EFFECTS OF LOW DENSITY LIPOPROTEINS AND OXIDIZED LOW DENSITY LIPOPROTEINS ON ENDOTHELIUM-DEPENDENT VASCULAR RELAXATION

By

Douglas Edwin Froese

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DOUGLAS EDWIN FROESE

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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Abstract

It was first determined by Furchgott and Zawadzki (1980) that the endothelium of a rabbit aortic ring preparation must be intact for endothelium-dependent relaxation (EDR) to occur in response to acetylcholine. Acetylcholine acts on muscarinic receptors of the endothelial cells, stimulating the release of the endothelium-dependent relaxing factor (EDRF), which has been identified as nitric oxide (NO) by Palmer et al (1987). Since 1980, it has been determined that the endothelium releases many other substances which stimulate vasodilation (nitric oxide, endothelium-dependent hyperpolarizing factor, prostacyclin, endothelium-dependent inhibitory factor) or vasoconstriction (endothelium-dependent constricting factor, endothelin).

Hyperlipidemia has been associated with an increase in the occurrence of atherosclerosis. The oxidation of low density lipoprotein (LDL) plays an important role in the initiation and progression of atherosclerosis, one of its effects being the inhibition of endothelium-dependent relaxation (Kugiyama et al 1990, Liu et al 1994). The objective of the present research is to examine how oxidized LDL interferes with the production or the effectiveness of vasoactive substances which are produced by the endothelium.

The elevated level of lysophosphatidylcholine (LPC) in oxidatively modified LDL has been shown to be the biochemical factor responsible for the impairment of endothelium-dependent relaxation in aortic ring preparations. The first objective of this project is to determine if the membrane perturbing effect of LPC is the molecular

mechanism by which LPC impairs endothelium-dependent relaxation. A concentration-dependent inhibition of acetylcholine-induced relaxation was observed with 5 and 10 μM LPC. Incubation with LPC (10 μM) for 15, 30 and 60 min produced similar impairment of relaxation suggesting the effect is maximal within 15 min. Palmitoyl carnitine (5 and 10 μM), a compound with similar structure and membrane perturbing properties, also inhibited relaxation rapidly in concentration dependent manner.

The effect of the PKC inhibitor Ro31-8220 on the ability of LPC and palmitoyl carnitine to inhibit endothelium-dependent relaxation in the rat aorta was examined. Ro31-8220 reduced the ability of both LPC and palmitoyl carnitine to inhibit endothelium-dependent relaxation, but did not completely prevent their effects. Therefore, it appears that PKC is involved in the mechanism by which LPC inhibits endothelium-dependent relaxation.

The reversibility of LPC-induced inhibition of EDR was examined by incubating the aortic rings in Krebs solution containing defatted bovine serum albumin (BSA; which is an acceptor for hydrophilic lipids) after exposure to LPC. BSA reversed the effect of LPC, restoring EDR in the aortic rings.

There is evidence that other endothelium-dependent factors are involved in vasodilation such as the endothelium-dependent hyperpolarizing factor (EDHF). In the aortic ring preparation, nitric oxide synthase was blocked nitro-L-arginine methyl ester (L-NAME) which prevented the production of EDNO. The effect of EDHF was blocked

by raising the K^+ concentration in the bath containing the aortic rings. Neither pathway could account for the entire inhibitory effect exerted by LPC on EDR. In conclusion, the mechanism by which LPC impairs endothelium-dependent relaxation appears to involve both EDRF (NO) and EDHF.

Our findings are consistent with the notion that LPC alters endothelium-dependent relaxation by altering the fluidity of the membrane, thus interfering in the G protein pathway. Interference of the G protein pathway may occur between the luminal endothelial receptor and G protein or via the activation of enzymes such as PKC. This interference reduces the production of NO and EDHF and inhibits the ability of the aortic ring to relax.

In view of the increased incidence of atherosclerosis in hyperlipidemia, the LPC composition of oxidized LDL samples from hyperlipidemic subjects and their effects on the impairment of endothelium-dependent relaxation was examined. Oxidatively modified LDL from hyperlipidemic subjects contained significantly greater levels of LPC than that from normal subjects, and was found to produce a higher degree of impairment of endothelium-dependent relaxation. Since LPC is composed of many molecular species, its composition in oxidized LDL samples was analyzed by gas liquid chromatography. LPC containing a higher proportion of long-chain acyl groups was observed in oxidized samples from hyperlipidemic subjects. Only long-chain LPC ($\geq C_{16:0}$) produced an impairment of endothelium-dependent relaxation. We conclude that the higher proportion

of long-chain LPC found in the oxidized LDL from hyperlipidemic subjects is responsible for the increased impairment of endothelium-dependent vascular relaxation. We propose that the high level of LDL found in the plasma of hyperlipidemic subjects, coupled with its enhanced ability to generate long chain species of LPC during oxidative modification, are important factors that contribute to the production of atherosclerosis in these subjects.

There is also an increased incidence of atherosclerosis in men compared to women. Another objective of this study was designed to examine the effect of hyperlipidemia and gender on susceptibility of LDL to oxidation in vitro and the effect of oxidation products on endothelium-dependent relaxation of rat aorta. The results showed that oxidatively modified LDL from hyperlipidemic subjects caused significantly greater impairment of endothelium-dependent relaxation in rat aortic rings and had significantly higher content of LPC than that from normal subjects. Correlation analysis indicated that the impairment of endothelium-dependent relaxation had a highly positive correlation with LPC formation for hyperlipidemic subjects. Further analysis of gender-related subgroups of the data showed that oxidized LDL from males caused greater impairment of endothelium-dependent relaxation and increased production of LPC compared with that from females in the hyperlipidemic group. The degree of impairment was correlated with production of LPC during the oxidation. These results are consistent with the notion that the increased LPC production and greater endothelial dysfunction in connection with oxidative

modification of LDL have a role, not only in the increased risk of atherosclerosis in hyperlipidemia but also for the different incidence of the disease in men and women.

Vascular endothelial cells produce another potent vasodilator, prostacyclin. Arachidonic acid (AA) is the precursor of eicosanoids and its release is essential for the biosynthesis of eicosanoids such as prostacyclin. The final objective of this study examined the effect of LDL on the release of arachidonic acid from human umbilical vein endothelial cells (HUVEC) to determine if this could be a third method by which LDL inhibits EDR.

Native-LDL (non-oxidized LDL) stimulated a significantly greater release of AA from HUVEC when compared to that produced by ox-LDL (oxidized LDL). When the ox-LDL was depleted of its LPC, ox-LDL stimulated a release of AA from HUVEC not significantly different from that of na-LDL. It appears that LPC inhibits the release of AA from HUVEC. This in turn would reduce the amount of prostacyclin produced by the cell and destabilizes the balance between prostacyclin (vasodilator) and thromboxane (vasoconstrictor), thus resulting in vasoconstriction.

The requirement for Ca^{2+} in the production of EDRF (NO), EDHF, and the release of AA from endothelial cells appears to be a common theme for the production of these three vasodilators. Nitric oxide synthase requires an increase of cytosolic Ca^{2+} to produce NO. The release of EDHF is regulated by cytosolic calcium and inhibited by calmodulin antagonists. The hydrolysis of the acyl chain at the sn-2 position of

glycerophospholipids by phospholipase A_2 (PLA_2) is regarded as the major source of AA release which is the initial step in the production of prostacyclin, and PLA_2 requires Ca^{2+} to function. In each case, if the cytosolic level of Ca^{2+} in the endothelial cell can not be increased, then the vasodilator will not be produced. We hypothesize that LPC from ox-LDL perturbs the endothelial membrane, resulting in an alteration of cytosolic Ca^{2+} within the endothelial cell.

In conclusion, these studies demonstrate LPC produced by the oxidation of LDL inhibits EDR by a generalized (no receptor involved) mechanism which is demonstrated by palmitoyl carnitine, a compound similar in structure, which also inhibits EDR. Evidence that inhibition of EDR by LPC occurs in the vascular endothelial membrane is provided by the ability to reverse the inhibitory effect of LPC by incubating the aortic ring with bovine serum albumin after incubation with LPC. This mechanism appears to involve both EDNO and EDHF vasorelaxing factors. Part of the inhibitory effect of LPC can be blocked by PKC inhibitors

In hyperlipidemic subjects, the high level of LDL found in the plasma, coupled with its enhanced ability to generate long chain species of LPC during oxidative modification, are important factors that contribute to the increased production of atherosclerosis in these subjects.

In male hyperlipidemic subjects the increased LPC production and greater endothelial dysfunction in connection with oxidative modification of LDL have a role, not

only in the increased risk of atherosclerosis in hyperlipidemia but also for the different incidence of the disease in men and women.

It has also been determined that ox-LDL can impair the release of arachidonic acid from HUVEC. This would prevent the production of prostacyclin, a potent vasodilator, by the vascular endothelial cell. This may also contribute to the vascular dysfunction that is associated with atherosclerosis.

I. Introduction

1. Regulation of Vascular Tone

Blood flow appears to be adjusted according to the metabolic activity of the tissue. At a constant tissue metabolism, any change in arterial blood pressure is met by a change in vascular resistance to maintain a constant blood flow. This mechanism is known as autoregulation of blood flow. Vascular resistance is determined by vascular tone, which is controlled by several mechanisms as discussed below.

1.1 The Role of the endothelium

It was first determined by Furchgott and Zawadzki (1980) that an intact endothelium in a rabbit aortic ring preparation is required for vasodilation to occur in response to acetylcholine. This astounding discovery opened up an entirely new area of research regarding the control of vascular tone. If the arterial endothelium is denuded, vasodilation in response to acetylcholine is abolished. Acetylcholine acts on muscarinic receptors of the endothelial cells, stimulating the release of a substance which causes the relaxation of the arterial smooth muscle (Furchgott and Zawadzki 1980). This substance was known as the endothelium-dependent relaxing factor (EDRF) and was subsequently identified as nitric oxide (NO) by Palmer et al (1987). Since 1980, it has been determined

that the endothelium releases other substances (some not yet identified) which cause vasodilation or vasoconstriction.

1.2 Endothelium-Dependent Relaxing Factor (EDRF)

EDRF has been identified as nitric oxide (NO) by Palmer et al (1987). An elevation of blood pressure in the artery results in an increase of blood flow and vessel diameter. The vasodilation occurs by EDRF which is released from the endothelium in response to shear stress (Yokoyama et al 1990), a result of the increased blood flow. If the arterial endothelium is denuded, vasodilation in response to shear stress is abolished (Furchgott and Zawadzki 1980).

There are many substances such as acetylcholine, adenosine triphosphate, bradykinin, serotonin, substance P, histamine, and thrombin which stimulate endothelial cells to release EDRF in vivo and cell culture (Vanhoutte and Houston 1985).

In view of the reproducibile endothelium-dependent relaxation produced by acetylcholine, its use has become common in the field and was chosen for this series of studies. The mechanism involves acetylcholine binding muscarinic receptors on the luminal side of the endothelial membrane, activating a G-protein. The activated G-protein activates the enzyme phospholipase C which cleaves phosphatidylinositol 4, 5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 moves into the cytosol of the endothelial cell and binds to receptors on the endoplasmic reticulum

causing the release of calcium from intracellular stores (Dinerman et al 1993). Calcium forms a complex with calmodulin which then binds and activates nitric oxide synthase (NOS) to produce nitric oxide (NO) (Bredt et al 1992). Nitric oxide diffuses from the endothelial cell to the smooth muscle and bind to iron at the active site of guanylyl cyclase enhancing the production of cGMP (Ignarro et al 1987). It has been hypothesized that cGMP elicits smooth muscle relaxation by activating a protein kinase which in turn phosphorylates a Na/Ca exchanger (Goy 1991). This reduces the calcium inside the smooth muscle cell resulting in relaxation.

1.3 Endothelial-Dependent Hyperpolarizing Factor (EDHF)

There is evidence that another endothelium-dependent factor is involved in vasodilation known as EDHF. Ach increases the production of NO (EDRF), but NO can not account for the actions of EDHF. Chen et al (1988) found that the blockade of the synthesis of NO had little effect on Ach-induced hyperpolarization. Blockade of the effects of NO by methylene blue resulted in almost complete abolishment of cGMP increases within the smooth muscle cell, yet it had no effect on Ach-induced hyperpolarization (Chen et al 1988). It must be concluded that endothelium-dependent hyperpolarization can not be attributed to nitric oxide (Vanhoutte et al 1995). The fact that Ach has a vasodilator effect, even in the presence of NOS inhibitors, leads us to believe an EDRF other than NO exists.

The action of NO is mediated by an increase in the production of cGMP, as discussed previously. EDHF is believed to act via the opening of K^+ channels resulting in hyperpolarization and relaxation of the vascular smooth muscle. The first evidence that K^+ channels are involved in the action of Ach was that lowering K^+ concentration in the tissue bath increased the magnitude of hyperpolarization while increasing the concentration of K^+ reduced or abolished the hyperpolarization (Kitamura and Kuriyama 1979, Kuriyama and Suzuki 1978). It has also been demonstrated that Ach causes an endothelium-dependent increase in efflux of K^+ (Ordway et al 1989). There is evidence that the K^+ channel blocker tetraethylammonium can inhibit hyperpolarization, but exactly which K^+ channels are involved is still not clear (Hasunuma et al 1991).

The release of EDHF is regulated by cytosolic calcium and inhibited by calmodulin antagonists (Nagao and Vanhoutte1993). Ach increases the endothelial cytosolic Ca^{2+} which activates Ca^{2+}-activated K^+ channels (K_{Ca}) inducing membrane hyperpolarization which increases the driving force of Ca^{2+} into the endothelial cell. The increase in Ca^{2+} results in an increase in production of NO and EDHF which diffuses to the vascular smooth muscle. EDHF is hypothesized to increase K_{Ca} channel activity on the smooth muscle membrane resulting in hyperpolarization and reduced Ca^{2+} entry via voltage-sensitive L-type Ca^{2+} channels, reducing cytosolic Ca^{2+}, resulting in relaxation (Waldron et al 1996) .

EDHF has not been identified, but one theory is it may be epoxyeicosatrienoic acid, a metabolite of arachidonic acid formed through the P-450 pathway (Komori and Vanhoutte 1990).

1.4 Endothelial-Derived Constricting Factor (EDCF)

The endothelium is very important to the regulation of vascular tone by producing vasodilators such as EDRF and EDHF which have been discussed above. The endothelium also produces vasoconstrictors such as endothelin and EDCF. It has been suggested that EDCF might be thromboxane A_2 (Buzzard et al 1993) or prostaglandin H_2 (Dai et al 1992). It is likely that several EDCFs exist, but their identity is still controversial.

1.5 Endothelium-Dependent Inhibitory Factor (EDIF)

The endothelium releases EDIF which antagonizes the release of noradrenaline preventing vasoconstriction. This can be demonstrated by the removal of the endothelium which results in increased noradrenaline release (Cohen et al 1988). It has also been proposed that angiotensin II can stimulate the release of EDIF (Thorin et Atkinson 1994). EDIF has not been identified yet.

1.6 Endothelin

Yanagisawa et al (1988) isolated endothelin, a potent endothelium-derived vasoconstrictor peptide, from the culture supernatant of porcine aortic endothelial cells. They determined its 21 amino-acid residue sequence and cloned the peptide precursor preproendothelin. It has been found to be a more potent vasoconstrictor than angiotensin II, vasopressin, and neuropeptide Y (Yanagisawa et al 1988).

Porcine aortic strips bathed in Krebs-Ringer solution free of Ca^{2+} inhibited endothelin-induced contraction. Ca^{2+} channel blockers also inhibited endothelin-induced vasoconstriction (Yanagisawa et al 1988). These findings suggest that an influx of extracellular Ca^{2+} is required for endothelin to cause vasoconstriction.

The production and release of endothelin has been linked to atherogenesis. The blockade of the endothelin A-receptor subtype decreases atherosclerosis in hypercholestrolemic hamsters (Kowala et al 1995). Endothelin possesses many properties indicating that it may be an atherogenic peptide. Endothelin is a potent vasoconstrictor which may result in dysfunction of EDR in the vasculature. It is also a strong chemoattractant for circulating monocytes, and activates macrophages (Haller et al 1991). Macrophages can cause injury to the endothelium and release platelet-derived growth factors (PDGF), both of which are important first steps in the pathogenesis of atherosclerosis. Overexpression of endothelin can also stimulate smooth muscle cell proliferation (Alberts et al 1994), another important event in atherogenesis. In fact,

oxidized low-density lipoproteins stimulate the production and release of endothelin (Boulanger et al 1992) from endothelial cells. These attributes point to a role for endothelin in atherogenesis and the dysfunction of EDR in the vasculature.

1.7 Metabolites of arachidonic acid

Vascular endothelial cells produce another potent vasodilator, prostacyclin, through the cylooxygenase pathway. Arachidonic acid (AA) is the precursor of the cyclooxygenase pathway and its release is essential for the biosynthesis of all eicosanoids, including prostacyclin. The final objective of this study examines the effect of LDL on the release of arachidonic acid from human umbilical vein endothelial cells (HUVEC) to determine if ox-LDL inhibits EDR by inhibiting the production of prostacyclin.

The eicosanoids are a group of oxygenated, twenty carbon fatty acids. There are three pathways involved in the biosynthesis of the eicosanoids which include the cyclooxygenase (prostaglandins and thromboxanes), lipoxygenase (leukotrienes), and epoxygenase (epoxides) pathways. These pathways are named after the enzyme involved in the first step of the respective pathways.

The hydrolysis of the acyl chain at the sn-2 position of glycerophospholipids by phospholipase A_2 (PLA_2) is regarded as the major source of AA release. PLA_2 is also responsible for the conversion of phosphatidylcholine into lysophosphatidylcholine upon oxidation of LDL and for this reason, it warrants further discussion. Many isoforms of

PLA_2 have been identified but only the 14 kDa secreted group II PLA_2 ($sPLA_2$) and the 85 kDa cytosolic PLA_2 ($cPLA_2$) have been purified and well characterized (Mayer and Marshall 1993, Clark et al 1995). The $sPLA_2$ requires extracellular (millimolar) Ca^+ for activation while $cPLA_2$ requires intracellular (micromolar) for translocation to the cell membrane. These isoforms are found in endothelial cells and are involved in AA release and PGI_2 production (Murakami et al 1993).

In the cyclooxygenase pathway, arachidonic acid is synthesized into prostaglandin endoperoxide (PGH_2) by the enzyme cyclooxygenase. PGH_2 is then metabolized into a biologically active product such as PGI_2 (prostacyclin), PGE_2, PGF_2, PGD_2, or TxA_2 (thromboxane) (Fig 1, Smith 1989). Prostacyclin, a potent vasodilator and an inhibitor of platelet aggregation (Moncada et al 1976), is the main prostanoid produced by vascular endothelial cells (Smith et al 1991b). Prostacyclin activates adenylate cyclase which increases the cyclic adenosine monophosphate (cAMP) concentration inside the cell (Gorman et al 1977). Thromboxane, which is the main prostanoid produced by the platelets (Smith et al 1991b), has biological actions which oppose prostacyclin, therefore a balance is required. Thromboxane is a strong contractor of blood vessels and induces platelet aggregation (Moncada and Vane 1979).

In the lipoxygenase pathway, the enzyme lipoxygenase first converts arachidonic acid into hydroperoxy eicosatetraenoic acids (HpETEs). HpETEs can then undergo several enzymatic transformations resulting in hydroxy eicosatetraenoic acids (HETEs),

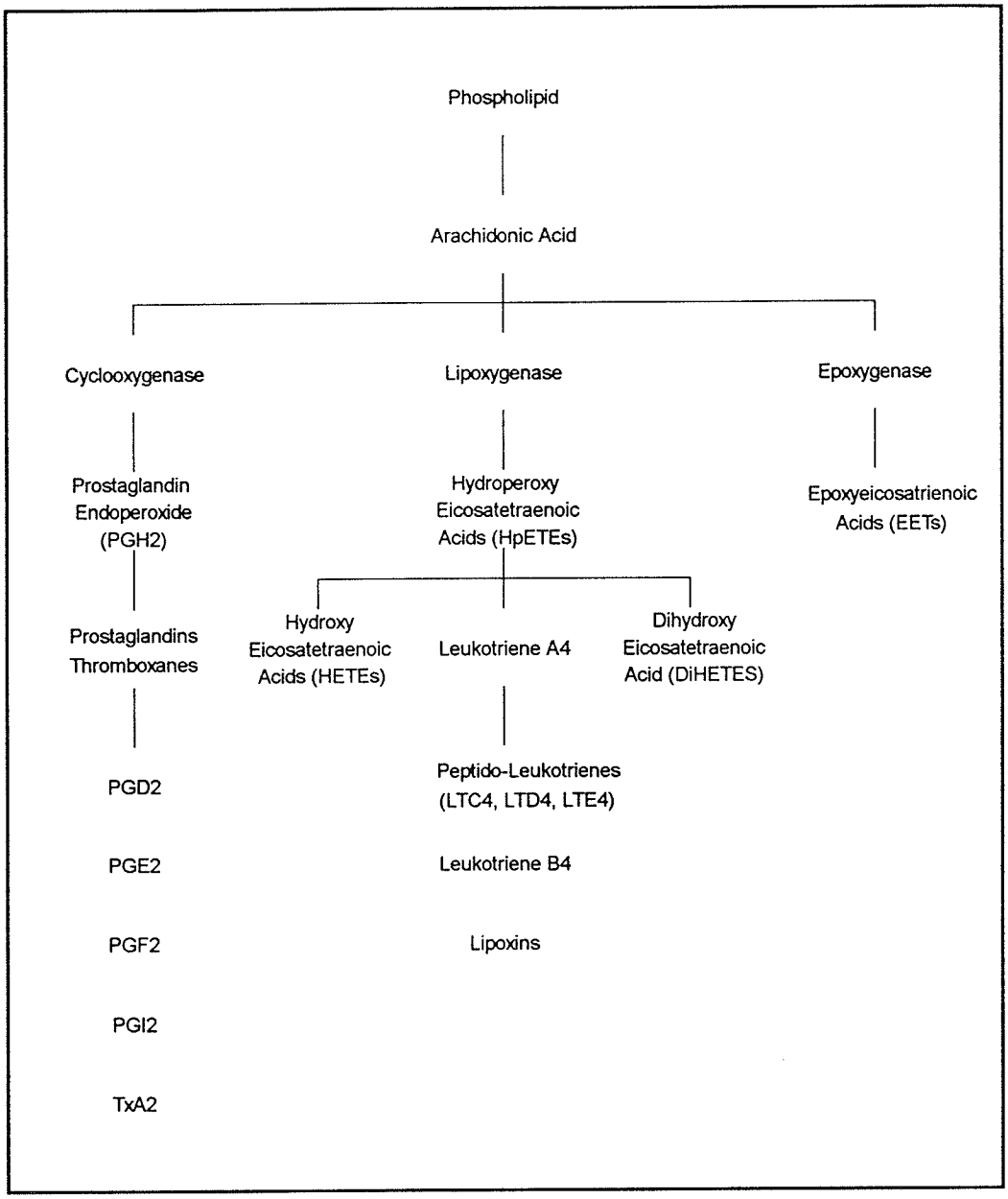

Figure 1. Pathways leading to the formation of eicosanoids from arachidonic acid (from Smith 1989)

dihydroperoxy eicosatetraenoic acids (DiHETEs), trihydroperoxy eicosatetraenoic acids (lipoxins), and leukotrienes (Fig. 1, Smith 1989). These products can be found in leukocytes such as neutrophils, eosinophils, monocytes, and macrophages (Smith et al 1991a). LTC_4, LTD_4, and LTE_4 are vasoconstrictors, increase vascular permeability, and are potent constrictors of respiratory smooth muscle (Smith et al 1991a). LTB_4 is highly chemotactic towards neutrophils and induces their adherence to the vascular endothelium (Smith et al 1991a). These actions are fundamental in the inflammation process.

In the epoxygenase pathway (Fig. 1), cytochrome P-450 transforms arachidonic acid into epoxyeicosatrienoic acids (EETs). Various EETs can be found in platelets and endothelial cells. A few of the biological actions of EETs include inhibition of cylooxygenase activity, inhibition of arachidonic acid-induced platelet aggregation, and vasodilation of arteries (Smith et al 1991a).

1.8 The Autonomic Nervous System

Most arteries and veins of the body are innervated by the sympathetic nervous system. Activation of the sympathetic nerves results in vasoconstriction (via norepinephrine release) which increases vascular resistance and decreases the blood flow. These fibers exert a tonic effect on blood vessels, therefore, cutting the sympathetic nerves of a vascular bed results in an increase in blood flow (Donald and Shepherd 1980). In

contrast, the parasympathetic nervous system does not play a role in regulation of blood flow, except in a few tissues such as the salivary gland.

1.9 Myogenic Mechanism

The vascular smooth muscle contracts in response to stretch and relaxes in response to a reduction of tension (Johnson 1980). An increase in arterial blood pressure will result in increased blood flow and distend the arterial wall. The smooth muscle will contract in response to this distention and return blood flow to its previous level over a period of several minutes.

1.10 Metabolic Regulation of Vascular Tone

This theory states blood flow is regulated by the metabolic activity of the tissue. Any activity that results in inadequate O_2 supply to a tissue causes the formation of vasodilator metabolites (Sparks 1980). An increase in O_2 tension (PO_2) causes contraction of the smooth muscle while a reduction in O_2 tension (PO_2) causes the smooth muscle to relax. Many substances have been proposed as metabolic vasodilators such as lactic acid, hydrogen ions, potassium ions, phosphate ions, adenosine, and prostaglandins.

2. The Role of Lipids in the Modulation of Vascular Control

Low density lipoprotein (LDL) is the main source and carrier of blood cholesterol (Brown and Goldstein 1976). In hyperlipidemic patients, plasma levels of LDL are associated with the occurrence of atherosclerosis (Farmer and Gotto 1992) and decreased endothelial function in arteries (Drexler and Zeiher 1991, Zeiher et al 1991). The proposed mechanism states monocytes adhere to the endothelium and migrate to the subendothelial space to become macrophages (Gerrity 1981a). They take up LDL very quickly and become transformed into foam cells . LDL must first be modified (eg. oxidized) before it can be taken up by macrophages.

One of the properties of oxidized LDL is that it impairs endothelium dependent relaxation (Parthasarathy et al 1992). The elevated level of lysophosphatidylcholine (LPC) in oxidatively modified LDL has been shown to be the biochemical factor responsible for the impairment of endothelium-dependent relaxation in aortic ring preparations (Liu et al 1994, Yokoyama et al 1990). There is a five fold increase in the LPC contained in oxidized LDL compared with native LDL upon in vitro oxidation with $CuSO_4$ at 37°C (Liu et al 1994). There is a corresponding decrease in phosphatidylcholine (PC) suggesting a conversion of PC into LPC by phospholipase A_2 (PLA_2) upon oxidation (Parthasarathy and Barnett 1990). PLA_2 hydrolyzes the ester bond which holds the fatty acid to the carbon of the glycerol at position 2. If oxidation is repeated at 20°C, less LPC is produced and impairment of endothelium dependent relaxation is not as severe (Liu et al

1994). Liu et al (1994) have demonstrated that impairment of endothelium dependent relaxation occurs in direct proportion to the amount of LPC produced in LDL during oxidation.

