Scavenger Receptor-BI Inhibits ATP-binding Cassette Transporter 1- mediated Cholesterol Efflux in Macrophages*

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Scavenger receptor BI (SR-BI) facilitates the efflux of cellular cholesterol to plasma high density lipoprotein (HDL). Recently, the ATP-binding cassette transporter 1 (ABC1) was identified as a key mediator of cholesterol efflux to apolipoproteins and HDL. The goal of the present study was to determine a possible interaction between the SR-BI and ABC1 cholesterol efflux pathways in macrophages. Free cholesterol efflux to HDL was increased (∼2.2-fold) in SR-BI transfected RAW macrophages in association with increased SR-BI protein levels. Treatment of macrophages with 8-bromo-cAMP (cAMP) resulted in a 4.1-fold increase in ABC1 mRNA level and also increased cholesterol efflux to HDL (2.2-fold) and apoA-I (5.5-fold). However, in SR-BI transfected RAW cells, cAMP treatment produced a much smaller increment in cholesterol efflux to HDL (1.1-fold) or apoA-I (3.3-fold) compared with control cells. In macrophages loaded with cholesterol by acetyl-LDL treatment, SR-BI overexpression did not increase cholesterol efflux to HDL but did inhibit cAMP-mediated cholesterol efflux to apoA-I or HDL. SR-BI neutralizing antibody led to a dose- and time-dependent increase of cAMP-mediated cholesterol efflux in both SR-BI transfected and control cells, indicating that SR-BI inhibits ABC1-mediated cholesterol efflux even at low SR-BI expression level. Transfection of a murine ABC1 cDNA into 293 cells led to a 2.3-fold increase of cholesterol efflux to apoA-I, whereas co-transfection of SR-BI with ABC1 blocked this increase in cholesterol efflux. SR-BI and ABC1 appear to have distinct and competing roles in mediating cholesterol flux between HDL and macrophages. In nonpolarized cells, SR-BI promotes the uptake of cholesterol actively effluxed by ABC1, creating a futile cycle.

Cholesterol efflux is the first step of reverse cholesterol transport, a process by which peripheral cells remove and deliver excess cholesterol to liver for secretion into bile or conversion into bile acids (1). Foam cells, resulting from the accumulation of excess cholesterol in arterial macrophages, play an important role in fatty streak formation and also in the formation of complex lesions and plaque rupture (2). Stimulation of arterial macrophage cholesterol efflux by high density lipoprotein (HDL)3 or apolipoprotein A-I (apoA-I) can inhibit foam cell formation.

It is generally accepted that there are at least two pathways for peripheral cells to remove excess cholesterol. The first one, proposed by Rothblat et al. (3), is a passive aqueous diffusion pathway. Cholesterol desorbs from the plasma membrane pool to extracellular phospholipid containing acceptor particles, for example, HDL, via a concentration gradient between membrane and acceptor (4, 5). Ji et al. (6) showed that scavenger receptor B type I (SR-BI) stimulated cholesterol efflux from SR-BI transfected Chinese hamster ovary cells and that the efflux rate was correlated with SR-BI expression levels in various cell lines. Subsequent experiments have suggested that the ability of SR-BI to stimulate cholesterol efflux is independent of binding of ligand to receptor and may reflect reorganization of membrane cholesterol domains, facilitating aqueous diffusion of cholesterol to HDL (7, 8). The second pathway, involves the transport of cholesterol to extracellular lipid-free apolipoprotein acceptors, such as apoA-I and apolipoprotein E (9, 10). Smith et al. (11) found that treatment of mouse macrophage RAW cells with 8-bromo-cAMP (cAMP) stimulated cholesterol efflux to apoA-I and apolipoprotein E. They proposed a model in which cAMP induces expression of a membrane receptor for apolipoproteins that transfers both cholesterol and phospholipid to the apolipoprotein acceptors and results in the net efflux of cholesterol from cells (11).

