Suppression of Endothelial Cell Apoptosis by High Density Lipoproteins (HDL) and HDL-associated Lysosphingolipids

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Apoptotic cell death following injury of vascular endothelium is assumed to play an important role in the pathogenesis of atherosclerosis. In this report, we demonstrate that high density lipoproteins (HDL), a major anti-atherogenic lipoprotein fraction, protect endothelial cells against growth factor deprivation-induced apoptosis. HDL blocked the mitochondrial pathway of apoptosis by inhibiting dissipation of mitochondrial potential (ΔΨm), generation of reactive oxygen species, and release of cytochrome c into the cytoplasm. As a consequence, HDL prevented activation of caspases 9 and 3 and apoptotic alterations of the plasma membrane such as increase of permeability and translocation of phosphatidylserine. Treatment of endothelial cells with HDL induced activation of the protein kinase Akt, an ubiquitous transducer of anti-apoptotic signals, and led to phosphorylation of BAD, a major Akt substrate. Suppression of Akt activity both by wortmannin and LY294002 or by a dominant negative Akt mutant abolished the anti-apoptotic effect of HDL. Two bioactive lysosphingolipids present in HDL particles, sphingosylphosphorylcholine and lysosulfatide, fully mimicked the survival effect of HDL by blocking the mitochondrial pathway of apoptosis and potently activating Akt. In conclusion, the present study identifies HDL as a carrier of endogenous endothelial survival factors and suggests that inhibition of endothelial apoptosis by HDL-associated lysosphingolipids may represent an important and novel aspect of the anti-atherogenic activity of HDL.

EXPERIMENTAL PROCEDURES

Materials—Antibodies to caspase-3, caspase-9, and cytochrome c were from PharMingen, San Diego, CA. Antibody to VDAC (voltage-dependent anion channel) was purchased by Calbiochem, Bad Nauheim, Germany. Anti-phospho-Akt and anti-phospho-BAD were from New England Biolabs, Schwalbach, Germany. YO-PRO, C2938 (6-carboxy-2', 7' dichlorodihydrofluorescein diacetate), and DiOC6 (3,3'-dihexyloxacarbocyanine iodide) were from Molecular Probes, Port Gebouw, The Netherlands. All other chemicals were from Sigma.

Cell Culture—Human umbilical vein endothelial cells (HUVEC) were purchased from PromoCell, Heidelberg, Germany, and maintained in RPMI 1640 medium supplemented with fetal bovine serum (20%, v/v), human basic fibroblast growth factor, hydrocortisone, porcine heparin, and endothelial cell growth supplement as recommended by the supplier. Growth factor deprivation was induced by changing the media to RPMI 1640 without supplements.

The abbreviations used are: ROS, reactive oxygen species; HDL, high density lipoproteins; VDAC, voltage-dependent anion channel; HUVEC, human umbilical vein endothelial cells; HPLC, high pressure liquid chromatography; LSFS, lysosulfatide; SPC, sphingosylphosphorylcholine; DiOC6 (3,3'-dihexyloxacarbocyanine iodide; ΔΨm, mitochondrial potential; GF, growth factors; PI3-K, phosphatidylinositol 3-kinase; dnAkt, dominant negative Akt; AMC, 7-amido-4-methylcoumarin; AFC, 7-amido-4-trifluoromethylcoumarin.
FIG. 1. HDL are an endothelial survival factor. HUVEC were exposed to GF deprivation in the absence or presence of HDL at indicated concentrations for 8 h or at a concentration of 1.0 g/liter for indicated time intervals. Apoptotic cell death was determined by flow cytometry with annexin V (A and C) or Yo-PRO-1 (B and C) or by counting cells detached from substratum (floaters) in the culture medium (D). The number of floaters in HUVEC cultured with GF was 1.2 ± 0.6 × 10³/ml (n = 3).