It has been demonstrated that vasodilators such as nitroglycerin, which act directly on the smooth muscle cells, are not inhibited by LPC (Kugiyama et al 1990). But vasodilators such as Ach which are regulated by G proteins (endothelial), are inhibited by LPC. Therefore, LPC likely perturbs the endothelial G protein pathway, reducing the production of NO and relaxation of the smooth muscle.

3. Epidemiology of Coronary Heart Disease

A brief discussion of atherosclerosis will clearly identify the need to examine the effects of lipids on vascular tone. Coronary heart disease in the form of heart attacks or coronary artery disease is the major cause of death in North America accounting for over half a million deaths annually (Monro and Cotran 1988). It is now well established that hypercholesterolemia is an important cause of coronary heart disease and that the lowering of elevated plasma cholesterol levels is therapeutic (Steinberg et al 1985).

One of the most important epidemiological studies is the Framingham Heart Study which began in 1949 (Kannel et al 1979; Anderson et al 1987). From 1951 to 1955, serum cholesterol levels were measured in 1959 men and 2415 women who were free of cardiovascular disease. A relationship between the concentration of serum cholesterol and

coronary heart disease was established in men under the age of 50 (fig. 2; Kannel et al 1971). The chance of developing CHD was 5.5 times greater if total serum cholesterol was above 260 mg/dL than if it was below 220 mg/dL. A follow up to the Framingham study concluded that the higher the level of cholesterol, the lower the probability of surviving 30 years (Anderson et al 1987). For men between 30 and 49 years of age, there was a relationship between CHD and low density lipoprotein (LDL) (fig.3; Kannel et al 1979).

The role of LDL in atherosclerosis can be easily demonstrated in a genetic disorder known as familial hypercholesterolemia. This disease is transmitted as an autosomal dominant trait that reduces the body's ability to produce functional LDL receptors. The result is that the liver can not bind, internalize, and degrade LDL as efficiently as a person with no genetic defect. If an individual is heterozygous, they will have one mutant gene and their plasma LDL levels will be twice the normal levels. They often have their first heart attack before they are 35 years old. If an individual is homozygous, plasma LDL levels will be six times higher than normal. Heart attacks can first occur as early as two years of age and almost always before 20 years of age (Brown and Goldstein 1984). This disease illustrates a causal relation between elevated plasma LDL and atherosclerosis.

HDL serum concentration is inversely correlated with total serum cholesterol (Miller and Miller 1975) in the body. It is involved in the removal of cholesterol from tissues by transporting it back to the liver for excretion. HDL competes with LDL

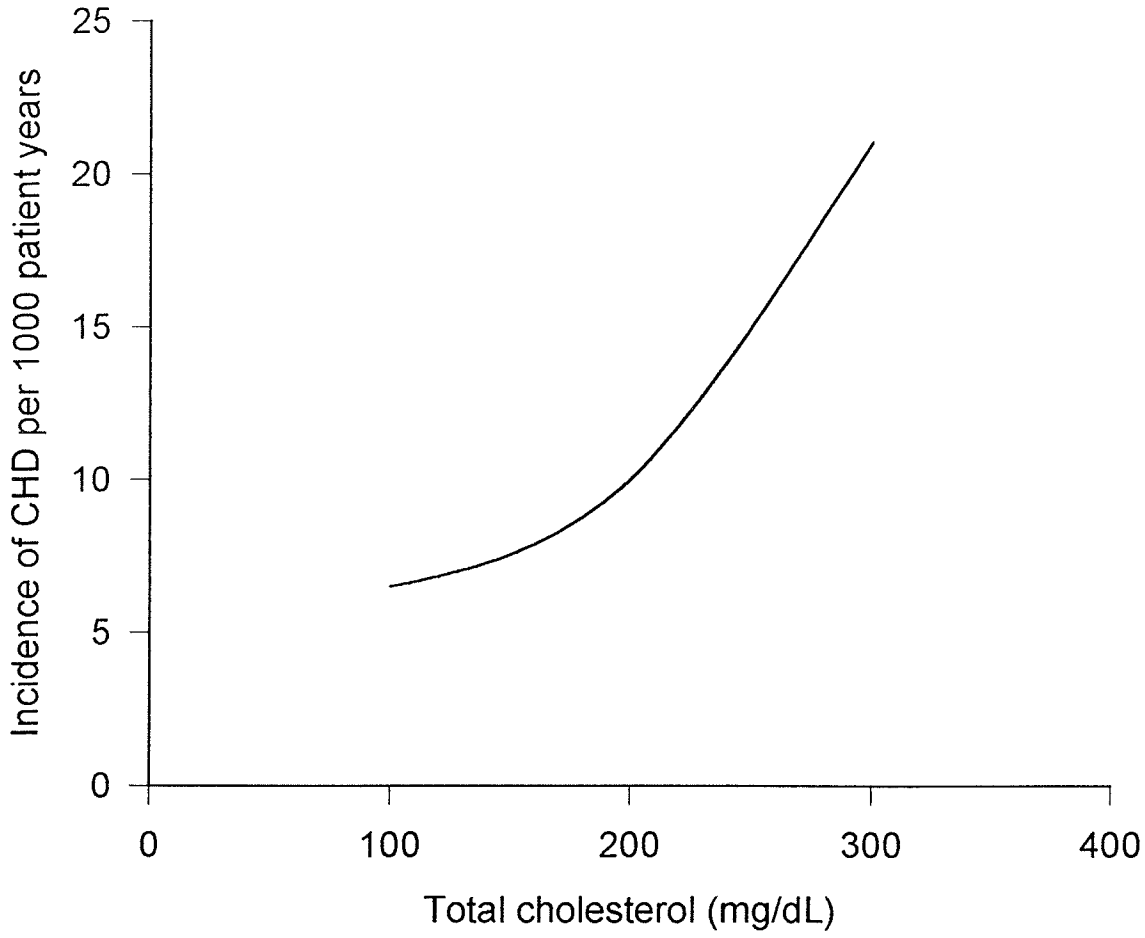

Figure 2. Relationship between total cholesterol and incidence of coronary heart disease (from Kannel et al 1971)

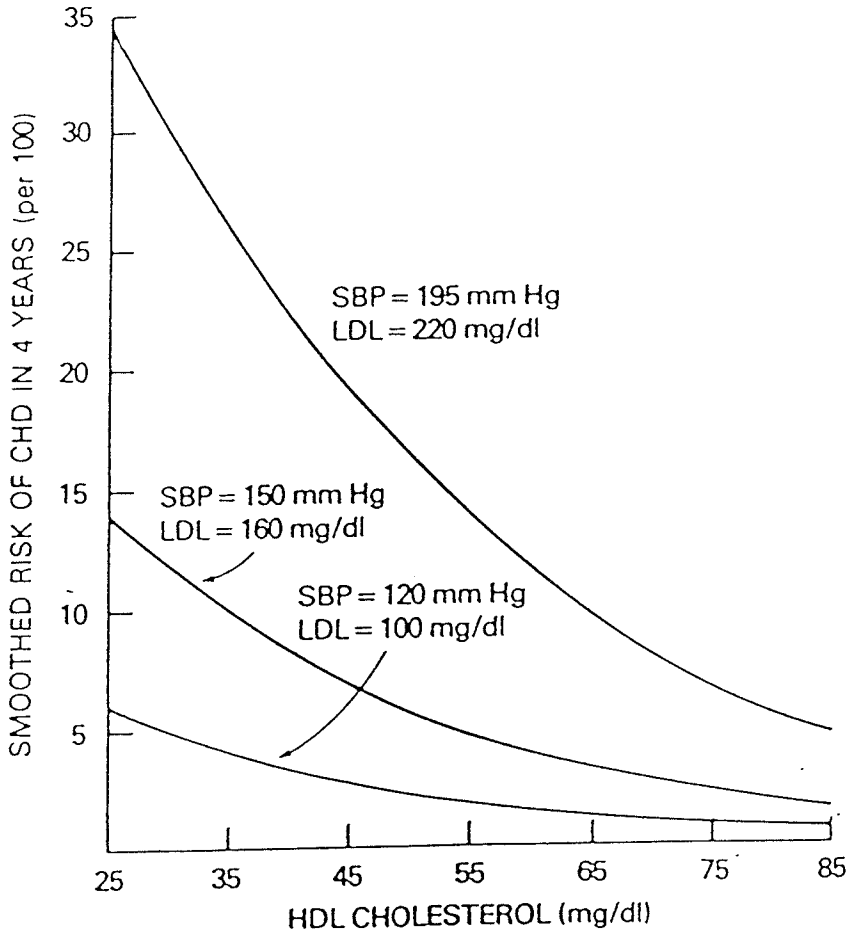

Figure 3. Risk of CHD in men aged 55 according to levels of HDL and LDL cholesterol from the Framingham Study. CHD = coronary heart disease, SBP = systolic blood pressure, LDL = low density lipoprotein, HDL = high density lipoprotein (from Kannel et al 1979).

for binding sites, thus interfering with the cellular uptake of LDL (Carew et al 1976, Stein et al 1976). HDL also possesses antioxidant properties (Mackness et al 1993). In figure 3 we can see that the higher the level of high density lipoprotein (HDL), the lower the risk of CHD.

4. Risk factors for Atherosclerosis

It has become clear that the alleviation of certain risk factors can lead to a reduction in mortality due to atherosclerosis (Glueck 1986). There is also evidence from treatment of hypercholesterolemia that regression of an atherosclerotic lesion may occur if the conditions that favor its development are removed (Blankenhorn 1978, Malinow 1981). Therefore, it would be very important to identify the risk factors so they may be reduced if possible (Table 1).

Hypercholesterolemia has been positively identified as a risk factor of coronary heart disease and is potentially reversible. There is a direct relationship between the serum level of cholesterol and the development of atherosclerosis (Fig. 2) (Rose and Shipley 1986). If the lipid profile is examined in more detail, there is also a positive correlation between the serum level of LDL and the risk of coronary heart disease (Fig. 3) (Freedman et al 1986). LDL concentrations are age dependent and rise significantly during the first year of life to remain at a constant level for approximately 20 years (Berenson et al 1979).

Table 1. Risk factors for atherosclerosis (adapted from Evans and Taylor 1988)

Unmodifiable	Age
	Male sex
	Family History
	Homocystinuria
Partially / Potentially Correctable	Hypercholesterolemia
	Hypertriglyceridemia
	Low HDL level
	Hypertension
	Diabetes mellitus
Totally Correctable	Smoking
	Obesity
	Physical activity

The serum levels of LDL will rise again between the ages of 20-30. Body weight, which can be modified, may affect the progression of LDL levels since races who do not become obese do not experience this rise (Evans and Taylor 1988).

Hypertension is another risk factor of atherosclerosis that is potentially reversible. There is a direct relationship between the increase in blood pressure and the increased risk of atherosclerosis (Lewis 1984). The risk may be reduced by antihypertensive treatment.

Hyperglycemia, a result of diabetes mellitus is also associated with premature atherosclerosis (Evans and Taylor 1988). It should be noted that the risk of atherosclerosis does not increase with the severity of the disease. With treatment, this risk factor is potentially reversible.

There are several risk factors that are totally reversible such as smoking, obesity, and lack of physical activity. The risk of coronary heart disease is three times higher in smokers and this risk increases proportionally with the amount smoked (Lewis 1984). Physical activity has a protective effect which may work through several mechanisms. Regular exercise reduces body weight which is beneficial because obesity is a risk factor of atherosclerosis (Evans and Taylor 1988). Prolonged training also elevates the serum levels of HDL, which is protective against coronary heart disease, and lowers total cholesterol, triglyceride, and LDL levels. Physical activity also has a beneficial effect in that it lowers blood pressure.

5. Pathogenesis of Atherosclerosis

5.1 Structure of the artery

Before atherogenesis is examined, it may be beneficial to recall the structure of a normal artery. The artery consists of three major layers which are the intima, media, and adventitia (Fig. 4). The intima is the innermost layer and is bound on the luminal side by a single continuous layer of endothelial cells. The outer edge is bound by the internal elastic lamina. The media, or middle layer consists entirely of smooth muscle cells surrounded by collagen, elastic fibers, and proteoglycans. The adventitia , or outer most layer consists of smooth muscle cells and fibroblasts mixed in loosely with collagen and proteoglycans. The external elastic lamina, which is a continuous sheet, separates the adventitia from the media (Ross and Glomset 1976).

5.2 The lesions of atherogenesis

Atherosclerosis involves muscular arteries such as the coronary, carotid, basilar, vertebral, aortic, iliac, and the femoral (Munro and Cotran 1988). The earliest lesion is the fatty streak and is commonly found in children before the age of 10 (Stary 1983). The fatty streak is a slightly raised yellow area which consists of subendothelial aggregates of foam cells. Foam cells are filled with lipids, mostly cholesteryl esters and free cholesterol. Studies involving monoclonal antibodies have identified that the majority of foam cells in

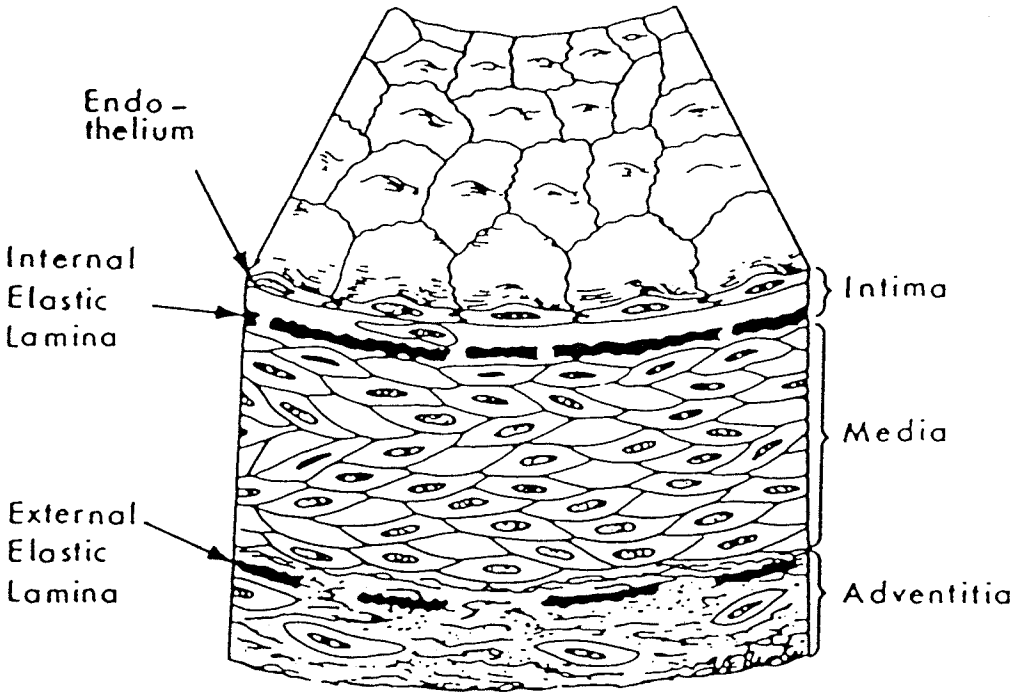

Figure 4. Structure of the artery (from Ross 1992).

the fatty streak are macrophages and some smooth muscle cells (Aqel et al 1985, Vedeler et al 1984). The presence of T lymphocytes has also been determined (Munro et al 1987).

The fibrous plaque is representative of advanced atherosclerosis. It causes narrowing of the artery, predisposes to thrombosis, calcifies, and leads to weakening of the muscle (Munro and Cotran 1988). These lesions are off-white, raised, and approximately one cm in diameter. They consist of a fibrous cap composed mostly of smooth muscles, leukocytes, and connective tissue. Beneath the cap is a cellular area consisting of macrophages, smooth muscle cells, and T lymphocytes (Jonasson 1986). Below these cells there may be an area of necrotic debris, cholesteryl esters, calcification, and foam cells (Ross 1986). The foam cells are of both macrophage and smooth muscle cell origin.

The relationship between fatty streaks and fibrous plaques is controversial. Fatty streaks cover approximately 10% of aortic intimal surface by the age of ten. But this area includes sites not particularly susceptible to fibrous plaques (McGill 1968). Another observation against a relationship between fatty streaks and fibrous plaques is that fatty streaks are present in individuals in countries where fibrous plaques are rare (Munro and Cotran 1988). On the other hand, fatty streaks do occur in the coronary artery at the same sites which are susceptible to fibrous plaques later in life (Stary 1987). In fact, it has been demonstrated that fatty streaks increase the area they occupy in coronary arteries prior to the development of fibrous plaques, (McGill 1984) which lends further support to

the relationship between the two lesions. It appears that fatty streaks are a universal occurrence which can disappear or simply remain harmless. In certain locations they can evolve into fibrous plaques , especially in predisposed individuals (Munro and Cotran 1988).

5.3 The response to endothelial injury hypothesis

The original endothelial injury hypothesis proposes that the initiating event in atherogenesis is the loss of endothelial cells (Ross and Glomset 1976). This hypothesis was based on two experimental observations. The first is that deliberate denudation of the endothelial cells can evoke an arterial lesion (Stemerman and Ross 1972, Moore 1973). The second is that platelets aggregating on a denuded arterial surface release platelet derived growth factor (PDGF) (Ross et al 1974). The injury may occur by mechanical, chemical, toxic, viral, and immunological agents resulting in denudation which will lead to platelet adhesion, aggregation, and the release of PDGF. PDGF causes smooth muscle cells to migrate into the intima and proliferate. It also stimulates the secretion of connective tissue. Repeated injury may result in the development of atherosclerotic plaque (Ross and Glomset 1976).

Since 1976, it has been determined that actual denudation is not always the first event of atherosclerosis that is initiated by hypercholesterolemia (Davies et al 1976) and that platelet adhesion is not sufficient to cause atherosclerotic lesions (Schwartz and Reidy

1987). As a result the hypothesis has had to be modified in the following ways. First, endothelial injury can include non-denuding alterations of permeability and the release of vasoactive substances and growth factors. Second, platelets are not the only sources of growth factors. Macrophages have been found to synthesize and secrete PDGF, FGF, EGF-like factor, and TGF beta (a growth inhibitor) (Ross 1992). Therefore, there are three possible sources of growth factors which are platelets, endothelial cells, and macrophages. Third, foam cells of the fatty streak which were once believed to be of smooth muscle cell origin are now recognized to be of monocyte/macrophage origin (Fowler et al 1979).

5.4 The role of low density lipoprotein (LDL) in atherosclerosis

Low density lipoprotein (LDL) is the main source and carrier of blood cholesterol (Brown and Goldstein 1976). In hyperlipidemic patients, plasma levels of LDL are associated with the occurrence of atherosclerosis (Farmer and Gotto 1992) and decreased endothelial function in arteries (Drexler and Zeiher 1991, Zeiher et al 1991).

One of the earliest events of atherogenesis is the increased adherence of circulating monocytes to the endothelium of the artery (Gerrity 1981a). These monocytes then migrate through the endothelium into the intima and transform into macrophages and foam cells. The migration of monocytes through the endothelium has been observed by

electron microscopy (Gerrity 1981a). As a result, the majority of foam cells in the fatty streak originate from circulating monocytes (Aqel et al 1984).

Monocytes contain virtually no lipids as they circulate but they do become heavily loaded with lipids in the subendothelial space (Steinberg and Witztum 1990). It is generally accepted that foam cells are derived from monocytes and macrophages engorged with LDL, resulting in atherosclerotic lesions. The LDL particle is internalized by most cells through the LDL receptor (Goldstein and Brown 1977). The LDL receptor can not account for the increased content of LDL inside the foam cell because as cholesterol content in the cell increases, the cellular need for cholesterol decreases and there is a down-regulation of LDL receptors (fig. 5). The latter should protect the cell from accumulating too much cholesterol and prevent its transformation into a foam cell. In fact, macrophage foam cells do not express LDL receptors (Yla-Herttuala et al 1991). Also, Watanabe rabbits and patients with familial hypercholesterolemia who lack functional LDL receptors still develop macrophage derived foam cells (Steinberg et al 1989). It would appear that the LDL receptor is not responsible for the enhanced uptake of LDL and the formation of foam cells.

Studies on the uptake of LDL in macrophages have demonstrated that plasma LDL must be modified prior to uptake by what is called the scavenger receptor pathway. Examples of such modification include the acetylation of LDL (Goldstein et al 1979), glycation of LDL apo-B (an increase in plasma sugar levels results in increased glycation

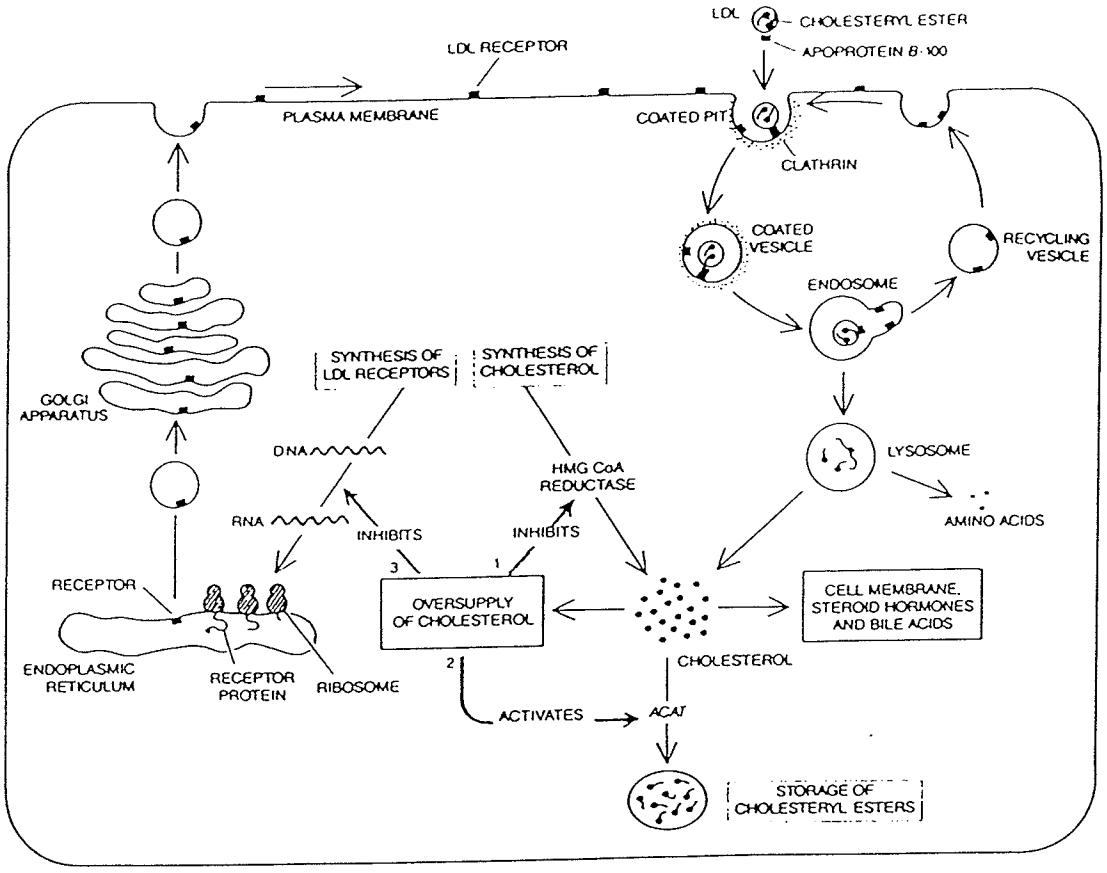

Figure 5. The regulation of cholesterol within a cell. As the level of cholesterol inside a cell increases, there is (1) a reduction in LDL receptor synthesis, (2) a reduction in cholesterol synthesis, and (3) an increase in storage of cholesteryl esters (from Brown and Goldstein 1984).

of LDL, this may be why diabetics have an increased risk of atherosclerosis) (Witztum et al 1982), the in vitro oxidation of LDL in the presence of transitional metals (Heinecke et al 1984), and other modifications of LDL by cell-mediated biochemical processes (Henricksen et al 1981, Morel et al 1984, Parthasarathy et al 1986). It seems that any modification of LDL which binds and masks lysine amino groups on apo-B of the LDL resulting in a negative charge will be taken up by the scavenger LDL receptor (Parthasarathy et al 1992). The presence of oxidized LDL in atherosclerotic lesions (Palinski et al 1990, Yla-Herttuala et al 1989) supports the notion that LDL oxidation is indeed an initial step in the development of atherosclerosis (Steinberg et al 1989).

Oxidative modification involves the peroxidation of phospholipids, followed by activation of the intrinsic phospholipase A_2 in LDL and the production of lysophospholipids (Steinbrecher et al 1984, Parthasarathy and Barnett 1990). LDL can be oxidativley modified when incubated with any of the principal cell types in the arterial wall (i.e. endothelial cells, vascular smooth muscle cells, macrophages, and fibroblasts) (Morel et al 1984, Parthasarathy et al 1986, Witztum 1993). Oxidation of LDL does not occur in the plasma because of the presence of anti-oxidants. The cells of the arterial wall may simply provide a micro environment free of anti-oxidants resulting in the oxidation of LDL (Steinberg et al 1989).

LDL can also be modified by some transitional metals such as copper and iron, which produce biochemical and functional alterations of LDL which are similar to those

produced by cells, and are commonly used to study the functional effects of lipid oxidation (Steinbrecher et al 1984). The effects of oxidation include the loss of endogenous antioxidants, changes in electrophoretic mobility of LDL, increases in lipid peroxides and the formation of lysophosphatidylcholine (LPC) (Esterbauer et al 1987, Parthasarathy et al 1985).

The oxidatively modified LDL has been shown to affect cellular functions associated with the regulation of vascular tone (Liao et al 1995, Kugiyama et al 1990), activation of inflammatory responses (Witztum and Steinberg 1991, Murohara et al 1994, Quinn et al 1988), and platelet aggregation (Aviram 1989, Kugiyama et al 1993). Table 2 lists the atherogenic properties of oxidized LDL. Although lipid peroxidation products (thiobarbituric acid reactive substances, TBARS) are often used to quantify the degree of oxidation (Ohkawa et al 1979), such analysis does not assess the effect of the change on its atherogenic properties (Liu et al 1994). A bioassay which has proven useful in the latter context is a measurement of the effect of oxidized LDL on endothelium-dependent relaxation in aortic rings (Liu et al 1994). The elevated level of LPC in oxidatively modified LDL has been shown to be the biochemical factor responsible for the impairment of endothelium-dependent relaxation in aortic ring preparations (Liu et al 1994, Yokoyama et al 1990).

