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University of Alberta

Effects of *ex vivo* and *in vivo* oxidation on high density lipoprotein structure and function.

by

Ryan James Perry 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Experimental Medicine

Department of Medicine

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If you're looking to find the key to the Universe, I have some bad news and some good news. The bad news is: there is no key to the Universe. The good news is: it has been left unlocked.

Something I read somewhere.

Abstract

To test the hypothesis activated human phagocytes oxidatively modify high density lipoprotein (HDL) to a form that enhances cellular cholesterol efflux, we investigated the effects of *ex vivo* and *in vivo* oxidation on HDL structure and function. We found that *ex vivo* oxidation of HDL by activated human neutrophils leads to crosslinking of apolipoprotein AI and AII to themselves and each other, in the absence of significant lipid peroxidation products. The oxidative crosslinking of HDL apolipoproteins was intraparticle, and the myeloperoxidase/hydrogen peroxide system was found to be involved in the oxidative crosslinking. The ability of HDL oxidized by activated neutrophils to deplete the acyl-CoA:cholesterol acyltransferase (ACAT)-accessible cholesterol pool in cholesterol-loaded human fibroblasts, was found to depend on the extent of apolipoprotein degradation and apolipoprotein crosslinking, which resulted in a decreased or increased ability of these HDL particles to deplete this pool, respectively. HDL modified *in vivo* at inflammatory sites also exhibited crosslinking of its apolipoproteins, and no significant apolipoprotein degradation. *In vivo*-oxidized HDL had at least a similar, or enhanced, ability to deplete intracellular cholesterol available for esterification by ACAT. These studies show that the *ex vivo* and *in vivo* oxidation of HDL results in crosslinking of its major apolipoproteins, and along with the absence of apolipoprotein degradation, results in an HDL particle that has at least a similar, or enhanced, ability to deplete the ACAT-accessible cholesterol pool compared with non-oxidized HDL. The oxidation of HDL *in vivo* may therefore explain a portion of the anti-atherogenic effects associated with increased plasma HDL levels.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Effects of *ex vivo* and *in vivo* oxidation on high density lipoprotein structure and function submitted by Ryan James Perry in partial fulfillment of the requirements for the degree of Master of Science in Experimental Medicine.

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Abbreviations

Buffers

A	50 mM NaCl, 5 mM Tris HCl supplemented with 1 mM EDTA, pH 7.4
B	150 mM NaCl supplemented with 1 mM EDTA
C	HBSS(-), supplemented with 100 μM DTPA, pH 7.4
D	RPMI-1640, 5 mM EDTA, 2 % FBS
E	PBS supplemented with 1 μM $CuSO_4$, pH 7.4
F	66 mM potassium phosphate buffer passed over Chelex 100 resin to remove any contaminating transition metal ions, then supplemented with 100 μM DTPA, pH 8.0
G	PBS supplemented with 100 μM DTPA, pH 8.0
H	HBSS(-) supplemented with 100 μM DTPA, 1 mg/mL D-glucose, 1 μg/mL aprotonin, 1μg/mL leupeptin, 1 μg/mL soy trypsin inhibitor, 0.5 mM PMSF, pH 7.4
I	Tris buffered saline supplemented with 0.1 % Tween 20

Diseases

CGD	chronic granulomatous disease

Enzymes

ACAT	acyl-CoA:cholesterol acyltransferase
CETP	cholesterol ester transfer protein
HRP	horseradish peroxidase
LCAT	lecithin:cholesterol acyltransferase
MPO	myeloperoxidase

NADPH oxidase	nicotinamide adenine dinucleotide phosphate oxidase
NCEH	neutral cholesterol ester hydrolase
PAF-AH	platelet activating factor-acetylhydrolase
SOD	superoxide dismutase

Lipids

CE	cholesterol ester
FA	fatty acid
FC	free cholesterol
MUFA	monounsaturated fatty acid
PC	phosphatidylcholine
PUFA	polyunsaturated fatty acid
TG	triglyceride

Lipoproteins

apo	apolipoprotein
CtlHDL	control high density lipoprotein
HDL	high density lipoprotein
IDL	intermediate density lipoprotein
LDL	low density lipoprotein
oxHDL	oxidized high density lipoprotein
oxLDL	oxidized low density lipoprotein
PlmHDL	plasma high density lipoprotein
TyrHDL	tyrosylated high density lipoprotein
SynHDL	synovial fluid high density lipoprotein

VLDL	very low density lipoprotein

Reagents

BSA	bovine serum albumin
CB	dihydrocytochalasin B
DFP	diisopropyl fluorophosphate
DMEM	Dulbecco's modified Eagle's medium
DTPA	diethylenetriaminepentaacetic acid
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
fMLP	N-formyl-methionyl-leucyl-phenylalanine
HBSS(-)	calcium and magnesium free Hank's balanced salt solution
H_2O_2	hydrogen peroxide
KBr	potassium bromide
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
P/S	penicillin/streptomycin
PMA	phorbol 12-myristate 13-acetate
SDS	sodium dodecyl sulfate
SFM	serum free media
TMB	3,3',5,5'-tetramethylbenzidine

Receptors

LDLR	low density lipoprotein receptor
LRP	low density lipoprotein receptor related protein

SR-BI	scavenger receptor class B type I

Technical Terms

GGE	gradient gel electrophoresis
MACS	magnetic cell separation system
SDS-PAGE	polyacrylamide gel electrophoresis including SDS
TLC	thin layer chromatography
RCT	reverse cholesterol transport

Weights and Measures

Amp	amperes
°C	degrees Celsius
Ci	Curies
cpm	counts per minute
d	density
Da	Daltons
ε	molar absorbance
g	acceleration due to gravity
g	grams
h	hours
kDa	kiloDaltons (10^3 Daltons)
L	litre
M	molar (moles per liter)
mAmp	milliamperes (10^{-3} amperes)
min	minutes

mL	milliliters (10^{-3} liters)
mM	millimolar (10^{-3} molar)
MWCO	molecular weight cut-off
nM	nanomolar (10^{-9} molar)
pH	-log of the concentration of H^+ in solution
rpm	revolutions per minute
SD	standard deviation
SE	standard error
V	volts
v/v	volume per volume
μCi	microCuries (10^{-6} Curies)
μg	micrograms (10^{-6} grams)
μL	microliters (10^{-6} liters)
μM	micromolar (10^{-6} molar)

Miscellaneous

ABC1	ATP-binding cassette transporter 1
H^+	hydrogen atom
HMW	high molecular weight
HSF	human skin fibroblasts
LMW	low molecular weight
NO	nitric oxide
O_2	molecular oxygen
O_2^-	superoxide

RNS	reactive nitrogen species
WBC	white blood cell

Chapter 1

Introduction

1.1 Introduction to Cardiovascular Disease

Cardiovascular disease is the leading cause of death worldwide [1]. Cardiovascular diseases also represent a huge economic burden, with an estimated $286.5 billion dollars in health care costs being spent on them in the United States this year [2]. The principle cause of cardiovascular disease is atherosclerosis, of which one hallmark is the unregulated accumulation of lipid in the artery wall. The protective actions of one class of lipoproteins, high density lipoproteins (HDL), are believed to be due mainly to their ability to prevent this accumulation of excess cholesterol in cells, including cells in the arterial wall. An understanding of the protective actions of HDL requires an understanding of the pathogenesis of atherosclerosis.

1.2 Introduction to Atherosclerosis

Atherosclerosis is an inflammatory disease that leads to accumulation of lipids and leukocytes in the artery wall, a decrease in arterial elasticity and eventual occlusion of the artery lumen [3]. The resulting obstruction of the blood vessel, when located in the coronary vessels, can result in a clinically significant event, a myocardial infarction or heart attack. The location of atherosclerotic lesion formation appears to be determined somewhat by blood flow, and generally occurs at arterial sites such as bifurcations and curvatures, where blood flow is turbulent and shear stress is variable [4, 5]. The change in blood flow can alter the expression of adhesion molecules on endothelial cells [6] as well as increase the interaction time of blood-borne particles (such as lipoproteins) with the arterial wall, resulting in increased transendothelial diffusion [7]. Moreover, the increased expression of

adhesion molecules on arterial endothelial cells at these atherosclerotic prone sites leads to rolling and adherence of blood leukocytes, with their eventual transmigration into the arterial wall (Figure 1.1, A) [8].

While the events that initiate atherosclerosis are still unknown, there are two main hypotheses to explain the pathogenesis of atherosclerosis: the response-to-injury hypothesis and the lipid hypothesis. Although the two hypotheses will be explained individually, it should be recognized that the two processes are most likely interrelated.

The response-to-injury hypothesis originally proposed that endothelial injury and denudation of the endothelial layer precedes intimal thickening [9-11]. The hypothesis was then modified to state that endothelial cells may be injured or activated, resulting in expression of leukocyte adhesion molecules and cytokine secretion, but remain intact [12]. This modification may have been in response to a number of studies which showed that fatty streak lesions can and do develop under an intact endothelial layer [13]. Factors that may lead to the initial endothelial injury or *dysfunction* include: elevated and modified low density lipoprotein (LDL), free radicals in cigarette smoke, hypertension, elevated blood glucose, elevated plasma homocysteine concentrations, infectious microorganisms such as herpes viruses or *Chlamydia pneumoniae*, and combinations of these or other factors [3]. The endothelial injury allows for the migration of leukocytes into the intimal layer, and the progression of the disease under the influence of inflammatory and proliferative stimulants released by leucocytes within this space.

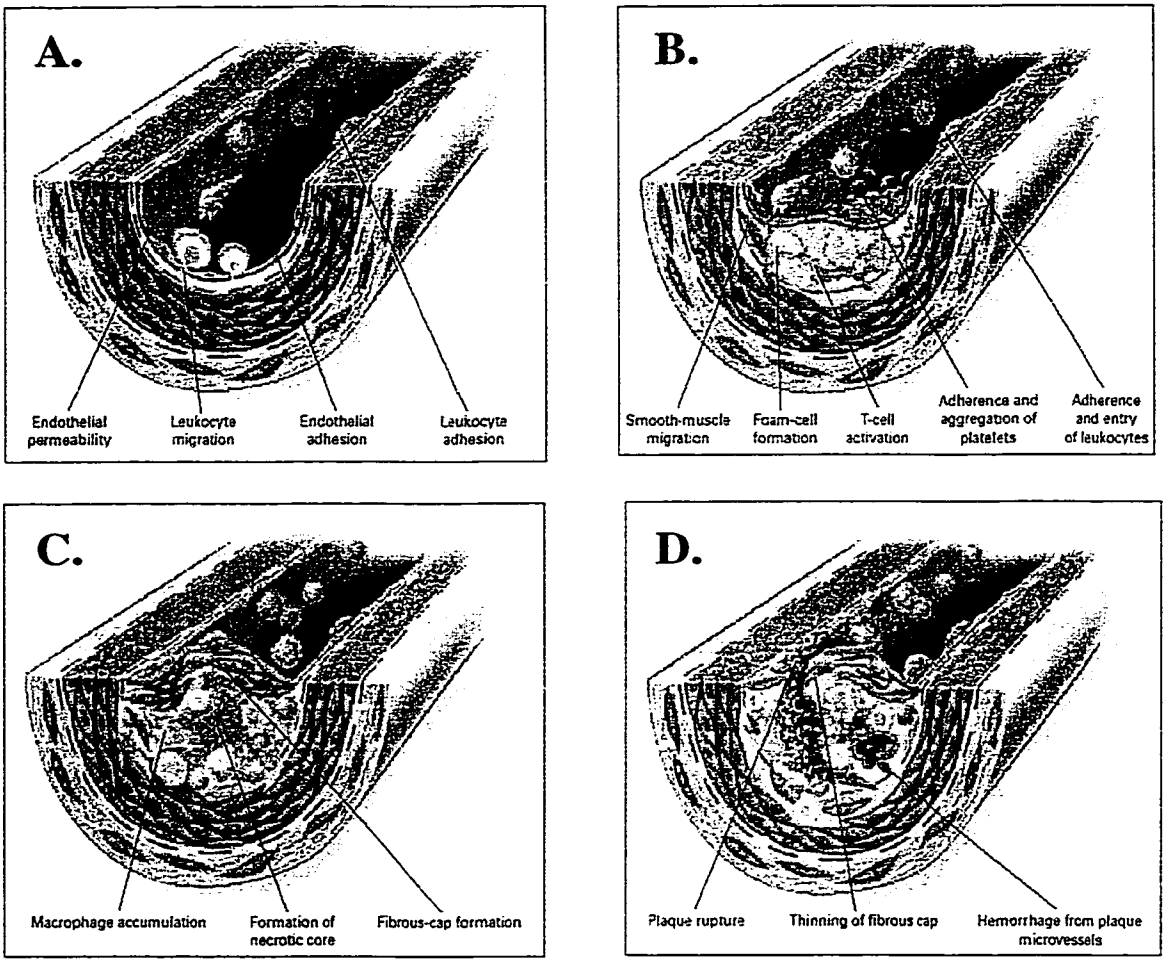


Figure 1.1 Lesion development in atherosclerosis.
A. Initiation of atherosclerosis: damage to the artery wall by various mechanisms (elevated cholesterol, viral or immune injury, sheer stress, etc.) leads to the adhesion and migration of white blood cells into the intimal space due to increased expression of adhesion molecules and increased endothelial permeability. **B. Early lesion:** fatty streaks are the first recognizable lesion of atherosclerosis. They consist of lipid-laden macrophages (foam cells) along with T lymphocytes and neutrophils (not shown). Medial smooth muscle cells may begin to infiltrate the intima due to growth factors released by activated T-cells, monocytes and macrophages. **C. Intermediate lesion:** as fatty streaks progress to intermediate lesions a fibrous cap of smooth muscle cells begins to form. The fibrous cap covers a mixture of leukocytes, lipid, and debris, which eventually leads to a necrotic core. The lesion expands at the shoulders by means of continued leukocyte adhesion and entry. The necrotic core results from a combination of apoptosis and necrosis, increased proteolytic activity and lipid accumulation. **D. Advanced lesion:** due to the release of proteases by activated leukocytes at the shoulder regions of the lesion the fibrous cap can eventually rupture. This can rapidly lead to thrombosis, hemorrhage and possible occlusion of the artery. Adapted from Ross [3].

The lipid hypothesis for atherosclerosis postulates that an elevation in plasma LDL levels results in the initial accumulation of LDL in the artery wall and the eventual formation of macrophage foam cells [14, 15]. The increase in LDL in the artery wall increases the likelihood of it being oxidized, and thereby becoming a ligand for uptake by macrophage scavenger receptors. As well, modified or oxidized LDL (oxLDL) is chemotactic to monocytes, promoting their migration into and retention in the artery wall [16, 17]. Oxidized LDL is also cytotoxic to endothelial cells, and may be responsible for their injury, dysfunction, and eventual loss from more advanced lesions [18, 19]. The lipid hypothesis is supported by the positive relationship between plasma LDL levels and cardiovascular risk [13], as well as a decrease in coronary heart disease with the use of lipid-lowering agents [20]. Moreover, oxLDL is present in atherosclerotic lesions of humans [21]. Regardless of their initial pathogenesis, atherosclerotic lesions represent different stages of a chronic inflammatory process, in which the dysfunction of lipid metabolism plays a key role.

Pathologically, the progression of atherosclerosis is generally defined in three stages: the early lesion, the intermediate lesion and the advanced lesion [22]. The early lesion can begin as early as childhood and is characterized by the formation of fatty streaks, the first recognizable lesion of atherosclerosis (Figure 1.1, B). Microscopically, fatty streaks consist primarily of monocyte-derived macrophages containing lipid deposits (known as foam cells), which are located in the subendothelial space or intima [23, 24]. These cells are the hallmark of atherosclerosis and are believed to result from unregulated uptake of oxidized and aggregated apolipoprotein B-containing lipoproteins by the macrophage scavenger

receptors [25]. Neutrophils have also been shown to be present in the early stages of atherosclerosis [26, 27], however they do not appear to accumulate lipid or become foam cells. Lastly, smooth muscle cells in the media and that have migrated into the intimal layer from the medial layer, may also contain lipid droplets [28, 29]. In contrast to more advanced lesions, most of the lipid deposits in the fatty streak are intracellular and consist of cholesteryl esters (CE), the storage form of cholesterol in cells [7].

The intermediate lesion results from further accumulation of lipid and foam cells (Figure 1.1, C). As well, lipid begins to deposit extracellularly, forming lipid pools just below the layers of macrophages and macrophage foam cells, creating a lipid core [7]. In contrast to early lesions where most of the cholesterol is present in the form of CE, intermediate lesions contain more free cholesterol (FC) along with the accumulation of extracellular lipids [30]. This may result from necrosis of foam cells followed by extracellular hydrolysis of CE to FC, LDL aggregation, or other unknown mechanisms [31]. Also, the intimal layer begins to thicken and a fibrous cap forms over the lipid core due to proliferation of smooth muscle cells, along with their production of collagen and proteoglycans. Monocyte recruitment into the lesion is further increased due to the inflammatory response of macrophages and production of cytokines by smooth muscle cells [3]. As the intermediate lesion progresses there is impairment of endothelium-dependent vasodilation [32-34] and stimulation of endothelial-dependent constriction [32, 35], and together with the increasing size of the intermediate lesion, this eventually results in the protrusion of the lesion into the arterial lumen.

The advanced lesion is characterized by a distinct acellular, necrotic core of dead cells plus extracellular lipid covered by a well-defined fibrous cap (Figure 1.1, D) [22]. Calcification of the artery wall occurs and is believed to result from the production of hydroxyapatite from a subpopulation of arterial wall cells, termed calcifying vascular cells, in response to lipid accumulation [36]. As well, macrophages concentrated in the shoulder regions of the lesion secrete proteases, which weaken the outside edge of the fibrous cap [3]. Increased blood flow due to narrowing of the artery lumen, and weakening of the fibrous cap at the shoulder regions by these proteases, increases the risk of plaque rupture, exposing the intima and creating a site for platelet adhesion, thrombosis, and possible occlusion of the blood vessel.

1.3 Introduction to Lipoproteins

The direct association between cholesterol carriers in the blood stream and atherosclerosis requires a basic introduction to lipoprotein structure and function. Lipoproteins are macromolecular complexes of lipids and specific proteins (termed apolipoproteins or apoproteins) that function to transport hydrophobic lipids, of dietary or endogenous origin, within the hydrophilic environment of plasma. The transported lipids are used by tissues for oxidative metabolism, triglyceride (TG) synthesis for energy storage, steroid hormone synthesis, and/or maintenance of cellular function and membrane integrity. Structurally, lipoproteins contain a central core of non-polar lipids, mainly TG and CE, surrounded by a monolayer of polar lipids, mainly phospholipids and some FC (Figure 1.2). Within the surface monolayer sit the apolipoproteins, which have amphipathic (hydrophilic plus hydrophobic)

properties similar to the phospholipids. Hydrophobic forces, whereby the fatty acyl chains and non-polar amino acid residues are excluded from the aqueous environment, thus drive the association of lipids and proteins within lipoproteins. Most apolipoproteins (excluding the B apolipoproteins) together with FC, have appreciable water solubility and can exchange readily between lipoprotein particles or with other lipid surfaces [37]. In contrast, non-polar lipids and phospholipids have little potential for spontaneous or passive exchange, but may be transferred between lipoproteins by specific transfer proteins [37].

The density of lipoproteins is inversely related to their size and reflects the relative amounts of low density lipid and high density surface protein present. Lipoproteins are classified from the least dense and largest particles to the most dense and smallest particles as follows: chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), LDL, and HDL.

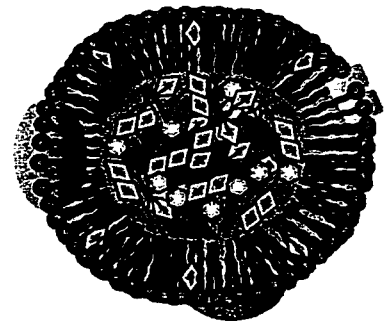


Figure 1.2. Basic structure of a plasma lipoprotein.
Outer monolayer consists mainly of phospholipid [polar head group (circles) with hydrophobic fatty acid (FA) chains facing inward] and some free cholesterol (diamond). Within the monolayer exist the apolipoproteins (ovals and rectangles). The hydrophobic inner core consists of cholesteryl esters (diamond with FA tail) and triglyceride (hexagon with 3 FA chains).

1.3.1 Apolipoprotein B-containing Lipoproteins

Apo B-containing lipoproteins include chylomicrons, VLDL, IDL, and LDL. Upon absorption of dietary fat the intestine synthesizes and secretes TG-rich chylomicrons [38]. Chylomicrons enter the circulation via the lymphatic system and are rapidly catabolized by lipoprotein lipase, located on the surface of capillary endothelial cells, to form TG-depleted particles called remnants. Chylomicron remnants are then cleared by the liver through the LDL receptor (LDLR), and possibly the LDL receptor related protein (LRP) pathway. VLDL is mainly synthesized and secreted by the liver and is TG-rich. When acted upon by LPL, IDL and LDL particles are generated and subsequently cleared from the circulation by either hepatic or peripheral cells through the LDLR pathway [39]. LDL is the major cholesterol-carrying lipoprotein in human plasma [37] and increased plasma LDL levels are correlated with increased risk for atherosclerosis [3].

1.4 Introduction to High Density Lipoproteins

1.4.1 High Density Lipoprotein Structure

HDL represent a heterogeneous population of particles in terms of their physicochemical properties and metabolic role, and are associated by the presence of at least one molecule of apolipoprotein AI (apo AI), the major apoprotein found in HDL (Figure 1.3) [40]. In general, plasma HDL can be classified into two main subfractions according to their density (d), namely HDL_2 (d = 1.063-1.125 g/mL) and HDL_3 (d = 1.125-1.21 g/mL). The smaller, denser HDL_3 fraction is thought to participate in cellular cholesterol efflux, whereas the larger, less dense HDL_2 fraction may act as 'cholesterol donor particles' to the liver and steroidogenic tissues via a

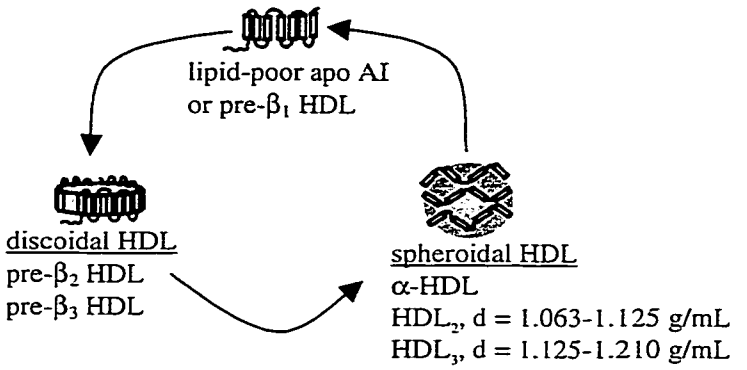


Figure 1.3. Models of high density lipoprotein particles.
Adapted from Fielding and Fielding [45].

receptor mediated process, as well as to apo B containing lipoproteins through the action of cholesteryl ester transfer protein (CETP) [41]. HDL particles can also be classified according to their electrophoretic mobility on agarose gels. The majority of HDL in plasma is of α-mobility and includes the spheroidal HDL_2 and HDL_3 subclasses. A smaller percentage of plasma HDL (2-14 % of total apo AI) has a similar mobility to VLDL and is therefore termed pre-β HDL [42-44]. Pre-β HDL can be further separated according to their size, generating three subfractions: pre-β_1, pre-β_2, and pre-β_3 and represent a continuum from a lipid poor apo AI particle to a discoidal HDL particle composed mainly of apo AI and polar lipid (phospholipid and FC) [41, 45]. Pre-β_1 HDL is believed to be the initial acceptor of cellular FC [43, 44].

The mechanisms by which HDL is generated *in vivo* are still not fully understood. The A apoproteins (apo AI, AII, and AIV) are the major protein components of plasma HDL, and can enter the circulation as components of either

chylomicrons secreted by the intestine or VLDL secreted by the liver [46]. This has led to the hypothesis that plasma HDL is assembled extracellularly as surface components of TG-rich lipoproteins (*e.g.*, phospholipids and FC) become dissociated during their lipolysis by lipoprotein lipase. It has been found, however, that inhibition of VLDL synthesis by the liver does not appear to affect HDL assembly [47-49], suggesting that lipolysis of chylomicrons alone can contribute to plasma HDL levels. Apo AI is also secreted by the liver as a proprotein, which is then converted, by proteolytic cleavage in the circulation, to mature apo AI with a molecular weight of approximately 28 000 daltons (Da) [50]. Secreted free apo AI is then thought to acquire lipids (phospholipids and FC) from cell membranes, as well as from lipolysis of TG-rich apolipoproteins, resulting in the formation of lipid-poor apo AI or pre-β HDL. Recent evidence from studies on Tangier disease (see below) suggests that the major mechanism of HDL formation is the acquisition of peripheral (non-hepatic) cell lipids by lipid free or lipid-poor apo AI [51, 52].

1.4.2 High Density Lipoprotein Function

In 1984, a review by Shlomo Eisenberg on HDL metabolism stated, "interest about HDL was revived in 1975 when Miller and Miller summarized existing data that demonstrated the putative role of HDL as a protective lipoprotein against premature development of atherosclerotic disease" [40]. Many epidemiological and prospective cohort studies have since supported this important beneficial effect of elevated levels of HDL in protection against atherosclerosis [53-56]. In fact, the Framingham Heart Study found plasma HDL levels to be a better predictor of

cardiovascular and coronary heart disease (CHD) risk than other CHD risk factors, including plasma LDL levels [57, 58].

1.4.2.1 Cardioprotective Functions of High Density Lipoprotein

The predominant mechanism by which HDL protects against the development of atherosclerosis is still not fully understood. *In vitro* and *in vivo* studies suggest HDL performs a variety of cardioprotective functions (reviewed in [59] and [60]). The most widely studied and best described of these is the ability of HDL to promote the removal of excess cellular cholesterol from peripheral cells, the initial step in a pathway referred to as "reverse cholesterol transport" (RCT) [45, 61]. HDL has also been shown to prevent the oxidation of LDL [62] and to neutralize the atherogenic effects of oxLDL [63]. These actions have been attributed to HDL-associated surface enzymes, which include paraoxonase, platelet activating factor-acetylhydrolase (PAF-AH), and lecithin:cholesterol acyltransferase (LCAT). HDL-associated paraoxonase was originally shown to retard the oxidation of LDL by preventing the generation of lipid peroxides [64, 65]. Subsequently, Watson *et al.* showed that purified paraoxonase could prevent the induction of endothelial cell adhesion molecules by oxLDL when incubated in a vascular cell co-culture system [66]. This effect was believed to be due to the cleavage by paraoxonase of sn-2 positioned fatty acids from oxLDL phospholipids. More recently, it was found that paraoxonase-deficient mice were more prone to atherosclerosis. These mice had HDL that was more readily oxidized and failed to prevent LDL oxidation *in vitro* [67]. This observation suggests that there is a cardioprotective role for HDL-associated paraoxonase. PAF-AH and LCAT also appear to have a similar function, both having the ability to inhibit the

oxidation of LDL by preventing the generation of phospholipid hydroperoxides [68-70]. However, recently it has been shown that the majority of PAF-AH *in vivo* is most likely associated with LDL rather than HDL [71]. HDL has also been shown to prevent endothelial cell production of adhesion molecules [63, 72, 73], which are important for the recruitment of monocytes to atherosclerosis-prone areas of the arteries (section 1.1). The major apolipoprotein associated with HDL, apo AI, may also have antioxidant properties as demonstrated by its ability to reduce cholesterol and phospholipid hydroperoxides [74]. Some other cardioprotective functions of HDL include its ability to bind bacterial endotoxin [75, 76] and its ability to prevent the formation of platelet aggregates at sites of endothelial injury [73, 77]. Clearly, HDL likely has an arsenal of mechanisms by which it can protect against the onset of atherosclerosis.

1.4.2.2 Reverse Cholesterol Transport

RCT refers to a multi-step pathway by which HDL removes and transports cholesterol from peripheral cells, such as macrophage foam cells in the artery wall, back to the liver for its ultimate excretion in bile [45, 78]. This pathway, first described by Glomset in 1968 [61], is critical for cholesterol homeostasis since non-hepatic cells other than those in steroidogenic tissues (adrenal glands, ovaries, testes) are unable to catabolize cholesterol they synthesize or take up from plasma lipoproteins. This pathway can be divided into four identifiable steps: (1) the efflux of cholesterol from peripheral cell membranes to HDL in the extracellular space; (2) esterification of HDL FC by LCAT; (3) transfer of some HDL-CE to apo B-

containing lipoproteins by CETP; and (4) the delivery of CE from HDL and apo B containing lipoproteins to the liver for the excretion of cholesterol in bile (Figure 1.4).

The first step of RCT is thought to be initiated by the interaction of lipid-poor apo AI or preβ-HDL with peripheral cells, such as cholesterol-laden macrophages and smooth muscle cells. These particles can traverse the endothelial layer of the vessel wall, entering the interstitial fluid [79]. HDL with the newly acquired cholesterol returns to the plasma via lymphatic channels or by traversing back across the endothelial layer of the vessel wall [80]. Under normal physiologic conditions, the initial removal of peripheral cell cholesterol is most likely the rate-limiting step of this pathway [59]. Moreover, the removal of peripheral cell lipids by HDL may be a key determinant of circulating HDL cholesterol levels [59, 81]. In support of this hypothesis, recent studies, using fibroblasts from patients with Tangier Disease, have shown that the block in apo AI-mediated cholesterol and phospholipid efflux from these cells is most likely responsible for the low plasma HDL levels found in these patients [51, 82, 83].

