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Changes in metabolism, composition, and function of high-density lipoproteins during the acute-phase response

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Changes in Metabolism, Composition, and Function of High-density Lipoproteins During the Acute-phase Response

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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of the

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To my parents, my brother, and my sisters

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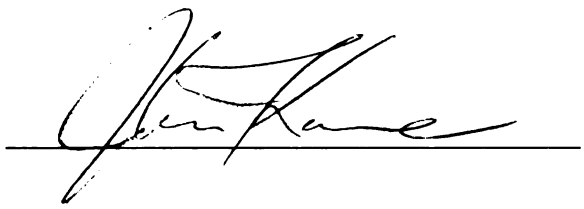
This thesis has been completed in fulfillment of the requirement of the Biomedical Sciences Graduate Program at the University of California in San Francisco. Part of the thesis is a reprint of the materials as they appear in Journal of Lipid Research. These works are comparable to a standard thesis. The co-authors listed in the publications helped direct and supervise the research that forms the basis for the thesis.

Changes in Metabolism, Composition, and Function of High-density Lipoprotein During the Acute-phase Response

Weerapan Khovidhunkit, M.D.



John P. Kane, M.D., Ph.D.

Chair, Thesis committee

ABSTRACT

Multiple changes in high-density lipoprotein (HDL) occur during the host response to infection and inflammation. Using in vivo models of hamsters and mice injected with endotoxin, and an in vitro model of cells treated with endotoxin and cytokines, changes in the metabolism, composition, and function of HDL during the acute-phase response (APR) were studied. Endotoxin decreased levels of two ATP-binding cassette (ABC) transporters involved in cholesterol efflux, ABCA1 and ABCG1, in J774 murine macrophages. Cholesterol flux experiments showed that acute-phase HDL, isolated from animals injected with endotoxin, was less efficient in promoting

cholesterol removal from cells. The defect was due to both decreased cholesterol efflux and increased cholesterol influx, causing cholesterol accumulation in cells. Lower activity of lecithin:cholesterol acyltransferase in acute-phase HDL was likely the mechanism. In rodent liver, endotoxin downregulated levels of scavenger receptor class B type I, which resulted in a decrease in cholesterol ester uptake into hepatocytes. In addition, endotoxin decreased hepatic mRNA levels of ABCG5 and ABCG8, two transporters proposed to efflux sterols into the bile. Collectively, these data show that many steps of reverse cholesterol transport are impaired during the APR. The protein composition of acute-phase HDL was also examined using a combination of two-dimensional gel electrophoresis, mass spectrometry, and immunoblot analysis. Acute-phase HDL had increased levels of apolipoprotein (apo) A-IV and A-V but decreased apo A-II. While levels of apo C-I, C-II, and C-III were decreased in acute-phase HDL, an increase was observed in acute-phase VLDL, suggesting a redistribution. Finally, hamster apo A-I immunoaffinity columns were developed to purify HDL. A protein not previously known to be associated with HDL, parotid secretory protein (PSP), was identified and the cDNA of hamster PSP was cloned. In addition to the salivary glands, PSP was expressed in the lung, skin, and gonads. PSP sequence contains a region homologous to an amino terminus of a family of lipid transfer proteins including bactericidal/permeability-increasing protein, lipopolysaccharide binding protein, cholesterol ester transfer protein, and phospholipid transfer protein. The levels of PSP were increased in hamster HDL after endotoxin injection, suggesting that PSP might be involved in the innate immune response.

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List of Abbreviations

ABC	ATP-binding cassette
Apo	Apolipoprotein
APR	Acute-phase response
BPI	Bactericidal/permeability-increasing protein
BSA	Bovine serum albumin
CE	Cholesterol ester
CETP	Cholesterol ester transfer protein
CID	Collision-induced dissociation
cpt-cAMP	8-(4-chlorophenylthio) adenosine 3', 5'-cyclic monophosphate
DME	Dulbecco's modified Eagle's minimum essential media
FCS	Fetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HDL	High-density lipoprotein
HL	Hepatic lipase
HSA	Human serum albumin
IL-1	Interleukin-1
IPG	Immobilized pH gradient
LBP	Lipopolysaccharide-binding protein
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low-density lipoprotein
LPDS	Lipoprotein-deficient serum

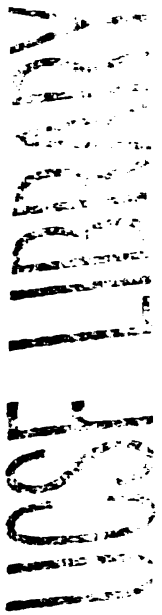
LPS	Lipopolysaccharide
LRP	LDL receptor-related protein
LXR	Liver X receptor
MALDI-MS	Matrix-assisted laser desorption ionization-mass spectrometry
NCBI	National Center for Biotechnology Information
NEM	N-ethyl maleimide
PAF-AH	Platelet-activating factor acetylhydrolase
pI	Isoelectric point
PLUNC	Palate, lung, and nasal epithelium clone
PON	Paraoxonase
PSP	Parotid secretory protein
RACE	Rapid amplification of cDNA ends
RCT	Reverse cholesterol transport
RXR	Retinoid X receptor
SAA	Serum amyloid A
SEM	Standard error of the mean
$sPLA_2$	Secretory phospholipase A_2
SR-BI	Scavenger receptor class B type I
TG	Triglyceride
TNF	Tumor necrosis factor
VLDL	Very low-density lipoprotein

CHAPTER 1

INTRODUCTION

Atherosclerosis is a major cause of morbidity and mortality in adults. It can be manifested as coronary artery disease, cerebrovascular disease, and peripheral vascular disease. The pathogenesis of atherosclerosis is complex and multiple risk factors for atherosclerosis have been identified (1, 2). Because a high level of low-density lipoprotein (LDL) cholesterol is an important risk factor for atherosclerosis, it is widely believed that high levels of LDL stimulate cellular uptake of cholesterol, thus promoting atherosclerosis.

Interests in the physiologic role of high-density lipoprotein (HDL) and its relationship with atherosclerosis emerged when several epidemiologic studies consistently found that plasma levels of HDL cholesterol were strongly and inversely correlated with the risk of coronary artery disease (3). HDL is, therefore, thought to protect against atherosclerosis, and several anti-atherogenic properties of HDL have been postulated. One of the most commonly accepted hypotheses implicates HDL as a key mediator in the removal of cholesterol from cells.



HDL, REVERSE CHOLESTEROL TRANSPORT, AND ATHEROSCLEROSIS

HDL is a group of heterogeneous lipoprotein particles that was initially described as lipoproteins in the density range of 1.063-1.21 g/mL when isolated by ultracentrifugation. Among lipoproteins, HDL particles are the smallest and have the highest density in the plasma. Because the particles float in the density range that was higher than 1.006-1.063 g/mL for low-density lipoproteins (LDL) and 0.9-1.006 g/mL for very low-density lipoproteins (VLDL), they are collectively called HDL. It is now known

that HDL particles are heterogeneous in terms of size, density, and composition. HDL metabolism is complex and its fate is regulated by a number of apolipoproteins, enzymes, and transfer proteins.

Similar to other lipoproteins, HDL is composed of lipids and proteins; however, the composition of both lipids and proteins in HDL is distinct from that of other lipoproteins. Approximately half of the HDL is composed of lipids whereas the other half is protein. The major lipids of HDL are cholesterol and phospholipids, whereas low amounts of triglycerides and sphingolipids are also present. The main protein of HDL is apolipoprotein (apo) A-I, which not only is a structural component of HDL but also plays a major role in the function of HDL. Besides apo A-I, more than 30 other proteins have been known to be associated with HDL. Each of them plays a distinct role in the metabolism and function of HDL.

Apo A-I is secreted by the liver and intestine. Interaction between apo A-I and phospholipids results in small, discoidal prebeta HDL particles. These particles are efficient acceptors of cholesterol from plasma membrane and can be converted into larger spherical HDL particles when free cholesterol is esterified into cholesterol ester by an enzyme lecithin:cholesterol acyltransferase (LCAT). Spherical HDL particles once in circulation are continuously remodeled by several enzymes and transfer proteins. Cholesterol ester transfer protein (CETP) exchanges cholesterol ester in HDL for triglycerides in triglyceride-rich lipoproteins. Phospholipid transfer protein (PLTP) transfers phospholipids from triglyceride-rich lipoproteins into HDL and promotes remodeling of HDL into larger and smaller particles. Hepatic lipase (HL) also hydrolyzes

triglycerides and phospholipids in spherical HDL and generates small prebeta particles, therefore, completing the HDL cycle.

One of the main functions of HDL in normal physiology is to remove cholesterol from peripheral tissues and transfer to the liver for catabolism and excretion. This process is known as reverse cholesterol transport (RCT, **Fig.1**) (4-6). One of the early steps in atherogenesis is lipid accumulation in macrophages, resulting in foam cell formation. By facilitating cholesterol removal from cells, HDL is believed to exert anti-atherogenic effects. RCT begins when free (unesterified) cholesterol is transferred from peripheral cells (i.e. macrophage foam cells) to the cholesterol acceptor in the process called cholesterol efflux. Two major mechanisms of cholesterol efflux have been proposed: an apolipoprotein-mediated mechanism and a diffusion mechanism (7, 8). The contribution of each pathway is dependent upon the type of HDL subspecies and the cell type as well as the cholesterol content and growth state of the cells. In the apolipoprotein-mediated mechanism, cholesterol in plasma membrane of peripheral cells is transported to free apolipoproteins or small discoidal prebeta HDL. This step requires apoA-I on HDL and an ATP-binding cassette transporter 1 (ABCA1) on the cell surface (9). Free cholesterol can also move from plasma membrane to HDL by the diffusion mechanism. In this pathway, fully lipidated HDL is the main cholesterol acceptor. Free cholesterol, which spontaneously desorbs from the cell surface, is taken up by HDL. However, cholesterol can also travel from HDL into cells (cholesterol influx); therefore in order for net cholesterol removal to occur, cholesterol efflux must be greater than cholesterol influx. LCAT plays a key role in cholesterol removal by converting free cholesterol on HDL into cholesterol ester, thereby maintaining the free cholesterol gradient. This gradient is

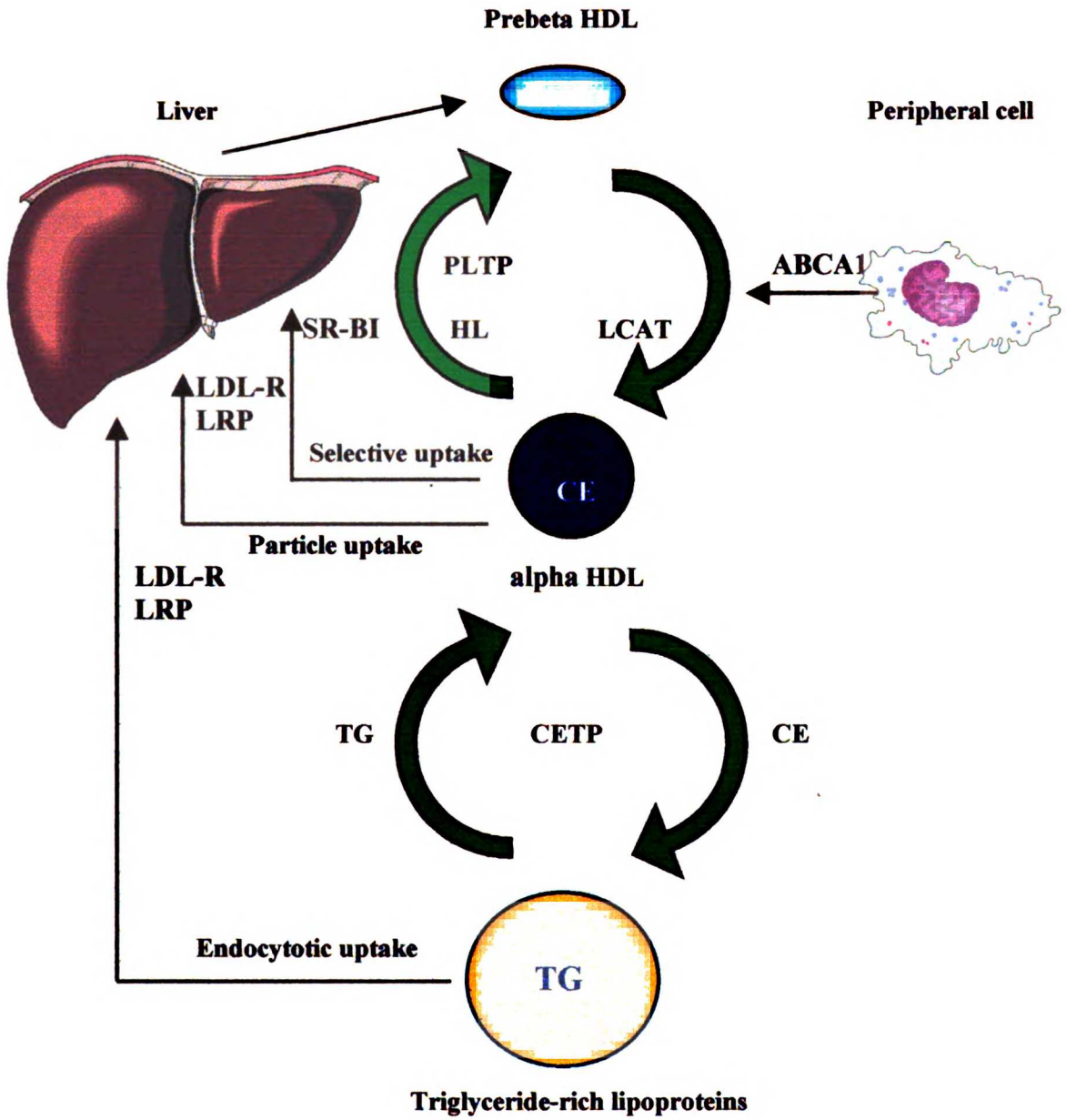
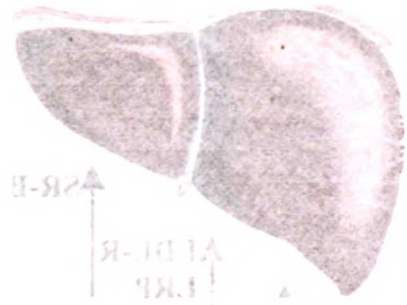


Fig. 1 Reverse cholesterol transport. The liver generates apo A-I, which acquires phospholipids and cholesterol from cells. ABCA1 mediates cholesterol efflux from cells to prebeta HDL. Lecithin:cholesterol acyltransferase (LCAT) esterifies free cholesterol so HDL becomes larger. Cholesterol ester (CE) in HDL is returned to the liver by 1) selective uptake of CE by scavenger receptor class B type I (SR-BI), 2) HDL particle uptake via LDL receptor (LDL-R) or LDL receptor-related protein (LRP), or 3) exchange of CE with triglyceride (TG) in triglyceride-rich lipoproteins by cholesterol ester transfer protein (CETP), and CE on TG-rich lipoproteins is endocytosed by LDL-R or LRP.



necessary for preventing cholesterol influx back into cells and allowing more cholesterol efflux to occur. Cholesterol ester generated by LCAT is accumulated in the core of HDL particles, resulting in larger α HDL.

After cholesterol ester is formed on HDL, it can be transported to the liver by several pathways. First, in the presence of CETP, cholesterol ester from HDL is transferred to triglyceride-rich lipoproteins in exchange for triglycerides. Cholesterol ester in triglyceride-rich lipoproteins can be delivered to the liver via the LDL receptor or the LDL receptor-related protein. Second, HDL can also be taken up as intact particles by the hepatocytes in an endocytotic process, therefore directly delivering cholesterol ester to the liver. Last, cholesterol ester in HDL can be preferentially taken up without protein degradation in a process called selective uptake of cholesterol ester. This process is mediated by the scavenger receptor class B type I (SR-BI) on the liver parenchymal cells. Once delivered to the hepatocytes, the majority of cholesterol is converted into bile acids by a series of enzymes in the liver and excreted into the bile. Some of the free cholesterol can also be directly transported into the bile. ABCG5 and ABCG8 are two ATP-binding cassette (ABC) transporters that have been proposed to mediate the transport of cholesterol and other sterols from hepatocytes into the bile.

By facilitating several steps along the RCT pathway, HDL plays a critical role in removing excess cholesterol from cells and returning it to the liver for catabolism and excretion, thus protecting against atherosclerosis.

INFECTION/INFLAMMATION, THE ACUTE-PHASE RESPONSE, AND ATHEROSCLEROSIS

Recently, growing evidence suggests a link between atherosclerosis and chronic infection and inflammation. Epidemiologic data showed that *Chlamydia pneumoniae*, *Helicobacter pylori*, cytomegalovirus, dental infections, chronic bronchitis, rheumatoid arthritis, systemic lupus erythematosus, and psoriasis are associated with an increased risk of atherosclerosis (10-17). Increased levels of plasma C-reactive protein, a marker of systemic inflammation, predict future risk of coronary events (18). A number of investigations have shown that certain infectious agents are found in the arterial wall and atherosclerotic lesions, however, in other infections and inflammatory states, it is unclear how they are associated with atherosclerosis.

During infection and inflammation, a wide range of alterations in plasma proteins occurs. These alterations are part of the body's reaction known as the acute-phase response (APR) (19). Levels of positive acute-phase proteins, such as C-reactive protein and serum amyloid A, increase during the APR, whereas levels of negative acute-phase proteins, such as albumin and transferrin, decrease. The increase in acute-phase proteins is aimed to modulate the inflammatory response by directly neutralizing foreign agents, minimizing the extent of tissue damage, and participating in tissue regeneration. However, the changes of APR, if present for a prolonged period of time, can lead to detrimental consequences on the host, a typical example of which is the development of secondary amyloidosis after chronic infection or inflammation. Cytokines, such as tumor necrosis factor (TNF) or interleukin-1 (IL-1), are now recognized to be prime mediators of these metabolic changes during infection and inflammation (19). The APR is species-

specific with both the magnitude and directions of changes varying from species to species.

Infection and inflammation can also perturb lipoprotein metabolism and produce a wide variety of changes in the plasma concentrations of lipids and lipoproteins as part of the APR (20). Increased triglyceride levels due to an increase in VLDL are characteristic changes during infection and inflammation (21, 22). Consequently, there is an appearance of small dense LDL (23), a particle believed to be more proatherogenic (24). A number of changes related to HDL occur during the APR. Because these lipoprotein changes are similar to those proposed to promote atherogenesis, they may initiate or aggravate atherosclerosis if the course of infection or inflammation is prolonged.

CHANGES IN HDL METABOLISM AND COMPOSITION DURING THE ACUTE-PHASE RESPONSE

Infection and inflammation are characteristically associated with a decrease in levels of HDL cholesterol (21, 22, 25, 26). The mechanism of this decrease of HDL has not been firmly established, and several hypotheses have been proposed including an increase in apo serum amyloid A (SAA), an increase in secretory phospholipase A_2 ($sPLA_2$), a decrease in LCAT, and a decrease in HL activity. All of these changes are known to occur during the APR (25, 27-35).

The increase in apo SAA content in HDL has been widely regarded as the cause of decreased apo A-I and HDL during infection and inflammation. Apo SAA is an HDL-associated apolipoprotein, the level of which can increase several fold during the APR. Several studies have shown that addition of apo SAA in vitro can displace apo A-I from

HDL (28, 36-38), and that apo SAA-rich HDL particles are more rapidly cleared from the circulation compared to normal HDL (39-41). However, a study in mice in which apo SAA was highly induced to the levels comparable to those seen in the APR but in the absence of other acute-phase changes has shown that there were no changes in HDL cholesterol or apo A-I levels (42). This study, therefore, suggests that high levels of apo SAA *per se* in the absence of the APR do not decrease HDL or apo A-I levels.

More recently, an increase in $sPLA_2$ has been proposed to be a cause of a reduction of HDL during the APR. $sPLA_2$ is a phospholipase enzyme that hydrolyzes phospholipids in HDL and it is highly induced during the APR (32). Mice overexpressing $sPLA_2$ have reduced HDL concentrations (43, 44), and HDL from these mice was catabolized more rapidly than HDL from normal mice (45). Although apo SAA in known to activate $sPLA_2$, overexpression of SAA in addition to $sPLA_2$ did not cause more reductions in levels of HDL cholesterol or apo A-I, further suggesting that the reduction of HDL during infection and inflammation is not caused by an increase in apo SAA (46).

Other changes of HDL metabolism during the APR have also been postulated to explain the reduction of HDL and apo A-I. Lower LCAT activity, for example, may result in a decrease in HDL cholesterol levels due to impaired esterification as observed in humans or animals with mutations in the LCAT gene. A decrease in HL may reduce preβ HDL generation. Which of these changes primarily responsible for the reduction of HDL and apo A-I during the APR, however, is not known.

Not only HDL cholesterol levels decrease during the APR, but other characteristics of HDL is also markedly different from those of normal HDL. HDL that circulates during the APR, called acute-phase HDL, appears to be larger than normal

HDL_3 with the radius extending into the HDL_2 range (28, 29, 36, 47), but has the density comparable to HDL_3 (28, 47). Recent reports show that there is a decrease in small and large apo A-I-containing particles, an increase in apo E-containing HDL, and an appearance of SAA-containing HDL devoid of apo A-I (47, 48).

Acute-phase HDL has distinct lipid and protein composition from normal HDL. Some conflicting reports regarding the composition of acute-phase HDL may be related to the difference among species and the methods of APR induction (31). In general, most investigators use endotoxin or cytokines to induce the APR in animals, which closely mimics acute infection in humans.

Compared with normal HDL, acute-phase HDL is depleted in cholesterol ester but enriched in free cholesterol, triglycerides, and free fatty acids (25, 33, 34, 49). Phospholipid content of acute-phase HDL was increased in some studies (25, 26) but decreased in others (28, 29), depending upon the different models of APR induction. In patients who underwent bypass surgery, acute-phase HDL had the same phospholipid/neutral lipids ratio (49). However, there was a selective decrease of phosphatidylethanolamine, and phosphatidylinositol, whereas there was an increase in isoprostane-containing phosphatidylcholine and lysophosphatidylcholine (49). There was also an exclusive decrease in sphingomyelin content.

Regarding the protein composition, the hallmark of acute-phase HDL is an increase in apo SAA (25, 27-31, 50). Apo A-I content of acute-phase HDL was decreased in most studies (25, 29-31, 49, 50). Apo E was increased in some, but not all studies (25, 48, 50). Apo J associated with HDL was also increased during the APR (51, 52). Besides apolipoproteins, other HDL-associated proteins of acute-phase HDL are decreased,

Table 1: Characteristics of acute-phase HDL

Size/Shape	Larger than normal HDL_3 with the radius in the HDL_2 range
Density	Comparable to HDL_3 (1.12-1.21 g/cm^3)
Particles	Decreased small and large apoA-I containing HDL particles
	Increased apoE-containing HDL
	SAA-containing HDL devoid of apoA-I
Lipid Composition	Decreased cholesterol ester content
	Increased free cholesterol content
	Increased triglyceride content
	Increased free fatty acid content
	Increased or decreased phospholipid content
	Decreased phosphatidylethanolamine and phosphatidylinositol
	Increased isoprostane-containing phosphatidylcholine
	Increased saturated and less polyunsaturated phosphatidylcholine
	Increased lysophosphatidylcholine
	Decreased sphingomyelin
Protein Composition	Increased apo SAA
	Decreased apo A-I
	Decreased apo A-II
	Decreased apo Cs
	Increased or decreased apo E
	Decreased LCAT
	Decreased CETP
	Decreased or increased PAF-AH
	Decreased PON1
	Increased ceruloplasmin

including LCAT (25, 33, 34), CETP (53, 54), and paraoxonase-1 (PON1) (30, 55). Platelet-activating factor acetylhydrolase (PAF-AH) activity in acute-phase HDL was decreased in some studies (30, 52), but increased in the other (56). Ceruloplasmin associated with HDL was increased (30, 52). The summary of these changes is in **Table 1**.

CHANGES IN HDL FUNCTION AND RCT DURING THE ACUTE-PHASE RESPONSE

Because of the dramatic changes in HDL metabolism during the APR, it has been postulated that the function of HDL and RCT may be perturbed (57).

Accumulating evidence suggests that, during the APR, RCT is impaired. First, levels of HDL and apo A-I decrease (29). Both apo A-I and HDL are main acceptors of cellular cholesterol; therefore, the decrease of apo A-I and HDL during the APR could potentially impair cholesterol removal from cells. Second, there are decreases in levels of several plasma proteins known to be involved in RCT pathway, including LCAT and CETP (25, 33, 34, 53, 54). The reduction in LCAT could limit cholesterol removal from cells because free cholesterol gradient is not maintained, whereas the decrease in CETP activity could limit the transfer of cholesterol to triglyceride-rich lipoproteins, thus further retarding the RCT pathway. Third, a series of enzymes that catabolize cholesterol into bile acids, and several proteins in the bile acid transport pathway, are downregulated (58-61). Collectively, the impairment of RCT is coordinately regulated at several steps during the APR.

Decreased RCT in response to injuries may help conserve cholesterol and redirect it to the peripheral sites where cholesterol is necessary. Acute-phase HDL, which is enriched in apo SAA, has been shown to have lower binding affinity to hepatocytes but increased affinity to macrophages that results in enhanced cholesterol ester uptake into cells (37, 62, 63). Therefore, cholesterol may be redirected from the liver for disposal to macrophages for host defense. However, prolonged stimulation of the APR, as seen in chronic infection and inflammation, may continually impair RCT and lead to more cholesterol deposition at the periphery, which could be deleterious in atherosclerosis.

OUTLINE OF THE THESIS

As reviewed above, there are a number of changes in the metabolism and composition of HDL during the APR, suggesting that RCT may be impaired. As new proteins in the RCT pathway and HDL metabolism were recently identified, including ABCA1, ABCG1, ABCG5, ABCG8, and SR-BI, we began by studying the regulation of these proteins during the APR in rodents. We then examined two important steps in RCT, cholesterol efflux from macrophages and cholesterol ester uptake into hepatocytes, during the APR. During the course of the study we also developed an alternative method of purifying HDL from hamsters using hamster apo A-I selected-affinity immunosorption columns. As a result, we investigated changes of HDL-associated proteins in acute-phase HDL, and identified a protein on HDL that has not previously been shown to be present in the circulation or associated with HDL.

Aims of the study:

Specific Aim 1. To determine the regulation of ABCG5, ABCG8, ABCA1, ABCG1, and SR-BI by endotoxin and cytokines

Specific Aim 2. To determine whether cholesterol efflux from cells is impaired during the APR

Specific Aim 3. To determine whether cholesterol ester uptake into hepatocytes is impaired during the APR

Specific Aim 4. To identify and characterize HDL-associated proteins that are increased or decreased during the APR

Overall Strategies:

We used Syrian hamsters and mice as animal models and sources of HDL. Both of these two rodent models have long been used to study changes in lipoproteins that occur during the APR. We chose to use hamsters for a number of reasons. First, serum lipoprotein levels in hamsters respond to dietary changes similar to humans (64). Second, hamsters, in contrast to other rodent species, have CETP activity in plasma like humans (54). Information gained from our study using two rodent species would complement each other and help avoid species-specific changes. The APR in animals was induced by endotoxin (lipopolysaccharide or LPS) injection, whereas control animals received normal saline injection.

We used two different methods of HDL isolation to minimize isolation artifacts: ulltracentrifugation and affinity column chromatography. Ultracentrifugation is the most common method for isolation of HDL because of its high yield, high concentration, and

relative purity, but it has disadvantages of dissociating several HDL-associated proteins, such as apo A-I, LCAT, and CETP (65, 66). Apo A-I immunoaffinity column chromatography is another method of HDL isolation (67). It does not dissociate proteins from HDL particles, however, until recently, only human apo A-I affinity columns were available. We constructed hamster apo A-I affinity columns and used them for our studies.

In **Chapter 2**, we examine the regulation of ABCG5 and ABCG8, two of the newly discovered ABC transporters in hepatocytes, by endotoxin. We also examine the regulation of ABCA1 and ABCG1, two key transporters involved in cholesterol efflux in macrophages. In **Chapter 3**, we demonstrate that acute-phase HDL has a decreased ability to remove cholesterol from cells and identify the mechanism. In **Chapter 4**, we examine the regulation of SR-BI by endotoxin and study cholesterol ester uptake into hepatocytes during the APR. In **Chapter 5**, we describe changes in levels of HDL-associated apolipoproteins during the APR, including apo A-II, apo A-IV, apo A-V, apo C-I, apo C-II, and apo C-III. In **Chapter 6**, we describe the construction of hamster apo A-I immunoaffinity columns and their use in our recent identification of a new HDL-associated protein, parotid secretory protein, in hamsters and subsequently in mice.

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CHAPTER 2

ENDOTOXIN DOWNREGULATES ABCG5 AND ABCG8 IN MOUSE LIVER AND ABCA1 AND ABCG1 IN J774 MURINE MACROPHAGES

ABSTRACT

Several of the ATP-binding cassette (ABC) transporters have recently been shown to play important roles in reverse cholesterol transport and atherosclerosis. In the liver, ABCG5 and ABCG8 have been proposed to efflux sterols into the bile for excretion. ABCG5 and ABCG8 also limit absorption of dietary cholesterol and plant sterols in the intestine. In macrophages, ABCA1 and ABCG1 mediate cholesterol removal from these cells to high-density lipoprotein (HDL). Many of these ABC transporters are regulated by the liver X receptor (LXR). We have previously shown that endotoxin downregulates LXR in rodent liver. In the present study, we examined the in vivo and in vitro regulation of these ABC transporters by endotoxin (LPS). We found that endotoxin significantly decreased mRNA levels of ABCG5 and ABCG8 in the liver, but not in the small intestine. When endotoxin or cytokines (tumor necrosis factor and interleukin-1) were incubated with J774 murine macrophages, the mRNA levels of ABCA1 were decreased. This effect was rapid and sustained, and was associated with a reduction in ABCA1 protein levels. Endotoxin and cytokines also decreased ABCG1 mRNA levels in J774 cells. Although LXR is a positive regulator of ABCA1 and ABCG1, we did not observe a reduction in protein levels of LXR in J774 cells. The decrease in ABCG5 and ABCG8 levels in the liver as well as a reduction in ABCA1 and ABCG1 in macrophages during the host response to infection and inflammation coupled with other previously described changes in the reverse cholesterol transport pathway may aggravate atherosclerosis.

INTRODUCTION

In epidemiological studies, plasma levels of high-density lipoprotein (HDL)-cholesterol are inversely correlated with the risk of coronary artery disease; therefore, HDL is postulated to protect against atherosclerosis. (1) Reverse cholesterol transport (RCT) is an HDL-mediated pathway by which cholesterol is removed from peripheral cells and transported to the liver for excretion and/or catabolism. (2, 3) Cholesterol efflux, the first step of RCT, begins when HDL or its major apolipoprotein, apo A-I, accepts cholesterol from cells. Cholesterol on HDL is returned to the liver by several routes. Once delivered to the liver, most cholesterol is metabolized by a series of hepatic enzymes into bile acids. The remaining cholesterol is excreted directly into the bile, along with bile acids.

Accumulating evidence has suggested a possible relationship between atherosclerosis and chronic infections and inflammatory diseases. (4, 5) How infections and inflammatory states, especially those outside the arterial wall, can promote atherosclerosis is not clear. However, it is known that the acute-phase response (APR) is induced during infection and inflammation. During the APR, multiple alterations in lipid and lipoprotein metabolism occur. (5) Plasma triglyceride levels increase, and there is an increase in small dense low-density lipoprotein (LDL).(6) These particular changes have been recognized as risk factors for atherosclerosis.

Considerable evidence also suggests that, during the APR, RCT is impaired. First, HDL cholesterol levels decrease. (7) Second, levels of several proteins involved in RCT pathway decrease, including lecithin:cholesterol acyltransferase, cholesterol ester transfer protein, phospholipid transfer protein, hepatic lipase, and scavenger receptor class B type

I. (8-16) Third, we and others have recently shown that, there is an impairment of both cholesterol efflux from cells and cholesterol ester uptake into the liver. (16-19) Fourth, a series of enzymes that catabolize cholesterol into bile acids, and several proteins in the bile acid transport pathway, are downregulated. (20-23) Collectively, several steps in RCT are impaired during the APR that may contribute to the increased risk of atherosclerosis.

Recently, several key proteins in the RCT pathway have been discovered, a number of which are members of the ATP-binding cassette (ABC) transporter superfamily. (24) ABCA1 and ABCG1 are ABC transporters that are involved in movement of cholesterol from cells to HDL and its apolipoproteins. (25-30) ABCG5 and ABCG8 have been proposed to regulate sterol absorption from the small intestine and its excretion from the liver into the bile. (31, 32)

Identification of factors that regulate ABC transporters should provide insights into the mechanisms by which RCT is affected in physiological and pathological states. Previously, our laboratory has shown that in rodents, hepatic mRNA levels of liver X receptor (LXR) and retinoid X receptor (RXR) were rapidly decreased in response to endotoxin. (33) LXR is a nuclear hormone transcription factor that, by heterodimerizing with RXR, increases levels of the mRNA for ABCA1, ABCG1, ABCG5, and ABCG8. (27, 31, 34-38) We therefore hypothesized that these ABC transporters would be decreased by endotoxin. In the present study, we report that endotoxin decreased mRNA levels of ABCG5 and ABCG8 in mouse liver. In J774 murine macrophages, endotoxin and cytokines decreased levels of ABCA1 and ABCG1. However, there was no reduction

in levels of LXR in macrophages. These data demonstrate that the effects of endotoxin on these proteins in macrophages may not be mediated solely through decreased LXR.

MATERIALS AND METHODS

Materials

Endotoxin (lipopolysaccharide or LPS from E. coli serotype 055:B5) was purchased from Difco Laboratories (Detroit, MI). Recombinant tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were purchased from R&D Systems (Minneapolis, MN). Polyclonal antibody against ABCA1 was purchased from Novus Biologicals (Littleton, CO). Antibodies against LXRα and LXRβ were purchased from Affinity Bioreagents (Golden, CO). Antibodies against RXRα, RXRβ, and RXRγ were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). 8-(4-chlorophenylthio) adenosine 3', 5'-cyclic monophosphate (cpt-cAMP), 22(R)-hydroxycholesterol and all other chemicals were obtained from Sigma (St. Louis, MO). Supplies for immunoblot analysis were purchased from Amersham Biosciences (Piscataway, NJ).

Animal experiments

C57BL/6 mice (approximately 6-8 weeks of age) were obtained from Jackson Laboratory (Bar Harbor, ME) and provided with rodent chow and water ad libitum. Animals were injected intraperitoneally with indicated doses of endotoxin whereas control animals were injected with normal saline. The highest dose of endotoxin used in our study (100 μg/animal) was able to induce the acute-phase response but is far below

the lethal dose (LD_{50} ~ 5 mg/100 g body weight) required to cause death in rodents in our laboratory. Because endotoxin can cause anorexia, food was withdrawn from endotoxin-injected mice and control mice after the injection. At indicated time points, the animals were euthanized using halothane, and the tissue was excised and stored at –80°C. The animal procedures were approved by the Animal Studies Subcommittee of the San Francisco Veterans Affairs Medical Center, and were performed in accordance with the guidelines.

