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Induction of Scavenger Receptor Class B Type I Is Critical for Simvastatin Enhancement of High-Density Lipoprotein-Induced Anti-Inflammatory Actions in Endothelial Cells

Takao Kimura,2* Chihiro Mogi,2* Hideaki Tomura,∗ Atsushi Kuwabara,∗† Doon-Soon Im,† Koichi Sato,* Hitoshi Kurose,§ Masami Murakami,† and Fumikazu Okajima3*

Changes in plasma lipoprotein profiles, especially low levels of high-density lipoprotein (HDL), are a common biomarker for several inflammatory and immune diseases, including atherosclerosis and rheumatoid arthritis. We examined the effect of simvastatin on HDL-induced anti-inflammatory actions. HDL and sphingosine 1-phosphate (SIP), a bioactive lipid component of the lipoprotein, inhibited TNF-α-induced expression of VCAM-1, which was associated with NO synthase (NOS) activation, in human umbilical venous endothelial cells. The HDL- but not SIP-induced anti-inflammatory actions were enhanced by a prior treatment of the cells with simvastatin in a manner sensitive to mevalonic acid. Simvastatin stimulated the expression of scavenger receptor class B type I (SR-BI) and endothelial NOS. As for SIP receptors, however, the statin inhibited the expression of SIP3 receptor mRNA but caused no detectable change in SIP1 receptor expression. The reconstituted HDL, a stimulator of SR-BI, mimicked HDL actions in a simvastatin-sensitive manner. The HDL- and reconstituted HDL-induced actions were blocked by small interfering RNA specific to SR-BI regardless of simvastatin treatment. The statin-induced expression of SR-BI was attenuated by constitutively active RhoA and small interfering RNA specific to peroxisome proliferator-activated receptor-α. Administration of simvastatin in vivo stimulated endothelial SR-BI expression, which was accompanied by the inhibition of the ex vivo monocyte adhesion in aortas from TNF-α-injected mice. In conclusion, simvastatin induces endothelial SR-BI expression through a RhoA- and peroxisome proliferator-activated receptor-α-dependent mechanism, thereby enhancing the HDL-induced activation of NOS and the inhibition of adhesion molecule expression. The Journal of Immunology, 2008, 181: 7332–7340.

High levels of low-density lipoprotein (LDL)4 and low levels of high-density lipoprotein (HDL) are thought to increase the risk of atherosclerosis and its associated cardiovascular disease (1, 2). Thus, an imbalance in the concentration of plasma LDL and HDL is a critical biomarker for atherosclerosis. Changes in plasma lipid profiles, particularly low levels of HDL, are associated with several inflammatory and immune diseases in addition to atherosclerosis, including rheumatoid arthritis (3), systemic lupus erythematosus (4), Sjögren’s syndrome (5), and ankylosing spondylitis (6). In some cases of these diseases, abnormal or proinflammatory HDL is accumulated (7). Reverse cholesterol transport is known to be an important mechanism allowing HDL to prevent the accumulation of cholesterol in leukocytes and the formation of foam cells in the intima of vessel walls (1, 2). Recent studies, however, have suggested that HDL exerts a variety of anti-atherogenic or anti-inflammatory actions independent of changes in cholesterol metabolism (1, 8). Leukocyte adhesion to endothelial cells and subsequent transmigration into the subendothelial space or outside the vascular system are commonly observed at early stages of inflammatory diseases and are thought to be critical in their initiation and progression (1, 8, 9). HDL has been shown to induce the inhibition of the adhesion of leukocytes to endothelial cells and their transmigration, the inhibition of proinflammatory gene expression in endothelial cells, and the activation of endothelial NO synthase (eNOS) (1, 8, 10). Furthermore, HDL protects normal endothelial cell functions by inhibiting apoptosis and stimulating re-endothelialization after injury (10). Thus, using the drugs not only to bring the plasma lipid profile to normal levels by lowering plasma LDL cholesterol and/or increasing plasma HDL cholesterol but also to enhance the ability of HDL to stimulate cholesterol metabolism-independent anti-inflammatory actions may be an effective way to protect against and treat inflammatory diseases.