The second and third steps in RCT involve the remodeling of pre-β HDL by plasma enzymes. In the second step, pre-β HDL returned to the plasma from the lymph is acted upon by LCAT [45, 84]. LCAT catalyzes the transfer of the 2-acyl group of phosphatidylcholine to the free hydroxyl residue of cholesterol, generating CE and lysophosphatidylcholine. The newly formed CE moves into the hydrophobic core of the pre-β HDL particle, resulting in the formation of spheroidal α-HDL and depletion of FC on the HDL surface [41, 85]. The depletion of surface cholesterol is thought to enable HDL to accept more cholesterol from cells upon recirculation into

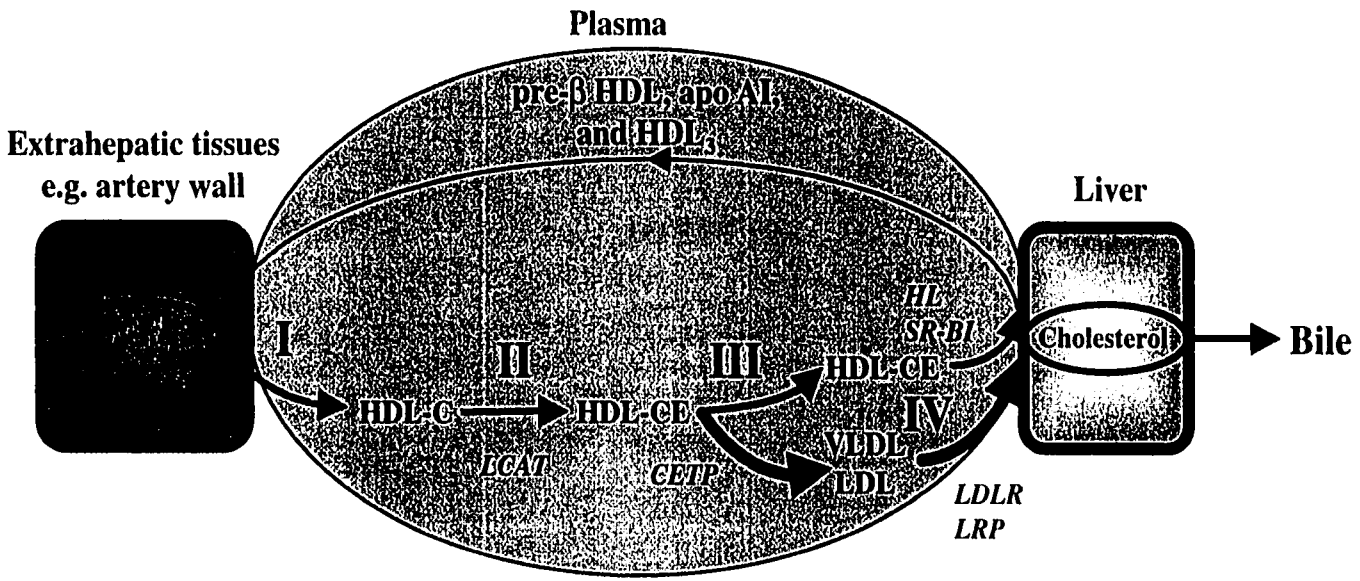


Figure 1.4 Reverse cholesterol transport pathway.
The steps involved are: (I) cholesterol efflux from extrahepatic tissues to high density lipoprotein (HDL-C) (pre-β HDL, apolipoprotein AI (apo AI), and HDL_3); (II) conversion of cholesterol (C) to cholesteryl ester (CE) by lecithin:cholesterol acyltransferase (LCAT); (III) transfer of cholesteryl ester to apo B-containing lipoproteins (very low density lipoprotein (VLDL), low density lipoprotein (LDL)) by cholesteryl ester transfer protein (CETP); (IV) uptake of cholesterol by liver either from HDL via hepatic lipase (HL)/scavenger receptor class B type I receptor (SR-BI), and from VLDL and LDL via the low density lipoprotein receptor (LDLR) or low density lipoprotein related protein receptor (LRP). Adapted from Francis and Perry [59]

the interstitial fluid. As CE accumulates in the core of HDL, large HDL_2 particles are formed. Once these particles reach a critical size they are no longer efficient acceptors of cellular cholesterol or effective substrates for LCAT [41, 45, 59]. The third step involves the transfer of some CE in HDL_2 to apo-B containing particles (VLDL, IDL, and LDL) by CETP in exchange for TG [86]. Some studies have shown that CETP action on HDL results in dissociation of apo AI from HDL and may represent another mechanism by which pre-β HDL is generated [87, 88]. Phospholipid transfer protein has also been implicated in the remodeling of HDL in the plasma, but experimental evidence is still lacking on its role in RCT [45].

The last step in the postulated RCT pathway is the delivery of cholesterol to the liver, where it may be either repackaged into newly synthesized VLDL particles or excreted in bile as either bile acid or FC. The delivery of cholesterol to the liver has been shown to occur by three different mechanisms. The first mechanism involves the uptake of apo B-containing lipoproteins through the LDLR and LRP pathways [39]. Due to the actions of CETP, it is believed that cholesterol originally removed by HDL from peripheral cells is thereby returned to the liver mainly by apo B-containing particles, implicating a role of these lipoproteins in RCT [45, 86, 89]. A second mechanism by which cholesterol is delivered to the liver is by endocytosis of large apo E-containing HDL, which also occurs via the LDLR and LRP pathway [90, 91]. However, the contribution of this pathway may not be significant since only a small fraction (15 %) of HDL contain apo E [59]. The final mechanism by which cholesterol is delivered to the liver is through a non-endocytotic process in which CE, and possibly FC, is selectively taken up from HDL by the liver [92, 93]. This process

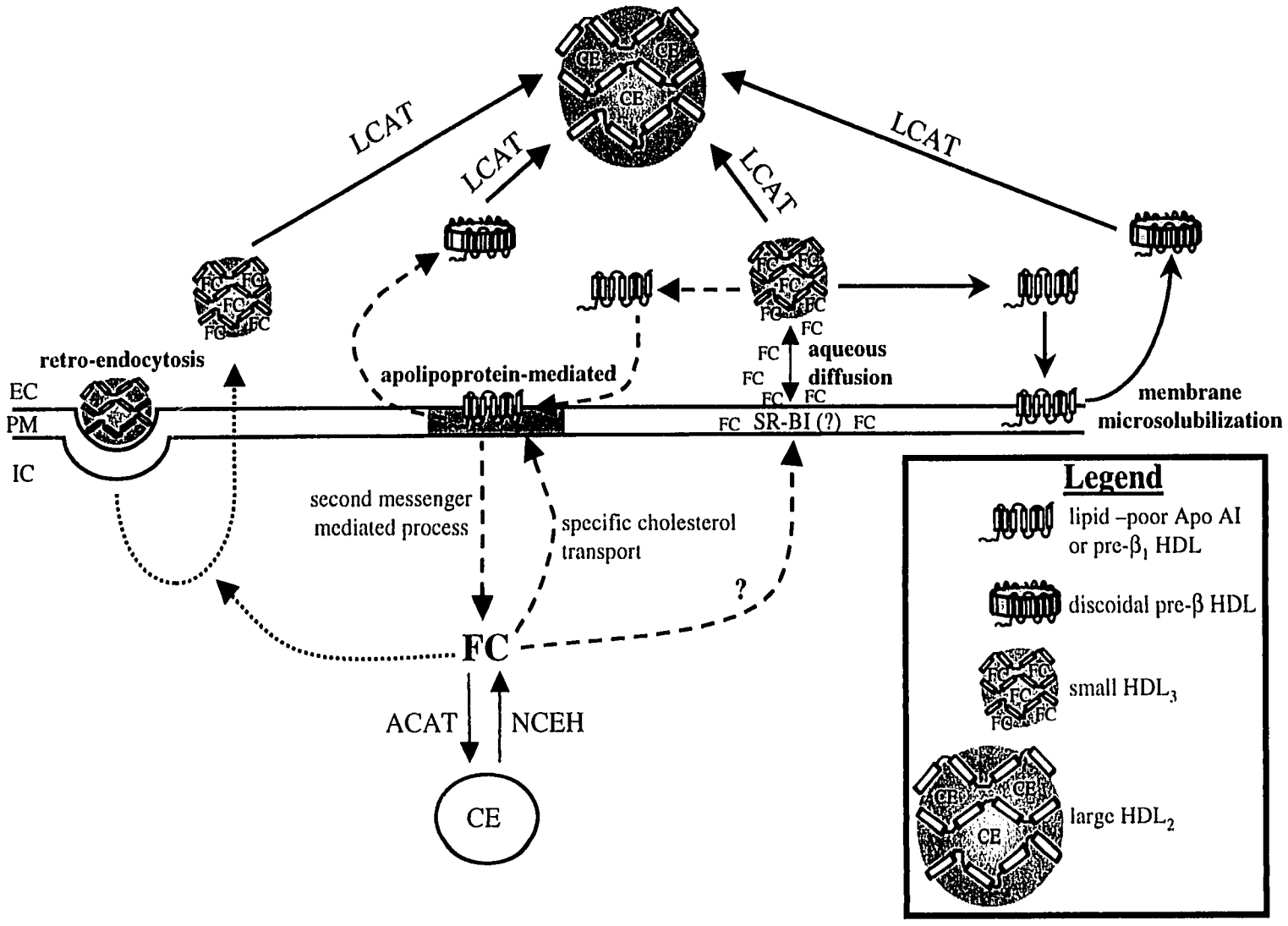
has been shown to be mediated through a recently identified HDL receptor, scavenger receptor class B type I (SR-BI), and may also involve the actions of hepatic lipase [93-96]. HDL cholesterol taken up by the liver this way is believed to be transported directly into bile for secretion [93, 97]. In fact, hepatic overexpression of SR-BI significantly decreased plasma HDL levels and increased biliary cholesterol concentrations in mice [98, 99]. Moreover, SR-BI knockout mice display increased plasma HDL cholesterol levels, which is consistent with the role of SR-BI in hepatic uptake of HDL cholesterol [100]. However, it should be noted that the importance of SR-BI in human HDL metabolism is still not fully known. Finally, the lipolytic action of hepatic lipase on HDL may be another mechanism for regeneration of pre-β HDL, which can then re-circulate in the RCT pathway.

1.4.2.3 Efflux of Cellular Cholesterol by High Density Lipoproteins

The physiological uptake of LDL cholesterol by peripheral cells was delineated through the Nobel Prize winning work of Michael Brown and Joseph Goldstein [101]. Although nearly two decades have past since their discovery, the understanding of how peripheral cells rid themselves of cholesterol is still limited. Three main mechanisms have been proposed for how cellular cholesterol is removed from peripheral cells by HDL in the first step of RCT: 1) aqueous diffusion; 2) apolipoprotein-mediated; and 3) retroendocytosis (Figure 1.5).

The first mechanism by which cholesterol is removed by HDL, aqueous diffusion, this is thought to occur in all cell types [102, 103]. Aqueous diffusion is a bi-directional, physiochemical process where cholesterol desorbs from the plasma membrane, diffuses through the unstirred water layer surrounding the cell, and is

Figure 1.5. Mechanisms of cellular cholesterol efflux.
Retro-endocytosis, involves whole high density lipoprotein (HDL) uptake, acquisition of intracellular cholesterol, followed by resecretion of intact, non-degraded HDL particles; **apolipoprotein-mediated efflux**, lipid-poor apolipoprotein AI (apo AI) or pre-β_1 HDL interact with a yet to be identified cell surface binding protein (shaded area) leading to second messenger events resulting in the active translocation of intracellular cholesterol to the plasma membrane where it may be off-loaded at the cell-surface binding site, aqueous diffusion and/or membrane microsolubilization; **aqueous diffusion**, plasma membrane free cholesterol (FC) desorbs from the cell surface, traversing the unstirred-water layer and absorbs to HDL. Scavenger receptor class B type I may help facilitate this process; **membrane microsolubilization**, lipid-poor apo AI or pre-β_1 HDL interacts with the cell surface, probably at specific lipid microdomains, "solubilizing" phospholipid and FC and removing it from the cell membrane. After acquisition of FC by each method lecithin:cholesterol acyltransferase (LCAT) esterifies FC forming cholesteryl esters (CE), which move to the inner core of HDL generating spheroidal particles. ACAT, acyl-CoA:cholesterol acyltransferase; EC, extracellular; IC, intracellular; NCEH, neutral cholesteryl ester hydrolase; PM, plasma membrane.


LCAT
LCAT
LCAT
LCAT
retro-endocytosis
apolipoprotein-mediated
aqueous
diffusion
membrane
microsolubilization
EC
PM
IC
SR-BI (?)
second messenger
mediated process
specific cholesterol
transport
?
FC
ACAT
NCEH
CE
Legend
lipid –poor Apo AI
or pre-β1 HDL
discoidal pre-β HDL
small HDL3
large HDL2

finally incorporated into HDL. The rate-limiting step for aqueous diffusion is desorption of cholesterol from the cell surface [104]. For the net removal of cellular cholesterol to occur, a concentration gradient must be established between the cell membrane and HDL where the ratio of FC to phospholipid must be greater in the donor membrane than on the HDL particle surface [102, 105]. Therefore, this mechanism most likely requires phospholipid-rich HDL particles containing LCAT to maintain a favorable concentration gradient between the cell membrane and HDL [102].

In addition to its role in mediating selective CE uptake, Ji *et al.* have shown that SR-BI is involved with cellular cholesterol efflux by aqueous diffusion [106]. This may occur by SR-BI tethering HDL to the cell surface, thereby decreasing the distance FC has to traverse through the unstirred water layer [106, 107]. It has also been shown that the phospholipid content of HDL correlates with SR-BI-mediated aqueous diffusion of cellular cholesterol [108]. In fact, apolipoprotein-free phospholipid vesicles can accept cholesterol via the SR-BI-mediated pathway [104, 108]. These findings, along with the ability of trypsinized HDL to accept cholesterol from cells [109], supports the concept that the aqueous diffusion mechanism is apolipoprotein independent. Finally, aqueous diffusion mainly removes plasma membrane cholesterol, and does not effectively mobilize intracellular cholesterol stores [83, 104]. As such, it is a relatively inefficient process for removing cholesterol from peripheral cells.

The second mechanism by which HDL promotes cellular cholesterol efflux is through the specific interaction of HDL-associated or free apo AI with the cell

surface. In contrast to aqueous diffusion, the movement of cholesterol is unidirectional from the cell membrane to these acceptors [83]. As the model of apoprotein-mediated cholesterol efflux is better understood it appears to describe two separate processes. The first process, which is classically referred to as apoprotein-mediated efflux, is postulated to involve the reversible binding of apo AI to a cell surface receptor, which results in the active translocation of cholesterol from a substrate pool for esterification, by the endoplasmic reticulum-associated enzyme acyl-CoA:cholesterol acyltransferase (ACAT), to sites available for cholesterol efflux located in the plasma membrane of the cell [83]. Many studies support this mobilization of the "ACAT-accessible" cholesterol pool to "efflux-available" sites on the plasma membrane in response to the apolipoprotein-cell interaction [82, 110-113]. This mechanism has been shown to be dependent on an intact Golgi apparatus and microtubule-dependent vesicular transport, further implicating a specific intracellular cholesterol trafficking pathway [80, 114, 115]. Cellular second messengers, including phospholipase C- and D-mediated generation of signaling molecules and activation of protein kinase C, have also been shown to be involved in apolipoprotein-mediated mobilization of intracellular cholesterol [116-122]. Finally, Tangier disease is characterized by very low plasma HDL cholesterol and apo AI concentrations along with the deposition of CE in tissues. Francis *et al.* demonstrated that the decrease in HDL and apo AI levels in these patients is a result of a defect in the apolipoprotein-mediated removal of intracellular cholesterol and phospholipids [51]. Passive diffusion of cholesterol to HDL is still intact in cultured fibroblasts from these patients [51]. These results demonstrate the importance of apolipoprotein-mediated

mobilization of intracellular cholesterol as an efficient means to remove excess cholesterol from peripheral cells, and as a (or *the*) major determinant of circulating HDL cholesterol levels.

Numerous potential HDL-binding proteins that would explain this specific interaction of HDL and apo AI with cells for cholesterol efflux have been described [94, 123-129]. Oram *et al.* [124, 130] showed that loading cells with cholesterol results in increased binding of HDL, suggesting that cells increase their expression of HDL-binding proteins to facilitate removal of excess cholesterol. As well, Li *et al.* showed that partial hydrolysis of macrophage surface proteins by proteases leads to a decrease in HDL binding [131]. More recently, Mendez and Oram showed that even limited proteolysis of HDL by trypsin abolished its interaction with cell surface high affinity binding sites on cholesterol-loaded fibroblasts, thereby preventing the removal of intracellular cholesterol [109]. Trypsin treatment of HDL did not, however, prevent the removal of cholesterol via the aqueous diffusion pathway [109, 111, 132]. Within the last year, five labs have identified the ATP-binding cassette transporter 1 (ABC1) as the defective gene in Tangier's disease [133-137]. Although the specific function of ABC1 is still not known, its expression was shown to be regulated by cellular cholesterol levels, and it has been shown to be localized to the plasma membrane, suggesting a potential HDL-binding role [133].

It has also been determined that the primary amino acid sequence of apo AI most likely does not determine its interaction with cell-surface binding proteins. Instead, it appears to be the amphipathic α-helical structure of apo AI that predicts its binding to cells. Synthetic peptides as short as 18 amino acids containing similar α-

helical motifs also interact with similar saturable binding sites on cholesterol-loaded fibroblasts, and stimulate cholesterol and phospholipid efflux [110, 138]. Moreover, all apolipoproteins containing amphipathic α-helices, including the exchangeable apolipoproteins apo AI, apo AII, apo AIV, apo E and insect apolipophorin III, are able to stimulate removal of cellular cholesterol and phospholipids from various cell types [139-142].

Recently a second mechanism for apo AI-mediated efflux has been demonstrated. Apo AI was shown to remove cellular cholesterol through membrane-microsolubilization, probably at specific lipid microdomains in the plasma membrane, where lipid-poor apo AI or pre-β HDL removes cell surface cholesterol and phospholipid by solubilizing a portion of the membrane then removing it [143, 144]. Other exchangeable apolipoproteins have also been shown to remove cell surface cholesterol via membrane-microsolubilization [143]. This mechanism does not appear to mobilize intracellular cholesterol stores or involve the interaction of cell-surface HDL binding proteins and therefore demonstrates a new mechanism by which apo AI can remove cholesterol from the cell surface.

A final, and less well-characterized mechanism by which HDL may remove peripheral cell cholesterol is through a process termed retroendocytosis. Retroendocytosis involves whole HDL particle uptake, acquisition of cellular cholesterol, followed by the resecretion of intact, non-degraded HDL particles [145, 146]. New data shows this process occurs through a calcium dependent pathway involving receptor-mediated endocytosis in coated pits [147]. This process appears to

occur in a limited number of cell types, however, and the overall contribution of this mechanism to cellular cholesterol efflux is not yet known.

It should be noted that the relative contribution of each cellular cholesterol efflux mechanism depends on the growth state of cells. Aqueous diffusion appears to account for most of the cellular cholesterol efflux that occurs from actively dividing cells, whereas apolipoprotein-mediated efflux appears to be the primary mechanism of cholesterol efflux from quiescent, cholesterol-loaded cells [83, 148]. As well, even though the identification of ABC1 as the mutated gene in Tangier disease is a significant finding, it is still not known how ABC1 is involved in apolipoprotein-mediated efflux of cell lipids. ABC1 may act as an HDL receptor, as well as an intracellular cholesterol trafficking molecule or a lipid flippase, flipping cholesterol and/or phospholipid(s) from the internal leaflet of the plasma membrane to the external leaflet. Finally, cholesterol efflux mechanisms may act in concert, whereby the interaction of apo AI with cell-surface binding proteins initiates the movement of intracellular cholesterol to the plasma membrane, where the actual off-loading of cholesterol may be by aqueous diffusion and/or membrane microsolubilization mechanisms. These plus many other questions regarding intracellular cholesterol transport remain to be clarified by further research.

1.5 Oxidation of Lipoproteins

Oxidative modification of LDL is believed to be partly responsible for the strong positive correlation between plasma LDL levels and atherosclerosis [149]. Normally, when LDL is incubated with macrophages they fail to accumulate excess cholesterol, even at high LDL concentrations. The uptake of LDL cholesterol by

peripheral cells is under strict feedback regulation, where an increase in cholesterol beyond a certain threshold leads to a decrease in the LDLR synthesis and number, inhibition of new cholesterol synthesis, and the formation of CE by ACAT, thereby preventing the pathologic accumulation of free (unesterified) cholesterol [101]. Despite these stringent regulatory mechanisms, cholesterol-laden macrophages (foam cells) are the hallmark of atherosclerosis. Studies have shown that the oxidative modification of LDL by cultured arterial wall cells converts it to a form that is recognized by scavenger receptors, whose expression is not regulated by cellular cholesterol content, therefore leading to the pathologic accumulation of cholesterol by macrophages as seen in atherosclerosis [150-152]. *In vivo* evidence supports the oxidation of LDL as a pathophysiologic event in atherosclerosis, since oxLDL has been isolated from human and animal atherosclerotic lesions [21, 153]. Since HDL contains lipids similar to LDL, and is present in interstitial fluid, it is conceivable that it can also be oxidatively modified *in vivo*. Indeed, numerous *in vitro* studies suggest HDL is equally or even more susceptible to oxidation than LDL (summarized in [60]). However, oxidized HDL (oxHDL) has not yet been isolated from humans, probably as a result of greater interest in oxLDL than oxHDL until recently.

Many *in vitro* models have been used to determine the effects of oxidation on the structure and function of HDL [60]. Oxidation of HDL has been found to impair its ability to promote passive efflux of cholesterol from cultured cells [154-165]. The possibility that oxidation of HDL may actually enhance its ability to protect against atherosclerosis, however, is suggested by the findings that tyrosyl radical oxidized HDL ("tyrosylated" or TyrHDL) markedly increases its ability to mobilize

intracellular cholesterol to the plasma membrane for removal from cells [166, 167]. These findings suggest that studies on HDL oxidation should focus on both the most likely mechanisms of HDL oxidation *in vivo*, as well as the most relevant mechanisms of cholesterol mobilization by HDL and oxHDL.

1.6 Oxidation of High Density Lipoproteins

1.6.1 Models of High Density Lipoprotein Oxidation

Several studies looking at the effects of oxidation on HDL function have used oxidation models that most likely do not represent physiologic mechanisms of lipoprotein oxidation *in vivo*. The most popular model used to oxidize HDL (and LDL) involves free metal ions, particularly copper ion [154-156, 158, 163, 165]. The high concentration of copper ion required to oxidize lipoproteins *in vitro* and the presence of numerous free radical scavengers and reducing agents in plasma, however, suggest it is highly unlikely that the oxidation of HDL by free metal ions represents a physiologically relevant model [168]. It has been shown that copper bound to ceruloplasmin, a plasma copper binding protein, can oxidize LDL [169]. As well, hemin, a low molecular weight chelate of iron, has also been shown to be a potent mechanism for LDL oxidation [170]. Nevertheless, premature atherosclerosis is not a prominent feature of hemochromatosis [171], a common genetic disorder that causes iron accumulation in plasma and liver, or Wilson's disease [172], a disorder that results in increased copper concentrations in liver, plasma, and brain. Evidence for free metal ion oxidation of LDL has been found in advanced atherosclerotic lesions [173]. The presence of free-metal ions in advanced lesions is most likely due

to necrosis of cells, and evidence for oxidation of lipoproteins by free metal ions is not found in the earlier stage lesions of atherosclerosis [149].

Physiologic mediators of lipoprotein oxidation of greater potential relevance include myeloperoxidase, peroxynitrite, lipoxygenase and tocopheroxyl radical [60, 149, 174]. Active myeloperoxidase has been found in human atherosclerotic lesions [175]. In addition, proteins modified by hypochlorite, an oxidant only believed to be generated by myeloperoxidase in humans [178], are present in atherosclerotic tissues [176, 177]. Moreover, *o,o'*-dityrosine, a product of myeloperoxidase-generated tyrosyl radical, is increased 100-fold in LDL isolated from atherosclerotic lesions compared to normal aortas, and is increased in both fatty streaks and advanced atherosclerotic lesions [173]. Peroxynitrite, which is generated by the reaction of nitric oxide (NO) with superoxide (O_2^-), also appears to oxidize lipoproteins *in vivo* [179-181]. Peroxynitrite is a potent protein-nitrating reagent that produces high levels of 3-nitrotyrosine *in vitro* [182]. Immunohistochemical studies have detected 3-nitrotyrosine in human atherosclerotic lesions [180]. Leeuwenburgh *et al.* showed, using isotope dilution gas chromatography-mass spectrometry, that LDL isolated from atherosclerotic lesions had an 80-fold increase in 3-nitrotyrosine compared to circulating LDL [181]. Lipoxygenase, produced by arterial wall cells, has also been implicated in the *in vivo* oxidation of lipoproteins. Both lipoxygenase mRNA and lipoxygenase protein have been detected in human atherosclerotic lesions [183]. Moreover, the major product of lipid peroxidation by this enzyme, 13*S*-hydroxy-9*Z*,11*E*-octadecadienoic acid, is somewhat increased in early atherosclerotic lesions [184]. Finally, tocopheroxyl radical, a product formed by oxidation of the antioxidant

α-tocopherol (vitamin E), may play a role in lipoprotein oxidation *in vivo*, since HDL has been shown to be oxidized by low rates of tocopheroxyl radical formation *in vitro* [185].

1.6.2 Effects of Oxidation on High Density Lipoprotein Structure

In general, all oxidation reactions follow a three-step process: initiation, propagation and termination [186, 187]. Initiation begins by hydrogen atom (H^+) abstraction, which is facilitated by the presence of multiple double bonds in the carbon chain. For example, a polyunsaturated fatty acid (PUFA) is more susceptible to H^+ abstraction then a monounsaturated fatty acid (MUFA). As well, amino acids such as tyrosine are also susceptible to H^+ abstraction from the hydroxyl group on the benzene ring. The propagation stage occurs in an autocatalytic manner where the newly formed radicals can generate more radicals in the same or adjacent molecules. Finally, termination of the radical reaction occurs when two radicals react together [186].

In HDL, the type of oxidant used has been found to determine which component of the lipoprotein is preferentially oxidized [60]. Both tyrosyl radical and low levels of hypochlorite have been shown to preferentially oxidize the apolipoproteins rather than the lipids of HDL, with both oxidants generating only low levels of lipid peroxidation products in HDL [161, 167]. Moreover, tyrosyl radical can oxidize HDL apolipoproteins in their lipid-free form [188]. In contrast, the initial site of oxidation by free metal ions and lipoxygenase appears to be lipid components of HDL, with the modification of apolipoproteins requiring the initial generation of lipid radicals [74, 156, 189]. With these models of lipoprotein oxidation, it appears

that methionine residues of apo AI are oxidized secondary to the generation of phospholipid and CE hydroperoxides, resulting in methionine sulfoxide and reduced hydroperoxides [74]. The requirement of initial lipid peroxidation for the oxidation of HDL apolipoproteins in these model systems is supported by the fact that these oxidants fail to oxidize lipid-free apo AI [60, 74, 189]. Further support for lipid peroxidation caused by free metal ions is seen by the increase in negative charge of copper-oxidized HDL [163], believed to be due to the masking of positively charged amino acid residues (*e.g.*, lysine) by decomposition products of oxidized lipid, as seen in oxLDL [190].

1.6.3 Effects of Tyrosyl Radical Oxidation on High Density Lipoprotein Function

As mentioned in section 1.5, the majority of studies looking at the effects of oxidation on HDL function have suggested this would have deleterious effects. The decreased ability of oxHDL particles to passively accept cholesterol from cultured cells has been attributed to the changes found in HDL lipid rather than apolipoprotein composition [60]. This is most likely due to a particular decrease in total phospholipid and the phospholipid/cholesterol ratio, that make HDL a less efficient acceptor of plasma membrane cholesterol through aqueous diffusion [102, 156, 158].

In contrast, our lab has shown that oxidation of HDL by peroxidase-generated tyrosyl radical markedly enhances the ability of HDL to mobilize the pool of cholesterol available for storage in cells, and to prevent the accumulation of cholesterol derived from LDL [166]. The active component of TyrHDL has subsequently been found to a crosslinked heterodimer of apo AI to apo AII [188].

The exact mechanism by which TyrHDL promotes cellular cholesterol efflux is not fully understood, but it is not due to it being a better passive acceptor of cell surface cholesterol than HDL [167]. It is postulated that the apo AI-apo AII crosslink results in the tethering of apo AI to HDL by apo AII, allowing apo AI to interact more effectively or longer with HDL binding sites on the cell surface (Figure 1.6) [59]. This interaction, likely via the same cellular second messenger events activated by HDL, results in the active translocation of intracellular (ACAT-accessible) cholesterol to sites available for efflux on the cell surface. The newly translocated cholesterol is then available to be offloaded by nascent and small (non-oxidized) HDL particles (lipid-poor apo AI, pre-β HDL, and/or HDL_3 *in vivo*), resulting in the enhancement of cellular cholesterol efflux. This effect of TyrHDL thus appears to enhance the specific apolipoprotein-mediated mechanism of cholesterol efflux (section 1.4.2.3). The increased availability of cell surface cholesterol induced by this oxidized form of HDL, if occurring *in vivo*, would thereby be expected to enhance the maturation of HDL, and increase plasma HDL levels.