Tangier disease is a rare genetic disorder of lipid metabolism. It is characterized by near or complete absence of circulating HDL and deposition of cholesterol esters in the reticuloendothelial system. A defect in apolipoprotein-mediated cholesterol and phospholipid removal has been demonstrated in Tangier disease fibroblasts (12–14). Recently, The ATP-binding cassette transporter 1 gene (ABC1) was shown to be defective in Tangier disease (15–18), and the ABC1 transporter was shown to mediate cholesterol efflux from cells to apoA-I or HDL (19, 20). In addition, the cAMP induction of cholesterol efflux was shown to be correlated with the up-regulation of ABC1 expression (19).

The current study was undertaken to determine a possible interaction between the SR-BI- and ABC1-mediated cholesterol efflux pathways in macrophages. We initially hypothesized that SR-BI and ABC1 might act synergistically on cholesterol efflux, because the nascent HDL particles formed from the ABC1-mediated cholesterol efflux to apoA-I might serve as an acceptor for SR-BI-mediated cholesterol efflux. To assess this

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¶ The abbreviations used are: HDL, high density lipoprotein; ABC1, ATP-binding cassette transport 1; AcLDL, acetylated-low density lipoprotein; apoA-I, apolipoprotein A-I; cAMP, 8-bromo-cyclic AMP; SR-BI, Scavenger receptor class B type I; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PIPES, 1,4-piperazine-diehtanesulfonic acid; BSA, bovine serum albumin.

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hypothesis, we stably transfected mouse macrophage RAW 264 cells with murine SR-BI cDNA and treated the cells with cAMP to induce ABC1 mRNA level. However, contrary to our expectation, we found that SR-BI inhibited ABC1-mediated cholesterol efflux. It appears that SR-BI promotes cellular reuptake of the cholesterol that initially undergoes ABC1-mediated cholesterol efflux to apoA-I, suggesting movement of cholesterol back down a concentration gradient initially created by ABC1.

**EXPERIMENTAL PROCEDURES**

**Stable Transfection of RAW 264 Cells with Murine SR-BI**—The murine SR-BI cDNA was subcloned into a mammalian expression vector pRc/CMV (Invitrogen) and transfected into RAW 264.7 cells (American Type Culture Collection) using the Effectene Transfection Reagent kit from Qiagen (Valencia, CA). Stable transformants were selected with 0.8 mg/ml of G418 (Life Technologies, Inc.) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Inc.). Individual colonies were picked and expanded for analysis of SR-BI expression. Three cell lines with different expression level of SR-BI were used in the present study. Their expression level was stable during the experimental period as monitored by densitometry analysis.

**Immunoblot Analysis of SR-BI**—For immunoblot analysis of SR-BI, RAW cells were washed and scrapped in phosphate-buffered saline and lysed in 10 mM Tris-HCl, pH 7.3, 1 mM MgCl2, and 0.5% Nonidet P-40 in the presence of protease inhibitors (0.5 µg/ml Leupeptin, 1 µg/ml aprotinin, and 1 µg/ml pepstatin A (Roche Molecular Biochemicals). Postnuclear supernatants from cell lysates were prepared by centrifugation at 3000 × g for 10 min at 4 °C. Samples containing the indicated amounts of protein were reduced with 2-mercaptoethanol in gel loading buffer, fractionated by 7.5% SDS-polyacrylamide gel electrophoresis, and transferred to 0.22-µm nitrocellulose membranes. Immunoblotting was performed using an anti-SR-BI antiserum (Novus, Littleton, CO) and ECL chemiluminescence detection (Amerham Pharmacia Biotech). The relative intensities of the bands were determined by densitometry (Molecular Dynamics, model 300A).

