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

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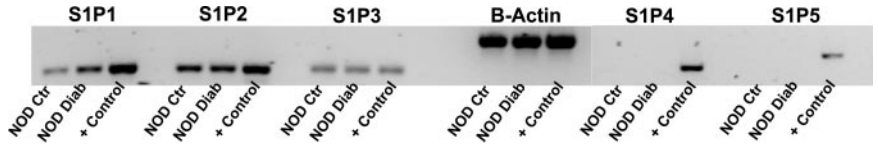

Figure 1. Expression of S1P receptors in nondiabetic and type 1 diabetic NOD mice. RNA isolated from type 1 diabetic (Diab) and nondiabetic (Ctr) aortic NOD endothelial cells was used in conventional RT-PCR to detect S1P receptors. There was no detectable expression of S1P4 or S1P5 mRNAs. Shown for each 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.

previously described.[21] 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.[21] Fluorescent microscopy for NF-κB was performed as described.[24] Immunoblotting for signaling molecules was per-

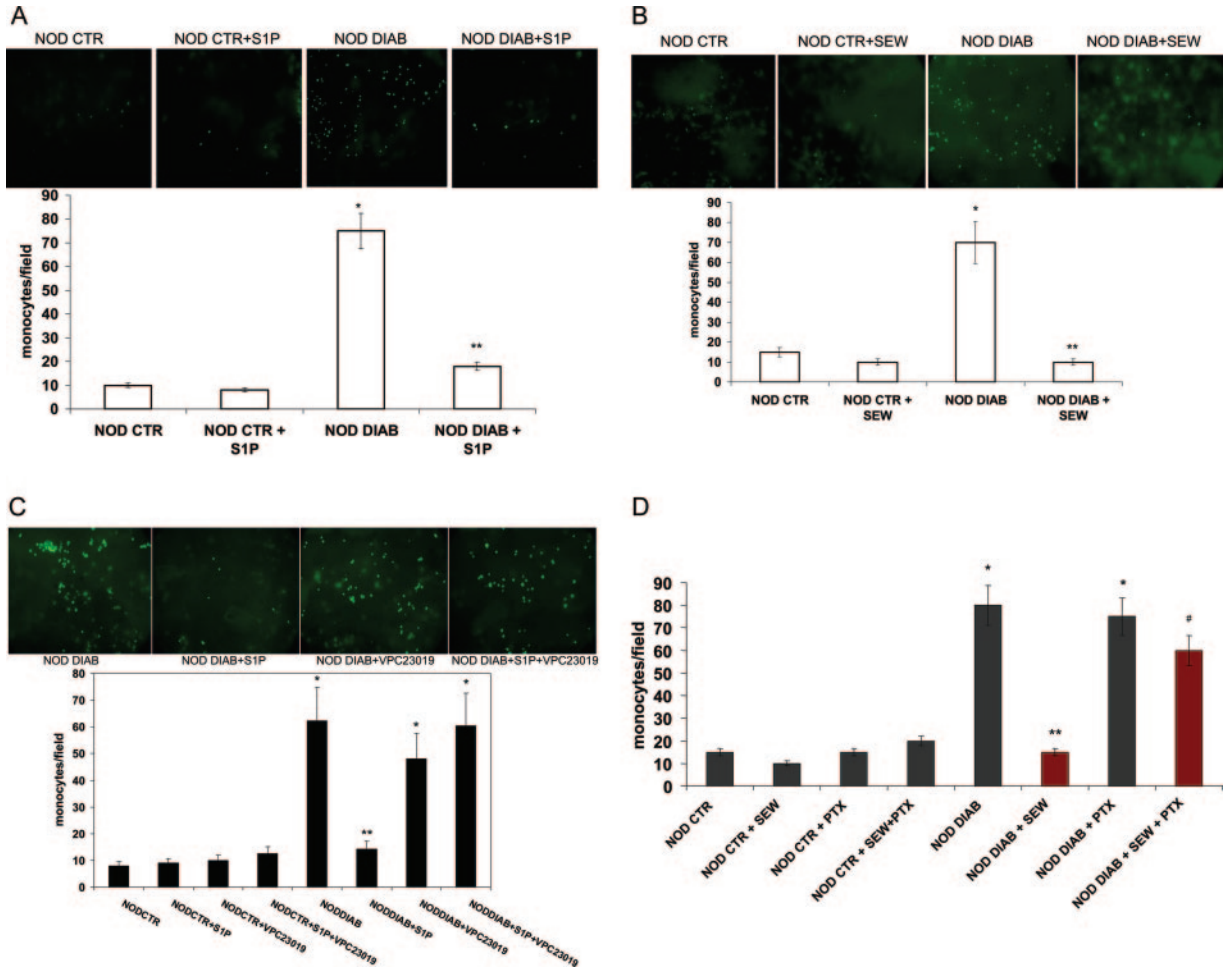

Figure 2. S1P and SEW2871 reduce monocyte adhesion to aorta in diabetic NOD mice. A, Studies using S1P. Aortas were isolated from nondiabetic NOD (CTR) and diabetic NOD (DIAB) mice and incubated for 4 hours in the absence and presence of 100 nmol/L S1P (+S1P). Fluorescently labeled monocytes were added 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 $G_{\alpha i}$ signaling. Aortas were isolated from nondiabetic (CTR) and diabetic (DIAB) NOD/LtJ mice and incubated overnight with 100 ng/mL PTX to uncouple $G_{\alpha i}$ 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 ($P<0.001$).

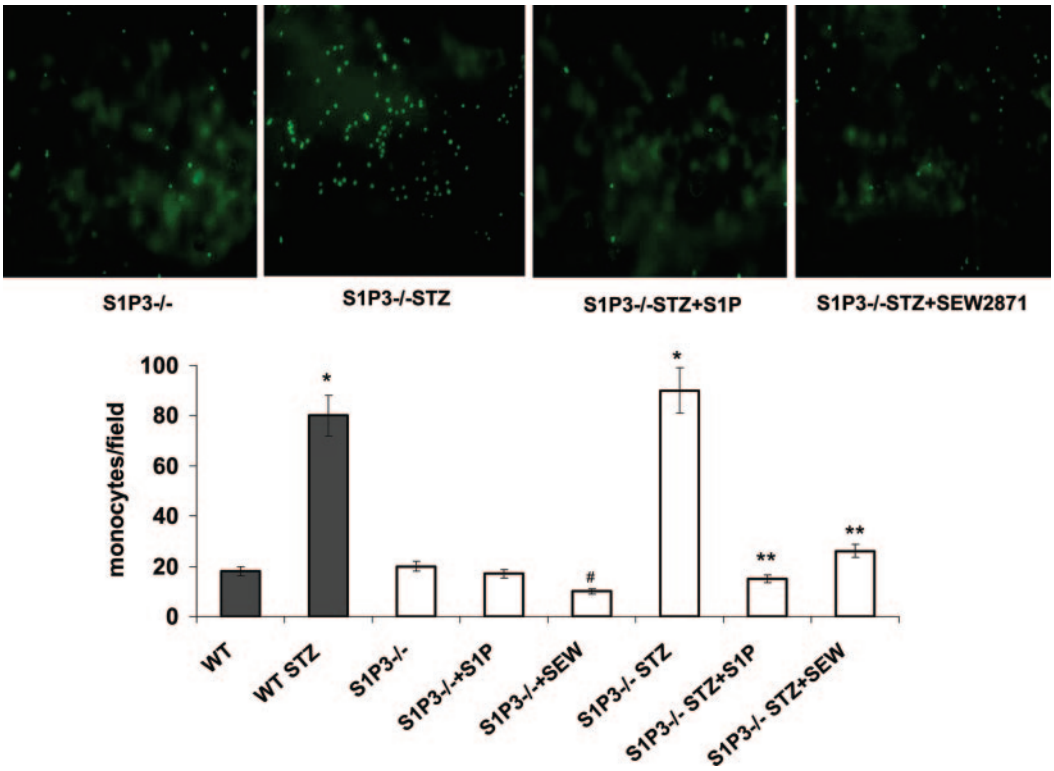

Figure 3. Absence of S1P3 receptor does not impact monocyte adhesion to diabetic aortic endothelium. S1P3-deficient mice (S1P3−/−) and wild-type (WT) littermates were rendered type 1 diabetic through the use of streptozotocin (+STZ). Mice were either injected with 2 mg/kg SEW2871 (+SEW), and aortas were isolated for monocyte adhesion assays, or aortas were treated with 100 nmol/L S1P (+S1P) ex vivo as described in Materials and Methods. Images represent monocyte adhesion to aorta in each experimental group. *Significantly higher than $S1P3^{-/-}$ ($P<0.001$); #significantly lower than $S1P3^{-/-}$ ($P<0.05$); **significantly lower than $S1P3^{-/-}$ +streptozotocin ($P<0.002$) by ANOVA. Data represent the mean±SE of 3 counted grids per aorta from 3 mice per group.

