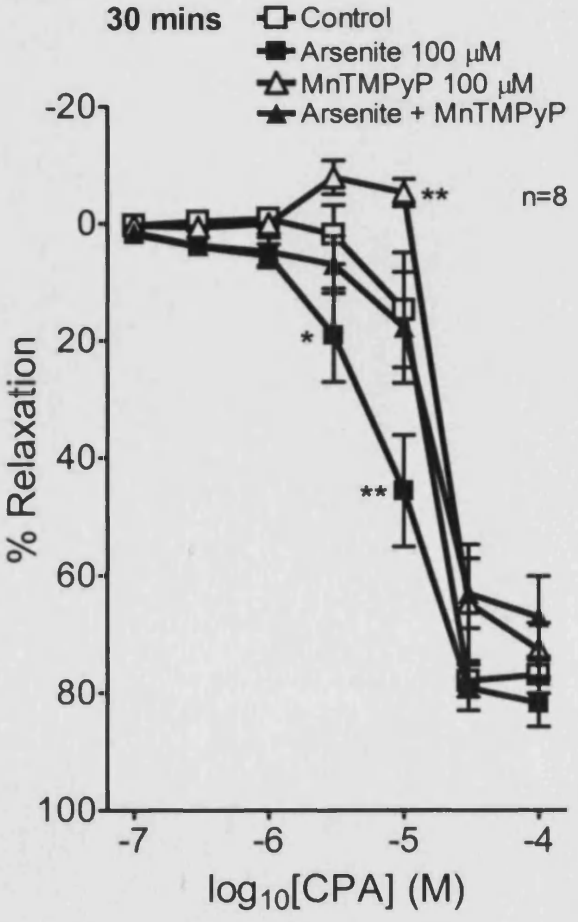
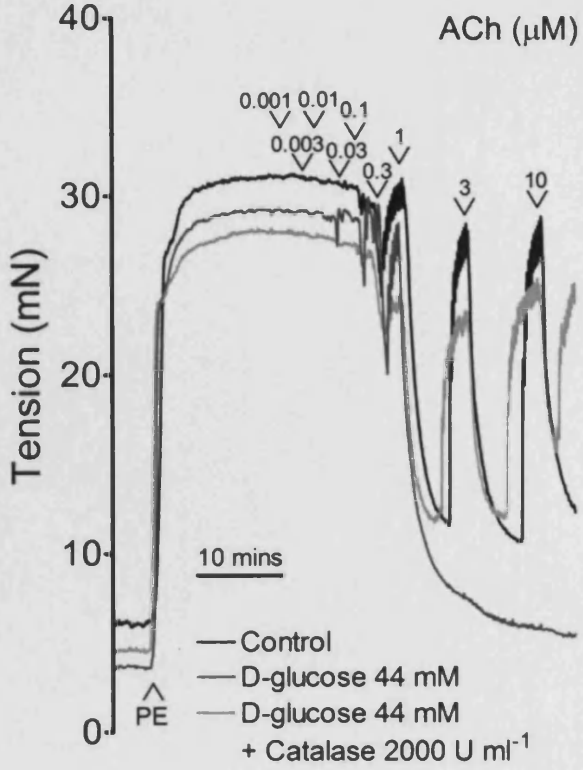

30 mins
Control
Arsenite 100 μM
MnTMPyP 100 μM
Arsenite + MnTMPyP
n=8
**
*
**
% Relaxation
-20
0
20
40
60
80
100
-7
-6
-5
-4
log10[CPA] (M)

Figure 5.4 Effect of catalase on D-glucose-augmented EDHF-type relaxations evoked by ACh. Original recording and concentration-response curves showing that catalase abolished the enhancing effect of D-glucose. In this example relaxation was more sustained in the presence of D-glucose, whereas in time-matched preparations relaxation was followed by a rapid constriction towards the level evoked by PE. * denotes $P<0.05$ compared with time-matched controls. n denotes the number of animals studied.


ACh (μM)
0.001
0.01
0.1
0.003
0.03
0.3
1
3
10
Tension (mN)
40
30
20
10
0
10 mins
PE
Control
D-glucose 44 mM
D-glucose 44 mM
+ Catalase 2000 U ml-1

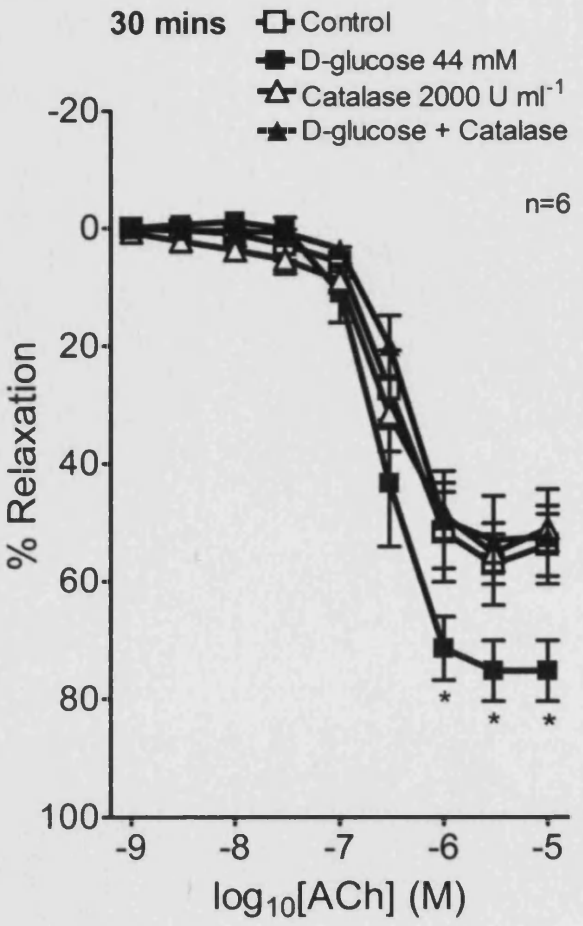

30 mins
Control
D-glucose 44 mM
Catalase 2000 U ml-1
D-glucose + Catalase
n=6
% Relaxation
-20
0
20
40
60
80
100
-9
-8
-7
-6
-5
log10[ACh] (M)
*
*
*

Table 5.2 Enhancing effects of arsenite and D-glucose on EDHF-type relaxations evoked by CPA are abolished by catalase. Subanalyses of total data were paired according to the threshold of relaxation to CPA in time-matched preparations. All experiments were performed in the presence of L-NAME (300 μM) and indomethacin (10 μM) and the effects of arsenite (100 μM), D-glucose (44 mM), catalase (2000 units ml^{-1}) were subsequently determined. Potency (negative log IC_{50}) and maximal responses (R_{max}) are given as means±SEM. *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$ compared with time-matched controls. †, †† and ††† denote $P<0.05$, $P<0.01$ and $P<0.001$ compared with arsenite/D-glucose alone. n denotes the number of animals studied.

Intervention	n	pIC_{50}	R_{max} %
Control (L-NAME+Indo) (combined)	9	4.92±0.05	79.5±2.3
Arsenite 100 μM		5.21±0.07***	81.0±2.5
Catalase 2000 U ml^{-1}		4.79±0.05	82.2±2.8
Arsenite + Catalase		4.80±0.04††	77.9±3.0
Control (L-NAME+Indo) (threshold 10 μM)	4	4.99±0.05	75.8±3.2
Arsenite 100 μM		5.28±0.07**	76.0±3.6
Catalase 2000 U ml^{-1}		4.78±0.03*	81.8±1.8
Arsenite + Catalase		4.80±0.05*†††	75.2±5.0
Control (L-NAME+Indo) (threshold 30 μM)	5	4.89±0.05	83.1±3.7
Arsenite 100 μM		5.07±0.05*	85.5±2.4
Catalase 2000 U ml^{-1}		4.79±0.03	82.3±4.9
Arsenite + Catalase		4.80±0.03†	85.9±3.3
Control (L-NAME+Indo) (combined)	11	4.85±0.04	82.2±3.6
D-glucose 44 mM		5.17±0.04**	85.1±2.6
Catalase 2000 U ml^{-1}		4.74±0.08	81.1±3.7
D-glucose + Catalase		4.81±0.04†	80.3±3.7
Control (L-NAME+Indo) (threshold 10 μM)	4	5.11±0.04	80.9±4.9
D-glucose 44 mM		5.15±0.06	84.7±2.6
Catalase 2000 U ml^{-1}		4.79±0.05**	84.2±7.3
D-glucose + Catalase		4.80±0.03**††	81.5±2.8
Control (L-NAME+Indo) (threshold 30 μM)	7	4.78±0.04	82.4±2.7
D-glucose 44 mM		5.19±0.09***	85.3±2.4
Catalase 2000 U ml^{-1}		4.57±0.08	75.3±6.7
D-glucose + Catalase		4.86±0.05†	79.3±3.7

Table 5.3 Enhancing effects of arsenite on EDHF-type relaxations evoked by CPA are abolished by MnTMPyP. All experiments were performed in the presence of L-NAME (300 μM) and indomethacin (10 μM) and the effects of arsenite (100 μM) and MnTMPyP (100 μM) were subsequently determined. Potency (negative $\log IC_{50}$) and maximal responses (R_{max}) are given as means±SEM. * denotes $P<0.05$ compared with time-matched controls. †† denotes $P<0.01$ compared with arsenite alone. n denotes the number of animals studied.

Intervention	n	pIC_{50}	R_{max} %
Control (L-NAME+Indo)	8	4.68±0.07	78.9±2.8
Arsenite 100 μM		5.05±0.05*	84.1±3.1
MnTMPyP 100 μM		4.59±0.07	73.3±4.5
Arsenite + MnTMPyP		4.69±0.10††	70.7±5.6

Table 5.4 Effects of arsenite, D-glucose, catalase and MnTMPyP on arterial tone induced by PE. It was determined that arsenite (100 μM), D-glucose (44 mM), catalase (2000 units ml^{-1}), MnTMPyP (100 μM) or combinations of these treatments did not affect PE-induced tone compared with time-matched controls. Data given as means±SEM. n denotes the number of animals studied (data pooled from Tables 5.2, 5.3 and 5.5).

Intervention	n	PE-induced tone (mN)
Control (L-NAME+Indo)	34	40.1±1.3
Arsenite 100 μM	17	37.4±3.4
D-glucose 44 mM	17	38.2±2.9
Catalase 2000 U ml^{-1}	26	42.7±1.7
Arsenite + Catalase	9	40.8±4.9
D-glucose + Catalase	17	37.3±2.6
MnTMPyP 100 μM	8	43.6±5.5
Arsenite + MnTMPyP	8	36.3±4.8

Table 5.5 Enhancing effects of D-glucose on EDHF-type relaxations evoked by ACh are abolished by catalase. All experiments were performed in the presence of L-NAME (300 μM) and indomethacin (10 μM) and the effects of D-glucose (44 mM) and catalase (2000 units ml^{-1}) were subsequently determined. Potency (negative $logEC_{50}$) and maximal responses (R_{max}) are given as means±SEM. ** denotes $P<0.01$ compared with time-matched controls. † denotes $P<0.05$ compared with D-glucose alone. n denotes the number of animals studied.

Intervention	n	pEC_{50}	R_{max} %
Control (L-NAME+Indo)	6	6.52±0.08	55.6±3.4
D-glucose 44 mM		6.57±0.06	77.1±2.7**
Catalase 2000 U ml^{-1}		6.43±0.06	53.8±1.6
D-glucose + Catalase		6.33±0.05	59.9±3.4†

5.4 Discussion

The principal finding of this Chapter was that both 100 μM arsenite and 44 mM D-glucose potentiate EDHF-type relaxation in the rabbit iliac artery via a mechanism that appears to involve H_2O_2. Results for each experimental group were analysed collectively and by subanalysis of data paired according to the threshold concentration of CPA required to evoke relaxation. Arsenite potentiated relaxation at 3 and 10 μM CPA in the combined data (as found in Chapter 3), a phenomenon that did not differ whether the threshold for relaxation was 10 or 30 μM CPA. D-glucose potentiated relaxation at 10 μM CPA in the combined data (as observed in Chapter 4) and in preparations responsive at 30 μM CPA, but not those responsive at 10 μM CPA. The enhancing effects of arsenite and D-glucose were reversed by the H_2O_2 scavenger catalase to a level comparable with time-matched control preparations and those incubated with catalase individually in the combined data.

