Prostaglandins and lipid peroxidation products in atherosclerosis

by

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ABSTRACT

Lipid peroxidation has been implicated in the development of atherosclerosis. It is responsible for modification of low density lipoprotein (LDL), injury to endothelial cells and has been reported to alter prostacyclin synthesis. We therefore investigated lipid peroxidation in human LDL and in LDL and aortae from rabbits fed cholesterol-supplemented diets. We also studied the correlation between lipid peroxidation and prostanoid production *in vivo* and *in vitro*.

Unsaturated fatty acids in human LDL are oxidized to their monohydroxy derivatives during the incubation with $CuSO_4$ and endothelial cells. Gas chromatographic-mass spectrometric analysis of monohydroxy fatty acids revealed a similar mechanism of peroxidation of LDL lipids by endothelial cells as that by $CuSO_4$, *i.e.*, autooxidation of LDL lipids.

Increased amounts of monohydroxy fatty acids in LDL and aorta were observed in rabbits fed cholesterol-supplemented diets. The increased amounts of monohydroxy derivatives of oleic and linoleic acids, but not those of arachidonic acid, were due to the increased amounts of their fatty acid precursors. The increased amounts of aortic monohydroxy derivatives of arachidonic acid, but not those of oleic and linoleic acids, were positively correlated with the severity of the atherosclerotic lesions.

Prostacyclin production by aortae was slightly increased after cholesterol-supplemented diets despite evidence for increased lipid peroxidation in this tissue. This may be because esterified hydroperoxy fatty acids in aorta are not very effective in inhibiting prostaglandin synthesis. In contrast to the moderate changes in aortic PGI_2 synthesis, there was a dramatic increase in the synthesis of thromboxane B_2 by aortae from hypercholesterolemic rabbits, suggesting a role for thromboxane A_2 in atherogenesis.

ABREGE

La peroxidation des lipides a été impliquée dans le développement de l'athérosclérose; elle est responsable de la modification des lipoprotéines de basse densité (LDL), de lésions aux cellules endothéliales et a été citée comme cause de l'altération de la synthèse des prostacyclines. Pour ces raisons nous avons étudié la peroxydation des lipides au niveau des LDL chez l'humain et des LDL et de l'aorte chez le lapin nourris avec une diète riche en cholestérol. Nous avons aussi étudié la corrélation entre la peroxydation des lipides et la production des prostanoïdes *in vivo* et *in vitro*.

Les acides gras insaturés des LDL de l'humain sont oxydés lors de l'incubation avec les cellules endothéliales ou avec du $CuSO_4$, générant des dérivés monohydroxylés. L'analyse des acides gras monohydroxylés par chromatographie gazeuse et spectrométrie de masse nous demontre que les mécanismes de la peroxydation des LDL lipidique par les cellules endothéliales ou le $CuSO_4$ est similaire, *i.e.*, une autooxydation des lipides des LDL.

Chez les lapins nourris avec une diète riche en cholestérol, on remarque au niveau des LDL et de l'aorte, une augmentation des acides gras monohydroxylés. L'augmentation des dérivés monohydroxylés de l'acide oléique et linoléique, mais non celle de l'acide arachidonique, est initialement causée par l'augmentation des précurseurs de ces acides gras. Au niveau de l'aorte, la sévérité des lésions produites par l'athérosclérose est directement associée à l'augmentation des dérivés monohydroxylés de l'acide arachdonique mais ne l'est pas avec celles des acides oléique et linoléique.

La production de prostacyclines par l'aorte rdy légèrement augmentée suite à la diète riche en cholestérol, et cela malgré l'augmentation évidente de la peroxydation des lipides dans ce tissu. Ce phénomène peut-être causé par l'inefficacité des acides gras hydroperoxydés estérifiés à inhiber la synthèse des prostaglandines. Par opposition aux changements modérés de la synthèse des PGI_2 aortique, nous avons observé une très forte augmentation de la synthèse de thromboxane B_2 par l'aorte chez le lapin hypercholestérolémique, suggérant un rôle de la thromboxane A_2 dans l'athérogénèse.

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ABBREVIATIONS

18:0	octadecanoic acid (stearic acid)
18:1	9-octadecenoic acid (oleic acid)
18:2	9,12-octadecadienoic acid (linoleic acid)
19:2	10,13-nonadecadienoic acid
20:4	n-eicosanoic acid (arachidic acid)
20:4	5,8,11,14-eicosatetraenoic acid (arachidonic acid)
6-oxoPGF$_{1\alpha}$	6-oxo-prostaglandin F$_{1\alpha}$
ACAT	acyl CoA:cholesterol acyltransferase
ACEH	acid cholesteryl ester hydrolase
ADP	adenosine 5'-diphosphate
ANOVA	analysis of variance
ATP	adenosine 5'-triphosphate
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
cAMP	cyclic adenosine-5'-monophosphate
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EI	electron impact
ETYA	5,8,11,14-eicosatetraynoic acid
FH	familial hypercholesterolemia
GC	gas chromatography
GSH	glutathione
GSH-Px	glutathione peroxidase
h-18:1	hydroxy octadecenoic acid
h-18:2	hydroxy octadecadienoic acid

h-20:4	hydroxy eicosatetraenoic acid
HDL	high density lipoprotein
HETE	hydroxy eicosatetraenoic acid
HMG CoA	3-hydroxy-3-methyl-glutaryl coenzyme A
HPETE	hydroperoxy eicosatetraenoic acid
HPLC	high-pressure liquid chromatography
LC	liquid chromatography
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
MDA	malondialdehyde
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MSTFA	*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide
NADPH	ß-nicotinamide adenine dinucleotide phosphate (reduced form)
NCEH	neutral cholesteryl ester hydrolase
NDGA	nordihydroguaiaretic acid
NP-HPLC	normal phase-high pressure liquid chromatography
ODS	octyldecyl salane
PAF	platelet activating factor
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
$PGF_{1\alpha}$	prostaglandin $F_{1\alpha}$
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
PH-GSH-Px	phospholipid hydroperoxide glutathione peroxidase
PUFA	polyunsaturated fatty acids
RP-HPLC	reversed phase-high pressure liquid chromatography
SIM	selected ion monitoring

SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	thiobarbituric acid-reactive substances
TXA_2	thromboxane A_2
TXB_2	thromboxane B_2
UV	ultraviolet
VLDL	very low density lipoprotein
WHHL	Watanabe heritable hyperlipidaemia

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INTRODUCTION

1.1. DEVELOPMENT OF ATHEROSCLEROSIS

Atherosclerosis is a common form of arteriosclerosis in which deposits of yellow plaques (atheromas) containing cholesterol, cholesteryl esters and other lipid materials are present in the large arteries. Coronary heart disease, stroke and other diseases related to atherosclerosis remain the major cause of death in the developed countries. For example, atherosclerosis accounts for a cumulative coronary incidence of about 50% and for 25-30% of death for population in North America [1]. The death rate due to atherosclerosis has recently had a tendency to decline, because of the extensive studies on this disease.

Before getting into the discussion of atherogenesis, a brief review of the morphological changes of the aorta due to atherosclerosis will help us to understand the pathogenesis of this disease.

1.1.1. Lesions of Atherosclerosis

The morphological appearance of atherosclerotic aorta depends on the stage of this disease. The first visible sign to the naked eye is the formation of fatty streaks in the intima followed by fibrous plaque and more severe complications. Using light or electron microscopy with conventional or immunocytochemical staining, cellular components of atherosclerotic lesions have been studied in great detail. In humans, these fatty streaks are composed of smooth muscle cells, monocyte-derived macrophages, increased amounts of elastin and collagen fibres, and other cellular components. Both smooth muscle cells and macrophages are capable of accumulating lipids and are transforming into foam cells which are characteristics of atherosclerotic lesions. Other characteristics of atherosclerotic lesions include diffused intimal thickening and proliferation of connective tissue. The hypercholesterolemic rabbit is frequently used as a model to investigate the initiation, expansion and maturation of the fatty streak. In the early stages of cholesterol feeding (ca. 1-2 weeks), mononuclear cell adhesion to the endothelial surface of the aorta can be observed. The monocytes start spreading or moving along the endothelium, and begin

to penetrate through the gap between endothelial cells into the intima. Lipid accumulation can be seen in endothelial cells, intimal smooth muscle cells and monocyte-derived macrophages in this stage. Cell proliferation may also be an early event in atherogenic process. In addition, there are increased amounts of elastic fibres, collagen, amorphous material and small vesicular structures present in the intimal space of the aorta. Longer periods of cholesterol-feeding (ca. 2-6 months) result in the formation of fibrous plaques in atherosclerotic lesions characterized by multiple layers of mixed populations of large foam cells and smooth muscle cells together with plasma insudates and connective tissue components. Accumulation of lipids is also seen within the extracellular spaces, probably due to the overloading of lipids within cells. In contrast to human atherosclerotic lesions, smooth muscle cells in rabbit aortic lesions do not account for as large a percentage of the cell volume of the early and mature fatty streak as do macrophages.

1.1.2. Pathogenesis of Atherosclerosis

The exact cause of atherosclerosis is still unclear even though several hypotheses have been postulated to explain the morphological changes observed in atherosclerotic lesions. The widely accepted ones are that atherosclerosis is initiated either (a) in response to vascular injury or (b) in response to changes in lipid metabolism. Although these two hypotheses emphasize different aspects of atherogenesis, they have some common features, including endothelial injury or dysfunction, monocyte/macrophage participation and modification of lipid metabolism.

1.1.2.1. Response to changes in lipid metabolism

(a) Cholesterol metabolism and atherosclerosis

The basic concept of the lipid hypothesis of atherogenesis is that an elevation of one or more of the cholesterol transport fractions of the blood predisposes the arteries to atherosclerosis. This hypothesis has been supported by several lines of evidence:

(1) Feeding high-cholesterol diet to certain non-human animals produces atherosclerotic plaques. This finding can be traced as far back as 1912 when Anitschkow reported that dietary cholesterol induced atherosclerotic plaques in rabbit which were

similar to those occurring in humans [2]. Over a period of nearly 80 years, cholesterol feeding has been shown to produce atherosclerotic lesions in pigeons, rats, swine, dogs and certain strains of monkey besides rabbits.

(2) The higher the blood total cholesterol level, the greater the chance of having atherosclerosis. Large scale epidemiological surveys have shown that the blood cholesterol level is a risk factor for atherosclerosis; a cholesterol level above 160 mg/dl is a prerequisite for cardiovascular disease [1]. In several countries where low-fat, high-carbohydrate diets are consumed, coronary heart disease rates are relatively low, appearing to be related to the low serum total cholesterol levels [3]. In contrast, only 5% of the population in the United States over 40 years of age has a serum or plasma total cholesterol level below 160 mg/dl [4].

(3) Lowering of total and low density lipoprotein cholesterol in clinical trials suggests that cholesterol reduction is associated with a decreased incidence of coronary disease proportional to the degree of lowering [5-8], probably due to an acceleration in the regression of atherosclerotic plaques [9]. Lovastatin, a competitive inhibitor of 3-hydroxy-3-methyl-glutaryl CoA reductase (HMG CoA reductase) decreases the extent of intimal surface involvement of atheroma by reducing cholesterol synthesis and thereby increasing the expression of LDL receptors on liver cells [10]. LAP-20, a synthetic peptide which removes cholesterol from the arterial wall and facilitates its transport to the liver, has antiatherosclerotic effect [11,12].

(b) Biosynthesis and metabolism of plasma lipoproteins

Cholesteryl esters are the major lipid components of both atherosclerotic lesions and plasma low density lipoprotein [13], suggesting a role of these substances in the development of atherosclerosis. Owing to their hydrophobicity, cholesteryl esters are transported in the circulation by lipoproteins. Based on their density, lipoproteins are classified as chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). Besides the density difference, each of these lipoproteins has a specific apolipoprotein composition (see **Table 1**). For example, LDL contains only apolipoprotein B-100 (apo B-100) whereas VLDL contains apo E and apo C besides apo B-100.

Dietary triglycerides and cholesterol absorbed from the intestine are incorporated into chylomicrons and then transported through the intestine lymphatic system and general circulation to the capillaries (**Fig. 1A**). There, free fatty acids and monoglycerides are released from chylomicrons by lipoprotein lipase and then taken up by tissues for energy generation or storage. The resulting chylomicron remnants travel to the liver where they are taken up via remnant receptors (apolipoprotein B/E receptors). This is referred as the exogenous fat-transport pathway [14]. The endogenous pathway starts with the synthesis and release of VLDL from the liver. VLDL undergoes a passage similar to chylomicrons in the circulation, but in this case, the VLDL remnants are taken up by either remnant receptors or by LDL receptors (apolipoprotein B receptors). VLDL remnants are also converted to cholesterol-enriched LDL in the circulation due to the extensive removal of triglycerides and apolipoproteins other than apo B-100 from the VLDL particles. LDL serve as the major carrier of cholesteryl esters to extrahepatic tissues.

Once it has entered the cells via receptors, absorptive endocytosis or pinocytosis, LDL is transported through the cytoplasm inside endocytosis vesicles which fuse with primary lysosomes. LDL is digested into apolipoprotein particles and cholesteryl esters, which are then hydrolysed to free cholesterol and free fatty acids by acid cholesteryl ester hydrolase (ACEH). Free cholesterol may serve as a regulator for intracellular cholesterol homeostasis. Increased cholesterol levels (1) suppress the production of lipoprotein receptors, preventing further uptake of plasma lipoproteins; (2) inhibit hydroxymethyl-glutaryl-CoA (HMG-CoA) reductase, therefore reducing the synthesis of endogenous cholesterol; (3) stimulate acyl CoA:cholesterol acyltransferase (ACAT) which catalyses cholesterol esterification (**Fig. 2**). These effects all contribute to reduce intracellular cholesterol levels. If the demand for cholesterol is increased, cholesteryl esters will be hydrolysed to cholesterol and fatty acids by neutral cholesteryl ester hydrolase (NCEH).

Plasma lipoprotein levels are maintained by the balance of their production and catabolism. The liver is the major site for lipoprotein production and their metabolism via lipoprotein receptors [14]. While chylomicron remnants are taken up by remnant receptors (apolipoprotein E receptors) and low density lipoprotein (LDL) by LDL

Table 1

Characteristics of Major Plasma Lipoproteins

	CHYLOMICRONS	VLDL	LDL	HDL
DENSITY	< 1.006	< 1.006	1.019 - 1.063	1.063 - 1.210
APOLIPOPROTEIN (% of total apolipoproteins)				
A-1	7			67
A-2	4			22
B-48	23			
B-100		37	100	
C-1	15	3		2
C-2	15	7		2
C-3	36	40		5
LIPID (% of total lipids)				
Cholesterol	1.5	4	9	11
Cholesteryl esters	3.5	11	43	25
Triglycerides	91	70	9	4
Phopholipids	4	15	29	60

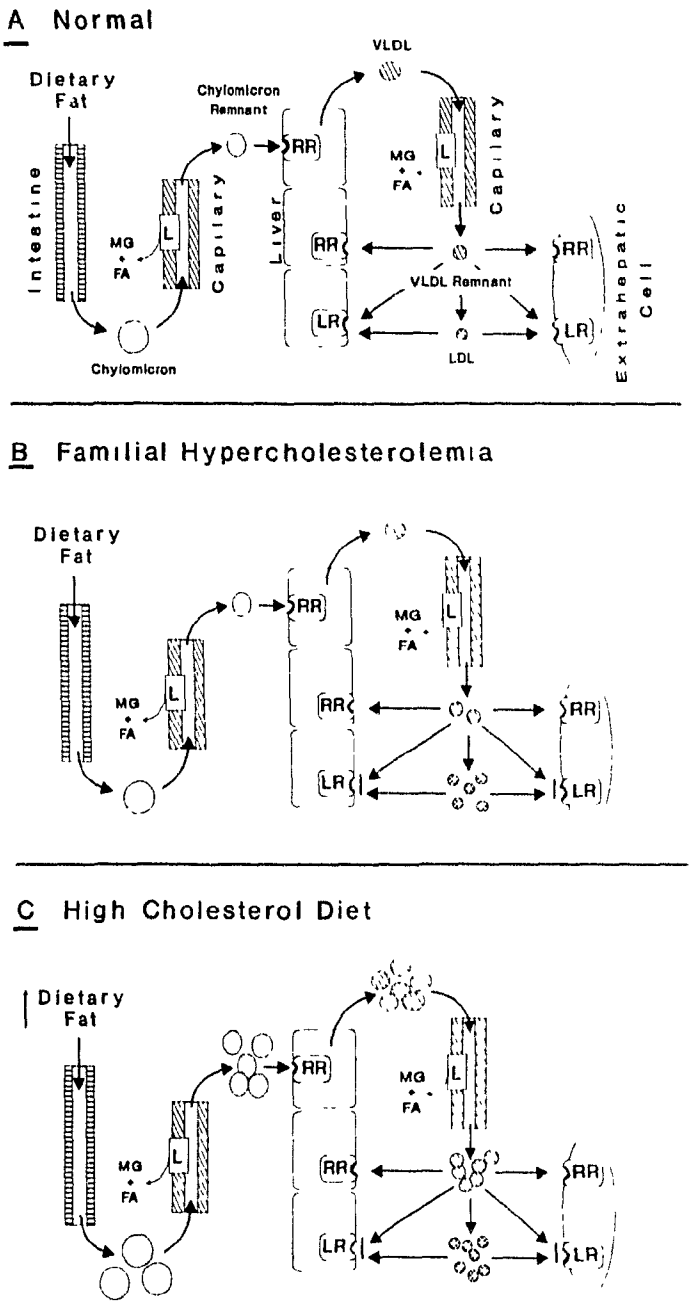


Fig. 1. Scheme for the receptor-mediated metabolism of plasma lipoproteins. The abbreviations are as follows. **MG**: monoglyceride; **FA**: fatty acids; **VLDL**: very low density lipoprotein; **LDL**: low density lipoprotein; **L**: lipoprotein lipase; **RR**: remnant receptors; **LR**: low density lipoprotein receptor.

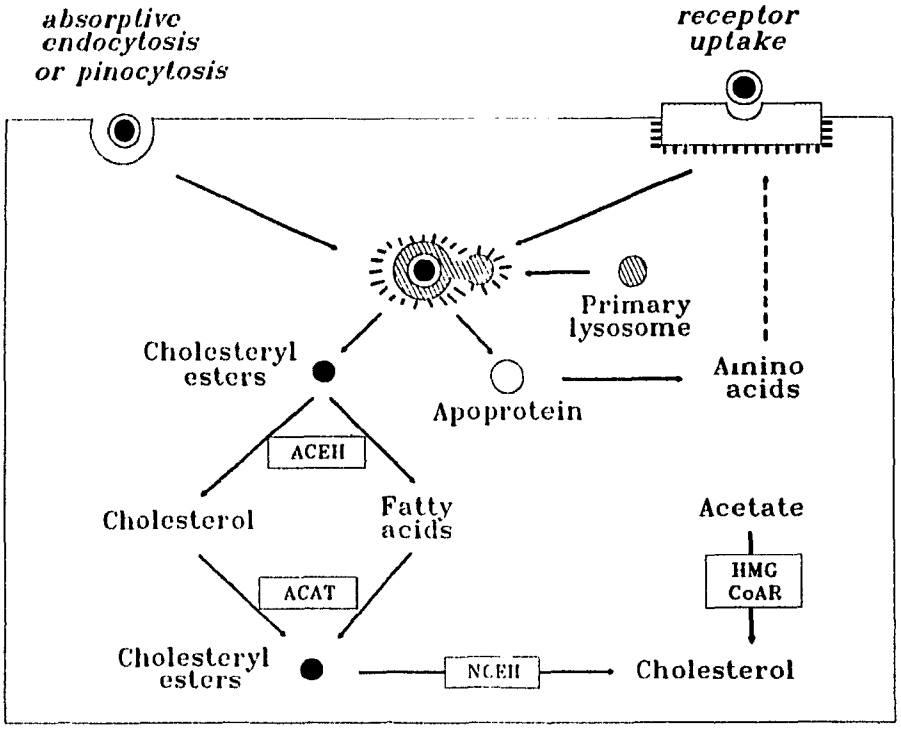


Fig. 2. Scheme showing cholesterol homeostasis in cells. **ACEH**: acid cholesteryl ester hydrolase; **ACAT**: acyl CoA:cholesterol acyltransferase; **NCEH**: neutral cholesteryl ester hydrolase; **HMG CoAR**: hydroxymethylglutaryl-CoA reductase.

receptors (apolipoprotein B receptors), VLDL and their remnants are taken up by both the remnant and LDL receptors. In physiological conditions, the LDL receptor pathway is the predominant mechanism for clearing circulating cholesteryl esters. This pathway is regulated by the availability of cholesterol and cholesteryl esters. When cells such as liver and adrenal cells grow, the demand for cholesterol increases, leading to increased production LDL receptors. On the other hand, when the need is low, excess cholesterol accumulates within the cells, which then make fewer LDL receptors and take up LDL at a reduced rate, protecting them from excessive cholesterol. However, this local protection results in decreased removal of LDL (and therefore cholesterol) from the circulation via the high-affinity LDL receptor pathway and consequently accelerates the development of atherosclerosis.

The importance of LDL receptors in atherogenesis is highlighted in a genetic disease called familial hypercholesterolemia (FH). Patients or rabbits (Watanabe heritable hyperlipidemic rabbits, WHHL rabbits) with this disease lack or have defective LDL receptors and have profound hypercholesterolemia (**Fig. 1B**) [14-17]. Heterozygous FH patients have twice the level of LDL as normal subjects and begin to have heart attacks at the age of 35; homozygous FH patients have circulating LDL levels more than six times higher than normal and inevitably have heart attacks by the age of 20.

Down-regulation of hepatic LDL receptors also results from increased dietary cholesterol. Unphysiologically high levels of cholesterol lead to increases of both chylomicrons and VLDL, coupled with a reduction in the production of LDL receptors. The combination of VLDL overproduction and retarded receptor-mediated catabolism of VLDL remnants results in cholesterol levels far above the normal level (**Fig. 1C**).

Hypercholesterolemia results in increased transport of cholesterol-rich blood components across the endothelium into the intima and subintimal spaces of the blood vessel wall, a necessary event for subsequent cholesterol deposition in this tissue. Interaction of LDL with its endothelial cell receptors do not enhance transendothelial transport of LDL across normal endothelium [18,19]. Therefore LDL must be transported across the endothelium by a low-affinity uptake process [20] or via a passive process which is the result of increased endothelial permeability [21,22].

1.1.2.2. Response to endothelial injury

Endothelial cells play a central role in the physiologic homeostasis of the blood vessel wall via their ability to function as a thrombo-resistant surface, a macromolecular barrier, and an active metabolic unit.

Development of atherosclerosis was observed in animals from which the endothelium was removed from the aorta by a balloon catheter [23]. This manipulation resulted in an intimal lesion characterized by smooth muscle cell proliferation, connective tissue formation and lipid deposition. Later, it was discovered that the smooth muscle cell proliferation in this model was caused by platelet-derived growth factor (PDGF) released from platelets adhering to the deendothelialized surface of the aorta [24]. The increased lipid deposition was the result of an increased entry of plasma components into the intima-media following endothelial denudation [25]. However, recent evidence indicates that endothelial denudation is extremely rare in both animal models of atherosclerosis [26,27] as well as in human [28]. Only in advanced atherosclerotic plaques have regions of vessel wall lacking intact endothelium been identified *morphologically* [29]. Moreover, platelet aggregation does not increase under conditions where endothelial cell death is drastically increased since the repair mechanisms for maintenance of endothelial continuity do not allow the exposure of the sub-endothelium even under pathological conditions [30].

However, the possibility of morphological changes and/or dysfunction of the endothelium in atherogenesis has been raised. The endothelium is a *single* layer of endothelial cells. Dysfunction or loss of one or more cells will result in increased permeability of the endothelium, allowing increased entry of cholesterol-rich lipoproteins into the arterial wall [31]. Lipoproteins can become anchored to the subendothelial matrix of the arterial tissue, and are then a better source of cholesterol to foam cells [32]. An *in vivo* study also showed that LDL bound to the arterial proteoglycans is more susceptible to oxidative modification and uptake by macrophages [33] (see **Section 1.1.2.3.** and **Section 1.3.6.4** for more detail). Hemodynamic [34-38], biochemical [39], immunological [40] and other factors known to be atherogenic can injure endothelial cells, causing increased endothelial cell turnover in specific regions of the artery [27,28].

The regenerated endothelium shows changes in gap and tight junction morphology associated with increased permeability [41]. The regenerated endothelium also leads to increased interaction of monocytes with endothelial cells [42], which is due to the release of chemotactic factors [43-45] and expression of binding sites for monocytes [42].

The heart of the "response to injury" hypothesis is that the lesions of atherosclerosis are initiated as a response to some form of injury to the arterial endothelium. The injury leads to a series of interactions between blood components and the arterial wall that result in the formation of atherosclerotic plaques. Among the blood components, lipoproteins are the most obvious since they are the carriers of lipids. However, the deposition of lipids in arteries is usually associated with monocytes/macrophages which are frequently seen in the early stages of atherosclerosis.

1. 1.2.3. Role of monocytes/macrophages

One of the early cellular responses in hypercholesterolemia is the attachment of clusters of leukocytes, principally monocytes, throughout the arterial tree. The potential importance of phagocytic cells in atherosclerosis was originally suggested by Leary [46] who suggested that "atherosclerosis in man and in the experimental rabbit is due to the presence of excess cholesterol esters within phagocytic cells, which first appear in the intima of the arterial wall". The appearance of monocytes on the surface of the endothelial surface of the arteries is the result of both monocyte activation [47] and endothelial injury [48,49] due to the hypercholesterolemia. Hypercholesterolemic monocytes are more adhesive [50], more responsive to chemotactic stimuli, and are more capable of penetrating areas of altered endothelium [47]. Once they have migrated into the arterial intima, they undergo activation and differentiation, and assume the structural and functional characteristics of macrophages [51].

Macrophages dominate the lipid-enriched core of the plaque. They have receptors for lipoproteins not usually found in substantial amount in the arterial tissues. These receptors include the VLDL receptor and the scavenger receptor for modified LDL (**Fig. 3**). VLDL, particularly ß-VLDL, is often elevated in animals with a high cholesterol diet, whereas modified LDL is the result of increased oxidative stress. In both cases,

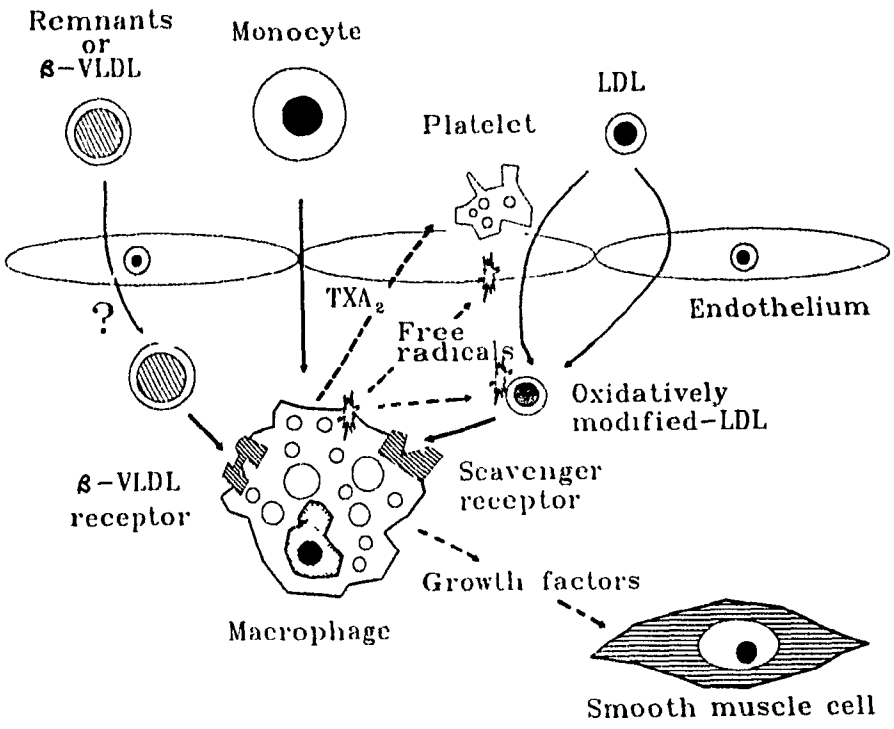


Fig. 3. Scheme showing role of macrophage in atherogenesis. Having entered the arterial wall, monocytes are transformed into macrophages which possess two distinct receptors for lipoproteins. One is the ß-VLDL receptor which takes up ß-VLDL or remnants of chylomicrons and VLDL; another is the scavenger receptor which binds LDL modified chemically or by cells such as platelets, endothelial cells, monocytes/macrophages or smooth muscle cells. After taking up excessive cholesterol, macrophages become macrophage-derived foam cells. Macrophages also damage cells by generating free radicals, activate platelets by releasing TXA_2, and stimulate smooth muscle cell proliferation by releasing growth factors.

macrophages serve as scavengers to take up excess lipids, preventing them accumulated in the interstitial spaces. Unlike LDL receptors, neither scavenger receptors nor VLDL receptors are regulated by intracellular cholesterol levels. In other words, macrophages will take up the above "unusual" lipoproteins in an unregulated manner.

Of the two type of receptors, the scavenger receptor is the one that is more interesting to researchers because of its role in atherogenesis in different species and in various situations. Scavenger receptors were discovered in the 1970's when researchers tried to learn why incubation of macrophages with high concentrations of LDL did not result in foam cell formation *in vitro* and how cholesterol from LDL accumulates in macrophages in atherosclerotic plaques of patients with familial hypercholesterolemia [52]. They found that monocytes/macrophages took up large amount of LDL, which had been modified chemically by acetylated reagents, via a specific receptor. This is the scavenger receptor. Besides taking up acetylated LDL, scavenger receptors also recognize LDL modified by transition metal ions and various type of cells including endothelial cells [45,53,54], arterial smooth muscle cells [54-56], monocytes [57-59] and activated platelets [60]. The underlining mechanism is the modification of lysine residues of the apolipoprotein B-100 by LDL lipid peroxidation products (also see **Section 1.3.6.4**). Oxidative or maleylated bovine serum albumin, polyvinyl sulphate and polyinosine also bind to these receptors [16,61].

Scavenger receptors were first partially purified from murine tumours obtained by injecting the murine macrophage cell line P388D1 into DBA/2 mice [62]. Purification of a triton-solubilized 250 kDa protein from rat liver that bound both malondialdehyde-modified LDL and maleic anhydride-modified bovine serum albumin on ligand blots was also reported [63]. In 1988, two groups reported considerable progress in the isolation and partial characterization of the scavenger receptors for acetyl LDL [64,65]. One group purified a 260 Kda protein to homogeneity using rabbit carrageenin-induced granulomas as an abundant source of macrophage receptor protein. This protein consisted of subunits between 60 and 70 Kda [64]. Another group obtained a similar receptor from bovine liver membranes. This receptor, a 220 kDa protein, appeared to be a trimer of 77-kDa subunits that contained asparagine linked carbohydrate chains [65]. The above finding

laid the foundation for the successful cloning of cDNA for two distinct macrophage scavenger receptors [66,67]. Both macrophage scavenger receptors, type I and type II, appear to be trimmers, consisting of highly glycosylated monomers of 453 amino acids (type I receptor) [66] or 349 amino acids (type II receptor) [67]. The receptors have one membrane-spanning region and have their N-terminal 50 amino acids in the cytoplasm and their C terminus outside the cell. When COS M6 cells were transfected with a scavenger receptor expression vector (plasmid pXSR7), the receptors found in this cell line had the same specificity as the macrophage scavenger receptor for acetylated LDL, maleylated bovine serum albumin and polyinosine. However, low density lipoprotein modified by lipid peroxidation products was not studied in these experiments. Therefore it is unclear if these two receptors account for all of the lipoprotein-scavenger activity of macrophages. Cross-competition experiments suggested macrophages expressed several scavenger receptors, at least one (probably some) of them recognizing only oxidatively modified but not acetylated LDL [68,69].

Besides taking up modified LDL via scavenger receptors, macrophages also accelerate atherogenesis by generating oxygen free radicals [70], synthesizing biologically active lipid derivatives such as thromboxane A_2 (TXA_2) [71] and releasing growth factors [72,73]. Oxygen free radicals such as superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen can promote membrane lipid peroxidation, leading to further cell damage. Oxygen free radicals also modify lipoproteins in the subendothelial spaces, increasing the availability of ligands for macrophage scavenger receptors. The release of biologically active lipid derivatives such as TXA_2 and platelet-activating factors (PAF) can promote platelet activation on the surface of atherosclerotic lesions. Growth factors are responsible for the proliferation of intimal smooth muscle cells and the formation of smooth muscle cell-derived foam cells within atherosclerotic lesions.

1.2. PROSTANOIDS

Prostanoids are a group of compounds derived from twenty-carbon polyunsaturated fatty acids, notably arachidonic acid, via prostaglandin endoperoxide synthase (also called cyclooxygenase) pathway. They are distributed in almost every organ in the body, and

are biologically active. However, they act only near their sites of synthesis since they are rapidly metabolized to biologically inactive compounds. Thus they generally have very short half-life in biological system and their plasma concentrations are very low. Prostanoids act on a variety of cells and tissues and have different biological effects, which are principally mediated via the adenylate cyclase and phosphoinositide pathways. Prostacyclin (PGI_2) and TXA_2 are two of the most important compounds in the prostanoid family in terms of atherogenesis.

1.2.1. Biosynthesis of Prostanoids

Upon stimulation. arachidonic acid is released from membrane-bound phospholipids by the action of phospholipase A_2 on phosphatidylcholine [74,75] or phospholipase C plus diacylglyceride lipase on phosphatidylinositol [76,77]. The released arachidonic acid is then metabolized by prostaglandin endoperoxide synthase to a 15-hydroperoxy-9,11-endoperoxide (PGG_2) (**Fig. 4**). The peroxidase activity of the same enzyme reduces PGG_2 to its 15-hydroxy analogue (PGH_2) [78]. PGH_2 has different fates in different tissues depending on the presence of various PGH_2-metabolizing enzymes. In large blood vessels, PGH_2 is metabolized to PGI_2 by prostacyclin synthase, whereas in platelets, it is metabolized to TXA_2 by thromboxane synthase. Both PGI_2 and TXA_2 are unstable and readily hydrolysed to 6-oxo$PGF_{1\alpha}$ and TXB_2, respectively. 6-oxo$PGF_{1\alpha}$ and TXB_2 are further metabolized *in vivo* to a variety of compounds that are excreted into the urine, the most abundant of these being 2,3-dinor-6-oxo$PGF_{1\alpha}$ [79] and 2,3-dinor-TXB_2 [80], respectively.

Prostaglandin endoperoxide synthase-catalysed fatty acid oxidation occurs slowly initially and later accelerates due to the requirement for hydroperoxides for enzyme activity [81-83]. Exogenous hydroperoxides eliminate the kinetic lag phase at concentrations of 10^{-7} to 10^{-8} M. When the availability of hydroperoxide activators was decreased by adding glutathione plus glutathione peroxidase, prostaglandin endoperoxide synthase activity was suppressed [84,85]. However, high concentrations of hydroperoxides, either formed during prostaglandin endoperoxide synthase-catalysed reactions or added as exogenous substances, inactivate the enzyme. Prostaglandin

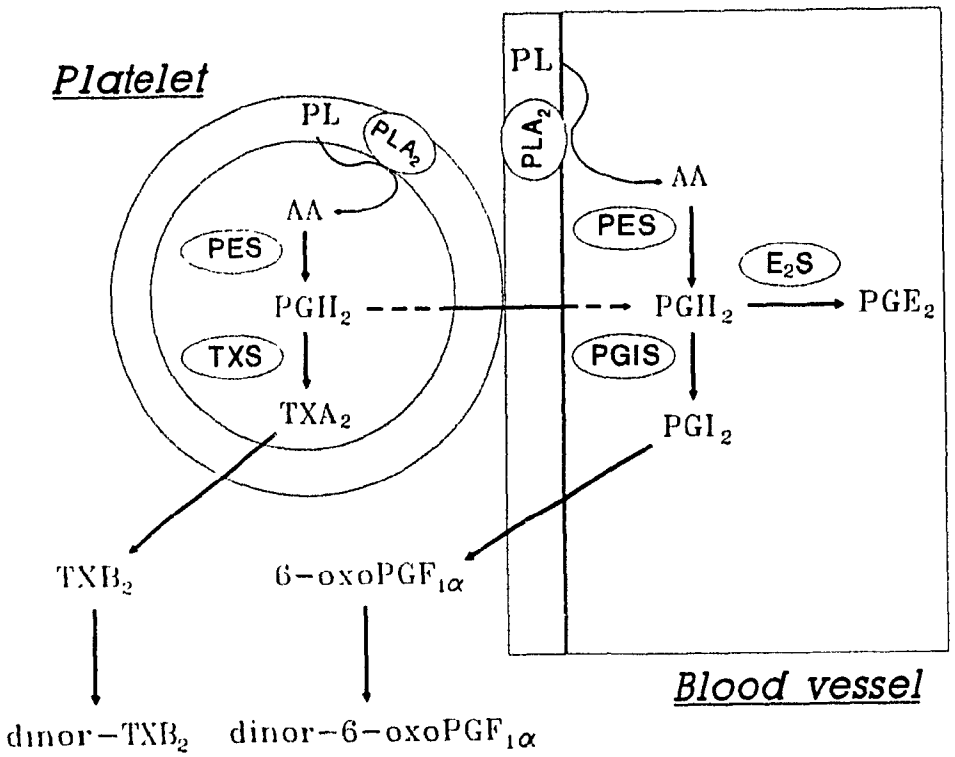


Fig. 4. Scheme showing arachidonic acid metabolism via the prostaglandin endoperoxide synthase pathway in platelets and blood vessels. Catalysed by phospholipase A_2 (PLA_2) or by phospholipase C and diacylglyceride lipase (not shown), arachidonic acid (AA) is released from membrane phospholipids (PL) and then oxidized to prostaglandin endoperoxide (PGH_2) by prostaglandin endoperoxide synthase (PES). PGH_2 is metabolized to thromboxane A_2 (TXA_2) in platelets or to prostacyclin (PGI_2) in vascular endothelial cells. Alternatively, PGH_2 is also metabolized to prostaglandin E_2 (PGE_2) by prostaglandin E_2 synthase (E_2S). When adhering to blood vessels, platelet PGH_2 is also transferred to blood vessels for PGI_2 formation. TXA_2 and PGI_2 are quickly catabolized nonenzymatically to TXB_2 and 6-oxo$PGF_{1\alpha}$, respectively, which are further metabolized to their dinor derivatives and eventually excreted into the urine. Other abbreviations are: TXS: thromboxane A_2 synthase; PGIS: prostacyclin synthase.

endoperoxide synthase-catalysed oxygen consumption declines to zero before complete consumption of the fatty acid substrate, and a second burst of oxygen consumption occurs upon addition of fresh enzyme [81]. Such self-inactivation of prostaglandin endoperoxide synthase appears to occur in intact cells as well as with purified enzyme preparations [86-88] and can be inhibited by the reversible prostaglandin endoperoxide synthase inhibitor ibuprofen [89]. Recently it has been speculated that the mechanism is due to the covalent binding of decomposition products of prostaglandin endoperoxides to the enzyme [90].

Besides being inhibited by its oxidation products, prostaglandin endoperoxide synthase can also be inhibited by nonsteroidal anti-inflammatory agents [91] and certain acetylenic fatty acids such as 5,8,11,14-eicosatetraynoic acid (ETYA). Prostanoid biosynthesis is also modulated by dietary fatty acids. Linoleic acid (18:2) inhibits arachidonic acid uptake by cells and therefore its oxidation to prostanoids [92]. Eicosapentaenoic acid (20:5) [93] and docosahexaenoic acid (22:6) [94], poor substrates for prostaglandin endoperoxide synthase, serve as competitive inhibitors of arachidonic acid oxygenation.

1.2.2. Prostacyclin and Atherosclerosis

Since the discovery of PGI_2 in 1977's, its relation to atherosclerosis has been postulated. PGI_2 is a potent vasodilator and a systemic vasodepressor. It inhibits platelet aggregation, decreases membrane permeability, inhibits release of growth factors from a variety of cells and accumulation of lipids in smooth muscle cells from blood vessels. On the other hand, PGI_2 production in blood vessels is modulated by factors related to atherogenesis such as increased hemodynamic stress, endothelial injury, growth factors and hypercholesterolemia.

1.2.2.1. Role of prostacyclin related to atherogenesis

(a) Regulation of cholesterol metabolism in blood vessel cells

PGI_2 regulates cholesterol metabolism in blood vessel cells by various mechanisms.

It modulates the number of LDL receptors on the cell surface [95], controlling the entry of LDL cholesterol into the blood vessels; it increases both acid [96] and neutral [97] cholesteryl ester hydrolase activity without affecting acyl CoA:cholesterol acyltransferase (ACAT) activity. The overall effect is therefore to enhance the efflux of cholesterol from cells [96] and decrease the intracellular levels of free cholesterol and cholesteryl esters. The increased hydrolysis of cholesteryl esters results in increased intracellular cholesterol levels, which prevents further cholesterol intake by suppressing LDL receptor synthesis and endogenous cholesterol production by inhibiting HMG CoA reductase. Excess amounts of free cholesterol are removed from the cells along a cholesterol gradient providing that the plasma components for accepting and metabolizing cholesterol, such as high density lipoprotein (HDL) and lecithin:cholesterol acyltransferase (LCAT), are in adequate supply [98]. The role of HDL in reducing intracellular cholesterol accumulation is augmented by its effect on stimulating PGI_2 production [99,100], mobilizing cellular arachidonic acid [101] and PGI_2 stabilization [102]. In addition to its effect on cholesterol, PGI_2 may also affect triglyceride metabolism since a stable analogue of PGI_2, carbocyclin, was found to decrease the triglyceride content of smooth muscle cells from fatty streaks of human aorta [103].

(b) Inhibition of platelet aggregation

PGI_2 is a potent endogenous inhibitor of platelet aggregation [104,105]. Intravenous infusion or local application of PGI_2 inhibits intravascular platelet aggregation in a model of coronary arterial thrombosis in dogs [106]. It also leads to platelet *disaggregation* in plasma or on collagen strips isolated from the achilles tendon of a rabbit [107]. The inhibition of platelet aggregation by PGI_2 is due to activation of the adenylate cyclase system, leading to a substantial increase in platelet intracellular cAMP levels [108,109] and a reduction in platelet cytoplasmic calcium levels due to uptake by the dense tubular system [110]. The final effect is the retardation of platelet shape change, aggregation and release. Interestingly, PGI_2 inhibits platelet aggregation (platelet-platelet interaction) at much lower concentrations than those needed to inhibit adhesion (platelet-collagen interaction) [111]. Direct contact between platelets and blood vessels provides a path for translocation of platelet PGH_2 to blood vessels for PGI_2 synthesis (see **Section 1.2.3.2.**

for more detail) which in turn limits thrombus formation and release of platelet granular contents, including platelet-derived growth factor (PDGF).

(c) Inhibition of growth factor release

PGI_2 has been reported to inhibit the release of growth factors from platelets [112,113], endothelial cells [112] and macrophages [112]. The effect is long-lasting despite of the short half-life of PGI_2. In addition, PGI_2 directly inhibits smooth muscle cell proliferation [114-117], probably by inhibiting DNA synthesis [118,119]. This in turn may be a direct nuclear event due to the location of PGI_2 synthase in the nuclear membrane [101]. By inhibiting smooth muscle cell proliferation and accumulation of lipids in these cells, PGI_2 has beneficial effects in preventing the formation of smooth muscle cell-derived foam cells [120]. On the other hand, there was a report showing a stimulatory effect of PGI_2 on smooth muscle cell proliferation in both static and superfusion cell cultures [112].

Because of its effects on endothelial cells, platelet activation, lipid metabolism and smooth muscle cell proliferation, sustained PGI_2 production by the vascular endothelium, its major site of synthesis, may be an important factor in preventing the onset of atherosclerosis.

1.2.2.2. Modulation of prostacyclin production by factors responsible for atherogenesis

(a) Shear stress and prostacyclin production

Atherosclerotic plaques have a tendency to occur in areas where shear stress is elevated [121]. In rabbits fed a cholesterol-supplemented diet for 4 months, atherosclerotic plaques were more extensive in the aortic arch and in the section of aorta near the diaphragm than in thoracic and abdominal aorta [122]. The aortic arch and the aorta near the diaphragm are subjected to higher shear stress than the rest of the aorta due to the change in the direction of blood flow and the narrowing of the aorta, respectively. Interestingly, PGI_2 synthesis, measured by its inhibitory activity on aggregated platelets, was higher in both of the above regions than in the rest of the aorta [122]. *In vitro*, 6-oxo$PGF_{1\alpha}$ production by cultured endothelial cells is also stimulated by

shear stress [123]. The mechanism may be due to the activation of the phosphoinositide system, which releases inositol triphosphate, by high shear stress [51]. Phosphoinositides have been found to stimulate PGI_2 release from cultured endothelial cells [124]. Increased shear stress also increases the number of LDL receptors on endothelial cells and enhances receptor-mediated LDL endocytosis [51], which in turn delivers more arachidonic acid for prostaglandin synthesis [125].

(b) Endothelial injury and prostacyclin production

According to the original response-to-injury model, injury of the vascular endothelium resulted in platelet adhesion, aggregation, and release of platelet-derived growth factor. This injury and the subsequent biological changes were responsible for the initiation of atherosclerosis. It has been demonstrated, at least *in vitro*, that damage to the endothelium by radiation [126,127] and antibodies to endothelial cells [128] is accompanied by a transient increase in PGI_2 synthesis. This may explain the elevated release of PGI_2 observed during vascular manipulation, cannulation and exposure to hypotonic solutions. It would appear that mild and continuous damage to vascular endothelium leads to increased release of PGI_2, which plays an important role in protecting the endothelium.

(c) Growth factors and prostacyclin production

Besides stimulating cell growth, various growth factors including epidermal growth factor (EGF), platelet derived growth factor (PDGF), and transforming growth factors also stimulate PGI_2 synthesis [129-134]. In subjects receiving aspirin treatment, growth factors accelerate the recovery of PGI_2 synthesis by increasing the amount of prostaglandin endoperoxide synthase induced by an increase in mRNA level [135-137].

(d) Hypercholesterolemia and prostacyclin production

Hypercholesterolemia, which is one of the major risk factors for atherosclerosis, affects PGI_2 synthesis. It has been reported to prevent the recovery of PGI_2 production after deendothelialization of rabbit aorta [138]. Sera from hypercholesterolemic human subjects [139] or rabbits [140] inhibit PGI_2 production by endothelial cells in vitro in a dose- and time-dependent manner [140]. The reduced PGI_2 production by blood vessels could be due to (1) direct inhibition of phospholipase A_2 by cholesterol; (2) competitive

inhibition by linoleic acid of prostaglandin endoperoxide synthase; and (3) reduced amounts of prostaglandin endoperoxide synthase [141,142] and PGI_2 synthase [142] proteins.

1.2.2.3. Prostacyclin production in atherosclerosis

There is considerable controversy in the literature about the production of PGI_2 by atherosclerotic blood vessels with several reports indicating considerably diminished production and others reporting unchanged or even enhanced production. The conclusions were based on studies of either cells from atherosclerotic lesions, or blood vessels isolated from atherosclerotic patients or animals, or urinary metabolites of prostacyclin.

In an experiment by Gryglewski and coworkers [143], rabbits were fed with 1 g of cholesterol and 3 g of olive oil daily for 1, 3 and 5 months. PGI_2 synthesis by slices of aorta from endogenous substrate, measured by bioassay of incubates on a strip of bovine coronary artery or platelet-rich plasma, were strongly suppressed after 1 and 3 months of the cholesterol diet. There was a tendency for PGI_2 synthesis to recover after 5 months. On the other hand, Beetens *et al* found that PGI_2 released (measured as 6-oxo$PGF_{1\alpha}$ by radioimmunoassay) from the luminal surface of aorta from cholesterol-fed rabbits was higher after 2 weeks on a cholesterol diet and the same as controls after 4 weeks. PGI_2 production subsequently declined continuously up to 10 weeks [144]. Unlike the luminal surface of aorta, these authors found no difference between atherosclerotic and control rabbits in PGI_2 release from slices of aorta [144].

