High Density Lipoproteins (HDL) Interrupt the Sphingosine Kinase Signaling Pathway

A POSSIBLE MECHANISM FOR PROTECTION AGAINST ATHEROSCLEROSIS BY HDL.*

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The ability of high density lipoproteins (HDL) to inhibit cytokine-induced adhesion molecule expression has been demonstrated in their protective function against the development of atherosclerosis and associated coronary heart disease. A key event in atherogenesis is endothelial activation induced by a variety of stimuli such as tumor necrosis factor-α (TNF), resulting in the expression of various adhesion proteins. We have recently reported that sphingosine 1-phosphate, generated by sphingosine kinase activation, is a key molecule in mediating TNF-induced adhesion protein expression. We now show that HDL profoundly inhibit TNF-stimulated sphingosine kinase activity in endothelial cells resulting in a decrease in sphingosine 1-phosphate production and adhesion protein expression. HDL also reduce TNF-mediated activation of extracellular signal-regulated kinases and NF-κB signaling cascades. Furthermore, HDL enhanced the cellular levels of ceramide which in turn inhibits endothelial activation. Thus, the regulation of sphingolipid signaling in endothelial cells by HDL provides a novel insight into the mechanism of protection against atherosclerosis.

Numerous evidence from epidemiological, clinical, and genetic studies have clearly shown a potential protective role of high density lipoproteins (HDL) against the development of atherosclerosis and associated coronary heart disease (1–4). Several mechanisms have been proposed for the cardioprotective function of HDL. These include the promotion of the efflux of cholesterol from atherosclerotic plaques and reducing the atherogenicity of LDL by inhibition of their oxidative modification (4–6). Recently, we and other groups have demonstrated an ability of HDL to inhibit endothelial adhesion protein expression, providing a new mechanistic explanation for its protective effect on atherosclerosis (7–11).

Atherosclerosis has been definitely characterized as an inflammatory disease (12). An important event in the initiation of atherosclerosis is adhesion of circulating monocytes to activated endothelial cells and their subsequent transendothelial migration to the subendothelium. This process is mediated by adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin (13, 14). The inappropriate expression of these adhesion proteins in response to the “injury” are induced by various inflammatory stimuli, including cytokines and noncytokines such as interleukin-1, tumor necrosis factor-α (TNF), and oxidized or native LDL (14–16). Pathological studies have shown increased adhesion molecule expression in several components of the atherosclerotic plaque (17–20), and there is also evidence for a role of adhesion molecules in the acute atherothrombotic process (21). Furthermore, a direct association between an increased plasma concentration of soluble adhesion molecules and the increase in risk of future cardiovascular diseases has recently been reported (22, 23).

The ability of HDL to inhibit the cytokines-induced adhesion protein expression has been well documented. It has been reported that human HDL profoundly inhibit the expression of VCAM-1, ICAM-1, and E-selectin in human umbilical vein endothelial cells (HUVEC) activated by TNF or interleukin-1 (7). Total native HDL together with both HDL₂ and HDL₃ subfractions, or the reconstituted HDL particles showed the inhibitory effect in a concentration-dependent manner, although considerable variation existed among different experiments (7–11). The phenotype of inhibition on adhesion molecule expression by HDL differs from their well known function in promoting cholesterol efflux and protecting against lipid peroxidation, suggesting a distinct mechanism exists. We recently demonstrated a novel signaling pathway, sphingosine kinase (SphK) pathway, through the generation of sphingosine 1-phosphate (SIP), which is critically involved in mediating adhesion protein expression and endothelial cell activation after TNF stimulation (24). The SphK pathway has also emerged as a signaling pathway in mediating a variety of cellular functions such as cell growth, proliferation, and inflammatory reactions (24–29). In the present report we show that HDL profoundly inhibit the TNF-induced SphK activity and SIP generation, and subsequently reduce the activation of ERK and NF-κB signaling cascades. Thus, demonstrate that HDL interrupt a signaling cascade, the SphK pathway, which results in inhibition of endothelial activation. This could provide a new potential mechanism by which HDL protect against atherosclerosis, a cardiovascular inflammatory disease.