1.7 Physiologic Model for Tyrosyl Radical Oxidation

Our *in vitro* model of peroxidase-generated tyrosyl radical oxidation is based on the known ability of activated human phagocytes to secrete myeloperoxidase (MPO) from its granules, and to produce superoxide (O_2^-), through the action of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase located in the cell plasma membrane (Figure 1.7). O_2^- spontaneously dismutates, or is actively converted by superoxide dismutase (SOD), into hydrogen peroxide (H_2O_2), which can be used by MPO to catalyze the oxidation of various substrates (*e.g.*, chloride ion, L-

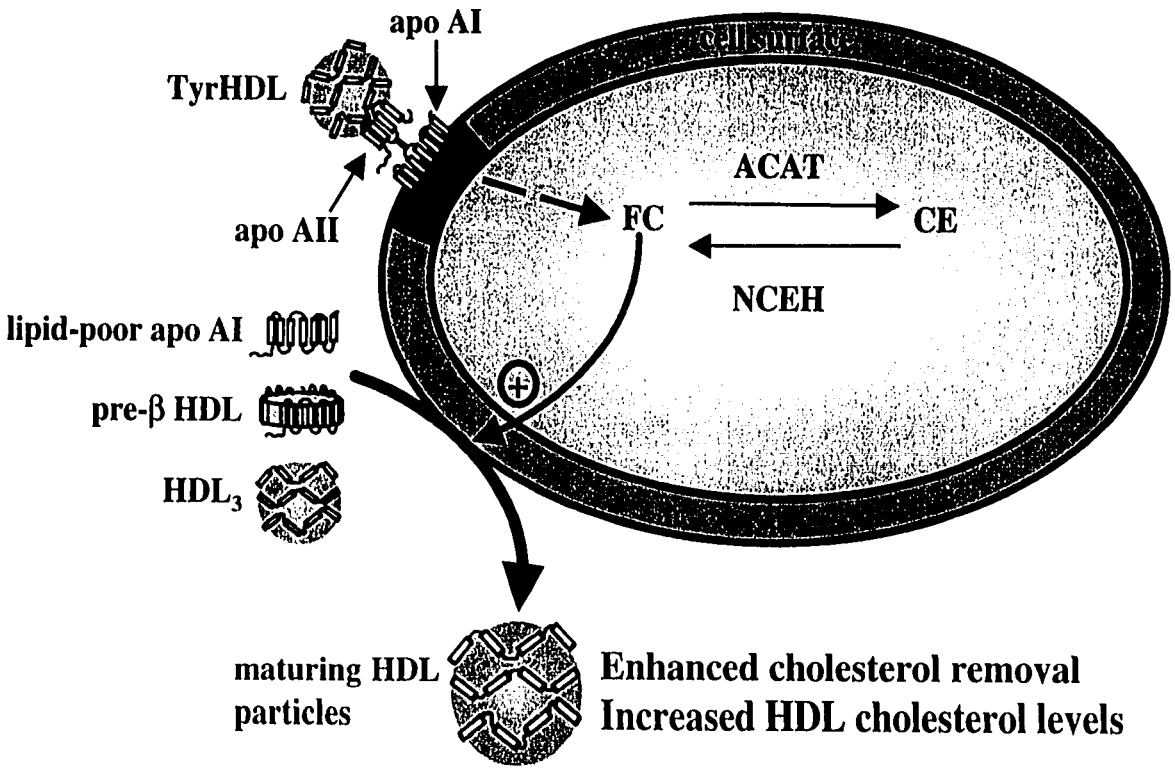


Figure 1.6 Proposed biologic action of tyrosylated high density lipoprotein (TyrHDL).
The apolipoprotein AI-AII heterodimer anchors apolipoprotein AI (apo AI) to high density lipoprotein (HDL) via apolipoprotein AII (apo AII) allowing apo AI to interact more effectively or longer with a yet to be identified cell surface binding domain (dark gray area). This interaction leads to enhancement of the delivery of acyl-CoA:cholesterol acyltransferase (ACAT)-accessible free cholesterol (FC) to efflux-accessible sites on the cell surface. Efflux-accessible cholesterol is now available to be removed by lipid-poor apo AI, pre-β HDL and/or HDL_3. In addition to depleting excess cellular cholesterol, the off-loading of cholesterol would enhance the maturation of HDL and increase plasma cholesterol levels. CE, cholesteryl esters; NCEH, neutral cholesterol ester hydrolase. Adapted from Francis and Perry [59]

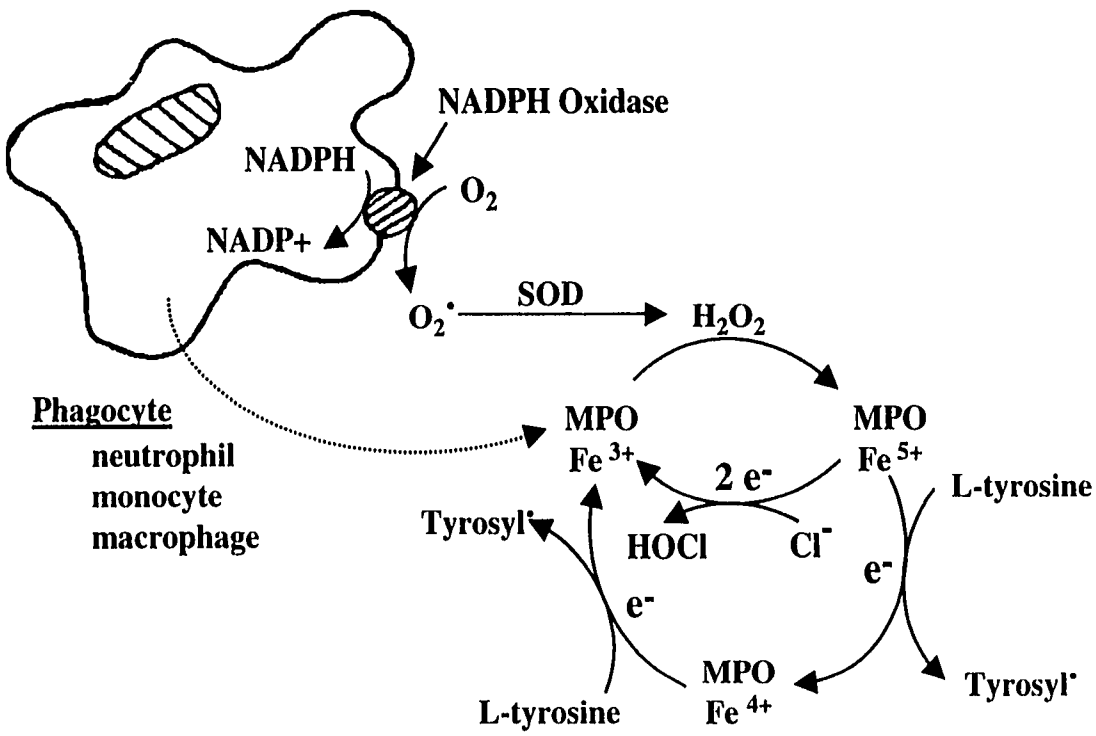


Figure 1.7 Model for tyrosyl radical generation *in vivo*.
Activated human phagocytes (neutrophils, monocytes, and macrophages) secrete the enzyme myeloperoxidase (MPO) and generate superoxide ($O_2^{\cdot}$) by the actions of the plasma membrane associated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Superoxide spontaneously dismutates or is enzymatically converted by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). H_2O_2 is used by MPO to catalyze the oxidation of its substrates in the extracellular or interstitial fluid to free radicals, which carry out the actual oxidative damage by activated phagocytes. One substrate for oxidation by MPO is the amino acid L-tyrosine which is converted to tyrosyl radical (Tyrosyl$^{\cdot}$). Cl^-, chloride ion; HOCl, hypochlorous acid; O_2, molecular oxygen; e^-, electron.

tyrosine) resulting in the production of free oxidizing radicals that carry out the actual damage of activated phagocytes. In support of L-tyrosine being an important substrate of MPO, it has been shown that activated neutrophils and macrophages generate tyrosyl radical via the MPO/H_2O_2 system at physiologic concentrations of L-tyrosine [191]. In addition, the generation of tyrosyl radical occurred in the presence of physiologic concentrations of chloride ion, another major substrate for oxidation by MPO. Dityrosine (formed by the phenolic coupling of two tyrosyl radicals) formation by activated phagocytes suggests that phagocyte-generated tyrosyl radicals may modify target proteins such as lipoproteins found in the artery wall [173].

Although controversial, human neutrophils have been shown to express nitric oxide synthase [192, 193] and synthesize NO [194, 195]. NO is considered relatively inactive, but its catabolism results in potent oxidants termed reactive nitrogen species (RNS) (reviewed in [196]). The RNS, nitrite and peroxynitrite, have been shown to cause dityrosine formation [197, 198]. Nitrite can be converted to nitryl chloride and nitrogen dioxide through MPO-dependent pathways [199], and nitrogen dioxide has been shown to react with hypochlorous acid (HOCl), forming intermediate species that generate tyrosyl radical and dityrosine [197]. As well, incubation of bovine serum albumin (BSA), human gamma-globulin, or bovine eye lens alpha-crystallin with nitrogen dioxide results in the crosslinking of the protein through dityrosine [200]. Lastly, low steady state concentrations of peroxynitrite has been shown to result in preferential formation of dityrosine over tyrosine nitration [198]. The relative contribution of RNS compared to MPO in the formation of protein tyrosyl radicals through free tyrosyl radical generation is not known. However, in the

absence of free L-tyrosine, the role of RNS for the generation of protein tyrosyl radicals may be significant, since MPO exhibits considerable steric hindrance at its active site, making it inaccessible to large macromolecules [201]. Therefore, oxidized low-molecular-weight intermediates, such as tyrosyl radical and HOCl, are thought to be required to convey oxidizing equivalents from its heme group to target sites (*e.g.*, apolipoproteins).

1.8 Neutrophils as a Cellular Model for Oxidation of High Density Lipoproteins

Atherosclerosis is an inflammatory disease believed to result mainly from the infiltration of monocytes into the artery wall (see section 1.1). However, neutrophils are also known to be involved with the initial aspects of the inflammatory process, and have been found to be present in early stage atherosclerotic lesions [26, 27]. Moreover, increased neutrophil adherence has been demonstrated in coronary arteries from cholesterol-fed rabbits [202]. Finally, it has been shown that activated neutrophils can oxidize LDL to a form that is recognized by scavenger receptors of macrophages [203-205], a mechanism believed to be partly responsible for foam cell formation in atherosclerosis [13, 149]. These factors, plus the increased ease of use and responsiveness of neutrophils, have led us to use these cells rather than monocytes in our studies on phagocyte modification of HDL.

1.9 Chronic Granulomatous Disease

Chronic granulomatous disease is an X-linked or autosomal recessive inherited disorder characterized by a functional defect in the neutrophil plasma membrane-associated NADPH oxidase [206]. In stimulated normal neutrophils

NADPH oxidase generates O_2^- by transferring one electron from intracellular NADPH to molecular oxygen (O_2) on the extracellular surface of the plasma membrane [207]. In the CGD neutrophil this system is non-functional, and O_2^- is not generated by NADPH oxidase. Since MPO uses the H_2O_2 produced from the dismutation of O_2^- to oxidize substrates in the extracellular space, a defect in O_2^- production would also be expected to lead to impaired production of MPO-generated radicals. CGD neutrophils therefore represent an additional system to (indirectly) investigate the role of MPO in the oxidation of HDL by activated phagocytes.

1.10 Rheumatic Disease as a Model for the Oxidation of High Density Lipoproteins *In Vivo*

The cellular interactions in atherogenesis are fundamentally no different from those in other chronic inflammatory-fibroproliferative diseases [3]. Rheumatic diseases contain a group of disorders that are characterized by the chronic and/or acute inflammation of joints. In many cases the offending agent is not known (*e.g.*, rheumatoid arthritis); in others the offensive agent is recognized (*e.g.*, sodium urate monohydrate crystals in gouty arthritis) [208]. The fluid contained in joints, synovial fluid, has been shown to be a rich source of plasma lipoproteins, including HDL [209]. Activated phagocytes are present in the synovial fluid of inflamed joints [208, 210] and therefore oxidizing agents such as myeloperoxidase are increased in inflammatory synovial fluid [211]. Isolated polymorphonuclear leukocytes from the synovial fluid of patients with rheumatoid arthritis show evidence of degranulation and increased responsiveness of NADPH-oxidase to stimulation by chemotactic peptide [212]. NO has also been shown to play a role in the oxidative damage that

occurs in rheumatoid arthritis joints [213, 214]. Finally, LDL isolated from synovial fluid of inflammatory joints is mildly oxidized [215, 216], exhibiting increased electronegative charge [217], a characteristic of oxLDL from atherosclerotic lesions [21]. Practically, the volume of synovial fluid that can be obtained from inflamed joints is significantly greater than can be isolated from atherosclerotic lesions, and synovial fluid lipoproteins can be isolated by standard ultracentrifugation techniques. Overall, the components found in the synovial fluid of rheumatic diseases are comparable to what is found in atherosclerotic lesions and thus serves as a suitable model for studying the effects of *in vivo* oxidation on HDL structure and function.

1.11 Hypothesis and Specific Aims of Thesis

Our lab has shown that oxidation of HDL by peroxidase-generated tyrosyl radical has a markedly enhanced ability to promote the mobilization of intracellular cholesterol to sites on the cell surface readily available for efflux [166, 167]. The active component of TyrHDL was found to be a crosslinked heterodimer of apo AI and apo AII [188]. My thesis aims to extend our *in vitro* model to a cell system, to investigate the effects of oxidation of HDL by activated phagocytes (similar to those found in atherosclerotic lesions) on its structure and function. Also, my thesis looks at the effect of *in vivo* oxidation on HDL isolated from inflammatory joints and atherosclerotic lesions. We hypothesize that activated phagocytes oxidatively modify HDL, and that this modification results in an HDL particle that has an enhanced ability to mobilize intracellular cholesterol to sites on the cell surface readily available for efflux.

Specific aims:

A. To investigate the ability of activated human neutrophils to initiate (dityrosine) crosslinking in HDL apolipoproteins by a myeloperoxidase-dependent pathway.

B. To investigate the ability of HDL modified by activated human neutrophils to promote the mobilization of intracellular cholesterol in cholesterol-laden fibroblasts.

C. To investigate the structural and functional characteristics of HDL obtained from inflammatory synovial fluid and human atherosclerotic lesions.

Chapter 2

Materials and Methods

2.1 Materials

Cholesterol, cholesteryl ester (CE), 1-monooleoyl-rac-glycerol, 1,2-distearoyl-rac-glycerol, triolein, oleate acid, aminotriazole, butylated hydroxytoluene (BHT), L-tyrosine, hydrogen peroxide (30 %, ACS grade), diethylenetriaminepentaacetic acid (DTPA; free acid form), essentially fatty acid-free bovine serum albumin (FAFA), bovine serum albumin (BSA), 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate, N-formyl-methionyl-leucyl-phenylalanine (fMLP), dihydrocytochalasin B (CB), phorbol 12-myristate 13-acetate (PMA), cytochrome C, superoxide dismutase, leupeptin, soybean trypsin inhibitor, and aprotinin were all purchased from Sigma. Ethylenediaminetetraacetic acid (EDTA) was purchased from BDH, Inc. Phenylmethylsulfonyl fluoride (PMSF) was purchased from ICN Biomedicals Inc. Heparin Sepharose CL-6B beads, Ficol Paque, ECL™ Western blotting detection system reagents, Rainbow™ molecular weight markers and [1-^{14}C]Oleate (55 mCi/mmol) were purchased from Amersham Pharmacia Biotech. BSA standard for protein assays was purchased from Pierce. Fine chemicals, Tween 20, sulphuric acid, Hema 3® Stain Set and PE SIL G polyester-backed silica thin layer chromatography (TLC) plates were purchased from Fisher Scientific. Tissue culture medium was purchased from Bio-Whittaker, and fetal bovine serum (FBS) was from HyClone. Reagents for polyacrylamide electrophoresis, nitrocellulose membranes for Western blotting, Chelex 100 resin and 10-DG size exclusion columns (6000 molecular weight cut off) were from Bio-Rad. Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS(-)), Trypan blue, Polymorphprep™, Tris HCl Trypsin-EDTA (0.5% trypsin,

5.3 mM EDTA) and penicillin/streptomycin (10000 units/mL penicillin G, 10000 μg/mL streptomycin sulfate) were purchased from GibcoBRL.

2.2 Cell Culture

2.2.1 Cell Culture Maintenance

Human skin fibroblasts (HSF) were cultured in 75 cm^3 tissue culture flasks (Falcon, Becton Dickinson) in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin G (100 U/mL medium), streptomycin (100 μg/mL), and 10% FBS v/v (DMEM/FBS). Cultured HSF were maintained at 37°C in a humidified atmosphere containing 95% air/5% carbon dioxide (CO_2). When the cells reached confluence they were trypsinized and either seeded in 75 cm^3 tissue culture flasks to maintain cell stocks (1 x 10^6 cells/flask) or seeded in 24 well × 16 mm tissue culture plates (Falcon, Becton Dickinson) at 15000-20000 cells/16 mm well for experimental use.

2.2.2 Cholesterol Loading of Human Skin Fibroblasts

HSF grown in 16 mm wells to confluence in DMEM/FBS (about 7 days), were washed twice with warm phosphate buffered saline containing 1 mg/mL fatty acid free albumin (PBS/FAFA), and incubated for 24-48 hr in 1 mL/well of DMEM/2 mg/mL FAFA/30 μg/mL cholesterol (cholesterol added from a 10 mg/mL solution in ethanol). To allow cell cholesterol pools to equilibrate, cells were washed twice with warm PBS/FAFA and incubated a further 24 hr in DMEM/1 mg/mL FAFA.

2.3 Isolation of Lipoproteins

2.3.1 Isolation of HDL_3 From Whole Blood

Lipoproteins were separated by sequential density gradient ultracentrifugation [218]. Blood donors were healthy male volunteers fasted overnight. Blood anti-coagulated with 1 mM EDTA was centrifuged at 450-500 x *g* for 20 min at 4°C to separate blood cells from plasma. Plasma was pooled and the protease inhibitor PMSF added to a final concentration of 10 μM to prevent potential proteolysis of apolipoproteins. Density of the pooled plasma was measured and adjusted to 1.125 g/mL with solid potassium bromide (KBr), then centrifuged at 302000 x *g* for 24 hr at 8°C to separate the LDL and HDL_2 fractions from the HDL_3 fraction. Following ultracentrifugation, the top layers (containing LDL and HDL_2) were removed. The bottom fractions (containing HDL_3) were pooled, the density adjusted to 1.21 g/mL with solid KBr, and the solution centrifuged at 302000 x *g* for 24 hr at 8°C. The top layers containing HDL_3 were then pooled, density adjusted again to 1.21 g/mL with solid KBr, and the solution centrifuged at 302000 x *g* for 24 hr at 8°C to ensure complete separation of HDL_3 from any remaining plasma proteins. Following this final spin the top layers, containing purified HDL_3, were pooled and dialyzed against buffer A [50 mM NaCl, 5 mM Tris HCl supplemented with 1 mM EDTA, pH 7.4] at 4°C using 12000-14000 molecular weight cut-off (MWCO) dialysis tubing (#4 SpectraPor, Spectrum Laboratories, Inc). Samples were dialyzed in 4 L of buffer for 24 hr, with 2 buffer changes. Following dialysis, the HDL_3 was incubated with heparin sepharose beads for 1 hr at 4°C to remove any apo B- and apo E-containing

particles [219]. HDL_3 was then re-isolated and dialyzed against buffer B [150 mM NaCl supplemented with 1 mM EDTA] at 4°C using 12000-14000 MWCO dialysis tubing (4 L × 24 hr × 2 buffer changes). Following this final dialysis step, HDL_3 was filtered sterilized using 0.22 μM syringe filters (Millipore) and stored under Argon at 4°C. HDL_3 was used within 8 weeks of isolation.

2.3.2 Isolation of HDL From Synovial Fluid

The University of Alberta Hospitals Ethics Committee approved the obtaining of knee joint synovial fluid and blood samples from patients with inflamed knee joints, and informed consent was obtained from each patient (Table 2.1). Synovial fluid was isolated in the Rheumatology Clinic (University of Alberta Hospitals) and placed into 0.1% EDTA and kept on ice. An aliquot was taken and sent to the University of Alberta Hospitals Laboratory for white blood cell (WBC) count and differential as well as analysis for crystals. After an aliquot was taken, the protease inhibitor PMSF (10 μM) and the antioxidants aminotriazole (10 mM), DTPA (100 μM), and BHT (100 μM) were added to the synovial fluid. Aliquots were taken and stored at -20°C for later analysis by mass spectrometry/gas chromatography. Lipoproteins were then isolated from the synovial fluid by sequential density gradient ultracentrifugation [218]. Synovial fluid was centrifuged at 450-500 x *g* for 30 min at 4°C to pellet any WBC, and then centrifuged at 543000 x *g* for 2 hr at 4°C in a Beckman TL-100 tabletop ultracentrifuge (Beckman, U.S.A.). The bottom fractions (containing LDL and HDL) were removed by submerged needle technique, pooled,

Table 2.1 Patient data for synovial fluid studies[a]

Patient	Age	Diagnosis	WBC count	PMN (%)	Lymphs (%)	Mono/Mac (%)	Eos (%)	Medication at time of isolation
1	70	Gout	16 300	92	3	3	2	allopurinol
2	64	Gout	15 100	96	1	3		oxaprozin, etodolac
3	40	Rheumatoid Arthritis	26 800	82	10	9		methotrexate, sulfasalazine
4	20	Reiter's Syndrome	15 300	31	49	20		cortisone injection (~ 1 week prior)

[a]WBC, white blood cells; Poly, polymorphonuclear cells; Lymphs, lymphocytes; Mono/Mac, monocytes/macrophages; Eos, eosinophils.

the density adjusted to 1.07 g/mL with solid NaBr, and the solution centrifuged at 543000 x *g* for 2.5 hr at 4°C. The top LDL fractions were removed. The bottom HDL fractions were obtained, pooled, the density adjusted to 1.21 g/mL with solid NaBr and the solution centrifuged at 543000 x *g* for 17 hr at 4°C. The top HDL fractions were removed, pooled, and dialyzed buffer B at 4°C using 12000-14000 MWCO dialysis tubing (4 L × 24 hr × 2 buffer changes). Following dialysis, synovial fluid HDL (SynHDL) was filtered sterilized using 0.22 μM syringe filters and stored under Argon at 4°C. SynHDL was used within 8 wk of isolation.

A plasma HDL (PlmHDL) sample was also obtained from each patient. Whole peripheral blood (20 mL) anti-coagulated with 0.1% EDTA was centrifuged at 450-500 *g* for 30 min at 4°C to obtain plasma. The same protease inhibitor and antioxidants were then added to the plasma as for the synovial fluid, and PlmHDL was isolated according to the protocol used to isolate SynHDL. PlmHDL was used within 8 wk of isolation.

2.3.3 Isolation of HDL From Atherosclerotic Aortas

Dr. Ute Panzenböeck, at the Heart Research Institute (Camperdown, NSW, Australia), isolated atherosclerotic aortic HDL as previously described [220]. Human aorta specimens were obtained at autopsy from the Institute of Forensic Medicine (Camperdown, NSW), with approval from the local Human Ethics Review Committee. Healthy and lesion areas of each single aorta were identified visually and by touch, and classified (Table 2.2). Aortic samples were then minced and gently agitated end-over-end overnight at 4°C in extraction buffer [PBS containing

Table 2.2 Classification of Atherosclerotic Aortas[a]

Lesion Stage	Classification	Description
I	Healthy	pink or yellow; no fatty streaks.
II	Early	yellow; fatty streaks; slightly raised.
III	Intermediate	early, soft calcification; surrounding lesions white/grey; raised and slightly gelatinous to firm
IV	Advanced	greenish hue; calcification; thrombosis; ulceration;

[a]Visual classification based on Upston, J.M. and Stocker, R. unpublished data.

0.3 mM EDTA, 0.1 mM DTPA, 10 mM aminotriazole, 20 μM BHT, 1 mM PMSF, .0005 % elastatinal, 2 mM benzamidine, 1 μM D-phenylalanyl-L-prolylyl-L-arginine chloromethyl ketone, 0.008 % gentamycin, and 0.008 % chloramphenicol pH 7.4]. The resulting "raw homogenate" was centrifuged and the resulting supernatant subjected to sequential density ultracentrifugation beginning in order with 1.019, 1.070, and 1.21 g/mL to collect VLDL/IDL, LDL and HDL fractions respectively. Samples were stored at -80°C.

2.4 Isolation of Neutrophils

Human whole blood was anti-coagulated with 1 mM EDTA, and neutrophils isolated by buoyant density centrifugation using Polymorphprep™, followed by hypotonic lysis of contaminating red blood cells [221]. Isolated neutrophils were resuspended in ice-cold buffer C [HBSS(-), supplemented with 100 μM DTPA, pH 7.4]. Neutrophils were counted using a hemocytometer and viability determined by trypan blue exclusion. Purity of neutrophils was determined by differential staining using Hema 3® Stain Set. Preparations contained > 96% neutrophils, < 4% eosinophils, and were > 97% viable. Neutrophils were used immediately for experiments.

Alternatively, neutrophils were isolated from heparinized human whole blood by buoyant density centrifugation using Ficol Paque following dextran sedimentation of RBC. Granulocytes were positively selected by resuspension in buffer D [RPMI-1640, 5 mM EDTA, 2% FBS] containing antibodies to CD16 (12 μl/5×10^7 cells), CD14 (1:200) and CD3 (1:200) (antibodies from Miltenyi Biotic Inc) bound to magnetic beads. The cell suspension was passed through a magnetic cell

separation system (MACS) to remove contaminating eosinophils, which do not bind to the antibodies [222]. The remaining granulocyte population was counted using a hemocytometer and checked for neutrophil purity using Hema 3® Stain Set and viability by Trypan blue exclusion. Preparations contained > 99% neutrophils, < 1% eosinophils, and were > 97% viable. Neutrophils were used immediately for experiments. Experimental outcome was the same for both types of neutrophil preparations.

For the CGD neutrophil experiments, informed consent was obtained and neutrophils were isolated from volunteer patients with CGD according to the first neutrophil isolation protocol outlined above. CGD patients 1 and 2 studied were both male and were 19 and 30 years of age respectively. The defect of NADPH oxidase for patient 1 is unknown. CGD molecular analysis on patient 2 showed an X-linked gene defect of the NADPH oxidase component gp-91 phox. Both patients were shown to have clinically abnormal NADPH oxidase activity and bacterial killing. Patient 1 was taking colony stimulating factor 1 mg 3 times a week, and interferon gamma 0.5 mg 3 times a week at the time of neutrophils isolation. Patient 2 was not taking any neutrophils stimulating factors. Patient treatment was not altered for our studies.

2.5 HDL_3 Oxidation

2.5.1 *In vitro* Oxidation of HDL_3 by Copper Ion

Copper oxidation of HDL_3 (1 mg/mL) was carried out at 37°C for 24 hr in buffer E [phosphate buffered saline (PBS) supplemented with 1 μM copper sulfate ($CuSO_4$), pH 7.4], following dialysis of stock HDL_3 against PBS for 24 hr to remove

EDTA. The reaction was stopped by addition of 2 mM EDTA, overlaying with nitrogen gas, and cooling to 4°C.

2.5.2 *In vitro* Oxidation of HDL_3 by Peroxidase-generated Tyrosyl Radical

The *in vitro* oxidation of HDL_3 by peroxidase-generated tyrosyl radical was carried out as previously described [167]. Briefly, reactions were carried out at 37°C for 16 hr in buffer F (66 mM potassium phosphate buffer passed over Chelex 100 resin to remove any contaminating transition metal ions, then supplemented with 100 μM DTPA, pH 8.0). The metal ion chelator, DTPA, was included to ensure that any oxidation seen in our cell free system was not due to contaminating transition metals. The reaction mixture contained 1 mg/mL HDL_3, 100 nM horseradish peroxidase (HRP) (Boehringer Mannheim), 100 μM H_2O_2, and 100 μM L-tyrosine. Following the incubation, an aliquot was saved for fluorescence and lipid peroxidation measurements and the remaining HDL_3 was subjected to size-exclusion chromatography on a 10-DG column equilibrated with buffer G [PBS supplemented with 100 μM DTPA, pH 8.0], to remove free dityrosine and other reaction components, prior to structural analysis or cell studies.

2.5.3 *Ex vivo* Oxidation of HDL_3 by Isolated Human Neutrophils

Human neutrophils (1×10^6 cells/mL) were incubated in polypropylene tubes with 0.5 mg/mL HDL_3 at 37°C in either buffer H [HBSS(-) supplemented with 100 μM DTPA, 1mg/mL D-glucose, 1 μg/mL aprotonin, 1 μg/mL leupeptin, 1 μg/mL soy trypsin inhibitor, 0.5 mM PMSF, pH 7.4] (incomplete) or buffer H supplemented with 2 mM L-tyrosine (complete). All buffers contained 100 μM DTPA to prevent any metal-ion catalyzed oxidation of HDL_3. Neutrophils were stimulated with either

200 nM PMA, or by priming the cells with 6.25 μg/mL CB for 5 min, followed by stimulation with 4×10^{-6} M fMLP, and the neutrophils maintained in suspension by gentle shaking (100 cycles/min). At the end of incubation, reactions were stopped by placing cells on ice, and the cells were pelleted by centrifugation at ~ 200 x *g* for 10 min at 4°C. The supernatant, containing HDL_3, was removed and an aliquot was saved for fluorescence and lipid peroxidation measurements. The remaining HDL_3 was subjected to size-exclusion chromatography on a 10-DG column equilibrated with buffer B to remove free dityrosine and other reaction components, prior to structural analysis or cell studies.

2.6 Determination of Dityrosine Fluorescence

Dityrosine fluorescence (Ex = 328 nm, Em = 410 nm) was determined using a fluorescence spectrophotometer (F-2000, Hitachi) for both pre- and post-10 DG column HDL samples. Pre-10 DG column sample dityrosine fluorescence was determined by adding 100 μl of sample to 900 μl buffer G. Post-10 DG column sample fluorescence was done using a 1 mL aliquot of sample.

2.7 Determination of Superoxide Generation

Generation of superoxide by activated human neutrophils was determined by measuring superoxide dismutase (SOD) inhibitable reduction of cytochrome C [223]. Briefly, neutrophils (1×10^6 cells/mL), activated by either 200 nM PMA or 6.25 μg/mL CB/fMLP (4×10^{-6} M), were incubated in buffer C supplemented with 90 μM cytochrome C in the presence or absence of 10 μg/mL SOD at 37°C for 20 min. Samples were then centrifuged at ~ 250 x *g* for 5 min at 4°C to pellet cells. Absorbance of reduced cytochrome C was measured at 550 nm against buffer C using

methylcrylate cuvettes (Hitachi spectrophotometer Model U-2000). Absorbance was converted to nmol superoxide generated using ε=21 $mM^{-1}cm^{-1}$ [224].

2.8 Determination of Myeloperoxidase Activity

Secretion of myeloperoxidase by activated human neutrophils was determined by measuring the oxidation of TMB [225]. Briefly, neutrophils (1×10^6 cells/mL), activated by either 200 nM PMA or 6.25 μg/mL CB/fMLP (4×10^{-6} M), was incubated in buffer C at 37°C for 1 hr. To measure myeloperoxidase activity, 150 μl of TMB liquid substrate was added to 50 μl of the aliquot in a 96 well plate and incubated at 37°C for 25 min. To stop the reaction 50 μl of 1 M sulphuric acid was added to the mixture. The plate was then read spectrophotometrically at 450 nm (SpectraMAX 250 plate reader, Molecular Devices, Sunnyvale, CA).

2.9 Conjugated Diene Assay

Conjugated dienes are an early marker of lipid peroxidation and were measured by the method of Dole and Meinertz [226]. A 500 μl or 250 μL aliquot was taken from HDL_3 samples of 0.5 mg/mL and 1 mg/mL respectively. Samples were acidified with concentrated hydrochloric acid. Lipids were extracted with isopropanol:heptane:1 N sulphuric acid (4:1:0.1, v/v/v), vortexed, and then 1 mL of water and 1.5 mL heptane added. The system was allowed to separate (~ 10 min), the organic layers were collected, dried under nitrogen, and then re-dissolved in 1 mL ice-cold heptane and read spectrophotometrically at 232 nm using heptane as blank. Absorbance was converted to μM conjugated dienes using ε=27 000 $M^{-1}cm^{-1}$ [227].

2.10 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed, under non-reducing conditions, using a Hoefer (20 cm × 20 cm) vertical slab gel apparatus by the method of Laemmli [228]. HDL apoproteins (10 μg) were resolved on a 7-20% gradient separation gel overlaid with a 3.5% stacking gel. Stacking of proteins was carried out at 25 mAmp/gel and separation at 35 mAmp/gel. Following SDS-PAGE, gels were fixed in 40% methanol/10% acetic acid (v/v) for 1 hr and proteins were then visualized by silver staining [229].

2.11 Western Blot Analysis

Proteins were transferred from SDS-PAGE gels to nitrocellulose overnight at 20 V, 4°C. Immunoblots were blocked in buffer I [Tris buffered saline supplemented with 0.1% Tween 20] plus 10% skim milk for 1 hr and 2 hr for the anti-apoAI and anti-apoAII immunoblots respectively. The membranes were washed with buffer I and incubated with either apo AI antibody (1:10000 dilution, rabbit anti-human, IgG fraction obtained by ammonium sulfate precipitation and DEAE-cellulose ion exchange chromatography, Calbiochem) or apo AII antibody (1:20000 dilution, goat anti-human, antiserum raised against purified apo AII, and antiserum defibrinated, delipidized, and absorbed by solid phase chromatography, Calbiochem) in buffer I plus 1% skim milk for 45 min. Membranes were then washed with buffer I and incubated with horseradish peroxidase-linked anti-rabbit (1:20000 dilution, IgG, whole molecule, IgG fraction of antiserum, Sigma) or anti-goat (1:20000 dilution, IgG, whole molecule, affinity isolated, Sigma) antibody for 30 min. Membranes

were then washed with buffer I and developed by ECL method and exposed to film (X-OMAT AR, Eastman Kodak Company, USA). Exposure time ranged from 5 sec to 5 min depending on the antibody used and the signal obtained. Specificity of binding for the primary and secondary antibodies was confirmed by blotting membranes with pre-immune sera and secondary antibody alone respectively. As well, no cross reactivity was found for the primary apo AI antibody to pure apo AII or the primary apo AII antibody to pure apo AI (data not shown).

2.12 Agarose Gel Electrophoresis

The Paragon® Electrophoresis System was used for agarose gel electrophoresis (Beckman, USA). Agarose gel electrophoresis was used to determine the relative electrophotometric mobility (REM) of HDL_3 following oxidation. A small aliquot (5-10 μL) of sample was added to each lane of the gel, allowed to dry for 5 min, placed onto a gel bridge assembly and the assembly placed into the Paragon Electrophoresis Cell. Electrophoresis was carried out at 100 V for 30 min. Gels were then fixed in 60% Methanol/30% deionized water/10% glacial acetic acid (v/v/v) for 5 min, placed in Paragon dryer to dry completely, stained with 55% Methanol/45% deionized water (v/v) plus 3 mL Lipo Stain for 5 min, and finally destained in 45% methanol/55% deionized water (v/v) for 5 min. A change in REM was determined by comparing the migration distance of oxidized HDL_3 samples to native HDL_3 towards the cathode from the origin.