Statins, inhibitors of HMG-CoA reductase, were initially developed to lower LDL cholesterol in plasma (9, 11). Showing a very

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clear therapeutic impact, they are now widely used to treat vascular diseases (12–14). In addition to their cholesterol-lowering effect, statins exert beneficial effects on not only vascular disease but also other inflammatory conditions, including kidney diseases and diabetes (9, 12–14). One target organ of statins is the vascular endothelium. For example, statins have been reported to decrease leukocyte adhesion to endothelial cells and subsequent transmigration, decrease proinflammatory gene expression, and increase eNOS expression (9, 11). Thus, exogenous statins and endogenous HDL exert similar actions, raising the possibility that statins act in part through modulating plasma HDL levels and/or its actions. In fact, statins have been shown to slightly but significantly increase HDL and apolipoprotein A-I (apoA-I) levels in vivo (15) through the up-regulation of apoA-I synthesis (16, 17), although the effect of statins is still controversial (18, 19). In contrast, it remains unclear whether statins modulate HDL-induced anti-inflammatory actions and, if so, how.

In the present study, we characterized the effects of simvastatin on HDL-induced actions on NOS activation, monocyte adhesion, and the expression of adhesion molecule VCAM-1 in human umbilical vein endothelial cell (HUVECs). We found that simvastatin enhanced HDL-induced anti-inflammatory actions, which was associated with the stimulation of scavenger receptor class B type I (SR-BI) expression. Experiments with a reconstituted HDL (rHDL), composed of delipidated apoA-I and phosphatidylcholine, and small interfering RNA (siRNA) specific to SR-BI indicated that the induction of SR-BI expression is critical for the enhancement of HDL actions. Thus, we propose endothelial SR-BI as a key molecule by which statins exhibit pleiotropic or anti-inflammatory actions.

Materials and Methods

Materials
SIP was purchased from Cayman Chemical; 15-deoxy-D-12,14-prostaglandin J2 (MK886), 11-NO2-nitroarginine methyl ester hydrochloride (t-NAME), and D-NAME-nitroarginine methyl ester hydrochloride were from BIOMOL Research Laboratories; Abs for eNOS, PPAR-α, and β-actin were from Cell Signaling Technology; andAbs for SR-BI and PDZK1 were from Chemicon International; and Abs for SR-BI and PDZK1 were from Santa Cruz Biotechnology. Simvastatin and fenofibrate were kindly provided by Merck & Co. and Kaken Pharmaceutical, respectively. The sources of all other reagents were the same as described previously (20–22). Plasma HDL (1.063–1.21 g/ml) was separated from freshly isolated plasma of healthy volunteers by sequential ultracentrifugation as described previously (23).

The reconstituted discoidal lipoprotein particle (rHDL) was prepared as previously described (22). In brief, HDL was delipidated and then the lipid-free apolipoprotein mixture was dialyzed against 5 × 1 liter Tris-buffered saline (0.01 M Tris buffer (pH 7.4) containing 0.15 M NaCl, 0.01% (v/v) EDTA, and 0.02% (v/v) Na3VO4). The discoidal lipoprotein particle was prepared by the sodium cholate dialysis method using apolipoprotein/palmito/oleoylphosphatidylcholine molar ratios of 1:80 (24).

Cell culture and siRNA transfection
HUVECs (passage number 3) were purchased from Whittaker Bioproducts.

The cells were cultured in RPMI 1640 medium supplemented with 15% (v/v) FBS and several growth factors as previously described (25, 26). We usually used 5–8 passage of the cells and checked the cobblestone-like cell shape before experiments. As for the transfection of siRNAs specific to SR-BI and peroxisome proliferator-activated receptor-α (PPAR-α), HUVECs were seeded with a density of 5 × 104 cells/cm2 on 96-well plates for adhesion molecule expression, 12-well plates for THP-1 monocye adhesion assay, and on 6-well plates for quantitative RT-PCR analysis, Western blotting, and NOS activation. Sixteen hours later, siRNA (100 nM) was introduced into cells using RNAiFect reagent (Qiagen) according to the manufacturer’s instructions. The cells were further cultured for 48 h. The nonsilencing siRNA (D-001206-13) and siRNAs targeted for SR-BI (M-010592-00) and PPAR-α (Hs003434-00) were obtained from Dharmacon. THP-1 monocyctic cells were cultured in RPMI 1640 medium containing 10% FBS. WEHI-274.1 mouse monocyes were purchased from ATCC and cultured with DMEM with 4.5 g/l glucose, 10% FBS, and 0.05 mM 2-ME.

Construction of adenoviral vector and infection of recombinant adenovirus

The recombinant adenovirus for a dominant-negative form of RhoA, T19NRhoA, and a constitutively active form of RhoA, G14VRhoA, was constructed (27) and infected (21) as described previously. In brief, 80% confluent HUVECs were infected with recombinant at a multiplicity of infection of 30 for 2 h at 37°C in RPMI 1640 containing 5% FBS. Cells were then cultured for an additional 48 h with RPMI 1640 containing 15% FBS and other supplements before treatment. Under these conditions, infection with adenovirus coding GFP resulted in almost 100% cells positive to GFP.

