THE EFFECTS OF LYSOPHOSPHOLIPIDS AND OXIDISED LOW-DENSITY LIPOPROTEINS ON THE L-ARGININE : NITRIC OXIDE PATHWAYS IN ISOLATED RABBIT AND RAT AORTA

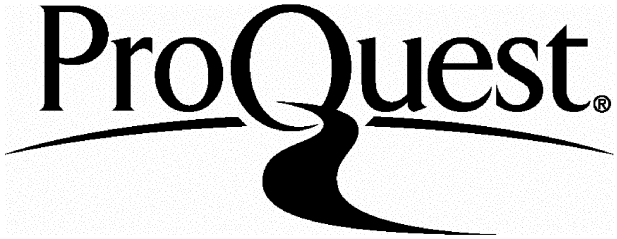
Paul Martin Kerr

Thesis submitted for the degree of Doctor of Philosophy

in the University of London

(Royal Free Hospital School of Medicine)

March 1993



ABSTRACT

Elevated plasma levels of low-density lipoproteins (LDL) are a major risk factor for the development of atherosclerosis. Atherosclerotic vessels from humans and animals show impaired endothelium-dependent relaxations (EDR) and altered responses to contractile agonists. Recent evidence suggests that oxidation of LDL is a key process in atherogenesis and plays an important role in the alterations in vascular reactivity. This study investigated the effects of a major component of oxidised LDL (OXLDL), lysophosphatidylcholine (LPC), on vascular responses in isolated rabbit and rat aortic rings. In addition, the effects of OXLDL on EDR in rat aortic rings was also examined. Furthermore, the possibility that impaired EDR in atherosclerotic vessels could be restored by L-arginine was investigated.

LPC caused immediate, dose dependent and partially reversible inhibition of EDR evoked by ACh, ATP and A23187 in rabbit aortic rings. This inhibition was decreased by serum albumin but not by L-arginine or indomethacin. Relaxations to exogenous NO and glyceryl trinitrate in endothelium-denuded tissues were unaffected by LPC, but responses were inhibited in endothelium-intact rings suggesting the release of an inhibitory factor from the endothelium. LPC also evokes EDR which are mediated by the release of NO. This dual effect of LPC can be demonstrated in the same tissue. Contractile responses to phenylephrine (PE) and 5-HT were unaffected in denuded tissues, but were inhibited in endothelium-intact tissues, again suggesting the release of a factor from the endothelium.

Relaxations evoked by L-arginine in isolated rat aortic rings were mediated by an inducible nitric oxide synthase (NOS). At a concentration that inhibited relaxations elicited by ACh, OXLDL did not influence L-arginine-evoked

relaxations, whereas LPC potentiated responses. NOS activity induced *in vivo* by endotoxin injection, was studied *ex vivo* by observation of PE-evoked contractions. Contractions were attenuated in rings from endotoxin-treated rats. However, the effect of this treatment was unaffected by the presence of OXLDL or LPC.

Aortic rings from WHHL rabbits which spontaneously develop atherosclerosis, showed impaired EDR. This impairment was not influenced by incubation of the tissues with L-arginine. Furthermore, rabbits fed a diet supplemented with L-arginine did not show improved endothelium-dependent responses *in vitro*. In addition, L-arginine feeding did not influence contractile responses to PE.

In conclusion, LPC can modulate EDR and contractile responses in isolated tissues although, these effects do not mimic those reported for OXLDL. In contrast, OXLDL and LPC do not inhibit the activity of an inducible form of NOS. Finally, the impairment of EDR observed in atherosclerotic vessels cannot be reversed by the administration of L-arginine.

ACKNOWLEDGEMENTS

I would like to thank the following people for their help during my Ph.D. :

Firstly, my supervisor, Dr. Mike Jacobs.

Those who gave blood, and Vernon for taking it.

The various members of the CBU whom I have pestered over the last three years.

Dr. Anwar Baydoun for the L-arginine measurements.

Finally, to my dearest Frances for her valued advice, and for being there from beginning to end.

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ABBREVIATIONS

ACAT	acyl CoA:cholesterol acyltransferase
ACD	acid citrate dextrose
ACh	acetylcholine
ADP	adenosine 5'-triphosphate
ANP	atrial naturietic peptide
apoB-100	apolipoprotein B-100
ATP	adenosine triphosphate
BK	bradykinin
BSA	bovine serum albumin
Ca^{2+}	calcium
cGMP	3'5'-cyclic guanosine monophosphate
Cu^{2+}	copper
EDHF	endothelium-derived hyperpolarising factor
EDNO	endothelium-derived nitric oxide
EDR	endothelium-dependent relaxation
EDRF	endothelium-derived relaxing factor
EDTA	ethylenediaminetetra acetic acid
FH[IIa]	familial hypercholesterolemia type IIa
GTN	glyceryl trinitrate
HDL	high-density lipoproteins
HMG CoA	3-hydroxy-3-methylglutaryl CoA
H_2O_2	hydrogen peroxide
5-HT	serotonin (5-hydroxytryptamine)
IP_3	inositol 1,4,5-trisphosphate
LCAT	lecithin:cholesterol acyltransferase

L-NMMA	L-N^G-monomethyl-arginine
L-NAME	L-nitro-L-arginine-methyl ester
LPC	lysophosphatidylcholine
MCP-1	monocyte chemotactic protein-1
NA	noradrenaline
NOS	nitric oxide synthase
NZW	New Zealand White
O_2^-	superoxide anion
OXLDL	oxidised low-density lipoproteins
PAF	platelet-activating factor
PC	phosphatidylcholine
PDGF	platelet-derived growth fctor
PE	phenylephrine
PGI_2	prostacyclin
PLA_2	phospholipase A_2
PLA_2-LDL	phospholipase A_2-treated LDL
PUFA	polyunsaturated fatty acid
S.E.M.	standard error of the mean
SOD	superoxide dismutase
VLDL	very low-density lipoproteins
WHHL	Watanabe heritable hyperlipidemic

CHAPTER ONE

GENERAL INTRODUCTION

1.1 ATHEROSCLEROSIS

Atherosclerosis is a vascular disease that is recognised to be a major cause of death in the United States and Western Europe but its origins and pathogenesis remain unclear. The disease involves complex cascades of interactions between environmental and biological factors which progress insidiously for many years before the clinical manifestations of myocardial and cerebral infarction and thrombosis are apparent.

1.1.1 MORPHOLOGY OF ATHEROSCLEROTIC LESIONS

(Reviewed by Steinberg *et al.*, 1989; Steinberg and Witztum, 1990; Woolf, 1990)

In morphological terms, atherosclerosis is characterised by patchy thickenings of the arterial intima, comprised of accumulations of fat and fibrous tissue, which can cause occlusion of the vessel.

In animal models of atherosclerosis, one of the earliest events is the adherence of monocytes to the endothelial surface followed by their migration into the sub-endothelial space (Gerrity, 1981a; 1981b; Joris *et al.*, 1983). The monocytes differentiate into macrophages, rapidly internalise cholesteryl esters derived from low-density lipoproteins (LDL) and become converted to "foam cells" characteristic of the first macroscopically recognisable lesion called fatty streaks (Ross and Glomset, 1976a). The age at which fatty steaks appear differs in different regions of the arterial tree, but they may be present in the aorta by 10 years of age (see Ross and Glomset, 1976a).

Development of the fatty streak is accompanied by the formation of

microthrombi in the vessel wall, accumulation of collagen-like fibres and limited migration of intimal smooth muscle cells into the sub-endothelial space (Geer, 1965). It has been reported that the endothelium undergoes morphological changes in responses to cholesterol feeding (Ingerman-Wojenski *et al.*, 1983) although other studies have suggested that the development of the fatty streak occurs under a morphologically intact endothelium (Davies *et al.*, 1976; Bondjers *et al.*, 1977; Taylor *et al.*, 1989)

As the fatty streak becomes established, smooth muscle cells become the predominant cell type, their proliferation a key event that determines how extensive the plaques will become. In addition to multiplying, smooth muscle cells are the source of much of the connective tissue matrix which is a major component of fibrous plaques and later lesions (Burk and Ross, 1979). The release of cytokines (Hansson *et al.*, 1989) and growth factors such as platelet-derived growth factor (PDGF; Ross *et al.*, 1974), stimulates cell proliferation and growth of the lesion. Further development of the plaque involves the formation of a fibrous cap covering the lesion under which calcification and crystalisation of cholesterol deposits may occur. This type of lesion often becomes associated occlusive disease. Eventually, rupture of the plaque due to necrosis of connective tissue at the base, may result in the release of cytotoxic components into the circulation, possibly leading to thrombosis (Falk, 1983; Davies and Thomas, 1984).

The distribution of atherosclerotic lesions is focal with the highest incidence occuring in areas where blood flow is slowed, such as branch points (Goldstein *et al.*, 1983; Packham and Mustard, 1986), where turbulance and alterations in shear stress may encourage endothelial damage and increase permeability of the vascular wall to lipid (Caro *et al.*, 1971; Zarins *et al.*, 1983).

1.1.2 RISK FACTORS FOR THE DEVELOPMENT OF ATHEROSCLEROSIS

The proposed role of hypercholesterolemia as a risk factor for development of atherosclerosis in general and ischaemic coronary heart disease is supported by a wealth of clinical, epidemiological, and pathological studies (see Tyroler, 1987b). Futhermore, intensive lipid-lowering regimes have been shown not only to slow the progression of coronary atherosclerosis and reduce the risk of coronary events (Tyroler, 1987a), but even in some cases to lead to absolute regression (Blankenhorn *et al.*, 1987).

Elevated plasma levels of LDL, the major carrier of cholesterol in the plasma, have been shown in many clinical and experimental studies to accelerate the development of atherosclerosis (Goldstein and Brown, 1977; Steinberg, 1983; Faggiotto and Ross, 1984; Faggiotto *et al.*, 1984; Tyroler, 1987a; 1987b) and the cholesterol which accumulates in atherosclerotic lesions is derived from circulating LDL (Newman and Zilversmith, 1962). The role of LDL in the development of atherosclerosis is most clearly defined in individuals with the genetic disorder Familiar Hypercholesterolemia Type IIa [FH (IIa)]. These patients have defective or absent LDL receptors leading to extremely elevated plasma levels of cholesterol and LDL and they develop premature cardiovascular disease (Goldstein and Brown, 1989). There now exists several genetic variants in animal models of atherosclerosis that closely resemble the human condition of FH IIa, which will be dicussed in more detail later (section 1.6), that further support the atherogenic role of elevated plasma LDL.

However, hypercholesterolemia is not the exclusive causative factor of atherosclerosis, as in patients with FH IIa, the expression of the disease varies between individuals and even within the same kindred (Piper and Orrild,

1956). Other factors have also been implicated in the progression of atherosclerosis notably hypertension (Roberston and Strong, 1968), cigarette smoking (Stamler, 1979), and diabetes (Kannel and McGee, 1979), although some minimum degree of hypercholesterolemia may be a pre-requisite before other factors become clinically important (Steinberg and Witztum, 1990).

1.1.3 THEORIES OF THE DEVELOPMENT OF ATHEROSCLEROSIS

Atherosclerosis is unlikely to be a single disease entity and there may be a variety of pathways leading to lesions that at the endpoint appear very much alike. As the endothelium provides a barrier between circulating blood and the arterial intima, it is hypothesised that loss of the endothelium is the initiating event in atherogenesis - the "Response to Injury" hypothesis (Ross and Glomset , 1976a; 1976b; Ross, 1986; Taylor *et al.*, 1990). This theory compares the formation of atherosclerotic lesions to the inflammatory response of arteries to mechanical endothelial denudation (Stemerman and Ross, 1972), and emphasises the role of PDGF released from aggregated platelets at injured sites which promotes the proliferation of smooth muscle cells (Ross *et al.*, 1974). Subsequent studies have demonstrated that atheroma develop under a morphologically intact endothelium (Davies *et al.*, 1976; Bondjers *et al.*, 1977; Faggiotto and Ross, 1984; Taylor *et al.*, 1989). However, endothelial morphology and structural changes following diet-induced hypercholesterolemia are claimed to preceed the lipid accumulation of initial lesions (reviewed by Taylor *et al.*, 1990).

Another hypothesis is the "Lipid Infiltration" theory of atherogenesis which focuses on the role of hypercholesterolemia alone in inducing the disease (reviewed by Steinberg, 1987). This theory suggests that cholesterol-carrying lipoproteins induce or favour the progression of atherosclerosis as a result of

increased uptake into the sub-endothelial space or, by causing endothelial damage.

These two hypotheses can now be regarded as complementary parts of the "Unified Hypothesis" (Steinberg, 1983; 1987) which recognises that an atheroma is extremely complex in structure and could be generated by different reaction sequences. Damage to the endothelium could result in increased rate of penetration resulting in the initiation of reactions proposed for both pathways. It provides links between the "Lipid Infiltration" theory, which may account for the development of fatty streaks, and the "Response to Injury" hypothesis, which may account for the progression to more advanced lesions (**Figure 1**). Recent reviews (Steinberg, 1989; 1990; Steinberg and Witztum, 1990; Witztum and Steinberg, 1991) have stressed that oxidatively modified LDL can play a part in atherogenesis in ways that need not be directly related to its role in lipid deposition, and will be discussed in section 1.5.1.

The processes of atheroma development are still a matter for debate, but early events include the migration of circulating monocytes into the vessel wall and subsequent differentiation into macrophages and accumulation of lipid to form foam cells, and endothelial dysfunction or damage. Smooth muscle proliferation and adherence and aggregation of platelets mark the progression of the lesion. The following sections will describe the physiological functions of lipoproteins and the vascular endothelium. The interactions of these two aspects of vascular biology in the initiation and progression of atherosclerosis will then be discussed.

1.2 THE PLASMA LIPOPROTEINS

Blood lipids are packaged into soluble protein complexes called lipoproteins,

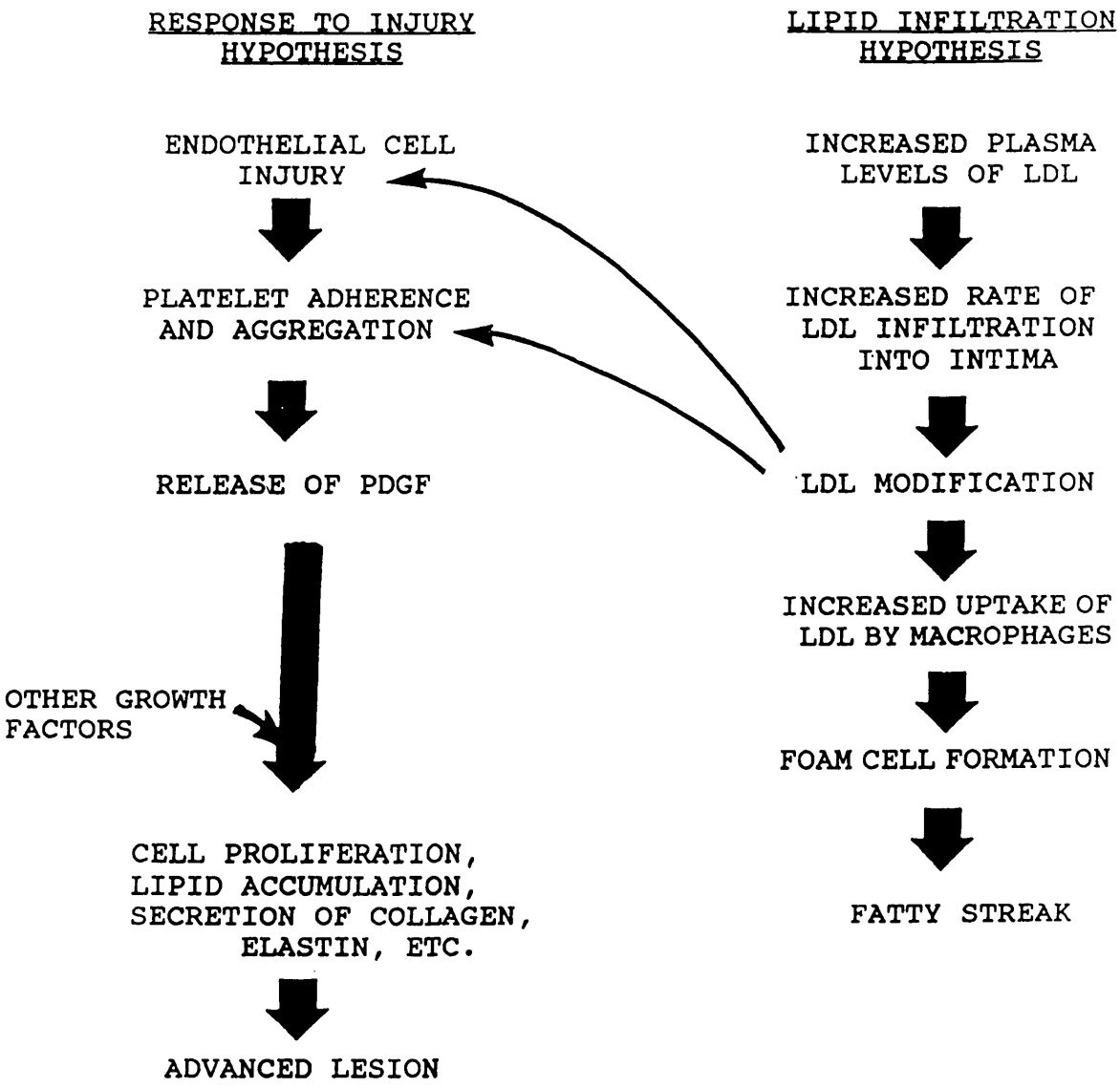

Figure 1 : The unified hypothesis of atherosclerosis
Interactions between "Response to Injury" and "Lipid Infiltration" hypothesis. (Adapted from Steinberg, 1989).

which transport essential cholesterol and triglyceride between the liver and extra-hepatic tissues. The four major classes of lipoproteins are LDL, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), and high-density lipoproteins (HDL). However, only LDL, the most abundant cholesterol-carrying lipoproteins in human plasma will be discussed in this thesis.

Plasma lipoproteins have essentially two functions:

(i) To supply cholesterol to extra-hepatic cells to satisfy the requirement of membrane synthesis and steroidogenesis.

(ii) To transfer cholesterol from cells to the liver for excretion.

1.2.1 STRUCTURE AND COMPOSITION OF LIPOPROTEINS

(Reviewed by Scanu and Spector, 1986)

Plasma lipoproteins have a common structural organisation, which is essentially an oily droplet composed of cholesterol esters and triglycerides, solubilised by a surface monolayer of phospholipid, such as phosphatidylcholine (PC), phosphatidylethanolamine and sphingomyelin, unesterfied cholesterol, and stabilised by protein. The protein constituents, called apoproteins (apo), differ between the classes of lipoprotein and regulate the metabolism of the lipoproteins by modulating the activity of a number of enzymes and acting as specific receptor ligands to mediate uptake into cells. For example, apo-B, the major or exclusive protein constituent of LDL and a major component of VLDL, suppresses the activity of 3-hydroxy-3-methylglutaryl co-enzyme A reductase (HMG-CoA reductase; Yang *et al.*, 1986), the rate limiting enzyme in cholesterol biosynthesis.

Each LDL particle has a single molecule of apo-B-100, an amphipathic, glycosylated protein with a relative molecular mass of 513-14 kDa (Yang *et al.*,

1986).

The major phospholipid component of LDL is phosphatidylcholine, commonly termed "lecithin" (Scanu and Spector, 1986). Position *sn*-1 in phosphatidylcholine is generally occupied by saturated fatty acids, mainly palmitic acid, while position *sn*-2 contains PUFAs, such as linoleic and arachidonic acid (Scanu, 1979). However, linoleic acid is predominately bound in the cholesterol esters, whereas arachdonic acid is mostly bound to the phospholipid (Esterbaurer *et al.*, 1990). LDL also contains antioxidants, such as vitamin E (α-tocopherol), the most abundant, and β-carotene (Esterbauer *et al.*, 1989).

1.2.2 METABOLISM OF LIPOPROTEINS

There are two separate pathways mediating the metabolism of cholesterol of dietary and hepatic origin as shown in **Figure 2** (Reviewed by Goldstein *et al.*, 1983; Scanu and Spector, 1986).

The exogenous pathway concerns the transport of dietary triglycerides and cholesterol esters to the liver and the endogenous pathway mediates the transport of cholesterol between the liver and extra-hepatic tissues.

1.2.3 RECEPTOR-MEDIATED ENDOCYTOSIS OF LDL

Receptor-mediated endocytosis is the term applied to the internalisation of surface membrane receptors following binding of a specific ligand. This is the mechanism for uptake of LDL particles from plasma (Reviewed by Brown and Goldstein, 1986). The existence of LDL receptors was first demonstrated in fibroblasts (Brown and Goldstein, 1974; Goldstein and Brown, 1974) and they were subsequently found on most mammalian cells, although the expression of the receptor varies with the cholesterol requirements of the cell. The LDL receptor is a cell surface single-chain transmembrane glycoprotein comprising

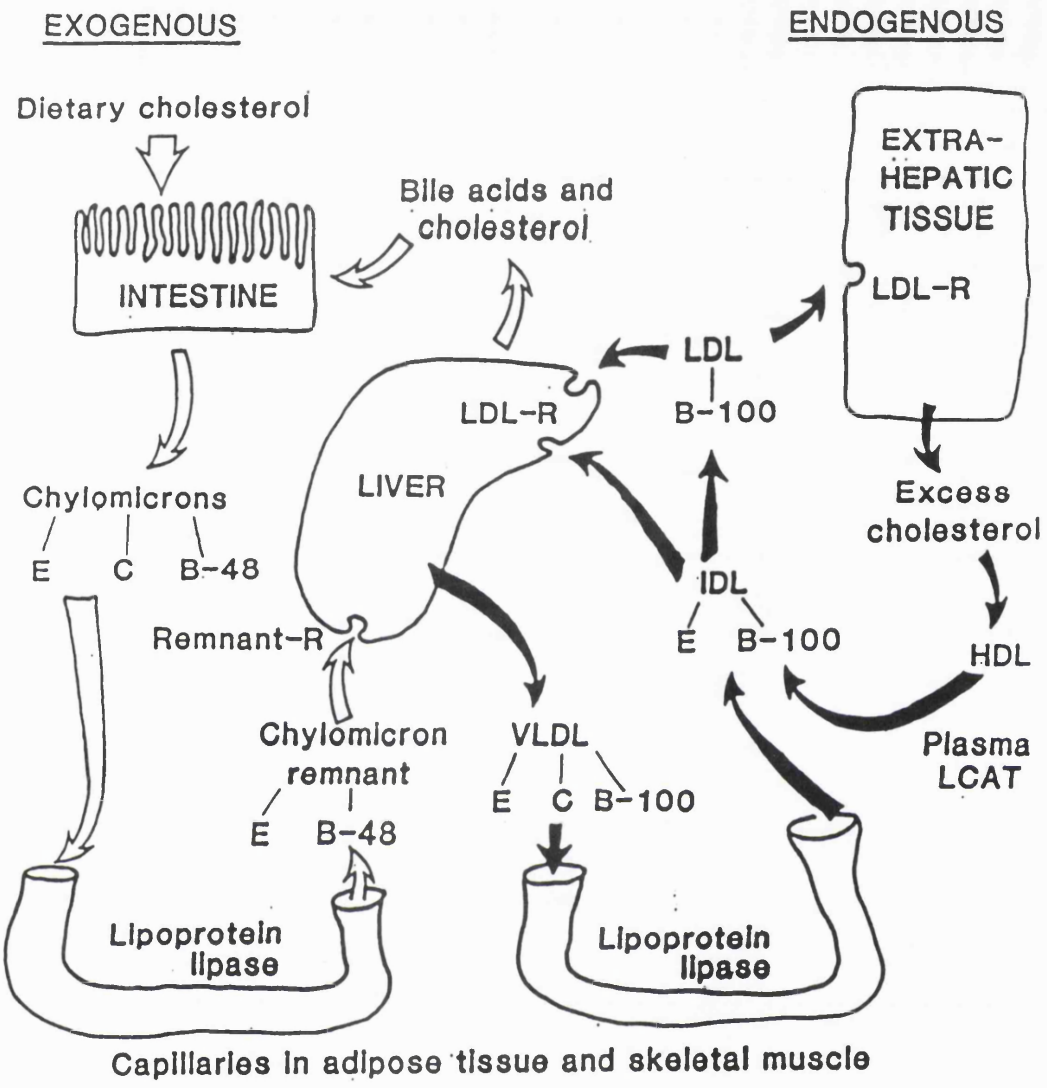

Figure 2 : The exogenous and endogenous pathways of lipoprotein metabolism

Abbreviations **VLDL**, very low-density lipoproteins; **IDL**, intermediate-density lipoproteins; **LDL**, low-density lipoproteins; **HDL**, high-density lipoproteins; **LDL-R**; LDL receptor; **Remnant-R**; remnant receptor; **LCAT**; lecithin:cholesterol acyltransferase.

of 5 distinct structural domains (Russell *et al.*, 1984; reviewed by Catapano, 1989), and which is specific for lipoproteins containing apo-B (LDL) or apo-E (VLDL).

Following LDL binding to the receptor, the complex is localised in clathrin-coated pits (Anderson *et al.*, 1982) which invaginate, forming coated vesicles, and fuse with one another to become endosomes. The receptor-ligand complex dissociates and the LDL is carried to lysosomes, where the apo-B moiety is broken down by proteases, the cholesterol ester component is hydrolysed and the free cholesterol is liberated for metabolic purposes (Brown and Goldstein, 1986). The receptor is recycled back to the plasma membrane, with each round trip taking about 10 mins (Goldstein *et al.*, 1979b).

Free cholesterol stimulates cholesterol esterification (Goldstein *et al.*, 1974), suppresses the activity of HMG-CoA reductase (Goldstein and Brown, 1973) and down regulates the transcription of the LDL-receptor gene (Brown *et al.*, 1975; Russell *et al.*, 1983). These negative feedback mechanisms act to protect against excessive accumulation of cholesterol or cholesteryl esters by a cell.

Receptor-independent, low affinity, non-saturable binding sites also take up LDL by a combination of fluid and absorptive endocytosis (Spady *et al.*, 1987). However, *in vivo* studies have shown that 2/3 of LDL clearance normally occurs through the high affinity receptor (Goldstein and Brown, 1977; Shepherd *et al.*, 1979; Thompson *et al.*, 1981) and under conditions of high plasma cholesterol, LDL accumulates in the plasma rather than being cleared and degraded by cells.

1.3 THE VASCULAR ENDOTHELIUM

The vascular endothelium forms a uniform layer of cells in all blood vessels, heart chambers and lymphatics of the body. Until quite recently, the

endothelium was thought of as a protective, nonadherent surface able to transport various substances from or into the bloodstream (Ross and Glomset, 1976a). However, it is now recognised that vascular endothelial cells participate in a number of homeostatic and cellular functions such as blood coagulation, the activity of leukocytes, the reactivity of platelets and regulation of vascular smooth muscle tone.

The various metabolic and homeostatic functions of the endotheliun will be briefly outlined and the biochemical and physiological properties of endothelium-derived relaxing factor (EDRF) will then be discussed in detail.

1.3.1 MORPHOLOGY OF THE ENDOTHELIUM

The vascular endothelium is a single, continuous layer of thin, flattened, rhomboidal cells, orientated in the direction of blood flow (Chambers and Zweifach, 1947). The luminal surface of this layer is covered by a glycoprotein coat, making a complex of projections and caveolae which greatly increase the surface area and promote the formation of a cell free layer of plasma over the endothelial surface. These structures also provide a wide range of specialised microenvironments which facilitate the binding, transport and processing of circulating molecules (reviewed by Ryan, 1986; Simionescu and Simionescu, 1986).

Junctions between endothelial cells range from "tight", as found in cerebral blood vessels (Betz and Goldstein, 1986), to "fenestrated", as in the glomerular capillaries of the kidney (Staehelin and Hull, 1978). Gap junctions of "myo-endothelial bridges" also exist between the endothelium and underlying smooth muscle cells and may be important for interactions between these two cell layers (Spagnoli *et al.*, 1982).

1.3.2 SYNTHETIC AND METABOLIC FUNCTIONS OF THE

ENDOTHELIUM

The endothelium synthesises a large number of vasoactive substances such as prostacyclin (PGI_2; Moncada *et al.*, 1976), adenosine (Pearson and Gordon, 1985) and endothelin (Yanagisawa *et al.*, 1988). The production of these substances is modulated by changes in the concentration of intracellular messengers such as Ca^{2+} and cyclic adenosine monophosphate.

Endothelial cells also have an important metabolic function with respect to vasoactive substances (Gerlach *et al.*, 1985) although activity differs between vessels. For example, PGI_2 is metabolised via 15-hydroxyprostaglandin dehydrogenase in the aorta (Sun and Taylor, 1978) but not in the pulmonary vasculature (Dusting *et al*, 1978). The lungs contain approximately half of the endothelial cells present in the body and, as they receive the entire cardiac output, they are central to maintaining and regulating the delivery of active substances to target organs (Vane, 1964; Bakhle and Vane, 1974).

The metabolic functions of the endothelium are fulfilled by a wide variety of enzymes present in the cells. For instance, the outer surface contains angiotensin-converting enzyme (Ryan *et al.*, 1976) which catalyses the formation of the potent vasoconstrictor angiotensin II from the relatively inactive precursor angiotensin I. The same enzyme also inactivates the vasodilator bradykinin (BK). Endothelial monoamine oxidases (MAO) breakdown biogenic amines such as serotonin (5-HT) and noradrenaline (NA; reviewed by Shepro and Durham, 1986).

1.3.3 THE ENDOTHELIUM AND BLOOD COAGULATION

In relation to the soluble coagulation system, the endothelium has both pro- and anti-coagulant roles (reviewed by Fajardo, 1989). Platelet activity is inhibited by the presence of an intact endothelium and both PGI_2 and EDRF

released from endothelial cells are potent anti-aggregatory agents (Moncada *et al.*, 1976; Palmer *et al.*, 1987; Radomski *et al.*, 1987a). EDRF also prevents the adhesion of platelets to the endothelial cell surface (Radomski *et al.*, 1987b).

However, platelets can adhere to the basal lamina and stroma which may become exposed if the endothelium becomes detatched (Povlishok and Rosenblum, 1988) leading to aggregation and the release of vasoactive substances such as adenosine nucleotides (ADP and ATP), thromboxane A_2 and PDGF (Jaffe, 1987). Furthermore, many of the factors involved in the initiation of coagulation can be synthesised by damaged endothelial cells (Jaffe *et al.*, 1974; Colucci *et al.*, 1983) and so promote clot formation in response to injury.

Thus, an intact endothelium presents both a physical and chemical barrier between circulating platelets and the pro-aggregatory matrix of the intimal layers of the vessel wall.

1.4 ENDOTHELIUM-DERIVED RELAXING FACTOR

(Reviewed by Furchgott, 1983; 1984; 1990; Vanhoutte and Houston, 1985; Vanhoutte *et al.*, 1986)

The paradox that acetylcholine (ACh), a potent hypotensive agent *in vivo* (Shepherd, 1963; Mellander and Jonansson, 1968), did not relax vascular preparations *in vitro*, was resolved when it was demonstrated that ACh-induced vascular relaxation was dependent on the presence of the endothelium (Furchgott and Zawadzki, 1980). It was proposed that the endothelial cells of blood vessels stimulated with ACh released a substance, called EDRF, which diffuses to the underlying smooth muscle to cause relaxation.

The essential role of EDRF in mediating endothelium-dependent relaxation (EDR) induced by a number of substances is now well documented. These substances include adenosine nucleotides (Furchgott, 1983), 5-HT (Cocks and Angus, 1983), substance P (Furchgott, 1983), A23187 (Furchgott, 1983), vasoactive intestinal polypeptide (Davies and Williams, 1984), calcitonin gene-related peptide (Brain *et al.*, 1985), BK (Cherry *et al.*, 1982) and melittin (Förstermann and Neufang, 1985). The endothelium is now recognised as playing a very important role in the regulation of vascular smooth muscle tone. However, many agents only elicit EDR in specific vessels and may cause contraction or endothelium-independent relaxations in other preparations. For example, 5-HT evokes endothelium-independent contraction of many isolated blood vessels but can only elicit EDR in canine (Cocks and Angus, 1983) and porcine (Houston *et al.*, 1985) coronary arteries.

In addition to chemical stimuli, EDR can be evoked by hypoxia (Pöhl and Busse, 1989), increases in flow (Rubanyi *et al.*, 1986), and electrical stimulation (Frank and Bevan, 1983).

The humoral, non-prostanoid nature of EDRF was first demonstrated using pharmacological techniques in which the biologically active substance was transferred from a donor to a detector bioassay by use of a sandwich preparation (Furchgott and Zawadzki, 1980). Indomethacin, a cyclooxygenase inhibitor, did not influence the activity of this factor, thus ruling out a role for prostanoids in these responses to ACh.

Subsequently, other more elegant approaches were devised to study the effects of physical and chemical manipulation on the generation, stability and actions of EDRF. These included superfusion bioassay cascades, in which the perfusate from either intact vessels (Förstermann *et al*, 1984; Griffith *et al.*,

1984a; 1984b; Rubanyi *et al.*, 1985) or vascular endothelial cells cultured on microcarrier beads (Cocks *et al.*, 1985; Grylewski *et al.*, 1986) induced relaxation of endothelium-denuded arterial rings or strips.

It was established, using techniques such as these, that EDRF was a very short-lived substance with a half-life of 3-6 secs in oxygenated physiological salt solutions (Förstermann *et al.*, 1984; Griffith *et al.*, 1984a; 1984b). Superoxide anions (O_2^-) may contribute to the instability of EDRF, as the effects of EDRF were prolonged by the addition of superoxide dismutase (SOD; Gryglewski *et al.*, 1986; Rubanyi and Vanhoutte, 1986).

Relaxation of vascular smooth muscle by EDRF is mediated by increases in intracellular levels of cyclic guanosine monophosphate (cGMP; Holzmann, 1982; Diamond and Chu, 1983; Rapoport and Murad, 1983b), possibly through binding to the haem moiety of the enzyme soluble guanylate cyclase (Ignarro, 1989a; 1989b). Increases in smooth muscle cGMP produce relaxation presumably by reduction of free intracellular Ca^{2+} (Schini *et al.*, 1987). Several mechanisms have been proposed to explain cGMP-evoked relaxations: (1) inhibition of inositol trisphosphate (IP_3) generation (Rapoport, 1986); (2) stimulation of the intracellular Ca^{2+} sequestration (Lincoln, 1983); (3) decrease of the myosin light chain phosphorylation (Rapoport *et al.*, 1983); (4) inhibition of receptor operated Ca^{2+} channels (Godfraind, 1986); (5) activation of cGMP-dependent protein kinases (Popescu *et al.*, 1985); (6) stimulation of membrane Ca^{2+}-ATPase (Fiscus, 1988); (7) increased K^+ permeability through K^+ channels causing membrane hyperpolarisation (Komori and Suzuki, 1987).

Methylene blue, an inhibitor of soluble or cytosolic guanylate cyclase (Murad *et al.*, 1978; Gruetter *et al.*, 1980), antagonises EDR of arteries and

veins and the associated increase in cGMP (Holzmann, 1982; Martin *et al*, 1985a; 1985b). Haemoglobin also inhibits vascular relaxation and increases in cGMP increases (Martin *et al.*, 1985a; 1985b). However, the interpretation of these results is complicated by the complex properties of these compounds. For example, haemoglobin can bind EDRF (Martin *et al.*, 1986b), inhibit activation of soluble guanylate cyclase (Murad *et al.*, 1978; Martin *et al.*, 1985a; 1985b) and generate O_2^-, which would directly inactivate EDRF (Misra and Fridovitch, 1972). Methylene blue can also generate free radicals (McCord and Fridovitch, 1970; Wolin *et al.*, 1990).

1.4.1 PHYSIOLOGICAL ROLE OF EDRF

(Reviewed by Moncada *et al.*, 1989; 1991a; Feng and Hedner, 1990a; 1990b)

Since the discovery of EDRF, the role the endothelium, and this substance, play in the mechanisms of autoregulation of blood pressure and blood flow to specific organs has been examined. The endothelial cells of blood vessels may act as transducers of several physical and chemical stimuli that modify vascular tone through the release of endothelial factors.

The physiological stimuli for EDRF release *in vivo* are unclear although many have been proposed from *in vitro* studies including changes in oxygen tension, changes in flow, thrombin, platelet products, such as 5-HT and ADP, and neurotransmitters, such as NA and ACh (Reviewed by Furchgott, 1983; Martin *et al.*, 1986a; Vanhoutte *et al*, 1986). Once released EDRF probably acts as a local mediator as its half-life *in vivo* may be only 0.1-1 secs (Kelm and Schrader, 1990).

(i) Basal EDRF Release

The endothelium continously releases EDRF to regulate basal levels of cGMP and blood vessel tone (Rapoport and Murad, 1983a). In endothelium-

denuded tissues, cGMP levels were shown to be higher than in intact tissues indicating a basal release of EDRF (Rapoport and Murad, 1983a; Martin *et al.*, 1985a; 1986a; 1986b; Ignarro *et al.*,1987a; 1987c). It was then demonstrated that exposure of endothelium-intact tissues to inhibitors of guanylate cyclase and endothelium removal cause an increase in basal tone and potentiation of contractile responses (Griffith *et al.*, 1984a; 1984b; Martin *et al.*, 1985a; 1986a). However, the most convincing evidence for the existence of basal EDRF release comes from studies using bioassay systems (Griffith *et al.*, 1984a; 1984b; Rubanyi *et al.*, 1985; Ignarro *et al.*, 1987a).

(ii) Flow-mediated dilatation

When the flow through large arteries is augmented, they dilate. Indeed, effluents from perfused rabbit aorta (Griffith *et al.*, 1984a) or canine femoral artery preparations with intact endothelium (Rubanyi *et al.*, 1985) produce relaxation of endothelium-denuded tissues. In addition, EDRF activity can influence the coordinated behaviour of an intact microvascular bed as has been demonstrated in the perfused rabbit ear (Griffith *et al.*, 1987; 1989). This illustrates the interdependence of different vessels within the bed and the need to consider the integrated behaviour of the whole vascular bed in controling flow, through changes in vessel calibre, and therefore pressure in the vascular bed.

(iii) Receptor-mediated dilatation

A vast array of substances can evoke EDR *in vitro* but the physiological significance of many of them is unclear. For example, ACh, the most classical agent that releases EDRF, does not circulate in the blood due to high levels of plasma cholinesterase and it seems unlikely, although not impossible, that it could activate muscarinic receptors on the endothelium. However, localisation

of choline acetyl transferase in endothelial cells (Parnavelas *et al.*, 1985; Burnstock, 1987) suggests that ACh may be released from the endothelium itself to modulate vascular tone. In addition, ATP, 5-HT and substance P have been found in certain endothelial cells which could be released in response to unknown stimuli and act on neighbouring endothelial cells to release EDRF causing relaxation of underlying smooth muscle (Burnstock, 1987).

Activated platelets release agents such as 5-HT and ADP which will stimulate an intact endothelium to release EDRF causing local vasodilation as has been demonstrated in isolated coronary arteries from dogs (Cohen *et al.*, 1983a; Houston *et al.*, 1985), pigs (Shimokawa *et al.*, 1987; 1988) and humans (Förstermann *et al.*, 1988a). This forms a negative feedback system to promote the flushing away of the aggregate as it forms, preventing occlusion of the vessel.

Receptor-mediated dilatation may be in response to physiological demands for increased blood flow to certain tissues or organs. For example, vasopressin released from the posterior pituitary in response to hypotension could favour redistribution of blood to the brain and heart as it relaxes cerebral and coronary vessels and constricts systemic arteries (Katusic *et al.*, 1984; Vanhoutte *et al.*, 1984)

(iv) EDRF modulation of platelet activity

EDRF inhibits platelet aggregation *in vitro* (Azuma *et al.*, 1986; Furlong *et al.*, 1987; Radomski *et al.*, 1987c), *ex vivo* (Hogan *et al.*, 1988) and *in vivo* (Bhardwaj *et al.*, 1988; Humphries *et al.*, 1990), can cause disaggregation of aggregated platelets (Radomski *et al.*, 1987a) and inhibit platelet adhesion to endothelial cell surfaces (Radomski *et al.*, 1987b).

EDRF and PGI_2 act synergistically to inhibit aggregation and to disaggregate

platelets (Radomski *et al.*, 1987a). Against a background of EDRF, the very low levels of PGI_2 may play a role in the regulation of platelet reactivity (Moncada *et al.*, 1988). PGI_2 has no effect on platelet adhesion to the endothelium even in the presence of EDRF (Radomski *et al.*, 1987b).

Thus, an intact endothelium has profound influence on platelet aggregation and the responsiveness to the agents released by aggregated platelets. However, under conditions leading to endothelial injury or dysfunction platelet aggregation and vasoconstriction in response to platelet-derived products such as 5-HT may be potentiated and intra-luminal thrombosis promoted.

1.4.2 CHEMICAL IDENTITY OF EDRF

Since the first demonstration of EDRF, the chemical identity of this labile, non-prostanoid substance had been keenly sought. The similarities between EDRF-elicited and NO-elicited relaxation of artery and vein and that EDRF is identical to NO were first reported at an international conference in 1986 (Furchgott, 1988; Ignarro *et al.*, 1988b). These similarities are summarised in **Table 1**. Confirmation of the chemical and pharmacological identification of EDRF as NO came from studies using cultured aortic endothelial cells (Palmer *et al.*, 1987) and isolated intact intrapulmonary artery and vein (Ignarro *et al.*, 1987a; 1987b).

There is now a vast body of evidence to support the proposal that EDRF is NO (EDNO; Reviewed by Ignarro, 1989a; 1989b; Angus and Cocks, 1989; Marin and Sanchez, 1990; Moncada *et al.*, 1991a). The release of NO has been shown in various preparations including isolated perfused rabbit heart (Amezcua *et al.*, 1988), rabbit aorta (Chen *et al.*, 1989) and human pulmonary artery (Greenberg *et al.*, 1987).

TABLE 1 **COMPARISON OF THE PHARMACOLOGICAL AND CHEMICAL PROPERTIES OF EDRF AND NO**

1. Chemically unstable with half-life of 3-6 secs under assay conditions
2. Spontaneous inactivation in presence of oxygen or superoxide anion
3. Chemical stabilisation by superoxide dismutase or acidic pH
4. React identically with sulfanilic acid and ozone
5. Lipophilic and readily able to permeate biological membranes
6. High binding affinity for and reactivity with haem iron in haemoglobin, myoglobin and soluble guanylate cyclase to form corresponding nitrosyl-haem adduct
7. Biological actions rapidily terminated by hemoglobin and myoglobin
8. Haem-dependent activation of soluble guanylate cyclase which is inhibited by methylene blue
9. Stimulation of cyclic GMP formation in vascular tissue and platelets
10. Relaxation of arterial and venous smooth muscle
11. Inhibition of platelet aggregation and adhesion to the endothelial cell surfaces

1.4.3 REGULATION OF EDRF SYNTHESIS AND RELEASE

The finding that the calcium ionophore A23187 is a powerful endothelium-dependent relaxing agent in isolated blood vessels (Furchgott *et al.*, 1981; Furchgott, 1983) and stimulates the release of EDRF from the perfused rabbit aorta (Griffith *et al*, 1984a) and cultured endothelial cells (Cocks *et al.*, 1985) led to the suggestion that an increase of calcium ions may be an early step in the reactions mediating release of EDRF. Later work clearly showed that extracellular Ca^{2+} is required for stimulation of EDRF release (Singer and Peach, 1982; Griffith *et al.*, 1986) whereas endothelium-independent responses to sodium nitroprusside are unaffected in Ca^{2+}-free medium (Holzmann, 1982; Rapoport and Murad, 1983b)

Numerous studies have shown that agents that interfere with certain pathways in phospholipid and polyunsaturated fatty acid metabolism such as quinacrine and 5,8,11,14-eicosatetraynoic acid cause attenuation of EDR (Furchgott, 1983), although the nonselective effects of many of the test agents cause problems in interpretation of these results (Ignarro and Kadowitz, 1985). Nevertheless, it has been suggested that cleavage of phospholipids with the formation of free fatty acids and lysophosphatides and/or the accompanying oxygen radical generation may play a role in triggering the formation and/or release of EDRF (Ignarro, 1989a).

A possible regulatory role for the protein kinase C agonist diacylglycerol in EDRF production has also been suggested. Activators of protein kinase C such as phorbol esters, have been shown to inhibit EDR from isolated blood vessels (Weinheimer *et al.*, 1986; Lewis and Henderson, 1987; Cherry and Gillis, 1988; Rubanyi *et al.*, 1989) and EDRF release from

cultured endothelial cells (Weinheimer *et al.*, 1986; Lewis and Henderson, 1987; Smith and Lang, 1990). These agents also attenuate agonist-induced increases in endothelial cell Ca^{2+} concentration (Ryan *et al.*, 1988) and IP_3 levels (Brock and Capasso, 1988).

Furthermore, pertussis toxin, an inhibitor of G-proteins, inhibits EDRF-mediated relaxations evoked by 5-HT and the α_2-adrenoceptor agonist UK 14,304, but not ADP, A23187 or BK (Flavanhan *et al.*, 1989). Responses to endothelium-independent relaxants remain unaffected by this toxin (Ignarro and Kadowitz, 1985), suggesting that there are at least two pathways leading to EDRF production, one of which involves G-proteins (Flavahan *et al.*, 1989).

Increases in EDRF production are associated with increases in cGMP levels in endothelial cells (Martin *et al.*, 1988) and this rise has been shown to inhibit the activation of the phosphoinositol pathway which is responsible for stimulating increases in intracellular Ca^{2+} (Collins *et al.*, 1986; Lewis *et al.*, 1988). In addition, increases in cGMP evoked by atrial natriuretic peptide (ANP; Martin *et al.*, 1988) and 8-bromo-cGMP inhibit the release of EDRF from cultured endothelial cells (Busse *et al.*, 1988; Evans *et al.*, 1988) and intact blood vessels (Hogan *et al.*, 1989). These findings indicate that there is a negative feedback system to control EDRF production with EDRF inhibiting its own release by increasing endothelial cell cGMP levels.

1.4.4 EDNO BIOSYNTHESIS

About a decade ago it was discovered that mammals, including humans, excrete more nitrate than they ingest (Green *et al.*, 1981a; 1981b), the amounts rising sharply during infection indicating that mammalian cells are

able to produce oxides of nitrogen from an endogenous source. This was first demonstrated when it was shown that cultured porcine aortic endothelial cells synthesised NO (Palmer *et al.*, 1987) via oxidation of one, or both, of the guanidino groups of L-arginine with the formation of L-citruiline (Schmidt *et al.*, 1988; Palmer and Moncada, 1989). Furthermore, activated macrophages which produce NO as a cytotoxic agent (Hibbs *et al.*, 1987a; 1987b; 1988) also do so from L-arginine (Marletta *et al.*, 1988).

L-arginine may be derived from several sources. Firstly it may be obtained exogenously from the diet, but it may also be generated endogenously from L-glutamate by the urea cycle. L-arginine generated by these routes is required not only for the synthesis of EDNO but also plays a role in amino acid metabolism, protein synthesis and as a precursor for polyamine formation (Moncada *et al.*, 1989). Futhermore, plasma arginine levels have no effect on resting blood pressure and therefore presumably have little or no effect on basal NO synthesis (Griffith *et al*, 1992), but the concentration of circulating L-arginine may influence the duration and magnitude of ACh-induced depressor responses (Aisaka *et al.*, 1989b).

The release of NO from L-arginine is specific, since a number of analogues of L-arginine, including its D-enantiomer, are not substrates. In addition, one analogue, N^G-monomethyl-L-arginine (L-NMMA), inhibits the synthesis of NO from endothelial cells (Palmer *et al*, 1988a), perfused rabbit aorta (Rees *et al.*, 1989a) and isolated rabbit heart (Amezcua *et al*, 1989) in a dose-dependent and enantiomerically specific manner. These effects could be reversed by L- but not D- arginine.

Other L-arginine analogues have been described as inhibitors of NO production in vascular tissues such as L-N^G-nitro-arginine (Moore *et al.*,

1990), N-imino-ethyl-L-ornithine (Mülsch and Busse, 1990) and N^G-nitro-L-arginine-methyl ester (Rees *et al.*, 1990b).

Recently, much effort has been concentrated on characterisation and purification of the several isoenzymes responsible for the production of NO from the vascular endothelium and different cell types and tissues (see Förstermann *et al.*, 1991b).

The similarities and differences between the constitutive and inducible NOS are outlined in **Table 2.**

i) CONSTITUTIVE NITRIC OXIDE SYNTHASE

The formation of NO from L-arginine in endothelial cell homogenates was shown to be dependent on the presence of NADPH (Palmer and Moncada, 1989). Furthermore, in L-arginine-depleted endothelial cell cytosol, L-arginine dependent increases in cGMP were found to require NADPH, and were accompanied by the formation of L-citrulline from L-arginine (Moncada and Palmer, 1990). Both the formation of L-citrulline and the increase in cGMP were inhibited by Ca^{2+} chelators, indicating that the enzyme, now called NO synthase (NOS), is calcium dependent (Mayer *et al.*, 1989; Moncada and Palmer, 1990). Futhermore, NOS in rat aortic endothelial cells was inhibited by calmodulin antagonists, suggesting that the Ca^{2+}-dependent stimulation of NOS in endothelial cells is mediated by calmodulin (Schini and Vanhoutte, 1992).

The enzyme that has been implicated in the synthesis of NO from endothelial cells, has been shown to be largely localised in the particulate fraction (Mitchell *et al*, 1991). For activity, this purified enzyme has also been shown to require Ca^{2+}/calmodulin, NADPH and tetrahydrobiopterin (Förstermann *et al.*, 1991a; 1991b). The purified NOS relaxed endothelium-

Table 2 SIMILARITIES AND DIFFERENCES BETWEEN THE TWO NO SYNTHASES

CONSTITUTIVE	INDUCIBLE
Cytosolic and particulate	Cytosolic
NADPH-dependent	NADPH-dependent
Inhibited by L-arginine analogues	Inhibited by L-arginine analogues
Ca^{2+}/calmodulin dependent	Ca^{2+}/calmodulin independent
Picomoles NO released	Nanomoles NO released
Short-lasting release	Long-lasting release
Unaffected by glucocorticoids	Induction inhibited by glucocorticoids
Unaffected by protein synthesis inhibitors	Induction inhibited by protein synthesis inhibitors

denuded rabbit aortic strips when added to the tissues together with substrate and cofactors.

The NOS found in freshly harvested endothelial cells is similiar to that present in brain and platelets (Moncada *et al.* 1991a).

ii) INDUCIBLE NITRIC OXIDE SYNTHASE

The enzyme responsible for the synthesis of NO from endothelial cells, brain and platelets is constitutive but another form does exist, the inducible type. The presence of an inducible NOS was first demonstrated in macrophages. This isoenzyme in the macrophage differs from the constitutive form in that it is not detectable in either macrophage cell lines or peritoneal macrophages that have not been activated by an immunological agent such as lipopolysaccharide (LPS), alone or in combination with interferon-γ (IFN-γ; Stuehr and Marletta, 1985; 1987a; 1987b) and requires *de novo* protein synthesis for its expression (Marletta *et al.*, 1988). Studies in a macrophage cell line showed that NOS activity was cytosolic (Marletta *et al.*, 1988), and that the enzyme required L-arginine and NADPH, but not Ca^{2+}/calmodulin (Steuhr *et al.*, 1991). Futhermore, NO production from activated macrophage cytosol has also been shown to be dependent on the presence of tetrahydrobiopterin (Tayeh and Marletta, 1989; Kwon *et al.*, 1990). In contrast to the constitutive enzyme, the inducible NOS enzymes releases NO over many hours, and will only decrease when the extracellular levels of L-arginine fall (Hibbs *et al.*, 1987b; Keller *et al.*, 1990)

Subsequent to these studies the inducible form of NOS has been located in several other cells and tissues including vascular smooth muscle (Busse and Mülsch, 1990b; Fleming *et al.*, 1990; Rees *et al.*, 1990a), endothelium

(Radomski *et al.* 1990; Gross *et al.*, 1992), hepatocytes (Billiar *et al.*, 1990) and lung (Knowles *et al.*, 1990). The activity of the induced enzyme can be inhibited in a stereospecific manner by the use of analogues of L-arginine. The expression of the inducible form of NOS requires the presence of endotoxin and/or cytokines and is time dependent and inhibited by cycloheximide suggesting a role for *de novo* protein synthesis. Glucocorticoids also inhibit the induction but have no effect once the enzyme has been expressed (Rees *et al.*, 1990a). Furthermore, the cytokine-induced NOS in endothelial cells has an absolute requirement for tetrahydrobiopterin and is completely unaffected by calmodulin antagonists (Gross *et al.*, 1991). Indeed it has been recently shown that tetrahydrobiopterin synthesis plays a pivotal role in the induction of NO production in vascular smooth muscle (Gross *et al.*, 1992). Therefore, it appears that this NOS is identical or very similiar to the one expressed in macrophages. However, there are reports that endotoxins can actually decrease EDRF activity and NO production from vascular endothelial cells (Myers *et al.*, 1992) and cytokines can down regulate the constitutive NOS to reduce NO synthesis (Schmidt *et al.*, 1992a).

iii) INDUCIBLE NITRIC OXIDE SYNTHASE *IN VIVO*

These recent findings give an insight into the factors and mechanisms underlying septic shock which is characterised by hypotension, hyporeactivity to vasoconstrictor agents (Parratt, 1974), inadequate tissue perfusion, vascular damage and disseminated intravascular coagulation leading to multiple organ dysfunction and death. Indeed, it has been shown that NO contributes to the hypotension induced by cytokines in the dog (Kilbourn *et al.*, 1990) and LPS in the rat (Thiemermann and Vane,

1990; Gray *et al.*, 1991).