Cell culture experiments

J774 murine macrophages were obtained from the American Type Culture Collection and maintained in minimum essential media supplemented with 10% fetal bovine serum under 5% CO_2. In some experiments, cells were incubated with 0.3 mM cpt-cAMP or 10 μM 22(R)-hydroxycholesterol before treated with LPS or cytokines. LPS or cytokines was incubated with cells in media containing human serum albumin in the absence of serum, and at indicated time points, cells were washed and harvested.

Isolation of RNA and RNA blot analysis

Isolation of RNA and RNA blot analyses were performed as previously described. (16) Total or poly $(A)^+$ RNA was quantified by measuring absorption at 260 nm and equal amounts of RNA were loaded on 1% agarose-formaldehyde gels and electrophoresed. The uniformity of sample applications was checked by ultraviolet visualization of the acridine orange-stained gels before transfer to Nytran membranes. Because endotoxin increased hepatic mRNA levels of actin, glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) and cyclophilin in rodent liver; (39-41) the mRNA levels of actin, GAPDH, and cyclophilin, which are widely used for normalizing data, cannot be used to study endotoxin regulation of proteins in the mouse liver. However, the differing directions of changes in mRNA levels (increased for some proteins, and decreased for some proteins), the magnitude of alterations (8 fold increase or up to 75% decrease), and the relatively small standard error of the mean (SEM) make it unlikely that the changes observed were due to unequal loading of mRNA. RNA blots were hybridized with ^{32}P-labeled cDNA probes generated by RT-PCR from the mouse liver (ABCG5, ABCG8, and ABCA1) or J774 murine macrophages (ABCG1) using the following primers: ABCG5 upper primer, 5'-TGC CCT TTC TGA GTC CAG AG-3' and lower primer, 5'-GTG CTC TTT CAA TGT TCT CCA G-3'; ABCG8 upper primer, 5'-ATG AGC TGG AAG ACG GGC TG-3' and lower primer, 5'-GCC AGT GAG AGC AAG GCT GA-3'; ABCA1 upper primer, 5'-TCT CTG CTA TCT CCA ACC TCA TC-3' and lower primer, 5'-ACG TCT TCA CCA GGT AAT CTG AA-3'; ABCG1 upper primer, 5'-GAA GAC CTG CAC TGC GAC ATC-3' and lower primer, 5'-GTT GCA TTG CGT TGC GTT AGT C-3'. After washing, the blots were exposed to X-Ray films for various durations to ensure that measurements were done on the linear portion of the curve, and the bands were quantified using the Bio-Rad imaging densitometer.

Immunoblot analysis

J774 cells were scraped into cold phosphate-buffered saline and centrifuged to get cell pellets. Lysis buffer containing 10 mM HEPES, pH 7.9, 0.5% Nonidet P40, 1.5 mM $MgCl_2$, 0.2 M sucrose, 10 mM KCl, 0.5 mM dithiothreitol, and 1% (v/v) protease

inhibitor cocktail (Sigma) was incubated with cells for 5 min at 4°C. The supernatant was collected after centrifugation and the protein concentrations were measured. For immunoblot analysis for LXR and RXR, nuclear proteins were isolated as previously described. (33) Proteins were resolved on polyacrylamide gels and transferred to Hybond-P PVDF membrane as previously described. (16) For immunodetection, the blots were blocked in phosphate-buffered saline-0.1% Tween containing 5% nonfat dry milk before incubation with a primary antibody and a secondary antibody conjugated with horseradish peroxidase. The blots were visualized using the ECL Plus chemiluminescence detection system and subjected to autoradiography. Quantification of the signals was performed by densitometry.

Electrophoretic mobility shift assay

Nuclear extracts were prepared as described above and an electrophoretic mobility shift assay was performed as previously described. (33) The oligonucleotides corresponding to the LXR response element of ABCA1 5'GCG CAG AGG TTA CTA TCG GTC AAA3' (36) and the mutated oligonucleotides 5' GCG CAG *TAG* TTA CTA TC*A* *CA*C AAA3' were used.

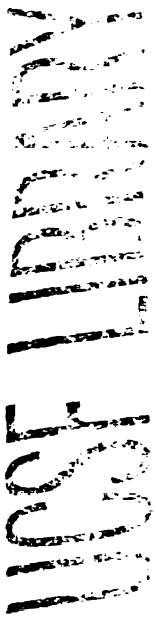
Statistics

Data are presented as mean ± SEM. Comparisons between groups were performed using a Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Effect of LPS on mRNA levels of ABCG5 and ABCG8 in mouse liver and small intestine

Our laboratory has previously shown that LPS decreased hepatic mRNA and protein levels of LXRα in hamsters. (33) We have obtained similar results in mouse liver (data not shown). Because ABCG5 and ABCG8 are regulated by LXRα, we examined whether these two transporters were affected by LPS.



A single dose of 100 μg of LPS rapidly decreased hepatic mRNA levels of ABCG5 and ABCG8 in mice as shown in **Fig. 1A&B**. The mRNA levels started to decrease at 4-8 hours and continued to be significantly suppressed for 24 hours after LPS administration. The chronological pattern and the magnitude of the reduction of hepatic mRNA levels of both ABCG5 and ABCG8 were relatively similar, suggesting that they were coordinately regulated.

The dose response curve of LPS effects on hepatic mRNA levels of ABCG5 and ABCG8 was examined at the 16-hour time point. LPS administration resulted in a dose-dependent decrease in hepatic mRNA levels of both ABCG5 and ABCG8 (**Fig. 2A&B**). The levels were significantly decreased by low doses of LPS. A half-maximal decrease in hepatic levels of ABCG5 and ABCG8 was produced by approximately 0.1 μg and 2.0 μg LPS, respectively.

In addition to the liver, ABCG5 and ABCG8 are also expressed in the small intestine. Therefore, we examined whether LPS injection decreased intestinal mRNA levels of these two transporters. In contrast to what observed in the liver, we found that

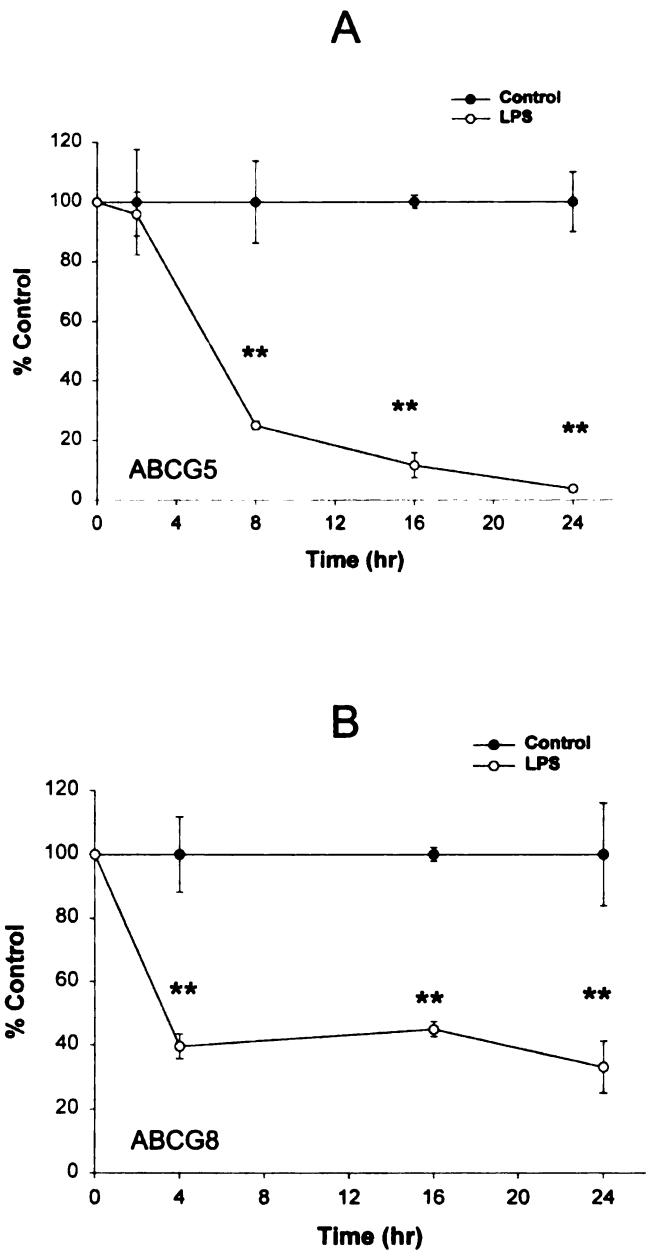


Fig. 1. Time course of the effect of LPS on ABCG5 mRNA (A) and ABCG8 mRNA (B) in mouse liver. Mice were injected with LPS (100 μg). At indicated time points, livers were harvested and RNA was isolated. Poly $(A)^+$ RNA was hybridized with a ^{32}P-labeled cDNA probe and bands were analyzed by agarose gel electrophoresis followed by autoradiography as described in Materials and Methods. Data are presented as percent change vs. control (mean ± SEM). N = 4-5 in each group at each time point. *: $P < 0.05$, **: $P < 0.01$.

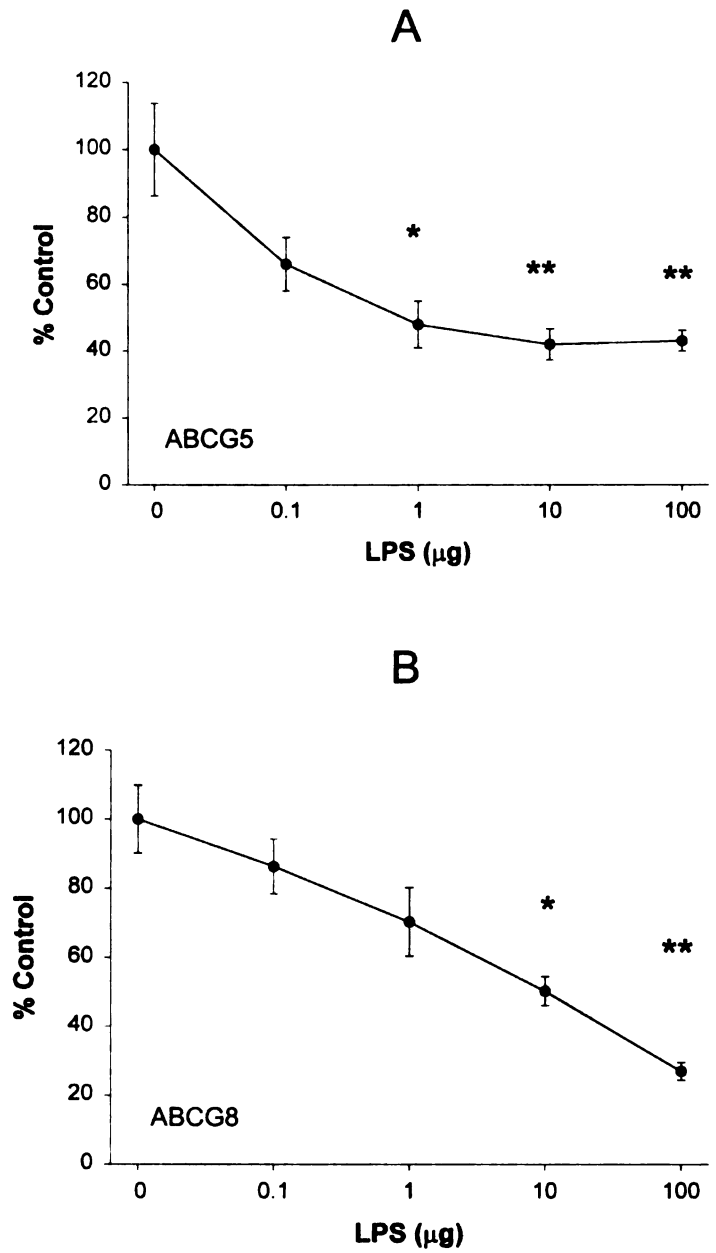


Fig. 2. Dose response curve of the effect of LPS on ABCG5 mRNA (A) and ABCG8 mRNA (B) in mouse liver. Mice were injected with LPS (100 μg). At indicated time points, livers were harvested and RNA was isolated. Poly $(A)^+$ RNA was hybridized with a ^{32}P-labeled cDNA probe and bands were analyzed by agarose gel electrophoresis followed by autoradiography as described in Materials and Methods. Data are presented as percent change vs. control (mean ± SEM). N = 4-5 in each group at each time point. *: $P < 0.05$, **: $P < 0.01$.

there were no changes in the mRNA levels of both ABCG5 and ABCG8 in the small intestine (data not shown).

Effect of LPS on mRNA levels of ABCA1 in J774 murine macrophages

In macrophages, ABCA1 plays a critical role in apolipoprotein-mediated cholesterol efflux. (25, 26) We found that incubation of LPS with J774 murine macrophages resulted in a decrease in ABCA1 mRNA levels (**Fig. 3**). The maximal decrease in mRNA levels of ABCA1 was observed at 8 hours and was relatively sustained until 24 hours (**Fig. 3A**). There was also a concentration-dependent decrease in mRNA levels of ABCA1 (**Fig. 3B**). It is of note that the half-maximal decrease in the mRNA levels of ABCA1 was produced by the concentrations of LPS between 1-10 ng/mL, which corresponds to the concentrations of LPS found in the circulation during sepsis. (42)

Effect of LPS on mRNA levels of ABCG1 in J774 cells

Besides ABCA1, ABCG1 is another ABC transporter that is expressed in macrophages and is involved in cholesterol efflux. (27) We examined whether LPS had similar effects on ABCG1. As shown in **Fig. 4A&B,** LPS decreased mRNA levels of ABCG1 in J774 cells in a time-dependent and concentration-dependent manner. The degree of reduction of mRNA levels of ABCG1, however, was relatively small compared to that observed with ABCA1 (**Fig. 3A&B**).

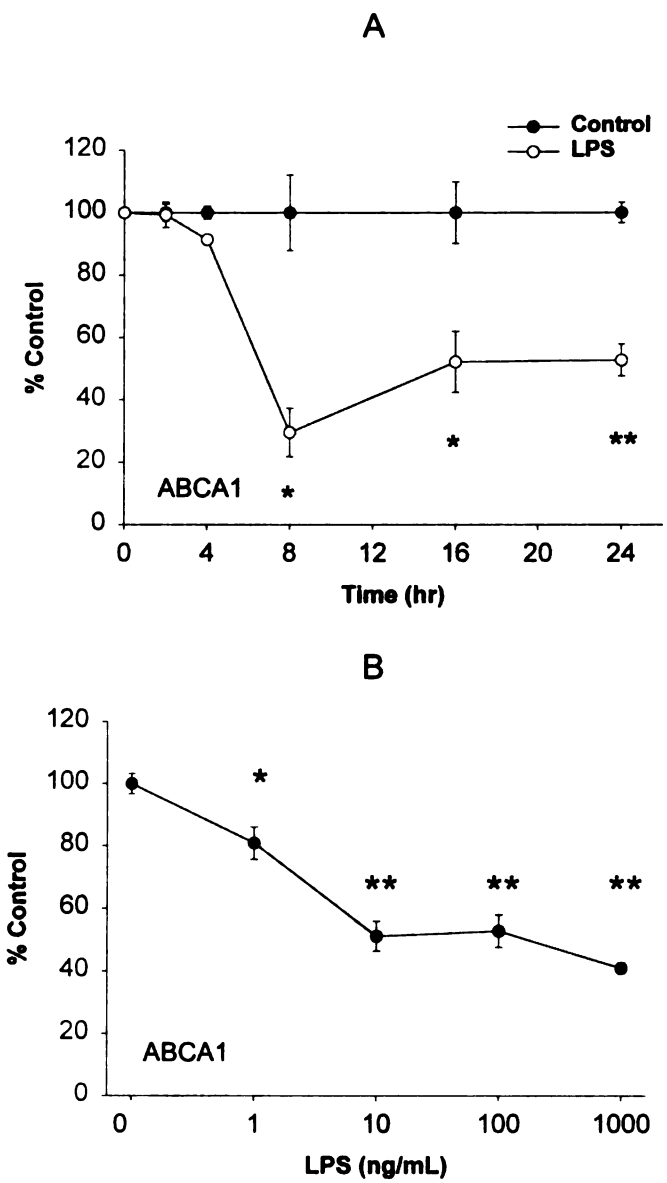


Fig. 3. Time course (A) and dose response curve (B) of the effect of LPS on ABCA1 mRNA in J774 macrophages. Cells were incubated with indicated concentrations of LPS, and at indicated time points, cells were harvested and RNA was isolated. Total RNA was hybridized with a ^{32}P-labeled cDNA probe and bands were analyzed by agarose gel electrophoresis followed by autoradiography as described in Materials and Methods. Data are presented as percent change vs. control (mean ± SEM). N = 3 in each group at each time point. *: $P < 0.05$, **: $P < 0.01$.

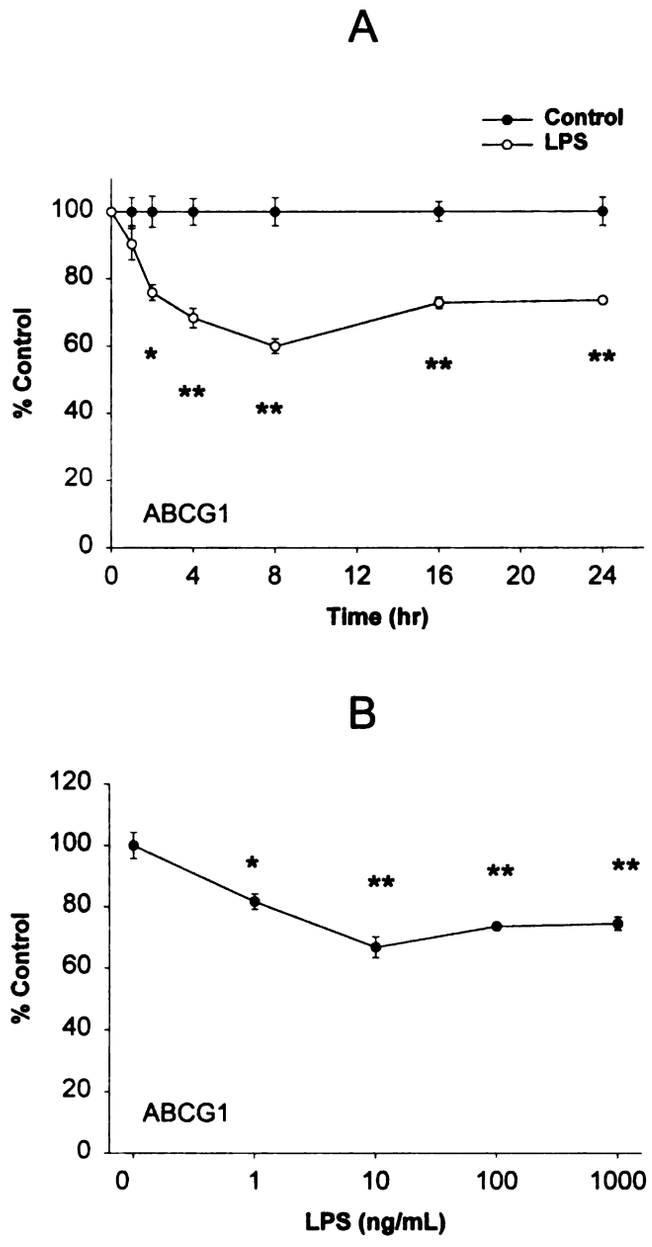


Fig. 4. Time course (A) and dose response curve (B) of the effect of LPS on ABCG1 mRNA in J774 macrophages. Cells were incubated with indicated concentrations of LPS, and at indicated time points, cells were harvested and RNA was isolated. Total RNA was hybridized with a ^{32}P-labeled cDNA probe and bands were analyzed by agarose gel electrophoresis followed by autoradiography as described in Materials and Methods. Data are presented as percent change vs. control (mean ± SEM). N = 3 in each group at each time point. *: $P < 0.05$, **: $P < 0.01$.

Effect of TNF and IL-1 on mRNA levels of ABCA1 and ABCG1 in J774 cells

Because cytokines mediate many of the effects of LPS, we next examined the effect of cytokines on mRNA levels of ABCA1. Both TNF and IL-1 (100 ng/mL) produced a ~ 50% decrease in ABCA1 mRNA levels (**Fig. 5A**). Similarly, TNF and IL-1 were able to decrease mRNA levels of ABCG1 (**Fig. 5B**).

Effect of cAMP, oxysterols, and LPS on mRNA levels of ABCA1 and ABCG1

Both cAMP and oxysterols are known inducers of ABCA1, however, the mechanism of induction is different. Oxysterols also induce ABCG1. To determine whether LPS can decrease mRNA levels of ABCA1 and ABCG1 in the presence of an inducer, we incubated J774 cells with cpt-cAMP, a cAMP analog, or 22(R)-hydroxycholesterol, an oxysterol, before treating cells with LPS. We found that cpt-cAMP and 22(R)-hydroxycholesterol both induced ABCA1 mRNA levels, but the magnitude was different. cpt-cAMP increased ABCA1 mRNA levels by 15-20 fold whereas 22(R)-hydroxycholesterol increased ABCA1 mRNA levels by approximately 40% (**Fig. 6A**). In our hands, the mRNA levels of ABCG1 were not significantly changed with either cpt-cAMP or 22(R)-hydroxycholesterol (**Fig. 6B**).

Treatment with LPS did not decrease ABCA1 mRNA levels when ABCA1 was induced by cpt-cAMP, but LPS was able to cause ~ 50% reduction in the mRNA levels of ABCA1 when ABCA1 was induced by 22(R)-hydroxycholesterol (**Fig. 6A**). LPS also

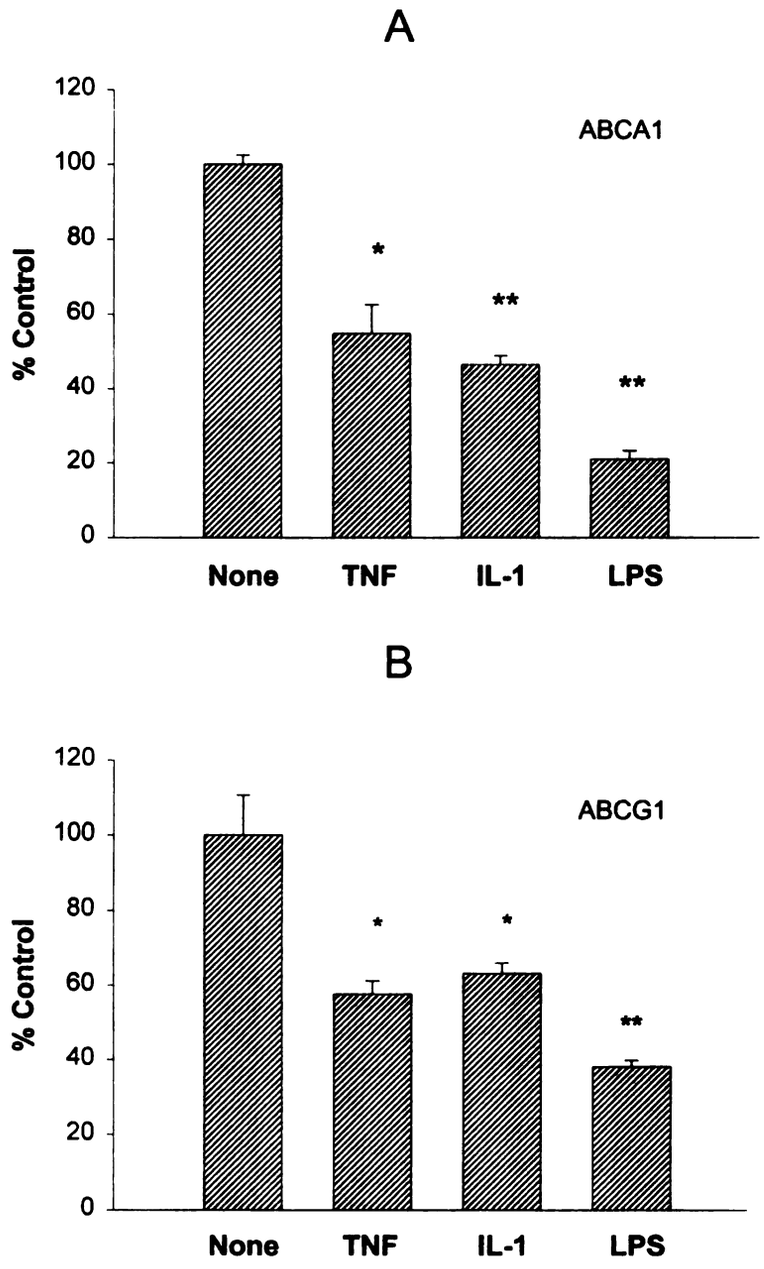


Fig. 5. The effect of TNF, IL-1, and LPS on ABCA1 mRNA (A) and ABCG1 mRNA (B) levels in J774 macrophages. TNF (100 ng/mL), IL-1 (100 ng/mL), or LPS (100 ng/mL) was incubated with cells in minimal essential media with 2.5% human serum albumin in the absence of serum, and 24 hours later, cells were harvested for RNA isolation as described in Materials and Methods. N = 3 in each group. *: $P < 0.05$, **: $P < 0.01$.

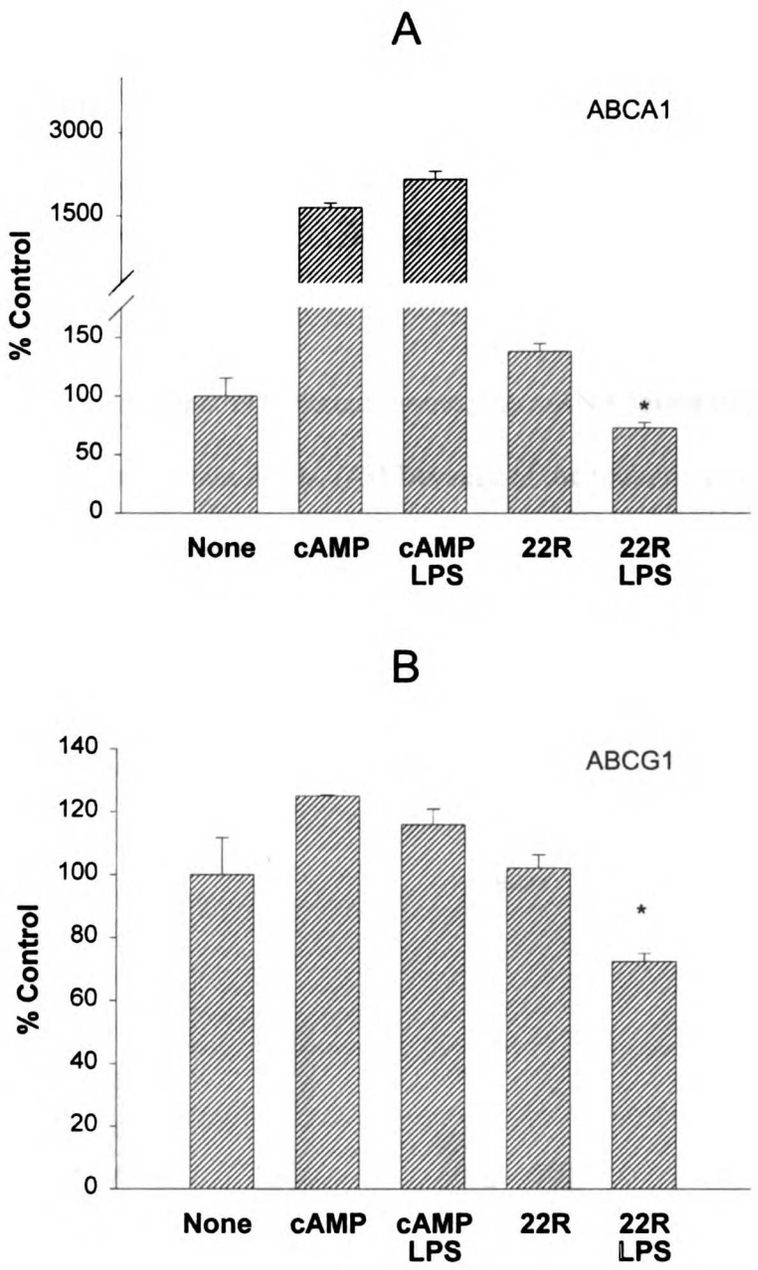


Fig. 6. The effect of cpt-cAMP and 22(R)-hydroxycholesterol in the presence or absence of LPS on ABCA1 mRNA (A) and ABCG1 mRNA (B) levels in J774 macrophages. J774 cells were pretreated with 0.3 mM cpt-cAMP or 10 μM 22(R)-hydroxycholesterol before treatment with LPS (100 ng/mL) for 24 hours. Cells were harvested for RNA isolation as described in Materials and Methods. N = 3 in each group. cAMP denotes cpt-cAMP and 22R denotes 22(R)-hydroxycholesterol. *: $P < 0.05$ compared to 22R alone.

decreased mRNA levels of ABCG1 when 22(R)-hydroxycholesterol was present, but not cpt-cAMP (**Fig. 6B**).

Effect of LPS, TNF and IL-1 on ABCA1 protein levels in J774 murine macrophages

It has been reported that in different tissues, the mRNA levels of ABCA1 do not always correlate with the protein levels. (43) Because of the reduction in mRNA levels of ABCA1 in macrophages, we investigated whether protein levels of ABCA1 were also decreased. As shown in **Fig. 7**, LPS, TNF, and IL-1, all produced a significant decrease in the protein levels of ABCA1. Due to lack of reactive antibodies against ABCG1, the protein levels in J774 cells were not investigated.

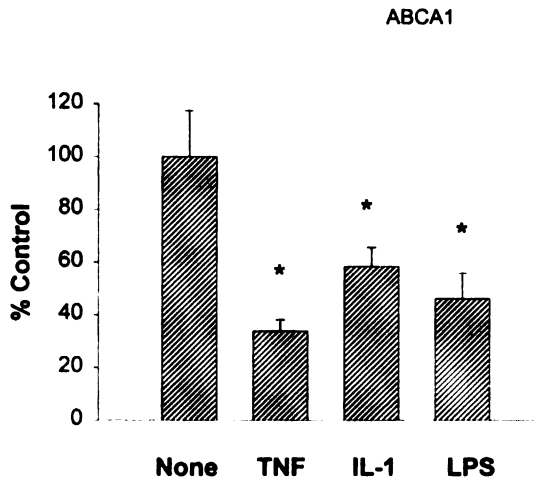


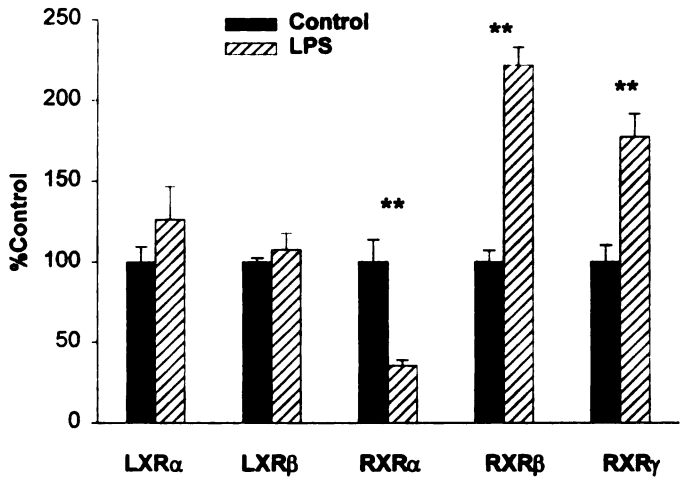
Fig. 7. The effect of LPS on ABCA1 protein levels in J774 macrophages. LPS (100 ng/mL) was incubated with cells in minimal essential media with 2.5% human serum albumin in the absence of serum, and 24 hours later, cells were harvested for protein isolation as described in Materials and Methods. N = 3 in each group. *: $P < 0.05$.

Effect of LPS on protein levels of LXR and RXR in J774 cells

Our laboratory has previously shown that, in the liver, LPS induced a reduction in mRNA levels, proteins levels, and binding activities of LXR and RXR. (33) Since ABCA1 and ABCG1 are activated by LXR, and LPS was able to block the induction of ABCA1 and ABCG1 by 22R-hydroxycholesterol, an LXR ligand, we examined whether a decrease in ABCA1 and ABCG1 in macrophages was due to a decrease in LXR. We, however, found no changes in the protein levels of either LXRα or LXRβ in J774 cells when cells were treated with LPS (**Fig. 8A**).

LXR is a nuclear hormone receptor that heterodimerizes with RXR. We also investigated whether LPS affected the protein levels of RXR in J774 cells. We detected all three isoforms of RXR in these cells, RXRα, RXRβ, and RXRγ. LPS decreased the protein levels of RXRα, whereas it increased the protein levels of RXRβ and RXRγ (**Fig. 8A**). We performed an electrophoretic mobility shift assay, using the oligonucleotides that correspond to the LXR response element found in the ABCA1 promoter region, to detect the binding of LXR:RXR heterodimers. However, we found that there was no difference between nuclei preparations from control cells or cells treated with LPS in the ability to bind the oligonucleotides (**Fig. 8B**). Altogether, these data did not support the hypothesis that the decrease in ABCA1 levels in J774 cells was solely mediated through a reduction in LXR.

A



B

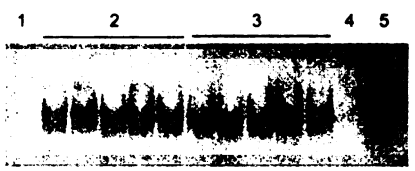


Fig. 8. The effect of LPS on protein levels of LXRα, LXRβ, RXRα, RXRβ, and RXRγ (A) and binding activities of LXR:RXR (B) in J774 macrophages. J774 cells were incubated with LPS (100 ng/mL) for 24 hours and nuclei fractions of the cells were prepared for immunoblot analysis and/or electrophoretic mobility shift assay as described in Materials and Methods. N = 3-5 in each group at each time point. **: $P < 0.01$. In Panel B, 1: no nuclear extract, 2: five different samples of nuclear extract from control cells, 3: five different samples of nuclear extract from LPS-treated cells, 4: Unlabeled specific oligonucleotides were included at 100-fold excess for competition, 5 Unlabeled nonspecific (mutated) oligonucleotides were included at 100-fold excess for competition.

