High Density Lipoprotein Binding to Scavenger Receptor, Class B, Type I Activates Endothelial Nitric-oxide Synthase in a Ceramide-dependent Manner*

Recently it has been demonstrated that high density lipoprotein (HDL) binding to scavenger receptors, class B, type I (SR-BI) stimulates endothelial nitric-oxide synthase (eNOS) activity. In the present studies we used a Chinese hamster ovary cell system and a human microvascular endothelial cell line to confirm that HDL stimulates eNOS activity in a SR-BI-dependent manner. Importantly, we have extended these studies to examine the mechanism whereby HDL binding to SR-BI stimulates eNOS. eNOS can be stimulated by an increase in intracellular calcium, by phosphorylation by Akt kinase, or by an increase in intracellular ceramide. Calcium imaging studies and experiments with the calcium chelator, 1,2-bis(o-aminophenoxy)ethane-N,N',N',N'-tetraacetic acid tetra(acetoxymethyl) ester demonstrated that HDL binding to SR-BI does not induce an increase in intracellular calcium. Antibodies specific for activated Akt kinase demonstrated that HDL binding to SR-BI does not induce Akt kinase activation. However, HDL binding to SR-BI caused a reversible increase in intracellular ceramide levels from 97 ± 14 pmol/mg of protein to 501 ± 21 pmol/mg of protein. In addition, C2-ceramide stimulated eNOS to the same extent as HDL, whereas C2-dihydroceramide did not stimulate eNOS. We conclude that HDL binding to SR-BI stimulates eNOS by increasing intracellular ceramide levels and is independent of an increase in intracellular calcium or Akt kinase phosphorylation.

The generation of nitric oxide by endothelial nitric-oxide synthase (eNOS) plays a major role in the normal physiological function of the cardiovascular system. A decrease in the bioavailability of nitric oxide is thought to be involved in the development of atherosclerotic lesions and the development of hypertension (1). Recently Yuhanna et al. (2) demonstrated that high density lipoprotein (HDL) binding to scavenger receptor, class B, type I (SR-BI) stimulates eNOS. HDL is generally considered to be cardioprotective primarily because of its role in reverse cholesterol transport, i.e. the transport of sterol to the liver were the sterol can be converted to bile acids and excreted (3). SR-BI is expressed most abundantly in the liver, adrenal, and ovaries, where it functions as a physiological HDL receptor that mediates the selective uptake of cholesteryl esters from HDL (3). We previously demonstrated that SR-BI is also expressed in endothelial cells where it, along with HDL, prevents oxidized low density lipoproteins from inhibiting agonist stimulation of eNOS (4, 5). The mechanism whereby HDL binding to SR-BI stimulates eNOS is not known.

The enzymatic activity of eNOS is regulated by multiple mechanisms and includes acylation (6, 7), localization (8), protein-protein interaction (9, 10), calcium (11), phosphorylation (12), and ceramide (13). Myristoylation and palmitoylation of eNOS targets the enzyme to plasma membrane regions called caveoleae (7). Once associated with caveoleae, a protein called caveolin directly binds to the catalytic domain of eNOS and maintains the enzyme in an inactive state (9, 10). An increase in intracellular calcium causes calcium-calmodulin complexes to displace caveolin from eNOS and stimulates the generation of nitric oxide (14). When intracellular calcium decreases, caveolin rebinds eNOS and the enzyme returns to an inactive state.

Although eNOS is clearly stimulated by calcium, recent studies have demonstrated that eNOS can also be stimulated in a calcium-independent manner (13). Estradiol-induced activation of phosphatidylinositol 3-kinase causes the translocation, phosphorylation, and activation of Akt kinase (12). Akt kinase phosphorylates serine 1177 in eNOS and activates the enzyme without an increase in intracellular calcium (12). In addition, Igarashi et al. (13) have demonstrated that bradykinin can cause an increase in cellular ceramide levels, which subsequently stimulates eNOS without increasing intracellular calcium.