Isolation of Lipoproteins, Apoproteins, and HDL-Lipid and HDL-Protein Fractions—HDL (d = 1.125–1.210 g/ml) and LDL (d = 1.019–1.250 g/ml) were isolated from human plasma as described by Havel et al. (14). LDL was oxidized with CuSO₄ as described previously (15). Lipid-free apoA-I and apoA-II were isolated by reverse phase HPLC (16). To obtain lipid and protein fractions, 0.1 ml of native HDL (10 g/liter) was diluted with 0.9 ml water, adjusted to pH 3.0 with sulfuric acid, and subsequently mixed with 1 ml acetonitrile. After addition of 0.5 g NaCl, the sample was centrifuged (5 min, 800 × g). The upper organic phase arbitrarily called HDL lipid fraction was dried, dissolved in ethanol, and rapidly injected into phosphate-buffered saline while vortexing. The lower aqueous phase containing apoproteins was arbitrarily called HDL protein fraction, collected, and lyophilized. Both fractions were used for cell stimulation in amounts indexed to original HDL concentration.

HPLC Characterization of Lipoprotein-associated Lysoosphingolipids—To characterize sphingosylphosphocholine (SPC) and lysosulfatide (LSF) contents in HDL and LDL, HPLC analysis of the lipid fractions obtained from each lipoprotein was performed on a Kontron (Neufahrn, Germany) liquid chromatograph with two Model 422 pumps and a Model 440 diode array detector. 0.03 ml of the HDL sample was introduced onto a column using a Rhodyne 7125 loop injector (Cotati, CA). The separation was carried out on a 5 µm Nucleosil 100-Si column (25 cm × 4 mm ID) (Macherey-Nagel, Düren, Germany), with a flow rate of 1.0 ml/min at an ambient temperature. Mobile phase component A was acetonitrile and component B was acetonitrile/water (80:20, v/v). The column was maintained at 30 °C. The elution began with pure component B and was followed after 5 min by a linear gradient to 30% A. Fractions were collected and subsequently freeze/thawed three times. The fractions were analyzed by HPLC for the presence of SPC and LSF.

Western Blotting—HDL were lyzed in 0.18 mol/liter Tris-HCl, 0.15 mol/liter NaCl, 10 % (v/v) Nonidet P-40, 5 % (v/v) sodium deoxycholate, 5 % (v/v) SDS, 100 mmol/liter NaF, 1 mmol/liter EGTA, 1 mmol/liter EDTA, 50 mmol/liter dithiothreitol, Complete protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany), subjected to four freeze/thaw cycles, and centrifuged. Caspase-3 and -9 activities were measured in the supernatant according to the protocol of the CaspACE Fluorometric Assay System (Promega, Mannheim, Germany). AE-DEVD-AMC (excitation wavelength: 460 nm; emission wavelength: 560 nm) and Ac-LEHD-AFC (excitation wavelength: 400 nm; emission wavelength: 505 nm) were used as substrates for caspase 3 and -9, respectively. Fluorescence was determined using a PerkinElmer Life Sciences LS70 fluorimeter. Data were expressed as picomoles of the fluorescence substrate AMC or AFC liberated per minute and µg protein.

Fluorimetric Determination of ROS and Mitochondrial Potential (ΔΨm)—Determination of ROS production and ΔΨm was performed as described by Quillet-Mary et al. (17). Briefly, production of ROS was detected with the fluorescent probe C2938. After labeling with 0.5 µmol/liter C2938 for 1 h floating and adherent cells were collected and washed twice, and fluorescence was determined using a PerkinElmer Life Sciences LS70 fluorimeter (excitation wavelength: 510 nm, emission wavelength: 534 nm). To evaluate ΔΨm cells were labeled with 40 nm DiOC6 and washed twice, and the fluorescence of the suspension was determined (excitation wavelength: 488 nm, emission wavelength: 500 nm).

Subcellular Fractionation and Cytochrome c Release—Cells were washed with cold phosphate-buffered saline, scraped, and centrifuged, and the cell pellet was resuspended in 20 mM Hepes-KOH, pH 7.5, 10 mM KCI, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, 250 mM sucrose, 100 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin and 1 µg/ml aprotinin. Subcellular fractionation was performed as described (15). Briefly, cells were homogenized using a glass Dounce (10 strokes) and were centrifuged at 15,000 × g for 15 min at 4 °C. The particulate represented the mitochondria-containing nuclear-heavy membrane fraction. The supernatant was centrifuged for 15 min at 20,000 × g at 4 °C. This second supernatant represented the cytosol (including the light membrane fraction).