Under control conditions, catalase attenuated relaxation at 10 μM CPA in preparations with a threshold of 10 μM CPA, but not those where the threshold was 30 μM CPA or in the combined data, suggesting that endogenous H_2O_2 production may contribute to the EDHF phenomenon in the rabbit iliac artery, as previously reported (Edwards *et al.*, 2008). The role of H_2O_2 in arsenite-augmented EDHF-type relaxations was confirmed by the demonstration that the cell-permeable SOD/catalase-mimetic MnTMPyP also abolished the enhanced component of responses evoked by CPA. Additionally, D-glucose-augmented EDHF-type relaxations evoked by ACh (characterized in Chapter 4) were also reversed by catalase. Thus, endogenous H_2O_2 production, in addition to receptor-independent relaxants, may also potentiate EDHF-type relaxations evoked by agonists acting via membrane-associated receptors in the rabbit iliac artery, as previously reported (Garry *et al.*, 2009).

The generality of these observations is substantiated by recent studies from our laboratory, in which EDHF-type relaxations evoked by either CPA or ACh were potentiated by exogenous H_2O_2 (100 μM) in the rabbit iliac artery (Edwards *et al.*, 2008; Garry *et al.*, 2009). It was also demonstrated in these studies that exogenous H_2O_2 promotes the mobilization of intracellular Ca^{2+} stores in rabbit endothelial cells, a mechanism that is likely to involve sensitization of the $InsP_3$ receptor via oxidation on the basis that (i) H_2O_2-potentiated elevations in $[Ca^{2+}]_i$ in response to CPA correlated with store depletion and was reversed by the thiol reductant glutathione monoethyl ester, (ii) this effect was mimicked by the thiol oxidant thimerosal which is known to sensitize the $InsP_3$ receptor to Ca^{2+} and basal levels of $InsP_3$ (Bootman *et al.*, 1992; Parekh & Penner, 1995; Montero *et al.*, 2001; Bultynck *et al.*, 2004), and (iii) the endothelium-dependent CPA-potentiated component of relaxation to H_2O_2 was reversed by combined inhibition of SK_{Ca} and IK_{Ca} channels (with apamin+TRAM). The proposal that H_2O_2 sensitizes the $InsP_3$ receptor was further substantiated by experiments showing that the phospholipase C inhibitor U-73122 did not affect the potentiation of EDHF-type relaxations, indicating that $InsP_3$ synthesis was not altered (Edwards *et al.*, 2008). Additionally, it has been reported that the endothelium-specific SERCA3 isoform is, unlike the smooth muscle SERCA2b isoform, insensitive to H_2O_2 at concentrations <500 μM, thus indicating that the effect of 100 μM H_2O_2 in the rabbit iliac artery is unlikely to be due to inhibition of the SERCA pump (Grover *et al.*, 1997; Edwards *et al.*, 2008).

Furthermore, in the same study, it was shown that endogenous H_2O_2 promoted the integrated activity of K_{Ca} channels secondary to the mobilization of endothelial Ca^{2+} stores in response to CPA. Correspondingly, a triple combination of K_{Ca} blockers (apamin+TRAM+iberiotoxin) essentially abolished EDHF-type relaxation in

preparations with a threshold of 30 μM CPA, whereas responses in preparations with a threshold of 10 μM were only attenuated to ~60% by this treatment and further attenuated to ~40% by catalase (Edwards *et al.*, 2008). Therefore, H_2O_2 may promote endothelial hyperpolarization in the rabbit iliac artery rather than acting as a freely diffusible EDHF, as has been suggested in studies of human and mouse mesenteric arteries, coronary microvessels of pigs, dogs and humans and piglet pial microvessels (Bény and von der Weid, 1991; Matoba *et al.*, 2000; 2002; Lacza *et al.*, 2002; Yada *et al.*, 2003; Matoba *et al.*, 2003; Larsen *et al.*, 2008; 2009).

One previous study has investigated the effects of arsenite on vascular function in the absence of eNOS activity, in which NO-dependent and –independent relaxations were preserved despite hypereactivity to phenylephrine mediated by reduced basal NO bioavailability (Bilszta *et al.*, 2006). Indeed, the only other data available are the inhibitory effects of arsenite on NO-dependent arterial relaxation to ACh which therefore reflect reduced NO bioavailability and subsequent cGMP signalling (Lee *et al.*, 2003; Srivastava *et al.*, 2007; Jindal *et al.*, 2007). The inhibitory effect of arsenite on ACh relaxations observed in the current study (see Chapter 3) cannot be attributed to H_2O_2 on the basis that: (i) D-glucose-mediated enhancement of EDHF-type relaxation to ACh is entirely mediated by H_2O_2, and (ii) exogenous H_2O_2 potentiates EDHF-type relaxation to ACh in this artery (Garry *et al.*, 2009). The involvement of H_2O_2 in EDHF-type relaxations potentiated by arsenite is consistent with previous reports that it activates NADPH oxidase in endothelial cells and increases the production of ROS (Barchowsky *et al.*, 1999; Smith *et al.*, 2001; Straub *et al.*, 2008). Indeed, arsenite has been reported to promote vascular smooth muscle Ca^{2+} mobilization via a mechanism involving the increased production of ROS, although a similar effect in endothelial cells remains to be demonstrated experimentally (Li *et al.*,

2009). Studies of isolated endothelial cells have provided compelling evidence that elevated D-glucose increases the production of ROS, including H_2O_2, and enhances agonist-evoked Ca^{2+} mobilization which could in theory potentiate the EDHF phenomenon (Wascher *et al.*, 1994; Graier *et al.*, 1996; 1997; 1998; 1999; Schaeffer *et al.*, 2003; Tamareille *et al.*, 2006). Indeed, the ability of H_2O_2 to enhance endothelial Ca^{2+} mobilization has been demonstrated in numerous studies conducted over the last twenty years, and has been variously proposed to involve increased $InsP_3$ synthesis or sensitization/activation of the $InsP_3$ receptor (Lewis *et al.*, 1988; Kimura *et al.*, 1992; Doan *et al.*, 1994; Siflinger-Birnboim *et al.*, 1996; Shimizu *et al.*, 1997; Hu *et al.*, 1998; Saeki *et al.*, 2000; Niwa *et al.*, 2001; Ji *et al.*, 2002; Zheng & Shen, 2005; Mergler *et al.*, 2005; Zhu *et al.*, 2008; Hecquet *et al.*, 2008; Edwards *et al.*, 2008).

While this Chapter has demonstrated the involvement of H_2O_2 in enhanced EDHF-type relaxation to CPA in an *in* vitro model of hyperglycaemia, the variability in the literature regarding modulation of the EDHF phenomenon during diabetes may reflect vessel- and species-specific responses to H_2O_2. For example, the increased production of H_2O_2 has been reported to promote endothelium-dependent thromboxane A_2-mediated contractions in the femoral artery of STZ-diabetic rats that are offset by an augmented apamin+charybdotoxin–sensitive EDHF-type component. The authors of this study also demonstrated the existence of augmented EDHF-type relaxations in the mesenteric artery of the same diabetic rat model (Shi *et al.*, 2006). Conversely, it has been reported that EDHF-type relaxations to ACh are attenuated in the mesenteric artery of STZ-diabetic rats, despite the same method of induction of diabetes and a similar disease duration prior to experimental analysis (Fukao *et al.*, 1997). These

confounding reports may be a reflection of the strain of rat (Sprague-Dawley and Wistar, respectively) studied (Fukao *et al.*, 1997; Shi *et al.*, 2006).

ROS have been suggested to contribute to impaired EDHF-type relaxations in healthy rat mesenteric arterial preparations acutely exposed to a high glucose medium, with responses being restored by SOD+catalase (Ozkan & Uma, 2005). However, the idea that oxidative stress induced by elevated glucose might differentially modulate the EDHF phenomenon in the rabbit iliac artery and rat mesenteric artery is consistent with recent demonstrations that ascorbic acid potentiates EDHF-type relaxation in the rabbit iliac artery (Garry *et al.*, 2009), but impairs these responses in the rat mesenteric artery, via its ability to generate H_2O_2 (Nelli *et al.*, 2009). Furthermore, in many non-vascular cell types H_2O_2 may variably augment (Rouach *et al.*, 2004; Saez *et al.*, 2005; Ramachandran *et al.*, 2007) or impair (Hu & Cotgreave, 1995; Upham *et al.*, 1997; Cho *et al.*, 2002) intercellular coupling via gap junctions constructed from connexin 43.

By contrast, EDHF-type relaxations in coronary arterioles isolated from type II diabetic mice are by abolished catalase or the combination of apamin+charybdotoxin, whereas these treatments only partially attenuate such responses in healthy mice (Park *et al.*, 2008). Furthermore, Malakul and co-workers (2008) reported that an apamin+charybdotoxin–sensitive EDHF-type relaxant response was unmasked (i.e. not observed in healthy controls) in the aorta of both diabetic and hypercholesterolaemic rats, with the combinatorial effects of these conditions being an additively augmented EDHF-type response and a synergistic increase in NADPH oxidase activity. Several other groups have also proposed that K_{Ca} channel activity is increased during diabetes and may contribute to augmented EDHF-type relaxations that serve to compensate for impaired NO bioavailability, although the direct

involvement of H_2O_2 in these responses remains to be determined (Thomsen *et al.*, 2002; Shen *et al.*, 2003; Pataricza *et al.*, 2008). Conversely, it remains to be determined whether oxidative stress contributes to the mechanisms that have been suggested to mediate the attenuation of EDHF-type relaxation in diabetes, which include reduced connexin and/or K_{Ca} channel expression (Ding *et al.*, 2005; Young *et al.*, 2008; Makino *et al.*, 2008) and impaired gap junctional communication mediated by impaired cAMP accumulation (Matsumoto *et al.*, 2004; 2005).

The results obtained in this Chapter have demonstrated that a mechanism involving H_2O_2 underpins the arsenite- and D-glucose- augmented components of EDHF-type relaxation in the rabbit iliac artery. As H_2O_2 is the end-product of the dismutation of $O_2^{\bullet-}$ anions, further analysis is required to determine the $O_2^{\bullet-}$-generating system involved in these responses. Indeed, NADPH oxidase has been reported to be directly activated by arsenite in endothelial cells, and its increased activity has been suggested to contribute to increased endothelial ROS production and impaired NO bioavailability in diabetes. The involvement of this enzyme in EDHF-type relaxations augmented by arsenite and D-glucose is the subject of the next Chapter.

CHAPTER 6

THE ROLE OF NADPH OXIDASE IN EDHF PHENOMENA AUGMENTED BY ARSENITE AND D-GLUCOSE

6.1 Introduction

In the previous Chapter it was shown that arsenite and D-glucose both potentiate EDHF-type relaxations evoked by CPA in the rabbit iliac artery via a mechanism sensitive to catalase. Additionally, catalase reversed the enhancing effect of D-glucose on EDHF-type relaxations to ACh, with the potentiation of CPA relaxations by arsenite also being reversed by the cell permeable SOD/catalase mimetic manganese porphyrin. These data suggest that the potentiating effects of arsenite and D-glucose are mediated by H_2O_2, which can potentiate EDHF-type relaxations to CPA and ACh in the rabbit iliac artery by promoting the mobilization of endothelial Ca^{2+} stores (Edwards *et al.*, 2008; Garry *et al.*, 2009).

The involvement of H_2O_2 in the potentiation of EDHF-type relaxations by arsenite appears to be consistent with findings from studies with isolated endothelial cells, where arsenite has been demonstrated to increase the production of ROS, including H_2O_2, by activating NADPH oxidase (Barchowsky *et al.*, 1999; Smith *et al.*, 2001; Qian *et al.*, 2005; Straub *et al.*, 2008). There is also evidence that the increased endothelial production of ROS during diabetes originates from NADPH oxidase, and impairs NO bioavailability and associated NO-dependent arterial relaxation (Table 6.1). However, it remains to be determined whether the associated increase in endothelial H_2O_2 levels augments the EDHF phenomenon in diabetes.

NADPH oxidases are classified according to the isoform of the large plasma membrane-bound catalytic subunit (NOX1-5, with NOX2 formerly known as

gp91^{phox}) (Brandes & Schroder, 2008). Also present is the smaller plasma membrane subunit, p22^{phox}, and different cytosolic subunits, variably p40^{phox}, p47^{phox}, p67^{phox} and the GTPase Rac1, which associate with the membrane subunits to activate the enzyme (Dworakowski *et al.*, 2008). NOX4 is believed to be the only constitutively active isoform, and does not require the cytosolic subunits for the oxidase activity, which is instead regulated by expression levels (Brandes & Schroder, 2008). Endothelial cells and vascular smooth muscle cells are widely known to express NOX2 and NOX4, with smooth muscle cells also thought to express NOX1 (Ray & Shah, 2005).

