EFFECTS OF LDL CHOLESTEROL ON VASCULAR FUNCTION

Mechanisms of action

by

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SUMMARY

1 Effects of native (LDL) and oxidised (ox-LDL) low density lipoproteins on cholesterol uptake have been investigated *in vitro*, by examination of the effects of exposure to LDL and ox-LDL on the cholesterol content of rat aortic vascular smooth muscle (VSM) cells. Hypertension and hypercholesterolemia were also examined as coexisting and interactive risk factors by comparison of effects in WKY and SHRSP VSM cells.

2 On exposure of SHRSP and WKY VSM cells to 20μg/ml LDL and ox-LDL, small increases in free membrane cholesterol content were observed. Incubation of SHRSP VSM cells with LDL and ox-LDL produced greater increases in free cholesterol content compared to the WKY normotensive reference strain.

3 Effects of the antioxidants vitamin E and N-acetyl-L-cysteine (NAC), on LDL and ox-LDL mediated cholesterol uptake were examined in WKY and SHRSP VSM cells. In the presence of vitamin E both LDL and ox-LDL mediated effects were significantly attenuated in both cell types. Vitamin E also reduced basal free membrane cholesterol levels in a

concentration-dependent manner, suggesting effects in addition to antioxidant properties. NAC produced only a small attenuation of LDL and ox-LDL mediated effects on cholesterol uptake in WKY and SHRSP VSM cells, and did not alter basal free cholesterol content.

4 The effects of cell stretch on ox-LDL induced cholesterol uptake were also examined in WKY VSM cells using a novel cell stretch apparatus. Results from this preliminary study were inconclusive, but demonstrated a potential model for future investigations of hemodynamic forces on cellular function.

5 Effects of LDL and ox-LDL on vascular reactivity were characterised *in vitro*, in the rat aorta. Rings of rat aorta were incubated in either vehicle, LDL, or ox-LDL for 5 hours before examination of vascular reactivity using a classical organ bath setup. Exposure to LDL and ox-LDL caused an impairment of carbachol-induced endothelium dependent relaxation and L-NAME induced contraction. Ox-LDL also impaired SNP-induced endothelium independent relaxation. LDL and ox-LDL exposure augmented phenylephrine-induced contraction and showed a tendency to increase

potassium chloride induced contraction, although the higher concentration of LDL (500μg/ml), caused attenuation of phenylephrine contraction and had no effect on potassium chloride induced contraction.

6 Possible mechanisms underlying LDL and ox-LDL effects on vascular ractivity in the rat aorta were examined.

7 In tissues not incubated with LDL/ox-LDL, removal of endothelium from the rings caused a significant reduction in L-NAME induced contraction by greater than 70%, demonstrating that nitric oxide assessed was mainly endothelium derived. Five hour incubation resulted in only a small induction of the L-NAME response, which was blocked in the presence of dexamethasone. Removal of the endothelium prior to incubation, caused a considerable increase in the level of induction observed. This increase was inhibited by dexamethasone.

8 The effect of LDL and ox-LDL on iNOS and eNOS activity was investigated by examination of the effects of dexamethasone on the L-NAME response+/-LDL and ox-LDL. Incubation with both LDL and

dexamethasone reduced L-NAME induced contraction to a similar degree as incubation with dexamethasone alone. Ox-LDL treated tissues showed a similar pattern, with a trend towards lower responses when incubation included both ox-LDL and dexamethasone.

9 Effects of vitamin E on LDL-induced alterations of vascular reactivity were examined. Vitamin E had no significant effect on LDL-induced alterations of vascular reactivity. Results suggested that only LDL-mediated attenuation of maximum carbachol induced relaxation may be altered in the presence of vitamin E (although this did not reach statistical significance).

10 Effects of superoxide dismutase (SOD) on LDL and ox-LDL induced alterations of vascular reactivity were examined. SOD alone, showed a tendency to reduce maximum phenylephrine induced contraction when compared with appropriate controls. Incubation in the presence of SOD did not significantly alter LDL and ox-LDL induced effects on vascular reactivity.

CONTENTS

7 INVESTIGATION INTO THE MECHANISMS UNDERLYING LDL/OX-LDL EFFECTS

8 GENERAL DISCUSSION (AND FUTURE DIRECTIONS)

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DECLARATION

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

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Katrine L. McPherson

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LIST OF FIGURES AND TABLES

List of Figures

CHAPTER 1

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

List of Tables

CHAPTER 2

CHAPTER 3

CHAPTER 4

ABBREVIATIONS

BHT	Butylated Hydroxytoluene
CI	95% Confidence Intervals
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagles Medium
DPPD	N,N'-Diphenyl-Phenylenediamine
EDRF	Endothelium Derived Relaxing Factor
EDTA	Ethylene-Diamine-Tetraacetic Acid
KBB	Krebs Bicarbonate Buffer
LDL	Low Density Lipoprotein
L-NAME	N^G-nitro-L-Arginine Methyl Ester
MDA	Malondialdehyde
NAC	N-Acetyl-L-Cysteine
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
eNOS	Endothelial/Constitutive Nitric Oxide Synthase
iNOS	Inducible Nitric Oxide Synthase
ox-LDL	Oxidised Low Density Lipoprotein
PBS	Phosphate Buffered Saline
Q1	1st Quartile
Q3	3rd Quartile
SBTI	Soybean Trypsin inhibitor
SDS	Sodium Dodecyl Sulphate
SHRSP	Stroke Prone Spontaneously Hypertensive Rats
SNP	Sodium Nitroprusside
SOD	Superoxide Dismutase

TBA	Thiobarbituric acid
V:V	Volume:Volume
Vit. E	Vitamin E
VLDL	Very Low Density Lipoproteins
WKY	Wistar-Kyoto Rats

1

GENERAL INTRODUCTION

1.1 ATHEROSCLEROSIS

Atherosclerosis is a condition involving arterial damage which plays an important role in the pathogenesis of numerous disease states affecting the coronary, cerebral, and peripheral vascular systems. Clinical consequences include ischaemic heart disease, myocardial and cerebral infarction, gangrene, and loss of function of the extremeties (Feher and Richmond, 1991; Ross, 1993).

Atherosclerosis has become of increasing concern in the later half of this century due to its escalating role in clinical disease, to the extent that it is now responsible for up to 75% of all deaths in western society.

Aortic fatty streaks are found in many children under three years, in almost all persons by the age of ten, and in approximately 90% of all individuals over thirty regardless of race, geography, or gender (Callow, 1991).

It has been concluded that atherosclerosis develops as a result of multiple complex interactions, and that such events involve both environmental and genetic factors.

Most of those clinically affected have several unfavourable associations, including a history of cigarette smoking, high blood pressure, and elevated levels of blood cholesterol. Of these associations elevated cholesterol levels appear prominent (Walton, 1975; The Pooling Project Research Group, 1978; Kannel and Sytkowski, 1987; Feher and Richmond, 1991).

1.2 CHOLESTEROL - AN IMPORTANT RISK FACTOR

Hypercholesterolaemia is well established as a major risk factor for atherosclerosis. This has been substantiated by numerous experimental and clinical investigations which show a concentration-related increase in the risk of atherosclerotic complications when plasma cholesterol levels exceed 4.1-4.7mmol/l (160-180mg/dl)(Witzum, 1993).

Plasma lipids such as cholesterol and triglyceride are poorly soluble and are transported through the body bound to proteins. These water soluble lipoproteins have become the focus of much interest in recent years.

Of the 6 known classes of lipoproteins low density lipoproteins (LDL) are of particular importance in the development of atherosclerotic disease. LDL's

are the major cholesterol carrying particles in the blood (making up 60-70% of the total plasma cholesterol). Responsible for uptake and transport of cholesterol from ingested fats and from cholesterol producing cells in the body, LDL's deliver cholesterol to cells in the blood vessels and muscles.

High levels of this lipoprotein type are recognised as salient in the development of atherosclerotic disease (Feher and Richmond, 1991; O'Brien *et al.*, 1991).

1.2.1 THE 'CHOLESTEROL HYPOTHESIS'

Many studies initially focused on the pathophysiological events involved in atherosclerosis such as intimal foam cell formation and development of the 'fatty streak'.

Discovery of the LDL receptor led to the logical assumption that this was the lipid uptake mechanism for macrophages, the cell type chiefly accounting for cholesterol laden 'foam cells' seen in the early stages of atherosclerosis.

The LDL receptor was to become a principal part of the original 'cholesterol hypothesis'.

1.2.2 THE 'MODIFIED LIPOPROTEIN HYPOTHESIS'

Subsequent studies however, showed that on incubation of cells with LDL, the rate at which native LDL uptake occurred was not sufficient to generate foam cell formation.

In 1979, Goldstein *et al.*, discovered that acetylation of LDL lead to a massive increase in macrophage uptake by a receptor dependent mechanism (the receptor involved was later to be known as the scavenger or acetyl LDL receptor).

This lead to the suggestion that LDL modification must be required, and that it was some modified form of LDL that was the actual ligand taken up by monocytes/macrophages (Goldstein *et al.*, 1979).

A number of chemical modifications were found to rapidly increase LDL uptake and accumulation (Goldstein *et al.*, 1979; Mahley *et al.*, 1979; Fogelman *et al.*, 1980; Henriksen *et al.*, 1981; Steinberg *et al.*, 1989), however of these, oxidation was found to have the strongest biological feasibility.

Such oxidative modification has been demonstrated *in vitro* by endothelial cells (Henriksen *et al.*, 1981; Steinbrecher *et al.*, 1984), vascular smooth muscle cells (Morel *et al.*, 1984), and monocytes/macrophages (Cathcart *et al.*, 1985; Hiramatsu *et al.*, 1987); the 3 major cell types of the arterial lesion.

This oxidative modification has also been demonstrated *in vivo* by groups such as Yla-Herttuala *et al.*, 1989. Studies of the physical and chemical properties of LDL from atherosclerotic lesions of rabbit and man have provided strong evidence for the presence and oxidative modification of LDL within these focal areas.

Exact mechanisms by which LDL is oxidatively modified are not, however, fully understood.

1.2.3 HYPERTENSION AS AN INTERACTIVE RISK FACTOR

As well as showing the multifactorial origin of atherosclerosis, epidemiological studies have made it clear that identifiable risk factors may act independently, or may reinforce one another when they coexist (sometimes synergistically) (Walton, 1975; Feher and Richmond, 1991).

Hypertension is one of the most prevalent and most potent determinants of atherosclerotic cardiovascular disease. In particular, hypertension is a well known risk factor when associated with elevated cholesterol levels (Hollander *et al.*, 1976; Chobanian, 1983; McGill *et al.*, 1985; Kannel and Sytkowski, 1987; Chobanian *et al.*, 1989).

Recently, Galle *et al.*, 1994, found that dose dependent infiltration of lipoprotein into the vessel wall and the accompanying attenuation of acetylcholine-induced dilations, only occurred under high pressure conditions.

This is similar to the findings of Curmi *et al.*, 1990, who showed a marked increase in LDL uptake with increasing transmural pressure.

Such studies have led to the suggestion that LDL uptake leading to atherosclerotic disease is dependent not only on oxidative modification, but also on transmural pressure.

A fuller understanding of such interactive mechanisms may be of importance to patients with both hypercholesterolemia and hypertension.

1.3 ATHEROGENIC EFFECTS OF LDL CHOLESTEROL

Since the formation of the 'modified lipoprotein hypothesis' numerous potentially atherogenic mechanisms have been ascribed to oxidised LDL (ox-LDL) including:-

1 Enhanced uptake by macrophages

2 Chemotactic attraction of circulating monocytes

3 Inhibition of motility of tissue macrophages

4 Cytotoxicity

5 Altered gene expression

6 Immunogenic effects

7 Adverse alteration of coagulation pathways

8 Adverse alteration of vascular reactivity

(Witzum and Steinberg, 1991).

The association of ox-LDL with various biological effects has however proved controversial, with conflicting results between different laboratories. Such discordance of results may be explained by the different preparation techniques used in laboratories.

Ox-LDL is not a single homogeneous entity, and differences may occur as a result of the cell type or exact metal ion concentration used to oxidise the

LDL, the time of incubation, the presence of differing amounts of endogenous antioxidants in the LDL particles, differences in the fatty acid composition, or other properties such as size or density of the LDL (Rosenfeld, 1991; Witztum and Steinberg, 1991).

Additionally considerable variability may occur from preparation to preparation even when every effort is made to hold conditions constant.

The vast majority of early studies investigating the role of oxidised lipoproteins in atherosclerotic progression focused on pathphysiological events such as scavenger receptor uptake and foam cell formation.

It is only relatively recently that emphasis has been placed on effects of oxidised lipoproteins, in particular their effect on the cellular functions associated with the regulation of vascular tone.

1.4 THE ENDOTHELIUM

The vascular endothelium is a dynamic participant in circulatory homeostasis due to its numerous physiological roles including the regulation of:-

1 Haemostasis and Thrombosis - via antiplatelet, anticoagulant, and fibrinolytic effects

2 Vascular Remodeling - via the production of growth promoting and inhibiting substances

3 Permeation - via the control of exchange, and active transport, of substances across the artery wall

(Ross, 1993; Daugherty *et al.*, 1995).

Importantly, the endothelium has also been shown to play a crucial role in the maintenance of vascular tone via the production and release of vasocontractile and vasorelaxant factors.

Known contractile factors include endothelin, angiotenisn II, and the cyclooxygenase pathway products thromboxane A_2, prostaglandin H_2, and superoxide, whereas relaxant factors identified to date include prostacyclin and nitric oxide (NO) (Luscher, 1993; Ross, 1993; Daugherty *et al.*, 1995).

Of these factors NO has proved of particular importance, having a definitive role in the normal control of basal tone.

1.5 NITRIC OXIDE

In 1980, Furchgott and Zawadzki first described a paracrine vascular hormone with potent vasodilatory activity, that was subsequently termed endothelium derived relaxing factor (EDRF), and is now known to be identical or similar to NO.

NO has since been implicated in a wide range of physiologic roles in the vasculature including:-

1 Preservation of endothelial permeability and integrity
2 Regulation of vascular smooth muscle proliferation
3 Regulation of vascular tone
4 Regulation of leucocyte-vessel wall interactions
5 Provision of antithrombic activity

(Welch *et al.*, 1995).

In particular NO is a primary determinant of resting vascular tone through continuous basal release, and causes vasodilation in response to various vasodilator agents such as acetylcholine, bradykinin, substance P, and increases in flow and shear stress (Calver *et al.*, 1993).

1.5.1 THE NITRIC OXIDE PATHWAY

Endothelium-dependent vasodilation, and basal vascular tone, are mediated via the synthesis, release, and effect of NO (Fig. 1.1).

NO is formed from L-arginine via the constitutive endothelial form of nitric oxide synthase (eNOS), and causes relaxation via activation of guanylyl cyclase in the vascular smooth muscle (Luscher *et al.*, 1995).

Inhibition of this NO synthesis can be acheived on exposure to nitric oxide synthase inhibitors such as L-NAME, or by removal of the endothelium.

Interestingly an inducible form of nitric oxide synthase (iNOS) also exists and has been found in various cell types including macrophages and smooth muscle cells (Welch *et al.*, 1995).

iNOS transcription may be activated by a range of substances including bacterial lipopolysaccharide (endotoxin), and various cytokines.

It is now thought that dysregulation of the NO pathway may contribute to the pathogenesis of a

The Nitric Oxide Pathway

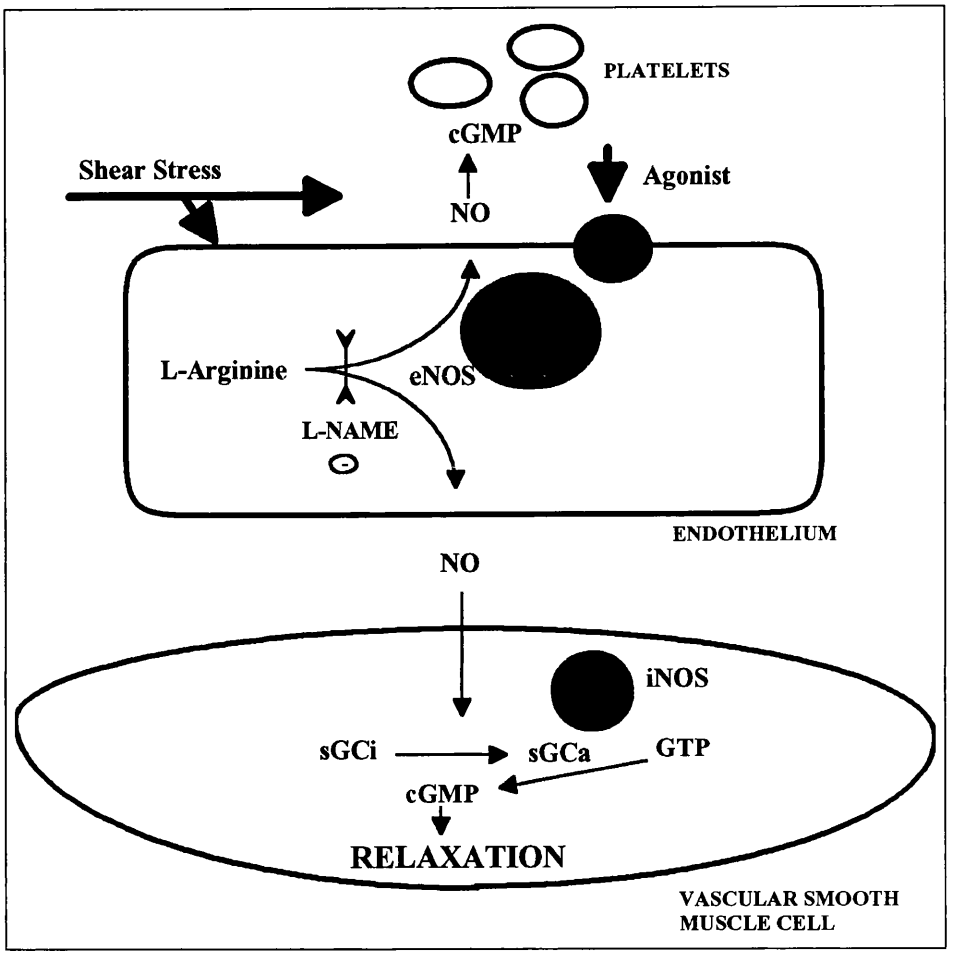


Fig. 1.1 - The nitric oxide pathway - the endothelium produces nitric oxide which causes relaxation.

eNOS = endothelial/constitutive nitric oxide synthase; iNOS = inducible nitric oxide synthase; sGCi/a = inactive/active soluble guanylyl cyclase; GTP = guanosine triphosphate, cGMP = cyclic guanosine monophosphate; L-NAME = L-nitro-arginine methylester.

(Luscher *et al.*, 1995)

number of vascular disorders including hypertension, reperfusion injury, and atherosclerosis.

1.6 EFFECTS OF LDL CHOLESTEROL ON THE REGULATION OF VASCULAR TONE

Alterations in vascular function are well documented in the development of atherosclerotic disease (Heistad *et al.*, 1984; Berkenboom *et al.*, 1987; Shimokawa and Vanhoutte, 1988; Casino *et al.*, 1995). Risk factors such as elevated cholesterol levels have been linked to these alterations, however exact mechanisms are not fully understood.

As a result of the proliferation of studies investigating the effects of LDL cholesterol on vascular tone, it has become apparent that LDL exerts 2 main effects:-

1 LDL profoundly affects the endothelium-dependent relaxation of isolated blood vessels

2 LDL affects the responsiveness of vascular smooth muscle cells to vasoconstrictor stimuli

These vascular effects have been shown to be primarily due to the oxidised form of LDL (although effects have been observed to a lesser extent on

exposure of tissues to native LDL - Andrews *et al.*, 1987; Jacobs *et al.*, 1990; Galle et al., 1991; Myers *et al.*, 1994).

1.6.1 EFFECTS OF LDL CHOLESTEROL ON ENDOTHELIUM-DEPENDENT RELAXATION

Atherosclerosis in animals and humans is associated with markedly reduced endothelium-dependent relaxation, and this attenuated responsiveness is thought to play an important role in associated disease states (Berkenboom *et al.*, 1987; Bossaller *et al.*, 1987; Forstermann *et al.*, 1988; Shimokawa and Vanhoutte, 1988).

Knowledge of this impairment of endothelium-dependent vasodilation has led to a focus of interest on the possible link between lipoproteins and impaired synthesis/effect of NO.

An increasing body of evidence has now accumulated to suggest that ox-LDL contributes significantly to this altered vasomotor responsiveness.

Ox-LDL-induced impairment of NO-dependent relaxation has been shown in various animal models including porcine coronary artery (Simon *et al.*,1990; Tanner

et al., 1991), and rabbit aorta (Jacobs *et al.*, 1990; Kugiyama *et al.*, 1990).

Studies in human and animal hypercholesterolaemic models have shown impaired NO-dependent vasorelaxation to a variety of substances including acetylcholine, substance P, histamine, and thrombin (Shimokawa and Vanhoutte, 1988; Flavahan , 1992; Casino et al., 1995).

Although impaired NO function is known to take place the mechanisms by which this dysfunction occurs remain controversial.

1.6.1.1 Nitric Oxide Synthase Inhibition

In recent years a limited number of studies have suggested that low-density lipoproteins affect vascular reactivity by inhibiting the enzyme nitric oxide synthase (NOS).

In 1995 Liao *et al.*, found that ox-LDL decreased the expression of endothelial NOS (eNOS) in human saphenous vein endothelial cells. This was thought to be through a combination of early transcriptional inhibition and post-transcriptional mRNA destabilisation.

Yang *et al.*, 1994, found that inducible NOS (iNOS) could also be inhibited by ox-LDL, on examination of iNOS activity in an activated macrophage cell line.

Such results indicate a potential mechanism for LDL/ox-LDL induced NO dysfunction via alteration of NOS activity.

1.6.1.2 Alteration of Membrane Physicochemical Structure and Calcium Influx

Various groups have suggested that the inhibitory effect of ox-LDL is due to lysophosphatidylcholine, which is generated in LDL during oxidation and transferred directly into the plasma membrane of endothelial cells.

Once integrated into the membrane lysophosphatidylcholine is thought to affect the functioning of membrane receptors specific for numerous vasodilator agonists (Kugiyama *et al.*, 1990; Yokoyama *et al.*, 1990; Murohara *et al.*, 1994).

Indeed, increased cholesterol content within the cell membrane has been suggested as an initiator of proatherogenic changes at the level of membrane physicochemical structure and calcium influx (Scott-Burden *et al.*, 1989; Yeagle, 1989; Bialecki

et al., 1991; Figueiredo *et al.*, 1991; Thorin *et al.*, 1995).

Changes observed following cholesterol exposure range from alterations in Na-H antiporter activity and intracellular calcium, to activation of phosphatidylinositol metabolism and induction of growth-related metabolism.

Deposition of lipids in atherosclerotic lesions suggests that the accumulation of cholesterol in cell membranes may be one of the earliest biochemical changes in the progression of the disease.

Since changes in membrane-bound cholesterol are known to alter the fluidity and permeability of lipid membranes, and the activation of excitable cells critically depends on the functional characteristics of this surface membrane, it seems possible that cholesterol-induced changes in the membrane function may be responsible for the altered vasoactivity observed.

Studies by Tanner *et al.*, 1991, suggest that the inhibitory effects of ox-LDL are derived from its ability to 'interfere with the receptor-operated

release of L-arginine from intracellular stores or the synthesis of the amino acid' (L-arginine being the precursor for NO).(Tanner *et al.* however, further suggest that the inhibitory effects of ox-LDL are not mimicked by addition of lysophosphatidylcholine to their coronary artery system).

Data by Jeserich *et al.*, 1992, also support this theory with studies showing an approximately 30% reduction of plasma L-arginine levels in patients with hypercholesterolemia.

Flavahan, 1992, has taken this theory further having observed complex selective dysfunction of agonist-induced relaxation in a number of different arterial preparations. This has led him to suggest that endothelial dysfunction occurs in 2 distinct phases (Fig. 1.2):-

1 Early in the disease process or after low concentrations of ox-LDL, endothelial dysfunction may be caused by a selective impairment in certain G proteins or G protein-dependent signal transduction pathways (in particular that of the pertussis toxin-sensitive G_i protein).

2 As the disease progresses or as the concentration of ox-LDL is increased, the dysfunction may spread to other signal transduction processes, causing inhibition by a non-specific action.
(Flavahan, 1992).

In endothelial cells this wider spread dysfunction may be due to inhibition of NO synthesis and release (eg. by decreased availability of L-arginine or NOS inhibition), and in the vascular smooth muscle by attenuation of NO effect (eg. decreased sensitivity to NO or increased NO breakdown following release).

1.6.1.3 Free Radical Neutralisation

Finally, a 3rd alternative theory accumulating considerable interest is that of 'free radical neutralisation'.

Recent observations of Galle *et al.*, 1991, suggest that both native and oxidised LDL inhibit endothelium-dependent vasodilation by inactivating NO released from endothelial cells. Galle *et al.*, have further theorised that inactivation may be due to the hydrophobic core of the lipoproteins acting as a 'sink' for NO.

Proposed mechanisms of Endothelial Dysfunction Associated with Atherosclerosis and Ox-LDL

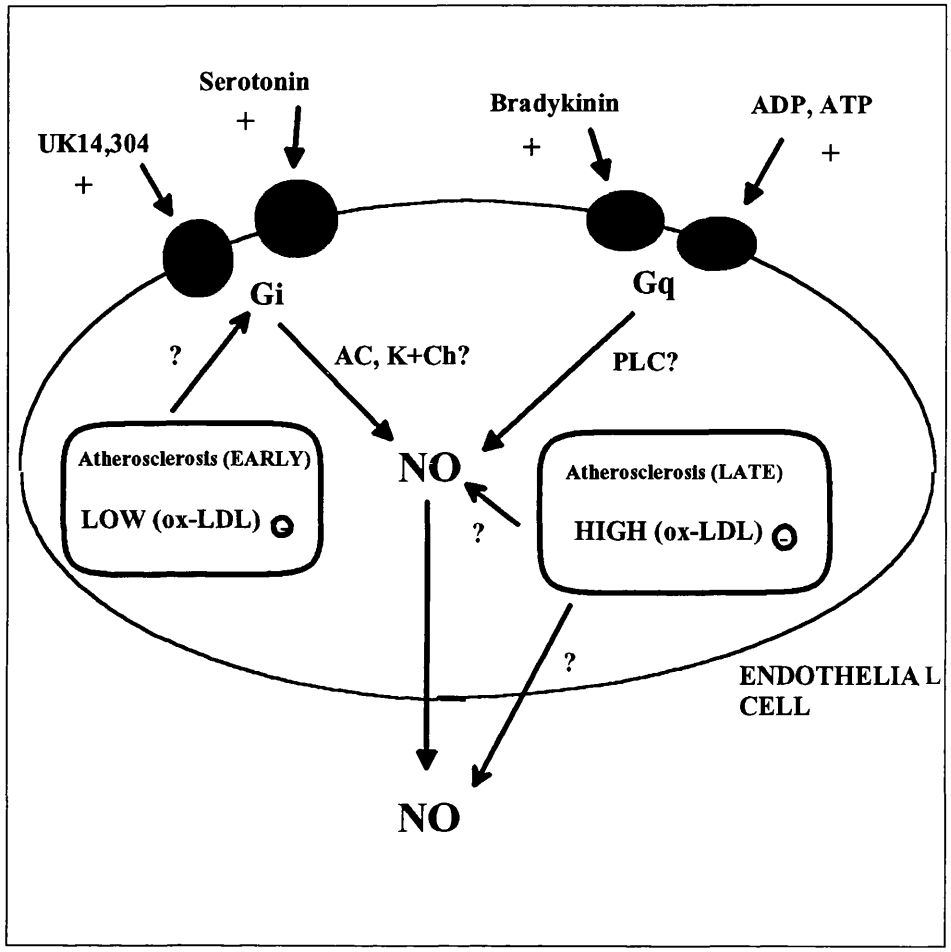


Fig. 1.2 - Proposed mechanisms of endothelial dysfunction associated with atherosclerosis and ox-LDL.

AC = adenyl cyclase, K+Ch = potassium channels, PLC = phospholipase C; NO = nitric oxide.

(Flavahan, 1992)

Further evidence to support this theory include the results of Jacobs *et al.*, 1990, who discovered that both LDL and ox-LDL reversibly inhibited relaxations evoked by exogenous NO in the rabbit aorta. Ox-LDL (but not LDL), also reversibly inhibited relaxations evoked by the NO donor compound glyceryl trinitrate.

Advancement of this theory has resulted in the development of the 'Free Radical Hypothesis' in which NO is thought to be inactivated by free radical neutralisation.

Araujo *et al.*, 1995, found that reactive oxygen species production by leucocytes was positively correlated with elevated VLDL or LDL levels in clinical studies.

Also strengthening this theory were the discoveries of Ohara *et al.*, 1995, who found that dietary correction of hypercholesterolaemia improved endothelium-dependent vascular relaxation but also normalised endothelial superoxide anion production (increased in the hypercholesterolaemic state).

Such results therefore indicate a potential mechanism for LDL/ox-LDL induced NO dysfunction, via

inactivation of the NO molecule by free radical neutralisation.

1.6.2 EFFECTS OF LDL CHOLESTEROL ON VASCULAR SENSITIVITY TO VASOCONTRACTILE STIMULI

Many studies have also examined the effect of LDL cholesterol on vascular responses to vasocontractile stimuli.

Results indicate that cholesterol potentiates vascular smooth muscle reactivity to several vasoconstrictor substances including norepinephrine and serotonin; and have shown that LDL may also elicite contractile responses itself (Bloom *et al.*, 1975; Yokoyama *et al.*, 1979; Rosendorff *et al.*, 1980; Heistad *et al.*, 1984; Broderick *et al.*, 1989; Galle *et al.*, 1990; Simon *et al.*, 1990).

Studies *in vitro* have suggested several mechanisms by which these contractile responses may be augmented.

1.6.2.1 Alteration of Membrane Physicochemical Structure and Calcium Influx

As previously mentioned, increased cholesterol content within the cell membrane is thought to be an initiator of proatherogenic changes at the level of membrane physicochemical chemical structure and calcium influx (Scott-Burden *et al.*, 1989; Yeagle, 1989; Bialecki *et al.*, 1991; Figeiredo *et al.*, 1991; Thorin *et al.*, 1995).

Yokoyama and Henry, 1979, found that in the presence of verapamil (an agent that acts primarily by inhibiting the inward movement of calcium into the cell), there was a potent inhibition of cholesterol-induced contractions.

Similarly, Galle *et al.*, 1990, found that preincubation with the calcium antagonists verapamil, diltiazem, and nitrendipine inhibited vasoconstrictions evoked by ox-LDL in the presence and in the absence of a contractile agonist.

Such results have led to the suggestion that increased cholesterol content of membranes may enhance cation permeability and therefore alter vascular responses to calcium and potassium.

1.6.2.2 Indirect Alteration as a Result of NO Dysfunction

More recently it has been suggested that impairment of NO synthesis/effect may be responsible for the augmented vasoconstrictor responses observed.

It is thought that due to NO dysfunction natural counterbalance mechanisms are depressed and vasoconstrictor responses go unchecked.

It therefore seems likely that ox-LDL-induced alteration of vascular reactivity will be found to occur as a result of a complex multidysfunctional mechanism, and that both impairment of endothelium-dependent relaxation and augmentation of contractile responses develop as a result of multiple dysfunctional sites.

Mechanisms by which oxidised LDL alter vasoactivity remain controversial. An increased knowledge of these mechanisms, and the complex interactions between the various atherogenic factors and dysfunctional sites will improve understanding of the physiology and pathophysiology of the vascular system. This in turn will provide a new outlook for the treatment of atherosclerosis and associated disease states.

1.7 ANTIOXIDANT PROTECTION

The formation of the 'modified lipoprotein hypothesis' (and more recently the suggestion that ox-LDL may exert its effect via a free radical induced dysfunctional site), has stimulated considerable interest in the possibility of administration of antioxidants to protect and prevent against LDL-mediated atherosclerotic disease.

Since the mid 1980's there has been an explosion in the number of studies investigating antioxidant protection, in particular against cardiac and circulatory disease.

Antioxidants studied include chemical antioxidants such as N,N'-diphenyl-phenylenediamine (DPPD), probucol (and structurally related compounds such as butylated hydroxytoluene (BHT)), and natural dietary antioxidants such as the vitamins A, C, and E, carotenoids (in particular beta-carotene), and the trace mineral selenium.

Numerous studies have been carried out examining the effectiveness of these various antioxidants, in reducing LDL oxidation, and protecting against

cardiovascular disease. Results have, however proved controversial.

β-carotene, vitamin C, and vitamin E have been shown to reduce the degree of LDL oxidation in clinical trials (Jialal and Grundy, 1993; Gilligan *et al.*, 1994; Fuller *et al.*, 1996), and evidence suggests that the presence of these antioxidant vitamins in the blood may have a protective role against cardiovascular disease (reviewed by Rice-Evans, 1995; Levine *et al.*, 1996; Stephens *et al.*, 1996).