The present studies confirm the findings of Yuhanna et al. (2) that HDL binding to SR-BI induces the stimulation of eNOS. We have extended these studies to examine the molecular mechanism responsible for the increase in nitric oxide production. Our studies demonstrate that HDL/SR-BI-dependent stimulation of eNOS is independent of an increase in intracellular calcium or Akt kinase-mediated phosphorylation of eNOS. We demonstrate that HDL binding to SR-BI causes an increase in intracellular ceramide levels and that this increase in ceramide appears to be responsible for the increase in eNOS activity.
trifugation as previously described (15). The HDL3 subfraction (g/ml) were isolated from human plasma by density gradient ultracentrifugation terminated by adding 500 (basal) or in the presence of acetylcholine (10 nM), 50 °C. The plates were incubated at 37 °C. The contents of each well were transferred to ice-cold glass test tubes. Ether extraction was performed three times with water-saturated ether to remove the trichloroacetic acid. The samples were neutralized with 1.5 ml of 25 mM HEPES, pH 8, applied to Dowex AG50WX-8 (Tris form) columns, and eluted with 1 ml of 40 mM HEPES buffer, pH 5.5, containing 2 mM EDTA and 2 mM EGTA. [3H]-Citrulline generated was quantified by high performance liquid chromatography counting. In individual experiments performed in 12-well plates, 4 wells were used for each treatment group. Findings were confirmed in at least three independent experiments. NOS activation in the intact cells was completely inhibited by 2 mM nitro-L-arginine methyl ester. To ensure that the treatments (HDL, LDL, ionomycin, ceramide, and nitro-L-arginine methyl ester) did not affect the loading of the CHO cells with tritiated eNOS, the amount of [3H]-arginine associated with each cell was determined. The cells contained 220,000 ± 9,184 dpm/well [3H]-arginine independent of the treatment.

Cytosolic Calcium Analysis—In each experiment, cytosolic calcium was measured simultaneously in 12–14 fura-2-loaded cells using a dual excitation spectrofluorometric system (Zeiss AttoFlour Ratio Vision Work station; Atto Instruments, Inc., Rockville, MD) (19). Prior to analysis, the cells were rinsed three times with PBS and loaded with 5 μM fura-2AM for 10 min at 37 °C. After loading, the cells were rinsed three times with PBS and allowed to recover for at least 15 min. The coverslip was mounted in a closed perfusion chamber (Warner Instruments Corp.), placed on the stage of a Zeiss Axiovert inverted microscope fitted with a 40× fluorescence oil immersion objective, and monitored by gravity-feed with ambient temperature buffer at 1–3 ml/min. In a typical experiment, basal (nonstimulated) fluorescence was monitored for 30–60 s prior to addition of HDL (50 μg/ml), which was added by the rapid change-out of the bathing medium. After 15 min exposure to HDL, cells were again washed thoroughly with PBS and then exposed to ionomycin (1 μg/ml) by the rapid change-out of the bathing medium. Fluorescence was determined using excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Intracellular calcium levels were expressed as a 340:380 ratio.

Ceramide Quantification—The lipids were extracted as described previously (20) and analyzed by thin layer chromatography on silica gel 60 plates (20 × 20 cm) using chloroform:methanol:triethylamine:2-propanol:potassium chloride (0.25%) (30:9:18:25:6, by volume) as the developing solvent. The regions migrating with a standard long chain ceramide were scraped from the plate and eluted from the silica with 1 ml of chloroform:methanol (1:1, v/v) followed by 1 ml of methanol. The combined eluted material was dried in vacuo, then 0.5 nml of N-acetyl-C20-sphinganine was added to the unknown ceramide sample, and the ceramide mass was quantified by high performance liquid chromatography of the long chain bases released after an acid hydrolysis in 0.5 M HCl at methanol at 65 °C for 15 h. Free long chain bases were analyzed as described by Merrill et al. (22).

Statistical Analysis—Least squares analysis of variance was used to evaluate the data with respect to cell fraction, time, and their interaction using the analysis of variance procedure of SigmaStat. When appropriate, fractions were compared within a given time using Tukey’s Honestly Significant Difference test. Means were considered different at p < 0.01.
generated, and the data are presented as a percentage of maximal eNOS activity (plus ionomycin) (Fig. 2A). Control experiments with L-NAME demonstrated that less than 1% of the generated citrulline was not the result of eNOS activity (data not shown). Cells not expressing eNOS did not have any measurable eNOS activity (data not shown), however, HDL increased eNOS activity in cells containing both SR-BI and eNOS in a dose-dependent manner. In contrast, HDL did not stimulate eNOS in cells that were not also expressing SR-BI. Because SR-BI can bind to other ligands, such as LDL (3), we determined whether the activation of eNOS was specific for HDL or solely related to the binding of ligands to SR-BI. The addition of 10 or 50 μg/ml LDL to cells expressing both SR-BI and eNOS did not stimulate eNOS activity (Fig. 2B).

