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High density lipoprotein-associated lysosphingolipids reduce E-selectin expression in human endothelial cells

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Abstract

Adhesion and recruitment of blood monocytes, processes mediated by cell adhesion molecules including E-selectin, represent an early event in atherogenesis. High density lipoproteins (HDLs) were shown to inhibit cytokine-induced expression of adhesion molecules, but mechanisms underlying this effect are not fully understood. We here investigated the effects of sphingosylphosphorylcholine (SPC) and lysosulfatide (LSF), two lysosphingolipids associated with HDL, on TNF-α-induced E-selectin expression in human umbilical endothelial cells. We found that HDL, SPC, and LSF inhibited E-selectin expression both on mRNA and protein level. In addition, all three agents reduced the number of E-selectin molecules present on endothelial cell surface. The inhibitory effects of HDL, SPC, and LSF on TNF-α-induced E-selectin expression were partially reverted in the presence of suramin, an antagonist of lysosphingolipid receptor EDG-3, or pertussis toxin, an inhibitor of trimeric G proteins. In addition, inhibition of activation of protein kinase Akt with LY294002 but not inhibition of phosphatidylinositol-specific phospholipase C (PI-PLC) with U73122 abolished the restrictive effects of HDL-, SPC-, or LSF on E-selectin expression. We conclude that HDL-associated lysosphingolipids may at least partially account for the inhibitory effects of HDL on cytokine-induced expression of adhesion molecules, and that activations of G-protein-coupled receptors and protein kinase Akt are involved in this process.
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Keywords: High density lipoproteins; Lysosphingolipids; E-selectin; Endothelial cells; Atherosclerosis

A strong inverse correlation between the concentration of high density lipoprotein (HDL) cholesterol and the incidence of coronary heart disease (CHD) has been established in a number of cross-sectional and prospective studies [1]. In addition, studies in animals demonstrated that rising HDL levels either by direct administration of native or reconstituted HDL particles or by overexpression of apolipoprotein A-I (apo A-I), the major protein component of HDL, confers protection against diet-induced atherosclerosis [1]. The negative correlation between CHD and plasma HDL-cholesterol has been traditionally attributed to the ability of HDL to take up cellular cholesterol from periphery and to mediate the transport of excess cholesterol to the liver [2]. However, a recent study suggests that this mechanism may operate only at the lower range of plasma HDL levels [3]. It is thus conceivable that mechanisms other than reverse cholesterol transport may additionally contribute to the anti-atherogenic effects of HDL.

Adhesion of mononuclear cells to the vascular endothelium and their subsequent transmigration into the arterial wall represent key events in the pathogenesis of atherosclerosis [4]. These processes depend on the coordinated expression of E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1), on the endothelial surface. Increased expression of endothelial adhesion molecules has been observed within atherosclerotic plaques and elevated concentrations of their soluble forms were reported in patients with coronary heart disease or dyslipidemia

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[5,6]. Several pro-atherogenic stimuli including inflammatory cytokines, reactive oxygen species, and homocysteine were demonstrated to augment the expression of endothelial cell adhesion molecules [7].

Several studies demonstrated reduced levels of cytokine-induced E-selectin, ICAM-1, and VCAM-1 in endothelial cells in the presence of HDL [8–10]. However, current understanding of mechanisms by which HDL suppresses the expression of endothelial cell adhesion molecules is incomplete. We and others have recently demonstrated that HDL serves as a carrier of several lysosphingolipids with potentially anti-atherogenic properties [11–13]. Here, we show that two HDL-associated lysosphingolipids, sphingosylphosphorylcholine (SPC) and lysosulfatide (LSF), reduce tumor necrosis factor α (TNF-α)-induced E-selectin expression in human umbilical vein endothelial cells (HUVECs) and that this effect depends on the activation of the protein kinase Akt.