Determination of cell surface expression of adhesion molecules by enzyme immunoassay

Total RNA was isolated using TRI REAGENT (Sigma-Aldrich) according to the instructions from the manufacturer. After DNase I (Promega) treatment, to remove possible traces of genomic DNA contaminating in the RNA preparations, 5 µg of the total RNA was reverse-transcribed using High Capacity cDNA Archive kit according to the instructions from the manufacturer (Applied Biosystems). To evaluate the expression level of mRNAs for SR-BI, PDZK1, S1P1, S1P3, PPAR-α, and eNOS, quantitative RT-PCR was performed using real-time TaqMan technology with a Sequence Detection System model 7700 (Applied Biosystems). The human probes specific to SR-BI, PDZK1, S1P1, S1P3, PPAR-α, eNOS, and GAPDH were obtained from TaqMan Gene Expression Assays (Applied Biosystems). The identification number of the products is Hs00194092 for SR-BI, Hs0042004 for PDZK1, Hs00173499 for S1P1, Hs00045464 for S1P3, Hs00211882 for PPAR-α, Hs00355860 for eNOS, and Hs00999905 for GAPDH. The expression level of the target mRNA was normalized to the relative ratio of the expression of GAPDH mRNA (28). Each RT-PCR assay was performed at least three times, and the results are expressed as mean ± SEM.

Western blotting

HUVECs were cultured and pretreated with several reagents as described above and then incubated for indicated times with test agents. For detection of eNOS, SR-BI, PDZK1, and β-actin, the reaction was terminated by washing twice with ice-cold PBS and adding 0.1 ml of lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 8 mM EGTA, 25 mM NaF, 10 mM Na2PO4, 1 mM NaVO3, 5 µg/ml leupeptin, 5 µg/ml pepstatin, 5 µg/ml aprotinin, and 0.5 mM PMSF. The lysate was separated by SDS-PAGE and analyzed by Western blotting with specific Abs as previously described (21). For detection of PPAR-α, HUVECs were lysed, and cytoplasmic protein and nuclear protein were separated by ReadyPrep Protein Extraction Kit (Cytoplasmic/Nuclear; Bio-Rad) according to the instructions from the manufacturer. In experiments as seen in Fig. 11, aortas were harvested from mice as described below. For detection of SR-BI protein expression, a part of aorta was treated with cotton stick to wipe off the endothelium. Aortas were homogenized and analyzed by Western blotting with SR-BI- or VCAM-1-specific Ab.

NOS enzymatic activity in cell lysate

NOS enzymatic activity was measured according to the method previously described (29). Briefly, HUVECs were grown to 80% confluence, and the cells were washed twice in ice-cold PBS, harvested from the dishes, and resuspended in ice-cold lysis buffer (29). The cells were disrupted by sonication (Branson Ultrasonics) three times for 10 s each. NOS enzymatic activity in the resulting cell lysates was determined by measuring the conversion of L-[^14]H]arginine to L-[^14]H]citrulline. Fifty microliters of cell lysate
FIGURE 1. Enhancement by simvastatin of HDL-induced inhibition of VCAM-1 expression and NOS activation. HUVECs were treated with or without 100 nM simvastatin for 24 h. A, HUVECs were then incubated for 8 h with the indicated concentrations of HDL in the presence or absence of TNF-α (60 pM) and l-NAME (1 μM). Cell surface expression of VCAM-1 was analyzed by enzyme immunoassay. l-NAME exerted no significant effect on the basal VCAM-1 expression without TNF-α (data not shown). Results are expressed as percentages of basal values in the absence of test agents. The value taken as 100% was 0.95 ± 0.04 (OD at 450 nm) for Control cells. Simvastatin treatment did not appreciably affect the TNF-α-induced activity. Data are the means ± SEM of three separate experiments. *, The effect of simvastatin was significant. B, HUVECs were incubated for 10 min with the indicated concentrations of HDL to measure NOS activity. The results are expressed as percentages of basal values in the absence of HDL. The basal enzymatic activity of NOS in control cells was 61 ± 7 pmol/mg protein/min and was unchanged by simvastatin treatment. Data are the means ± SEM of three separate experiments. *, The effect of simvastatin was significant.

were added to 50 μl of reaction mixtures containing 2 μM cold l-arginine and 2 μCi/ml l-3H]arginine. After incubation at 37°C for 1 h, the assay was terminated by the addition of 400 μl of 40 mM HEPES buffer (pH 5.5) with 2 mM EDTA and 2 mM EGTA. The terminated reaction samples were applied to 1-ml columns of Dowex AG50Wx-8 (Tres form) and eluted with 1 ml of 40 mM HEPES buffer. The l-3H]citrulline generated was collected into scintillation vials and quantified by liquid scintillation spectrometry.