Studies of different morphological types of human atherosclerotic lesions ranging from fatty streak to fibrous plaque demonstrated significantly decreased PGI_2 formation by all types of lesions [145,146]. The inhibition of PGI_2 synthesis in atherosclerosis is further supported by the finding that smooth muscle cells from atherosclerotic rabbit aorta produces less PGI_2 than healthy smooth muscle cells [147].

In contrast to the above studies, there are also a number of reports suggesting that PGI_2 synthesis is higher, rather than lower, in atherosclerosis. In a rabbit model which the aortic endothelium had been stripped by balloon catheter followed by 3 months on a cholesterol diet, both endogenous and arachidonate-induced PGI_2 biosyntheses in aortic

segments from atherosclerotic rabbits were increased compared to control rabbits [148]. When pieces of tissues were punched out of the aorta and incubated in small volumes of phosphate buffer, PGI_2 synthesis, measured by its antiaggregatory effect on platelets, was significantly higher in atherosclerotic regions than that in non-lesion regions in the same aorta [122]. The increase was also observed in homogenates of cholesterol-induced atherosclerotic aorta of rabbits [149]. Increased prostacyclin production in atherosclerosis in humans was supported by the increased excretion of its major urinary metabolite, 2,3-dinor-6-oxo$PGF_{1\alpha}$, by atherosclerotic patients [150].

1.2.3. Thromboxanes and Atherosclerosis

TXA_2, another member of the prostanoid family, has actions which oppose those of PGI_2 on many system including platelets and vasculature. These effects are physiologically useful in stopping acute haemorrhage. Pathologically, however, TXA_2 is regarded as a proatherosclerotic compound.

1.2.3.1. Physiological effects of thromboxane A_2

TXA_2 and its pro-aggregatory effect on platelets were first reported by researchers in Karolinska Institute in mid 1970's [151,152] when they tried to identify a compound which had a stronger but shorter contractile activity on the rabbit aorta than the prostaglandin endoperoxides released from aggregated platelets or guinea-pig lungs by arachidonic acid [153]. Subsequent studies showed that TXA_2 mediates platelet aggregation and release stimulated by low concentrations of such endogenous agents as collagen [154]. This is relevant to atherogenesis since injury to the vascular endothelium leads to the exposure of subendothelial matrices and platelet adhesion to them. Upon contact with collagen, one of the components of the subendothelial matrix, platelets are activated and undergo shape change, aggregation and release of granule contents including platelet-derived growth factor, which stimulates smooth muscle cell proliferation [155-157] as discussed above (**Section 1.1.2.2.** on P. 9). However, the role of platelet-generated TXA_2 in initiating atherosclerosis is questioned due to the lack of endothelial denudation in early stages of this disease. It therefore appears more plausible

that platelet-generated TXA_2 is an important factor responsible for the onset of complications of atherosclerosis [158,159] rather than atherogenesis.

TXA_2 constricts various blood vessels including aorta, and coronary and mesenteric arteries via a decrease of intracellular cAMP [160]. Even though this effect does not contribute to atherogenesis, it certainly causes such complications of atherosclerosis as angina [161-163]. Direct evidence is the release of TXA_2 into coronary sinus observed in patients with recent episodes of angina [164].

1.2.3.2. Balance of Prostacyclin/Thromboxane A_2

An interesting aspect of PGH_2 metabolism is that the prostaglandin endoperoxide produced by one cell type can be used by another to produce prostaglandins. An example of this is the translocation of PGH_2 from platelets to blood vessels [165-168]. However, translocation in the opposite direction was not observed [167]. The translocation of PGH_2 from platelets to endothelial cells is accentuated by thromboxane synthase inhibitors [87]. This *unidirectional* translocation is particularly important in the case of endothelial damage to the arteries which promotes adherence of platelets to the damaged surface. PGH_2 released from activated platelets on the surface of the injured arteries can thus be utilized by undamaged endothelial cells and smooth muscle cells of the blood vessel to generate prostacyclin [169], which prevents excessive activation of platelets and thrombus formation.

Inhibition of TXA_2 synthesis by platelets can be achieved by inhibiting prostaglandin endoperoxide synthase by non-steroidal anti-inflammatory agents. However, this could also inhibit the production of PGI_2 by blood vessels. Use of thromboxane synthase inhibitors can overcome this problem due to aspirin by redirecting PGH_2 from platelets to endothelial cells of the blood vessel where it is converted to PGI_2 [170]. The balance of PGI_2/TXA_2 can also be shifted to favour PGI_2 synthesis by irreversible inhibitors of prostaglandin endoperoxide synthase. Platelets do not have nuclei and therefore can not synthesize new prostaglandin endoperoxide synthase once it has been irreversibly inhibited. On the other hand, prostaglandin endoperoxide synthase in blood vessels can be regenerated as soon as inhibitors are removed [171]. The balance of biologically

active PGI_2 and TXA_2 is also affected by the consumption of fish oil, which contains ω-3 polyunsaturated fatty acids. The Eskimo population of Greenland have mildly prolonged bleeding times, reduced reactivity to aggregating agents and, notably, a low incidence of ischemic heart disease [172]. TXA_3 from ω-3 polyunsaturated fatty acids appears to have little pro-aggregatory activity [173-175] whereas PGI_3 maintain antiaggregatory effect as PGI_2 [173-175]. Thus the balance is shifted in favour of antiaggregatory prostacyclin [175,176]. As a result of fish oil consumption, endogenous production of PGI_3 (measured as urinary Δ^{17}-2,3-dinor-6-oxo$PGF_{1\alpha}$) is higher in Greenland Eskimos than in age matched Danish controls [176]. Surprisingly, PGI_2 production (measured as urinary 2,3-dinor-6-oxo$PGF_{1\alpha}$) is also higher, whereas TXA_2 is lower, in Eskimos. Similar results were also observed in healthy volunteers taking fish oils [177].

1.2.3.3. Thromboxane production in atherosclerosis

Unlike PGI_2, TXA_2 production was uniformly found to be higher in aorta of atherosclerotic rabbits [143,148,178-180]. It seems that the increase is associated with hypercholesterolemia in atherosclerosis [181]. *In vitro*, 'cholesterol-enriched' platelets released more labelled arachidonic acid and more TXB_2 than control platelets [182]. Atherosclerosis and its risk factors, such as smoking [183], diabetes [184-186], and hyperlipoproteinemia [187-189], are all associated with decreased platelet sensitivity to antiaggregatory prostaglandins. Furthermore, inhibitors of TXA_2 synthesis were found to suppress atherosclerotic lesions in hypercholesterolemic rabbits [190,191].

1.3. LIPID PEROXIDATION

Lipid peroxidation has been loosely defined as the "oxidative deterioration of polyunsaturated lipids (PUFA)" [192]. Lipid peroxidation may play an important role in many diseases, including atherosclerosis, tissue damage due to reperfusion of oxygen after ischemia, and cancer. A better understanding of the role of lipid peroxidation in these diseases may result in a better treatment.

1.3.1. Mechanism of Lipid Peroxidation

Lipid peroxidation is a chain reaction consisting of three events: initiation, propagation and termination.

Initiation of the peroxidation of polyunsaturated fatty acids (LH) is caused by the attack of any species (X·) that has sufficient reactivity to abstract a hydrogen atom from a *bis*-allylic methylene group to form a *alkyl radical* (L·, **Fig. 5**, step [1]). The presence of a double bond in the fatty acid weakens the C-H bonds on the adjacent carbon atom and so makes hydrogen atom removal easier. The alkyl radical tends to be stabilized by a molecular rearrangement to produce a *conjugated diene*, which then reacts with an oxygen molecule to give a *peroxyl radical* (LOO·, **Fig. 5**, step [2]). The peroxyl radical is then converted to a lipid hydroperoxide (LOOH) by abstracting a hydrogen atom from *another* molecule of unsaturated fatty acid (**Fig. 5**, step [3]), leading to *propagation* of the lipid peroxidation process.

The position of oxygen insertion depends on the position of hydrogen-abstraction. Each 1,4-*cis,cis*-pentadiene unit will give rise to two hydroperoxy isomers. For example, two isomers (9- and 13-hydroperoxy derivatives) are formed from linoleic acid (**Fig. 6A**) and six isomers (5-, 8-, 9-, 11-, 12- and 15-hydroperoxy derivatives) are generated from arachidonic acid (**Fig. 6B**). Two isomers of each of the hydroperoxy fatty acids are formed, one of which has a *cis-trans* conjugated diene chromophore, and the other a *trans-trans* conjugated diene chromophore.

In the case of monounsaturated fatty acids, L· is a radical formed as a result of abstraction of an allylic hydrogen. Oxygenation can occur at either end of the allylic radical. Therefore four positional isomers, 8-, 9-, 10- and 11-hydroperoxides, will be formed from oleic acid (**Fig. 7**).

Termination of lipid peroxidation can be achieved by combination of radicals:

(1) $L\cdot + LOO\cdot \longrightarrow LOOL$

(2) $2\ LOO\cdot \longrightarrow LOOL + O_2$

(3) $2\ L\cdot \longrightarrow L\text{-}L.$

In some areas of the membrane where PUFA molecules are not adjacent to vitamin E molecules (The role of vitamin E will be further discussed in **Section 1.4.1.**), forming

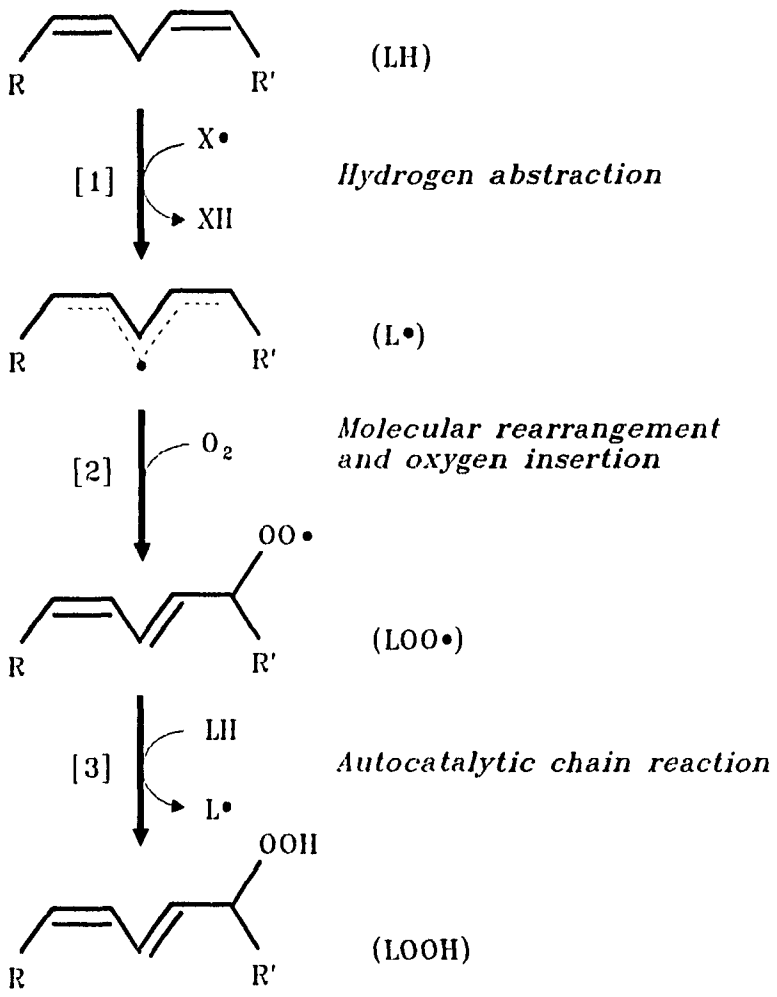


Fig. 5. Scheme for the formation of hydroperoxy derivatives of polyunsaturated fatty acids by lipid peroxidation.

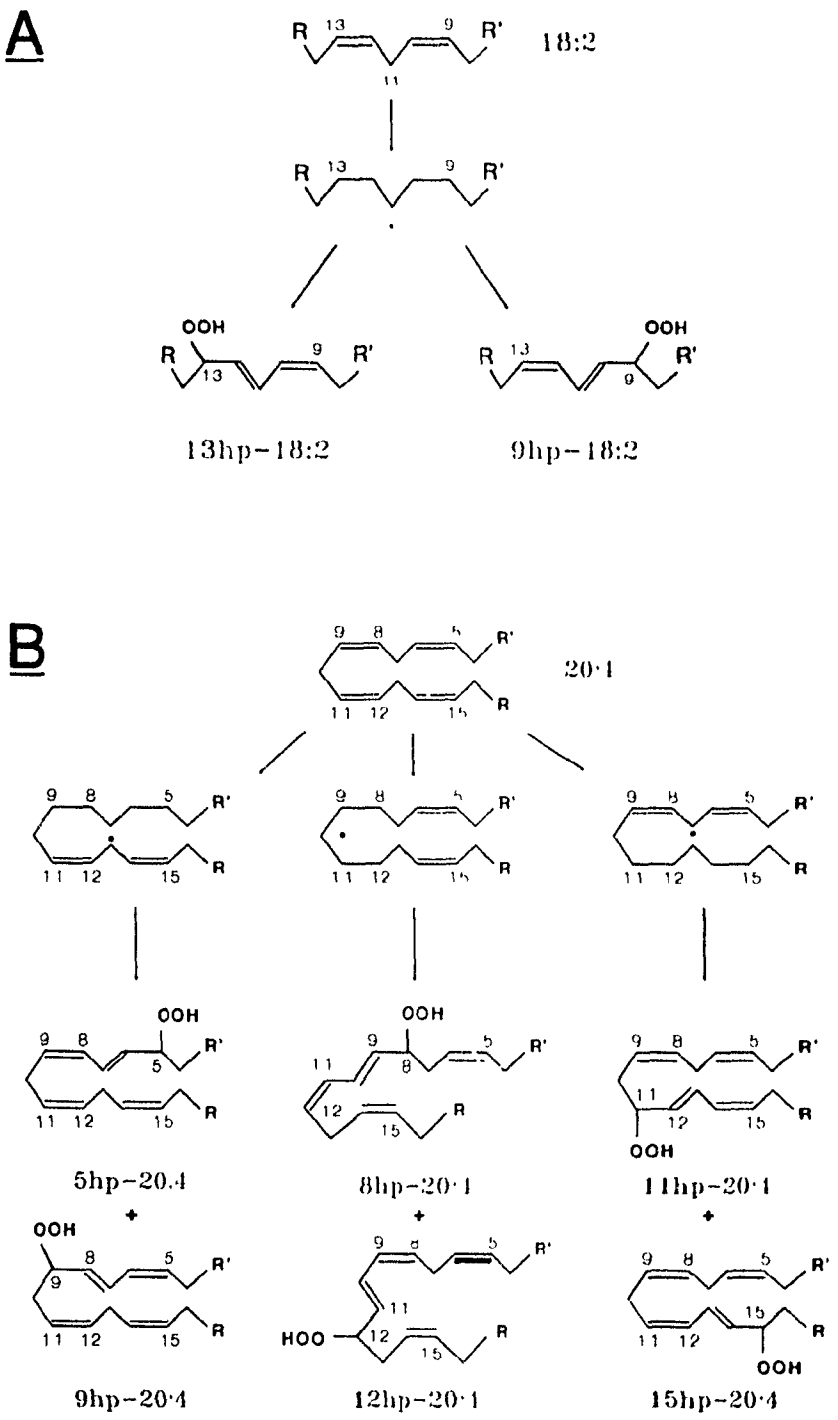


Fig. 6. Scheme for the formation of positional isomers of hydroperoxy derivatives of linoleic (A) and arachidonic (B) acids.

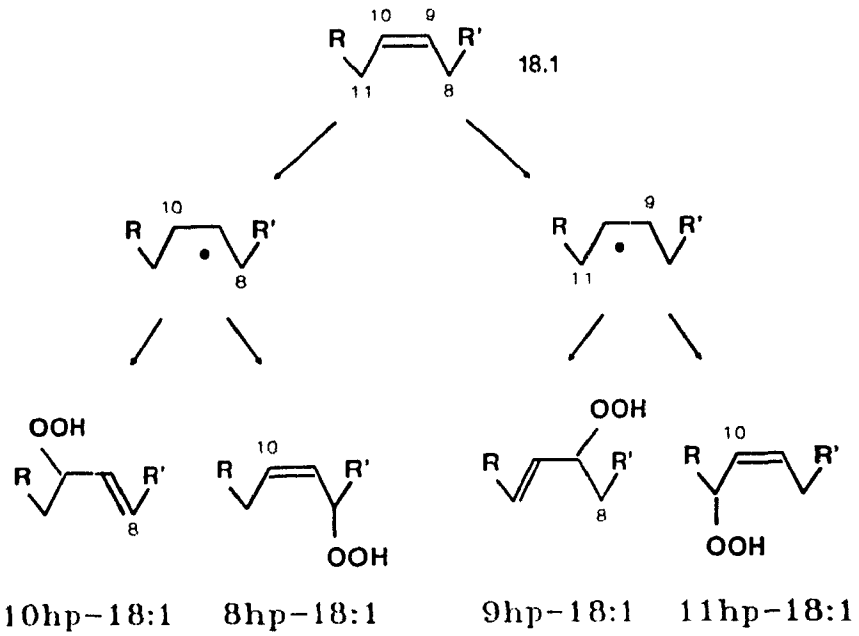


Fig. 7. Scheme for the formation of positional isomers of hydroperoxy derivatives of oleic acid.

dimers with neighbouring free radicals (**reaction 2 and 3**) could be the best way to prevent propagation of lipid peroxidation and therefore keep the damage to a minimum [193].

1.3.2. Lipid Peroxidation in Biological System

Formation of hydroperoxy metabolites of polyunsaturated fatty acids *in vivo* is more complicated than *in vitro* since lipid peroxidation can be initiated by autoxidation as discussed above or by enzymatic catalysis, either directly or indirectly.

1.3.2.1. Superoxide, hydroxyl radical and lipid peroxidation

In vivo lipid peroxidation can be initiated by the passage of electrons from substrates of certain enzymes to a system containing O_2 and iron (**Fig. 8**) [194,195]. One of these enzymes involves the NADPH-cytochrome P-450 reductase system which reduces ADP-Fe^{3+} to ADP-Fe^{2+} (step [1]); another is xanthine oxidase (step [2]) [196,197]. The resulting electrons are transformed directly to oxygen, reducing it to superoxide, O_2^- (step [3]) [198]. O_2^- itself is insufficiently reactive to abstract a hydrogen atom from membrane lipids and it is not expected to enter the interior of the membrane because of its charged nature [199]. However O_2^- can be metabolized to hydrogen peroxide H_2O_2 and ground state oxygen by superoxide dismutase (step [4]) [197,198]. In the presence of ferrous irons, hydroxyl radical (OH·) will be formed by homolysis of H_2O_2 (Haber-Weiss reaction; step [5]) [200]. Alternatively, OH· can also be formed by the combination of O_2^- and H_2O_2 [201]:

$$O_2^- + H_2O_2 \longrightarrow OH\cdot + OH^- + O_2.$$

OH· is a very strong oxidant, abstracting hydrogen atoms from membrane lipids to give alkyl radicals and water (step [6]) [200].

1.3.2.2. Lipoxygenases and lipid peroxidation

Lipid hydroperoxides can also be formed by enzymatic oxidation of polyunsaturated fatty acids by lipoxygenases (**Fig. 9**). Lipoxygenases catalyse dioxygenation of polyunsaturated fatty acids containing a least one 1,4-*cis*,*cis*-pentadiene to yield a

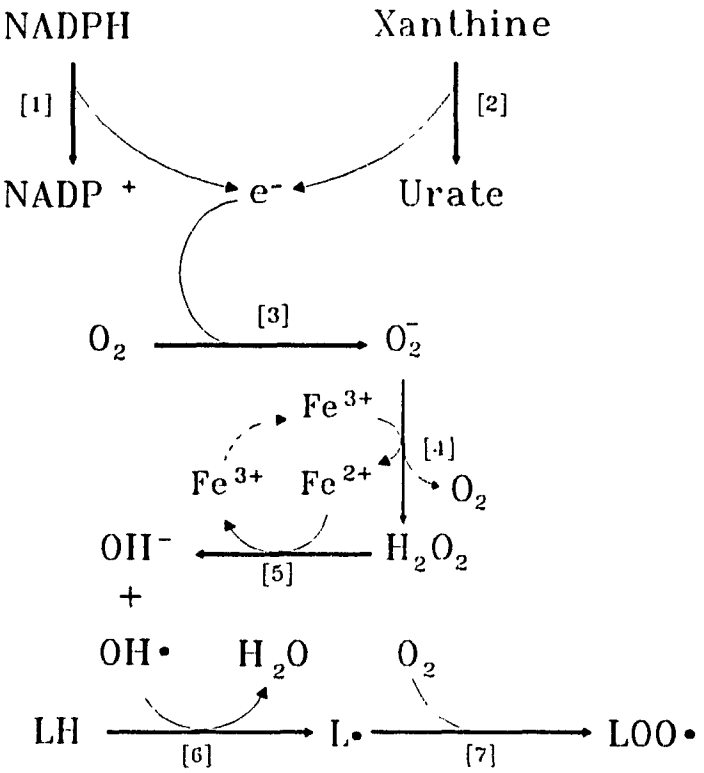


Fig. 8. Scheme for the initiation of lipid peroxidation in biological system via superoxide and hydroxyl radicals.

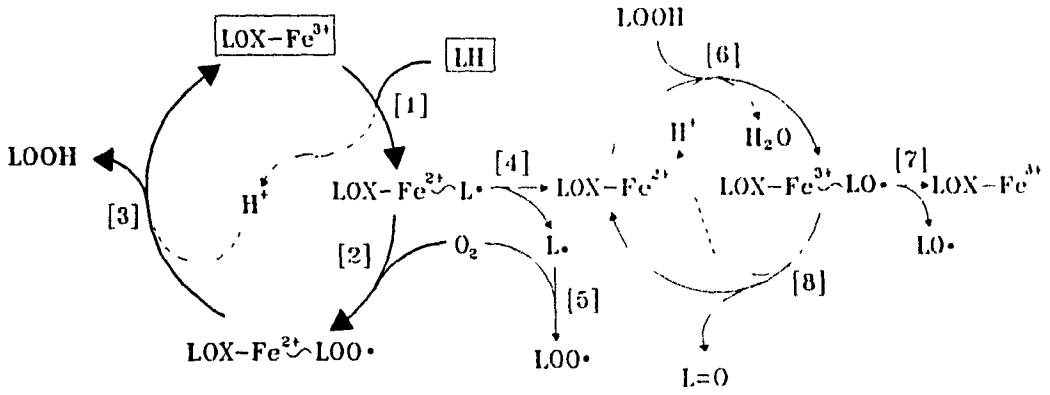


Fig. 9. Scheme showing the role of lipoxygenases (LOX) on lipid peroxidation. After forming an enzyme-substrate complex, LOX catalyses stereospecific dioxygenation of polyunsaturated fatty acids (LH) to yield conjugated hydroperoxy metabolites (Steps [1] and [2]). The enzyme can be recycled after releasing the final products (Step [3]). Under anaerobic condition, alkyl radical (L•) is dissociated from the enzyme (Step [4]) and the dioxygenation of this fatty acid radicals results in a racemic mixture of hydroperoxy fatty acids (Step [5]). The enzyme, in the Fe^{2+} form rather than the original Fe^{3+} form, then catalyses a reaction called lipohydroperoxidation in which the initial-formed the hydroperoxy fatty acids are converted to the corresponding alkoxyl radical (LO•, Step [6]). LO• is then dissociated from the enzyme, which is then free to bind another polyunsaturated fatty acid molecule (Step [7]).

conjugated 1-hydroperoxy-2,4-*trans,cis*-pentadiene product. The initial step in lipoxygenase catalysis is the *regiospecific* and *stereospecific* removal of a hydrogen atom from the substrate (step [1]), followed by rearrangement of double bond and the insertion of molecular oxygen (step [2]). A hydroperoxy fatty acids is formed by attracting the hydrogen ion released during the initial hydrogen abstraction step (step [3]).

Various lipoxygenases differ in three substantial features: (1) the site of hydrogen abstraction; (2) the direction of the double bond shift in the primary radical leading to the 1-hydroperoxy-2,4-*trans,cis*-pentadiene system and (3) the stereospecificity of both hydrogen abstraction and dioxygen insertion. For example, platelet lipoxygenase abstracts a pro *S* (L) hydrogen atom from the C-10 position of the arachidonic acid with subsequent antarafacial insertion of molecular oxygen in the C-12 position, generating 12(S)-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) [202,203]. On the other hand, both reticulocyte and polymorphonuclear leukocyte lipoxygenase abstract a pro *S* (L) hydrogen atom from the C-13 carbon of 20:4 to yield 15(S)-HPETE or from the 11-carbon of 18:2 to yield 13(S)-hydroperoxy 18:2 [204,205]. In both cases, however, the hydrogen is abstracted from the ω-8 carbon.

Most lipoxygenases are not very active in directly oxidizing cellular phospholipids. However, some lipoxygenases, such as the 15-lipoxygenases from soybean [206], human polymorphonuclear leukocytes [204] and reticulocytes [205,207,208], catalyse not only the oxygenation of free polyunsaturated fatty acids but also the oxygenation of fatty acids esterified to phospholipids. In contrast, no oxygenation of esterified arachidonic acid was observed with either rat basophilic leukaemia cell 5-lipoxygenase or rabbit platelet 12-lipoxygenase [204].

Lipoxygenases also catalyse another reaction called lipohydroperoxidation under circumstances where the oxygen supply is limited (**Fig. 9**). In this case, an alkyl radical (L·) disassociates from the enzyme and may be oxygenated non-enzymatically to form LOO· (step [5]). In sharp contrast to LOOH formed by the action of lipoxygenases, which is stereospecific, the LOO· formed by lipohydroperoxidation is *racemic* and the reaction is readily inhibited by antioxidants, such as butylated hydroxytoluene (BHT). Under the same conditions of low oxygen concentrations, lipoxygenases in the ferrous

form catalyse the homolytic scission of the O-O bond of LOOH formed in the previous reaction (step [6]). Either an alkoxyl radical (step [7]) or a keto fatty acid (step [8]) will be generated by lipohydroperoxygenation. Alkoxyl radicals will decompose to various products as discussed in the following section.

1.3.3. Catabolism of Lipid Hydroperoxides

During the propagation of lipid peroxidation, decomposition of lipid hydroperoxides becomes important. Lipid hydroperoxides are decomposed, especially in the presence of transition metal ions, to alkoxyl and peroxyl radicals:

$$LOOH + M^{n+1} \longrightarrow LOO\cdot + H^{+} + M^{n}$$

$$LOOH + M^{n} \longrightarrow LO\cdot + OH^{-} + M^{n+1}.$$

LOO· and LO· then undergo reactions such as intramolecular rearrangement, intermolecular addition, oxidation-reduction, cyclization, ß-scission, *etc.*, to generate varieties of products, including polymers (dimers, trimers and oligomers), hydroxy fatty acids, epoxy fatty acids, epoxy-hydroxy fatty acids, ketones and aldehydes (**Fig. 10**). Some of these metabolites have detrimental effects in cellular functions (see **Section 1.3.5**).

Besides being decomposed to products as discussed above, hydroperoxy fatty acids are also reduced to hydroxy fatty acids by cytosolic peroxidases [209,210], which are part of the body's defense system against lipid peroxidation (more in **Section 1.4.2.**).

1.3.4. Measurement of Lipid Peroxidation in Biological Systems

Peroxidation of fatty acids results in many different products ranging from high-molecular weight polymers to volatile products. Detailed analysis of the *complete spectrum* of lipid peroxidation products is not feasible. However, combinations of several methods is useful to study lipid peroxidation in biological systems [211,212] because different assays measure lipid peroxidation products at different stages of the reaction. Different assays also have different characteristics and can not be replaced by the others. The methods most frequently used in studies of lipid peroxidation are discussed below.

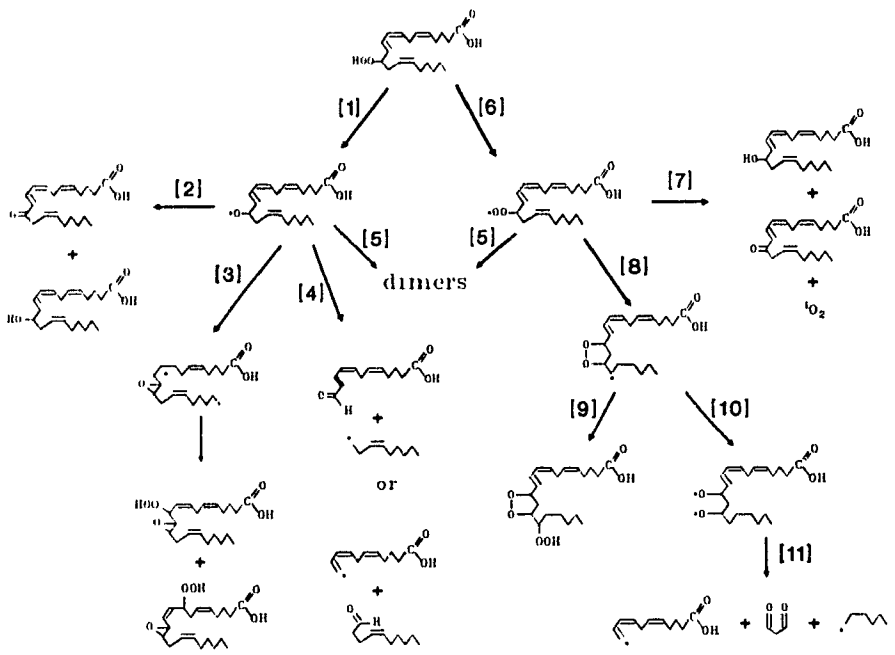


Fig. 10. Scheme for the degradation of 12-hydroperoxyeicosatetraenoic acid in the presence of transition metal ions. During lipid peroxidation, 12-hydroperoxyeicosatetraenoic acid can be converted either to an alkoxyl radical (Step [1]) or to a peroxyl radical (Step [6]). Both these radicals can undergo radical combination to form dimers and other polymers (Step [5]). ß-Scission of hydrogen from the alkoxyl radical formed by Step [1] results in the formation of the corresponding oxodiene and the reduction of the alkoxyl radical to a hydroxy product (Step [2]). Alkoxyl radicals tend to rearrange into epoxyallylic radicals and consequently form epoxyhydroperoxy metabolites (Step [3]). Alternatively, alkoxyl radicals undergo ß-scission, resulting in cleavage of a C-C bond adjacent to the alkoxyl group (Step [4]). By comparison, intramolecular rearrangement of peroxyl eicosatetraenoic acid leads to the formation of a cyclic peroxide (Step [8]) which is further metabolized either to an epidioxy-hydroperoxy product (Step [9]) or to a dialkoxyl radical (Step [10]). The latter radical then undergoes hydrolysis to form malondialdehyde (MDA) [Step 11]. Peroxyl radical combination followed by decomposition of tetraoxide (dimer of peroxyl radicals) through a cyclic intermediate (Russel mechanism) results in the formation of a molecule each of alcohol, ketone and singlet oxygen (Step [7]).

(a) Measurement of oxygen uptake

Initiation of lipid peroxidation is characterized by the abstraction of a hydrogen atom from unsaturated fatty acids followed by insertion of molecular oxygen. Measurement of oxygen consumption using an oxygen electrode in the incubation chamber can give us the rate of oxidation of lipids. This method however lacks specificity and will overestimate the reaction rate since oxygen uptake can also be the result of oxidation of other biological substances such as cholesterol and amino acids rather than fatty acids [213]. It can also result from secondary decomposition of lipid hydroperoxides.

(b) Measurement of fatty acids content

Lipid peroxidation leads to extensive conversion of unsaturated fatty acids to their oxygenated metabolites. Measuring the loss of fatty acids during lipid peroxidation can therefore provide such information as overall reaction rate and the specific fatty acids involved. Fatty acids with different carbon lengths and double bond positions can be easily separated and quantified using gas chromatography.

(c) Measurement of conjugated dienes

Spectrophotometric detection around 235 nm can be used to measure conjugated dienes which are formed during lipid peroxidation products. The molecular extinction coefficient is around 30,500 [214]. This method is widely used to quantify hydroperoxy as well as hydroxy lipids. However, the maximum absorption of a conjugated diene (around 235 nm) is in the region in which other components of the reaction mixture also show high end absorption. Some decomposition products of lipid hydroperoxides also possess the conjugated diene chromophores and therefore have the same spectrophotometric properties as lipid hydroperoxides themselves. To avoid these problems, spectrophotometric detection is usually coupled with chromatography, especially high performance liquid chromatography (HPLC) to measure specific compounds.

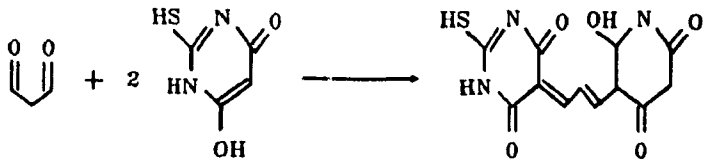
(d) Measurement of oxygenated products by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS)

Even though HPLC coupled to a UV detector provides a very useful tool to separate

and detect oxygenated products, problems may be encounted in biological samples because of various interfering substances as well as the presence of positional isomers of the oxygenated metabolites of polyunsaturated fatty acids. Coupling of the HPLC or GC column with a mass spectrometer provides a solution to this problem since the separation of various lipid peroxidation products by chromatography is followed by a highly selective mass spectrometric method. Each compound has characteristic fragment ions in mass spectrometry, especially when the functional groups of the lipid peroxidation products are properly derivatized and electron impact mass spectrometry is employed. This high selectivity gives mass spectrometry an advantage over other conventional methods. However, the operational cost and requirement for cumbersome preparation of samples limit the use of this method for routine analysis of lipid peroxidation products.

(c) Measurement of malondialdehyde (MDA)

MDA is one of the homolytic cleavage products formed from lipid hydroperoxides (**Fig. 10**, step [11]) which can be measured by the thiobarbituric acid (TBA) assay. This assay has been widely used by many investigators to study lipid peroxidation in tissues because of its simplicity. This test presumably depends on the formation of MDA and its reaction with thiobarbituric acid to give an intensely coloured product with a λ_{max} of 530 nm [215]:



The TBA assay has three major disadvantages, however. Firstly, MDA is a very minor peroxidation product (**Fig. 10**). Secondly, the TBA assay is rather non-specific, since many other substances such as carbonyl compounds, sucrose, pyrimidines, amino acids and peptides also generate chromophores under the assay conditions employed [216-218]. Therefore, the chromophores formed during the assay are usually referred to as "TBA reactive substances" or TBARS. This problem, however, can be overcome by quantification of the TBA conjugates by HPLC [219,220] or by gas chromatography [221]. Thirdly, MDA is generated only from fatty acids with three or more double

bonds, not from linoleic acid, the major polyunsaturated fatty acid in most tissues [222].

(f) Measurement of fluorescence

Aldehydes such as malondialdehyde react with side-chain amino groups of proteins, free amino acids or nucleic acids to produce products known as *Schiff bases*, which can be measured spectrofluorometrically after extraction from biological samples. Fluorescent products can also be formed by the polymerization of aldehydes.

Fluorescence is very sensitive and correlates well with other methods [31,223]. However, it has several disadvantages. Firstly, measurement at a single wavelength does not accurately represent all the Schiff bases or polymerization products since the exact wavelength for the maximal emission of each end product may not be the same. Secondly, only the end products of lipid peroxidation are measured by fluorescence, so the amounts of fluorescent materials will depend on the extent of decomposition of lipid hydroperoxides rather than the initial formation of these products. In normal cells where lipid hydroperoxides are rapidly reduced to lipid hydroxides, lipid peroxidation will be underestimated.

(g) Measurement of expired hydrocarbons in the breath

In the presence of transition metal ions, alkoxyl radicals undergo ß-scission reactions to generate both aldehydes and alkyl radicals (**Fig. 10**). The latter will react with a hydrogen donor to form alkanes which are volatile and therefore can be detected in exhaled air. Even though this is the only noninvasive method to measure lipid peroxidation in biological systems, it has certain drawbacks. Firstly, alkanes are minor metabolites of lipid hydroperoxides. Secondly, they are metabolized by liver and therefore their production may be affected by agents which affect liver function. Thirdly, measurement of expired hydrocarbons does not give any information about lipid peroxidation in specific organs. Finally, these hydrocarbons can also be formed by bacteria in the intestine, which is not relevant to *in vivo* lipid peroxidation.

1.3.5. Lipid Peroxidation and Human Diseases

Lipoxygenase-catalyzed lipid peroxidation have an important biological role in the conversion of reticulocyte to erythrocyte and other physiological processes. However,

most of the effects of lipid peroxidation products are detrimental due to their atherogenic and carcinogenic effects as well as their effects on the development of other diseases. The underlining mechanism involves the alteration of membrane structure, modification of proteins and nucleic acids.

The effects of lipid peroxidation products on membrane structure include decreased membrane fluidity, change of phase properties of membranes, inhibition of cellular metabolic processes, loss of secretory functions, breakdown of transmembrane ion gradients and increased membrane permeability [224]. Particularly, peroxidation of membrane phospholipids increases their hydrophilicity, favouring the aggregation of these molecules and may result in the formation of phospholipid polymers or lipid peroxide clusters [193,225]. This may lead to membrane fragmentation and increased membrane permeability (**Fig. 11**). Recent evidence showed that membrane fluidity in liposomes was increased when as little as 3.5% of the phosphatidylcholine contained one HETE chain [226]. This effect was more pronounced for 15-HETE than 5-HETE probably because the hydroxy group of 5-HETE was located closer to the polar surface of the liposome and therefore disturbed the usual packing of the hydrocarbon phase to a lesser extent.

The modification of proteins by lipid peroxidation products involves the formation of fluorescent chromophores, lipid-protein adducts, protein-protein cross-links, protein scission and amino acid damage [227], resulting in the loss of activities of receptors and enzymes. Of the lipid peroxidation products, unsaturated aldehydes are the most probable ones to be involved in the modification of proteins because of their high reactivity with thiols and amino groups (**Fig. 12**, A-C) [228,229]. 4-Hydroxyalkenals originating from the peroxidation of liver microsomal lipids were reported to inhibit calcium sequestration activity by binding to the microsomal proteins [230]. The same compounds were reported to modify LDL in such way that it was taken up and degraded by macrophages to a greater extent (see **Section 1.3.6.4** for more detail). Glucose-6-phosphatase, Ca^{2+}-ATPase and monoamine oxidase are a few of the many enzymes inhibited during lipid peroxidation due to the modification of proteins [231-234]. Hormone receptors such as ß-adrenergic and acetylcholine receptors are also inactivated during lipid peroxidation [235,236].

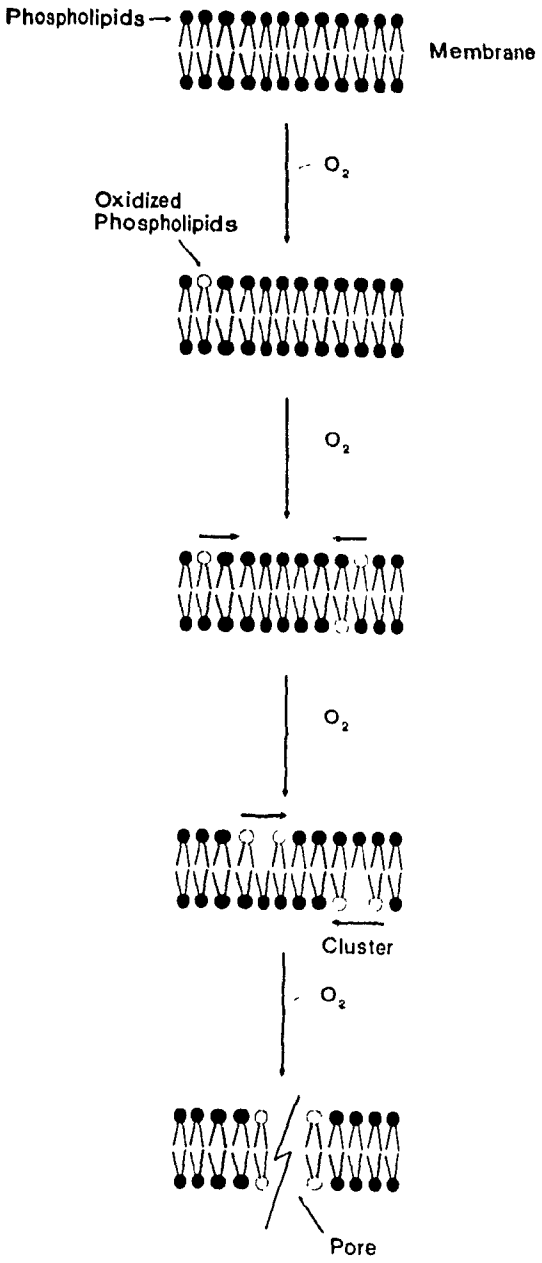


Fig. 11. Scheme showing the formation of lipid peroxide clusters and membrane fragmentation upon induction of lipid peroxidation.

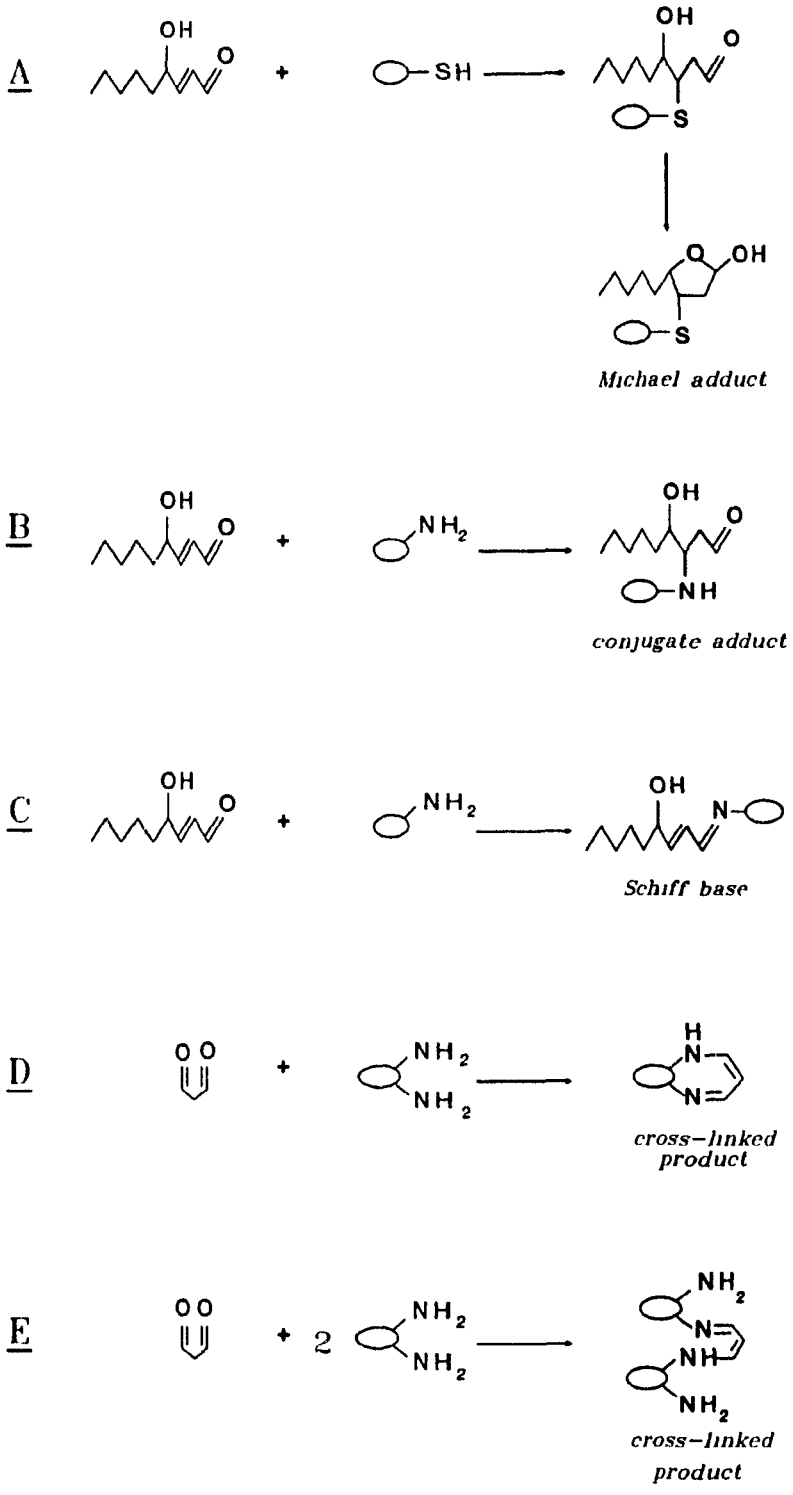


Fig. 12. Scheme showing the interactions between proteins and unsaturated aldehydes.

Dialdehydes such as malondialdehyde can also react with amino groups on proteins to form both intramolecular and intermolecular cross-links (**Fig. 12**, D-E) [237]. These high-molecular-weight protein polymers can be seen in malondialdehyde-treated erythrocytes or aging erythrocytes in the circulation [238]. Proteins can also be modified by keto metabolites of fatty acids via covalent binding [239].

1.3.6. Lipid Peroxidation and Atherosclerosis

Lipid peroxidation products can mediate the formation of atherosclerotic lesion by (1) injuring endothelial cells or causing membrane malfunctions; (2) attracting monocyte into the blood vessels; (3) inhibiting prostacyclin synthesis and activating platelets; (4) modifying LDL trapped inside the intima of the arteries; and (5) stimulating smooth muscle cell proliferation to form smooth muscle cell-derived foam cells.

1.3.6.1. Lipid peroxidation and endothelial cell injury

Because of its constant exposure to blood components and prooxidants, the endothelium is very susceptible to oxidative stress and free radical-mediated reactions. Free radicals can damage endothelial cells or alter their functions leading to the loss of selective permeability of the endothelium, and consequently, the increased entry of cholesterol-rich lipoproteins into the arterial wall. It was found that linoleic acid-derived hydroperoxides, but not linoleic acid itself, increased endothelial cell permeability to albumin [240] and LDL [241] in vitro. This was probably the result of rapid and irreversible cell injury caused by lipid hydroperoxides or their decomposition products since they caused a change of the shape of endothelial cells [242], a decrease in the electron density of the mitochondrial matrix, a dilation of the rough-surfaced endoplasmic reticulum, and cellular release of lactate dehydrogenase into the culture medium [240,243]. Peroxides and other oxygen radicals generated by lipoxygenase and cyclooxygenase [244], as well as oxidized LDL [245], have also been shown to damage endothelial cells. Furthermore, injecting linoleic acid-derived hydroperoxides into rabbits led to damage of aortic endothelial cells as well as to the adhesion and aggregation of platelets on the injured site, as shown by scanning electron microscopy [246,247]. The

morphological changes were correlated with increased level of lipid peroxides (TBARS) in serum and in aorta.

1.3.6.2. Lipid peroxidation and leukocyte activation

It has recently been reported that oxidized LDL stimulated monocyte-endothelium interactions [248,249], probably due to the induction of a monocyte-chemotactic protein in endothelium [250] and/or an adhesion protein in monocytes. Lysophosphatidylcholine [44,45] and 4-hydroxyalkenals [251], which are released during oxidative modification of LDL, also have chemoattractant effects. Oxidized LDL also stimulated the differentiation of monocytes to macrophages [249]. The attraction and subsequent activation of monocytes on the endothelium or in blood vessels results in an increased generation of oxygen free radicals such as superoxide, hydrogen peroxide and hydroxyl radical [252,253], which can propagate lipid peroxidation in blood vessels. Furthermore, activation of monocytes/macrophages is also associated with release of proteolytic enzymes such as elastase and collagenase [254] which are destructive to vascular tissues [255]. Obviously, activation of leukocytes due to lipid peroxidation products will augment the damage to endothelial cells and accelerate the development of atherosclerotic lesions.

