Sphingosine 1-Phosphate May Be a Major Component of Plasma Lipoproteins Responsible for the Cytoprotective Actions in Human Umbilical Vein Endothelial Cells*

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Sphingosine 1-phosphate (S1P), a novel lipid mediator, is concentrated in the fraction of lipoproteins that include high density lipoprotein (HDL) and low density lipoprotein (LDL) in human plasma. Here, we show that oxidation of LDL resulted in a marked reduction in the S1P level in association with a marked accumulation of lysophosphatidylcholine (LPC). We therefore investigated the role of the lipoprotein-associated lipids especially S1P in the lipoprotein-induced cytoprotective or cytotoxic actions in human umbilical vein endothelial cells. The viability of the cells gradually decreased in the absence of serum or growth factors in the culture medium. The addition of oxidized LDL (ox-LDL) accelerated the decrease in the cell viability. LPC and 7-ketocholesterol mimicked ox-LDL actions. On the other hand, HDL and LDL almost completely reversed the serum deprivation- or ox-LDL-induced cytotoxicity. Exogenous S1P mimicked cytoprotective actions. Moreover, the S1P-rich fraction and chromatographically purified S1P from HDL exerted cytoprotective actions, but the rest of the fractions did not. The cytoprotective actions of HDL and S1P were associated with extracellular signal-regulated kinase (ERK) activation and were almost completely inhibited by pertussis toxin and PD98059, an ERK kinase inhibitor. The HDL-induced action was specifically desensitized in the S1P-pretreated cells. Taken together, these results indicate that the lipoprotein-associated S1P and the lipid receptor-mediated signal pathways may be responsible for the lipoprotein-induced cytoprotective actions. Furthermore, the decrease in the S1P content, in addition to the accumulation of cytotoxic substances such as LPC, may be important for the acquisition of the cytotoxic property to ox-LDL.

Plasma lipoproteins are responsible for lipid transport to cells and control of cholesterol synthesis. Low-density lipoprotein (LDL)1 provides cholesterol to cells through LDL receptors, and this lipoprotein is thought to play an important role in atherosclerosis after undergoing oxidative modifications (1–4). Thus, ox-LDL is present in atherosclerotic lesions and exerts a variety of biological actions, including cytotoxicity on the cells of the artery wall, potentially involved in atherogenesis (1–4). Recent studies show that LPC mimics some of ox-LDL-induced actions (5–8). On the other hand, HDL levels have been shown to be inversely correlated with the risk of cardiovascular disease (1–4). Several mechanisms have been proposed for the anti-atherogenic functions of HDL. These include the promotion of the efflux of cholesterol from atherosclerotic plaques, inhibition of the oxidative modification of LDL, and inhibition of the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) (1–4). HDL has also been shown to protect endothelial cells from serum deprivation- and ox-LDL-induced cytotoxicity (1–4, 9, 10), but the mechanisms by which HDL exerts cytoprotective action are not fully understood.

S1P, one of the sphingolipid metabolites, has been shown to participate in a variety of cellular responses including proliferation, differentiation, adhesion, motility, and apoptosis (11–16). These cellular responses elicited by S1P were first thought to be mediated through an intracellular target(s), but extracellular mechanisms through G-protein-coupled S1P receptors have also been suggested. Supporting the latter extracellular mechanisms, several isoforms of S1P receptors have been identified (11–16). These S1P receptor subtypes are expressed and functioning in a variety of cells including endothelial cells. In vascular endothelial cells, S1P has been shown to regulate a wide range of cellular activities involved in angiogenesis, wound healing, apoptosis, and atherosclerosis (17–20). Thus, S1P induces cell migration, expression of several cell adhesion molecules, DNA synthesis, and cell survival (17–20).