2.13 Gradient Gel Electrophoresis

Gradient gel electrophoresis was performed using a Hoefer (20 cm × 20 cm) vertical slab gel apparatus [230]. HDL particle size was resolved on a 4-20%

gradient separation gel overlaid with a 3.5% stacking gel electrophoresed at 150 V (constant voltage) for 24 hr at 4°C. Following gradient gel electrophoresis, gels were stained with Coomassie blue to visualize particles.

2.14 Acyl-CoA:Cholesterol Acyltransferase (ACAT) Assay

To assess the ability of oxidized HDL_3 to deplete cellular cholesterol available for esterification by ACAT, cholesterol-loaded cells were incubated for 16-20 hr in DMEM/1 mg/mL FAFA (serum free medium, SFM) containing the indicated concentrations of control, cell-modified or *in vitro*-oxidized HDL_3, or patient SynHDL and PlmHDL, or isolated aortic HDL. Cells were then washed once with warm PBS and incubated for 1 hr in DMEM containing 9 μM [^{14}C]oleate complexed to 3 μM bovine serum albumin (BSA). After 1 hr, cells were chilled on ice, rinsed twice with ice-cold PBS/2 mg/mL BSA and twice with ice-cold PBS, then stored at -20°C until lipids were extracted. To extract cellular lipids, 1 mL of hexane:isopropanol (3:2, v/v) was added to each well and incubated for 30 min at room temperature. Extracts were added to individual 13×100 mm test tubes containing 20 μL of complete carrier [0.5 mg/mL cholesteryl ester (CE), 1 mg/mL free cholesterol (FC), 1 mg/mL 1-monooleoyl-rac-glycerol (MG), 1 mg/mL 1,2-distearoyl-rac-glycerol (DG), 1 mg/mL triolein (TG), and 1 mg/mL oleate acid dissolved in chloroform] along with two 1 mL washes of hexane:isopropanol. Extracts were dried down under air then resuspended in 110 μL ice-cold chloroform. 100 μL of each extract was spotted onto a thin layer chromatography (TLC) plate using an autospotter (TLC AutoSpotter model 10, Romer Labs Inc, USA). The TLC plates were then developed in hexane:diethyl ether:acetic acid (130:40:1.5, v/v/v)

until the solvent front was 0.5 cm from the top (12-16 min) to separate cell neutral lipids. Neutral lipids were visualized by exposure to iodine vapors and identified by comparison with authentic standards (complete carrier). The CE spot was cut and counted for radioactivity as described [166]. Cell protein was determined by the method of Lowry *et al.* [231] using BSA as the standard.

2.15 Other Methods

Estimation of HDL protein was determined by the method of Bradford [232] (Bio-Rad), using BSA as the standard, to minimize potential errors in estimation due to alterations in tyrosine residues [167].

2.16 Statistical Analysis

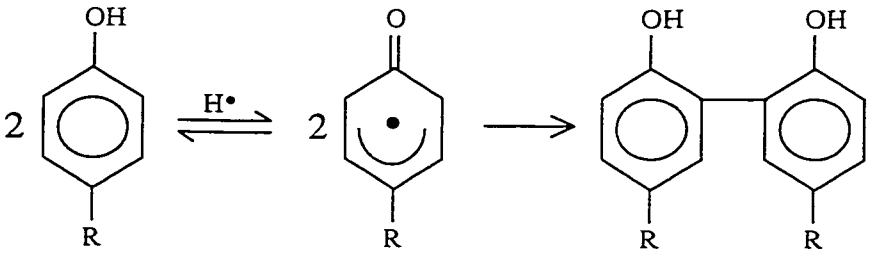
All statistical analyses (either two-sided Student's t-test or single variable ANOVA) were done using Microsoft® Excel 2000.

Chapter 3

Results

3.1 Introduction

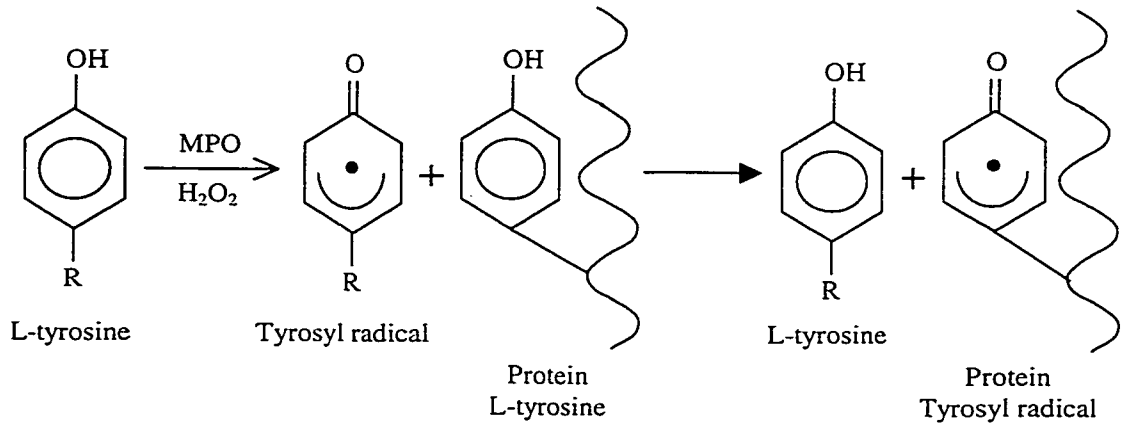
Our lab has previously shown that HDL_3 oxidized by peroxidase-generated tyrosyl radical is modified to a form that has an enhanced ability to mobilize cholesterol for removal from cells [166, 167]. Subsequently, the component of tyrosyl radical oxidized HDL_3 (TyrHDL) responsible for this action was found to be a crosslinked heterodimer of apo AI and apo AII on the particle surface [188]. Oxidation of HDL_3 by peroxidase-generated tyrosyl radical was not dependent on free metal ions, since oxidation was carried out in the presence of the potent metal ion chelator DTPA. A unique oxidation product of tyrosyl radical generation is o,o′-dityrosine, which results from the phenolic coupling of two tyrosyl radicals as shown by Scheme I.



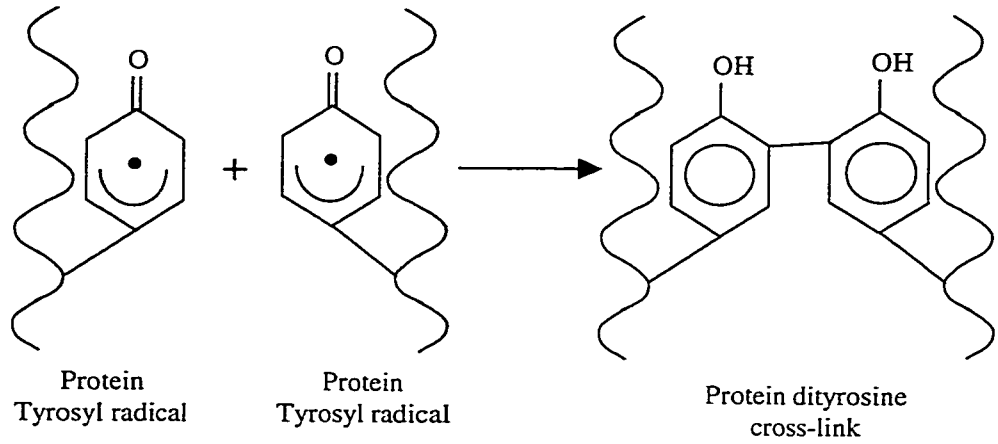
Scheme I

The physiological relevance of our *in vitro* model for HDL_3 oxidation is supported by the known ability of activated human phagocytes to generate tyrosyl radical [191, 233], and the presence of dityrosine in atherosclerotic lesions [173, 234]. When activated, human phagocytes secrete MPO, a heme enzyme, from its granules and produce $O_2^{\cdot}$, through the action of NADPH oxidase located in the plasma membrane. $O_2^{\cdot}$ spontaneously dismutates, or is actively converted by SOD, into

H_2O_2, which can be used by MPO to catalyze the oxidation of various substrates (*e.g.*, chloride ion and L-tyrosine to HOCl and tyrosyl radical, respectively). These MPO-generated radicals then carry out the actual oxidative damage of the activated phagocyte. In support of L-tyrosine being an important substrate of MPO, it has been shown that activated neutrophils and macrophages can generate tyrosyl radical via the MPO/H_2O_2 system at physiologic concentrations of L-tyrosine [191]. Moreover, the generation of tyrosyl radical occurs in the presence of physiologic concentrations of chloride ion, another major substrate for oxidation by MPO [178]. Dityrosine synthesis by activated phagocytes suggests that phagocyte-generated tyrosyl radicals may modify target proteins such as lipoproteins found in the artery wall [173], as shown by Scheme II and Scheme III.



Scheme II



Scheme III

Based on this observation, and the previous demonstration of HDL oxidation by peroxidase-generated tyrosyl radical in a cell-free system [166], we were interested in determining if HDL_3 apolipoproteins could serve as a target for dityrosine crosslinking by peroxidase-generated tyrosyl radical produced by activated neutrophils. Moreover, since we have previously found that HDL_3 oxidized by tyrosyl radical has an increased ability to mobilize cellular cholesterol for efflux, we were interested if HDL_3 oxidized by activated neutrophils would have a similarly enhanced effect. Finally, we investigated the oxidation of HDL *in vivo* by characterizing structural changes and biological activity of HDL isolated from human inflammatory joint fluid and atherosclerotic lesions.

3.2 Overview of Chapter

The experimental results shown here have been broken down into two main groups. The first group of results contains *ex vivo* experiments where we used isolated human neutrophils to oxidize HDL_3. Within the *ex vivo* experiments, normal

and CGD neutrophils were used to investigate the mechanism(s) of HDL oxidation by activated phagocytes. As well, the *ex vivo* experiments are further broken down according to the stimulant used for activating isolated neutrophils, in order to demonstrate and highlight differences seen in HDL_3 oxidized by neutrophils activated in different ways. The second group of results contains experiments where we investigated the structural and biological changes seen in *in vivo*-modified HDL isolated from inflammatory joint fluid and atherosclerotic aortas. Experiments on HDL isolated from inflammatory joint fluid were done in collaboration with the Division of Rheumatology at the University of Alberta Hospitals, and experiments on HDL isolated from atherosclerotic aortas were performed in collaboration with Drs. Roland Stocker and Ute Panzenböeck from the Biochemistry Group at the Heart Research Institute in Camperdown, NSW, Australia.

3.3 Effects of Oxidation of HDL_3 by Neutrophils in *ex vivo* Systems

This section has been divided into three main parts. The first two parts cover results found from the oxidation of HDL_3 by PMA-activated or by CB/fMLP-activated neutrophils, and the third part examines the ability of neutrophils from CGD patients to oxidize HDL when stimulated by CB/fMLP.

Superoxide production and MPO secretion are vital components for the modification of substrates by activated phagocytes. PMA and CB/fMLP were significantly different from each other in inducing these responses. As shown in Figure 3.1, neutrophils activated with PMA produced significantly greater amounts of O_2^- than did neutrophils stimulated by CB/fMLP, as expected based upon published results [235]. In contrast, the secretion of MPO by PMA-activated neutrophils was

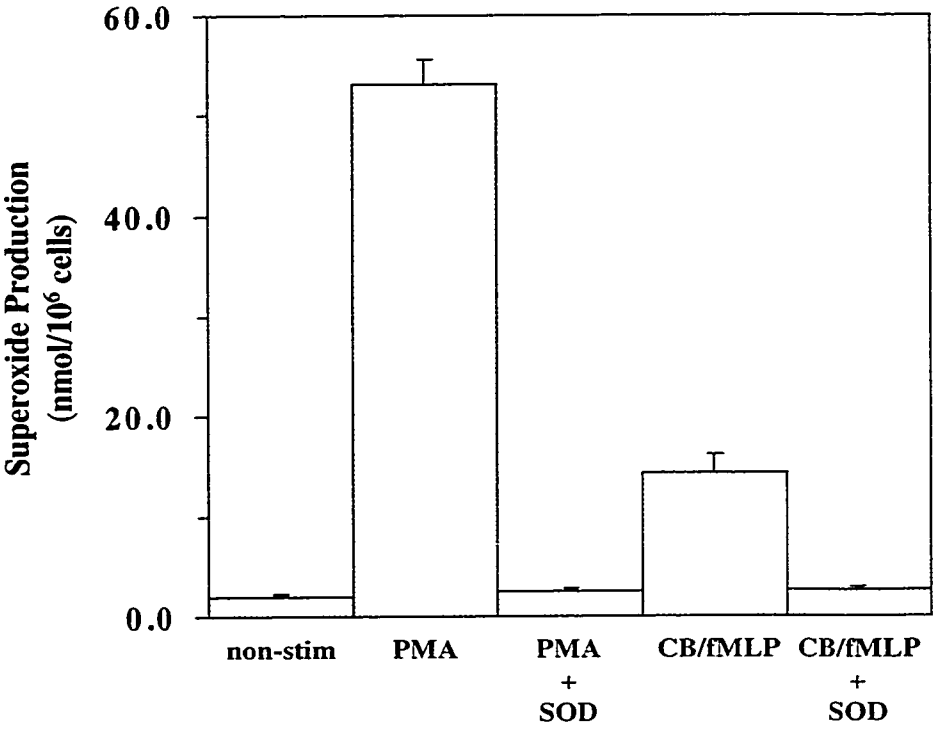


Figure 3.1 Effect of stimulant on superoxide production by activated neutrophils. Superoxide generation, by PMA (200 nM)- or CB (6.25 μg/mL)/fMLP (1×10^{-5} M)-activated neutrophils (1×10^6 cells/mL), was determined by measuring the SOD (10 μg/mL)-inhibitable reduction of cytochrome C (90 μM) (see Methods). Values are means ± SE from at least four values from two or more independent experiments.

significantly less when compared to CB/fMLP-activated neutrophils (Figure 3.2). These results indicate that there are significant differences in the response of neutrophils to different activators. Similarly, we found that *ex vivo* oxidation of HDL_3 by activated neutrophils resulted in changes in HDL function that were stimulant-dependent. Therefore, for ease of interpretation, we have separated the following data according to the activator utilized.

3.3.1 Oxidation of HDL_3 by PMA-Activated Neutrophils

3.3.1.1 Dityrosine Fluorescence

As outlined in section 3.1, a unique oxidation product of tyrosyl radical generation is o,o′-dityrosine, a highly fluorescent compound which can be detected at specific fluorescence excitation (Ex) and emission (Em) wavelengths, Ex=328 nm, Em=410 nm [236]. We therefore used dityrosine fluorescence as a measure of tyrosyl radical oxidation. To determine if HDL_3 apolipoproteins exposed to phagocyte-generated tyrosyl radicals undergo phenolic coupling, we exposed HDL_3 to PMA-activated neutrophils in the presence of added L-tyrosine in a Cl^--containing buffer. Following the incubation, we measured HDL_3-associated dityrosine fluorescence after passing HDL_3 over a size exclusion column to remove free dityrosine.

Consistent with the results of Heinecke *et al.* showing production of free dityrosine by activated neutrophils [191], we also found that activated neutrophils generate free dityrosine (Figure 3.3). Oxidation of HDL_3 by activated neutrophils resulted in a time-dependent increase in HDL_3-associated dityrosine fluorescence (Figure 3.4). Incubations were not extended beyond 3 hr due to the clumping of neutrophils. This data shows that HDL_3 apolipoproteins can serve as a target for

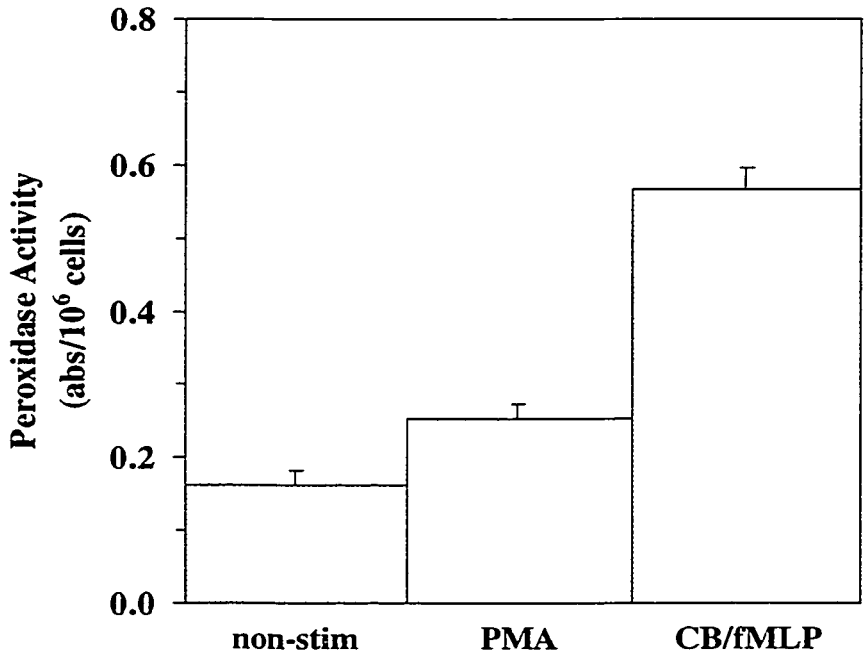


Figure 3.2 Effect of stimulant on myeloperoxidase secretion by activated neutrophils. Incubation media were collected from non-activated human neutrophils (1 x 10^6 cells/mL), PMA (200nM)-activated, or CB (6.25 μg/mL)/fMLP (1 x 10^{-5} M)-activated, and incubated for 1 hr at 37°C in buffer C. Supernatants were then assayed for myeloperoxidase (MPO) activity using the TMB-substrate system (see Methods). Values are means ± SD of three replicates, representative of three independent experiments. MPO secretion was significantly greater from activated neutrophils ($p<0.001$) compared to non-activated neutrophils, and was significantly greater from CB/fMLP-activated neutrophils compared to PMA-activated neutrophils ($p<0.001$) determined by Student's t-test.

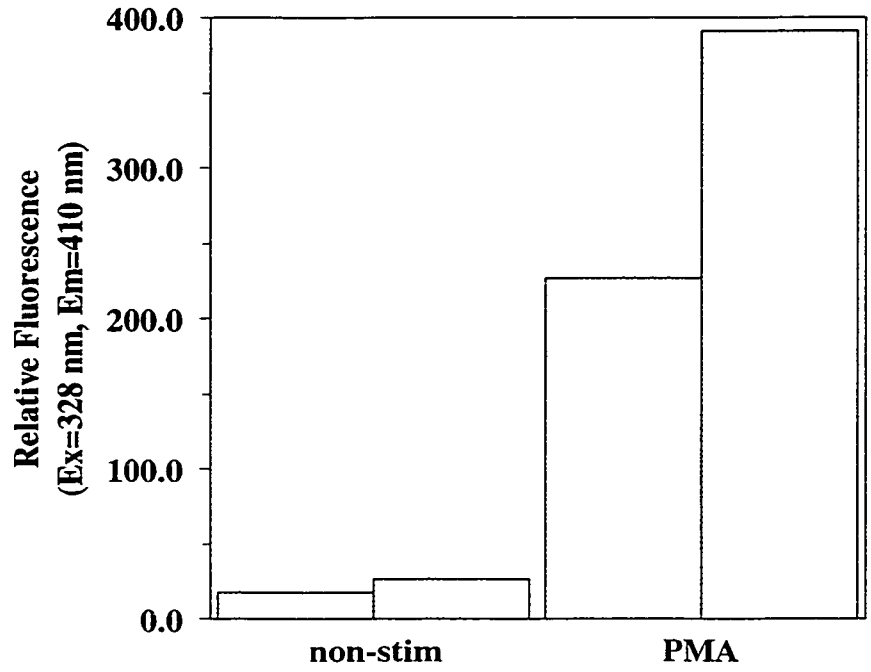


Figure 3.3 Generation of dityrosine by activated neutrophils. Isolated human neutrophils were incubated in buffer H plus 100 μM L-tyrosine at 37°C for 60 min. Non-activated neutrophils; PMA (200 nM)-activated neutrophils (1 x 10^6 cells/mL). Values are from two independent experiments.

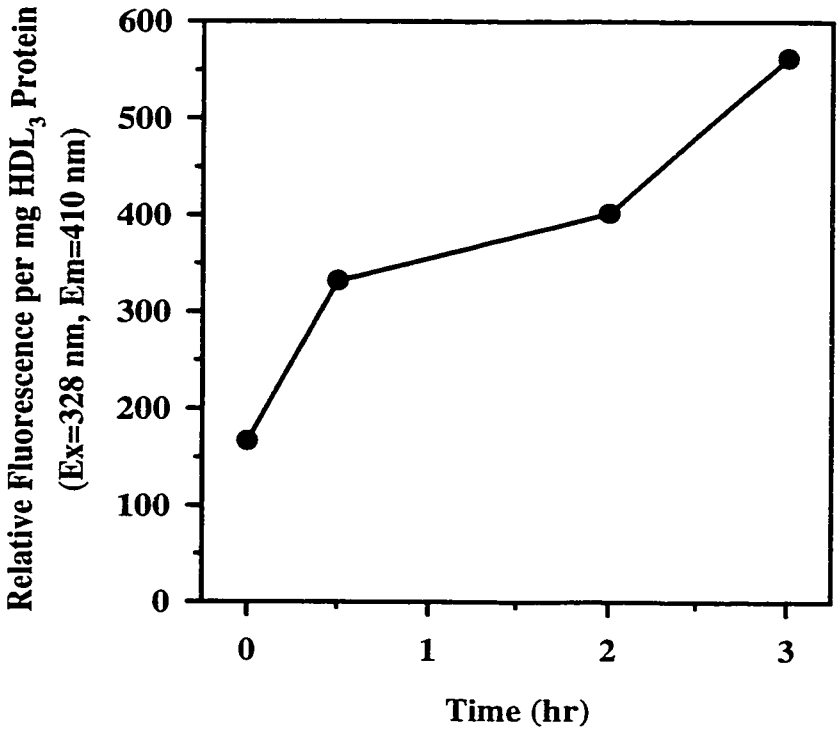


Figure 3.4 Time course of dityrosine formation in HDL_3 oxidized by PMA-activated neutrophils. Post 10-DG column fluorescence of 0.5 mg/mL HDL_3 incubated for indicated times in buffer H containing PMA (200 nM)-activated neutrophils (2 x 10^6 cells/mL) in the presence of 2 mM L-tyrosine. Single values representative of 2 independent experiments.

tyrosyl radical generated by activated phagocytes. We next wanted to determine the importance of the MPO/H_2O_2 system for the generation of tyrosyl radical and dityrosine in neutrophil-modified HDL_3. Since the ability of MPO to generate tyrosyl radical is dependent on the presence of H_2O_2, we assessed the effects of SOD and catalase to increase and decrease the availability of H_2O_2, respectively. SOD increases H_2O_2 availability by catalyzing the dismutation of O_2^- to H_2O_2, whereas catalase decreases H_2O_2 availability by scavenging it [237, 238]. As expected, the addition of SOD resulted in a significant increase in HDL_3-associated dityrosine fluorescence (Figure 3.5, complete + SOD). In contrast, the addition of catalase significantly decreased HDL_3-associated dityrosine fluorescence (Figure 3.5, complete + catalase). These results support the involvement of the MPO/H_2O_2 system in the generation of tyrosyl radical by activated neutrophils, leading to dityrosine formation in HDL_3.

3.3.1.2 Crosslinking of HDL_3 Apolipoproteins by PMA-activated Neutrophils

Our lab has previously shown that the oxidation of HDL_3 *in vitro* by peroxidase-generated tyrosyl radical results in the crosslinking of its major apolipoproteins, apo AI and apo AII, to themselves and each other [166, 188]. We examined whether neutrophil-generated tyrosyl radical also resulted in the oxidative crosslinking of apo AI and apo AII in HDL_3. HDL_3 was incubated with PMA-activated neutrophils in the presence of L-tyrosine with and without the addition of either SOD or catalase, and the extent of apolipoprotein crosslinking was monitored by non-reducing SDS-PAGE. As shown in Figure 3.6, apo AI and apo AII crosslinked bands were formed (lane 3). Consistent with the protein crosslinks

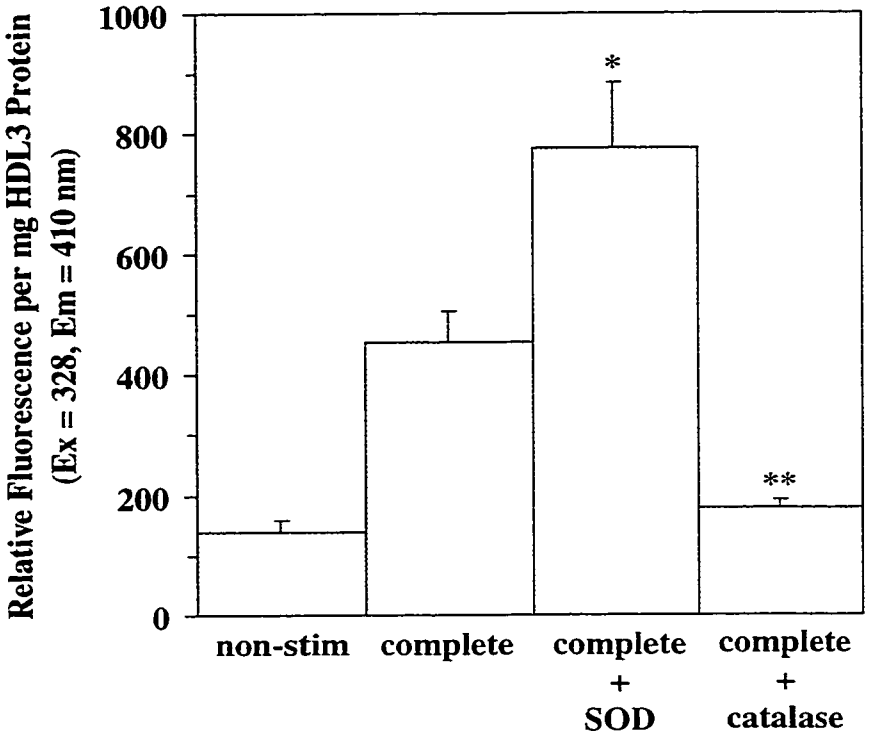


Figure 3.5 Effect of H_2O_2 availability on dityrosine formation in HDL_3 oxidized by PMA-activated neutrophils. Non-stim, 0.5 mg/mL of HDL_3 incubated with non-activated neutrophils in buffer H at 37°C for 3 hr; complete, 0.5 mg/mL of HDL_3 incubated with PMA (200 nM)-activated cells (1 x 10^6 cells/mL) in buffer H plus 2 mM L-tyrosine at 37°C for 3 hrs; SOD, superoxide dismutase (10 μg/mL); and catalase (10 μg/mL). Values are means ± SD of three independent experiments. *, $p<0.02$ vs complete, **, $p<0.01$ vs complete based on Student's t-test.

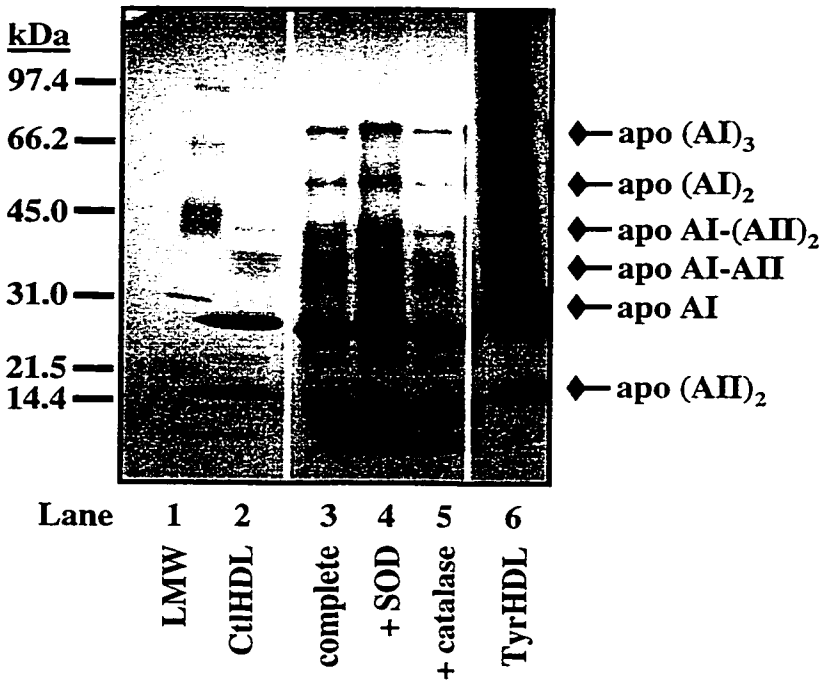


Figure 3.6 Non-reducing SDS-PAGE analysis of crosslinked HDL_3 apolipoproteins oxidized by PMA-activated neutrophils. Neutrophil-oxidized HDL_3 apolipoproteins were separated by 7-20% non-reducing SDS-PAGE and visualized by silver stain. LMW, low molecular weight standard; CtlHDL, 1 mg/mL HDL_3 incubated in buffer F at 37°C overnight; complete, 0.5 mg/mL HDL_3 incubated with PMA (200 nM)-activated neutrophils (1 x 10^6 cells/mL) in buffer H plus 2 mM L-tyrosine at 37°C for 3 hrs; SOD, superoxide dismutase (10 μg/mL); catalase (10 μg/mL); and TyrHDL, 1 mg/mL HDL_3 incubated with 100 nM HRP, 100 μM L-tyrosine, and 100 μM H_2O_2 in buffer F at 37°C overnight. All lanes shown were from the same SDS-PAGE.

generated in TyrHDL [188], the more prominent crosslinks seen had apparent molecular masses of 36.5, 45, 56, and 84kDa, indicating the formation of apo AI-AII heterodimers, apo AI-$(AII)_2$ heterodimers, apo AI dimers and apo AI trimers, respectively [188]. The addition of SOD resulted in an increase in appearance of these crosslinked proteins, whereas the addition of catalase resulted in a decrease in appearance of crosslinked bands compared to the complete system (Figure 3.6, lanes 4 and 5 respectively). Moreover, the increase and decrease in apolipoprotein crosslinking by SOD and catalase (respectively) was similar to their effect on HDL_3-associated dityrosine fluorescence, suggesting the involvement of dityrosine in the crosslinking of HDL_3 apolipoproteins. These results also support the involvement of the MPO/H_2O_2 system in the oxidative crosslinking of HDL_3 apolipoproteins by PMA-activated neutrophils. Although it is known that silver staining cannot be used to compare the abundance of different proteins, it has been shown that changes in abundance of the same protein is proportional to its relative density in silver-stained gels [239].