DISCUSSION

A number of proteins in the ABC transporter superfamily have recently been discovered to play a role in cholesterol efflux and RCT. (24) ABCA1 is a transporter on the plasma membrane that translocates cholesterol and phospholipid out of the cells. Mutations of ABCA1 result in defects in apolipoprotein-mediated cholesterol efflux as found in Tangier disease and familial HDL deficiency. (25, 44-47) Those patients have low levels of HDL, and in some cases, premature atherosclerosis has been reported. (48) ABCG1 is another ABC transporter that is involved in cholesterol efflux. (27) In contrast to ABCA1, ABCG1 is located primarily inside the cells. ABCG5 and ABCG8 are two ABC half-transporters that have been proposed to form a heterodimer in the small intestine and in the liver. (31) In the small intestine, the heterodimers efflux plant sterols as well as dietary cholesterol out of the intestinal cells, whereas in hepatocytes, they efflux those sterols into the bile. (49) Mutations in ABCG5 or ABCG8 are a cause of sitosterolemia, a disorder characterized by xanthomatosis and premature atherosclerosis due to uncontrolled absorption of sterols and failure to excrete them into the bile. In addition to defects in plant sterol metabolism, patients with sitosterolemia do have abnormal cholesterol metabolism. An increase in cholesterol absorption as well as a defect in cholesterol excretion into the bile has been noted in these patients. (50, 51)

Our laboratory has previously reported that endotoxin decreased LXR and RXR in rodent liver. Because ABCG5 and ABCG8 are positively regulated by LXR, (31, 38) we investigated the regulation of ABCG5 and ABCG8 by endotoxin in the liver. As expected, we found that both ABCG5 and ABCG8 were coordinately downregulated.

Our data suggest that cholesterol excretion into the bile could be impaired during the APR.

In macrophages, ABCA1 and ABCG1 are involved in cholesterol efflux. Previous studies from our laboratory and others have shown that during the APR, changes in HDL particles resulted in impairment of cholesterol efflux from cells to HDL. (17, 18) Treatment of mouse peritoneal macrophages with interferon-γ caused a decrease in ABCA1 mRNA levels and cholesterol efflux. (52) A recent study in RAW cells also reported that endotoxin treatment downregulated ABCA1 mRNA levels, (53) however, data on protein levels of ABCA1 were not provided. A study comparing mRNA and protein levels of ABCA1 in different tissues has shown that in some tissues, the abundance of mRNA levels and protein levels are not well correlated. (43) For example, in spleen and thymus, the mRNA levels of ABCA1 were low, but the protein levels were found moderate. (43) Therefore, in our study using J774 cells, we examined both mRNA levels and protein levels of ABCA1 in response to endotoxin treatment. We found that endotoxin decreased both ABCA1 mRNA and protein levels. We also provided new information that TNF and IL-1, like endotoxin, could decrease ABCA1 mRNA and protein levels. Collectively, our data on ABCA1 as well as ABCG1 provided the mechanism for previous findings that cholesterol efflux was impaired during the APR. (17, 18, 53)

In macrophages, both ABCA1 and ABCG1 are upregulated by LXR, but we found that the effect could not be explained by a decrease in LXR. Data from immunoblot analysis showed that both isoforms of LXR, LXRα and LXRβ, did not decrease with endotoxin. When RXR, a partner of LXR heterodimerization, was

examined in J774 cells, changes in levels of three isoforms of RXR were different. Endotoxin decreased RXRα but increased RXRβ and RXRγ isoforms. Because the relative abundance of different LXR and RXR isoforms vary among different cell types, an electrophoretic mobility shift assay was performed to detect the binding of LXR:RXR heterodimers. Again, we did not find differences between nuclei preparations from control cells or cells treated with LPS. Therefore, our data did not support the hypothesis that a decrease in ABCA1 levels in J774 cells was mediated through a reduction in LXR. A recent study using an inhibitor of NF-κB reported that NF-κB might be involved in the downregulation of ABCA1 by endotoxin. (53)

Our current study demonstrates that endotoxin, TNF, and IL-1 decreased ABCA1 mRNA and protein levels in J774 macrophages. A study in RAW264.7 cells using endotoxin also reported similar changes in mRNA levels. (53) Another study using mouse peritoneal macrophages also reported that interferon-γ decreased ABCA1. (52) However, in human THP-1 cells, ABCA1 was found upregulated by endotoxin. (54) It is not clear whether the discrepancy is due to different cell types or other unknown factors. It is of note that the upregulation of ABCA1 by endotoxin in THP-1 cells was also reported to be mediated by an LXR-independent mechanism. (54)

In summary, our study showed that endotoxin downregulated ABCG5 and ABCG8 in the liver. This effect likely impairs cholesterol excretion from hepatocytes into the bile. Endotoxin also decreased ABCA1 and ABCG1 in macrophages, thus providing the mechanism for previous findings that cholesterol efflux from macrophages was impaired during the APR. Although LXR is an activator of several ABC transporters, the downregulation of ABCA1 by endotoxin may not be mediated by LXR. The present

study adds to the accumulating evidence that several steps in RCT are impaired during the APR. Although decreased RCT may redirect cholesterol to peripheral cells for host defense, prolongation of impaired RCT may contribute to an increased risk of atherosclerosis observed in chronic infections and inflammatory states.

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CHAPTER 3

CHOLESTEROL EFFLUX BY ACUTE-PHASE HIGH-DENSITY LIPOPROTEIN

ROLE OF LECITHIN:CHOLESTEROL ACYLTRANSFERASE

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ABSTRACT

High-density lipoprotein (HDL) plays an initial role in reverse cholesterol transport by mediating cholesterol removal from cells. During infection and inflammation, several changes in HDL composition occur that may affect the function of HDL, therefore, we determined the ability of acute-phase HDL to promote cholesterol removal from cells. Acute-phase HDL was isolated from plasma of Syrian hamsters injected with lipopolysaccharide. Cholesterol removal from J 774 murine macrophages by acute-phase HDL was less efficient than that by control HDL due to both a decrease in cholesterol efflux and an increase in cholesterol influx. Lecithin:cholesterol acyltransferase (LCAT) activity of acute-phase HDL was significantly lower than that of control HDL. When LCAT activity of control HDL was inactivated, cholesterol efflux decreased and cholesterol influx increased to the level observed in acute-phase HDL. Inactivation of LCAT had little effect on acute-phase HDL. In GM 3468A human fibroblasts, the ability of acute-phase HDL to remove cholesterol from cells was also lower than that of normal HDL. The impaired cholesterol removal, however, was primarily a result of an increase in cholesterol influx without changes in cholesterol efflux. When control HDL in which LCAT had been inactivated was incubated with fibroblasts, cholesterol influx increased to the level comparable to that of acute-phase HDL, without any change in cholesterol efflux. These results suggest that the ability of acute-phase HDL to mediate cholesterol removal was impaired compared to that of control HDL and the lower LCAT activity in acute-phase HDL may be responsible for this impairment. The decreased ability of acute-phase HDL to remove cholesterol from

cells may be one of the mechanisms that account for the well-known relationship between infection/inflammation and atherosclerosis.

INTRODUCTION

Plasma HDL-cholesterol levels are inversely correlated with the risk of coronary artery disease in epidemiological studies; therefore, HDL is postulated to exert protective effects against atherosclerosis (1). One of the most commonly accepted antiatherogenic properties of HDL is the removal of cellular cholesterol and transport of this cholesterol to the liver for excretion and/or catabolism (2). This pathway, known as reverse cholesterol transport (RCT), is a multi-step process (3). Cholesterol removal from cells is the first step of RCT, and it begins when HDL takes up free (unesterified) cholesterol from cells. Because cholesterol flux between HDL and cells can be bidirectional (i.e. influx and efflux), cholesterol removal (or net efflux) occurs when cholesterol efflux is greater than cholesterol influx (4). Free cholesterol on HDL then becomes esterified by the enzyme lecithin:cholesterol acyltransferase (LCAT), allowing further removal of free cholesterol. Subsequently, cholesterol ester can be returned to the liver by several routes.

Recently, several lines of evidence have suggested the possible relationship between infection/inflammation and atherosclerosis. For example, epidemiological studies have shown that chronic infections and inflammatory diseases, such as *Chlamydia pneumoniae*, Cytomegalovirus, *Helicobacter pylori*, dental infections, chronic bronchitis, rheumatoid arthritis, systemic lupus erythematosus, and psoriasis, are associated with an increased risk of atherosclerosis (5, 6). Plasma levels of C-reactive protein, a marker for

inflammation, are a strong predictor of future cardiovascular events (7). Moreover, a pilot study of patients with coronary artery disease who received antichlamydial macrolide therapy demonstrated reductions in recurrent coronary events (8). Although certain infectious agents have been found in atheromatous lesions, it is unclear how other infections and inflammatory states promote atherosclerosis.

During infection and inflammation, a systemic reaction occurs in order to protect the host from further injury and help in the repair process. This cytokine-mediated response, known as the acute-phase response (APR), induces marked changes in the concentrations of specific plasma proteins. During the APR, multiple alterations in lipid and lipoprotein metabolism also occur (6). Plasma triglyceride levels increase, and plasma HDL-cholesterol levels decrease. In addition, there is an increase in small dense LDL, a particle that is believed to be more proatherogenic (9). Moreover, during the APR, levels of several plasma proteins proposed to be involved in RCT pathway decrease, including LCAT, cholesterol ester transfer protein, phospholipid transfer protein, and hepatic lipase (10-17).

During the APR, not only do HDL-cholesterol levels decrease, but the composition of HDL is also altered. HDL that occurs during the APR, called acute-phase HDL, is depleted in cholesterol ester but enriched in free cholesterol, triglyceride, and phospholipids (10-12, 18). In addition, there are changes in levels of several proteins associated with HDL; apolipoprotein J and serum amyloid A (SAA) levels increase, whereas apolipoprotein A-I levels decrease (19-21). Although biochemical characteristics of acute-phase HDL have been determined, the biologic effects of acute-phase HDL are relatively unknown.

Because of the marked changes in HDL composition during the APR, we hypothesized that the acute-phase HDL may be functionally different from normal HDL in terms of its protective effects against atherosclerosis. Specifically, we postulated that acute-phase HDL might be less capable of supporting cholesterol removal from cells. In this study, we determined the ability of acute-phase HDL to mediate cholesterol efflux in comparison to that of normal (control) HDL. Recent data support the concept that acute-phase HDL is less effective in removing cholesterol from cells (22), and the authors attributed the difference to the presence of SAA on HDL. We now report that acute-phase HDL is indeed less able to mediate cholesterol efflux from cells, that cholesterol influx is increased, and that much of the change is due to a decrease of LCAT in acute-phase HDL. Defective cholesterol removal due to acute-phase HDL may provide one of the mechanisms underlying the relationship between infection/inflammation and atherosclerosis.

MATERIALS AND METHODS

Materials

[4-^{14}C]-cholesterol (51 mCi/mmol) and [1,2-^{3}H]-cholesterol (53.3 Ci/mmol) were purchased from NEN Life Sciences (Boston, MA); Lipopolysaccharide (LPS, E. coli strain 055:B5) was purchased from Difco Laboratories (Detroit, MI). Whatman glass microfiber filters were purchased from Fisher Scientific (Santa Clara, CA); 0.45 μ filters were from Corning (Corning, NY). N-ethyl maleimide (NEM), chemicals for the determination of LCAT activity and DNA content, and all other chemicals were

purchased from Sigma (St. Louis, MO). Tissue culture media and supplies were purchased from GIBCO BRL (Life Technologies, Gaithersburg, MD).

Cell cultures

J 774 murine macrophages were obtained from American Type Culture Collection and maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's minimum essential media (DME) containing 10% fetal calf serum (FCS). In the experiments using HDL from NEM-incubated plasma, FCS was heat-inactivated at 55-60°C for 2 hours to inactivate serum LCAT activity.

GM 3468A normal human fibroblasts were obtained from NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). Cells were maintained in Eagle's Minimum Essential Medium (MEM) with Earle's salts containing 20% FCS, 2 mM glutamine, and 2 × concentrations of non-essential amino acids, and vitamins.

Because different types of cells exhibit different mechanisms of control of cholesterol homeostasis, we used two types of cells for the cholesterol flux experiments. It has been proposed using control HDL that cholesterol homeostasis in macrophages is regulated primarily through cholesterol influx whereas that of fibroblasts is mainly regulated by cholesterol efflux (23).

Isolation and labeling of HDL

We used Syrian hamsters as a source of HDL for the experiments because HDL metabolism in hamsters has been shown to resemble that in humans in several aspects (24, 25). Moreover, lipoprotein changes that occur during the APR induced by LPS

injection have been demonstrated in hamsters (12, 18). The animal protocol was approved by the Animal Studies Subcommittee of our institution.

Male Syrian hamsters (approximately 140-180 g) were purchased from Charles River Laboratories (Wilmington, MA). Intraperitoneal injection of LPS (100 μg/100 g body weight) was used to induce the APR and normal saline was used in the control group. Because LPS can cause anorexia, food was withdrawn after the injection in both groups. Sixteen hours after the injection, blood was drawn using sterile syringes containing EDTA at a final concentration of 1.7 mg/ml, and plasma was isolated. In some experiments, plasma was immediately mixed with NEM (final concentration of 5-10 mM) to inactivate LCAT activity. Control and acute-phase HDL (d = 1.063-1.225) were isolated from pooled plasma of hamsters injected with normal saline or LPS, respectively, by sequential ultracentrifugation. KBr was used to adjust for the desired density, and ultracentrifugation was performed using the Beckman L8-70M ultracentrifuge. HDL was extensively dialyzed against normal saline containing 0.01% EDTA, pH 7.4, filtered with a 0.45 μ filter, and used within 2 weeks. Characteristics of control and acute-phase HDL in hamsters have been reported by our laboratory previously (12, 18). Special precautions during isolation and handling of HDL were used to avoid contamination with LPS as previously described (26). Materials used, including glassware, tubes, solutions, and chemicals, if not apyrogenic when obtained commercially, were sterilized and/or depyrogenated using ethylene oxide or an autoclave followed by dry heating at 180°C for at least 4 hours.

HDL was radiolabeled with [1,2-^{3}H]-cholesterol using a glass filter exchange method (27). Briefly, 50 μCi of [1,2-^{3}H]-cholesterol was applied onto the glass

microfiber filter in a glass scintillation vial and dried with N_2. HDL was added and the vial was rotated gently overnight at 4°C. HDL was recovered and filter sterilized before use. The specific activity of the labeled HDL varied from experiment to experiment. The average specific activity of the control HDL was 6.10 ± 0.9 cpm/ng cholesterol and that of the acute-phase HDL was 6.37 ± 0.7 cpm/ng cholesterol (from 6 separate experiments).

Cholesterol flux experiments

For J 774, 3.75×10^5 cells/ml were plated in 35-mm wells and incubated overnight in DME containing 10% FCS. Cells were then washed with phosphate buffered saline (PBS) and incubated in the media containing 10% FCS and [4-^{14}C]-cholesterol (1 μCi/ml) for 2 days. After 2-day incubation, cells were washed with PBS and incubated with DME containing 0.3% human serum albumin (HSA) for 1-2 hours for equilibration. Following several washes with PBS, efflux was initiated by the addition of control or acute-phase HDL in DME media containing 2.5% HSA. At different time points, an aliquot of media was removed, centrifuged to remove cells, and counted for radioactivity. At the end of the experiments, media were collected; cells were washed, scraped, and resuspended in deionized water. Cell suspension was sonicated for 15 seconds and an aliquot was used for the measurement of radioactivity, total and free cholesterol concentrations, and DNA content. A double labeling program of the Beckman LS 8501 scintillation counter was used to differentiate the radioactivity between ^{3}H (0-275) and ^{14}C (475-670). Cholesterol efflux was measured by the appearance of [4-^{14}C]-cholesterol in the media, and cholesterol influx was measured by the disappearance of [1,2-^{3}H]-

cholesterol from the media or the appearance of [1,2-^{3}H]-cholesterol in cells. Fractional cholesterol efflux was calculated as the amount of ^{14}C radioactivity present in the media divided by the total (media + cell) ^{14}C radioactivity in each well. Efflux mediated by HDL was calculated after subtraction of the ^{14}C radioactivity of the wells without HDL. Fractional cholesterol influx was calculated as the amount of ^{3}H radioactivity present in cells at the end of the experiment divided by the total ^{3}H radioactivity in each well. Additionally in some experiments, the amount of cholesterol influx was calculated by multiplying fractional influx by the amount of cholesterol present in HDL. In each experiment, cholesterol flux between cells and HDL was performed in triplicate. This system is a well-established in vitro model to study bidirectional flux of cholesterol between cells and lipoproteins (4, 27, 28).

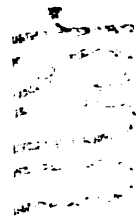
For GM 3468A cells, a similar cholesterol flux protocol was used except that 5×10^{4} cells/ml were plated in 35-mm wells, and MEM was used as described above.

Biochemical assays

LCAT activity in HDL was determined using the exogenous assay as previously described (12). Artificial substrate liposomes consisting of human apoA-I, egg yolk lecithin, and cholesterol at a molar ratio of 0.8:250:12.5 were prepared by the cholate dialysis method and the volume adjusted to 4.0 mL. Each assay mixture containing 100 µL of the liposome substrate, 235 µL of the assay buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4), and 125 µL of 2% (w/v) HSA was preincubated at 37°C. After 15 min, 2.5 µmol of β-mercaptoethanol and 15 µL of hamster HDL were added to a final volume of 0.5 mL. The assay mixture was vortexed and incubated in a shaking water bath

at 37°C for 30 min. The reaction was stopped by the addition of 2 mL ethanol. The lipids were extracted twice with 4 mL hexane containing 50 μg unlabeled cholesterol and cholesterol oleate as carriers. The extract was dried under nitrogen and redissolved in chloroform. The lipids were then separated by thin layer chromatography using a hexane-ethyl acetate 9:1 (v/v) solvent system. The cholesterol ester and free cholesterol were identified, scraped off, and the radioactivities were measured by liquid scintillation counting. LCAT activity was determined from the conversion of free cholesterol to cholesterol ester, and expressed as fractional cholesterol esterification rate (% esterified/hr) and molar esterification rate (nmol esterified/mL/hr).

Protein concentrations of HDL were measured using a modified Lowry assay (Pierce, Rockford, IL). Total and free cholesterol concentrations of HDL and cells were determined using enzymatic kits from Wako (Richmond, VA). DNA content of cells was determined using a fluorometric method (29).



Statistics

The results are presented as mean ± SEM. Statistical significance was determined using a two-tailed Student's *t* test. The Mann-Whitney test was performed when values were not in normal distribution.

RESULTS

Characteristics of control and acute-phase HDL

Control and acute-phase HDL were isolated from hamster plasma sixteen hours after injection of normal saline and LPS, respectively. The composition of control and acute-phase HDL was similar to that previously reported by our laboratory (12, 18). Compared with control HDL, acute-phase HDL had a lower content of total cholesterol and a higher content of phospholipids (18). Although HDL cholesterol levels were decreased after LPS treatment, there was a disproportionate increase in free cholesterol content of acute-phase HDL compared to that of control HDL (12, 18). Free cholesterol was 23.3 ± 1.5 % of total cholesterol in control HDL and 33.9 ± 2.1 % of total cholesterol in acute-phase HDL ($p = 0.003$, from 5 separate experiments). The protein content of acute-phase HDL was not significantly different from that of control HDL (18), however, we observed changes in individual proteins associated with HDL, including an increase in SAA in acute-phase HDL.

Cholesterol efflux, influx, and mass in J 774 cells

When cells are incubated with HDL, bidirectional transport of cholesterol between HDL and cells can occur. Therefore, we differentially labeled cells with [4-^{14}C]-cholesterol and HDL with [1,2-^{3}H]-cholesterol in order to study cholesterol efflux and influx, respectively (27). Both cholesterol efflux from J 774 cells and cholesterol influx from HDL increase with increasing concentrations of HDL (**Fig. 1A&B**). At various HDL concentrations, cholesterol efflux mediated by acute-phase HDL was significantly

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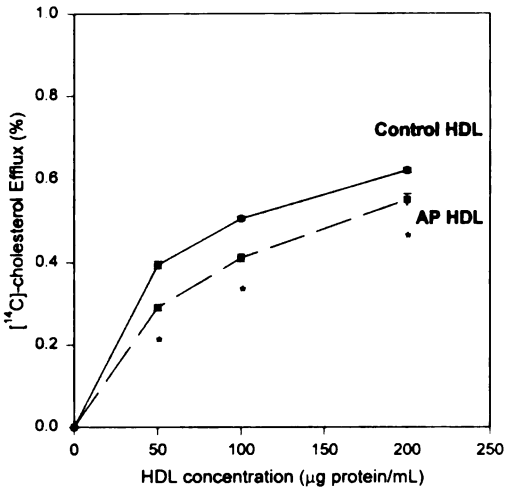

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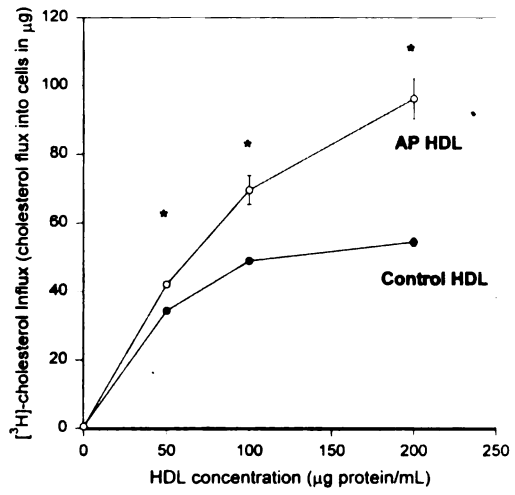

Fig. 1. Dose response curve of cholesterol efflux (**A**) and cholesterol influx (**B**) by control HDL and acute-phase HDL in J 774 cells. J 774 cells were labeled with [4-^{14}C]-cholesterol and HDL was labeled with [1,2-^{3}H]-cholesterol. Cholesterol flux was performed as described in Materials and Methods for 24 hours. Fractional cholesterol flux was calculated as the amount of radioactivity present in the media (efflux) or cells (influx) divided by the total (media + cell) radioactivity in each well. Additionally in cholesterol influx, the amount of cholesterol influx into cells was calculated by multiplying fractional influx by the amount of cholesterol present in HDL. The total specific activity of cholesterol efflux was 44,884 ± 1,095 cpm/well. The total specific activity of cholesterol influx using 200 μg/mL HDL was 2,075 ± 56 cpm/well for control HDL and 1,714 ± 37 cpm/well for acute-phase HDL. AP HDL: acute-phase HDL; *: $p < 0.01$ compared to control HDL.

lower than that by control HDL. Furthermore, cholesterol influx was significantly higher. With lower cholesterol efflux and higher cholesterol influx, the reduced net efflux mediated by acute-phase HDL was confirmed by measurements of cholesterol content in cells. At the end of the experiment, cells incubated with acute-phase HDL showed higher cholesterol content (**Fig. 2A**). Because our protocol did not preload cells with cholesterol-rich media, free cholesterol accounted for the majority (>85%) of cellular total cholesterol. Accordingly, higher total cholesterol content in cells was primarily due to high free cholesterol content, and there was no significant difference in cholesterol ester content of cells (**Fig. 2B**).

LCAT activity of acute-phase HDL

LCAT is a plasma enzyme that catalyzes the formation of cholesterol ester from free cholesterol. It is found associated with HDL and is thought to play a key role in cholesterol removal from peripheral cells. During the APR, total plasma activity of LCAT is decreased (10-13). In this study, we measured LCAT activity of control and acute-phase HDL, and found that the LCAT activity was 73% lower in acute-phase HDL than in control HDL. The fractional esterification rate of the control HDL was 20.40 ± 0.13 % esterified/hr whereas that of the acute-phase HDL was 5.61 ± 1.55 % esterified/hr (**Fig. 3**). Similarly, the molar esterification rate of the control HDL was 247.5 ± 1.7 nmol esterified/mL/hr and that of the acute-phase HDL was 68.19 ± 18.7 nmol esterified/mL/hr. Lower LCAT activity in acute-phase HDL could potentially limit the cholesterol removal from cells. In order to study the role of decreased LCAT activity of acute-phase HDL in cholesterol removal, we used NEM, a known LCAT inhibitor

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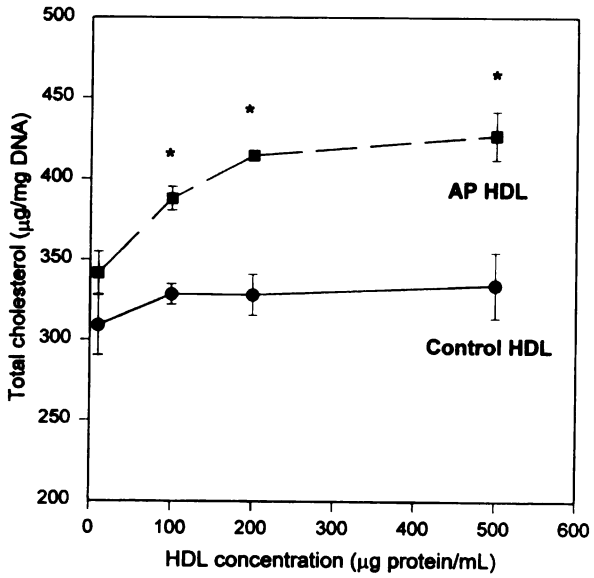

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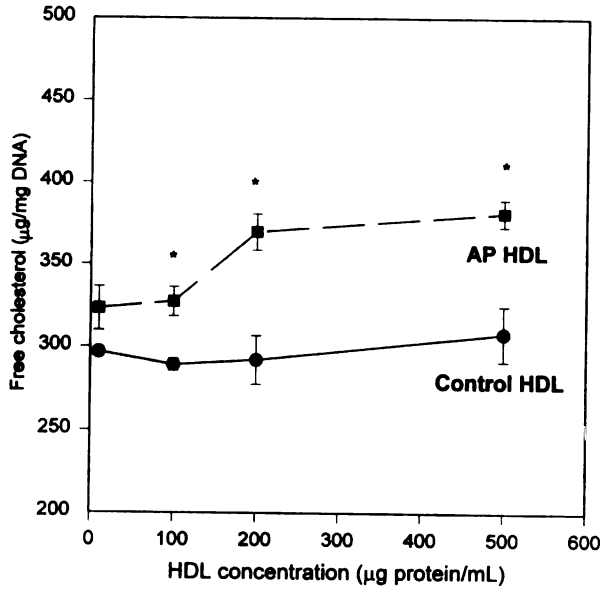


Fig. 2. Total cholesterol mass (**A**) and free cholesterol mass (**B**) (µg/mg DNA) of J 774 cells 24 hours after the addition of HDL. Cells were washed with PBS, scraped into deionized water, sonicated for 15 seconds, and assayed for total cholesterol and DNA content as described in Materials and Methods. AP HDL: acute-phase HDL. *: $p < 0.025$ compared to control HDL.

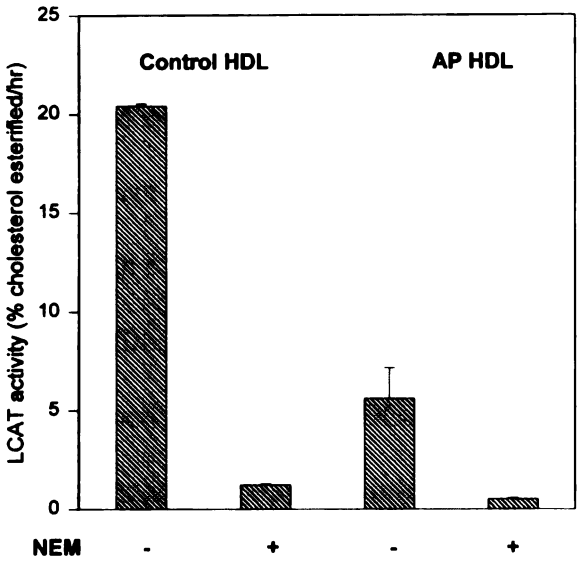


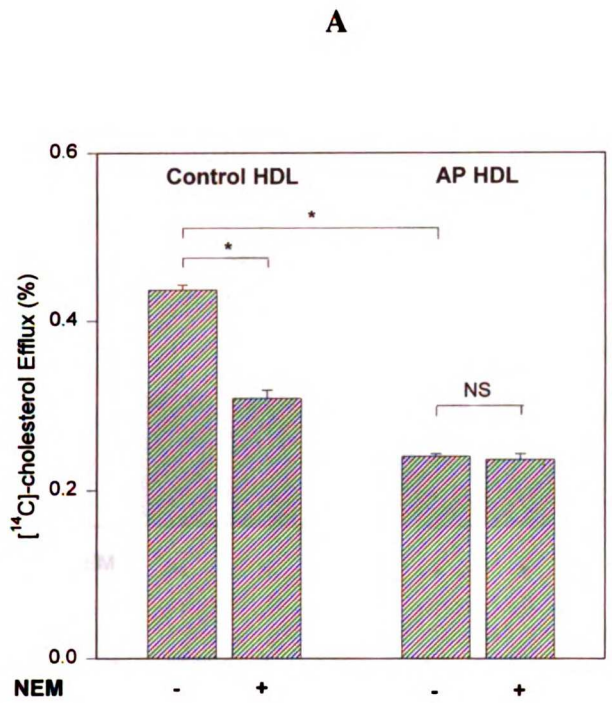
Fig. 3. LCAT activity of HDL preparations. Control and acute-phase HDL were isolated from hamster plasma 16 hours after injection with normal saline or LPS, respectively. NEM was used to inactivate plasma and HDL LCAT activity. Five or 10 mM of NEM in the plasma produced a similar degree of inhibition of LCAT activity in HDL (85-95% inhibition). LCAT activity was determined as described in Materials and Methods and expressed in fractional esterification rates.

(30-32), to inhibit LCAT activity. NEM at the final plasma concentration of 5 or 10 mM almost completely abolished LCAT activity of both control and acute-phase HDL (**Fig. 3**). In agreement with LCAT activity data and the previous report by our laboratory (12), free cholesterol content of HDL was higher and cholesterol ester content was lower in acute-phase HDL or HDL in which LCAT activity was inhibited. As described above, free cholesterol content was 23.3 ± 1.5 % of total cholesterol in control HDL and 33.9 ± 2.1 % in acute-phase HDL. In addition, free cholesterol content in control HDL in which NEM had been added was 31.9 ± 1.2 % and in acute-phase HDL in which NEM had been added was 40.2 ± 2.9 %.

LCAT inactivation experiments using J 774 cells

We next determined cholesterol efflux and influx using control and acute-phase HDL with and without LCAT inactivation. As previously observed, cholesterol efflux was lower and cholesterol influx was higher when J 774 cells were incubated with acute-phase HDL than with control HDL (**Fig. 4A&B**). In addition, when J 774 cells were incubated with control HDL in which LCAT activity had been inhibited, fractional cholesterol efflux was lower than that seen with control HDL (0.31 ± 0.01 vs 0.44 ± 0.01 for LCAT-inactivated control HDL and control HDL, respectively, $p < 0.001$) (**Fig. 4A**). Moreover, fractional cholesterol influx was higher with LCAT-inactivated control HDL compared to control HDL (0.58 ± 0.003 vs 0.35 ± 0.002, respectively, $p < 0.001$) (**Fig. 4B**). In fact, both cholesterol efflux and cholesterol influx of LCAT-inactivated control HDL were comparable to those observed with acute-phase HDL (**Fig. 4A&B**). Inactivation of LCAT activity in acute-phase HDL resulted in a small (<5%) change in cholesterol influx, but not in cholesterol efflux, compared to those of acute-phase HDL with intact LCAT activity (**Fig. 4A&B**).

Cholesterol content of cells was also determined at the end of the cholesterol flux experiments. Total cholesterol content of cells incubated with control HDL in which LCAT activity had been inactivated was significantly higher than in cells incubated with control HDL (337.4 ± 10.5 vs 263.9 ± 11.4 μg total cholesterol/mg DNA for LCAT-inactivated control HDL and control HDL, respectively, $p < 0.05$) (**Fig. 5**). Free cholesterol content primarily accounted for the differences of total cholesterol content (data not shown). The values for total and free cholesterol in cells treated with control HDL in which LCAT was inactivated were not significantly different from those found



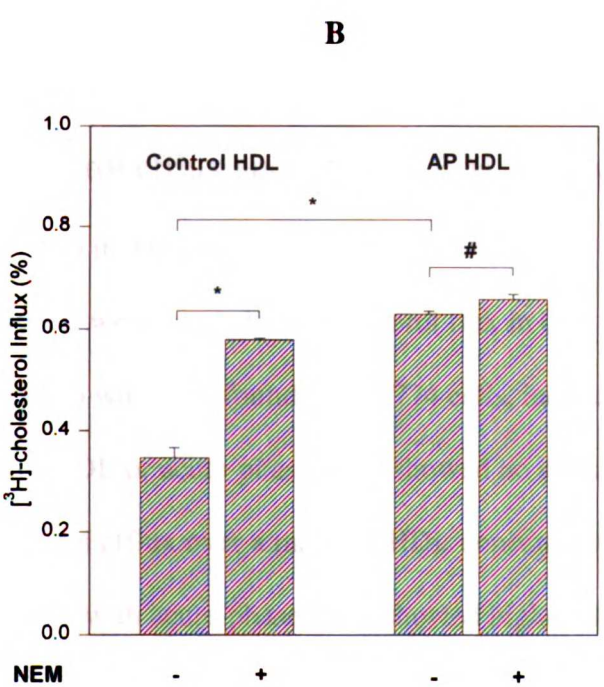


Fig. 4. Cholesterol efflux (**A**) and cholesterol influx (**B**) of J 774 cells at 24 hours after addition of different HDL preparations (100 µg/mL). Cholesterol flux was performed as described in Materials and Methods, and fractional cholesterol flux was calculated as the amount of radioactivity present in the media (efflux) or cells (influx) divided by the total (media + cell) radioactivity in each well. Data represent the average of the two independent experiments. For the first experiment, the total specific activity of cholesterol efflux was 168,742 ± 1,992 cpm/well and the specific activity of cholesterol influx was 3,475 ± 239 cpm/well. For the second experiment, the total specific activity of cholesterol efflux was 127,846 ± 2,593 cpm/well and the specific activity of cholesterol influx was 2,792 ± 806 cpm/well. *: $p < 0.001$; #: $p < 0.05$; NS: nonsignificant.

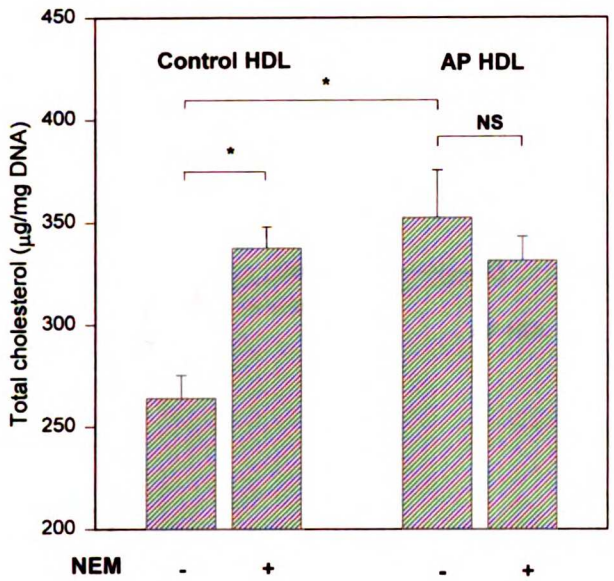


Fig. 5. Total cholesterol mass of J 774 cells at 24 hours after incubation with different HDL preparations (100 µg/mL). *: $p < 0.05$; NS: nonsignificant.

with acute-phase HDL. In addition, the cholesterol content of cells incubated with acute-phase HDL in which LCAT activity had been further inhibited was not significantly different from those with acute-phase HDL (**Fig. 5**).

