Role of Scavenger Receptor Class B Type I and Sphingosine 1-Phosphate Receptors in High Density Lipoprotein-induced Inhibition of Adhesion Molecule Expression in Endothelial Cells*

Received for publication, June 19, 2006, and in revised form, September 15, 2006 Published, JBC Papers in Press, October 16, 2006

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We characterized the molecular mechanisms by which high density lipoprotein (HDL) inhibits the expression of adhesion molecules, including vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, induced by sphingosine 1-phosphate (S1P) and tumor necrosis factor (TNF) in endothelial cells. HDL inhibited S1P-induced nuclear factor κB activation and adhesion molecule expression in human umbilical vein endothelial cells. The inhibitory HDL actions were associated with nitric-oxide synthase (NOS) activation and were reversed by inhibitors for phosphatidylinositol 3-kinase and NOS. The HDL-induced inhibitory actions were also attenuated by down-regulation of scavenger receptor class B type I (SR-BI) and its associated protein PDZK1. When TNF was used as a stimulant, the HDL-induced NOS activation and the inhibitory action on adhesion molecule expression were, in part, attenuated by the down-regulation of the expression of S1P receptors, especially S1P₁, in addition to SR-BI. Reconstituted HDL composed mainly of apolipoprotein A-I and phosphatidylcholine mimicked the SR-BI-sensitive part of HDL-induced actions. Down-regulation of S1P₃ receptors severely suppressed the stimulatory actions of S1P. Although G₁/o-proteins may play roles in either stimulatory or inhibitory S1P actions, as judged from pertussis toxin sensitivity, the coupling of S1P₃ receptors to G₁₂/₁₃-proteins may be critical to distinguish the stimulatory pathways from the inhibitory ones. In conclusion, even though S1P alone stimulates adhesion molecule expression, HDL overcomes S1P₃ receptor-mediated stimulatory actions through SR-BI/PDZK1-mediated signaling pathways involving phosphatidylinositol 3-kinase and NOS. In addition, the S1P component of HDL plays a role in the inhibition of TNFα-induced actions through S1P receptors, especially S1P₁.

The plasma level of HDL has been shown to be inversely correlated with the risk of atherosclerosis and associated cardiovascular disease (1, 2). HDL can remove excess cholesterol from arterial and nonliver cells, transport it to the liver, and excrete it as bile acids. The so-called reverse cholesterol transport is thought to be an important anti-atherogenic action of HDL (1, 2). In recent studies, however, HDL has been shown to exert a variety of actions that are independent of cholesterol metabolism. For example, HDL inhibits LDL oxidation, smooth muscle cell migration, platelet aggregation, and endothelial dysfunction (3, 4). The inhibition of endothelial dysfunction may be achieved by several responses to HDL, including the stimulation of proliferation, cell survival, migration, and NO synthesis, or the inhibition of apoptosis and of the expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (3–5). An increase in the expression of the adhesion molecules stimulates monocyte interaction with endothelial cells and cell penetration into subendothelial space or the intima of arterial walls. Thus, the expression of adhesion molecules is thought to be one of the early steps in the onset of atherosclerosis (3–5). However, the molecular mechanisms whereby HDL inhibits adhesion molecule expression are poorly defined.

We have recently shown that S1P is accumulated in lipoprotein fractions, especially HDL fractions (6–9), and demonstrated that HDL-induced cell survival and migration responses are mediated by S1P and its receptors, S1P₁ and S1P₃ (9, 10).

The abbreviations used are: HDL, high density lipoprotein; LDL, low density lipoprotein; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; S1P, sphingosine 1-phosphate; S1P₁, S1P₃, EDG-1 receptor; S1P₃, S1P₃/EDG-3 receptor; PI, phosphatidylinositol; NOS, nitric-oxide synthase; eNOS, endothelial NOS; NF-κB, nuclear factor κB; TNF, tumor necrosis factor; SR-BI, scavenger receptor class B type I; NAME, N°-nitroarginine methyl ester hydrochloride; PTX, pertussis toxin; PBS, phosphate buffered saline; siRNA, small interfering RNA; HUVECs, human umbilical vein endothelial cells; rHDL, reconstituted HDL; RT, reverse transcription.