**Ribonuclease Protection Assay**—Reverse transcription–polymerase chain reaction was used to obtain a fragment of the murine ABC1 cDNA. Murine ABC1 and β-actin antisense riboprobes were prepared by *in vitro* transcription using murine ABC1 and β-actin cDNA plasmid constructs. The protected hybrid fragments for ABC1 and β-actin were 290 and 160 base pairs, respectively. In brief, 20 µg of total RNA were hybridized with 10 × 106 cpm ABC1 and β-actin riboprobes at 48 °C overnight in 30 µl of a buffer consisting of 40 mM PIPEs, pH 6.0, 400 mM NaCl, 1 mM EDTA, and 80% formamide. The hybridization mixture was digested with 20 units of T2 ribonuclease (Life Technologies, Inc.) at 37 °C for 1 h, extracted with phenol/chloroform, precipitated with ethanol, and dissolved in 5 µl RNA loading buffer. The protected RNA hybrid-form protect fragment was resolved on a 6% polyacrylamide/urea gel and subjected to autoradiography.

**[3H]Cholesterol Efflux Study**—Cells were labeled with [3H]cholesterol by two procedures: cholesterol-normal and cholesterol-loaded conditions. With the cholesterol-normal condition, cell monolayers in 24-well plate were cholesterol-labeled with 0.5 µCi/ml [1,2-3H(N)]cholesterol (NEC Life Science Products) in 0.5 ml of DMEM supplemented with 10% FBS. Following 24 h of labeling, cells were equilibrated overnight in DMEM containing 0.2% fatty acid-free bovine serum albumin (BSA) (Sigma) with or without 0.3 mM cAMP (Sigma). After the labeling and equilibrium procedures, the specific activity of the cholesterol pool was similar for control and SR-BI-transfected cells ([3H]cholesterol cpm/µg of total cellular cholesterol: control cells with and without cAMP treatment: 4747 ± 258 and 4853 ± 111; SR-BI-transfected cells with and without cAMP treatment: 4892 ± 558 and 4909 ± 193). With the cholesterol-loaded condition, cells were labeled overnight by 0.5 µCi/ml [1,2-3H(N)]cholesterol in 0.5 ml of 0.2% BSA and DMEM supplemented with 50 µg/ml acetylated low density lipoprotein (AcLDL) and then equilibrated in 0.2% BSA and DMEM. After the labeling and equilibration procedures, the specific activity of the cholesterol pool was also similar for control and SR-BI-transfected cells ([3H]cholesterol cpm/µg of total cellular cholesterol: control cells with and without cAMP treatment: 19483 ± 420 and 18596 ± 226; SR-BI-transfected cells with and without cAMP treatment: 21307 ± 505 and 19548 ± 426). Following two washes with phosphate-buffered saline and 0.2% BSA, cells were incubated by 150 µg/ml human HDL or 10 µg/ml purified human apoA-I (PerImmune) in 0.5 ml of 0.2% BSA and DMEM with or without 0.5 mM cAMP. At different time points, 55 µl of medium was taken, and after precipitation at 6000 × g for 10 min to remove cell debris and cholesterol crystall, radioactivity in a 50-µl aliquot of supernant was determined by liquid scintillation counting. The cells were finally lysed in 0.5 ml of 0.1 M sodium hydroxide and 0.1% SDS, and the radioactivity in an aliquot was determined. Cholesterol efflux was expressed as the percentage of the radioactivity released from cells into the medium relative to the total radioactivity in cells and medium.

**Cholesterol Mass Analysis**—Cells in 6-well plates were [3H]cholesterol-labeled and treated with or without cAMP as described above. After 6 h of incubation with apoA-I, media were collected, and cells were washed twice in phosphate-buffered saline and 0.2% BSA and incubated with 150 µg/ml human HDL or 10 µg/ml apoA-I in 0.5 ml of 0.2% BSA and DMEM with or without 0.3 mM cAMP. At different time points, 55 µl of medium was taken and precipitated at 6000 × g for 10 min to remove cell debris. Then [3H]phospholipid in a 50-µl aliquot of supernant was first extracted by chloroform:methanol (2:1), and then the radioactivity determined by scintillation counting. The cells were finally lysed in 0.5 ml of 0.1 M sodium hydroxide, 0.1% SDS, and the radioactivity in an aliquot after lipid extraction was determined. The percentage of secreted [3H]phospholipid was calculated by dividing the medium-derived counts by the sum of the total.

