

**EFFECTS OF ATORVASTATIN TREATMENT ON THE OXIDATIVELY MODIFIED LOW  
DENSITY LIPOPROTEIN IN HYPERLIPIDEMIC PATIENTS**

By

**Quansheng Zhu**

A Thesis Submitted to Faculty of Graduate Studies  
in partial fulfillment of the requirements for the degree of

Master of Science

Department of Physiology

University of Manitoba

**Quansheng Zhu©1999**



National Library  
of Canada

Acquisitions and  
Bibliographic Services

395 Wellington Street  
Ottawa ON K1A 0N4  
Canada

Bibliothèque nationale  
du Canada

Acquisitions et  
services bibliographiques

395, rue Wellington  
Ottawa ON K1A 0N4  
Canada

*Your file Votre référence*

*Our file Notre référence*

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-41653-4

**Canada**

**THE UNIVERSITY OF MANITOBA**  
**FACULTY OF GRADUATE STUDIES**  
**\*\*\*\*\***  
**COPYRIGHT PERMISSION PAGE**

**EFFECTS OF ATORVASTATIN TREATMENT ON THE OXIDATIVELY MODIFIED  
LOW DENSITY LIPOPROTEIN IN HYPERLIPIDEMIC PATIENTS**

**BY**

**Quansheng Zhu**

**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**

**Quansheng Zhu©1999**

**Permission has been granted to the Library of The University of Manitoba to lend or sell copies of this thesis/practicum, to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film, and to Dissertations Abstracts International to publish an abstract of this thesis/practicum.**

**The author reserves other publication rights, and neither this thesis/practicum nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.**

## ACKNOWLEDGEMENTS

I would like to thank my supervisor and friend Dr. Edwin A. Kroeger. Ed, I am not only grateful for your excellent supervision in my research, but also for your great help in my English as well as many other things in my life. I will be always in debt to you.

I would like to thank my co-supervisor Dr. Patrick C. Choy. Pat, I enjoyed very much the time when I did research in your lab. I look to you as a great scientist and friend. I have learned a lot from you, about scientific research, life, and other matters. I will always feel a debt of gratitude.

I would especially like to thank Stella Choy, whose kind help so materially benefited this research. I very much appreciate her kindness and generosity.

I would like to thank Dr. Grant Hatch for his generous help with the gas-chromatography.

I would like to thank my colleagues Jason McMaster, Douglas Lee, Munroe Chan and Monica Skrzypczak for their good-hearted friendship which made the laboratory an enjoyable place to work. I am also very grateful for their technical help and collegial advice.

I would like to acknowledge my committee advisors, Dr. E.A. Kroeger, Dr. P.C. Choy, and Dr. P. Zahradka, for their critical assessment of this thesis.

I would also like to thank my wife, Hong Tang, for her love, support and encouragement. I would also like to thank my mother for her love and inspiration toward excellence. I dedicate this thesis to my wife and mother.

## TABLE OF CONTENTS

<b>LIST OF FIGURES</b>	vii
<b>LIST OF TABLES</b>	viii
<b>LIST OF ABBREVIATIONS</b>	ix
<b>ABSTRACT</b>	xi

### **I. INTRODUCTION**

<b>1. Regulation of vascular tone</b>	1
1.1 Endothelium-mediated regulation	1
1.1.1 The role of endothelium	1
1.1.2 Endothelium-dependent vasodilation	2
1.1.2.1 Endothelium derived-relaxing factor (EDRF)	2
1.1.2.1.1 Discovery of EDRF	2
1.1.2.1.2 Nitric oxide synthesis	2
1.1.2.1.3 Functions of EDRF	3
1.1.2.1.4 Regulation EDRF synthesis	4
1.1.2.2 Prostacyclin	6
1.1.2.3 EDHF	7
1.1.3 Endothelium-dependent vasoconstriction	10
1.1.3.1 Endothelin-1	10

1.1.3.2	<b>Vasconstrictor prostanoids and renin-angiotensin system</b>	10
1.2	<b>Metabolic regulation</b>	11
1.3	<b>Neural influences</b>	11
1.4	<b>Autoregulation</b>	12
1.5	<b>Humoral factors</b>	12
<b>2.</b>	<b>Pathology of atherosclerosis</b>	12
2.1	<b>Structure of the artery</b>	13
2.2	<b>The lesions of atherosclerosis</b>	15
2.2.1	<b>The fatty streak</b>	15
2.2.2	<b>The fibrofatty lesion</b>	15
2.2.3	<b>The fibrous plaque</b>	16
<b>3.</b>	<b>Risk factors of atherosclerosis and the vascular endothelium</b>	16
3.1	<b>Modified response-to-injury hypothesis</b>	16
3.2	<b>Hyperlipidemia</b>	20
3.3	<b>Hypertension</b>	22
3.4	<b>Smoking</b>	22
3.5	<b>Male gender</b>	23
3.6	<b>Aging</b>	23
3.7	<b>Diet</b>	24
3.8	<b>Diabetes mellitus</b>	25
3.9	<b>Exercise</b>	25

<b>4.</b>	<b>Effect of oxidized LDL on vascular tone and its role in atherosclerosis</b>	<b>26</b>
4.1	Oxidative modification of LDL	26
4.2	Effect of oxidized LDL on vascular tone	29
4.3	Occurrence of oxidized LDL <i>in vivo</i>	30
4.4	Role of oxidized LDL in atherogenesis	30
<b>5.</b>	<b>The metabolism of cholesterol in the human body</b>	<b>35</b>
5.1	Metabolism of cholesterol	35
5.1.1	Synthesis and excretion of cholesterol	35
5.1.2	HMG-CoA reductase	36
5.2	Metabolism of chylomicron and very low density lipoprotein	40
5.3	Metabolism of low density lipoprotein	40
5.4	Metabolism of high density lipoprotein	43
<b>6.</b>	<b>The metabolism of triacylglycerol in the human body</b>	<b>45</b>
<b>7.</b>	<b>The metabolic pathway of lysophosphatidylcholine</b>	<b>45</b>
<b>8.</b>	<b>Clinical classifications of hyperlipidemia</b>	<b>48</b>
<b>9.</b>	<b>The pharmacological basis of atorvastatin treatment</b>	<b>50</b>
<b>10.</b>	<b>Atorvastatin treatment and oxidatively modified LDL in hyperlipidemic patients: statement of hypothesis</b>	<b>52</b>

## **II. MATERIALS and METHODS**

<b>1. Materials</b>	<b>53</b>
1.1 Drugs	53
1.2 Experimental animals	53
1.3 Chemical reagents	53
<b>2. Methods</b>	
2.1 Recruitment of hyperlipidemic patients	54
2.2 Dietary counseling of hyperlipidemic patients	54
2.3 Atorvastatin treatment of hyperlipidemic patients	54
2.4 Measurement of plasma lipid and lipoprotein contents in hyperlipidemic patients	56
2.5 Isolation of LDL samples from hyperlipidemic patients plasma	56
2.6 Protein determination	56
2.7 Oxidative modification of LDL	56
2.8 Assessment of endothelium-dependent relaxation in aortic rings	57
2.9 Biochemical analysis of native and oxidized LDL	60
2.10 Lipid phosphorus determination	60
2.11 Determination of acyl groups in LPC	62
2.12 Statistical analyses	62

### **III. RESULTS**

1. Effects of dietary counseling and atorvastatin treatment on plasma lipid levels in type IIa hyperlipidemic patients 63
2. Effects of dietary counseling and atorvastatin treatment on the oxidized LDL from hyperlipidemic patients: impairment of endothelium-dependent relaxation 67
3. Effects of dietary counseling and atorvastatin treatment on phospholipid contents in native and oxidized LDL from hyperlipidemic patients 71
4. Effects of dietary counseling and atorvastatin treatment on the distribution of acyl groups in LPC in native and oxidized LDL from hyperlipidemic patients 74

### **VI. DISCUSSION**

1. Effect of atorvastatin treatment on plasma lipid levels 78
2. Effect of atorvastatin treatment on LPC levels and endothelium-dependent relaxation 81
3. Other benefits of atorvastatin treatment 85

**V. REFERENCES**

87

## **LIST OF FIGURES**

Figure 1.	Endothelium derived relaxing factors.	9
Figure 2.	Structure of the artery.	14
Figure 3.	Relation between serum cholesterol levels and risk of coronary disease.	21
Figure 4.	Structure of LDL.	28
Figure 5.	Proposed mechanism by which oxidized LDL causes atherosclerosis.	34
Figure 6.	Proposed structure of HMG-CoA reductase.	38
Figure 7.	Regulation of HMG-CoA reductase.	39
Figure 8.	Metabolism of LDL.	42
Figure 9.	Overview of the metabolism of plasma lipoproteins.	44
Figure 10.	Combined pathway of triglyceride and LPC metabolism.	47
Figure 11.	Structural formula of atorvastatin.	51
Figure 12.	Endothelium-dependent relaxation.	59
Figure 13.	Bartlett lipid phosphorus analysis.	61
Figure 14.	Effect of oxidized LDL from patients before and after dietary intervention on the impairment of endothelium-dependent relaxation.	69
Figure 15.	Effect of oxidized LDL from patients after dietary intervention and after atorvastatin treatment on the impairment of endothelium-dependent relaxation.	70

## LIST OF TABLES

Table 1.	Functions of vascular endothelium in normal and injured states.	19
Table 2.	Atherogenic effects of oxidized LDL.	33
Table 3.	Classifications of hyperlipidemia and their genetic basis.	49
Table 4.	The American Heart Association and the National Cholesterol Education Program Step I diet.	55
Table 5.	Plasma lipid and lipoprotein contents in hyperlipidemic patients before and after dietary counseling.	65
Table 6.	Plasma lipid and lipoprotein contents in hyperlipidemic patients after dietary counseling and after atorvastatin treatment.	66
Table 7.	Phospholipid contents in native and oxidized LDL from hyperlipidemic patients before and after dietary counseling.	72
Table 8.	Phospholipid contents in native and oxidized LDL from hyperlipidemic patients after dietary counseling and after atorvastatin treatment.	73
Table 9.	Distribution (%) of acyl groups in lysophosphatidylcholine in native and oxidized LDL from hyperlipidemic patients before treatment and after dietary counseling.	76
Table 10.	Distribution (%) of acyl groups in lysophosphatidylcholine in native and oxidized LDL from hyperlipidemic patients after dietary counseling and after atorvastatin treatment.	77

## **LIST OF ABBREVIATIONS**

<b>ACAT</b>	<b>acyl CoA: cholesterol acyltransferase</b>
<b>apoB-100</b>	<b>apoproteinB-100</b>
<b>BH<sub>4</sub></b>	<b>(6R)-5,6,7,8-tetrahydrobiopterin</b>
<b>cAMP</b>	<b>cyclic adenosine 3,5-monophosphate</b>
<b>cGMP</b>	<b>cyclic guanosine 3,5-monophosphate</b>
<b>EDRF</b>	<b>endothelium derived relaxing factor</b>
<b>EDHF</b>	<b>endothelium derived hyperpolarizing factor</b>
<b>eNOS</b>	<b>endothelial nitric oxide synthase</b>
<b>FAD</b>	<b>flavin adenine dinucleotide</b>
<b>FMN</b>	<b>flavin mononucleotide</b>
<b>HDL</b>	<b>high density lipoprotein</b>
<b>4-HNE</b>	<b>4-hydroxynonenal</b>
<b>ICAM-1</b>	<b>intercellular adhesion molecule-1</b>
<b>IDL</b>	<b>intermediate density lipoprotein</b>
<b>IL-1</b>	<b>interleukin-1</b>
<b>iNOS</b>	<b>macrophage inducible nitric oxide synthase</b>
<b>IP<sub>3</sub></b>	<b>inositol-1,4,5-trisphosphate</b>
<b>LCAT</b>	<b>lecithin: cholesterol acyltransferase</b>
<b>LDL</b>	<b>low density lipoprotein</b>
<b>LPC</b>	<b>lysophosphatidylcholine</b>

MCP-1	monocyte chemoattractant protein-1
M-CSF	macrophage-colony stimulating factor
MDA	malondialdehyde
MRFIT	Multiple Risk Factor Intervention Trial
NADPH	nicotinamide adenine dinucleotide phosphate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
PC	phosphatidylcholine
PDGF-B	platelet-derived growth factor-B
PGI <sub>2</sub>	prostacyclin
PLA <sub>1</sub>	phospholipase A <sub>1</sub>
PLA <sub>2</sub>	phospholipase A <sub>2</sub>
PLC	phospholipase C
TGF-β1	transforming growth factor-β1
tPA	tissue plasminogen activator
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low density lipoprotein

## **ABSTRACT**

Hyperlipidemia is one of the major risk factors for atherosclerosis, the leading cause of death in North America. High levels of cholesterol in the plasma can increase the retention time of low density lipoprotein (LDL) in the intima, thus increasing the risk of LDL oxidation by free radicals produced from endothelial or smooth muscle cells. It has been confirmed that oxidatively modified LDL plays a critical role in atherogenesis. Oxidized LDL, in contrast with native LDL, is taken up by macrophages to form foam cells and also causes the secretion of cytokines and growth factors from the arterial wall. In addition, the endothelium-dependent relaxation of blood vessel is impaired by oxidized LDL, even before histological evidence of atherosclerotic plaque formation. The active inhibitory component of oxidized LDL is found in the phospholipid fraction, and lysophosphatidylcholine (LPC), which accumulates in the LDL particle during the oxidative process, has been identified as an agent responsible for this action.

The endothelium is a single layer of cells positioned at the interface between the blood and the vessel wall. It performs multiple function including the regulation of vascular smooth muscle tone, modulation of platelet adhesion and aggregation, local clotting, and vascular growth. The endothelium serves a dual role in the control of vascular tone. It secretes relaxing factors such as nitric oxide (NO), prostacyclin, endothelium-derived hyperpolarizing factor (EDHF) and adenosine, as well as constricting factors such as the endothelin-1 and prostanoids. The balance between these dilators and constrictors is very important in regulating the normal vascular tone. Dysfunction of the endothelium is considered to be a critical factor in the pathogenesis of vascular disease.

Atorvastatin is a new synthetic lipid-lowering drug. It is a selective, competitive inhibitor of HMG-CoA reductase, which is the rate-limiting enzyme in the biosynthesis of cholesterol. Atorvastatin lowers plasma cholesterol and lipoprotein levels both by inhibiting hepatic cholesterol synthesis and by increasing its cellular uptake as well as influencing the catabolism of LDL through increasing the number of LDL receptors. Atorvastatin can reduce both LDL-cholesterol, VLDL-cholesterol and triacylglycerol (TG), as well as the number of apolipoprotein B containing particles.

The objective of the present study is to investigate whether treatment with atorvastatin alters the biochemical content of oxidized LDL in hyperlipidemic patients and the ability of oxidized LDL in hyperlipidemic patients to impair the endothelium-dependent relaxation. The hypothesis that atorvastatin treatment of hyperlipidemic patients attenuates the ability of oxidized LDL to impair the endothelium-dependent relaxation of the blood vessels forms the basis of this investigation, and we are interested in determining whether such an alteration arises from changes in the content and composition of lysophosphatidylcholine in the oxidized LDL.

Atorvastatin treatment is effective in reducing total cholesterol, LDL-cholesterol, as well as triglyceride levels in the plasma in hyperlipidemic patients. It is also associated with a slight increase in high density lipoprotein (HDL) levels. With atorvastatin (10mg/day) treatment for 4 weeks, the following, statistically significant changes were observed: total cholesterol level was lowered by 23%, LDL-cholesterol was lowered by 32% and triacylglycerol was lowered by 19% as compared with dietary therapy alone. Interestingly, HDL level was found to increase by about 9% in these type IIa hyperlipidemic patients. No changes in glucose levels were seen after the drug treatment.

The impairment of endothelium-dependent relaxation by oxidized LDL from hyperlipidemic patients before and after dietary therapy and after atorvastatin treatment was assessed by the stepwise

addition of acetylcholine to phenylephrine-precontracted rat aortic rings *in vitro*. The results show that the ability of oxidized LDL from hyperlipidemic patients after atorvastatin treatment to impair the endothelium-dependent relaxation was significantly reduced as compared with dietary intervention alone. The aortic rings incubated with oxidized LDL from atorvastatin-treated patients were almost completely relaxed by  $10^{-5}$  M acetylcholine, (comparable to the control rings exposed to native LDL or no lipid).

Analysis of the biochemical contents of oxidized LDL from this group revealed that there is an 11% reduction in LPC as compared with the group that received only dietary counseling. Interestingly, the drug also caused a decrease in the C16:0 moiety with a corresponding increase in the C18:0 moiety in LPC in oxidized LDL. We propose that the reduction of LPC observed in the oxidized LDL of the atorvastatin-treated group results from a combination of the continued dietary treatment as well as drug therapy. Our postulate is based on the notion that patients under dietary treatment should consume more unsaturated- than saturated fatty acids, and this might increase the resistance of LDL to oxidative modification. In view of the observation that both LPC species are equally potent in the impairment of endothelium-dependent relaxation of the aortic rings, we feel that the reduced level of LPC in the oxidized LDL produced by atorvastatin treatment is partially responsible for the improvement in endothelium control of vascular tone.

# **I. INTRODUCTION**

## **1. Regulation of Vascular Tone**

Blood circulation is controlled by both the nervous system and local factors. In the brain, heart and resistance vessels, however, local factors are more important in this control than is the nervous system. Blood flow throughout the body is regulated by resistance vessels. The main component of the resistance vessel wall is smooth muscle and the latter is always contracted to some degree (basal tone). Thus factors which influence vascular smooth muscle have the potential to regulate vascular tone (Berne and Levy 1996).

### **1.1 Endothelium-mediated regulation**

#### **1.1.1 The role of endothelium**

The endothelium is uniquely positioned at the interface between the blood and the vessel wall. Its function has been considered within the context of providing a semipermeable membrane for macromolecules and a non-clotting surface. Recent evidence clearly shows that the endothelium performs multiple functions: it is involved in the regulation of vascular smooth muscle tone, platelet adhesion and aggregation, local clotting, and vascular growth (Anderson et al. 1994; Furchgott 1993). It is a dynamic tissue secreting and modifying vasoactive substances, influencing the behaviour of other cell types, and regulating extracellular matrix production and composition. The endothelium is now considered to be a multifunctional organ whose health is essential to normal vascular physiology,

and whose dysfunction can be a critical factor in the pathogenesis of vascular disease (Dicorleto et al. 1996).

The endothelium serves a dual role in the control of vascular tone. It secretes relaxing factors such as nitric oxide (NO), prostacyclin, endothelium-derived hyperpolarizing factor (EDHF), and adenosine. In addition, the endothelium also secretes constricting factors such as endothelin-1 and prostanoids. The interaction between these dilators and constrictors provides a local control mechanism that regulates vascular tone (Dicorleto et al. 1996).

## **1.1.2 Endothelium-dependent vasodilation**

### **1.1.2.1 Endothelium-derived relaxing factor (EDRF)**

#### **1.1.2.1.1 Discovery of EDRF**

In 1980, Furchgott and Zawadzki found the first evidence for release by the endothelium of a potent vasodilator upon stimulation with acetylcholine, and termed this unidentified substance “endothelium-derived relaxing factor” (EDRF, Furchgott and Zawadzki 1980). Nitric oxide (NO) was later proposed as the chemical entity of EDRF (Ignarro et al. 1987; Furchgott 1988). In 1987, Palmer and others demonstrated that NO was responsible for the biological activity of this factor (Palmer et al. 1987).

#### **1.1.2.1.2 Nitric oxide Synthesis**

NO is produced from L-arginine by the action of a family of isoenzymes called nitric oxide synthase (NOS) (Palmer et al. 1988). Required cofactors are the presence of calcium, calmodulin and

four cofactors including flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), (6R)-5,6,7,8-tetrahydrobiopterin (BH<sub>4</sub>) and nicotinamide adenine dinucleotide phosphate (NADPH) (Nathan 1992). The family of isoenzymes in mammalian tissues have widespread cellular and subcellular distribution (Nathan 1992) and have a tightly bound heme center (Pufahl and Marletta 1993). The isoforms of NOS are composed of medium-sized subunits (80-125 kDa), usually forming a homodimer (Bredt et al. 1991; Marsden et al. 1992; Nunokawa et al. 1993; Muruganandam and Mutus 1994). Three isoforms of NOS have been found: endothelial NOS (eNOS), neuronal NOS (nNOS) and macrophage inducible NOS (iNOS) (Cooke and Dzau 1997). These three different isoforms are the products of distinct genes located on different chromosomes (Marsden et al. 1993; Nadaud et al. 1994; Sessa et al. 1992), and have been classified according to the calcium dependency of the enzyme and whether they are constitutively expressed. Both eNOS and nNOS are calcium-dependent enzymes (Griendling et al. 1996). Endothelium NOS isoform is a constitutively expressed 135 kD membrane-bound protein which is predominantly associated with the particulate subcellular fraction (Pollock et al. 1991; Boje and Fung 1990; Busconi and Michel 1993; Hecker et al. 1994).

#### **1.1.2.1.3 Functions of EDRF**

Following its synthesis, NO activates its intracellular receptor both in endothelial cells and in the adjacent smooth muscle cells. It has been demonstrated that the intracellular receptor for NO is soluble guanylyl cyclase. NO binds to the heme iron in the catalytic domain of guanylyl cyclase and, by increasing its activity, leads to an enhanced formation of cyclic GMP (cGMP) (Drexler et al. 1989; Martin et al. 1988). In smooth muscle cells, cGMP activates the cGMP -dependent kinase and leads to the phosphorylation and modulation of the activity of proteins involved in Ca<sup>2+</sup> homeostasis. The

end result of this activation is a reduction in the amount of  $\text{Ca}^{2+}$  available for contraction and subsequently relaxation of smooth muscle (Kobayashi et al. 1985; Hassid 1986; Kai et al. 1987). In addition, NO has also been shown to activate charybdotoxin (CTX)-sensitive, calcium-dependent potassium channels and induce hyperpolarization in vascular smooth muscles (Bolotina et al. 1994; Archer et al. 1994).

In addition to its vasorelaxing properties, NO has also been shown to control haemostasis by inhibiting cell-cell contact at the sensitive interface between platelets and endothelial cells (Radomski and Moncada 1993). Furthermore, NO appears to regulate leukocyte adhesion and activation on endothelial cells (Kubes et al. 1991; Moilanen et al. 1993).

NO may also have a role in controlling the function of the heart. It has also been suggested that NO as well as NO donors enhance myocardial relaxation, decreasing diastolic tone and slightly reducing peak contraction (Shah et al. 1994). When generated by the coronary vasculature, NO increases the coronary blood flow and thus supply to the myocardium (Amezcuca et al. 1989). In addition, there is now evidence that NO formed both by endocardium and myocardium (Schulz et al. 1991; De Belder et al. 1993; Balligand et al. 1993) exerts a negative inotropic effect on heart contractility. Recently, NO has been found to modulate vascular function by altering the expression of genes encoding certain endothelial proteins, which underlines the significance of endothelium-derived NO, both in short-term and long-term vascular homeostasis (Busse et al. 1995).

#### **1.1.2.1.4 Regulation of EDRF synthesis**

In the vascular system, eNOS is constitutively expressed in endothelial cells and modulated by physiological stretch and a variety of receptor-activated agonists. The integrity and release of NO

are crucial to the normal function of the endothelium (Griendling et al. 1996).

The physiological forces that result in vascular stretch and stimulate NO release from endothelium include shear stress, blood flow velocity and pulsatile blood flow (Traub et al. 1998). The shear stress refers to the dragging frictional force created by blood flow. A steady laminar shear stress is essential for maintaining the NO production at a constant state (Griffith et al. 1990; Pohl et al. 1991; Busse et al. 1993). It has been shown that both mRNA and protein levels of eNOS were markedly increased in the sheared cells (Nishida et al. 1992). The mechanism is supposed to involve a shear response element in the promoter of genes which encode many proteins (Resnick et al. 1993). A 6 bp sequence was identified that confers responsiveness to shear stress. This element, GAGACC, has been termed the shear stress response element (SSRE). It is present in the promoter of platelet-derived growth factor-B (PDGF-B), tissue plasminogen activator (tPA), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), intercellular adhesion molecule-1 (ICAM-1), and NOS, among others (Resnick et al. 1993). The signal transduction pathway which is responsive to stress likely involves the cytoskeleton and its associated enzymes (Hecker et al. 1993; Langille et al. 1991; Thurston et al. 1994; Franke et al. 1984; Wechezak et al. 1985). The intracellular signal transduction pathway that is initiated by an increase in shear stress has been reported to involve the activation of phospholipase C (PLC) (Bhagyalakshmi et al. 1992) and a rapid increase in the intracellular level of inositol-1,4,5-trisphosphate (IP<sub>3</sub>) (Nollert et al. 1990; Prasad et al. 1993), enhanced release of NO (Rubanyi et al. 1986; Pohl et al. 1986) and increased cellular levels of cGMP (Ohno et al. 1993). Phosphoinositide hydrolysis by PLC is also linked to the cytoskeleton via association of the lipid substrates with profilin (Goldschmidt-Clermont et al. 1990).

Receptor-activated agonists responsible for the release of NO from endothelium include

acetylcholine, thrombin, serotonin and adenosine diphosphate (Luscher et al. 1987). They interact with their receptors on the luminal surface of endothelial cells. The receptor is G protein linked to PLC which causes production of IP<sub>3</sub> and diacylglycerol. IP<sub>3</sub> interacts with its receptor on endoplasmic reticulum to release calcium (Dinerman et al. 1993), and subsequently increase the synthesis of NO (Bredt et al. 1992).

#### **1.1.2.2 Prostacyclin**

Another vasodilator factor secreted by the endothelium is prostacyclin (PGI<sub>2</sub>). It is produced from arachidonic acid by the activation of cyclooxygenase and prostacyclin synthase. Prostacyclin induces relaxation of vascular smooth muscle by activating adenylate cyclase and increasing the production of cyclic AMP (cAMP) (Moncada et al. 1979), which stimulates the extrusion of Ca<sup>2+</sup> from the cytosol (Raeyemaekers et al. 1990) and decreases the sensitivity of the contractile apparatus to Ca<sup>2+</sup> (Morgan et al. 1984). cAMP also induces the activation of ATP-dependent potassium channels which cause the hyperpolarization of smooth muscle cells (Siegel et al. 1989; Nakashima et al. 1995). Prostacyclin is released in response to shear stress, hypoxia, and several mediators that also release NO. Its release depends mainly upon Ca<sup>2+</sup> release from intracellular stores (Parsaee et al. 1992). The release of prostacyclin induced by shear stress in cultured human endothelial cells was found to be mediated by a pertussis toxin-sensitive G protein (Berthiaume and Frangos 1992). In most blood vessels, the contribution of prostacyclin to endothelium-dependent relaxation is negligible, and its effect is essentially additive to that of NO (Vanhoutte 1997).