Under normal conditions, NADPH oxidase-derived H_2O_2 may contribute physiologically to EDHF-type dilatations evoked by bradykinin in the human coronary microcirculation, as indicated by the similar abilities of apocynin or gp91ds-tat and catalase to inhibit bradykinin-evoked endothelial H_2O_2 generation and EDHF-type dilatations in these vessels (Larsen *et al.*, 2008; 2009). Apocynin is a methoxyphenol plant extract that is widely acknowledged to inhibit NADPH oxidase activity in phagocytic and non-phagocytic cells (Yu *et al.*, 2008). This compound is known to inhibit the association of the cytosolic subunits (e.g. p47^{phox}, p67^{phox} and Rac1) with the membrane-bound subunits following intracellular bioactivation by ROS and peroxidase, although clear details of the mechanism of action are currently unavailable (Yu *et al.*, 2008). Gp91ds-tat is a recently developed peptide inhibitor that specifically prevents the association of the cytosolic p47^{phox} subunit with the plasma membrane-bound gp91phox subunit (Rey *et al.*, 2001).

The aim of the current Chapter was to determine the role of NADPH oxidase in arsenite- and D-glucose-augmented EDHF-type relaxations. Based on the evidence highlighted above, it was hypothesized that the H_2O_2 previously found (Chapter 5) to

underpin these augmented relaxations is the end product of NADPH oxidase-derived $O_2^{\bullet-}$.

Table 6.1 Oxidative stress-mediated modulation of NO/prostanoid-dependent arterial relaxation in experimental diabetes. Note that the majority of evidence supports NADPH oxidase as the ROS-generating system involved in reduced NO bioavailability and impaired relaxant responses. The lack of studies into the role of the enhanced activity of this enzyme in modulation of the EDHF phenomenon in experimental diabetes warrants its investigation in the current study.

db/db: murine model of type 2 diabetes; GK: Goto-Kakizaki, rat model of type 2 diabetes; OLETF: Otsuka Long-Evans Tokushima Fatty, rat model of obese type 2 diabetes; streptozotocin evokes pancreatic ß cell necrosis to mimic type 1 diabetes; alloxan evokes pancreatic ß cell necrosis and insulin resistance to mimic type II diabetes; HG: high glucose; EDR: endothelium-dependent relaxation. *groups: glucose tolerant, non-glucose tolerant, diabetic glucose-tolerant

Study	Species	Induction of diabetes	Artery	Stimulant	Effect on EDR	Attributed to:
Lopez-Lopez *et al*, 2008	Rat	streptozotocin	pulmonary	ACh	↓	NADPH oxidase, ↓NO
Shukla *et al*, 2008	Rabbit	alloxan	aorta	ACh	↓	NADPH oxidase, ↓NO
Matsumoto *et al*, 2008	Rat	OLETF	basilar	ACh	↓	NADPH oxidase, ↓NO
Su *et al*, 2008	Mouse	db/db	mesenteric	ACh/shear stress	↓	↑NADPH oxidase expression, ↓NO
Elmi *et al*, 2008	Mouse	db/db	aorta	ACh	↓	cytochrome P_{450}-derived ROS, ↓NO
Moien-Ashfari *et al*, 2008	Mouse	db/db	coronary	ACh	↓	oxidative stress, ↓SOD expression
Baluchnejadmojarad and Roghani, 2008	Rat	streptozotocin	aorta	ACh	↓	oxidative stress, ↓SOD expression
Natali *et al*, 2008	Human	*	subcutaneous microvessels	ACh	↔ (all groups)	oxidative stress evident
Reyes-Toso *et al*, 2007	Rat	pancreatomy	aorta	ACh	↓	oxidative stress
Tawfik *et al*, 2006	Rat	HG medium	coronary	ACh	↓	NADPH oxidase, ↓NO
Rosen and Wiernsperger, 2006	Rat	GK	aorta	ACh	↔	mitochondrial-derived ROS increased
Hayashi *et al*, 2005	Rat	OLETF	aorta	ACh	↓	NADPH oxidase, ↓NO
Bitar *et al*, 2005	Rat	GK	aorta	ACh	↓	NADPH oxidase, ↓NO
Ling *et al*, 2005	Mouse	non-obese diabetic Lc7	aorta	ACh	↓	NADPH oxidase, constrictor prostanoids
Bagi *et al*, 2004	Rat	HG medium	skeletal muscle arterioles	flow-induced	↓	mitochondrial ROS, ↓NO, ↓BH_4
Sercombe *et al*, 2004	Rabbit	HG medium	basilar	ACh	↑/↓	↑sensitivity to NO/NADPH oxidase, ↓NO
Coppey *et al*, 2003	Rat	streptozotocin	sciatic nerve arterioles	ACh	↓	NADPH oxidase, complex I, ↓NO
Kim *et al*, 2002	Rat	OLETF	aorta	ACh	↓	NADPH oxidase
Kanie *et al*, 2002	Rat	streptozotocin	aorta	ACh	↓	endothelin-1, NADPH oxidase activity
Pieper *et al*, 1996	Rat	streptozotocin	aorta	ACh/A23187	↓/↔	hydroxyl radicals

6.2 Methods

Male New Zealand White rabbits were sacrificed and arterial rings studied isometrically as described in 2.1 and 2.2. The rings were maintained at a resting tension of 1 mN over a 1 hour equilibration period the buffer repeatedly replaced, with any subsequent alteration in baseline tension due to stress relaxation corrected. All preparations were then incubated with L-NAME (300 μM) and indomethacin (10 μM), and some preparations were also incubated with apocynin (100 μM), for 30 minutes. D-Glucose (total buffer concentration of 44 mM) or arsenite (100 μM) was then added for 30 minutes prior to constriction with PE (1 μM). Once PE-induced tone had reached a stable plateau (after approximately 15 minutes) relaxant responses were studied by constructing cumulative concentration-response curves to CPA (100 nM – 100 μM). Data was collected and analysed as described in 2.3 and 2.4.

6.3 Results

6.3.1 Effects of arsenite and D-glucose on EDHF-type responses to CPA

EDHF-type relaxations evoked by CPA were potentiated by 30 minutes prior incubation with either 100 μM arsenite or 44 mM D-glucose, shifting the concentration-response curve to the left in each case (pIC_{50}: from 4.99±0.07 to 5.31±0.05 and from 4.84±0.06 to 5.11±0.04; n=14 and 8; $P<0.01$ and $P<0.001$; Figure 6.1A and 6.1B; Table 6.2). Subanalysis of this data revealed that arsenite also potentiated relaxations in preparations more sensitive to CPA (threshold 10 μM), shifting the concentration-response curve to the left (pIC_{50}: from 5.03±0.04 to 5.32±0.03; $P<0.01$; n=9; Figure 6.2A; Table 6.2), whereas D-glucose was ineffective in such preparations (n=4; Figure 6.2B; Table 6.2). In preparations less responsive to

CPA (threshold 30 μM), both arsenite and D-glucose potentiated relaxation, shifting the concentration-response curve to the left in each case (pIC_{50}: from 4.83±0.06 to 5.25±0.08 and from 4.74±0.07 to 5.00±0.02; n=5 and 4; $P<0.001$ and $P<0.05$; Figure 6.2A and 6.2B; Table 6.2). Neither the R_{max} to CPA (Table 6.2) nor PE-induced tone (Table 6.3) was affected by incubation with either arsenite or D-glucose.

6.3.2 Effect of apocynin on arsenite and D-glucose-augmented EDHF-type responses to CPA

Apocynin (100 μM) did not affect relaxations evoked by CPA, in either the arsenite or D-glucose groups, under control conditions in the combined data (n=14 and n=8; Figures 6.1A and 6.1B, respectively) or in preparations responsive at 30 μM CPA (n=5 and n=4; Figure 6.2A and 6.2B). However, apocynin attenuated relaxation in preparations that were responsive at 10 μM CPA, shifting the concentration-response curve to the right in each case (pIC_{50}: from 5.03±0.04 to 4.78±0.02 and from 5.05±0.08 to 4.72±0.02; $P<0.01$ for each; n=9 and 4; Figure 6.2A and 6.2B; Table 6.2). Apocynin also reversed the enhancing effects of both arsenite and D-glucose in the combined data (Figure 6.1A and 6.1B; Table 6.2) and in preparations responsive at 30 μM CPA (Figure 6.2A and 6.2B; Table 6.2). Additionally, in preparations responsive at 10 μM CPA, apocynin reversed the enhancing effect of arsenite to a level comparable with preparations incubated with apocynin individually (Figure 6.2A; Table 6.2). Neither the R_{max} to CPA (Table 6.2) nor PE-induced tone (Table 6.3) was affected by apocynin individually, or in combination with arsenite or D-glucose.

Figure 6.1 Effect of apocynin on arsenite- and D-glucose-augmented EDHF-type relaxations evoked by CPA. Original recordings and concentration-response curves showing that apocynin abolished the enhancing effects of (A) 100 μM arsenite and (B) 44 mM D-glucose. ** and *** denote $P<0.01$ and $P<0.001$ compared with time-matched control preparations incubated in the absence of arsenite, elevated D-glucose or apocynin. n denotes the number of animals studied.

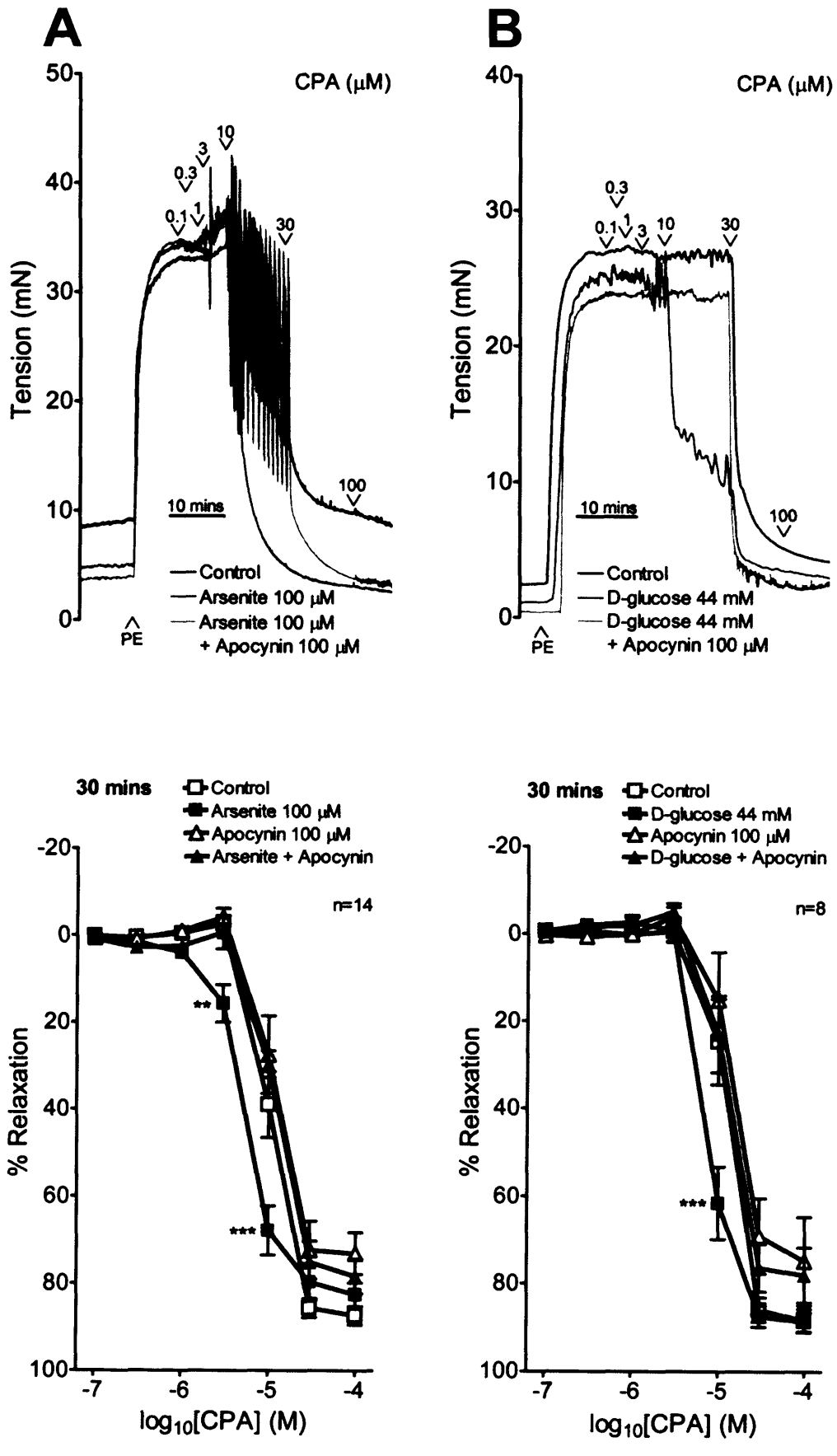

A
B
CPA (μM)
Tension (mN)
10 mins
Control
Arsenite 100 μM
Arsenite 100 μM
+ Apocynin 100 μM
D-glucose 44 mM
D-glucose 44 mM
+ Apocynin 100 μM
PE
30 mins
Apocynin 100 μM
Arsenite + Apocynin
D-glucose + Apocynin
n=14
n=8
% Relaxation
log10[CPA] (M)

