THE IMPAIRMENT OF ENDOTHELIUM-DEPENDENT RELAXATIONS IN ISOLATED RABBIT AORTA AND CORONARY ARTERIES BY LOW-DENSITY LIPOPROTEINS AND OXIDISED FATTY ACIDS.

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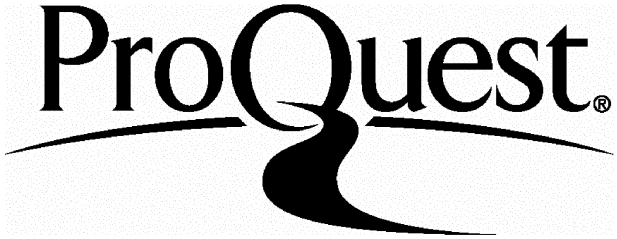

ABSTRACT

Vasodilation mediated by endothelium-derived relaxing factor (EDRF) is accounted for by the release of nitric oxide from the endothelium in response to acetylcholine and other agents. In isolated arteries from atherosclerotic and hypercholesterolaemic animals endothelium-dependent responses are attenuated. This dysfunction may result from the accumulation of oxidised low-density lipoproteins (OXLDL) within the vessel wall, a process known to contribute to the pathogenesis of atherosclerosis. Previous studies have shown that LDL oxidised by Cu^{2+} inhibits endothelium-dependent relaxation in isolated rabbit aorta and porcine coronary arteries. This study investigated the effects of Cu^{2+}-oxidised LDL on relaxations in rabbit large coronary arteries and small resistance vessels which do not exhibit overt signs of atherosclerosis. The effects of lipoxygenase metabolites of linoleic and arachidonic acid, which are present in OXLDL, on vascular reactivity of rabbit aorta were also investigated.

Oxidised, but not native, LDL caused a reversible inhibition of endothelium-dependent relaxations evoked by ACh in isolated rabbit coronary arteries precontracted with either $PGF_{2\alpha}$ or KCl. Furthermore, the extent of the inhibitory effect was similar in both large and small coronary arteries. Relaxations evoked by the nitrovasodilator SNP were unaffected by the presence of OXLDL.

Hydroperoxy and hydroxy derivatives of linoleic and arachidonic acid, which have been identified in OXLDL, caused an immediate and reversible attenuation of ACh-evoked relaxations in isolated rabbit aorta. The inhibition was prevented by the addition of the protein kinase C inhibitor chelerythrine chloride suggesting the inhibition is mediated through a mechanism

involving the activation of protein kinase C. Metabolites of arachidonic but not linoleic acid inhibited endothelium-independent relaxations evoked by GTN. In addition, arachidonic acid oxidation products caused a direct contraction of rabbit aortic rings which was not altered by the presence of the endothelium.

LDL modified by treatment with lipoxygenase (LO-LDL) also inhibited endothelium-dependent relaxations in rabbit aorta by a mechanism involving protein kinase C and not dependent on uptake via the scavenger receptor. Relaxations evoked by GTN were reversibly attenuated by the presence of LO-LDL.

In conclusion, the inhibitory effect of OXLDL in coronary vessels suggests that the effects of atherosclerosis on vascular function may extend into the microcirculation. Products of fatty acid peroxidation may contribute, with other constituents of OXLDL, to the impairment of coronary vasodilation observed in atherosclerosis and hypercholesterolaemia. Arachidonic acid metabolites may also be involved in enhanced vasoconstrictor responses and vasospasm.

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ABBREVIATIONS

ACAT	acyl CoA : cholesterol acyl transferase
ACD	acid citrate dextrose
ACh	acetylcholine
ADP	adenosine 5'-diphosphate
ANP	atrial naturietic peptide
Apo-B-100	apolipoprotein B-100
ATP	adenosine 5'-triphosphate
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular calcium
cAMP	3'5'-cyclic adenosine monophosphate
cGMP	3'5'-cyclic guanosine monophosphate
Cu^{2+}	copper
EDHF	endothelium-derived hyperpolarising factor
EDRF	endothelium-derived relaxing factor
EDTA	ethylenediamine tetra-acetic acid
ETYA	eicosatetrayenoic acid
FH(IIa)	familial hypercholesterolaemia type IIa
GTN	glyceryl trinitrate
HDL	high-density lipoprotein
15-HETE	15-hydroxy-5,8,11,13-eicosatetraenoic acid
5-HT	5-hydroxytryptamine (serotonin)
HMG-CoA	3-hydroxy-3-methylgluteryl CoA
H_2O_2	hydrogen peroxide
9-HODE	9-hydroxy-10,12-octadecadienoic acid
13-HODE	13-hydroxy-9,11-octadecadienoic acid
15-HPETE	15-hydroperoxy-5,8,11,13-eicosatetraenoic acid
9-HPODE	9-hydroperoxy-10,12-octadecadienoic acid
IP_3	inositol 1,4,5-trisphosphate
K^+	potassium

L-CAT	lecithin: cholesterol acyltransferase
LDL	low-density lipoprotein
L-NMMA	L-N^G-monomethyl arginine
LO-LDL	lipoxygenase-treated low-density lipoprotein
LPC	lysophosphatidylcholine
MCP-1	monocyte chemotactic protein-1
mRNA	messenger ribonucleic acid
NA	noradrenaline
NADPH	nicotinamide adenine dinucleotide phosphate
NDGA	nordihydroguaiaretic acid
NO	nitric oxide
NZW	New Zealand White
O_2^-	superoxide anion
OXLDL	oxidised low-density lipoprotein
PC	phosphatidylcholine
PDGF	platelet derived growth factor
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostacyclin
Poly I	polyinosinic acid
PUFA	polyunsaturated fatty acid
S.E.M	standard error of the mean
SNP	sodium nitroprusside
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
VLDL	very-low-density lipoprotein
WHHL	watanabe heritable hyperlipidaemic

CHAPTER 1

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 many aspects of its pathogenesis remain unclear. It affects large arteries, mainly the aorta and coronary, carotid, iliac and femoral arteries, and is characterised by the accumulation of lipid-rich deposits. In advanced stages of the disease, the artery may become occluded resulting in myocardial or cerebral infarction, or peripheral vascular disease. These clinical manifestations are the final stages of a complex process involving damage to vascular cells and disruption in the mechanisms which regulate vascular tone and blood flow.

1.1.1 STRUCTURE OF THE NORMAL ARTERY WALL

(Reviewed by Ross and Glomset, 1976; Badimon *et al.,* 1993).

Normal arterial vessels are composed of three morphologically distinct layers: the intima, media and adventitia. The intima is the innermost layer and is in direct contact with the flowing blood. It consists of a continuous monolayer of endothelial cells, the subendothelial layer, containing components of extracellular connective tissue matrix and some smooth muscle cells, and the internal elastic lamina. With increasing age intimal smooth muscle cells and extracellular matrix components slowly accumulate.

The media consists entirely of smooth muscle cells, surrounded by a varied amount of collagen, small elastic fibrils and proteoglycans and generally does not alter with age. It is separated from the adventitia by the external elastic lamina.

The adventitia is the outermost layer of the vascular wall and consists mainly of fibroelastic tissue. It stabilises the vascular wall by connecting the vessel to the surrounding tissue and carries nutrients to the medial smooth muscle cells.

1.1.2 MORPHOLOGY OF ATHEROSCLEROTIC LESIONS

(Reviewed by Steinberg and Witztum, 1990; Woolf, 1990; Badimon *et al.,* 1993)

In experimental atherosclerosis, one of the earliest events is an increase in the adherence of circulating monocytes to the arterial endothelium and their migration into the subendothelial space (Gerrity, 1981a; 1981b; Joris *et al.,* 1983). Once in the intima, the monocytes are transformed into macrophages and rapidly accumulate cholesterol and its esters derived from plasma low-density lipoproteins (LDL) to form "foam cells", characteristic of the earliest macroscopically recognisable lesion known as fatty streaks (Fowler *et al.,* 1979).

Development of the fatty streak is accompanied by the formation of microthrombi in the vessel wall, deposition of fibrin and the migration of some intimal smooth muscle cells into the subendothelial space (Geer, 1965). Morphological changes to the endothelium have been described in hypercholesterolaemic rabbits (Ingerman-Wojenski *et al.,* 1983) although

other studies have demonstrated that fatty streaks can develop under an intact endothelium (Davies *et al.,* 1976; Bondjers *et al.,* 1977; Taylor *et al,.* 1989).

As the fatty streak becomes established the smooth muscle cell becomes the dominant cell type and is the source of much of the connective tissue matrix which is a major component of fibrous plaques and later lesions (Burke 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), stimulate smooth muscle cell proliferation which determines the growth of the lesion. Smooth muscle cells are also capable of lipid uptake to become foam cells although this may occur at a later stage than the formation of macrophage-derived foam cells (Ross and Glomset, 1976a). The advanced lesion is covered by a fibrous cap composed of macrophages, smooth muscle cells and extracellular matrix components with a necrotic core containing cellular debris, extracellular lipids, cholesterol crystals and calcium. The plaques usually cause some degree of stenosis, narrowing the vascular lumen. However, continued growth of the fibrous plaque may lead to rupture due to necrosis of connective tissue at the base resulting 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 occurring where blood flow is slowed, such as branch points and bifurcations (Goldstein *et al.,* 1983; Packham and Mustard, 1986), where turbulence 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).

1.1.3 RISK FACTORS FOR THE DEVELOPMENT OF ATHEROSCLEROSIS

It is now well established that hypercholesterolaemia is an important contributory factor in coronary heart disease (Kannel *et al.,* 1979). 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).

Recent clinical and experimental studies have shown that elevated plasma levels of LDL, the major carrier of cholesterol in the plasma, are associated with accelerated atherosclerosis (Goldstein and Brown, 1977; Steinberg, 1983; Faggiotto and Ross, 1984; Faggiotto *et al.,* 1984; Tyroler, 1987a; 1987b), and furthermore that the cholesterol which accumulates in atherosclerotic lesions is derived primarily from circulating LDL (Newman and Zilversmit, 1962). The central role of LDL in the development of atherosclerosis can be clearly shown in individuals with the genetic disorder Familial Hypercholesterolaemia Type IIa [FH (IIa)] (Brown and Goldstein, 1986). These patients have either non-functional or absent LDL receptors leading to extremely high levels of circulating LDL, and can often experience heart attacks before the age of 20 (Goldstein *et al.,* 1983). Further evidence came from studies using the Watanabe heritable hyperlipidaemic (WHHL) rabbit, an animal model of atherosclerosis which resembles the human condition of FH (IIa) (Watanabe, 1980; Goldstein *et al.,* 1983).

However, hypercholesterolaemia is not the only causative factor in the development of atherosclerosis, since in patients with FH (IIa) with closely matched cholesterol levels there is considerable variation in the clinical

expression of the disease (Piper and Orrild, 1956). Other risk factors implicated in the progression of atherosclerosis include hypertension (Robertson and Strong, 1968), cigarette smoking (Stamler, 1979) and diabetes (Kannel and McGee, 1979), although it is possible that some minimum degree of hypercholesterolaemia is a pre-requisite before the adverse effects of other factors become clinically significant (Steinberg and Witztum, 1990).

1.1.4 THEORIES FOR THE DEVELOPMENT OF ATHEROSCLEROSIS

As the endothelium provides a barrier between circulating blood and the arterial intima, it was proposed that the initiating event in atherogenesis was the loss of endothelial cells from the arterial surface, as described in the "response to injury" hypothesis (Ross and Glomset, 1976a; 1976b; Ross, 1986; Taylor *et al.,* 1990). This theory is based on the similarities between the formation of atherosclerotic lesions and the inflammatory response of arteries to mechanical endothelial denudation (Stemerman and Ross, 1972). Injury to the arterial endothelium resulted in platelet aggregation and the accompanying release of PDGF which stimulates the proliferation of smooth muscle cells and the development of fibrous lesions (Ross *et al.,* 1974). However, subsequent studies have shown that early lesions can develop under a structurally intact endothelium (Davies *et al.,* 1976; Bondjers *et al.,* 1977; Faggiotto and Ross, 1984; Taylor *et al.,* 1989). Furthermore, the dominant lipid-laden cells in early lesions of atherosclerosis are primarily derived from circulating monocytes not from smooth muscle cells as originally hypothesised (Fowler *et al.,* 1979; Gerrity *et al.,* 1979; Gerrity, 1981a; 1981b).

Another hypothesis is the "lipid infiltration" theory which proposed that hypercholesterolaemia is the major contributing cause of atherosclerosis (Steinberg, 1987). This theory suggests that the cholesterol-carrying lipoproteins induce or favour the progression of the atherosclerotic lesion as a result of an increased uptake of the lipoproteins into the arterial wall or, by causing endothelial damage.

These two hypotheses have several points of interaction and can be linked into a "unified hypothesis" (Steinberg, 1983; 1987) which recognises that an atheroma is extremely complex in structure and could be generated by different reaction sequences. It provides links between the "Lipid Infiltration" theory, which may account for the development of the fatty streak, and the "Response to Injury" hypothesis, which may account for the progression of more advanced lesions **(Figure 1)**. Reviews of the "Unified Hypothesis" (Steinberg, 1990; Steinberg and Witztum, 1990; Witztum and Steinberg, 1991) have emphasised the importance of oxidatively modified LDL in the progression of atherosclerosis in processes other than in lipid deposition; this will be discussed in section 1.5.

The interactions between cells in the vessel wall, plasma lipoproteins and platelets and their role in atherogenesis have been extensively studied. It is now clear that early events include the migration of monocytes into the vessel wall, where they ingest large amounts of lipid to become foam cells, endothelial damage or dysfunction, the adherence and aggregation of platelets and the proliferation of smooth muscle cells. The following sections will describe the normal functions of lipoproteins and the vascular endothelium. The interactions of these two components of the vascular system and their role in the initiation and progression of atherosclerosis will then be discussed.

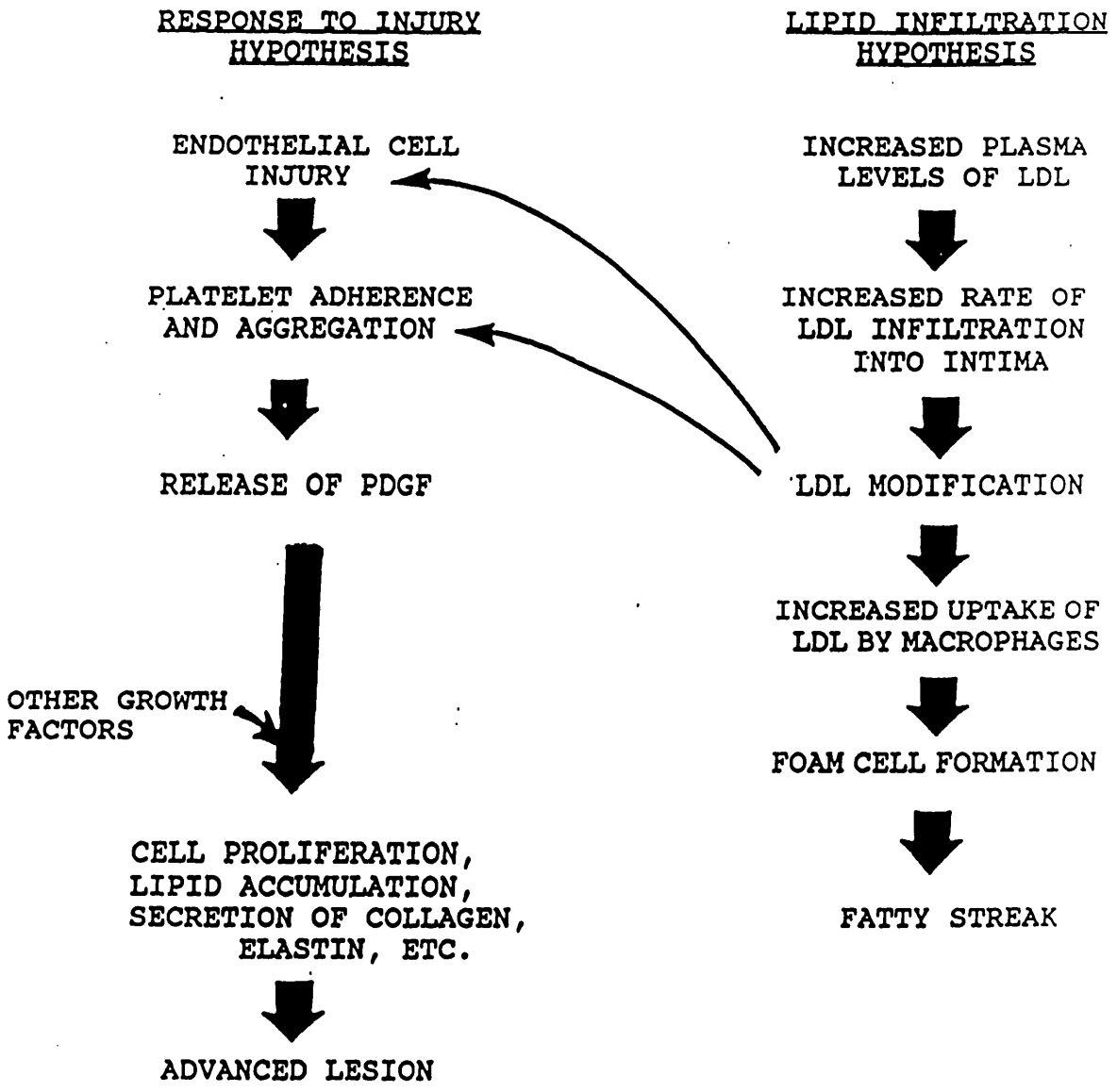


FIGURE 1

The Unified Hypothesis of Atherosclerosis.

Interactions between the "Response to Injury" and "Lipid Infiltration" hypotheses. (Adapted from Steinberg, 1989).

1.2 THE PLASMA LIPOPROTEINS

Blood lipids are transported in soluble lipid-protein complexes called lipoproteins, which play an essential role in the transport of cholesterol, triglycerides and phospholipids between the liver and extra-hepatic tissues. The principal classes of lipoproteins, in increasing order of density are; chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). The lipoproteins can be distinguished physically on the basis of their hydrated density and can be separated by ultracentrifugation. There is also a high degree of heterogeneity within each class (Hatch and Lees, 1968).

Plasma lipoproteins have essentially three 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.

(iii) To supply adipose tissue and muscle cells with free fatty acids.

1.2.1 THE STRUCTURE AND COMPOSITION OF LIPOPROTEINS

(Reviewed by Scanu and Spector, 1986)

Plasma lipoproteins have a common structural organisation which consists of a central oily core of triglyceride and cholesteryl esters enclosed in a layer of phospholipids, such as phosphatidylcholine, phosphatidylethanolamine and sphingomyelin, and unesterified cholesterol. The phospholipids are arranged so that the polar heads are on the outside contributing to the solubility of the lipoprotein particle in aqueous solution. Embedded in the outer phospholipid monolayer are the apolipoproteins

which stabilise the molecule. Each class of lipoprotein contains a characteristic profile of apolipoproteins which regulate the metabolism of the lipoproteins by modulating the activity of several enzymes, or by mediating the uptake of lipoproteins by specific apolipoprotein receptors.

Each LDL particle has a single molecule of apolipoprotein-B-100 (apo-B-100), an amphipathic, glycosylated protein with a relative molecular weight of 513 - 514 kDa (Yang *et al.,* 1986). Apo-B-100, the major or exclusive protein constituent of LDL, and also a major component of VLDL, suppresses the activity of 3-hydroxy-3-methylgluteryl co-enzyme A reductase (HMG-Co A reductase; Yang *et al.,* 1986), the rate-limiting enzyme in cholesterol biosynthesis.

The lipids of LDL contain large amounts of both linoleic acid, which is predominantly bound in the cholesterol esters, and arachidonic acid which is mainly bound to the phospholipid (Esterbauer *et al.,* 1990). LDL also contains antioxidants such as vitamin E (α-tocopherol) and β-carotene (Esterbauer *et al.,* 1989).

1.2.2 LIPOPROTEIN METABOLISM

There are two separate pathways mediating the metabolism of cholesterol, derived from 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.

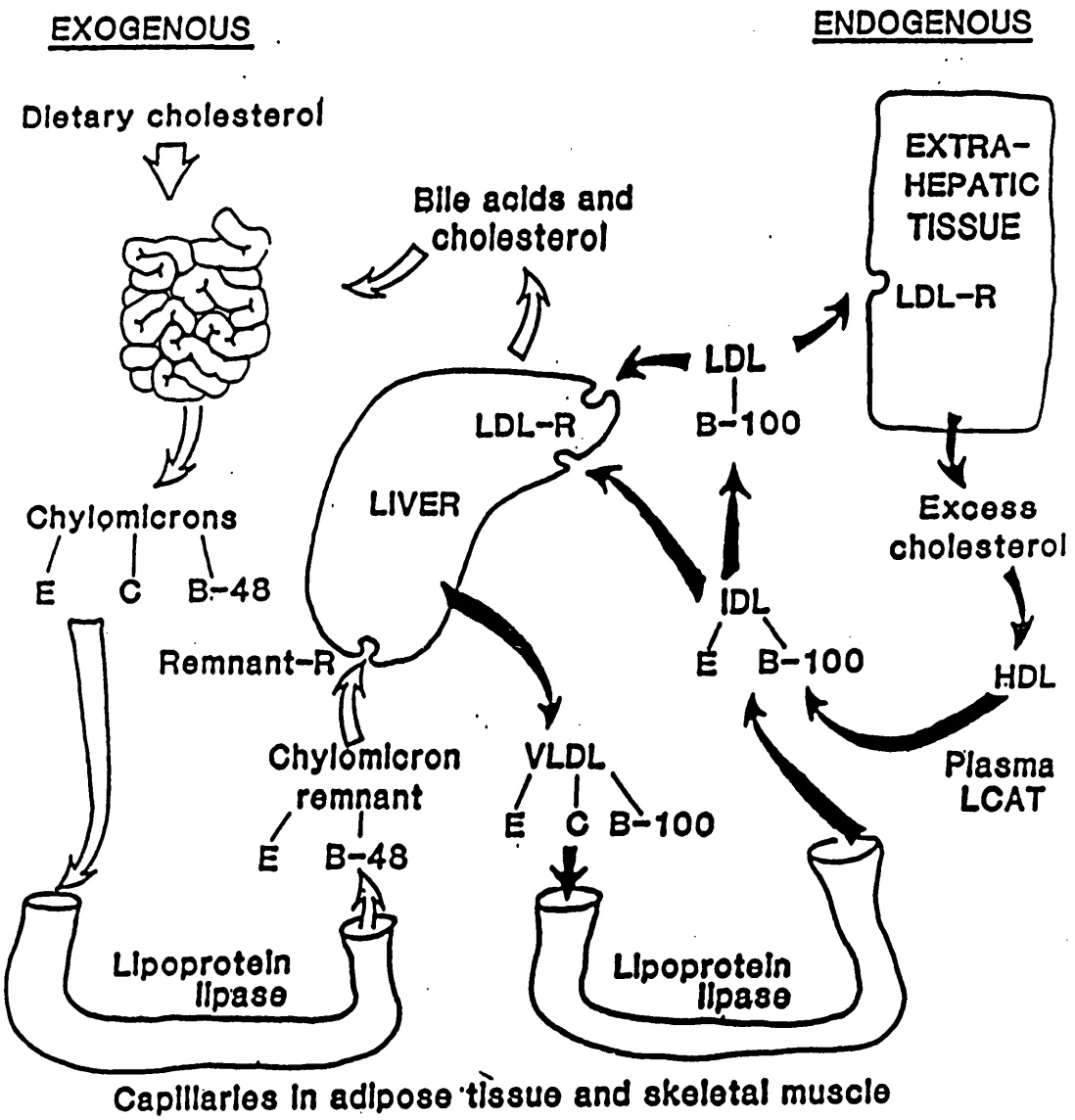


FIGURE 2

The exogenous and endogenous pathways of lipoprotein metabolism.

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

1.2.3 RECEPTOR-MEDIATED ENDOCYTOSIS OF LDL

Receptor-mediated endocytosis is the term applied to the uptake of LDL following binding to specific high-affinity receptors on the cell surface (reviewed by Brown and Goldstein, 1986). The existence of the LDL receptor was first demonstrated in fibroblasts (Brown *et al.,* 1973; Goldstein and Brown, 1973) but was later shown to be present on most mammalian cell types. LDL is taken into the cells and broken down yielding cholesterol which is required for membrane synthesis. The number of receptors on the surface of cells varies with the cholesterol requirements of the cell. The human LDL receptor is a single-chain glycoprotein of 839 amino acids comprising of 5 structurally distinct transmembrane domains (Russell *et al.,* 1984; reviewed by Catapano, 1989). The receptor is specific for lipoproteins containing apo-B-100 or the related protein apo-E.

Following the binding of LDL to the cell surface receptor, the LDL-receptor complex is localised in clathrin-coated pits (Anderson *et al.,* 1982) which invaginate to form coated endocytic vesicles and fuse to become endosomes. The LDL then dissociates from its receptor and is delivered to a lysosome containing enzymes where the apo-B is broken down by proteases and the cholesterol ester component is hydrolysed. The receptor then returns to the cell surface (Goldstein *et al.,* 1985).

The free cholesterol migrates into the cytoplasm where it is used by the cell to maintain cholesterol homeostasis (Brown and Goldstein, 1986). It suppresses the activity of HMG-CoA reductase (Goldstein and Brown, 1973), activates the enzyme acyl cholesteryl acyl transferase (ACAT) enabling excess cholesterol to be stored as cholesterol ester droplets (Goldstein *et al.,* 1974) and can also down-regulate the transcription of the

LDL-receptor gene (Brown *et al.,* 1975; Russell *et al.,* 1983). The number of receptors controls the uptake of cholesterol to maintain cellular functions, but the feedback mechanisms exist to protect the cell against excessive accumulation of cholesterol and cholesterol esters.

Non-receptor, low affinity, non-saturable mechanisms also exist for the uptake of LDL by a combination of fluid and absorptive endocytosis (Spady *et al.,* 1987). These mechanisms are not adequate for the normal maintenance of cholesterol homeostasis and *in vivo* studies have demonstrated 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). However, under conditions of high plasma cholesterol, LDL accumulates rather than being cleared and degraded by cells.

1.3 THE VASCULAR ENDOTHELIUM

The vascular endothelium is a layer of squamous cells which is in direct contact with the blood. Until quite recently the endothelium was thought of as a uniform layer of cells that provided a protective, nonadherent surface and transported various substances from or into the bloodstream (Ross and Glomset, 1976a). However, it is now clear that the endothelial system is a highly active metabolic and endocrine organ which has an important role in the control of homeostasis and participates in cellular functions such as the coagulation of blood, the activity of leukocytes, the reactivity of platelets and the regulation of vascular smooth muscle tone (Gerlach *et al.,* 1985).

1.3.1 STRUCTURE 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, and is made up of 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 "fenestered", as in the glomerular capillaries of the kidney (Staehelin and Hull, 1978). Gap junctions or "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 METABOLIC FUNCTIONS OF THE ENDOTHELIUM

Endothelial cells have an important metabolic function with respect to vasoactive substances (Gerlach *et al.,* 1985), although the activity varies between vessels. For example, prostacyclin (PGI_2) is metabolised by 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 number of enzymes present in the endothelial cells. The outer surface of the endothelial cell contains angiotensin-converting enzyme (Ryan *et al.,* 1976) which catalyses the formation of the vasoconstrictor angiotensin II from the less active precursor angiotensin I. The same enzyme, also known as kininase II, inactivates bradykinin, a potent vasodilator. Biogenic amines such as 5-hydroxytryptamine (5-HT), noradrenaline (NA) and adenosine are metabolised by endothelial monoamine oxidases and adenosine deaminase (reviewed by Shepro and Durham, 1986).

To maintain the patency of the blood vessels and the fluidity of blood, endothelial cells are able to synthesise many vasoactive substances such as PGI_2 (Moncada *et al.,* 1976a), endothelium-derived relaxing factor (EDRF; Furchgott and Zawadzki, 1980) adenosine (Pearson and Gordon, 1985), endothelin-1 (Yanagisawa *et al.,* 1988), 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE; Takayama *et al.,* 1987) and 13-hydroxy-9,11-octadecadienoic acid (13-HODE; Buchanan *et al.,* 1985a). The production of these substances is modulated by changes in the concentrations of intracellular messengers such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and calcium, and by interactions between the endothelium and white blood cells, platelets or plasma constituents.

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.,* 1976a; 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 detached (Povlishock 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).

In 1980, Furchgott and Zawadzki demonstrated that acetylcholine (ACh) evoked relaxations of precontracted rabbit aortic rings which were dependent on the presence of an intact layer of endothelial cells. This observation resolved the paradox that ACh was a potent vasodilator agent *in vivo* (Shepherd, 1963; Mellander and Johansson, 1968) and yet caused vasoconstriction of arterial strip preparations when studied *in vitro* (Furchgott, 1955; Vanhoutte, 1977). It was proposed that stimulation of endothelial muscarinic receptors with ACh lead to the release of a relaxing substance, known as EDRF, which diffused to the underlying smooth muscle cells to induce relaxation.

Later, it was observed that other substances also elicited endothelium-dependent relaxation of arteries *in vitro* including ATP and ADP (Furchgott, 1983), substance P (Zawadzki *et al.,* 1981), bradykinin (Cherry *et al.,* 1982), 5-HT (Cocks and Angus, 1983), mellitin (Förstermann and Neufang, 1985) and the calcium ionophore A23187 (Zawadzki *et al.,* 1980). The endothelium is now recognised as playing an important role in the modulation of vascular smooth muscle tone. However, many agents only elicit endothelium-dependent relaxations in certain species, or in specific vessels from certain species, and may cause contraction or endothelium-independent relaxations in other preparations. For example, 5-HT evokes endothelium-independent contractions of many isolated arterial preparations but can also elicit endothelium-dependent relaxations in canine (Cocks and Angus, 1983) and porcine (Houston *et al.,* 1985) coronary arteries.

The endothelium has also been shown to modulate vascular reactivity in resistance vessels by the release of EDRF (Furchgott *et al.,* 1987; Bhardwaj and Moore, 1988; Myers *et al.,* 1989; Tschudi *et al.,* 1991). EDRF may therefore have a role in controlling the tone of resistance blood vessels and also in the distribution of blood flow within organs and vascular beds to regulate blood pressure.

In addition to chemical stimuli, EDRF release is also induced by increases in flow (Rubanyi *et al.,* 1986), hypoxia (Pöhl and Busse, 1989) 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). The activity of EDRF was not affected by indomethacin, an inhibitor of cyclo-oxygenase, thus ruling out a role for prostanoids in these responses.

Further evidence that endothelial cells exposed to ACh released a diffusable vasodilator substance came from 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; Gryglewski *et al.,* 1986) induced relaxation of endothelium-denuded arterial strips or rings. These approaches allowed for more direct studies of the physical and chemical manipulation on the generation, stability and actions of EDRF. For example, it was demonstrated using such techniques that EDRF was a very short-lived substance with a half-life of 4-6 secs in oxygenated physiological salt solutions (Förstermann *et al.,* 1984; Griffith *et al.,* 1984a; 1984b) and that the effects of EDRF were prolonged by the addition of superoxide

dismutase (SOD), suggesting that superoxide anions (O_2^-) contributed to the instability of the factor (Gryglewski *et al.,* 1986; Rubanyi and Vanhoutte, 1986).

Relaxation of vascular smooth muscle by EDRF is mediated by the activation of soluble guanylate cyclase leading to an increase in intracellular cGMP levels (Holzmann, 1982; Diamond and Chu, 1983; Rapoport and Murad, 1983b). EDRF is thought to activate guanylate cyclase by interacting with a haem moiety on the enzyme (Ignarro *et al.,* 1982). The mechanism by which cGMP produces relaxation is unclear although probably involves a reduction in intracellular free Ca^{2+} levels (Schini *et al.,* 1987). Possible mechanisms to explain the cGMP-mediated relaxations have been proposed including inhibition of inositol trisphosphate (IP_3) generation (Rapoport, 1986), stimulation of intracellular Ca^{2+} sequestration (Lincoln, 1983), inhibition of receptor operated Ca^{2+} channels (Godfraind, 1986), decrease in the myosin light chain phosphorylation (Rapoport *et al.,* 1983), stimulation of membrane Ca^{2+}-ATPase (Fiscus, 1988) and 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) was shown to inhibit EDRF-mediated relaxations and the associated increase in cGMP (Holzman, 1982; Martin *et al.,* 1985a; 1985b). The effects of EDRF and increases in cGMP are also blocked by haemoglobin (Martin *et al.,* 1985a; 1985b). The exact mechanism mediating the inhibition is unclear due to the complex properties of these compounds. Haemoglobin can bind EDRF (Martin *et al.,* 1986b), inhibit the activation of soluble guanylate cyclase (Murad *et al.,* 1978; Martin *et al.,* 1985a; 1985b) and generate O_2^-, which would inactivate EDRF

(Misra and Fridovich, 1972). However, the effects of haemoglobin are not always reduced by SOD (Hutchinson *et al.,* 1987) suggesting that its main action is to bind EDRF (Moncada *et al.,* 1988). Methylene blue can also generate free radicals (McCord and Fridovich, 1970; Wolin *et al.,* 1990).

1.4.1 PHYSIOLOGICAL ROLE OF EDRF

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

Since the discovery of EDRF, the ability of the vascular endothelium to modulate the tone of underlying smooth muscle in response to physiological or pharmacological stimuli has been extensively studied. The endothelium may be involved in local mechanisms to regulate blood flow of different organs depending on their specific metabolic needs. Endothelial cells may act as transducers of physical and chemical stimuli that are able to modify vascular tone and blood flow through the release of relaxant or contractile factors from the endothelium.

The exact physiological stimuli for the release of EDRF *in vivo* is not clear although *in vitro* studies have proposed that changes in oxygen tension, changes in flow, thrombin, platelet products, such as 5-HT and ADP, and neurotransmitters, such as NA and ACh, may be involved (reviewed by Furchgott, 1983; Martin *et al.,* 1986a; Vanhoutte *et al.,* 1986).

(i) Basal EDRF Release

The endothelium continuously releases EDRF to regulate basal levels of cGMP and blood vessel tone (Rapoport and Murad, 1983a). Basal release of EDRF was indicated by the demonstration that cGMP levels were higher in endothelium-intact tissues than in denuded tissues (Rapoport and Murad,

1983a; Martin *et al.,* 1985a; 1986a; 1986b; Ignarro *et al.,* 1987a; 1987c). It was then demonstrated that exposure of intact tissues to guanylate cyclase inhibitors or endothelium removal caused an increase in the basal level of tone and potentiated contractile responses (Griffith *et al.,* 1984a; 1984b; Martin *et al.,* 1985a; 1986a). In bioassay systems the release of EDRF under basal conditions has been shown directly (Griffith *et al.,* 1984a; 1984b; Rubanyi *et al.,* 1985; Ignarro *et al.,* 1987a).

(ii) Flow-Mediated Dilatation

When the blood flow though large arteries is augmented, they dilate. This flow-induced vasodilatation can be attributed to the release of EDRF. Effluents from perfused rabbit aorta (Griffith *et al.,* 1984a) or canine femoral artery preparations with an intact endothelium (Rubanyi *et al.,* 1985) produce relaxation of endothelium-denuded bioassay preparations. The release of EDRF by flow has been shown to be dependent upon activation of a calcium-dependent potassium channel present on the endothelial cell membrane (Cooke *et al.,* 1991b). In addition, the activity of EDRF has been shown to be greatest in large arterioles in which hydraulic resistance and shear stress are highest (Griffith and Edwards, 1990).

(iii) Receptor-Mediated Dilatation

Although many substances have now been shown to evoke endothelium-dependent relaxations *in vitro* the physiological significance of many of them is unclear. For example, ACh, the first agent known to evoke relaxations mediated by EDRF does not circulate in the blood due to high levels of plasma cholinesterase so that activation of endothelial muscarinic receptors is unlikely. However, in resistance vessels, ACh released from adventitial

nerves may diffuse through the thin vessel wall and stimulate the release of EDRF producing vascular relaxation (Myers *et al.,* 1989). It has been shown that some endothelial cells contain the enzyme choline acetyltransferase (Parnavelas *et al.,* 1985; Burnstock, 1987) suggesting that ACh itself may be released from the endothelium. The presence of ATP, 5-HT and substance P has also been demonstrated in certain endothelial cells which may be released following stimulation and act on neighbouring endothelial cells to release EDRF causing relaxation of the underlying smooth muscle (Burnstock, 1987).

5-HT, thrombin and ADP, released from activated platelets, evoke endothelium-dependent relaxation 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). *In vivo,* this endothelium-dependent relaxation in response to platelet aggregation may promote the flushing away of the aggregate as it forms, preventing occlusion of the vessel.

Receptor-mediated dilatation may occur in response to adverse stimuli such as shock and haemorrhage to increase blood flow to specific tissues or organs. For example, vasopressin released from the posterior pituitary in response to hypotension evokes endothelium-dependent relaxation in cerebral and coronary arteries but causes vasoconstriction in peripheral vessels so favouring redistribution of blood to the cerebral and coronary circulations (Katusic *et al.,* 1984; Vanhoutte *et al.,* 1984).

(iv) EDRF Modulation of Platelet Reactivity

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 can inhibit the adhesion of platelets to endothelial cell surfaces (Radomski *et al.,* 1987b). Additionally, EDRF can act synergistically with PGI_2 to inhibit platelet aggregation and to disaggregate platelets (Radomski *et al.,* 1987a). It has been suggested that the very low levels of PGI_2 found in the plasma may be important in the regulation of platelet reactivity against a background release of EDRF from the endothelium (Moncada *et al.,* 1988). However, PGI_2 has no effect on platelet adhesion even in the presence of EDRF (Radomski *et al.,* 1987b).

Thus, an intact endothelium has a protective role by exerting an inhibitory effect on platelet reactivity and on vascular responses to agents released by aggregated platelets. However, in conditions of endothelium dysfunction or injury, platelet aggregation and vasoconstriction in response to platelet-derived products such as 5-HT may be potentiated and luminal thrombus formation promoted.

1.4.2 CHEMICAL IDENTITY OF EDRF

Since the first demonstration that ACh-evoked relaxation of vascular smooth muscle was dependent on the presence of an intact endothelium, the chemical nature of the labile, non-prostanoid mediator was sought. In 1986 it was suggested that EDRF may be nitric oxide (NO) or a closely related compound (Furchgott, 1988; Ignarro *et al.,* 1988b), since both EDRF and NO had similar pharmacological effects on vascular strips (Hutchinson *et al.,* 1987) and on platelets (Radomski *et al.,* 1987c). A comparison of their chemical and pharmacological properties is summarised in **Table 1.** The proposal that EDRF was NO was confirmed by the demonstration that

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

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

cultured vascular endothelial cells release sufficient nitric oxide to account for the effects of EDRF on vascular strips (Palmer *et al.,* 1987) and on platelet aggregation (Radomski *et al.,* 1987a) and adhesion (Radomski *et al.,* 1987b).

A vast body of evidence now exists to support the proposal that EDRF is NO (reviewed by Ignarro, 1989a; 1989b; Angus and Cocks, 1989; Marin and Sanchez-Ferrer, 1990; Moncada *et al.,* 1991; Feelisch *et al.*, 1994). The release of NO has been demonstrated in a number of vascular preparations including isolated perfused rabbit (Amezcua *et al.,* 1988) or guinea pig (Kelm and Schrader, 1988) hearts, rabbit aorta (Chen *et al.,* 1989), human pulmonary artery (Greenberg et al., 1987) and bovine intrapulmonary artery and vein (Ignarro *et al.,* 1987a; 1987b; 1988a; 1988b).

1.4.3 REGULATION OF EDRF SYNTHESIS AND RELEASE

The calcium ionophore A23187, which facilitates Ca^{2+} entry into cells, evokes endothelium-dependent relaxations 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). In addition, the absence of extracellular Ca^{2+} was shown to rapidly and completely inhibit endothelium-dependent relaxations in blood vessels in response to ACh, bradykinin and thrombin (Furchgott and Zawadzki, 1980; Singer and Peach, 1982; Long and Stone, 1985), whereas endothelium-independent responses to sodium nitroprusside (SNP) were unaltered in Ca^{2+}-free buffer (Holzmann, 1982; Rapoport and Murad, 1983b). These findings led to the suggestion that an agonist-induced influx of Ca^{2+} from the extracellular space contributes to an

increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and is an important early step in the release of EDRF.

Numerous studies have shown that agents which interfere with the metabolism of phospholipids and polyunsaturated fatty acids (PUFA) such as quinacrine, which inhibits phospholipase A_2, 5,8,11,14-eicosatetrayenoic acid (ETYA), an inhibitor of both cyclo-oxygenase and lipoxygenase, and nordihydroguaiaretic acid (NDGA) which inhibits lipoxygenase, inhibit relaxations mediated by EDRF (reviewed by Furchgott, 1983). These observations led to the suggestion that the cleavage of phospholipids with the formation of free fatty acids and lysophosphatides and/or the accompanying generation of oxygen free radicals may be associated with the formation and/or release of EDRF (Ignarro, 1989a). However, the non-selective effects of some of these agents may cause problems in the interpretation of these results (Ignarro and Kadowitz, 1985; Moncada *et al.,* 1986).

Phorbol esters, which activate protein kinase C, inhibit agonist-mediated release of EDRF from isolated blood vessels (Weinheimer *et al.*, 1986; Lewis and Henderson, 1987; Cherry and Gillis, 1988; Rubanyi *et al.*, 1989) and cultured endothelial cells (Weinheimer *et al.*, 1986; Lewis and Henderson, 1987; Smith and Lang, 1990). These agents also inhibit agonist-induced increases in the endothelial cell Ca^{2+} concentrations (Ryan *et al.,* 1988) and IP_3 levels (Brock and Capasso, 1988) suggesting a possible role for diacylglycerol, the endogenous agonist of protein kinase C in the regulation of EDRF synthesis.

Furthermore, pertussis toxin, an inhibitor of G_i-proteins, inhibits EDRF mediated responses evoked by 5-HT and the α_2-adrenoceptor agonist UK 14,304, but not ADP, A23187 or bradykinin (Flavahan *et al.,* 1989). Responses to endothelium-independent relaxants are unaltered by this toxin

(Ignarro and Kadowitz, 1985), suggesting that there may be at least two pathways leading to the formation of EDRF, one of which involves G-proteins (Flavahan *et al.,* 1989).

Increases in the production of EDRF are associated with a rise in cGMP levels in endothelial cells (Martin *et al.,* 1988) which has been shown to inhibit the activation of the phosphoinositol pathway responsible for stimulating increases in $[Ca^{2+}]_i$ (Collins *et al.,* 1986; Lewis *et al.,* 1988). Increases in cGMP evoked by atrial natriuretic peptide (ANP; Martin *et al.,* 1988) and 8-bromo-cGMP also inhibit the release of EDRF from intact blood vessels (Hogan *et al.,* 1989) and cultured endothelial cells (Busse *et al.,* 1988; Evans *et al.,* 1988). These observations may indicate a negative-feedback mechanism by which EDRF inhibits its own release *via* increases in endothelial cGMP levels.

1.4.4 BIOSYNTHESIS OF EDRF

After the demonstration that EDRF was NO (Palmer *et al.,* 1987) it was shown that cultured porcine aortic endothelial cells synthesised NO by oxidation of the terminal guanidino atom of L-arginine (Palmer *et al.,* 1988a; 1988b). In addition, activated macrophages were shown to produce NO as a cytotoxic agent from L-arginine (Hibbs *et al.,* 1987a; 1987b; 1988; Marletta *et al.,* 1988). Endothelial cells, cultured in the absence of L-arginine showed a decrease in EDRF release in response to bradykinin which could be restored by L- but not D-arginine demonstrating the stereospecificity of the enzyme (Palmer *et al.,* 1988a).

L-arginine may be derived from several sources. 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 EDRF but also for its role in amino acid metabolism, protein synthesis and as a precursor for polyamine formation (Moncada *et al.,* 1989).

The arginine analogue L-N^G-monomethyl-arginine (L-NMMA), an inhibitor of NO synthesis in macrophages (Hibbs *et al.,* 1987b), was shown to inhibit 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 concentration-dependent and enantiomerically specific manner. These effects were reversed by L- but not D-arginine.

Other analogues of L-arginine including L-N^G-nitro-arginine (Moore *et al.,* 1990), N-imino-ethyl-L-ornithine (Mülsch and Busse, 1990) and L-N^G-nitro-arginine-methyl-ester (Rees *et al.,* 1990) have now also been shown to inhibit the production of NO.

Endothelial homogenates form L-citrulline from L-arginine by a mechanism dependent on NADPH and inhibited by L-NMMA (Palmer and Moncada, 1989). Furthermore, in endothelial cell cytosol, depleted of L-arginine, there was an L-arginine-dependent increase in cGMP which required NADPH and was accompanied by the formation of L-citrulline from L-arginine (Moncada and Palmer, 1990). Both the production of L-citrulline and the increases in cGMP were inhibited by L-NMMA. These findings suggested that NO and L-citrulline were co-products of the same enzymatic reaction. The formation of L-citrulline and the increase in cGMP were also shown to be inhibited by Ca^{2+} chelators, indicating that the enzyme, now known as NO synthase, is Ca^{2+}-dependent (Mayer *et al.,* 1989; Mülsch *et al.,* 1989; Moncada and Palmer, 1990). Furthermore, the synthesis of NO from endothelial cell cytosol was inhibited by calmodulin-binding peptides and

antagonists, an effect which was reversed by calmodulin, suggesting that the Ca^{2+}-dependent stimulation of NO synthase in endothelial cells is mediated by calmodulin (Busse and Mülsch, 1990).

Two types of NO synthase have now been identified. The isoform found in freshly harvested endothelial cells and also in brain and platelets is constitutive (Moncada *et al.,* 1991) and is responsible for the release of NO in physiological transduction purposes. The formation of the other isoform is induced by cytokines and endotoxins and releases NO as part of the immunological response (Curran *et al.,* 1989; Hibbs *et al.,* 1990; Knowles *et al.,* 1990; Werner-Felmayer *et al.,* 1990). Inducible NO synthase is present in activated macrophages, endothelial cells and smooth muscle cells. It requires *de novo* protein synthesis for its expression (Marletta *et al.*, 1988) and is dependent on the presence of NADPH and tetrahydrobiopterin (Tayeh and Marletta, 1989; Kwon *et al.,* 1990). It has been demonstrated that calmodulin is bound tightly to the inducible enzyme (Cho *et al.*, 1992) so that its activity is not susceptible to control through elevated Ca^{2+} levels and is independent of exogenous Ca^{2+} and calmodulin (Steuhr *et al.*, 1991).

1.4.5 METABOLISM OF EDRF

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 salt solutions can be accounted for by spontaneous oxidation of NO to nitrite (NO_2^-):

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

At least 90% of the NO is converted to NO_2^-, with little or no formation of nitrate (NO_3^-; Feelisch and Noack, 1987; Kelm *et al.,* 1988; Ignarro *et al.*, 1993). The half-life of NO varies inversely as a function of oxygen tension and the concentration of O_2^- (Förstermann *et al.,* 1984; Rubanyi *et al.,* 1985; Gryglewski *et al.,* 1986) and directly with the concentration of NO in aqueous solution. NO concentrations of 10 - 50 nM have half-lives of 3 - 5 secs whereas concentrations in excess of 300 mM have a half-life of over 30 secs (Ignarro, 1990).

Reaction with O_2^- results in the rapid and almost complete inactivation of NO (Gryglewski *et al.,* 1986; Moncada *et al.,* 1986; Rubanyi and Vanhoutte, 1986; Ignarro *et al* , 1988a) and this is reduced in the presence of SOD (Gryglewski *et al.,* 1986; Rubanyi and Vanhoutte, 1986; Ignarro *et al* , 1988a). However, it is unlikely that O_2^- contributes significantly to the inactivation of EDRF *in vivo* due to the ubiquitous distribution of SOD, although pathological conditions may provide a role for O_2^- (Ignarro, 1990).

1.4.6 EVIDENCE THAT EDRF IS NOT NO

Despite the evidence supporting NO as EDRF and the similarities between the two agents, several studies have questioned this conclusion and suggested that EDRF may not be 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).

S-Nitrosothiols are unstable compounds which spontaneously decompose yielding NO. They are pharmacologically similar to 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, cause vascular smooth muscle relaxation (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).

It has been suggested that EDRF may be S-nitrosocysteine rather than free NO. This finding was based on comparisons of the potencies of the two agents on vascular strips and by using a chemiluminescence procedure together with a bioassay system to measure EDRF release from bovine aortic endothelial cells (Myers *et al.,* 1990). Furthermore, studies comparing the O_2^--generating effects of xanthine plus xanthine oxidase on relaxation of the rabbit aorta by ACh, free NO and *S*-nitrosocysteine, suggested that EDRF released abluminally by ACh is more like nitrosocysteine than NO (Furchgott *et al.,* 1992a; 1992b). More recent evidence has, however, demonstrated that EDRF is NO. Using a bioassay system to compare the potency, stability, inhibition by oxyhaemoglobin and also the effect of cysteine on the activity of EDRF, NO, *S*-nitrosothiols and other compounds proposed to account for the relaxant properties of EDRF, it was shown that EDRF released from endothelial cells behaves like NO and not *S*-nitrosocysteine or any of the other proposed EDRF candidates (Feelisch *et al.*, 1994).