Gilligan *et al.*, 1994, however, found that although antioxidant administration reduced suceptibility of LDL to oxidation, impaired endothelial function in hypercholesterolemic patients remained unaltered.

Additionally, Kok *et al.*, 1987, found no clear association between low serum selenium, vitamin A, and vitamin E levels and mortality from cardiovascular disease.

Further research is therefore required to clearly elucidate effects and associations of these antioxidants individually and in combination, and to

establish their utility in the prevention of coronary artery disease.

1.8 AIMS

The aims of this thesis were as follows:-

1 To characterise the effects of LDL and ox-LDL on vascular reactivity *in vitro*.

2 To investigate the mechanisms underlying LDL and ox-LDL induced effects on vascular reactivity *in vitro*.

3 To examine hypertension and hypercholesterolemia as coexisting risk factors *in vitro*, and elucidate combined effects on cholesterol uptake as a possible interactive mechanism.

4 To examine the effects, and possible benefits of antioxidants, in the prevention of LDL and ox-LDL induced vascular dysfunction.

2

MATERIALS AND GENERAL METHODS

MATERIALS AND METHODS

2.1 Materials

See appendix 1.

2.2 METHODS

2.2.1 PREPARATION OF VASCULAR SMOOTH MUSCLE CELLS

Vascular smooth muscle cells were prepared via enzymatic dissociation as described by Devlin *et al.*, 1995.

Male WKY and SHRSP rats were sacrificed by overdose of sodium pentobarbitone administered by intraperitoneal injection. The thoracic aorta was dissected under sterile conditions and immediately placed in ice cold serum free DMEM. Following dissection all work was carried out aseptically in a laminar flow tissue culture cabinet (class II) where possible. Excess fat and connective tissue was carefully removed, the clean artery placed in 2.5ml digest mix (see appendix 3), and incubated for 30 minutes at 37°C, 5%CO_2/95% air.

Following digestion the artery was placed in serum free DMEM and the adventitia carefully removed. The vessel was then cut open longitudinally to expose the endothelial layer, and placed in 2.5ml fresh digest mix for a further 30 minutes as previously.

The vessel was then cut into very small pieces (1-2mm in size) and placed in fresh digest mix. The tissue was incubated at 37°C, 5%CO_2/95% air for a further 30 minutes or until a single cell suspension was obtained (NOTE- tissue was periodically titurated using a fine-tipped pastette during this time).

Once a sufficient yield of cells had been obtained, (as judged by observation under a microscope) 2mls ice cold VSM growth medium (see appendix 4) was added, the suspension dispersed using a fine tipped pasteur pipette, and centrifuged in a Sorvall RT600B refrigerated centrifuge at 900rpm (120g) for 10 minutes at 4°C. The digest mix was poured off and 5mls of fresh VSM growth medium added. The solution was dispersed for a final time and the VSM cells (1 x 10^4cells/ml) seeded into a 25cm^2 cell culture flask, which was incubated at 37°C in 5%CO_2/95%air overnight.

The following day cells were checked to ensure attachment had occured. The VSM growth medium was removed, the cells washed with 5mls PBS, 5mls of fresh growth medium added, and the cells reincubated at 37°C, 5%CO_2/95%air.

Medium was changed every 2 days until cells were confluent, at which time cells were subcultured into a 260cm^2 flask from which a cell line was established.

2.2.2 MAINTENANCE OF CELLS

2.2.2.1 Cell Culture

All cell culture procedures were carried out in a laminar flow tissue culture cabinet (class II) using sterile medium, buffers, and equipment unless otherwise stated.

2.2.2.2 Cell Passage

On reaching confluence, cells were passaged into flasks as per standard procedure using trypsin EDTA. Subculture and harvesting of cells was carried out by washing the cells briefly in a calcium and magnesium free balanced salt solution/PBS and detaching the cells with 0.05% trypsin in 0.02% EDTA (V:V).

For experimentation, cells were passed into 6-well multiwell culture plates (2ml/well) at a plating density of 0.1x10^{6}cells/ml growth medium (NOTE- for cell stretch experiments see 2.2.5).

Cells were only used for experimentation prior to their 12th subculture.

2.2.2.3 Changing of Medium

Medium in flasks was removed and replaced with fresh solution every 2 days (medium in 6-well multiwell culture plates (2ml/well), and cell stretch apparatus changed every day).

2.2.3 LDL/OX-LDL PREPARATION

30mls of whole blood was collected into tubes containing EDTA (1mg/ml), and centrifuged in a Sorvall RT600B refrigerated centrifuge at 3,000rpm (1,250g) for 10 minutes, at 4°C.

The plasma/top layer was removed and 4ml volumes aliquoted into Sorvall 93237 ultracentrifuge tubes. 0.32mls of 1.182g/ml density solution (see appendix 2) was added to each tube and tubes mixed with a pasteur pipette. 2mls of 1.019g/ml density solution was overlayed and the plasma samples ultracentrifuged at 47,000rpm (150,000g), at 4°C overnight.

The top 2mls of each ultracentrifuge sample was removed and discarded. 1.47mls of 1.182g/ml density solution was added to the remaining solution, and tubes mixed with a pasteur pipette. 0.53mls of 1.063g/ml density solution was slowly overlayed, and the ultracentrifugation repeated.

The top 1-2mls (yellow layer) was removed from each tube and the protein value estimated by reading 1ml of solution in a quartz cuvette at 280nm against distilled water (dH_2O) ie. 0.230 = 230µg/ml. The LDL solution was then diluted to a protein concentration of approx. 200µg/ml using 1/10 dialysis buffer (NOTE - for organ bath experiments LDL solution was not diluted at this stage, as required at high concentrations).

The LDL solution was dialysed in 0.01M phosphate buffer containing 0.015M NaCl, pH7.4 (ie. 2 litres 1/10 dialysis buffer). Dialysis was carried out in the dark at 4°C for 40 hours. Four changes of buffer were made during this time.

Following dialysis protein content was measured as previously and solutions diluted using 1/10 dialysis buffer if required.

The solution was split into 2 equal amounts and treated as follows:-

LDL CHOLESTEROL SOLUTION

Dialysed LDL solution was poured into tubes containing EDTA (1mg/ml) to protect against oxidation, and stored in the dark at 4°C until required.

OX-LDL CHOLESTEROL SOLUTION

20µls of copper chloride solution was added per millilitre of dialysed LDL solution to aid oxidation, and the solution left at room temperature for approx. 8 hours. Following oxidation the solution was stored in the dark at 4°C until required.

LDL and ox-LDL solutions were kept for a maximum of 4 weeks.

2.2.4 CHOLESTEROL UPTAKE IN CULTURED VASCULAR SMOOTH MUSCLE CELLS FROM SHRSP AND WKY RATS

Cells were grown in 6-well multiwell culture plates (2ml/well). On reaching confluence cells were incubated at 37°C, 5%CO_2/95% air, overnight as follows:-

Incubation for Cholesterol Uptake Experiments

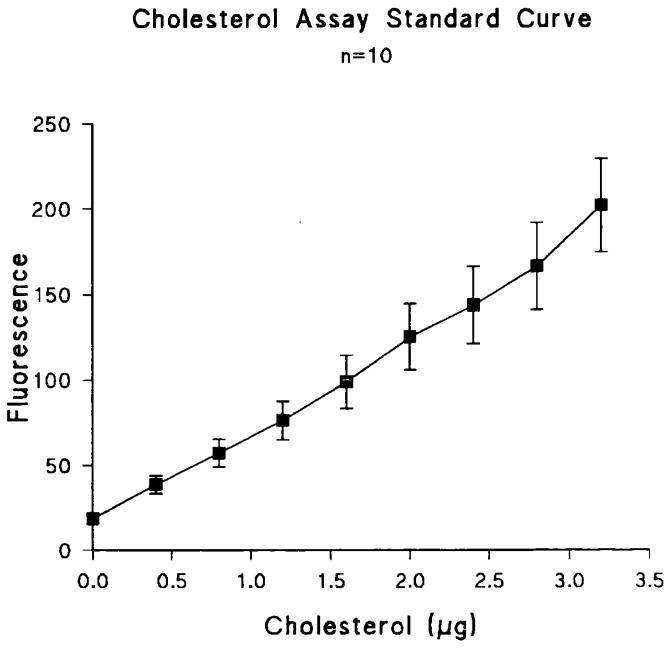
Plate No.	1	1	1
Incubation Medium	Medium 199 + control volume PBS +/- Vit. E NALC	Medium 199 + 20µg/ml LDL +/- Vit. E NALC	Medium 199 + 20µg/ml ox-LDL +/- Vit. E NALC
Final Volume	2ml	2ml	2ml

TABLE 2.1 - Incubation set up for 'cholesterol uptake' experiments in cultured WKY and SHRSP vascular smooth muscle cells

Following incubation plates were washed twice with 2mls PBS buffer (pH7.4). Cells were scraped from wells into 250µls of buffer, and transfered into 1ml eppendorf tubes (2 wells/500µls per eppendorf).

Cholesterol was extracted and free and total cholesterol determined using the method described by Gamble *et al.*, 1978. 500µls of chloroform:methanol (2:1) was added to each tube, the samples vortex mixed, and left to extract overnight at 4°C.

The tubes were then centrifuged at 13,000rpm (9,000g) for 15 minutes. The bottom layer (275µls chloroform) was removed into polypropylene



Example of standard curve for cholesterol assay. In general, samples fell on lower half of the curve.

test-tubes and the supernatant stored for protein analysis at a future date. The chloroform was evaporated at 37°C under a stream of air, following which 250µls of 95% ethanol was added, and the tubes vortex mixed thoroughly. Samples were bath sonicated on ice for 15 minutes to dissolve the cholesterol.

Assay solutions were prepared as follows:-

Stock solution	***Free Cholesterol***	***Total Cholesterol***
K_2HPO_4 buffer	20ml	16ml
Cholesterol esterase	0ml	4ml
Cholesterol oxidase	4ml	4ml
Peroxidase	4ml	4ml
Triton X 100	2ml	2ml
Na cholate	2ml	2ml
p-OH-phenylacetic acid	6ml	6ml

NOTE - Glass tubes used for assay

(see appendix 5)

The cholesterol standard stock solution was diluted 1 in 10 and a standard curve prepared. The points on the standard curve were 0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, and 3.2 µg cholesterol content.

100µls of each standard and sample was added to both free and total cholesterol assay tubes (note -

standards were assayed in duplicate). 1ml of the assay reagent (free or total) was added to each tube, and the tubes were vortex mixed.

TOTAL CHOLESTEROL

Total cholesterol samples were incubated for 15 minutes at 37°C in a shaking water bath. Following incubation samples were left for 15 minutes and fluorescence read at 325-415nm.

FREE CHOLESTEROL

Free cholesterol samples were incubated for 15 minutes at 37°C in a shaking water bath and left to sit for a minimum of 5 minutes before fluorescence was read (325-415nm).

Calculated values were multiplied by a factor of 2.5 to give total sample values.

2.2.5 EFFECT OF CELL STRETCH ON CHOLESTEROL UPTAKE IN VASCULAR SMOOTH MUSCLE CELLS

Vascular smooth muscle cells were prepared as previously described.

2.2.5.1 Preparation of Cell Stretch Apparatus

The perspex components of the cell stretch apparatus were sterilised overnight in 70% methanol and allowed to dry within a laminar flow hood.

PRETREATMENT OF SILICONE SHEETING

Silicone sheets were cut into rectangles 110 x 100mm in area, cleaned by washing in 0.1% Decon 75 solution, and rinsed thoroughly in dH_2O. The silicone was then transferred to aluminium foil and autoclaved at 121°C.

Apparatus was assembled under sterile conditions within a laminar flow hood. Autoclaved silicone sheets were mounted on vertially aligned screws, the apparatus fully constructed, and sheeting stretched to a precalculated resting tension.

Following assembly 10mls of a 25µg/ml fibronectin solution dissolved in serum-free medium was applied to the silicone and incubated at room temperature for 20 minutes. Silicone was rinsed with serum-free medium and allowed to dry at room temperature (Deehan, 1994).

Cells were plated on the dried silicone at a density of 2.5 x 10^6 cells/ml and grown in a serum enriched

medium (as per vascular smooth muscle growth medium - see appendix 3 - with 10% newborn calf serum in place of horse serum).

2.2.5.2 Cell Stretch Protocol

DAY 0 Construction of apparatus - cells passaged and left to attach overnight

DAY 1 Medium changed

DAY 2 Cells incubated in 20µg/ml ox-LDL in medium 199 for 24 hours +/- 20% stretch

DAY 3 Incubation terminated, wells scraped and samples assayed as previously described

2.2.6 PROTEIN DETERMINATION - Cell Culture

Protein was measured by a method adapted from Lowry *et al.*, 1951.

Protein samples were evaporated dry using a Cryovap centrifuge at 60°C for 3 hours. 250µls of 1M sodium hydroxide was added to each sample, the samples vortex mixed, and left for 48 hours at 4°C.

A standard curve was prepared using bovine serum albumin (BSA) as the protein standard. Points on the standard curve were 0, 2.5, 5, 10, 20, 40, 50, 60, 70, 80, and 90µg/200µls.

200µls of each sample (neat + 1/10 dilution) and standard (in duplicate) were added to assay tubes. 1.5mls of sodium copper tartrate reagent (see appendix 6) was added to each tube, and the tubes vortex mixed.

After 10 minutes 150µls of Folins phenol reagent was added to each tube. The tubes were vortex mixed and incubated at room temperature for 30 minutes.

Samples were centrifuged in a Sorvall RT600B refrigerated centrifuge at 3,000rpm (1,250g) for 5 minutes, at 4°C. Protein measurements were read using a spectrophotometer at a wavelength of 750nm.

Calculated values were multiplied by a factor of 1.25 to give total protein content of samples.

2.2.7 EFFECT OF CHOLESTEROL ON VASCULAR RESPONSES

Male Sprague Dawley rats (>6 weeks) were sacrificed by exposure to an overdose of halothane. The thoracic aorta was immediately removed into ice cold Krebs bicarbonate buffer, cleaned of connective tissue, and dissected into 2-3mm long rings.

The aortic rings were then randomly placed in 3 different solutions for incubation:

Incubation for Vascular Response Experiments

Approx. No. Rings Per Experiment	3	2	3
Solution	Krebs Control	ox-LDL/LDL	ox-LDL/LDL
Contents	Krebs bicarbonate buffer (KBB) +/- Vit. E Dex. SOD	100µg/ml ox-LDL/LDL + KBB	500µg/ml ox-LDL/LDL + KBB +/- Vit. E Dex. SOD
Volume	1ml	1ml	1ml

TABLE 2.2 - Incubation set up for experiments studying the effect of cholesterol on vascular responses

NOTE - where controls had no additional compound, appropriate concentration of vehicle added.

Experiments were also conducted using a 20µg/ml ox-LDL solution. Preincubation control rings were examined, and dexamethasone experiments were carried out in the presence and absence of endothelium.

Rings were incubated in the above solutions for 5 hours at 37°C in 95%Air/5%CO_2. Tubes were mixed by

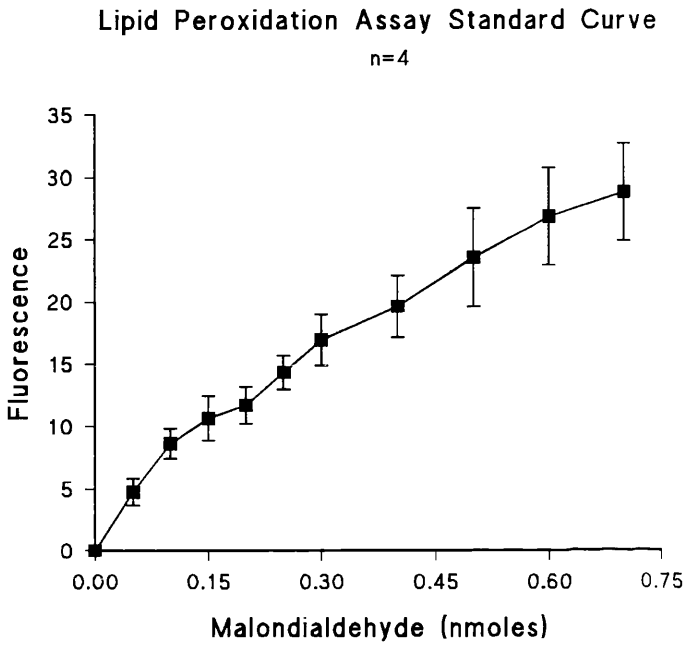
inversion every 30 minutes to ensure adequate oxygenation.

In vitamin E/LDL studies 4μM final concentration of EDTA and BHT was added to solutions immediately following incubation and removal of rings to prevent further oxidation. Levels of incubation-induced oxidation were measured using a modified TBA assay with appropriate ox-LDL and preincubation controls (see 2.2.8.).

Rings were suspended in 10ml organ baths containing Krebs bicarbonate buffer (see appendix 7) at 37°C, and continuously gassed with 95%O_2/5%CO_2.

Isometric tension was recorded using a force transducer (Grass model FT03) connected to a chart recorder (Grass polygraph model 7B). Tissues were stretched to a resting tension of 1g and were left to equilibrate for 1 hour. During this time tissues were washed and restretched every 15 minutes.

Tissues were 'primed' with a $5x10^{-7}$M concentration of phenylephrine. Cumulative concentration-response curves were constructed to phenylephrine, carbachol, SNP, and potassium chloride. L-NAME induced



Example of standard curve for lipid peroxidation (TBA) assay. In general, samples fell on lower half of the curve.

contraction was also measured. (NOTE- L-NAME and SNP responses measured in different tissues).

Following experimentation tissues were removed, blotted dry and weighed. Their wet weight was used to calculate results 'per 0.1g tissue'.

2.2.8 ASSAY FOR LIPID PEROXIDATION LEVELS (TBA TEST)

Oxidation was measured by a modification of Ohkawa's spectrophotometric method (Ohkawa et al., 1979, Shad, 1993).

A standard curve was prepared using malondialdehyde (MDA) to calibrate the reaction. Points on the standard curve were 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, 0.6, 0.7nmoles MDA.

500µls of each sample and standard were added to assay tubes, 800µls of sodium dodecyl sulphate (SDS) (see appendix 8) added, and the tubes vortex mixed. 3mls of 0.8% TBA acetic acid was then added to each tube, the tubes vortex mixed, and heated at 95°C for 60 minutes.

Following incubation 1ml of dH_2O followed by 2.5mls buton-1-ol was added, tubes mixed, and centrifuged at 10°C, 2000rpm (500g) for 5 minutes.

The top layer (organic) was removed and measured for fluorescence at 515-553nm.

3

MEMBRANE CHOLESTEROL ALTERATIONS IN WKY AND SHRSP VASCULAR SMOOTH MUSCLE CELLS ON EXPOSURE TO NATIVE AND OXIDISED LDL

3.1 INTRODUCTION

The first indication of atherosclerosis is the accumulation of lipid in the intima of susceptible arteries. These fatty streaks classified by the World Health Organisation as stage 1 of atherosclerosis, are characterised by extracellular and intracellular lipids and are usually covered by intact endothelium. The lipid is predominantly derived from LDL of the plasma (Cardona-Sanclemente and Born, 1996).

Smooth muscle cells are the predominant cell type in the intima and media of large arteries and are also the cell type which proliferates early in the development of atheroma. During the progression of atherosclerotic disease they become an increasingly important cell type, accounting for a substantial proportion of cells within the atherosclerotic plaque.

Various groups have shown that increased cholesterol content within the cell membrane may be an initiator of proatherogenic changes at the level of membrane physicochemical structure and calcium influx (Scott-Burden *et al.*, 1989, Yeagle, 1989; Bialecki *et al.*, 1991, Figueiredo *et al.*, 1991; Thorin *et al.*, 1995). Changes observed following cholesterol

exposure range from alterations in Na-H antiporter activity and intracellular calcium, to activation of phosphatidylinositol metabolism and induction of growth-related metabolism.

The mechanism(s) responsible for cholesterol accumulation within arterial cells are not fully understood.

Most cells are known to have specific apolipoprotein (apo) B and apo E receptors on their surface (ie. the LDL receptor), however the activity of this receptor is inversely proportional to the cholesterol level in the cell. It is therefore unlikely that LDL receptor-mediated uptake can solely account for the observed cholesterol accumulation in cells (Slotte *et al.*, 1988).

Macrophages (the dominant cell type of atherosclerotic 'foam cells') have acetyl LDL receptors (also known as scavenger receptors) that bind and take up several modified forms of LDL, of which oxidative modification is prominent. The activity of this receptor does not alter with changes in cell cholesterol content, and leads to massive cholesterol accumulation and foam cell formation. Groups such as Brown *et al.*, 1983, and

Slotte *et al.*, 1988, have suggested that arterial smooth muscle cells lack such acetyl LDL receptors, and that cholesterol uptake is via a receptor-independent mechanism. This would allow arterial smooth muscle cells to take up LDL in both its native and oxidised form to a similar extent. Indeed, work by Fischman *et al.*, 1985, showed that the distribution and intensity of native and methylated LDL accumulation in the arterial wall of rabbit aorta was the same.

Acetyl LDL receptors are, however, expressed on cell types other than macrophages.

Stein and Stein, 1980, and more recently Thorin *et al.*, 1995, have shown preferential uptake of modified LDL over native LDL in bovine endothelial cells. Thorin *et al.*, found that free cholesterol content increased by 35% and 100% in LDL and ox-LDL treated cells respectively, suggesting the presence of acetyl LDL receptors on this cell type.

More interestingly, Pitas, 1990, found acetyl LDL receptor expression on both rabbit fibroblasts and smooth muscle cells. This expression could be up-regulated by both serum and phorbol esters, suggesting that vascular smooth muscle cells can express the acetyl LDL receptor, and that receptor expression can be regulated.

Hypertension is also a well known risk factor, especially when associated with hypercholesterolemia (Hollander *et al.*, 1976; Chobanian, 1983; McGill *et al.*, 1985; Kannel and Sytkowski, 1987; Chobanian *et al.*, 1989). Mechanisms by which hypertension contributes to atherogenesis have not been established, however a synergy between the two risk factors has been suggested. Since uptake of LDL is an important primary event in the atherosclerotic process it is possible that hypertension could accelerate the disease process by increasing cholesterol uptake by cells.

Studies by Dominiczak *et al.*, 1993, found that membrane microviscosity was elevated (ie. decreased membrane fluidity) in vascular smooth muscle cells from stroke-prone spontaneously hypertensive (SHRSP) rats when compared with cells from Wistar-Kyoto (WKY) rats, a normotensive reference strain.

This, and other studies (McLaren *et al.*, 1993, Devynck *et al.*, 1982), suggest that hypertension causes alterations in physicochemical structure of the lipid bilayer. As mentioned previously, similar alterations have also been observed following increased cholesterol content of cell membranes.

3.2 AIMS

The aim of this study was to investigate vascular smooth muscle cholesterol uptake by comparing the effects of native and oxidised LDL on free and total membrane cholesterol levels.

Hypertension and hypercholesterolemia were also examined as coexisting and interactive risk factors using a multiple risk model.

3.3 METHODS

Cells were cultured, LDL and ox-LDL prepared, and experimental protocols carried out as described previously (see 2.2.1-2.2.4, 2.2.6).

On reaching confluence WKY and SHRSP VSM cells were incubated for 24 hours in medium 199 containing either vehicle control, 20µg/ml LDL, or 20µg/ml ox-LDL.

Following incubation cells were scraped from plates, and free membrane cholesterol measured by enzymatic fluorescence assay determination. Protein content was measured using a modified Lowry assay.

3.4 DATA CALCULATION AND STATISTICS

Results were expressed in 'μg cholesterol per mg protein'. The mean for each incubation group was calculated +/- standard error of the mean (SEMEAN).

Statistical analysis was carried out using repeated measures ANOVA, and Bonferroni paired multiple comparison tests (differences being considered significant when $p<0.05$).

Results were also calculated as a '% increase in cell cholesterol content'. The median for each incubation group was calculated and 1st and 3rd quartile values given.

Statistical analysis was carried out using 1-sample Wilcoxon signed rank confidence interval tests, and Mann-Whitney 2-sample tests (with Bonferroni correction for multiple comparisons).

(Note - in cell culture experiments cells were derived from a minimum of 4 rats per experimental set).

3.5 RESULTS

Small increases in free membrane cholesterol were observed on exposure of WKY and SHRSP vascular smooth muscle cells to both LDL and ox-LDL (Fig. 3.1).

Both WKY and SHRSP cells had similar basal cholesterol levels (no significant difference), which increased following a 24 hour incubation with 20μg/ml LDL or ox-LDL. This reached significance in the SHRSP cells following LDL and ox-LDL treatment, while WKY cells showed a similar trend.

'Percentage increase in free membrane cholesterol content' was also examined following LDL and ox-LDL treatment (Table 3.1).

Free cell cholesterol content of SHRSP VSM cells increased significantly after LDL and ox-LDL treatment by 31.15% and 43.35% respectively. WKY VSM cells showed a smaller but similar trend (18.27% and 17.6% respectively).

In both cell types there was no significant difference between LDL and ox-LDL treatment.

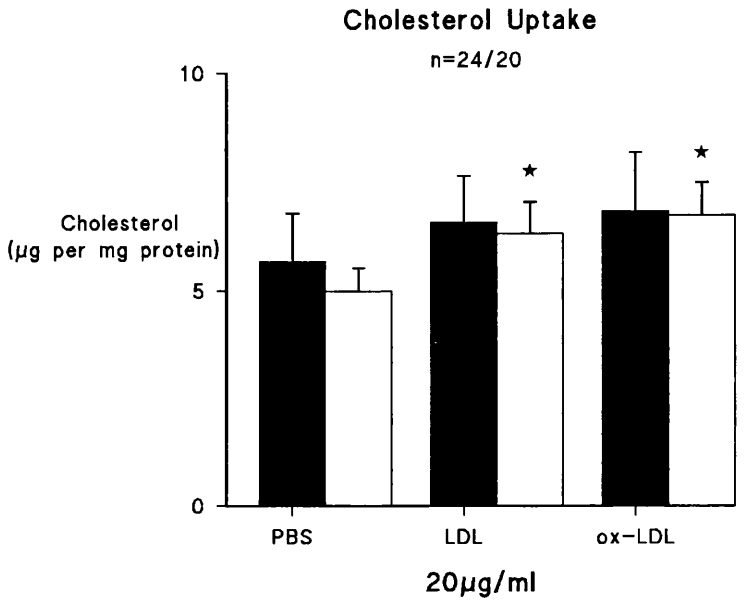


FIG. 3.1 - Effect of LDL and ox-LDL (20µg/ml) on free membrane cholesterol content in WKY and SHRSP cultured vascular smooth muscle cells. Solid bars represent WKY and open bars represent SHRSP.

* Significant difference from PBS/control ($p<0.05$)

Percentage Change in free membrane Cholesterol Content

	WKY		SHRSP	
	LDL	ox-LDL	LDL	ox-LDL
Median	18.2	17.6	31.15*	43.35*
Q1	-6.4	3.8	2.55	12.75
Q3	47.4	42.1	53.45	55.8
C.I.	-0.7	-2.6	3.6	15.6
	47	51.9	56.8	67.5

TABLE 3.1 - Percentage increase in free membrane cholesterol in cultured vascular smooth muscle cells on exposure to 20μg/ml LDL/ox-LDL.

* Significant increase from control ($p<0.05$)

C.I. 95% confidence intervals

Total membrane cholesterol content was also measured (not shown) and expressed a similar trend.

3.6 DISCUSSION

On exposure of vascular smooth muscle cells to 20μg/ml LDL/ox-LDL small increases in free membrane cholesterol content were observed.

This equated to 18.27% and 17.6% in WKY VSM cells following LDL and ox-LDL treatment respectively. These results confirm those of groups such as Alam *et al.*, 1980, who observed an increase in free membrane cholesterol content of 44.8% following a 24 hour incubation of human VSM cells with plasma LDL at the higher concentration of 400μg/ml.

Having carried out studies with plasma LDL concentrations ranging from 200μg/ml-800μg/ml, Alam *et al.*, noted that cellular cholesterol content was dose dependent, which may explain the higher value observed when compared with the present study. The following study therfore substantiates the findings of such groups.

As already mentioned treatment of WKY VSM cells with LDL and ox-LDL produced similar increases in free membrane cholesterol content.

This is similar to the findings of Fischman *et al.*, 1985, who found that distribution and intensity of native and methylated LDL accumulation were the same in the rabbit aortic wall. Results from this study, therefore support the theory that vascular smooth muscle cells may not normally express acetyl LDL receptors, but can accumulate cholesterol via a receptor-independent mechanism.

The aim of this study was also to examine hypertension and hypercholesterolemia as coexisting and interactive risk factors using a multiple risk model.

Incubation of SHRSP VSM cells with LDL and ox-LDL produced increases in free cholesterol content of 31.15% and 43.35% respectively, suggesting greater changes in cellular cholesterol content following LDL/ox-LDL exposure than observed in VSM cells from WKY rats, a normotensive reference strain.

Numerous studies have shown structural alterations in the cell membranes of SHRSP rats.

For example, decreased lateral diffusion (decreased membrane fluidity) has been observed in SHRSP cells when compared with WKY cells - their normotensive control (Dominiczak *et al.*, 1993; McLaren *et al.*, 1993).

Berk *et al.*, 1989, found that cultured VSM cells from spontaneously hypertensive rats exhibited increased growth and Na^+/H^+ exchange.

Such data are consistent with alterations in membrane physicochemical structure observed following increased cholesterol content.

Since changes in cell membrane cholesterol content have been shown to alter membrane physiochemical structure (see introduction 3.1), these results indicate a possible interactive mechanism between hypertension and hypercholesterolemia, whereby hypertension could accelerate atherosclerotic progression by increasing cholesterol uptake by cells.

Incubation of SHRSP VSM cells with ox-LDL also tended to result in a greater % increase in cellular free cholesterol content when compared with LDL

treatment (43.35% v's 31.15% respectively), although this was not statistically significant.

A possible theory which would explain such results may be that SHRSP VSM cells express a small number of acetyl LDL receptors, however further work would be required to substantiate any such theory.

4

EFFECTS OF VITAMIN E AND n-ACETYL L CYSTEINE ON MEMBRANE CHOLESTEROL

4.1 INTRODUCTION

In recent years considerable international interest has grown in the possibility that some of the major chronic disease states that afflict mankind worldwide, may be preventable by increasing intake of antioxidants.

Free radicals have been implicated as a contributing factor to the pathophysiology of diseases ranging from stroke and cancer to respiratory and cardiovascular disease. Antioxidant neutralisation of such free radicals may therefore prevent and protect against these associated disease states.

Since the mid 1980's there has been an explosion in the number of studies investigating antioxidant protection, in particular against cardiac and circulatory disease. Antioxidants studied include chemical antioxidants such as DPPD, probucol (and structurally related compounds such as BHT), and natural dietary antioxidants such as the vitamins A, C, and E, carotenoids (in particular beta-carotene), and the trace mineral selenium.

DPPD, BHT, and probucol have all been shown to decrease the degree of LDL oxidation and to protect against the progression of atherosclerotic disease

in animal models (Carew *et al.*, 1987; Kita *et al.*, 1988; Steinberg *et al.*, 1988; Bjorkhem *et al.*, 1991; Sparrow *et al.*, 1992; Plane *et al.*, 1993).

Although effective in the prevention of atherosclerosis DPPD has been shown to be mutagenic (Rannug *et al.*, 1984), and BHT can cause considerable acute toxicity in animals (although studies also suggest negligible chronic toxicity at the maximum acceptable daily intake for man) (Hirose *et al.*, 1981).

Probucol, a hypolipidemic drug, and the most extensively studied of the chemical antioxidants, was found to have marked antioxidant activity in humans, however also caused substantial lowering of HDL cholesterol (Reaven *et al.*, 1992; Walldius *et al.*, 1993). The significance of this lowering is unknown, however HDL is thought to protect against atherosclerotic disease and studies have shown that a low HDL cholesterol level is linked to an increased risk of coronary artery disease (Kannel *et al.*, 1979).

Given the toxicity and potential side effects of the chemical antioxidants their use as preventative agents in man is very limited.

As a result interest has turned towards the natural dietary antioxidants. High doses of these nutrients are well tolerated and without serious adverse reactions (Bendich and Machlin, 1988; Hathcock *et al.*, 1990; Walter, 1991; Diplock, 1995).

Evidence suggests that the presence of the antioxidant vitamins C and E, and beta-carotene in the blood may have a protective role against cardiovascular disease (reviewed by Rice-Evans, 1995).

Numerous studies have been carried out examining effects and actions of these antioxidants individually and in combination with varying results.

Data collected from the World Health Organisation MONICA study for example, found that plasma vitamin E exhibited a strong inverse correlation with the risk of ischaemic heart disease. Vitamin C also showed a moderately strong statistically significant inverse

correlation, whereas vitamin A and carotene showed only a weak inverse correlation (Gey *et al.*, 1991).

