Sphingosine-1-phosphate inhibits high glucose-mediated ERK1/2 action in endothelium through induction of MAP kinase phosphatase-3

Angela M. Whetzel, David T. Bolick, and Catherine C. Hedrick

Robert M. Berne Cardiovascular Research Center and Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, Virginia

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Whetzel AM, Bolick DT, Hedrick CC. Sphingosine-1-phosphate inhibits high glucose-mediated ERK1/2 action in endothelium through induction of MAP kinase phosphatase-3. Am J Physiol Cell Physiol 296: C339–C345, 2009. First published December 17, 2008; doi:10.1152/ajpcell.00293.2008.—Endothelial activation is a key early event in vascular complications of Type 1 diabetes. The nonobese diabetic (NOD) mouse is a well-characterized model of Type 1 diabetes. We previously reported that Type 1 diabetic NOD mice have increased endothelial activation, with increased production of monocyte chemoattractant protein (MCP)-1 and IL-6, and a 30% increase of surface VCAM-1 expression leading to a fourfold increase in monocyte adhesion to the endothelium. Sphingosine-1-phosphate (SIP) prevents monocyte:endothelial interactions in these diabetic NOD mice. Incubation of diabetic NOD endothelial cells (EC) with SIP (100 nmol/l) reduced ERK1/2 phosphorylation by 90%, with no significant changes in total ERK1/2 protein. In the current study, we investigated the mechanism of SIP action on ERK1/2 to reduce activation of diabetic endothelium. SIP caused a significant threefold increase in mitogen-activated kinase phosphatase-3 (MKP-3) expression in EC. MKP-3 selectively regulates ERK1/2 activity through dephosphorylation. Incubation of diabetic NOD EC with SIP and the SIP₃-selective agonist SEW2871 significantly increased expression of MKP-3 and reduced ERK1/2 phosphorylation, while incubation with the SIP₁/SIP₃ antagonist VPC23019 decreased the expression of MKP-3, both results supporting a role for SIP₁ in MKP-3 regulation. To mimic the SIP-mediated induction of MKP-3 diabetic NOD EC, we overexpressed MKP-3 in human aortic endothelial cells (HAEC) cultured in elevated glucose (25 mmol/l). Overexpression of MKP-3 increased surface VCAM-1 expression leading to a fourfold increase in monocyte adhesion in a static monocyte adhesion assay. Finally, we used small interfering RNA to MKP-3 and observed increased monocyte adhesion. Moreover, SIP was unable to inhibit monocyte adhesion in the absence of MKP-3. Thus, one mechanism for the anti-inflammatory action of SIP in diabetic EC is inhibition of ERK1/2 phosphorylation through induction of MKP-3 expression via the SIP₁ receptor axis.

mitogen-activated protein kinase phosphatase-3; extracellular signal-regulated kinase 1/2; monocyte adhesion

Sphingosine-1-phosphate (SIP), a biologically active sphingolipid, plays an important role in the regulation of a variety of cellular processes, including cell survival and vascular maturation, by binding to a family of G-protein-coupled receptors (termed SIP₁,₃) (46). SIP is generated in mammalian cells primarily from the degradation of ceramide to sphingosine (28, 33). Sphingosine is phosphorylated by sphingosine kinases to generate SIP (1, 29, 30). SIP is secreted from leukocytes, erythrocytes, platelets, and endothelial cells (EC) in the vasculature (16). SIP is present in nanomolar concentrations and resides on albumin and lipoproteins, particularly HDL, in the circulation (23, 49).

Atherosclerosis development is accelerated several-fold in patients with both Type 1 and Type 2 diabetes (8, 9, 12, 14). Monocyte:endothelial interactions are a key early event in atherosclerosis development. We and others have shown that diabetes increases endothelial activation and monocyte:endothelial interactions in both human and mouse models (5–7, 17, 19, 26, 39, 40, 52). The nonobese diabetic (NOD/LtJ) mouse is a spontaneous model of Type 1 diabetes that develops autoimmune destruction of the pancreatic β-cells resulting in insulin and spontaneous hyperglycemia (18, 22, 25, 31). Susceptibility to Type 1 diabetes in this mouse is polygenic, and within 16–20 wk, ~60% of female NOD mice develop frank Type 1 diabetes.