To ensure that these findings were not solely the result of the heterologous expression system, we used SR-BI blocking antibodies (5) and human microvascular endothelial (HME) cells to confirm that HDL stimulates eNOS in a SR-BI-dependent manner. HME cells were pretreated with buffer only, 50 μg/ml blocking SR-BI IgG, or 50 μg/ml isotype-matched nonspecific IgG for 15 min at 37 °C. The cells were then incubated with 0.75 μCi/ml [3H]arginine and 50 μg/ml HDL for 15 min at 37 °C. Fig. 3 demonstrates that HDL stimulated eNOS in cells pretreated with buffer or nonspecific IgG. In contrast, cells pretreated with blocking SR-BI IgG did not increase eNOS activity in response to the addition of HDL.

Mechanism of eNOS Stimulation—eNOS is stimulated by an increase in intracellular calcium, and it has been reported that HDL can induce an increase in intracellular calcium in cells grown in culture (23). Therefore, to determine whether HDL binding to SR-BI causes an increase in intracellular calcium, we loaded CHO cells expressing SR-BI and eNOS with fura-2/AM for 10 min at 37 °C. The cells were then incubated with 50 μg/ml HDL for 10 min while continuously monitoring for calcium-induced fluorescence changes. Fig. 4A demonstrates that the addition of HDL to the cells did not cause an increase in intracellular calcium. However, the addition of the calcium ionophore, ionomycin, induced a calcium influx. To further confirm that intracellular calcium is not involved in HDL-mediated stimulation of eNOS, we treated HME cells with buffer only or 20 μM BAPTA/AM for 20 min to chelate intracellular calcium prior to the addition of buffer, 50 μg/ml HDL, or 10 nM acetylcholine. Fig. 4B demonstrates that pretreatment with BAPTA/AM completely inhibited acetylcholine-induced (calcium-dependent) eNOS stimulation but did not inhibit HDL-induced eNOS activation.

Phosphorylation of eNOS by the protein kinase Akt has been shown to stimulate eNOS independent of an increase in intracellular calcium (12). Active Akt kinase is phosphorylated (12). To determine whether HDL binding to SR-BI promoted Akt activation, we treated CHO cells expressing both SR-BI and eNOS with buffer (15 min), 50 μg/ml HDL (5 or 15 min), or 50 nM PDGF (15 min). The growth factor, PDGF, induced the phosphorylation of Akt kinase (Fig. 5) but did not stimulate eNOS activity (data not shown). In contrast, HDL did not promote Akt kinase phosphorylation, suggesting that Akt kinase is not involved in HDL-mediated stimulation of eNOS. To further confirm this finding, we measured eNOS activity in CHO cells expressing both SR-BI and eNOS in the presence or absence of 100 nM wortmannin and 50 μg/ml HDL (15 min, 37 °C). Wortmannin inhibits phosphatidylinositol-3-kinase, which subsequently inhibits the activation of Akt kinase (12). Cells treated with HDL but not wortmannin generated 72 ± 8 femtoliter of [3H]citrulline.
Ceramide-induced eNOS Stimulation

Fig. 3. HDL-induced eNOS stimulation in human microvascular endothelial cells. Human microvascular endothelial cells were pretreated with buffer only, 50 μg/ml blocking SR-BI IgG, or 50 μg/ml isotype-matched nonspecific IgG for 15 min at 37 °C. The cells were then incubated with 0.75 μCi/ml [3H]arginine and 50 μg/ml HDL or 1 μg/ml ionomycin for 15 min at 37 °C. The cells were processed to quantify the amount of l-citrulline generated. Control experiments with l-NAME demonstrated that less than 1% of the generated citrulline was not the result of eNOS activity (data not shown). The data are presented as a percentage of maximal eNOS stimulation defined as the activity of eNOS in the presence of the calcium ionophore, ionomycin. The eNOS activity obtained with ionomycin was 256 ± 21 fmol of [3H)citrulline/well. The data are from three independent experiments (mean ± S.E.), *, significant difference with respect to buffer.

