Cholesterol efflux by acute-phase high density lipoprotein: role of lecithin:cholesterol acyltransferase[1]

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Abstract 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 because of both a decrease in cholesterol efflux and an increase in cholesterol influx. 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 a 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 with 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.—Khovidhunkit, W., J. K. Shigenaga, A. H. Moser, K. R. Feingold, and C. Grunfeld. **Cholesterol efflux by acute-phase high density lipoprotein: role of lecithin:cholesterol acyltransferase.** ***J. Lipid Res.*** **2001.** 42: **967–975.**

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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 multistep 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 LCAT, allowing further removal of free cholesterol. Subsequently, cholesteryl ester can be returned to the liver by several routes.

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). Al-

Abbreviations: APR, acute-phase response; DME, Dulbecco's modified Eagle's minimum essential medium; FCS, fetal calf serum; HSA, human serum albumin; LPS, lipopolysaccharide; NEM, *N*-ethyl maleimide; RCT, reverse cholesterol transport; SAA, serum amyloid A.

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though 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 the RCT pathway decrease, including LCAT, CETP, 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 cholesteryl 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 with 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 in 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, *Escherichia coli* 055:B5) was purchased from Difco (Detroit, MI). Whatman glass microfiber filters were purchased from Fisher Scientific (Santa Clara, CA); 0.45 μm pore size 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 (Manassas, VA) and maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's minimum essential medium (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 h to inactivate serum LCAT activity.

GM 3468A normal human fibroblasts were obtained from the National Institute of General Medical Sciences 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 nonessential 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 the San Francisco Veterans Affairs Medical Center (San Francisco, CA).

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 with 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 with a Beckman (Fullerton, CA) L8-70M ultracentrifuge. HDL was extensively dialyzed against normal saline containing 0.01% EDTA, pH 7.4, filtered with a 0.45 μm pore size 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 with ethylene oxide or an autoclave followed by dry heating at 180°C for at least 4 h.

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 six 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 PBS and incubated in the medium containing 10% FCS and [4-^{14}C]cholesterol (1 μCi/ml) for 2 days. After A 2-day incubation, cells were washed with PBS and incubated with DME containing 0.3% human serum albumin (HSA) for 1–2 h for equilibration. After several washes with PBS, efflux was initiated by the addition of control or acute-phase HDL in DME containing 2.5% HSA. At various time points, an aliquot of medium was removed, centrifuged to remove cells, and counted for radioactivity. At the end of the experiments, media were collected; and cells were washed, scraped, and resuspended in deionized water. Cell suspension was sonicated for 15 s 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 medium, and cholesterol influx was measured by the disappearance of [1,2-^{3}H]cholesterol from the medium 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 medium divided by the total (medium plus 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. In addition, in some experiments, the amount of cholesterol influx was calculated by multiplying the 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 by the exogenous assay as previously described (12). Artificial substrate liposomes consisting of human apolipoprotein A-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 was 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 2-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 of ethanol. The lipids were extracted twice with 4 ml of hexane containing 50 μg of unlabeled cholesterol and cholesterol oleate as carriers. The extract was dried under nitrogen and redissolved in chloroform. The lipids were then separated by TLC, using a hexane–ethyl acetate 9:1 (v/v) solvent system. The cholesteryl ester and free cholesterol were identified and scraped off, and the radioactivities were measured by liquid scintillation counting. LCAT activity was determined from the conversion of free cholesterol to cholesteryl ester, and expressed as fractional cholesterol esterification rate (percent esterified per hour) and molar esterification rate (nanomoles esterified per milliliter per hour).

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

Statistics

The results are presented as means ± SEM. Significance was determined by 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 16 h 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 with 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 five 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 (data not shown).

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** and **B**). At various HDL concentrations, cholesterol efflux mediated by acute-phase HDL was significantly 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. 2**). Because our protocol did not preload cells with cholesterol-rich medium, 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 cholesteryl ester content of cells (data not shown).