It should be noted that LPC in LDL is not a single homogenous entity, but a combination of molecular species with different acyl groups. The level of LPC is normally

Table 2. Atherogenic effects of oxidized LDL (Ox-LDL) (Parthasarathy et al 1992)

1. Ox-LDL is rapidly taken up by macrophages.
2. Ox-LDL is chemotactic for monocytes and inhibits macrophage mobility.

   (Attributed to production of LPC)
3. Ox-LDL is cytotoxic.
4. Ox-LDL increases expression of adhesion molecules at the endothelial surface.
5. Ox-LDL inhibits endothelium-dependent relaxing factor (nitric oxide).
6. Ox-LDL increase binding to type I collagen.
7. Ox-LDL adversely affects coagulation pathways.
8. Ox-LDL is immunogenic.

low in native LDL, but becomes significantly higher in oxidized LDL (Quinn et al 1988, Liu et al 1994). If LPC is an important factor for the production of atherosclerotic lesions, its level and/or composition must be altered in the LDL of hyperlipidemic patients after oxidative modification.

Until now, we have only discussed the fatty streak, which can develop under an intact layer of endothelial cells (Steinberg et Witzum 1990). This does not agree with the original response-to-injury hypothesis discussed earlier, which postulates endothelial denudation as the first step of atherogenesis. The original response to injury hypothesis can be linked to the more recent lipid infiltration hypothesis (Fig. 6). As LDL levels increase, so do the levels of oxidized LDL, which is a chemoattractant of monocytes. Once monocytes adhere and migrate to the subendothelial space of an intact endothelium (lipid infiltration), they take up oxidized-LDL at an accelerated rate through the scavenger receptor pathway to become foam cells and the fatty streak. This accumulation of foam cells beneath the endothelium contains large amounts of oxidized LDL which is cytotoxic. These cytotoxic factors (superoxide anions, proteolytic enzymes, and lipolytic enzymes) can damage and cause the loss of the overlying endothelial cells (Steinberg and Witztum 1990). It has been observed that endothelial cells do lift off from populations of foam cells by electron microscopy (Gerrity 1981b). Therefore, the progression of the fatty streak to the fibrous plaque may be initiated by the loss of endothelial cells. At this point, the original response to injury hypothesis is relevant (Steinberg and Witztum 1990) (Fig. 6).

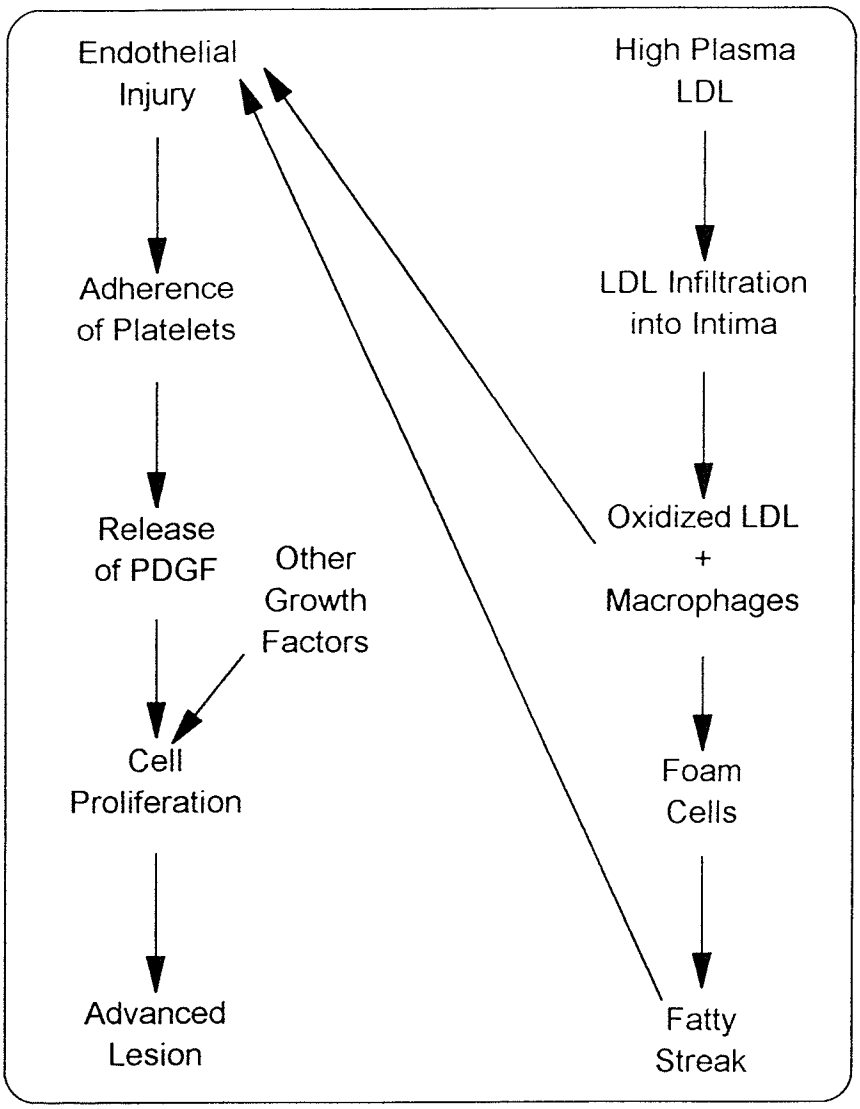

Figure 6. Postulated linkage between the lipid-infiltration hypothesis and the response to endothelial injury hypothesis. The lipid-infiltration hypothesis may account for fatty streaks, and the response to injury hypothesis may account for the progression to more advanced lesions (from Steinberg et al 1989).

Platelets adhere to the arterial wall and release PDGF causing smooth muscle cells to proliferate and the secretion of connective tissue which produces an advanced fibrous plaque.

6. Antioxidant Therapy

Antioxidants are thought to prevent atherosclerosis by blocking the oxidative modification of LDL which promotes the disease. There is epidemiological evidence that antioxidants may reduce atherosclerosis, but only a small number of intervention trials have been completed.

A group of 34 486 postmenopausal women (Kushi et al 1996) and 39 910 men (Rimm et al 1993) completed a questionnaire about their daily intakes of vitamins A, C, E, and beta carotene. Follow up analysis lasted for the next seven years. In both studies, the intake of vitamins A, C, and beta carotene were not associated with coronary heart disease (CHD). But, vitamin E had an inverse relationship with CHD. These studies demonstrated an inverse relationship but not a causal relationship between vitamin E intake and CHD.

In the Cambridge Heart Antioxidant Study (CHAOS), subjects in the experimental group were given vitamin E supplements while the control group received a placebo (Stephens et al 1996). They found that vitamin E significantly reduced non-fatal myocardial infarctions but the effect on death by cardiovascular disease requires further

study. In a trial where 22 071 men (Hennekens et al 1996) were given beta-carotene supplementation, there was no benefit in terms of cardiovascular disease.

Another study in Sweden (Walldius et al 1994) examined the effect of probucol supplementation to reduce atherosclerotic plaque. Probucol is a drug that lowers both LDL and HDL marginally. This drug may also inhibit atherogenesis by a mechanism other than lowering blood lipids. It has antioxidant properties which may protect lipoproteins from oxidation and prevent the formation of foam cells. It has been effective in reducing the progression of macrophage rich fatty streak lesions in Watanabe rabbits deficient of LDL receptors (Kita et al 1987, Carew et al 1987). But in the human intervention study (Walldius et al 1994), probucol did not prevent the lesions from progressing.

In summary, it appears that probucol can reduce atherosclerotic plaque in animal models (Watanabe rabbits), but vitamin E can not. While in human models, vitamin E appears to reduce coronary heart disease but vitamins A, C, beta carotene, or the drug probucol are ineffective for this purpose.

The notion that antioxidant administration might provide protection against coronary artery disease is very exciting but not yet proven. This can only be accomplished by long term intervention studies.

7. Objective

It was first determined by Furchgott and Zawadzki (1980) that the endothelium of a rabbit aortic ring preparation must be intact for endothelium-dependent relaxation (EDR) to occur in response to acetylcholine. This astounding discovery opened up an entirely new area of research which examines the control of vascular tone by the endothelium. If the arterial endothelium is denuded, the vasodilator response to acetylcholine is abolished. Acetylcholine acts on muscarinic receptors of the endothelial cells, stimulating the release of the endothelium-dependent relaxing factor (EDRF), which has been identified as nitric oxide (NO) by Palmer et al (1987). Since 1980, it has been determined that the endothelium releases many other substances which stimulate vasodilation (eg. nitric oxide, EDHF, prostacyclin, EDIF) or vasoconstriction (eg. EDCF, endothelin).

The oxidation of low density lipoprotein (LDL) plays an important role in the initiation and progression of atherosclerosis, one of its effects being the inhibition of EDR (Kugiyama et al 1990, Liu et al 1994). The elevated level of lysophosphatidylcholine (LPC) in oxidatively modified LDL has been shown to be the biochemical factor responsible for the impairment of endothelium-dependent relaxation in aortic ring preparations (Liu et al 1994, Yokoyama et al 1990).

It has been demonstrated that vasodilators such as nitroglycerin, which act directly on the smooth muscle cells, are not inhibited by LPC (Kugiyama et al 1990). Vasodilators

such as Ach, however, which are regulated by G proteins (endothelial), are inhibited by LPC. Therefore, LPC likely perturbs the endothelial G protein pathway, reducing the production of NO and the subsequent signal to the smooth muscle.

The present research will attempt to determine how oxidized LDL and LPC affect the endothelium, inhibiting its function to effect endothelium-dependent relaxation. Specifically, LPC may be altering the fluidity of the endothelial membrane altering the G-protein pathway. This in turn effects the production of vasodilators such as EDNO, EDHF, and prostacyclin. The role of these three vasodilators and the involvement of the enzyme PKC in LPC-induced EDR impairment will be examined. These studies are also extended to examine variations of the lipid profile and LPC species between men and women or normal and hyperlipidemic subjects.

II. Materials and Methods

1. Materials

Phenylephrine hydrochloride was obtained from the pharmacy of the Health Sciences Centre, Winnipeg. Reagents for lipid phosphorus determination, EDTA and acetylcholine were purchased from the Sigma Company, St Louis, MO. Molecular species of LPC and all lipid standards were obtained from Serdary Company, London, Ontario. L-lyso-3-phosphatidylcholine, 1-[1-^{14}C]palmitoyl (56 mCi/mmol) was obtained from Amersham (Oakville, ON). Ro31-8220 was a generous gift from Roche Research Center (Welwyn Garden City, Hertfordshire). NCS tissue solubilizer for liquid scintillation counting was obtained from Amersham (Arlington Heights, IL). N-nitro-L-arginine methyl ester was obtained from Sigma Chemical Co. (St. Louis, MO). Thin-layer chromatographic plates (G-25) were the product of Macherey-Nagel and purchased through Brinkman Instruments, Rexdale. BF_3 methanol (12% w/w) was obtained from Supelco Canada Ltd., Oakville, Ontario. All other chemicals were of reagent grade and were obtained through the Canlab Division of Travenol Canada Inc., Mississauga, Ontario. Mature male Sprague-Dawley rats, 250 ± 50 g were obtained from Charles River Canada Inc., St. Constante, Quebec.

Medium 199 with Hank's salt and L-glutamine, heat inactivated fetal calf serum, and other standard culture reagents were obtained from Gibco (Burlington, ON). Type I

collagenase was obtained from Worthington Biochemical Corp. (Freehold, NJ). Endothelial cell growth supplement (ECGS) was obtained from Collaborative Biomedical Products (Bedford, MA). [5,6,8,11,12,14,15-^{3}H(N)]arachidonic acid (230.5 Ci/mmol) was obtained from Dupont NEN (Boston, MA), 1-stearoyl-2-[1-^{14}C]arachidonoyl-L-3-phosphatidylcholine (55 mCi/mmol) was obtained from Amersham (Oakville, ON). Thin layer chromatographic plates (silica gel G) were products of Fisher Scientific Co. (Edmonton, AB). All glassware used for cell culture was silanized before use.

2. Isolation of LDL and Preparation of Oxidatively Modified LDL

Blood samples were obtained after informed consent from healthy volunteers (total cholesterol < 5.2 mmol/l) and hyperlipidemic patients (total cholesterol > 6.2 mmol/l, enrolled at the Lipid Clinic, Health Sciences Center, Winnipeg). The blood was collected in EDTA containing tubes (4.0 mM), after subjects had fasted overnight. Concentrations of total serum cholesterol, high density lipoprotein (HDL) cholesterol, and triglyceride were determined enzymatically with commercial test kits (Boehringer Mannheim). LDL cholesterol was calculated by the Friedewald equation (Friedewald et al 1972). LDL (density = 1.019-1.063 g $\cdot$ ml^{-1}) was isolated from freshly prepared plasma by sequential ultracentrifugation (Havel et al 1955). EDTA (0.3 mM) was placed in buffers in order to prevent the auto-oxidation of the lipoproteins. The LDL sample was dialysed for 24 h at 4° C against three changes of phosphate buffered saline (composition: NaCl 140 mM, KCl

3 mM, Na_2HPO_4 8 mM, KH_2PO_4 2 mM) prior to use. The LDL samples were used separately for the various experiments described below instead of being pooled. Thus these results represent truly independent experiments (whereas the use of pool samples would represent replications of the same experiment).

The LDL was dialysed against an EDTA-free phosphate buffered saline prior to oxidative modification. Modification of LDL was initiated by the addition of $CuSO_4$, a condition known to produce modification of LDL similar to that produced by endothelial cells (Steinbrecher et al 1984) and was the preferred procedure in most recent studies. Aliquots of LDL samples (containing 1.0 mg LDL protein/ml) were treated with 5 μM $CuSO_4$ at 37°C for 24 h with constant agitation to initiate oxidation. The duration of incubation and the concentration of the $CuSO_4$ used in this study have been optimized previously (Liu et al 1994). The vials containing the sample were not capped tightly so that there was no oxygen limitation for the oxidation process. The oxidation of LDL was terminated by the addition of EDTA (0.6 mM) followed by dialysis for 24 h with four changes of Krebs-Henseleit buffer (composition in mmol · $litre^{-1}$: NaCl 120, KCl 4.76, $MgCl_2$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25.0, NaH_2PO_4 1.18, and glucose 5.5). The dialysis buffer was saturated with N_2 and 0.3 mM EDTA was added to prevent further oxidation of the LDL sample. These samples were referred to as oxidized LDL. For comparison, paired LDL samples were processed in an identical manner, but in the absence of $CuSO_4$.

These LDL samples were referred to as native LDL. LDL samples were stored at 4° C under N_2, and used within two weeks of preparation.

3. Biochemical Analysis of Native and Oxidized LDL

Proteins in the LDL samples were determined by the method of Lowry (Lowry et al 1951). Lipids in the LDL samples were extracted by a chloroform/ methanol mixture (2:1, v/v) and total lipid phosphorous were determined by the method of Bartlett (1959) and Zhou and Arthur (1992). The lipid fraction was separated by thin layer chromatography using a solvent system of chloroform/methanol/acetic acid/water (70:30:2:4, v/v). The phospholipid fractions on the thin layer chromatography plate were visualized by iodine vapor, and the lipid phosphorus content in each fraction was determined (Bartlett 1959, Zhou and Arthur 1992).

Lipid peroxidation was determined by measuring the amount of thiobarbituric acid reactive substances (TBARS) according to the procedure of Ohkawa et al (1979) and tetramethoxypropane was used as a standard. The results are expressed as nmol malondialdehyde (MDA) equivalent per mg LDL protein.

4. Determination of Acyl Groups in LPC

The acyl content in the LPC sample was determined by gas-liquid chromatography. The acyl groups in the sample were converted into methyl esters in the presence of boron

trifluoride (Tardi et al 1992). The solvent in the sample was evaporated under a stream of nitrogen, and 0.5 ml of BF_3 reagent was added to the dried sample. Methylation of the sample took place in a screw-capped tube which was heated at 96° C for 5 min. Subsequent to heating, 0.5 ml water and 1.5 ml petroleum ether were added to the reaction mixture. The methyl esters formed were recovered in the petroleum ether phase and the efficiency of the methylation was over 95 %. The methyl esters were analyzed by a Shimadzu Mini-2 gas-liquid chromatograph (Tekscience; Oakville, Ontario, Canada) equipped with 15 % DEGS (on 80/100 Chromosorb W/AW) columns. Heptadecanoic acid methyl ester was used as a standard for quantitation. The data obtained were analyzed by a Shimadzu Chromatopac CR601 integrator.

5. Loading of LDL With LPC

Native LDL was incubated with 0.5 mM 1-lauroyl-sn-glycero-3-phosphocholine ($C_{12:0}$-LPC) or 1-palmitoyl-sn-glycero-3-phosphocholine ($C_{16:0}$-LPC) for 24 h at room temperature. The LDL was then dialyzed for 24 h with three changes of buffer to remove the free LPC in the solution as described in a previous report from this laboratory (Liu et al 1994). The amount of LPC incorporated into the LDL sample was determined prior to use.

6. Preparation of LPC-Depleted LDL

LDL was first isolated by sequential ultracentrifugation and then oxidatively modified by incubation with $CuSO_4$ as described above. Native LDL was incubated in a $CuSO_4$-free mixture but otherwise treated the same as oxidized LDL. Both native LDL and oxidized LDL were then divided into 2 parts. The first part was incubated for 2 h at 37 °C with 100 fold excess of fatty acid free albumin, an acceptor for hydrophilic lipids (i.e., 0.01 mg LDL protein : 1 mg albumin) (Murohara et al 1994). The oxidized LDL was recovered from the supernatant by sequential ultracentrifugation for 17 h at 15°C (density = 1.21). The second part remained the control receiving no albumin.

All LDL samples were then dialyzed for 48 h with four changes of PBS containing 30 mM EDTA, for 24 h with three changes of PBS without EDTA, and for 24 h with three changes of Krebs-Henseleit buffer before use in the organ chamber experiments.

7. Assessment of Endothelium Dependent Relaxation in Aortic Rings

These experiments were performed using Sprague-Dawley rats (250-350 g). The protocol was reviewed and approved by the University of Manitoba animal care committee, and the animals were cared for in accordance to the guidelines of the Canadian Council on Animal Care (Ottawa, Canada). After intraperitoneal administration of an anticoagulant and sedative (800 units of heparin and 0.65 mg diazepam), rats were killed by cervical dislocation. The thoracic aorta was removed and each aortic ring (3 mm in

length) was suspended isometrically between two horizontal steel wires in an organ bath. A resting tension of 2.0 g was applied to the aortic ring and changes in tension were measured with a Grass Ft.03 force-displacement transducer. Due to the visco\plastic\elastic nature of blood vessels, the aortic rings required an equilibration period of 90 min., which involved a series of tension adjustments until the resting tension remained stable at 2.0 g. The organ bath contained 10 ml of Krebs-Henseleit solution which was maintained at 37° C saturated with a 95 % O_2 - 5 % CO_2 gas mixture. After an equilibration period of 90 min, the aortic ring was precontracted with phenylephrine (10^{-6} M). When the contraction had become stable, acetylcholine (10^{-8}-10^{-5} M) was added cumulatively to produce endothelium dependent relaxation. The relaxation produced by acetylcholine was expressed as a percentage of the phenylephrine contraction. Criteria for acceptability of the endothelium/vascular ring preparation included active contraction of 1.5 g and acetylcholine induced relaxation of 80 %. After the washout of phenylephrine and acetylcholine, the preparation was incubated with oxidized LDL (0.3 mg LDL protein · ml^{-1}) for 1 h. The oxidized LDL was removed from the bath prior to the determination of contraction by phenylephrine and relaxation by acetylcholine. The removal of LDL from the bath was necessary since the presence of LDL has been shown to inactivate the endothelium derived relaxing factor (Galle et al 1991). For comparison, native LDL (0.3 mg LDL protein · ml^{-1}) and/or no addition were

used in separate experiments during the incubation period prior to the determination of contraction by phenylephrine and relaxation by acetylcholine.

8. Protein Determination

The protein content in each fraction was determined by the modified method of Lowry et al (1951). Standards containing 0, 10, 20, 30, 50, and 100 μg of bovine serum albumin were prepared as standards. NaOH and solution E (containing 263.47 μM $CuSO_4$, 466.23 μM Na-K tartrate, 328.95 μM NaOH, 106.11 mM Na_2CO_3) were added to the sample/standard. The reaction was allowed to proceed for 10 minutes at room temperature and then 0.5 ml of 1 M Folin's reagent was added. The mixture was incubated for 20 min at 37° C, allowed to cool for 30 min and the absorbence was determined at 650 nm.

9. Tissue Solubilization

The aortic ring was placed in a scintillation counting vial and moistened to speed up the digestion process. NCS solubilizer for liquid scintillation counting was added in the ratio 6ml/g dry tissue sample. The vial was capped and heated at 37° C in a water bath under constant agitation for 16 h. The reaction was stopped by the addition of 30 μl glacial acetic acid per ml of NCS and allowed to cool. The radioactivity was determined by liquid scintillation counting.

10. Culture Medium

Medium 199 (pH 7.4) was supplemented with heparin (90 μg/ml), HEPES (25 nM), gentamicin sulfate (40 μg/ml), sodium penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml), fungizone (2.5 mg amphotericin B / ml) and heat-inactivated fetal bovine serum (10%). The endothelial cell growth supplement (30 μg/ml) was added into the culture dishes after each feeding.

The stock medium 199 (pH 7.4) containing heparin, HEPES and gentamicin sulfate was prepared in large quantity and stored after being sterilized by filtering through a 0.22 μm filter purchased from Millipore (Bedford, MA).

The culture medium supplemented with antibiotic/antimycotic and fetal bovine serum was used within two weeks.

The stock of endothelial cell growth supplement (ECGS) was prepared by dissolving lyophilized ECGS in serum-free medium 199 to a concentration of 3 mg/ml. The ECGS solution was stored in small volume at -20° C. At each time of changing new medium, 10 μl of stock ECGS was added to each ml of medium to obtain a final concentration of 30 μg/ml.

11. Culture of Endothelial Cells

Endothelial cells were isolated and cultured from human umbilical veins according to the method of Jaffe (1984). Fresh human umbilical cords were obtained from the

Health Sciences Center (Winnipeg, MB). The portions of the cords having any clamp marks or needle holes were removed. The cord vein was cannulated with the tubing of butterfly needles and was flushed with 50 ml of warm phosphate-buffered saline (PBS), pH 7.4, to remove any residual blood. The vein was then filled with 2-5 ml of 0.2% collagenase (type IV) in PBS, and was incubated in the tissue culture incubator for 15 min. The collagenase/cell mixture was flushed with PBS into a 50 ml plastic conical centrifuge tube containing 10 ml of culture medium. The cells were sedimented by light centrifugation at 1000 rpm (Beckman TJ-6) for 10 min; the cell pellet was resuspended in 10 ml of culture medium and plated on a 0.2% gelatin-precoated 100 mm petri dish. Maximum attachment of the cells to the bottom of the dish was obtained after overnight incubation in a cell culture incubator (5% CO_2 / 95% air); unattached cells and any contaminating red blood cells were removed by rinsing the dish with warmed PBS and 10 ml fresh medium plus 100 ml of stock ECGS was added. The medium was changed every 2-3 days.

Gentle massage to the cord after incubation with collagenase increased cell yield and confluency was reached in 5-7 days.

Cells were detached by using trypsin-EDTA and subcultured in a 1:3 ratio. Briefly, the culture dish was rinsed twice with warm PBS, and the cell monolayer was incubated with 2 ml of 0.05% trypsin - 0.53 mM EDTA for 3-5 min at 37° C. Culture medium (approximately 10 ml) was added to stop trypsin digestion, the cells were

collected, sedimented by centrifugation and subcultured as described above. Cells used in all experiments were at passage 1. The cells were identified as endothelial origin by the appearance of cobblestone shape using phase contrast microscopy and by the presence of factor VIII-related antigen by immunofluorescent microscopy (Chan and Tran 1990).

12. Radiolabelling and Stimulation of Cells

Cell monolayers grown to approximately 80% confluency in 35 mm culture dishes were supplied with complete growth medium 24 h prior to incubation in 1 μCi/ml [^{3}H]arachidonic acid in Medium 199 containing 1% fetal calf serum for 20 h. The cells were washed three times with HEPES-buffered saline (HBS) (140 mM NaCl, 4 mM KCl, 5.5 mM glucose, 10 mM HEPES, 1.5 mM $CaCl_2$, and 1.0 mM $MgCl_2$; pH 7.4) containing 0.025% or otherwise indicated concentrations of essentially fatty-acid free bovine serum albumin (BSA). The cells were then challenged with the indicated concentrations of lysophospholipids or other agonists. Aliquots of LPC or other lysophospholipids dissolved in chloroform/methanol (2:1, by volume) were pipetted into test tubes, the solvent was evaporated to dryness under N_2 gas, and the lysophospholipids were resuspended on HBS containing the appropriate concentrations of BSA. The cells were challenged with 1 ml of the stimulating solution kept at 37°C.

13. Measurement of Arachidonic Acid Release

Following the challenge, the buffer solution was collected and acidified with 50 μl glacial acetic acid. The mixture was centrifuged for 5 min at 800 x g and a 0.8 ml aliquot was used for lipid extraction with a final solvent mixture consisting of chloroform/methanol/water (4:3:2, by volume). Oleic acid (60 μg) was added to the extraction mixture as a fatty acid carrier. The free fatty acid in the organic phase was resolved by thin-layer chromatography with a solvent system consisting of hexane/diethyl ether/acetic acid (70:30:1, by volume). The fatty acid band was visualized by iodine vapor and its radioactivity was determined by liquid scintillation counting.

14. Determination of Phospholipase A_2 Activity

Cells were homogenized by sonication in buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.25 M sucrose, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Aliquots of cell lysate containing 30-40 μg protein were used for the phospholipase A_2 assay. Phospholipase A_2 activity was determined by the release of radiolabelled arachidonic acid from the sn-2 position of 1-stearoyl-2-[1-^{14}C]arachidonyl-sn-glycero-3-phosphocholine. The assay mixture contained 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.02% BSA, 5 mM $CaCl_2$, 5 mM dithiothreitol, and 1 μM 1-stearoyl-2-[1-^{14}C]arachidonyl-sn-glycero-3-phosphocholine (25000 dpm/assay) in a final volume of 0.2 ml. The substrate was prepared by evaporating the solvent containing the

required amount of labeled phosphatidylcholine under N_2. The labeled phosphatidylcholine was resuspended in a small amount (0.25% of the final volume) of DMSO, vortexed for 2 min, mixed with Tris-buffer containing NaCl, and sonicated for 10 min in a water bath sonicator. The reaction was initiated by the addition of the cell lysate and the reaction mixture was incubated at 37 °C for 5 min. The reaction was quenched by the addition of 1.0 ml of chloroform/methanol (2:1, by volume). Total lipid was extracted and the radioactivity of the arachidonic acid released was determined as described in the preceding section.

15. Statistical Analysis

Student's t test for paired and unpaired data was used where appropriate. For multiple groups, analysis of variance (ANOVA) was used followed by Student-Neuman-Keuls' test to detect individual differences. Pearson Product Moment Correlation was used to compute correlation coefficients. The level of significance was defined as p value < 0.05.