We also found that incubation of HDL_3 with PMA-activated neutrophils resulted in the degradation of both apo AI and apo AII. This is indicated by the presence of protein bands beneath the native apo AI (28 kDa) and apo AII (17 kDa) bands (Figure 3.6, lanes 3, 4 and 5). Western blot analysis with polyclonal antibodies supported this conclusion, showing a significant degree of degradation of the HDL_3 apo AI even after 1 hr of incubation with PMA-activated neutrophils (Figure 3.7, lane 2). The degradation of HDL_3 apo AI by PMA-activated neutrophils was inhibited by the addition of a potent non-specific serine protease inhibitor, diisopropyl

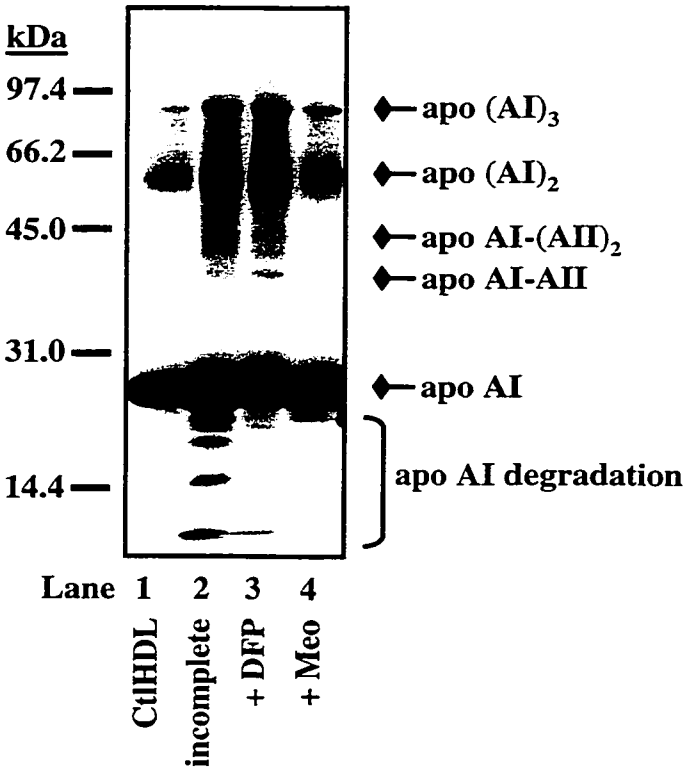


Figure 3.7 Apolipoprotein AI degradation in HDL_3 incubated with PMA-activated neutrophils. Neutrophil oxidized HDL_3 apolipoproteins were separated on a 7-20 % non-reducing SDS-PAGE, transferred to nitrocellulose, and the nitrocellulose blotted with an antibody to apo AI (see Methods). CtlHDL, 1 mg/mL HDL_3 incubated in buffer F at 37°C overnight; incomplete, 0.5 mg/mL HDL_3 incubated with PMA (200 nM)-activated neutrophils (1 x 10^6 cells/mL) in buffer H at 37°C for 1 hr; incomplete system plus DFP, diisopropyl fluorophosphate (1 mM); and incomplete system plus Meo, N-Methoxysuccinyl-Ala-Ala-Pro-Val (0.6 mM). apo, apolipoprotein.

fluorophosphate (DFP) (Figure 3.7, lane 3). The addition of a more specific protease inhibitor, N-methoxysuccinyl-Ala-Ala-Pro-Val, which inhibits elastase, also prevented the degradation of HDL_3 apo AI by PMA-activated neutrophils (Figure 3.7, lane 4). These results are consistent with previous findings showing elastase released by activated neutrophils leads to apolipoprotein degradation [240, 241].

3.3.1.3 Ability of HDL_3 Oxidized by PMA-activated Neutrophils to Deplete the ACAT Substrate Pool.

It has been previously shown that TyrHDL has a markedly enhanced ability to mobilize intracellular cholesterol for efflux from cultured cells [59, 166, 167]. Our lab showed that cholesterol-loaded fibroblasts incubated with TyrHDL had a marked increase in the translocation of cholesterol available for esterification by the cell (the "ACAT-accessible" cholesterol pool), to a pool available for efflux on the cells' surface ("efflux-accessible" cholesterol), compared to cells incubated with control HDL [167]. This effect was not due to direct inhibition of the ACAT enzyme, nor to activation of neutral cholesteryl ester hydrolase (NCEH), but rather to depletion of the substrate pool of cholesterol available to ACAT [167]. This enhanced effect of TyrHDL was later found to be due to the presence of apo AI-AII heterodimers on the modified HDL surface [188]. The direct correlation of enhanced cholesterol mobilization and efflux from cells with depletion of the pool of cholesterol available for esterification in these earlier studies led us to use this esterification or "ACAT" assay as our principal determinant of cellular cholesterol mobilization by neutrophil- and *in vivo*-modified HDL.

In the ACAT assay, the decrease in [^{14}C]oleate incorporation into cholesteryl[^{14}C]oleate esters following incubation with HDL is a measure of the individual HDL species' ability to deplete the regulatory pool of cholesterol available for esterification by ACAT [83]. In contrast to the effects of HDL oxidized by tyrosyl radical in our cell-free system [166], HDL_3 oxidized by PMA-activated neutrophils showed a diminished capacity to deplete cultured fibroblasts of ACAT-accessible cholesterol compared to control HDL_3 (Figure 3.8). Even in the presence of SOD, where there was an increase in apolipoprotein crosslinking, there was no decrease observed in cholesterol esterification, which would have been expected. As well, the addition of catalase did not affect the ability of HDL_3 oxidized by PMA-activated neutrophils to deplete the ACAT cholesterol pool, despite a decrease in the crosslinking of apo AI to apo AII.

These results suggested that other modifications of HDL_3 by PMA-activated neutrophils were responsible for the loss in ability to mobilize ACAT-accessible cholesterol. The release of proteases and degradation of HDL apoproteins, despite protein crosslinking, by PMA-activated neutrophils (Figs. 3.6 and 3.7), was felt to be the most likely explanation for this finding. In support of this hypothesis, Mendez and Oram showed that even minimal degradation of HDL_3 proteins results in a markedly impaired ability of these HDL to deplete ACAT-accessible cholesterol [109]. To test this hypothesis in our system, we investigated the ability of HDL_3 oxidized by PMA-activated neutrophils in the presence of the protease inhibitors DFP or N-methoxysuccinyl-Ala-Ala-Pro-Val to promote cellular cholesterol efflux. We found that inhibition of apo AI degradation by PMA-activated neutrophils resulted in

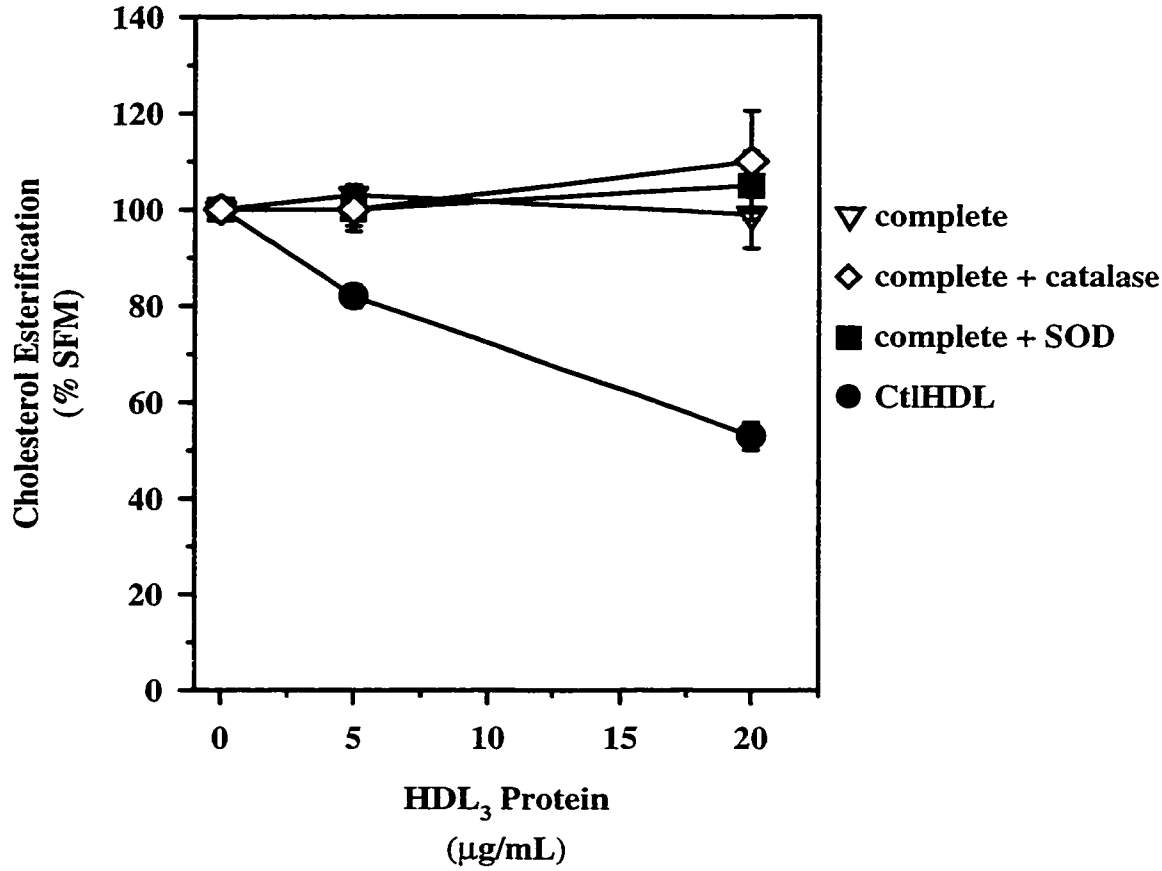


Figure 3.8 Effect of HDL_3 oxidized by PMA-activated neutrophils on cholesterol esterification in cholesterol-loaded fibroblasts. Human skin fibroblasts loaded with non-lipoprotein cholesterol were incubated for 16 hr with serum free media (SFM) plus the indicated concentration of: $CtlHDL_3$, 1 mg/mL HDL_3 incubated in buffer F at 37°C overnight; complete, 0.5 mg/mL HDL_3 incubated with PMA (200 nM)-activated neutrophils (1 x 10^6 cells/mL) in buffer H plus 2 mM L-tyrosine at 37°C for 1 hr; + SOD, complete plus 10 μg/mL SOD; or + catalase, complete plus 10 μg/mL catalase. Cells were then washed and incubated for 1 hr with [^{14}C]oleate, and cellular cholesteryl [^{14}C]oleate formed was measured as described under Methods. Results are mean ± SE of three independent experiments containing three or more replicates for each condition, expressed as percentage of picomoles of [^{14}C]oleate incorporated into cholesteryl esters/mg cell protein/hr in cells treated with SFM alone. Error bars not shown are within the symbol dimensions. Cholesterol esterification by cells incubated with HDL_3 oxidized by PMA-activated neutrophils was significantly greater than cells incubated with CtlHDL at all concentrations and conditions studied (p<0.002) as determined by multiple Student's t-tests.

HDL_3 particles that had an increased ability to deplete ACAT-accessible cholesterol compared to CtlHDL (Figure 3.9). These results support our hypothesis that apolipoprotein degradation, not oxidation, resulted in the decreased ability of neutrophil-modified HDL_3 to mobilize cellular cholesterol. Moreover, these results show that oxidation of HDL_3 by PMA-activated neutrophils in the absence of protein degradation results in an HDL_3 particle that has an enhanced ability to promote cellular cholesterol efflux.

3.3.2 Oxidation of HDL_3 by CB/fMLP-activated Neutrophils

The differences in response of neutrophils to PMA and CB/fMLP indicated above (Figures 3.1 and 3.2), and the presence of non-oxidative proteolysis of HDL_3 induced by PMA-activated neutrophils, led us to explore changes in HDL_3 structure and function induced by CB/fMLP-activated neutrophils.

3.3.2.1 Effects of CB/fMLP-activated Neutrophils on HDL_3 Apolipoprotein Degradation

We first wanted to investigate whether oxidation of HDL_3 by CB/fMLP-activated neutrophils also resulted in significant amounts of apolipoprotein degradation. To test this, we incubated HDL_3 with CB/fMLP-activated neutrophils and assessed the extent of apolipoprotein degradation by non-reducing SDS-PAGE. We found a time-dependent increase in HDL_3 apolipoprotein degradation; both apo AI and apo AII were degraded over time (Figure 3.10). Moreover, degradation of the apolipoprotein crosslinks was also observed as seen by the downward shift of these bands over time. However, incubation of HDL_3 with CB/fMLP-activated neutrophils for up to 60 min resulted in no demonstrable native or crosslinked apolipoprotein

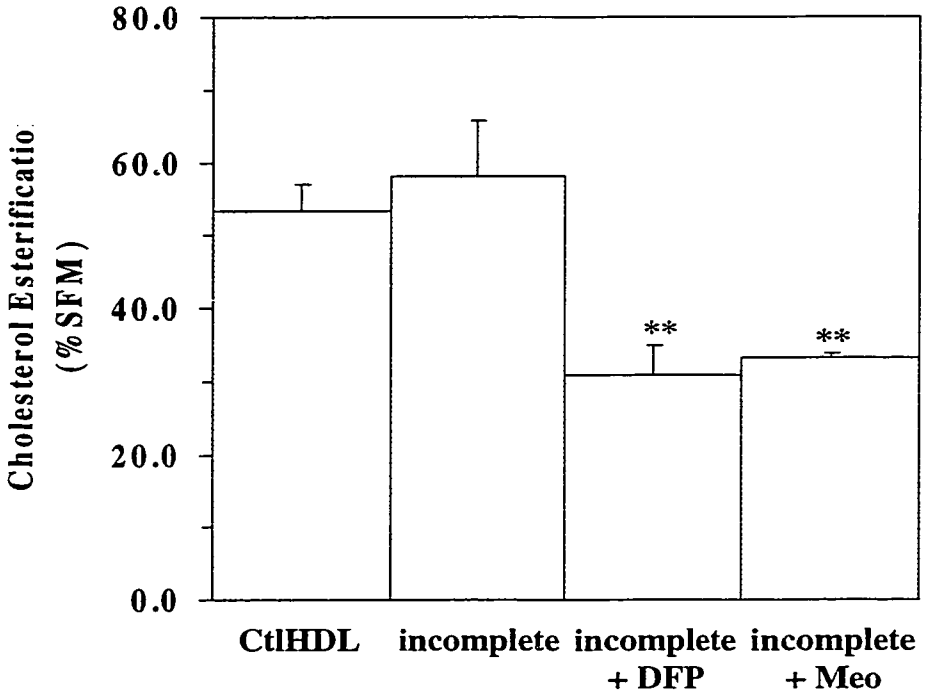


Figure 3.9 Effect of protease inhibitors on HDL_3 oxidized by PMA-activated neutrophils to mobilize cholesterol for esterification in cholesterol-loaded fibroblasts. Human skin fibroblasts loaded with non-lipoprotein cholesterol were incubated for 16 hr with serum free media (SFM) plus 2.5 μg/mL of: CtlHDL, 1 mg/mL HDL_3 incubated in buffer F at 37°C overnight; incomplete, 0.5 mg/mL HDL_3 incubated with PMA (200 nM)-activated neutrophils (1 x 10^6 cells/mL) in buffer H at 37°C for 1 hr; DFP, diisopropyl fluorophosphate (1 mM); Meo, N-methoxysuccinyl-Ala-Ala-Pro-Val (0.6 mM). Cells were then washed and incubated for 1 hr with [^{14}C]oleate, and cellular cholesteryl [^{14}C]oleate formed was measured as described under Methods. Results are mean ± SE of three determinations expressed as percentage of picomoles of [^{14}C]oleate incorporated into cholesteryl esters/mg cell protein/hr in cells treated with SFM alone. **, $p<0.01$ vs CtlHDL as determined by Student's t-test.

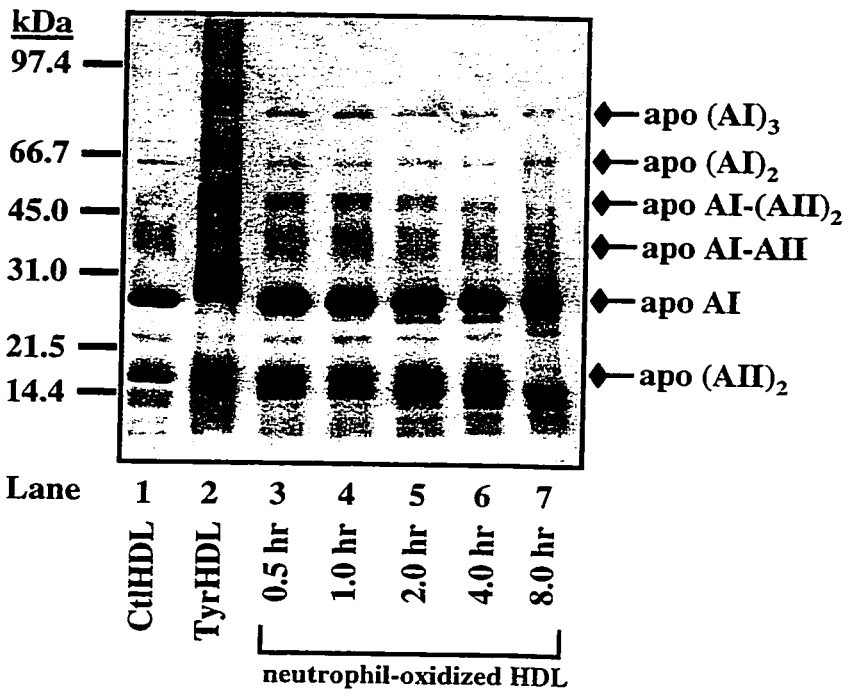


Figure 3.10 Non-reducing SDS-PAGE analysis of time-dependent HDL_3 apolipoprotein degradation by CB/fMLP-activated neutrophils. Neutrophil oxidized HDL_3 apolipoproteins were separated by 7-20 % non-reducing SDS-PAGE and visualized by silver stain. CtlHDL, 1 mg/mL HDL_3 incubated in buffer F at 37°C overnight; TyrHDL, 1 mg/mL HDL_3 incubated with 100 nM HRP, 100 μM L-tyrosine, and 100 μM H_2O_2 in buffer F at 37°C overnight; and 0.5 mg/mL HDL_3 incubated with CB (6.25 mg/mL)/fMLP (1×10^{-5} M)-activated neutrophils (1×10^6 cells/mL) in buffer H plus 2 mM L-tyrosine at 37°C for indicated time. apo, apolipoprotein.

degradation using this sensitive silver-staining method (Figure 3.10, lane 4). This was further confirmed by Western blot analysis using polyclonal antibodies to apo AI (Figure 3.11). These results confirm protein crosslinking in the absence of protein degradation in HDL_3 incubated with CB/fMLP-activated neutrophils for 60 min or less.

3.3.2.2 Dityrosine Fluorescence

Dityrosine fluorescence was also determined as described in section 3.3.1.1. Figure 3.12 shows that CB/fMLP-activated neutrophils in the presence of exogenously added L-tyrosine generate tyrosyl radical, and oxidize HDL as evidenced by increased HDL_3-associated dityrosine fluorescence. The addition of SOD significantly increased HDL_3-associated dityrosine fluorescence; addition of catalase decreased HDL_3-associated dityrosine fluorescence relative to the complete system, however this decrease was not found to be statistically significant.

To further investigate the role of MPO in tyrosyl radical and dityrosine production by activated neutrophils, azide was used to inhibit MPO directly. Maximum inhibition of MPO activity occurred at 3.5 mM azide (Figure 3.13). This concentration of azide was found to have no effect on O_2^- generation by CB/fMLP-activated neutrophils (Figure 3.14). MPO inhibition by 3.5 mM azide resulted in a decrease in HDL_3-associated dityrosine fluorescence to a level similar to the minus L-tyrosine sample (Figure 3.12), further supporting the role of MPO-dependent tyrosyl radical formation in the oxidation of HDL_3 by activated neutrophils.

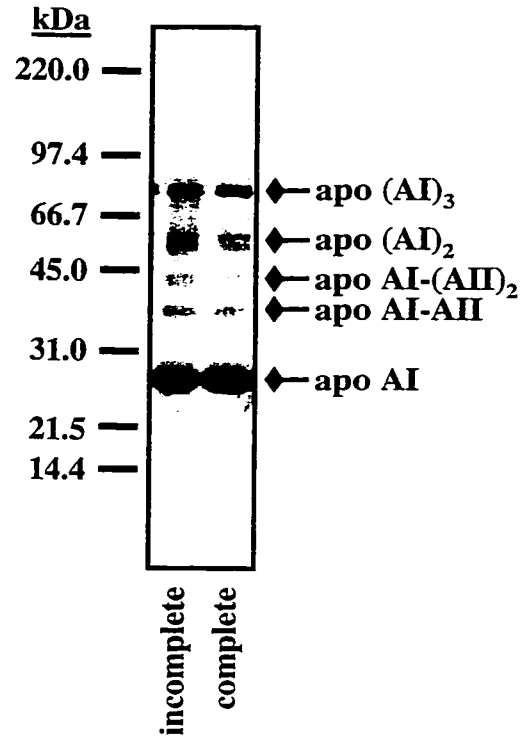


Figure 3.11 Lack of apolipoprotein AI degradation in HDL_3 oxidized by CB/fMLP-activated neutrophils for 1 hr. Neutrophil-oxidized HDL_3 apolipoproteins were separated by 7-20 % non-reducing SDS-PAGE, transferred to nitrocellulose, and the nitrocellulose blotted with an antibody to apo AI (see Methods). Incomplete, HDL_3 (0.5 mg/mL) incubated with CB (6.25 μg/mL)/fMLP (1×10^{-5} M)-activated neutrophils (1×10^6 cells/mL) in buffer H at 37°C for 1 hr; and complete, incomplete plus L-tyrosine (2 mM). apo, apolipoprotein.

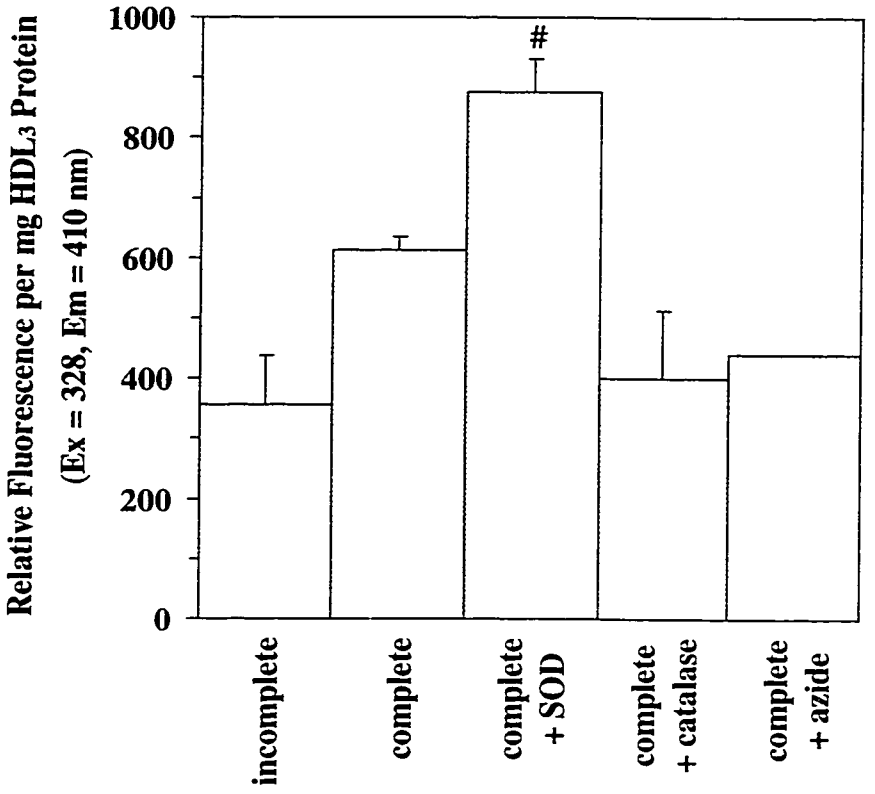


Figure 3.12 Effect of H_2O_2 availability and MPO activity on dityrosine formation in HDL_3 oxidized by CB/fMLP-activated neutrophils. HDL_3 (0.5 mg/mL) was incubated with CB (6.25 μg/mL)/fMLP(1 x 10^{-5} M)-activated neutrophils (1 x 10^6 cells/mL) in buffer H At 37°C for 1 hr. Incomplete, HDL_3 plus activated neutrophils; complete, HDL_3 plus activated neutrophils plus 2 mM L-tyrosine; SOD, superoxide dismutase (10 μg/mL); catalase (10 μg/mL); and azide (3.5 mM). Values are means ± SD of three independent experiments, except for complete system + azide which is the average of two experiments (values = 427 and 455). #, $p<0.05$ vs complete as determined by Student's t-test.

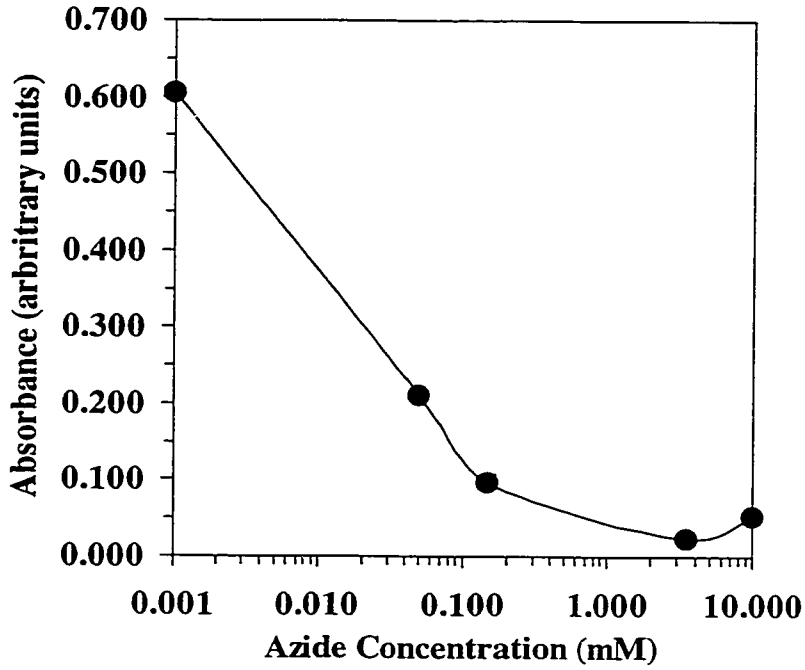


Figure 3.13 Inhibition of myeloperoxidase activity with azide. Supernatants containing myeloperoxidase secreted from CB (6.25 μg/mL)/fMLP (1 x 10^{-5} M)-activated neutrophils (1 x 10^{6} cells/mL) incubated for 1 hr at 37°C hr in buffer C were collected and assayed for azide inhibition of myeloperoxidase activity using the TMB-substrate system (see Methods). Values are means ± SD of triplicates, representative of two independent experiments. No azide addition value = 0.670 ± 0.004. Error bars not shown are contained within symbol dimensions.

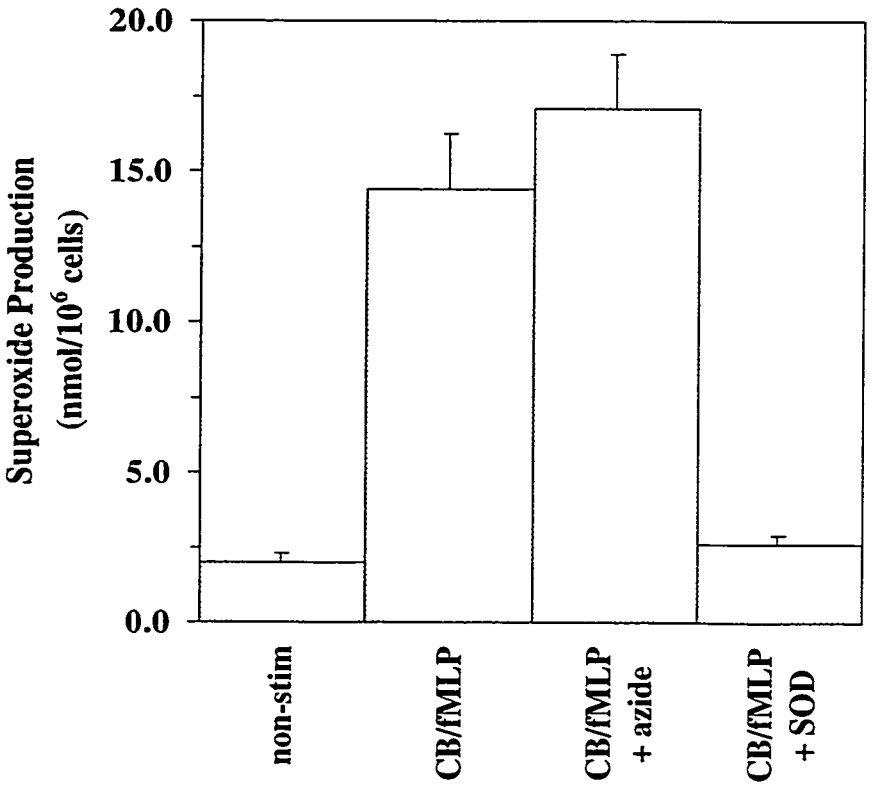


Figure 3.14 Effect of azide on superoxide production by CB/fMLP-activated neutrophils. Superoxide generation was determined by measuring the SOD-inhibitable reduction of cytochrome C (see Methods). Non-activated neutrophils (1×10^6 cells/mL) (non-stim); CB (6.25 μg/mL)/fMLP (1×10^{-5} M)-activated neutrophils (CB/fMLP); activated neutrophils plus 3.5 mM azide (CB/fMLP + azide); and activated neutrophils plus 10 μg/mL SOD (CB/fMLP + SOD); were incubated for 20 min at 37°C in buffer C containing cytochrome C (90 μM). Values are means ± SE of four observations from two independent experiments.

3.3.2.3 Crosslinking of HDL_3 Apolipoproteins by CB/fMLP-activated Neutrophils

Similar to our analysis of apolipoprotein crosslinks in HDL_3 oxidized by PMA-activated neutrophils, the crosslinking of HDL proteins by CB/fMLP-activated neutrophils was assessed. HDL_3 was incubated with CB/fMLP-activated neutrophils for 60 min in the presence or absence of L-tyrosine, and the extent of apolipoprotein crosslinking was determined by Western blot analysis using polyclonal antibodies to apo AI and apo AII. Similar to our *in vitro* oxidation model [188] and our results with HDL_3 oxidized by PMA-activated neutrophils, apolipoprotein crosslinks with apparent molecular masses of 36.5, 45, 56, and 84 kDa were generated to varying degrees in HDL_3 oxidized by CB/fMLP-activated neutrophils (Figure 3.15). Interestingly, we found that these crosslinks were formed in the presence or absence of added L-tyrosine (Figure 3.15, lanes 1 and 2). This suggests that even in the absence of added L-tyrosine (and apparently free tyrosyl radical – see discussion), activated neutrophils can oxidatively crosslink HDL_3 apolipoproteins.