Therefore, it is likely that the hypotension of endotoxemia in animal models is mediated, at least in part, by the induction of the NOS in the vasculature. The acute cardiovascular effects of endotoxin in man are similar to those described in animals: intravenous administration of endotoxin to healthy volunteers leads to a fall in systemic vascular resistance and blood pressure and an increased heart rate (Suffredini *et al.*, 1989).

The study of cirrhotic patients, who have hyperdynamic circulation, high heart rate and cardiac output, and low blood pressure and systemic vascular resistance, has provided an opportunity to investigate circulatory shock in humans. Interestingly, these patients have increased levels of endotoxins (Lumsden *et al*, 1988; Yomota *et al.*, 1989) and increased urinary excretion of cGMP (Miyase *et al.*, 1990) indicating that induction of NO synthesis in the peripheral circulation may explain their hyperdynamic state (Vallance and Moncada, 1991). More recently, cancerous patients receiving interleukin-2 (IL-2) therapy, which is known to induce IFN-γ, TNF and IL-1, have been shown to have elevated serum and urinary NO_3^- levels (Hibbs *et al.*, 1992).

The mechanism by which L-arginine is used as substrate to produce NO is still incompletely understood. However, the initial step in macrophages and endothelial cells is that a guanidino nitrogen of L-arginine undergoes oxidation to yield the gaseous radical NO via an Nω-hydroxyl-L-arginine intermediate, a reaction catalysed by NOS, a dioxygenase enzyme (Nathan, 1992).

1.4.5 METABOLISM OF EDNO

The termination of the biological actions of NO is rapid, occurring within seconds of its release, indicating that it is a locally acting modulator.

The half-life of EDRF under bioassay conditions is identical to that of NO superfused over vascular tissues (Palmer *et al.*, 1987; Ignarro *et al.*, 1987a). The biological inactivation of NO in oxygenated physiological salts solutions can be accounted for by spontaneous oxidation of NO to nitrite (NO_2^-):

$$2NO + O_2 \longrightarrow 2NO_2^-$$

At least 90% of the NO is converted to NO_2^-, with little or no formation of NO_3^- (Feelisch and Noack, 1987; Kelm *et al.*, 1988). The biological half-life of NO varies inversely as a function of oxygen tension and superoxide anion concentration (Förstermann *et al.*, 1984; Rubanyi *et al.*, 1985; Gryglewski *et al.*, 1986) and directly with NO concentration in aqueous solution, with concentrations over 300 mM having a half life of over 30 secs (Ignarro, 1990).

There is abundant evidence to show that O_2^- results in the rapid and nearly complete inactivation of NO (Gryglewski *et al.*, 1986; Moncada *et al.*, 1986; Rubanyi and Vanhoutte, 1986; Ignarro *et al.*, 1988a) which is reduced in the presence of SOD (Gryglewski *et al.*, 1986; Rubanyi and Vanhoutte, 1986; Ignarro *et al.*, 1988a). The relative importance of EDNO inactivation by O_2^- *in vivo* is unknown, but it seems unlikely to occur to any extent due to the ubiquitious distribution of SOD in tissues, although pathological conditions may provide a role for O_2^-.

1.4.6 EVIDENCE THAT EDRF IS NOT NO

Although the evidence supporting the proposal that EDRF is NO is very strong, several lines of research have questioned this conclusion. EDR-

mediated responses may not mediated by free NO but an unstable NO-releasing compound with the same bioreactivity as free NO, such as a S-nitrosothiol (Angus and Cocks, 1989; Myers *et al.*, 1990).

The pharmacology of S-nitrosothiols is essentially that of NO as they activate cytosolic guanylate cyclase (Ignarro *et al.*, 1980; Mellion *et al.*, 1983) by a haem-dependent mechanism (Ignarro *et al.*, 1984; Mellion *et al.*, 1983), elevate vascular (Ignarro *et al.*, 1981; Ignarro and Kadowitz, 1985) and platelet (Mellion *et al.*, 1983) cGMP levels, relax arteries and veins (Ignarro *et al.*, 1981; Ignarro and Kadowitz, 1985), inhibit platelet aggregation (Mellion *et al.*, 1983) and elicit potent vasodilator responses *in vivo* (Ignarro *et al.*, 1981).

Using a chemiluminescence procedure together with a bioassay system to characterise the properties of EDRF released from bovine aortic endothelial cells , it has been reported that EDRF is more like S-nitroso-cysteine than free NO (Myers *et al.*, 1990). These findings have been supported by work using the rabbit aorta that compared the O_2^- generating effects of xanthine plus xanthine oxidase on ACh, free NO, and nitrosocysteine-evoked relaxations, which suggested that EDRF released abluminaly by ACh behaves more like nitrosocysteine than like NO (Furchgott *et al.*, 1992a; 1992b)

Recently, it has been suggested that NO is synthesised and released under basal conditions but agents such as A32187 or bradykinin, stimulate the release of a labile nitroso compound perhaps from acidic lysosome-like granules in endothelial cells (Rubanyi *et al.*, 1989; Ignarro, 1990).

Evidence from electron paramagnetic resonance spectroscopy has also recently questioned the identification of NO as EDRF (Rubanyi *et al.*,

1990; Vedernikov *et al.*, 1990). These measurements are based on the reaction between haemoglobin and NO to form nitrosyl-haemoglobin and showed that NO, but not EDRF, generated a spectrum that was characteristic for such a species. Additionally, NO and EDRF are reported to be differentially retained by anionic exchange resins (Long *et al.*, 1987; Shikano *et al.*, 1988), and EDRF, but not NO, shows stability during chromatography and lyophilisation (Angus and Cocks, 1989).

1.4.7 MULTIPLE EDRFs

The term EDRF refers to the mediator(s) released upon stimulation of the endothelium which evoke relaxation of vascular smooth muscle, one of which is almost certainly NO. One such other possible substance released on its own or together with NO is endothelium-dependent hyperpolarising factor (EDHF). It has been reported that ACh-evoked EDR is accompanied by hyperpolarisation of the smooth muscle (Bolton *et al.*, 1984; Feletou and Vanhoutte, 1988; Komori *et al.*, 1988; Rand and Garland, 1992), whereas, NO causes vascular relaxation and increases in cGMP with no change in membrane potential (Huang *et al.*, 1988; Komori *et al.*, 1988; Rand and Garland, 1992). However, NO has recently been shown to cause hyperpolarisation of some arteries (Tare *et al.*, 1990; Garland and McPherson, 1992).

Although there is little doubt that free NO or a labile nitrosothiol accounts for EDRF activity, the term EDRF will be used to describe the mediator of EDR throughout the rest of this thesis.

1.4.8 *IN VIVO* ACTIVITY OF EDRF

(Reviewed by Feng and Hedner, 1990a; 1990b; Marshall and Kontos, 1990; Moncada *et al.*, 1991a)

There is abundant evidence for the existence of EDRF activity in large vessels *in vivo*. The dilation of canine femoral artery induced by ACh and substance P was abolished after endothelial damage by mechanical or chemical means (Angus *et al.*, 1983; Pöhl *et al.*, 1986a; 1986b) without affecting the response to nitrovasodilators. In humans, ACh evoked vasodilation of coronary arteries (Ludmer *et al.*, 1986) and it was suggested that this was due to the release of EDRF, as indicated by the inhibition of ACh-mediated dilation by methylene blue (Hodgson and Marshall, 1989).

Further evidence of a physiological role of EDRF *in vivo* stems from experiments with the inhibitors of the L-arginine : NO pathway. In anaesthetised rabbits, L-NMMA induced a substantial increase in blood pressure (Rees *et al.*, 1989b), strongly suggesting that basal release of EDRF is involved in the homeostatic regulation of arterial blood pressure. L-NMMA also causes dose dependent increases in blood pressure in guinea pigs (Aisaka *et al.*, 1989a; 1990) and rats (Whittle *et al.*, 1989; Gardiner *et al.*, 1990a; 1990b; Tolins *et al.*, 1990) which was prevented by L-arginine but not D-arginine. The increase in blood pressure induced by L-NMMA was accompanied by a decrease in vascular conductance in the renal, mesenteric, carotid, and hindquarters vascular beds of conscious instrumented rats (Gardiner *et al.*, 1990b), which could be sustained for up to six hours by maintained infusion of L-NMMA (Gardiner *et al*, 1990b). This not only indicates the maintenance of a continuous vasodilator tone by EDRF but that the vasculature are unable to reaccommodate the flow to normal levels.

In humans, the vasodilation responses induced by ACh and BK but not glyceryl trinitrate (GTN) in the brachial artery or dorsal hand vein of the

hand were attenuated by infusion of L-NMMA (Vallance *et al.*, 1989a; 1989b). Furthermore, L-NMMA induced direct vasoconstriction in the brachial artery, but had no such direct effect on the hand veins, suggesting that on the arterial side of the circulation, but not on the venous side, NO maintains a dilator tone (Vallance 1989b).

In conclusion, inhibition of NO biosynthesis by L-arginine analogues demonstrates that NO plays a pivotal role in the regulation of blood flow and blood pressure and in the maintenance of a continuous vasodilator tone in whole vascular beds.

1.5 OXIDISED LDL AND ATHEROSCLEROSIS

(Reviewed by Heinecke, 1987; Steinberg *et al*, 1989; Steinberg, 1990; Steinberg and Witztum, 1990; Steinbrecher *et al.*, 1990; Witztum and Steinberg, 1991).

It is very clear that hyperlipoproteinemia plays a causative role in the development of atherosclerosis. Futhermore, it has recently been proposed that the occurrence of early atherosclerotic lesions, the fatty streaks, can be accounted for solely by elevated plasma levels and the oxidative modification of LDL in the vessel wall (Steinberg *et al.*, 1989; Steinberg, 1990). The potential for OXLDL to initiate and contribute to atherogenesis will be discussed, and is illustrated in **Figure 3**.

(i) Oxidative modification of LDL and uptake by macrophages

The development of atherosclerosis is first apparent as the fatty streak, characterised by the accumulation of foam cells loaded with cholesteryl esters, just beneath the endothelium. Most foam cells are derived from circulating monocytes (Gerrity, 1981a; 1981b), and therefore their migration into the subendothelial space may be a key step in atherogenesis.

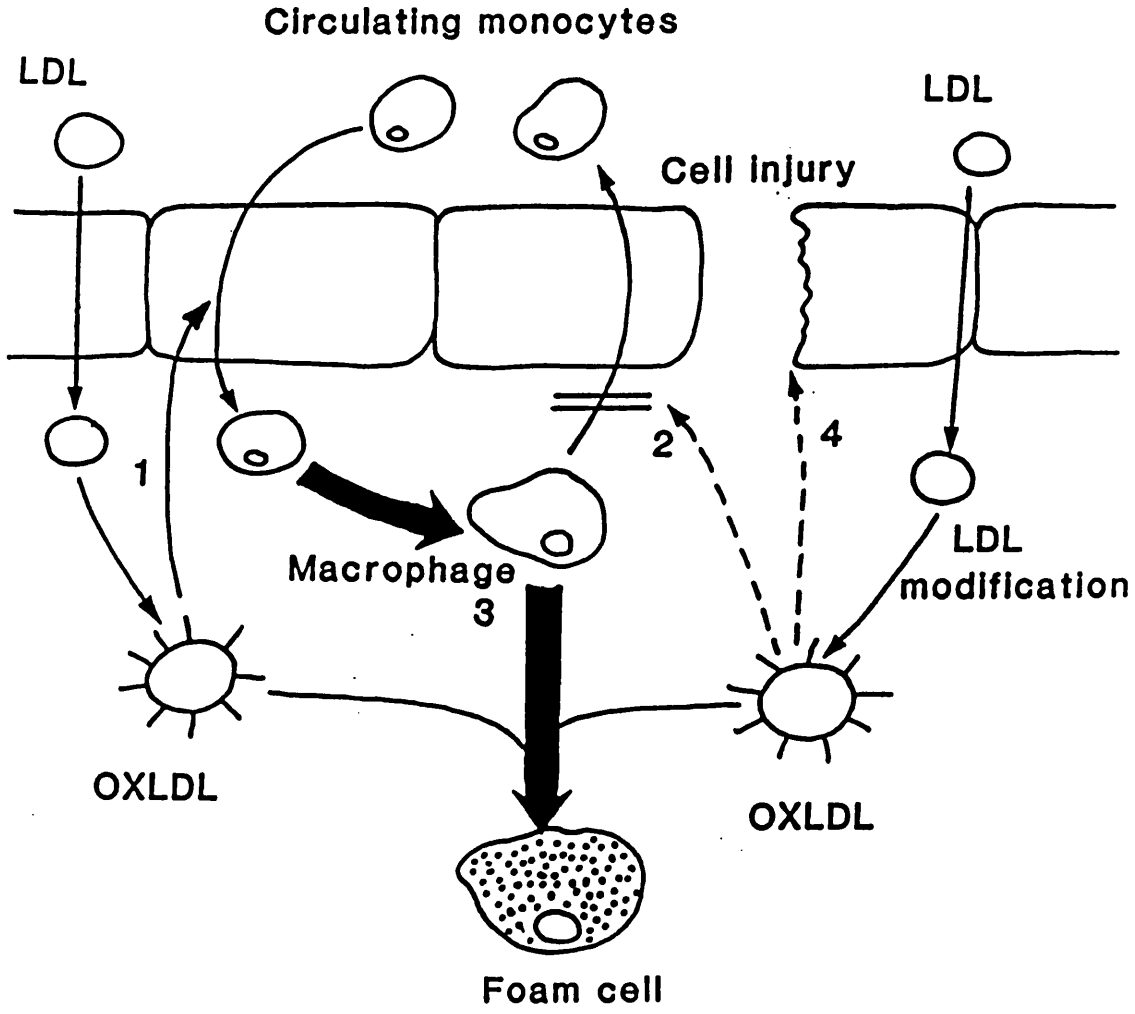

Figure 3 : Mechanisms by which OXLDL may contribute to atherogenesis.
(1) Recruitment of circulating monocytes to the sub-endothelial space
(2) Inhibition of macrophage motility
(3) Uptake by macrophages leading to foam cell formation
(4) Cytotoxicity leading to endothelial cell damage and loss
(Modified from Steinberg *et al.*, 1989)

However, *in vitro* studies revealed a paradox in that cultured macrophages take up native LDL only at a very low rate (Goldstein *et al.*, 1979a; Fogelman *et al.*, 1980), even when incubated with high concentrations. Further work revealed that LDL must undergo some form of modification in structure and biological properties before being taken up by macrophages (Goldstein *et al.*, 1979b; Brown and Goldstein, 1983). Chemical modification, such as acetylation, which neutralises the positively charged residues on the apo-B moiety essential for recognition by the classical high-affinity LDL receptor, enabled macrophages to take up LDL at a rate sufficient to generate foam cells (Steinberg *et al.*, 1989). This uptake was shown to be saturable and specific, and attributed to the "acetyl-LDL receptor" or "scavenger receptor" (Goldstein *et al.*, 1979b; Brown and Goldstein, 1983), which does not recognise native LDL and not regulated by cellular cholesterol levels.

There is, however, no evidence that acetylation occurs to any extent *in vivo* but an analogous biological modification of LDL may facilitate foam cell formation. All of the three major cell types of the arterial lesion, endothelial cells (Henriksen *et al.*, 1981; 1983; Morel *et al.*, 1984), vascular smooth muscle cells (Morel *et al.*, 1984; Heinecke *et al.*, 1986), and monocytes-macrophages (Parthasarathy *et al.*, 1986a), can oxidise LDL in cell culture leading to rapid uptake by macrophages, partially mediated by the acetyl-LDL receptor. More recent studies suggest that there may be more than one macrophage receptor capable of internalising cell-modified LDL (Steinberg, 1990). It is apparent, therefore, that if these modifications do occur *in vivo* they could provide a mechanism for foam cell formation within the vascular wall.

The oxidative modification of LDL by cultured cells is absolutely dependent on low concentrations of transition metal ions in the medium (Steinberg *et al.*, 1989). Moreover, cell-induced oxidation can be mimicked by simply incubating LDL with sufficiently high concentrations of Cu^{2+} or Fe^{3+} (Steinbrecher *et al.*, 1984; 1987; Parthasarathy *et al.*, 1986a). This method was used in the experiments described in Chapter 4 of this thesis, as it has been shown to generate OXLDL particles similar to those obtained by exposure to cultured cells (Steinbrecher *et al.*, 1984; 1987).

During the oxidative modification of LDL, the particles undergo many physiochemical changes. A striking change that accompanies LDL oxidation is the hydrolysis of phosphatidylcholine (PC), with up to 50% being converted to lysophosphatidylcholine (LPC; Steinbrecher *et al.*, 1984) mediated by a phospholipase A_2 enzyme associated with LDL (Parthasarathy *et al.*,1985). LPC has only one of the two positions of the glycerol backbone esterified and this is to a saturated fatty acid, predominately palmitic and less commonly stearic acid, in the *sn*-1 position. It is PUFAs in the *sn*-2 position which are liberated by the action of the phospholipase enzyme which then undergo peroxidation. Many of the physiochemical changes are the result of the peroxidation of PUFAs, mainly linoleic and arachidonic acid, in the LDL lipids, yielding an array of low molecular weight fragments such as ketones, aldehydes and alkanes (Esterbauer *et al.*, 1987), some of which form covalent bonds with the apo-B moiety (Steinbrecher *et al.*, 1987). This lipid-protein conjugation is crucial in generating a form of apo-B which is recognised by the scavenger receptor (Jürgens *et al.*, 1986; Parathasarathy , 1987).

The properties of cell-modified LDL, as compared to those of native LDL,

are described in **Table 3**.

(ii) Recruitment and retention of macrophages

Adhesion of circulating monocytes to the vascular endothelium is one of the earliest events in atherogenesis (Gerrity *et al.*, 1979). It has been demonstrated that treatment of rabbit aortic and human umbilical vein endothelial cells with minimally oxidised LDL stimulates the adherence of monocytes to the endothelium, possibly via increased expression of specific adherence molecules (Berliner *et al.*, 1990). In addition, minimally oxidised LDL stimulates the expression and secretion of monocyte chemotactic protein-1 (MCP-1) by cultured endothelial cells and smooth muscle cells (Cushing *et al.*, 1990).

OXLDL, but not native LDL, is a potent chemoattractant for circulating monocytes (Quinn *et al.*, 1985; 1987; 1988), a property attributable to LPC generated during the oxidation of LDL (Quinn *et al.*, 1988). Therefore, it appears that OXLDL are important because they promote the recruitment of monocytes in the subendothelial space, and will also induce differentiation into macrophages (Frostegard *et al.*, 1990).

Although OXLDL is a chemoattractant for circulating monocytes, it is also a potent inhibitor of both basal and stimulated macrophage motility (Quinn *et al.*, 1985), and thus the ability of OXLDL to promote the retention of macrophages in the vessel wall may contribute to the atherogenicity of these lipoproteins.

(iii) Cytotoxicity of OXLDL

OXLDL has been shown to be cytotoxic to endothelial cells and fibroblasts in culture (Henriksen *et al.*, 1979; Hessler *et al.*, 1979; Morel *et al.*, 1984), and could conceivably induce functional changes in the

TABLE 3 **PROPERTIES OF CELL-MODIFIED LDL**

1. Increased electrophoretic mobility on agarose gel due to increased net negative charge (Henriksen *et al.*, 1983; Steinbrecher *et al.*, 1984).
2. Increased density and fragmentation of the apo-B moiety with decreased

histidine, lysine and proline content (Parthasarathy *et al.*, 1986a).

3. Increased thiobarbituric acid reactive substances (TBARS) content and PC converted to LPC (Morel *et al.*, 1984; Steinbrecher *et al*, 1984; Heinecke *et al.*, 1986; Parthasarathy *et al.*, 1986a).
4. Increased uptake by macrophages *in vitro* (Henriksen *et al.*, 1983; Morel *et al.*, 1984; Heinecke *et al.*, 1986).
5. Derivatisation of lysine groups on apo-B-100 and generation of fluorescent adducts due to the covalent bonding of lipid oxidation products to the apo-B-100 moiety (Henriksen *et al.*, 1983; Steinbrecher *et al.*, 1984; 1987; Heinecke *et al.*, 1986).

endothelium that favour the migration of circulating monocytes as well as injuring vascular smooth muscle cells (Jürgens *et al.*, 1987; Steinberg *et al.*, 1989), and thus accelerate the formation of the fatty streak.

The cytotoxicity of oxidatively modified LDL has been shown to reside in the lipid component, with agents such as fatty acids, lipid hydroperoxides and 2-alkenals proposed to mediate this effect (Esterbaurer *et al.*, 1987; Jürgens *et al.*, 1987).

1.5.1 EVIDENCE THAT OXLDL EXISTS *IN VIVO*

Only fragmentary evidence exists to suggest that oxidative modification of LDL occurs in plasma, the degree of which is probably restricted by circulating antioxidants such as ascorbate. However, a subfraction of LDL from Lp(a)-negative human plasma which was more electronegative than the bulk of LDL isolated indicating that oxidation of this fraction had occured (Avogarro *et al.*, 1988). Furthermore, detection of fragments of apo-B (Schuh *et al.*, 1978), increased levels of lipid peroxides (Yagi, 1987) and a low level of modified LDL immunoreactivity (Salmon *et al.*, 1987) have suggested that some degree of oxidation of LDL can take place in the circulation. Further support for a role of oxidatively modified LDL in atherogenesis has recently been provided with the demonstration of autoantibodies against OXLDL in human plasma (Salonen *et al.*, 1992), and, in addition, this report claimed that autoantibodies against OXLDL can be used to predict the progression of carotid atherosclerosis.

Several lines of evidence suggest the presence of oxidatively modified LDL in atherosclerotic lesions. LDL eluted from human lesions and those from WHHL rabbits showed an increased electromobility, increased fragmentation of apo-B, and an increased hydrated density (Daugherty *et*

al., 1988; Ylä-Herttuala *et al*, 1989). This is consistent with the physical and chemical properties of OXLDL.

Studies using monoclonal antibodies have shown immunoreactivity against modified apo-B (Haberland *et al.*, 1988; Palinski *et al.*, 1989) or against OXLDL itself (Palinski *et al.*, 1989) in atherosclerotic lesions of rabbits and humans. In addition, monoclonal antibodies to human OXLDL reacted with atheromatous lesions from WHHL rabbits but not with arterial tissue from normal rabbits (Boyd *et al.*, 1989) and monoclonal antibodies raised to WHHL rabbit arterial plaque homogenate were specific for OXLDL (Mowri *et al.*, 1988).

The occurrence *in vivo* of OXLDL does not necessarily establish a pathogenically important role for the oxidation of LDL. Studies documenting the effects of probucol, a lipid lowering drug with powerful antioxidant properties (Parthasarathy *et al.*, 1986b), have lent further support to the hypothesis that oxidation of LDL is a prerequisite for rapid uptake into macrophages, and formation of foam cells in the artery wall. Treatment of cholesterol-fed and WHHL rabbits with probucol has been shown to slow the progression of atherosclerosis (Tawara *et al.*, 1986; Carew *et al.*, 1987; Kita *et al*, 1987; Steinberg *et al*, 1988; Daugherty *et al*, 1991) and may promote the regression of established plaques (Nagano *et al.*, 1992), independent of its lipid-lowering effect (Carew *et al*, 1987; Nagano *et al.*, 1992). A recent study has shown that butylated hydroxytolulene (BHT), a close chemical homologue of probucol and an equally effective antioxidant, also slows the progression of atherosclerosis in cholesterol-fed rabbits (Björkhem *et al.*, 1991). However, one study has failed to demonstrate an anti-atherosclerotic effect of probucol in

cholesterol-fed rabbits (Stein *et al.*, 1989). The antioxidant properties of probucol were highlighted in a recent study which showed that it can reduce plasma lipid peroxides in hyperlipidemic patients (Paterson *et al.*, 1992). However, probucol may have antiatheromatous properties other than acting as an antioxidant or as a lipid lowering drug. One study has demonstrated that administration of probucol suppresses intimal thickening of the carotid artery after injury by a balloon catheter, and this could be mediated via the inhibition of cell migration and/or cell proliferation (Shimomiya *et al.*, 1992).

The mechanisms by which *in situ* modification of LDL in the vessel wall might lead to foam cell formation has not been established. Clearly, if extensive oxidative modification takes place, then uptake via the scavenger pathway might occur. Indeed, LDL isolated from human atherosclerotic lesions was degraded by macrophages in culture via the scavenger pathway at a much higher rate than native LDL (Ylä-Herttuala *et al.*, 1989). Furthermore, macrophage-derived foam cells isolated from rabbit atherosclerotic plaques degrade OXLDL, promote oxidation of LDL and contain oxidation-specific lipid protein adducts. This indicates that *in vivo*, arterial wall macrophages express receptors for modified LDL and are capable of oxidising LDL even when maximally loaded with cholesterol (Rosenfeld *et al.*, 1991).

In another study, however, degradation of human atherosclerotic LDL by macrophages was found to occur via a low affinity non-scavenger receptor mechanism (Morton *et al.*, 1986). and extracts of atherosclerotic plaque could modify LDL *in vitro* in a way that led to increased nonsaturable degradation in cultured macrophages (Hoff and O'Neil, 1988). The findings

of the latter study suggest that this modification did not solely involve oxidation, but reactions between the apo-B, and aldehydes or formation of complexes with arterial proteoglycans may provide a mechanism for uptake by macrophages.

In summary, a number of different lines of evidence from *in vivo* studies support the presumption that oxidation does take place *in vivo* and that oxidation enhances the rate of progression of atherosclerotic lesions.

1.6 ANIMAL MODELS OF ATHEROSCLEROSIS

There are several animal models of atherosclerosis and this section will briefly describe the relative merits of the models most commonly in use at the present.

The New Zealand white (NZW) rabbit is the most common animal used for diet-induced atherosclerosis as it readily develops fatty streaks in responses to increased cholesterol in the diet. However, in this model increases in plasma cholesterol are transported in VLDL rather than LDL, causing a rise in atherogenic VLDL.

There now exists several genetic variants of rabbits which offer expanded possibilities in exploring the relationship of lipids to lesion formation and development. The best kown is the Watanabe strain (Watanabe, 1980), whose lipoprotein pattern on a standard chow diet is a fairly close approximation of monoclonal human familiar hypercholesterolemia (Havel *et al.*, 1989). The lesions in the Watanabe rabbit are also considered close approximations of their human counterparts (Buja *et al.*, 1983). The Watanabe heritable hyperlipidemic (WHHL) rabbit has been used in this study (Chapter 5).

Swine are also good models of atherosclerosis. Both full-sized and

miniature swine have been extensively studied (e.g. Fritz *et al.*, 1980; Reitman *et al.*, 1982). Lesions occur to some extent in free-living animals, and can be induced with atherogenic diet to an important degree.

Birds are relatively hypercholesterolemic animals, with some tendency to develop atherosclerotic lesions in the absence of dietary induction. Pigeons are currently the main bird species of atherosclerotic lesions. However, the myocardial infarctions that develop in pigeons have been ev aluated and found to be the result of atheromatous embolism (Pritchard *et al.*, 1963), in contrast to the atherothrombotic pathogenesis in humans and so other differences may exist this model and the human condition.

Nonhuman primates have been studied in detail but the future use of these models is uncertain because of their decreasing availability and the expense in providing proper maintenance.

Animal models continue to have important roles in atherosclerosis research. During the past 25 years the actual choice of animals for study have narrowed, and animals now uncharacterised are unlikely to be superior models.

1.7 ATHEROSCLEROSIS AND VASCULAR REACTIVITY

Hypercholesterolemia and atherosclerosis alters the normal homeostatic mechanisms that control the vasomotor tone in blood vessels. This includes the pertubation of EDRF activity which plays a vital role in the control of blood pressure and the antiaggregatory effect on platelets, and potentiation of some vasomotor responses.

The effects of elevated plasma cholesterol and atherosclerosis on vascular relaxations and contractions and the possible pathological consequences will now be discussed.

1.7.1 ENDOTHELIUM-DEPENDENT RESPONSES

A wide range of hyperlipidemic models of atheroma have consistently shown impairment of EDRF-mediated responses. These include hypercholesterolemic and atherosclerotic rabbits (Sreeharan *et al.*, 1986; Verbeuren *et al.*, 1986; 1990; Bossaller *et al*, 1987a; 1987b; Jayakody *et al*, 1987; Osborne *et al.*, 1989), monkeys (Armstrong *et al*, 1982; Frieman *et al*, 1986; Harrison *et al.*, 1987) and pigs (Shimokawa *et al.*, 1987; Yamamoto *et al.*, 1987; Cohen *et al.*, 1988; Shimokawa and Vanhoutte, 1989). It has also been suggested that hypercholesterolemia alone can attenuate EDRF-mediated responses since animals on a high cholesterol diet show altered vascular reactivity before macroscopic changes associated with atherosclerosis occurs (Cohen *et al*, 1988; Shimokawa and Vanhoutte, 1989; Merkel *et al*, 1990).

Studies of isolated atherosclerotic human coronary arteries have shown attenuated EDR responses (Bossaller *et al.*, 1987b; Berkenboom *et al*, 1989; Förstermann *et al.*, 1988b) and responses to ACh show a shift from dilation in normal arteries to constriction (Ludmer *et al.*, 1986). Recent studies have provided further evidence of impaired EDRF responsiveness in the human vasculature with blunted increases in coronary arterial flow induced by ACh (Drexler *et al.*, 1990). Futhermore, in clinical studies, paradoxical vasoconstriction of angiographically normal coronary arteries after ACh injection or infusion has been reported (Horio *et al*, 1986; Werns *et al.*, 1989; Vita *et al.*, 1990; Drexler *et al.*, 1991).

The patterns of inhibition of EDR in the presence of hypercholesterolemia and atherosclerosis are fairly well described although the mechanisms involved remain unclear. Discussed below are possible factors leading to

the observed alterations in EDR:

(i) Selective inhibition of receptor-mediated pathway

A defect in receptor-operated release of EDRF has been suggested because in the rabbit aorta and human coronary arteries with atherosclerosis, EDR is blunted in response to ACh, but that relaxations to histamine, substance P, and the non-receptor-mediated responses to the Ca^{2+} ionophore A23187 remain intact (Bossaller *et al.*, 1987a). Atherosclerotic iliac arteries from monkeys (Frieman *et al.*, 1986) and coronary arteries from hypercholesterolemic pigs (Yamamoto *et al.*, 1987; Cohen *et al.*, 1988) have also been reported to exhibit selective inhibition of receptor-induced EDR.

However, many reports have demonstrated impairment of both receptor- and non-receptor -mediated relaxations, indicating that atherosclerosis and hypercholesterolemia can cause a general impairment of responses to endothelium-dependent relaxing agents rather than a selective inhibition of endothelial receptor pathways (Jayakody *et al.*, 1985; Habib *et al.*, 1986; Verbeuren *et al.*, 1986; Harrison *et al*, 1987; Förstermann *et al.*, 1988b; Guerra *et al.*, 1989; Shimokawa and Vanhoutte, 1989).

(ii) Endothelial denudation of vessels

Loss of endothelial cells in response to a cholesterol-rich diet has been demonstrated in monkeys (Faggiotto *et al.*, 1984) and in WHHL rabbits, a gradual attenuation of endothelium-dependent responses was associated with arterial denudation (Kolodgie *et al.*, 1990), suggesting that the inhibition of EDR was the result of endothelial loss.

However, many investigators have demonstrated that even in the most severely affected atherosclerotic vessels, the integrity of the endothelium

was preserved (Jayakody *et al.*, 1985; Frieman *et al.*, 1986; Verbeuren *et al.*, 1986; 1990), although shape changes, altered surface projections, and a reduction in surface charge have been noted (Taylor *et al.*, 1990). These morphological alterations may accompany inhibition of EDR, thus reflecting functional changes in addition to the structural modifications (Jayakody *et al.*, 1988; 1989).

(iii) Decreased availability of L-arginine

As discussed in section 1.4.4, L-arginine is the precursor of EDRF and it has been proposed that decreased availability of L-arginine may account for the attenuation of EDR in atherosclerotic vessels and arteries exposed to hypercholesterolemia. In support of this hypothesis, it has been reported that attenuation of relaxations evoked by cholinergic agents in the hind quarters of hypercholesterolemic rabbits (Girerd *et al.*, 1990) and in the aorta (Cooke *et al.*, 1991) can be reversed by infusion of L-arginine *ex vivo.*

(iv) Reduction in EDRF generation

Bioassay studies have demonstrated that the response of detector tissues is decreased with superfusion from an atherosclerotic donor tissue as compared to control donor tissue (Sreeharan *et al.*, 1986; Guerra *et al*, 1989; Jayakody *et al.*, 1989), indicating that the release of EDRF is decreased in diseased vessels.

In contrast, other studies have shown that the intraluminal release of EDRF from atherosclerotic vessels is normal, although production may be reduced in the most severely diseased vessels. Futhermore, EDRF release may be normal even when EDR were absent in intact isolated tissues (Verbeuren *et al.*, 1986; 1990). This indicates that the inhibition of EDR

may not be the result of decreased production but that the mechanisms of relaxation subsequent to release are also altered.

(v) Increased rate of EDRF inactivation

Atherosclerosis is characterised by thickening of the intimal layer due to the presence of foam cells and smooth muscle cells. This could provide a significant diffusion barrier to the short-lived EDRF. Several investigators have shown that cholesterol-fed animals returned to a normal diet show regression of atherosclerotic lesions with the reabsorption of intimal lipids and inflammatory cells (Armstrong *et al.*, 1970; 1982; Adams and Morgan, 1977; Harrison *et al.*, 1987) but significant intimal thickening persists due to smooth muscle cells (Armstrong *et al.*, 1982). However, the thickened intima may not play a major role as a distance barrier because endothelium-dependent responses are restored after regression of atherosclerotic lesions (Harrison *et al.*, 1987), and unaffected in arteries with intimal hyperplasia (Cocks *et al.*, 1987), although others have found EDR to remain impaired after regression (Armstrong *et al.*, 1982; Jayakody *et al.*, 1987; 1989)

The atherosclerotic lesion, with lipid-laden cells, may represent a functional barrier for the lipophilic EDRF, and thus by acting as a sink for EDRF, will decrease the amount reaching the underlying smooth muscle layers. Alternatively, free radicals produced by inflammatory cells present in lesions could increase the inactivation of EDRF.

(vi) Decreased responsiveness of vascular smooth muscle

Many studies have shown that inhibition of EDRF-mediated responses in atherosclerotic tissues is not accompanied by decreased sensitivity to nitrovasodilators such as GTN that also act via the activation of soluble guanylate cyclase (Jayakody *et al.*, 1985; Frieman *et al.*, 1986; Sreeharan *et*

al., 1986; Bossaller *et al.*, 1987a; Harrison *et al.*, 1987; Guerra *et al.*, 1989). However, in severely atherosclerotic vessels, GTN-evoked relaxations have been shown to decrease indicating that the responsiveness of smooth muscle becomes impaired as the disease progresses (Verbeuren *et al.*, 1986; 1990; Förstermann *et al.*, 1988b; Berkenboom *et al.*, 1989).

In summary, inhibition of EDR in atherosclerotic vessels could be the result of several possible mechanisms, the relative importance of each perhaps depending on the stage of the disease. In early atherosclerosis, endothelial dysfunction and lipid deposition may account for this attenuation, whereas advanced states may also cause decreased sensitivity of the vascular smooth muscle, together, resulting in the abolition of EDR in diseased vessels.

1.7.2 CONTRACTILE RESPONSES IN ATHEROSCLEROSIS

Many investigators have studied the effects of hypercholesterolemia and atherosclerosis on the vasoconstrictor properties of vessels. Coronary vasospasm can be provoked by agents such as ergonovine in patients with coronary atherosclerosis, which fail to evoke any response in normal subjects (Schroeder *et al.*, 1977; Cipriano *et al.*, 1979; Waters *et al.*, 1983; Kaski *et al.*, 1986). Similarly, 5-HT and histamine relax normal porcine coronary arteries in an endothelium-dependent manner, but cause vasospasm in atherosclerotic miniture swine (Shimokawa *et al.*, 1983; 1985a; 1985b).

Several mechanisms have been proposed to account for the altered contractile responses in hypercholesterolemia and atherosclerosis, and are oulined briefly below :

(i) Accumulation of cholesterol in cell membranes

Increased cholesterol in the membrane fraction of arteries constitutes one of the earliest demonstrable biochemical changes during atherogenesis (Small and Shipley, 1974). Incorporation of cholesterol into membranes has been shown to augment cation permeability (Wiley and Cooper, 1975) and inhibit β-adrenoceptor-mediated responses in erythrocyte membranes (Lurie *et al.*, 1985). However, changes in membrane cholesterol content would be expected to alter vasoconstrictor responses irrespective of the agent used but, specific changes in sensitivity have been reported in many studies suggesting that this mechanism does not play a large role (see for example, Yokoyama *et al.*, 1983; Heistad *et al.*, 1984; Lopez *et al.*, 1989; Merkel *et al.*, 1990).

(ii) Increase in receptor number

Potentiation of 5-HT-evoked constrictor responses in hypercholesterolemic and atherosclerotic animals have been reported by many investigators (Shimokawa *et al*, 1983; 1985a; Yokoyama *et al*, 1983; Heistad *et al.*, 1984; Verbeuren *et al.*, 1986; Wines *et al*.1989; Kolodgie *et al.*, 1990). Increased sensitivity to the 5-HT in atherosclerotic rabbit aorta could be the result of an increased number of serotonergic receptors (Nanda and Henry, 1982). However, studies of altered responses to adrenergic agonists are mixed but most report decreased contractile responses (Rossendorf *et al.*, 1981; Verbeuren *et al*, 1986; Wines *et al*, 1989; Kolodgie *et al.*, 1990; Asada *et al.*, 1992).

(iii) Endothelial dysfunction

As described previously endothelium-dependent responses are attenuated in atherosclerotic animals and humans, and agonist-induced contractions are potentiated in tissues denuded of endothelium due to removal of

basally released EDRF (Cocks and Angus, 1983; Cohen *et al*, 1983b; Martin *et al*., 1985a; 1985b). Additionally, agents such as ACh and 5-HT can evoke EDR in many vessels as well as having direct vasoconstrictor actions on smooth muscle. Thus removal or dysfunction of the endothelium could be particularly significant to such agents by removing their endothelium-dependent relaxing action (see for example, Lamping *et al.*, 1985; Shimokawa and Vanhoutte, 1989).

(iv) Release of contracting factors

The endothelium can release factors that constrict vascular smooth muscle and these have been implicated in mediating the potentiation of contractions in atherosclerotic vessels. Products of the cyclo-oxygenase pathway have been shown to augment 5-HT-evoked contractions, and inhibit 5-HT and ADP- induced EDR, in atherosclerotic porcine coronary arteries in an endothelium-dependent manner (Shimokawa and Vanhoutte, 1989). However, endothelial removal did not influence the potentiation of contractions observed in other studies (Verbeuren *et al.*, 1986).

1.7.3 PATHOPHYSIOLOGICAL IMPLICATIONS

Patients with atherosclerosis are prone to spontaneous vasospasm (Schroeder *et al.*, 1977) which can lead to myocardial ischaemia and sudden death (Maseri *et al.*, 1978). The occurrence of spasm is not dependent on the presence of atherosclerotic lesions as angiographically normal arteries also experience spasm (Horio *et al.*, 1986; Werns *et al.*, 1989; Vita *et al.*, 1990) indicating that alterations in vascular reactivity can preceed macroscopic changes.

The link between EDRF-mediated actions and coronary vasospasm in humans remains elusive. The occurrence of spasm related to coronary

angioplasty (Dorros *et al*, 1983) suggests the important inhibitory effect of an intact endothelium on vascular smooth muscle constriction. More direct evidence of endothelial dysfunction comes from the finding that intracoronary injection of ACh in human subjects vasoconstricts atherosclerotic, but relaxes normal, coronary arteries (Ludmer *et al.*, 1986).

As a consequence of endothelial dysfunction, products of aggregated platelets may also play a pathological role in coronary vasospasm. The endothelium mediates vasodilation to many platelet products under normal conditions (Cohen *et al.*, 1983a; Houston *et al.*, 1985; Shimokawa *et al.*, 1987) but in diseased arteries the altered vascular response may favour vasoconstriction.

In conclusion, altered vascular reactivity under conditions of hypercholesterolemia and atherosclerosis will be significantly influenced by endothelial dysfunction resulting in the dcreased production of EDRF both under basal conditions and upon stimulation.

1.7.4 ROLE OF LIPOPROTEINS

In hypercholesterolemia, the vascular endothelium is exposed to elevated plasma levels of LDL and many recent studies have investigated the effects of native and oxidatively modified LDL on EDR.

Native LDL has been shown to inhibit endothelium-dependent responses by a rapid and reversible mechanism (Jacobs *et al.*, 1990; Tomita *et al*, 1990) which may be the result of direct inactivation of EDRF by LDL (Galle *et al.*, 1991). However, others have failed to demonstrate any effect of native LDL on EDRF release in isolated tissues (Kugiyama *et al.*, 1990; Yokoyama *et al.*, 1990; Simon *et al.*, 1990) or in bioassay systems (Galle *et al.*, 1990; Chin *et al.*, 1992).

In contrast, OXLDL has been widely reported to inhibit EDR. The extent of inhibition of EDRF-mediated responses were inhibited varied from complete inhibition (Kugiyama *et al.*, 1990; Yokoyama *et al.*, 1990) to much lesser effects (Galle *et al.*, 1990). However, OXLDL has shown both to inhibit (Chin *et al.*, 1992) or have no effect (Galle *et al.*, 1991) on EDRF generated from cultured endothelial cells. The effects of OXLDL on EDR in these studies may be complicated by the variability in the degree of inhibition between LDL preparations from different donors (Jacobs *et al.*, 1990; Plane, 1992).

The mechanism of action of OXLDL on EDRF responses in unclear. Direct inactivation of EDRF by OXLDL in a manner similar to that described for native LDL has been proposed (Galle *et al.*, 1991). Inhibition of the signal transduction pathways involved in the production of EDRF, such as G_i proteins, has also been suggested (Tanner *et al.*, 1991; see Flavahan, 1992). A direct action of OXLDL on vascular smooth muscle has also been demonstrated by the attenuation of GTN-evoked relaxations. In addition, OXLDL was shown to inhibit the activation of partially purified soluble guanylate cyclase by both nitrovasodilators and NO (Schmidt *et al.*, 1990; 1992b).

Many studies in atherosclerotic vessels and those exposed to diet-induced hypercholesterolemia have demonstrated alterations in the vasoconstrictor responses to certain agonists as discussed in section 1.7.2. Thus the effects of isolated lipoproteins on contractile responses have also been investigated. Direct vasoconstrictor properties of LDL have been reported in some studies (Sachinidis *et al.*, 1989; Simon *et al.*, 1990; Weisser *et al.*, 1991) although others have failed show a similar effect (Galle *et al.*,

1991; Plane, 1992). Incubation of isolated tissues with OXLDL potentiates responses to 5-HT (Galle *et al.*, 1991; Plane, 1992), but descrepancies exist as to the effects on contractions evoked by potassium, NA and PE in these two reports.

In conclusion, it appears that the potential of LDL to inhibit EDRF-mediated responses in isolated tissues is increased by oxidation, but the effect OXLDL has on smooth muscle contraction is less well defined.

1.8 AIMS

(1) During oxidative modification of LDL particles, many chemical and structural changes take place including the conversion of PC to LPC. This study investigated the vascular effects of LPC in isolated aortic rings from NZW rabbits.

(2) Hypercholesterolemia may induce the expression of an isoform of NOS distinct from that constitutinely present in endothelial cells. Presented here are studies of the responses of isolated aortic rings from Wistar rats examining the influence of OXLDL and LPC on NOS induced both *in vitro* and *in vivo.*

(3) Attenuation of EDR in atherosclerotic arteries has been suggested to result from a defiency of L-arginine in endothelial cells. The aim of this study was to investigate whether increasing the L-arginine supply to the endothelium, by oral or *in vitro* administration, would improve EDR and alter contractions in aortae from WHHL rabbits.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Chemicals for density solutions, Krebs' and other buffers were of Analar grade and were obtained from BDH.

Phenylephrine, 5-hydroxytryptamine creatine sulphate, calcium ionophore A23187, L- and D-arginine hydrochloride, L-nitroarginine methylester, phosphatidylcholine dipalmitoyl, L-α-lysophosphatidylcholine palmitoyl, stearoyl, and caproyl, and bovine serum albumin (fatty acid free) were supplied by Sigma Chemical Company.

Acetylcholine and glyceryl trinitrate were supplied by the Pharmacy, Royal Free Hospital.

2.1.2 Drugs

All drugs were prepared in Krebs' buffer unless otherwise stated in the text.

2.1.3 Nitric Oxide Gas

Nitric oxide gas was supplied by Cambrian Gases.

2.1.4 Preparation of Nitric Oxide Solutions

Solutions of nitric oxide were prepared using the following procedure, modified from Palmer *et al.* (1987). Double distilled water was boiled for 30 mins and then placed on ice and bubbled with helium for 30 mins. The degassed water was then transferred to a gas bulb, under vacuum, which had been sealed at one end with a rubber septum (Phase Separation Ltd.) and heat-shrink tubing (RS Components Ltd.). The water was then bubbled with helium for a further 15 mins before the bulb was sealed. Nitric oxide gas was

added to the solution of degassed water in the sealed gas bulb using a gas tight syringe. After preparation, the nitric oxide solutions were kept on ice.

2.1.5 Animals

New Zealand White rabbits (3 kg, 6 months old), Watanabe Heritable Hyperlipidaemic (WHHL) rabbits and Wistar rats (10-12 weeks old) were supplied by the Comparative Biology Unit, Royal Free Hospital.

2.2 BUFFERS AND DENSITY SOLUTIONS

2.2.1 Buffers

Acid-Citrate-Dextrose (ACD)

113.8 mM glucose, 29.9 mM trisodium citrate, 72.6 mM NaCl, 2.8 mM citric acid, pH 6.4.

Tris Buffer for dialysis of LDL

67.5 mM Tris, 25.8 mM NaCl, pH 7.4

Krebs' Buffer

118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2.6H_2O$, 2 4 mM $NaHCO_3$, 1.2 mM $MgSO_4.7H_2O$, 1.2 mM KH_2PO_4, 11 mM glucose, 0.3 mM EDTA, pH 7.4.

2.2.2 Density Solutions

Density of stock solution 1.006 g/ml : 195 mM NaCl, 1 mM NaOH, 0.34 EDTA.

All other density solutions were prepared from this stock solution by the addition of solid NaBr as determined by the equation below :

$$M = \frac{V(p_2 - p_1)}{1 - (v \times p_2)}$$

where : M = mass of NaBr to be added (g)

V = initial volume (ml)

p_1 and p_2 = initial and final densities (g/ml)

v = partial specific volume of NaBr => 0.2434

The density of all solutions were checked using a Paar refractometer to measure the refractive index.

2.3 ISOLATION OF LDL

2.3.1 Collection of blood

Venous blood was collected from apparently healthy volunteers into sterile plastic universal tubes (Sterilin) containing ACD anticoagulant (5:1, v/v). Sterile polypropylene syringes for the withdrawl of blood were from Plastipak (Becton Dickinson Ltd.) and butterfly needles (21 gauge) were from Venisystems.

Immediately after collection, the blood was centifuged at 1800 g for 20 mins. at 20^{0}C in a Centra-7R bench centrifuge (International Equipment Co. , USA.) to separate the blood cells from the plasma. Normally, 120 mls of whole blood was collected from each donor which gave approximatley 70 mls of plasma (plus ACD).

2.3.2 Preparation of LDL

The method used for the isolation of LDL (1.019-1.063 g/ml) was based on that of Chung *et al.* (1980), using discontinuous gradient ultra-centifugation. LDL was prepared separately from the plasma of each donor. The density of the plasma was adjusted to 1.3 g/ml by the addition of solid NaBr (31 g per 70 mls of plasma) and 10 to 12 mls were layered under 0.9% (w/v) saline in polypropylene centrifuge tubes. The tubes were capped before being placed in a fixed angle rotor (Kontron TFT 70-38 or Beckman 70 Ti) and spun at 200,000 g for 2.5 hrs at 16^{0}C in a Centrikon T-2070 or a Beckman XL-70 ultracentrifuge.

Following centifugation the lipoprotein fractions were banded in the tubes,

VLDL and chylomicrons at the top, LDL in the middle and HDL at the base of the tube. The yellow LDL band was carefully removed and trasferred to clean tubes to which 6.5 mls of a solution of density 1.151 g/ml was added. The tubes were filled with a solution of density 1.063 g/ml, capped, placed in the fixed angle rotor and spun at 200,000 g for 14 hrs at 16°C to give a clearly defined band of LDL at the top of the tube which was carefully removed.

2.3.3 Concentration of LDL

LDL samples were concentrated by centrifugation at 8,000 rpm in a Sorval RC-5B refrigerated centrifuge (Dupot Instruments, USA) in tubes containing nitrocellulose ultrafiltration membranes (Diaflo, Amicon Corp. , USA) which retain the lipoproteins but allow the buffer to pass through. The LDL were concentrated to a volume of 2 to 4 mls before being placed in 2 cm wide dialysis tubing (Scientific Industries International Incorporated, UK) and dialysed for 12 hrs against Tris buffer at 4°C.

Finally, the LDL samples were tranferred to plastic eppendorf vials, spun in a Sorvall microspin 24S mini-centrifuge (Dupont Instuments, USA) at 13,000 rpm for 5 mins and then filtered through a sterile 0.2 μM filter (Acrodisc, Gelmen Sciences, UK) to remove any impurities. The protein concentration was determined as described below (section 2.3.4) and the lipoproteins were stored at 4°C until use.

LDL prepared by this process was defined as native LDL (Jacobs *et al.*, 1990; Plane, 1992).

2.3.4 Protein Assay

The protein concentration of LDL samples was determined by a modification (Markwell *et al.*, 1978) of the Lowry method (Lowry *et al.*, 1951) using Folin-Ciocalteau reagent (BDH). Bovine serum albumin (BSA fraction V, Sigma) was

used as a standard and the concentration of the standard stock solution was checked by measuring the absorption at 279 nm against distilled water on a DU-70 Beckman spectrophotometer. The concentration of the standard solution was then calculated using the following equation :

BSA conc. (mg/ml) = absorbance at 279 nm x 13/9

A typical standard curve is shown in **Figure 4**. All standards and samples were prepared in triplicate and the absorbance was read at 660 nm against distilled water.

2.4 PREPARATION OF OXLDL

2.4.1 Cu^{2+}-Oxidation of LDL

Before modification, LDL was dialysed for 12 hrs at 4°C against EDTA-free Tris buffer.

OXLDL was prepared by incubating native LDL with 1 nmole $Cu_2SO_4.5H_20$ per mg of LDL at room temperature for 24 hrs. The Cu^{2+} ions were removed by extensive dialysis against several changes of Tris buffer containing EDTA (0.3 mM). This method was based on that of Steinbrecher *et al.* (1985). All OXLDL was filtered before use as described above (section 2.3.3).

2.5 VASCULAR REACTIVITY STUDIES

Aortic rings were mounted in organ baths containing oxygenated Krebs' buffer as described below (sections 2.5.1 and 2.5.2).

2.5.1 Preparation of Rabbit and Rat Aortic Rings

New Zealand White rabbits (6 months old, 3 kg body weight), WHHL rabbits (ages as stated in Chapter 5) and Wistar rats (10-12 weeks old) were killed by cervical dislocation. The thoracic aorta was carefully removed and transferred to oxygenated Krebs' buffer at room temperature. The vessel was rapidly cleaned of adhering fat and connective tissue and cut into 2 mm wide

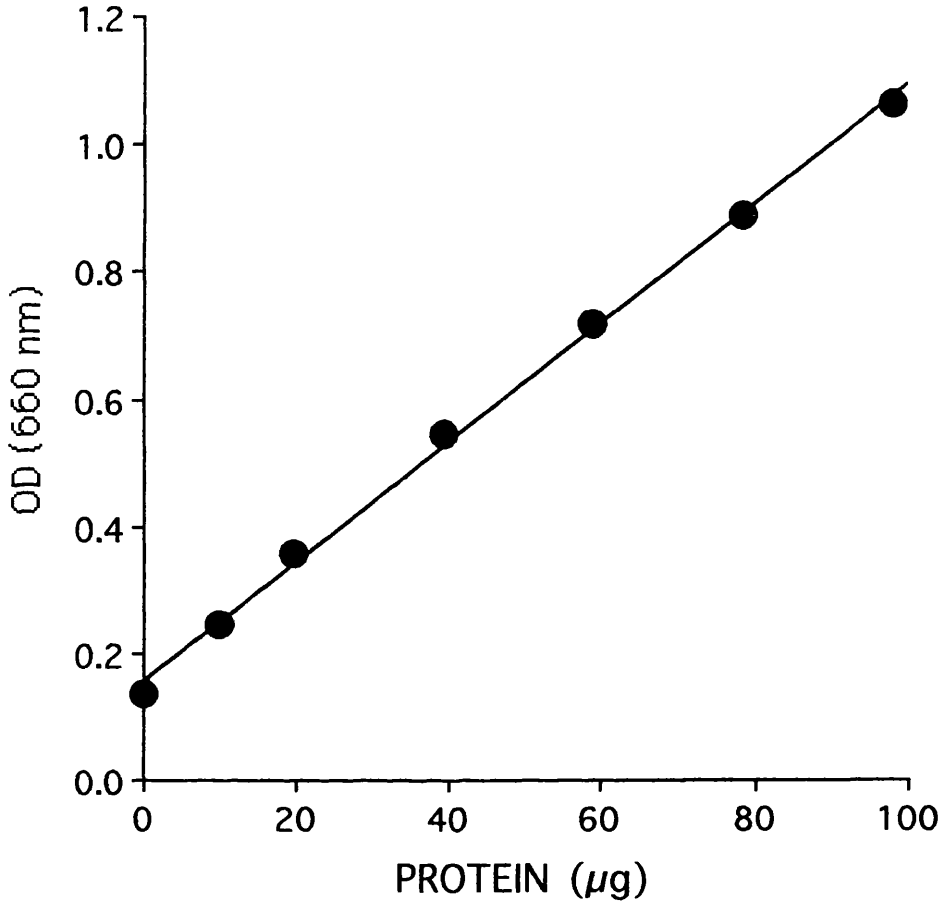

Figure 4 : Bovine serum albumin (BSA) standard curve for protein assay
The protein concentration of LDL samples was determined as described in section 2.3.4 and expressed as mg LDL protein/ml.

transverse rings. For some experiments the endothelium was gently removed by rubbing the luminal surface of the ring.