Static THP-1 cell adhesion assay
THP-1 monocytic cells were washed twice and resuspended in RPMI 1640 containing 0.1% BSA. The cell suspensions were overlaped (1.5 × 10^6 cells/ml) on the confluent monolayers of HUVECs that had been grown in 12-well plates and treated with various reagents. After incubation for 15 min at 37°C, nonadherent THP-1 monocytic cells were removed by washing four times with prewarmed RPMI 1640 medium containing 0.1% BSA. The number of THP-1 monocytic cells on the HUVECs was counted in four places under microscopy at × 400 magnification (4HPF) as adhering cells. Unless otherwise stated, the static adhesion assay was used in the present study.

THP-1 cell adhesion assay under the shear stress
HUVECs were cultured on coverglass, stimulated with the indicated agents for 8 h, and then placed onto the flow chamber apparatus. The cells were washed by perfusion of RPMI 1640 medium containing 0.1% BSA at 37°C for 10 min. THP-1 monocytic cell suspension at the concentration of 10^6 cells/ml was then allowed to flow over HUVECs at the shear stress of 0.75 dynes/cm^2 as described (30). Five minutes later, the number of THP-1 monocytic cells attached to HUVECs was counted.

Mouse aorta isolation and ex vivo monocyte adhesion assay
Male C57BL/6J mice were purchased from Japan SLC. Mice were fed rodent chow and housed in microisolator cages in a pathogen-free facility. All experiments followed guidelines from the Association for Assessment of Laboratory Animal Care guidelines, and approval for use of rodents was obtained from the Gunma University. The mice were administrated i.p. with 40 mg simvastatin/kg/day for 7 days and then injected i.v. with 500 ng recombinant murine TNF-α or PBS as a vehicle. At 2 h postinjection, aortas were harvested from mice and immediately placed into 10% FBS containing DMEM. The ex vivo monocyte adhesion assay was performed as described by Bolick et al. (30). In brief, the aortas were opened up longitudinally and pinned onto sterile agar. All aortas were incubated for 15 min with 1 × 10^6 WEHI-241.1 mouse monocytes that were fluorescently labeled with Calcein-AM (Molecular Probes). After incubation, unbound monocytes were rinsed away, and the number of monocytes firmly bound to aorta was counted in four fields using fluorescent microscopy. Data are represented as the mean ± SEM of four areas of aorta.

Data presentation
All experiments were performed in duplicate or triplicate. The results of multiple observations are presented as the mean ± SEM or as a representative result of more than three different separate experiments, unless otherwise stated. Statistical significance was assessed by ANOVA or the Student’s t test; values were considered significant at p < 0.05 (*).

Results
Simvastatin enhanced HDL-induced inhibition of adhesion molecule expression in association with NOS activation
As shown in Fig. 1A, HDL inhibited the TNF-α-induced expression of VCAM-1, an adhesion molecule, in a dose-dependent manner. Treatment of HUVECs for 24 h with simvastatin did not affect TNF-α-induced VCAM-1 expression but significantly enhanced the inhibitory HDL action especially at lower concentrations of the lipoprotein. As a result, the dose-response curve of HDL was shifted to the left by simvastatin treatment. HDL also inhibited TNF-α-induced ICAM-1 expression, and simvastatin similarly enhanced HDL-induced actions (data not shown). We have shown that NO synthesis is involved in the inhibitory action of HDL on
adhesion molecule expression (22). In fact, the HDL-induced action was almost completely reversed by L-NAME, an NOS inhibitor, regardless of the statin treatment (Fig. 1A). Moreover, HDL induced NOS activation in a dose-dependent manner, and the HDL action was again shifted to the left by simvastatin pretreatment (Fig. 1B). Thus, simvastatin enhanced HDL-induced NOS activation and thereby modulated the HDL-induced inhibitory action on adhesion molecule expression.