LCAT activity of acute-phase HDL

LCAT is a plasma enzyme that catalyzes the formation of cholesteryl ester from free cholesterol. It is found associated with HDL and is thought to play a key role in cho-

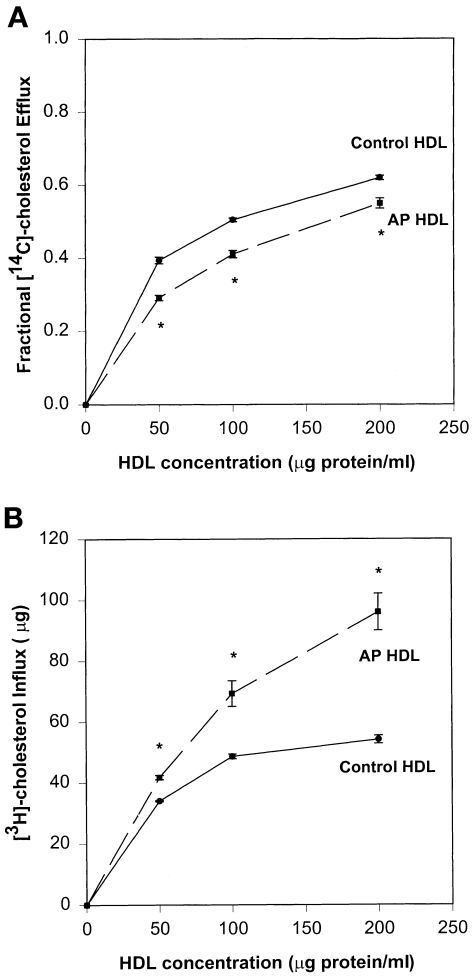

Fig. 1. Dose-response curve of cholesterol efflux (A) and cholesterol influx (B) by control HDL and acute-phase HDL in J 774 cells. J 774 cells were labeled with [4-^{14}C]cholesterol and HDL was labeled with [1,2-^{3}H]cholesterol. Cholesterol flux was performed as described in Materials and Methods for 24 h. Fractional cholesterol flux was calculated as the amount of radioactivity present in the medium (efflux) or cells (influx) divided by the total (medium plus cell) radioactivity in each well. In addition, 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 HDL at 200 μg/ml, 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 with control HDL.

lesterol 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/h whereas that of the acute-phase HDL was 5.61 ± 1.55% esterified/h (**Fig. 3**). Similarly, the molar esterification rate of the control HDL was 247.5 ± 1.7 nmol esterified/ml/h and that of the acute-phase HDL was 68.19 ± 18.7 nmol esterified/ml/h. Lower LCAT activity in acute-phase HDL could potentially limit the cho-

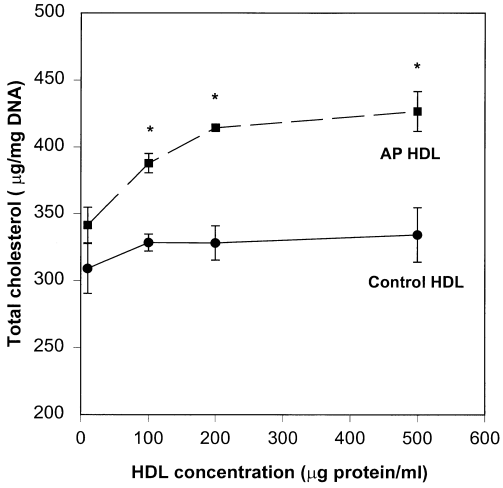

Fig. 2. Total cholesterol mass (μg/mg DNA) of J 774 cells 24 h after the addition of HDL. Cells were washed with PBS, scraped into deionized water, sonicated for 15 s, and assayed for total cholesterol and DNA content as described in Materials and Methods. * $P < 0.025$ compared with control HDL.

lesterol removal from cells. To study the role of decreased LCAT activity of acute-phase HDL in cholesterol removal, we used NEM, a known LCAT inhibitor (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 cholesteryl 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

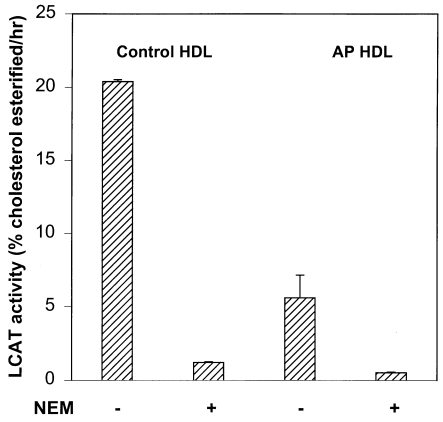

Fig. 3. LCAT activity of HDL preparations. Control and acute-phase HDL were isolated from hamster plasma 16 h after injection with normal saline or LPS, respectively. NEM was used to inactivate plasma and HDL LCAT activity. Five or 10 mM NEM in the plasma produced a similar degree of inhibition of LCAT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

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** and **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 with 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 and B). Inactivation of LCAT activity in acute-phase HDL resulted in a small (<5%) change in cholesterol influx, but not in cholesterol efflux, compared with those of acute-phase HDL with intact LCAT activity (Fig. 4A and B).

