Sphingosine-1 Phosphate Prevents Monocyte/Endothelial Interactions in Type 1 Diabetic NOD Mice Through Activation of the S1P1 Receptor

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Abstract—Monocyte recruitment and adhesion to vascular endothelium are key early events in atherosclerosis. We examined the role of sphingosine-1-phosphate (S1P) on modulating monocyte/endothelial interactions in the NOD/LtJ (NOD) mouse model of type 1 diabetes. Aortas from nondiabetic and diabetic NOD mice were incubated in the absence or presence of 100 nmol/L S1P. Fluorescently labeled monocytes were incubated with the aortas. Aortas from NOD diabetic mice bound 7-fold more monocytes than nondiabetic littermates (10±1 monocytes bound/field for nondiabetic mice vs 74±12 monocytes bound/field for diabetic mice, P<0.0001). Incubation of diabetic aortas with 100 nmol/L S1P reduced monocyte adhesion to endothelium by 90%. We found expression of S1P1, S1P2, and S1P3 receptors on NOD aortic endothelial cells. The S1P1 receptor-specific agonist SEW2871 inhibited monocyte adhesion to diabetic aortas. Studies in diabetic S1P3-deficient mice revealed that the S1P3 receptor did not play a pivotal role in this process. S1P reduced endothelial VCAM-1 induction in type 1 diabetic NOD mice, most likely through inhibition of nuclear factor κB translocation to the nucleus. Thus, S1P activation of the S1P1 receptor functions in an antiinflammatory manner in type 1 diabetic vascular endothelium to prevent monocyte/endothelial interactions. S1P may play an important role in the prevention of vascular complications of type 1 diabetes. (Circ Res. 2006;99:731-739.)

Key Words: endothelial □ NF-κB □ type 1 diabetes □ sphingosine-1-phosphate □ adhesion molecules

Atherosclerosis development is accelerated several-fold in patients with both type 1 and type 2 diabetes.1,2 Endothelial activation, monocyte recruitment, and monocyte adherence to activated endothelium are key early events in atherosclerosis.3,4 We and others have identified multiple mechanisms through which hyperglycemia in diabetes activates endothelium and increases monocyte/endothelial interactions in the vessel wall.5–10

Sphingosine-1-phosphate (S1P) is generated in mammalian cells primarily from the degradation of ceramide to sphingosine.11,12 Sphingosine is phosphorylated by sphingosine kinases to generate S1P.13 S1P is secreted from leukocytes, platelets, and endothelial cells in the vasculature. S1P is present in nanomolar concentrations and resides on albumin and lipoproteins, particularly high-density lipoprotein, in the circulation.14 The functions of S1P in the vasculature include promotion of endothelial migration, upregulation of endothelial NO synthase (eNOS), inhibition of platelet aggregation, regulation of CD4+ T-lymphocyte trafficking, and regulation of smooth muscle cell proliferation.15–20 We recently reported that S1P reduces endothelial activation in response to tumor necrosis factor (TNF) α challenge in mice.21 S1P binds to 5 G protein–coupled receptors, named S1P1 to S1P5.22

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.23 Susceptibility to type 1 diabetes in this mouse is polygenic, and by 20 weeks, approximately 60% of female NOD mice develop type 1 diabetes. In the current study, we report that S1P prevents monocyte/endothelial adhesion in NOD diabetic mice in vivo through binding to the S1P1 receptor. Activation of this antiinflammatory signaling pathway results in induction of Akt/eNOS signaling, and inhibition of nuclear factor κB (NF-κB).

Materials and Methods

Detailed methods and reagents used can be found in the online data supplement, available at http://circres.ahajournals.org. NOD/LtJ mice were obtained from The Jackson Laboratory (Bar Harbor, Me; stock no. 001976). NOD diabetic or nondiabetic littermates were injected with 2 mg/kg SEW2871 or aortas removed and incubated in vitro with 100 nmol/L S1P and/or 10 μmol/L VPC23019. Subsequently, aortas were opened, pinned on agar, and used in an ex vivo monocyte adhesion assay as
previously described. Alternatively, aortic endothelium (endothelial cells [ECs]) was freshly isolated from NOD diabetic and nondiabetic mice. Conventional RT-PCR was performed for S1P receptor expression, and flow cytometry was performed for intercellular adhesion molecule (ICAM)-1 and vascular cellular adhesion molecule (VCAM)-1 surface expression on ECs. NOD diabetic and nondiabetic ECs were used in a flow chamber assays after incubation with SEW2871, S1P, or BAY11-7085 as described. Fluorescent microscopy for NF-κB was performed as described. Immunoblotting for signaling molecules was performed as described.

Figure 1. Expression of S1P receptors in nondiabetic and type 1 diabetic NOD mice. RNA isolated from type 1 diabetic (Diab) and nondiabetic (Ctr) aortic NOD endothelial cells was used in conventional RT-PCR to detect S1P receptors. There was no detectable expression of S1P4 or S1P5 mRNAs. Shown for each receptor is a positive control; the positive control for S1P1, S1P2, and S1P3 is whole mouse cDNA; the positive control for S1P4 is T-lymphocyte cDNA; and the positive control for S1P5 is EL4-IL2 T-cell line cDNA. β-Actin is shown as a loading control. Data represent pooled RNA samples from 8 mice per group.