Cholesterol efflux and influx in GM 3468A cells

To investigate whether cellular cholesterol removal would be impaired in other cell types, we next studied cholesterol flux in GM 3468A normal human fibroblasts. In contrast to what was found with J 774 cells, incubation of GM 3468A cells with either control HDL or acute-phase HDL showed no significant change in HDL-mediated cholesterol efflux over a range of HDL concentrations (**Fig. 6A**). However, cells incubated with acute-phase HDL showed higher cholesterol influx compared to those incubated with control HDL at each HDL concentration (**Fig. 6B**). The increase in cholesterol influx without significant change in cholesterol efflux indicates that *net* efflux from fibroblasts is lower with acute-phase HDL compared to control HDL.

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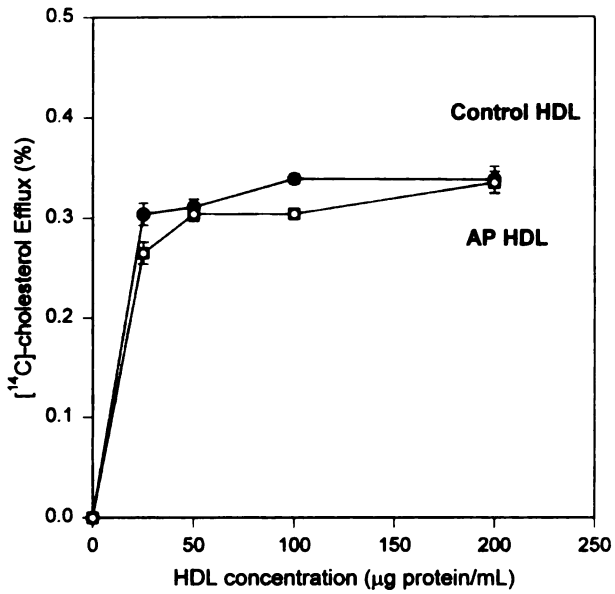

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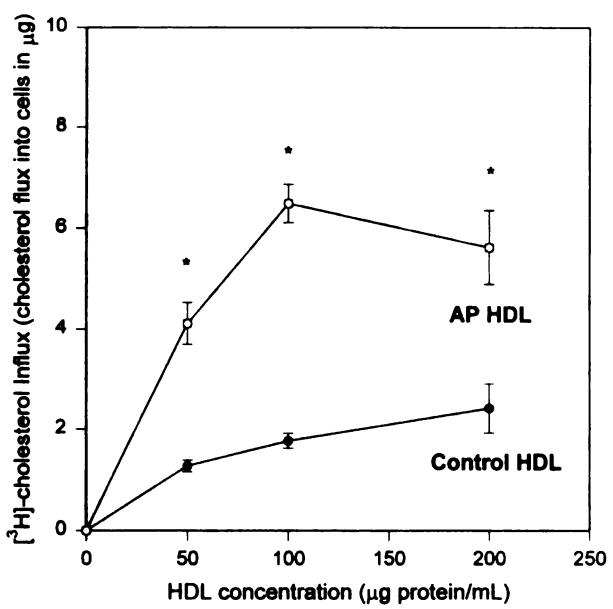


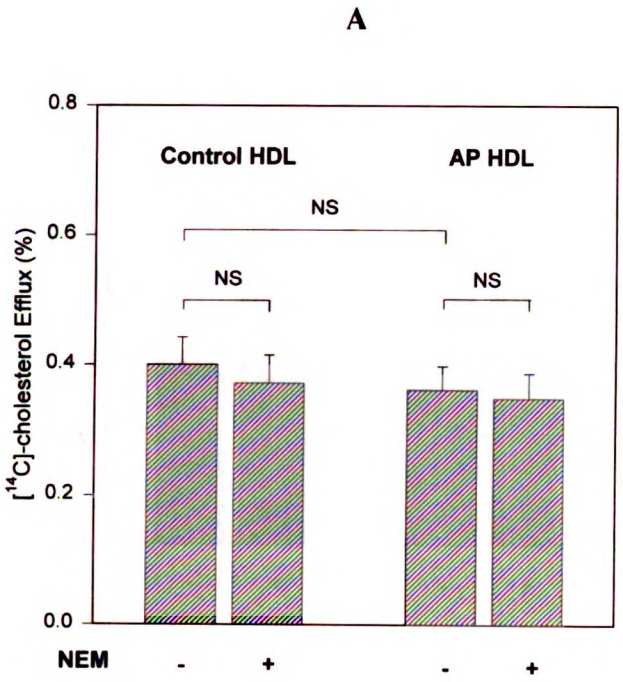
Fig. 6. Dose response curve of cholesterol efflux (**A**) and cholesterol influx (**B**) by control HDL and acute-phase HDL in GM 3468A cells. GM 3468A cells were labeled with [4-^{14}C]-cholesterol and HDL was labeled with [1,2-^{3}H]-cholesterol. Cholesterol flux was performed as described in Materials and Methods for 48 hours. Fractional cholesterol flux was calculated as the amount of radioactivity present in the media (efflux) or cells (influx) divided by the total (media + cell) radioactivity in each well. Additionally in cholesterol influx, the amount of cholesterol influx into cells was calculated by multiplying fractional influx by the amount of cholesterol present in HDL. The total specific activity of cholesterol efflux was 42,981 ± 1,656 cpm/well. The total specific activity of cholesterol influx using 200 µg/mL HDL was 1,260 ± 6 cpm/well for control HDL and 1,182 ± 37 cpm/well for acute-phase HDL. *: $p < 0.025$ compared to control HDL.

LCAT inactivation experiments using GM 3468A cells

To determine whether LCAT also plays a role in HDL-mediated cholesterol removal in GM 3468A cells, we inhibited LCAT activity of HDL with NEM and performed the cholesterol flux experiments. There was no significant difference in cholesterol efflux from cells incubated with either control HDL or control HDL in which LCAT activity had been inactivated (**Fig. 7A**). Similarly, cholesterol efflux was not different when cells were incubated with either acute-phase HDL or acute-phase HDL in which LCAT activity had been inhibited (**Fig. 7A**).

In contrast, cholesterol influx into cells was higher when cells were incubated with control HDL in which LCAT had been inactivated compared to control HDL (0.24 ± 0.02 vs 0.14 ± 0.01 for LCAT-inactivated control HDL and control HDL, respectively, $p < 0.001$) (**Fig. 7B**). Cholesterol influx into cells from control HDL with LCAT inactivation was comparable to that from acute-phase HDL (**Fig. 7B**). There was no significant difference in cholesterol influx when cells were incubated with acute-phase HDL or acute-phase HDL in which LCAT had been inactivated (**Fig. 7B**).

Cholesterol content of GM 3468A cells was also determined and the results were in agreement with the cholesterol flux experiments. Total cholesterol content of cells incubated with control HDL was lower than those with acute-phase HDL or control HDL in which LCAT activity had been inactivated (**Fig. 8**). Free cholesterol accounted for most of the differences (data not shown). There was no significant difference in cholesterol content of cells incubated with acute-phase HDL or acute-phase HDL in which LCAT activity had been inactivated (**Fig. 8**).



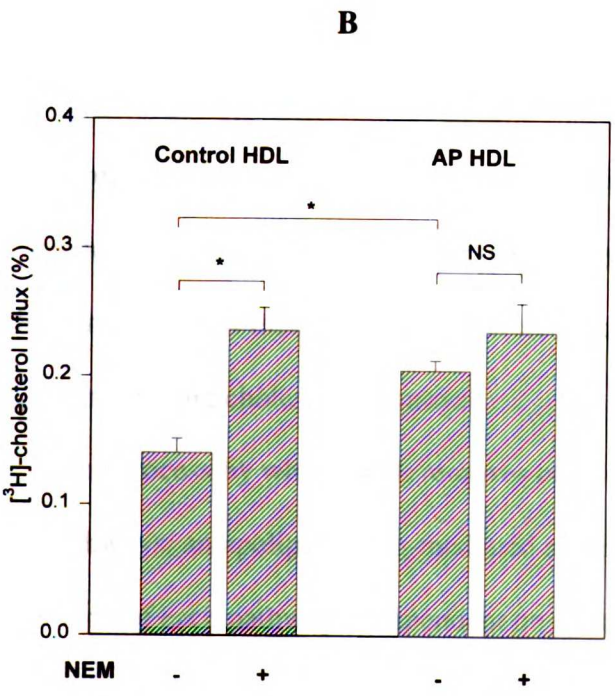


Fig. 7. Cholesterol efflux (**A**) and cholesterol influx (**B**) of GM 3468A cells at 48 hours after addition of different HDL preparations (100 µg/mL). Cholesterol flux was performed as described in Materials and Methods, and fractional cholesterol flux was calculated as the amount of radioactivity present in the media (efflux) or cells (influx) divided by the total (media + cell) radioactivity in each well. Data represent the average of the two independent experiments. For the first experiment, the total specific activity of cholesterol efflux was 17,137 ± 963 cpm/well and the specific activity of cholesterol influx was 425 ± 4 cpm/well. For the second experiment, the total specific activity of cholesterol efflux was 48,474 ± 817 cpm/well and the specific activity of cholesterol influx was 1,858 ± 26 cpm/well. *: $p < 0.001$; NS: nonsignificant.

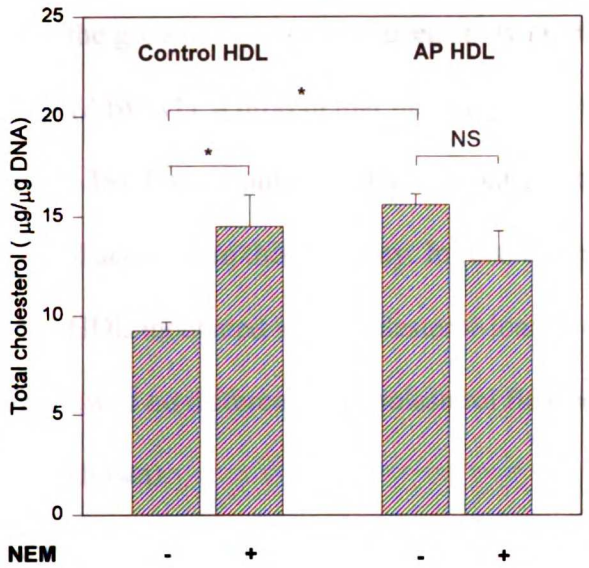


Fig. 8. Total cholesterol mass of GM 3468A cells at 48 hours after incubation with different HDL preparations (100 µg/mL). *: $p < 0.05$; NS: nonsignificant.

DISCUSSION

Cholesterol is important for the normal function of cells and multiple mechanisms are utilized in order to maintain cellular cholesterol homeostasis. Different cell types use different mechanisms to regulate cholesterol flux into and out of cells. Although most cells cannot metabolize or secrete cholesterol, they can use cholesterol efflux as a mechanism to control their cellular cholesterol content. HDL acts as a cholesterol acceptor, mediating cholesterol removal.

The process by which HDL removes cellular cholesterol involves at least two major pathways: an apolipoprotein-mediated mechanism and aqueous diffusion (33). The contribution of each pathway is dependent on the type of HDL subspecies and the cell type, as well as the cholesterol content and growth state of the cells (23, 34). Free apolipoproteins and lipid-poor preβ HDL are the main cholesterol acceptors in

apolipoprotein-mediated efflux. Cholesterol loading, raising cAMP content of cells, and arresting the growth of cells have been shown to increase the levels of ATP-binding cassette1 (ABCA1), a protein that plays a pivotal role in the apolipoprotein-mediated efflux (35-38). Fully-lipidated HDL, in contrast to lipid-poor HDL, is not a major cholesterol acceptor in this pathway. In fact, our preliminary experiments using fully-lipidated HDL incubated with cholesterol-loaded cells that were treated with a cAMP analog showed no difference in cholesterol flux between control and acute-phase HDL (data not shown).

Fully-lipidated HDL, however, is the main cholesterol acceptor in the aqueous diffusion pathway. In proliferating cells, the aqueous diffusion pathway accounts for most of the cholesterol efflux. Our experiments used an established protocol that studies mainly the aqueous diffusion mechanism and the bidirectional flux of cholesterol between cells and HDL (27). In aqueous diffusion, free cellular cholesterol spontaneously desorbs from the cell surface and is taken up by HDL. However, cholesterol can also travel from HDL into the cells; therefore, both directions must be measured to quantitate net flux. The enzyme LCAT plays a role in this pathway by converting free cholesterol into cholesterol ester on HDL particles, thus creating a free cholesterol gradient in HDL so that more diffusion of cholesterol from cells can occur with less diffusion from HDL back into cells.

This study demonstrates that the ability of acute-phase HDL to remove cholesterol from cells was lower than that of control HDL. Compared with control HDL, incubation of acute-phase HDL with J 774 macrophages resulted in lower cholesterol efflux and higher cholesterol influx. In GM 3468A fibroblasts, acute-phase HDL caused

only an increase in cholesterol influx without changes in cholesterol efflux. In both cells, the impairment of *net* cholesterol efflux due to acute-phase HDL led to higher cellular cholesterol content. Although net efflux of cholesterol can be affected by the selective uptake of cholesterol from HDL by the scavenger receptor class B type I (39), incubation of maleylated bovine serum albumin in the presence of HDL had no effect on efflux in our experiments (data not shown).

We (12) and others (10, 11, 13) have previously reported a decrease in both total plasma cholesterol esterification activity and total plasma LCAT activity during the APR. Now, we report that acute-phase HDL has lower LCAT activity compared to that of control HDL. By using NEM as an LCAT inhibitor, we found that LCAT on HDL plays a key role in promoting cholesterol efflux and preventing cholesterol influx in J 774 cells. In GM 3468A cells, however, LCAT on HDL is important only in preventing cholesterol influx, and has no role in cholesterol efflux. Furthermore, we found that inhibition of LCAT in control HDL led to a similar change in cholesterol flux as was found in acute-phase HDL with its decrease in LCAT. LCAT may play a direct role by actively promoting cholesterol flux during the flux experiment. In addition, our data on cholesterol composition of HDL suggest that LCAT may also play an indirect role by modifying HDL particles to keep the lipoprotein surface depleted of free cholesterol, thus making them better cholesterol acceptors.

Our results are consistent with several studies that demonstrate the role of LCAT in cholesterol transport from cells. Studies using LCAT inhibitors to inactivate LCAT activity have shown that LCAT can prevent cholesterol influx and promote *net* cholesterol efflux mediated by plasma or serum in FU5AH hepatoma cells and in human

fibroblasts (31, 40). LCAT, however, seems to have a lesser role in promoting cholesterol efflux from cells. For example, in human fibroblasts, LCAT appeared to have no effects on cholesterol efflux because when serum LCAT was inactivated, cholesterol efflux was unchanged (30, 40).

Although most of the studies using fibroblast cells have shown that LCAT is important for preventing cholesterol influx and has no major role in enhancing cholesterol efflux, results from studies using other cells differ. In erythrocytes, for example, LCAT appeared to increase cholesterol efflux and prevent cholesterol influx, resulting in a decrease in cellular cholesterol content (41, 42). Our results in J774 macrophages showing that LCAT promotes HDL-mediated cholesterol efflux as well as preventing cholesterol influx are similar to the studies in erythrocytes. It has been postulated that in cultured cells in which the cholesterol exchange rate is slow and cholesterol efflux is rate-limiting, cholesterol esterification does not play a role, e.g. LCAT does not increase cholesterol efflux from cells. On the other hand, in cells where the cholesterol exchange rate is high, cholesterol esterification plays an important role and thus LCAT can increase cholesterol efflux from cells (42).

A recent study has shown that acute-phase HDL is associated with a decrease in cholesterol efflux from J 774 cells (22). Although it was suggested that the presence of SAA in acute-phase HDL might be related to the decreased cholesterol efflux, no experimental data to support such a hypothesis was provided. Another study found no difference in cholesterol efflux from monocytic THP-1 cells between normal and acute-phase HDL (43). When normal HDL was markedly remodeled in vitro such that the HDL contained SAA greater than 50% of its total protein, this resulted in decreases in

cholesterol efflux (43). However, this degree of remodeling does not typically occur during the APR.

Our study differs from those two studies in that we examined both cholesterol efflux and influx in two different types of cells that have been extensively used for reverse cholesterol transport experiments (27, 37, 39, 44). We showed that the ability of acute-phase HDL to remove cholesterol from cells was impaired. More importantly, we provided evidence that the decreased LCAT activity in acute-phase HDL might be the underlying mechanism of decreased cholesterol removal from cells. During the APR, the lower plasma LCAT activity is preceded by a decrease in hepatic LCAT mRNA (12), suggesting that decreased LCAT protein may be responsible for the decrease in plasma LCAT activity. However, an inhibitory effect on LCAT activity by other factors cannot be excluded. For example, SAA and lipid hydroperoxides, both of which increase during the APR (20, 45), have been reported to inhibit LCAT activity (46, 47).

The APR has profound effects on HDL. Acute-phase HDL is unable to protect against LDL oxidation (48), which could be related to the decrease in paraoxonase seen in acute-phase HDL (17, 48). The data presented here provide further support for the hypothesis that acute-phase HDL becomes proatherogenic by decreasing cholesterol removal from cells due to decreased LCAT activity in acute-phase HDL. Defective cholesterol removal as well as the proinflammatory effects of acute-phase HDL during the APR could provide potential mechanisms that contribute to the observations linking chronic infection and inflammation and atherosclerosis.

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CHAPTER 4

REGULATION OF SCAVENGER RECEPTOR CLASS B TYPE I (SR-BI) IN HAMSTER LIVER AND HEP3B CELLS BY ENDOTOXIN AND CYTOKINES

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ABSTRACT

Multiple changes in high-density lipoprotein (HDL) metabolism occur during infection and inflammation that could potentially impair the anti-atherogenic functions of HDL. Scavenger receptor class B type I (SR-BI) promotes cholesterol efflux from peripheral cells and mediates selective uptake of cholesterol ester into hepatocytes, thereby playing a pivotal role in reverse cholesterol transport. We studied the effect of endotoxin (LPS) and cytokines (TNF and IL-1) on hepatic SR-BI mRNA and protein levels in Syrian hamsters. LPS significantly decreased SR-BI mRNA levels in the hamster liver. This effect was rapid and sustained, and was associated with a decrease in hepatic SR-BI protein levels. High cholesterol diet did not change hepatic SR-BI mRNA levels, and LPS was able to decrease SR-BI mRNA levels during high cholesterol feeding. TNF and IL-1 decreased SR-BI mRNA levels in the liver, and the effects of TNF and IL-1 were additive. TNF and IL-1 also decreased SR-BI levels in Hep3B hepatoma cells. More importantly, TNF and IL-1 decreased the uptake of HDL cholesterol ester into Hep3B cells. In addition, we studied the effect of LPS on SR-BI mRNA in RAW 264.7 cells, a macrophage cell line. LPS rapidly decreased SR-BI mRNA levels in RAW 264.7 cells, but the effect was not sustained and did not lead to a reduction in SR-BI protein levels. Our results suggest that the decrease in hepatic SR-BI levels due to LPS and cytokines during infection and inflammation may decrease selective uptake of cholesterol ester into the liver and result in impaired reverse cholesterol transport.

INTRODUCTION

Lipoproteins are a heterogeneous group of lipid-transporting particles whose levels are associated with atherosclerosis. While plasma levels of low-density lipoprotein (LDL)-cholesterol directly correlate with the risk of coronary artery disease, the relationship between plasma levels of high-density lipoprotein (HDL)-cholesterol and risk is inverse indicating that HDL is anti-atherogenic. There are several mechanisms by which HDL may protect against atherogenesis, and one of the most extensively studied and accepted hypotheses suggests that HDL plays a role in removing excess cholesterol from peripheral cells and returning it to the liver for excretion (1). This process, known as reverse cholesterol transport (RCT), consists of multiple steps and involves a number of enzymes, transfer proteins, and cell surface receptors (2, 3).

RCT begins when HDL removes cholesterol from cells in an unesterified form. Once transferred onto HDL, unesterified cholesterol is converted to cholesterol ester by the enzyme lecithin:cholesterol acyltransferase (LCAT). Cholesterol ester in HDL particles may be returned to the liver by several mechanisms. First, cholesterol ester in HDL may be exchanged for triglyceride in triglyceride-rich lipoproteins by cholesterol ester transfer protein (CETP). Cholesterol ester in triglyceride-rich lipoproteins can then be taken up by the hepatocyte via the LDL receptor and/or LDL receptor-related protein (LRP). Second, cholesterol ester in HDL may be delivered directly into hepatocytes by endocytosis of HDL particles through the LDL receptor, LRP, or yet to be elucidated HDL receptors. Lastly, cholesterol ester in HDL may be preferentially taken up by hepatocytes without degradation of apolipoproteins, leaving the particle intact. This latter process is known as selective uptake of cholesterol ester.

Selective uptake of cholesterol ester from HDL has been documented in several in vitro and in vivo studies (4-6), and is now known to be mediated by scavenger receptor class B type I (SR-BI) (7). SR-BI is a cell surface receptor initially shown to bind modified LDL (8). It was later demonstrated that SR-BI could bind HDL and mediate the selective uptake of cholesterol ester from HDL into hepatocytes (7). Moreover, SR-BI may play an additional role in cholesterol removal from peripheral cells (9, 10). Animal studies have shown that manipulating SR-BI levels affects HDL metabolism and the development of atherosclerosis (11-18), making SR-BI one of the key proteins in these processes. Identification of factors that regulate SR-BI in vivo should provide insights into the mechanisms by which HDL metabolism and RCT are affected in physiological and pathological conditions.

Recent evidence has suggested a possible relationship between chronic infection/inflammation and atherosclerosis (19). During infection and inflammation, a variety of changes in HDL metabolism occur (20). HDL cholesterol levels decrease during infection and inflammation. In addition, we and others have shown that endotoxin and cytokines decrease plasma levels and activities of several key proteins involved in HDL metabolism and RCT, including LCAT, CETP, hepatic lipase, and phospholipid transfer protein (21-26). In contrast, circulating levels of secretory phospholipase A_2, serum amyloid A, and apolipoprotein J increase (27-29). All of these changes can have profound effects on HDL metabolism and potentially affect RCT. Although SR-BI is one of the key receptors in RCT, how SR-BI is regulated in vivo by endotoxin and cytokines is unknown. In this study, we examined whether endotoxin and cytokines, major inducers

of inflammation, affect SR-BI levels in hamster liver, in Hep3B human hepatoma cells, and in RAW 264.7 cells, a murine macrophage cell line.

MATERIALS AND METHODS

Materials

Endotoxin (lipopolysaccharide or LPS from E. coli serotype 055:B5) was purchased from Difco Laboratories (Detroit, MI). Recombinant human tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal antibody against the last 14 amino acid of the carboxy terminus of the mouse SR-BI (7) was kindly provided by Dr. Helen H. Hobbs (Dallas, TX). In some experiments, anti SR-BI antibody was purchased from Novus Biologicals (Littleton, CO). Supplies for immunoblot analysis and [^{3}H] cholesteryl oleyl ether (58.0 Ci/mmol) were purchased from Amersham-Pharmacia (Piscataway, NJ).

Animal experiments

We used hamsters as an animal model because HDL metabolism in hamsters resembles that of humans (30). In addition, changes of lipid and lipoprotein metabolism during infection/inflammation in hamsters have already been characterized (31). Male Syrian hamsters (approximately 140-180 g) were obtained from Charles River Laboratories (Wilmington, MA) and provided with rodent chow and water ad libitum. In cholesterol feeding experiments, animals were fed with 3% cholesterol diet for 4 days. Animals were injected intraperitoneally with indicated doses of endotoxin, TNF, IL-1, or

TNF and IL-1. Control animals were injected with normal saline. At the dose of 100 μg LPS/100 g BW, animals become toxic, serum amyloid A is induced, and circulating cytokine levels are expected to be in the septic range (32). However, this dose is far below the lethal dose (LD_{50} ~ 5 mg/100 g BW) required to cause death in rodents in our laboratory. Because LPS and cytokines can cause anorexia, food was withdrawn after the injection. At indicated time points, the animals were euthanized using halothane, plasma was collected, and the tissue was excised and stored at –80°C. The animal procedures were approved by the Animal Studies Subcommittee of the San Francisco Veterans Affairs Medical Center, and were performed in accordance with the guidelines.

Cell culture experiments

Hep3B human hepatoma cells and RAW 264.7 murine macrophages were obtained from the American Type Culture Collection and maintained in Minimum Essential Media (MEM) supplemented with 10% fetal bovine serum under 5% CO_2. For the mRNA and protein experiments, cells were plated until confluent. LPS or cytokine was added into MEM media containing bovine serum albumin (BSA) in the absence of serum, and at indicated time points, cells were washed and harvested.

Isolation of RNA and RNA blot analysis

Isolation of RNA and RNA blot analyses were performed as previously described (33). Poly A+ RNA was quantified by measuring absorption at 260 nm and 10 micrograms of RNA were loaded on 1% agarose-formaldehyde gels and electrophoresed. The uniformity of sample applications was checked by ultraviolet visualization of the

acridine orange-stained gels before transfer to Nytran membranes. We and others have found that LPS, TNF, and IL-1 increase actin mRNA levels in the liver by 2-5 fold in rodents (31, 34). LPS also produced a twofold increase in hepatic mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and a 2.6 fold increase in cyclophilin mRNA (35); therefore, the mRNA levels of actin, GAPDH, and cyclophilin, which are widely used for normalizing data, cannot be used to study LPS or cytokine-induced regulation of proteins in the liver. However, the differing direction of the changes in mRNA levels (increased for some proteins, decreased for some proteins, and no change for other proteins), the magnitude of alterations (up to 90% decrease), and the relatively small standard error of the mean make it unlikely that the changes observed were due to unequal loading of mRNA. RNA blots were hybridized with a ^{32}P-labeled SR-BI cDNA probe generated by RT-PCR from the mouse liver (oligonucleotide primers: upper, 5'-ATG CAG GTC CAT GAA GCT GAC-3' and lower, 5'CTA TAG CTT GGC TTC TTG CAG C-3'). After washing, the blots were exposed to X-Ray films for various durations to ensure that measurements were done on the linear portion of the curve, and the bands were quantified using the Bio-Rad imaging densitometer.

Isolation of membranes and Immunoblot analysis

Frozen hamster tissues were homogenized in the homogenization buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.2 M sucrose, and 5% (v/v) protease inhibitor cocktail, and centrifuged at 8,000 g for 15 min. For the cultured cells, after extensive washing, scraped cells were repeatedly frozen and thawed in the homogenization buffer using liquid N_2 and centrifuged to remove cell debris. The

supernatant was collected and centrifuged at 100,000 g for 60 min. The membrane pellets were resuspended in 0.1 M phosphate buffer pH 7.5, and protein concentration measured. Membrane proteins were resolved on a 7.5% polyacrylamide gel and transferred to Hybond-P PVDF membrane. For immunodetection, the blots were blocked in phosphate buffer saline-0.1% Tween containing 5% nonfat dry milk before incubation with an anti SR-BI antibody. Donkey anti-rabbit antibody conjugated with horseradish peroxidase was used as a secondary antibody. The blots were visualized using the ECL Plus chemiluminescence detection system and subjected to autoradiography. Quantification of the signals was performed by densitometry.

Isolation and labeling of HDL_3

HDL_3 (d = 1.125-1.21 g/ml) and lipoprotein-deficient serum (LPDS, d > 1.25 g/ml) were isolated from fasting sera of normal human subjects by ultracentrifugation and extensively dialyzed as previously described (36). HDL_3 was radiolabeled with [^{3}H] cholesteryl oleyl ether, a nondegradable, intracellularly-trapped marker, using a glass fiber exchange method. HDL labeled with a cholesterol ether analog has been shown to have similar metabolic and biological functions as native HDL (4). Briefly, 100 μCi of [^{3}H] cholesteryl oleyl ether was applied onto the glass microfiber filter in a glass scintillation vial and dried with N_2. 1 ml of HDL_3 (~ 5 mg/ml) and 5 ml of LPDS (as a source of CETP) were added and the vial was rotated gently for 24 hours at 37°C. HDL_3 was recovered after ultracentrifugation and dialysis. The cholesterol/protein ratio of HDL_3 was ~ 0.27, and the ^{3}H activity of radiolabeled HDL_3 was ~ 29,000 cpm/μg cholesterol.

Cholesterol ester uptake experiments

Uptake of radiolabeled HDL_3 was performed as previously described (37). Hep3B human hepatoma cells were plated overnight in serum-containing media. On the following day, cells were incubated in MEM containing 10% LPDS with or without cytokines or LPS for 48 hours. Cells were then preincubated in MEM containing 0.1% BSA for 30 min before incubation with radiolabeled HDL_3 (20 μg protein/ml) for 4 hours. 1 mM EDTA was added to the media to inhibit the activity of classical lipoprotein receptors. Unlabeled HDL_3 (60-100 μg/ml) was added for chase incubation for 2 hours to remove reversibly cell-associated tracers. Cells were extensively washed with phosphate-buffered saline and dissolved in 0.1 N NaOH for 30 min followed by sonication. Aliquots were used to determine ^3H radioactivity and DNA content. The uptake of cholesterol ester was expressed as ng cholesterol ester/μg DNA.

Biochemical determinations

Total and free cholesterol concentrations were measured using enzymatic assays (Wako, Osaka, Japan). Protein concentrations were determined using a modified Lowry protein method (Pierce, Rockford, IL). DNA content of cells was determined using a fluorometric method (38).

Statistics

Data are presented as mean ± SEM. Comparisons between groups were performed using a Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Effect of LPS on lipids and lipoprotein levels

LPS administration to hamsters (100 μg/100 g body weight (BW)) resulted in a significant decrease in plasma HDL cholesterol levels at 16 hours (58 ± 3 mg/dl in control group, n = 6, and 44 ± 3 mg/dl in LPS-treated group, n = 7, 25% decrease, $P < 0.005$). In our previous study using 50 μg of LPS/100 g BW (21), we showed that HDL cholesterol levels decreased by 41%, 35%, 13% and 17% at 4, 8, 16, and 24 hours, respectively, after LPS injection. Similarly, both total and LDL cholesterol levels in this study also increased significantly after LPS injection as previously observed (21) (total cholesterol = 94 ± 8 mg/dl in control group and 146 ± 13 mg/dl in LPS-treated group, $P < 0.01$; LDL cholesterol = 13 ± 5 mg/dl in control group and 77 ± 12 mg/dl in LPS-treated group, $P < 0.001$). Plasma triglyceride levels increased after LPS injection but did not reach statistical significance (109 ± 10 mg/dl in control group and 128 ± 21 mg/dl in LPS-treated group, $P = 0.45$).

Effect of LPS on hepatic SR-BI mRNA and protein levels in hamsters

A single dose of 100 μg of LPS/100 g BW rapidly decreased hepatic SR-BI mRNA levels in hamsters as shown in **Fig. 1A&B**. LPS produced a maximum decrease in SR-BI mRNA levels at 8-16 hours (75% decrease), and the levels continued to be significantly suppressed at 24 hours after LPS administration.

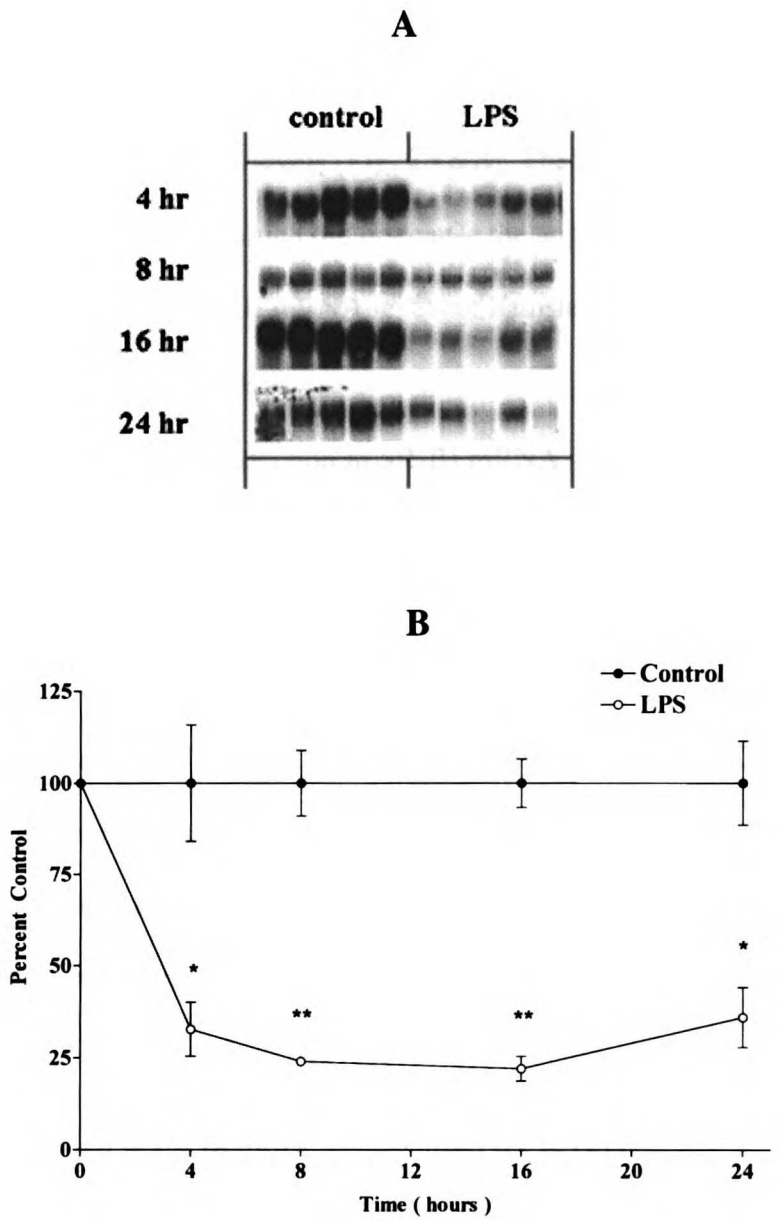

Fig. 1. Time course of the effect of LPS on SR-BI mRNA in the hamster liver. Syrian hamsters were injected with LPS (100 μg/100 g BW). At indicated time points, livers were harvested and RNA was isolated. **A**: Poly A^+ RNA (10 μg in each lane) was hybridized with ^{32}P-labeled cDNA probes and bands were analyzed by agarose gel electrophoresis followed by autoradiography as described in Materials and Methods. **B**: The SR-BI bands were quantitated by densitometry. Data are presented as percent change vs. control (mean ± SEM). N = 5 in each group at each time point. *: $P < 0.005$, **: $P < 0.001$.

