Sphingosine 1-Phosphate Protects Human Umbilical Vein Endothelial Cells from Serum-deprived Apoptosis by Nitric Oxide Production

Received for publication, December 19, 2000, and in revised form, December 28, 2000
Published, JBC Papers in Press, December 28, 2000, DOI 10.1074/jbc.M011449200

Young-Guen Kwon‡§, Jeong-Ki Min‡, Ki-Mo Kim¶, Doo-Jae Lee‡, Timothy R. Billiar, and Young-Myeong Kim‡¶

From the ‡Department of Biochemistry, College of Natural Sciences, Kangwon National University, Chuncheon, Kangwon-do 200-701, Korea and the ¶Department of Surgery, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

Endogenous nitric oxide (NO) is synthesized from L-arginine by catalytic reaction of three isotypes of NO synthases (NOS), the neuronal or type I isoform (nNOS), the inducible or type II isoform (iNOS), and the endothelial or type III isoform (eNOS) (7, 8). Endothelium-derived NO is formed by eNOS, which is constitutively expressed in endothelial cells and localized in plasmalemmal caveolae through association with the caveolea integral membrane structural protein caveolin-1 (9). Binding of eNOS with caveolin-1 inhibits the eNOS catalytic activity, and the inhibitory effect of caveolin-1 on the eNOS activity can be reversed by Ca\textsuperscript{2+}-calmodulin (10). Thus, increase in [Ca\textsuperscript{2+}]\textsubscript{i}, elicited by diverse extracellular signals (stimuli), including shear stress (11), bradykinin (12), and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (12, 13), leads to activation of eNOS (8), resulting in increased NO production.

Numerous previous studies have demonstrated the role of eNOS in controlling blood pressure, vascular remodeling, angiogenesis, and apoptosis (14–17). In particular, NO is implicated as a cytoprotective effector molecule, protecting some cell types from apoptotic cell death induced by TNF\textalpha, anti-Fas antibody, LPS, and trophic factor withdrawal. These apopgenic stimuli activate a series of tightly controlled intracellular sig-
naling events that induced mitochondrial cytochrome c release into cytosol and the activation of cysteine proteases known as caspases (18). Inappropriate endothelial cell apoptosis may be linked to several cardiovascular diseases. For example, eNOS-deficient mice exhibit delayed angiogenesis thought to be due to decreases in endothelial cell migration, proliferation, and differentiation, compared with wild type controls (19). In addition, we have shown that transduction with NOS gene into aortic allografts suppresses the development of allograft atherosclerosis (20), possibly in part via suppression of endothelial apoptosis (21). The antiapoptotic effects of NO are associated with direct inhibition of caspase activity by S-nitrosylation of the catalytic cysteine residue of the enzymes, as well as through cGMP-dependent mechanism (22). Therefore, eNOS-activating agents are capable of protecting endothelial cells from apoptosis. We hypothesized that S1P would protect endothelial cells by Ca^{2+}-dependent activation of eNOS. Here we show that S1P increases NO production in HUVECs by elevating [Ca^{2+}], through the EDG-1 and -3/G_{q/11}PLC pathway. This increased NO production prevents serum-deprived apoptosis by inhibiting mitochondrial cytochrome c release and release caspase-3 activation/activity.

MATERIALS AND METHODS

Chemicals and Reagents—SIP, U73122, and BAPTA-AM were purchased from Biomol (Plymouth Meeting, PA). Pertussis toxin was from Research Biochemical International, M199, penicillin, streptomycin, l-glutamate, and heparin were obtained from Life Technologies, Inc. Basic fibroblast growth factor was from Upstate Biotechnology (Lake Placid, NY). N-Monomethyl-L-arginine (NMA), N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) and z-VAD-fmk were obtained from Alexis Corp. (San Diego, CA). Antibodies against caspase-3 and eNOS were purchased from Transduction Laboratories (Lexington, KY), and a mouse monoclonal anti-cytochrome c oxidase antibody was from Molec- ulular Probes. 1H-1,2-4oxadiazolo[4,3-e]-quinoxaline-1-one (ODQ) was purchased from Promega (Madison, WI). N-Nitroso-N-acetyl-l-1,4-penicillamine (SNAP) was synthesized, as described previously (23). The following 18-mer phosphothiolate oligonucleotides were synthesized to block the expression of EDG-1 and EDG-3: antisense EDG-1, 5'-GAC GCT GGT GGG CCC CAT-3'; sense EDG-1, 5'-ATG GGG CCC ACC AGG CAG GCA-3'; antisense EDG-3, 5'-GAG TGC CAT-3'; sense EDG-3, 5'-ATG GCA ACT GCC CTC CCG-3'. All other reagents were purchased from Sigma, unless indicated otherwise.