Figure 6.2 Subanalyses showing differential effects of arsenite, D-glucose and apocynin on EDHF-type relaxations according to the threshold of the response to CPA. Where the threshold was 10 μM CPA, (A) arsenite, but not (B) D-glucose, augmented relaxation at 10 and 30 μM CPA. In these preparations, apocynin attenuated relaxation at 10 μM CPA under control conditions (A and B) and also reversed the enhancing effect of arsenite to a level indistinguishable from apocynin alone (A). Apocynin was ineffective in preparations responsive at 30 μM CPA under control conditions (A and B) but abolished the enhancing effect of both (A) arsenite and (B) D-glucose. *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$ compared with time-matched control preparations incubated in the absence of arsenite, elevated D-glucose and apocynin. n denotes the number of animals studied.

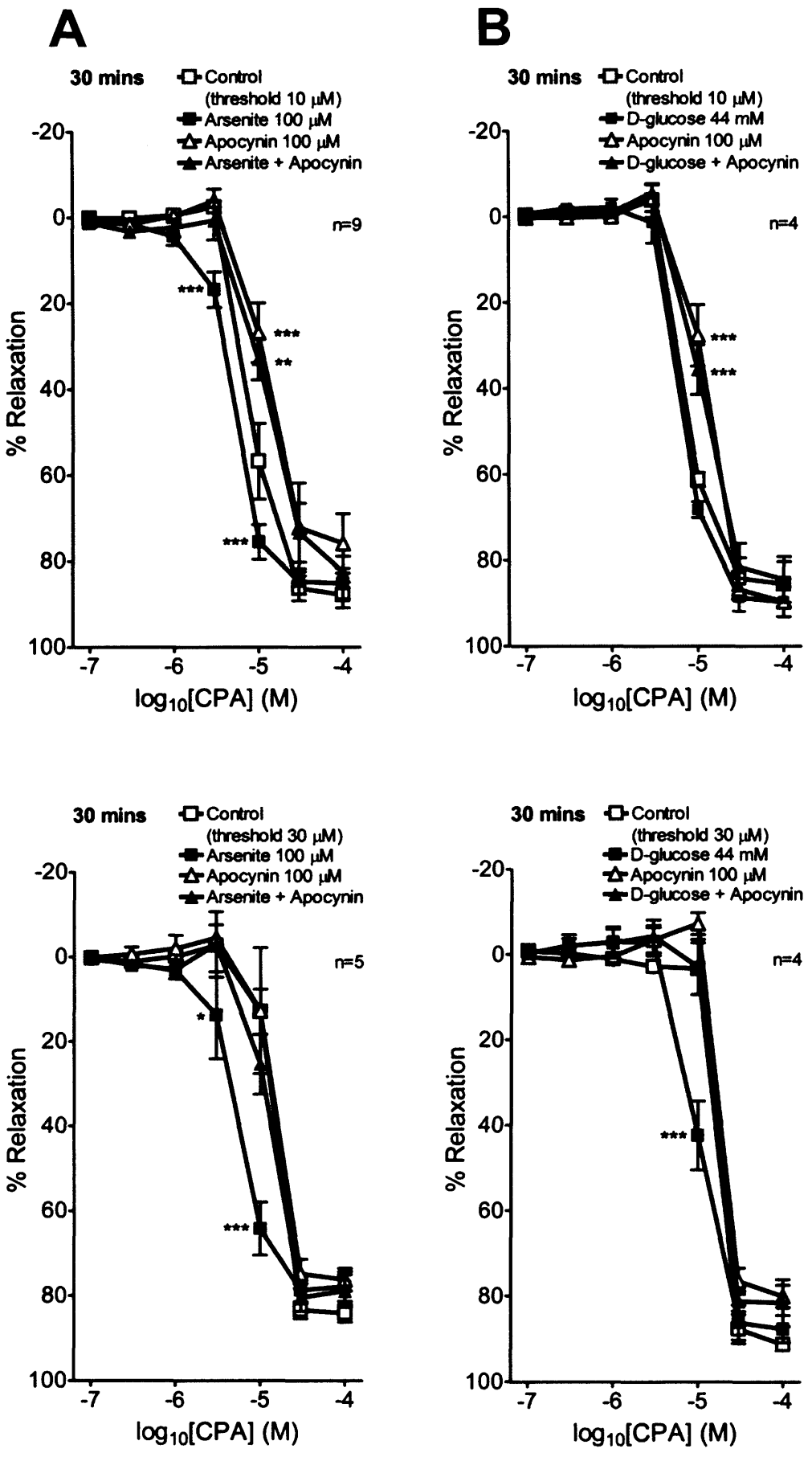

A
30 mins
Control
(threshold 10 μM)
Arsenite 100 μM
Apocynin 100 μM
Arsenite + Apocynin
n=9
% Relaxation
log10[CPA] (M)
B
30 mins
Control
(threshold 10 μM)
D-glucose 44 mM
Apocynin 100 μM
D-glucose + Apocynin
n=4
% Relaxation
log10[CPA] (M)
30 mins
Control
(threshold 30 μM)
Arsenite 100 μM
Apocynin 100 μM
Arsenite + Apocynin
n=5
% Relaxation
log10[CPA] (M)
30 mins
Control
(threshold 30 μM)
D-glucose 44 mM
Apocynin 100 μM
D-glucose + Apocynin
n=4
% Relaxation
log10[CPA] (M)

Table 6.2 Enhancing effects of arsenite and D-glucose on EDHF-type relaxations evoked by CPA are abolished by apocynin. All experiments were performed in the presence of L-NAME (300 μM) and indomethacin (10 μM) and the effects of arsenite (100 μM), D-glucose (44 mM) and apocynin (100 μM) were subsequently determined. Subanalyses of combined data were paired according to the threshold of relaxation to CPA in time-matched preparations. Potency (negative log IC_{50}) and maximal responses (R_{max}) are given as means±SEM. *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$ compared with time-matched control preparations incubated in the absence of arsenite, elevated D-glucose and apocynin. †, †† and ††† denote $P<0.05$, $P<0.01$ and $P<0.001$ compared with arsenite/D-glucose alone. n denotes the number of animals studied.

Intervention	n	pIC_{50}	R_{max} %
Control (L-NAME+Indo) (combined)	14	4.99±0.07	86.2±1.9
Arsenite 100 μM		5.31±0.05**	83.4±2.1
Apocynin 100 μM		4.89±0.07	78.7±3.7
Arsenite + Apocynin		5.04±0.06†	82.9±3.1
Control (L-NAME+Indo) (threshold 10 μM)	9	5.03±0.04	87.9±3.0
Arsenite 100 μM		5.32±0.03**	85.8±3.2
Apocynin 100 μM		4.78±0.02**	79.2±5.9
Arsenite + Apocynin		4.82±0.02**†††	83.7±2.9
Control (L-NAME+Indo) (threshold 30 μM)	5	4.83±0.06	84.4±2.1
Arsenite 100 μM		5.25±0.08***	79.0±3.2
Apocynin 100 μM		4.81±0.03	77.3±2.8
Arsenite + Apocynin		4.97±0.04††	81.0±3.7
Control (L-NAME+Indo) (combined)	8	4.84±0.06	88.6±2.7
D-glucose 44 mM		5.11±0.04***	88.8±2.2
Apocynin 100 μM		4.70±0.09	84.5±5.6
D-glucose + Apocynin		4.77±0.04†††	84.2±2.8
Control (L-NAME+Indo) (threshold 10 μM)	4	5.05±0.08	85.5±5.2
D-glucose 44 mM		5.12±0.02	89.7±3.5
Apocynin 100 μM		4.72±0.02**	89.7±3.4
D-glucose + Apocynin		4.76±0.03**†††	84.5±5.3
Control (L-NAME+Indo) (threshold 30 μM)	4	4.74±0.07	91.7±1.0
D-glucose 44 mM		5.00±0.02*	87.9±3.3
Apocynin 100 μM		4.64±0.08	81.4±2.5
D-glucose + Apocynin		4.79±0.09†	84.0±3.7

Table 6.3 Effects of arsenite, D-glucose and apocynin on arterial tone induced by PE in the presence of L-NAME and indomethacin. It was determined that arsenite (100 μM), D-glucose (44 mM), apocynin (100 μM) or combined treatments did not affect PE-induced tone compared with time-matched control preparations incubated in the absence of these treatments. Data given as means±SEM. n denotes the number of animals studied (data pooled from Table 6.2).

Intervention	n	PE-induced tone (mN)
Control (L-NAME+Indo)	22	38.4±3.1
Arsenite 100 μM	14	34.2±4.8
D-glucose 44 mM	8	36.3±2.9
Apocynin 100 μM	22	40.1±3.1
Arsenite + Apocynin	14	35.3±4.0
D-glucose + Apocynin	8	38.7±5.7

6.4 Discussion

The principal finding of this Chapter was that the mechanism through which arsenite and D-glucose potentiate EDHF-type relaxations evoked by CPA in the rabbit iliac artery is sensitive to apocynin. Indeed, apocynin completely abolished the arsenite- and D-glucose-augmented components of relaxation. Consistent with the effects of catalase described in Chapter 5, subanalysis showed that the ability of apocynin to suppress relaxation in control preparations correlated with the threshold concentration of CPA required to evoke relaxation. Apocynin was thus ineffective under control conditions in preparations responsive to 30 μM CPA, but attenuated relaxations in preparations responsive to 10 μM CPA. The similar abilities of catalase and apocynin to attenuate control EDHF-type relaxations and their potentition by arsenite and D-glucose suggest that (i) the endogenous H_2O_2 known to contribute to EDHF-type relaxations in the rabbit iliac artery under control conditions (Chapter 5; Edwards *et al.*, 2008) may originate from NADPH oxidase, and (ii) the activity of this enzyme may be increased by arsenite and elevated D-glucose, thus potentiating EDHF-type relaxations to CPA via increased H_2O_2 production.