Differences between NO and EDRF have been demonstrated, for example EDRF and NO are reported to be differentially retained by anionic exchange resins (Long *et al.,* 1987; Shikano *et al.,* 1988). In addition, EDRF, but not NO, shows stability during chromatography and lyophilisation (Angus and Cocks, 1989) and, following passage through a haemoglobin-agarose

column, NO, but not EDRF, gave a nitrosyl-haemoglobin signal detectable by electron paramagnetic resonance spectroscopy (Rubanyi *et al.,* 1990; Vedernikov *et al.,* 1990).

1.4.7 MULTIPLE EDRFs

The term EDRF refers to the mediator(s) released from the endothelium to evoke relaxation of vascular smooth muscle, one of which is NO. NO cannot, however, explain all the endothelium-dependent responses of isolated arteries. ACh is known to evoke endothelium-dependent relaxations which are accompanied by hyperpolarisation of the smooth muscle due to the release from the endothelium of a diffusable factor other than NO (Bolton *et al.,* 1984; Feletou and Vanhoutte, 1988; Komori *et al.,* 1988, Rand and Garland, 1992). This factor, known as endothelium-dependent hyperpolarising factor (EDHF) evokes relaxation *via* an increase in K^+ conductance of the cell membrane (Bolton *et al.,* 1984; Komori and Suzuki, 1987; Chen and Suzuki, 1989) whereas NO causes vascular relaxation *via* an increase in cGMP with no change in membrane potential (Huang *et al.,* 1988; Komori *et al.,* 1988; Rand and Garland, 1992). However, NO has been shown to cause hyperpolarisation of some arteries (Tare *et al.,* 1989; Garland and McPherson, 1992). In addition, L-NMMA, which inhibits the synthesis of NO, has been shown to inhibit ACh-evoked relaxation and the accompanying hyperpolarisation in some arteries indicating that NO can mediate both responses (Tare *et al.,* 1990a; 1990b).

1.4.8. *IN VIVO* ACTIVITY OF EDRF

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

There is substantial evidence for the existence of EDRF activity in large vessels *in vivo.* The dilation of canine femoral arteries in response to substance P, ACh and increases in blood flow are abolished after endothelial damage by mechanical or chemical means (Angus *et al.,* 1983; Pöhl *et al.,* 1986a; 1986b), whereas the responses to nitrovasodilators are unaffected. In humans, ACh evoked vasodilation of coronary arteries (Ludmer *et al.,* 1986) which was inhibited by methylene blue (Hodgson and Marshall, 1989) indicating that the vasodilation was due to EDRF.

Studies using inhibitors of the L-arginine : NO pathway have demonstrated basal and stimulated release of EDRF *in vivo.* In anaesthetised rabbits, L-NMMA induced an increase in blood pressure (Rees *et al.,* 1989b) suggesting that basal release of EDRF is important in the homeostatic regulation of arterial blood pressure. L-NMMA also caused a concentration-dependent increase in blood pressure in rats (Whittle *et al.,* 1989; Gardiner *et al.,* 1990a; 1990b) and guinea pigs (Aisaka *et al.,* 1989; 1990) which was prevented by L- but not D-arginine. In conscious rats, the increase in blood pressure induced by L-NMMA was accompanied by a decrease in vascular conductance in renal, mesenteric, carotid and hindquarter vascular beds (Gardiner *et al.,* 1990a), which was sustained for up to six hours if the L-NMMA infusion was maintained (Gardiner *et al.,* 1990b). This finding indicates that EDRF plays an essential role in maintaining vascular tone and that the vascular beds are unable to reaccommodate the flow to normal levels.

In humans, an infusion of L-NMMA into the brachial artery or dorsal hand vein attenuated vasodilator responses induced by ACh and bradykinin but not glyceryl trinitrate (GTN; Vallance *et al.,* 1989a; 1989b). In addition, in the brachial artery, but not in the dorsal hand vein, L-NMMA induced a direct vasoconstriction suggesting that on the arterial side of the circulation, but not on the venous side, a continuous release of NO maintains dilator tone (Vallance *et al.,* 1989b).

In conclusion, by using analogues of L-arginine to inhibit NO biosynthesis *in vivo* it can be demonstrated that NO plays a critical role in the regulation of arterial blood flow and in the maintenance of a continuous vasodilator tone in whole vascular beds (Rees *et al.,* 1989b).

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)

There is no longer any doubt about the causative role of hypercholesterolaemia in the development of atherosclerosis. It has been proposed that the formation of early atherosclerotic lesions can be explained solely by the presence of elevated plasma levels of LDL and the oxidative modification of LDL in the vessel wall (Steinberg *et al.,* 1989; Steinberg, 1990). The potential role for oxidised LDL (OXLDL) in the pathogenesis of atherosclerosis is illustrated in **Figure 3** and is discussed below.

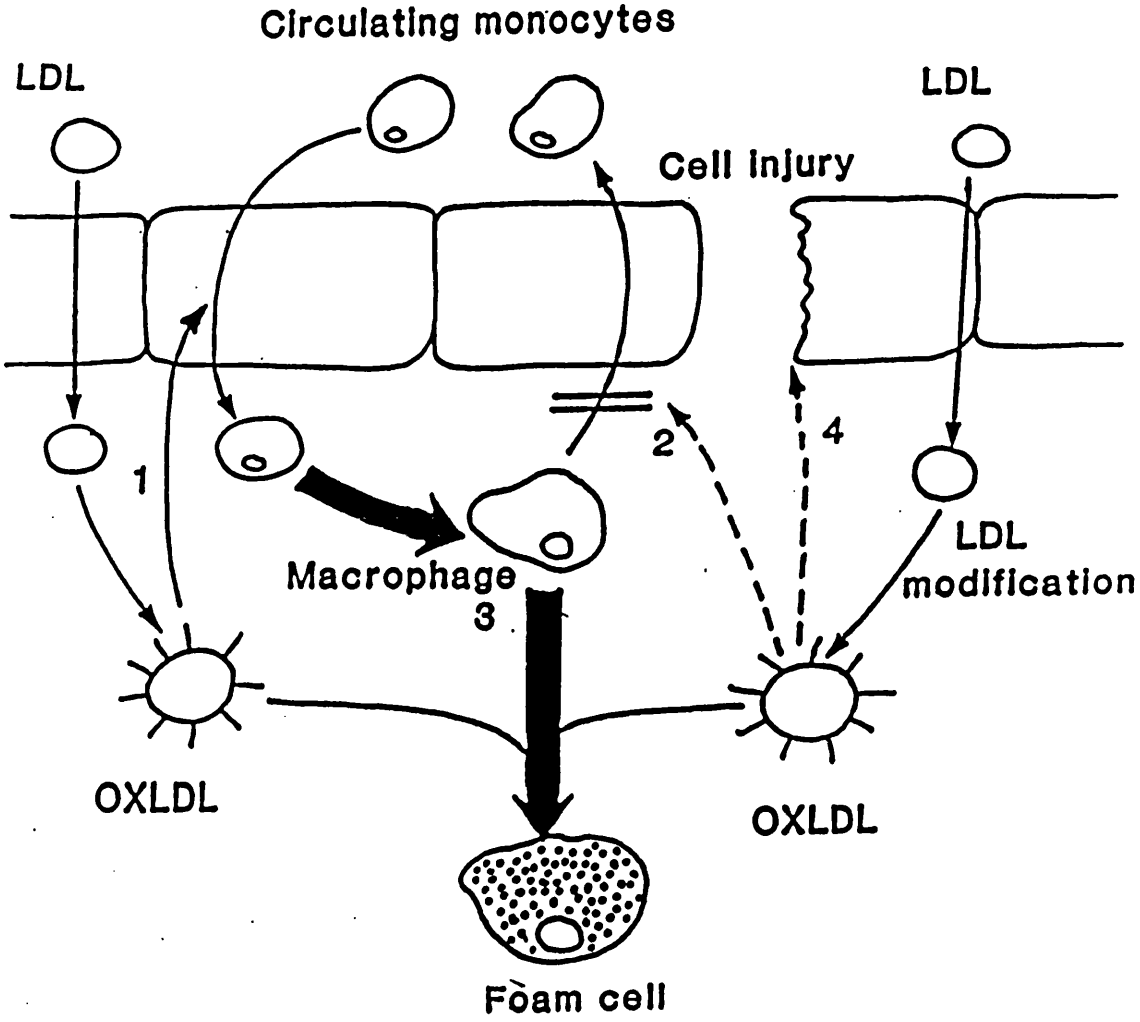


FIGURE 3

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)

(i) Oxidative Modification of LDL and Uptake by Macrophages

One of the earliest events in the formation of atherosclerotic lesions is the accumulation of cholesterol in foam cells in the sub-endothelial space. Foam cells in early lesions are derived predominantly from macrophages (Gerrity, 1981a; 1981b). The mechanism by which increased LDL levels lead to the accumulation of cholesterol in macrophages was not readily apparent since cultured macrophages did not accumulate cholesterol ester even when exposed to high concentrations of native LDL (Goldstein *et al.,* 1979; Fogelman, *et al.,* 1980). It was therefore proposed that chemical modification of LDL was a requisite for macrophage uptake (Goldstein *et al.,* 1979). Acetylation of LDL, which neutralises the positively charged residues on the apo-B moiety essential for recognition by the high-affinity LDL receptor, resulted in a rapid uptake by macrophages at a rate sufficient to generate foam cells (Steinberg *et al.,* 1989). This uptake was shown to be *via* a saturable and specific receptor termed the "acetyl LDL receptor" or "scavenger receptor" which does not recognise native LDL and is not regulated by cellular cholesterol levels (Goldstein *et al.,* 1979; Brown and Goldstein, 1983).

Other similarly chemically modified forms of LDL, such as malondialdehyde-treated LDL, were found to be recognised by the same receptor, and it was suggested that analogous modifications may occur *in vivo* to facilitate foam cell formation. All three of the major cell types in the arterial lesion, endothelial cells (Henriksen *et al.,* 1981; 1983; Morel *et al.,* 1984), vascular smooth muscle cells (Morel *et al.,* 1984; Heineke *et al.,* 1986), and monocytes-macrophages (Parthasarathy *et al.,* 1986a), were subsequently shown to convert LDL to a form rapidly taken up by macrophages, partly mediated by the scavenger receptor.

The modification of LDL was shown to be an oxidative process involving the peroxidation of polyunsaturated fatty acids, mainly linoleic and arachidonic acid, in the LDL lipids yielding hydroperoxy and hydroxy derivatives, which could be further broken down to produce 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; Parthasarathy, 1987). Many studies have now demonstrated that OXLDL, produced by a variety of different techniques, undergoes enhanced uptake in macrophages, and therefore, if these modifications were to occur *in vivo* they could provide a mechanism for the accumulation of cholesterol ester and the formation of foam cells within the vascular wall.

Oxidative modification of LDL by cultured cells results in a number of compositional and structural changes to the LDL molecule which are summarised in **Table 2.**

The oxidative modification of LDL by cultured cells is absolutely dependent on the presence of trace amounts of transition metal ions in the media (Steinberg *et al.,* 1989). Furthermore, it has now been demonstrated that similar modifications to LDL can be induced in the absence of cells by incubation of LDL with transition metal ions such as Cu^{2+} or Fe^{3+} (Steinbrecher *et al.,* 1984; 1987; Parthasarathy *et al.,* 1986). Such Cu^{2+}-oxidised LDL is recognised by the scavenger receptor and has similar biological properties to LDL obtained by exposure to cultured cells (Steinbrecher *et al.,* 1984; 1987), and is used in the experiments described in Chapter 3 of this thesis. LDL incubated in the presence of Fe^{3+} is usually

TABLE 2 **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.

3. Increased thiobarbituric acid reactive substances (TBARS) content and hydrolysis of phosphatidylcholine (PC) to lysophosphatidylcholine (LPC; Morel *et al.,* 1984; Steinbrecher *et al.,* 1984; Heinecke *et al.,* 1986; Parthasarathy *et al.,* 1986).

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).

termed "minimally modified LDL" and is still recognised by the high-affinity LDL receptor not by the scavenger receptor (Berliner *et al.,* 1990).

(ii) Recruitment and Retention of Macrophages

OXLDL differs from native LDL in that it is chemotactic for circulating monocytes (Quinn *et al.,* 1985; 1987; 1988), a property attributable in part to lysolecithin formed during LDL oxidation (Quinn *et al.,* 1988). It has been shown that rabbit aortic and human umbilical vein endothelial cells treated with minimally oxidised LDL stimulate the adherence of monocytes to the endothelium, possibly through an increased expression of monocyte specific adherence molecules (Berliner *et al.,* 1990). Furthermore, minimally oxidised LDL stimulates the transcription and secretion of monocyte chemotactic protein-1 (MCP-1) by cultured endothelial cells and smooth muscle cells (Cushing *et al.,* 1990). Therefore, accumulation of OXLDL in the subendothelial space, promotes the recruitment of monocytes and their differentiation into macrophages (Frostegard *et al.,* 1990). Furthermore, OXLDL is a potent inhibitor of both basal and stimulated macrophage motility (Quinn *et al.,* 1985), and so may play a role in the retention of macrophages in the vessel wall and contribute to the pathogenesis of atherosclerosis.

(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 so may play a role in disrupting a variety of cellular processes including macrophage motility and injuring vascular smooth muscle cells (Jürgens *et al.,* 1987; Steinberg *et al.,* 1989). Damage to endothelial cells

overlying fatty streaks could help bring about the formation of more advanced lesions.

The lipid components of OXLDL are thought to be involved in the cytotoxicity of OXLDL since fatty acids and products of oxidation including lipid hydroperoxides and 2-alkenals have been shown to have cytotoxic effects (Esterbauer *et al.,* 1987; Jürgens *et al.,* 1987).

1.5.1 THE INVOLVEMENT OF LIPOXYGENASES IN LDL OXIDATION

The oxidative modification of LDL by cultured cells involves the peroxidation of LDL lipids although the cellular mechanisms involved remain unclear. It has been demonstrated that LDL can be modified by incubation with purified 15-lipoxygenase in the absence of cells or transition metal ions to produce a particle which closely resembles LDL oxidised by incubation with cells (Sparrow *et al.,* 1988; Cathcart *et al.,* 1991). This observation suggested that the modification of LDL may involve cellular 15-lipoxygenases and it has been shown that inhibitors of lipoxygenase can prevent the modification of LDL by endothelial cells (Parthasarathy *et al.,* 1989; Derian and Lewis, 1992) and macrophages (McNally *et al.,* 1990; Rankin *et al.,* 1991). In addition, mRNA for 15-lipoxygenase has been found to be co-localised with epitopes characteristic for OXLDL in macrophage-rich areas of human and WHHL rabbit atherosclerotic lesions (Ylä-Herttuala *et al.,* 1990; 1991). 15-lipoxygenase could contribute to the modification of LDL through the generation of intracellular hydroperoxy lipids or peroxy radicals which could then initiate the extracellular oxidation of LDL.

Lipoxygenases have been implicated in the pathogenesis of atherosclerosis and induction of 15-lipoxygenase has been demonstrated in

the aortas of cholesterol-fed (Henriksson *et al.,* 1985; Simon *et al.,* 1989b) and WHHL (Pfister *et al.,* 1988; Simon *et al.,* 1989b) rabbits during atherogenesis. In addition, atherosclerotic lesions from both cholesterol-fed and WHHL rabbits have increased levels of the lipoxygenase-derived product 15-HETE which may directly affect the atherogenic process since it has been shown to be chemotactic for smooth muscle cells (Nakao *et al.,* 1982) and mitogenic for endothelial cells (Setty *et al.,* 1987).

1.5.2 EVIDENCE THAT OXLDL EXISTS *IN VIVO*

It is unlikely that the oxidation of LDL occurs to any great extent in the circulation due to the presence of antioxidants such as ascorbate. However, the presence of fragments of apo-B (Schuh *et al.,* 1978), increased levels of lipid peroxides and thiobarbituric acid-reactive substances (TBARS; Yagi, 1987) and a low level of modified LDL immunoreactivity (Salmon *et al.,* 1987) has been demonstrated in human plasma, indicating that some degree of oxidative modification of LDL occurs in plasma. Furthermore, a modified LDL fraction has been isolated from the plasma of Lp(a)-negative subjects which was more electronegative that the bulk of LDL and was more rapidly internalised by macrophages indicating that the fraction represented oxidised LDL (Avogaro *et al.,* 1988).

Several lines of evidence exist to suggest the presence of oxidatively modified LDL in atherosclerotic lesions. LDL extracted from aortas of WHHL rabbits and from human atherosclerotic lesions showed an increased electrophoretic mobility, increased fragmentation of apo-B and an increased hydrated density indicating that oxidation had occurred (Daugherty *et al.,* 1988; Ylä-Herttuala *et al.,* 1989). In addition, monoclonal antibodies against

modified residues found in apo-B of OXLDL (Haberland *et al.,* 1988; Palinski *et al.,* 1989) or against OXLDL itself (Palinski *et al.,* 1989) show immunoreactivity in rabbit aortic atherosclerotic lesions, but not in normal areas of aortic tissue. Furthermore, monoclonal antibodies against 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 homogenates were specific for OXLDL (Mowri *et al.,* 1988).

Although the demonstration of OXLDL in lesions *in vivo* supports the hypothesis that oxidation of LDL is important in atherosclerosis, it does not prove that OXLDL plays a causal role. Studies using probucol, a lipid-lowering drug with powerful antioxidant properties (Parthasarathy *et al.,* 1986), have provided more convincing evidence for the role of OXLDL in the pathogenesis of atherosclerosis. Treatment of WHHL rabbits with probucol has been shown to inhibit the rate of uptake and modification of native LDL in areas with lesions and to decrease the rate of progression of atherosclerosis (Tawara *et al.,* 1986; Carew *et al.,* 1987; Kita *et al.,* 1987; Steinberg *et al.,* 1988; Daugherty *et al.,* 1991). In addition, probucol may promote the regression of established plaques (Yamamoto *et al.,* 1986; Nagano *et al.,* 1992). The protective effect of probucol was found to be independent of its lipid-lowering properties and was attributed to its antioxidant action (Carew *et al.,* 1987; Nagano *et al.,* 1992). It has been demonstrated that the antioxidant butylated hydroxytoluene (BHT), a close chemical analogue of probucol, can also slow the progression of atherosclerosis in cholesterol-fed rabbits (Björkhem *et al.,* 1991). Probucol has also been shown to reduce plasma lipid peroxide levels in hyperlipidaemic patients (Paterson *et al.,* 1992). However, in contrast, one

study found no protective role of probucol on atherosclerosis in cholesterol-fed rabbits when the cholesterol-lowering effect was controlled for (Stein *et al.,* 1989).

It is possible that probucol may have actions other than its antioxidant or lipid-lowering effects which contribute to its effectiveness in decreasing the rate of atherosclerosis. It has been suggested that probucol directly affects macrophages to inhibit the uptake of LDL (Yamamoto *et al.,* 1986), or may increase the rate of cholesterol efflux from lipid-laden macrophages (Goldberg and Mendez, 1988). Furthermore, administration of probucol has been shown to suppress intimal thickening of the carotid artery following balloon catheter injury which may be due to the inhibition of cell migration or cell proliferation (Shinomiya *et al.,* 1992).

The mechanism by which *in situ* modification of LDL in the arterial intima may lead to the formation of foam cells has not been established. If extensive modification occurs then uptake by the scavenger receptor pathway may be involved. It has been demonstrated that modified LDL from human atherosclerotic plaques was more susceptible to degradation by the scavenger receptor pathway in cultured macrophages (Ylä-Herttuala *et al.,* 1989). In addition, macrophage-derived foam cells isolated from rabbit atherosclerotic lesions degrade OXLDL, promote the oxidation of LDL and contain oxidation-specific lipid-protein adducts, indicating 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 contrast, another study demonstrated that degradation of LDL from human atherosclerotic lesions by macrophages occurred *via* a low affinity non-scavenger receptor mechanism (Morton *et al.,* 1986), and that extracts of

atherosclerotic plaques could modify LDL *in vitro* in a way which led to an increased non-saturable, non-receptor mediated degradation in cultured macrophages (Hoff and O'Neil, 1988). It was concluded that the modification of LDL by plaque components which was responsible for the interaction with macrophages did not involve oxidation, since the uptake by macrophages persisted in the presence of BHT (Hoff and O'Neil, 1988), but reactions between the apo-B and aldehydes or the formation of complexes with arterial proteoglycans may be involved.

1.6 ATHEROSCLEROSIS AND VASCULAR REACTIVITY

The endothelium plays a critical role in the control of vasomotor tone and blood pressure through the release of EDRF (see section 1.3). Vasodilator responses mediated by the endothelium are markedly impaired in atherosclerosis and hypercholesterolaemia, and atherosclerotic blood vessels have been found to be very susceptible to vasospasm.

The effects of hypercholesterolaemia and atherosclerosis on vascular responses and the possible pathological consequences will be discussed.

1.6.1 ENDOTHELIUM-DEPENDENT RESPONSES

Previous studies have demonstrated that relaxations mediated by EDRF are impaired in isolated arteries of hypercholesterolaemic 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; Merkel *et al.*, 1990), monkeys (Armstrong et al., 1982; Freiman 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 been suggested that hypercholesterolaemia alone, without accompanying atherosclerosis, is sufficient to alter vascular reactivity since resistance vessels, which do not exhibit gross atherosclerotic lesions, also show impaired endothelium-dependent responses (Osborne *et al.,* 1989; Sellke *et al.,* 1990; Simonsen *et al.,* 1992). In addition, impaired endothelium-dependent relaxations have been observed in coronary resistance vessels downstream from atherosclerotic lesions indicating that the pathophysiological consequences of atherosclerosis may extend into the microcirculation (Kuo *et al.,* 1992). During the development of atherosclerosis, there is a progressive impairment of endothelium-dependent responses suggesting that other mechanisms may be involved (Verbeuren *et al.,* 1986; 1990; Shimokawa and Vanhoutte, 1989; Zeiher *et al.,* 1991).

In isolated atherosclerotic human arteries, endothelium-dependent responses evoked by a number of agonists are attenuated (Bossaller *et al.,* 1987b; Berkenboom *et al.,* 1989; Förstermann et al., 1988b). Furthermore, vasoconstriction, rather than a normal vasodilator effect, is observed *in vivo* in response to ACh in atherosclerotic human coronary arteries (Ludmer *et al.,* 1986; Zeiher *et al.,* 1991). This vasoconstriction following infusion or injection of ACh has been demonstrated in hypercholesterolaemic patients with angiographically normal coronary arteries providing further evidence that endothelial dysfunction may occur in response to hypercholesterolaemia without macroscopic changes associated with atherosclerosis (Horio *et al.,* 1986; Werns *et al.,* 1989; Vita *et al.,* 1990, Drexler *et al.,* 1991).

The mechanisms underlying the abnormal endothelium-dependent relaxations observed in hypercholesterolaemia and atherosclerosis remain

unclear although several factors have been proposed which will now be discussed.

(i) Selective Inhibition of Receptor-Mediated Pathways

The observation that in atherosclerotic rabbit aorta and human coronary arteries endothelium-dependent relaxations evoked by ACh were impaired, but those mediated by histamine, substance P and the non-receptor-mediated responses to A23187 remained unaltered, led to the proposal that a selective defect in receptor-operated release of EDRF may account for the attenuation of responses (Bossaller *et al.,* 1987a). Responses of atherosclerotic iliac arteries from monkeys (Freiman *et al.,* 1986) and coronary arteries from hypercholesterolaemic pigs (Yamamoto *et al.,* 1987; Cohen *et al.,* 1988) have also been shown to exhibit selective impairment of endothelial function. It has been suggested that the dysfunction may occur distal to the endothelial receptors at the level of endothelial G-proteins and that the selective impairment of receptor-mediated responses could be explained by an inhibition of specific G-protein-dependent signal transduction pathways (Shimokawa *et al.,* 1991; Flavahan, 1992).

However, many studies have demonstrated that both receptor- and non-receptor-mediated relaxations are attenuated in isolated vessels from hypercholesterolaemic animals indicating a general impairment of endothelium-dependent responses rather than a selective impairment of receptor pathways (Jayokady *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). This non-specific impairment of endothelium-dependent responses may represent a later stage in the

atherosclerotic disease process when mechanisms other than the inhibition of G-proteins are involved.

(ii) Denudation of Endothelial Cells

In monkeys, a loss of endothelial cells in the iliac artery has been demonstrated in response to cholesterol feeding (Faggiotto *et al.,* 1984), and in WHHL rabbits a progressive impairment of endothelium-dependent relaxations was observed with an associated loss of endothelial cells (Kolodgie *et al.,* 1990), suggesting that the attenuation of vascular relaxation in atherosclerosis was a result of endothelial denudation or damage. However, many studies have demonstrated the presence of an intact endothelium even in severely atherosclerotic vessels in which endothelium-dependent responses were inhibited (Jayakody *et al.*, 1985; Freiman *et al.,* 1986; Verbeuren *et al.,* 1986; 1990; Schuschke *et al.,* 1990). Morphological changes such as vacuoles, shape changes and altered surface projections and a reduction in the surface charge have been observed in atherosclerotic vessels (Taylor *et al.,* 1990) and may be associated with the inhibition of endothelium-dependent relaxations, thus reflecting functional changes in addition to the structural modifications (Jayakody *et al.,* 1988; 1989).

(iii) Decreased Availability of L-arginine

It has been proposed that a decreased availability of the precursor of EDRF, L-arginine, may explain the inhibitory influence of atherosclerosis and hypercholesterolaemia on endothelium-dependent relaxation. In some studies, *in vivo* administration of L-arginine reversed the attenuation of endothelium-dependent relaxations in the hind-limb resistance vessels (Girerd *et al.,* 1990), isolated aorta (Cooke *et al.,* 1991a) and cerebral vessels

(Rossitch *et al.,* 1991) from hypercholesterolaemic rabbits. However, in other investigations, L-arginine did not reverse endothelial dysfunction *in vitro* in atherosclerotic rabbit aorta (Mugge and Harrison, 1991). In hypercholesterolaemic patients, administration of L-arginine was shown to restore endothelial function in coronary resistance vessels in which no structural alterations had occurred although, in larger vessels of the coronary circulation which showed some evidence of atherosclerosis, L-arginine had no effect on ACh-induced responses (Drexler *et al.,* 1991).

(iv) Decreased Production of EDRF

Bioassay studies have demonstrated that the vasodilation and accompanying increases in cGMP in endothelium-denuded detector tissues is decreased when superfused using a donor vessel from an atherosclerotic or hypercholesterolaemic animal compared with a normal donor (Sreeharan *et al.,* 1986; Guerra *et al.,* 1989; Jayakody *et al.,* 1989; Shimokawa and Vanhoutte, 1989; Schuschke *et al.,* 1991), indicating a decreased release of EDRF from atherosclerotic and hypercholesterolaemic vessels. However, other studies have demonstrated that the intraluminal release of EDRF from atherosclerotic rabbit aorta is normal except in the most severely diseased tissues and that the release of EDRF may be normal even when relaxations of intact tissues are completely abolished (Verbeuren *et al.,* 1986; 1990). Furthermore, in bioassay experiments, it was shown that the impaired vasodilator activity in cholesterol-fed rabbits was associated with an increased production of nitrogen oxides, as measured by chemiluminescence (Minor *et al.*, 1990).

These findings suggest that the attenuation of endothelium-dependent relaxations may not be due to a decreased production of EDRF but by a mechanism occurring subsequent to the release of EDRF.

(v) Increased Rate of Inactivation of EDRF

During the progression of atherosclerosis the intimal layer of the arterial wall becomes thickened due to the infiltration and accumulation of foam cells and the infiltration of lipid-laden smooth muscle cells. The lesions may act as a diffusion barrier and will alter the sequestration properties of the vessel so may prevent the short-lived EDRF from reaching the vascular smooth muscle cells. The intimal layer of the lesion containing lipid-laden cells may be important by acting as a sink for the lipophilic EDRF molecule and so reducing the amount reaching the vascular smooth muscle. Free-radicals produced by the inflammatory cells in the lesion may also play a role by inactivating EDRF.

Several studies have demonstrated that by returning cholesterol-fed animals to a normal diet some regression of the atherosclerotic lesions occurs with the reabsorption of intimal lipids and inflammatory cells (Armstrong *et al.,* 1970; 1982; Adams and Morgan, 1977; Harrison *et al.,* 1987). The intimal layer remains thickened due to smooth muscle cell proliferation (Armstrong *et al.,* 1982) but this may not provide a significant diffusion barrier since endothelium-dependent relaxations are restored following regression of atherosclerotic lesions (Harrison *et al.,* 1987). However, other studies have shown that endothelium-dependent responses remain impaired following regression (Armstrong *et al.,* 1982; Jayakody *et al.,* 1987; 1989).

(vi) Decreased Smooth Muscle Responsiveness

Many studies have demonstrated that in atherosclerosis and hypercholesterolaemia endothelium-dependent relaxations are impaired whereas endothelium-independent responses evoked by nitrovasodilators such as GTN or SNP remain intact (Jayakody *et al.,* 1985; Freiman *et al.,* 1986; Sreeharan *et al.,* 1986; Bossaller *et al.,* 1987a; Harrison *et al.,* 1987; Guerra *et al.,* 1989; Simonsen *et al.,* 1992). In severely atherosclerotic vessels from humans and rabbits, however, responses to nitrovasodilators are also decreased suggesting that as the disease progresses, the responsiveness of the smooth muscle becomes impaired (Verbeuren *et al.,* 1986; 1990; Förstermann *et al.,* 1988b; Berkenboom *et al.,* 1989).

In summary, several possible mechanisms may underly the inhibition of endothelium-dependent relaxations in hypercholesterolaemia and atherosclerosis, their relative importance determined by the stage of the disease process. Early in the disease, endothelial dysfunction may result from an impairment of selective receptor or G-protein-mediated pathways. As the disease progresses the dysfunction may spread to other signal-transduction pathways in the endothelial cell, and a non-specific attenuation of endothelium-dependent relaxations exists which may involve a reduction in the release of EDRF or an increased breakdown of the factor. In advanced stages of atherosclerosis a decreased sensitivity of the vascular smooth muscle to EDRF may contribute leading to a complete abolition of relaxations in severely diseased vessels.

1.6.2 CONTRACTILE RESPONSES IN ATHEROSCLEROSIS

Many studies have demonstrated that vasoconstrictor responses of vessels may be potentiated by hypercholesterolaemia and atherosclerosis. Coronary vasospasm can be provoked in patients with coronary atherosclerosis by several agents such as ergonovine which have no response in normal subjects (Schroeder *et al.,* 1977; Cipriano *et al.,* 1979; Waters *et al.,* 1983; Kaski *et al.,* 1986). Similarly, in atherosclerotic miniature swine, 5-HT and histamine can cause vasospasm whereas in normal porcine coronary arteries these agents produce endothelium-dependent vasodilation (Shimokawa *et al.,* 1983; 1985a; 1985b).

Several mechanisms have been proposed to account for the altered vascular contractility observed in atherosclerosis and hypercholesterolaemia and will now be discussed.

(i) Accumulation of Cholesterol in Vascular Cell Membranes

One of the earliest changes to occur during the development of atherosclerosis is an increased cholesterol content of cell membranes (Small and Shipley, 1974). The incorporation of cholesterol into cell membranes has been shown to augment cation permeability (Wiley and Cooper, 1975) and inhibit responses mediated by the activation of β-adrenoceptors in erythrocyte membranes (Lurie *et al.,* 1985). However, changes in membrane cholesterol content would be expected to non-specifically alter vasoconstrictor responses, but changes only in response to certain agonists have been observed, suggesting that this mechanism does not play a large role (see Yokoyama *et al.,* 1983; Heistad *et al.,* 1984; Lopez *et al.,* 1989a; 1989b; Merkel *et al.,* 1990).

(ii) Increase in Receptor Number

Many studies have demonstrated a potentiation of 5-HT-evoked constrictions in hypercholesterolaemic and atherosclerotic vessels (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). Radioligand binding studies have shown an increased number of both serotonergic and adrenergic receptors in the atherosclerotic rabbit aorta (Nanda and Henry, 1982) which may explain these observations. However, most studies of altered responses to adrenergic agonists report a decrease in contractile response (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 relaxations are attenuated in atherosclerotic and hypercholesterolaemic animals and humans and, due to the removal of basally released EDRF, tissues denuded of endothelium show enhanced agonist induced contractions (Cocks and Angus, 1983; Cohen *et al.,* 1983b; Martin *et al.,* 1985a; 1985b). In addition, agents such as ACh and 5-HT can evoke endothelium-dependent relaxations and have a direct vasoconstrictor effect on smooth muscle. Therefore, removal or dysfunction of the endothelium may lead to potentiated constrictor responses to these agents by removing their endothelium-dependent relaxant effect (Lamping *et al.,* 1985; Shimokawa and Vanhoutte, 1989).

(iv) Release of Contracting Factors

It has been suggested that the enhanced vasoconstrictor responses in atherosclerotic vessels are due to the release of cyclo-oxygenase-derived

The endothelium-derived vasoconstrictor peptide, endothelin-1, has been shown to potentiate contractions evoked by noradrenaline and 5-HT (Yang *et al.*, 1990) and may therefore play a role in the altetred contractile responses observed in atherosclerosis. In addition, elevated plasma concentrations of endothelin have been demonstrated in humans with advanced atherosclerosis (Lerman *et al.*, 1991).

vasoconstrictor products from the endothelium (Miller and Vanhoutte, 1985; Lüscher and Vanhoutte, 1986; Shimokawa and Vanhoutte, 1989). Products of the cyclo-oxygenase pathway have been shown to augment 5-HT-induced contractions and inhibit 5-HT and ADP-evoked relaxations of atherosclerotic porcine coronary arteries in an endothelium-dependent manner (Shimokawa and Vanhoutte, 1989). However, other studies have demonstrated potentiation of contractions which is not dependent on the endothelium (Verbeuren *et al.,* 1986).

1.6.3 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). Spasm can occur in angiographically normal arteries as well as in arteries with atherosclerotic lesions (Horio *et al.,* 1986; Werns *et al.,* 1989; Vita *et al.,* 1990) suggesting that alterations in vascular reactivity precede macroscopic alterations.

A direct link between the inhibition of relaxations mediated by EDRF and coronary vasospasm is not clear although the occurrence of spasm related to coronary angioplasty demonstrates the important inhibitory effect of an intact endothelium on vascular smooth muscle constriction (Dorros *et al.,* 1983). More direct evidence of endothelial dysfunction came from the finding that intracoronary injection of the endothelium-dependent vasodilator ACh caused vasoconstriction in atherosclerotic human coronary arteries but dilated normal arteries (Ludmer *et al.,* 1986).

The endothelium mediates vasodilation to a number of products of aggregated platelets and so endothelial dysfunction may alter the vascular

responses of these products to favour vasoconstriction (Cohen *et al.,* 1983a; Houston *et al.,* 1985; Shimokawa *et al.,* 1987) indicating a role for aggregating platelets in coronary vasospasm.

In conclusion, the release of EDRF under basal conditions and on stimulation plays an important role in the control of vascular homeostasis. Altered vascular reactivity caused by hypercholesterolaemia and atherosclerosis may therefore have pathological consequences (reviewed by Vanhoutte and Shimokawa, 1989).

1.6.4 THE ROLE OF LIPOPROTEINS

Hypercholesterolaemia is associated with an increase in circulating LDL which is recognised as a major risk factor for atherosclerosis. Several studies have investigated the effects of both native and oxidatively modified LDL on endothelium-dependent relaxations.

Native LDL has been shown to inhibit endothelium-dependent relaxations by a rapid and reversible mechanism that may result from a direct interaction between LDL and EDRF (Jacobs *et al.,* 1990; Tomita *et al.,* 1990). A bioassay study demonstrated that LDL inactivated EDRF possibly by sequestration and inactivation within the hydrophobic core of the lipoprotein molecule (Galle *et al.,* 1991). Not all studies, however, have demonstrated an inhibitory effect of native LDL on endothelium-dependent relaxations 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). It has been shown that the inhibition of relaxations by native LDL is dependent on the agonist used to precontract the tissues (Jacobs *et al.,* 1990).

Many studies have shown that OXLDL causes inhibition of endothelium-dependent relaxations of isolated tissues. The extent of the inhibition varies from complete abolition of responses (Kugiyama *et al.,* 1990; Yokoyama *et al.,* 1990) to much lesser effects (Galle *et al.,* 1990; Tanner *et al.,* 1991). These variations in effects may be explained by the finding that the degree of inhibition varies between LDL preparations from different donors (Jacobs *et al.,* 1990; Plane, 1992).

The mechanism of action of OXLDL in causing inhibition of relaxations is not clear. It has been suggested that OXLDL inhibits endothelium-dependent relaxations by inactivation of EDRF after its release from cultured endothelial cells (Galle *et al.,* 1991; Chin *et al.,* 1992). Low concentrations of OXLDL have been shown to inhibit endothelium-dependent relaxations evoked by receptor-mediated agonists but not by receptor-independent stimuli whereas at higher concentrations OXLDL was found to cause a non-specific inhibition of endothelium-dependent relaxations (Kugiyama *et al.,* 1990). Therefore, OXLDL may induce endothelial dysfunction by selectively inhibiting endothelial receptor signal transduction pathways and at higher concentrations cause a more non-specific inhibition. It was suggested that inhibition of the G_i protein-dependent pathway may contribute in part to the attenuation of relaxations by OXLDL (Tanner *et al.,* 1991). A direct action of OXLDL on vascular smooth muscle has been proposed to account for the inhibitory effects of OXLDL since the modified lipoprotein has been shown to inhibit relaxations evoked by GTN (Jacobs *et al.,* 1990) and to inhibit the activation of partially purified soluble guanylate cyclase by nitrovasodilators and NO (Schmidt *et al.,* 1990). However, other studies have shown that OXLDL has no effect on endothelium-independent relaxations (Tanner *et al.,* 1991).

The inhibitory effects of OXLDL on endothelium-dependent relaxations are associated with the lipid fraction of the molecule. During the modification of LDL, phosphatidylcholine (PC) is converted to lysophosphatidylcholine (LPC) which has been shown to cause inhibition of endothelium-dependent relaxations (Kugiyama *et al.,* 1990; Yokoyama *et al.,* 1990; Mangin *et al.,* 1993) and the inhibitory properties of OXLDL have been attributed to LPC. However, differences in the inhibitory properties of LPC and OXLDL (Plane *et al.,* 1992) suggest that lipid factors present in OXLDL other than LPC must contribute to the inhibition of relaxations by OXLDL.

In conclusion, the alterations in vascular reactivity produced by OXLDL are similar to those associated with hypercholesterolaemia and atherosclerosis although the exact mechanisms underlying the inhibitory effects remain unclear. The potential of LDL to inhibit endothelium-dependent relaxations is increased by oxidation but the inhibitory factors generated during the oxidative modification are not known.

1.7 AIMS

(1) It has been previously demonstrated that OXLDL inhibits endothelium-dependent relaxations of isolated rabbit aortic rings and that this inhibitory effect may contribute to the attenuation of vasodilator responses observed in atherosclerosis. This study investigated the effects of native and OXLDL on relaxations of isolated rabbit coronary arteries which do not exhibit gross atherosclerotic lesions.

(2) Cellular 15-lipoxygenases have been implicated in the oxidative modification of LDL and 15-lipoxygenase activity is increased in aortas from atherosclerotic rabbits. The aim of this investigation was to examine the effects of LDL modified by treatment with lipoxygenase on the responses of isolated rabbit aortic rings.

(3) The constituents of oxidatively modified LDL responsible for the attenuation of relaxations are yet to be identified. In this study the vascular effects of lipoxygenase-derived products of arachidonic and linoleic acid were investigated in isolated rabbit aortic rings.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICALS

Chemicals for density solutions and buffers were from BDH and of Analar grade.

Acetylcholine (ACh), phenylephrine, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), sodium nitroprusside (SNP), soybean lipoxidase (Type V), indomethacin, ascorbic acid and polyinosinic acid (poly I) were obtained from the Sigma Chemical Company.

Glyceryl trinitrate (GTN) was supplied by the Pharmacy, Royal Free Hospital.

Chelerythrine chloride was from Calbiochem.

Dextran sulphate (MW = 1,000,000) was obtained from Fluka Biochemika.

Other suppliers are named in the text where appropriate.

2.1.2 ANIMALS

New Zealand White rabbits weighing 2.7 - 3.5 kg (6 months old) were supplied by the Comparative Biology Unit, Royal Free Hospital or by Zeneca Pharmaceuticals.

2.1.3 NITRIC OXIDE

Nitric oxide gas was supplied by Cambrian Gases.

2.1.4 PREPARATION OF NITRIC OXIDE SOLUTIONS

Nitric oxide solutions were prepared in degassed water by the following method (Palmer *et al.*, 1990). Double distilled water was boiled for 30 mins then placed on ice and bubbled with nitrogen for 30 mins. The degassed water was transferred, under vacuum, to a gas bulb which had been sealed at one end with a rubber septum (Phase Separations Ltd.) and heat shrink tubing (RS Components Ltd.). The water was then bubbled with nitrogen for a further 30 mins before the bulb was sealed.

Nitric oxide gas was injected into the solution of degassed water using a gas tight syringe. After preparation, the nitric oxide solutions were kept on ice. Solutions were prepared fresh for each experiment at a concentration of 44.6μM.

2.1.5 HYDROPEROXY AND HYDROXY FATTY ACIDS

Hydroperoxy and hydroxy derivatives of arachidonic and linoleic acids were supplied by Cascade Biochem. Ltd.

They were provided dissolved in ethanol at concentrations between 4 and 5 mM. The fatty acid metabolites were stored in silanised vials at -70°C under nitrogen.

2.2 BUFFERS AND DENSITY SOLUTIONS

Krebs' Buffer

119 mM NaCl, 4.7 mM KCl, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11 mM glucose, 0.03 mM EDTA, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, pH 7.4

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 the dialysis of LDL

140 mM NaCl, 12.5 mM tris, pH 7.4.

Density Solutions

A stock solution of density 1.006 g/ml of composition 195 mM NaCl, 1 mM NaOH, 0.34 mM EDTA was used to prepare all other density solutions. Solid NaBr was added to this stock solution according to the equation below:

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

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 was checked using a Paar densitometer to measure the refractive index.

2.3 ISOLATION OF LIPOPROTEINS

2.3.1 COLLECTION OF BLOOD

Venous blood was taken from healthy volunteers using sterile polypropylene syringes (Plastipak, Becton Dickinson Ltd) and 19 gauge butterfly needles (Venisystems). Blood was immediately transferred into sterile plastic universal tubes (Sterilin) containing ACD anticoagulant (5:1 v/v) and centrifuged at 1800 *g* for 20 mins at 20°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 taken from each donor which gave approximately 80 mls of plasma (plus ACD).

2.3.2 PREPARATION OF LDL

LDL (density 1.019 - 1.063 g/ml) was isolated separately from the plasma of each donor by discontinuous gradient ultracentrifugation in the presence of 0.3 mM EDTA to avoid autoxidation (Chung *et al.*, 1980). The density of the plasma was adjusted to 1.3 g/ml by the addition of solid NaBr (31 g per 70 ml plasma). 10 - 15 mls of plasma were carefully layered underneath 0.9% (w/v) saline in centrifuge tubes (Beckman). The tubes were then capped, placed in a fixed angle rotor (Beckman 70-Ti) and spun at 200,000 *g* for 2.5 hrs at 16°C in a Beckman XL-70 ultracentrifuge.

Following centrifugation, the lipoproteins were banded in the tubes with VLDL and chylomicrons at the top, LDL in the middle and HDL at the bottom of the tubes. The middle LDL band was carefully removed using a syringe and needle and evenly distributed between clean centrifuge tubes containing 6.5 mls of density solution 1.151 g/ml. The tubes were filled with a solution of density 1.063 g/ml, capped and gently mixed before being

placed in a fixed angle rotor and spun at 200,000 *g* for 16 hrs at 16°C. This produced a yellow band at the top of the tubes which was carefully removed.

2.3.3 CONCENTRATION OF LDL

LDL samples were concentrated by centrifugation in a Sorval RC-5B refrigerated centrifuge (Dupont Instruments, USA) at 8000 rpm at a precooled temperature of 4°C in tubes containing nitro-cellulose ultrafiltration membranes (Diaflo, Amicon Corp., USA) which retained LDL but allowed the buffer to pass through. LDL samples were concentrated until the volume was reduced to 2 - 4 mls and were then transferred into 2 cm wide dialysis tubing (Scientific Industries International Incorporated) and dialysed against 5 changes of Tris buffer containing 0.3 mM EDTA for 18 hrs at 4°C. The LDL was finally filtered through a sterile 0.2 μm filter (Acrodisc, Gelman Sciences, UK) to remove any impurities. LDL prepared in this way is referred to as native LDL and was stored in the dark at 4°C and used within 2 weeks.

2.3.4 PROTEIN ASSAY

The protein concentration of the LDL samples was estimated using a modification (Markwell *et al.*, 1978) of the Lowry method (Lowry *et al.*, 1951) using Folin-Ciocalteu's pholin reagent. Bovine serum albumin (BSA) was used to prepare a standard curve. The exact concentration of the BSA stock solution was determined by measuring the UV-absorbance at 279 nm against distilled water. The concentration of the solution was then calculated using the equation below:

BSA Conc. (mg/ml) = Absorbance at 279 nm x 13/9

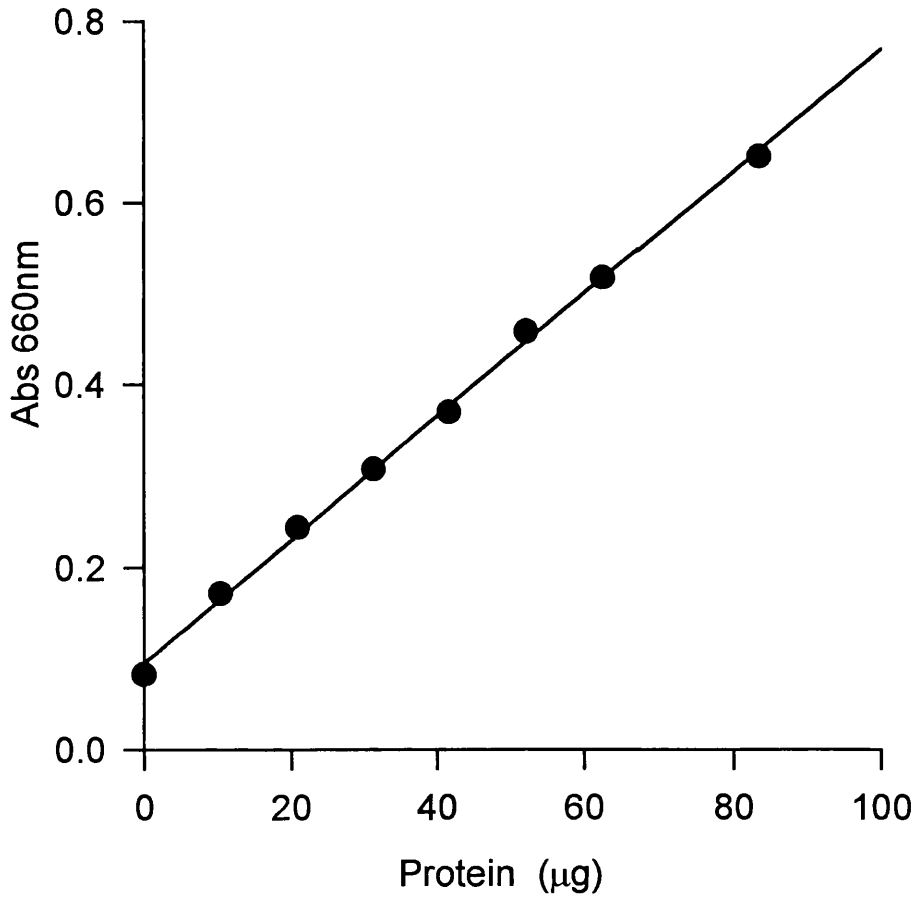


FIGURE 4

Bovine serum albumin (BSA) standard curve for protein assay.

The protein concentration of all LDL samples was determined as described in section 2.3.4 and expressed as mg LDL protein/ml.

Standards and samples were prepared in triplicate and the absorbance read at 660 nm against distilled water on a Beckman DU-70 spectrophotometer.

A typical standard curve is shown in **Figure 4** . Normally LDL samples were prepared at a concentration of between 5 and 8 mg protein/ml.

2.3.5 MODIFICATION OF LDL

Prior to modification, LDL was dialysed against 5 changes of Tris buffer for 18 hrs at 4°C to remove EDTA from the LDL sample. LDL was dialysed at a ratio of 1 part LDL to 1000 parts dialysis buffer.

(a) Cu^{2+}-oxidised LDL (OXLDL)

Oxidised LDL was prepared by incubating LDL (5 - 8 mg protein/ml) with $CuSO_4$ at a concentration of 1 nmol per mg of LDL for 24 hrs at room temperature. The OXLDL was then extensively dialysed against Tris buffer containing 0.3 mM EDTA to remove the excess Cu^{2+} ions and filtered using a sterile filter as described previously (section 2.3.3).