Sachinidis et al. (21) were the first to show that S1P-like lipids are associated with plasma lipoproteins (21). Recently, we specified one of the S1P-like lipids as S1P (22). We also succeeded in quantifying the S1P content in plasma components: this lipid was concentrated per unit amount of protein in lipoprotein fractions with the rank order of HDL > LDL = VLDL > lipoprotein-deficient plasma (albumin fraction) (22). These results raise the possibility that S1P mediates some of the biological actions.
the lipoprotein-induced actions in endothelial cells. In the present
study, we show that S1P may mediate the lipoprotein-
induced cytoprotective actions through S1P receptors and their
intracellular signaling pathways. We also found that oxidation of
LDL markedly reduced its S1P content in association with a
marked increase in cytoplxic LPC content. Thus, plasma lipoprotein-associated S1P may be an important factor for deter-
mining whether these actions are cytoprotective or cytotoxic.

EXPERIMENTAL PROCEDURES

Materials—SIP was purchased from Cayman Chemical Co., and
1-oleoyl-sn-glycero-3-phosphate (lyso phosphatidic acid; LPA), 7-keto-
cholesterol, 25-hydroxycholesterol, 1-palmitoyl (C16:0) lysophosphati-
dylcholine (LPC), and other lipids were purchased from Sigma unless
otherwise noted. A p44/p42 MAP kinase (ERK1/2) enzyme assay kit was
purchased from Amersham Pharmacia Biotech and an ERK-specific
antibody (K-23, amino acids 305–327 of rat ERK1, which recognizes
both ERK1 and ERK2) was from Santa Cruz Biotechnology. 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 human plasma by sequential ultracentrifugation as
described previously (22). Human plasma was collected from normal
healthy volunteers. ox-LDL was prepared by oxidizing for 20 h with 10
μM CuSO4 after extensive dialysis against 150 μM NaCl/PBS (9:1) (5, 6).
For preparation of paraformaldehyde-treated lipoproteins, the lipoproteins (2.5
mg proteins in 1 ml) were treated with 250 mg of BSA-pretreated
charcoal, which was prepared by mixing with 1% BSA, subsequent
washing with PBS, and filtering (0.45 μm). The sources of all other reagents were the same as described previously (15, 19, 22–24).

Cell Culture—HUVECs with passages of 3 were purchased from
Whittaker Bioproducts (Walkersville, MD). The cells (passage number
between 5 and 12) were cultured in RPMI 1640 medium supplemented with 15%
(v/v) FBS (Sigma) and several growth factors as previously
described (19). Where indicated, PTX (100 ng/ml) or its vehicle (final 2
μM) was added to the culture medium 24 h before experiments, unless
otherwise stated. CHO cells, which express Edg-1 or Edg-3, were
cultured as previously described (15, 19, 22–24).

Cell Survival Assay—HUVECs were cultured for 24 h with test
agents in fresh RPMI 1640 medium containing 0.1% BSA unless oth-
erwise specified. In the experiments with PD98059 (10 μM) or
SB203580 (1 μM), the cells were pretreated with these inhibitors for 1 h
and then cultured for another 24 h with test agents in the presence of
these inhibitors. All experiments were performed in duplicate or
tricuplicate. The results of multiple observations were presented as
means ± S.E. of at least three separate experiments unless otherwise
stated. Statistical significance was assessed by Student’s t test.

RESULTS

Oxidation of LDL Resulted in a Decrease in the Lipoprotein-
associated SIP Content—In Fig. 1A, we measured the SIP content in the lipoprotein particles of human plasma by a
radio receptor binding assay, which we recently established
(23). For this quantitative measurement, SIP was extracted
from lipoproteins. Consistent with the previous result (22), SIP
contents in LDL and HDL reached about 100–200 pmol/mg protein,
respectively, which are 20–40 times higher than the SIP con-
tent in the lipoprotein-deficient plasma (22). Because oxidation
of LDL is thought to be a major risk factor for the development of
atherosclerosis, we examined the effect of oxidation on the
SIP content in LDL. The CuSO4 treatment of LDL induced
degradation of Apo B (Fig. 1B). The copper treatment also
induced a marked accumulation of LPC at an expense of reduc-
tion of phosphatidylcholine (Fig. 1C) (5, 6). Under these condi-
tions, the SIP content was reduced to about 25% of the initial value
(Fig. 1A).

