

# Sphingosine 1-phosphate activates Weibel–Palade body exocytosis

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**Sphingosine 1-phosphate (S1P) not only regulates angiogenesis, vascular permeability and vascular tone, but it also promotes vascular inflammation. However, the molecular basis for the proinflammatory effects of S1P is not understood. We now show that S1P activates endothelial cell exocytosis of Weibel–Palade bodies, releasing vasoactive substances capable of causing vascular thrombosis and inflammation. S1P triggers endothelial exocytosis in part through phospholipase C- $\gamma$  signal transduction. However, S1P also modulates endothelial cell exocytosis by activating endothelial nitric oxide synthase production of nitric oxide, which inhibits exocytosis. Thus S1P plays a dual role in regulating endothelial exocytosis, triggering pathways that both promote and inhibit endothelial exocytosis. Regulation of endothelial exocytosis may explain part of the proinflammatory effects of S1P.**

nitric oxide | ceramide | endothelial | von Willebrand factor

Sphingosine-1-phosphate (S1P) is a lysophospholipid secreted by platelets, monocytes, and mast cells that acts as an extracellular messenger molecule (1–3). Extracellular S1P regulates cardiac precursor cell migration, vascular smooth muscle cell migration, platelet activation, and also endothelial cell migration, proliferation, and survival (4). The endothelial differentiation gene (EDG) family of G protein-coupled receptors (GPCR) are receptors for S1P (1–3). The EDG receptor family (also called the S1P receptor family) is comprised of at least eight independent subtypes, EDG1–8 (also called S1P<sub>1–8</sub>). S1P interaction with EDG receptors leads to the activation of various G proteins, which in turn activate a set of signal transduction pathways, including the mitogen-activated protein kinase pathway, various small GTPases such as Rho and Rac, and phospholipase C (PLC), which liberates inositol-3 phosphate and elevates intracellular calcium. Furthermore, S1P binding to the EDG-1 receptor promotes Akt-dependent phosphorylation of the endothelial nitric oxide (NO) synthase (eNOS), activating eNOS production of NO (5–11).

S1P also activates vascular inflammation, but the molecular basis of its proinflammatory effect is unclear (12). Lysophospholipids increase endothelial cell surface expression of E-selectin and vascular cell adhesion molecule-1 in endothelial cells (13). Lysophospholipids also activate the transcription factor NF- $\kappa$ B in endothelial cells, increasing the transcription of E-selectin, intracellular adhesion molecule-1, IL-8, and monocyte chemoattractant protein-1 (13–16). These findings suggest that S1P may activate the endothelium, triggering endothelial expression of selectins and adhesion molecules, thus initiating vascular inflammation.

Endothelial activation has two stages: the initial rapid translocation of preformed P-selectin to the endothelial surface and the subsequent slower synthesis and expression of adhesion molecules such as intracellular adhesion molecule-1. P-selectin is stored in Weibel–Palade bodies, endothelial storage granules that also contain von Willebrand factor (VWF) and tissue plasminogen activator (17–24). Rapid exocytosis of Weibel–Palade bodies is activated by thrombin, histamine, and other agonists. The protein machinery that mediates exocytosis includes *N*-ethylmaleimide-sensitive factor (NSF) and soluble NSF

attachment protein receptors (25–27). NO can inhibit exocytosis by covalently modifying NSF (28). Exocytosis of Weibel–Palade bodies causes rapid translocation of P-selectin from within granules to the endothelial surface, where P-selectin then interacts with P-selectin glycoprotein ligand-1 on the surface of leukocytes, triggering leukocyte rolling, the first step in leukocyte trafficking (29). Weibel–Palade body exocytosis also releases VWF, which mediates platelet rolling along the endothelium. Thus exocytosis of Weibel–Palade bodies is a critical early step in vascular inflammation and thrombosis.

We now show that S1P has two opposing effects on Weibel–Palade body exocytosis. S1P triggers Weibel–Palade body exocytosis in part by activating the PLC- $\gamma$  pathways. However, S1P also modulates Weibel–Palade body exocytosis by activating the phosphatidylinositol 3-kinase (PI3-K) pathway, which increases NO synthesis.