EXPERIMENTAL PROCEDURES

Materials—TNF was purchased from R&D Systems. C₂-Ceramide, SIP, sphingosine, N,N-dimethylsphingosine (DMS), and dihydrosphingosine were from Biomol (Plymouth Meeting, PA). [3H]Serine and [cho-
line-methyl-\(^{14}C\) sphingomyelin were from NEN Life Science Products. \(\gamma^{\text{32P}}\)ATP was purchased from Bresatec (Adelaide, Australia), and \(1^{25}\text{I}\)-TNF was from Amersham Pharmacia Biotech (United Kingdom). Escherichia coli diacylglycerol kinase was from Calbiochem (La Jolla, CA). Anti-ERK/2 antibodies were purchased from Zymed Laboratories Inc. (South San Francisco, CA). Other chemicals were from Sigma.

**Cell Culture and Flow Cytometry Analysis**—HUVEC were isolated as described previously (30) and cultured on gelatin-coated culture flasks in Dulbecco's modified Eagle's medium containing 20% fetal calf serum, endothelial growth supplement (Collaborative Research) and heparin. In some experiments, cells were treated in Opti-MEM (Life Technologies), containing at appropriate acid-free bovine serum albumin as a serum-free medium. The expression of cell-surface adhesion molecules was measured as described previously (24) by use of a Coulter Epics Profile XL flow cytometer.

**Isolation and Preparation of Lipoproteins**—As described previously (10), the lipoproteins were isolated from normal healthy adult donors by sequential ultracentrifugation in their appropriate density range: total HDL 1.07 < d < 1.21, HDL3 1.13 < d < 1.21, and LDL 1.019 < d < 1.063 g/ml. The resulting preparations of lipoproteins were dialyzed against endotoxin-free PBS (pH 7.4) prior to use. Oxidized LDL was obtained by incubating LDL (500 \(\mu\)g/ml) with confluent cultures of HUVEC in Dulbecco's modified Eagle's medium containing 10 \(\mu\)M CuSO\(_4\) for 24 h, and the oxidation was assessed by the increase of mobile ceramide. Discoidal-reconstituted HDL containing apoA-I and 1-palmityl-2-oleylphosphatidylcholine (POPC) were prepared by the cholate dialysis method described by Matz et al. (31).

**Measurement of Sphingosine Kinase (SphK) Activity**—As described above, cellular ceramide was quantitated by the diacylglycerol kinase reaction as described previously (32). Briefly, the cellular lipids were extracted with chloroform/methanol (2:1) and then solubilized in 1\(\times\)NaOH, 1% SDS and radioactivity was determined in a \(\gamma\)-counter. Specific binding is defined as the difference between total binding and nonspecific binding with excess unlabeled TNF.

**Metabolic Labeling and Sphingolipids Assay**—HUVEC were labeled with \([^{3}H]\)serine (10 \(\mu\)Ci/ml) for 48 h as described previously (24). The cells were then washed three times and incubated for an additional 2 h in the presence or absence of HDL3. After treatment with TNF for the indicated times, cellular lipids were extracted and resolved by thin layer chromatography (TLC) in two different solvent systems: (a) chloroform/methanol/acidic acid/water (50:30:8:5, \(v/v\)) and (b) 1-butanol:acetic acid/water (3.1.1, \(v/v\)). The samples were concomitantly run with standard ceramide standards including sphingomyelin, ceramide, sphingosine, and S1P. Sphingolipid spots were visualized by fluorography, quantitated by scintillation spectrometry, and normalized by radioactivity recovered in total cellular lipids.

**Ceramide Measurement**—In addition to metabolic labeling assay as described above, cellular ceramide was quantitated with the diacylglycerol kinase reaction as described previously (32). Brieﬂy, the cellular lipids were extracted with chloroform/methanol/CH\(_2\)OH/HCl (10) and resuspended into a sample buffer containing 7.5% n-octyl-\(\beta\)-D-glucopyranoside, 5 mM cardiolipin, and 1 mM diethylenetriamine-pentaacetic acid. The samples were reacted with diacylglycerol kinase and \([\gamma^{\text{32P}}]\)ATP in enzyme buffer containing 20 mM Tris/HCl (pH 7.4), 10 mM dithiothreitol, and 15 mM glycerol for 30 min at 22 °C. The product of the phosphorylation reaction, ceramide 1-phosphate, was extracted and resolved by TLC using CHCl\(_3\)/CH\(_2\)OH/H\(_2\)O (65:15:5) as solvent, detected and quantified by the Phosphoimage system. To exclude a possible error caused by some factors in the extracts affecting diacylglycerol kinase (33), synthetic C\(_2\)-ceramide was added in assays as an internal control. There were no changes in the phosphorylated C\(_2\)-ceramide in this assay system.