Western Blotting—HUVeC were lysed in 0.18 mol/liter Tris-HCl, 0.15 mol/liter NaCl, 10 % (v/v) Nonidet P-40, 5 % (v/v) sodium deoxycholate, 5 % (v/v) SDS, 100 mmol/liter NaF, 1 mmol/liter EGTA, 1 mmol/liter EDTA, 50 mmol/liter dithiothreitol, Complete protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany), subjected to four freeze/thaw cycles, and centrifuged. Caspase-3 and -9 activities were measured in the supernatant according to the protocol of the CaspACE Fluorometric Assay System (Promega, Mannheim, Germany). AE-DEVD-AMC (excitation wavelength: 460 nm; emission wavelength: 560 nm) and Ac-LEHD-AFC (excitation wavelength: 400 nm; emission wavelength: 505 nm) were used as substrates for caspase 3 and -9, respectively. Fluorescence was determined using a PerkinElmer Life Sciences LS70 fluorimeter. Data were expressed as picomoles of the fluorescence substrate AMC or AFC liberated per minute and µg protein.

Transfection of HUVEC with Dominant Negative Akt (dnAkt) Constructs—HUVEC were transfected with 11 µg DNK (c-myc-tagged dominant negative Akt1 (K179M)) in pUSEamp, Upstate Biotechnology) and a control vector, respectively, using lipid pF7 (Invitrogen) in Opti-mem (Life Technologies, Inc.) as described earlier (19, 20). After 24 h of recovery by culture in complete growth medium, cells transfected with control vector or dnAkt were exposed to growth factor (GF) deprivation in the presence of 1.0 g/liter HDL for 24 h. Floating apoptotic and adherent survived cells were collected and fixed in 3.7% (w/v) paraformaldehyde. Separate analysis of c-myc-positive cells (apoptotic and surviving) in both transfected cell populations (vector control and dnAkt) was performed by fluorescence-activated cell sorter analysis using a primary anti-c-myc-antibody (Upstate Biotechnology) and a secondary fluorescein isothiocyanate-labeled anti-mouse antibody. Apoptosis was calculated as percent c-myc-positive apoptotic cells from all c-myc-positive cells in vector control and dnAkt-transfected cell populations.

RESULTS

HDL Inhibit Apoptosis of Human Endothelial Cells—HUVEC undergo apoptosis when deprived of GF (19, 20). Some of the early molecular events in apoptosis include alterations of the plasma membrane properties such as translocation of phosphatidylserine from the endosomal to the external leaflet and increased permeability. To test the effect of HDL on endothelial cell apoptosis in our system, we exposed HUVEC to GF deprivation in the presence or absence of HDL and quantified phosphatidylserine-positive cells using annexin V staining as a measure of apoptosis. GF deprivation led to a significant increase of annexin V-positive cells over time, which was consistently inhibited by 40–50% in the presence of 1.0 g/liter HDL at any time point measured (Fig. 1A). The time-dependent in-
increase of apoptosis-associated membrane permeability measured with the dye YO-PRO-1 was also substantially inhibited by 1.0 g/liter HDL (Fig. 1B). The blocking effect of HDL on endothelial apoptosis was concentration-dependent, with a maximal suppression at 0.5–1.0 g/liter HDL (Fig. 1C). After undergoing apoptosis, HUVEC detach from the substratum and appear in the supernatant as apoptotic “floaters” (20). HDL suppressed the number of these apoptotic floaters in the conditioned medium in a concentration-dependent manner as well, with an 50–60% inhibition of apoptosis at 1.0 g/liter HDL (Fig. 1D).