Figure 2. S1P and SEW2871 reduce monocyte adhesion to aorta in diabetic NOD mice. A, Studies using S1P. Aortas were isolated from nondiabetic NOD (CTR) and diabetic NOD (DIAB) mice and incubated for 4 hours in the presence of 100 nmol/L S1P (+S1P). Fluorescently labeled monocytes were added to the aortas for an adhesion assay and counted using a fluorescent microscope. *Significantly higher than nondiabetic control (P < 0.0001); **significantly lower than NOD diabetic (P < 0.0002) by ANOVA. B, Studies using SEW2871, a S1P1 receptor-specific agonist. Nondiabetic NOD (CTR) and diabetic NOD (DIAB) mice were injected intravenously with 2 mg/kg SEW2871 (+SEW). Aortas were harvested and fluorescently labeled monocytes were incubated with the aortas and counted using a fluorescent microscope. *Significantly higher than nondiabetic control (P < 0.0001); **significantly lower than NOD diabetic (P < 0.0001) by ANOVA. C, Studies using VPC23019, a S1P1 receptor antagonist. Aortas were isolated from nondiabetic NOD (CTR) and diabetic NOD (DIAB) mice and incubated for 4 hours in the absence and presence of 10 μmol/L VPC23019 (+VPC23019) with or without 100 nmol/L S1P (+S1P). Aortas were harvested and fluorescently labeled monocytes were added to the aortas and counted using a fluorescent microscope. *Significantly higher than nondiabetic control (P < 0.0001); **significantly lower than NOD diabetic (P < 0.0001) by ANOVA. D, Studies using PTX to uncouple G<sub>i</sub> signaling. Aortas were isolated from nondiabetic (CTR) and diabetic (DIAB) NOD/LtJ mice and incubated overnight with 100 ng/mL PTX to uncouple G<sub>i</sub> signaling. After treatment, aortas were incubated in the absence or presence of 1 μmol/L SEW2871 (+SEW) for 4 hours. Fluorescently labeled monocytes were added and counted using a fluorescent microscope. *Significantly higher than NOD control (P < 0.0001), **significantly lower than NOD diabetic (P < 0.0001) by ANOVA; #significantly higher than NOD diabetic - SEW2871 (P < 0.001).
formed on protein extracts isolated from nondiabetic and diabetic ECs after incubation with S1P or SEW2871 for various times.21,24

**Results**

**Expression of S1P Receptors in Mouse Aortic Endothelium of Type 1 Diabetic Mice**

Expression of S1P receptor mRNA in aortic ECs of NOD mice was analyzed by RT-PCR. NOD nondiabetic and diabetic aortic ECs express mRNA for S1P1, S1P2, and S1P3 receptors (Figure 1). There is no expression of either S1P4 or S1P5 mRNA in mouse aortic endothelium.

**SIP Blocks Monocyte/Endothelial Interactions in Type 1 Diabetic Mouse Aorta Through S1P1**

We have shown that TNFα-mediated activation of endothelium was blocked by S1P and by the S1P1-specific receptor agonist 5-(4-phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylphenyl)-1,2,4-oxadiazole (SEW2871).21 In the current study, we examined whether monocyte adhesion to intact type 1 diabetic mouse aorta could be reduced by S1P or SEW2871. Aortas from control and type 1 diabetic NOD mice were incubated with fluorescently labeled monocytes. Representative images are shown in Figure 2. The hazy background in the images is a result of autofluorescent structures in the aortic wall. Type 1 diabetic mouse aortas displayed significant elevations in adhesion of monocytes to endothelium (Figure 2A). As nondiabetic, nonactivated endothelium binds very few monocytes,21,25 these data suggest that the endothelium of type 1 diabetic NOD mice is highly activated to bind monocytes. Incubation with 100 nmol/L S1P ex vivo dramatically reduced monocyte adhesion to diabetic NOD aorta (Figure 2A). To test whether specific activation of the S1P1 receptor could prevent monocyte adhesion to aorta, NOD control and diabetic mice were injected intravenously with 2 mg/kg SEW2871. SEW2871 is a selective agonist for S1P1 and is 30-fold less potent than S1P at S1P1, with no agonist activity at S1P2 or S1P3 at concentrations up to 10 μmol/L.26 Subgroups of mice were injected with saline +0.2% fatty acid–free BSA (FABBSA) as a vehicle control. Aortas were harvested and immediately incubated with WEHI 78/24 mouse monocyte cells ex vivo. SEW2871 completely blocked monocyte adhesion to aorta (Figure 2B), suggesting that activation of the endothelial S1P1 receptor prevents monocyte/endothelial adhesion. The fact that SEW2871 does not act on either S1P2 or S1P3 receptors at the concentration used in our study strongly suggests that S1P1 is the receptor causing inhibition of monocyte/endothelial adhesion in the diabetic NOD mouse.

We next used VPC23019, a S1P receptor antagonist; VPC23019 is approximately 50-fold less potent in blocking S1P3, but is completely inactive at S1P2.27 VPC23019 (10 μmol/L) inhibited the ability of S1P to reduce monocyte adhesion to NOD diabetic aorta by 70% (Figure 2C), further supporting a role for S1P1 and indicating that S1P2 is not playing a major role in this process. We also used pertussis inhibitors
toxin (PTX) to uncouple $G_{ai}$ receptor signaling.28 S1P1 is known to couple solely through the G protein $G_{ai}$.29 Subgroups of aortas were treated with 100 ng/mL PTX overnight to uncouple $G_{ai}$, and the aortas were treated with 1 $\mu$mol/L SEW2871 or 100 nmol/L S1P. PTX reversed the inhibitory action of SEW2871 on monocyte adhesion to aorta by approximately 75% (red bars in Figure 2D). PTX also reversed the inhibitory action of S1P by approximately 70% (data not shown). PTX alone had no effect on monocyte adhesion to aorta (Figure 2D). The PTX experiments indicate that S1P and SEW2871 work through a $G_{ai}$-coupled receptor to inhibit monocyte/endothelial adhesion. Thus, the notion that S1P1 is the receptor regulating monocyte/endothelial interactions by S1P is strengthened by the facts that (1) the S1P1 receptor–specific agonist SEW2871 inhibits monocyte/endothelial adhesion in aorta of type 1 diabetic NOD mice; (2) the S1P1 receptor antagonist VPC23019 blocks the inhibitory action of S1P; and (3) PTX inhibits the antiinflammatory action of both S1P and SEW2871.

