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

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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 monocyte:endothelial interactions, with increased production of monocyte chemotactic agonist (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 SIP1-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 in glucose-cultured HAEC decreased ERK1/2 phosphorylation and resulted in decreased monocyte:endothelial interactions 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/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 important roles 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 S1P1–5) (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 an 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 insulitis 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, extracellular signal-regulated kinase (ERK), 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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**SIP and MKP-3 induction in EC**

**Resuspension of S1P and SEW2871.** S1P was dissolved in 95% DMSO-5% 1 N 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% acetylated DMSO. Further dilutions of this 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% acetylated DMSO to yield a final concentration of 1 mmol/l. 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).

**Results for other cell types isolated from diabetic animals (24, 27).** 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 NOD mice were incubated in the absence or presence of S1P (100 mmol/l), VPC23019 (10 μmol/l), and SEW2871 (1 μmol/l) for 4 h at 37°C. After incubation, EC were washed and incubated for 30 min with 1 × 10⁶/ml fluorescently labeled (using calcein AM) WEHI 78/24 mouse monocytes. Monocytes were labeled with calcein AM (Molecular Probes) according to the manufacturer’s instructions. After incubation, unbound monocytes were rinsed away, and the number of monocytes firmly bound to the EC monolayer was counted in three consistent fields within a 10 × 10 grid using fluorescent microscopy (50).

**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 EGCS, and 30 ng/l heparin. Cells were used from passages 3 to 5 only. Use of HAEC was approved by the University of Virginia Institutional Review Board (IRB), and all procedures were performed in accordance with University of Virginia IRB guidelines.

**Human monocyte adhesion assay.** HAEC were cultured for 7 days under normal glucose (NG, 5 mmol/l) and high glucose (HG, 25 mmol/l) conditions. We have previously reported that this dose and time of EC culture in elevated glucose stimulates monocyteendothelial interactions (48). S1P (100 mmol/l) and SEW2871 (1 μmol/l) was added to NG and HG-cultured EC for 4 h at 37°C. MonOMac 6 cells (MM6), a human monocyte cell line, were labeled with calcein AM, and 50,000/well monocytes were labeled with calcein AM (Molecular Probes) according to the manufacturer’s instructions. After incubation, unbound monocytes were rinsed away, and the number of monocytes firmly bound to the EC monolayer was counted in three consistent fields within a 10 × 10 grid using fluorescent microscopy (50).

**Immunoblotting for MKP-3, phospho-ERK1/2, and ERK1/2.** Cytosolic extracts were collected from mouse aortic endothelial cells and HAEC using the NE-PER kit (Pierce) according to the manufacturer’s instructions. Protein (50 μg) was analyzed by SDS-PAGE on 4–12% gels (Invitrogen) in MOPS running buffer and transferred to nitrocellulose.Blocking agent used was 5% nonfat milk in Tris-buffered saline plus 0.1% Tween-20 (TBS-T, Sigma). MKP-3 antibody (Santa Cruz) was used at 1:500 dilution. Blots were stripped and reprobed with ERK1/2 (Santa Cruz) and phospho-ERK1/2 (R&D Systems) antibodies, used at 1:1,000 dilution. Horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham) was used at 1:5,000 dilution. All antibodies were diluted in 1% nonfat milk in TBS-T. Blots were quantitated using densitometry and ZeroD-Scan software.

**Transfections.** Primary HAEC were transfected using a Nucleofector II (Amaza Technologies) using the M3 setting and the basic endothelial transfection kit (Amaza) according to the manufacturer’s instructions. Transfection rates of HAEC were 75% of cells (data not shown). For MKP-3 overexpression studies, HAEC were transfected with 1 μg of the control vector, pCMV-SPORT6 or 500 ng of a vector containing the full-length human MKP-3 cDNA (American Type Culture Collection MGC-35368). Cells were used in experiments at 24 h after transfection.

For MKP-3 knockdown studies, HAEC were transfected with 2 μg of the Dharmacon ON-TARGET plus SMARTpool small interfering RNA (siRNA) for Human MKP-3 (L-003964-00-0005) according to the manufacturer’s instructions. As a control, HAEC were transfected with a negative control siRNA (catalog no. 4611, Ambion). Cells were used in experiments at 72 h after transfection. For monocyte adhesion studies, cells were passaged into 48-well plates 48 h after transfection, and adhesion was performed at 72 h.

**Quantitative real-time PCR.** Primary 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'-TTACCTGGCTTGC-GTCTG-3' and reverse: 5'-GGTCTGACCTATCT-3'; β-actin: forward 5'-CATGTGGAGACCTTACAC-3' and reverse 5'-CT-GTTGCTGATCACACT-3'. The PCR conditions were 95°C for 10 min, 95°C for 4 min, followed by 50 cycles of 95°C for 15 s, 58°C for 30 s, 72°C for 30 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:

\[
\text{Relative expression} = 2^{-\Delta \Delta C_T}
\]

where ΔΔCT 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**

**SIP 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 ($P < 0.002$ by ANOVA). Thus, S1P has the ability to regulate MKP-3 expression and ERK1/2 phosphorylation in EC.