Cell Culture—HUVECs were isolated as described previously (6). The cells were grown on a gelatin-coated 75-cm² flask in M199 with 20% fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin, 3 mg/ml basic fibroblast growth factor, and 5 units/ml heparin at 37 °C in 5% CO2. To induce apoptosis, serum-free medium containing 500 mM sucrose solution and carefully homogenized in a Dounce tissue grinder with a loose pestle. Cytosol was obtained for measuring cytochrome c release by centrifugation at 100,000 × g for 1 h. For Western blot of caspase-3, cells (2 × 10⁶ cells) were suspended in 100 mM Tris-EDTA buffer (pH 7.4) and lysed by three freeze-thaw cycles. Cytosolic proteins were obtained by centrifugation at 12,000 × g for 20 min at 4 °C. Proteins (40 μg) were separated on 14% SDS-PAGE for caspase-3 and then transferred to nitrocellulose. The membranes were hybridized with antibodies against caspase-3 and cytochrome c, and protein bands were visualized by exposing to x-ray film, as described previously (23).

Assay for DEVDase Activity—The cell pellets were washed with ice-cold phosphate-buffered saline and resuspended in 100 mM HEPES buffer, pH 7.4, containing protease inhibitors (5 μg/ml aprotinin and pepstatin, 10 μg/ml leupeptin, 0.5 mM phenylmethanesulfonyl fluoride). The cell suspension was lysed by three freeze-thaw cycles, and the cytosolic fraction was obtained by centrifugation at 12,000 × g for 20 min at 4 °C. DEVDase activity was assayed in the presence or absence of 20 mM DTT by measuring the increased absorbency at 405 nm after cleavage of 150 μM Ac-DEVD-pNA (22).

In Vitro Cleavage of PARP—[35S]Methionine-labeled PARP was synthesized using a transcription/translation-coupled transcription and translation system (Promega). Aliquots (4 μl) of in vitro translated [35S]-labeled PARP were incubated with 4 μg of cytosolic protein in 10 μl of the total reaction volume at 37 °C for 1 h. The reaction was stopped by mixing with an equal volume of 2× SDS sample buffer and heating the mixture for 2 min. Cleavage profiles of PARP were examined by electrophoresis on 10% SDS-PAGE and protein visualized by fluorography.

Determination of NOS Activity—Cells were cultured in 60-mm culture dishes with SIP for different time periods, washed twice, and equilibrated for 20 min with HEPES buffer, pH 7.4, containing: 10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM CaCl2, and 1 mM glucose. Cells were incubated for 40 min with 1 μM of L-[3H]arginine plus 8 mM L-arginine. To assess the calcium dependence of NOS activation, experiments were performed in calcium-free HEPES buffer containing 20 μM BAPTA-AM. The reaction mixture was removed, and ice-cold ethanol (0.5 ml) was added. After allowing for evaporation of ethanol, 2 ml of 10 mM HEPES-NA, pH 5.5, was added and kept for 20 min. The supernatant was collected and applied onto a 2 ml cation-exchange column (50W-XS resin converted to Na+ form, Bio-Rad). [3H]Citrulline product was collected in the eluate by washing the column with 4 ml of H2O and then quantified by scintillation counting. Enzyme activity was normalized to protein concentration. NOS activity was expressed as pmol/mg protein.

DNA Fragmentation—DNA fragmentation was demonstrated by harvesting total cellular DNA and agarose gel electrophoresis. After the indicated treatment, both adherent and detached cells were harvested, washed with phosphate-buffered saline, and lysed in 50 mM Tris-EDTA buffer, pH 7.4, 1% SDS, and 0.5 mg/ml proteinase K for 3 h at 50 °C. Samples were then extracted with phenol/chloroform and precipitated with ethanol. The pellet was resuspended in 20 mM Tris-EDTA, pH 8.0. After digesting RNA with RNase (0.1 mg/ml) at 37 °C for 1 h, DNA was separated by electrophoresis on a 1.2% agarose gel stained with ethidium bromide.

Other Analysis—Protein concentration was determined with the BCA assay (Pierce). Data are presented as mean ± S.D. of at least three separate experiments, except where results of blots are shown in which case a representative experiment is depicted in the figures. Comparisons between two values analyzed using Student's t test. Differences were considered significant when p < 0.05.