**Inhibition of S1P3 Does Not Interfere With The Antiinflammatory Action of S1P on Endothelium**

To formally rule out a role for S1P3, we performed studies in diabetic S1P3-deficient mice in vivo. S1P3-deficient mice are viable and exhibit no obvious phenotypic abnormalities.30 S1P3-deficient mice were rendered type 1 diabetic through IP injection of streptozotocin. Fasting blood glucose values of the diabetic S1P3-deficient mice averaged 295±25 mg/dL (control S1P3-deficient mouse blood glucose values averaged 110±5 mg/dL). Two weeks after the mice developed hyperglycemia, aortas were isolated from the mice and used in an ex vivo monocyte adhesion study. As expected, monocyte adhesion was increased by almost 5-fold in diabetic S1P3-deficient endothelium compared with nondiabetic S1P3-deficient endothelium (Figure 3), again supporting the concept that diabetes increases monocyte/endothelial interactions in aorta. If the S1P3 receptor is required for S1P to inhibit monocyte/endothelial interactions in type 1 diabetic mice,
then S1P should not inhibit monocyte adhesion to diabetic S1P3-deficient endothelium. In support of this hypothesis, S1P and SEW2871 were able to completely inhibit monocyte adhesion to aorta in diabetic S1P3-deficient mice (Figure 3). To rule out changes in S1P1 receptor expression in response to the knockout of S1P3, we found no differences in S1P1 receptor mRNA levels between wild-type and S1P3-deficient mice (data not shown). Taken together, our data indicate that the antiinflammatory effect of S1P on monocyte/endothelial interactions appears to be dependent on S1P1 and is clearly independent of S1P3.

SIP Reduces Monocyte Adhesion to Type 1 Diabetic Endothelium

Using a flow chamber system, we examined whether S1P could prevent monocyte adhesion to diabetic endothelium. Type 1 diabetic NOD endothelium showed increased adhesion of monocytes in the flow chamber (Figure 4A). Treatment of ECs with 100 nmol/L S1P for 4 hours blocked monocyte adhesion (Figure 4A).

SIP Modulates Endothelial Adhesion Molecule Expression in Diabetic NOD Mice

Surface expression of ICAM-1 and VCAM-1 was dramatically increased on aortic endothelium of type 1 diabetic NOD mice (Figure 4B). E-selectin expression was unchanged in diabetic NOD endothelium and there was little expression of P-selectin in either control or diabetic NOD endothelium (data not shown). Incubation of diabetic ECs either with 100 nmol/L S1P or with 1 μmol/L SEW2871 for 4 hours reduced endothelial surface expression of VCAM-1 approximately 30% (Figure 4C). Surprisingly, neither S1P nor SEW2871 reduced ICAM-1 surface expression (Figure 4C). We confirmed decreased expression of VCAM-1 after 4 hours of incubation of ECs with S1P using immunoblotting of total cell lysates (Figure 4C). We also found decreased levels of ICAM-1 after 4 hours, although surface expression changed little, suggesting that there may be redistribution of ICAM-1 on the cell surface in response to S1P.

Blocking antibodies to ICAM-1 and VCAM-1 blocked monocyte adhesion to diabetic NOD ECs by 80% and 90%, respectively (data not shown). Because S1P and SEW2871 reduce expression of VCAM-1 on diabetic NOD endothelium, this explains, in part, the antiinflammatory effects of S1P on monocyte/EC interactions. However, these data do not preclude the possibility that other molecules, such as chemokines or lipid mediators, influence monocyte/endothelial interactions.

SIP Does Not Decrease Endothelial Production of Either Monocyte Chemoattractant Protein-1 or IL-6 in Diabetic NOD Mice

Monocyte chemoattractant protein (MCP)-1 secretion was increased by approximately 50% in diabetic NOD ECs compared with control ECs. Values for MCP-1 were 325±20 pg/mL per milligram of protein for control mice versus 484±50 pg/mL per milligram of protein for NOD diabetic mice (P<0.01). Values for IL-6 tripled from 65±6 pg/mL per milligram of protein for control mice to 196±58 pg/mL per milligram of protein for diabetic mice (P<0.03). S1P did not reduce endothelial secretion of either MCP-1 or IL-6 (values averaged 499±47 pg/mL per milligram of protein for MCP-1 and 212±52 pg/mL per milligram of for IL-6 in diabetic NOD EC with S1P incubation).