**Cholesterol Efflux in ABC1 and SR-BI Transiently Transfected 293 Cells**—The murine ABC1 cDNA was cloned into a mammalian expression vector pCDNA 3.1 (Invitrogen). The cDNA contains an additional N-terminal 60 amino acids (21), compared with the earlier reported cDNA sequences (22). Transient transfections of ABC1 alone, SR-BI alone, and ABC1+SR-BI (co-transfection) were performed in 24-well plates using LipofectAMINE reagent according to manufacturer's instructions (Life Technologies, Inc.). 5 h after transfection, media were changed to containing 0.2% BSA and DMEM containing 0.5 µCi/ml [1,2-3H(N)]cholesterol, and the cells were labeled for 24 h. After equilibration in 0.2% BSA and DMEM overnight, the cells were incubated with 10 µg/ml apoA-I for 6 h in 0.2% BSA and DMEM. Cholesterol efflux was measured at the end of the incubation and expressed as the medium [3H]cholesterol radioactivity as a percentage of total [3H]cholesterol radioactivity (cells plus medium).

**Statistical Analysis**—Results are presented as the means ± S.D. Tests for the significant differences between groups were performed by Student's t test.

**RESULTS**

To compare the SR-BI and ABC1-mediated cholesterol removal pathways, we stably transfected mouse macrophage RAW 264 cells with the murine SR-BI cDNA. We also treated the cells with cAMP to induce ABC1 expression.

Three cell lines with different SR-BI expression levels were isolated and used in the experiments. Overexpression of SR-BI resulted in an up to 2.2-fold increase of cholesterol efflux to HDL in mouse macrophage RAW cells (Fig. 1). The increment of cholesterol efflux because of SR-BI increased with increasing expression levels of SR-BI, as shown in three cell lines with different expression levels (Fig. 1, A and C). In contrast, SR-BI did not stimulate cholesterol efflux to apoA-I (Fig. 1B).

Treating control RAW cells with cAMP induced cholesterol efflux both to HDL and apoA-I during a 6-h incubation period. At the 6-h point, efflux to HDL was increased 2.2-fold, whereas efflux to apoA-I was increased 5.5-fold by cAMP treatment (data not shown). These results were similar to the earlier report of Smith et al. (11). Fig. 2A shows that cAMP induced 3–4-fold increase of ABC1 mRNA expression in both mock vector transfected (mk) and SR-BI-transfected cells (SR2), as determined by RNase protection assay.

The combined effects of SR-BI and cAMP on cholesterol efflux were next examined. Surprisingly, cAMP treatment failed to increase cholesterol efflux to HDL in SR-BI-transfected cells (Fig. 2B), whereas in vector control cells in the same
experiment, the efflux was increased 2.2-fold. After cAMP treatment, efflux to apoA-I was increased 3.3-fold in SR-BI transfected cells, compared with a 5.5-fold increase in control cells (Fig. 2C). The specific activity of [3H]cholesterol was similar in control and SR-BI transfected cells (see “Experimental Procedures”) and could not account for these differences. Moreover, measurement of free cholesterol mass in the medium confirmed the inhibition of cAMP-mediated efflux by SR-BI expression (Fig. 3A). Thus SR-BI overexpression inhibited ABC1-mediated cholesterol efflux to HDL and apoA-I.

Both HDL and apoA-I can bind to SR-BI (23, 24), and this binding might lead to a lower amount of acceptor available for the ABC1 pathway in SR-BI transfected cells, causing decreased cholesterol efflux. To check this possibility, an efflux experiment was conducted using higher concentrations of apoA-I. No further obvious increment of cholesterol efflux was found when 4-fold higher concentration of apoA-I was used as acceptor in cAMP-treated cells (Fig. 4), suggesting that competition for acceptor was not the underlying mechanism.