1.3.6.3. Lipid peroxidation and prostacyclin production

Even though low concentrations of hydroperoxides are required to activate prostaglandin endoperoxide synthase, high concentrations of lipid hydroperoxides could diminish PGI_2 biosynthesis [256] at the level of either prostaglandin endoperoxide synthase [257], or prostacyclin synthase [258-260], or both [261]. Oxidized LDL is also reported to inhibit PGI_2 production [262]. The mechanism of the inhibition could be the conjugation of lipid peroxidation products to prostaglandin endoperoxide synthase, resulting in inactivation of this enzyme [90]. Inhibition of PGI_2 production may result in increased interaction between platelets and blood vessels, promoting atherosclerotic lesion formation.

1.3.6.4. Modification of LDL by lipid peroxidation products

Modified LDL has several physical, chemical and biological differences from native LDL, including increased electrophoretic mobility [54], increased buoyant density of the LDL particles [53,54], decreased fatty acid contents [263,264], decreased cholesterol/protein and phospholipid/protein ratios [54], fragmentation of apo B [265], increased uptake by macrophages *in vitro* [45,53] and toxicity to proliferating cells [54].

The following evidence indicates that modification of LDL is initiated by the peroxidation of polyunsaturated fatty acids in LDL lipids [45,56,263]:

(1) Incubation of LDL with copper or iron, which can initiate lipid peroxidation, resulted in its modification [45,55,266,267].

(2) Phospholipase A_2 plus soybean lipoxygenase [268,269] or lipoxygenase itself [270] mimic most of the changes induced by the incubation of LDL with endothelial cells. Lipoxygenases may also be required for the modification of LDL by endothelial cells and macrophages *in vitro* [271] and *in vivo* [272].

(3) Thiobarbituric acid reactive substances (TBARS) and various aldehydes, which are known to be formed as a result of lipid peroxidation, are formed during the modification of LDL [60,273,274]. The rate of uptake of modified LDL by macrophages is proportional to the amounts of the above products formed during its modification.

(4) TBARS and aldehydes [60,274-276] as well as hydroxyl radical [265] directly modify LDL *in vitro*.

(5) Antioxidants such as butylated hydroxytoluene (BHT) and vitamin E [45] as well as superoxide dismutase [277] inhibit the modification of LDL by cells and transition metal ions. Lipoxygenase inhibitors such as nordihydroguaiaretic acid (NDGA) and eicosatetraynoic acid (ETYA) [268] also have the similar effects.

Peroxidation of LDL lipids results in formation of oxygenated intermediates, probably unsaturated aldehydes, which react with lysine residues of apolipoprotein B-100 [61,278]. The derivatized lysine residues may be the epitopes recognized by macrophage scavenger receptors.

In addition to promoting the formation of foam cells, oxidatively modified LDL may

be involved in the atherogenic process due to its toxicity to endothelial and smooth muscle cells. Products released during the oxidative modification of LDL, such as lysophosphatidylcholine and 4-hydroxynonenal, have chemotactic effects on monocytes/macrophages, therefore augmenting the recruitment of monocytes into subendothelial spaces of the arteries and preventing macrophages from leaving the arteries.

1.3.6.5. Increased lipid peroxidation in atherosclerosis

Even though the link between lipid peroxidation and atherosclerosis has been suggested for about 40 years [279], convincing evidence did not emerge until the past 20 years. One of the earliest pieces of evidence was the identification of oxidized derivatives of cholesteryl linoleate in atherosclerotic plaques by GC-MS [280]. These substances were not observed in non-atherosclerotic human aorta. *In vitro*, exogenous arachidonic acid was selectively converted to 15-HETE by homogenates [281] or slices [282] of aortae from cholesterol-fed [281,282] or WHHL [282] rabbits at a rate higher than the controls, suggesting 15-lipoxygenase activity was higher in atherosclerotic lesions. Recent evidence has shown that aorta of WHHL rabbits had increased levels of mRNA for 15-lipoxygenase [272]. The role of lipid peroxidation in atherogenesis is further supported by studies in which probucol was administrated to Watanabe heritable hyperlipidemic rabbits [206,283,284]. Probucol appears to prevent the progression of atherosclerosis in these rabbits by inhibiting the oxidation of LDL, rather than by lowering the plasma cholesterol level. Another antioxidant, BHT, was also reported to suppress the development of atherosclerotic lesions in cholesterol-fed rabbits [285].

Besides the existence of lipid peroxidation products in atherosclerotic lesions, *in vivo* studies on the oxidative modification of LDL also support a role for lipid peroxidation in atherogenesis. LDL extracted from atherosclerotic lesions possesses similar properties to LDL modified *in vitro* [286,287]. Histostaining using a monoclonal antibody to 1-amino-3-iminopropene, a conjugated imine produced by intramolecular cross-linking of peptidyl lysines by MDA in a 2:1 molar ratio, indicated the existence of such modified proteins in aorta of WHHL rabbits [288]. Similar results in WHHL rabbits [289-291] and

humans [291] have been confirmed using immunohistochemical assays or Western blots of materials from atherosclerotic lesions. Recent evidence indicates that macrophage-derived foam cells within, or freshly isolated from, rabbit atherosclerotic lesions contain lipid-protein adducts derived from lipid peroxidation products which were identified using monoclonal antibodies against MDA or 4-hydroxynonenal conjugated to LDL [292]. This suggests that macrophages might take up and degrade oxidized LDL *in vivo* in the same way as *in vitro*.

1.4. PROTECTION AGAINST LIPID PEROXIDATION

Lipid peroxidation is a chain reaction involving hydrogen abstraction from polyunsaturated fatty acids by free radicals. *In vitro* lipid peroxidation can be prevented or suppressed by one of the following mechanisms: (1) scavenging initiating radicals such as $OH\cdot$, (2) scavenging intermediate radicals ($LO\cdot$, $LOO\cdot$, *etc.*) by chain-breaking antioxidants, or (3) destroying radicals by specific enzymes. Biological systems possess additional protective mechanisms due to the structure of biological membrane and the existence of binding proteins which limit the availability of transition metal ions and free radicals derived from fatty acids [293,294]. For example, phospholipids in biological membranes are more resistant to lipid peroxidation than their counterparts in organic solvents or in simple micelles [192]. Phospholipids in biological membrane are organized in such way that the fatty acid side-chains face the interior and the hydrophillic heads form the boundary with the aqueous environment. When a fatty acid is oxidized, the fatty acid side-chain containing hydroperoxy group tends to fold towards the surface of the bilayer due to its increased hydrophilicity, thus reducing the efficiency of initializing peroxidation of neighbouring fatty acids.

1.4.1. Antioxidants

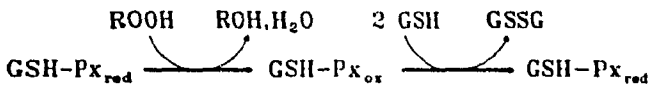
Antioxidants are better hydrogen donors than polyunsaturated fatty acids so that free radicals will preferentially abstract hydrogen from antioxidants. Unlike polyunsaturated fatty acids, however, antioxidant radicals are not reactive enough to abstract hydrogen atoms from another molecule, thus breaking the chain reaction of lipid peroxidation.

Antioxidants acting by this mechanism are usually called *chain-breaking antioxidants.*

Based on their solubility, antioxidants are classified into two categories, water-soluble antioxidants and lipid-soluble antioxidants. In biological systems, the most important ones in these two categories are ascorbic acid (vitamin C) and α-tocopherol (vitamin E), respectively. Ascorbic acid, located primarily in plasma and the cytosol, reacts readily with superoxide and peroxyl and hydroxyl radicals, protecting lipids from free-radical attack (**Fig. 13**, step [3]). Lipid peroxidation does not occur until the ascorbic acid is completely consumed. Other antioxidants, such as urate, bilirubin and protein thiol groups can only partially trap aqueous peroxyl radicals. α-Tocopherol, localized in membranes due to its hydrophobicity, plays an important role in the resistance of biological membranes to lipid peroxidation. α-Tocopherol is also concentrated in LDL and plays an important role in preventing LDL from oxidative modification. However, like other chain-breaking antioxidants, α-tocopherol will be oxidized to its radical form (**Fig. 13**, step [1]) which undergoes decomposition unless it can be reduced back to α-tocopherol. One of these reducing compounds *in vivo* is ascorbic acid (**Fig. 13**, step [2]). Ascorbic acid prolonged the lag-phase of lipid peroxidation of human LDL but did not affect the rate of peroxidation during the propagation phase [295]. The prolonged lag-phase is due to the delayed consumption of α-tocopherol in LDL. Thus, ascorbic acid and α-tocopherol act synergistically due to the regeneration of α-tocopherol by ascorbic acid, resulting in a delay in the onset of lipid peroxidation even though the radicals are initially generated within the LDL [296].

1.4.2. Enzymatic Defence System

The action of α-tocopherol is to convert the initially-formed lipid peroxyl radicals (LOO·) to lipid hydroperoxides (LOOH). In the presence of transition metal ions, however, LOOH can be decomposed to both alkoxyl (LO·) and peroxyl radicals (LOO·) as discussed in **Section 1.3.3** on P. 32. Fortunately, semistable LOOH can be reduced to less reactive hydroxy lipids (LOH) by a group of enzymes called glutathione peroxidases (GSH-Px). GSH-Px catalyze the reaction of hydroperoxy fatty acids with reduced glutathione to form oxidized glutathione disulphide and hydroxy fatty acids:



In this way, GSH-Px prevent the formation of LO· or LOO· from LOOH and therefore retard the propagation of lipid peroxidation. This enzyme is specific for its hydrogen donor, GSH and nonspecific for the hydroperoxides. The lack of substrate specificity extends the range of substrates from H_2O_2 to a variety of hydroperoxides derived from fatty acids, nucleotides, and steroids.

There are at least three types of glutathione peroxidases: selenium-dependent glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase (PH-GSH-Px) and selenium-independent glutathione peroxidase. The major form of these enzymes is selenium-dependent and is important in endothelial cells. PH-GSH-Px is a membrane-bound enzyme that can reduce phospholipid hydroperoxides as well as other hydroperoxides in membranes [297,298] (**Fig. 13**, step [4]). If hydroperoxy fatty acids are released from membranes by the action of phospholipase A_2, or if fatty acids are oxidized in the cytosol, they can be reduced by the other two forms of GSH-Px (**Fig. 13**, step [5]). Therefore, the cooperative effects of these GSH-Px are sufficient to protect cells against free radical damage.

The actions of α-tocopherol and GSH-Px are not just sequential. GSH-Px decrease the amount of α-tocopherol required to inhibit lipid peroxidation. On the other hand, the inhibition of microsomal lipid peroxidation by PH-GSH-Px requires vitamin E since the protective effect of PH-GSH-Px is dramatically reduced after α-tocopherol has been depleted [299]. The actual mechanism is still unknown, however.

Superoxide dismutase (SOD), located in the cytosolic fraction of the cell, can dismute superoxide (O_2^-) to hydrogen peroxide (H_2O_2) (**Fig. 13**, step [6]), which in turn is reduced to H_2O by GSH-Px or catalase (**Fig. 13**, steps [7] and [8]). Without the coupling to GSH-Px or catalase, the dismutation of superoxide to H_2O_2 would be harmful because the combination of superoxide and H_2O_2 could give rise to the more reactive hydroxy radical. GSH-Px and catalase are complementary in their locations. GSH-Px (except PH-GSH-Px) is located primarily in the cytosol and mitochondria but not in

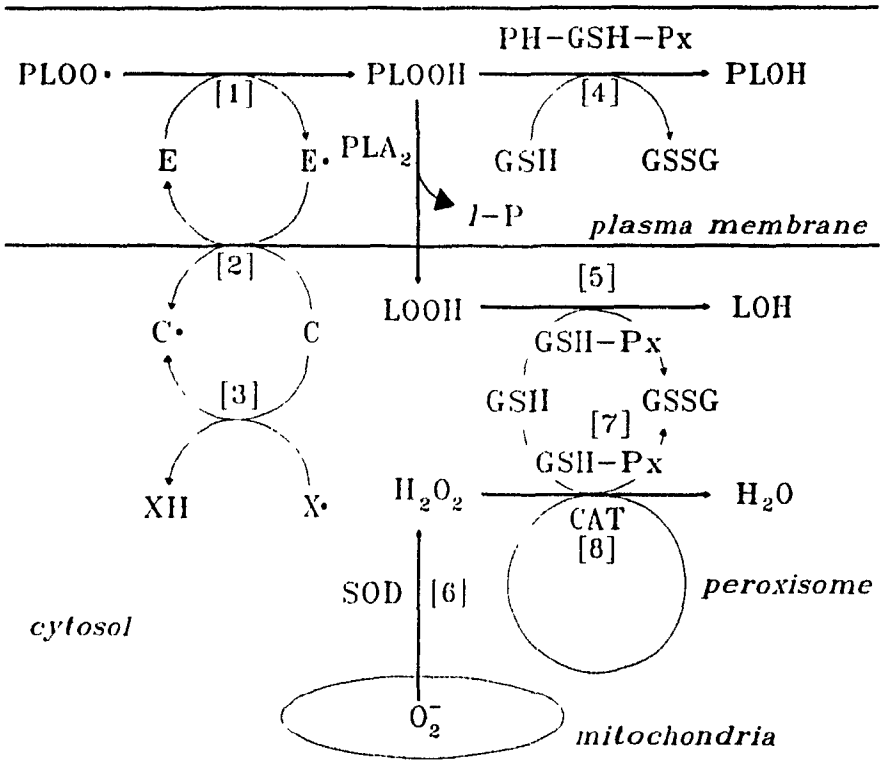


Fig. 13. Scheme showing the interactions of components of the free radical defence system. The abbreviations are as follows: **PLOO•**: phospholipid peroxy radicals; **l-P**: lysophospholipids; **LOOH**: lipid hydroperoxides; **E**: α-tocopherol (vitamin E); **E•**: α-tocopherol radical; **C**: ascorbate (vitamin C); **C•**: ascorbate radical; **X•**: water soluble free radicals; **GSH**: reduced glutathione; **GSSG**: oxidized glutathione, **GSH-Px**: glutathione peroxidase; **PH-GSH-Px**: phospholipid-hydroperoxide glutathione peroxidase; **SOD**: superoxide dismutase; **CAT**: catalase.

peroxisomes [300]. In contrast, catalase is primarily localized in peroxisomes. Mitochondria and endoplasmic reticulum contain little, if any, catalase activity [301].

1.5. STUDIES IN OUR LABORATORY

Previous investigations on the metabolism of polyunsaturated fatty acids in blood vessels from normal animals have laid the foundation for the current research [302-304]. The effects of selenium-deficient diets on the formation of oxygenation products of polyunsaturated fatty acids in aorta from rats and rabbits [305] also aided in the interpretation of the results obtained from my studies on hyperlipidemic rabbits.

Exogenous arachidonic acid is mainly converted to PGI_2 along with small amounts of monohydroxy products by particulate fractions and homogenates of fetal calf aorta [302]. In comparison, linoleic acid was primarily converted to 9- and 13-hydroxy metabolites [303]. Preincubation of particulate fractions with indomethacin or acetylsalicylic acid inhibited the formation of 6-oxo$PGF_{1\alpha}$ and monohydroxy derivatives of linoleic acids. On the other hand, nordihydroguaiaretic acid had no effect on the formation of these oxygenation products except at very high concentrations. This suggested that the formation of monohydroxy derivatives of linoleic acid in this study was catalzed by prostaglandin endoperoxide synthase even though linoleic acid was a much poorer substrate for this enzyme than arachidonic acid. The same study also showed that substantial amounts of monohydroxy derivatives of linoleic acid were formed from endogenous substrate during incubation of slices of fetal calf aorta. A method using GC-MS was then developed to quantify the major oxygenated metabolites of 18:2 and 20:4 formed by blood vessels [304]. In rabbit aorta, the major oxygenated products derived from endogenous 18:2 were its 9- and 13-hydroxy derivatives. On the other hand, the amounts of monohydroxy derivatives of 20:4 in aorta were much smaller than the amount of 6-oxo$PGF_{1\alpha}$ released from this tissue. The distribution of these metabolites were different in cellular lipids. The amounts of monohydroxy derivatives of 18:2 in esterified lipids were about 8 to 10 folds times greater than the amounts released into the incubation medium. In comparison, the difference between the amounts of esterified and free forms of monohydroxy derivatives of 20:4 was not as great as the difference found

for monohydroxy derivatives of linoleic acid, even though they were found predominantly in esterified lipids. Interestingly, the percentage of esterified 20:4 in its monohydroxy form was much less than the percentage of esterified 18:2 which is oxygenated. This phenomenon was observed in all species of animals studied as well as at different ages of the same species. It was hypothesized that monohydroxy derivatives of different fatty acids may be located in different lipid fractions in aorta. Efforts have been made to investigate the origin of the esterified hydroxy fatty acids in blood vessels in which vascular endothelial and smooth muscle cells were incubated with radioactively labelled linoleic acid or its 13-hydroxy metabolite. Although 18:2 itself was well incorporated into both the neutral and polar lipid fractions from endothelial and smooth muscle cells, very little 13h-18:2 was incorporated into either of these fractions in endothelial cells, whereas the amount of the latter substance incorporated into polar lipids in smooth muscle cells was only 11 % of that of 18:2. This suggested that the hydroxy derivatives of linoleic acid derived from the direct oxygenation of esterified linoleic acid in the tissues rather than the incorporation of oxygenated products into lipids.

The effects of lipid peroxidation products on prostaglandin synthesis were also investigated. 13-Hp-18:2 was found to inhibit both prostaglandin endoperoxide synthase and PGI_2 synthase in particulate fractions from aorta [261]. Formation of monohydroxy derivatives of 20:4 was not affected. To investigate this interaction *in vivo*, oxygenation products derived from both 18:2 and 20:4 were measured in aorta from rabbits or rats fed either control or selenium-deficient diets in order to deplete the selenium-dependent form of glutathione peroxidases [305]. This could lead to increased amounts of hydroperoxy fatty acids in aorta, which in turn could affect PGI_2 synthesis. Glutathione peroxidase activity in rat aorta was reduced to 17 % of control levels after 4 weeks and to undetectable levels after 6 weeks. 6-Oxo$PGF_{1\alpha}$ production by slices of aorta from rats with selenium-deficient diets was identical to that from control rats after 4 weeks, but it was decreased by about 20 % after 6 weeks on the diet. The amounts of monohydroxy derivatives of linoleic acid esterified to lipids were higher in aorta from selenium-deficient rats after 6 weeks, but the differences were statistically significant only in the case of 9h-19:2. Glutathione peroxidase activity in rabbit aorta was reduced by less than

50% as a result of administration of a selenium-deficient diet for 7 weeks. Consequently, no significant changes were observed in the amounts of 6-oxo$PGF_{1\alpha}$ or other metabolites of 20:4 and 18:2 released into the medium by slices of aorta from control and selenium-deficient rabbits. Neither were there significant changes in the amounts of esterified hydroxy products derived from linoleic and arachidonic acids in rabbit aorta.

1.6. BASIS FOR THE CURRENT STUDY

Oxidative modification of LDL appears to be atherogenic, since it leads to the uptake of modified LDL by macrophages, forming macrophage-derived foam cells. In most studies, the peroxidation of LDL had been evaluated by measuring TBARS, aldehydes and conjugated dienes. However, these are not entirely satisfactory procedures because they give little information concerning the nature of the fatty acids being oxidized. Therefore, we used GC-MS to measure monohydroxy derivatives, early peroxidation products, of the LDL fatty acids as an alternative and much more specific approach.

The role of lipid peroxidation in atherogenesis has been hypothesized to be due to injury of the blood vessels and inhibition of PGI_2 biosynthesis in this tissue. However, the specific oxidation of individual fatty acids in LDL and aorta had not yet been comprehensively studied. Therefore, we studied the formation of monohydroxy derivatives of oleic, linoleic and arachidonic acids in both LDL and aorta, using cholesterol-fed rabbits as an experimental model for atherosclerosis. The time course for the effects of cholesterol-feeding on the formation of these derivatives was also studied.

Following the investigation of the formation monohydroxy fatty acids in atherosclerotic rabbit aorta, we hypothesized that fatty acids in different lipid fractions would undergo different degrees of peroxidation during atherogenesis. If the lipid peroxidation occurred in membranes, the effect would be more detrimental. Therefore, we decided to study the distribution of monohydroxy fatty acids in polar lipid (primarily phospholipids) and neutral lipid (mainly cholesteryl esters and triglycerides) fractions in atherosclerotic rabbit aorta.

The increased lipid peroxidation in atherosclerotic aorta has been suggested to be responsible for the decreased PGI_2 production in this tissue. However, there is

considerable controversy in the literatures with some reports of decreased PGI_2 synthesis and others of increased PGI_2 synthesis in atherosclerosis. We hypothesized that one reason for these discrepancies could be due to the manipulation of the sample during the experiment because prostaglandin endoperoxide synthase could be autoinactivated due to the large amounts of substrate released during tissue preparation. We therefore tested the effects of adding ibuprofen, a reversible prostaglandin endoperoxide synthase inhibitor, during tissue preparation on the subsequent production of PGI_2. To obtain as much information as possible concerning the production of prostanoids in atherosclerosis, we simultaneously measured 6-oxo$PGF_{1\alpha}$ (for PGI_2), TXB_2 (for TXA_2) and PGE_2 released from slices and synthesized by the luminal surface of aorta, as well as the urinary metabolites of PGI_2 (measured as 2,3-dinor-6-oxo$PGF_{1\alpha}$) and TXA_2 (measured as 2,3-dinor-TXB_2).

Hydroperoxy derivatives of linoleic and arachidonic acids were reported to inhibit both prostaglandin endoperoxide synthase and prostacyclin synthase. But the effects of hydroperoxy fatty acids esterified to lipids on prostacyclin biosynthesis had not been studied. Measurement of monohydroxy derivatives of fatty acids in aorta indicated that these products were primarily located in the esterified lipid fraction. Therefore, we investigated the effects of hydroperoxy fatty acids esterified to phospholipids, cholesterol and triglycerides on prostaglandin endoperoxide synthase and prostacyclin synthase activities.

Table 2

Chemicals and Reagents

NAME	COMPANY OR INSTITUTION
Chemicals	
N-methyl-*N*-nitroso-*p*-toluene-sulfonamide (Diazald)	Aldrich Chemical Company
Methyl formate	
Platinum (IV) dioxide	
Propyl gallate	
Rhodium on alumina	
Sodium methoxide	
MSTFA	Pierce
EDTA, disodium salt	Fisher Scientific Company
norganic salts	
Bovine serum albumin	Sigma Chemical Company
Butylated hydroxyanisole	
Ibuprofen	
Papaverine	
Organic Solvents	
Organic solvents (HPLC grade)	Fisher Scientific Company
Lipids & Fatty Acids	
5ß-Cholestane-3α-ol	Aldrich Chemical Company
Cholesterol	
Eicosanoic acid (20:0)	NuChek Prep. Inc.
Arachidonic acid (20:4)	
10,13-Nonodecadienoic acid (19:2)	
Linoleic acid (18:2)	
Oleic acid (18:1)	
PGs & Their Antibodies	
6-Oxo$PGF_{1\alpha}$	Dr. J.E.Pike, Upjohn Company
PGE_2	
TXB_2	
6-oxo[5,8,9,11,12,14,15-^{3}H]$PGF_{1\alpha}$	New England Nuclear
[5,6,8,11,12,14,15-^{3}H]PGE_2	
[5,6,8,9,11,12,14,15-^{3}H]TXB_2	
Anti-6-oxo$PGF_{1\alpha}$	Dr. L.Levine, Bradeis Univ.
Anti-PGE_2	Advanced Magnetics
Anti-TXB_2	Dr. F.Fitzpatrick
[9α-^{3}H]$PGF_{1\beta}$	Our Lab

MATERIALS AND METHODS

2.1. CHEMICALS AND SOLVENTS

The main chemicals and reagents used in these experiments are listed in **Table 2.**

2.2. PREPARATION OF STANDARDS

2.2.1. 14-Hydroxy Nonadecadienoic Acid (Internal Standard for GC-MS)

14-Hydroxy nonadecadienoic acid (14h-19:2), the internal standard used for the quantification of monohydroxy fatty acids, was prepared by incubating 10,13-nonadecadienoic acid (19:2) with soybean lipoxygenase [306]. In detail, 19:2 (5 mg) was dried down on one edge of the bottom of 60 ml beaker. The residue was then dissolved in 150 μl of 0.019 M ammonium hydroxide. Soybean lipoxygenase solution (10 ml; 220,000 units/30 ml 0.1 M sodium borate buffer, pH 9.0) was added to initiate the reaction which was carried out in an ice-water bath. Addition of enzyme (10 ml) was repeated after 15 and 30 min. The reaction was stopped at 45 min by adding 5.3 ml of ethanol and the products were extracted using Sep-Pak cartridges containing ODS-silica [307]. Briefly, the mixture was acidified with concentrated HCl and then loaded onto the ODS silica Sep-Pak which had been pre-washed with methanol and H_2O. The ODS-silica Sep-Pak was washed with 15% methanol (20 ml), H_2O (20 ml) and petroleum ether (10 ml) followed by elution of 14hp-19:2 with methyl formate (10 ml). The hydroperoxy metabolite was reduced with sodium borohydride (about 1 mg) in methanol (0.3 ml) for 20 min at 0 °C followed by 20 min at room temperature. Sodium borohydride (about 1 mg) was added to the solution as long as the previously added sodium borohydride had been completely dissolved and no bubbles could be seen. The reduction was arrested by adding 1.2 ml of methanol and 8.5 ml of H_2O. The sample was acidified and extracted using an ODS-silica Sep-Pak cartridge as described above.

The hydroxylated 19:2 was first purified by an open column procedure. Briefly, having been dried down in a test tube under nitrogen, the hydroxylated 19:2 was taken up in 10 μl of isopropanol, 50 μl of ethyl acetate, 3 ml of hexane and 3 μl of acetic acid and then loaded onto a glass column containing 0.5 g of silicic acid (Biosil HA, BioRad

Laboratories, Richmond, CA). The column was washed with 5 ml of ethyl acetate:hexane:acetic acid (6:94:0.1) and the hydroxylated 19:2 was then eluted with 5 ml of ethyl acetate:hexane:acetic acid (20:80:0.1). The hydroxylated 19:2 was further purified by normal phase-high pressure liquid chromatography (NP-HPLC) using a mobile phase consisting of isopropanol:hexane:acetic acid (1:99:0.1). 14-Hydroxy 19:2 was quantified by UV spectrophotometry at 234 nm using a value of 30,500 for the extinction coefficient.

2.2.2. Monohydroxy Derivatives of Oleic Acid

Hydroxy derivatives of octadecenoic acid (h-18:1) were prepared by autoxidation of 9-octadecenoic acid (oleic acid) by a procedure similar to that described in the literature for the preparation of hydroxy derivatives of arachidonic acid [308]. Oleic acid (10 mg) was dissolved in methanol (40 ml), followed by the addition of 10 ml of 0.2 M Tris buffer containing $CuSO_4$ (0.5 mM). The reaction mixture was stirred for 5 hours at room temperature in the presence of hydrogen peroxide (0.36 mmol, 400 μl of a 30% solution) which was added at 0, 1 and 3 hour. The reaction mixture was then diluted with H_2O (100 ml) and extracted on an ODS silica Sep-Pak as described above. The hydroperoxy products were reduced with sodium borohydride and purified using the open column procedure as described in the previous section. This was followed by NP-HPLC using a mobile phase consisting of isopropanol:hexane:acetic acid (0.9:99:0.1) (**Fig. 14**). Fractions containing either 10h-18:1 or 11h-18:1 (underlined with separated solid bars) were further purified by reversed phase-high pressure liquid chromatography (RP-HPLC) using a gradient between H_2O:acetonitrile:acetic acid (60:40:0.02) and H_2O:acetonitrile: acetic acid (35:65:0.02) over 30 min followed by H_2O:acetonitrile:acetic acid (35:65:0.02). The two derivatives were well separated using these conditions (**Fig. 14**, insert). The identities of all monohydroxy derivatives of oleic acid were confirmed by gas chromatography-mass spectrometry (GC-MS) using linear scanning (**Fig. 15**) and quantified by gas chromatography (GC) using stearic acid (18:0) as an internal standard.

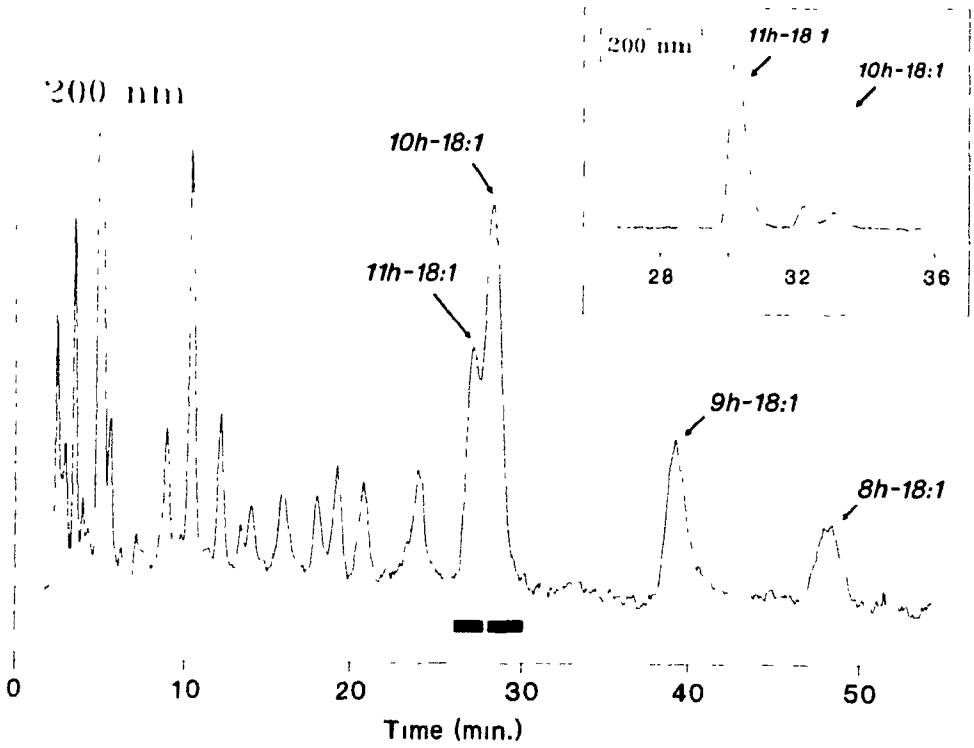


Fig. 14. High-pressure liquid chromatograms of monohydroxy C_{18} fatty acids formed by autoxidation. Oleic acid (10 mg; 99% purity) from *Nu Chek* was autoxidized in the presence of 100 μM $CuSO_4$ in methanol:0.2 M Tris buffer (4:1; 50 ml) for 5 hours at room temperature. Hydrogen peroxide (0.35 mmol; 400 μl) was added at 0, 1 and 3 hour. The medium was then diluted with H_2O (100 ml), extracted on an ODS-silica Sep-Pak cartridge and the products were separated by open column chromatography. Monohydroxy derivatives of C_{18} fatty acids were purified by NP-HPLC with a mobile phase of isopropanol:hexane:acetic acid (0.9:99:0.1; 2 ml/min). Fractions containing either 10h-18:1 or 11h-18:1 (underlined fractions) were further purified by RP-HPLC (insert) using a linear gradient between H_2O:acetonitrile:acetic acid (60:40:0.02) and H_2O:acetonitrile:acetic acid (35:65:0.02). Fractions containing 8h-18:1 were also collected.

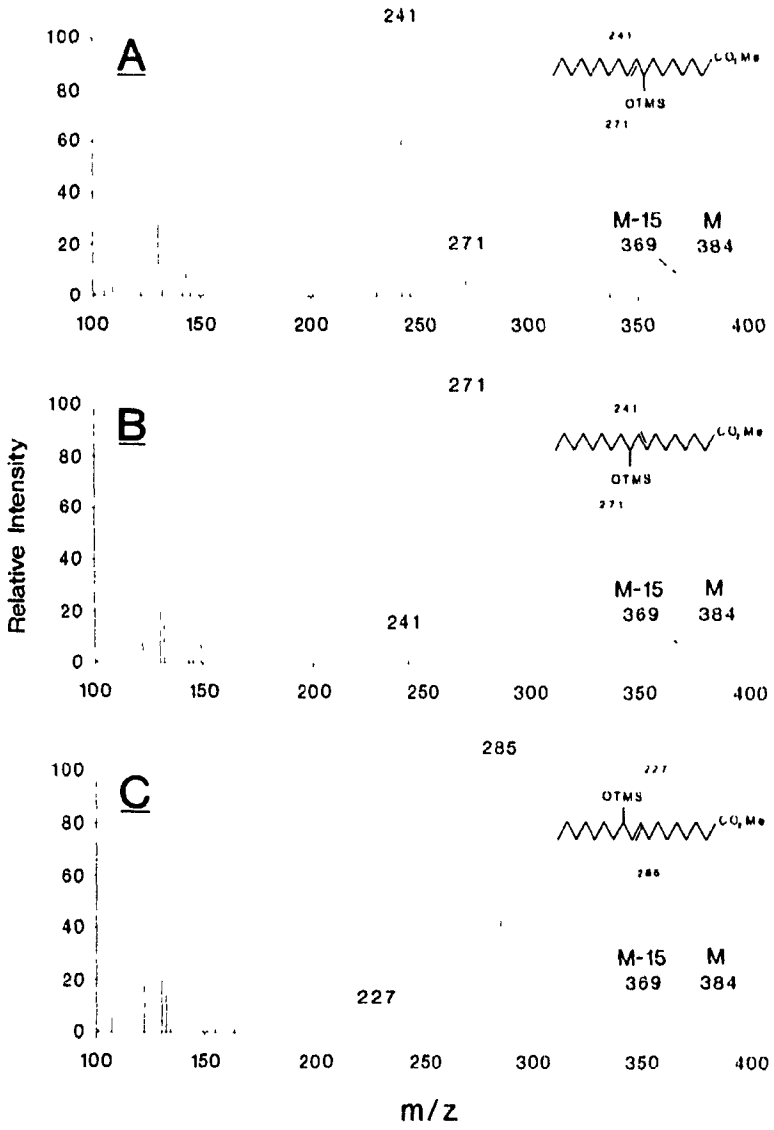


Fig. 15. Mass spectra of the trimethylsilyl ether derivatives of the methyl esters of hydroxy derivatives of oleic acid. The 8, 10 and 11-hydroxy derivatives of oleic acid (A, B and C, respectively) were methylated using diazomethane, and converted to their trimethyl ether by MSTFA before GC-MS analysis. The products were analyzed by mass spectrometry using electron impact ionization (EI) and linear scanning.

2.2.3. Monohydroxy Derivatives of Linoleic Acid

9-Hydroxy-10,12-octadecadienoic acid (9h-18:2) was prepared by incubating linoleic acid (18:2) with tomato lipoxygenase [309]. In detail, a firm, red tomato was peeled and, after the seeds were removed, the flesh was diced and washed in water at 0 °C. The slices (10 g) were homogenized in 35 ml of 0.1 M sodium acetate buffer, pH 5.5. 18:2 (5 mg) was dissolved in 0.019 M ammonium hydroxide (0.25 ml) and added to the homogenate and the mixture was shaken for 20 minutes at room temperature. Oxygen was bubbled through the mixture for 15 seconds every two minutes. Ethanol (1 volume) was added to stop the reaction and the products were then extracted using an ODS-silica Sep-Pak as described above. Hydroperoxides were reduced with sodium borohydride and 9h-18:2 was purified by NP-HPLC using a mobile phase of hexane:isopropanol:acetic acid (98.75:1.25:0.1).

13-Hydroxy-9,11-octadecadienoic acid (13h-18:2) was prepared using soybean lipoxygenase as described in **Section 2.2.1.**.

2.2.4. Monohydroxy Derivatives of Arachidonic Acid

5-Hydroxy-6,8,11,14-eicosatetraenoic acid (5h-20:4) and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12h-20:4) were prepared by incubating 20:4 and A23187 with porcine leukocytes in the presence or absence, respectively, of 5,8,11,14-eicosatetraynoic acid (ETYA) [310]. The monohydroxy products were purified by RP-HPLC as described [310].

8-Hydroxy-5,9,11,14-eicosatetraenoic acid (8h-20:4), 9-hydroxy-5,7,11,14-eicosatetraenoic acid (9h-20:4) and 11-hydroxy-5,8,12,14-eicosatetraenoic acid (11h-20:4) were prepared by autoxidation of 20:4 in the presence of $CuSO_4$ and hydrogen peroxide [308] and then purified and quantified as described in Section **2.2.1.**.

15-Hydroxy-5,8,11,13-eicosatetraenoic acid (15h-20:4) was prepared using soybean lipoxygenase in the same way as described for 14h-19:2.

2.3. LIPID PEROXIDATION OF HUMAN LOW DENSITY LIPOPROTEIN

2.3.1. Preparation of Low Density Lipoprotein

Human low density lipoprotein (LDL) was prepared according the method of Havel [311]. In detail, 100 ml to 200 ml of blood were withdrawn from male volunteers who fasted overnight. Plasma was prepared by centrifugation at 200 x g for 20 min at 4 °C. Propyl gallate was added to the plasma at a final concentration of 50 μM to protect LDL from autoxidation. Plasma was mixed well with a solution of potassium bromide (KBr)/sodium chloride (NaCl) (d=1.071) in a ratio of 4:1 to give a final density of 1.019. The final volume was then brought up to 9 ml with a solution of KBr/NaCl (d=1.019). The first ultracentrifugation was carried out at 125,000 x g for 18 hours. The infranant (middle layer) was withdrawn with a syringe and mixed well a solution of KBr/NaCl (d=1.239) in a ratio of 4:1 to give to a final density of 1.063. The final volume was then brought up to 9 ml with a solution of KBr/NaCl (d=1.063). Propyl gallate was also added at this stage to maintain a concentration of at least 50 μM. After ultracentrifugation at 105,000 x g for 20 hours, LDL was retrieved from the top layer. LDL was then dialysed against 30 volumes of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 9.5 mM NaH_2PO_4; pH 7.4) containing 0.01% EDTA in the dark for 20 hours with two buffer changes at 3 and 10 hours. The buffer had been degassed and bubbled with argon for at least 30 minutes before use in order to minimize lipid peroxidation during dialysis. LDL was passed through a filter with pore sizes of 0.2 μm before use.

The protein concentration of the LDL fraction was measured using a modified Lowry protein assay [312]. Briefly, 100 μl of LDL were diluted in 900 μl of saline and then mixed with 3 ml of reagent C, which was freshly prepared by mixing 100 parts of reagent A (283 mM sodium carbonate, 52 mM sodium dodecyl sulphate, 10 mM sodium tartrate and 15 mM sodium hydroxide) and 1 part of reagent B (240 Mm copper sulphate). The mixture was allowed to stand at room temperature for 10 minutes. Phenol solution (Folin Ciocalteu reagent; 1 N) was added to the LDL/reagent A mixture *while the latter solution was being vortexed vigorously.* The mixture was then allowed to sit at room temperature for 45 minutes and then the protein concentration was measured

using a Hitachi model 2000 spectrophotometer with a wavelength at 660 nm.

2.3.2. Oxidation of Human LDL

Human LDL was oxidized either in the presence of endothelial cells or copper ions.

Endothelial cells from human umbilical vein along with endothelium growth medium were purchased from Clonetics (San Diego, CA.). Endothelial growth medium (EGM-UV) was sterile-filtered liquid culture medium formulation based on MCDB 131 medium supplemented with epidermal growth factor (10 ng/ml), bovine brain extract containing heparin (2 ml aliquot), hydrocortisone (1 μg/ml), fetal bovine serum (2%), gentamicin (50 μg/ml) and amphotericin (0.5 μg/ml). On the second day after receiving the proliferating cells, endothelial cells were subcultured onto Falcon culture dishes (32 cm^2 area) at a density of 5000 cells/cm^2. The cells were then grown in EGM-UV until about 70% confluence as suggested in the literature [45]. Before the experiment, endothelial cells were washed twice with serum-free F10 medium and incubated with human LDL (2 ml; 200 μg/ml). Control incubations were carried out in the absence of endothelial cells. After incubation for 1, 5 and 20 hours, LDL was withdrawn and the endothelial cells were washed with 1 ml of PBS which was combined with the LDL previously withdrawn for analysis.

To investigate the effects of copper ions on the formation of monohydroxy fatty acid in LDL, human LDL (2 ml; 200 μg/ml) were incubated in Ham's F10 medium or in Ham's F10 medium supplemented with $CuSO_4$ at a final concentration of 20 μM. After 1, 5 and 20 hours, LDL was drawn and the test tube was washed with 1 ml of PBS which was then combined with the LDL previously withdrawn for analysis as shown in **Fig. 16**.

2.3.3. Measurement of Conjugated Dienes and Thiobarbicuric Acid-Reactive Substances

Aliquots (1 ml) were taken from the LDL solutions and mixed with 0.5 ml of PBS and the ultraviolet spectra were recorded between 200 and 300 nm using a Hitachi model 2000 spectrophotometer. The absorbance at 234 nm was selected for the

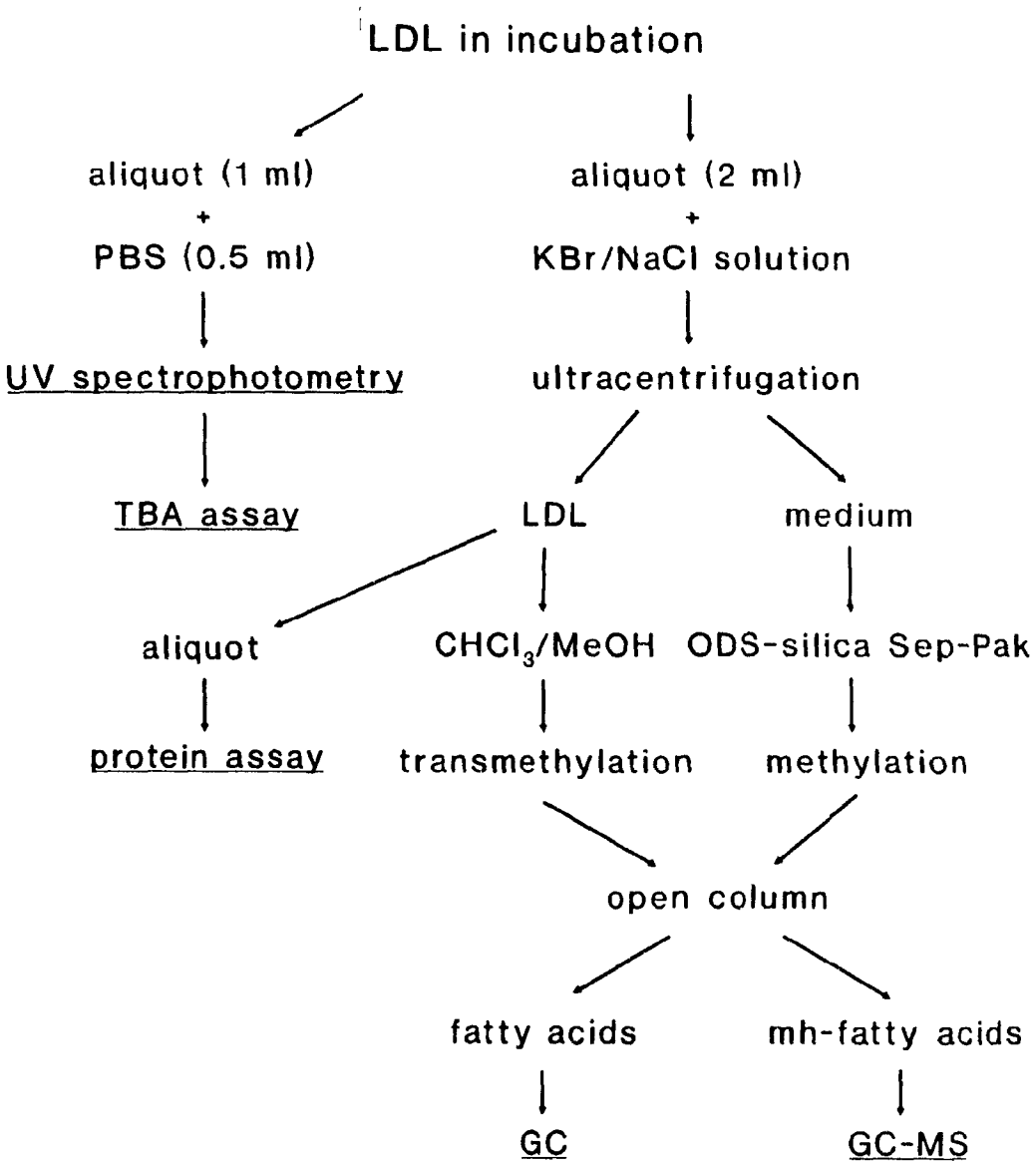


Fig. 16. Scheme for the analysis of lipid peroxidation products from human LDL lipids.

quantification of products with conjugated dienes using a value of 30,500 as the extinction coefficient.

The same aliquot (1 ml) was then assayed for TBARS according to Yagi [313]. Briefly, the LDL (1 ml) was mixed with 10% BHT in ethanol (8 μl), 8.1% sodium dodecyl sulphate (0.2 ml), 20% acetic acid in water (1.5 ml, adjusted to pH 3.5 by addition of NaOH) and then 0.8% aqueous thiobarbituric acid (1.5 ml). The test tubes were put into boiling water for one hour and then cooled with tap water. Water (1 ml) and n-butanol (5 ml) were added to the sample which was then vortexed vigorously. After centrifugation at 200 x g for 10 min, the TBARS in the *n*-butanol layer (top layer) were measured using an Aminco-Bowman spectrophotofluorometer (American Instrument Company, Inc.) with the excitation wavelength at 515 nm and the emission wavelength at 565 nm. A standard curve was constructed from freshly diluted 1,1,3,3-tetramethoxypropane.

2.3.4. Measurement of Fatty Acids and Their Monohydroxy Derivatives

2.3.4.1. Measurement of monohydroxy derivatives of fatty acids

(a) Reisolation of LDL

Propyl gallate was added to the remainder of the LDL sample (2 ml) to give a final concentration of at least 50 μM. A KBr/NaCl solution containing 0.1 % EDTA was added to the LDL to give a final density of 1.15 [314] and then reisolated by ultracentrifugation at 125,000 x g for 16 hours using a Beckman type 50 Ti rotor. The top layer (about 1.5 ml) contained reisolated LDL.

(b) Extraction of fatty acids and their monohydroxy derivatives

Fatty acids and their derivatives esterified to LDL lipids were extracted using the Folch method [315] with 14h-19:2 (200 ng) of the methyl ester and 20:0 (10 μg) of the methyl ester as internal standards for monohydroxy derivatives of fatty acids and fatty acids, respectively. In detail, LDL was mixed with propyl gallate, internal standards, and then extracted with 4 volumes of chloroform:methanol (2:1). The bottom fraction was aspirated and then dried under a stream of nitrogen. The residue was then taken up in chloroform:methanol (2:1) and washed with a salt solution (0.04% $CaCl_2$, 0.034%

$MgCl_2$, 0.58% NaCl) in a 4:1 ratio to remove some non-lipid contaminants.

Monohydroxy fatty acids released into the medium were extracted using an ODS-silica Sep-Pak cartridge as described in **Section 2.2.1** (P. 53) with 14h-19:2 (200 ng) of the free acid as an internal standard. In this case, 30% methanol was used for loading the sample onto an ODS-silica Sep-Pak and for the first wash since this gives a better recovery of monohydroxy fatty acids in high salt solution.

(c) Reduction of hydroperoxy fatty acids

Lipids from either LDL or fatty acids from medium were treated with triphenyl phosphine (1 mg in 1 ml of diethyl ether) for 1 hour at room temperature to reduce hydroperoxides to the corresponding hydroxy fatty acids. The latter products are far more stable than their hydroperoxy precursors during the workout described in the following sections.

(d) Preparation of methyl esters of fatty acids and hydroxylated fatty acids

Esterified fatty acids and their monohydroxy derivatives were hydrolyzed and converted to their methyl esters in a single step by transmethylation. Lipid extracts were transferred to a Reacti-vial (Pierce), dried down under a stream of nitrogen and then taken up in 50 μl of methylene chloride. 0.3 N sodium methoxide in methanol (0.6 ml) was added and the reaction was allowed to proceed under argon at 60 °C for 30 min. 50 mM Tris-HCl (3.4 ml) and 1 N HCl (100 μl) were added to the reaction mixture to adjust the pH to around 7.5 and the sample was extracted with 3 x 5 ml of methylene chloride. A pH of 7.5 is required to prevent the extraction of some contaminants into the organic fraction and, at the same time, maximize the recovery of fatty acids and their monohydroxy derivatives.