* This work was supported by a Grants-in-Aid for scientific research from the Japan Society for the Promotion of Science (to F. O., H. T., K. S., and A. K.); Pusan National University research grant (to D. S. I.); a grant of the 21st Century Center of Excellence (COE) Program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to C. M. and A. D.); and grants from Uehara Memorial Foundation (to F. O.), Yamamoto Foundation for Research on Metabolic Disorders (to F. O.), Takeda Science Foundation (to H. T. and F. O.), and the Life Science Foundation of Japan (to H. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: HDL, high density lipoprotein; LDL, low density lipoprotein; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; S1P, sphingosine 1-phosphate; S1P₁, S1P₃, EDG-1 receptor; S1P₃, S1P₃/EDG-3 receptor; PI, phosphatidylinositol; NOS, nitric-oxide synthase; eNOS, endothelial NOS; NF-κB, nuclear factor κB; TNF, tumor necrosis factor; SR-BI, scavenger receptor class B type I; NAME, N°-nitroarginine methyl ester hydrochloride; PTX, pertussis toxin; PBS, phosphate buffered saline; siRNA, small interfering RNA; HUVECs, human umbilical vein endothelial cells; rHDL, reconstituted HDL; RT, reverse transcription.
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Other groups also have demonstrated that the S1P_3 receptor mediates HDL-induced relaxation through NO synthase (NOS) activation and NO synthesis in mouse artery (11). Thus, we proposed the S1P component of HDL as an anti-atherogenic mediator (6, 7, 10). However, S1P action on adhesion molecule expression is not simple; S1P alone stimulates the expression of VCAM-1 and ICAM-1 through NF-κB activation (12–15), whereas the lysolipid inhibits the TNFα-induced adhesion of monocytes (15, 16). These results suggest that both S1P and HDL have opposing potentials for atherogenesis. The inhibitory action of HDL on adhesion molecule expression could be explained by the S1P component of HDL; however, a previous study (17) and our preliminary study did not show any stimulatory effect on adhesion molecule expression by HDL alone. More interestingly, the S1P-induced stimulatory action was lost in the presence of HDL, suggesting that HDL may have mechanisms in addition to those mediated by S1P to inhibit adhesion molecule expression. In the present study, we characterized the molecular mechanisms involved in the HDL-induced inhibitory action and found a critical role of scavenger receptor class B type 1 (SR-BI) and its associated protein, termed PDZK1, which contains four PSD-95/Dlg/ZO-1 (PDZ) domains (18, 19). Furthermore, as expected, we demonstrated the involvement of S1P receptors, in addition to SR-BI, in the HDL-induced inhibition of adhesion molecule expression when TNFα was used as a pro-atherogenic stimulant.

EXPERIMENTAL PROCEDURES

Materials—S1P was purchased from Cayman Chemical Co.; wortmannin was from Calbiochem-Novabiochem; L-arginine methyl ester hydrochloride (L-NAME), and d-NG-nitroarginine methyl ester hydrochloride (D-NAME) were from BIOMOL Research Laboratories Inc.; anti-endothelial nitric-oxide synthase (eNOS) antibody, anti-Ser(P)1177 eNOS antibody, and anti-β-actin antibody were from Cell Signaling Technology Inc.; primary mouse antibodies for VCAM-1 and ICAM-1 were from Chemicon International; and anti-SR-BI and anti-PDZK1 were from Santa Cruz Biotechnology, Inc. YM-254890 was generously provided by Dr. Masatoshi Taniguchi of Astellas Co. (Tsukuba, Japan). The sources of all other reagents were the same as described previously (15, 20). Plasma lipoproteins were prepared by density gradient centrifugation; LDL (1.019–1.063 g/ml) and HDL (1.063–1.21 g/ml) were separated from freshly isolated plasma of healthy volunteers by sequential ultracentrifugation as described previously (8). In the present study, we used HDL isolated from nine different donors; the protein content was estimated to be 1.92 ± 0.12 mg proteins (mainly apolipoprotein A-I)/ml plasma, and S1P content (21) was 204 ± 10 pmol/mg protein (within the range from 168 to 248 pmol/mg protein). Thus, S1P content in HDL samples is rather constant. We usually used HDL at 1 mg/ml, which corresponds to ~0.2 μM S1P.

Preparation of Reconstituted HDL—The reconstituted discoloid lipoprotein particle (rHDL) was prepared as follows. HDL was delipidated as described (22), 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% (w/v) EDTA, and 0.02% (w/v) NaN_3). The discoloid lipoprotein particle was prepared by the sodium cholate dialysis method as previously described (23), using apolipoprotein/palmitoyloleoylphosphatidylcholine molar ratios of 1:80 (24). The rHDL samples thus prepared were diluted to roughly 50% with respect to protein content compared with native HDL samples. To avoid the loss of protein, we used the rHDL samples without further concentration. Thus, in the experiments using rHDL, we used 500 μg/ml of rHDL or HDL (Fig. 9). The S1P content in the rHDL was undetectable by our S1P assay method (21).

