High-Density Lipoprotein Stimulates Endothelial Cell Migration and Survival Through Sphingosine 1-Phosphate and Its Receptors

Takao Kimura, Koichi Sato, Enkhzol Malchinkhuu, Hideaki Tomura, Kenichi Tamama, Atsushi Kuwabara, Masami Murakami, Fumikazu Okajima

Objective—Plasma high-density lipoprotein (HDL) level is inversely correlated with the risk of atherosclerosis. However, the cellular mechanism by which HDL exerts antiatherogenic actions is not well understood. In this study, we focus on the lipid components of HDL as mediators of the lipoprotein-induced antiatherogenic actions.

Methods and Results—HDL and sphingosine 1-phosphate (S1P) stimulated the migration and survival of human umbilical vein endothelial cells. These responses to HDL and S1P were almost completely inhibited by pertussis toxin and other specific inhibitors for intracellular signaling pathways, although the inhibition profiles of migration and survival were different. The HDL-stimulated migration and survival of the cells were markedly inhibited by antisense oligonucleotides against the S1P receptors EDG-1/S1P₁ and EDG-3/S1P₃. Cell migration was sensitive to both receptors, but cell survival was exclusively sensitive to S1P₁. The S1P-rich fraction and chromatographically purified S1P from HDL stimulated cell migration, but the rest of the fraction did not, as was the case of the cell survival.

Conclusions—HDL-induced endothelial cell migration and survival may be mediated by the lipoprotein component S1P and the lipid receptors S1P₁ and S1P₃. (Arterioscler Thromb Vasc Biol. 2003;23:1283-1288.)

Key Words: high-density lipoprotein • sphingosine 1-phosphate • migration • EDG • endothelial cell

Plasma lipoproteins are responsible for the transport of cholesterol to cells and the control of cholesterol synthesis. Low-density lipoprotein (LDL) provides cholesterol to cells through LDL receptors, whereas high-density lipoprotein (HDL) has been shown to remove excess cholesterol from the cells. The so-called reverse transport of cholesterol is thought to be an important mechanism for the antiatherogenic actions of HDL. Recent studies have shown that HDL induces cytoprotective actions, proliferation, and migration in endothelial cells, activities presumably independent of cholesterol metabolism, although the mechanism by which HDL induces these antiatherogenic actions has not been well characterized. It has been reported recently that HDL activates endothelial nitric oxide (NO) production through the scavenger receptor-BI (SR-BI). NO production has been shown to be involved in the cytoprotective action of endothelial cells.

In endothelial cells, sphingosine 1-phosphate (S1P) has been shown to regulate a wide range of cellular activities associated with angiogenesis, wound healing, apoptosis, and atherosclerosis. S1P promotes cell migration, DNA synthesis, cell survival, cell barrier integrity, NO production, and the expression of several cell adhesion molecules. We recently reported that S1P accumulates in the lipoprotein fraction, especially the HDL fraction, and that HDL-associated S1P mediates the cytoprotective actions of HDL in human umbilical vein endothelial cells (HUVECs). Nofer et al reported that sphingosylphosphorylcholine (SPC) and lysosulfatide (LSF) were major components of HDL responsible for these cytoprotective actions. Thus, lipoprotein-associated lipids may also be involved in some HDL-induced actions independent of cholesterol metabolism; however, the species of lipid components are controversial. In the present study, an extension of the previous study, we examined the role of the lipid components of HDL in the stimulation of endothelial cell migration, which may be an important antiatherogenic action of lipoproteins. We also reexamined the role of S1P in the cytoprotective activity of HDL by comparing it with the roles of SPC, LSF, and lysophosphatidic acid (LPA), which are reported to be present in HDL and other lipoproteins. We found that S1P may mediate HDL-induced migration as well as the cytoprotection of HUVECs through the G-protein–coupled lipid receptors EDG-1/S1P₁ and EDG-3/S1P₃.