RESULTS

SIP Protects HUVECs from Serum-deprived Apoptosis—Previous studies showed that trophic factor deprivation induces apoptotic cell death in several cell types, including endothelial cells (24), and that SIP protects endothelial cells from Cε-ceramide- and TNFa-induced apoptotic cell death (25, 26). To investigate whether SIP protects HUVECs from serum deprivation-induced cell death, HUVECs were cultured in serum-free M199 medium with or without SIP, and cell viability was assayed by trypan blue exclusion after 24 h. Serum deprivation decreased cell viability to ~30% of control cells grown in the presence of serum. The addition of SIP (0.1 μM) reversed the serum-deprived effects associated with cell death in a dose-dependent manner (Fig. 1). Cells can die by either necrosis or apoptosis, and the inhibition of caspase activation/activity can protect cells from apoptotic cell death, but not from necrotic cell death (27). HUVECs were cultured in serum-free medium with or without the caspase inhibitor z-VAD-fmk. z-VAD-fmk prevented serum-deprived cell death (Fig. 1), indicating that serum deprivation causes caspase-dependent apoptosis.

NMA Suppresses the Inhibitory Effects of SIP on Serum-
deprived Apoptosis—It has been shown that NO production from eNOS or exogenous NO donor protects endothelial cells from apoptosis induced by TNFα (22) and LPS (21). To investigate the involvement of NO in the protective effect of S1P, HUVECs were treated with S1P in the presence of the specific inhibitor of NOS NMA, and cell viability was measured. The protective effect of S1P was significantly reduced by the addition of NMA (Fig. 2A). DNA fragmentation typical of apoptosis was identified in serum-deprived HUVECs (Fig. 2B). S1P prevented DNA fragmentation, and the protective effect of S1P was blocked by the addition of NMA. These results suggest that the cytoprotective effect of S1P on serum-deprived apoptosis is mediated by NO production from HUVECs.

Antisense Oligonucleotides of EDG-1 and -3 Suppress S1P-Mediated Survival and NO Production—Exogenous S1P regulates cellular responses such as angiogenesis (2), morphogenesis (2, 4), cell migration (2, 3), and cell survival (28) through the activation of EDG-1 and -3 receptors or through the activation of sphingosine kinase, which synthesizes endogenous formation of S1P. We next examined the involvement of these signaling pathways in the S1P-mediated increase in cell survival and NO production by treating the HUVECs with antisense oligonucleotides for EDG-1 and -3 or the sphingosine kinase inhibitor dihydrosphingosine. Pretreatment of HUVECs with the antisense, but not sense, oligonucleotide to EDG-1 significantly suppressed S1P-mediated cell survival and S1P-induced NO production (Fig. 3, A and B). In contrast, pretreatment with antisense, but not sense, oligonucleotide to EDG-3 only partially suppressed the effects of S1P (Fig. 3, C and D). A combination of antisense oligonucleotides to EDG-1 and -3 suppressed the effects of S1P to about 90% (data not shown). Treatment with the sphingosine kinase inhibitor dihydrosphingosine did not inhibit S1P-mediated cell survival or NO production (data not shown). These results suggest that S1P requires both EDG-1 and -3 for full activation of the cellular signal needed to promote cell survival and NO production in HUVECs.

S1P Inhibits Caspase Activity/Activation and Cytochrome c Release in a NO-dependent Manner—Since serum deprivation induces apoptosis by activating the caspase signal cascade and mitochondrial cytochrome c release (29), we examined the effect of S1P on caspase activation/activity and mitochondrial cytochrome c release in serum-deprived HUVECs. HUVECs were treated with or without S1P in serum-free medium, and DEVDase (caspase-3-like protease) activity was measured in the cytosol by a colorimetric assay using the tetrapeptide sub-

![Fig. 1. S1P protects HUVECs from serum deprivation-induced cell death. HUVECs (1 × 10⁶ cells/well) were plated onto six-well plates in 1 ml of M199 containing 20% fetal bovine serum. The next day, the cells were switched to serum-free M199 with or without 20% fetal bovine serum or with 0–5 μM S1P or 100 μM z-VAD-fmk. After 24 h, cell viability was determined by trypan blue exclusion. Data represent mean ± S.D. (n = 4).](http://www.jbc.org/)

![Fig. 2. NMA blocks S1P-mediated HUVECs survival. HUVECs were incubated in serum-free medium containing S1P (5 μM) in the absence or presence of NMA. Cell viability was measured by trypan blue after 24 h (A), and DNA fragmentation was determined by isolation of DNA and agarose gel electrophoresis after 18 h (B). Data represent mean ± S.D. of three experiments. *, p < 0.01 versus treatment with S1P alone.](http://www.jbc.org/)