## Materials and Methods

**Materials.** S1P, dihydro-S1P, and D-erythro-*N,N*-dimethylsphingosine (DMS) were purchased from Biomol (Plymouth Meeting, PA). S1P and dihydro-S1P were resuspended in methanol with acetylated BSA according to the manufacturer's instructions (Biomol). Ceramide from Matreya (State College, PA) was resuspended in DMSO. Thrombin, acetylpenicillamine, *N*-nitro-L-arginine methyl ester (L-NAME), 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis (acetoxymethyl ester), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) U73211, calphostin C, and LY294002, SB203580, and PD98059 were purchased from Sigma; DMEM and DMEM without calcium and PBS were purchased from GIBCO; VWF ELISA kit was from American Diagnostica (Stamford, CT); soluble P-selectin (sP-selectin) ELISA kit was from R & D Systems; and *S*-nitrosothiol-penicillamine (SNAP) was from Cayman Chemical (Ann Arbor, MI); endothelium-based medium 2 with growth factors (FBS, hydrocortisone, R3-IGF-1, ascorbic acid, gentamicin amphotericin, and heparin) was from Clonetics (Walkersville, MD). Rabbit polyclonal antibody to eNOS and mouse monoclonal antibody to phospho-eNOS (S1177) were purchased from BD Biosciences (San Diego). Antibody to EDG receptor isoforms 1–8 was purchased from Exalpha Biologicals (Watertown, MA).

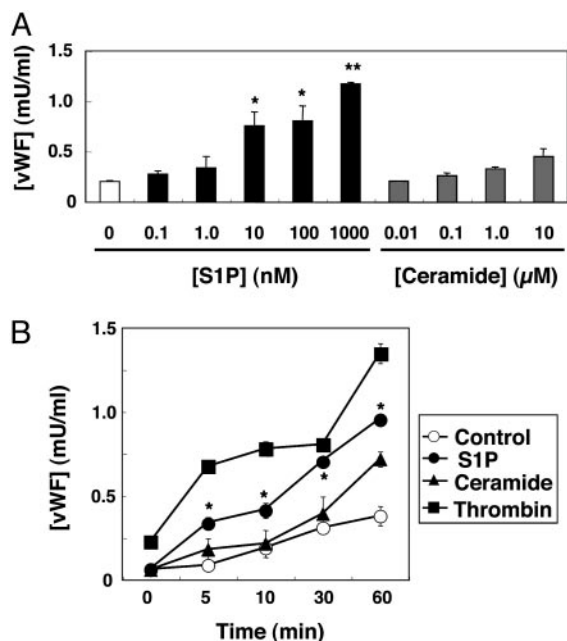
**Cell Culture and Analysis of VWF Exocytosis.** Human aortic endothelial cells (HAEC) were obtained from Clonetics and grown in EGM-2 media with supplements (Clonetics catalog CC-3162). To measure the effect of S1P upon VWF release, HAEC were washed and incubated in EGM-2 media without serum. HAEC were stimulated with various concentration of S1P, and the amount of VWF released into the media was measured by

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Abbreviations: S1P, sphingosine-1-phosphate; GPCR, G protein-coupled receptor; PLC, phospholipase C; eNOS, endothelial NO synthase; VWF, von Willebrand factor; DMS, D-erythro-*N,N*-dimethylsphingosine; TNA- $\alpha$ , tumor necrosis factor  $\alpha$ ; SNAP, *S*-nitrosothiol-penicillamine; HAEC, human aortic endothelial cells; L-NAME, *N*-nitro-L-arginine methyl ester; PTX, pertussis toxin; sP-selectin, soluble P-selectin; PI3-K, phosphatidylinositol 3-kinase.

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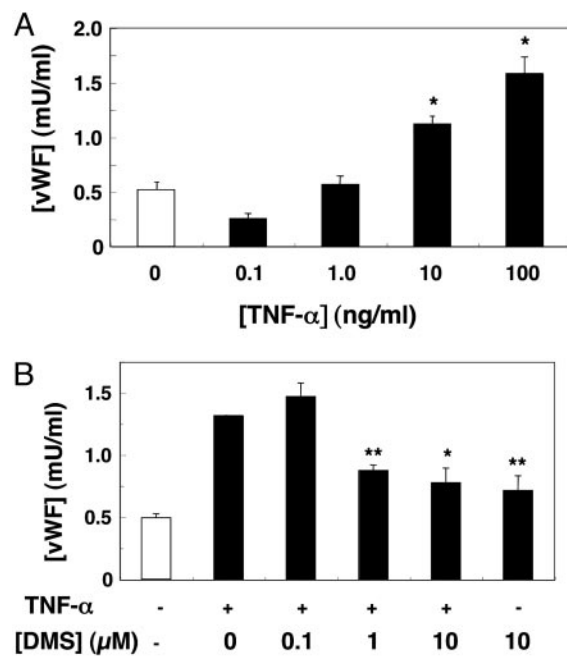