SIP Prevents NF-κB Nuclear Translocation in Diabetic Endothelium

When NF-κB is activated, it mobilizes from the cytosol to the nucleus, where it initiates inflammatory gene transcription. Using an Alexa 594 fluorescent antibody that recognizes the p65 subunit of NF-κB, we found a significant increase in nuclear localization of the p65 subunit of NF-κB in NOD diabetic endothelial cells, indicating that these cells were activated compared with their corresponding nondiabetic controls (Figure 5A). Treatment of cells with either S1P or SEW2871 significantly reduced p65 mobilization to the nucleus (Figure 5A). There was a significant 3-fold induction in the percentage of nuclei that were positive for nuclear p65 staining in diabetic NOD mice (Figure 5B). Both S1P and SEW2871 reduced p65 nuclear localization in diabetic NOD mice by 60% (Figure 5B). As NF-κB becomes activated, cytosolic IκB is degraded to allow NF-κB to translocate to the nucleus to initiate inflammatory gene transcription. Endothelial cell extracts from diabetic NOD mice show more p65 expression in the nucleus and less IκB expression in the cytosol compared with control NOD mice (Figure 5C). In fact, there is little IκB expression in cytosol of diabetic NOD mice (Figure 5C). We confirmed this finding using total EC protein lysates (Figure 5C). These data imply degradation of IκB and mobilization of NF-κB to the nucleus in diabetic NOD mice. S1P- and SEW2871-treated diabetic NOD ECs display less nuclear p65 expression and greater IκB expression in the cytosol (Figure 5C), indicating that S1P and SEW2871 prevent NF-κB activation. We performed a time course of S1P addition to ECs and found that IκB protein expression begins to be restored in the cytosol of diabetic NOD ECs within 30 minutes to 1 hour of S1P treatment (Figure 5C and Figure 6). These data suggest that S1P rapidly induces IκB synthesis to inhibit NF-κB activation. Interestingly, we observed a slight reduction in IκB expression in control mouse ECs after 1 hour of S1P treatment (Figure 5C). This was not observed in diabetic mouse ECs. This may be an initial protective or survival response of control ECs to lipid loading. However, this response appeared to be rapid and transient and disappeared within 1 to 2 hours (Figure 5C). Finally, to confirm that NF-κB is a primary signaling pathway for mediating monocyte adhesion to type 1 diabetic NOD endothelium, we examined monocyte adhesion using a flow chamber in the presence of BAY11-7085, a commonly used pharmacological inhibitor of NF-κB. As shown in Figure I of the online data supplement, pretreatment of type 1 diabetic NOD ECs with BAY11-7085 reduced monocyte adhesion approximately 80%, indicating that NF-κB is a primary mediator of monocyte/endothelial interactions in diabetic NOD ECs. We also examined phosphorylation of Ser536 of NF-κB p65, which stimulates maximal transcriptional activation of NF-κB.11 We found significant reduction of p65 phosphorylation after 30 minutes of S1P treatment of
ECs isolated from diabetic NOD mice (Figure 6). We did not observe decreased phosphorylation in control NOD mice. Thus, S1P binding to the S1P1 receptor inhibits NF-κB signaling in endothelial cells via several mechanisms, thereby reducing diabetes-mediated endothelial activation and monocyte/endothelial interactions in the vessel wall.

Next, we examined Akt pathway and eNOS activation by S1P in endothelium. Sessa and colleagues reported that S1P activates Akt and eNOS through interactions with S1P1.19 In diabetic NOD endothelium, both eNOS and Akt are phosphorylated within 10 minutes of S1P treatment of ECs (Figure 6). Akt is also phosphorylated by S1P in nondiabetic ECs within 10 minutes. To determine whether S1P acted via eNOS to inhibit monocyte adhesion to endothelium, we treated diabetic NOD ECs with N\textsubscript{G}-nitro-L-arginine methyl ester (L-NAME), a well-characterized inhibitor of eNOS. Treatment of diabetic NOD ECs with L-NAME reduced the action of S1P by approximately 45% (supplemental Figure II). Thus, it is quite plausible that S1P induces eNOS in endothelium through Akt activation and that eNOS activation contributes to the anti-inflammatory action of S1P.

To correlate S1P effects on signaling pathways with monocyte adhesion to endothelium, we performed a time course of S1P treatment on monocyte adhesion. Control nondiabetic and diabetic NOD ECs were treated with S1P for 10 minutes, 30 minutes, 1 hour, and 4 hours, followed by a monocyte adhesion assay using the flow chamber. The time course of S1P incubation resulted in a step-wise decrease in monocyte adhesion (supplemental Figure III). Thus, reductions in monocyte adhesion to endothelium can be detected as early as 30 minutes of S1P incubation; we routinely chose 4 hours for our studies, as that time point appeared to provide maximal reduction in adhesion (supplemental Figure III). Fitting with these data, we observed reductions in VCAM-1 and ICAM-1 protein at 4 hours of S1P incubation, but not before (Figure 4C). The reduction in monocyte adhesion to endothelium that occurred within 30 minutes of incubation of ECs with S1P was most likely attributable to chemokine or lipid mediators and not to changes in adhesion molecule expression (Figure 4C).