Studies of isolated endothelial cells have demonstrated that arsenite increases NADPH oxidase-derived ROS production in endothelial cells, possibly by promoting the translocation of Rac1 to membrane fractions (Barchowsky *et al.*, 1999; Smith *et al.*, 2001). Additionally, there is strong evidence that the increased activity of NADPH oxidase during diabetes, which appears to be mediated by a PKC-dependent mechanism involving phosphorylation of Rac1, contributes to endothelial dysfunction characterized by impaired NO bioavailability and NO-dependent relaxation (Hink *et al.*, 2001; Inoguchi *et al.*, 2003; Ceolotto *et al.*, 2007; Table 6.1). In contrast to this pathophysiological role of NADPH oxidase, it has also been suggested that ROS

derived from this enzyme contributes to physiological signalling in the vasculature under normal conditions (Gao & Lee, 2005; Larsen *et al.*, 2009). However, while a number of authors have suggested that NADPH oxidase is the predominant source of ROS in the healthy vasculature, it is now apparent that the ROS-generating systems involved in endothelium-dependent relaxation may differ depending on the vessel under study and the mode of endothelial stimulation employed. For example, flow-induced dilatations in the human coronary microcirculation appears to involve H_2O_2 derived from the mitochondrial electron transport chain, whereas dilatations evoked by bradykinin are mediated by NADPH oxidase-derived H_2O_2 (Liu *et al.*, 2003; Larsen *et al.*, 2008; 2009). By contrast, endothelial H_2O_2 production in response to ACh in mouse mesenteric arteries, which has been demonstrated to be involved in the EDHF phenomenon in this artery, has been suggested to originate from eNOS, whereas ACh-evoked endothelial H_2O_2 accumulation in the rat renal artery may derive from NADPH oxidase (Matoba *et al.*, 2000; Gao & Lee, 2005). Furthermore, shear stress-induced H_2O_2 production in isolated bovine aortic endothelial cells appears to involve xanthine oxidase (McNally *et al.*, 2003). Thus, the endothelial ROS-generating systems involved in the physiological control of arterial tone remain controversial, with considerable effort still required to clarify underlying mechanisms. Furthermore, the potential non-specific effects of the classical NADPH oxidase inhibitor diphenylene iodonium, which has been suggested to inhibit of ROS production by complex I (Li & Trush, 1998; Hutchinson *et al.*, 2007), cytochrome P_{450} (Zhukov & Ingleman-Sundberg, 1999), xanthine oxidase (Dambrova *et al.*, 2000) and eNOS (Stuehr *et al.*, 1991), have led to calls for more specific inhibitors of this enzyme to be employed. Additionally, there is still controversy as to whether apocynin forms dimers (and therefore becomes activated to specifically inhibit

NADPH oxidase rather than act as a generalised antioxidant) in endothelial and vascular smooth muscle cells (Johnson *et al.*, 2002; Heumuller *et al.*, 2008).

The demonstration by our laboratory that H_2O_2 contributes to the EDHF phenomenon in the rabbit iliac artery, probably by sensitizing the $InsP_3$ receptor and promoting endothelial Ca^{2+} mobilization, and the data obtained in the current Chapter suggesting that NADPH oxidase may be the source of functionally relevant levels of H_2O_2 in this artery, appear consistent with studies of isolated endothelial cells where the increased activity of NADPH oxidase was found to evoke Ca^{2+} oscillations. Here, NADPH (the substrate of NADPH oxidase that serves to reduce molecular O_2 to $O_2^{\bullet-}$) potentiated $InsP_3$-stimulated Ca^{2+} release from internal stores via a mechanism sensitive to both catalase and diphenylene iodonium that was not observed in cells transiently lacking Rac1 (Hu *et al.*, 2000).

These findings may provide a novel insight into the mechanisms that underpin the augmentation of the EDHF phenomenon in diabetes that has been reported in dogs, rats, mice and humans (Timar-Peregrin & Guy, 2001; Pannirselvam *et al.*, 2002; Yousif *et al.*, 2002; Shen *et al.*, 2003; Thomsen *et al.*, 2002; Shi *et al.*, 2006; MacKenzie *et al.*, 2008; Malakul *et al.*, 2008; Pataricza *et al.*, 2008). Additionally, it remains to be determined whether the beneficial effects of ascorbic acid and the eNOS co-factor tetrahydrobiopterin against endothelial dysfunction during diabetes, that are usually attributed to the restoration of NO-mediated relaxations, involve mechanisms attributable to their ability to generate H_2O_2, which has recently been demonstrated to underpin the potentiation of the EDHF phenomenon by these agents (Garry *et al.*, 2009).

CHAPTER 7

GENERAL DISCUSSION

This thesis has provided novel insights into the mechanisms through which vascular responsiveness may be altered in diabetes and inorganic arsenic toxicity. It was demonstrated that arsenite and elevated D-glucose both potentiate EDHF-type relaxations evoked by CPA in the rabbit iliac artery via a mechanism sensitive to catalase and apocynin. Differential effects of arsenite and elevated D-glucose against EDHF-type relaxations evoked by ACh were identified in that arsenite attenuated relaxation in a time- and concentration-dependent manner, whereas D-glucose potentiated relaxation via a mechanism that was sensitive to catalase. By contrast, arsenite and elevated D-glucose did not affect endothelium-dependent relaxations observed in the absence of L-NAME+indomethacin.

Taken together, these findings suggest that in the rabbit iliac artery arsenite and elevated D-glucose (i) increase NADPH oxidase-derived ROS production, (ii) augment the EDHF phenomenon via a mechanism involving H_2O_2, while (iii) this augmented component may compensate for NO-dependent responses impaired by oxidative stress (Figure 7.1). These findings may represent an important advance in understanding the mechanisms by which the EDHF phenomenon is able to compensate for the loss of NO in diabetes. Additionally this study is the first to study the effects of arsenite on the EDHF phenomenon.

7.1 Hydrogen Peroxide and Compensatory EDHF Phenomena

Superoxide radicals are known to rapidly react with NO, thereby attenuating its bioavailability and associated NO-dependent relaxations in diabetes (see Table 6.1 for references). This mechanism may also contribute to impaired endothelium-dependent

relaxations mediated by arsenic toxicity (Srivastava *et al.*, 2007; Jindal *et al.*, 2007). Correspondingly, there is now evidence suggesting that both arsenite (Barchowsky *et al.*, 1999; Smith *et al.*, 2001; Qian *et al.*, 2005; Straub *et al.*, 2008) and elevated glucose (Kim *et al.*, 2002; Kanie & Kamata, 2002; Coppey *et al.*, 2003; Sercombe *et al.*, 2004; Hayashi *et al.*, 2005; Bitar *et al.*, 2005; Ling *et al.*, 2005; Tawfik *et al.*, 2006; Matsumoto *et al.*, 2007; Lopez-Lopez *et al.*, 2008; Shukla *et al.*, 2008; Su *et al.*, 2008) increase the activity of NADPH oxidase leading to increased ROS production in endothelial cells.

The possibility that elevated levels of H_2O_2, produced by the dismutation of $O_2^{\bullet-}$, may compensate for impaired NO bioavailability via its ability to contribute to or augment the EDHF phenomenon has been suggested in recent studies (Phillips *et al.*, 2007; Park *et al.*, 2008). Phillips and colleagues (2007) have provided evidence that a catalase-sensitive EDHF-type mechanism replaces NO in arterioles from patients with a history of coronary artery disease (various underlying causes), thereby preserving flow-induced dilatations. Additionally, Park and colleagues (2008) have shown that EDHF-type relaxations evoked by ACh in coronary arterioles from type II diabetic mice are abolished by catalase or apamin+charybdotoxin, whereas responses in healthy animals were only partially attenuated by these treatments. These findings led the authors to suggest that the contribution of H_2O_2 to K_{Ca} channel functionality and the EDHF phenomenon may become increasingly significant in diabetes, and serve to maintain dilator function when NO-dependent responses are impaired (Park *et al.*, 2008).

The generality of the effects of arsenite and elevated D-glucose demonstrated in this thesis is consistent with recent findings showing that EDHF-type relaxations evoked by CPA and ACh in the RIA are potentiated by exogenous H_2O_2, which was found to

enhance endothelial Ca^{2+} mobilization and promote the opening of endothelial K_{Ca} channels (Edwards *et al.*, 2008; Garry *et al.*, 2009). This novel action of H_2O_2 (in the context of the EDHF phenomenon) is consistent with previous studies of isolated endothelial cells showing that exogenous H_2O_2 enhances Ca^{2+} mobilization (Lewis *et al.*, 1988; Kimura *et al.*, 1992; Doan *et al.*, 1994; Siflinger *et al.*, 1996; Shimizu *et al.*, 1997; Hu *et al.*, 1998; Saeki *et al.*, 2000; Niwa *et al.*, 2001; Ji *et al.*, 2002; Mergler *et al.*, 2005; Zheng *et al.*, 2005; Edwards *et al.*, 2008; Zhu *et al.*, 2008; Hecquet *et al.*, 2008), and that the enhanced activity of NADPH oxidase potentiates $InsP_3$-evoked Ca^{2+} mobilization via a mechanism sensitive to catalase (Hu *et al.*, 2000; 2002). Correspondingly, studies of isolated endothelial cells have demonstrated that elevated glucose potentiates agonist/SERCA inhibitor-evoked elevations in $[Ca^{2+}]_i$ via a mechanism sensitive to various antioxidants, while a number of authors have suggested that augmented EDHF-type arterial relaxations in diabetic animal models are associated with increased endothelial K_{Ca} channel activity (Graier *et al.*, 1996; 1997; Thomsen *et al.*, 2002; Shen *et al.*, 2003; Dragomir *et al.*, 2004; Tamareille *et al.*, 2006; Shi *et al.*, 2006; Malakul *et al.*, 2008). Additionally, arsenite-evoked Ca^{2+} mobilization has been suggested to involve ROS in vascular smooth muscle cells, although similar studies of endothelial cells have not yet been undertaken (Li *et al.*, 2009).

However, it is unlikely that the increased endothelial production of H_2O_2 in diabetes and arsenic toxicity universally augments EDHF-types relaxations on the basis of recent studies showing that H_2O_2 generated from ascorbic acid (in well-oxygenated buffer in the presence of trace metal ions) differentially modulates EDHF-type relaxations in the rabbit iliac artery (augmented; Garry *et al.*, 2009) and the rat mesenteric artery (impaired; Nelli *et al.*, 2009). Indeed, impaired EDHF-type

relaxations evoked by ACh observed in rat mesenteric arteries acutely exposed to elevated D-glucose (22.2 mM for 360 minutes) are restored by SOD+catalase (Ozkan & Uma, 2005). It remains to be determined, however, whether ROS participate in the mechanisms that have been proposed to contribute to impaired EDHF-type responses in diabetic animals models, which include impaired expression and/or functionality of connexin proteins and K_{Ca} channels (Matsumoto *et al.*, 2003; 2004; 2005; Ding *et al.*, 2005; Burnham *et al.*, 2006a; Young *et al.*, 2008; Makino *et al.*, 2008).

7.2 Diverse Functional Roles of Hydrogen Peroxide in Diabetes

While the results in this thesis are the first to provide evidence that H_2O_2 may contribute to augmented EDHF-type relaxations in diabetes, previous studies have suggested that the increased production of H_2O_2 by endothelial cells, that is characteristic of the condition, may contribute to alterations in vascular responsiveness through mechanisms other than those involved in the EDHF phenomenon.

For example, the increased production of EDCFs, such as thromboxane A_2, has been suggested to contribute to impaired endothelium-dependent relaxations in diabetes in animals (Mayhan *et al.*, 1991; Kobayashi *et al.*, 2002; Pannirselvam *et al.*, 2005; Cheng *et al.*, 2007; Erdei *et al.*, 2007; Shi *et al.*, 2007) and humans (Angulo *et al.*, 2006), the mechanism of which has been suggested to involve H_2O_2 (Cosentino *et al.*, 2003; Shi *et al.*, 2007). For example, the increased production of thromboxane A_2 in STZ-diabetic rat femoral artery is attenuated by catalase, and is functionally offset by an augmented EDHF-type response involving the enhanced activity of endothelial K_{Ca} channels (Shi *et al.*, 2006; 2007). Additionally, Cosentino and colleagues (2003) have shown that elevated glucose increases thromboxane A_2 synthesis in isolated

endothelial cells via a mechanism involving enhanced NADPH oxidase-derived H_2O_2 production. Furthermore, other workers have suggested that increased H_2O_2-mediated thromboxane A_2 synthesis contributes to increased basal tone in gracilis muscle arterioles of type II diabetic mice, and augmented noradrenaline-induced contractions in the STZ-diabetic rat aorta (Kobayashi *et al.*, 2002; Erdei *et al.*, 2007). By contrast, endogenous H_2O_2 attenuates indomethacin-sensitive angiotensin II-induced contractions in the superior mesenteric artery of STZ-diabetic rats (Chin *et al.*, 2007). A distinct functional role of increased H_2O_2 production in diabetes was proposed by Karasu (2000), who showed that H_2O_2 compensates for impaired NO bioavailability during ACh-evoked relaxations of aortae from STZ-diabetic rats. However, that author did not investigate whether an EDHF-type mechanism was involved in these responses. Additionally, it has been demonstrated that authentic H_2O_2 evokes more potent relaxation in aortae from STZ-diabetic rats, the mechanism of which remains to be identified (Pieper & Gross, 1988, Karasu *et al.*, 1999).