To examine whether the change in the SIP content is re-
lected in the functional activity, we measured SIP receptor-
mediated phospholipase C-stimulating activity by the intact
lipoprotein samples without the extraction procedure of SIP. In
the vector-transfected CHO cells, inositol phosphate produc-
tion in response to lipoproteins regardless of the lipoprotein
species was very small (Fig. 1D, upper panel). On the other
respectively (number of observations was four). For other experimental
conditions, see "Experimental Procedures.” In A and D, data are means ± S.E. of four separate experiments. In B and C, a representative result from four separate experiments is shown.

hand, in the S1P receptor Edg-3-overexpressing CHO cells, HDL and LDL markedly stimulated inositol phosphate production reflecting activation of phospholipase C, whereas ox-LDL exerted only a small effect on the activity (Fig. 1D, lower panel). It is reasonable to assume that the increase in the activity induced by the receptor transfection may be mediated by the S1P receptor. Thus, the change in the S1P content in lipoprotein particles seems to reflect their ability to stimulate the S1P receptor.

HDL and LDL Protect HUVECs from Cytotoxicity Induced by Serum-deprivation and ox-LDL—When HUVECs were cultured without serum or growth factors, the cells gradually lost their viability and were detached from the dishes. At 24 h after serum deprivation, only 50% of the cells had survived (Fig. 2A). Under these conditions, both HDL and LDL at 100 μg/ml almost completely reversed the serum deprivation-induced cytotoxicity (Fig. 2A). On the contrary, ox-LDL accelerated the cytotoxicity (Fig. 2A). As shown in Fig. 2B, the oxidative lipids, including 7-ketocholesterol, 25-hydroxycholesterol, and LPC, which were accumulated during LDL oxidation (5, 6, 27, 28) mimicked the ox-LDL-induced action. The cytotoxicity induced by these agents including ox-LDL was reversed by HDL (Fig. 2B). LDL was also effective for inhibiting the ox-LDL-induced action (Fig. 2C).

S1P has been shown to protect HUVECs from cytotoxicity or apoptosis induced by serum deprivation (18-20). We confirmed this observation (Fig. 2D). Furthermore, we found that S1P also inhibited the ox-LDL-induced cytotoxicity (Fig. 2D). Thus, S1P mimicked cytoprotective action of HDL or LDL. LPA has also been shown to regulate the variety of functions of HUVECs (29), but this lipid was ineffective for cytoprotection of HUVECs at concentrations less than 10 μM and exerted a rather cytotoxic effect at higher concentrations (data not shown). We also examined the effects of other lipids including platelet-activating factor, phosphatidic acid, phosphatidylinerine, phosphatidylglycerol, and phosphatidylethanolamine, but we could not detect any significant cytoprotective effect at concentrations lower than 10 μM (data not shown).

HDL and S1P-induced Cytoprotective Actions May Be Mediated by G i/Go Protein-regulated ERK Pathways—We next examined the signaling pathways involved in the S1P and HDL-induced cytoprotective actions. For this, we used PTX, an inhibitor for G i/Go protein functions; PD98059, an inhibitor for MAP kinase. Any drug treatment minimally affected the viability of the cells in the presence of serum (Fig. 3A). Among these agents, a prior treatment of the cells with PTX or PD98059, but not SB203580, almost completely inhibited S1P- or HDL-induced cytoprotective actions against the cytotoxicity induced by serum deprivation and ox-LDL (Fig. 2B). When LDL was used instead of HDL, we observed a similar cytoprotective action that was sensitive to both PTX and PD98059 (data not shown).