**Discussion**

Monocyte/endothelial interactions are key initiating events in atherosclerosis. We demonstrate that monocyte/endothelial interactions are significantly increased in the NOD mouse model of type 1 diabetes. S1P in low nanomolar concentrations inhibits monocyte adhesion to endothelium in type 1 diabetic NOD mice through specific activation of the endothelial S1P1 receptor. Thus, S1P could provide a beneficial therapeutic effect for diabetes-induced vascular complications through specific activation of endothelial S1P1.
Our data strongly support the concept that S1P1 is the primary receptor mediating the “antiinflammatory” effects of S1P. Our studies in S1P3-deficient mice (Figure 3 and supplemental Figure IV) indicate that S1P3 does not participate significantly to this process. Supplemental Figure IV illustrates that there are no major differences in monocyte adhesion between wild-type and S1P3-deficient mice in response to physiological concentrations of S1P. Our data obtained from the use of pharmacological agonists (SEW2871) and antagonists of the S1P1 receptor (VPC23019)—both of which have no action on S1P2—strongly suggest that S1P2 does not mediate the effects of S1P on adhesion. Ideally, the best model for these studies is the S1P1-deficient mouse. This mouse displays embryonic lethality, as do endothelial-specific S1P1 KO mice, resulting from the critical developmental role for S1P1 in vascular maturation. If these mice were viable, we speculate that S1P would not be able to reduce monocyte/endothelial interactions in these mice and that these mice would develop accelerated atherosclerosis, particularly in the setting of diabetes or hyperlipidemia. Our data indicate that S1P1 is required by S1P for reducing monocyte/endothelial interactions in type 1 diabetic mice.

We suspect that signaling pathways are modulated by S1P in monocytes as well as in endothelium. However, for the purposes of the current study, we focused entirely on S1P action on aortic endothelium. S1P has a striking ability to block endothelial NF-κB signaling most likely attributable to induction of 1kB synthesis. The induction of 1kB synthesis prevents NF-κB mobilization to the nucleus to initiate inflammatory gene transcription. We also found decreased p65 phosphorylation in response to S1P (Figure 6). Phosphorylation of p65 is a posttranslational modification that allows for maximal activation of NF-κB. We confirmed that NF-κB was a primary regulator of monocyte adhesion to diabetic NOD endothelium. Using the NF-κB inhibitor BAY11-7085, we reduced adhesion in type 1 diabetic NOD mice by approximately 80% (supplemental Figure I). However, addition of S1P to BAY11-treated ECs showed a further reduction in monocyte adhesion (supplemental Figure I). These data imply that (1) there are additional mechanisms involved in S1P action in diabetic ECs and (2) S1P is a more effective inhibitor of NF-κB than the BAY11-7085 compound. Our data indicate that S1P blocks NF-κB activation at several steps in the pathway, including 1kBα synthesis, NF-κB translocation, and p65 subunit phosphorylation, all of which make S1P quite an effective NF-κB inhibitor. However, there are additional mechanisms of action of S1P in endothelium. Sessa and colleagues have shown that S1P activates eNOS in endothelium through Akt phosphorylation. We found rapid phosphorylation of eNOS and Akt within 10 minutes of treating ECs with S1P. The phosphorylation of Akt was apparent in both control and diabetic NOD mouse ES, whereas the phosphorylation of eNOS seemed to be more apparent in diabetic NOD ECs (Figure 6). Although the phosphorylation of eNOS appears somewhat subtle, we found that L-NAME reduces the ability of S1P to block monocyte adhesion to diabetic NOD endothelium by approximately 45%. Thus, we concur from these data that eNOS is indeed activated by S1P in endothelium, most likely through S1P1, and that eNOS activation contributes to the antiinflammatory action of S1P on endothelium.

We found that it takes approximately 4 hours of S1P treatment to reduce VCAM-1 expression; thus, there is not rapid turnover of adhesion molecule expression (Figure 4C). However, we do not know whether S1P interferes with the physical interaction of monocyte integrins and endothelial adhesion molecules. We anticipate that this is not the case, because maximal effects on adhesion occur after 4 hours of incubation with S1P and ECs are rinsed before incubation with monocytes. Despite only a 30% reduction in VCAM-1 expression by S1P, our data suggest that the majority of S1P inhibitory action on monocyte adhesion to endothelium after 4 hours is attributable to decreased VCAM-1 expression. However, our results in Figure 6 and supplemental Figure III indicate that there are additional factors contributing to the antiinflammatory action of S1P on monocyte/endothelial interactions at early time points (between 30 minutes and 4 hours). We have previously shown that CS-1 fibronectin impacts monocyte/endothelial interactions. We did not examine CS-1 expression in the current study. Moreover, ECs secrete multiple chemokines and lipid mediators that impact monocyte/endothelial adhesion. These include the chemokines IL-8/KC, RANTES, macrophage inflammatory protein (MIP)-1α, and lipid mediators produced by endothelial cyclooxygenase and 12/15 lipoxigenase (LO) enzymes. Indeed, we have reported upregulation of 12/15 LO activity in endothelium of diabetic db/db mice, as well as in human aortic endothelial cells cultured in elevated glucose. We have found that the 12/15 LO products 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) and 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE) stimulate monocyte/endothelial interactions. Thus, inhibition of 12/15 LO product function may be an additional mechanism of action of S1P. Studies are
underway to identify additional factors that mediate monocyte/endothelial interactions that are modified by S1P.

Previous studies have reported that S1P increases endothelial VCAM-1 and E-selectin expression. These investigators used high concentrations of S1P (5 to 20 μmol/L). We observed increased monocyte adhesion to aortic mouse ECs at concentrations of S1P greater than 5 μmol/L (supplementary Figure IV). In the bloodstream, physiological levels of free S1P are in the nanomolar range, and the reported Kᵢ₃ values for S1P binding to the S1P1 receptor are 1 to 10 nmol/L. Therefore, it is quite probable that high micromolar concentrations of S1P act on receptors other than S1P1.

In summary, we have found that S1P prevents monocyte adhesion to type 1 diabetic mouse aorta through activation of the S1P1 receptor on endothelium. Activation of S1P1 by S1P decreases NF-κB nuclear translocation and p65 phosphorylation, resulting in reduced monocyte adhesion to diabetic NOD endothelium. Therapies to upregulate the S1P1 signaling pathway in type 1 diabetes may be useful for prevention of diabetic vascular complications, including atherosclerosis.