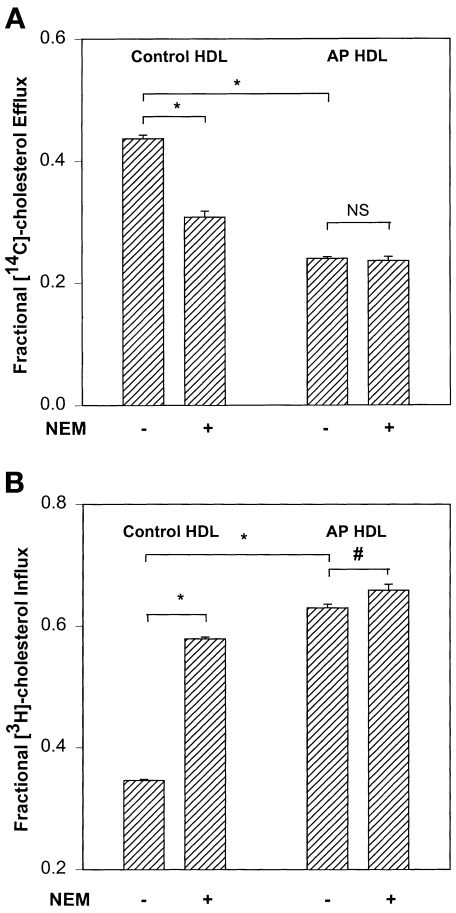

Fig. 4. Cholesterol efflux (A) and cholesterol influx (B) of J 774 cells 24 h after addition of various HDL preparations (100 μg/ml). Cholesterol flux was performed as described in Materials and Methods, and fractional cholesterol flux was calculated as the amount of radioactivity present in the medium (efflux) or cells (influx) divided by the total (medium plus 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.

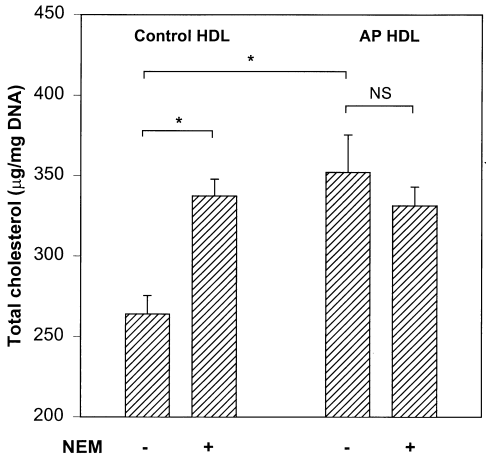

Fig. 5. Total cholesterol mass of J 774 cells 24 h after incubation with various HDL preparations (100 μg/ml). * $P < 0.05$; NS, nonsignificant.

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 in 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 in cells treated 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 that of cells treated 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 with those incubated with control HDL at each HDL concentration (Fig. 6B). The increase in