These results suggest involvement of G i/Go proteins and ERK in the S1P- and HDL-induced actions. Actually, S1P and HDL induced the phosphorylation of the ERK 1/2 as evidenced by the gel mobility-shift (Fig. 4A) and activated the enzyme as evidenced by the phosphorylation of the ERK-specific substrate peptide (Fig. 4, B and C). As expected, the activation of ERK was completely suppressed by the treatment of the cells with
PTX and PD98059 (Fig. 4, A and D). These results indicate that HDL and S1P-induced cytoprotective actions may be mediated by ERK signaling pathways that are regulated by Gi/Go protein-coupled receptors.

**S1P May Be a Major Component Mediating the HDL-induced Cytoprotective Actions**—Thus, we could not discriminate the action mode of HDL from that of S1P. This suggests that HDL-induced cytoprotective actions may be mediated by S1P. To demonstrate this possibility, we performed desensitization experiments as shown in Fig. 5. When the cells were treated with S1P, the ERK activity peaked at around 5 min and then gradually decreased to the initial level at around 5 h (data not shown). After the S1P pretreatment, the cells no longer responded to the second applied S1P, but ATP-induced ERK activation was little affected by the S1P pretreatment (Fig. 5). Thus, the cells were undergoing homologous desensitization when the cells were pretreated with S1P. Under these conditions, HDL-induced ERK activation was also completely lost (Fig. 5). Thus, S1P seems to mediate HDL-induced ERK activation and hence the cytoprotective action of the lipoprotein.

The participation of S1P in the HDL action was further confirmed in Fig. 6. In this experiment, 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 soluble under an alkaline aqueous solution such as S1P and LPA; fraction c, substances soluble in an aqueous solution. The cytoprotective activity (Fig. 6A) and ERK-activating activity (Fig. 6B) of HDL were recovered in the S1P-rich fraction b but not in fraction a or fraction c. The lipid components of fraction b were further separated by an HPTLC (Fig. 6E), in which S1P was mostly recovered in the fraction 4. The S1P-containing fraction 4 clearly induced the cytoprotective action (Fig. 6C) and ERK activation (Fig. 6D).

**Charcoal Treatment Attenuated Not Only Cytoprotective Actions of HDL and LDL but Also Cytotoxic Action of ox-LDL**—Finally, we examined the effects of charcoal treatment, which would eliminate low molecular weight substances such as S1P and LPC from lipoprotein action. Charcoal treatment reduced the S1P content to 10–20% of initial value in either LDL or ox-LDL (Fig. 7A) without any significant change in the Apo composition (Fig. 7B). This treatment also markedly removed LPC from the lipoprotein particles (Fig. 7C). Under these conditions, not only cytoprotective action of LDL but also cytotoxic action of ox-LDL was reversed (Fig. 7D). In the case of HDL, however, charcoal treatment only partially (50%) removed S1P from the lipoprotein particles (Fig. 7A) probably because of its tight binding to the lipoprotein (22). Thus, the charcoal treatment exerted a small but significant inhibitory effect on the cytoprotective action of HDL (Fig. 7D).
activity (E) of each fraction corresponding to 200 g/ml HDL measured. Data are means ± S.D. of three values from a representative experiment. Other two experiments gave similar results.

**DISCUSSION**

HDL has been shown to exhibit a wide range of anti-atherogenic functions, including cytoprotective action against cytotoxicity or apoptosis induced by several cytokines, Fas, growth factor-deprivation, and ox-LDL (1–4, 9, 10). Consistent with previous studies, 100 g/ml HDL protected HUVECs from serum deprivation- and ox-LDL-induced cytotoxic action. We also found that HDL exerted the cytoprotective action to an extent comparable with HDL. Considering the characteristics of LDL as a risk factor for atherogenesis, one might wonder if this observation was anomalous. In previous studies, ox-LDL has been repeatedly shown to be cytotoxic, but, to our knowledge, there is no report showing the cytotoxic action of the native LDL. Thus, we postulate that native LDL itself possesses potentially cytotoxic function, although this lipoprotein might acquire cytotoxic character during its oxidation.