The dose response curve of LPS effects on hepatic SR-BI mRNA levels was examined at the 8-hour time-point. LPS administration resulted in a dose-dependent decrease in hepatic SR-BI mRNA levels (**Fig. 2A&B**). The levels were significantly decreased by low doses of LPS, which did not cause the animals to appear ill. A half-maximal decrease in hepatic SR-BI mRNA levels of hamsters was produced by approximately 0.1 μg LPS/100 g BW.

Because LPS induced a reduction in SR-BI mRNA in the hamster liver, we next investigated whether SR-BI protein levels in the liver also are decreased. We determined the protein levels of hepatic SR-BI at the time of the maximal decrease of mRNA levels. Sixteen hours after LPS injection (100 μg/100 g BW), SR-BI protein levels in the liver were significantly decreased (80% decrease) compared to controls (**Fig. 3A&B**). In contrast, SR-BI protein levels in the adrenal and the testis, two tissues that highly express SR-BI, increased in response to LPS but the increases did not achieve the statistical significance (data not shown).

Effect of diet and LPS on hepatic SR-BI mRNA levels

A high cholesterol diet has been shown to decrease hepatic SR-BI mRNA levels in rats (39), therefore, we next investigated whether a high cholesterol diet had a similar effect on hepatic SR-BI mRNA levels in hamsters. After 4 days of a 3% cholesterol diet, the hepatic levels of SR-BI mRNA were not significantly different from those on regular chow diet (**Fig. 4A&B**). As described above, LPS significantly decreased hepatic SR-BI mRNA levels in hamsters fed regular chow. In addition, LPS also significantly decreased hepatic SR-BI mRNA levels in animals fed a high cholesterol diet (**Fig. 4A&B**).

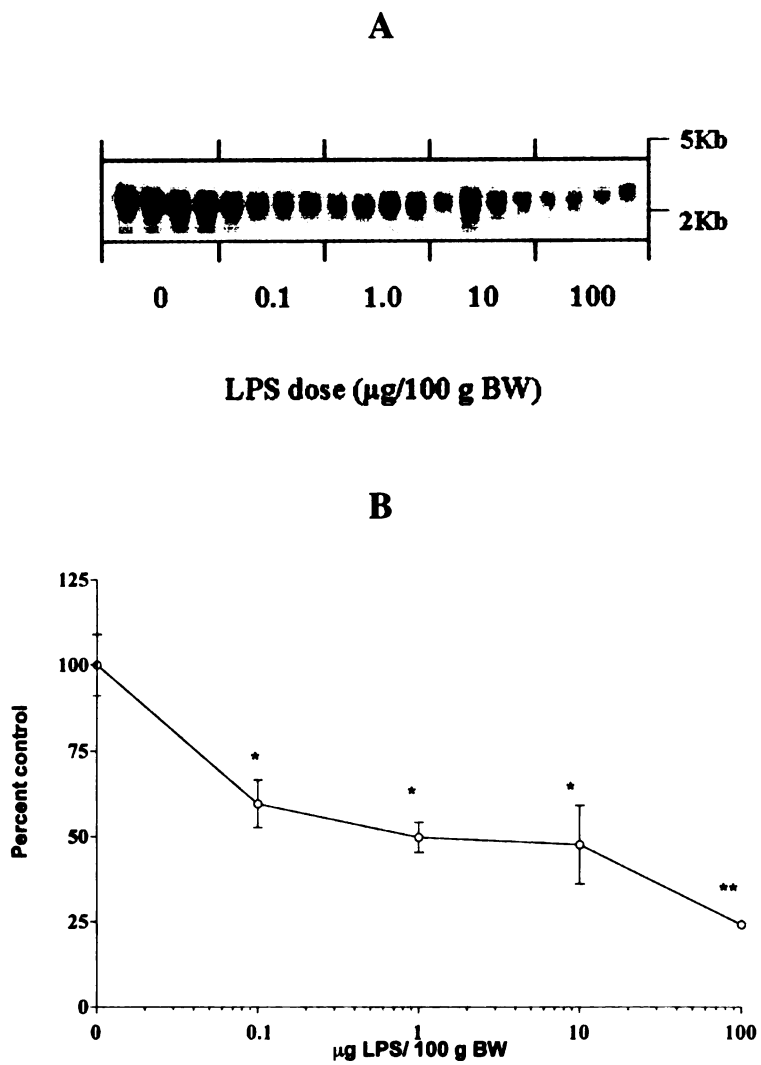

Fig. 2. Dose-response curve of the effect of LPS on SR-BI mRNA in the hamster liver. Syrian hamsters were injected with indicated doses of LPS and livers were harvested for mRNA isolation eight hours later. **A**: RNA blot showing the effect of different doses of LPS on hepatic SR-BI mRNA levels. **B**: The SR-BI bands were quantitated by densitometry and data are presented as percent change vs. control (mean ± SEM). N = 4 in each group. *: $P < 0.025$, **: $P < 0.001$.

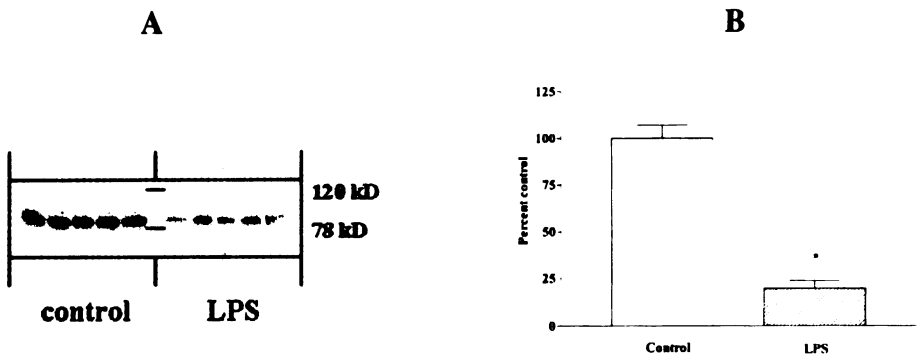

Fig. 3. The effect of LPS on SR-BI protein in the hamster liver. Syrian hamsters were injected with normal saline or 100 μg LPS/100 g BW and sixteen hours later, livers were harvested for preparation of liver membranes. **A**: Fifty micrograms of the liver membranes were loaded in each lane and immunoblot analysis was performed as described in Materials and Methods. **B**: The SR-BI bands were quantitated by densitometry and data are presented as percent change vs. control (mean ± SEM). N = 5 in each group. *: $P < 0.001$.

Effect of TNF and IL-1 on hepatic SR-BI mRNA levels

Cytokines mediate many of the effects of LPS, therefore, we next examined the effect of cytokines on hepatic SR-BI mRNA levels. The effects of TNF (25 μg/100 g BW), IL-1 (1 μg/100 g BW), and a combination of TNF and IL-1 (25 μg TNF and 1 μg IL-1/100 g BW) on hepatic SR-BI mRNA levels are shown in **Fig. 5A&B**. TNF produced a 50% decrease in SR-BI mRNA levels; IL-1 induced a 60% reduction in hepatic SR-BI mRNA levels. In addition, the combination of TNF and IL-1 administration resulted in a further decrease in SR-BI mRNA levels (90%), suggesting an additive effect.

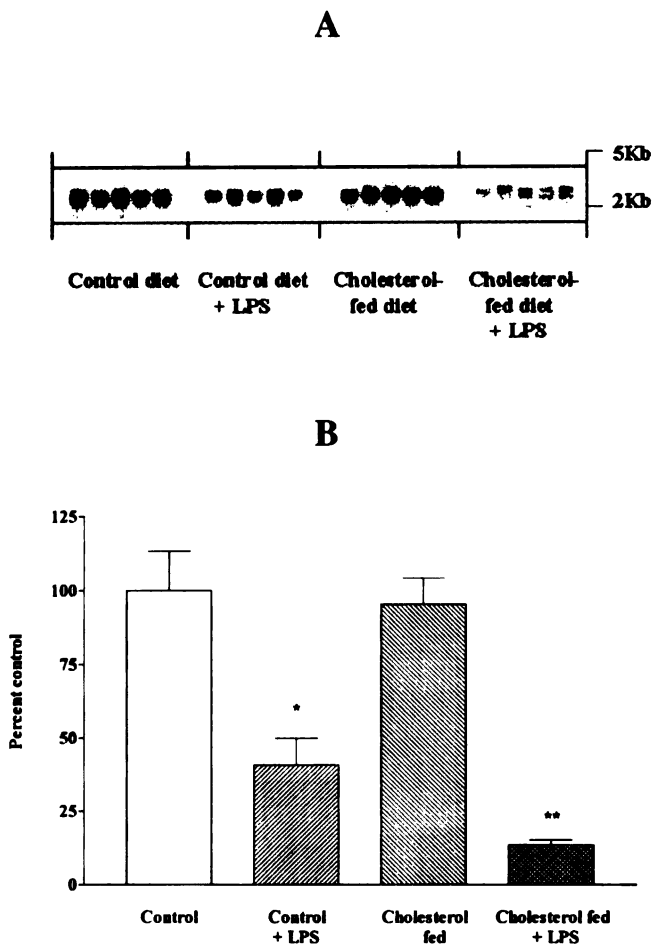

Fig. 4. The effect of diet and LPS on SR-BI mRNA in the hamster liver. Syrian hamsters were fed with regular chow or 3% cholesterol diet for 4 days, followed by injection with normal saline or LPS (100 μg/100 g BW). The liver was harvested sixteen hours later for mRNA isolation. **A**: RNA blots showing the effect of a high cholesterol diet and LPS on hepatic SR-BI mRNA levels. **B**: The SR-BI bands were quantitated by densitometry and data are presented as percent change vs. control (mean ± SEM).) N = 5 in each group. The difference between the control group and the cholesterol-fed group is not statistically significant ($P = 0.8$), *: $P < 0.01$, **: $P < 0.001$.

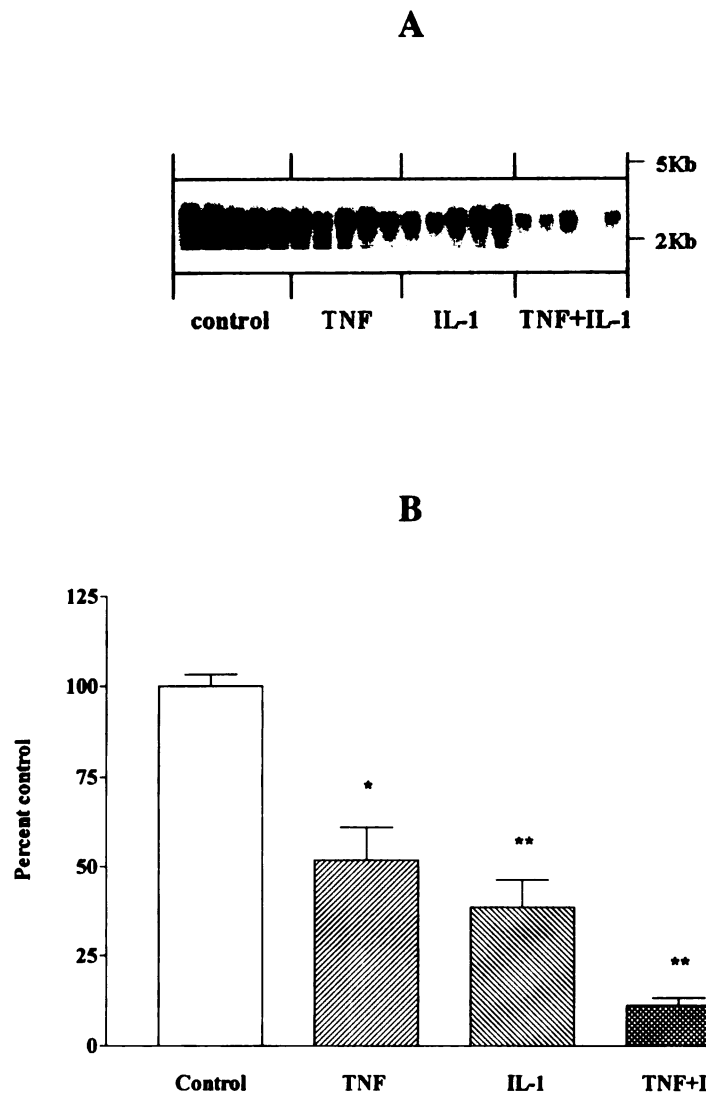

Fig. 5. The effect of cytokines on SR-BI mRNA in the hamster liver. Syrian hamsters were injected with normal saline, TNF-α (25 μg/100 g BW), IL-1β (1 μg/100 g BW), or a combination of TNF-α and IL-1β (25 μg TNF-α and 1 μg IL-1β/100 g BW) and livers were harvested eight hours later for mRNA isolation. **A**: RNA blot showing the effect of TNF and/or IL-1 on hepatic SR-BI mRNA levels. **B**: The SR-BI bands were quantitated by densitometry and data are presented as percent change vs. control (mean ± SEM). N = 5 in each group except in the TNF + IL-1, N = 4. *: $P = 0.001$, **: $P < 0.001$.

Effect of TNF and IL-1 on SR-BI levels and cholesterol ester uptake in Hep3B cells

In the liver, SR-BI is expressed in both hepatocytes and Kupffer cells (39). To determine whether the decrease of SR-BI levels in the liver was due to a decrease of SR-BI in the hepatocyte, we studied the effect of cytokines in Hep3B human hepatoma cells. In agreement with our in vivo data, treatment of Hep3B cells with TNF (10 ng/ml) resulted in a significant decrease of SR-BI mRNA and proteins levels. There was a 12% decrease in SR-BI mRNA levels at 4 hours and an 82% decrease at 24 hours (**Fig. 6**). For SR-BI protein levels, there was a 36% decrease at 24 hours and a 76% decrease at 48 hours (**Fig. 6**). A similar degree of the reduction of SR-BI levels between the hamster liver and Hep3B cells suggests that the decrease in vitro reflects the in vivo changes.

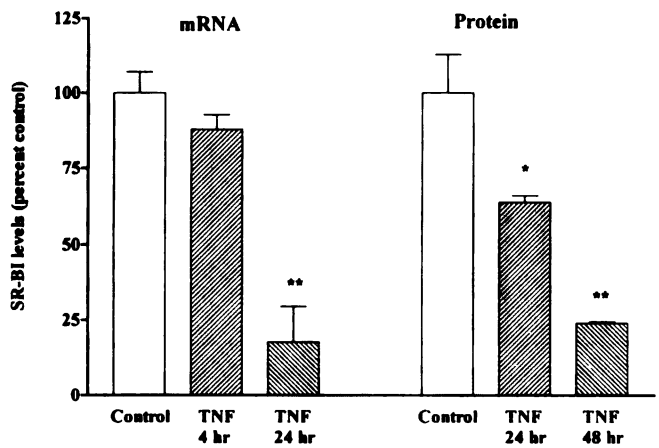

Fig. 6. The effect of TNF on SR-BI mRNA and protein levels in Hep3B cells. Hep3B cells were grown until confluent. TNF (10 ng/ml) was incubated with cells in MEM media with 0.1% BSA in the absence of serum, and at indicated time points, cells were harvested. A: the effect of TNF on SR-BI mRNA levels at 4 and 24 hours. N = 4 in each group at each time point. B: the effect of TNF on SR-BI protein levels at 24 and 48 hours. N = 3 in each group at each time point. *: $P < 0.05$, **: $P < 0.01$.

SR-BI on the hepatocyte promotes cholesterol ester uptake from HDL. To determine the significance of the decrease of SR-BI levels in the liver and in Hep3B cells, we performed cholesterol ester uptake experiments using HDL_3 that had been labeled with a cholesterol ether analog. Preincubation of TNF (10 ng/ml), IL-1 (10 ng/ml), or a combination of TNF and IL-1 (10 ng/ml each) with Hep3B cells resulted in a decrease in uptake of HDL_3 cholesterol ether analog (**Fig. 7**), indicating that the cytokine-induced decrease of SR-BI resulted in a decrease in cholesterol ester uptake by cells. Preincubation of LPS (10-1,000 ng/ml) with Hep3B cells, in contrast, did not decrease the uptake of cholesterol ester from HDL_3 (**Fig. 7**), confirming previous studies that immortalized cell lines, such as Hep3B and HepG2 cells typically respond to cytokines, but not LPS (40-42).

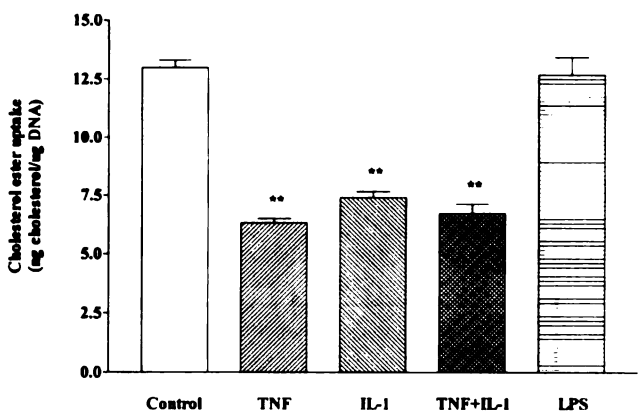

Fig. 7. The effect of cytokines on cholesterol ester uptake from HDL. HDL_3 was isolated and radiolabeled as described in Materials and Methods. Hep3B cells were incubated with TNF-α (10 ng/ml), IL-1β (10 ng/ml), TNF-α and IL-1β (10 ng/ml each), LPS (1 μg/ml), or no cytokine/LPS for 48 hours before incubation with radiolabeled HDL_3 (20 μg/ml) for 4 hours. Unlabeled HDL_3 (60 μg/ml) was added for 2 hours and cells were lysed in 0.1 N NaOH and sonicated. Aliquots were used to determined radioactivity and DNA content. **: $P < 0.001$ compared with control.

Effect of LPS on SR-BI mRNA levels in RAW 264.7 cells

Recent studies have implicated SR-BI in promoting cholesterol movement out of cells (9, 10). This process of cholesterol efflux is the initial step of RCT and helps prevent cholesterol accumulation in peripheral cells, such as macrophages (43). We therefore studied the effect of LPS in RAW 264.7 cells, a murine macrophage cell line. As shown in **Fig. 8,** LPS rapidly decreased SR-BI mRNA levels (40% decrease by 2 hours) and the maximum effect was reached by 4 hours (90% decrease). In contrast to the LPS-induced decrease in SR-BI levels in the liver, the decrease in SR-BI mRNA levels in RAW 264.7 cells was not sustained. In addition, only high doses of LPS (100 and 1,000 ng/ml), but not lower doses (1 and 10 ng/ml), significantly decreased SR-BI mRNA levels (data not shown). Moreover, we did not detect a decrease in SR-BI protein levels after cells were incubated with LPS (up to 1,000 ng/ml) at 4, 8, or 24 hours (data not shown). Overall, the results suggest that, in contrast to the effect on the liver, the reduction of SR-BI levels in RAW 264.7 cells in response to LPS was transient and did not lead to a reduction in SR-BI protein levels.

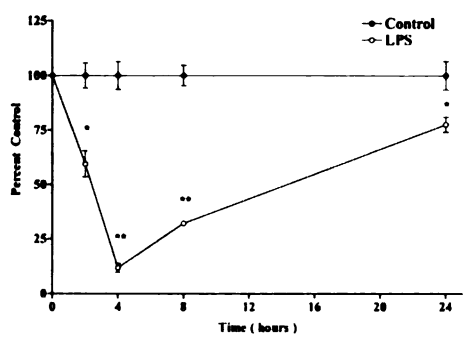

Fig. 8. The effect of LPS on SR-BI mRNA in RAW 264.7 cells. RAW 264.7 cells were grown until confluent. LPS (100 ng/mL) was incubated with cells in MEM media with 2% bovine serum albumin in the absence of serum, and at indicated time points, cells were harvested for RNA isolation. N = 4 in each group at each time point. *: $P < 0.025$, **: $P < 0.001$.

DISCUSSION

SR-BI is a scavenger receptor initially shown to bind native and modified LDL (8). Later, SR-BI was shown to bind HDL and mediate selective uptake of HDL cholesterol ester (7). SR-BI is abundantly expressed in the liver, adrenal glands, and gonads, the tissues in which selective uptake of HDL cholesterol ester has been previously demonstrated (6). In steroidogenic tissues, such as the adrenals and gonads, SR-BI plays a role in delivering cholesterol ester for steroid hormone synthesis. In the liver, SR-BI is involved in selective uptake of cholesterol ester, one of the key steps in RCT. Moreover, SR-BI may play an additional role in RCT by mediating the removal of cholesterol from peripheral tissues.

The role of SR-BI in HDL metabolism and RCT is supported by a number of in vivo studies (11-15). Overexpression of SR-BI in the liver resulted in a profound reduction of plasma levels of HDL cholesterol in mice (11-13). Concomitantly, there is an increase in selective uptake of HDL cholesterol ester into the liver resulting in an increase in biliary cholesterol secretion (11). In addition, mice in which SR-BI had been deleted or dramatically reduced showed increased plasma HDL cholesterol levels (14, 15). Furthermore, the reduction of hepatic SR-BI expression was associated with decreased selective uptake of HDL cholesterol ester (15). Collectively, these studies support an important role of SR-BI in RCT by mediating selective uptake of HDL cholesterol ester at the hepatic level.

Previously, it has been shown that there are multiple changes of proteins involved in HDL metabolism and RCT during the acute phase response (APR) (21-29). Specifically, a decrease of two proteins, LCAT and CETP, could potentially lead to a

decrease in RCT. Because SR-BI is one of the key proteins in RCT, it was logical to determine whether SR-BI expression was also decreased during the APR. We hypothesized that SR-BI levels might be decreased during the APR, and, therefore, studied the hepatic expression of SR-BI using LPS and cytokines, known inducers of the APR.

In this study, we demonstrated that LPS reduced the hepatic mRNA levels of SR-BI in hamsters, and the reduction was rapid and sustained. The decrease in hepatic mRNA levels of SR-BI was also associated with a decrease in SR-BI protein levels. In addition, treatment of hamsters with TNF or IL-1 produced a reduction in hepatic mRNA levels of SR-BI. When TNF and IL-1 were simultaneously given, the reduction of hepatic levels of SR-BI was additive and the degree of the reduction was similar to that observed with LPS treatment.

Diet has been shown to regulate hepatic SR-BI levels in some studies (39, 44). In rats, feeding a diet enriched with cholesterol, olive oil, and cholic acid for 2 weeks led to a decrease in SR-BI protein levels in hepatocytes (39). In hamsters, a diet enriched with polyunsaturated fatty acids resulted in an increase in hepatic SR-BI protein levels (44). In another study in which mice were fed high cholesterol or high fat diet for 4 weeks, however, there was no significant change in hepatic SR-BI levels (45). Our study in which hamsters were fed high cholesterol diet for 4 days did not show changes in hepatic SR-BI mRNA levels. The difference between our results from others could represent species differences, differences in diet composition, or possibly a shorter period of cholesterol feeding. It is of note, however, that our protocol of cholesterol feeding has been shown to regulate a number of genes involved in cholesterol metabolism in the

hamster liver previously (46, 47). Nevertheless, LPS was able to decrease hepatic SR-BI mRNA levels in animals fed a high cholesterol diet, similar to that observed with regular diet.

In the liver, both hepatocytes and Kupffer cells express SR-BI (39). SR-BI on the hepatocyte is responsible for the selective uptake of HDL cholesterol ester, however, the role of SR-BI on the Kupffer cells in cholesterol ester uptake and RCT is unknown. In this study, we have shown that the decrease in SR-BI levels in the hamster liver could be accounted for, at least in part, by a decrease in the hepatocyte. More importantly, the reduction in SR-BI levels in the hepatocyte was associated with the decrease in the uptake of cholesterol ester from HDL.

The reduction of SR-BI due to LPS and cytokines may have several consequences. During the APR, the reduction in hepatic expression of SR-BI may decrease selective uptake of cholesterol ester into hepatocytes (as demonstrated by our in vitro studies of hepatocytes), limiting cholesterol removal by the liver, and thus making cholesterol available to peripheral cells for host defense. For example, cholesterol has been shown to regulate lymphocyte activation and proliferation (48, 49). In addition, the APR is associated with cellular damage, and the processes of repair and regeneration of peripheral cells require cholesterol for new membrane synthesis. Decreasing RCT could facilitate the cellular responses required for surviving acute injuries, infection, or inflammation.

Although the acute decrease in hepatic SR-BI levels during the APR may be beneficial to the host, a prolonged reduction of SR-BI in the liver may result in undesirable consequences. SR-BI deficient mice are susceptible to atherosclerosis, which

may be related to decreased RCT (16, 17). In agreement with the concept that SR-BI protects against atherosclerosis, hepatic overexpression of SR-BI significantly reduced atherosclerosis in cholesterol-fed LDL receptor-deficient mice (18). It is possible that the acute reduction of SR-BI in the liver during the APR may decrease RCT and redirect cholesterol to peripheral cells for host defense, but a prolonged reduction of hepatic SR-BI, which might occur in chronic infection or inflammation, may impair RCT which could result in an increased risk for atherosclerosis.

Recently, SR-BI has been demonstrated to promote cholesterol efflux from peripheral cells (9, 10). Overexpression of SR-BI in CHO cells increased cholesterol efflux, and the efflux rates of different types of cells correlated with the levels of SR-BI expression. However, the significance of SR-BI in mediating cholesterol efflux in vivo is presently unknown. In this study, we demonstrated that LPS decreased mRNA levels of SR-BI in RAW 264.7 cells. However, the reduction was rather short-lived, and high doses of LPS were required to produce a reduction (**Fig. 8**), which is in contrast to the effect of LPS on the hepatic SR-BI mRNA, which was prolonged and sensitive to low doses of LPS (**Fig. 1&2**). Additionally, it did not decrease the protein levels. A study in human monocyte-derived macrophages recently showed that LPS decreased the protein levels of Cla-1, a human homolog of SR-BI (50). Nevertheless, incubation of LPS with macrophages did not result in a change in cholesterol efflux compared with control cells (51). Failure to detect the effect of LPS on cholesterol efflux may, in fact, be due to a transient effect of LPS on SR-BI levels.

In summary, we have demonstrated that LPS and cytokines decreased SR-BI levels in the liver and in hepatocyte and macrophage cultures. These changes, which may

be beneficial initially for the host, may impair selective uptake of HDL cholesterol ester and thereby decrease RCT increasing the risk for atherosclerosis.

Acknowledgment: We thank Dr. Helen H. Hobbs for providing antibody to mouse SR-BI.

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CHAPTER 5

CHANGES IN HDL-ASSOCIATED APOLIPOPROTEINS INDUCED BY ENDOTOXIN

INCREASE IN APOLIPOPROTEINS A-IV AND A-V AND DECREASE IN APOLIPOPROTEINS A-II, C-I, C-II, AND C-III IN ACUTE-PHASE HDL

ABSTRACT

A number of proteins associated with high-density lipoprotein (HDL) play different roles in the function and metabolism of HDL. During infection and inflammation, there are changes in the levels of several HDL-associated proteins. Using two-dimensional gel electrophoresis and mass spectrometry, we found that apolipoprotein (apo) A-IV and apo A-V were increased in acute-phase HDL isolated from mice injected with endotoxin. We also found an increase in hepatic mRNA levels of apo A-IV and apo A-V. Additionally, we demonstrated that the levels of apo A-II, apo C-I, C-II, and C-III in acute-phase HDL were decreased. In contrast, the levels of apo C-I, C-II, and C-III in acute-phase very low-density lipoprotein (VLDL) were increased. As a consequence, there were no significant changes in the circulating serum levels of apo Cs, suggesting that there may be a redistribution of apo C from HDL to VLDL. Enrichment of HDL with apo serum amyloid A (SAA) has previously been shown to displace apo A-I from HDL, but we found no significant changes in the apo C levels in these apo SAA-enriched HDL particles or in VLDL when VLDL was present during the incubation. These results suggest that changes in levels of apo Cs in acute-phase HDL and acute-phase VLDL were not simply due to an increase in apo SAA in acute-phase HDL. The changes in the levels of apolipoproteins after endotoxin administration reflect continuous remodeling of lipoprotein particles, which may explain the alterations of functions of lipoproteins during the acute-phase response.

INTRODUCTION

Infection and inflammation initiate a cascade of reactions in the host collectively known as the acute-phase response (APR) (1). As part of the host response to injury, the APR induces changes in the concentration of specific plasma proteins that modulate the inflammatory response. Levels of certain positive acute-phase proteins (e.g., C-reactive protein and serum amyloid A) increase whereas there are decreases in levels of negative acute-phase proteins (e.g., albumin). The APR is also associated with systemic changes in lipids and lipoproteins (2). Serum triglyceride levels typically increase during infection and inflammation. Consequently, there is an appearance of small dense low-density lipoprotein (LDL) (3), a particle believed to be particularly proatherogenic.

High-density lipoprotein (HDL) is a group of lipoprotein particles, the level of which inversely correlates with the development of atherosclerosis (4). A number of proteins associated with HDL play distinct roles in the metabolism and function of HDL (5, 6). During the APR, HDL metabolism is severely perturbed (7). The levels of HDL cholesterol decrease early after the onset of infection and inflammation. In addition, there is a reduction in levels of several plasma proteins involved in HDL-mediated reverse cholesterol transport, such as lecithin:cholesterol acyltransferase (LCAT), cholesterol ester transfer protein, phospholipid transfer protein, and hepatic lipase (8-13). As a consequence, the composition of HDL that circulates during infection and inflammation is altered, both in the lipid and protein components. These HDL, also known as acute-phase HDL, are enriched in free cholesterol and triglyceride and depleted in cholesterol ester (8, 14, 15). The levels of apolipoprotein J (apo J or clusterin), apo serum amyloid A

(SAA), and secretory phospholipase A_2 increase several fold in acute-phase HDL, whereas apo A-I, paraoxonase, and LCAT levels decrease (16-21).

Because of the marked changes in HDL during the APR, it has been proposed that acute-phase HDL behaves differently than normal HDL in terms of its protective effects against atherosclerosis (7, 20, 22). In fact, our laboratory and others have recently shown that acute-phase HDL was less effective in removing cholesterol from macrophages, a defect that might be due to decreased LCAT levels (21, 23, 24). Moreover, lower levels of paraoxonase in acute-phase HDL resulted in its inability to protect LDL from oxidation (20). These studies provide evidence that changes in the levels of proteins associated with HDL have a major impact on the various biological functions of HDL.

Identification of other HDL-associated proteins that are increased or decreased in acute-phase HDL could provide new insights into the alterations of the function of HDL during the APR. In order to examine changes in the levels of HDL-associated proteins in a systematic fashion, we subjected control and acute-phase HDL to two-dimensional gel electrophoresis and identified proteins by a combination of their isoelectric points/molecular weight, mass spectrometry, and/or immunoreactivity. With recent improvement in isoelectric focusing and mass spectrometers, as well as the availability of genome and protein databases, this combined approach has become a powerful tool to assess a global picture of proteins in samples with higher sensitivity, accuracy, and reproducibility. In this study, we found increased levels of apo A-IV and apo A-V in acute-phase HDL. Levels of apo A-II were, however, decreased. Although we also found a decrease in the levels of apo C-I, C-II, and C-III in acute-phase HDL, it was

accompanied by an increase in apo C-I, C-II, and C-III in acute-phase very low-density lipoprotein (VLDL).

EXPERIMENTAL PROCEDURES

Materials

Endotoxin (lipopolysaccharide or LPS) from E. coli serotype 055:B5 was purchased from Difco Laboratories (Detroit, MI). Centricon centrifugal devices (molecular weight cutoff 10,000 Dalton) and C18 ZipTip were obtained from Millipore (Bedford, MA). Immobiline DryStrip, pH 3-10 NL (non-linear) and immobilized pH gradient buffer were purchased from Amersham Biosciences (Piscataway, NJ). Sequencing-grade modified trypsin was purchased from Promega (Madison, WI). Chemical supplies were purchased from Sigma (St. Louis, MO) or Amersham Biosciences. Rabbit polyclonal antibodies against mouse apo C-I, apo C-II, and apo C-III were produced as previously described (25). These apo C antibodies do not cross react with the other apo C proteins. Rabbit antibodies against mouse apo A-II were purchased from Biodesign (Saco, ME). Recombinant human apo SAA was purchased from Peprotech (Rocky Hill, NJ).

Animal experiments

C57BL/6 mice (approximately 6-8 weeks old, weight 18-20 g) were obtained from Jackson Laboratory (Bar Harbor, ME) and provided with rodent chow and water ad libitum. Animals were injected intraperitoneally with indicated doses of endotoxin,

whereas control animals were injected with normal saline. At the dose of 100 μg of endotoxin, animals become toxic, SAA is induced, and circulating cytokine levels are expected to be in the septic range. However, this dose is far below the lethal dose (LD_{50} ~ 5 mg/100 g body weight) required to cause death in mice in our laboratory (26). Because endotoxin can cause anorexia, food was withdrawn from endotoxin-treated and control mice after the injection. At indicated time points, the animals were euthanized using halothane, serum was collected, and the tissue was excised and stored at –80°C. The animal procedures were approved by the Animal Studies Subcommittee of the San Francisco Veterans Affairs Medical Center, and were performed in accordance with the guidelines.

Lipoprotein isolation

Control and acute-phase lipoproteins were isolated from pooled sera of 8-10 mice injected with normal saline or endotoxin, respectively, by sequential preparative ultracentrifugation as previously described (21). KBr was used to adjust for the desired density (VLDL: d < 1.006 g/ml, LDL: d = 1.006-1.063 g/ml, HDL: d = 1.063-1.21 g/ml). Lipoproteins were extensively dialyzed in 5 mM Tris HCl, 1 mM EDTA, pH 7.4 and concentrated using Centricon cartridges. Protein concentrations of lipoproteins were measured by a modified Lowry assay (Pierce, Rockford, IL).

Two-dimensional gel electrophoresis

The first dimension was performed using immobilized pH gradient (IPG) strips (Immobiline DryStrip, pH 3-10 NL) according to the manufacturer's instructions. Briefly,

IPG strips were rehydrated overnight in HDL mixed with 8 M urea, 4% CHAPS, 65 mM dithiothreitol, and 0.5 % IPG buffer. Rehydrated IPG strips were subjected to isoelectric focusing for a total of 50,000-60,000 V-hr at 20°C. After isoelectric focusing, IPG strips were equilibrated in 50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and trace bromophenol blue for 15 minutes. The second dimension was performed in a vertical system using 10% polyacrylamide gels at 40 mA. Two-dimensional gels were then fixed and stained with silver nitrate as described by Shevchenko et al (27). Protein spots were visualized using the BioRad imaging densitometer.