The effect of simvastatin on adhesion molecule expression was accompanied by a change in the adhesion of THP-1 monocyte to HUVECs, as shown in Fig. 2A. TNF-α/H9251 facilitated THP-1 cell adhesion. We used the threshold concentration of HDL of 0.1 mg/ml, which slightly inhibited the TNF-α/H9251-induced action in control cells (Fig. 2A; b vs d). However, the same concentration of HDL markedly inhibited the TNF-α/H9251 action in the simvastatin-treated cells (Fig. 2A; f vs h). The results are summarized in Fig. 2B.

Since endothelial cells are situated at the interface between blood and the vessel wall and always undergo the shear stress at the physiological conditions, the adhesion of monocytes to HUVECs was also examined in the flow chamber. As shown in Fig. 3, we observed similar effects of TNF-α/H9251, HDL, and simvastatin to those under the static conditions. Thus, simvastatin treatment of the cells enhanced the HDL-induced inhibition of THP-1 cell adhesion under the shear stress as well.

We have recently shown that HDL induced the stimulation of NOS and the inhibition of adhesion molecule expression through both apoA-I/SR-BI and the lipoprotein-associated S1P/S1P receptors (22). To clarify whether these receptor systems are modulated by statin treatment, we first examined the effects of rHDL, which was prepared by reconstituting delipidated HDL with phosphatidylcholine. Since the rHDL contains apoA-I, a ligand protein of SR-BI, we used rHDL to examine the effect of simvastatin on adhesion molecule expression.

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SR-BI, but not bioactive lipid components, including S1P, the activity induced by rHDL is supposed to reflect the activity of SR-BI and its intracellular signaling pathway (22, 24). As in the previous study (22), rHDL inhibited TNF-α-induced VCAM-1 expression (Fig. 4A), which was associated with NOS activation (Fig. 4C). Treatment of simvastatin significantly enhanced the rHDL-induced inhibition of VCAM-1 expression and NOS activation (Figs. 4A and C), as was the case for HDL (Fig. 1).

We next examined the effects of simvastatin on S1P actions in VCAM-1 expression (Fig. 4B) and NOS activation (Fig. 4D). It has been shown that the inhibition of adhesion molecule expression by S1P is mediated mainly by S1P1 receptors (21). In contrast to rHDL, however, the inhibitory action of S1P on TNF-α-induced VCAM-1 expression was not appreciably affected by simvastatin treatment (Fig. 4B), while S1P-induced NOS activation was slightly but not significantly enhanced (Fig. 4D). In the absence of TNF-α, S1P stimulated VCAM-1 expression, possibly through S1P3 receptors, which was consistent with previous results (21).

S1P3 receptor-mediated VCAM-1 expression was significantly suppressed by simvastatin treatment (Fig. 4B). Simvastatin treatment may have enhanced SR-BI-mediated actions, while the statin affected S1P-induced actions in a manner dependent on S1P receptor subtypes. Thus, the statin suppressed S1P1 receptor-mediated stimulatory action on VCAM-1 expression but had only a small effect, if any, on S1P3 receptor-mediated inhibitory action.

Association of change in expression of HDL receptors and eNOS with the change in cellular activities

We examined the expression profile of receptors, including SR-BI, S1P1 receptor, and S1P3 receptor, and signaling molecules, including eNOS and PDZK1. As shown in Fig. 5, changes in the expression profiles were dependent on receptor types. Thus, the expression of SR-BI mRNA and protein was increased, that of S1P3 receptor mRNA was decreased, and that of S1P1 receptor mRNA was unchanged (Fig. 5). The expression of eNOS mRNA and protein was also increased, while that of PDZK1 was unchanged. The increase in SR-BI and eNOS expression can explain the enhancement of SR-BI-mediated responses (Fig. 4, A and C), while the decrease in S1P3 receptors may account for the attenuation of S1P-induced VCAM-1 expression (Fig. 4B). Although eNOS expression was significantly enhanced by the statin treatment, the treatment affected the S1P1-mediated inhibition of adhesion molecule expression and NOS activation only slightly (Figs. 4, B and D).
suggesting that the induction of eNOS expression was not as important as SR-BI for the enhancement of HDL-induced actions under the experimental conditions.

**Critical role of SR-BI in enhancement of HDL-induced actions**

To further confirm the role of SR-BI, we used the siRNA strategy. As shown in Fig. 6A, siRNA against SR-BI markedly inhibited SR-BI expression without a change in eNOS and PDZK1 expression. Under the conditions used, simvastatin enhancement of inhibitory HDL- and rHDL-induced VCAM-1 expression was almost completely attenuated by SR-BI-siRNA (Fig. 6B). The simvastatin effect on HDL- and rHDL-induced NOS activation was also inhibited by SR-BI siRNA (Fig. 6C). However, the S1P-induced inhibition of VCAM-1 and NOS activation was not appreciably affected by siRNA, indicating the specificity of siRNA (Fig. 6, B and C).

