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MODIFICATION OF HUMAN HIGH DENSITY LIPOPROTEIN BY CIGARETTE SMOKE EXTRACT AND ITS IMPACT ON REVERSE CHOLESTEROL TRANSPORT

by

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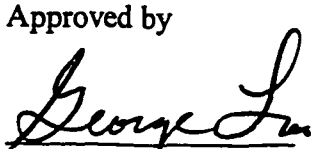

Dissertation Advisor

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High density lipoprotein (HDL) normally functions with lecithin:cholesterol acyltransferase (LCAT) to facilitate reverse cholesterol transport whereby excess cholesterol is removed from peripheral tissue sites, such as arteries. Cigarette smoke contains reactive chemicals which may modify HDL and, therefore, impair its antiatherogenic function in reverse cholesterol transport. We tested this hypothesis by first comparing modification of HDL by cigarette smoke extract (CSE) and acrolein, a potent reactive aldehyde known to be present in cigarette smoke. Both CSE and acrolein caused structural modification of HDL in a concentration-dependent manner, as evidenced by diminution of free amino groups, increase in electrophoretic mobility, and apolipoprotein crosslinking. We then evaluated the capacity of modified HDL to activate LCAT, which requires apolipoprotein A-I as a cofactor, and also to stimulate cholesterol efflux from THP-1 monocytic cells. After incubation with either CSE or acrolein, HDL had decreased capacity to activate LCAT. The effects of CSE and acrolein were both time- and concentration-dependent. Incubation of HDL with 20% (v/v) CSE for 0.5-6 hours caused a progressive decrease (21-48%) in the capacity of HDL to activate LCAT. With the same range of incubation times, acrolein caused a 24-63% decrease in LCAT activation capacity of HDL. After incubation with increasing concentrations of CSE (2.5-20%) for 6 hours, HDL had a 21-59% decrease in LCAT activation capacity. Similarly, incubation of HDL with increasing concentrations (0.1-0.8 mM) of acrolein for 6 hours

resulted in a 31-63% decrease in LCAT activation capacity. When present during incubation, both aminoguanidine and glutathione totally prevented modification of HDL and subsequent decrease in LCAT activation capacity caused by acrolein. Both aminoguanidine and glutathione totally prevented the increase in electrophoretic mobility but not apolipoprotein crosslinking caused by CSE. Moreover, they each partially prevented the decrease in LCAT activation capacity caused by CSE. However, modification of HDL by either CSE or acrolein did not change its capacity to stimulate cholesterol efflux from the cells. The data suggest that reactive aldehydes are involved in the modification of HDL by CSE. But other reactive chemicals in cigarette smoke are also implicated. Furthermore, it is suggested that modification of HDL by cigarette smoke may impair HDL's function in reverse cholesterol transport.

APPROVAL PAGE

This dissertation has been approved by the following committee of the faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

Coronary heart disease (CHD) is the number one killer in the United States and cigarette smoking is the single most preventable cause of death. Each year, cigarette smoking claims more than 400,000 lives, and it also contributes to more than 20% of death from heart disease in this country.

The pathogenesis of atherosclerosis, a form of CHD, is not fully understood despite extensive investigation. Blood cholesterol associated with different lipoprotein species has been implicated in the pathogenesis and progression of atherosclerosis. Epidemiological data have revealed a positive relationship between the levels of low density lipoprotein (LDL) and the incidence of atherosclerosis, and a negative relationship between the levels of high density lipoprotein (HDL) and the incidence of atherosclerosis. Normally, LDL delivers cholesterol to peripheral tissues via the LDL receptor pathway [1], whereas HDL removes excess cholesterol from peripheral tissues via the reverse cholesterol transport pathway [2].

Modification of either LDL or HDL may compromise their biological functions and promote atherogenesis. Recent studies have implicated oxidative modification of LDL in the development of atherosclerosis [3 for review]. Oxidized LDL is more atherogenic than native LDL because it is avidly taken up by macrophages via scavenger

receptors. Unlike the LDL receptor pathway, the unregulated scavenger receptor pathway leads to accumulation of cholesterol and eventually the formation of foam cells, the presence of which marks the initial onset of atherosclerosis. HDL can be also oxidatively modified. Modification of HDL may impair its functions in reverse cholesterol transport and, therefore, accelerate the development of atherosclerosis.

As an independent risk factor for atherosclerosis, cigarette smoking adversely affects the lipoprotein profile by increasing LDL and total blood cholesterol and decreasing HDL cholesterol [4]. Cigarette smoking may also increase the risk of atherosclerosis via another mechanism. Inhalation of toxic chemicals in cigarette smoke, such as free radicals and reactive aldehydes, may result in modification of lipoproteins. For example, cigarette smoke modifies LDL into a form that promotes foam cell formation from macrophages [5]. However, modification of HDL by cigarette smoke and the biological consequences of HDL modification by cigarette smoke have not been systematically studied.

In an attempt to further elucidate the higher risk of atherosclerosis in cigarette smokers, this study was undertaken to characterize the modification of human HDL by an aqueous extract of cigarette smoke, *i.e.* cigarette smoke extract (CSE), and to evaluate the impact of this modification on HDL-mediated reverse cholesterol transport.

CHAPTER II

REVIEW OF LITERATURE

Introduction

To facilitate full understanding of the rationale behind this research project, the literature review begins with a general discussion of the HDL-mediated reverse cholesterol transport pathway. The discussion focuses on the major components and their participation in different stages of reverse cholesterol transport. The second part of the review introduces the concept of lipoprotein modification and its implication in atherogenesis. The research findings by other researchers on oxidative modification of lipoproteins provide a solid scientific foundation to study lipoprotein modification by cigarette smoke. The third part of the review deals with previous work on lipoprotein modification by cigarette smoke. Modification of LDL by cigarette smoke is discussed in great detail because of its logical connection to HDL modification by cigarette smoke. Limited data on HDL modification by cigarette smoke is also discussed with identification of the areas that need further research. In the final part of this review, studies on the inhibition of lecithin:cholesterol acyltransferase (LCAT) activity are discussed. The research in this area is informative to enable the design of a hypothesis-driven study aimed at evaluating the possible mechanism of HDL modification by cigarette smoke.

Overview of Reverse Cholesterol Transport

Cholesterol has significant biological functions in the human body. Overall, it supports biosynthesis of cell membranes, steroid hormones and bile acids. However, excess cholesterol associated with peripheral tissues, such as arteries, is implicated in the pathogenesis of atherosclerosis. The body can use both endogenous and exogenous cholesterol. In contrast to celluiar cholesterol biosynthesis, cholesterol absorption in the intestine is not actively regulated. Therefore, removal of excess cholesterol from peripheral tissues for transport to the liver and subsequent excretion via bile acids are critical processes for cholesterol homeostasis.

The concept of reverse cholesterol transport was first postulated by Glomset in 1968 [2] . Since then, extensive research has supported such a metabolic process to transport peripheral cholesterol back to the liver. Figure 1 provides an overview of an current understanding of the reverse cholesterol transport pathway. HDL, LCAT and cholesteryl ester transport protein (CETP) each play a central role in reverse cholesterol transport. HDL removes free cholesterol from peripheral tissues. LCAT converts cholesterol into cholesteryl esters that can be transferred to other lipoprotein species by CETP. Eventually, LDL and HDL deliver cholesterol to the liver by receptor-mediated and also receptor-independent pathways.

The most important lipoprotein species participating in the reverse cholesterol transport pathway is HDL. HDL is a highly heterogeneous family of particles with hydrated densities ranging from 1.07 to 1.21 g/ml [6]. The general structure of a HDL

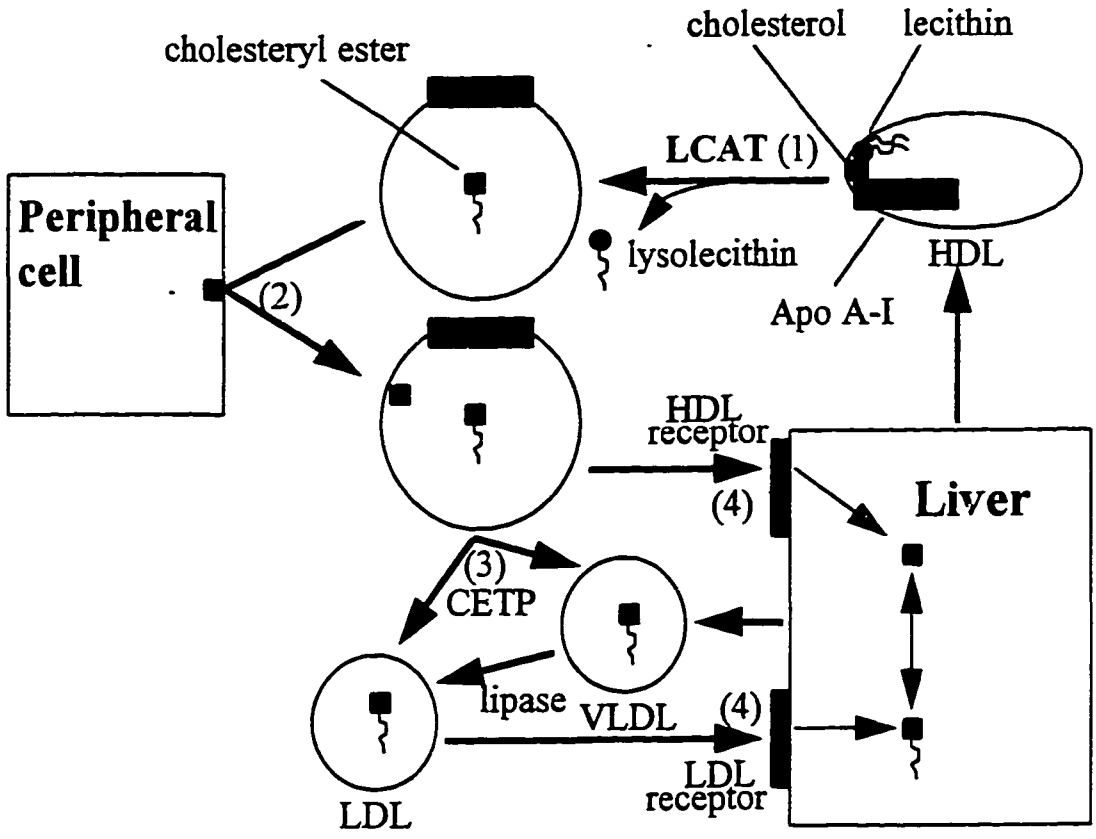

Figure 1. Schematic representation of the reverse cholesterol transport pathway. (1) Using Apo A-I as a cofactor, LCAT transfers a fatty acyl group from lecithin to free cholesterol and generates lysolecithin and cholesteryl ester. (2) HDL removes free cholesterol from peripheral cell membrane via cholesterol efflux. (3) CETP transfers cholesteryl ester to VLDL or LDL. (4) The liver takes up LDL and HDL via LDL and HDL receptors.

particle consists of a core of hydrophobic lipids (triglycerides and cholesteryl esters) surrounded by a shell of polar lipids (phospholipids and free cholesterol) and apolipoproteins. Apolipoproteins A-I and A-II are the major proteins in HDL. Other apolipoproteins, such as apolipoproteins A-IV, C, D and E, may also be present in certain HDL species.

HDL is primarily synthesized in the liver and then secreted into the circulation [7]. The nascent HDL matures in the circulation by obtaining more lipid components from different sources including other lipoprotein species. Nascent HDL can also be derived in the circulation either from the surface of other lipoprotein species, such as very low density lipoprotein (VLDL) remnant, or by formation of a complex between apolipoproteins and phospholipids. Twelve HDL subclasses have been identified, based upon a variety of properties, including hydrated density, apolipoprotein composition and charge. These HDL particles have different sizes and either discoidal or spherical shapes.

Heterogeneity of HDL is implicated in removal of cellular cholesterol. Small, lipid-poor HDL particles which only contain apolipoprotein A-I as protein component are much more effective in removing cellular cholesterol than other HDL species [8]. The mechanism of cholesterol transfer from peripheral tissue membrane to HDL is still not fully elucidated. Two major hypotheses to explain the phenomenon of cholesterol efflux from cell membrane to HDL have been proposed.

The first hypothesis is that cholesterol efflux is a simple diffusion process [9]. With limited aqueous solubility, free cholesterol can move between cell membranes and

blood plasma. However, this movement is a bidirectional exchange process. Thermodynamically, HDL is a much more effective acceptor of free cholesterol than other plasma components because it has a small size and an ideal phospholipid/protein ratio. When there is a chemical gradient of cholesterol between cells and HDL, net flux of free cholesterol may occur in either direction.

The second hypothesis is that HDL-mediated cholesterol efflux occurs via a receptor-dependent mechanism. An HDL-binding protein has been identified in many cell types, including fibroblasts, hepatocytes and macrophages [10]. Enrichment of free cholesterol in cells increased abundance of HDL-binding protein on the cell surface and also cholesterol efflux by HDL [10,11]. It is believed that binding of HDL to the receptor signals the cell to release free cholesterol to HDL via passive transport.