### **1.1.2.3 EDHF**

Although NO and prostacyclin are the main factors to cause a reduction of vascular tone, they can not account quantitatively for the full actions of Ach-induced endothelium-dependent relaxation. An extensive body of evidence has shown that there exists a non-prostanoid and non-nitric oxide synthesis product which can mediate the hyperpolarization of vascular smooth muscle. Blockade of NO synthesis has been shown to have little or no effect on the Ach-induced hyperpolarization (Chen et al. 1988; Nagao et al. 1991; Fujii et al. 1992; Chen et al. 1991). Similarly, blockade of the effects of NO by hemoglobin or methylene blue almost completely abolished the increases in cGMP, but had no effect upon the Ach-induced hyperpolarization in rat aorta (Chen et al. 1988) or mesenteric artery (Fujii et al. 1992). However, the effect of this factor can be abolished by raising  $K^+$  concentration (Mombouli and Vanhoutte 1997).

Electrophysiological studies in various arteries have demonstrated that endothelium-dependent hyperpolarization and relaxation induced by acetylcholine and other agonists is due to a diffusible EDHF (Feletou et al. 1988; Vanhoutte 1996). The chemical identity of EDHF is still not known. In some blood vessels, epoxyeicosatrienoic acids formed from arachidonic acid by the action of cytochrome P-450 may correspond to EDHF (Campbell et al. 1996; Komori et al. 1990; Nagao et al. 1993). The hyperpolarization of smooth muscle cells induced by EDHF is mediated by an increased movement of potassium ions. The type of  $K^+$  channel involved is more likely to be calcium-dependent rather than ATP-dependent  $K^+$  channels (Cohen et al. 1995; Taylor et al. 1988). Activation of endothelial cells requires an increase in their intracellular calcium concentration (Flavahan et al. 1995), and the resulting release of EDHF produces a reduction in the intracellular calcium concentration in vascular smooth muscle cells (Fukuta et al. 1996).

The vascular relaxation induced by EDHF is more prominent in smaller than large arteries. (Hasunuma et al. 1991; Mugge et al. 1991; Nagao et al. 1992) In large arteries, both NO and EDHF can contribute to endothelium-dependent relaxations, but the role of NO predominates under normal circumstances (Vanhoutte 1997). In these arteries, if the synthesis of NO is inhibited, EDHF can still mediate near normal endothelium-dependent relaxations (Vanhoutte 1996; Kilpatrick et al. 1994). In diseases such as atherosclerosis, when the production and the activity of NO is reduced, this preservation may be important for the regulation of vascular tone.

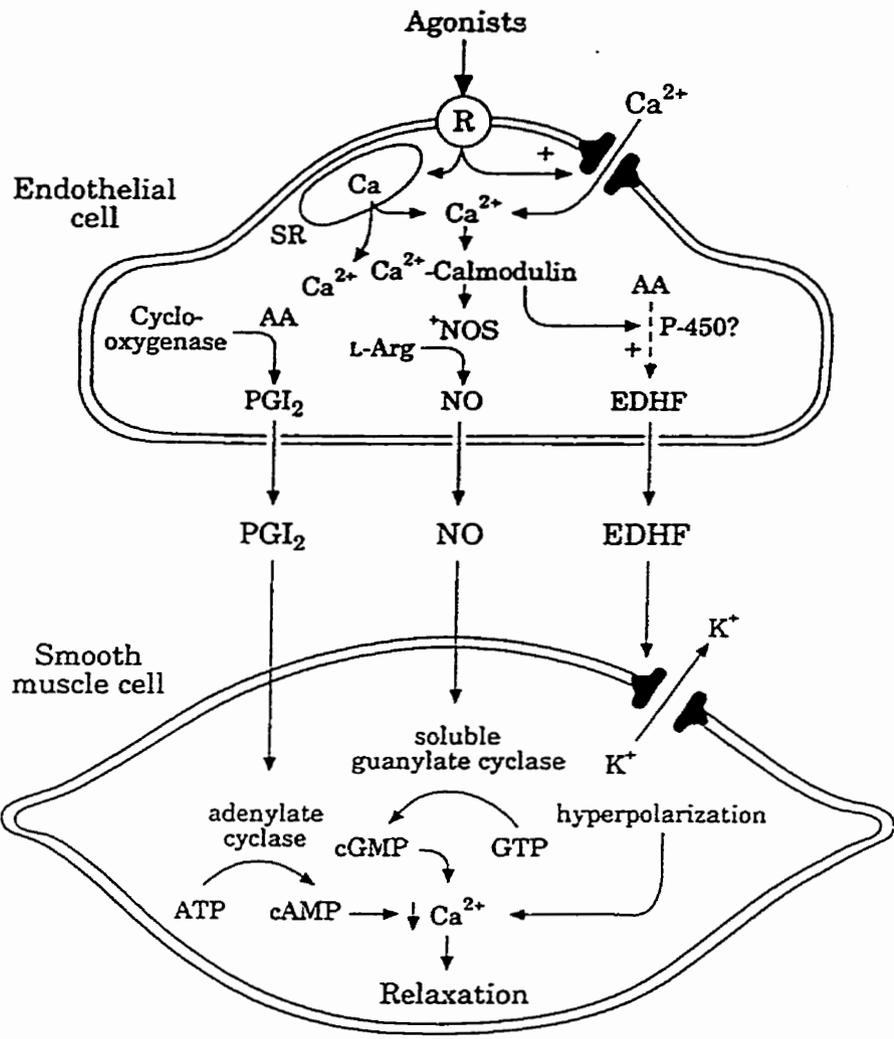


Figure 1. Endothelium derived relaxing factors. (Adapted from Vanhoutte 1997.)

### **1.1.3 Endothelium-dependent vasocontraction**

#### **1.1.3.1 Endothelin-1**

Endothelin-1 is one of three endothelin isoforms which is produced by endothelial cells (Yanagisawa et al. 1988). It is a 21 amino acid peptide and has two receptors which are the endothelin -A receptor on smooth muscle cells and endothelin-B receptor on endothelial cells (Arai et al. 1990; Sakurai et al. 1990). Endothelin-1 can cause vasodilation at lower, but sustained contractions at higher, concentrations (Yanagisawa et al. 1988, Kiowski et al. 1991). In the heart, intramyocardial vessels are more sensitive to endothelin-1 than their corresponding arteries (Kung et al. 1995), therefore high concentrations of endothelin-1 will eventually lead to ischemia, arrhythmia and death. Increased expression of endothelin-1 messenger RNA and production of the peptide have been found in the presence of thrombin, TGF- $\beta$ 1, interleukin-1 (IL-1), epinephrine, angiotension II, arginine vasopressin, calcium ionophore, and phorbol ester (Yanagisawa et al. 1988, Boulanger et al. 1990). In addition, hypoxia is also found to be associated with increase in plasma endothelin level (Goerre et al. 1995). However, it has been found that circulating levels of endothelin-1 are low, suggesting that little of the peptide is formed physiologically (Luscher 1992). Three inhibitory mechanisms regulating endothelin production have been proposed: (1) cGMP-dependent inhibition (Boulanger et al. 1990); (2) cAMP-dependent inhibition (Yokokawa et al. 1991), and (3) an inhibitory factor produced by vascular smooth muscle cells (Stewart 1990).

#### **1.1.3.2 Vasoconstrictor prostanoids and renin-angiotensin system**

Thromboxane A<sub>2</sub> or prostaglandin H<sub>2</sub>, the product of arachidonic acid catalyzed by

cyclooxygenase can mediate endothelium-dependent contraction (Luscher et al. 1990). Thromboxane  $A_2$  and prostaglandin  $H_2$  can also counteract NO and prostacyclin by activating the thromboxane receptor in vascular smooth muscle and platelets (Luscher and Noll 1995).

Angiotensin II, which is synthesized from angiotensin I by the action of angiotensin converting enzyme in endothelial cells, can activate endothelial angiotensin receptors which increase the production of endothelin and other mediators (Luscher et al. 1995).

## **1.2 Metabolic Regulation**

In most tissues, blood flow is closely paralleled by the metabolic activity. An increase in metabolic rate of the tissue or decrease of oxygen supply will result in more vasodilator substance formation and relaxation of smooth muscle, which leads to increases in blood flow (Berne and Levy 1996). In contrast, decreased metabolic rate will cause the contraction of smooth muscle. Many substances have been assumed to be vasodilator substance, such as lactic acid, carbon dioxide, hydrogen ions, potassium ions, inorganic phosphate ions, adenosine.

## **1.3 Neural influences**

Most arteries and veins are innervated by the vasoconstrictor fibers of the sympathetic nervous system. The sympathetic nerve terminal at the blood vessels releases norepinephrine which causes the constriction of vessels. Differential responsiveness is seen in the veins which are more responsive to sympathetic nerve stimulation than arteries. The influence of the sympathetic nervous system on the large vessels is also far less important than that on the microcirculation. In contrast, the effect of the parasympathetic nervous system on total vascular resistance is very small.

#### **1.4 Autoregulation**

Autoregulation refers to the process by which vascular resistance is altered so as to match blood flow to metabolic demand despite changes in perfusion pressure. This is also called the myogenic response mechanism. According to this mechanism, the vascular smooth muscle contracts in response to stretch and relaxes in response to a reduction in stretch (Berne and Levy 1996). This regulation, for example, is mainly responsible for maintaining a constant blood pressure when a person changes from a recumbent to a standing position.

#### **1.5 Humoral factors**

The adrenal gland can release epinephrine and some norepinephrine into the blood stream when it is stimulated. Both of these amines have effects on the peripheral blood vessels. In skeletal muscle, low concentrations of epinephrine can cause vasodilation while high concentrations cause vasoconstriction. However, the main effect of norepinephrine in all vascular beds is vasoconstriction (Berne and Levy 1996).

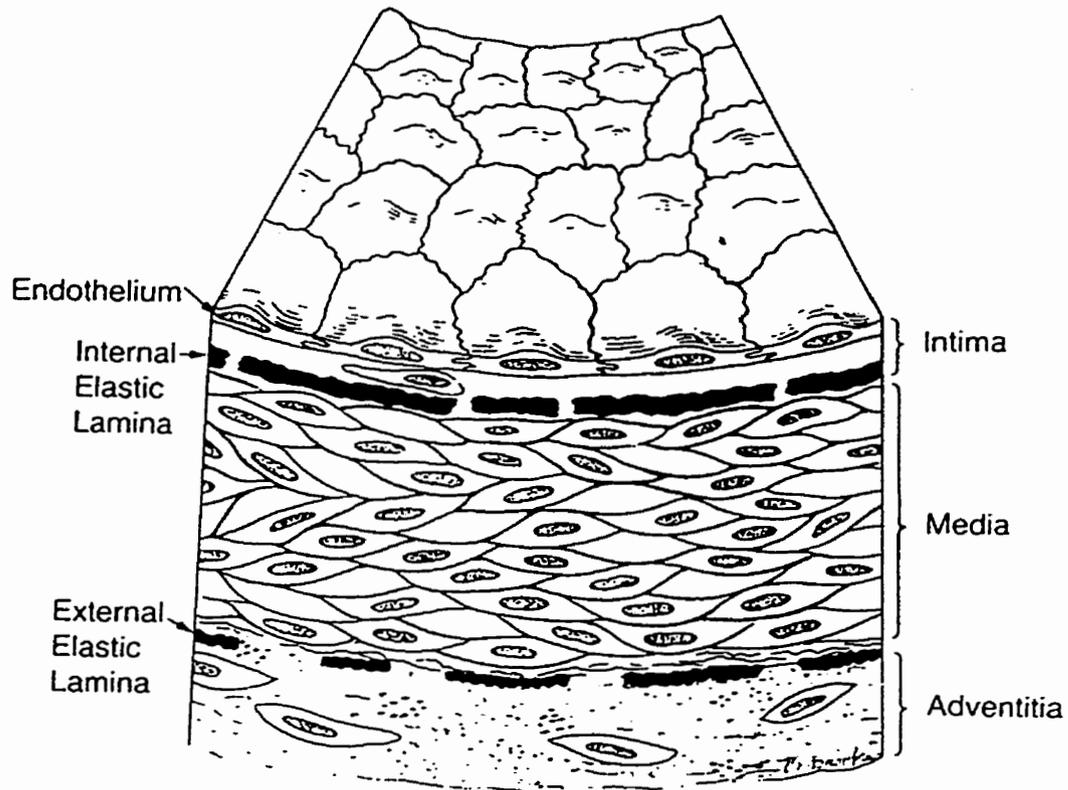
### **2. Pathology of Atherosclerosis**

Atherosclerosis is the leading cause of death in North America. The unusual thickening of artery wall caused by atherosclerotic plaque decreases the blood flow, sometimes even totally blocking the blood flow through the artery, which leads to the heart attack. Endothelial dysfunction has been found in the early stages of atherosclerotic plaque formation (Harrison 1994). It is generally thought that atherosclerotic plaque is the result of the endothelial impairment caused by risk factors

of atherosclerosis (Ross 1995; Consigny 1995; Harrison 1994).

## **2.1 Structure of the artery**

The arterial wall is composed of three major layers: the intima, media and adventitia. 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 numerous bundles of collagen fibrils, elastic fibers and fibroblasts together with some smooth muscle cells. The external elastic lamina, which is a continuous sheet, separates the adventitia from the media.



**Figure 2. Structure of the artery. (Adapted from Ross 1992.)**

## **2.2 The lesions of atherosclerosis**

The lesions of atherosclerosis has been divided into three categories: the fatty streak, the fibrofatty lesion and the fibrous plaque (Ross 1995).

### **2.2.1 The fatty streak**

The fatty streak is the earliest visible atherosclerotic lesion and is commonly found at sites where blood flow is altered, including sites that have decreased flow, back currents, or eddy currents at branches, bifurcations, and curves in the vascular system (Cornhill et al. 1985; Wissler et al. 1983). It consists of lipid-filled macrophages and a varying number of T lymphocytes (Faggiotto et al. 1984; Stary 1989; Ku et al. 1985). The accumulation of lipid filled macrophages occupies two to five or six layers of the intima of the artery and gives the lesion a yellow colour, thus it is termed “fatty streak”.

### **2.2.2 The fibrofatty lesion**

The next step in the development of an atherosclerotic lesion is the conversion of fatty streak into a fibrofatty lesion. It contains layers of lipid-filled macrophages and T cells, joined with a varying number of smooth muscle cells. It is surrounded by poorly developed connective tissue matrix of fine collagen fibrils, elastic fibers, and proteoglycans. Some of the smooth muscle cells may also contain lipid droplets (Ross 1995). It is suggested that not all fatty streaks develop into fibrofatty lesions. Experimental studies show that fatty streaks can regress (Small 1988; Blankenhorn et al. 1989). As the lesion progress, the cells undergo a re-arrangement that leads to the formation of fibrous plaque.

### **2.2.3 The fibrous plaque**

The fibrous plaque is characterized by a fibrotic cap composed of smooth muscle cells which are embedded in connective tissue matrix, made up principally of collagen with some elastic fibers (Adams et al. 1989; Stary et al. 1994; Faggiotto et al. 1984). The fibrous cap may also contain macrophages and some T lymphocytes (Faggiotto et al. 1984). Beneath the fibrous cap, the lesion consists of foam cells originating from macrophages and smooth muscle cells that are often associated with a core of lipid and necrotic material (Ross 1995). When the lesion becomes thick, small microvascular channels, vasa vasora, may be present. If the lesion is poorly formed, turbulence or mechanical stress may cause rupture or ulceration, which leads to secondary hemorrhage (Fuster et al. 1992; Richardson et al. 1989; Falk 1992; Loree et al. 1994). Subsequently, thrombosis ensues and may lead to occlusion of the artery.

## **3. Risk Factors of Atherosclerosis and the Vascular Endothelium**

### **3.1 Modified response-to-injury hypothesis**

The original response-to-injury hypothesis was formulated in 1973 in order to explain the accumulated experimental and clinical observations on the process of atherogenesis (Ross and Glomset 1973). Since then, it has been tested, modified, retested, and remodified several times. The most recent version suggests that endothelial injury is the earliest event in the formation of atherosclerotic lesion (Ross 1993), which results from various associated risk factors. The injured endothelial cells appear morphologically different from normal endothelial cells, they are typically not

aligned in the direction of blood flow, and they have fewer intercellular attachments, resulting in increased permeability (Faggiotto et al. 1984). Injured endothelial cells are thrombogenic in that they reduce the production of PGI<sub>2</sub> and EDRF-NO (Groves et al. 1993). Injured endothelial cells also promote vascular smooth muscle cell migration and proliferation by releasing less EDRF-NO and by secreting PDGF and endothelin-1 (Garg et al. 1989). Finally, injured endothelial cells promote the recruitment of macrophages by secreting monocyte chemoattractant protein-1 (MCP-1), and by expressing cell surface receptors, including E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and ICAM-1, to which monocytes can bind (Ross 1993; Bath et al. 1991). After attaching and adhering, the monocytes migrate into the intima of the artery between endothelial cells due to the generation of chemoattractants by the endothelium, by smooth muscle, or both. Once in the intima, the monocytes become activated as macrophages, ingest the modified lipids that precede them into the artery wall, and can also generate molecules that are chemotactic for additional monocytes (Ross 1995). The macrophages become large foam cells through lipid accumulation and with the T-cell and smooth muscle, form a fatty streak. The fatty streak can then progress to an intermediate, fibrofatty lesion and ultimately to a fibrous plaque.

The risk factors which are proposed to be responsible for the development of atherosclerosis include hypercholesterolemia, hypertension, diabetes, obesity, smoking, physical inactivity, age and gender (Averbook et al. 1989). Considerable evidence indicates that these risk factors initiate the atherosclerotic process by blunting the release of EDRF-NO from the endothelium (Flavahan 1992). Clinical studies have also demonstrated that endothelial cell release of EDRF-NO is diminished with aging (Zeicher et al. 1993), smoking (Nitenberg et al. 1993), hypercholesterolemia (Creager et al. 1990), essential hypertension (Panza et al. 1993), and diabetes (Saenz et al. 1989). Furthermore,

animal studies have revealed diminished endothelial cell production of EDRF-NO in hypercholesterolemic animals before any morphologic changes take place in the artery (Cohen et al. 1988).

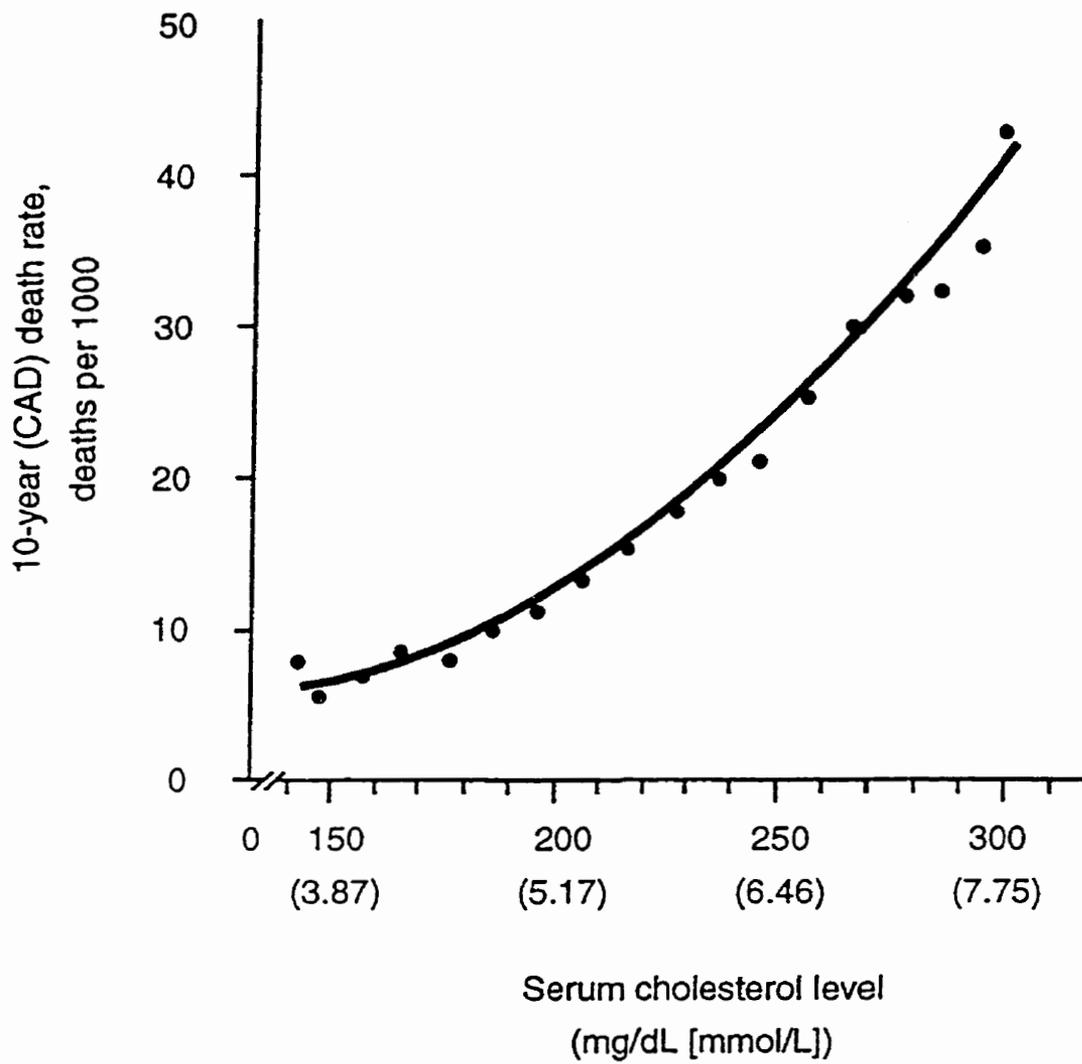
**Table 1. Functions of vascular endothelium in normal and injured states (Adapted from Consigny 1995)**

Function	Normal Endothelium	Injured Endothelium
Permeability	Tight endothelium cell junctions prevent passage of large molecules into subendothelium.	Loss of tight junctions increases penetration of large molecules, e.g., low-density lipoprotein, into subendothelial space.
Thrombogenicity	Platelets are repelled by negative surface charge of endothelium cells. Platelet aggregation is inhibited by endothelium secretion of PGI <sub>2</sub> and EDRF-NO. Thrombolysis is promoted by secretion of tPA.	Function is converted from antithrombotic to prothrombotic. PGI <sub>2</sub> , EDRF-NO, and tPA secretions are diminished. Plasminogen activator inhibitor and tissue factor secretions are increased.
Vasomotor tone	Vasodilation is promoted by secretion of PGI <sub>2</sub> and EDRF-NO.	Vasoconstriction is promoted by diminished secretion of PGI <sub>2</sub> and EDRF-NO and by increased secretion of endothelin-1
Vascular smooth muscle migration and proliferation	Smooth muscle cell migration and proliferation are inhibited by secretion of heparan sulfate and EDRF-NO.	Smooth muscle proliferation is promoted by diminished secretion of EDRF-NO and increased secretion of platelet-derived growth factor and endothelin-1
Inflammation	Inflammatory cells fail to adhere to normal endothelium.	Leukocytes are recruited to sites of injury by expression on cell surface of proteins (endothelium-leukocyte adhesion molecule and intercellular adhesion molecule). T lymphocytes are recruited by endothelium cells expressing major histocompatibility complex class II proteins.

### **3.2 Hyperlipidemia**

Hyperlipidemia has been confirmed to be a major risk factor of atherosclerosis in the Framingham study (Anderson et al. 1987) and the Multiple Risk Factor Intervention Trial (MRFIT) (Martin et al. 1986) in 1950s. Data from the two studies showed that there is a proportional increase in the risk of coronary disease corresponding to the increase of plasma cholesterol. Evidence from both animal experiments and clinical data have demonstrated that hypercholesterolemia can cause damage to endothelial function. Several studies have shown that endothelium-dependent relaxation is impaired in animals with experimentally induced hypercholesterolemia (Verbeuren et al. 1986; Freiman et al. 1987; Harrison et al. 1987). Habib and co-workers studied aortic rings from cholesterol-fed rabbits and found that relaxations to acetylcholine were markedly abnormal (Habib et al. 1986). Jayakody and co-workers also showed that cholesterol feeding of rabbits decreased aortic relaxation to acetylcholine (Jayakody et al. 1987). In most of these studies, the abnormal vasodilation is seen well before any morphological changes of endothelium .

The mechanism responsible for causing impairment of endothelium-dependent relaxation by hypercholesterolemia has been suggested to be an intracellular deficiency of arginine, modification of the expression of the activity of NO synthase or increased degradation of the EDRF by the superoxide anion (Luscher et al. 1990; Harrison 1995).



**Figure 3. Relation between serum cholesterol levels and risk of coronary disease.**  
 (Adapted from Farnier 1998.)

### **3.3 Hypertension**

Hypertension has been demonstrated to be another independent indicator of increased risk of atherosclerosis. In hypertensive patients with high renin levels or left ventricular hypertrophy, reduced EDRF has been found (Panza et al. 1994). As EDRF plays a major role in the regulation of systemic vascular resistance, these patients are prone to develop large and small vessel vasoconstriction, reduced blood flow, and myocardial ischemia. This process can be reversed by treatment which lowers blood pressure (Panza et al. 1993). However, if treatment is not initiated soon enough, or, it is the primary problem rather than a consequence, the endothelial defect can become irreversible (Panza et al. 1993).

### **3.4 Smoking**

Cigarette smoking is a major risk factor for the development of atherosclerosis and coronary events. Research has shown that there is a dose-dependent impairment of endothelial dysfunction in asymptomatic young smokers (Celermajet et al. 1993). In addition, endothelial dysfunction may be worsened by cigarette smoke. Kiowski and co-workers studied the effect of short- and long- term smoking on vascular tone. They found that long-term smoking is associated with vasodilation and further that short-term smoking is associated with enhanced endothelin-1 induced vasoconstriction (Kiowski et al. 1994). Studies have demonstrated a direct toxic effect of tobacco smoke on human endothelium associated with an increase in the number of endothelial cells with nuclear damage in the circulating blood (Davis et al. 1985). One theory is that components of cigarette smoke may be toxic by inducing oxidative stress and thereby causing endothelial damage. This risk factor, however, is reversible. Smokers who discontinue smoking decrease their risk of developing coronary disease.

### **3.5 Male gender**

Men have a greater risk of heart attack than women, and they have attacks earlier in life. These observations suggest a protective effect of estrogen. Several studies have demonstrated that estrogens can improve the endothelium-dependent relaxation of atherosclerotic coronary arteries in ovariectomized female monkeys (Losordo et al. 1994; Lieberman et al. 1994; Williams et al. 1994). It was also found that treatment with conjugated equine estrogen augmented endothelium-dependent dilation of atherosclerotic arteries (Williams et al. 1992). Further, the addition of cyclic or continuous medroxyprogesterone acetate to the conjugated estrogen regimen diminished the endothelium-mediated dilation. A hypothesis has been tested in a rabbit model that the sex hormone status influences responsiveness of the coronary artery to exogenous estrogen (Collins et al. 1994). The results showed a significant difference in the relaxing sensitivity of aortic rabbit rings in oophorectomized animals but sensitivity in animals with estrogen replacement were comparable with acutely hormone-deprived animals. Therefore, estrogen can beneficially affect coronary vasomotion, and this may explain some of the protective effects of estrogens.