7.3 Insights from Studies of the Physiological Role of Hydrogen Peroxide

The postulation in a number of recent reviews that H_2O_2 could in theory play a role in maintaining dilator function when NO bioavailability and associated NO-dependent relaxations are impaired by oxidative stress (Cai *et al.*, 2005; Ardanez & Pagano, 2006; Rush & Ford, 2007; Bagi *et al.*, 2009) is based on numerous demonstrations that this ROS may contribute physiologically to dilator signalling mechanisms. For example, H_2O_2 may contribute to the EDHF phenomenon in a number of arteries, either by (i) acting as a freely diffusible EDHF (Matoba *et al.*, 2000; 2002; Kimura *et al.*, 2002; Lacza *et al.*, 2002; Matoba *et al.*, 2003; Miura *et al.*, 2003; Yada *et al.*, 2003; Fujiki *et al.*, 2005; Liu *et al.*, 2006; Yada *et al.*, 2008; Park *et al.*, 2008; Larsen

et al., 2008; 2009), (ii) by promoting the release of a chemically distinct EDHF (Hatoum *et al.*, 2005), or (iii) by enhancing endothelial hyperpolarizations that are then transmitted to the vascular media via gap junctions (Edwards *et al.*, 2008; Hercule *et al.*, 2009; Garry *et al.*, 2009).

Additionally, H_2O_2-evoked relaxations in mammalian vessels have been variously suggested to involve (i) increased NO synthesis (Yang *et al.*, 1998; 1999; Hirai *et al.*, 2000; Cseko *et al.*, 2004; Samora *et al.*, 2008), (ii) activation of sGC (Burke & Wolin *et al.*, 1987; Burke-Wolin *et al.*, 1991; Fujimoto *et al.*, 2001; Leung *et al.*, 2006; Drouin *et al.*, 2007), (iii) activation of PKG (Burgoyne *et al.*, 2007), (iv) activation of cyclooxygenase (Wolin *et al.*, 1987; Fujimoto *et al.*, 2001; Thengchaisri *et al.*, 2003), (v) decreased sensitivity of the smooth muscle contractile apparatus to Ca^{2+} (Iesaki *et al.*, 1996), and (vi) activation of hyperpolarizing K^+ efflux channels (variously K_{Ca}, K_v and K_{ATP}) located on vascular smooth muscle cells (Matoba *et al.*, 2000; Fujimoto *et al.*, 2001; Miura *et al.*, 2003; Ellis *et al.*, 2003; Gao *et al.*, 2003; Cseko *et al.*, 2004; Rogers *et al.*, 2006; Leung *et al.*, 2006; Samora *et al.*, 2008). It has also been demonstrated in studies of rat arteries (with an exception possibly being the femoral artery) that low concentrations of exogenous H_2O_2 may evoke endothelium-dependent and/or –independent arterial contractions (succeeded by relaxations at higher concentrations of H_2O_2) by increasing the synthesis of thromboxane A_2 (Gao *et al.*, 2003; Cseko *et al.*, 2004; Leung *et al.*, 2006; Samora *et al.*, 2008; Garcia-Redondo *et al.*, 2009).

However, the endothelial sources of H_2O_2 that contribute to vascular responsiveness under normal conditions are poorly understood, with separate groups having variously proposed eNOS, nNOS, NADPH oxidase, the mitochondrial electron transport chain and cytochrome P_{450} (Matoba *et al.*, 2000; Fleming *et al.*, 2001; Liu *et al.*, 2003; Gao

& Lee, 2005; Leung *et al.*, 2006; Drouin *et al.*, 2007; Capettini *et al.*, 2008; Larsen *et al.*, 2009). Indeed, in the rabbit iliac artery NADPH oxidase-derived H_2O_2 may contribute to EDHF-type relaxations evoked by CPA under control conditions (Chapter 6). These preliminary studies have collectively indicated that the source of H_2O_2 may depend on the mode of endothelial stimulation and the vessel under study. Taken together, the findings discussed in this section suggest that increased arterial production of H_2O_2 in diabetes and arsenic toxicity could in theory compensate for impaired NO-dependent relaxations via a number of potential mechanisms.

7.4 Compensatory EDHF Phenomena Associated with Other Cardiovascular Risk Factors

In addition to diabetes and the novel effects of arsenite demonstrated in the current study, augmented EDHF-type relaxations have also been reported in models of other cardiovascular disease states that are also characterized by oxidative stress and impaired NO bioavailability.

Consistent with studies of diabetic animal models, augmented EDHF-type relaxations in models of hypertension, hyperlipidaemia, chronic heart failure and ischaemia-reperfusion are associated with the increased activity of K_{Ca} channels (Najibi *et al.*, 1994; Brandes *et al.*, 1997; Malmsjo et al., 1999; Bocker *et al.*, 2000; Marrelli, 2002; Moroe *et al.*, 2004; Sendao-Oliveira & Bendhack, 2004; Ueda *et al.*, 2005; Taniguchi *et al.*, 2005; Taddei *et al.*, 2006; Malakul *et al.*, 2008). For example, hyperlipidaemia augments EDHF-type relaxations in rabbit renal arteries via a mechanism involving increased production of cytochrome P_{450}-derived EETs, which have been proposed as freely diffusible EDHFs in certain artery types but are devoid of direct smooth muscle relaxant actions in rabbit arteries, where instead they mimic gap junction-dependent

EDHF-type relaxations evoked by ACh (Hecker *et al.*, 1994; Campbell *et al.*, 1996; Brandes *et al.*, 1997; Fisslthaler *et al.*, 1999; Hutcheson *et al.*, 1999; Taylor *et al.*, 2001; Gauthier *et al.*, 2002; Archer *et al.*, 2003; Moroe *et al.*, 2004; Taniguchi *et al.*, 2005). Indeed, it has been suggested that nanomolar concentrations of 5,6-EET may promote SOCE in response to agonists or SERCA inhibitors in endothelial cells (Graier *et al.*, 1995; Hoebel *et al.*, 1997; Rzigalinski *et al.*, 1999; Xie *et al.*, 2002), which, through increasing intracellular Ca^{2+} levels, would be expected to increase the open state probability of all endothelial K_{Ca} channel subtypes. Furthermore, EDHF-type relaxations are also augmented by oxLDL in the rabbit aorta and rat mesenteric arteries, although the mechanisms involved have not yet been identified (Seppo *et al.*, 2000; Bocker *et al.*, 2001).

It has also been demonstrated that the combination of diabetes (STZ-induced) and hyperlipidaemia (diet) cumulatively augments an apamin+charybdotoxin–sensitive component of EDHF-type relaxations in the rat aorta, whereas a separate group demonstrated that the EDHF phenomenon is impaired in small mesenteric arteries from STZ-diabetic ApoE($^{-/-}$) mice (Morikawa *et al.*, 2005; Malakul *et al.*, 2008). Furthermore, enhanced EDHF-type relaxations in renal arteries of spontaneously hypertensive rats maintained on a high cholesterol diet are associated with decreased smooth muscle accumulation of cGMP following agonist stimulation, which again suggests a compensatory role of the EDHF phenomenon under conditions where NO-dependent responses are impaired (Kagota *et al.*, 1999).

While the underlying mechanisms contributing to these augmented EDHF phenomena are not yet clear, Cosentino and colleagues (1998) and Landmesser and colleagues (2003) have suggested that H_2O_2, rather than NO, mediates endothelium-dependent relaxations evoked by ACh in aortae from spontaneously hypertensive rats (SHR) and

deoxycorticosterone acetate–salt-induced hypertensive mice. These groups demonstrated that H_2O_2 generated following the uncoupling of eNOS (resulting from $ONOO^-$-mediated oxidation of BH_4) served to almost fully preserve endothelium-dependent relaxations, whereas supplementation with BH_4 abolished the catalase-sensitive component of relaxation and restored NO-dependent responses (Cosentino *et al.*, 1998; Landmesser *et al.*, 2003). Furthermore, Cosentino and colleagues (1995; 2001) also demonstrated that this compensatory role of H_2O_2 could be mimicked in BH_4-deficient mice or by depleting BH_4 levels pharmacologically in wild-type mice. However, these groups did not determine whether the increase in H_2O_2 production by uncoupled eNOS contributed to endothelium-dependent relaxations via an EDHF-type mechanism. Indeed, Yada and colleagues (2008) have recently suggested that the increased production of H_2O_2 following ischaemia-reperfusion contributes to reactive hyperaemia via an EDHF-type mechanism in the mouse mesenteric circulation *in vivo*, while the study of Marrelli (2002) demonstrated that the potentiation of EDHF-type relaxations following ischaemia-reperfusion in the rat middle cerebral artery may involve enhanced endothelial Ca^{2+} mobilization.

However, as is the case with models of diabetes, augmented EDHF phenomena are unlikely to be universal in models of the cardiovascular risk factors discussed above, with other studies having demonstrated impaired responses. For example, both EDHF-type and NO-mediated relaxations evoked by ACh are impaired in mesenteric arteries from SHRs, with the latter being restored by fluvastatin (Kansui *et al.*, 2004). Furthermore, in the aorta of rats with chronic heart failure impaired EDHF-type relaxations are restored by ACE inhibitors, which have been shown to augment the EDHF phenomenon by generating H_2O_2 (Westendorp *et al.*, 2005; Fujiki *et al.*, 2005). Additionally, $O_2^{\bullet-}$ appears to underpin impaired NO-dependent and EDHF-type

relaxations in goat cerebral arteries following ischaemia/reperfusion (Sanchez *et al.*, 2006), while NADPH oxidase-derived $O_2^{\bullet-}$ may contribute to impairments in the EDHF phenomenon in mesenteric arteries from high cholesterol-fed mice (Matsumoto *et al.*, 2006).

7.5 Stimulation-Specific Modulation of Endothelial Function by Arsenite – Methodical Considerations

Previous investigations of the modulation of endothelial function by arsenite have employed only ACh as a mode of endothelial stimulation (Lee *et al.*, 2003; Bilszta *et al.*, 2006; Srivastava *et al.*, 2007; Jindal *et al.*, 2008). Indeed, experiments conducted in Chapter 3 are, to the author's knowledge, the first to study the effects of arsenite against endothelium-dependent relaxations evoked by a mode of endothelial stimulation other than ACh. The results demonstrated that arsenite impaired EDHF-type relaxations evoked by ACh in a time- and concentration-dependent manner, whereas responses to CPA were potentiated via a mechanism that may involve NADPH oxidase-derived H_2O_2. These confounding effects indicate that arsenite may exert an antagonistic effect on the muscarinic receptor (Figure 7.1). However, it remains to be determined whether arsenite similarly impairs relaxations evoked by other endothelial agonists, such as bradykinin, histamine and ATP, and may be an important focus for future research. Indeed, the findings of this thesis may indicate a necessity to rule out such effects of arsenite in all future studies by employing receptor-independent modes of endothelial stimulation, such as SERCA inhibitors, in addition to agonists.

7.6 Unaltered Vascular Function: Unaltered Endothelial Function?

In the experiments conducted in Chapters 3 and 4, it was apparent that arsenite and D-glucose augmented EDHF-type responses, but had no net effect on endothelium-dependent relaxations observed in the absence of L-NAME and indomethacin. Indeed, Sofola and colleagues (2002) reported similar findings in mesenteric arteries from rats maintained on a high salt diet, whereby overall responses were not altered but were resistant to L-NAME and abolished by apamin+charybdotoxin, thus indicating that the EDHF phenomenon compensated for the loss of the NO-dependent component of relaxation. Their study received an editorial by Katusic, who stated "*more importantly, this observation illustrates that, in diseased arteries, 'normal' endothelium-dependent relaxations in response to acetylcholine should not be interpreted as if there is no alteration in endothelial function*". Additionally, Pataricza and colleagues (2008) demonstrated that while ACh-evoked relaxations of carotid arteries from alloxan-diabetic dogs did not differ from healthy controls overall, responses were attenuated by apamin+charybdotoxin, thus indicating an enhanced functional role of K_{Ca} channels in endothelium-dependent relaxation.

Therefore, investigations into the mechanisms that underpin endothelium-dependent relaxations in disease states could potentially unmask alterations in endothelial function that may otherwise be obscured.