Both hypothetical models recognize the importance of LCAT in facilitating cholesterol efflux from cellular membranes to HDL. LCAT is a plasma enzyme primarily associated with HDL [2]. Using HDL as substrate, LCAT catalyzes the transfer of a fatty acyl group from the sn-2 position of phosphatidylcholine to free cholesterol, generating lysophosphatidylcholine and cholesteryl esters as products. As a result, LCAT decreases surface cholesterol concentration and increases cholesterol concentration in the core of HDL. LCAT catalysis creates a concentration gradient necessary for cellular cholesterol to diffuse to HDL. In addition, the LCAT reaction represents the first step in remodeling HDL to form small, lipid-poor HDL. Other enzymatic steps in the remodeling process

include transfer of cholesteryl esters to triglyceride-rich lipoproteins by CETP and removal of triglycerides by lipoprotein lipase.

The contribution of CETP to reverse cholesterol transport is not limited to the remodeling of HDL. CETP catalyzes exchange of neutral lipids, particularly triglycerides and cholesteryl esters, among different lipoprotein species [12]. Under normal physiological conditions, CETP activity results in net transfer of cholesteryl esters from HDL to VLDL, and triglycerides from VLDL to HDL. VLDL is metabolized into LDL via lipoprotein lipase in the circulation. LDL can then deliver its cholesterol to the liver via the LDL receptor pathway [1]. Therefore, CETP apparently plays a role in cholesterol transport from plasma to the liver.

HDL may also deliver cholesterol to the liver via a receptor-dependent pathway. Numerous studies have demonstrated the existence of so-called HDL-binding proteins. The determined sizes and proposed functions of HDL-binding proteins vary from these studies. However, a recent study identified a specific HDL receptor in the liver and nonplacental steroidogenic tissues [13]. This receptor binds HDL with high affinity and mediates selective uptake of cholesteryl esters possibly by retroendocytosis, during which apolipoproteins A-I and A-II are not degraded. The identification of a cell surface HDL receptor provides an important mechanism for the final delivery of cell-derived cholesterol from plasma to the liver, and further demonstrates the critical role of HDL in reverse cholesterol transport.

Lipoprotein Modification and Atherosclerosis

Although high blood LDL and low blood HDL are the major risk factors for atherosclerosis, cholesterol accumulation in arterial wall cannot be fully explained by blood lipoprotein levels alone. During the past decade, intensive research has focused on oxidative modification of LDL. It is now widely accepted that LDL becomes fully atherogenic only after being modified. Lipoprotein modification in general may provide a biochemical basis for the development of atherosclerosis.

It has long been known that LDL can be chemically modified *in vitro*. However, Goldstein and colleagues [14] were the first to find that acetylated LDL, a form of LDL modified chemically by acetylation, is recognized and taken up by monocytes/macrophages at a rate many times that of normal LDL. The receptor responsible for this uptake is distinct from the classical LDL receptor, and is recognized as a so-called scavenger receptor. This scavenger receptor is expressed in large amounts in monocytes/macrophages, and is not down-regulated by the cellular cholesterol content as is the LDL receptor. Therefore, unregulated uptake of modified LDL by monocytes/macrophages will eventually lead to the formation of foam cells, which is the hallmark of early development of atherosclerosis.

The scavenger receptor is also able to recognize and take up other chemically modified forms of LDL, including acetoacetyl LDL and malondialdehyde-conjugated LDL [3]. Oxidation of LDL can be induced by incubation with transition metal such as

copper and iron, and the oxidized LDL is also recognized and taken up by the scavenger receptor.

The concept of biological, as opposed to chemical, modification of LDL initially came from a study in which LDL was incubated with cultured endothelial cells [15]. Under such conditions, LDL undergoes oxidative modification similar to that induced by transition metals. To date, it is known that LDL can be oxidatively modified by all the major cells of the arterial wall, including endothelial cells, smooth muscle cells, and monocyte-derived macrophages. Regardless of the type of oxidative modification, LDL undergoes profound structural changes, and assumes certain properties that normal LDL does not have [3].

The initial step of oxidative modification involves the peroxidation of polyunsaturated fatty acids in LDL [16]. During oxidative modification of LDL, lipid peroxidation proceeds as naturally occurring antioxidants, such as vitamin E, are depleted. There is a loss of polyunsaturated fatty acids and formation of aldehydic lipid peroxidation products [17]. The reactive fragments from lipid peroxidation, such as malondialdehyde, can form adducts with apolipoprotein B-100 via lysine ε-amino groups. As a result, there is neutralization of the positively charged region of LDL, disrupting the recognition of LDL by the LDL receptor.

Free radicals are also able to oxidize LDL cholesterol and fragment apolipoprotein B-100 [18, 19]. In addition, phosphatidylcholine is extensively degraded to lysophosphatidylcholine as oxidized LDL is formed [16]. The degradation may be due

to an intrinsic phospholipase A_2 activity of apolipoprotein B-100 [20]. Lysophosphatidylcholine is cytotoxic and chemotactic for circulating human monocytes [21]. These physicochemical changes are important cues leading to oxidatively-modified LDL being taken up avidly by macrophages via scavenger receptors.

There is evidence indicating that modification of LDL does happen *in vivo.* Oxidized LDL has been identified in atherosclerotic plagues from humans and Watanabe Heritable Hyperlipidemic (WHHL) rabbits by using anti-malondialdehyde-LDL antibodies [22]. In coronary heart disease patients, oxidized cholesterol was found to be significantly increased in LDL, relative to the LDL of control subjects [23]. Other evidence for the role of oxidized LDL in atherogenesis comes from studies demonstrating that the antioxidant, probucol, can inhibit atherosclerotic lesion formation in WHHL rabbits and also cholesterol-fed rabbits [24].

By analogy, oxidative modification of HDL has also been studied intensively. Incubation of HDL with copper induces lipid peroxidation of polyunsaturated fatty acids. As a result, oxidized HDL had significantly increased levels of conjugated dienes and thiobarbituric acid-reactive substances (TBARS) [25-27], which are intermediate- and end-products of lipid peroxidation. Similar to LDL, HDL lost significant amounts of intact lysine and tyrosine residues upon oxidative modification [28]. HDL also had increased electrophoretic mobility on agarose gel [26, 28], presumably via the neutralization of positively charged amino residues of apolipoproteins.

In contrast to LDL oxidation, oxidative modification of HDL does not result in fragmentation of apolipoproteins. Instead, there is cross-linking of apolipoproteins as demonstrated by several studies [26, 28]. The nature of apolipoprotein cross-linking in oxidized HDL is not fully understood. However, dimerization among different amino residues may be the result of oxidation or linkage with products of lipid peroxidation, such as reactive aldehydes. One study also suggested that there was proteolytic degradation of apolipoprotein A-I during oxidation [25].

Many studies also examined the biological functions of HDL in reverse cholesterol transport after oxidative modification of HDL. One study demonstrated that HDL lost much of its capacity to activate LCAT after being oxidatively modified by copper [28]. Another study showed that oxidized HDL formed by either copper- or gamma radiolysis-generated oxyradicals had decreased ability to stimulate cholesterol efflux from cholesterol-loaded fibroblasts [29]. In two other studies, researchers generated foam cells by loading mouse peritoneal macrophages with acetylated LDL, and found that copper-oxidized HDL lost its capacity to stimulate cholesterol efflux from these foam cells [25, 26]. Yet another study demonstrated that incubation of human monocytes with copper-oxidized HDL increased intracellular cholesterol content [30]. The researchers of this study reported that in concurrence with its reduced capacity in promoting cholesterol efflux, oxidized HDL was recognized and degraded by scavenger receptors. Therefore, accumulation of intracellular cholesterol of monocytes/macrophages could result from decreased efflux together with increased cholesterol uptake by the cells.

HDL can be also modified chemically by many other compounds. Reactive aldehydes are a class of such compounds that have been studied because of their physiological relevance. Modification of HDL by reactive aldehydes, such as malondialdehyde, caused structural changes that are similar to those occurring upon HDL oxidation. These changes include decreased lysine and tryptophan amino residues, increased TBARS and agarose gel electrophoretic mobility, and the formation of cross-linked apolipoproteins [28]. More importantly, aldehyde-modified HDL has impaired ability to activate LCAT and to stimulate cholesterol efflux from cholesterol-laden cells [28,31].

Another chemical crosslinker that has been widely used in studying the function of HDL is tetranitromethane (TNM). TNM specifically modified tyrosine amino residues of HDL apolipoproteins and caused apolipoprotein cross-linking [26, 32]. It also appeared to induce lipid peroxidation and cause lipid degradation of HDL. The modified HDL had increased electrophoretic mobility on agarose gel. In addition, cross-linking of apolipoproteins by TNM led to a loss of ligand binding activity of HDL to both macrophages and fibroblasts, coincident with reduced ability of HDL to promote cholesterol efflux from these cells.

Modification of Lipoproteins by Cigarette Smoke

Cigarette smoke is noxious in that it contains many free radicals including hydroxyl radicals and alkoxyl radicals [33]. In addition to its adverse effects on the blood

lipid profile, cigarette smoking is speculated to promote atherogenesis by inducing oxidative modification of LDL.

Cigarette smoking was demonstrated to render LDL more susceptible to peroxidative damage and enhance the metabolism of LDL by macrophages [34, 35]. Macrophage-mediated lipid peroxidation of LDL isolated from blood of cigarette smokers was significantly increased in comparison to macrophage-treated LDL from nonsmokers. The uptake of LDL isolated from smokers by macrophages was also significantly increased. Supplementation of cigarette smokers with the antioxidant vitamin C decreased the susceptibility of their LDL to copper-induced oxidation [36].

Some *in vitro* studies have examined the mechanism of LDL modification by cigarette smoke. Yokode and colleagues reported that rabbit LDL treated with an aqueous cigarette smoke extract (CSE) migrated further on agarose gel after electrophoresis than native LDL [5]. The treated LDL also showed extensive fragmentation of apolipoprotein B-100 on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The metabolism of treated LDL by macrophages was significantly increased, which led to massive accumulation of cholesteryl esters in macrophages. However, TBARS levels did not increase in treated LDL, indicating lipid peroxidation was not involved in the modification of LDL by CSE. Superoxide dismutase, but not catalase, partially inhibited these changes. The authors concluded that superoxide anion produced in CSE might directly damage the protein moiety of HDL.

In another study, lipid peroxidation in human plasma was detected after the plasma was exposed to gas phase cigarette smoke that had been passed through a 0.02 μm filter [37]. There was also a slight increase of electrophoretic mobility of plasma LDL on agarose gel. These changes were only found after endogenous ascorbate was depleted, and exogenous ascorbate was able to delay such effects.

Whole cigarette smoke, however, did not induce lipid peroxidation of LDL in plasma in the absence of ascorbate, nor did it change electrophoretic mobility of LDL. Nevertheless, structural modification of plasma proteins by whole cigarette smoke was found as measured by protein carbonyl formation [38]. Such modification was partially inhibited by glutathione, but not ascorbate. The investigators suggested that phenolic compounds in whole cigarette smoke actually protected LDL from lipid peroxidation by arresting free radicals, and that the protein modification as detected by carbonyl formation could be caused by reactive aldehydes in cigarette smoke.

A recent study by the author found that CSE did not cause oxidative modification of LDL [39]. Our data suggested that CSE possesses antioxidant activity and actually inhibited oxidative modification of LDL caused by either copper or a peroxyl-radical generating compound, 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH). A low concentration of CSE (0.25%, in the final incubation volume) inhibited the formation of TBARS when LDL was incubated with copper. CSE also prevented copper- and AAPH-induced . degradation of phosphatidylcholine to lysophosphatidylcholine and fragmentation of apolipoprotein B-100. In addition, CSE partially blocked the increased

electrophoretic mobility of LDL, as well as the loss of immunoreactivity of apolipoprotein B-100 against a murine monoclonal antibody caused by treatment with either copper or AAPH. Despite the apparent antioxidant potential of CSE that protected LDL against copper- and AAPH-mediated oxidation, CSE itself did induce modification of LDL. When LDL was incubated with CSE alone, structural modification of apolipoprotein B-100 was indicated by a slight increase in electrophoretic mobility, slight decrease of immunoreactivity with the monoclonal antibody against apolipoprotein B-100, and marked increase of protein carbonyls in LDL. Since lipid peroxidation did not occur under these experimental conditions, we proposed that reactive aldehydes in CSE directly modified certain amino residues of apolipoprotein B-100, specifically, free amino groups and free sulfhydryl groups.

There is evidence that HDL also can be modified by cigarette smoke. It was first reported by Frei *et al.* [37] that the electrophoretic mobility of HDL on agarose gel was slightly increased after human plasma was treated with filtered gas phase cigarette smoke. Recent work by McCall *et al.* [40] confirmed this finding and further demonstrated crosslinking of HDL apolipoproteins by filtered gas-phase cigarette smoke. Although gas-phase cigarette smoke is known to induce lipid peroxidation in plasma, lipid peroxidation was not detected by TBARS assay in LDL and HDL isolated from cigarette smoke-treated plasma. Fatty acid analysis of LDL and HDL found little change upon exposure of plasma to cigarette smoke, further indicating lipid peroxidation was not involved in the modification of LDL and HDL. It was suggested by the researchers [40]

that reactive chemicals in cigarette smoke, particularly reactive aldehydes, directly modified HDL apolipoproteins.