Materials and methods

Materials. Enzyme-linked immunosorbent assay (ELISA) for determination of E-selectin, E-selectin-specific forward and reverse oligonucleotide primers, and FITC-conjugated anti-E-selectin antibody were from R&D Systems, Minneapolis, USA. Anti-phospho-specific antibodies against Akt were obtained from New England Biolabs, Schwalbach, EU. RNeasy RNA isolation kit was from Qiagen, Hilden, EU. Superscript II Polymerase, cell culture media, and bovine pituitary extract were purchased by Gibco Life Technologies, Karlsruhe, EU. FURA-2-AM was from Molecular Probes, Port Gebouw, EU. All other reagents were from Sigma, Taufkirchen, EU, and were of the highest purity available.

Cell culture. HUVECs were harvested exactly as described [14]. Cells (2–5 passage) were cultured in gelatin-precoated tissue flasks in RPMI 1640 medium supplemented with FCS (15% v/w), heparin (50 µg/mL), bovine pituitary extract (50 µg/mL), and ciprofloxacin (10 µg/mL). The cells were characterized morphologically by growth contact inhibition and typical cobblestone pattern at confluence.

Isolation of HDL. HDL ($d = 1.125$–1.210 g/mL) was isolated from human plasma by sequential isopycnic ultracentrifugation as described previously [11].

Isolation of RNA and RT-PCR analysis. After treatment with agonists and/or inhibitors HUVECs were washed three times and total RNA was isolated by Qiagen RNeasy kit and further purified by DNase digestion according to the manufacturer's protocol. Two micrograms of total RNA was than reverse transcribed into cDNA using the Superscript II Polymerase. The sequences specific for E-selectin forward and reverse oligonucleotide primers for PCR were designed by R&D Systems. PCR products were visualized by agarose gel electrophoresis. The size of the cDNA fragment specific for E-selectin was 485 bp.

Quantitative determination of E-selectin. For determination of E-selectin protein concentration an enzyme-linked immunosorbent assay (ELISA) recognizing recombinant and natural human soluble and cell-surface bound protein at a concentration range of 0.5–10 ng/mL was used. After treatment with agonists and/or inhibitors, HUVECs were washed three times with PBS and solubilized on ice in 0.2–0.3 mL of 0.5% (w/v) Brij35 in PBS containing protease inhibitors. The cell lysate was centrifuged at 10,000g for 10 min at 4 °C and the clear supernatant was immediately used for determination of E-selectin according to the manufacturer's instructions. Absorbances were read at 450 nm in a 96-well microplate reader (Dynatech) against a blank substrate.

Cell-surface expression of E-selectin. For the flow cytometric analysis for cell-surface E-selectin HUVECs were harvested by mild trypsinization, washed in PBS, and fixed for 30 min with 1% (w/v) paraformaldehyde in PBS. Cells were then incubated for 30 min at 4 °C with a FITC-conjugated anti-E-selectin antibody (10 µL/mL). After washing, membrane antigen expression was measured by fluorescence activated cell sorting (FACS) with the Coulter EPICS XL cytofluorimeter (Coulter Electronics, Hialeigh, FL) in a standard configuration with a 530 nm bandpass filter using excitation by an argon laser at 488 nm. Cells were gated and data were obtained from fluorescence channels in a logarithmic mode. A total of 5000 events were analyzed. Specific binding of monoclonal antibody was calculated by subtracting non-specific binding as determined with a FITC-labeled isotype-specific, non-relevant IgG antibody.

Determination of the intracellular Ca^{2+} concentration. Intracellular Ca^{2+} measurements were performed using the Ca^{2+}-sensitive fluorescence probe FURA2-AM according to established methods [12]. Briefly, HUVECs (1×10^6 cells/mL) were loaded with 5 µmol/L cell-permeant FURA2-AM for 60 min at 37 °C. The fluorescence intensity was recorded at 37 °C using the F2000 spectrophotometer (Hitachi, Tokyo, Japan) with alternate excitation wavelengths of 340 and 380 nm (bandwidth, 5 nm) and the emission wavelength of 510 nm (bandwidth, 5 nm). The intracellular calcium concentration was calculated as previously described [11].