Fig. 4. HDL does not increase intracellular calcium in CHO cells. A, CHO cells expressing SR-BI and eNOS were loaded with fura-2AM for 10 min at 37 °C. The cells were then incubated with 50 μg/ml HDL for 15 min while continuously monitoring for calcium-induced fluorescence changes. Ionomycin (1 μg/ml) was added after the HDL incubation as a control to demonstrate that the system can measure a change in intracellular calcium. Lines represent fluorescence ratios recorded simultaneously in 12–14 individual cells from one coverslip. Comparable responses were observed in three separate experiments. B, human microvascular endothelial cells were incubated with buffer only or 20 μM BAPTA/AM for 20 min to chelate intracellular calcium prior to the addition of buffer, 50 μg/ml HDL, 10 nM acetylcholine, or 1 μg/ml ionomycin for 15 min at 37 °C. The cells were then processed to quantify the amount of l-citrulline generated. Control experiments with l-NAME demonstrated that less than 1% of the generated citrulline was not the result of eNOS activity (data not shown). The data are presented as a percentage of maximal eNOS stimulation defined as the activity of eNOS in the presence of the calcium ionophore, ionomycin. The eNOS activity obtained with ionomycin was 261 ± 22 fmol of [3H)citrulline/well. The data are from four independent experiments (mean ± S.E.), *, significant difference with respect to buffer.

Igarashi et al. (13) recently demonstrated that ceramide could stimulate eNOS-independent of calcium. To determine whether ceramide can stimulate eNOS in our system, CHO cells expressing eNOS only or eNOS and SR-BI were incubated with C2-ceramide (0.1–10 μM) or C2-dihydroceramide (10 μM) (inactive ceramide) for 0–15 min at 37 °C. Fig. 6 demonstrates that C2-ceramide stimulates eNOS in both cells, indicating that ceramide can activate eNOS and that ligand binding to SR-BI is not necessary when ceramide is added directly. The inactive analog of ceramide, C2-dihydroceramide, did not stimulate eNOS.

We next determined if HDL binding to SR-BI increased intracellular ceramide levels by treating the appropriate CHO cells with buffer or 50 μg/ml HDL as described above. Following the incubation, the cells were processed to quantify ceramide (see “Experimental Procedures”). Fig. 7 demonstrates that the addition of HDL to CHO cells expressing SR-BI increased intracellular ceramide levels 5-fold. Importantly, this increase in ceramide did not lead to cell death because, when the HDL was removed and the cells incubated for an additional 4 h, the cells remained viable and ceramide levels returned to unstimulated levels (the cells were viable for more than 24 h after HDL treatment; data not shown).

DISCUSSION

These data demonstrate that HDL binding to SR-BI stimulates endothelial nitric-oxide synthase in both a CHO heterologous expression system and in human microvascular endothelial cells. Yuhanna et al. (2) recently published similar data demonstrating that eNOS is activated by HDL binding to SR-BI. However, Yuhanna et al. (2) did not identify the mechanism of eNOS activation. eNOS is generally thought to be a calcium-dependent enzyme; however, recently both Akt kinase-mediated phosphorylation (12) and an increase in cellular ceramide (13) have been shown to activate eNOS in a calcium-independent manner.

The primary means of increasing eNOS activity is by increasing the intracellular concentration of calcium (1). Consequently, we first determined whether HDL binding to SR-BI increased intracellular calcium. An increase in intracellular calcium was not seen in cells loaded with Fura-2 and then treated with HDL; however, treatment of the same cells with ionomycin caused an increase in intracellular calcium. Although this suggested that eNOS was activated without an increase in calcium, it was possible that a small and/or localized calcium transient was responsible for the stimulation. To further confirm that an increase in calcium was not involved in HDL-mediated stimulation of eNOS, the cells were treated with the calcium chelator BABPTA/AM and then incubated with HDL or acetylcholine. Acetylcholine-mediated stimulation of eNOS is calcium-dependent and was completely inhibited by BABPTA/AM, whereas HDL-induced stimulation of eNOS was unaffected by BABPTA/AM. Taken together these data strongly imply that HDL binding to SR-BI does not increase intracellular calcium. This finding was surprising because HDL has been shown to promote an increase in intracellular...
calcium in a variety of cells, including endothelial cells (23). It is unclear why HDL did not induce an increase in intracellular calcium in our CHO and HME cells. However, we used two independent methods to demonstrate that HDL stimulation of eNOS does not require an increase in intracellular calcium.