Cell Culture and Transfection—HUVECs (passage number 3) were purchased from Whittaker Bioproducts. The cells were cultured in RPMI 1640 medium supplemented with 15% (v/v) fetal bovine serum and several growth factors as previously described (9, 10). We usually used 5–8 passages of the cells and checked the cobblestone-like cell shape before experiments. Where indicated, pertussis toxin (PTX, 100 ng/ml) or its vehicle (PBS) was added to the culture medium 24 h before experiments, unless otherwise stated. Transfection of antisense oligonucleotides to block the expression of S1P_1 and S1P_3 receptors was performed using NovaFECTORTM reagent (VennNova) according to the method of Paik et al. (25). 18-mer phosphothioate oligonucleotides used are as follows: antisense EDG-1/S1P_1, 5'-GAC GCT GGT GGG CC C CAT-3' and antisense EDG-3/S1P_3, 5'-CGG GAG GGC AGT TGC CAT-3'. The expression of these S1P receptor mRNAs was measured at 12 h, and experiments were started at 16 h after the transfection. Although we have not succeeded in observing S1P receptor protein expression by Western blotting, we confirmed almost complete inhibition of the expression of both S1P receptor mRNAs by Northern blotting under these conditions (10). For the transfection of siRNAs specific to SR-BI, PDZK1, S1P_1, S1P_3, Go12, and Go13, HUVECs were seeded with a density of 5.0 × 10^4 cells/cm^2 on 96-well plates for adhesion molecule expression and on 6-well plates for quantitative RT-PCR analysis. Western blotting, Northern blotting, and NF-κB transcription assay. Sixteen h 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), PDZK1 (M-010615-01), S1P_1 (M-003655-01), S1P_3 (M-005208-01), Go12 (M-008435-00), and Go13 (M-009948-00) were from Dharmacon Inc. (Lafayette, CO). THP-1 monocytic cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum.

Determination of Cell Surface Expression of Adhesion Molecules by Enzyme Immunoassay—HUVECs were plated on 96-well plates and transfected with antisense oligonucleotides or siRNA as described above. The cells were then washed twice and incubated in RPMI 1640 containing 0.1% bovine serum albumin with test agents for 8 h. Thereafter cells were washed with PBS twice and fixed with PBS containing 3% formaldehyde under 4°C. The plates were blocked at 4°C overnight with 5% skim milk powder in PBS. Cell surface expression of adhesion molecules was determined by primary binding with specific mouse antibody for VCAM-1 or ICAM-1, followed by secondary binding with a horseradish peroxidase-conjugated goat anti-mouse IgG antibody as described previously (15). Quantification was performed by determination of colorimetric con-
version at an optical density at 450 nm of 3,3',5,5'-tetramethylbenzidine using TMB peroxidase ELA substrate kit (Bio-Rad).

Quantitative RT-PCR Analysis—Total RNA was isolated using TRI REAGENT (Sigma-Aldrich) according to the instructions from the manufacturer. After DNase I (Promega, Madison, WI) treatment to remove possible traces of genomic DNA contaminating in the RNA preparations, 5 μg of the total RNA was reverse-transcribed using a 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, Gα12, and Gα13, quantitative RT-PCR was performed using real time TaqMan technology with sequence detection system model 7700 (Applied Biosystems). The human probes specific to SR-BI, PDZK1, S1P1, S1P3, Gα12, and Gα13 were obtained from TaqMan Gene Expression Assays (Applied Biosystems). The ID numbers of the products were Hs00194092 for SR-BI, Hs0042004 for PDZK1, Hs00173499 for S1P1, Hs00245464 for S1P3, Hs00170899 for Gα12, Hs00183573 for Gα13, and Hs99999905 for glyceraldehyde-3-phosphate dehydrogenase. The expression level of the target mRNA was normalized to the relative ratio for glyceraldehyde-3-phosphate dehydrogenase. The expression level of the target mRNA was normalized to the relative ratio.

NF-κB Transcription Assay—HUVECs were transfected with the Lipofectin/nucleic acid mixture including 1 μg of Lipofectin reagent (Invitrogen), 1 μg of pNFκB-Luc (Stratagene), and 240 ng of pRL (Renilla luciferase)-SV40 as described previously (15). Luciferase reporter assay was performed 48 h after transfection using a dual luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Renilla luciferase activity was used to normalize transfection efficiencies among experiments.