**HDL Suppress the Mitochondrial Pathway of Apoptosis**—The execution of the apoptotic program requires the activation of the caspase family of cysteine proteases, which disassemble cells through the selective proteolysis of key protein substrates. One of the principal mechanisms of caspase activation requires the release of cytochrome c from mitochondria, which together with Apaf-1 and dATP/ATP form the “apoptosome,” the complex that recruits and activates the “initiator” caspase-9. In turn, caspase-9 recruits, cleaves, and activates “effector” caspases such as caspase-3 (21). Therefore, we examined the effect of HDL on the activation of caspase-9 and caspase-3 by measuring cleavage of their fluorogenic substrates, Ac-LEHD-AFC and Ac-DEVD-AMC, respectively. HDL dramatically suppressed the activity of both caspase-9 and caspase-3 measured 8 h after withdrawal of GF in a concentration-dependent manner (Fig. 2A). As a biochemical marker for caspase activation, we also determined the effect of HDL on the proteolytic processing of pro-caspases-3 and -9, which is required for the full caspase activity. 1.0 g/liter HDL almost completely abolished the generation of the active 23 kDa caspase-3 subunit and markedly reduced the generation of the active 37 kDa caspase-9 subunit after GF deprivation (Fig. 2B).

To elucidate the impact of HDL on the molecular pathways upstream of caspase activation, we tested the effect of HDL on the dissipation of the \( \Delta \psi_m \) and the generation of ROS, which are associated with the release of cytochrome c into the cytoplasm. After GF deprivation of HUVEC for 8 h a marked decrease of \( \Delta \psi_m \) from 620 ± 162 to 78 ± 17 (n = 3) was observed. This \( \Delta \psi_m \) collapse was reverted in the presence of increasing concentrations of HDL (Fig. 2C). Because \( \Delta \psi_m \) dissipation leads to production of ROS at the ubiquinone site of the respiratory chain, we also investigated ROS generation during endothelial apoptosis. Withdrawal of GFs for 8 h increased ROS levels from 18.2 ± 4.1 (n = 5) to 62.3 ± 8.5 (n = 5), which was inhibited by HDL in a concentration-dependent manner (Fig. 2D). These protective effects of HDL on the mitochondrion were associated with the inhibition of mitochondrial cytochrome c release into the cytoplasm, the initial step for the activation of caspases (Fig. 2E), while cytoplasmic cytochrome c levels gradually increased over time after GF deprivation in the absence of HDL, addition of 1.0 g/liter HDL to the culture medium substantially reduced cytochrome c levels released into the cytoplasm. As expected, the release of cytochrome c into the cytoplasm was accompanied by a decrease in the cytochrome c content in mitochondria, which occurred between 2 and 8 h after GF withdrawal. No such decrease was observed in endothelial cells preincubated with 1.0 g/L HDL at this time. To investigate the specificity of cytochrome c release during apoptosis we examined the effect of GF deprivation on VDAC, a membrane channel expressed in the mitochondrion as described previously (18). As shown in Fig. 2E, no VDAC could be detected in the cytoplasmic fraction of endothelial cells either in the presence or absence of HDL, thus excluding contamination of the cytosolic fraction with mitochondria.

**Akt Is Essential for HDL-mediated Cell Survival**—The protein kinase Akt was identified as an important survival factor that suppresses the mitochondrial pathway of apoptosis in a number of experimental systems (22). To assess the involve-
The percentage of apoptotic cells was determined by flow cytometry with annexin V. Caspase-3 activity in HUVEC lysates was measured as in Fig. 2A. B, HUVEC were starved for 12 h in medium containing 1% fetal calf serum and treated with 1.0 g/liter HDL for indicated times. Cell lysates (50 μg/tube) were subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis using antibodies directed against phosphoserine 473 of Akt (P-Akt) or phosphoserine 136 of BAD (P-BAD). Blots were subjected to densitometric analysis (C and D). Increases in phosphorylation are expressed as fold increase over the basal level in unstimulated cells. Data points represent the average ± S.D. of three independent experiments. E, HUVEC were pre-incubated with 100 nmol/liter wortmannin (WT) or 10 μmol/liter LY-294002 (LY) and then treated with 1.0 g/liter HDL for 8 h as in B. Akt phosphorylation was determined by densitometric analysis of Western blots with phosphospecific antibodies. Data points represent the average ± range from two separate experiments. F, HUVEC were transfected with an expression vector encoding a c-myc-tagged dominant negative form of Akt (dnAkt) or with an empty vector (mock) as described under “Experimental Procedures”. Cells were allowed to recover for 24 h in complete medium and then exposed to GF deprivation in the presence of 1.0 g/liter HDL. Transfected cells were assessed by flow cytometry with anti-c-myc antibodies. The percentage of apoptotic cells was calculated by dividing the number of c-myc-positive floaters by the total number of c-myc-positive endothelial cells. Data points represent the average ± S.D. of three separate determinations.