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DISCLOSURES

None.

REFERENCES


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Materials and Methods

Reagents: WEHI 78/24 mouse monocytes\textsuperscript{1} were a gift of Dr Judy Berliner (UCLA). Pertussis toxin was a gift of Dr Erik Hewlett (University of Virginia). Cytometric bead arrays (#552364) were purchased from BD Biosciences. Antibodies used were: PE-labeled ICAM-1 (clone 3E2), FITC-labeled VCAM-1 (clone 429), FITC-labeled P-selectin (clone GMP140), and PE-labeled E-selectin (clone 10E9.6) for flow cytometry (all from BD Biosciences); ICAM-1 blocking antibody (clone LTF653) and VCAM-1 blocking antibody (clone M/K2) (both from Chemicon). Isotype antibodies included rat IgG2b (control for ICAM-1 antibody), and rat IgG1\(\kappa\) (control for VCAM-1 antibody). Alexa 488-conjugated and Alexa 594-conjugated secondary antibodies were purchased from Molecular Probes, ERK1 (sc-94), NF\(\kappa\)B p65 antibody (sc-109), ICAM-1 (sc-1511), VCAM-1 (sc-8304) and Histone H1 antibody (sc-8030) were purchased from Santa Cruz; I\(\kappa\)B\(\alpha\) antibody (#9242), phospho-eNOS (ser1177; #9571S), total p38 (#9212), and phospho-NF\(\kappa\)B p65 (ser536; #3031S) were obtained from Cell Signaling. Phospho-ERK1/2 (T202/Y204; #AF1018), phospho-Akt (S473; #AF887), phospho-p38 (T180/Y182; #AF869) were obtained from R&D Systems, total Akt1 antibody (#07-416) was obtained from Upstate, S1P was obtained from Cayman Chemicals, BAY11-7085 and L-NAME were purchased from Biomol, SEW2871 was from Sigma, and VPC23019 was synthesized in the laboratory of Dr Timothy MacDonald in the Department of Chemistry at University of Virginia.

Resuspension of S1P and SEW2871. S1P was dissolved in 95\%DMSO/5\%1N HCl in \(H_2O\) at a concentration of 20 mM S1P. This 20 mM solution was further diluted 20:1 into 3\% fatty acid free BSA (FAFBSA) in 1X PBS to yield a final stock concentration of 1
mM S1P in 3% FAFBSA/5% acidified DMSO. Further dilutions of the 1 mM S1P stock solution for cell culture use were diluted into 0.2% FAFBSA/PBS and added directly to cells. The final solution contained 0.001% DMSO. DMSO at this concentration had no effect on monocyte adhesion (data not shown).

SEW2871 was dissolved in PBS with 3% FAFBSA/5% acidified DMSO to yield a final concentration of 1mM. Further dilutions of this 1 mM SEW2871 stock solution were diluted into 3% FAFBSA/sterile saline and injected into mice, or diluted into 3% FAFBSA/PBS and added directly to cells.

Mouse Aorta Isolation and Monocyte Adhesion Assay: NOD/LtJ mice (stock #001976) were purchased from Jackson Laboratories and S1P3-deficient mice were obtained from Dr. Richard Proia\textsuperscript{2,3}. All mice were maintained on rodent chow. All animal studies were performed following approved guidelines of the University of Virginia Animal Care and Use Committee. In some studies, mice were injected intravenously with 2 mg/kg of the S1P1 agonist SEW2871. At 2h post-injection, aortas were harvested from the mice, and immediately placed into DMEM + 1% heat-inactivated FBS. The aortas were opened up longitudinally, and pinned onto sterile agar. Aortas were incubated for 30 mins with 1x10^6 fluorescently-labeled (using Calcein AM) WEHI 78/24 mouse monocytes. Monocytes were labeled with Calcein–AM (Molecular Probes) according to manufacturer’s instructions. After incubation, unbound monocytes were rinsed away, and the number of monocytes firmly bound to aorta was counted in 3 fields using fluorescent microscopy\textsuperscript{1,4,5}. Data are represented as the mean±SE of 3 areas of aorta. The areas counted were within a grid, and the same locations per aorta were counted. In other studies, aortas were isolated and immediately incubated with either
100nM S1P for 4h at 37C, or with 100nM S1P plus 10µM VPC23019, a S1P1 receptor antagonist\(^6\) for times indicated in the Figure Legends. As controls for injection, mice were injected with 150 µl saline+0.2% FAFBSA intravenously.

**Isolation of mouse aortic endothelial cells:** Aortic EC from NOD diabetic and control mice (normoglycemic littermates) were harvested from mouse aorta under sterile conditions as outlined previously \(^7\). Mouse endothelial cells were cultured in DMEM containing 15% heat-inactivated FBS, 30 µg/ml ECGS, and 50 µg/ml heparin, and were used in experiments from passages 2 to 4.