We also assessed the role of MPO and H_2O_2 in the oxidative crosslinking of HDL_3 apolipoproteins by CB/fMLP-activated neutrophils in this experiment. Again, HDL_3 was incubated with CB/fMLP-activated neutrophils for 60 min in the presence or absence of added L-tyrosine with and without the addition of SOD, catalase or azide. The results were similar for both the plus and minus L-tyrosine additions (Figure 3.15). Interestingly, we found that when SOD was added (increasing H_2O_2 availability and indirectly MPO activity) there was a decrease in apo AI crosslinking and an increase in apo AII crosslinking compared to HDL_3 incubated with CB/fMLP-

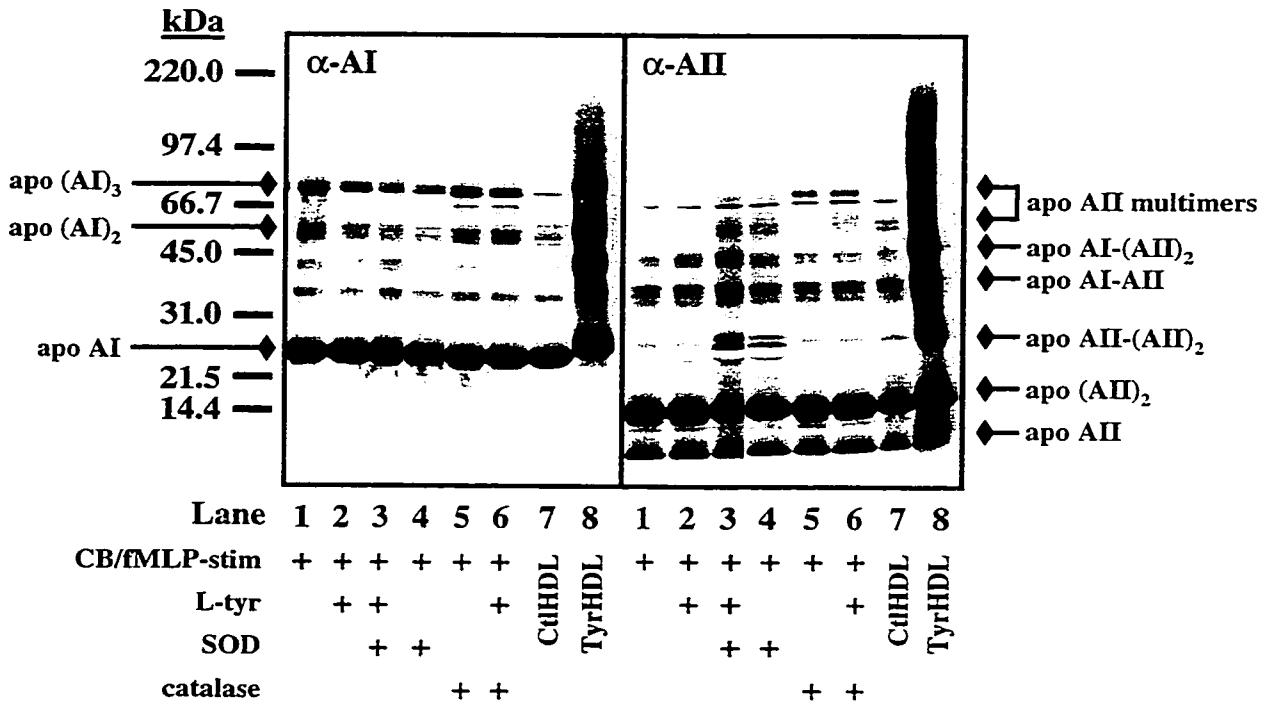


Figure 3.15 Western blot analysis of HDL_3 oxidized by CB/fMLP-activated neutrophils. Apolipoproteins of HDL_3 (0.5 mg/mL) oxidized by CB (6.25 μg/mL)/fMLP (1×10^{-5} M)-activated neutrophils were separated by 7-20 % non-reducing SDS-PAGE, the apolipoproteins transferred to nitrocellulose, and the nitrocellulose blotted with an antibody to apolipoprotein AI (α-AI) or apolipoprotein AII (α-AII) (see Methods). L-tyr, L-tyrosine (2 mM); SOD, superoxide dismutase (10 μM); catalase (10 μg/mL); CtlHDL, 1 mg/mL HDL_3 incubated in buffer F at 37°C overnight; and TyrHDL, 1 mg/mL HDL_3 incubated with 100 nM HRP, 100 μM L-tyrosine, and 100 μM H_2O_2 in buffer F at 37°C overnight. apo, apolipoprotein. Representative of three independent experiments.

activated neutrophils alone (Figure 3.15, lanes 3 and 4). The addition of catalase to decrease H_2O_2 availability (and indirectly MPO activity) did not appear to have much affect on the crosslinking of apo AI, but did diminish apo AII crosslink formation to itself and apo AI (Figure 3.15, lanes 5 and 6). Inhibition of MPO activity by azide showed similar results to that of catalase addition (data not shown). The lack of increased apo AI crosslinking by addition of SOD may have resulted from the masking of apo AI epitopes (recognized by the polyclonal antibody) due to increased crosslinking of apo AII to apo AI, although this needs to be confirmed. These results do not fully support the absolute requirement for MPO in the oxidative crosslinking of HDL_3 apolipoproteins by activated neutrophils, although they do show that increased MPO activity does lead to increased crosslinking of apo AII in the apo AI-AII complexes.

3.3.2.4 Oxidative Crosslinking of HDL_3 Apolipoproteins by Neutrophils is Intraparticle Rather Than Interparticle

Initial studies on the oxidation of HDL by tyrosyl radical in a cell-free peroxidase system indicated the crosslinks between apoproteins occurred on individual particles rather than between particles, and that HDL did not aggregate in this system [166]. In the case of LDL, however, oxidation has been reported to lead to particle aggregation [242]. To test whether the oxidative crosslinking of HDL_3 apolipoproteins by activated neutrophils resulted in the crosslinking or aggregation of HDL_3 particles, we assessed particle size by non-denaturing gradient gel electrophoresis (Figure 3.16). An increase in particle size would suggest that the crosslinking of HDL_3 apolipoproteins was interparticle rather than intraparticle.

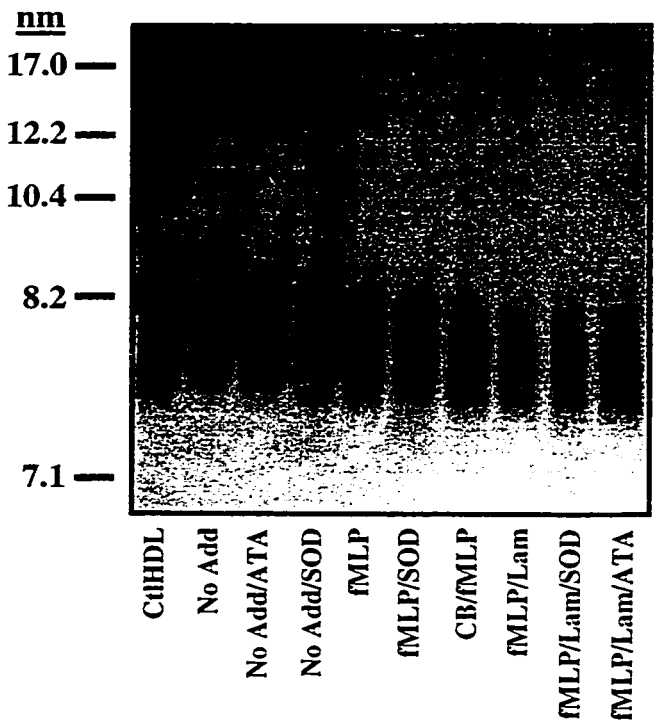


Figure 3.16 Non-denaturing gradient gel electrophoresis analysis of particle size of HDL_3 oxidized by activated neutrophils. Neutrophil oxidized HDL_3 apolipoproteins were separated by a 4-20 % gradient gel and the apolipoproteins visualized by Coomassie stain. (see Methods). CtlHDL, 1 mg/mL HDL_3 incubated in buffer F at 37°C overnight; No Add, 0.5 mg/mL HDL_3 incubated non-activated neutrophils (0.5 x 10^6 cells/mL) in buffer H at 37°C for 3 hr; ATA, aminotriazole (an MPO inhibitor, 20 mM); SOD, superoxide dismutase (5 µg/mL); fMLP, HDL_3 incubated with fMLP (1 x 10^{-7} M)-activated neutrophils; CB/fMLP, cytochalasin B (2.5 µg/mL)/fMLP (1 x 10^{-7} M)-activated neutrophils; fMLP/Lam, HDL_3 incubated with fMLP (1 x 10^{-7} M)-activated neutrophils plated on laminin (Lam)-coated wells. nm, Stokes diameter.

Similar to HDL_3 oxidized in the cell-free system [166], we found no change in neutrophil-modified-HDL_3 particle size compared to non-oxidized HDL, suggesting that the crosslinking was intraparticle, *i.e.*, occurring between apolipoproteins on single HDL_3 particles.

3.3.2.5 Lack of Significant Lipid Peroxidation in HDL_3 Oxidized by Activated Neutrophils

Many oxidants used in *in vitro* models of lipoprotein oxidation induce significant amounts of lipid peroxidation. The resultant loss of intact phospholipids is felt to be in part responsible for the decreased ability of oxHDL to passively accept cholesterol from the cell surface [60, 149]. We have previously reported that oxidation of HDL_3 by peroxidase-generated tyrosyl radical *in vitro* results in only low levels of lipid peroxidation [167]. To assess the relative levels of lipid versus protein oxidation products in neutrophil-modified HDL_3, we first determined whether any changes in particle charge were present in the cell-modified particles, as assessed by relative electrophoretic mobility (REM) of HDL_3 on agarose gels. An increase in negative charge suggests that positively charged amino acid (*e.g.*, lysine) residues have been blocked due to generation of advanced lipid peroxidation products [243]. Figure 3.17 shows that there was no change in REM of neutrophil-oxidized HDL_3 compared to HDL_3 incubated in the absence of neutrophils, suggesting that formation of lipid peroxidation products was minimal. In contrast, copper oxidation, which is known to oxidize HDL through the generation of lipid peroxidation products [60], showed an increase in REM. This result suggests that oxidation of HDL_3 by activated

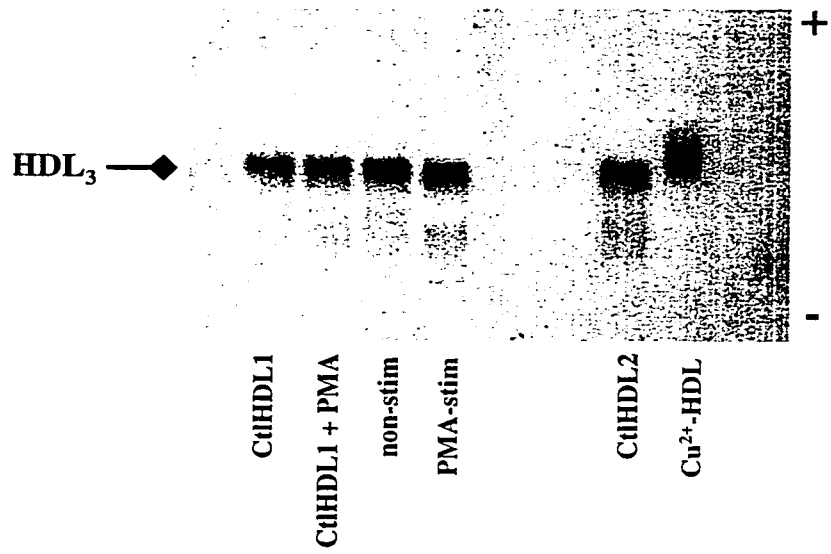


Figure 3.17 Agarose gel analysis of particle charge of HDL_3 oxidized by activated neutrophils. Agarose gel electrophoresis was performed as described in Methods. HDL_3 (0.5 mg/mL) was incubated in buffer H at 37°C for 1 hr alone (CtlHDL1); plus 200 nM PMA (CtlHDL1 + PMA); plus non-activated neutrophils (1 x 10^6 cells/mL) (non-stim); or plus 200 nM PMA-activated neutrophils (PMA-stim); HDL_3 (0.5 mg/mL) incubated in buffer C at 37°C for 1 hr (CtlHDL2); and copper oxidized HDL, HDL_3 incubated with buffer E for 24 hr (Cu^{2+}-HDL).

neutrophils preferentially oxidizes protein rather than lipid components of the HDL particles.

A second measure we used to determine the extent of lipid peroxidation in HDL_3 oxidized by activated neutrophils was conjugated dienes, a marker of early lipid peroxidation. Oxidation of HDL_3 by activated neutrophils under all conditions studied resulted in no significant increase in conjugated dienes, when compared to HDL_3 incubated in buffer alone (Figure 3.18). This further supports the hypothesis that HDL_3 oxidized by activated neutrophils results in the preferential oxidation of HDL_3 apolipoproteins rather than lipids.

3.3.2.6 Ability of HDL_3 Oxidized by CB/fMLP-activated Neutrophils to Deplete the ACAT Substrate Pool.

The decrease in the ability of HDL_3 oxidized by PMA-activated neutrophils to deplete ACAT-accessible cholesterol was found to be due to degradation of HDL apoproteins (Figs. 3.7 and 3.9). Protein degradation was not present in HDL_3 oxidized by CB/fMLP-activated neutrophils for up to 1 hr (Figs. 3.10 and 3.11). The presence of apo AI-AII heterodimers – the active component of *in vitro* TyrHDL [188] – in the absence of apolipoprotein degradation in the CB/fMLP-activated system, provided us with a model to investigate the effects of cell-mediated oxidation on HDL_3 without the confounding effect of proteolysis.

As seen in HDL_3 modified by PMA-activated neutrophils in the presence of protease inhibitors, HDL_3 modified by CB/fMLP-activated neutrophils showed an increased ability to deplete ACAT-accessible cholesterol compared to control HDL (Figure 3.19). Initially, we were interested in the effects of HDL_3 oxidized by

Figure 3.18 Lack of conjugated diene formation in HDL_3 oxidized by CB/fMLP-activated neutrophils. Conjugated diene formation was measured as described in Methods. CB/fMLP, CB (6.25 μg/mL)/fMLP (1 x 10^{-5} M)-activated neutrophils (1 x 10^6 cells/mL); SOD, superoxide dismutase (10 μg/mL); catalase (10 μg/mL); azide (3.5 mM); non-stim, non-activated neutrophils; CtlHDL, 1 mg/mL HDL_3 incubated in buffer F at 37°C overnight; TyrHDL, 1 mg/mL HDL_3 incubated with 100 nM HRP, 100 μM L-tyrosine, and 100 μM H_2O_2 in buffer F at 37°C overnight; and Cu^{2+}-HDL, HDL_3 incubated with buffer E for 24 hr. Values are means ± SD of three independent experiments, except for the plus azide conditions, without (4.78 and 5.41) or with L-tyrosine (4.26 and 4.04), which are an average of two independent experiments. At 24 hr the peak in conjugated dienes in Cu^{2+}-HDL is already declining with progression to more advanced lipid peroxidation products underway (data not shown). This is not seen in TyrHDL (Francis *et al.* unpublished observations).

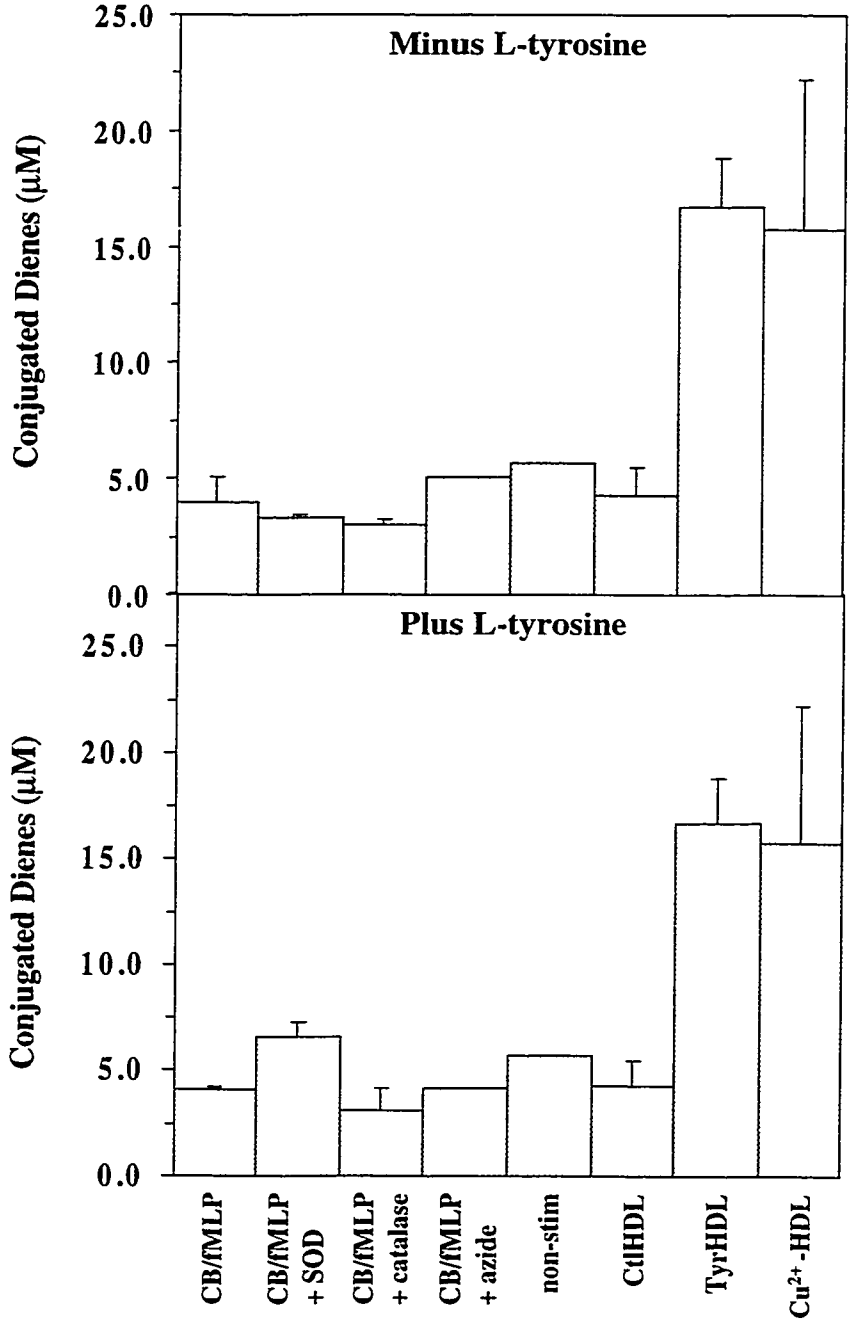
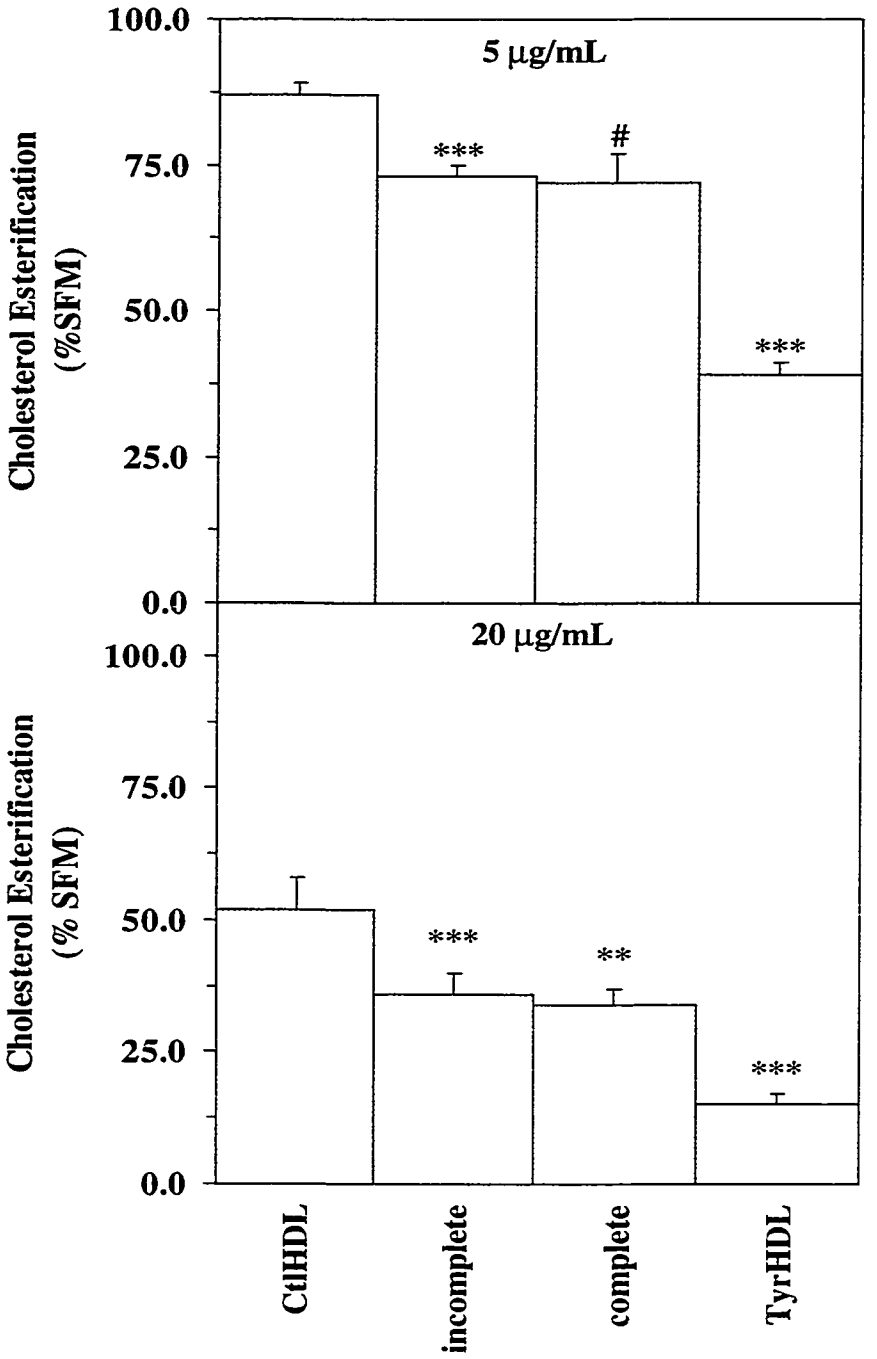

Minus L-tyrosine
Plus L-tyrosine
Conjugated Dienes (μM)
25.0
20.0
15.0
10.0
5.0
0.0
CB/fMLP
CB/fMLP + SOD
CB/fMLP + catalase
CB/fMLP + azide
non-stim
CtlHDL
TyrHDL
Cu2+ -HDL

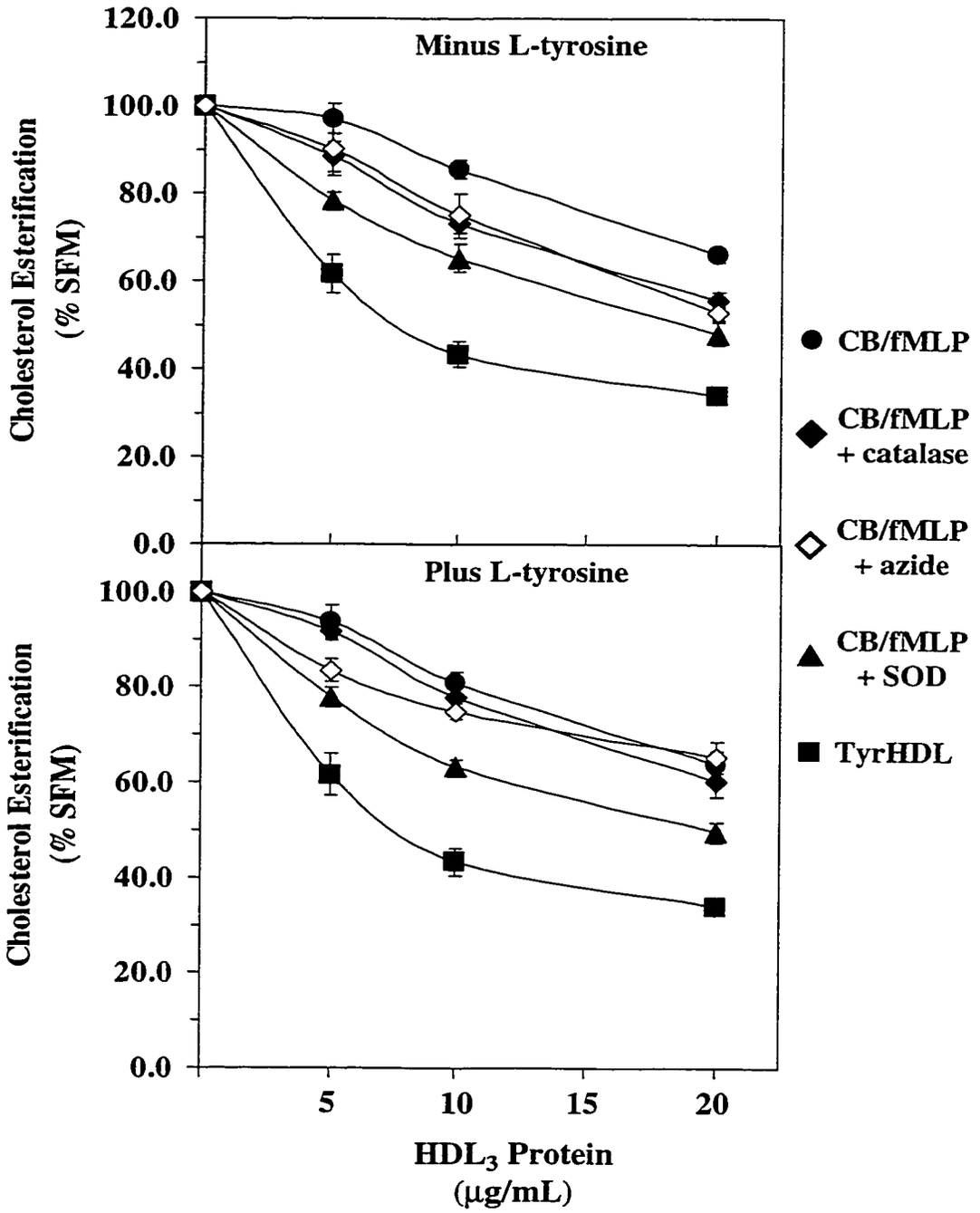
Figure 3.19 Effect of the presence of L-tyrosine on HDL_3 oxidized by CB/fMLP-activated neutrophils to mobilize cholesterol for esterification in cholesterol-loaded fibroblasts. Human skin fibroblasts loaded with non-lipoprotein cholesterol were incubated for 16 hr with serum free media (SFM) plus: CtlHDL, 1 mg/mL HDL_3 incubated in buffer F at 37°C overnight; incomplete, 0.5 mg/mL HDL_3 incubated with CB (6.25 μg/mL)/fMLP (1×10^{-5} M)-activated neutrophils (1×10^6 cells/mL) in buffer H at 37°C for 1 hr; complete, incomplete plus 2 mM L-tyrosine; or TyrHDL, 1 mg/mL HDL_3 incubated with 100 nM HRP, 100 μM L-tyrosine, and 100 μM H_2O_2 in buffer F at 37°C overnight. Cells were then washed and incubated for 1 hr with [^{14}C]oleate, and cellular cholesteryl [^{14}C]oleate formed was measured as described under Methods. Results are mean ± SD of three determinations from two independent experiments, expressed as percentage of picomoles of [^{14}C]oleate incorporated into cholesteryl esters/mg cell protein/hr in cells treated with SFM alone. #, $p<0.05$ vs CtlHDL, *, $p<0.02$ vs CtlHDL, and ***, $p<0.001$ vs CtlHDL as determined by Student's t-test.


5 µg/mL
20 µg/mL
Cholesterol Esterification (%SFM)
Cholesterol Esterification (% SFM)
100.0
75.0
50.0
25.0
0.0
***
#
**
CtlHDL
incomplete
complete
TyrHDL

neutrophil-generated tyrosyl radical, since we know tyrosyl radical is involved in the oxidation of TyrHDL, which has an increased ability to promote mobilization of cellular cholesterol. Interestingly, we found that HDL_3 oxidized by CB/fMLP-activated neutrophils, in the presence or absence of added L-tyrosine, had an enhanced ability to deplete the pool of cholesterol available for esterification by ACAT (Figure 3.19). This result was unexpected, since we hypothesized that tyrosyl radical would be necessary for the oxidation of HDL_3 to a form that promotes cellular cholesterol efflux. These results suggest that other oxidative mechanisms of CB/fMLP-activated neutrophils, besides free tyrosyl radical generation, may oxidize HDL_3 to a form that depletes the ACAT substrate pool.

Regardless of an absolute requirement for free tyrosyl radical in the oxidized HDL generated, we assessed whether an active MPO/H_2O_2 system was necessary for this oxidative modification. Similar to the PMA-activated neutrophil experiments, we used SOD and catalase to modulate H_2O_2 availability. Consistent with the role of the MPO/H_2O_2 system in the oxidative mechanism(s) of activated neutrophils, the addition of SOD led to a further increase in the ability of CB/fMLP-activated neutrophil-oxidized HDL_3 to deplete the ACAT cholesterol pool compared to HDL_3 incubated with activated neutrophils alone (Figure 3.20). This result was the same in the presence or absence of added L-tyrosine. Interestingly, the addition of catalase to the reaction conditions resulted in an HDL_3 particle, which showed an increase (Figure 3.20, minus L-tyrosine) or no difference (Figure 3.20, plus L-tyrosine) compared to the CB/fMLP system alone, to deplete the ACAT cholesterol pool. The same was true for HDL_3 oxidized by CB/fMLP-activated neutrophils in the presence

Figure 3.20 Depletion of ACAT-accessible cholesterol by HDL_3 oxidized by CB/fMLP-activated neutrophils: Effects of SOD, catalase and azide. Human skin fibroblasts loaded with non-lipoprotein cholesterol were incubated for 16 hr with serum free media (SFM) plus the indicated concentration of HDL (0.5 mg/mL) oxidized by CB (6.25 μg/mL)/fMLP (1×10^{-5} M)-activated neutrophils (1×10^6 cells/mL) in buffer H. Cells were then washed and incubated for 1 hr with [^{14}C]oleate, and cellular cholesteryl [^{14}C]oleate formed was measured as described under Methods. Results are mean ± SE of at least two independent experiments containing three or more replicates for each condition, expressed as percentage of picomoles of [^{14}C]oleate incorporated into cholesteryl esters/mg cell protein/hr in cells treated with SFM alone. Error bars not shown are within the symbol dimensions. All conditions depleted ACAT-accessible cholesterol more effectively than HDL incubated with activated neutrophils alone, excluding the minus L-tyrosine plus azide condition (5 and 10 μg/mL), and the plus L-tyrosine plus catalase or plus azide conditions. SOD, superoxide dismutase (10 μg/mL); catalase (10 μg/mL); azide (3.5 mM); and TyrHDL, 1 mg/mL HDL_3 incubated with 100 nM HRP, 100 μM L-tyrosine, and 100 μM H_2O_2 in buffer F at 37°C overnight. TyrHDL was more effective at depleting the ACAT-accessible cholesterol pool than HDL incubated with activated neutrophils alone in the absence or presence of L-tyrosine ($p<0.05$) as determined by single factor ANOVA.


Minus L-tyrosine
Plus L-tyrosine
Cholesterol Esterification (% SFM)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
5
10
15
20
HDL3 Protein (µg/mL)
CB/fMLP
CB/fMLP + catalase
CB/fMLP + azide
CB/fMLP + SOD
TyrHDL

of the MPO inhibitor azide (Figure 3.20, CB/fMLP + azide). Although we would have expected HDL_3 oxidized by CB/fMLP-activated neutrophils in the presence catalase and azide to be less active then HDL_3 oxidized with activated neutrophils alone, the results were anticipated since the extent of apolipoprotein crosslinking was similar under these three conditions. These results suggest that other, MPO-independent, oxidative mechanisms may also be involved with the oxidation of HDL_3 by CB/fMLP-activated neutrophils. An increase in H_2O_2 availability (thereby increasing MPO activity), however, does result in increased oxidation of HDL_3 to a form that is better at promoting cellular cholesterol efflux compared to HDL_3 oxidized by CB/fMLP-activated neutrophils alone. This may be due to the increased crosslinking of apo AII in the apo AI-AII complexes. Another possibility is that low levels of baseline crosslinking in this control HDL preparation were sufficient to increase the activity to a level unaffected by azide or catalase activities (see discussion).