Rimm et al., 1993, using data generated from the Health Professionals Study, also found a strong association between higher intake of vitamin E and a lower risk of coronary artery disease. However, beta-carotene was associated with a significantly lower risk of coronary artery disease among current and former smokers, wheras vitamin C showed no association with lower risk among any of the population groups.

As a result the cardiovascular protective effects of these antioxidants remains controversial, however vitamin E stands out - producing the most consistent results, for the prevention of LDL oxidation and atherosclerotic development and progression (Belcher *et al.*, 1993; Ferns *et al.*, 1993; Reaven *et al.*, 1993[1]; Rimm *et al.*, 1993; Stampfer *et al.*, 1993; Andersson *et al.*, 1994; Jialal and Fuller, 1995; Stephens *et al.*, 1996).

α-tocopherol is the most prevalent and biologically active form of vitamin E. It is the predominant lipid soluble antioxidant in tissues, and is present

in blood plasma in the LDL fraction. Vitamin E is therefore, optimally placed to prevent free radical mediated modification of LDL and tissue damage (Ferns *et al.*, 1993).

There is increasing evidence indicating that LDL oxidation, which occurs during atherosclerosis and is thought to be involved in lesion initiation and progression, is inhibited by vitamin E (Reaven *et al.*, 1993[1], 1993[2]; McDowell *et al.*, 1994; Suzukawa *et al.*, 1994; Parker *et al.*, 1995).

Recent studies have also suggested that vitamin E may have other anti-atherogenic effects.

Physiological concentrations of alpha-tocopherol have been shown to inhibit smooth muscle cell proliferation and protein kinase C activity (Ozer *et al.*, 1993; Boscoboinik *et al.*, 1994, 1995).

Thorin *et al.*, 1995, observed that increased free cholesterol content in LDL and ox-LDL treated bovine aortic endothelial cells could be prevented by addition of vitamin E during the treatment period. Ox-LDL mediated alterations in membrane microviscosity were also blocked by addition of vitamin E (and the microviscosity of all cells

reduced). In addition vitamin E was shown to affect basal and stimulated intracellular calcium concentrations in normal cells. These results suggest an additional role of vitamin E in cholesterol flux alterations of the cell membrane.

Investigation into the non-antioxidant anti-atherogenic effects of vitamin E is however, relatively recent, and much work is still required before its full effects and actions are uncovered.

Another antioxidant of increasing interest in recent years is that of N-acetyl-l-cysteine (NAC).

A potential therapeutic agent in antioxidant mediated disorders, NAC has been widely used as a mucolytic drug for the treatment of chronic obstructive lung disease, and has been shown to reduce the suceptibility of lung tissue to free radical-induced damage by potentiating antioxidant defence systems (Sala *et al.*, 1993).

4.2 AIMS

The aim of this study was to investigate the effects of the antioxidants vitamin E and NAC on LDL and ox-LDL mediated cholesterol uptake in WKY and SHRSP vascular smooth muscle cells.

4.3 METHODS

Cells were cultured, LDL and ox-LDL prepared, and experimental protocols carried out as described previously (see 2.2.1-2.2.4, 2.2.6).

On reaching confluence WKY and SHRSP VSM cells were incubated for 24 hours in medium 199 containing 20μg/ml LDL or ox-LDL. Experiments were also carried out in the presence and absence of 10 and 100μM vitamin E or NAC. Appropriate controls were used for each experiment.

Following incubation cells were scraped from plates, and free membrane cholesterol measured by enzymatic fluorescence assay determination. Protein content was measured using a modified Lowry assay.

4.4 DATA CALCULATION AND STATISTICS

Results were expressed in 'μg cholesterol per mg protein'. The mean for each incubation group was calculated +/- standard error of the mean (SEMEAN).

Statistical analysis was carried out using repeated measures ANOVA, and Bonferroni paired multiple comparison tests (differences being considered significant when $p<0.05$).

Results were also calculated as a '% increase/decrease in cell cholesterol content'. The median for each incubation group was calculated and 1st and 3rd quartile values given.

Statistical analysis was carried out using 1-sample Wilcoxon signed rank confidence interval tests, and Mann Whitney 2-sample tests (with Bonferroni correction for multiple comparisons).

4.5 RESULTS

4.5.1 EFFECTS OF VITAMIN E ON LDL-INDUCED CHOLESTEROL UPTAKE

Vitamin E abolished any LDL effect at both 10 and 100μM concentrations, but also appeared to reduce basal free membrane cholesterol.

At the 10μM concentration vitamin E abolished any LDL-mediated increase in cholesterol content (Fig. 4.1). A significant reduction in free cholesterol content was observed when both WKY and SHRSP VSM cells were incubated with LDL in the presence of 10μM vitamin E when compared with LDL alone.

Interestingly, vitamin E also showed a tendency to reduce basal free cholesterol levels. Results

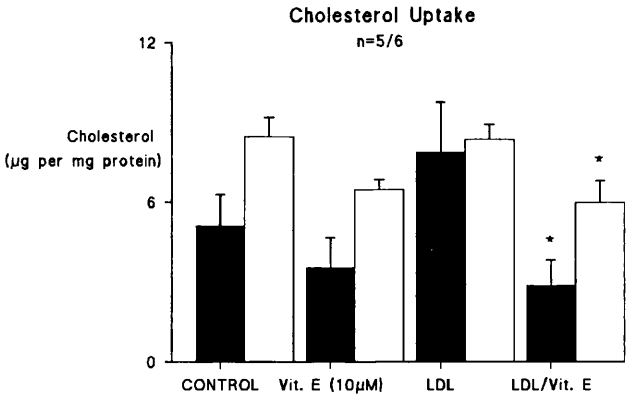


FIG. 4.1 - Effect of LDL (20µg/ml) +/- 10µM vitamin E on free membrane cholesterol content in WKY and SHRSP cultured vascular smooth muscle cells. Where solid bars represent WKY and open bars represent SHRSP.

* significant difference from LDL sample set ($p<0.05$).

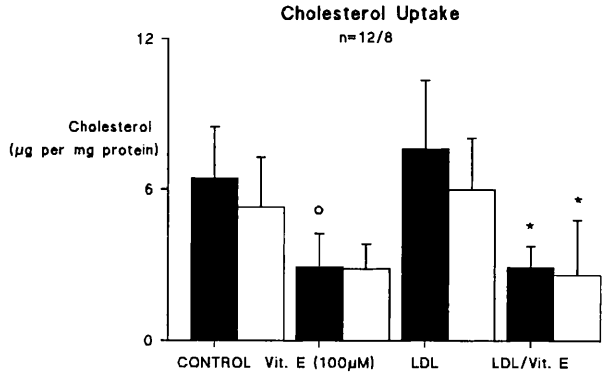


FIG. 4.2 - Effect of LDL (20µg/ml) +/- 100µM vitamin E on free membrane cholesterol content in WKY and SHRSP cultured vascular smooth muscle cells. Where solid bars represent WKY and open bars SHRSP.

O significant difference from control ($p<0.05$)

* significant difference from LDL sample set ($p<0.05$)

suggest that vitamin E-treated WKY and SHRSP VSM cells had a lower free cholesterol content than that of control cells, however this did not reach significance.

Incubation with a higher (100μM) concentration of vitamin E showed a similar trend (Fig. 4.2).

A significant reduction in free cholesterol content was again observed when both WKY and SHRSP VSM cell were incubated with LDL and vitamin E compared with LDL alone.

Vitamin E showed a tendency to reduce basal free cholesterol levels in both cell types, reaching significance in WKY cells.

Results were also expressed as a 'percentage increase/decrease in free membrane cholesterol content' following LDL +/- vitamin E treatment (Tables 4.1 & 4.2).

As previously, any increase in free cholesterol following LDL treatment in WKY VSM cells was again, abolished by vitamin E at both 10 and 100μM concentrations. SHRSP VSM cells appeared to show a similar trend (however no % increase in free

Percentage Change in Free Membrane Cholesterol Content

10μM Vitamin E

	WKY			SHRSP		
	vit. E	LDL	LDL/E	vit. E	LDL	LDL/E
Median	-13.6	40.9	-39.3*0	-25.5	1.8	-24.7
Q1	-62.4	7.9	-66.9	-36.4	-19.4	-46.8
Q3	-4.2	119.5	-25.5	-8.4	16.2	-9.8
C.I.	-72.1	7	-93	-40.9	-19.5	-69
	0	180	-14.3	21.5	28.9	7.9

TABLE 4.1 - Percentage increase/decrease in free membrane cholesterol in cultured vascular smooth muscle cells on exposure to 20μg/ml LDL +/- 10μM vitamin E.

0 significant difference from control ($p<0.05$)

***** significant difference from LDL sample set ($p<0.05$)

C.I. 95% confidence intervals

Percentage Change in Free Membrane Cholesterol Content

100μM Vitamin E

	WKY			SHRSP		
	vit. E	LDL	LDL/E	vit. E	LDL	LDL/E
Median	-69.8 0	10.5	-65.8*0	-55.5	-13.2	-67
Q1	-79.5	-30.5	-78.8	-74.6	-52.4	-84.1
Q3	-48.3	40.8	-41.1	-35.9	50.1	-9.3
C.I.	-86.5	-40	-82.8	-79	-83	-89
	-32.7	65	-17.9	84	226	65

TABLE 4.2 - Percentage increase/decrease in free membrane cholesterol in cultured vascular smooth muscle cells on exposure to 20μg/ml LDL +/- 100μM vitamin E.

0 significant difference from control ($p<0.05$)

* significant difference from LDL sample set ($p<0.05$)

C.I. 95% confidence intervals

cholesterol content was observed following incubation of SHRSP VSM cells with LDL in these experimental subgroups).

WKY VSM cells treated with LDL and vitamin E (10 and 100 μM concentrations), also had significantly lower free cholesterol content than that of control cells (39.3% and 65.8% decrease in free cholesterol content respectively).

Treatment of WKY and SHRSP VSM cells with 10μM vitamin E only, resulted in a decrease in free cholesterol content of 13.6% and 25.5% respectively. At the higher treatment concentration of 100μM vitamin E, free cholesterol content was reduced by 69.8% in WKY, and 55.5% in SHRSP cells, suggesting a possible concentration-dependent effect. Reduction of free cholesterol content in WKY cells at this concentration was statistically significant.

4.5.2 EFFECTS OF VITAMIN E ON OX-LDL-INDUCED CHOLESTEROL UPTAKE

Vitamin E attenuated any effect at both 10 and 100μM concentrations, but also appeared to reduce basal free membrane cholesterol levels.

At the 10μM concentration vitamin E attenuated any ox-LDL-mediated increase in cholesterol content (Fig. 4.3). A significant reduction in free cholesterol content was observed when SHRSP VSM cells were incubated with ox-LDL in the presence of 10μM vitamin E (when compared to ox-LDL alone), while WKY VSM cells showed a similar trend.

Vitamin E also showed a tendency to reduce basal free cholesterol levels; results suggesting that vitamin E-treated WKY and SHRSP VSM cells had a lower free cholesterol content than that of control cells.

Incubation with a higher (100μM) concentration of vitamin E showed a more prominent trend (Fig. 4.4).

A significant reduction in free membrane cholesterol content was observed when both WKY and SHRSP VSM cells were incubated with ox-LDL and vitamin E, compared with ox-LDL alone.

Vitamin E significantly reduced basal free cholesterol levels in both WKY and SHRSP VSM cells.

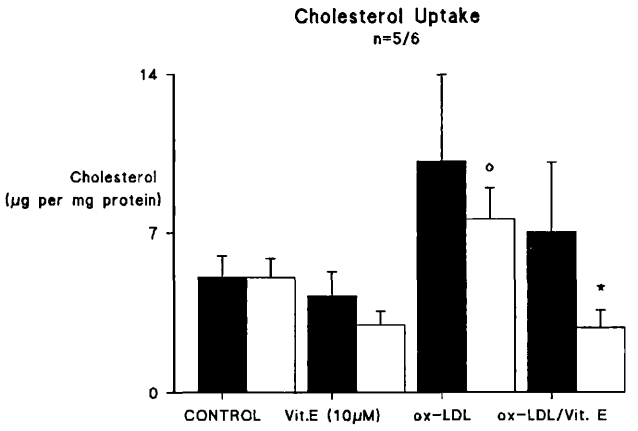


FIG. 4.3 - Effect of ox-LDL (20µg/ml) +/- 10µM vitamin E on free membrane cholesterol content in WKY and SHRSP cultured vascular smooth muscle cells. Where solid bars represent WKY and open bars represent SHRSP.

O significant difference from control ($p<0.05$)

* significant difference from LDL sample set ($p<0.05$).

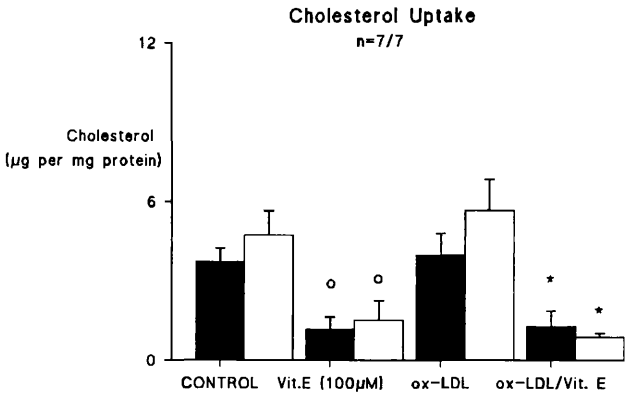


FIG. 4.4 - Effect of ox-LDL (20µg/ml) +/- 100µM vitamin E on free membrane cholesterol content in WKY and SHRSP cultured vascular smooth muscle cells. Where solid bars represent WKY and open bars SHRSP.

O significant difference from control ($p<0.05$)

* significant difference from LDL sample set ($p<0.05$)

Results were also expressed as a 'percentage increase/decrease in free membrane cholesterol content' following ox-LDL +/- vitamin E treatment (Tables 4.3 & 4.4).

As previously, results showed that any increase in free cholesterol content following ox-LDL treatment, in both WKY and SHRSP VSM cells, was attenuated by vitamin E at both 10 and 100μM concentrations.

At the 10μM vitamin E concentration a significant attenuation of ox-LDL-mediated change in free membrane cholesterol was observed in SHRSP VSM cells. WKY VSM cells showed a similar trend.

At the higher (100μM) concentration a significant attenuation of ox-LDL-mediated effects were observed in both WKY and SHRSP VSM cells.

Treatment of WKY and SHRSP VSM cells with 10μM vitamin E alone, suggested a decrease in free cholesterol content (-15.5% and -33.9% respectively).

Percentage Change in Free Membrane Cholesterol Content

10μM Vitamin E

	WKY			SHRSP		
	vit. E	ox-LDL	ox/E	vit. E	ox-LDL	ox/E
Median	-15.5	52.7 o	-7.8	-33.9	50.3 o	-39.3*o
Q1	-35.2	23.5	-22.7	-63.9	14.3	-87.4
Q3	-4.3	147.6	75.7	-14.1	75.9	-22.5
C.I.	-38.5	19	-35	-65.3	14.3	-96.4
	1.6	218	142	9.8	78.6	-4.3

TABLE 4.3 - Percentage increase/decrease in free membrane cholesterol in cultured vascular smooth muscle cells on exposure to 20μg/ml ox-LDL +/- 10μM vitamin E.

o significant difference from control ($p<0.05$)

* significant difference from LDL sample set ($p<0.05$)

C.I. 95% confidence intervals

Percentage Change in Free Membrane Cholesterol Content

100μM Vitamin E

	WKY			SHRSP		
	vit. E	ox-LDL	ox/E	vit. E	ox-LDL	ox/E
Median	-67.2 0	7.41	-56.5*	-89.9 0	13.9	-73.5*
Q1	-88.9	-4.04	-92.2	-92.8	6.1	-91.8
Q3	-30.8	29.4	-9.1	-38.2	68.4	-52
C.I.	-92	-20	-98	-97.1	-21	-93
	-11.2	31.7	7	-10.2	90	81

TABLE 4.4 - Percentage increase/decrease in free membrane cholesterol in cultured vascular smooth muscle cells on exposure to 20μg/ml ox-LDL +/- 100μM vitamin E.

0 significant difference from control ($p<0.05$)

* significant difference from LDL sample set ($p<0.05$)

C.I. 95% confidence intervals

This reached significance in the 100μM vitamin E treated cells (-67.2% and -89.9% respectively), suggesting a concentration-dependent effect.

4.5.3 EFFECTS OF NAC ON LDL-INDUCED CHOLESTEROL UPTAKE

The effects of NAC on LDL-mediated increases in free cholesterol content were minimal at both 10 and 100μM concentrations. No significant alteration in basal free cholesterol content was observed following NAC treatment alone.

Data suggested a very small attenuation of LDL-mediated free cholesterol changes at 10μM NAC in SHRSP VSM cells, and to a greater extent at 100μM NAC in both cell types (Figs 4.5 & 4.6). observed changes were minimal and did not reach significance.

No effect on WKY or SHRSP basal free cholesterol content was observed following NAC-treatment at either concentration.

Results were also expressed as a 'percentage increase/decrease in free membrane cholesterol content' following LDL +/- NAC treatment (Tables 4.5 & 4.6).

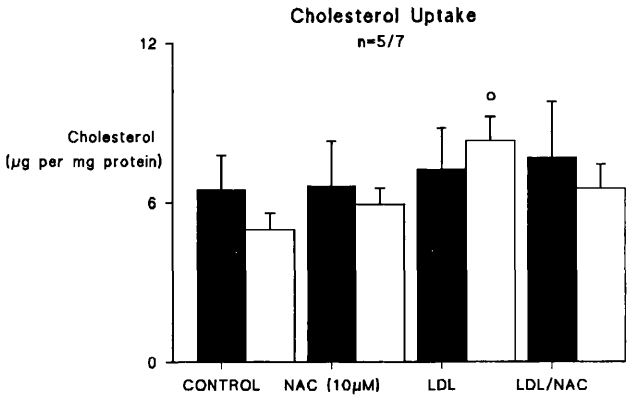


FIG. 4.5 - Effect of LDL (20µg/ml) +/- 10µM NAC on free membrane cholesterol content in WKY and SHRSP cultured vascular smooth muscle cells. Where solid bars represent WKY and open bars represent SHRSP.

o significant difference from control ($p<0.05$)

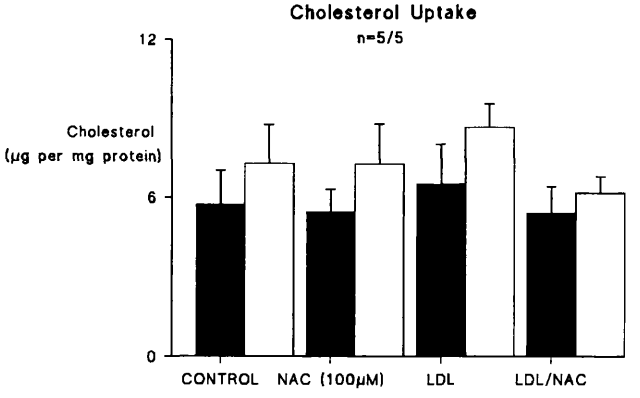


FIG. 4.6 - Effect of LDL (20µg/ml) +/- 100µM NAC on free membrane cholesterol content in WKY and SHRSP cultured vascular smooth muscle cells. Where solid bars represent WKY and open bars SHRSP.

Percentage Change in Free Membrane Cholesterol Content

10μM NAC

	WKY			SHRSP		
	NAC	LDL	LDL/N	NAC	LDL	LDL/N
Median	7.9	7.1	29.8	26.2	52.5 0	17.1
Q1	-44.9	-16.2	-51.4	2.6	11.3	2.2
Q3	18.7	51.6	65.8	35.5	145.6	56.8
C.I.	-14.9	-35.1	-29.9	-16	11	-14
	72.7	59	25	74.3	261	158

TABLE 4.5 - Percentage increase in free membrane cholesterol in cultured vascular smooth muscle cells on exposure to 20μg/ml LDL +/- 10μM NAC.

0 significant difference from control ($p<0.05$)

C.I. 95% confidence intervals

Percentage Change in Free Membrane Cholesterol Content

100μM NAC

	WKY			SHRSP		
	NAC	LDL	LDL/N	NAC	LDL	LDL/N
Median	-9.5	27	3.5	6.8	12	-20
Q1	-13.7	-13.2	-20.65	-13	-19.6	-31.4
Q3	35	50	18.05	16.3	147.6	40.2
C.I.	-14.9	-35.1	-29.9	-28.4	-20	-36
	72.7	59	25	22.6	261	93

TABLE 4.6 - Percentage increase/decrease in free membrane cholesterol in cultured vascular smooth muscle cells on exposure to 20μg/ml LDL +/- 100μM NAC.

C.I. 95% confidence intervals

As previously, data suggested a minimal reduction in LDL-mediated effects following treatment with 10μM NAC in SHRSP VSM cells (LDL 52.5%, LDL/NAC 17.1% change in free cholesterol content).

Following 100μM NAC treatment a minimal reduction was observed in both cell types (WKY: LDL 27, LDL/NAC 3.5; SHRSP: LDL 12, LDL/NAC -20%). Such changes were not significant.

Data suggested a minimal reduction in basal free cholesterol following 10μM NAC treatment in SHRSP and 100μM NAC treatment in SHRSP and WKY VSM cells. Such changes were not significant.

4.5.4 EFFECTS OF NAC ON OX-LDL-INDUCED CHOLESTEROL UPTAKE

NAC attenuated any ox-LDL effect at the 100μM concentration only. No alteration in free cholesterol content was observed following NAC treatment alone.

10μM NAC had no observable effect on ox-LDL-mediated increases in free cholesterol content in either WKY or SHRSP VSM cells (Fig. 4.7).

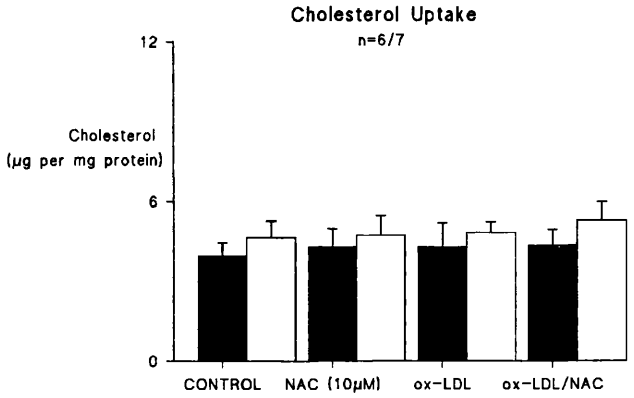


FIG. 4.7 - Effect of ox-LDL (20µg/ml) +/- 10µM NAC on free membrane cholesterol content in WKY and SHRSP cultured vascular smooth muscle cells. Where solid bars represent WKY and open bars represent SHRSP.

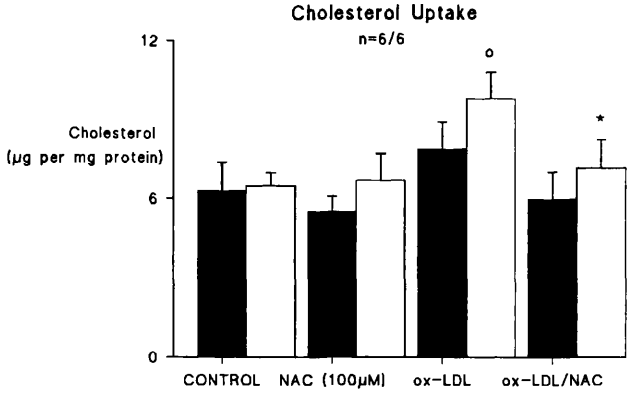


FIG. 4.8 - Effect of ox-LDL (20µg/ml) +/- 100µM NAC on free membrane cholesterol content in WKY and SHRSP cultured vascular smooth muscle cells. Where solid bars represent WKY and open bars SHRSP.

o significant difference from control ($p<0.05$)

* significant difference from LDL sample set ($p<0.05$)

At the higher (100μM) concentration of NAC a significant reduction in free cholesterol content was observed when SHRSP VSM cells were incubated with ox-LDL in the presence of 100μM NAC when compared with ox-LDL alone (Fig. 4.8). WKY VSM cells showed a similar trend.

No effect on basal free cholesterol content was observed following NAC treatment at either concentration.

Results were also expressed as a 'percentage increase/decrease in free membrane cholesterol content' following ox-LDL +/- NAC treatment (Tables 4.7 & 4.8).

As previously, data suggested a small reduction in ox-LDL mediated effects following treatment with the higher (100μM) NAC concentration only. Small reductions in ox-LDL mediated effects were observed in both WKY and SHRSP VSM cells (as a % increase/decrease in free cholesterol content; WKY: ox-LDL 28.7, ox/NAC -0.1; SHRSP: ox-LDL 32.7, ox/NAC 13.1), but did not reach significance.

Percentage Change in Free Membrane Cholesterol Content

10μM NAC

	WKY			SHRSP		
	NAC	ox-LDL	ox/N	NAC	ox-LDL	ox/N
Median	18.3	9.3	13.1	2	12.2	34.7
Q1	-39.9	-13	-34.8	-14.3	-20.6	-18.4
Q3	77.4	30	60.9	12.5	32.6	44.3
C.I.	-48	-40.4	-45	-26.7	-29	-38
	79	31.7	158	28.7	90	73

TABLE 4.7 - Percentage increase in free membrane cholesterol in cultured vascular smooth muscle cells on exposure to 20μg/ml ox-LDL +/- 10μM NAC.

C.I. 95% confidence intervals

Percentage Change in Free Membrane Cholesterol Content

100μM NAC

	WKY			SHRSP		
	NAC	ox-LDL	ox/N	NAC	ox-LDL	ox/N
Median	-4.83	28.7	-0.1	1.8	32.7 **0**	13.1
Q1	-28.95	-2.7	-46.1	-25.1	17.8	-14.3
Q3	17.28	64	53.4	34.6	111.2	41.7
C.I.	-29.7	-14	-47	-42.1	5	-49
	17.5	98	82	49.6	153	52

TABLE 4.8 - Percentage increase/decrease in free membrane cholesterol in cultured vascular smooth muscle cells on exposure to 20μg/ml ox-LDL +/- 100μM NAC.

0 significant increase from control ($p<0.05$)

C.I. 95% confidence intervals

Data suggested a minimal reduction in basal free cholesterol following 10μM NAC treatment in SHRSP, and 100μM NAC treatment in SHRSP and WKY VSM cells, however, no significant difference was observed.

4.6 DISCUSSION

In chapter 3 it was shown that exposure of vascular smooth muscle cells to 20μg/ml LDL/ox-LDL produced small increases in free membrane cholesterol content.

The aim of the present study was to investigate the effects of the antioxidants vitamin E and NAC on LDL and ox-LDL mediated cholesterol uptake in WKY and SHRSP VSM cells.

Results were expressed in 'μg cholesterol per mg protein' and as a 'percentage increase/decrease in cell cholesterol content' to give a more critical interpretation of data, and allow a clearer analysis of results.

Due to smaller experimental subsets, and variation in responses observed to LDL and ox-LDL, results did not always reach statistical significance. This made interpretation of data harder, however, by

expression of data in 2 ways certain patterns clearly emerged.

Variation in responses observed to LDL and ox-LDL may be due to differences in cell source (*ie.* intraspecies variation), or rate of growth and health of cells at the time of incubation.

Alternatively, interbatch differences in LDL and ox-LDL could account for such variation in results.

As previously mentioned, LDL and ox-LDL were regularly prepared from numerous healthy normolipidaemic volunteers, and it is likely that genetic variation and differences in lifestyle will cause considerable intersample diversity.

Additionally, ox-LDL is not a single homogeneous entity, and considerable variation may occur from preparation to preparation even when every effort is made to hold conditions constant.

The present study demonstrated that in the presence of either 10 or 100μM vitamin E, LDL and ox-LDL mediated effects were attenuated.

These results are similar to those of Thorin *et al.*, 1995, who found that when vitamin E (100μM) was added together with LDL/ox-LDL to bovine aortic endothelial cells for a 3 day incubation period, the increase in cell free cholesterol content in both LDL and ox-LDL treated cells was completely reversed. However, 10μM concentrations of vitamin E reversed ox-LDL induced increases in cell free cholesterol content only.

Due to the action of vitamin E on both LDL and ox-LDL mediated increases in cell free cholesterol content, effects cannot be attributed to vitamin E's antioxidant properties, and therefore suggest other mechanisms of action.

Traber and Kayden, in 1984, showed that vitamin E uptake by fibroblasts occured via the high affinity LDL receptor, but could also be transported into the cells via a receptor-independent mechanism.

It is therefore possible that vitamin E could prevent LDL and ox-LDL uptake by directly competing for transport mechanisms.

Viamin E was found to have similar effects in both WKY and SHRSP vascular smooth muscle cells.

Results therefore suggest that in the present study, any additional effects of genetic hypertension on cellular function in relation to cholesterol uptake, occurred via enhancement of, or similar mechanisms to, those observed in hypercholesterolemia (as both cell types were equally affected by vitamin E action).

Interestingly, 10 and 100μM concentrations of vitamin E also reduced basal free membrane cholesterol levels in a concentration-dependent manner.

Data by Thorin *et al.*, 1995, found a negligible effect in bovine aortic endothelial cells.

Goldberg and Mendez, 1988, however, found that incubation of human skin fibroblasts with probucol (2-5μM) led to cholesterol efflux. In the presence of HDL, probucol caused an approximate 20% reduction in cell cholesterol content. This effect on HDL-mediated cholesterol efflux was concentration-dependent and was capable of producing

a 2-fold increase in the amount of cholesterol leaving the cells.

Results from this study suggest that vitamin E may have similar non-antioxidant anti-atherogenic properties to probucol, and may explain why both these antioxidants have been particularly successful and consistent in preventing atherosclerotic development and progression in comparison to other antioxidants.

It is therefore possible that vitamin E may also reduce cholesterol overload and acculmulation through enhancement of cholesterol efflux from tissues.

The present study also examined the effects of the antioxidant NAC on LDL and ox-LDL mediated cholesterol uptake in WKY and SHRSP vascular smooth muscle cells.

NAC produced only a small attenuation of LDL and ox-LDL mediated effects in both cell types at the higher (100μM) concentration. At the lower (10μM) concentration no effect was observed.

Due to the action of NAC on both LDL and ox-LDL mediated cholesterol uptake effects of NAC cannot be attributable to its antioxidant properties alone, and sugggest another mechanism of action. It is possible that NAC may have a similar action to that previously described for vitamin E, whereby it prevents LDL and ox-LDL uptake by directly competing for transport mechanisms. Further work will be required to elucidate such mechanisms.

In the present study NAC did not alter basal free cholesterol content. This suggests that NAC does not, however, share all of vitamin E's mechanisms of action.

5

EFFECTS OF CELL STRETCH ON OX-LDL INDUCED MEMBRANE CHOLESTEROL ALTERATIONS IN CULTURED WKY VASCULAR SMOOTH MUSCLE CELLS

5.1 INTRODUCTION

Increased awareness of vascular biology has led to a deeper understanding of the development and progression of atherosclerotic disease.

Foam cells are now known to be a result of LDL oxidation, and uptake by the scavenger receptors of monocytes/macrophages that have migrated into the intima. A prior requirement to this process appears to be the accumulation of plasma lipid in the intima of suceptible arteries.

The mechanisms of transport and distribution of these plasma macromolecules into the arterial wall is not fully understood, however, increased knowledge of the processes involved may contribute to the understanding of atherosclerotic disease.

Interest in blood flow and vascular hemodynamics have been motivated by a possible role in the uptake and accumulation of plasma lipid, and localization of early atherosclerotic lesions (Nerem *et al.*, 1993).

Hypertension is one of the most prevalent and most potent determinants of atherosclerotic disease.

In particular, hypertension is a well known risk factor when associated with hypercholesterolemia (Hollander *et al.*, 1976; Chobanian, 1983; McGill *et al.*, 1985; Kannel and Sytkowski, 1987; Chobanian *et al.*, 1989).

The mechanisms by which hypertension contributes to atherosclerotic disease has not been established, however, since uptake of LDL is an important primary event in atherosclerosis it is possible that hypertension could accelerate the disease process by increasing cholesterol uptake by cells.

Several groups have examined the effect of transmural pressure on low density lipoprotein transport with varying results.

Curmi et al., 1990, found that increased transmural pressure (from 70mmHg to 160mmHg) in the rabbit thoracic aorta, caused a 44-fold increase in the concentration of LDL in the inner layers of the arterial wall, and a much lower (10-fold) increase in the subsequent layers.

Similarly, Rosati and Garay in 1991 found that turbulent flow stimulated cell uptake of exogenous cholesterol in rat aortic VSM cells.

Cardona-Sanclemente and Born, 1996, discovered that accumulation of LDL by rat aorta was increased by adrenaline or by angiotensin II at concentrations which raised blood pressure progressively and significantly. Previous work by this group (1994) however, suggested that aortic accumulation of LDL was increased by angiotensin II even when pressor effects were small and transient, suggesting mechanisms independent of pressure.