**Measurement of SpHk Activity**—As described previously (24), cells were homogenized in 20 mM Tris buffer (pH 7.4) containing 20% glycerol, 1 mM diethiothreitol, 1 mM EDTA, 20 \(\mu\)M ZnCl\(_2\), 1 mM Na\(_2\)VO\(_4\), 15 mM NaF, 10 \(\mu\)g/ml leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxypyridoxine. After centrifugation at 13,000 \(\times\)g for 30 min, SpHk activity was measured in the supernatant by incubating [\(\gamma^{\text{32P}}\)]ATP in the serum albumin complex and [\(\gamma^{\text{32P}}\)]ATP for 20 min at 37 °C. The labeled lipids were extracted and resolved two times by TLC in the solvent of CHCl\(_3\)/CH\(_2\)OH/NH\(_2\)OH (65:35:5, \(v/v\)) and 1-butanol:acetic acid/water (3:1.1, \(v/v\)), respectively. The radioactive spots corresponding to authentic S1P were visualized and quantitated by the Phosphoimage system. For kinetic studies, cell extracts were prepared from HUVEC treated with TNF for 5 min after preincubation with an increasing concentration of HDL3 for 4 h. The kinase assay was performed with various concentrations of sphingosine (0, 2.5, 5, and 10 \(\mu\)M).

To measure the SpHk activity in vivo, the formation of S1P was measured in the permeabilized cells as described previously (25).

**Electrophoretic Mobility Shift Assay**—Nuclear extracts were prepared from HUVEC treated for 30 min with vehicle or the indicated agents after preincubation with or without HDL3. The double-stranded oligonucleotides used as a probe in these experiments included 5'-GGATGCATTGGGGATCTTTTACTGAGT-3', which contains a consensus NF-\(\kappa\)B binding site in the E-selectin promoter that is underlined (34). Gel mobility shift of a consensus NF-\(\kappa\)B oligonucleotide was measured by incubating \([\gamma^{\text{32P}}]\)labeled NF-\(\kappa\)B probe with nuclear extracts. The specific DNA-protein complexes were completely abolished by addition of a 50-fold molar excess of unlabeled E-selectin NF-\(\kappa\)B oligonucleotides. RESULTS

**HDL\(_3\) Inhibits Adhesion Molecule Expression and Synthesis in Response to TNF**—In order to minimize possible confounding effects of the variations between the distinct subfractions of total HDL, HDL\(_3\) (d = 1.13–1.21 g/ml) was used in the present study. As shown in Fig. 1A, HDL\(_3\) inhibited by \(\sim70\%\) the TNF-induced expression of VCAM-1 and E-selectin in HUVEC, which was consistent with our previous reports (7, 10). The inhibitory effect of HDL3 was further identified by its reduction of E-selectin mRNA levels (Fig. 1B). To determine whether the inhibitory effect of HDL3 on TNF-induced adhesion protein expression result from alterations of TNF access to its receptors, \(1^{25}\text{I}\)-TNF binding assay was performed. Fig. 1C shows that HDL3 at concentrations of 0.25–1.0 mg/ml (apo A-I) did not significantly affect binding of TNF to HUVEC, suggesting that the effect of HDL3 is not secondary to perturbation of subsequent signaling pathways at the receptor sites.

**HDL\(_3\) Inhibits SpHk Activation and S1P Formation**—Since we have recently identified the SpHk pathway as a potent signaling pathway in mediating TNF-induced endothelial activation, the effect of HDL\(_3\) on this pathway was determined. Consistent with our previous report (24), TNF stimulation of
HUVEC caused a significant increase in cytosolic SphK activity. This activity was profoundly inhibited by HDL₃ pretreatment at a physiological concentration (p < 0.01, Fig. 2A white bars). The inhibitory effect of HDL₃ was dose-dependent with half-maximal inhibition at about 0.41 mg/ml apoA-I (Fig. 2B). To characterize the inhibition of SphK by HDL₃, Lineweaver-Burk plots revealed that HDL₃ treatment altered the kinetics of SphK by decreasing its Vₘₐₓ without significant changes in the Kₘ (Fig. 2B, inset). Moreover, TNF-induced increases in S1P formation and its levels in intact cells were also markedly inhibited by HDL₃ to comparable levels as obtained by N, N-dimethylsphingosine (DMS), a competitive inhibitor of SphK (Fig. 2A, gray and dark bars). These data further confirm the inhibitory effect of HDL₃ on activation of SphK in endothelial cells.