### **3.6 Aging**

Aging causes a decrease in vascular compliance. This effect may be caused by the decrease in elastin along with collagen degeneration and increased intima-media thickness in the arterial wall with age. In addition, another potentially important factor occurring in the arterial system with aging might be the loss of endothelium-dependent vasorelaxation. Several studies have shown that increasing age is associated with progressive coronary endothelial dysfunction (Egashira et al. 1993; Cohn et al. 1992). The mechanisms leading to the loss of EDRF-mediated vasodilation seen with

aging are probably related to age-related decreases in EDRF production or responsiveness, increased degradation of EDRF in the blood vessel wall, or an increased production or responsiveness to vasoconstricting factors (Glasser et al. 1996).

### **3.7 Diet**

The role of diet, separate from its effects on lipids and blood pressure, is currently being evaluated. One proposal is that providing L-arginine as a substrate for increased NO production may be beneficial because NO is an end product of L-arginine metabolism. Recently, both short-term and long-term studies have shown support for this concept (Dubois-Rande et al. 1992; Hamon et al. 1994). The beneficial effects of long-term L-arginine administration on the degree of neointimal thickening and response to acetylcholine after balloon denudation of iliac rabbit arteries has been demonstrated by Hamon et al.(1994). Another interesting area is the role of antioxidant therapy in primary and secondary prevention. In the isolated vascular ring model, an antioxidant can normalize endothelium-dependent relaxation when vascular tone is rendered abnormal by ischemia (Mehta et al. 1993). Also, epidemiologic studies support the idea that dietary antioxidants are useful in preventing coronary events (Gilligan et al. 1994). The results of a study on the effect of antioxidant vitamins on the susceptibility of low density lipoprotein (LDL) to oxidation and endothelium-dependent vasodilator responsiveness in patients with hypercholesterolemia showed that antioxidant vitamin supplements can reduce the susceptibility of LDL to oxidation, but impairment of endothelial function was unimproved (Gilligan et al. 1994).

### **3.8 Diabetes mellitus**

Diabetes is clinically associated with impaired endothelial function which is worsened by severe hyperglycemia (Johnstone et al. 1993). Chronic hyperglycemia associated with diabetes can modify protein structure and function due to growth factors such as insulin which stimulate the proliferation of smooth muscle cells. Diabetes is also associated with elevation of plasminogen activator inhibitor-1 and alterations in the lipid profile, including elevated triglycerides and LDL cholesterol and reduction in high density lipoprotein (HDL) cholesterol. Extensive studies of endothelial dysfunction in diabetic patients and animals and studies of the effect of elevated concentrations of glucose on normal blood vessels and cultured endothelial cells have been reported. These results suggest that elevated glucose can cause characteristic dysfunction of the endothelium in the regulation of vascular tone (Cohen 1993).

### **3.9 Exercise**

Lack of physical exercise is a recognized risk factor for atherosclerosis. It is generally thought to be independent of its effects on body weight, blood pressure, or lipids. Endothelium-dependent vasorelaxation in athletes was investigated and the results suggested that it was enhanced with chronic exercise (Kingwell et al. 1995). Also, an increase in arterial compliance was observed in 13 previously sedentary young men who received a 4-week exercise training program (Cameron et al. 1994). Furthermore, in endurance-trained male athletes, arterial stiffness was significantly less than in sedentary age peers (Vaitkevicius et al. 1993).

## **4. Effect of Oxidized LDL on Vascular Tone and Its Role in Atherosclerosis**

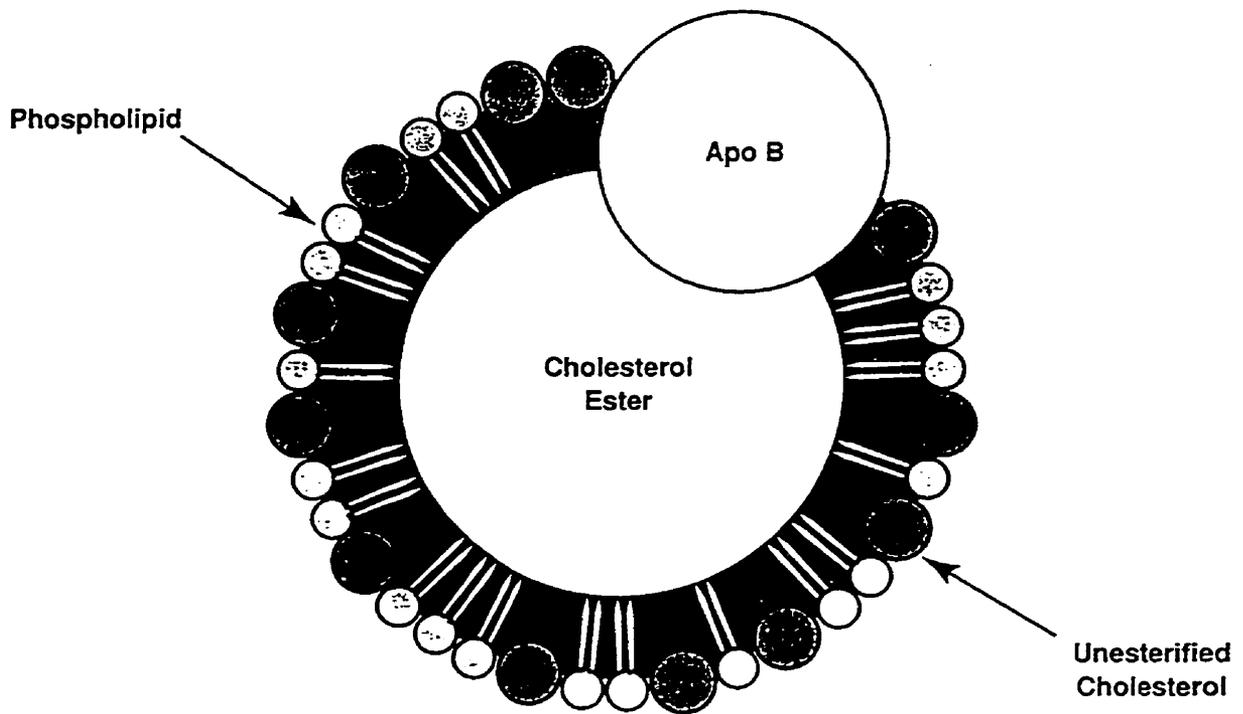
### **4.1 Oxidative modification of LDL**

LDL is a large spherical particle with a molecular weight of about 2.5 million daltons. It has a central core which is composed of cholesteryl ester and triglyceride molecules, and is surrounded by an outer monolayer shell composed of phospholipids and free cholesterol molecules. Embedded in the outer monolayer is a large protein termed apoprotein B-100 (apoB-100). In native LDL it is this protein that is recognized by the LDL receptor.

LDL oxidation can be initiated *in vitro* by incubating it with macrophages (Parthasarathy et al. 1986), endothelial cells (Morel et al. 1984; Steinbrecher et al. 1984), smooth muscle cells (Heinecke et al. 1984), lymphocytes (Cathcart et al. 1985) as well as in the presence of transition metal ions (Hessler et al. 1979). Oxidation of LDL causes dramatic changes in both the apoB-100 and all of the lipid moieties of the lipoprotein. There are two prominent changes that take place in the apoB-100. One is the single polypeptide chain of apoB-100 is fragmented into pieces but still associated with the surface of the LDL particle (Fong et al. 1987). The other is that reactive residues of the apoprotein, lysine and histidine, bind to lipid peroxidation products, including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (Jurgens et al. 1987), both of which are aldehyde breakdown products of polyunsaturated fatty acyl moieties. These changes increase the net negative charge of the lipoprotein from its native state, alter receptor recognition, and thereby increase the rate at which oxidized LDL is removed from plasma (Steinbrecher et al. 1987; Nagelkerke et al. 1984). Fragmented apoB-100 is no longer recognized by the LDL receptor but by the scavenger receptor. Numerous new lipid products are also formed when LDL is oxidized, including oxysterols, MDA, 4-HNE and many

others (Jurgens et al. 1987). In addition to numerous lipid oxidation products, another bioactive lipid, lysophosphatidylcholine (LPC) is formed indirectly, secondary to the oxidation of LDL. A phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activity has been discovered in association with LDL during exogenous oxidation (Steinbrecher et al. 1984) and has been described as an enzymatic activity inherent to apoB-100 which hydrolyses preferentially the 2-position unsaturated fatty acid of phosphatidylcholine (Steinbrecher et al. 1989; Parthasarathy et al. 1985; Stafforini et al. 1989; Parthasarathy et al. 1990; Stafforini et al. 1987).

It has been reported that LDL which is modified by copper sulfate *in vitro*, produces biochemical and functional alterations of LDL which are similar to those by cells, and is commonly used to study the functional effects of lipid oxidation (Steinbrecher et al. 1984). The effects of Cu<sup>2+</sup> induced oxidation include the loss of endogenous antioxidants, changes in electrophoretic mobility of LDL, increases in lipid peroxides and the formation of lysophosphatidylcholine (Esterbauer et al. 1987; Parthasarathy et al. 1985).



**Figure 4. Structure of LDL.**

## 4.2 Effect of oxidized LDL on vascular tone

Normal arterial tone is directly influenced by the endothelium lining the vessel via endothelial cell production and release of EDRF such as NO. However, endothelium dependent vasodilation is seriously impaired in atherosclerotic vessels (Bossaller et al. 1987; Forstermann et al. 1988; Tomoike et al. 1989). It has been demonstrated that oxidized LDL has many effects on the vascular tone of arteries, including effects on the nitric oxide pathway, calcium ion exchange, and prostaglandin and endothelin production.

Oxidized LDL has been demonstrated to have an overall negative effect on the EDRF-mediated relaxation of arterial smooth muscle cells. Studies have shown that oxidized LDL may interfere with multiple steps, including the synthesis of nitric oxide synthetase, the transport of nitric oxide from the endothelial cell, and the responsiveness of the smooth muscle cell (Chisolm et al. 1996). Oxidized LDL, but not native LDL, is a potent inhibitor of EDRF-mediated vasodilation (Chin et al. 1992; Yokoyama et al. 1990). The mediator of the effect of oxidized LDL were shown to be contained in the lipid moiety and absent in the protein portion of the lipoprotein (Yokoyama et al. 1990).

Lysophosphatidylcholine (LPC), whose level is dramatically elevated during oxidative modification of LDL, has been shown to be responsible for the biological effects of oxidized LDL (Chin et al. 1992; Yokoyama et al. 1990; Ohgushi et al. 1993; Kugiyama et al. 1990; Matsuda et al. 1993). Liu and co-workers found that there is a five fold increase in the amount of LPC contained in oxidized LDL compared with native LDL upon oxidation *in vitro* with CuSO<sub>4</sub> at 37 °C (Liu et al. 1994), and they also demonstrated that impairment of endothelium dependent relaxation occurs in direct proportion to the amount of LPC produced in LDL during oxidation. Removal of LPC from

oxidized LDL with phospholipase B or albumin abolished its impairment on endothelium-dependent relaxation (Kugiyama et al. 1990; Mangin et al. 1993; Yokoyama et al. 1990). Direct treatment of endothelium-intact arterial rings with LPC mimics the inhibitory effects of oxidized LDL on endothelium-dependent relaxation (Kugiyama et al. 1990; Mangin et al. 1993; Tanner et al. 1991). It has been shown that the ability of LPC to impair endothelium-dependent relaxation is also dependent on the chain length of the acyl group in LPC - only long-chain LPC has the ability to impair endothelium-dependent relaxation (Chen et al. 1997).

#### **4.3 Occurrence of oxidized LDL *in vivo***

Several lines of evidence suggest the presence of oxidatively modified LDL *in vivo*. First, immunocytochemical evidence for the presence of oxidatively modified LDL in atherosclerotic lesions but not in normal arteries has been found by using antibodies that recognize epitopes of oxidized LDL. Second, LDL eluted from lesions, but not from normal arterial tissue, has all the physical, immunologic, and biologic properties observed with LDL oxidized *in vitro*, and can be rapidly taken up in macrophages. Third, oxidized LDL is immunogenic, and autoantibodies against epitopes of oxidized LDL can be found in the sera of rabbits and humans. Fourth, IgG in atherosclerotic lesions recognizes *in vitro* oxidized LDL. Fifth, treatment of hypercholesterolemic rabbits with antioxidants leads to inhibition of the progression of atherosclerosis, independent of any effects on plasma lipoprotein levels (Reaven et al. 1995; Witztum 1993; Witztum et al. 1991; Reaven et al. 1996).

#### **4.4 Role of oxidized LDL in atherosclerosis**

A high concentration of circulating LDL is a major risk factor for atherosclerosis. Whereas

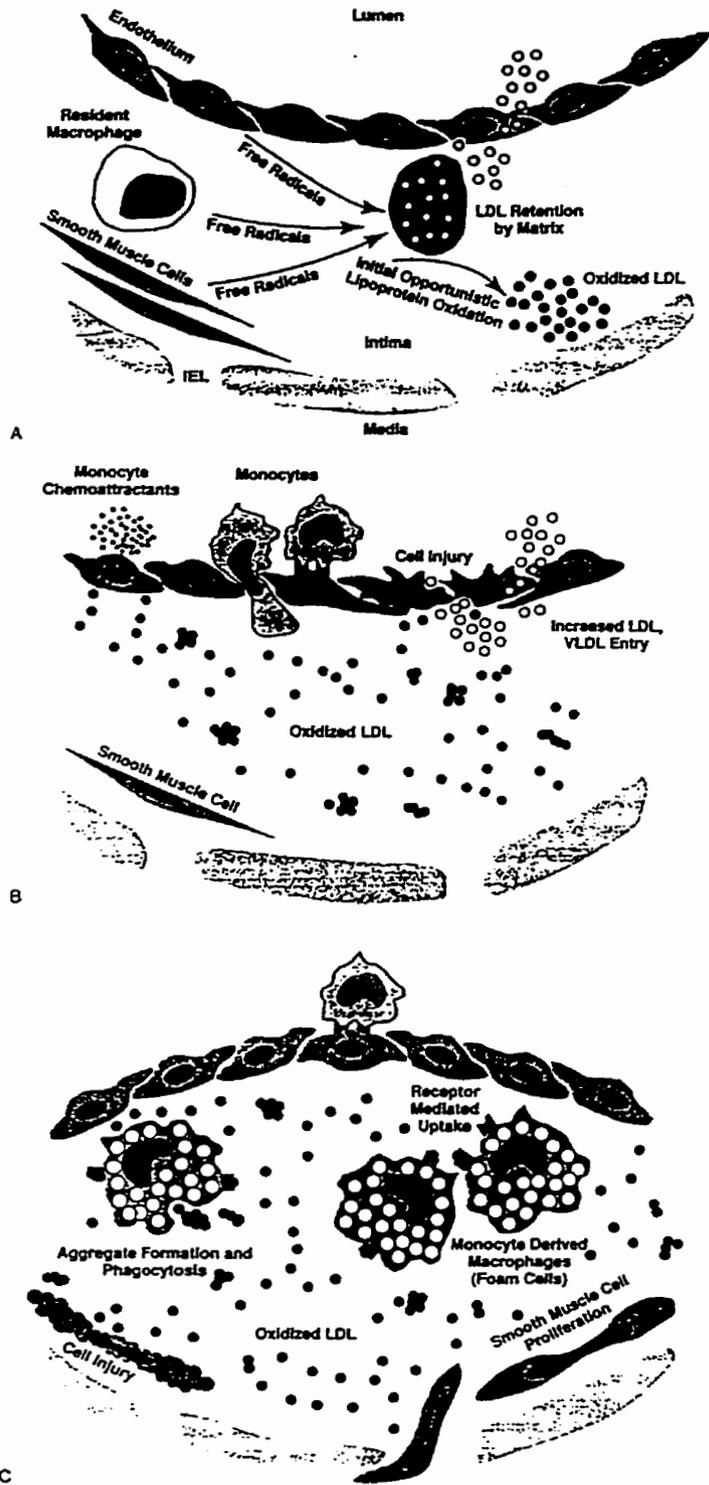
it was initially thought that the earliest event of atherosclerosis was the dysfunction of endothelium, it is now recognized that this is not the initial event, but rather a subsequent event that contributes importantly to the development and progression of the fatty streak (Gerrity et al. 1979; Faggiotto et al. 1984; Ross 1986). The focal accumulation of LDL within the subintimal space of an apparently “normal” artery is now considered to be the earliest response to cholesterol feeding (Schwenke et al. 1989). The weight of evidence suggests that oxidatively modified LDL plays a critical role in atherogenesis. Modified LDL, in comparison with native LDL, demonstrates enhanced cellular uptake by macrophages, foam cell formation and also causes the secretion of cytokines and growth factors from arterial wall cells. A theory of atherosclerosis based on LDL oxidation has been constructed, with oxidized LDL playing a central role that causes injury to, or dysfunction of the endothelium as an initial step in the formation of the fatty streak (Chisolm et al. 1996). Briefly, focal accumulation of LDL due to high plasma LDL concentration (Bratzler et al. 1977) increases the retention time of LDL in the intima (Schwenke and Carew 1989), and this increases the probability of LDL being oxidized by free radicals produced by adjacent endothelium or smooth muscle cells or isolated macrophages (Morel et al. 1984; Steinbrecher et al. 1984; Cathcart et al. 1985; Parthasarathy et al. 1986). The oxidation of LDL could also be enhanced after it binds to proteoglycans (Hurt-Camajo et al. 1992). In addition, small dense LDL, which has been shown to signal increased risk of atherosclerosis and to be more readily oxidized than other LDL subfractions (Tribble et al. 1992; Chait et al. 1993), may be able to pass the endothelium more readily. Once LDL is oxidized, it causes injury to the endothelium and leads to enhanced entry of LDL. Oxidized LDL can stimulate endothelial cells to produce MCP-1 (Cushing et al. 1990) and monocyte binding proteins (Berliner et al. 1990; Frostegard et al. 1990; Kume et al. 1992) which promote monocyte adhesion (Quinn et

al. 1987). Also, oxidized LDL itself can act as a chemoattractant for circulating human monocytes. The monocytes not only adhere to the endothelial cells but migrate along the MCP-1 gradient into the subendothelial space ( Navab et al. 1988; Navab et al. 1991). After entering the intima, monocytes differentiate into macrophages under the influence of macrophage-colony stimulating factor (M-CSF) ( Rajavashisth et al. 1990). Macrophages can internalize oxidized LDL by their scavenger receptors, which are not subject to negative feedback regulation (Witztum et al. 1991; Wu et al. 1992). As this modified LDL uptake is not saturable, large amounts of modified LDL can be incorporated into macrophages, resulting in the formation of foam cells. Smooth muscle cells are also found in the intima, which may be facilitated by a chemoattractant effect (Autio et al. 1990) as well as by the proliferative influence of oxidized LDL (Chatterjee 1992; Lafont et al. 1995). Smooth muscle cells can develop into foam cells by engulfing oxidized LDL through scavenger receptors (Pitas et al. 1983; Li et al. 1995). Taken together, the accumulation of foam cells which develop from macrophages and smooth muscle cells by internalizing oxidized LDL in the intima is the first important step in the formation of a atherosclerotic lesion.

**Table 2. Atherogenic effects of oxidized LDL (Adapted from Parthasarathy et al. 1992)**

---

1. Oxidized LDL is rapidly taken up by macrophages and leads to cholesterol accumulation
  2. Oxidized LDL is chemotactic for monocytes, yet inhibits macrophage motility
  3. Oxidized LDL is cytotoxic
  4. Minimally oxidized LDL can alter gene expression in arterial cells, for example, including endothelium cells to express colony-stimulating factors and monocyte chemotactic protein-1 (MCP-1)
  5. Oxidized LDL increases expression of adhesion molecules at the endothelium cell surface
  6. Oxidized LDL inhibits endothelium-dependent relaxation
  7. Oxidized LDL exhibits increased binding to type-1 collagen
  8. Oxidized LDL can adversely affect coagulation pathways
  9. Oxidized LDL is immunogenic.
-



**Figure 5. Proposed mechanism by which oxidized LDL causes atherosclerosis. (Adapted from Chisolm et al. 1996.)**

## **5. The Metabolism of Cholesterol in the Human Body**

Cholesterol is present in tissues and in plasma lipoproteins either as free cholesterol or, combined with a long-chain fatty acid, as cholesteryl ester. It is not only an important component of cell membranes, but also the precursor of adrenal steroids and vitamin D and a substrate for the synthesis of bile acids in the body. The input and output of cholesterol must be balanced in the human body such that any defects in cholesterol metabolism may cause disease, including cardiovascular disease.

### **5.1 Metabolism of cholesterol**

#### **5.1.1 Synthesis and excretion of cholesterol**

The cholesterol pool of the human body is derived from two sources: absorption of dietary cholesterol and *de novo* biosynthesis in the liver. The diet provides half of the cholesterol of the body, and the remainder derives from synthesis (Mayes 1993). Dietary cholesteryl esters can not be utilized by the body directly, but must first undergo hydrolysis into free cholesterol by pancreatic cholesterol esterase in the small intestine, and then be absorbed by passive diffusion in the small intestine. Within the intestinal mucosal cells, cholesterol is reacylated into cholesteryl esters by acyl CoA: cholesterol acyltransferase (ACAT), and then cholesteryl esters, triacylglycerols, phospholipids and some free cholesterol are packaged with specific proteins to form chylomicrons. Chylomicrons are exported into the lymphatic system by exocytosis and enter the blood at the angle of the left jugular and subclavian vein (Marinetti 1990).

The *de novo* synthesis of cholesterol in the body occurs primarily in the liver. It starts from

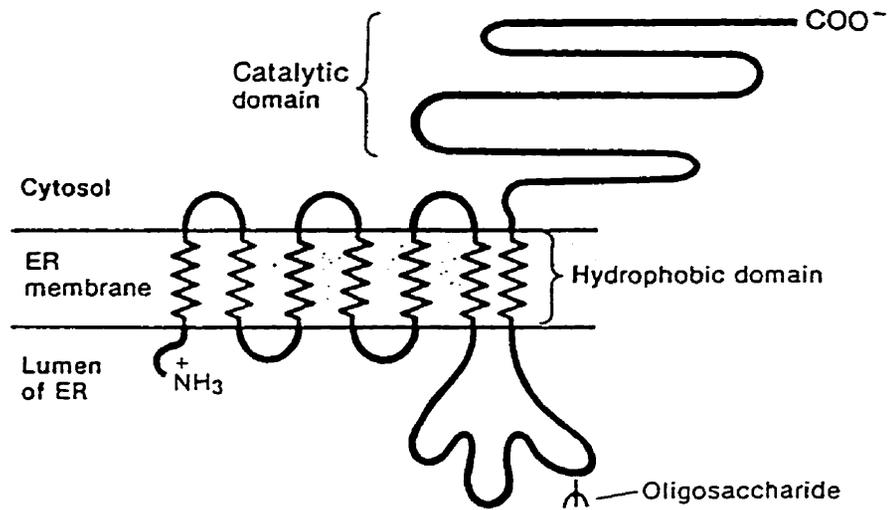
acetyl-CoA via a complex pathway in the cytosol of liver cells. Briefly, three molecules of acetyl-CoA form mevalonate. This is the rate-limiting step of cholesterol synthesis and is catalyzed by HMG-CoA reductase. A 5-carbon isoprenoid unit is formed from mevalonate by loss of CO<sub>2</sub> and six isoprenoid units condense to form squalene (C<sub>30</sub>). Squalene undergoes cyclization to form the parent steroid lanosterol, which, after the loss of 3 methyl groups, forms cholesterol (C<sub>27</sub>) (Mayes 1993).

Cholesterol is eliminated from the body by conversion to bile acids which are excreted in the feces, and secretion of cholesterol into the bile which transports it to the intestine for elimination. A small portion of cholesterol is also lost from the skin cells and through urine (Champe et al. 1994).

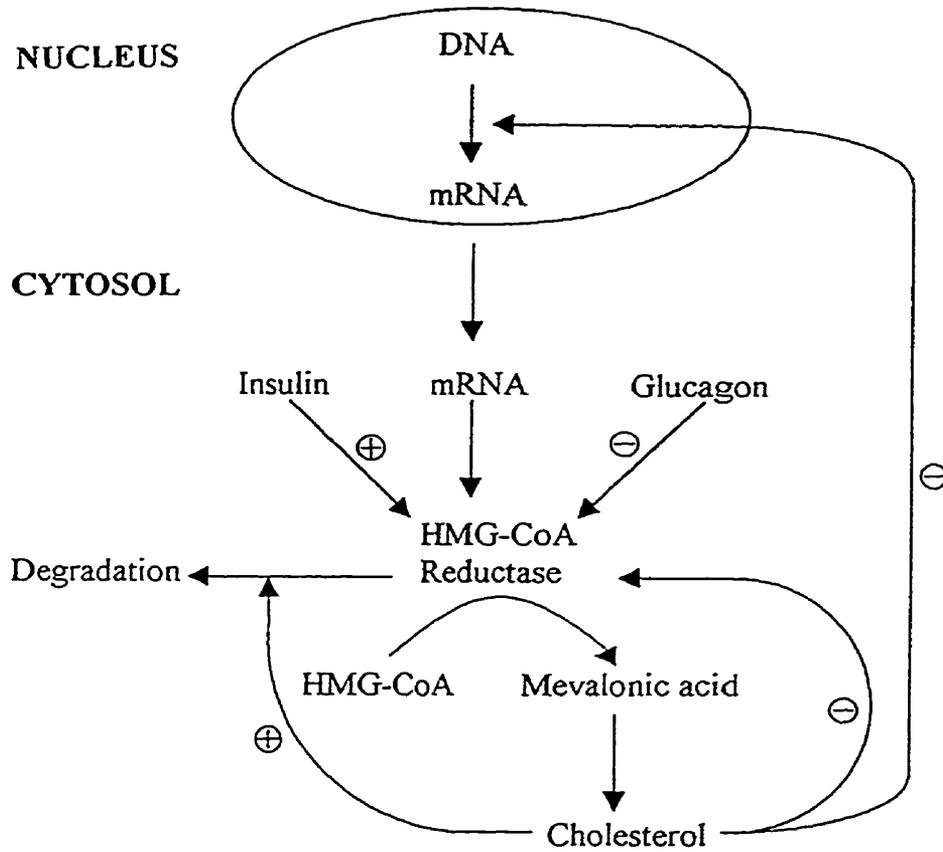
### 5.1.2 HMG-CoA Reductase

HMG-CoA reductase is the rate-limiting enzyme in cholesterol biosynthesis and therefore may be the primary regulatory site on the *de novo* synthesis pathway of cholesterol. HMG CoA reductase is located on the endoplasmic reticulum. The molecular weight is 97,092 D and consists of 887 amino acids in a single polypeptide chain. The enzyme has two domains. The hydrophobic domain is embedded in the membrane and catalytic domain protrudes into the cytosol (Vance 1988). The regulation of HMG CoA reductase can be achieved in several ways: (1) cholesterol effects feedback inhibition of its own synthesis by inhibiting the activity of pre-existing HMG CoA reductase; (2) increased cholesterol concentrations decrease the amount of mRNA for coding HMG-CoA reductase; (3) increased cholesterol concentrations increase the rate of the degradation of HMG-CoA reductase; and (4) the activity of HMG CoA reductase is modulated by phosphorylation and dephosphorylation. Phosphorylation inhibits and dephosphorylation activates the enzyme. Insulin can stimulate the dephosphorylation of HMG CoA reductase, whereas epinephrine and glucagon stimulate the

phosphorylation of HMG CoA reductase (Champe et al. 1994).