Methods

Please see the online data supplement (which can be accessed at http://atvb.ahajournals.org) for materials, preparation of reconsti-
HDL Contains Adequate Amount of S1P to Duplicate Lipoprotein-Induced Cell Migration

Consistent with the previous report, HDL stimulated the migration of HUVECs in a dose-dependent manner. S1P also stimulated the cell migration (Figure 1). In these experiments, HDL or S1P was placed in the lower chamber and then cells were loaded on the upper chamber of the Boyden apparatus. We also used Transwell chemotaxis chambers, where the cells were first attached on the filers, and then the migration activity in response to HDL and S1P was determined. In this system as well, we observed a significant migration activity by HDL and S1P; the migration activity was increased 305±5% by 100 μg/mL HDL and 345±38% by 100 nmol/L S1P. In the separate experiments, we observed no significant effect of HDL and S1P on the adhesion of the cells on the dishes; the degree of the cells attached on the dishes 1 hour after plating was 80±9% by control, 83±8% by 100 μg/mL HDL, and 81±11% by 100 nmol/L S1P. These results clearly indicate that HDL and S1P enhance migratory activity of the cells but not their adhesion activity to the filter or extracellular matrix.

LDL also stimulated the migration by Boyden chamber method, but to a lesser extent. Oxidation of these lipoproteins markedly modulated their migration activity: oxidized HDL (oxHDL) reduced its activity to the level of LDL and oxLDL failed to exert any significant effect. Reconstituted HDL (rHDL), which was prepared by delipidation of HDL and subsequent reconstitution with phosphatidylcholine and cholesterol, was ineffective (Figure 1, inset), suggesting that lipid components rather than apolipoprotein components may be important for the induction of migration. The S1P content in these lipoproteins was 118±6 pmol/mg protein in HDL, 41±5 pmol/mg protein in oxHDL, and 48±12 pmol/mg protein in LDL (3 observations). The lipid content in oxLDL and rHDL was out of the range of determination (less than 8 pmol/mg protein). Thus, the migration activity induced by lipoproteins was roughly parallel to their S1P content. Based on the S1P content in HDL, the dose-response curve of S1P was overlaid on the same figure (Figure 1). The dose-response curves of HDL and S1P almost completely overlapped, suggesting that the amount of S1P in HDL is high enough to account for the migration activity of HDL. The reconstitution of the inactive rHDL with the corresponding amount of S1P recovered its activity to the level of the native HDL (Figure 1, inset).

HDL and S1P Stimulate Migration Through G1-Proteins and Intracellular Signaling Pathways Involving Phosphatidyl Inositol 3-Kinase and Rho Kinase

The migration response to HDL and S1P was completely inhibited by PTX, suggesting an involvement of toxin-sensitive G1-proteins (Figure 2). Whereas the cytoprotective response to HDL and S1P was markedly inhibited by PD98059, the extracellular signal-regulated kinase (ERK) kinase inhibitor failed to inhibit the migration response. In contrast, the p38 MAPK inhibitor SB203580 greatly inhibited it (Figure 2). In accordance with these observations, HDL and S1P activated p38 MAPK in a PTX-sensitive manner, as shown in online Figure IA (please see http://atvb.ahajournals.org). These results suggest that p38 MAPK is critical for HDL-induced cell migration. Phosphatidyl inositol 3-kinase (PI3-K) and Rho kinase have also been shown to be involved in endothelial cell migration.11,12,16,17 The migration response to HDL and S1P was markedly inhibited by wortmannin, a PI3-K inhibitor, and Y-27632, a Rho kinase inhibitor, which supports the above findings. Wortmannin inhibited the activation of p38 MAPK, but Y-27632 did not (see online Figure IA). Thus, p38 MAPK seems to be located downstream of G1-proteins and PI3-K but seems to be independent of Rho kinase.
In addition to p38 MAPK, Akt is located downstream of PI3-K and is involved in S1P-induced migration. We examined the relationship of p38 MAPK and Akt in the signaling pathways. Both HDL and S1P stimulated the formation of the active phosphorylated Akt in a manner sensitive to PTX and wortmannin (online Figure IB). However, the phosphorylation of Akt was insensitive to either SB203580 or Y-27632. These results suggest that Akt activation is dependent on Gi-proteins and PI3-K but not on Rho kinase, as was the case for p38 MAPK activation, but is independent of p38 MAPK itself. Thus, p38 MAPK and Akt are regulated by the same upstream Gi and PI3-K signaling pathways, but they seem to independently control downstream migration activity. In addition to the PI3-K pathways, Rho kinase may participate in the cell migration response to HDL and S1P.