**Simvastatin effects are mediated by the inhibition of HMG-CoA reductase and the subsequent inhibition of Rho activity**

Statin effects are not always mediated by the inhibition of HMG-CoA reductase, although the molecular mechanisms of the enzyme-independent actions of statins remain unknown (9, 11). As shown in Fig. 7, simvastatin enhancement of the HDL-induced inhibition of VCAM-1 expression (Fig. 7, A and B) and the activation of NOS (Fig. 7, C and D) were completely reversed by mevalonic acid, a product of HMG-CoA reductase. These results suggest that the simvastatin modulation of HDL-induced actions on VCAM-1 expression and NOS activation is dependent on the inhibition of HMG-CoA reductase.

The inhibition of HMG-CoA reductase inhibits cholesterol synthesis and induces LDL receptor expression, thereby decreasing plasma LDL cholesterol (9, 11). Recent studies, however, have shown that, in addition to changes in cholesterol metabolism, the inhibition of HMG-CoA reductase is associated with the inhibition of the posttranslational prenylation of a variety of important cell signaling proteins, including Rho, Rac, and Cdc42, through the inhibition of the formation of farnesyl pyrophosphate and geranylgeranyl pyrophosphate, downstream products of mevalonate (9, 11). As shown in Fig. 8A, dominant-negative RhoA, T19NRhoA, induced SR-BI expression; furthermore, constitutively active RhoA, G19VRhoA, inhibited simvastatin-induced SR-BI expression. The infection of dominant-negative RhoA showed the enhancement of the HDL- and rHDL-induced inhibition of VCAM-1 expression (Fig. 8B) and the stimulation of NOS activation (Fig. 8D). Consistent with these results, the infection of constitutively active RhoA attenuated simvastatin-induced effects (Fig. 8, C and E). These results suggest that simvastatin may inhibit Rho activity possibly by inhibiting its prenylation and thereby induce SR-BI expression.

**FIGURE 8.** Involvement of RhoA in the simvastatin-induced actions. HUVECs were infected with dominant-negative RhoA adenovirus (DN-RhoA), constitutively active RhoA adenovirus (CA-RhoA), or empty vector 48 h before experiments. Twenty-four hours before experiments, 100 nM simvastatin (Simva) or vehicle was added to the culture medium. A, Protein expression of SR-BI, RhoA, and β-actin was analyzed by Western blotting with Abs specific to the respective protein. B and C, HUVECs were incubated for 8 h with or without HDL (0.1 mg/ml) or rHDL (0.1 mg/ml) in the presence or absence of TNF-α (60 pM) to measure VCAM-1 expression. Results are expressed as percentages of TNF-α-induced activity in the absence of test agents. The treatment of DN-RhoA and CA-RhoA hardly affected the TNF-α-induced activity. Data are the means ± SEM of three separate experiments. *, The effect of DN-RhoA (B) and simvastatin (C) was significant. D and E, HUVECs were incubated for 10 min with HDL (0.1 mg/ml) or rHDL (0.1 mg/ml) to measure NOS activity. Results are expressed as percentages of the basal values in the absence of test agents. The treatment of DN-RhoA and CA-RhoA hardly affected the basal value. Data are the means ± SEM of three separate experiments. *, The effect of DN-RhoA (D) and simvastatin (E) was significant.

**FIGURE 9.** Fenofibrate enhanced HDL-induced actions in association with induction of SR-BI expression. A, HUVECs were infected with constitutively active RhoA adenovirus (CA-RhoA) or empty vector (Control) 48 h before experiments. Twenty-four hours before experiments, 100 nM simvastatin (Simva), 1 µM fenofibrate (Feno), or vehicle was added to the culture medium. Protein expression of SR-BI, RhoA, and β-actin was analyzed by Western blotting with Abs specific to the respective protein. A representative of three separate experiments is shown. B, The cells were treated for 24 h with or without 1 µM fenofibrate (Feno) and then incubated for 8 h with the indicated concentrations of HDL in the presence or absence of TNF-α (60 pM). Cell surface expression of VCAM-1 was analyzed by enzyme immunossay. Results are expressed as percentages of TNF-α-induced activity in the absence of test agents. The treatment of fenofibrate did not appreciably affect the TNF-α-induced activity. Data are the means ± SEM of three separate experiments. **+**, The effect of fenofibrate was significant.
Involvement of PPAR-α in simvastatin-induced actions