**Figure 6. Proposed structure of HMG-CoA reductase. (Adapted from Vance 1988.)**



**Figure 7. Regulation of HMG-CoA reductase. (⊕, Stimulation; ⊖, Inhibition.)**

## **5.2 Metabolism of chylomicron and very low density lipoprotein**

Chylomicron is an exogenous triacylglycerol-rich lipoprotein. It is synthesized in the small intestine utilizing dietary cholesterol, triacylglycerol and phospholipids, and carries primarily triacylglycerols and cholesteryl esters via the lymphatics to the blood. Chylomicron is degraded in the blood by lipoprotein lipase, which hydrolyzes the triacylglycerol to free fatty acids and glycerol, and converts chylomicron to a smaller particle called the chylomicron remnant, which contains apoB-48 and apoE. The chylomicron remnant is taken up by liver cells through LDL receptor mediated endocytosis. In liver cells, chylomicron remnants are degraded by lysosomal proteases, lipases and phosphodiesterases to free amino acids, fatty acids, glycerol, and free cholesterol (Mainetti 1990).

Very low density lipoprotein (VLDL) is an endogenous lipoprotein which is produced in the liver. It is synthesized in the rough endoplasmic reticulum, and assembled, packaged, and transported via secretory vesicles to the plasma. The function of VLDL is to transport triacylglycerol, phospholipids, and cholesterol from the liver to other tissues of the body. The nascent VLDL in the plasma is stepwise degraded by lipoprotein lipase, yielding intermediate density lipoprotein (IDL) and then LDL. IDL contains both apoB-100 and apoE. It can be taken up more rapidly by liver cells through apoB/E receptors than can LDL, which contains only apoB-100. Thus, IDL do not normally accumulate in the plasma, and consequently LDL represents the major degradation end product of VLDL metabolism (Mainetti 1990).

## **5.3 Metabolism of low density lipoprotein**

LDL is the major carrier of cholesterol in the plasma. It is composed of free cholesterol, phospholipids, triacylglycerol, cholesteryl esters and apoB-100. More than 50 percent of LDL is

cholesterol and cholesteryl esters (Champe et al. 1994). LDL derives from the endogenous lipoprotein VLDL by the degradation of lipoprotein lipase. The removal of LDL from plasma is mainly dependent on receptor-mediated endocytosis. Briefly, LDL particles are bound by cell-surface membrane receptors which specifically recognize apoB-100. LDL particles are then internalized by endocytosis in the form of clathrin-coated vesicles. Intracellularly, the coated vesicle loses its clathrin and fuses with lysosomes. LDL is broken down in the lysosome by the action of hydrolytic enzymes. The LDL receptors are not destroyed but separated from LDL and return to the cell surface (Glew 1992).

The LDL receptors are negatively charged glycoprotein molecules which are clustered in pits on the cell surface opposite the clathrin-coated cytosolic side. Each receptor has five domains and consists of 839 amino acids. The binding domain of the receptor contains clusters of negatively charged amino acids that can bind to the positively charged lysine and arginine residues of apoB and apoE. It is synthesized in the rough endoplasmic reticulum, modified in the Golgi, transported to the membrane and gathered in coated pits (Mainetti 1990).

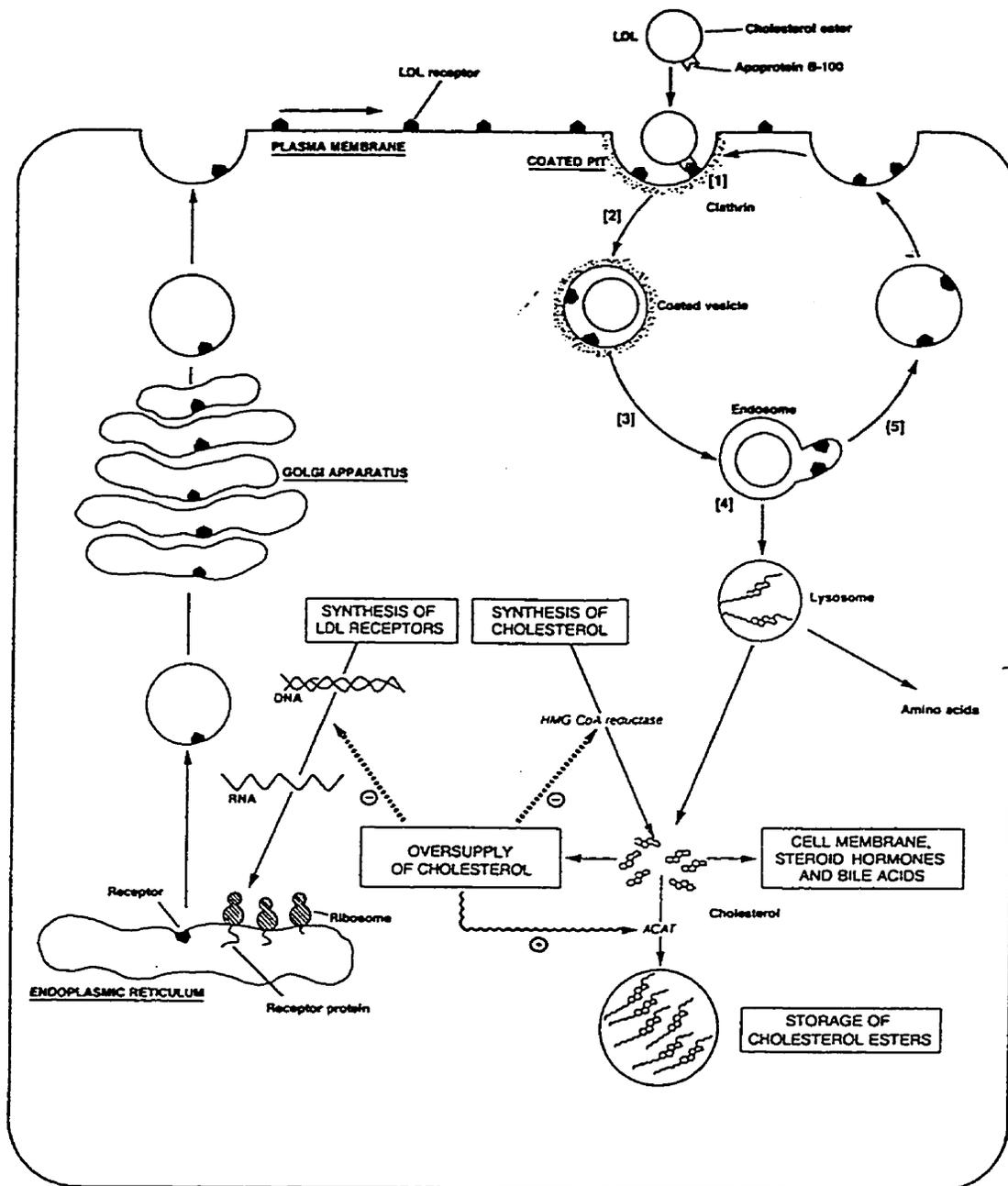
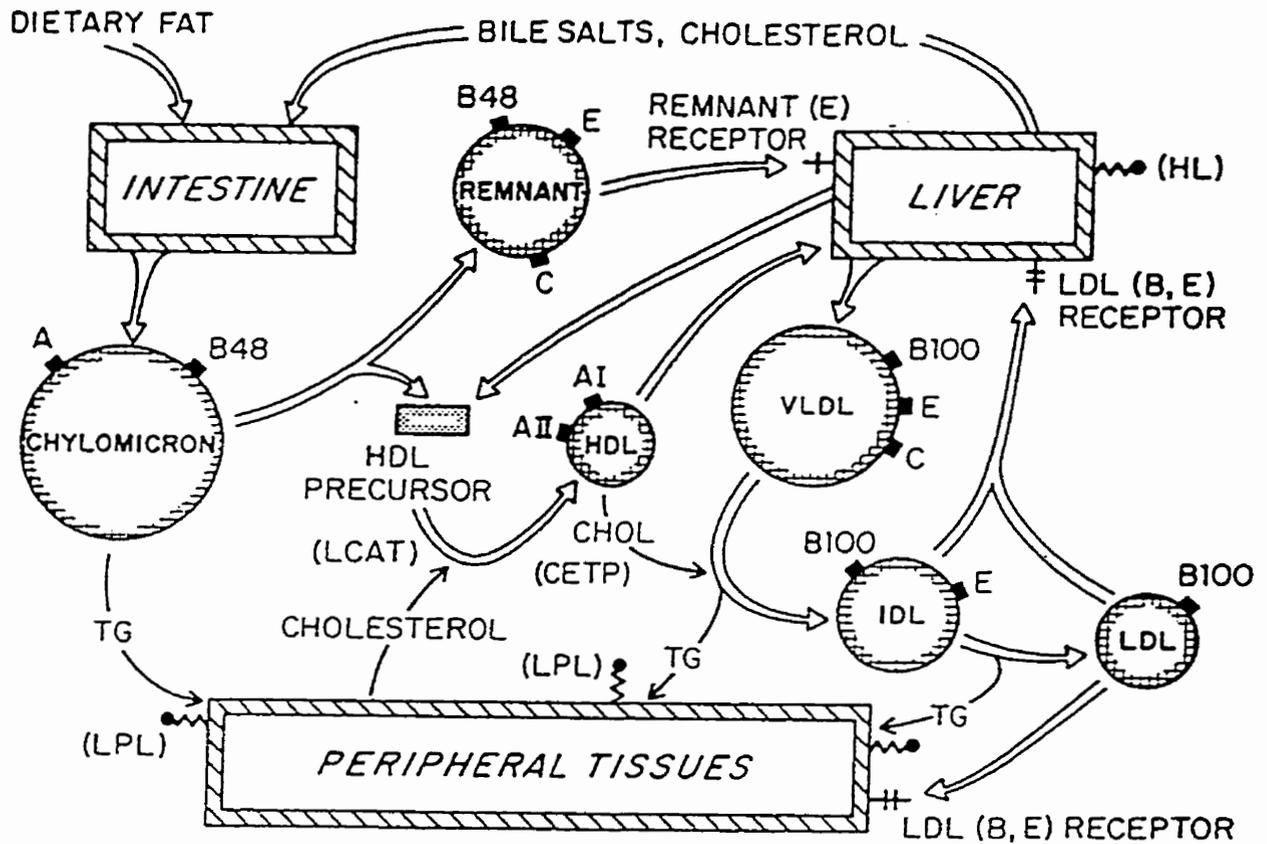


Figure 8. Metabolism of LDL. (Adapted from Marinetti 1990.)

#### **5.4 Metabolism of high density lipoprotein**

High density lipoprotein (HDL) is synthesized in the liver and is released into the bloodstream by exocytosis. Newly secreted HDL consists of disklike particles containing predominantly unesterified cholesterol, phospholipids and a number of apolipoproteins including apoE, apoA and apoC. ApoC and E are transferred between HDL and VLDL and chylomicrons in the plasma because they are essential in the metabolism of chylomicrons and VLDL. Once free cholesterol is taken up by the HDL, it is immediately esterified by lecithin: cholesterol acyltransferase (LCAT). LCAT can transfer an unsaturated fatty acid from the 2-position of PC to the hydroxyl group of cholesterol, producing cholesteryl ester and lysophosphatidylcholine. The nonpolar cholesteryl esters move into the hydrophobic interior of the bilayer, whereas LPC is transferred to plasma albumin. During this process, the HDL particle changes shape from disk to sphere. Spherical HDL particles are taken up by the liver by receptor-mediated endocytosis, and the cholesteryl esters are degraded. The cholesterol thus released can be either repackaged in lipoproteins, converted into bile acids, or secreted into bile for removal from the body (Mayes 1993).

HDL particles perform a number of important functions in the metabolism of cholesterol. First, they serve as a circulating reservoir of apoC and apoE for VLDL and chylomicron. Second, they can transfer cholesteryl esters to VLDL and LDL in exchange for triacylglycerol and can carry cholesteryl esters to the liver by itself. The most important function of HDL is that it can accumulate cholesterol from peripheral tissues and transport it to the liver, which is the major mechanism for the body to eliminate excess cholesterol (Mainetti 1990).



**Figure 9. Overview of the metabolism of plasma lipoproteins. (Adapted from Marinetti 1990.)**

## **6. The Metabolism of Triacylglycerols in the Human Body**

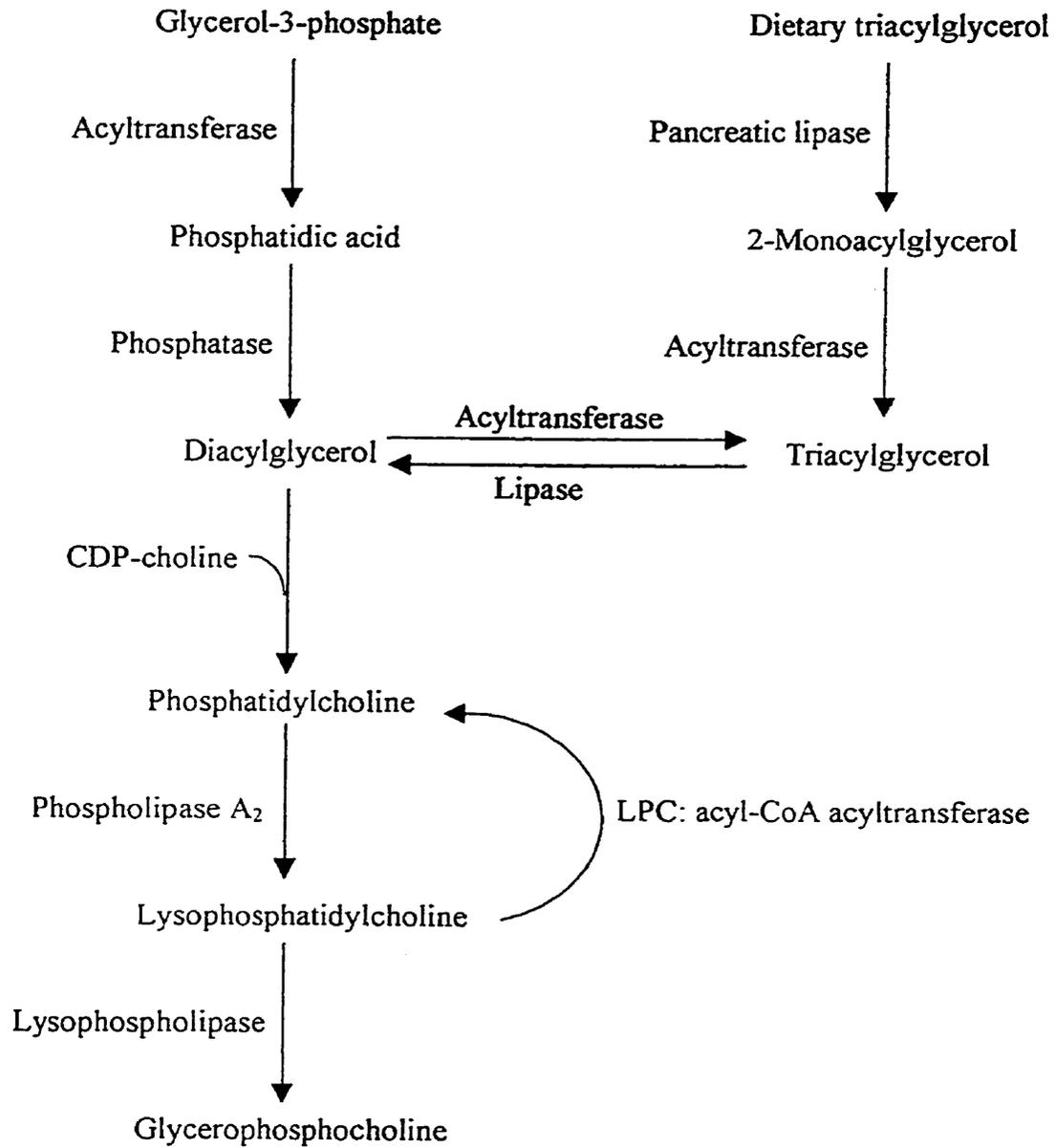
Triacylglycerols (TG) are the major energy storage source and transport form of fatty acids in animals. The major sites of synthesis are in the intestine, liver and adipose tissues. There are two separate synthetic pathways of triacylglycerol in the human body. The intestine utilizes a pathway beginning with 2-monoacylglycerol which accounts for 80 percent of triacylglycerol metabolism in the body. Other tissues utilize a pathway beginning with glycerol 3-phosphate (Montgomery et al. 1990).

In the intestinal lumen, dietary triacylglycerol is degraded by pancreatic lipase to 2-monoacylglycerol and fatty acids. These products enter the epithelial cells of the small intestine and are resynthesized to triacylglycerol by direct acylation of monoacylglycerol which is catalysed by acyltransferase. In the liver and adipose tissue, the biosynthesis of triacylglycerol starts from glycerol 3-phosphate which normally comes from glucose metabolism. Glycerol 3-phosphate is first acylated to phosphatidic acid by the action of acyltransferase. Then the phosphate group is hydrolysed by phosphatidate phosphatase to form diacylglycerol, and later diacylglycerol is further acylated to triacylglycerol (McGarry 1992).

## **7. The Metabolic Pathway of Lysophosphatidylcholine**

Lysophosphatidylcholine derives from phosphatidyl choline by the action of phospholipase A<sub>1</sub> (PLA<sub>1</sub>) or phospholipase A<sub>2</sub> (PLA<sub>2</sub>). PLA<sub>1</sub> selectively hydrolyses the sn-1 fatty acyl bond, which is usually esterified with saturated fatty acids. In contrast, PLA<sub>2</sub> selectively hydrolyse the sn-2 fatty acyl bond, which is usually esterified with unsaturated fatty acids (Choy and Arthur 1989). In mammalian cells, PLA<sub>2</sub> is the major phospholipase responsible for the formation of LPC. However, in plasma,

LPC is mainly produced by lecithin: cholesterol acyltransferase, which cleaves the sn-2 acyl ester in PC and transfers it to cholesterol to form cholesteryl ester (Stein 1968). LPC is a detergent-like phospholipid, which can disturb the integrity of the cell membrane. It is important that the amount of LPC be controlled in the cell. LPC can be either degraded to glycerophosphocholine by lysolipase or reacylated to form PC by the action of LPC-acyltransferase (Choy and Arthur 1989).



**Figure 10. Combined pathway of triglyceride and LPC metabolism.**

## **8. Clinical Classifications of Hyperlipidemia**

Hyperlipidemia can be classified as primary and secondary hyperlipidemia. Primary hyperlipidemia describes elevated cholesterol levels that are independent of other health problems, such as familial hyperlipidemia. Secondary hyperlipidemia is associated with other health problems, such as diabetes mellitus and obesity. Primary hyperlipidemia is generally caused by genetic defects. There may be a defective synthesis of the apoproteins, a lack of receptors, defective receptors, or defects in the handling of cholesterol within the cells.

**Table 3. Classifications of hyperlipidemia and their genetic basis (Adapted from Cotran et al. 1994)**

Type	Familiar Name	Lipoprotein abnormality	Known underlying genetic defects
1	Exogenous dietary hypertriglyceridemia	Elevated chylomicrons and triglycerides	Mutation in lipoprotein lipase gene
2a	Familial hypercholesterolemia	Elevated LDL-cholesterol	Mutation in LDL receptor gene or in apolipoprotein B gene
2b	Combined hyperlipidemia	Elevated LDL, VLDL, and triglycerides	Mutation in LDL receptor gene or in apolipoprotein B gene
3	Remnant hyperlipidemia	Increased remnants (chylomicrons), IDL triglycerides, and cholesterol	Mutation in apolipoprotein E gene
4	Endogenous hypertriglyceridemia	Elevated VLDL and triglycerides	Unknown
5	Mixed hypertriglyceridemia	Elevated VLDL, chylomicrons, cholesterol, triglycerides greatly elevated	Mutation in apolipoprotein C II gene

## 9. The Pharmacological Basis of Atorvastatin (Lipitor®) Treatment

Atorvastatin is a synthetic lipid-lowering agent. The chemical name for atorvastatin is [R-(R\*,R\*)]-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic acid, and the molecular weight is 1209.42. It is a selective, competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is the key enzyme in the biosynthesis of cholesterol. Atorvastatin lowers plasma cholesterol and lipoprotein levels by inhibiting HMG-CoA reductase and cholesterol synthesis in the liver and by increasing the number of hepatic low density lipoprotein receptors on the cell surface for enhanced uptake and catabolism of LDL. Atorvastatin can reduce both LDL-cholesterol, VLDL-cholesterol and TG, as well as the number of apolipoprotein B containing particles.

Before receiving atorvastatin treatment, patients should be placed on a standard cholesterol lowering diet, and should continue this diet during treatment. The recommended dose of atorvastatin is 10 mg once a day. The significant response is within 2 weeks, and maximum response is usually achieved within 2-4 weeks.

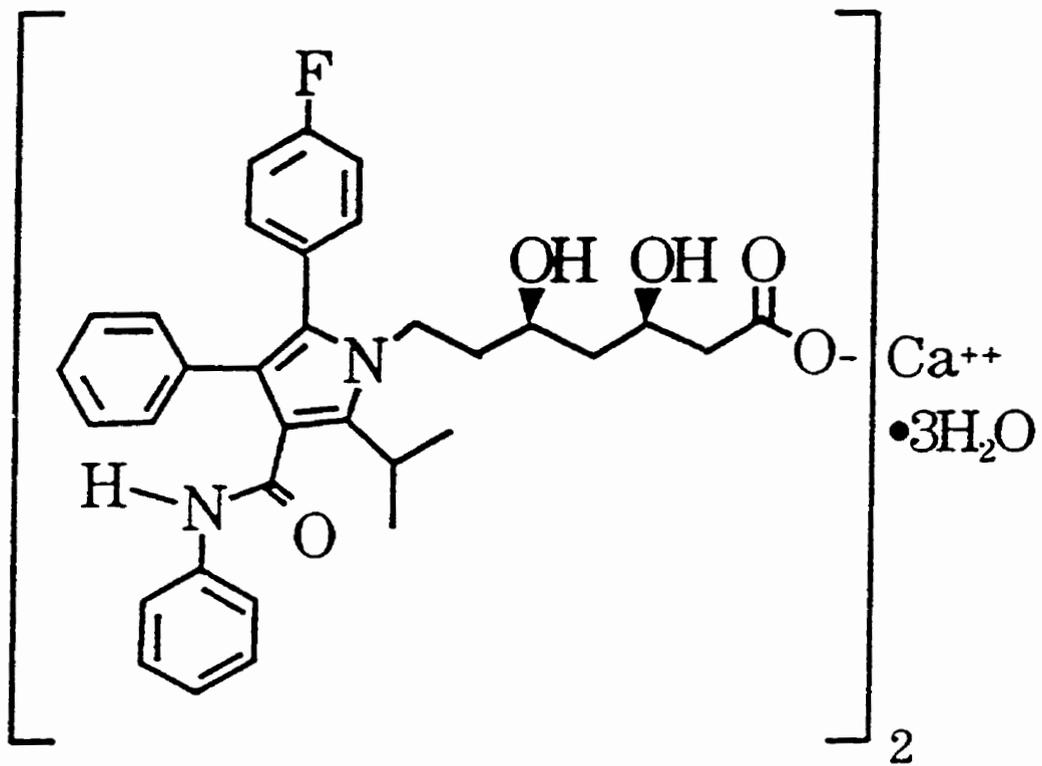


Figure 11. Structural formula of atorvastatin.

## **10. Atorvastatin Treatment and Oxidatively Modified LDL in Hyperlipidemic Patients:**

### **Statement of Hypothesis**

Atorvastatin is an HMG-CoA reductase inhibitor which is highly effective in reducing LDL-cholesterol levels in hyperlipidemic patients. This drug may also be able to lower triglyceride and elevate HDL-cholesterol levels in these patients. The objective of the present research project was to investigate the effect of atorvastatin treatment on the biochemical content of oxidized LDL and its ability to impair the endothelium-dependent relaxation in hyperlipidemic patients. The hypothesis was that atorvastatin treatment of hyperlipidemic patients attenuates the ability of their oxidized LDL to impair the endothelium-dependent relaxation of blood vessels. Such an alteration may arise from changes in the content and composition of lysophosphatidylcholine in the oxidized LDL by drug treatment.

## **II. MATERIALS AND METHODS**

### **1. Materials**

#### **1.1 Drugs**

Atorvastatin (Lipitor<sup>®</sup>, 10mg tablet) was donated by the Pfizer company.

#### **1.2 Experimental animals**

Mature male Sprague-Dawley rats ( $250 \pm 50$ g) were used for the studies of endothelium dependent relaxation. The rats were purchased from Charles River Canada Inc., St. Constante, Quebec, Canada. The rats were cared for in accordance with the guidelines of the Canadian Council on Animal Care.

#### **1.3 Chemical reagents**

Sodium bromide, copper sulfate, were purchased from Fisher Scientific. Heparin sodium and Diazepam were obtained from the pharmacy of the Health Science Centre, Winnipeg, Manitoba. Phenylephrine hydrochloride and acetylcholine were purchased from the Sigma Chemical Company, St. Louis, MO. Thin layer chromatographic plates (G-25), chloroform, methanol, acetic acid were purchased from Fisher Scientific. Reagents for lipid phosphorus determination were purchased from the Sigma Chemical Company, St. Louis, MO.  $\text{BCl}_3$ , methanol was obtained from Supelco Canada Ltd., Oakville, Ontario. High grade nitrogen, hydrogen and compressed air were from Welders Supplies,

Winnipeg, Manitoba. All other chemicals were of reagent grade and were obtained through the Canlab Division of Travenol Canada Inc., Mississauga, Ontario.

## **2. Methods**

### **2.1 Recruitment of hyperlipidemic patients**

Twenty-five patients with type IIa hyperlipidemia (total cholesterol > 6.2 mmol/L) were recruited at the Lipid Clinic, Health Sciences Centre, Winnipeg, Manitoba. None of the patients was on active treatment with cholesterol-lowering agents or antioxidant drugs. A blood sample was taken after informed consent from the hyperlipidemic patient upon recruitment.

### **2.2 Diet counseling of hyperlipidemic patients**

After recruitment, hyperlipidemic patients were counseled to follow the American Heart Association Step I diet. The duration of the dietary treatment was 6 weeks and subsequently a second sample was taken.

### **2.3 Atorvastatin treatment of hyperlipidemic patients**

Nineteen of the above patients were found still to have high plasma lipids after dietary treatment, and these patients were treated with atorvastatin (10mg/day) for the subsequent 4 weeks (diet continued). A blood sample was then taken after drug treatment.

**Table 4. The American Heart Association and the National Cholesterol Education Program Step I diet**

Nutrient*	Recommended Intake as Percent of Total Calories
	Step I Diet
Total Fat	30% or less
Saturated Fatty Acids	8-10%
Polyunsaturated Fatty Acids	up to 10%
Monounsaturated Fatty Acids	up to 15%
Carbohydrate	55% or more
Protein	Approximately 15%
Cholesterol	Less than 300 mg per day
Total Calories	To achieve and maintain desired weight

\* Calories from alcohol not included.