**Fig. 1.** Exogenous S1P activates Weibel-Palade body exocytosis. (A) Dose-response. Increasing amounts of S1P or ceramide were added to HAEC for 1 h, and the concentration of VWF released into the media was measured by ELISA ( $n = 3 \pm \text{SD}$ ; \*,  $P < 0.05$  vs. 0 ng/ml; \*\*,  $P < 0.01$  vs. 0 ng/ml). (B) Time course. S1P 1  $\mu\text{M}$ , ceramide 10  $\mu\text{M}$ , or thrombin 1 unit/ml was added to HAEC for 1 h, and the concentration of VWF released into the media was measured by ELISA ( $n = 3 \pm \text{SD}$ ; \*,  $P < 0.01$  for S1P vs. control).

ELISA (American Diagnostica). To clarify the mechanism by which S1P induces VWF exocytosis, HAEC were washed, cultured in EGM-2 media without serum, and pretreated for 10 min with inhibitors (except for U73122 given for 60 min) and then stimulated with 1  $\mu\text{M}$  S1P for 1 h. The amount of VWF released into the media was measured by an ELISA. To explore the effects of Ca, HAEC were washed, placed in EGM-2 media without serum, and pretreated with 10  $\mu\text{M}$  1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis (acetoxymethyl ester) for 30 min in DMEM or  $\text{CaCl}_2$ -free DMEM. The cells were then stimulated with S1P as above. The supernatants were collected, and the concentration of VWF released into the media was measured by ELISA. To measure the effect of NO on VWF release by S1P, HAEC in EGM-2 and serum were pretreated with SNAP for 4 h. The cells were washed, refed with EGM-2 without serum, stimulated with 1 unit/ml thrombin, and the amount of VWF released into the media measured by an ELISA. To measure the effect of inhibiting endogenous NO production on exocytosis, HAEC in EGM-2 with serum were pretreated for 16 h with L-NAME before S1P stimulation.

**Western Blot Analysis of eNOS Phosphorylation.** Confluent HAEC were serum-starved in serum-free EGM-2 media for 18 h. Cell were rinsed and stimulated with various concentration of S1P for 60 min in PBS. The supernatant was removed, and SDS/PAGE sample buffer (Bio-Rad) was added. Cell lysates were fractionated on a 7.5% SDS/PAGE and immunoblotted with antibodies to eNOS and phospho-eNOS.

**Measurement of Endothelial NO Production.** Confluent HAEC were serum-starved in serum-free EGM-2 media for 18 h. Cell were rinsed and stimulated with various concentration of S1P for 60 min in PBS. NO content in the supernatants was then measured by the Griess reaction (30).



**Fig. 2.** Endogenous S1P induces VWF release. (A) TNF- $\alpha$  induces VWF exocytosis. HAEC were incubated with various concentration of TNF- $\alpha$  for 1 h. The amount of VWF released from cells into the media was measured by ELISA ( $n = 3 \pm \text{SD}$ ; \*,  $P < 0.01$  vs. 0 ng/ml). (B) The sphingosine kinase inhibitor DMS blocks TNF- $\alpha$ -induced VWF release. HAEC were pretreated with DMS for 30 min and then incubated with 10 ng/ml of TNF- $\alpha$  for 1 h. The amount of VWF released from cells into the media was measured by ELISA ( $n = 2 \pm \text{SD}$ ; \*,  $P < 0.05$  vs. 0  $\mu\text{M}$  DMS; \*\*,  $P < 0.01$  vs. 0  $\mu\text{M}$  DMS).

**In Vivo Measurements of Exocytosis.** Mice were injected i.v. with 10 pmol S1P in 0.1 ml of saline. Blood was collected retroorbitally 1 h after S1P treatment and analyzed for sP-selectin with an ELISA.