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.

S1P 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[26] 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 (FAFBSA) 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

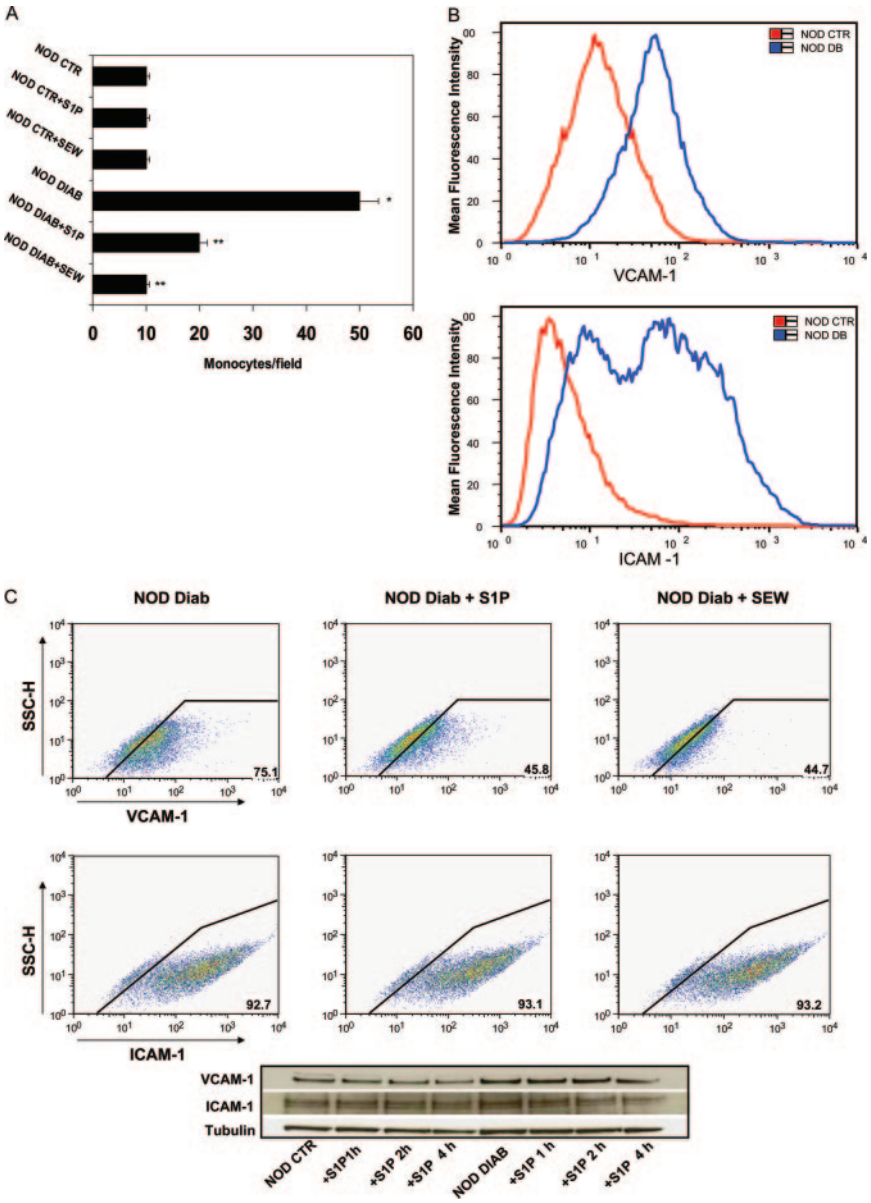

Figure 4. S1P and SEW2871 prevent monocyte adhesion to diabetic NOD endothelium. A, Monocyte adhesion to endothelium. Mouse aortic endothelial cells (MAECs) from nondiabetic (CTR) and diabetic (DIAB) NOD/LtJ mice were treated for 4 hours with 100 nmol/L S1P (+S1P) or 1 μmol/L SEW2871 (+SEW). Cells were plated in a parallel plate flow chamber system, and WEHI 78/24 mouse monocytes in medium 199 containing 1% heat-inactivated FBS were allowed to flow over confluent monolayers of MAECs at a shear stress of 0.75 dyne/cm^2 for 5 minutes. The number of firmly adherent monocytes during the 5 minute flow assay were counted. Data represent the mean±SE of 4 experiments performed in duplicate. *Adhesion significantly higher than NOD control ($P<0.0002$); **adhesion significantly less than NOD diabetic ($P<0.0003$) by ANOVA. B, Endothelial adhesion molecule expression. Nondiabetic NOD (CTR) and diabetic NOD (DB) aortic endothelial cells were harvested and analyzed by flow cytometry. Endothelial cells were gated by size using forward and side scatter. Data are representative of three experiments performed in duplicate dishes. C, S1P and adhesion molecule expression. Flow cytometry: diabetic (DIAB) NOD aortic ECs were cultured with either 100 nmol/L S1P (+S1P) or 1 μmol/L SEW2871 (+SEW) for 4 hours. Cells were harvested and analyzed by flow cytometry. Data are representative of three experiments performed in duplicate dishes. Numbers shown indicate the percentage of VCAM-1 or ICAM-1 on the EC surface. Immunoblotting: total protein lysates from nondiabetic NOD (CTR) and diabetic NOD (DIAB) aortic ECs treated with S1P for various time points were harvested and used for immunoblotting. Tubulin was run as a control for gel loading.

toxin (PTX) to uncouple $G_{\alpha i}$ receptor signaling.[28] S1P1 is known to couple solely through the G protein $G_{\alpha i}$.[29] Subgroups of aortas were treated with 100 ng/mL PTX overnight to uncouple $G_{\alpha i}$, and the aortas were treated with 1 μmol/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 $G_{\alpha i}$-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 to 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.

S1P 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).

S1P 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.

S1P 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).

S1P 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.[31] We found significant reduction of p65 phosphorylation after 30 minutes of S1P treatment of