## **2.4 Measurement of plasma lipid and lipoprotein contents in hyperlipidemic patients**

All blood samples were taken from patients after fasting overnight. The samples were collected in Vacutainer tubes containing EDTA (4.0 mM). Plasma was separated by low speed (2,500 rpm) centrifugation at 4°C. Concentrations of total serum cholesterol, high density lipoprotein-cholesterol, triglycerides and glucose were determined by enzymatic analysis using commercial test kits (Boehringer Mannheim, Mannheim, Germany). LDL-cholesterol was calculated by the Friedewald equation (Friedewald et al. 1972).

## **2.5 Isolation of LDL samples from hyperlipidemic patients plasma**

LDL ( $d=1.019-1.063$  g/ml) was isolated from freshly prepared plasma by sequential ultracentrifugation (Havel et al. 1955). Sodium bromide was used to adjust the density of the plasma. EDTA (0.3 mM) was placed in all buffers in order to prevent auto-oxidation of the lipoproteins. LDL samples were dialyzed for 48 h at 4°C against 3 changes of phosphate-buffer saline (composition: NaCl 140 mM, KCl 3 mM,  $\text{Na}_2\text{HPO}_4$  2 mM and EDTA 0.3 mM) and immediately used for the next step.

## **2.6 Protein determination**

The protein content in the LDL sample was determined by the method of Lowry (Lowry et al. 1951).

## **2.7 Oxidative modification of LDL**

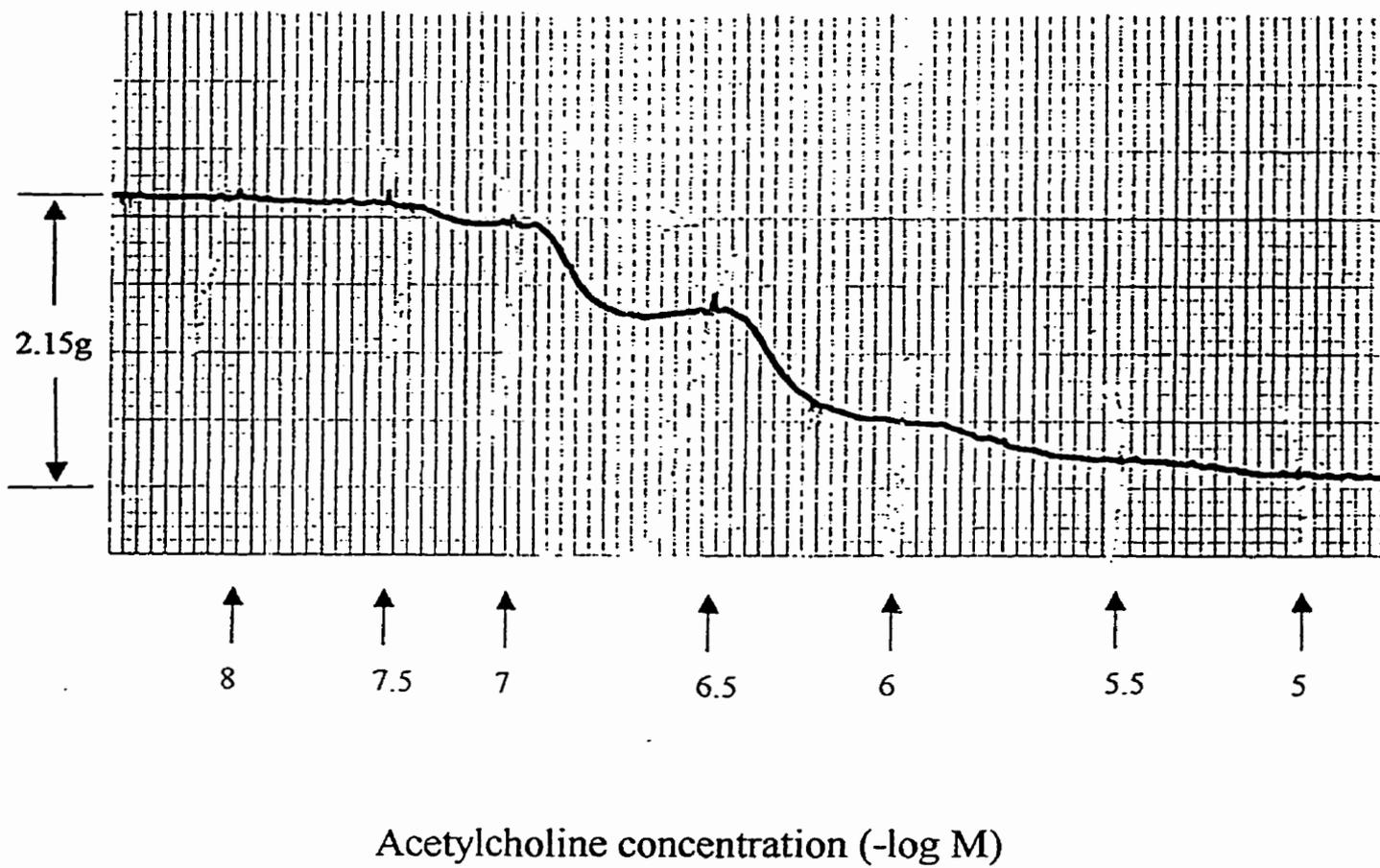
Oxidative modification of LDL was effected *in vitro* by the addition of 5 $\mu\text{M}$   $\text{CuSO}_4$ , an agent

known to produce a modification of LDL similar to that produced by endothelium cells (Steinbrecher et al. 1984) and was the preferred procedure in most recent studies. The isolated LDL was dialyzed for 24 h at 4°C against 3 changes of an EDTA-free phosphate-buffered saline prior to oxidative modification. After measuring the volume, protein concentration in the sample was determined by the method of Lowry and was adjusted to 1mg/ml by EDTA-free phosphate -buffered saline. Oxidation was initiated by addition of 5µM CuSO<sub>4</sub> and samples were constantly agitated at 37°C in an incubation bath for 24 h (Liu et al. 1994). The oxidation process was terminated by the addition of EDTA (0.6 mM). A portion of native and oxidized LDL samples were dialyzed at 4°C for 24 h with 4 changes of Krebs-Henseleit buffer (composition: NaCl 120 mM, KCl 4.76 mM, MgCl<sub>2</sub> 1.18 mM, CaCl<sub>2</sub> 1.25 mM, NaHCO<sub>3</sub> 25.0 mM, NaH<sub>2</sub>PO<sub>4</sub> 1.18 mM and glucose 5.5 mM). The dialysis buffer was saturated with N<sub>2</sub>. These *in vitro* oxidized LDL samples are referred to as oxidized LDL throughout the thesis. For comparison, LDL samples were processed in an identical manner in the absence of CuSO<sub>4</sub>, which are referred as native LDL. All LDL samples were stored at 4°C under N<sub>2</sub> and used within 2 weeks. Liu and co-workers (1994) have demonstrated that the oxidative modification produced by this method is complete. The products of this oxidation process include high amounts of lipid peroxides (thiobarbituric acid reactive substances, TBARS), high amounts of LPC and the electrophoretic mobility of LDL is altered as well (Liu et al. 1994).

## **2.8 Assessment of endothelium-dependent relaxation in aortic rings**

After the administration of an anticoagulant and sedative (800 units of heparin and 0.65mg diazepam, respectively), male Sprague-Dawley rats ( 250 ± 50g ) were killed by cervical dislocation. The thoracic aorta was removed, carefully cleared of adhering connective and fatty tissue and cut into

3 mm sections. Each aortic ring was mounted 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 connected to a Gould Brush 2400 chart recorder. The organ bath contained 10 ml of Krebs-Henseleit solution which was maintained at 37°C and bubbled with a gas mixture containing 95% O<sub>2</sub> and 5% CO<sub>2</sub>. After an equilibration period of 90 min, with tension being adjusted periodically until resting tension remained stable at 2.0 g, the aortic ring was precontracted with phenylephrine (10<sup>-6</sup> M). When the contraction had become stable, acetylcholine (Ach, 10<sup>-8</sup>-10<sup>-5</sup> M) was added cumulatively to produce endothelium-dependent relaxation. The relaxation produced by acetylcholine was expressed as a percentage of the phenylephrine-induced contraction. Criteria for acceptability of the endothelium/vascular ring preparation included active contraction of at least 1.5g and acetylcholine-induced relaxation of at least 80%. After the washout of phenylephrine and Ach, the aortic ring was incubated with oxidized LDL (0.3 mg LDL protein/ml) 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 as the presence of LDL may inactivate the endothelium-derived relaxing factor. For comparison, controls treated with native LDL (0.3 mg LDL protein/ml) or no addition were also studied.



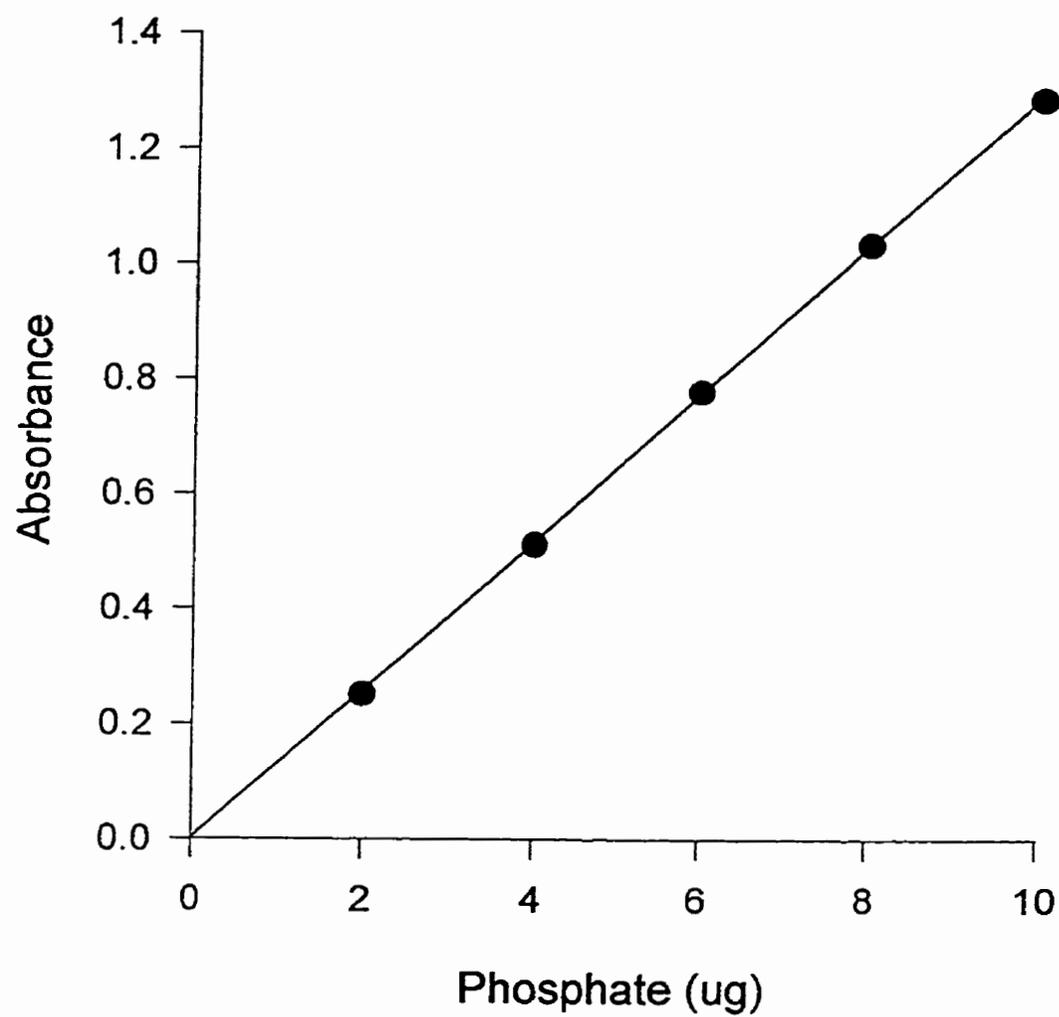
**Figure 12. Endothelium-dependent relaxation.** The aortic ring was precontracted by phenylephrine, then relaxed by stepwise addition of acetylcholine.

## **2.9 Biochemical analysis of native and oxidized LDL**

The protein concentration in the LDL samples was determined by the method of Lowry et al. (1951). Lipids in the LDL samples were extracted by a chloroform/methanol mixture (2:1, v/v), and the total lipid phosphorus in each sample was determined by the method of Bartlett (1959). The lipid extract was separated into phospholipid groups by thin layer chromatography (TLC) using a solvent system of chloroform-methanol-acetic acid-water (70:30:2:4, v/v). Phospholipid fractions on the TLC plate were visualized by iodine vapor. Bands corresponding to PC, SM and LPC were scraped into tubes after the evaporation of iodine. Phospholipids were eluted from silica gel and the lipid phosphorus content in each fraction was determined by the method of Bartlett (1959).

## **2.10 Lipid phosphorus determination**

The lipid phosphorus content in the LDL sample was determined by Bartlett method (Bartlett 1959).



**Figure 13. Bartlett lipid phosphorus analysis.** Absorbance was measured at 830 nm.

### **2.11 Determination of acyl groups in LPC**

After separation by TLC, LPC was eluted from silica gel with GLC grade chloroform and methanol (1:1). The solvent was evaporated under a stream of nitrogen. Subsequently, 0.5 ml of  $\text{BCl}_3$  reagent was added to the dried sample. Methylation of the sample took place in a screw-capped tube which was heated at  $96^\circ\text{C}$  for 5 min. The reaction was stopped by adding 0.5 ml distilled water. Then 1.5 ml petroleum ether was added to the reaction mixture to extract methylated esters. The mixture was then vortexed briefly and centrifuged at 2000rpm for 5 min. The methyl esters were recovered in the petroleum ether phase and the efficiency of methylation was over 95%. The methyl esters were analyzed with a Shimadzu gas-liquid chromatograph (Tekscience; Oakville, Ontario, Canada) equipped with a 15% DEGS columns. Heptadecanoic acid methyl ester was used as a standard for quantitation. The data obtained were analyzed with a Shimadzu Chromatopac CR601 integrator.

### **2.12 Statistical analyses**

Paired t test was used to compare data between recruitment and after diet treatment, after diet treatment and after atorvastatin treatment. All values are expressed as mean  $\pm$  standard error of the mean (SEM). P value  $< 0.05$  was considered to be statistically significant.

### **III. RESULTS**

#### **1. Effects of dietary counseling and atorvastatin treatment on the plasma lipid levels in type IIa hyperlipidemic patients**

Twenty-five patients with type IIa hyperlipidemia were recruited for the study. A blood sample was taken immediately upon recruitment. The cholesterol, triglyceride and glucose concentrations in blood samples were determined. The levels of total cholesterol and LDL-cholesterol were significantly elevated in the plasma (Table 5) compared with the Canadian Cholesterol Consensus Guidelines (values in  $\mu\text{mol/ml}$ , total cholesterol  $\leq 4.5$ - $5.0$ , LDL-cholesterol  $\leq 3.4$ , HDL-cholesterol  $\geq 0.9$ , triglyceride  $\leq 2.0$ ). The levels of HDL-cholesterol and triglycerides were within the normal range. These data confirmed the type IIa hyperlipidemic status of these patients.

The American Heart Association and the National Cholesterol Education Program Step I diet were the basis for dietary counselling given to these patients immediately upon their recruitment. The step I diet is recommended by the American Heart Association and the National Cholesterol Education Program for the treatment of hypercholesterolemia. The primary aim of this dietary therapy is to reduce the risk of coronary heart disease. The diet is designed to help patients decrease their intakes of saturated fat and cholesterol and restore appropriate calorie balance. In most trials, patients are recommended to follow the NCEP Step 1 diet for several weeks before they receive any drug treatment and are encouraged to maintain this diet throughout the study (NECP 1994). After six weeks of dietary therapy, the cholesterol, triglyceride and glucose concentrations in blood samples

of these patients were determined. Six of these patients were found to respond to the dietary therapy. Their total plasma cholesterol and LDL-cholesterol levels decreased to within the normal range (data not shown). This shows that dietary therapy is effective in decreasing the plasma cholesterol level in some patients. However, 19 of the patients failed to respond to the dietary counseling and their lipid levels remained elevated (Table 5).

The type IIa hyperlipidemic patients who were resistant to dietary therapy (19/25) were treated with atorvastatin for the subsequent 4 weeks. A blood sample was taken after drug treatment and the cholesterol, triglyceride and glucose concentration were measured. Atorvastatin treatment showed good effectiveness in reducing total cholesterol, LDL-cholesterol, and triglyceride levels in the plasma of these patients (Table 6). It also produced a slight increase in HDL levels. After treatment with atorvastatin (10mg/day) for 4 weeks, total cholesterol levels were found to be lowered by 23%, LDL-cholesterol was lowered by 32%, and triacylglycerol was lowered by 19% compared with dietary therapy alone. Interestingly, HDL level was also found to increase by about 9% in these type IIa hyperlipidemic patients. No change was observed in the glucose level after the drug treatment.

**Table 5. Plasma lipid and lipoprotein contents in hyperlipidemic patients before and after dietary counseling**

---

	Pre-diet	Diet
	( $\mu\text{mol.ml}^{-1}$ plasma)	
Total cholesterol	7.14 $\pm$ 0.24	7.19 $\pm$ 0.24
LDL cholesterol	4.79 $\pm$ 0.22	4.82 $\pm$ 0.22
HDL cholesterol	1.43 $\pm$ 0.06	1.39 $\pm$ 0.06
Triglyceride	2.03 $\pm$ 0.19	2.06 $\pm$ 0.16
Glucose	4.98 $\pm$ 0.15	5.22 $\pm$ 0.25

---

Blood samples were taken from patients before any treatment (pre-diet) and after 6 weeks dietary counseling (diet). LDL was obtained from the plasma by density centrifugation. All values are expressed as mean  $\pm$ SEM (n=25). No significant differences between means of groups for any of the measured lipids, pre- and post dietary counseling were found. Paired t-test was used.

**Table 6. Plasma lipid and lipoprotein contents in hyperlipidemic patients after dietary counseling and after atorvastatin treatment**

	Diet	Atorvastatin
	( $\mu\text{mol.ml}^{-1}$ plasma)	
Total cholesterol	7.62 $\pm$ 0.24	5.84 $\pm$ 0.27**
LDL cholesterol	5.21 $\pm$ 0.23	3.53 $\pm$ 0.27**
HDL cholesterol	1.38 $\pm$ 0.07	1.50 $\pm$ 0.10*
Triglycerides	2.19 $\pm$ 0.20	1.78 $\pm$ 0.12*
Glucose	5.46 $\pm$ 0.34	5.17 $\pm$ 0.26

Blood samples were taken from patients after 6 weeks dietary counseling (diet) and after 4 weeks atorvastatin treatment (10mg/day, diet continued). LDL was obtained from the plasma by density centrifugation. All values are expressed as mean  $\pm$ SEM (n=19). \*\*p< 0.001, \*p< 0.02 when compared with subjects after dietary counseling. Paired t-test was used.

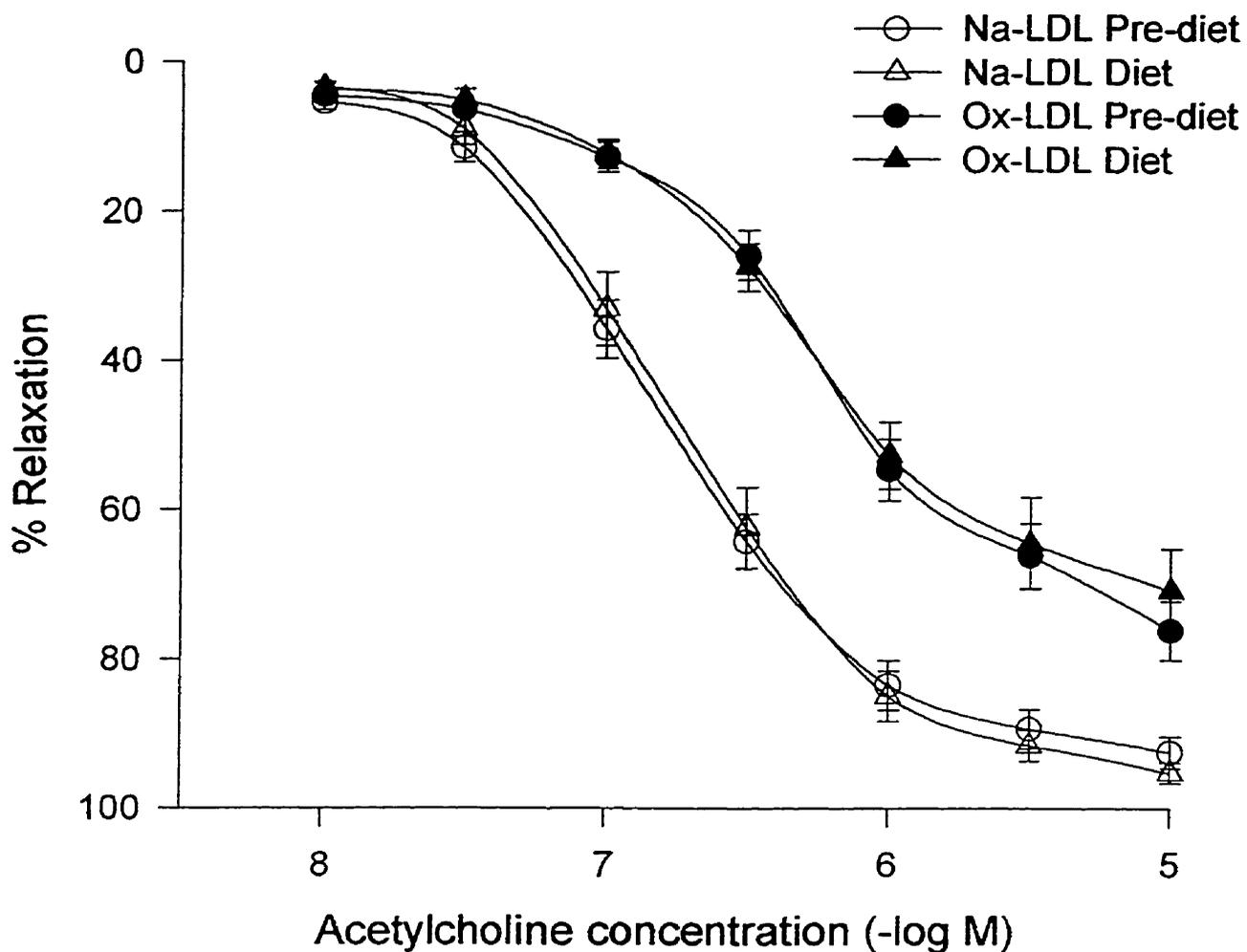
## **2. Effects of dietary counseling and atorvastatin treatment on the oxidized LDL from hyperlipidemic patients: impairment of endothelium-dependent relaxation**

In the present research, we focused on studying the effects of LDL which had been oxidized *in vitro*. The term “oxidized LDL” used throughout this thesis represents exogenously oxidized LDL. The LDL-cholesterol from patients upon recruitment, after dietary therapy and after atorvastatin treatment were isolated from the plasma by sequential ultracentrifugation and oxidized in the presence of 5  $\mu$ M copper sulfate at 37°C for 24 hour with a constant agitation. The ability of oxidatively modified LDL from these patients to impair the endothelium-dependent relaxation of aortic rings was investigated.

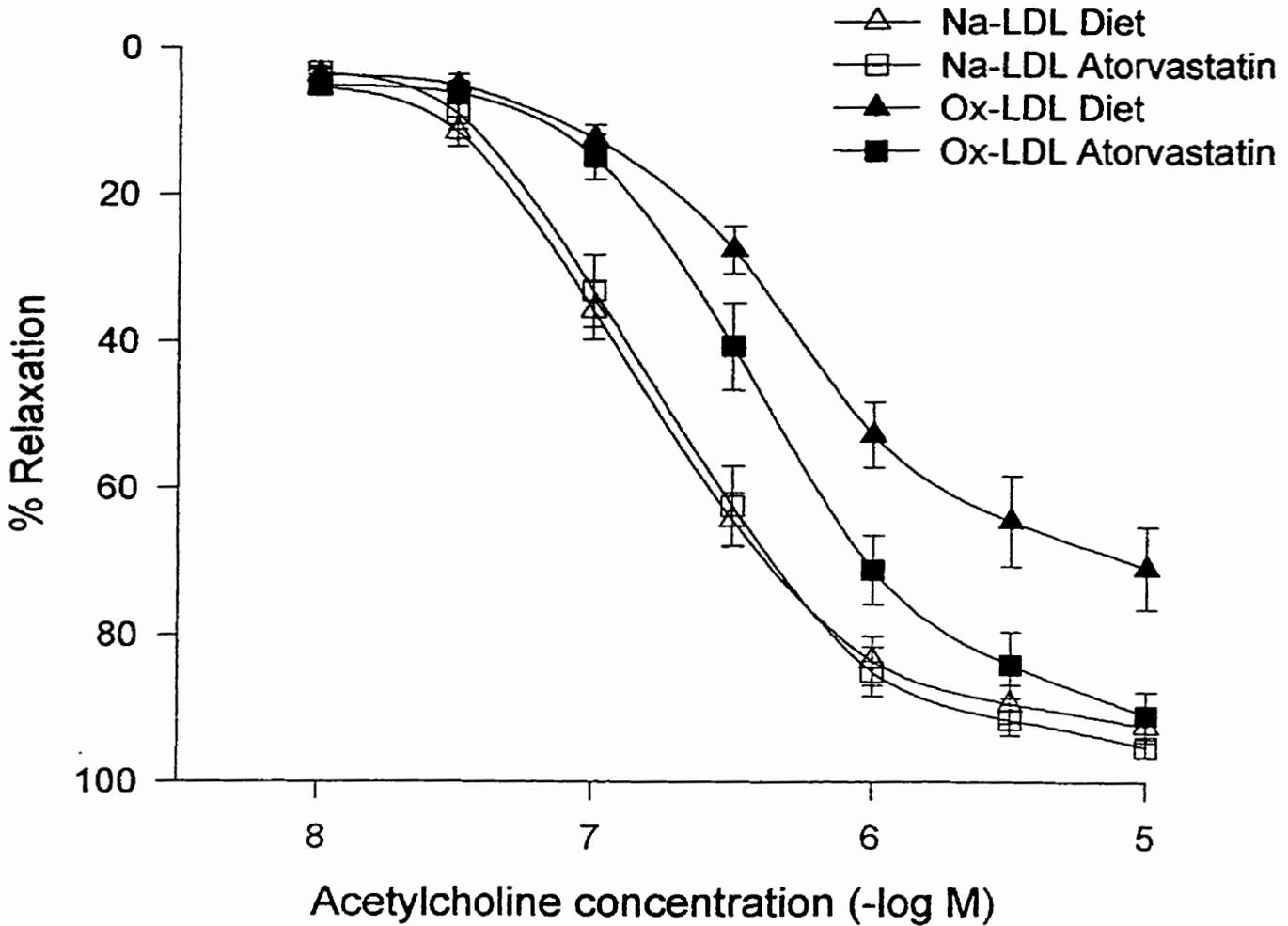
The effect of LDL oxidation on the endothelium-dependent relaxation was assessed by stepwise addition of acetylcholine to phenylephrine-precontracted, endothelium-intact rat aortic rings. After incubation with 0.3mg/ml LDL for 30 mins, segments exposed to oxidized LDL from patients both upon recruitment and after dietary therapy showed strong impairment of the endothelium-dependent relaxation of aortic rings compared with native LDL and controls. It should be noted that the oxidized LDL samples from patients after dietary therapy did not show any improvement on the impairment of endothelium-dependent relaxation compared with samples obtained from patients upon recruitment (Fig. 14).