Fenofibrate, a PPAR-α stimulator, has been shown to enhance SR-BI expression via PPAR-α activation in human macrophages (31, 32). We therefore examined the effects of fenofibrate on SR-BI expression and HDL-induced actions. As shown in Fig. 9, fenofibrate was as effective as simvastatin in causing enhancement of SR-BI expression, which was accompanied by the enhancement of HDL-induced inhibition of VCAM-1 expression (Fig. 9B). In contrast to simvastatin, however, the fenofibrate effect was not appreciably affected by a constitutively active RhoA mutant (Fig. 9A), suggesting that fenofibrate acts on the downstream signaling of RhoA.

To confirm the involvement of a fenofibrate target molecule, PPAR-α, in simvastatin actions, we used siRNA against PPAR-α and MK886, a PPAR-α inhibitor (33). SiRNA against PPAR-α inhibited the enhancement of SR-BI expression induced by simvastatin as well as fenofibrate (Fig. 10A), in association with the inhibition of the expression of PPAR-α mRNA and protein (Fig. 10B). Moreover, MK886 also inhibited simvastatin- and fenofibrate-induced SR-BI expression (Fig. 10A). As expected, the simvastatin-induced enhancement of the inhibitory actions of HDL and rHDL on VCAM-1 expression (Fig. 10C) and THP-1 monocytic cell adhesion (Fig. 10D), as well as the enhancement of NOS activation (Fig. 10E) by HDL and rHDL were blocked by siRNA against PPAR-α. These results suggest that PPAR-α is involved in downstream of simvastatin-regulated and RhoA-mediated signaling pathways.

Administration of simvastatin inhibited the ex vivo monocye adhesion in aortas from TNF-α-injected mice

C57BL/6J mice were administrated with simvastatin or its vehicle for 7 days and the expression of SR-BI was examined. As shown in Fig. 11A, the statin treatment clearly increased the SR-BI expression in the whole aortas, whereas the endothelium-removed aortas showed a marginal expression of SR-BI regardless of the statin treatment. These results suggest that SR-BI is mainly expressed in endothelium and endothelial SR-BI expression is stimulated by statin treatment. The mice treated with or without simvastatin were then injected with TNF-α. Two hours later, aortas were harvested and ex vivo adhesion of WEHI 241.1 mouse monocytes to the aortas was examined. TNF-α stimulated the monocyte adhesion (Fig. 11C), which
was associated with VCAM-1 expression (Fig. 1B). In contrast to the in vitro study, where simvastatin hardly affected the TNF-α-induced VCAM-1 expression (Fig. 1A) and monocyte adhesion (Figs. 2 and 3), the statin treatment in vivo clearly inhibited the cytokine-induced VCAM-1 expression (Fig. 1B) and ex vivo monocyte adhesion (Fig. 1C). However, these results are expected because HDL is circulating in blood during TNF-α treatment in vivo. Thus, simvastatin also increases endothelial SR-BI expression in vivo, thereby inhibiting monocyte adhesion to endothelium.

Discussion

The results of the present study indicated that simvastatin enhanced the HDL-induced activation of NOS and the inhibition of VCAM-1 expression in HUVECs. We have previously shown that the HDL-induced inhibition of adhesion molecule expression is mediated by NOS activation through both SR-BI and S1P receptors (22). Our results suggest that simvastatin induced SR-BI expression and thereby enhanced HDL-induced anti-inflammatory actions. To our best knowledge, the present study is the first indication that endothelial SR-BI plays a critical role as a receptor of HDL in anti-inflammatory actions of the statin. This conclusion is based on the following observations. First, simvastatin clearly stimulated endothelial SR-BI expression not only in vitro but also in vivo. In addition to the induction of SR-BI expression, simvastatin stimulated eNOS expression. However, S1P-induced NOS activation was not appreciably affected by the statin treatment, suggesting that the induction of eNOS by simvastatin may not be as important as SR-BI for the enhancement of NOS activation by HDL. Second, reconstituted apoA-I-rich HDL (rHDL), which stimulates SR-BI, mimicked anti-inflammatory HDL actions, and its actions were enhanced by simvastatin. Finally, siRNA against SR-BI reversed the effects of the statin on HDL- and rHDL-induced actions without a change in the SR-BI-induced actions.