III. Results

1. Effect of Lysophosphatidylcholine (LPC) on endothelium-dependent relaxation.

The dramatic increase of LPC in oxidized LDL has been postulated to be a biochemical factor in the impairment of endothelium-dependent relaxation. The effect of LPC on acetylcholine (Ach)-induced (endothelium-dependent) relaxation was assessed in phenylephrine-precontracted rat aortic rings. Incubation of the aortic rings with 5 μM LPC for 60 min significantly ($p < 0.05$) impaired endothelium-dependent relaxation when compared to relaxation in the absence of LPC (controls, Fig. 7). Incubation with 10 μM LPC for 60 min produced a significantly ($p < 0.05$) greater impairment of endothelium-dependent relaxation when compared with 5 μM LPC (Fig. 7) at Ach concentrations of 10^{-6} to 10^{-5} M.

The effect of time was examined by incubating the aortic rings with LPC (10 μM) for 15, 30, and 60 minutes (Fig. 8). LPC significantly impaired endothelium-dependent relaxation ($p < 0.05$), an effect which was stable within 15 min.

The effect of palmitoyl carnitine on Ach-induced (endothelium-dependent) relaxation was assessed in phenylephrine-precontracted rat aortic rings and compared with the effects of LPC (see above). Incubation with 5 μM palmitoyl carnitine for 60 min significantly ($p < 0.05$) impaired endothelium-dependent relaxation when compared with relaxation in the absence of palmitoyl carnitine (controls, Fig. 9). Incubation with 10 μM palmitoyl carnitine for 60 min produced a significantly ($p < 0.05$) greater impairment of

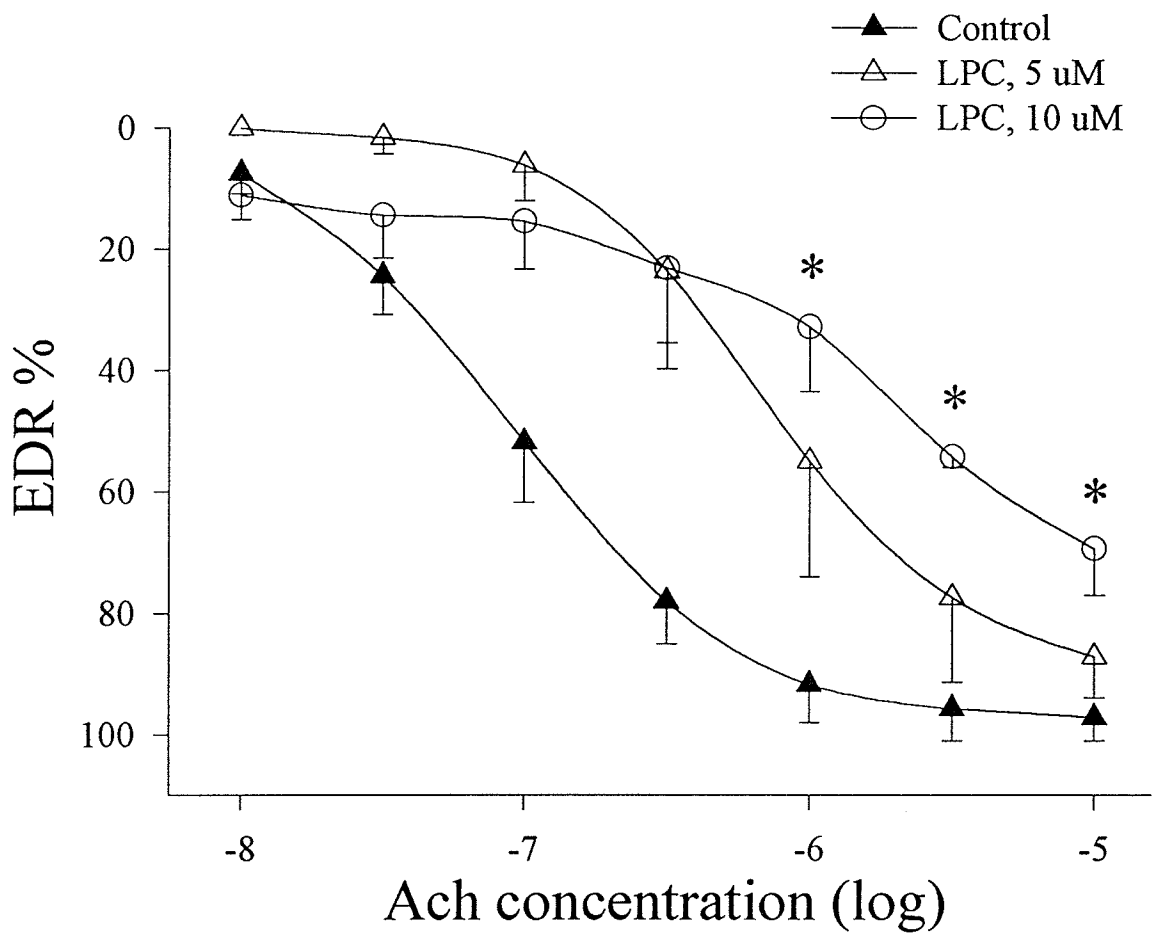

Figure 7. Effect of LPC concentration on acetylcholine-induced endothelium-dependent (EDR) relaxation. The impairment of EDR of the rat aortic ring was studied in the absence (control) or presence of 5 μM LPC or 10 μM LPC after 60 min of incubation. Values are means and error bars represent S.D. (n = 10, 6, 5 respectively). * indicates pairs (5 vs 10 μM LPC) of means which are significantly different ($p < 0.05$).

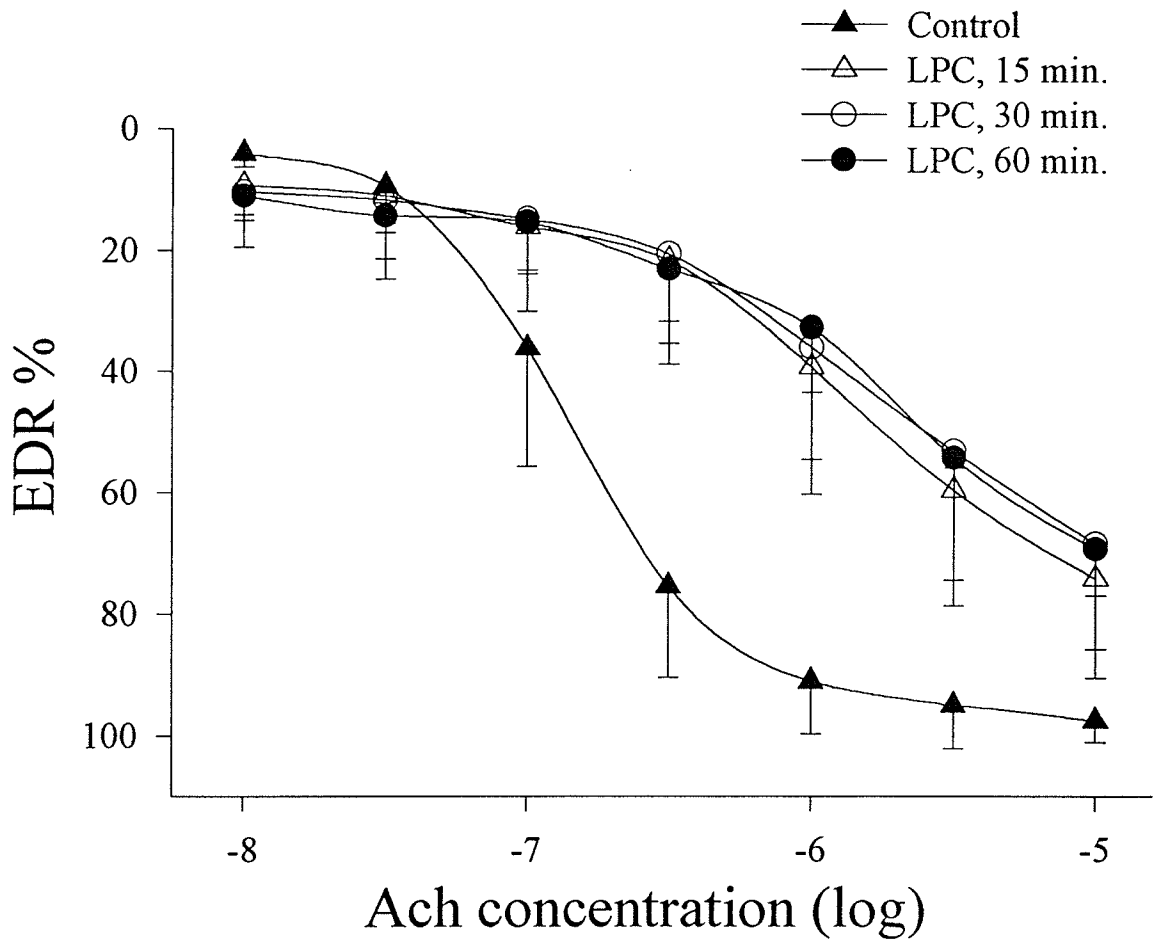

Figure 8. Effect of incubation time with LPC on acetylcholine-induced EDR. The aortic rings were incubated with 10 μM LPC for 0, 15, 30, and 60 min (n = 10, 7, 20, 5 respectively). LPC significantly impaired endothelium-dependent relaxation ($p < 0.05$) but not in a time dependent manner. Values are means, and error bars represent S.D.

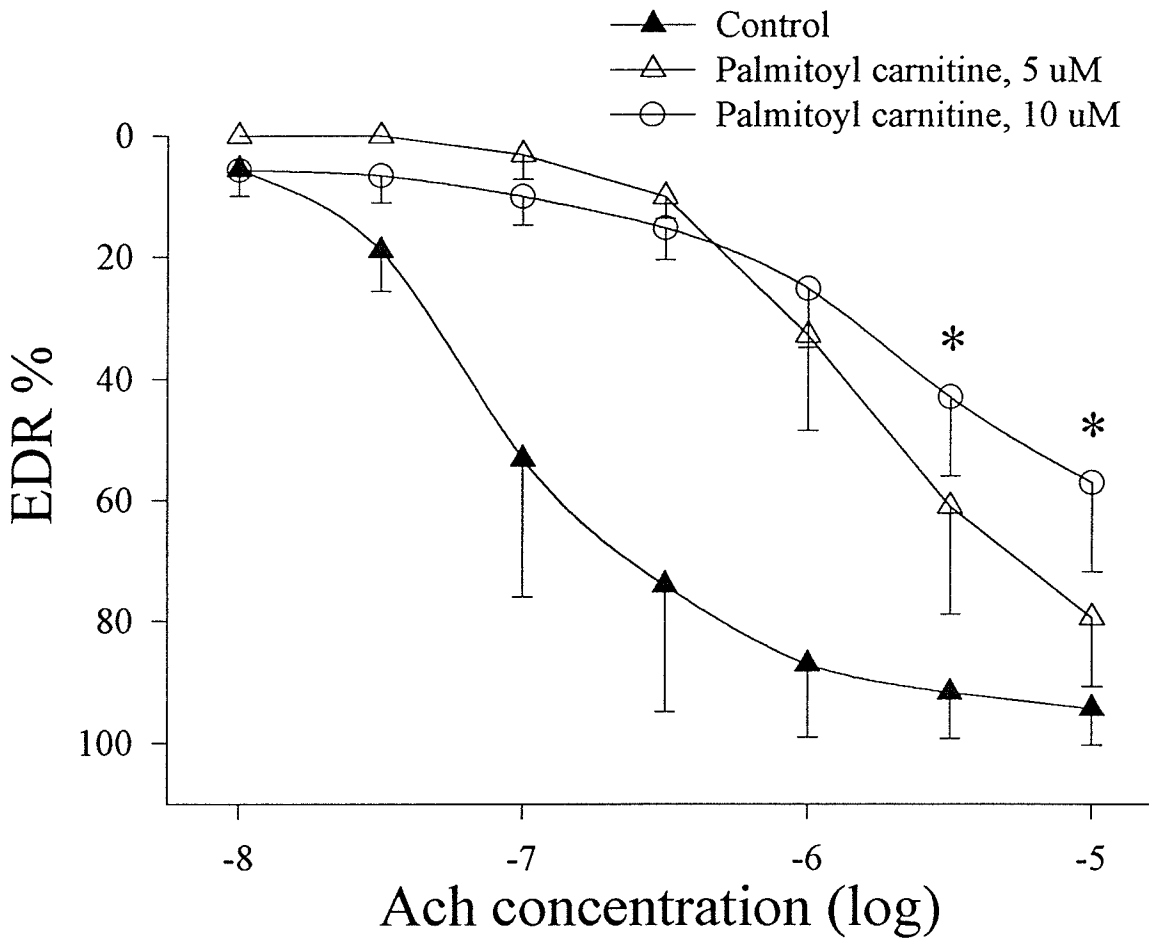

Figure 9. Effect of palmitoyl carnitine concentration on acetylcholine-induced EDR.

The impairment of EDR of the rat aortic ring was studied in the absence (control) or presence of 5 μM or 10 μM palmitoyl carnitine after 60 min of incubation. Values are means and error bars represent S.D. (n = 7, 7, 5 respectively).

* indicates pairs (5 vs 10 μM palmitoyl carnitine) of means which are significantly different ($p < 0.05$).

endothelium-dependent relaxation when compared with 5 μM palmitoyl carnitine (Fig. 9).

The effect of time was examined by incubating the aortic rings with palmitoyl carnitine (10 μM) for 15, 30, and 60 minutes (Fig. 10). Palmitoyl carnitine significantly impaired endothelium-dependent relaxation ($p < 0.05$) when compared to the control, an effect which was stable within 15 min. The effect of concentration and time of incubation on EDR were similar for LPC and palmitoyl carnitine.

2. Effect of protein kinase C (PKC) inhibitor Ro31-8220 on impairment of endothelium-dependent relaxation.

The ability of LPC to impair endothelium-dependent relaxation in the presence of a protein kinase C inhibitor (Ro31-8220) was investigated. Incubation with 10 μM LPC for 15 min significantly ($p < 0.05$) impaired endothelium-dependent relaxation when compared with the control (Fig. 11). Incubation of the aortic rings with 0.5 μM Ro31-8220 for 15 min prior to incubation with LPC reduced the effect of LPC significantly ($p < 0.05$, Fig. 11).

To determine whether Ro31-8220 acts directly on endothelium-dependent relaxation or on the effect of LPC, the aortic rings were incubated with 0.5 μM Ro31-8220 for 30 min. Ro31-8220 did not impair Ach induced endothelium-dependent relaxation (data not shown).

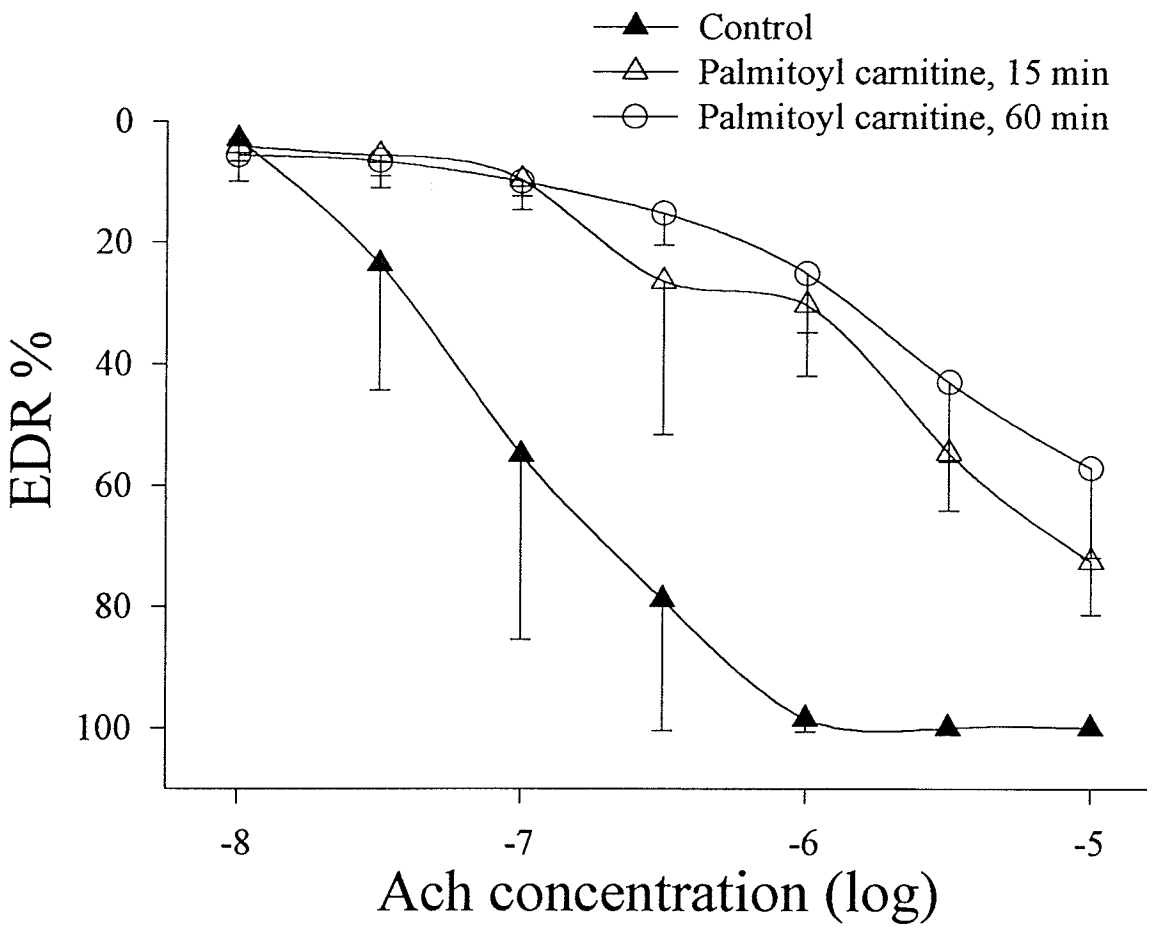

Figure 10. Effect of incubation time with palmitoyl carnitine on acetylcholine induced EDR. The aortic rings were incubated with 10 μM palmitoyl carnitine for 0, 15, and 60 min (n = 7, 5, 5 respectively). Palmitoyl carnitine significantly impaired endothelium-dependent relaxation ($p < 0.05$). Values are means, and error bars represent S.D.

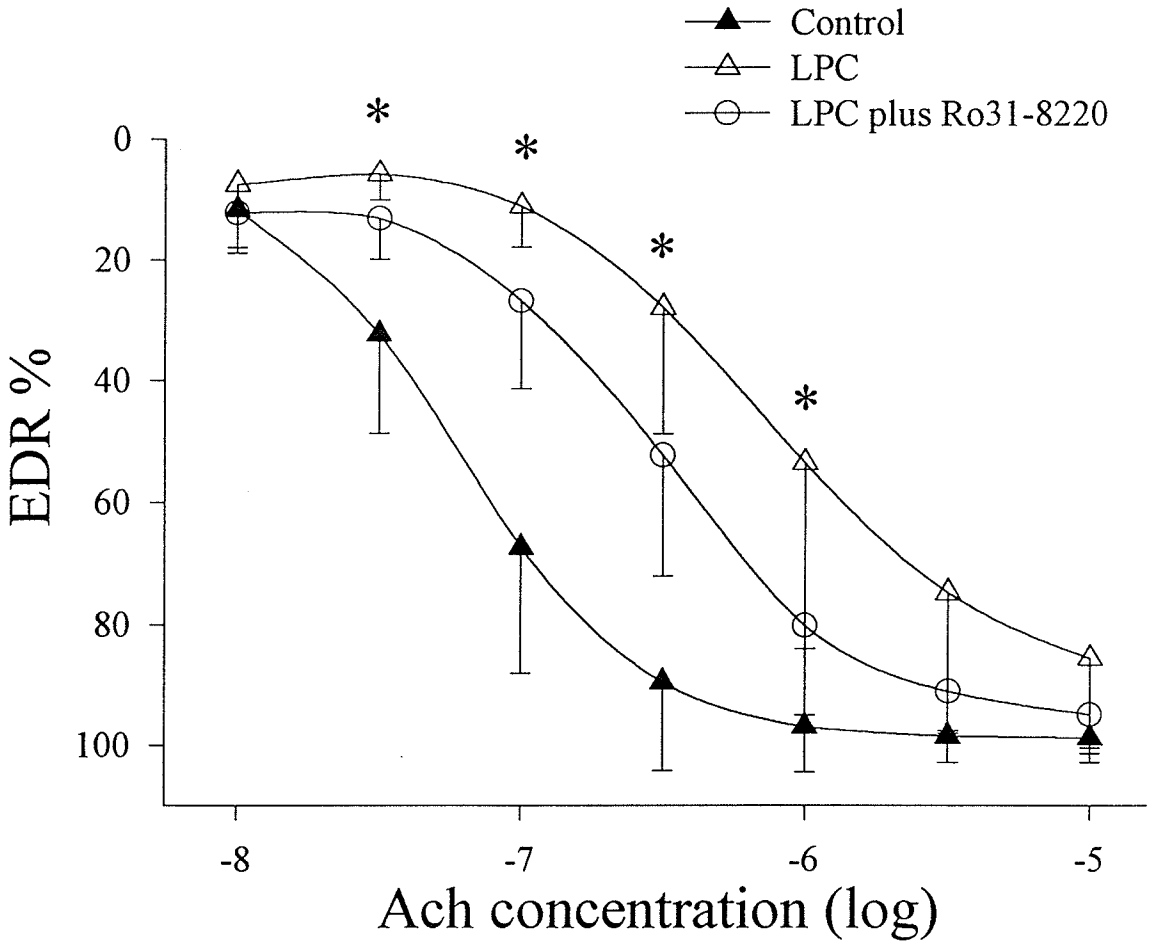

Figure 11. Effect of protein kinase C inhibitor (Ro31-8220) on LPC induced impairment of EDR. The fractional EDR was calculated on the basis of maximal relaxation produced at 10^{-5} M Ach. Solid triangles represent relaxation under control conditions (no addition), open triangles represent relaxation after 15 min of incubation with 10 μM LPC, and open circles represent relaxation after 15 min of incubation with 0.5 μM Ro31-8220 prior to incubation with LPC. Values are means, and error bars represent S.D.(n = 8).

*indicates pairs (LPC vs Ro31-8220 plus LPC) of means which are significantly different ($p < 0.05$).

If LPC and palmitoyl carnitine exert their effects via the same general mechanism, Ro31-8220 should reduce the ability of palmitoyl carnitine to impair endothelium-dependent relaxation. Incubation of the aortic rings with 10 μM palmitoyl carnitine for 15 min significantly ($p < 0.05$) impaired endothelium-dependent relaxation when compared to the control (Fig. 12). The impairment of endothelium-dependent relaxation by palmitoyl carnitine was significantly reduced ($p < 0.01$) in rings pretreated with 0.5 μM Ro31-8220 for 30 min (Fig. 12).

3. Effect of LDL on Endothelium Dependent Hyperpolarizing Factor (EDHF)

The role of EDHF was assessed on Ach induced endothelium-dependent relaxation of phenylephrine precontracted rat aortic rings. To prevent hyperpolarization, the aortic rings were incubated with 15 mM KCl for 30 min. Endothelium-dependent relaxation was significantly ($p < 0.01$) reduced when compared to endothelium-dependent relaxation in the absence of KCl (Fig. 13). Incubation with 5 μM lysophosphatidylcholine (LPC) for 30 min significantly ($p < 0.01$) impaired endothelium-dependent relaxation (Fig. 14) when compared to the control (no addition). The exposure to both 15 mM KCl and 5 μM LPC for 30 min significantly ($p < 0.01$) impaired endothelium-dependent relaxation when compared to LPC alone (Fig. 14). The two treatments potentiate impairment of endothelium-dependent relaxation, therefore the mechanism of action of LPC appears to involve EDNO (endothelium-derived nitric oxide).

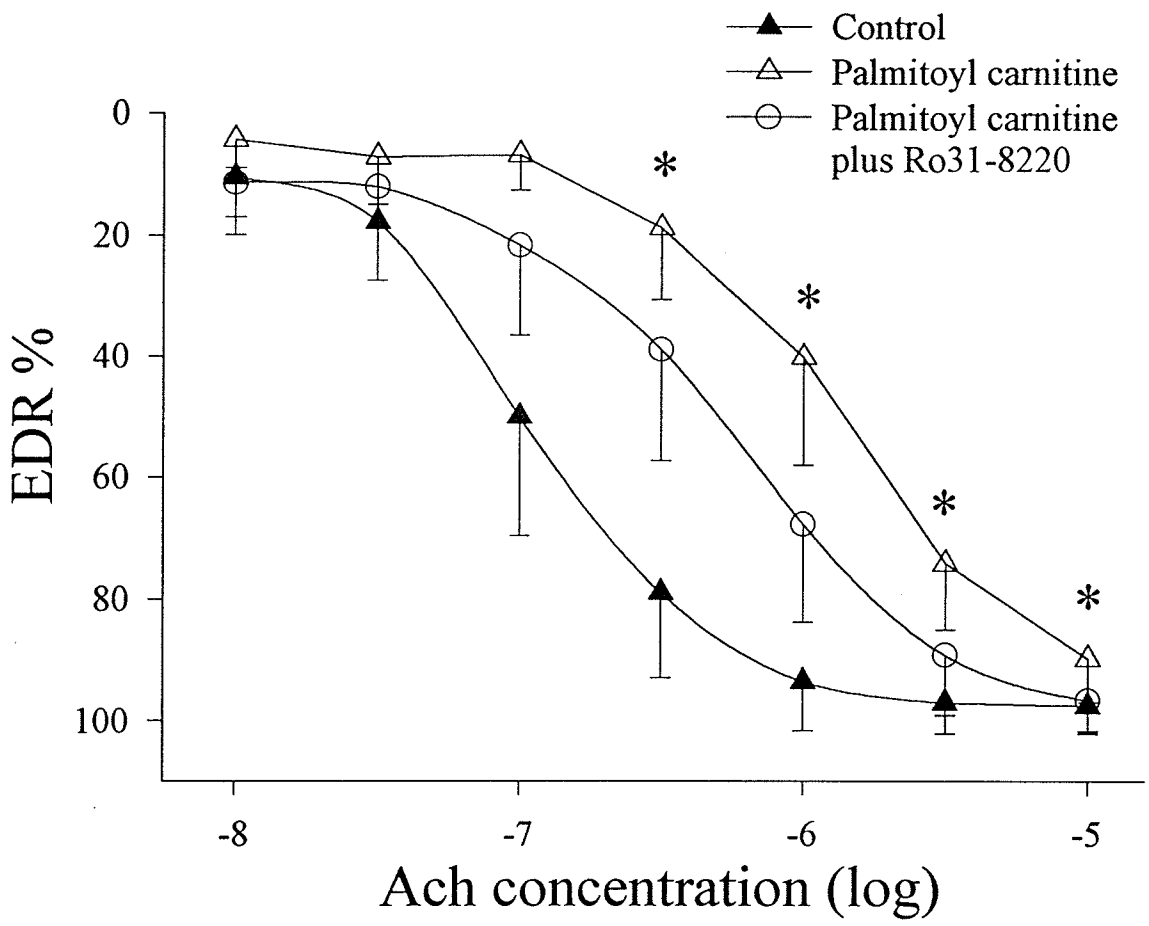

Figure 12. Effect of protein kinase C inhibitor (Ro31-8220) on palmitoyl carnitine induced impairment of EDR. The fractional EDR was calculated on the basis of maximal relaxation produced at 10^{-5} M Ach. Solid triangles represent relaxation under control conditions (no addition), open triangles represent relaxation after 15 min of incubation with 10 μM palmitoyl carnitine, and open circles represent relaxation after 15 min of incubation with 0.5 μM Ro31-8220 prior to incubation with palmitoyl carnitine. Values are means, and error bars represent S.D.(n = 14).