The present studies indicate that S1P and its receptor-mediated signaling pathways are important for HDL- and LDL-induced cytoprotective action. First, the S1P-rich fraction and HPTLC-purified S1P from HDL exerted the cytoprotective action, but the rest of the fraction did not (Fig. 6). Second, the removal of S1P by charcoal treatment of HDL and LDL inhibited the cytoprotective action of these lipoproteins, although the effect was small in the case of HDL because of the insufficient removal of S1P (Fig. 7). Third, S1P- and HDL-induced cytoprotective actions were associated with the activation of ERK, and these responses were suppressed by PTX, an inhibitor of Gs/Gi-protein function or PD98059, an inhibitor of ERK kinase (Figs. 3 and 4). These results suggest that Gs/Gi-protein-regulated ERK activation may play an important role in the cytoprotective actions of S1P and HDL. The role of Ca2+ signaling and/or ERK pathway in the S1P-induced cell survival has recently been proposed by other groups (18, 20). Fourth, S1P or HDL-induced, but not ATP-induced, ERK activation was specifically desensitized by a prior stimulation of the cells with S1P, suggesting an involvement of S1P receptors in the HDL action (Fig. 5). In relation to this, it has been reported that TNF-α increases the intracellular S1P level by activation of sphingosine kinase and thereby induces anti-apoptotic action in HUVECs (17). This suggests that an accumulation of intracellular S1P may also exhibit cytoprotective action. However, the same authors also reported that HDL decreased rather than increased the intracellular level of S1P by inhibiting sphingosine kinase (30). Thus, it would be a minor mechanism, if not negligible, that HDL-associated S1P would be incorporated into the cells and thereby induce cytoprotective action. Although we did not specify the subtype of the S1P receptor involved in the HDL actions in the present study, both Edg-1 and Edg-3 may be responsible for the cytoprotective action. Although we did not specify the subtype of the S1P receptor involved in the HDL actions in the present study, both Edg-1 and Edg-3 may be responsible for the cytoprotective action.

In the previous study (9), Apo A as well as HDL exhibited cytoprotective action against ox-LDL-induced cytotoxicity in endothelial cell lines, although HDL was more effective than Apo A. This suggests that not only the lipid component, probably S1P as shown here, but also Apo A may possess the potential cytoprotective activity against cytotoxicity of ox-LDL. However, in that study, the endothelial cell lines seem to be stable for serum deprivation and ox-LDL; the cells survived for at least 48 h even without serum, and more than 24 h was required for the induction of significant cytotoxic effect by ox-LDL. This was somehow different from our system using HUVECs; about 50% of the cells lost their viability during 24-h culture without serum or growth factors even in the absence of ox-LDL. Similar susceptibility to serum deprivation of HUVECs has been observed by other groups (10, 18, 20). Thus, Apo A might participate in the cytoprotective action of HDL against predominantly late or chronic phase of cytotoxicity. Alternatively, the cytoprotective mechanisms might differ with different sources of endothelial cells.

The present study indicates that S1P mediates the HDL-induced cytoprotective actions through ERK-involving pathways, but it should be noted that there was a considerably large difference in their potency between ERK activation (about 3 nM, see Fig. 4B) and cytoprotective action (30–100 nM, see Fig. 2D), when exogenous S1P effects were compared. On the other
hand, in the case of HDL, the difference was small; 10 μg/ml for ERK activation (Fig. 4C) versus 30 μg/ml for cytoprotective action (Fig. 2A). This peculiar observation may be explained by the notion that S1P is metabolized very fast especially in the absence of lipoproteins. Under the present assay conditions using HUVECs, we observed that the half-life of HDL-associated S1P was about 2 h at 100 μg/ml HDL (which corresponds to ~20 nM S1P), whereas the half-life of exogenous S1P was about 30 min at the same concentration in the absence of HDL but the presence of 0.1% BSA (data not shown). For the ERK assay, the activity was measured 5 min after the addition of test agents, whereas it was measured 24 h after for the cytoprotective activity. Thus, it is reasonable to speculate that a higher concentration of S1P is necessary to observe the long term cytoprotective action compared with the short term ERK activation especially in the absence of HDL.