2.5.2 Organ Bath Studies

This method is based on that of Furchgott and Zawadzki (1980). The aortic rings were suspended between metal hooks in Krebs' buffer bubbled with 95% O_2/5% CO_2 maintained at 37°C (**Figure 5**) and set at the following tensions :

a) NZW Rabbit 2g

b) WHHL Rabbit 4 g

c) Wistar Rat 1.5 g

One hook was fixed in position and the other was linked via a Grass FT03 or Dyanamometer 4F1 force displacement transducer to a model 7 Grass polygraph amplifier and chart recorder or Lectromed Multitrace 4-P recorder. Alternatively, LKB or Rikadenki flat-bed recorders were used in conjunction with a pre-amplfier. In each experiment, 8-10 tissues were mounted. Following a 60 mins period, the tension was reset to initial levels. A further 30 mins equilibration period was then allowed before the experiment was started. The experimental procedure followed for each of the animal groups was as described below.

2.5.3 NZW Rabbit Aortic Rings

A contraction of approximately 2 g was induced by addition of PE (0.05-0.1 μM) until a stable plateau was obtained. The integrity of the endothelium was assessed by the addition of a bolus dose of ACh (1 μM) to relax the tissues. Following washout and equilibration, the tissues were recontracted and relaxed to cumulative concentrations of ACh, calcium ionophore A23187, ATP or to the endothelium-independent relaxants GTN or NO.

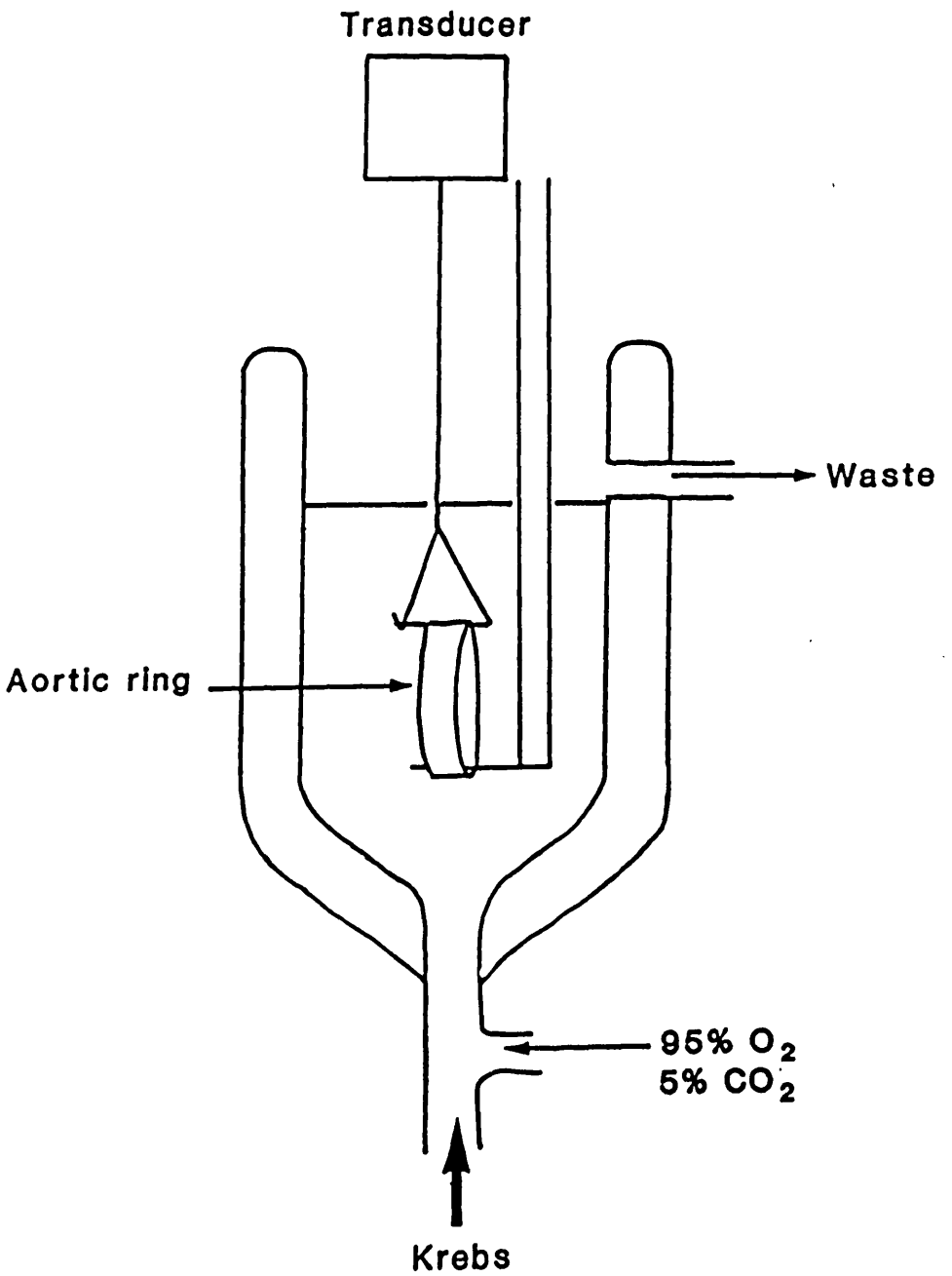

Figure 5 : Organ Bath Apparatus For Isometric Tension Recording Studies With Isolated Aortic Rings

2 mm wide aortic thoracic aortic rings suspended between two hooks, one linked to a transducer, the other fixed in position. Organ bath contains Krebs' buffer maintained at 37°C and bubbled with 95% O_2/5% CO_2. Tissues were washed and allowed to equilibrate for 90 mins.

In experiments investigating the effect of lysophospholipids on contractions, tissues were contracted with cumulative doses of PE or 5-HT. The protocol for these experiments was then as described for relaxing agents.

Following contraction and relaxation the tissues were washed and allowed to equilibrate for 30 mins. The lysophospholipid was then added to the bath and the contraction/relaxation cycle was then repeated. In some experiments a preincubation period with the tissues was employed. After washout the rings were equilibrated for a further 30 mins before the contraction/relaxation cycle was again repeated to determine the reversiblility of any effects. The tissues were contracted to the same level of tone (approximately 2 g) in each contraction/relaxation cycle in a bath volume of 5 mls.

2.5.4 WHHL Rabbit Aortic rings

From each animal, 2 rings from the arch and 6-7 rings from the descending aorta were mounted in organ baths. The mean responses from the two regions of the aorta were calculated separately.

The integrity of the endothelium was tested and the protocol used for the experiments was as described for aortic rings from NZW rabbits.

L-Arginine Feeding of WHHL Rabbits : Age-matched WHHL rabbits were placed into two groups. One group acted as control and the other was supplied with 2.2 g of L-arginine hydrochloride in their drinking water *ad libitum* each day for 5 days. The animals were then sacrificed and the aorta removed as described above.

2.5.5 Wistar Rats

The protocol for the testing integrity of the endothelium of aortic rings from Wistar rats was as described for tissues from NZW rabbits except a contraction of approximately 1 g was achieved by PE (0.1 μM).

Relaxations to L-arginine : Responses to L-arginine could be obtained by incubating the suspended tissues in the Krebs' buffer for 4 hrs. After this period the tissues were preconstricted and tested for relaxations to a bolus dose of L-arginine (100 μM). The rings were equilibrated for 30 mins and the experimental regime described for tissues from NZW rabbits was used with cumulative concentrations of L-arginine added to relax the tissues.

LPS-Treated Wistar Rats : Rats were injected intraperitoneally with sterile LPS (Salmonella typhosa, Difco) at a dosage of 4 mg/kg in 0.2-0.25 mls volumes. Control rats were injected with sterile vehicle (0.9% NaCl) in equal volumes. After 4 hrs the rats were sacrificed and the aorta quickly removed. Some tissues were denuded of endothelium by gently rubbing with forceps and then set up as described above. The tissues were mounted, and following equilibration, the rings were constricted with PE (0.1 μM) and challenged with L-arginine (100 μM) and ACh, to test for relaxation. After 30 mins LPC, Lω-NAME, or OXLDL, or equivalent volume of Tris buffer, was added to the baths. The lipoproteins and Tris buffer controls were incubated with the tissues for a 30 min period. The rings were then contracted with cumulative doses of PE.

2.5.6 Expression of Results

Results of relaxation studies are expressed as % relaxation of PE-induced tone or % inhibition of maximum relaxation. The results of the contraction studies are expressed as % of control maximum or in g tension.

Data is expressed as mean ± standard error of the mean for n separate experiments and analysed by Students' t-tests for unpaired samples where $p<0.05$ was considered significant.

CHAPTER THREE

THE EFFECTS OF LPC ON VASCULAR REACTIVITY

3.1 INTRODUCTION

Previous studies in this laboratory demonstrated that oxidatively modified LDL inhibits EDR in isolated rabbit aortic rings (Jacobs *et al.*, 1990). This inhibition has been attributed to LPC generated from PC in LDL during oxidation (Kugiyama *et al.* , 1990; Plane *et al.*, 1990; Yokoyama *et al.*, 1990). The aim of the present study was to investigate further the vasoactive properties of synthetic LPC, and in particular, that of palmitoyl LPC, the most common lysophospholipid in OXLDL (see section 1.5).

3.2 EFFECT OF LPC ON BASAL AND INDUCED TONE

3.2.1 LPC AND BASAL TONE

1-palmitoyl LPC did not alter basal tone when added to endothelium-denuded aortic rings (10-300 μM) as shown in **Figure 6a**. In endothelium-intact rings, high concentrations of 1-palmitoyl LPC evoked small contractions (**Figure 6b**) .

3.2.2 LPC AND INDUCED TONE

When added to PE-contracted (0.1 μM) tissues, 1-palmitoyl LPC (0.1-50 μM) caused dose-dependent relaxations of endothelium-intact aortic rings (**Figure 7a**). Maximum relaxation of PE-induced by LPC was 68.9 ± 6.7%, (n=4; **Figure 8**). No relaxations were observed in endothelium-denuded tissues as shown in **Figure 7b**.

Relaxations evoked by 1-palmitoyl LPC in endothelium-intact tissues were almost abolished in the presence of 100 μM L$^{\omega}$-NAME (**Figure 8**). In contrast, pre-incubation of tissues with the cyclo-oxygenase inhibitor

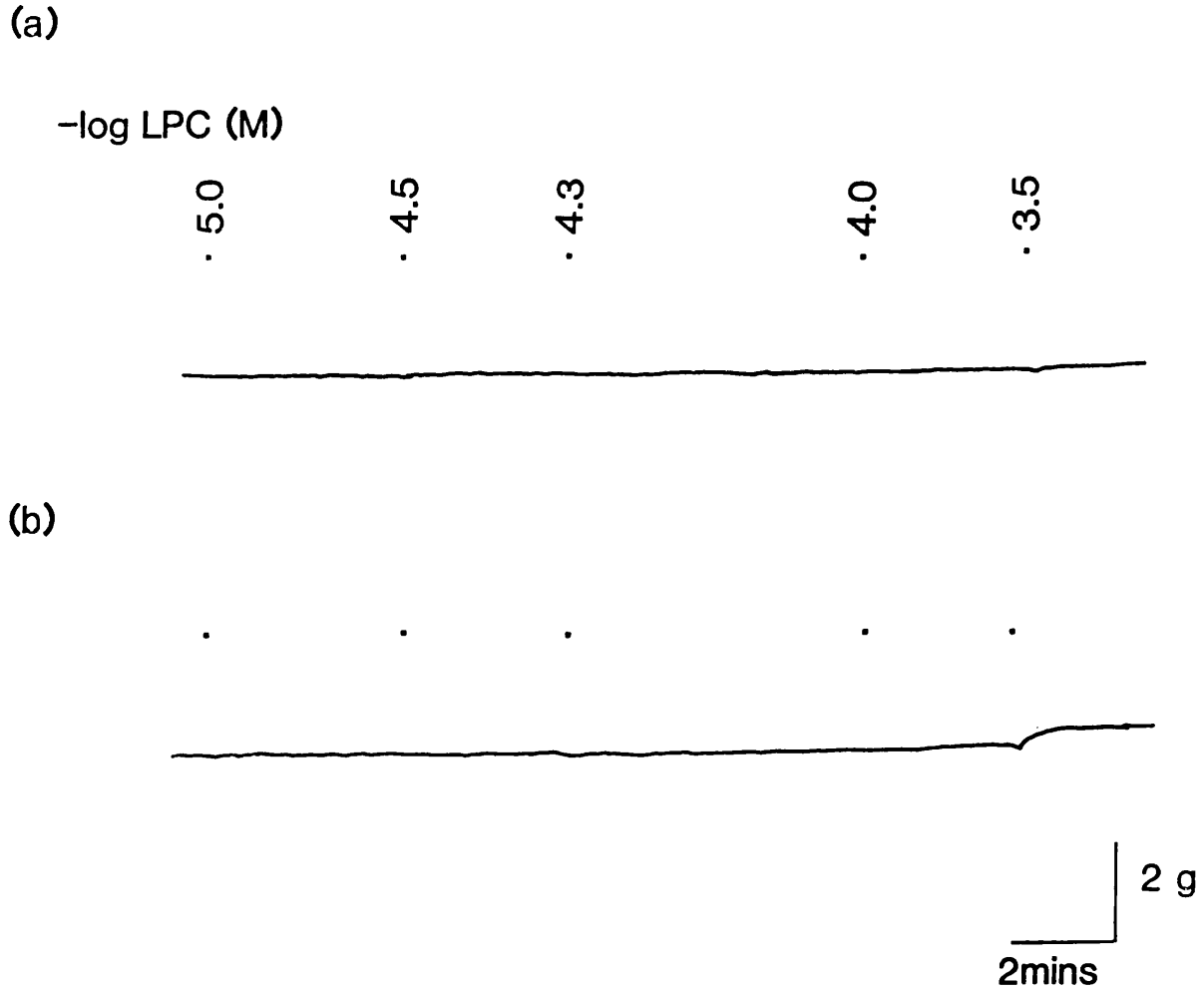

Figure 6 : Addition of 1-palmitoyl LPC to resting tone
(a) Addition of 1-palmitoyl LPC (10-300 μM) to basal tone of endothelium-denuded tissues
(b) Addition of 1-palmitoyl LPC (10-300 μM) to basal tone of endothelium-intact tissues

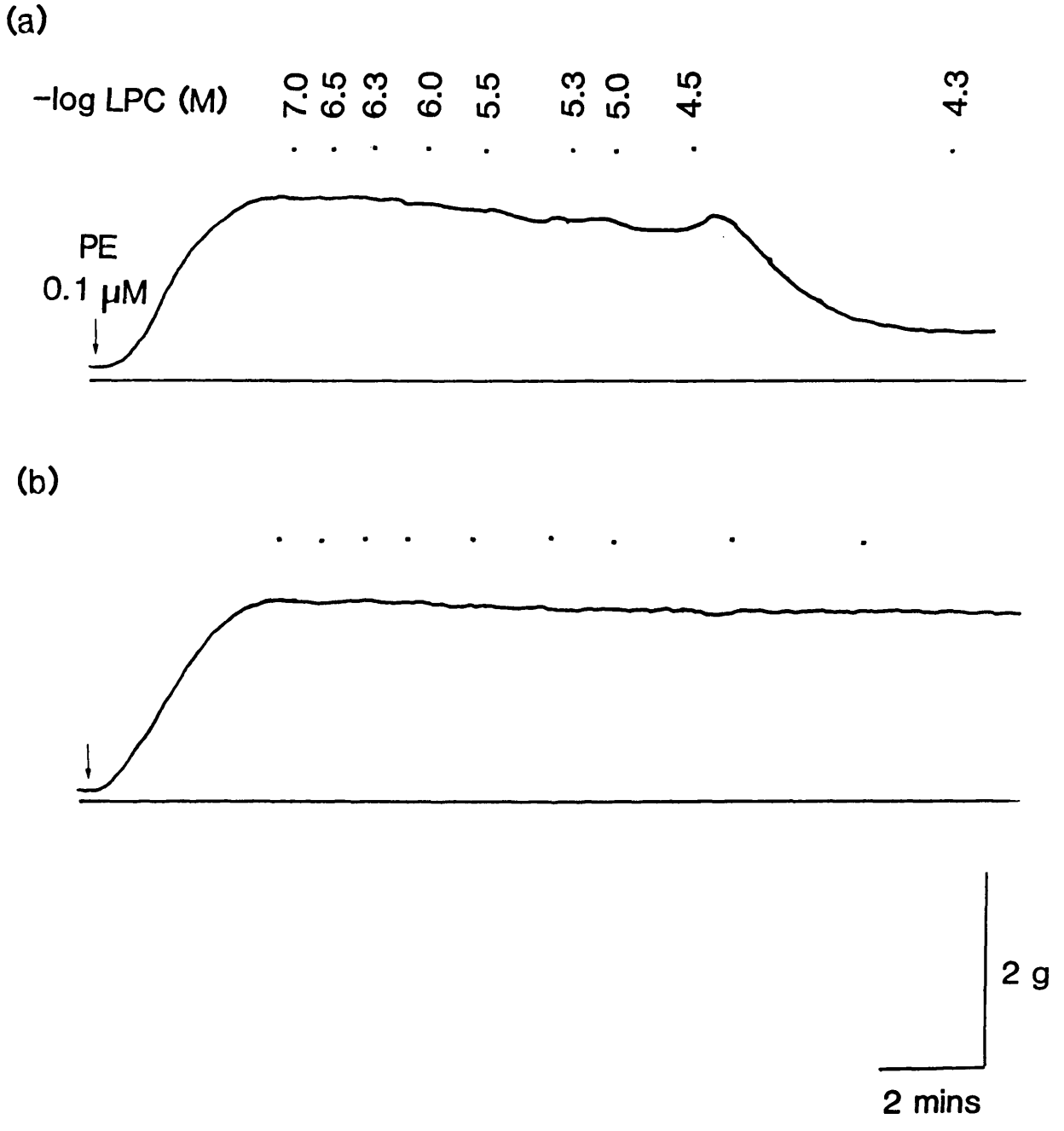

Figure 7 : Addition of 1-palmitoyl LPC to pre-contracted tissues
(a) Addition of 1-palmitoyl LPC (0.1-500 μM) to PE-contracted (0.1 μM) endothelium-intact tissues
(b) Addition of 1-palmitoyl LPC (0.1-500 μM) to PE-contracted (0.1 μM) endothelium-denuded tissues

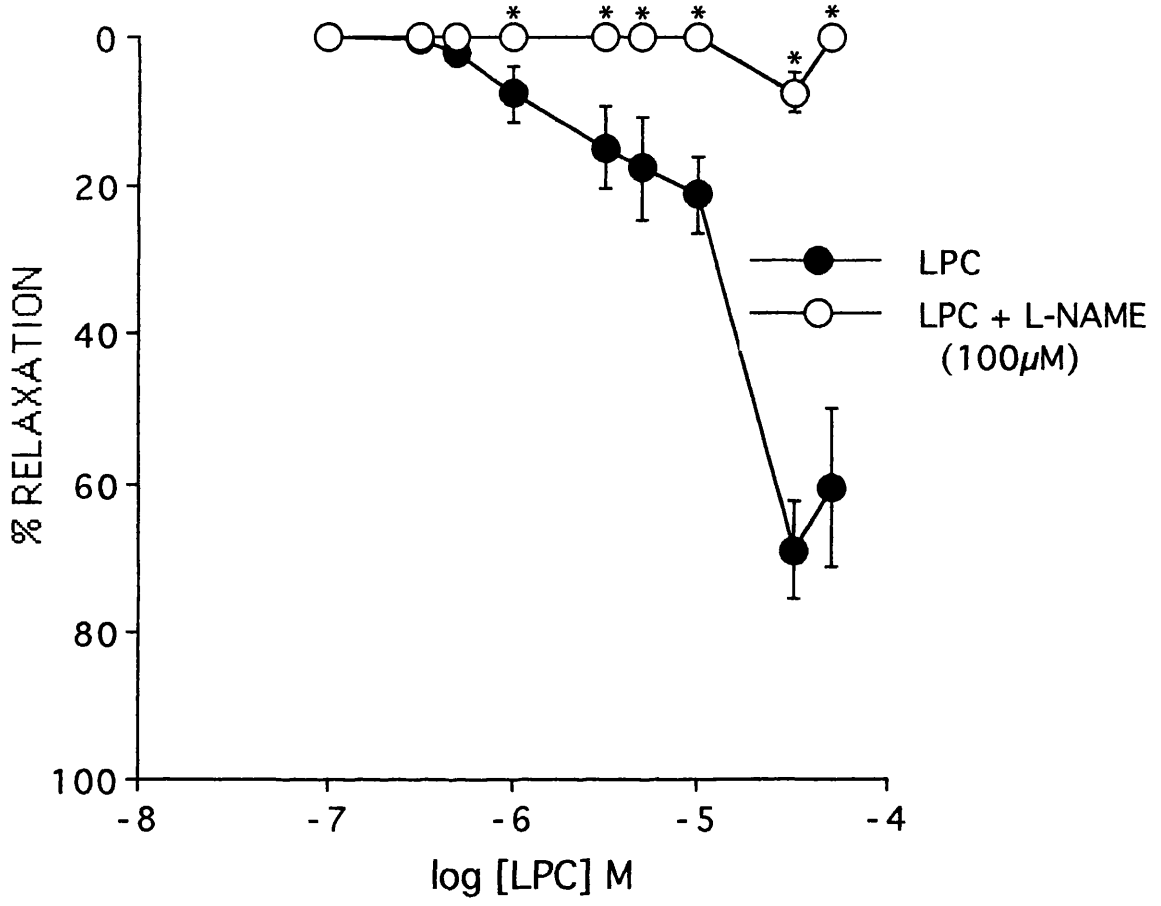

Figure 8 : 1-palmitoyl LPC-evoked relaxations in endothelium-intact PE-contracted rings and the effect of L-NAME

Tissues pre-contracted with PE (0.1 μM) and relaxed to cumulative doses of 1-palmitoyl LPC (0.1-500 μM) in the absence and presence of L-NAME (100 μM; n=4)

Students' t-test compared to control : * $p<0.05$

indomethacin (10 μM) for 15 mins did not alter the sensitivity to LPC (maximum relaxation 71.7 ± 8.8%; n=4).

In contrast, when 1,2-dipalmitoyl phosphatidylcholine was used instead of 1-palmitoyl LPC, no effect on PE-induced tone was observed (n=3).

1-stearoyl LPC (0.1-50 μM) also evoked relaxations which were dependent on an intact endothelium with maximum relaxation 53.8 ± 12.0% (n=4) of induced tone. This response was completely inhibited in the presence of L-NAME (100 μM; n=3).

In two separate experiments, 1-caproyl LPC (0.1-50 μM) failed to induce any response in PE-contracted rabbit aortic rings.

3.3 THE EFFECT OF LPC ON EDR

3.3.1 THE EFFECT OF LPC ON ACh-EVOKED RELAXATIONS

As shown in **Figure 9a**, ACh (0.01-1 μM) evokes dose-dependent relaxations of intact PE-contracted (0.1 μM) rabbit aortic rings. ACh-evoked relaxations remain unchanged, even after 30 mins pre-incubation with drug vehicles such as water, as shown in **Figure 9b**.

In the presence of 10 μM 1-palmitoyl LPC added immediately before the tissues were contracted with PE, ACh-evoked relaxations were almost completely inhibited. A representive trace of four experiments is shown in **Figure 10** and the mean of pooled data from 4 experiments is shown in **Figure 11a**. LPC (10 μM) inhibited maximum relaxation by 94 ± 5.9% (n=4) which was reduced to 10 ± 2.0% (n=4) after washout. In contrast, dipalmitoyl phosphatidylcholine (10 μM) was ineffective against ACh-evoked relaxations (n=3; **Figure 11b**).

1-palmitoyl LPC at concentrations of 0.1 μM and 1 μM failed to exert any inhibitory effect on ACh-evoked relaxations without a pre-incubatory period

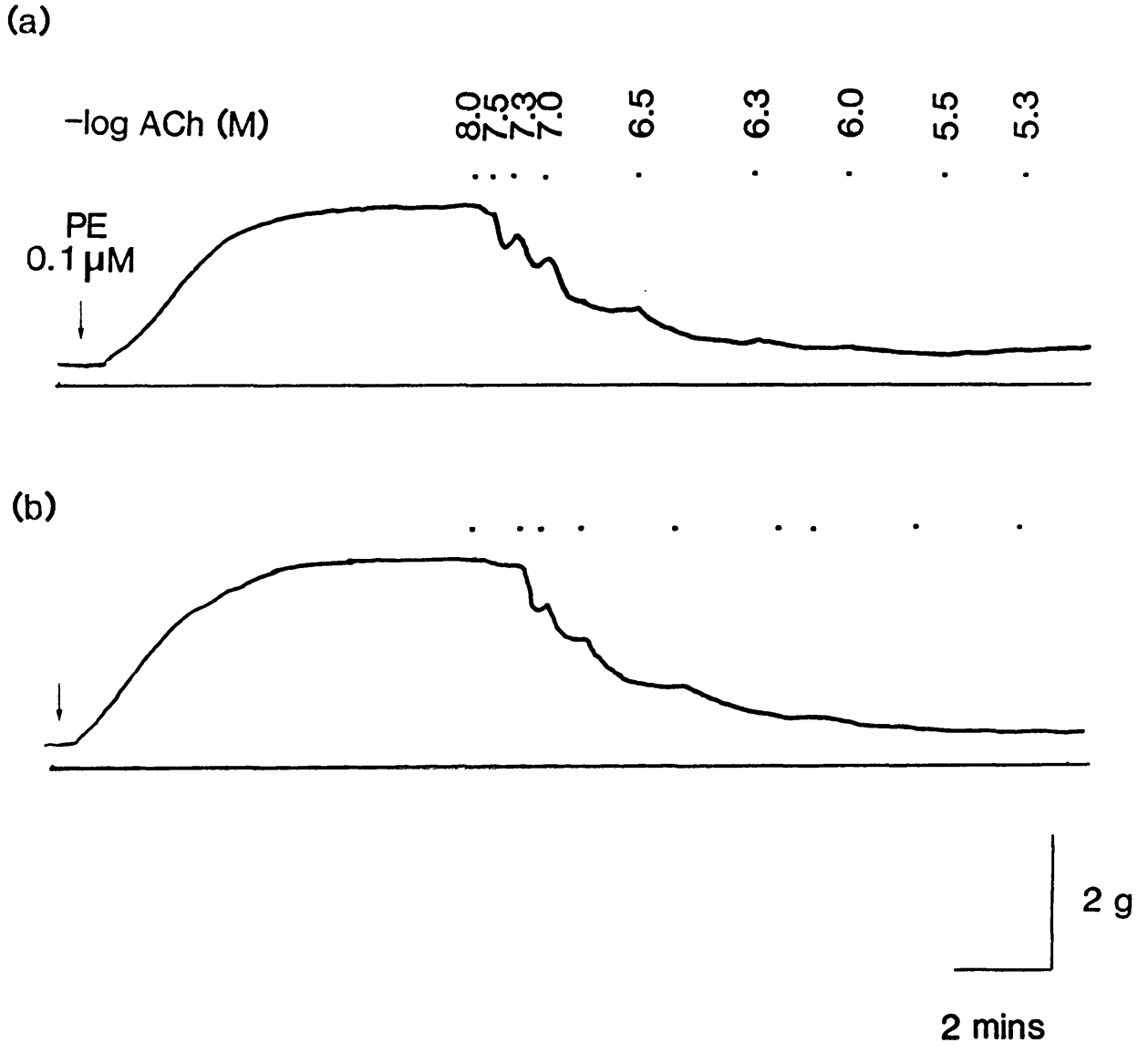

Figure 9 : The influence of vehicle pre-incubation on ACh-evoked relaxations

Tissues pre-contracted with PE (0.1 μM) and relaxed to cumulative doses of ACh (0.01-5μM)

(a) Control

(b) After 30 mins pre-incubation with vehicle (water)

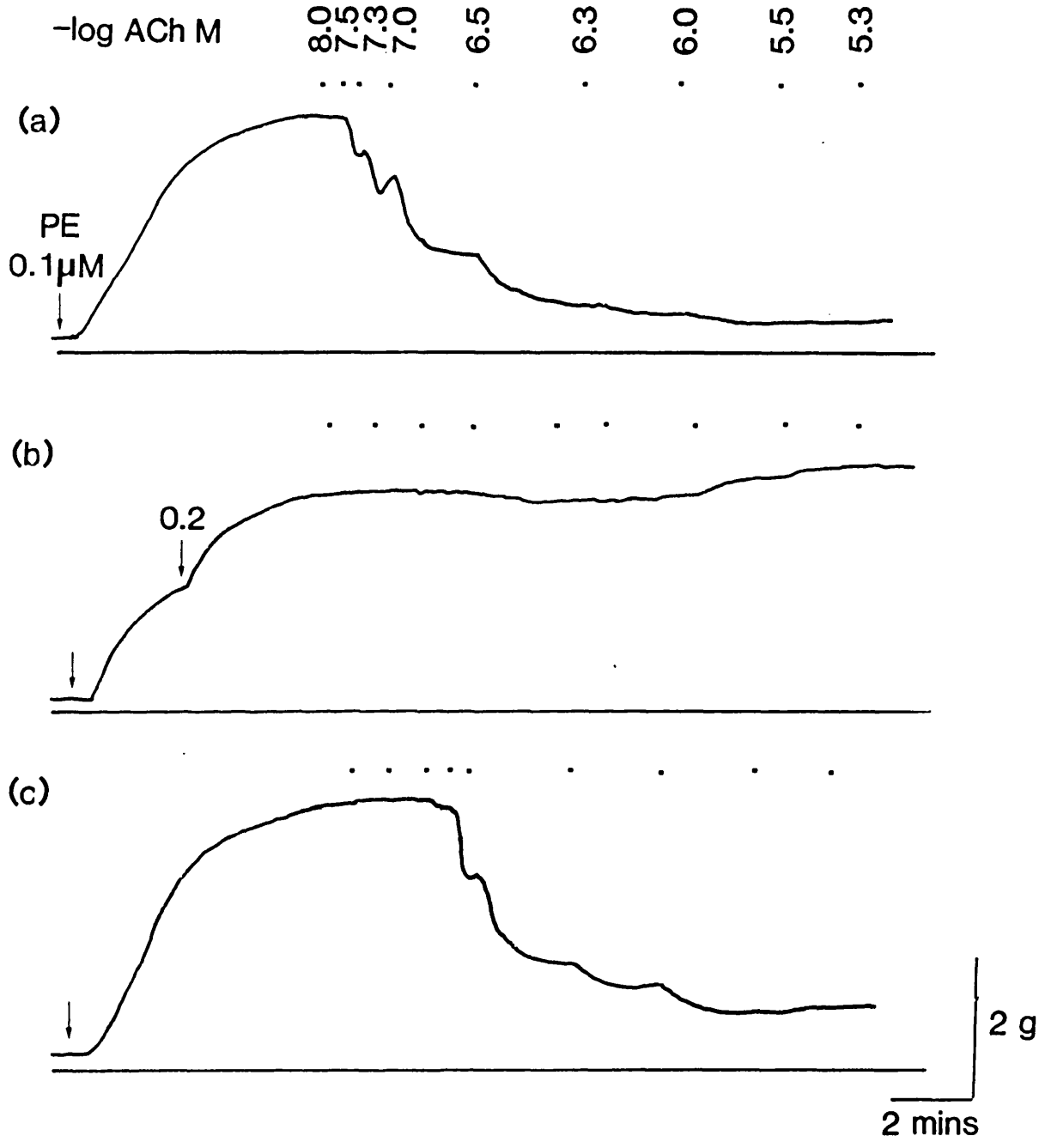

Figure 10 : ACh-evoked relaxations in the absence and presence of 1-palmitoyl LPC

Tissues pre-contracted with PE (0.1-0.2 μM) and relaxed to cumulative doses of ACh (0.01-5 μM)

(a) Control

(b) In the presence of 10 μM 1-palmitoyl LPC

(c) After washout

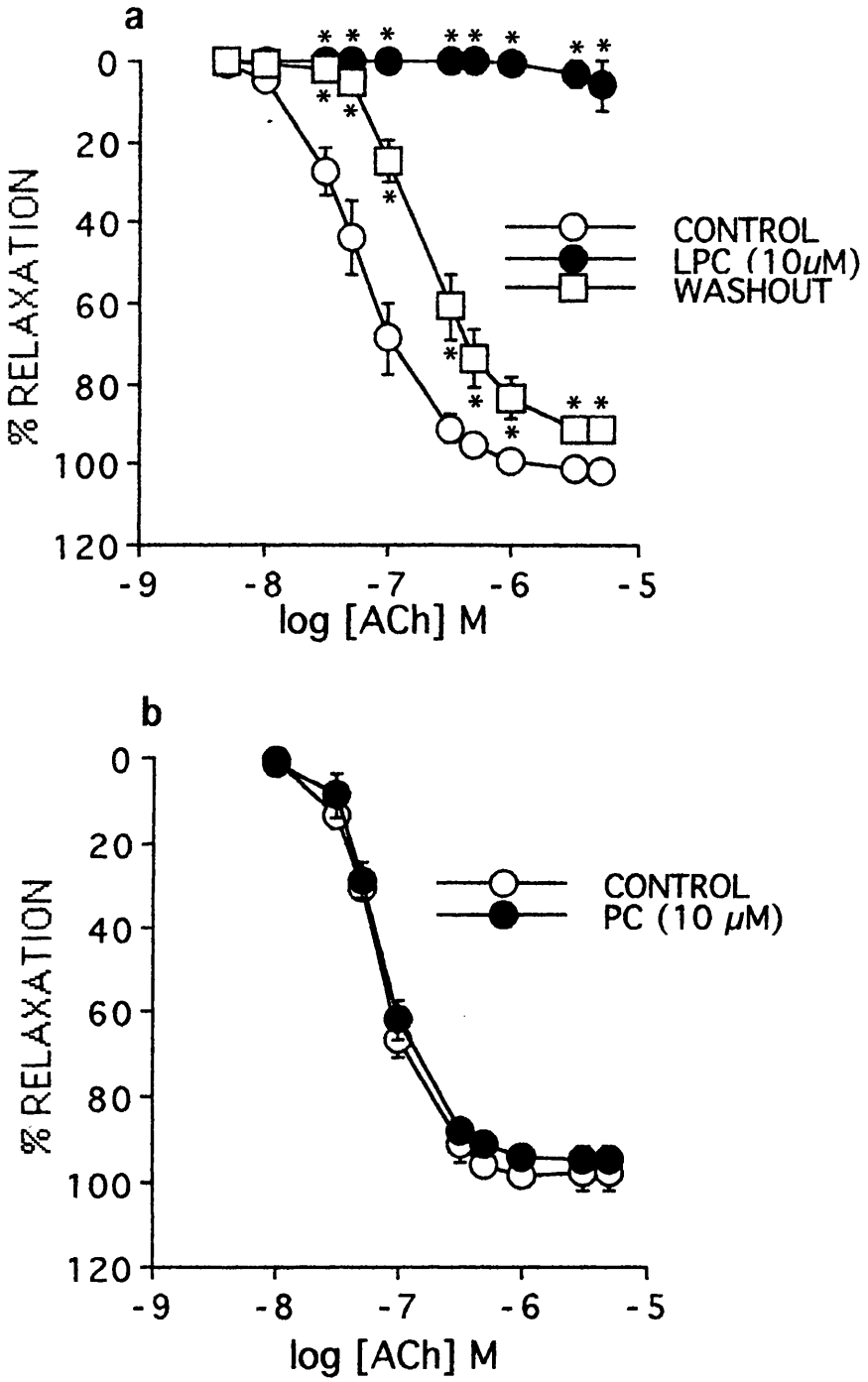

Figure 11 : The influence of 1-palmitoyl LPC and dipalmitoyl PC on ACh-evoked relaxations

Tissues were pre-contracted with PE (0.1-0.2 μM) and relaxed to cumulative doses of ACh (0.01-5 μM). Following washout and equilibration, 10 μM LPC or 10 μM PC added to the tissues and contraction/relaxation cycle repeated. After further washout and equilibration, rings re-contracted and re-challenged with ACh (n=4 for each).

(a) 10 μM 1-palmitoyl LPC

(b) 10 μM dipalmitoyl PC

Students' t-test compared to control : * $p<0.05$.

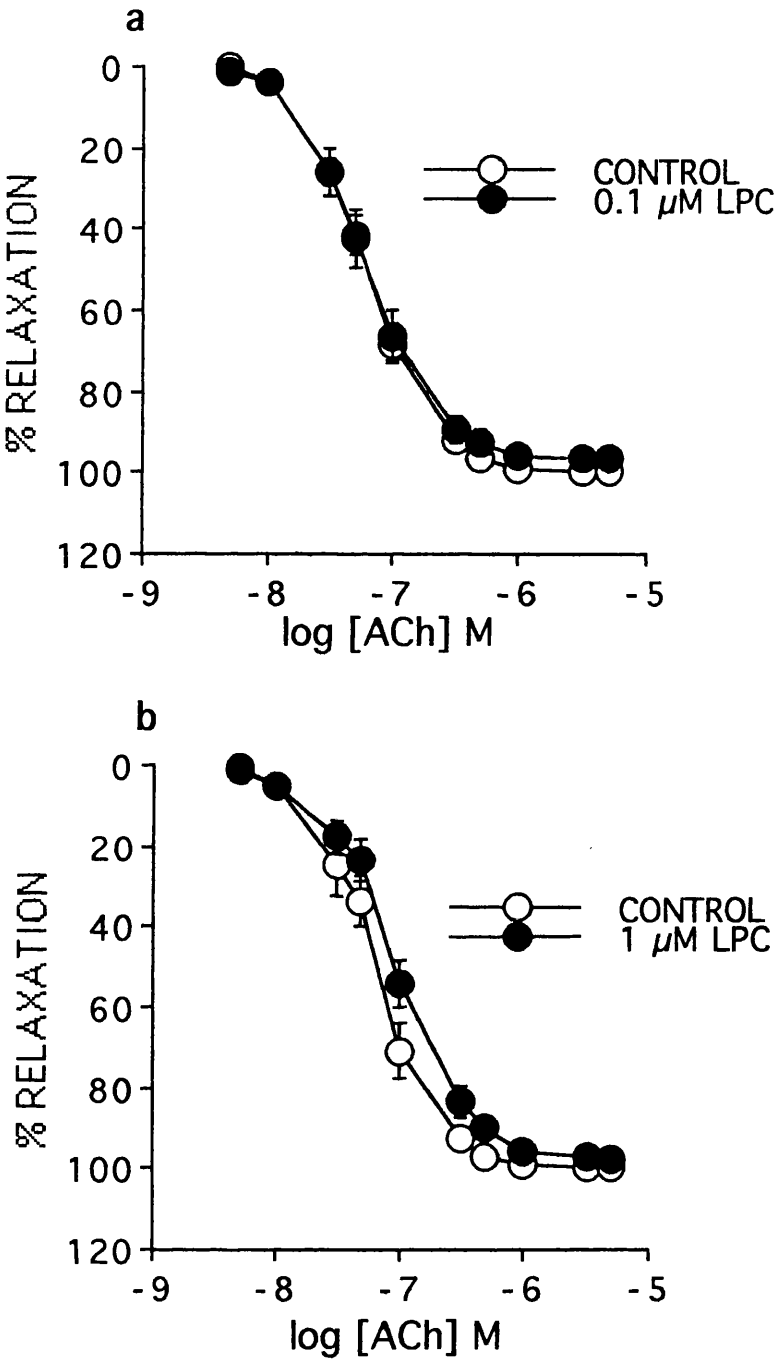

Figure 12 : ACh dose response curves in the absence and presence of 1-palmitoyl LPC (0.1 μM and 1 μM)

Aortic rings pre-contracted with PE (0.1 μM) and relaxed to cumulative doses of ACh (0.01-5 μM). Following washout and equilibration, 0.1 μM or 1 μM 1-palmitoyl LPC added to tissues and contraction/relaxation cycle repeated. After further washout and equilibration, tissues re-challenged with PE and ACh (n=4 for each).

(a) 0.1 μM 1-palmitoyl LPC

(b) 1 μM 1-palmitoyl LPC

Students' t-test compared to control : * $p<0.05$.

as shown in **Figure 12 a** and **b** respectively. After 30 mins pre-incubation, the maximal relaxation to ACh was significantly decreased by 23 ± 3% (**Figure 13b;** n=4) in the presence of 1 μM 1-palmitoyl LPC. However, even after 30 mins, 0.1 μM 1-palmitoyl LPC failed to inhibit relaxations evoked by ACh (**Figure 13a**).

The effect of 1-stearoyl LPC on ACh-evoked relaxations was also investigated. In the presence of 1 μM 1-steroyl LPC responses to ACh were unaffected (**Figure 14a**) whereas at a concentration of 10 μM, relaxations were abolished, as shown in **Figure 14b**. This effect was partially reversed after washout. This pattern of effects closely resembles that of 1-palmitoyl LPC.

In contrast, 1-caproyl LPC (10 μM) had no effect on ACh-evoked relaxations in tissues pre-contracted with PE (n=2).

For the remainder of the experiments in this chapter and those in chapter 4, the term LPC will refer solely to 1-palmitoyl LPC.

3.3.2 DUAL EFFECTS OF LPC IN PRE-CONTRACTED TISSUES

As shown in **Figure 11a**, ACh-evoked relaxations are inhibited by pre-incubating the tissues with 10 μM LPC. A representative trace of 4 experiments shown in **Figure 15a**, illustrates that LPC can also reverse maximal relaxation elicited by 1μM ACh. In addition, these experiments show that the time course of inhibition of EDR evoked by ACh by LPC is rapid (~ 1 min).

The dual effects of LPC, EDR of pre-contracted rings and inhibition of ACh-evoked responses, can be demonstrated in the same tissue. As shown in **Figure 15b**, 10 μM LPC partially relaxed a pre-contracted endothelium-intact ring, with the subsequent response of 1 μM ACh abolished in the presence of

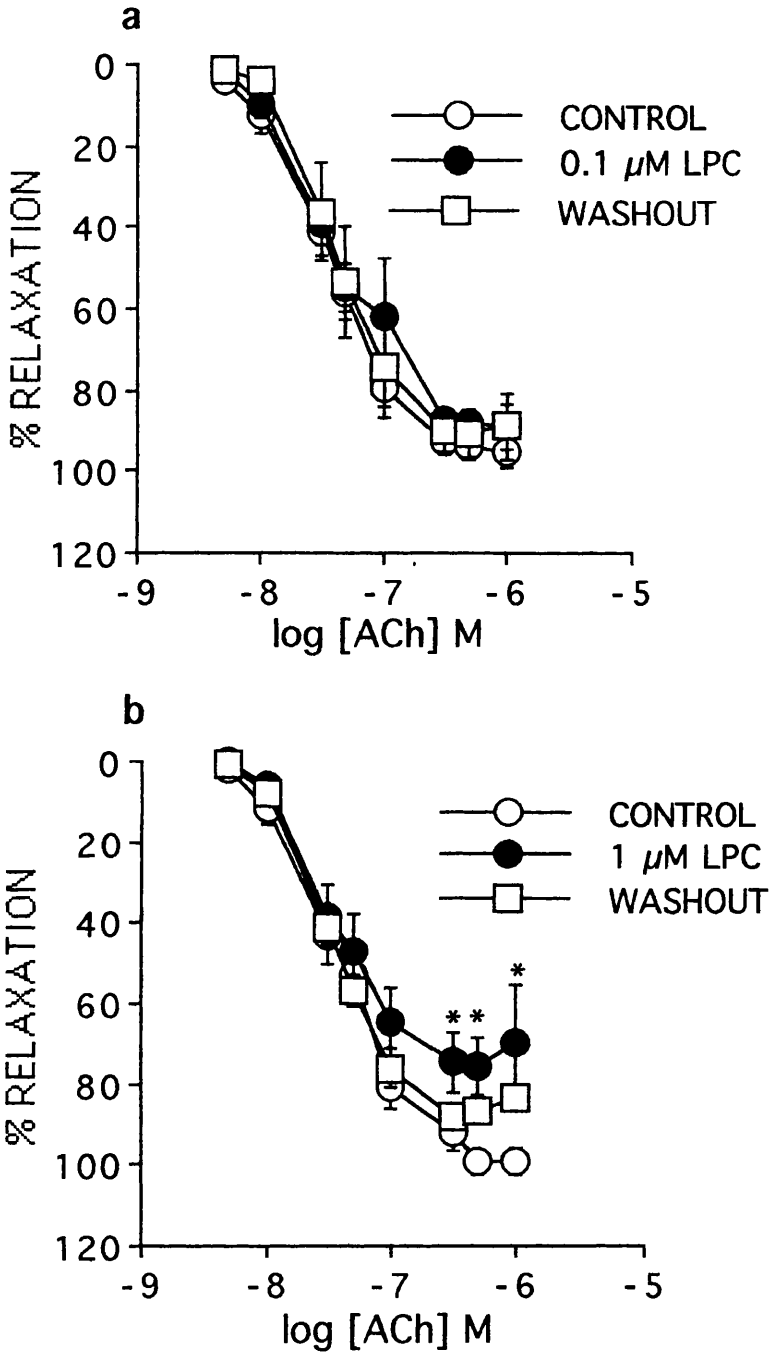

Figure 13 : ACh dose-response curves in the absence and presence of 1-palmitoyl LPC pre-incubated for 30 mins

Aortic rings pre-contracted with PE (0.1 μM) and relaxed to cumulative doses of ACh (0.01-5 μM). Following washout and equilibration, tissues pre-incubated with 0.1 μM or 1 μM LPC for 30 mins before contraction/relaxation cycle repeated. After further washout and equilibration, tissues re-challenged with PE and ACh (n=4 for each).

(a) 0.1 μM 1-palmitoyl LPC

(b) 1 μM 1-palmitoyl LPC

Students' t-test compared to control : * $p<0.05$.

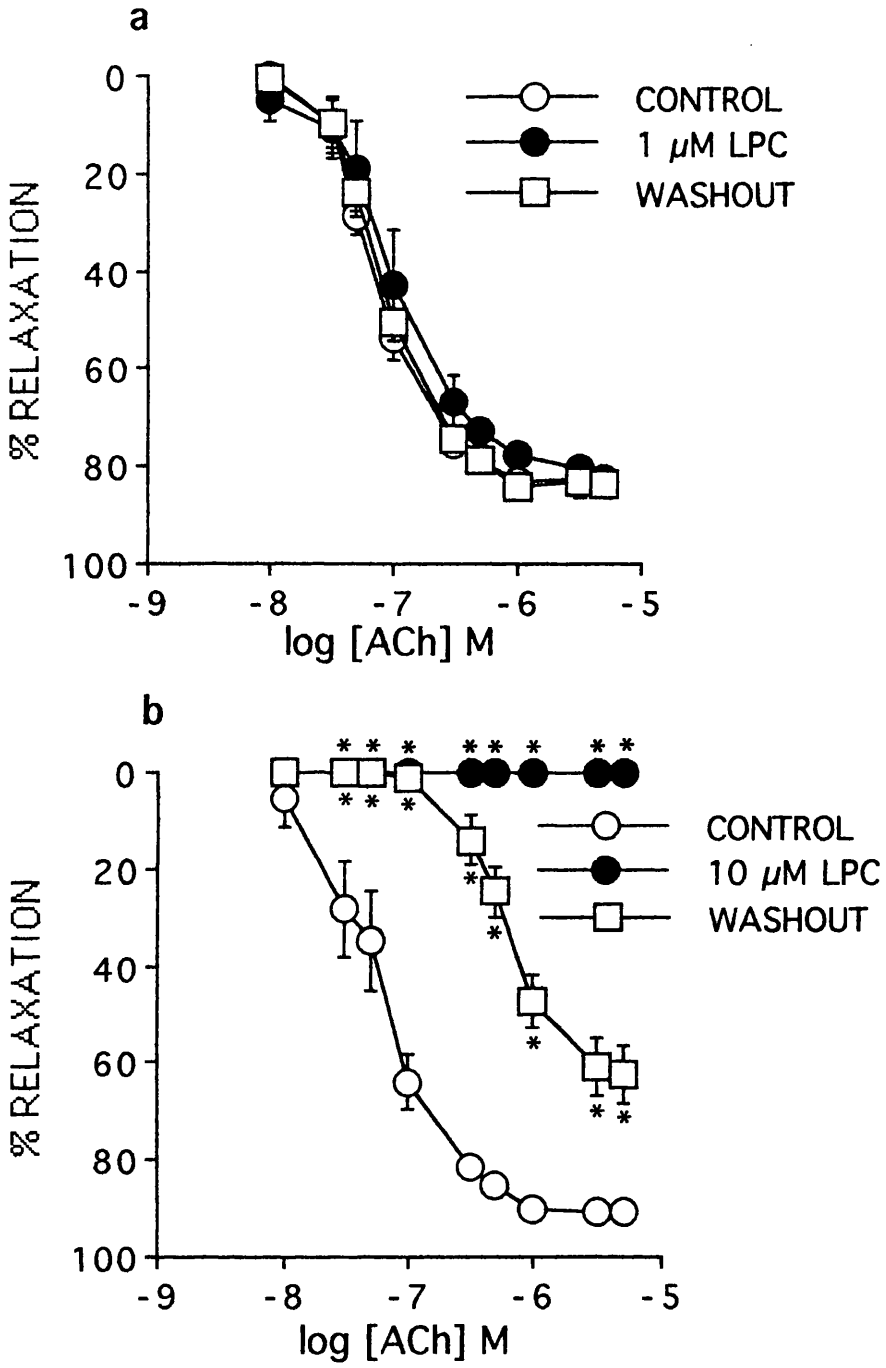

Figure 14 : The influence of 1-stearoyl LPC on ACh-evoked relaxations
Aortic rings pre-contracted with PE (0.1-0.2 μM) and relaxed to cumulative doses of ACh (0.01-5 μM). Following washout and equilibration, 1 μM or 10 μM 1-stearoyl LPC added to tissues and contraction/relaxation cycle repeated. After further washout and equilibration, tissues re-challenged with PE and ACh (n=4 for each).
(a) 1 μM 1-stearoyl LPC
(b) 10 μM 1-stearoyl LPC
Students' t-test compared to control : * $p<0.05$.

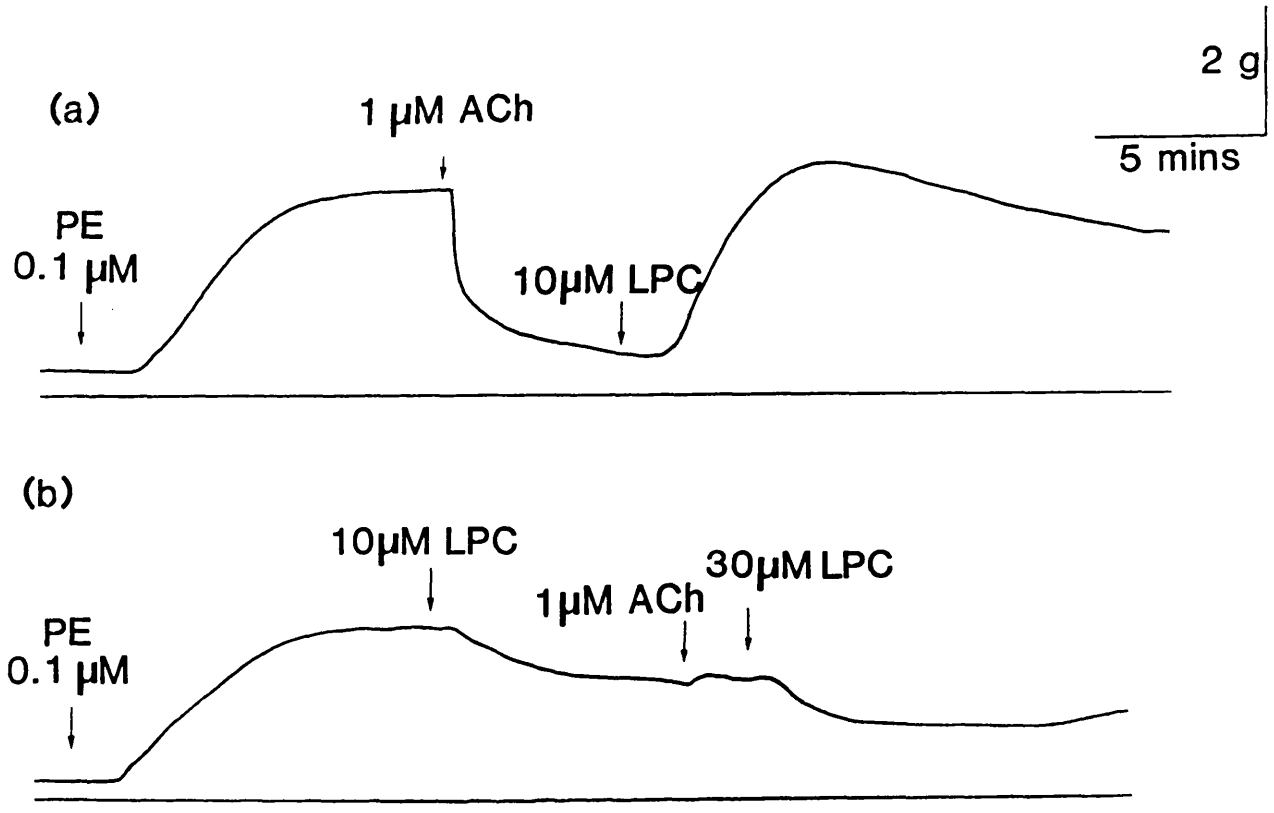

Figure 15 : Reversal of ACh-evoked relaxation by LPC and dual effects of LPC in pre-contracted tissues

(a) Tissue pre-contracted with PE (0.1 μM) followed by addition of ACh (1 μM), and reversal of relaxation by 10 μM LPC

(b) 10 μM LPC added to tissue pre-contracted with PE (0.1 μM). At plateau of response, 1 μM ACh was added, and then LPC concentration cumulatively increased to 30 μM LPC.

the lysophospholipid. When the concentration of LPC was cumulatively increased to 30 μM, further relaxation of the tissue was observed.