(b) Lipoxygenase-treated LDL (LO-LDL)

LDL modified by treatment with lipoxygenase was prepared by incubating LDL (5 mg protein/ml) with 200,000 U/mg of affinity purified soybean lipoxygenase in 50 mM borate buffer pH 9.0 for 24 hrs at room temperature (Cathcart *et al.*, 1991). In order to remove the enzyme following oxidation, the LO-LDL was transferred into centrifuge tubes containing density solutions as described in section 2.3.2 and spun at 200,000 *g* for 4 hrs in a Beckman XL-70 ultracentrifuge. The yellow band of LO-LDL could be seen at the top of the tubes. Lipoxygenase alone spun following the same procedure and stained with Coomassie Brilliant Blue R-250 protein

stain (Demacker *et al.*, 1983) was located in the bottom part of the tubes. Following recentrifugation of LO-LDL which had been stained with Coomassie Blue, a band of protein could be seen at the top of the tube and also in the bottom part of the tube showing the separation of the enzyme from LO-LDL. The LO-LDL was then removed from the tubes and extensively dialysed against Tris buffer containing 0.3 mM EDTA and filtered as described previously (section 2.3.3)

2.4 VASCULAR REACTIVITY STUDIES

The effects of lipoproteins on vascular reactivity was studied using isolated rabbit coronary arteries mounted in a double myograph or isolated rabbit aortic rings suspended in organ baths.

2.4.1 PREPARATION OF RABBIT CORONARY ARTERIES

New Zealand White rabbits weighing 2.7 - 3.5 kg were anaesthetised with sodium pentobarbital (60 mg/kg), the hearts were rapidly removed and placed in ice cold Krebs' buffer. Segments of the left circumflex coronary artery were dissected from the heart and cleaned of adhering cardiac muscle. In each experiment a large (first order branch) and a small (third order branch) artery from the same heart were prepared.

The vessels were mounted on two 40 μm wires in a double myograph system for isometric force measurements and fixed in place by attaching one of the wires to a force transducer and the other to a micrometer as shown in **Figure 5** (Mulvany and Halpern, 1976). The force transducers were connected via a preamplifier to a recorder. The vessels were allowed to

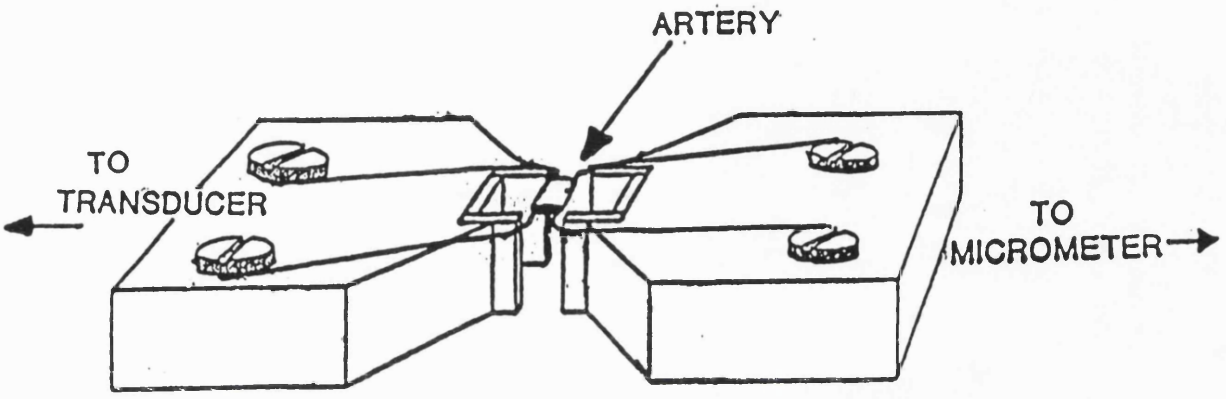


FIGURE 5

Myograph apparatus for isometric tension recording studies with isolated rabbit coronary arteries.

Coronary arteries mounted on two wires, one connected to a transducer, the other to a micrometer. The bath contained Krebs' buffer maintained at 37°C and gased with 95% O_2/5% CO_2.

equilibrate in Krebs' buffer maintained at 37°C and gassed with 95% O_2, 5% CO_2 for 30 mins.

The vessels were then normalised in order to set them at lumen diameters at which they developed maximum or near maximum active tension. The normalisation procedure used the passive wall tension-internal circumference relationship, which for vascular smooth muscle produces a dome-shaped curve (Mulvany and Halpern, 1977). The vessels underwent a series of stretches by turning the micrometer screw until they had been stretched to the point when the effective internal pressure was equal to or greater than 100 mmHg. L_{100} was defined as the internal circumference of the vessel when relaxed and under an effective transmural pressure of 100 mmHg. The effective lumen diameter, l_{100}, was calculated by $l_{100} = L_{100} / \pi$ and by using the micrometer the vessels were set to a normalised diameter, l_0, which corresponded to 90% of l_{100}, and at this diameter the active tension development has been shown to be maximal or near maximal (Mulvany and Halpern, 1977). The effective lumen diameter of large coronary arteries used was 809.9 ± 20.3 μm (n=45) and of small coronary arteries was 280.8 ± 11.4 μm (n=35). The vessels were left to equilibrate under their normalised tension for 30 mins.

To assess the mechanical condition of the vessels, they were successively activated four times with 125 mM KCl which was prepared from normal Krebs' buffer with an equimolar substitution of KCl for NaCl. The vessels were washed and allowed to relax between each activation. Following this procedure a 30 min equilibration period was allowed before the beginning of the experiment.

2.4.2 PREPARATION OF RABBIT AORTIC RINGS

New Zealand White rabbits weighing 2.7 - 3.5 kg were killed by cervical dislocation. The aorta was rapidly removed and transferred to oxygenated Krebs' buffer. The vessel was carefully cleaned of adhering fat and connective tissue and cut into 2 mm wide transverse rings. In some experiments the endothelium was removed from the luminal surface by gently rubbing with a buffer soaked pipe cleaner. Aortic rings were mounted in organ baths as shown in **Figure 6** and described by Furchgott and Zawadzki (1980). The vessels were suspended between metal hooks in organ baths containing oxygenated Krebs' buffer at 37°C under a resting tension of 2 g for isometric force measurements. One hook was fixed in position and the other was attached via a force displacement transducer (Grass FT-03 or Dynamometer 4F1) to a model 7 Grass Polygraph amplifier and recorder or to a Lectromed Multitrace 4-P recorder. Alternatively LKB, Rikadenki or Gould flat bed recorders were used in conjunction with a pre-amplifier. In each experiment 9 - 11 vessels were set up simultaneously.

The tissues were washed and equilibrated for 60 mins in Krebs' buffer after which time the tension was reset to 2 g, tissues were allowed to equilibrate for a further 30 mins.

To test the integrity of the endothelium a contraction of approximately 2 g was induced with phenylephrine (0.1 - 0.3 μM) and, when a plateau had been reached, the tissues were relaxed with a bolus dose of acetylcholine (1 μM). Tissues were then washed and left to equilibrate for 30 mins before the experiment was started.

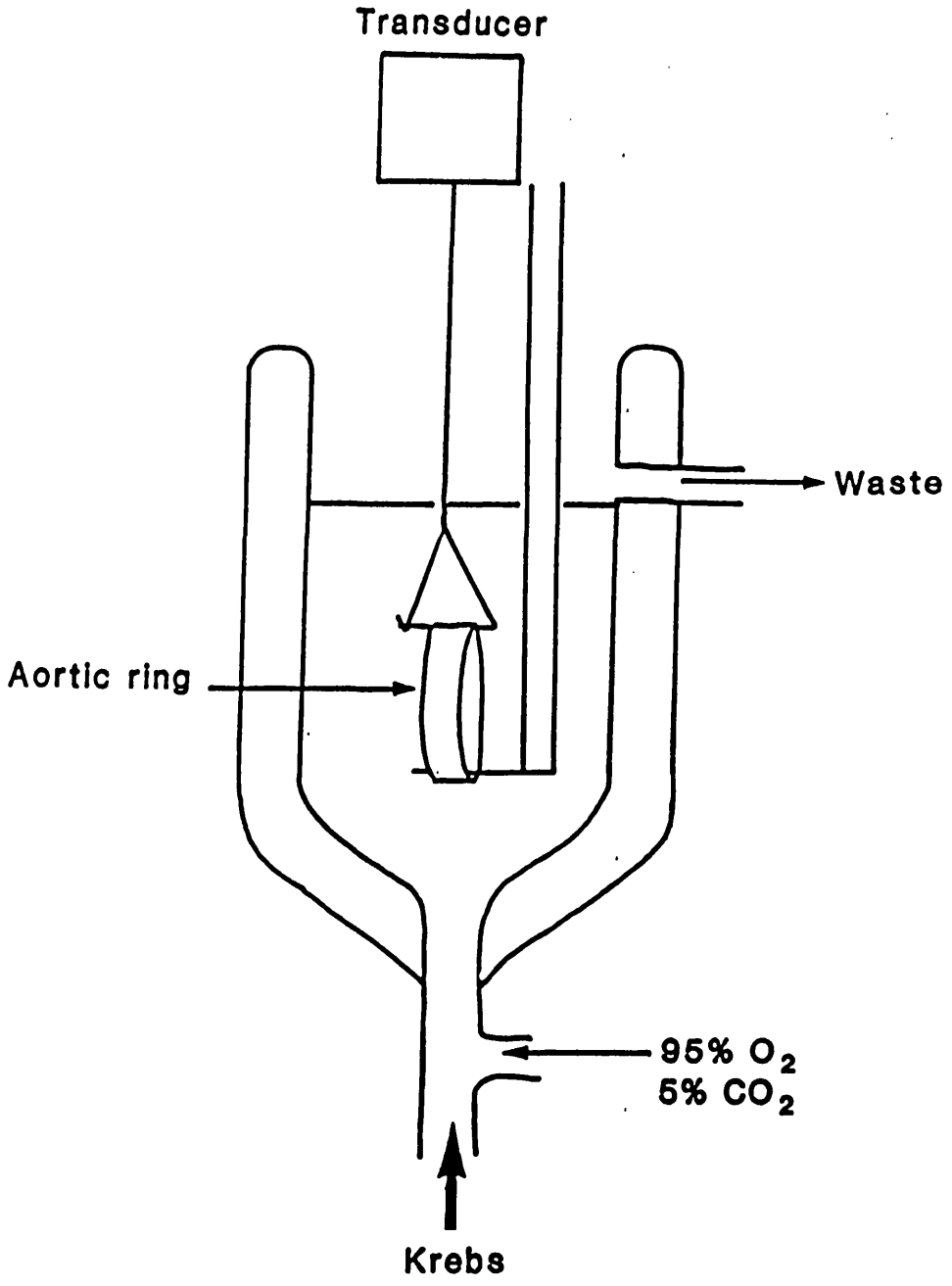


FIGURE 6

Organ bath apparatus for isometric tension recording studies with isolated rabbit aortic rings.

Aortic rings suspended between two hooks, one connected to a transducer, the other fixed in position. The bath contained Krebs' buffer maintained at 37°C and gased with 95% O_2/5% CO_2.

2.4.3 ORGAN BATH STUDIES

Tissues were contracted with either phenylephrine (0.1 - 0.3 μM), $PGF_{2\alpha}$ (5 - 8 μM) or KCl (30 mM). Once a stable plateau had been reached the tissues were relaxed with cumulative concentrations of ACh, or the endothelium-independent relaxants GTN, NO or SNP. The tissues were then washed and allowed to equilibrate for 30 mins before the addition of lipoprotein or fatty acid product. An equivalent volume of Tris buffer or appropriate vehicle was added to some tissues in each experiment as controls. The contraction-relaxation cycle was then repeated in the presence of the lipoprotein either immediately after the lipoprotein was added to the tissue or following a 30 min preincubation period. The lipoprotein was then washed out and the tissues equilibrated for a further 30 mins before a third relaxation curve was carried out to examine the reversibility of any effects.

The tissues were contracted to the same level of tone for each contraction-relaxation cycle and all drug additions were made to bath volumes of 2 ml for aortic ring experiments and 12 ml for coronary artery experiments.

2.4.4 EXPRESSION OF RESULTS

Results are expressed as % relaxation of induced tone or % inhibition of the maximal relaxation, *i.e.* the relaxation in the presence of the lipoprotein as a percentage of the relaxation prior to the addition of the lipoprotein:

$$\text{\% inhibition of maximal relaxation} = \frac{\text{\% relaxation before exposure to LDL} - \text{\% relaxation during exposure to LDL}}{\text{\% relaxation before exposure to LDL}} \times 100\%$$

Data is expressed as mean ± standard error of the mean (SEM) for n separate experiments. The concentration of an agonist causing half maximal relaxation (EC_{50}) was calculated for each experiment. Statistical evaluation of the data was performed by Student's t -test for unpaired samples where $p < 0.05$ was considered significant.

The inhibition of relaxations obtained with lipoprotein preparations from different donors was tested for significance by two-way analysis of variance (ANOVA) with the donor as the independent variable.

CHAPTER 3

THE EFFECTS OF NATIVE AND OXIDISED LDL ON VASCULAR RELAXATION OF CORONARY VESSELS.

3.1 INTRODUCTION

Many studies have now demonstrated that endothelium-dependent relaxations of large conduit arteries such as the rabbit aorta are impaired in the presence of OXLDL (see section 1.6.4). However, little is known about the effects of lipoproteins on endothelial function of coronary resistance vessels which do not exhibit overt signs of atherosclerosis but are nevertheless exposed to high levels of circulating lipids.

The aim of this study was, therefore, to investigate the effects of native and OXLDL on endothelium-dependent and independent relaxations of both large and small isolated rabbit coronary arteries.

3.2 LDL AND VASCULAR TONE

No change in basal tone was observed when large or small coronary vessels with an intact endothelium were exposed to either native or OXLDL (1 mg protein/ml) for up to 30 mins. This is illustrated in **Figure 7** which shows the addition of OXLDL to basal tone in a small coronary vessel.

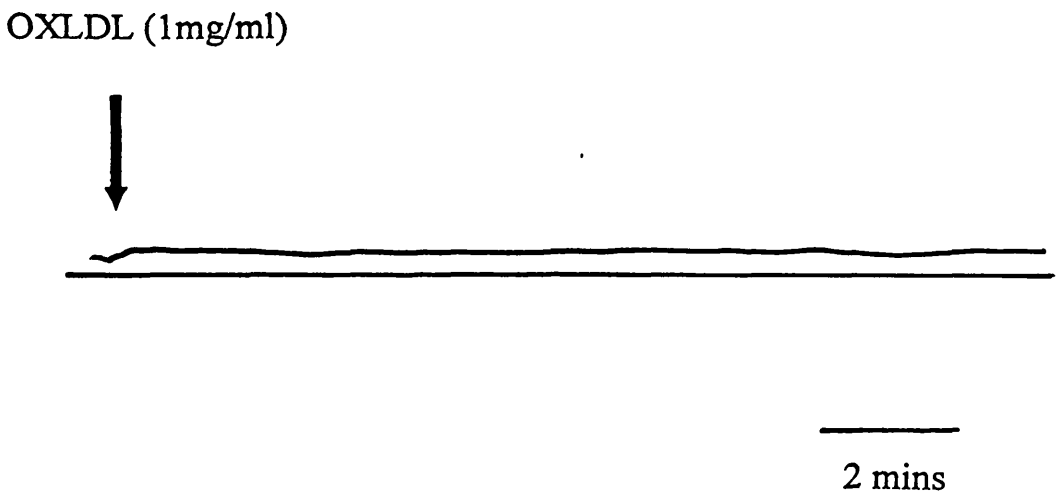


FIGURE 7

The addition of OXLDL to resting tone.

The addition of OXLDL (1 mg protein/ml) to the basal tone of a small coronary artery.

3.3 THE EFFECT OF NATIVE LDL ON ACh-EVOKED RELAXATIONS

$PGF_{2\alpha}$ evoked a sustained contraction in both large and small coronary arteries. The vessels were preincubated for 15 mins with indomethacin (10 μM) prior to the addition of $PGF_{2\alpha}$ as this was found to lead to the production of more reproducible contractions. Under these conditions 38 out of 46 large vessels and 25 out of 46 small vessels contracted in response to $PGF_{2\alpha}$. ACh (1 nM - 3μM) evoked concentration-dependent relaxations in isolated coronary arteries precontracted with $PGF_{2\alpha}$ (5 - 8 μM). The maximal relaxation to ACh (3 μM) was 94.9 ± 1.6% (n=23) in large vessels and 95.7 ± 2.1% (n=16) in small vessels. The EC_{50} values for ACh were 48.6 ± 9.1 nM (n=23) and 89.5 ± 10.5 nM (n=16) in large and small vessels, respectively. Relaxations of both large and small control tissues were unchanged throughout the experiment as shown in **Figure 8**.

Preincubation with native LDL (1 mg protein/ml) for 30 mins had no effect on ACh-evoked relaxations of either large or small coronary arteries precontracted with $PGF_{2\alpha}$. The EC_{50} values for ACh before treatment with native LDL were 64.8 ± 8.6 nM (n=4) and 124.2 ± 12.7 nM (n=3) and following incubation with LDL were 72.4 ± 9.5 nM (n=4; p>0.05) and 115.2 ± 10.9 nM (n=3; p>0.05) in large and small vessels, respectively. **Figure 9** shows concentration-response curves for ACh-evoked relaxations of large and small coronary arteries in the presence and absence of native LDL.

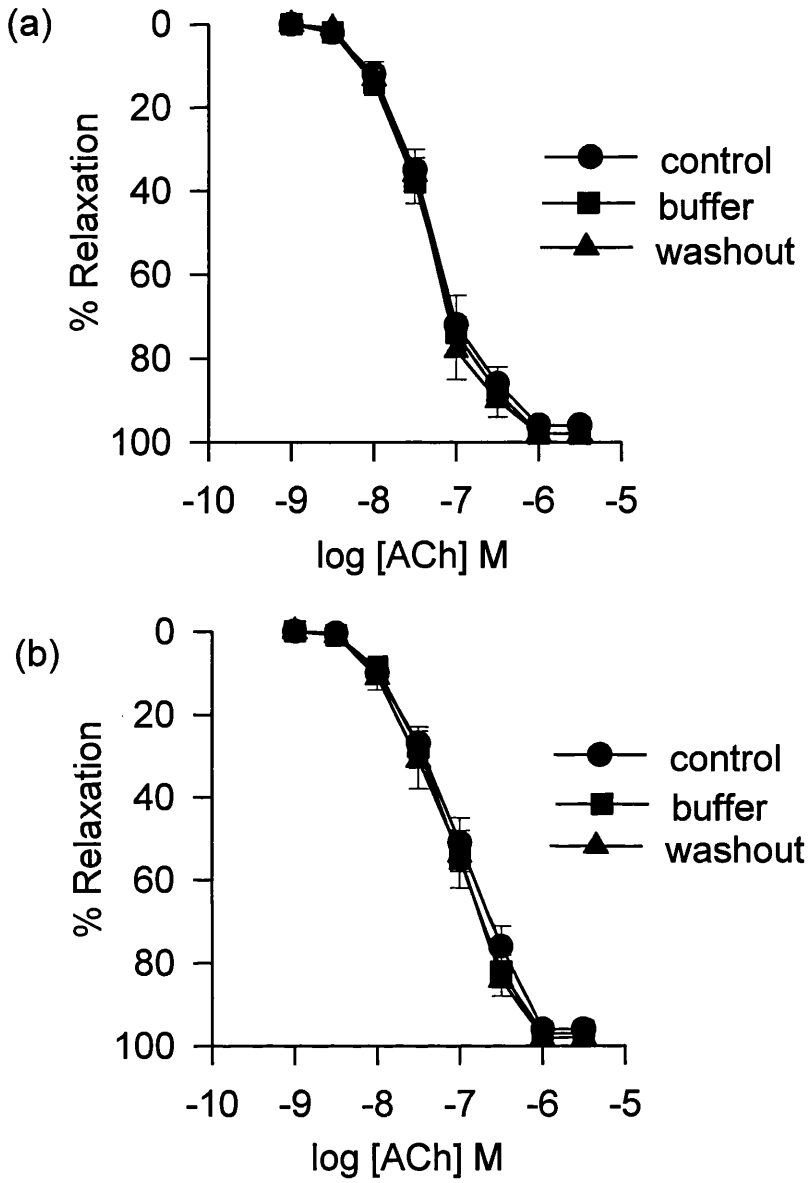


FIGURE 8

The effect of vehicle preincubation on ACh-evoked relaxations of coronary arteries precontracted with $PGF_{2\alpha}$.

Tissues were precontracted with $PGF_{2\alpha}$ (5 - 8 μM) in the presence of indomethacin (10 μM) and relaxed with cumulative concentrations of ACh (1 nM - 3 μM). Following washout, tissues were exposed to Tris buffer for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out.

(a) Large coronary arteries (n=6)

(b) Small coronary arteries (n=4)

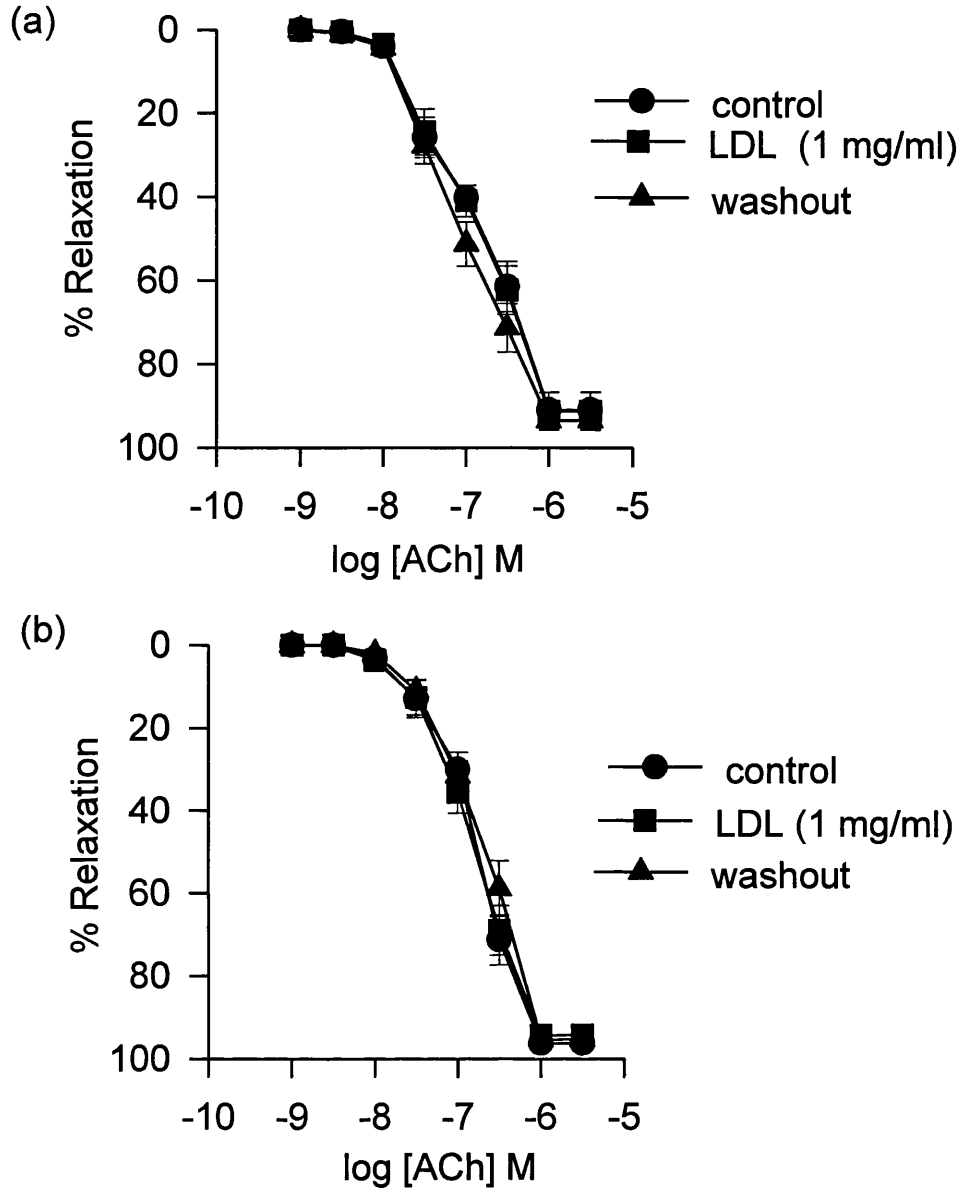


FIGURE 9

The influence of Native LDL on ACh-evoked relaxations.

Coronary arteries were precontracted with $PGF_{2\alpha}$ (5 - 8 μM) in the presence of indomethacin (10 μM) and relaxed with cumulative concentrations of ACh (1 nM - 3μM). Following washout, tissues were exposed to native LDL (1 mg protein/ml) and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out.

(a) Large coronary arteries (n=4)

(b) Small coronary arteries (n=3)

3.4 THE EFFECT OF OXLDL ON ACh-EVOKED RELAXATIONS

3.4.1 OXLDL IN $PGF_{2\alpha}$ PRECONTRACTED VESSELS

Following a 30 min preincubation period with OXLDL (0.5 mg protein/ml) endothelium-dependent relaxations of both large and small coronary arteries precontracted with $PGF_{2\alpha}$ were attenuated as shown in **Figure 10b**. The EC_{50} values for ACh were significantly increased from 50.4 ± 10.2 nM (n=8) to 122.7 ± 14.1 nM (n=8; p<0.05) in large vessels and from 91.0 ± 11.5 nM (n=4) to 200.9 ± 29.3 nM (n=4; p<0.05) in small vessels. This is reflected by a significant rightward shift in the concentration-response curves for ACh in the presence of the lipoprotein as shown in **Figure 11**. OXLDL had no effect on the maximal relaxation to ACh which was 96.1 ± 1.0% (n=8) before OXLDL treatment and 94.3 ± 1.8% (n=8; p>0.05) during exposure to OXLDL in large vessels. Similarly, the maximal relaxation to ACh was unaltered by OXLDL treatment in small vessels: 89.8 ± 5.2% (n=4) in control tissues and 88.5 ± 3.8% (n=4; p>0.05) following treatment with OXLDL. The sensitivity to ACh was restored on washout of the lipoproteins in both large and small vessels.

Incubation of the tissues with 1 mg protein/ml OXLDL did not significantly further attenuate ACh-evoked relaxations. Following exposure to OXLDL (1 mg protein/ml) for 30 mins the EC_{50} values for ACh increased from 52.4 ± 8.5 nM to 151.3 ± 9.2 nM (n=5; p<0.05) in large vessels and from 94.7 ± 6.7 nM to 229.4 ± 9.6 nM (n=4; p<0.05) in small vessels. **Figure 12** shows the effect of 1 mg protein/ml OXLDL on ACh-evoked relaxations in small coronary arteries. The degree of inhibition of endothelium-dependent relaxations did not significantly differ between OXLDL samples prepared from the plasma of 6 different donors (ANOVA).

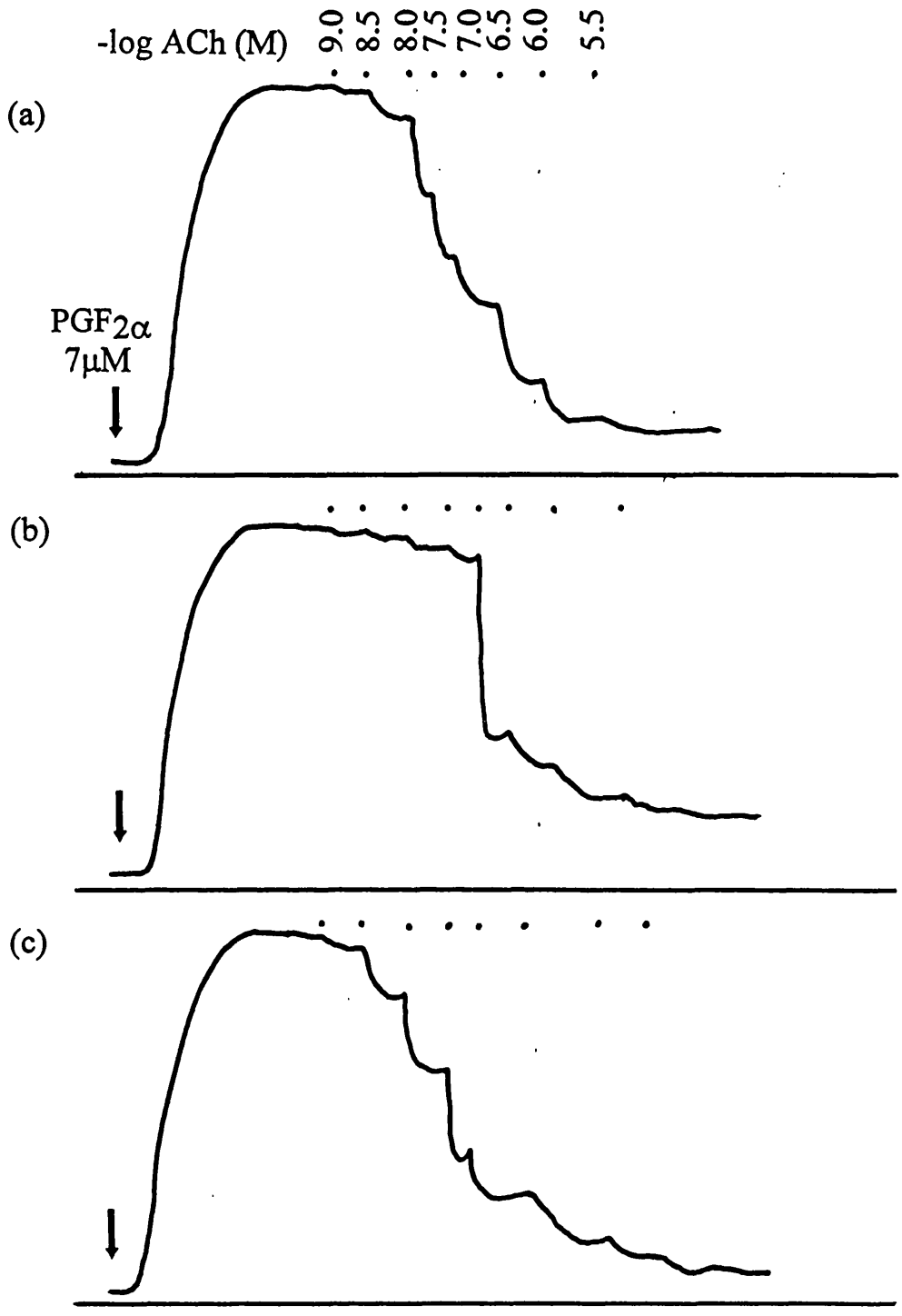


FIGURE 10

ACh-evoked relaxations of small coronary arteries in the absence and presence of OXLDL.

Coronary arteries were precontracted with $PGF_{2\alpha}$ (7μM) and relaxed with cumulative concentrations of ACh (1nM - 3μM).

(a) Control
(b) Following exposure to OXLDL (0.5 mg protein/ml)
(c) After washout

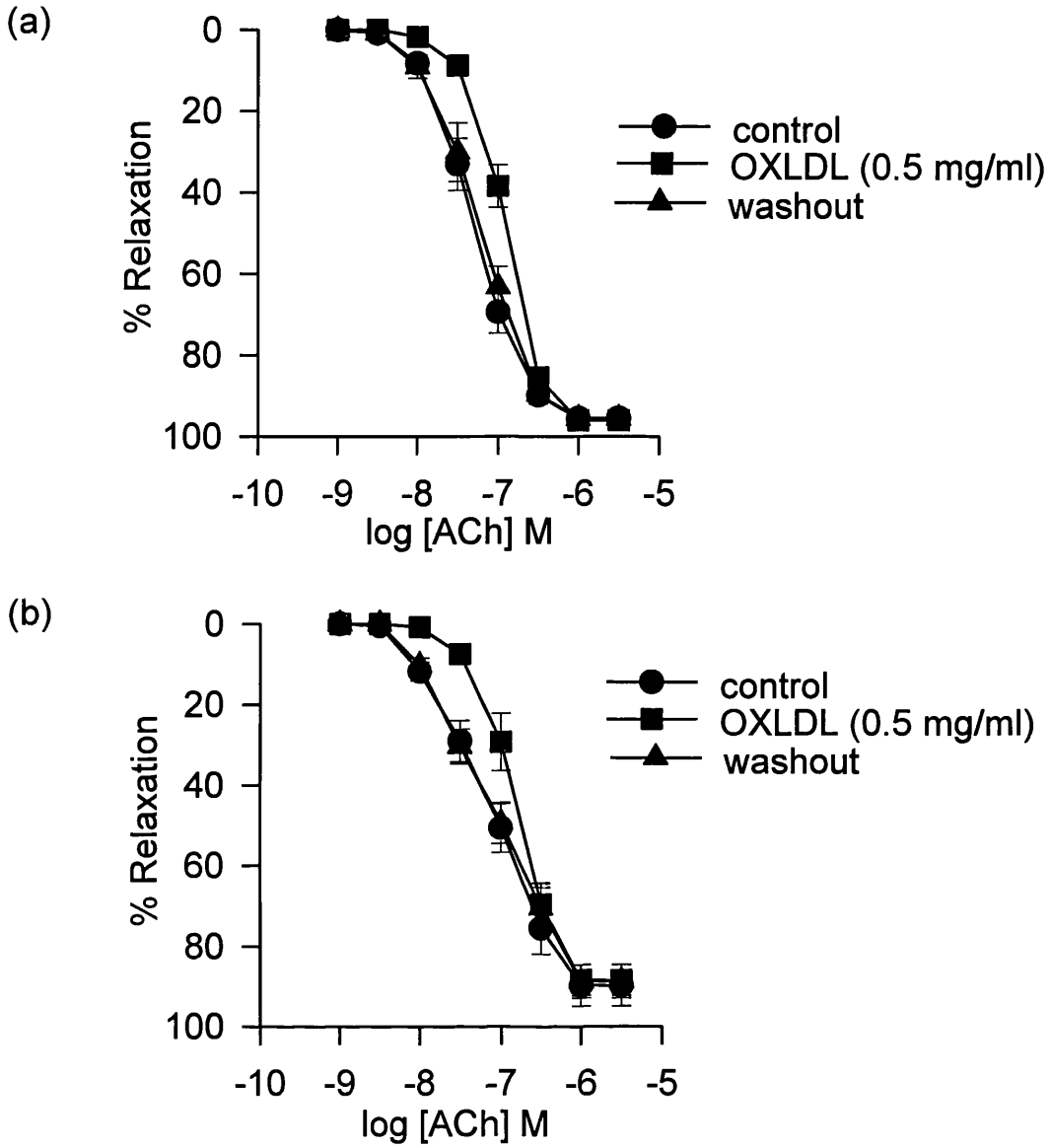


FIGURE 11

The effect of OXLDL on ACh-evoked relaxations of large and small coronary arteries.

Coronary arteries were precontracted with $PGF_{2\alpha}$ (5 - 8 μM) in the presence of indomethacin (10 μM) and relaxed with cumulative concentrations of ACh (1 nM - 3μM). Following washout, tissues were incubated with OXLDL (0.5 mg protein/ml) for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out.

(a) Large coronary arteries (n=8)

(b) Small coronary arteries (n=4)

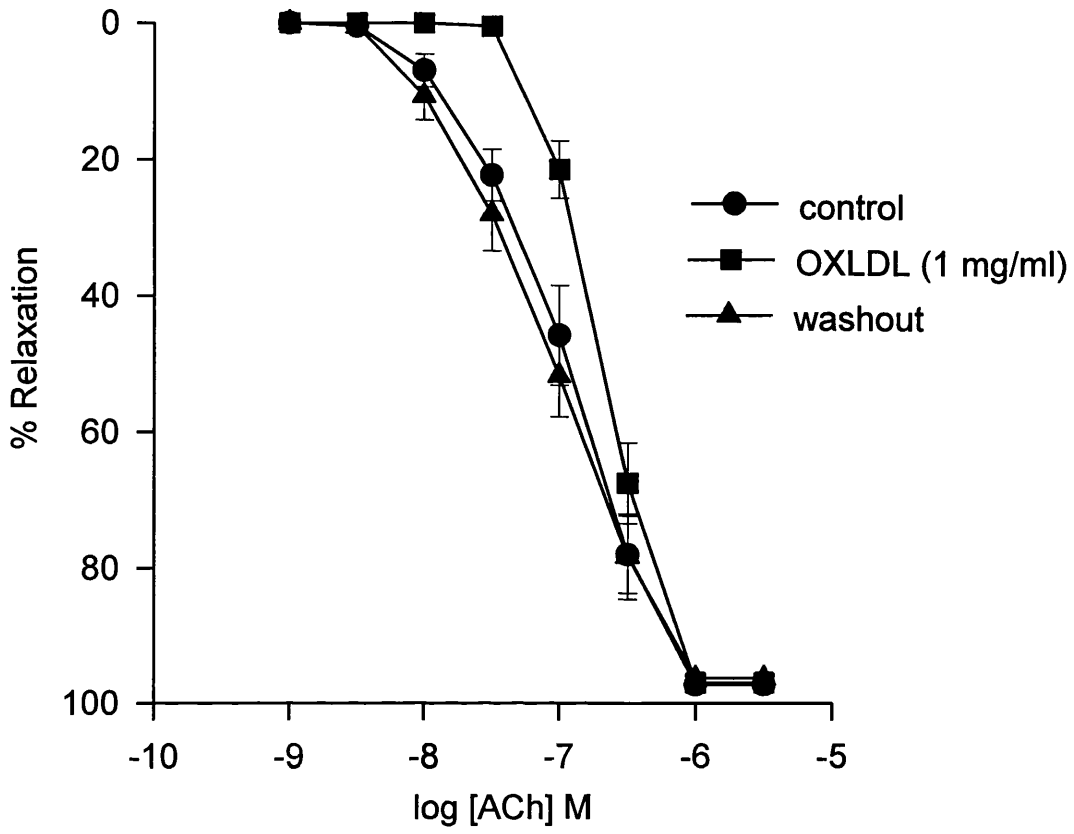


FIGURE 12

ACh-evoked relaxations of small coronary arteries in the absence and presence of OXLDL.

Small coronary arteries were precontracted with $PGF_{2\alpha}$ (5 - 8 μM) in the presence of indomethacin (10 μM) and relaxed with cumulative concentrations of ACh (1 nM - 3μM). Following washout, tissues were preincubated with OXLDL (1 mg protein/ml) for 30 mins and the contraction/relaxation cycle repeated. After a further washout a third relaxation curve to ACh was carried out (n=4).

In some experiments, large coronary arteries and aortic rings from the same rabbit were mounted in organ baths, as described in section 2.4.2, to directly compare the effect of OXLDL, prepared from the plasma of the same donor, on endothelium-dependent relaxations. **Figure 13a** shows concentration-response curves for ACh in aortic rings in the presence and absence of OXLDL (1 mg protein/ml). The degree to which ACh responses were inhibited in the aorta was not significantly different from that observed in coronary arteries, with a similar increase in the EC_{50} value for ACh from 38.8 ± 5.2 nM (n=4) prior to OXLDL treatment to 139.6 ± 12.7 nM (n=4; p<0.05) in the presence of OXLDL. Preincubation of OXLDL (1 mg protein/ml) with large coronary arteries mounted in organ baths caused a decrease in sensitivity to ACh which was similar to that observed when the vessels were mounted in a myograph, with an increase in the EC_{50} value from 47.4 ± 10.3 nM (n=4) before incubation with OXLDL to 152.2 ± 11.4 nM (n=4; p<0.05) in the presence of OXLDL. **Figure 13b** shows concentration-response curves for ACh-evoked relaxations of large coronary arteries in the presence and absence of OXLDL.

3.4.2 OXLDL IN KCl PRECONTRACTED VESSELS

In both large and small coronary arteries, KCl (30 mM) induced contractions which were not significantly different to those induced by $PGF_{2\alpha}$ (5 - 8 μM). The level of contraction evoked by KCl was 8.37 ± 0.81 mN/mm (n=11) in large vessels and 2.38 ± 0.33 mN/mm (n=13) in small vessels. This compares to $PGF_{2\alpha}$ induced contractions of 8.63 ± 0.40 mN/mm (n=32) and 2.77 ± 0.24 mN/mm (n=19) in large and small vessels, respectively. ACh (1 nM - 1 μM) evoked endothelium-dependent relaxations of KCl contracted tissues but the maximal relaxations

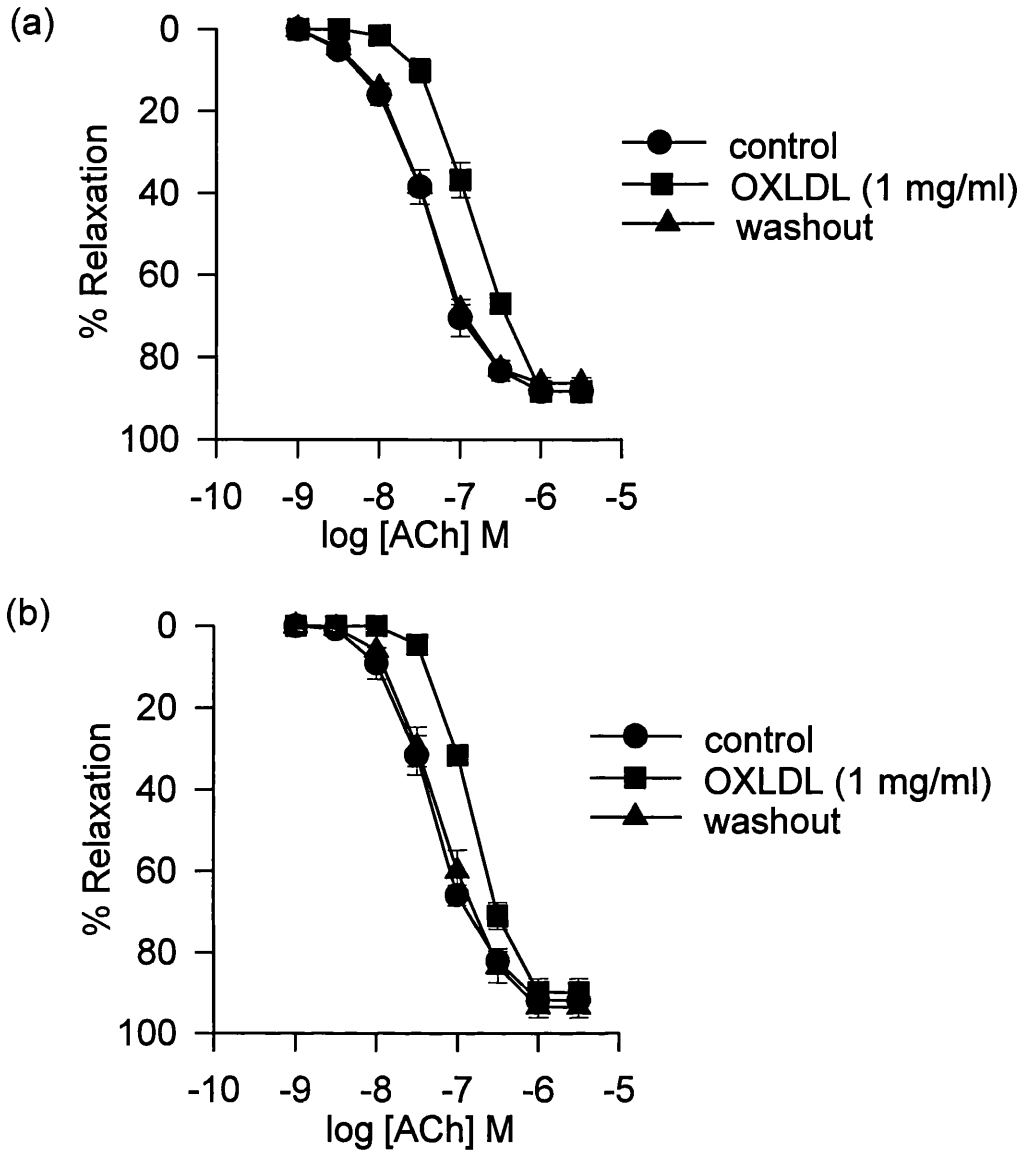


FIGURE 13

The influence of OXLDL on ACh-evoked relaxations of large coronary arteries and aortic rings.

Tissues were precontracted with $PGF_{2\alpha}$ (5 - 8 μM) in the presence of indomethacin (10μM) and relaxed with cumulative concentrations of ACh (1 nM -3 μM). Following washout, tissues were exposed to OXLDL (1 mg protein/ml) and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out.

(a) Aortic rings (n=4)

(b) Large coronary arteries (n=4)

obtained were depressed compared to the maximum response in the presence of $PGF_{2\alpha}$. The maximal relaxation to ACh (1 μM) in KCl contracted tissues was 48.4 ± 10.8% (n=5) in large vessels and 54.3 ± 9.1% (n=5) in small vessels. However, the EC_{50} values for ACh were not significantly different to those obtained in $PGF_{2\alpha}$ precontracted vessels: 48.5 ± 7.2 nM (n=5) in large vessels and 96.8 ± 7.4 mM (n=5) in small vessels. At concentrations of ACh above 1 μM a marked vasoconstrictor response was seen. **Figure 14** shows concentration-response curves for ACh-evoked relaxations in tissues precontracted with $PGF_{2\alpha}$ and KCl. Concentration-response curves to ACh in control tissues were unchanged throughout the experiment as shown in **Figure 15**.

Following a 30 min preincubation period with OXLDL (0.5 mg protein/ml), relaxations to ACh were attenuated in both large and small coronary vessels precontracted with KCl as shown in **Figure 16b**. This is demonstrated by a significant rightward shift in the concentration-response curve for ACh (**Figure 17**). The EC_{50} values for ACh increased from 48.4 ± 7.1 nM (n=4) and 96.5 ± 7.1 nM (n=5) to 94.2 ± 8.3 nM (n=4) and 180.9 ± 7.6 nM (n=5) in large and small vessels, respectively (p<0.05). In contrast to the effect observed in $PGF_{2\alpha}$ precontracted tissues, there was also a significant decrease in the maximal level of relaxation in the presence of OXLDL. The maximal relaxation was reduced from 56.2 ± 11.4% (n=4) and 47.4 ± 10.3% (n=5) before incubation with OXLDL to 42.1 ± 7.3% (n=4) and 33.2 ± 6.5% (n=5) during exposure to OXLDL in large and small vessels, respectively (p<0.05).

In the presence of an increased lipoprotein concentration (1 mg protein/ml) a further decrease in sensitivity to ACh was observed as shown in **Figure 18.** The EC_{50} values for ACh increased from

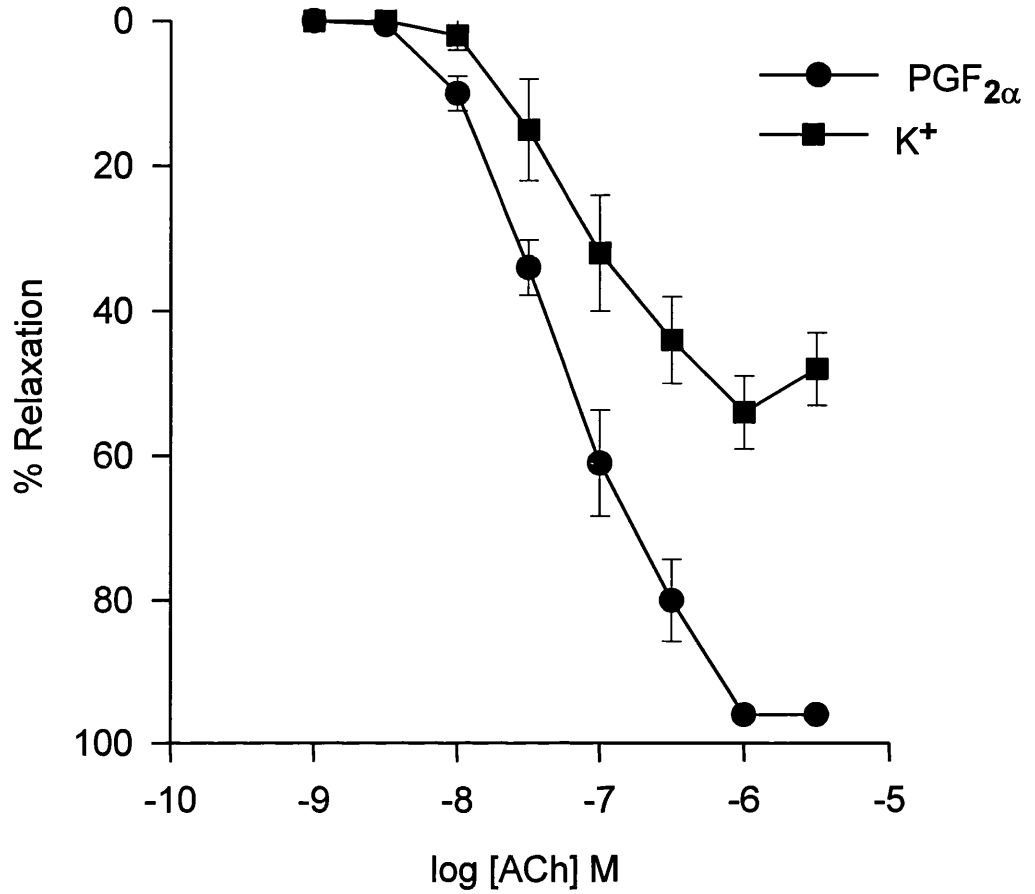


FIGURE 14

ACh-evoked relaxations of small coronary arteries precontracted with $PGF_{2\alpha}$ and K^+.