Regarding the effect of atorvastatin treatment, the LDL was also isolated from patients who received the atorvastatin treatment, oxidized by addition of 5 $\mu$ M CuSO<sub>4</sub>, and the effect of this oxidized LDL on the endothelium-dependent relaxation was examined. Oxidized LDL from patients after atorvastatin treatment still showed considerable impairment of endothelium dependent relaxation

compared with native LDL and controls. There was, however a significant difference as compared with samples from patients after diet therapy (alone). The ability of oxidized LDL from patients after atorvastatin treatment was greatly reduced, especially, at the higher concentrations of acetylcholine (Fig. 15).



**Figure 14. Effect of oxidized LDL from patients before and after dietary intervention on the impairment of endothelium-dependent relaxation.** The aortic ring preparation was exposed to native and oxidized LDL (0.3 mg/ml) from hyperlipidemic patients (O- native LDL from pre-diet patients, ●- oxidized LDL from pre-diet patients, Δ- native LDL from diet patients, ▲- oxidized LDL from diet patients) for 1 hour. Subsequently, the aortic ring preparation was washed with buffer and the acetylcholine-induced endothelium-dependent relaxation was determined. Each point represents the mean of 25 patients tested. The vertical bar represents the SEM.



**Figure 15. Effect of oxidized LDL from patients after dietary intervention and after atorvastatin treatment on the impairment of endothelium-dependent relaxation.** The aortic ring preparation was exposed to native and oxidized LDL (0.3 mg/ml) from hyperlipidemic patients ( $\Delta$ - native LDL from diet patients,  $\blacktriangle$ - oxidized LDL from diet patients,  $\square$ - native LDL from atorvastatin treated patients,  $\blacksquare$ - oxidized LDL from atorvastatin treated patients) for 1 hour. Subsequently, the aortic ring preparation was washed with buffer and the acetylcholine-induced endothelium-dependent relaxation was determined. Each point represents the mean of 19 patients tested. The vertical bar represents the SEM.

### **3. Effects of dietary counseling and atorvastatin treatment on phospholipid contents in native and oxidized LDL from hyperlipidemic patients**

The major phospholipids in native LDL are choline-containing phospholipids, which are phosphatidylcholine (PC), sphingomyelin and very small amounts of LPC. They comprise a monolayer with free cholesterol on the outer surface of LDL. In the human body, phospholipids come from two sources. One is from the diet and the other is from endogenous synthesis.

The biochemical factor leading to the impairment of endothelium-dependent relaxation by oxidized LDL has been confirmed to reside in the lipid components. It is reported that there is a five-fold increase of LPC content in the oxidized LDL compared with native LDL (Chen et al. 1997). Thus the phospholipid content of oxidized LDL from these patients upon recruitment, after dietary therapy and after atorvastatin treatment was examined. Lipids were extracted from native and oxidized LDL and the content of each phospholipid class was analyzed by thin layer chromatography.

No significant difference was detected in the total phospholipid content of the native and oxidized LDL from patients upon recruitment. A substantial decrease in the level of PC and a concomitant increase in the level of LPC were detected in the LDL after oxidization (Table 7). After 6 weeks of dietary therapy, no significant changes were found in the composition of phospholipids both in native and oxidized LDL compared with those obtained upon recruitment (Table 7). Further analysis of the LPC content in the oxidized LDL from patients after atorvastatin treatment showed that after 4 weeks of treatment there were no significant changes in the LPC content of native LDL, but there was an 11% decrease in LPC content of LDL between the two groups after oxidative modification (Table 8).

**Table 7. Phospholipid contents in native and oxidized LDL from hyperlipidemic patients before and after dietary counseling**

	Pre-diet	Diet
	( $\mu\text{mol}\cdot\text{mg}^{-1}$ LDL protein)	
<b>Phospholipids (total)</b>		
native LDL	1.347 $\pm$ 0.043	1.351 $\pm$ 0.030
oxidized LDL	1.250 $\pm$ 0.013	1.251 $\pm$ 0.012
<b>Phosphatidylcholine</b>		
native LDL	0.799 $\pm$ 0.021	0.800 $\pm$ 0.019
oxidized LDL	0.411 $\pm$ 0.016	0.415 $\pm$ 0.020
<b>Lysophosphatidylcholine</b>		
native LDL	0.068 $\pm$ 0.007	0.066 $\pm$ 0.009
oxidized LDL	0.401 $\pm$ 0.021	0.398 $\pm$ 0.018

Blood samples were taken from patients upon recruitment (pre-diet) and after 6 weeks dietary counseling (diet). Oxidized LDL was prepared *in vitro* by addition of 5  $\mu\text{M}$   $\text{CuSO}_4$  with constant agitation at 37°C for 24 h. All values are expressed as mean  $\pm$ SEM (n=25). No significant differences between means of groups for any of the measured lipids, pre- and post dietary counseling were found ( $p < 0.05$ , paired t-test).

**Table 8. Phospholipid contents in native and oxidized LDL from hyperlipidemic patients after dietary counseling and after atorvastatin treatment**

	Diet	Atorvastatin
	( $\mu\text{mol}\cdot\text{mg}^{-1}$ LDL protein)	
<b>Phospholipids (total)</b>		
native LDL	1.350 $\pm$ 0.053	1.346 $\pm$ 0.033
oxidized LDL	1.250 $\pm$ 0.043	1.251 $\pm$ 0.021
<b>Phosphatidylcholine</b>		
native LDL	0.789 $\pm$ 0.042	0.802 $\pm$ 0.029
oxidized LDL	0.410 $\pm$ 0.016	0.435 $\pm$ 0.035
<b>Lysophosphatidylcholine</b>		
native LDL	0.068 $\pm$ 0.011	0.063 $\pm$ 0.009
oxidized LDL	0.400 $\pm$ 0.012	0.358 $\pm$ 0.014*

Blood samples were taken from patients after 6 weeks dietary counseling (diet) and after 4 weeks atorvastatin treatment (10mg/day, diet continued). Oxidized LDL was prepared *in vitro* by addition of 5  $\mu\text{M}$   $\text{CuSO}_4$  with constant agitation at 37°C for 24 h. All values are expressed as mean  $\pm$ SEM (n=19). \*p<0.001 when compared with subjects in the after dietary counseling group. Paired t-test was used.

#### **4. Effects of dietary counseling and atorvastatin treatment on the distribution of acyl groups in LPC in native and oxidized LDL from hyperlipidemic patients**

It has been reported that LPC in oxidized LDL from hyperlipidemic patients has more long chain acyl groups compared with samples from normal people (Chen et al. 1997). It has also been confirmed that only long chain LPC can cause impairment of endothelium-dependent relaxation (Chen et al. 1997). In order to investigate whether dietary therapy and atorvastatin treatment can change the structure of LPC in LDL before and after oxidatively modification, LPC was isolated from other phospholipids and chain length was determined by GLC.

The acyl groups in LPC in native LDL from patients upon recruitment are mainly 16:0 and 18:0, of which 16:0 accounts for 55% and 18:0 accounts for 40% (Table 9). After LDL undergoes oxidative modification, the 16:0 group increases to 63%, and correspondingly the 18:0 group decreases to 34%.

After these patients received 6 weeks dietary intervention, acyl groups in LPC from native and oxidized LDL were measured again. In oxidized LDL, no significant changes were found in acyl groups of LPC between patients after dietary therapy and upon recruitment. A significant difference, however, was seen in LPC acyl groups in native LDL compared with patients upon recruitment with 6% decrease in 16:0 acyl group. The latter provides a measure of patient compliance and indicates that patients did effect changes in their diets, a factor which is very important for the validity of this study (Table 9).

The acyl groups in LPC in both native and oxidized LDL were also determined in the nineteen patients who received atorvastatin treatment. Compared with dietary therapy alone, the acyl groups

in LPC from native LDL show no change in patients after atorvastatin treatment. However, after oxidation of LDL, a significant difference was found in acyl groups in LPC compared with diet therapy: there was a decrease of about 4% in the 16:0 group, with a small increase in 18:0 group (Table 10).

As LPC with either 16:0 or 18:0 groups is equally potent in the impairment of endothelium-dependent relaxation of aortic rings (Chen et al, 1997), the physiological significance of this change in acyl group profile of LPC in oxidized LDL is unknown.

**Table 9. Distribution (%) of acyl groups in lysophosphatidylcholine in native and oxidized LDL from hyperlipidemic patients before treatment and after dietary counseling**

	Pre-diet	Diet
<b>Native LDL</b>		
Acyl Group	%	%
16:0	54.784±1.800	49.222±1.957*
16:1	0.725±0.058	0.783±0.061
18:0	39.996±1.837	42.490±2.670
18:1	7.566±0.379	6.873±0.542
18:2	2.508±0.257	3.179±0.494
<b>Oxidized LDL</b>		
Acyl Group	%	%
16:0	62.963±0.979	63.020±0.937
16:1	0.682±0.062	0.662±0.047
18:0	33.940±0.939	33.913±0.762
18:1	6.367±0.456	6.847±0.859
18:2	----	----

Blood samples were taken from patients before any treatment (pre-diet) and after 6 weeks dietary counseling (diet). Oxidized LDL was prepared *in vitro* by addition of 5  $\mu$ M CuSO<sub>4</sub> with constant agitation at 37°C for 24 h. Numbers represent the mean  $\pm$ SEM (n=25). \*P< 0.002 when compared with untreated subjects. Paired t-test was used.

**Table 10. Distribution (%) of acyl groups in lysophosphatidylcholine in native and oxidized LDL from hyperlipidemic patients after dietary counseling and after atorvastatin treatment**

	Diet	Atorvastatin
<b>Native LDL</b>		
Acyl Group	%	%
16:0	48.548±2.488	49.154±1.926
16:1	0.801±0.071	0.908±0.083
18:0	43.685±3.282	42.789±2.594
18:1	6.845±0.689	7.317±0.646
18:2	3.057±0.516	2.966±0.377
<b>Oxidized LDL</b>		
Acyl Group	%	%
16:0	63.633±1.025	60.068±0.769*
16:1	0.615±0.043	0.766±0.085
18:0	33.740±0.776	36.638±0.798*
18:1	6.548±1.273	7.270±0.773
18:2	----	----

Blood samples were taken from patients after 6 weeks dietary counseling (diet) and after 4 weeks atorvastatin treatment (10mg/day). Oxidized LDL was prepared *in vitro* by addition of 5  $\mu$ M CuSO<sub>4</sub> with constant agitation at 37°C for 24 h. Numbers represent the mean  $\pm$ SEM (n=19). \*p< 0.001 when compared with after dietary counseling subjects. Paired t-test was used.

## **VI. DISCUSSION**

### **1. Effect of atorvastatin treatment on plasma lipid levels**

Based on the results of our study, the administration of atorvastatin (10mg/day) for 4 weeks caused a significant reduction in total cholesterol (23%), LDL-cholesterol (32%) as well as plasma triglyceride levels (19%) in type IIa hyperlipidemic patients. The drug treatment was also associated with a slight increase in HDL (9%). Similar results have also been reported by others (Cilla et al. 1996; Le et al. 1996).

Atorvastatin is a synthetic reversible competitive inhibitor of HMG-CoA reductase which is the rate-limiting enzyme of cholesterol biosynthesis. HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate, a rate-limiting step in the formation of endogenous cholesterol. The inhibition of HMG- CoA reductase leads to the decrease in the intracellular stores of cholesterol, and this results in the up-regulation of the number of low density lipoprotein receptors on the cell membrane, thus increasing the clearance of LDL cholesterol from plasma (Blum 1994; Rackely 1996). The elevated cholesterol levels in hepatic cells results from the increased internalization of LDL and produces a feedback inhibition on the activity of the pre-existing HMG-CoA reductase. In addition to the feedback inhibition, the elevated cholesterol in cells will cause a decrease in the amount of mRNA for coding HMG-CoA reductase, and accelerate the rate of degradation of the enzyme in the cytosol. Taken together, these effects increase the utilization of plasma LDL by cells, and reduce the level of LDL in the plasma.

It is interesting to note that atorvastatin may also lower plasma cholesterol levels by inhibiting hepatic synthesis of cholesterol in very low density lipoprotein which is the source of LDL cholesterol, resulting in the reduced levels of LDL cholesterol (Haria et al. 1997). Since atorvastatin diminishes the synthesis of cholesterol in hepatic cells, and cholesterol is essentially required in the normal production of VLDL particles, the inhibition of cholesterol synthesis may impair VLDL particle assembly and secretion, decrease the VLDL levels in plasma, and further decrease the LDL level in plasma (Bakker et al. 1996).

The major source of cholesterol for the human body is primarily dietary. The effect of statins in inhibiting the absorption of dietary cholesterol in the small intestine has also been suggested (Miettinen 1991). It is reported that the efficiency of dietary cholesterol absorption was reduced 7.3% after 3 weeks, 16.8% after 6 month treatment by pavastatin. The mechanisms for reduced cholesterol absorption during statin treatment are thought not to involve a lack of intestine bile acid. One proposed mechanism is that statins may reduce the cholesterol content in the intestinal mucosal cells, with a subsequent reduction in ACAT activity and thus decrease the absorption of cholesterol (Miettinen 1991).

It is generally accepted that HMG CoA reductase does not play a direct role in the regulation of TG levels. Atorvastatin administration, however, produces marked reduction in TG levels in hyperlipidemic patients. Two indirect mechanisms have been suggested to explain the effect of atorvastatin on TG levels. The drug has been shown to inhibit the assembly of apoB and TG into VLDL (Arad et al. 1992, Ginsberg 1998). Since VLDL is the major transporting molecule of TG, suppressing the incorporation of TG into this molecule will impede the formation of VLDL particle and minimize the VLDL particles available for secretion, resulting in a lower TG level. A second

possibility is that atorvastatin induces a marked reduction in hepatic cholesterol levels, leading to an increase in LDL receptor expression, which in turn causes a reduction in the TG levels through the increased binding of VLDL particles and LDL (Conde et al. 1996). Furthermore, LDL receptors recognize apoB and E and as various VLDL particles contain both of these apolipoproteins (Grundy. 1991), and VLDL remnants may have greater affinity than LDL for these receptors (Ma et al. 1986).

The exact mechanism for the increase in HDL cholesterol levels caused by atorvastatin treatment remains unclear. It is possible that atorvastatin treatment alters the production of HDL by the liver or gastrointestinal tract, or that the increase is associated with the decrease in VLDL and LDL levels. The increased level of HDL would facilitate an increase the clearance of free cholesterol which is produced by peripheral tissues from plasma, and thus help to further decrease the cholesterol level in plasma.

In type IIa hyperlipidemia, which is also called familial hypercholesterolemia, the high level of LDL is caused by a genetic defect in LDL receptors. It results from a mutation in the gene which specifically codes for the LDL receptor. It is also possible that the defect in LDL receptor synthesis renders it unable to bind with LDL. Alternatively, it is possible that LDL receptors are completely absent in these patients. Therefore, the effect of atorvastatin to upregulate LDL receptors on the cell membrane seems inadequate to explain the reduced LDL level in these patients. We interpret the effect of atorvastatin treatment in reducing plasma LDL-cholesterol levels in these patients to be dependent on its effectiveness in inhibiting the activity of HMG-CoA reductase, thus leading to a decreased production of cholesterol, the assembly and secretion of VLDL from liver and the absorption of dietary cholesterol in the small intestine. On the basis of the observed reduction in LDL cholesterol in this study, we conclude that atorvastatin may be particularly suitable for patients with

heterozygous or homozygous familial hypercholesterolemia.

## **2. Effect of atorvastatin treatment on LPC levels and endothelium-dependent relaxation**

Oxidized LDL used in this study was prepared *in vitro* by the addition of 5 $\mu$ M CuSO<sub>4</sub>, this agent being known to produce a modification of LDL similar to that produced by endothelium cells and is commonly used to study the functional effects of lipid oxidation (Steinbrecher et al. 1984). Several lines of evidence suggest the presence of oxidatively modified LDL *in vivo*. At this time, it is unclear if the oxidized LDL produced by CuSO<sub>4</sub> is the same as that produced *in vivo* oxidation.

This study shows that the ability of oxidized LDL from hyperlipidemic patients after atorvastatin treatment to impair the endothelium-dependent relaxation has been significantly reduced compared with dietary therapy alone. The aortic rings incubated with oxidized LDL from patients after atorvastatin treatment can be almost completely relaxed by 10<sup>-5</sup> M acetylcholine (similar to rings incubated in the absence of oxidized LDL). Analysis of the biochemical content of LDL from this group revealed that 11% less LPC was present after oxidation when compared with the group that received only dietary counseling. Interestingly, the drug also caused a decrease in the C16:0 moiety with a corresponding increase in the C18:0 moiety in LPC in oxidatively modified LDL in the drug treated group. We think this might result from a combination of continued dietary treatment and drug therapy. In view of our observation that both LPC species are equally potent in the impairment of endothelium-dependent relaxation of the aortic rings, we feel that the atorvastatin-induced reduction in the level of LPC in the oxidized LDL is partially responsible for the improvement of endothelium-dependent relaxation of the aortic ring.

Endothelium-dependent relaxation of blood vessels can be inhibited early in the atherosclerosis process, even before histological evidence of plaque formation (Cohen et al. 1988, Shimokawa et al. 1989). Oxidized LDL has been confirmed to be responsible for this inhibition (Simon et al. 1990; Galle et al. 1991; Steinberg et al. 1989; Parthasarathy et al. 1989). The inhibitory action of oxidized LDL, in turn, can be partially attributed to LPC, which accumulates in the LDL particle during the oxidative process (Chen et al. 1997). It has been reported that the aortic rings from hypercholesterolemic rats after lipid-lowering treatment showed improved responsiveness to acetylcholine, and the latter was attributed to the lowered level of LDL in the plasma (Harrison 1994). In the present study, we were surprised to find that atorvastatin could also alter the ability of oxidized LDL to impair endothelium-dependent relaxation. It thus appears that atorvastatin treatment may provide beneficial effects, not only in the reduction of LDL-cholesterol, but also in diminishing the impairment of endothelium function when the LDL is oxidized under physiological conditions.

LPC is a phospholipid with detergent properties. It has a positively charged hydrophilic choline head, and one hydrophobic fatty acid tail. It is very effective in disrupting membranes by intercalation into the membrane matrix and, at high concentrations, may cause the solubilization of the membrane-associated lipids and proteins. LPC can solubilize membrane components by forming detergent-lipid and detergent-lipid-protein mixed micelles because of its detergent properties and its molecular configuration (Jacobson et al. 1988). The inhibition of endothelium-dependent relaxation by LPC is probably caused by its ability to alter the fluidity of the endothelium membrane, but not due to its cytotoxicity towards the endothelial cell (Cowan et al. 1995), since the endothelium-dependent relaxation of the aortic ring can be reversed after the removing of LPC from the bathing solution. As

acetylcholine induces the release of EDRF from endothelium cells through activating its membrane receptors leading to increased intracellular calcium concentration, LPC may interrupt the signal transduction pathway of EDRF release which is thought to occur within, or in close proximity to the cell membrane (Cowan et al. 1995). In separate studies, LPC has been shown to have a broad inhibitory effect on intracellular signaling processes, including the inhibition of IP<sub>3</sub> production and calcium elevation (Kugiyama et al. 1992; Inoue et al. 1992) and inhibition of G-protein-mediated signal transduction (Flavahan 1993; Liao 1994). LPC may act to alter the membrane environment with which membrane-bound proteins interact and thereby alter their affinity for substrates or cofactors. Alteration of membrane fluidity by LPC could provide an explanation for the diverse reported effects of LPC (Bing 1993). It is speculated that the effect of LPC on the impairment of endothelium-dependent relaxation is due to alteration of membrane fluidity in endothelium cells, inhibition of G-protein-mediated signal transduction, and thus inhibition of the release of NO and, perhaps, other endothelium-derived relaxant substances.

There is limited information on the modulation of phospholipid metabolism by atorvastatin. Yanagita and co-workers have shown that statins can inhibit phospholipid synthesis in HepG2 cells (Yanagita et al. 1994). In this study, we are the first to report that atorvastatin modulates the hydrolysis of PC to LPC when LDL is being oxidized. It is clear that the content of LPC and the distribution of acyl groups in LPC in native LDL were not significantly changed among the three study groups. When LDL was oxidized by Cu<sup>2+</sup> for 24 hours, the content of LPC in oxidized LDL from the atorvastatin-treated group was found to decrease about 11%, the distribution of acyl groups in LPC molecule was significantly changed with a decrease in 16:0 group and a corresponding increase of 18:0 group. The group treated with diet only produced a slight decrease in the 16:0 group

of LPC compared with the non-treated group. It is possible that the reduction of LPC observed in the oxidized LDL of the atorvastatin-treated group resulted from a combination of the continued dietary treatment as well as drug therapy. Our postulate is based on the notion that patients under dietary treatment would consume more unsaturated- than saturated fatty acids. It has been confirmed that double bonds in unsaturated fatty acids can act as an antioxidant, thus high amounts of unsaturated fatty acids in LDL might increase its resistance to the oxidative modification. Therefore, under the same conditions of oxidative modification, a lesser degree of LDL from patients after atorvastatin treatment is oxidized and thus less LPC is formed, because the amount of LPC generated in oxidized LDL is proportional to the degree of oxidation (Liu et al. 1994). In the liver, the increased consumption of unsaturated fatty acids might increase the utilization of unsaturated fatty acid to synthesize PC. The latter could lead to the substitution of saturated fatty acids with unsaturated fatty acids at sn-1 position in the PC molecule. The major class of unsaturated fatty acids in foods are oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) (Marinetti 1990). Thus LPC, which is generated from PC in LDL after oxidative modification, has increased amounts of the 18:0 group. In the present research, we noticed that there is more LPC with C16:0 group formed in oxidized LDL compared with native LDL. It appears that PC, with a saturated C16:0 group at its sn-1 position, is more vulnerable to hydrolysis during oxidation. It has been suggested that apoprotein-B has intrinsic PLA<sub>2</sub> activity which can act to hydrolyze PC to LPC during the oxidative modification of LDL. We propose that PC, with unsaturated C18:1, C18:2 or C18:3 groups at the sn-1 position, might not be hydrolyzed efficiently by this PLA<sub>2</sub>, causing the decreased content of LPC observed in oxidized LDL after atorvastatin treatment. This needs to be further explored.

### **3. Other benefits of atorvastatin treatment**

Recently, atorvastatin, like other drugs of its class, has been suggested to have additional antiatherogenic effects beyond its significant capacity to lower plasma LDL cholesterol level. Soma and co-workers reported that atorvastatin treatment can inhibit the focal accumulation of LDL and thus prevent the formation of the fatty streak (Soma et al. 1996). Also, atorvastatin has been reported to inhibit the *in vitro* proliferation and migration of vascular smooth muscle cells (Negre et al. 1996; Ortego et al. 1996; Soma et al. 1996). Interestingly, a significant reduction of atherosclerotic lesion size by the treatment of atorvastatin has been shown in cholesterol-fed rabbits (Bocan et al. 1994). Furthermore, in patients with hyperlipidemia, atorvastatin showed significant ability to reduce plasma viscosity, factor VII activity, red blood cell sedimentation rate and arachidonic acid-induced whole blood aggregation (Dujovne et al. 1996). There is also evidence that atorvastatin treatment can reduce the plasma level of E-selectin, a soluble adhesion molecule which may have some relevance to the development of atherosclerosis (Hackman et al. 1996).

As mentioned in previous sections, the major risk factor for atherosclerosis is hyperlipidemia. Increased plasma cholesterol can increase the retention of LDL in the intima and subsequently increase the opportunity for LDL to be exposed to free radicals. The latter, which are generated by endothelial- or smooth muscles cells, cause the oxidative modification of LDL. Oxidized LDL is atherogenic and can damage the normal function of the endothelium. High levels of cholesterol in the plasma can also increase the viscosity of the blood; this may decrease the velocity and change the laminar state of the blood flow, causing decreased shear stress rate on the endothelial cells (Deng et al. 1995). This change of blood flow can further impair the release of EDRF from the endothelium

cell and increase the tendency toward vasospasm. Atorvastatin can effectively inhibit HMG-CoA reductase, decrease the LDL levels in the circulation and initiate the sequence of events identified above. The increased blood flow can reduce the retention of LDL in the intima, and reduce the oxidation of LDL. In addition, atorvastatin has been confirmed to have antiatherogenic effects leading to the regression of atherosclerotic lesions. Therefore, atorvastatin can be used as both primary management drug to prevent atherosclerosis and as the first choice drug to treat atherosclerosis.

In conclusion, atorvastatin treatment can benefit hyperlipidemic patients from many ways, including reduce LDL-cholesterol levels in the blood; change the biochemical content of oxidized LDL; attenuate the ability of oxidized LDL to impair the endothelium-dependent relaxation of blood vessels; and prevent the formation of atherosclerotic plaque. We think it is the best drug for treating both hyperlipidemia and atherosclerosis available so far.

While the amount of oxidized LDL in the blood of patients, as measured by its LPC content, is likely quite low, the parameter of physiological interest would be the oxidation of LDL in the sub-endothelial space. At present, there are no methods of analysis available for estimating the oxidation of LDL in that functionally important area.