Western blotting. HUVECs were washed with PBS and lysed in 0.18 mol/L Tris–HCl, 0.15 mol/L NaCl, 10% (v/v) Nonidet P-40, 5% (v/v) sodium deoxycholate, 1% (v/v) SDS, 50 mmol/L NaF, 1 mmol/L EGTA, 1 mmol/L orthovanadate, and the protease inhibitor cocktail (Complete, Roche, Germany). Cell lysates (50 µg/lane) were subjected to SDS–PAGE. Thereafter, proteins were transferred to nitrocellulose membranes, which were blocked overnight in Tris-buffered saline containing 5% non-fat dried milk prior to incubations with antibodies. Loading controls were performed with an antibody against an ubiquitously expressed protein (α-actin).

Results

Effect of HDL, SPC, and LSF on E-selectin mRNA levels

We examined the effect of HDL and HDL-associated lysosphingolipids, SPC and LSF, on TNF-α-induced E-selectin gene expression level. To this purpose, confluent HUVECs were incubated for 15 min in the absence or presence of HDL (1 g/L), SPC (10 µmol/L), and LSF (20 µmol/L) and then subjected to stimulation with TNF-α (10 ng/mL) for further 6 h. As shown in Fig. 1, a large amount of E-selectin-specific PCR product could be detected in HUVECs stimulated with TNF-α but not in untreated cells. The E-selectin expression level was decreased in HUVECs pretreated with HDL, SPC, or LSF prior to cytokine stimulation.

Effect of HDL, SPC, and LSL on E-selectin protein levels

To verify the mRNA expression data shown above, an ELISA was used for quantification of both cell-surface and intracellular E-selectin in confluent HUVECs. Basal expression of E-selectin was low, with the anti-E-selectin signal equivalent to that of the isotype control (not shown). Stimulation with 10 ng/mL TNF-α led to a time-dependent elevation of total E-selectin with a maximum
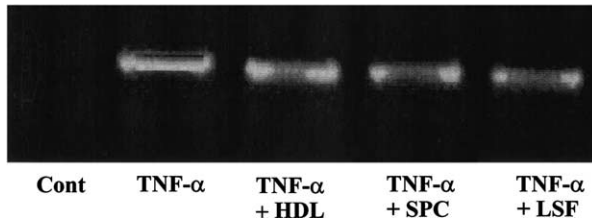
Fig. 1. Effect of HDL, SPC, and LSF on TNF-α-induced E-selectin gene expression level. HUVECs were preincubated for 15 min with HDL (1 g/L), SPC (10 μmol/L), or LSF (20 μmol/L) prior to stimulation with TNF-α (10 ng/mL) for further 6 h. Total RNA was isolated from confluent cells, reverse transcribed into cDNA, and submitted to PCR using E-selectin-specific primers. PCR products were visualized by agarose gel electrophoresis. Shown is typical result from one experiment out of three.

$(2.4 \pm 0.3$ ng/10^5 cells) reached within 4–8 h. E-selectin induction decreased thereafter and reached basal levels by 24–48 h (not shown). Neither HDL nor SPC and LSF affected total E-selectin levels in unstimulated cells. However, preincubation of HUVECs for 15 min with HDL (1 g/L), SPC (10 μmol/L), and LSF (20 μmol/L) prior to cytokine stimulation for 6 h markedly restricted TNF-α-induced E-selectin induction (Fig. 2A). As shown in Fig. 2B, the effects of HDL, SPC, and LSF were concentration-dependent. To investigate whether HDL, SPC, or LSF inhibit TNF-α-induced E-selectin cell-surface expression, confluent HUVECs were pretreated for 15 min with HDL (1 g/L), SPC (10 μmol/L), and LSF (20 μmol/L) before addition of the cytokine for further 6 h and analysis by flow cytometry. As illustrated in Figs. 2C and D, both HDL and lysosphingolipids markedly reduced the amount of E-selectin present on endothelial cell surface. As a result of TNF-α stimulation, $44.9 \pm 11.5\%$ ($n = 3$) cells were positive for E-selectin surface expression. Pretreatment with HDL, SPC, or LSF reduced E-selectin positive cells by $69.1 \pm 4.2\%$ ($n = 3$), $31.6 \pm 2.7\%$ ($n = 3$), and $44.7 \pm 11.1\%$ ($n = 3$), respectively.