Small coronary arteries were precontracted with either $PGF_{2\alpha}$ (5 - 8 μM) or KCl (30 mM) and relaxed with cumulative concentrations of ACh (1 nM - 3 μM).

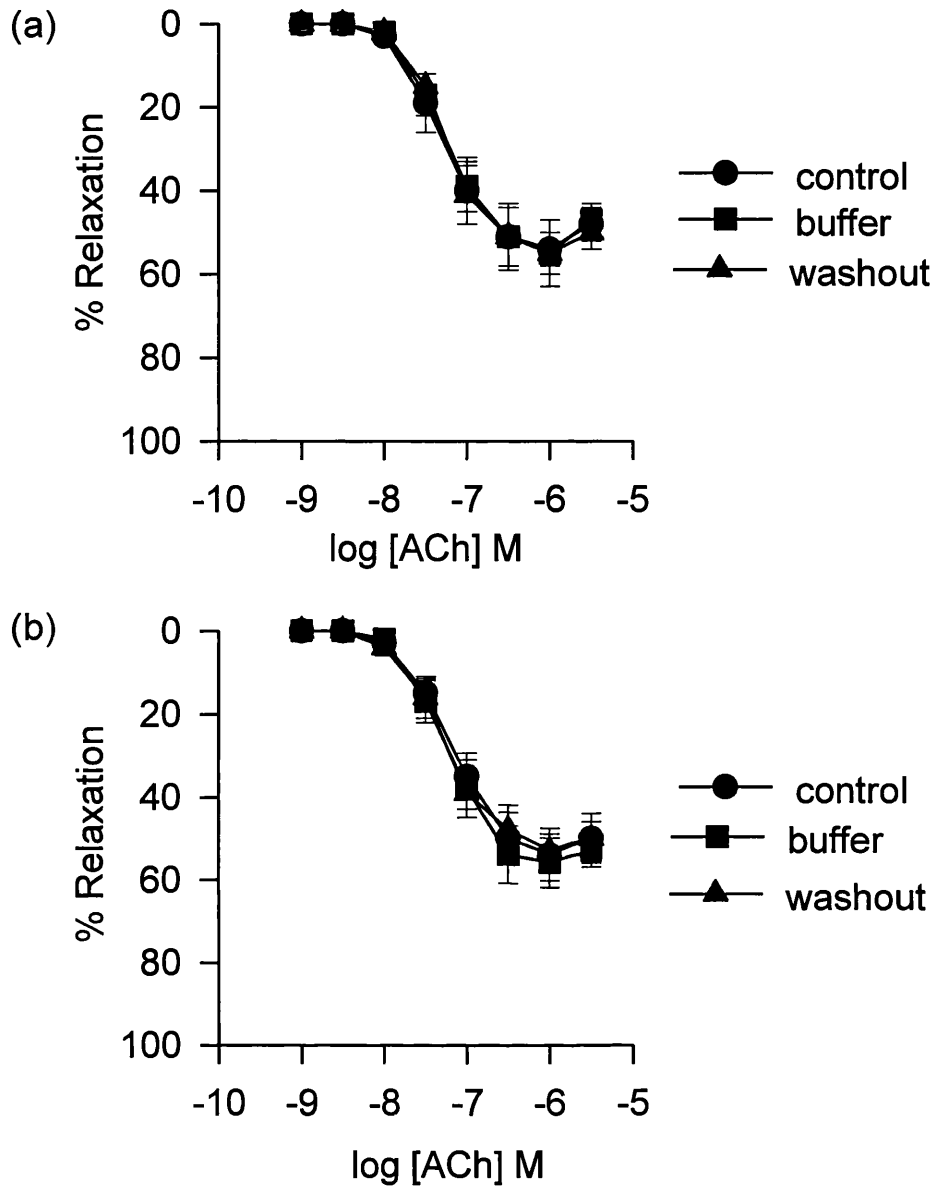


FIGURE 15

The effects of vehicle preincubation on ACh-evoked relaxations of coronary arteries precontracted with K^+.

Tissues were precontracted with KCl (30 mM) and relaxed with cumulative concentrations of ACh (1 nM - 3 μM). Following washout, tissues were exposed to Tris buffer for 30 mins and the contraction/relaxation cycle repeated. After further washout, a third relaxation curve to ACh was carried out.

(a) Large coronary arteries (n=5)

(b) Small coronary arteries (n=4)

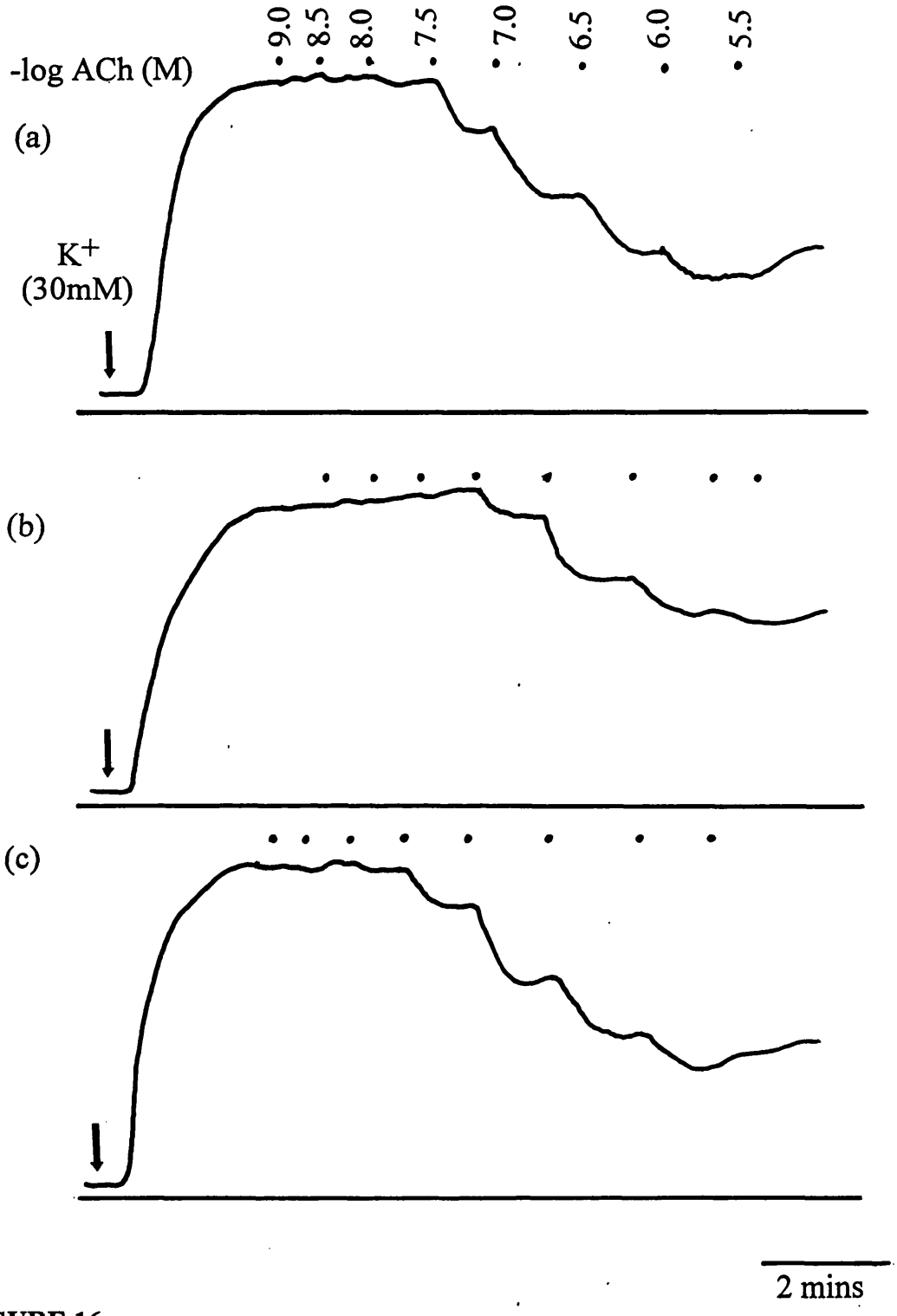


FIGURE 16

ACh-evoked relaxations of small coronary arteries in the absence and presence of OXLDL.

Coronary arteries were precontracted with KCl (30mM) and relaxed with cumulative concentrations of ACh (1nM - 3μM).

(a) Control

(b) Following exposure to OXLDL (0.5 mg protein/ml)

(c) After washout

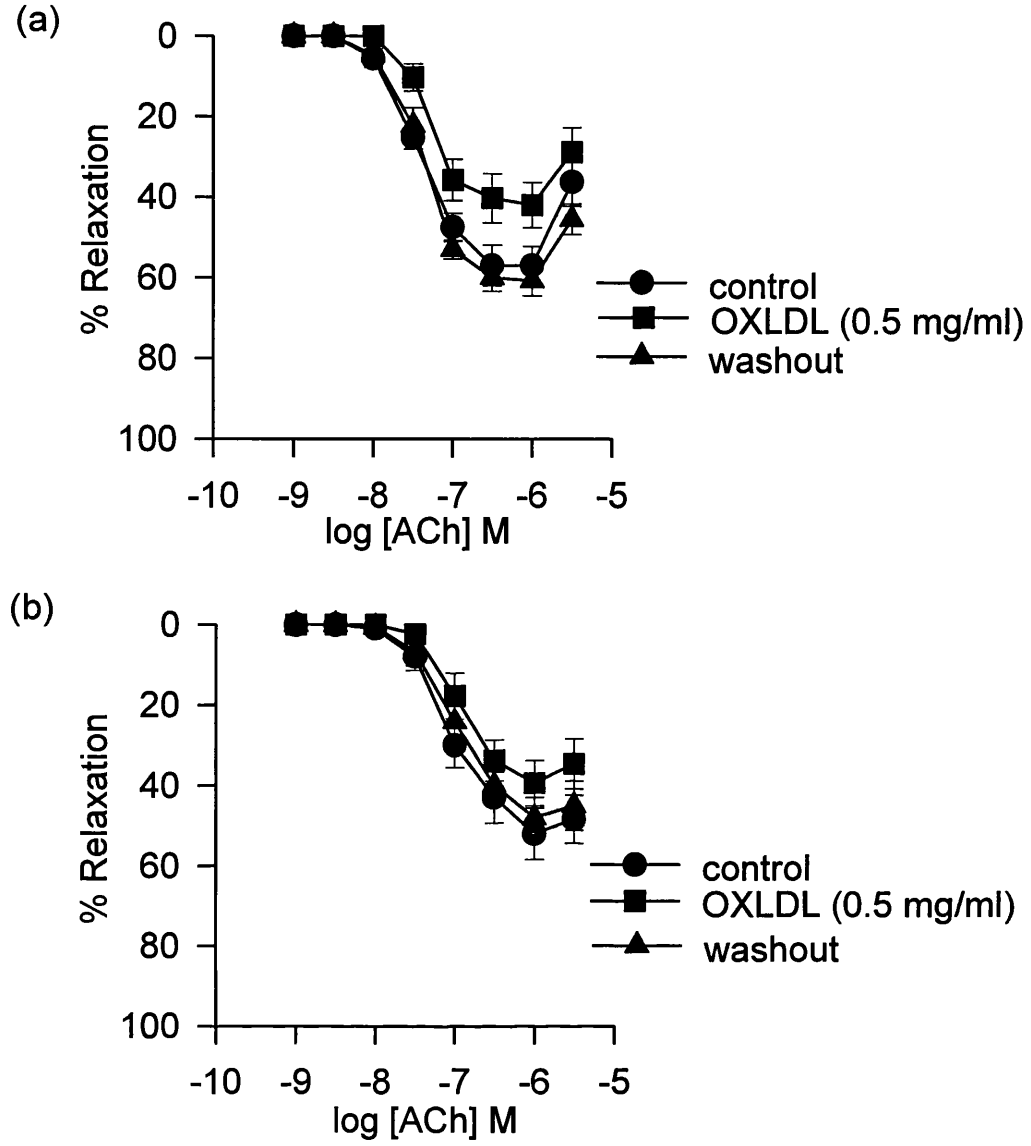


FIGURE 17

The influence of OXLDL on ACh-evoked relaxations of large and small coronary arteries.

Coronary arteries were precontracted with KCl (30 mM) and relaxed with cumulative concentrations of ACh (1 nM - 3 μM). Following washout, tissues were preincubated with OXLDL (0.5 mg protein/ml) for 30 mins and the contraction/relaxation cycle was repeated. After further washout a third relaxation curve was carried out.

(a) Large coronary arteries (n=4)

(b) Small coronary arteries (n=5)

51.0 ± 6.2 nM (n=4) before OXLDL treatment to 140.8 ± 7.6 nM (n=4; p<0.05) in the presence of OXLDL in large vessels and from 92.6 ± 8.5 nM (n=4) to 269.4 ± 6.9 nM (n=4; p<0.05) in small vessels. The maximal relaxation evoked by ACh was also further reduced to 32.4 ± 8.3% (n=4) and 19.2 ± 7.8% (n=4) in large and small coronary vessels, respectively (p<0.05). After removal of the lipoproteins, relaxations to ACh were restored to control levels in all cases. **Figure 19** shows the effect of 1 mg protein/ml OXLDL on ACh-evoked relaxations in small coronary arteries precontracted with KCl. The degree of inhibition of endothelium-dependent relaxations evoked by ACh did not significantly differ between OXLDL samples prepared from the plasma of 4 different donors (ANOVA).

In some experiments, large coronary arteries were mounted in individual organ baths, as described in section 2.4.2, with aortic rings from the same rabbit to directly compare the effects of OXLDL, prepared from plasma from the same donor, on endothelium-dependent responses of the two tissues precontracted with KCl. Following incubation with OXLDL (1 mg protein/ml), ACh evoked relaxations of isolated aortic rings were inhibited to a similar extent as in coronary arteries. **Figure 20a** shows concentration-response curves for ACh-evoked relaxations in aortic rings precontracted with KCl. The EC_{50} values for ACh in the aorta increased from 43.6 ± 5.4 nM (n=6) before treatment with OXLDL to 121.8 ± 14.4 nM (n=6; p<0.05) during exposure to OXLDL. The maximal level of relaxation was reduced from 73.7 ± 4.2% (n=6) to 48.5 ± 7.4% (n=6; p<0.05). Preincubation of OXLDL with large coronary arteries mounted in organ baths caused a similar degree of inhibition to that observed when the vessels were mounted in a myograph as shown in **Figure 20b**.

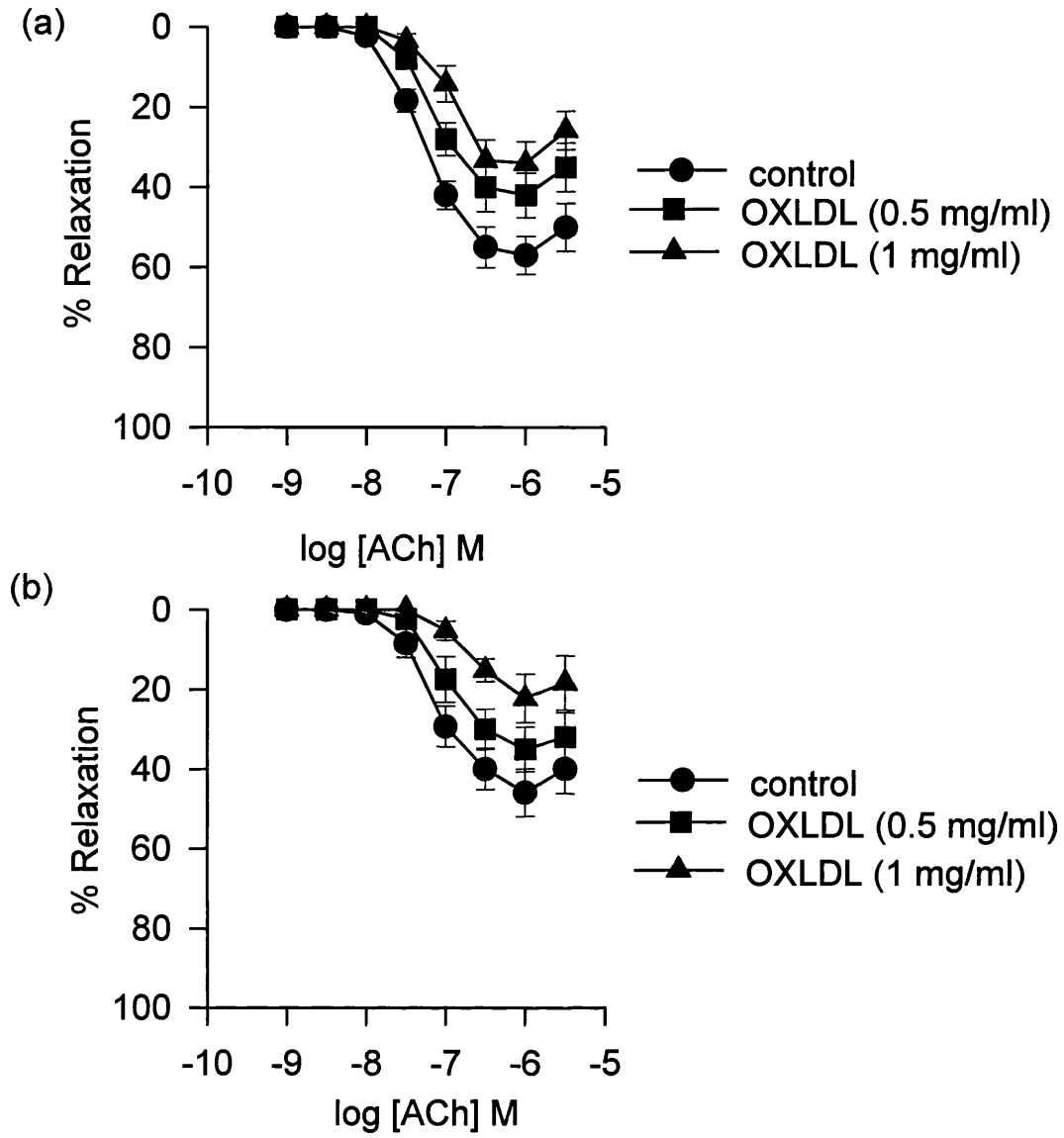


FIGURE 18

The influence of 0.5 mg/ml and 1 mg/ml OXLDL on ACh-evoked relaxations.

Coronary arteries were precontracted with KCl (30 mM) and relaxed with cumulative concentrations of ACh (1 nM - 3 μM). Following washout, tissues were exposed to OXLDL (0.5 or 1.0 mg protein/ml) for 30 mins and the contraction/relaxation cycle repeated.

(a) Large coronary arteries (*n*=4)

(b) Small coronary arteries (*n*=4)

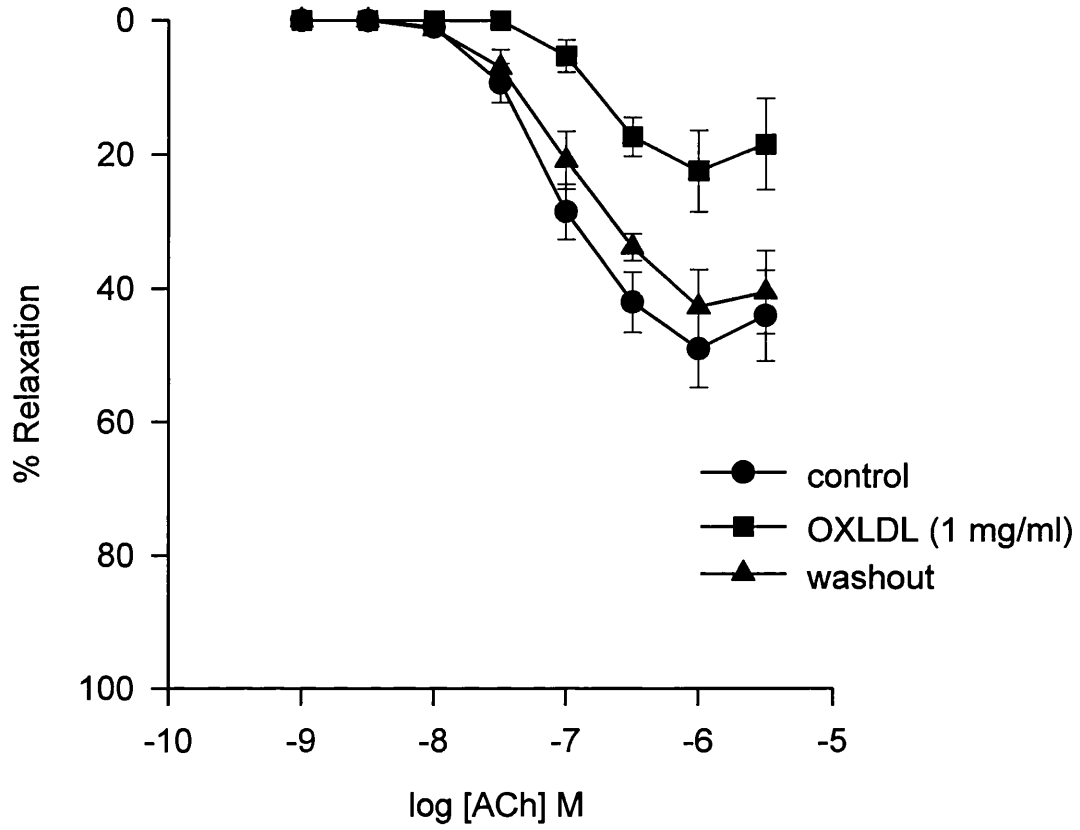


FIGURE 19

The effect of OXLDL on ACh-evoked relaxations of small coronary arteries.

Tissues were precontracted with KCl (30 mM) and relaxed with cumulative concentrations of ACh (1 nM - 3 μM). Following washout, tissues were exposed to OXLDL (1 mg protein/ml) for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out.

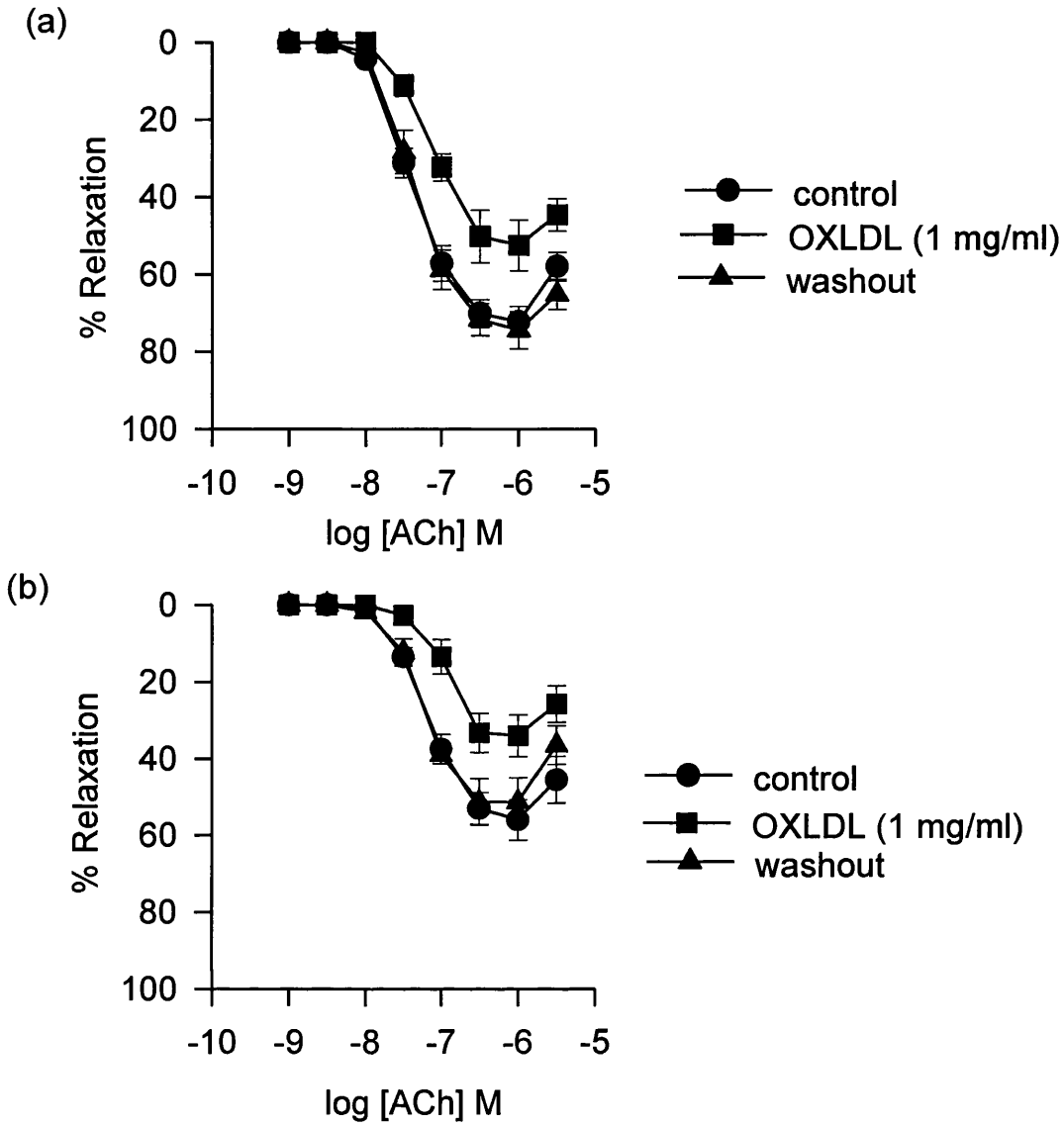


FIGURE 20

The effect of OXLDL on ACh-evoked relaxations of large coronary arteries and aortic rings.

Tissues were precontracted with KCl (30 mM) and relaxed with cumulative concentrations of ACh (1 nM - 3 μM). Following washout, tissues were exposed to OXLDL (1 mg protein/ml) and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out.

(a) Aortic rings (n=4)

(b) Large coronary arteries (n=4)

3.5 THE EFFECT OF OXLDL ON SNP-EVOKED RELAXATIONS

The nitrovasodilator SNP evokes endothelium-independent relaxations by the direct activation of the enzyme soluble guanylate cyclase to increase cGMP levels within the vascular smooth muscle cells. SNP spontaneously releases NO which is thought to be the final active species that activates the soluble guanylate cyclase. In order to determine whether the inhibitory effects of OXLDL could be due to an action on the soluble guanylate cyclase, the effects of OXLDL on relaxations evoked by SNP were assessed in both large and small coronary arteries.

SNP (10 nM - 0.1 mM) evoked concentration-dependent relaxations of both small and large coronary vessels precontracted with $PGF_{2\alpha}$ (**Figure 21**). The EC_{50} values for SNP-evoked relaxations in large and small vessels were 1.68 ± 0.37 μM (*n*=4) and 2.18 ± 0.62 μM (*n*=4) respectively. The maximal relaxation to SNP was 83.3 ± 2.6% (*n*=4) and 78.2 ± 4.5% (*n*=4) in large and small vessels, respectively.

Following incubation with OXLDL (1 mg protein/ml) for 30 mins, there was no change in the sensitivity to SNP of either large or small vessels precontracted with $PGF_{2\alpha}$. **Figure 22** shows concentration-response curves to SNP in the presence and absence of OXLDL (1 mg protein/ml). In the presence of OXLDL the EC_{50} value for SNP was 2.29 ± 0.64 μM (*n*=6) and the maximal relaxation 86.2 ± 2.3 % (*n*=6) compared with an EC_{50} value of 2.03 ± 0.48 μM (*n*=6) and a maximal relaxation of 87.4 ± 3.1 % (*n*=6) in the absence of OXLDL.

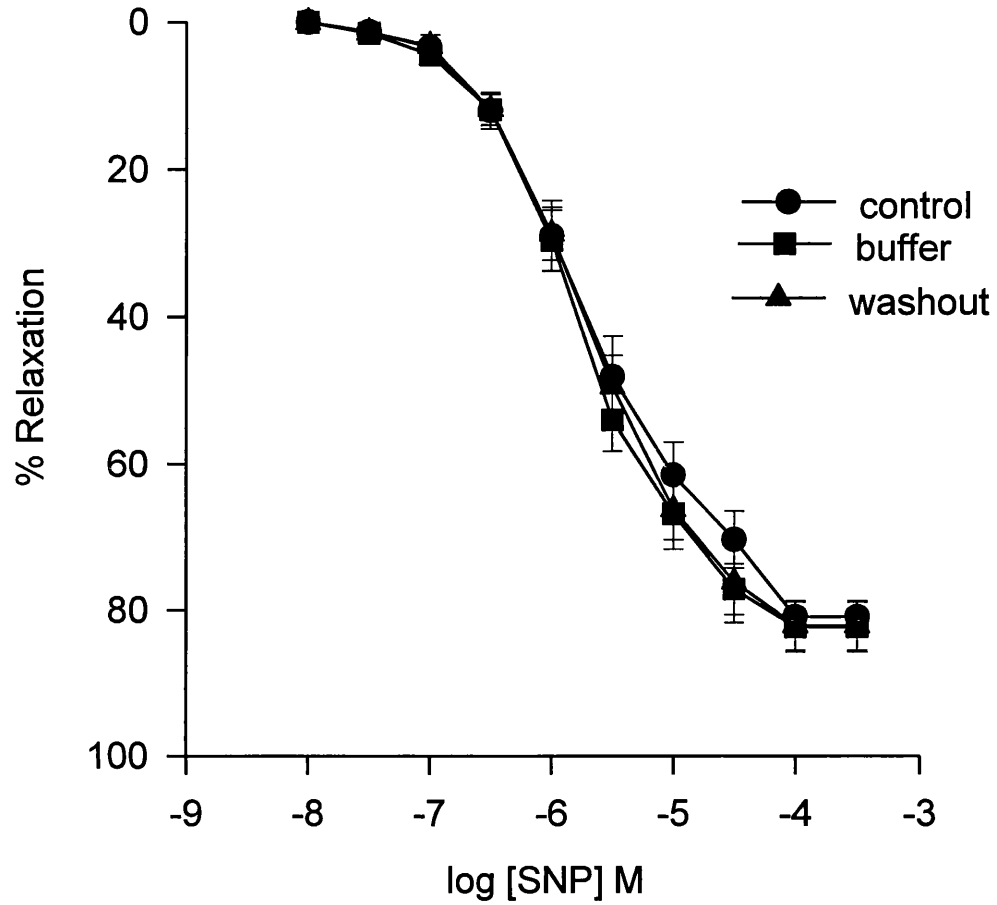


FIGURE 21

The influence of vehicle preincubation on SNP-evoked relaxations.

Coronary arteries were precontracted with $PGF_{2\alpha}$ (5 - 8 μM) in the presence of indomethacin (10 μM) and relaxed with cumulative concentrations of SNP (10 nM - 30 μM). Following washout, tissues were exposed to Tris buffer for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to SNP was carried out (n=6).

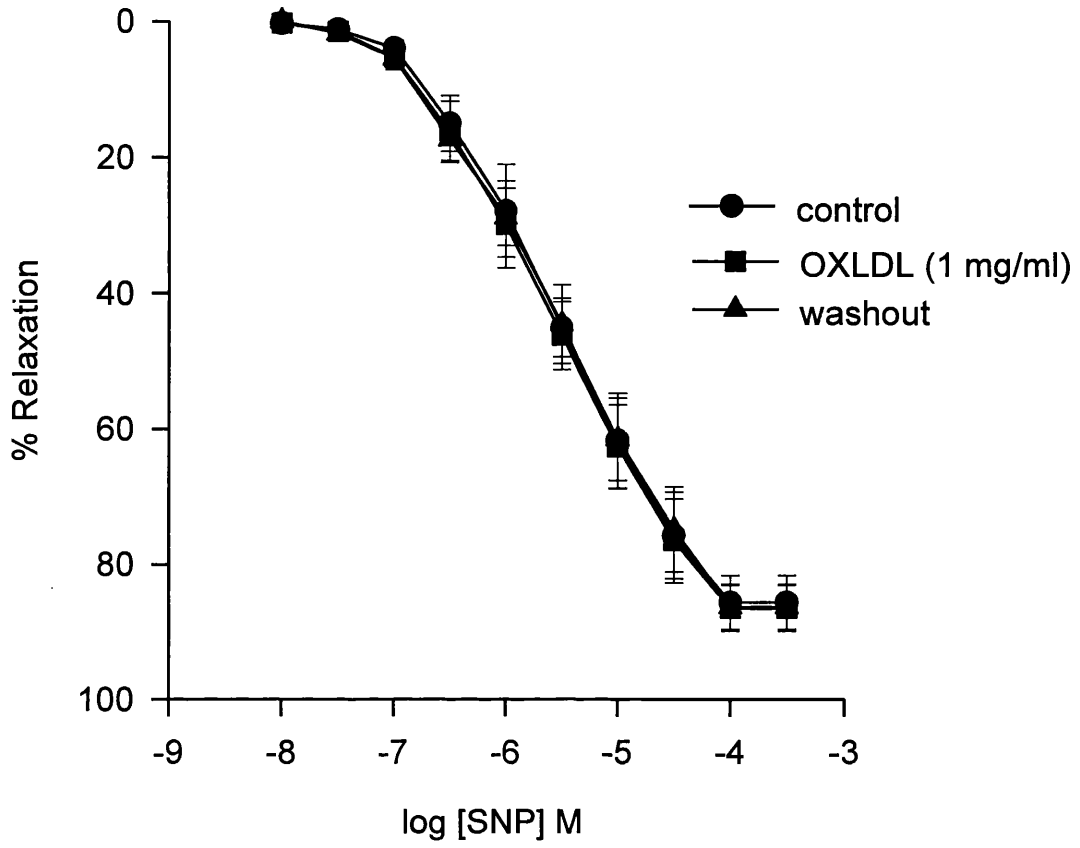


FIGURE 22

SNP-evoked relaxations in the absence and presence of OXLDL.

Coronary arteries were precontracted with $PGF_{2\alpha}$ (5 - 8 μM) in the presence of indomethacin (10 μM) and relaxed with cumulative concentrations of SNP (10 nM - 30μM). Following washout, tissues were incubated with OXLDL (1 mg protein/ml) for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to SNP was carried out (*n*=6).

3.6 DISCUSSION

Results from several studies have indicated that in atherosclerotic arteries from a variety of experimental animals, as well as humans, endothelium-dependent relaxations are severely impaired, and in some cases, completely abolished (Verbeuren *et al.*, 1986; Shimokawa and Vanhoutte, 1989). It has been suggested that this attenuation of responses mediated by nitric oxide may result from an accumulation of OXLDL within the vessel wall, a process known to occur in the early stages of atherosclerosis (Steinberg *et al.*, 1989).

Although atherosclerotic lesions are confined to large arteries, the functional consequences of atherosclerosis may extend into the microcirculation. Recent studies have demonstrated an impaired vasodilation to ACh in angiographically normal small coronary resistance vessels from cholesterol-fed rabbits (Simonsen *et al.*, 1992) and monkeys (Chilian *et al.*, 1990).

Very few studies have investigated the direct effects of LDL on relaxations of coronary resistance vessels to determine whether lipoproteins can modulate vascular reactivity and contribute to the impairment of endothelium-dependent relaxations observed in atherosclerosis and hypercholesterolaemia.

In the present study native LDL had no effect on endothelium-dependent relaxations of either large or small coronary arteries precontracted with $PGF_{2\alpha}$. In contrast, in the rabbit aorta native LDL was shown to inhibit ACh-evoked relaxations although the inhibition was found to be dependent on the agonist used to precontract the tissues (Jacobs *et al.*, 1990; Plane, 1992). The inhibitory effect of native LDL could be demonstrated in rabbit

aortic rings precontracted with noradrenaline and 5-HT but not in tissues contracted with phenylephrine (Jacobs *et al.*, 1990; Plane, 1992). Several other studies have reported the effects of native LDL on vascular reactivity of large vessels such as the aorta and large coronary arteries with varying results (Tomita *et al.*, 1990; Hayashi *et al.*, 1991). For example, in isolated porcine large coronary arteries precontracted with $PGF_{2\alpha}$, native LDL was found to cause a marked inhibition of endothelium-dependent relaxations (Tomita *et al.*, 1990; Hayashi *et al.*, 1991), whereas other studies have demonstrated that native LDL has no effect on endothelium-dependent relaxations, in the same tissue (Simon *et al.*, 1990; Tanner *et al.*, 1991). In addition, endothelium-dependent relaxations mediated by ACh were unaltered following luminal exposure of rabbit femoral artery segments to native LDL (Galle *et al.*, 1991).

The results from the present study have shown that, in contrast to native LDL, OXLDL can cause a reversible inhibition of endothelium-dependent relaxations evoked by ACh in isolated rabbit coronary arteries precontracted with either $PGF_{2\alpha}$ or KCl. Furthermore, the extent of the inhibitory effect was similar in large coronary arteries and in small resistance vessels. This indicates that the inhibitory effects of OXLDL previously reported in the rabbit aorta can be reproduced in rabbit coronary arteries. This study demonstrated that responses of aortic rings and large coronary arteries taken from the same rabbit exhibit a similar degree of inhibition when exposed to OXLDL.

The mechanism by which OXLDL inhibits endothelium-dependent relaxations is unknown. It may be due to the release of vasoconstrictor products together with a reduced amount of EDRF (Shimokawa and Vanhoutte, 1989). A study by Galle *et al.* (1990) demonstrated that low

concentrations of OXLDL enhanced agonist induced vasoconstrictions in rabbit femoral arteries and, at higher concentrations, evoked vasoconstriction by a direct action on the vascular smooth muscle. This direct vasoactive effect of OXLDL has also been demonstrated in porcine coronary arteries and may contribute to the abnormal vascular reactivity observed during atherosclerosis and hypercholesterolaemia (Simon *et al.*, 1990). In this study however, OXLDL was found to have no direct contractile action in resting tissues nor did it have any effect on contractions evoked by either $PGF_{2\alpha}$ or KCl indicating that the inhibitory effects of OXLDL on endothelium-dependent relaxations are not due to enhanced vasoconstriction nor to the inhibition of basal EDRF release.

It has been suggested that OXLDL may inhibit endothelium-dependent responses by interfering with receptor-operated signal transduction mechanisms linked to the formation of EDRF, and specifically with the intracellular availability of L-arginine, the substrate for EDRF formation in endothelial cells (Tanner *et al.*, 1991). Furthermore, administration of L-arginine either by intravenous infusion *in vivo* or by *in vitro* exposure has been shown to normalise endothelium-dependent relaxations in both conduit and resistance vessels of hypercholesterolaemic animals but has no effect in normal animals (Girerd *et al.*, 1990; Rossitch *et al.*, 1991).

The proposed mediator of endothelium-dependent relaxations in the rabbit aorta is nitric oxide and it has been suggested that the attenuation of these responses by OXLDL may be due to an increased inactivation of NO before it reaches the vascular smooth muscle (Lopez *et al.*, 1989a; 1989b). Bioassay studies have demonstrated that OXLDL can directly inactivate EDRF after its release from cultured endothelial cells (Galle *et al.*, 1991; Galle and Bassenge, 1990). The synthesis of EDRF in cultured endothelial

cells and in isolated arterial segments was, however unaffected following incubation with OXLDL (Galle *et al.*, 1991). In contrast, Tanner *et al.* (1991) suggested that in porcine coronary arteries OXLDL inhibits the formation of EDRF. In addition, relaxations of isolated rabbit aorta evoked by exogenously added NO are inhibited by the presence of OXLDL suggesting a direct sequestration or inactivation of NO by the lipoprotein particle (Jacobs *et al.*, 1990).

The impairment of endothelium-dependent relaxations by OXLDL may be due to a reduced vascular responsiveness to EDRF at the level of t he smooth muscle. The nitrovasodilator SNP evokes endothelium-independent relaxations mediated by the direct activation of soluble guanylate cyclase. In this study, relaxations evoked by SNP were not altered following incubation with OXLDL indicating that smooth muscle function is not impaired and also that OXLDL does not affect the activity of the soluble guanylate cyclase. Similarly other studies have reported that relaxations mediated by nitrovasodilators are unaltered in porcine coronary arteries following exposure to OXLDL (Tanner *et al.*, 1991) and in coronary resistance vessels from cholesterol-fed rabbits and pigs (Osborne *et al.*, 1989; Kuo *et al.*, 1992). However, in severe atherosclerosis endothelium-independent relaxations mediated by GTN were diminished suggesting that there is an impairment in vascular smooth muscle function in advanced stages of the disease (Förstermann *et al.*, 1988; Berkenboom *et al.*, 1989).

When the endothelium is stimulated by an agonist such as ACh, the intracellular free calcium concentration ($[Ca^{2+}]_i$) increases with an initial peak due to a mobilisation of Ca^{2+} from intracellular stores followed by a sustained plateau which is dependent on the presence of extracellular Ca^{2+} (Lückhoff and Busse, 1986; Colden-Stanfield *et al.*, 1987). Many studies

have now demonstrated that increases in $[Ca^{2+}]_i$ in vascular endothelial cells are essential for the synthesis/release of EDRF (Furchgott and Zawadzki, 1980; Singer and Peach, 1982; Long and Stone, 1985). In cultured endothelial cells, membrane depolarisation caused by raising the extracellular $[K^+]$ inhibits the sustained increase in $[Ca^{2+}]_i$ associated with Ca^{2+} influx (Laskey *et al.*, 1990; Lückhoff and Busse, 1990). Furthermore, agonist-induced release of EDRF from isolated segments of rabbit aorta is reduced in a K^+-rich medium (Lückhoff and Busse, 1990) indicating that Ca^{2+} influx is essential for the continued release of EDRF (Lückhoff *et al.*, 1988; Laskey *et al.*, 1990).

These findings may explain the results of the present study in which relaxations evoked by ACh were reduced in vessels precontracted with KCl compared to those contracted to a similar extent with $PGF_{2\alpha}$. This reduction in endothelium-dependent relaxations in the presence of a high $[K^+]$ has been demonstrated previously (Furchgott, 1983; Lincoln, 1983). However, in other studies, endothelium-dependent relaxations evoked by ACh were similar when the tissues were precontracted with K^+ or other agonists (Parsons *et al.*, 1991; Plane and Garland, 1993). The reduced relaxation in vessels precontracted with KCl in this study cannot be due to functional antagonism since the contractions induced by KCl and $PGF_{2\alpha}$ were not significantly different in size.

NO, the proposed mediator of endothelium-dependent responses induces relaxation of vascular smooth muscle by activating soluble guanylate cyclase to increase levels of cGMP. Other studies have suggested that the reduced relaxation in KCl-contracted vessels is due to a greater elevation of cGMP in agonist-contracted vessels than in those contracted with KCl (Rapoport *et al.*, 1985; Collins *et al.*, 1988).

The results described in the present investigation demonstrated differences in the inhibitory effect of OXLDL in vessels precontracted with $PGF_{2\alpha}$ and KCl. In coronary arteries precontracted with $PGF_{2\alpha}$, preincubation with OXLDL caused a rightward shift in the ACh concentration-response curve whereas KCl-precontracted vessels demonstrated a reduced sensitivity to ACh as well as a reduction in the maximum relaxation evoked by ACh. Since elevated concentrations of KCl have been shown to inhibit the release of EDRF, a further inhibition of EDRF synthesis and/or release by OXLDL may be sufficient to cause the reduction in maximum relaxation to ACh and the greater inhibitory effect at higher concentrations of OXLDL observed in KCl-precontracted tissues. In vessels precontracted with $PGF_{2\alpha}$ more EDRF is released in response to ACh and the inhibition of synthesis and/or release by OXLDL may be sufficient only to produce a shift in the ACh concentration-response curve.

OXLDL has also been shown in other studies to reversibly inhibit endothelium-dependent relaxations without influencing endothelium-independent responses to either SNP or SIN-1 in porcine large coronary arteries (Tomita *et al.*, 1990; Tanner *et al.*, 1991). However, the concentration of OXLDL required to cause inhibition of relaxations is, in some cases, much lower than that used in the experiments described here. Simon *et al.*, (1990) demonstrated inhibition of endothelium-dependent relaxations in porcine coronary arteries by OXLDL at a concentration of 100 μg /ml and in another study, OXLDL at concentrations as low as 30 - 300 μg/ml was reported to inhibit the formation of EDRF (Tanner *et al.*, 1991). The study by Tanner *et al.*, (1991) demonstrated inhibition of endothelium-dependent relaxations in large coronary arteries but found OXLDL, at concentrations up to 300 μg/ml to have no effect on relaxations

of small coronary arteries. This is in contrast to the present study in which incubation with OXLDL at a concentration of 500 μg/ml resulted in a significant reduction in sensitivity to ACh in both large and small coronary arteries. Comparison of these studies is difficult due to the many differences in the methods used both in isolation and modification of the lipoprotein as well as in the experimental protocol. In the study by Tanner *et al.*, (1991) the lipoproteins were incubated with the tissue for 2 hours and then washed out for 10 mins before the relaxations of the tissues were assessed. Since, in the present study, the effects of OXLDL were found to be reversible after washout, this may explain the lack of effect of the lipoprotein in small coronary vessels described by Tanner *et al.* (1991).

This study has shown that OXLDL inhibits endothelium-dependent relaxations in both large and small coronary arteries to a similar degree. This may suggest that, despite the heterogeneity in vascular susceptibility to develop atherosclerotic lesions, exposure to oxidised lipids causes impairment of vascular relaxation in both conductance and resistance vessels. Other studies have also reported impaired relaxations in coronary resistance vessels from cholesterol-fed atherosclerotic animals (Osborne *et al.*, 1989; Chilian *et al.*, 1990; Sellke *et al.*, 1990). Furthermore, a recent study by Kuo *et al.*, (1992) demonstrated that small coronary vessels taken from atherosclerotic pigs showed no signs of vascular lesions or intimal thickening themselves, but did exhibit reduced vasodilator responses to endothelium-dependent agonists, indicating that pathophysiological consequences of atherosclerosis in the coronary circulation extend downstream into small coronary resistance vessels.

No structural alterations within the vessel wall of small coronary arteries from cholesterol-fed animals have been observed as examined by

light microscopy (Osborne *et al.*, 1989). However, transmission electron microscopy revealed the presence of vacuoles possibly indicating lipid droplets within the endothelium of coronary resistance vessels from cholesterol-fed primates with impaired endothelium-dependent responses (Sellke *et al.*, 1990). Therefore, the incorporation of lipids within the vessel wall represents an early stage of atherosclerosis and may contribute to the impairment of endothelium-dependent responses in the microcirculation. The involvement of OXLDL in the attenuated dilator responses in hypercholesterolaemia is supported by the observation that a similar inhibitory pattern of vasodilator responses was obtained in vessels exposed to OXLDL and in arteries from cholesterol-fed animals. In addition, impaired vasodilator responses to ACh and A23187 in the coronary microcirculation of cholesterol-fed rabbits can be prevented by dietary supplementation with the antioxidant vitamin E (Anderson *et al.*, 1993). These results indicate that an oxidative process, possibly due to the presence of OXLDL in the vessel wall, is involved in the attenuation of relaxations in hypercholesterolaemia and early atherosclerosis prior to the formation of lesions associated with the gross accumulation of oxidised lipids.

The results of the present study may have important implications not only in coronary artery disease but also in myocardial perfusion. The regulation of coronary perfusion has been shown to be regulated predominantly by resistance arteries (Chilian *et al.*, 1986) and up to 50% of coronary resistance resides in coronary microvessels. The inhibition of endothelium-mediated vasodilation in coronary microvessels observed in hypercholesterolaemia may, therefore contribute to the pathogenesis of myocardial ischaemia.

CHAPTER 4

THE EFFECTS OF LIPOXYGENASE TREATED LDL ON RELAXATIONS OF ISOLATED RABBIT AORTIC RINGS.

4.1 INTRODUCTION

The presence of OXLDL in the artery wall is thought to play a major role in the pathogenesis of atherosclerosis (see section 1.5). The exact mechanism by which LDL becomes oxidised remains unclear but it has been suggested that cellular 15-lipoxygenases may be involved (see section 1.5.1). It has beem demonstrated that purified soybean lipoxygenases can convert LDL to an oxidised and cytotoxic form (Sparrow *et al.*, 1988; Cathcart *et al.*, 1991). Lipoxygenases are enzymes that catalyse the incorporation of molecular oxygen into both unesterified and esterified polyunsaturated fatty acids (reviewed by Yamamoto, 1992). Soybean 15-lipoxygenase can oxidise both arachidonic and linoleic acid present in LDL yielding the corresponding hydroperoxy acid which are major constituents of OXLDL.