Free fatty acids and their monohydroxy derivatives, on the other hand, were converted to their methyl esters by treatment with 1 ml of ethereal diazomethane for 10-15 minutes at room temperature.

(e) Separation of fatty acids and their monohydroxy derivatives

The sample after transmethylation or methylation was dried down, taken up in 10 μl of isopropanol, 50 μl of ethyl acetate, and 3 ml of hexane and then loaded onto a glass column containing 0.5 g of silicic acid, which had been washed with hexane (5 ml). The

column was first eluted with 6% ethyl acetate in hexane (5 ml) and then with 20% ethyl acetate in hexane (5 ml). Fatty acids were recovered in the initial elute and the fraction containing 6% ethyl acetate in hexane whereas monohydroxy fatty acids were recovered in the fraction containing 20% ethyl acetate in hexane.

(f) Purification of monohydroxy fatty acids

Monohydroxy fatty acids were further purified by RP-HPLC on a Novapak ODS-silica column (150 x 3.9 mm; Waters-Millipore) which was eluted with a gradient between H_2O/acetonitrile (70:30) and H_2O/acetonitrile (25:75) over 30 minutes followed by a gradient to H_2O/acetonitrile (15:85) in 8 minutes. A single fraction containing all the monohydroxy fatty acids of interest was collected between 26 and 33 minutes (**Fig. 17**).

(g) Hydrogenation of monohydroxy fatty acids

Hydrogenation of monohydroxy fatty acids was performed by one of two methods. In the first method, methanol (300 μl) containing 1 mg of platinum(IV) oxide (Adam's catalyst) was bubbled with hydrogen for 30 seconds. Then a mixture of monohydroxy fatty acids (in 150 μl methanol) was added to the tube and hydrogen was bubbled for an additional 90 seconds. According to second method, methanol (300 μl) containing 1 mg of rhodium on alumina was initially bubbled with hydrogen for 90 seconds and then for an additional 5 minutes after the addition of the sample. In both cases, the sample after hydrogenation was passed through a column (1 x (n) of silicic acid and eluted with a further 1.5 ml of methanol.

(h) Preparation of trimethylsilyl ethers

Prior to GC-MS analysis, monohydroxy fatty acids were converted to their trimethylsilyl ethers by treatment with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA· 20 μl) for 30 min at room temperature. Having been dried down under nitrogen, the samples were taken up in 100 μl of iso-octane for GC-MS analysis.

(i) Quantification of monohydroxy fatty acids

Monohydroxy fatty acids were quantified on a Hewlett-Packard model 5890 gas chromatograph with a 12 m DB-1 fused capillary column coupled to a model 5988 mass spectrometer using electron impact ionization. The temperature gradient for eluting

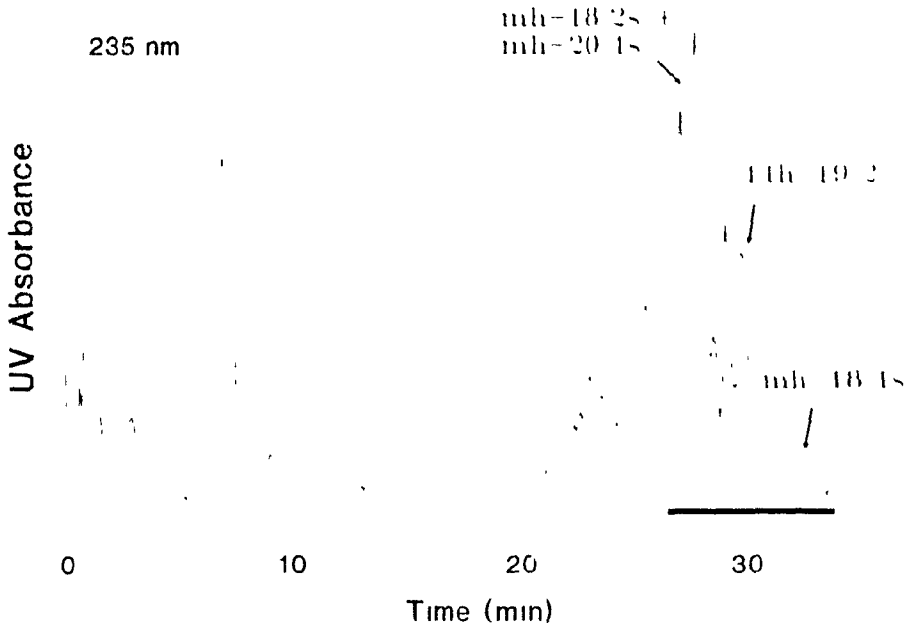


Fig. 17. High-pressure liquid chromatogram of monohydroxy derivatives of fatty acids from human LDL. The fractions containing monohydroxy derivatives of fatty acids from an open column of silicic acid were further purified by RP-HPLC on a Novapak ODS-silica column with a linear gradient between water·acetonitrile (70.30) and water.acetonitrile (25 75) over 30 minutes followed by water·acetonitrile (25:75) followed by a gradient to H_2O/acetonitrile (15 85) in 8 minutes. The elute collected during the time indicated by the solid line contained all of the monohydroxy derivatives of interest and was combined and analyzed by GC-MS

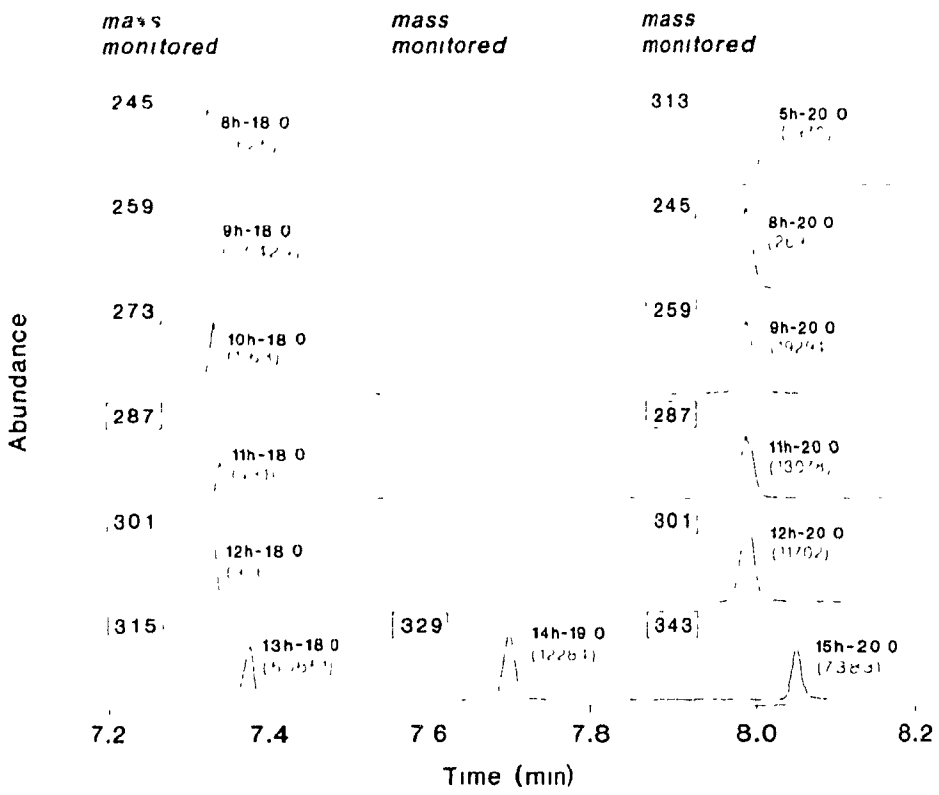


Fig. 18. Selected ion chromatograms of monohydroxy fatty acids in human LDL. After purification and derivatization, aliquots of monohydroxy derivatives of fatty acids from human LDL were measured by GC-MS using electron impact ionization (EI) and selected ion monitoring (SIM). The ions monitored (enclosed in boxes) were changed at 7.6 and 7.81 minutes (indicated by vertical dotted lines) to measure the specified monohydroxy compounds eluted from the GC column. The area of each peak was compared to the area of the internal standard (14h-19:0) and the ratio was compared to a standard curve constructed for each experiment. Note that the peak heights are normalized according to the highest peak for each ion monitored.

monohydroxy fatty acids was 50 °C for 1 minute followed by a rapid increase in oven temperature at 25 °C/min to 240 °C. The column was then purged at 280 °C for 15 minutes before the next injection. Monohydroxy fatty acids (C_{18} - C_{20}) were divided into three groups according to their carbon numbers. The ions monitored by the mass spectrometer were changed between each group (**Fig. 18**). The peak area of each compound was compared to the peak area of the internal standard, 14h-19:0, and the ratio was compared to a calibration curve constructed for each experiment.

2.3.4.2. Measurement of fatty acids and cholesterol

Fractions of open column chromatography containing fatty acids (**Section 2.3.4.1. (e)** on P. 62) were quantified using a Varian model 3300 gas chromatograph with a flame ionization detector. Fatty acids and cholesterol from rabbit LDL and aorta were extracted and transmethylated as discussed in the previous section. But in this case, open column chromatography was omitted and the fatty acids and cholesterol were treated with MSTFA prior to GC analysis. A 30 meter DB-25 fused silica capillary column was used for the analysis. The temperature program was 50 °C to 220 °C at a rate of 20 °C/min followed by a rate of 2 °C/min. The column was purged at 250 °C for 15 min before the next injection. The gas chromatogram of fatty acids and cholesterol from rabbit LDL is shown in **Fig. 19**.

2.4. LIPID PEROXIDATION AND PROSTANOID PRODUCTION IN ATHEROSCLEROTIC RABBITS

2.4.1. Source of Animals and Diets

Male New Zealand white rabbits (2 - 2.2 Kg) were purchased from La Ferme Lapro Inc., Stukely-Sud, Quebec. In studies on the effects of high cholesterol and high fat diets on the formation of monohydroxy derivatives of fatty acids and prostanoids, rabbits were fed a daily ration of either standard rabbit chow (60 g; Purina Canada, catalog No. LC-5301) or standard rabbit chow (53 g) supplemented with cholesterol (0.5 g), peanut oil (6 ml; Planters), ethanol (0.5 ml) and butylated hydroxyanisole (BHA; 0.5 g) as described by Weigensberg *et al.* [316]. To prevent the rabbits from becoming sick, the

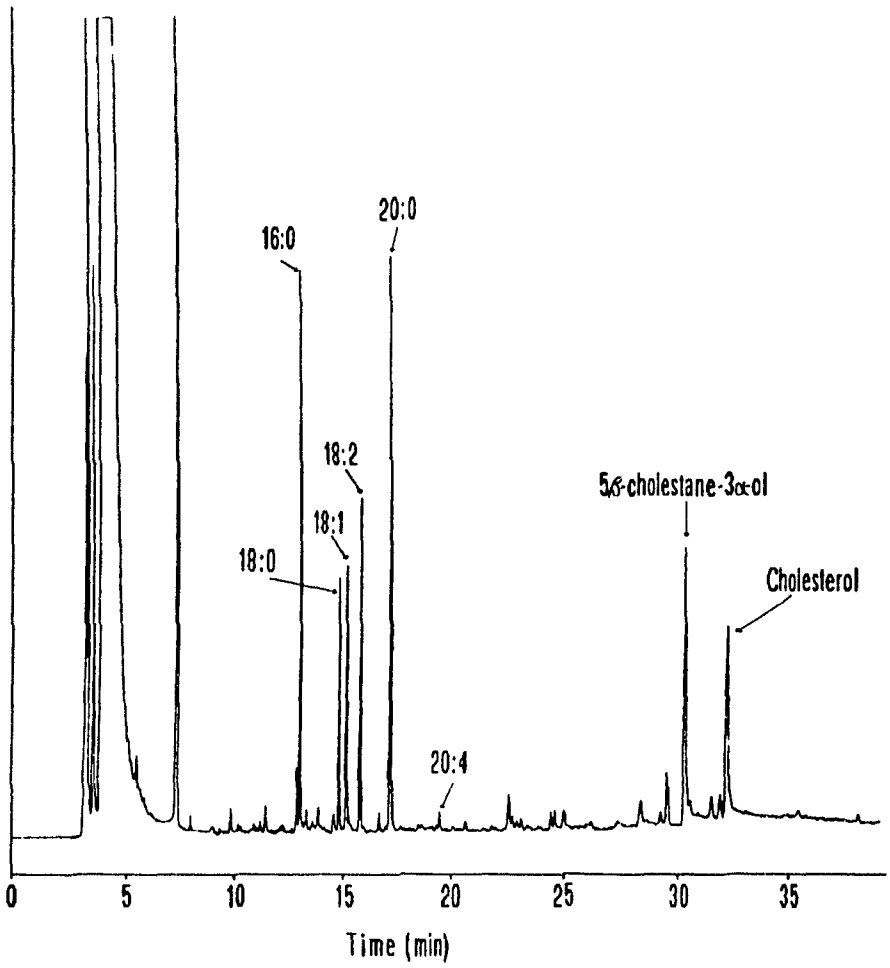


Fig. 19. Gas chromatogram of fatty acids and cholesterol in rabbits LDL. LDL lipids from a control rabbit was extracted using the Folch method with 16 μg of nonadecadienoic acid (19:2) and 5ß-cholestane-3α-ol as the internal standards for fatty acids and cholesterol, respectively. They were transmethylated, treated with MSTFA and then analyzed by gas chromatography on a J&W DB-23 fused capillary column (30 m x 0.25 mm) with a flame isonization detector. The temperature program was 50 °C for 1 min followed by a temperature program to 220 °C at a rate of 20 °C/min. The increasing rate was then changed to 2 °C/min until the oven temperature reached 250 °C.

cholesterol-supplemented diet was replaced by standard chow two days a week (on weekends) starting on the 8th week of the 15-week experiment. The composition of fatty acids and their monohydroxy derivatives in the experimental and control diets is listed in **Table 3**. In a second study on the distribution of monohydroxy derivatives of fatty acids in different lipid fractions, rabbits were fed a daily ration of standard rabbit chow (100 g), or standard chow supplemented with either cholesterol alone (1 g), or cholesterol (1 g) plus peanut oil (12 ml), butylated hydroxytoluene (1 g) and ethanol (1 ml) for 13 weeks.

2.4.2. Preparation of Samples

Two days before the aorta samples were to be collected, rabbits were placed in metabolic cages. After 24 hours of stabilization, urine was collected over a 24-hour period. Urinary creatinine content was measured at the Department of Clinical Biochemistry, Royal Victoria Hospital. Dinor metabolites of prostacyclin and thromboxane A_2 (2,3-dinor-6-oxo$PGF_{1\alpha}$ and 2,3-dinor-TXB_2, respectively) were measured by GC-MS with the collaboration of Dr. Falardeau at the Clinical Research Institute of Montreal [317,318]. The purification procedure is summarized in **Fig. 20**.

On the morning of the day when the rabbits were to be sacrificed, blood was withdrawn from the ear vein into two VACUTAINER blood collection tubes (Becton Dickinson) containing 10.5 mg EDTA (tripotassium salt) and 0.014 mg potassium sorbate per tube and the LDL was prepared as described in **Section 2.3.1.** on P. 58. Rabbits were then injected with papaverine (4 mg/kg body weight) and heparin (200 U/kg body weight) to minimize the effects of vessel contraction and thrombin formation, respectively [319]. Two minutes later, they were sacrificed by inhalation of carbon dioxide. After rapidly opening the thoracic cavity, the aorta was immediately perfused in situ with Ca^{2+} and Mg^{2+}-free modified Krebs-Ringer-Tris medium containing 127 mM NaCl, 5 mM KCl, 1.27 Mm KH_2PO_4, 5.5 mM glucose, 2 mM EDTA disodium salt, 10 μM ibuprofen, 10 μM papaverine, and 15 Mm Tris-HCl, pH 7.4 (medium A). The aorta was then removed and placed in medium A in which it was cut into 4 pieces as shown in **Fig. 21**. Adhering fatty tissue was removed from each of the pieces. The area of aorta covered

Table 3

Daily Intake of Fatty Acids and Their Monohydroxy Derivatives

Control and High cholesterol, high fat rabbit chow were grounded into powders and then extracted by 20 volumes of chloroform/methanol (2:1). An amount equal to one ten thousandth of the daily intake was mixed with 20 μg of 19:2 and 200 ng of 14h-19:2 as internal standards. After reduction by triphenyl phosphine, the lipid extracts were hydrolyzed by 0.3 N KOH at 60 °C for 45 minutes. The free fatty acids and their monohydroxy derivatives were acidified with concentrated HCl and then extracted with methylene chloride followed by methylation with diazomethane. Fatty acids and their monohydroxy derivatives were then separated from each other by an open column procedure. Fatty acids were quantified by a gas chromatography (3A) whereas their monohydroxy metabolites were quantified by GC-MS after RP-HPLC purification and hydrogenation (3B). The values are the averages of triplicate analyses.

Table 3A

	18:0	18:1	18:2	18:3	20:0	20:4
Control	30	294	680	176	46	6
High-fat	102	1723	1606	163	44	96

Table 3B

	18:0		20:0		
	9h	13h	11h	12h	15h
Control	874	512	0.92	1.28	1.16
High-fat	3041	1274	1.80	1.99	1.76

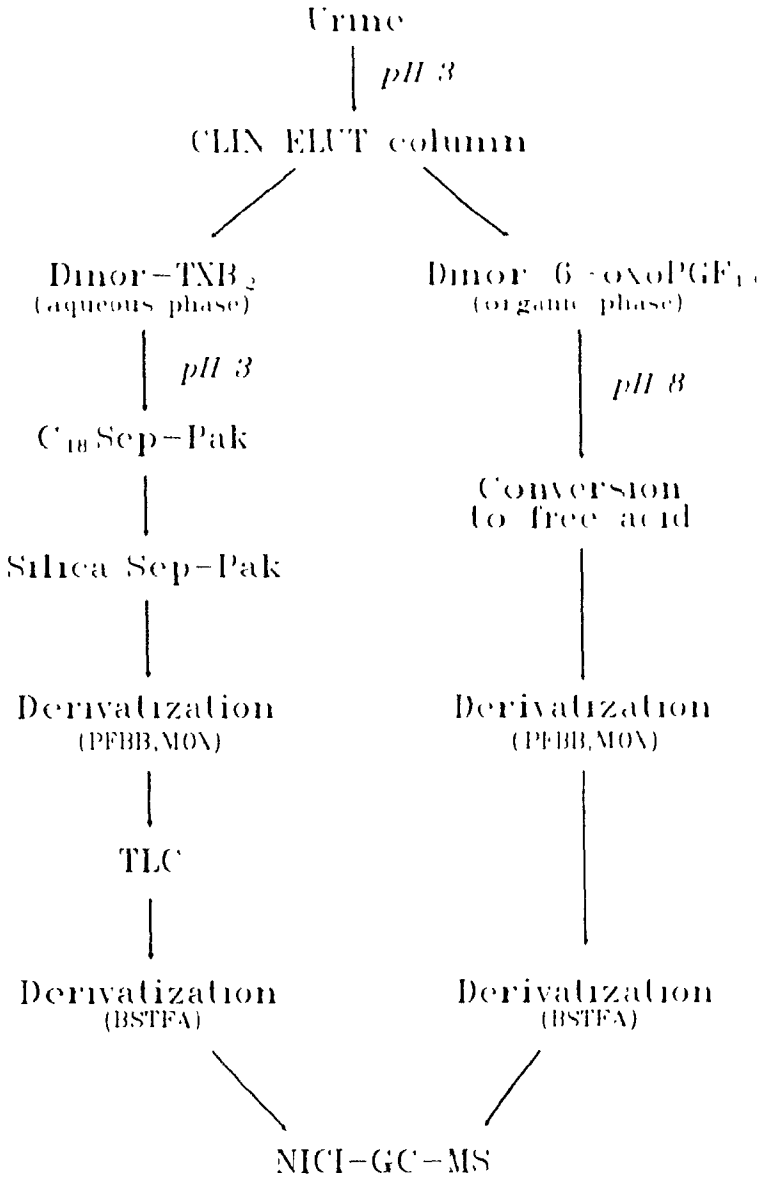


Fig. 20. Scheme showing the purification of dinor metabolites of PGI_2 and TXA_2 for GC-MS analysis. The abbreviations are as the follows: **PFBB**: pentafluorobenzyl bromide; **MOX**: methyoxyamine hydrochloride; **BSTFA**: N,N-bis(trimethylsilyl)trifluoroacetamide.

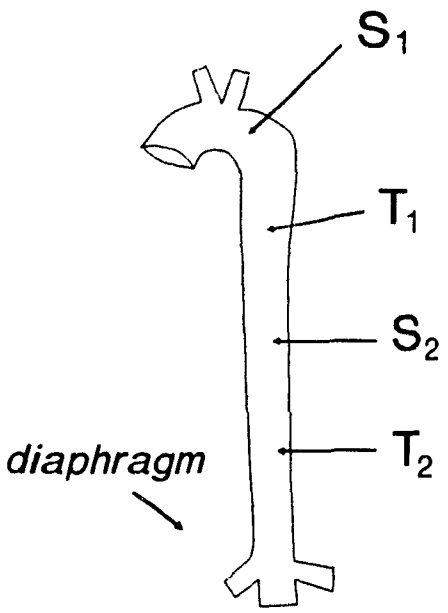


Fig. 21. Preparation of aortic tissue for incubation. After opening the thoracic cavity, rabbit aorta was immediately perfused *in situ* with Ca^{2+}, Mg^{2+}-free buffer containing ibuprofen and EDTA. The aorta was then removed and freed of adhering fatty tissue in the same buffer. Two sections (S_1 and S_2) were cut into small slices and then incubated at 37 °C for 30 min. The two remaining sections (T_1 and T_2) were mounted in a template as shown in next figure.

with lesions was evaluated visually by two individuals and expressed as a percentage of the total area.

2.4.3. Incubation of Aortic Tissues

2.4.3.1. Slices of aorta

The aortic arch (S_1) and the middle section of the thoracic aorta (S_2) were cut into small slices (about 3 mm^2). To remove the EDTA, ibuprofen and papaverine, the slices were washed 5 times at 0 °C with Krebs-Ringer-Tris medium containing 127 mM NaCl, 5 mM KCl, 1.27 mM $MgSO_4$, 1.27 mM KH_2PO_4, 2.7 mM $CaCl_2$, 5.5 mM glucose, and 15 mM Tris-HCl, pH 7.4 (medium B). The slices were then incubated in 10 ml of medium B for 30 min at 37 °C in the absence of exogenous fatty acid substrate. After incubation, aortic slices were removed from the incubation medium and extracted with chloroform/methanol for analysis of monohydroxy fatty acids. An aliquot (1 ml) of the medium was withdrawn for radioimmunoassay and the rest was extracted with an ODS-silica Sep-Pak cartridge for measurement of the monohydroxy fatty acids released from aorta.

2.4.3.2. Luminal surface of aorta

Sections (T_1 and T_2) of the aorta were placed vertically between two lucite blocks, one of which contained a small incubation chamber (height, 27 mm; diameter, 10 mm), which allowed the medium to come in contact with only the luminal surface of the aorta (see **Fig. 22**). The chamber extended below the aorta, which was mounted on the side, to permit stirring with a magnetic stirring bar during the incubation [319] The template was kept in a chamber with a constant temperature of 37 °C. The luminal surface (30 mm^2) of the aorta was incubated for 5 min with medium A and then washed for 5 periods of 5 min with medium B. The aorta was then incubated for 10 min at 37 °C in the presence of exogenous arachidonic acid (25 μM).

Aliquots from the incubations of the rabbit aorta slices or the aortic lumen were assayed for 6-oxo$PGF_{1\alpha}$, PGE_2 and TXB_2 by radioimmunoassay [305].

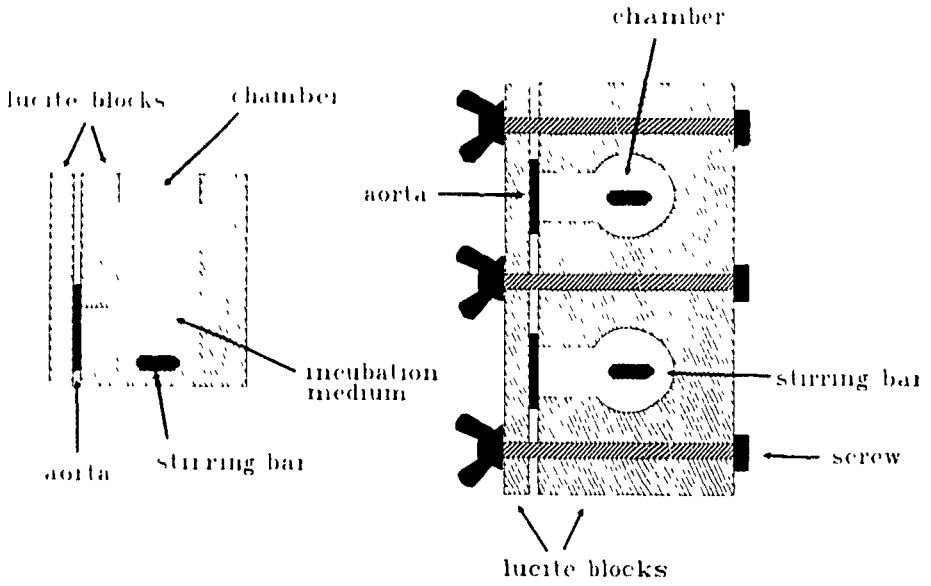


Fig. 22. Cross-section of incubation template for measuring products released from the luminal surface of the aorta. A side view (left) and a top view (right) of an incubation template are shown. Sections of rabbit aorta were placed vertically between two lucite blocks, one of which contains a small incubation chamber, which allows the medium to come in contact with only the luminal surface of the aorta. The chamber extended below the aorta, which is mounted on the side, to permit stirring with a magnetic stirring bar during incubation.

2.4.4. Measurement of Fatty Acids and Their Monohydroxy Derivatives in Aorta and LDL from Atherosclerotic Rabbits

A small portion of the rabbit aortic slices (about 20 mg) from each section of aorta was homogenized in saline (4 ml) and an aliquot was taken for measurement of protein. The remaining aortic slices (about 200 mg) were homogenized in $CHCl_3/CH_3OH$ (2:1) containing 14h-19:2 (200 ng) of the methyl ester as an internal standard for monohydroxy fatty acids, and extracted using the Folch method. The $CHCl_3/CH_3OH$ mixture was bubbled with argon and cooled in an acetone/dry ice bath in which the extraction procedure was carried out. Medium from the incubation was extracted on an ODS-silica Sep-Pak cartridge with 200 ng 14h-19:2 (free acid) as the internal standard. The extracts were then derivatized and purified as described previously in **Section 2.3.4.1.**.

For measurement of esterified fatty acids and total cholesterol, pieces of rabbit aorta (about 50 mg) from upper part of the thoracic aorta (T_1) were homogenized in saline (4 ml). Aliquots of homogenates or LDL were mixed with 16 μg of 19:2 (methyl ester) and 5ß-cholestane-3α-ol as the internal standards for fatty acids and cholesterol, respectively, and extracted using the Folch method. The lipid extracts were transmethylated and the cholesterol and 5ß-cholestane-3α-ol were converted to their trimethylsilyl ethers with MSTFA before GC analysis (see **Fig. 19** on P. 67).

The experimental protocol is summarized in **Fig. 23.**

2.4.5. Measurement of Fatty Acids and Their Monohydroxy Derivatives in Neutral and Polar Lipids

To investigate the lipid class containing the esterified hydroxylated fatty acids and their fatty acid precursors, lipid extracts of aortae from either normal rabbits or from rabbits fed either 1% cholesterol or 1% cholesterol, peanut oil and BHT for 13 weeks were separated into fractions containing neutral and polar lipids by chromatography on a silicic acid column [304]. In detail, the residue from the lipid extracts was dissolved in chloroform (3 ml) and passed through a glass column containing 0.3 g of silicic acid. The column was eluted with a further 7 ml of chloroform. The 10 ml chloroform fraction contained neutral lipids (triglycerides and cholesteryl esters). Polar lipids (phospholipids)

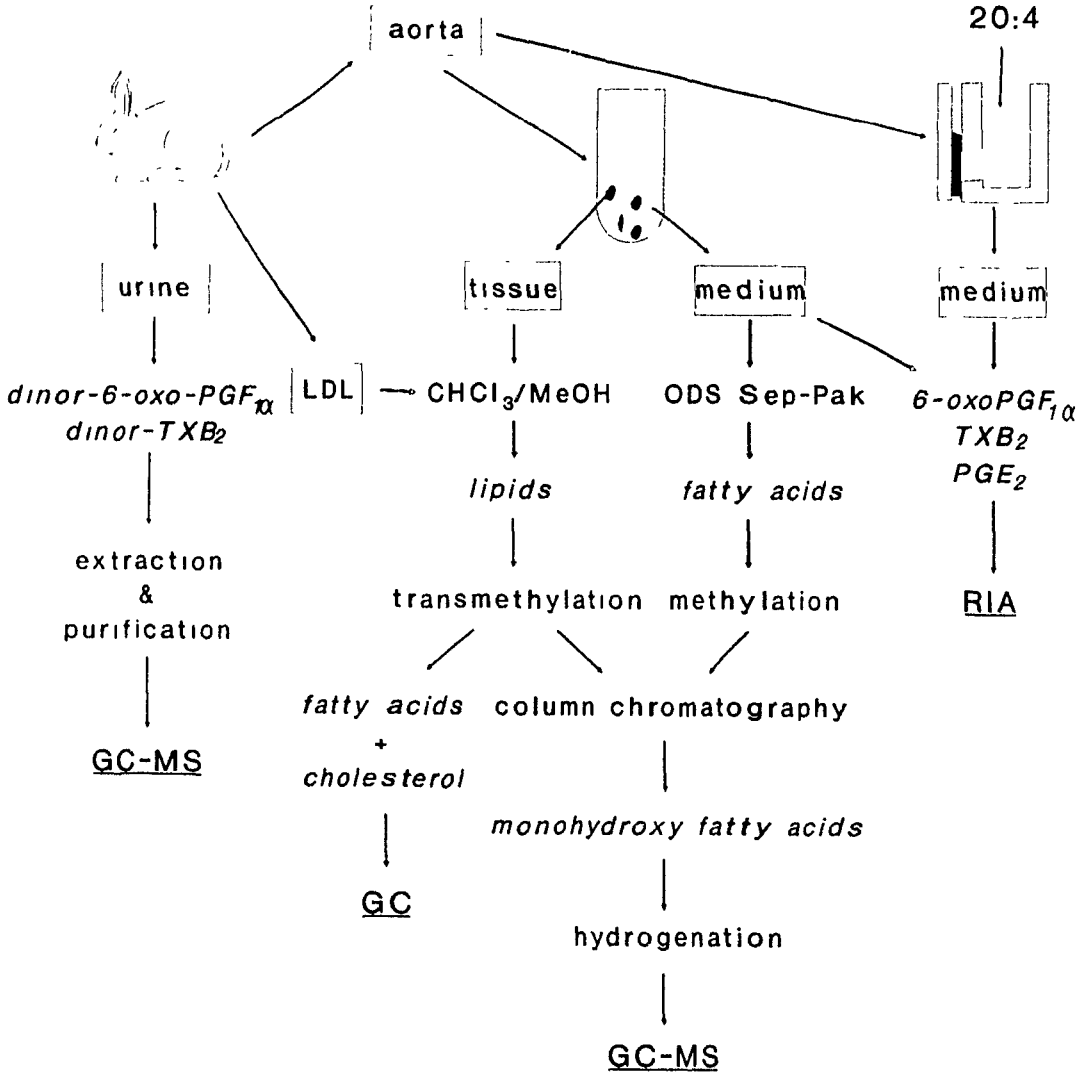


Fig. 23. Scheme for the analysis of monohydroxy derivatives of fatty acids and prostanoids from rabbits.

were then eluted with methanol (4 ml) followed by 2 ml of methanol:H_2O (1:1), which were combined. 14H-19:2 and 19:2 (methyl esters) were added to the neutral and polar lipid fractions as internal standards for monohydroxy fatty acids and their fatty acid precursors, respectively. After transmethylation and purification as described in **Section 2.3.4.1.**, the amounts of esterified monohydroxy fatty acids and fatty acids in these two fractions were measured by GC-MS and GC, respectively.

2.5. EFFECTS OF HYDROPEROXY LIPIDS ON THE FORMATION OF PROSTAGLANDINS BY PARTICULATE FRACTION FROM FETAL CALF AORTA

2.5.1. Preparation of Particulate Fractions from Fetal Calf Aorta

Fetal calf aorta was cleaned and the adventitia removed. The remaining tissue was minced in 0.05 M Tris-HCl, pH 7.5, (4 ml/g tissue) and homogenized in an ice-water bath with a Vir-Tis homogenizer (6 x 10 seconds with 50 seconds in between to allow for cooling). The supernatant was filtered through one layer of cheesecloth and the pellet was resuspended to the original volume of the homogenate by addition of Tris buffer, and rehomogenized with a Vir-Tis homogenizer (3 x 10 seconds). This homogenate was centrifuged for 10 min at 400 x g and, after filtration, the two filtered supernatants were combined and centrifuged at 100,000 x g for 60 min at 4 °C using a Beckman type 50.2 Ti rotor. The pellet was resuspended in Tris buffer (0.25 ml/g tissue).

2.5.2. Preparation of PGH_2

Ram seminal vesicle microsomes (2 ml) were mixed with 1% liquid phenol in water (20 μl) and then kept in an ice bath for 30 min. P-hydroxymercuribenzoate (a PGE_2 synthase inhibitor; 2.2 mg) was then added and the microsomes were placed in a water bath at 37 °C for 5 min. The reaction was started by adding [1-^{14}C] 20:4 (3.5 x 10^6 cpm with 200 μg unlabelled substrate). One minute later, the reaction was stopped by acidification with 1N HCl and the mixture was extracted twice with 8 ml of hexane:diethyl ether (1:4). The organic and inorganic phases were separated by rapid centrifugation. The organic fraction was washed with water and the excess water was

removed by addition of $MgSO_4$. The purity of the preparation was checked by reducing an aliquot of PGH_2 with triphenyl phosphine (1 mg in 1 ml of ether) for 1 hour at room temperature. The products were analyzed by thin-layer chromatography using a mobile phase consisting of petroleum ether:diethyl ether:acetic acid (15:85:0.1) and the radioactivity on the plate was measured using a radioactivity scanner.

If the hexane:diethyl ether extract contained significant amount of other oxygenation products, purification of PGH_2 was performed using column chromatography (0.5 g silicic acid) with 15 ml of ethyl acetate:hexane (1:4) followed by 15 ml of ethyl acetate:hexane (1:3). The last fraction was kept at -40 °C before use.

2.5.3. Preparation of Hydroperoxy Lipids

Solutions of cholesteryl linoleate (2 mg) in toluene (2 ml), trilinolein (2 mg) in toluene (2 ml) or 1-palmitoyl-2-linoleoyl-phosphatidylcholine (2 mg) in chloroform:methanol (1:1; 2 ml), all containing dl-α-tocopherol (50 μg) to retard the decomposition of peroxyl radicals (see **Section 1.3.3** on P. 32 and **Section 1.4.1** on P. 44), were concentrated to dryness in round bottom flasks using a rotary evaporator. The round bottom flasks were flushed with oxygen and kept in the dark for 70 hours at 40 °C.

Hydroperoxides prepared from cholesteryl linoleate and trilinolein were purified by NP-HPLC using hexane:isopropanol (99.7:0.3) with a flow rate of 5 ml/min. Hydroperoxides prepared from phosphatidylcholine were purified by RP-HPLC using methanol:water (95:5) with a flow rate of 2 ml/min (**Fig. 24**).

The hydroperoxy nature of the samples was confirmed by their capability to reduce oxidized iodide (I^-) ions to iodine (I_2). For hydroperoxy derivatives of trilinolein, the products were reduced with sodium borohydride and then rechromatographed by NP-HPLC using the same condition as described above. The resulting hydroxy lipids had longer retention time than their hydroperoxy counterparts. Hydroperoxy lipids were dried down and taken up in acetone just prior to the experiment.

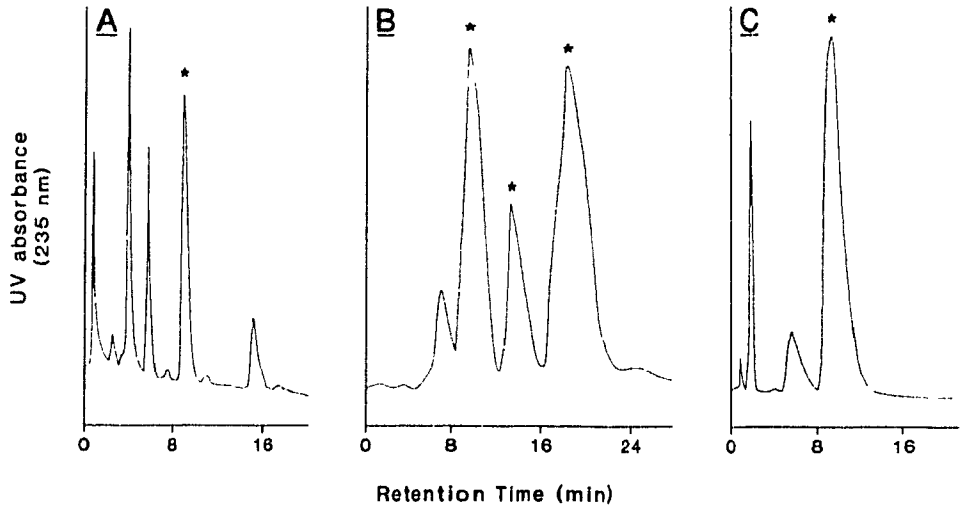


Fig. 24. Purification of hydroperoxy derivatives of lipids by high pressure liquid chromatography. Hydroperoxy derivatives prepared from cholesteryl linoleate (A) and trilinolein (B) were purified by NP-HPLC using a mobile phase of hexane:isopropanol (99.7:0.3) at 5 ml/min. Hydroperoxy derivatives prepared from 1-palmitoyl-2-linoleoyl-phosphatidylcholine (C) were purified by RP-HPLC using a mobile phase of methanol:water (95:5) at 2 ml/min. The peaks marked with asterisks were collected for the experiment.

2.5.4. Experimental Protocol

2.5.4.1. Effects on prostaglandin endoperoxide synthase

Prostaglandin endoperoxide synthase activity was evaluated by incubating particulate fractions from fetal calf aorta with arachidonic acid followed by measurement of the total amounts of oxygenation products. Particulate fractions (0.5 ml) were preincubated at 37 °C for 1 min. A solution of 0.04% Triton X-100 in Tris-HCl (0.5 ml, pH 7.5; final concentration, 0.02%) was added, followed by lipid hydroperoxides in acetone (10 μl). After incubation for a further 3 min, 20:4 (30,000 cpm or 1.71 μg/incubation) was added in 3 μl of ethanol and the incubation was continued for 10 min. The reaction was stopped by addition of ethanol (2 ml). [9α-^{3}H]$PGF_{1\alpha}$ (20,000 cpm) was added as an internal standard and the mixture was centrifuged. The supernatant was extracted on an ODS-silica Sep-Pak cartridge as described in **Section 2.2.1.** on P. 53 and the methyl formate fraction was dried down under a stream of nitrogen. The residue was dissolved in 10 μl of methanol and then 5 ml of a solvent consisting of hexane:toluene:acetic acid (95:5:0.1) was added. The sample was then loaded onto a column of silicic acid (0.5 g), which was eluted with 13 ml of hexane:ether:acetic acid (95:5:0.1) to remove unconverted 20:4. Total oxygenation products of 20:4 were then eluted with 10 ml of methyl formate:methanol:acetic acid (80:20:0.1). This fraction was evaporated in a fume hood overnight and the radioactivity due to the ^{14}C-labelled products was measured using a beta-counter with a detection window range of 350-655. Recovery was calculated by counting the radioactivity due to [9α-^{3}H]$PGF_{1\alpha}$ in the tritium window (0-250). Inhibition of the enzyme was calculated by dividing the radioactivity due to ^{14}C in the samples containing hydroperoxide (corrected for recovery) by the radioactivity in the control incubations.

2.5.4.2. Effects on prostacyclin synthase

Prostacyclin synthase activity was evaluated by measuring the amounts of prostaglandins formed from PGH_2 by particulate fractions from fetal calf aorta. Particulate fraction (0.5 ml) were preincubated at 37 °C for 1 min. A solution of 0.04% Triton X-100 in Tris-HCl (0.5 ml, pH 7.5; final concentration, 0.02%) was added,

followed by lipid hydroperoxides in acetone (10 μl). After incubation for a further 3 min, PGH_2 (30,000 cpm or 17.1 μg/incubation) in 3 μl of ethanol was added and the incubation was continued for another 3 min. The reaction was stopped by addition of ethanol (2 ml). [9α-^{3}H]$PGF_{1\beta}$ (20,000 cpm) was added as an internal standard and the mixture was centrifuged. The supernatant was extracted on an ODS-silica Sep-Pak cartridge and the methyl formate fraction was dried down under a stream of nitrogen. Prostaglandins formed in the reaction were separated by RP-HPLC using acetonitrile:water:acetic acid (31:69:0.02) for 5 min with a flow rate of 1.5 ml/min followed by a linear gradient to acetonitrile:water:acetic acid (80:20:0.02) over 6 min with a flow rate of 3 ml/min, and then to acetonitrile:acetic acid (100:0.02) over 5 min. The radioactivity in fractions collected every minute for the first 10 minutes was measured using a beta-counter and the amount of radioactivity in the peak corresponding to 6-oxo[1-^{14}C]$PGF_{1\alpha}$ was calculated and corrected for recovery.

2.6. STATISTICAL ANALYSIS

Statistical analyses (**Table 4**) were performed using SPSS-X (release 3.0) for IBM OS/MVS at the computing centre of McGill University.

2.6.1. Comparison between Two Means

Comparison of two means was performed using Student's t-test. There are two situations for which this test is used -- independent samples or paired samples. The arrangement of data is quite different in these two situations:

Treatment	Measurement	
	Y1	Y2
I	*a*	*b*
II	*c*	*d*

For independent sample, the two measurements are being considered independent of each other. For example, when the means for control rabbits (*a*) and cholesterol-fed

Table 4

Statistical Analyses Used in This Thesis

Statistical analyses were performed using SPSS/X (release 3.0) for IBM/MVS at the computing centre of McGill University. Dependent variables (Y) are the response to other variables. Independent variables (X) are the predictor or grouping variables. The level of independent variables is included in the bracket.

INDEPENDENT VAR. (X)	DEPENDENT VAR. (Y)	PROCEDURE
1 category (2) -- independent	1 continuous	Student's T-Test (independent)
1 category (2) -- paired	1 continuous	Student's T-Test (paired)
1 category (>2)	1 continuous	Analysis of Variance & Multiple Comparison
2 categories (>1)	1 continuous	Analysis of Variance (two-way ANOVA)
1 category (>2)	1 continuous -- repeated measures on the same sample	Analysis of Variance with repeated measures
1 continuous	1 continuous	Pearson product-moment Correlations

rabbits (*b*) of the amounts of TXB_2 released by slices of aorta (Y1) were to be compared, the mean and variance of each group (*a* and *c*) were calculated separately and then compared. The control line of SPSS-X was

T-TEST GROUPS = treatment (I,II) / *VARIABLES* = Y1.*

For paired samples, the *same* sample was measured twice on different occasions. For example, when the difference between the aortic arch (Y1) and the middle section of the thoracic aorta (Y2) from the same aorta (I) were to be compared in their production of TXB_2, the differences in the values between pairs (*a* and *b*) were used in the calculation and therefore the variance between different animals for the same section of aorta was removed. The control line of SPSS-X was

T-TEST PAIRS = Y1 Y2.

2.6.2. Comparison among Several Means

In biomedical research, the effects of multiple factors on experimental variables are often investigated. In our study on the effect of cholesterol feeding on TXB_2 production by aorta, the length of time on the diets was also a factor of interest:

Treatment	Length of Time on Diets		
	2	8	15
I	*a*	*b*	*c*
II	*d*	*e*	*f*

In this case, two-way analysis of variance (ANOVA) was utilized to test the difference between treatments (group effect) and the differences among several time periods (time effect). The interaction of these two effects was also investigated. The control line for SPSS-X was

ANOVA VARIABLES = txb2 BY treatment (I,II) time (1,3)
/METHOD=UNIQUE.

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*. SPSS/X procedures are shown in italics and in upper cases. Keywords for each procedure are shown in upper cases. Variables are all shown in lower cases even though some of them should have been shown in upper cases (*i.e.*, txb2 for TXB_2).

The method used to decompose sum of squares was UNIQUE, which should be used in situations where there are unequal number of cases in each group. If all the groups had equal number of cases, the EXPERIMENTAL approach could have been used.

If there existed a significant interaction between two factors, i.e., treatment and time, post-hoc tests were performed to test the differences between groups due to treatment in a particular time period, since an interaction would suggest that the magnitude of the difference between groups due to treatment would change with time.

In a two-way experimental design discussed above, different subjects (rabbits in this case) were used in each group. However, it may be possible to measure a variable for the same subjects or the same tissue at different points. In this case, *univariate repeated measures F-test* should be utilized to remove the variance due to different subjects and increase the power of analysis. For example, to test the effect of ibuprofen/EDTA on PGI_2 production by slices of aorta, the same pieces of aorta were used in the incubation and aliquots were taken at 0, 5, 10, 20, 30 and 40 min for analysis. The data was arranged as follows

Treatment	Length of Time on Diets					
	0	5	10	20	30	40
I	*a*	*b*	*c*	*d*	*e*	*f*
II	*g*	*h*	*i*	*j*	*k*	*l*

The control line for SPSS-X was

```
MANOVA  pgi_0 TO pgi_40 BY treatment (I,II)
   /WSFACTOR = time (6)
   /CONTRAST (time) = POLYNOMIAL (0,5,10,20,30,40)
   /WSDESIGN = time
   /PRINT = TRANSFORM
            SIGNIF (AVERF)
   /DESIGN
```

In this analysis, the PGI_2 production at different time points was used as the dependent variable rather than one single independent variable as was the case in two-way ANOVA. We tested the difference in PGI_2 production by two groups of rabbit aortae (I for controls and II for aortae treated with ibuprofen/EDTA). Since more than two dependent variables were involved, multivariate analysis or repeated measure F-test was used. WSFACTOR was to assign the *time factor* as a within-subject factor. CONTRAST indicated a trend analysis, which reported the statistical significances based on linear, quadratic, cubic or higher polynomial trends. The numbers in the brackets after the keyword POLYNOMIAL indicates of the spaces between different variables; otherwise equal distances would have been applied to all variables The subcommand PRINT reported the transformation information since all the original values were transformed for consequent analysis and the keyword SIGNIF (AVERF) reported the repeated measure F-test along with multivariate analysis with repeated measures. The subcommand DESIGN indicated that the treatment (I and II) was the between-subject factor.

2.6.3. Correlation between Two Variables

The association between two dependent variables was measured using Pearson's correlation coefficient (Pearson *r*) with the control line for SPSS/X

CORRELATIONS Y1 Y2.

It is important to note that a strong correlation does not necessarily indicate a cause-effect relationship. For example, the fact that Y1 and Y2 are correlated does not necessarily mean the change in Y2 is caused by the change in Y1 or vice versa.

RESULTS

3.1. MEASUREMENT OF LIPID PEROXIDATION IN HUMAN LDL

Modification of LDL by lipid peroxidation products has been hypothesized as an important event leading to atherosclerosis. Incubation of LDL *in vitro* with transition metal ions and cells, including endothelial cells, leads to increased formation of TBARS, modification of apolipoprotein B-100 and the consequent uptake and degradation of LDL by macrophages. Even though lipid peroxidation plays an important role in the modification of LDL, there were no reports in the literature on the measurement of monohydroxy derivatives of polyunsaturated fatty acids, which are initial products of the lipid peroxidation *. We therefore decided to measure these derivatives and compare the changes in their levels during oxidation of LDL with changes in other parameters of lipid peroxidation such as loss of LDL fatty acids, formation of conjugated dienes and TBARS.