3.3.3 Oxidation of HDL_3 by CB/fMLP-activated Chronic Granulomatous Disease (CGD) Neutrophils

CGD is an x-linked and recessive hereditary disorder in which NADPH-oxidase is defective, resulting in lack of O_2^- generation in response to neutrophil activators (Figure 3.21) [244]. Since MPO uses H_2O_2 produced by the spontaneous or catalytic dismutation of O_2^-, the MPO/H_2O_2 generation of oxidizing radicals is also defective. Thus, we used CGD neutrophils to further investigate the MPO/H_2O_2 system in the oxidation of HDL_3 by CB/fMLP-activated neutrophils.

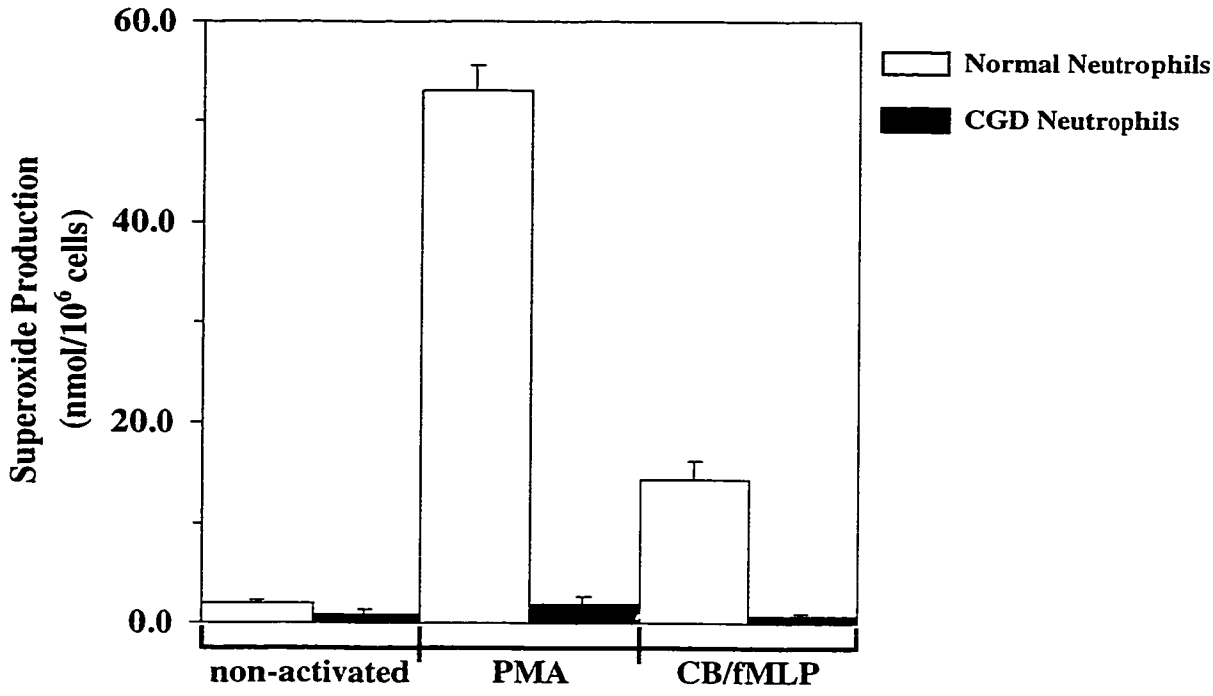
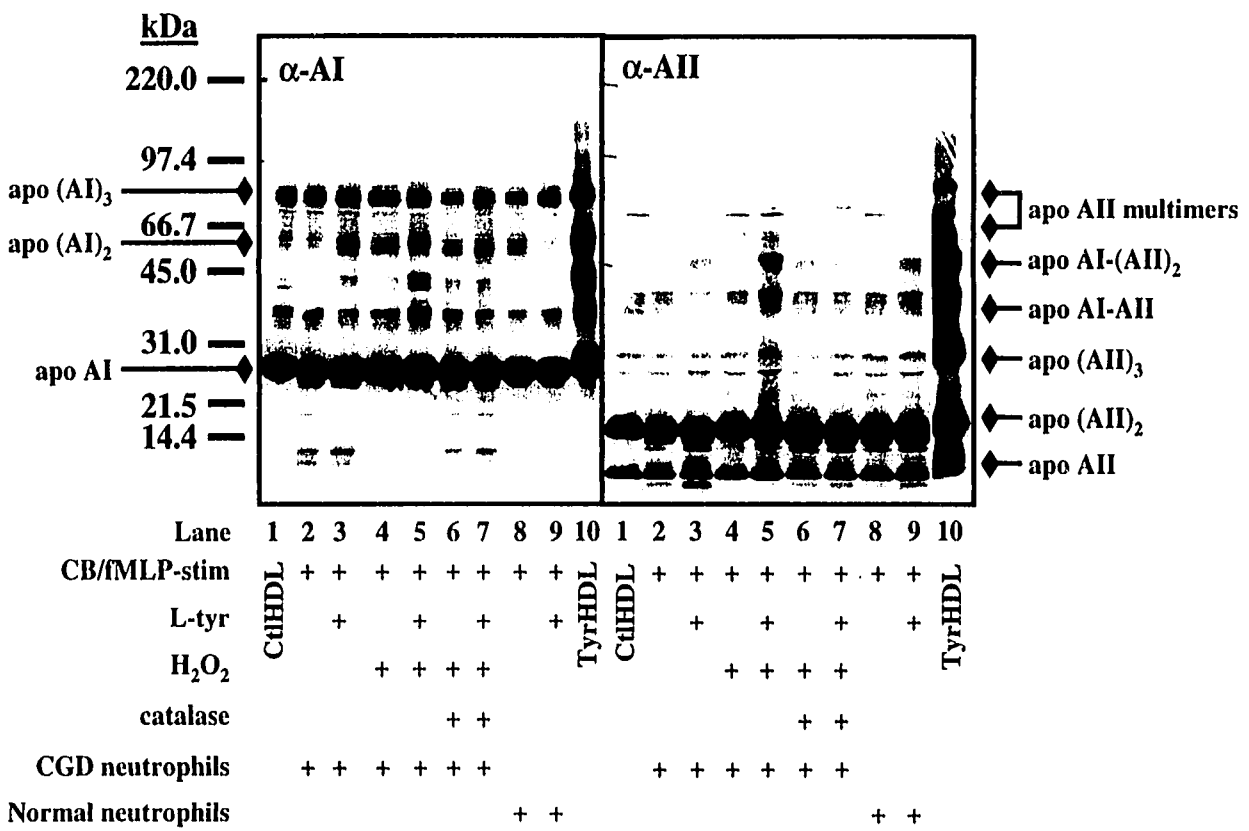


Figure 3.21 Superoxide generation by CGD neutrophils. Normal neutrophils; and CGD neutrophils; were incubated for 20 min at 37°C in buffer C containing 90 μM cytochrome C (see Methods) and activated with either PMA (200 nM) or CB (6.25 μg/mL)/fMLP (1 x 10^{-5} M). Values are means ± SE of four observations from at least two independent experiments.

Non-reducing SDS-PAGE and Western blotting were used to assess the extent of apolipoprotein crosslinking in HDL_3 oxidized by activated CGD neutrophils. Interestingly, oxidation of HDL_3 by activated CGD neutrophils resulted in crosslinking of HDL_3 apolipoproteins in the absence or presence of added L-tyrosine (Figure 3.22, lanes 2 and 3). This was most likely due to the presence of apolipoprotein crosslinks in the original HDL sample (Figure 3.22, lane 1). However, when we reconstituted the system by the addition of H_2O_2, we found increased oxidative crosslinking of HDL_3 apolipoproteins, suggesting an increase in MPO-dependent oxidative mechanisms results in increased crosslinking of HDL_3 apolipoproteins (Figure 3.22, lanes 4 and 5). Further support for MPO-dependent oxidative mechanisms in the crosslinking of HDL_3 apolipoproteins, was shown by a decrease in apolipoprotein crosslinking by the addition of catalase to the reconstituted system to scavenge H_2O_2 (Figure 3.22, lanes 6 and 7). These results further support our findings that an increase in MPO activity results in increased oxidative crosslinking of HDL_3 apolipoproteins. It should also be noted that HDL_3 oxidized by activated CGD neutrophils exhibited degradation of its apolipoproteins (Figure 3.22, lanes 2 and 3), which was prevented by the addition of H_2O_2 (Figure 3.22, lanes 4 and 5). This suggests that an active MPO/H_2O_2 system also protects HDL_3 apolipoproteins from neutrophil-dependent proteolysis, possibly by oxidative damage to proteases.

We also assessed the requirement for superoxide generation in the alteration of cholesterol mobilization by HDL_3 incubated with CGD neutrophils. HDL_3 incubated with CGD neutrophils showed a decreased ability to promote cellular

Figure 3.22 Western blot analysis of HDL_3 oxidized by CB/fMLP-activated CGD neutrophils. Apolipoproteins of HDL_3 (0.5 mg/mL) oxidized by CB (6.25 μg/mL)/fMLP (1×10^{-5} M)-activated CGD neutrophils (1×10^6 cells/mL) in buffer H at 37°C for 1 hr, were separated by 7-20 % non-reducing SDS-PAGE, the apolipoproteins transferred to nitrocellulose, and the nitrocellulose blotted with an antibody to apolipoprotein AI (α-AI) or apolipoprotein AII (α-AII) (see Methods). L-tyr, L-tyrosine (2 mM); H_2O_2, hydrogen peroxide (100 μM); catalase (10 μg/mL); CtlHDL, 1 mg/mL HDL_3 incubated in buffer F at 37°C overnight; and TyrHDL, 1 mg/mL HDL_3 incubated with 100 nM HRP, 100 μM L-tyrosine, and 100 μM H_2O_2 in buffer F at 37°C overnight. CGD, chronic granulomatous disease; and apo, apolipoprotein.

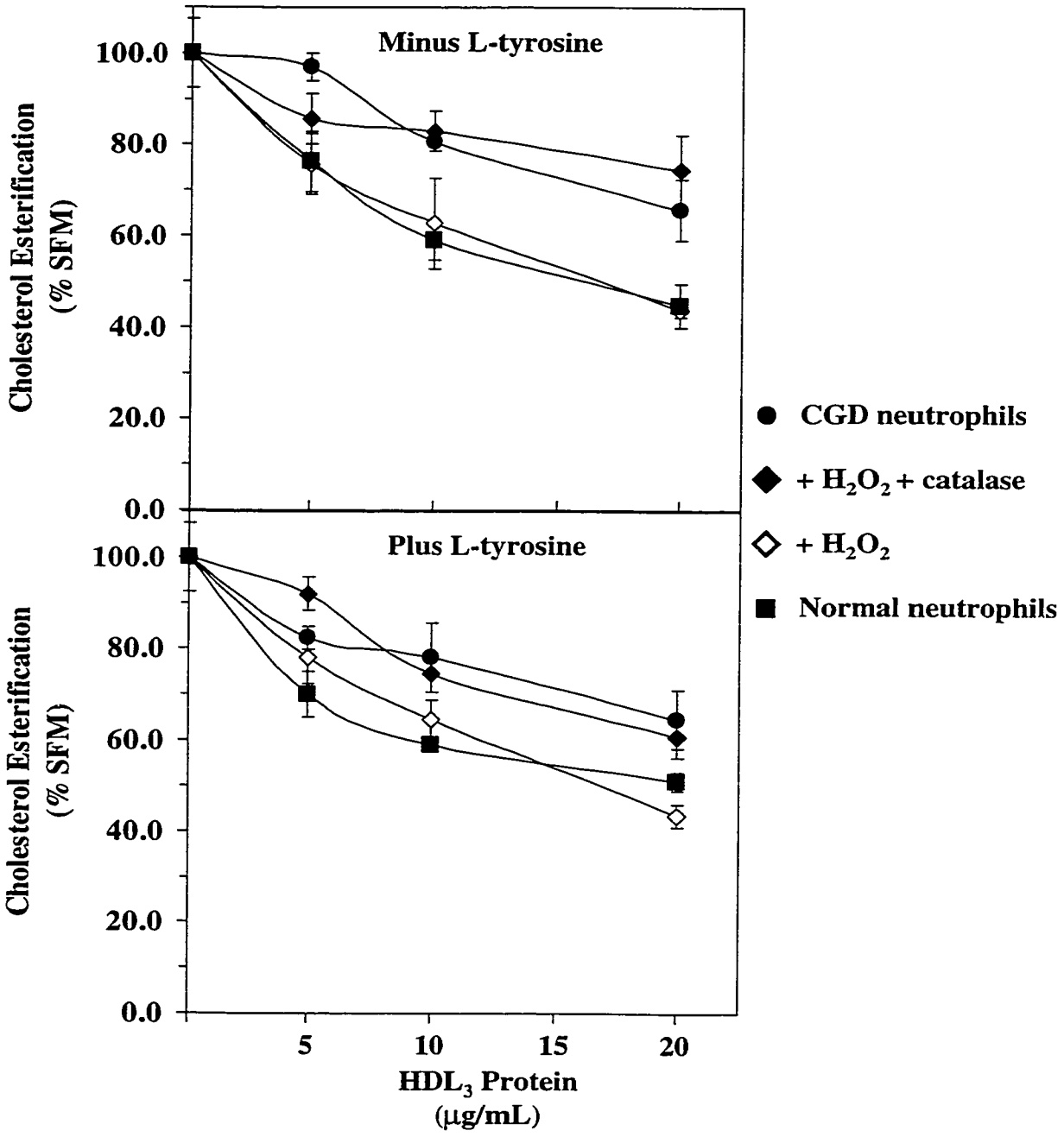

kDa
220.0
97.4
66.7
45.0
31.0
21.5
14.4
apo (AI)3
apo (AI)2
apo AI
α-AI
α-AII
apo AII multimers
apo AI-(AII)2
apo AI-AII
apo (AII)3
apo (AII)2
apo AII
Lane 1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10
CB/fMLP-stim
L-tyr
H2O2
catalase
CGD neutrophils
Normal neutrophils
CtlHDL
TyrHDL

cholesterol efflux compared to HDL_3 incubated with activated normal neutrophils (Figure 3.23). This is probably related to the degradation of apo AI in HDL_3 by activated CGD neutrophils (Figure 3.22, lanes 2 and 3). In contrast, reconstitution of the CGD system with H_2O_2 resulted in HDL_3 particles that had a similar ability to promote cellular cholesterol efflux compared to normal neutrophil-modified HDL. Moreover, the addition of catalase to the reconstituted system inhibited this beneficial modification of HDL_3 by activated CGD neutrophils, again probably due to the degradation of HDL_3 apo AI. These results further implicate the involvement of the MPO/H_2O_2 system in the oxidation of HDL_3 by activated neutrophils to a form that promotes cellular cholesterol efflux, as well as protecting HDL_3 apolipoproteins from neutrophil-mediated proteolysis.

3.4 Effects of *In Vivo* Oxidation on HDL Structure and Function

Numerous studies have provided evidence for the oxidation of LDL *in vivo* [149]. Interestingly, the pursuit of oxidized HDL *in vivo* has not been nearly as active, and a convincing demonstration of HDL oxidized *in vivo* has not yet been published. Based on our *ex vivo* studies, we know that HDL_3 can be oxidized by activated phagocytes to a form that enhances cellular cholesterol mobilization. We extended our investigations to look at potential *in vivo* oxidation of HDL, and how this would affect its structure and function. The most appropriate site to isolate oxidized HDL from, based on our proposed model that this modification of and enhanced cholesterol mobilization by HDL would occur in the artery wall, would be the inflammatory atherosclerotic lesion itself. Since our lab was not set up to isolate HDL from atherosclerotic lesions, we instead initiated the isolation of HDL from

Figure 3.23 Depletion of ACAT-accessible cholesterol by HDL_3 oxidized by activated CGD neutrophils: Effect of H_2O_2 addition. A, minus L-tyrosine; B, plus L-tyrosine. Human skin fibroblasts loaded with non-lipoprotein cholesterol were incubated for 16 hr with serum free media (SFM) plus the indicated concentration of: 0.5 mg/mL HDL_3 incubated with CB (6.25 μg/mL)/fMLP (1×10^{-5} M)-activated CGD neutrophils (1×10^6 cells/mL) in buffer H (CGD neutrophils); plus 100 μM H_2O_2 (+ H_2O_2); plus H_2O_2 and 10 μg/mL catalase (+ H_2O_2 + catalase); or HDL_3, incubated with CB (6.25 μg/mL)/fMLP (1×10^{-5} M)-activated normal neutrophils (1×10^6 cells/mL) in buffer H (Normal neutrophils). Cells were then washed and incubated for 1 hr with [^{14}C]oleate, and cellular cholesteryl [^{14}C]oleate formed was measured as described under Methods. Results are mean ± SD of three determinations for each condition from one representative experiment, expressed as percentage of picomoles of [^{14}C]oleate incorporated into cholesteryl esters per mg cell protein per hour in cells treated with SFM alone. Error bars not shown are within the symbol dimensions.


Minus L-tyrosine
Plus L-tyrosine
Cholesterol Esterification (% SFM)
100.0
80.0
60.0
40.0
20.0
0.0
5
10
15
20
HDL3 Protein (μg/mL)
CGD neutrophils
+ H2O2 + catalase
+ H2O2
Normal neutrophils

inflamed knee joint synovial fluid as a surrogate inflammatory site. The rationale for using inflammatory knee joints as an alternative source of *in vivo*-oxidized HDL is several fold. First, synovial fluid is known to be a rich source of lipoproteins, particularly HDL, and contains similar concentrations of L-tyrosine compared to plasma [209]. Moreover, neutrophils and monocytes in inflammatory joint fluid are a rich source of MPO [210]. As well, isolation of synovial fluid HDL (SynHDL) can be carried out by standard ultracentrifugation techniques since significant amounts of synovial fluid can be obtained from inflamed knee joints. Our study was also enhanced by the ability to obtain a blood sample, and therefore plasma HDL (PlmHDL), as a control for each patient from which we obtained synovial fluid. These blood samples were taken immediately following knee joint aspiration. Therefore, the ease of isolation, the much larger amount of HDL available, and the ability to obtain this internal PlmHDL control for each patient, compared to HDL isolated from atherosclerotic lesions of deceased individuals, led us to use inflammatory SynHDL in our initial studies of *in vivo*-oxidized HDL.

3.4.1 Synovial Fluid

3.4.1.1 Structural Characterization of Synovial Fluid HDL Isolated From Inflammatory Joints

Isolated SynHDL from inflammatory joint fluid demonstrated crosslinked apolipoproteins on SDS-PAGE under non-reducing conditions (Figure 3.24). The more prominent crosslink bands had apparent molecular masses similar to our *in vitro* TyrHDL and *ex vivo* neutrophil-oxidized HDL, indicating the formation of apo AI-$(AII)_2$ heterodimers, apo AI dimers and apo AI trimers. Interestingly, there was

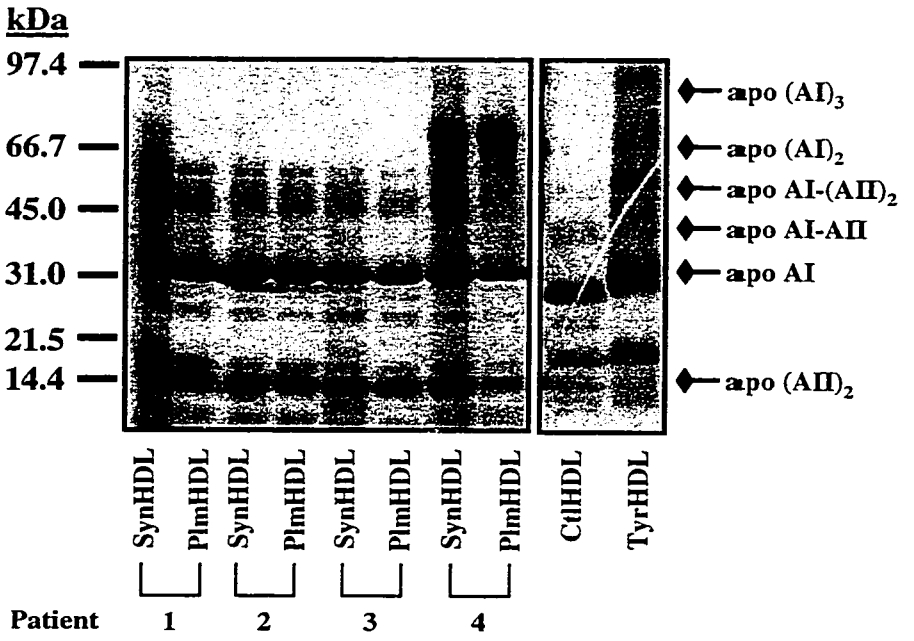


Figure 3.24 Non-reducing SDS-PAGE analysis of crosslinked synovial and plasma HDL apolipoproteins. Apolipoproteins were separated by a 15 % non-reducing SDS-PAGE and visualized by silver stain. SynHDL, synovial fluid HDL; PlmHDL, matched plasma HDL sample for each patient. For comparision CtlHDL, 1 mg/mL HDL_3 incubated in buffer F at 37°C overnight; and TyrHDL, 1 mg/mL HDL_3 incubated with 100 nM HRP, 100 µM L-tyrosine, and 100 µM H_2O_2 in buffer F at 37°C overnight are shown on a 7-20 % SDS-PAGE gel, silver stained.

crosslinking also seen in PlmHDL samples (although generally to a lesser degree than the corresponding SynHDL), suggesting that oxidized HDL may recirculate back into the plasma from sites of inflammation. This is the first demonstration that we know of for *in vivo*-oxidized HDL in synovial fluid or plasma.

Although analysis of lipid peroxidation products was not done directly, particle charge of SynHDL and PlmHDL was determined as an indirect measure of lipid peroxidation. SynHDL and PlmHDL were found to have no change in REM compared to CtlHDL, suggesting no or minimal lipid peroxidation had occurred (Figure 3.25).

3.4.1.2 Functional Characterization of Synovial Fluid HDL Isolated From Inflammatory Joints

Preliminary experiments were done to assess the ability of SynHDL to deplete the ACAT-accessible cholesterol pool of cultured fibroblasts compared to matched PlmHDL controls. As well, our *in vitro* CtlHDL and TyrHDL were incorporated as our benchmark negative and positive controls, respectively. Combined results from four patients showed both patient SynHDL and PlmHDL were better than CtlHDL at promoting the depletion of ACAT-accessible cholesterol (Figure 3.26). Moreover, SynHDL was better than PlmHDL at depleting this pool at higher concentrations. These results suggest that *in vivo*-oxidation of HDL is potentially beneficial, since SynHDL showed an increased ability to mobilize ACAT-accessible cholesterol compared to our pooled control HDL obtained from healthy male volunteers. Moreover, PlmHDL from these patients also had an increased ability to mobilize this cholesterol, and may reflect the recycling of oxidized SynHDL back into plasma. In

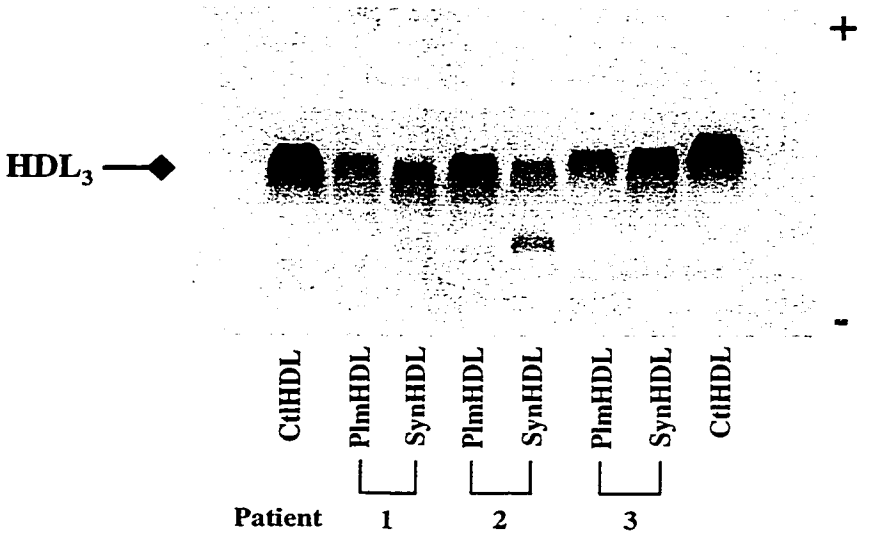


Figure 3.25 Agarose gel analysis of particle charge of synovial and plasma HDL. Agarose gel electrophoresis was performed as described in Methods. CtlHDL, 1 mg/mL HDL_3 incubated in buffer F at 37°C overnight; PlmHDL, patient plasma HDL; and SynHDL, synovial fluid HDL.

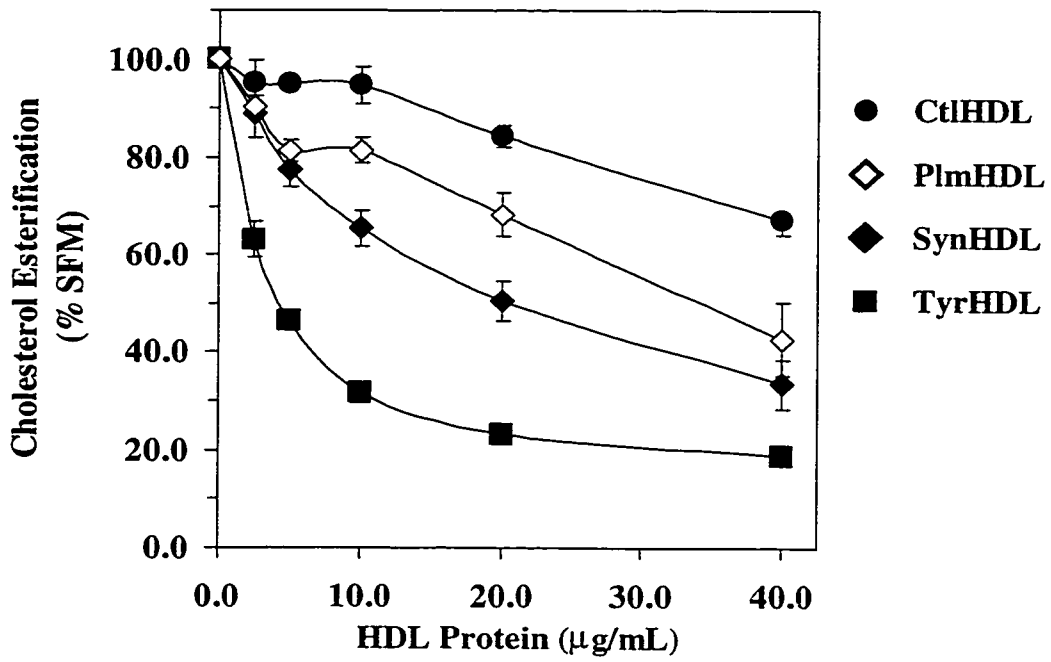


Figure 3.26 Effect of synovial and plasma HDL to mobilize cholesterol for esterification in cholesterol-loaded fibroblasts. Human skin fibroblasts loaded with non-lipoprotein cholesterol were incubated for 16 hr with serum free media (SFM) plus the indicated concentration of: CtlHDL, 1 mg/mL HDL_3 incubated in buffer F at 37°C overnight; PlmHDL, patient plasma HDL; SynHDL, synovial fluid HDL; or $TyrHDL_3$, 1 mg/mL HDL_3 incubated with 100 nM HRP, 100 μM L-tyrosine, and 100 μM H_2O_2 in buffer F at 37°C overnight. Cells were then washed and incubated for 1 hr with [^{14}C]oleate, and cellular cholesteryl [^{14}C]oleate formed was measured as described under Methods. Results are mean ± SE of three determinations from three independent experiments, expressed as percentage of picomoles of [^{14}C]oleate incorporated into cholesteryl esters/mg cell protein/hr in cells treated with SFM alone. Error bars not shown are within the symbol dimensions. SynHDL and TyrHDL depleted ACAT-accessible cholesterol more effectively than CtlHDL (p<0.02) as determined by single factor ANOVA.

no case did our individual synovial fluid HDL samples from patients exhibit a diminished capacity to deplete ACAT-accessible cholesterol compared with the patients' own plasma HDL or our pooled control HDL.

3.4.2 Characterization of HDL Isolated From Human Atherosclerotic Aortas

During the course of these studies, collaboration with Drs. Roland Stocker and Ute Panzenböeck at the Heart Institute in Sydney, Australia also allowed us to examine the structural and functional characteristics of HDL isolated from human atherosclerotic lesions.

3.4.2.1 Identification of Crosslinks in HDL Isolated From Atherosclerotic Aortas

On non-reducing SDS-PAGE, aortic HDL samples presented a variety of high molecular weight (HMW) and low molecular weight (LMW) proteins. To delineate intact and potentially degraded HDL apolipoprotein species amongst these proteins, Western blot analyses with polyclonal anti-apo AI and anti-apo AII antibodies were performed (Figure 3.27, A). The anti-apo AI Western blot showed minimal degradation of apo AI, as seen by the absence of small peptide fragments below the native 28 kDa apo AI band. These results suggest effective anti-proteolytic mechanisms within the interstitial fluid of the artery wall to counteract the numerous proteases known to exist in atherosclerotic lesions [3]. Also, there were crosslinks of HDL apolipoproteins, as seen by the presence of HMW immunoreactive bands above the native apo AI band on the immunoblot. The more prominent crosslinked bands had apparent molecular masses identical to those seen in *in vitro* TyrHDL, neutrophil-modified HDL, and synovial fluid HDL (Figure 3.27). The anti-apo AII Western blot

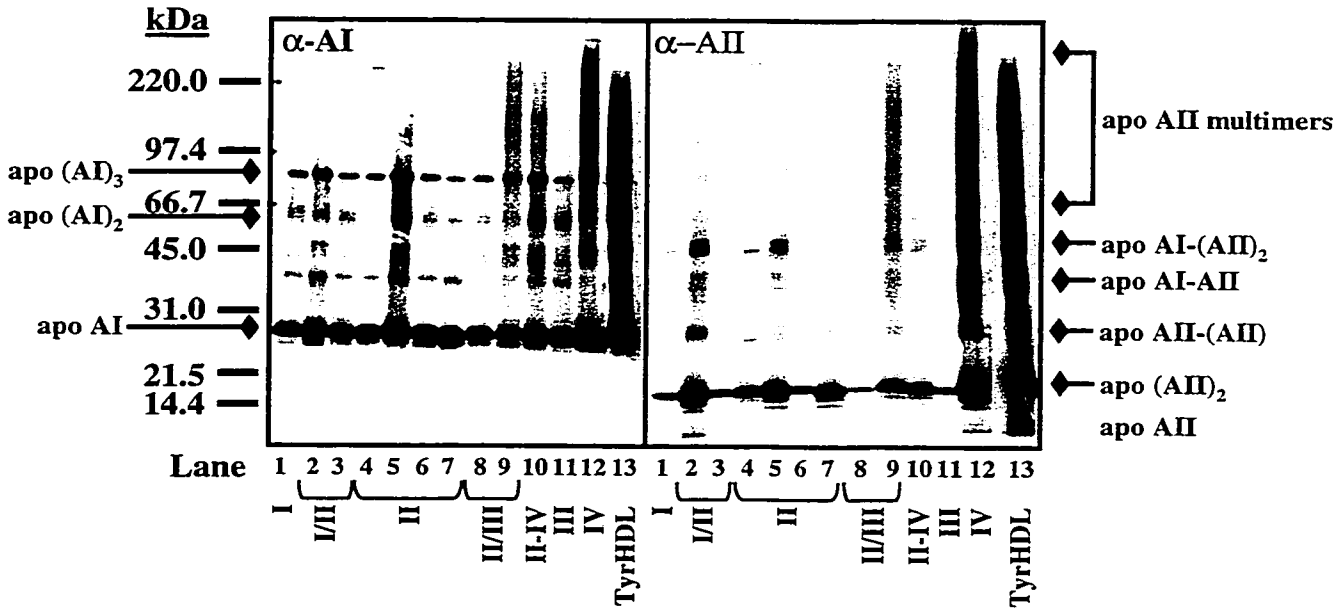


Figure 3.27 Western blot analysis of HDL isolated from atherosclerotic aortas. Apolipoproteins of HDL isolated from atherosclerotic aortas were separated by 7-20 % non-reducing SDS-PAGE, transferred to nitrocellulose, and blotted with an antibody to apolipoprotein AI (α-AI) or apolipoprotein AII (α-AII) (see Methods). Roman numerals represent lesion stage (see Table 2.2) and TyrHDL, 1 mg/mL HDL_3 incubated with 100 nM HRP, 100 μM L-tyrosine, and 100 μM H_2O_2 in buffer F at 37°C overnight.

indicated only minimal amounts of apo AII degradation products beneath the native 17 kDa dimeric apo AII band; the 8.5 kDa band most likely represents the native monomeric form of apo AII (Figure 3.27, B). Crosslinks of apo AII were indicated by the presence of immunoreactive bands above the native apo AII band. The more prominent crosslink bands had apparent molecular masses of 25.5 kDa, 36.5 kDa, and 45 kDa. Based on immunoblot analysis and apparent molecular mass, the 25.5 kDa, 36.5 kDa, 45 kDa, 56 kDa and 84 kDa bands seen in the immunoblots were due to the formation of apo AII-$(AII)_2$ complexes, apo AI-AII heterodimers, apo AI-$(AII)_2$ heterodimers, apo AI dimers and apo AI trimers, respectively. These results along with the synovial fluid results support the hypothesis that HDL is oxidized *in vivo* at sites of inflammation. Interestingly, the extent of crosslinking was not related to the lesion stage, although the most extensive crosslinking of HDL apolipoproteins was seen in HDL isolated from a stage IV (late) atherosclerotic lesion (Figure 3.27, A and B lane 12). The reason for the lack of correlation between apolipoprotein crosslinking and lesion stage is unknown, however it may have resulted from differences in plasma HDL levels, residence time of HDL in the artery wall, and/or the composition of the inflammatory site.