The aims of the present study were to investigate the effects of LDL modified by treatment with lipoxygenase on endothelium-dependent relaxations in isolated rabbit aorta.

4.2 LIPOXYGENASE AND ACh-EVOKED RELAXATIONS

Following 30 mins preincubation with lipoxygenase, endothelium-dependent relaxations of aortic rings precontracted with phenylephrine

(0.1 - 0.3 μM) were attenuated. Lipoxygenase (16,000 U), equivalent to that present in 0.4 mg protein/ml LO-LDL had no effect on ACh-evoked relaxations. Enzyme concentrations higher than this, equivalent to the amount present in 0.8, 1.5 and 2.0 mg protein/ml LO-LDL, resulted in a concentration-dependent inhibition of relaxations evoked by ACh as shown in **Figure 23**.

Due to this inhibitory effect of lipoxygenase on ACh-evoked relaxations, the enzyme was removed from the LDL following oxidation by recentrifugation of the modified LDL as described in section 2.3.5(b).

4.3 LO-LDL AND VASCULAR TONE

No change in basal tone was observed when endothelium-intact or denuded aortic rings were exposed to LO-LDL (0.4 - 2 mg protein/ml) for up to 30 mins. This is illustrated in **Figure 24a** which shows the addition of 2 mg protein/ml LO-LDL to a resting tissue.

Furthermore, LO-LDL (0.4 -2 mg protein/ml) had no effect on tone when added to precontracted tissues in the presence or absence of endothelium. This is illustrated in **Figure 24b** which shows the addition of 2 mg protein/ml LO-LDL to a tissue precontracted with phenylephrine (0.3 μM).

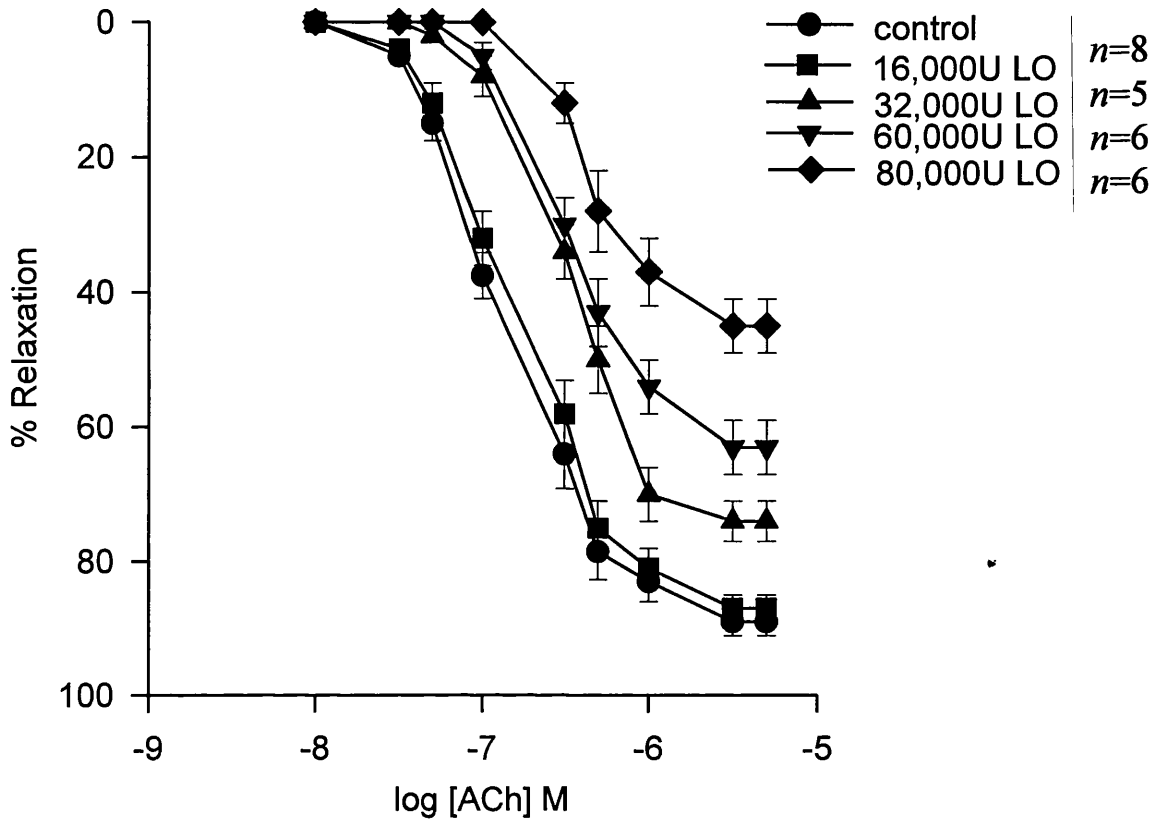


FIGURE 23

Lipoxygenase and ACh-evoked relaxations.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were preincubated with lipoxygenase (16000, 32000, 60000 or 80000 U) for 30 mins and the contraction/relaxation cycle repeated.

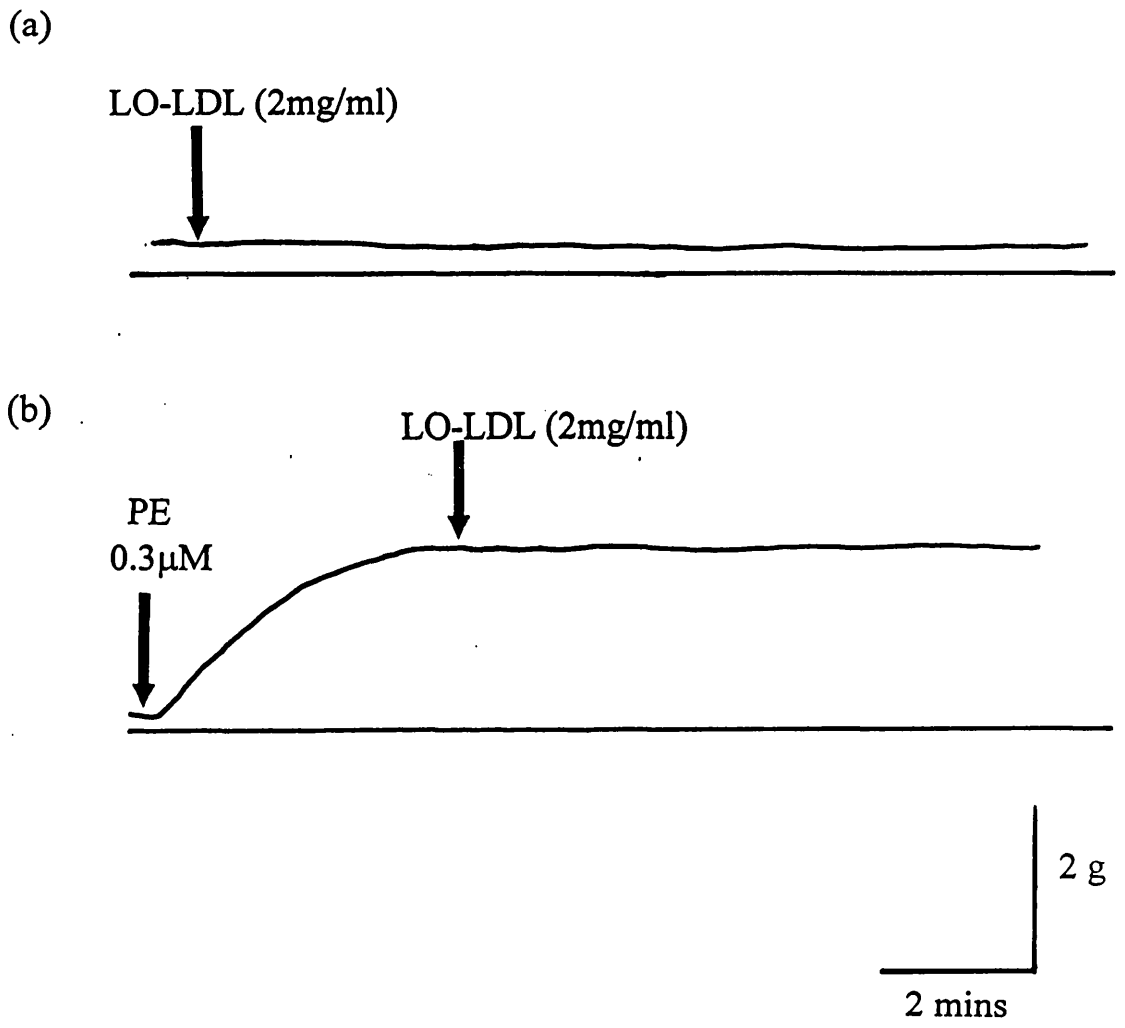


FIGURE 24

Addition of LO-LDL to resting and induced tone.

(a) The addition of LO-LDL (2 mg protein/ml) to basal tone
(b) The addition of LO-LDL (2 mg protein/ml) to an aortic ring precontracted with PE (0.3μM)

4.4 THE EFFECT OF LO-LDL ON ENDOTHELIUM-DEPENDENT RELAXATIONS.

4.4.1 THE EFFECT OF LO-LDL ON ACh-EVOKED RELAXATIONS

As shown in **Figure 25a,** ACh (10 nM - 5 μM) evoked concentration-dependent relaxations in intact phenylephrine precontracted (0.1 - 0.3 μM) rabbit aortic rings. Following a 30 min preincubation period with LO-LDL (2 mg protein/ml), relaxations evoked by ACh were attenuated as shown in **Figure 25b**. This is reflected by a rightward shift in the concentration-response curve for ACh and a significant reduction in the maximum level of relaxation observed in the presence of the lipoprotein. **Figure 26** shows concentration-response curves for ACh in the presence and absence of LO-LDL (2 mg protein/ml). The maximum relaxation was reduced from 91.1 ± 1.8% (n=17) in control tissues to 77.8 ± 2.2% (n=17; p<0.05) following exposure to LO-LDL. The degree of inhibition of endothelium-dependent relaxations did not significantly differ between LO-LDL samples prepared from the plasma of 10 different donors (ANOVA). After removal of the lipoprotein, the inhibition of relaxations was partially reversed and a maximum relaxation of 85.9 ± 1.9% (n=17) was obtained. However, this reduction in the inhibitory effect was not significant (p>0.05).

The inhibition of ACh-evoked relaxations by LO-LDL was concentration-dependent with a threshold concentration of 0.8 mg protein/ml. **Figure 27** shows the concentration-dependent effect of LO-LDL on endothelium-dependent relaxations following a 30 min preincubation period.

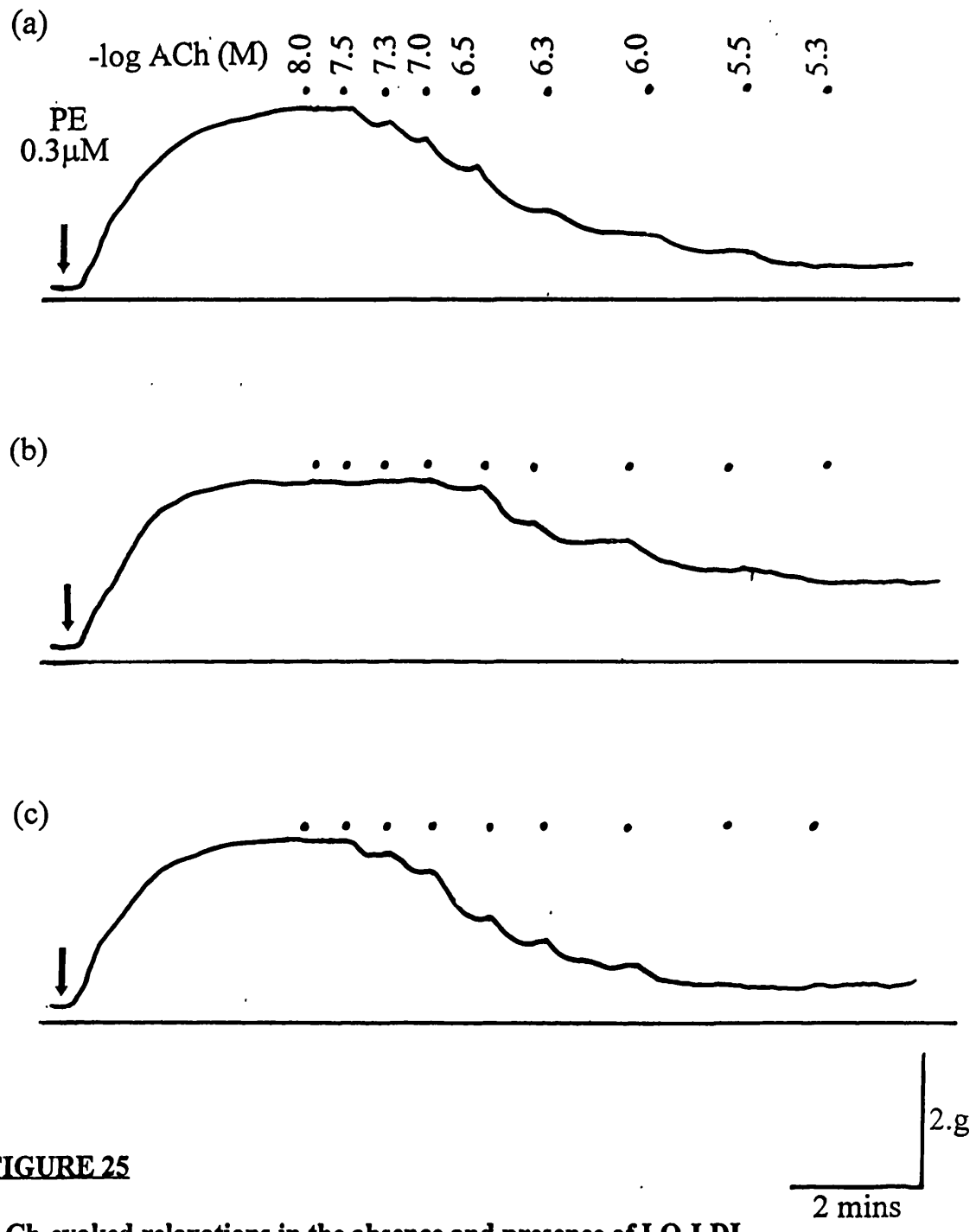


FIGURE 25

ACh-evoked relaxations in the absence and presence of LO-LDL.

Tissues were precontracted with PE (0.3μM) and relaxed with cumulative concentrations of ACh (10nM - 5μM).

(a) Control
(b) Following 30 mins incubation with LO-LDL (2 mg protein/ml)
(c) After washout

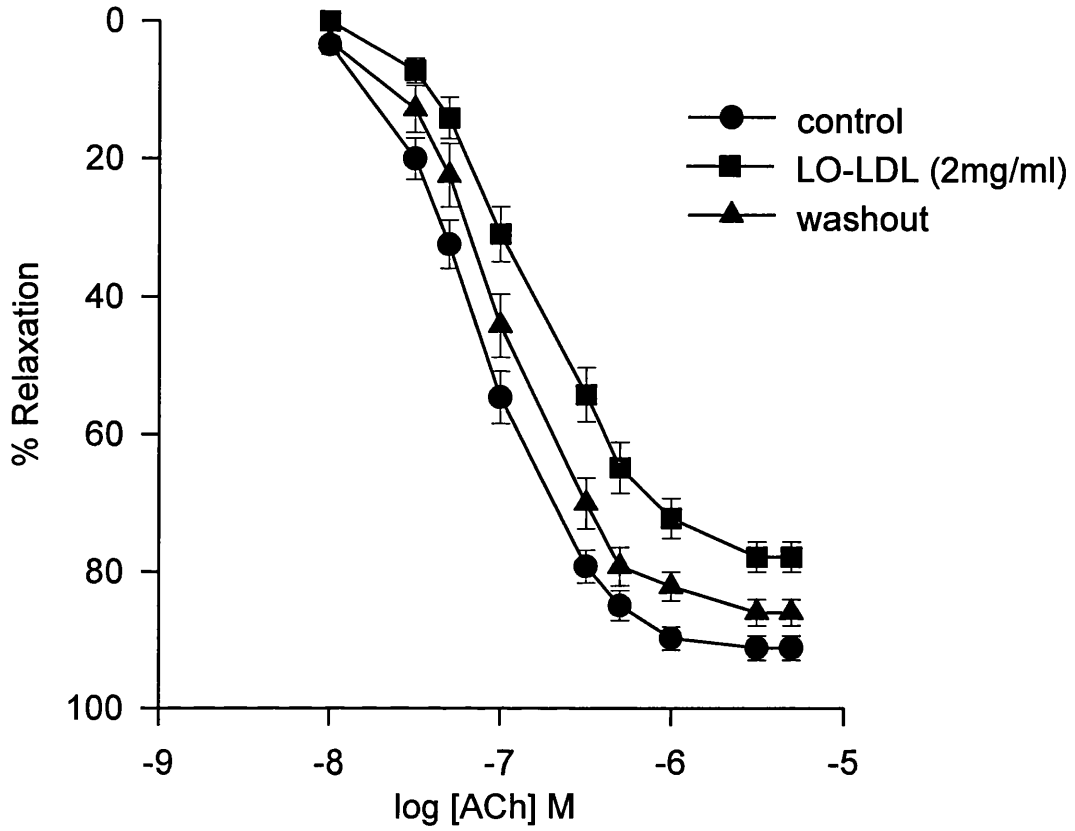


FIGURE 26

The influence of LO-LDL on ACh-evoked relaxations.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were exposed to LO-LDL (2 mg protein/ml) for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out (n=17).

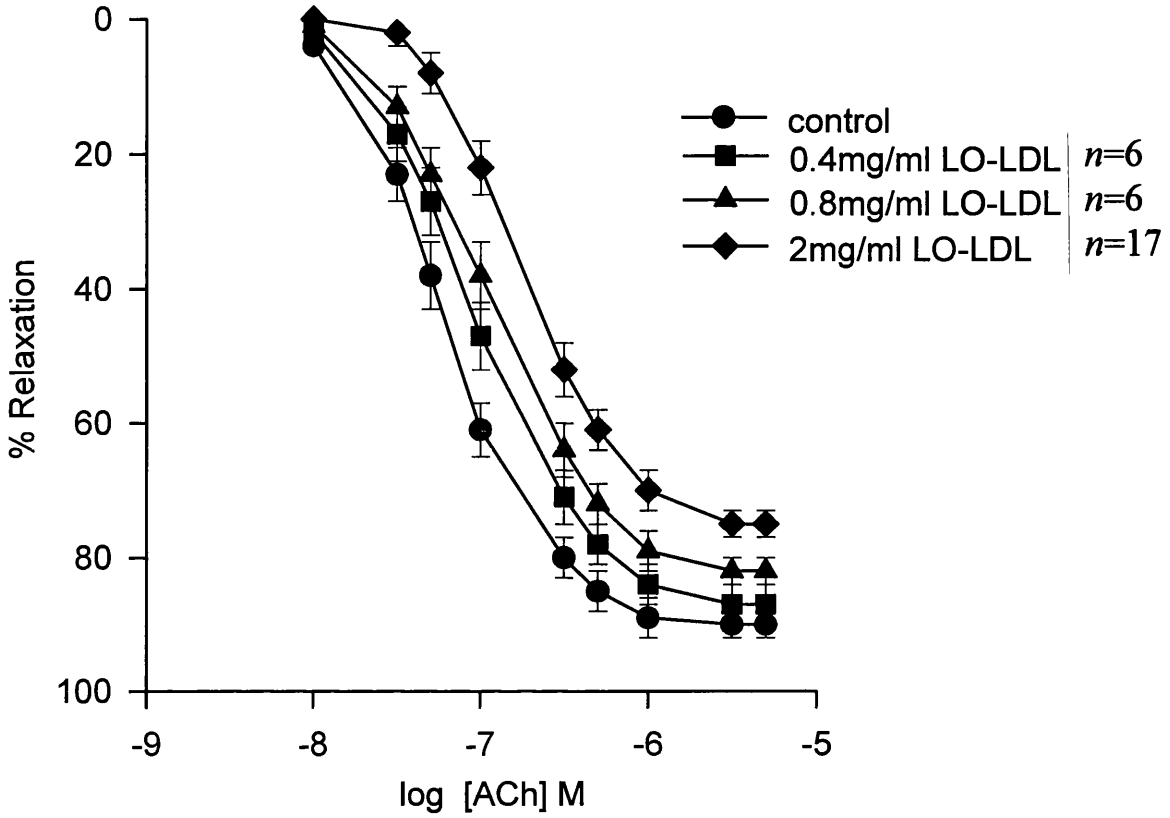


FIGURE 27

Concentration-dependent inhibition of ACh-evoked relaxations by LO-LDL.

Tissues were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were exposed to LO-LDL (0.4, 0.8 or 2 mg protein/ml) for 30 mins and the contraction/relaxation cycle repeated.

4.4.2 THE INFLUENCE OF INDOMETHACIN ON THE INHIBITION OF ACh-EVOKED RELAXATIONS BY LO-LDL

The effect of the cyclo-oxygenase inhibitor, indomethacin, on the LO-LDL-induced attenuation of endothelium-dependent relaxations was assessed. Preincubation of the tissues with indomethacin (10 μM dissolved in 2% Na_2CO_3) for 15 mins did not alter the sensitivity of phenylephrine-contracted tissues to ACh as shown in **Figure 28a**. In addition, indomethacin did not have any influence on the attenuation of ACh-evoked relaxations observed in the presence of LO-LDL (2 mg protein/ml). **Figure 28b** shows the effect of indomethacin on ACh concentration-response curves following treatment with LO-LDL. The maximum level of relaxation was reduced by 14.6 ± 1.7% (n=17; p<0.05; section 4.4.1) following exposure to LO-LDL alone and by 13.0 ± 1.9% (n=5; p<0.05) in the presence of LO-LDL and indomethacin.

These findings suggest that cyclo-oxygenase products released by the endothelium do not play a role in the inhibition of endothelium-dependent relaxations caused by LO-LDL.

4.4.3 THE EFFECT OF DEXTRAN SULPHATE AND POLYINOSINIC ACID ON THE INHIBITION OF ACh-EVOKED RELAXATIONS BY LO-LDL

Dextran sulphate and poly I block the scavenger receptors present on endothelial cells and macrophages which are responsible for the uptake of modified LDL leading to the formation of foam cells within the vascular wall.

Dextran sulphate (10 μg/ml) had no effect on ACh-evoked relaxations when added to the organ bath as shown in **Figure 29a**. Furthermore,

preincubation of the tissues for 15 mins with dextran sulphate before the addition of LO-LDL (2 mg protein/ml) did not have any influence on the inhibition of endothelium-dependent relaxations caused by the presence of LO-LDL alone. The maximum relaxation to ACh was reduced by 12.1 ± 2.2% (n=5; p<0.05) in the presence of LO-LDL and dextran sulphate compared with a reduction of 14.6 ± 1.7% (n=17; p<0.05) following exposure to LO-LDL alone. **Figure 29b** shows the effect of dextran sulphate on the concentration-response curves to ACh following treatment with LO-LDL.

Preincubation of the tissues with poly I (50 μg/ml) for 15 mins did not alter the sensitivity of phenylephrine contracted tissues to ACh as shown in **Figure 30a.** Furthermore, poly I had no influence on the inhibition of ACh-evoked relaxations caused by the presence of LO-LDL (2 mg protein/ml). **Figure 30b** shows the effect of poly I on the concentration-response curves to ACh following treatment with LO-LDL. The maximum relaxation to ACh was reduced from 91.1 ± 1.8% (n=17) to 77.8 ± 2.2% (section 4.4.1; n=17; p<0.05) in the presence of LO-LDL alone and from 92.2 ± 2.6% (n=5) to 78.6 ± 1.7% (n=5; p<0.05) following exposure to LO-LDL and poly I.

These findings indicate that the inhibition of ACh-evoked relaxations caused by LO-LDL is not mediated through the scavenger receptor.

4.4.4 THE INFLUENCE OF CHELERYTHRINE CHLORIDE ON THE LO-LDL INDUCED INHIBITION OF ACh-EVOKED RELAXATIONS

To investigate the involvement of protein kinase C in the inhibition of ACh-evoked relaxations caused by LO-LDL, experiments were carried out in the presence of the protein kinase C inhibitor chelerythrine chloride.

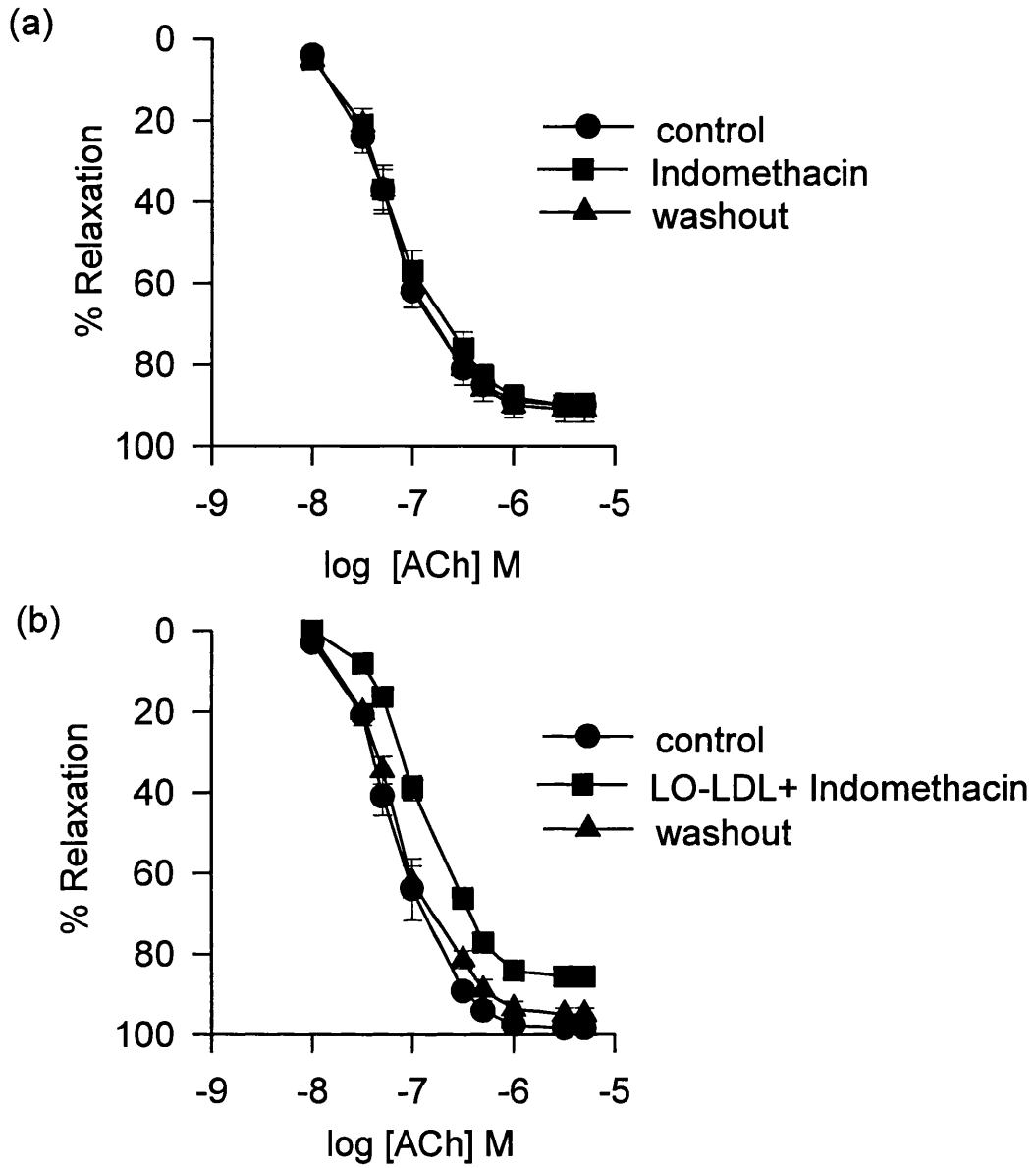


FIGURE 28

The influence of indomethacin on the inhibition of ACh-evoked relaxations by LO-LDL.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were incubated with indomethacin (10 μM) for 15 mins before exposure to LO-LDL (2 mg protein/ml) for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out.

(a) Indomethacin alone (n=5)

(b) Indomethacin and LO-LDL (n=5)

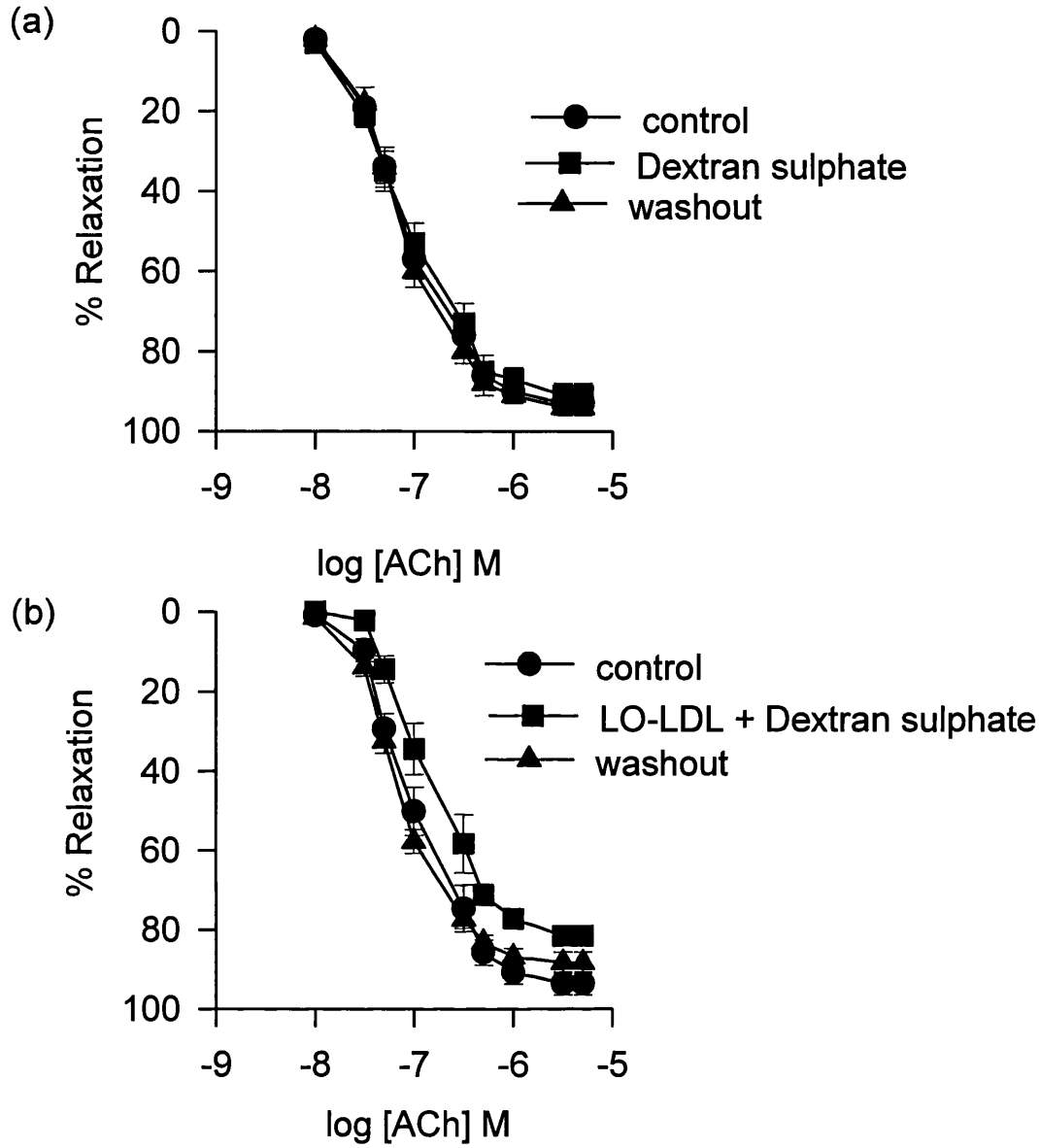


FIGURE 29

The influence of dextran sulphate on the attenuation of ACh-evoked relaxations by LO-LDL.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were incubated with dextran sulphate (10 μg/ml) for 15 mins before exposure to LO-LDL for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out.

(a) Dextran sulphate alone (n=6)

(b) Dextran sulphate and LO-LDL (n=5)

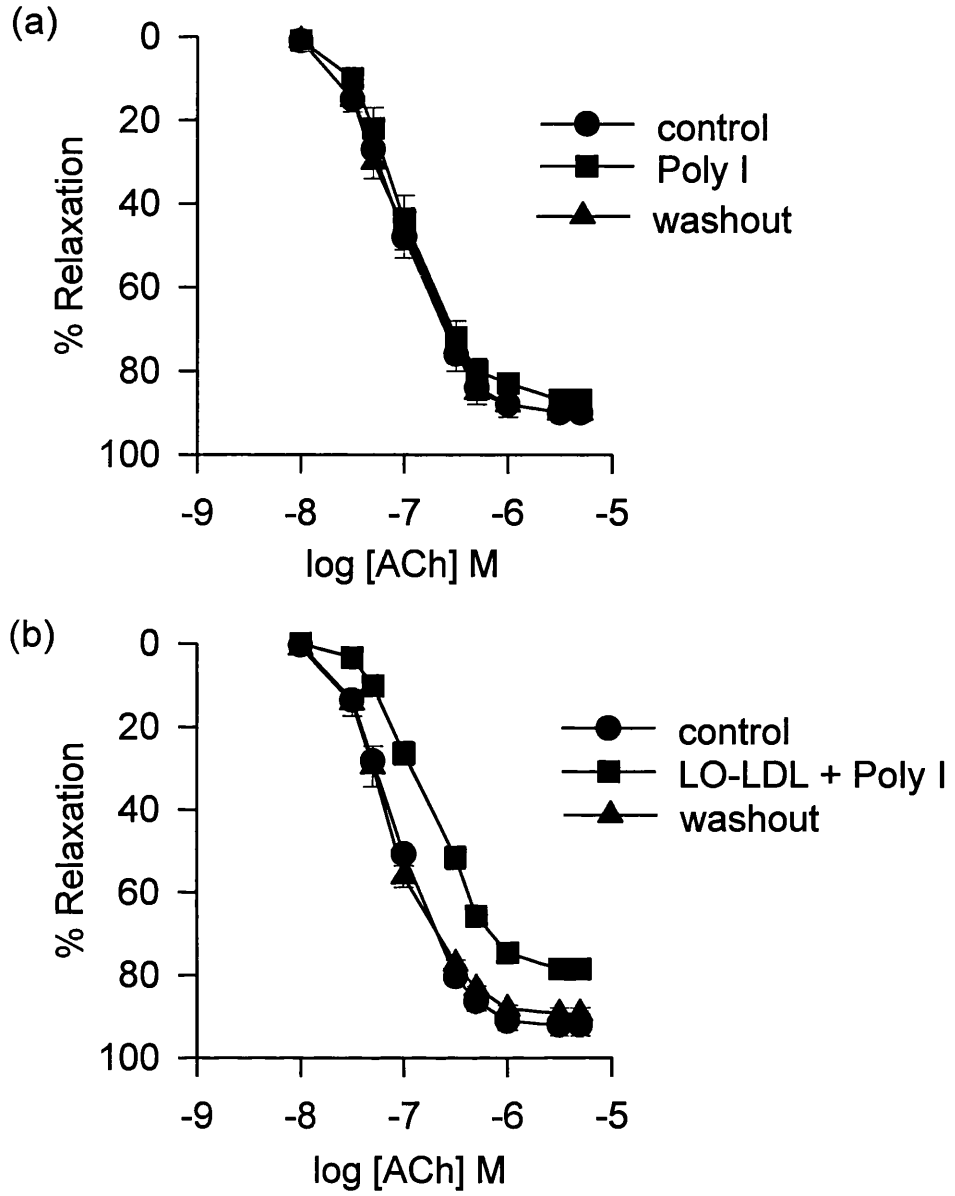


FIGURE 30

The effect of poly I on the attenuation of ACh-evoked relaxations by LO-LDL.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were incubated with poly I (50 μg/ml) for 15 mins before exposure to LO-LDL (2 mg protein/ml) for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out.

(a) Poly I alone (n=5)

(b) Poly I and LO-LDL (n=5)

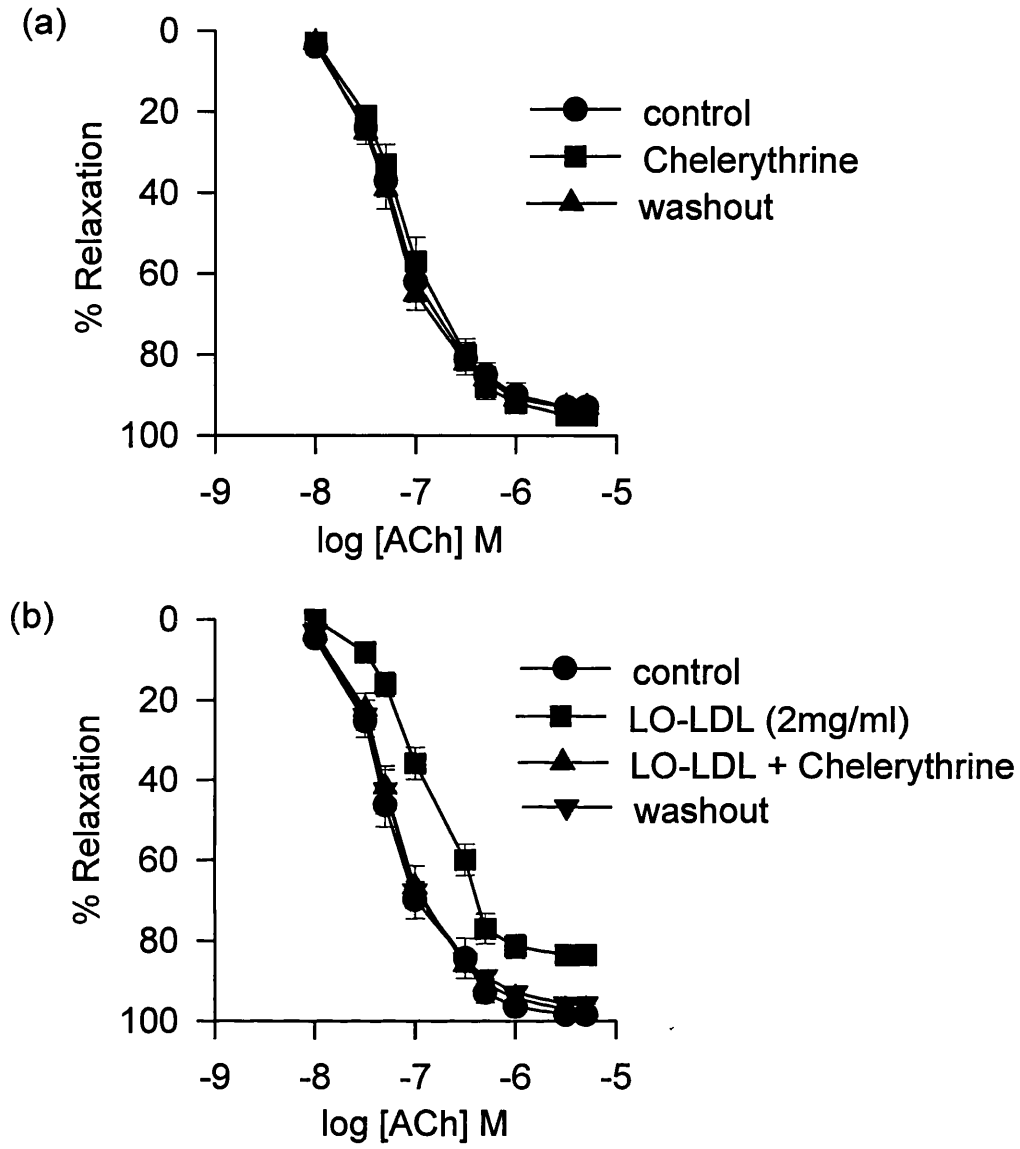


FIGURE 31

The effect of chelerythrine chloride on the inhibition of ACh-evoked relaxations by LO-LDL.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were incubated with chelerythrine chloride (1 μM) for 15 mins before exposure to LO-LDL (2 mg protein/ml) for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out.

(a) Chelerythrine chloride alone (n=8)

(b) Chelerythrine chloride and LO-LDL (n=4)

Chelerythrine chloride (1 μM) was added to the tissues 15 mins prior to the addition of LO-LDL and had no effect on ACh-induced responses as shown in **Figure 31a**. However, preincubation of the tissues with chelerythrine chloride before the addition of LO-LDL (2 mg protein/ml) prevented the attenuation of endothelium-dependent relaxations observed in the presence of LO-LDL alone. **Figure 31b** shows concentration-response curves for ACh following treatment with LO-LDL in the presence and absence of chelerythrine chloride. The maximum level of relaxation to ACh in control tissues was 98.5 ± 1.5% (n=4) compared with 97.3 ± 1.5% (n=4) in the presence of LO-LDL and chelerythrine chloride. In the presence of LO-LDL alone the maximum response to ACh was reduced by 14.6 ± 1.6% (n=17; p<0.05).

This suggests that the inhibitory effect of LO-LDL on endothelium-dependent relaxations is mediated through a mechanism involving protein kinase C.

4.5 LO-LDL AND NITRIC OXIDE EVOKED RELAXATIONS

EDRF, released from the rabbit aorta has been identified as NO or a NO-releasing compound. The addition of exogenous NO to the organ bath elicits concentration-dependent, transient relaxations of phenylephrine contracted tissues which are independent of the endothelium. **Figure 32** shows relaxations evoked by NO in endothelium-intact and denuded tissues. The maximum relaxation to NO in endothelium-intact tissues was 81.6 ± 3.1% (n=5) and in denuded tissues was 83.5 ± 3.0% (n=11).

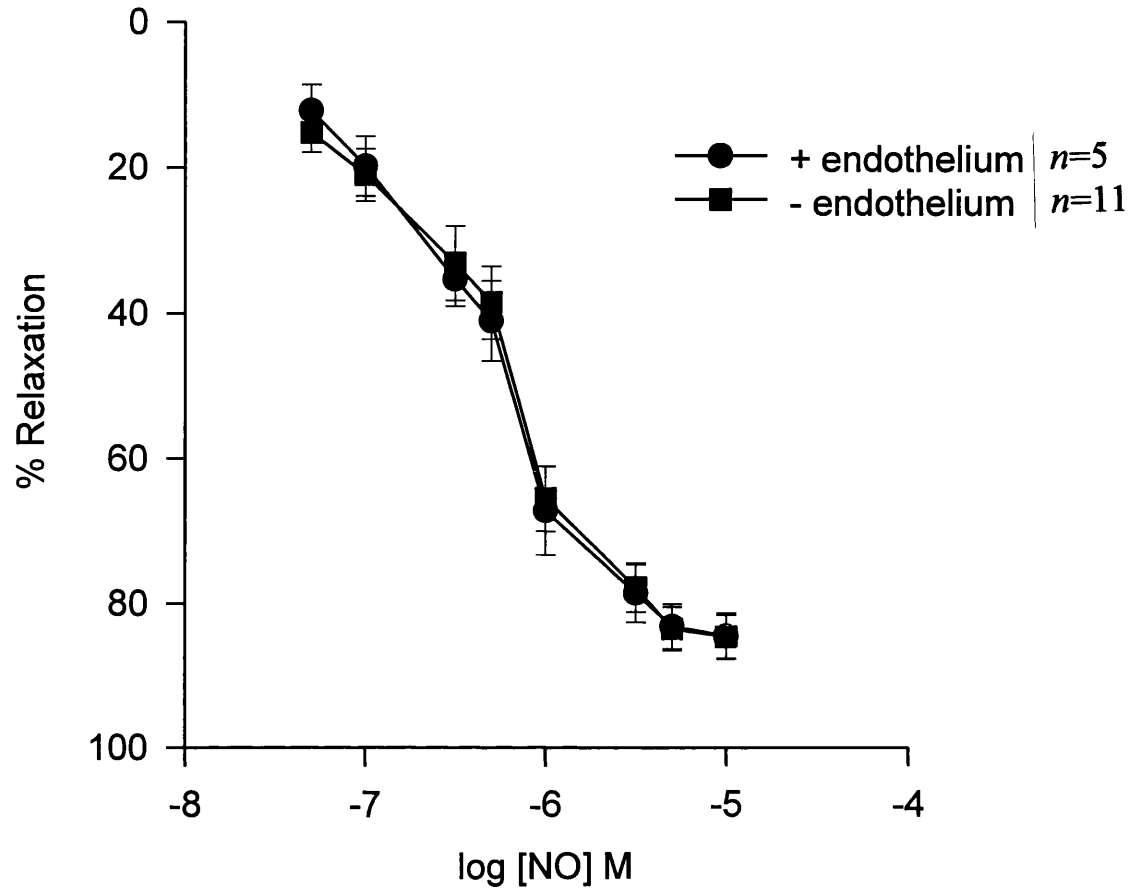


FIGURE 32

NO-evoked relaxations in endothelium-intact and denuded tissues.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of NO (50 nM - 10 μM).

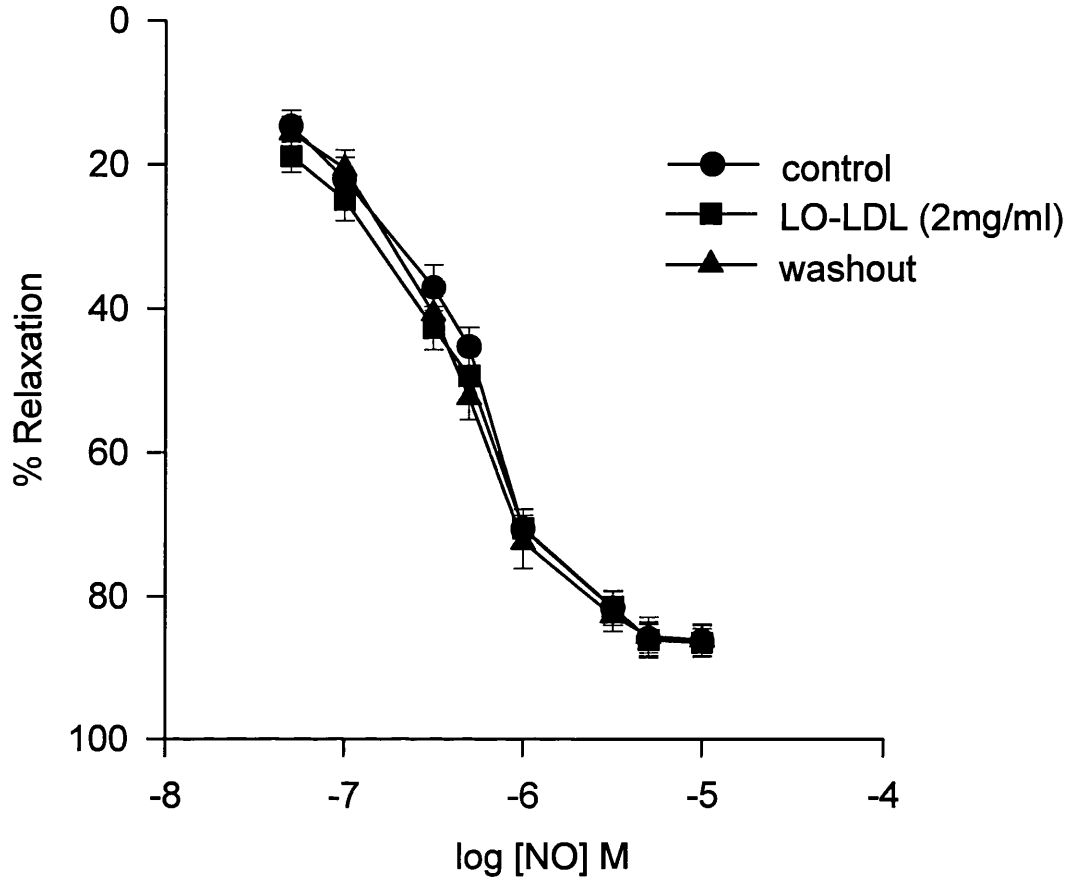


FIGURE 33

The influence of LO-LDL on NO-evoked relaxations in endothelium-denuded tissues.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of NO (50 nM - 10 μM). Following washout, tissues were incubated with LO-LDL (2 mg protein/ml) for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to NO was carried out (n=10).

To examine whether the inhibitory effect of LO-LDL could be due to a direct interaction with NO, the influence of LO-LDL on relaxations evoked by exogenous NO was investigated.

In endothelium-denuded aortic rings, preincubation with LO-LDL (2 mg protein/ml) for 30 mins had no effect on NO-evoked relaxations as shown in **Figure 33**. The maximum relaxation evoked by NO was 86.3 ± 2.2% (n=10) in control tissues and 86.5 ± 2.0% (n=10; p>0.05) following exposure to LO-LDL.

This finding indicates that the attenuation of endothelium-dependent relaxations by LO-LDL is not due to a direct interaction with NO.

4.6 LO-LDL AND GTN-EVOKED RELAXATIONS

The nitrovasodilator GTN evokes endothelium-independent relaxations by activation of soluble guanylate cyclase of smooth muscle to increase cGMP levels. To determine whether LO-LDL could exert its inhibitory effect by an action on soluble guanylate cyclase, the effect of LO-LDL on relaxations mediated by GTN was examined.