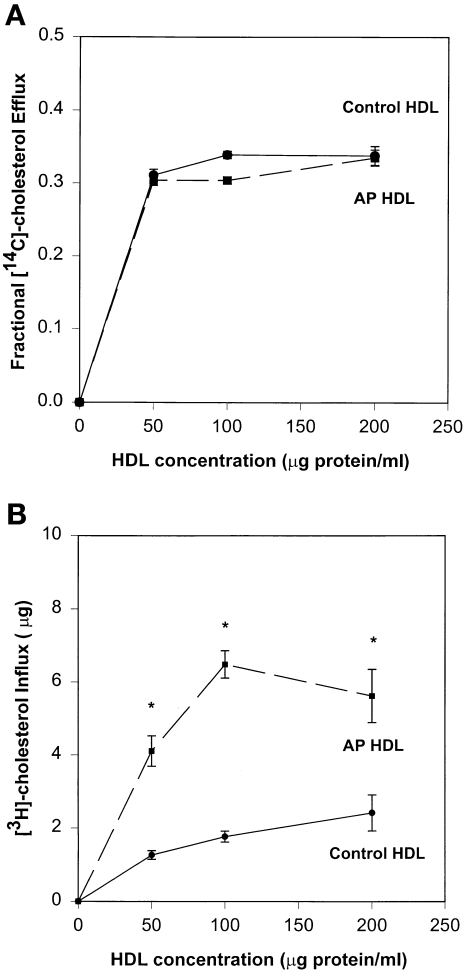

Fig. 6. Dose-response curve of cholesterol efflux (A) and cholesterol influx (B) by control HDL and acute-phase HDL in GM 3468A cells. GM 3468A cells were labeled with [4-^{14}C]cholesterol and HDL was labeled with [1,2-^{3}H]cholesterol. Cholesterol flux was performed as described in Materials and Methods for 48 h. Fractional cholesterol flux was calculated as the amount of radioactivity present in the medium (efflux) or cells (influx) divided by the total (medium plus cell) radioactivity in each well. In addition, 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 HDL at 200 μg/ml, was 1,260 ± 6 cpm/well for control HDL and 1,182 ± 37 cpm/well for acute-phase HDL. * $P <$ 0.025 compared with control HDL.

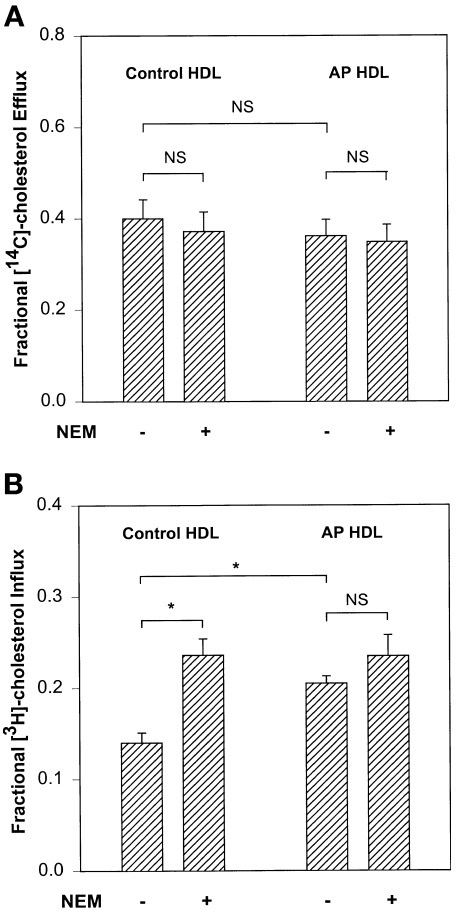

Fig. 7. Cholesterol efflux (A) and cholesterol influx (B) of GM 3468A cells 48 h after addition of various HDL preparations (100 μg/ml). Cholesterol flux was performed as described in Materials and Methods, and fractional cholesterol flux was calculated as the amount of radioactivity present in the medium (efflux) or cells (influx) divided by the total (medium plus 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.

cholesterol influx without significant change in cholesterol efflux indicates that net efflux from fibroblasts is lower with acute-phase HDL compared with 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 with 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 choles-

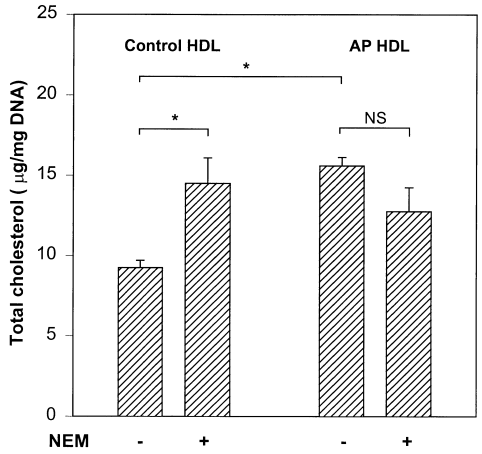

Fig. 8. Total cholesterol mass of GM 3468A cells 48 h after incubation with various HDL preparations (100 μg/ml). * $P < 0.05$; NS, nonsignificant.

terol flux experiments. Total cholesterol content of cells incubated with control HDL was lower than that of cells incubated 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).

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 pre-β-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 cassette 1, 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 cholesteryl 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 with 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 the 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 J 774 macrophages showing that LCAT promotes HDL-mediated cholesterol efflux as well as prevents cholesterol influx are similar to the erythrocyte study results. 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, for example, LCAT does not increase cholesterol efflux from cells. On the other hand, in cells in which the cholesterol exchange rate is high, cholesterol esterification plays an important role and thus LCAT can increase cholesterol efflux from cells (42).

One 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 were 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 an amount of 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 RCT 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 (48). The data presented here provide further support for the hypothesis that acute-phase HDL becomes proatherogenic by decreasing cholesterol removal from cells through 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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