Fry *et al.*, 1992, however, found that normalized transendothelial uptake of LDL across normal intact minipig aortic endothelial surfaces were insensitive to pressure, in a dual pressure/flow model.

Differing results may be due to the various different cell preparations used, or a result of variations in the methods and techniques used and the haemodynamic forces simulated.

In vivo, cells are continuously exposed to forces existing within the vessel. Such forces can be resolved into 2 components:-

1 Pressure

- acting perpendicular to the surface

2 Shear Stress

- a tangential dragging component resulting from friction with the endothelial surface

The VSM cell constituent of the vessel wall absorbs most of the pressure, whereas the endothelium is subjected to the shear stress (Davies, 1989).

It is therefore practical to study the effects of these forces separately.

Cell culture experiments represent a model which allows the study of the specific mechanisms involved in biological responses, under well-defined mechanical conditions.

Stretch/pressure is a significant hemodynamic factor of blood pressure (particularly in hypertension). Limited studies have looked at the effects of pressure alone on the vascular system. These have been of a preliminary nature but have suggested that further investigations are warranted (Nerem *et al.*, 1993).

5.2 AIMS

The aim of this study was to examine the effects of cell stretch on ox-LDL-induced cholesterol uptake in WKY VSM cells using a novel cell stretch apparatus.

5.3 METHODS

Cells were cultured, ox-LDL prepared, and experimental protocols carried out as described previously (see 2.2.1-2.2.6).

Cells were grown on fibronectin-treated silicone sheeting mounted on a novel cell stretch apparatus developed by Deehan, 1994 (Fig. 5.1).

To mimic the pressure component *in vitro* the apparatus was designed to expose cells to a controlled uniform stretching force, resulting in an increase in the length of the cells.

On day 2 WKY VSM cells were either:-

1 carefully exposed to a stretch of 20%

2 allowed to remain at a control tension

Cells were then incubated for 24 hours in medium 199 containing 20μg/ml ox-LDL.

Cell Stretch Apparatus

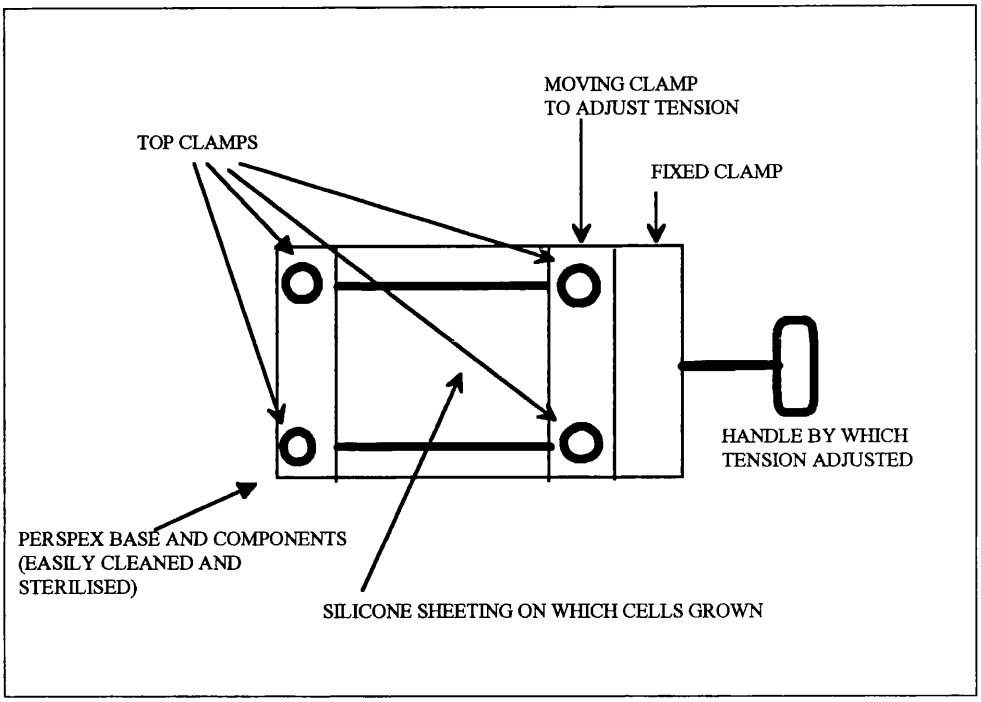


FIG. 5.1 - Sketch of cell stretch apparatus

Following incubation cells were scraped from the silicone and free membrane cholesterol measured by enzymatic fluorescence assay determination. Protein content was measured using a modified Lowry assay.

5.4 DATA CALCULATION AND STATISTICS

Results were expressed in 'μg cholesterol per mg protein'. The mean for each incubation group was calculated +/- standard error of the mean (SEMEAN).

Statistical analysis was carried out using a 2-sample (paired) t test (differences being considered significant when $p<0.05$).

5.5 RESULTS

Ox-LDL-mediated cholesterol uptake was compared between WKY VSM cells which were incubated at a control tension and those which were exposed to a single uniform stretch of 20% (Fig. 5.2).

No significant difference in free cholesterol content was observed between control and 20% stretch VSM cells following incubation with 20μg/ml ox-LDL. Similar levels of ox-LDL-mediated cholesterol uptake were observed in both groups.

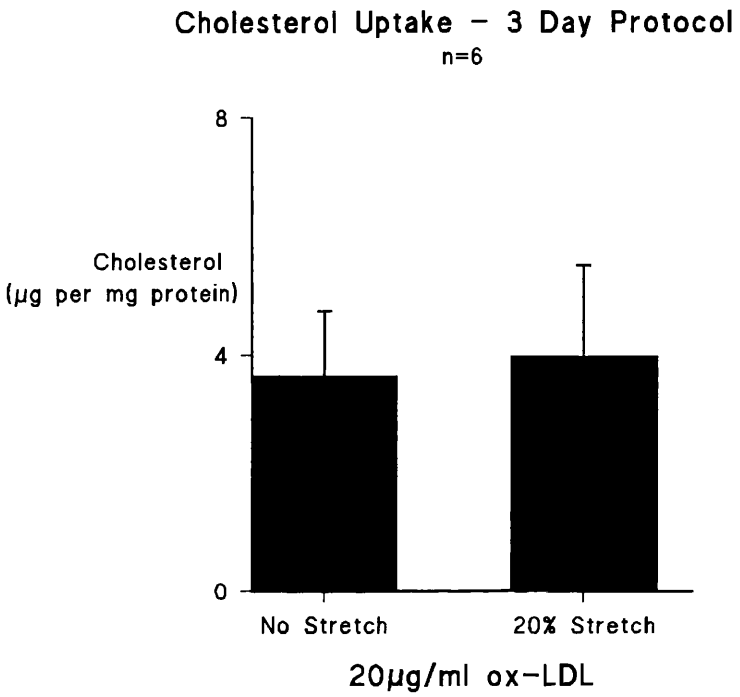


FIG. 5.2 - Effect of 20µg/ml ox-LDL +/- 20% cell stretch on free membrane cholesterol content in WKY cultured vascular smooth muscle cells

5.6 DISCUSSION

In the present preliminary study, no significant difference was observed in ox-LDL-mediated cholesterol uptake by VSM cells exposed to a uniform stretch of 20%, compared to those incubated at a control tension.

Such results differ from that of Curmi *et al.*, 1990 and Meyer *et al.*, 1996, who have investigated the effects of pressure on cholesterol uptake and accumulation in the vessel wall. Both groups observed an increased uptake of LDL cholesterol with increasing pressure.

Studies by these groups were carried out using isolated aortic segments as opposed to cells in culture. Results may therefore differ due to the considerably different methods of pressure application and experimentation used.

Negative results obtained in the present study may also be due to a variety of reasons.

Initial experimental conditions were developed by Deehan, in 1994, for the use of rat mesenteric VSM cells with the cell stretch apparatus. These conditions, although modified, were not ideal for

the growth of rat aortic VSM cells, however complete resetting of conditions was outwith the range of this Ph.D.

As previously mentioned, cells were grown on fibronectin-coated silicone sheeting.

In 1995 Beppu *et al.*, found that substrate-bound fibronectin enhanced the binding and uptake of ox-LDL by macrophage scavenger receptors. Although VSM cells are not thought to express scavenger receptors under normal conditions, further investigation is required to ascertain fully the effect of fibronectin on cholesterol uptake in this cell type.

Such investigation would be required to validate any results already achieved under the present conditions, and to substantiate methods before any further studies are carried out.

Also of interest in the future would be the adaption of stretch apparatus to expose cells to a pulsatile cell stretch. Cyclical stretch in preference to a single fixed stretch (as used in the present study), would allow examination of pressure effects under conditions nearer to those observed physiologically.

Results from the present study are therefore inconclusive, however demonstrate a potential model for future investigations of hemodynamic forces on cellular function, in the absence of other humoral influences.

6

EFFECTS OF NATIVE AND OXIDISED LDL ON VASCULAR REACTIVITY IN THE RAT AORTA

6.1 INTRODUCTION

For years the arterial system was viewed as a static network of resistance vessels responsible for the maintenance of arterial pressure.

However, in the past 16 years the discovery of the ability of the vascular endothelium to regulate vascular tone via the production of contractile and relaxant factors, has revolutionised, and brought about a new understanding, of vascular biology.

Known contractile factors include endothelin, angiotensin II, and the cyclooxygenase pathway products thromboxane A_2, prostaglandin H_2, and superoxide, whereas relaxant factors identified to date include prostacyclin and nitric oxide (NO) (Luscher, 1993; Ross, 1993; Daugherty *et al.*, 1995).

Of these factors NO has been extensively studied, and has proved to be of particular importance.

In 1980 Furchgott and Zawadzki demonstrated that acetylcholine-induced vasodilation in isolated preparations of rabbit aorta required intact endothelium, and resulted from the release of an endothelium-derived relaxing factor (EDRF), now known to be identical or similar to NO.

NO has since been implicated in a wide range of physiological and pathophysiological actions from roles in nonspecific immunity and endotoxic shock, to actions as a neurotransmitter.

In the vasculature NO has numerous actions including preservation of endothelial permeability and integrity, regulation of VSM proliferation and leucocyte-vessel wall interactions, and provision of antithrombic activity (Loscalzo and Welch, 1995).

In particular NO is a primary determinant of resting vascular tone through basal release, and causes vasodilation in response to various vasodilator agents such as acetylcholine, bradykinin, substance P, and increases in flow and shear stress (Calver *et al.*, 1993).

It is now thought that dysregulation of NO production/effect may contribute to the pathogenesis of a number of vascular disorders, including hypertension, reperfusion injury, and atherosclerosis.

Alterations in vascular function are well documented in the development of atherosclerotic disease (Heistad *et al.*, 1984; Berkenboom *et al.*, 1987;

Forstermann et al., 1988; Shimokawa and Vanhoutte, 1988). Risk factors such as elevated cholesterol levels have been linked to these alterations, however exact mechanisms are not fully understood.

As a result of numerous investigations examining the effects of LDL cholesterol on vascular tone, it has become apparent that LDL exerts 2 main effects:-

1 LDL impairs endothelium-dependent relaxation

(Andrews *et al.*, 1987; Jacobs *et al.*, 1990; Kugiyama *et al.*, 1990; Galle *et al.*, 1991; Tanner *et al.*, 1991; Chin *et al.*, 1992; Myers *et al.*, 1994).

2 LDL alters reactivity of vascular smooth muscle to contractile agonists

(Broderick *et al.*, 1989; Galle *et al.*, 1990)

These vascular effects have been shown to be primarily due to ox-LDL (although effects have been observed to a lesser extent on exposure to native LDL - Andrews *et al.*, 1987; Jacobs *et al.*, 1990; Galle *et al.*, 1991; Myers *et al.*, 1994).

Dysfunction of the NO pathway is thought to account for the impairment of stimulated NO release or

endothelium-dependent relaxation by agonists such as acetylcholine (NOTE - NO release has been used as a generalisation to cover NO action, which may be impaired at any point of the NO pathway).

More recently it has been suggested that modification of basal NO release may also occur, and may contribute to the changes in vascular reactivity observed. Few studies to date have examined this possibility.

6.2 AIMS

The aim of this study was to investigate the effects of native and oxidised LDL on vascular reactivity, in particular basal and stimulated NO release.

More specifically the aim of the present study was to examine the effects of native and oxidised LDL on:-

1 receptor-dependent contraction

2 receptor-independent contraction

3 stimulated NO release

4 basal NO release

5 VSM sensitivity to exogenous NO

6.3 METHODS

LDL and ox-LDL were prepared, and experiments carried out, as previously described (see 2.2.3, 2.2.7, 2.2.8).

Aortic rings 3-4mm wide were prepared from male Sprague Dawley rats (>6 weeks). Rings were incubated for 5 hours, at 37°C, 5% CO_2 95% air, in either Krebs bicarbonate buffer (control), 100μg/ml, or 500μg/ml LDL or ox-LDL solutions. Experiments were also conducted using 20μg/ml ox-LDL.

Following incubation rings were mounted under 1g tension for recording of isometric tension.

Cumulative concentration-response curves were constructed to phenylephrine (10^{-8}-$3x10^{-5}$M), carbachol (10^{-8}-$3x10^{-5}$M), SNP (10^{-11}-10^{-7}M), and potassium chloride (10^{-2}-10^{-1}M). Tissues were then contracted to the EC_{20} phenylephrine and the nitric oxide synthase inhibitor L-NAME (200μM) added at the plateau of phenylephrine contraction.

Following experimentation tissues were blotted dry and their wet weight taken.

(Note - all experiments were carried out in endothelium intact rings in the presence of indomethacin, β-estradiol, and cocaine, unless otherwise stated).

In seperate experiments with LDL 4μM final concentration EDTA and BHT were added to solutions immediately following incubation to prevent further oxidation. Levels of incubation-induced oxidation were measured using a modified TBA assay with appropriate ox-LDL and preincubation controls.

6.4 DATA CALCULATION AND STATISTICS

Data were calculated 'g tension per 0.1g tissue', and results expressed as mean +/- standard error of the mean (SEMEAN).

Statistical analysis was carried out using repeated measures ANOVA, and Bonferroni paired multiple comparison tests (differences being considered significant when $p<0.05$).

6.5 RESULTS

6.5.1 EFFECTS OF LDL AND OX-LDL ON PHENYLEPHRINE-INDUCED CONTRACTION IN THE RAT AORTA

Effects of LDL and ox-LDL on receptor-mediated contraction was investigated by examinination of responses to the α_1 adrenoceptor agonist phenylephrine.

Ox-LDL treatment at both the 100μg/ml and 500μg/ml concentration had no significant effect on tissue sensitivity to phenylephrine (Fig. 6.1).

Similarly no alterations in phenylephrine concentration-response curve sensitivity was observed following 100μg/ml LDL treatment (Fig. 6.2).

Aortic rings incubated in 500μg/ml LDL however, showed a decreased sensitivity to phenylephrine, and had significantly higher EC_{50} values than equivalent controls.

Maximum phenylephrine-induced contractions were significantly increased following incubation with 100μg/ml LDL and ox-LDL cholesterol (Fig.s 6.3, 6.4, 6.5, & 6.6). 500μg/ml ox-LDL treated rings showed a similar trend.

Interestingly, on incubation with 500μg/ml LDL a significant decrease in maximum phenylephrine induced contraction was observed.

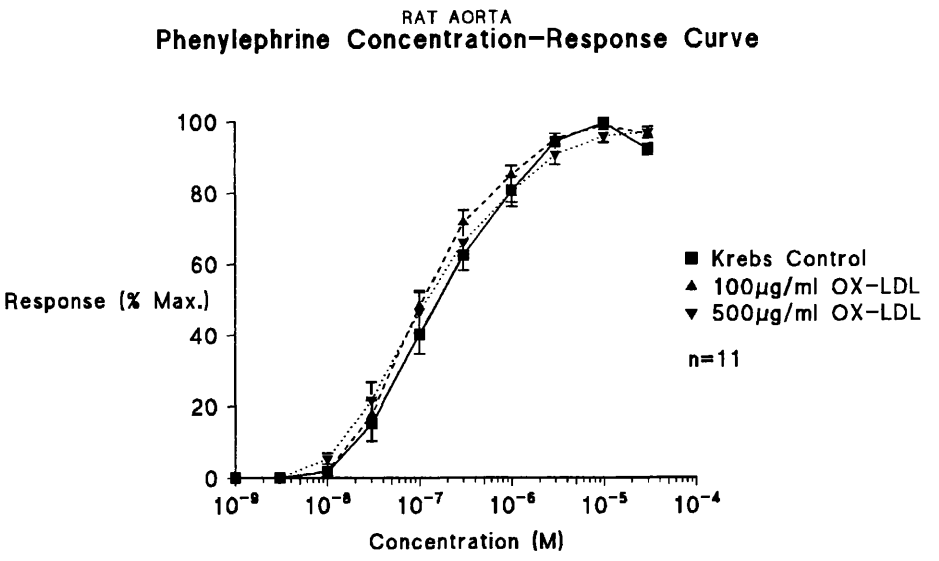


FIG. 6.1 - Effect of ox-LDL (100 & 500µg/ml) on phenylephrine concentration-response curve in the rat aorta.

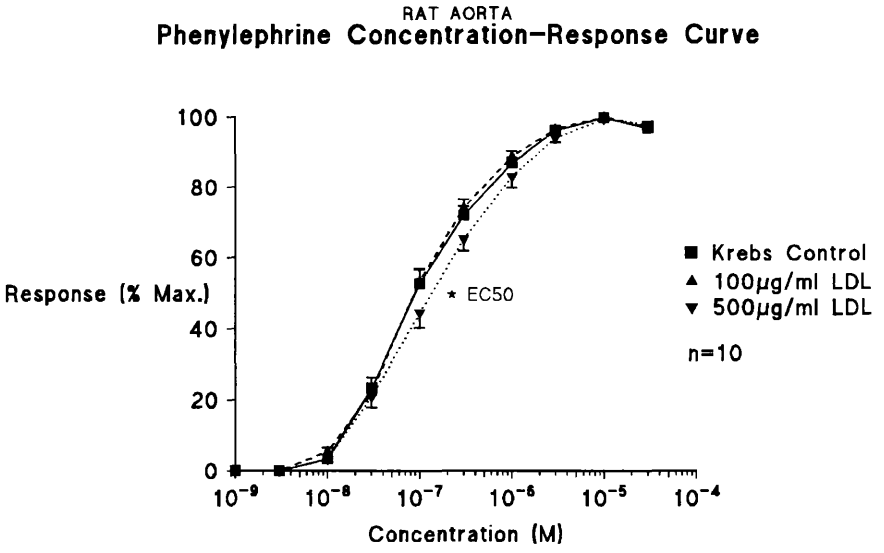


FIG. 6.2 - Effect of LDL (100 & 500µg/ml) on phenylephrine concentration-response curve in the rat aorta.

* significant difference from control ($p<0.05$)

Note - % Max. = percentage of maximum phenylephrine response

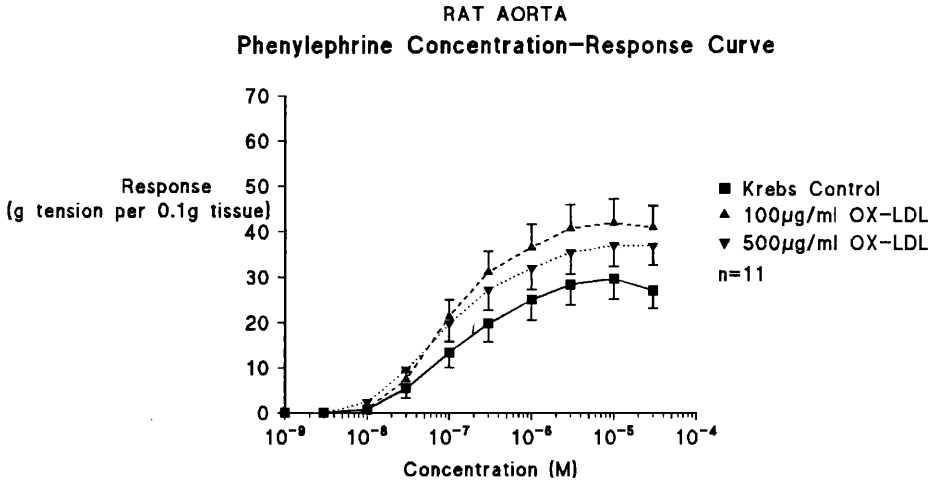


FIG. 6.3 - Effect of ox-LDL (100 & 500µg/ml) on phenylephrine concentration-response curve in the rat aorta.

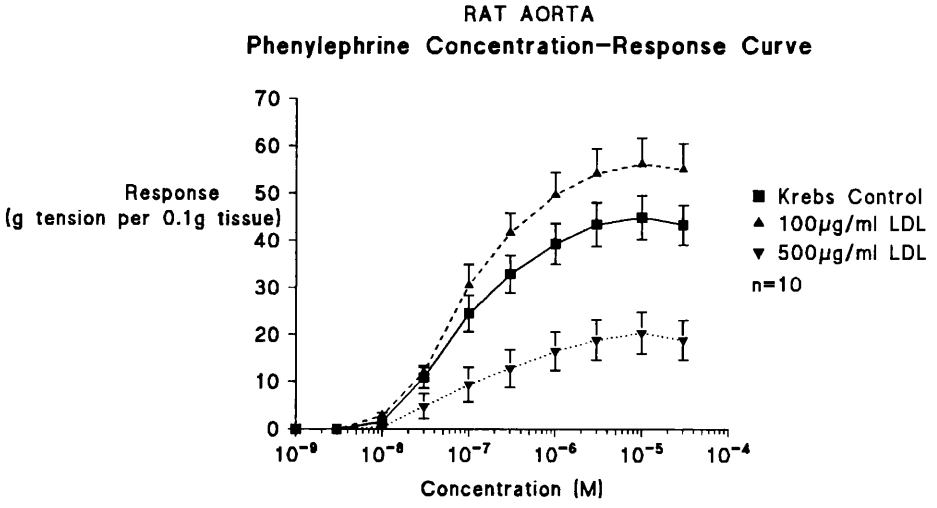


FIG. 6.4 - Effect of LDL (100 & 500 µg/ml) on phenylephrine concentration-response curve in the rat aorta.

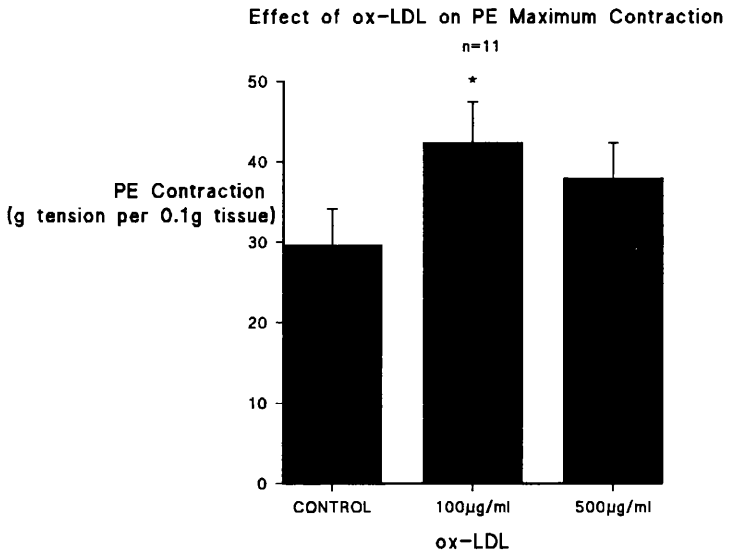


FIG. 6.5 - Effect of ox-LDL (100 & 500µg/ml) on maximum phenylephrine-induced contraction in the rat aorta.

* significant difference from control ($p<0.05$)

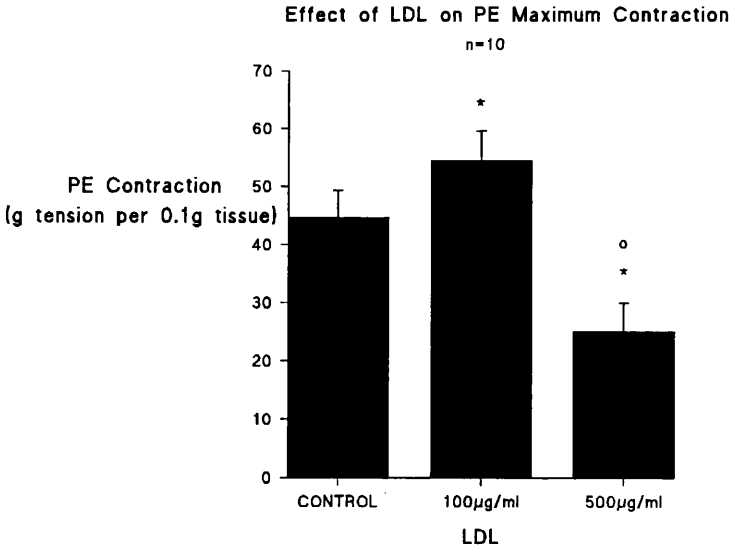


FIG. 6.6 - Effect of LDL (100 & 500µg/ml) on maximum phenylephrine-induced contraction in the rat aorta.

* significant difference from control ($p<0.05$)

O significant difference from LDL sample set ($p<0.05$)

6.5.2 EFFECTS OF LDL AND OX-LDL ON CARBACHOL-MEDIATED ENDOTHELIUM-DEPENDENT RELAXATION

Effects of LDL and ox-LDL on stimulated nitric oxide release were investigated by examination of responses to the muscarinic receptor agonist carbachol.

100μg/ml ox-LDL treatment had no significant effect on tissue sensitivity to carbachol (Fig. 6.7).

Incubation with 500μg/ml ox-LDL however, was found to decrease tissue sensitivity to carbachol; treated tissues having significantly higher EC_{50} values compared with their equivalent controls.

On incubation of tissues with 100μg/ml and 500μg/ml LDL cholesterol, results suggested a dose-dependent decrease in carbachol concentration-response curve sensitivity, however this did not reach significance (Fig. 6.8).

100μg/ml ox-LDL treatment had no significant effect on maximum carbachol-induced relaxation (Fig. 6.9).

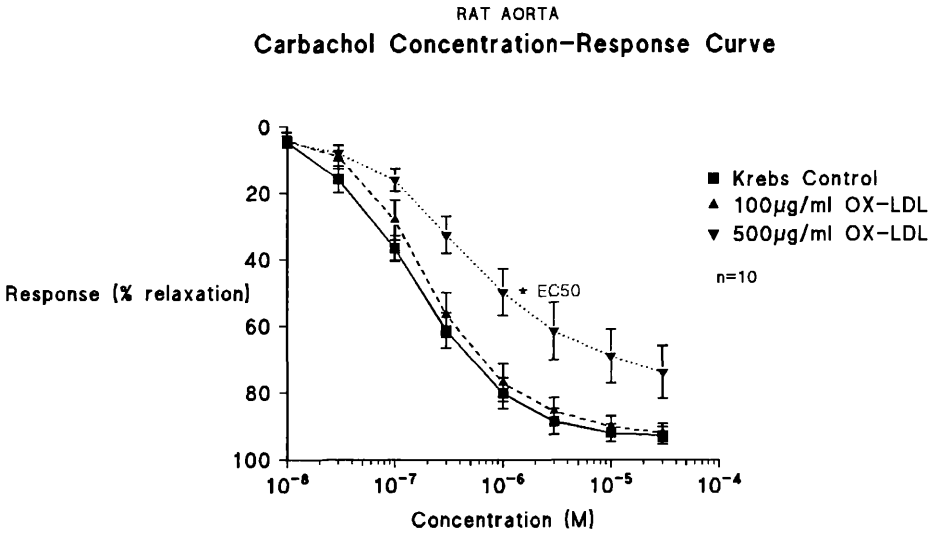


FIG. 6.7 - Effect of ox-LDL (100 & 500µg/ml) on carbachol concentration-response curve in the rat aorta.

* significant difference from control ($p<0.05$)

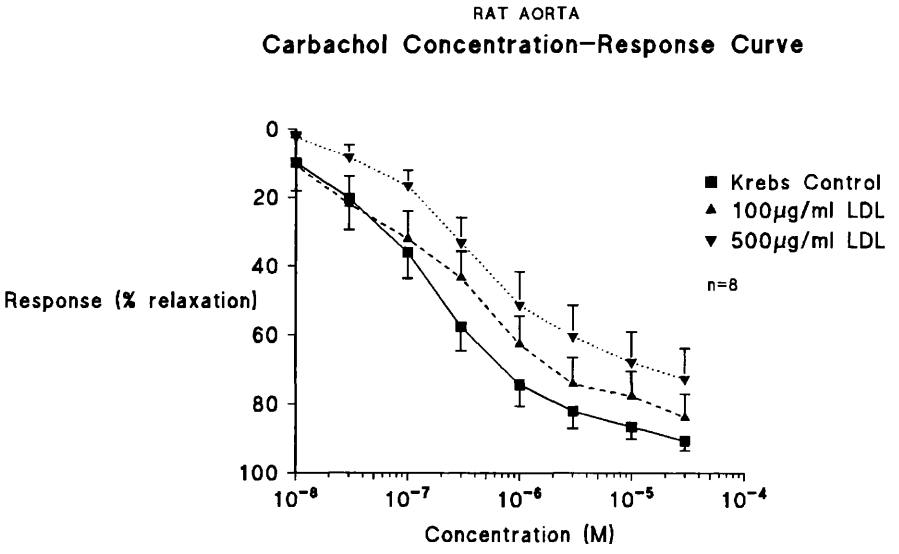


FIG. 6.8 - Effect of LDL (100 & 500µg/ml) on carbachol concentration-response curve in the rat aorta.

Incubation with 500μg/ml ox-LDL, however, was found to significantly reduce the maximum relaxation obtained to carbachol.

On incubation of tissues with 100μg/ml and 500μg/ml LDL, results suggested a dose-dependent reduction in carbachol-induced maximum relaxation, reaching significance in tissues incubated with the higher (500μg/ml) concentration (Fig. 6.10).

6.5.3 EFFECTS OF LDL AND OX-LDL ON SNP-INDUCED RELAXATION IN THE RAT AORTA

Effects of LDL and ox-LDL on VSM sensitivity to exogenous NO were investigated by examination of responses to the NO donor compound SNP.

Ox-LDL treatment, resulted in a dose-dependent reduction in SNP concentration-response curve sensitivity; both 100μg/ml and 500μg/ml ox-LDL treated tissues having significantly higher EC_{50} values compared with their equivalent controls (Fig. 6.11).

On incubation of tissues with 100μg/ml and 500μg/ml LDL, no significant effect on tissue sensitivity to SNP was observed (Fig. 6.12).

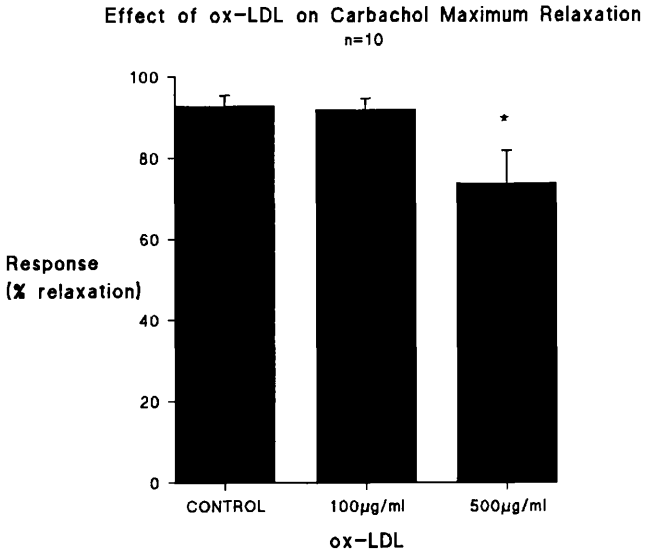


FIG. 6.9 - Effect of ox-LDL (100 & 500µg/ml) on maximum carbachol-induced relaxation in the rat aorta.

* significant difference from control ($p<0.05$)

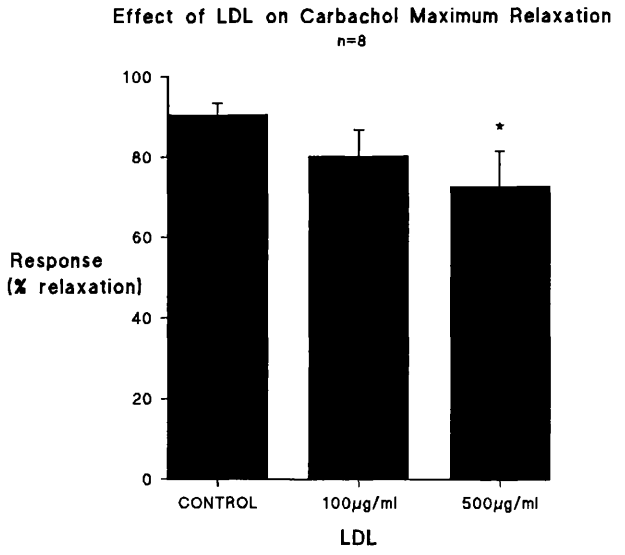


FIG. 6.10 - Effect of LDL (100 & 500µg/ml) on maximum carbachol-induced relaxation in the rat aorta.