![Fig. 3. Antisense oligonucleotides to EDG-1 and -3 block S1P-mediated cell survival and NO production. HUVECs were pretreated with antisense or sense oligonucleotide (5 μM) to EDG-1 or -3 for 12 h. The medium was replaced with serum-free fresh medium containing 5 μM S1P and 5 μM oligonucleotide. After 24 h, cell viability and NO production were measured. Antisense oligonucleotide of EDG-1 decreases HUVEC survival (A) and NO production (B). Antisense oligonucleotide of EDG-3 decreases HUVEC survival (C) and NO production (D). Data represent mean ± S.D. of three experiments.](http://www.jbc.org/)
HUVECs, while cytosols from z-VAD-fmk and S1P-treated cells did not cleave PARP (Fig. 4B). This effect of S1P was also reversed by NMA.

To further determine whether the release of mitochondrial cytochrome c into cytosol and the activation of caspase-3 by cytochrome c oxidation are affected by treatment with S1P, we measured appearance of the cytosolic cytochrome c (Cyt c) and the active fragment (p17) of caspase-3 by Western blot analyses. As shown in Fig. 4C, serum deprivation induced the release of cytochrome c to cytosol and the cleavage of pro-caspase-3 (p32) into a 17-kDa fragment. These events were suppressed by the addition of S1P or z-VAD-fmk. The S1P effects were reversed by NMA. However, cytochrome c oxidase as an indicator of mitochondrial contamination was not detected in the cell lysates. Studies in several cell-free systems of apoptosis have revealed that cytosolic cytochrome c interacts with Apaf-1 and procaspase-9 and forms an apoptosis in the presence of dATP. This results in the activation of caspase-9, which leads to activation of caspase-3 (18). Therefore, we next determined whether mitochondrial cytochrome c release precedes increases in DEVDase activity (Fig. 4D). Mitochondrial cytochrome c release into cytosol and DEVDase activity increased 12 and 16 h following serum withdrawal, respectively. Cytochrome c release was maximal at 16 h, and DEVDase activity peaked at 20 h. This is consistent with the view that cytochrome c release is upstream of caspase-3 activation in the apoptotic signal cascade of serum-deprived HUVECs. These results suggest that the antiapoptotic effect of S1P in serum-deprived HUVECs includes the suppression of cytochrome c release and the inhibition of caspase-3 activation/activity.

**NO-mediated Protective Effect of S1P**—The antiapoptotic action of NO is mediated at least in part through cGMP in hepatocytes (22), PC12 cells (29), and cultured neuronal cells (31). To examine the role of NO/cGMP pathway in S1P-mediated protection, the effect of S1P on cell viability was examined in the presence of the specific inhibitor of soluble guanylate cyclase ODQ (Fig. 5A). ODQ did not inhibit the protective effect of S1P, while NMA did. Furthermore, treatment with an exogenous NO donor SNAP (100 μM) protected cells from serum-deprived cell death, and this protection would not be blocked by ODQ. As expected a NO scavenger hemoglobin (400 μM as heme concentration) prevent the protective effects of SNAP (Fig. 5B). In addition, the membrane-permeable cGMP analog 8-Br-cGMP did not inhibit serum-deprived apoptotic cell death. These results indicate that the protective effect of S1P is NO-dependent, but cGMP-independent.

**S1P Increases NO Synthesis in HUVECs**—If S1P protects via NO then it should be possible to measure increases in NO formation following S1P exposure. The formation of NOx as stable oxidized products of NO was measured in culture medium. S1P increased NO production in a dose-dependent manner reaching about 2-fold over controls at a concentration of 5 μM S1P (Fig. 6A). The accumulation of NOx in the medium was detectable 4 h following S1P treatment (Fig. 6B). To examine mechanism possible for S1P-induced NO production, we examined the NOS activity in response to S1P. The NOS activity was increased to about 1.5- and 2-fold at 4 and 12 h, respectively, following treatment with S1P (Fig. 6C). The increased NOS activity was suppressed by addition of the Ca2+-chelator BAPTA-AM. Western blot analysis revealed no significant difference of eNOS protein levels between 0 and 4 h (Fig. 6D). These data suggest that increased NO production by S1P is due to the activation of Ca2+-sensitive eNOS.