Identification of proteins by mass spectrometry

Silver-stained proteins were reduced with 1 mM dithiothreitol, alkylated with 2 mM iodoacetamide, and in-gel digested with trypsin. The peptides were extracted and purified using C18 ZipTip as described at http://donatello.ucsf.edu/ingel.html. An aliquot of each peptide digest was analyzed by LC/MS/MS performed on a QSTAR Pulsar quadrupole-orthogonal-acceleration-time-of-flight tandem mass spectrometer equipped with an electrospray source. (MDS Sciex, Toronto, Canada). The peptides were separated by nanoHPLC (Ultimate HPLC system with Famous autosampler, LC Packings, San Francisco, CA) developing a 5%-50% B linear gradient over 30 min, on a 75 μm ID Pepmap column (LC Packings, San Francisco, CA) at a flow rate of ~300 nL/min. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile. The eluent was directly introduced in the mass spectrometer, and was monitored in an information-dependent acquisition mode; 1 sec mass measurements were followed by 5 sec collision-induced dissociation (CID) experiments throughout the

analysis. Multiply charged precursor ions were computer-selected and the collision energy was adjusted according to the mass and charge of the ion fragmented. The identity of the proteins was determined by database searches with the uninterpreted CID data against all the proteins in the National Center for Biotechnology Information database using the appropriate Bioanalyst Script (MDS Sciex) and the Mascot software (www.matrixscience.com).

Isolation of RNA and RNA blot analysis

Isolation of RNA and RNA blot analyses were performed as previously described (28). Because endotoxin produced a twofold increase in hepatic mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a 2-5 fold increase in hepatic actin mRNA, and a 2.6 fold increase in hepatic cyclophilin mRNA in rodents (14, 29, 30), the mRNA levels of GAPDH, actin, and cyclophilin, which are widely used for normalizing data, cannot be used to study endotoxin regulation in the mouse liver. However, the differing direction of the changes in mRNA levels (increased for some proteins, decreased for some proteins, and no change for other proteins), the magnitude of alterations (5 fold increase or 40% decrease), and the relatively small standard error of the mean (SEM) make it unlikely that the changes observed were due to unequal loading of mRNA or toxic effects on the mouse liver. RNA blots were hybridized with ^{32}P-labeled cDNA probes. Apo A-IV, apo A-V, and apo A-II cDNA probes were generated by RT-PCR from the mouse liver using the following primers: apo A-IV 5'AATCTGCACAGGGACACAG3' and 5'TTCTCAGTTGCTTTATTGAGTAG3'; apo A-V 5'GCGCGTGGTGGGAGAAGACA3' and 5'TCGCGCAGCTGGTCCAGGTT3';

apo A-II 5'CTGCTCGCAATGGTCGCAC3' and 5'ACATCTCACTTAGCCGCAGG3'. After washing, the blots were exposed to X-Ray films for various durations to ensure that measurements were done on the linear portion of the curve, and the bands were quantified using a Bio-Rad imaging densitometer.

Immunoblot analyses

Immunoblot analyses were performed as previously described (28). Equal amount of total proteins in HDL or VLDL was loaded in each lane. Rabbit polyclonal antibodies against mouse apo A-II, apo C-I, apo C-II, and apo C-III were used as primary antibodies and a donkey anti-rabbit antibody conjugated with horseradish peroxidase (Amersham Biosciences) was used as a secondary antibody.

Enrichment of HDL with SAA

Normal mouse HDL (2 mg protein) and VLDL (0.8 mg triglyceride) were incubated in the absence or presence of recombinant SAA (0, 100, and 500 μg) in 0.15 M NaCl, 1 mM EDTA, pH 7.4 (total volume of 1.0 mL) at room temperature for 6 hours as previously described (31). The concentrations of HDL, VLDL and SAA used were calculated to reflect those of lipoproteins and SAA found in mouse serum in the absence or presence of the APR after endotoxin injection. VLDL and HDL were reisolated by sequential ultracentrifugation and analyzed for the levels of apo C-I, C-II, and C-III using immunoblot analysis as described above.

Statistics

The results are presented as means ± SEM. Significance was determined by a two-tailed Student's *t*-test.

RESULTS

Apo A-IV and apo A-V are increased in acute-phase HDL

Control and acute-phase HDL were isolated from pooled mouse sera (8-10 mice) 16 hr after injection of normal saline or endotoxin, respectively. The two-dimensional gel electrophoresis patterns of proteins associated with control and acute-phase HDL were visualized and compared using silver staining. A representative set is shown in **Fig. 1**. Certain protein spots that were consistently and unequivocally increased in acute-phase HDL compared to those in control HDL (labeled # 1-5 in **Fig. 1**) were chosen (based on four different sets of HDL). These protein spots were in-gel digested and identified by mass spectrometry. The identifications were performed once or twice and were based on multiple peptide sequences determined by MS/MS experiments for each protein (data not shown). In agreement with previous reports (17, 32), we found an increase in apo SAA, a major positive acute-phase reactant protein, in acute-phase HDL (**Fig. 1**, spots 1 and 2 which represent two isoforms of acute-phase SAA). Moreover, we found that apo E was also increased in acute-phase HDL (**Fig. 1**, spot 3). Spot 4 was identified as mouse apolipoprotein A-IV, while spot 5 corresponded to mouse apolipoprotein A-V. Mouse apo A-IV has a molecular mass of 44-46 kDa and an isoelectric point (pI) of ~ 5.3, whereas mouse apo A-V has a predicted molecular mass of 41 kDa and a predicted pI of ~ 5.9. Therefore, the locations of apo A-IV and apo A-V spots on two-dimensional gels

are in agreement with the expected molecular mass and pI of both proteins. Densitometric scanning of apo A-IV spots showed that there were more than 2 fold increases in acute-phase HDL (data not shown). For apo A-V, however, quantification was not possible because the spot was not detectable in control HDL.

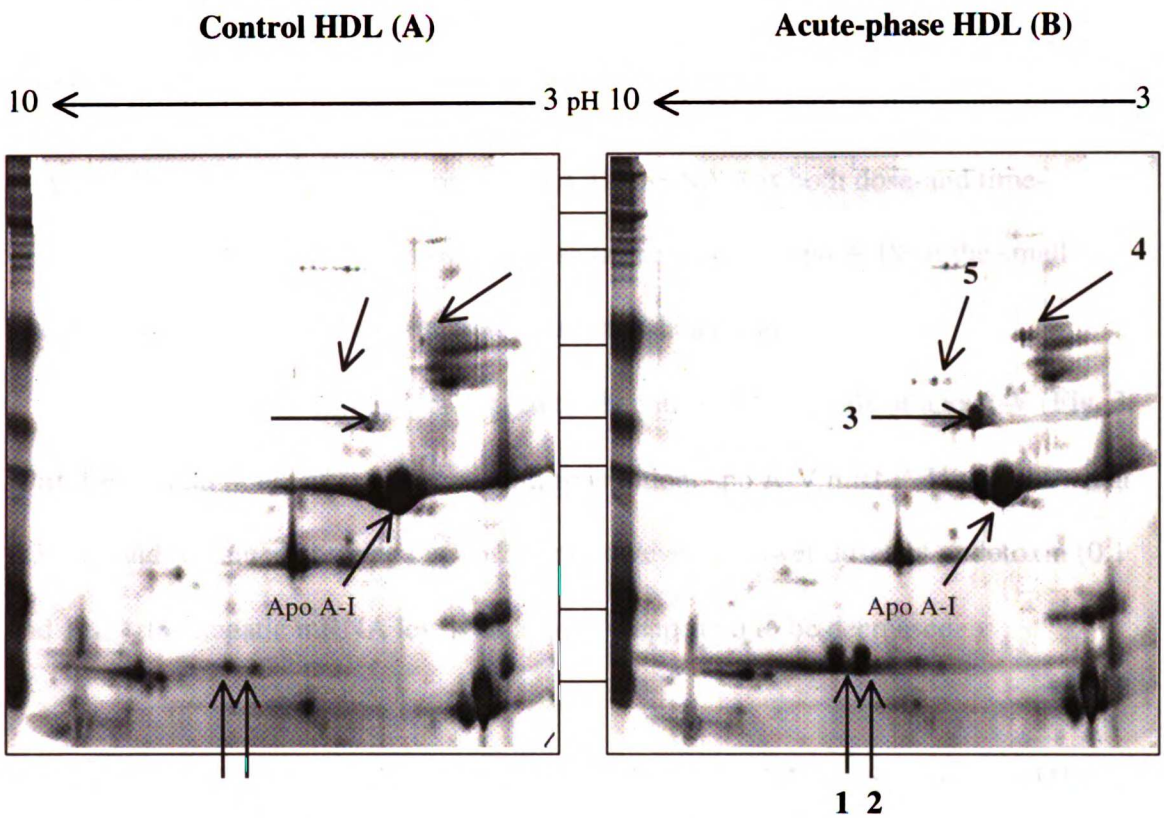


FIG. 1. Representative two-dimensional gel electrophoresis results of HDL-associated proteins of control HDL (A) and acute-phase HDL (B). Control and acute-phase HDL were isolated by ultracentrifugation and subjected to two-dimensional gel electrophoresis. Protein spots were visualized using silver stain and the identity of the protein was identified using mass spectrometry as described in Experimental Procedures. Only protein spots that were consistently and unequivocally increased in acute-phase HDL were chosen for identification based on four different sets of HDL. The non-linear pH gradient was from pH 3 (right) to pH 10 (left). Molecular mass markers are shown between the two gels: 203, 115, 93, 48, 34, 28, 21, and 7.2 kDa. Spots 1 and 2 are two isoforms of apo SAA, spot 3 is apo E, spot 4 is apo A-IV, and spot 5 is apo A-V.

Endotoxin increased hepatic mRNA levels of apo A-IV and apo A-V

To determine whether an increase in protein levels of apo A-IV and apo A-V in acute-phase HDL is associated with increased synthesis, we next examined mRNA levels of these two proteins in mouse tissues. Apo A-IV is secreted by the small intestine and the liver, whereas apo A-V is expressed only in the liver. We found that endotoxin injection resulted in an increase in the levels of apo A-IV mRNA in the mouse liver (**Fig. 2, A and B**). The induction of hepatic apo A-IV mRNA was both dose-and time-dependent. In contrast, there was no increase in the levels of apo A-IV in the small intestine in response to endotoxin administration (data not shown).

Endotoxin (100 μg) also increased the hepatic mRNA levels of apo A-V (**Fig. 3, A and B**). Eight hours after endotoxin administration, apo A-V mRNA levels peaked at 2.3 fold and remained sustained up to 24 hr. However, at lower doses of endotoxin (0.1 and 1 μg), the hepatic mRNA levels of apo A-V appeared to be decreased.

Changes of apolipoproteins A-II, C-I, C-II, and C-III in acute-phase HDL

Apo A-I, a major apolipoprotein of HDL, has previously been shown to be decreased in acute-phase HDL, (8, 33) and our data confirmed that finding. We next investigated changes of other apolipoproteins associated with HDL. Apo A-II, C-I, C-II, and C-III are associated with HDL. Quantification of these apolipoproteins by comparing corresponding spots from silver-stained gels could be misleading because each protein has multiple isoforms that may be separated in different locations. In addition, these apolipoproteins, unlike apo A-IV and apo A-V, are relatively abundant and appear as

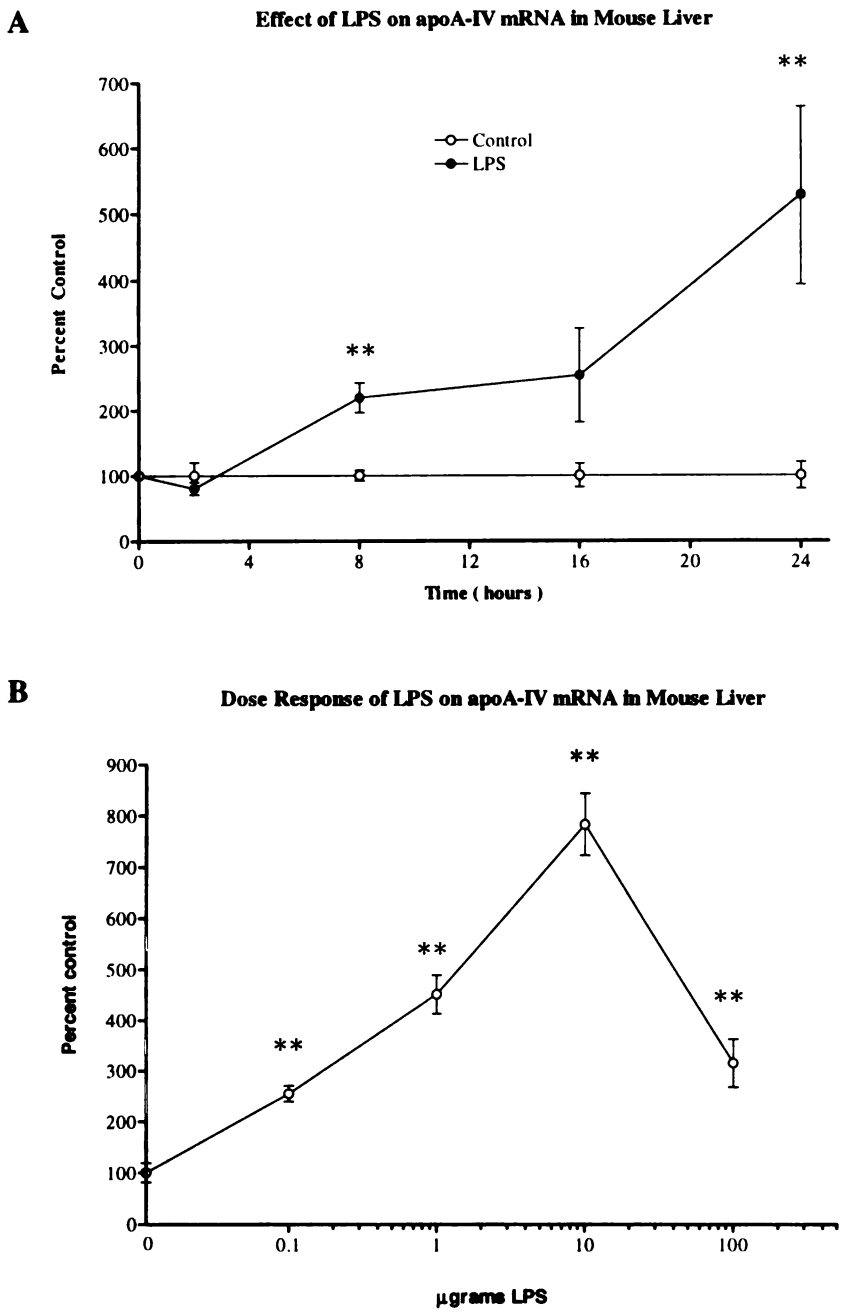


FIG. 2. Time course (A) and dose response curve (B) of the effect of endotoxin (LPS) on apo A-IV mRNA levels in mouse liver. C57BL/6 mice were injected with endotoxin and at each time points, livers were harvested and RNA was isolated. Poly (A)+ RNA was hybridized with ^{32}P-labeled cDNA probes and bands were analyzed by agarose gel electrophoresis followed by autoradiography as described in Experimental Procedures. Data are presented as percent change versus control (mean ± SEM). N = 4-5 in each group at each dose of endotoxin and at each time points. **: $P<0.01$.

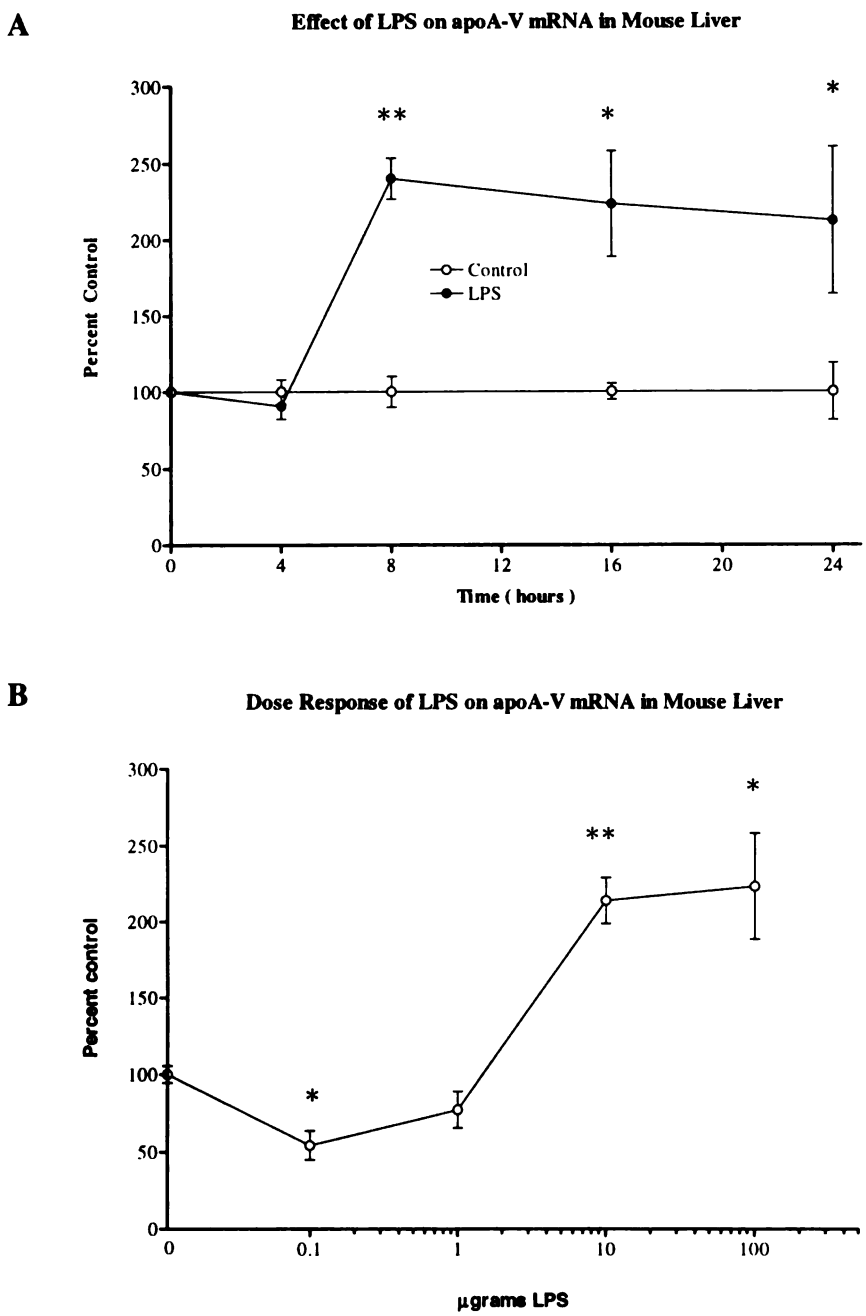


FIG. 3. Time course (A) and dose response curve (B) of the effect of endotoxin (LPS) on apo A-V mRNA levels in mouse liver. C57BL/6 mice were injected with endotoxin and at each time points, livers were harvested and RNA was isolated. Poly (A)+ RNA was hybridized with ^{32}P-labeled cDNA probes and bands were analyzed by agarose gel electrophoresis followed by autoradiography as described in Experimental Procedures. Data are presented as percent change versus control (mean ± SEM). N = 4-5 in each group at each dose of endotoxin and at each time points. *: $P<0.05$, **: $P<0.01$.

overloaded spots in gels stained with sensitive methods such as silver staining. Therefore, we chose to examine changes of these apolipoproteins in acute-phase HDL using immunoblot analyses in a one-dimensional system.

In contrast to an increase in apo A-IV and apo A-V in acute-phase HDL, we found that the levels of apo A-II were decreased in acute-phase HDL (**Fig. 4, A and B**). Because apo A-II is mainly associated with HDL, the levels of apo A-II in total serum were also decreased (data not shown). We next examined hepatic mRNA levels of apo A-II and found that endotoxin decreased apo A-II mRNA levels in mouse liver (**Fig. 4C**).

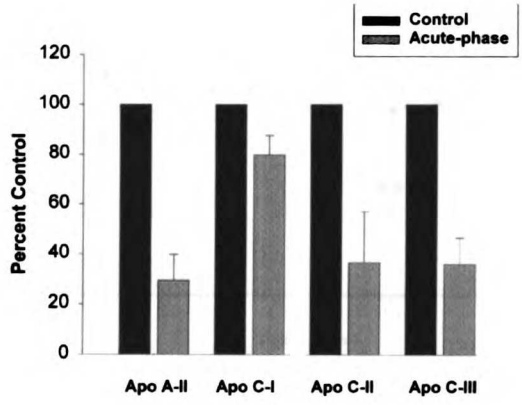
Besides apo A-II, we also found that the levels of apo C-I, C-II, and C-III were all decreased in acute-phase HDL compared to those in control HDL (**Fig. 4, A and B**). The degree of reduction of apo C-I levels was relatively small compared to that of apo C-II and apo C-III.

Changes of apolipoproteins C-I, C-II, and C-III in acute-phase VLDL

In serum, apo A-I and apo A-II are almost exclusively associated with HDL. However, apo C-I, C-II, and C-III can be found associated with both HDL and VLDL. We therefore examined the levels of apo C-I, C-II, and C-III in acute-phase serum and acute-phase VLDL. Despite a decrease in levels of apo C-I, C-II, and C-III in acute-phase HDL, we found that there were no significant changes in levels of apo C-I, C-II, or C-III in acute-phase serum (data not shown). These results suggested that there might be an increase in other serum fractions rather than HDL. Indeed, we found that there was an increase in the levels of all apo Cs in acute-phase VLDL (**Fig. 4B**). Again, the degree of

increase in apo C-I levels in acute-phase VLDL was also small compared to that of apo C-II and apo C-III.

A



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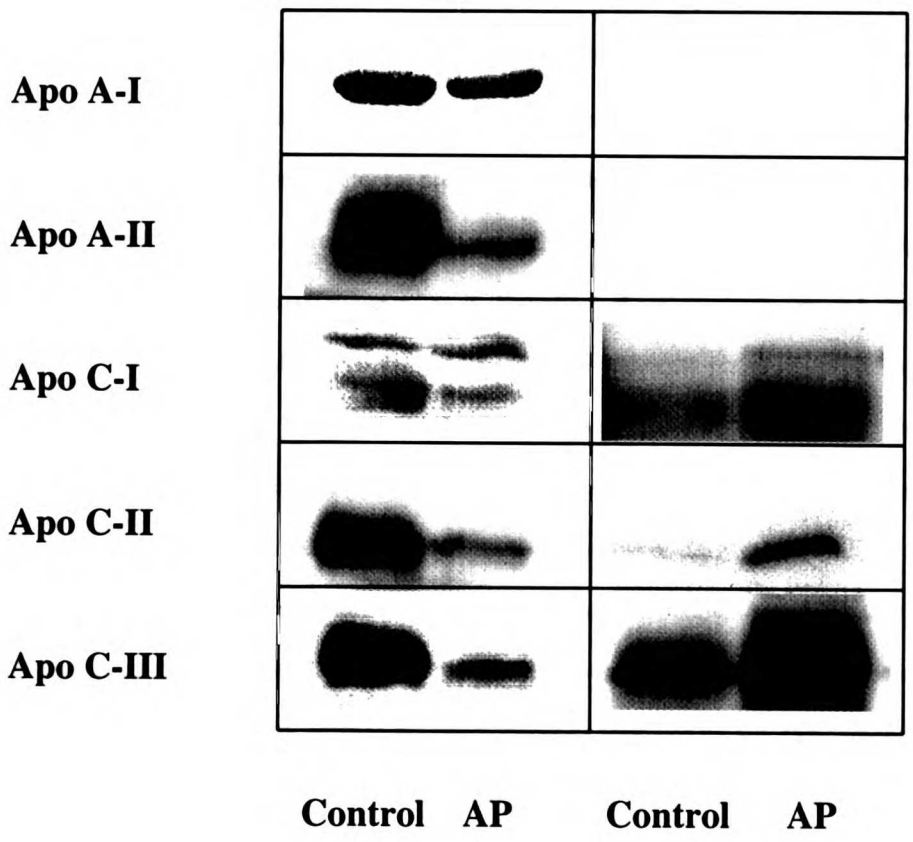

C

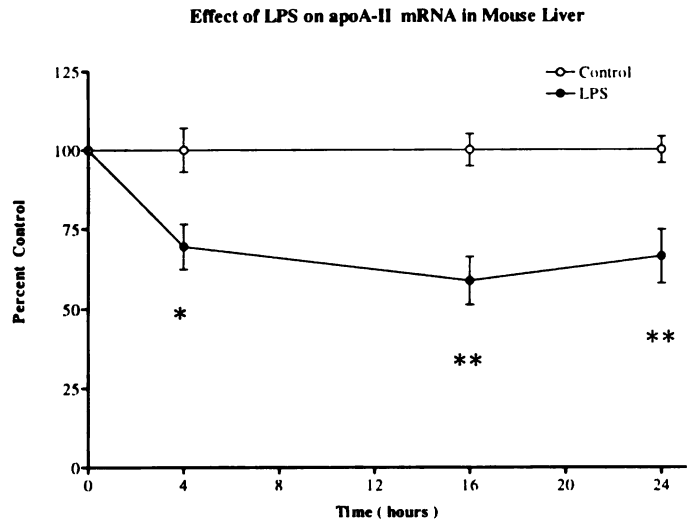

FIG. 4. The effect of endotoxin on protein levels of apo A-II, C-I, C-II, and C-III in acute-phase HDL (A), acute-phase VLDL (B), and on apo A-II mRNA levels in mouse liver (C). *A*, Apo A-II, C-I, C-II, and C-III levels were decreased in acute-phase HDL. Control and acute-phase HDL were isolated from pooled mouse sera by ultracentrifugation. Five micrograms of HDL proteins were loaded onto a polyacrylamide gel and immunoblot analysis was performed as described in Experimental Procedures. The results represent the average of three different preparations of HDL. There were variations in the levels of each apolipoprotein from different preparations, therefore, the level of an individual apolipoprotein in the control HDL was set at 100% in each preparation. *B*, The effect of endotoxin on apo A-I, A-II, C-I, C-II, and C-III in acute-phase HDL and acute-phase VLDL. Control and acute-phase lipoproteins were isolated from pooled mouse sera 16 hours after normal saline or endotoxin injection, respectively, and immunoblot analysis was performed as described in Experimental Procedures. Immunoblot analysis for apo A-I and apo A-II in VLDL was not performed. AP: acute-phase. *C*, Time course of the effect of endotoxin (LPS) on apo A-II mRNA levels in mouse liver. C57BL/6 mice were injected with endotoxin and at each time points, livers were harvested and RNA was isolated. Poly (A)+ RNA was hybridized with ^{32}P-labeled cDNA probes and bands were analyzed by agarose gel electrophoresis followed by autoradiography as described in Experimental Procedures. Data are presented as percent change versus control (mean ± SEM). N = 5 in each group at each dose of endotoxin and at each time points. *: $P<0.05$, **: $P<0.01$.

Enrichment of HDL by apo SAA alone did not alter the levels of apo C-I, C-II, or C-III in HDL or VLDL

Apo SAA is a major positive acute-phase protein, which is mainly associated with HDL. Enrichment of HDL by apo SAA has been shown to cause dissociation of apo A-I and apo A-II from HDL particles (23, 31, 32, 34, 35). We hypothesized that apo Cs on acute-phase HDL might be dissociated from HDL due to an increase in apo SAA, and that apo Cs could be transferred to VLDL, which acts as an acceptor of these free proteins. After an incubation of apo SAA with HDL and VLDL, we found that HDL was enriched with apo SAA comparable to that on acute-phase HDL and apo A-I levels decreased (data not shown). However, there was no significant changes in the amount of apo C-I, C-II, or C-III proteins in apo SAA-enriched HDL or in VLDL (data not shown). This experiment suggests that an enrichment of apo SAA in HDL alone, in the absence of the APR, does not result in redistribution of apo Cs from HDL to VLDL.

DISCUSSION

A number of proteins are associated with HDL particles that play different roles in the function and metabolism of HDL (5, 6). During the APR, there are changes in levels of several of these HDL-associated proteins. For example, apo J and apo SAA are increased (16, 17), whereas LCAT and paraoxonase are decreased (20, 21). These changes could potentially explain the altered functions of acute-phase HDL. Lower LCAT in acute-phase HDL appears to limit cholesterol removal from macrophages (21),

whereas lower paraoxonase may convert anti-oxidant HDL into pro-inflammatory HDL (20).

In order to define the changes of other HDL-associated proteins in acute-phase HDL, we used a combination of two-dimensional gel electrophoresis and mass spectrometry. Using this approach, we confirmed previous reports of increased levels of apo SAA and apo E in acute-phase HDL (8, 17, 32, 36). In addition, our study identified two other apolipoproteins to be present in higher quantities: apo A-IV and apo A-V.

Apo A-IV is an apolipoprotein previously known to be associated with HDL. Changes of apo A-IV during the APR have been investigated only in a few studies. In rats injected with turpentine, a decrease in hepatic, but not intestinal, apo A-IV mRNA levels was observed (37, 38). However, the protein levels were reported unchanged. The relevance of these findings is not clear because turpentine injection is a model of inflammation that does not occur in diseases. Using endotoxin, a more pathophysiologically relevant inducer of the APR, we showed that apo A-IV levels were increased in acute-phase HDL. Furthermore, we found that endotoxin also increased hepatic, but not intestinal, mRNA levels of apo A-IV. In line with our findings, apo A-IV levels were increased in HDL isolated from mice overexpressing apo SAA (39).

A variety of physiological functions of apo A-IV have been proposed. In vitro studies have implicated apo A-IV in activation of LCAT, promotion of cholesterol efflux from cells, and inhibition of oxidative processes (40-42). It is of note that previous studies from our laboratory and others showed that acute-phase HDL, in fact, had lower LCAT activity, and was less effective than normal HDL in removing cholesterol from cells (21, 23, 24). Moreover, acute-phase HDL lost its ability to protect LDL from

oxidation (20). Because other HDL-associated proteins also possess similar functions proposed for apo A-IV (i.e., apo A-I can activate LCAT and promote cholesterol removal from cells, and paraoxonase can inhibit LDL oxidation), it is possible that changes of these proteins, rather than that of apo A-IV, in acute-phase HDL may play a more significant role and account for the effects observed during the APR.

Clinical observations and animal experiments also suggested that apo A-IV plays an important role in fat and fat-soluble vitamin absorption and satiety regulation (43, 44). However, recent experiments from apo A-IV transgenic and knockout mice have cast some doubt on the role of apo A-IV in fat absorption and food intake regulation. Both apo A-IV transgenic and knockout mice had no defect in intestinal lipid absorption or feeding behavior (45, 46). It is now accepted that the process of satiety regulation is complex and many redundant pathways are involved. We, however, cannot rule out a role for the acute elevation of apo A-IV after endotoxin injection in anorexia commonly observed during infection and inflammation.

Apo A-V is a newly discovered HDL-associated protein recently identified independently by two different groups (47, 48). Pennacchio et al. identified apo A-V gene near the apo A-I/C-III/A-IV gene cluster (47). The product of this gene, apo A-V, is most homologous to apo A-IV, but is expressed only in the liver. The exact function of apo A-V is presently unknown although it affects triglyceride metabolism. Transgenic mice overexpressing apo A-V had serum triglyceride levels that were one-third of those of control mice, whereas mice deficient in apo A-V exhibited hypertriglyceridemia (47, 49). The apo A-V gene was also identified by van der Vliet et al. as one of the genes upregulated in the liver of rats that underwent subtotal hepatectomy (48). Plasma levels

of apo A-V were increased shortly after hepatectomy. Although, plasma levels of triglyceride were not reported in the study, it is well established that after subtotal hepatectomy, there is a rapid increase in hepatic triglyceride synthesis causing accumulation of triglyceride in hepatocytes (50-52). Most studies also reported an increase in VLDL secretion and an increase in serum triglyceride levels after subtotal hepatectomy (51-53).

Our result showed that apo A-V levels were increased in acute-phase HDL after injection with endotoxin. Previous studies have shown that the APR induced by endotoxin and/or cytokines in rodents is associated with an increase in hepatic triglyceride synthesis, accumulation of triglyceride in the liver, enhanced VLDL secretion, and hypertriglyceridemia (2). Although several similarities in alterations of hepatic triglyceride metabolism exist between endotoxin injection and hepatectomy, the role of increased apo A-V induced by endotoxin is not clear. Apo A-V could play a role in the increased triglyceride synthesis and enhanced VLDL production. On the other hand, apo A-V may be synthesized in response to an increase in triglyceride content in hepatocytes or it could blunt the hypertriglyceridemic effect of endotoxin. Our results indicate that more studies of modulation of apo A-V levels in the physiological range are needed.

Apo A-I and apo A-II are major apolipoproteins of HDL. Both apo A-I and apo A-II have been found to be decreased in acute-phase HDL after a wide range of injurious stimuli (8, 33, 54). In this study, we found that apo A-II were decreased in acute-phase HDL, similar to what had been reported.

Furthermore, we also found that the amount of apo C-I, C-II, and C-III were all decreased in acute-phase HDL. Changes in the levels of apo C-I, however, were relatively small compared to those of apo C-II and apo C-III. Only a few studies have reported changes of apo C levels in HDL during the APR. In patients after surgery, apo C-I levels were decreased in HDL (36). In mice injected with Salmonella endotoxin, apo C-II was decreased (55). In rats injected with turpentine, apo C-III in HDL was also decreased (38). In addition to decreases in acute-phase HDL, we further showed that there was a concurrent increase in the levels of apo Cs in acute-phase VLDL. Because the total levels of apo Cs in acute-phase serum were not significantly different from those in normal serum, we postulated that apo Cs might be redistributed from acute-phase HDL to acute-phase VLDL.

The mechanism of transfer of apo C between HDL and VLDL has recently been proposed to involve aqueous diffusion (56). Therefore, a reduction of apo Cs in acute-phase HDL may be due to displacement of apo Cs from the HDL particles and the dissociated apo Cs are diffused to VLDL. It is known that during the APR, apo SAA is increased several fold in acute-phase HDL, and addition of apo SAA to HDL causes displacement of other apolipoproteins, such as apo A-I and apo A-II, from HDL (31, 32, 35). After incubation of apo SAA with normal HDL, HDL became enriched in apo SAA. However, there was no change in apo Cs levels in apo SAA-enriched HDL. In addition, levels of apo Cs in VLDL remained unchanged. We interpret these data to indicate that, in the absence of the APR, increased apo SAA in acute-phase HDL alone does not cause changes in apo Cs levels in HDL or VLDL, and that other factors occurring during the APR, other than increased apo SAA, could be responsible for these changes.

Secretory phospholipase A_2 ($sPLA_2$), another positive acute-phase reactant protein associated with HDL, has recently been proposed to play a role in remodeling of HDL during the APR by hydrolyzing phospholipids and reducing the particle size of HDL (19, 57, 58). However, C57BL/6 mice that we used in the study lack this protein due to a mutation in mouse $sPLA_2$ gene similar to other inbred mouse strains (59). Therefore, it is unlikely that $sPLA_2$ plays a role in redistribution of apo C proteins observed in our study. Indeed, a variety of alterations in both the lipid and protein composition of acute-phase HDL and acute-phase VLDL have been documented. Which of these biophysical or biochemical changes resulting in the redistribution of apo C proteins during the APR should be a subject of further investigations.