GTN (1 nM - 0.5 μM) caused concentration-dependent relaxations of phenylephrine contracted tissues which were independent of the endothelium as shown in **Figure 34**. In endothelium-intact aortic rings the maximum relaxation evoked by GTN was 98.4 ± 1.6% (n=5) and in endothelium-denuded tissues the maximum response was 100 ± 0.0% (n=5).

In the presence of LO-LDL (2 mg protein/ml), relaxations of endothelium-intact tissues evoked by GTN were attenuated. This is demonstrated by a reduction in the maximum relaxation evoked by GTN in

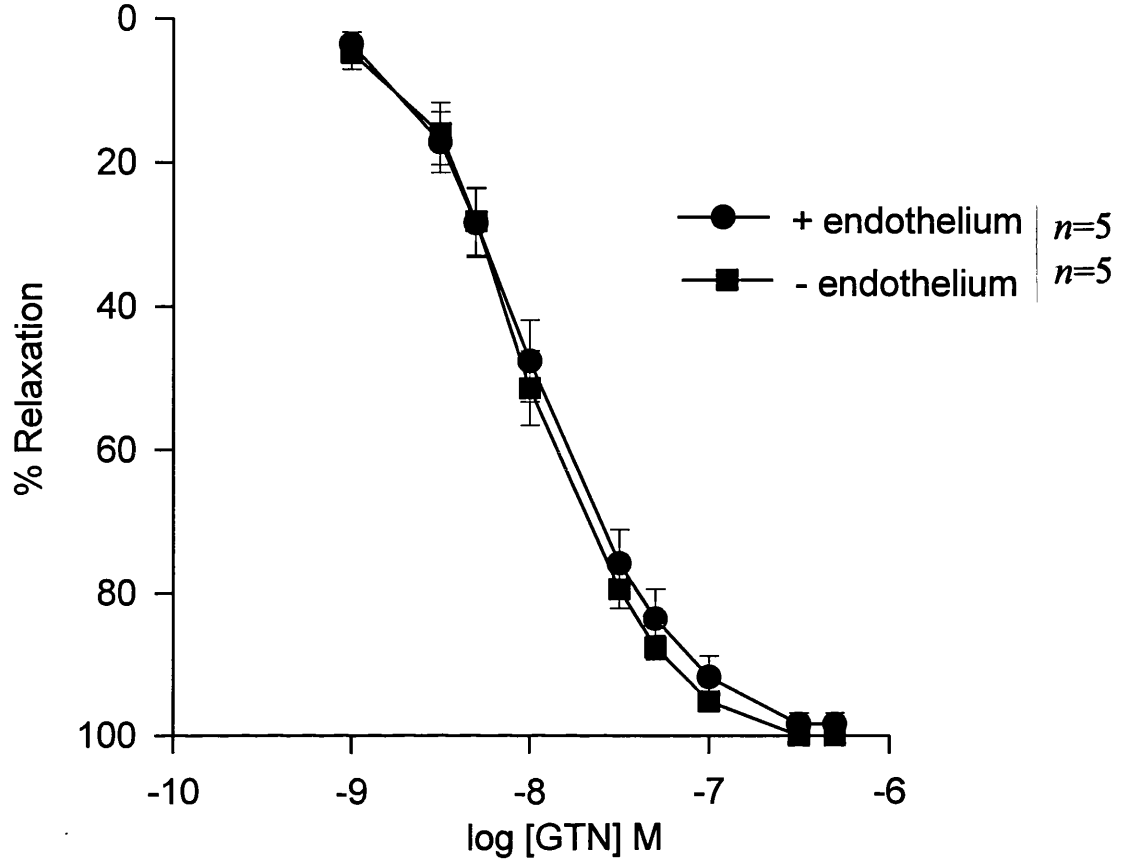


FIGURE 34

GTN-evoked relaxations in endothelium-intact and denuded tissues.

Tissues were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of GTN (1 nM - 0.5 μM).

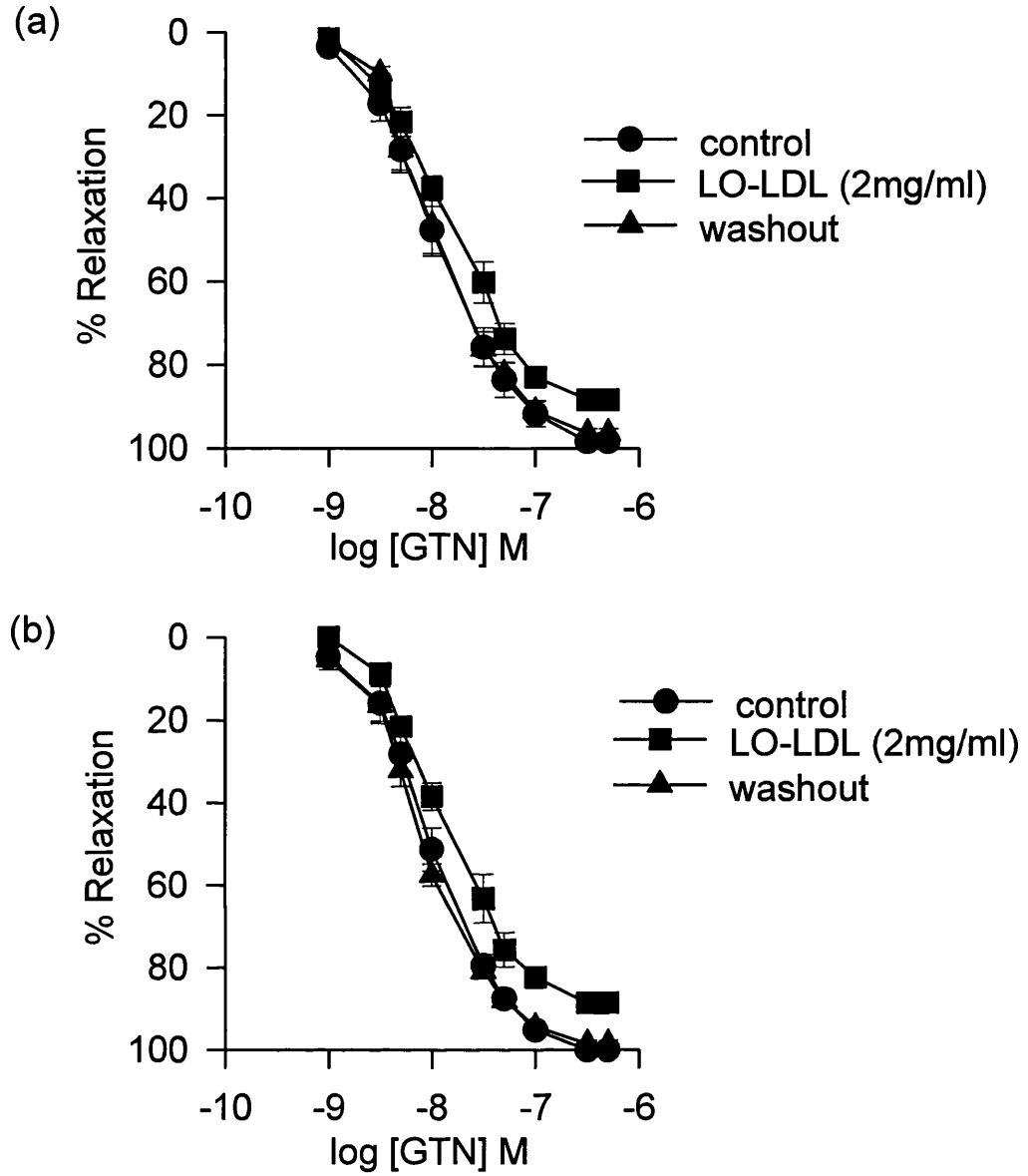


FIGURE 35

The influence of LO-LDL on GTN-evoked relaxations in endothelium-intact and denuded tissues.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of GTN (1 nM - 0.5 μM). Following washout, tissues were exposed to LO-LDL (2 mg protein/ml) and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to GTN was carried out.

(a) Endothelium-intact tissues (n=5)

(b) Endothelium-denuded tissues (n=5)

the presence of LO-LDL as shown in **Figure 35a**. The maximum relaxation to GTN was 98.4 ± 1.6% (n=5) in control tissues and 88.4 ± 2.3% (n=5; p<0.05) following 30 mins exposure to LO-LDL.

Following preincubation with LO-LDL (2 mg protein/ml), GTN-evoked relaxations in endothelium-denuded aortic rings were inhibited to a similar degree as in intact tissues as shown in **Figure 35b**. The maximum relaxation to GTN following treatment with LO-LDL was 89.6 ± 1.8% (n=5) which was significantly reduced compared to control tissues which were completely relaxed in response to GTN (n=5; p<0.05).

These findings indicate that LO-LDL may affect the activity of soluble guanylate cyclase within the smooth muscle or a mechanism subsequent to the elevation of cGMP levels.

4.7 DISCUSSION

Previous studies have shown that LDL can be readily oxidised by a number of cell types including endothelial cells, smooth muscle cells and macrophages (Morel *et al.*, 1984; Parthasarathy *et al.*, 1986; Leake *et al.*, 1990). The mechanisms of cell-mediated oxidation of LDL are unknown although recent evidence has implicated cellular lipoxygenases in the process. Incubation of LDL with purified lipoxygenase can produce some of the alterations to LDL which are observed during cell-mediated oxidation (Sparrow *et al.*, 1988). This study further investigated the role of lipoxygenases in the modification of LDL by examining the vascular effects of LDL modified by treatment with lipoxygenase and comparing them with the inhibitory effects of OXLDL on endothelium-dependent relaxations

previously described (Jacobs *et al.*, 1990; Simon *et al.*, 1990; Galle *et al.*, 1991).

Endothelium-dependent relaxations evoked by ACh are mediated by activation of endothelial cell muscarinic receptors, and in the present study, LDL modified by treatment with lipoxygenase was shown to inhibit this pathway. It has been suggested that the inhibition of endothelium-dependent relaxations observed in isolated porcine atherosclerotic vessels is due to the release of vasoconstrictor products of cyclo-oxygenase from the endothelium (Shimokawa and Vanhoutte, 1989). Furthermore, OXLDL has been shown to evoke contractions in porcine coronary arteries (Simon *et al.*, 1990), although other studies have failed to demonstrate a direct contractile effect of OXLDL in the rabbit aorta (Plane, 1992). In the present study LO-LDL had no direct contractile action in resting tissues, and the inhibition of ACh-evoked relaxations was not dependent on products of the cyclo-oxygenase pathway since indomethacin had no influence on the inhibitory effect.

The inhibitory effect of LO-LDL on ACh-evoked relaxations was unaltered by the presence of dextran sulphate or poly I which act as competitive antagonists of modified forms of LDL for the scavenger receptor (Brown and Goldstein, 1983). This suggests that LDL modified by lipoxygenase does not exert its inhibitory effect on endothelium-dependent relaxations by activating the scavenger receptor present on endothelial cells. This is in contrast to OXLDL, which caused inhibition of endothelium-dependent relaxations of isolated porcine coronary arteries which was completely prevented by the presence of dextran sulphate (Tanner *et al.*, 1991). Other studies have reported that unlike OXLDL, LDL modified by treatment with lipoxygenase alone is not recognised by macrophage scavenger receptors although when LDL is incubated with lipoxygenase in

the presence of phospholipase A_2 the modified lipoprotein is recognised by the scavenger receptor (Sparrow *et al.*, 1988). The purified lipoxygenase used in the present study and in that of Cathcart *et al.* (1991) was found to have no detectable phospholipase A_2 activity (Cathcart *et al.*, 1991). Oxidation of LDL is a complex process and it has been suggested that the modification by lipoxygenase eliminates many complex reactions and focuses on lipid peroxidation and the introduction of lipid hydroperoxides into the LDL molecule. Further oxidation may be required to cause the modification of the apo-B moiety leading to the generation of structures recognised by the scavenger receptor.

To further study the mechanism underlying the inhibition of endothelium-dependent relaxations by LO-LDL, experiments were carried out in the presence of the protein kinase C inhibitor, chelerythrine chloride. Chelerythrine chloride is a benzophenanthridine alkaloid and has been shown to be a potent and highly specific inhibitor of protein kinase C (Herbert *et al.*, 1990). The potency and selectivity of chelerythrine chloride have been reported to be greater than for the more commonly used inhibitors of protein kinase C, staurosporine and H-7 (Herbert *et al.*, 1990). In the presence of chelerythrine chloride, the inhibition of ACh-evoked relaxations by LO-LDL was completely prevented. This suggests that the inhibitory effect of LO-LDL is mediated through the activation of protein kinase C. It is well documented that phorbol esters, activators of protein kinase C inhibit relaxations mediated by EDRF (Weinheimer *et al.*, 1986; Lewis and Henderson, 1987). These agents are known to alter Ca^{2+} signaling and homeostasis in endothelial cells by inhibiting agonist-induced increases in

intracellular Ca^{2+} (Ryan *et al.,* 1988), which is required for the release of EDRF. Furthermore, recent studies have demonstrated that the inhibitory effects of OXLDL are dependent on the activation of protein kinase C (Smith and Turner, 1992; Ohgushi *et al.*, 1993; Smith *et al.,* 1993). In addition, OXLDL has been shown to directly activate protein kinase C in porcine aortic cultured endothelial cells (Smith and Babuji, 1994) An increased protein kinase C activity has also been shown to phosphorylate nitric oxide synthase resulting in a decreased production of nitric oxide (Bredt *et al.,* 1992). These findings may suggest that the inhibitory effect of LO-LDL on endothelium-dependent relaxations is mediated by an inhibition of intracellular Ca^{2+} stimulation resulting in a reduction in the release of EDRF and therefore a reduced vascular relaxation.

The proposed mediator of endothelium-dependent relaxations in the rabbit aorta is NO which evokes relaxation through the activation of soluble guanylate cyclase. To examine whether the inhibition of endothelium-dependent relaxations by LO-LDL could be due to inactivation of NO or the inhibition of soluble guanylate cyclase activity, the effects of LO-LDL on relaxations evoked by exogenous NO and the nitrovasodilator GTN were investigated. In endothelium-denuded tissues, responses elicited by NO were unaffected by exposure to LO-LDL indicating that the inhibition of endothelium-dependent relaxations by the modified lipoprotein is not mediated by interactions with NO. In contrast, LDL oxidised by incubation with Cu^{2+} was found to inhibit relaxations evoked by exogenous NO (Jacobs *et al.*, 1990) indicating that direct sequestration or inactivation of NO by the lipoprotein particle may be responsible for the inhibition.

In endothelium-intact and denuded tissues relaxations evoked by GTN were attenuated following exposure to LO-LDL. Similarly, OXLDL has been

shown to inhibit endothelium-independent relaxations evoked by GTN in isolated endothelium-intact and denuded tissues (Jacobs *et al.*, 1990) and also to inhibit the activation of partially purified soluble guanylate cyclase by both nitrovasodilators and NO (Schmidt *et al.*, 1990). However, in the present study the inhibition of soluble guanylate cyclase cannot account for the inhibitory effect of LO-LDL since relaxations evoked by NO were unaltered. The inhibition of GTN-evoked relaxations by LO-LDL with a lack of effect on NO-evoked relaxations may suggest that the modified lipoprotein exerts its inhibitory effect prior to the activation of soluble guanylate cyclase, possibly by interfering with the intracellular metabolism of GTN. Unlike SNP, which spontaneously releases NO, organic nitrate esters such as GTN undergo an enzymztic and/or nonenzymatic reaction with tissue thiol groups forming NO_2^- (Ignarro *et al.* 1981). NO_2^- then reacts with H^+ to form NO and/or *S*-nitrosothiol which can activate soluble guanylate cyclase to evoke vascular relaxation (Ignarro *et al.* 1981). LO-LDL may therefore cause the attenuation of endothelium-dependent responses by inhibiting this reaction pathway.

The results described here have demonstrated that LDL modified by treatment with purified lipoxygenase can inhibit endothelium-dependent relaxations of isolated rabbit aorta. There are however, differences between the inhibitory properties of LO-LDL and Cu^{2+}-OXLDL. The degree of inhibition of ACh-evoked relaxations by OXLDL has been shown to be dependent on the donor from which the LDL was prepared (Jacobs *et al.*, 1990). In contrast, the present study demonstrated no differences in the inhibition caused by LO-LDL prepared from different donors.

This investigation has shown that purified soybean lipoxygenase can act directly on LDL generating a modified form which has similar effects on vascular reactivity as OXLDL. Oxidised fatty acids formed during the modification of LDL by lipoxygenase may therefore contribute to the

inhibition of relaxations caused by the presence of Cu^{2+}-OXLDL. While other mechanisms may also be involved in the oxidative modification of LDL, the results described here do support a role for lipoxygenase in the formation of modified lipoproteins and therefore in the pathogenesis of atherosclerosis.

CHAPTER 5

THE EFFECTS OF 15-LIPOXYGENASE PRODUCTS OF LINOLEIC AND ARACHIDONIC ACID ON RELAXATIONS OF ISOLATED RABBIT AORTIC RINGS.

5.1 INTRODUCTION

The accumulation of OXLDL in the artery wall is thought to play a major role in the pathogenesis of atherosclerosis (see section 1.5). The exact mechanism by which LDL becomes oxidised is unclear but appears to involve peroxidation of fatty acids producing hydroperoxy and hydroxy derivatives. One of the major fatty acids present in LDL is linoleic acid which is converted to the hydroperoxy acids 9- and 13-HPODE. These intermediates are then further reduced to their corresponding hydroxy derivatives 9- and 13-HODE. Similarly, arachidonic acid can be converted to 15-HPETE and is then reduced to the more stable 15-HETE.

The aims of the present study were to investigate the effects of hydroperoxy and hydroxy derivatives of both linoleic and arachidonic acid on endothelium-dependent and independent relaxations of isolated rabbit aortic rings to determine whether they might contribute to the inhibition of relaxations observed in the presence of OXLDL.

5.2 METABOLITES OF LINOLEIC ACID AND VASCULAR TONE

Exposure of endothelium-intact or denuded aortic rings to 9-HODE, at concentrations between 10 nM and 5 μM for up to 30 mins did not alter the basal level of tone. Similarly, 13-HODE (5 μM) or 9-HPODE (5 μM) had no effect on basal tone in either intact or denuded tissues. This is illustrated in **Figure 36a** which shows the addition of 5 μM 9-HODE to a resting tissue with an intact endothelium.

Furthermore, neither 9-HODE, nor 13-HODE, nor 9-HPODE (10 nM - 5 μM) had any effect on tone when added to precontracted vessels in the presence or absence of endothelium. **Figure 36b** shows the addition of 5 μM 9-HODE to an endothelium-intact vessel precontracted with phenylephrine (0.3 μM).

5.3 THE EFFECT OF LINOLEIC ACID METABOLITES ON ENDOTHELIUM-DEPENDENT RELAXATIONS

5.3.1 THE EFFECT OF 9-HODE ON ACh-EVOKED RELAXATIONS

ACh (10 nM - 5 μM) evoked concentration-dependent relaxations of rabbit aortic rings precontracted with phenylephrine (0.1 - 0.3 μM). Relaxations to ACh were unchanged in control tissues throughout the experiment even after 30 mins incubation with vehicle (ethanol) as shown in **Figure 37a**. The volume of ethanol added to the bath was never more than 0.2% of the total bath volume.

The addition of 9-HODE (5μM) to the tissues immediately prior to contraction and approximately 4-5mins before the first dose of ACh resulted in a reversible attenuation of ACh-evoked relaxations (**Figure 38b**). This is

demonstrated as a rightward shift in the ACh dose-response curve and by a significant reduction in the maximum relaxation to ACh in the presence of 9-HODE (**Figure 37b**). The maximum relaxation was reduced from 91.6 ± 1.6% (n=7) in control tissues to 80.4 ± 2.4% (n=7; p<0.05) in the presence of 9-HODE. After washout, responses were restored to control values (92.0 ± 1.9%; n=7) as shown in **Figure 38c.**

Following preincubation with 9-HODE (5 μM) for 30 mins, relaxations evoked by ACh were inhibited to a similar degree to that observed when the hydroxy acid was added immediately prior to the contraction (**Figure 39**). The maximum level of relaxation was reduced from 92.3 ± 2.3% (n=8) in control tissues to 80.0 ± 3.6% (n=8; p<0.05) following exposure to 9-HODE. Relaxations to ACh were partially restored on washout of the hydroxy acid with a maximum relaxation of 85.5 ± 2.3% (n=8) although this reversal of the inhibitory effect was not significant (p>0.05).

The threshold concentration of 9-HODE required to attenuate ACh-evoked relaxations was 1 μM. The concentration-dependent inhibition of endothelium-dependent relaxations by 9-HODE following a 30 min preincubation period is illustrated in **Figure 40** which shows the effect of 0.5, 1.0 and 5 μM 9-HODE on relaxations evoked by ACh. The concentration of the stock hydroxy acid restricted the use of concentrations higher than 5 μM due to the large volumes of ethanol which would be added to the bath.

The inhibitory effect of 9-HODE on endothelium-dependent relaxations could also be observed when the 9-HODE was added to tissues after they had been contracted with phenylephrine and immediately before the first concentration of ACh as shown in **Figure 41.** The degree of inhibition was similar to that observed when the 9-HODE was added prior to the

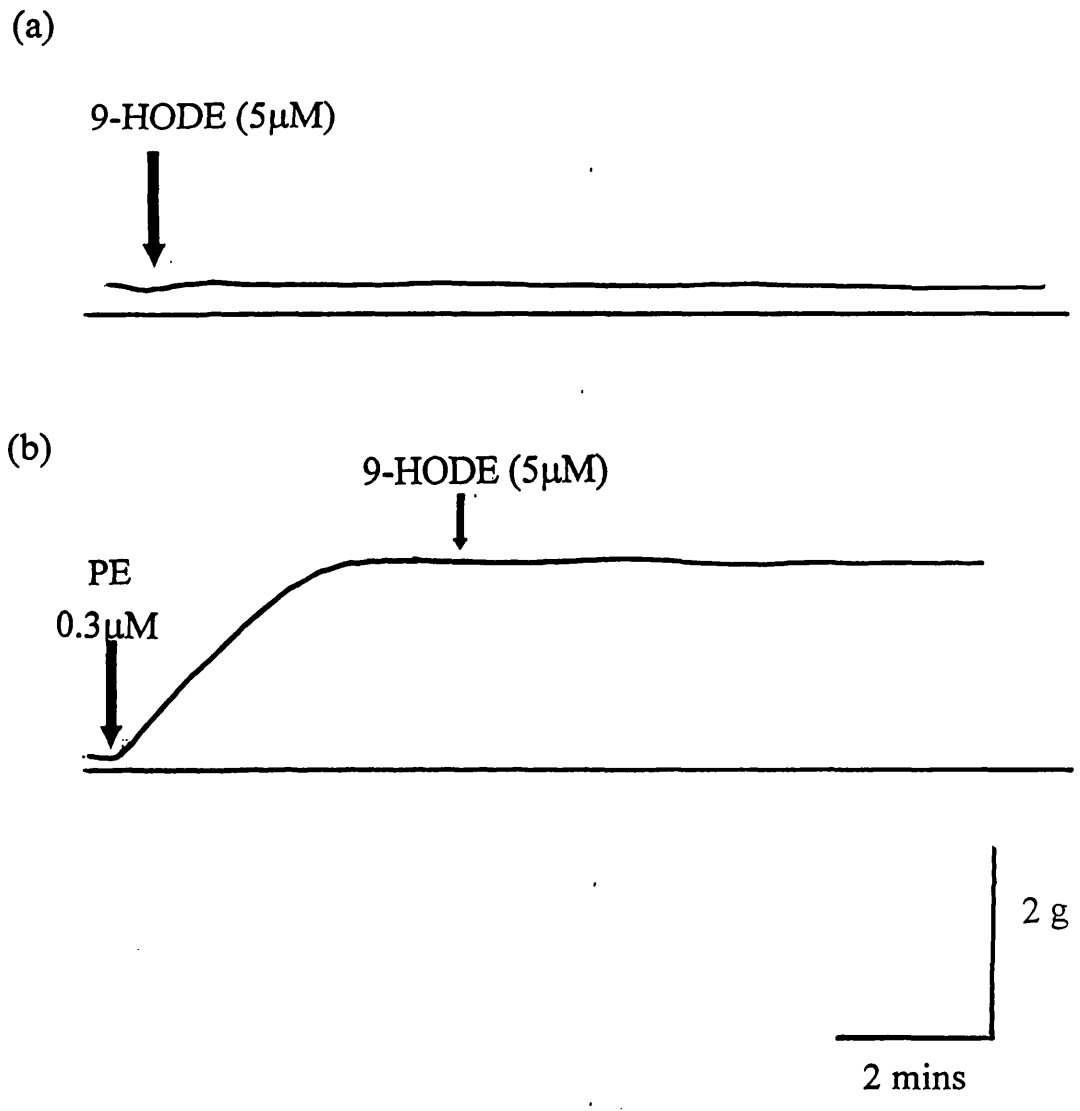


FIGURE 36

Addition of 9-HODE to basal and induced tone.

(a) The addition of 9-HODE (5μM) to basal tone
(b) The addition of 9-HODE (5μM) to an aortic ring precontracted with PE (0.3μM)

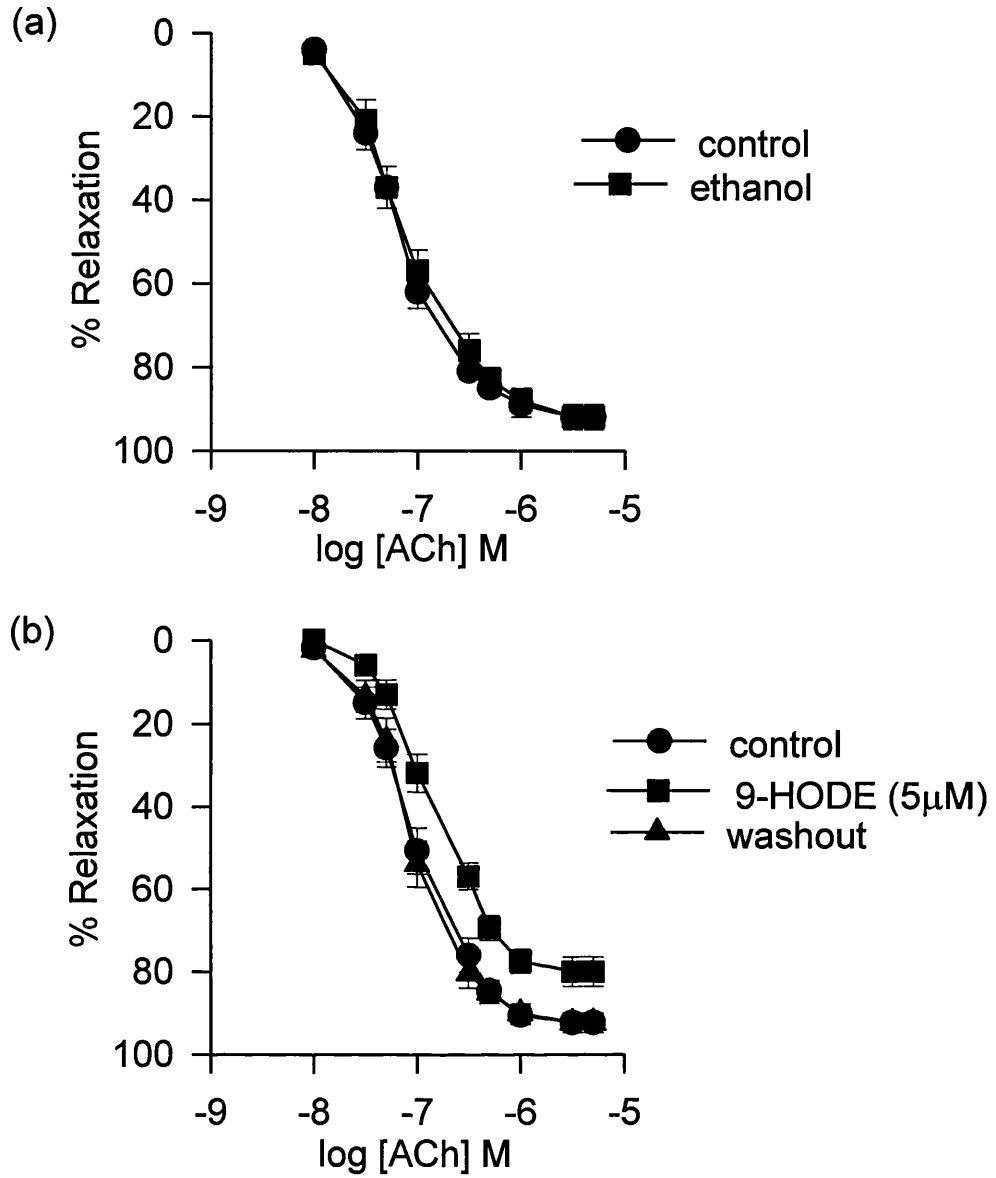


FIGURE 37

ACh-evoked relaxations in the absence and presence of 9-HODE.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were exposed to 9-HODE (5 μM) or an equivalent volume of ethanol and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out.

(a) Control tissues

(b) 9-HODE (5μM; n=7)

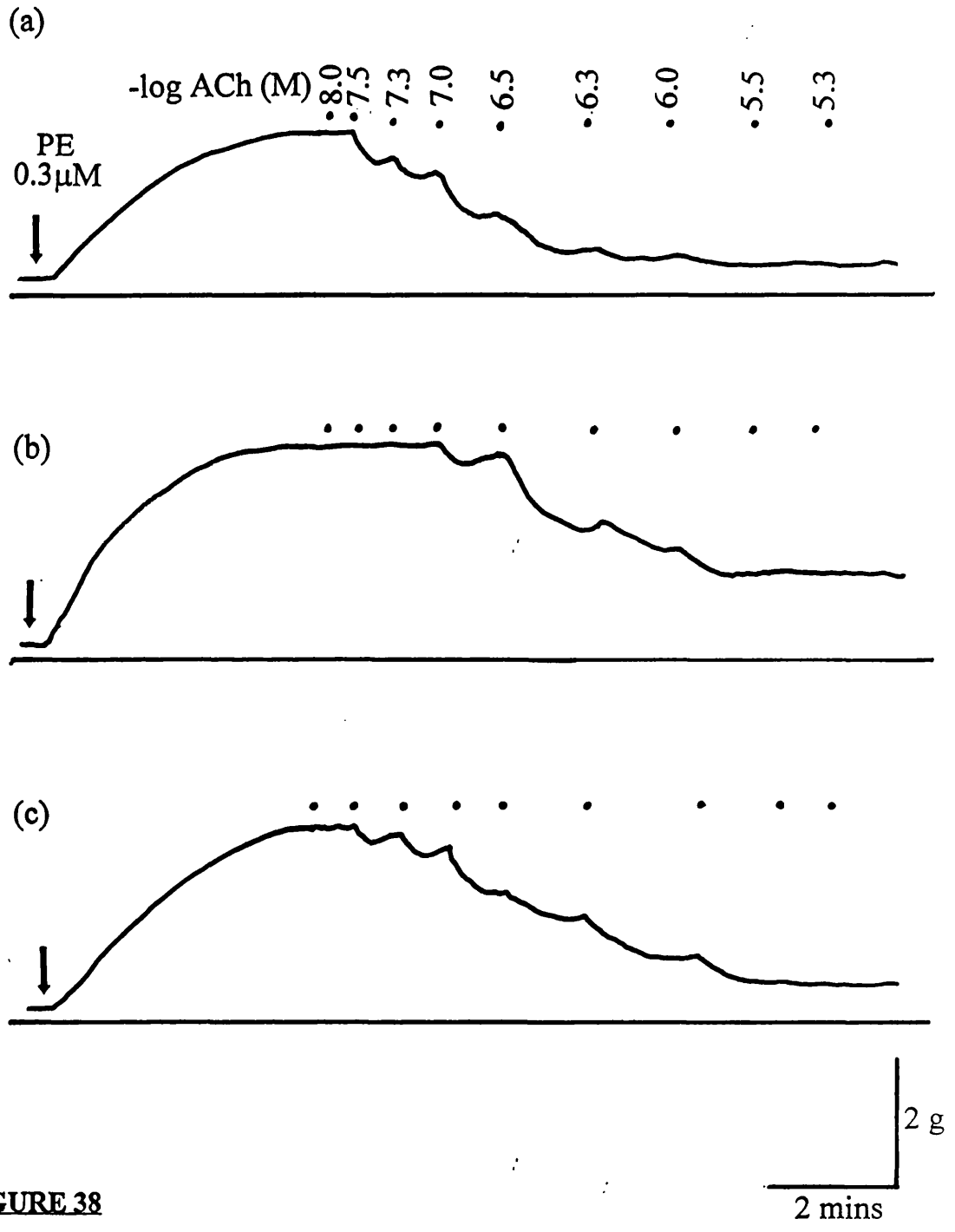


FIGURE 38

ACh-evoked relaxations in the absence and presence of 9-HODE.

Tissues were precontracted with PE (0.3μM) and relaxed with cumulative concentrations of ACh (10nM - 5μM).

(a) Control
(b) In the presence of 9-HODE (5μM)
(c) After washout

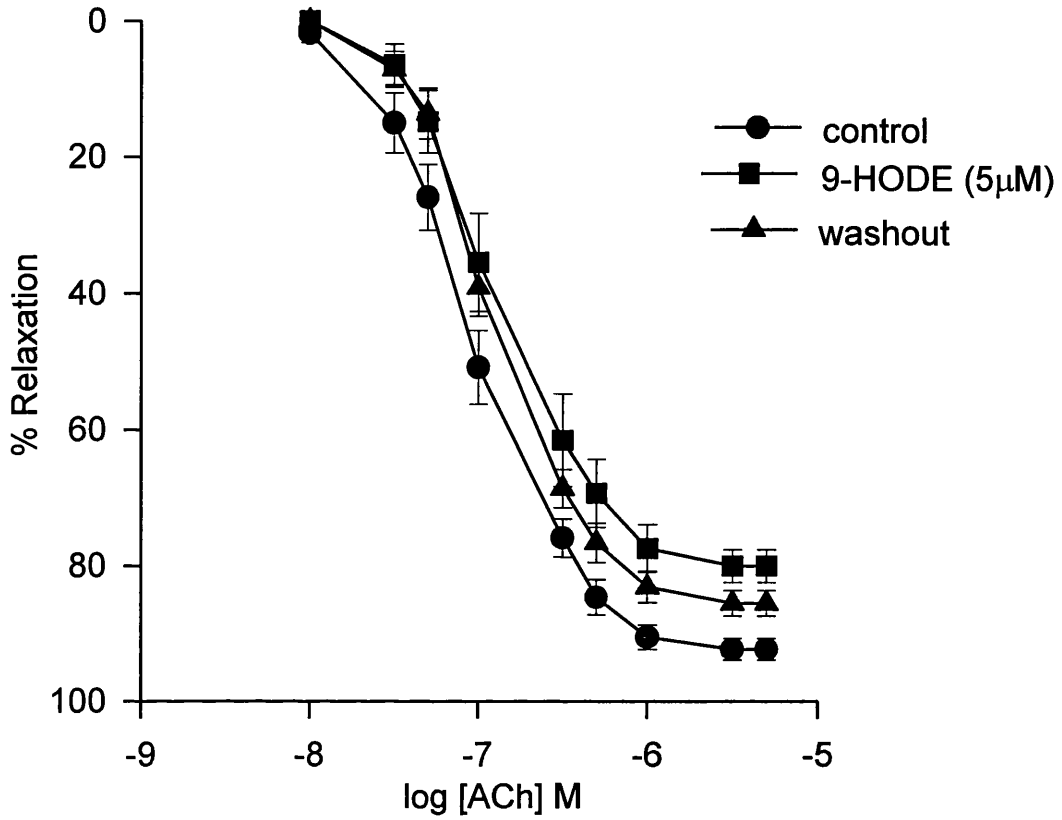


FIGURE 39

The effect of 9-HODE on ACh-evoked relaxations following 30 mins preincubation.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were preincubated with 9-HODE (5 μM) for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out (*n*=8).

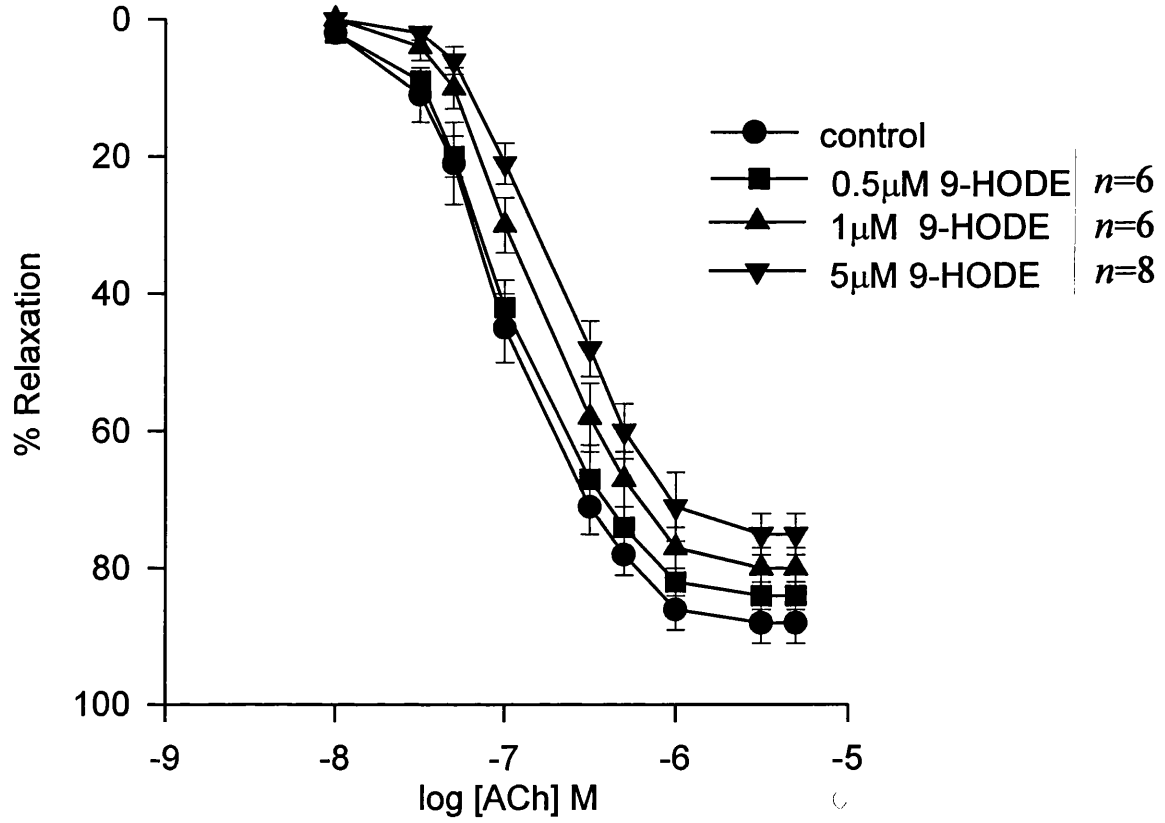


FIGURE 40

Concentration-dependent inhibition of ACh-evoked relaxations by 9-HODE.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were exposed to 9-HODE (0.5, 1 or 5 μM) and the contraction/relaxation cycle repeated.

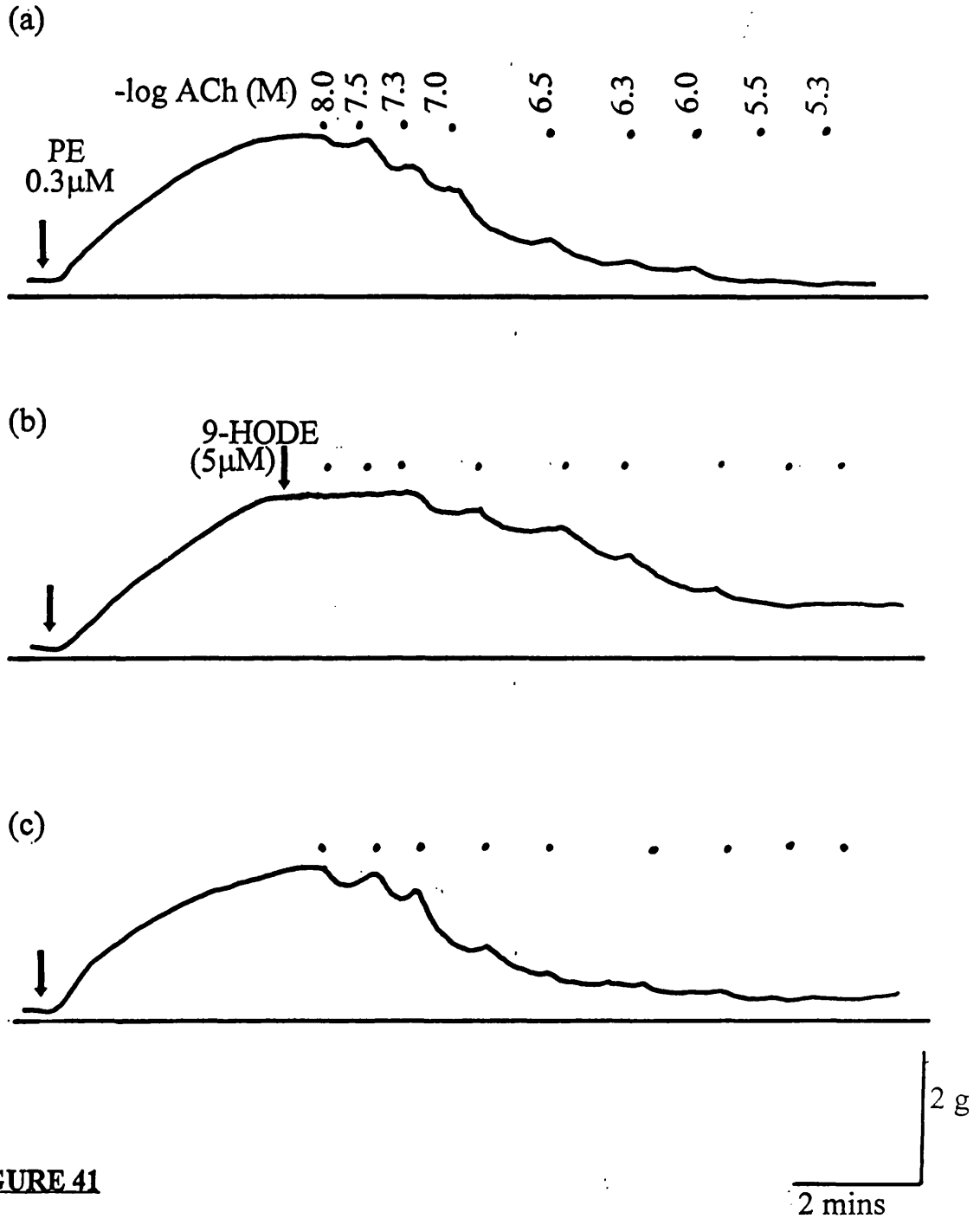


FIGURE 41

Inhibition of ACh-evoked relaxations following the addition of 9-HODE to the precontracted tissue.

Tissues were precontracted with PE (0.3μM) and relaxed with cumulative concentrations of ACh (10nM - 5μM)

(a) Control

(b) In the presence of 9-HODE (5μM) added immediately before the first concentration of ACh

(c) After washout

contraction with a reduction in the maximum level of relaxation from 85.0 ± 3.0% (n=4) to 73.3 ± 2.5% (n=4; p<0.05). Relaxations were restored to control values (82.3 ± 2.8%; n=4) after the hydroxy acid was washed out.

5.3.2 THE EFFECT OF OTHER LINOLEIC ACID METABOLITES ON ACh-EVOKED RELAXATIONS

The effects of 9-HPODE, the hydroperoxy precursor of 9-HODE, and 13-HODE on endothelium-dependent relaxations evoked by ACh were also studied. The fatty acid metabolites were added immediately before the tissues were precontracted.

In the presence of 9-HPODE (5 μM), ACh-evoked relaxations of aortic rings precontracted with phenylephrine were inhibited. The maximum relaxation to ACh was reduced from 79.0 ± 3.0% (n=4) to 69.5 ± 1.9% (n=4; p<0.05) following exposure to 9-HPODE. After washout relaxations were restored to control values (76.3 ± 2.7%; n=4). **Figure 42** shows concentration-response curves to ACh in the presence and absence of 9-HPODE.

The addition of 13-HODE (5 μM) to aortic rings resulted in a similar attenuation of ACh-evoked relaxations which was reversible on washout of the hydroxy acid as shown in **Figure 43**. The maximum level of relaxation was reduced from 89.0 ± 2.8% (n=5) to 76.7 ± 3.4% (n=5; p<0.05) in the presence of 13-HODE.

5.3.3 THE EFFECT OF ASCORBIC ACID ON THE ATTENUATION OF ACh-EVOKED RELAXATIONS BY 9-HODE

The influence of the antioxidant, ascorbic acid on the attenuation of endothelium-dependent relaxations by 9-HODE was assessed.

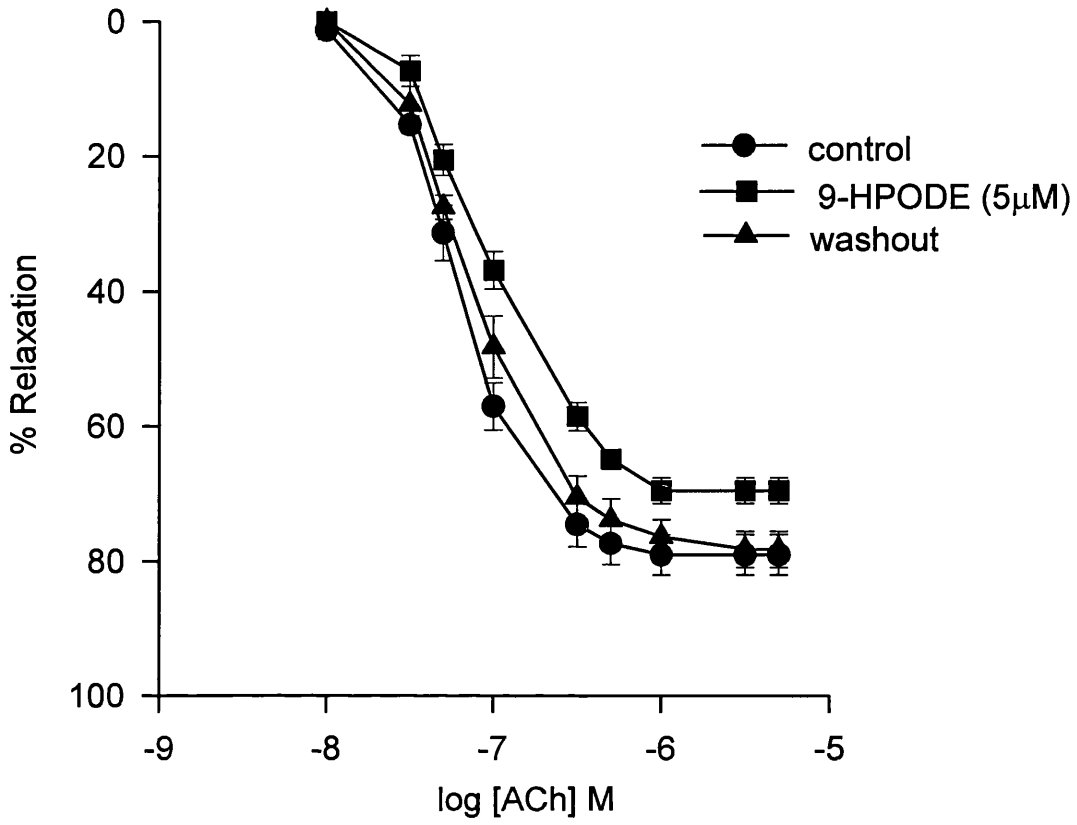


FIGURE 42

The influence of 9-HPODE on ACh-evoked relaxations.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were exposed to 9-HPODE (5 μM) and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out (n=4).