*S1P signaling through the S1P$_1$ 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 S1P$_1$ receptor as playing a potent anti-inflammatory role in EC (54). Since S1P$_1$ receptor-deficient mice show embryonic lethality, we used pharmacological reagents that are selective for S1P$_1$. SEW2871 {5-[4-phenyl-5-(trifluoromethylthiophen-2-yl)-3-[3-(trifluoromethylphenyl)-1,2,4-oxadiazole} is a selective S1P$_1$ receptor agonist that is 30-fold less potent than S1P at S1P$_1$, with no agonist activity at S1P$_2$ at concentrations up to 10 μmol/l (55). VPC23019 is a S1P receptor antagonist with a $K_i$ for S1P$_1$ of 25 nmol/l; the molecule is about 50-fold less potent in blocking the S1P$_2$ receptor but is not active at the S1P$_2$ 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 S1P$_1$ 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

**Fig. 4. Decreased monocyte adhesion to EC with MKP-3 overexpression.** Primary HAEC cultured in 5 mmol/l (HAEC) or 25 mmol/l glucose (HG) for 7 days were transfected with a plasmid expressing human MKP3 (+pCMV-MKP3) as described in Methods. After 24 h, HG-EC were treated with either 100 mmol/l S1P (+S1P) or 1 μmol/l SEW2871 (+SEW) for 4 h. A: Cytosolic protein extracts were collected and analyzed by SDS-PAGE for MKP-3 and p-ERK. B: in an in vitro static monocyte adhesion assay, HG-EC bound 3-fold more monocytes than control HAEC; *P < 0.001 by ANOVA. Overexpression of MKP-3 reduced monocyte adhesion in high glucose EC; #P < 0.005 by ANOVA. S1P and SEW2871 further reduced monocyte adhesion in EC overexpressing MKP-3; ##P < 0.002 by ANOVA.

**Fig. 3. Overexpression of MKP-3 in EC reduces ERK1/2 phosphorylation.** Primary human aortic EC (HAEC) were transfected with the plasmid pCMV-MKP3 to overexpress human MKP-3. At 24 h after transfection, cells were incubated with 100 nmol/l S1P (+S1P). Top: cytosolic protein extracts were collected and analyzed by SDS-PAGE for MKP-3, p-ERK, and ERK. A representative gel is shown. Bottom: densitometry shows mean ± SE of 4 mice per group from the above study. There was an induction of MKP-3 with addition of S1P or through overexpression of MKP-3; **P < 0.05 by ANOVA. Addition of S1P and overexpression of MKP-3 resulted in a 3-fold reduction in ERK phosphorylation; *P < 0.005 by ANOVA.
no further effect on reducing monocyte adhesion, suggesting that S1P–S1P1 receptor axis acts through the MKP-3-ERK1/2 pathway in EC to reduce monocyte adhesion (Fig. 4B).

Finally, to further illustrate specificity of MKP-3 in mediating the S1P action on monocyte:endothelial interactions, we used siRNA approaches. We transfected HAEC with either human MKP-3 siRNA or scrambled control siRNA. As shown in Fig. 5A, transfection of HAEC with MKP-3 siRNA reduced MKP-3 expression by ~60%. This resulted in a significant increase in ERK1/2 phosphorylation (Fig. 5B) in HAEC. In the presence of MKP-3 siRNA, treatment of HAEC with S1P did not further alter ERK1/2 phosphorylation, suggesting that the action of S1P in HAEC was MKP-3 pathway specific. Moreover, treatment of HAEC with MKP-3 siRNA significantly increased monocyte adhesion to EC, and S1P was unable to modulate this adhesion (Fig. 5C). Taken together, these data suggest that S1P acts through MKP-3 to regulate ERK1/2 phosphorylation and monocyte:endothelial interactions.

**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-α association in NF-κB 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. It has been reported that MKP-3 has the ability to shuttle between the nucleus and cytoplasm, giving it an important role in deter-

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**Fig. 5.** MKP-3 small interfering RNA (siRNA) inhibits S1P-mediated monocyte adhesion. Primary HAEC were cultured in 5 mmol/l glucose and were transfected with either control siRNA (Neg Trnsft Ctr) or human MKP-3 siRNA (siMKP3). A: quantitative mRNA expression. Expression of MKP-3 was measured using quantitative RT-PCR in untransfected HAEC (HAEC Ctr), in HAEC transfected with a control siRNA (HAEC Neg Trnsft Ctr), and in HAEC transfected with siRNA to human MKP-3. UnRxed, untreated. *Significantly lower than HAEC Neg Trnsft Ctr; P < 0.001 by Student’s unpaired t-test. B: Immunoblotting. ERK1/2 phosphorylation was measured by SDS-PAGE and immunoblotting in untransfected HAEC (HAEC Ctr), in HAEC transfected with a control siRNA (HAEC Neg Trnsft Ctr), and in HAEC transfected with siRNA to human MKP-3. C: monocyte adhesion. HAEC were transfected with siRNA as described above and, at 72 h, were incubated in the absence or presence of S1P (+S1P) for 4 h. At the end of the incubation period, cells were washed and used in a monocyte adhesion assay. *Significantly higher than Neg Trnsft Ctr or Neg Trnsft Ctr + S1P; P < 0.0001 by ANOVA.
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 phosphorylase and phosphotyrosine 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 enhances cell growth and survival while inhibiting apoptosis through activation of members of the Bcl family (13). ERK1/2 phosphorylation is correlated with constitutive 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 cytokine production, adhesion molecule expression, and monocyte adhesion (54). Moreover, we have shown that S1P reduces NF-κB activation, at least in part, through regulation of IkB expression (3). Inhibition of NF-κB has been shown to decrease monocyte recruitment and adhesion in murine models of atherosclerosis (2). Thus, action of MKP-3 on ERK1/2 phosphorylation may also reduce NF-κB activation, providing an additional anti-inflammatory mechanism for regulation of NF-κB in endothelium by S1P.

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 S1P is another anti-inflammatory mechanism through which S1P acts in endothelium to regulate monocyte/endothelial interactions associated with early atherosclerotic plaque development in the vascular complications of diabetes.

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