Polyunsaturated fatty acids are readily oxidized in the presence of oxygen, therefore rigorous efforts were made to minimize lipid peroxidation during preparation of the samples. All procedures performed prior to the separation of monohydroxides from their fatty acid precursors were performed under argon or nitrogen. Addition of propyl gallate prior to the extraction could enhance this protection since copper-catalyzed oxidation of LDL lipids was nearly completely inhibited by propyl gallate evaluated by all the parameters of lipid peroxidation which were measured.

3.1.1. Measurement of Monohydroxy Derivatives of Fatty Acids

The initial products of lipid peroxidation of fatty acids are hydroperoxides which can be reduced to more stable hydroxy derivatives by triphenyl phosphine. Measurement of hydroxy derivatives of different fatty acids could give us an indication of the rate of oxidation of each individual fatty acid.

---

*. We started the experiment on human LDL in the summer of 1989 and had all the results from incubation of LDL with copper in the spring of 1990. However, before we got the chance to publish it, a paper appeared in the 1990 July issue of *Journal of Lipid Research* in which monohydroxy derivatives of 18:2 and 20:4 were measured by HPLC in LDL subjected to Cu^{2+} oxidation.

3.1.1.1. Monohydroxy fatty acids in human LDL after peroxidation induced by copper

Monohydroxy fatty acids in LDL were quantified by gas chromatography/mass spectrometry (GC-MS) using a method similar to one previously developed in our laboratory [304]. The samples were hydrogenated prior to analysis to increase the specificity of the assay with respect to the position of the hydroxyl group, since the hydrogenated compounds have relatively simple fragmentation patterns. Peroxidation of polyunsaturated fatty acids results in the formation of conjugated double bonds from 1,4-*cis,cis*-pentadiene groups (see **Fig. 9** on P. 30). Although the predominant configuration of these conjugated double bond are *trans-cis*, monohydroxy fatty acids also have conjugated double bonds with *trans-trans* configurations especially when they are formed by non-enzymatic peroxidation. The *cis-trans* and *trans-trans* configurations of the same positional isomer will give identical products after hydrogenation, yielding a simpler gas chromatographic profile of monohydroxy fatty acids. However the disadvantage of hydrogenation is that is not possible to know how many double bonds were present in the parent compound. This is not the major problem for hydroxylated fatty acids with 20 carbons since they come almost exclusively from arachidonic acid. But it is a problem for monohydroxy derivatives of fatty acids with 18 carbons since they can be derived from either oleic acid or linoleic acid, two major fatty acid components in human LDL. This problem, however, can be partly solved by studying the dynamic changes of these monohydroxy fatty acids under conditions in which they undergo decomposition at different rates as in the presence of moderately high concentrations of copper ions. **Fig. 25** shows the changes in monohydroxy C_{18} fatty acids in LDL incubated with 20 μM of $CuSO_4$. There were basically two patterns -- one was represented by 8h, 10h, and 11h-18:0 (**Fig. 25**; A, C and E, respectively) in which the amounts of monohydroxy derivatives increased continuously up to 20 hours; another was represented by 9h and 13h-18:0 (**Fig. 25**; B and D, respectively), the amounts of which were elevated after 5 hours but declined significantly after 20 hours of incubation. Since oleic acid can be oxidized to 8, 10 and 11-hydroxy derivatives (see **Section 1.3.1** on P 24), it is reasonable to conclude that 8h, 10h and 11h-18:0 are coming from hydroxylated derivatives of oleic

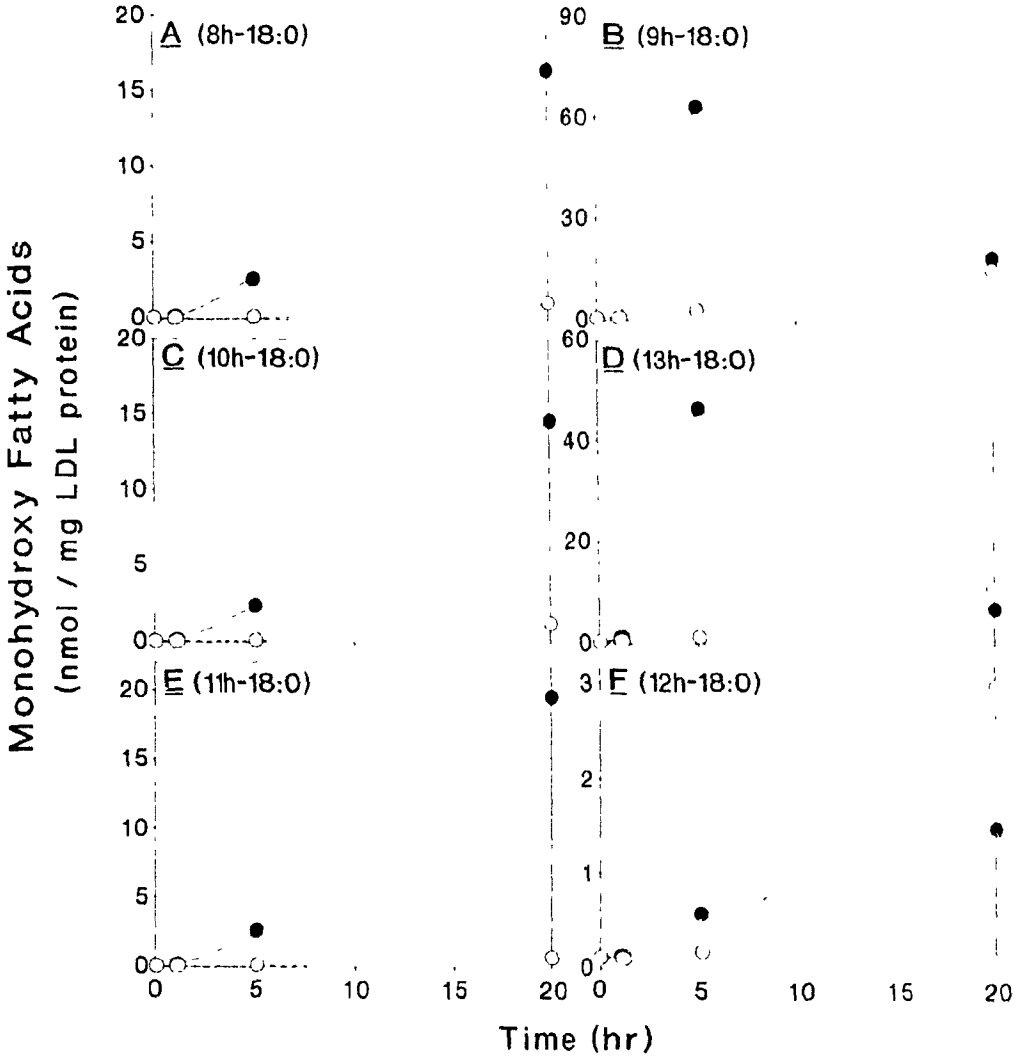

Fig. 25. Changes in the amounts of monohydroxy derivatives of C_{18} fatty acids in LDL incubated with $CuSO_4$. Human LDL was incubated in Ham's F10 medium (o o) or in Ham's F10 medium containing 20 μM of $CuSO_4$ (•——•) for 1, 5 and 20 hours. Monohydroxy derivatives to C_{18} fatty acids esterified to LDL lipids were then purified and measured by GC-MS as described in MATERIALS AND METHODS. The values are averages of triplicates.

acid. 9H-18:0 and 13h-18:0, on the other hand, are presumably coming from hydroxylated derivatives of linoleic acid which contain 2 double bonds and would therefore more easily be further oxidized to secondary products. Only a relatively small amount of 12h-18:0 was detected (**Fig. 25F**). Although the amount increased with time, the difference between the control and the sample incubated with copper was much less than those for other hydroxy C_{18} products. Furthermore, the origin of 12h-18:0 is not clear [304]. We therefore decided not to include the values for 12h-18:0 in the following results. All monohydroxy fatty acids derived from arachidonic acid had similar patterns to 9h-18:0 and 13h-18:0.

Based on the information in **Fig. 25**, monohydroxy fatty acids in LDL were classified into three groups: 8h, 10h and 11h-18:0 in group 1; 9h and 13h-18:0 in group 2; 5h, 8h, 9h, 11h, 12h and 15h-20:0 in group 3. The total amounts in each group are shown in **Fig. 26**. The amounts of monohydroxy derivatives of oleic acid (8h, 10h and 11h-18:0) were the same as the control after 1 hour in the presence of 20 μM $CuSO_4$, but increased compared to the controls after incubation for 5 hours (**Fig. 26A**). This increase was time-dependent up to 20 hours. In comparison, the amounts of monohydroxy derivatives in group 2 and 3, *i.e.*, monohydroxy derivatives of polyunsaturated fatty acids, were dramatically increased after 5 hours of incubation but declined significantly after 20 hours (**Fig. 26**; C and E).

The amounts of individual monohydroxy fatty acids in LDL after 5 hours of incubation with $CuSO_4$ are presented in **Fig. 27A**. All monohydroxy fatty acids from the same fatty acid precursors increased to approximately the same extent, consistent with the pattern expected for the autoxidation of lipids.

3.1.1.2. Monohydroxy fatty acids in human LDL incubated with endothelial cells

The mechanism of oxidation of LDL lipids in the presence of endothelial cells may be different from the oxidation of LDL induced by copper due to the presence of lipoxygenases in the endothelial cells. If the oxidation is mediated by lipoxygenases, 12h-20:4 and/or 15h-20:4 would increase. On the other hand, if the oxidation is due to

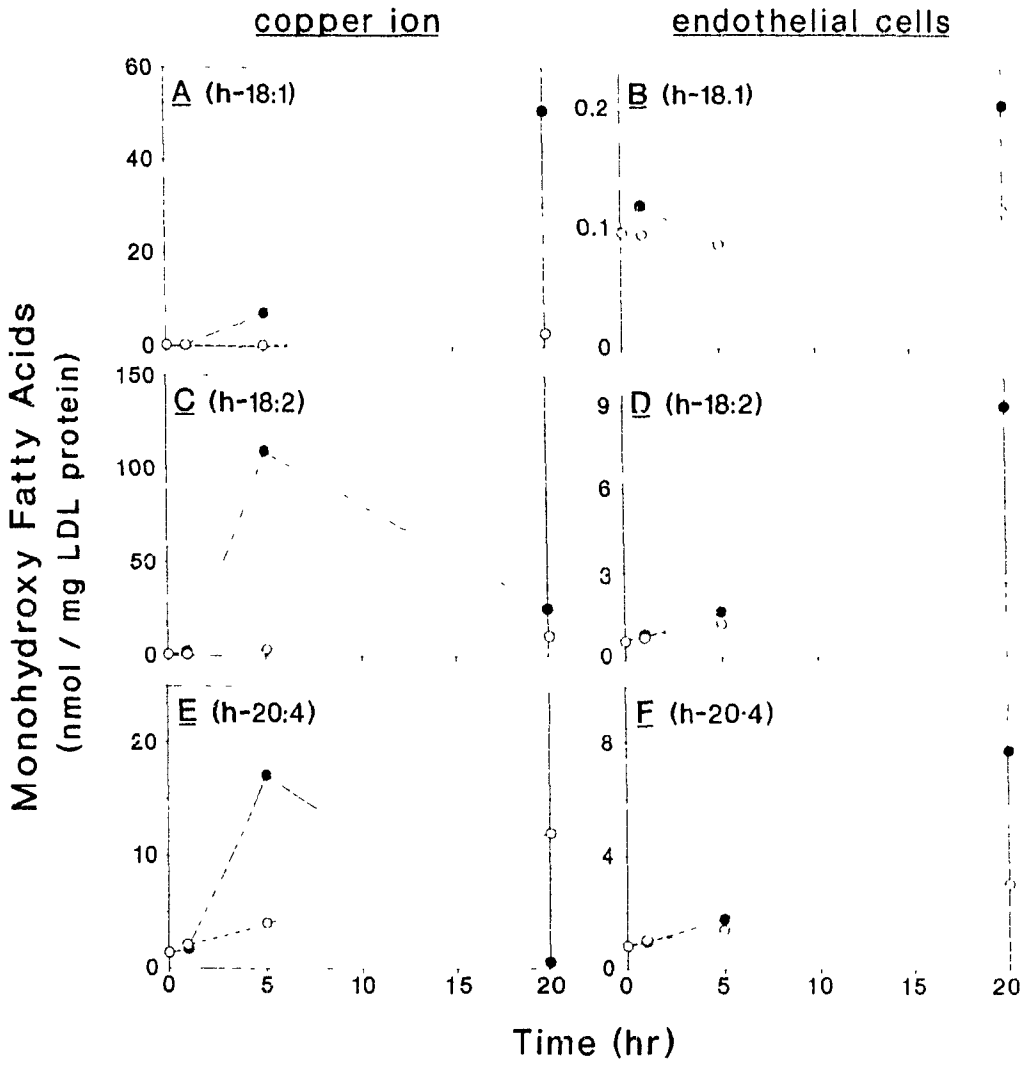

Fig. 26. Changes in the amounts of monohydroxy derivatives of unsaturated fatty acids in LDL incubated with $CuSO_4$ or with endothelial cells. Human LDL was incubated in Ham's F10 medium (○-- --○), or in Ham's F10 medium supplemented with 20 μM of $CuSO_4$ (●——●, A, C and E), or in Ham's F10 medium with human umbilical vein endothelial cells (●——●, B, D and F) for 1, 5 and 20 hours. Monohydroxy derivatives of oleic (A and B), linoleic (C and D) and arachidonic (E and F) acids esterified to LDL lipids were then prepared and measured by GC-MS. The values are averages of triplicates ($CuSO_4$) or duplicates (endothelial cells).

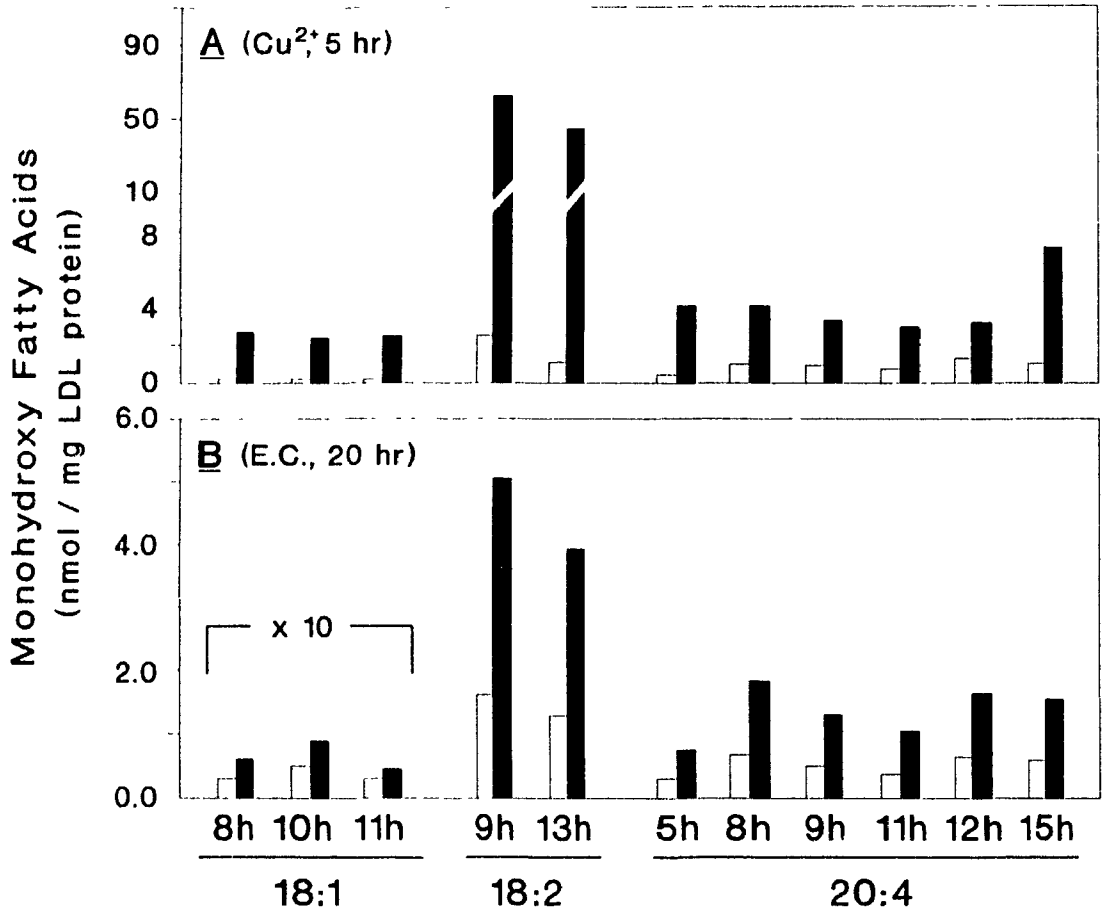

Fig. 27. Formation of individual monohydroxy derivatives of unsaturated fatty acids in human LDL incubated with $CuSO_4$ or with endothelial cells. Human LDL was incubated in Ham's F10 medium (open bars), or in Ham's F10 medium supplemented with 20 μM of $CuSO_4$ for 5 hours (A, solid bars), in Ham's F10 medium with human umbilical vein endothelial cells (B, solid bars) for 20 hours. Monohydroxy derivatives of unsaturated fatty acids esterified to LDL lipids were prepared and measured by GC-MS. The values are averages of triplicates (A) or duplicates (B). Note the change of scale above 10 nmol/mg protein in panel A.

autoxidation, a pattern similar to the one obtained in the previous experiment would be expected.

The amounts of monohydroxy fatty acids in LDL derived from 18:2 and 20:4 increased by 2 to 3 times after incubation with endothelial cells for 20 hours compared to the controls (**Fig. 26**; D and F) whereas the amounts of monohydroxy fatty acids derived from 18:1 were less than twice the amounts in the control after the same period (**Fig. 26B**). No differences were observed after either 1 or 5 hours of incubation.

The amounts of each individual monohydroxy fatty acid in LDL after incubation with endothelial cells for 20 hours are shown in **Fig. 27B**. The pattern was similar to that obtained when LDL was incubated with $CuSO_4$ for 5 hours (**Fig. 27A**). No selective increase of one positional isomer over other derived from the same fatty acid was observed.

3.1.2. Measurement of Other Parameters of Lipid Peroxidation in Oxidized LDL

To compare the measurement of monohydroxy fatty acids with other methods used for the evaluation of lipid peroxidation, peroxidation of LDL lipids in the presence of copper ions and endothelial cells was also evaluated by measurement of fatty acid content, as well as the formation of conjugated dienes and thiobarbituric acid-reactive substances (TBARS).

3.1.2.1. Loss of fatty acids in human LDL during oxidation

Fatty acids in LDL were extracted, separated from their monohydroxy derivatives by column chromatography and quantified by gas chromatography with a flame-ionization detector. Oxidation of human LDL in the presence of 20 μM $CuSO_4$ was accompanied by moderate decreases in stearic (**Fig. 28A**) and oleic (**Fig. 28C**) acids. In contrast, polyunsaturated fatty acids (linoleic and arachidonic acids) were completely depleted after 20 hours of incubation (**Fig. 28**; E and G). Incubation of LDL with endothelial cells resulted in only slight decreases in the amounts of all fatty acids in LDL (**Fig. 28**; B, D, F and H).

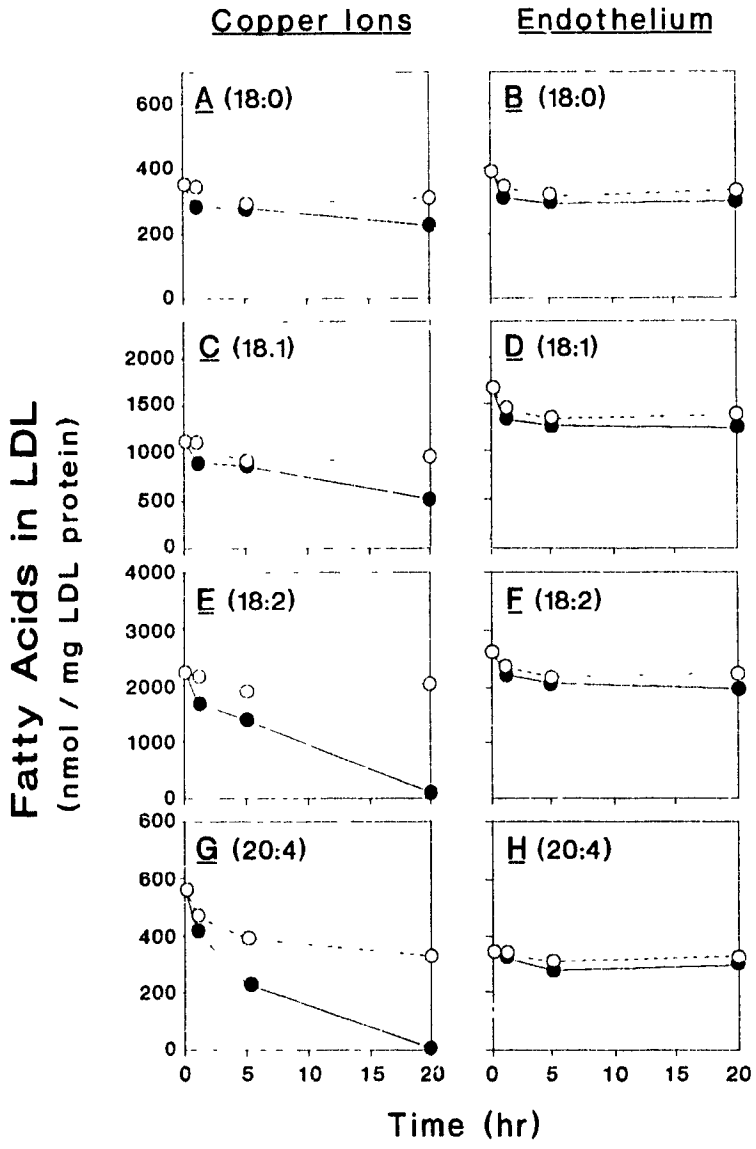

Fig. 28. Loss of fatty acids from human LDL during lipid peroxidation. Human LDL was incubated in Ham's F10 medium (o---------o), or in Ham's F10 medium supplemented with $CuSO_4$ (20 μM; •———•, A, C, E, G), or in Ham's F10 medium with endothelial cells (•———•, B, D, F, H) for 1, 5 and 20 hours. Fatty acids esterified to LDL lipids were extracted by the Folch method with methyl arachidic acid (20:0) as an internal standard, and measured by gas chromatography. Quantification of fatty acids was performed by comparing the peak areas for LDL fatty acids to the peak area for internal standard (20:0). The results are averages of triplicates ($CuSO_4$) or duplicates (endothelial cells).

3.1.2.2. Formation of conjugated dienes during oxidation of human LDL

Incubation of LDL with 20 μM $CuSO_4$ resulted in continuous increases in ultraviolet (UV) absorption around 234 nm and between 260 and 280 nm after 5 and 20 hours (**Fig. 29**). However, no increase in UV absorption at these wavelengths was observed in the case of incubation of LDL with endothelial cells up to 20 hours (data not shown).

3.1.2.3. Formation of TBARS during oxidation of human LDL

Malondialdehyde, considered as one of the end products formed during the oxidation of polyunsaturated fatty acids, was measured by the thiobarbituric acid (TBA) assay using spectrophotofluorometry. Since lipid peroxidation products other than malondialdehyde also react with TBA, the results are expressed as "thiobarbituric acid-reactive substances" (TBARS). TBARS increased continuously up to 20 hours in LDL incubated both with copper ions and with endothelial cells (**Fig. 30**).

3.1.3. Different Distribution of Monohydroxy Fatty Acids and TBARS

To investigate the distribution of lipid peroxidation products after oxidation of LDL, LDL was incubated with $CuSO_4$ and then reisolated by ultracentrifugation. Due to the loss of fatty acids, the density of LDL increased after the oxidation. Therefore, a solution with a density of 1.15 was necessary to recover the modified LDL [314]. Monohydroxy fatty acids in LDL and in incubation media were extracted and purified as described in **Section 2.3.4** (P. 61). TBARS were also measured in these two fractions. The results showed that over 98% of the monohydroxy fatty acids were found esterified to LDL lipids whereas about 80% of TBARS were present in the incubation medium (**Table 5**).

3.2. MONOHYDROXY DERIVATIVES OF FATTY ACIDS IN HYPERLIPIDEMIC RABBITS

It has been reported that atherosclerosis is associated with elevated lipid peroxidation in LDL and aorta as well as altered production of prostanoids by blood vessels. We therefore investigated the formation of monohydroxy fatty acids derived from oleic, linoleic and arachidonic acids in aorta and LDL and the relationship between

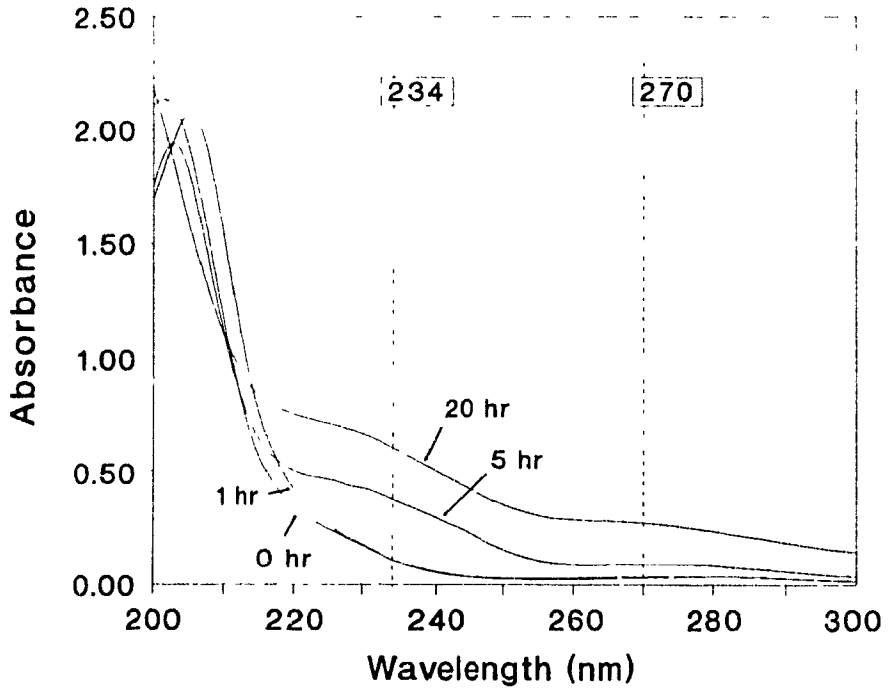

Fig. 29. Ultraviolet spectra of LDL incubated with $CuSO_4$. Human LDL was incubated in the presence of $CuSO_4$ (20 μM) for 1, 5 and 20 hours. LDL was diluted with phosphate-buffered saline and the ultraviolet spectra were recorded. The baseline was established by recording the spectrum of same incubation medium without LDL.

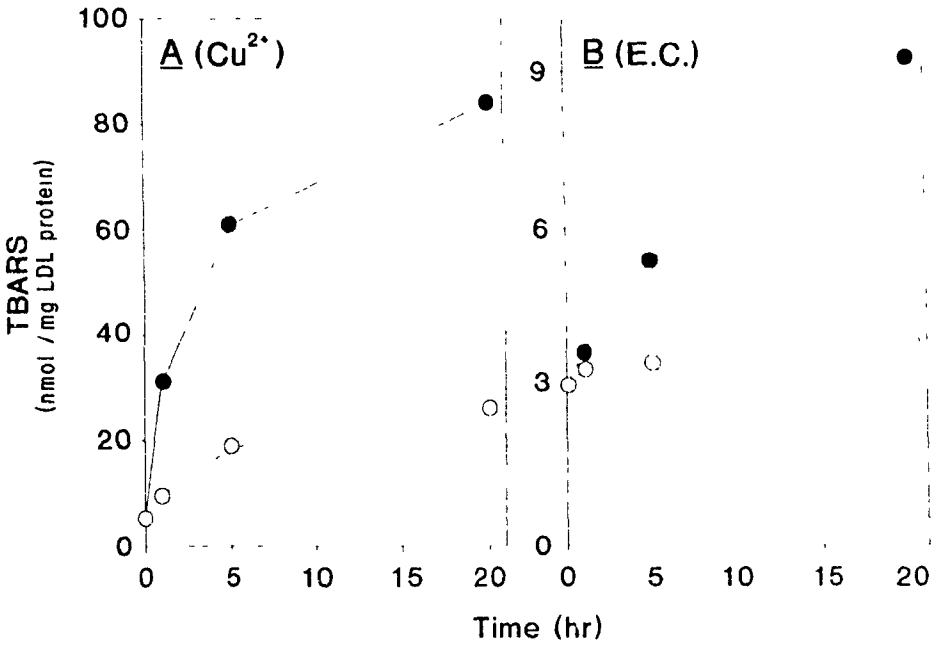

Fig. 30. Increase in the formation of thiobarbituric acid-reactive substances (TBARS) from LDL during lipid peroxidation. Human LDL was incubated in F10 medium (o o), in F10 medium supplemented with $CuSO_4$ (20 μM; •———•, A), or in F10 medium with endothelial cells (•———•, B) for 1, 5 and 20 hours The incubates were diluted with phosphate-buffered saline and the TBARS were measured using spectrophotofluorometry with an excitation wavelength at 515 nm and emission wavelength at 565 nm. The concentration of TBARS was calculated against a freshly-prepared calibration curve of 1,1,3,3-tetramethoxypropane. The values are averages of triplicates ($CuSO_4$) or duplicates (endothelial cells).

Table 5

Distribution of Monohydroxy Fatty Acids in LDL and Medium after Incubation with $CuSO_4$

LDL was incubated with 20 μM $CuSO_4$ for either 5 hours (a) or 20 hours (b) and then reisolated by ultracentrifugation. Monohydroxy derivatives of fatty acids in LDL and in medium were extracted and purified as described in **Section 2.3.4**. TBARS were also measured in these two fractions. All values are averages of three measurements and expressed as "nmole/mg LDL proteins".

	h-18:1/(b)		h-18:2(a)		h-20:4(a)		TBARS(b)	
	LDL	Medium	LDL	Medium	LDL	Medium	LDL	Medium
Native	0.15	0.20	0.72	0.17	1.50	0.13	0.30	1.82
Oxidized	50.26	0.35	109.56	0.19	17.13	0.30	11.95	48.35

lipid peroxidation and aortic prostanoid production in hypercholesterolemic rabbits.

Rabbits were fed either control diets (standard rabbit chow) or diets supplemented with cholesterol, peanut oil and BHA. After 2, 8 and 15 weeks, rabbits were housed in metabolic cages for 48 hours. The urine collected during the second 24 hours was extracted and the 2,3-dinor metabolites of PGI_2 and TXA_2 were measured. On the morning of the day when the rabbits were to be sacrificed, blood was collected from an ear vein and LDL was prepared. Aorta samples were then obtained after sacrificing the rabbits. The final numbers of rabbits from which samples were obtained for analysis are listed in **Table 6**. The area of aorta covered with lesions was evaluated visually by two individuals and the scores were averaged (**Table 7**).

3.2.1. Monohydroxy Fatty Acids in LDL from Cholesterol-fed Rabbits

Monohydroxy derivatives of fatty acids were measured in LDL from rabbits fed a high cholesterol and high fat diet. Among the monohydroxy fatty acids measured, those derived from 18:2 were the most abundant. The levels of monohydroxy derivatives of 20:4 in control LDL were too low to be detected at the time of this experiment. The total amounts of monohydroxy derivatives of 20:4, however, were 1.06, 2.97 and 19.26 ng/mg LDL protein, after 2, 8, and 15 weeks on a cholesterol-supplemented diet, respectively.

Monohydroxy derivatives of C_{18} fatty acids in LDL are shown in **Fig. 31**. The amounts of 9- and 13-hydroxy derivatives of linoleic acid in LDL reached about 100 ng/mg LDL protein after 2 weeks on a high cholesterol/high fat diet, and were about 2 to 3 times higher than controls (**Fig. 31A**). The amounts of monohydroxy derivatives from 18:2 in LDL from cholesterol-fed rabbits were also about twice the control values at 8 and 15 weeks (**Fig. 31**; B and C). In contrast, monohydroxy derivatives of oleic acid in LDL were not increased in the cholesterol-fed rabbit except for 8h-18:1 after 2 weeks (**Fig. 31A**). These derivatives were actually decreased after 8 weeks on a cholesterol diet (**Fig 31B**) and then returned to levels similar to the controls after 15 weeks (**Fig. 31C**).

When the above results were expressed as "ng monohydroxy fatty acids in LDL/ml plasma" to represent of total amounts of these products in plasma, the differences

Table 6

Numbers of Rabbits on Control and Cholesterol/Peanut Oil Diets from Which Samples Were Obtained

Rabbits were fed a control diet or a diet supplemented with 1% cholesterol, 10% peanut oil and 1% BHA (CHOL + PO) for 2, 8 and 15 weeks. Urine was collected for a 24-hour period for the analysis of dinor metabolites of PGI_2 and TXA_2. LDL was prepared from these rabbits for the measurement of fatty acids and their monohydroxy derivatives. Sections of aortic tissues (S_1, T_1, S_2 and T_2, see **Fig. 21** for the location of these sections) were also used to measure fatty acids and their monohydroxy derivatives as well as prostanoids. The numbers of rabbits from which each of the above samples were taken are shown in this table.

		2 week	8 week	15 week
Rabbits (survived/purchased)				
	Control	9/10	7/7	7/7
	CHOL + PO	7/8	5/8	7/12
Aorta				
S_1, S_2	Control	9	6	7
	CHOL + PO	8	5	7
T_1, T_2	Control	7	4	7
	CHOL + PO	6	5	7
LDL				
	Control	9	6	7
	CHOL + PO	8	5	7
Urine				
	Control	9	6	6
	CHOL + PO	6	5	7

Table 7

Lesions in Aorta from Hyperlipidemic Rabbits

Rabbits were fed a diet supplemented with 1% cholesterol, 10% peanut oil, 1% BHA and 1% ethanol for 2, 8 and 15 weeks, respectively. The areas covered with lesions in different sections of the aortae were evaluated visually by two individuals and were averaged. The values are averages of 5 - 7 samples with the range shown in brackets. Aortic total cholesterol concentration was measured by gas chromatography with 16 μg of 5ß-cholestane-3α-ol as an internal standard. The values are means $\pm$ S.E.M.. * $p < 0.05$, ** $p < 0.001$, compared to the corresponding controls using Student's t-test.

	TIME OF CHOLESTEROL DIET	
	8 Weeks	15 Weeks
Visual Evaluation (%)		
S_1	56 (20 - 90)	94 (80 - 100)
T_1	38 (10 - 90)	93 (80 - 100)
S_2	26 (10 - 50)	53 (10 - 100)
T_2	14 (10 - 20)	50 (10 - 100)
Total Cholesterol (µg/mg protein)		
Control	10.66 ± 1.21	16.43 ± 1.65
Cholesterol	123.98 ± 41.46 *	378.93 ± 38.66 **

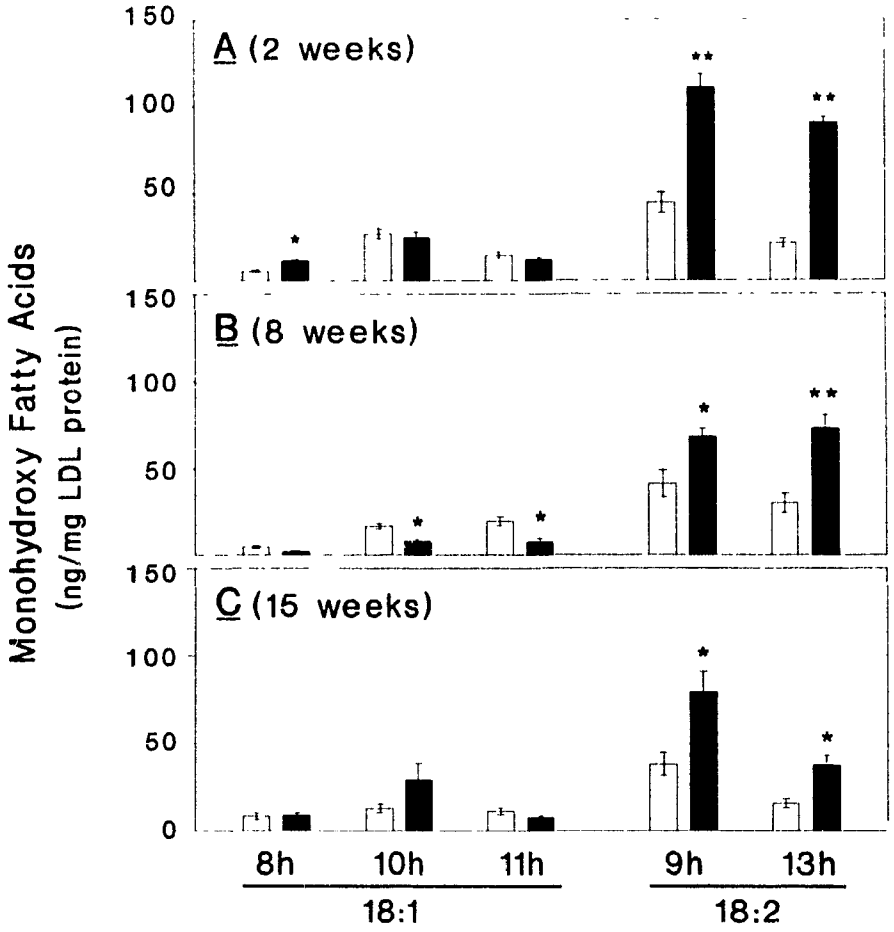

Fig. 31. Amounts of monohydroxy derivatives of C_{18} fatty acids in LDL from control and hyperlipidemic rabbits. LDL from rabbits on either a control (open bars) or a cholesterol-supplemented (solid bars) diet for either 2 (A), 8 (B) or 15 weeks (C) was extracted using the Folch method. Monohydroxy derivatives of fatty acids esterified to LDL lipids were measured by GC-MS. The values are means ± S.E.M.. Statistical differences between control and hyperlipidemic rabbits in the amounts of monohydroxy derivatives of fatty acids in LDL were calculated using Student's *t*-test. * $P < 0.05$, ** $P < 0.01$.

between control and cholesterol treatment became more profound, especially after 8 and 15 weeks (**Fig. 32**) because of the elevation of LDL in plasma as a result of the cholesterol diet (**Table 8**). This was true for monohydroxy derivatives of both oleic and linoleic acids.

3.2.2. Monohydroxy Fatty Acids in Aorta from Hyperlipidemic Rabbits

Slices of aortic arch (S_1) were incubated at 37 °C for 30 minutes and monohydroxy fatty acids in the medium and in the aortic slices were extracted on an ODS-silica Sep-Pak and by Folch method, respectively. Internal standards were added prior to extraction in both cases. The monohydroxy fatty acids were then derivatized and purified for GC-MS analysis. Unlike the study on oxidation of human LDL, only 11h-20:4, 12h-20:4 and 15h-20:4 rather than all six HETEs were measured in this study since previous studies done in our laboratory showed that the dominant monohydroxy derivatives of arachidonic acid in rabbit aorta were 11-20:4, 12-20:4 and 15-20:4, along with smaller amounts of 5-20:4, 8h-20:4 and 9h-20:4.

3.2.2.1 Release of Monohydroxy Fatty Acids into the Medium

The amounts of monohydroxy derivatives of fatty acids released into the medium after incubation of slices of aortic arch for 30 min at 37 °C are shown in **Fig. 33**. There were no differences between control and treated rabbits after 2 weeks on a cholesterol diet (**Fig. 33A**). After 8 weeks on the cholesterol diet, the amounts of monohydroxy derivatives of linoleic and arachidonic acids were higher in the cholesterol-treated group. However, the differences between the two groups were statistically significant only in the cases of the 11- and 15-hydroxy derivatives of arachidonic acids (**Fig. 33B**). Similar results were obtained after 15 weeks on the cholesterol diet, but in this case, only 9h-18:2, 13h-18:2 and 15h-20:4 were significantly higher in the cholesterol-fed group (**Fig. 33C**). There were no differences between the control and cholesterol-treated group in the amounts of monohydroxy derivatives of oleic acid released into the medium at all time investigated.

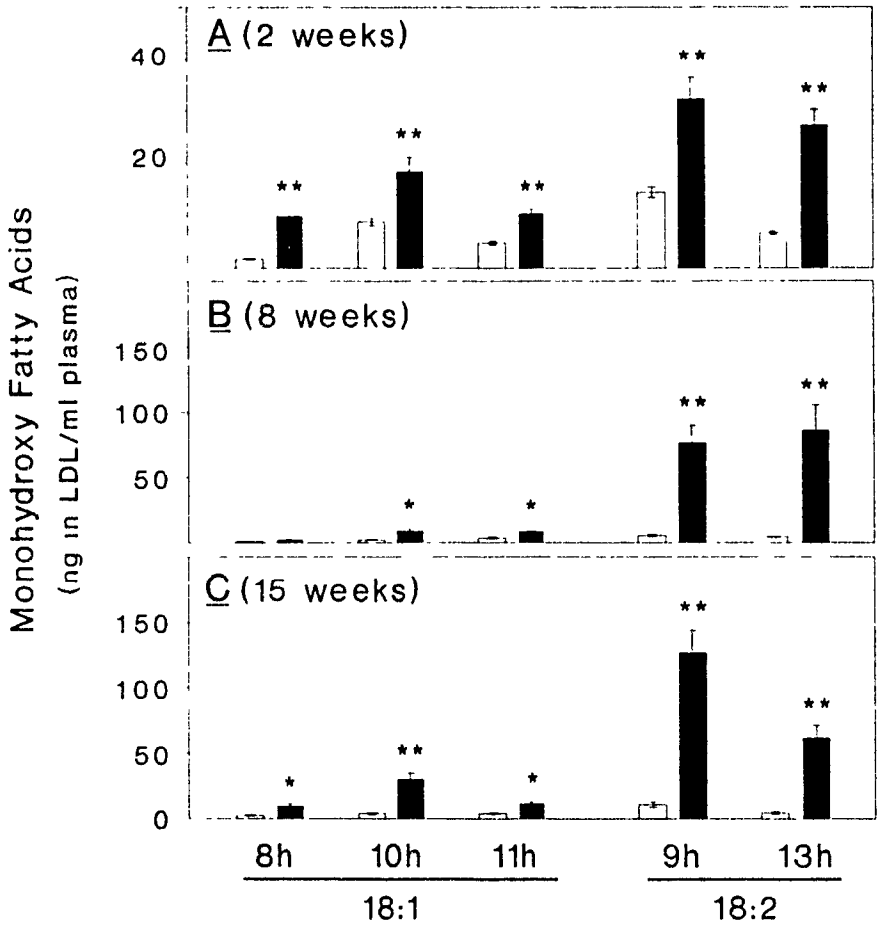

Fig. 32. Amounts of LDL-associated monohydroxy derivatives of C_{18} fatty acids in plasma from control and hyperlipidemic rabbits. Monohydroxy derivatives of C_{18} fatty acids in LDL from rabbits on either a control (open bars) or a cholesterol-supplemented (solid bars) diet for either 2 (**A**), 8 (**B**) or 15 weeks (**C**) were quantified by GC-MS. The concentrations of monohydroxy derivatives are expressed as "ng in LDL per ml of plasma" instead of "ng per mg of LDL protein" as shown in Fig. 31 to represent the concentrations of these LDL-associated derivatives in plasma. Statistical differences between control and hyperlipidemic rabbits in the amounts of these derivatives were calculated using Student's *t*-test. * $P < 0.05$, ** $P < 0.01$.

Table 8

LDL Concentration in Plasma from Hyperlipidemic Rabbits

Rabbits were fed a control diet or a diet supplemented with 1% cholesterol, 10% peanut oil, 1% BHA and 1% ethanol for 2, 8 and 15 weeks. LDL protein concentration was quantified by a modification of the Lowry method. The values, expressed as "mg LDL protein/ml plasma", were corrected according the final volume of LDL and the original volume of plasma. Statistical analysis was performed by 2-way ANOVA followed by a post-hoc test (simple contrast) to compare the means of pairs at the same time. All three effects in 2-way ANOVA, *i.e.*, group effect, time effect and group x time effect are highly significant ($P < 0.01$).

	LDL proteins (mg/ml plasma)	
	Control	Cholesterol
2 weeks	0.18 ± 0.02	0.43 ± 0.04
8 weeks	0.21 ± 0.03	1.47 ± 0.25
15 weeks	0.32 ± 0.06	1.67 ± 0.21

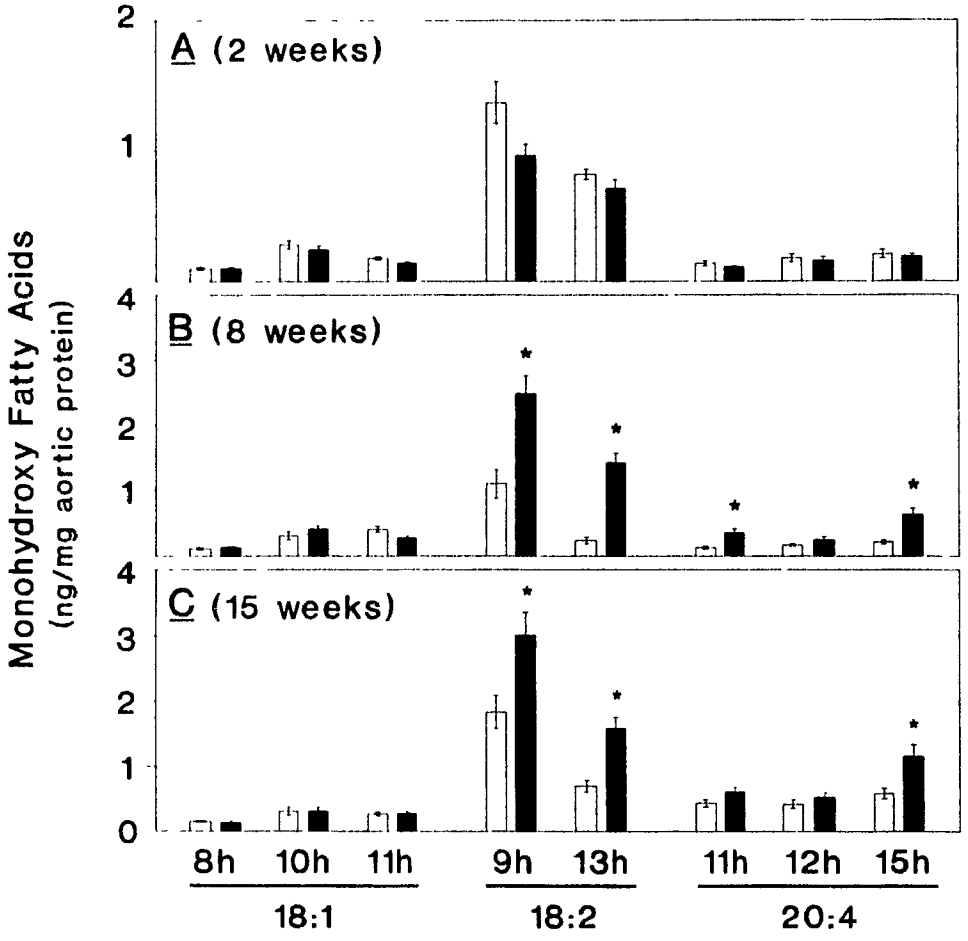

Fig. 33. Amounts of monohydroxy derivatives of fatty acids released by aortae of control and hyperlipidemic rabbits. After 2, 8 and 15 weeks on a cholesterol diet (A, B and C, respectively), slices of aortic arches (S_1) from control (open bars) or cholesterol-fed rabbits (solid bars) were incubated in Tris-HCl buffer for 30 minutes at 37 °C. Monohydroxy derivatives of fatty acids in the incubation medium were extracted on an ODS-silica Sep-Pak and the quantified by GC-MS. The values are expressed as means ± S.E.M.. Statistical differences between control and hyperlipidemic rabbits in the amounts of these derivatives were calculated using Student's *t*-test. * $P < 0.05$. Note that the scale of panel A is only half that of the other panels.