Inhibition of LCAT Activity by Cigarette Smoke and Reactive Aldehydes

Cigarette smoke is rich in reactive aldehydes, including acetaldehyde, propanal, acrolein, crotonaldehyde, formaldehyde, and isovaleraldehyde [38]. Although no study has measured blood aldehyde levels in cigarette smokers, it is known that more than 90% of some hydrophilic volatile compounds, including acetaldehyde, is retained in human lungs during cigarette smoking [41]. Reactive aldehydes may react with different chemical groups on amino acid residues of proteins. In oxidative modification of LDL, reactive aldehydes generated from decomposition of lipid hydroperoxides covalently react with free amino groups on lysine residues to form Schiff's bases via nucleophilic addition [42]. Many α,β-unsaturated aldehydes, such as acrolein and crotonaldehyde in cigarette smoke, can also react with free amino groups or free sulfhydryl groups to form protein carbonyls via conjugated addition [43].

In a previous study [44], we found that CSE inhibited plasma LCAT activity. The decrease of LCAT activity upon incubation of plasma with CSE was both time- and dose-dependent. When different antioxidants were included in the incubation, only glutathione was effective in inhibiting the loss of LCAT activity caused by CSE. We proposed that glutathione protected LCAT from losing its activity by two possible mechanisms. First, glutathione preserves free sulfhydryl groups of LCAT. These free sulfhydryl groups

reside within the active site region of the enzyme and are required for LCAT activity [45]. Second, glutathione reacts with reactive chemicals in cigarette smoke, including aldehydes, and keep them from attacking free sulfhydryl groups of LCAT.

We further tested five reactive aldehydes, namely acrolein, hexanal, formaldehyde, malondialdehyde and acetaldehyde, for their effects on LCAT activity. All five aldehydes inhibited LCAT activity in a dose-dependent manner. Acrolein, the only α,β-unsaturated aldehyde tested, was 10 to 50 times more potent in inhibiting LCAT activity than the other reactive aldehydes. When included in the incubation, both glutathione and dihydrolipoic acid were each able to block the loss of LCAT activity caused by acrolein.

Similar inhibition of LCAT activity with gas phase cigarette smoke and reactive aldehydes have been reported by McCall's group [46, 47]. Besides glutathione, they also showed that two other free sulfhydryl compounds, *N*-acetyl cysteine and 5,5'-dithiobis-nitrobenzoic acid (DTNB), were able to block LCAT inhibition caused by cigarette smoke.

CHAPTER III

SPECIFIC AIMS AND RATIONALE

Specific Aims

Modification of human HDL by cigarette smoke may provide an explanation for the increased risk of atherosclerosis in cigarette smokers. To partially address this hypothesis, the mechanism by which CSE causes HDL modification and the impact of this modification on HDL function were investigated in this study. The specific aims are as follow:

(1) to characterize modification of HDL by CSE with special emphasis on structural changes of apolipoproteins;

(2) to determine if acrolein, the most potent reactive aldehyde known to be present in cigarette smoke, can directly modify HDL;

(3) to examine whether CSE-mediated modification of HDL decreases the capacity of HDL to activate LCAT;

(4) to determine whether CSE-modified HDL, as compared to native HDL, exhibits decreased capacity to stimulate cholesterol efflux from macrophages;

(5) and, to evaluate the capacity of glutathione and aminoguanidine to prevent modification of HDL and also any subsequent impairment of HDL function.

Rationale

Our previous study of LDL modification demonstrated that CSE possesses antioxidant activity and does not induce lipid peroxidation. We suggested that reactive aldehydes, instead of free radicals, in cigarette smoke might be responsible for LDL modification. Therefore, the initial study of the present project focused on structural modification of HDL lipoproteins by CSE and acrolein. SDS-PAGE was used in combination with Western-blot analysis to characterize structural modification of certain apolipoproteins, such as apolipoprotein A-I. Lysine residues of HDL apolipoproteins were analyzed because reactive aldehydes can form different adducts with free amino groups, particularly the ε-amino group of lysine. Modification of HDL by acrolein was compared with modification of HDL by CSE to ascertain the possible role of reactive aldehydes in CSE-mediated HDL modification.

The next study was to evaluate the impact of HDL modification on its biological function in reverse cholesterol transport. Since apolipoprotein A-I of HDL is a cofactor of LCAT and might be modified by CSE, the capacity of HDL to activate LCAT was examined. Removal of intracellular cholesterol by HDL, another major function of HDL in reverse cholesterol transport, might be also decreased by CSE-mediated modification. In a third study, a cellular model was used to examine this possibility.

There has been increasing interest in identifying compounds that can inhibit different types of lipoprotein modifications. Both glutathione and aminoguanidine readily react with reactive aldehydes [43, 48]. Inclusion of these compounds in the incubation

should protect HDL from structural modification induced by acrolein. In addition, they might inhibit HDL modification by CSE if reactive aldehydes are indeed involved in the modification process. As such, these two possibilities were tested. Ascorbate, a classical antioxidant known to inhibit LDL oxidation, was also tested for its ability to inhibit CSE-mediated HDL modification in certain experiments. Identification of compounds that inhibit HDL modification could provide us with valuable information to understand mechanistically the basic biochemistry of HDL modification. More importantly, such compounds might be used as either dietary or medicinal agents to prevent HDL modification and, therefore, decrease the risk of atherosclerosis.

CHAPTER IV

EXPERIMENTAL METHODOLOGY

Chemicals

[4-^{14}C]-cholesterol (51.40 mCi or 1.9 Gbq/mmol) and [^{14}C]-sodium acetate (57 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Acrolein and glutathione were obtained from Sigma Chemical Co. (St. Louis, MO). Aminoguanidine was obtained from Fisher Scientific Co. (Pittsburgh, PA). All other reagents were also obtained from either Fisher Scientific or Sigma Chemical Co.

Isolation of HDL and Semi-purified LCAT Preparation from Plasma

Blood samples were obtained from healthy, normolipidemic, adult male nonsmokers. Fasting blood was drawn by arm venipuncture using Vacutainer tubes with disodium ethylenediamine tetraacetic acid (EDTA) as anticoagulant. After centrifugation of the blood at 1,500 x *g* for 30 minutes at 4°C, plasma was recovered. Plasma density was raised to 1.21 g/ml with potassium bromide (0.3265 g/ml). Density-adjusted plasma was centrifuged at 114,000 x *g* for 40 hours at 4 °C in a Beckman L7-65 ultracentrifuge/50.3 Ti rotor. The total lipoprotein fraction was then recovered from the top of each centrifuge tube. The clear zone in the middle of the tubes was also collected and used as a semi-purified LCAT source [28, 49] in LCAT activation experiments. HDL

in the total lipoprotein fraction was separated from the other lipoprotein species by a density gradient procedure [50]. Briefly, 3.4 ml of the total lipoprotein fraction was overlaid with 8.5 ml of a 0.9% sodium chloride solution containing 0.01% (w/v) EDTA (density = 1.006 g/ml) in Beckman 50 Ti rotor tubes. Tubes were centrifuged at 105,000 x *g* for 4 hours at 18 °C. After recovery, the HDL was dialyzed against phosphate-buffered saline (PBS) containing 1 mM EDTA, pH 7.4, at 4 °C for 24 hours. The semi-purified LCAT preparation was dialyzed against LCAT assay buffer (10 mM Tris, 140 mM NaCl and 1 mM EDTA, pH 7.4 [51]) at 4 °C for 20 hours. Protein concentrations of HDL and LCAT preparation were determined by the Bradford protein assay (Bio-Rad). After dialysis, aliquots of LCAT preparation were frozen at -20 °C for later use in the LCAT activation experiments.

Preparation of Cigarette Smoke Extract (CSE)

CSE was prepared using a procedure somewhat similar to that reported in another study [39]. Smoke from one burning University of Kentucky research cigarette (2R1) was continuously bubbled through 1 ml PBS, pH 7.4, at room temperature (Figure 2). The vacuum needed to "smoke" the cigarettes was supplied by a water-driven aspirator pump. It took about 2-3 minutes to consume one cigarette at a constant flow rate of 1.0 standard cubic foot per hour (SCFH), which was monitored with an air flow meter. PBS that was bubbled with plain air served as the control. Both preparations were individually

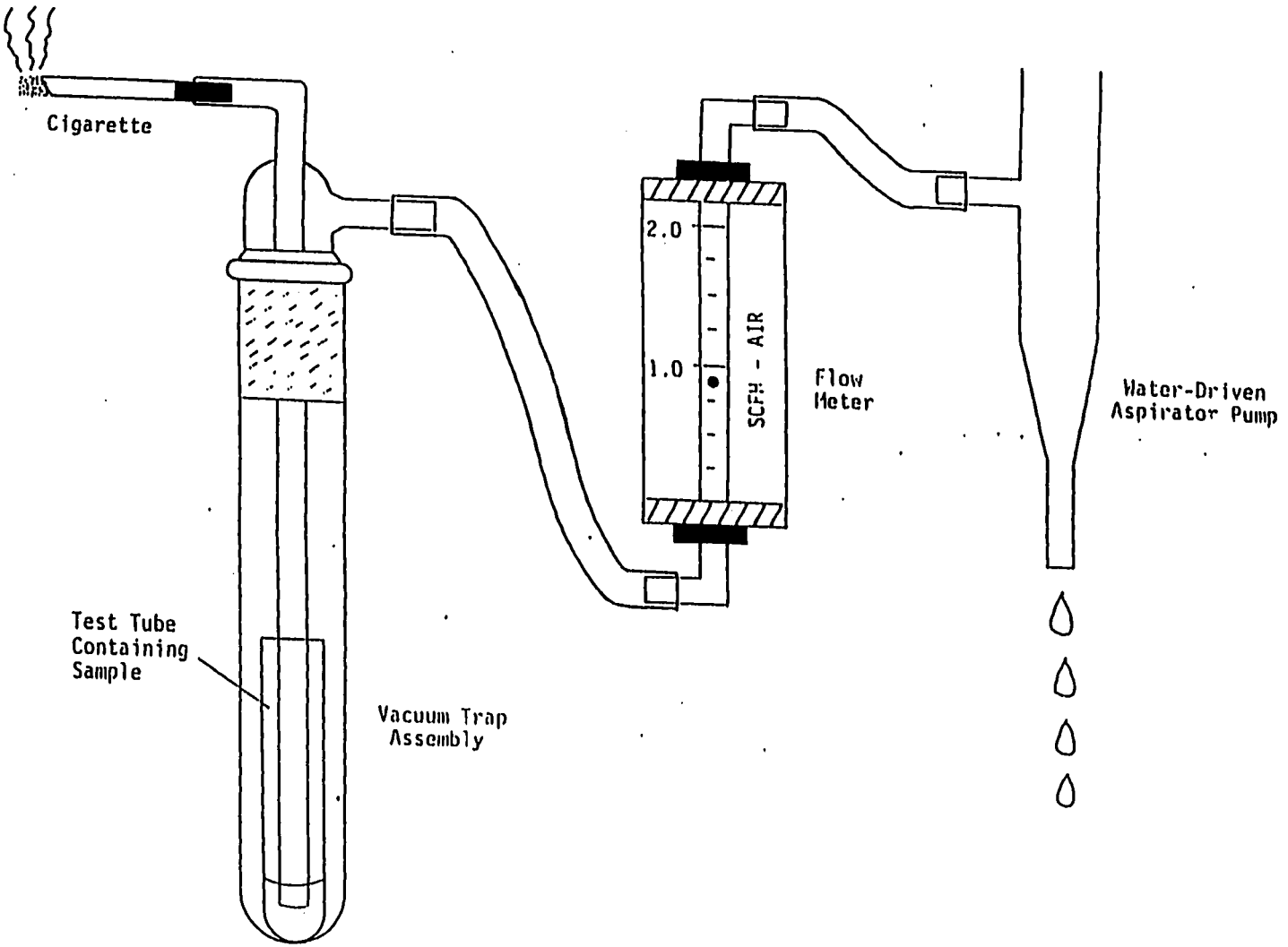

Figure 2. Diagram of apparatus used to prepare cigarette smoke extract.

filtered through 0.2 micron filters, and aliquots of the stock filtrates were immediately incubated with HDL as described below.

Experimental Treatment of HDL

HDL modification was induced by incubation of HDL (1 mg/ml) with different concentrations of either CSE (2.5-20%, v/v, volume of stock CSE comprising total incubation volume) or acrolein (0.1-0.8 mM) in the presence or absence of aminoguanidine or glutathione at 37 °C for 6 hours. When used in the subsequent LCAT activation and cholesterol efflux experiments, the treated HDL samples were immediately dialyzed against the LCAT assay buffer or PBS (pH 7.4), respectively, at 4 °C. The dialysis buffer was changed twice over 20 hours. After dialysis, protein concentration of each HDL sample was determined as before.

Determination of Reactive Amino Residues in HDL Samples

Levels of the free amino groups in apolipoproteins of HDL were determined with trinitrobenzenesulfonic acid (TNBS) using valine as a standard [52]. Briefly, 50 µl of HDL sample was mixed with 1 ml of 4% $NaCO_3$, pH 8.4, and 50 µl of 0.1 % TNBS. Samples were then incubated at 37 °C for 1 hour, and the absorbance at 340 nm was measured spectrophotometrically.