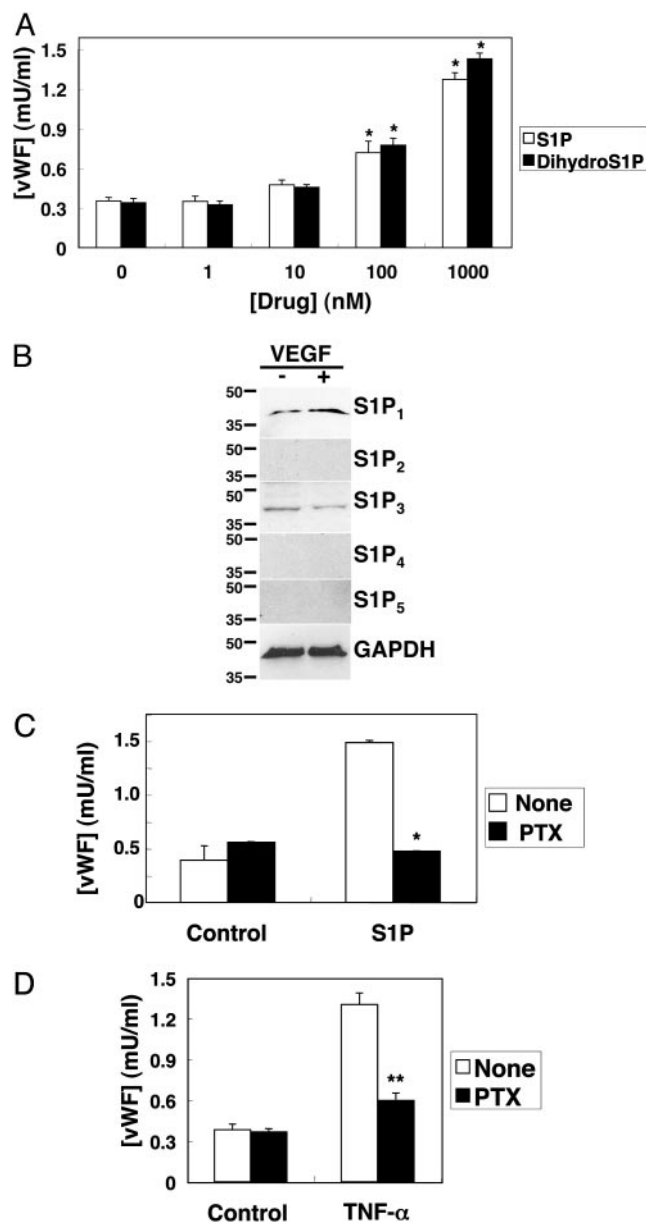
**Statistical Analysis.** Results are expressed as mean  $\pm$  SD. Significance between mean values was determined by Student's *t* test, with a value of  $P < 0.05$  considered significant.

## Results

**S1P Triggers Weibel-Palade Body Exocytosis.** To explore the effect of S1P on Weibel-Palade body exocytosis, we treated HAEC with S1P for 1 h and measured the concentration of VWF in the media by ELISA. S1P activated VWF release from endothelial cells in a dose-dependent manner (Fig. 1A Left). This range of concentrations of S1P fits within the plasma concentrations of S1P in humans, which can be as high as 400 nM (31). S1P induced a greater release of VWF than did ceramide (Fig. 1A Right). S1P rapidly induced VWF release, starting within 5 min of treatment and continuing through 60 min after treatment (Fig. 1B).