The effect of SR-BI and cAMP treatment on phospholipid efflux was also examined. As shown in Fig. 5, cAMP stimulated phospholipid efflux to HDL and apoA-I, whereas SR-BI overexpression had no such effect. Importantly, SR-BI overexpression did not inhibit the induction of phospholipid efflux by cAMP. This indicates a specific inhibition of the active cholesterol efflux pathway by SR-BI.

Cholesterol efflux to HDL and apoA-I in three cell lines with different SR-BI expression levels is shown in Fig. 6. With increasing SR-BI expression levels (SR3 > SR2 > SR1; see Fig. 1), the increment in cholesterol efflux to HDL induced by cAMP was progressively smaller (Fig. 6A). The total efflux after cAMP induction was similar in the three cell lines. In contrast, total cholesterol efflux to apoA-I (Fig. 6B) was approximately inversely related to the expression level of SR-BI in the three lines treated with cAMP.

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SR-BI Inhibits ABC1-mediated Cholesterol Efflux

Control and SR-BI transfected RAW cells were \(^{3}H\)cholesterol-labeled in 10% FBS and DMEM as described under "Experimental Procedures." After equilibration in 0.2% BSA and DMEM, with or without 0.3 mM cAMP overnight, the cells were incubated with 10 or 40 \(\mu\)g/ml of apoA-I for 6 h in 0.2% BSA and DMEM. Cholesterol efflux was expressed as the medium \(^{3}H\)cholesterol radioactivity as a percentage of total \(^{3}H\)cholesterol radioactivity (cells plus medium). Representative data are from one of two independent experiments. Values are the means \(\pm\) S.D. (\(n = 3\)).

Control and SR-BI transfected cells were \(^{3}H\)cholesterol-labeled (1 \(\mu\)Ci/ml) and treated with or without cAMP as described under "Experimental Procedures." \(^{3}H\)Phospholipid was first extracted by chloroform:methanol (2:1), and the radioactivity was determined by scintillation counting.

Three cell lines with different SR-BI expression level.

Cholesterol efflux was expressed as the medium \(^{3}H\)cholesterol radioactivity as a percentage of total \(^{3}H\)cholesterol radioactivity (cells plus medium). Representative data are from one of two independent experiments. Values are the means \(\pm\) S.D. (\(n = 3\)).

SR-BI Inhibits ABC1-mediated Cholesterol Efflux

Cells were \(^{3}H\)cholesterol-labeled and cholesterol-loaded by 50 \(\mu\)g/ml AcLDL as described under "Experimental Procedures." After equilibration in 0.2% BSA and DMEM, with or without 0.3 mM cAMP overnight, the cells were incubated with 150 \(\mu\)g/ml HDL (A) or 10 \(\mu\)g/ml apoA-I (B) for 6 h in 0.2% BSA and DMEM. Cholesterol efflux was expressed as the medium \(^{3}H\)cholesterol radioactivity as a percentage of total \(^{3}H\)cholesterol radioactivity (cells plus medium). Values are the means \(\pm\) S.D. (\(n = 3\)).

Because cAMP treatment might stimulate cholesterol efflux by several different mechanisms, we carried out further experiments to directly examine the role of SR-BI on ABC1-mediated cholesterol efflux. Thus, 293 cells were transfected with ABC1 alone, SR-BI alone, and ABC1 plus SR-BI (co-transfection) (Fig. 8). ABC1 transfection led to a 2.3-fold increase of cholesterol efflux to apoA-I. SR-BI transfection had no effect on cholesterol efflux to apoA-I. The decreased efflux to apoA-I in cells expressing SR-BI was also confirmed by direct mass measurement (Fig. 3B). The data indicate that when cells are cholesterol-loaded, SR-BI does not increase efflux from intracellular cholesterol stores but does inhibit the ABC1-mediated cholesterol efflux.