3.3.3 EFFECT OF INDOMETHACIN ON THE ATTENUATION OF ACh-EVOKED RELAXATIONS BY LPC

Pre-incubation of aortic rings with indomethacin (10 μM) for 15 mins did not alter the sensitivity of PE-contracted tissues to ACh as shown in **Figure 16a**. In addition, indomethacin did not reduce the inhibition of ACh-evoked relaxations caused by the presence of LPC (10 μM). **Figure 16b** shows ACh dose response curves in the absence and presence of LPC (10 μM) plus indomethacin. This observation suggests that cyclo-oxygenase products do not play a role in the endothelium-dependent responses of ACh nor in the inhibition of these relaxations caused in the presence of LPC.

3.3.4 EFFECT OF L-ARGININE ON THE ATTENUATION OF ACh-EVOKED RELAXATIONS BY LPC

As discussed in section 1.4.4, the basic amino acid L-arginine is the precursor of EDRF, the mediator of ACh-evoked relaxations. Pre-incubation of the tissues with L-arginine (100 μM) for up to 30 mins did not influence responses to ACh (**Figure 17a**). Furthermore, L-arginine (100 μM) incubated for 30 mins did not prevent or reduce the attenuation of ACh-evoked relaxations by LPC (10 μM; **Figure 17b**). The lack of effect of L-arginine on control ACh dose response curves suggests that L-arginine availibility is not rate-limiting for EDRF production in the rabbit aorta. In addition, depletion of cellular stores of L-arginine by LPC seems an unlikely explanation for the observed effects of LPC.

3.3.5 INFLUENCE OF BSA ON THE ATTENUATION OF ACh-EVOKED RELAXATIONS BY LPC

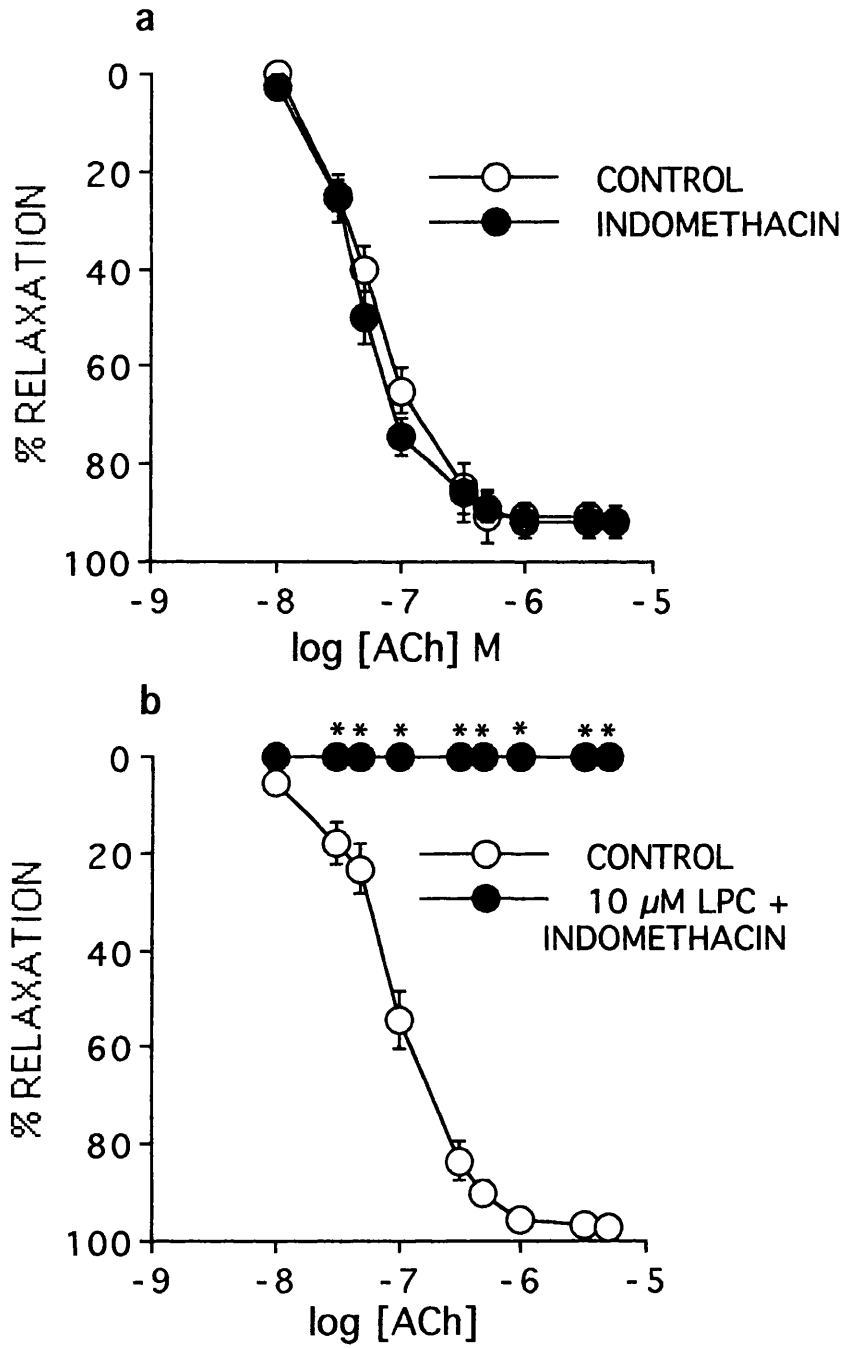

Figure 16 : The influence of indomethacin on the inhibition of ACh-evoked relaxations by LPC

Aortic rings pre-contracted with PE (0.1-0.2 μM) and relaxed to cumulative doses of ACh (0.01-5 μM). Following washout and equilibration, indomethacin (10 μM) added to the baths and incubated for 15 mins. LPC (10 μM) then added to bath and and contraction/relaxation cycle repeated. After further washout and equilibration, tissues re-challenged with PE and ACh (n=4 for each).

(a) Indomethacin (10 μM)

(b) LPC (10 μM) and indomethacin (10 μM)

Students' t-test compared to control : * $p<0.05$.

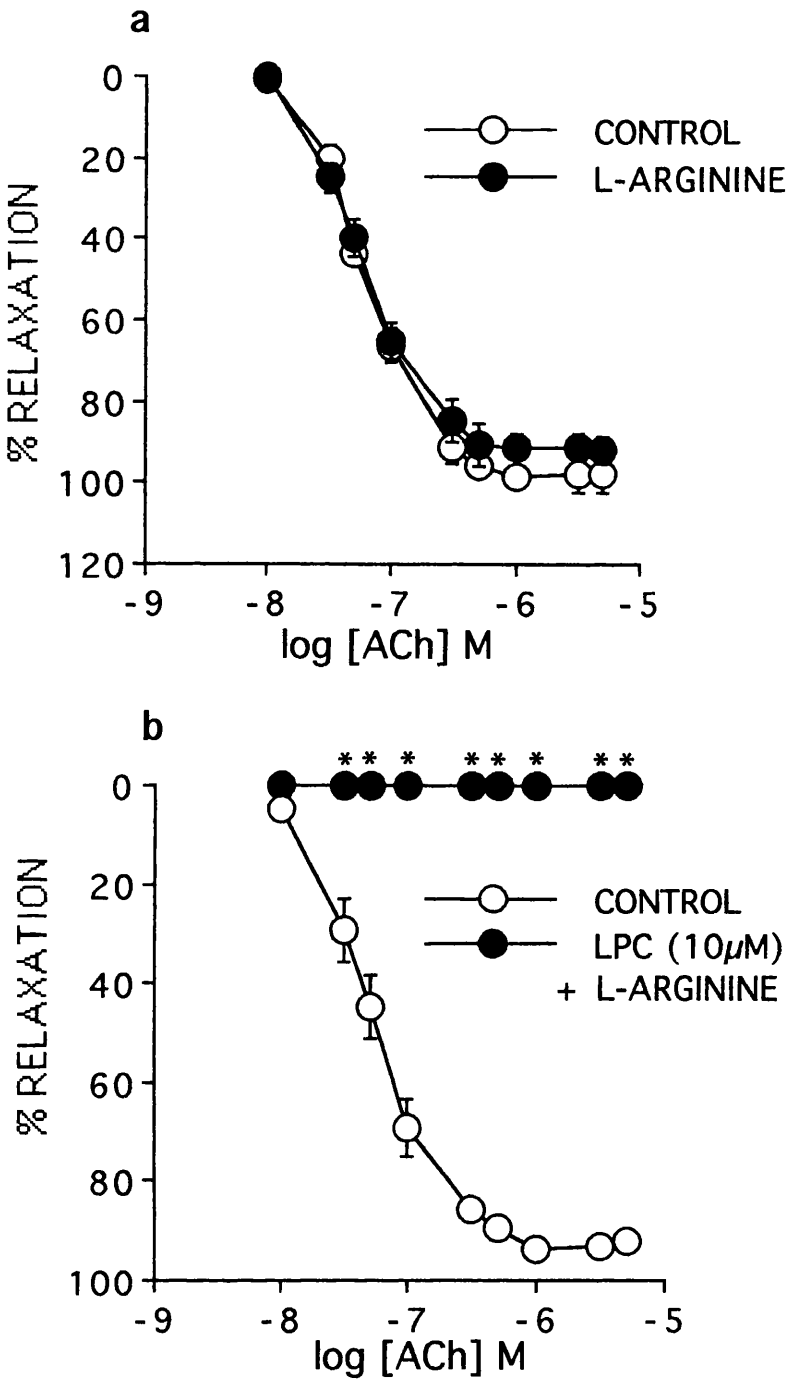

Figure 17 : The influence of L-arginine on the inhibition of ACh-evoked relaxations by LPC

Aortic rings pre-contracted with PE (0.1-0.2 μM) and relaxed to cumulative doses of ACh (0.01-5 μM). Following washout and equilibration, L-arginine (100 μM) added to the bath and incubated for 30 mins. LPC (10 μM) then added to the bath and contraction/relaxation cycle repeated. After further washout and equilibration, tissues re-challenged with PE and ACh (n=4 for each).

(a) L-arginine (100 μM)

(b) LPC (10 μM) and L-arginine (100 μM)

Students' t-test compared to control : * $p<0.05$.

In these experiments, the ability of BSA (fatty acid free) to prevent the inhibitory effects of LPC was investigated. BSA (0.1-1 mg protein/ml) did not alter the sensitivity of the tissues to ACh as shown in the dose response curves (DRCs) in **Figure 18a**. However, BSA did decrease the inhibition of ACh responses by LPC (10 μM). The reduction in the inhibitory action of LPC (10 μM) by BSA is concentration dependent over the range 0.1-1 mg protein/ml, as shown in **Figure 18b**. As can be seen from these findings, the sensitivity of the tissues to ACh can be partially or fully restored in the presence of BSA suggesting that LPC is a lipophilic substance and is readily taken up into the albumin molecule.

3.3.6 EFFECT OF LPC ON OTHER ENDOTHELIUM-DEPENDENT RELAXANTS

To investigate whether the inhibitory effects of LPC are the result of a specific action on endothelial cell muscarinic receptors, the influence of LPC on other endothelium-dependent agonists was investigated.

ATP, like ACh, evokes receptor-linked EDR relaxations in the rabbit aorta. Responses elicited by ATP (1-300 μM) were unaltered in the presence of 1 μM LPC; maximum relaxation in control tissues (66.5 ± 3.3%; n=4) did not significantly differ from treated rings (67.5 ± 1.0%; n=4). LPC at a concentration of 5 μM inhibited ATP-elicited responses only at the highest dose (300 μM), reducing relaxations from 64.0 ± 3.7% (n=4) to 46.7 ± 5.7% (n=4), as shown in **Figure 19a**. In the presence of 10 μM LPC, ATP-evoked relaxations were almost abolished (**Figure 19b**). This effect was partially reversed by washing the tissues. This pattern of effects closely resembles the inhibition of ACh-evoked responses.

The effect of LPC on endothelium-dependent relaxations was also

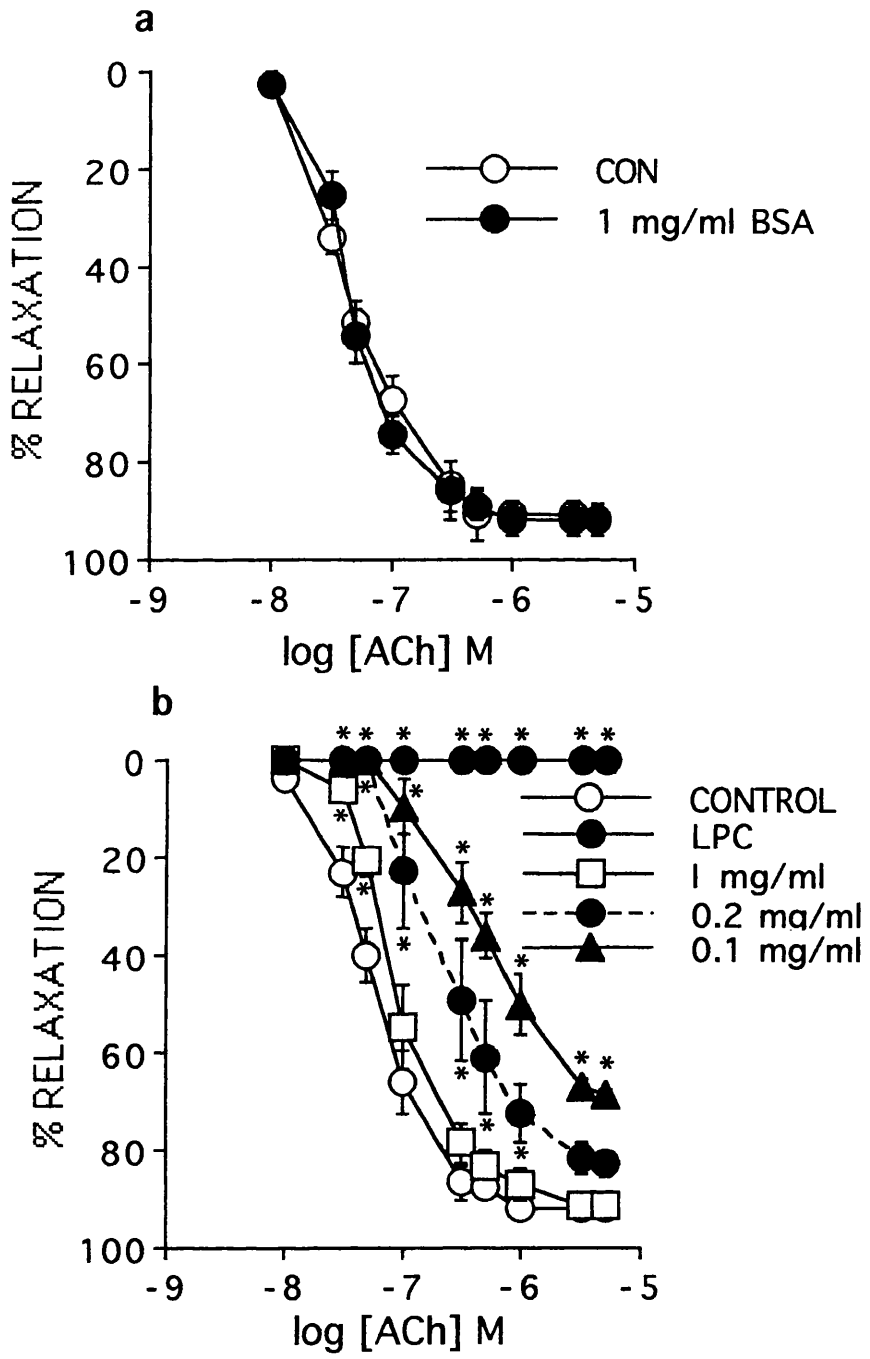

Figure 18 : The influence of BSA on the attenuation of ACh-evoked relaxations by LPC

Aortic rings pre-contracted with PE (0.1-0.2 μM) and relaxed to cumulative doses of ACh (0.01-5 μM). Following washout and equilibration, BSA (0.1-1 mg) was added to the bath, and LPC (10 μM) immediately after, and contraction/relaxation cycle repeated (n=4 for each).

(a) 1 mg/ml BSA

(b) LPC (10 μM) with BSA (0.1-1 mg)

Students' t-test compared to control : * $p<0.05$.

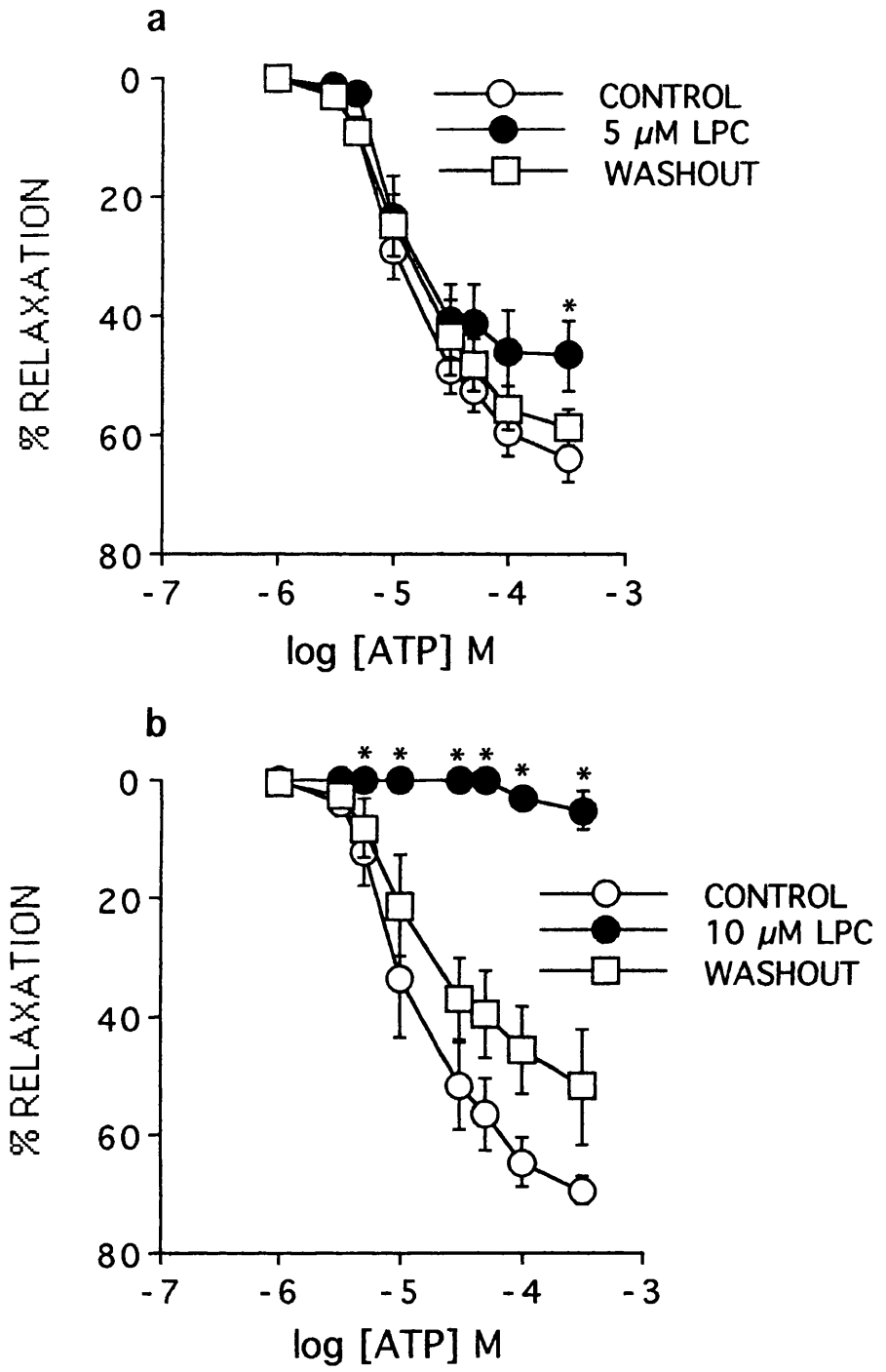

Figure 19 : The influence of LPC on ATP-evoked relaxations
Aortic rings pre-contracted with PE (0.1-0.2 μM) and relaxed to cumulative doses of ATP (1-300 μM). Following washout and equilibration, 5 μM and 10 μM LPC added to the tissues and contraction/relaxation cycle repeated. After further washout and equilibration, tissues re-challenged with PE and ATP (n=4 for each).
(a) 5 μM LPC
(b) 10 μM LPC
Students' t-test compared to control : * $p<0.05$.

investigated using the calcium ionophore A23187. This agonist acts by increasing intracellular Ca^{2+} concentration via a receptor-independent mechanism. A typical trace showing dose-dependent A23187-evoked relaxations of a PE (0.1 μM) pre-constricted aortic ring is shown in **Figure 20a**. In the presence of 1 μM LPC, added to the bath immediately before pre-constriction, A23187-induced relaxations were unaltered (maximum relaxation control 95.2 ± 4.8 vs LPC-treated 93.8 ± 3.6; n=4). As shown in **Figure 21a**, A23187-evoked responses were significantly attenuated by 5 μM LPC, with maximum responses reduced from 91.2 ± 1.6% (n=4) to 64.5 ± 6.7% (n=4). A representive trace of the effect of 10 μM LPC on A23187-evoked relaxations is shown in **Figure 20b.** DRCs from 4 separate experiments showed that the maximal relaxations were inhibited by 53.0 ± 3.1% (n=4; **Figure 21b**). Washing the tissues partially reversed the effects of 10 μM LPC (**Figures 20c** and **21b**).

Although the responses to A23187 were greatly reduced by LPC, they were not completely inhibited as were ACh and ATP-evoked relaxations.

3.3.7 LPC AND NO-EVOKED RELAXATIONS

EDRF, released from the rabbit aorta in response to various physical and chemical stimuli, has been identified as NO or a NO-releasing compound (section 1.4.2) which activates soluble guanylate cyclase in the underlying smooth muscle to increase cGMP levels and evoke relaxation. Dose-dependent, endothelium-independent transient relaxations of pre-contracted rabbit aortic rings can be elicited by addition of exogenous NO to the organ bath. Responses to NO are not altered by an intact endothelium (**Figure 22**). The maximal relaxation to NO in endothelium-intact tissues was 77.8 ± 3.3% (n=5) and 82.7 ± 3.5% (n=4) in denuded tissues.

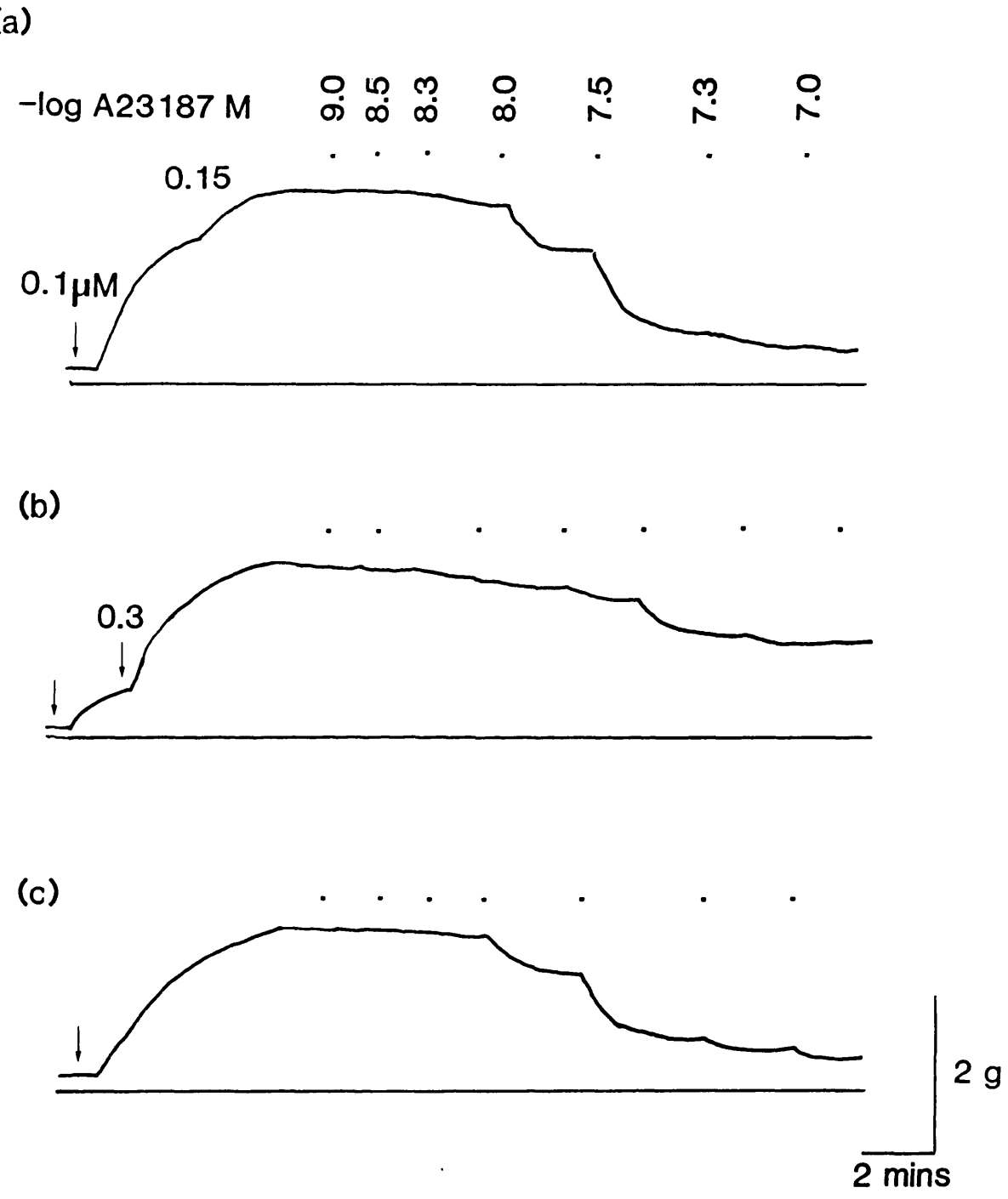

Figure 20 : A23187-evoked relaxations in the absence and presence of LPC

Tissues pre-contracted with PE (0.1-0.3 μM) and relaxed to cumulative doses of A23187 (1-100 nM)

(a) Control
(b) In the presence of LPC (10 μM)
(c) After washout

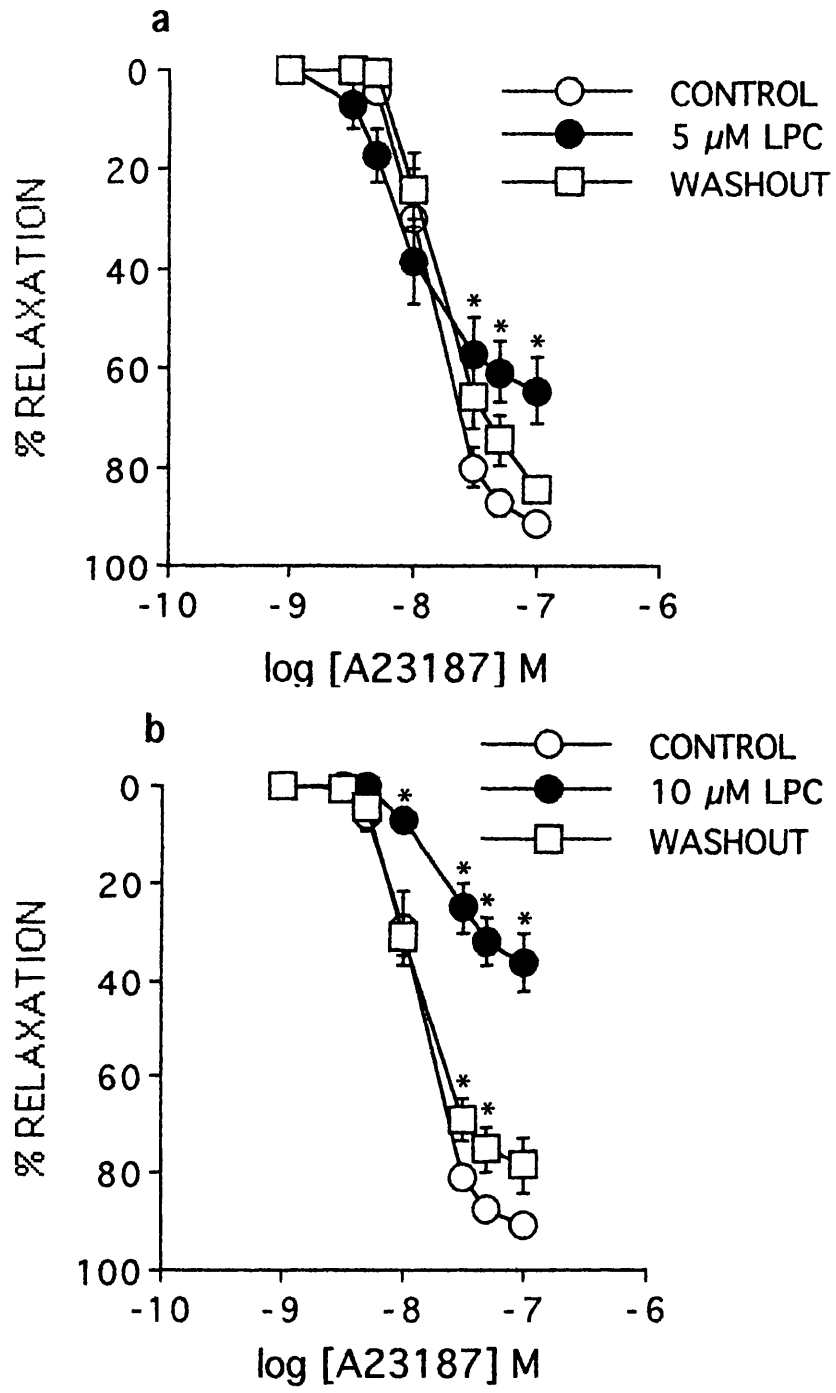

Figure 21 : The influence of LPC on A32187-evoked relaxations
Aortic rings pre-contracted with PE (0.1-0.3 μM) and relaxed to cumulative doses of A23187 (1-100 nM). Following washout and equilibration, 5 μM and 10 μM LPC added to the tissues and contraction/relaxation cycle repeated. After further washout and equilibration, tissues re-challenged with PE and A23187 (n=4 for each).
(a) 5 μM LPC
(b) 10 μM LPC
Students' t-test compared to control : * $p<0.05$.

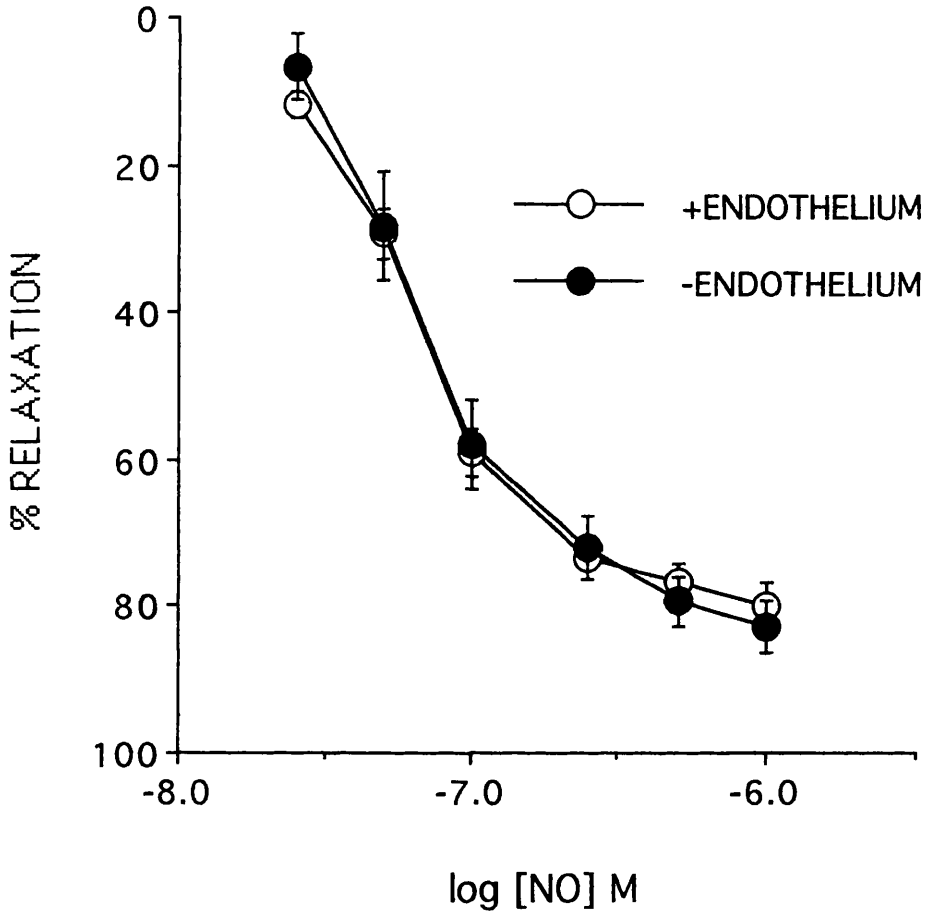

Figure 22 : NO-evoked relaxations in endothelium-intact and denuded tissues

Endothelium-intact and denuded aortic tissues pre-contracted with PE (0.1 μM) and relaxed to increasing doses of NO (0.025-1 μM; n=4-5 for each).

To examine whether the inhibition of EDR by LPC could be due, at least in part, to direct interaction between NO and LPC, the effect of LPC on responses to exogenous NO was investigated.

In endothelium-intact, PE (0.1 μM) contracted tissues, NO (0.025-1 μM) evokes relaxations which were significantly inhibited in the presence of 10 μM LPC as compared to control. Relaxations to NO were restored to control levels after washing. This attenuation is illustrated in **Figure 23a** showing a rightward shift in the dose response curve to NO but without significant effect on the maximal relaxation. Before LPC treatment, the maximal relaxation evoked by NO was 79.8 ± 3.3% (n=5) and in the presence of LPC the mean response was 69.8 ± 4.2% (n=5).

The effect of LPC on relaxations to exogenous NO in rabbit aortic rings denuded of endothelium was also examined. In contrast to endothelium-intact tissues, dose-dependent relaxations of pre-constricted rings to NO remained unchanged in the presence of LPC (10 μM) as shown in **Figure 23b**.

These findings seem to exclude a direct interaction between NO and LPC as a possible mechanism for the observed inhibition of EDR (see **Figures 11a, 14b, 19b, 20b**). Furthermore, the inhibition of NO-evoked responses by LPC in endothelium-intact rings appears to be due to the release of a LPC-induced inhibitory factor from the endothelium (**Figure 23a**).

3.3.8 LPC AND GTN-EVOKED RELAXATIONS

The nitrovasodilator GTN evokes endothelium-independent relaxations by direct activation of guanylate cyclase of smooth muscle to increase cGMP levels. NO released intracellularly is believed to be an intermediate in the action of GTN. To investigate further the possible sites at which LPC acts to inhibit endothelium-dependent responses and those to exogenous NO, the

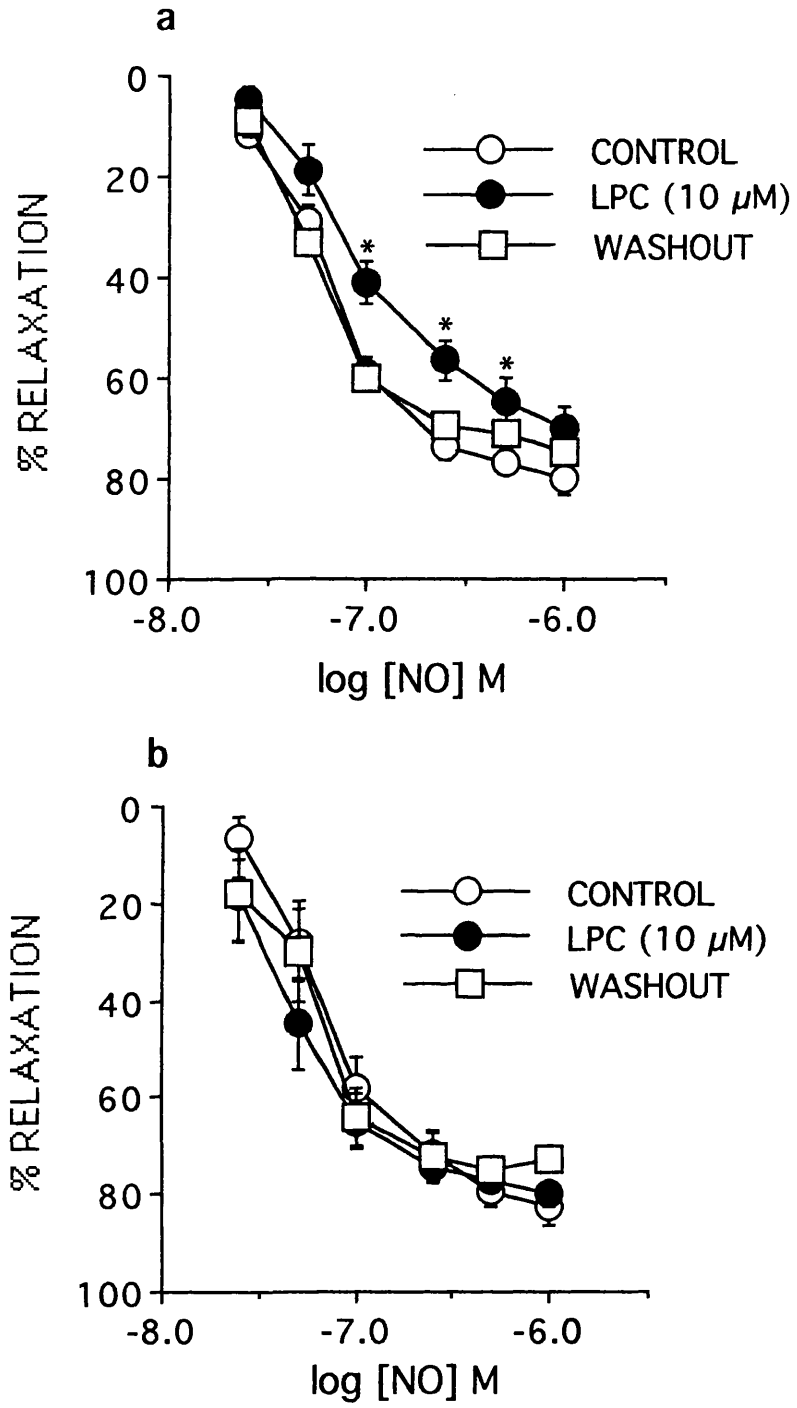

Figure 23 : The influence of LPC on NO-evoked relaxations in endothelium-intact and denuded tissues

Aortic rings pre-contracted with PE (0.1-0.2 μM) and relaxed to increasing doses of NO (0.025-1 μM). Following washout and equilibration, 10 μM LPC added to the tissues and contraction/relaxation cycle repeated. After further washout and equilibration, tissues re-challenged with PE and NO.
(a) LPC (10 μM) in endothelium-intact tissues (n=5)
(b) LPC (10 μM) in endothelium-denuded tissues (n=4)
Students' t-test compared to control : * $p<0.05$.

effect of LPC on GTN-evoked relaxations was assessed.

As shown in **Figure 24** GTN-evoked relaxations (0.1 nM-0.5 μM) of pre-constricted aortic rings was not influenced by an intact endothelium. Maximal response of PE-contracted endothelium-intact tissues to GTN was 99.2 ± 1.0% (n=4) and in denuded tissues was 98.0 ± 1.8% (n=4).

In tissues with an intact endothelial cell layer, the presence of LPC (10 μM) attenuated the relaxations induced by cumulative doses of GTN (50 nM-0.5 μM). The mean results of 4 experiments are shown in **Figure 25a**, with LPC inhibited the maximal relaxation by 26.0 ± 4.4% (n=4) and this effect was reversed on washout.

The possibility that the endothelium releases an inhibitory factor in response to LPC which could be EDRF (NO) was examined by pre-incubating the tissues exposed to L-NAME (100 μM) for 15 mins before LPC was added to the bath. In tissues with L-NAME (100 μM) only, responses evoked by GTN were unaltered (98.7 ± 1.2%; n=4). However, L-NAME (100 μM) increased GTN-evoked responses in the presence of LPC (10 μM) from 73.4 ± 5.4% (n=4) to 86.9 ± 3.4% (n=4), but this was not statistically significant.

In contrast, GTN-evoked relaxations in endothelium-denuded rings remained unchanged in the presence of 10 μM LPC, with the maximum relaxation in control dose responses curves 98.0 ± 1.8% (n=4) not significantly different to those in the presence of LPC 91.8 ± 2.6% (n=4; **Figure 25b**).

These findings agree with those from experiments in which NO was used to relax pre-constricted rings in that LPC inhibits endothelium-independent relaxations in endothelium-intact tissues only, indicating that LPC stimulates the release of an inhibitory factor from the endothelium.

3.4 EFFECT OF LPC ON AGONIST-INDUCED CONTRACTIONS

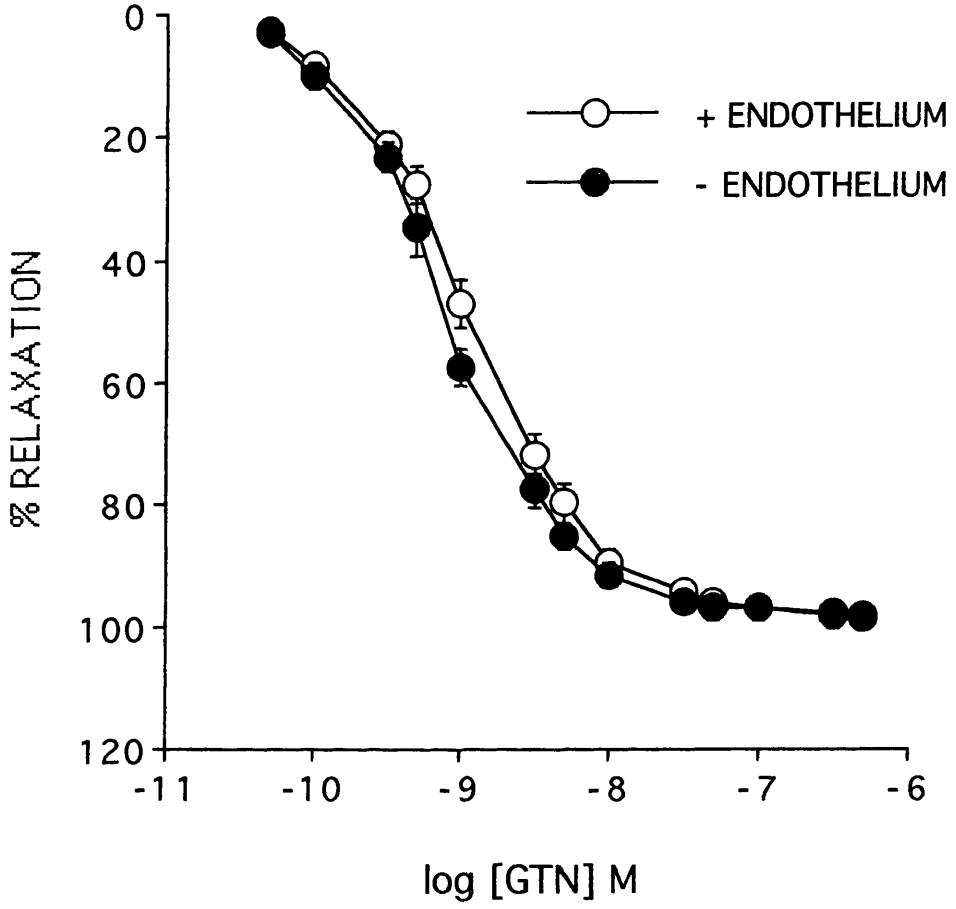

Figure 24 : GTN-evoked relaxations in endothelium-intact and denuded tissues

Endothelium-intact and denuded aortic tissues pre-contracted with PE (0.1 μM) and relaxed to increasing doses of GTN (0.1 nM-0.5 μM; n=4 for each).

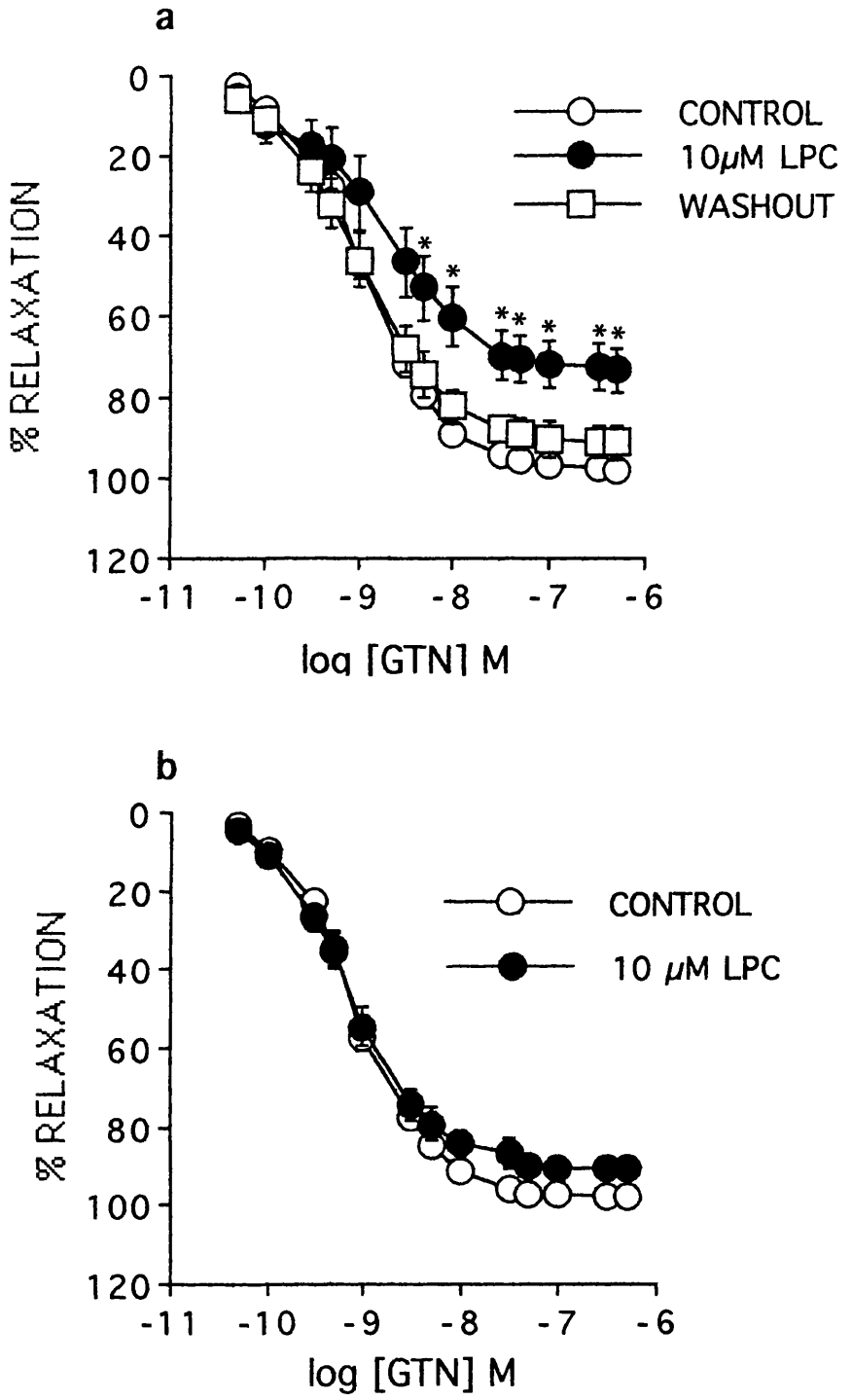

Figure 25 : The influence of LPC on GTN-evoked relaxations in endothelium-intact and denuded tissues

Aortic rings pre-contracted with PE (0.1-0.3 μM) and relaxed to cumulative doses of GTN (0.1 nM-0.5 μM). Following washout and equilibration, 10 μM LPC added to the tissues and contraction/relaxation cycle repeated. After further washout and equilibration, tissues re-challenged with PE and GTN (n=4 for each).

(a) LPC (10 μM) in endothelium-intact tissues

(b) LPC (10 μM) in endothelium-denuded tissues

Students' t-test compared to control : * $p<0.05$.

Cumulative concentrations of PE (0.01-50 μM) and 5-HT (0.01-10 μM) evoke endothelium-independent contractions of rabbit aortic rings. An intact endothelial layer did not alter responses as assessed by EC_{50} and maximum contraction values, shown graphically in **Figure 26a** and **b**. The maximum contraction evoked by PE in endothelium-intact rabbit aortic rings was 4.8 ± 0.19 g (n=5) and in denuded tissues, 4.3 ± 0.33 g (n=5) with EC_{50} values 147 ± 22 nM (n=5) and 112 ± 17 nM (n=5), respectively. 5-HT elicited a maximum contraction of 3.1 ± 0.39 g (n=4) in intact rings, with an EC_{50} of 220 ± 40 nM (n=4) compared to 3.6 ± 0.5 g (n=4) and 224 ± 30 nM (n=4) in denuded tissues.

3.4.1 EFFECT OF LPC ON PE-EVOKED CONTRACTIONS

In endothelium-intact rings, LPC (10 μM) caused a small but significant rightward shift in the dose response curve to PE (**Figure 27a**) with a control EC_{50} of 147 ± 22 nM compared to a value of 505 ± 130 nM in the presence of LPC. The maximal contraction to PE was unchanged; 4.76 ± 0.19 g (n=5) in control tissues and 4.56 ± 0.37 g (n=5) in LPC-treated tissues. The effect of LPC on PE-induced contractions was reversed after washout.

When the experiments were repeated in tissues denuded of endothelium, LPC had no effect on PE-induced contractions as shown in **Figure 27b**. The maximum PE-induced tone was unchanged (control, 4.34 ± 0.33 g, n=5; LPC-treated, 4.63 ± 0.5 g, n=5) as were the EC_{50} values (control, 112 ± 17 nM, n=5; LPC-treated, 120 ± 25 nM, n=5).