7.7 Future Directions

The novel findings of the current study suggest a number of possibilities for future research into the compensatory role of the EDHF phenomenon in endothelial dysfunction characterized by oxidative stress-impaired NO bioavailability. Such roles for EDHF-type responses have been proposed in animal models of diabetes,

hyperlipidaemia, hypertension, ischaemia-reperfusion and chronic heart failure, and while it has been suggested that the increased activity of endothelial K_{Ca} channels contributes to these responses, the mechanisms involved are not yet clear. Furthermore, the demonstration in the current study that H_2O_2 underpins augmented EDHF phenomena evoked by arsenite and elevated D-glucose may need to be substantiated in future studies by direct measurements of its production in endothelial cells.

The group of Shimokawa quantified endothelial generation of H_2O_2 to substantiate their claims that H_2O_2 was an EDHF. These authors employed electron spin resonance (ESR) spectroscopy and detected a bradykinin-evoked 2-fold increase in H_2O_2 accumulation (as a stable nitroxide radical formed following the reaction of H_2O_2 with *p*-acetamidophenol in the presence of horseradish peroxidase using the spin trap 1-hydroxy-2,2,5,5,-tetramethyl-3-imidazoline-3-oxide) in the perfusate collected from their organ chamber experiments. The group of Gutterman also employed ESR to quantify mitochondrial-derived H_2O_2 accumulation in response to flow in human coronary resistance vessels (Liu *et al.*, 2003).

Furthermore, while this thesis has demonstrated a potential role for the EDHF phenomenon in preserving arterial responsiveness in arsenic toxicity and diabetes *in vitro*, its significance *in vivo* in patients with diabetes and those at risk of arsenic toxicity, and its vasculoprotective role in attenuating atheroma formation (that is known to correlate with impaired NO bioavailaility), is unclear and requires further investigation. From a translational perspective, the effect of NADPH oxidase inhibitors, such as apocynin, against endothelial dysfunction and atherosclerosis in patients is unclear and requires clarification. Indeed, clinical trials exploring the beneficial effects of antioxidants on cardiovascular outcomes are often unsuccessful

and have led researchers to question either the pathophysiological role of ROS or the specificity of the antioxidants employed (Widlansky *et al.*, 2004; Kinlay *et al.*, 2004; Kelemen *et al.*, 2005; Magliano *et al.*, 2006). It also remains to be determined whether the beneficial effects of K_{Ca} channel openers, such as the SK_{Ca} and IK_{Ca} channel opener NS309, against endothelial dysfunction, which have recently been afforded to the improvement of EDHF-type responses in type II diabetic rats (Brondum *et al.*, 2009), include protection against atheroma formation *in vivo* as hyperpolarization of the endothelium following activation of these channels is known to contribute to NO formation by electrogenically favouring extracellular Ca^{2+} influx (Feletou, 2009). Indeed, novel insights into the vasculoprotective role of the EDHF phenomenon have recently been provided by demonstrations that EETs, the production of which is increased in hyperlipidaemic rabbits and compensates for impaired NO bioavailbility (Brandes *et al.*, 1997; Moroe *et al.*, 2004; Taniguchi *et al.*, 2005), inhibit platelet-endothelium interactions *in vitro* and *in vivo* (Krotz *et al.*, 2010).

Additionally, considering that our laboratory has recently provided evidence that ascorbic acid and BH_4 potentiate the EDHF phenomenon via a mechanism involving H_2O_2 (that is distinct from their more widely recognized ability to restore NO bioavailability) (Garry *et al.*, 2009), further studies investigating their ability to restore EDHF-type responses in animal models of diabetes may provide a novel focus for their clinical potential. Indeed, BH_4 prevents endothelial dysfunction in the human forearm circulation following ischaemia/reperfusion via mechanisms that appear to be distinct from its role as a co-factor for eNOS (Mayahi *et al.*, 2007). Furthermore, the concentration of ascorbic acid required to potentiate the EDHF phenomenon in the RIA *in vitro* (1 mM) (Garry *et al.*, 2009) is lower than venous concentrations (1.5-3.2

mM) (achieved following intra-brachial arterial administration of the vitamin) that restore responses to ACh/methacholine in the human forearm in patients with hypertension or peripheral arterial disease (Sherman *et al.*, 2000; Pleiner *et al.*, 2008). Additionally, systemic administration of ascorbic acid in the rat elevates arterial interstitial fluid [H_2O_2] to 25-150 μM (with H_2O_2 remaining undetectable in the luminal compartment due scavenging by erythrocyte catalase and glutathione peroxidase) (Chen *et al.*, 2007; 2008; Garry *et al.*, 2009), which correlates with the potentiation of CPA- and ACh-evoked EDHF-type dilatations in perfused RIA preparations following selective adventitial administration of 100 μM H_2O_2 that nonetheless remains undetectable in the perfusate (Garry *et al.*, 2009).

It should also be noted that in studies of non-vascular cells H_2O_2 has been shown to variably potentiate or attenuate intercellular communication via gap junctions constructed from connexin 43 (Garry *et al.*, 2009). However, analogous studies of vascular gap junctions have not yet been undertaken and further analyses are therefore required to evaluate the effects of competing pro- and anti-oxidant mechanisms on myoendothelial and homocellular communication, and, indeed, the significance of these mechanisms in diabetes mellitus and arsenic toxicity when the production of ROS is elevated.

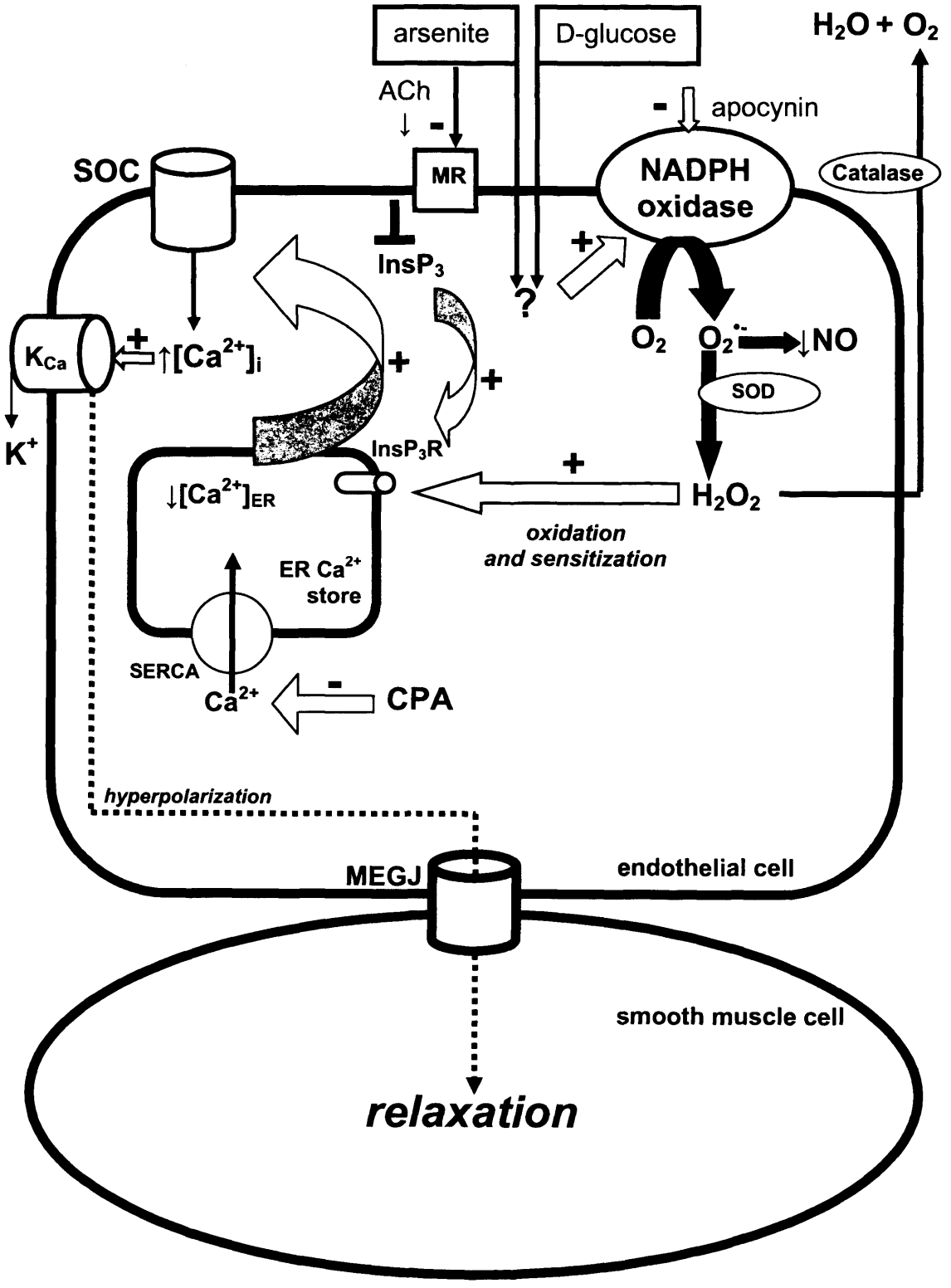
Finally, in comparison to agonist/relaxant-evoked vascular responses there is a distinct lack of studies investigating the modulation of endothelium-dependent dilatations evoked by flow/shear stress, arguably the most significant mode of endothelial stimulation *in vivo*, in models of cardiovascular disease states, which may also provide an important focus for future research. Indeed, the mechanisms contributing to endothelial control of arterial tone, such as the functionally significant sources of H_2O_2 and the relative roles of NO and the EDHF phenomenon, may differ

during shear stress-induced responses from those evoked by agonists and SERCA inhibitors (see sections 1.4.3, 1.5, 1.6 and 7.3).

7.8 Concluding Remarks

This thesis has identified a novel role of H_2O_2 in endothelium-dependent relaxations modulated by arsenite and elevated glucose, whereby this ROS may compensate for the loss of NO by augmenting the EDHF phenomenon. These findings may provide insights into the mechanisms that underpin the augmentation of the EDHF phenomenon in diabetes mellitus and other cardiovascular disease states characterized by oxidative stress and impaired NO bioavailability. Considering that the EDHF phenomenon has been proposed as a novel therapeutic target for the treatment of endothelial dysfunction and microvascular complications, and that angiotensin converting enzyme inhibitors, tetrahydrobiopterin and ascorbic acid may offset endothelial dysfunction via H_2O_2-mediated augmentation of the EDHF phenomenon, further insight into the mechanisms that underpin compensatory EDHF phenomena may provide an important focus for pharmaceutical effort.

Figure 7.1 The role of H_2O_2 in arsenite- and D-glucose-augmented EDHF-type relaxations in the RIA. Augmented EDHF-type relaxations contributed to endothelium-dependent responses that were unaltered overall, indicating that the EDHF phenomenon compensated for impaired NO bioavailability mediated by oxidative stress. The augmented components of relaxation were abolished by the H_2O_2 scavenger catalase or the NADPH oxidase inhibitor apocynin. H_2O_2-augmented EDHF-type relaxations in this artery are mediated by enhanced endothelial Ca^{2+} mobilization, which likely involves sensitization of the $InsP_3$ receptor (Edwards *et al.*, 2008). Note that the augmentatory effect of arsenite on EDHF-type relaxations is masked when these responses are evoked by ACh, indicating that this agent may inhibit the muscarinic receptor. Abbreviations: CPA, cyclopiazonic acid; ER, endoplasmic reticulum; $InsP_3R$, inositol 1,4,5-trisphosphate receptor; K_{Ca}, calcium-activated K^+ channel; MEGJ, myoendothelial gap junction; MR, muscarinic receptor; SERCA, sarcoendoplasmic reticulum Ca^{2+} ATPase; SOC, store-operated Ca^{2+} channel; SOD, superoxide dismutase.


arsenite
D-glucose
H2O + O2
ACh
apocynin
SOC
MR
NADPH oxidase
Catalase
InsP3
?
KCa
↑[Ca2+]i
O2
O2
↓NO
SOD
K+
InsP3R
↓[Ca2+]ER
H2O2
oxidation and sensitization
ER Ca2+ store
SERCA
Ca2+
CPA
hyperpolarization
MEGJ
endothelial cell
smooth muscle cell
relaxation

APPENDIX

A POTENTIAL LIMITATION FOR THE USE OF CATALASE DURING NO-DEPENDENT ARTERIAL RELAXATION

Introduction

Catalase is widely used pharmacologically for scavenging H_2O_2 and thus dissociating the role of this ROS in cellular signalling mechanisms, including those that mediate endothelium-dependent arterial relaxation (Wolin, 1996; Faraci, 2006). However, a number of reports have suggested that catalase may also reversibly bind NO to form ferrous-nitrosyl complexes, thus questioning its use as a specific H_2O_2 scavenger in biological systems where NO also features as an important signalling molecule (Brown, 1995; Kim & Han, 2000; Brunelli *et al.*, 2001). Indeed, ferrous interactions underpin the principal NO signal transduction mechanism, e.g. activation of sGC (Cooper, 1999; Koesling, 1999). Furthermore, it has also been demonstrated that the binding of NO to catalase may inhibit its H_2O_2-scavenging activity (Brown, 1995; Mohazzab *et al.*, 1996; Kim & Han, 2000; Brunelli *et al.*, 2001).