## V. REFERENCES

- Adams DH, Schoen FJ. (1989) Contemporary concepts in atherosclerosis pathology. In *Atherosclerosis and arteriosclerosis: human pathology and experimental animal methods and models* (White RA, ed.), Boca Raton, FL: CRC, pp 49-86.
- Amezcuca JL, Palmer RMJ, de Souza BM, Moncada S. (1989) Nitric oxide synthesized from L-arginine regulates vascular tone in the coronary circulation of the rabbit. *Br J Pharmacol*, 97, 1119-1124.
- Anderson KM, Castelli WP, Levy D. (1987) Cholesterol and mortality. 30 years of follow-up from the Framingham study. *JAMA*, 257, 2176-2180.
- Anderson TJ, Meredith IT, Ganz P, Selwyn AP, Yeung AC. (1994) Nitric oxide and nitrovasodilators: similarities, differences and potential interactions. *J Am Coll Cardiol*, 24, 555-566.
- Arad Y, Ramakrishnan R, Ginsberg H. (1992) Effects of lovastatin therapy on very-low-density lipoprotein triglyceride metabolism in subjects with combined hyperlipidemia: evidence for reduced assembly and secretion of triglyceride-rich lipoproteins. *Metabolism*, 41, 487-493.
- Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S. (1990) Cloning and expression of a cDNA encoding an endothelin receptor. *Nature*, 348, 730-732.
- Archer SL, Huang JMC, Hampl V, Nelson DP, Shultz PJ, Weir EK. (1994) Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. *Proc Natl Acad Sci USA*, 91, 7583-7587.
- Autio I, Jaakkola O, Solakivi T, Nikkari T. (1990) Oxidized low-density lipoprotein is chemotactic for arterial smooth muscle cells in culture. *FEBS-Lett*, 277, 1-2, 247-249.
- Averbook A, White GH, Wilson SE. (1989) Epidemiology and anatomic distribution of atherosclerosis in man. In *Atherosclerosis and Arteriosclerosis: human pathology and experimental animal methods and models* (White RA, ed.), CRC, Boca Raton, FL, pp 17-46.
- Bakker-Arkema RG, Davidson MH, Goldstein RJ. (1996) Efficacy and safety of a new HMG-CoA reductase inhibitor, atorvastatin, in patients with hypertriglyceridemia. *JAMA*, 275, 128-133.
- Balligand JL, Kelly RA, Marsden PA, Smith TW, Michel T. (1993) Control of cardiac muscle cell function by an endogenous nitric oxide signalling system. *Proc Natl Acad Sci USA*, 90, 347-351.
- Bartlett GR. (1959) Phosphorus assay in column chromatography. *J Biol Chem*, 234, 466-468.

Bath PMM, Hassall DG, Gladwin A-M, Palmer RMJ, Martin JF. (1991) Nitric oxide and prostacyclin: divergence of inhibitory effects on monocyte chemotaxis and adhesion to endothelium in vitro. *Arterioscler Thromb*, 11, 254-260.

Berliner JA, Territo MC, Sevanian A, Ramin S, Kim JA, Bamshad B, Esterson M, Fogelman AM. (1990) Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J Clin Invest*, 85, 1260-1266.

Berne RM, Levy MN. (1996) Cardiovascular system. In *Principles of Physiology* (2<sup>nd</sup> edition, Berne RM, Levy MN, eds.), Mosby, St. louis, pp 313-322.

Berthiaume F, Frangos JA. (1992) Flow-induced prostacyclin production is mediated by a pertussis toxin-sensitive G protein. *FEBS Letters*, 308, 277-279.

Bhagyalakshmi A, Berthiaume F, Reich KM, Frangos JA. (1992) Fluid shear stress stimulates membrane phospholipid metabolism in cultured human endothelium cells. *J Vasc Res*, 29, 443-449.

Bing RJ, Termin A, Conforto A, Dudek R, Hoffman MJ. (1993) Membrane function and vascular reactivity. *Biosci Rep*, 13, 61-67.

Blankenhorn DH, Kramsh DM. (1989) Reversal of atherosclerosis and sclerosis: the two components of atherosclerosis. *Circulation*, 79, 1-7.

Blum CBB. (1994) Comparison of properties of four inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Am J Cardiol*, 73, Suppl D, 3D-11D.

Bocan TMA, Mazur MJ, Mueller SB. (1994) Antiatherosclerotic activity of inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase in cholesterol-fed rabbits: a biochemical and morphological evaluation. *Atherosclerosis*, 111, 127-142.

Boje KM, Fung H. (1990) Endothelial nitric oxide generating enzymes in the bovine aorta: subcellular localization and metabolic characterization. *J Pharmacol Exp Ther*, 253, 20-26.

Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, Cohen RA. (1994) Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature*, 368, 850-853.

Bossaller C, Habib GB, Yamamoto H. (1987) Impaired muscarinic endothelium-dependent relaxation and cyclic guanosine 5'-monophosphate formation on an atherosclerotic human coronary artery and rabbit aorta. *J Clin Invest*, 79, 170-174.

Boulanger C, Luscher TF. (1990) Release of endothelin from the porcine aorta. Inhibition of endothelium-derived nitric oxide. *J Clin Invest*, 85, 587-590.

Bratzler RL, Chisolm GM, Colton CK, Smith KA, Lees RS. (1977) The distribution of labeled low-

density lipoprotein across the rabbit thoracic aorta in vivo. *Atherosclerosis*, 28, 289-307.

Bredt DS, Ferris CD, Snyder. (1992) Nitric oxide synthase regulatory sites. *J Biol Chem*, 267, 10976-10981.

Bredt DS, Hwang PH, Glatt C. (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature*, 351, 714-718.

Busconi L, Michel T. (1993) Endothelial nitric oxide synthase: N-terminal myristoylation determines sub cellular localization. *J Biol Chem*, 268, 8410-8413.

Busse R, Fleming I. (1995) Regulation and functional consequences of endothelial nitric oxide formation. *Ann Med*, 27, 331-340.

Busse R, Mulisch A, Fleming I, Hecker M. (1993) Mechanisms of nitric oxide release from the vascular endothelium. *Circulation*, 87, Suppl V, V18-25.

Cameron JD, Dart AM. (1994) Exercise training increases total systemic compliance in humans. *Am J Physiol*, 266, 693-701.

Campbell WB, Gebremedhin D, Pratt RF, Harder DR. (1996) Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing. *Circ Res*, 78, 415-423.

Cathcart MK, Morel DW, Chisolm GM. (1985) Monocytes and neutrophils oxidize low density lipoprotein making it cytotoxic. *J Leukocyte Biol*, 38, 341-350.

Celermajer DS, Sorensen KE, Georgakopoulos D, Bull C, Thomas O, Robinson J, Deanfield JE. (1993) Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. *Circulation*, 88, part I, 2149-2155.

Chait A, Brazg RL, Tribble DL, Krauss RM. (1993) Susceptibility of small, dense, low-density lipoproteins to oxidative modification in subjects with the atherogenic lipoprotein phenotype, pattern B. *Am J Med*, 94, 350-356.

Champe PC, Harvey RA. (1994) Cholesterol and steroid metabolism. In *Lippincott's illustrated reviews: Biochemistry* (2<sup>nd</sup> edition, Champe PC, Harvey RA, eds.), J.B. Lippincott company, Philadelphia, pp 205-228.

Chatterjee S. (1992) Role of oxidized human plasma low density lipoproteins in atherosclerosis: Effects on smooth muscle cell proliferation. *Mol Cell Biochem*, 111, 143-147.

Chen G, Suzuki H, Weston AH. (1988) Acetylcholine releases endothelium derived hyperpolarizing factor and EDRF from rat blood vessels. *Br J Pharmacol*, 95, 1165-1174.

- Chen G, Yamamoto Y, Miwa K, Suzuki H. (1991) Hyperpolarization of arterial smooth muscle induced by endothelial humoral substances. *Am J Physiol*, 260, H1888-1892.
- Chen L, Liang BH, Froese DE, Liu SY, Wong JT, Tran K, Hatch GM, Mymin D, Kroeger EA, Man RYK, Choy PC. (1997) Oxidative modification of low density lipoprotein in normal and hyperlipidemic patients: effect of lysophosphatidylcholine composition on vascular relaxation. *J Lipid Res*, 38, 546-553.
- Chin JH, Azhar S, Hoffman BB. (1992) Inactivation of endothelial derived relaxing factor by oxidized lipoprotein. *J Clin Invest*, 89, 10-18.
- Chisolm GM, Penn MS. (1996) Oxidized lipoproteins and atherosclerosis. In *Atherosclerosis and Coronary Artery Disease* (Fuster V, Ross R and Topol EJ, eds.), Lippincott-Raven Publisher, Philadelphia, New York, pp129-149.
- Choy PC, Arthur G. (1989) Phosphatidylcholine biosynthesis from lysophosphatidylcholine. In *Phosphatidylcholine Metabolism* (Vance DE, ed.), CRC Press, Inc., Boca Raton, pp 87-101.
- Cilla jr DD, Gibson DM, Whitfield LR. (1996) Pharmacodynamic effects and pharmacokinetics subjects in the morning and evening. *J Clin Pharmacol*, 36, 604-609.
- Cohen RA. (1993) Dysfunction of vascular endothelium in diabetes mellitus. *Circulation*, 87, Suppl, V67-76.
- Cohen RA, Vanhoutte PM. (1995) Endothelium-dependent hyperpolarization: beyond nitric oxide and cyclic GMP. *Circulation*, 92, 3337-3349.
- Cohen RA, Zitnay KM, Haudenschid CC, Cunningham LD. (1988) Loss of selective endothelial cell vasoactive functions in pig coronary arteries caused by hypercholesterolemia. *Circ Res*, 63, 903-910.
- Cohn JN, Finkelstein SM. (1992) Abnormalities of vascular compliance in hypertension, aging and heart failure. *J Hypertens*, 10, Suppl, S61-64.
- Collins P, Shay J, Jiang C, Moss J. (1994) Nitric oxide accounts for dose-dependent estrogen-mediated coronary relaxation after acute estrogen withdrawal. *Circulation*, 90, 1964-1968.
- Conde K, Vergara-Jinenez M, Krause ER. (1996) Hypercholesterolemic actions of atorvastatin are associated with alterations on hepatic cholesterol metabolism and lipoprotein composition in the guinea pig. *J Lipid Res*, 37, 2372-2382.
- Consigny PM. (1995) Pathogenesis of atherosclerosis. *AJR*, 164, 553-558.
- Cooke JP, Dzau VJ. (1997) Nitric oxide synthase: role in the genesis of vascular disease. *Annu Rev Med*, 48, 489-509.

Cornhill JF, Barrett WA, Herderick EE, Mahley RW, Fry DL. (1985) Topographic study of sudanophilic lesions in cholesterol-fed minipigs by image analysis. *Arteriosclerosis*, 5, 415-426.

Cotran RS, Kumar V, Robbins SL. (1994) *Robbins pathologic basis of disease* (5<sup>th</sup> edition, Cotran RS, Kumar V, Robbins SL, eds.), WB, Philadelphia, pp481-482.

Cowan CL, Steffen RP. (1995) Lysophosphatidylcholine inhibits relaxation of rabbit abdominal aorta mediated by endothelium-derived nitric oxide and endothelium-derived hyperpolarizing factor independent of protein kinase C activation. *Arterioscler Thromb Vasc Biol*, 15, 2290-2297.

Creager MC, Cooke JP, Mendelsohn ME. (1990) Impaired vasodilation of forearm resistance vessels in hypercholesterolemic humans. *J Clin Invest*, 86, 228-234.

Cushing SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, Gerrity R, Schwartz CJ, Fogelman AM. (1990) Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc Acad Sci USA*, 87, 5134-5138.

Davis JW, Shelton L, Eigenberg DA, Hignite CE, Watanated IS. (1985) Effects of tobacco and non-tobacco cigarette smoking on endothelium and platelets. *Clin Pharmacol Ther*, 37, 529-533.

De Belder AJ, Radomski MW, Why HJF. (1993) Nitric oxide synthase activities in human myocardium. *Lancet*, 341, 84-85.

Deng X, Marois Y, How T, Merhi Y, King M, Guidoin R, Karino T. (1995) Luminal surface concentration of lipoprotein (LDL) and its effect on the wall uptake of cholesterol by canine arteries. *J Vasc Surg*, 21, 135-145.

Dicorleto PE, Michael A, Gimbrone JR. (1996) Vascular Endothelium. In *Atherosclerosis and coronary artery disease* (Volume 1, Fuster V, Ross R, Topol EJ, eds.), Lippincott-Raven Publishers, Philadelphia, pp 387-399.

Dinerman JL, Lowenstein CJ, Snyder SH. (1993) Molecular mechanism of nitric oxide regulation, potential relevance to cardiovascular disease. *Circ Res*, 73, 217-222.

Drexler H, Zeiher AM, Wollschlager R, Meinertz T, Just H, Bonzel T. (1989) Flow dependent coronary artery dilation in humans. *Circulation*, 80, 466-474.

Dubois-Rande JL, Zelinsky R, Roudot F, Chabrier PE, Castaigne A, Geschwind H, Adnot S. (1992) Effects of infusion of L-arginine into the left anterior descending coronary artery on acetylcholine-induced vasoconstriction of human atheromatous coronary arteries. *Am J Cardiol*, 70, 1269-1275.

Dujovne CA, Harris WS, Altman R, Overhiser RW, Cunningham JJ, Black DM. (1996) Changes in hemorheology and atherothrombotic parameters by improving serum lipids in atorvastatin-treated hyperlipidemics (abstract). In *66<sup>th</sup> Congress of the European Atherosclerosis Society Abstract Book*,

July 13-17, Florence (Italy), 169.

Egashira K, Inou T, Hirooka Y, Kai H, Sugimachi M, Suzuki S, Kuga T, Urabe Y, Takeshita A. (1993) Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. *Circulation*, 88, 77-81.

Esterbauer H, Jurgens G, Quehenberger O, Koller E. (1987) Autooxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. *J Lipid Res*, 28, 495-509.

Faggiotto A, Ross R. (1984) Studies of hypercholesterolemia in the nonhuman primate. I. Changes that lead to fatty streak formation. *Arteriosclerosis*, 4, 323-340.

Faggiotto A, Ross R. (1984) Studies of hypercholesterolemia in the nonhuman primate. II. Fatty streak conversion to fibrous plaque. *Arteriosclerosis*, 4, 341-356.

Falk E. (1992) Why do plaque rupture? *Circulation*, Suppl III, 30-42.

Farnier M, Davignon J. (1998) Current and future treatment of hyperlipidemia: The role of statins. *Am J Cardiol*, 82, 3J-10J.

Feletou M, Vanhoutte PM. (1988) Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br J Pharmacol*, 93, 515-524.

Flavahan NA. (1993) Lysophosphatidylcholine modifies G protein-dependent signaling in porcine endothelial cells. *Am J Physiol*, 264, H722-H727.

Flavahan NA. (1992) Atherosclerosis or lipoprotein-induced endothelial dysfunction. Potential mechanisms underlying reduction in EDRE/nitric oxide activity. *Circulation*, 85, 1927-1938.

Flavahan NA, Vahoutte PM. (1995) Endothelial cell signaling and endothelial dysfunction. *Am J Hypertens*, 8, 28S-41S.

Fong LG, Parthasarathy S, Witztum JL, Steinberg D. (1987) Nonenzymatic oxidative cleavage of peptide bonds in apoprotein B-100. *J Lipid Res*, 28, 1466-1477.

Forstermann U, Mudge A, Alheid U. (1988) Selective attenuation of endothelium-mediated vasodilation in atherosclerotic human coronary arteries. *Circ Res*, 62, 185-190.

Franke R-P, Grafe M, Schnittler H, Seiffge D, Mittermayer C, Drenckhahn D. (1984) Induction of human vascular endothelial stress fibres by fluid shear stress. *Nature*, 307, 648-649.

Freiman PC, Mitchell GC, Heistad DD, Armstrong ML, Harrison DG. (1987) Atherosclerosis impairs endothelium-dependent vascular relaxation to acetylcholine and thrombin in primates. *Circ Res*, 58,

783-789.

Friedewald WT, Levy RI, Frederickson DS. (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem*, 18, 499-502.

Frostegard J, Nilsson J, Haegerstrand A, Hamsten A, Wigzell H, Gidlund M. (1990) Oxidized low density lipoprotein induces differentiation and adhesion of human monocytes and the monocytic cell line U937. *Proc Natl Acad Sci USA*, 87, 904-908.

Fujii K, Tominaga M, Ohmori S, Kobayashi K, Koga T, Takata Y. (1992) Decreased endothelium-dependent hyperpolarization to acetylcholine in smooth muscle of the mesenteric artery of spontaneously hypertensive rats. *Circ Res*, 70, 660-669.

Fukuta H, Miwa K, Hozumi T, Yamamoto Y, Suzuki H. (1996) Reduction by EDHF of the intracellular calcium concentration in vascular smooth muscle. In *Endothelium-Derived Hyperpolarizing Factor* (Vanhoutte PM, ed.), Harwood Academic Publishers, Amsterdam, pp143-153.

Furchgott RF. (1993) The discovery of endothelium-dependent relaxation. *Circulation*, 87 (suppl), V3-V8.

Furchgott RF. (1988) Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activable inhibitory factor from bovine retractor penis is organic nitrite and the endothelium-derived relaxing factor is nitric oxide. In *Mechanism of Vasodilation* (Vanhoutte PM, ed.), Raven Press, New York, pp 401-418.

Furchgott RF and Zawadzki JV. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, 228, 373-376.

Fuster V, Badimon L, Badimon JJ, Chesebro JH. (1992) The pathogenesis of coronary artery disease and the acute coronary syndromes. *N Engl J Med*, 326, 242-250, 310-318.

Galle J, Mulsch A, Busse R, Bassenge E. (1991) Effects of native and oxidized low density lipoprotein on formation and inactivation of endothelium-derived relaxing factor. *Arterioscler Thromb*, 11, 198-203.

Garg UC, Hassid A. (1989) Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest*, 83, 1774-1777.

Gerrity RG, Naito HK, Richardson M, Schwartz CJ. (1979) Dietary-induced atherogenesis in swine. *Am J Pathol*, 95, 775-793.

- Gilligan DM, Sack MN, Guetta V, Casino PR, Quyyumi AA, Rader DJ, Panza JA, Cannon RO. (1994) Effect of antioxidant vitamins on low density lipoprotein oxidation and impaired endothelium-dependent vasodilation in patients with hypercholesterolemia. *J Am Coll Cardiol*, 24, 1611-1617.
- Ginsberg HN. (1998) Effects of statins on triglyceride metabolism. *Am J Cardiol*, 81, 4A, 32B-35B.
- Glasser SP, Selwyn AP, Ganz P, Fla T, Mass B. (1996) Atherosclerosis: risk factors and the vascular endothelium. *Am Heart J*, 131, 379-384.
- Glew RH. (1992) Lipid metabolism II: pathways of metabolism of special lipids. In *Text Book of Biochemistry with Clinical Correlations* (3<sup>rd</sup> edition, Devlin TM, ed.), Wiley-Liss, New York, pp 438-448.
- Goerre S, Wenk M, Bartsch P, Luscher TF, Niroomand S, Hohernhaus H, Oelz O, Reinhart WH. (1995) Endothelin-1 in pulmonary hypertension associated with high altitude. *Circulation*, 91, 359-364.
- Goldschmidt-Clermont PJ, Machesky LM, Baldassare JJ, Pollard TD. (1990) The actin-binding protein profilin binds to PIP2 and inhibits its hydrolysis by phospholipase C. *Science*, 247, 1575-1578.
- Griendling KK, Alexander RW. (1996) Endothelial control of the cardiovascular system: recent advances. *FASEB J*, 10, 283-292.
- Griffith TM, Edward DH. (1990) Myogenic autoragulation of flow may be inversely related to endothelium-derived relaxing factor activity. *Am J Physiol*, 258, H1171-1180.
- Groves PH, Lewis MJ, Cheadle, HA, Penny WJ. (1993) SIN-1 reduces platelet adhesion and platelet thrombus formation in a porcine model of balloon angioplasty. *Circulation*, 87, 590-597.
- Grundy SM. (1991) HMG-CoA reductase inhibitors: clinical applications and therapeutic potential. In *Drug treatment of hyperlipidemia* (Rifkind BM, ed.), MerceL Dekker Inc, New York, pp139-167.
- Habib JB, Bossaler C, Wells S, William C, Morrisett JD, Henry PD. (1986) Preservation endothelium-dependent vascular relaxation in cholesterol-fed rabbit by treatment with the calcium blocker PN 200110. *Circ Res*, 58, 305-309.
- Hackman A, Abe Y, Insull W jr. (1996) Levels of soluble cell adhesion molecules in patients with dyslipidemia. *Circulation*, 93, 1334-1338.
- Hamon M, Vallet B, Bauters C, Werntert N, McFadden E, LaBlanche J-M, Dupuis B, Bertrand ME. (1994) Long-term oral administration of L-arginine reduces intimal thickening and enhances neoendothelium-dependent acetylcholine-induced relaxation after arterial injury. *Circulation*, 90, 1357-1362.

- Haria M, McTavish D. (1997) Pravastatin: a reappraisal of its pharmacological properties and therapeutic use in the management of coronary heart disease. *Drugs*, 53, 2, 299-336.
- Harrison DG. (1994) Endothelial dysfunction in atherosclerosis. *Basic Res Cardiol*, 89, Suppl, 1, 87-102.
- Harrison DG. (1995) Alteration of vasomotor regulation in atherosclerosis. *Cardiovasc Drugs Ther*, 9, 55-63.
- Harrison DG, Freiman PC, Armstrong ML, Macus ML, Heistad DD. (1987) Alterations vascular reactivity in atherosclerosis. *Circ Res*, 61, II74-80.
- Hassid A. (1986) Atriopeptin II decreases cytosolic free  $Ca^{2+}$  in cultured vascular smooth muscle cells. *Am J Physiol*, 251, C681-686.
- Hasunuma K, Yamaguchi T, Rodman D, O'Brien R, McMurtry I. (1991) Effects of inhibitors of EDRF and EDHF on vasoreactivity of perfused rat lungs. *Am J Physiol*, 260, L97-104.
- Havel RJ, Eder HA, Bragdon JH. (1955) The distribution and chemical composition of ultracentrifugally separated lipoprotein in human serum. *J Clin Invest*, 34, 1345-1353.
- Hecker M, Mulsch A, Bassenge E, Busse R. (1993) Vasoconstriction and increased flow: two principal mechanisms of shear stress-dependent endothelial autacoid release. *Am J Physiol*, 265, H828-833.
- Hecker M, Mulsch A, Bassenge E, Forstermann U, Busse R. (1994) Subcellular localization and characterization of nitric oxide synthase(s) in endothelial cells: physiological implications. *Biochem J*, 299, 247-252.
- Heinecke JW, Rosen H, Chait A. (1984) Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J Clin Invest*, 74, 1890-1894.
- Hessler JR, Robertson AL Jr, Chisolm GM. (1979) LDL-induced cytotoxicity and endothelial cells in culture. *Arteriosclerosis*, 32, 213-229.
- Hurt-Camejo E, Camejo G, Rosengren B, Lopez F, Ahlstrom C, Fager G, Bondjers G. (1992) Effect of arterial proteoglycans and glycosaminoglycans on low density lipoprotein oxidation and its uptake by human macrophages and arterial smooth muscle cells. *Arterioscler Thromb*, 12, 569-583.
- Ignarro LJ, Byrns R, Buga GM, Wood RS, Chaudhuri G. (1987) Pharmacological evidence that endothelium-derived relaxing factor is nitric oxide: use of pyrogallol and superoxide dismutase to study the endothelium-dependent and nitric oxide elicited vascular smooth muscle relaxation. *J Pharmacol Exp Ther*, 244, 181-189.

Inoue N, Hirata K, Yamada M, Hamamori Y, Matsuda Y, Akita H, Yokoyama M. (1992) Lysophosphatidylcholine inhibits bradykinin-induced phosphoinositide hydrolysis and calcium transients in cultured bovine aortic endothelial cells. *Circ Res*, 71, 1410-1421.

Jacobson GR, Saier jr MH. (1988) Structure and assembly of biological membranes. In *Biochemistry* (2<sup>nd</sup> edition, Zubay G, ed.), Macmillan Publishing Company, New York, pp185-191.

Jayakody L, Senaratne M, Thomson A, Kappagoda T. (1987) Endothelium-dependent relaxation in experimental atherosclerosis in the rabbit. *Circ Res*, 60, 251-264.

Johnstone MT, Creager SJ, Scales KM, Cusco JA, Lee BK, Creager MA. (1993) Impaired endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. *Circulation*, 88, 2510-2516.

Jurgens G, Hoff HF, Chisolm GM, Esterbauer H. (1987) Modification of human serum low density lipoprotein by oxidation- characterization and pathophysiologic implications. *Chem Phys Lipids*, 45, 315-336.

Kai H, Kanaide H, Matsumoto T, Nakamura M. (1987) 8-bromoguanosine 3': 5' -cyclic monophosphate decreasea intracellular free calcium concentrations in cultured vascular smooth muscle cells from the rat aorta. *FEBS Lett*, 221, 284-288.

Khan B, Wilcox HG, Heimberg M. (1989) Cholesterol is required for secretion of very low density lipoprotein by rat liver. *Biochem J*, 258, 807-816.

Kilpatrick EV, Cocks TM. (1994) Evidence for differential roles of nitric oxide (NO) and hyperpolarization in endothelium-dependent relaxation of pig isolated coronary artery. *Br J Pharmacol*, 112, 557-565.

Kingwell BA, Tran B, Jennings GL, Dart AM. (1995) Endothelium dependent vasorelaxation is enhanced in athletes. In *Abstracts of the Scientific Conference on the Functional and Structural Aspects of the Vascular Wall*, Feb, 149.

Kiowski W, Linder L, Stoschitzky K, Pfisterer M, Burckhardt D, Burkart F, Buhler F. (1994) Diminished vascular response to inhibition of endothelium-derived nitric oxide and enhanced vasoconstriction to exogenously adminished endothelin-1 in clinically healthy smokers. *Circulation*, 90, 27-34.

Kiowski W, Ltischer TF, Linder L, Buhler FR. (1991) Endothelin-1-induced vasoconstriction in humans. Reversal by calcium channel blockade but not by nitrovasodilators or endothelium-derived relaxing factor. *Circulation*, 83, 469-475

Kobayashi S, Kanaide H, Nakamura M. (1985) Cytosolic free calcium transients in cultured vascular smooth muscle cells: microfluorometric measurements. *Science*, 229, 533-536.

Komori K, Vanhoutte PM. (1990) Endothelium-derived hyperpolarizing factor. *Blood Vessels*, 27, 238-245.

Krause BR, Newton RS. (1997) Lipid-lowering activity of atorvastatin and lovastatin in rodent species: triglyceride-lowering in rats correlates with efficacy in LDL animal model. *Atherosclerosis*, 117, 237-244.

Ku DN, Giddens DP, Zarins CK, Glagov S. (1985) Pulsatile flow and atherosclerosis in the human carotid bifurcation: positive correlation between plaque location and low oscillating shear stress. *Arteriosclerosis*, 5, 293-302.

Kubes P, Suzuki M, Granger DN. (1991) Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci USA*, 88, 4651-4655.

Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD. (1990) Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature*, 344, 160-162.

Kugiyama K, Ohgushi M, Sugiyama S, Murohara T, Fukunaga K, Miyamoto E, Yashue H. (1992) Lysophosphatidylcholine inhibits surface receptor-mediated intracellular signals in endothelium cells by a pathway involving protein kinase C activation. *Circ Res*, 71, 1422-1428.

Kume N, Cybulsky MI, Gimbrone MA Jr. (1992) Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. *J Clin Invest*, 90, 1138-1144.

Kung CF, Tschudi MR, Noll G, Clozel J-P, Luscher TF. (1995) Differential effects of calcium antagonist mibefradil in epicardial and intramyocardial coronary arteries. *J Cardiovasc Pharmacol*, 26, 312-318.

Lafont A, Chai YC, Cornhill JF, Whitlow PL, Howe PH, Chisolm GM. (1995) Effect of alpha-tocopherol on restenosis after angioplasty in a model of experimental atherosclerosis. *J Clin Invest*, 95, 1018-1025.