Agarose Gel Electrophoresis of HDL Samples

Electrophoretic mobility of HDL on agarose gel was evaluated by using a Ciba-Corning complete electrophoresis system and pre-cast gels. More specifically, 2 µl of HDL sample was applied to each lane, and the gel was electrophoresed in the Universal Electrophoresis Cell base unit with the two chambers filled with 95 ml of Universal Buffer. After a 35 minute run, the gel was dried in the dryer unit at 55°C. The gel was then incubated with Fat Red 7B staining solution for 5 minutes and destained in methanol/water solution (5:2,v/v). The gel was dried again and photographed for a permanent record.

SDS-PAGE of HDL Samples

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the HDL samples was performed under non-reducing conditions with a Mini-PROTEAN II electrophoresis system (Bio-Rad) using 4-20% Mini-PROTEAN gradient gels. HDL samples were diluted 1:1 (v/v) with sample buffer (a mixture of 4.4 ml of deionized water, 1.0 ml of 0.5 M Tris-HCl at pH 6.8, 0.8 ml of glycerol, 1.6 ml of 10% SDS, and 0.2 ml 1% bromophenol blue). The diluted HDL samples were heated at 95°C for 5 minutes. Then, 0.035 ml of each sample was loaded onto the gel. The gel was processed in the electrophoresis cell unit at a constant 200 volts for about 30 minutes to allow the tracking dye to migrate to 5 mm from the bottom of the gel. The gel was stained with a coomassie blue staining solution (2.5 g in 1 liter of methanol/acetic acid/water (4:1:5))

for 30 minutes. Destaining of the gel was done using the same solvent system for the dye. The gel was then photographed for a permanent record.

Western-blot of HDL Samples

In some experiments, proteins in SDS-PAGE gels were Western-blotted to nitrocellulose membrane sheets with a Genie electrophoretic blotter (Idea Scientific Co.) following the manufacturer's instruction. The transfer was made in Tris-Glycine buffer (20% methanol), pH 8.3, at 24 volts for about 45 minutes. The nitrocellulose membrane was incubated with a blocking solution consisting of 3% gelatin in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) to block other protein-binding sites on the membrane. This step was performed on an orbital shaker at room temperature for an hour. Next, the membrane sheet was washed with a TTBS wash solution (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5) for 10 minutes, again with gentle agitation at room temperature.

The proteins on the nitrocellulose membranes were then immunodetected with murine monoclonal antibodies against human apolipoprotein A-I and A-II (Cappel) used in conjunction with an Immun-Blot assay kit (Bio-Rad). The murine monoclonal antibody against human apolipoprotein A-I or A-II was diluted by 1000-fold with an antibody buffer (1% gelatin in TTBS). The membrane sheet was incubated with 20 ml of the primary antibody solution overnight with gentle agitation. On the next morning, the membrane sheet was washed twice with TTBS for 5 minutes. The membrane sheet was then incubated with secondary antibody (goat anti-mouse IgG/alkaline phosphatase

conjugate) in 30 ml of antibody buffer for 2 hours. The membrane sheet was washed twice with TTBS. Just before color development, the membrane sheet was washed with TBS. Finally, the membrane sheet was incubated with 30 ml of color development solution supplied with the assay kit. The incubation was stopped by immersing the membrane sheet in deionized water for 10 minutes with gentle agitation with at least one change of water. The membrane sheet was air-dried and then photodocumented.

Measurement of HDL-mediated LCAT Activation

Extent of LCAT activation by HDL was measured by a method derived from others [28, 51, 53, 54]. The albumin-stabilized [4-^{14}C]-cholesterol was initially prepared according to Stokke and Norum [55]. Briefly, 5 μCi [4-^{14}C]-cholesterol (51.40 mCi/mmol) was transferred into a small vial and the original solvent for the radiolabel was evaporated off by blowing with plain air. The "hot" residue was redissolved in 0.125 ml of acetone. Using a Hamilton syringe, the radiolabelled cholesterol in acetone was added slowly, drop by drop, into 1.25 ml of 5% (w/v) albumin in 0.2 M phosphate buffer (pH 7.4) with continuous stirring. The vial was washed using additional acetone that was added to the albumin-containing solution to ensure quantitative transfer. The acetone was evaporated by carefully directing a stream of air over the albumin-containing solution.

The LCAT activation assay was performed in an incubation mixture containing 25 μg HDL, 10 μl of semi-purified LCAT preparation (15 μg protein), 13 μl of albumin-stabilized [4-^{14}C]-cholesterol (10^5 dpm), 5μl of 0.1 M β-mercaptoethanol, and LCAT

assay buffer to give a total incubation volume of 100 µl. After 30 minutes of incubation at 37 °C, the reaction was terminated by adding 2 ml of chloroform-methanol (1:1, v/v). The samples were incubated overnight at room temperature to allow complete lipid extraction.

The incubation mixture was filtered the next day through a 10 cc plastic syringe barrel fitted with a fritted disc filter, and the precipitate was washed three times with chloroform/methanol. The combined filtrate was evaporated by directing a stream of plain air in a ventilation hood. The lipid residue was redissolved in 0.1 ml chloroform, and 0.05 ml was applied to a previously activated TLC plate with a TLC Multi-Spotter (Alltech). The plate was developed in a TLC developing tank containing 186 ml of light petroleum (b.p. 40-60 °C) - diethyl ether - acetic acid , 85:15:3 (v/v/v). Free cholesterol and cholesteryl esters were visualized on the plate by exposure to iodine vapor. The TLC gel bands containing free cholesterol and cholesteryl esters were scraped into 20 ml liquid scintillation vials using a razor-blade scraper. To each liquid scintillation vial, 10 ml of liquid scintillation cocktail (Scinti Verse™ I from Fisher Scientific) was added. After thoroughly mixing, the samples were counted with a 2% counting error in a Beckman LS 6000SE liquid scintillation system.

This assay procedure excluded a preincubation period, normally to allow for equilibration between [4-^{14}C]-cholesterol and endogenous HDL cholesterol, because uniform equilibration might not be possible among the different samples of HDL especially after modification. Therefore, extent of LCAT activation by HDL was

determined as amount of radioactivity in the cholesteryl ester fraction and expressed as percentage of control. The assay was linear for at least an hour.

Assay of Cholesterol Efflux by HDL Samples

The THP-1 human monocyte, a transformed monocytic leukemic cell, has been used in the study of lipoprotein-mediate cholesterol efflux previously [56]. THP-1 cells were cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS), 10 mM Hepes, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 2.5 units/ml penicillin, 2.5 units/ml streptomycin, and 0.5 μg/ml fungizone. Cell culture was maintained in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C.

Efflux experiments were performed by modifying a published procedure [56]. Briefly, $3x10^5$ cells were seeded in each well of a 24-well culture dish with 1 ml of serum-free medium in which 10% FCS was replaced by 1% Nutridoma-HU (Boehringer Mannheim). Each well also received 5 μCi [^{14}C]-sodium acetate (57 mCi/mmol). Non-radioactive sodium acetate was added in the incubation to give a final concentration of 0.5 mM acetate. Cells were incubated for 48 hours to allow incorporation of ^{14}C-acetate into cholesterol and cholesteryl esters. Medium was removed at the end of the incubation and cells were washed twice with PBS, pH 7.4. Different cholesterol acceptors (0.1 mg/ml), including control HDL and modified HDL, were then added for a further 24-hour incubation. Control cells received albumin instead of any lipoproteins. Native LDL and

oxidized HDL were used as negative and positive controls, respectively, in the experiments. Cells were then collected by centrifugation and washed with PBS, pH 7.4.

Cellular cholesterol was extracted by incubating cell pellet with 1 ml of hexane-isopropanol mixture (3:2, v/v) for one hour. The cell pellet was washed twice with hexane-isopropanol mixture, and the solvent was collected for cholesterol determination. The combined solvent was evaporated by directing a stream of air over the solvent surface in a ventilation hood. Cholesterol and cholesteryl esters were separated by thin-layer chromatography using a two-solvent system. The TLC plate was placed in a TLC tank with first solvent, isopropyl ether-acetic acid (96:4), which was allowed to run to the middle of the plate. The plate was then dried and placed in another TLC tank with second solvent, hexane-diethyl ether-acetic acid (83:16:1), for complete development. The TLC bands containing free cholesterol and cholesteryl esters were identified and their radioactivities were determined with a Beckman LS 6000SE scintillation spectrometer.

After lipid extraction, each cell pellet was incubated with 0.2 N NaOH for an hour and cellular protein was determined as above. The cellular cholesterol was calculated and expressed as DPM/μg protein.

Statistical Analysis of Experimental Data

All experiments were performed in at least triplicate and repeated three times unless otherwise stated. Since multiple incubations were conducted, one-way analysis of

variance (ANOVA) followed by Duncan's multiple range test was performed using SAS program to analyze data for significant differences (P,0.05).

CHAPTER V

RESULTS

Relative Levels of Reactive Amino Groups in HDL Samples

When incubated with HDL for 6 hours, CSE, in a concentration-dependent manner, decreased HDL reactive amino groups relative to control HDL which contained 1.3 nmol reactive amino groups per mg of protein (Table I). At 2.5% (v/v), CSE caused a 6% decrease in HDL reactive amino groups. When the concentrations of CSE was increased to 5%, 10% and 20%, reactive amino groups in HDL decreased 14%, 24% and 33%, respectively. The effect of acrolein on HDL reactive amino groups followed the same pattern (Table I). When HDL was incubated with 0.1, 0.2, 0.4 and 0.8 mM acrolein, HDL reactive amino groups decreased by 6%, 12%, 28% and 33%, respectively.

Agarose Gel Electrophoresis of HDL Samples

As shown in Figure 3, HDL samples that were incubated with increasing concentrations of either CSE (lanes 2-5) or acrolein (lanes 8-11) progressively migrated further to the anode than the respective control HDL samples (lane 1 and lane 7). Although the increases in electrophoretic mobility of HDL caused by CSE or acrolein appeared to be relatively small, they were reproducible and concentration-dependent.

Table I. Relative levels of reactive amino groups in HDL samples after incubation with either CSE or acrolein.

Sample	Reactive amino group (%)
Control HDL	100 ± 3^{A}
HDL/2.5% CSE	94 ± 2^{B}
HDL/5% CSE	86 ± 3^{C}
HDL/10% CSE	76 ± 4^{D}
HDL/20% CSE	67 ± 3^{E}
Control HDL	100 ± 3^{a}
HDL/0.1 mM acrolein	94 ± 1^{b}
HDL/0.2 mM acrolein	88 ± 1^{c}
HDL/0.4 mM acrolein	72 ± 2^{d}
HDL/0.8 mM acrolein	67 ± 2^{e}

HDL (1 mg/ml) was incubated for 6 hours at 37 °C with increasing concentrations of either CSE or acrolein in a total incubation volume of 100 µl. After incubation, levels of free amino groups in 50 µl of HDL samples were determined. Results are the mean±S.E.M. of triplicate incubations using the same CSE preparation. Superscipts represent Duncan groupings. The experiment was repeated three times. Level of free amino groups in control HDL was 1.3 nmol/mg protein.

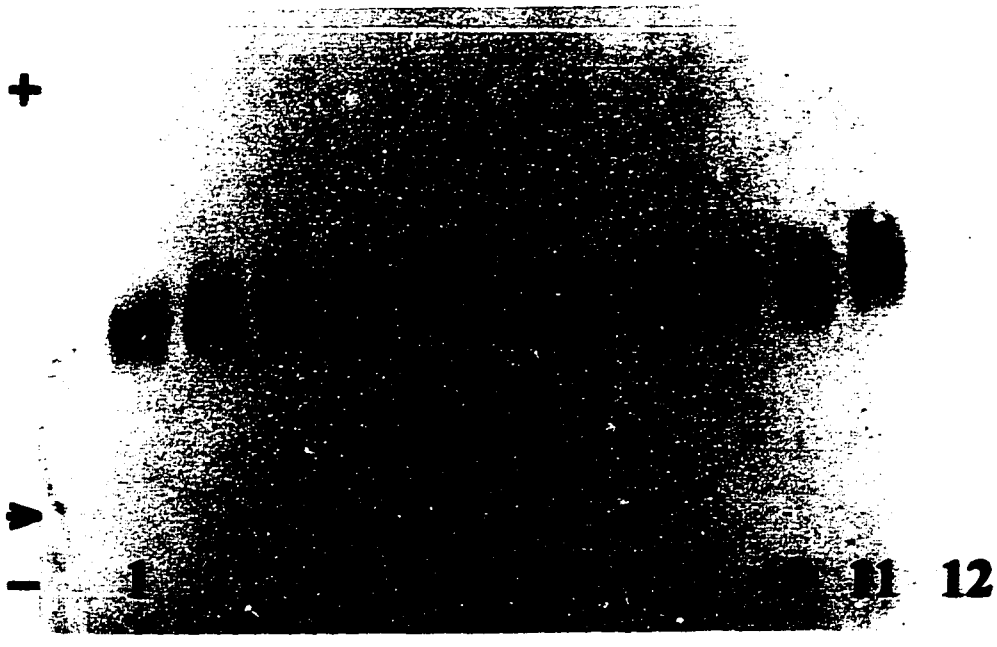

Figure 3. Agarose gel electrophoretic mobility of HDL incubated with different concentrations of either CSE or acrolein. HDL (1 mg/ml) was incubated with increasing concentrations of either CSE or acrolein at 37 °C for 6 hours. After incubation, samples were subjected to agarose gel electrophoresis and then stained. The arrow indicates the well position for sample application. The samples in the gel lanes are as follow: lane 1, control HDL; lane 2, HDL incubated with 2.5% CSE; lane 3, HDL incubated with 5% CSE; lane 4, HDL incubated with 10% CSE; lane 5, HDL incubated with 20% CSE; lane 6, no sample applied; lane 7, control HDL; lane 8, HDL incubated with 0.1 mM acrolein; lane 9, HDL incubated with 0.2 mM acrolein; lane 10, HDL incubated with 0.4 mM acrolein; lane 11, HDL incubated with 0.8 mM acrolein; lane 12, no sample applied. The gel is a representation of three separate experiments.