3.4.2 EFFECT OF LPC ON 5-HT-EVOKED CONTRACTIONS

5-HT-evoked dose-dependent contractions of endothelium-intact tissues which were depressed in the presence of 10 μM LPC but only significantly at the dose of 0.5 μM (**Figure 28a**). The control EC_{50} value was 220 ± 40 nM

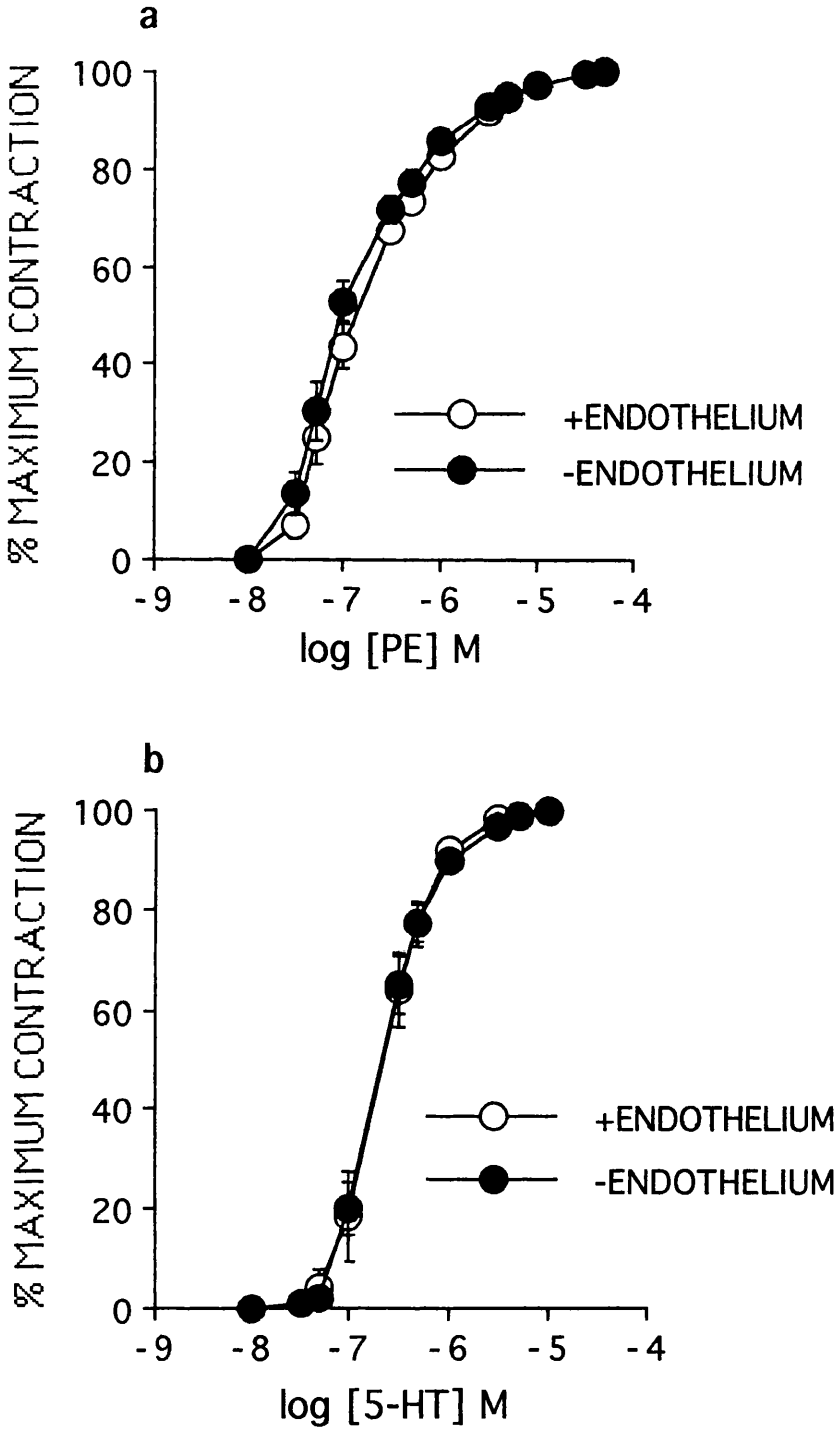

Figure 26 : The influence of the endothelium on contractions to PE and 5-HT

Endothelium-intact and denuded aortic rings contracted with cumulative doses of PE (0.01-50 μM) and 5-HT (0.01-10 μM) until maximal level of induced tone was attained.
(a) PE in endothelium-intact and denuded tissues (n=5)
(b) 5-HT in endothelium-intact and denuded tissues (n=4)
Students' compared to control : * $p<0.05$.

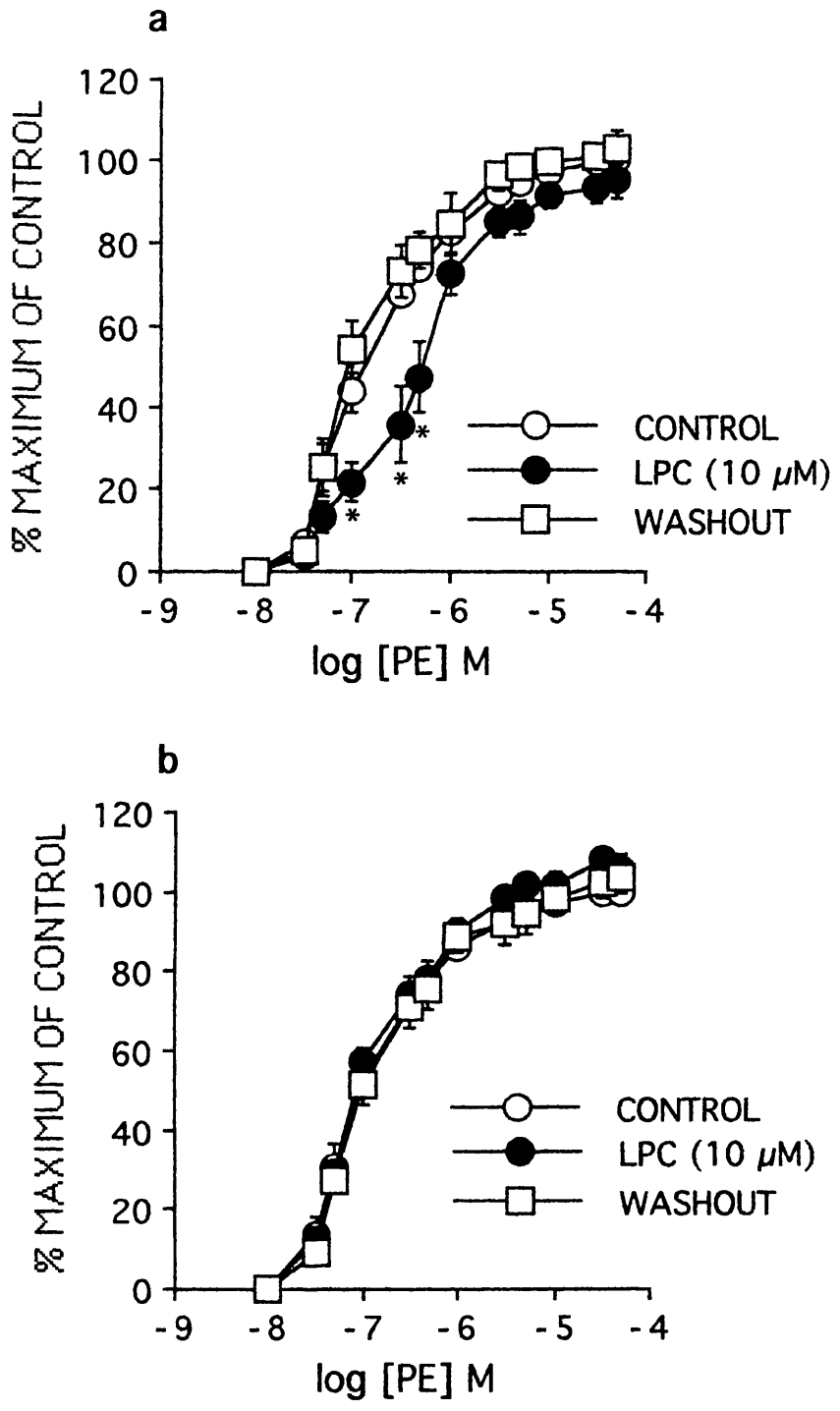

Figure 27 : The influence of LPC on PE-evoked contractions in endothelium-intact and denuded tissues

Endothelium-intact and denuded aortic rings contracted with cumulative doses of PE (0.01-50 μM) until maximal level of induced tone was attained. Following washout and equilibration, 10 μM LPC added to the tissues and cumulative additions of PE repeated. After further washout and equilibration, tissues re-challenged with PE (n=5 for each).

(a) LPC (10 μM) in endothelium-intact tissues

(b) LPC (10 μM) in endothelium-denuded tissues

Students' t-test compared to control : * $p<0.05$.

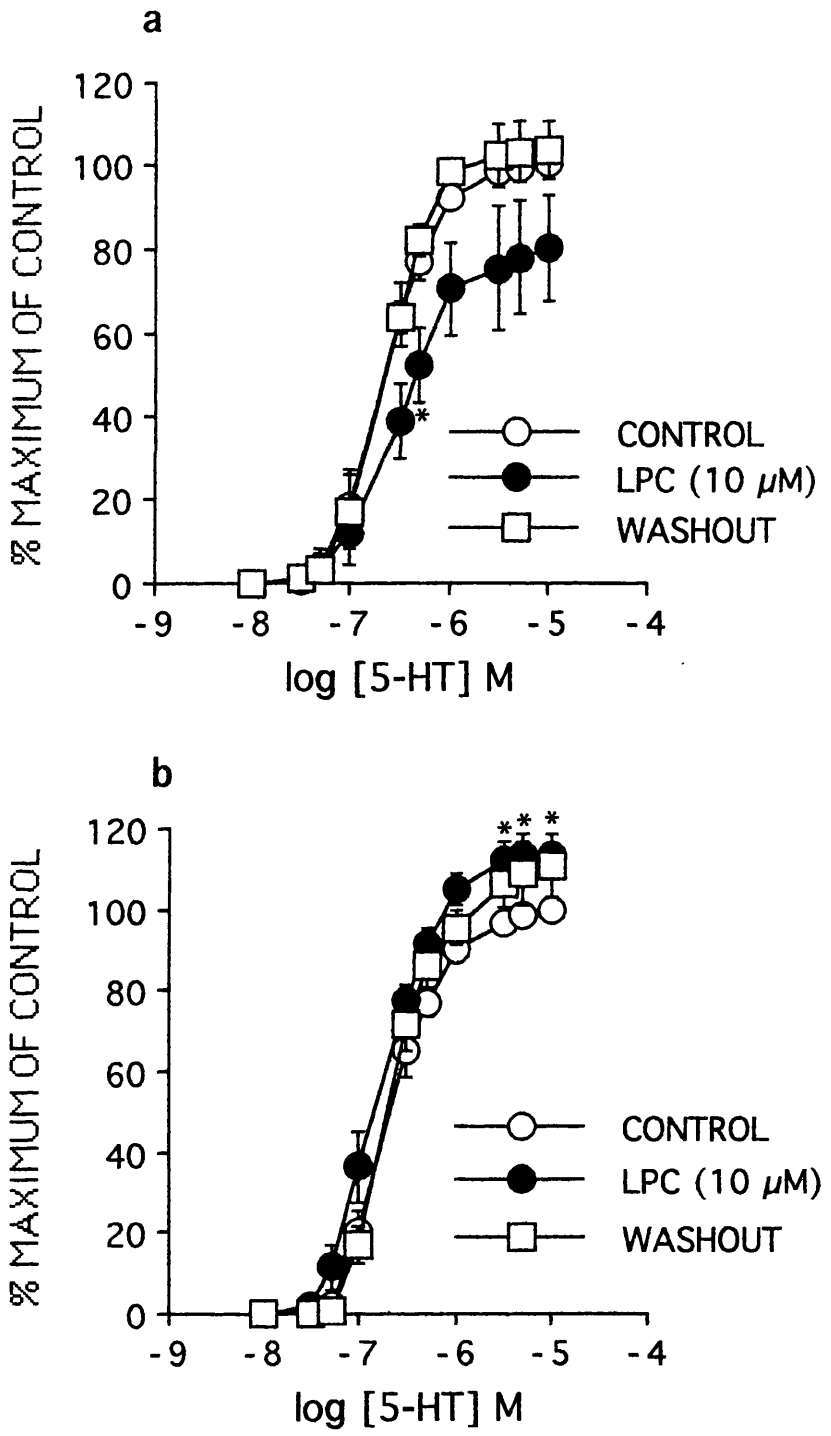

Figure 28 : The influence of LPC on 5-HT-evoked contractions in endothelium-intact and denuded tissues

Endothelium-intact and denuded aortic tissues contracted with cumulative doses of 5-HT (0.01-10 μM) until maximal level of induced tone was attained. Following washout and equilibration, LPC (10 μM) added to the tissues and cumulative additions of 5-HT repeated. After further washout and equilibration, tissues re-challenged with 5-HT (n=4 for each).

(a) LPC (10 μM) in endothelium-intact tissues

(b) LPC (10 μM) in endothelium-denuded tissues

Students' t-test compared to control : * $p<0.05$.

(n=4) and that for LPC-treated rings was 288 ± 96 nM (n=4). In addition, the control maximum contraction of 2.95 ± 2.3 g (n=4) was not significantly different to 2.3 ± 0.3 g (n=4) in LPC-treated rings. After washing, the contractions induced by 5-HT returned to control levels.

Figure 28b shows the effect of LPC (10 μM) on 5-HT-evoked contractions in endothelium-denuded aortic rings. The control EC_{50} value for 5-HT was 224 ± 30 nM (n=4) and 191 ± 32 nM (n=4) in the presence of LPC (10 μM) showing that there was no significant alteration in the sensitivity of the tissues to 5-HT. The maximal contraction to 5-HT in the abscence of LPC (10 μM) was 3.12 ± 0.39 g (n=4) compared to 3.57 ± 0.5 g (n=4) in the presence of LPC showing no significant difference. However, when the responses are expressed as a % of control maximum as in **Figure 28b**, 5-HT-evoked responses are significantly potentiated at doses 3-10 μM.

The effect of LPC on the contractile agents PE and 5-HT suggest that LPC stimulates the release of an endothelium-derived factor to reduce the efficacy of the agents since this effect is not observed in endothelium-denuded tissues. LPC also selectively potentiated responses to 5-HT in denuded tissues.

3.5 DISCUSSION

Lysophospholipids are amphiphilic molecules present in cell membranes and phospholipid layers (Tay *et al.*, 1969; Scanu, 1979). One of these molecules, LPC, has been implicated in a number of physiological and pathophysiological processes, acting via diverse mechanisms.

Inhibition of EDRF-mediated relaxations by OXLDL has been demonstrated in many laboratories (Jacobs *et al.*, 1990; Kugiyama *et al.*, 1990; Yokoyama *et al.*, 1990; Simon *et al.*, 1990; Galle *et al.*, 1991; Tanner *et al.*, 1991; Chin *et al.*, 1992). This effect on relaxation by OXLDL has been attributed to LPC,

generated from PC during oxidative modification (Kugiyama *et al.*, 1990; Plane *et al.*, 1990; Yokoyama *et al.*, 1990). This study further investigated the vasoactive properties of LPC, and considers whether LPC can account for the inhibitory effects of OXLDL.

LPC evoked relaxations in isolated, pre-contracted rabbit aortic rings which were dependent on the presence of an intact endothelium. The mediator was non-prostanoid in nature as indomethacin, a cyclo-oxygenase inhibitor, did not affect these responses.

LPC has also been shown to evoke EDR responses in several other isolated tissue preparations (Saito *et al.*, 1988; Yokoyama *et al.*, 1990; Hirayama *et al*, 1992; Saito *et al.*, 1992), and *in vivo*, to increase coronary blood flow and decrease blood pressure in rabbits (Wolf *et al.*, 1991). The present study has demonstrated that 1-palmitoyl (C=16) and 1-stearoyl (C=18) LPC induce relaxations, whereas 1-caproyl (C=6) LPC failed to evoke any response. The ability of different LPCs to evoke relaxations seems to dependent on the length of the fatty acid bound to the glycerol backbone of the molecule, with those with longer chains able to elicit a response. This may be related to the weaker detergent action of LPCs with longer aliphatic chains (Saito *et al*, 1988).

The role of an endothelium-derived diffusable factor in LPC-evoked relaxations was demonstrated when it was shown that denuded bovine pulmonary arteries relaxed after superfusion by a LPC-stimulated intact pulmonary artery (Menon *et al.*, 1989). The present study has demonstrated that LPC-evoked relaxations were inhibited by L-NAME, suggesting NO mediates this response. However, the role of NO in LPC-evoked EDR is controversial. Several reports have proposed that the relaxing factor was

something other than NO (Saito *et al.*, 1988; Bing and Menon, 1990; Menon and Bing, 1991) but, more recently, it was suggested that LPC-evoked relaxations are mediated by NO (Hirayama *et al.*, 1992; Saito *et al.*, 1992). Indeed, a recent report has shown that 1-palmitoyl and 1-stearoyl, but not 1-caproyl, LPC induced a biphasic increase in intracellular Ca^{2+} in cultured endothelial cells (Inoue *et al.*, 1992), with the initial phase dependent on an intracellular store without IP_3 formation, and the second phase dependent on extracellular Ca^{2+}. This rise in Ca^{2+} could activate the constitutive NOS, and catalyse the release of NO from L-arginine.

The mechanism by which LPC stimulates a rise in intracellular Ca^{2+} and EDNO release is unknown. However, LPC has been reported to act as a Ca^{2+} ionophore (Serhan *et al.*, 1981; Ungemach, 1987; Locher *et al*, 1992). LPC is also known to cause nonselective membrane leakage and changes in fluidity (Shier *et al.*, 1976; Weltzien, 1979), and it is possible that LPC-evoked relaxations are related to alterations in endothelial cell membranes. A further possibility arises from the similarity in the chemical structure of LPC and platelet-activating factor (PAF), a potent endothelium-dependent relaxant. It has been shown that LPC-induced relaxations may, at least in part, involve the activation of the PAF receptor (Hirayama *et al.*, 1992; Saito *et al*, 1992). Whatever the mechanism of release, relaxations by LPC are mediated through activation of guanylate cyclase and increases in cGMP in vascular smooth muscle (Saito *et al.*, 1988).

The ability of LPC to evoke relaxations is in contrast to the lack of effect of modified LDL in pre-contracted tissues. Neither OXLDL or LDL treated with PLA_2, the enzyme responsible for endogenous production of LPC, influenced the level of induced tone in rabbit aortic rings (Plane, 1992). This could be due

to LPC being more tightly held within the lipoprotein particle with insufficent amount of LPC able to transfer to endothelial cells to stimulate EDNO production.

ACh-evoked EDR are mediated by activation of endothelial muscarinic receptors, and in the present study, LPC was shown to inhibit this pathway. This is in agreement with other reports (Kugiyama *et al.*, 1990; 1992; Yokoyama *et al.*, 1990; Mangin *et al.*, 1993). LPC also inhibited relaxations mediated by the receptor-dependent agonist ATP, and to a less extent the Ca^{2+} ionophore A23187, which acts through a receptor-independent mechanism. The latter finding is in agreement with a recent study (Mangin *et al.*, 1993), but not with the conclusions of another (Kugiyama *et al.*, 1992). However, in the latter study, the effect of LPC on Ca^{2+} ionophore-evoked responses was tested only after washing the tissues, and thus removing LPC from the bath. The attenuation of EDR shown in the present study suggests that the inhibitory action of LPC is not mediated by an action on individual receptor-dependent pathways, and that the inhibitory effect could occur, in part, subsequent to receptor activation and elevation of intracellular Ca^{2+}. This may point to two possible sites of action for LPC, one before and one after the rise in intracellular Ca^{2+} levels.

The mechanism(s) by which LPC inhibits relaxations is(are) unknown. However, as shown in the present study, LPC can both evoke relaxations and inhibit ACh-induced responses in the same tissue, suggesting that the site of action of LPC on receptor-mediated relaxations is prior to the rise in intracellular Ca^{2+} concentration, since LPC must also cause a similar increase in Ca^{2+} to stimulate EDNO release. Therefore, it is possible this may reflect an action of LPC within the endothelial cell membrane, preventing the activation

of receptor-operated Ca^{2+} influx. This hypothesis is supported by the findings of a recent study which demonstrated that palmitoyl-LPC inhibited BK-evoked increases in Ca^{2+} influx in cultured endothelial cells (Inoue *et al.*, 1992). LPC has previously been shown to modify the activity of membrane associated enzymes, such as guanylate cyclase, adenylate cyclase (Shier *et al.*, 1976), sialytransferase (Shier and Troffer, 1976) and galactosyltransferase (Mookerjea and Yung, 1974). In addition, LPC may be involved in membrane transduction by diffusing through the lipid portion of membranes, thus activating enzymes at various locations in the cell (Shier *et al.*, 1976).

It is well documented that phorbol esters, activators of protein kinase C (PKC), inhibit EDRF-mediated responses (see for examples; Weinheimer *et al.*, 1986; Lewis *et al.*, 1987). It has also been demonstrated that LPC can regulate the activity of PKC (Oishi *et al.*, 1990), and possibly act as a second messenger synergistic to diacylglycerol and calcium (Asaoka *et al.*, 1991; 1992). Therefore, it is possible that the inhibitory actions of LPC could be mediated through the activation of PKC. Indeed, a recent report has suggested that LPC activates PKC to inhibit Ca^{2+} influx into cultured endothelial cells and inhibit EDR in coronary arteries (Kugiyama *et al.*, 1992). Furthermore, OXLDL may exert its inhibitory action on EDR through activation of PKC (Smith and Turner, 1992). Interestingly, increased PKC activity has also been shown to phosphorylate NOS leading to reduced NO production (Bredt *et al.*, 1992). Since LPC has been implicated in signal transduction in cells, this could be a further mechanism by which LPC can inhibit responses to endothelium-dependent relaxants.

Alternatively, LPC may cause inhibition of EDR elicited by receptor-dependent and independent agonits by elevating intracellular Ca^{2+} to such an

extent that the endothelial cells become refractory to stimulation by ACh and ATP, but is less effective against A23187. It has been shown that A23187 is more effective at raising intracellular Ca^{2+} than ACh or ATP (Singer and Peach, 1982), and thus A23187 could still raise Ca^{2+} levels further to elicit a response, albeit a reduced one.

L-arginine is the substrate for EDRF formation in endothelial cells. *In vitro* studies have shown that depletion of L-arginine stores causes inhibition of EDRF-mediated responses which can be reversed by addition of exogenous L-arginine (Gold *et al.*, 1989). In the present study, however, LPC-induced inhibition of EDR in isolated aortic rings was not prevented by pre-incubation with L-arginine, indicating that in this preparation reversible inhibition of L-arginine availability cannot account for the actions of LPC.

Endothelium-derived constrictor products of cyclo-oxygenase have been suggested to contribute to the attenuation of EDR in isolated atherosclerotic porcine vessels (Shimokawa and Vanhoutte, 1989). Additionally, OXLDL itself can induce endothelium-dependent contractions (Simon *et al*, 1990) although, others have not observed such actions of OXLDL (Plane, 1992). However, as shown in the present study, the mechanism by which LPC inhibits EDR does not seem to involve the release of products of cyclo-oxygenase as the presence of indomethacin did not influence the action of LPC.

Albumin and HDL have been shown to reduce the inhibitory effects of OXLDL on EDR (Plane, 1992; Plane *et al.*, 1992) and guanylate cyclase activity (Schmidt *et al.*, 1992b). This suggests the involvement of a lipid component of OXLDL, which is readily transferable between the lipoprotein particles and the endothelial cells. In agreement with these findings, the

present study has shown that the inhibition of EDR by LPC was prevented in a concentration-dependent manner by defatted serum albumin as would be expected since fatty-free albumin has a high avidity for lysophospholipids (Mohandas *et al.*, 1982). The protective effects of HDL and albumin *in vitro* has led to the suggestion that these lipophilic particles may reduce the potential for OXLDL to inhibit EDRF-mediated responses in atherosclerotic plaques. However, *in vivo* fatty acids are bound to serum albumin which may reduce the capacity to bind the inhibitory lipid components of OXLDL.

The proposed mediator of EDR in the rabbit aorta is NO (section 1.4.4) and the possiblity that the inhibition of EDR by LPC may be due to increased inactivation of NO or inhibition of soluble guanylate cyclase activity was investigated. In denuded tissues, responses to exogenous NO and to the nitrovasodilator GTN were unaffected by the presence of LPC. This suggests that the attenuation of EDR by LPC is not mediated by interactions with NO nor by direct inhibition of smooth muscle guanylate cyclase. It has been suggested that LPC can inhibit the activity of partially purified guanylate cyclase (Schmidt *et al.*, 1992b). However, at concentrations identical to those used in this study (10 μM), only very slight inhibition of guanylate cyclase was observed and this was under conditions where LPC was incubated directly with the enzyme. Therefore, it appears likely that LPC-induced inhibition of EDR observed in this study is through action on the endothelium with no effect on the mediator of EDR or its site of action in vascular smooth muscle.

In contrast, in endothelium-intact tissues, LPC did attenuate responses to exogenous NO and GTN, which could contribute to the inhibition of EDR. This suggests that LPC may stimulate the release of a substance from the

endothelium which inhibits relaxation. As LPC can release EDNO, the effect of L-NAME on GTN-evoked relaxations was investigated. L-NAME reduced the inhibition of GTN-evoked responses by LPC although this was not statistically significant. Nevertheless, it does hint at an involvement of EDNO which may reduce the sensitivity of the smooth muscle to GTN. Indeed, L-arginine analogues and endothelial denudation potentiated relaxations evoked by GTN in rat aorta (Moncada *et al.*, 1991b), suggesting release of NO desensitises the soluble guanylate cyclase in the smooth muscle.

Whether LPC alone can account for the inhibitory properties of modified LDL is questionable since there exists large differences in the pattern of effects. The reported effects of oxidatively modified LDL on EDRF-mediated relaxations range from abolition of responses (Kugiyama *et al.*, 1990; Yokoyama *et al.*, 1990) to lesser effects (Simon *et al.*, 1990; Tanner *et al*, 1991). Furthermore, the degree of inhibition has been shown to be dependent on the donor from whom the LDL was prepared (Jacobs *et al.*, 1990), whereas the LPC content of OXLDL preparations from different donors was not found to be significantly different (Plane *et al.*, 1992). OXLDL also inhibits endothelium-independent relaxations in responses to exogenously applied NO (Jacobs *et al.*, 1990) and in bioassay studies (Galle *et al.*, 1991; Chin *et al.*, 1992) suggesting a direct sequestration or inactivation of NO, a lipophilic molecule, by the lipoprotein particle. The present study has shown that LPC does not interact with NO. In addition, LPC does not inhibit relaxations evoked by nitrovasodilators in endothelial-denuded tissues, whereas OXLDL attenuates activation of guanylate cyclase in isolated denuded tissues (Jacobs *et al.*, 1990) and of the partially purified enzyme (Schmidt *et al.*, 1990; 1992b), highlighting a further difference between the inhibitory properties of LPC

and oxidatively modified LDL. However, both OXLDL and LPC inhibit BK-induced phosphoinositide hydrolysis and Ca^{2+} transients in cultured endothelial cells (Inoue *et al.*, 1990; 1992; Hirata *et al.*, 1991).

Nevertheless, the fact that LPC is present in significant amounts in modified LDL and able to inhibit EDR does make it, at least, a potential contributing factor to the inhibitory properties of OXLDL.

In the present study LPC was shown to attenuate PE and 5-HT-mediated contractile responses in endothelium-intact tissues, but did not effect PE-evoked responses, and potentiated 5-HT contractions in denuded tissues. This suggests that LPC stimulates the release of an inhibitory factor from the endothelium, possibly NO, which would functionally antagonise contractions. This hypothesis appears to contradict the one proposed for the inhibition of GTN-evoked relaxations in intact tissues, in which EDNO relaxations decreases responses. However, in the same report as discussed above (Moncada *et al.*, 1991b), L-arginine analogues and removal of the endothelium in rat aortic rings potentiated PE-evoked contractions, suggesting that basally released EDNO mediates this effect. Therefore, it is possible that LPC-stimulated release of EDNO may cause the attenuation of contractions and inhibition of GTN-evoked relaxations.

The effect of LPC on PE and 5-HT-evoked contractions in endothelium-denuded tissues resembles the actions of OXLDL, since it also potentiated 5-HT-evoked contractions, although this was observed in both intact and denuded rabbit aortic rings, but had no effect on responses elicited by PE (Plane, 1992). The mechanism of action of LPC on 5-HT-evoked contractions in denuded tissues is unclear. LPC may act to increase intracellular Ca^{2+}, perhaps through its ionophore action (Serhan *et al.*, 1981; Ungemach, 1987;

Locher *et al.*, 1992) in smooth muscle cells to potentiate responses. However, this may also be expected to potentiate contractions evoked by PE, rather than having a specific action on 5-HT.

In conclusion, the present study has, for the first time, recognised the dual effects of LPC in evoking EDR mediated by EDRF as well as inhibition of relaxations elicited by endothelium-dependent agonists. These findings confirm that LPC may contribute to the inhibitory actions of OXLDL, but also suggest that LPC alone cannot account for all the properties of OXLDL on EDR and contractile responses.

CHAPTER FOUR

EFFECTS OF OXLDL AND LPC ON THE INDUCIBLE NOS

4.1 INTRODUCTION

The progression of atherosclerosis is characterised by the recruitment of monocytes into the subendothelial space. The production of cytokines by inflammatory cells, such as macrophages, in atherosclerotic lesions may stimulate *de novo* enzyme synthesis in the major cell types in the artery wall.

In vitro, induction of NOS by endotoxins and cytokines has been demonstrated in macrophages, vascular smooth muscle and endothelial cells (see section 1.4.4). This study investigated whether OXLDL or LPC could influence the production of NO via this pathway.

In this study, aortic rings from Wistar rats were used, as attempts to induce NOS in isolated aortic rings from NZW rabbits were unsuccessful.

RESULTS

4.2 INDUCTION OF NOS *IN VITRO*

4.2.1 L-ARGININE-EVOKED RELAXATIONS INDUCED *IN VITRO*

Rat aortic rings, some denuded of endothelium, were mounted in organ baths as described in Chapter 2 (section 2.5.2). An attempt was made to investigate the effects of L-arginine in tissues with and without endothelium. However, in denuded tissues, relaxation of pre-contracted rings to L-arginine were not consistent and therefore the experiments in this section were carried out in endothelium-intact rings.

Following the equilibration period of 90 mins, the tissues were challenged with a bolus dose of L-arginine (100 μM), and subsequently with ACh (1 μM)

to test the integrity of the endothelium, as illustrated in **Figure 29a**. This time point was considered zero time. The tissues were then tested after 60, 120, 240, and 360 mins to determine the time dependency of responses. Relaxations to bolus doses of L-arginine (100 μM) were shown to be time dependent as illustrated in **Figure 30**, reaching a plateau 4 hrs after the equilibration period, with relaxations 85 ± 5.3% of the induced tone. The relaxation evoked by L-arginine (100 μM) of a PE-contracted rat aortic ring after 4 hrs is shown in **Figure 29b**. PE-contracted (0.1 μM) tissues incubated with cycloheximide (10 μM) from the time they were mounted until the end of the experiment, did not respond to repeated challenges of L-arginine (100 μM) even after 6 hrs, as shown in **Figure 30**. **Figure 29c** shows a representative trace illustrating the effect of cycloheximide on L-arginine-evoked (100 μM) relaxations after 4 hrs. In addition, **Figure 29c** also shows that cycloheximide (10 μM) did not alter the contraction to PE (0.1 μM) nor the response to ACh (1 μM).

Addition of D-arginine (100 μM) to pre-contracted aortic rings did not affect the induced tone in tissues that responded to a 100 μM bolus dose of L-arginine (**Figure 31**) demonstrating the stereospecificity of the relaxations to arginine. Addition of L-NAME (100 μM) to the bath after the relaxation to 100 μM L-arginine reversed the response. This inhibition of relaxation could be overcome by increasing L-arginine concentration to 1 mM as shown in **Figure 31**. L-NAME (100 μM) added to basal tone in rat aortic rings did not have any effect (n=4).

Therefore these results suggest that relaxations to L-arginine are mediated by an inducible enzyme that is stereospecific.

4.2.2 EFFECT OF OXLDL ON L-ARGININE AND ACh -EVOKED

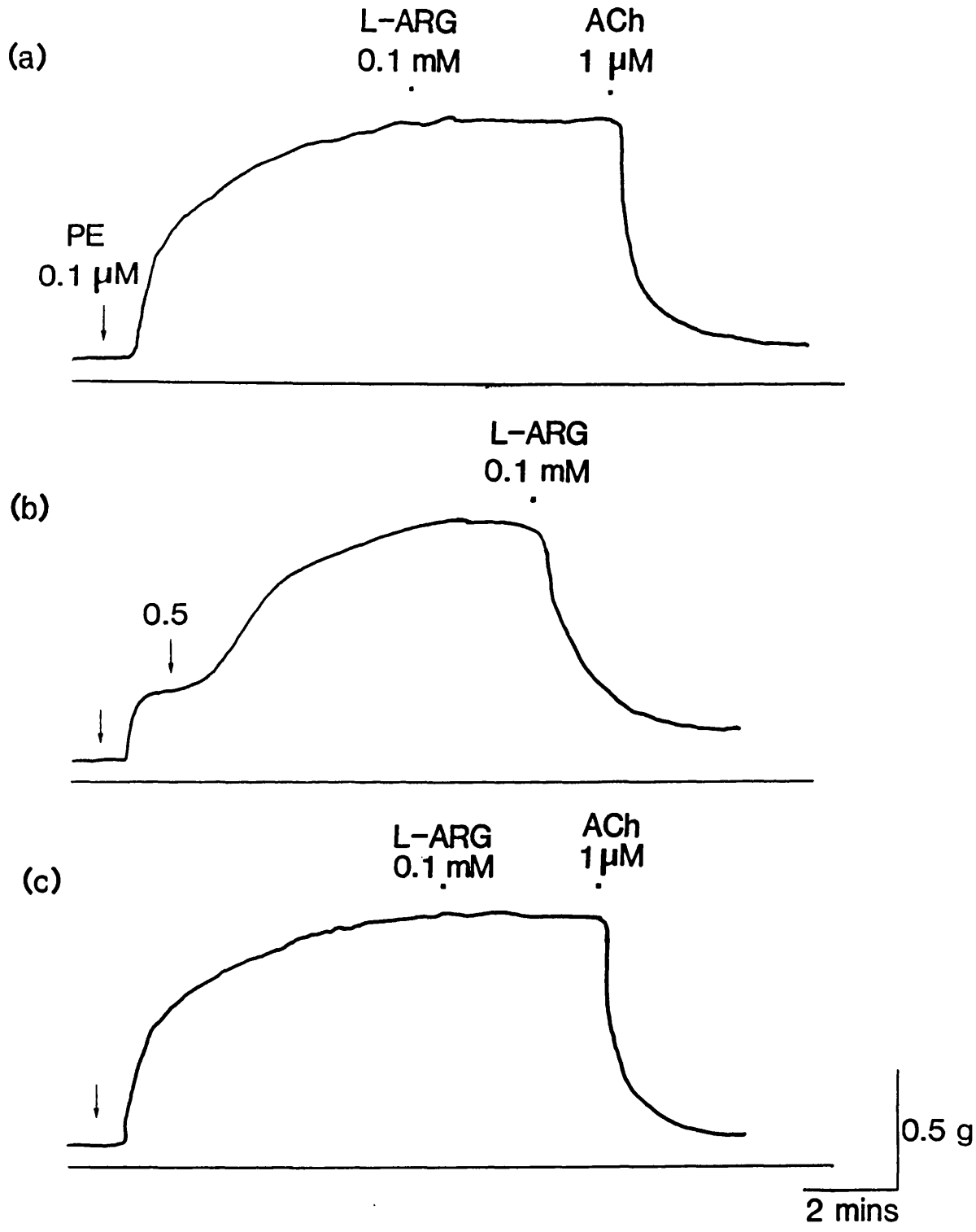

Figure 29 : Time-dependent relaxations to L-arginine
Aortic tissues precontracted with PE (0.1-0.5 μM) were challenged with bolus doses of L-arginine (100 μM) and ACh (1 μM).
(a) At 0 time
(b) After 4 hrs
(c) After 4 hrs with cycloheximide (10 μM)

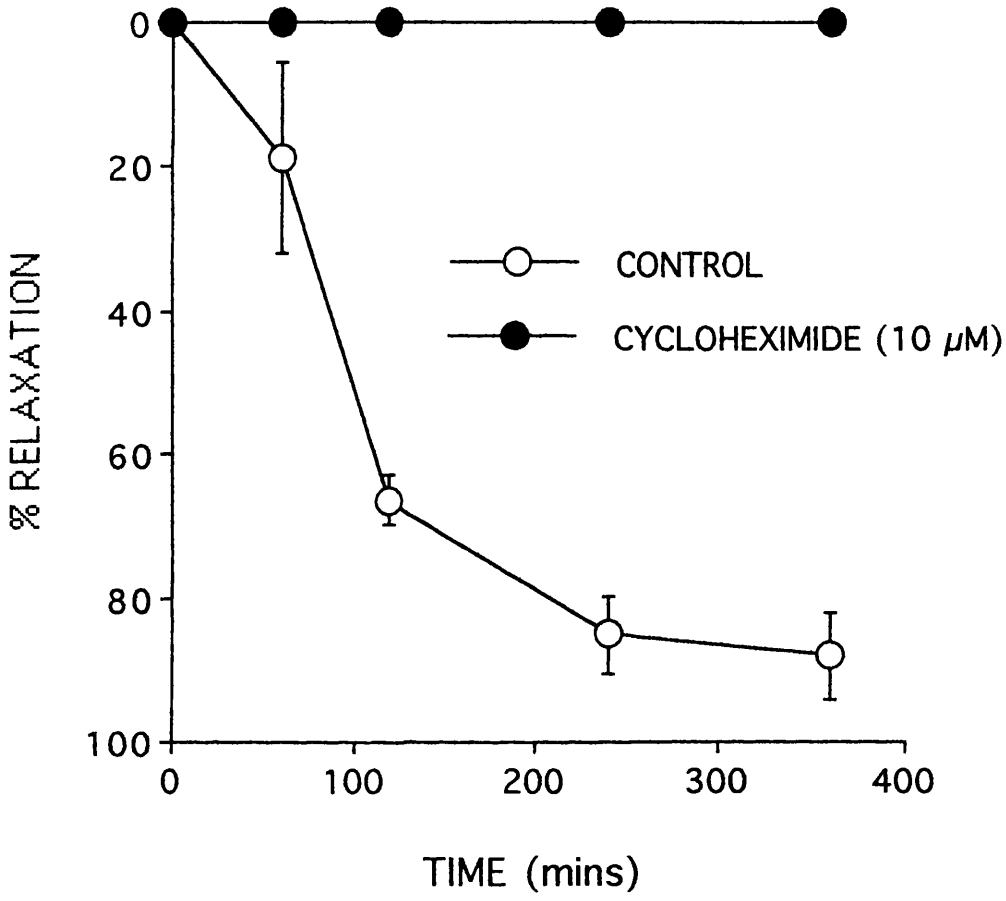

Figure 30 : The influence of time and cycloheximide on L-arginine-evoked relaxation

Aortic tissues pre-contracted with PE (0.1-0.6 μM) and challenged with bolus doses of L-arginine (100 μM) at time 0, 60, 120, 240, and 360 mins in the absence and presence of cycloheximide (10 μM).

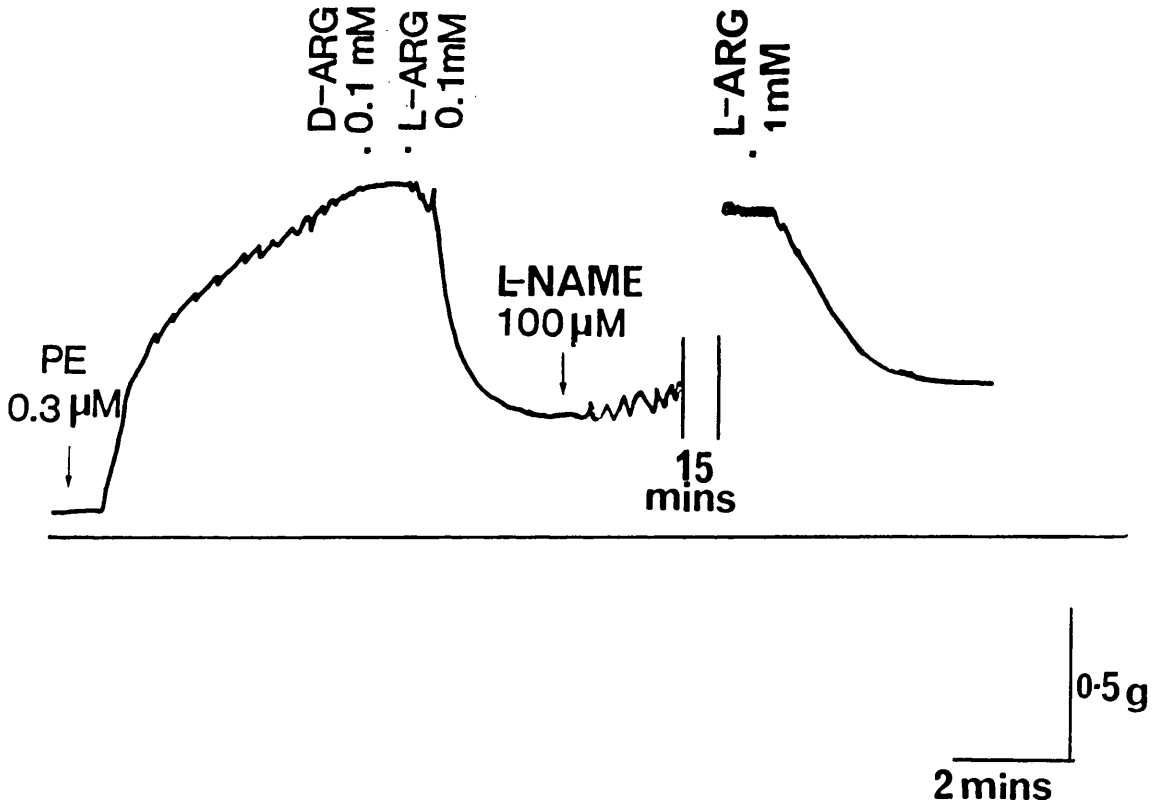

Figure 31 : The influence of D-arginine on induced tone and L-NAME on L-arginine-evoked relaxations

Aortic ring incubated in Krebs' buffer for 4 hrs contracted with PE (0.3 μM) and challenged with D-arginine (0.1 mM) followed by L-arginine (0.1 mM). At plateau of relaxation, L-NAME (100 μM) added to tissue. After 15 mins, re-contraction reached plateau and tissue challenged with cumulatively increasing L-arginine concentration to 1 mM.

RELAXATIONS

In the experiments described in sections 4.2.2 and 4.2.3, endothelium-intact tissues were challenged with L-arginine and ACh, as illustrated in **Figure 29a**. Rings were incubated in Krebs' buffer for 4 hrs, then washed and pre-contracted with PE and challenged with L-arginine (100 μM) to test for the induction of NOS (see **Figure 29b**). The contraction-relaxation cycle used for NZW rabbit aortic rings was repeated for these experiments using PE to induce tone and L-arginine to relax the tissues in a dose dependent manner.

Previous work from this laboratory has shown that OXLDL inhibits endothelium-dependent relaxations of rabbit aortic rings evoked by ACh and A23187 in a time dependent manner. In addition, the extent and the reversibility of this effect was dependent on the donor of the plasma from which the OXLDL was prepared.

In this study, the effect of OXLDL from two different donors on dose-dependent L-arginine-evoked relaxations was assessed. The effect of OXLDL on ACh-evoked responses was used as a reference for the extent of inhibition of EDRF-mediated responses. These tissues were also incubated in Krebs' buffer for 4 hrs to induce NOS as assessed by L-arginine-evoked relaxations in pre-constricted tissues.

Figure 32a shows ACh-evoked relaxations of intact rat aortic rings pre-contracted with PE, and the effect of OXLDL (2 mg protein/ml) from donor 1 after a 30 min pre-incubation period. Responses to ACh are significantly attenuated in the presence of OXLDL with the maximum response reduced from 95.7 ± 4.3 to 67.7 ± 8.9% (n=4). This effect was reversed after removal of the lipoproteins. However, following incubation with the same preparation of OXLDL, L-arginine-evoked relaxations were unchanged (**Figure 32b**). The

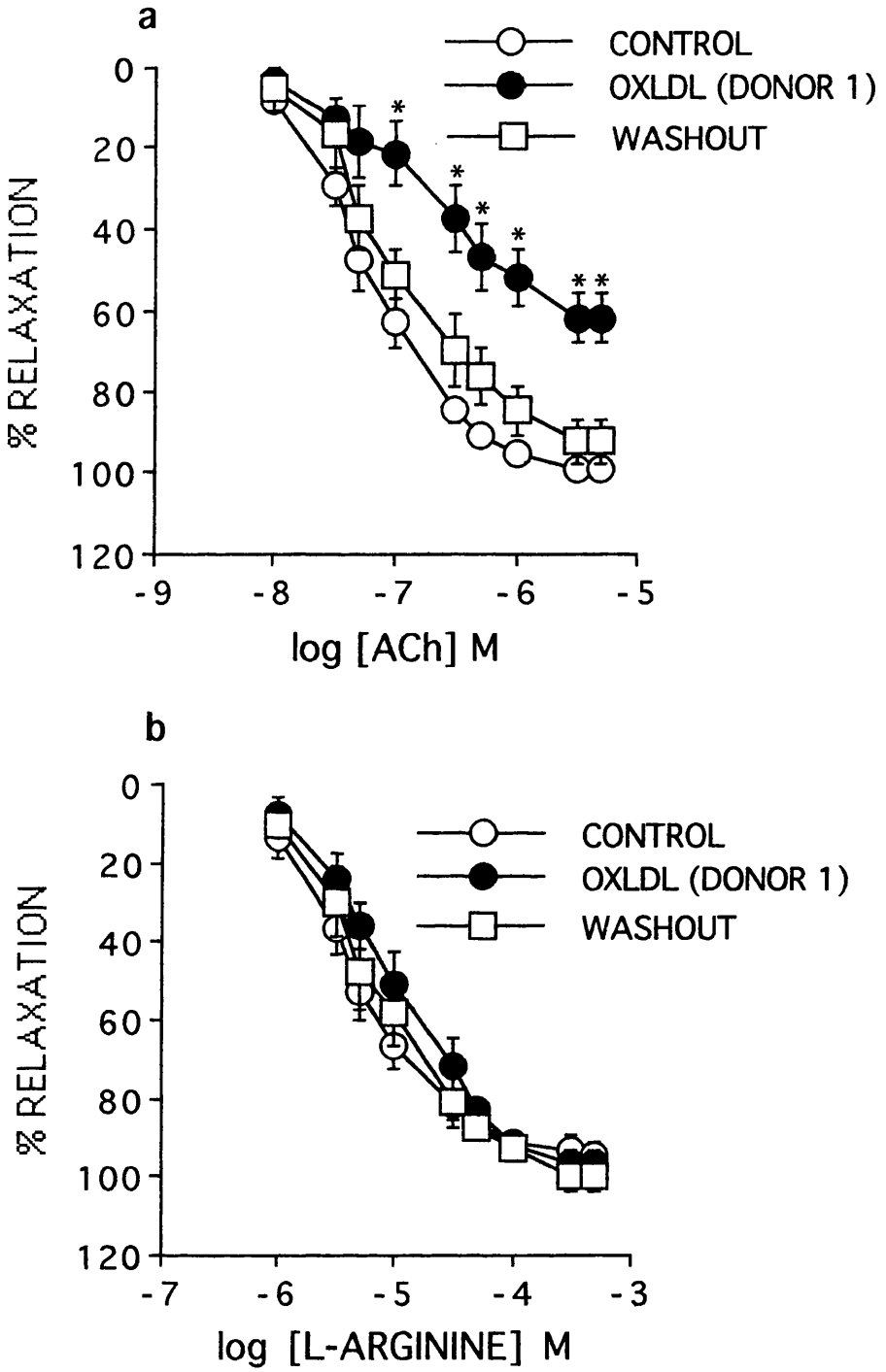

Figure 32 : The influence of OXLDL from donor 1 on ACh and L-arginine -evoked relaxations

Aortic rings pre-contracted with PE (0.1-0.5 μM) and relaxed to cumulative doses of ACh (0.01-5 μM) or L-arginine (1-500 μM). Following washout and equilibration, tissues pre-incubated with OXLDL (2 mg protein/ml) from donor 1 for 30 mins before contraction/relaxation repeated. After further washout and equilibration, tissues re-challenged with PE and ACh or L-arginine (n=4 for each).

(a) ACh and OXLDL

(b) L-arginine and OXLDL

Students' t-test compared to control : * $p<0.05$.

maximum response to L-arginine in controls was 90.0 ± 3.8% (n=4) and in the presence of the lipoproteins (2mg protein/ml) for 30 mins, 91.2 ± 4.6% (n=4) relaxation was observed.

As shown in **Figure 33a** the exposure of rat aortic tissues to OXLDL (2mg protein/ml) from donor 2 for 30 mins had essentially the same effect. This preparation of lipoproteins also inhibited ACh-evoked relaxations, decreasing maximum responses from 99.0 ± 1.0% to 62.0 ± 6.2% (n=4) which was reversed on washing. **Figure 33b** shows that relaxations evoked by L-arginine were unchanged in the presence of OXLDL (2 mg protein/ml) with maximum responses in control tissues 94.3 ± 3.2 (n=4) and in the presence of OXLDL, 96.7 ± 3.3% (n=4).

4.2.3 EFFECT OF LPC ON L-ARGININE-EVOKED RELAXATIONS

The effect of LPC, a proposed inhibitory factor of OXLDL, on L-arginine-evoked relaxations was also investigated.

LPC (10 μM) added to the the tissues immediately before PE-induced pre-contraction completely inhibited relaxations evoked by ACh (1 μM), decreasing responses from 90.4 ± 2.1% to 0% (n=5).

In the presence of LPC (10 μM), L-arginine-evoked relaxations of PE-contracted tissues were significantly potentiated as shown by a leftward shift in the DRC to L-arginine (**Figure 34**). The maximum relaxation to L-arginine was increased from 76.7 ± 3.8% (n=5) in controls to 91.5 ± 1.9% (n=5) in LPC-treated rings. This effect was immediate and fully reversed after washing.

The results from experiments with OXLDL and LPC indicate that they have differential effects on relaxations evoked by ACh and L-arginine despite the fact that both responses are mediated by EDNO. This finding points to the differences in mechanism of synthesis/release of EDNO from the constitutive

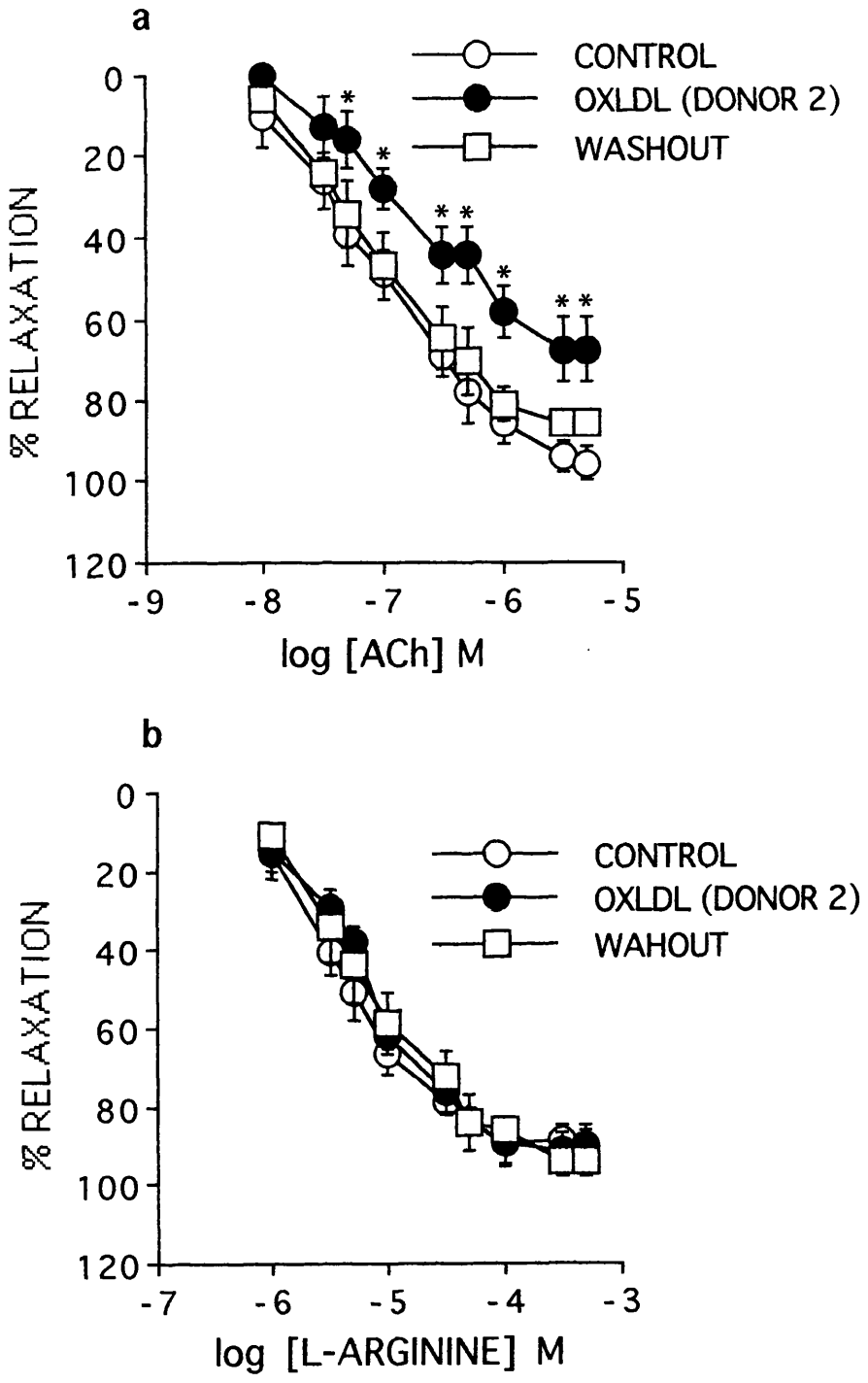

Figure 33 : The influence of OXLDL from donor 2 on ACh and L-arginine -evoked relaxations

Aortic rings pre-contracted with PE (0.1-0.5 μM) and relaxed to cumulative doses of ACh (0.01-5 μM) or L-arginine (1-500 μM). Following washout and equilibration, tissues pre-incubated with OXLDL (2 mg protein/ml) from donor 2 for 30 mins before contraction/relaxation repeated. After further washout and equilibration, tissues re-challenged with PE and ACh or L-arginine (n=4 for each).

(a) ACh and OXLDL

(b) L-arginine and OXLDL

Students' t-test compared to control : * $p<0.05$.