3.2.2.2. Esterified Monohydroxy Fatty Acids in Rabbit Aorta

The amounts of monohydroxy C_{18} fatty acids esterified to aortic lipids were about 10 times greater than the amounts released into the medium. After 2 weeks, monohydroxy derivatives of oleic acid (11h-18:1) and linoleic acid (9h-18:2 and 13h-18:2) were lower in aorta from cholesterol-fed rabbits than from controls (**Fig. 34A**). However, after 8 weeks, the amounts of all hydroxy derivatives of C_{18} fatty acids except for 11h-18:1 were about 2 to 3 times higher than the corresponding control (**Fig. 34B**). The amounts of 8h-18:1, 10h-18:1 and 9h-18:2 were still considerably elevated after 15 weeks on a cholesterol diet, but there was no significant difference in aortic 13h-18:2 between hypercholesterolemic and control rabbits (**Fig. 34C**).

Unlike the situation with the hydroxy derivatives of C_{18} fatty acids, the amounts of esterified monohydroxy derivatives of arachidonic acid were only about twice as high as the amounts measured in the incubation medium. Furthermore, there were no significant differences in the amounts of esterified monohydroxy derivatives of 20:4 in aorta from the two groups until 15 weeks on a cholesterol-supplemented diet, at which time the amounts of all of the monohydroxy derivatives of arachidonic acid were markedly higher in the cholesterol-fed rabbits (**Fig. 34C**).

3.2.3. Esterified Fatty Acids in Aorta

The above results immediately pose a question: can the changes in esterified monohydroxy fatty acids in aortae from cholesterol-fed rabbits be explained solely by changes in the levels of their fatty acid precursors? To answer this question, the amounts of esterified fatty acids in aortae from control and cholesterol-fed rabbits were measured by gas chromatography using a DB-23 capillary column.

Fig. 35 shows typical GC chromatograms of the fatty acid compositions of aortae from control and cholesterol-fed rabbits after 15 weeks on the diets. At this time, it is evident that there is an increased percentage of 18:1 and a decreased percentage of 20:4 compared to other fatty acids in the aortae from cholesterol-fed rabbits.

The amounts of oleic, linoleic and arachidonic acids are shown in **Fig. 36** (D, E and F). The value for the monohydroxy derivatives of 18:1 (the combined amounts of

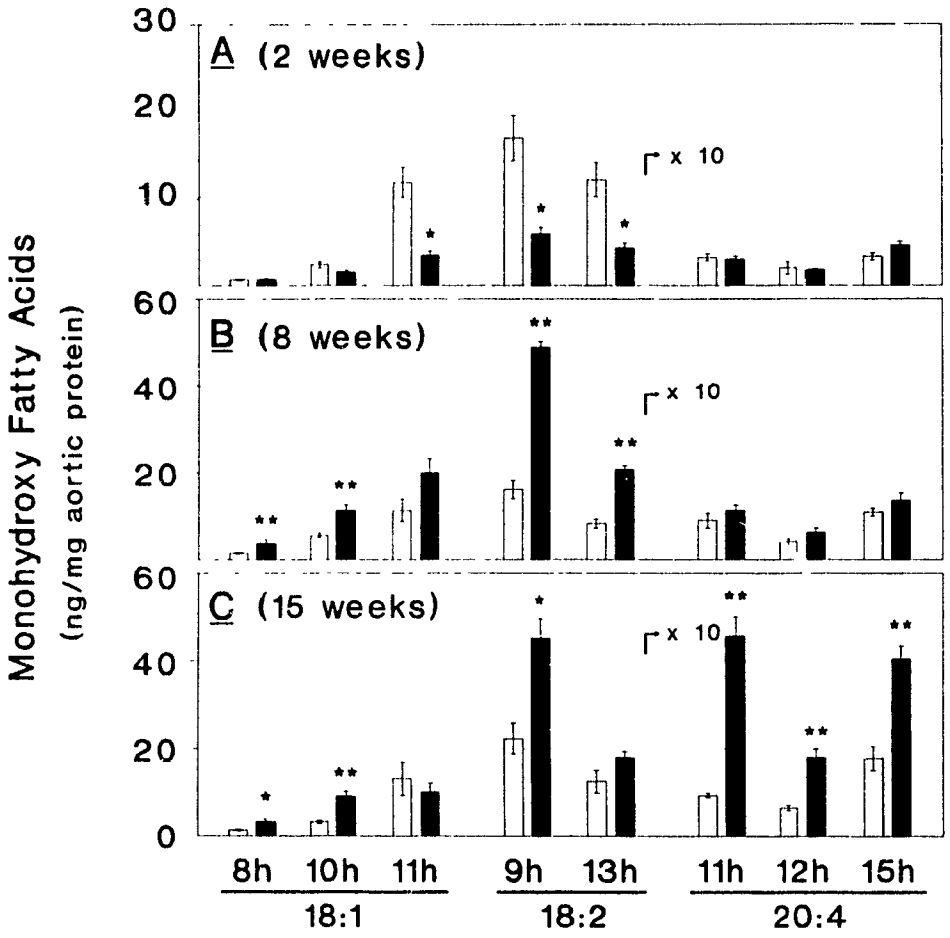

Fig. 34. Amounts of esterified monohydroxy derivatives of fatty acids in aortae of control and hyperlipidemic rabbits. After 2, 8 and 15 weeks on a cholesterol-supplemented diet (A, B and C, respectively), slices of aortic arches (S_1) of control (open bars) or cholesterol-fed rabbits (solid bars) were incubated in Tris-HCl buffer for 30 minutes at 37 °C. Monohydroxy derivatives of fatty acids esterified to the aortic lipids were extracted using the Folch method and then quantified by GC-MS. The values are expressed as means ± S.E.M.. Statistical differences between control and hyperlipidemic rabbits in the amounts of these derivatives were calculated using Student's *t*-test. * $P < 0.05$, ** $P < 0.01$. Note that the scale of panel A is only half that of the other panels and that the values for monohydroxy derivatives of arachidonic acid have been multiplied by 10.

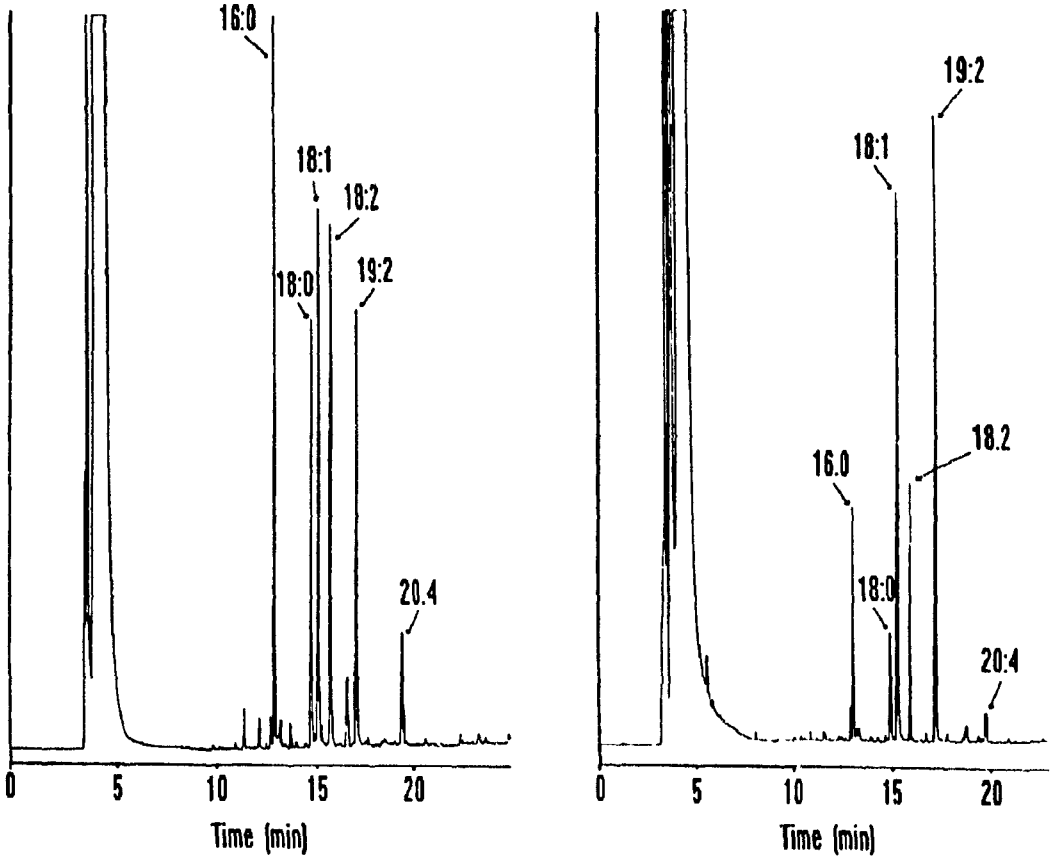

Fig. 35. Gas chromatograms of fatty acids esterified to aortic lipids from control and hyperlipidemic rabbits. Aortic lipids from control (left) and hyperlipidemic (right) rabbits were extracted and analyzed by gas chromatography. The temperature program was identical to that in the legends to **Fig. 19** on P. 67.

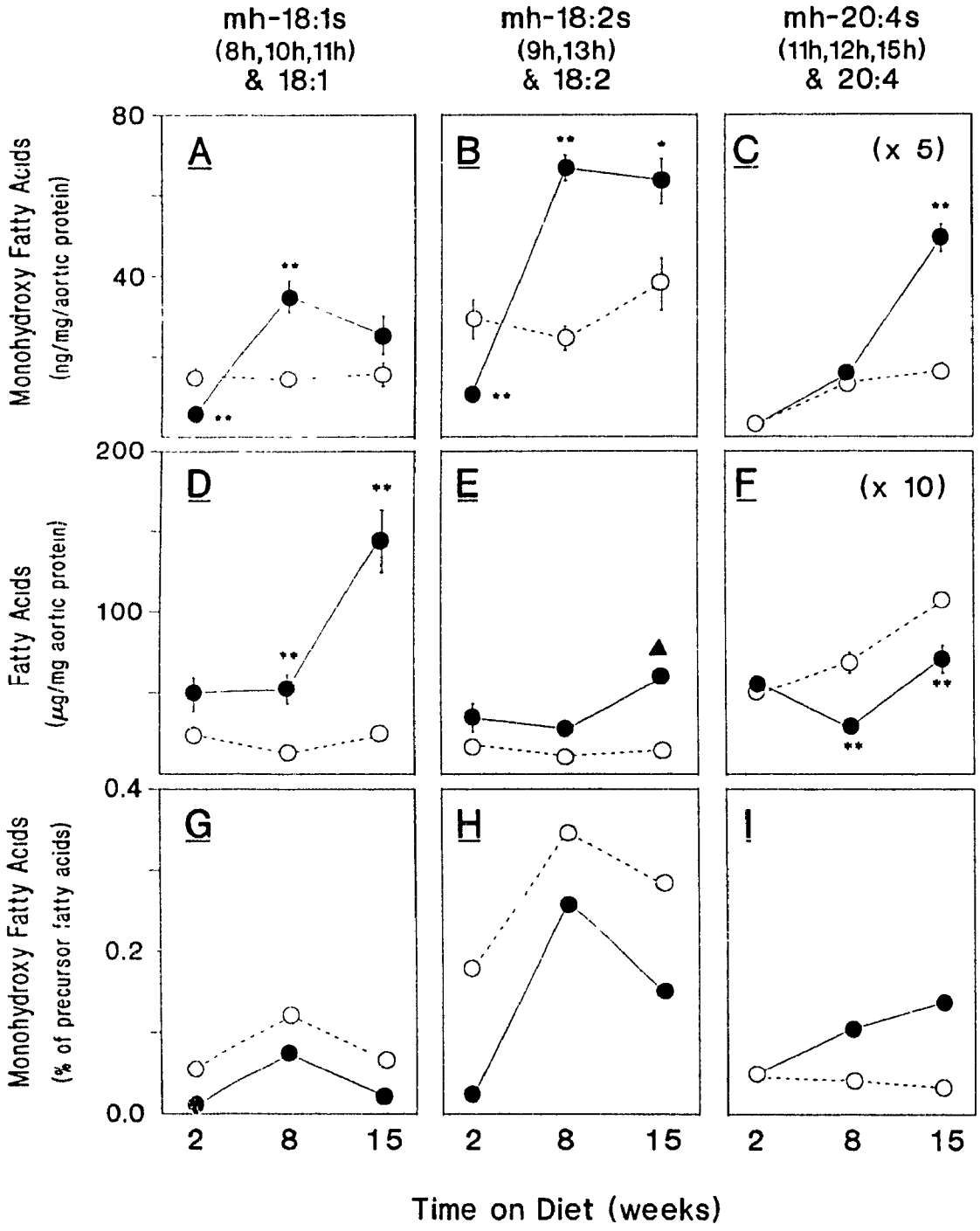
mh-18:1s
(8h,10h,11h)
& 18:1
mh-18:2s
(9h,13h)
& 18:2
mh-20:4s
(11h,12h,15h)
& 20:4
Monohydroxy Fatty Acids
(ng/mg/aortic protein)
Fatty Acids
(μg/mg aortic protein)
Monohydroxy Fatty Acids
(% of precursor fatty acids)
A
B
C
(x 5)
D
E
F
(x 10)
G
H
I
80
40
200
100
0.4
0.2
0.0
2
8
15
Time on Diet (weeks)

Fig. 36. Amounts of esterified fatty acids and their monohydroxy derivatives in aortae from control and hyperlipidemic rabbits. The total amounts of esterified monohydroxy derivatives of 18:1 (A), 18:2 (B) and 20:4 (C) in aorta from control (○–○) and cholesterol-fed (●–●) rabbits were calculated by adding the values for each of the individual products shown in **Fig. 34**. The amounts of their fatty acid precursors in the same tissue are shown in panels D, E and F. The percentage of esterified fatty acids present in the forms of their monohydroxy metabolites in aorta from the two groups of rabbits are shown in panels G, H and I. Data are expressed as means ± S.E.M. for fatty acids and their monohydroxy derivatives (panels A to F), and as averages of the ratios of monohydroxy derivatives to their fatty acid precursor (panels G to I). The statistical differences between control and cholesterol-fed rabbits in fatty acids and their monohydroxy derivatives were assessed by two-way analysis of variance (using treatment and time on diets as two independent variables) using the regression approach. Post-hoc tests were done using simple contrast based on design matrix to compare the differences between control and treated groups at each particular time period.

8h-18:1, 10h-18:1 and 11h-18:1), 18:2 (the combined amounts of 9h-18:2 and 13h-18:2) and 20:4 (the combined amounts of 11h-20:4, 12h-20:4 and 15h-20:4) are also shown in **Fig. 36** (A, B and C). The percentages of oleic, linoleic and arachidonic acids in the form of their monohydroxy derivatives were calculated from the above data and are also shown in **Fig. 36** (G, H and I).

The amounts of 18:1 and 18:2 were about 3 to 4 times higher in cholesterol-fed rabbits than in the control rabbits in all time points (**Fig. 36**; D and E). Consequently, the percentages of 18:1 and 18:2 present as their monohydroxy derivatives were lower in the cholesterol-fed group even though the absolute amounts of these monohydroxy fatty acids were higher after 8 and 15 weeks on the diet (**Fig. 36**; G and H). In comparison, the aortic content of arachidonic acid was not changed after 2 weeks on the diet and significantly lower in the cholesterol-fed group after 8 and 15 weeks (**Fig. 36F**). Thus, although there were no significant differences in the absolute amounts of monohydroxy C_{20} fatty acids after 8 weeks (**Fig. 36C**), the percentage of 20:4 present in the form of its monohydroxy derivatives was significantly higher in aortae from hypercholesterolemic rabbits (**Fig. 36I**). After 15 weeks, increased amounts of monohydroxy 20:4 were accompanied by decreased amounts of their fatty acid precursor so that the percentage of 20:4 oxidized in aorta from cholesterol-fed rabbits was the highest after 15 weeks (**Fig. 36I**). Interestingly, the percentage of arachidonic acid in the form of its monohydroxy derivatives in control rabbit aorta was rather low compared to that of linoleic acid in the same tissue. However, after 15 weeks on a cholesterol diet, the percentages of linoleic and arachidonic acids as their monohydroxy derivatives were about the same.

3.2.4. Correlation of the Amounts of Monohydroxy Derivatives of Arachidonic Acid in Aorta with the Severity of Atherosclerotic Lesions

As mentioned above, the percentage of 20:4 present in the form of its monohydroxy derivatives was significantly higher in aortae from hypercholesterolemic rabbits after 8 and 15 weeks on the diet whereas the opposite was true for 18:1, 18:2 and their

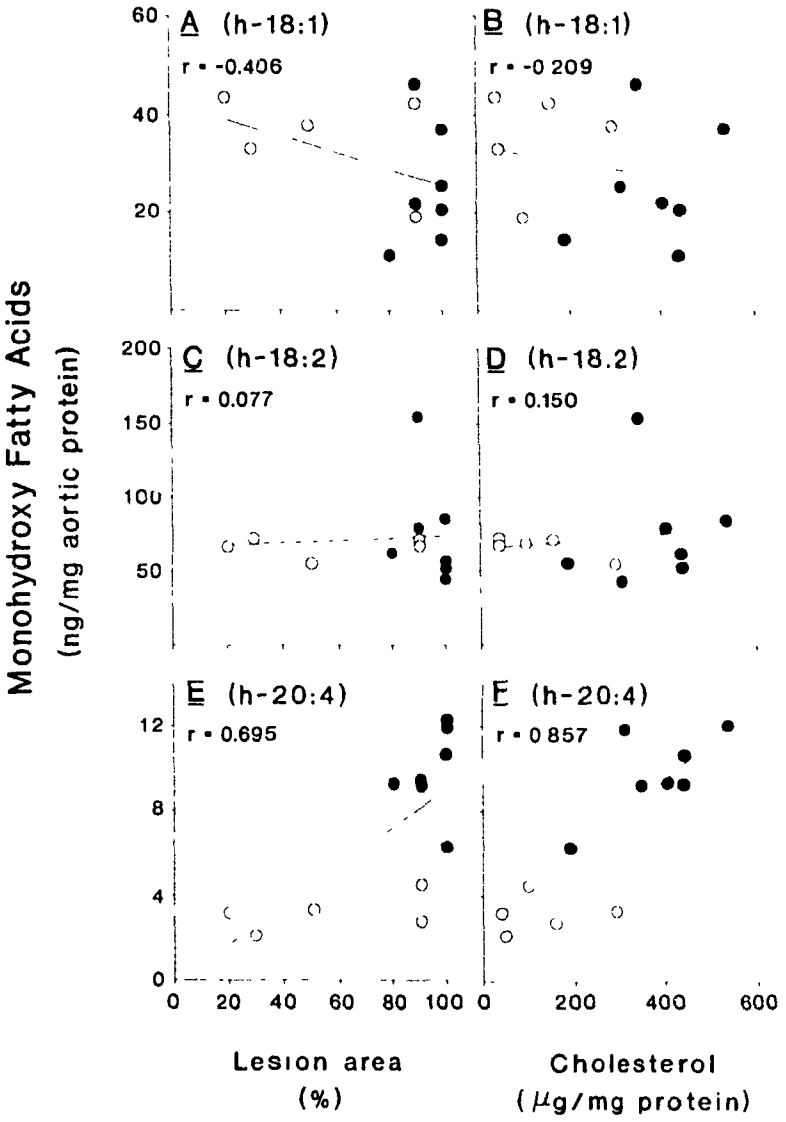

Fig. 37. Correlations between monohydroxy derivatives of fatty acids esterified to aortic lipids and severity of atherosclerotic lesions in aorta from hyperlipidemic rabbits. Rabbits were fed a cholesterol-supplemented diet for 8 (open circles) or 15 (closed circles) weeks. Monohydroxy metabolites of fatty acids esterified to the aortic lipids were quantified by GC-MS. Severity of atherosclerotic lesions was evaluated either by visual estimation of the areas of aortic lumen covered by lipid plaque (panels A, C and E) or by measurement of total aortic cholesterol (panels B, D and F). Correlation between monohydroxy derivatives of fatty acids and severity of atherosclerotic lesions was calculated using Pearson's product-moment correlations (Pearson's *r*).

monohydroxy derivatives. This would suggest that there is a closer relationship between monohydroxy derivatives of arachidonic acid and the severity of atherosclerotic lesions.

The severity of atherosclerotic lesions was assessed by two methods, visual evaluation of the area of the aortic arch covered with atherosclerotic lesions and the total cholesterol content of the aortic arch. Pearson product-moment correlations (Pearson *r*) were used to estimate the correlations between the amounts of esterified monohydroxy fatty acids in aorta and the severity of atherosclerotic lesions. Scatter plots with regression lines for the total monohydroxy derivatives of each fatty acid are shown in **Fig. 37**. The amounts of esterified monohydroxy derivatives of 18:2 were not related (**Fig. 37**; C and D), whereas monohydroxy derivatives of 18:1 were weakly negatively correlated (**Fig. 37**; A and B) to the severity of atherosclerotic lesions. In other words, in aortae with atherosclerotic lesions, the amounts of monohydroxy C_{18} derivatives were either not changed (18:2), or tended to drop (18:1) as the severity of the atherosclerotic lesions increased. In contrast, positive correlations were observed between the amounts of esterified monohydroxy 20:4 in aorta and the severity of atherosclerotic lesions, particularly with regard to total aortic cholesterol.

3.2.5. Fatty Acids in LDL from Cholesterol-fed Rabbits

Typical GC chromatograms of fatty acids from LDL of controls and cholesterol-fed rabbits after 15 weeks on the diets, respectively, are shown in **Fig. 38**. The total amounts of monohydroxy derivatives of 18:1, 18:2 and 20:4 and their fatty acid precursors in LDL are listed in **Table 9**. Fatty acids in LDL after 2 weeks on the diet were, unfortunately, not measured.

In spite of a 100% increase in 18:1 in LDL from cholesterol-fed rabbits, there were no changes in the amounts of monohydroxy derivatives of 18:1 in LDL. Therefore the ratio of monohydroxy derivatives of 18:1 to its fatty acid precursor 18:1 was decreased after the cholesterol diet, as was observed for aorta. On the other hand, an increase of 18:2 in LDL from cholesterol-fed rabbits was accompanied by a parallel increase of monohydroxy 18:2 in the same sample. Consequently there was no difference in the ratio

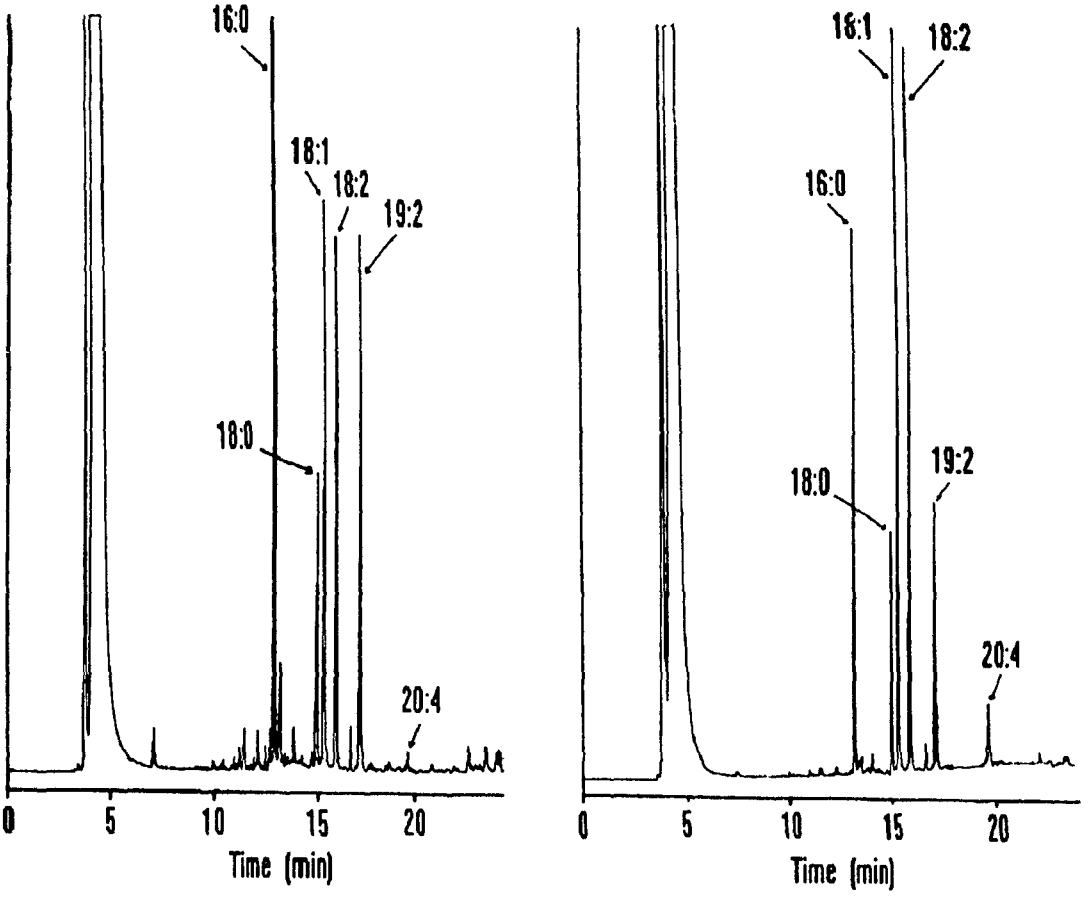

Fig. 38. Gas chromatograms of fatty acids esterified to lipids in LDL from control and hyperlipidemic rabbits. Lipids in LDL from control (left) and hyperlipidemic (right) rabbits were extracted and then analyzed by gas chromatography. The temperature program was shown in the legend to **Fig. 19** on P. 67.

Table 9

Fatty Acids and Their Monohydroxy Derivatives in LDL from Rabbits Fed Diets Supplemented with Cholesterol and Peanut Oil

Rabbits were fed a control diet or a diet supplemented with cholesterol, peanut oil and BHT for 8 and 15 weeks. Fatty acids and their monohydroxy derivatives in LDL were prepared, derivatized, purified and measured by GC and GC-MS, respectively. Data are averages of 5 - 7 LDL samples with S.E.M.. Statistical analyses on the effects of the cholesterol diet over different time periods were performed by two-way analysis of variance (ANOVA) on either the original data or the natural logarithmic-transformed data depending on the variances of the groups. The sum of squares were decomposed using the regression approach because of the unequal group sizes of the experiment. N.D., not detectable. N.A., not available. § $P < 0.01$, GROUP effect and INTERACTION or ¶ $P < 0.01$, GROUP effect using two-way ANOVA. * $P < 0.01$, compared to the control group at the same period using Student's *t* -test as a post-hoc test. (a) μg/mg LDL protein. (b) ng/mg LDL protein.

	8 week		15 week	
	Control	Cholesterol	Control	Cholesterol
18:1 & h-18:1				
18:1 (a)¶	401 ± 53	957 ± 31	402 ± 83	1080 ± 47
h-18:1 (b)§	39.7 ± 6.0	17.3 ± 2.8*	35.6 ± 7.9	34.3 ± 5.5
Ratio (‰)¶	.102 ± .016	.018 ± .000	.127 ± .036	.032 ± .005
18:2 & h-18:2				
18:2 (a)¶	292 ± 34	738 ± 48	447 ± 69	868 ± 58
h-18:2 (b)¶	70.9 ± 12.9	140.9 ± 12.1	58.9 ± 9.0	129.6 ± 14.9
Ratio (‰)	.242 ± .040	.194 ± .019	.145 ± .031	.187 ± .030
20:4 & h-20:4				
20:4 (a)	N.D.	29.6 ± 3.1	28.2 ± 3.7	45.1 ± 2.1
h-20:4 (b)	N.D.	2.97 ± .37	N.D.	19.3 ± 5.6
Ratio (‰)	N.A.	.101 ± .017	N.A.	.312 ± 071

of monohydroxy 18:2 to 18:2 between the control and cholesterol-fed rabbits. Cholesterol feeding led to an elevation of both 20:4 and its monohydroxy derivatives in LDL. Since monohydroxy derivatives of arachidonic acid could not be detected in control LDL, we could not calculate the percentage of arachidonic acid as its monohydroxy derivatives. However, the percentage of arachidonic acid as its monohydroxy derivatives double between 8 and 15 weeks on a cholesterol diet (**Table 9**).

3.2.6. Distribution of Esterified Monohydroxy Fatty Acids in Neutral and Polar Lipids from Rabbit Aorta

One question to be answered is what causes the differences between monohydroxy derivatives from C_{18} fatty acids and from arachidonic acid in terms of the percentage of the fatty acid which is oxidized and the correlation between formation of monohydroxy fatty acids and progression of atherosclerotic lesions. One of the possible reasons is that there may be differences in the distribution of these fatty acids in different lipid classes. Fatty acids in different lipid fractions, probably due to their localization in the cells or tissues, may have different susceptibilities to free radical attack. Another question which we needed to answer was whether the peanut oil, which was included with cholesterol in the high cholesterol diet in the above experiment, contributed to the changes which we observed in monohydroxy fatty acids To answer these questions, I conducted an experiment in collaboration with Dr. Wen-qui Yu in our laboratory in which rabbits were fed diets consisting of (1) standard rabbit chow, (2) chow supplemented with 1% cholesterol, and (3) cholesterol supplemented with 1% cholesterol, 10% peanut oil and 1% BHT for 13 weeks. Lipids were extracted from the aortae of the rabbits and then separated into neutral (cholesteryl esters and triglycerides) and polar (mainly phospholipids) fractions by chromatography on a silicic acid column. The amounts of esterified 18:2 and 20:4 and their monohydroxy derivatives in these two fractions were measured by GC and GC-MS, respectively.

Similar to the previous experiment discussed in **Section 3.2.2.2.** (see P. 105), the total amounts of aortic monohydroxy derivatives (combination of monohydroxy derivatives in neutral lipids and in polar lipids) of linoleic and arachidonic acids were

increased after 13 weeks on diets supplemented with cholesterol and peanut oil (**Fig. 39B**). Total monohydroxy fatty acids were also increased to a similar extent in rabbits on a diet supplemented with cholesterol alone, suggesting that the increased amounts of monohydroxy derivatives of fatty acids in aortae from rabbits on diets supplemented with cholesterol and peanut oil are not due to the increased dietary intake of fatty acids or their hydroxy derivatives in the peanut oil.

Fig. 39 also shows the distribution of aortic linoleic and arachidonic acids and their monohydroxy derivatives in neutral and polar lipid classes. In control aorta, linoleic acid was almost exclusively localized in neutral lipids (**Fig. 39A**; 18:2 (Ctrl)), but its monohydroxy derivatives were evenly distributed between neutral and polar lipids (**Fig. 39B;** h-18:2 (Ctrl)). Therefore, the proportion of linoleic acid as its monohydroxy derivatives was much greater in polar lipids (0.448% $\pm$ 0.116%) than in neutral lipids (0.049% $\pm$ 0.008%) (**Table 10**). After cholesterol supplementation, the amounts of linoleic acid in both polar and neutral lipids were elevated 6 times over the controls (**Fig. 39A**; 18:2 (C) and 18:2 (C/P)). The amounts of its monohydroxy derivatives, however, were increased to a less extent (about 4 folds) in neutral lipids and unchanged in polar lipids in cholesterol-fed rabbits (**Fig. 39**; h-18:2 (C/P). The proportion of linoleic acid as its monohydroxy derivatives in both neutral and polar lipid fractions was therefore decreased after cholesterol or cholesterol/peanut oil-supplemented diets (**Table 10**).

Both arachidonic acid (**Fig. 39A**, 20:4 (Ctrl)) and its monohydroxy derivatives were almost exclusively localized in polar lipids (**Fig. 39B**; h-20:4 (Ctrl)) of aortae from control rabbits. The proportion of arachidonic acid as its monohydroxy derivatives was 0.014% $\pm$ 0.023% in polar lipids whereas the proportion in neutral lipids was not calculated since the amounts of monohydroxy derivatives of arachidonic acid in neutral lipids were under the detection limit of the assay (**Table 10**), which was about 0.01 ng/mg protein. After cholesterol treatment, the amounts of aortic arachidonic acid were increased slightly in neutral lipids but either unchanged (**Fig. 39A**; 20:4 (C)) or decreased (**Fig. 39A**; 20:4(C/P)) in polar lipids. The amounts of monohydroxy derivatives of arachidonic acid in polar lipids were slightly increased as was the case for the monohydroxy derivatives of linoleic acid. Therefore, although the proportion of

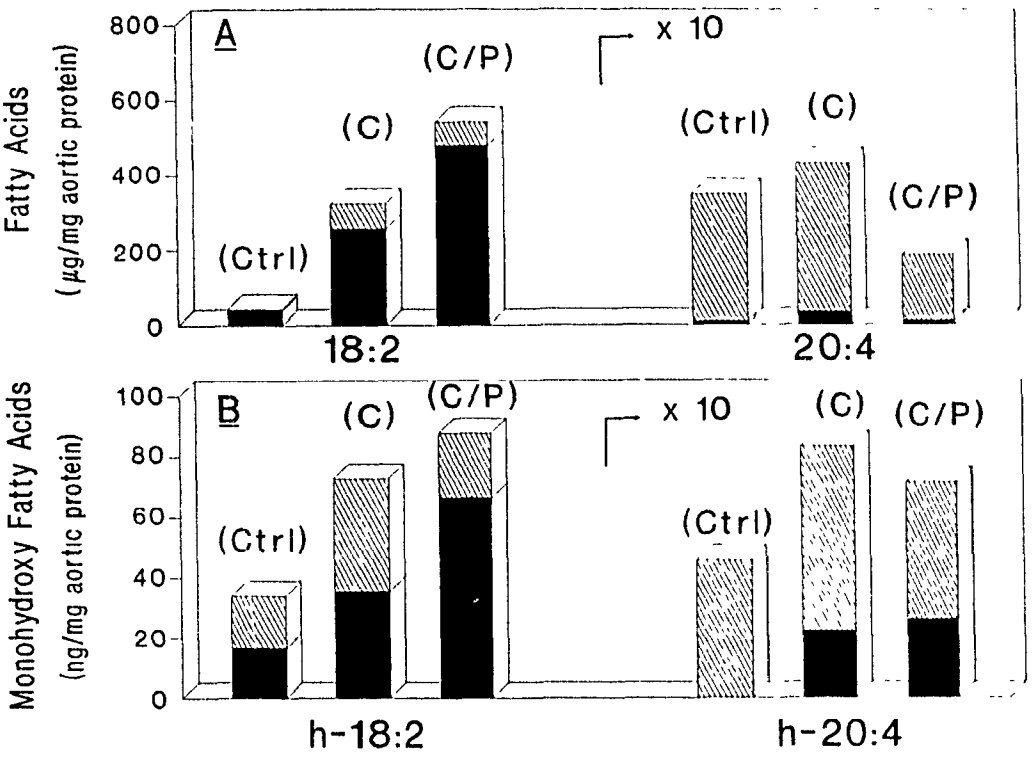

Fig. 39. Distribution of 18:2 and 20:4 and their monohydroxy derivatives in aortae from control and hypercholesterolemic rabbits. Rabbits were fed a control diet (Ctrl; n = 4), or a diet supplemented with 1% cholesterol (C; n = 3), or a diet supplemented with 1% cholesterol plus peanut oil (C/P; n = 5) for 13 weeks. Aortic lipids were extracted using the Folch method and then separated into neutral (solid bars) and polar (hatched bars) fractions on an open column of silicic acid. 20:0 And 14h-19:2 (methyl esters) were added to these two fractions as internal standards for fatty acids and their monohydroxy derivatives, respectively. Fatty acids were quantified by GC (panel A) and their monohydroxy derivatives were analysed by GC-MS (panel B). Note that the values for 20:4 and its monohydroxy derivatives have been multiplied by 10.

Table 10

Percentages of Fatty Acids in the Form of Their Monohydroxy Derivatives in Different Lipid Fractions from Rabbit Aorta

18:2 and 20:4 and their monohydroxy derivatives in aortae from 3 groups of rabbits were measured as described in the legend to **Fig. 39**. The ratios of hydroxy 18:2 or hydroxy 20:4 to their fatty acid precursors in either polar or neutral lipid fractions in aorta were obtained from the data presented in **Fig. 39**. The values are expressed as Mean ± S.E.M.. Original data were transformed using natural logarithm to archive equal variances for the groups. The overall differences among three groups of rabbits were estimated by ANOVA (♥ $P < 0.01$). Differences between any two groups were estimated by Duncan's multiple comparison test (♣ $P < 0.01$, compared to control; ¶ $P < 0.01$, compared to CHOL). U.D.: under detection limit. CHOL: cholesterol; PO: peanut oil.

	mh-18:2/18:2		mh-20:4/20:4	
	Polar	Neutral	Polar	Neutral
CONTROL (N = 4)	0.448±0.116♥	0.049±0.008	0.014±0.002	U.D.♥
CHOL (N = 3)	0.063±0.016♣	0.014±0.003♣	0.023±0.010	0.082±0.023♣
CHOL/PO (N = 5)	0.031±0.009♣	0.014±0.002♣	0.027±0.005	0.353±0.108♣¶

arachidonic acid as its monohydroxy derivatives in polar lipids tended to be higher in aortae of rabbits on cholesterol or cholesterol/peanut oil-supplemented diets compared to the controls, the differences were not significant (**Table 10**). On the other hand, the amounts of monohydroxy derivatives were dramatically elevated in neutral lipids, suggesting that arachidonic acid was selectively oxidized to its monohydroxy derivatives in neutral lipids after the cholesterol diets.

3.3. BIOSYNTHESIS OF PROSTAGLANDINS AND THROMBOXANES BY AORTA IN VIVO AND IN VITRO BY HYPERLIPIDEMIC RABBITS

We evaluated prostanoid synthesis by rabbit aorta using two different methods. Incubation of tissue slices enables us to measure prostanoids synthesized by all parts of the aorta, including the intima, media and adventitia. The template (**Fig. 22** on P. 73) was used to measure prostanoids synthesized primarily by the cells of the aortic lumen. Since the basal release of prostanoids from luminal surface was quite low under our conditions, exogenous arachidonic acid was added to the medium. *In vivo* production of PGI_2 and TXA_2 was also estimated by measuring their dinor metabolites in urine.

3.3.1. Effects of Preincubation with Ibuprofen and EDTA on the Synthesis of 6-OxoPGF$_{1\alpha}$

The production of prostanoids by aorta could be diminished due to the partial autoinactivation of prostaglandin endoperoxide synthase caused by a rapid burst in the release of arachidonic acid during preparation of the tissue. We attempted to minimize this problem by perfusing the aorta *in situ* with medium containing EDTA and the reversible prostaglandin endoperoxide synthase inhibitor, ibuprofen (10 μM). Tissue sections were then prepared in the same medium, and mounted in a template. Having been washed several times with calcium-containing medium to remove the ibuprofen and EDTA, the aortic lumen was incubated with arachidonic acid (25 μM). Pretreatment of aortae with ibuprofen/EDTA resulted in a decrease in the basal release of 6-oxo$PGF_{1\alpha}$ by the aortic lumen, and an approximately 2-fold increase in the amount of this substance synthesized from exogenous arachidonic acid (25 μM), added after 40 min (**Fig. 40B**).

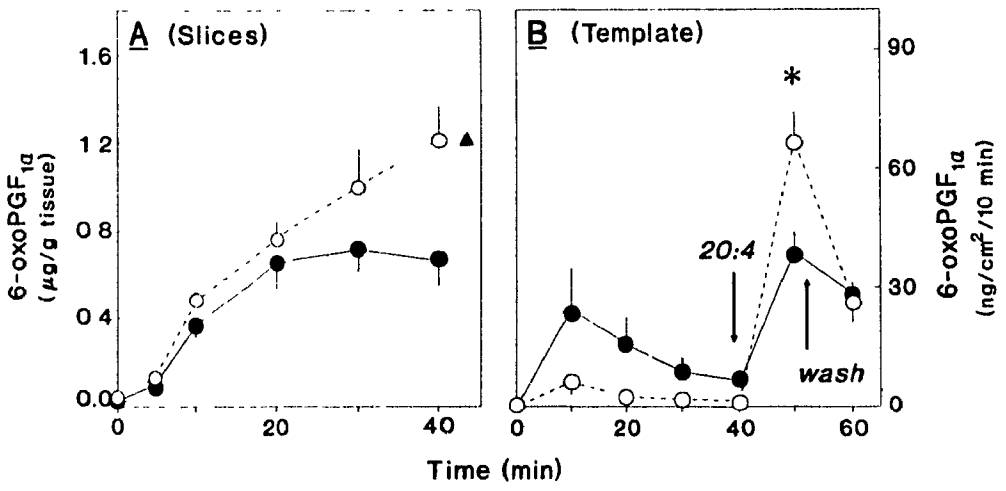

Fig. 40. Release of 6-oxoPGF$_{1\alpha}$ from rabbit aorta prepared in the presence and absence of ibuprofen/EDTA. Aortae from normal New Zealand White rabbits were prepared either in medium containing calcium and magnesium (medium B, ●—●; see Material and Methods) or in calcium and magnesium-free medium (Medium A) containing EDTA (2 mM) and ibuprofen (10 μM) (○ -○). Panel A: Aorta arch (S_1; see **Fig. 21** on P. 71) were cut into slices in the same medium in which they were prepared, and then washed 5 times with medium B. The slices were then incubated in medium B (10 ml) for 40 min, with aliquots (300 μl) taken every 10 min to measure of 6-oxoPGF$_{1\alpha}$. Statistical difference between two treatment was calculated using univariate analysis of variance with repeated measures. ▲ $p < 0.05$, within subject and interaction effects. Panel B: Segments taken from the upper part of the aortae (T_1) were mounted in a template in medium A in which they were prepared and incubated for a further 10 min. Then medium A was replaced by medium B and the aorta was incubated for a further 10 min. This was repeated twice, followed by the addition of medium containing arachidonic acid (25 μM) at 40 min. After incubation with arachidonic acid for 10 min, the medium was replaced by medium B (without arachidonic acid) for an additional 10 min. Statistical difference between two treatment in synthesizing 6-oxoPGF$_{1\alpha}$ from exogenous 20:4 was calculated using Student's *t*-test. * $p < 0.05$. All values are means ± S.E.M. with 4 rabbits in each of the two groups.

The initial burst in the release of 6-oxoPGF$_{1\alpha}$ in the first 10 min, which was much greater in non-pretreated aortae, was presumably due to stimulation caused by mounting the tissue in the template.

Pretreatment with ibuprofen/EDTA had a relatively small effect on the amount of 6-oxoPGF$_{1\alpha}$ initially synthesized from endogenous substrate by slices of aorta (**Fig. 39A**). However, the synthesis of this substance by the non-pretreated aortae virtually ceased after incubation for 20 min, whereas it continued almost linearly with time up to 40 min after pretreatment with ibuprofen/EDTA (**Fig. 40A**). It should be noted that in the experiment using the template, depicted in **Fig. 40B**, the medium was changed every 10 min, whereas in the experiment performed on aortic slices, the values are the cumulative amounts of 6-oxoPGF$_{1\alpha}$ released, measured in aliquots removed from the incubation mixture every 10 min.

3.3.2. Biosynthesis of Prostaglandins and Thromboxane A_2 by Blood Vessels from Cholesterol-fed Rabbits

The amounts of 6-oxoPGF$_{1\alpha}$, TXB$_2$, and PGE$_2$ formed from endogenous substrate by slices of aortic arch (S$_1$; see **Fig. 21** on P. 71) from rabbits fed cholesterol for different periods of time are shown in **Fig. 41** (A, C, and E). The same figure (B, D, and F) also shows the amounts of these substances synthesized from exogenous arachidonic acid (25 μM) by the luminal surface of the thoracic aorta (T$_1$) as evaluated by the template technique. In both cases, aortae were prepared in medium containing ibuprofen and EDTA as described above. We also compared the amounts of these three prostanoids synthesized by the upper (S$_1$ and T$_1$) and lower (S$_2$ and T$_2$) parts of the aorta (**Fig. 42**; A, C, E for slices and B, D, F for the luminal surface).

3.3.2.1. Formation of 6-oxoPGF$_{1\alpha}$

The amounts of 6-oxoPGF$_{1\alpha}$ synthesized by the luminal surface of aorta (**Fig. 41B**) were almost the same for control and cholesterol-fed rabbits at all time points investigated. Neither was there any difference between the control and cholesterol-fed groups in the ability of the lumen (**Fig. 42B**) from the lower part of the aorta (T$_2$) to synthesize this product after 15 weeks on the diets.

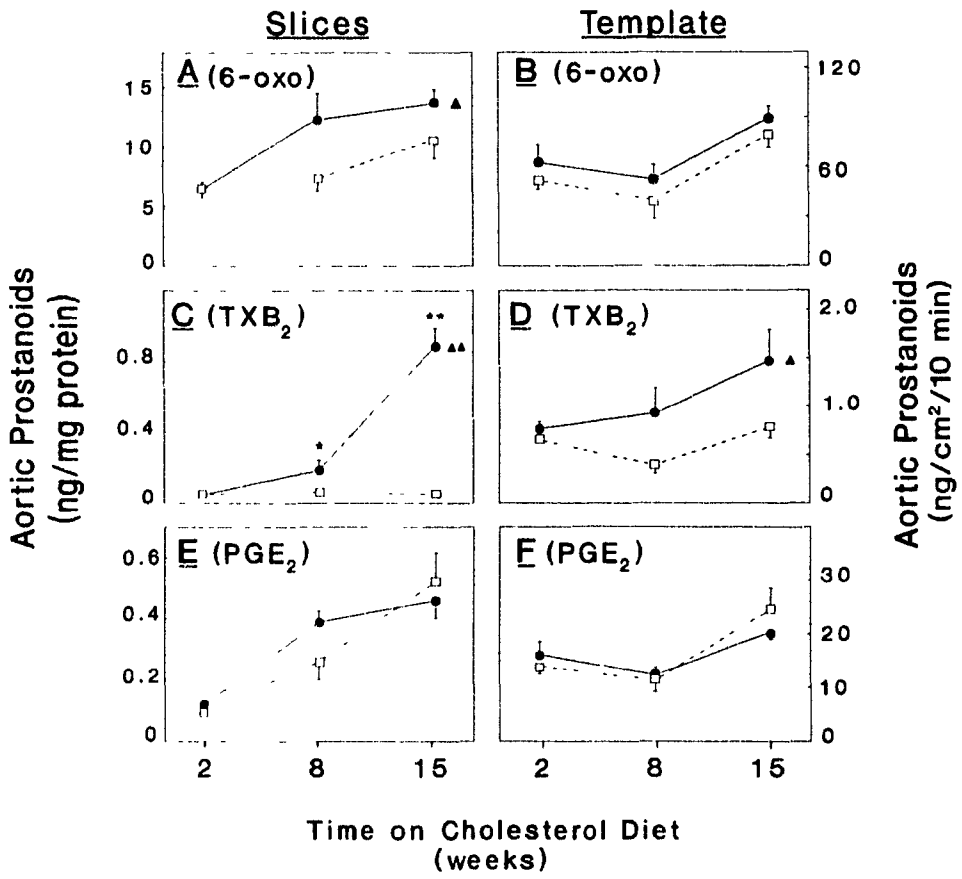

Fig. 41. Synthesis of 6-oxoPGF$_{1\alpha}$, TXB$_2$, and PGE$_2$ by aortae from rabbits fed control and cholesterol-supplemented diets for different periods of time. Aortae from rabbits fed either control (□ ┐) or cholesterol-supplemented (●–●) diets were either cut into small pieces (S_1; left panels) or were mounted in a template (T_1; right panels). Prostanoid production was from endogenous substrate in the incubations with aortic slices, and was stimulated by addition of exogenous arachidonic acid (25 μM) in the template procedure. Aortae were pretreated in both cases with ibuprofen and EDTA. 6-OxoPGF$_{1\alpha}$, TXB$_2$, and PGE$_2$ were measured by radioimmunoassay. All values are means ± S.E.M.. Statistical differences between control and cholesterol-supplementation in prostanoid production were calculated using two-way ANOVA with the regression approach (with treatment and time as two independent variables). Post-hoc tests were done using simple contrast based on design matrix control and treated groups at each particular time period. ▲ $p < 0.05$ (2-way ANOVA; between subject effect), ▲▲ $p < 0.001$ (2-way ANOVA; all effects), ** $p < 0.001$ (post-hoc test).