*indicates pairs (palmitoyl carnitine vs Ro31-8220 plus palmitoyl carnitine) of means which are significantly different ($p < 0.05$).

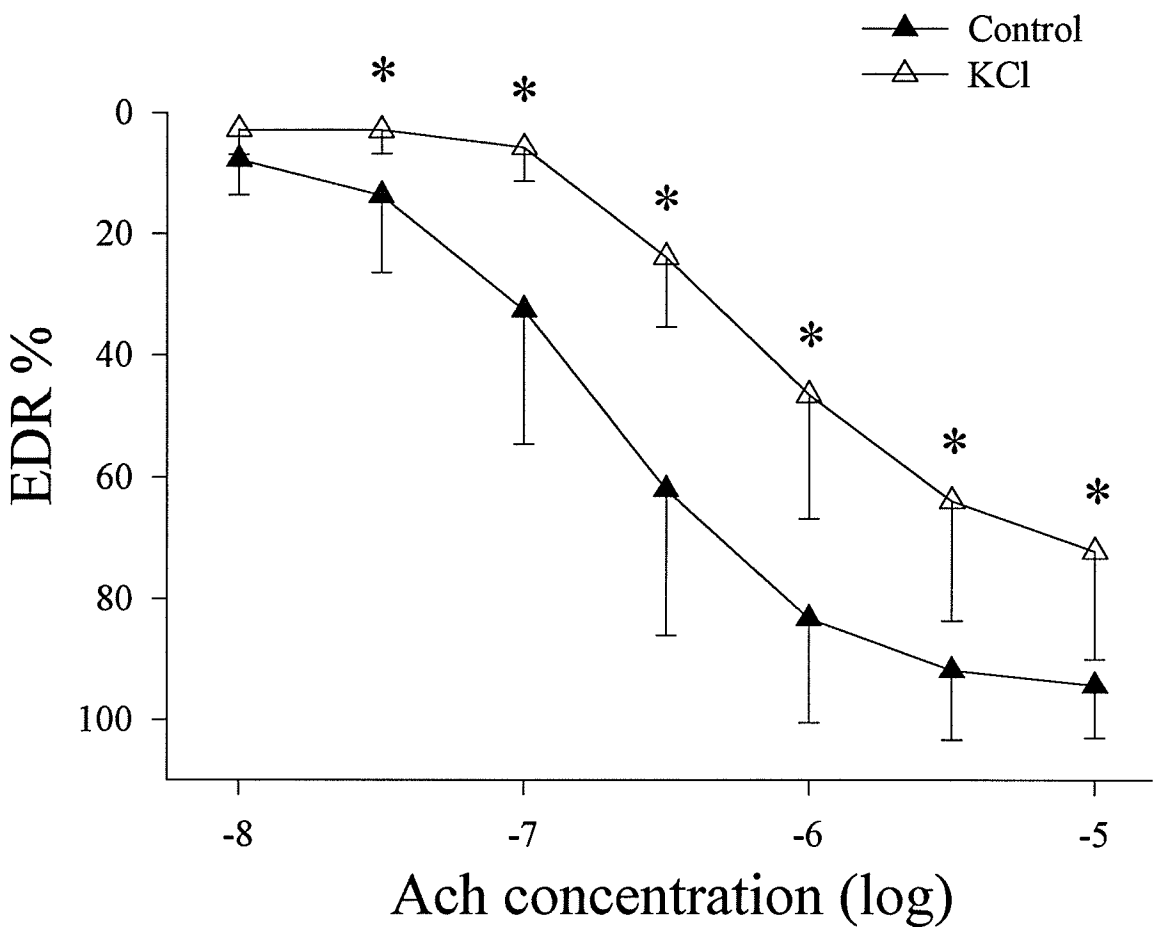

Figure 13. Effect of KCl on Ach induced endothelium-dependent relaxation of phenylephrine precontracted rat aortic rings. The fractional EDR was calculated on the basis of maximal relaxation produced at 10^{-5} M Ach. Closed triangles represent EDR under control conditions (no addition). Open triangles represent EDR after incubation with 15 mM KCl for 30 min. Values are means, error bars = S.D. (n = 12 for all data points).

* indicate pairs (control vs. KCl) of means which are significantly different ($p < 0.01$).

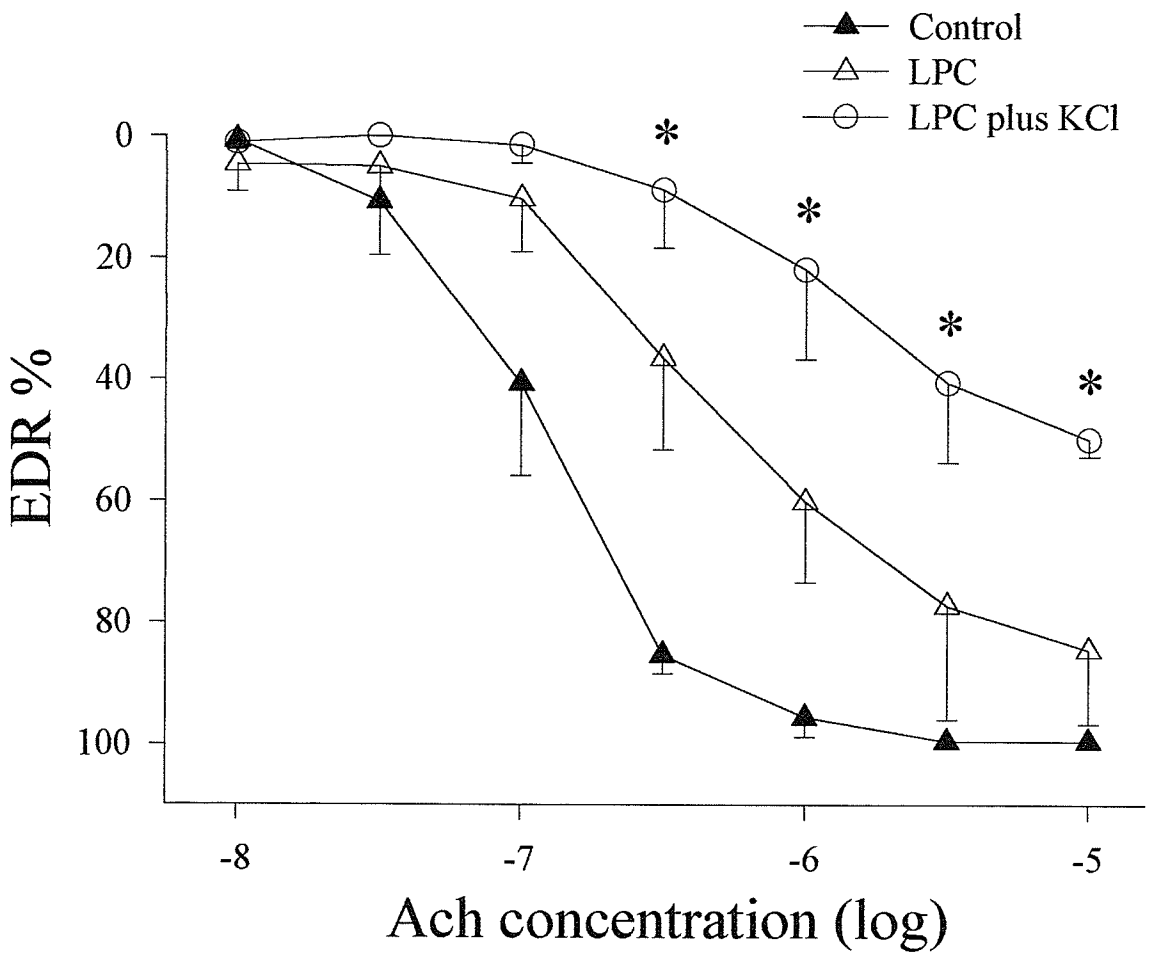

Figure 14. Effect of KCl and LPC on Ach induced endothelium-dependent relaxation of phenylephrine precontracted rat aortic rings. The fractional EDR was calculated on the basis of maximal relaxation produced at 10^{-5} M Ach. Closed triangles represent EDR under control conditions (no addition). Open triangles represent EDR after incubation with 5 μM LPC for 30 min. Open circles represent EDR after incubation with 15 mM KCl and 5 μM LPC for 30 min. Values are means, error bars = S.D. (n = 4 for all data points). * indicate pairs (LPC vs. KCl plus LPC) of means which are significantly different ($p < 0.01$).

To isolate Ach induced endothelium-dependent relaxation mediated by EDHF, the rat aortic rings were incubated with 6.8 μM N^{W}-nitro-L-arginine methyl ester (L-NAME; a NOS inhibitor) for 30 min. The addition of L-NAME significantly ($p < 0.01$) impaired endothelium-dependent relaxation when compared to the control (no addition) (Fig. 15). Incubation with both 20 μM LPC and 6.8 μM L-NAME for 30 min impaired endothelium-dependent relaxation significantly ($p < 0.01$) more when compared to L-NAME alone (Fig. 15). The mechanism of action of LPC appears to also involve EDHF.

4. Effect of bovine serum albumin (BSA) on LPC induced impairment of endothelium-dependent relaxation in aortic rings.

The ability of BSA to prevent LPC induced impairment of endothelium-dependent relaxation was examined. The aortic ring preparations were equilibrated in Krebs-Henseleit solution or Krebs-Henseleit solution containing 0.25% BSA. The control rings were maintained in Krebs containing 0.25% BSA. One group of experimental rings were incubated with 10 μM LPC for 15 min in Krebs (no BSA). This resulted in significant impairment of endothelium-dependent relaxation ($p < 0.01$) when compared to the control group. A second group of experimental rings was incubated with 10 μM LPC for 15 min in Krebs containing 0.25% BSA (Fig. 16). In the latter group, no impairment of endothelium-dependent relaxation occurred.

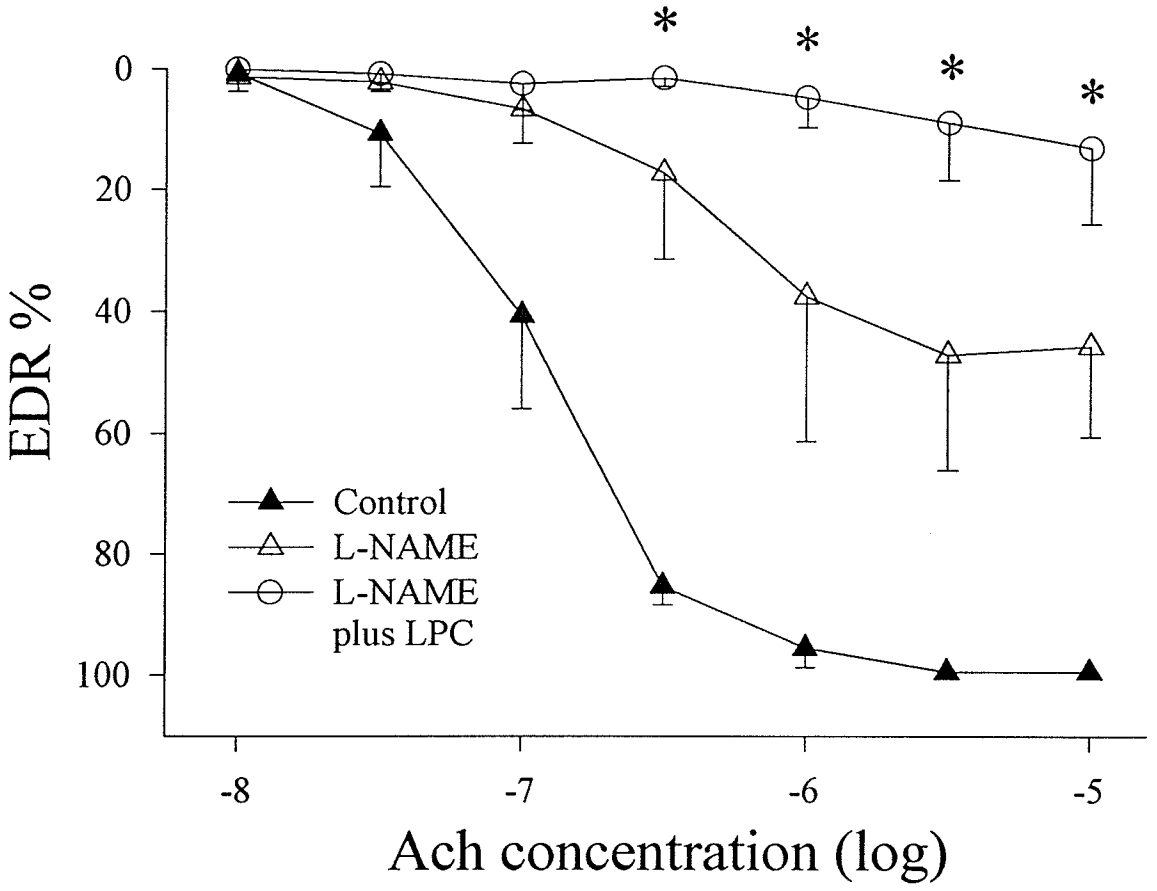

Figure 15. Effect of L-NAME and LPC on Ach induced endothelium-dependent relaxation of phenylephrine precontracted rat aortic rings. The fractional EDR was calculated on the basis of maximal relaxation produced at 10^{-5} M Ach. Closed triangles represent EDR under control conditions (no addition). Open triangles represent EDR after incubation with 6.8 μM L-NAME for 30 min. Open circles represent EDR after incubation with 6.8 μM L-NAME and 20 μM LPC for 30 min. Values are means, error bars = S.D. (n = 4 for all data points).

* indicate pairs (L-NAME vs. L-NAME plus LPC) of means which are significantly different ($p < 0.01$).

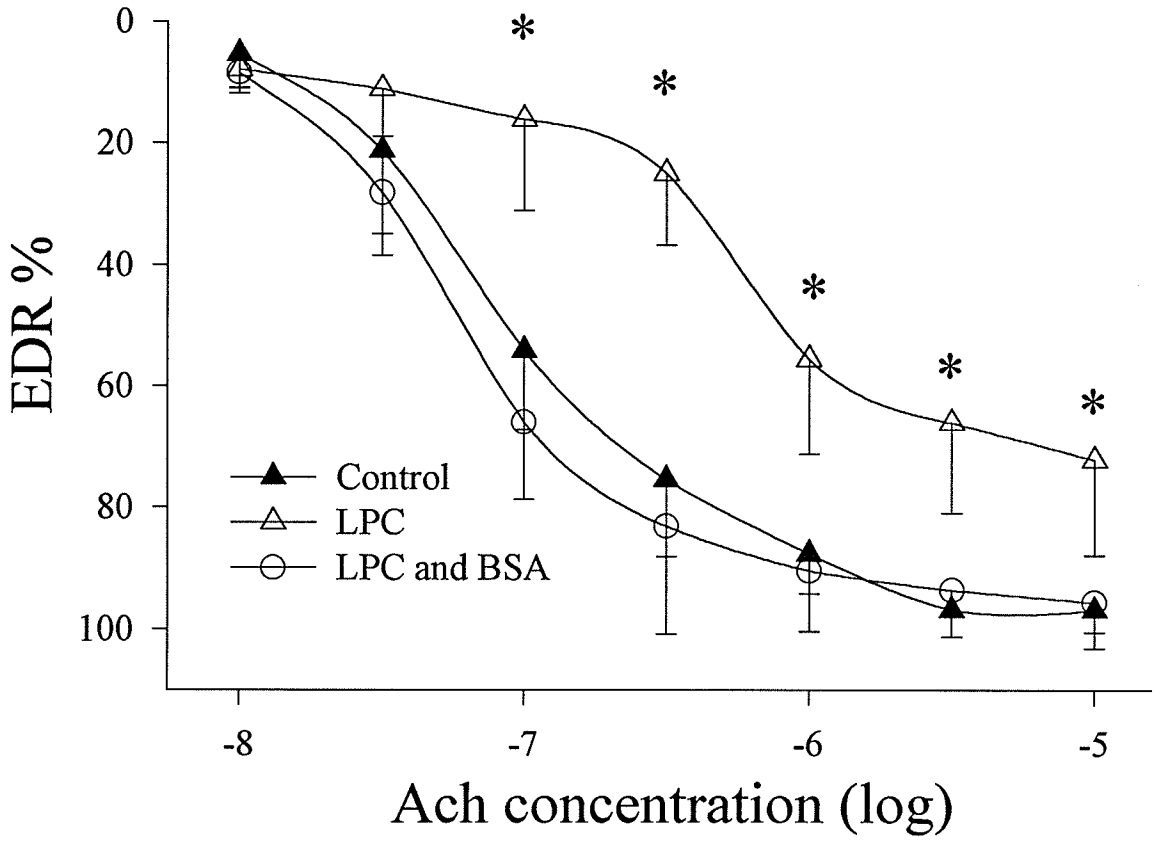

Figure 16. Effect of Bovine serum albumin (BSA) prevents LPC induced impairment of EDR in aortic rings. The fractional EDR was calculated on the basis of maximal relaxation produced at 10^{-5} M Ach; drugs had no effect on strength of contraction. Solid triangles represent relaxation under control conditions (no addition) and open triangles represent relaxation after 15 min of incubation with 10 μM LPC in Krebs (no BSA). Open circles represent relaxation after 30 min of equilibration in Krebs-Henseleit solution containing 0.25% BSA and 15 min of incubation with 10 μM LPC. Values are means, and error bars represent S.D.(n = 5). *indicates pairs (LPC vs LPC plus Krebs containing 0.25% BSA) of means which are significantly different ($p < 0.01$).

The ability of BSA to reverse LPC induced impairment of endothelium-dependent relaxation was also examined. The aortic ring preparation was incubated with 10 μM LPC for 30 min. and a significant impairment of Ach induced endothelium-dependent relaxation on phenylephrine pre-contracted aortic rings was observed. The aortic rings were then washed and equilibrated for 30 min with either Krebs-Henseleit solution or Krebs-Henseleit solution containing 0.25% BSA. Assessment of Ach induced endothelium-dependent relaxation on phenylephrine pre-contracted aortic rings was then repeated. The ability of LPC to impair EDR decreased by approximately 50% after the 30 min equilibration period in Krebs-Henseleit solution (Fig. 17) . The Krebs-Henseleit solution containing 0.25% BSA reduced the effect of LPC significantly ($p < 0.01$), completely restoring the ability of the aortic ring to relax.

To determine if the BSA actually removed LPC from the membrane, the above protocol was repeated with L-lyso-3-phosphatidylcholine, 1-[1-^{14}C] palmitoyl (LPC ^{14}C). The aortic rings were incubated with LPC ^{14}C (25 nCi/ml) and then washed and equilibrated with either Krebs-Henselleit solution or Krebs-Henselleit solution containing 0.25% BSA. The rings were contracted and relaxed as before. Radioactivity was determined by liquid scintillation counting for both the aortic rings and the bathing solution. There was a significant ($p < 0.01$) decrease in tissue ^{14}C-LPC in muscles equilibrated in Krebs containing 0.025% BSA (3.20 ± 0.81 dpm x 10^3 / mg) when compared with tissue equilibrated in Krebs without BSA (5.84 ± 0.44 dpm x 10^3 / mg)

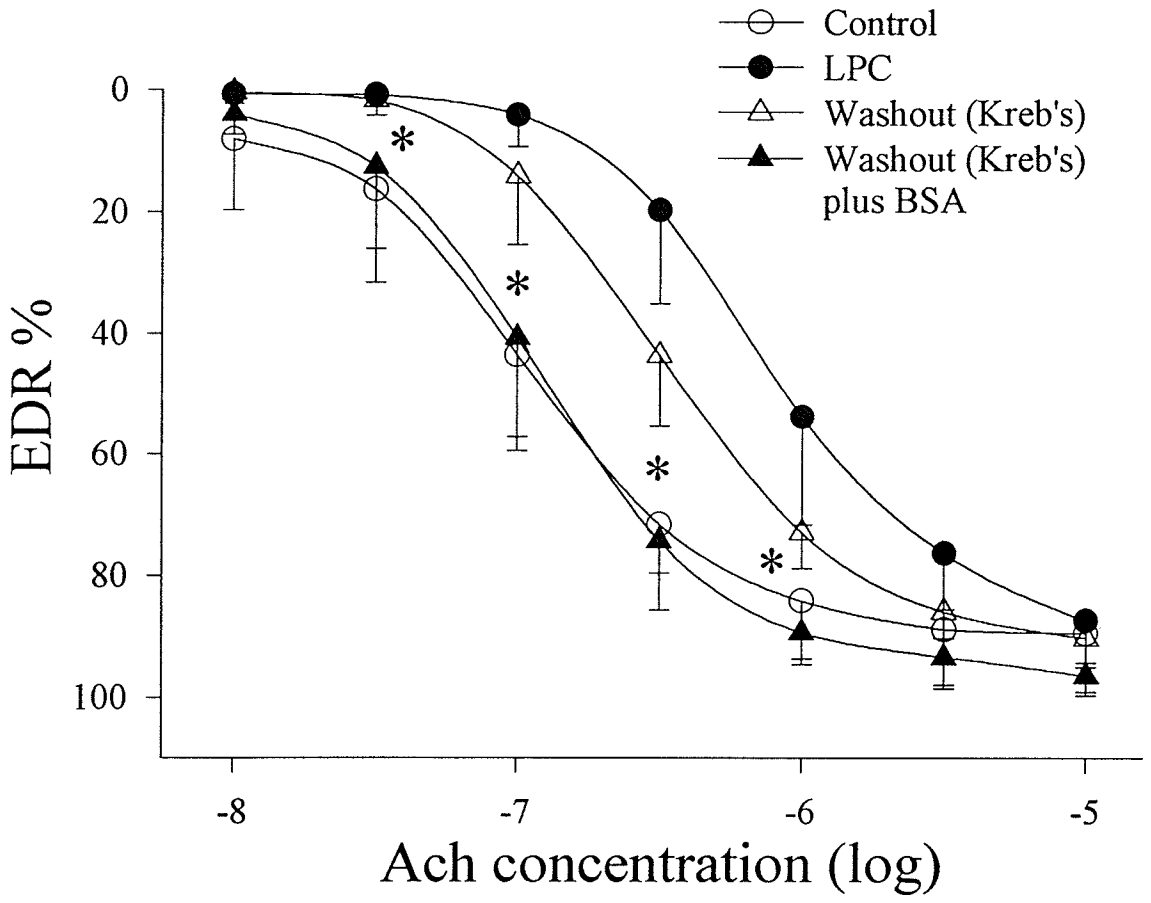

Figure 17. Bovine serum albumin (BSA) reverses LPC induced impairment of EDR in aortic rings. The fractional EDR was calculated on the basis of maximal relaxation produced at 10^{-5} M Ach. Open circles represent relaxation under control conditions (no addition) and solid circles represent relaxation after 15 min of incubation with 10 μM LPC. Triangles represent rings exposed to LPC (solid circles) which are then equilibrated for 30 min in Krebs-Henseleit (open triangles) or equilibrated for 30 min in Krebs-Henseleit containing 0.25% BSA (solid triangles). Values are means, and error bars represent S.D.($n = 5$). *indicates pairs (Krebs vs Krebs containing 0.25% BSA) of means which are significantly different ($p < 0.01$).

(Fig. 18). There was also a significant ($p < 0.01$) increase in dpm / ml in the bathing solution Krebs 0.025% BSA (94.8 ± 23.4 dpm x 10^3 / ml) when compared to Krebs without BSA (40.4 ± 9.3 dpm x 10^3 / ml). BSA removed LPC from the aortic ring and reversed the impairment of EDR. This would imply that LPC affects EDR at the outer membrane of the endothelial cells. It should, however, be noted that the increase of radioactivity in the bathing solution can be largely attributed to BSA binding ^{14}C-LPC which adhered to the glass (50.33 ± 24.74 dpm x 10^3 / ml).

5. Effect of hyperlipidemia and acyl chain length on endothelium-dependent relaxation.

The cholesterol and triglyceride concentrations in blood samples of normal individuals were determined and compared with those obtained in samples from hyperlipidemic patients. The levels of total cholesterol, LDL cholesterol, and triglyceride were significantly elevated in the plasma of the hyperlipidemic patients (Table 3). The amount of LDL estimated from LDL protein was also elevated in these patients. The data confirmed the hyperlipidemic status of these patients. The values obtained from normal individuals were within the desirable range defined by the Canadian Cholesterol Consensus Guidelines.

The ability of oxidized LDL from hyperlipidemic patients to impair the endothelium-dependent relaxation of aortic rings was investigated. Native LDL

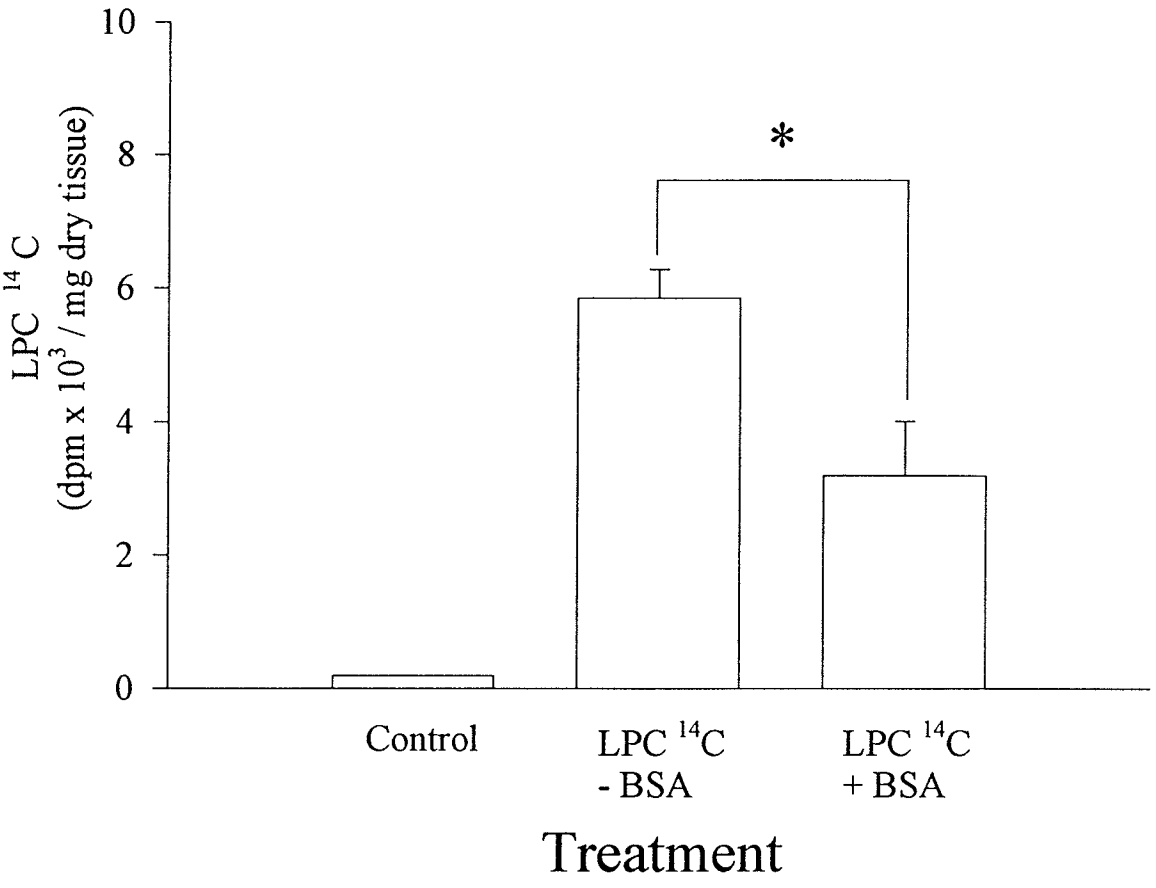

Figure 18. Effect of 0.25% BSA exposure on LPC C^{14} content of the aortic ring.