Effect of PTX or suramin on HDL-, SPC-, or LSF-induced reduction of E-selectin expression

Previous studies have shown that responses to SPC are at least in some cells mediated by the binding of SPC to the EDG-3 receptor [15,16]. As SPC-induced signaling via the EDG-3 receptor is antagonized by suramin or pertussis toxin (PTX) [15,16], we investigated the effect of both compounds on HDL-, SPC-, and LSF-mediated inhibition of E-selectin expression in endothelial cells. To this end, HUVECs were pretreated with 1 g/L suramin for 10 min or with 100 nmol/L PTX for 16 h and then stimulated for 6 h with 10 ng/mL TNF-α in the presence or absence of HDL (1 g/L), SPC (10 μmol/L), or LSF (20 μmol/L). In control experiments, neither suramin nor PTX affected cytokine-in-
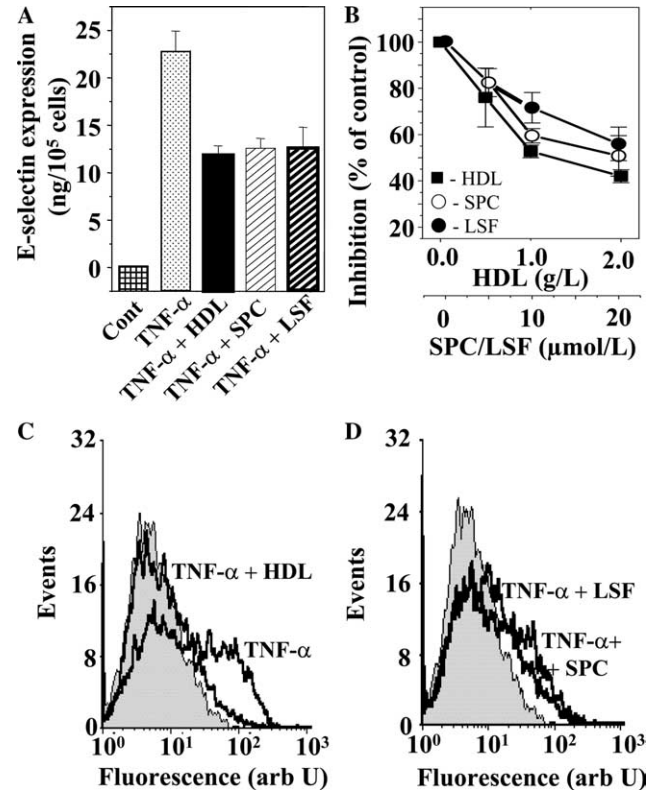

Fig. 2. Effect of HDL, SPC, and LSF on TNF-α-induced E-selectin protein expression level HUVECs were preincubated for 15 min with HDL (1 g/L), SPC (10 μmol/L), or LSF (20 μmol/L) (A, C, and D) or with increasing concentrations of HDL, SPC, and LSF (B) prior to stimulation with TNF-α (10 ng/mL) for further 6 h. (A, B) Cells were solubilized in PBS containing 0.5% (w/v) Brij35 and protease inhibitors and the amount of E-selectin in cell lysates was determined using E-selectin-specific immunoassay system as described under Materials and methods. Shown are results from three to five independent experiments. (C, D) Cells were collected by trypsinization, fixed, and analyzed by flow cytometry with FITC-conjugated anti-E-selectin antibody as described under Materials and methods. Shown are results representative for one experiment out of three.

duced expression of E-selectin (not shown). However, we repeatedly observed reversal of inhibitory effects of HDL or lysosphingolipids on E-selectin expression in HUVECs pretreated with suramin or PTX (Fig. 3).