Fig. 3. Akt mediates the survival signal induced by HDL. A, HUVEC were pre-incubated for 0.5 h with increasing concentrations of wortmannin (left panel) and LY-294002 (right panel) and then exposed to GF deprivation in the presence of 1.0 g/liter HDL and inhibitors for 8 h. The percentage of apoptotic cells was determined by flow cytometry with annexin V. Caspase-3 activity in HUVEC lysates was measured as in Fig. 2A. B, HUVEC were starved for 12 h in medium containing 1% fetal calf serum and treated with 1.0 g/liter HDL for indicated times. Cell lysates (50 μg/tube) were subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis using antibodies directed against phosphoserine 473 of Akt (P-Akt) or phosphoserine 136 of BAD (P-BAD). Blots were subjected to densitometric analysis (C and D). Increases in phosphorylation are expressed as fold increase over the basal level in unstimulated cells. Data points represent the average ± S.D. of three independent experiments. E, HUVEC were pre-incubated with 100 nmol/liter wortmannin (WT) or 10 μmol/liter LY-294002 (LY) and then treated with 1.0 g/liter HDL for 8 h as in B. Akt phosphorylation was determined by densitometric analysis of Western blots with phosphospecific antibodies. Data points represent the average ± range from two separate experiments. F, HUVEC were transfected with an expression vector encoding a c-myc-tagged dominant negative form of Akt (dnAkt) or with an empty vector (mock) as described under “Experimental Procedures”. Cells were allowed to recover for 24 h in complete medium and then exposed to GF deprivation in the presence of 1.0 g/liter HDL. Transfected cells were assessed by flow cytometry with anti-c-myc antibodies. The percentage of apoptotic cells was calculated by dividing the number of c-myc-positive floaters by the total number of c-myc-positive endothelial cells. Data points represent the average ± S.D. of three separate determinations.

The protein kinase Akt is activated by binding of PI3-K-phosphorylated phospholipids and by direct phosphorylation at threonine 308 and serine 473. To determine whether HDL regulate the activity of Akt in endothelial cells, we analyzed whole-cell lysates of HDL-treated HUVEC for Akt activation using a phosphospecific antibody to serine 473 of Akt. In time-course experiments, we found Akt to be activated 5–10 min after exposure to HDL (Fig. 3, B and C). This response gradually decreased after prolonged incubation. Densitometric analysis of signals revealed a maximal 3.4 ± 0.3-fold (n = 3) stimulation by HDL at 1.0 g/liter (Fig. 3C). In addition, HDL induced a time-dependent phosphorylation of BAD, a major substrate of Akt, at serine 136 (Fig. 3B). The maximal 4.2 ± 1.3-fold (n = 2) increase of BAD phosphorylation occurred 30 min after exposure to HDL and decreased thereafter (Fig. 3D). To test the involvement of PI3-K in the activation of Akt in response to HDL, we pretreated cells with 100 nmol/liter wortmannin or 10 μmol/liter LY-294002. Both inhibitors completely abolished the phosphorylation of Akt (Fig. 3E).

To examine whether Akt is directly responsible for the survival effect of HDL, we transfected HUVEC with a c-myc-tagged catalytically inactive Akt mutant, which acts in a dominant negative fashion to suppress the activity of endogenous Akt. We then exposed the transfected HUVEC to GF deprivation in the presence of HDL and determined the percentage of apoptotic cells carrying the c-myc-tagged dominant negative Akt compared with vector controls using fluorescein isothiocyanate-labeled anti-c-myc antibodies and flow cytometry. We observed that almost twice as many endothelial cells expressing dominant negative Akt underwent apoptosis in the presence of HDL as control-transfected cells (Fig. 3F). This experiment documents that HDL mediate survival in endothelial cells via activation of Akt.