HDL was indistinguishable from S1P with respect to the migration signaling mechanism. In addition, HDL contains a high amount of S1P to duplicate the lipoprotein action (Figure 1). When the cells were pretreated with S1P, they did not respond to S1P that was subsequently applied. Under these conditions, HDL-induced Akt phosphorylation was also completely lost without any reduction of the activity by vascular endothelial growth factor (VEGF) (online Figure IC). These results suggest that S1P or its related lipid mediator may mediate HDL-induced Akt activation and the downstream migration response to the lipoprotein.

SPC, LPA, and S1P Are Potential Lipid Mediators for Endothelial Cell Migration and Survival

As shown in Figure 3A, SPC, LPA, and S1P significantly stimulated migration in a dose-dependent manner, although the potency of SPC was roughly 2 orders lower than that of S1P and the potency of LPA was 4 orders lower. We could not detect any significant stimulation by LSF. S1P and SPC also stimulated cell survival, but neither LPA nor LSF was effective (Figure 3B). We have previously shown that S1P and HDL activate ERK and its activation is critical for their cytoprotective actions. Consistent with cell survival activity, SPC, but not LSF or LPA, induced the activation of ERK (data not shown).

Involvement of S1P1 and S1P3 in HDL-Stimulated Cell Migration and Survival

Transfection of antisense oligonucleotides against S1P1 and S1P3 resulted in a dramatic decrease in the expression of the respective S1P receptor mRNA in a specific manner (Figure 4A and online Figure IIA). The migration response to HDL and S1P was approximately 50% inhibited by the respective antisense oligonucleotide and 90% inhibited by their combination (Figure 4B). Under these conditions, the VEGF-induced activity was unchanged, indicating that the antisense strategy is efficacious and selective. Neither S1P1 nor S1P3 antisense oligonucleotide hardly affected the LPA-induced migration (Figure 4B), whereas they were effective for the SPC action (online Figure IIB). These results suggest that both S1P1 and S1P3 are necessary for the full migration response to HDL, S1P, and SPC but that LPA is not a ligand for these S1P receptors and is not a major mediator of lipoprotein-induced migration.

For cell survival activity, S1P may be a major receptor, as evidenced by the marked inhibition by S1P1, but the insignificant inhibition by S1P3 antisense oligonucleotides of HDL- and S1P-induced cell survival activity (Figure 4C). Likewise, S1P may be crucial to SPC-induced survival (online Figure IIC).
S1P Is a Major Component of HDL Responsible for Endothelial Cell Migration

Although SPC is a potential mediator of HDL-induced actions, its participation may be ruled out (Figure 5). In this experiment, components of HDL were separated into 3 fractions: fraction a, lipid fractions containing the majority of lipids, including SPC; fraction b, lipids soluble in an alkaline aqueous solution, such as S1P and LSF; and fraction c, substances soluble in an aqueous solution. In the previous study, we showed that cell survival activity was recovered in fraction b.4 Migration activity of HDL was also recovered in the S1P-rich fraction b but not in the potentially SPC-rich fraction a or c (Figure 5A). The lipid fraction b was additionally separated by high-performance thin-layer chromatography (Figure 5B), in which authentic S1P, SPC, and LSF migrated to apparently different positions. Although the S1P-rich fraction 5 clearly stimulated cell migration and cell survival,4 neither the potentially SPC-rich fraction 1 nor the potentially LSF-rich fraction 6 induced migration (Figure 5B). These results strongly suggest that the HDL-associated component responsible for cell migration and survival may be S1P but not SPC or LSF.