In other agarose gel electrophoretic experiments, we examined the protective effects of aminoguanidine and glutathione against HDL modification caused by CSE and acrolein. Aminoguanidine at concentrations of 5, 25 or 50 mM and glutathione at concentrations of 1, 5 or 10 mM were tested. After HDL was incubated with either 20% (v/v) CSE or 0.8 mM acrolein in the absence or presence of either aminoguanidine or glutathione, the relative electrophoretic mobility (REM) was calculated for each HDL sample. The results are shown in Table II. Incubation of HDL with either CSE or acrolein increased the REM values of HDL from 1.0 to 1.2 and 1.3, respectively. At the tested concentrations, both aminoguanidine (5.0 mM) and glutathione (1.0 mM) were very effective in preventing the increase of electrophoretic mobility of HDL caused by acrolein. Glutathione at 1 mM also completely inhibited the increase of electrophoretic mobility of HDL caused by CSE. Aminoguanidine at 5 mM did not have any inhibitory effect on the increase of electrophoretic mobility of HDL caused by CSE. When its concentrations was increased to 25 mM, aminoguanidine decreased REM of CSE-treated HDL to 1.1. At the highest concentration (50 mM), aminoguanidine completely inhibited the increase of electrophoretic mobility of HDL caused by CSE.

Table II. Relative electrophoretic mobility (REM) of HDL incubated with either CSE or acrolein in the presence or absence of aminoguanidine (AG) or glutathione (GSH).

Sample	REM
Control HDL	1.0
HDL/50 mM AG	1.0
HDL/10 mM GSH	1.0
HDL/20% CSE	1.2
HDL/20% CSE/5.0 mM AG	1.2
HDL/20% CSE/25 mM AG	1.1
HDL/20% CSE/50 mM AG	1.0
HDL/20% CSE/1.0 mM GSH	1.0
HDL/0.8 mM acrolein	1.3
HDL/0.8 mM acrolein/5.0 mM AG	1.0
HDL/0.8 mM acrolein/1.0 mM GSH	1.0

HDL (1 mg/ml) was incubated with either 20% (v/v) CSE or 0.8 mM acrolein in the presence or absence of different concentrations of aminoguanidine or glutathione at 37 °C for 6 hours. After incubation, HDL samples were subjected to 1% agarose gel electrophoresis. REM was calculated as the ratio of migration distance (from the gel well origin) of treated HDL to that of control HDL. The results are a representation of three separate experiments.

SDS-PAGE and Western-Blot of HDL Samples

As presented in Figures 4 and 5, results of SDS-PAGE show that apolipoproteins are modified after incubation of HDL with CSE (Figure 4) or acrolein (Figure 5). Apolipoproteins A-I and A-II were the major proteins in control HDL after SDS-PAGE (Lane 1 in both Figures). Incubation of HDL with 20% CSE (Figure 4, Lane 2) or 0.8 mM acrolein (Figure 5, Lane 2) resulted in apolipoprotein crosslinking as indicated by the presence of multiple high molecular weight bands in both of the gel lanes. Moreover, the intensity of both apolipoprotein A-I and A-II bands was noticeably decreased by CSE and acrolein. After apolipoproteins on duplicate gels were Western-blotted to nitrocellulose membranes, all high molecular weight bands cross-reacted with both a murine monoclonal antibody against apolipoprotein A-I (Figure 6, Membrane I) and a murine monoclonal antibody against apolipoprotein A-II (Figure 6, Membrane II).

As further seen on the gels, aminoguanidine (Figure 5, Lanes 3-5) and glutathione (Figure 5, Lanes 6-8) inhibited acrolein-induced apolipoprotein crosslinking in a concentration-dependent manner. At the highest concentrations used, aminoguanidine (50 mM, Lane 5) and glutathione (10 mM, Lane 8) almost completely protected apolipoproteins from crosslinking caused by acrolein. Glutathione also reduced apolipoprotein A-II dimer (D) to monomeric form (M). To a lesser extent, glutathione also inhibited CSE-induced apolipoprotein crosslinking in a concentration-dependent manner (Figure 4, Lanes 6-8). The protective effect of aminoguanidine on CSE-induced apolipoprotein crosslinking was minimal (Figure 4, Lanes 3-5).

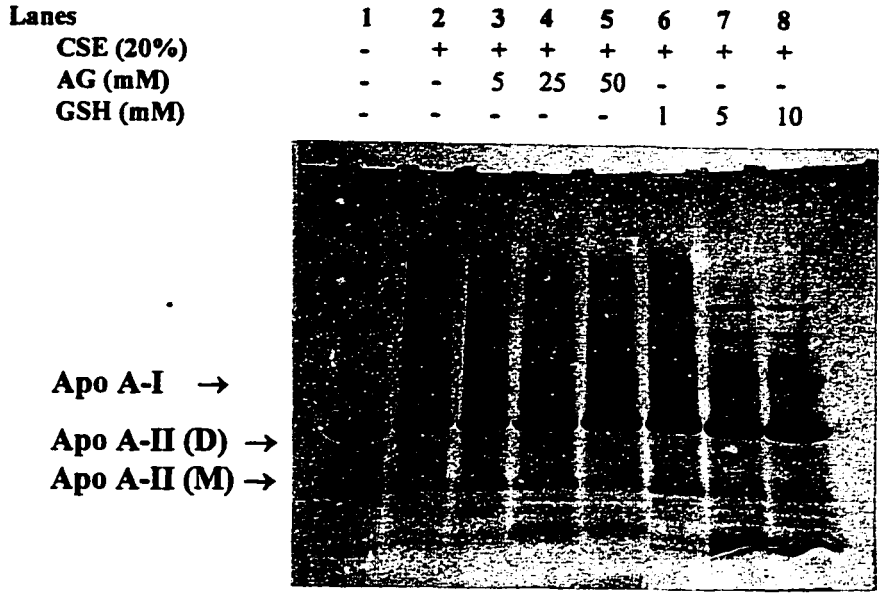

Figure 4. SDS-PAGE of HDL after incubation with CSE in the presence or absence of aminoguanidine (AG) or glutathione (GSH). HDL (1 mg/ml) was incubated with 20% CSE in the presence or absence of different concentrations of either aminoguanidine or glutathione at 37 °C for 6 hours. After incubation, samples containing equal amounts of protein were subjected to SDS-PAGE and then stained. The gel is a representation of three separate experiments.

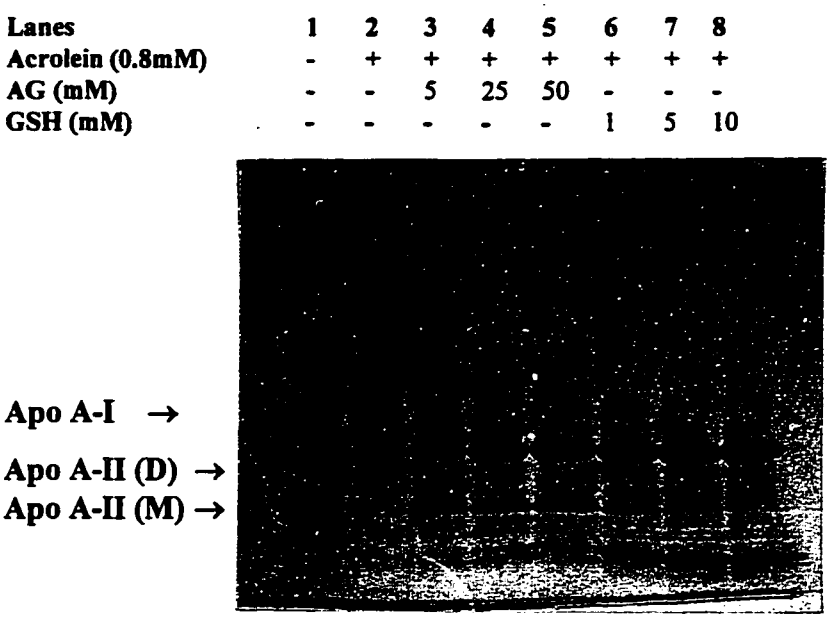

Figure 5. SDS-PAGE of HDL after incubation with acrolein in the presence or absence of aminoguanidine (AG) or glutathione (GSH). HDL (1 mg/ml) was incubated with 0.8 mM acrolein in the presence or absence of different concentrations of either aminoguanidine or glutathione at 37 °C for 6 hours. After incubation, samples containing equal amounts of protein were subjected to SDS-PAGE and then stained. The gel is a representation of three separate experiments.

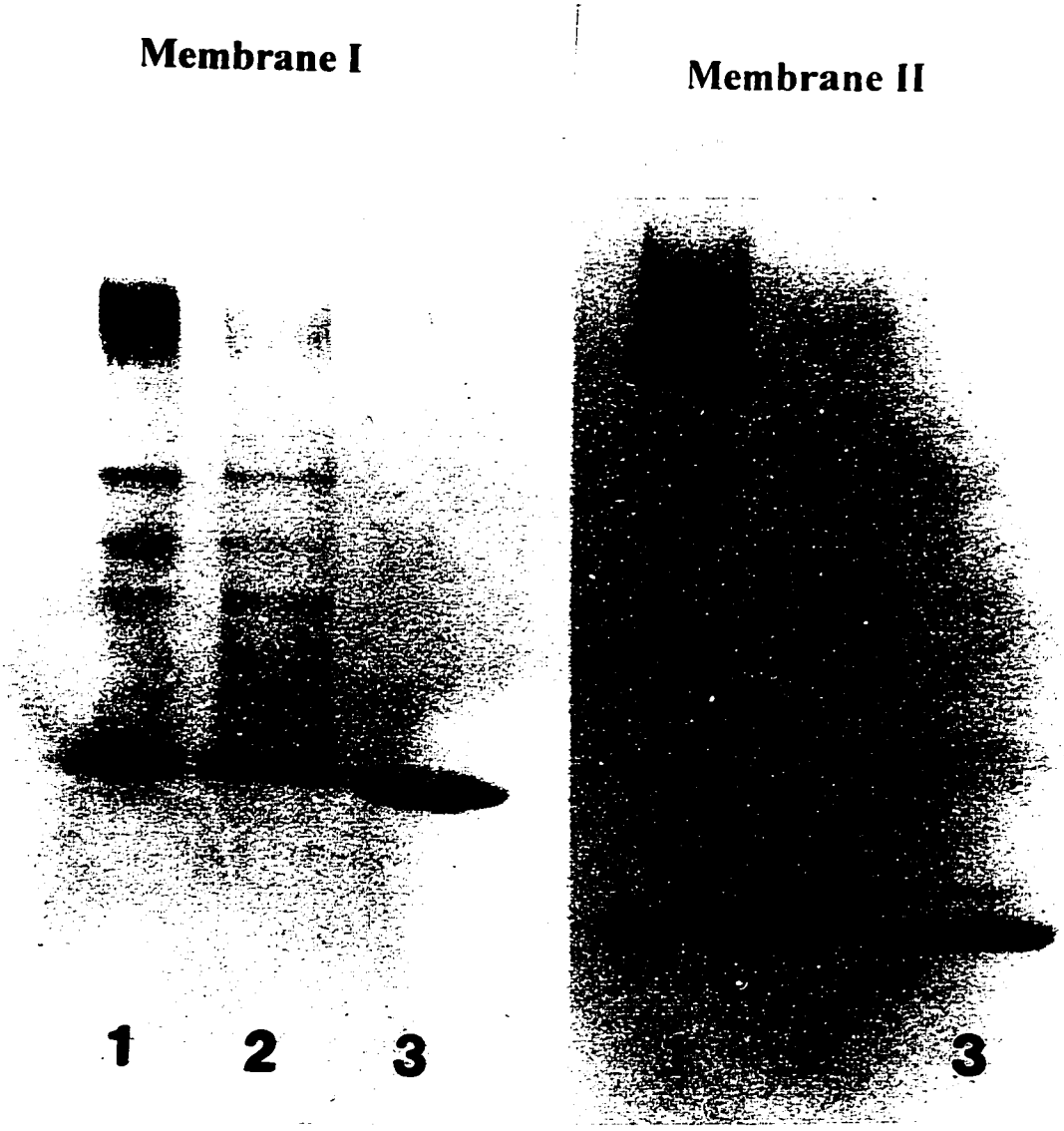

Figure 6. Western-blots of HDL samples after SDS-PAGE. HDL (1 mg/ml) was incubated with either 20% CSE or 0.8 mM acrolein at 37 °C for 6 hours. After incubation, samples containing equal amounts of protein were subjected to SDS-PAGE. Proteins were then Western-blotted to nitrocellulose membranes and probed with either a murine monoclonal antibody against apolipoprotein A-I (Membrane I) or a murine monoclonal antibody against apolipoprotein A-II (Membrane II). The samples on both nitrocellulose membranes are as follow: Lane 3, control HDL; Lane 2, HDL incubated with 20% CSE; Lane 1, HDL incubated with 0.8 mM acrolein.