Mitogen-activated protein kinase phosphatases (MKPs) are a family of dual-specificity phosphatases that dephosphorylate both tyrosine and serine/threonine residues in mammalian cells. MKPs thus play important roles in the regulation of p38, ERK1/2, and JNK signaling pathways that are induced by growth factors, cellular stress, and inflammatory cytokines (21, 32). Endothelial cells express several MKPs, including MKP-1 and MKP-3 (44). MKP-3 is a cytosolic phosphatase that targets ERK1/2. Nitric oxide production in endothelium has been shown to downregulate endothelial MKP-3 expression (43). Vollmar and colleagues have reported that MKP-1 induction is anti-inflammatory in endothelium (15), and Fogelman and colleagues have shown that MKP-1 is rapidly induced in aortic endothelium on exposure to oxidized phospholipids (41). We have recently shown that SIP reduces monocyte adhesion to Type 1 diabetic NOD EC (54). In the current study, we examined the role of MKP-3 in regulating monocyte:endothelial interactions in diabetic NOD endothelium. We show that incubation of aortic EC with SIP triggers rapid induction of MKP-3, which dephosphorylates ERK1/2. This pathway contributes to the regulation of monocyte:endothelial interactions in Type 1 diabetes.

METHODS

Reagents. WEHI mouse monocytes were a gift of Dr. Judy Berliner (University of California, Los Angeles, CA). Antibodies used were MKP-3 (Santa Cruz; sc-28902), phospho-ERK1/2 (R&D Systems; AF1018), and ERK1/2 (Santa Cruz; sc-94). SIP was obtained from BioMol, and SEW2871 was from Sigma. VPC23019 was a gift of Dr. Kevin Lynch and Dr. Timothy Macdonald (University of Virginia, Charlottesville, VA).

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Address for reprint requests and other correspondence: C. C. Hedrick, Cardiovascular Research Center, Univ. of Virginia, P. O. Box 801394, 415 Lane Rd., MR5 Rm. G123, Charlottesville, VA 22908 (e-mail: cch6n@virginia.edu).

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Resuspension of S1P and SEW2871. S1P was dissolved in 95% DMSO-5% 1N HCl in H2O at a concentration of 20 mmol/l S1P. This 20 mmol/l solution was further diluted 20:1 into 3% fatty acid free BSA (FABFSA) in 1× PBS to yield a final stock concentration of 1 mmol/l S1P in 3% FABFSA-5% acidified DMSO. Further dilutions of the 1 mmol/l S1P stock solution for cell culture use were diluted into 0.2% FABFSA-PBS and added directly to cells.

SEW2871 was dissolved in PBS with 3% FABFSA-5% acidified DMSO to yield a final concentration of 1 mmol. Further dilutions of this 1 mmol SEW2871 stock solution were diluted into 3% FABFSA-PBS and added directly to cells. In our studies, we used this same concentration and dilution strategy of FABFSA-PBS-DMSO in our control samples to account for the addition of DMSO. We have observed no differences in responsiveness of the endothelium in DMSO-treated cells (data not shown).

Mouse. NOD/LtJ mice were purchased from Jackson Laboratories and maintained on rodent chow. Nondiabetic littermate mice were used as controls. All animal studies were approved by the University of Virginia Animal Care and Use Committee.

Isolation of mouse aortic endothelial cells. Aortic EC from NOD diabetic and control normoglycemic littermate mice were harvested from mouse aorta under sterile conditions as previously reported (3). Aortic endothelial cell cultures were cultured in DMEM containing 15% heat-inactivated FBS, 30 ng/l endothelial cell growth supplement from mouse aorta under sterile conditions as previously reported (3).

Diabetic and control normoglycemic littermate mice were harvested from 6-8 weeks old diabetic NOD mice. Control NOD/LtJ mice were purchased from Jackson Laboratories. Mice were weaned at 4 weeks and housed in a barrier facility. All experiments were approved by the University of Virginia Institutional Review Board (IRB), and all procedures were performed in accordance with University of Virginia IRB guidelines.

Human endothelial cell culture. Human aortic endothelial cells (HAEC) were a kind gift of Dr. Judith Berliner (UCLA). HAEC were cultured in medium 199 containing 20% heat-inactivated FBS (HyClone), 30 ng/l ECGS, and 50 ng/l heparin. Cells were used from passages 2 to 4. We have previously shown that aortic EC isolated from NOD diabetic mice retain a “metabolic memory” for short periods of time in culture (54). This has recently been shown to be the case for other cell types isolated from diabetic animals (24, 27).

Mouse monocyte adhesion assay. Aortic EC from nondiabetic and diabetic mouse cultures were incubated in the absence or presence of S1P (100 nmol/l), VPC23019 (10 μmol/l) for 4 h at 37°C. After incubation, EC were washed and incubated for 30 min with a 10 × 10 grid using fluorescent microscopy (50).