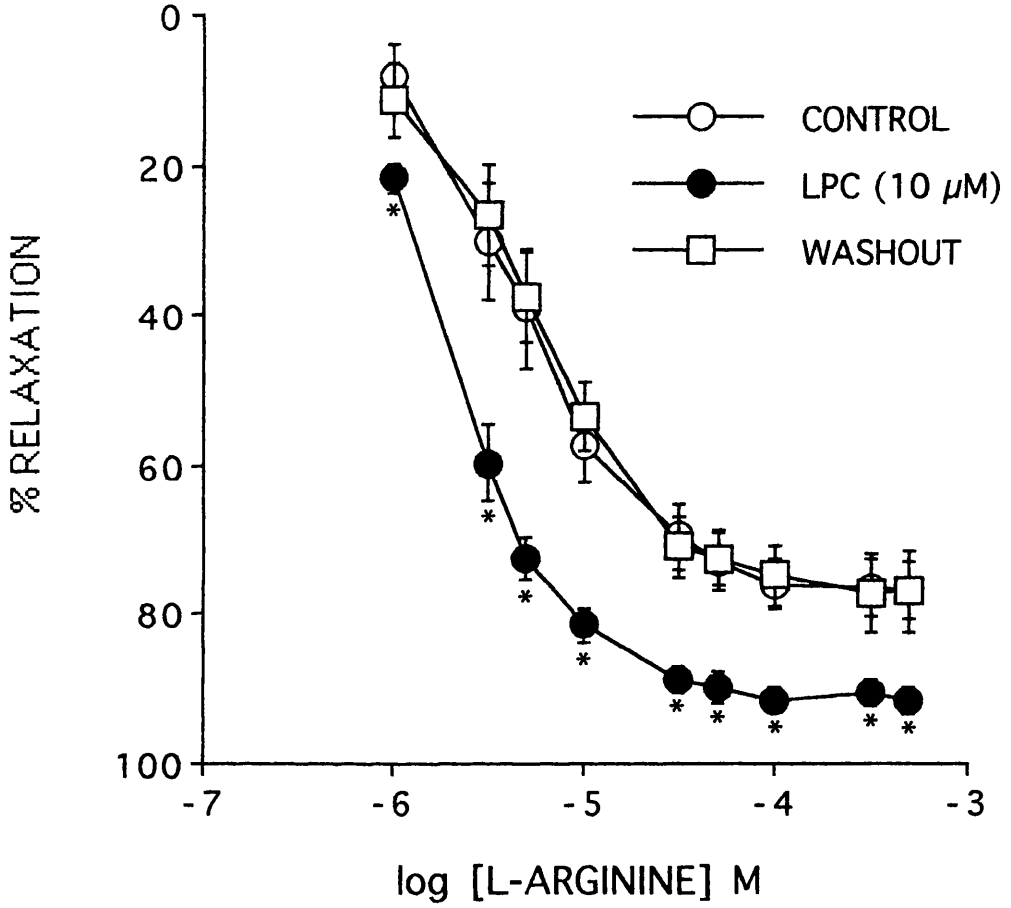

Figure 34 : The influence of LPC on L-arginine-evoked relaxations
Aortic tissues pre-contracted with PE (0.1-0.6 μM) and relaxed to cumulative doses of L-arginine (1-500 μM). Following washout and equilibration, LPC (10 μM) added to tissues and contraction/relaxation cycle repeated. After further washout and equilibration, tissues re-challenged with PE and L-arginine (n=4).
Students' t-test compared to control : * $p<0.05$.

and inducible NOS enzymes as the site of action of LPC.

4.3 LPS TREATMENT OF RATS

Wistar rats were injected with LPS (4 mg/kg) i.p. as described in section 2.5.5. After 1-2 hrs animals treated with LPS became apathetic and sluggish while rats injected with 0.9% NaCl appeared healthy. After 4 hrs the animals were sacrificed and the aorta quickly removed.

4.3.1 THE EFFECT OF ENDOTHELIAL DENUDATION ON L-ARGININE - EVOKED RELAXATIONS

Folowing the equilibration period, tissues from both LPS-treated and control (0.9% NaCl)-injected rats were contracted with PE (0.1 μM) and challenged with L-arginine (100 μM). This was to test for the induction of a NOS in LPS-treated rats and was followed by addition of ACh (1 μM) to test for the integrity of the endothelium.

In tissues from control rats, a bolus dose of L-arginine (100 μM) failed to evoke any response in PE-contracted endothelium-intact (n=8) or denuded (n=8) aortic rings. In contrast, in tissues from LPS-treated rats L-arginine-evoked relaxations were observed. In endothelium-intact tissues, PE (1-3 μM) induced a contraction of 0.6 ± 0.18 g (n=8) and in denuded tissues 0.72 ± 0.2 g (n=8). In endothelium-intact rings, L-arginine (100 μM) relaxed PE-induced tone by 84.4 ± 6.8% (n=8) and in denuded tissues the response was 60.6 ± 3.4% (n=8), demonstrating that removal of the endothelium significantly attenuated the relaxation response to L-arginine. This reduced response to L-arginine in denuded tissues was not due to decreased ability of the tissue to relax since GTN (0.5 μM) was able to further relax the ring to 96.6 ± 4.2% (n=8) of the induced tone, which did not differ from intact tissues (93.8 ± 5.3%; n=8).

4.3.2 THE EFFECT OF CYCLOHEXIMIDE ON L-ARGININE-EVOKED RELAXATIONS

The effect of cycloheximide on the relaxations to L-arginine was investigated to exclude any *ex vivo* protein synthesis that may occur in the organ bath similar to that demonstrated in section 4.2.1. Cycloheximide (10 μM) was incubated with the rings immediately after excision from the animal and for the remainder of the experiment.

No response to L-arginine (100 μM) was observed in rings from saline-injected animals incubated with cycloheximide (10 μM), as in untreated rings from the same animal (section 4.3.1). The presence of cycloheximide in the bath did not inhibit relaxations to ACh (1 μM) in endothelium-intact tissues (92.4 ± 1.8% n=8 in controls; 94.0 ± 4.9% n=8 in presence of cycloheximide) or relaxations to GTN (0.5 μM) in denuded tissues (90.9 ± 5.2%, n=8 in controls; 89.9 ± 3.5%, n=8 in presence of cycloheximide).

Cycloheximide (10 μM) influenced the responses of aortic rings from endotoxin-treated rats to L-arginine. Relaxations evoked by bolus doses of L-arginine (100 μM) in pre-contracted endothelium-intact aortic rings were significantly decreased from 84.4 ± 6.8% (n=7) to 57.0 ± 9.0% (n=7). In denuded tissues from endotoxin-treated rats, the response to 100 μM L-arginine (60.6 ± 3.4%; n=7) was abolished by cycloheximide (10 μM; n=7).

4.3.3 THE EFFECT OF LPS ON PE-EVOKED CONTRACTIONS

Treatment of rats with LPS may also cause depression of contractile responses due to the induction of a NOS and resulting production of NO. This hypothesis was tested by constructing DRCs to PE in aortic rings from saline (control) and LPS injected animals

DRCs to PE (1 nM-10 μM) were carried out on endothelium-intact and

denuded rings from both saline and endotoxin -treated rats. **Figure 35** shows the effect of 4 mg/kg LPS treatment on responses to PE in rat aortic rings with an intact endothelium (**a**) and tissues denuded of endothelium (**b**). These responses were greatly decreased compared to contractions of tissues from control rats. In rings with endothelium, maximum contractions to PE decreased from 1.28 ± 0.1 g (control animals; n=8) to 0.21 ± 0.08 g (LPS-treated animals; n=7). In tissues denuded of endothelium the results were very similiar (**Figure 35b**). The maximum PE-evoked response was reduced from 1.67 ± 0.07 g (n=8) in controls to 0.44 ± 0.1 g (n=7) in endotoxin-treated rats.

The maximum contractions attained in intact and denuded tissues in these experiments are less than PE-induced pre-contractions used to test for relaxations to L-arginine described above (section 4.3.1) as the contractions decreased with time.

4.3.4 THE EFFECT OF ENDOTHELIAL DENUDATION ON PE-EVOKED CONTRACTIONS

Figure 36a shows the effect of removal of the endothelium on PE-evoked contractions in tissues from saline-treated rats. The responses to PE were significantly potentiated with a decrease in the EC_{50} value from 63.8 ± 17.4 nM (n=8) to 7.8 ± 1.8 nM (n=8) and an increase in the maximum contraction from 1.28 ± 0.1 g (n=8) to 1.67 ± 0.07 g (n=8). As shown in **Figure 36b**, in rings from LPS-treated rats the maximum response to PE was increased by denuding the tissues of endothelium but this was not statistically significant. The maximum contraction in tissues with intact endothelium was 0.21 ± 0.08 g (n=7) as compared to 0.44 ± 0.1 g (n=7) in denuded tissues.

4.3.5 THE EFFECT OF L-NAME ON PE-EVOKED CONTRACTIONS

L-NAME (100 μM) added to resting tissues had no effect on tone either in

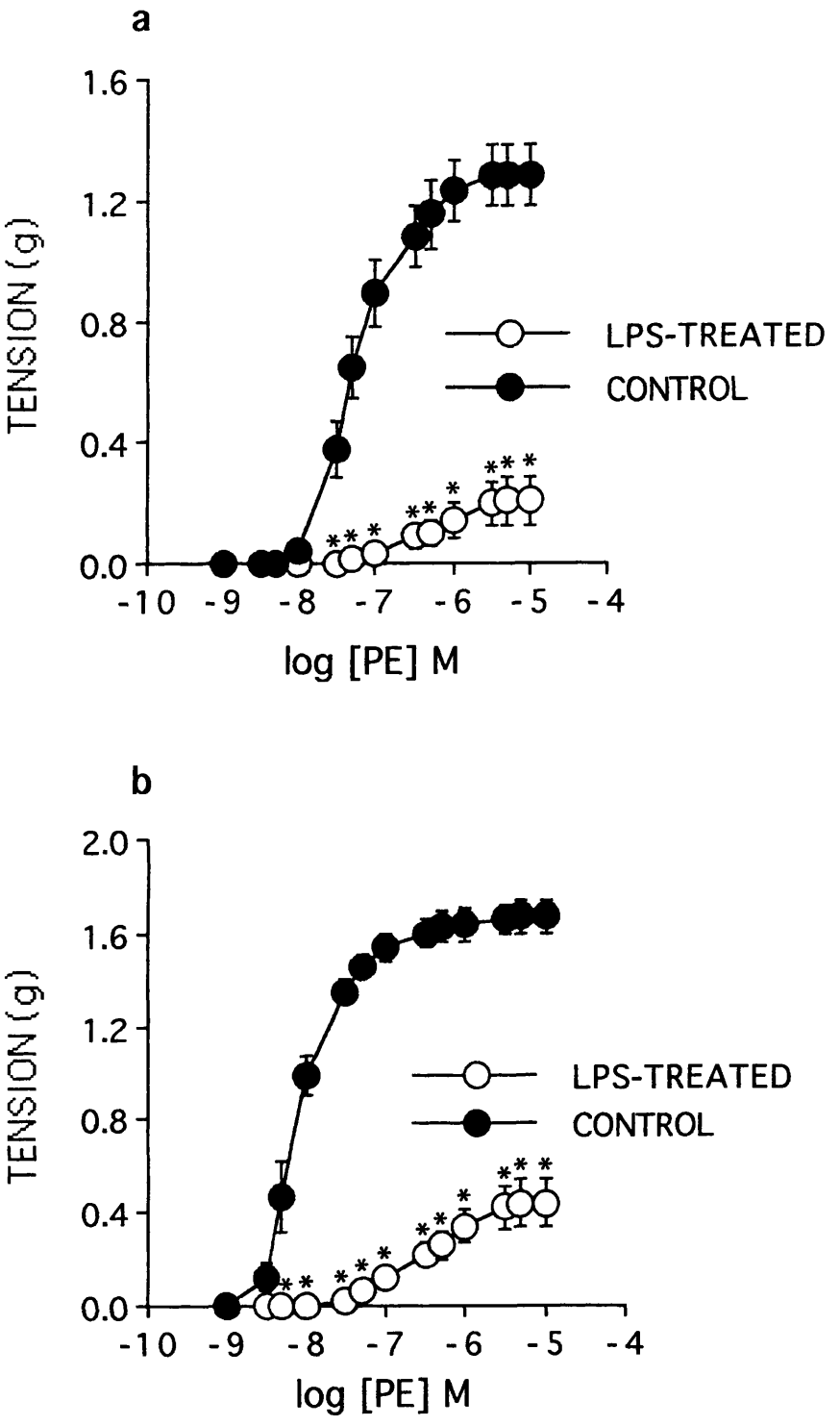

Figure 35 : PE-evoked contractions in endothelium-intact and denuded aortic rings from saline and LPS-treated rats

Comparison of endothelium-intact and denuded aortic tissues from saline and LPS-treated rats contracted with cumulative doses of PE (1 nM-10 μM) until maximum level of induced tone was attained.

(a) Endothelium-intact tissues from saline (n=8) and LPS-treated (n=7) rats

(b) Endothelium-denuded tissues from saline (n=8) and LPS-treated (n=7) rats

Students' t-test compared to control : * $p<0.05$.

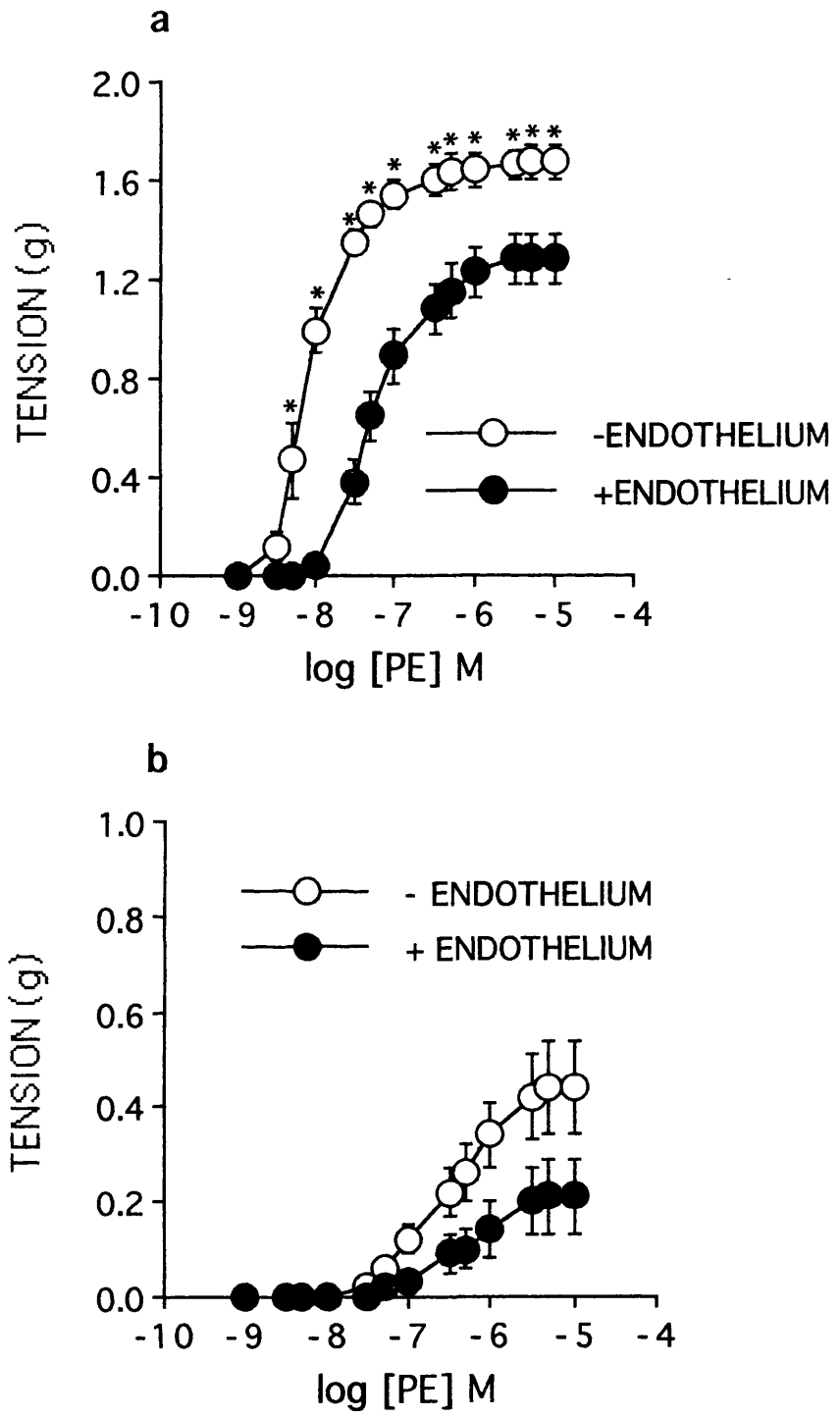

Figure 36 : The influence of endothelial removal on PE-evoked contractions in aortic rings from saline and LPS-treated rats

Aortic tissues from saline and LPS-treated rats with and without endothelium contracted with cumulative doses of PE (1 nM-10 μM) until maximum level of induced tone was attained.

(a) Endothelium-intact and denuded tissues from saline-treated rats (n=8)

(b) Endothelium-intact and denuded tissues from LPS-treated rats (n=7)

rings from control or endotoxin-treated rats.

Figure 37 shows the effect of L-NAME on the contractions evoked by PE in endothelium intact tissues from control and LPS-treated animals. In tissues from saline-treated rats (**Figure 37a**), L-NAME (100 μM) potentiated the responses to PE causing a decrease in the EC_{50} value from 63.8 ± 17.4 nM (n=8) to 18.4 ± 2.6 nM (n=3) and an increase in the maximum response from 1.28 ± 0.1 g (n=8) to 1.6 ± 0.03 g (n=3). In tissues from LPS-treated rats, L-NAME (100 μM) also significantly potentiated PE-evoked contractions of endothelium-intact rings (**Figure 37b**). Before addition of L-NAME, the maximum level of contraction and EC_{50} value was 0.21 ± 0.08 g (n=7) and 628 ± 186 nM (n=7), and in the presence of the inhibitor the maximal response was 0.68 ± 0.19 g and the EC_{50} value 178 ± 5.5 nM (n=3).

It is noteworthy that the effect of L-NAME on contractions to PE in endothelium-intact tissues from saline-treated rats increased responses to values close to those of denuded tissues. The maximum contraction and EC_{50} values in endothelium-denuded rings were 1.67 ± 0.07 g (n=8) and 7.8 ± 1.8 nM (n=8) respectively, and in L-NAME (100 μM)-incubated rings 1.6 ± 0.03 g (n=3) and 18.4 ± 2.6 nM (n=3), respectively. This suggests that at this concentration L-NAME effectively reduces NO produced via the constitutive pathway in these experiments.

As shown in **Figure 38a**, pre-incubation of 100 μM L-NAME did not alter contractions elicited by PE in endothelium-denuded tissues from saline-injected rats (maximum response 1.58 ± 0.25 g; EC_{50} 8.7 ± 4.7 nM; n=3). However, in denuded tissues from endotoxeamic rats, L-NAME (100 μM) significantly increased maximum PE responses. **Figure 39** shows a typical trace of the effect of L-NAME (100 μM) on PE-evoked contractions in a

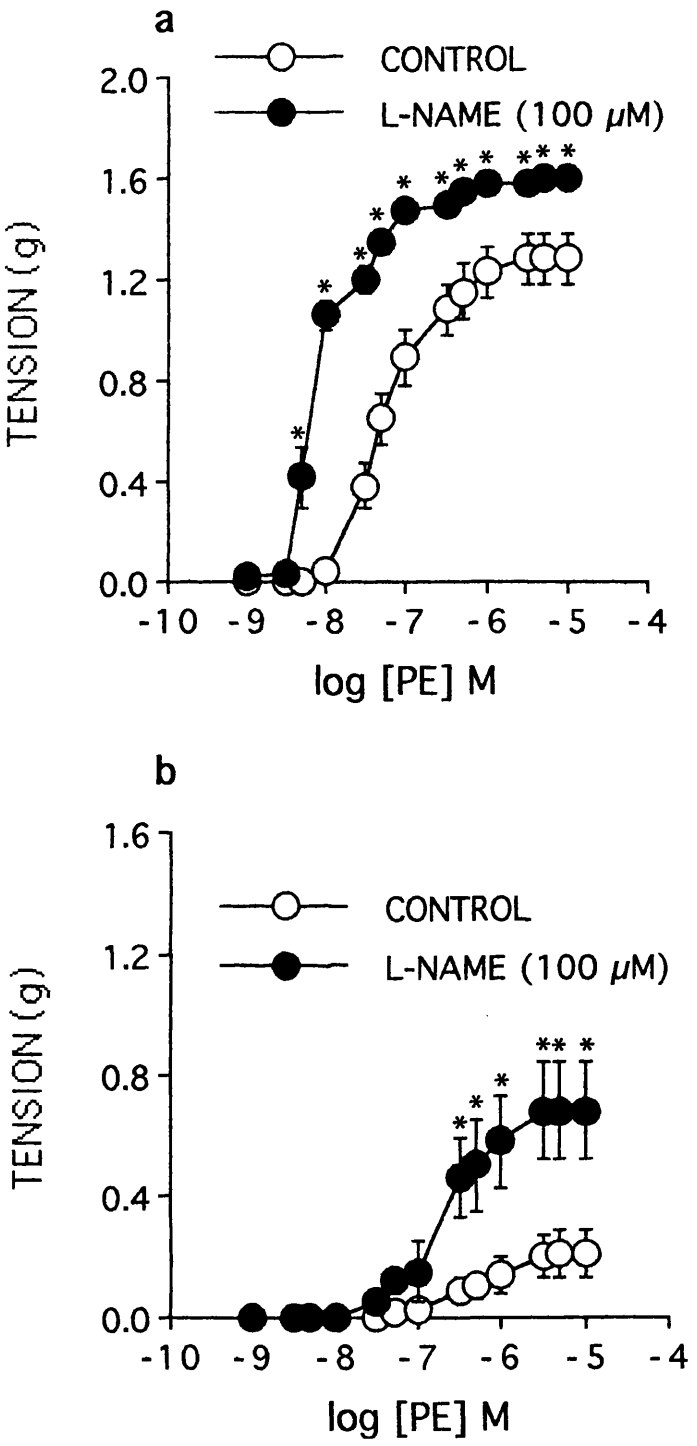

Figure 37 : The influence of L-NAME on PE-evoked contractions in endothelium-intact aortic rings from saline and LPS-treated rats
Endothelium-intact aortic tissues from saline and LPS-treated rats in the absence and presence of L-NAME (100 μM) contracted with cumulative doses of PE (1 nM-10 μM) until maximum level of induced tone was attained.
(a) L-NAME (100 μM) in tissues from saline-treated rats (n=3)
(b) L-NAME (100 μM) in tissues from LPS-treated rats (n=7)
Students' t-test compared to control : * $p<0.05$.

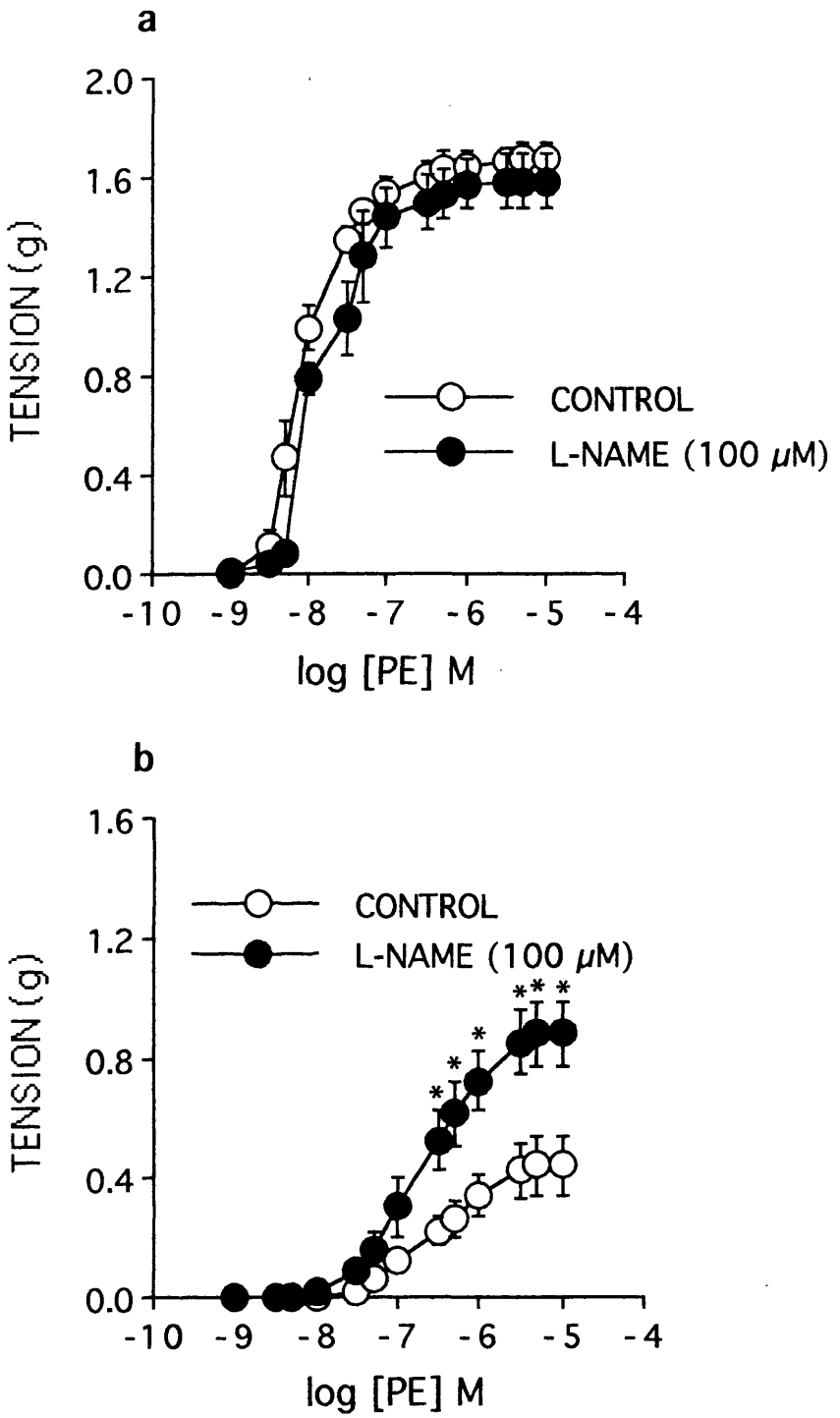

Figure 38 : The influence of L-NAME on PE-evoked contractions in endothelium-denuded aortic rings from saline and LPS-treated rats
Endothelium-denuded aortic tissues from saline and LPS-treated rats in the absence and presence of L-NAME (100 μM) contracted with cumulative doses of PE (1 nM-10 μM) until maximum level of induced tone was attained.
(a) L-NAME (100 μM) in tissues from saline-treated rats (n=3)
(b) L-NAME (100 μM) in tissues from LPS-treated rats (n=6)
Students' t-test compared to control : * $p<0.05$.

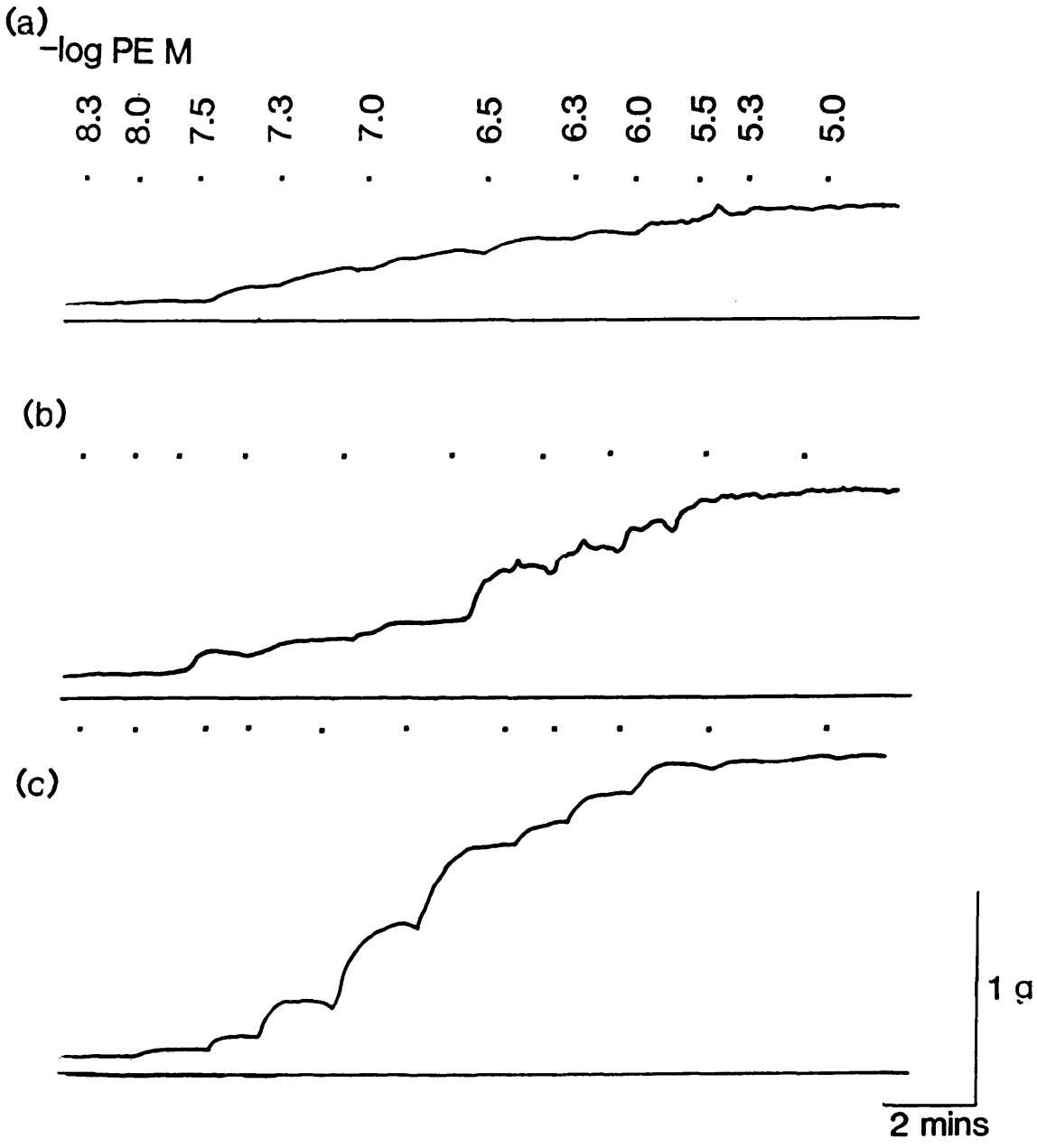

Figure 39 : The influence of L-NAME and cycloheximide on PE-evoked contractions in endothelium-denuded tissues from LPS-treated rats

Endothelium-denuded aortic tissues from LPS-treated in the absence and presence of L-NAME (100 μM) or cycloheximide (10 μM) contracted with cumulative doses of PE (5 nM-10 μM) until maximum level of induced tone was attained.

(a) Endothelium-denuded tissues from LPS-treated rats
(b) Endothelium-denuded tissues from LPS-treated rats in the presence of L-NAME (100 μM)
(c) Endothelium-denuded tissues from LPS-treated rats in the presence of cycloheximide (10 μM)

denuded aortic ring from a LPS-treated rat. The mean maximum response to PE was increased from 0.44 ± 0.1 (n=7) to 0.88 ± 0.16 g (n=6). However, the sensitivity of the tissues to PE was not significantly altered with the EC_{50} for control tissues 399 ± 136 nM (n=7) and 222 ± 55 nM (n=6) for tissues from endotoxin-treated rats (**Figure 38b**).

4.3.6 THE EFFECT OF CYCLOHEXIMIDE ON PE-EVOKED CONTRACTIONS

Figure 40 shows DRCs to PE in aortic rings with an intact endothelium incubated with cycloheximide (10 μM) from control (**a**) and endotoxin-treated (**b**) rats. As can be seen, cycloheximide had no effect on PE-induced contractions in rings from control animals with a maximal response of 1.34 ± 0.12 g (n=8) and an EC_{50} value of 48.2 ± 9.9 nM (n=8; **Figure 40a**). In contrast, the contractility of rings from LPS-treated rats in response to PE was markedly augmented in the presence of cycloheximide (10 μM) with the maximum contraction increased from 0.21 ± 0.08 g (n=7) to 0.94 ± 0.12 g (n=6) and the EC_{50} decreased from 628 ± 186 nM (n=7) to 199 ± 63 nM (n=6; **Figure 40b**)

Essentially similar results were obtained in tissues denuded of endothelium. Cycloheximide (10 μM) had no effect on contractions (EC_{50} 8.9 ± 2.6 nM; max. contraction 1.71 ± 0.08 g; n=7) evoked by PE in rings from control rats as shown in **Figure 41a**. However, in the presence of cycloheximide responses to PE in tissues from endotoxin-treated rats greatly increased from 0.44 ± 0.1 g (n=7) to 1.46 ± 0.1 g (n=7; **Figure 41b**). A representative trace illustrating the effect of cycloheximide (10 μM) on PE-evoked contractions in a denuded ring from a LPS-treated rat is shown in **Figure 39c**.

Figure 42 illustrates the marked effect cycloheximide (10 μM) has on

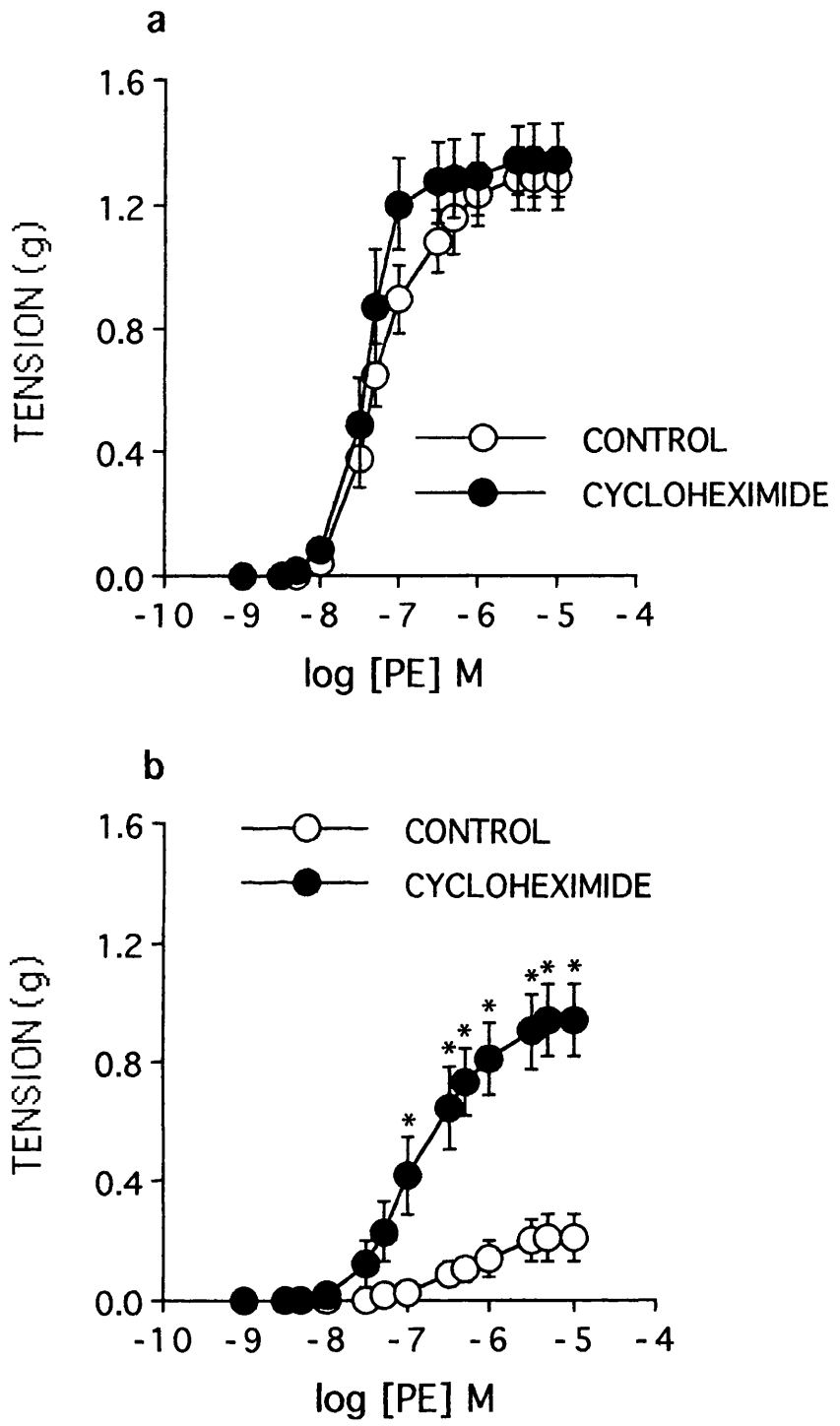

Figure 40 : The influence of cycloheximide on PE-evoked contractions in endothelium-intact tissues from saline and LPS-treated rats

Endothelium-intact aortic tissues from saline and LPS-treated rats in the absence and presence on cycloheximide (10 μM) contracted with cumulative doses of PE (1 nM-10 μM) until maximum level of induced tone was attained.

(a) Cycloheximide (10 μM) in endothelium-intact tissues from saline-treated rats (n=8)

(b) Cycloheximide (10 μM) in endothelium-intact tissues from LPS-treated rats (n=7)

Students' t-test compared to control : * $p<0.05$.

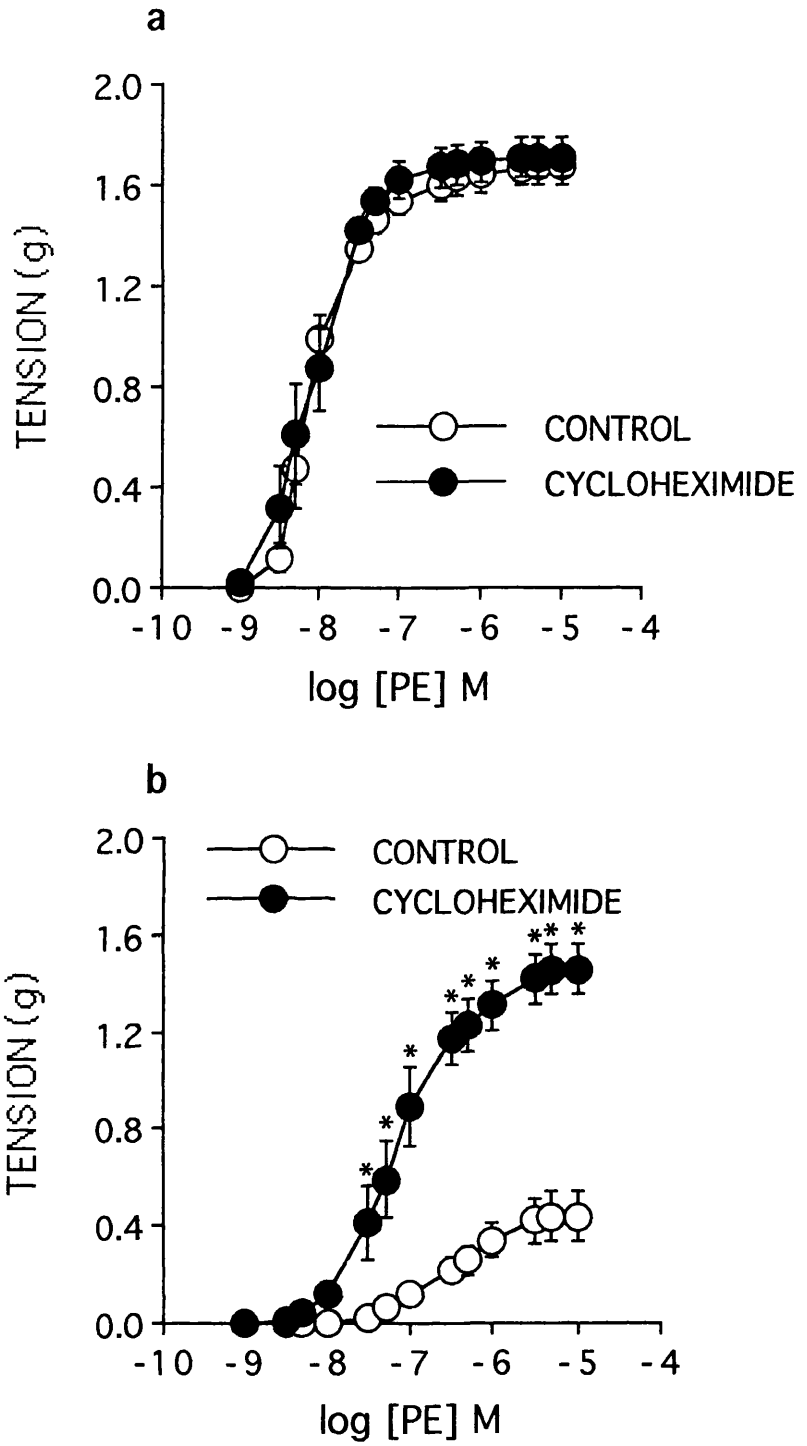

Figure 41 : The influence of cycloheximide on PE-evoked contractions in endothelium-denuded tissues from saline and LPS-treated rats

Endothelium-denuded aortic tissues from saline and LPS-treated rats in the absence and presence of cycloheximide (10 μM) contracted with cumulative doses of PE (1 nM-10 μM) until maximum level of induced tone was attained (n=7 for each).

(a) Cycloheximide (10 μM) in endothelium-denuded tissues from saline-treated rats

(b) Cycloheximide (10 μM) in endothelium-denuded tissues from LPS-treated rats

Students' t-test compared to control : * $p<0.05$.

contractions to PE in (**a**) intact and (**b**) denuded aortic rings from LPS-treated as compared to saline-treated rats. The maximum responses to PE in intact rings were still significantly depressed, whereas in denuded tissues maximum contractions returned to control levels, although a significant rightward shift in the DRC to PE remained.

4.3.7 THE EFFECT OF OXLDL ON PE-EVOKED CONTRACTIONS

The effect of OXLDL on the contractions induced by PE in endothelium-denuded tissues from LPS-treated rats was investigated. OXLDL (2 mg protein/ml), or the equivalent volume of Tris buffer, was incubated with the tissues for 30 mins before the DRCs to PE were initiated. As shown in **Figure 43**, the OXLDL from donors 3 and 4 had no significant effect on the responses to PE as compared to controls (n=4 for each donor).

4.3.8 EFFECT OF LPC ON PE-EVOKED CONTRACTIONS

The effect of LPC on the responses to PE in tissues from endotoxin-treated rats was investigated.

LPC (10 μM) added to the bath immediately before the DRC to PE in endothelium-intact tissues from control animals had an inhibitory effect on these responses as shown in **Figure 44a**. The maximum contraction to PE was reduced from 1.28 ± 0.1 g (n=8) to 0.83 ± 0.1 g (n=8) and significantly increased the EC_{50} value from 63.8 ± 17.4 nM (n=8) to 168.0 ± 45.5 nM (n=8). However, as shown in **Figure 44b**, LPC (10 μM) had no significant effect on contractions to PE in endothelium-intact tissues from endotoxin-treated rats (maximum contraction 0.15 ± 0.09 g; n=5)

Figure 45 shows the effect of 10 μM LPC on PE-evoked contractions in tissues denuded of endothelium from (**a**) control and (**b**) endotoxin-treated rats. There was no significant change in sensitivity to the contractile agonist in

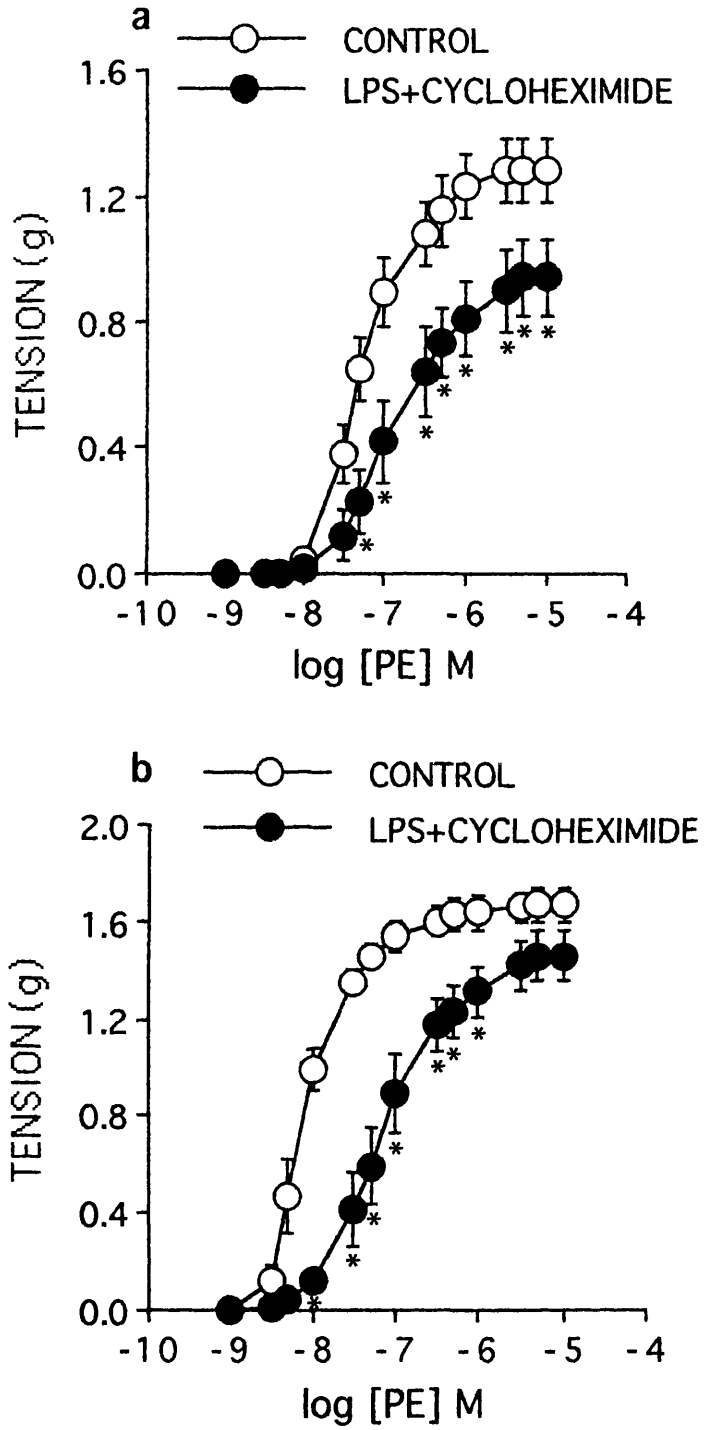

Figure 42 : Comparison of PE-evoked contractions in endothelium-intact and denuded tissues from saline, and LPS-treated rats with cycloheximide
Endothelium-intact and denuded aortic tissues from saline and LPS-treated rats contracted with cumulative doses of PE (1 nM-10 μM) until maximum level of induced tone was attained.

(a) Endothelium-intact tissues from saline-treated rats compared to intact tissues from LPS-treated rats incubated with cycloheximide (10 μM; n=7-8)

(b) Endothelium-denuded tissues from saline-treated rats compared to intact tissues from LPS-treated rats incubated with cycloheximide (10 μM; n=7).

Students' t-test compared to control : * $p<0.05$.

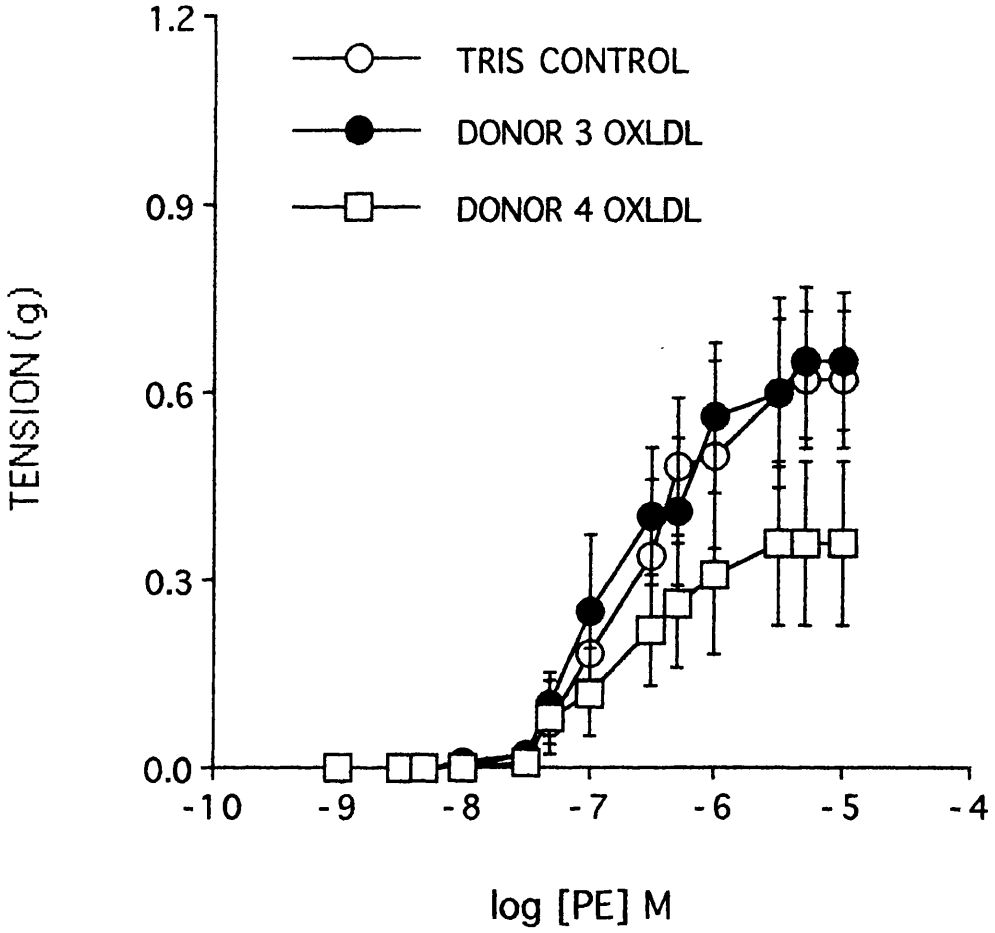

Figure 43 : The influence of OXLDL on PE-evoked contractions in endothelium-denuded tissues from LPS-treated rats

Tissues incubated with OXLDL (2 mg protein/ml) from donors 3 and 4, or equivalent volume of Tris buffer, for 30 mins and contracted with cumulative doses of PE (1 nM-10 μM) until maximum level of induced tone was attained (n=4 for each donor).

Students' t-test compared to control : * $p<0.05$.

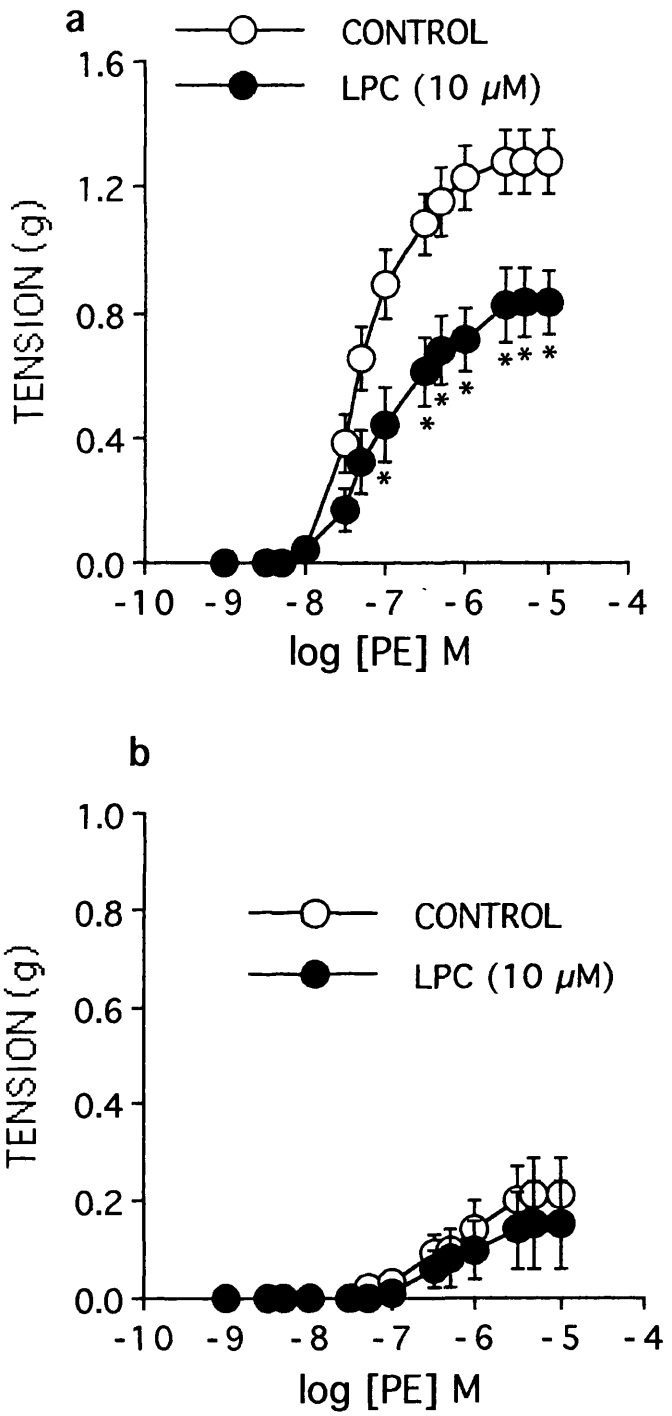

Figure 44 : The influence of LPC on PE-evoked contractions in endothelium-intact tissues from saline and LPS-treated rats

Endothelium-intact aortic tissues from saline and LPS-treated rats in the absence and presence of LPC (10 μM) contracted with cumulative doses of PE (1 nM-10 μM) until maximum level of induced was attained (n=8 for each).
(a) LPC (10 μM) in endothelium-intact tissues from saline-treated rats
(b) LPC (10 μM) in endothelium-denuded tissues from LPS-treated rats
Students' t-test compared to control : * $p<0.05$.