Although the present study is the first indication of SR-BI expression by statin in endothelial cells, recent studies have reported that SR-BI expression is stimulated by statins in other cell types, including macrophages (34), adipocytes (35), and keratinocytes (36). In these cells, the induction of SR-BI expression was associated with a change in cholesterol metabolism. However, previous studies (34–36) did not examine the cholesterol metabolism-independent actions of HDL through SR-BI. Our results showed that statin induction of SR-BI in endothelial cells facilitated HDL-induced NOS activation and the subsequent inhibition of adhesion molecule expression. Thus, statins have the potential to stimulate SR-BI-mediated cholesterol metabolism-independent actions as well as cholesterol metabolism in vivo. In the present study, we showed crucial roles of RhoA and PPAR-α on SR-BI expression by simvastatin. PPAR-α activation has been shown to play an essential role in anti-inflammatory actions by simvastatin in macrophages and neutrophils (37), that supports our findings. Moreover, it has been suggested that PPAR-α is involved in the transcriptional regulation of SR-BI expression (31, 32). However, how RhoA regulates PPAR-α activity remains unclear. Further study is needed to clarify the relation of RhoA and PPAR-α in simvastatin-induced SR-BI expression.

A recent study has shown that S1P1 receptors are up-regulated by pitavastatin and atorvastatin, resulting in the enhancement of S1P- and HDL-induced NOS activation in cultured bovine aortic endothelial cells (38). In our experimental condition, we failed to detect the up-regulation of S1P1 receptor mRNA; instead, we observed the down-regulation of S1P3 receptor mRNA. Since we could not successfully show the results of Western blotting of S1P receptors, the real expression profile of S1P receptors under the statin treatment in the present study remains unknown. Nevertheless, S1P1 receptor protein up-regulation, if it exists, might be limited because S1P-induced NOS activation was only slightly potentiated by simvastatin treatment. The small enhancement of the S1P-induced action probably reflects the up-regulation of eNOS protein. The cause of the discrepancy between these results remains unknown, but different sources of endothelial cells and/or the different types of statins used might explain it. It is worth noting, however, that both studies suggest anti-inflammatory actions of statins through modified actions of HDL-associated components, i.e., apoA-I and S1P.

Numerous studies have shown that statins affect adhesion molecule expression, but the results are controversial (9, 39). In accordance with reduction of cell infiltration in vivo (40–42), the expression of adhesion molecules, including VCAM-1 and ICAM-1, in response to cytokines has been prevented by statin treatment in vitro (9, 39). In contrast, the enhancement of adhesion molecule expression has also been reported in some in vitro experiments (9, 39). In the present study, statin alone did not show any significant effect on TNF-α-induced VCAM-1 expression but inhibited its expression in the presence of HDL regardless of the shear stress in vitro. At present, there is no clear explanation for the differences between the results of the in vitro experiments, but they may be explained in part by the concentrations of statin used. In fact, we observed that simvastatin alone inhibited TNF-α-induced VCAM-1 expression at concentrations higher than the 100 nM used in the present study (data not shown). In contrast, administration of statin and TNF-α in vivo significantly inhibited the cytokine-induced adhesion of monocytes to the endothelium ex vivo. Although further characterization is required to explain the discrepancies in the experimental results in vitro, the enhancement of HDL-induced inhibition of adhesion molecule expression is consistent with the anti-inflammatory actions of statins and hence might contribute in part to statin actions in vivo.

To determine the NOS-regulated response in endothelial cells, in the present study, we evaluated adhesion molecule expression. In addition to inhibiting cell adhesion, NOS is known to be involved in other anti-atherogenic actions. For example, NO leads to the inhibition of platelet aggregation, the inhibition of smooth muscle cell migration and proliferation, and the stimulation of vascular relaxation (9, 11). Moreover, SR-BI stimulation may also activate ERK and Rac in an NO-independent manner, leading to the stimulation of cell migration and the repair of injured endothelial cells (10). Thus, the stimulation of SR-BI and subsequent eNOS and ERK activation may exert a variety of beneficial effects on vascular system in addition to the inhibition of adhesion molecule expression.

In conclusion, simvastatin treatment of vascular endothelial cells induced SR-BI expression and thereby enhanced the activation of HDL-induced NOS activity and the inhibition of adhesion molecule expression. This novel action mechanism of simvastatin may in part account for the beneficial cholesterol metabolism-independent statin actions in patients with inflammatory and immune diseases, including atherosclerosis and rheumatoid arthritis, who experience changes in plasma lipid profiles, especially low levels of HDL.

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