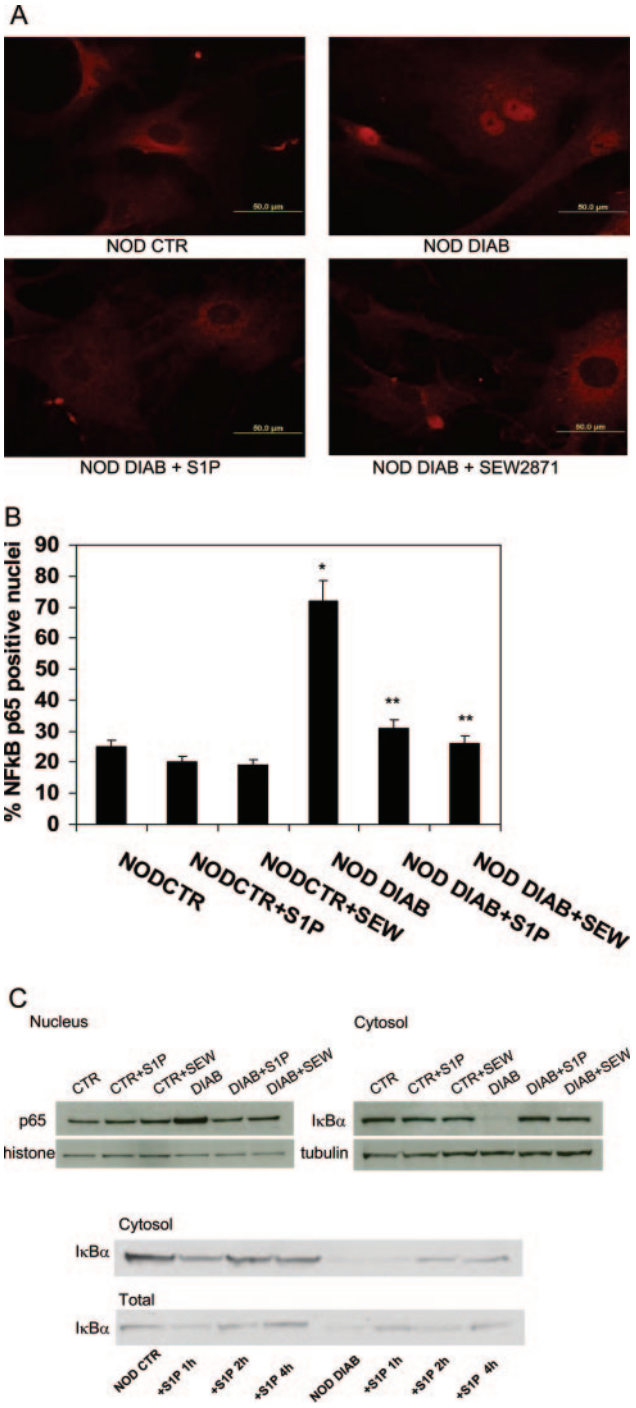

Figure 5. SEW2871 and S1P reduce NF-κB nuclear translocation in diabetic NOD aortic endothelium. A, Fluorescence microscopy of nuclear p65 staining. Nondiabetic (CTR) and diabetic (DIAB) NOD ECs were treated with 100 nmol/L S1P (+S1P) or 1 μmol/L SEW2871 (+SEW) for 4 hours. After treatment, NF-κB translocation was visualized by fluorescent microscopy using an Alexa 594–conjugated antibody specific for the p65 subunit of NF-κB. B, Quantification of nuclear p65 staining. Cells were scored by blinded observers as positive or negative for nuclear p65 in fluorescent images of NOD ECs, as illustrated in A, and the percentage positive for nuclear p65 was determined. *Significantly higher than NOD control ($P<0.0001$); **significantly lower than NOD diabetic ($P<0.0001$). Data represent the mean±SEM of 4 separate experiments. C, S1P restores IκBα. Murine aortic ECs from NOD diabetic (DIAB) and nondiabetic (CTR) were treated in the absence or presence of 100 nmol/L S1P (+S1P) or 1 μmol/L SEW2871 (+SEW) for 4 hours in medium 199 containing 1% heat-inactivated FBS. Total cell extracts were collected from NOD diabetic and nondiabetic ECs and analyzed by SDS-PAGE for IκBα, and cytosolic and nuclear extracts were collected from NOD diabetic and nondiabetic ECs and analyzed by SDS-PAGE for IκBα and NF-κB p65, respectively. Data shown are representative of 4 separate experiments.

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^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.