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 phosphorylation (15), 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 Na3PO4, 1 mM Na2VO4, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml apro tinin, and 0.5 mM phenylmethylsulfonyl fluoride. The lysate was separated by 6% SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with eNOS-specific and eNOS-Ser(P)1177 antibody. As for the detection of SR-BI, PDZK1, and β-actin, the procedures were essentially the same as those for eNOS phosphorylation except for the primary antibody.

NOS Enzymatic Activity in Cell Lysate—NOS enzymatic activity was measured according to the method previously described (27). Briefly, HUVECs were grown to 80% confluence, and the cells were rinsed twice in ice-cold PBS, harvested from the dishes, and resuspended in ice-cold lysis buffer (27). The cells were disrupted by sonication (Branson Ultrasonics, Chicago, IL) three times for 10 s each. NOS enzymatic activity in the resulting cell lysates was determined by measuring the conversion of 1-[3H]arginine to 1-[3H]citrulline. Fifty microliters of cell lysate were added to 50 μl of reaction mixtures containing 2 μM cold l-arginine and 2 μCi/ml of 1-[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 reactions were applied to 1-ml columns of Dowex AG50WX-8 (Tris form) and eluted with 1 ml of 40 mM HEPES buffer. The 1-[3H]citrulline generated was collected into scintillation vials and quantified by liquid scintillation spectroscopy.

THP-1 Cell Adhesion Assay—THP-1 monocytes cells were washed twice and resuspended in RPMI 1640 containing 0.1% bovine serum albumin. The cell suspensions were overlaid (1.5 × 106 cells/ml, 500 μl/well) 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 monocytes cells were removed by washing four times with prewarmed RPMI 1640 medium containing 0.1% bovine serum albumin. The number of THP-1 monocytes cells on the HUVECs was counted in four places under microscopy at 400× magnification as adhering cells.

Data Presentation—All of the experiments were performed in duplicate or triplicate. The results of multiple observations are presented as the means ± S.E. of a representative experiment or as a representative result from more than three different batches of cells, unless otherwise stated. Statistical significance was assessed by Student’s t test; the values were considered significant at p < 0.05.

RESULTS

HDL Inhibits S1P-induced Adhesion Molecule Expression through eNOS Activation—TNFα, a well known cytokine for adhesion molecule expression, is more potent than S1P. In the present study, however, we first characterized in detail the action of HDL on exogenous S1P-induced adhesion molecule expression to exclude the possible role of the S1P component of HDL. In accordance with previous results (15), S1P promoted THP-1 monocyte cell adhesion to HUVECs, and S1P-induced cell adhesion was almost completely inhibited by HDL (Fig. 1, A and B). The change in cell adhesion was associated with the change in the expression of VCAM-1 (Fig. 1C, left panel) and ICAM-1 (Fig. 1C, right panel). Thus, HDL inhibited S1P-induced adhesion molecule expression without any significant effect alone. In contrast to HDL, LDL was ineffective for the suppression of adhesion molecule expression (Fig. 1C).

The inhibitory action of HDL was associated with the inhibition of NF-κB activation (Fig. 1D), which has been shown to be critical for adhesion molecule expression (5, 15). Because we previously showed that S1P-induced adhesion molecule expression was inhibited by NO donor S-nitroso-N-acetylpenicillamine (15), we investigated the effect of NOS inhibitor l-NAME. The HDL-induced inhibition of VCAM-1 expression (Fig. 1E) and NF-κB activation (Fig. 1D) was completely reversed by the NO donor l-NAME, whereas an inactive derivative d-NAME was ineffective (data not shown). HDL-induced inhibitory actions were also inhibited by the PI 3-kinase inhibitor wortmannin (Fig. 1, D and E). The inhibitory action of HDL on ICAM-1 expression was also reversed by l-NAME and wortmannin (data not shown). These results suggest that PI
3-kinase and NOS are involved in the HDL-induced inhibition of NF-κB-mediated adhesion molecule expression.

HDL stimulated the phosphorylation of eNOS (Fig. 2A), reflecting the activation of enzyme activity (Fig. 2B) and thus supporting this proposal. As in a previous study (15), S1P also activated NOS, but the activation was rather transient compared with HDL-induced activation. The HDL action was still effective even at 6 or 8 h after stimulation, whereas the S1P action was ineffective at these time points. HDL-induced enzyme activation was completely inhibited by wortmannin (Fig. 2, C and D), as was the short term S1P-induced NOS activation (15). These results are consistent with those shown in Fig. 1.