Involvement of Akt and Ca^{2+} signaling in HDL-, SPC-, or LSF-induced reduction of E-selectin expression

We were further interested whether activation of intracellular signaling pathways accounts for the inhibitory effects of HDL or lysosphingolipids on cytokine-induced E-selectin expression. Both HDL and SPC/LSF were previously shown to activate serine/threonine kinase Akt as well as phosphatidylinositol-specific phospholipase C (PI-PLC)-dependent Ca^{2+} signaling in endothelial cells [11,16]. Therefore, we examined the effect of inhibition of Akt or PI-PLC on the TNF-α-induced E-selectin expression in the presence or absence of HDL, SPC, or LSF. First, the action of inhibitors was tested in our
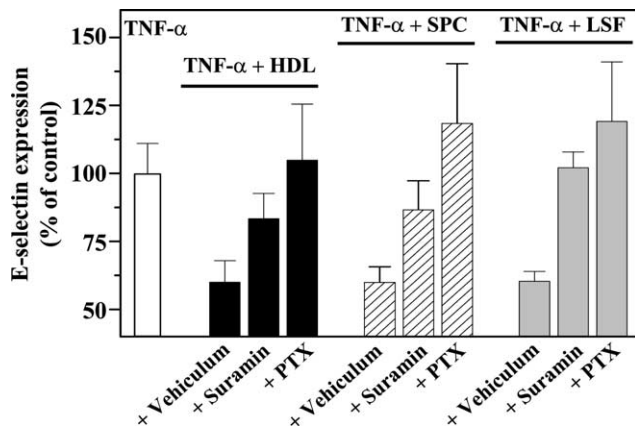
Fig. 3. Reversal of the inhibitory effects of HDL, SPC, and LSF on TNF-α-induced E-selectin protein expression by suramin and PTX. HUVECs were preincubated with 1 g/L suramin for 10 min or with 100 nmol/L PTX for 16 h and then stimulated for 6 h with 10 ng/mL TNF-α in the presence or absence of HDL (1 g/L), SPC (10 μmol/L), or LSF (20 μmol/L). Cells were solubilized in 0.5% (w/v) Brij35 containing protease inhibitors and the amount of E-selectin in cell lysates was determined using E-selectin-specific immunoassay system as described under Materials and methods. Shown are results from three to five independent experiments.

Fig. 4. Reversal of the inhibitory effects of HDL, SPC, and LSF on TNF-α-induced E-selectin protein expression by suramin and PTX (A) HUVECs loaded with FURA2-AM were pretreated for 30 min with U73122 (10 μmol/L) and then exposed to HDL (1 g/L), SPC (10 μmol/L), or LSF (20 μmol/L). Fluorescence was recorded as described under Materials and methods. Original tracings obtained from one representative experiment out of three to four were superimposed for comparison. (B) HUVECs were pretreated for 20 min with LY294002 (20 μmol/L) and then exposed to HDL (1 g/L), SPC (10 μmol/L), or LSF (20 μmol/L). Cell lysates were subjected to SDS–polyacrylamide gel electrophoresis and Western blot analysis using antibodies directed against phosphoserine 473 of Akt (p-Akt). Shown are blots representative for one experiment out of two. (C) HUVECs were preincubated with 10 μmol/L U73122, 20 μmol/L LY294002, or vehiculum (DMSO) for 30 min and then stimulated for 6 h with 10 ng/mL TNF-α in the presence or absence of HDL (1 g/L), SPC (10 μmol/L), or LSF (20 μmol/L). Cells were solubilized with PBS containing Brij35 (0.5% (w/v)) and protease inhibitors and the amount of E-selectin in cell lysates was determined using E-selectin-specific immunoassay system as described under Materials and methods. Shown are results from three independent experiments.