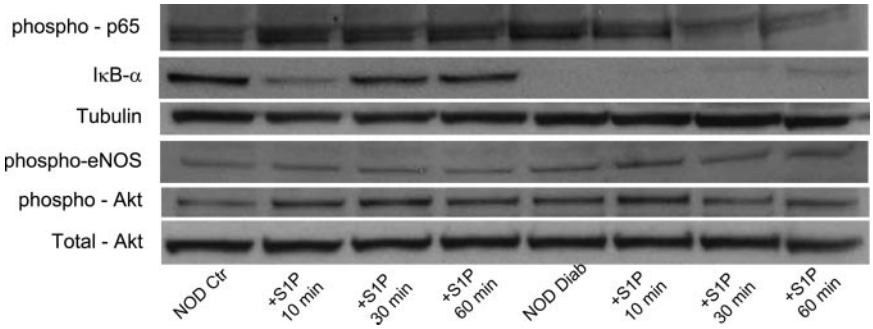

Figure 6. Signaling pathway regulation by S1P in type 1 diabetic NOD ECs. Nondiabetic (CTR) and diabetic (DIAB) NOD ECs were treated with 100 nmol/L S1P (+S1P) for the times indicated in the legend. Total cell lysates were isolated and used in immunoblotting as described in Materials and Methods.

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)[26,32] and antagonists of the S1P1 receptor (VPC23019)[27]—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 IκB synthesis. The induction of IκB 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.[31] 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 IκBα 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 ECs, 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,[19] 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.[25] 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 lipoxygenase (LO) enzymes. Indeed, we have reported upregulation of 12/15 LO activity in endothelium of diabetic db/db mice,[5] as well as in human aortic endothelial cells cultured in elevated glucose.[6,7] 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.[25] 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[33,34] 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 (supplemental Figure IV). In the bloodstream, physiological levels of free S1P are in the nanomolar range,[14,35] and the reported K_d 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.

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