SPC and LSF Are Agonists Responsible for the Survival Activity of HDL—To determine specificity of the apoptosis-suppressing effect of HDL we compared it with native and oxidized LDL. As shown in Fig. 4, native LDL exerted no significant inhibitory effects on annexin V binding or caspase-3 activity at physiological concentrations (0.5 g/liter). Similarly, oxidized LDL had no effect on the above parameters of apoptosis during the time monitored (8 h). At longer incubation times (24–48 h) oxidized LDL initiated apoptosis as expected and previously published (5, 6; and data not shown). HDL are complex molecules containing several biologically active proteins and lipids. To determine HDL entities responsible for the inhibition of endothelial apoptosis, we tested different components of HDL for their anti-apoptotic potential. We observed no effect of the HDL protein fraction (0, 8 g/liter), purified apoA-I (0, 25 g/liter), or purified apoA-II (0, 25 g/liter) on annexin V binding or caspase-3 activity after induction of apoptosis (Fig. 4A). In contrast, the HDL lipid fraction exerted a potent anti-apoptotic effect in our system (Fig. 4A). Recently, we demonstrated that HDL serve as a carrier for bioactive lysosphingolipids, such as SPC and LSF (23). The content of SPC and LSF in HDL used in the present study was 1.24 ± 0.38 μg/mg (n = 5) and 4.71 ± 1.12 μg/mg (n = 5), respectively. As shown in Fig. 4B, these two substances are abundantly present in HDL and to much lesser extent in LDL as characterized by HPLC. Therefore, we tested the effect of these two substances on endothelial apoptosis. Both SPC and LSF inhibited the increase of annexin V binding and cell permeability to YO-PRO-1 after GF depri-
vation in a concentration-dependent manner (Fig. 4C). Moreover, caspase-3 and caspase-9 activities after induction of apoptosis were dose-dependently reduced in the presence of SPC and LSF (Fig. 4D). The maximal inhibitory effect of both compounds on apoptosis and caspase activation was observed in concentrations close to those present in HDL particles in serum. Finally, both SPC and LSF induced a time-dependent Akt phosphorylation in endothelial cells with similar kinetics to those of HDL (Fig. 4E). Hence, SPC and LSF represent HDL entities responsible for the inhibition of HUVEC apoptosis by HDL.

**DISCUSSION**

Low HDL cholesterol levels are one of the most predictive coronary heart risk factors (24, 25). The negative correlation between coronary heart disease and plasma HDL cholesterol has been attributed to the ability of HDL to take up excess cellular cholesterol from the periphery and transport it to the liver, a process termed reverse cholesterol transport (26). In addition, several other activities exerted by HDL such as inhibition of monocyte adhesion to endothelial cells, inhibition of platelet activation, or inhibition of LDL oxidation are potentially antiatherogenic (27). In this study, we demonstrate that in contrast to native and oxidized LDL, HDL potently promote endothelial cell survival and protect against apoptosis. HDL suppress the mitochondrial pathway of apoptosis by preserving mitochondrial integrity and thus inhibiting cytochrome c release as well as the subsequent activation of the caspase cascade. As mediator of the anti-apoptotic effect of HDL upstream of the mitochondrion we identify the protein kinase Akt, an important survival factor in a number of experimental systems. We demonstrate that HDL activate Akt via a PI3-K-dependent pathway similarly to growth factors such as insulin (14), vasoendothelial growth factor (28), and angiotropein-1 (29) because the inhibitors of this pathway, wortmannin and LY-294002, abolished HDL-mediated Akt activation. Furthermore, both inhibitors as well as overexpression of dominant negative Akt were found to block the anti-apoptotic activity of HDL in endothelial cells. These findings constitute the direct demonstration that the PI3-K/Akt pathway mediates the anti-apoptotic biological activity of HDL. Although cytoprotective effects of HDL against cell death induced by oxLDL (30) or TNF-α (31) were reported previously, neither the role of mitochondrial apoptosis nor the involvement of Akt were tested, and extremely long incubation times (>20 h) were necessary to demonstrate a protective effect, much in contrast to the HDL-mediated protection already visible 3 h after induction of apoptosis in our system. Moreover, the cytoprotective effects of HDL against oxLDL and TNF-α have been found to be partially mimicked by free apolipoprotein A-I, whereas none of the HDL apoproteins examined in this study had a protective effect in our system. These differences may be due to the different apoptotic stimuli used or may reflect the complexity of HDL-induced intracellular signaling, which we and others have previously shown to involve several independent pathways (32, 33).