The mechanism by which ox-LDL induces a variety of responses involved in the development of atherosclerosis was recently extensively investigated although it is still not completely defined (1–4). During oxidation of LDL, several products such as lipid hydroperoxides, oxysterols, and LPC are produced (5, 6, 27, 28). In addition, the production of lipid mediators such as LPA and platelet-activating factor has also been reported (29, 32). Among these oxidative lipid products, LPC has been shown to duplicate a variety of ox-LDL-induced actions including monocyte migration and expression of adhesion molecules on endothelial cells (5–8). As for cytotoxicity, LPC and oxysterols such as 7-ketocholesterol have been shown to mimic the ox-LDL-induced action in vascular endothelial cells (8, 28). Thus, these lipids may be components of ox-LDL responsible for the induction of cytotoxicity, although their molecular targets and the mechanisms causing cytotoxicity remain unknown. This conclusion is further supported by the observation that charcoal treatment of ox-LDL reversed its cytotoxic activity in an association with a marked decrease in LPC content without any apparent change in Apo components (Fig. 7).

In vascular smooth muscle cells, LDL- and HDL-associated S1P-like lipids stimulated DNA synthesis (21). Based on these results, the investigators postulated that the S1P-like lipids might behave as atherogenic mediators and might be increased by oxidation of lipoproteins (21). However, in the present study, we demonstrated that oxidation of LDL markedly reduced, but not increased, its S1P content. The reduction of S1P content by copper treatment was blocked by an antioxidant butylated hydroxytoluene, indicating an oxidation-dependent reaction (data not shown). At present, however, the metabolic pathway of S1P degradation and its mechanism remains uncharacterized. This is an important future subject for investigation. In any event, during LDL oxidation, the contents of cytotoxic LPC and cytoprotective S1P changed reciprocally. The decrease in the S1P content may also be involved in the acquisition of cytotoxicity to ox-LDL. Thus, we propose that the balance between the contents of cytotoxic lipids including LPC and cytoprotective S1P may be an important factor that determines whether plasma lipoproteins are cytotoxic or cytoprotective. This balance might also be an important determinant for lipoproteins to be atherogenic or anti-atherogenic. In this proposal, S1P is postulated to be an atherogenic mediator. In the endothelial cells, S1P has been shown to stimulate tumor cell migration, ox cell proliferation, and cell polarization (18–20, 33). Furthermore, in vascular smooth muscle cells, S1P is a potent inhibitor of cell migration (34). These responses in addition to cytoprotective action seem to favor anti-atherogenic properties. On the other hand, S1P has been shown to induce expression of adhesion molecules such as VCAM-1 and E-selectin in endothelial cells (16). These actions suggest rather atherogenic properties of S1P. Thus, further experiments are necessary to conclude whether S1P is atherogenic or anti-atherogenic.

In conclusion, HDL-associated S1P is a major component of the lipoprotein-induced cytoprotective action in HUVECs. This action is probably mediated by ERK pathways that are regulated by S1P receptors such as Edg-1 and Edg-3. Oxidation of LDL resulted in a marked decrease in S1P content in association with a marked increase in LPC content. Such a reciprocal change in the lysosphospholipid composition may be important for cytotoxicity to ox-LDL.
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J. Biol. Chem. 2001, 276:31780-31785.
doi: 10.1074/jbc.M104353200 originally published online June 26, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104353200

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