As shown in Fig. 1C, the inhibitory action of HDL on adhesion molecule expression was not detected until an HDL concentration of 300 μg/ml was reached. The dose-response curve of HDL was comparable with the HDL concentrations previously reported to inhibit VCAM-1 expression (17) and platelet aggregation (28). If NOS activation is involved in the inhibitory role of HDL, we should expect a similar dose-response of NOS activation. Recent studies, however, have shown that HDL significantly stimulated NOS activity at 10–50 μg/ml (24, 29, 30), concentrations comparable with the affinity of HDL to SR-BI, one of the HDL receptors (31). Thus, there is a clear discrepancy between the potencies for the inhibition of VCAM-1 expression and NOS activation. This discrepancy may be explained by the different time at which the two activities were measured. The expression of adhesion molecules, such as VCAM-1 and ICAM-1, requires transcriptional regulation, and it takes 6 – 8 h to detect significant activity, whereas NOS activation is regulated by a minute order of early change in signaling events involving phosphorylation. As shown in Fig. 2E, 30 μg/ml HDL effectively activated NOS at 10 min. However, the enzyme activation was transient and declined to the basal level within 4 – 6 h. On the other hand, NOS activity by 1 mg/ml HDL was also maximal at 10 min, but the activation was long acting, as shown in Fig. 2 (B and E). As a result, the apparent dose-response curves were dependent on the incubation time and shifted to the right with longer incubation (Fig. 2F). Thus, the duration of NOS activation may be one of the key mechanisms whereby HDL inhibits adhesion molecule expression.

Involvement of SR-BI/PDZK1 System in HDL-induced Stimulation of NOS and Inhibition of Adhesion Molecule Expression—Recent studies have indicated the HDL-induced stimulation of eNOS through more than one mechanism, including S1P receptors (11) and SR-BI (24, 29, 30). We first examined the role of SR-BI on HDL-induced eNOS activation and adhesion molecule expression. Transfection of siRNA specific to SR-BI
markedly inhibited the expression of SR-BI mRNA (Fig. 3A) and protein (Fig. 3B) in HUVECs. Under the conditions of the present study, the SR-BI-specific siRNA reversed the HDL-induced inhibitory action on VCAM-1 expression (Fig. 3C), ICAM-1 expression (data not shown), and NF-κB activation (Fig. 3D) without any significant effect on the S1P-induced actions.

PDZK1 was initially isolated as an SR-BI-associated protein and has been shown to be involved in the regulation of SR-BI protein levels (18, 19). To confirm the role of SR-BI in HDL-induced actions on adhesion molecule expression, we used siRNA specific to PDZK1. We supposed that a reduction of PDZK1 expression might decrease SR-BI expression (18) and thereby inhibit scavenger receptor-mediated actions. Down-regulation of the expression of PDZK1 mRNA (Fig. 4A) and protein (Fig. 4B) by siRNA treatment reversed the HDL-induced inhibition of adhesion molecule expression (Fig. 4E) and NF-κB activation (Fig. 4F). However, the siRNA treatment hardly affected the expression of SR-BI mRNA (Fig. 4C) and protein (Fig. 4D). These results suggest that PDZK1 may act as an adapter or a scaffolding protein (19) to communicate SR-BI to downstream effector molecules.

The roles of SR-BI and PDZK1 in HDL signaling were further confirmed in NOS activation; the treatment of siRNA specific to either SR-BI or PDZK1 significantly inhibited HDL-induced NOS activation without any significant effect on S1P-induced action regardless of the incubation time (Fig. 5).

Involvement of S1P Receptors, in Addition to SR-BI, in HDL Signaling—Although siRNA specific to SR-BI or PDZK1 effectively inhibited HDL-induced NOS activation, HDL still significantly activated the enzyme in the presence of these siRNAs (Fig. 5), suggesting the involvement of SR-BI/PDZK1-independent mechanisms in HDL-induced actions. Indeed, the treatment of cells with either PTX or antisense oligonucleotide against S1P receptors also partially inhibited NOS activation, and their combination with SR-BI siRNA completely abolished it (Fig. 6). These results seem strange because the effect of exogenous S1P was very small at ~6 h after stimulation (Fig. 2); however, the results may be accounted for by the fact that the metabolism of HDL-associated S1P is slower than that of albumin-associated S1P (9).