Human monocyte adhesion assay. Human aortic endothelial cells (HAEC) were cultured as described above in 100-mm cell dishes, and cells collected 72 h after transfection. Total cellular RNA was isolated from HAEC using TRIzol according to the manufacturer’s instructions. cDNA was synthesized using the Iscript cDNA synthesis kit (Bio-Rad). Total cDNA was diluted 1:8, and 4 μl of this dilution were used for each real-time condition using a Bio-Rad MyIQ Single Color Real-Time PCR Detection Systems and iQ SYBR Green supermix (Bio-Rad). Primers for human MKP-3 are forward: 5′-TTACTCTGGCTCTGTCG-3′ and reverse: 5′-GGTCTGAGCGTATCTATC-3′; β-actin: forward 5′-CATGTTTGGACCTTAAAC-3′ and reverse 5′-CT-GCTGTGCTGATCCACATCT-3′. The PCR conditions were 95°C for 10 min, 95°C for 4 min, followed by 50 cycles of 95°C for 10 s, 58°C for 10 s, 72°C for 10 s, followed by a final extension at 81°C for 15 s. Data were analyzed and presented on the basis of the relative expression method. This formula for calculation is:

\[
\text{Relative expression} = 2^{-(ΔCt/ΔCt)}
\]

where ΔCt is the difference in threshold cycle between the gene of interest (MKP-3) and the housekeeping gene (β-actin). HAEC control samples were normalized to β-actin.

Statistical analyses. Data for all experiments were analyzed using the StatView 6.0 software program. Comparisons between groups were performed using one-way analysis of variance (ANOVA) methods. Data are graphically represented as means ± SE, in which each mean consists of four experiments performed in triplicate (unless noted otherwise in the figure legends) using three to six mice per group. Comparisons between groups and tests of interactions were made assuming a two-factor analysis with the interaction term testing each main effect with the residual error testing the interaction. All comparisons were made using Fisher’s least significant difference procedure, so that multiple comparisons were made at the 0.05 level only if the overall F-test from the ANOVA was significant at \( P < 0.05 \).

RESULTS

S1P inhibits ERK1/2 phosphorylation and stimulates MKP-3 expression in aortic endothelial cells. To examine MKP-3 activation by S1P in the endothelium, we performed a time course of S1P treatment to endothelium. Aortic EC were isolated from nondiabetic littermate and diabetic NOD mice, and MKP-3 protein expression was measured by immunoblotting. Control nondiabetic and diabetic NOD EC were treated with S1P for 1 h, 2 h, and 4 h. First, we observed a dramatic reduction of MKP-3 levels in NOD diabetic mice compared with nondiabetic littermates (Fig. 1A). Incu-
bation of EC with 100 nmol/l S1P for 4 h significantly upregulated MKP-3 protein expression compared with earlier time points, although there was a slight increase in MKP-3 expression after only 1 h of S1P incubation (Fig. 1A). MKP-3 has been shown to cleave the phosphate from ERK1/2 in the cytoplasm, thereby preventing its mobilization to the nucleus to initiate gene transcription (36). Thus, we chose to measure levels of phosphorylated ERK1/2 in the cytosol of diabetic EC, expecting to observe increased phospho-ERK1/2 expression due to decreased MKP-3 activity. Indeed, as shown in Fig. 1B, concomitant with the reduction in MKP-3 levels, there was a significant increase in ERK1/2 phosphorylation in the cytosol of diabetic NOD EC. Incubation of diabetic EC with S1P significantly reduced ERK phosphorylation \( (P < 0.002 \text{ by ANOVA}) \). Thus, S1P has the ability to regulate MKP-3 expression and ERK1/2 phosphorylation in EC.