experimental setting. As shown in Fig. 4A, intracellular Ca^{2+} concentration rose from 66 ± 9 nmol/L ($n = 11$) to 217 ± 21 nmol/L ($n = 3$), 233 ± 28 nmol/L ($n = 4$), and 178 ± 15 nmol/L ($n = 4$) in HUVECs stimulated with HDL (1 g/L), SPC (10 μmol/L), and LSF (20 μmol/L), respectively. The increase of intracellular Ca^{2+} concentration brought about by HDL, SPC, or LSF was almost completely abolished in HUVECs pretreated for 30 min with U73122 (10 μmol/L) (Fig. 4A). Fig. 4B demonstrates that HDL (1 g/L), SPC (10 μmol/L), and LSF (20 μmol/L) induced phosphorylation of Akt in HUVECs, which was inhibited in cells pretreated for 30 min with LY294002 (20 μmol/L). To investigate the involvement of PI-PLC or Akt in the inhibitory effects of HDL or lysosphingolipids on E-selectin expression, HUVECs were pretreated with 10 μmol/L U73122 for 30 min or with 20 μmol/L LY294002 for 30 min and then stimulated for 6 h with 10 ng/mL TNF-α in the presence or absence of HDL (1 g/L), SPC (10 mmol/L), or LSF (20 μmol/L). In control experiments, neither U73122 nor LY294002 affected cytokine-induced expression of E-selectin (not shown). Moreover, no effect on E-selectin expression was exerted by U73122 in the presence of HDL, SPC, or LSF. However, the reversal of inhibitory effects of HDL or lysosphingolipids on E-selectin expression was observed in HUVECs pretreated with LY294002 (Fig. 4C).

Discussion

HDL are complex molecules known to exert various potentially anti-atherogenic activities, for which differ-
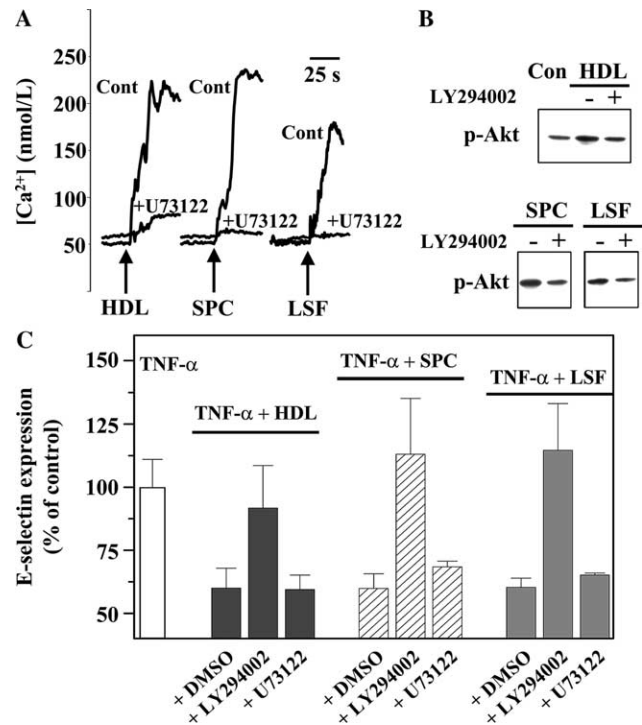
ent components of HDL have been made responsible. We previously demonstrated that while HDL-associated apolipoproteins (apo A-I and apo A-II) accounted for the HDL-induced mobilization of cellular cholesterol, they could not substitute for the intact HDL particle to induce proliferation and to promote endothelial survival [11]. In contrast, we showed that both cell growth and inhibition of apoptosis in response to HDL could be mimicked by two lysosphingolipids associated with native HDL, namely SPC and LSF [11,12]. The present study demonstrates that even a broader spectrum of activity can be attributed to HDL-associated lysosphingolipids. Similarly to native lipoproteins, SPC and LSF potently downregulated levels of TNF-α-induced

E-selectin in HUVECs. The inhibition took place at the transcriptional level as both E-selectin mRNA expression and protein synthesis were suppressed in the presence of lysosphingolipids. As a consequence, SPC and LSF markedly diminished the number of E-selectin molecules present on the endothelial surface.