In the experiments described above, the action of the S1P component of HDL on adhesion molecule expression is hidden by exogenous S1P. To disclose the role of the S1P component of the lipoprotein, S1P was replaced with TNFα as a stimulant of adhesion molecule expression. HDL inhibited the TNFα-induced expression of VCAM-1 (Fig. 7A) and ICAM-1 (Fig. 7B), which was associated with the inhibition of NF-κB activation (Fig. 7C). This result is consistent with that reported previously (17). As expected based on the results shown in Fig. 6, the HDL-induced inhibition of the expression of VCAM-1 (Fig. 7A), ICAM-1 (Fig. 7B), and NF-κB activation (Fig. 7C) was partly attenuated by the down-regulation of either S1P receptors or SR-BI and was completely inhibited by the down-regulation of both receptors. These results suggest that S1P receptors, in addition to SR-BI, are critical for the inhibitory role of HDL in TNFα-induced adhesion molecule expression.

Differential Roles of S1P Receptor Subtypes in Stimulatory and Inhibitory Signaling in Adhesion Molecule Expression—The finding that HDL inhibited TNFα-induced adhesion molecule expression partly through S1P receptors (Fig. 7) is consistent
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with the previous observation that S1P itself inhibited TNFα-induced adhesion molecule expression (15). In the previous study (15), we showed that the S1P1 receptor antisense oligonucleotide was more effective than the S1P3 receptor antisense oligonucleotide for NOS activation and that the S1P1 receptor antagonist VPC23019 but not the S1P3 receptor antagonist CAY10444 reversed the S1P inhibition of TNFα/H9251-induced adhesion molecule expression, suggesting that the role of the S1P1 receptor is more important than that of the S1P3 receptor in the inhibitory pathway. Confirmation of the dominant role of the S1P1 receptor in the inhibitory actions by HDL-associated S1P is shown in Fig. 8: siRNAs specific to the S1P1 and S1P3 receptors were used to down-regulate S1P receptors. Under conditions in which siRNAs specifically inhibited the respective S1P receptor expressions (Fig. 8A), siRNA for the S1P1 receptor but not for the S1P3 receptor significantly suppressed the HDL-induced inhibition of the TNFα action (Fig. 8B).

The roles of lipid and protein components of HDL and the specificity of siRNAs were further confirmed, as shown in Fig. 9. rHDL, which was prepared by the delipidation of HDL and...
subsequent reconstitution with phosphatidylcholine, clearly stimulated NOS activity, although less effectively than HDL (Fig. 9A). As expected, the rHDL-induced action was hardly affected by S1P1 receptor siRNA but was almost completely inhibited by SR-BI siRNA when the respective siRNA specifically inhibited the HDL- and S1P-induced actions to the extent expected (Fig. 9A). It is worth noting that S1P1 siRNA inhibited the HDL-induced NOS activation to the level of rHDL-induced activation without any effect on the rHDL-induced action. These results further support not only the specificity of S1P1 siRNA but also the role of both an S1P component and a nonlipid component, probably apolipoprotein A-I of HDL.

Consistent with the change in the NOS activity, rHDL inhibited TNFα/H9251-induced VCAM-1 expression, and the inhibitory action of rHDL was reversed by SR-BI siRNA but not by S1P1 siRNA (Fig. 9B).
As shown in Fig. 1, S1P alone stimulated adhesion molecule expression in association with NF-κB activation, although the magnitude of the action was much less than that of TNFα. In the previous study, S1P-induced adhesion molecule expression and NF-κB activation were markedly inhibited by the S1P3 receptor-specific antisense oligonucleotide but were only partly inhibited by the S1P1 receptor-specific antisense oligonucleotide (15), suggesting that the S1P3 receptor is a dominant receptor responsible for adhesion molecule expression. However, the stimulatory S1P action was also PTX-sensitive, as was S1P-induced NOS activation, an obligatory process of the inhibitory pathway (15). These results suggest that PTX-sensitive G proteins play roles in both stimulatory and inhibitory pathways to adhesion molecule expression. The apparently strange observation may be explained by the involvement of G12/13 proteins or Gq proteins in the stimulatory pathways because previous studies have shown that S1P3 receptors are coupled to multiple G proteins, including Gq/11, G12/13, and Gi proteins (32). We first examined the possible involvement of Gq proteins in S1P3 receptor-mediated actions. For this purpose, we employed YM-254890, a Gq/11-selective inhibitor (33). Even though YM-254890 inhibited the S1P-induced increase in intracellular Ca2+ concentration, it exerted no significant effect on S1P-induced VCAM-1 expression and NF-κB activation (data not shown). On the other hand, siRNA specific to either Gα12 or Gα13 significantly inhibited S1P-induced VCAM-1 expression (Fig. 10B) and NF-κB activation (Fig. 10C). A combination of both siRNAs markedly inhibited the S1P-induced actions. However, siRNA for Gα12, siRNA for Gα13, or their combination did not affect S1P-induced NOS activation (Fig. 10D). These results suggest that both Gq/11 proteins and G12/13 proteins may be necessary for the stimulation of S1P3 receptor-mediated NF-κB activation and adhesion molecule expression.