**Flow chamber studies.** MAEC were cultured to confluency in a parallel plate flow chamber (Glycotech). WEHI 78/24 mouse monocytes (1x10\(^6\) cells/ml), a mouse monocyte cell line that has been fully characterized by McEvoy and colleagues\(^1;8\), were labeled with Calcein-AM (Molecular Probes) according to manufacturer’s instructions. The WEHI cells were allowed to flow over the endothelium at a shear stress of 0.75 dynes/cm\(^2\). Data was recorded for 5 minutes using a video recorder as previously described \(^9;10\). The total number of rolling and firmly adherent WEHI monocytes to the endothelium at the end of the 5 minute period were counted by fluorescent microscopy using a 10mmx10mm eyepiece grid and a 10X objective\(^10\). Triplicate plates were performed for each experimental condition. For studies using S1P and SEW2871, MAEC were treated with 100 nM S1P or 1 µM SEW2871 in DMEM + 1% HIFBS for the times indicated in the Figure Legends prior to performing the assay. For studies using blocking antibodies, MAEC were treated for 4h with either 5 µg/mL of blocking antibody directed against murine ICAM or VCAM or with 5µg/ml isotype control antibodies in DMEM + 1% HIFBS in the presence of absence of 100nM S1P prior to performing the
flow chamber assay. For studies using L-NAME or BAY11-7085, MAEC were treated for 4h with either 1µM L-NAME or 10µM BAY11-7085 in DMEM + 1% HIFBS prior to performing the flow chamber assay.

**NFκB translocation studies.** MAEC were maintained as described above and incubated in DMEM +1% HIFBS with 100 nM S1P or 1 µM SEW2871 for the times indicated in the Figure legends. After treatment, media was removed and cells were fixed in PBS + 2% formaldehyde for 20 mins at room temp. Cells were then permeabilized by the addition of PBS + 0.2% Triton for 10 minutes. Permeabilized cells were incubated for 1 hour in PBS containing 1% FAFBSA and 10% goat serum to block non-specific binding. Cells were incubated for 1h at room temperature with 1:200 dilution of rabbit anti-NFκB p65 in blocking buffer. Cells were rinsed with PBS and incubated for 1h with 1 µg/ml Alexa 594-conjugated goat anti-rabbit IgG in blocking buffer. After incubation, cells were rinsed 3 times with PBS and coverslips were mounted using Fluoromount media for microscopy.

**Immunoblotting for NFκB.** MAEC were incubated in DMEM +1% HIFBS with 100 nM S1P or 1 µM SEW2871 for the times indicated in the Figure legends. Cytosol and nuclear extracts were collected from MAEC using the NE-PER kit (Pierce) according to the manufacturer’s instructions. 50 µg protein was analyzed by SDS-PAGE on 4-12% gels (Invitrogen). Cytosol was probed for IκBα 1:2000 (Cell Signaling) and nuclear extract for NFκB p65 1:2000 (Santa Cruz). Cytosol and nuclear extracts were normalized to tubulin 1:5000 (Sigma) or histone H1 1:3000 (Santa Cruz) respectively.

**Immunoblotting for signaling molecules.** MAEC were incubated in DMEM +1% HIFBS with 100 nM S1P for the times indicated in the Figure Legends. Cells were
collected using 1x lysis buffer containing 50mM TrisHCl (pH8.0), 150mM NaCl, 1% NP40, 10mM NaF, 2mM Na3VO4, and protease inhibitors (Sigma). 35 µg protein was analyzed by SDS-PAGE on 4-12% gels (Invitrogen). Pierce Blocker BLOTTO in TBS was used as a blocking agent. Membranes were probed using 1:1000 dilutions of VCAM-1, ICAM-1, phospho and total Akt, phospho p65, phospho eNOS, and IκBα antibodies. Proteins were detected using 1:5000 dilutions of appropriate secondary antibodies and chemiluminescence.

**RT-PCR for murine S1P receptors.** MAEC were freshly isolated from aorta and cultured as described above in 100 mm cell dishes. Total cellular RNA was obtained from MAEC using Trizol according to manufacturer’s instructions. The RNA was treated with DNAse I at rt for 10 min. The reaction was stopped by the addition of EDTA. Reverse transcription of 2 µg of total RNA was performed in a total volume of 30 µl using iScript RT (Biorad) and random hexamers. For measurement of S1P receptor mRNA abundance, 2 µl cDNA from each experimental group were utilized. Primer sequences used were as follows: S1P1: sense 5’- caccggcccatgtactattt-3’ antisense 5’-gactgccttggagatgttc-3’, S1P2: sense 5’-gggcatgtcactctgtcctt-3’ antisense 5’-gacgggacaagggtgagtcta-3’, S1P3: sense 5’-gagcaaccttggctacttgc-3’ antisense 5’-agcttcgggttcagtgagaa-3’, S1P4: sense 5’ - GGCTACTGGCAGCTATCCTG - 3’ antisense 5’ - GCTGAGTGACCGAGAAGTCC - 3’, S1P5 sense 5’ - GCCGGTGAGTGAGGTTATTG - 3’ antisense 5’ - CGCGACATCCAGTAATAGCA - 3’, and β-actin: sense 5’-catgtttgagaccttcaacac-3’ antisense 5’-ctgcttgctgatccacatct-3’. PCR conditions for S1P1, S1P2, S1P3, and β-actin were as follows: 94°C 2min, followed by 30 cycles of 94°C 30 sec, 55°C 30 sec, 72°C 45 sec, with a final extension
time of 5 min at 72°C. Bands were analyzed on a 1.0% agarose gel in 1X TAE buffer. PCR for \( \beta \)-actin was performed as a control for normalization purposes.

**Flow cytometry.** MAEC at passage 2 were collected in PBS containing 1% FAFBSA using Accutase (MP Biomedicals). 150,000 cells per sample were analyzed for each antibody. Cells were incubated for 30 min at 4C with PE-labeled or FITC-labeled primary antibodies for mouse adhesion molecules (ICAM-1, E-selectin, VCAM-1, P-selectin) as described previously \(^{11} \). Samples were analyzed at the University of Virginia Flow Cytometry Core using a Becton Dickinson FACSCalibur™ instrument. Analyses were performed using FL1 channel histograms from which the mean fluorescence intensity (MFI) channel was calculated for each sample.