To examine whether the inhibiting effect of SR-BI on ABC1-mediated cholesterol efflux could be reversed by an SR-BI neutralizing antibody, we used an antibody to the extracellular domain of SR-BI,\(^{2}\) which inhibits selective uptake of HDL cholesterol ester (25). First, SR-BI transfected and control RAW cells were \(^{3}H\)cholesterol-labeled and incubated with cAMP to induce ABC1. Then they were incubated with apoA-I plus different concentrations of SR-BI neutralizing antibody, and cellular cholesterol efflux was measured after 6 h of incubation. Fig. 9 shows that the SR-BI neutralizing antibody treatment resulted in a dose-dependent and time-dependent in-

\(^{2}\) From Novus, Littleton, CO, whole rabbit serum.
SR-BI Inhibits ABC1-mediated Cholesterol Efflux

Fig. 8. Cholesterol efflux to apoA-I in ABC1 and SR-BI transiently transfected 293 cells. 293 cells were transiently transfected with ABC1 alone, SR-BI alone, and ABC1 plus SR-BI (co-transfection). 5 h after transfection, cells were \[^3\text{H}\]cholesterol-labeled in 10% FBS and DMEM for 24 h. After equilibration in 0.2% BSA and DMEM overnight, the cells were incubated with 10 \(\mu\text{g} / \text{ml}\) apo-I for 6 h in 0.2% BSA and DMEM. Cholesterol efflux was expressed as the medium \[^3\text{H}\]cholesterol radioactivity as a percentage of total \[^3\text{H}\]cholesterol radioactivity (cells plus medium).

Fig. 9. SR-BI neutralizing antibody increases cholesterol efflux to apoA-I in cAMP-treated cells. Control (A) and SR-BI transfected (B) RAW cells were \[^3\text{H}\]cholesterol-labeled and cholesterol-loaded by 50 \(\mu\text{g} / \text{ml}\) AcLDL as described under “Experimental Procedures.” After equilibration in 0.2% BSA and DMEM with 0.3 mM cAMP overnight, different concentrations of SR-BI neutralizing antibody were added, \(10 \text{mg} / \text{ml}\) AcLDL was added for 4 h, and the cells were incubated for 6 h. Efflux was measured during a subsequent 6 h incubation period. Representative data are from one of two independent experiments. Values are the means \(\pm\) S.D. (n = 3).

Fig. 10. SR-BI neutralizing antibody does not inhibit cholesterol efflux in cells not treated with cAMP. Control and SR-BI transfected RAW cells were \[^3\text{H}\]cholesterol-labeled as described under “Experimental Procedures.” After equilibration in 0.2% BSA and DMEM with 0.3 mM cAMP overnight, SR-BI neutralizing antibody (1:50 dilution) was added as well as 150 \(\mu\text{g} / \text{ml}\) HDL and the cells were incubated for 6 h. Efflux was measured at the end of this period. Values are the means \(\pm\) S.D. (n = 3).

Stimulation of free cholesterol efflux by SR-BI is independent of HDL binding to SR-BI (7, 8). These findings suggest that the stimulation of cholesterol efflux by the antibody (Fig. 9) is related to the antibody blocking reuptake of nascent cholesterol effluxed via ABC1.

To directly examine this futile cycle model, ABC1 transfected 293 cells were \[^3\text{H}\]cholesterol-labeled and incubated with apo-I for 4 h. Then the whole media were collected and concentrated to isolate nascent HDL particles formed from ABC1-mediated cholesterol efflux. Subsequently, the whole concentrated media were placed onto SR-BI transfected and control cells to measure cholesterol reuptake during 1 h of incubation. Fig. 11 showed that the reuptake of cholesterol from nascent HDL particles in SR-BI transfected cells was 27% higher than that in control cells. Similar results were obtained when the nascent particles were isolated from control RAW cells treated with cAMP. The data indicate that the reuptake of the nascent HDL cholesterol at least partly accounts for the inhibition of ABC1-mediated cholesterol efflux by SR-BI.