Several downstream targets of Akt may account for the anti-apoptotic effect of HDL. We show that HDL induce phosphorylation of the pro-apoptotic protein BAD, an important substrate of Akt. This phosphorylation is known to promote the association of BAD with BCL-XL, which is then free to suppress mitochondrial apoptosis. Because in this study HDL was found to inhibit mitochondrial apoptosis, it is possible that the anti-apoptotic effect of HDL is associated with the phosphorylation of BAD. In addition, HDL inhibited the activity of caspase-9, which is another negatively regulated direct substrate of Akt (22). Recent studies suggest that in some cell systems Akt controls cell survival via regulation of the transcription factor NF-κB (34, 35).
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35. However, because HDL neither induce the translocation of NF-κB to the nucleus nor the binding of NF-κB to specific DNA sequences (36), it is unlikely that this pathway contributes to the anti-apoptotic effect of HDL. Finally, Akt stimulation may enhance endothelial NO synthesis and promote endothelial cell survival by inhibiting caspasas via nitrosylation (37). In this context, it is of interest that HDL counteract inhibitory effects of oxidized LDL on NO production in endothelial cells (38).

HDL are complex molecules known to induce a multitude of intracellular signals, for which different components of HDL have been made responsible. We previously demonstrated that free apolipoproteins A-I and A-II are responsible for the HDL-mediated activation of phosphatidylincholine-specific phospholipase D but not for the activation of phosphatidylinositol-specific phospholipase C (32). Furthermore, neither apoA-I nor apoA-II could substitute for the intact HDL particle in mobilizing intracellular calcium in fibroblasts and endothelial cells (23, 39). In contrast, we showed that both phosphatidylinositol-specific phospholipase C activation, Ca2+ mobilization, and cell proliferation in response to HDL can be mimicked by two lysosphingolipids associated with native HDL, namely SPC and LSF (23). The present study extends the unique biological activities of SPC and LSF to the ability to mediate the anti-apoptotic effect of HDL. Both substances inhibited activation of caspasas and biochemical features of apoptosis in endothelial cells. In addition, SPC and LSF potently stimulated Akt in concentrations similar to those physiologically present in HDL particles in serum. In contrast, neither free apoproteins nor the protein fraction isolated from HDL had any effect. As 93 and 72% of the total SPC and LSF plasma levels, respectively, are present in HDL, these lipoproteins serve as the primary physiological carrier of the two bioactive lysosphingolipids. Consequently, no significant anti-apoptotic effect was observed with other lipoproteins such as LDL.

The intracellular signaling events initiated by SPC and LSF were the focus of several recent studies. In addition to their ability to induce phosphatidylinositol-specific phospholipase C activation and Ca2+ mobilization, SPC has been shown to activate both mitogen-activated protein kinases (Erk-1 and Erk-2) and protein kinase C in several cell lines (40–42). To our knowledge, SPC-induced activation of Akt and its anti-apoptotic activity has not been reported to date. However, sphingosine-1-phosphate, a compound structurally closely related to SPC was recently demonstrated to promote endothelial cell viability, although no signaling pathway has been identified (43). Both SPC and sphingosine-1-phosphate interact with the same receptor termed endothelial differentiation gene 3 (EDG-3) (44). Due to their lipophilicity, SPC and LSF were suggested to act primarily in an autocrine fashion, but their physiological function has been unknown. The present study identifies SPC and LSF as endogenous plasma-derived anti-apoptotic factors, and reveals that the endothelium may constitute an important physiological target for SPC and LSF delivered by HDL. Therefore, maintenance of endothelial cell integrity and protection against potentially deleterious proatherogenic stimuli may constitute a new biological function of HDL and thus may define a new mechanism that contributes to their atheroprotective function.

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