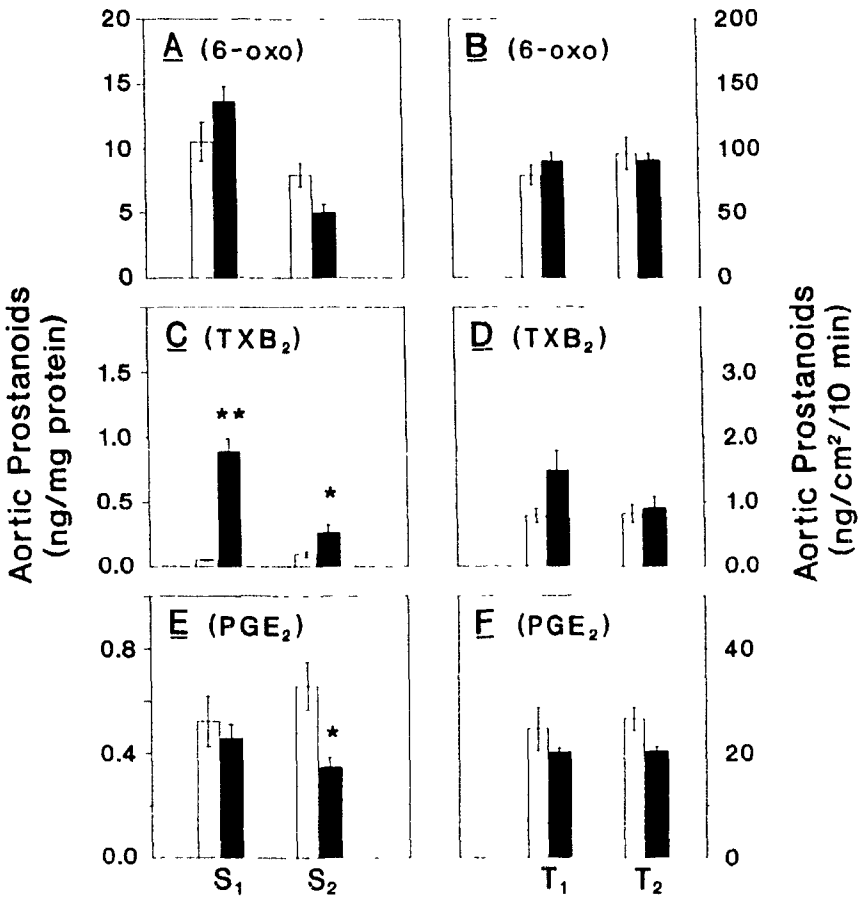

Fig. 42. Synthesis of 6-oxoPGF$_{1\alpha}$, TXB$_2$, and PGE$_2$ by different regions of aorta from control and hyperlipidemic rabbits. After 15 weeks on a cholesterol diet, the amounts of 6-oxoPGF$_{1\alpha}$ (A, B), TXB$_2$ (C, D), and PGE$_2$ (E, F) synthesized by slices and the luminal surface of aorta from either control (open bars) or hyperlipidemic (solid bars) rabbits were determined by radioimmunoassay. All values are means ± S.E.M.. Statistical significance of differences between two groups was calculated by Student's *t*-test. * $p < 0.05$; ** $p < 0.01$. The regions of the aortae used in these experiments (S_1 and S_2 for slices and T_1 and T_2 for the template procedure) are shown in **Fig. 21** on P. 71.

The synthesis of 6-oxoPGF$_{1\alpha}$ by slices of aorta from control and cholesterol-fed rabbits were not significantly different from one to another at any of the 3 individual time points investigated (**Fig. 41A**). However, two-way analysis of variance using treatment and time as two independent variables indicated that the production of 6-oxoPGF$_{1\alpha}$ was significantly higher in aorta from the cholesterol-fed rabbits (group effect: $p = 0.023$; time effect: $p = 0.000$). No interaction effect was observed ($p = 0.160$).

3.3.2.2. Formation of TXB_2

The release of TXB_2 by slices of aortic arch (S_1) was the same after 2 weeks, over 2-fold higher after 8 weeks and about 10 fold higher after 15 weeks on a cholesterol-supplemented diet compared to controls (**Fig. 41C**). Statistical analysis showed very significant differences due to treatment, time and interactions. A strong interaction effect indicated that the differences between the control and cholesterol-fed groups in TXB_2 production changed over the time. Slices from the lower part of aorta (S_2) from cholesterol-fed rabbits also released significantly more TXB_2 than controls, but the difference was much less pronounced than observed for the aortic arch (**Fig. 42C**).

TXB_2 synthesis by the aortic lumen was also higher in the cholesterol-fed group, but the difference was much less than with slices (**Fig. 41D**). Two-way analysis of variance led to the same conclusion as it did for the analysis of 6-oxoPGF$_{1\alpha}$ production by slices of aorta. The amounts of TXB_2 synthesized by the luminal surface of the aorta from cholesterol-fed rabbits were not significantly different from the controls at each individual time point. The between-subject effect became significant only if all three time points were pooled together for analysis. There were no differences in TXB_2 synthesis by the lumen of the lower part of the aorta (T_2) between the control and experimental groups (**Fig. 42D**).

3.3.2.3. Formation of PGE_2

PGE_2 synthesis by aortic slices and the aortic lumen tended to increase with age, but was nearly the same in the two groups of animals (**Fig. 41**; E and F. **Fig. 42F**). The only exception was in the release of PGE_2 from slices of the lower part of the aorta, in

which case cholesterol feeding resulted in significant decrease in PGE_2 production (**Fig. 42E**).

3.3.3. Correlation between the Severity of Atherosclerotic Lesion and Aortic Prostanoid Synthesis

As we did for the amounts of esterified hydroxy 20:4 in aorta from cholesterol-fed rabbits, we also investigated the correlation between PGI_2 production by slices of aorta from cholesterol-fed rabbits and the severity of the atherosclerotic lesions (**Fig. 43**). In this way, we attempted to determine whether or not the increased amount of PGI_2 produced by atherosclerotic rabbit aorta was associated with this disease. The analysis revealed that there existed a moderate positive correlation between PGI_2 production and atherosclerotic development in aorta especially when the area of aorta covered with lesions was taken as an index of severity. Thus, PGI_2 biosynthesis tended to increase slightly with the development of atherosclerotic lesions.

The degree of correlation between TXB_2 synthesis by either aortic slices or aortic lumen and the severity of atherosclerotic lesions was also estimated (**Fig. 44**). TXB_2 synthesis by both slices and lumen of the atherosclerotic aorta was moderately associated with the development of atherosclerosis in terms of area covered with lesions (**Fig. 43**; A and C). However, when total cholesterol content in aorta was used as a parameter to evaluate the severity of atherosclerotic lesions, a much stronger correlation with TXB_2 synthesis by slices of atherosclerotic aorta was observed (**Fig. 44B**). In comparison, the correlation between TXB_2 released from the luminal surface of atherosclerotic aorta and total cholesterol in this tissue was much weaker (**Fig. 44D**).

Since both TXB_2 (**Fig. 44B**) and esterified monohydroxy derivatives of arachidonic acid (**Fig. 37F**) were correlated strongly with the content of cholesterol in atherosclerotic lesions, we also determined the degree of correlation between TXA_2 synthesis and the amounts of esterified monohydroxy 20:4 in atherosclerotic aortae from cholesterol-fed rabbit. A highly positive correlation ($r = 0.926$) can be seen (**Fig. 45A**). As expected from **Fig. 43** and **Fig. 37F**, there is also a correlation between PGI_2 synthesis and the amounts of esterified monohydroxy 20:4 in atherosclerotic aortae (**Fig. 45B**). But in this

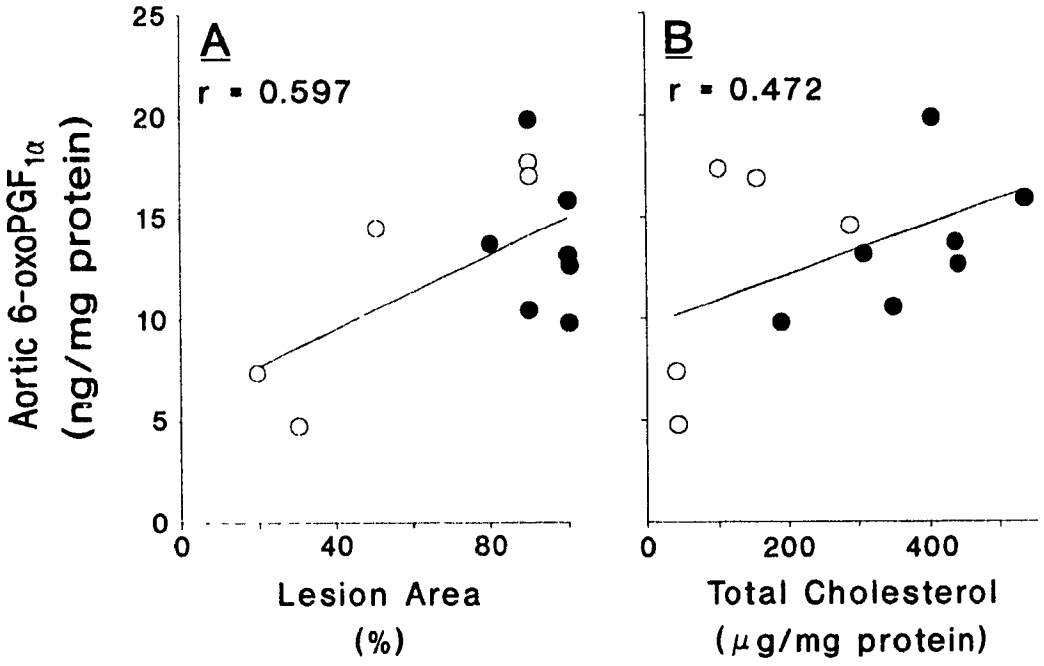

Fig. 43. Correlations between 6-oxoPGF$_{1\alpha}$ synthesis and the severity of atherosclerotic lesions in aortae from hyperlipidemic rabbits. Rabbits were fed a cholesterol-supplemented diet for 8 (open circles) or 15 (closed circles) weeks. Slices of aortic arch (S_1) were incubated for 30 min at 37 °C and then aliquots of the incubation medium were assayed for 6-oxoPGF$_{1\alpha}$ by radioimmunoassay. The severity of atherosclerotic lesions in aorta was evaluated either by visual estimation of area covered with lipid plaque (A) or by measuring total cholesterol (B) using gas chromatography. The correlation between severity of atherosclerotic lesions and 6-oxoPGF$_{1\alpha}$ synthesis in aorta of cholesterol-fed rabbits was computed using Pearson product-moment correlations (Pearson *r*).

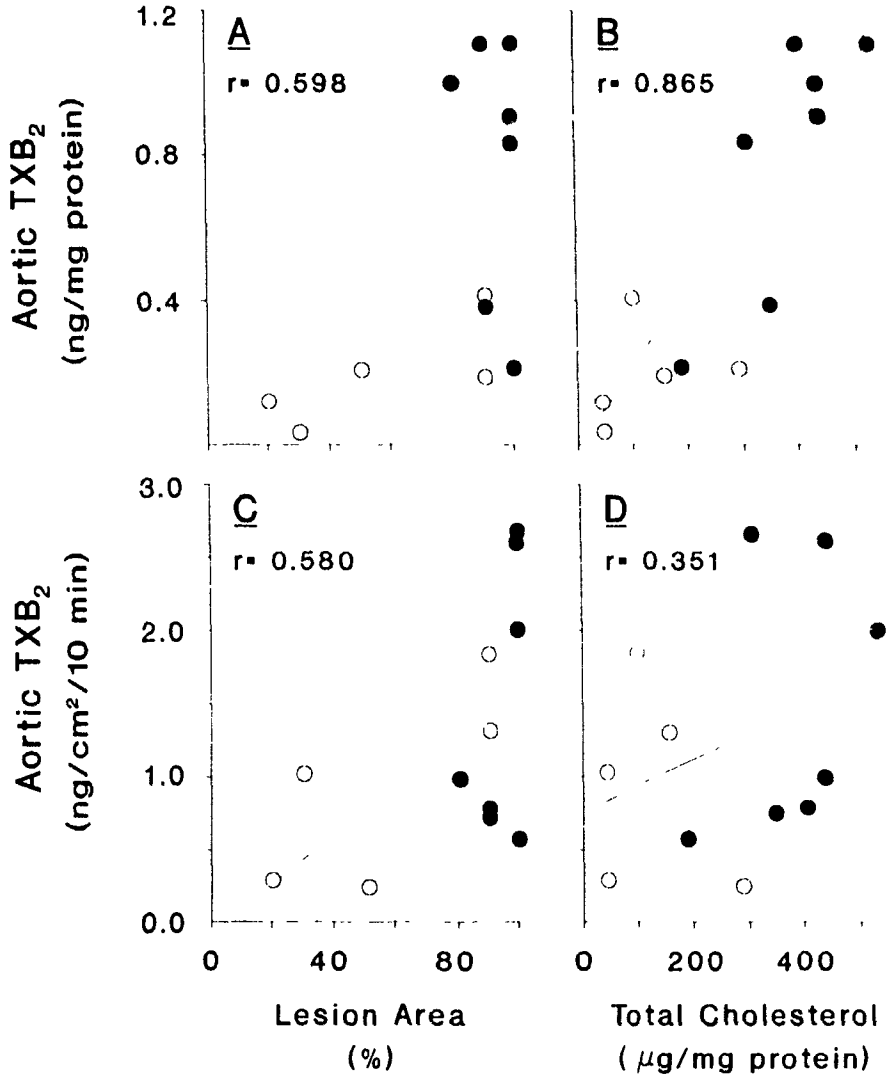

Fig. 44. Correlation between TXB_2 synthesis and the severity of atherosclerotic lesions in aortae from hyperlipidemic rabbits. Rabbits were fed a cholesterol-supplemented diet for 8 (open circles) or 15 (closed circles) weeks. TXB_2 production by slices (A, B) or by luminal surface (C, D) of the aortae were measured by radioimmunoassay. The severity of atherosclerotic lesions in aorta was estimated either by visual estimation of the area covered with lipid plaque (A, C) or by measuring total cholesterol (B, D) using gas chromatography. Correlations between the severity of atherosclerotic lesions and TXB_2 synthesis in aorta of cholesterol-fed rabbits was computed using Pearson product-moment correlations (Pearson *r*).

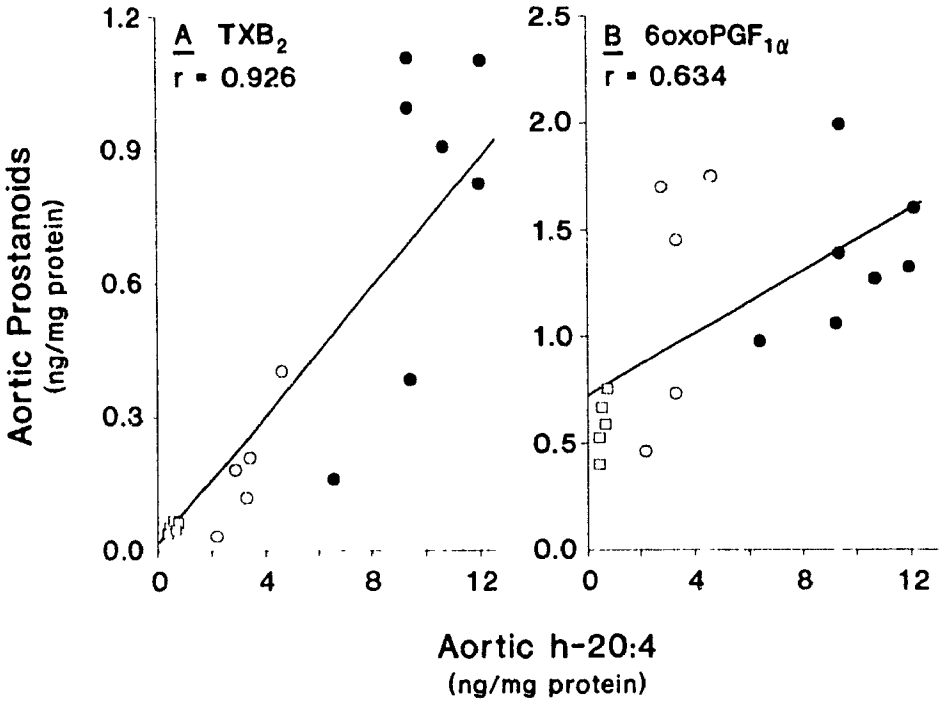

Fig. 45. Correlation between monohydroxy derivatives of 20:4 esterified to lipids and 6-oxoPGF$_{1\alpha}$ and TXB$_2$ formation by slices of aorta from hyperlipidemic rabbits. Rabbits were fed a diet supplemented with cholesterol and peanut oil for 2 (open squares), 8 (open circles) and 15 (closed circles) weeks. Monohydroxy derivatives of 20:4 esterified to aortic lipids (T_1) were analyzed by GC-MS. TXB$_2$ (A) and 6-oxoPGF$_{1\alpha}$ (B) released from the same tissue were measured by RIA. Correlations between monohydroxy derivatives of 20:4 and prostanoids were computed using Pearson product-moment correlations (Pearson *r*).

case, the degree of correlation is weaker compared to that between TXA_2 and monohydroxy 20:4 (0.634 vs. 0.926).

3.3.4. Biosynthesis of PGI_2 and TXA_2 in vivo by Control and Cholesterol-fed Rabbits

To estimate the production of PGI_2 and TXA_2 *in vivo*, we measured the amounts of their principal urinary metabolites, 2,3-dinor-6-oxo$PGF_{1\alpha}$ and 2,3-dinor-TXB_2, in rabbits fed control or cholesterol/peanut oil-supplemented diets (**Fig. 46**). This work was done in collaboration with Dr. Falardeau at the Clinical Research Institute of Montreal. There were no overall differences in the amounts of urinary 2,3-dinor-6-oxo$PGF_{1\alpha}$ between hypercholesterolemic rabbits and controls at any of the times we measured (**Fig. 46A**). Somewhat unexpectedly, the levels of 2,3-dinor-TXB_2 in urine were the same for controls and cholesterol-fed rabbits and the levels were decreased rather than increased with age (**Fig. 46B**).

3.4. EFFECTS OF LIPID HYDROPEROXIDES ON PROSTAGLANDIN ENDOPEROXIDE SYNTHASE AND PROSTACYCLIN SYNTHASE IN FETAL CALF AORTA

13-Hydroperoxy 18:2 and 15-hydroperoxy 20:4 can inhibit both prostaglandin endoperoxide synthase and prostacyclin synthase and can thereby diminish prostanoid synthesis. We have shown that atherosclerotic aortae contain large amounts of monohydroxy products derived from the above hydroperoxides. However, these products exist primarily in the esterified form, rather than as free monohydroxy fatty acids. It was therefore of interest to compare the effects of esterified and free fatty acid hydroperoxides on the enzymes required for PGI_2 synthesis.

Particulate fractions of fetal calf aorta provided a good source of both prostaglandin endoperoxide synthase and prostacyclin synthase. ^{14}C-labelled 20:4 or PGH_2 were used as substrates for prostaglandin endoperoxide synthase and prostacyclin synthase, respectively. Incubations were carried out in the presence of 0.02% Triton X-100 and in the absence or presence of 13-hydroperoxy-18:2 or hydroperoxides prepared from

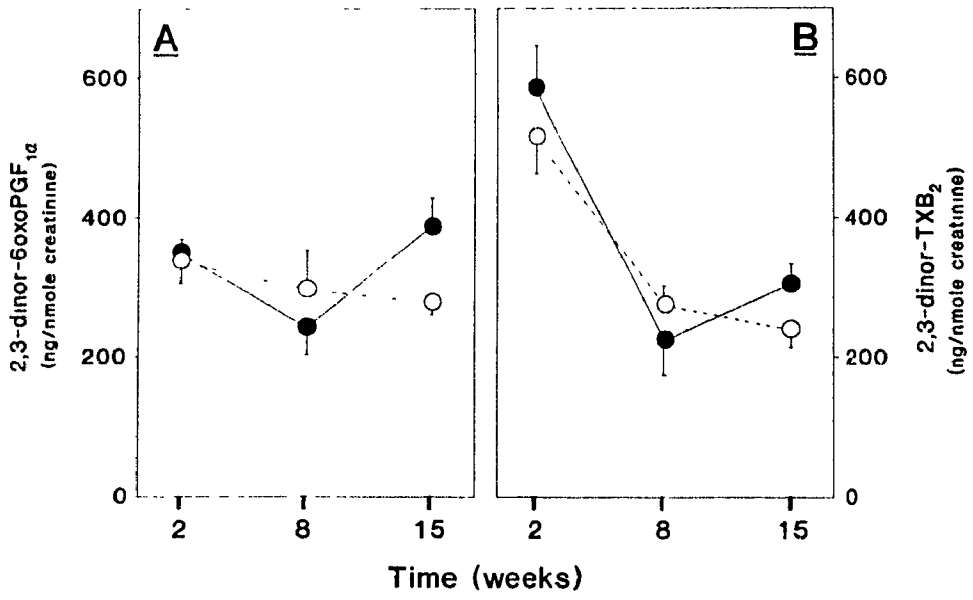

Fig. 46. Amounts of 2,3-dinor-6-oxoPGF$_{1\alpha}$ and 2,3-dinor-TXB$_2$ in urine from control and hypercholesterolemic rabbits. After 2, 8, or 15 weeks on either control (o-o) or cholesterol-supplemented (● ●) diets, rabbits were placed in metabolic cages and the urine was collected during a 24-hour period. Urinary 2,3-dinor-6-oxoPGF$_{1\alpha}$ and 2,3-dinor-TXB$_2$ were quantified by GC-MS using negative ion chemical ionization and selected ion monitoring. Values are means ± S.E.M..

cholesteryl linoleate, trilinolein, or 1-palmitoyl-2-linoleoyl-phosphatidylcholine as inhibitors. Due to the low yield of hydroperoxy lipids prepared, the three major isomers of hydroperoxide derived from trilinolein were pooled together for the experiment (see **Fig. 24B** on P. 78). A single peak of hydroperoxide derived from cholesteryl linoleate and 1-palmitoyl-2-linoleoyl-phosphatidylcholine was obtained after RP-HPLC and used in the experiment. However, the positional isomers of phosphatidylcholine hydroperoxides may not been resolved by the chromatographic method we used. However, the inhibitory effects of fatty acid hydroperoxides are not affected to a significant extent by the position of the hydroperoxy group [320].

13-Hydroperoxy-18:2 inhibited prostaglandin endoperoxide synthase and prostacyclin synthase by 53% and 70% at 2 μM and by 78% and 92% at 10 μM, respectively (**Fig. 47**). In contrast, at a concentration of 2 μM, all 3 of the hydroperoxy derivatives of lipids inhibited these enzymes by only 20 %. However, this effect was not concentration-dependent for the hydroperoxides of cholesteryl linoleate or trilinolein since higher concentrations (10 and 25 μM) did not increase their inhibitory effects. Hydroperoxy phosphatidylcholine, on the other hand, inhibited prostaglandin endoperoxide synthase in a dose-dependant manner whereas prostacyclin synthase activity was not affected by this hydroperoxide.

Since hydroperoxy derivatives of esterified lipids are not very soluble in aqueous solution, efforts were made to solubilize these products with detergent. However, the detergent initially used, Triton X-100, had inhibitory effects on prostaglandin endoperoxide synthase (**Fig. 48**). On the other hand, Triton X-100 was not inhibitory to prostacyclin synthase at these concentrations. To eliminate the inhibitory effects due to Triton X-100, digitonin, which did not inhibit prostaglandin endoperoxide synthase at concentrations up to 0.1% (not shown), was used as the detergent in another experiment. In this case, prostaglandin endoperoxide synthase was inhibited by hydroperoxy phosphatidylcholine (25 μM) to a similar extent as observed in the experiment using Triton X-100 (**Table 11**). Phosphatidylcholine itself inhibited prostaglandin endoperoxide synthase to some extent at this concentration, but not nearly as much as its hydroperoxy derivatives.

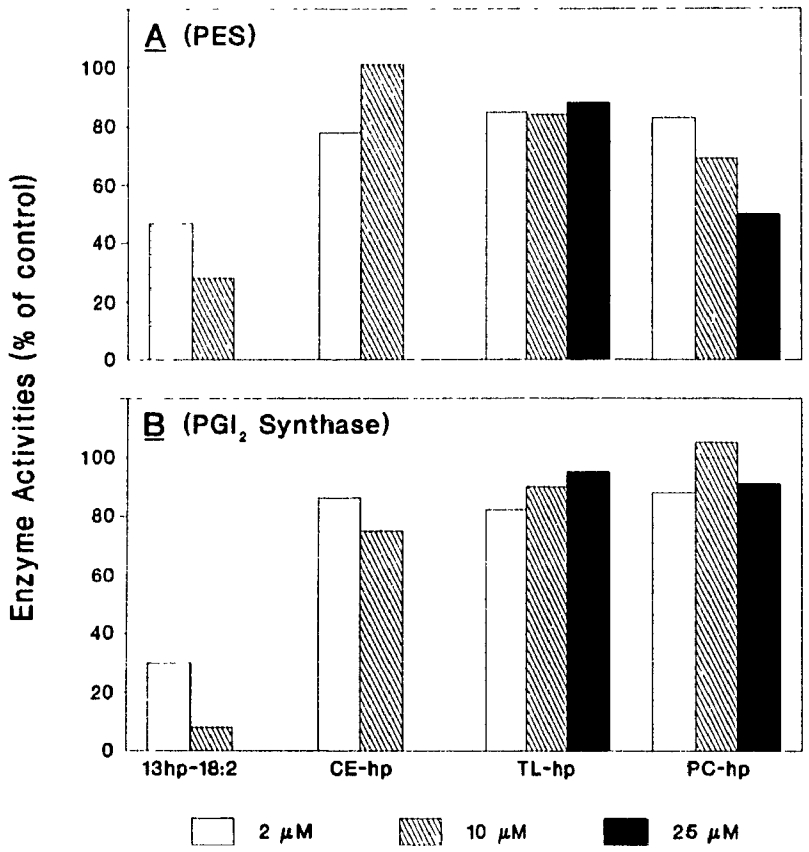

Fig. 47. Effects of free and esterified hydroperoxy derivatives of linoleic acid on prostaglandin endoperoxide synthase and prostacyclin synthase activities. Particulate fractions from fetal calf aorta (0.5 ml) were preincubated at 37 °C for 1 min. A solution of 0.04% Triton X-100 in Tris-HCl (pH 7.5; 0.5 ml) was added, followed by hydroperoxy derivatives at concentrations of 2 (open bars), 10 (hatch bars) or 25 (solid bars) μM in acetone (10 μl) and the incubation was continued for 3 min. [1-^{14}C]20:4 (A) or [1-^{14}C]PGH_2 was added and the incubation was continued for 10 min (A) or 3 min (B). Prostaglandin endoperoxide synthase (PES) activity was estimated by measuring the conversion of [1-^{14}C]20:4 to total oxygenation products (A), whereas PGI_2 synthase activity was estimated by measuring the conversion of [1-^{14}C]PGH_2 to 6-oxo$PGF_{1\alpha}$ (B). The values are averages of duplicate incubations and are expressed as percentage of the control values. **13hp-18:2**: 13-hydroperoxy 18:2; **CE-hp**: hydroperoxy 18:2 esterified to cholesteryl linoleate; **TL-hp**: hydroperoxy 18:2 esterified to trilinolein; **PC-hp**: hydroperoxy 18:2 esterified to phosphatidylcholine.

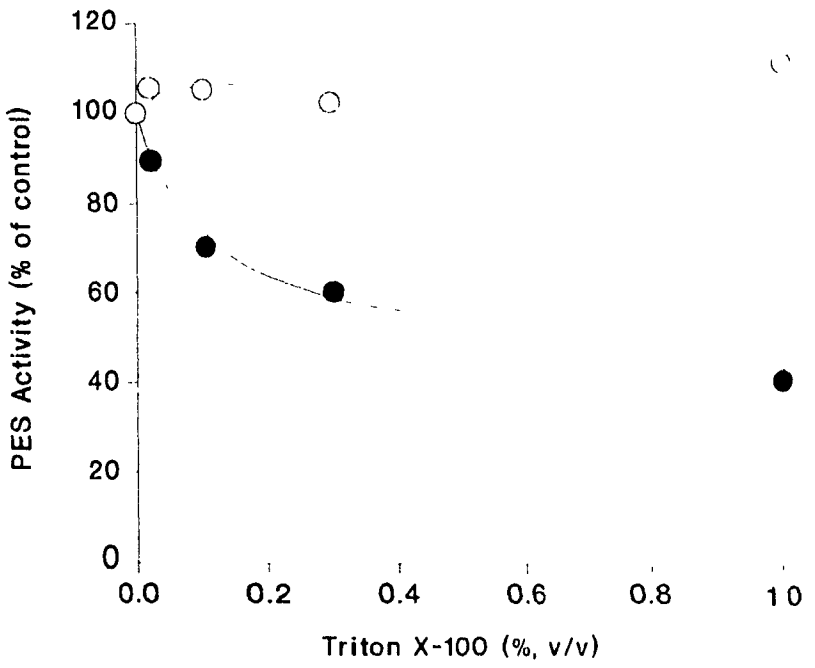

Fig. 48. Effects of Triton X-100 on prostaglandin endoperoxide synthase activity. Particulate fractions of fetal calf aorta (0.5 ml) were incubated at 37 °C for 1 min. Solutions of Tris-HCl (0.5 ml) with different concentrations of Triton X-100 were added to the incubation media, giving final concentrations of 0.02%, 0.08%, 0.3% and 0.1%, and the incubation was continued for 3 min. [1-^{14}C]20:4 or [1-^{14}C]PGH_2 were added and the incubation was continued for a further 10 min or 3 min, respectively. Prostaglandin endoperoxide synthase (•———•) or PGI_2 synthase (○---------○) activities were estimated by measuring total oxygenase products from radiolabelled 20:4 or 6-oxo$PGF_{1\alpha}$ from radiolabelled PGH_2, respectively. All values are averages of duplicate incubations and expressed as a percentage of the value obtained from incubations without Triton X-100.

Table 11

Effects of Hydroperoxy-phosphatidylcholine on Prostaglandin Endoperoxide Synthase

Particulate fractions from fetal calf aorta (0.5 ml) were incubated at 37 °C for 1 min. Tris-HCl (0.5 ml) containing 0.1% of digitonin was added to the incubation media, followed by the hydroperoxy derivatives of 1-palmitoyl-2-linoleoyl-phosphatidylcholine (25 μM) or unoxidized phosphatidylcholine (25 μM). The incubation was continued for 3 min. [1-^{14}C]20:4 (30,000 cpm) was added was added and the incubation was continued for a further 10 min. Prostaglandin endoperoxide synthase activity was estimated by measuring total oxygenation products from radiolabelled 20:4 as described in MATERIALS AND METHODS (P. 79). The values are the averages of duplicate incubations.

	Products (cpm)	Inhibition (%)
Control	3304	
Phosphatidylcholine	2666	19.3
Hydroperoxy Phosphatidylcholine	1741	47.3

DISCUSSION

Lipid peroxidation has been implicated in the development of atherosclerosis. Products of lipid peroxidation can cause damage to the endothelium, modification of low density lipoprotein (LDL), movement of monocytes into the arteries as well as inhibition of PGI_2 synthesis. All these effects may combine to accelerate the development of atherosclerotic lesions. Even though increases of various lipid peroxidation products have been observed in atherosclerosis, little was known concerning the peroxidation of specific fatty acids in LDL and aorta in atherosclerosis. Study of the peroxidation of specific fatty acids during atherogenesis could lead to a better understanding of the role of lipid peroxidation in the development of atherosclerosis. Therefore, we decided to investigate the formation of monohydroxy derivatives of fatty acids, early lipid peroxidation products, in human LDL incubated with copper ions and endothelial cells as well as the formation of these products in LDL and aorta from cholesterol-fed rabbits. The correlation between lipid peroxidation and prostanoid production in these rabbits was also studied.

4.1. MEASUREMENT OF MONOHYDROXY DERIVATIVES OF FATTY ACIDS BY GC-MS

To achieve a better understanding of the role of lipid peroxidation in atherogenesis, sensitive and selective methods should be employed. Prior to this study, a gas chromatographic-mass spectrometric method had been developed in our laboratory to measure the monohydroxy derivatives of polyunsaturated fatty acids in blood vessels. Using gas chromatography, monohydroxy derivatives of fatty acids can be separated based on their carbon numbers; using mass spectrometry, positional isomers of the monohydroxy derivatives of fatty acids can be detected because of the unique fragmentation of each product. Unlike the conventional TBA assay which measures end products of lipid peroxidation, measurement of monohydroxy derivatives of fatty acids, early lipid peroxidation products, could help us to understand the mechanism of this process. For example, selective increases of 11h-20:4 and 15h-20:4 may implicate the

action of prostaglandin endoperoxide synthase; selective increases of 12h-20:4 or 15h-20:4 may implicate 12- and 15-lipoxygenases, respectively; whereas increases of all isomers of monohydroxy derivatives of fatty acids to the same extent would suggest that autoxidation is the mechanism.

The method used in these studies has been modified from the original method in several ways. Firstly, hydroperoxy compounds were reduced to their hydroxy products by triphenyl phosphine before derivatization and purification. This prevented decomposition of hydroperoxy fatty acids during preparation of the samples prior to analysis by GC-MS. Secondly, hydrolysis of esterified lipids and methylation of their carboxyl groups is done in a single step by transmethylation, in stead of alkaline hydrolysis followed by methylation with diazomethane. Thirdly, purification of monohydroxy derivatives of fatty acids is performed by RP-HPLC instead of NP-HPLC. This eliminates the use of radiolabelled internal standards to verify the retention time of monohydroxy fatty acids since monohydroxy derivatives of polyunsaturated fatty acids can easily be monitored by a UV detector at 235 nm. It also reduces the purification time by over one-third for each sample.

The method has also been extended to measure monohydroxy derivatives of oleic acid. The application of this method to the study of lipid peroxidation of LDL *in vitro* and *in vivo* provided us with a tool to explore the mechanism of oxidative modification of LDL. It also enabled us to investigate the peroxidation of individual fatty acids in aorta from cholesterol-fed rabbits. Even though this is not the first report of monohydroxy fatty acids in LDL and atherosclerotic aorta, it is the first on the oxidation of oleic acid and on the relative amounts of monohydroxy derivatives from monounsaturated and polyunsaturated fatty acids in these tissues.

Peroxidation products of oleic acid cannot easily be measured by conventional techniques since they do not contain conjugated diene chromophore and are not decomposed to malondialdehyde. On the other hand, monohydroxy fatty acids with conjugated diene chromophore can be analyzed in biological samples using HPLC with a UV detector [264,321,322], although the selectivity of this method is not nearly as good as GC-MS. Using GC-MS, however, monohydroxy derivatives of oleic acid can

be measured at the same time as monohydroxy derivatives of polyunsaturated fatty acids.

4.2. MEASUREMENT OF LIPID PEROXIDATION IN HUMAN LDL

Oxidative modification of LDL has been hypothesized to be an important step in atherogenesis. LDL, which has been modified by transition metal ions, endothelial cells, arterial smooth muscle cells, monocytes/macrophages, platelets, is readily taken up and degraded by macrophages, resulting in the formation of macrophage-derived foam cells. The involvement of lipid peroxidation in the modification of LDL is supported by measurement of increases in lipid peroxidation products in LDL during the modification process and the inhibition of modification by antioxidants. However, measurement of TBARS and conjugated dienes reveal little concerning the mechanism of lipid peroxidation. Therefore, we decided to investigate the formation of monohydroxy derivatives of fatty acids in LDL oxidized in the presence of copper ions or in the presence of endothelial cells, and to compare the pattern of these products formed in these two conditions. Other parameters such as loss of fatty acids, formation of conjugated dienes and trienes, and formation of TBARS were also monitored for comparison with the GC-MS method.

4.2.1. Measurement of Monohydroxy Fatty Acids in Human LDL by GC-MS

We found that incubation of LDL with Cu^{2+} or endothelial cells results in increased amounts of monohydroxy derivatives of linoleic and arachidonic acids (**Figs. 26 and 27** on P. 89 and P. 90 respectively). These monohydroxy derivatives were dramatically increased after 5 hours in the presence of 20 μM $CuSO_4$ but dropped after 20 hours of incubation (**Fig. 26**; C and E). This pattern is similar to the time course for lipid autoxidation postulated by Gardner [211]. Polyunsaturated fatty acids are continuously oxidized to hydroperoxy metabolites which are then converted to either high molecular weight or low molecular weight compounds after the concentrations of hydroperoxides reaches a certain level. Consistent with this hypothesis, the reduction in the concentrations of monohydroxy derivatives of linoleic and arachidonic acids after 20 hours of incubation is due to the exhaustion of their fatty acid precursors (**Fig. 28**; E and

G) and the continuous decomposition of hydroperoxides to substances such as malondialdehyde (**Fig. 30**).

Monohydroxy products derived from linoleic and arachidonic acids were shown to be increased in LDL incubated with copper ions [264]. Our study however is much more extensive than other studies since we measured *all* the major monohydroxy derivatives of linoleic and arachidonic acids in LDL with much better selectivity than that can be obtained by HPLC with a UV detector. Moreover, we have measured the monohydroxy derivatives of oleic acid which could not be detected using HPLC with a UV detector because they lack a conjugated diene chromophore.

Oleic acid has been postulated to have a beneficial role in preventing atherosclerosis. Oleic acid lowers the serum LDL cholesterol as much as does linoleic acid. Oleic acid, however, does not lower HDL cholesterol as does linoleic acid. An epidemiological study showed that in the Mediterranean, where large quantities of oleic acid are consumed in the form of olive oil, coronary heart disease rates are relatively low [323]. It is possible that this could be due, at least in part, to the substitution of oleic acid for the more easily oxidized linoleic acid in LDL and tissue lipids. Laboratory evidence has shown that retina membrane [324] and LDL [325] from rabbits fed an oleic acid-supplemented diet are more resistant to oxidative modification. Our results indicate that oleic acid in LDL is oxidized in the presence of copper ions, on the basis of the loss of oleic acid from LDL (**Fig. 28C**) and the increase in the amounts of its monohydroxy derivatives (**Figs. 26A and 27A**). However, the time course for the formation of monohydroxy derivatives of oleic acid is quite different from that for monohydroxy derivatives of polyunsaturated fatty acids, in that the former increased linearly with time over a period of 20 hours even though the initial rate of formation was slower (**Fig. 26A**). The presence of only one double bond in oleic acid makes this molecule relatively resistant to oxidation compared to fatty acids with 1,4-*cis,cis*-pentadiene units because more energy is required for hydrogen abstraction [326]. Furthermore, the decomposition of monohydroperoxy derivatives of oleic acid would also be expected to be slower than that of the polyunsaturated hydroperoxides, since they would be both metabolically and chemically more stable [326].

It is hypothesized that it is the decomposition products of hydroperoxy fatty acids rather than the hydroperoxides themselves which modify apolipoprotein B of the LDL [274]. No matter how the lipids are oxidized, by endothelial cells or by the sequential actions of phospholipase A_2 and lipoxygenases on LDL, LDL is modified only in the presence of transition metal ions [45]. Transition metal ions promote the decomposition of lipid hydroperoxides to various compounds including unsaturated aldehydes, such as 4-hydroxynonenal and malondialdehyde, which can modify LDL directly [60,274-276] via conjugation with lysine amino groups in apolipoprotein B [288]. Our results combined with those of Parthsarathy on oleate-fed rabbits [325] suggest that, even though oleic acid can be oxidized to hydroperoxides just like polyunsaturated fatty acids, the relative resistance to further decomposition to 1,2-unsaturated aldehydes and other products in the presence of transition metal ions make the modification of oleate-enriched LDL less likely.

The oxidation of LDL by copper ions was compared to its oxidation by endothelial cells. Unlike the oxidation of LDL in the presence of copper ions, oxidation of LDL in the presence of endothelial cells resulted in an increase in the amounts of monohydroxy fatty acids only after 20 hours (**Fig. 26**; B, D and F). This difference could be the result of the incubation conditions. In the presence of Cu^+ and Cu^{2+}, hydroperoxides will be rapidly decomposed to either alkoxyl and peroxyl radicals, respectively [327]. These two radicals are in turn rapidly decomposed or polymerized to form low molecular weight or high molecular weight products [328,329]. The increased formation of unsaturated aldehydes and ketones from LDL incubated in the presence of copper ions is evidenced by the increased UV absorption between 260 - 280 nm (**Fig. 29**). With milder conditions, such as in the presence of endothelial cells or lower concentrations of copper [264], the formation of hydroperoxides may exceed the decomposition of these compounds, resulting in an increase over a longer period of time.

Modification of LDL by endothelial cells [268,269] or macrophages [271] was postulated to be the result of lipoxygenase-catalyzed oxidation since the modification could be retarded by inhibitors of lipoxygenases. The mechanism could be the direct oxidation of LDL lipids by endothelial lipoxygenase (**Fig. 49**; step [1]). Parthasarathy *et. al.* showed that modification of LDL by endothelial cells required direct contact of

LDL and endothelial cells, since no modification occurred when LDL was added to endothelial cell cultures within a dialysis bag [268]. Cathcart *et. al.* further demonstrated that incubation of soybean lipoxygenase (15-lipoxygenase) with human LDL *in vitro* resulted in increased formation of conjugated dienes and TBARS as well as increased cytotoxicity of LDL [270]. According to this hypothesis, there would be a selective increase of 13-hydroxy derivatives of linoleic acid and 15-hydroxy derivatives of arachidonic acid in LDL. However, our results on the oxidation of human LDL by human endothelial cells indicate otherwise. The pattern of monohydroxy derivatives of arachidonic acid in LDL incubated with endothelial cells was similar to that in LDL incubated with 20 μM $CuSO_4$ (**Fig. 27**) in that there was no selectivity with respect to the position of oxygenation, suggesting an autoxidation process.

Therefore, an alternative hypothesis must be considered. Even though 15-lipoxygenase can oxidize arachidonic acid esterified to phospholipid, it is unlikely that cytosolic lipoxygenases of endothelial cells could directly catalyze oxidation of LDL phospholipids. Moreover, lipoxygenases oxygenate free arachidonic acid more efficiently than esterified arachidonic acid. In contrast to Cathcart's results, Sparrow *et. al.* found that oxidative modification of human LDL resulted from the sequential action of phospholipase A_2 and soybean lipoxygenase, rather than from the action of phospholipase A_2 or lipoxygenase alone [269]. Therefore, unlike the first hypothesis, endothelial cell lipoxygenases presumably oxidize arachidonic acid released from cellular phospholipid by phospholipase A_2 to its hydroperoxy derivatives (**Fig. 49**; step [2] and [4]), which are then transferred to LDL upon LDL-endothelial cell contact (**Fig. 49**; step [5]). During this LDL-endothelial cell contact, arachidonic acid from LDL (by the action of LDL phospholipase A_2-like protein) can also be used by endothelial lipoxygenases as a substrate (**Fig. 49**; step [3]). In the presence of transition metal ions, hydroperoxy derivatives of arachidonic acid are converted to alkoxyl or peroxyl radicals, the latter serving as hydrogen abstractors to initiate a chain of lipid peroxidation in LDL. According to the second hypothesis, endothelial cell lipoxygenases are the initiator for lipid peroxidation in LDL only. Any site-specific oxidation would be masked by the following propagation of lipid peroxidation.

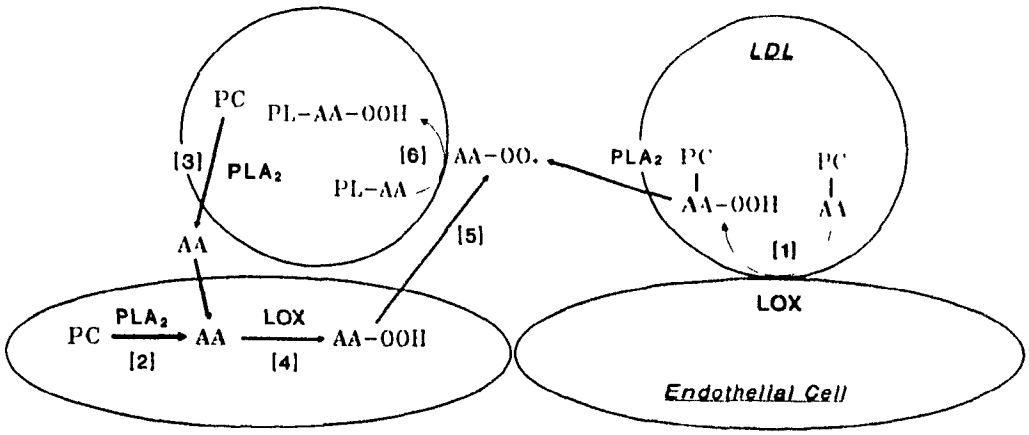

Fig. 49. Hypothetical schemes for the oxidation of LDL lipids in the presence of endothelial cells. PC: Phosphatidylcholine; PLA_2: phospholipase A_2; AA: arachidonic acid; LOX: lipoxygenases; AA-OOH: hydroperoxy derivatives of arachidonic acid; AA-OO·: peroxy radicals derived from arachidonic acid.

4.2.2. Measurement of Other Lipid Peroxidation Products in LDL

In addition to measurement of hydroxy derivatives of fatty acids by GC-MS, we also evaluated lipid peroxidation in LDL by three other methods including (1) fatty acid analysis, (2) measurement of conjugated dienes and trienes, and (3) measurement of TBARS. These methods each give information about different stages of lipid peroxidation (see **Section 1.3.4.** on P. 32).

Oxidation of LDL has been reported to be associated with a loss of fatty acids [263,264], cholesteryl esters and triglycerides in LDL [330] due to conversion to their oxygenated products. Consistent with these observations, lipid peroxidation in LDL is accompanied by the loss of fatty acids after incubation with 20 μM of $CuSO_4$ (**Fig. 28**; C, E and G). Even though measurement of fatty acids can provide us with information on the extent of oxidation of each fatty acid, it is not sensitive enough to detect the small amount of lipid peroxidation occurring in LDL incubated with endothelial cells (**Fig. 28**; D, F and H). Furthermore, measurement of fatty acids does not provide any information on the nature of the oxidation products subsequently formed.

After hydrogen abstraction, alkyl radicals from polyunsaturated fatty acids can be stabilized by addition of molecular oxygen and rearrangement of the double bond (**Fig. 5** on P. 25) to produce conjugated dienes. Therefore, conjugated diene formation can be one of the signs of peroxidation of *polyunsaturated fatty acids*. The results obtained from measurement of conjugation dienes, however, are not the same as those obtained by measuring hydroperoxy/hydroxy fatty acids by GC-MS as can be seen by comparison of **Fig. 26** (C and E) on P. 89 and **Fig. 29** on P. 94. The decreases in the amounts of monohydroxy derivatives of linoleic and arachidonic acids were not associated with corresponding decreases in UV absorbance around 234 nm (conjugated dienes). The reason for this is that some subsequently formed oxidized products of hydroperoxy/hydroxy fatty acids, both low and high molecular weight, still bear the conjugated diene chromophore (**Fig. 11** on P. 38). This problem can be solved by separation of the conjugated diene products by HPLC as used by some investigators [264,271]. However, the GC-MS analysis has advantages over HPLC methods since it is much more sensitive and selective and it enables us to quantify monohydroxy

derivatives of oleic acid, which otherwise could not be detected.

Another commonly-used method to study lipid peroxidation of LDL is the thiobarbituric acid assay, which theoretically measures malondialdehyde (MDA), an end product formed during the degradation of hydroperoxides with at least three double bonds (**Fig. 10**). However, this assay also gives rise to positive results due to interference from some other non-MDA products. Therefore, the results can only be interpreted as "thiobarbituric acid-reactive substances" (TBARS). Our results show that TBARS continuously increase, as expected, when LDL is incubated with both copper and endothelial cells (**Fig. 30** on P. 95).

Compared to other methods for measuring lipid peroxidation, the GC-MS method is much more selective. It is this high selectivity that provides with a new tool to study the mechanism of oxidative modification of LDL. However, when interpreting of the results, it is important to keep in mind that monohydroperoxy/monohydroxy derivatives are rapidly decomposed in the presence of transition metal ions. Furthermore, the different distribution of monohydroxy derivatives of fatty acids and TBARS in LDL and medium (**Table 3**) reminds us that the results depend on the source of the sample. Dynamic measurement of monohydroxy fatty acids in LDL combined with other methods selective for different stages of lipid peroxidation is important to obtain meaningful information [192].