The control represents the background radioactivity (no addition). Aortic rings exposed to 0.25% BSA for 30 min had significantly ($p < 0.01$) reduced radioactive content (dpm /mg of tissue) when compared to rings not exposed to 0.25% BSA. Values are means, error bars represent S.D ($n = 6$).

* indicates pairs (Krebs vs Krebs containing 0.25% BSA) of means which are significantly different ($p < 0.01$).

Table 3. Plasma lipid and lipoprotein contents in normal and hyerlipidemic subjects.

	Normal	Hyperlipidemic
	(μmol/ml plasma)	
Total Cholesterol	4.5 ± 0.9	7.2 ± 1.0
LDL Cholesterol	2.6 ± 0.7	4.7 ± 0.9
HDL Cholesterol	1.4 ± 0.2	1.2 ± 0.2
Triglyceride	1.2 ± 0.3	2.7 ± 1.0
LDL protein (mg/ml)	0.6 ± 0.2	1.1 ± 0.1

Blood samples for analysis were taken from normal volunteers and hyperlipidemic patients attending a local lipid clinic. Results represent the means ± SEM for 8 subjects in each group. Means for data from hyperlipidemic patients are significantly ($p < 0.05$) different from normal subjects in all categories except HDL cholesterol.

preparations obtained from normal and hyperlipidemic subjects were not significantly different in their effect on endothelium-dependent relaxation of the aortic ring (Fig. 19). Oxidized LDL from both groups, however, was found to produce significant impairment of endothelium-dependent relaxation. The oxidized LDL obtained from hyperlipidemic patients was significantly more effective in impairing the endothelium-dependent relaxation as compared with that obtained from normal individuals.

The biochemical change leading to the greater impairment of endothelium-dependent relaxation by oxidized LDL from hyperlipidemic patients was examined. Lipids were extracted from native and oxidized LDL preparations from both subject groups, and the content of each phospholipid class in the extract was analyzed by thin-layer chromatography. No significant differences in the total phospholipid or sphingomyelin contents were detected in the native LDL preparations of either subject group (Table 4). The total phospholipid and sphingomyelin contents were also not different in the two groups after the LDL had been subjected to oxidation. A substantial decrease (45%) in the level of phosphatidylcholine (PC) and a concomitant increase (6 fold) in the level of LPC were measured in oxidized LDL samples from both normal and hyperlipidemic subjects.

The dramatic increase in LPC in oxidized LDL has been postulated to be a biochemical factor in the enhanced impairment of endothelium-dependent relaxation. The oxidized LDL samples of hyperlipidemic patients contained a significantly ($p < 0.05$)

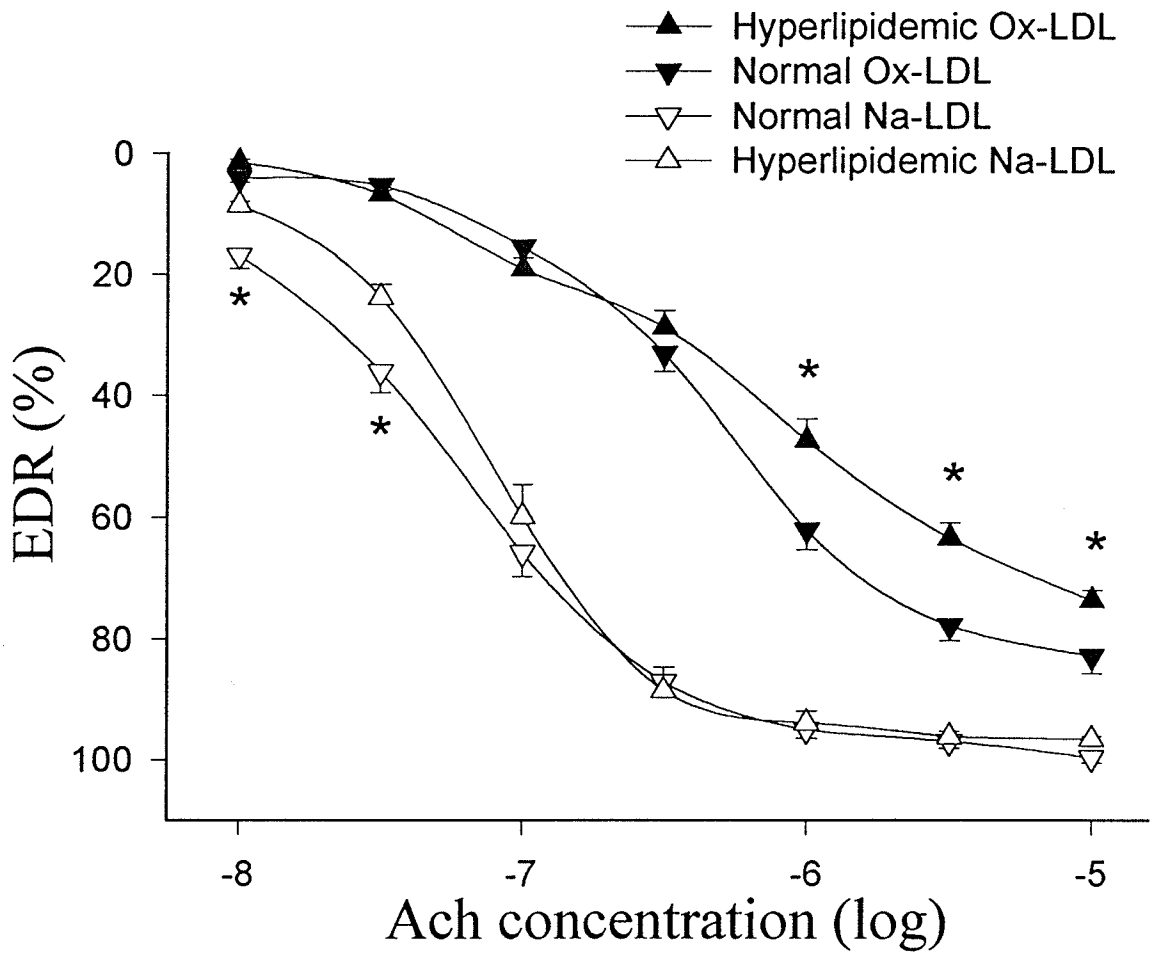

Figure 19. Effect of LDL from normal and hyperlipidemic subjects on Ach-induced EDR. LDL was obtained and oxidized as described in the Methods. The fractional EDR was calculated on the basis of maximal relaxation produced at 10^{-5}M Ach. Solid and open symbols represent data obtained with oxidized- and native LDL (resp). Inverted and upright triangles represent data from normal and hyperlipidemic subjects (resp). Values are means ± SEM (n=29 for hyperlipidemic subjects; n=13 for normal subjects).

* indicate pairs (normal vs. hyperlipidemic) of means which are significantly different ($p < 0.05$).

Table 4. Effect of hyperlipidemia on TBARS and LPC in LDL.

	Normal (13)	Hyperlipidemic (29)
TBARS[1]		
Native LDL	0.93 ± 0.59	2.29 ± 1.02*
Oxidized LDL	69.88 ± 17.43	56.65 ± 11.85*
Phosphatidylcholine[2]		
Native LDL	0.79 ± 0.04	0.80 ± 0.04
Oxidized LDL	0.47 ± 0.04	0.42 ± 0.06*
Sphingomyelin[2]		
Native LDL	0.32 ± 0.03	0.31 ± 0.05
Oxidized LDL	0.33 ± 0.02	0.31 ± 0.04
Lysophosphatidylcholine[2]		
Native LDL	0.06 ± 0.01	0.06 ± 0.01
Oxidized LDL	0.35 ± 0.03	0.41 ± 0.05*

Results represent means ± S.D. for each group.

* indicates that means for data from the hyperlipidemic group are significantly ($p < 0.05$) different from the normal group.

[1]nmol malondialdehyde equivalents / mg LDL protein

[2]micromoles / mg LDL protein.

higher level of LPC as compared with that from normal subjects (Table 4). The biochemical basis for the enhanced impairment of endothelium-dependent relaxation in the oxidized LDL from hyperlipidemic patients was investigated. Our approach was to determine the acyl profiles of LPC in the native and oxidized LDL from the normal and hyperlipidemic subjects. The LPC fraction was isolated from the LDL preparation and the acyl composition was analyzed by gas-liquid chromatography. In both normal and hyperlipidemic subjects there was a preponderance of short- and medium-chain ($< C_{16}$) acyl groups in the native LDL and an increase in the proportion of long-chain ($> C_{16}$) acyl groups after oxidative modification (Table 5). The increased content of long-chain LPC groups in oxidized LDL from hyperlipidemic patients (from 41.5% to 56.5%), however, was significantly greater ($p < 0.05$) than that found in samples from normal subjects (from 41.5% to 49.5%).

A change in acyl composition could provide a biochemical mechanism for the differential endothelium-related effect of the oxidized LDL from hyperlipidemic and normal subjects. In evaluating this possibility, the direct effect of medium- ($C_{12\text{-}14}$) and long-chain ($C_{16\text{-}18}$) LPC on the impairment of endothelium-dependent relaxation was investigated. The presence of 10 μM lauroyl-LPC ($C_{12:0}$) did not impair the endothelium-dependent relaxation of the aortic ring whereas 10 μM palmitoyl-LPC ($C_{16:0}$) caused a strong impairment of that relaxation (Fig. 20). Myristoyl-LPC ($C_{14:0}$) did not impair endothelium-dependent relaxation, whereas stearoyl-LPC ($C_{18:0}$) and

Table 5. Distribution (%) of acyl groups in lysophosphatidylcholine in native and oxidized LDL from normal and hyperlipidemic subjects.

Acyl group	Normal		Hyperlipidemic	
	Native	Oxidized	Native	Oxidized
$C_{8:0}$	0.5 ± 0.4	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.2
$C_{10:0}$	12 ± 0.4	10 ± .4	11 ± 0.8	8 ± 0.7
$C_{12:0}$	24 ± 0.7	20 ± .5	25 ± 1.1	18 ± 1.0
$C_{12:1}$	2 ± 0.4	2 ± 0.3	3 ± 0.7	2 ± 0.6
$C_{14:0}$	19 ± 0.5	17 ± 0.4	18 ± 0.9	14 ± 0.8
$C_{14:1}$	1 ± 0.5	1 ± 0.2	1 ± 0.5	1 ± 0.6
$C_{16:0}$	10 ± 0.6	17 ± .7	14 ± 1.7	22 ± 1.8
$C_{16:1}$	13 ± 0.6	14 ± 0.4	13 ± 1.0	11 ± 1.1
$C_{18:0}$	8 ± 0.6	9 ± 0.6	9 ± 1.0	13 ± .1
$C_{18:1}$	8 ± 0.3	7 ± 0.4	4 ± 1.0	6 ± 1.0
$C_{18:2}$	ND	ND	0.5 ± 0.7	2 ± 0.6
$C_{18:3}$	0.5 ± 0.3	0.5 ± 0.2	ND	0.5 ± 0.3
$C_{20:2}$	2 ± 0.5	2 ± 0.5	2 ± 0.5	2 ± 0.7
Short & medium chain ($C_{8:0}$ - $C_{14:1}$)	58.5 ± 0.7	50.5 ± 0.9*	58.5 ± 1.2	43.5 ± 1.5*+
Long chain ($C_{16:0}$ - $C_{20:2}$)	41.5 ± 0.6	49.5 ± .7*	41.5 ± 0.9	56.5 ± 1.3*+

Results represent means ± SEM for 8 subjects in each group.
* indicates that means for data from the oxidized LDL are significantly ($p < 0.05$) different from the native LDL in the same subject group.
+ indicates that the means for data from the hyperlipidemic group are significantly ($p < 0.05$) different from the normal group.

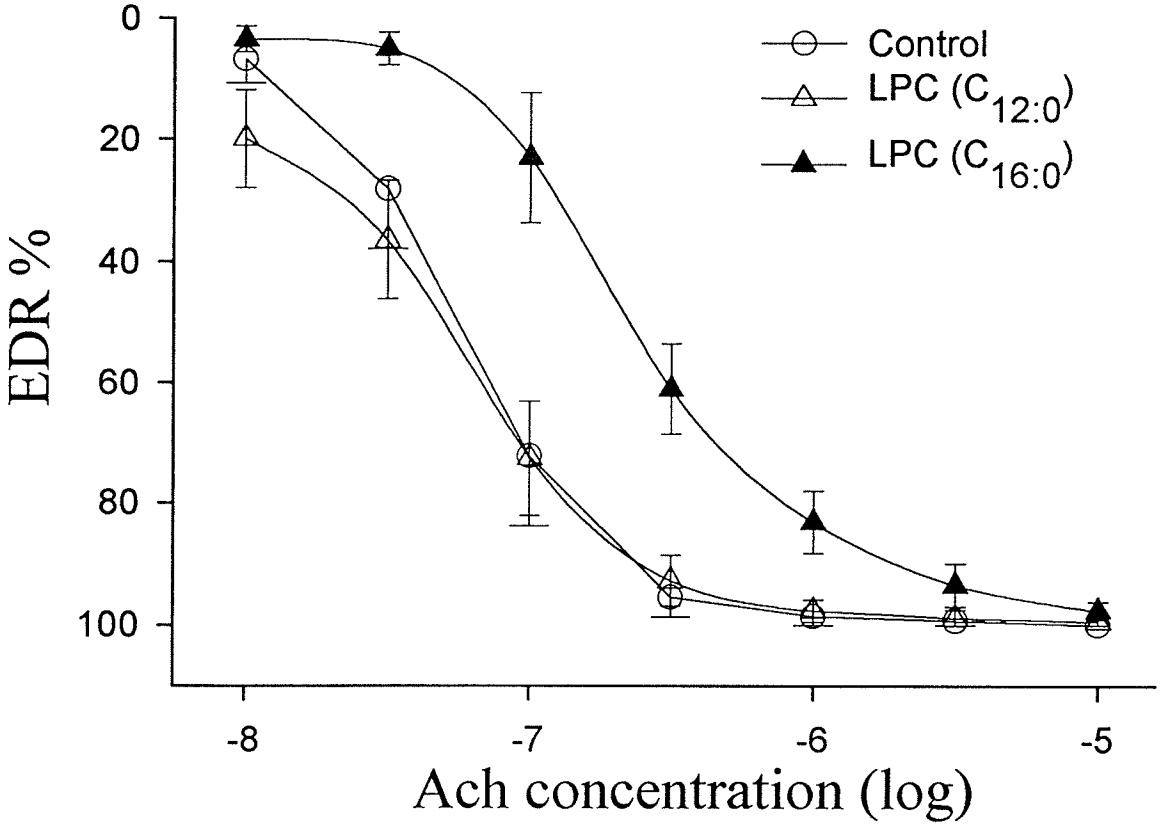

Figure 20. Direct effects of medium- (C_{12} and C_{14}) and the long-chain (C_{16} and C_{18}) LPC on EDR of the rat aortic ring. The impairment of EDR of the rat aortic was studied in the absence (control) or presence of 10 μM of 1-lauroyl-sn-glycero-3-phosphocholine ($C_{12:0}$-LPC) or 1-palmitoyl-sn-glycero-3-phosphocholine ($C_{16:0}$-LPC). Each point represents the mean of at least three separate experiments, each done in duplicate. The vertical bars represent the SEM.

oleoyl-LPC ($C_{18:1}$) produced a more effective impairment of endothelium-dependent relaxation than that produced by palmitoyl-LPC (data not shown).

The effect of acyl chain length within the LPC component of LDL was examined by loading LDL with lauroyl- or palmitoyl-LPC and monitoring their effects on endothelium-dependent relaxation. Native LDL was incubated in a buffer containing 0.5 mM of either palmitoyl- or lauroyl-LPC for 24 h, and the LDL was then dialyzed for 24 h with three changes of buffer to remove the free LPC in the solution. The amount of LPC in the LDL sample after treatment was found to increase from 0.08 μmol/mg LDL protein to 1.56 μmol/mg LDL protein. The amounts of PC and sphingomyelin in the LDL sample were not altered by this treatment. The LDL containing the lauroyl-LPC did not display any increase in the impairment of endothelial relaxation when compared with the native LDL (control, Fig. 21). The LDL containing the palmitoyl-LPC, however, produced a significantly greater impairment of the endothelium-dependent relaxation than did the native LDL or LDL loaded with medium-chain LPC.

6. Impairment of endothelium-dependent relaxation by oxidatively modified LDL is gender-dependent.

Subject-related data and blood lipid levels are shown in Table 6. As is shown in the table, all subjects classified as normal had normal cholesterol and triglyceride levels as defined by the Canadian Cholesterol Consensus Guidelines. In contrast, the

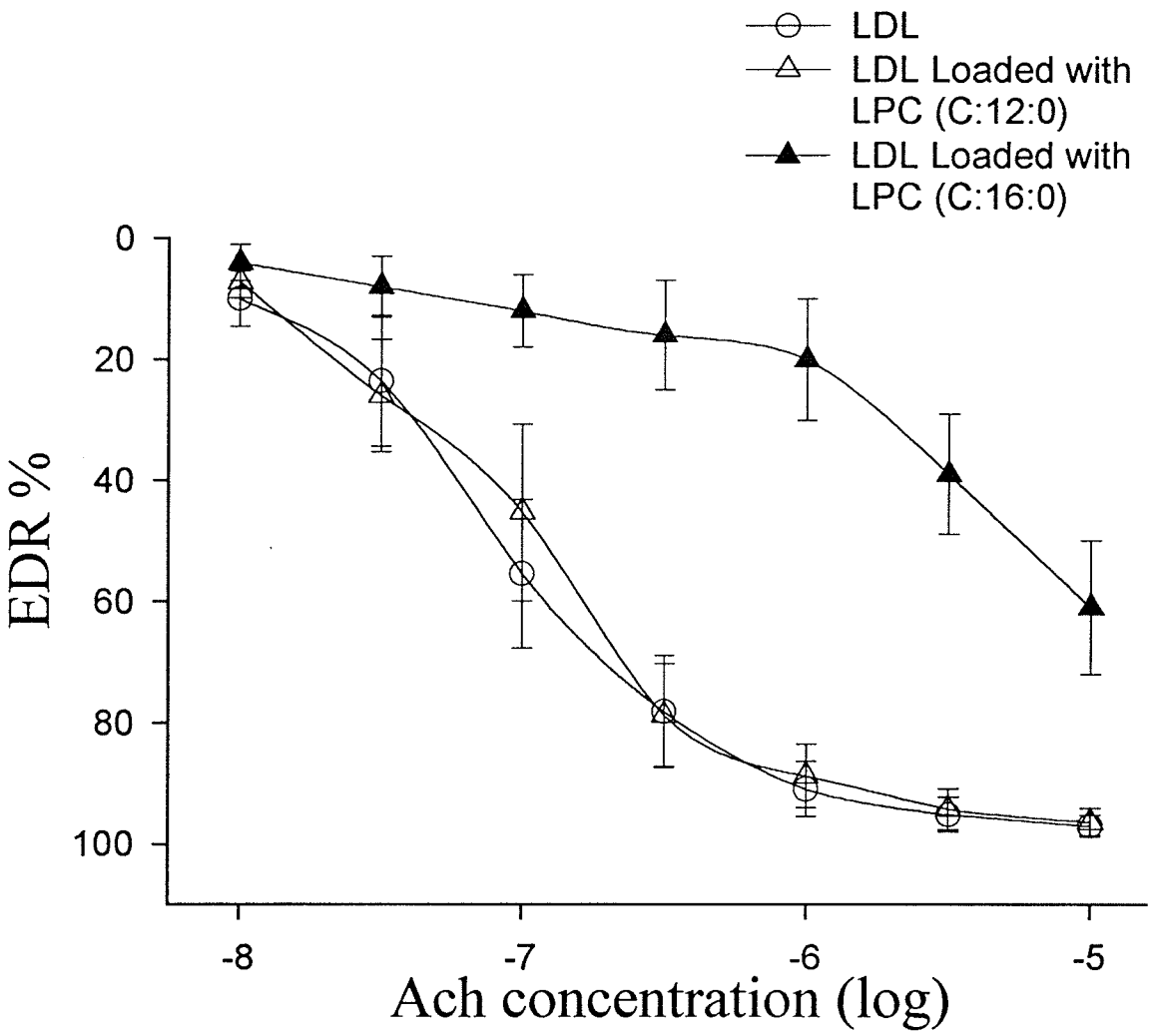

Figure 21. Effect of LPC-treated native low density lipoprotein on EDR of the rat aortic ring. The aortic ring preparation was exposed to LDL loaded with 1-lauroyl-sn-glycero-3-phosphocholine ($C_{12:0}$-LPC) or 1-palmitoyl-sn-glycero-3-phosphocholine ($C_{16:0}$-LPC) for 1 h. Native LDL (without LPC incubation) was used as control. Each point represents the mean of at least three experiments, each done in duplicate. The vertical bar represents the SEM.

Table 6. Subject data and blood lipid levels.

Normal Subjects			
	Male	Female	All
Age (years)	29 ± 10 (7)	34 ± 6 (6)	31 ± 9 (13)
BMI (kg/m^2)	24.7 ± 1.8	22.7 ± 1.5	23.8 ± 2.0
Total cholesterol (mmol/L)	4.12 ± 0.68	4.00 ± 0.31	4.07 ± 0.54
LDL cholesterol (mmol/L)	2.19 ± 0.25	2.10 ± 0.24	2.15 ± 0.25
HDL cholesterol (mmol/L)	1.24 ± 0.28	1.56 ± 0.35	1.39 ± 0.35
Triglyceride (mmol/L)	1.11 ± 0.31	0.73 ± 0.22	0.93 ± 0.33
Hyperlipidemic Subjects			
Age (years)	50 ± 12 (15)	53 ± 12 (14)	52 ± 12 (29)
BMI (kg/m^2)	28.5 ± 3.6	31.0 ± 8.9	29.7 ± 6.8
Total cholesterol (mmol/L)	7.40 ± 1.91	7.79 ± 2.31	7.59 ± 2.12
LDL cholesterol (mmol/L)	4.96 ± 1.65	4.31 ± 0.82	4.61 ± 1.31
HDL cholesterol (mmol/L)	1.19 ± 0.33	1.28 ± 0.30	1.23 ± 0.32
Triglyceride (mmol/L)	3.75 ± 2.43	4.62 ± 4.01	4.17 ± 3.32

Blood samples were drawn after subjects had fasted overnight and were collected in EDTA-containing tubes (4.0 mM). In contrast to normal subjects, hyperlipidemic subjects had elevated total and LDL cholesterol and triglyceride levels as defined by the Canadian Cholesterol Consensus Guidelines. Values are mean ± S.D.

hyperlipidemic subjects had markedly elevated total and LDL cholesterol and triglyceride levels. The elevated blood cholesterol and triglyceride levels did not show any gender-related difference. Although the mean ratio of LDL/HDL in men was slightly greater for both subject groups, these differences were not statistically significantly.

The effect of oxidatively modified LDL from normal and hyperlipidemic subjects on endothelium-dependent relaxation was assessed in phenylephrine precontracted rat aortic rings (Fig. 19). A slight inhibition of relaxation by native LDL from hyperlipidemic subjects was noted at the lowest Ach concentrations used. Oxidized LDL from both groups produced significant depression of endothelium-dependent relaxation at all Ach concentrations tested. The impairment induced by the oxidized LDL from hyperlipidemic subjects, however, was significantly greater than that from normal individuals at Ach concentrations from 10^{-6} to 10^{-5} M.

Table 4 provides TBARS and phospholipid contents in native and oxidized LDL from both subject groups. Regarding TBARS levels, these were elevated in native LDL and diminished in oxidized LDL from hyperlipidemic subjects (as compared with normal subjects). The principal phospholipids in LDL particles include phosphatidylcholine (PC), sphingomyelin and lysophosphatidylcholine (LPC). In native LDL, none of these were altered by hyperlipidemia. Upon oxidation, the concentration of LPC increased linearly with the time until 24-48 h after the initiation of oxidative modification (data not shown). The present results show that the concentration of LPC was increased 6-7 fold upon

oxidation of LDL and that this increase was significantly greater in LDL from hyperlipidemic subjects. A concomitant and quantitatively equivalent decrease in PC was also noted. Oxidation had no effect on sphingomyelin content in either subject group.

In order to ascertain whether the impairment of endothelium-dependent relaxation was related to LPC formed during the oxidation of LDL, correlation analysis was done for the data from normal and hyperlipidemic subjects (Fig. 22). The results showed good correlation between the impairment of endothelium-dependent relaxation and LPC formation in LDL from hyperlipidemic subjects (correlation coefficient = 0.740, $p < 0.05$); data from normal subjects showed no statistical correlation between the two parameters (correlation coefficient = 0.137, $p > 0.05$).