Recently it has been demonstrated that estradiol can activate eNOS without an increase in cytosolic calcium (13). Additional studies demonstrated that estradiol activates phosphatidylinositol 3-kinase, which subsequently activates the enzyme Akt kinase (12). Akt kinase resides in the cytosol and translocates to the plasma membrane when an agonist stimulates phosphatidylinositol 3-kinase (12). Once at the plasma membrane, Akt kinase is activated by serine and threonine phosphorylation. Activated Akt kinase then can phosphorylate human eNOS on serine 1177, causing enhanced eNOS activity (24). A standard method of assessing if Akt kinase is active is to determine whether the kinase has been phosphorylated by using an anti-phospho-Akt kinase antibody. We could not detect any HDL-dependent phosphorylation of Akt kinase; however, the addition of PDGF induced significant Akt kinase phosphorylation. It is critical to note that PDGF did not stimulate eNOS, which suggests that different pools of Akt kinase exist in the cells. In contrast to our results, Nofer et al. (25) have demonstrated that HDL can activate Akt kinase in human umbilical vein endothelial cells. Furthermore, the studies by Nofer et al. (25) demonstrated that HDL contains two bioactive lipids, sphingosylphosphorylcholine and lysosulfatide, that caused the activation of Akt kinase. In these studies the authors used HDL at 1000 µg/ml, which is 20-fold higher than the concentration that we used. It is possible that the lower concentration of HDL that we used did not have enough bioactive lipid to activate Akt kinase.

Recently, Igarashi et al. (13) demonstrated that ceramide could activate eNOS independent of calcium. These authors demonstrated that bradykinin could induce eNOS activation without an increase in intracellular calcium. In the present study, we demonstrate for the first time that HDL binding to SR-BI will increase the intracellular concentration of ceramide. The accumulation of ceramide is relatively slow, taking up to 15 min for maximal generation (data not shown). The activation of eNOS parallels the generation of ceramide. When HDL was removed from the cells, ceramide returned to unstimulated levels, which was paralleled by a decrease in eNOS activity. Importantly, the generation of ceramide did not induce cell death because the cells remained viable for up to 24 h. In addition, incubation of cells with C_{2}-ceramide activated eNOS independent of HDL binding to SR-BI, whereas the inactive form of ceramide, C_{2}-dihydroceramide, did not stimulate eNOS.

These data strongly suggest that HDL binding to SR-BI increases intracellular ceramide, which subsequently activates eNOS. The source of the ceramide is not clear. Both caveolae and lipid rafts are enriched in sphingomyelin (26), and several laboratories have reported sphingomyelinase activity in isolated caveolae and/or rafts (27). Furthermore, Yuhanna et al. reported that a C-terminal anti-SR-BI antibody inhibited the ability of SR-BI to stimulate eNOS. When these data are taken together, it is tempting to speculate that HDL binding to SR-BI activates a caveola-localized sphingomyelinase that generates the ceramide that activates eNOS. Alternatively, it is possible that the ceramide is coming directly from the HDL particles. SR-BI can internalize cholesteryl esters (3, 22) and phospholipids (28) from HDL; therefore, it is formally possible that SR-BI is internalizing HDL-ceramide that then stimulates eNOS activity. If this latter possibility is true, then different
incubated with buffer or 50 μg/ml HDL for 15 min at 37 °C. The cells were then processed to quantify the amount of ceramide generated as described under "Experimental Procedures." The data are presented as picomoles of ceramide/mg of protein (n = 3, mean ± S.E.). *, significant difference with respect to buffer only control.

HDL particles should have different effects on vascular reactivity. This may explain why relatively high HDL levels in humans do not always protect against cardiovascular disease.

In summary, we have demonstrated that HDL binding to SR-BI stimulates eNOS, which is in agreement with the studies of Yuhanna et al. (2). In addition, we have demonstrated that the mechanism of HDL-SR-BI activation of eNOS is independent of an increase in intracellular calcium or Akt kinase phosphorylation. HDL binding to SR-BI induces an increase in intracellular ceramide, which appears to stimulate eNOS.

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