Discussion

The goal of the present study was to test the hypothesis that SR-BI and ABC1 might have synergistic roles in promoting cholesterol efflux from macrophages. Overexpression of SR-BI in RAW macrophages did result in increased cholesterol efflux to HDL; under basal conditions, however, in agreement with earlier findings (6, 7, 28), SR-BI did not increase cholesterol efflux to apoA-I. In cholesterol-loaded cells, SR-BI facilitated efflux neither to HDL nor to apoA-I. Unexpectedly, SR-BI markedly inhibited the increase in cholesterol efflux to both HDL and apoA-I attributable to ABC1 when cells were treated with cAMP. These results suggest that SR-BI and ABC1 have distinct, competitive roles in mediating cholesterol flux between HDL and macrophages.

SR-BI overexpression did facilitate cholesterol efflux to HDL and the magnitude of the effect increased as cellular SR-BI expression was increased (Fig. 1). These results are similar to those obtained by transfection of Chinese hamster ovary (6) and COS cells (7) with SR-BI. These earlier studies indicated that SR-BI facilitates cholesterol efflux to both HDL and phosphatidylcholine vesicles but not to apoA-I (6, 7, 28). Because phosphatidylcholine vesicles bind poorly to SR-BI (29), the enhancement of cholesterol efflux does not require binding to SR-BI. A dissociation of efflux from binding is also indicated by the finding that CD36 can bind HDL but it does not facilitate cholesterol efflux (7). The stimulation of cholesterol efflux by SR-BI could result from a reorganization of plasma membrane cholesterol that leads to a lowering of the activation energy for...
desorption of cholesterol from the cellular plasma membrane, i.e. SR-BI might facilitate efflux by the aqueous diffusion pathway (7, 8). SR-BI appears to be localized in cholesterol-rich regions of the plasma membrane, such as caveolae (30–32), and SR-BI increases the cholesterol oxidase sensitive, possibly caveolar pool of plasma membrane cholesterol (8). Consistent with a local effect in the plasma membrane, SR-BI does not facilitate efflux when intracellular cholesterol stores are increased (Fig. 7). When the cells are cholesterol-loaded by AcLDL, the AcLDL particles are taken by scavenger receptor A and stored in an intracellular pool that appears to be inaccessible to SR-BI. In contrast to the SR-BI-mediated mechanism of cholesterol efflux, ABC1 is thought to actively mediate cholesterol and phospholipid efflux onto apoA-I or HDL utilizing both plasma membrane and intracellular sources. Like a typical ABC transporter, the multidrug resistance P-glycoprotein ABC1 might form a pore that is open to the outside of the cell (33). Cholesterol might be translocated from intracellular sites onto apoA-I or small HDL sitting in the ABC1 pore.

The major novel finding was that SR-BI markedly inhibits ABC1-mediated cholesterol efflux to both HDL and apoA-I. In three different cell lines, the degree of inhibition was roughly proportionate to SR-BI expression levels. In control RAW cells treated with cAMP, an SR-BI neutralizing antibody increased cholesterol efflux to apoA-I, suggesting that SR-BI inhibits ABC1-mediated cholesterol efflux even at physiological expression levels. Control RAW cells express appreciable amounts of SR-BI, comparable to that found in fresh human monocyte-derived macrophages.3 SR-BI overexpression reduced the increment in cholesterol efflux attributable to ABC1 but did not decrease total cholesterol efflux to HDL (Fig. 6). Thus, SR-BI might play a role in facilitating cholesterol efflux to HDL, under basal conditions, i.e. when macrophages are not yet cholesterol-loaded and ABC1 is not up-regulated or when ABC1 is down-regulated. Such conditions could perhaps prevail early after entry of macrophages into the arterial wall (34) or perhaps in lesion with an active inflammatory component (35). However, when macrophages are cholesterol-loaded, ABC1 is up-regulated (19), and SR-BI inhibits cholesterol efflux to HDL. Moreover, SR-BI inhibits total cholesterol efflux to apoA-I under all conditions examined. These findings suggest that SR-BI promotes uptake or retention of cholesterol in a specific cellular cholesterol pool, opposing the ABC1 cholesterol efflux pathway.