* significant difference from control

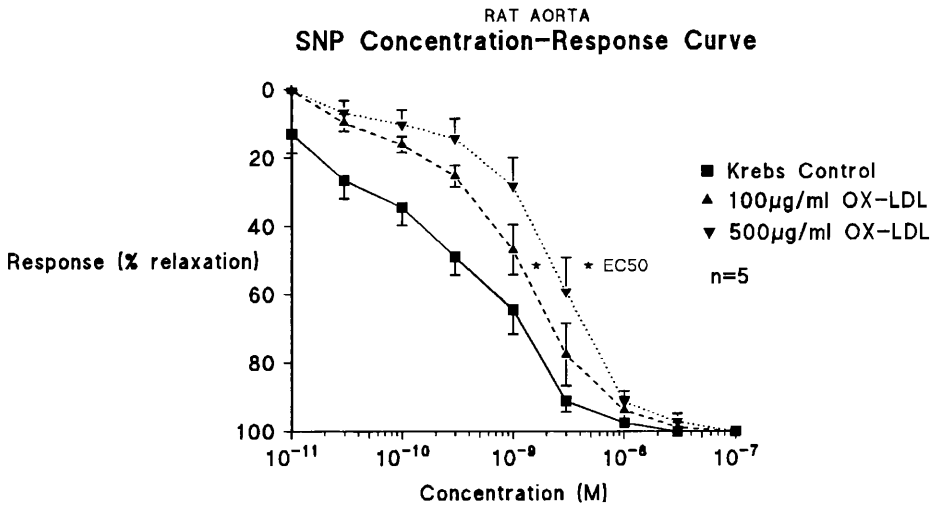


FIG. 6.11 - Effect of ox-LDL (100 & 500µg/ml) on SNP concentration-response curve in the rat aorta.

* significant difference from control ($p<0.05$)

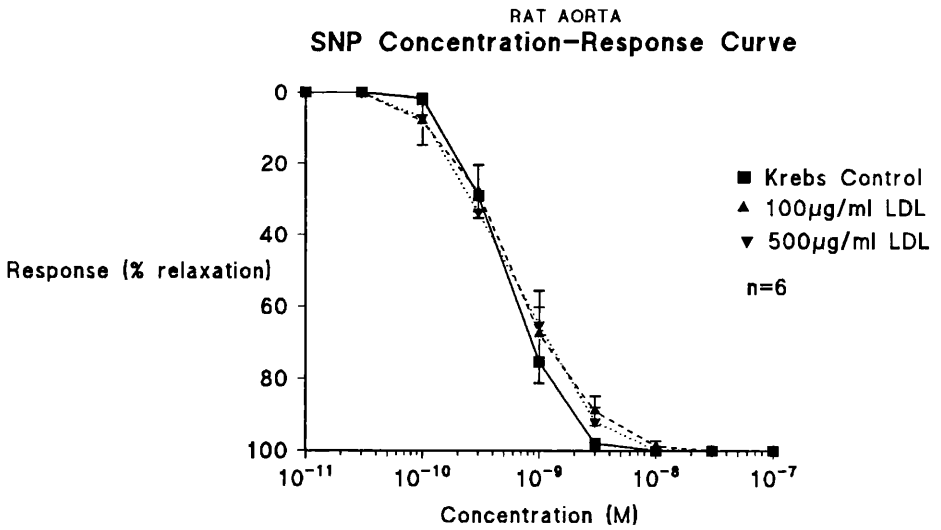


FIG. 6.12 - Effect of LDL (100 & 500µg/ml) on SNP concentration-response curve in the rat aorta.

LDL and ox-LDL had no significant effect on maximum SNP-induced relaxation; 100% relaxation being acheived in all ring preparations.

6.5.4 EFFECTS OF LDL AND OX-LDL ON L-NAME-INDUCED CONTRACTION IN THE RAT AORTA

Effects of LDL and ox-LDL on basal NO release were investigated by examination of contractile responses following exposure to the nitric oxide synthase inhibitor L-NAME (NOTE- contraction obtained following L-NAME treatment was taken as representative of basal NO release).

Incubation of tissues with 100μg/ml and 500μg/ml ox-LDL resulted in a significant dose-dependent reduction in contraction following exposure to L-NAME (Fig. 6.13).

100μg/ml and 500μg/ml LDL treated tissues showed a similar trend, reaching significance at the higher 500μg/ml concentration (Fig. 6.14).

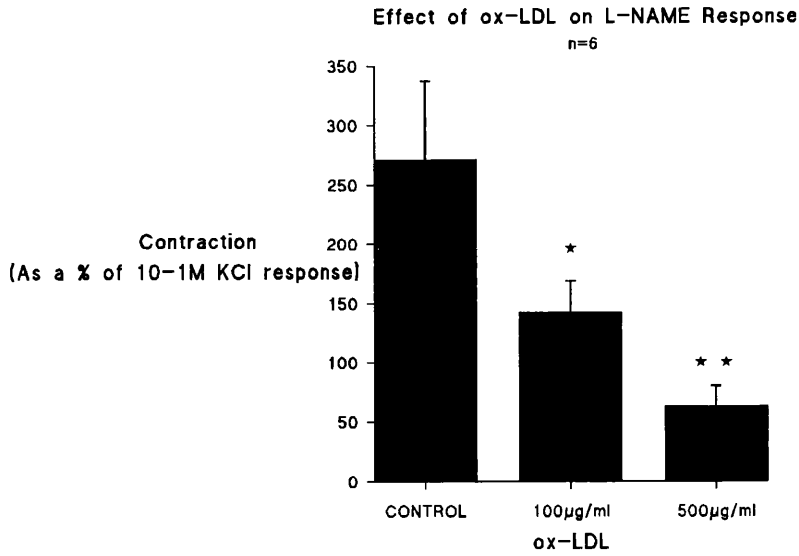


FIG. 6.13 - Effect of ox-LDL (100 & 500µg/ml) on L-NAME-induced contraction in the rat aorta.

* significant difference from control ($p<0.05$)

** significant difference from control ($p<0.01$)

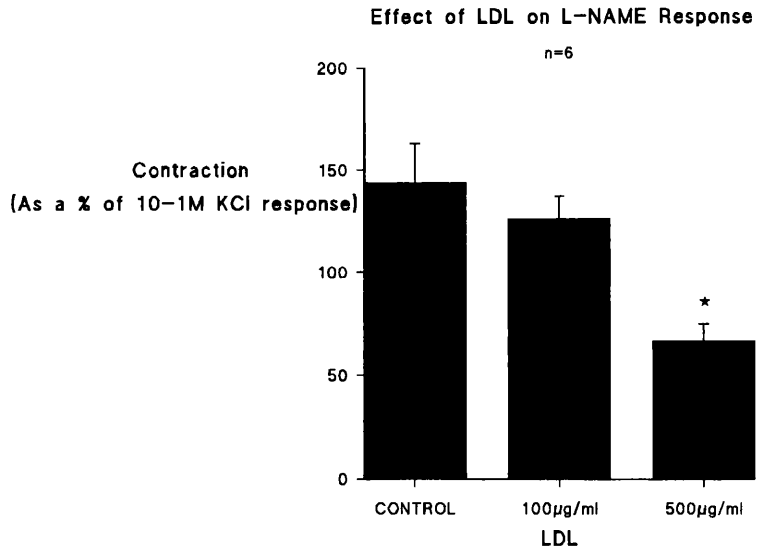


FIG. 6.14 - Effect of LDL (100 & 500µg/ml) on L-NAME-induced contraction in the rat aorta.

* significant difference from control ($p<0.05$)

6.5.5 EFFECTS OF LDL AND OX-LDL ON POTASSIUM CHLORIDE MEDIATED CONTRACTION IN THE RAT AORTA

Effects of LDL and ox-LDL on receptor-independent contraction were investigated by examination of responses to potassium chloride (KCl).

LDL and ox-LDL treatment had no effect on tissue sensitivity to KCl (Fig.s 6.15 & 6.16).

Results suggested that 100μg/ml and 500μg/ml ox-LDL treatment caused a slight increase in the maximum contraction obtained as a result of 10^{-1}M KCl, however, this did not reach significance (Fig. 6.17).

100μg/ml LDL caused a significant increase in maximum KCl-induced contraction, whereas 500μg/ml LDL had no significant effect (Fig. 6.18).

6.5.6 EFFECTS OF 20μg/ml OX-LDL ON VASCULAR REACTIVITY IN THE RAT AORTA

Initial experiments were also carried out using a 20μg/ml concentration of ox-LDL.

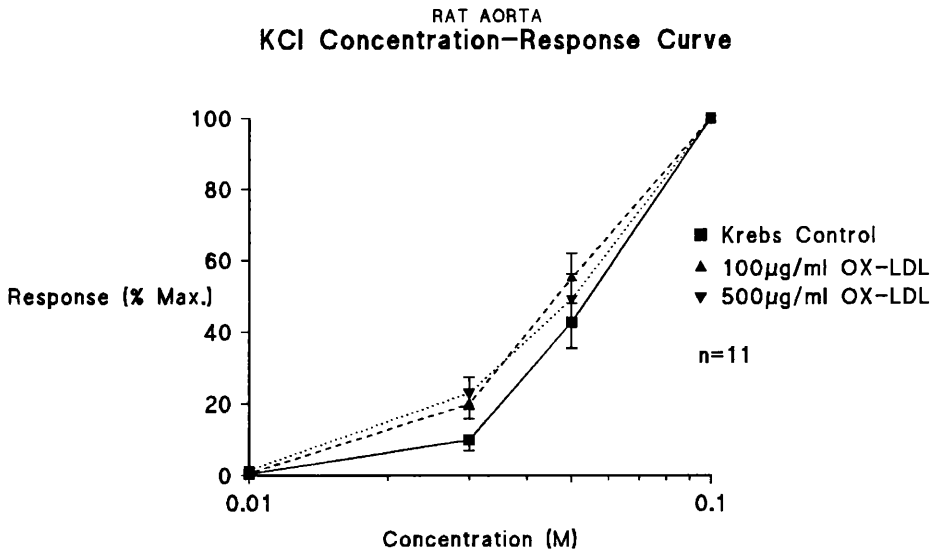


FIG. 6.15 - Effect of ox-LDL (100 & 500µg/ml) on potassium chloride concentration-response curve in the rat aorta.

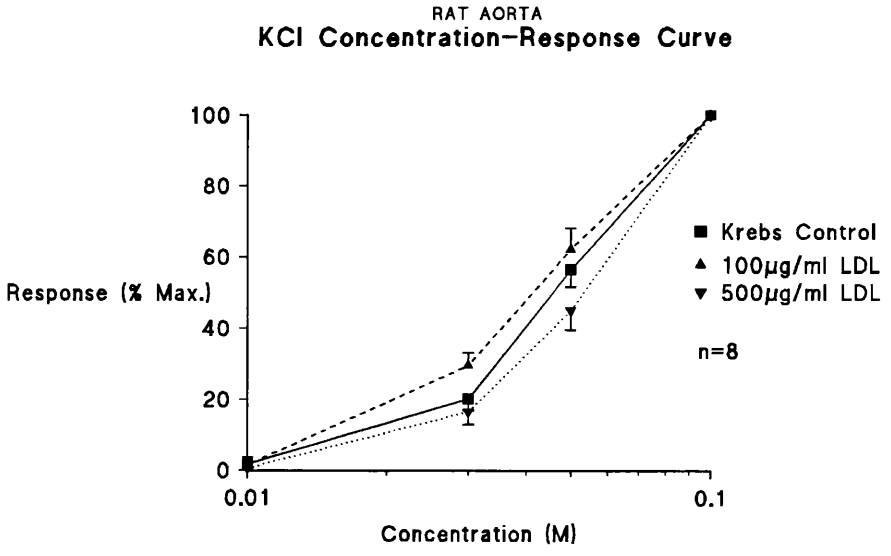


FIG. 6.16 - Effect of LDL (100 & 500µg/ml) on potassium chloride concentration response curve in the rat aorta.

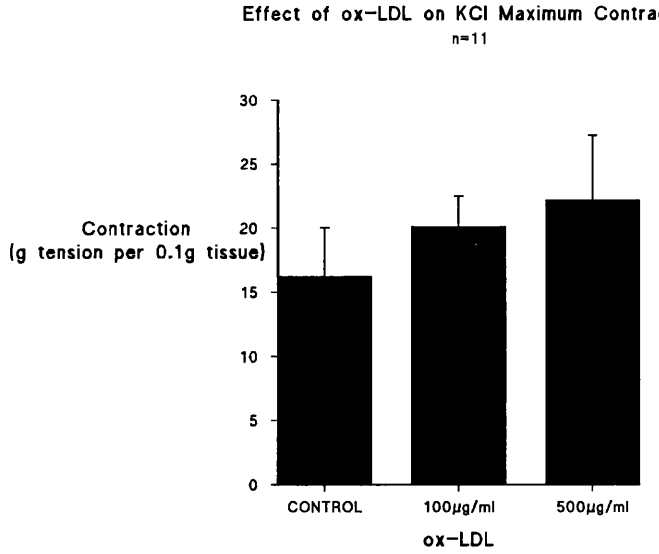


FIG. 6.17 - Effect of ox-LDL (100 & 500µg/ml) on maximum potassium chloride induced contraction in the rat aorta.

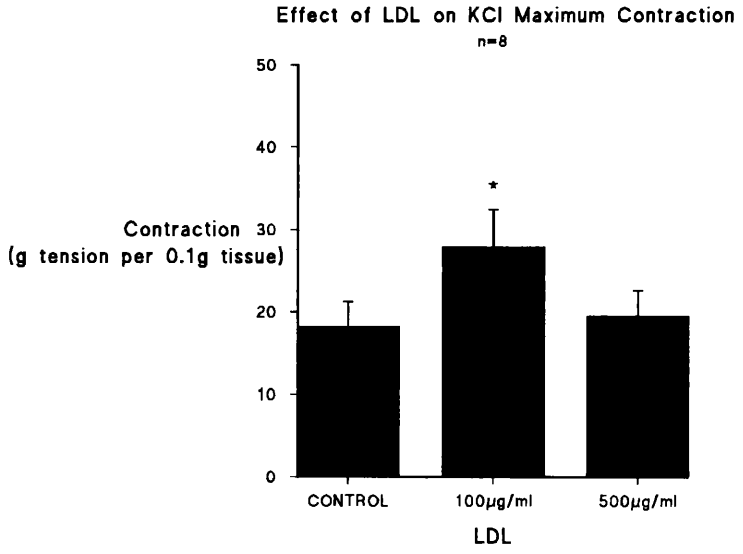


FIG. 6.18 - Effect of LDL (100 & 500µg/ml) on maximum potassium chloride induced contraction in the rat aorta.

* significant difference from control

No effect on vascular reactivity was observed following tissue exposure to this concentration (results not shown).

6.5.7 EFFECT OF 5 HOUR INCUBATION ON LDL OXIDATION

Lipid peroxidation of native LDL was examined following 5 hours incubation to ascertain whether the standard incubation procedure used in the present study would cause a significant increase in oxidative modification.

Incubation of LDL for 5 hours under standard incubation conditions (see 6.3), resulted in only a small increase in oxidative modification which was not significant (oxidative modification as a '% of 500μg/ml ox-LDL response' - preincubation control 0.98%, LDL 1.54%; postincubation control 0.74%, LDL 3.42%) (Fig. 6.19).

6.6 DISCUSSION

The aim of the present study was to investigate cholesterol-induced alterations of vascular function *in vitro*, by exposure of rat aortic ring preparations to LDL and ox-LDL.

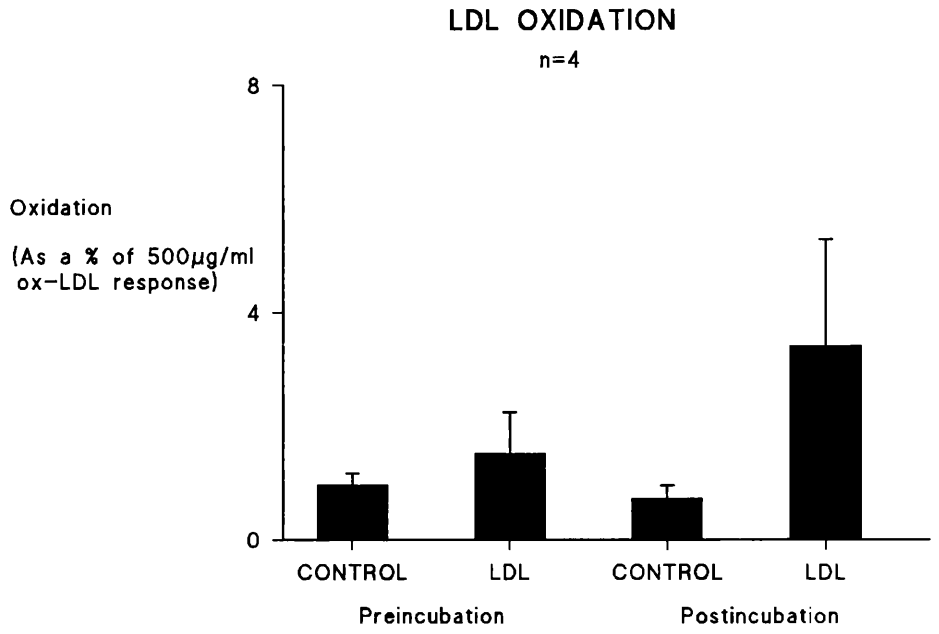


FIG. 6.19 - Effect of 5 hour incubation on LDL oxidation

6.6.1 LDL AND OX-LDL EFFECTS ON RECEPTOR-DEPENDENT CONTRACTION

LDL and ox-LDL cholesterol (excluding 500μg/ml LDL), caused a significant increase in maximum receptor-dependent contraction to phenylephrine, but had no effect on tissue sensitivity.

Similarly, Galle *et al.*, 1990, observed augmented contractile responses in tissues perfused with ox-LDL in the presence of low concentrations of receptor-dependent contractile agonists.

Galle *et al.*, however, found that ox-LDL (80μg/ml) also increased tissue sensitivity to the agonists norephinephrine and phenylephrine, and LDL had no effect on either maximal responses or tissue sensitivity.

Reasons for such discordance in results may be the considerable difference in experimental procedures used.

Galle *et al.* examined responses immediately following exposure, and in the presence of, ox-LDL/LDL. As a result experimentation by this

method would measure acute effects only. Results may therefore be attributable to the agonist effects which are known to occur on LDL and ox-LDL exposure (Simon *et al.*, 1990; Murohara *et al.*, 1994).

In the present study, ring preparations were incubated for 5 hours in the presence of LDL/ox-LDL. Following incubation extracellular LDL/ox-LDL was removed and tissues allowed to equilibrate for an hour prior to examination of responses.

The present study was therefore designed to examine the chronic effects of LDL and ox-LDL on vascular function, and excluded any possiblility of acute agonist effects.

Interestingly, 500μg/ml LDL caused a decrease in sensitivity of aortic ring preparations to phenylephrine, and significantly reduced the maximum receptor-dependent contractions achieved.

Reasons for this LDL-specific effect could not be explained, however this did not appear to be due to tissue toxicity, as potassium chloride responses remained unaffected.

6.6.2 LDL AND OX-LDL EFFECTS ON STIMULATED NO RELEASE

500µg/ml ox-LDL reduced tissue sensitivity to carbachol, and caused a decrease in the maximum relaxation observed. Incubation with LDL cholesterol showed a similar trend.

These results substantiate those of numerous groups who found that ox-LDL (and to a lesser extent LDL), attenuated stimulated NO release (Jacobs *et al.*, 1990; Simon *et al.*, 1990; Andrews *et al.*, 1987).

6.6.3 LDL AND OX-LDL EFFECTS ON VASCULAR SMOOTH MUSCLE SENSITIVITY TO EXOGENOUS NO

Ox-LDL (but not LDL), caused a dose-dependent reduction in tissue sensitivity to the NO donor SNP, although maximum relaxation was still achieved.

This substantiates the results of Jacobs et al., 1990, who found that ox-LDL (but not LDL), evoked a small but significant rightward shift in the dose response curve to the NO donor glyceryl trinitrate.

SNP is known to act as an nitric oxide donor, and therefore directly acts on the vascular smooth muscle to stimulate a relaxation effect.

The data from the present study suggests that ox-LDL can affect the relaxation pathway after NO synthesis and release, but provided weak evidence that ox-LDL-induced dysfunction is at least partially due to free radical neutralisation of the NO molecule (Ohara *et al.*, 1993, 1995; Pritchard *et al.*, 1995).

6.6.4 LDL AND OX-LDL EFFECTS ON BASAL NO RELEASE

In the present study L-NAME contraction was studied as a measurement of basal NO release and was found to be the most sensitive system to dysfunction.

Incubation of tissues with ox-LDL resulted in a significant dose-dependent reduction in L-NAME-mediated contraction, with a similar trend being observed following LDL treatment.

Attenuation of basal NO release by LDL and ox-LDL may account for the increased responses to phenylephrine observed, and potentiation of agonist responses reported during the early development of atherosclerotic disease (Kolodgie *et al.*, 1990).

In the present study basal release was shown to be more sensitive to LDL and ox-LDL, than carbachol-stimulated release, consistent with suggestions that alterations in contractile responses precede decreased endothelium-dependent relaxation in the atherosclerotic disease process.

Interestingly, Mian and Martin, 1995, recently found that basal NO activity was more sensitive to superoxide anion inactivation than acetylcholine-stimulated activity.

Along with results from the present study this would also suggest 'free radical neutralisation' as a possible mechanism of ox-LDL action.

6.6.5 LDL AND OX-LDL EFFECTS ON RECEPTOR-INDEPENDENT CONTRACTION

Results from the present study have shown that 100µg/ml LDL treatment causes a significant increase in the maximum KCl-induced contraction observed. 100µg/ml and 500µg/ml ox-LDL treatment showed a similar trend, while 500µg/ml LDL had no significant effect.

Incubation with ox-LDL and LDL did not alter tissue sensitivity.

As previously mentioned (see 3.1), it has been suggested that cholesterol may cause dysfunction effects by altering the cell membrane at the level of membrane physicochemical structure. Increased cell cholesterol content is known to decrease membrane fluidity, which in turn may alter membrane proteins leading to a modification of function. It is therefore possible that alterations in KCl-mediated contractions may be due to modification of ion channel sensitivity.

No alteration was observed following incubation with the higher (500μg/ml) LDL concentration, however this LDL concentration was found to have unique effects on other aspects of contractile reactivity as mentioned previously (see 6.6.1).

6.6.6 EFFECT OF 5 HOUR INCUBATION ON LDL OXIDATION

Incubation of LDL for 5 hours under standard incubation conditions had no significant effect on the level of oxidative modification of native LDL.

LDL effects observed in the present study are therefore, not attributable to oxidative

modification occuring during the incubation procedure.

Caution must however, be taken when interpreting data as although TBARS are a commonly used laboratory technique for the measurement of lipid peroxidation levels, the LDL particle may have undergone numerous stages of oxidative damage, while still being outwith the peroxidation levels required for measurement.

Such partially oxidised or 'minimally modified' LDL has been shown to have various effects, as seen with fully oxidised LDL (Witztum and Steinberg, 1991; Parhami et al., 1995).

It is likely that much of the controversy surrounding LDL effects, and variation in results, is due to such minimal oxidative modification which will vary in degree depending on experimental procedures.

6.6.7 GENERAL DISCUSSION

The following study has therefore shown that both LDL and ox-LDL effect numerous aspects of vascular reactivity *in vitro*.

In summary:-

Ox-LDL

1 Maximum phenylephrine induced contractions were significantly increased following incubation with ox-LDL

2 ox-LDL significantly reduced tissue sensitivity to carbachol, and maximum relaxation obtained

3 Ox-LDL treatment resulted in a significant dose-dependent reduction in SNP concentration-response curve sensitivity

4 Ox-LDL treatment resulted in a significant dose-dependent reduction in L-NAME-induced contraction

5 Results suggested that ox-LDL caused a small increase in maximum potassium chloride induced contraction (however this was not significant)

LDL

1 LDL significantly reduced tissue sensitivity to phenylephrine

2 Maximum phenylephrine-induced contraction was significantly increased following incubation with low concentration LDL, whereas higher concentration LDL treatment caused a significant reduction in response

3 Results suggested that LDL caused a dose-dependent reduction in carbachol concentration-response curve sensitivity (although did not reach significance), and a similar reduction in the maximum carbachol-induced relaxation obtained

4 Results suggested that LDL treatment caused a dose dependent reduction in L-NAME-induced contraction

5 Low concentration LDL treatment caused a significant increase in maximum potassium chloride induced contraction

Further studies using human arterial preparations would be of much interest in the future, particularly with regards to the effects of LDL cholesterol on basal and stimulated NO pathways.

The activity of both these pathways has been shown to vary considerably between species, and also intraspecies, between arteries (Christie and Lewis, 1991; Christie *et al.*, 1989).

Studies examining effects within the human arterial system will therefore prove vital in substantiating the results of previous animal models.

7

INVESTIGATION INTO MECHANISMS UNDERLYING LDL AND OX-LDL EFFECTS

7.1 INTRODUCTION

As previously mentioned, LDL cholesterol exerts two main effects on vascular reactivity:-

1 LDL impairs endothelium-dependent relaxation

(Andrews *et al.*, 1987; Jacobs *et al.*, 1990; Kugiyama *et al.*, 1990; Galle *et al.*, 1991; Tanner *et al.*, 1991; Chin *et al.*, 1992; Myers *et al.*, 1994).

2 LDL alters reactivity of vascular smooth muscle to contractile agonists

(Broderick *et al.*, 1990; Galle *et al.*, 1990).

Knowledge of this impairment of endothelium-dependent vasodilation and augmentation of contractile responses, has led to a focus of interest on the possible link between lipoproteins and impaired synthesis/effect of NO.

Indeed, in chapter 6, ox-LDL (and to a lesser extent LDL) was found to affect both basal and stimulated NO release. Ox-LDL also attenuated tissue sensitivity to the exogenous NO donor SNP.

Although impairment of NO function is known to take place the mechanisms by which this dysfunction occurs remain controversial.

7.1.1 NITRIC OXIDE SYNTHASE INHIBITION

In recent years a limited number of studies have suggested that low-density lipoproteins affect vascular reactivity by inhibiting the enzyme nitric oxide synthase (NOS).

In 1995 Liao *et al.*, found that ox-LDL decreased the expression of endothelial NOS (eNOS) in human saphenous vein endothelial cells. This was thought to be through a combination of early transcriptional inhibition and post-transcriptional mRNA destabilisation.

Yang *et al.*, 1994, found that on examination of iNOS activity in an activated macrophage cell line, inducible NOS (iNOS) could also be inhibited by ox-LDL.

Such results indicate a potential mechanism for LDL and ox-LDL induced NO dysfunction via alteration of NOS activity.

Due to the massive accumulation of cholesterol unique to macrophages, and differences in the NO pathway and activity between the venous and arterial system (Thorin-Trescases et al., 1995), these studies only provide limited knowledge of possible LDL and ox-LDL mechanisms in arteriovascular dysfunction. Further investigation using arterial preparations are now required to elucidate LDL and ox-LDL effects on arterial eNOS and iNOS activity.

7.1.1.1 The Glucocorticoids

Since as early as 1969, glucocorticoids have been known to potentiate vasoconstrictor responses to contractile agonists in animal models (Kalsner, 1969; Yard and Kadowitz, 1972).

In recent years it has been discovered that glucocorticoids, such as dexamethasone exert their action by inhibition of expression of inducible, but not constitutive, NOS (Rees *et al.*, 1990; Radomski *et al.*, 1990; Pfeilschifter, 1991; Baydoun *et al.*, 1993).

Glucocorticoids therefore provide an ideal tool for the study of iNOS and eNOS activity in isolated tissue preparations.

7.1.2 FREE RADICAL NEUTRALISATION

Another theory rapidly accumulating interest is that of 'free radical neutralisation'. An increasing number of studies have provided evidence to indicate that ox-LDL induced NO dysfunction may, at least partially, be due to free radical neutralisation of the NO molecule.

In 1990, Galle *et al.*, suggested that ox-LDL enhanced agonist-induced vasoconstrictions by a direct effect on the vascular smooth muscle. This group was later to suggest that both LDL and ox-LDL inhibited endothelium-dependent vasodilation by inactivating NO released from the endothelial cells (Galle *et al.*, 1991).

Around the same time, Jacobs *et al.*, 1990, discovered that both LDL and ox-LDL reversibly inhibited relaxations evoked by exogenous NO in rabbit aorta. Ox-LDL (but not LDL), also reversibly inhibited relaxations evoked by the NO donor compound glyceryl trinitrate.

Initial studies such as those above, therefore suggested that LDL and ox-LDL could effect the NO pathway at a point following endothelial release, and several investigators began to focus on the

possibility that NO was being inactivated after release, and prior to vascular smooth muscle stimulation.

7.1.2.1 The Superoxide Anion

The inactivation of EDRF by superoxide anion was recognised before the demonstration that EDRF was NO in 1987 (Wei et al., 1985; Palmer et al., 1987; Marin and Rodriguez-Martinez, 1995).

Suggestions that NO was being inactivated following release and possibly prior to smooth muscle activation therefore led to the development of a 'free radical neutralisation' hypothesis in which the superoxide anion was the 'potential culprit'.

Dowell *et al.*, 1993, discovered that exposure of rabbit aortic ring preparations to superoxide anions and hydroxyl radicals (via a xanthine oxidase/hypoxanthine generating system) caused impairment of endothelium-dependent carbachol induced relaxation. Similar results were observed by Furchgott *et al.* (1994[1], 1994[2]).

Ohara *et al.*, 1993, demonstrated that an excess generation of superoxide anions occurred within hypercholesterolemic vessels, from cholesterol fed

New Zealand white rabbits. Dietary correction of hypercholesterolemia improved endothelium-dependent vascular relaxation, and normalised endothelial superoxide anion production.

More recently, Araujo et al., 1995, found that reactive oxygen species production by leucocytes was positively correlated with elevated VLDL or LDL levels in clinical studies, and in the same year, Pritchard *et al.*, 1995, found that native LDL increased endothelial NOS generation of the superoxide anion in cultured human umbilical vein endothelial cells.

Finally, Mian and Martin, 1995, found that basal NO activity was more sensitive to superoxide anion inactivation than acetylcholine-stimulated activity. This is similar to the observations in chapter 6, whereby basal NO release was shown to be more sensitive to LDL and ox-LDL, than carbachol stimulated release.

Recent results, therefore suggest that LDL and ox-LDL induced NO dysfunction may occur as a result of enhanced superoxide anion generation, resulting in an increased inactivation of the NO molecule.

Interestingly, *in vivo* superoxide may act as a precursor for the formation of numerous other free radical species, and several groups have suggested that it may be the resultant radicals such as hydrogen peroxide and peroxinitrite (+ resultant hydroxyl radicals), that act as the primary cytotoxic reactive species (Link and Riley, 1988; Beckman *et al.*, 1990; Beckman and Tsai, 1994; Darley-Usmar and Radomski, 1994; Harrison and Ohara, 1995).

7.1.2.2 Antioxidant Protection

Indication of a potential mechanism for LDL and ox-LDL induced NO dysfunction via inactivation of the NO molecule by free radical neutralisation, has stimulated interest in the possibility of additional benefits of antioxidants not previously realised.

If true, this would suggest a 2nd site of action at which antioxidants could protect against the atherosclerotic effects of low-density lipoproteins.

Antioxidants, such as vitamin E, could not only prevent oxidative modification of LDL into its more potent atherogenic form, but could also reduce NO dysfunction by protecting against free radical neutralisation of the NO molecule.

Investigation into the effect of vitamin E on LDL and ox-LDL induced alterations of vascular reactivity, would therefore be of much interest.

7.1.2.3 Superoxide Dismutase

All cells in eukaryotic organisms contain powerful antioxidant defense systems against free radicals. If it were not for these protective antioxidant defenses generation of free radical chain reactions would quickly cripple and destroy cellular functions (Sies, 1993).

Within the human body the natural antioxidant defense systems include catalase, glutathione peroxidase, and superoxide dismutase (SOD).

SOD is a large water soluble molecule, which provides the main line of defence against the superoxide anion. As a preventive antioxidant, SOD rapidly converts superoxide anion to hydrogen peroxide, which may then be scavenged by catalase (DeBono, 1994).

Three isoforms of SOD are known to exist; Mitochondrial Mn-SOD, intracellular (or cytosolic) CuZn-SOD, and extracellular (or secreted) CuZn-SOD.

In 1995 Stralin *et al.*, noted that human blood vessel walls (particularly those of arteries), contained exceptionally large amounts of extracellular SOD, indicating the probable importance of this extracellular enzyme.