**HDL₃ Does Not Inhibit Sphingomyelinase Activation by TNF**—Given the evidence that HDL₃ inhibited SphK activity and S1P production, we tested the possibility that this inhibition was due to a reduction in sphingomyelin-ceramide turnover, an essential upstream event in S1P metabolic pathway. We previously reported that TNF stimulation of HUVEC rapidly reduced sphingomyelin content and consistently increased cellular ceramide levels by approximately 2 fold peaking at 30 min with return to near basal levels by 2 h (24). Pretreatment with HDL₃ did not interrupt the TNF-promoted sphingomyelinase activation, but significantly delayed the reversion of post TNF sphingomyelin levels to base line and sustained the increased ceramide levels (Fig. 3A). There was a significant difference in the comparison of both sphingomyelin and ceramide levels between pretreatment with and without HDL₃ after 2 h of TNF stimulation (p < 0.01, three separate experiments). The parallel delay in sphingomyelin reversion and accumulation of ceramide strongly argued against the proposition that HDL₃ decreased S1P production by inhibiting the response of sphingomyelin-ceramide cycle to TNF. Interestingly, the addition of exogenous cell-permeable ceramide (C₂-ceramide) induced a dose-dependent inhibition of TNF-induced adhesion protein expression (Fig. 3B). Thus, the increased ceramide levels could be at least partly responsible for the inhibitory effect of HDL₃.

**Intact HDL Particles Are Required for Their Inhibitory Effect**—To gain an insight into which components of HDL₃ are responsible for the inhibitory activity, the effect of apoA-I and lipids isolated from HDL₃ were investigated. In marked contrast to intact HDL₃ particles, lipid-free apoA-I, at the same concentration as HDL₃, did not inhibit SphK activity. Similarly, HDL lipid constituents such as POPC in the form of small unilamellar vesicle also had no inhibitory effect (Fig. 4A). However, when apoA-I and POPC were reconstituted into discoidal HDL, the resulting complexes inhibited the TNF-induced SphK activation comparable to that seen with native HDL particles (Fig. 4A). In parallel, neither apoA-I nor POPC had any significant inhibitory activity on the TNF-induced expression of E-selectin (Fig. 4A, bottom panel), which was in agreement with our previous observations on VCAM-1 expression (10, 11).

As the cardioprotective ability of HDL in vivo appears to be dependent on the presence of LDL (3, 5), we tested whether the inhibitory effect of HDL on SphK is due to the interaction of LDL or other unknown factors in the serum. When cells were incubated in the serum-free conditions, the SphK activities in response to TNF in the presence or absence of LDL were to the same extent as that in the cells cultured with normal growth medium containing 20% fetal calf serum (compare Fig. 4B and 4A). The presence of LDL (250 μg/ml of apoB) in the cultures did not influence SphK activities either at the basal levels or post TNF stimulation. Further LDL had no effect on the inhibitory activity of HDL on SphK activation (Fig. 4B). Additionally, in the presence of oxidized LDL (250 μg/ml of apoB) HDL retained its ability to inhibit SphK activation (data not shown). Thus it is unlikely that the inhibitory effect of HDL resulted from the interaction of LDL, oxLDL or other unknown factors in the serum.

**HDL₃-induced Reduction of Adhesion Protein Expression Is Related to the Inhibition of SphK Activity**—Having shown that HDL inhibited SphK activity and the production of S1P, a novel identified inducer of adhesion protein expression, we further examined the linkage between the inhibition of SphK and reduction of endothelial activation. In the experiment illustrated in Fig. 5A, the HDL₃-induced dose-dependent inhibition of SphK activity was plotted against the reduction of E-selectin expression. There was a significant linear correlation between the inhibitory effects of HDL₃ on SphK activity and E-selectin expression (r = 0.953, Fig. 5A). Furthermore, when the formation of intracellular S1P was inhibited by DMS, a competitive inhibitor of SphK, the TNF-induced adhesion protein expression was also reduced (Fig. 5B). Conversely, both the HDL₃ and DMS inhibitory effects on TNF action were reversed by the
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The expression of adhesion proteins on activated endothelial cells plays an essential role for the inflammatory processes in the pathogenesis of atherosclerosis. The inhibition of adhesion molecules in atherogenesis is strongly supported by several lines of evidence: (i) adhesion molecules are present in atherosclerotic plaques; (ii) increased plasma levels of adhesion molecules are associated with the risks of atherosclerosis; and (iii) a deficiency of adhesion molecules significantly reduces the formation of atherosclerotic fatty streaks in knockout mice lacking the genes of ICAM-1, P-selectin, or both ICAM-1 and P-selectin.