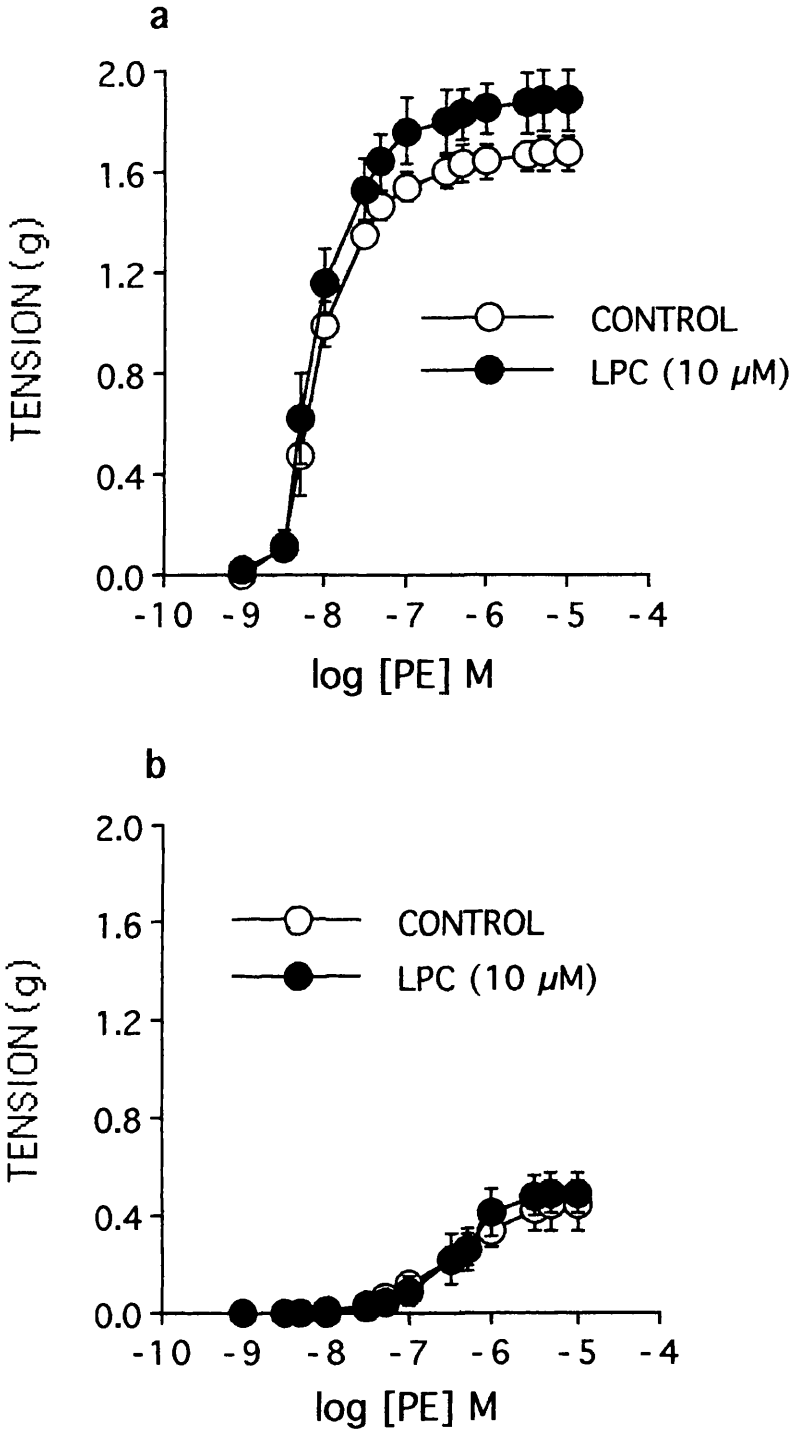

Figure 45 : The influence of LPC on PE-evoked contractions in endothelium-denuded tissues from saline and LPS-treated rats

Endothelium-denuded aortic tissues from saline and LPS-treated rats in the absence and presence of LPC (10 μM) contracted with cumulative doses of PE (1 nM-10 μM) until maximum level of induced was attained.
(a) LPC (10 μM) in endothelium-intact tissues from saline-treated rats (n=7)
(b) LPC (10 μM) in endothelium-denuded tissues from LPS-treated rats (n=5)
Students' t-test compared to control : * $p<0.05$.

the presence of LPC in tissues from control (EC_{50} 12.2 ± 2.2 nM; n=7) or endotoxin-treated (EC_{50} 628 ± 187 nM; n=4) animals and the maximal level of contraction was unaltered (control 1.88 ± 0.15 g, n=7; LPS-treated 0.49 ± 0.08, n=4).

4.4 DISCUSSION

L-arginine has been shown to act as the precursor for EDRF/NO (Palmer *et al.*, 1988a; 1988b; Sakuma *et al.*, 1988; Schmidt *et al.*, 1988), although bolus doses failed to relax isolated segments of the rat thoracic aorta (Thomas and Ramwell, 1988) or porcine mammary artery (Thomas *et al.*, 1989), and produced negligible relaxation in rabbit aorta (Palmer *et al.*, 1988b; Rees *et al.*, 1989a). More recently, L-arginine has been shown to evoke relaxations in a time dependent manner in bovine intrapulmonary artery and rat aorta, both in the absence and presence of the endothelium (Wood *et al.*, 1990; Julou-Schaeffer *et al.*, 1991; Moritoki *et al.*, 1991; 1992; Schini and Vanhoutte, 1991a; 1991b). The time dependence of these responses indicated that there may be induction of a new pathway to metabolise L-arginine. Indeed, incubation of isolated tissues with cycloheximide and other protein synthesis inhibitors, prevented responses to L-arginine (Moritoki *et al.*, 1992).

The present study also demonstrated that L-arginine-evokes time-dependent relaxations of isolated rat thoracic aortic rings, which were inhibited by prophylactic treatment with cycloheximide implicating *de novo* protein synthesis.

The sources of NO in this study are likely to be the endothelium and/or the smooth muscle although blood constituents which remain attached to or infiltrate the vessel wall cannot be excluded. Electron microscopic (Freudenberg and Riese, 1976) and histological studies (Hibbs *et al.*, 1987)

have demonstrated the presence of a substantial population of lymphocytes and monocytes within the aortic intima of rats, and monocytes have been shown to synthesise NO when stimulated by LPS *in vitro* (Hibbs *et al*, 1987a; Grisham *et al.*, 1988).

Responses to L-arginine were found to be more reproducible in endothelium-intact than denuded rings. Other investigations have reported that relaxations to L-arginine are independent of the endothelium (Wood *et al.*, 1990; Julou-Schaeffer *et al.*, 1991; Moritoki *et al.*, 1991; 1992) although the responses are induced more quickly in intact tissues (Schini and Vanhoutte, 1991a; 1991b). These discrepancies may be due to differences in laboratory conditions and the rather uncontrolled method of induction, but the time for responses to appear and reach maximum in the present study is similar to those reported in other investigations (Moritoki *et al*, 1991; 1992; Schini and Vanhoutte, 1991a; 1991b). The relaxations to L-arginine obtained in this study were stereospecific since D-arginine did not evoke any responses in pre-contracted tissues, and were also reversed by addition of the L-arginine analogue, L-NAME. Therefore, responses to L-arginine in precontracted rat aortic rings may be mediated by an inducible NOS, similar to the one reported in other vascular reactivity studies.

The presence of endotoxins in the bathing solutions has been linked to the induction of NOS in *in vitro* studies of vascular reactivity (Rees *et al.*, 1990a) and it seems likely that in the present study and others (Wood *et al.*, 1990; Schini and Vanhoutte, 1991a; 1991b) the induction of a NOS, rather than depletion of L-arginine, accounts for the relaxations to L-arginine.

Many investigators have demonstrated inhibition of relaxations mediated via the constitutive NOS by oxidatively modified LDL (e.g. Jacobs *et al.*, 1990;

Tanner *et al.*, 1991). The present study investigated whether OXLDL could inhibit relaxations to L-arginine mediated via an inducible NOS. At concentrations that attenuated responses to the endothelium-dependent agonist ACh, OXLDL did not affect L-arginine-evoked relaxations. Additionally, it was found that LPC, a proposed inhibitory component of OXLDL (Kugiyama *et al.*, 1990; Plane *et al.*, 1990; Yokoyama *et al.*, 1990), at concentrations which abolished ACh-evoked relaxations in rat and rabbit aortic rings, had no inhibitory effect on responses to L-arginine. This indicates that the mechanisms of NO synthesis and release by the constitutive and inducible L-arginine : NO pathways are significantly different. Futhermore, in contrast to OXLDL, LPC was found to potentiate relaxations to L-arginine, further highlighting the differences between the two pathways. The reason for this effect is unclear but may reside in the fact that LPC also has vasorelaxing properties (see section 3.2.2), and thus may lessen the functional antagonism to L-arginine relaxation caused by the pre-constricting agent rather than being a specific action of LPC on the activity of the inducible NOS.

The differences between the constitutive and inducible NOS, with regard to ion and co-factor dependencies, enzyme modulation, or location within the cell may also explain how OXLDL and LPC are able to inhibit one pathway but not the other. For example, the constitutive enzyme is Ca^{2+}/calmodulin dependent whereas the activity of the inducible enzyme is independent of intracellular Ca^{2+}. This hypothesis is supported by recent evidence showing LPC inhibits BK-induced increases in Ca^{2+} and EDRF release in cultured endothelial cells (Inoue *et al.*, 1992). Additionally, LPC and OXLDL have also been proposed to inhibit EDR via a pathway involving activation of PKC

(Kugiyama *et al.*, 1992; Smith and Turner, 1992) which can regulate IP_3 formation and subsequent $[Ca^{2+}]_i$ mobilisation and thus may not interfere with the activity of the inducible enzyme. Finally, the constitutive form of NOS is predominantly located in the particulate fraction of endothelial cells while the inducible form is largely cytosolic (see Förstermann *et al.*, 1991b). As lipid soluble components of OXLDL such as LPC can easily be incorporated into plasma membranes and modify the activity of membrane associated enzymes (Mookerjea and Yung, 1974; Shier *et al.*, 1976; Shier and Troffer, 1976), the differentiation of effects may possibly reflect the location of the NOS and the access LPC and other possible inhibitory constituents of OXLDL have to these enzymes. Therefore, it is clear from these experiments that sequestration of EDNO or attenuation of soluble guanylate cyclase activity in vascular smooth muscle could not account for the actions of OXLDL and LPC on the L-arginine : NO systems but point to the interesting differences between two distinct pathways.

Many recent studies have demonstrated that NOS can also be induced by injection of bacterial endotoxin into rats (see for example, Knowles *et al.*, 1990; Guc *et al.*, 1990; Fleming *et al.*, 1991) and rabbits (Smith *et al.*, 1991; Vallance *et al.*, 1992) and vascular reactivity can be assessed *in vivo*, or *ex vivo* when tissue from treated animals are isolated and mounted in organ chambers, as performed in this study.

Administration of L-arginine analogues increased mean arterial blood pressure in LPS-treated rats (Gray *et al.*, 1991) and the resting tone of jugular vein rings from endotoxin-treated rabbits (Vallance *et al.*, 1992) which suggests that basal NO release is increased after LPS stimulation. However, no increase in basal tone was observed in the present study after the

administration of L-NAME to isolated rat aortic rings from LPS-treated animals indicating that there was no significant release of NO in these tissues, although the reasons for this are unclear.

In the present study, aortic rings from endotoxin-treated rats showed marked hyporesponsiveness to the contractile agent PE, both in the presence and absence of the endothelium. This attenuation was partially reversed by L-NAME suggesting that the production of NO from the smooth muscle and possibly the endothelium can, in part, account for the hyporeactivity to contractile agents. These findings are largely in agreement with those of other *in vitro*, and also *in vivo* studies (Fleming *et al*, 1991; Julou-Schaeffer *et al*, 1991; Smith *et al.*, 1991; 1992; Auguet *et al*, 1992; Vallance *et al*, 1992). It has been suggested that the release of NO from an inducible enzyme can fully account for the hyporesponsiveness to contractile agents as responses are restored in the presence of L-arginine analogues (Fleming *et al.*, 1990; 1991; Gray *et al.*, 1991). However, the present findings indicate that other mechanisms may contribute. Recent evidence has suggested that endotoxin treatment impairs PI hydrolysis in rat aorta (Suba *et al.*, 1992), and therefore likely to decrease mobilisation of intracellular Ca^{2+} and PKC-mediated responses, both of which would contribute to an impaired contractile mechanism. Indeed, decreased availability of Ca^{2+} for the contraction process has been demonstrated in aorta from septic rats (Litten *et al.*, 1988)

The induction of a NOS is further confirmed by the observation that addition of L-arginine to precontracted tissues from LPS-treated but not control rats, resulted in a rapid relaxation. This is in agreement with other studies (Fleming *et al.*, 1990; Julou-Schaeffer *et al.*, 1991) and LPS treatment has been shown to increase cGMP levels in isolated rat aortic rings (Fleming *et al.*, 1990; 1991).

The response to L-arginine was greater in intact rings possibly indicating differences between enzymes induced in endothelium and smooth muscle; if the NOS isoforms were identical, tissue denudation would not be expected to significantly alter the amount of induced enzyme due to the small proportion of endothelial cells in an intact aortic ring.

Incubation of tissues with cycloheximide immediately after excision from the LPS-treated rats and throughout the course of the experiment greatly reduced the hyporeactivity to contractile agents, and completely inhibited responses to L-arginine in endothelium-denuded tissues. This may reflect a high turnover of the induced enzyme in the smooth muscle cells stimulated by endotoxin treatment, with protein synthesis *ex vivo* inhibited by cycloheximide. The finding that the contractions to PE decrease with time may also reflect continued induction in the tissues *ex vivo*. Similar findings have recently been reported (Auguet *et al.*, 1992). Alternatively, synthesis of NOS may be induced entirely in the organ bath by endotoxins in the Krebs' buffer (Rees *et al.*, 1990a) in a manner similar to that described in this study. This seems unlikely, however, since expression of the induced enzyme *in vitro* is time-dependent while tissues from LPS-treated rats display immediate hyporesponsiveness to contractile agents and relaxations to L-arginine.

In contrast, cycloheximide only partially prevented the effects of *in vivo* endotoxin treatment on PE-evoked contractions and responses to L-arginine in endothelium-intact rings. This suggests that NOS was induced *in vivo* and preserved *in vitro*, and illustrates that an endotoxin-induced NOS is present in the endothelium, which agrees with studies using cultured endothelial cells (Radomski *et al.*, 1990; Palmer *et al.*, 1992). A reduced turnover rate of the induced NOS in the endothelium as compared to that in smooth muscle cells

may explain the differential effects of cycloheximide between endothelium-intact and denuded tissues.

The present study investigated whether OXLDL and LPC could influence the endotoxin-induced hyporeactivity to contractile agents in vascular smooth muscle studied *ex vivo*. At concentrations that alter contractile responses in the rabbit aorta (Plane, 1992), the hyporesponsiveness to PE in denuded rings from endotoxaemic rats remained unaltered in the presence of OXLDL and LPC. This suggests that modified lipoproteins or LPC do not influence the vascular effects of endotoxemia studied *ex vivo*. As shown in this study and others, (Fleming *et al.*, 1991; Julou-Schaeffer *et al.*, 1991; Smith *et al.*, 1991; 1992; Auguet *et al.*, 1992; Vallance *et al*, 1992), endotoxemia has been shown to cause a sustained overproduction of NO from an inducible NOS. As demonstrated here, OXLDL and LPC do not inhibit the production of NO from this enzyme. This supports other findings of this chapter, where OXLDL and LPC attenuated responses mediated via the constitutive NOS but were ineffective against the activity of an inducible isoform. Some possible explanations for this lack of effect are highlighted above.

During atherogenesis, inflammatory cell products such as cytokines may induce the synthesis of an isoform of NOS in the major cell types in the artery wall, which is distinct from the constitutive enzyme present in endothelial cells. This study has shown that oxidatively modified LDL, which are also present in atherosclerotic lesions, and LPC, a major component of OXLDL, can differentially inhibit responses mediated by the induced and constitutive enzymes. OXLDL and LPC inhibit agonist-evoked EDR which are mediated by NO generated by the constitutive NOS whereas L-arginine-evoked relaxations mediated via the inducible form of the enzyme are unaffected.

Furthermore, LPS-induced depression of contractile responses, which can be partly accounted for by NO produced by an inducible NOS, is also unaffected by OXLDL and LPC.

In conclusion, the differential effects of OXLDL and LPC on the activity of the constitutive and inducible isoforms of NOS further illustrates fundamental differences between the two pathways of NO formation in vascular endothelial and smooth muscle cells.

CHAPTER FIVE

INFLUENCE OF L-ARGININE ON VASCULAR RESPONSES IN WHHL RABBIT AORTA

5.1 INTRODUCTION

Many studies have demonstrated inhibition of EDR and altered contractile responses to several agonists in atherosclerotic vessels. The mechanisms which underly these alterations are unclear, although several have been proposed (see section 1.7).

The present study investigated whether deficiency of L-arginine, the precursor of EDRF, could account for the decreased response to endothelium-dependent relaxants. In addition, the effect of an L-arginine supplemented diet on PE-evoked contractions of isolated tissues was also investigated.

5.2 RESULTS

All experiments in this chapter were carried out in endothelium-intact aortic rings.

5.2.1 ENDOTHELIUM-DEPENDENT RESPONES

As shown in **Figure 46b** and **Figure 47**, ACh-evoked responses in descending aortic rings from 6 month-old homozygous WHHL rabbits are markedly attenuated as compared to responses in the same vessel from 6 month-old NZW rabbits (**Figure 46a**).

ACh evoked EDR in descending thoracic aortic rings (n=6-7 rings from each animal), with the mean response of all rings being 30.8 ± 14.2% (n=4 separate experiments) relaxation of PE-induced tone, as shown in **Figure 47a**. However, relaxations to low doses of ACh were significantly greater in rings from the aortic arch (2 rings from each animal) compared to the responses in

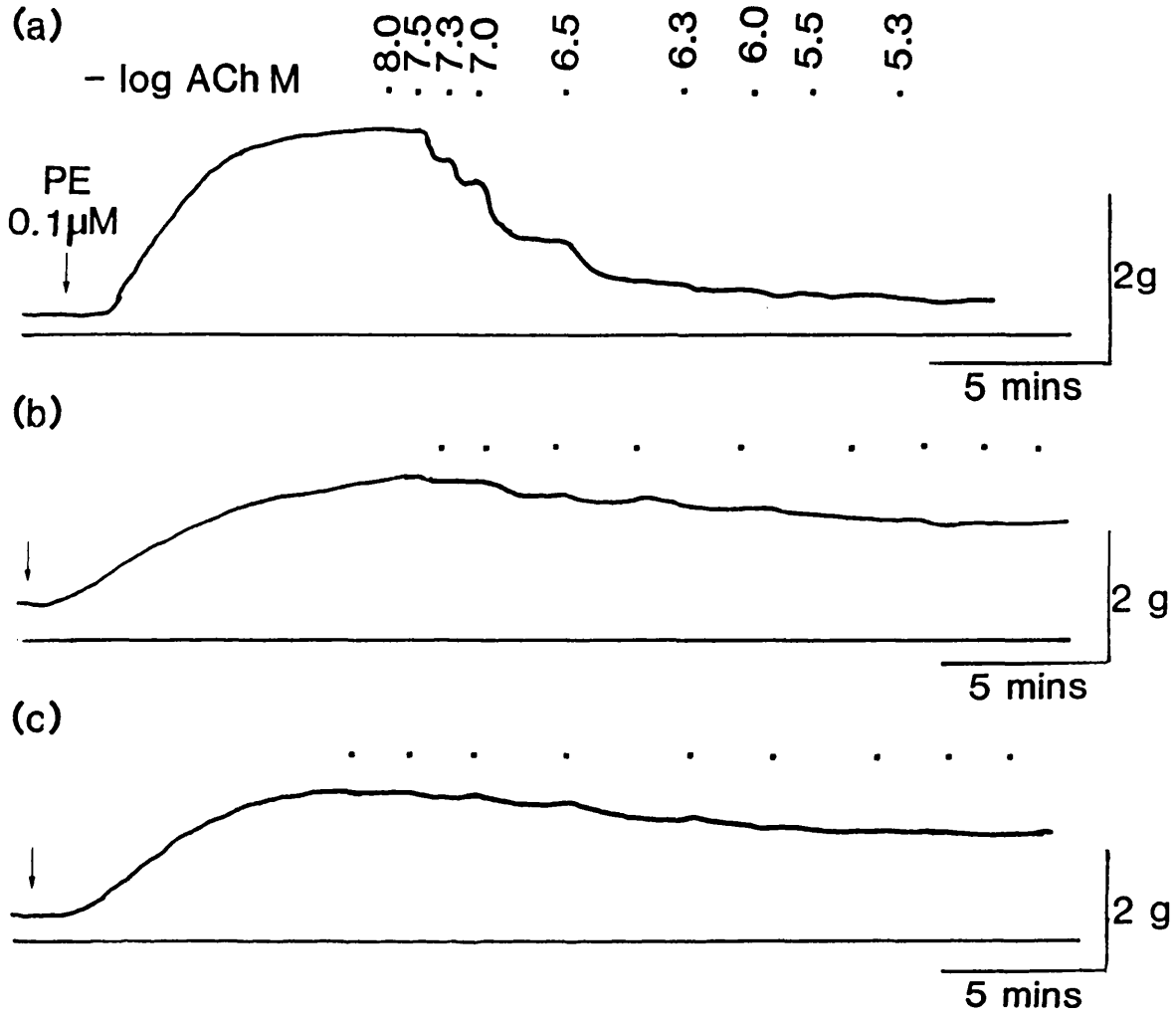

Figure 46 : Comparison of ACh-evoked relaxations in aortic rings from NZW and homozygous WHHL rabbits and the influence of L-arginine incubation on responses in WHHL rabbits

Descending aortic rings from 6-month old NZW and WHHL rabbits pre-contracted with PE (0.1 μM) and relaxed to cumulative doses of ACh (0.01-5 μM)

(a) NZW rabbit

(b) WHHL rabbit

(c) WHHL rabbit and L-arginine (100 μM) incubated for 1 hr.

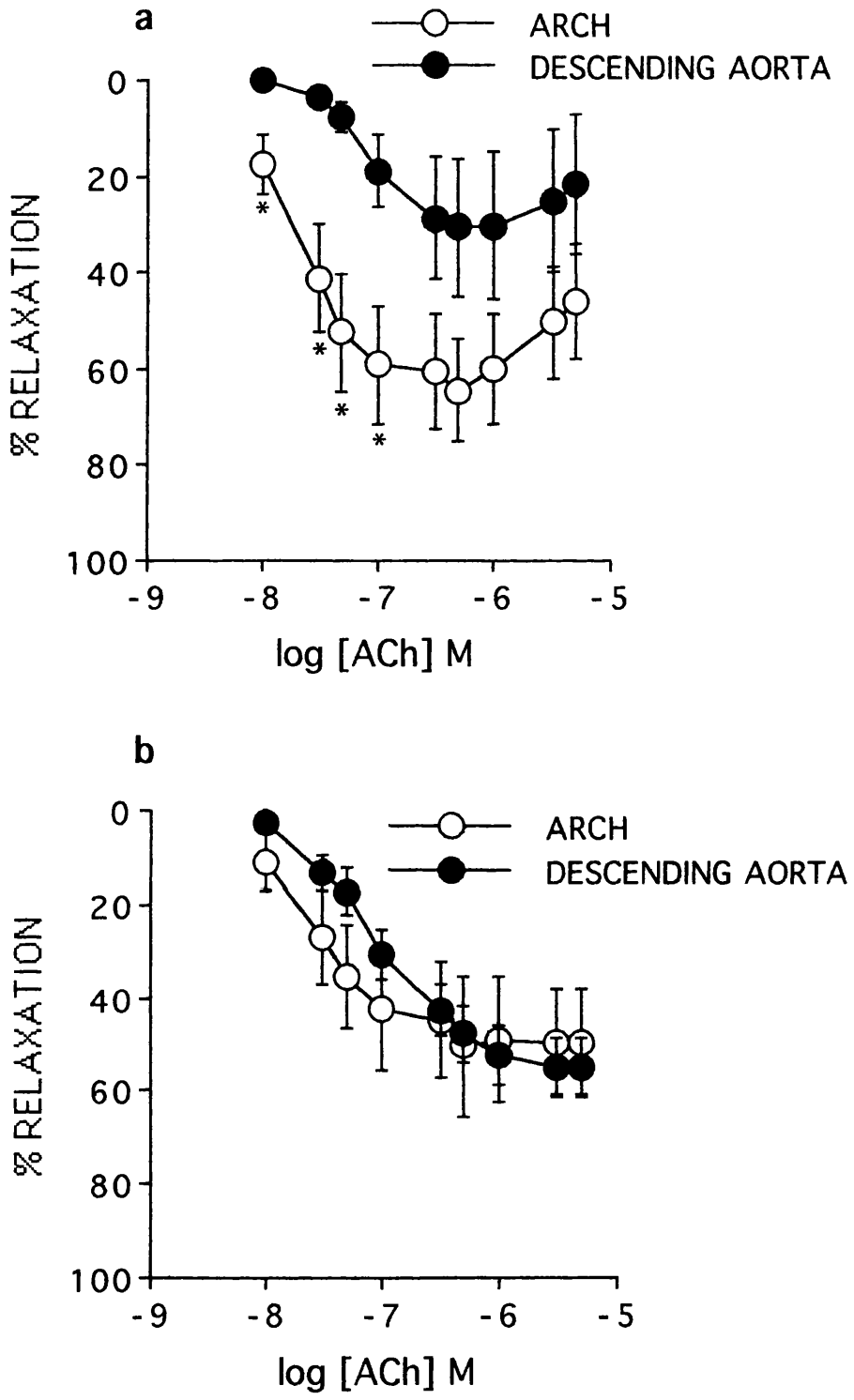

Figure 47 : ACh-evoked relaxations in aortic arch and descending aortic tissues from young and old homozygous WHHL rabbits

Arch and descending aortic tissues from young (6-month old) and old (18-30-month old) homozygous WHHL rabbits pre-contracted with PE (0.1-0.2 μM) and relaxed to cumulative concentrations of ACh (0.01-5 μM; n=4).
(a) Aortic arch and descending tissues from young rabbits
(b) Aortic arch and descending tissues from old rabbits.
Students' t-test compared to descending aortic tissues : * $p<0.05$.

rings from the descending aorta. However, the maximal relaxation in the arch to ACh (64.6 ± 10.7%; n=4 separate experiments) was not significantly different from responses in descending aortic segments (**Figure 47a**).

In older homozygous WHHL rabbits (18-30 months), no significant difference between ACh-evoked relaxations in tissues from the arch and descending aorta was observed. The maximal relaxation elicited by ACh in the arch was 50.5 ± 15.2% (n=4) and in the descending aorta, 55.0 ± 6.4% (**Figure 47b**; n=4).

As shown in **Figure 48**, comparison of the relaxations elicited by ACh in isolated aortic rings from younger and older homozygous WHHL rabbits show that the attenuation of EDR did not increase as the age of the animal increased, and that the impairment was maximal at the age of 6 months.

In contrast, descending aortic rings from heterozygous WHHL rabbits, which have modestly elevated plasma cholesterol (Atkinson *et al.*, 1989), showed almost normal responses to ACh but ACh-evoked relaxations in rings from the aortic arch were depressed (**Figure 49**). The maximal response in the descending aortic rings (96.3 ± 0.9%; n=3) was significantly greater than in arch rings (77.3 ± 4.8%; n=3).

5.2.2 EFFECT OF L-ARGININE ON PRE-CONTRACTED TISSUES

To determine whether the inducible form of NOS was present in freshly isolated descending aortic rings from young WHHL rabbits, the effect of L-arginine on pre-contracted tissues was examined. Bolus doses of L-arginine (100 μM) added to tissues pre-contracted with PE (0.1-0.3 μM) did not alter induced tone (n=4).

5.2.3 EFFECT OF L-ARGININE INCUBATION ON EDR

This study was carried out to determine whether exposure of tissues to L-

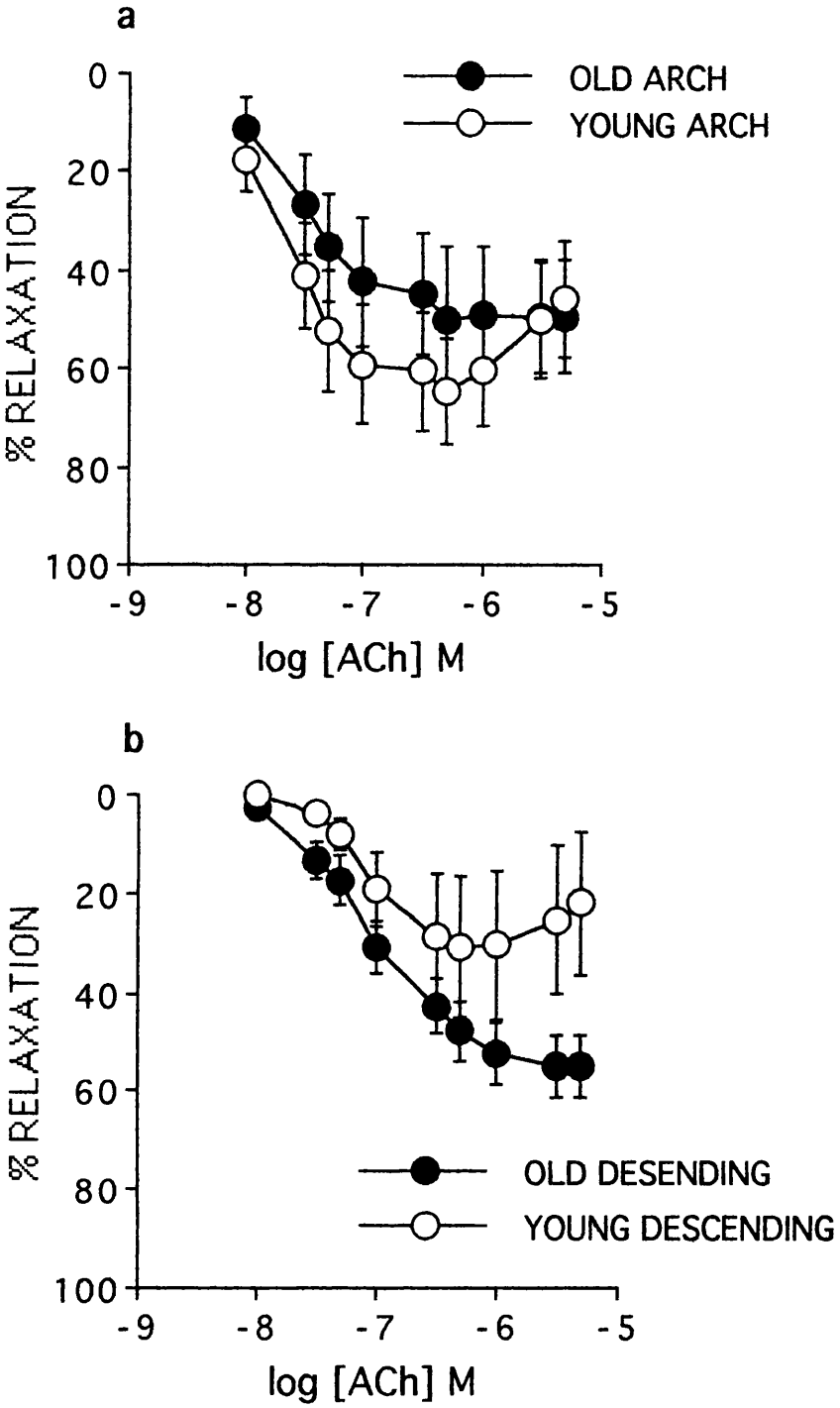

Figure 48 : Comparison of ACh-evoked relaxations in arch and descending aortic tissues from young and old homozygous WHHL rabbits

Arch and descending aortic tissues from young (6-month old) and old (18-30-month old) homozygous WHHL rabbits pre-contracted with PE (0.1-0.2 μM) and relaxed to cumulative doses of ACh (0.01-5 μM; n=4 for each).
(a) Aortic arch tissues from young and old WHHL rabbits
(b) Descending aortic tissues from young and old WHHL rabbits
Students' t-test compared to young tissues : * $p<0.05$.

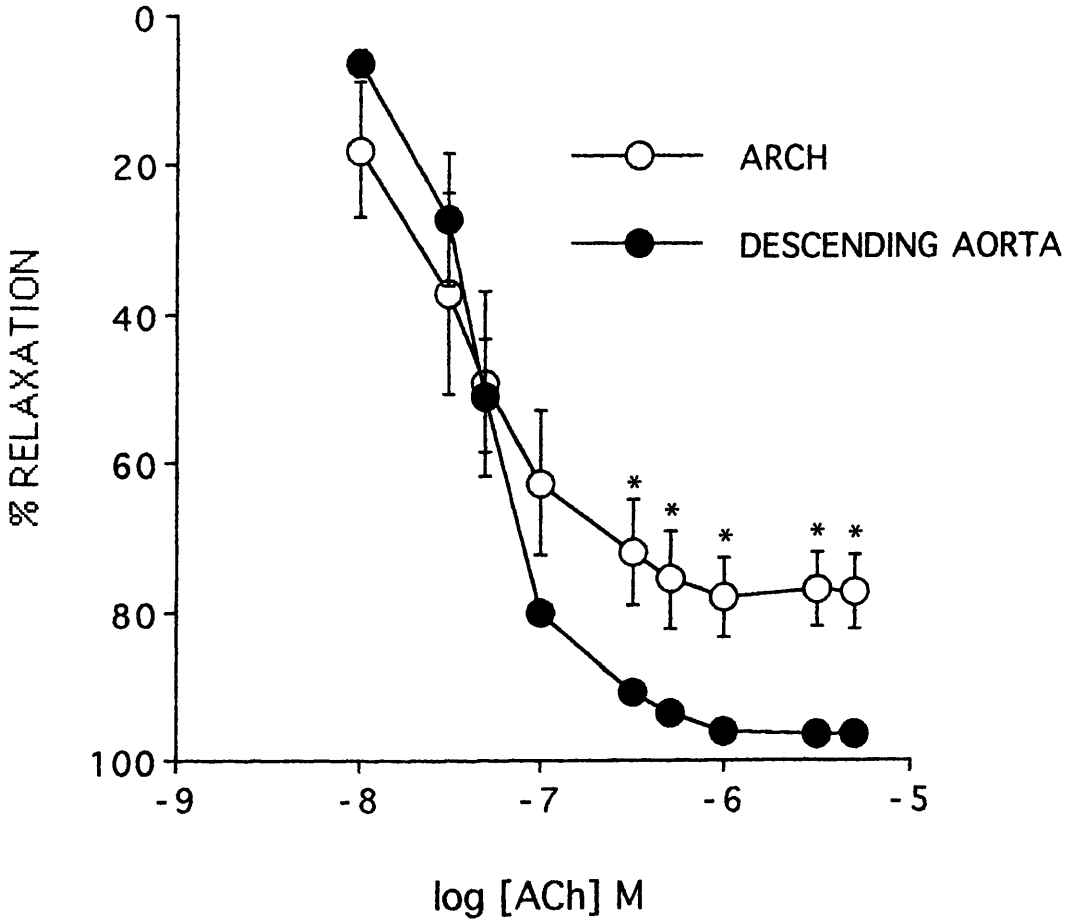

Figure 49 : Comparison of ACh-evoked relaxations in arch and descending aortic tissues from heterozygous WHHL rabbits

Aortic arch and descending tissues from heterozygous WHHL rabbits pre-contracted with PE (0.1 μM) and relaxed to cumulative doses of ACh (0.01-5 μM; n=3).

Students' t-test compared to descending aortic tissues : * $p<0.05$.

arginine could reverse the depression of ACh-evoked relaxations in atherosclerotic aortic rings from young (6-month old) and old (18-30 -month old) homozygous WHHL rabbits. As shown in **Figure 50**, responses to cumulative concentrations of ACh in pre-constricted (**a**) arch and (**b**) descending aortic rings from young rabbits were unchanged in the presence of L-arginine (100 μM) for 1 hr.

Similar results were obtained in arch and descending aortic tissues from old WHHL rabbits as illustrated in the representative trace in **Figure 46**. The mean of 4 experiments is shown in **Figure 51**.

These results demonstrate that, as in NZW rabbits (section 3.3.4), acute administration of L-arginine does not influence ACh-evoked endothelium-dependent responses and suggests that the availability of L-arginine is not a rate-limiting factor for EDRF synthesis and release in this tissue.

5.3 EFFECT OF L-ARGININE FEEDING ON VASCULAR RESPONSES

This study investigated whether placing WHHL rabbits on an L-arginine supplemented diet could improve endothelium-dependent responses or alter contractions of aortic tissues *in vitro*. WHHL rabbits were fed a standard chow diet, with L-arginine in the drinking water available *ad libitum* as described in section 2.5.2. After five days, the animals were sacrificed and aortic rings were mounted (section 2.5.1). Two homozygous (6 months and 18-30 months) and one heterozygous (9 months) WHHL rabbit groups were used in this study.

5.3.1 EFFECT OF L-ARGININE FEEDING ON PLASMA L-ARGININE LEVELS

L-arginine levels in the plasma of two control and two treated younger homozygous WHHL rabbits were measured by high performance liquid

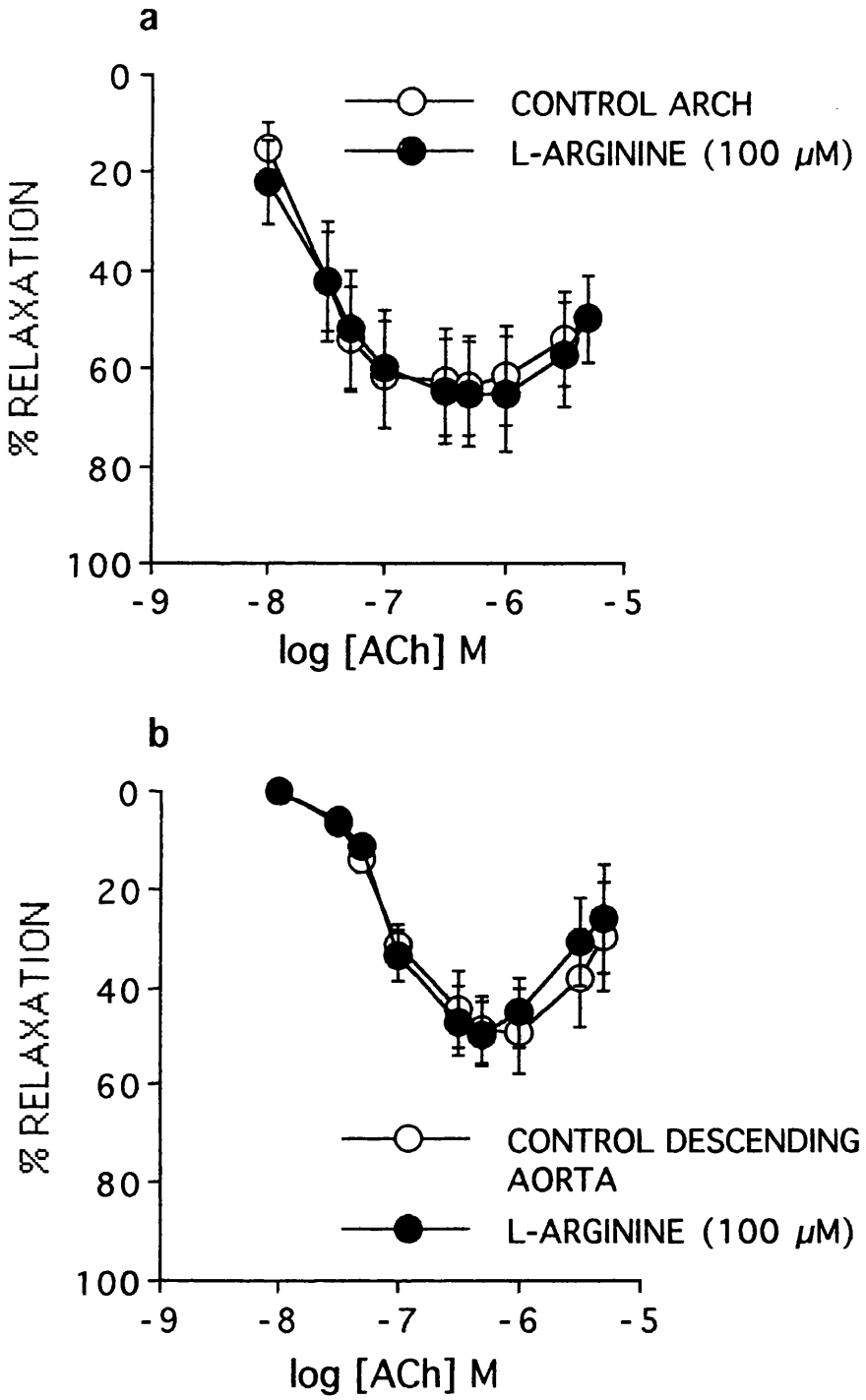

Figure 50 : The influence of L-arginine on ACh-evoked relaxations in tissues from young homozygous WHHL rabbits

Arch and descending aortic tissues from young (6-month old) homozygous WHHL rabbits pre-contracted with PE (0.1-0.3 μM) and relaxed to cumulative doses of ACh (0.01-5 μM). After washout and eqilibration, L-arginine (100 μM) added to the tissues for 1 hr before contraction/ relaxation cycle repeated (n=4 for each).

(a) Aortic arch tissues with L-arginine (100 μM) for 1 hr

(b) Descending aortic tissues with L-arginine (100 μM) for 1 hr.

Students' t-test compared to control : * $p<0.05$.

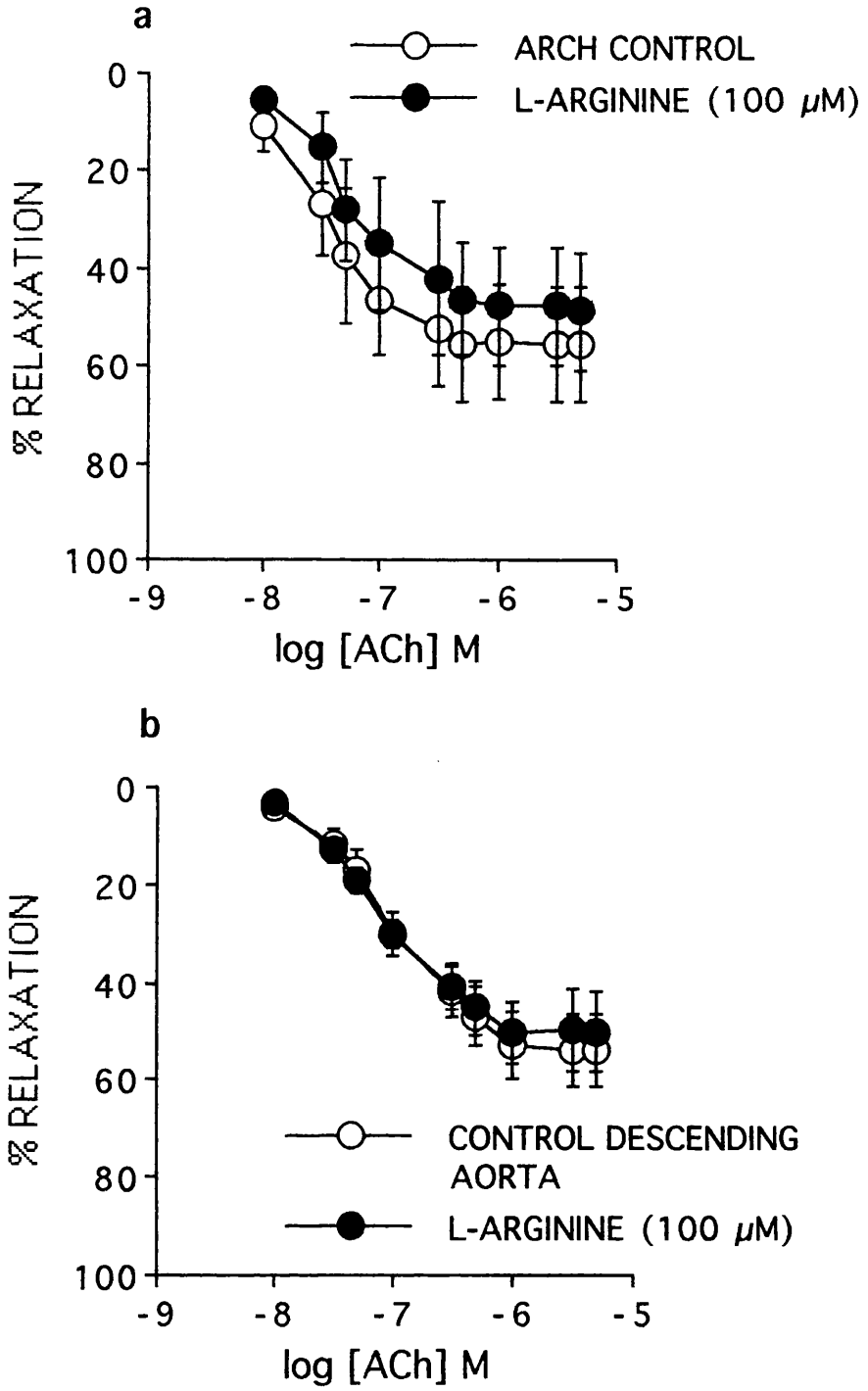

Figure 51 : The influence of L-arginine on ACh-evoked relaxations in tissues from old homozygous WHHL rabbits

Arch and descending aortic tissues from old (18-30-month old) homozygous WHHL rabbits pre-contracted with PE (0.1-0.3 μM) and relaxed to cumulative doses of ACh (0.01-5 μM). After washout and eqilibration, L-arginine (100 μM) added to the tissues for 1 hr before contraction/ relaxation cycle repeated (n=4 for each).

(a) Aortic arch tissues with L-arginine (100 μM) for 1 hr

(b) Descending aortic tissues with L-arginine (100 μM) for 1 hr.

Students' t-test compared to control : * $p<0.05$.

chromotography. In control rabbits, the concentrations were 0.12 and 0.15 mM. In L-arginine-fed rabbits, the concentrations were 0.40 and 0.27 mM, showing an approximate two-fold increase above controls.

5.3.2 EFFECT OF L-ARGININE FEEDING ON EDR

Figure 52 shows the effect of L-arginine feeding on ACh-evoked relaxations in aortic rings from young (6 month) homozygous WHHL rabbits. In rings taken from the aortic arch (**a**) the sensitivity to ACh was unaffected and the maximal relaxation was unchanged with respect to controls (control, 64.6 ± 10.7% vs L-arginine-fed, 64.0 ± 12.0%; n=4). In tissues from the descending aorta (**b**), the maximal response to ACh increased from 30.8 ± 14.2% (n=4) to 48.2 ±3.9% (n=4) of PE-induced tone. This increase was not significant.

In the older homozygous WHHL rabbits, L-arginine-feeding also had no significant effect on the responses to ACh in tissues from either the arch or descending aorta (**Figure 53 a** and **b**). In the arch, the control maximal relaxation was 50.5 ± 15.2% (n=4) whereas in L-arginine-fed animals the maximal response was 27.0 ± 11.6% (n=4). In the descending aorta, the maximum response in controls was 55.0 ± 6.4% (n=4) and 29.5 ± 13.4% (n=4) in tissues from L-arginine-fed rabbits.

Concentration-response curves to ACh in heterozygous WHHL rabbits were also not influenced by the L-arginine-supplemented diet, as shown in **Figure 54**. The maximal relaxation to ACh in (**a**) arch (**b**) descending aortic rings in control animals (78.0 ± 5.3%, n=3; 96.3 ± 0.9%, n=3 respectively) did not significantly differ from L-arginine-fed rabbits (68.3 ± 11.5%, n=3; 95.3 ± 0.7%, n=3 respectively).

5.3.3 PE-EVOKED CONTRACTIONS

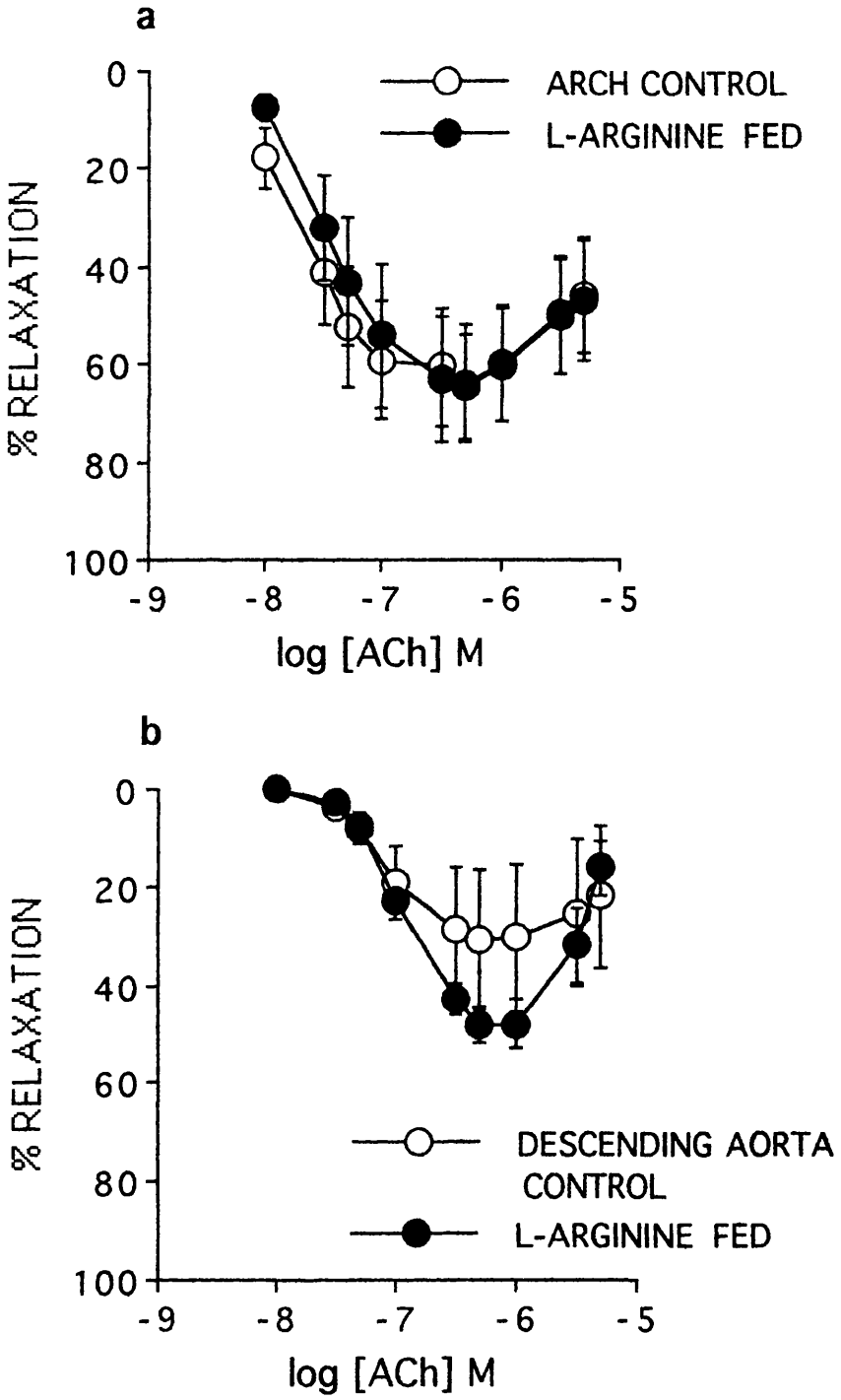

Figure 52 : The influence of L-arginine feeding on ACh-evoked relaxations in tissues from young homozygous WHHL rabbits

Arch and descending aortic tissues from control and L-arginine-fed young (6-month old) homozygous WHHL rabbits pre-contracted with PE (0.1-0.3 μM) and relaxed to cumulative doses of ACh (0.01-5 μM; n=4 for each).

(a) Aortic arch tissues

(b) Descending aortic tissues.

Students' t-test compared to control : * $p<0.05$.