**S1P Induces NO via the G, Protein-PLC Signaling Pathway**—To examine the signaling mechanism by which S1P stimulates NO production in HUVECs, the effects of various inhibitors on S1P signaling were evaluated (Fig. 7). We (2, 6) and...
Inhibition of Endothelial Cell Apoptosis by S1P

This study was undertaken to determine the molecular mechanism by which S1P protects endothelial cells from apoptosis. We provide evidence that S1P protects HUVECs from serum-deprived apoptosis and mitochondrial cytochrome c release. Increases in cell survival and NO production by S1P were significantly suppressed by pretreatment with antisense oligonucleotides of EDG-1, and to a lesser extent, by EDG-3 antisense, indicating that the cellular effects of S1P involve the activation of EDG-1 and -3. The antiapoptotic effects of S1P could not be inhibited by ODQ, indicating that the protective effect does not involve cGMP production. This study also provides evidence that the increase in NO production by S1P occurred through the involvement of EDG-1 and -3, G protein, PLC, and Ca2+. Thus, S1P increases eNOS activity via intracellular Ca2+ mobilization, and the resulting increases in NO protect endothelial cells from apoptosis by suppression of apoptotic signaling cascade.

Apoptotic cell death by cytotoxic stimuli and serum or growth factor withdrawal is induced by tightly controlled intracellular signaling events, which require serial activation of caspase family proteases and mitochondrial cytochrome c release. Caspase-deficient animals or cells are resistant to apoptosis induced by anti-Fas antibody (32) and DNA-damaging agents (33). Treatment with caspase inhibitors reduces fulminant liver injury and animal mortality induced by administration of anti-Fas antibody (34) or LPS (35). These studies indicate that inhibition of caspase activation/activity is a potential approach for the prevention of apoptosis. Our data show that serum deprivation induced HUVEC apoptosis, as indicated by increased DNA fragmentation, DEVDase activity, and cytochrome c release. Treatment with S1P and the caspase inhibitor z-VDAD-fmk protected cells from serum-deprived apoptosis by suppressing these effects. These results raise the possibility that S1P is an endogenous protectant against endothelial cell apoptosis through a mechanism of suppressed cytochrome c release and caspase-mediated signaling.

In this study, our major question was how S1P prevents serum-deprived apoptotic cell death in HUVECs. We (21) and others (17) have previously shown that NO protects endothelial cells from LPS- and TNFα-induced apoptosis. These studies directed our attention toward NO as a potential mediator of the S1P effect. Importantly, the present study revealed that S1P increased NO production from HUVECs by enhancing Ca2+-sensitive eNOS activity without significant increase in the eNOS protein. eNOS is normally located at the cell membrane within caveolae in an inactive state (36). Increases in intracellular Ca2+ promote calmodulin binding to the enzyme, leading to transient eNOS activation and NO production. Other interaction and modification can alter eNOS activity, including binding to caveolin-1, which inhibits eNOS activity (37), and phosphorylation (38) and binding of heat shock protein 90 (39) that enhance NO production. Our studies suggest that the dominant action of S1P is to increase Ca2+ levels; however influence on these other regulatory processes cannot be excluded.

NO has been shown to regulate either induction of apoptosis in some cells (40) or prevention of apoptosis in others (22, 29). High levels of NO have been shown to be a cytotoxic through the inhibition of ATP synthetic enzymes, including mitochondrial aconitase and electron transfer complexes I and II (41) or through DNA damage (42). NO also suppresses cell proliferation through the inhibition of ribonucleotide reductase (43) and eukaryotic initiation factor-2α (44). NO can also prevent apoptosis in several cell types, including hepatocytes (22, 45, 46), human B lymphocytes (47), PC12 cells (29), splenocytes (48), eosinophils (49), ovarian follicles (50), neuronal cells (31), MCF7 (51), Jurkat cells (52), and endothelial cells (17, 21). Several mechanisms for the antiapoptotic effect of NO have been identified, including the up-regulation of protective pro-
In these cases, an acute increase in [Ca\(^{2+}\)] was determined by trypan blue exclusion. Data represent mean ± S.D. (n = 4).

Fig. 7. S1P-induced NO production is dependent on the Gi protein-PLC signaling pathway. HUVECs in serum-free M199 were pretreated with or without pertussis toxin (PTX, 100 ng/ml), U73122 (U, 5 μM), and BAPTA-AM (BATA, 5 μM) for 1 h and then incubated with or without S1P (5 μM) for additional 12 h for NOx measurement or 24 h for cell viability. NMA (1 mM) was cotreated with S1P. A, NOx was measured in the culture medium by chemiluminescence. B, cell viability was determined by trypan blue exclusion. Data represent mean ± S.D.

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