**S1P signaling through the S1P1 receptor in EC induces MKP-3 expression.** We next wished to examine the receptor signaling pathway triggered by S1P that modulates MKP-3 expression in EC. Our previous studies have identified the S1P1 receptor as playing a potent anti-inflammatory role in EC (54). Since S1P1 receptor-deficient mice show embryonic lethality, we used pharmacological reagents that are selective for S1P1. SEW2871 \( \{5-[4 \text{-phenyl-5-(trifluoromethylthiophen-2-yl)-3-[3-(trifluoromethylphenyl)-1,2,4-oxadiazole}\} \) is a selective S1P1 receptor agonist that is 30-fold less potent than S1P at S1P1, with no agonist activity at S1P2 at concentrations up to 10 \( \mu \text{mol/l} \) (55). VPC23019 is a S1P receptor antagonist with a \( K_i \) for S1P1 of 25 \( \mu \text{mol/l} \); the molecule is about 50-fold less potent in blocking the S1P3 receptor but is not active at the S1P2 receptor (11). As shown in Fig. 2A, incubation of diabetic NOD mouse EC with either S1P or the S1P receptor-specific agonist SEW2871 restored MKP-3 expression to levels similar to that found in nondiabetic NOD EC. Pretreatment of EC with the S1P1 receptor antagonist VPC23019 blocked the ability of S1P to restore MKP-3 expression (Fig. 2A).
together, these results suggest that the S1P-S1P₁ receptor axis stimulates MKP-3 expression in aortic EC.

MKP-3 activation in EC is associated with changes in monocyte adhesion. Monocyte adhesion to endothelium is a key early inflammatory event associated with atherosclerosis. We have previously shown that S1P reduces monocyte:endothelial interactions both in vitro and in vivo in diabetic NOD mice (54). As shown in Fig. 2B, the expression of MKP-3 is associated with changes in monocyte adhesion. There was a significant threefold increase in monocyte adhesion to diabetic NOD EC compared with control EC, and this was inhibited by both S1P and SEW2871 (Fig. 2B). Pretreatment of EC with VPC23019 to inhibit S1P₁ receptor activation prevented the anti-inflammatory effects of S1P on monocyte adhesion (Fig. 2B). Although highly correlative with the level of expression of MKP-3, these data do not conclusively demonstrate a causal role for MKP-3 in mediating monocyte:endothelial interactions. To directly test this, we performed overexpression studies of MKP-3 using EC cultured in elevated glucose and examined both ERK1/2 phosphorylation and monocyte adhesion. Using nucleofection, we obtained approximately a twofold increase in MKP-3 expression in HAEC using an expression plasmid for human MKP-3. This increase in MKP-3 expression was concomitant with significant reductions in ERK1/2 phosphorylation (Fig. 3). Moreover, this approximate twofold increase in the level of MKP-3 expression was similar to what we observed for S1P treatment of EC (Fig. 3). Overexpression of MKP-3 also resulted in a significant 50% reduction in ERK1/2 phosphorylation in HAEC cultured in elevated glucose (Fig. 4A).

Next, we tested the direct effect of MKP-3 overexpression on monocyte adhesion. Using human aortic EC cultured in 25 mmol/l glucose, we found a significant increase in monocyte adhesion to EC as we have reported previously (48). Overexpression of MKP-3 significantly reduced glucose-mediated monocyte adhesion by ~70% (Fig. 4B). S1P and SEW2871 had
regulated in part through endothelial MKP-3. We demonstrate monocyte:endothelial interactions in diabetic NOD mice are products in endothelium (2). In the current study, we show that PKC-\(\gamma\) (17, 37). We have recently shown the importance of RhoA and diabetes and contribute to monocyte:endothelial interactions products of arachidonic acid metabolism are increased in groups, including ours, have shown that 12/15-lipoxygenase ing pathways are altered in diabetic endothelium (51). Several shown that the phosphatidylinositol 3-kinase and AKT signal-ings mediating endothelial activation in diabetes. Studies have King et al. (10) have shown the importance of PKC enzymes in been implicated in the pathogenesis of diabetic endothelium. ENDOTHELIAL ACTIVATION AND MONOCYTE:ENDOTHELIAL INTERACTIONS are key early events in atherogenesis. We have shown that these processes are amplified, or accelerated, in diabetic endothelium (47, 48). Several key signaling cascades have been implicated in the pathogenesis of diabetic endothelium. King et al. (10) have shown the importance of PKC enzymes in mediating endothelial activation in diabetes. Studies have shown that the phosphatidylinositol 3-kinase and AKT signaling pathways are altered in diabetic endothelium (51). Several groups, including ours, have shown that 12/15-lipoxygenase products of arachidonic acid metabolism are increased in diabetes and contribute to monocyte:endothelial interactions (17, 37). We have recently shown the importance of RhoA and PKC-\(\alpha\) association in NF-kB activation by 12/15-lipoxygenase products in endothelium (2). In the current study, we show that monocyte:endothelial interactions in diabetic NOD mice are regulated in part through endothelial MKP-3. We demonstrate a direct link between MKP-3 expression, ERK1/2 phosphorylation, and endothelial activation in the setting of Type 1 diabetes. Moreover, we show that S1P stimulates MKP-3 expression and reduces monocyte:endothelial interactions in Type 1 diabetes. We cannot rule out some contribution of Akt signaling in mediating anti-inflammatory effects of S1P in endothelium in the current study (34); however, it is clear that ERK1/2 and MKP-3 aid in regulation of monocyte:endothelial interactions by S1P.