The data describing LDL from normal and hyperlipidemic groups and their effect on endothelium-dependent relaxation were further subgrouped and analyzed on the basis of subject gender. Regarding oxidation products (Table 7), there was no gender based difference on TBARS levels in either of the groups for native- or oxidized LDL. Native LDL contained similar levels of LPC in males and females of both subject groups. The oxidized LDL from male subjects, however, produced more LPC than that from females in both hyperlipidemic and normal subjects ($p < 0.05$). Concerning their physiological effects, for native LDL, there was no effect of gender on endothelium-dependent relaxation in either subject group (fig 23 and 24). The effect of gender was demonstrated with oxidized LDL in both the normal and hyperlipidemic groups. Oxidized LDL from

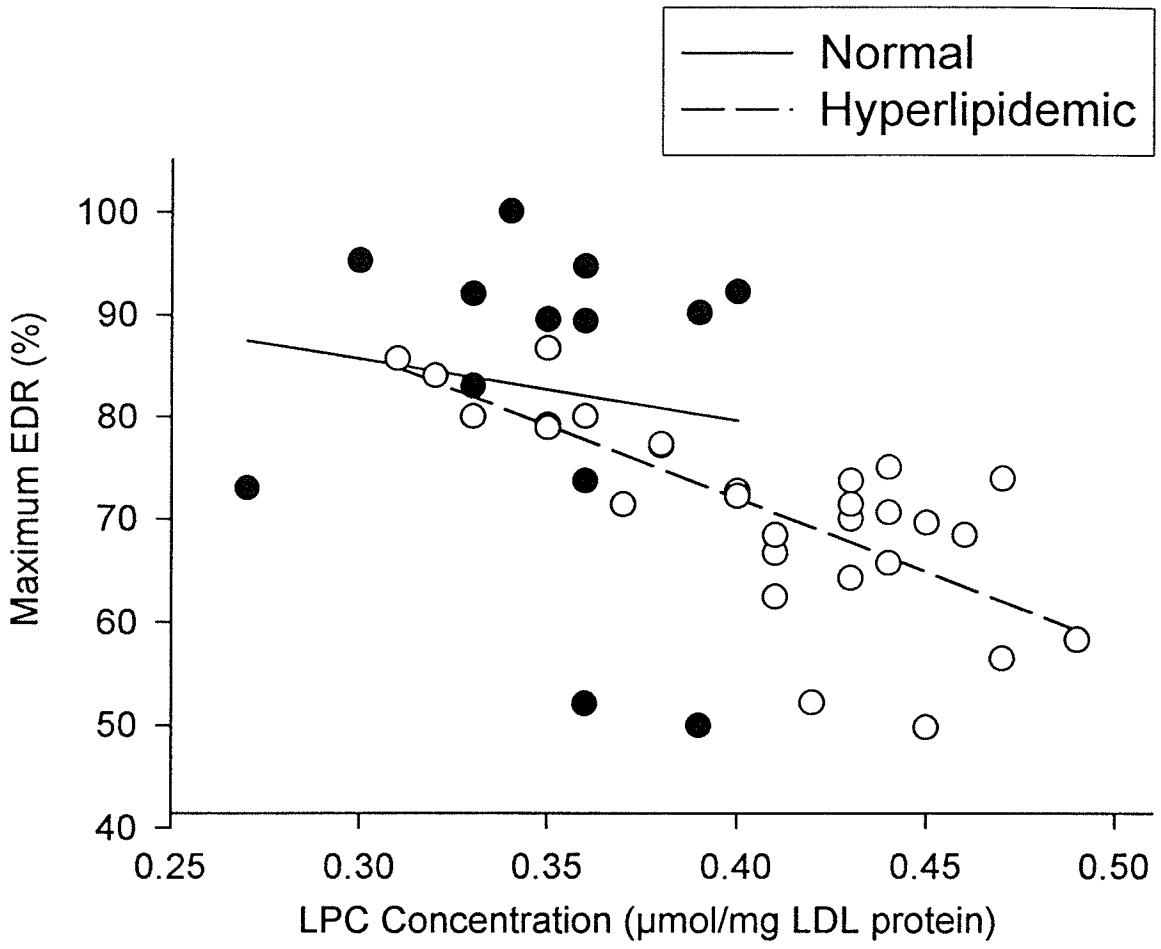

Figure 22. Correlation between the maximal EDR and LPC content in oxidized LDL from normal and hyperlipidemic subjects. The fractional maximum EDR (at 10^{-5} M Ach) of vessels equilibrated in the presence of oxidized LDL from normal (solid circles) and hyperlipidemic (open circles) subjects was calculated (as a fraction of that obtained by the vessels in the absence of oxidized LDL) and plotted as a function of LPC concentrations measured in the respective LDL samples. Statistical treatment utilized the Pearson product moment analysis and yielded a correlation coefficient of 0.137 ($p > 0.05$) for normal subjects and 0.740 ($p < 0.05$) for hyperlipidemic subjects.

Table 7. Effect of gender on TBARS and LPC in LDL from hyperlipidemic and normal subjects.

	Normal (13)	
	Male (7)	Female (6)
TBARS[1]		
Native LDL	0.85 ± 0.59	1.03 ± 0.58
Oxidized LDL	4.94 ± 19.41	75.65 ± 12.55
LPC[2]		
Native LDL	0.06 ± 0.01	0.05 ± 0.01
Oxidized LDL	0.37 ± 0.02	0.33 ± 0.03*
	Hyperlipidemic (29)	
TBARS[1]		
Native LDL	2.43 ± 1.00	2.14 ± 1.06
Oxidized LDL	58.02 ± 13.09	55.19 ± 10.15
LPC[2]		
Native LDL	0.06 ± 0.01	0.06 ± 0.02
Oxidized LDL	0.42 ± 0.04	0.39 ± 0.04*

Oxidized LDL from male subjects produced more LPC than that from females in both hyperlipidemic and normal subjects. Results represent means ± S.D. for each group.
* indicates that means for data from the female subjects are significantly ($p < 0.05$) different when compared to corresponding male subjects.
[1]nmol malondialdehyde equivalents / mg LDL protein
[2]micromoles / mg LDL protein.

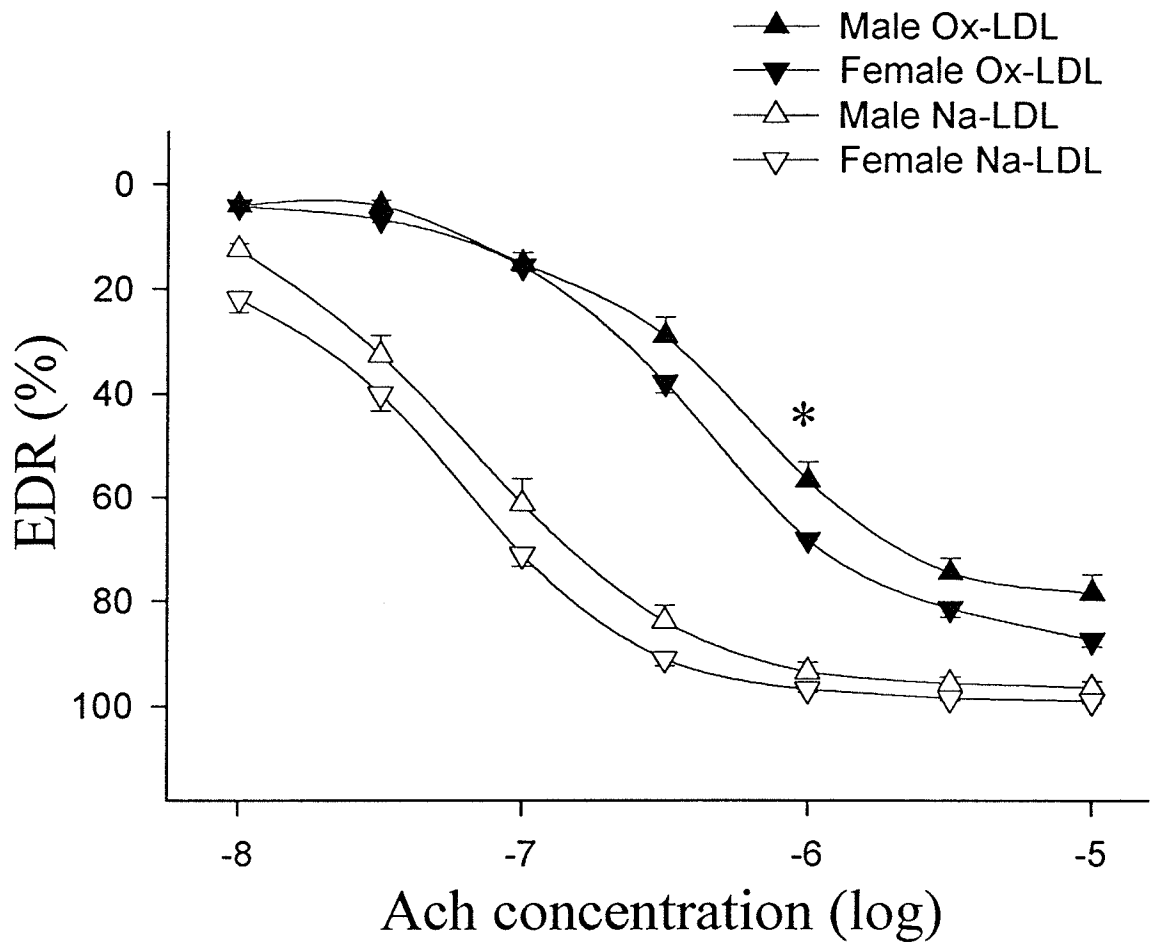

Figure 23. Effect of oxidized LDL from normal male and female subjects on Ach-induced endothelium-dependent relaxation (EDR). Fractional EDR was calculated on the basis of maximal relaxation produced at 10^{-5}M Ach. Solid and open symbols represent data obtained with oxidized- and native LDL (resp). Inverted and upright triangles represent data from female and male subjects (resp). Values are means ± SEM (n = 6 for female subjects; n = 7 for male subjects).

* indicate pairs (male ox-LDL vs. female ox-LDL) of means which are significantly different ($p < 0.05$).

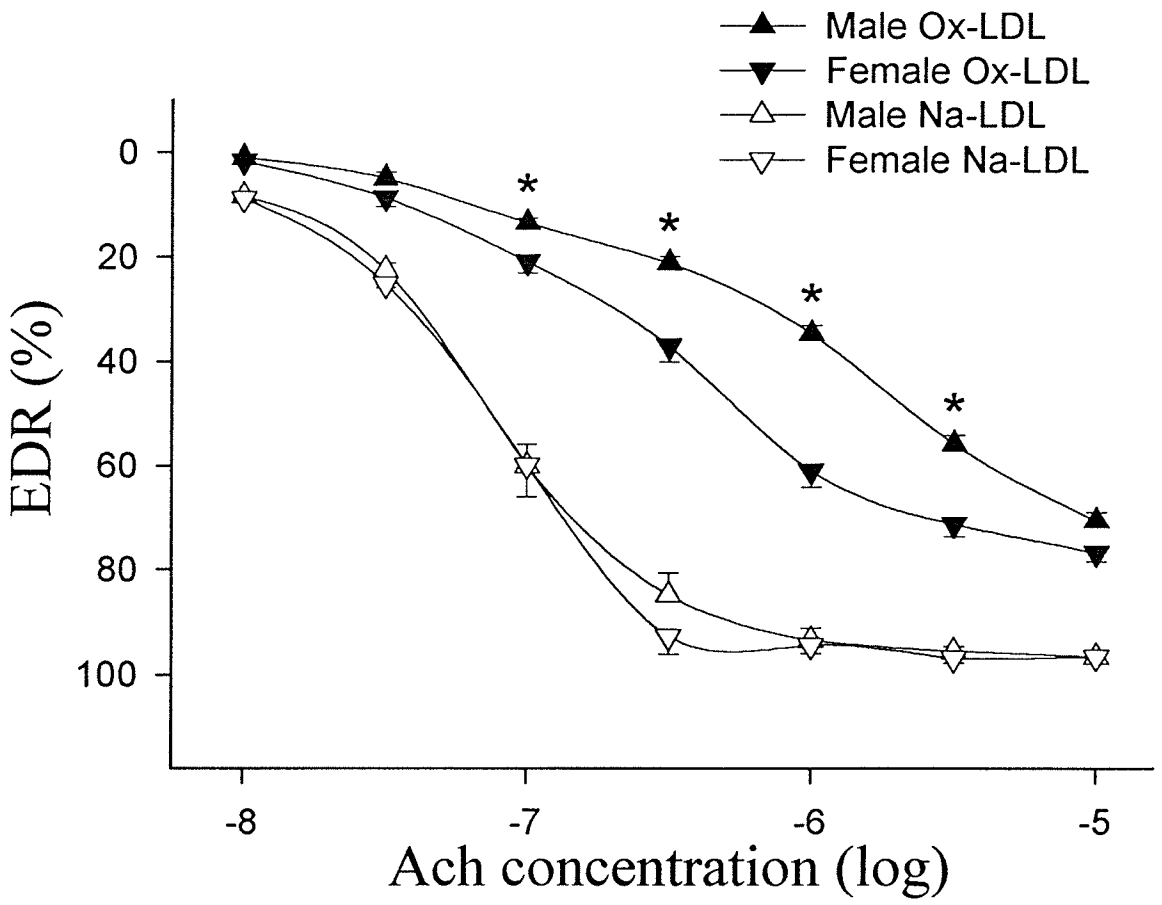

Figure 24. Effect of oxidized LDL from hyperlipidemic male and female subjects on Ach-induced EDR. The fractional EDR was calculated on the basis of maximal relaxation produced at 10^{-5}M Ach. Solid and open symbols represent data obtained with oxidized- and native LDL (resp). Inverted and upright triangles represent data from female and male subjects (resp). Values are means ± SEM (n = 14 for female subjects; n = 15 for male subjects).

* indicate pairs (male ox-LDL vs. female ox-LDL) of means which are significantly different ($p < 0.05$).

normal males produced significantly ($p < 0.05$) greater inhibition of aortic rings compared with that from females at Ach concentration 10^{-6} M (fig 23). In the hyperlipidemic group, oxidized LDL from males showed significantly greater inhibition of the endothelium-dependent relaxation than that from females at Ach concentrations 10^{-5} to 10^{-7} M (fig 24).

Correlation analysis was carried out for endothelium-dependent relaxation as a function of LPC content to compare the relationships for male and female subjects. Although the relationship for female subjects showed a higher correlation coefficient than that for males, (0.816 vs. 0.592), the oxidized LDL from males produced more LPC and greater impairment of endothelium-dependent relaxation (fig 25).

In view of the difference in mean age of the subjects available to this investigation through the Lipid Clinic and within the volunteer pool (respectively), the data for EDR and LPC content were replotted as a function of subject age for both normal and hyperlipidemic groups. The data (not shown) do not show any correlation with age for either of the parameters.

7. Effect of LDL on the release of arachidonic acid from human umbilical vein endothelial cells (HUVEC).

The release of arachidonic acid from the endothelial cells is indicative of the arachidonic acid contained within the cell. Arachidonic acid is the precursor of many

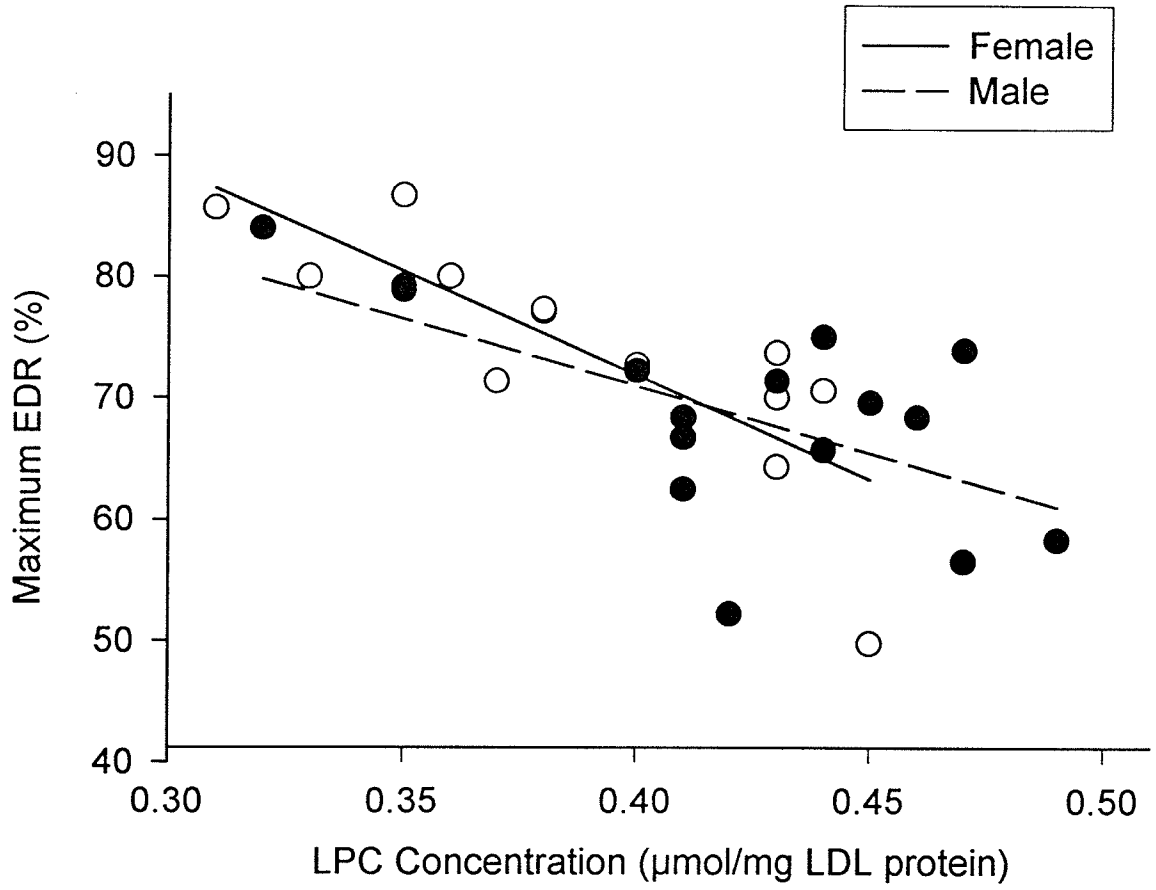

Figure 25. Correlation between the maximal EDR and LPC content in oxidized LDL from hyperlipidemic male and female subjects. The fractional maximum EDR (at 10^{-5}M Ach) of vessels equilibrated in the presence of oxidized LDL from hyperlipidemic male (solid circles) and female (open circles) subjects was calculated (as a fraction of that obtained by the vessels in the absence of oxidized LDL) and plotted as a function of LPC concentrations measured in the respective LDL samples. Statistical treatment utilized the Pearson product moment analysis and yielded a correlation coefficient of 0.592 ($p < 0.05$) for males and 0.816 ($p < 0.05$) for females.

vasoactive substances such as prostacyclin, which is a potent vasodilator. If the oxidation of LDL which results in the production of LPC affects the release of arachidonic acid, this may be another method by which ox-LDL affects the endothelial control of blood vessel tone. The effect of LDL concentration on arachidonic acid release from human umbilical vein endothelial cells (HUVEC) was examined. The cells were challenged with 1 ml of native- or oxidized-LDL (0, 0.1, 0.5, 1.0 mg/ml) for 10 min. Figure 26 illustrates that as the concentration of native or oxidized LDL increases there is an increase in the arachidonic acid released from the endothelial cells. Native LDL (0.5 and 1.0 mg/ml) stimulated a significantly ($p < 0.01$) greater release of arachidonic acid from endothelial cells when compared to the corresponding concentrations of oxidized LDL (Fig. 26).

The effect of LDL incubation time on the release of arachidonic acid from HUVEC was also examined. The cells were incubated with 1.0 mg/ml LDL for 5, 10, 20, and 30 min. The release of arachidonic acid from endothelial cells proceeds continuously. Native LDL stimulated a significantly ($p < 0.05$) greater amount of arachidonic acid release from the endothelial cells when compared to the control or to oxidized LDL (Fig. 27 and 28).

In Figure 28, lipids were removed from native- and oxidized-LDL by incubating the LDL for 2 h in the presence of 100 fold excess of fatty acid free albumin (0.01 mg LDL protein : 1 mg albumin). Incubation with albumin does not alter the release of arachidonic acid stimulated by native LDL (Fig. 28). The removal of lipids does significantly increase the release of arachidonic acid stimulated by oxidized LDL. To

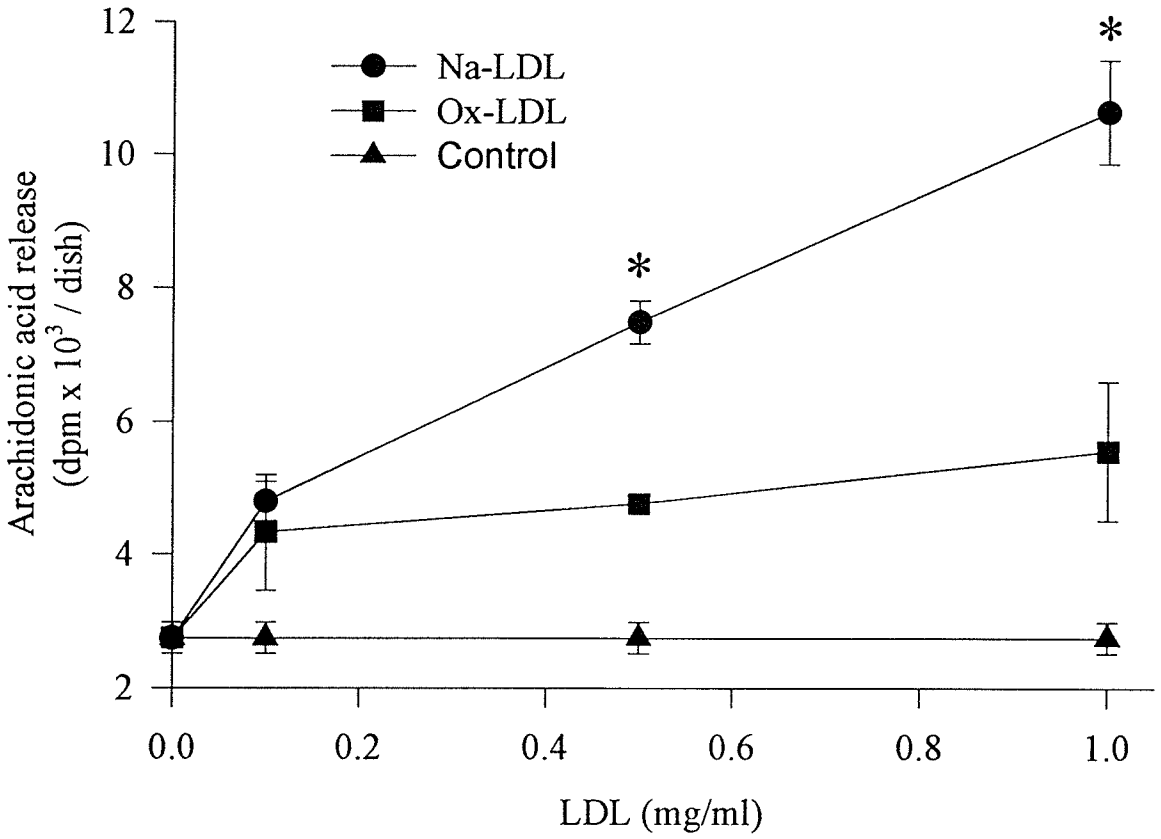

Figure 26. Effect of LDL concentration on arachidonic acid release from human umbilical vein endothelial cells (HUVEC). HUVEC were exposed to unoxidized (native) or oxidized LDL for 10 min to stimulate the release of arachidonic acid. Circles represent data obtained with native LDL. Squares represent data obtained with oxidized LDL. Values are means, error bars = SEM (n = 3, for all data points).

* indicates pairs (native vs. oxidized) of means which are significantly different ($p < 0.01$).

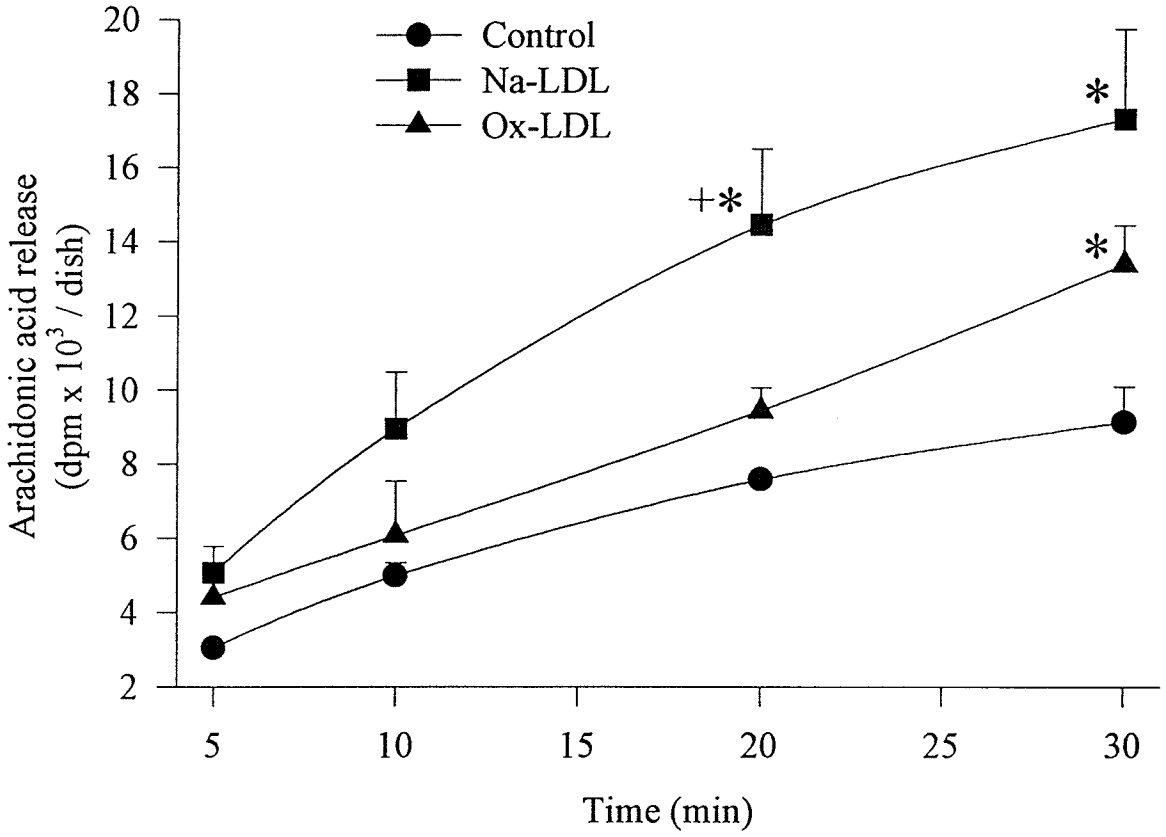

Figure 27. Effect of LDL incubation time on the release of arachidonic acid from human umbilical vein endothelial cells (HUVEC). HUVEC were exposed to unoxidized (native) or oxidized LDL (1.0 mg/ml) for 5, 10, 20, and 30 min to stimulate the release of arachidonic acid. Circles represent data obtained from the control (no addition), squares represent data obtained with unoxidized (native) LDL, and triangles represent data obtained with oxidized LDL. Values are means, error bars = SEM (n = 4, 6, 6 respectively).

* indicates pairs of means which are significantly different from the control ($p < 0.05$).

+ indicates pairs (native vs. oxidized) of means which are significantly different ($p < 0.05$).