**DISCUSSION**

HDL has been shown to exhibit a wide range of anti-atherogenic actions, one of which is the inhibition of the expression of adhesion molecules, including VCAM-1 and ICAM-1 (3–5). Although S1P is a bioactive lipid component of HDL that mediates endothelial cell survival, endothelial cell migration (9, 10),
and vasodilation (11), the lipid mediator has also been shown to stimulate the expression of pro-atherogenic adhesion molecules, such as VCAM-1 and ICAM-1 (12–15). This S1P action has been listed as evidence that S1P is pro-atherogenic in the vascular system (6, 7, 12, 34). However, the S1P-induced pro-atherogenic action disappears in the presence of HDL. In the present study, we have demonstrated for the first time that SR-BI plays a critical role in the HDL-induced inhibition of adhesion molecule expression. In addition to the scavenger receptor, S1P receptors are in some cases also involved in HDL signaling. Thus, HDL seems to inhibit adhesion molecule expression through dual mechanisms involving SR-BI and S1P receptors, especially S1P1. Our proposal is shown in Fig. 11.

Although S1P stimulated the adhesion of monocytes to endothelial cells in association with the expression of VCAM-1 and ICAM-1 (12–15), the lysolipid simultaneously inhibited the TNFα-induced adhesion of monocytes on endothelial cells (15, 16). Both stimulatory and inhibitory actions may be mediated by S1P receptors; the stimulatory action involves NF-kB activation predominantly through the S1P3 receptor, and the inhibitory action involves PI 3-kinase/eNOS predominantly through the S1P1 receptor (Refs. 15 and 16 and the present paper). Thus, when the cells are exposed to exogenous S1P, the level of adhesion molecule expression may be determined by the balance of the "stimulatory signal" through NF-kB activation and the "inhibitory signal" through NOS activation.

The role of S1P3 receptors in S1P-induced NF-kB activation is further supported by the finding that S1P2 and S1P3 receptors, but not S1P1 receptors, can activate NF-kB in HEK293 cells transfected with the respective S1P receptor subtype (35). S1P2 receptors have not been detected in HUVECs (20, 36). Based on PTX sensitivity, it can be inferred that the toxin-sensitive G12/g protein may play roles in both the S1P1 receptor-mediated activation of the PI 3-kinase/NOS system and S1P3 receptor-mediated NF-kB activation. The distinction between the stimulatory S1P3 receptors and the inhibitory S1P1 receptors may be determined by the additional coupling of S1P3 receptors to G proteins, e.g. Gq/11 or G12/13 proteins, other than...
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G_{i/o} proteins (32). Although it has been suggested that G_q proteins are involved in S1P-induced NF-κB activation in S1P_3 receptor-transfected HEK293 cells (35), our results suggest the involvement of G_{12/13} proteins rather than G_{q/11} proteins in HUVECs. The down-regulation of G_{12/13} proteins suppressed S1P-induced NF-κB activation and VCAM-1 expression (Fig. 10), whereas a selective inhibitor of G_{q/11} proteins, YM-254890, was ineffective (data not shown). This finding is very consistent with those of recent studies (37, 38) in which G_{12} proteins or G_{13} proteins have been reported to mediate NF-κB activation through RhoA in endothelial cells.

The HDL-triggered inhibitory pathway may involve the SR-BI system. The involvement of SR-BI was suggested by the inhibition of HDL-induced actions by SR-BI siRNA (Figs. 3 and 5–7). Moreover, reconstituted HDL composed of delipidated lipoprotein (mainly apolipoprotein A-I) and phosphatidylcholine stimulated NOS activity and inhibited VCAM-1 expression in an SR-BI-specific manner (Fig. 9). SR-BI mediates the HDL-induced inhibitory pathway through PI 3-kinase and NOS, as evidenced by the complete inhibition of HDL-induced actions by the enzyme-specific inhibitors (Fig. 1). PDZK1, an SR-BI-associated protein, may play an important role in the scavenger receptor-mediated activation of the PI 3-kinase/NOS system. PDZK1 was initially isolated as an SR-BI-associated protein and was thought to be involved in the regulation of SR-BI protein levels (18, 19). In the present study, however, we observed that the down-regulation of PDZK1 expression by siRNA specific to PDZK1 inhibited HDL-induced NOS activation and reversed the lipoprotein-induced inhibition of adhesion molecule expression without any significant effect on SR-BI mRNA and protein expression (Fig. 4). These results suggest that PDZK1 plays a scaffolding role to maintain SR-BI in the correct place on the plasma membrane to mediate HDL-induced actions. The role of the PDZ-interacting domain of SR-BI has recently been suggested in HDL-induced eNOS activation in bovine aortic endothelial cells (24).