Although the mechanism of changes on apo Cs is not clear, increases in apo C levels in acute-phase VLDL could have important functional consequences. During infection and inflammation, serum levels of triglyceride increase due to enhanced VLDL synthesis and impaired VLDL clearance (7). Apo C-I has been shown to inhibit binding and uptake of VLDL to hepatic receptors, whereas apo C-III has been reported to inhibit lipoprotein lipase-mediated lipolysis (60). Increases in apo C-I and apo C-III levels in acute-phase VLDL therefore could result in delayed clearance of VLDL particles. It is of note that although apo C-II is known as a cofactor for lipoprotein lipase, high levels of apo C-II have been shown to inhibit lipoprotein lipase activity (61). Therefore, an increase in apo C-I, apo C-II and apo C-III in acute-phase VLDL could potentially impair VLDL clearance resulting in hypertriglyceridemia.

In conclusion, our study showed that in acute-phase HDL, there was an increase in apo A-IV and apo A-V levels. Moreover, there was a reduction in levels of apo A-II,

apo C-I, C-II, and C-III. Changes of the protein composition of these acute-phase lipoproteins likely affect their functions and metabolism during the APR. HDL has been shown to be beneficial during the APR by ameliorating the effects of endotoxin (62). Studies to investigate altered functions of these acute-phase lipoproteins are ongoing in our laboratory. Identification of other HDL-associated proteins using an alternative method of HDL isolation is also underway.

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CHAPTER 6

PAROTID SECRETORY PROTEIN IS ASSOCIATED WITH HDL AND EXPRESSED IN RESPIRATORY EPITHELIUM, KERATINOCYTES, SEBACEOUS GLANDS, AND GONADS

ABSTRACT

Proteins associated with high-density lipoprotein (HDL) play different roles in the function and metabolism of HDL. Identification of HDL-associated proteins could provide new insights into the function of HDL. From a two-dimensional gel of hamster HDL isolated by a hamster apo A-I immunoaffinity column, we identified an amino acid sequence in a hamster protein that showed homology to rat and mouse parotid secretory protein (PSP), a salivary protein secreted from the parotid glands. We cloned the cDNA encoding a putative hamster homologue of rat and mouse PSP. The predicted amino acid sequence is homologous to rat PSP (59% identity) and mouse PSP (57% identity). Searches for conserved domains of the protein showed that a carboxy terminus of hamster PSP contains a region homologous to the amino terminus of a family of proteins including lipopolysaccharide binding protein (LBP), bactericidal/permeability-increasing protein (BPI), cholesteryl ester transfer protein (CETP), and phospholipid transfer protein (PLTP). Weak homology to von Ebner's salivary gland protein and PLUNC protein was also observed. In mice, PSP was also associated with HDL isolated by ultracentrifugation, but was not detected in very low-density lipoprotein, low-density lipoprotein, or lipoprotein-deficient sera. In addition to salivary glands, we found that PSP mRNA was expressed in lung, skin, testis, and ovary. Immunofluorescence studies showed that PSP was localized in the respiratory epithelium of the bronchioles, keratinocytes and sebaceous glands of the skin, Leydig cells of the testis, and stromal cells of the ovary. Removal of all major salivary glands or testes in mice did not significantly decrease the protein level of PSP in HDL, suggesting that many tissues may contribute to the circulating levels of PSP. The level of PSP in HDL was increased after

endotoxin injection in hamsters, but not in mice. In summary, our results showed that PSP is associated with HDL in circulation, and is produced by a number of tissues including lung, skin, and gonads.

INTRODUCTION

High-density lipoproteins (HDL) are heterogeneous lipoprotein particles in the density range of 1.063-1.21 g/mL that contain lipids, mainly cholesterol and phospholipids, and a variety of proteins, including apolipoprotein (apo) A-I, apo A-II, apo C, lecithin:cholesterol acyltransferase, and cholesterol ester transfer protein. HDL plays a role in a wide variety of biological functions. It is best known as a key factor in protecting against atherosclerosis by facilitating the removal of excess cholesterol from peripheral cells and the movement of cholesterol to the liver in a pathway known as reverse cholesterol transport. HDL can also protect low-density lipoprotein (LDL) from oxidative modification, a process believed to be crucial in the development of atherosclerotic lesions. Additionally, HDL can inhibit cytokine-induced adhesion molecule expression on endothelial cells, thus preventing the entry of macrophages into the arterial wall. Other anti-inflammatory and anti-thrombotic effects of HDL have been observed (1). HDL also plays a role in innate immunity by scavenging endotoxin and ameliorating its deleterious effects (2-5).

The biochemically diverse effects of HDL are carried out by a number of proteins found on HDL particles. More than 30 different proteins have been shown to associate with HDL, and each of these proteins plays a distinct role in the function and the

metabolism of HDL (6, 7). Identification of new proteins associated with HDL will eventually unravel the biological roles HDL plays in a variety of physiological and pathological states.

To characterize proteins associated with HDL, the optimal method of HDL isolation needs to be taken into consideration. Ultracentrifugation is a method widely used by different laboratories because of its simplicity and high yield. However, it has been shown that certain HDL-associated proteins can be dissociated from the HDL particles during ultracentrifugation (8, 9). Apo A-I immunoaffinity column chromatography is another method of HDL isolation. It does not dissociate proteins from HDL, however until now, apo A-I columns have been available in a few laboratories for purification of only human apo A-I-containing HDL.

In this study, we report the development of hamster apo A-I immunoaffinity columns and their use in isolation of HDL from hamster plasma. We reasoned that the use of these columns might help us detect certain proteins that are dissociated from HDL during ultracentrifugation. In addition, the use of both apo A-I columns and ultracentrifugation may help avoid isolation artifacts. One of the advantages of developing *hamster* apo A-I columns is that the conditions of animals can be more easily manipulated than with humans, and changes in HDL and its protein composition can be studied accordingly. Using such approach, we found a salivary protein not previously known to be in the circulation or associated with HDL. In the present study, we describe the identification of parotid secretory protein (PSP) as an HDL-associated protein. We also report the molecular cloning of a hamster homologue of rat and mouse PSP and its tissue distribution.

EXPERIMENTAL PROCEDURES

Materials

ImmunoPure Plus immobilized protein G was obtained from Pierce (Rockford, IL). Cyanogen bromide-activated Sepharose 4B, thiopropyl Sepharose 4B, Immobiline DryStrip, pH 3-10 NL (non-linear), and immobilized pH gradient buffer were purchased from Amersham Biosciences (Piscataway, NJ). Rabbit anti-hamster albumin antiserum used to construct anti-hamster albumin columns was obtained from Accurate Chemicals (Westbury, NY). Ultrafree centrifugal devices and C18 ZipTip were obtained from Millipore (Bedford, MA). Sequencing-grade modified trypsin was purchased from Promega (Madison, WI). Chemical supplies were purchased from Sigma (St. Louis, MO) or Amersham Biosciences. Endotoxin (lipopolysaccharide or LPS) from E. coli serotype 055:B5 was purchased from Difco (Detroit, MI). RT-PCR and rapid amplification of cDNA ends (RACE) kits were purchased from Clontech (Palo Alto, CA). Gel extraction kit was obtained from Qiagen (Valencia, CA).

Animal experiments

Syrian hamsters (8-12 weeks old) were obtained from Charles River (Wilmington, MA) and C57BL/6 mice (6-8 weeks old) were obtained from Jackson Laboratory (Bar Harbor, ME). Sialectomized mice (from which all major salivary glands were removed), castrated mice, and sham-operated mice were purchased from Charles River. Rodent chow and water were provided ad libitum. In some experiments, animals were injected intraperitoneally with endotoxin, whereas control animals were injected with normal saline. The dose of endotoxin was 100 μg/100 g body weight for hamsters and 100

μg/animal for mice. At these doses of endotoxin, the acute-phase response was induced and animals became toxic, however, these doses are far below the lethal dose (LD_{50} ~ 5 mg/100 g body weight) required to cause death in rodents in our laboratory (10). Sixteen hours after injection, the animals were euthanized using halothane, plasma or serum was collected, and the tissues were excised and stored at –80°C for RNA isolation or fixed in 4% paraformaldehyde (Fisher, Pittsburgh, PA) at 4°C for immunohistochemistry and/or immunofluorescence microscopy. The animal procedures were approved by the Animal Studies Subcommittee of the San Francisco Veterans Affairs Medical Center, and were performed in accordance with the guidelines.

HDL isolation by ultracentrifugation

HDL (d=1.063-1.21 g/mL) was isolated from pooled sera or plasma of animals by sequential ultracentrifugation as previously described (11). A protease inhibitor cocktail (final concentration: 0.3 mg/mL benzamidine, 10 μg/mL phenylmethylsulfonyl fluoride, 0.13% ε-aminocaproic acid, 0.04% EDTA) and NaN_3 (0.05%) was added to the whole blood before isolation of plasma. HDL was extensively dialyzed in 5 mM Tris HCl, 1 mM EDTA, pH 7.4 and concentrated. Other fractions of lipoproteins and lipoprotein-deficient sera were isolated by ultracentrifugation as previously described (11). Protein concentrations of lipoproteins were measured by a modified Lowry assay (Pierce).

Construction of columns for purifying HDL by selected-affinity immunosorption

Hamster HDL isolated by ultracentrifugation was electrophoresed in polyacrylamide gels and apo A-I bands identified by copper staining (BioRad, Hercules,

CA) were cut and proteins eluted using an electro-eluter (BioRad). Hamster apo A-I was then used to produce antiserum in a goat (Animal Pharm, Healdsburg, CA). Goat antibodies were initially isolated using ImmunoPure protein G columns, and a subpopulation of low-affinity antibodies against hamster apo A-I was further purified using a hamster HDL protein-Sepharose column as previously described (12). These selected hamster apo A-I antibodies were eluted with 0.2 M acetic acid, 0.15 M NaCl (pH 3.0). The eluate was immediately neutralized to pH 7.4 using 2 M Tris and used to construct hamster apo A-I columns by crosslinking to cyanogen bromide-activated Sepharose 4B at a concentration of ~ 5.4 mg IgG per 1 mL gel according to the manufacturer's instructions.

Isolation of column-purified HDL using hamster apo A-I immunoaffinity columns

Hamster plasma was applied to the hamster apo A-I column and washed with 0.01 M Tris, 0.15 NaCl, 0.04% EDTA, 0.05% NaN_3 (pH 7.3) at 4°C until there was no detectable absorption at 280 nm. Hamster apo A-I-containing HDL was eluted with 0.2 M acetic acid, 0.15 M NaCl (pH 3.0) and immediately neutralized with 2 M Tris to pH 7.4 and concentrated using Ultrafree centrifugal devices. We found that column-purified HDL also contained albumin (data not shown), therefore, we constructed anti-hamster albumin columns and routinely passed column-purified HDL through the anti-hamster albumin columns to remove contaminated albumin.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed as previously described. Briefly, immobilized pH gradient (IPG) strips (Immobiline DryStrip, pH 3-10 NL) were rehydrated with HDL mixed with 8 M urea, 4% CHAPS, 65 mM dithiothreitol, and 0.5 % IPG buffer overnight. We intentionally overloaded the IPG strips (50-200 μg protein) in order to allow us to visualize low abundance proteins. Rehydrated IPG strips were subjected to isoelectric focusing for a total of 50,000-60,000 V-hr at 20°C. After isoelectric focusing, IPG strips were equilibrated in 50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and trace bromophenol blue for 15 minutes. The second dimension was performed in a vertical system using 10% polyacrylamide gels at 40 mA. Two-dimensional gels were then fixed and stained with silver nitrate as described by Shevchenko et al (13). Protein spots were visualized using the BioRad imaging densitometer. We also created a database of known HDL-associated proteins and used published two-dimensional gel patterns of HDL in several animal species for references.

Identification of proteins by mass spectrometry

Silver-stained proteins were reduced with 1 mM dithiothreitol, alkylated with 2 mM iodoacetamide, and in-gel digested with trypsin as described at http://donatello.ucsf.edu/ingel.html. The peptides were extracted and purified using C18 ZipTip. An aliquot of each digest was subjected to matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) as well as to LC/MS/MS analysis. The MALDI-MS and MS-MS, i.e. high energy collision-induced dissociation (CID) analyses were performed on a 4700 Proteomics Analyzer (Applied Biosystems, Framingham,

MA), using 4-OH-αCN-cinnamic acid as matrix (14). LC/MS/MS was performed on a QSTAR Pulsar quadrupole-orthogonal-acceleration-time-of-flight tandem mass spectrometer equipped with an electrospray source. (MDS Sciex, Toronto, Canada). The peptides were separated by nanoHPLC (Ultimate HPLC system with Famous autosampler, LC Packings, San Francisco, CA) developing a 5%-50% B linear gradient over 30 min, on a 75 μm ID Pepmap column (LC Packings, San Francisco, CA) at a flow rate of ~300 nL/min. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile. The eluent was directly introduced in the mass spectrometer, and was monitored in an information-dependent acquisition mode; 1 sec mass measurements were followed by 5 sec CID experiments throughout the analysis. Multiply charged precursor ions were computer-selected and the collision energy was adjusted according to the mass (m) and charge (z) of the ion fragmented. The identity of the proteins is usually determined by database searches with the uninterpreted CID data against all the proteins in the National Center for Biotechnology Information (NCBI) database using the appropriate Bioanalyst Script (MDS Sciex) and the Mascot software (www.matrixscience.com) or Protein Prospector MS-Tag (www.prospector.ucsf.edu). De novo sequencing was performed without the aid of computer programs. BLAST and MS-Pattern searches were performed with the sequences determined.

Cloning of hamster PSP cDNA

A hamster homologue of rat and mouse PSP cDNA was cloned from the hamster parotid gland RNA by RT-PCR using the rat PSP primers: 5'ATG TTC CAA CTT GGG AGC C3' and 5'CCC AAC AAG GAA ATG GAG AT3'. The band was gel purified and

submitted for sequencing. 5' RACE and 3' RACE were performed following the manufacturer's instructions using hamster PSP-specific primers: 5'CCC AAC AAG GAA ATG GAG ATT TTG TCT G3' and 5'CTT ATC ACC TAA GAA TGG TTT AGG GTT GC3'. All of the nucleotide sequencing was performed at Sequetech (Mountain View, CA).

Isolation of RNA and RT-PCR

Isolation of RNA and RT-PCR were performed as previously described (15). RT-PCR to detect the expression of mouse PSP mRNA in various tissues was performed using the mouse primers: 5'CCT CTG AAG CTG TCC CTC A3' and 5'CCC AAC AAG GAA ATG GAG ATT TT3'.

Immunoblot analyses

Immunoblot analyses were performed as previously described (15). The peptide CSSNTDKISISLLGRR, which corresponds to the amino acids 161-176 of the predicted mouse PSP protein, was synthesized (AnaSpec, San Jose, CA) and used to raise polyclonal antibodies in rabbits. PSP-specific antibodies were isolated and affinity-purified using ImmunoPure protein G columns followed by a thiopropyl Sepharose 4B column coupled with the PSP peptide. The eluted rabbit polyclonal anti-mouse PSP IgG was then used as a primary antibody.

Immunofluorescence microscopy and immunohistochemistry

Tissue paraffin sections were prepared for immunofluorescence microscopy using standard methods. Deparaffinized sections were blocked with blocking buffer (0.1% fish gelatin, 0.8% bovine serum albumin, and 0.01% Tween in phosphate-buffered saline) for 30 min as described previously (16). Sections were then incubated with rabbit anti-mouse PSP antibody overnight at 4°C, followed by a secondary antibody (Alexa Fluor chicken anti-rabbit IgG from Molecular Probes, Eugene, OR). Counterstaining for nuclei was performed with 1 mg/mL propidium iodide. After washing, slides were mounted and viewed using a confocal microscope (Zeiss, Heidelberg, Germany). Sections incubated with normal rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) during primary antibody incubation served as a negative control.

In some experiments, immunohistochemistry was also performed. Endogenous peroxidase activity was blocked with 1% H_2O_2 in methanol for 15 min before the blocking buffer. After overnight incubation with a primary antibody, sections were incubated with a secondary antibody (biotinylated goat anti-rabbit IgG fromVector Lab, Burlingame, CA) followed by Vecta stain and DAB peroxidase substrate kit (Vector Lab). Slides were viewed with a light microscope.

Bioinformatic analyses

BLAST and PSI-BLAST were performed using the non-redundant database through the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST). Searches for conserved domains and predicted secondary and tertiary structures were performed using

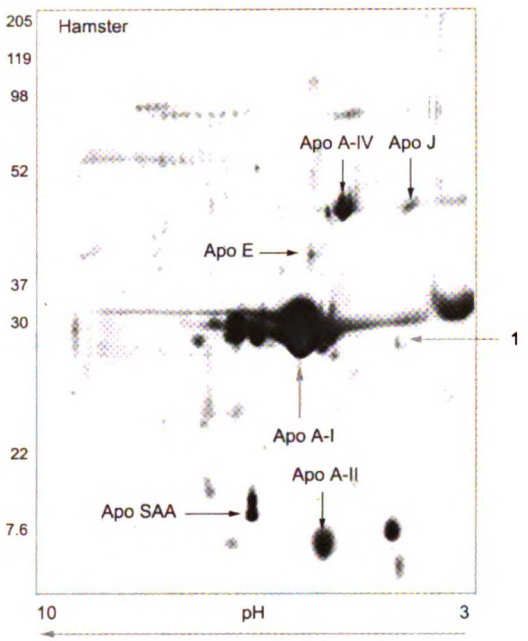
3DPSSM at http://www.sbg.bio.ic.ac.uk as previously described (17). Multiple sequence alignments were performed using ClustalW and Boxshade at http://www.ch.embnet.org.

RESULTS

Identification of parotid secretory protein as an HDL-associated protein in hamsters

Column-purified HDL from hamster plasma was subjected to two-dimensional gel electrophoresis and HDL-associated proteins were visualized using silver staining. A representative two-dimensional gel of proteins is shown in **Fig. 1A**. Using a database of HDL-associated proteins that we created and the two-dimensional gel patterns of HDL published in the literature, we selected certain protein spots for identification. For confirmation, we first selected the spots to represent known HDL-associated proteins. As a result, apo A-I, apo A-II, apo A-IV, apo E, apo J, and apo serum amyloid A (SAA) were all identified by mass spectrometry (**Fig. 1A**). We then selected some "unknown protein" spots, the location of which did not match the molecular weight and isoelectric point of the known HDL-associated proteins. We, however, did not attempt to identify all proteins spots seen in the two-dimensional gels. Spot 1 was among those "unknown protein" spots selected and submitted for identification by mass spectrometry. MALDI-MS analysis of the unfractionated digest showed a single peptide at m/z 1651.78. Manual interpretation of the high energy CID spectrum of this peptide yielded a partial sequence of Q/KISISLLGR (**Fig. 2**). High energy CID fragmentation permitted the unambiguous

1A



1B

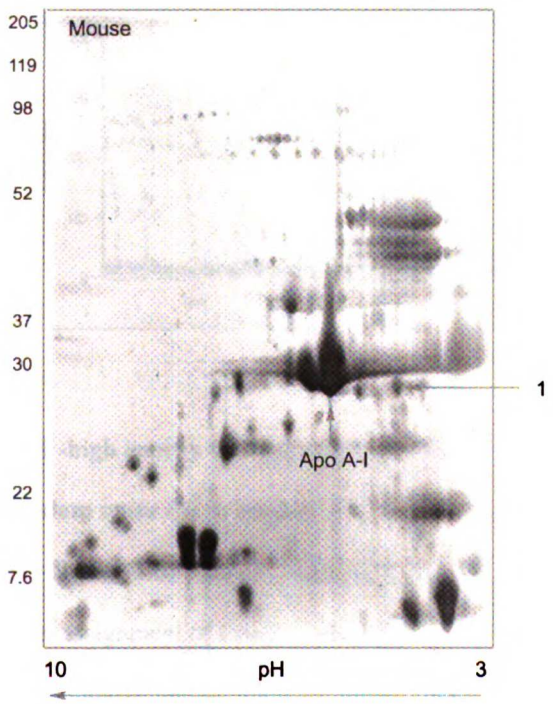


FIG. 1. Representative two-dimensional gel electrophoresis results of HDL-associated proteins from hamster column-purified HDL (A) and mouse ultracentrifuged HDL (B). Hamster HDL was isolated by hamster apo A-I column chromatography, whereas mouse HDL was isolated by ultracentrifugation. Both hamster and mouse HDL were subjected to two-dimensional gel electrophoresis and protein spots were visualized using silver staining. The identity of the proteins was identified using mass spectrometry as described in Experimental Procedures. The non-linear pH gradient was from pH 3 (right) to pH 10 (left). Molecular mass markers (kDa) are shown on the left of the gel. Spot 1 in Fig. 1A was identified as a hamster homologue of rat and mouse parotid secretory protein. Spot 1 in Fig. 1B was identified as mouse parotid secretory protein.

assignments of the isomeric Leu/Ile residues (18). LC/MS/MS analysis with low energy CID yielded only one additional sequence of TGI/LPTI/LTI/LGK (m/z 500.82^{2+}). In low energy CID, Leu and Ile residues are indistinguishable.

BLAST and MS-Pattern searches were performed with the two sequences determined. Both sequences showed homology to a salivary protein from rats and mice known as parotid secretory protein (**Fig. 3A**).

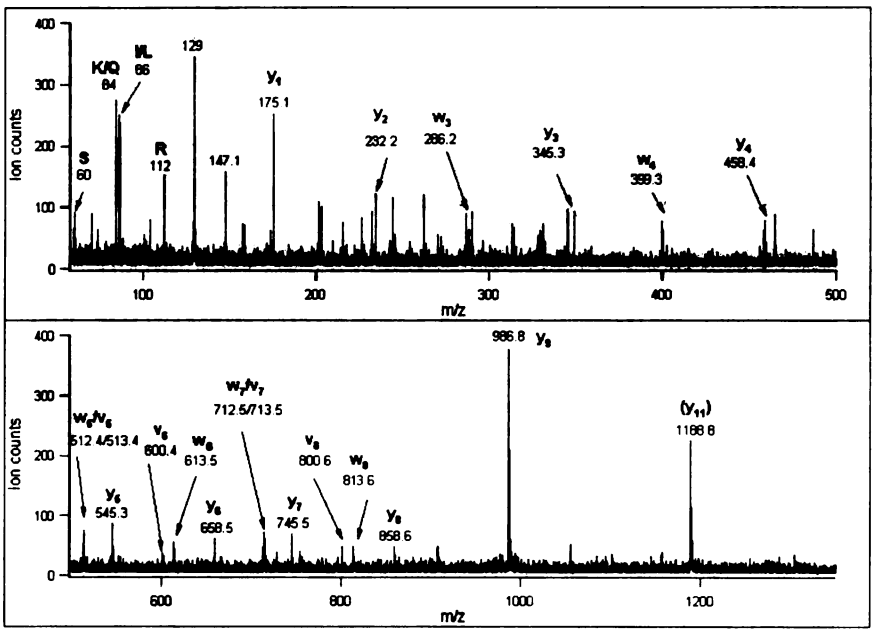


FIG. 2. MALDI -high energy CID spectrum of m/z 1651.78 obtained on a 4700 Proteomics Analyzer (TOF-TOF tandem mass spectrometer). These data permitted the determination of a partial sequence as Gln/Lys-**Ile**-Ser-**Ile**-Ser-**Leu**-**Leu**-Gly-Arg. High energy CID fragmentation, i.e. w-ion formation permitted the unambiguous assignment of Leu/Ile residues. (Nomenclature as in (46)) The full sequence is: Cys-Ser-Ser-Asp-Ser-Asp-Lys-**Ile**-Ser-**Ile**-Ser-**Leu**-**Leu**-Gly-Arg, and its calculated MH^+ value is indeed 1651.83 with an acrylamide-modified Cys – a common byproduct in gel-separated proteins.

In order to ensure that parotid secretory protein (PSP) is truly an HDL-associated protein, and that its protein spot found on the two-dimensional gel was not due to isolation artifacts from the columns, we prepared hamster HDL by ultracentrifugation and subjected them to two-dimensional gel electrophoresis. A spot corresponding to hamster PSP was also found in ultracentrifuged HDL (data not shown).

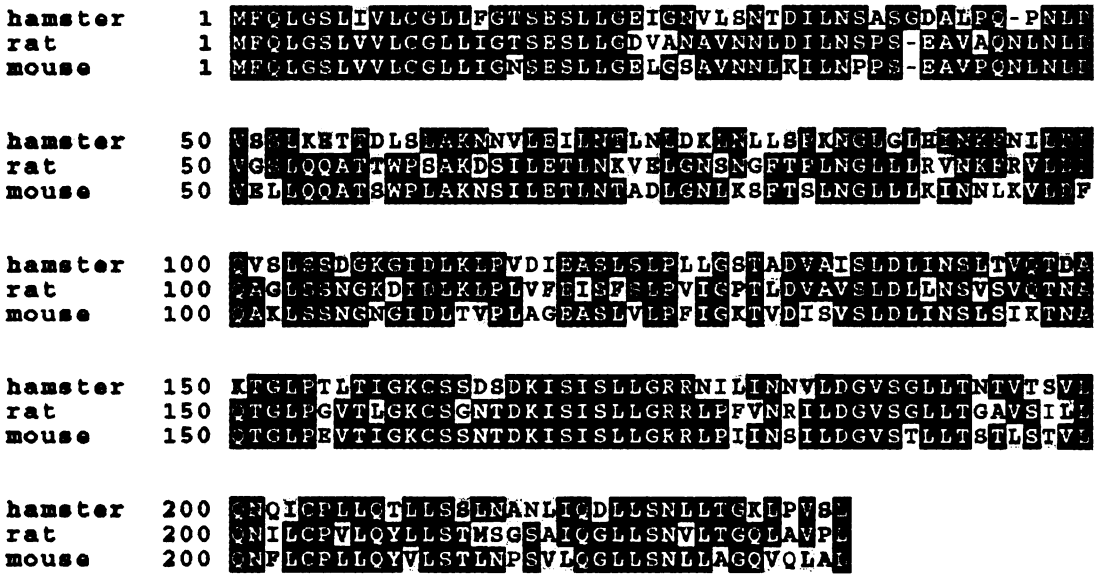


FIG. 3A. Predicted amino acid sequence of hamster PSP and its alignment with rat and mouse PSP. Amino acid residues identical in the alignment are shown in black boxes, whereas similar residues are shown in gray.

Cloning of the putative hamster PSP cDNA

After we putatively identified the hamster homologue of rat and mouse PSP in HDL, we next cloned the hamster PSP cDNA. Based on close homology to rat PSP, we amplified the coding sequence of hamster PSP by RT-PCR using primers derived from

the rat nucleotide sequence. The complete sequence of the putative hamster PSP contained an open reading frame of 705 base pairs (**Fig. 3B**) predicted to encode a protein of 235 amino acids (**Fig. 3A**). The first 60 base pairs were predicted to encode a highly hydrophobic region, which is likely a signal peptide of 20 amino acids. Similar to rat and mouse PSP, hamster PSP is leucine rich (23.8%). A predicted molecular mass of the mature peptide after the signal sequence is cleaved is ~ 22,600 kDa and the isoelectric point is expected to be ~ 4.5. The predicted molecular mass is lower than that expected from the location of the spot found on the gel, suggesting that hamster PSP may be post-translationally modified. Several sites for protein kinase C phosphorylation, casein kinase II phosphorylation, N-myristoylation, and amidation were predicted, but no glycosylation site has been found. We also performed 5' RACE and 3' RACE, which identified at least 68 base pairs of the 5' untranslated sequence and 242 base pairs of the 3' untranslated sequence (**Fig. 3B**).

Analysis of the NCBI protein database revealed that hamster PSP shared 59% amino acid identity with rat PSP, and 57% identity with mouse PSP. The three cysteines are also conserved among hamster, rat, and mouse PSP (**Fig. 3A**). In addition, hamster PSP also showed homology to murine neonatal submandibular gland protein B (19) (42% identity), BSP30, a putative bovine PSP homologue (20) (30% identity), von Ebner's salivary gland protein (21) (25% identity), and PLUNC (palate, lung, and nasal epithelium clone) (22) (25% identity).

GGGGG GAGAC TGTGT AGAGA TAGAA GCATT
TCCTG TACAT CTTTC TCCAA CCTGT GTCAG
AACCA AAG**AT** **G**TTCC AACTT GGGAG CCTCA
TTGTC TTGTG TGGCT TGCTC TTCGG GACCT
CAGAA TCACT TTTGG GTGAA ATTGG CAATG
TCCTG AGCAA CACGG ACATT CTGAA TTCTG
CCTCT GGAGA TGCCC TTCCA CAACC AAACC
TGGAT GTGAG CTCAC TTAAG GAAAC CACGG
ATTTG TCATT GGCCA AGAAC AACGT ACTGG
AAATT TTGAA CACAC TGAAC CTTGA CAAGT
TAAAC CTCTT ATCAC CTAAG AATGG TTTAG
GGTTG CACAT CAACA AATTC AACAT CCTGG
ACTTG CAAGT TAGCT TGTCT TCTGA TGGCA
AAGGC ATCGA TCTGA AGTTG CCTGT GGACA
TAGAA GCCTC TTTGT CTCTG CCTCT TCTTG
GGTCC ACGGC TGATG TTGCT ATTTC CTTGG
ACCTC ATAAA CTCAC TCACT GTTCA AACCG
ATGCC AAGAC TGGCC TTCCC ACCCT GACCA
TAGGG AAATG CTCAA GTGAT TCAGA CAAAA
TCTCC ATTTC CTTAT TGGGC AGGCG AAACA
TCCTT ATCAA CAACG TACTG GATGG TGTAT
CTGGC CTCCT TACAA ACACA GTGAC AAGCG
TGCTG CAAAA CCAAA TATGT CCACT GCTCC
AGACA CTCCT CAGCA GCCTG AATGC AAATC
TTATT CAAGA CCTCC TCTCT AATCT ACTGA
CAGGA AAGTT ACCAG TCTCC CTT**TG** **A**AGAG
GAAGG AGCAG AAGGA GGAAT GCCAT GACTC
CTGCT GGGTA GTCCC CAGTG GCTTC ATCTG
ACTCC CTGTG GCATT TCCCT CTAGA AATTA
CAACT ATCAC CCACA CAAGG TCTGC TTGAG
CCCAC CTGAA GGAGC CTCCC AGACT CTTTT
CTTCC CTCAG TGGTT GCCAC TCCTG TTTAC
CACCC TCCAA GCATT AAAAT CCTAA CTGCA
AAAAA AAAAA AAAAA AAANA AAAAG TNC

FIG. 3B. cDNA sequence of hamster PSP. RT-PCR, 5' RACE, and 3' RACE were performed as described in Experimental Procedures. Sequence in the 5' untranslated region is not complete and only 68 base pairs are shown. ATG start codon and TGA stop codon are underlined and in bold.

In order to get insight into the potential function of hamster PSP, searches for conserved functional domains as well as secondary and tertiary structure were performed (17). The searches revealed that the carboxy terminus of hamster PSP is homologous to a region in the amino terminus of a family of proteins including lipopolysaccharide binding protein (LBP), bactericidal permeability-increasing protein (BPI), cholesterol ester transfer protein (CETP), and phospholipid transfer protein (PLTP). The sequence alignment between hamster PSP and human BPI is shown in **Fig. 3C**. The two cysteines found in this region of hamster, rat, and mouse PSP are conserved with those in BPI (Cys^{135} and Cys^{175}). These cysteines are known to form a single disulfide bond in BPI that is critical to its function (23, 24). In addition, these two cysteines are conserved among LBP, CETP, and PLTP (24), all of which are HDL-associated proteins (25, 26)

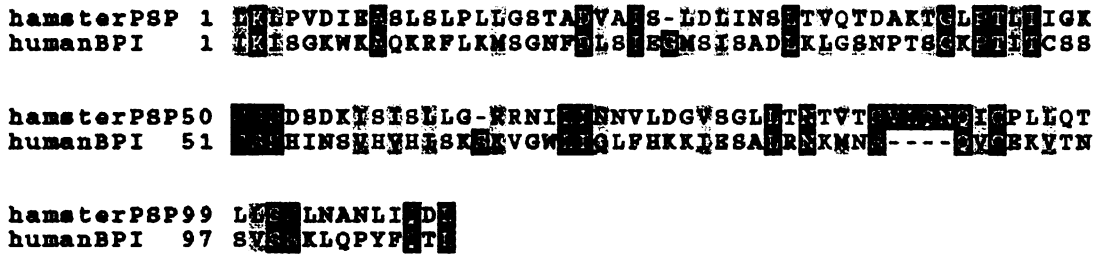


FIG. 3C. Amino acid sequence alignment of hamster PSP with human BPI. Amino acid sequence alignment of the carboxy terminus of hamster PSP (start from amino acid residue 113) with the amino-terminal domain of human BPI (start from amino acid residue 135). Amino acid residues identical in the alignment are shown in black boxes, whereas similar residues are shown in gray.

Hamster PSP was also predicted to have a tertiary structure similar to that of the amino terminus of human BPI. The expectation value (E value) for the prediction was 0.0005, which suggested > 95% certainty. Similar result was also found for mouse PSP (E value = 0.0036). Because BPI is consisted of two domains (amino terminus and carboxy terminus) of similar size, secondary structure, and topology, the predicted structure of hamster PSP represents only half of the BPI molecule. It is of note that the efficacy of the recombinant amino terminal fragment of BPI in binding LPS and neutralizing its effects is similar to that of the full-length protein (23, 27). Homologies to the amino terminal domain of BPI (SMART database accession number 00328, BPI1) have previously been found in von Ebner's salivary gland protein and PLUNC, two proteins closely related to PSP (28, 29).

PSP is also an HDL-associated protein in mice

To determine if PSP is an HDL-associated protein in other species, we isolated ultracentrifuged HDL from mouse sera and subjected mouse HDL to two-dimensional gel electrophoresis and mass spectrometry. Because the sequence of mouse PSP has been known and present in the NCBI database, the spot corresponding to mouse PSP should be easily identified by mass spectrometry if it is associated with HDL. Indeed, mouse PSP (Spot 1 in **Fig. 1B**) was independently identified from the two-dimensional gel without prior knowledge of the result from the hamster. Eight peptides selected randomly from the mass spectra (m/z range from ~1100 to ~ 6300) showed that they matched 59% of the amino acid sequence of the mouse PSP (data not shown).

To examine whether PSP is associated with other lipoprotein fractions, we isolated mouse lipoproteins by ultracentrifugation and performed immunoblot analysis using an anti-peptide antibody against mouse PSP. As shown in **Fig. 4**, PSP was detected only in HDL, and not in very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), or lipoprotein-deficient serum.

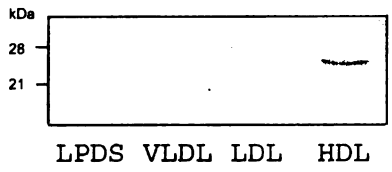


FIG. 4. Immunoblot analysis of PSP in different fractions of lipoproteins in mouse serum. Lipoproteins and lipoprotein-deficient serum were isolated by ultracentrifugation and electrophoresed in 10-20% gel. Thirty μg of VLDL and LDL proteins were loaded whereas 50 μg proteins of HDL and LPDS were loaded. Immunoblot analysis was performed as described in Experimental Procedures.