From a functional perspective, Mohazzab *et al* reported that physiologically relevant concentrations of exogenously applied NO (~50 nM) abolished endothelium-independent relaxant responses to H_2O_2 in calf pulmonary arteries by binding to endogenous catalase and inhibiting the formation of compound I (the active form of catalase produced during the metabolism of H_2O_2), a process necessary for activation of sGC and relaxation to H_2O_2 in this artery (Wolin & Burke, 1987; Mohazzab *et al.*, 1996). However, it remains to be determined whether exogenous catalase is capable of attenuating NO-mediated arterial relaxations.

The aim of the current study was to characterize the effect of catalase on endothelium-independent relaxant responses to the NO donor MAHMA NONOate. The main focus was to establish whether the use of catalase during studies of L-NAME-sensitive endothelium-dependent relaxations can conclusively dissociate the role of H_2O_2 in these responses.

Methods

Male New Zealand White rabbits were sacrificed and endothelium-denuded iliac arterial rings prepared for isometric analysis as described previously (2.1 and 2.2). After the successful removal of the endothelium was confirmed (see 2.2) preparations were incubated with catalase (500, 1000 or 2000 units ml^{-1}) for 30 minutes. Tone was induced by PE (1 μM) and allowed to reach a stable plateau (taking approximately 15 minutes) before endothelium-independent NO-mediated relaxant responses were studied by constructing cumulative concentration-response curves to MAHMA NONOate (1 nM – 10 μM). Data was collected and analysed as described in 2.3 and 2.4.

Results

Endothelium-independent relaxations to MAHMA NONOate were maximally equivalent to 93.3±1.4% of the constrictor response to PE with a pEC_{50} value of 7.32±0.06 (n=11; Figure 1). At 500 units ml^{-1}, catalase attenuated relaxation at a single concentration of MAHMA NONOate (100 nM; $P<0.01$) but did not alter either the pEC_{50} value or R_{max} (Figure 1A; Table 1). At 1000 units ml^{-1}, catalase further attenuated relaxation, shifting the concentration-response curve to the right (pEC_{50}: 6.97±0.09; $P<0.05$) but did not alter R_{max} (Figure 1B; Table 1). At 2000 units ml^{-1},

catalase further attenuated relaxation, shifting the concentration-response curve to the right relative to time-matched controls (pEC_{50}: 6.71±0.08; $P<0.001$) and also relative to preparations incubated in the presence of 500 units ml^{-1} catalase ($P<0.05$; Table 1), but did not alter R_{max} (Figure 1C; Table 1).

Figure 1 Mechanical studies of endothelium-denuded rabbit iliac artery rings with catalase and MAHMA NONOate. Concentration-response curves revealed that catalase attenuated endothelium-independent relaxation to MAHMA NONOate in a concentration-dependent manner. *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$ compared to control. Note that controls are common to each graph and the effect of each concentration of catalase illustrated separately for clarity.

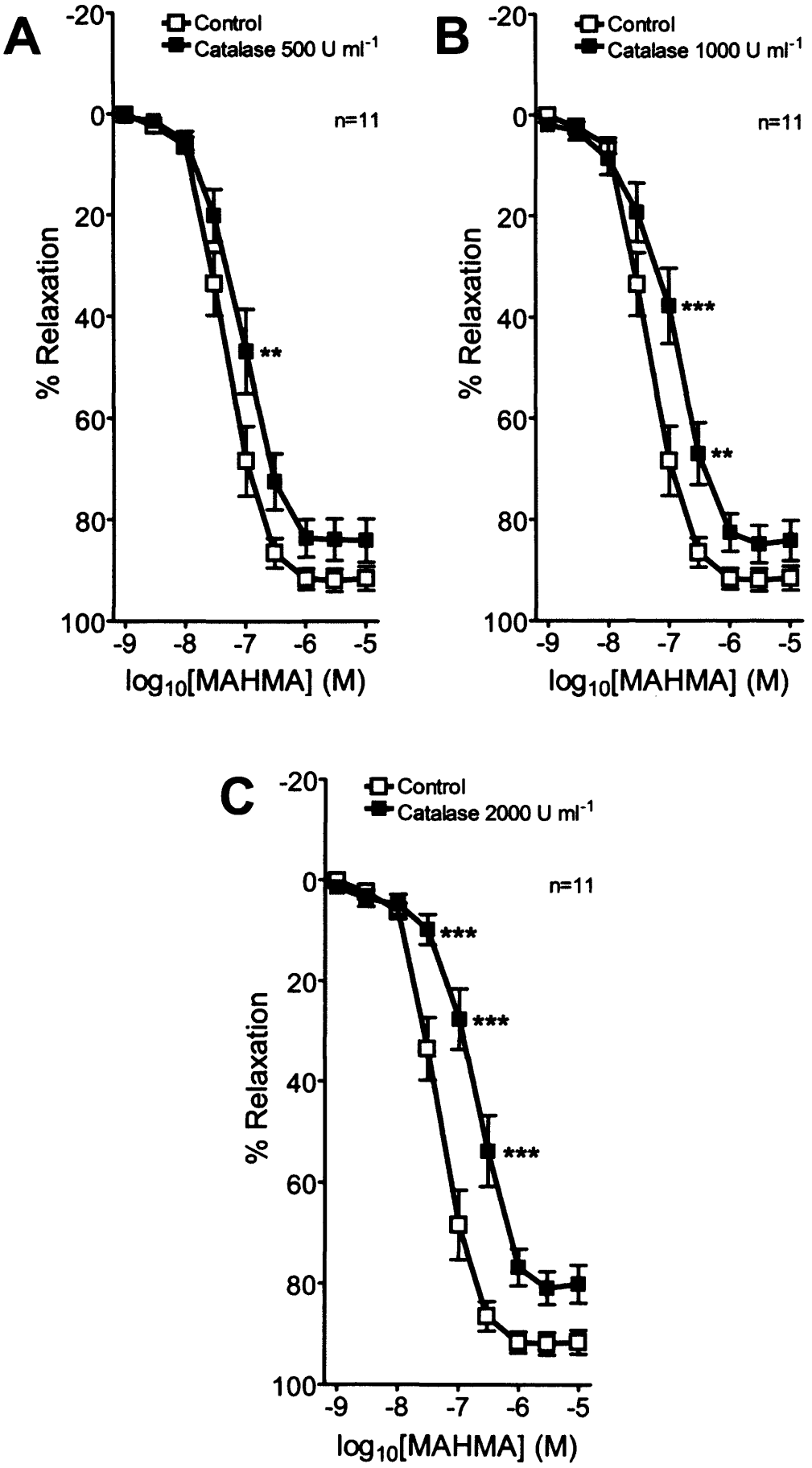

A
Control
Catalase 500 U ml-1
n=11
% Relaxation
log10[MAHMA] (M)
B
Control
Catalase 1000 U ml-1
n=11
% Relaxation
log10[MAHMA] (M)
C
Control
Catalase 2000 U ml-1
n=11
% Relaxation
log10[MAHMA] (M)

Table 1 The effect of catalase on endothelium-independent relaxation to MAHMA NONOate in the rabbit iliac artery. Potency (negative log EC_{50}) and maximal responses (R_{max}) are a function of the constrictor response to PE and given as means±SEM. * and *** denote $P<0.05$ and $P<0.001$ compared to time-matched control preparations. † denotes $P<0.05$ compared to preparations incubated with catalase 500 units ml^{-1}. n denotes the number of animals studied.

Intervention	n	R_{max}	pEC_{50}
Control	11	93.7±1.7	7.32±0.06
Catalase 500 units ml^{-1}		89.2±3.7	7.03±0.10
Catalase 1000 units ml^{-1}		87.0±3.2	6.97±0.09*
Catalase 2000 units ml^{-1}		83.4±2.8	6.71±0.08***†

Discussion

The results of the current study have demonstrated that increasing concentrations of catalase progressively attenuate NO-mediated smooth muscle relaxant responses to MAHMA NONOate in the rabbit iliac artery. These results are consistent with previous reports that catalase binds NO by forming ferrous-nitrosyl complexes (Brown, 1995; Kim & Han, 2000; Brunelli *et al.*, 2001). This additional effect of catalase may be of particular pharmacological relevance during studies of endothelium-dependent arterial relaxations that are sensitive to inhibitory L-arginine analogues.

Catalase has been widely used in organ bath-type experiments to dissociate the role of H_2O_2 in endothelium-dependent relaxant responses, and is generally regarded as a specific scavenger of this ROS (Faraci, 2006). However, the present findings question the sole attribution of H_2O_2 to the effects of catalase. By contrast, previous reports have shown that endothelium-independent relaxant responses to SNP are unaffected by concentrations of catalase that caused attenuation of responses to MAHMA NONOate in the current study (Leung *et al.*, 2006; Capettini *et al.*, 2008). A possible explanation may be because SNP requires intracellular bioactivation to donate NO, whereas MAHMA NONOate (and NONOates in general) release NO spontaneously at physiological pH (Wanstall *et al.*, 2001). Thus, NO released by SNP and MAHMA NONOate may be differentially susceptible to extracellular interactions with catalase. However, Capettini *et al* reported that, in addition to SNP, catalase (at 2400 units ml^{-1}) did not alter relaxant responses of the mouse aorta to diethylamine (DEA) NONOate (Capettini *et al.*, 2008), whereas relaxations to MAHMA NONOate in the current study were progressively attenuated by catalase at concentrations $\geq$500 units ml^{-1}. Furthermore, relaxant responses to DEA NONOate are unaltered in aortae of

catalase-overexpressing mice (Suvorava *et al.*, 2005). A possible explanation may be that the affinity of catalase for NO increases in the presence of H_2O_2 (Brown, 1995; Brunelli *et al.*, 2001). Thus, the basal levels of H_2O_2 present in such bioassays may determine the extent of NO binding by catalase. Indeed, overexpression of catalase in RINm5F cells protects against NO toxicity induced by the NO donors S-nitroso-N-acetyl penicillamine (SNAP) and 5-amino-3-(4-morphonilyl)-1,2,3-oxadiazolium chloride (SIN-1), which also release $O_2^{\bullet-}$ intracellularly that is later dismutated to H_2O_2, but not DEA NONOate which donates NO but does not release ROS (Tiedge *et al.*, 1999).

In conclusion, the current study has demonstrated that catalase may be capable of binding NO in arterial organ bath-type experiments, thus reducing its bioavailability to smooth muscle. As a result, the use of catalase during the course of this thesis to dissociate the role of H_2O_2 in endothelium-dependent relaxant responses in the rabbit iliac artery was restricted to those insensitive to L-NAME.

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PUBLICATIONS IN PROGRESS

1. Ellinsworth D. C., Edwards, D. H., Griffith, T. M (2010)

Hydrogen Peroxide Underpins the Potentiation of the EDHF Phenomenon by Inorganic Arsenic

2. Ellinsworth D. C., Edwards, D. H., Griffith, T. M (2010)

Hydrogen Peroxide Mediates the Augmentation of the EDHF Phenomenon by Glucose

INVITED SPEAKER

June 16th 2008

Cardiovascular Sciences Interdisciplinary Research Group (CVS-IRG) Seminar Series

Sir Geraint Evans Wales Heart Research Institute

School of Medicine

Heath Park

Cardiff University

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"The Comparative Effects of Inorganic Arsenic and Glucose on Endothelium-Dependent Vasorelaxation"

November 23rd 2007

22nd Annual Postgraduate Research Open Day

School of Medicine

Heath Park

Cardiff University

Cardiff CF14 4XN

"Inorganic Arsenic Potentiates NO/Prostanoid-Independent Vasorelaxation via Activation of NADPH Oxidase and Production of Reactive Oxygen Species (H_2O_2)"

AWARD

Sion Rhodri Enyon Evans Bequest Prize (June 2008)