Our experiments provide some insight into the potential mechanisms by which SR-BI expression inhibits ABC1-mediated cholesterol efflux in macrophages. This was a direct result of the inhibition of ABC1-mediated cholesterol efflux by SR-BI, as shown in the experiment where SR-BI and ABC1 were co-transfected in 293 cells (Fig. 8). SR-BI overexpression did not affect ABC1 mRNA (Fig. 2A). It is possible that ABC1 protein expression was decreased as a result of SR-BI overexpression. However, the finding that SR-BI specifically inhibited cholesterol efflux but did not inhibit phospholipid efflux caused by ABC1 up-regulation (Fig. 5) is inconsistent with this or other general mechanisms involving ABC1 expression or function. For the same reason, competition between ABC1 and SR-BI for binding efflux substrate (apoA-I or HDL) appears unlikely. Moreover, inhibition was observed with high concentrations of HDL (150 µg/ml) and apoA-I (40 µg/ml) in medium, when competition for substrate should not be a major factor (Fig. 4). It is unlikely that inhibition simply reflected an approach to equilibrium at high cholesterol efflux levels, because the time course of efflux showed that inhibition was active at the earliest time points measured (Fig. 2).

Strikingly SR-BI neutralizing antibody reversed the inhibition on ABC1-mediated cholesterol efflux by SR-BI, and this effect was observed even in control cells with low SR-BI expression level (Fig. 9). This SR-BI neutralizing antibody inhibits HDL cholesterol ester-selective uptake and cell association of HDL particles (25). However, the antibody does not inhibit SR-BI-mediated cholesterol efflux (Fig. 10). Based on this observation, a futile cycle mechanism is proposed. In such a model, ABC1 would pump cholesterol and phospholipid onto apoA-I to form nascent HDL particles, and the SR-BI would facilitate movement of cholesterol (but not phospholipid) from the nascent HDL back into the cell. This model explains why the SR-BI antibody is so effective at stimulating ABC1-mediated cholesterol efflux (Fig. 9), i.e. it blocks reuptake of newly effluxed cholesterol back into the cell. To assess this futile cycle mechanism, nascent HDL particles formed from ABC1-lipidated apoA-I was isolated and then placed back onto control and SR-BI transfected cells. A 27% increase of cholesterol uptake was observed in SR-BI transfected cells compared with control cells (Fig. 11). This represents a minimal estimate for reuptake, because SR-BI is expressed in 293 cells and was likely mediating reuptake from the nascent HDL during the first phase of the experiment.

Although SR-BI catalyzes a bi-directional flux of cholesterol across cellular plasma membranes (7, 8), the present findings emphasize the role of SR-BI in mediating cellular cholesterol retention. In vivo, SR-BI is highly expressed in steroidogenic tissues and liver (36, 37), where the major role appears to be in the uptake of both cholesterol ester and free cholesterol for steriogenesis or excretion into bile, respectively (38–40). Likewise, in the macrophage, it appears that the predominant effect of SR-BI may be to mediate cholesterol retention. It might be important for macrophages and other cells to retain a minimum level of cholesterol in plasma membranes, particularly under conditions where ABC1-mediated cholesterol efflux is active. Recently, macrophages have been shown to have the ability to selectively remove free cholesterol and cholesterol ester from aggregated LDL (41). It is even possible that macrophage SR-BI expression could under certain conditions promote foam cell formation. SR-BI overexpression in liver is anti-atherogenic (42–44), and the global knock-out of SR-BI is pro-atherogenic (45, 46). The present study suggests that these findings may be primarily influenced by the role of SR-BI in the liver rather than in macrophages. An important caveat to the interpretation of these experiments is that they were conducted

3 Y. Sun and A. Tall, unpublished results.
in nonpolarized cells. In polarized cells, such as hepatocytes, it is possible that SR-BI and ABC1 could be expressed on different sides of the cell, and together might promote movement of cholesterol across the cell.

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