LCAT Activation Capacity by HDL Samples

Modification of HDL by either CSE or acrolein caused a statistically significant decrease in HDL's capacity to activate LCAT. This reduction in LCAT activation capacity caused by CSE or acrolein was both time-dependent (Figure 7) and concentration-dependent (Figures 8 and 9). As shown in Figure 7, incubation of HDL with 20% CSE for 0.5 hour resulted in a sharp 21% decrease in LCAT activation capacity of HDL. As incubation time increased from 0.5 to 6 hours, there was a progressive decrease in HDL's capacity to activate LCAT. However, the rate of decline in LCAT activation capacity of HDL was not as sharp, especially up to 4 hours. After incubation with CSE for 6 hours, HDL lost 48% of its capacity to activate LCAT. Incubation of HDL with 0.8 mM of acrolein also caused a sharp decrease (24%) in LCAT activation capacity at 0.5 hour (Figure 7). This trend continued for 2 hours, with 33% and 53% decrease of LCAT activation capacity at the end of 1 and 2 hours of incubation, respectively. However, during the next 4 hours, there was only 10% additional decrease of LCAT activation capacity of HDL. As shown in Figure 8, HDL was also sensitive to low concentrations of CSE with respect to being modified and losing some of its capacity to activate LCAT. After incubation with 2.5% CSE for 6 hours, HDL lost 21% of its capacity to activate LCAT. The loss of LCAT activation capacity of HDL increased as HDL was incubated with increasing CSE concentrations. The decreases of LCAT activation capacity were 33%, 39% and 59% after HDL was incubated with 5%, 10% and 20% CSE, respectively, for 6 hours. Acrolein from 0.1 to 0.8 mM produced similar

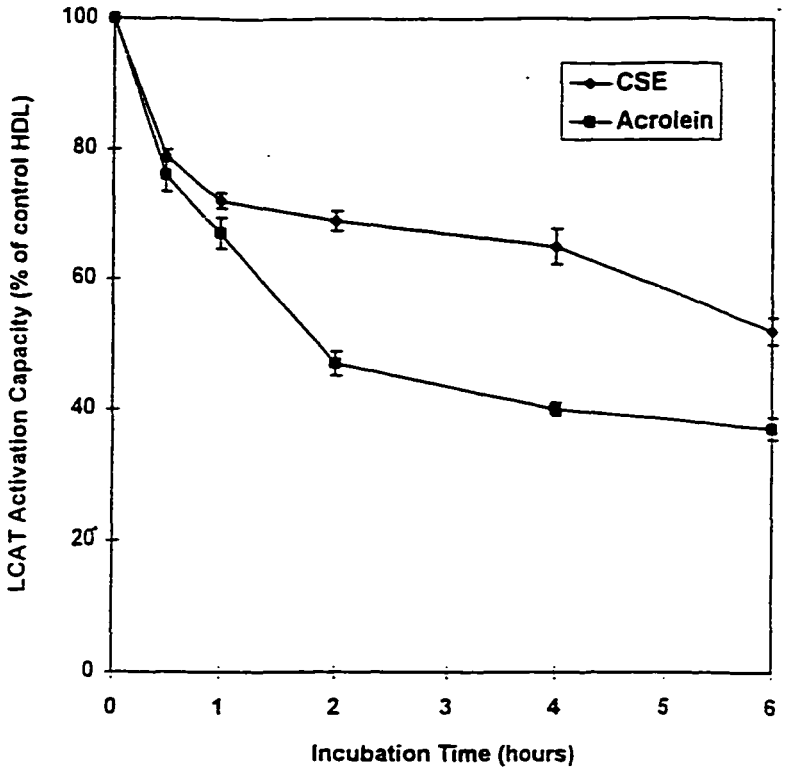

Figure 7. Loss of LCAT activation capacity of HDL after incubation of HDL with either CSE or acrolein. HDL (1 mg/ml) was incubated with either 20% CSE or 0.8 mM acrolein for different time periods up to 6 hours. LCAT activation capacity of treated HDL was then determined. The assay mixture in a total incubation volume of 100 µl contained 25 µg of treated HDL, 10 µl of LCAT enzyme preparation (15 µg protein), 13 µl of albumin-stabilized [4-^{14}C]-cholesterol (10^5 dpm), and 5µl of 0.1 M β-mercaptoethanol. After 30 minutes of incubation at 37 °C, the reaction was terminated by adding 20 volumes of chloroform-methanol (1:1,v/v). Following lipid extraction and then separation of cholesteryl ester from unesterified cholesterol by thin-layer chromatography, radioactivity in the TLC fractions was measured by liquid scintillation spectrometry. LCAT activation capacity was determined as amount of radioactivity detected in the cholesteryl ester fraction and expressed as percentage of control. The values are the means of triplicate incubations using the same batch of CSE. The experiment was repeated three times.

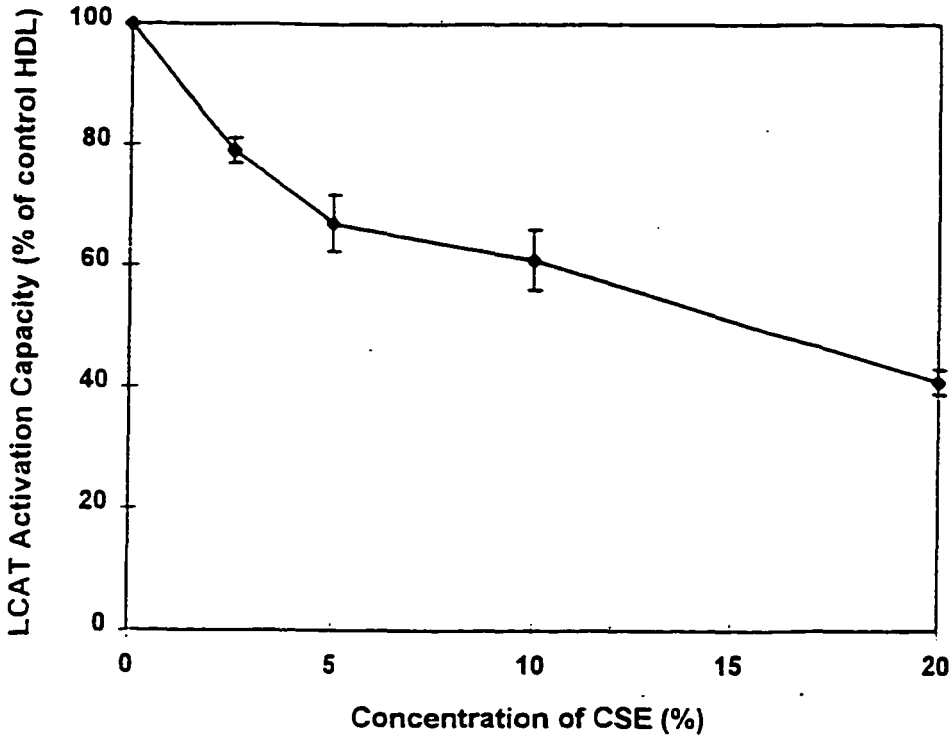

Figure 8. Loss of LCAT activation capacity of HDL after incubation of HDL with different concentrations of CSE. HDL (1 mg/ml) was incubated with different concentrations of CSE for 6 hours. LCAT activation capacity of treated HDL was then determined. The assay mixture in a total incubation volume of 100 µl contained 25 µg of treated HDL, 10 µl of LCAT enzyme preparation (15 µg protein), 13 µl of albumin-stabilized [4-^{14}C]-cholesterol (10^5 dpm), and 5µl of 0.1 M β-mercaptoethanol. After 30 minutes of incubation at 37 °C, the reaction was terminated by adding 20 volumes of chloroform-methanol (1:1,v/v). Following lipid extraction and then separation of cholesteryl ester from unesterified cholesterol by thin-layer chromatography, radioactivity in the TLC fractions was measured by liquid scintillation spectrometry. LCAT activation capacity was determined as amount of radioactivity detected in the cholesteryl ester fraction and expressed as percentage of control. The values are the means of triplicate incubations using the same batch of CSE. The experiment was repeated three times.

reduction of LCAT activation capacity of HDL with 6 hours of incubation (Figure 9). Inhibition of LCAT activation capacity increased from 31% with 0.1 mM acrolein to 39%, 48%, and 63% as acrolein concentrations increased to 0.2, 0.4 and 0.8 mM, respectively.

In accord with results from the previous experiments, both aminoguanidine (Figure 10) and glutathione (Figure 11) prevented loss of LCAT activation capacity of HDL caused by acrolein. When 5 mM aminoguanidine was present as HDL incubated with 0.8 mM acrolein, 89% of HDL's capacity to activate LCAT remained in comparison to 34% LCAT activation capacity of HDL incubated with acrolein in the absence of aminoguanidine (Figure 10). It required a much higher concentration of aminoguanidine (50 mM) to totally prevent the loss of LCAT activation capacity of HDL caused by acrolein. Glutathione at 1 mM provided a similar degree of protection as 5 mM aminoguanidine against acrolein-induced loss of LCAT activation capacity of HDL (Figure 11). A higher concentration of glutathione (5 mM) completely prevented the loss of LCAT activation capacity of HDL caused by acrolein.

Aminoguanidine and glutathione inhibited the loss of LCAT activation capacity of HDL caused by CSE as well (Figures 10 and 11). However, the inhibitory effect of both compounds was less pronounced in comparison to that of acrolein-induced loss of LCAT activation capacity of HDL. Presence of 5, 25 and 50 mM aminoguanidine as HDL was incubated with CSE resulted in an increase in LCAT activation capacity from 41% to 50%, 56% and 62%, respectively. Similarly, with 1, 5 and 10 mM glutathione,

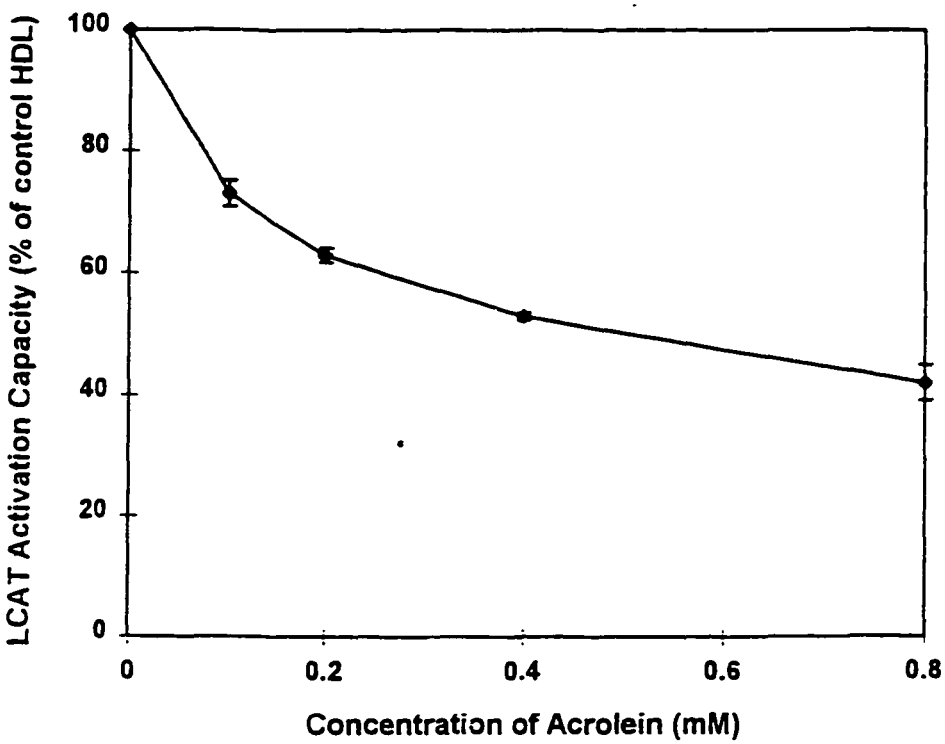

Figure 9. Loss of LCAT activation capacity of HDL after incubation of HDL with different concentrations of acrolein. HDL (1 mg/ml) was incubated with different concentrations of acrolein for 6 hours. LCAT activation capacity of treated HDL was then determined. The assay mixture in a total incubation volume of 100 µl contained 25 µg of treated HDL, 10 µl of LCAT enzyme preparation (15 µg protein), 13 µl of albumin-stabilized [4-^{14}C]-cholesterol (10^5 dpm), and 5µl of 0.1 M β-mercaptoethanol. After 30 minutes of incubation at 37 °C, the reaction was terminated by adding 20 volumes of chloroform-methanol (1:1,v/v). Following lipid extraction and then separation of cholesteryl ester from unesterified cholesterol by thin-layer chromatography, radioactivity in the TLC fractions was measured by liquid scintillation spectrometry. LCAT activation capacity was determined as amount of radioactivity detected in the cholesteryl ester fraction and expressed as percentage of control. The values are the means of triplicate incubations using same batch of CSE. The experiment was repeated three times.