**Cytometric bead array for inflammatory cytokines.** MAEC from diabetic and control NOD were cultured to confluency using DMEM containing 15% heat-inactivated FBS, 30\( \mu \)g/ml ECGS, and 50\( \mu \)g/ml heparin. MAEC were incubated in DMEM +1% HIFBS ±100nM S1P for 4h at 37C. Cell culture supernatant was collected from EC from both mouse groups. The collected supernatant was quantitatively measured for interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), interferon-\( \gamma \) (IFN-\( \gamma \)), TNF\( \alpha \), and interleukin-12p70 (IL-12p70) protein levels using a Cytometric Bead Array according to manufacturer’s instructions. Triplicate plates were performed for each experimental condition. Values for IFN\( \gamma \), IL-10, IL-12, and TNF\( \alpha \) ranged below the level of detection of the assay, indicating that endothelial cells produce little of these cytokines.

**Streptozotocin Injections.** S1P3-deficient mice were a kind gift of Dr. Richard Proia (NIH). 8-10 week old S1P3-deficient mice or C57BL/6J control mice were injected intraperitoneally daily with 40 mg/kg streptozotocin (Sigma) dissolved in freshly
prepared 0.05 M sodium citrate pH 4.5, for 4 consecutive days. After 2 weeks, random blood glucose levels were measured using a OneTouch Ultra glucometer (Life Scan). Mice with random blood glucose levels of ≥250 mg/dl were considered diabetic and were used in experiments.

**Statistical Analyses:** Data for all experiments were analyzed by ANOVA using the Statview 6.0 software program. Comparisons between groups were performed using analysis of variance (ANOVA) methods. Data are graphically represented as mean ± SE, in which each mean consists of four experiments performed in triplicate (unless noted otherwise in the figure legends) using 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 LSD 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.
References


Figure Legends

**Online Figure I. NFκB inhibition in Type 1 Diabetic NOD EC reduces monocyte adhesion.** Aortic EC from non-diabetic (black bars) and Type 1 diabetic (red bars) NOD mice were incubated for 4h with 100nM S1P (+S1P), 10µM BAY11-7085 (+BAY11), or DMSO as a vehicle control (+DMSO). EC were maintained in media as a baseline control (No Rx). Cells were used in a parallel plate flow chamber system to measure monocyte adhesion. Note there is very little adhesion to the non-diabetic EC. Data represent the mean±SEM of 3 experiments performed in duplicate dishes.

*Significantly higher than NOD CTR, p<0.0001; **significantly lower than diabetic NOD No Rx, p<0.005, #significantly lower than diabetic NOD No Rx, p<0.0001 by ANOVA.

**Online Figure II. eNOS is involved in S1P action on endothelium.** Aortic EC from non-diabetic (black bars) and diabetic (red bars) NOD mice were incubated for 4h with 100nM S1P (+S1P), L-NAME (+LNAME), or S1P and LNAME (+LNAME+S1P). EC were maintained in media as a baseline control (No Rx). Cells were used in a parallel plate flow chamber system to measure monocyte adhesion. Note there is very little adhesion to the non-diabetic EC. Data represent the mean±SEM of 2 experiments performed in duplicate dishes. *Significantly lower than diabetic NOD No Rx, p<0.003, #significantly higher than +S1P, p<0.01 by ANOVA.

**Online Figure III. Timecourse of S1P action on monocyte:endothelial adhesion to NOD aortic EC.** Aortic EC from non-diabetic (CTR) and diabetic (DIAB) NOD mice were incubated with 100nM S1P (+S1P) for the times indicated in the figure and were used in a parallel plate flow chamber system to measure monocyte adhesion. Note there is very little adhesion to the non-diabetic EC. *Significantly higher than NOD CTR,
p<0.0001; #significantly lower than NOD diab, p<0.01, $significantly lower than NOD diab, p<0.007, **significantly lower than NOD diab, p<0.0001 by ANOVA.

**Online Figure IV. Monocyte adhesion to S1P3-deficient mouse EC.** Aortic EC from control (white bars) and S1P3-deficient (blue bars) mice were incubated with S1P (+S1P) at the doses indicated in the figure for 4h and were used in a parallel plate flow chamber system to measure monocyte adhesion. Left panel shows cells incubated in the absence of TNFα addition; the right panel shows EC treated with 10ng/ml TNFα for 4h together with S1P. Note the increase in monocyte adhesion in EC treated with TNF.*Significantly higher than No Rx, p<0.001; #significantly lower than TNF, p<0.01; **significantly lower than TNF, p<0.001 by ANOVA.
Online Figure I
Online Figure II

The bar chart illustrates the number of monocytes/field across different conditions:

- **No Rx**: A high number of monocytes/field.
- **+S1P**: A moderate number of monocytes/field.
- **+LNAME**: A high number of monocytes/field.
- **+LNAME+S1P**: A moderate number of monocytes/field.

Significance markers are present:
- *: Indicates statistical significance.
- #: Indicates another significant difference.
Figure III

Monocytes/Field

NOD CTR
NOD CTR + STP 10min
NOD CTR + STP 30min
NOD CTR + STP 1 hr
NOD CTR + STP 4 hr
NOD DIAB
NOD DIAB + STP 10min
NOD DIAB + STP 30min
NOD DIAB + STP 1 hr
NOD DIAB + STP 4 hr

Online

Figure III