Discussion

The present study showed that HDL stimulated endothelial cell migration and survival through S1P, and S1P-specific receptors. First, both HDL and S1P stimulated early intracellular signaling pathways in association with cell migration and survival. The involvement of intracellular signaling pathways in these antiatherogenic actions was supported by their inhibition by several specific inhibitors for G- proteins, PI3-K, Rho kinase, ERK kinase, and p38 MAPK, although the inhibition profiles of migration and survival differed. Second, desensitization of the S1P receptors led to the disappearance of the HDL- and S1P-induced phosphorylation of ERK4 and Akt (online Figure IC); the activation of these enzymes seems to be essential to survival and migration, respectively (see below). Third, the inhibition of the expression of S1P1 and S1P1 by the antisense oligonucleotide against the respective receptor resulted in the inhibition of HDL- and S1P-induced survival and migration (Figure 4).

Fourth, HDL contains an adequately high amount of S1P to account for lipoprotein action. Finally, the S1P-rich fraction and chromatographically purified S1P from HDL stimulated cell migration, but the other fractions did not (Figure 5), which was also true for cytoprotective action.4

Nofer and colleagues20,22 recently reported that SPC and LSF were concentrated in HDL and that these lipids mediate HDL-induced cytoprotective actions. In the present study, we confirmed that exogenous SPC mimics antiatherogenic mi-
against S1P1 and S1P3 did not exert any significant effect on the induction of significant migration of H9262 cells. However, Nofer et al. stated that LSF plus SPC were major lipid components of HDL. In the present study, LPA was also partially recovered. However, HDL contains at most 100 pmol/mg protein LPA, as estimated based on its ability to inhibit cAMP accumulation in LPA1-expressing RH7777 cells (data not shown). We usually used 100 μg protein/mL HDL, which corresponds to 10 nmol/L LPA. However, the induction of significant migration activity required ≈100 to 1000 nmol/L LPA (Figure 3A), and no significant effect was detected at up to 10 μmol/L LPA on survival (Figure 3B). Furthermore, antisense oligonucleotides against S1P1 and S1P3 did not exert any significant effect on exogenous LPA-induced migration (Figure 4). These results may rule out the possibility that SPC, LSF, and LPA mediate HDL-induced actions.

The reason for the discrepancy between the present results and those of Nofer et al. remains unclear. However, it should be noted that the content of LSF and SPC in HDL estimated by Nofer et al. is extremely high, exceeding the sum of major phospholipid components, ie, phosphatidylcholine and sphingomyelin. The chemical composition of HDL (by weight) is roughly 20% in cholesterol, 10% in triglyceride, 20% in phospholipids, and 50% in protein. Phosphatidylcholine and sphingomyelin account for approximately 80% of the total phospholipids. Based on these values, the content of these phospholipids in HDL is roughly 0.32 mg/mg protein. In accordance with these observations, we noticed that phosphatidylcholine and sphingomyelin are major phospholipids in HDL by HPTLC (data not shown). On the other hand, Nofer et al. stated that LSF plus SPC reached approximately 10 μmol/mg of protein, corresponding to approximately 5 mg of LSF plus SPC/mg of protein in HDL based on their molecular weight of approximately 500. Mysteriously, in Nofer et al.'s procedure by extraction with acetone-tritrate and separation by high-performance liquid chromatography of lipid components of HDL, they did not seem to recognize major phospholipid components phosphatidylcholine and sphingomyelin in HDL; instead, they mentioned that LSF and SPC were major lipid components of HDL.

S1P is concentrated in LDL, although to a lesser degree than in HDL. During oxidation, the S1P content in LDL markedly decreases. This fact may in part explain why LDL significantly induced cell migration but oxLDL did not (Figure 1). In addition, lysophosphatidylcholine accumulates during the oxidation of LDL and this lipid is known to inhibit the migration of endothelial cells. Lysophosphatidylcholine accumulation might also be partially involved in the loss of migration activity by oxLDL.