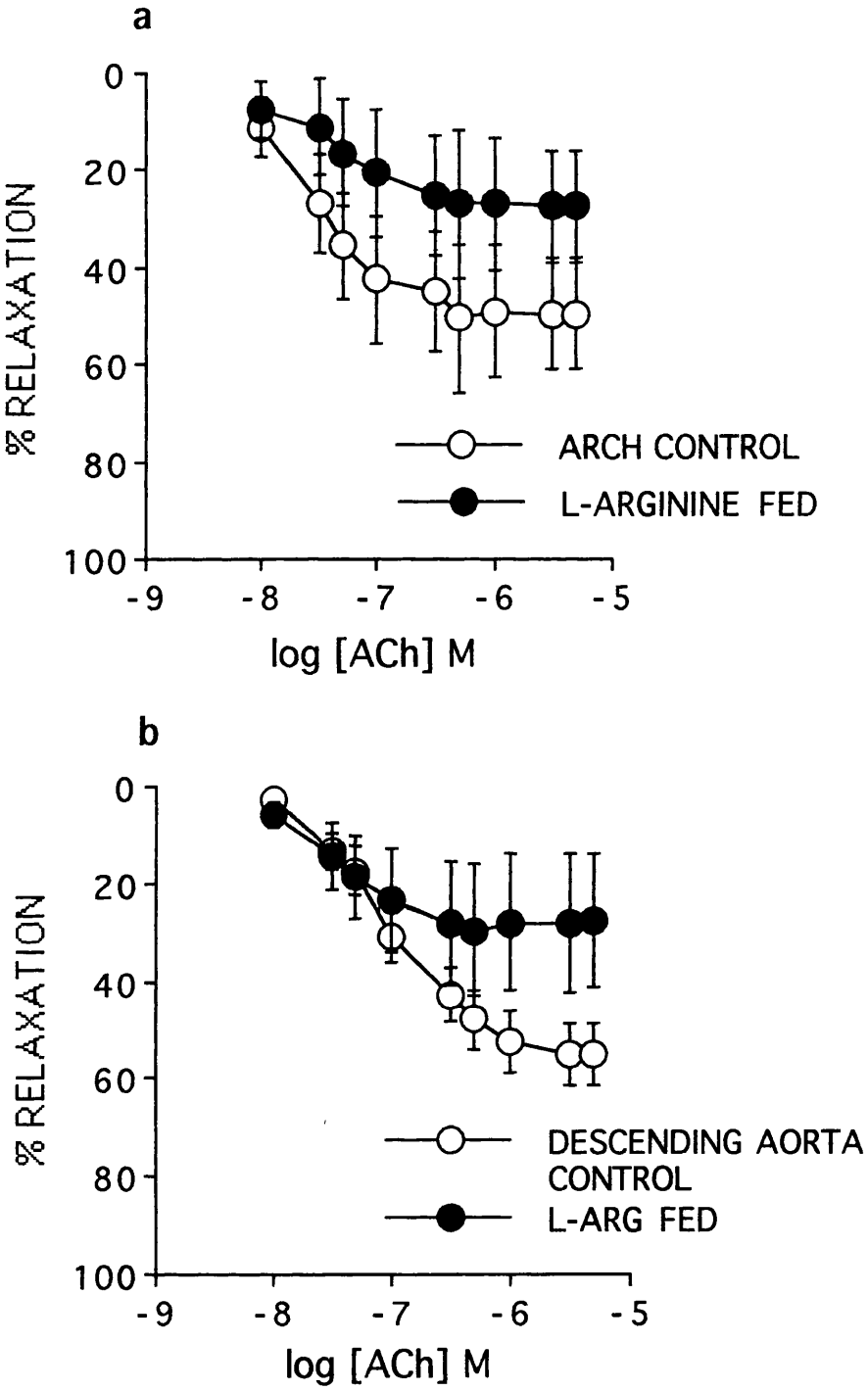

Figure 53 : The influence of L-arginine feeding on ACh-evoked relaxations in tissues from old homozygous WHHL rabbits

Arch and descending aortic tissues from control and L-arginine-fed old (18-30-month old) homozygous WHHL rabbits pre-contracted with PE (0.1-0.3 μM) and relaxed to cumulative doses of ACh (0.01-5 μM; n=4 for each).

(a) Aortic arch tissues

(b) Descending aortic tissues.

Students' t-test compared to control : * $p<0.05$.

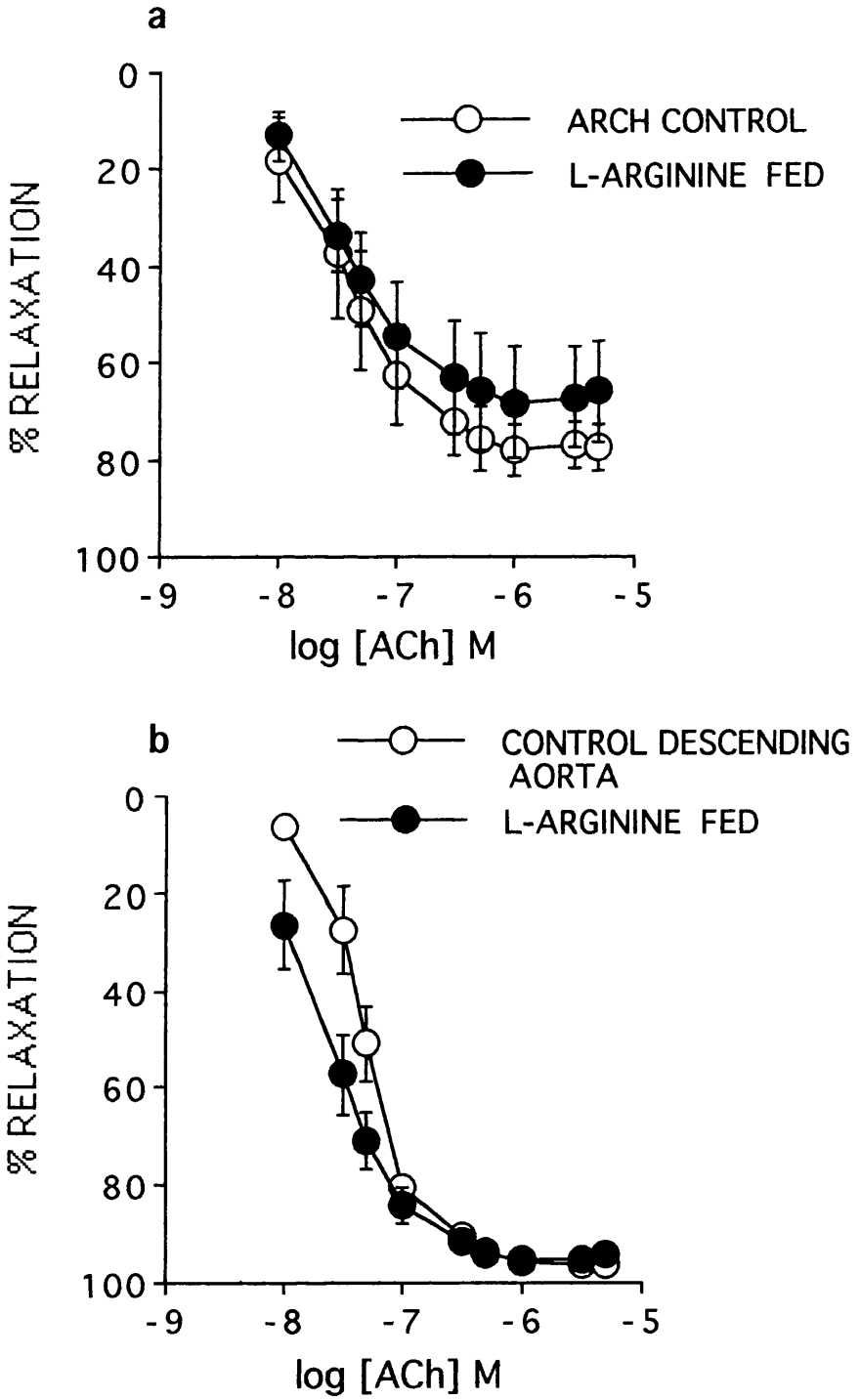

Figure 54 : The influence of L-arginine feeding on ACh-evoked relaxations in tissues from heterozygous WHHL rabbits

Arch and descending aortic tissues from control and L-arginine-fed heterozygous WHHL rabbits pre-contracted with PE (0.1-0.3 μM) and relaxed to cumulative doses of ACh (0.01-5 μM; n=3 for each).

(a) Aortic arch tissues

(b) Descending aortic tissues.

Students' t-test compared to control : * $p<0.05$.

Endothelium-independent contractions induced by the α_1-adrenoceptor agonist PE (0.01-50 μM) were compared in aortic tissues from young and old homozygous, and heterozygous WHHL rabbits.

In all three groups, PE-evoked contractions were significantly greater in the arch tissues compared to descending aortic rings. In young homozygous animals, the maximum contraction in tissues from the arch were 8.0 ± 0.4 g (n=4) and in the descending thoracic aorta, 5.4 ± 0.09 g (n=4). The EC_{50} values were significantly greater in the arch (arch, 788 ± 268 nM; n=4 vs descending aorta, 320 ± 26 nM; n=4; **Figure 55a**).

Similarly, in older rabbits the maximal contraction evoked by PE was significantly greater in arch tissues (7.6 ± 1.1 g; n=4) than the descending thoracic aorta (5.2 ± 0.27 g; n=4), with greater EC_{50} values (arch 706 ± 189 nM; n=4 vs descending aorta 320 ± 42 nM; n=4; **Figure 55b**).

When PE-evoked contractions of isolated aortic tissues from younger and older homozygous WHHL rabbits are compared, it is clear that they remain consistent as the age of the animals increases, as shown in **Figure 56**.

As shown in **Figure 57**, contractions to PE in arch rings from heterozygous rabbits (8.1 ± 1.2 g; n=3) were also significantly greater than those evoked in rings from the descending aorta (5.3 ± 0.09 g; n=3) with the EC_{50} value decreased from 540 ± 30 nM (n=3) in aortic arch rings to 160 ± 18 nM (n=3).

5.3.4 EFFECT OF L-ARGININE FEEDING ON PE-EVOKED CONTRACTIONS

Responses to PE in aortic rings from L-arginine-fed homozygous and heterozygous WHHL rabbits were compared to contractions in tissues from age-matched controls to assess the effects of L-arginine diet supplementation on contractile responses.

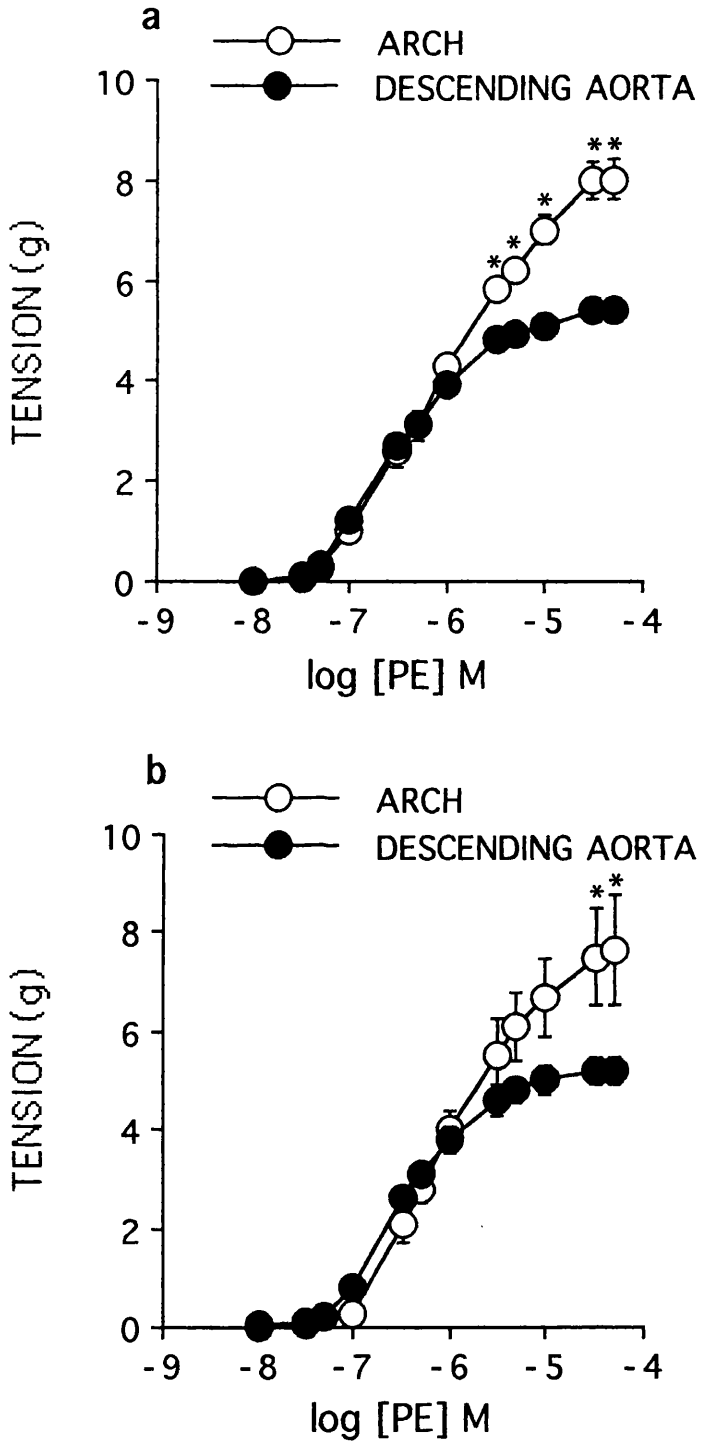

Figure 55 : Comparison of PE-evoked contractions between arch and descending aortic tissues from young and old homozygous WHHL rabbits
Arch and descending aortic tissues from young (6-month old) and old (18-30 month old) homozygous WHHL rabbits contracted with cumulative doses of PE (0.01-50 μM) until maximum level of induced tone was attained (n=4 for each).
(a) Arch and descending aortic tissues from young rabbits
(b) Arch and descending aortic tissues from old rabbits.
Students' t-test compared to descending aortic tissues : * $p<0.05$.

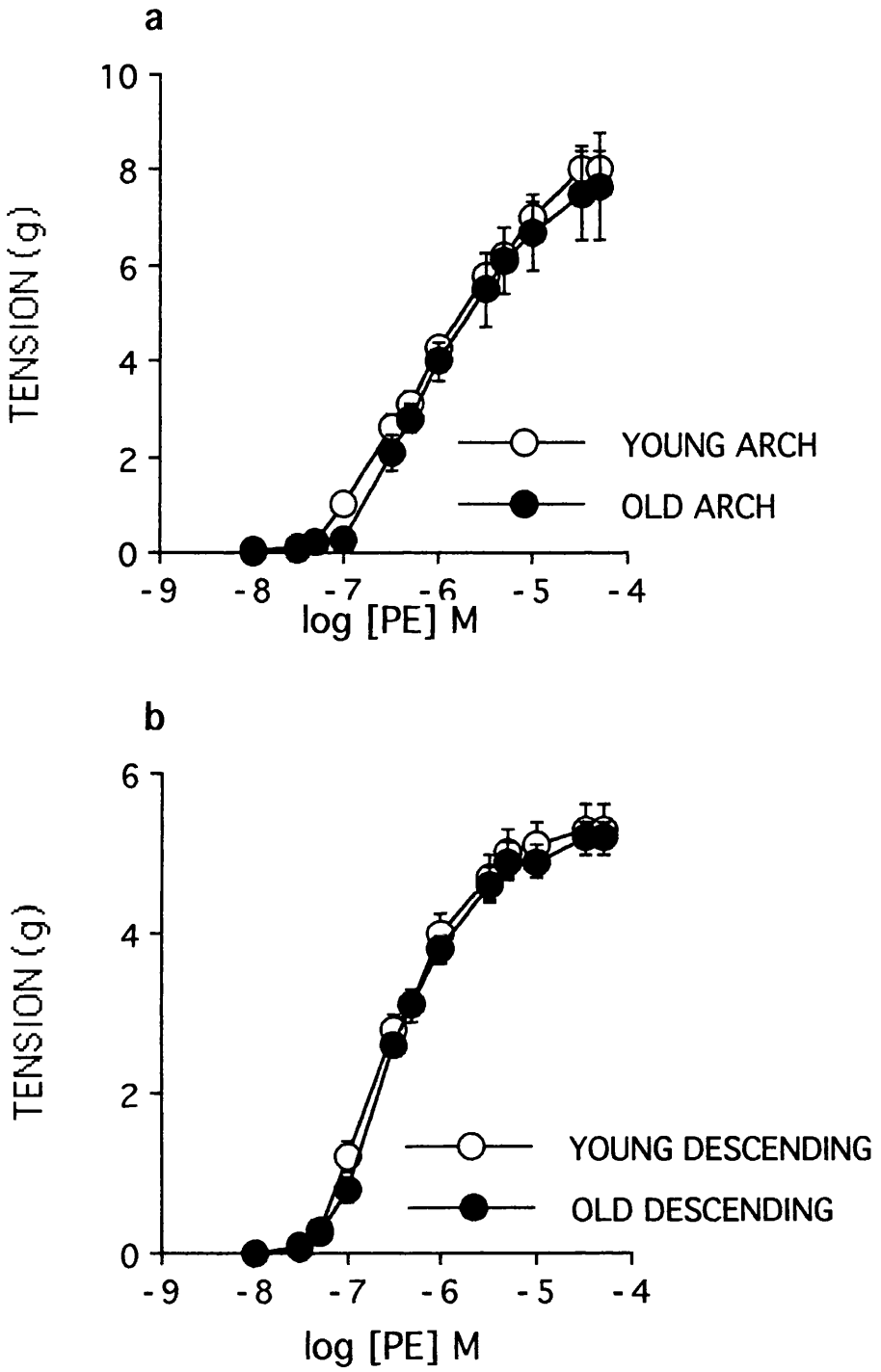

Figure 56 : Comparison of PE-evoked contractions in arch and descending aortic tissues between young and old homozygous WHHL rabbits

Arch and descending aortic tissues from young (6-month old) and old (18-30 month old) homozygous WHHL rabbits contracted with cumulative doses of PE (0.01-50 μM) until maximum level of induced tone was attained (n=4 for each).

(a) Aortic arch tissues from young and old rabbits

(b) Descending aortic tissues from young and old rabbits.

Students' t-test compared to young tissues : * $p<0.05$.

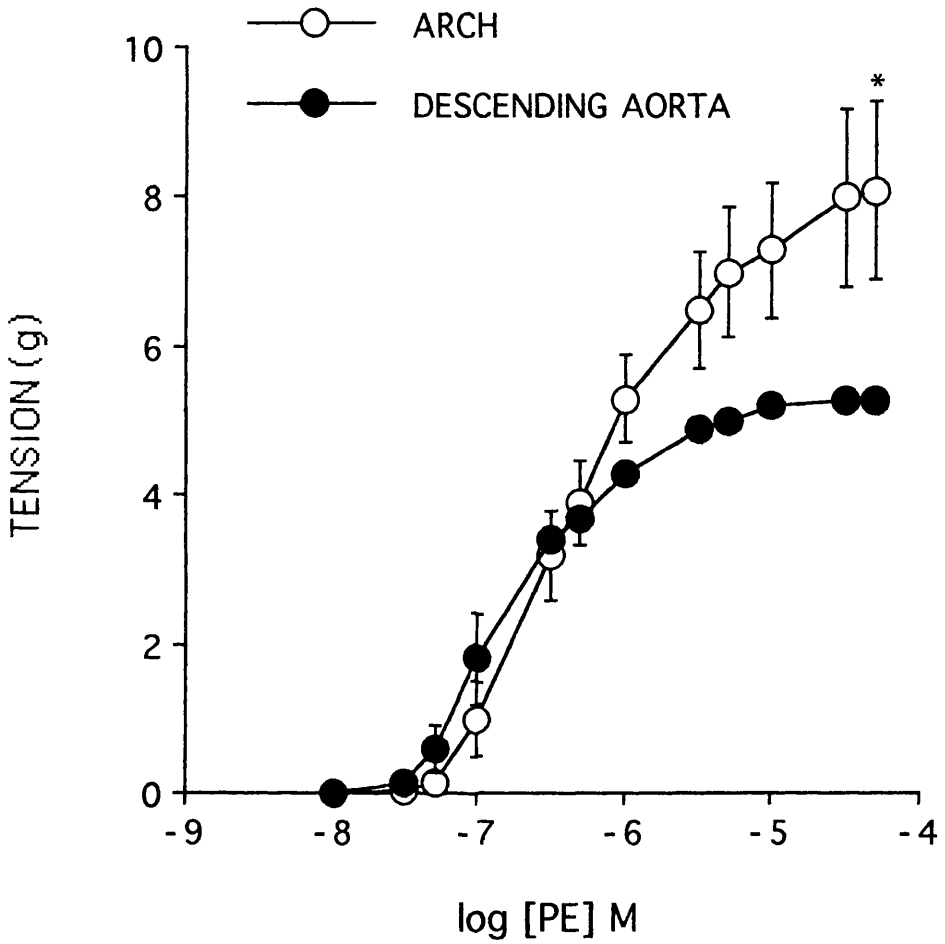

Figure 57 : PE-evoked contractions in arch and descending aortic tissues from heterozygous WHHL rabbits

Arch and descending aortic tissues from heterozygous WHHL rabbits contracted with cumulative doses of PE (0.01-50 μM) until maximum level of induced tone was attained (n=3).

Students' t-test compared to descending aortic tissues : * $p<0.05$.

The effect of L-arginine feeding on PE-evoked contractions in decending aortic rings from 6 month-old homozygous WHHL rabbits are shown **Figure 58**. In tissues from both the arch (**Figure 59a**) and descending aorta (**Figure 59b**), L-arginine-feeding had no effect on the sensitivity or the maximum contraction elicited by PE. The EC_{50} value for PE in arch rings from control animals (788 ± 268 nM; n=4) was not significantly different from the EC_{50} in arch rings from L-arginine-fed rabbits (1466 ± 406 nM; n=4). Also, the maximal contraction was unaltered (control, 8.0 ± 0.4 g n=4; L-arginine fed, 8.3 ± 0.34 g, n=4). In descending aortic rings, the sensitivity ($EC_{50\ control}$ 320 ± 26 nM, n=4; $EC_{50\ L\text{-}arginine}$ 535 ± 145 nM, n=4) and maximal contraction (control 5.4 ± 0.09 g, n=4; L-arginine-fed 6.0 ± 0.27 g, n=4) were not significantly altered by L-arginine feeding.

Similarly, placing animals on a L-arginine supplemented diet had no significant effect on contractions evoked by PE in aortae from older homozygous WHHL rabbits, as shown in **Figure 60**. Maximal responses in arch tissues (7.6 ± 1.1 g; n=4) and descending aortic rings (5.2 ± 0.27 g; n=4) did not significantly differ from those obtained in rings from L-arginine-fed rabbits (arch, 6.5 ± 1.1 g, n=4; descending aorta, 4.4 ± 0.52 g, n=4). Furthermore, the sensitivity of the tissues did not alter in L-arginine-fed rabbits as indicated by the EC_{50} values for the arch and descending aortic rings (802 ± 115 nM, n=4; 465 ± 30 nM, n=4 respectivly).

Tissues from heterozygous WHHL rabbits fed an L-arginine enriched diet showed non-significant reductions in responses evoked by PE (**Figure 61**). In the aortic arch, the maximal contraction in control tissues was 8.1 ± 1.2 g (n=3) and in rings from treated animals the maximum response was 5.8 ±1.7 g (n=3). Additionally, EC_{50} values increased from 540 ± 30 nM (n=3) to 750 ±

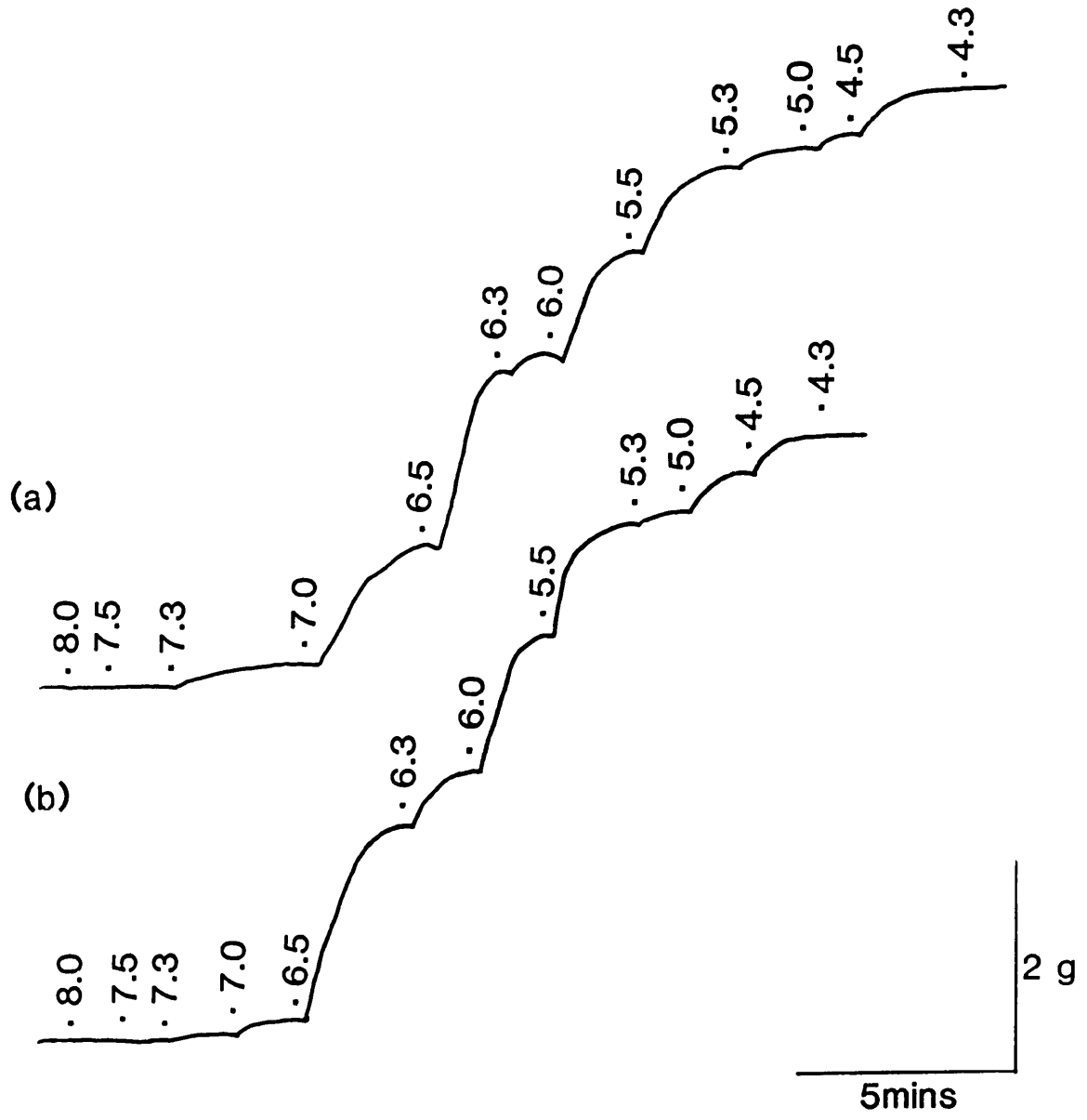

Figure 58 : The influence of L-arginine feeding on PE-contractions in tissues from young homozygous WHHL rabbits

Descending aortic rings from control and L-arginine-fed young (6-month old) homozygous WHHL rabbits contracted with cumulative doses of PE (0.01-50 μM) until maximum level of tone was attained (n=4).

(a) Control

(b) L-arginine-fed

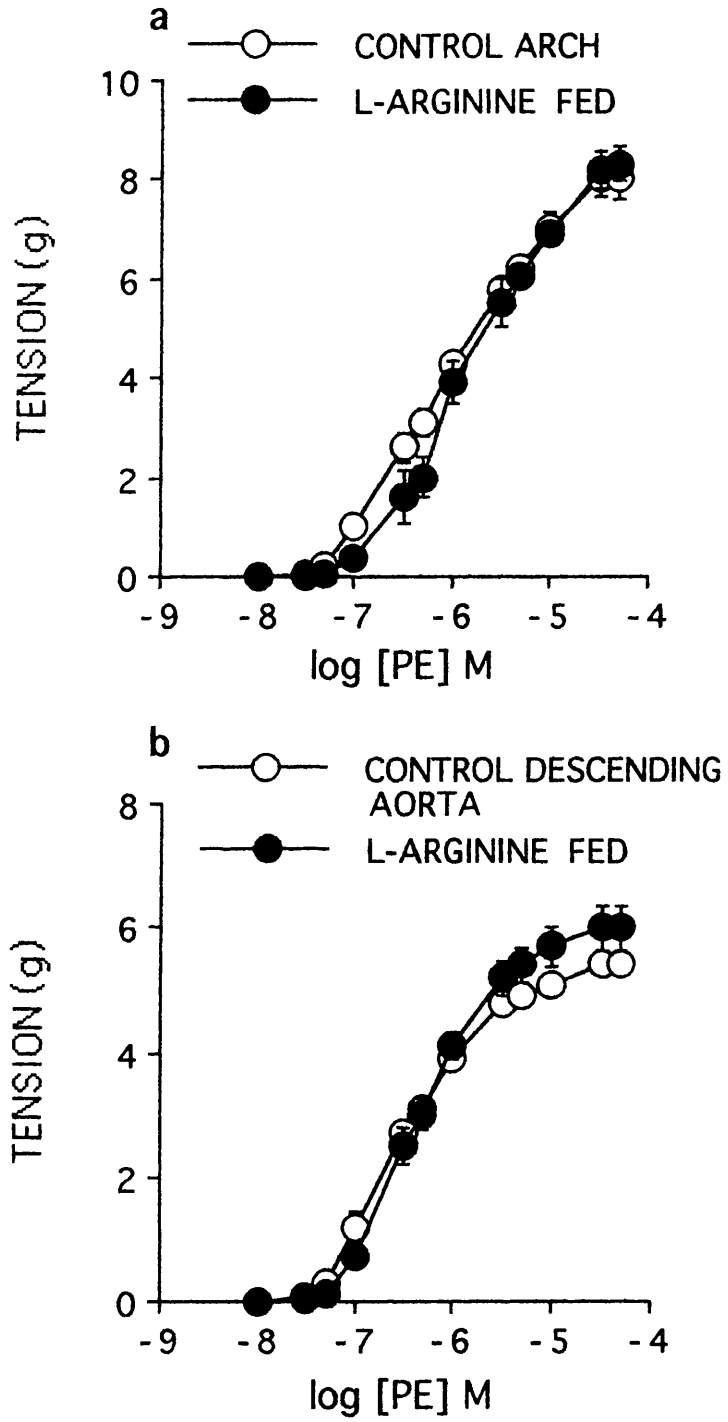

Figure 59 : The influence of L-arginine feeding on PE-evoked contractions in tissues from young homozygous WHHL rabbits

Arch and descending aortic tissues from control and L-arginine-fed young (6-month old) homozygous WHHL rabbits contracted with cumulative doses of PE (0.01-50 μM) until maximum level of tone was attained (n=4).

(a) Aortic arch tissues

(b) Descending aortic tissues

Students' t-test compared to control : * $p<0.05$.

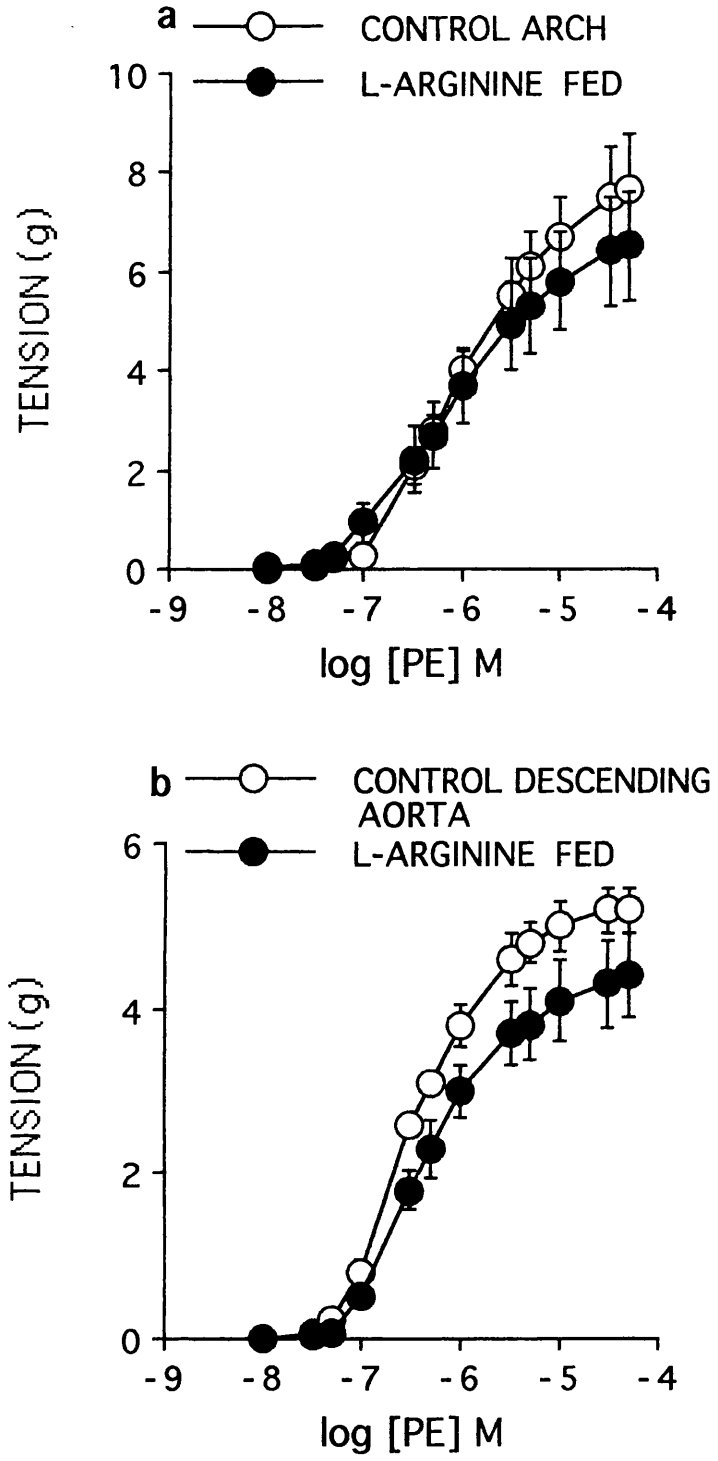

Figure 60 : The influence of L-arginine feeding on PE-evoked contractions in tissues from old homozygous WHHL rabbits

Arch and descending aortic tissues from control and L-arginine-fed old (18-30-month old) homozygous WHHL rabbits contracted with cumulative doses of PE (0.01-50 μM) until maximum level of tone was attained (n=4).

(a) Aortic arch tissues

(b) Descending aortic tissues

Students' t-test compared to control : * $p<0.05$.

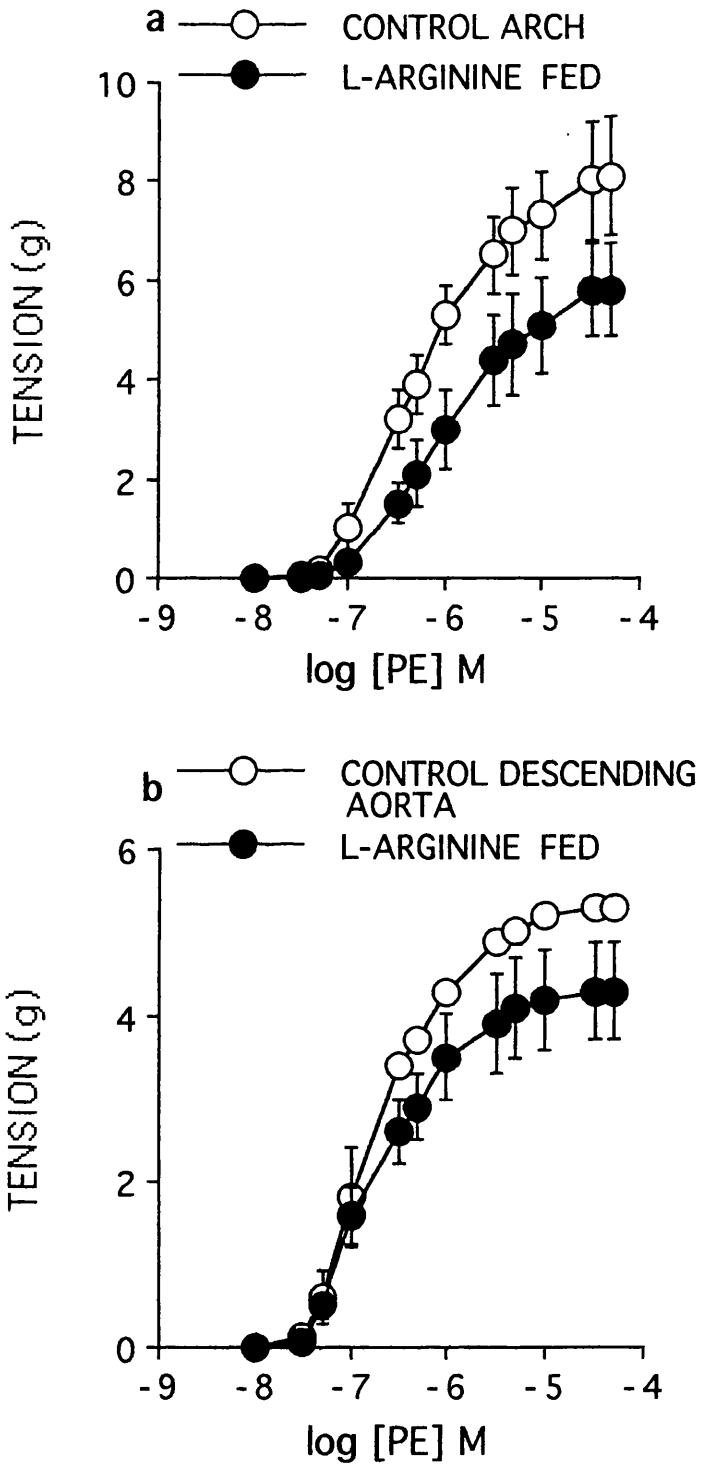

Figure 61 : The influence of L-arginine feeding on PE-evoked contractions in tissues from heterozygous WHHL rabbits

Arch and descending aortic tissues from control and L-arginine-fed heterozygous WHHL rabbits contracted with cumulative doses of PE (0.01-50 μM) until maximum level of tone was attained (n=3).

(a) Aortic arch tissues

(b) Descending aortic tissues

Students' t-test compared to control : * $p<0.05$.

210 nM (n=3). The maximal response to PE showed a non-significant decrease in descending aortic rings from 5.3 ± 0.09 g (n=3) to 4.3 ± 0.58 g in treated rabbits and EC_{50} values increased from 160 ± 18 nM (n=3) to 190 ± 40 nM (n=3).

5.4 DISCUSSION

Many reports have demonstrated impairment of EDR in atherosclerotic vessels and those exposed to hypercholesterolemia (Armstrong *et al.*, 1982; Sreeharan *et al.*, 1986; Verbeuren *et al.*, 1986; 1990; Shimokawa *et al.*, 1987; Yamamoto *et al.*, 1987). However, the precise mechanisms underlying this inhibition are unclear. The present study investigated whether the attenuation of EDR in the WHHL rabbit, an animal model of atherosclerosis that spontaneously develops atheroma on a standard diet can be accounted for by the decreased availability of L-arginine, the precursor of EDRF.

In this investigation, responses in isolated aortic tissues from homozygous WHHL rabbits showed severely attenuated EDR compared to tissues from NZW rabbits. Furthermore, the extent of inhibition of EDR was not significantly different between the younger (6 months) and older (18-30 months) rabbits, indicating that the mechanisms involved in this inhibition are fully expressed at a very young age. This is in contrast to previous reports that impairment of responses increases with the age of the animal (Kolodgie *et al.*, 1990; Stewart-Lee and Burnstock, 1991).

Addition of L-arginine failed to evoke any response in pre-contracted tissues suggesting that the inducible form of NOS was not present in either the endothelium or vascular smooth muscle. This is despite the presence of inflammatory cells, such as macrophages, in atherosclerotic lesions (Steinberg *et al.*, 1989) which would be expected to release cytokines, known to induce NOS in several cell

types, including macrophages themselves (see Moncada *et al.*, 1991a).

Acute administration of L-arginine to aortic tissues *in vitro* did not influence endothelium-dependent responses suggesting that the availability of the amino acid is not rate limiting for the production of EDRF. This is in agreement with the findings of a study using the aorta from cholesterol-fed rabbits (Müggе and Harrison, 1991). However, in contrast, *in vitro* exposure to L-arginine was shown to restore responses to endothelium-dependent relaxants in cerebral arteries (Rossitch *et al.*, 1991) and coronary microvessels (Kuo *et al.*, 1992) from hypercholesterolemic animal models. The reasons for these discrepencies are unclear, but cannot be attributed to the length of exposure time of the tissues to L-arginine since in this study a 1 hour period was employed which was longer than that used in either of the other two studies which showed restoration of responses. The concentration of L-arginine used in reports by Rossitch *et al.*(1991) and Kuo *et al.* (1992; 3 mM) was greater than used in the present study (0.1 mM) but was less than used by Müggе and Harrison (1991; 5 mM), therefore, this also seems unlikely to account for the differences reported. However, a major difference between the studies is the size of the vessels used, and may indicate that smaller arteries are more responsive to L-arginine administration, and thus more able to restore endothelium-dependent responses.

Another factor which may be of major importance in explaining the lack of effect of L-arginine could be the severity of the disease. In the present study, the degree of inhibition of endothelium-dependent responses may have been too great to be overcome by L-arginine. If younger rabbits than those used in this investigation (6 months) were studied, the severity of the disease, and EDR impairment would be less, and incubation of the tissues with L-arginine

in vitro maybe more effective in restoring responses.

The uptake and transport of L-arginine in cultured endothelial cells from human umbilical veins and porcine aorta has been demonstrated (Mann *et al.*, 1989; Bogle *et al.*, 1991). However, the transport of L-arginine in endothelial cells of vessels from WHHL rabbits may be altered as a result of the chronic exposure of these cells to elevated levels of cholesterol. If this was to occur in the aorta, it could lead to a deficiency of L-arginine in endothelial cells and impairment of EDR, and thus increasing L-arginine concentrations *in vitro* or *in vivo* may not be effective in restoring responses.

A second approach used in this study to restore responses to endothelium-dependent agonists in aortae from WHHL rabbits was supplementation of the diet with L-arginine. However, under this regime, responses to ACh did not change in either of the homozygous groups or the heterozygous group of WHHL rabbits. This supports the other finding of this study that decreased availability of L-arginine may not account for the impairment of endothelium-dependent responses, as assessed by this *ex vivo* technique. The L-arginine daily requirement of rabbits has been reported to be between 0.6-0.7 g/day (Adamson and Fisher, 1973). In the present study, supplementation with L-arginine (2.2 g/day for 5 days) was administered, a more than three-fold increase in daily intake, which resulted in a two-fold increase in plasma free L-arginine levels.

These results contrast with those of a recent investigation which showed that impaired EDR in isolated aortic rings from cholesterol-fed rabbits could be partially restored by oral administration of L-arginine (Cooke *et al.*, 1992). In the latter study a different model of atherosclerosis was used, and a larger amount of L-arginine added to the diet (2.25 g/100 mls for 10 wks). However,

the plasma L-arginine levels in control and treated rabbits were very similar to those obtained in the present study, and thus may not account for the contrasting results. Furthermore, L-arginine was administered concominantly with the cholesterol-rich diet used to induce atheroma whereas in the present study, L-arginine was given after the disease was macroscopically evident.

Elevation of plasma levels of L-arginine by direct infusion into the blood stream has also been shown to improve endothelium-dependent responses in cholesterol-fed rabbits (Girerd *et al.*, 1990; Cooke *et al.*, 1991) and humans (Drexler *et al.*, 1991; Creager *et al.*, 1992). These findings support the hypothesis that decreased availability of this amino acid may, at least in part, explain the impairment of relaxation responses. This method of administration will increase plasma L-arginine concentrations to high levels in a short period of time, and therefore it may be important to consider the route of administration when comparing results from the various studies that elevate plasma levels of L-arginine *in vivo*.

The interest in the relationship between atherosclerosis and L-arginine has recently extended to patients with hypercholesterolemia. It was suggested that decreased plasma L-arginine levels may contribute to attenuation of EDR, as patients with hypercholesterolemia were shown to have significantly lower levels than normocholesterolemic controls (Jeserich *et al.*, 1992). However, other studies have failed to demonstrate a similar reduction in plasma L-arginine (Oleesky *et al.*, 1992; Pasini *et al.*, 1992), and therefore an inverse causal relationship between cholesterol and L-arginine has still to be established.

Vascular contraction elicited by PE is mediated by activation of α_1-adrenoceptors located on smooth muscle cells. In the present study, the

contractile responses of isolated aortic arch tissues of WHHL rabbits were greater than the descending aortic rings, although the arch segments were less sensitive. Other investigators have found that responses to PE in arch and thoracic aortic tissues from WHHL rabbits are very similar in sensitivity and the maximal levels of contraction to this agonist (Kolodgie *et al.*, 1990). However, they also showed that the contractile responses to PE did not differ between younger and older rabbits which is in agreement with the findings of the present study. Additionally, it has been reported that contractions to PE are not altered in atherosclerotic aortae and coronary vessels from WHHL rabbits (Henry and Yokoyama, 1980; Yokoyama *et al.*, 1983). The consistency of responses to PE in atherosclerotic vessels is in contrast to those reported for 5-HT which are widely reported to be potentiated in both *in vivo* and *in vitro* (see for example, Yokoyama *et al.*, 1983; Verbeuren *et al.*, 1986). The contractile response to the non-specific adrenoceptor agonist NA have been shown to be potentiated (Rossendorff *et al.*, 1981; Heistad *et al.*, 1984) and decreased (Verbeuran *et al.*, 1986; Wines *et al.*, 1989; Asada *et al.*, 1992) by exposure to hypercholesterolemia.

In view of these inconsistencies, and the apparent reproducibility of PE contractions in atherosclerotic vessels from animals of different ages, PE was chosen as the agonist of choice to investigate the effect of L-arginine feeding on the contractions of aortae from WHHL rabbits. Oral administration of L-arginine had no significant effect on contractions to PE in either the arch or descending thoracic aorta from homozygous and heterozygous WHHL rabbits. This suggests that this amino acid does not interefere with the mechanisms of vascular smooth muscle contracture mediated via α_1-adrenoceptors. Furthermore, it supports the finding that L-arginine feeding

does not potentiate responses to endothelium-dependent relaxants in this vessel as increased release of EDRF may depress responses to a contractile agent such as PE.

In conclusion, the findings of the study suggest that the decreased availability of the amino acid L-arginine, the precursor of EDRF, does not contribute to impaired endothelium-dependent responses in isolated atherosclerotic aortic tissues from WHHL rabbits. Furthermore, this indicates that inhibition of EDR in diseased vessels may, therefore, be due to a dysfunction at some point proximal or distal to the intracellular levels of L-arginine required for NO production in endothelial cells.

CHAPTER SIX

GENERAL DISCUSSION

Atherosclerosis is a vascular disease resulting from abnormal interactions between plasma lipoproteins, platelets, monocytes/macrophages and cells of the vessel wall. The development of atherosclerotic lesions involves localised damage to the endothelial cell layer allowing, or caused by, the invasion of LDL and platelets.

The suggestion that oxidatively modified LDL plays an important role in the attenuation of EDR in atherosclerotic vessels has been extensively studied (see for example, Jacobs *et al.*, 1990; Galle *et al.*, 1991). In particular, interest has focused on LPC, a component of OXLDL which has been shown to have complex physiological and pathophysiological roles in cell function, such as signal transduction, and to cause arrhythmias in the ischemic myocardium (Joshua *et al.*, 1992)). In addition, LPC accumulates in the wall of atherosclerotic arteries (Portman and Alexander, 1969; Vidaver *et al.*, 1985) where it may act as a potent chemoattractant for circulating monocytes and inhibitor of macrophage motility (Quinn *et al.*, 1988). In the present investigation, the vasoactive properties of LPC were examined to determine whether it could contribute to the inhibition of EDR by OXLDL.

The possible sites of action of LPC and OXLDL in the arterial wall are summarised in **Figure 62**. Both OXLDL and LPC inhibit EDR mediated by receptor-dependent and independent agonists which stimulate the constitutive NOS. However, in contrast to OXLDL, which appears to have multiple sites of action, the inhibition of EDR by LPC was mediated by a direct action on the endothelium to reduce synthesis/release of NO, with little or no effect on the underlying smooth muscle or NO itself. This suggests that

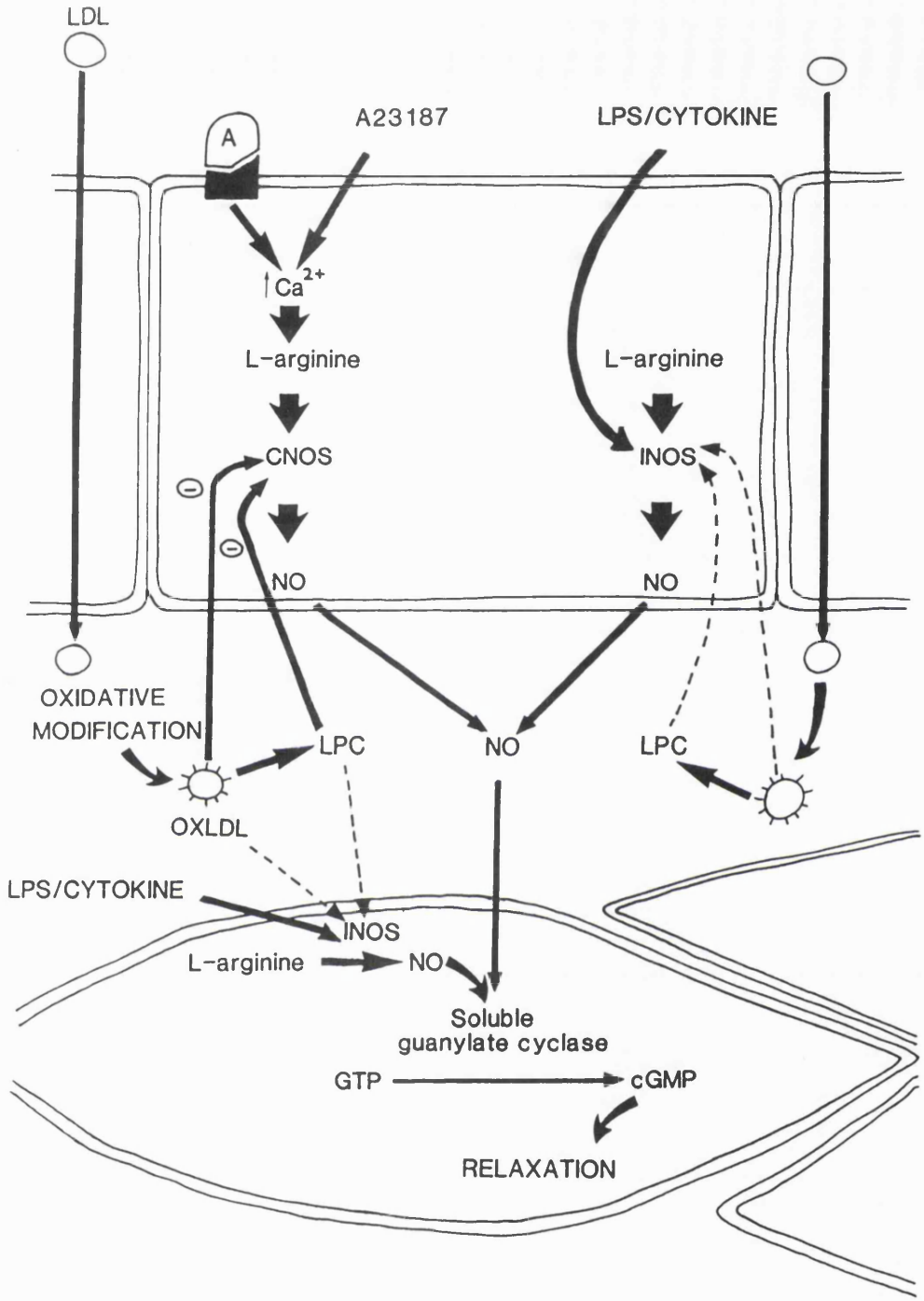

Figure 62 : The possible mechanisms by which OXLDL and LPC may contribute to the alterations in vascular reactivity observed in hypercholesterolemia and atherosclerosis

Abbrevations : **A**, agonist; **CNOS**, constitutive NOS; **INOS**, inducible NOS.

-------- , no effect

_____ , inhibitory effect

other factors present in the OXLDL particle, such as oxidised fatty acids, may be involved in the inhibitory action.

In addition, LPC also activates the constitutive NOS to cause the release of NO and thus smooth muscle relaxation, whereas OXLDL has no direct effect on vascular tone.

During the progression of atherosclerosis, cytokines released from inflammatory cells within the arterial wall, may stimulate the induction in macrophages, endothelial and smooth muscle cells, of a NOS distinct from the constitutive form of the enzyme present in endothelial cells. If this were to occur *in vivo*, induction of the enzyme may serve as a protective mechanism to maintain dilator tone in atherosclerotic vessels. Production of NO via this pathway would be resistant to the inhibitory effects of OXLDL and LPC. This observation also highlights a difference between the two pathways of NO production mediated by the constitutive and inducible NOS enzymes.

The present study has also shown that decreased availability of L-arginine in atherosclerotic vessels does not seem to account for the reduced EDR observed in these vessels. Futhermore, it also suggests that the attenuation of responses is due to some other dysfunction in the metabolism of L-arginine to NO or a point distal to release of NO, such as increased inactivation. Therefore, increasing plasma levels of L-arginine in patients with atherosclerosis may not prove to be beneficial in relieving the consequences of the atherosclerosis, at least once the disease has become established.

In conclusion, the results presented here demonstrate additional properties of LPC which may contribute to the attenuation of EDR characteristic of atherosclerotic vessels. Furthermore, the inhibition of endothelium-dependent responses in diseased vessels is not caused by the reduced availability of L-

arginine in the vascular endothelium.

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