The nature of the inflammatory signals and associated molecular mechanisms that activate adhesion molecule expression in endothelial cells in the atherogenic lesion are unknown. Factors such as TNF and interleukin-1 that are commonly found in inflammatory atherogenic lesions induce the expression of adhesion molecules in cultured endothelial cells. Thus, TNF-stimulated adhesion molecule expression on HUVEC provides a useful model to investigate the signaling pathways involved in the regulation of endothelial cell activation. In this model we have recently identified a novel signaling pathway, the SphK pathway, in mediating TNF-induced adhesion protein expression and endothelial cell activation (24). We found that TNF consistently stimulated SphK activity and the generation of S1P which could account for the reduction of adhesion protein expression. Furthermore, HDL3 treatment did not inhibit the phorbol ester-promoted NF-κB activation (data not shown), indicating a specificity of HDL3 effect in this pathway.

**DISCUSSION**

In this report, we show a novel mechanism of atheroprotection by HDL. In this model, HDL interrupt a signal transduction pathway, the SphK pathway, which is critically involved in endothelial cell activation and adhesion protein expression. The expression of adhesion proteins on activated endothelial cells plays an essential role for the inflammatory processes in the pathogenesis of atherosclerosis (12). The importance of adhesion molecules in atherogenesis is strongly supported by several lines of evidence: (i) adhesion molecules are present in atherosclerotic plaques; (ii) increased plasma levels of adhesion molecules are associated with the risks of atherosclerosis; and (iii) a deficiency of adhesion molecules significantly reduces the formation of atherosclerotic fatty streaks in knockout mice lacking the genes of ICAM-1, P-selectin, or both ICAM-1 and P-selectin (36, 37).

We have previously demonstrated a role of SphK pathway for TNF-promoted NF-κB activation (24) that is essential for the transcription of adhesion molecule genes (35). Fig. 6B shows that HDL3 significantly inhibited the TNF-induced activation of NF-κB by 51 ± 17% (p < 0.01), but did not inhibit that induced by S1P. This inhibition was comparable to that induced by DMS, the SphK inhibitor, suggesting that HDL3 inhibited the SphK pathway resulting in an inhibition of NF-κB which could account for the reduction of adhesion protein expression. Furthermore, HDL3 treatment did not inhibit the phorbol ester-promoted NF-κB activation (data not shown), indicating a specificity of HDL3 effect in this pathway.

**FIG. 4.** A, confluent monolayers of HUVEC were preincubated for 4 h with lipid-free apoA-I (1 mg/ml), POPC (5 mM), or discoidal reconstituted HDL (rHDL, 1 mg/ml apoA-I), followed by TNF (100 units/ml) stimulation. SphK activity (top panel) and the cell-surface expression of E-selectin (bottom panel) were measured, respectively. Values are mean ± S.D.; n = 3, *p < 0.01; †, p < 0.001 versus TNF stimulation alone. B, HUVEC were preincubated with the serum-free medium in the absence (Nil) or presence of rHDL (1 mg/ml apoA-I), LDL (250 μg/ml apoB), or rHDL + LDL for 16 h, and treated with (dark bars) or without (gray bars) TNF (100 units/ml) for 10 min. SphK activity was then measured. Data are presented as mean of two separate experiments.

**FIG. 5.** The reduction of adhesion protein expression is related to inhibition of SphK. A, linear regression plot between the HDL3-induced inhibition of SphK activity and the reduction of E-selectin expression. The cells were pretreated with an increasing concentration of HDL3, and then SphK activity and E-selectin expression were measured after TNF stimulation, respectively. B, the HUVEC were pre-treated with a vehicle, DMS (5 μM), S1P (5 μM), and/or HDL3 (1 mg/ml), followed by TNF (100 units/ml) stimulation for 4 h. The expression of VCAM-1 (gray bars) or E-selectin (dark bars) was then measured. Values are mean ± S.D.; n = 3, *p < 0.01; †, p < 0.001, versus TNF stimulation alone.

addition of S1P (Fig. 5B). This demonstrated that the inhibition of SphK is an important event in the HDL3-mediated reduction of endothelial activation. As a control, neither DMS nor HDL3 prevented S1P-induced adhesion protein expression (Fig. 5B), indicating a specific inhibitory effect on SphK. In addition, DMS did not change cellular ceramide levels (data not shown), suggesting the inhibition of SphK activity was not associated with altered ceramide levels.