Results of the experiments using specific inhibitors for the signaling pathways suggest that S1P-induced migration involves G-proteins, PI3-K, small G-proteins such as Rho and Rac, and Akt. The pathways may be composed of 2 major pathways: the PI3K/Akt and Rac pathway, which leads to the formation of cortical actin assembly, and the Rho/Rho kinase pathway, which leads to the formation of focal contact and stress fiber. S1P1 and S1P3 may prefer the former pathway and the latter pathway, respectively, although their coupling is not always strict. The formation of cortical actin assembly may also function as an antiatherosclerosis event by enhancing the endothelial barrier integrity and reducing endothelial permeability. The present results are consistent with this scheme and indicate that HDL signals migration through its component S1P and the lipid receptors S1P1 and S1P3. In addition to these signaling events, p38 MAPK may play an obligatory role in the migration response to HDL and S1P, as evidenced by complete inhibition by a specific inhibitor to the enzyme. p38 MAPK is suggested to be involved in the migration of endothelial cells10,13,26 and other cell types.27–29 The activation of p38 MAPK was sensitive to PTX and wortmannin but not to Y-27632, suggesting that its activity is regulated by G-proteins and PI3-K but is independent of Rho kinase. Rac has recently been shown to be involved in p38 MAPK-dependent migration28,29 and might function as an intermediate signaling molecule to transduce signals from PI3-K to p38 MAPK.

On the other hand, p38 MAPK may play little role in S1P- and HDL-induced cell survival, as suggested by a finding that a p38 MAPK inhibitor failed to inhibit the cell survival response to S1P and HDL. Instead, ERK activation may be essential for this pathway. In addition, PI3-K/Akt-dependent NO synthase activation might play a critical role in the survival of HUVECs, although the involvement of NO in cell survival is controversial. The ERK pathway and the PI3-K/Akt pathway may be independent. The inhibition of PI3-K by wortmannin hardly affected the S1P-induced activation of ERK. We also found that wortmannin did not affect the HDL-induced activation of ERK (data not shown). Although both S1P1 and S1P3 seem to participate in the migration response to HDL and S1P, S1P1 is likely to be a major S1P receptor involved in cytoprotective activity, as suggested by the finding that antisense oligonucleotides against S1P1 but not S1P3 markedly attenuated the cell survival activity of HDL and S1P (Figure 4C).

The proposed mechanisms by which HDL induces antiatherogenic cell migration and survival in addition to the well-known action on cholesterol metabolism in endothelial cells are shown in Figure 6. As for cell survival and migration, the lipid component S1P in HDL may play an important role through the S1P receptors S1P1 and S1P3, which couple to G-proteins. The S1P/ERK pathway is essential for cell survival, and both S1P1 and S1P3 are required for cell migration through the PI3-K/Akt and p38 MAPK pathway and the Rho/Rho kinase pathway. A similar mechanism mediated by S1P may account for other HDL-induced antiatherogenic actions independent of cholesterol metabolism. Cholesterol metabolism, on the other hand, is mediated by apolipoprotein receptors such as SR-B1 and.
Figure 6. Proposed mechanism by which HDL induces antiatherogenic actions on cholesterol metabolism, cell migration, and cell survival in endothelial cells. See text for more detail.

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7. HDL exerts antiatherogenic actions through multiple mechanisms involving more than 2 kinds of receptors and their intracellular signaling systems.
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ONLINE SUPPLEMENT

Methods

Materials

S1P was purchased from Cayman Chemical Co., L-α-Dipalmitoylphosphatidylcholine, LSF and lysophosphatidic acid (LPA) were from Sigma, SB203580 and wortmannin were from Calbiochem-Novabiochem, SPC was from Biomol Research Laboratories Inc., and p38 mitogen activated protein kinase (p38 MAPK) and the Akt assay kit were from New England Biolabs. Y-27632 was kindly provided by Welfide. Plasma lipoproteins were prepared by density gradient centrifugation as previously described. Human plasma was collected from normal healthy volunteers. OxLDL was prepared by oxidizing with 10 µmol/L CuSO₄ as described previously. Extent of its oxidation was assessed by measuring the thiobarbituric acid-reactive substances (TBARS), which reached more than 10 nmol malondialdehyde equivalents/mg protein. In the case of HDL, 20 µmol/L CuSO₄ was used to produce oxHDL with the same amount of TBARS as oxLDL. Under these conditions, apolipoprotein B in LDL was cleaved to the small
molecular size, but no apparent cleavage of apolipoprotein A in HDL was not detected. The sources of all other reagents were the same as described previously.\textsuperscript{1,2,4,5}