4.3. LIPID PEROXIDATION IN ATHEROSCLEROTIC RABBITS

Our studies indicated that LDL and aortae from hypercholesterolemic rabbits contain increased amounts of esterified hydroxylated derivatives of 18:2 and 20:4 compared to controls. Although we have previously measured esterified 11h-20:4, 12h-20:4, and 15h-20:4 in aortic lipids from normal rabbits [304], this would appear to be the first report of the occurrence of these substances in lipids from LDL and aortae of cholesterol-fed rabbits containing atherosclerotic lesions.

4.3.1. Increased Formation of Monohydroxy Fatty Acids in LDL from Atherosclerotic Rabbits

Increased levels of TBARS in plasma have been observed in rabbits fed a cholesterol diet [331,332] as well as in hyperlipidemic patients [333]. Oxidation of LDL may be especially important in the development of atherosclerosis, since it results in the recognition and uptake of LDL by the macrophage scavenger receptor, and could thus promote the conversion of macrophages to foam cells [45]. Moreover, oxidized LDL has been reported to exhibit chemotactic activity for human monocytes and to inhibit the chemotactic response of mouse resident macrophages [44,251]. It could therefore be capable of attracting monocytes to the site of the lesion, and, once they have been transformed into macrophages, preventing them from leaving it [334].

Our results on the oxidation of human LDL indicate that linoleic and arachidonic acids in LDL are oxidized to monohydroperoxy derivatives upon incubation with copper ions or endothelial cells *in vitro*. Our results with rabbits show that linoleic acid in LDL is also oxidized to its 9- and 13-hydroperoxy derivatives *in vivo*, with the amounts of these products increasing after feeding rabbits with a cholesterol-enriched diet (**Fig. 31** on P. 100). We also found that LDL from hypercholesterolemic rabbits contains derivatives of 20:4 oxygenated in the 11, 12, and 15 positions. Unlike monohydroxy derivatives of polyunsaturated fatty acids, monohydroxy derivatives of oleic acid were not increased in LDL from cholesterol-fed rabbits (**Fig. 31**). This, combined with the formation of these products in LDL incubated with endothelial cells (**Fig. 26B**; P. 89), further supports the beneficial role of oleic acid in preventing atherosclerosis because of its resistance to lipid peroxidation both *in vivo* and *in vitro*.

4.3.2. Increased Formation of Monohydroxy Fatty Acids in Aortae of Atherosclerotic Rabbits

4.3.2.1. Increased formation of monohydroxy fatty acids as the results of hypercholesterolemia

The formation of monohydroxy fatty acids in aortae of hyperlipidemic rabbits is rather complex. It depends not only on the type of fatty acid oxidized but also on the

length of the treatment. The amounts of monohydroxy products derived from C_{18} fatty acids change differently with the length of dietary supplementation with cholesterol than those derived from C_{20} fatty acids (*i.e.*, arachidonic acid). Aortae of rabbits after 8 and 15 weeks on a cholesterol/peanut oil-supplemented diet contained about twice as much esterified monohydroxy C_{18} fatty acids oxygenated in positions 8, 9 and 10 compared to controls (**Fig. 34**; B and C; see P. 106). The amount of 13-hydroxy-18:2 was increased to a similar extent after 8 weeks, but fell after 15 weeks of this diet. The 11-hydroxy derivative of oleic acid, was not affected by the cholesterol diet. Somewhat unexpectedly, after 2 weeks, the amounts of monohydroxy derivatives of C_{18} fatty acids were lower in aortae from hyperlipidemic rabbits compared to controls (**Fig. 34A**). This decrease could possibly be the result of increased aortic superoxide dismutase and glutathione peroxidase activities in aortae of rabbits on cholesterol diet [335]. The amounts of monohydroxy derivatives of arachidonic acid in aorta, in contrast to those derived from C_{18} fatty acids, were not increased before 15 weeks on a cholesterol diet (**Fig. 34C**).

Hydroxylated polyunsaturated fatty acids have also been detected in aortae from Watanabe heritable hyperlipidemic (WHHL) rabbits. Products with UV absorbance indicative of conjugated dienes were detected by HPLC after hydrolysis of the lipids in lipid droplets from aortae of WHHL rabbits. One of these products had a retention time identical to that of 13h-18:2 [322]. Slices of aortae from WHHL rabbits were also found to convert both exogenous [282,336] and endogenous [336] 20:4 to monohydroxy derivatives. In this case, however, the amounts of immunoreactive 15h-20:4 released into the medium by aortae from 12 month old rabbits (exhibiting fatty streaks and raised lesions) were lower than those released by aortae from 1 month old rabbits [336]. On the other hand, increased amounts of immunoreactive 12h-20:4 were released by aortae from 12 month old WHHL rabbits [336].

Exogenous free radioactively labelled 20:4 was shown to be converted to 15h-20:4 by aortae from New Zealand white rabbits which had been fed a diet supplemented with cholesterol, but little or none was formed by normal aorta [281,282]. However, we have shown that unesterified 15h-20:4 is synthesized from *endogenous* substrate and released by aortic slices from both normal and, to a greater extent, cholesterol-fed rabbits (**Figs.**

33C and 34C). The discrepancy between these results could possibly be explained by the fact that we have measured mass rather than radioactivity, and our results would not be affected by changes in the specific activity of the radioactive substrate due to the release of endogenous 20:4.

Even though hypercholesterolemia has been shown to be responsible for the increased formation of monohydroxy derivatives of arachidonic acid in the aorta, one question still remains. It may be possible that the increased amounts of monohydroxy fatty acids observed in aorta and LDL are the result of increased intake of these metabolites of fatty acids due to the peanut oil which accompanied the cholesterol in the diet (**Table 6A**; see P. 98). In a subsequent study in collaboration with Dr. Wen-gui Yu, rabbits were divided into 3 groups which were fed diet consisted of (1) rabbit chow, (2) rabbit chow supplemented with 1% cholesterol and (3) rabbit chow supplemented with 1% cholesterol, 10% peanut oil, 1% BHA and 1% ethanol. Similar increases of monohydroxy fatty acids derived from linoleic and arachidonic acids were demonstrated in aortae from rabbits with either of the two cholesterol-supplemented diets. Thus, hypercholesterolemia alone can be responsible for the increased lipid peroxidation in LDL and aorta observed in our study. Cholesterol could inhibit GSH-Px activity by an yet as unknown mechanism [337] and therefore result in increased lipid peroxidation. Further evidence that cholesterol results in increased lipid peroxidation *in vivo* is also available from other studies [338,339].

4.3.2.2. Selective peroxidation of arachidonic acid in aortae of cholesterol-fed rabbits

Another possibility is that the increase in the formation of monohydroxy fatty acids in aortae of hypercholesterolemic rabbits is due to the increase in their fatty acid precursors in the aortae. Peanut oil supplementation provided more dietary fatty acids for the cholesterol-fed rabbits compared to controls (**Table 6A**). Cholesterol feeding also resulted in an increase in the amounts of linoleic acid in aorta (**Figs. 36A and 38A**). However, adequate levels of the factors for lipid peroxidation, which include the availability of oxygen, transition metal ions and enzyme activities, are required to convert

the increased amounts of 18:2 to monohydroxy products. Our results suggested that these factors could be the limiting factors in aortae from cholesterol-fed rabbits, especially after 8 and 15 weeks on the diet, when the percentage of C_{18} fatty acids present as their monohydroxy derivatives was lower than that in controls (**Fig. 36**; G and H), even though the absolute amounts of monohydroxy derivatives were higher (**Fig. 36**; D and E).

In contrast to the increase in aortic 18:2 in hyperlipidemic rabbits, there was a reduction in the amount of aortic 20:4 (**Fig. 36F**). Consequently, the percentage of 20:4 which had been converted to monohydroxy derivatives was higher in aortae from rabbits fed cholesterol-supplemented diets. The loss of arachidonic acid, especially in polar lipids (**Fig. 38A**), could be the result of increased phospholipase A_2 activity due to increased lipid peroxidation, and the subsequent reacylation by linoleic and oleic acids as suggested by van Kuijk *et. al.* [340]. This becomes more feasible when the dietary supply of these fatty acid is high due to addition of peanut oil to the diet.

The hypothesis that 20:4 is selectively oxidized in aortae from hypercholesterolemic rabbits is further supported by the high degree of positive correlation between monohydroxy derivatives of this fatty acids and the total cholesterol content observed in this tissue (**Fig. 38F**). Monohydroxy derivatives of oleic acid were negatively correlated to, whereas monohydroxy derivatives of linoleic acid were not correlated to, the total cholesterol in the lesions.

Our results suggest that in hypercholesterolemic rabbits, there may be specific changes resulting in increased oxygenation of 20:4. This could be due to changes either in the distribution of 20:4 in aortic lipids or to increases in its oxygenation by specific processes. In aortae from control rabbits, the percentage of esterified 18:2 present as monohydroxy C_{18} fatty acids is 5-10 times higher than the percentage of 20:4 present as monohydroxy C_{20} fatty acids (comparing **Fig. 36H** and **Fig. 36I**). In contrast, in aortae from rabbits fed a cholesterol/peanut oil-supplemented diet for 15 weeks, the percentages of 18:2 and 20:4 which were present as monohydroxy derivatives were nearly equal to one another. The differences in the proportions of 18:2 and 20:4 which are oxidized could possibly be explained by differences in the distribution of these fatty acids in

different lipid classes. In aortae of control rabbits, both 20:4 and its monohydroxy derivatives are present almost exclusively in polar lipids whereas 18:2 is predominantly in neutral lipids; monohydroxy derivatives of 18:2 are evenly distributed between neutral and polar lipids. After 13 weeks on cholesterol diets, however, the percentage of 20:4 oxidized in the polar lipid fraction is much lower than the percentage oxidized in the neutral lipid fraction (**Table 10**). This may be due to the combined effects of different free-radical defence systems to which polar membrane lipids would be exposed. Lipid peroxidation in the neutral-lipid fraction (presumably in intracellular or extracellular lipid droplets) may be higher because of inaccessibility to the free radical defence system. In lipid droplets, the defence against peroxidation might rely entirely on lipid-soluble chain-breaking antioxidants such as vitamin E.

4.3.2.3. Mechanisms for the increased formation of monohydroxy fatty acids in aorta of hyperlipidemic rabbits

It is possible that the monohydroxy derivatives of fatty acids esterified to aortic lipids could have originated via the oxidation of esterified 18:2 and 20:4 by the action of lipoxygenases. It has been demonstrated that aorta contains 12-lipoxygenase [341] and 15-lipoxygenase [342] activities. Macrophages, which contain 5, 12, and 15-lipoxygenases [343], could also contribute to the lipoxygenase-catalyzed oxidation of polyunsaturated fatty acids in atherosclerotic lesions. Lipoxygenase activities in macrophages were shown to be increased after treatment with acetyl-LDL to promote cholesterol uptake [344,345]. Recently, 15-lipoxygenase mRNA has been shown to be present in aorta of WHHL rabbits and co-localized with proteins which interact with an antibody to MDA-modified LDL (MAL-2) [272]. Although lipoxygenases normally catalyze the oxygenation of free polyunsaturated fatty acids, lipoxygenases from several sources have been shown to directly oxidize polyunsaturated fatty acids in phosphatidylcholine to give esterified hydroperoxy metabolites [205,206,346]. This raises the possibility that lipoxygenases could directly catalyze the oxidation of arachidonic acid in phospholipid to monohydroperoxy metabolites. Another possibility is that, as discussed in **Section 4.2.1.** on P. 135, free polyunsaturated fatty acids are first oxidized by

lipoxygenases, followed by the incorporation into lipids [347-351]. However, our laboratory demonstrated that 13h-18:2 was very poorly incorporated into both neutral and polar lipids from vascular endothelial and smooth muscle cells compared to 18:2 [304]. It is also possible that increased lipoxygenase-catalyzed oxidation of free polyunsaturated fatty acids could result in the formation of reactive intermediates which could propagate the oxidation of esterified polyunsaturated fatty acids. Increased amounts of 15-hydroxy derivatives of arachidonic acid were released into the incubation medium by aortae from rabbits after 8 and 15 weeks on a cholesterol-supplemented diet, suggesting that 15-lipoxygenase activity may be increased in atherosclerotic aorta (**Fig. 33**; B and C; P. 104). Arachidonic acid has been reported to be converted predominantly to its 15-hydroxy metabolite by aorta from WHHL [282,336] and cholesterol-fed rabbits [281,282]. The formation of this substance can be reversed by lipoxygenase inhibitors (NDGA or ETYA) [282,336] but not by prostaglandin endoperoxide synthase inhibitors (aspirin) [282].

A more likely explanation for the increased amounts of esterified monohydroxy fatty acids in aorta from atherosclerotic rabbits is that they are formed by increased nonenzymatic oxidation of fatty acids. This is supported by several lines of evidence. Firstly, even though lipoxygenases can oxidize polyunsaturated fatty acids esterified to phospholipid, there is little evidence that they can oxidize these fatty acids when they are esterified to cholesterol. Secondly, the fact that the changes in the monohydroxy derivatives of linoleic acid are paralleled by similar changes in the amounts of monohydroxy compounds derived from oleic acid in atherosclerotic aorta, suggesting that both of these fatty acids are oxidized by the same mechanism. Even though lipoxygenases catalyze the oxidation of polyunsaturated fatty acids, they do not catalyze the oxidation of fatty acids with only one double bond. Thirdly, cortisone acetate significantly suppressed atherosclerotic plaque formation in both WHHL and cholesterol-fed WHHL/NZW heterozygous rabbits [352]. However, this suppression was not associated with decreased 15-lipoxygenase activity. Instead, the suppression of lesion development can be achieved by antioxidants such as probucol for WHHL rabbits [283,353] and BHT for cholesterol-fed rabbits [285]. Finally, 9- and 13-hydroperoxy derivatives of

cholesteryl linoleate were identified in post-mortem samples of atherosclerotic plaques from human aorta. Gas chromatographic analysis of the methyl ester of 13-hydroxy derivatives of linoleate isolated from arterial cholesteryl esters revealed a 1:1 ratio of the 13(S)- and 13(R)-derivatives, suggesting that they had been formed by autoxidation of cholesteryl linoleate in atherosclerotic aorta [280]. The increased lipid peroxidation in atherosclerotic aorta could therefore be initiated by the action of superoxide generated by activated macrophages in the lesions [343].

4.3.2.4. An unanswered question

It has been demonstrated that lipid peroxidation has detrimental effects on atherogenesis. The relation of some risk factors, such as hypercholesterolemia [332,333,339] and smoking, to atherosclerosis have been shown to increase lipid peroxidation *in vivo*. However, is lipid peroxidation the only answer? The answer is "No".

Even though hypercholesterolemia results in both increased lipid peroxidation and formation of atherosclerotic lesions in rabbits, a clear cause-effect relationship between lipid peroxidation and atherogenesis has only been shown in WHHL rabbits, a special model for atherosclerosis. Oxidatively modified LDL and other proteins were observed in aorta of WHHL rabbit exhibiting atherosclerotic lesions [288-290]. LDL extracted from aortic lesions of WHHL rabbits greatly resembled LDL that had been oxidatively modified *in vitro* [291]. However, none of the above evidence has been observed in cholesterol-fed rabbits. Probucol inhibited the development of atherosclerosis in WHHL rabbits by inhibiting lipid peroxidation rather than by lowering plasma cholesterol levels [283,353]. *In vitro* experiments showed that probucol inhibits oxidative modification of LDL and its subsequent uptake and degradation by macrophages [284]. On the other hand, the beneficial effects of antioxidants in preventing atherogenesis in cholesterol-fed rabbits are still controversial. Two reports showed that antioxidants suppress lesion development [285,354], whereas another report showed that probucol failed to prevent the lesion formation [355], in cholesterol-fed rabbits even though it lowered the level of TBARS in plasma in the early phase of the treatment [355].

The above discrepancy might be due to the different mechanisms of atherogenesis in these two rabbit models. WHHL rabbits resemble homozygous familial hypercholesterolemic human characterized by missing or nonfunctional LDL receptors. Lack of LDL receptors results in the diminished removal of LDL from the circulation and consequently increased plasma cholesterol levels. However, WHHL rabbits clear chylomicron remnants normally [356] and rapidly clear an appreciable fraction of VLDL particles from the circulation [357], achieved by a newly-discovered receptor called low density lipoprotein receptor-related protein [358,359]. Therefore, in WHHL rabbits, LDL (or more specifically, modified LDL) is the only lipoprotein responsible for the development of atherosclerotic lesions. Probucol, being hydrophobic, is transported in LDL particles and therefore protects LDL from free-radical attack and serves as an anti-atherosclerotic agent.

Unlike WHHL rabbits, cholesterol-fed rabbits have increased dietary cholesterol intake and the consequently their LDL receptors are down-regulated. The hyperlipidaemia in cholesterol-fed rabbits is characterized by elevated levels of ß-VLDL. Unlike normal rabbits, ß-VLDL from cholesterol-fed rabbits is enriched in cholesterol rather than triglycerides. This cholesterol-enriched VLDL can be taken up by macrophages via ß-VLDL receptor. Like scavenger receptor, ß-VLDL receptors are not down-regulated by the excessive amounts of cholesterol accumulated within the macrophages. Even though ß-VLDL oxidatively modified by copper ions *in vitro* was reported to be taken up by macrophages at a greater rate than that of native ß-VLDL [360,361], little is known about the lipid peroxidation of ß-VLDL and the contribution of modified ß-VLDL (and/or modified LDL) to atherogenesis in cholesterol-fed rabbits compared to native ß-VLDL. Furthermore, besides being accumulated within macrophages, lipids are also accumulated within arterial smooth muscle cells, forming smooth muscle cell-derived foam cells. Unlike macrophages, arterial smooth muscle cells do not accumulate oxidatively modified LDL [362]. Therefore, modification of LDL may not be responsible for the formation of smooth muscle cell-derived foam cells in atherosclerotic lesions.

At this time it is too early to say that increased lipid peroxidation is the main factor

responsible for the formation of atherosclerotic lesion in cholesterol-fed rabbits. We can only conclude that hyperlipidaemia causes atherosclerosis which is associated with increased lipid peroxidation.

4.4. PROSTANOID PRODUCTION IN ATHEROSCLEROTIC RABBITS

4.4.1. Prostacyclin Production in Atherosclerotic Rabbits

The major metabolite of arachidonic acid released by both slices and the luminal surface of rabbit aorta was prostacyclin, which was measured as its stable degradation product, 6-oxoPGF$_{1\alpha}$. The synthesis of 6-oxoPGF$_{1\alpha}$ by slices of aorta from hypercholesterolemic rabbits, particularly after 8 and 15 weeks on the cholesterol-supplemented diet, was higher than that from the control rabbits when both time and diet were considered to be independent variables (**Fig. 41A**). On the other hand, 6-oxoPGF$_{1\alpha}$ synthesis by the aortic lumen was unaffected by the diet (**Fig. 41B**). When the synthesis of 6-oxoPGF$_{1\alpha}$ by different sections of the aorta was compared at a single time point (15 weeks) with the diet as the only dependent variable, cholesterol-treatment did not have significant effect (**Fig. 42**; A and B) in spite of the fact that the upper section (S_1) was almost completely covered with lesions (**Table 7**). The poor correlation between PGI_2 synthesis and atherosclerosis in cholesterol-fed rabbits is further illustrated by **Fig. 43**.

Our results are not in accord with a number of reports in the literature on vascular prostacyclin synthesis in hypercholesterolemic rabbits. The synthesis of PGI_2 by slices of aorta, measured using a bioassay, was reported to be reduced by about 80% in rabbits fed a diet supplemented with cholesterol for 4 weeks [143]. PGI_2 synthesis remained at this level for up to 13 weeks, but subsequently increased, so that it was only about 35% lower than controls after 22 weeks on the diet [143]. Similar results were recently reported by another group, who used radioimmunoassay to measure 6-oxoPGF$_{1\alpha}$ production by aortic slices from rabbits fed a cholesterol-supplemented diet for 9 weeks, followed by a normal diet for an additional 5 weeks [332]. In a slightly different model, diet-induced hypercholesterolemia was shown to prevent the *recovery* of PGI_2 synthesis by rabbit aorta after removal of the endothelium with a balloon catheter [138]. Human atherosclerotic arteries were found to synthesize less PGI_2 than similar control arteries

[145]. Smooth muscle cells from cholesterol-fed rabbits [147] or even smooth muscle cells enriched with cholesterol [141] were reported to produce less PGI_2 from exogenous arachidonic acid.

However, decreased PGI_2 synthesis by blood vessels from cholesterol-fed rabbits has not been observed in all studies. Production of PGI_2 by atherosclerotic aorta, ranging from the arch to the abdominal aorta was increased on the basis of a bioassay of PGI_2 using rat platelets [122]. Homogenates of atherosclerotic aortae of rabbits fed a 1% cholesterol-supplemented diet for 32 weeks produced more 6-oxo$PGF_{1\alpha}$ compared to control homogenates [149]. 6-Oxo$PGF_{1\alpha}$ synthesis was moderately correlated to total aortic cholesterol content (r = 0.643). In another model in which rabbits were fed a cholesterol-supplemented diet for 2 weeks followed by deendothelialization and a continuous cholesterol-supplemented diet for 3 months thereafter, aortic 6-oxo$PGF_{1\alpha}$ was higher than in rabbits which underwent deendothelialization but had normal diets [148].

The reason for the discrepancy between our results and some of those described in the literature is not clear, but the experimental protocol we used was somewhat different from those used in the above studies. In our study, we attempted to minimize autoinactivation of prostaglandin endoperoxide synthase caused by manipulation of the aorta prior to incubation by pretreatment with ibuprofen and EDTA. Thus our results should more accurately reflect the ability of the aorta to synthesize prostacyclin *in vivo*.

There are substantial differences between the amounts of PGI_2 produced by normal, unmanipulated blood vessels and the large amounts released by isolated or perfused vessels [86,87]. The marked increase in PGI_2 production in the latter cases appears to be a response to injury or mechanical stimulation. It is now known that injury and mechanical manipulation, along with other factors, stimulate the release of arachidonic acid from membrane phospholipid, providing substrate for prostaglandin endoperoxide synthase and other enzymes involving in prostanoid synthesis. Prostaglandin endoperoxide synthase [86,257,363,364], prostacyclin synthase [260] and thromboxane synthase [365,366] have all be shown to be inactivated during catalysis probably via hydroperoxide-related mechanism [260,364,367,368]. The self-inactivation of prostaglandin endoperoxide synthase in the presence of substrate can be prevented by

reversible prostaglandin endoperoxide synthase inhibitors such as ibuprofen [89,369]. After being stabilized by inhibitors during tissue preparation, prostaglandin endoperoxide synthase can be activated on demand as soon as the inhibitors are removed. By doing so, we preserve both prostaglandin endoperoxide synthase and prostacylin synthase activities in blood vessels. Even though the use of reversible prostaglandin endoperoxide synthase inhibitors in preparing blood vessels is unique to our study, the use of reversible inhibitors in preparing sensitive samples for subsequent studies has also been used by others. For example, preparation of human platelets from whole blood in the presence of prostacyclin, which prevents platelet activation by increasing cAMP level, results in stable platelet suspensions which respond to agonists and inhibitors readily and remain morphologically normal and physiologically active for long periods [370,371].

The absence of a reduction in prostacyclin synthesis in hypercholesterolemic rabbits was confirmed by measurement of its urinary metabolite, 2,3-dinor-6-oxo$PGF_{1\alpha}$ (**Fig. 46A**; P. 128). To our knowledge, this is the first report on the urinary levels of this substance in hypercholesterolemic rabbits. *In vivo* production of PGI_2 has been reported to be higher in atherosclerotic humans than in healthy control subjects, on the basis of measurement of urinary 2,3-dinor-6-oxo$PGF_{1\alpha}$ [150,372].

Those who reported decreased PGI_2 production in atherosclerosis postulated that this was due to the inhibition of prostaglandin endoperoxide synthase and prostacyclin synthase by lipid hydroperoxides which were increased in this disease [262,331,332,373,374]. Fatty acid hydroperoxides inhibit both prostaglandin endoperoxide synthase [261,320] and prostacyclin synthase [258,261] *in vitro*. Oxidized LDL was also reported to inhibit PGI_2 synthesis by endothelial cells [375]. However, our results do not support this hypothesis. Cholesterol-supplementation resulted in increased lipid peroxidation, measured as monohydroxy derivatives of the fatty acids, but did not decrease PGI_2 production in aorta. The lack of an inhibitory effect of increased lipid peroxidation on prostacyclin synthesis could be due to differences in the cellular or subcellular localization of the lipid peroxides and the enzymes required for PGI_2 synthesis. Moreover, our data indicated that fatty acids esterified to lipids are the primary site of lipid peroxidation and that hydroperoxy derivatives of these esterified lipids are

not nearly as effective as the corresponding unesterified hydroperoxy fatty acids in inhibiting either prostaglandin endoperoxide synthase or prostacyclin synthase (**Fig. 47**; P. 130).

The slight increase in 6-oxo$PGF_{1\alpha}$ from hyperlipidemic aorta might be due to the increase of free hydroxy fatty acids in the aortic tissue. Low concentrations of lipid hydroperoxides stimulate whereas high concentrations inhibit prostaglandin endoperoxide synthase *in vitro*. Free hydroperoxy/hydroxy fatty acids were only slightly higher in aortae from cholesterol-fed rabbits compared to control rabbits in this experiment. The increase in free hydroperoxy fatty acid formation within aorta may be stimulatory rather than inhibitory to prostaglandin production. It has been reported that the amounts of hydrogen peroxide released by activated neutrophils stimulate PGI_2 production by endothelial cells [376,377]. If this is the case for macrophages in atherosclerotic lesions, PGI_2 production would be expected to increase.

In contrast to the report mentioned above in which oxidized LDL was found to inhibit PGI_2 synthesis by endothelial cells [375], others have reported the opposite results for both endothelial cells [378,379] and by macrophages [380]. Uptake of malondialdehyde-modified LDL via the scavenger receptor of monocytes and macrophages promotes the release of arachidonic acid from these cells [381]. 13-Hydroxy-18:2, and possibly other hydroxy fatty acids, could increase 6-oxo$PGF_{1\alpha}$ production by endothelial cells by enhancing arachidonic acid release from phospholipid [382]. It is also possible that increased lipid peroxidation could result in elevated phospholipase A_2 activity [340] and therefore provide more arachidonic acid for prostaglandin synthesis.

PGI_2 synthesis may also be increased in atherosclerotic tissue by a mechanism not related to hydroperoxides. The synthetic phenotype of arterial smooth muscle cells, which is present only in atherosclerotic lesions, synthesized more PGI_2 *de novo* than did the contractile phenotype of these cells [383]. Increased PGI_2 production by modulated smooth muscle cells might be the result of increased amounts of growth factors in atherosclerotic tissues due to the accumulation of macrophages. Growth factors stimulate the synthesis of enzymes required for prostaglandin synthesis [132-135]. Therefore, even

though prostaglandin endoperoxide synthase could be covalently inhibited by lipid peroxidation products, the inactivated enzymes would be readily replaced by newly synthesized prostaglandin endoperoxide synthase.

The discrepancies in the literatures concerning PGI_2 synthesis by atherosclerotic aorta could also be due to the type of lesions studied. When compared to unaffected areas in human aorta, both PGI_2 (measured by bioassay on rabbit platelets) and its stable degradation products 6-oxo$PGF_{1\alpha}$ (measured by RIA) in the same human subjects were slightly increased in fatty streaks but dramatically decreased in advanced plaques [146].

4.4.2. Thromboxane Production in Atherosclerotic Rabbits

The most dramatic change in prostanoid synthesis observed in our study was in TXB_2 formation, which was about 10 times higher in slices from the aortic arch (S_1) of hypercholesterolemic rabbits compared to controls after 15 weeks (**Fig. 41C**; P. 120). There was also a large increase in the release of TXB_2 by slices from the lower part of the thoracic aorta (S_2), but the difference was less than with the aortic arch (**Fig. 42C**). In contrast to aortic slices, the differences between TXB_2 formation by the luminal surface of aortae from control and hypercholesterolemic rabbits were quite modest for aortic arch, whereas no difference was observed for the lower part of the aorta (**Figs. 41D and 42D**).

Modest increases (ca. 30 to 200% above control values) in the production of TXB_2 by whole aorta [179] or aortic slices [332] from hypercholesterolemic rabbits have recently been reported in the literature. Production of TXB_2 from exogenous arachidonic acid by slices of deendothelialized aortae from hypercholesterolemic rabbits was also recently reported to be about 70% higher after 30 min than in controls [148]. However, production of TXB_2 by different regions of the aorta or by the luminal surface was not reported in the above studies.

The source of TXB_2 in atherosclerotic aorta is of interest. Only very small amounts of this product were synthesized by normal aorta. Platelets produces substantial amounts of TXB_2. Even though platelets can be seen in atherosclerotic lesions [384,385], they are mostly located near the aortic lumen if platelets were the source of the increased TXB_2

production by atherosclerotic aorta, the aortic lumen might be expected to produce more of this substance than aortic slices. However, our data indicates that TXB_2 synthesis is increased much more with aortic slices than with the lumen. The amounts of TXB_2 released from slices of aorta were positively correlated to the total cholesterol content in atherosclerotic lesions (**Fig. 44**; see P. 125). This correlation became weaker when the lesion area was used to evaluated the severity of atherosclerosis (**Fig. 44A**). The correlations were even weaker when the luminal TXB_2 was plotted against the severity of atherosclerosis (**Fig. 44**; C and D). The discrepancy in the results between aortic slices and the aortic lumen would suggest that the major site of increased TXB_2 synthesis in aorta from hypercholesterolemic rabbits is not the endothelium, but rather the underlying cells. Two types of cells within atherosclerotic lesions which could be responsible for producing TXB_2 would be monocyte/macrophages and modified smooth muscle cells. It has not been shown that secretory smooth muscle cells produce increased amount of TXB_2. On the other hand, TXB_2 is the major prostaglandin endoperoxide synthase product of arachidonic acid metabolism in monocytes/macrophages [71,386,387]. It is therefore likely that monocytes/macrophages are the source of TXB_2 in atherosclerotic lesions.

The large increase in TXB_2 synthesis by the aortic arch that we observed raises the possibility that aortic TXA_2 could be involved in the development of atherosclerosis, due to its potent effects on platelets. An inhibitor of thromboxane synthase has been shown to reduce the area of aorta covered by atherosclerotic lesions in cholesterol-fed rabbits [191]. However, it must be kept in mind that TXA_2 production by blood vessels, even in atherosclerosis, would be much less than by platelets, the major site of its production. We did not observe any significant differences in the excretion of the urinary TXA_2 metabolite, 2,3-dinor-TXB_2, between control and cholesterol-fed rabbits, suggesting that in this model there are no large alterations in TXA_2 production by platelets *in vivo*. This is in contrast to human studies in which the *in vivo* production of TXA_2 was shown to be considerably elevated in atherosclerotic patients [177].

The high degree of correlation between TXB_2 and monohydroxy derivatives of arachidonic acid in aorta (r = 0.926) raises a very interesting question: where are

increased monohydroxy derivatives of arachidonic acid produced? The correlation between TXB_2 and monohydroxy derivatives of arachidonic acid (**Fig. 45**) is similar to the correlation between aortic cholesterol and TXB_2 (r = 0.865; **Fig. 44**) and between aortic cholesterol and monohydroxy derivatives of arachidonic acid (r = 0.857; **Fig. 37F**). Does this suggest that the increase in both TXB_2 and h-20.4 are due to the same cell type, possibly macrophage-derived foam cells, in atherosclerotic lesions? If this is the case, it may suggest that membrane phospholipid of macrophages are the source of substrate for TXB_2 synthesis, whereas neutral lipids in lipid droplets within or around macrophages or macrophage-derived foam cells are the source of monohydroxy derivatives of fatty acids.

4.4.3. Prostaglandin E_2 Production by Aortae from Atherosclerotic Rabbits

PGE_2 was used as a parameter to determine whether aortic prostacyclin synthase was selectively inhibited in cholesterol-fed rabbits. Inhibition of prostaglandin endoperoxide synthase would lead to a decrease in the synthesis of both 6-oxo$PGF_{1\alpha}$ and PGE_2, whereas inhibition of prostacyclin synthase alone would result in a decrease in 6-oxo$PGF_{1\alpha}$, but an increase in PGE_2 due to the redirection of PGH_2 to the enzymatic or nonenzymatic synthesis of PGE_2. Feeding cholesterol did not result in inhibition of PGI_2 synthesis by aorta (**Fig. 41**; A and B) and had little effect on PGE_2 synthesis by slices (**Fig. 41E**) or by luminal surface (**Fig. 41F**) Even though only small amounts of PGE_2 are synthesized from endogenous substrate, the proportion was much larger when exogenous arachidonic acid was used as a substrate. In incubations with slices of aorta, where prostanoids are produced from endogenous substrate, the amount of 6-oxo$PGF_{1\alpha}$ is 20-50 times higher than that of PGE_2 (**Fig. 41A** *vs.* **Fig. 41E**). On the other hand, for incubations utilizing the template, where prostanoids are produced from exogenous substrate, the amount of 6-oxo$PGF_{1\alpha}$ is only 3-4 times higher than that of PGE_2 (**Fig. 41B** *vs* **Fig. 41F**). The relatively higher level of PGE_2 production from exogenous substrate, which has also been reported by others [86], may suggest that the capacity of prostacyclin synthase is smaller than that of prostaglandin endoperoxide synthase in cells of the aortic lumen. PGH_2 or PGG_2 may accumulate under these conditions,

and be converted to PGE_2. Moreover, the concentration of hydroperoxide and other reactive intermediates generated by prostaglandin endoperoxide synthase in the presence of an excess of arachidonic acid may result in the selective inactivation of PGI_2 synthase, which is somewhat more sensitive to hydroperoxides than prostaglandin endoperoxide synthase. An alternative explanation could be the different catabolism of PGH_2 in incubation with slices of aorta and in incubation using template. Vascular endothelial cells have high levels of both prostaglandin endoperoxide synthase and PGI_2 synthase whereas the levels of prostacyclin synthase are higher than that of prostaglandin endoperoxide synthase in vascular smooth muscle cells [388]. In incubations with slices of aorta, PGH_2 produced by the endothelial cells can be used by PGI_2 synthase in both endothelial cells and smooth muscle cells. One the other hand, in incubations using the template, PGH_2 produced by endothelial cells can only be used by PGI_2 synthase in endothelial cells. A combination of excessive production of PGH_2 from exogenous arachidonic acid and limited catabolism of PGH_2 by PGI_2 synthase could then result in the redirection of PGH_2 to PGE_2 by PGE_2 synthase or by nonenzymatic isomerization.

4.5. EFFECTS OF LIPID HYDROPEROXIDES ON PROSTANOID PRODUCTION IN VITRO

Hydroxy derivatives of fatty acids in aorta, both from control and cholesterol-fed rabbits, are predominantly esterified to lipids. Free monohydroxy 18:2 accounts less than 10% of the total monohydroxy 18:2. Although free monohydroxy 20:4 are approximately equal to their esterified counterparts in control rabbit aorta, the increased amounts of monohydroxy 20:4 in aortae from cholesterol-fed rabbits are primarily in esterified lipids. Unesterified hydroperoxy polyunsaturated fatty acids have been found to inhibit both prostaglandin endoperoxide synthase and PGI_2 synthase [256,258-261]. It was therefore of interest to investigate the effects of esterified hydroperoxy polyunsaturated fatty acids on prostaglandin endoperoxide synthase and PGI_2 synthase.

Esterified hydroperoxides are much less potent than their free acid counterparts in inhibiting both prostaglandin endoperoxide synthase and prostacyclin synthase from

particulate fractions of bovine aorta (**Fig. 47**). Among the three hydroperoxy lipids investigated, hydroperoxy derivative of 1-palmitoyl-2-linoleoyl-phosphatidylcholine inhibited prostaglandin endoperoxide synthase in a dose-dependent manner whereas hydroperoxy derivatives of cholesteryl linoleate and trilinolein did not. This may be because hydroperoxy derivatives of phospholipids are more accessible to prostaglandin endoperoxide synthase than the hydroperoxy derivatives of neutral lipids. The differences in the inhibitory effects of hydroperoxy fatty acids and hydroperoxy lipids could also be due to the insolubility of hydroperoxy lipids in aqueous solution in spite of the fact that we used detergent in our studies. Two of the commonly-used methods to solubilize lipids in aqueous solution are the utilization of detergents and liposomes. However, there are drawbacks to these approaches in studies related to the effect of lipid hydroperoxide on enzyme activity. Even though nonionic detergents have been widely used for solubilizing membranes and lipids, they may modify the enzyme structure and subsequently their activity. One of the examples is the inhibitory effects on prostaglandin endoperoxide synthase (**Fig. 48** on P. 131). Consequently, a concentration of 0.02% Triton X-100 was used in our experiment. Even though Triton X-100 has been shown to generate hydroperoxides which are as highly reactive as hydrogen peroxide [389], there is no evidence showing that it interferes our experiment. Firstly, this detergent has no effect on prostacyclin synthase (**Fig. 48**) even though the enz .e is more sensitive to 13hp-18:2 (**Fig. 47**) Secondly, when digitonin, a structurall ela d detergent which did not inhibit prostaglandin endoperoxide synthase at a concentration of 0.1% was used, hydroperoxy phosphatidylcholine at a concentration of 25 μM inhibited prostaglandin endoperoxide synthase to a similar extent in the presence of 0.02% Triton X-100 and 0.05% digitonin (44% and 47% inhibition, respectively; see **Fig. 47A** and **Table 11**). This suggests that the inhibitory effect of hydroperoxy phosphatidylcholine on prostaglandin endoperoxide synthase is not the result of Triton X-100. The results in **Table 11** also reveal that hydroperoxy group is responsible for the inhibitory effect of hydroperoxy phosphatidylcholine since phosphatidylcholine itself had a much smaller effect on prostaglandin endoperoxide synthase. Even though we used detergents to facilitate the access of hydroperoxy lipids to the enzymes, we still can not be sure exactly

how the presence of the detergent affects this interaction. Another approach to the solublization of lipids in aqueous solutions is to form liposomes. However, sonication, even performed in an ice bath for a very short period, resulted in decomposition of lipid hydroperoxides.

Our study on the effects of hydroperoxy derivatives of fatty acids esterified to lipids agrees our experiment on the prostanoid production in aortae from hypercholesterolemic rabbits. Prostanoid synthesis is not decreased even though there is an increase in the amounts of monohydroperoxy derivatives of fatty acids esterified to aortic lipids. The increased amounts of monohydroperoxy derivatives of polyunsaturated fatty acids, however, are primarily located in neutral lipid fractions which consisted of cholesteryl esters and triglycerides (**Fig. 39** on P. 116). Hydroperoxy fatty acids esterified to neutral lipids are much weaker inhibitors to both prostaglandin endoperoxide synthase and prostacyclin synthase (**Fig. 47**). Furthermore, the enhanced production of TXA_2 is highly correlated to the increased formation of monohydroxy derivatives of 20:4 in aortic lipids. If hydroperoxy derivatives of 20:4 in lipids were potent inhibitors of prostaglandin endoperoxide synthase, TXA_2 would not increase at such drastic rate after the cholesterol-supplemented diet. Therefore the inhibitory effects of lipid peroxidation products on prostaglandin synthesis in atherosclerotic aorta can not be supported by our experiment.

SUMMARY AND CONCLUSIONS

The formation of monohydroxy derivatives of unsaturated fatty acids in human LDL and LDL and aorta from hypercholesterolemic rabbits has been studied. The effects of lipid peroxidation on PGI_2 synthesis and atherogenesis have been discussed.

We have developed a mass spectrometric procedure to measure the amounts of specific hydroperoxy fatty acids formed by oxidation of the major unsaturated fatty acids, such as oleic acid, linoleic acid, and arachidonic acid, in human LDL. Oxidation of human LDL in the presence of a relatively strong stimulus (20 μM $CuSO_4$) resulted in very large increases in the amounts of the major monohydroxy derivatives of linoleic acid (9 and 13-hydroxy derivatives) and arachidonic acid (5, 8, 9, 11, 12, and 15-hydroxy derivatives) in LDL lipids in the early stages of the reaction. After 20 hours, the amounts of these products declined due to substrate depletion. Large amounts of monohydroxy derivatives of oleic acid (8, 10, and 11-hydroxy derivatives) were also detected after incubation for 5 or 20 hours. Unlike the monohydroxy derivatives of linoleic and arachidonic acids, the amounts of monohydroxy derivatives of oleic acid increased in a time-dependent manner up to 20 hours. Although TBARS clearly increased under these conditions, the changes were not nearly as dramatic as those observed for monohydroxy fatty acids Oxidation of LDL in the presence of endothelial cells, a much milder stimulus, resulted in 2.5 to 3-fold increases in the amounts of monohydroxy derivatives of linoleic and arachidonic acids, as well as TBARS, with more modest increases in the amounts of hydroxylated derivatives of oleic acid No positional specificity was observed in the oxidation of any of the above fatty acids in the presence of either stimulus. This suggests that oxidation of LDL in the presence of endothelial cells may be primarily due to autooxidation, although a role for lipoxygenase(s) in the initiation of those reaction cannot be excluded. Our results also reveal that oxidation of LDL results in the formation of a large number of monohydroxy derivatives of oleic, linoleic, and arachidonic acids. The relative amounts of products formed from each of these fatty acids depends on the strength of the stimulus as well as the incubation time.

Increased oxidation of LDL lipids was also observed in rabbits fed a

cholesterol/peanut oil-supplemented diet. After 2, 8 or 15 weeks on a cholesterol diet, the amounts of monohydroxy derivatives of linoleic and arachidonic acids in LDL increased by over 2-folds compared to controls. When the increases were expressed in terms of the amounts of these LDL lipid oxidation products per millilitre of plasma, the differences were much greater because of the higher LDL concentrations in plasma from hypercholesterolemic rabbits, consistent with our observation on the relative resistance of oleic acid to oxidation in human LDL *in vitro*, oleic acid in rabbit LDL appeared to be more resistant to oxidation *in vivo*, since there were no increases in the amounts of monohydroxy derivatives of this fatty acid in LDL from cholesterol-fed rabbits.

Increased amounts of the 9-hydroxy, and, to a lesser extent, the 13-hydroxy derivatives of 18:2 were observed in aortae from cholesterol-fed rabbits at both 8 and 15 weeks. The amounts of esterified 11, 12, and 15-hydroxy derivatives of 20:4 in aortae from cholesterol-fed rabbits were similar to controls after 2 or 8 weeks, but about 3-fold higher after 15 weeks. The greater amounts of hydroxy derivatives of C_{18} fatty acids in the cholesterol-fed group could be explained by an approximately 2 to 6-fold increase in C_{18} fatty acids in the aorta. However, this would not explain the increased levels of hydroxy-20:4 observed after 15 weeks, since aortic 20.4 levels were lower in the cholesterol-fed group. The selectively increased oxidation of 20:4 in aortae from cholesterol-fed rabbits was further supported by the highly positive correlations between monohydroxy derivatives of this fatty acid and the aortic total cholesterol and the area of aorta covered with atherosclerotic lesions. The discrepancy between the oxidation of C_{18} fatty acids and 20:4 could be due to differences in the distribution of these fatty acids in cellular lipids. Our results suggest that increased peroxidation of lipids, especially the ones containing 20:4, could be involved in the development of atherosclerotic lesions in cholesterol-fed rabbits.

In contrast to several early reports, PGI_2 synthesis by slices and luminal surface of the aorta as well as *in vivo* production, was not decreased in atherosclerotic rabbits, despite the increased oxidation of fatty acids esterified to aortic lipids. This may be because hydroperoxy fatty acids esterified to aortic lipids was less inhibitory to the enzymes required for PGI_2 synthesis. In agreement with this, we found that lipid

hydroperoxy derivatives of cholesteryl linoleate, trilinolein and 1-palmitoyl-2-linoleoyl-phosphatidylcholine are less potent than 13-hydroperoxy-18:2 in inhibiting both prostaglandin endoperoxide synthase and prostacyclin synthase. For our experiment on prostanoid synthesis by rabbit aorta, we developed conditions designed to minimize the autoinactivation of prostaglandin endoperoxide synthase during removal of the tissue. Thus, treatment of aortae with medium containing ibuprofen and EDTA during removal and preparation of the tissue resulted in an approximately 2-fold increase in 6-oxo$PGF_{1\alpha}$ production upon subsequent incubation. The preservation of prostaglandin endoperoxide synthase during tissue preparation may explain in part the discrepancy between our results and some of the other reports in the literature.

In contrast to aortic 6-oxo-$PGF_{1\alpha}$ synthesis, there was a dramatic 10-fold increase in TXB_2 released from slices of thoracic aorta after rabbits were fed a cholesterol/peanut oil-supplemented diet for 15 weeks. Although TXB_2 synthesis by the luminal surface of the aorta was also higher than controls, the difference was not as dramatic as observed in slices of aorta, suggesting that the primary site of TXB_2 synthesis in the aorta is the inner part of the blood vessel. Aortic TXB_2 production also has highly positive correlations with aortic total cholesterol and the area of aorta covered with atherosclerotic lesions. It is possible that aortic TXA_2 could contribute to the development of atherosclerosis due to its potent effects on platelets.

CLAIMS TO ORIGINAL RESEARCH

- A GC-MS assay with selected ion monitoring has been employed to quantify monohydroxy derivatives of fatty acids in human LDL as well as LDL and aorta from rabbits.
- In the presence of high concentrations of copper ions, monohydroxy derivatives of polyunsaturated fatty acids in LDL increase dramatically in the early phases of the incubation but are converted to other products during the late phases of the reaction. On the other hand, the rate of oxidation of oleic acid to its monohydroxy derivatives and the conversion of the latter to other products are much lower than those of the polyunsaturated fatty acids and their monohydroxy products. Incubation of LDL with endothelial cells also results in increased oxidation of polyunsaturated fatty acids to their monohydroxy derivatives. Comparison of different fatty acids oxidized to their monohydroxy derivatives in LDL has not been previously studied.
- The amounts of monohydroxy derivatives of fatty acids are elevated in LDL and aortae from hypercholesterolemic rabbits. No previous report of such a comprehensive study on the peroxidation of different fatty acids in LDL and aortae from hypercholesterolemic rabbits has been published.
- Even though increases in the amounts of monohydroxy C_{18} fatty acids were observed in aortae from hypercholesterolemic rabbits, this appeared to be due to amounts of substrate fatty acids. An increased percentage of fatty acids being oxidized to their monohydroxy derivatives was only observed with arachidonic acid. Only the amounts of monohydroxy derivatives of arachidonic acid were correlated positively to the severity of the atherosclerotic lesions of the hypercholesterolemic rabbits. This appears the first study demonstrating the direct correlation between lipid peroxidation and the severity of atherosclerosis.
- Monohydroxy derivatives of linoleic and arachidonic acids were increased selectively in the neutral lipids in the aortae from hypercholesterolemic rabbits. No studies on the distribution of lipid peroxidation products in different lipid fractions from atherosclerotic aorta has been reported.

- We found that it is important to prepare aorta samples under conditions in which the activities of enzymes for prostaglandin biosynthesis have been preserved by preventing enzyme autoinactivation. This approach has not previously been used in studies on prostanoid production.
- Thromboxane production is dramatically higher in aortae from hypercholesterolemic rabbits and this increase was much more pronounced for the slices than the lumenal surface of the aorta.
- There was a very significant degree of correlation between the formation of monohydroxy derivatives of arachidonic acid and synthesis of TXB_2 in aortae from hypercholesterolemic rabbits. Even though the correlation between TXA_2 and severity of atherosclerosis has been studied, the correlation between lipid peroxidation products and TXA_2 synthesis in atherosclerosis has not been explored.
- Even though dinor metabolites of PGI_2 and TXA_2 have been studied in the urine from other species, we are the first to report the amounts of these products in the urine from rabbits.
- Hydroperoxy fatty acids esterified to lipids are not as good inhibitors as free hydroperoxy fatty acids of prostaglandin endoperoxide synthase and prostacyclin synthase.

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