Since studies have suggested that superoxide anion is responsible for LDL/ox-LDL induced NO dysfunction, investigation into the effects of extracellular SOD on LDL and ox-LDL mediated alterations of vascular reactivity would be of great interest.

7.2 AIMS

The aim of the present study was to investigate possible mechanisms underlying LDL and ox-LDL effects on vascular reactivity in the rat aorta.

More specifically the aim of the present study was to examine:-

1 Basal iNOS and eNOS activity (and the effect of 5 hour incubation on)

2 The effect of LDL and ox-LDL on iNOS and eNOS activity

3 The effect of vitamin E on LDL-induced alterations of vascular reactivity

4 The effect of SOD on LDL and ox-LDL induced alterations of vascular reactivity

7.3 METHODS

LDL and ox-LDL were prepared, and experimental protocols carried out, as previously described (see 2.2.3, 2.2.7, 2.2.8).

Aortic rings 3-4mm wide were prepared from male Sprague Dawley rats (>6 weeks).

7.3.1 DEXAMETHASONE EXPERIMENTS

Rings were either immediately mounted under 1g tension for recording of isometric tension (-inc.) or incubated for 5 hours, at 37°C, 5%CO_2 95% air, in either vehicle control or 500µg/ml LDL/ox-LDL (+/- 0.5µM dexamethasone (dex.))(+inc.). All experiments were carried out in the presence (+E) or absence (-E) of endothelium.

Tissues were checked for endothelial integrity (by examination of carbachol response following preconstriction with EC_{70} phenylephrine), and cumulative concentration-response curves constructed to phenylephrine (10^{-8}-$3x10^{-5}$M) and potassium chloride (10^{-2}-10^{-1}M). Tissues were then contracted to the EC_{20}

phenylephrine and the nitric oxide synthase inhibitor L-NAME (200μM) added at the plateau of phenylephrine contraction.

7.3.2 VITAMIN E EXPERIMENTS

Rings were incubated for 5 hours, at 37°C, 5% CO_2 95% air, in either control or 500μg/ml LDL cholesterol (+/- 100μM vitamin E). Following incubation rings were mounted under 1g tension for recording of isometric tension.

Cumulative concentration-response curves were constructed to phenylephrine (10^{-8}-$3x10^{-5}$M), carbachol (10^{-8}-$3x10^{-5}$M), SNP (10^{-11}-10^{-7}M), and potassium chloride (10^{-2}-10^{-1}M). Tissues were then contracted to the EC_{20} phenylephrine and the nitric oxide synthase inhibitor L-NAME (200μM) added at the plateau of phenylephrine contraction.

In separate experiments with vitamin E 4μM final concentration EDTA and BHT was added to solutions immediately following incubation to prevent further oxidation. Levels of incubation-induced oxidation were measured using a modified TBA assay with appropriate vitamin E, ox-LDL, and preincubation controls.

7.3.3 SUPEROXIDE DISMUTASE EXPERIMENTS

Rings were incubated for 5 hours, at 37°C, 5% CO_2 95% air, in either vehicle control, or 500μg/ml LDL/ox-LDL (+/- 45 units/ml SOD). Following incubation rings were mounted under 1g tension for the recording of isometric tension.

Cumulative concentration-response curves were constructed to phenylephrine (10^{-8}-$3x10^{-5}$M), carbachol (10^{-8}-$3x10^{-5}$M), SNP (10^{-11}-10^{-7}M), and potassium chloride (10^{-2}-10^{-1}M). Tissues were then contracted to the EC_{20} phenylephrine and the nitric oxide synthase inhibitor L-NAME (200μM) added at the plateau of phenylephrine contraction.

Following experimentation all tissues were blotted dry and their wet weight taken.

7.4 DATA CALCULATION AND STATISTICS

Data were calculated 'per 0.1g tisssue' and results expressed as mean +/- standard error of the mean.

Statistical analysis was carried out using repeated measures ANOVA, and Bonferroni paired multiple comparison tests (differences being considered significant when $p<0.05$).

7.5 RESULTS

7.5.1 RESULTS FROM DEXAMETHASONE EXPERIMENTS

As previously described, basal NO release was investigated by examination of contractile responses following exposure to the nitric oxide synthase inhibitor L-NAME.

7.5.1.1 Preincubation L-NAME Responses +/- Endothelium

L-NAME induced-contraction was examined in aortic ring preparations with and without endothelium, immediately following removal from the animal, and prior to incubation.

On removal of the endothelium L-NAME-induced contraction was significantly reduced (>70%)(Fig. 7.1).

7.5.1.2 Effect of 5 Hour Incubation +/- Dexamethasone on L-NAME Responses in the Rat Aorta

The effect of 5 hour incubation on L-NAME-induced contraction in the presence and absence of 0.5μM dexamethasone was examined in both endothelium intact and endothelium denuded ring preparations.

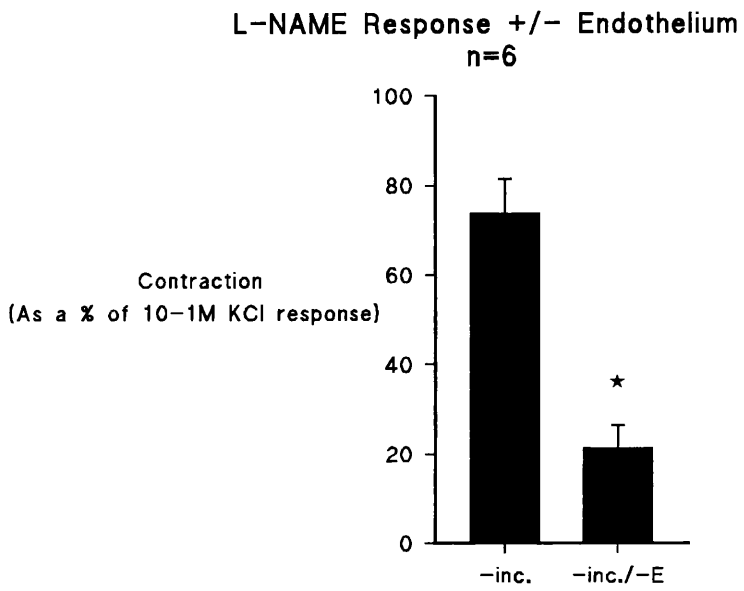


FIG. 7.1 - L-NAME-induced contraction in preincubation rat aortic ring preparations +/- endothelium.

* significant difference from control ($p<0.05$)

In endothelium intact rings, there was some indication that 5 hour incubation caused a small increase in L-NAME-induced contraction, however this did not reach statistical significance (Fig. 7.2).

Dexamethasone appeared to abolish any incubation-mediated induction of L-NAME response.

In endothelium denuded tissues, induction was considerably greater; 5 hour incubation resulting in a significant increase in L-NAME-induced contraction (Fig. 7.3).

Incubation-mediated induction of L-NAME responses was blocked by the presence of dexamethasone.

7.5.1.3 Effect of Dexamethasone on L-NAME Response +/- LDL and Ox-LDL

The effect of 0.5μM dexamethasone on L-NAME-induced contraction was examined in the presence and absence of 500μg/ml LDL and ox-LDL.

Results suggested that incubation with both LDL and dexamethasone reduced L-NAME-induced contraction to a similar degree as incubation with dexamethasone alone (p=0.0511)(Fig. 7.4).

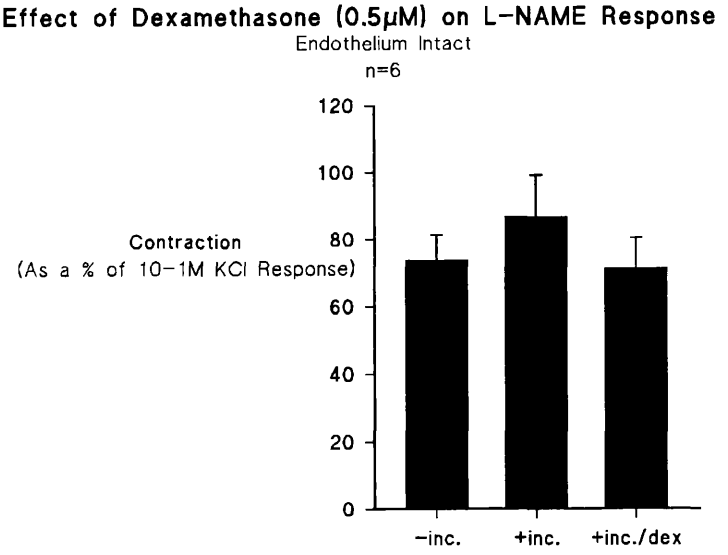


FIG. 7.2 - Effect of 5 hour incubation on L-NAME-induced contraction +/- 0.5μM dexamethasone in the rat aorta (endothelium intact).

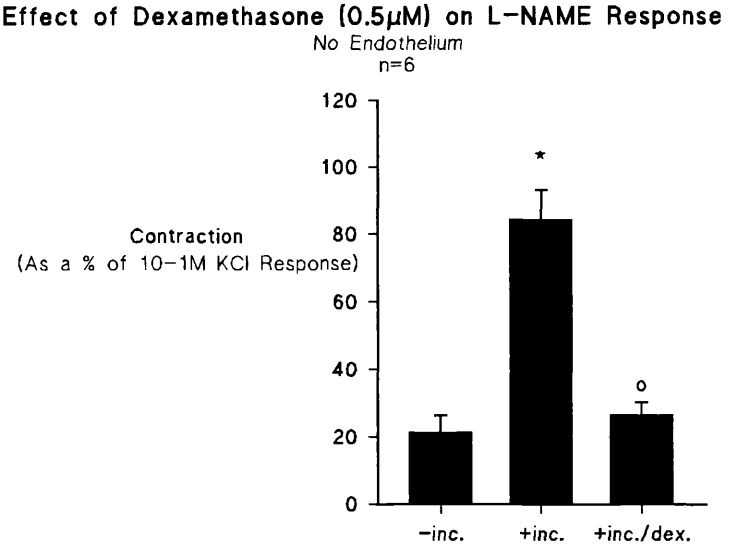


FIG. 7.3 - Effect of 5 hour incubation on L-NAME-induced contraction +/- 0.5μM dexamethasone in the rat aorta (endothelium denuded).

* significant difference from control ($p<0.05$)

O significant difference from +inc. sample group ($p<0.05$)

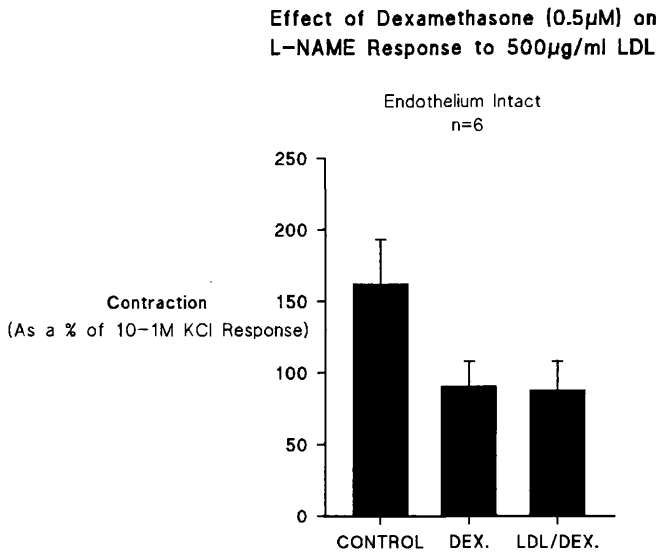


FIG. 7.4 - Effect of dexamethasone (0.5μM) on L-NAME response +/- 500μg/ml LDL (endothelium intact).

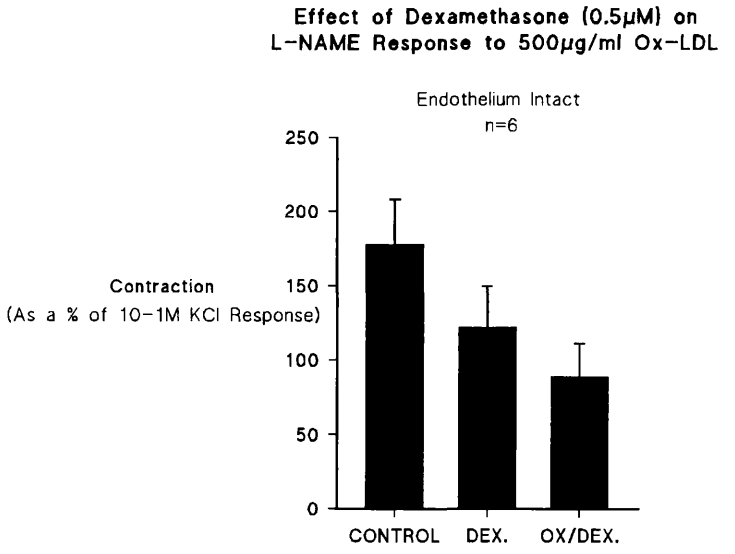


FIG. 7.5 - Effect of dexamethasone (0.5μM) on L-NAME response +/- 500μg/ml ox-LDL (endothelium intact).

Ox-LDL treated tissues showed a similar pattern, with a trend towards lower responses when incubation included both ox-LDL and dexamethasone compared with dexamethasone alone (p=0.0597)(Fig. 7.5).

7.5.2 RESULTS FROM VITAMIN E EXPERIMENTS

Effects of 500μg/ml LDL +/- 100μM vitamin E on vascular reactivity was examined in the rat aorta.

7.5.2.1 Effect of LDL +/- Vitamin E on Phenylephrine-induced Contraction in the Rat Aorta

The effect of LDL +/- vitamin E on receptor-mediated contraction was investigated by examination of tissue responses to the α_1 adrenoceptor agonist phenylephrine.

As previously, results suggested a small decrease in sensitivity to phenylephrine following incubation with 500μg/ml LDL (although in the present study this did not reach significance)(Fig.7.6).

Vitamin E appeared to have no effect on this LDL-induced decrease in phenylephrine sensitivity.

Incubation with LDL significantly reduced maximum phenylephrine-induced contraction (Fig.s 7.7 & 7.8).

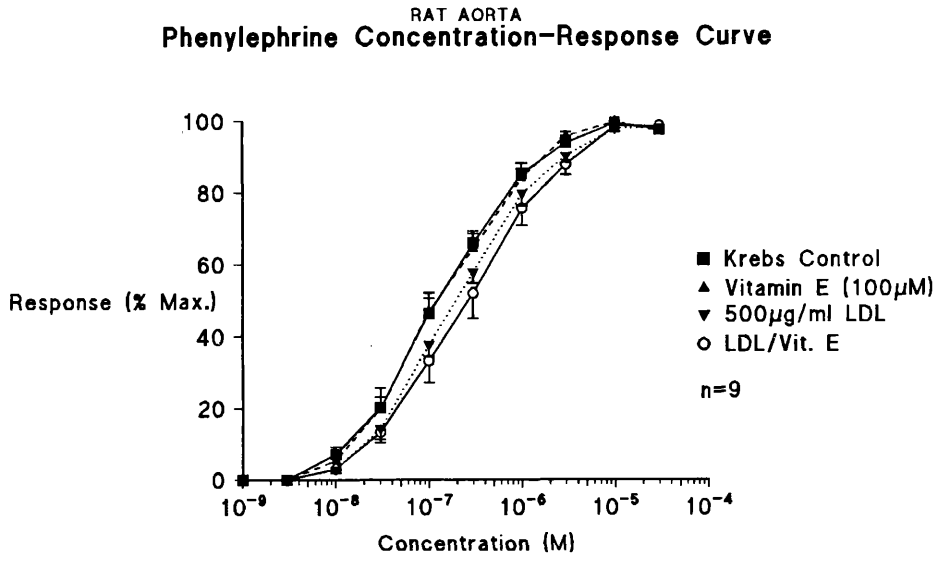


FIG. 7.6 - Effect of LDL (500μg/ml) +/- 100μM vitamin E on phenylephrine concentration-response curve in the rat aorta.

NOTE - vitamin E curve cannot be seen as coincides, and falls directly beneath, Krebs control curve

Note - % Max. = percentage of maximum phenylephrine response

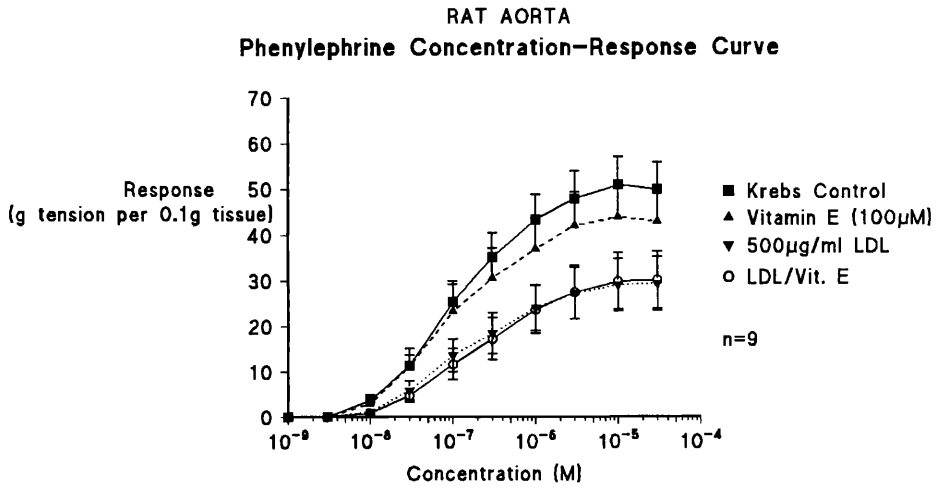


FIG. 7.7 - Effect of LDL (500μg/ml) +/- 100μM vitamin E on phenylephrine concentration-response curve in the rat aorta.

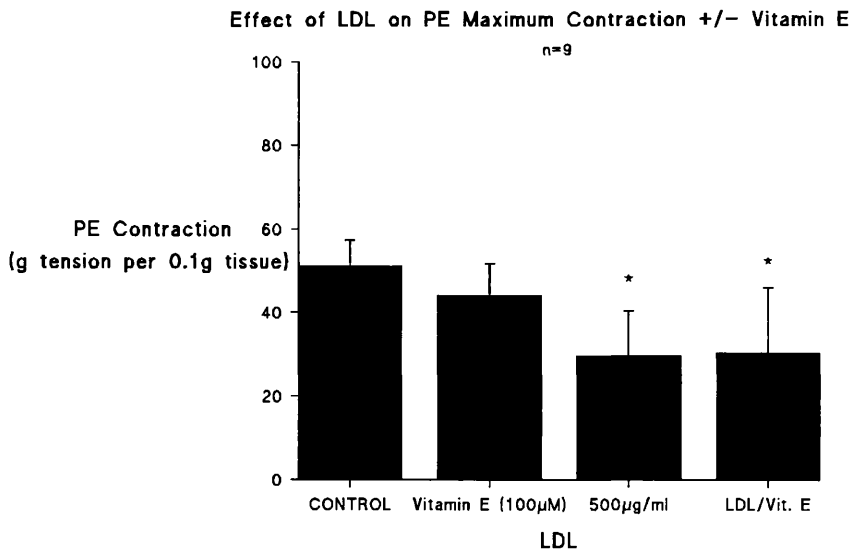


FIG. 7.8 - Effect of LDL (500μg/ml) +/- 100μM vitamin E on maximum phenylephrine-induced contraction in the rat aorta.
* significant difference from control ($p<0.05$)

No alteration in this LDL-mediated reduction of response was observed on incubation in the presence of vitamin E.

Vitamin E alone had no significant effect on tissue responses to phenylephrine, when compared with appropriate controls.

7.5.2.2 Effect of LDL +/- Vitamin E on Carbachol-mediated Endothelium-dependent Relaxation

The effect of LDL +/- vitamin E on stimulated NO release was investigated by examination of responses to the muscarinic agonist carbachol.

In the present study incubation of tissues with 500µg/ml LDL had no effect on carbachol concentration-response curve sensitivity (Fig. 7.9).

On incubation of tissues with LDL, results did however, suggest a reduction in maximum carbachol-induced relaxation (as seen previously in chapter 6)(Fig. 7.10).

Incubation in the presence of vitamin E appeared to attenuate this LDL-mediated reduction in maximum carbachol-induced relaxation (although this did not reach statistical significance, p=0.1376).

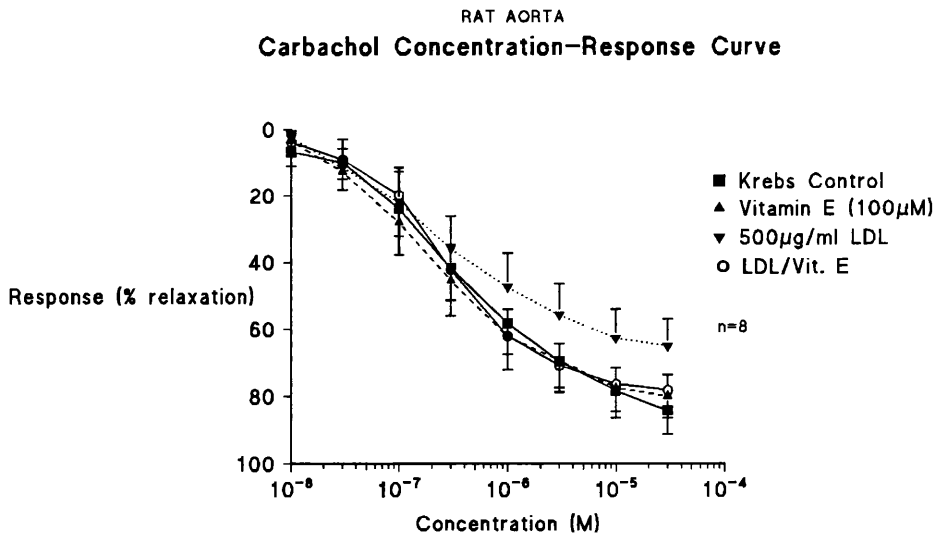


FIG. 7.9 - Effect of LDL (500μg/ml) +/- 100μM vitamin E on carbachol concentration-response curve in the rat aorta.

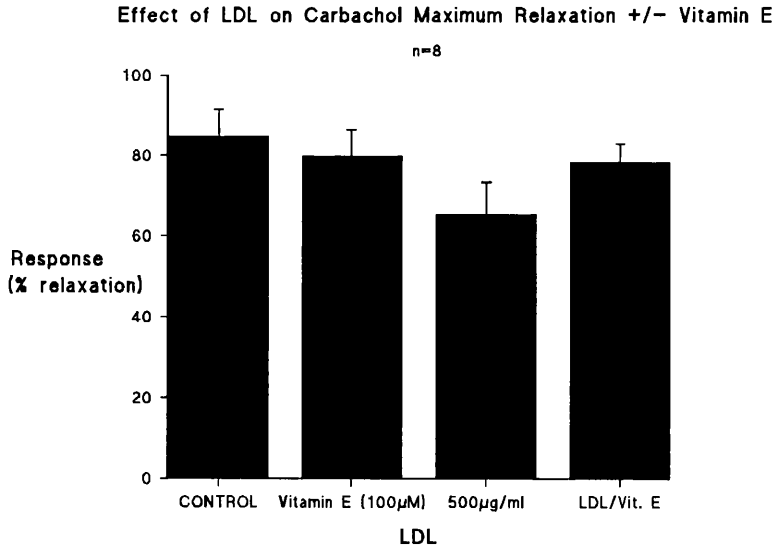


FIG. 7.10 - Effect of LDL (500μg/ml) +/- 100μM vitamin E on maximum carbachol-induced relaxation in the rat aorta.

Vitamin E alone had no significant effect on tissue responses to carbachol, when compared with appropriate controls.

7.5.2.3 Effect of LDL +/- Vitamin E on SNP-induced Relaxation

The effect of LDL +/- vitamin E on VSM sensitivity to exogenous NO was investigated by examination of responses to the NO donor compound SNP.

As previously, 500μg/ml LDL had no significant effect on tissue sensitivity to SNP or maximum relaxation achieved (Fig. 7.11).

Vitamin E alone had no significant effect on SNP-mediated tissue responses.

7.5.2.4 Effect of LDL +/- Vitamin E on L-NAME-induced Contraction in the Rat Aorta

The effect of LDL +/- vitamin E on basal NO release was investigated by examination of contractile responses following exposure to the nitric oxide synthase inhibitor L-NAME.

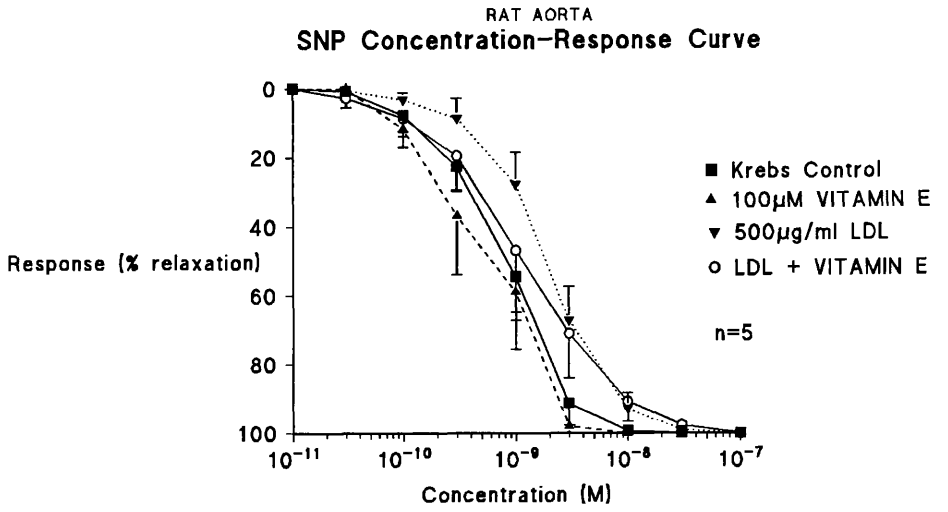


FIG. 7.11 - Effect of LDL (500μg/ml) +/- 100μM vitamin E on SNP concentration-response curve in the rat aorta.

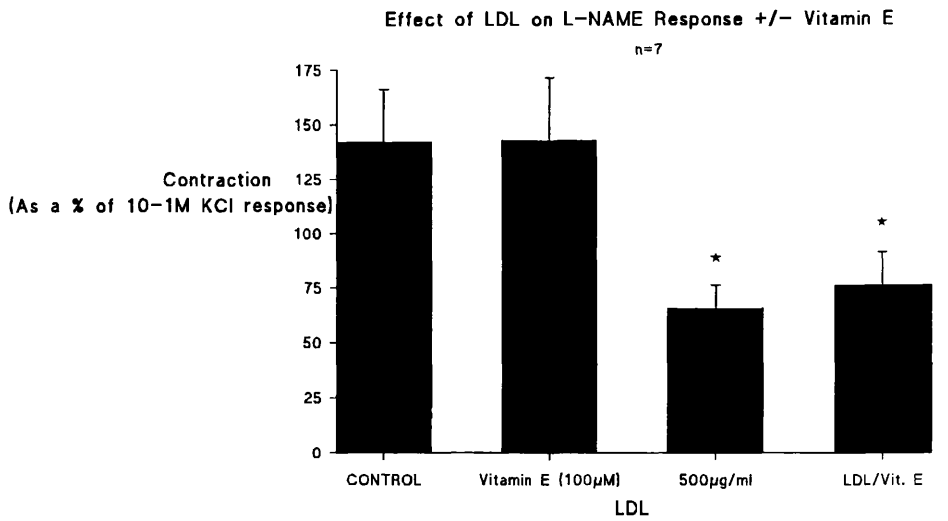


FIG. 7.12 - Effect of LDL (500μg/ml) +/- 100μM vitamin E on L-NAME-induced contraction in the rat aorta.

* significant difference from control ($p<0.05$)

Incubation of tissues with 500μg/ml LDL caused a significant reduction in L-NAME-mediated contraction (Fig. 7.12).

Incubation in the presence of vitamin E had no significant effect on this LDL-mediated decrease in L-NAME response.

Vitamin E alone did not alter tissue responses to L-NAME, when compared with appropriate controls.

7.5.2.5 Effect of LDL +/- Vitamin E on Potassium Chloride Mediated Contraction in the Rat Aorta

The effect of LDL +/- vitamin E on receptor-independent contraction was investigated by examination of responses to potassium chloride (KCl).

As previously, 500μg/ml LDL treatment had no significant effect on tissue sensitivity to KCl (Fig. 7.13), or maximum contraction achieved (Fig. 7.14).

Vitamin E alone had no effect on KCl-induced tissue responses.

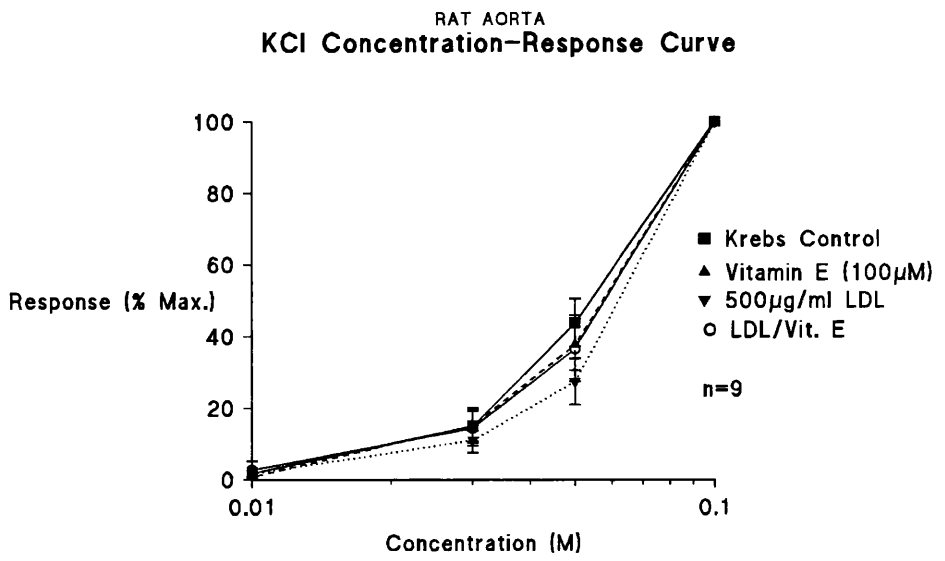


FIG. 7.13 - Effect of LDL (500μg/ml) +/- 100μM vitamin E on potassium chloride concentration-response curve in the rat aorta.

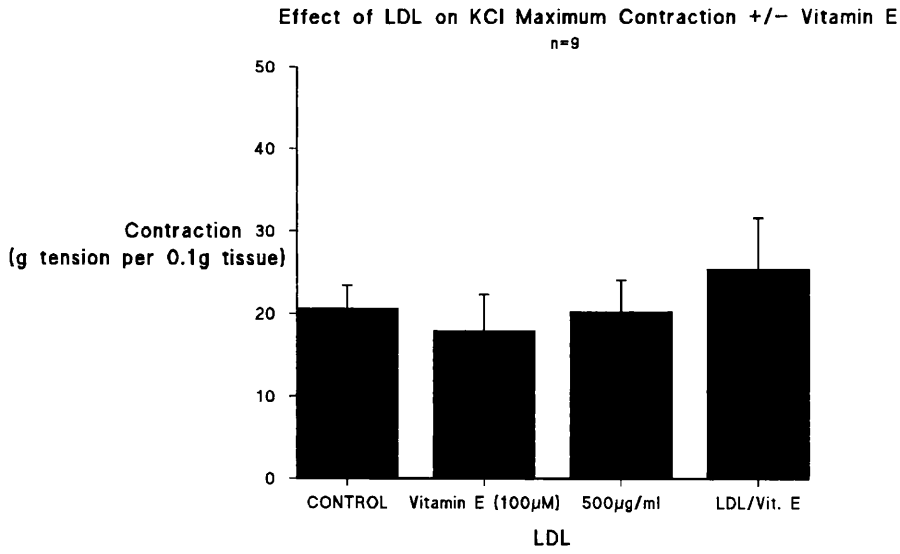


FIG. 7.14 - Effect of LDL (500μg/ml) +/- 100μM vitamin E on maximum potassium chloride induced contraction in the rat aorta.

7.5.2.6 Effect of 5 Hour Incubation +/- Vitamin E on LDL Oxidation

Lipid peroxidation of native LDL was examined following 5 hour incubation in the presence and absence of vitamin E.

Incubation of LDL for 5 hours under standard incubation conditions (see 7.3.2), resulted in only a small increase in oxidative modification, which was not statistically significant (Fig. 7.15).

Results suggested that incubation in the presence of vitamin E reduced any oxidative modification of native LDL.

7.5.3 RESULTS FROM SUPEROXIDE DISMUTASE EXPERIMENTS

Effects of 500μg/ml LDL and ox-LDL +/- 45 units SOD on vascular reactivity were examined in the rat aorta.