HDL-induced NOS activation may also be partially explained by the S1P component of the lipoprotein, which is reflected in the partial sensitivity to PTX and the down-regulation of S1P receptors (Figs. 6 and 9). Under the experimental conditions, exogenous S1P can be partly associated with HDL and thereby the bioactivity of S1P to stimulate the S1P_3 receptor and the subsequent NF-κB/VCAM-1 pathway might be somehow reduced. This assumption is attractive because it explains, in part, the lack of exogenous S1P to stimulate the NF-κB/VCAM-1 pathway in the presence of HDL. However, it is also true that HDL-associated S1P maintains significant bioactivity. We previously reported that HDL-associated S1P stimulated both S1P_1 and S1P_3 receptors, promoting the migration of HUVECs (10).

Taken together, HDL has the ability to stimulate the PI 3-kinase/NOS system either by S1P receptors through its S1P component or by SR-BI, probably through its apolipoprotein A-I component. The finding that apolipoprotein B-rich LDL failed to inhibit S1P-induced VCAM-1 expression (Fig. 1C) despite LDL binding to SR-BI (39) supports the role of apolipoprotein A-I as a critical component of HDL to stimulate SR-BI. The involvement of dual receptor systems in the HDL inhibition of adhesion molecule expression was clearly observed when S1P was replaced with TNFα. Thus, inhibitory actions of HDL on TNFα-induced adhesion molecule expression and NF-κB activation were partially suppressed by either SR-BI siRNA or S1P receptor antisense oligonucleotides and were completely abolished by their combination (Fig. 7).

The findings observed in the present study are consistent with those of recent studies (24, 29, 30) showing that apolipoproteins in the HDL particle may play a critical role in the stimulation of SR-BI-mediated NOS activation, leading to the relaxation of thoracic aortic rings (29). Furthermore, Nofer et al. (11) recently reported that HDL-induced and NO-mediated relaxation of the thoracic aortic rings was inhibited by ~50% in aortic rings derived from the S1P_3 receptor knock-out mouse. It remains unclear whether the divergence in the receptor subtype involved in NOS activation between the present study (human umbilical vein) and the mouse study (aorta) is explained by the different species or the different vessels; however, in the mouse study, the participation of the S1P_1 receptor was not addressed (11). The predominant role of the S1P_1 receptor in the activation of the NOS and PI 3-kinase/Akt pathways has already been reported in HUVECs (15, 40, 41), bovine aortic endothelial cells (42), and lung microvascular endothelial cells (43). The role of S1P receptor subtypes in NOS activation should be addressed in detail in future studies.

The mechanism by which NO attenuates NF-κB activation, a critical step in the expression of adhesion molecules, remains unclear. Xia et al. (12, 17) reported the involvement of sphingosine kinase in HDL action; HDL inhibited TNFα-induced NF-κB activation and adhesion molecule expression through the inhibition of sphingosine kinase-mediated intracellular S1P accumulation. At the present stage, it is not easy to include sphingosine kinase as one of the signaling molecules in the action mechanism of HDL leading to the inhibition of adhesion molecule expression as proposed in the present study (Fig. 11). There is still controversy concerning the primary target of S1P. Our results suggest the involvement of the cell surface of S1P receptors, whereas Xia et al. (12, 17) suggested unidentified intracellular targets. Several recent reports have supported the role of the cell surface of S1P receptors at least for exogenously applied S1P (13–16). The relationship of NO synthesis and sphingosine kinase activity may be a key to solving the problems underlying the HDL-induced inhibition of NF-κB activity and adhesion molecule expression and should be addressed in future research.

In conclusion, although S1P alone stimulates the expression of adhesion molecules, such as VCAM-1 and ICAM-1, its proatherogenic action is lost in the presence of HDL. SR-BI plays a critical role in HDL-induced actions. In addition to SR-BI, S1P receptors, especially S1P_1, may mediate the HDL-induced inhibition of adhesion molecule expression when an inflammatory cytokine such as TNFα is a pro-atherogenic stimulant.

Acknowledgments—We are grateful to Dr. Masatoshi Taniguchi (Astellas Co., Tsukuba, Japan) for the generous gift of YM-254890 and Chisuko Uchiyama for technical assistance.
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