**Preparation of Reconstituted HDL**

HDL was delipidated as described.\textsuperscript{6} The lipid-free apolipoprotein mixture was dialysed against 5 x 1 L Tris-buffered saline (0.01 mol/L Tris buffer (pH 7.4) containing 0.15 mol/L NaCl, 0.01% (w/v) EDTA and 0.02% (w/v) NaN\textsubscript{3}). Reconstituted HDL (rHDL) was prepared by the sodium cholate dialysis method as previously described,\textsuperscript{7} using apolipoprotein mixture/cholesterol/L-\alpha-dipalmitoylphosphatidylcholine molar ratios of 1:5:100.

**Cell Culture and Transfection**

HUVECs (passage number 3) were purchased from Whittaker Bioproducts. The cells (passage number 5-12) were cultured in RPMI 1640 medium supplemented with 15% (v/v) fetal bovine serum (FBS) (Sigma) and several growth factors as previously described.\textsuperscript{4} Where indicated, pertussis toxin (PTX, 100 ng/ml) or its vehicle (2 mmol/L urea) was added to the culture medium 24 h before experiments, unless otherwise stated. Transfection of antisense oligonucleotides to block the expression of S1P\textsubscript{1} and S1P\textsubscript{3} was performed using NovaFECT\textsuperscript{TM} reagent (VennNova) according to the methods of Paik \textit{et al.}\textsuperscript{8} 18-mer phosphothioate oligonucleotides used are as follows: antisense EDG-1/S1P\textsubscript{1}, 5’-GAC GCT GGT GGG CC C CAT-3’; sense EDG-1/S1P\textsubscript{1}, 5’-ATG GGG CCC ACC AGC GTC-3’; antisense EDG-3/S1P\textsubscript{3}, 5’-CGG GAG GGC AGT TGC CAT-3’; and sense EDG-3/S1P\textsubscript{3}, 5’-ATG GCA ACT GCC ACT GCC CTC CCG-3’. The expression of these S1P receptor mRNAs was measured at 12 h and the migration and survival experiments started at 16 h after the transfection.

**Extraction of Active Components of HDL**

This was performed exactly as described previously.\textsuperscript{1} In brief, the components of HDL were separated into three fractions: fraction a, lipid fractions containing the majority of lipids including fatty acids, neutral lipids and phospholipids; fraction b, lipids such as
S1P soluble under an alkaline aqueous solution; and fraction c, substances soluble in an aqueous solution. SPC was recovered in fraction a approximately 90% and S1P was recovered in fraction b approximately 90% as determined by including [\( ^3\)H]SPC or [\( ^3\)H]S1P as an internal standard in the lipid purification procedure. In the case of LSF, most of the lipid was recovered visually in fraction b based on the ninhydrin staining. Fraction b was further processed by a silica gel high-performance thin layer chromatography (HPTLC) (Merck) using a solvent system consisting of 1-butanol/acetic acid/water (3:1:1). The silica gel with the resolved lipids (about 1-cm length each) was scraped off to obtain lipids covering the entire area of migration. The lipids were eluted and dried by evaporation. All fractions thus separated were dissolved in PBS containing 0.4% BSA and were used at the final concentration corresponding to 200 µg proteins/ml HDL.

**Cell Survival Assay**

HUVECs were cultured for 24 h with test agents in fresh RPMI 1640 medium containing 0.1% bovine serum albumin (BSA) unless otherwise specified. The cells were then washed twice with PBS and harvested with trypsin. The viable cells were determined by trypan blue (0.2%) exclusion assay as previously described. The result was expressed as a percentage of the activity obtained in the presence of 15% FBS.