7.5.3.1 Effects of LDL and Ox-LDL +/- Superoxide Dismutase on Phenylephrine-induced Contraction in the Rat Aorta

Effects of LDL and ox-LDL +/- SOD on receptor-mediated contraction were investigated by

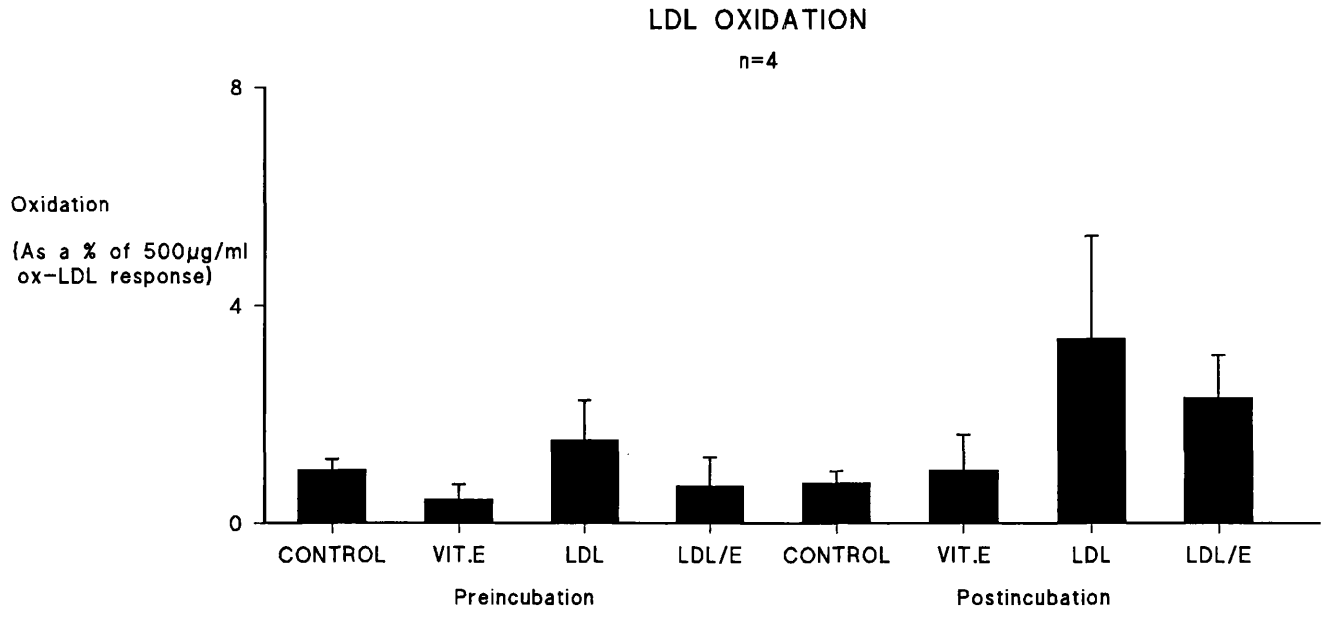


FIG. 7.15 - Effect of 5 hour incubation on LDL oxidation +/- 100µM vitamin E

examination of responses to the α_1 adrenoceptor agonist phenylephrine.

In the present study 500μg/ml LDL and ox-LDL had no significant effect on tissue sensitivity to phenylephrine (Fig.s 7.16 & 7.17).

SOD alone had no significant effect on phenylephrine concentration-response curve sensitivity.

As previously, results suggested that ox-LDL treatment caused an increase in maximum phenylephrine induced contraction (Fig.s 7.18 & 7.20).

This increase in maximum phenylephrine-induced contraction was unaffected by incubation in the presence of SOD.

In the present study 500μg/ml LDL treatment had no effect on maximum phenylephrine-induced contraction (Fig.s 7.19 & 7.21).

In both LDL and ox-LDL experiments incubation with SOD alone showed a tendency to reduce maximum

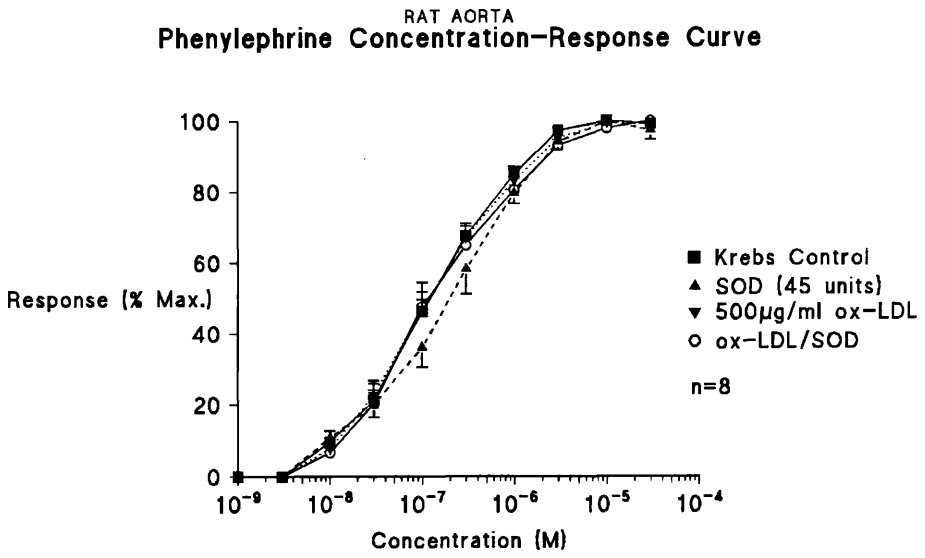


FIG. 7.16 - Effect of ox-LDL (500µg/ml) +/- 45 units/ml SOD on phenylephrine concentration-response curve in the rat aorta.

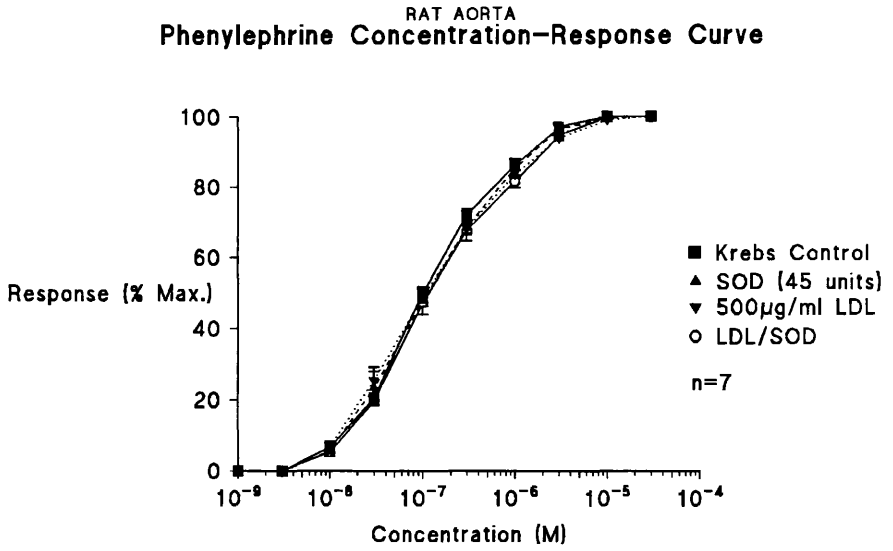


FIG. 7.17 - Effect of LDL (500µg/ml) +/- 45 units/ml SOD on phenylephrine concentration-response curve in the rat aorta.

Note - % Max. = percentage of maximum phenylephrine response

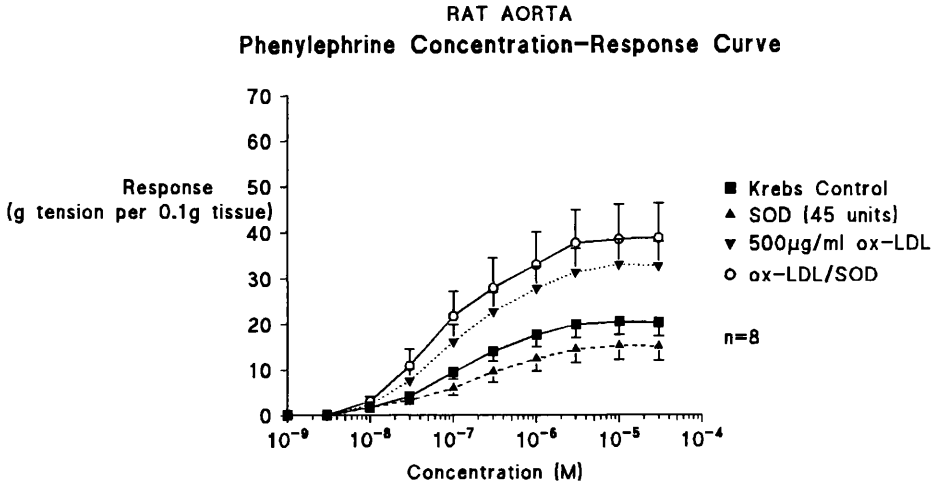


FIG. 7.18 - Effect of ox-LDL (500µg/ml) +/- 45 units/ml SOD on phenylephrine concentration-response curve in the rat aorta.

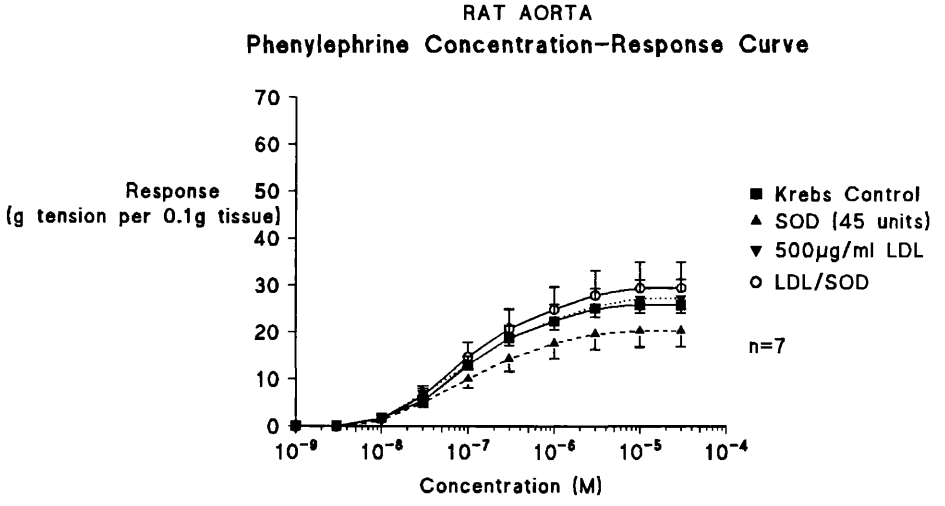


FIG. 7.19 - Effect of LDL (500µg/ml) +/- 45 units/ml SOD on phenylephrine concentration-response curve in the rat aorta.

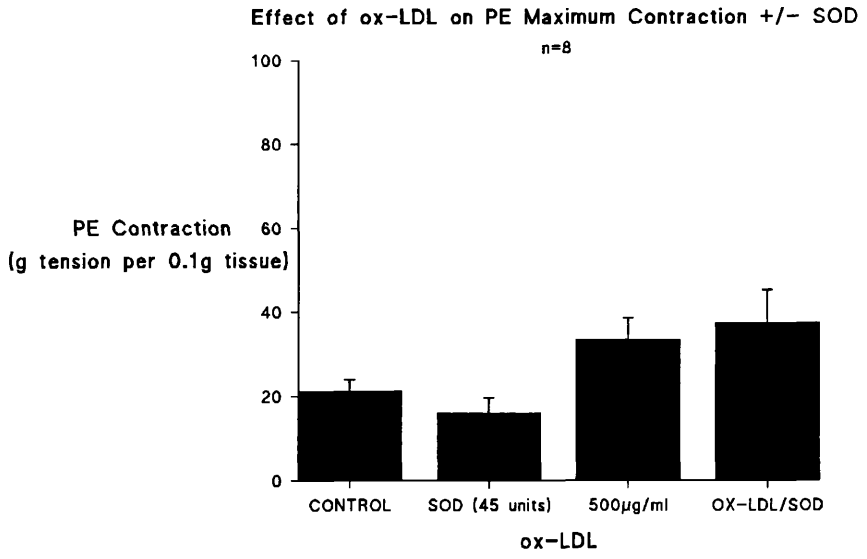


FIG. 7.20 - Effect of ox-LDL (500µg/ml) +/- 45 units/ml SOD on maximum phenylephrine-induced contraction in the rat aorta.

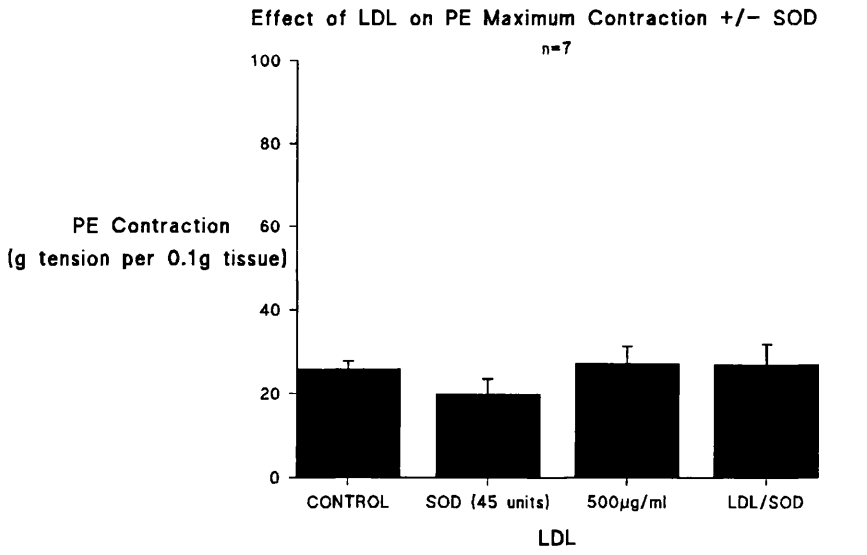


FIG. 7.21 - Effect of LDL (500µg/ml) +/- 45 units/ml SOD on maximum phenylephrine-induced contraction in the rat aorta.

phenylephrine-induced contraction when compared with appropriate controls.

7.5.3.2 Effects of LDL and Ox-LDL +/- Superoxide Dismutase on Carbachol-mediated Endothelium Dependent Relaxation in the Rat Aorta

Effects of LDL and ox-LDL +/- SOD on stimulated NO release were investigated by examination of responses to the muscarinic agonist carbachol.

In the present study, incubation of tissues with 500μg/ml LDL or ox-LDL had no significant effect on carbachol concentration-response curve sensitivity (Fig.s 7.22 & 7.23).

As observed in chapter 6, results suggested that both LDL and ox-LDL (at a concentration of 500μg/ml) caused a reduction in maximum carbachol-induced relaxation (Fig.s 7.24 & 7.25).

This was unaffected by incubation in the presence of SOD.

SOD alone had no significant effect on tissue responses to carbachol, when compared with

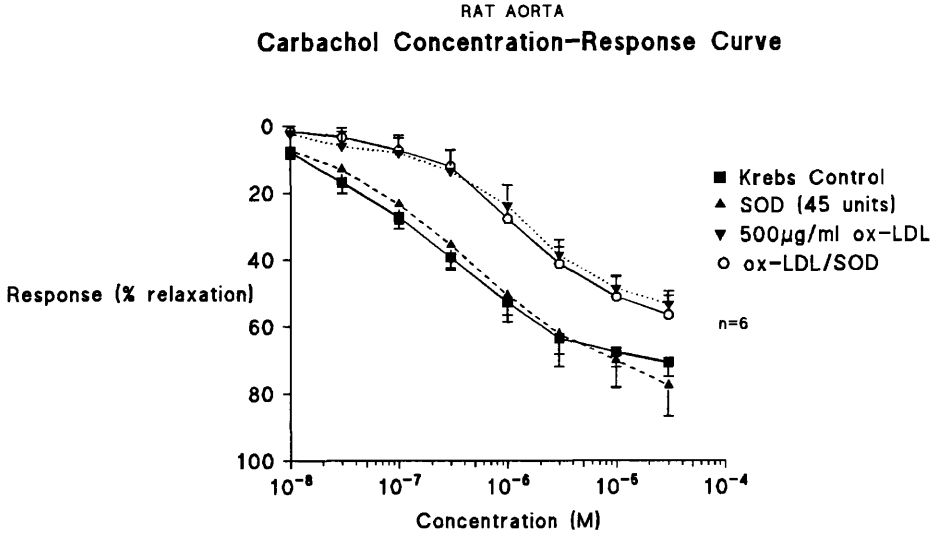


FIG. 7.22 - Effect of ox-LDL (500µg/ml) +/- 45 units/ml SOD on carbachol concentration-response curve in the rat aorta.

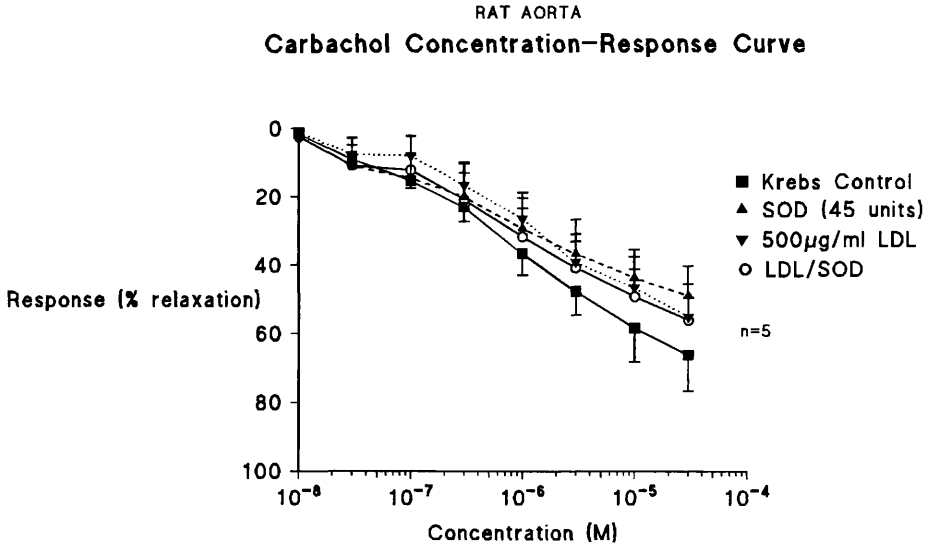


FIG. 7.23 - Effect of LDL (500µg/ml) +/- 45 units/ml SOD on carbachol concentration-response curve in the rat aorta.

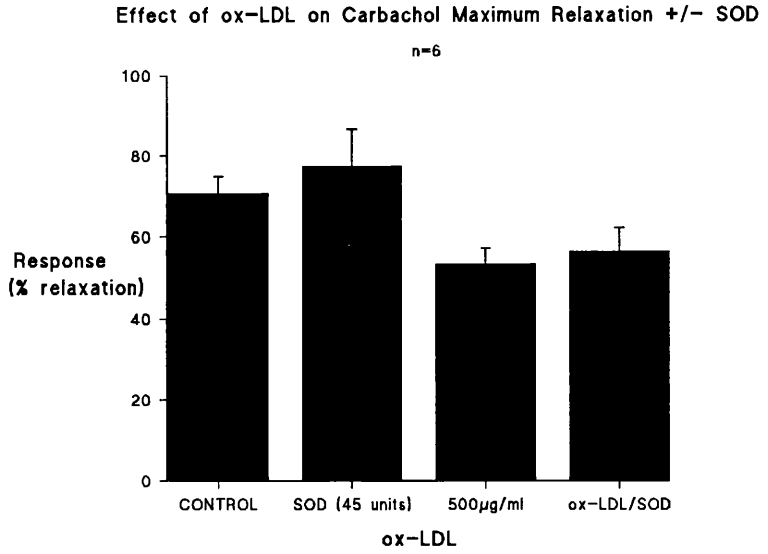


FIG. 7.24 - Effect of ox-LDL (500µg/ml) +/- 45 units/ml SOD on maximum carbachol-induced relaxation in the rat aorta.

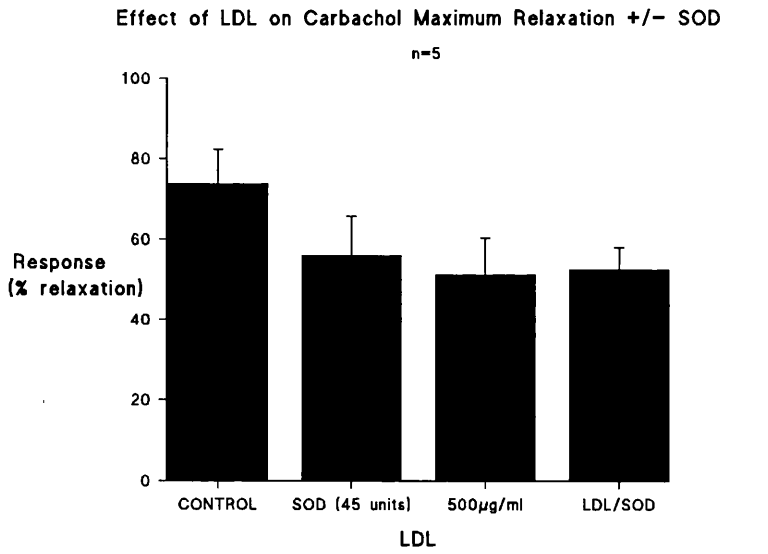


FIG. 7.25 - Effect of LDL (500µg/ml) +/- 45 units/ml SOD on maximum carbachol-induced relaxation in the rat aorta.

appropriate controls (although maximum relaxation responses were variable).

7.5.3.3 Effects of LDL and Ox-LDL +/- Superoxide Dismutase on SNP-induced Relaxation in the Rat Aorta

Effects of LDL and ox-LDL +/- SOD on VSM sensitivity to exogenous NO was investigated by examination of responses to the NO donor compound SNP.

As previously, 500μg/ml ox-LDL caused a significant reduction in SNP concentration-response curve sensitivity (Fig. 7.26).

This reduction in tissue sensitivity to SNP appeared greater in tissues incubated with both ox-LDL and SOD (although this did not reach statistical significance).

A similar trend was observed on incubation with 500μg/ml LDL; data suggesting a reduction in SNP concentration-response curve sensitivity (p=0.6189)(Fig. 7.27).

This appeared unaffected by incubation in the presence of SOD.

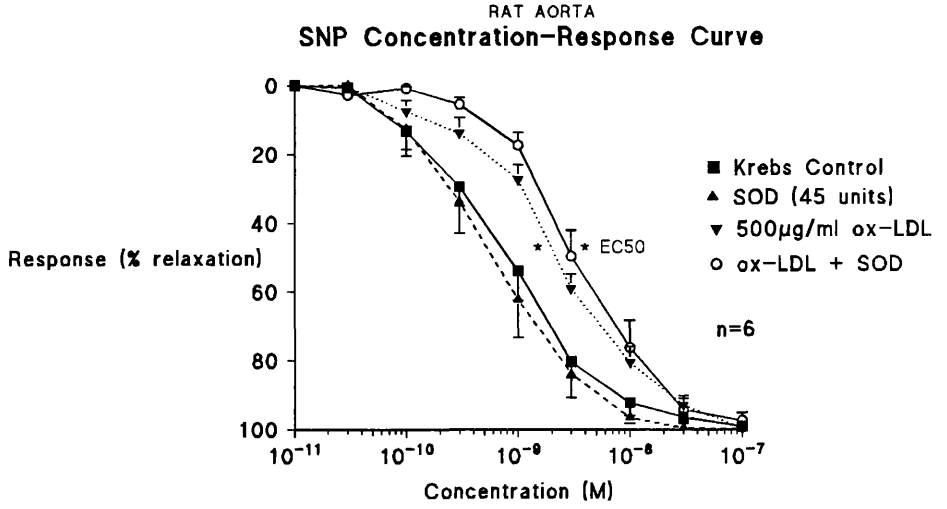


FIG. 7.26 - Effect of ox-LDL (500µg/ml) +/- 45 units/ml SOD on SNP concentration-response curve in the rat aorta.

* significant difference from control ($p<0.05$)

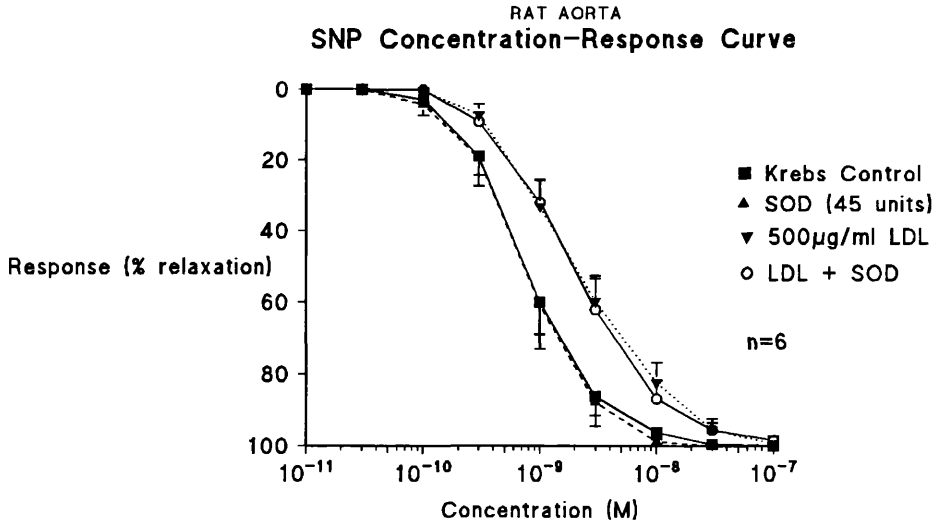


FIG. 7.27 - Effect of LDL (500µg/ml) +/- 45 units/ml SOD on SNP concentration-response curve in the rat aorta.

SOD alone had no effect on tissue responses to SNP, when compared with appropriate controls.

7.5.3.4 Effects of LDL and Ox-LDL +/- Superoxide Dismutase on L-NAME-induced Contraction in the Rat Aorta

Effects of LDL and ox-LDL +/- SOD on basal NO release was investigated by examination of contractile responses following exposure to the nitric oxide synthase inhibitor L-NAME.

As in chapter 6, 500μg/ml ox-LDL caused a significant reduction in contraction following L-NAME exposure (Fig. 7.28).
LDL treatment showed a similar trend (Fig. 7.29).

Incubation in the presence of SOD had no significant effect on this LDL/ox-LDL mediated reduction in L-NAME response.

SOD alone showed a tendency to decrease L-NAME-induced contraction however, this was minimal and did not reach statistical significance.

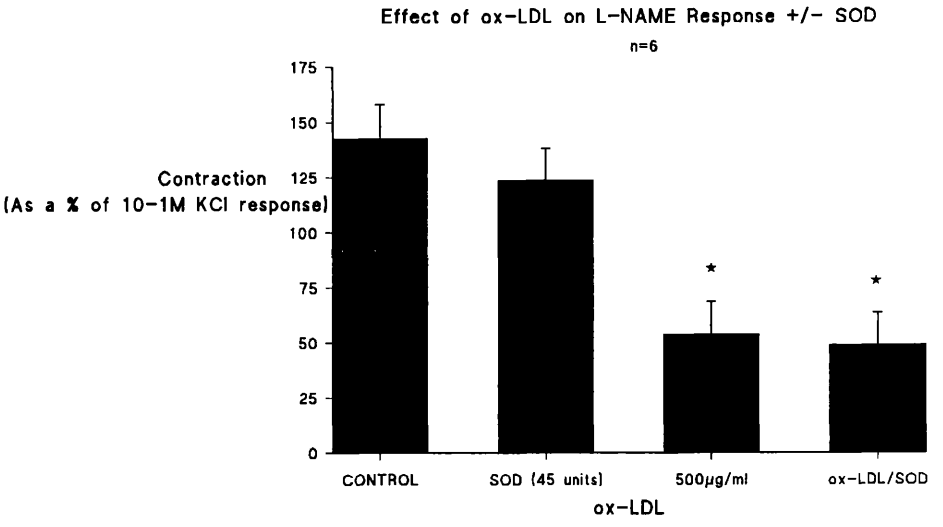


FIG. 7.28 - Effect of ox-LDL (500µg/ml) +/- 45 units/ml SOD on L-NAME-induced contraction in the rat aorta.

* significant difference from control ($p<0.05$)

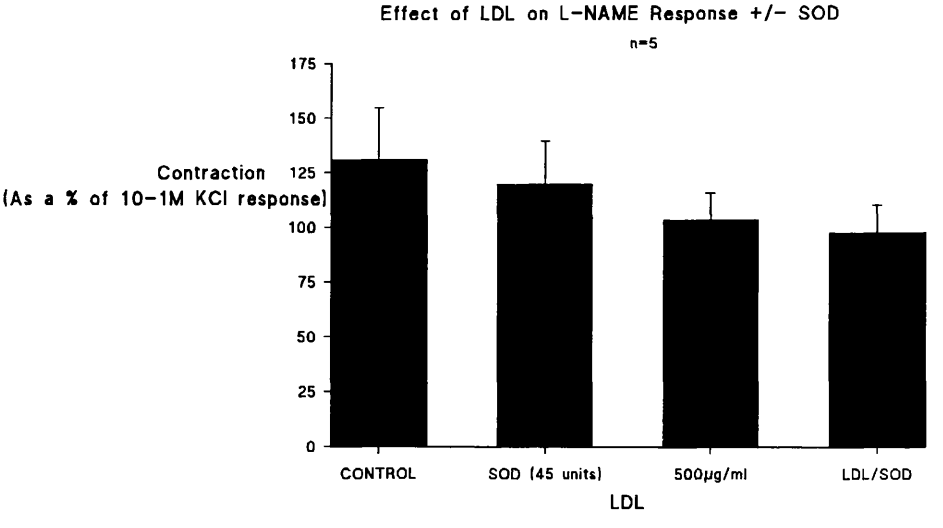


FIG. 7.29 - Effect of LDL (500µg/ml) +/- 45 units/ml SOD on L-NAME-induced contraction in the rat aorta.

7.5.3.5 Effects of LDL and Ox-LDL +/- Superoxide Dismutase on Potassium Chloride Mediated Contraction in the Rat Aorta

Effects of LDL and ox-LDL +/- SOD on receptor-independent contraction were investigated by examination of responses to potassium chloride (KCl).

As previously, LDL and ox-LDL treatment had no significant effect on tissue sensitivity to KCl (Fig.s 7.30 & 7.31).

In the present study, 500μg/ml ox-LDL caused a significant increase in maximum KCl-induced contraction; a similar (although minimal) trend being observed following LDL treatment (Fig.s 7.32 & 7.33).

This was unaffected by incubation in the presence of SOD.

SOD alone had no effect on tissue responses to KCl, when compared with appropriate controls.

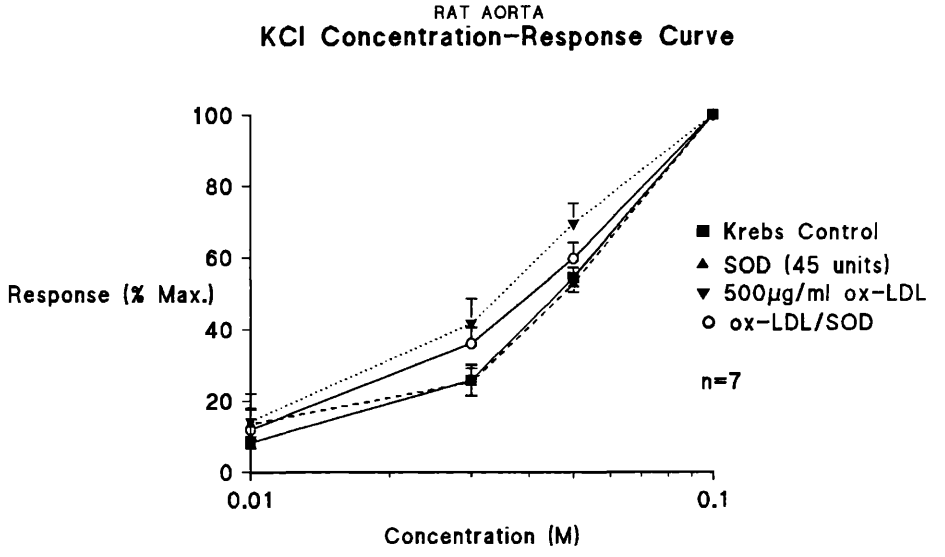


FIG. 7.30 - Effect of ox-LDL (500µg/ml) +/- 45 units/ml SOD on potassium chloride concentration-response curve in the rat aorta.