HDL3 Inhibits TNF-promoted ERK and NF-κB Activation—The MAP kinase, ERK, has been proposed to be a downstream target in the SphK pathway mediating a variety of cellular functions including adhesion protein expression (24, 26, 29). Fig. 6A showed that both TNF and S1P were approximately equipotent in stimulating ERK activities. Treatment with DMS significantly inhibited TNF-activated ERK, indicating the involvement of SphK in the TNF-activated ERK signal cascade. Preincubation of HUVEC with HDL3 also reduced TNF-stimulated ERK activation by 49 ± 6.2% (p < 0.01), consistent with its effect on reducing cellular levels of S1P.

**FIG. 6.** Effect of HDL3 on ERK and NF-κB activation. A, HUVEC were preincubated with or without HDL3 (1 mg/ml) for 4 h and treated with the indicated agents for 30 min. ERK activities were then assayed with myelin basic protein (MBP) as substrate after immunoprecipitation with antibodies against p42/p44ERK. The kinase reaction products were separated on 10% SDS-PAGE. In parallel, an aliquot of the same samples was analyzed by Western blotting using antibodies against p42/p44ERK and anti-ERK antibodies. The positions of the subunits of active ERK are indicated. B, NF-κB binding activity was measured by electrophoretic mobility shift assay after treatment with a vehicle (lane 1), TNF (100 units/ml, lane 2), S1P (5 μM, lane 3), DMS (5 μM) + TNF (lane 4), HDL3 (1 mg/ml) + TNF or + S1P (lanes 5 and 6) for 30 min. The specific NF-κB binding complexes were identified by competition analyses with the addition of a 50-fold molar excess of unlabeled NF-κB oligonucleotides (lane 7). Results in A and B are representative of at least three similar experiments.

In this report, we show a novel mechanism of atheroprotection by HDL. In this model, HDL interrupt a signal transduction pathway, the SphK pathway, which is critically involved in endothelial cell activation and adhesion protein expression. The expression of adhesion proteins on activated endothelial cells plays an essential role for the inflammatory processes in the pathogenesis of atherosclerosis (12). The importance of adhesion molecules in atherogenesis is strongly supported by several lines of evidence: (i) adhesion molecules are present in atherosclerotic plaques; (ii) increased plasma levels of adhesion molecules are associated with the risks of atherosclerosis; and (iii) a deficiency of adhesion molecules significantly reduces the formation of atherosclerotic fatty streaks in knockout mice lacking the genes of ICAM-1, P-selectin, or both ICAM-1 and P-selectin (36, 37).
HDL inhibited (i) SphK activity, (ii) S1P generation, (iii) S1P levels, (iv) ERK activation and (v) nuclear translocation of NF-κB. Moreover, HDL3-induced inhibition of SphK activity is linear correlating with the reduction of adhesion protein expression, and the inhibitory effects of HDL3 were reversed by the addition of S1P (Fig. 5). Taken together, these results strongly indicated that the inhibition of SphK activation by HDL3 could account for its inhibitory effect on adhesion protein expression and endothelial activation.

The finding that HDL3 not only inhibited the activity and the V_{max} of SphK in a dose-dependent manner but also the generation of S1P and its levels in intact cells (Fig. 2) indicated a primary inhibitory effect of HDL3 on the SphK pathway. On the other hand, it is possible that HDL3 may affect endothelial phenotype by an effect on the sphingomyelin-ceramide turnover since HDL3 increased the TNF-dependent ceramide generation and inhibited the reaccumulation of sphingomyelin (Fig. 3A). It is uncertain whether the ceramide accumulation is primarily due to prolonged hydrolysis by sphingomyelinase or to inefficient metabolism by downstream catalysis. The inhibition of adhesion molecule expression by exogenous ceramide (Fig. 3B) indicated a two-pronged inhibition on endothelial activation by HDL: reduction of S1P formation and increase in HDL-dependent S1P and its levels in intact cells (Fig. 2) indicated a

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