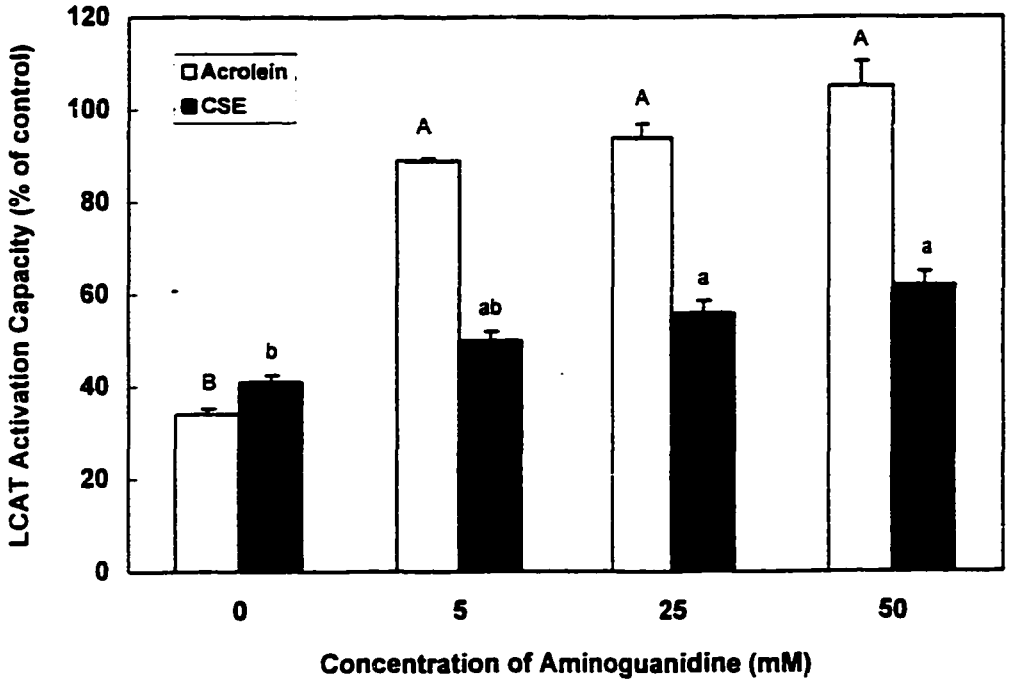

Figure 10. Effects of aminoguanidine on loss of LCAT activation capacity of HDL caused by either CSE and acrolein. HDL (1 mg/ml) was incubated for 6 hours with either 0.8 mM acrolein or 20% CSE in the absence or presence of aminoguanidine. LCAT activation capacity of treated HDL was then determined. The assay mixture in a total incubation volume of 100 µl contained 25 µg of treated HDL, 10 µl of LCAT enzyme preparation (15 µg protein), 13 µl of albumin-stabilized [4-^{14}C]-cholesterol (10^5 dpm), and 5µl of 0.1 M β-mercaptoethanol. After 30 minutes of incubation at 37 °C, the reaction was terminated by adding 20 volumes of chloroform-methanol (1:1,v/v). Following lipid extraction and then separation of cholesteryl ester from unesterified cholesterol by thin-layer chromatography, radioactivity in the TLC fractions was measured by liquid scintillation spectrometry. LCAT activation capacity was determined as amount of radioactivity detected in the cholesteryl ester fraction and expressed as percentage of control. The values are the means of triplicate incubations using the same batch of CSE. The experiment was repeated three times.

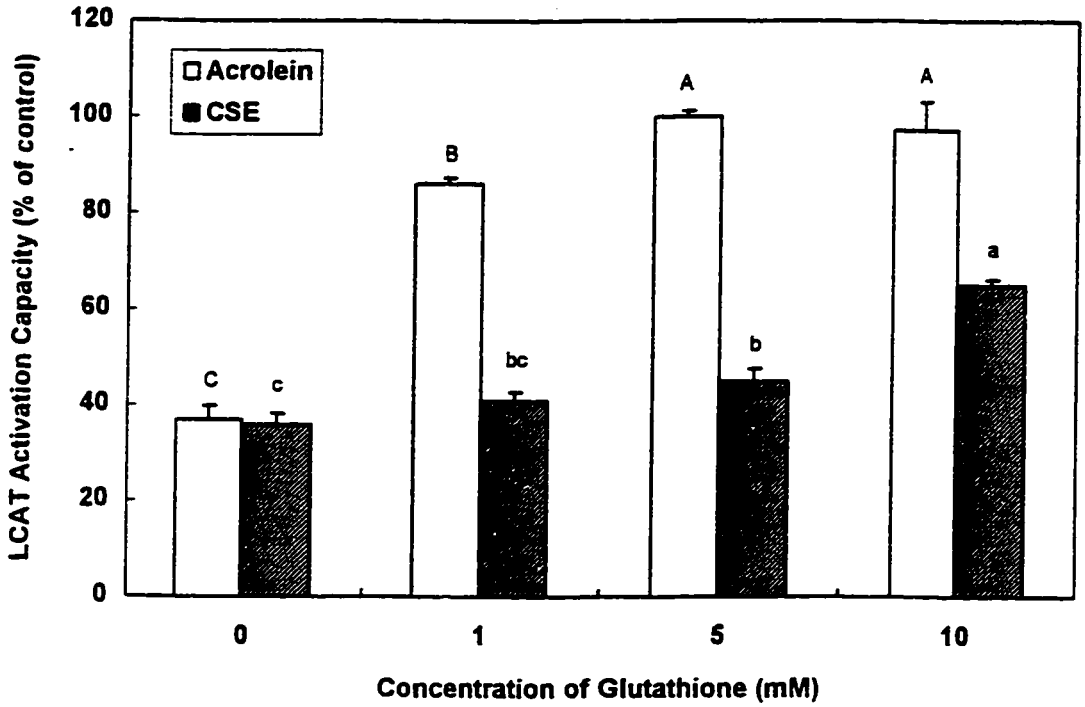

Figure 11. Effects of glutathione on loss of LCAT activation capacity of HDL caused by either CSE and acrolein. HDL (1 mg/ml) was incubated for 6 hours with either 0.8 mM acrolein or 20% CSE in the absence or presence of glutathione. LCAT activation capacity of treated HDL was then determined. The assay mixture in a total incubation volume of 100 µl contained 25 µg of treated HDL, 10 µl of LCAT enzyme preparation (15 µg protein), 13 µl of albumin-stabilized [4-^{14}C]-cholesterol (10^5 dpm), and 5µl of 0.1 M β-mercaptoethanol. After 30 minutes of incubation at 37 °C, the reaction was terminated by adding 20 volumes of chloroform-methanol (1:1,v/v). Following lipid extraction and then separation of cholesteryl ester from unesterified cholesterol by thin-layer chromatography, radioactivity in the TLC fractions was measured by liquid scintillation spectrometry. LCAT activation capacity was determined as amount of radioactivity detected in the cholesteryl ester fraction and expressed as percentage of control. The values are the means of triplicate incubations using the same batch of CSE. The experiment was repeated three times.

LCAT activation capacity of HDL increased from 36% to 41%, 45% and 65%, respectively. LCAT activation capacity was not affected when HDL was incubated with either aminoguanidine or glutathione alone at any tested concentration (data not shown).

Cellular Cholesterol Efflux by HDL samples

After THP-1 cells were incubated with [^{14}C]-sodium acetate for 48 hours and treated with PBS for another 24 hours, the radioactivity associated with total cellular cholesterol was 884±33 DPM/μg protein. Cellular [^{14}C]-cholesteryl esters and [^{14}C]-free cholesterol were 178±14 DPM/μg protein and 706±19 DPM/μg protein, respectively (Figure 12). Incubation of THP-1 cells with bovine serum albumin (BSA, 0.1 mg/ml) in PBS caused a slight decrease in both cellular [^{14}C]-cholesteryl esters and [^{14}C]-free cholesterol to 133±15 DPM/μg and 669±37 DPM/μg protein, respectively. The total cellular [^{14}C]-cholesterol was 802±45 DPM/μg protein. In comparison to the BSA control, LDL (0.1 mg/ml)caused little change in total cellular [^{14}C]-cholesterol (773±28 DPM/μg protein). However, cellular [^{14}C]-cholesteryl esters increased to 282±13 DPM/μg protein and cellular [^{14}C]-free cholesterol decreased to 491±26 DPM/μg protein.

THP-1 cells incubated with control HDL (0.1 mg/ml) had significantly less total cellular [^{14}C]-cholesterol than cells incubated with BSA. HDL caused a 25% decrease in cellular [^{14}C]-cholesteryl esters (from 133±15 to 102±1 DPM/μg protein) and a 14% decrease in cellular [^{14}C]-free cholesterol (from 669±37 to 572±22 DPM/μg protein), resulting in a 16% decrease in total cellular [^{14}C]-cholesterol (from 802±45 to 674±23 DPM/μg protein). After incubation with 20% CSE for 6 hours, HDL did not lose any of

Table III. Cellular [^{14}C]-cholesterol after incubation of THP-1 cells with various experimental components.

Treatment	CE	FC	TC
	(DPM/μg protein)		
PBS	178±13.7^{b}	706±19.2^{a}	884±32.9^{a}
BSA	133±14.7^{cd}	669±37.0^{ab}	802±44.9^{ab}
LDL	282±13.0^{a}	491±25.5^{d}	773±27.5^{bcd}
HDL	102±0.83^{e}	572±22.1^{cd}	674±22.7^{d}
HDL/CSE	107±8.55^{de}	591±19.9^{bc}	698±24.8^{cd}
HDL/Acrolein	112±2.66^{de}	582±16.9^{bc}	694±17.0^{cd}
HDL/Copper	155±11.4^{bc}	623±41.0^{abc}	778±36.7^{bc}

HDL (1 mg/ml) was incubated with either 20% CSE, 0.8 mM acrolein, or 10 μM cupric chloride at 37 °C for 6 hours. THP-1 cells were cultured as described in the "Experimental Methodology") and exposed to the various experimental components for 24 hours. Cellular total [^{14}C]-cholesterol (TC) was determined by liquid scintillation spectrometry following separation of free cholesterol (FC) and cholesteryl esters (CE). The values are the means ± SEM of triplicate cultures. The experiment was repeated three times. Superscripts represent Duncan groupings. Means with the same letter in the column are not statistically significantly different ($P<0.05$).

its capacity to promote cholesterol efflux. THP-1 cells incubated with CSE-treated HDL (0.1 mg/ml) had 107±9 DPM/μg protein [^{14}C]-cholesteryl esters, 591±20 DPM/μg protein [^{14}C]-free cholesterol and 698±25 DPM/μg protein total cellular [^{14}C]-cholesterol. These values are comparable to those in THP-1 cells treated with control HDL. Incubation of HDL with 0.8 mM acrolein for 6 hours also did not have any effect on HDL's capacity to promote cholesterol efflux. THP-1 cells incubated with acrolein-treated HDL (0.1 mg/ml) had 112±3 DPM/μg protein [^{14}C]-cholesteryl esters, 582±17 DPM/μg [^{14}C]-free cholesterol, and 694±17 DPM/μg total cellular [^{14}C]-cholesterol. On the other hand, HDL oxidized with 10 μM copper for 6 hours lost some of its capacity to promote cholesterol efflux from THP-1 cells. Compared to BSA treatment, THP-1 cells incubated with copper-treated HDL (0.1 mg/ml) did not show any decrease in cellular cholesterol with [^{14}C]-cholesteryl esters, [^{14}C]-free cholesterol and total [^{14}C]-cholesterol being 155±11, 623±41 and 778±37 DPM/μg protein , respectively.

CHAPTER VI

DISCUSSION

Epidemiological data indicate that there is an inverse relationship between blood HDL levels and incidence of atherosclerosis [4]. The antiatherogenic effect of HDL has been attributed to its important function in reverse cholesterol transport [2]. Therefore, it is conceivable that modification of HDL may impair reverse cholesterol transport and increase risk of atherosclerosis.

HDL can be oxidized *in vitro* by exposure to different oxidative agents including copper, AAPH and ionizing radiation. During HDL oxidation, lipid peroxidation occurs and results in the formation of fatty acid epoxides, oxidized cholesterol derivatives and lysophophatidylcholine [57]. Lipid peroxidation can be detected by noting the formation of conjugated diene followed by TBARS [27]. Similar to oxidized LDL, oxidized HDL has decreased lysine amino groups coincident with increased agarose gel electrophoretic mobility. Unlike LDL oxidation, HDL oxidation causes apolipoprotein crosslinking instead of apolipoprotein fragmentation. Several studies [25-28] have found that after HDL is oxidatively modified by copper, it loses its capacity to activate LCAT and to stimulate cholesterol efflux.

In the present study, HDL incubated with CSE also had decreased reactive amino groups, increased agarose gel electrophoretic mobility, and crosslinked apolipoproteins.

However, it seems that CSE modifies HDL in a non-oxidative manner based on previous work demonstrating that CSE has antioxidant activity by virtue of its ability to inhibit LDL oxidation [39]. Furthermore, ascorbate from 0.1 to 10 mM was not able to inhibit modification of HDL by CSE (data not shown). In another study, plasma HDL modified by gas-phase cigarette smoke shared similar physicochemical properties, including increased electrophoretic mobility and apolipoprotein crosslinking [40]. Also, lipid peroxidation was not detected by measuring TBARS and fatty acid composition in HDL. Therefore, modification of HDL by cigarette smoke does not appear to be mediated by any free radicals.