Endothelial cells express multiple MKP family members. We observed the presence of mRNA for MKPs 1–4 in both murine and human aortic EC. Castillo et al. (4) found that S1P significantly raised MKP-1 levels in fibroblasts. Several studies have reported the anti-inflammatory effects of MKP-1 in endothelial cells (21, 53). However, MKP-1 has also been localized to atherosclerotic lesions in mice and is suggested to contribute to atherosclerosis development through regulation of MCP-1 synthesis and monocyte recruitment (42). Thus, the role of MKP-1 in regulating early events in atherogenesis is unclear. In the current study, we found that S1P caused induction of only MKP-3 mRNA in murine and aortic EC (data not shown); thus, we focused only on MKP-3 function. Although we cannot rule out some contribution of MKP-1 in reducing ERK activation in EC, our data suggest that S1P acts through MKP-3 in EC to reduce monocyte:endothelial interactions (Figs. 2 and 4).

MKP-3 is unique within the MAP kinase phosphatase family because it is exclusively located in the cytosol, indicating a specific regulatory role in inactivating MAP kinases by targeting cytoplasmic substrates or by blocking nuclear localization (35, 36). MKP-3 binds to ERK1/2 independently of its phosphorylation state, suggesting that once it has targeted and dephosphorylated ERK1/2, it could anchor the inactive ERK1/2 within the cytoplasm to limit competition with MAP kinase kinase, another activator of ERK1/2 signaling in mediating anti-inflammatory effects of S1P.

**DISCUSSION**

Endothelial activation and monocyte:endothelial interactions are key early events in atherogenesis. We have shown that these processes are amplified, or accelerated, in diabetic endothelium (47, 48). Several key signaling cascades have been implicated in the pathogenesis of diabetic endothelium. King et al. (10) have shown the importance of PKC enzymes in mediating endothelial activation in diabetes. Studies have shown that the phosphatidylinositol 3-kinase and AKT signaling pathways are altered in diabetic endothelium (51). Several groups, including ours, have shown that 12/15-lipoxygenase products of arachidonic acid metabolism are increased in diabetes and contribute to monocyte:endothelial interactions (17, 37). We have recently shown the importance of RhoA and PKC-\(\alpha\) association in NF-kB activation by 12/15-lipoxygenase products in endothelium (2). In the current study, we show that monocyte:endothelial interactions in diabetic NOD mice are regulated in part through endothelial MKP-3. We demonstrate...
mining the subcellular localization of ERK1/2 as well as inhibiting its activity (20). We did not examine nuclear versus cytoplasmic localization of MKP-3 in the current study.

The MKP family of phosphatases acts on both phosphoryl-
rosine and phosphothreonine residues to reverse ERK1/2 activation. Of the MKP family members, it is MKP-3 that specifically inactivates ERK1/2. We observed significant regulation of ERK1/2 phosphorylation in both murine and aortic EC by MKP-3 (Figs. 4 and 5). The ERK1/2 signaling cascade has diverse effects in a broad range of cell types (45). ERK1/2 phosphorylation promotes cell growth and survival while in-
dering apoptosis through activation of members of the Bcl-
family (13). ERK1/2 phosphorylation is correlated with con-
stitutive activation of NF-κB in endothelium (38, 56). We have previously reported that endothelial cells from diabetic mice have increased NF-κB activation, resulting in increased cyto-
rine production, adhesion molecule expression, and monocyte adhesion (54). Moreover, we have shown that SIP reduces NF-κB activation, at least in part, through regulation of IkB expression (3). Inhibition of NF-κB has been shown to de-
crease monocyte recruitment and adhesion in murine models of atherosclerosis (2). Thus, action of MKP-3 on ERK1/2 phos-
phorylation may also reduce NF-κB activation, providing an additional anti-inflammatory mechanism for regulation of NF-κB in endothelium by SIP.

In conclusion, our data suggest that expression of MKP-3 is important in minimizing endothelial activation in an elevated glucose environment such as occurs in Type 1 and Type 2 diabetes. Induction of MKP-3 by SIP is another anti-inflam-
atory mechanism through which SIP acts in endothelium to regulate monocyte/endothelial interactions associated with early atherosclerotic plaque development in the vascular com-
plications of diabetes.

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