Several studies demonstrated that in addition to native HDL also reconstituted HDL particles containing solely apo A-I and phosphatidylcholine reduced endothelial expression of adhesion molecules [9,10,17]. These studies would imply that the main HDL component responsible for the inhibition of TNF-α-induced E-selectin expression is identical with apo A-I. However, available information concerning the extent of inhibition brought about by native and reconstituted HDL is inconsistent. Thus, while the original authors suggested that downregulatory effects of reconstituted HDL were comparable to those of native HDL [8], there are other reports showing that reconstituted HDL only partially reduced expression of adhesion molecules in HUVECs [9,17]. Furthermore, a great variation in the ability to modulate VCAM-1 expression was observed between two major HDL subfractions, HDL_2 and HDL_3, and between HDL preparations obtained from different donors despite the fact that HDL used in these studies for pretreatment of endothelial cells contained equal amounts of apo A-I [10]. These findings suggest that other entities in HDL distinct from apo A-I may additionally contribute to the inhibition of the expression of endothelial adhesive molecules. Our observation that HDL-associated lysosphingolipids inhibit E-selectin expression is consistent with this interpretation. There is still lack of systematic studies concerning plasma lysosphingolipid levels in human population. Nevertheless, preliminary evidence points to a considerable inter-individual variability [13]. Various amounts of SPC and LSF present in HDL subjects could account for the marked differences in the inhibitory activities observed among HDL subfractions and HDL preparations from different human subjects.

Several pleiotropic physiological activities were demonstrated to be exerted both by HDL and lysosphingolipids [2,13,18]. These include stimulation of cell proliferation, inhibition of growth factor deprivation-induced apoptosis, and induction of nitric oxide synthase (eNOS) phosphorylation, NO production, and arterial vasorelaxation. The functional similarity between HDL and lysosphingolipids is further reflected by the fact that common intracellular signaling pathways including PI-PLC activation, Ca^{2+} mobilization, and Akt activation are utilized by these compounds. To our knowledge, this is the first report demonstrating that lysosphingolipids inhibit cytokine-induced expression of endothelial adhesive molecules. Furthermore, based on the observation that inhibition of E-selectin expression could be reverted in the presence of LY294002 but not

U73122, we suggest that Akt activation rather than PI-PLC activation and Ca^{2+} mobilization accounts for the HDL and lysosphingolipid-induced inhibition of E-selectin expression. We are unaware of any studies of Akt effects on the expression of adhesion molecules. However, Akt was shown to restrict several signaling pathways such as intracellular generation of reactive oxygen species (ROS) or activation of NF-κB, which are localized upstream to cytokine-induced transcription of E-selectin and other adhesion molecules [19,20]. In this context, it is worth to notice that both ROS generation and NF-κB activity were demonstrated to be attenuated in human endothelial cells in the presence of HDL [21].

Lysosphingolipids including SPC are known to interact with "endothelial differentiation genes" (EDG), a family of heptahelical receptors coupling to several trimeric G proteins including G_i. Accordingly, we found that the inhibitory effects of HDL, SPC, and LSF on TNF-α-induced E-selectin expression were reverted in cells pretreated with PTX, which ADP-ribosylates and thereby irreversibly blocks G_i activation. In addition, we observed that both HDL- and lysosphingolipid-induced inhibitory effects were antagonized by an anionic polycyclic compound, suramin. Since suramin is believed to specifically compete with SPC for EDG-3- but not for EDG-1 or EDG-5-binding [15], these observations may indicate that EDG-3 acts as a preferential binding partner for HDL-associated lysosphingolipids.

There is a great deal of discussion whether lysosphingolipids in plasma exert anti-antherogenic activities or whether the opposite is true. Tamama and Okajima [13] proposed that these compounds promote development of atherosclerosis only while acting as intracellular messengers. However, extracellular lysosphingolipids acting via specific receptors are thought to be potent anti-atherogenic compounds [13]. Our results add further support to this contention by showing that exogenous lysosphingolipids inhibit expression of E-selectin in endothelial cells and thereby potentially restrict transmigration of leukocytes into arterial wall. It is additionally suggested that these compounds may significantly contribute to anti-atherogenic activities exerted by HDL in endothelial cells.

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