The most important finding of the present study is that HDL lost a significant extent of its capacity to activate LCAT after modification by CSE. The LCAT activation capacity of HDL was very sensitive to the CSE treatment. After incubation with a low concentration of 2.5% CSE for 6 hours, HDL lost 21% of its capacity to activate LCAT. Incubation of HDL with 20% CSE for half an hour decreased its capacity to activate LCAT by 24%. In another study by the author [44], it was demonstrated that LCAT activity decreased rapidly upon incubation of plasma with CSE. It is possible that modification of HDL in the plasma by CSE also contributed to the loss of LCAT activity observed in that study [44]. Since LCAT activity *per se* was not decreased in cigarette smokers as reported in several studies [58-60], this new finding concerning an effect of CSE on HDL, as opposed to an effect of CSE on LCAT, provides the rationale to further examine possible impairment of LCAT function in cigarette smokers.

It is apparent that other chemical species in CSE caused non-oxidative modification of HDL. Cigarette smoke contains many reactive aldehydes [38]. Reactive aldehydes can react covalently with free amino groups, such as ε-amino groups of lysine, via nucleophilic addition to form Schiff's base products [43]. Lysine and arginine residues make up a significant portion of the amino acid composition of apolipoprotein A-I [61]. Reaction of reactive aldehydes in CSE with free amino groups of lysine and arginine in apolipoprotein A-I could reduce overall positive charge on this protein, which would lead to increased electrophoretic mobility of HDL on agarose gel. It has been suggested that positive charges on the lysine and arginine residues of apolipoprotein A-I are important for LCAT activation [62]. Reactive aldehydes can also cause apolipoprotein crosslinking in HDL [28, 46].

To provide support to the hypothesis that reactive aldehydes are involved in the modification of HDL by CSE, we compared CSE with acrolein, a major reactive aldehyde in cigarette smoke, in terms of their abilities to modify HDL. Acrolein and CSE had similar effects on HDL. Both CSE and acrolein caused a decrease in free amino groups, increase in electrophoretic mobility, and apolipoprotein crosslinking. In addition, both CSE and acrolein modified HDL in such a way as to cause HDL to have decreased capacity to activate LCAT. Thus, the data indicate that acrolein and CSE modified HDL in a similar manner, resulting in loss of apolipoprotein A-I-dependent LCAT activation capacity of HDL.

Although reactive aldehydes are apparently implicated, other chemical components in cigarette smoke may also be involved in the modification of HDL by CSE. This possibility is supported by other experiments we performed. More specifically, aminoguanidine and glutathione were tested to determine if either compound could inhibit HDL modification caused by CSE and acrolein. Both aminoguanidine and glutathione are very reactive toward aldehydes that can modify protein amino groups [43, 48]. It has been shown that aminoguanidine can prevent protein crosslinking initiated by glycosylation in diabetes [63]. Glutathione can inhibit loss of LCAT activity in human plasma exposed to either cigarette smoke or CSE [40, 44, 47]. Thus, the presence of aminoguanidine and glutathione during incubation of HDL with CSE should protect free amino groups of HDL from attack by reactive aldehydes. As expected, both aminoguanidine and glutathione were able to exert this effect and thus completely prevented HDL modification and subsequent loss of LCAT activation capacity of HDL caused by acrolein. However, when tested against CSE-mediated HDL modification, both aminoguanidine and glutathione completely prevented only the increase in electrophoretic mobility but not apolipoprotein crosslinking of HDL. The inhibitory effects of aminoguanidine and glutathione on loss of LCAT activation capacity of HDL caused by CSE were also incomplete. Hence, other class(es) of chemicals in CSE besides reactive aldehydes remains to be examined for capacity to modify HDL and cause loss of LCAT activation capacity of HDL.

Structural characterization of apolipoprotein crosslinking of HDL after incubation with CSE has not been attempted. Besides lysine and arginine, other amino acids of apolipoproteins might be affected by cigarette smoke components. Cigarette smoke is able to modify tyrosine and generate a dimeric product referred to as dityrosine [64]. The modification of tyrosine by cigarette smoke is thought to be mediated by nitrogen compounds, such as NO and NO_2. This type of modification was partially inhibited by certain antioxidants, including glutathione, ascorbic acid and uric acid. In our work, it is possible that tyrosine residues of HDL apolipoproteins were modified and crosslinked by CSE which might contain NO/NO_2 [65]. The importance of tyrosine residues in LCAT activation by apolipoprotein A-I is not known. On the other hand, modification of HDL by copper and malondialdehyde decreases tryptophan residues, in addition to free amino groups, of HDL apolipoproteins, and the modified HDL had decreased capacity to activate LCAT [28].

Although apolipoproteins are modified when HDL is incubated with CSE, phospholipids of HDL could possibly be modified also in a manner not involving lipid peroxidation. Phosphatidylethanolamine (PE) contains a free amino group that can react with glucose-derived carbonyls to form Schiff's base products [66]. Therefore, PE of HDL might react with reactive aldehydes in CSE as well. Since PE is implicated in the binding of LCAT to HDL [67], CSE-induced modification of PE could limit availability of HDL substrate to LCAT enzyme molecules. In addition, two other studies [68, 69] have found that PE and also phosphatidylserine can crosslink with apolipoproteins when

HDL is incubated with either dimethylsuberimidate (DMS) or tetranitromethane (TMN). However, it is not known if these compounds are present in cigarette smoke. Nevertheless, it is possible that reactive chemicals in cigarette smoke, including reactive aldehydes, may also cause crosslinking of apolipoproteins to phospholipids in HDL.

Although CSE-mediated modification decreased HDL's capacity to activate LCAT, it did not change HDL's capacity to promote cholesterol efflux from THP-1 cells. In contrast, HDL oxidized by copper showed a decreased capacity to mediate cholesterol efflux. The phenomenon of HDL-mediated cholesterol efflux has been studied for more than a decade and the mechanism is still under debate. Cholesterol efflux may be mediated by either HDL lipids or HDL apolipoproteins or both. In the diffusion-based efflux model, HDL phospholipid content and its ratios to HDL apolipoproteins and cholesterol determine the efficiency of cholesterol removal. In the receptor-dependent efflux model, the binding of HDL to its receptor on cell surface trigger the release of intracellular cholesterol to HDL particles.

Based on the data in the current study, it might be concluded that CSE did not affect HDL to cause either lipid/protein ratio changes or loss of binding affinity to HDL receptors. Oxidation modifies both lipid and protein moieties of HDL particles. Oxidized HDL also has reduced ligand binding activity to macrophages [26]. It is very difficult to differentiate the effects of lipid and protein oxidation on HDL-mediated cholesterol efflux. However, Morel [70] demonstrated that cholesterol efflux capacity of HDL decreased before HDL apolipoprotein crosslinking occurred under mild oxidation

conditions with copper. There were only a decrease in total phospholipids and an increase in TBARS. Agarose gel electrophoretic mobility also did not change. Rifici *et al.* [27] further demonstrated a negative linear correlation ($r = -0.97$) between cholesterol efflux and TBARS formation in oxidized HDL. Since lipid-free apolipoproteins are known to promote cholesterol efflux [71], they isolated apolipoproteins from oxidized HDL and compared its capacity to promote cholesterol efflux with apolipoproteins isolated from control HDL. The oxidized apolipoproteins had similar effect on cholesterol efflux as the control apolipoproteins. Therefore, HDL lost its capacity to stimulate cholesterol efflux because of lipid oxidation but not protein oxidation. In the present work, CSE-modified HDL did not seem to have elevated levels of TBARS and did not have reduced capacity to stimulate cholesterol efflux.

Some studies demonstrated that an HDL binding protein increased after cells were enriched with cholesterol [10, 11, 26]. This seems to suggest that an HDL receptor facilitates cholesterol removal from the cells. One study [26] found that crosslinking of HDL apolipoproteins by TNM caused a parallel decrease of HDL binding activity to macrophages and HDL-mediated cholesterol efflux from the macrophages. However, TNM also induces lipid peroxidation of HDL which may contribute to the decrease in cholesterol efflux. On the other hand, DMS also caused crosslinking of HDL apolipoproteins but did not induce lipid peroxidation [69, 72]. The apolipoprotein crosslinking was associated with decreased HDL ligand binding activity to fibroblasts but without any change in cholesterol efflux from the fibroblasts. Although CSE caused

apolipoprotein cross-linking, it did not induce lipid peroxidation of HDL. Therefore, modification of HDL by CSE did not decrease HDL-mediated cholesterol efflux from macrophages.

In summary, we demonstrated in the present study that CSE modified HDL into a form with decreased capacity to activate LCAT. It is conceivable that such modification might impair HDL's antiatherogenic function in reverse cholesterol transport in cigarette smokers. Three studies have found that LCAT activity is not decreased in cigarette smokers [58-60]. However, there has been no study examining possible HDL modification and functional impairment of HDL in cigarette smokers. Therefore, data from this study warrant further research on HDL metabolism and function in cigarette smokers.

CHAPTER VII

EPILOGUE

The present study demonstrated that CSE modified HDL *in vitro* and the modified HDL had decreased capacity to activate LCAT. Although HDL oxidation has been studied intensively *in vitro*, there is no direct evidence that oxidized HDL exists *in vivo*. HDL has antioxidant activity and protects LDL against lipid oxidation [73, 74]. Therefore, it could be very difficult to demonstrate that oxidative modification of HDL occurs *in vivo*. However, glycosylation of HDL occurs in diabetic patients [75]. Glucose is an aldehyde and "reactive aldehydes" are present in cigarette smoke. Therefore, aldehyde addition can occur even though HDL is resistant to oxidation. Since cigarette smoke modifies HDL *in vitro* rapidly, it is possible that similar modification may occur *in vivo* and be detectable in heavy smokers.

Two approaches might be used to detect modified HDL in cigarette smokers. First, autoantibodies against HDL modified by cigarette smoke could be measured in cigarette smokers. The human immune system may produce autoantibodies against modified HDL since modified HDL may be highly immunogenic, as in the case of oxidized LDL [76]. Two groups of human subjects would be needed for the study. One would be cigarette smokers and the other non-smoking control subjects. To quantify autoantibodies, a solid-phase radioimmunoassay that was developed to measure

autoantibodies against oxidized LDL [77] could be adapted. In this assay, antigens, such as HDL modified by cigarette smoke *in vitro*, would be coated at the bottom of microtiter plate wells. After remaining binding sites are blocked with 2% BSA, serum dilutions from human subjects would be added in the wells for an overnight incubation. The antigen-antibody complexes would be then detected by adding an iodinated monoclonal antibody specific for human IgG, IgA, or IgM. To increase the sensitivity, immunoglobulins could be purified from human serum by the conventional method [78] and used in the assay.

The second approach would be to conduct a sensitive functional test of HDL and compare the results from cigarette smokers with those from nonsmokers. We found in this study that LCAT activation capacity of HDL decreased when HDL was modified. Since HDL lost its LCAT activation capacity very rapidly upon the incubation with CSE, the functional test may be sensitive enough to detect a difference in LCAT activation capacity of HDL between cigarette smokers and the non-smoking control subjects. Because of its importance in reverse cholesterol transport, LCAT enzyme itself has been studied in cigarette smokers. Although cigarette smokers have less LCAT mass [79], their LCAT activity did not change compared to their nonsmoking counterparts [58-60]. The assay of LCAT activation capacity of HDL might be more appropriate than the LCAT activity assay in studying HDL-mediated reverse cholesterol transport in cigarette smokers.

The present study did not examine the impact of HDL modification on the final step of reverse cholesterol transport, which is the transport of cholesterol back to the liver for disposal. The recent identification of a specific HDL receptor [13] makes this research possibility feasible in a future study. The identified HDL receptor is expressed in the liver and nonplacental steroidogenic tissues including ovary, adrenal gland, testis and mammary gland. The major function of the receptor is to facilitate selective delivery of cholesterol esters to the tissues, finishing the reverse cholesterol transport by removal of HDL cholesterol from the circulation. Therefore, this HDL receptor is different from other HDL receptors expressed in fibroblasts and macrophages. These latter HDL receptors may be involved in facilitating cholesterol efflux or intracellular signaling [80]. To measure selective cholesterol delivery via the liver HDL receptor, HDL (native or modified) can be radiolabelled with [^{3}H]-cholesteryl oleate and then incubated with hepatocytes. Following the incubation, cellular [^{3}H]-cholesterol and total cholesterol can be determined.

Modification of HDL by cigarette smoke might result in the impairment of reverse cholesterol transport and reduce the protective effects of HDL against atherosclerosis in cigarette smokers. It is of practical importance to identify compounds that can inhibit HDL modification by cigarette smoke. In the present study, the antioxidant ascorbate was not effective. Although aminoguanidine and glutathione protected HDL from CSE-induced modification to a large extent, the concentrations were very high and not physiological. More importantly, each compound only provided limited

protection against CSE-mediated loss of LCAT activation capacity of HDL even at such high concentrations. To identify effective compounds that inhibit HDL modification by cigarette smoke, a better understanding of the mechanism of the modification is indispensable. Future study is in need to identify the toxic chemicals in cigarette smoke that are responsible for the modification of HDL. Only with that information, a strategy to inhibit this process can be developed. In the meantime, curtailment of cigarette smoking is recommended to decrease the risk of coronary heart disease.

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