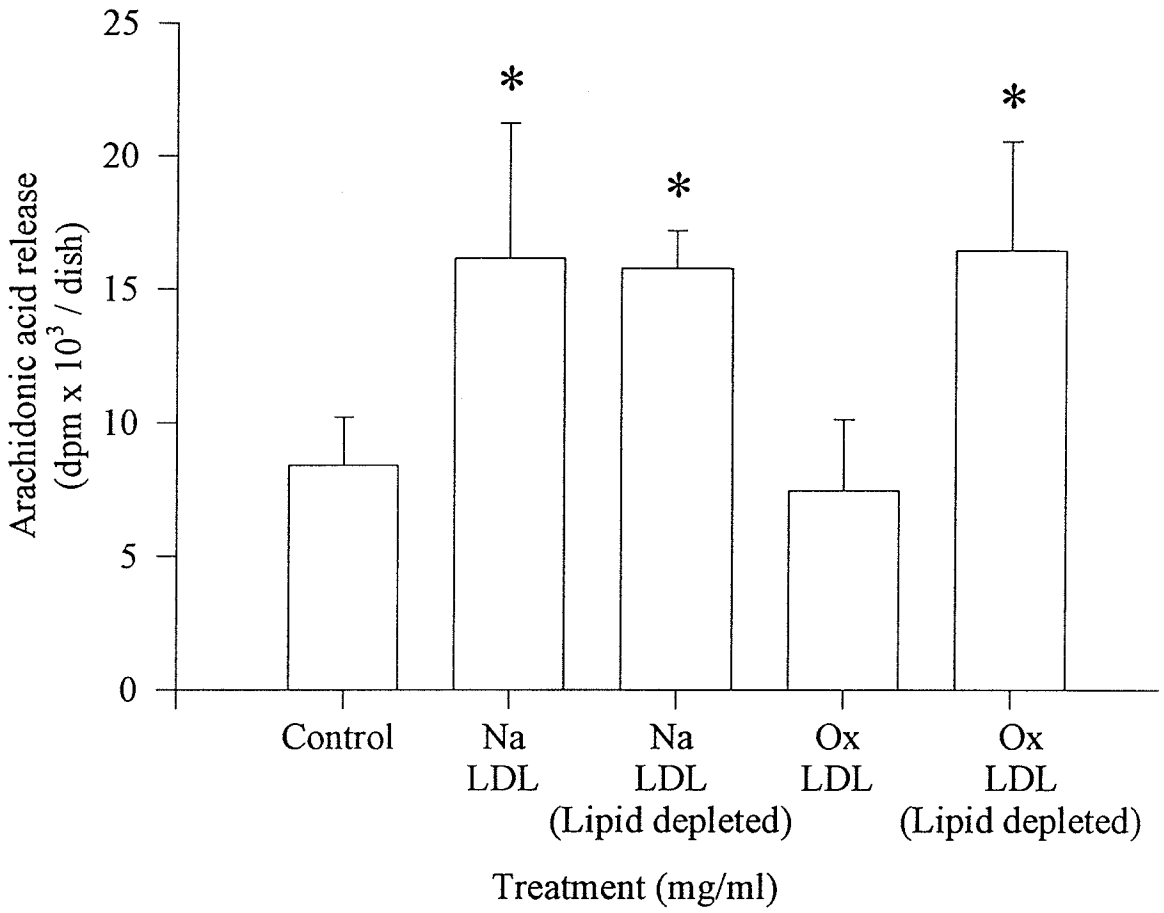

Figure 28. Effect of lipid removal from native and oxidized LDL on the release of arachidonic acid from human umbilical vein endothelial cells (HUVEC). HUVEC were exposed to unoxidized (naitve) LDL, lipid depleted native LDL, oxidized LDL, and lipid depleted oxidized LDL (1.0 mg/ml) for 20 min to stimulate the release of arachidonic acid. Values are means, error bars = S.D. (n = 6, for all data points).

* indicates pairs of means which are significantly different from the control and oxidized LDL ($p < 0.01$).

determine which lipids (if any) were being removed, lysophosphatidylcholine, sphingomyelin, and phosphatidylcholine were isolated by thin-layer chromatography and lipid phosphorus was determined by the method outlined by Zhou and Arthur (1992) using malachite green. There was statistically significant removal of lysophosphatidylcholine, sphingomyelin, and phosphatidylcholine from native and oxidized LDL by albumin (Table 8). Table 8 also illustrates the increase of lysophosphatidylcholine and the decrease of phosphatidylcholine upon oxidation that was seen earlier in the paper.

A phospholipase A_2 assay was used to determine if this was the enzyme responsible for the increase in arachidonic acid release from the endothelial cells by the native LDL when compared to oxidized LDL. Phospholipase A_2 activity for the control was 38.6 picomol/min/mg, phospholipase A_2 activity for native LDL was 30.8 picomol/min/mg, and phospholipase A_2 activity for oxidized LDL was 38.8 picomol/min/mg. Phospholipase A_2, therefore, does not appear to be responsible for the release of arachidonic acid in these assays.

Table 8. The depletion of lipids from native and oxidized LDL by albumin.

	Native LDL	Native LDL (lipid depleted)	Oxidized LDL	Oxidized LDL (lipid depleted)
		(nmol lipid phosphate / mg LDL)		
LPC	6.78 ± 0.96*	0.96 ± 0.44	19.22 ± 7.08*	1.37 ± 0.54
Sphingomyelin	9.38 ± 0.47*	2.71 ± 0.97	7.77 ± 3.32*	2.31 ± 0.50
PC	33.75 ± 1.19*	6.14 ± 7.61	14.51 ± 4.96*	2.92 ± 0.18

Results represent means ± S.D. (n = 3 for each group).

* indicates that means for data from LDL are significantly ($p < 0.01$) different from lipid depleted LDL.

LPC = lysophosphatidylcholine, PC = phosphatidylcholine.

IV. Discussion

1. The membrane perturbing effects of lysophosphatidylcholine (LPC).

Low density lipoprotein (LDL) is the main source and carrier of blood cholesterol (Brown and Goldstein 1976). In hyperlipidemic patients, plasma levels of LDL are associated with the occurrence of atherosclerosis (Farmer and Gotto 1992) and decreased endothelial function in arteries (Drexler and Zeiher 1991, Zeiher et al 1991). The elevated level of lysophosphatidylcholine (LPC) in oxidatively modified LDL has been shown to be the biochemical factor responsible for the impairment of endothelium-dependent relaxation in aortic ring preparations (Liu et al 1994, Yokoyama et al 1990).

Ach-induced vasodilation is endothelium dependent in that the endothelium must be intact to occur (Furchgott and Zawadzki 1980). Acetylcholine binds receptors on the luminal side of the endothelial membrane, activating a G-protein, which results in the production and release of nitric oxide from the endothelial cell. Nitric oxide diffuses from the endothelial cell to the smooth muscle and binds to iron at the active site of guanylyl cyclase, enhancing the production of cGMP (Ignarro et al 1987) which results in the relaxation of the smooth muscle cell.

Liu et al (1994) have demonstrated that impairment of endothelium dependent relaxation occurs in direct proportion to the amount of LPC produced in LDL during

oxidation. These results were confirmed by our findings in which 10 μM LPC impaired endothelium-dependent relaxation more than 5 μM LPC.

It has been demonstrated that vasodilators such as nitroglycerin, which act directly on the smooth muscle cells, are not inhibited by LPC (Kugiyama et al 1990). But vasodilators such as Ach which are regulated by G proteins (endothelial), are inhibited by LPC. Therefore, LPC likely perturbs the endothelial G protein pathway reducing the production of NO, subsequently, inhibiting the smooth muscle. To help determine where in the G protein pathway LPC might be causing this interference, we examined the effect of exposure time to LPC on endothelium dependent relaxation. If LPC is acting in the cytosol of the endothelial cell, it would be expected that the longer the aortic ring is exposed to LPC, the more EDR will be inhibited. The rationale relates to the time required to cross the phospholipid bilayer and enter the cytosol. We found that inhibition of EDR by LPC is rapid and it appears, therefore, to act at the endothelial membrane.

How could LPC interfere with the production of EDRF at the endothelial membrane? Fink and Gross (1984) found that the incorporation of LPC into the sarcolemmal membrane resulted in an increase of transmembrane fluidity by increasing the mean squared amplitude of motion in the bilayer . The alteration of membrane fluidity by detergents such as LPC alters the activity of many membrane bound enzymes such as guanylate cyclase (Menon et al 1989) and protein kinase C (Bing et al 1993). We hypothesize that LPC may be interfering with the G protein pathway at the membrane of

the endothelium by altering the fluidity of the membrane. This theory is supported by the fact that inhibition of EDR by LPC is rapid and dependent upon the concentration, with the fluidity change relating directly to LPC concentration.

To determine if LPC inhibits EDR by a generalized mechanism, such as altering the fluidity of the membrane, the experiment was repeated with palmitoyl carnitine which is a compound similar in structure to LPC. Although palmitoyl carnitine does not have a choline, glycerol, or phosphate group, it does possess a quaternary ammonium which gives it a charged polar head capable of interacting with water. The other similarity is that palmitoyl carnitine has one long chain fatty acid. Palmitoyl carnitine inhibited EDR at the same concentrations as LPC rapidly and in a concentration dependent manner. This lends further support for the theory that LPC may simply be altering the fluidity of the membrane resulting in interference in the G protein pathway and reducing the production of NO.

The next question concerns the identity of the enzyme(s) which might be affected by membrane fluidity. LPC has been shown to activate protein kinase C (PKC) (Bing et al 1993) which can phosphorylate nitric oxide synthase (NOS) and reduce its ability to produce NO (Bredt et al 1992). In fact, NOS is phosphorylated by cAMP dependent protein kinase (PKA), PKC, and calcium / calmodulin dependent protein kinase at different serine sites, resulting in multiple means of regulation (Bredt et al 1992). PKC can also phosphorylate the enzyme trisphosphate-5'-phosphomonoesterase which breaks

down IP_3 (Connolly et al 1986). A reduction of IP_3 will reduce the availability of intracellular calcium to form a complex with calmodulin, the latter complex is required for NOS activation.

Assuming Ro31-8220 is blocking PKC, it appears that PKC is involved in the mechanism by which LPC inhibits endothelium-dependent relaxation. Ro31-8220 reduced the ability of both LPC and palmitoyl carnitine to inhibit endothelium-dependent relaxation.

It has also been suggested that LPC selectively inhibits a G_i protein dependent pathway by disrupting receptor-G protein interactions (Flavahan 1993). Flavahan (1993) found that LPC did not affect endothelium-dependent relaxations evoked by direct activation of G_i protein stimulated by flouride ions. Therefore, LPC may disrupt the interaction between G proteins and membrane bound receptors which may account for the inhibition not attributable to PKC.

If LPC is altering the fluidity of the membrane, the ability of the aortic ring to relax (endothelium-dependent) should return if LPC is removed from the membrane. This was accomplished by incubating the aortic rings in Krebs solution containing defatted bovine serum albumin (BSA; which is an acceptor for hydrophilic lipids, Murohara et al 1994) after exposure to LPC. It should be noted that once LPC is incorporated into the endothelial membrane, it is slowly reacetylated into phosphatidylcholine (Stoll et al 1990). This may account for the reduction in inhibition of EDR by LPC in the absence of BSA.

BSA did eliminate the remaining effect of LPC returning the function of the endothelium to control vascular tone. BSA could only remove LPC that is in the membrane, not in the cytosol. It would, therefore, appear that LPC inhibits EDR at the membrane.

Our findings suggest that LPC alters endothelium-dependent relaxation by altering the fluidity of the membrane, thus interfering in the G protein pathway via interaction between the receptor and G protein or via enzymes such as PKC. The latter interference reduces the production of NO and the ability of the aortic ring to relax.

There is evidence that another endothelium-dependent factor, EDHF, is involved in vasodilation. Ach increases the production of NO (EDRF), but NO can not account for the actions of EDHF. Chen et al (1988) found that the blockade of the synthesis of NO had little effect on Ach-induced hyperpolarization. The fact that Ach has a vasodilator effect, even in the presence of NOS inhibitors, leads us to believe an EDRF other than NO exists (Vanhoutte et al 1995).

In our rat aortic ring preparation, the rings were also exposed to nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, and/or LPC. L-NAME impaired endothelium-dependent relaxation induced by acetylcholine. The two treatments (L-NAME and LPC) together potentiate impairment of endothelium-dependent relaxation. In a second series of aortic ring experiments, the concentration of K^+ was increased in an effort to block hyperpolarization resulting in impairment of EDR. If LPC was added to this preparation, EDR was impaired significantly more. In conclusion, the mechanism by

which LPC impairs endothelium-dependent relaxation appears to involve both EDRF (NO) and EDHF.

2. Oxidative modification of low density lipoprotein in normal and hyperlipidemic patients: Effect of LPC composition on vascular relaxation.

The increased incidence of atherogenic lesions in hyperlipidemic patients has been well documented (Ross 1992). The production of atherosclerotic lesions in hyperlipidemia is initiated by an elevated level of plasma LDL and the enhanced uptake of LDL by macrophages (Steinberg et al 1989). Our initial studies were designed to document the elevated LDL content in hyperlipidemic patients and to investigate the effect of oxidatively modified LDL preparations on endothelium-dependent relaxation. As was expected, high levels of total cholesterol, LDL cholesterol and triglyceride were detected in the plasma of hyperlipidemic patients. Unmodified LDL from hyperlipidemic patients did not impair the endothelium-dependent relaxation. Of particular interest, however, was the greater impairment of endothelium-dependent relaxation by the oxidatively modified LDL from hyperlipidemic patients as compared with that from the control group. These results are functionally consistent with previous findings that LDL levels in hyperlipidemic patients are associated with loss of endothelial function and constriction of blood vessels (Zeiher 1991).

Analysis of the lipid content in the oxidatively modified LDL from hyperlipidemic patients revealed that the level of LPC was greatly elevated, with a corresponding decrease in the level of PC (phosphatidylcholine). Changes in the phospholipid composition were significantly different from the values obtained with LDL samples from normal subjects under the same treatment. LDL from hyperlipidemic subjects produced more LPC than that from normals. This finding was expected since (a) the oxidatively modified LDL from hyperlipidemic patients has been shown to produce a stronger impairment of the endothelium-dependent relaxation and (b) an elevated level of LPC has been postulated to be a factor responsible for that impairment (Kugiyama et al 1990, Murohara et al 1994). Our hypothesis, therefore, was that a higher level of LPC would be present in the oxidized LDL of the hyperlipidemic group. Although the biochemical event leading to the formation of LPC from PC during oxidative modification remains unclear, it could be mediated via the hydrolysis of PC by the action of a phospholipase A_2 (Parthasarathy and Barnett 1990).

LPC is not a single entity, but is composed of molecular species with different acyl groups (White 1973). The physical and biological properties of LPC are dependent on the chain length of the constituent acyl groups (Weltzien 1979). Molecular species with long acyl chains are more hydrophobic (Weltzien 1979) and any increase in their proportion may alter the biological properties of the LDL. The findings of this study are novel in providing the first evidence that oxidative modification of LDL produces changes in the

acyl composition of LPC. These changes are characterized by the elevated long-chain acyl content which is further increased in the LDL from hyperlipidemic subjects.

This study is also the first to demonstrate that the ability of LPC to impair endothelium-dependent relaxation is a function of the chain length of the acyl group. In the native LDL, the very small amount of LPC, together with the preponderance of the short- and medium-chain acyl groups present, would predict a minimal effect on endothelium-dependent relaxation. Upon oxidative modification, however, the dramatic increase of LPC content, together with a higher proportion of long-chain ($\geq C_{16}$) acyl content, would provide the oxidized LDL with an enhanced effectiveness in impairing endothelium-dependent relaxation. The different effect of the molecular species of LPC on the impairment of the endothelium-dependent relaxation has been demonstrated with both free LPC and native LDL loaded with LPC. The result obtained in this study provides a reasonable explanation for the greater impairment of endothelium-dependent relaxation by the oxidized LDL of hyperlipidemic patients. In view of the similar LPC contents found in the LDL of both subject groups, it is clear that the higher proportion of the long-chain moiety found in the oxidized LDL from hyperlipidemic patients is responsible for its enhanced ability to impair endothelium-dependent relaxation of the vascular preparation. We propose that the high level of LDL found in the plasma of hyperlipidemic patients, coupled with the enhanced ability to generate long chain species

of LPC during oxidative modification, are important factors that contribute to the production of atherosclerosis in these patients.

3. Impairment of endothelium-dependent relaxation by oxidatively modified LDL is gender-dependent in hyperlipidemic patients.

In view of the increased risk of coronary artery disease (CAD) in hyperlipidemic patients, the important role of the endothelium in the regulation of vascular tone and the inhibitory effect of oxidatively modified LDL on endothelial function, the present studies investigated the possibility that LDL from hyperlipidemic subjects specifically modulates the control of vascular tone by the endothelium. An additional aspect of the study was to test the hypothesis that the increased risk of CAD in men could be linked to alterations in blood lipids. Our findings indicate that upon oxidation, 1) LDL from hyperlipidemic subjects produces more LPC than that from normals, 2) LDL from men produces more LPC than that from women, 3) LPC is correlated with inhibition of endothelium-dependent relaxation in hyperlipidemic, but not in normal subjects and 4) LDL from men produces greater inhibition of the endothelium and this effect is exacerbated in hyperlipidemia.

The importance of oxidative modification of LDL has been recognized not only through its association with endothelial control of vascular tone but also through its association with hypercholesterolemia and the development of atherosclerotic plaque

(Morel et al 1984, Parthasarathy et al 1986, Steinbrecher et al 1984, Andrews et al 1987, Galle et al 1991, Simon et al 1990, Plane et al 1992, Sorensen et al 1994, Sreeharan et al 1986, Mangin et al 1993). The determination of susceptibility to oxidation by LDL, however, has been frequently made on the basis of indicators such as TBARS formation or lag time in conjugated diene production (Raal et al 1995, Rabini et al 1994, Bruckdorfer et al 1995, Regnstrom et al 1992, Andrews et al 1995, Stalenhoef et al 1994, Miwa et al 1995) which have no pathophysiological significance. In view of the role of LPC, both as a biological oxidation product of phosphatidylcholine (PC) in LDL (Steinbrecher et al 1984, Liu et al 1994) and as a potent inhibitor of endothelial function which has been linked to CAD, the present study focused on this metabolite in measuring oxidative modification of LDL and assessing its impact on endothelium-dependent vascular relaxation.

The present findings show enhanced susceptibility to oxidation in LDL from hyperlipidemic subjects and that the effect is strongest in men. Susceptibility to oxidation can be related to antioxidant content (Princen et al 1995, Princen et al 1992, Jialal 1992), activation of an intrinsic phospholipase A_2 (Parthasarathy and Barnett 1990), fatty acid composition of LDL (Corby et al 1993, Croft et al 1995) and the presence of small dense LDL (Chait et al 1993, Tribble et al 1995). Concerning the effect of hyperlipidemia on oxidative LPC formation, this is unlikely to be a result of differential antioxidant content as the dialysis procedure (required to remove Cu^{2+} in sample preparation) removes most

of the intrinsic antioxidants, principally vitamin E (Packer 1991, Scheek et al 1995). Although fatty acid composition was not systematically documented in this study, the high cholesterol and triglyceride profile of the hyperlipidemic subjects has been associated with a high content of small dense LDL which are more susceptible to oxidative modification (Chait et al 1993, Tribble et al 1995).

The finding of greater LDL oxidation in hyperlipidemic men, despite the similar blood cholesterol and triglyceride levels in the hyperlipidemic men and women subjects was surprising. The benefits of estrogen, in terms of reducing risk of CAD, include decreased LDL:HDL ratios (Manson 1994) and direct antioxidant effects (Knopp et al 1994, Sack et al 1994) in addition to the indirect antioxidant effect of increased HDL. In the present subject sample, the female component of which likely included a majority of post-menopausal women (none of whom reported being on estrogen replacement therapy), gender-related differences between ratios of LDL:HDL were of borderline statistical significance ($p > 0.05$). Nevertheless, the strong evidence of estrogen benefit in terms of therapeutic use (Manson 1994, Brinton 1996 and including specific observations of an inhibition of LDL oxidation, ref Sack et al 1994), elevated coronary artery disease (CAD) risk associated with oophorectomy (Stampfer and Colditz 1990) and advantageous lipoprotein profile attributed to hormonal differences between the genders (Manson 1994, Tikkanen 1990) would permit interpretation of the present data in terms of residual estrogen benefit regarding LDL oxidation.

The present study also demonstrated significant inhibitory effects of LPC on endothelium-dependent relaxation, an effect which was more severe both in hyperlipidemics and in males. Linkage of enhanced LDL oxidation with inhibition of endothelial function includes the observations that 1) the formation of LPC from PC in LDL oxidation is the most prominent lipid-related event (Steinbrecher et al 1984, Liu et al 1994), 2) exposure to LPC in vitro causes inhibition of endothelial-dependent vascular relaxation (Kugiyama et al 1990, Murohara et al 1994, Yokoyama et al 1990) and 3) oxidized LDL lipids inhibit inducible NO synthase in activated macrophages (Yang et al 1994). Although the present results do not support speculation regarding the reason for the lack of correlation between LPC content and inhibition of EDR in normal subjects, a qualitative change, with significant correlation between the two variables in both gender groups of hyperlipidemic subjects was noted. Further, the relationship was significantly stronger in LDL samples from men, thus underscoring the regulatory significance of the enhanced oxidative LPC formation in these samples. The latter findings are consistent with the correlation of coronary vasomotor response to acetylcholine with the male gender in patients having angiographically smooth coronary arteries (Vita et al 1990). The biochemical basis for these differences, in terms of lipid composition and interaction with the endothelium deserve further investigation. In view of the significant differences noted in the hyperlipidemic group, these suggest a systematic study regarding estrogen levels in samples from females and age of subjects.

We conclude that the changes in LDL oxidation in hyperlipidemic subjects and particularly in men are consistent with greater inhibitory effect on endothelial function and a role in the increased risk of arterial vasospasm of these subpopulations.

4. Effect of LDL on the release of arachidonic acid from human umbilical vein endothelial cells (HUVEC).

Arachidonic acid (AA) is the precursor of eicosanoids (Smith 1989) and its release is essential for the biosynthesis of eicosanoids such as prostacyclin. Prostacyclin, a potent vasodilator and an inhibitor of platelet aggregation (Moncada et al 1976), is the main prostanoid produced by vascular endothelial cells (Smith et al 1991b). Thromboxane, which is the main prostanoid produced by the platelets (Smith et al 1991b), has biological actions which oppose prostacyclin, therefore a balance is required. Thromboxane is a strong contractor of blood vessels and induces platelet aggregation (Moncada and Vane 1979).

We found that na-LDL stimulated a significantly greater release of AA from HUVEC when compared to ox-LDL. When the ox-LDL was depleted of its LPC, ox-LDL stimulated a release of AA from HUVEC not significantly different from that produced by na-LDL. It appears that LPC inhibits the release of AA from HUVEC. This in turn would reduce the amount of prostacyclin produced within the cell and destabilize the balance between prostacyclin (a vasodilator produced by the endothelial cells) and thromboxane (a vasoconstrictor produced by the platelets), thus resulting in

vasoconstriction. It has been demonstrated that exposure to ox-LDL abolishes the production of prostacyclin by cultured bovine endothelial cells (Thorin et al 1994). This inability to produce prostacyclin may also be involved in the progression of the fatty streak to a more advanced lesion.

One of the properties of ox-LDL is that it inhibits endothelium-dependent relaxation (Parthasarathy et al 1992). The elevated level of LPC in oxidatively modified LDL has been shown to be the biochemical factor responsible for the impairment of endothelium-dependent relaxation in aortic ring preparations (Yokoyama et al 1990, Liu et al 1994). LPC, a natural amphiphile, has been shown to readily incorporate into lipid membranes (Kawashima and Bell 1987) and change physical properties of the lipid bilayer such as membrane fluidity and permeability (Kitigawa et al 1976, Fink and Gross 1984, Weltzien 1979). We postulate that the effect of ox-LDL on AA release is mediated through the membrane perturbing property of LPC.

The hydrolysis of the acyl chain at the sn-2 position of glycerophospholipids by phospholipase A_2 (PLA_2) is regarded as the major source of AA release. Many isoforms of PLA_2 have been identified but only the 14 kDa secreted group II PLA_2 ($sPLA_2$) and the 85 kDa cytosolic PLA_2 ($cPLA_2$) have been purified and well characterized (Mayer and Marshall 1993, Clark et al 1995). The $sPLA_2$ requires extracellular (millimolar) Ca^+ for activation while $cPLA_2$ requires intracellular (micromolar) Ca^+ for translocation to the cell

membrane. These isoforms are found in endothelial cells and are involved in AA release and PGI_2 production (Murakami et al 1993).

An increase in PLA_2 enzyme activity would be expected in HUVEC exposed to na-LDL compared with HUVEC exposed to ox-LDL because na-LDL stimulated a greater release of AA. The PLA_2 enzyme assay did not indicate any increase when HUVEC was exposed to na-LDL. It is believed that this is simply a limitation of our model.

The requirement of Ca^{2+} for the production of EDRF (NO), EDHF, and the release of AA from endothelial cells appears to be a unifying theme. In the case of EDRF stimulated by acetylcholine, acetylcholine binds receptors on the luminal side of the endothelial membrane, activating a G-protein. The activated G-protein will turn on the enzyme phospholipase C which cleaves phosphatidylinositol 4, 5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 will move into the cytosol of the endothelial cell and bind to receptors on the endoplasmic reticulum causing the release of Ca^{2+} from intracellular stores (Dinerman et al 1993). Ca^{2+} will form a complex with calmodulin which then binds and activates nitric oxide synthase (NOS) to produce nitric oxide (NO) (Bredt et al 1992).

The release of EDHF is regulated by cytosolic calcium and inhibited by calmodulin antagonists (Nagao and Vanhoutte1993). Ach increases the endothelial cytosolic Ca^{2+} which activates Ca^{2+}-activated K^+ channels (K_{Ca}) inducing membrane hyperpolarization

which increases the driving force of Ca^{2+} into the cell. The increase in Ca^{2+} results in an increase in production of NO and EDHF which diffuses to the vascular smooth muscle. NO increases the production of cGMP which reduces the Ca^{2+}inside the smooth muscle (possibly by a $Na^{+}\backslash Ca^{2+}$ exchanger) cell causing it to relax. EDHF is hypothesized to increase K_{Ca} channel activity on the smooth muscle membrane resulting in hyperpolarization and reduced Ca^{2+} entry via voltage-sensitive L-type Ca^{2+} channels, reducing cytosolic Ca^{2+}, resulting in relaxation (Waldron et al 1996) .

In conclusion, we hypothesize that LPC from ox-LDL perturbs the fluidity of the endothelial membrane, resulting in an alteration of cytosolic Ca^{2+} within the endothelial cell. EDRF (NO), EDHF, and prostacyclin are all potent vasodilators. In each case, if the cytosolic level of Ca^{2+} in the endothelial cell can not be increased, then the vasodilator will not be produced. The final result being dysfunction of endothelium-derived relaxation of the vasculature which is more pronounced in the hyperlipidemic and male subgroups.

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