**Migration Assay**

The migration experiment was performed using a blind Boyden chamber apparatus as previously described. In brief, HUVECs were cultured for 4 h with fresh RPMI1640 containing 0.1% BSA. The cells were then washed once, preincubated for 20 min without or with wortmannin, Y-27632, PD98059, or SB203580 at 37 °C in a Hepes-buffered medium containing 0.1% BSA, pH 7.5. These cells were loaded on the upper chamber and test agents were placed in the lower chamber. The number of cells that had migrated during 4 h to the lower surface was determined by counting the cells in four
places under microscopy at x 400 magnification (4HPF).

**Measurement of S1P Content in Lipoproteins**

This was performed by a radioreceptor assay based on the competition on EDG-1, one of S1P receptors, expressed on Chinese hamster ovary cells.

**Evaluation of p38 MAPK and Akt Kinase Activity**

HUVECs were cultured and pretreated with several inhibitors in a way similar to that shown in the migration experiments. The cells were then incubated for 5 min with test agents. For measurement of p38 MAPK and Akt activity, the reaction was terminated by washing twice with ice-cold PBS and adding 0.5 mL of lysis buffer, as previously described. The lysate was electrophoresed and analyzed by Western blotting with an Akt-specific and phospho-Akt-Ser\(^{473}\) antibody. For measurement of p38 MAPK activity, the active (phospho) p38 MAPK in the lysate was immunoprecipitated and its activity was evaluated by its ability to phosphorylate the activating transcription factor (ATF)-2 fusion protein as previously described.

**RNA Extraction and Northern Blot Analysis**

Total RNA was prepared from HUVECs according to the manufacturer's instructions for TRIZOL reagent (Life Technologies). Northern blot analysis was performed as described previously. The bands were quantified by a densitometer.

**Data Presentation**

All experiments were performed in duplicate or triplicate. The results of multiple observations were presented as means ± SD or means ± SEM of at least three separate experiments unless otherwise stated. In the case of Western or Northern blotting, a representative result from at least three separate experiments was shown unless otherwise stated.

**References for Online Supplement**

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**Figure I.** Effects of PTX and other inhibitors for intracellular signaling pathways. In A and B, the cells were pretreated without (Control) or with PTX (24 h with 100 ng/mL); SB203580, an inhibitor for p38 MAPK, (20 min with 1 μmol/L, SB); wortmannin, an inhibitor for PI3-K, (20 min with 100 nmol/L, W); or Y-27632, an inhibitor for Rho kinase, (20 min with 1 μmol/L, Y) and then assayed for 5 min without (None), with S1P (1 μmol/L), or with HDL (100 μg proteins/mL) to evaluate the p38 MAPK activity (A) and the Akt phosphorylation (B). p38 MAPK activity (A) was inhibited by PTX and wortmannin but not by Y-27632, suggesting that p38 MAPK seems to be located downstream of Gi and PI3-K but appears to be independent of Rho kinase. Akt activity (B) was also inhibited by PTX and wortmannin but not by Y-27632 similarly to p38 MAPK, whereas it was not susceptible to SB203580, suggesting that Akt also seems to be located downstream of Gi and PI3-K but independent of p38 MAPK. C, Effect of S1P pretreatment on the HDL action. After the usual 4 h-culture, the cells were further cultured for 5 h with or without S1P (1 μmol/L) in RPMI1640 medium containing 0.1% BSA, and then assayed for 5 min without (None), with S1P (1 μmol/L), or with HDL (100 μg proteins/mL) to measure phosphorylation of Akt. In A–C, a representative result from three separate experiments is shown. The other two experiments gave similar results.
Figure II. Inhibition of S1P receptor mRNA expression by antisense oligonucleotide transfection (A) and modulation of SPC-induced actions (B and C). HUVECs were treated with sense (s) or antisense (as) oligonucleotides. A, 12 h after the transfection, Northern blot was performed for S1P1, S1P3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs. Quantitation of the autoradiograph was done based on the ratio of S1P1/GAPDH mRNA or S1P3/GAPDH mRNA. Data are means±SEM of three separate experiments. Sixteen h after the transfection, migration (B) and survival (C) response to SPC (10 µmol/L) was measured. Please see Methods for more detail (on line supplement).