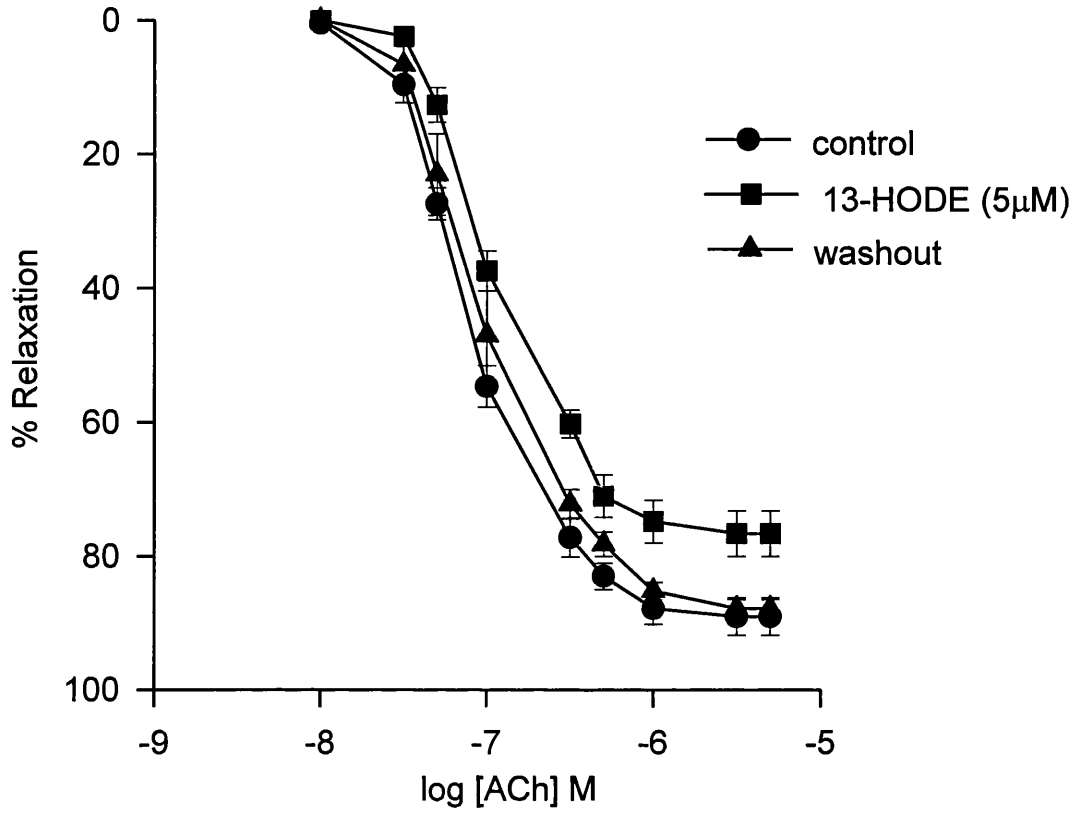


FIGURE 43

The influence of 13-HODE on ACh-evoked relaxations.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were exposed to 13-HODE (5 μM) and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out (n=5).

Ascorbic acid alone had no effect on ACh-evoked relaxations of aortic rings precontracted with phenylephrine as shown in the concentration-response curves in **Figure 44a**. When added together with 9-HODE (5 μM), ascorbic acid did not potentiate nor reduce the inhibition of relaxations caused by the presence of 9-HODE. Ascorbic acid (100 μM) was added to the organ bath at the same time as the 9-HODE. **Figure 44b** shows the influence of ascorbic acid on the concentration-response curves to ACh in the presence of 9-HODE (5 μM). The maximum level of relaxation was significantly reduced by 13.3 ± 1.2% (n=7; p<0.05) following exposure to 9-HODE alone and by 14.3 ± 1.6% (n=5; p<0.05) in the presence of ascorbic acid and 9-HODE.

This finding indicates that the inhibition of ACh-evoked relaxations observed following treatment with 9-HODE is not due to the oxidation of the hydroxy acid in the organ bath and that 9-HODE is the active compound.

5.3.4 THE INFLUENCE OF INDOMETHACIN ON THE INHIBITION OF ACh-EVOKED RELAXATIONS BY 9-HODE

Some of the vascular effects of hydroperoxy and hydroxy acids have been reported to be partly due to the production of cyclo-oxygenase products (Van Diest *et al.*, 1991; De Meyer *et al.*, 1992). Therefore, the cyclo-oxygenase inhibitor, indomethacin was used to investigate whether products of the cyclo-oxygenase pathway may be involved in the inhibition of endothelium-dependent responses to ACh by 9-HODE.

Indomethacin (10 μM) alone had no effect on ACh-evoked relaxations in aortic rings precontracted with phenylephrine (see section 4.4.2). Furthermore, preincubation of the tissues with indomethacin for 15 mins prior to the addition of 9-HODE did not have any influence on the

attenuation of ACh-evoked relaxations observed in the presence of 9-HODE alone. **Figure 45** shows concentration-response curves to ACh following treatment with 9-HODE (5μM) in the presence of indomethacin. The maximum relaxation to ACh was significantly reduced from 91.6 ± 1.6% (n=7) to 80.0 ± 2.4% (n=7; p<0.05; section 5.3.1) in the presence of 9-HODE alone and from 94.5 ± 2.3% (n=4) to 80.5 ± 4.7% (n=4; p<0.05) in the presence of 9-HODE and indomethacin.

This finding suggests that the production of cyclo-oxygenase products by the endothelium do not contribute to the inhibition of ACh-evoked relaxations of phenylephrine precontracted tissues observed in the presence of 9-HODE.

5.3.5 THE EFFECT OF CHELERYTHRINE CHLORIDE ON THE INHIBITION OF ACh-EVOKED RELAXATIONS BY 9-HODE

The influence of the protein kinase C inhibitor, chelerythrine chloride on the attenuation of endothelium-dependent relaxations of aortic rings by 9-HODE was assessed.

Chelerythrine chloride alone had no effect on endothelium-dependent relaxations in tissues precontracted with phenylephrine (see section 4.4.4). Preincubation of the tissues with chelerythrine chloride (1 μM) for 15 mins before the addition of 9-HODE prevented the inhibition of ACh-evoked relaxations observed in the presence of the hydroxy acid alone. **Figure 46** shows the concentration response curves to ACh following treatment with 9-HODE (5 μM) in the presence and absence of chelerythrine chloride. The maximum relaxation to ACh in the presence of 9-HODE and chelerythrine chloride was 92.3 ± 1.9% (n=4) compared with 93.8 ± 2.4% (n=4) in control

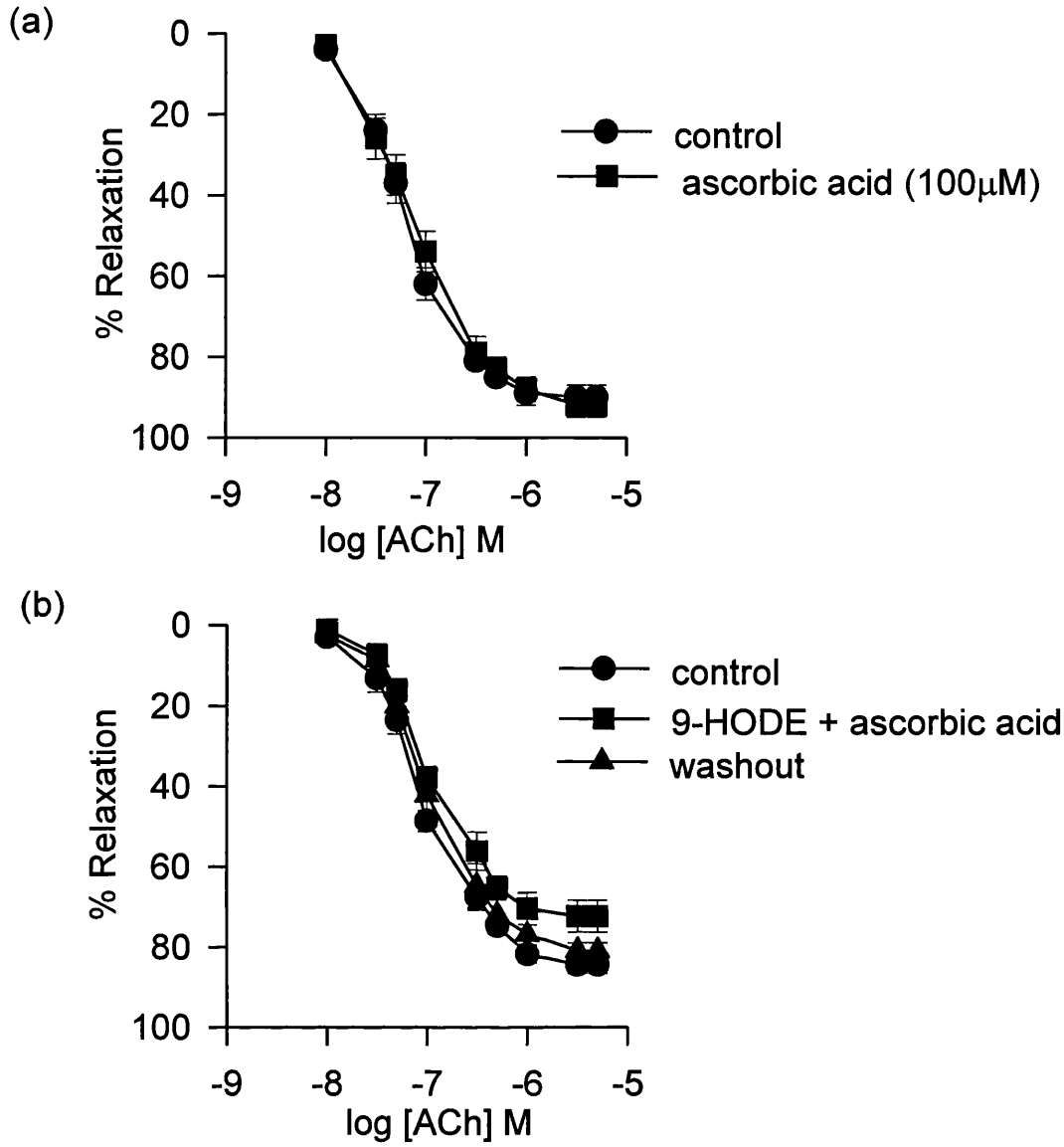


FIGURE 44

The effect of ascorbic acid on the inhibition of ACh-evoked relaxations by 9-HODE.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were exposed to ascorbic acid (100 μM) and 9-HODE (5 μM) and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out.

(a) Ascorbic acid alone (n=5)

(b) Ascorbic acid and 9-HODE (n=5)

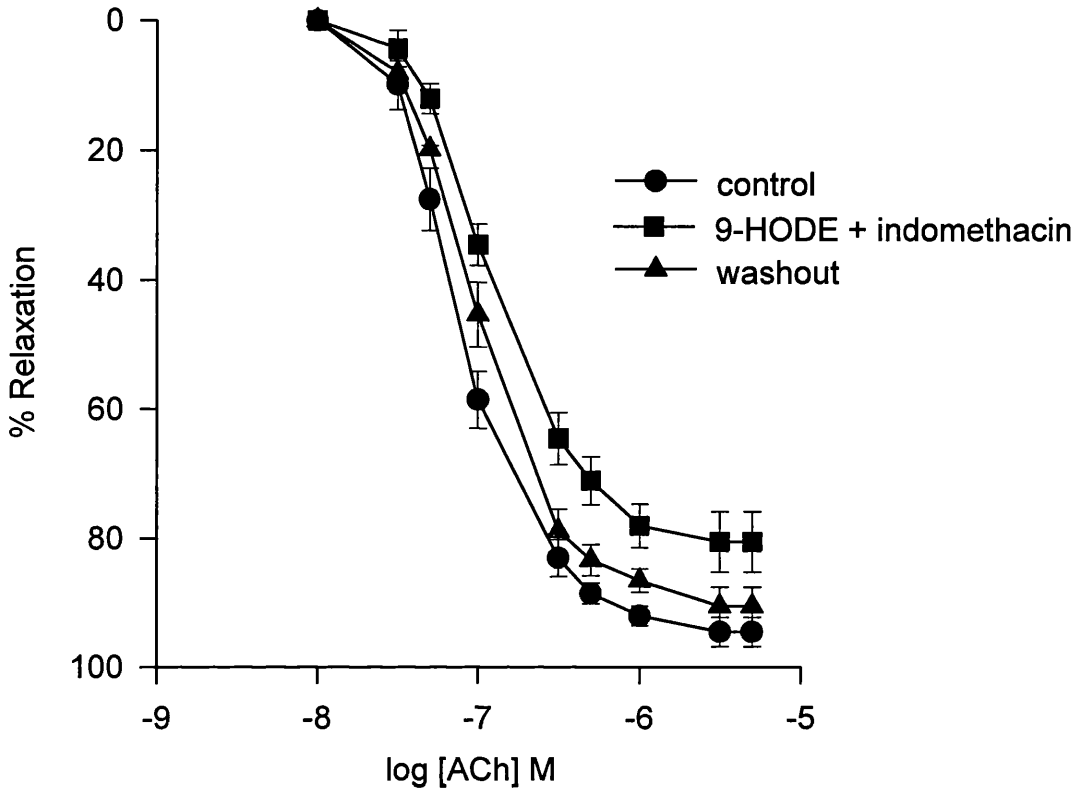


FIGURE 45

The influence of indomethacin on the inhibition of ACh-evoked relaxations by 9-HODE.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were incubated with indomethacin (10 μM) for 15 mins before exposure to 9-HODE (5 μM) and the contraction/relaxation cycle was repeated. After further washout a third relaxation curve to ACh was carried out (n=4).

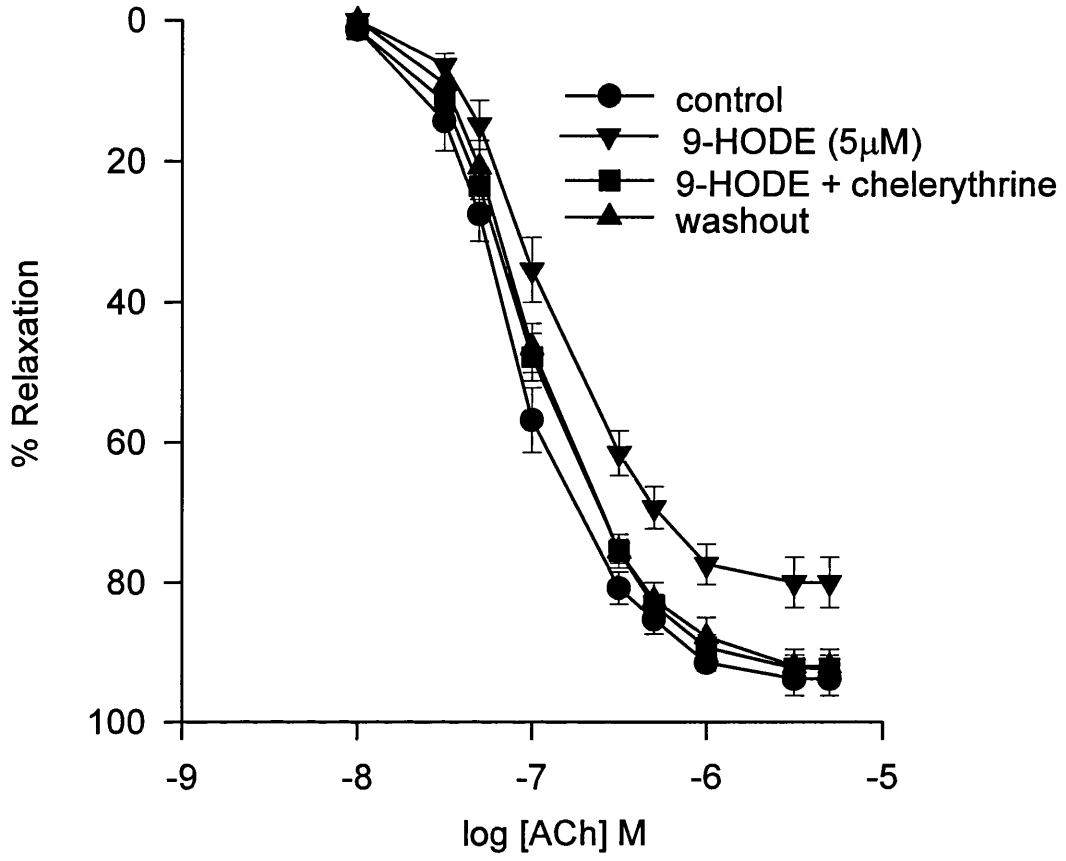


FIGURE 46

The influence of chelerythrine chloride on the attenuation of ACh-evoked relaxations by 9-HODE.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were incubated with chelerythrine chloride (1 μM) for 15 mins before exposure to 9-HODE (5 μM) and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out (n=4).

tissues. In the presence of 9-HODE alone the maximum response to ACh was inhibited by 12.7 ± 1.7% (n=7; p<0.05).

This finding indicates that 9-HODE inhibits ACh-evoked relaxations of phenylephrine precontracted tissues by a mechanism involving protein kinase C.

5.4 THE EFFECT OF LINOLEIC ACID METABOLITES ON GTN-EVOKED RELAXATIONS

The nitrovasodilator GTN evokes endothelium-independent relaxations of vascular smooth muscle by activation of soluble guanylate cyclase leading to an increase in cGMP. To assess whether the inhibition of ACh-evoked responses of phenylephrine precontracted tissues by hydroperoxy and hydroxy derivatives of linoleic acid was due to a direct action on the vascular smooth muscle, the effects of linoleic acid metabolites on relaxations evoked by GTN were investigated.

Endothelium intact or denuded tissues were preincubated with 9-HODE, 9-HPODE or 13-HODE for 30 mins before the addition of phenylephrine and cumulative concentrations of GTN (1 nM - 0.5 μM). None of hydroperoxy and hydroxy acids studied had any effect on GTN-evoked relaxations in either endothelium intact or denuded tissues precontracted with phenylephrine. **Figure 47** shows concentration-response curves to GTN in intact and denuded tissues in the presence and absence of 9-HODE. The maximum response to GTN in endothelium intact and denuded tissues, respectively, was 96.4 ± 2.2% (n=8) and 98.9 ± 0.9% (n=7) in the absence of 9-HODE and 92.6 ± 3.4% (n=8; p>0.05) and 97.1 ± 1.9% (n=7; p>0.05)

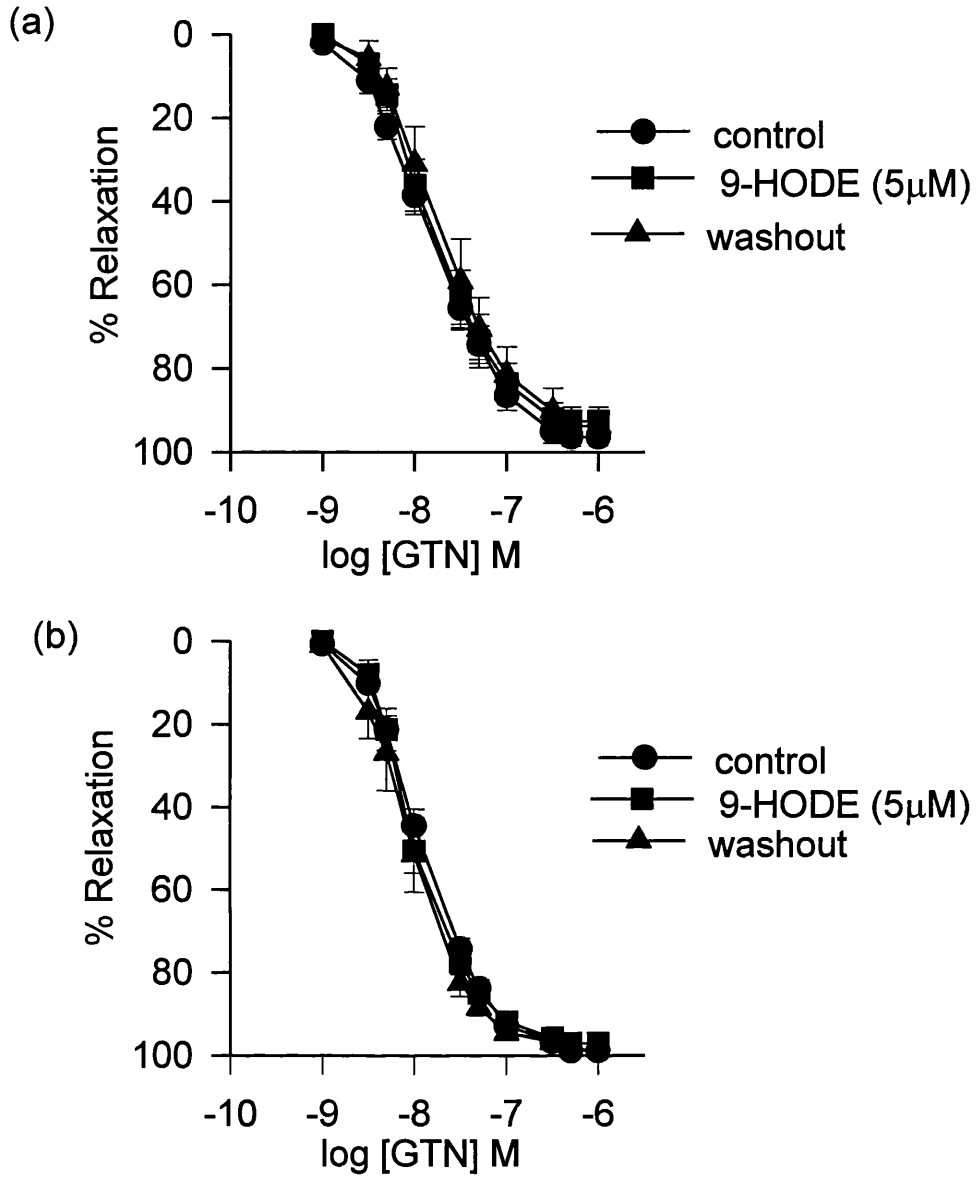


FIGURE 47

The effect of 9-HODE on GTN-evoked relaxations in endothelium-intact and denuded tissues.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of GTN (1 nM - 1 μM). Following washout, tissues were exposed to 9-HODE (5 μM) for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to GTN was carried out.

(a) Endothelium-intact tissues (n=8)

(b) Endothelium-denuded tissues (n=7)

following exposure to 9-HODE. Similar results were obtained when tissues were preincubated with either 9-HPODE or 13-HODE.

5.5 METABOLITES OF ARACHIDONIC ACID AND VASCULAR TONE

5.5.1 ARACHIDONIC ACID METABOLITES AND BASAL TONE

Endothelium intact or denuded aortic rings were exposed to increasing concentrations of 15-HPETE (10 nM - 5 μM) and 15-HETE (5 μM). 15-HPETE induced contractions of the tissues with a threshold concentration of 1 μM. The reactivity of the vessels was not altered by the presence of the endothelium. In endothelium-intact tissues, 15-HPETE (5 μM) induced contractions of 0.66 ± 0.07 g (n=14; p<0.05) compared with 0.73 ± 0.11 g (n=8; p<0.05) in endothelium-denuded aortic rings. **Figure 48a** shows the effect of 5 μM 15-HPETE on the basal level of tone of an endothelium-intact tissue.

The addition of 15-HETE to resting tissues resulted in a contraction which was significantly greater than that obtained with 15-HPETE. 15-HETE (5 μM) evoked a contraction of 1.13 ± 0.24 g (n=4; p<0.05) in endothelium-intact tissues and 1.13 ± 0.06 g (n=4; p<0.05) in endothelium-denuded preparations. The effect of 5 μM 15-HETE on the resting tone of an intact tissue is illustrated in **Figure 48b**.

5.5.2 15-HPETE AND INDUCED TONE

The addition of 15-HPETE (5 μM) to aortic rings in which the tone was raised with phenylephrine (0.1 - 0.3 μM) produced a further contraction in

both endothelium-intact and -denuded tissues. 15-HPETE caused a significant increase in tension from 1.85 ± 0.37 g (n=4) to 2.60 ± 0.42 g (n=4; p<0.05) when added to precontracted tissues with an intact endothelium and from 1.97 ± 0.32 g (n=4) to 2.85 ± 0.21 g (n=4; p<0.05) when added to endothelium-denuded tissues. The effect of 5 μM 15-HPETE on a precontracted intact tissue is shown in **Figure 49.**

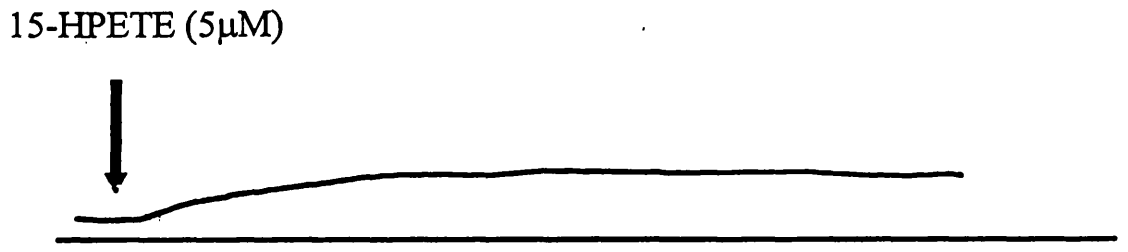
The size of the contractions evoked by 15-HPETE in resting tissues and in aortic rings with induced tone were not significantly different in endothelium-intact or denuded preparations (p>0.05).

5.6 THE EFFECT OF METABOLITES OF ARACHIDONIC ACID ON ENDOTHELIUM-DEPENDENT RELAXATIONS

5.6.1 THE EFFECT OF 15-HPETE AND 15-HETE ON ACh-EVOKED RELAXATIONS

Aortic rings were precontracted with phenylephrine to the same level of tone both in the presence and absence of 15-HPETE and 15-HETE. In the presence of 15-HPETE (5 μM), endothelium-dependent relaxations of phenylephrine precontracted tissues were inhibited (**Figure 50b**). This is shown as a rightward shift in the concentration-response curve to ACh and by a significant reduction in the maximum relaxation from 91.7 ± 2.0% (n=10) before the addition of 15-HPETE to 80.3 ± 2.7% (n=10; p<0.05) during exposure to 5 μM 15-HPETE. **Figure 51** shows the concentration-response curves to ACh in the presence and absence of 15-HPETE. The inhibition was reversible and the maximum relaxation restored to control

(a)



(b)

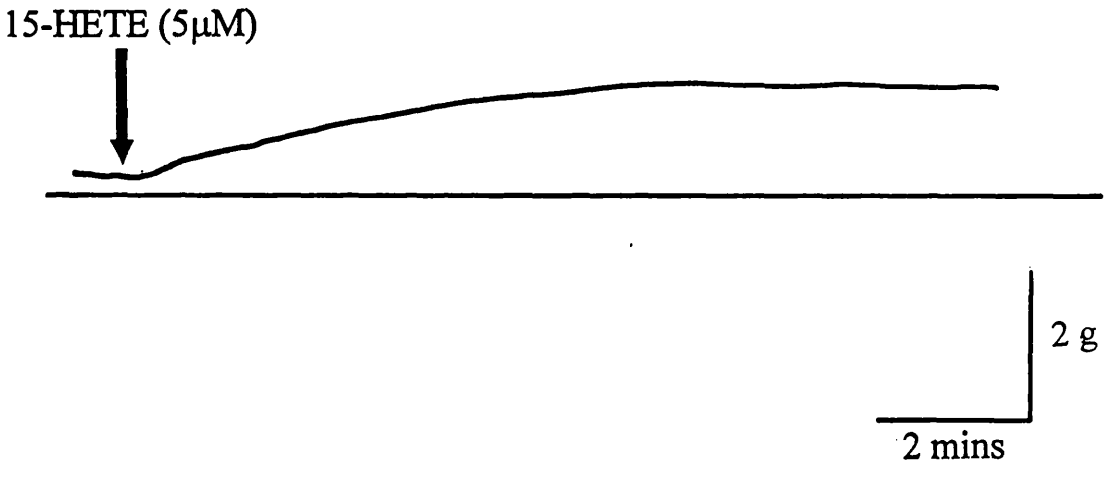


FIGURE 48

Addition of 15-HPETE and 15-HETE to resting tone.

(a) The addition of 15-HPETE (5μM) to basal tone
(b) The addition of 15-HETE (5μM) to basal tone

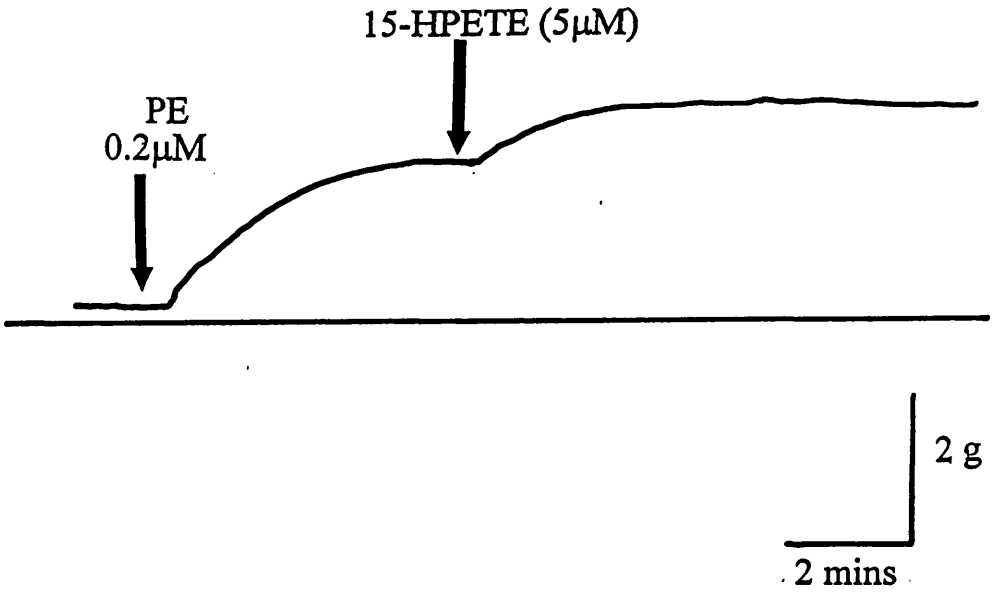


FIGURE 49

Addition of 15-HPETE to precontracted tissues.

The addition of 15-HPETE (5μM) to an aortic ring precontracted with PE (0.2μM)

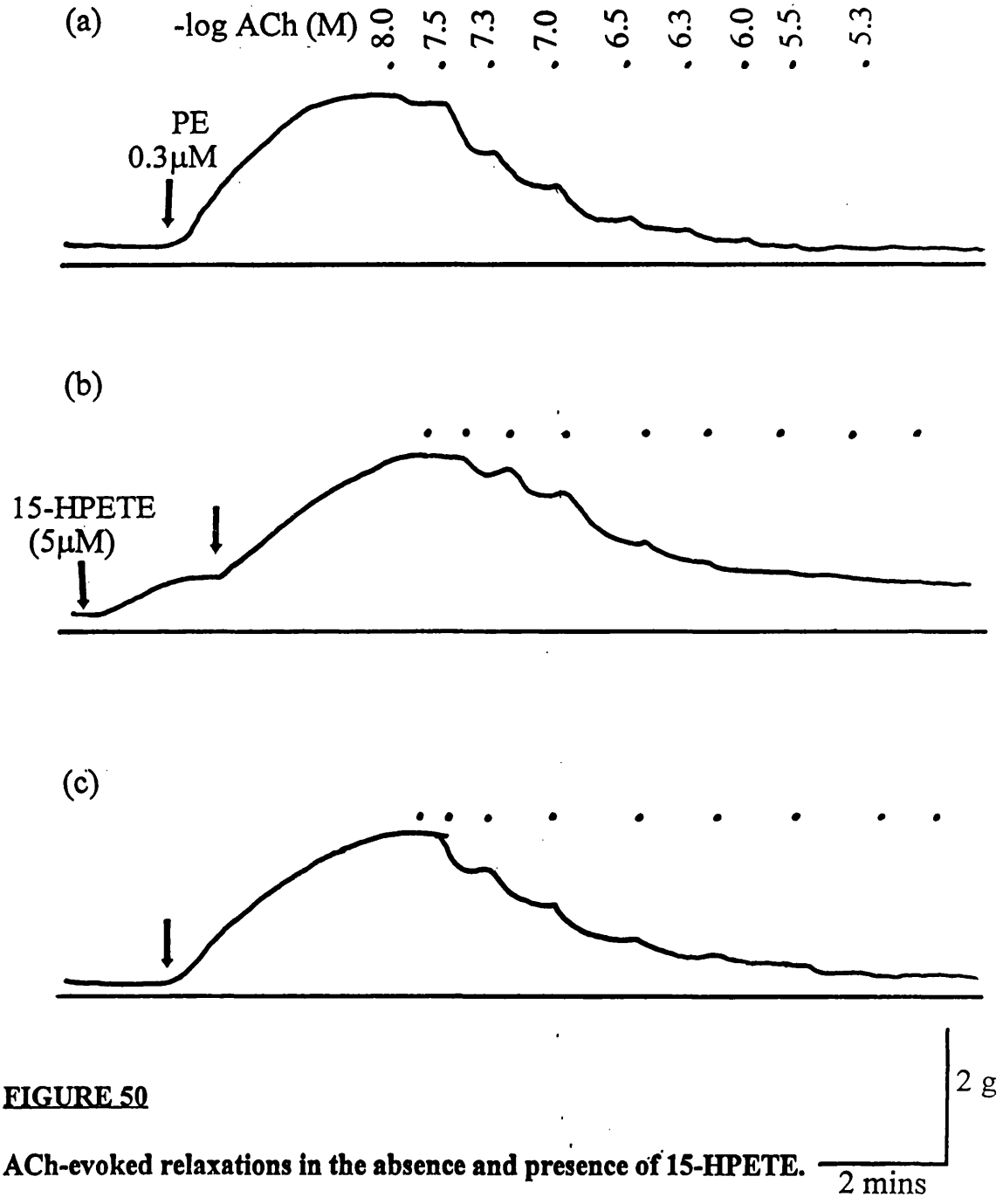


FIGURE 50

ACh-evoked relaxations in the absence and presence of 15-HPETE.

Tissues were precontracted with PE (0.3μM) and relaxed with cumulative concentrations of ACh (10nM - 5μM).

(a) Control
(b) In the presence of 15-HPETE (5μM)
(c) After washout

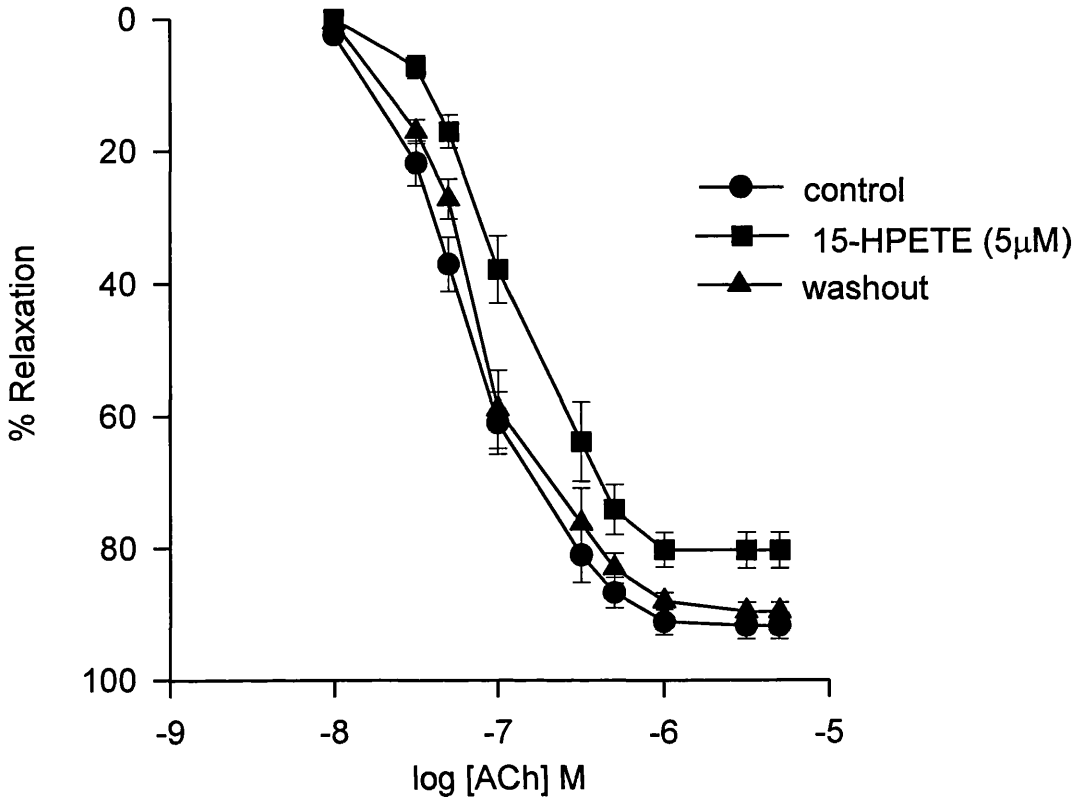


FIGURE 51

The influence of 15-HPETE on ACh-evoked relaxations.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, 15-HPETE (5μM) was added to tissues immediately prior to contraction and the relaxation curve repeated after approximately 5 mins exposure to 15-HPETE. After further washout a third relaxation curve to ACh was carried out. (n=10).

values (88.6 ± 2.4%; n=10) on washout of the hydroperoxy acid (**Figure 50c**).

Preincubation of the tissues with 15-HPETE for 30 mins prior to relaxation with ACh did not enhance the degree of inhibition of endothelium-dependent responses (**Figure 52**). The reduction in the maximum level of relaxation, from 89.6 ± 3.2% (n=6) to 79.4 ± 2.5% (n=6; p<0.05) in the presence of 15-HPETE was similar to that observed when the hydroperoxy acid was added immediately before the tissues were contracted. However, when a 30 min preincubation period was used the inhibitory effect was only partially reversible and the maximum level of relaxation after washout was 82.1 ± 0.6% (n=6; p>0.05).

The inhibitory effect of 15-HPETE could also be demonstrated when the hydroperoxy acid was added to precontracted aortic rings immediately before the addition of ACh. **Figure 53** shows ACh-evoked relaxation of a phenylephrine precontracted tissue following the addition of 15-HPETE to the contracted tissue. The maximum relaxation was reduced from 89.8 ± 2.8% (n=4) in control tissues to 77.5 ± 2.5% (n=4; p<0.05) during exposure to 15-HPETE. After washout the relaxations were restored to control values (87.0 ± 3.4%; n=6).

The threshold concentration for the attenuation of ACh-evoked relaxations by 15-HPETE was 1 μM. The inhibitory effect of 0.5, 1 and 5 μM 15-HPETE on endothelium-dependent relaxations is shown in **Figure 54.**

The addition of the hydroxy derivative of arachidonic acid, 15-HETE (5 μM) immediately before the tissues were contracted resulted in a similar degree of inhibition of ACh-evoked relaxations as its hydroperoxy precursor,

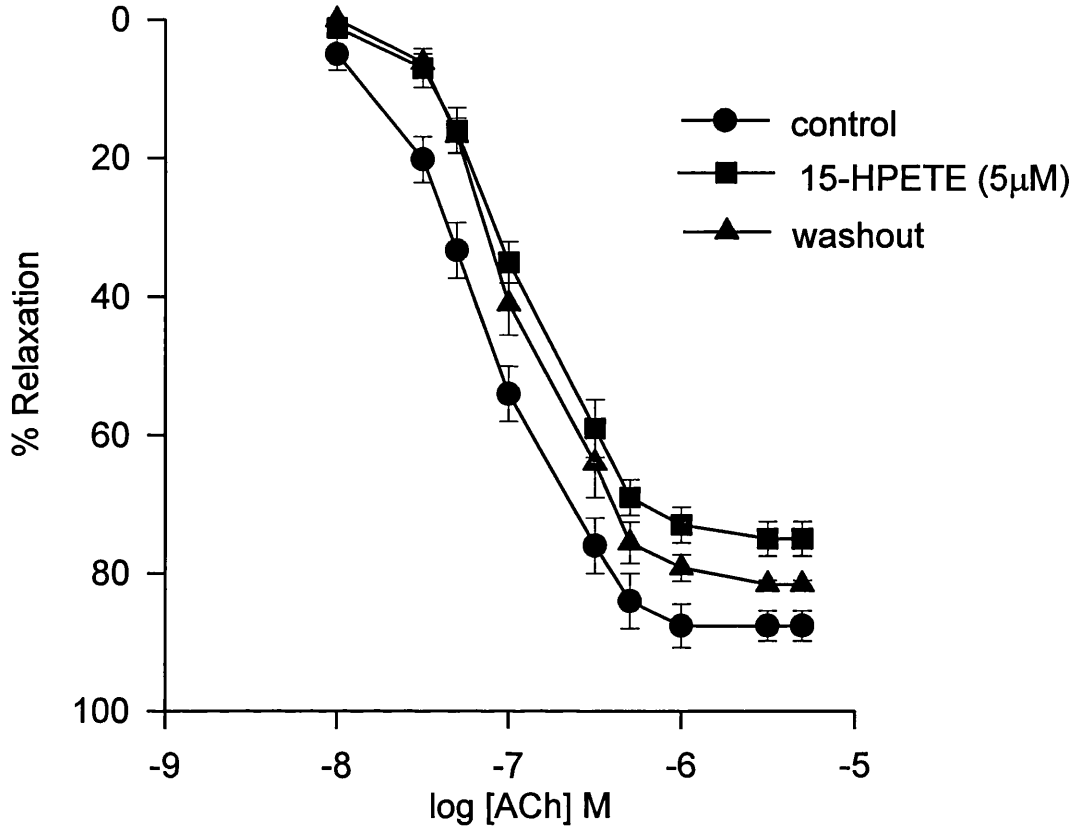


FIGURE 52

ACh concentration-response curves in the absence and presence of 15-HPETE preincubated for 30 mins.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were incubated with 15-HPETE (5 μM) for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out (*n*=6).

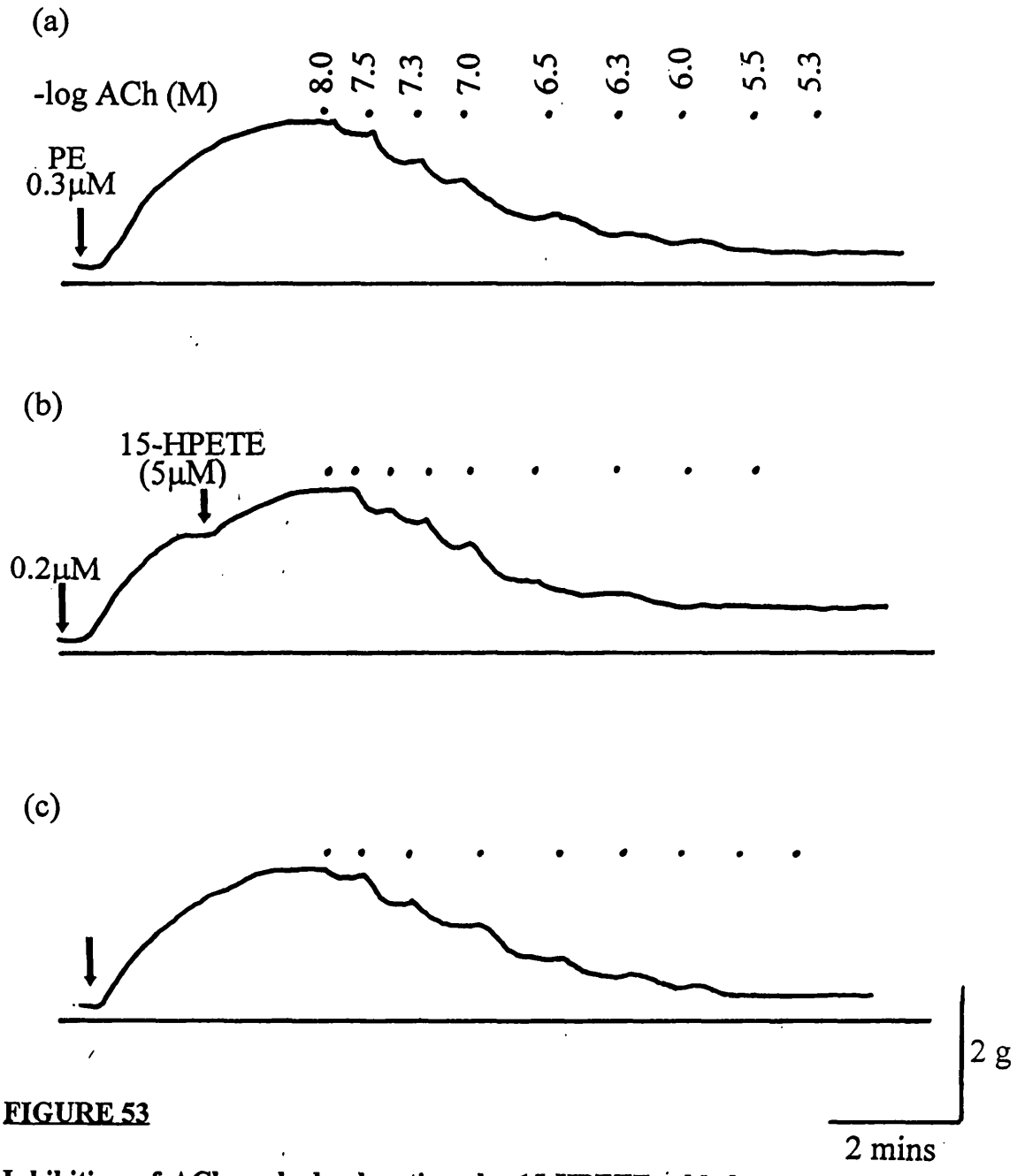


FIGURE 53

Inhibition of ACh-evoked relaxations by 15-HPETE added to precontracted tissues.

Tissues were precontracted with PE (0.3μM) and relaxed with cumulative concentrations of ACh (10nM - 5μM).

(a) Control

(b) In the presence of 15-HPETE (5μM) added immediately before the first concentration of ACh

(c) After washout

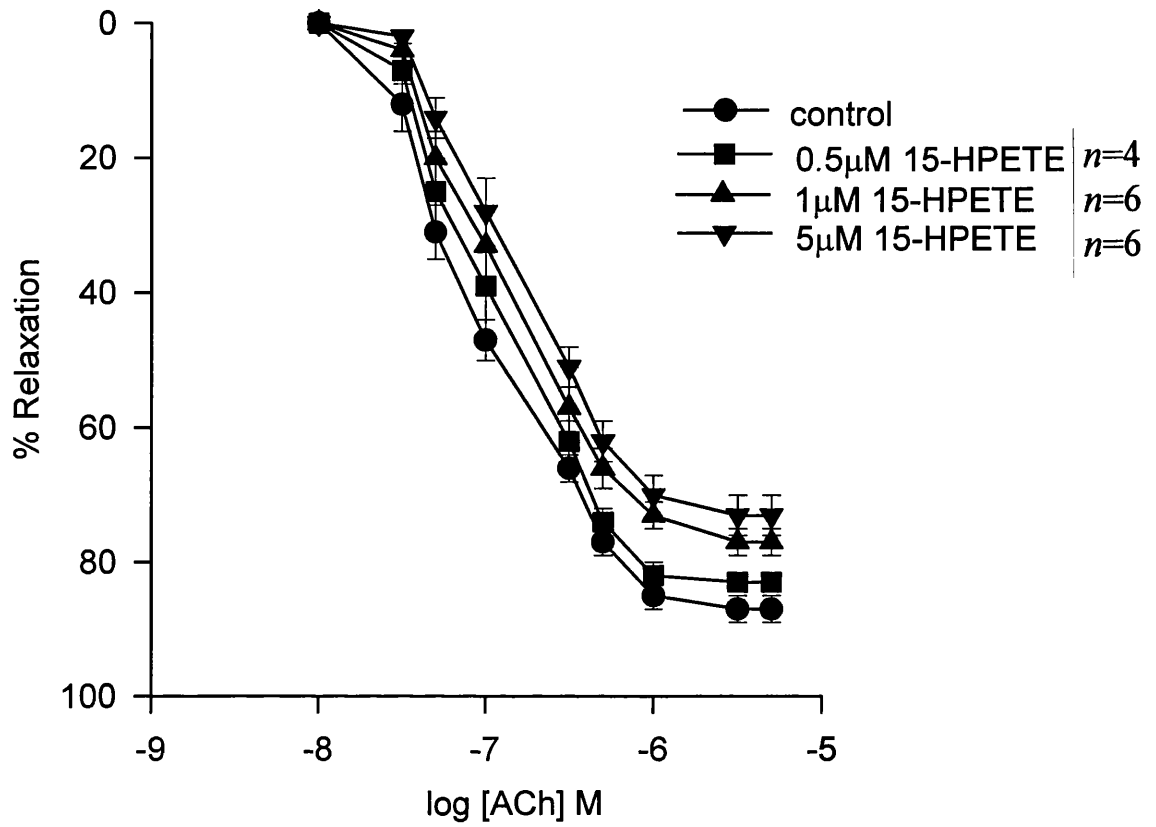


FIGURE 54

Concentration-dependent inhibition of ACh-evoked relaxations by 15-HPETE.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were exposed to 15-HPETE (0.5, 1 or 5μM) and the contraction/relaxation cycle repeated.