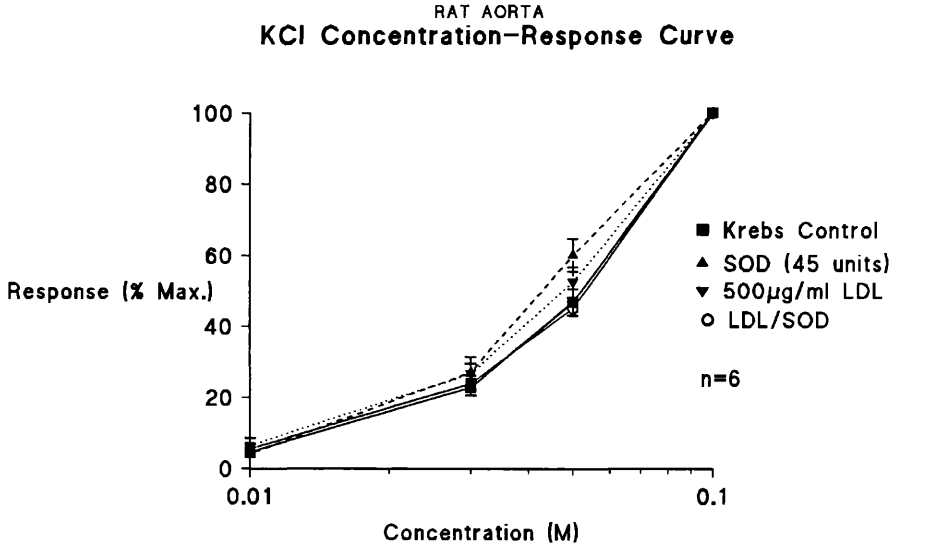


FIG. 7.31 - Effect of LDL (500µg/ml) +/- 45 units/ml SOD on potassium chloride concentration-response curve in the rat aorta.

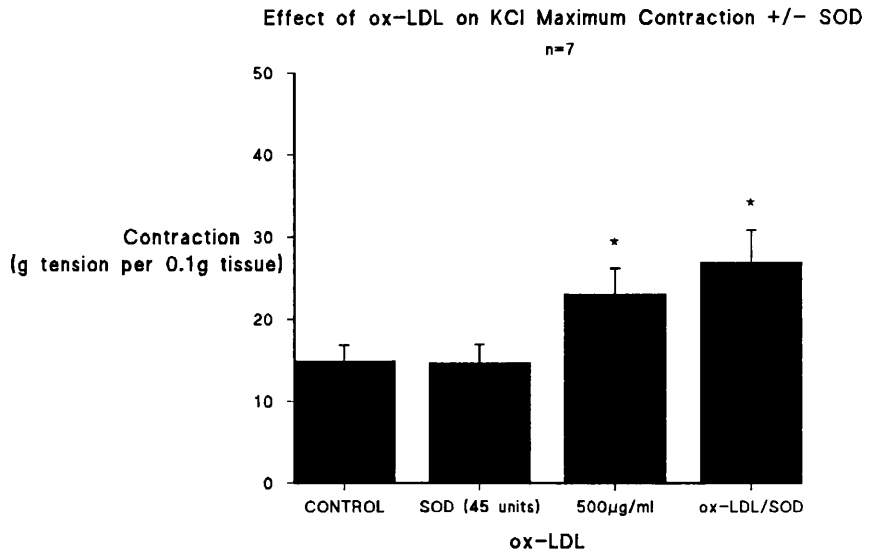


FIG. 7.32 - Effect of ox-LDL (500µg/ml) +/- 45 units/ml SOD on maximum potassium chloride induced contraction in the rat aorta.

* significant difference from control ($p<0.05$)

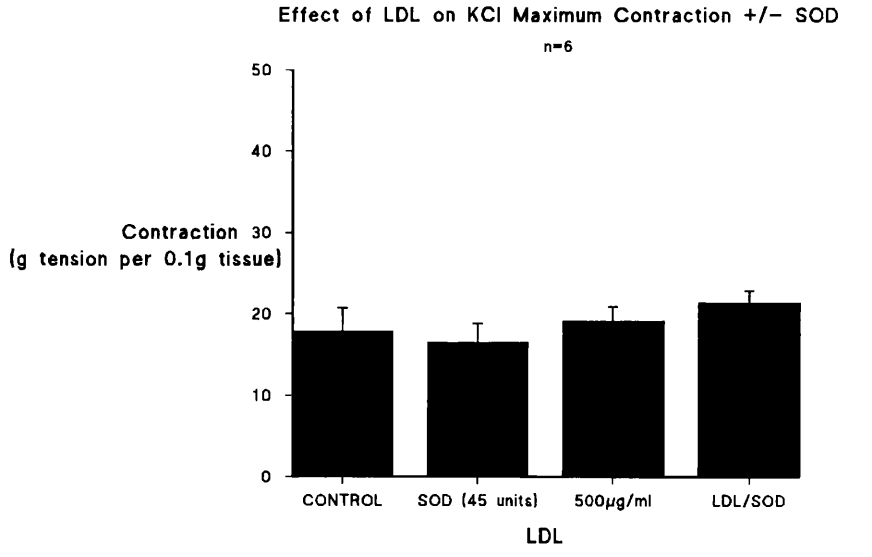


FIG. 7.33 - Effect of LDL (500µg/ml) +/- 45 units/ml SOD on maximum potassium chloride induced contraction in the rat aorta.

7.6 DISCUSSION

The aim of the present study was to investigate possible mechanisms underlying LDL and ox-LDL effects on vascular reactivity in the rat aorta.

7.6.1 DEXAMETHASONE EXPERIMENTS

In chapter 6, LDL and ox-LDL were found to attenuate contractile responses to the NO synthase inhibitor L-NAME, suggesting a decrease in basal NO levels.

Induction of inducible NO synthase (iNOS) is however, known to occur readily in the rat aorta.

In the present study it was hoped to elucidate the relative contributions of iNOS and endothelial eNOS to L-NAME-mediated responses, and establish the effect of LDL and ox-LDL on their individual activity.

7.6.1.1 Substantiation of Basal NO Measurement

Removal of the endothelium in preincubation rings caused a significant reduction in L-NAME-induced contraction by greater than 70%, demonstrating that NO activity measured was mainly endothelium dependent (eNOS).

Following a 5 hour incubation only a small induction of iNOS occurred, which did not reach statistical significance. This was blocked in the presence of dexamethasone.

These results therefore substantiate our previous assumption that L-NAME-induced responses were mainly representative of basal NO release from the endothelium.

Interestingly, on removal of the endothelium prior to incubation, induction of iNOS increased considerably (returning NO activity to endothelium intact levels), and could be specifically inhibited by dexamethasone.

Similar to this Moritoki *et al.*, 1992[1,2], also found induction of NO synthesising mechanisms in the vascular smooth muscle over similar time periods. L-arginine induced relaxation was found to occur irrespective of whether the endothelium was present or absent. Detection of responses were possible 2 hours after removal of the endothelium and the start of experimentation, and increased progressively to reach a maximum at 6 hours.

Other groups such as Adeagbo and Triggle, 1993, have also suggested that endothelial cell removal/damage triggers the induction of a smooth muscle NO synthase.

This upregulation of iNOS activity suggests a possible regulatory defense pathway, whereby endothelial damage and the resultant reduction of eNOS activity, triggers upregulation of iNOS activity in the vascular smooth muscle and restoration of NO to previous levels.

7.6.1.2 The Effect of LDL and Ox-LDL on iNOS and eNOS activity in the Rat Aorta

The effect of LDL and ox-LDL on iNOS and eNOS activity was investigated by examination of the effects of dexamethasone on L-NAME responses +/- LDL and ox-LDL.

Since dexamethasone specifically inhibits iNOS, comparisons were made between tissues incubated in dexamethasone only, and those incubated in dexamethasone and LDL/ox-LDL. Similar measurements for example, would suggest inhibition of iNOS only, whereas greater inhibition in dexamethasone and LDL/ox-LDL tissues would suggest inhibition of eNOS also.

Results suggested that incubation with both LDL and dexamethasone reduced L-NAME-induced contraction to a similar degree as incubation with dexamethasone alone.

Ox-LDL treated tissues showed a similar pattern, with a trend towards lower responses when incubation included both ox-LDL and dexamethasone.

LDL and ox-LDL therefore appear to have their main effect on iNOS (although ox-LDL may also have a small effect on eNOS).

These results suggest that LDL and ox-LDL may help accelerate the atherosclerotic process by causing dysfunction in regulatory defense pathways, which would result in abnormally low pathophysiological levels of NO (see Fig. 7.34).

7.6.2 VITAMIN E EXPERIMENTS

The second aspect of the present study, was an examination of the effects of vitamin E on LDL-induced alterations of vascular reactivity.
Vitamin E alone had no significant effect on vascular reactivity when compared with appropriate controls.

Possible NO Regulatory Feedback Pathway and Site of LDL/Ox-LDL Action

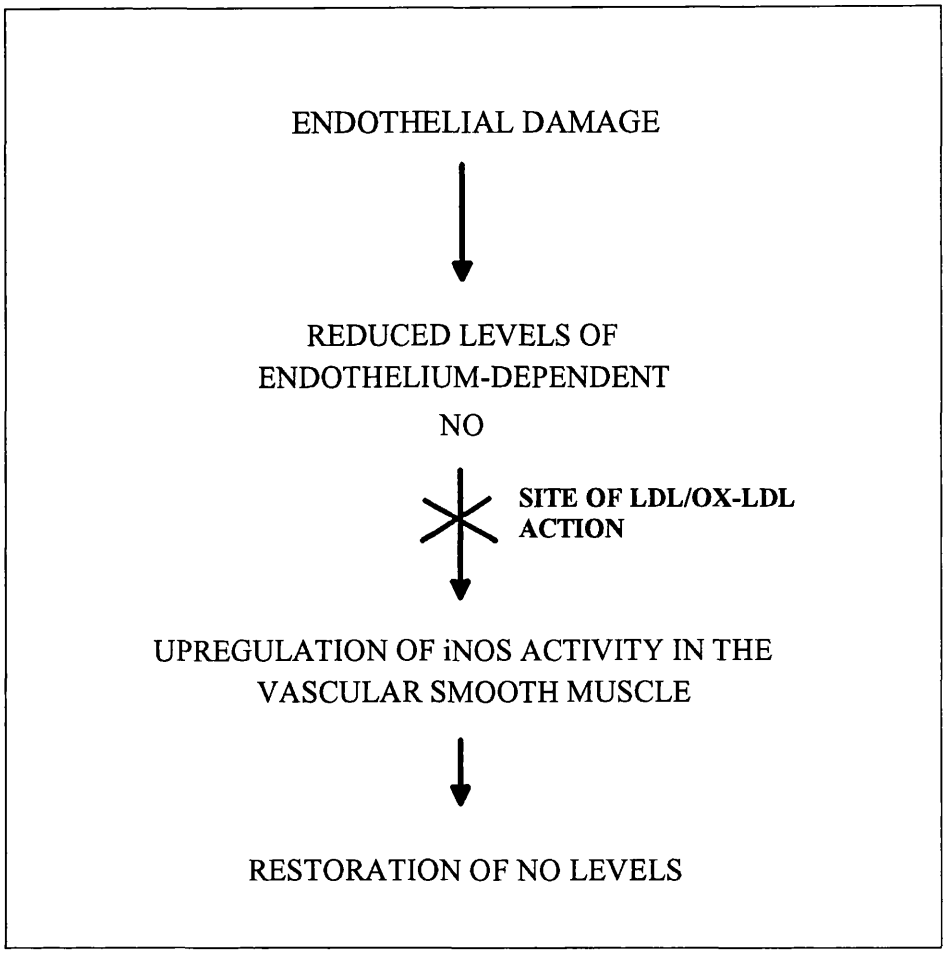


FIG. 7.34 - Possible NO regulatory feedback pathway and site of action of LDL and ox-LDL.

Incubation in the presence of vitamin E had few effects on LDL-induced alterations of vascular reactivity.

Results suggested that only LDL-mediated attenuation of maximum carbachol-induced relaxation may be reduced in the presence of vitamin E.

On measurement of lipid peroxidation levels following incubation, vitamin E showed a tendancy to reduce any oxidative modification of native LDL. However, using the present techniques measurement of minimal oxidation and elucidation of possible vitamin E effects on this partial oxidation process was not possible.

Various explanations for vitamin E's lack of effect may be proposed, including the concentration of vitamin E used.

Alternatively, combination of vitamin E into the LDL particle (as seen *in vivo*) may be required before vitamin E can exert a protective effect.

These results highlight the controversy surrounding the exact beneficial effects, and mechanisms of

action, of antioxidants in the prevention of atherosclerotic disease.

It can therefore be concluded from the present study that 100μM vitamin E did not affect any of the alterations in vascular reactivity observed following exposure to LDL. Vitamin E did however, show a tendancy to alleviate LDL specific attenuation of carbachol-stimulated NO release.

Mechanisms of action of LDL and ox-LDL could not be elucidated from this part of the study.

7.6.3 SUPEROXIDE DISMUTASE EXPERIMENTS

The final part of the present study examined the effects of SOD on LDL and ox-LDL induced alterations of vascular reactivity.

SOD alone, showed a tendency to reduce maximum phenylephrine-induced contraction when compared with appropriate controls.

Similarly, Mian and Martin, 1995, found that SOD (1-300 u ml^{-1}) induced a concentration-dependent relaxation of phenylephrine-induced tone in endothelium intact rat aortic ring preparations.

These results support suggestions that free radicals, in particular superoxide, are natural regulators of NO. SOD inactivation of superoxide anion would therefore reduce NO neutralisation, leading to increased NO levels and greater suppression of contractile responses.

Apart from this tendency to reduce maximum receptor-dependent contraction SOD alone had no other effect on vascular responses studied.

Incubation in the presence of SOD had few effects on LDL and ox-LDL induced alterations of vascular reactivity.

Interestingly, results suggested that ox-LDL-induced reduction in tissue sensitivity to SNP was enhanced to a small extent, in tissues incubated in the presence of SOD.

This substantiates results of Dowell, 1993, who found accentuated impairment of carbachol-stimulated relaxation, following incubation with a xanthine oxidase/hypoxanthine free radical/reactive oxygen species generating system in the presence of SOD.

SOD is known to rapidly convert superoxide anion to hydrogen peroxide, which may then be scavenged by catalase.

In the present study, incubation with SOD may have caused accumulation of hydrogen peroxide (which is itself a free radical) in the absence of sufficient catalase activity.

Groups such as Link and Riley, 1988, have suggested that hydrogen peroxide is the major cytotoxic product formed by the xanthine/xanthine oxidase system, and not the superoxide anion as first thought.

Indeed Dowell, 1993, concluded that hydrogen peroxide was the primary reactive oxygen species responsible for impairment of carbachol-induced endothelium-dependent relaxation in aortic rings (although superoxide may also have a detrimental effect).

Additionally, reaction of superoxide anion and hydrogen peroxide leads to the formation of highly reactive hydroxyl radicals which are known to set off toxic free radical chain reactions (Sinatra and DeMarco, 1995).

Of future interest will be the study of ox-LDL effects in the presence of SOD and catalase, but also catalase alone, to further elucidate the importance of superoxide anion and hydrogen peroxide radicals in this system.

Incubation with SOD had no other effects on LDL and ox-LDL induced alterations of vascular reactivity.

Reasons for this may be the inability of SOD to enter the cell. As a result further studies using free radical scavengers such as tiron, which are capable of acting both intra and extracellularly would be of considerable interest.

Alternatively, dysfunctional effects may be due to any of the other numerous free radicals thought to be formed including hydrogen peroxide, peroxynitrite and hydroxyl species.

It can therefore be concluded from the present study that 45 units/ml SOD showed a tendency to enhance ox-LDL-induced alterations in tissue sensitivity to SNP, but had no other effect on LDL and ox-LDL mediated changes in vascular reactivity.

In the present study there was only weak evidence to suggest that induction of NO dysfunction by low density lipoproteins may be partially due to free radical neutralisation of the NO molecule. Incubation with SOD alone, had few effects on LDL/ox-LDL mediated alterations of vascular reactivity, and further studies (such as those suggested above) will be required to substantiate (or disprove) this theory.

8

GENERAL DISCUSSION

(AND FUTURE DIRECTIONS)

8.1 DISCUSSION

Hypercholesterolemia is an important risk factor in the development and progression of atherosclerotic disease.

The initial aims of this thesis were to characterise effects of LDL and ox-LDL on vascular reactivity *in vitro*, and to investigate possible mechanisms of action.

8.1.1 CHOLESTEROL UPTAKE

Various groups have suggested that increased cholesterol content within the cell membrane may be an initiator of proatherogenic changes at the level of membrane physicochemical structure and calcium influx (Scott-Burden *et al.*, 1989; Yeagle, 1989; Bialecki *et al.*, 1991; Figueiredo *et al.*, 1991; Thorin *et al.*, 1995).

VSM cells are one of two cell types predominant in the atherosclerotic lesion, however mechanisms by which increased cholesterol accumulation may occur in these cells are controversial.

VSM cholesterol uptake was therefore investigated by comparison of the effects of LDL and ox-LDL on free

and total membrane cholesterol levels in cultured cells.

On exposure of VSM cells to LDL and ox-LDL, similar small increases in free and total membrane cholesterol content were observed, supporting theories that VSM cells may not normally express acetyl LDL receptors, but can accumulate cholesterol via a receptor-independent mechanism.

Further studies could have been carried out to investigate the consequences of increases in cholesterol content on biochemical pathways, however, it would have been difficult to relate such findings in cell culture directly to changes in vascular function. Further investigations were therefore carried out in isolated ring preparations.

8.1.2 LDL AND OX-LDL EFFECTS

Several previous studies examining effects of LDL and ox-LDL on vascular reactivity were flawed due to the use of exceptionally high concentrations of ox-LDL (Jacobs *et al.*, 1990; Galle *et al.*, 1991), which is thought to represent only a small proportion of LDL in the body (Yla-Herttuala et al., 1989). Other studies measured responses in the presence of the effector lipoproteins (Jacobs *et*

al., 1990; Simon *et al.*, 1990), therefore excluding the separation of lipoprotein-induced acute agonist responses known to occur, and longer term effects leading to vascular dysfunction (Simon et al., 1990; Murohara et al., 1994).

In the present study, effects on vascular reactivity were characterised *in vitro* in the rat aorta following 5 hour incubation with, and removal of, low and more realistic physiological concentrations of LDL and ox-LDL respectively. Concentrations of LDL and ox-LDL used equated to 20μg/ml (2mg/dl), 100μg/ml (10mg/dl), and 500μg/ml (50mg/dl) (ie. a person with cholesterol levels of 160-180mg/dl (4.2-4.7mmol/l) total cholesterol, will have approximately 104-117mg/dl plasma LDL (Feher and Richmond, 1991)).

Thus concentrations of LDL and ox-LDL used were physiological and pathophysiological rather than pharmacological.

On examination of NO-dependent responses LDL and ox-LDL exposure caused impairment of carbachol-induced endothelium dependent relaxation, and attenuation of L-NAME mediated contraction.

Ox-LDL (but not LDL) also impaired SNP-induced endothelium independent relaxation.

On investigation of contractile responses LDL and ox-LDL exposure augmented phenylephrine-induced contraction, and showed a tendancy to increase maximum contractions due to potassium chloride.

LDL and ox-LDL were therefore found to effect numerous aspects of vascular reactivity and have a wide ranging effect on vascular function (effects included alteration of both endothelium and VSM dependent pathways; and modification of contractile, as well as relaxant responses).

8.1.3 MECHANISMS OF ACTION

Several possible mechanisms underlying LDL and ox-LDL effects on vascular reactivity were investigated.

8.1.3.1 NOS Inhibition

Studies using dexamethasone to inhibit iNOS, indicated that LDL and ox-LDL could cause dysfunction of the NO pathway by inhibiting the inducible form of NOS (although ox-LDL may also have a small effect on eNOS).

Interestingly during this part of the study, removal or damage of the endothelium was found to considerably increase iNOS induction (returning NO activity to endothelium intact levels).

Results therefore point to a novel hypothesis whereby LDL and ox-LDL may accelerate the atherosclerotic process by causing dysfunction of regulatory defense pathways, which would result in abnormally low pathophysiological levels of NO.

8.1.3.2 Free Radical Neutralisation

Free radical neutralisation of the NO molecule was also investigated as a possible mechanism of LDL and ox-LDL induced vascular dysfunction.

Effects of SOD on LDL and ox-LDL induced alterations of vascular reactivity were examined, due to numerous suggestions that the damaging free radical species involved is the superoxide anion (Ohara *et al.*, 1993; Araujo *et al.*, 1995; Pritchard *et al.*, 1995).

Incubation in the presence of SOD did not significantly alter LDL and ox-LDL mediated effects on vascular reactivity, and results suggested that

SOD may even enhance ox-LDL-induced reduction of tissue sensitivity to SNP.

Interestingly SOD alone, showed a tendency to reduce maximum phenylephrine-induced contraction when compared with appropriate controls, suggesting a constant basal release of superoxide in the vasculature.

In the present study, there was therefore at the most only weak evidence to suggest that LDL and ox-LDL mediated dysfunction may be partially due to free radical neutralisation. However such results may simply indicate that the damaging reactive species involved is not in fact superoxide (a theory already suggested by numerous groups).

8.1.4 ADDITIONAL INVESTIGATIONS

8.1.4.1 Risk Factor Interactions

Hypertension and hypercholesterolemia were also examined as coexisting and interactive risk factors using a multiple risk model.

Mechanisms by which hypertension contributes to atherogenesis have not been established, however a synergy between the two risk factors has been suggested (Walton, 1975; Feher and Richmond, 1991).

Results suggested that incubation of SHRSP VSM cells with LDL and ox-LDL produced greater increases in free cholesterol content (on comparison with their WKY normotensive reference strain), and that this was marginally greater in ox-LDL treated cells.

These results indicate a possible interactive mechanism between hypertension and hypercholesterolemia, whereby hypertension could accelerate atherosclerotic progression by increasing cholesterol uptake by cells.

Preliminary examination of the effects of cell stretch on ox-LDL-induced cholesterol uptake, using a novel cell stretch apparatus, were inconclusive, however demonstrated a potential model for future investigations of hemodynamic forces on cellular function.

8.1.4.2 Antioxidant Effects

Another additional aim of this thesis was to examine the effects, and possible benefits of antioxidants, in the prevention of LDL and ox-LDL induced vascular dysfunction.

Vitamin E has recently been suggested to have additional non-antioxidant anti-atherogenic effects,

including an additional role in cholesterol flux alteration of the cell membrane (Ozer *et al.*, 1993; Boscoboinik *et al.*, 1994, 1995; Thorin *et al.*, 1995).

As a result the effects of the antioxidants vitamin E and NAC on LDL and ox-LDL mediated cholesterol uptake were examined in cultured WKY and SHRSP VSM cells.

In the presence of vitamin E both LDL and ox-LDL mediated effects were attenuated in both cell types. Interestingly, vitamin E also reduced basal free membrane cholesterol levels in a concentration dependent manner.

Results indicate that the main observed effects of vitamin E are not a result of its antioxidant properties, and suggest that other mechanisms of action are involved.

NAC produced only a small attenuation of LDL and ox-LDL mediated effects on cholesterol uptake in WKY and SHRSP VSM cells, and did not alter basal free cholesterol content of cells.

NAC's effects were also not attributable to its antioxidant properties alone, however results showed that NAC did not share all vitamin E's mechanisms of action.

Numerous groups have also suggested that observed LDL mediated effects are mainly attributable to LDL which has been oxidatively modified.

As a result the effects of vitamin E on LDL-induced alterations of vascular reactivity were examined in isolated aortic ring preparations from rat aorta. Vitamin E was found to have no significant effect on LDL-induced alterations of vascular reactivity (although results suggested that LDL-mediated attenuation of maximum carbachol induced relaxation may be reduced to a small extent in the presence of vitamin E).

Such results highlight the controversy already surrounding the actual actions and effects of antioxidants in atherosclerotic protection.

8.2 CONCLUSIONS

In conclusion, the work presented in this thesis has provided novel information on the effects of LDL

cholesterol on vascular function, and the possible mechanisms of action involved.

LDL and ox-LDL were demonstrated to have wide ranging effects on vascular function, which could be mainly attributed to dysfunction of the NO pathway (at both basal and stimulated levels).

Studies involving investigation of possible mechanisms of action indicate that LDL and ox-LDL induced vascular effects probably occur via a complex multidysfunctional mechanism, resulting from a combination of interference at several mechanistic sites.

Hypertension and hypercholesterolemia were investigated as coexisting and interactive risk factors and a possible site of interaction demonstrated.

Finally, additional non-antioxidant properties of the antioxidants vitamin E (and to a lesser extent NAC), were elucidated.

8.3 FUTURE WORK

The potential for future work resulting from this thesis are wide ranging, and many have been discussed previously in the individual chapters.

Increased knowledge and understanding of the mechanisms of action of LDL cholesterol, and the individual importance of the various dysfunctional sites will be of value in the future to allow possible targeting for preventative medicine, and reduction of cholesterol enhanced atherosclerotic damage.

Such knowledge will also be invaluable in improving understanding of the physiology of the vascular system.

Increased investigation of risk factor interactions, with particular reference to hypertension and hypercholesterolemia, will also be of much interest.

Free radical involvement has been suggested in the pathobiology of both essential hypertension and hypercholesterolemic enhancement of atherosclerotic disease (Auch-Schwelk et al., 1989; Kumar and Das, 1993; Ohara *et al.*, 1993; Araujo *et al.*, 1995;

Keaney and Vita, 1995), suggesting another potential site of interaction.

Further examination of interactions between hypertension and hypercholesterolemia could be carried out by comparison of LDL and ox-LDL mediated effects in isolated aortic ring preparations from WKY and SHRSP rats.

Alternatively, establishment of the effects of perfusion pressure on LDL and ox-LDL induced vascular alterations in isolated arterial preparations, would also be of great interest.

Finally, examination of LDL and ox-LDL effects on human vascular function will be of prime importance in the future.

APPENDIX

1 Materials

CHEMICALS

GIBCO/LIFE TECHNOLOGIES LTD

All cell culture chemicals, buffers and reagents

SIGMA

Butylated Hydroxytoluene (BHT)	B1378
Carbachol	C4382
Collagenase Type II	C6885
Cholesterol	C8667
Dexamethasone	D1756
Elastase Type IV	E0258
17β Estradiol	E8875
Folin & Ciocalteu's Phenol Reagent	F9252
Fibronectin	F1141
Indomethacin	I7378
Lauryl Sulfate	L4509
L-NAME	N5751
NALC	A7250
Phenylephrine	P6126
Soybean Trypsin Inhibitor (SBTI)	T6522
Superoxide Dismutase (SOD)	S2515
2-Thiobarbituric Acid (TBA)	T5500
1,1,3,3 - Tetramethoxypropane	T1642
Vitamin E	T3634

Chemicals used in cholesterol and protein assays

Sodium nitroprusside and cocaine hydrochloride were obtained from the Western Infirmary (General Stores). All other chemicals were obtained from BDH.

2 LDL/OX-LDL Preparation Solutions

SOLUTIONS

1. Density Solution - **1.006g/ml**

0.195M NaCl

11.4g NaCl + 0.1g EDTA-Na_2 + 500mls dH_2O

2. Density Solution - **1.182g/ml**

0.195M NaCl / 2.44M NaBr

24.98g NaBr + 100mls 1.006g/ml density solution

3. Density Solution - **1.019g/ml**

8mls 1.182g/ml density solution + 100mls NaCl/Saline

4. Density Solution - **1.063g/ml**

1.006g/ml	:	1.182g/ml	
2	**:**	**1**	**Ratio**

5. Density Solution - **1.478g/ml**

0.195M NaCl / 7.65M NaBr

78.32g NaBr + 100mls 1.006g/ml density solution

6. Density Solution - **1.21g/ml**

1.063g/ml : 1.478g/ml

2 : 1 Ratio

7. Phosphate Buffer / Stock Dialysis Buffer

i/ **NaCl / Saline** ie. 8.76g + 1l dH_2O

ii/ **Na_2HPO_4 (Anhyd.)** ie. 10.65g + 750mls NaCl/Saline

iii/ **$NaH_2PO_42H_2O$** ie. 3.9g + 250mls NaCl/Saline

iv/ Adjust pH of Na_2HPO_4(ii) to 7.4 with $NaH_2PO_42H_2O$ (iii)

v/ Dilute 1/10 before dialysis

8. Copper Chloride Solution - **make fresh**

4μM

i/ 0.00269g + 100mls dH_2O = 200μM

ii/ Add 20μl/ml LDL solution = 4μM $CuCl_2$

3 Preparation of VSM cells - Digest Mix

Digest mix was prepared as follows:-

i/ 5mgs elastase type IV was dissolved in 2ml serum free DMEM, and 100μls of soya bean trypsin inhibitor solution (ie.10mgs SBTI + 1ml serum free DMEM) added.

ii/ 1ml of Collagenase type II solution (ie.25mgs COLL + 1ml serum free DMEM) was then added and the solution gently mixed by swirling.

iii/ The solution was made up to a final volume of 20mls, mixed, and filtered through a 0.22 millipore filter.

4 Vascular Smooth Muscle Growth Medium

CONTENTS	**CONCENTRATION**	**QUANTITY**
DMEM		500mls
Foetal Calf Serum	10% (V:V)	50mls
Horse Serum	10% (V:V)	50mls
Gentamicin (10mg/ml)	100µg/ml	5mls
Glutamine (200mM)	2mM	5mls
Penicillin/Streptomycin (10,000IU/10,000µg/ml)	100IU/100µg/ml	5mls
Fungizone (250µg/ml)	5µg/ml	10mls

5 Cholesterol Assay Stock Solutions -store at 4°C

1. Na Cholate

FW= 430.6

20mM

eg. 86.12mg in 100mls dH_2O

2. Triton X 100

0.5%

eg. 0.5ml in 100mls dH_2O

3. Di Potassium Hydrogen Phosphate(K_2HPO_4 buffer) -

pH7.4

FW= 174.18

0.1M

eg. 1.7418g in 100mls dH_2O

4. Ethanol

95%

eg. 475mls absolute ETOH + 25mls dH_2O

5. Cholesterol standard

0.4mg/ml

eg. 20mg in 50mls 95% ETOH

Note - Sonicate for 15 minutes to insure proper dissolution

Make the following up in 0.1M K_2HPO_4 buffer (pH7.4):-

6. Cholesterol esterase

0.1u/ml

eg. 5 units in 1ml = 5u/ml stock solution

200µls stock solution in 10mls = 0.1u/ml

7. Cholesterol oxidase

1u/ml

eg. 100 units in 1ml = 100u/ml stock solution

100µls stock solution in 10mls = 1u/ml

8. Horseradish peroxidase

10u/ml

eg. 5000 units in 10mls = 500u/ml stock solution

200µls stock solution in 10mls = 10u/ml

9. p-hydroxyphenylacetic acid

4mg/ml

eg. 40mg in 10 mls

6 Protein Assay Solutions

1. Bovine Serum Albumin

To be made up 24 hours prior to assay

25mg BSA in 50ml 1M Sodium Hydroxide = 500µg/ml stock solution

Store at 4°C

Make up as required:-

2. Solution A

2g Na_2CO_3 in 100mls 0.1M NaOH

3. Solution B

100mg $CuSO_4$ + 200mg NaK Tartrate in 20mls dH_2O

NOTE - Must dissolve $CuSO_4$ completely in approx. 1/2 volume before adding NaK Tartrate and making up to end volume

4. Sodium Copper Tartrate Solution

100mls solution A + 20mls Solution B

5. Folins Solution

4mls Folins reagent + 4mls dH_2O **ie.**1:1 dilution

7 Krebs Bicarbonate Buffer pH 7.4

CONTENTS	**CONCENTRATION (mM)**	**QUANTITY (g/5l)**
Ca EDTA	0.05	0.0935
Glucose	11.1	10.0
KCl	4.7	1.75
KH_2PO_4	1.2	0.80
$MgSO_4$	1.2	1.50
NaCl	118.3	34.55
$NaHCO_3$	25.0	10.50
$CaCl_2$	2.5	12.5ml (1M)
17β Estradiol	0.01	0.0135

Indomethacin	0.01	0.0180
Cocaine HCl	0.01	5ml (10^{-2}M)

NOTE - 17β estradiol and indomethacin dissolved in 1ml DMSO prior to addition.

8 TBA Assay Reagents

1. 8.1% Sodium Dodecyl Sulphate (SDS)

i/ 8.1g SDS + 100ml dH_2O

ii/ Dilute 1:3 with dH_2O prior to use

2. 20% Acetic Acid

i/ 20ml pure acetic acid + 80ml dH_2O

ii/ pH adjust to 3.5 and make up to 100ml

3. 0.8% TBA Reagent

i/ 0.8g TBA + 50ml dH_2O

ii/ Add 50ml pure acetic acid

iii/ Dilute with 20% acetic acid 1:1

NOTE - Must be prepared daily. Protect from light.

4. Standard Solution

i/ 0.1642g MDA + 100ml dH_2O = 10^{-2}M

ii/ Dilute to 10^{-5}M

PUBLICATIONS AND PRESENTATIONS CONTAINING THE WORK UNDERTAKEN FOR THIS THESIS

McPherson,KL; Jardine,E; Hamilton,CA; Devlin,AM; Dominiczak,AF; Reid,JL (1995): Membrane cholesterol alterations in cultured WKY and SHRSP vascular smooth muscle cells on exposure to LDL, Ox-LDL, and vitamin E. Br. J. Pharmacol. 115, 151P.

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