Langille BL, Graham JJK, Kim D, Gotlieb AL. (1991) Dynamics of shear-induced redistribution of F-actin in endothelial cells in vivo. *Arterioscler Thromb*, 11, 1814-1820.

Le N-A, Li X, Innis-Whitehouse W. (1996) Lipid and apolipoprotein levels and distribution in patients with hypertriglyceridemia: effect of atorvastatin. *Ann Arbor*, Parke-Davis, Michigan, USA.

Li H, Freeman MW, Libby P. (1995) Regulation of smooth muscle cell scavenger receptor expression in vivo by atherogenic diets and in vitro by cytokins. *J Clin Invest*, 95, 122-133.

Liao JK. (1994) Inhibition of Gi proteins by low density lipoprotein attenuates bradykinin-stimulated

- release of endothelial-derived nitric oxide. *J Biol Chem*, 269, 12987-12992.
- Lieberman EH, Gerhard MD, Uehata A, Walsh BW, Selwyn AP, Ganz P, Yeung A, Creager M. (1994) Estrogen improves endothelium-dependent, flow-mediated vasodilation in postmenopausal women. *Ann Intern Med*, 121, 936-941.
- Liu SY, Lu X, Choy S, Dembinski TC, Hatch GM, Mymin D, Shen X, Angel A, Choy PC, Man RYK. (1994) Alteration of lysophosphatidylcholine content in low density lipoprotein after oxidative modification: relationship to endothelium dependent relaxation. *Cardiovas Res*, 28, 1476-1481.
- Loree HM, Tobias BJ, Gibson LJ, Kamm RD, Small DM, Lee, RT. (1994) Mechanical properties of model atherosclerotic lesion lipid pools. *Arterioscler Thromb*, 14, 230-234.
- Losordo DW, Kearney M, Kim EA, Jekanowski J, Isner JM. (1994) Variable expression of the estrogen receptor in normal and atherosclerosis coronary arteries of premenopausal women. *Circulation*, 89, 1501-1510.
- Lowry OH, Roseborough NJ, Farr AL, Randall RJ. (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem*, 193, 265-275.
- Luscher TF, Boulanger CM, Dohi Y, Yang ZH. (1992) Endothelium-derived contracting factors. *Hypertension*, 19, 117-130.
- Luscher TF, Cooke JP, Houston BS. (1987) Endothelium-dependent relaxation in human arteries. *Mayo Clinic Proc*, 62, 601-606.
- Luscher TF, Noll G. (1995) The pathogenesis of cardiovascular disease: role of the endothelium as a target and mediator. *Atherosclerosis*, 118, Suppl, S81-90.
- Luscher TF, Vanhoutte PM. (1990) The endothelium: modulator of cardiovascular function. Vol. FL, Boca Raton, CRC Press, pp 1-228.
- Ma PTS, Gil G, Sudhof TC. (1986) Mevinolin, an inhibitor of cholesterol synthesis, induces mRNA for low density lipoprotein receptor in livers of hamsters and rabbits. *Proc Natl Acad Sci*, 88, 8370-8374.
- Mangin EL, Kugiyama K, Nguy JH, Kerns SA, Henry PD. (1993) Effects of lysolipids and oxidatively modified low density lipoprotein on endothelium-dependent relaxation of rabbit aorta. *Circ Res*, 72, 161-166.
- Marinetti GV. (1990) Metabolism of high-density lipoproteins. In *Disorders of Lipid Metabolism* (Marinetti GV, ed.), Plenum Press, New York, pp 94-98.
- Marinetti GV. (1990) LDL, LDL receptor, and HMGR. In *Disorders of Lipid Metabolism* (Marinetti

GV, ed.), Plenum Press, New York, pp 98-105.

Marinetti GV. (1990) Dietary management of elevated blood lipids. In *Disorders of Lipid Metabolism* (Marinetti GV, ed.), Plenum Press, New York, pp 135-168.

Marsden PA, Heng HHQ, Scherer SW. (1993) Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *J Biol Chem*, 268, 17478-17488.

Marsden PA, Schappert KT, Chen HS. (1992) Molecular cloning and characterization of human endothelial nitric oxide synthase. *FEBS Lett*, 307, 287-293.

Martin MJ, Hulley SB, Browner WS, Kuller LH, Wentworth D. (1986) Serum cholesterol, blood pressure and mortality: implications from a cohort of 361,662 men. *Lancet*, 2, 933-936.

Martin W, White DG, Henderson AH. (1988) Endothelium-derived relaxing factor and atriopeptin II elevate cyclic GMP levels in pig aortic endothelial cells. *Br J Pharmacol*, 93, 229-239.

Matsuda Y, Hirata K, Inoue N, Suematsu M, Kawashima S, Akita H, Yokoyama M. (1993) High density lipoprotein reverses inhibitory effect of oxidized low density lipoprotein on endothelium-dependent arterial relaxation. *Circ Res*, 72, 1103-1109.

Mayes PA. (1993) Lipid transport & storage. In *Harper's Biochemistry* (23<sup>rd</sup> edition, Murray RK, Granner DK, Mayes PA, Rodwell VW, eds.), Appleton & Lange, Norwalk, pp 250-265.

Mayes PA. (1993) Cholesterol synthesis, Transport, & excretion. In *Harper's Biochemistry* (23<sup>rd</sup> edition, Murray RK, Granner DK, Mayes PA, Rodwell VW, eds.), Appleton & Lange, Norwalk, Connecticut, pp 266-278.

Mcgarry JD. (1992) Lipid metabolism I: utilization and storage of energy in lipid form. In *Text Book of Biochemistry with Clinical Correlations* (3<sup>rd</sup> edition, Devlin TM, ed.), Wiley-Liss, New York, pp 387-422.

Mehta JL, Yang BC, Nichol WW. (1993) Free radicals, antioxidants, and coronary artery disease. *J Myocardial Ischemia*, 5, 31-41.

Miettinen TA. (1991) Inhibition of cholesterol absorption by HMG-CoA reductase inhibitor. *Eur J Clin Pharmacol*, 40, Suppl I, S19-S21.

Moilanen E, Vuorinen P, Metsa-Ketela T, Vapaatalo H. (1993) Inhibition by nitric oxide donors of human polymorphonuclear leukocyte functions. *Br J Pharmacol*, 109, 852-858.

Mombouli JV, Vanhoutte PM. (1997) Endothelium-derived hyperpolarizing factor(s): updating the unknown. *Tips*, 18, 252-256.

- Moncada S, Vane JR. (1979) Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A<sub>2</sub> and prostacyclin. *Pharmacol Rev*, 30, 293-298.
- Montgomery R, Conway TW, Spector AA. (1990) Lipid metabolism. In *Biochemistry A case-oriented Approach* (15<sup>th</sup> edition, Montgomery R, Conway TW, Spector AA, eds.), Mosby, St. Louis, pp 409-463.
- Morel DW, Dicorleto PE, Chisolm GM. (1984) Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. *Arteriosclerosis*, 4, 357-364.
- Morgan JP, Morgan KG. (1984) Alteration of cytoplasmic ionized calcium levels in smooth muscle by vasodilators in the ferret. *J Physiol*, 357, 539-551.
- Mugge A, Lopez JAG, Piegors DJ, Breese KR, Heistad DD. (1991) Acetylcholine-induced vasodilatation in rabbit hindlimb in vivo is not inhibited by analogues of L-arginine. *Am J Physiol*, 260, H242-247.
- Murphy TJ. (1992) Molecular cloning and characterization of the constitutive bovine aortic endothelial nitric oxide synthase. *J Clin Invest*, 90, 2092-2096.
- Muruganandam A, Mutus B. (1994) Isolation of nitric oxide synthase from human platelets. *Biochim Biophys Acta*, 1200, 1-6.
- Nadaud S, Bonnardeaux A, Lathrop M, Soubrier F. (1994) Gene structure, polymorphism and mapping of the human endothelial nitric oxide synthase gene. *Biochem Biophys Res Commun*, 198, 1027-1033.
- Nagao T, Illiano SC, Vanhoutte PM. (1992) Heterogeneous distribution of endothelium-dependent relaxation resistant to N<sup>G</sup>-nitro-L-arginine in rats. *Am J Physiol*, 263, H1090-1094.
- Nagao T, Vanhoutte PM. (1993) Endothelium-derived hyperpolarizing factor and endothelium-dependent relaxations. *Am J Respir Cell Mol Biol*, 8, 1-6.
- Nagao T, Vanhoutte PM. (1991) Hyperpolarization contribution to endothelium-dependent relaxation to acetylcholine in femoral veins of rats. *Am J Physiol*, 261, H1034-1037.
- Nagelkerke JF, Havekes L, van Hinsbergh VWM, van Berkel TJC. (1984) In vivo catabolism of biologically modified LDL. *Arteriosclerosis*, 4, 256-264.
- Nakashima M, Vanhoutte PM. (1995) Isoproterenol causes hyperpolarization through opening of ATP-sensitive potassium channels in vascular smooth muscle of the canine saphenous vein. *J Pharmacol Exp Ther*, 272, 379-384.
- Nathan C. (1992) Nitric oxide as a secretory product of mammalian cells. *FASEB J*, 6, 3051-3064

National Cholesterol Education Program. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. (1994) Second report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel II): II. Dietary therapy and physical activity. *Circulation*, 89, 1363-1404, 1432-1445.

Navab M, Hough GP, Stevenson LW, Drinkwater DC, Laks H, Fogelman AM. (1988) Monocyte migration into the subendothelial space of a coculture of adult human aortic endothelial and smooth muscle cells. *J Clin Invest*, 82, 1853-1863.

Navab M, Imes SS, Hama SY, Hough GP, Ross LA, Bork RW, Valente AJ, Berliner JA, Drinkwater DC, Laks H, Fogelman AM. (1991) Monocyte transmigration induced by modification of low density lipoprotein in cocultures of human aortic wall cells is due to induction of monocyte chemotactic protein-1 synthesis and is abolished by high density lipoprotein. *J Clin Invest*, 88, 2039-2046.

Negre-Aminou P, van Erck M, Cohen LH. (1996) Anti-proliferative protection of 6 vastatins in cultured human cells; involvement of the ras-mediated signalling pathway (abstract). In *66<sup>th</sup> Congress of the European Atherosclerosis Society Abstract Book*, July 13-17, Florence (Italy), 120.

Nishida K, Harrison DG, Navas JP, Fisher AA, Dockery SP, Uematsu M, Nerem RM, Alexander RW, Murphy TJ. (1992) Molecular cloning and characterization of the constitutive bovine aortic endothelial nitric oxide synthase. *J Clin Invest*, 90, 2092-2096.

Nitenberg A, Antony I, Foulst JM. (1993) Acetylcholine-induced coronary vasoconstriction in young, heavy smokers with normal coronary arteriographic findings. *Am J Med*, 95, 71-77.

Nollert MU, Eskin SG, McIntyre LV. (1990) Shear stress increases inositol triphosphate levels in human endothelium cells. *Biochem Biophys Res Commun*, 170, 281-287.

Nunokawa Y, Ishida N, Tanaka S. (1993) Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells. *Biochem Biophys Res Commun*, 191, 89-94.

Ohgushi M, Kugiyama K, Fukunaga K, Murohara T, Sugiyama S, Miyamoto E, Yasue H. (1993) Protein kinase C inhibitors prevent impairment of endothelium-dependent relaxation by oxidatively modified LDL. *Arterioscler Thromb*, 13, 1525-1532.

Ohno M, Gibbons GH, Dzau VJ, Cooke JP. (1993) Shear stress elevates endothelium cGMP. Role of a potassium channel and G protein coupling. *Circulation*, 88, 193-197.

Ortego M, Bustos C, Hernandez-Presa M. (1996) The HMG-CoA reductase inhibitor atorvastatin reduces NF- $\kappa$ B activation and MCP-1 gene expression in vascular smooth muscle cells and mononuclear cells (abstract). In *66<sup>th</sup> Congress of the European Atherosclerosis Society Abstract Book*, July 13-17, Florence (Italy), 66.

Palmer RM, Ashton DS, Moncada S. (1988) Vascular endothelial cells synthesize nitric oxide from

L-arginine. *Nature*, 333, 664-666.

Palmer RMJ, Ferrige AG, Moncada S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, 327, 524-526.

Panza JA, Casino PR, Kilcoyne CM, Quyyumi AA. (1994). Impaired endothelium-dependent vasodilation in patients with essential hypertension: Evidence that the abnormality is not at muscarinic receptor level. *J Am Coll Cardiol*, 23, 1610-1616.

Panza JA, Casino PR, Kilcoyne CM, Quyyumi AA. (1993) Role of endothelium-derived nitric oxide in abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *Circulation*, 87, 1468-1474.

Panza JA, Casino PR, Kilcoyne CM, Quyyumi AA. (1993). Role of endothelium-derived nitric oxide in the abnormal endothelium-dependent vascular relaxation of patients with essential hypertension. *Circulation*, 87, 1468-1474.

Panza JA, Quyyumi AA, Callahan TS, Epstein SE. (1993). Effect of antihypertensive treatment on endothelium-dependent vascular relaxation in patients with essential hypertension. *J Am Coll Cardiol*, 21, 1145-1151.

Parsaee H, McEwan JR, Sunil J, McDermot J. (1992) Differential sensitivities of the prostacyclin and nitric oxide biosynthetic pathways to cytosolic calcium in bovine aortic endothelial cells. *Br J Pharmacol*, 107, 1013-1019.

Parthasarathy S, Barnett J. (1990) Phospholipase A2 activity of low density lipoprotein: Evidence for an intrinsic phospholipase A2 activity of apoprotein B-100. *Proc Natl Acad Sci USA*, 87, 9741-9745.

Parthasarathy S, Steinberg D, Witztum JL. (1992) The role of oxidized low-density lipoproteins in the pathogenesis of atherosclerosis. *Annu Rev Med*, 43, 219-225.

Parthasarathy S, Printz DJ, Boyd D, Joy L, Steinberg D. (1986) Macrophage oxidation of low density lipoprotein generates a form recognized by the scavenger receptor. *Arteriosclerosis*, 6, 505-510.

Parthasarathy S, Rankin SM. (1992) Role of oxidized low density lipoprotein in atherogenesis. *Prog Lipid Res*, 31, 127-143.

Parthasarathy S, Steinbrecher UP, Barnett J, Witztum JL, Steinberg D. (1985) Essential role of phospholipase A2 activity in endothelial cell-induced modification of low density lipoprotein. *Proc Natl Acad Sci USA*, 82, 3000-3004.

Pitas RE, Innerarity TL, Mahley RW. (1983) Foam cells in explants of atherosclerotic rabbit aortas have receptors for  $\beta$ -very low density lipoproteins and modified low density lipoproteins. *Arteriosclerosis*, 3, 2-12.

- Pohl U, Herlan K, Huang A, Bassenge E. (1991) EDRF-mediated shear-induced dilation opposes myogenic vasoconstriction in small rabbit arteries. *Am J Physiol*, 261, H2016-2023.
- Pohl U, Holtz J, Busse R, Bassenge E. (1986) Critical role of endothelium in the vasodilator response to increased flow in vivo. *Hypertension*, 8, 37-44.
- Pollock JS, Forstermann U, Mitchell JA. (1991) Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovin aortic endothelial cells. *Proc Natl Acad Sci USA*, 88, 10480-10484.
- Prasad ARS, Logan SA, Nerem RM, Schwartz CJ, Sprague EA. (1993) Flow-related responses of intracellular inositol phosphate levels in cultured aortic endothelium cells. *Circ Res*, 72, 827-836.
- Pufahl RA, Marletta MA. (1993) Oxidation of N<sup>G</sup>-hydroxy-L-arginine by nitric oxide synthase: Evidence for the involvement of the heme in catalysis. *Biochem Biophys Res Commun*, 193, 963-970.
- Quinn MT, Parthasarathy S, Fong LG, Steinberg D. (1987) Oxidatively modified low density lipoproteins: A potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc Natl Acad Sci USA*, 84, 2995-2998.
- Rackley CE. (1996) Monotherapy with HMG-CoA reductase inhibitors and secondary prevention in coronary artery disease. *Clin Cardiol*, 19, 683-689.
- Radomski MW, Moncada S. (1993) Regulation of vascular homeostasis by nitric oxide. *Thromb Haemost*, 70, 36-41.
- Raeymaekers L, Eggermont JA, Wuytack F, Casteels. (1990) Effects of cyclic nucleotide dependent protein kinases on the endoplasmic reticulum Ca<sup>2+</sup> pump of bovine pulmonary artery. *Cell Calcium*, 11, 261-268.
- Rajavashisth TB, Andalibi A, Territo MC, Berliner JA, Navab M, Fogelman AM, Lusis AJ. (1990) Induction of endothelial expression of granulocyte and macrophage colony-stimulating factors by modified low density lipoproteins. *Nature*, 344, 254-257.
- Reaven P, Witztum JL. (1995) The role of oxidation of LDL in atherogenesis. *Endocrinologist*, 5, 44-54.
- Reaven PD, Witztum JL. (1996) Oxidized low density lipoprotein in atherogenesis: role of dietary modification. *Annu Rev Nutr*, 1996, 16, 51-71.
- Resnick N, Collins T, Atkinson W, Bonthron DT, Dewey CFJ, Gimbrone MAJ. (1993) Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element. *Proc Natl Acad Sci USA*, 90, 4591-4585.

Richardson PD, Davies MJ, Born GVR. (1989) Influence of plaque configuration and stress distribution on fissuring of coronary atherosclerotic plaques. *Lancet*, 2, 941-944.

Ross R. (1995) Cell biology of atherosclerosis. *Annu Rev Physiol*, 57, 791-804.

Ross R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*, 362, 801-809.

Ross R. (1986) The pathogenesis of atherosclerosis- and update. *New Engl J Med*, 314, 418-500.

Ross R. (1992) The pathogenesis of atherosclerosis. In *Heart Disease* (4<sup>th</sup> edition, Braunwald E, Sauder WB, eds.), Philadelphia, pp 1106-1124.

Ross R, Glomset JA. (1973) Atherosclerosis and the arterial smooth muscle cell. *Science*, 180, 1332-1339.

Rubanyi GM, Romero JC, Vanhoutte PM. (1986) Flow-induced release of endothelium-derived relaxing factor. *Am J Physiol*, 250, H145-149.

Saenz de Tejada I, Goldstein I, Azadoi K, Drane RJ, Cohen RA. (1989) Impaired neurogenic and endothelium-mediated relaxation of penile smooth muscle from diabetic men with impotence. *N Engl J Med*, 320, 1025-1030.

Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, Masaki T. (1990) Cloning of a cDNA encoding a non-isopeptide selective subtype of the endothelin receptor. *Nature*, 348, 732-735.

Schulz R, Smith JA, Lewis MJ, Moncada S. (1991) Nitric oxide synthase activities in cultured endocardial cells of the pig. *Br J Pharmacol*, 104, 21-24.

Schwenke DC, Carew TE. (1989) Initiation of atherosclerotic lesions in cholesterol-fed rabbits. I. Focal increases in arterial LDL concentration precede development of fatty streak lesions. *Arteriosclerosis*, 9, 895-907.

Schwenke DC, Carew TE. (1989) Initiation of atherosclerotic lesions in cholesterol-fed rabbits. II. Selective retention of LDL vs. selective increases in LDL permeability in susceptible sites of arteries. *Arteriosclerosis*, 9, 908-918.

Sessa WC, Harrison JK, Barber CM. (1992) Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase. *J Biol Chem*, 267, 15274-15276.

Shah AM, Spurgeon H, Sollott SJ, Talo A, Lakatta EG. (1994) 8-bromo cyclic GMP reduces the myofilament response to calcium in intact cardiac myocytes. *Circ Res*, 74, 970-978.

Shimokawa H, Vanhoutte PM. (1989) Impaired endothelium-dependent relaxation to aggregating platelets and related vasoactive substances in porcine coronary arteries in hypercholesterolemia and atherosclerosis. *Circ Res*, 1989, 900-914.

Siegel G, Carl A, Adler A, Stock G. (1989) Effect of the prostacyclin analogue iloprost on K<sup>+</sup> permeability in the smooth muscle cells of the canine carotid artery. *Eicosanoids*, 2, 213-222.

Simon B, Cunningham LD, Cohen RA. (1990) Oxidized low-density lipoproteins cause contraction and inhibit endothelium-dependent relaxation in the pig coronary artery. *J Clin Invest*, 86, 75-79.

Small DM. (1988) Progression and regression of atherosclerotic lesions: insights from lipid physical biochemistry. *Arteriosclerosis*, 8, 103-129.

Soma M, Pilego T, Seregini R, Donetti E, Paoletti R, Fumagalli R. (1996) Effect of atorvastatin in intimal carotid thickening induced by perivascular manipulation in normocholesterolemic rabbits (abstract). In *66<sup>th</sup> Congress of the European Atherosclerosis Society Abstract Book*, July 13-17, Florence (Italy), 231.

Stafforini DM, Carter ME, Zimmerman GA, McIntyre TM, Prescott SM. (1989) Lipoproteins alter the catalytic behavior of the platelet-activating factor acetylhydrolase in human plasma. *Proc Natl Acad Sci USA*, 86, 2393-2397.

Stafforini DM, Prescott SM, McIntyre TM. (1987) Human platelet activating factor acetylhydrolase: Association with lipoprotein particles and role in the degradation of platelet-activating factor. *J Biol Chem*, 262, 4223-4230.

Stary HC. (1989) Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults. *Arteriosclerosis*, 9, Suppl I, 119-132.

Stary HC, Bleakley A, Glagov S. (1994) A Definition of initial, fatty streak, and intermediate lesions of atherosclerosis: a report from the committee on vascular lesions of the council on atherosclerosis, American Heart Association. *Arterioscler Thromb*, 14, 840-856.

Stein Y and Stein O. (1968) Metabolism of labelled lysolecithin, lysophosphatidylethanolamine and lecithin in the rat. *Biochim. Biophys. Acta*, 116, 95-100.

Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. (1989) Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N Engl Med*, 320, 915-924.

Steinbrecher UP, Parthasarathy S, Leake DS, Witztum LJ, Steinberg D. (1984) Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci USA*, 81, 3883-3887.

Steinbrecher UP, Pritchard PH. (1989) Hydrolysis of phosphatidylcholine during LDL oxidation is

mediated by platelet-activating factor acetyl-hydrolase. *J Lipid Res*, 30, 305-315.

Steinbrecher UP, Witztum JL, Parthasarathy S, Steinberg D. (1987) Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL. Correlation with changes in receptor-mediated catabolism. *Arteriosclerosis*, 7, 135-143.

Stewart DJ, Langleben D, Cernacek P, Cianflone K. (1990) Endothelin release is inhibited by coculture of endothelial cells with cells of vascular media. *Am J Physiol*, 259, H1928-1932.

Tanner FC, Noll G, Boulanger CM, Luscher TF. (1991) Oxidized low density lipoproteins inhibit relaxation of porcine coronary arteries. *Circulation*, 83, 2012-2020.

Taylor SG, Weaton AH. (1988) Endothelium-derived hyperpolarizing factor: a new endogenous inhibitor from the vascular endothelium. *Trends Pharmacol Sci*, 9, 272-274.

Thurston G, Baldwin AL. (1994) Endothelial actin cytoskeleton in rat mesentery microvasculature. *Am J Physiol*, 266, H1896-1909.

Tomoike H, Egashira K, Yamamoto Y, Nakamura M. (1989) Enhanced responsiveness of smooth muscle, impaired endothelium-dependent relaxation and the genesis of coronary spasm. *Am J Cardiol*, 63, 33E-39E.

Traub O, Berk BC. (1998) Laminar shear stress: mechanisms by which endothelium cells transduce an atheroprotective force. *Arterioscler Thromb Vasc Biol*, 18, 677-685.

Tribble DL, Holl LG, Wood PD, Krauss RM. (1992) Variations in oxidative susceptibility among six low density lipoprotein subfractions of differing density and particle size. *Atherosclerosis*, 93, 189-199.

Vaitkevicius PV, Fleg JL, Engel JH, O'Conner FC, Wright JG, Lakatta LE, Yin FC, Lakatte EG. (1993) Effects of age and aerobic capacity on arterial stiffness in healthy adults. *Circulation*, 88, 1456-1462.

Vance DE. (1988) Cholesterol and related derivatives. In *Biochemistry* (2<sup>nd</sup> edition, Geoffrey Zubay, ed.), Macmillan publishing company, New York, pp 726-729.

Vanhoutte PM. (1997) Endothelial dysfunction and atherosclerosis. *Eur Heart J*, 18, Suppl E, E19-E29.

Vanhoutte PM. (1996) Endothelium-derived hyperpolarizing factor. Harwood Academic Publishers, The Netherlands.

Verbeuren TJ, Jordaens FH, Zonnekeyn LL, Van Hove CE, Coene MC, Herman, D. (1986) Effects of hypercholesterolemia on vascular reactivity in the rabbit. *Circ Res*, 58, 560-564.

- Wechezak AR, Viggers RF, Sauvage LR. (1985) Fibronectin and F-actin redistribution in cultured endothelial cells exposed to shear stress. *Lab Invest*, 53, 639-647.
- Williams JK, Adams MR, Herrington DM, Clarkson TB. (1992) Short-term administration of estrogen and vascular responses of atherosclerotic coronary arteries. *J Am Coll Cardiol*, 20, 452-457.
- Williams JK, Shively CA, Clarkson TB. (1994) Determinants of coronary artery reactivity in premenopausal female cynomolgus monkeys with diet-induced atherosclerosis. *Circulation*, 90, 983-987.
- Wissler RW, Vesselinovitch D. (1983) Atherosclerosis-relationship to coronary blood flow. *Am J Cardiol*, 52, 2, 2A.
- Witztum JL. (1993) Role of oxidized low density lipoprotein in atherogenesis. *Br Heart J*, 69, 512-518.
- Witztum JL, Steinberg D. (1991) Role of oxidized LDL in atherogenesis. *J Clin Invest*, 88, 1785-1792.
- Wu H, Moulton KS, Glass CK. (1992) Macrophage scavenger receptors and atherosclerosis. *Trends Cardiovasc Med*, 2, 220-225.
- Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, 332, 411-415.
- Yanagita T, Yamamoto K, Ishida S, Sonda K, Morito F, Saku K, Sakai T. (1994) Effects of simvastatin, a cholesterol synthesis inhibitor, on phosphatidylcholine synthesis in HepG2 cells. *Clin Ther*, 16, 200-208.
- Yokokawa K, Kohno M, Yasunari K, Murakawa K, Takeda T. (1991) Endothelin-3 regulates endothelin production in cultured human endothelial cells. *Hypertension*, 18, 304-315.
- Yokoyama M, Hirata K, Miyake R, Akita H, Ishikawa Y, Fukuzaki H. (1990) Lysophosphatidylcholine: essential role in the inhibition of endothelium-dependent vasorelaxation by oxidized low density lipoprotein. *Biochem Biophys Res Commun*, 168, 301-308.
- Zeiger AM, Crexler H, Saurbier B, Just H. (1993) Endothelium-mediated coronary blood flow modulation in humans: effects of age, atherosclerosis, hypercholesterolemia, and hypertension. *J Clin Invest*, 92, 652-662.