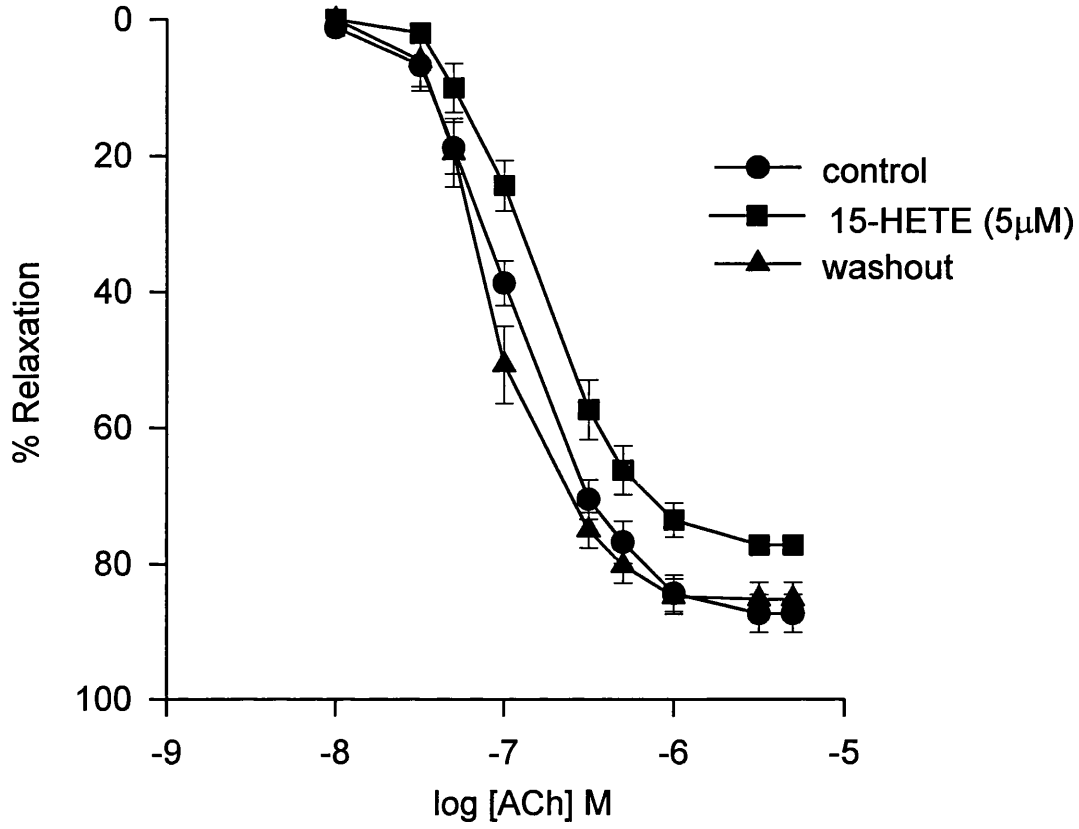


FIGURE 55

The influence of 15-HETE on ACh-evoked relaxations.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were exposed to 15-HETE (5 μM) and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out (n=6).

15-HPETE, as shown in **Figure 55**. The maximum relaxation to ACh was reduced from 87.3 ± 2.8% (n=6) before the addition of 15-HETE to 77.2 ± 1.5% (n=6; p<0.05) during exposure to the hydroxy acid. The relaxations to ACh were restored to control values (85.2 ± 2.5%; n=6) on washout of the 15-HETE.

5.6.2 THE INFLUENCE OF ASCORBIC ACID ON THE INHIBITION OF ACh-EVOKED RELAXATIONS BY 15-HPETE

To investigate whether the inhibition of endothelium-dependent relaxations by 15-HPETE was due to oxidation in the organ bath, the influence of the antioxidant, ascorbic acid was assessed.

Ascorbic acid (100 μM) had no effect on the attenuation of ACh-evoked relaxations of phenylephrine precontracted tissues caused by the presence of 15-HPETE (5 μM) as shown in **Figure 56**. The maximum level of relaxation in the presence of 15-HPETE was 80.3 ± 2.7% (n=10; p<0.05) and in the presence of 15-HPETE and ascorbic acid the maximum relaxation to ACh was 81.2 ± 1.9% (n=4; p<0.05) compared with 92.3 ± 1.1% (n=4) in control tissues.

This finding suggests that the inhibition of endothelium-dependent relaxations caused by 15-HPETE is not due to oxidation in the organ bath and 15-HPETE is the active compound.

5.6.3 THE EFFECT OF INDOMETHACIN ON THE INHIBITION OF ACh-EVOKED RELAXATIONS BY 15-HPETE

The effect of indomethacin on the attenuation of endothelium-dependent relaxations evoked by ACh in the presence of 15-HPETE was assessed.

Preincubation of the tissues with indomethacin (10 μM) for 15 mins prior to the addition of 15-HPETE (5 μM) had no effect on the degree of inhibition caused by the presence of the hydroperoxy acid. The maximum level of relaxation was reduced by 12.5 ± 1.9% (n=10; p<0.05) following exposure to 15-HPETE and by 11.7 ± 0.6% (n=4; p<0.05) in the presence of 15-HPETE and indomethacin. **Figure 57** shows the influence of indomethacin on the concentration-response curves to ACh in the presence of 15-HPETE.

This lack of effect of indomethacin indicates that cyclo-oxygenase products are not involved in the inhibition of ACh-evoked relaxations observed in the presence of 15-HPETE.

5.6.4 THE INFLUENCE OF CHELERYTHRINE CHLORIDE ON THE 15-HPETE INDUCED INHIBITION OF ACh-EVOKED RELAXATIONS

Preincubation of the tissues with the protein kinase C inhibitor, chelerythrine chloride (1 μM) for 15 mins before the addition of 15-HPETE (5 μM) prevented the attenuation of endothelium-dependent responses observed in the presence of the hydroperoxy acid alone as shown in **Figure 58**. The maximum relaxation to ACh in control tissues was 82.9 ± 3.5% (n=7) and in the presence of 15-HPETE and chelerythrine chloride was 82.3 ± 2.5% (n=7; p>0.05). In the presence of 15-HPETE alone, the maximum response to ACh was reduced by 12.5 ± 1.9% (n=10; p<0.05).

This suggests that the inhibitory effect of 15-HPETE on endothelium-dependent relaxations may be mediated *via* a protein kinase C dependent pathway.

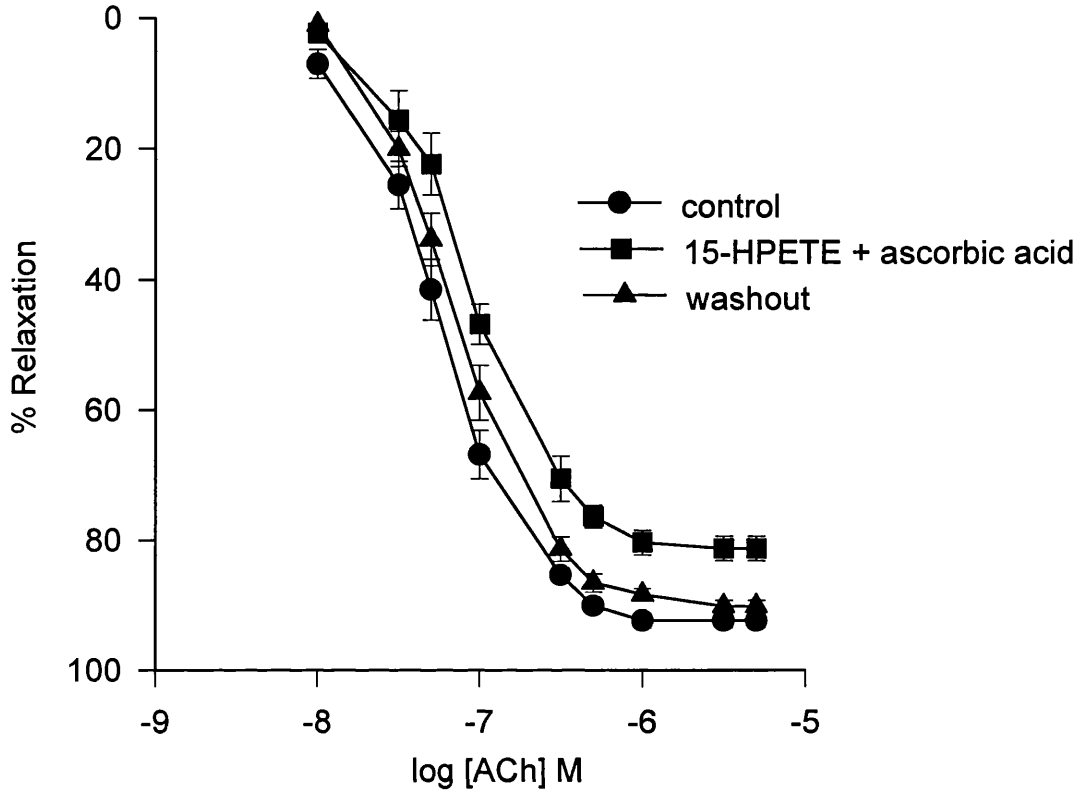


FIGURE 56

The influence of ascorbic acid on the attenuation of ACh-evoked relaxations by 15-HPETE.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were exposed to 15-HPETE (5 μM) in the presence of ascorbic acid (100 μM) and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out (n=4).

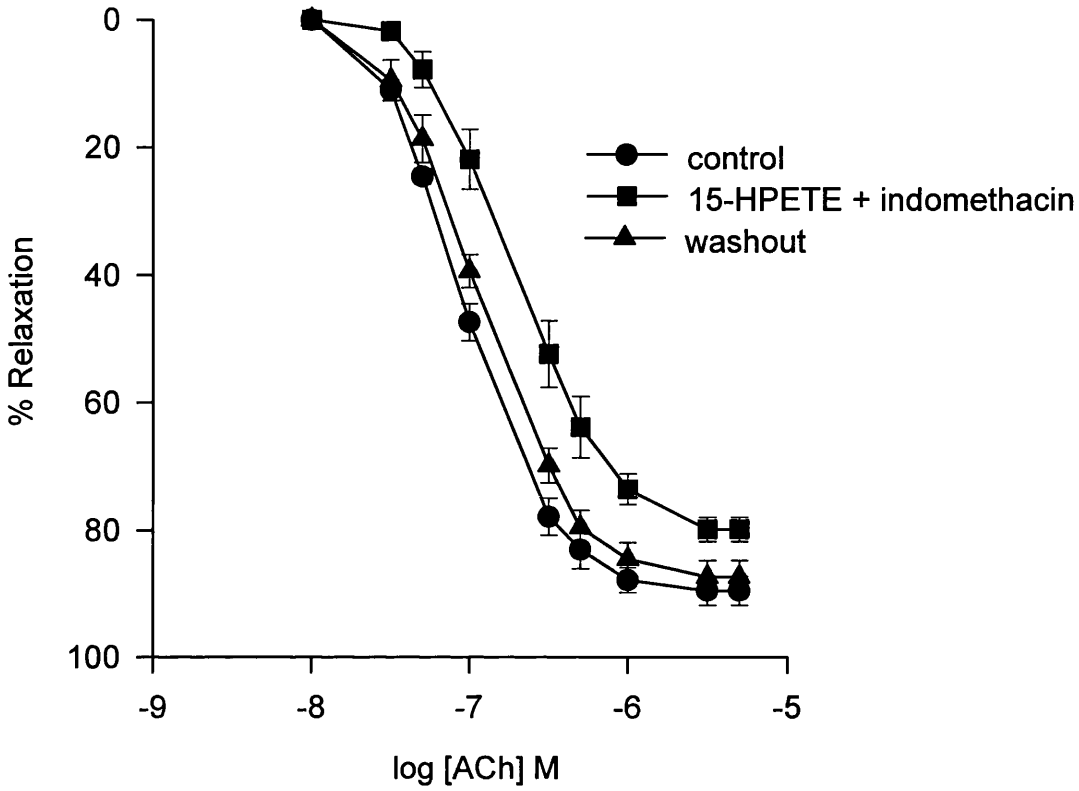


FIGURE 57

The effect of indomethacin on the inhibition of ACh-evoked relaxations by 15-HPETE.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were incubated with indomethacin (10 μM) for 15 mins before exposure to 15-HPETE (5 μM) and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out (n=4).

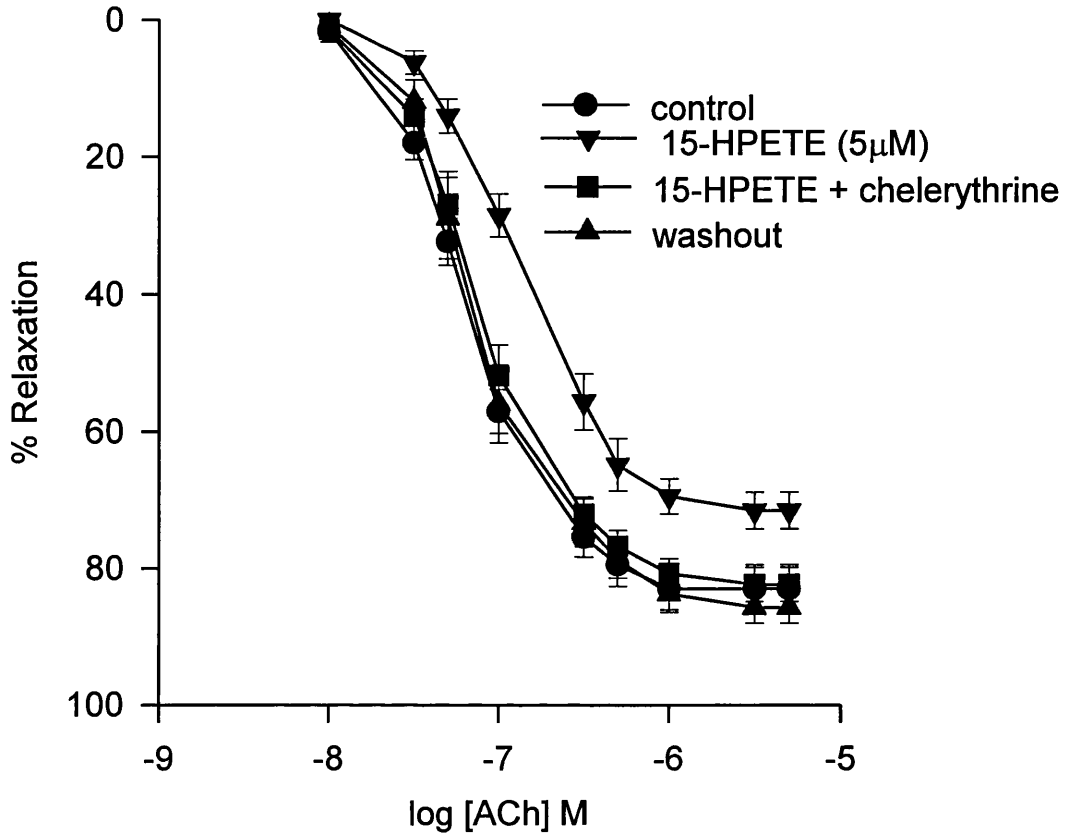


FIGURE 58

The effect of chelerythrine chloride on the attenuation of ACh-evoked relaxations by 15-HPETE.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of ACh (10 nM - 5 μM). Following washout, tissues were incubated with chelerythrine chloride (1 μM) for 15 mins before exposure to 15-HPETE (5 μM) and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to ACh was carried out (n=7).

5.7 15-HPETE AND NITRIC OXIDE EVOKED RELAXATIONS

Endothelium-dependent relaxations evoked by ACh are mediated by the release of NO from the endothelium. NO elicits concentration-dependent, transient relaxations of phenylephrine precontracted tissues which are independent of the endothelium. To investigate whether a direct interaction between 15-HPETE and NO could account for the inhibitory effect of 15-HPETE on ACh-evoked relaxations, the effect of the hydroperoxy acid on relaxations evoked by exogenous NO was assessed.

As shown in **Figure 59** exposure to 15-HPETE (5 μM) had no effect on relaxations to NO in endothelium-denuded aortic rings indicating that the attenuation of endothelium-dependent responses in the presence of 15-HPETE cannot be due to a direct interaction with NO. The maximum relaxation evoked by ACh was 84.7 ± 2.1% (n=10) in control tissues and 83.2 ± 2.9% (n=10; p>0.05) following treatment with 15-HPETE.

5.8 ARACHIDONIC ACID METABOLITES AND GTN-EVOKED RELAXATIONS

The effects of the arachidonic acid metabolites on endothelium-independent relaxations evoked by GTN were investigated.

Preincubation of tissues for 30 mins with 15-HPETE (5 μM), resulted in a significant inhibition of GTN-evoked relaxations in endothelium-intact and denuded aortic rings precontracted with phenylephrine. In endothelium intact tissues the maximum relaxation to GTN was reduced from 100.0% (n=4) to 90.3 ± 3.1% (n=4; p<0.05) following preincubation with 15-HPETE. A

similar degree of inhibition was observed following treatment with 15-HPETE in endothelium denuded tissues. The maximum level of relaxation was reduced from 100.0% (n=4) before the addition of 15-HPETE to 90.0 ± 3.2% (n=4: p<0.05) during exposure to the hydroperoxy acid. The relaxations to GTN were restored to control values on washout of the 15-HPETE. **Figure 60** shows the concentration response curves to GTN in the presence and absence of 15-HPETE in endothelium intact and denuded aortic rings.

The hydroxy derivative, 15-HETE (5 μM), also inhibited relaxations evoked by GTN following 30 mins preincubation. The maximum level of relaxation in tissues with an intact endothelium was reduced from 94.0 ± 2.1% (n=6) to 82.7 ± 3.0% (n=6; p<0.05) in the presence of 15-HETE. In endothelium denuded tissues, a similar degree of inhibition of GTN-evoked relaxations was observed following preincubation with 15-HETE with the maximum relaxation reduced from 93.0 ± 3.2% (n=6) to 83.0 ± 2.9% (n=6; p<0.05). Concentration-response curves for GTN in the presence and absence of 15-HETE in intact and denuded aortic rings are shown in **Figure 61.**

5.9 DISCUSSION

The inhibition of endothelium-dependent relaxations of rabbit aortic rings by OXLDL is well documented (see section 1.6.4). However, the specific constituents of the modified lipoprotein responsible for the altered vascular responses are unknown. Cellular 15-lipoxygenases have been implicated in the oxidative modification of LDL (Parthasarathy *et al.,* 1989) and an increase in 15-lipoxygenase activity has been demonstrated in aortic tissue

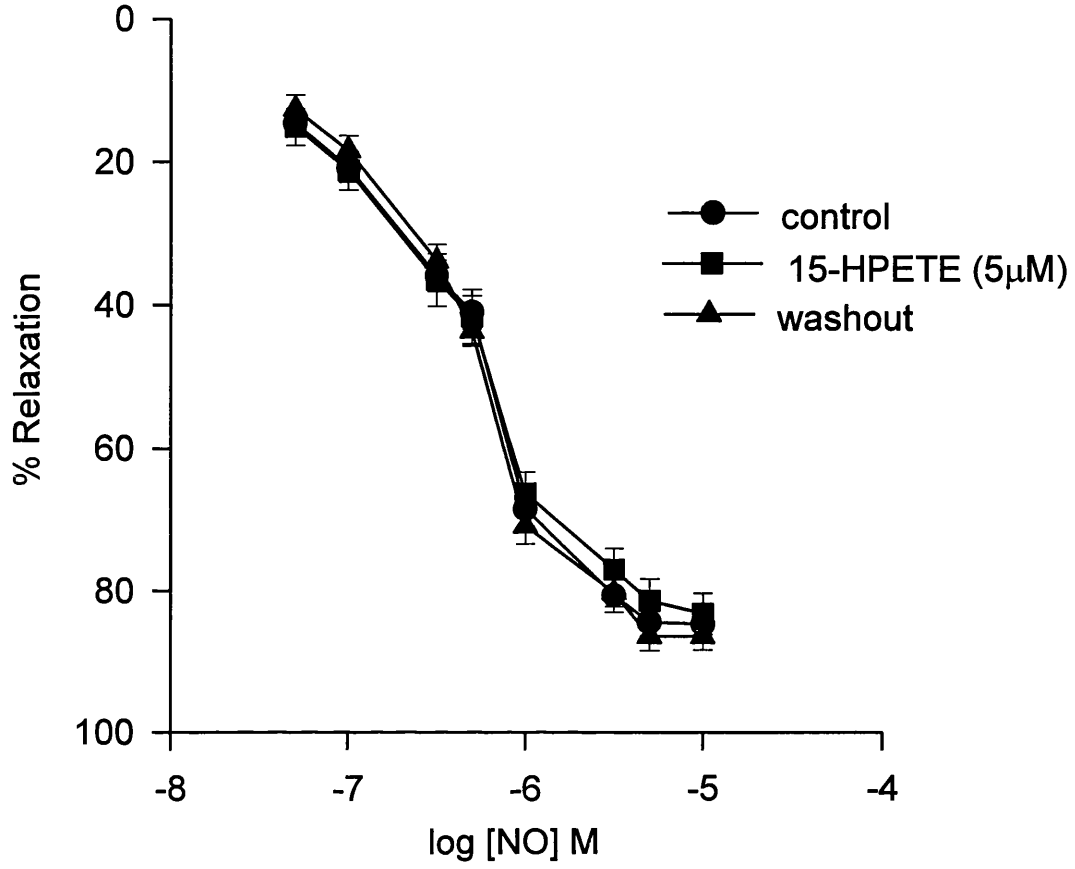


FIGURE 59

The influence of 15-HPETE on NO-evoked relaxations in endothelium-denuded tissues.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of NO (50 nM - 10 μM). Following washout, tissues were exposed to 15-HPETE (5 μM) and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to NO was carried out (*n*=10).

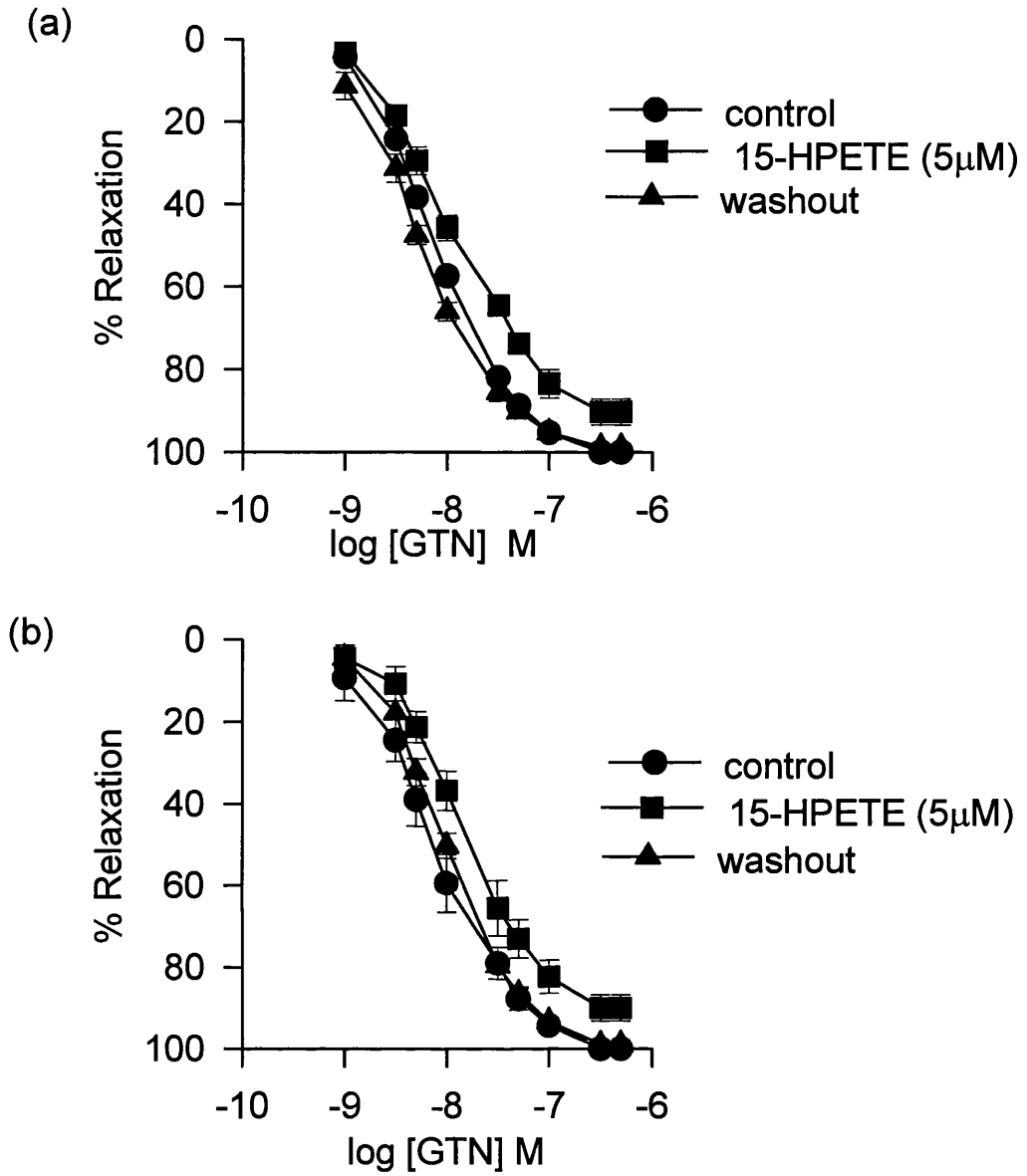


FIGURE 60

The influence of 15-HPETE on GTN-evoked relaxations in endothelium-intact and denuded tissues.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of GTN (1 nM - 0.5 μM). Following washout, tissues were incubated with 15-HPETE (5 μM) for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to GTN was carried out.

(a) Endothelium-intact tissues (n=4)
(b) Endothelium-denuded tissues (n=4)

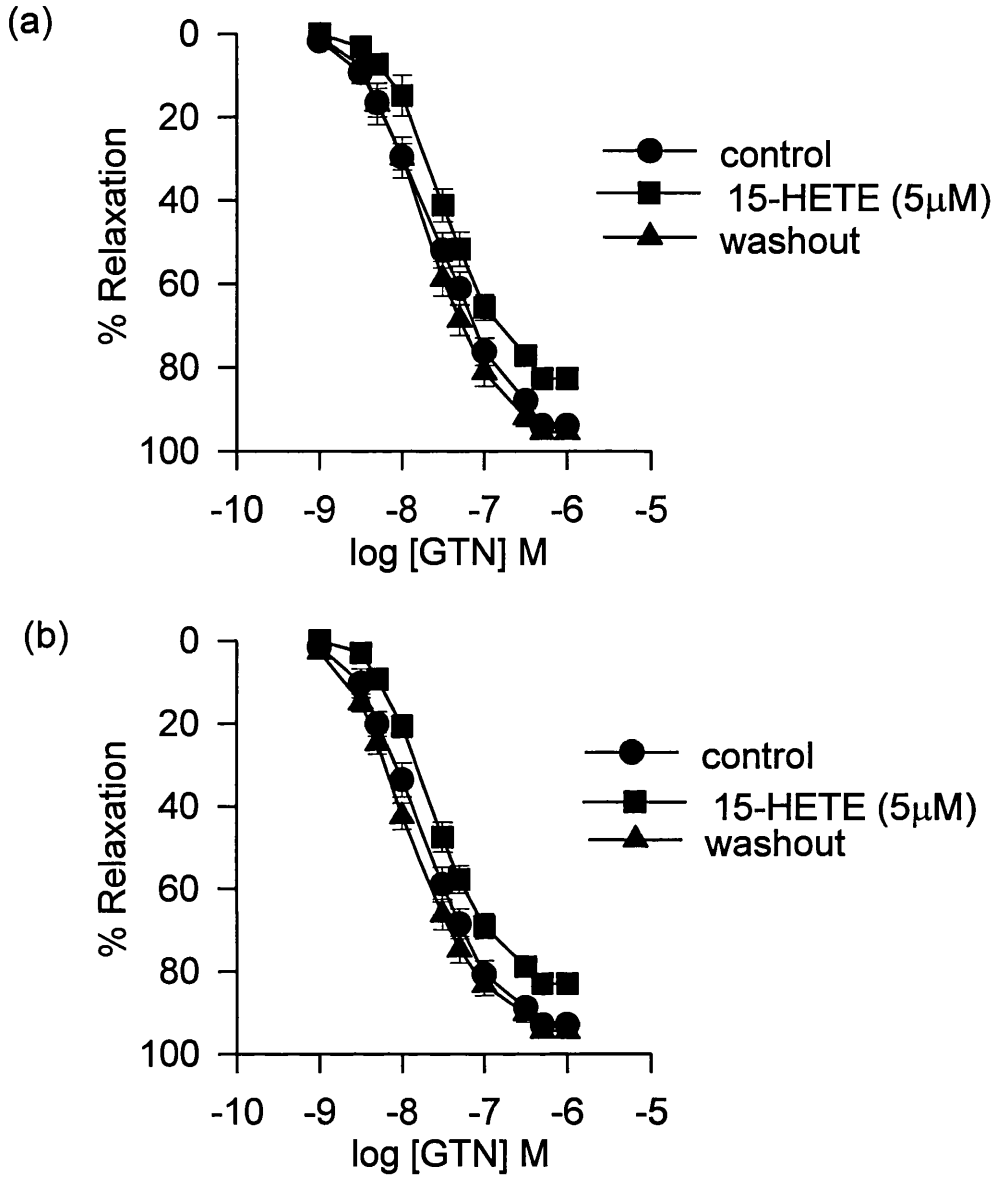


FIGURE 61

The influence of 15-HETE on GTN-evoked relaxations in endothelium-intact and denuded tissues.

Aortic rings were precontracted with PE (0.1 - 0.3 μM) and relaxed with cumulative concentrations of GTN (1 nM - 1 μM). Following washout, tissues were incubated with 15-HETE (5 μM) for 30 mins and the contraction/relaxation cycle repeated. After further washout a third relaxation curve to GTN was carried out.

(a) Endothelium-intact tissues (n=6)

(b) Endothelium-denuded tissues (n=6)

from atherosclerotic rabbits (Simon *et al.,* 1989b). Therefore, it has been suggested that lipoxygenase-derived products may play a role in the attenuation of endothelium-dependent responses caused by the presence of OXLDL and observed in atherosclerosis and hypercholesterolaemia.

The predominant fatty acids present in LDL are linoleic and arachidonic acids and their hydroperoxy and hydroxy derivatives are major constituents in OXLDL. In the present study, the effects of lipoxygenase metabolites of these two fatty acids on endothelium-dependent relaxations of isolated rabbit aortic rings were examined to investigate whether these oxidation products may contribute to the impaired vascular reactivity observed in the presence of OXLDL.

In a previous study, lipoxygenase metabolites of arachidonic acid have been shown to evoke contractions in isolated strips of rabbit aorta (Asano and Hidaka, 1979) and in isolated canine arteries (Takahashi *et al.,* 1985; Van Diest *et al.,* 1986, 1991). Similarly, in the experiments described here, products derived from arachidonic acid, 15-HPETE and 15-HETE evoked contraction in unstimulated isolated rabbit aortic rings which was not influenced by the presence of the endothelium. 15-HETE-evoked contractions were of greater magnitude than those evoked by 15-HPETE. The mechanisms of action of 15-HPETE and 15-HETE are unclear. However, a previous study (Van Diest *et al.,* 1991) suggested that contractions of isolated canine splenic and coronary arteries, by 15-HPETE and 15-HETE may be, in part, mediated by activation of thromboxane A_2 receptors on the vascular smooth muscle cells.

When added to tissues precontracted with phenylephrine, 15-HPETE caused a further increase in tone which was not dependent on the presence of the endothelium. This is in contrast to other studies which have demonstrated

concentration-dependent relaxations to 15-HPETE and 15-HETE (Thomas and Ramwell 1986; Van Diest *et al.,* 1991). However, Uotila *et al.* (1987) and Van Diest *et al.* (1991) demonstrated that higher concentrations of 15-HPETE, similar to those used in the present study, could induce further contractions in vessels with induced tone either with or without an intact endothelium.

The 15-HPETE and 15-HETE induced contractions in vessels with and without active tone may be explained by the inhibition of vasodilator prostanoid synthesis by the arachidonic acid metabolites in addition to the direct vasoconstrictive action of 15-HPETE and 15-HETE (Takahashi *et al.,* 1985).

In contrast to metabolites of arachidonic acid, hydroperoxy and hydroxy derivatives of linoleic acid had no effect on either basal or induced tone of endothelium intact or denuded aortic rings. Similarly, in the rat aorta, linoleic acid metabolites, at the same concentrations as used in this investigation, had no effect when added to phenylephrine precontracted vessels either with or without endothelium although higher concentrations caused a further contraction (Uotila *et al.,* 1987). However, Asano and Hidaka (1979) showed that fatty acid peroxides, produced by pretreatment of linoleic acid with lipoxygenase, contracted strips of rabbit aorta although the specific products formed were not identified. In contrast to the present study, De Meyer *et al.* (1992) demonstrated concentration-dependent relaxations in precontracted isolated canine coronary arteries in response to 13-HPODE and 13-HODE and suggested that this effect was due to the stimulation of prostacyclin biosynthesis by the linoleic acid derivatives.

Hydroperoxy and hydroxy derivatives of linoleic acid, 9-HPODE, 9-HODE and 13-HODE in addition to metabolites of arachidonic acid, 15-

HPETE and 15-HETE, each inhibited endothelium-dependent relaxations evoked by ACh in aortic rings. A similar degree of inhibition was caused by exposure to each of the fatty acid products. Furthermore, following a short exposure to the lipoxygenase metabolites, all effects were reversible on washout.

Hydroperoxy metabolites of linoleic and arachidonic acids are unstable intermediates in the conversion of the fatty acid to its hydroxy derivative. It may be possible, therefore, that the inhibitory effects of the hydroperoxy acids 9-HPODE and 15-HPETE are due to their conversion to 9-HODE and 15-HETE, respectively in the organ bath. The hydroxy acids are also susceptible to further oxidation. However, the addition of the antioxidant ascorbic acid to the fatty acid oxidation products prior to their addition to the organ bath had no influence on the inhibitory effect, suggesting that oxidation is not responsible for the attenuation of ACh-evoked relaxations by the hydroperoxy and hydroxy fatty acids.

It has been reported that some of the vascular effects of metabolites of linoleic and arachidonic acid may be in part mediated by products of the cyclo-oxygenase pathway (Van Diest *et al.,* 1991; De Meyer *et al.,* 1992). Furthermore, 13-HPODE and 13-HODE have been shown to stimulate the biosynthesis of prostacyclin in endothelial cells (Setty *et al.,* 1987; De Meyer *et al.,* 1992). However, in contrast, 15-HPETE has been shown to inhibit the production of prostacyclin from rabbit vessel wall microsomes (Moncada *et al.,* 1976b) and 15-HETE can inhibit cyclo-oxygenase activity in human umbilical arteries (Setty and Stuart, 1986).

In the present study, however, the inhibition of endothelium-dependent relaxations caused by 9-HODE and 15-HPETE was unaffected by the presence of indomethacin, indicating that cyclo-oxygenase products do not play a role in the inhibitory effect.

To further investigate the mechanism underlying the inhibitory effect of lipoxygenase-derived products on endothelium-dependent relaxations of isolated rabbit aortic rings, the effect of the protein kinase C inhibitor, chelerythrine chloride on the inhibition was investigated. The inhibition of ACh-evoked relaxations caused by 9-HODE and 15-HPETE was prevented by the presence of chelerythrine chloride indicating that these fatty acid products inhibit vascular relaxation through a mechanism involving the activation of protein kinase C. Similarly, recent studies have suggested that OXLDL may exert its inhibitory effect by a mechanism involving protein kinase C (Smith and Turner, 1992; Ohgushi *et al.*, 1993; Smith *et al.*, 1993). As discussed in section 4.7, phorbol esters, which activate protein kinase C, inhibit EDRF-mediated responses (Weinheimer *et al.,* 1986; Lewis and Henderson, 1987) possibly by inhibiting agonist-induced increases in endothelial cell intracellular Ca^{2+} levels (Ryan *et al.,* 1988) and by inhibiting Ca^{2+} influx into cultured endothelial cells.

Other studies have also demonstrated that oxidative stress alters receptor-mediated Ca^{2+} signaling and homeostasis in vascular endothelial cells (Elliott and Schilling, 1991; Schilling and Elliott, 1992). *Tert*-butyl hydroperoxide (*t*-BOOH) inhibited bradykinin-induced increases in Ca^{2+} influx in cultured endothelial cells (Elliott *et al.,*1989; Elliott and Doan, 1993). Furthermore, after prolonged incubation basal intracellular Ca^{2+} levels progressively rose and cytosolic Ca^{2+} failed to increase in response to agonist stimulation (Elliott and Schilling, 1991). This rise in resting intracellular Ca^{2+} levels in cultured endothelial cells has also been demonstrated in response to H_2O_2 (Kimura *et al.,* 1992). These results indicating altered transmembrane signalling mechanisms in response to oxidative stress may be important in endothelial dysfunction in diseased

states and may in part explain the inhibitory effect of fatty acid metabolites on endothelium-dependent relaxations described in the present study. As a result of vascular endothelial cell receptor activation, release of Ca^{2+} from internal stores and stimulation of Ca^{2+} influx each contribute to an increase in intracellular free Ca^{2+} levels which is now known to be necessary for the release of EDRF (Furchgott and Zawadzki, 1980; Singer and Peach, 1982; Long and Stone, 1985). The hydroperoxy and hydroxy fatty acids used in the present study may therefore exert their inhibitory effect on endothelium-dependent relaxations of isolated aortic rings by inhibiting agonist-induced increases in endothelial-cell intracellular Ca^{2+} resulting in a decreased release of EDRF.

Endothelium-dependent relaxations are mediated by activation of smooth muscle guanylate cyclase. To investigate whether the inhibition of endothelium-dependent relaxations by linoleic and arachidonic acid oxidation products was due to inhibition of soluble guanylate cyclase activity or an action at the level of the smooth muscle, the effects of these fatty acid products on relaxations evoked by the nitrovasodilator, GTN were studied. Linoleic acid derived metabolites, 9-HODE, 9-HPODE and 13-HODE had no effect on endothelium-independent relaxations evoked by GTN in intact or denuded aortic rings. This suggests that the attenuation of endothelium-dependent relaxations by these lipoxygenase metabolites is not mediated by a direct action on the vascular smooth muscle.

In contrast to linoleic acid-derived products, 15-HETE and 15-HPETE derived from arachidonic acid caused a reversible attenuation of GTN-evoked relaxations in endothelium-intact and denuded tissues. This may suggest that arachidonic acid oxidation products are important in more severe atherosclerosis since many studies have shown that responses to GTN are

attenuated only in advanced stages of the disease (Verbeuren *et al.* 1986; 1990). The attenuation of GTN-evoked relaxations indicates that these products may effect the action of GTN on the vascular smooth muscle, which may contribute to the inhibition of ACh-evoked relaxations. However, 15-HPETE had no effect on endothelium-independent relaxations evoked by exogenous NO in endothelium-denuded aortic rings suggesting that the inhibitory effects of the hydroperoxy acid are not due to an interaction with NO or by inhibiting the activation of soluble guanylate cyclase. In contrast OXLDL was shown to inhibit endothelium-independent relaxations evoked by exogenous NO suggesting a direct sequestration or inactivation of NO by the lipoprotein (Jacobs *et al.*, 1990). It has also been demonstrated that OXLDL inhibits the activation of guanylate cyclase in the rabbit aorta (Jacobs *et al.*, 1990) and of the partially purified enzyme (Schmidt *et al.*, 1990). The inhibition of GTN-evoked relaxations by 15-HPETE with a lack of effect on relaxations evoked by NO may suggest that the fatty acid derivative exerts its inhibitory effect prior to the activation of guanylate cyclase, possibly by interfering with the intracellular metabolism of GTN as discussed in section 4.7.

The results of this study illustrate that 15-lipoxygenase metabolites of arachidonic acid, 15-HPETE and 15-HETE and of linoleic acid, 9-HPODE, 9-HODE and 13-HODE can affect the reactivity of isolated rabbit aortic rings. Since these lipid hydroperoxy and hydroxy derivatives are major products in modified LDL these findings indicate that they may have an important role in atherosclerosis.

Linoleic acid is the most abundant fatty acid present in arterial tissues and plasma suggesting that this may be the natural substrate for 15-lipoxygenase *in vivo* (Simon *et al.,* 1989a). 13-HODE has been suggested to have a

protective role in the vessel wall since it has been identified as a platelet chemorepellant factor produced intracellularly by endothelial cells (Buchanan *et al.,* 1985b) and it has been shown to increase prostacyclin production by endothelial cells (Setty *et al.,* 1987). Furthermore, 13-HODE levels in atherosclerotic rabbit aorta and plasma linoleic acid levels in humans have been found to have an inverse correlation with the degree of fatty streaks and the risk of coronary heart disease (Wood *et al.,* 1987; De Meyer *et al.,* 1991). However, other studies have demonstrated increased levels of linoleic acid-derived hydroperoxides in atherosclerotic lesions and noted a positive correlation between the concentrations of these products and the degree of atherosclerosis (Harland *et al.,* 1971; Yalcien *et al.,* 1989). It is possible that 13-HODE is produced from linoleic acid by the increased 15-lipoxygenase activity in vascular tissues as a protective response to hypercholesterolaemia (Simon *et al.,* 1989a). However, 15-HETE was found to be the most abundant lipoxygenase-derived product formed in aortae from atherosclerotic rabbits with more severe lesions (Henriksson *et al.,* 1985; Simon *et al.,*1989b). During early fatty streak formation an increased synthesis of prostacyclin has been demonstrated in endothelial cells, possibly due to the presence of 13-HODE whereas synthesis of prostacyclin by endothelial cells is suppressed as the disease progresses (Beetens *et al.,* 1986). This latter finding supports the involvement of 15-HETE in more advanced atherosclerosis since lipoxygenase-derived products of arachidonic acid have been shown to inhibit the production of prostacyclin (Moncada *et al.,* 1976; Setty *et al.,* 1986). 15-HETE has been shown to be a

chemoattractant for smooth muscle cells and a mitogen for endothelial cells indicating a role in the cellular events of atherogenesis (Setty *et al.,* 1987). The accumulation of lipid hydroperoxy and hydroxy acids in the arterial wall with chemotactic activity is thought to be important in the development of early atherosclerotic plaques (Faggiotto *et al.,* 1984).

Whether the hydroperoxy and hydroxy derivatives of arachidonic and linoleic acid examined in the present study have a major role in the attenuation of vascular responses observed in hypercholesterolaemia and atherosclerosis is not clear. The fact that oxidised fatty acids are present in atherosclerotic lesions and are major products in OXLDL and are able to inhibit endothelium-dependent relaxations suggests that they may contribute to the impaired vascular reactivity associated with hypercholesterolaemia and atherosclerosis.

CHAPTER 6

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 endothelium as a result of, or allowing, the invasion of LDL and platelets.

Oxidatively modified LDL is thought to play a major role in the pathogenesis of atherosclerosis. Furthermore, the endothelial dysfunction associated with hypercholesterolaemia and atherosclerosis and that produced by oxidised LDL in the rabbit aorta are similar. In the present investigation the effects of native and OXLDL on endothelium-dependent relaxations of isolated rabbit large and small coronary arteries were studied.

Oxidised, but not native LDL, inhibited endothelium-dependent responses evoked by ACh to a similar extent in both small resistance vessels and in large coronary arteries and aortic rings. Endothelium-independent relaxations evoked by SNP were unaltered by the presence of OXLDL indicating that the inhibition of ACh-evoked relaxations in this study may be through an action on the endothelium and not on the vascular smooth muscle as shown in **Figure 62**. These results further support the finding that endothelial dysfunction occurs in the microcirculation and that alterations in vascular reactivity preceed the development of detectable atherosclerotic lesions.

The inhibitory effect of OXLDL on endothelium-dependent relaxation is associated with the lipid fraction of the molecule and this study examined the vasoactive properties of oxidised fatty acids formed during the modification

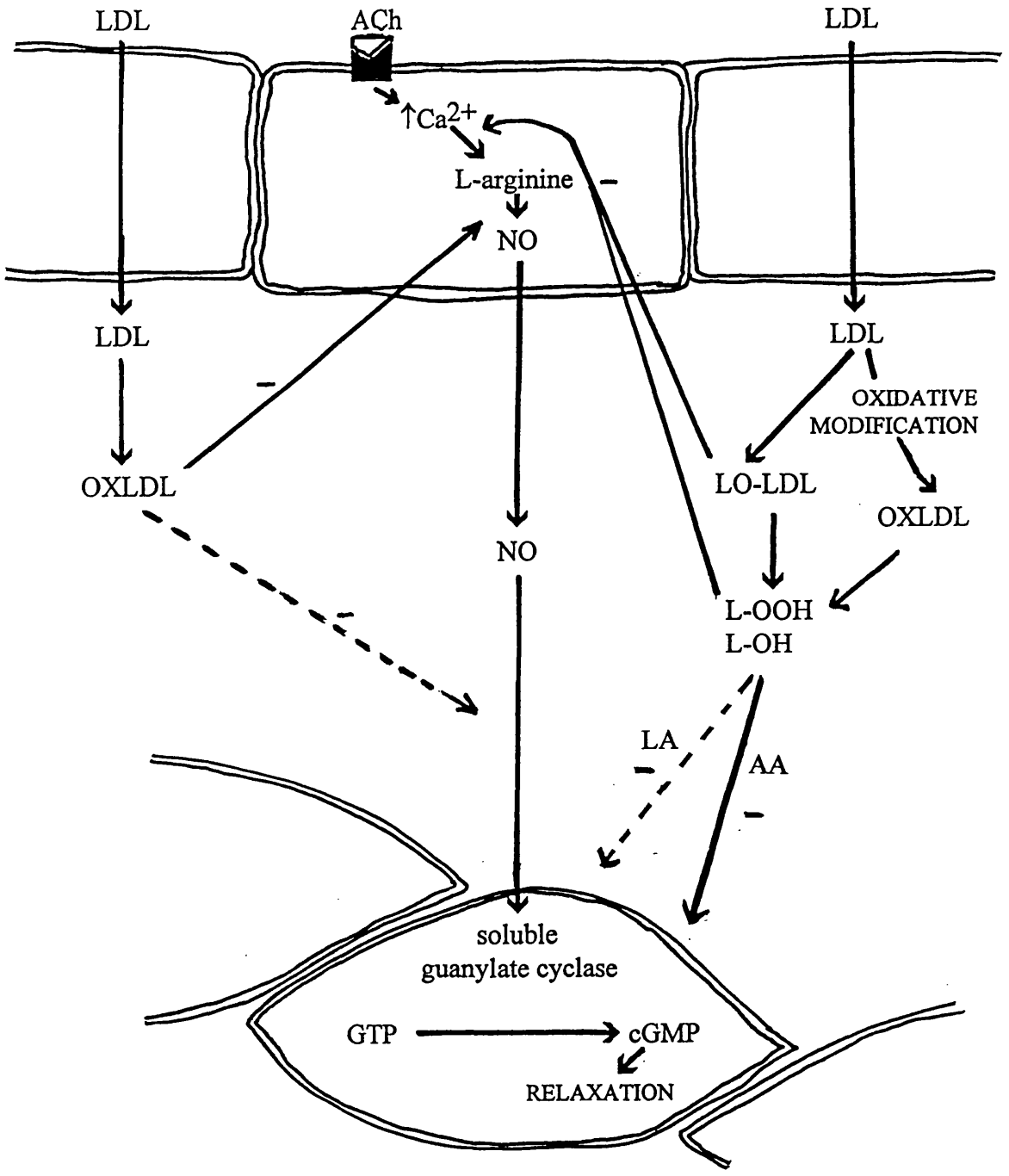


FIGURE 62

The possible mechanisms by which OXLDL, LO-LDL and fatty acid oxidation products may contribute to the alterations in vascular reactivity observed in atherosclerosis and hypercholesterolaemia.

Abbreviations: L-OOH, fatty acid hydroperoxide; L-OH, fatty acid hydroxide; AA, Arachidonic acid derived products; LA, Linoleic acid derived products.

——— inhibitory effect

--------- no effect

of LDL to determine whether they could contribute to the inhibition of relaxations by OXLDL. The possible sites of action of OXLDL, LO-LDL and hydroperoxy and hydroxy metabolites of fatty acids in the arterial wall are summarised in **Figure 62**. Arachidonic and linoleic acid-derived hydroperoxides and hydroxides inhibited endothelium-dependent relaxations suggesting that these oxidised fatty acids may play a role in the attenuation of relaxations by OXLDL. In addition, some of the effects of OXLDL could be mimicked by using LDL modified by treatment with lipoxygenase, supporting the hypothesis that lipoxygenase is involved in the oxidative modification of LDL *in vivo*. The main site of action of linoleic acid-derived products appears to be at the endothelium, possibly by inhibiting Ca^{2+} influx through the activation of protein kinase C, with little or no effect on the underlying smooth muscle. Metabolites of arachidonic acid, in addition to LO-LDL, may also have some action on the vascular smooth muscle since GTN-evoked responses were attenuated by these compounds. GTN is metabolised in smooth muscle cells and 15-HPETE and LO-LDL may affect this process.

Lipoxygenase-derived products of fatty acids have been implicated in atherogenesis since hydroxy acids derived from arachidonic acid have chemoattractant activity for smooth muscle cells and 15-HETE is a mitogen for endothelial cells. Furthermore, an increased formation of hydroperoxy and hydroxy fatty acids has been demonstrated in hypercholesterolaemic animals due to an increase in 15-lipoxygenase activity. In addition, linoleic acid hydroperoxides have been shown to be cytotoxic to fibroblasts and endothelial cells. Lipid hydroperoxides and hydroxy acids present in the vessel wall may therefore contribute to the cellular events in early stages of atherosclerosis by chemoattractant activities and futher accumulation could

result in cytotoxic effects leading to necrosis and the development of more advanced lesions.

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