PSP is expressed in mouse lung, skin, and gonads

PSP is one of the major proteins found in the saliva of rats and mice (30, 31). Previous studies have reported that PSP mRNA is expressed only in the salivary glands and the lacrimal glands (32, 33). Because our study showed that PSP was found in HDL in the circulation, we hypothesized that PSP might have a wider tissue distribution than previously thought. Using RT-PCR, we found that in addition to the salivary glands, PSP mRNA was expressed in the mouse lung and ovary (**Fig. 5**). Northern analysis also revealed that PSP was additionally found in the testis and skin, albeit at the relatively low levels (data not shown). Using a combination of RT-PCR and Northern analysis, we did

not detect signals from the liver, spleen, kidney, heart, stomach, small intestine, pancreas, urinary bladder, and brain (data not shown).

FIG. 5. The expression of mouse PSP in different tissues. RNA was isolated from different tissues and RT-PCR was performed as described in Experimental Procedures. Lane 1: 1 kb ladder, 2: liver, 3: lung, 4: pancreas, 5: stomach, 6: small intestine, 7: ovary, 8: skin, 9: placenta, 10: heart, 11: salivary gland. An arrow indicates the expected PCR product of PSP. Placental RNA was included in the RT-PCR reaction as a positive control (positive band not shown) and the primers used were not for the detection of PSP.

Because of the relatively low levels of PSP detected in certain tissues, we hypothesized that PSP might be restrictively expressed only in a subset of cells. In order to localize the cellular origin of the PSP in tissues, we examined mouse organ sections using immunofluorescence and/or immunohistochemistry. As a positive control, we detected abundant immunoreactivity in the glandular epithelium of the parotid and submandibular glands (data not shown). In agreement with the mRNA data, we detected PSP immunoreactivity in the lung, skin, testis, and ovary (**Fig. 6**). We also screened several other tissues and did not detect specific immunoreactive signals in the liver, spleen, kidney, heart, stomach, small intestine, colon, pancreas, prostate, or uterus (data not shown).

In the lung, PSP was exclusively localized to the respiratory epithelium in the bronchioles (**Fig. 6**). It is of note that the immunoreactivity in the respiratory epithelium was discontinuous and only a subset of cells lining the respiratory epithelium of the bronchioles expressed PSP. We did not detect specific immunoreactivity in the alveoli.

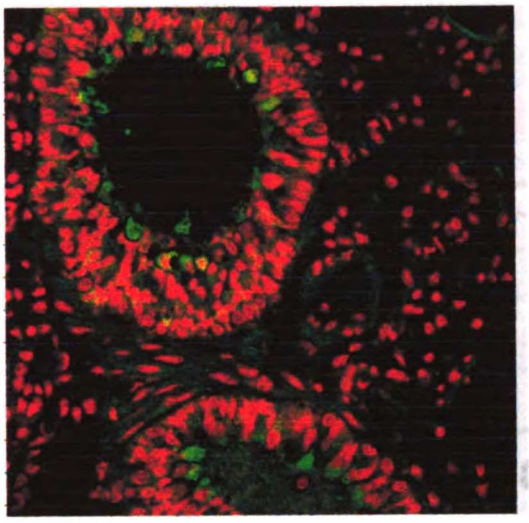
In the skin, PSP immunoreactivity was found in the keratinocytes, around the hair follicles, and in the sebaceous gland (**Fig. 6**).

In the testis, PSP immunoreactivity was found only in Leydig cells, and not in the seminiferous tubules (**Fig. 6**). In the ovary, PSP immunoreactivity was localized mainly to the stromal cells, and was not found in the ovarian follicles (**Fig. 6**). However, we could not rule out that PSP might be expressed in the theca cells.

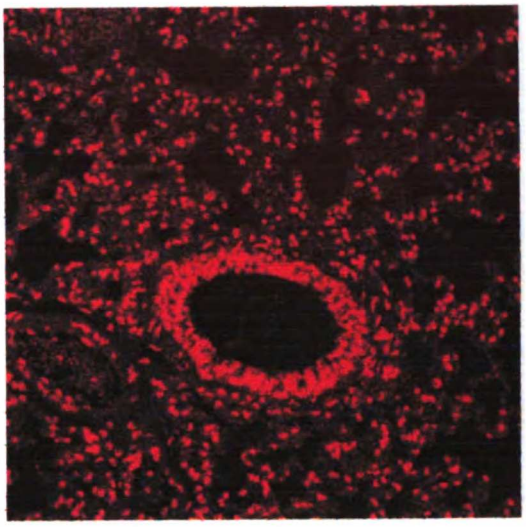
FIG. 6 (next pages). The immunoreactivity of mouse PSP in different tissues. Mouse tissues were prepared as described in Experimental Procedures. Confocal microscopy visualized a strong green signal for PSP localized to different cell types in various tissues in Panel A (using anti mouse PSP antibody). In contrast, almost no green signal was observed in Panel B, which were negative controls (using normal rabbit antibody). Strong red signals showed nuclei staining by propidium iodide.

Lung

A

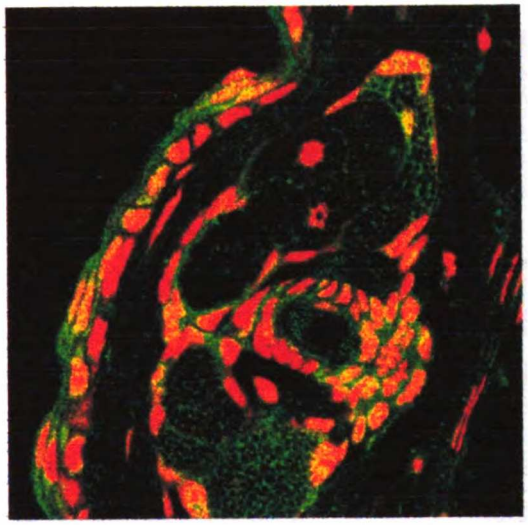


B



Skin

A



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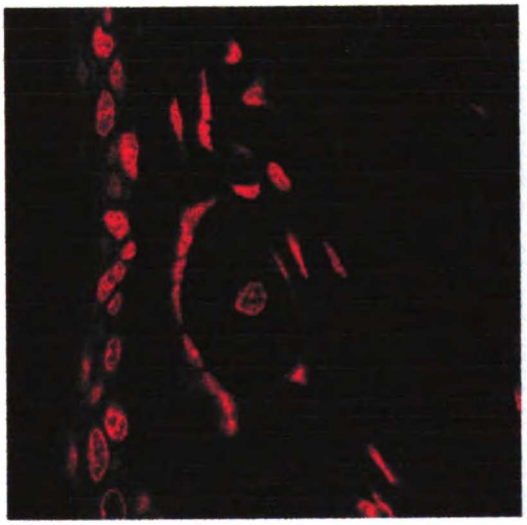
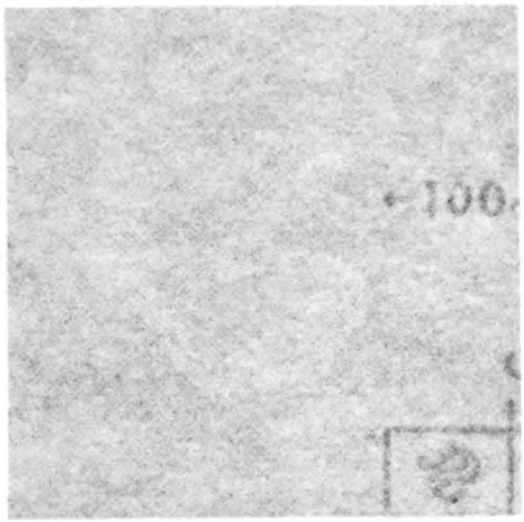
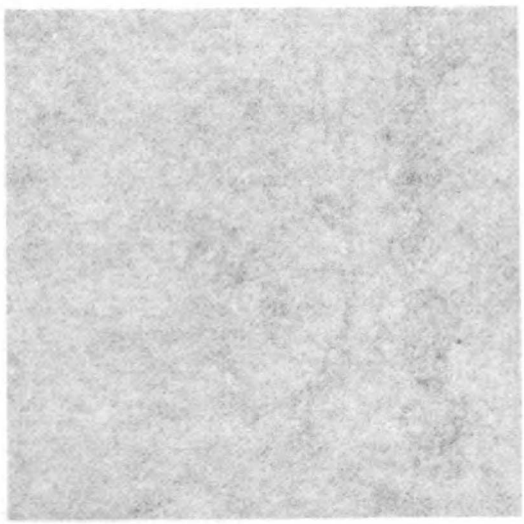
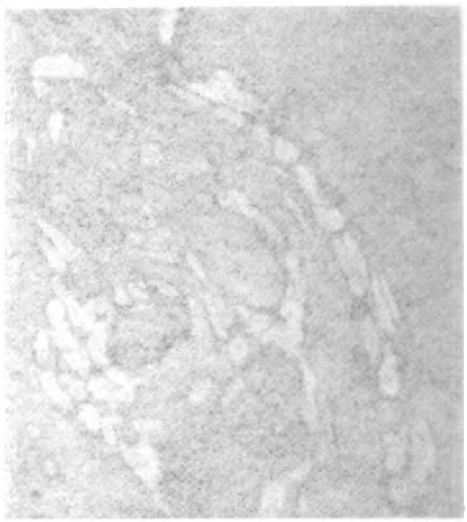


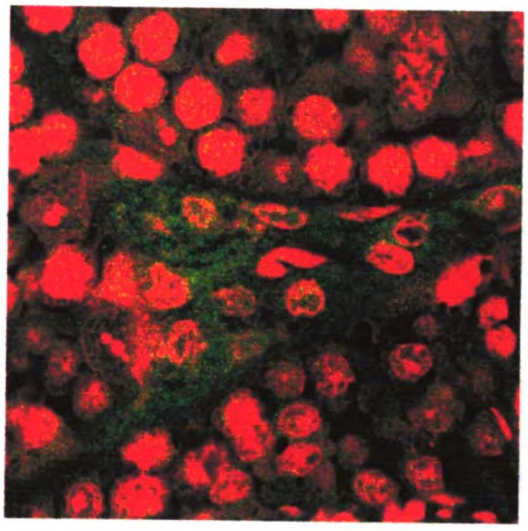






Testis

A



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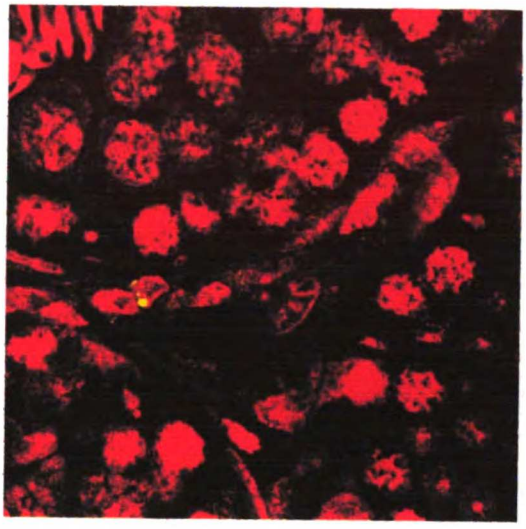
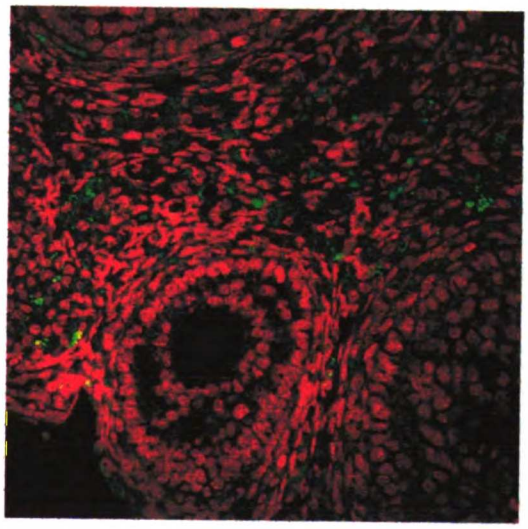


Ovary

A



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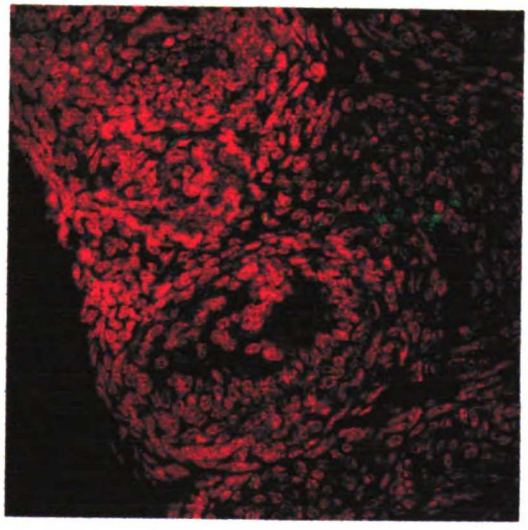

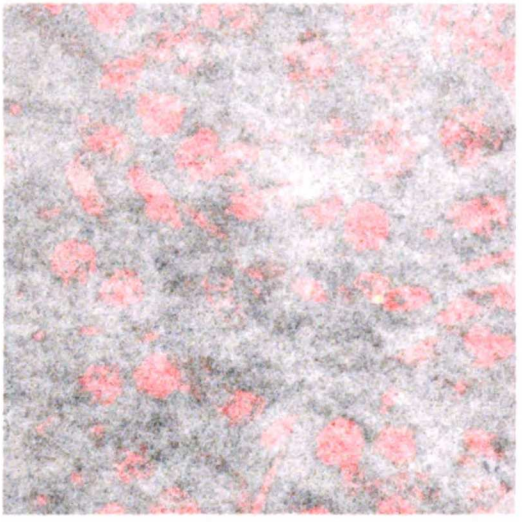

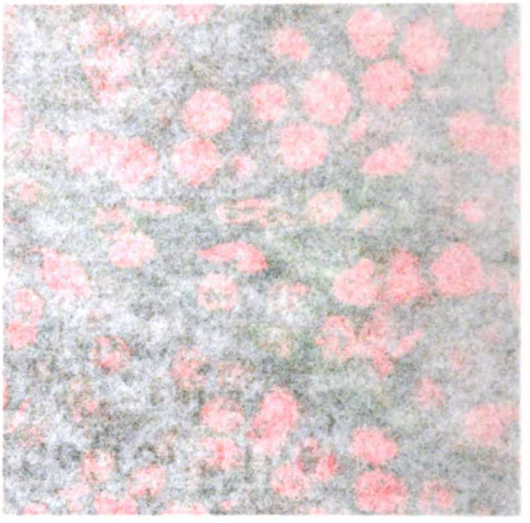

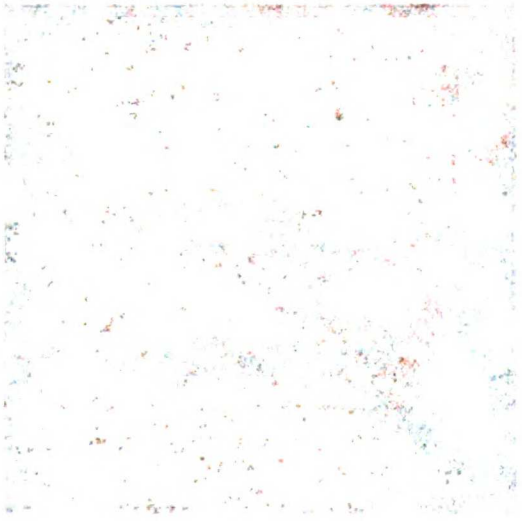

Collectively, our study showed that PSP is expressed in several organs, and not restricted only to the salivary glands and the lacrimal glands as previously reported (32, 33). Because PSP is a secreted protein, each of the organs that express PSP could potentially secrete PSP into the circulation and contribute to the presence of the protein found in HDL. To examine the contribution by various organs, we selectively removed either all of the major salivary glands (as in sialectomized mice) or the testes (as in castrated mice). Approximately two weeks after surgery, HDL was isolated from pooled mouse sera of sialectomized or castrated mice and immunoblot analyses for PSP were performed using HDL from sham-operated mice as a control. We found that the level of PSP in HDL was not decreased despite the removal of either the salivary glands or testes (data not shown). The result suggests that other tissues or multiple tissues may contribute to the level of PSP in HDL. Less likely, the half-life of PSP in the circulation may be prolonged and a period of two weeks after removal of the tissues producing PSP might not have been long enough to detect the decrease of PSP levels in HDL.

Changes in PSP levels in HDL in response to endotoxin

Because of the LBP/BPI/CETP N-terminal domain found in the PSP sequence, we hypothesized that PSP might be involved in the innate immune response, and that PSP levels might be regulated by endotoxin. Both LBP and BPI, which play a role in innate immunity, are upregulated by endotoxin (34, 35). In contrast, CETP and PLTP levels are decreased after endotoxin administration (36, 37).

In order to determine whether the levels of PSP in HDL were affected by endotoxin, hamsters were injected with endotoxin, whereas control animals were injected

with normal saline. Acute-phase HDL from plasma of endotoxin-injected hamsters and control HDL from plasma of saline-injected hamsters were separately purified using hamster apo A-I immunoaffinity columns followed by anti-hamster albumin columns. Both preparations of HDL were subjected to two-dimensional gel electrophoresis and the amount of PSP in HDL was compared using silver staining. As shown in **Fig. 7**, we found that hamster PSP was present in higher amount in acute-phase HDL compared to

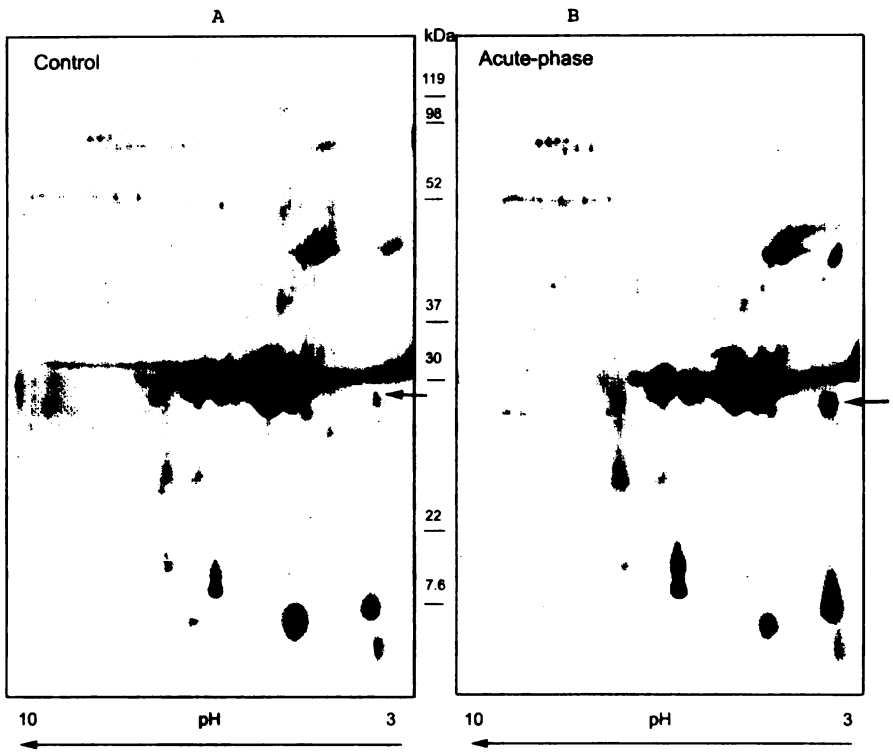

FIG. 7. Representative two-dimensional gel electrophoresis results of hamster control (A) and acute-phase (B) HDL isolated from apo A-I immunoaffinity columns Control hamster HDL and acute-phase hamster HDL were isolated by apo A-I column chromatography and subjected to two-dimensional gel electrophoresis. Protein spots were visualized using silver staining as described in Experimental Procedures. The non-linear pH gradient was from pH 3 (right) to pH 10 (left). Molecular mass markers are shown between the two gels. Arrows indicate parotid secretory protein. Five different sets of control and acute-phase HDL gave similar results.

that in control HDL. However, when we performed similar experiments in mice using ultracentrifuged HDL, we did not find a consistent increase in the amount of PSP in mouse acute-phase HDL (data not shown). These data suggest that there may be species-specific changes in the level of PSP in response to endotoxin as seen with many other serum proteins (38).

DISCUSSION

A number of proteins are associated with HDL particles that play different roles in the function and metabolism of HDL (6, 7). In this study, we identified parotid secretory protein (PSP) as an HDL-associated protein. PSP is one of the major proteins found in the saliva of mice and rats. However, it has not previously been shown to be present in the circulation or associated with HDL. Using two independent methods of HDL isolation, ultracentrifugation and apo A-I immunoaffinity column chromatography, we identified a hamster homologue of mouse and rat PSP as an HDL-associated protein. Data from mice also confirmed that PSP is associated with mouse HDL. Besides HDL, PSP was not associated with other lipoproteins or other plasma fractions. We also cloned a hamster homologue of rat and mouse PSP, performed sequence analysis, and studied the tissue distribution.

PSP was initially discovered in rat and mouse parotid saliva (30, 31). Both rat and mouse PSP have a high content (~ 20%) of leucine, which suggests that PSP may be involved in protein-protein interaction. Although the exact function of PSP is currently unknown, it has been reported that PSP can bind bacterial membranes (33). In cows, low

levels of salivary BSP30, a bovine PSP homologue, are correlated with high susceptibility to bloat, a disorder of cattle in which bacterial fermentation in the gut causes gas accumulation (20). A human homologue of PSP has recently been identified (29).

Our searches for conserved domains revealed that part of the carboxy terminus of hamster and mouse PSP is homologous to the amino terminal domain of a family of proteins including LBP, BPI, CETP, and PLTP. The fact that PSP has this conserved domain is of special interest for a number of reasons. First, this protein family includes LBP, BPI, CETP, and PLTP. Except for BPI, which is found in neutrophil granules, LBP, CETP, and PLTP are all known to be HDL-associated proteins (25, 26, 39). Thus, it is not surprising that PSP is associated with HDL like other proteins in the family. Second, LBP, CETP, and PLTP are lipid transfer proteins (26). LBP binds LPS and transfers it to soluble CD14 (40) and HDL (25). CETP mediates cholesterol ester exchange with triglyceride between HDL and triglyceride-rich lipoproteins, whereas PLTP facilitates phospholipid transfer between lipoproteins. Whether PSP could function as a lipid transfer protein in HDL requires further investigations. Third, HDL has been recognized to play a role in innate immunity. HDL is known to bind endotoxin and ameliorate its deleterious effects (2-5). LBP and PLTP are two HDL-associated proteins that can bind endotoxin of bacteria and neutralize its effects (25, 41). With regard to PSP, one study has shown that PSP binds bacterial membranes (33). Data in cows also suggested that high levels of BSP30, a bovine PSP homologue in saliva, conferred protection against the bacterial disease of bloat (20). The fact that PSP is now known to be an HDL-associated

protein and its levels increased in acute-phase HDL in hamsters raises the question whether PSP could contribute to the endotoxin-neutralizing effects of HDL.

Previous studies have reported that PSP expression was limited only to the salivary glands and the lacrimal glands (32, 33). In this study, we provide evidence that PSP is also expressed in several additional tissues including lung, skin, testis, and ovary. Immunofluorescence studies showed that PSP is produced by a certain subset of cells in the pseudostratified columnar epithelium of the bronchioles. The pattern of the PSP expression suggests that it may be secreted by goblet cells or submucosal glands of the respiratory epithelium. Keratinocytes and sebaceous glands of the skin also express PSP. Keratinocytes and sebocytes may secrete PSP into stratum corneum and the sebum, respectively. In the sebaceous gland, alternatively, PSP may bind lipid substances in the sebum and may be involved in the organization of lipids. In the testis and the ovary, however, a functional role for PSP cannot be postulated at this time. Altogether, the sites of tissue expression of PSP support the hypothesis that PSP is a protein produced by a variety of epithelial cells, and that it might be involved in the host defense against microbes encountered at those sites. In certain tissues, PSP may also play a role in the organization of lipophilic substances.

The sequence of PSP is homologous to two other proteins, von Ebner's salivary gland protein and PLUNC. Von Ebner's salivary gland protein, also known as tear lipocalin, is a protein found in human saliva, tears, nasal mucus, and sweat (42). The tissue expression of von Ebner's gland protein in humans is overlapping with PSP; however, no murine homologue exists (21). Of note, von Ebner's salivary gland protein

can bind a number of lipid substances, including fatty acids, cholesterol, and lipid peroxidation products (43).

PLUNC is a group of proteins that also has an overlapping tissue distribution with PSP (29). PLUNC is found in nasal secretion and mucus, but not in saliva or tears (28). PLUNC expression is induced by a variety of stimuli, suggesting that PLUNC may also be an acute-phase protein. For example, the levels of PLUNC in human nasal lavage fluid were increased in workers exposed to an airway irritant (44). PLUNC proteins also share a tertiary structure similar to an N-terminal domain of LBP/BPI/CETP, and it has been proposed that PLUNC is involved in host defense in the respiratory tract where pathogens are encountered (29).

Our experiment in hamsters showed that PSP levels were increased in acute-phase HDL after injection with endotoxin. This is agreement with data from PLUNC showing that PLUNC is a positive acute-phase protein (28, 44). However, we did not find an increase in PSP in mice after endotoxin injection. We think that the discordant results between hamsters and mice could represent species-specific differences. It is well known that the acute-phase response in different animal species can be different both in the direction and magnitude of changes (45). For example, C-reactive protein is a positive acute-phase protein in humans, but its levels change only a little in rats (38); whereas α_2-macroglobulin increases only in rats, but not in humans (38).

In summary, we identified PSP as an HDL-associated protein in both hamsters and mice. PSP is expressed in lung, skin, and gonads, in addition to the salivary glands. The identification of the LBP/BPI/CETP domain in the PSP sequence and its close relationship with von Ebner's salivary gland protein and PLUNC suggest that these

secreted proteins may be involved in the host defense in the various parts of the body. Whether PSP contributes to the endotoxin-neutralizing effect of HDL requires further investigations.

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CHAPTER 7

SUMMARY

Plasma concentrations of HDL cholesterol are inversely correlated with the risk of coronary artery disease. As a result, HDL is thought to protect against atherosclerosis and several anti-atherogenic properties of HDL have been proposed. One of the most commonly studied and accepted hypotheses implicates HDL as a key mediator in the pathway known as reverse cholesterol transport. In this pathway, HDL facilitates cholesterol removal from peripheral cells and transports cholesterol to the liver for excretion.

Several lines of evidence suggest a link between atherosclerosis and chronic infection and inflammation. The acute-phase response induced during infection and inflammation is associated with multiple changes in HDL. Previous observations indicate that during the acute-phase response, reverse cholesterol pathway may be impaired.

My thesis was focused on changes in the metabolism, structure, and function of HDL during the acute-phase response. In particular, changes in several steps of reverse cholesterol transport were examined. Hamsters and mice were chosen as animal models and the acute-phase response was induced by injection of endotoxin.

Chapter 1 reviews the general background on the subject and outlines the experimental work. In **Chapter 2**, I began by studying the regulation of two ATP-binding cassette transporters, ABCG5 and ABCG8, by endotoxin. These two ATP-binding cassette (ABC) transporters are proposed to form a heterodimer which effluxes sterols into the bile in the liver and into the intestinal lumen in the small intestine. I showed that endotoxin decreased mRNA levels of ABCG5 and ABCG8 in the liver, but not in the small intestine. These changes would likely impair cholesterol excretion into the bile, the final step in reverse cholesterol transport. The regulation of two other ABC transporters

involved in cholesterol efflux from cells, ABCA1 and ABCG1, were also examined in J774 macrophages. Both endotoxin and cytokines (tumor necrosis factor and interleukin-1) decreased mRNA levels in these cells in a concentration-dependent and time-dependent manner. Protein levels of ABCA1 were also decreased. These reductions would likely affect cholesterol efflux from cells, the first step of reverse cholesterol transport.

Chapter 3 demonstrates that in addition to changes in the transporter proteins of cells, alterations in HDL during the acute-phase response can also impair cholesterol efflux. In this chapter, I isolated acute-phase HDL from animals injected with endotoxin and used them in cholesterol flux studies. The results showed that in J774 macrophages, cholesterol removal by acute-phase HDL was less efficient compared with that of control HDL. The defect was due to both decreased cholesterol efflux and increased cholesterol influx, thus causing cholesterol accumulation in cells. Experiments using an inhibitor of lecithin:cholesterol acyltransferase (LCAT) suggested that lower LCAT activity in acute-phase HDL was a likely mechanism.

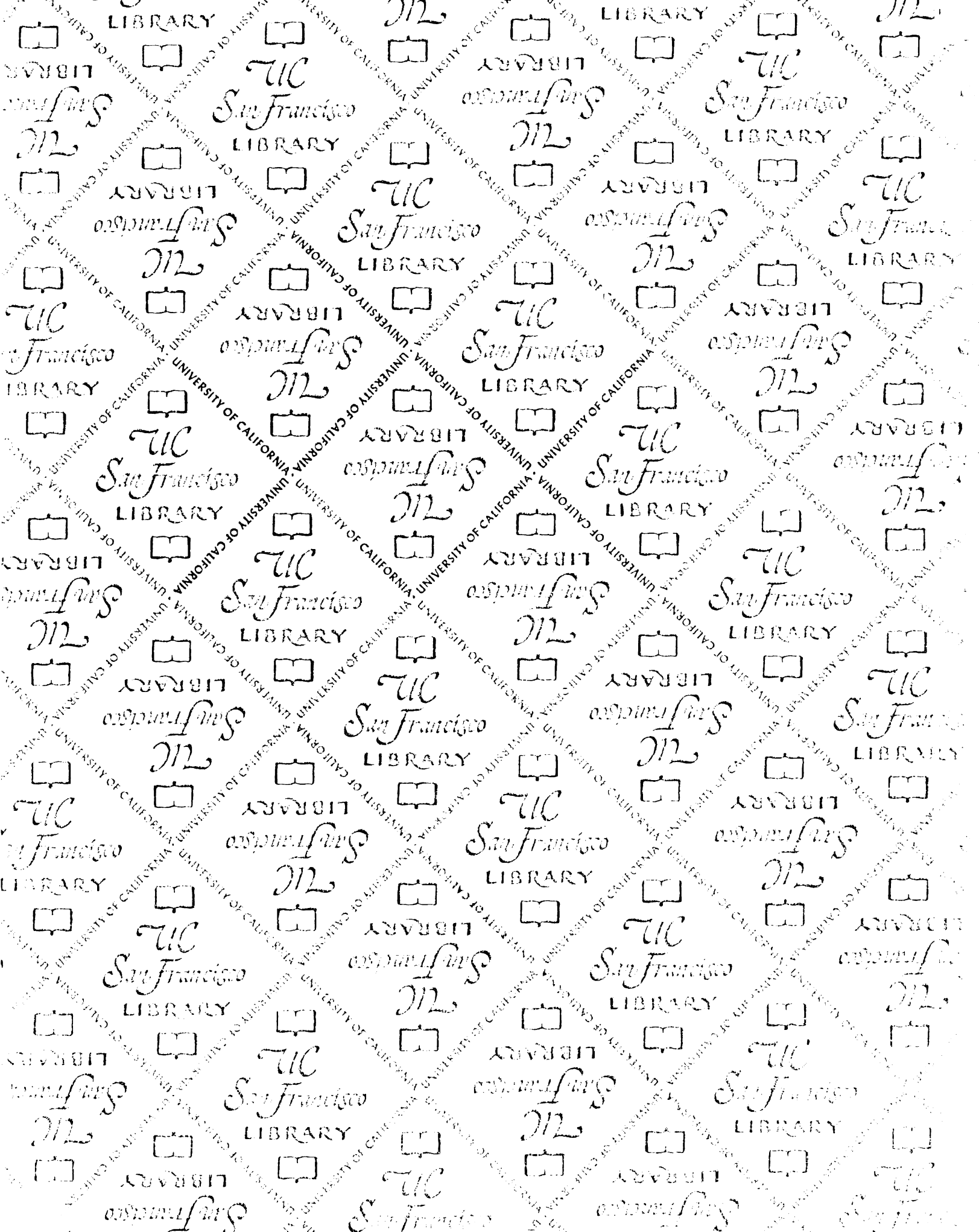
Chapter 4 describes the regulation of scavenger receptor class B type I (SR-BI) by endotoxin. SR-BI is a receptor on hepatocytes that mediates cholesterol ester uptake from HDL into the liver, one of the final steps in reverse cholesterol transport. I demonstrated that levels of SR-BI in the liver were downregulated by endotoxin and cytokines. The reduction was also associated with decreased cholesterol ester uptake into hepatocytes. Altogether, results from **Chapters 2-4** conclusively show that many steps of reverse cholesterol transport are impaired during the acute-phase response. These changes may help conserve cholesterol at peripheral sites for host defense, however, if

the acute-phase response is prolonged, as seen in chronic infections and inflammatory states, impaired reverse cholesterol transport may result in acceleration of atherogenesis.

In **Chapter 5**, I examined the differences in the protein composition of acute-phase HDL. Using a combination of two-dimensional gel electrophoresis, mass spectrometry, and immunoblot analysis, I identified several proteins associated with HDL that were increased or decreased. Apolipoprotein A-IV and apolipoprotein A-V were found increased in acute-phase HDL, whereas apolipoproteins A-II, C-I, C-II, and C-III were decreased. Because apolipoprotein C are also present in very low-density lipoprotein (VLDL), their levels were also investigated. I found that in acute-phase VLDL, the amount of apolipoproteins C-I, C-II, and C-III were all increased. As a consequence, there was no increase in the total amount in the plasma, suggesting a redistribution of apolipoproteins C from HDL to VLDL during the acute-phase response.

During the course of the study, I also developed an alternative method of purifying HDL from hamster plasma using hamster apo A-I immunoaffinity column chromatography. **Chapter 6** describes the development of these selected-affinity immunosorption columns and also reports their use in identification of a protein not previously known to be associated with HDL. Parotid secretory protein (PSP), one of the major salivary proteins in rodents, was identified in column-purified hamster HDL. The cDNA of the hamster homologue of rat and mouse PSP was cloned, and subsequently mouse PSP was also identified in mouse HDL. I showed that in addition to the salivary glands, PSP was also expressed in the lung, skin, testis and ovary. Only a subset of cells in the organs expresses PSP, for example, respiratory epithelium of the bronchioles, keratinocytes and sebaceous glands of the skin, Leydig's cells of the testis, and stromal

cells of the ovary. Database searches revealed that part of PSP protein contains a region homologous to an amino terminus of a family of proteins including bactericidal/permeability-increasing protein (BPI), lipopolysaccharide binding protein (LBP), cholesterol ester transfer protein (CETP), and phospholipid transfer protein (PLTP). This finding suggested that PSP might be involved in the innate immune response and/or lipid transfer. In support of this hypothesis, I found that the levels of PSP were increased in hamster HDL after endotoxin injection. PSP therefore could be considered to be one of the acute-phase proteins, the levels of which change during the acute-phase response.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