3.4.2.2 Ability of HDL Isolated From Atherosclerotic Aortas to Deplete the ACAT Substrate Pool

The ability of aortic HDL from the various stage lesions to deplete ACAT-accessible cholesterol from cultured fibroblasts was determined. We found no difference between HDL isolated from early (stages I and II) or advanced lesions (stages II-III, III, II-IV, and IV) to deplete this cholesterol pool (Figure 3.28). This

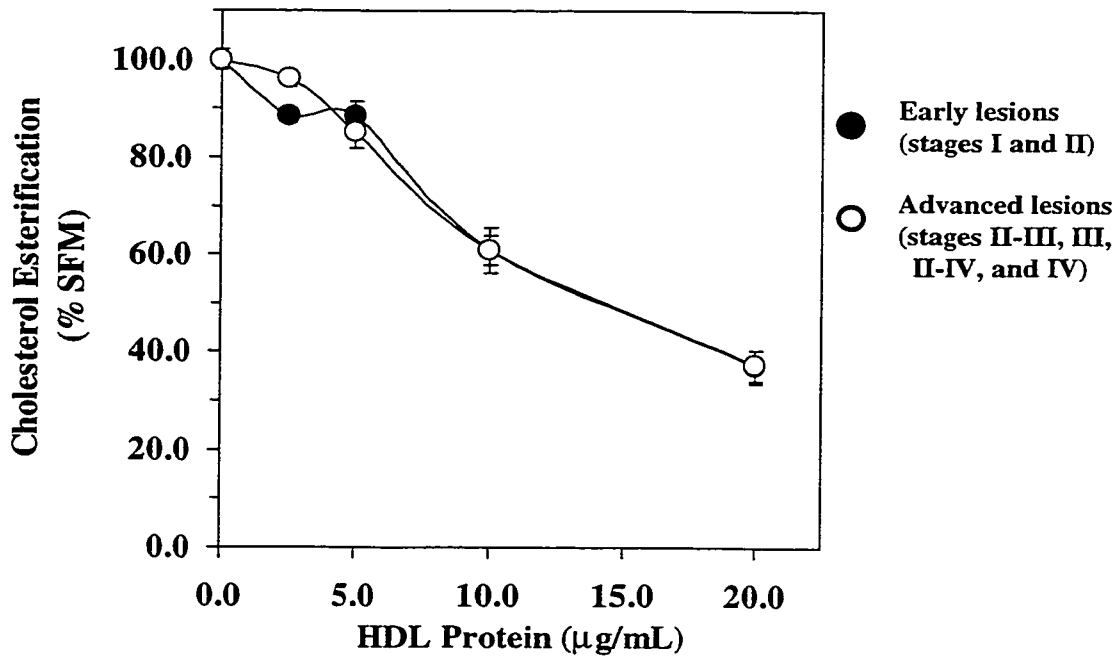


Figure 3.28 Effect of HDL isolated from early and advanced atherosclerotic lesions to mobilize cholesterol for esterification in cholesterol-loaded fibroblasts. Human skin fibroblasts loaded with non-lipoprotein cholesterol were incubated for 16 hr with serum free media (SFM) plus the indicated concentration of: HDL isolated from early lesions (stage I and II) or from advanced lesions (II-III, III, II-IV, and IV). Cells were then washed and incubated for 1 hr with [^{14}C]oleate, and cellular cholesteryl [^{14}C]oleate formed was measured as described under Methods. Results are mean ± SE of four determinations each from 5 (early lesions) and 6 samples (advanced lesions) expressed as percentage of picomoles of [^{14}C]oleate incorporated into cholesteryl esters/mg cell protein/hr in cells treated with SFM alone. Error bars not shown are within the symbol dimensions.

suggests that the oxidative modification of HDL in atherosclerotic lesions, at the very least, does not decrease its ability to deplete the ACAT-accessible cholesterol pool. The apparent lack of significant degradation of HDL apolipoproteins, and the retention of the ability of these particles to promote cholesterol mobilization, is in striking contrast to our results with HDL_3 modified by PMA-activated neutrophils in the absence of protease inhibitors, and again suggests the presence of very effective anti-proteolytic mechanisms in artery wall interstitial fluid that still allow oxidative crosslinking of apolipoproteins to occur.

Chapter 4

Discussion and Future Directions

4.1 Discussion

The *in vitro* susceptibility of HDL to oxidation (reviewed in [60]) and its ability to infiltrate the artery wall [79, 245, 246] suggests HDL would be prone to similar oxidative mechanisms thought to modify LDL in the interstitial space. These oxidative modifications may markedly impact the ability of HDL to mobilize cellular cholesterol, as well as its other roles in RCT. Previous studies investigating the oxidation of HDL have shown this decreases its ability to induce passive efflux of cholesterol from cells [154-165]. However, these studies did not investigate the effects of oxidation on the ability of HDL to mobilize intracellular cholesterol. Our laboratory has shown that HDL oxidized by peroxidase-generated tyrosyl radical *in vitro* has a markedly enhanced ability to mobilize intracellular cholesterol, independent of cholesterol efflux by passive diffusion [166, 167]. The active component of TyrHDL was subsequently identified to be the crosslinked apo AI-apo AII heterodimers generated following tyrosyl radical oxidation [188].

In the current studies we investigated the effects of neutrophil oxidation on HDL structure and function. We also isolated HDL from inflammatory sites of the human body and investigated the effects of *in vivo*-oxidation on HDL structure and function. The results from the studies presented here suggest that: 1) *ex vivo* oxidative modification of HDL by activated neutrophils results in an HDL particle that has an enhanced ability to mobilize intracellular cholesterol; 2) HDL isolated from inflammatory sites of the human body are modified *in vivo* to a form that has at least a similar (or better) ability to mobilize intracellular cholesterol; and 3) the

increased activity of *ex vivo*- and *in vivo*-oxidized HDL was related to the presence of apolipoprotein crosslinks.

Consistent with the formation of apolipoprotein crosslinks in HDL oxidized *in vitro* by peroxidase-generated tyrosyl radical [188], we found that oxidation of HDL by activated neutrophils resulted in the crosslinking of apo AI and apo AII to each other and themselves. The increase in HDL-associated dityrosine fluorescence following its oxidation by activated neutrophils (in the presence of added L-tyrosine) suggested the participation of tyrosyl radical in the crosslink formation. This was further supported by the increase in HDL apolipoprotein crosslinking along with increased dityrosine fluorescence when SOD was added to the system. Conversely, HDL-associated dityrosine fluorescence decreased along with HDL apolipoprotein crosslinking when catalase was added. The increased HDL-associated dityrosine fluorescence may have resulted from the crosslinking of free tyrosyl radical with a protein tyrosyl radical and/or from the crosslinking of two protein tyrosyl radicals [166, 247]. Interestingly, the crosslinking of HDL apolipoproteins also occurred in the absence of added L-tyrosine, suggesting the involvement of other oxidative mechanisms in the crosslinking of HDL apolipoproteins. It is unlikely that MPO oxidizes tyrosine of HDL apolipoproteins or other amino acid residues directly, since there is considerable steric hindrance in its active site, making it inaccessible to large macromolecules [201]. Low-molecular-weight intermediates are therefore thought to be required to convey oxidizing equivalents from the MPO heme group to targets. Since the oxidation of HDL by activated neutrophils was carried out in a chlorine-containing buffer, HOCl generated from chloride ion could act as another low-

molecular-weight intermediate in addition to tyrosyl radical generated from free L-tyrosine [178]. HOCl has also been shown to induce crosslinking of HDL apolipoproteins *in vitro* [161], and this may account for the crosslinking of apolipoproteins in the absence of added L-tyrosine in our activated neutrophil system. As well, neutrophils have a pool of free intracellular L-tyrosine [248], which may be a source for free tyrosyl radical generation even in the absence of added L-tyrosine. NO can also be released from activated neutrophils [193, 249, 250], and peroxynitrite and other reactive nitrogen species generated from NO have also been shown to crosslink proteins [200, 251]. Although the crosslinking of HDL apolipoproteins in the absence of added L-tyrosine did not result in a measurable increase in HDL-associated dityrosine fluorescence under the conditions of our assay, we cannot rule out the possible formation of protein dityrosine crosslinks since protein tyrosyl radicals could have been generated by these other oxidants [197-199].

Although there was not a requirement for added free L-tyrosine for the crosslinking of HDL apolipoproteins by activated neutrophils, several lines of evidence suggest the involvement of the MPO/H_2O_2 system. First, the extent of HDL-associated dityrosine fluorescence, when exogenous L-tyrosine was present, was modulated by the availability of H_2O_2. This change in HDL-associated fluorescence was related to the extent of apolipoprotein crosslinking. Second, even in the absence of exogenously added L-tyrosine, addition of SOD increased crosslinking of apo AII in the apo AI-apoAII heterodimer complexes. In contrast, addition of catalase to scavenge H_2O_2, or azide to directly inhibit MPO, decreased the crosslinking of HDL apolipoproteins. This suggests MPO oxidizes other low-

molecular-weight intermediates, which then oxidize HDL apolipoproteins resulting in their crosslinking. Finally, although HDL incubated with activated CGD neutrophils exhibited crosslinks, the addition of H_2O_2 increased apolipoprotein crosslink formation, and the addition of catalase to the reconstituted system inhibited this increase in crosslink formation. These results indicate that the presence of H_2O_2, and therefore increased MPO activity, leads to increased oxidation of HDL apolipoproteins. The presence of crosslinks in HDL incubated with CGD neutrophils in the absence of H_2O_2 was unexpected, and was most likely due to the initial presence of apolipoprotein crosslinks in the original HDL sample before incubation with CGD neutrophils. Further experiments with CGD neutrophils will be required, using a control HDL preparation lacking any evidence of crosslinking, to determine whether these cells catalyze the oxidation of HDL by superoxide-independent mechanisms. Despite some anomalies, taken together, these data indicate that the neutrophil MPO/H_2O_2 system is involved with the oxidative crosslinking of HDL apolipoproteins.

As with *in vitro*-generated TyrHDL [166], the crosslinking of HDL apolipoproteins by activated neutrophils was found to be intraparticle rather than between particles, as no change in particle size or evidence of aggregation was found on non-denaturing gradient gels. As well, we found no evidence of elevated lipid peroxidation products in HDL oxidized by activated neutrophils. These results are consistent with our previous studies of HDL oxidized *in vitro* by tyrosyl radical, and with other reports investigating the effects of polymorphonuclear cells (PMNs) on HDL structure [240, 252]. Even after a 24 hr incubation of HDL with activated

PMNs, in the absence of free metal ions, there was no detectable increase in advanced lipid peroxidation products [252]. This suggests that the battery of oxidants generated by activated neutrophils results in the preferential oxidation of the protein component of HDL particles.

Incubation of HDL with PMA-activated neutrophils (and CB/fMLP-activated neutrophils at longer time points) resulted in the degradation of apo AI. It has been shown that oxidation of proteins can result in protein fragmentation (reviewed in [253]). It is unlikely, however, that the degradation of apo AI by activated neutrophils was due to oxidative fragmentation, since it was prevented by the addition of potent protease inhibitors. More specifically, the elastase inhibitor N-methoxysuccinyl-Ala-Ala-Pro-Val, prevented the degradation of apo AI by activated neutrophils, which is consistent with previous reports demonstrating the involvement of elastase in the degradation of apolipoproteins by these cells [240, 241]. As well, HDL incubated with activated CGD neutrophils, which do not generate O_2^-, resulted in the degradation of apo AI. Interestingly, addition of H_2O_2 in the CGD cell incubations prevented apo AI degradation, suggesting that an MPO/H_2O_2 system with "normal" activity may inactivate secreted proteases of stimulated neutrophils [254-257]. The finding that apo AI degradation was present when catalase was added to the reconstituted CGD system further supports this hypothesis.

Oxidation of HDL by activated neutrophils resulted in HDL particles that had a markedly increased ability to deplete ACAT-accessible cholesterol. Although studies were not directly done to identify the active component of neutrophil-oxidized HDL, several lines of evidence suggest the apo AI-apo AII heterodimer crosslinks are

also the active component in HDL oxidized by activated neutrophils. First, the addition of SOD resulted in neutrophil-oxidized HDL particles that had an increased ability to deplete ACAT-accessible cholesterol compared to HDL particles oxidized by activated neutrophils alone. Although there was not an increase in apo AI crosslinking there was an increase in the crosslinking of apo AII in the apo AI-apo AII heterodimer complex. Addition of catalase did not decrease the activity of neutrophil-oxidized HDL below that of HDL incubated with activated neutrophils alone, and both of these HDL particles showed similar amounts of apo AI-apo AII crosslinked heterodimers. This raises the possibility that even small amounts of the active apo AI-apo AII heterodimers are sufficient to markedly enhance the mobilization of ACAT-accessible cholesterol. The reconstitution of the CGD neutrophil system with H_2O_2 resulted in increased crosslinking of apo AI to apo AII, and an increased ability of this HDL to deplete the ACAT-accessible cholesterol pool. The increase in activity was similar to that of HDL oxidized by normal neutrophils, supporting the involvement of the MPO/H_2O_2 system in this beneficial oxidation of HDL. Conversely, the addition of catalase to the reconstituted system prevented the increase in apolipoprotein crosslinking caused by the addition of H_2O_2, and these HDL had a similar ability to deplete ACAT-accessible cholesterol as HDL incubated with activated CGD-neutrophils alone.

Although there was crosslinking seen in HDL oxidized by PMA-activated neutrophils and CGD neutrophils, the presence of apolipoprotein degradation most likely accounts for the decreased ability of these particles to deplete the ACAT-accessible cholesterol pool. This is supported by the results of Mendez and Oram,

who showed that degradation of HDL apolipoproteins results in a markedly impaired ability of these particles to deplete ACAT-accessible cholesterol [109]. Taken together, these results suggest the active components of HDL oxidized by activated neutrophils, as with in vitro TyrHDL, are the apo AI-apo AII heterodimers, but that these complexes need to be intact to retain their enhanced activity.

Our finding of oxidized HDL in human inflammatory synovial fluid and atherosclerotic aortas is the first definite demonstration of *in vivo*-oxidized HDL that we know of. HDL isolated from inflammatory joints and atherosclerotic lesions displayed increased crosslinking of HDL apolipoproteins. Interestingly, plasma HDL isolated from patients with inflamed joints also showed apolipoprotein crosslinking, suggesting HDL oxidized at inflammatory sites can recirculate to some extent back into the general circulation. The crosslinks found in oxidized HDL isolated from human inflammatory sites were identical to those found in TyrHDL [188], and HDL oxidized *ex vivo* by activated neutrophils. Lipid peroxidation in SynHDL was found to be minimal, suggesting *in vivo* oxidative mechanisms also target the protein component of the HDL particle preferentially. HDL isolated from atherosclerotic lesions did not display a definite incremental increase in apolipoprotein crosslinking with lesion stage. This may reflect differences in residence time of HDL particles in atherosclerotic lesions of different individuals.

A striking finding in our aortic HDL studies was the absence of significant degrees of apolipoprotein degradation in these samples. Since many types of proteases are known to be present in atherosclerotic lesions [3], extensive apolipoprotein degradation might be expected to occur. Our findings of mainly intact

HDL apoproteins in these samples suggest the presence of very effective anti-proteolytic mechanisms in the artery wall interstitial fluid. These results also suggest that, similar to HDL oxidized by PMA-activated neutrophils in the presence of protease inhibitors, the beneficial cross-linking of HDL apoproteins may occur *in vivo* in the absence of significant protein degradation. It is possible that HDL protein degradation products are formed *in vivo* and removed from the inflammatory site, or that the isolation procedure for aortic HDL stripped the particles of any shorter apoprotein fragments. The *complete* absence of any degradation products of apo AI or apo AII on Western blotting, however, suggests effective protection against proteolysis of these proteins. At least some protein degradation products would have been expected to be seen by this sensitive analysis if they were present.

We found an increase in the ability of crosslinked HDL isolated from in vivo sites to deplete the substrate pool of cholesterol for ACAT. Synovial fluid HDL was found to be more effective at depleting this pool than plasma HDL from the same patients, which also correlated generally with greater crosslinking in synovial than plasma HDL samples. We also found that plasma HDL from patients with inflamed joints was more effective at mobilizing cholesterol than our pooled plasma HDL from healthy volunteers. These results further suggest that the presence of even low levels of apoprotein crosslinks (specifically apo AI-apo AII) leads to more active HDL particles.

When the ability of HDL isolated from early stage atherosclerotic lesions to deplete the ACAT substrate pool was assessed compared to HDL isolated from advanced lesions, no differences were found. This similar ability of HDL isolated

from early and advanced atherosclerotic lesions to deplete the ACAT-accessible cholesterol pool further supports the hypothesis that even small amounts of apolipoprotein crosslinks results in a more active HDL particle. As well, these results suggest that even in advanced stages of atherosclerosis, the oxidation of HDL does not impair its ability to mobilize cholesterol available for esterification by ACAT.

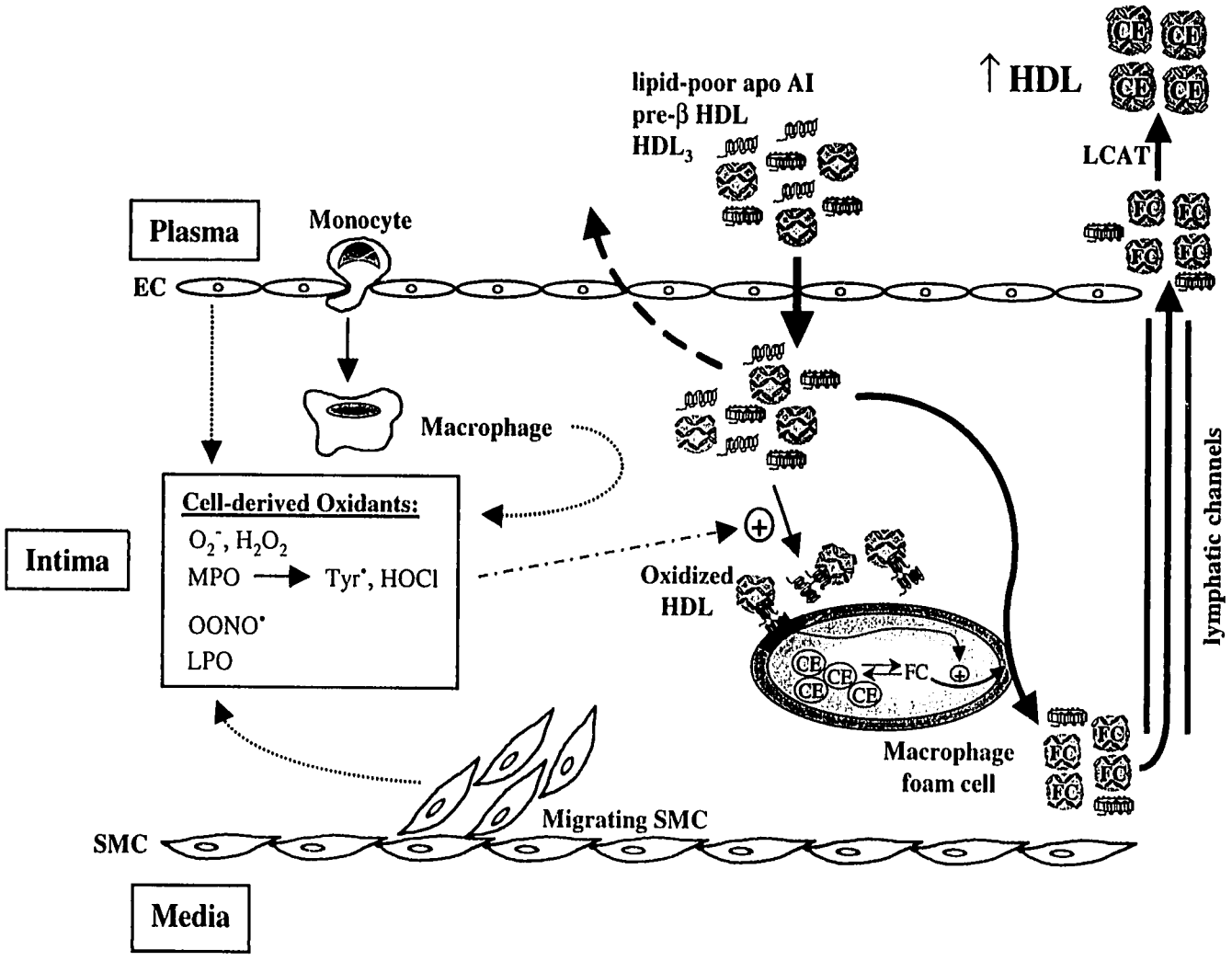
Our results with HDL isolated from inflammatory sites do not allow us to infer directly what the *in vivo* consequences of HDL oxidation would be, but suggest that this oxidized HDL at minimum retains, and more likely has an enhanced capacity to mobilize intracellular cholesterol compared to unoxidized HDL. Although we did not determine directly whether neutrophil- or *in vivo*-oxidized HDL increased the availability of cholesterol for efflux from cultured cells, the similarity in the structural features and mobilization of ACAT-accessible by these particles and *in vitro* TyrHDL suggest *in vivo*-oxidized HDL would also enhance overall mobilization and efflux of cholesterol from peripheral cells. The increased cell-surface cholesterol induced by these oxidized HDL particles would be available for removal by nascent, non-oxidized HDL particles, thereby enhancing their maturation to larger HDL. The longer half-life of larger HDL [258] suggests this would result in markedly increased plasma HDL levels. Preliminary evidence for the ability of TyrHDL to raise plasma HDL levels has recently been found in our lab, where mice injected with TyrHDL twice weekly for 8 weeks had a 160 % increase in HDL as percent of total cholesterol over baseline (Macdonald *et al.*, manuscript in preparation). Although this was a treatment study, the current results indicate HDL oxidized *in vivo* would have a

similar HDL-raising effect, and that this may explain a portion - possibly a major portion - of the protective effect of HDL against atherosclerosis.

Unfortunately, most studies investigating the effects of oxidation on HDL function, including neutrophil-modified HDL [159, 252] have assessed only its ability to passively remove cholesterol from cell membranes, and not its ability to mobilize intracellular cholesterol. Although the ability of *ex vivo*- and *in vivo*-oxidized HDL to efflux cholesterol from cell membranes was not investigated, any decreased ability of these particles to passively remove cholesterol from cells would most likely be offset by their ability to mobilize cholesterol to cell surface sites available for removal by nascent, non-oxidized HDL.

A model from our studies for the effects of *in vivo*-oxidation on HDL structure and function is shown in Figure 4.1. Since HDL crosses the arterial wall and enters the interstitial space [79, 245, 246], it would be exposed to similar oxidants believed to be involved with LDL oxidation. From the results presented here, HDL in the interstitial space could be oxidized by various oxidants generated by activated phagocytes, mediated by the MPO/H_2O_2 system, resulting in the intraparticle crosslinking of HDL apolipoproteins. The interaction of oxHDL crosslinked apolipoproteins with foam cells would result in the active translocation of intracellular cholesterol to sites on the cell surface available for efflux by nascent, non-oxidized HDL particles. These non-oxidized HDL particles would then recirculate back to the general circulation and participate in the remainder of the RCT pathway. The increase in cholesterol available for efflux would increase circulating

Figure 4.1 Model of HDL oxidation in the atherosclerotic lesion.
HDL diffuse readily into the artery wall, where they can diffuse back into the plasma or are oxidized by artery wall cell-derived oxidants (*e.g.*, migrating smooth muscle cells (SMC), endothelial cells (EC), and macrophages) within the intimal space. The oxidized HDL particles would then interact with a proposed cell surface binding site (shaded box on cell surface) on macrophage foam cells, leading to cell signaling events that would turn on intracellular cholesterol trafficking molecules, resulting in the active mobilization of intracellular free cholesterol (FC) to cell surface sites. The increased pool of FC on the cell surface would then be available for removal by nascent, non-oxidized HDL particles (lipid-poor apo AI, pre–β HDL and HDL_3) resulting in enhanced removal of cholesterol from these cells. FC-enriched HDL particles may then be returned to the plasma, partly via lymphatic channels, where HDL-FC is acted upon by lecithin:cholesterol acyltransferase (LCAT), converting it to cholesteryl ester (CE). The larger HDL particles have a longer half-life in plasma, leading to an overall increase in plasma HDL levels. The increased removal of cellular FC by HDL would also increase the proposed reverse cholesterol transport pathway. apo AI, apolipoprotein AI; MPO, myeloperoxidase; $Tyr^{\cdot}$, tyrosyl radical; HOCl, hypochlorite; $ONOO^{\cdot}$, peroxynitrite; LPO, lipoxygenase; O_2^{-}, superoxide; H_2O_2, hydrogen peroxide. Adapted from Francis [60].


lipid-poor apo AI
pre-β HDL
HDL3
↑ HDL
LCAT
Plasma
Monocyte
EC
Macrophage
Cell-derived Oxidants:
O2-, H2O2
MPO → Tyr•, HOCl
OONO•
LPO
Intima
Oxidized HDL
CE
FC
Macrophage foam cell
lymphatic channels
Migrating SMC
SMC
Media

HDL levels, thereby enhancing RCT and all of the other proposed cardioprotective actions of HDL.

By this model, the balance between the detrimental effects of LDL oxidation and beneficial effects of HDL oxidation would be determined by the relative availability of these particles in the arterial intima. Although unlikely, if the enhanced cholesterol mobilization induced by oxidized HDL were not balanced by the availability of acceptor particles to remove this cholesterol, cell free cholesterol levels could rise to toxic levels. Our model suggests, however, that oxidation of even a fraction of total HDL particles would markedly increase the pool of cholesterol available for removal by the larger pool of nascent, non-oxidized HDL particles or apo AI in the interstitial space.

4.2 Future Directions

4.2.1 *Ex vivo* Experiments

Further studies would help confirm whether or not neutrophil-oxidized HDL mobilize cholesterol by the same mechanisms demonstrated for *in vitro* TyrHDL. Future cell culture studies would include the assessment of new cholesterol synthesis in cells treated with neutrophil-oxidized HDL. An increase in new cholesterol synthesis would confirm the movement of cholesterol away from the same regulatory pool that supplies ACAT. As well, experiments directly investigating mass transfer of CE to FC would be used to determine depletion of the intracellular CE storage pool of cholesterol, indicating cholesterol is being mobilized from storage pools to efflux-available pools on the cell surface [167]. Finally, more direct chase experiments determining the ability of *ex vivo*-oxidized HDL to mobilize intracellular cholesterol

to sites on the plasma membrane available for efflux by non-oxidized HDL particles should be done to confirm our working model. It would be of interest to determine whether the crosslinks formed in the absence of added L-tyrosine are similar to those formed in the presence of added L-tyrosine. More sensitive measures of dityrosine formation (*e.g.*, mass spectrometry) would help determine whether the crosslinks formed in the absence of added L-tyrosine are due to crosslinking of protein tyrosine radicals. If they were found to be the same, this might explain the similar abilities of these oxHDL particles to mobilize intracellular cholesterol.

Unambiguous confirmation of the role of the MPO/H_2O_2 system in the oxidation of HDL by activated neutrophils to a form that promotes the mobilization of cholesterol is needed. Results from the CGD neutrophil experiments were not as conclusive as hoped, and as indicated further experiments with a completely non-oxidized control HDL sample are required to confirm whether CGD neutrophils may induce crosslinking of HDL apoproteins in the absence of secreted superoxide. In addition, the CGD experiments only indirectly investigated the participation of MPO in the oxidative modification of HDL. A more definitive model would include neutrophils isolated from patients diagnosed with MPO deficiency. Lastly, the degradation of HDL apolipoproteins by activated neutrophils and its effect on the mobilization of intracellular cholesterol needs to be more clearly defined.

4.2.2 *In vivo* Experiments

More samples are needed to study HDL isolated from inflammatory joints. The assessment of apolipoprotein crosslinks and potential apoprotein degradation products needs to be confirmed by Western blot analysis using antibodies to apo AI

and apo AII. Moreover, analysis of particle size of SynHDL and PlmHDL should be done to see if there are differences that may influence the particle's ability to mobilize intracellular cholesterol (*e.g.*, pre-β HDL versus α-HDL). A larger sample size is also needed to confirm preliminary results. As well, isolation of HDL from non-inflammatory joint effusions would provide a control for studying the effects of phagocyte oxidation on synovial HDL compared to non-oxidized HDL particles isolated from synovial fluid. Further assays of cholesterol mobilization would also help to confirm a definite beneficial effect of *in vivo*-oxidized HDL, as indicated above for the *ex vivo* neutrophil studies.

The investigation of HDL isolated from atherosclerotic sites was also in the preliminary phase, and more HDL samples are needed from each lesion stage. Investigation of the extent of lipid peroxidation in HDL isolated from atherosclerotic lesions would provide further insight into the mechanisms involved in the oxidation of HDL in the artery wall.

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