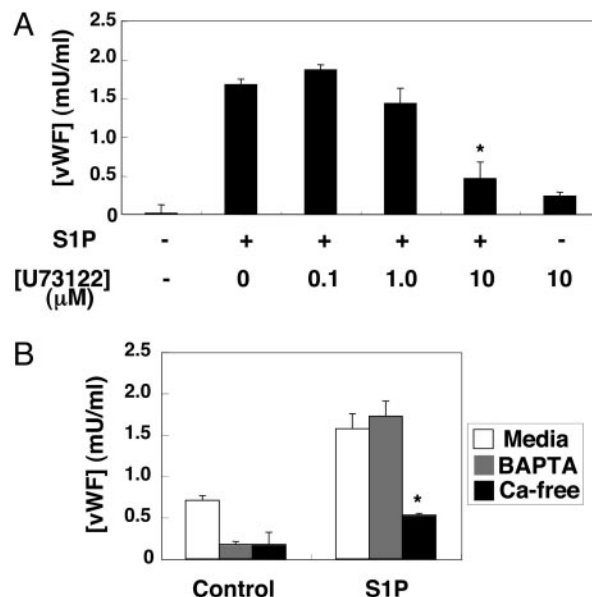
To examine the effect of endogenous S1P on endothelial exocytosis, we exposed HAEC to TNF- $\alpha$ , which activates sphingosine kinase to generate S1P. TNF- $\alpha$  stimulated VWF release in a dose-dependent manner (Fig. 2A). This effect was blocked by DMS, an inhibitor of sphingosine kinase (Fig. 2B). These data suggest that exogenous and endogenous S1P trigger endothelial cell granule exocytosis.

S1P is a ligand for a family of S1P receptors, which are members of the superfamily of GPCRs. To explore the role of GPCRs in the S1P-triggered signal transduction cascade leading to exocytosis, we first compared the effects of dihydro-S1P to S1P on exocytosis. Dihydro-S1P, which lacks any intracellular effects but activates S1P receptors, activated exocytosis to the



**Fig. 3.** PTX-sensitive receptors mediate S1P-induced VWF release. (A) Dihydro-S1P activates exocytosis. Increasing amounts of S1P or dihydro-S1P were added to HAEC for 1 h, and the concentration of VWF released into the media was measured by ELISA ( $n = 3 \pm \text{SD}$ ; \*,  $P < 0.01$  vs. 0 nM). (B) Expression of S1P receptors in HAEC. HAEC were treated with or without 50 ng/ml vascular endothelial growth factor for 2 h, and cell lysates were immunoblotted with antibodies to S1P receptor subtypes 1–5. (C) Exogenous S1P. HAEC were pretreated with 100 ng/ml of PTX for 16 h, washed twice, and then incubated with 1  $\mu\text{M}$  S1P for 1 h. The amount of VWF released from cells into the media was measured by ELISA ( $n = 3 \pm \text{SD}$ ; \*,  $P < 0.01$  vs. no PTX). (D) Endogenous S1P. HAEC were pretreated with 100 ng/ml of PTX for 16 h, washed twice, and then incubated with 10 ng/ml of TNF- $\alpha$  for 1 h. The amount of VWF released into the media was measured by ELISA ( $n = 3 \pm \text{SD}$ ; \*\*,  $P < 0.01$  vs. TNF- $\alpha$ ).

same extent as S1P, suggesting that the S1P receptors can mediate activation of exocytosis (Fig. 3A). HAEC express the S1P1 and S1P3 receptor subtypes, and vascular endothelial growth factor increases S1P1 expression as reported (Fig. 3B) (32). We used pertussis toxin (PTX) as a tool to confirm that pertussis-sensitive GPCRs are necessary for S1P to trigger exocytosis. We pretreated HAEC with 100 ng/ml PTX for 16 h, then incubated HAEC with 1  $\mu\text{M}$  S1P for 1 h, and measured the



**Fig. 4.** PLC and calcium mediate S1P-activated VWF exocytosis. (A) The PLC inhibitor U73122 blocks S1P-induced VWF release from endothelial cells. HAEC were pretreated with U73122 for 30 min and then incubated with 1  $\mu\text{M}$  S1P for 1 h. The amount of VWF released from cells into the media was measured by ELISA ( $n = 2 \pm \text{SD}$ ; \*,  $P < 0.01$  vs. 0  $\mu\text{M}$ ). (B) Calcium pools and VWF release from endothelial cells. HAEC were pretreated with DMEM or with 20  $\mu\text{M}$  1,2-bis(2-aminophenoxy)ethane- $N,N,N',N'$ -tetraacetic acid tetrakis (acetoxymethyl ester) for 10 min in DMEM or with calcium-free DMEM and then incubated with 1  $\mu\text{M}$  S1P for 1 h. The amount of VWF released from cells into the media was measured by ELISA ( $n = 3 \pm \text{SD}$ ; \*,  $P < 0.01$  vs. media).

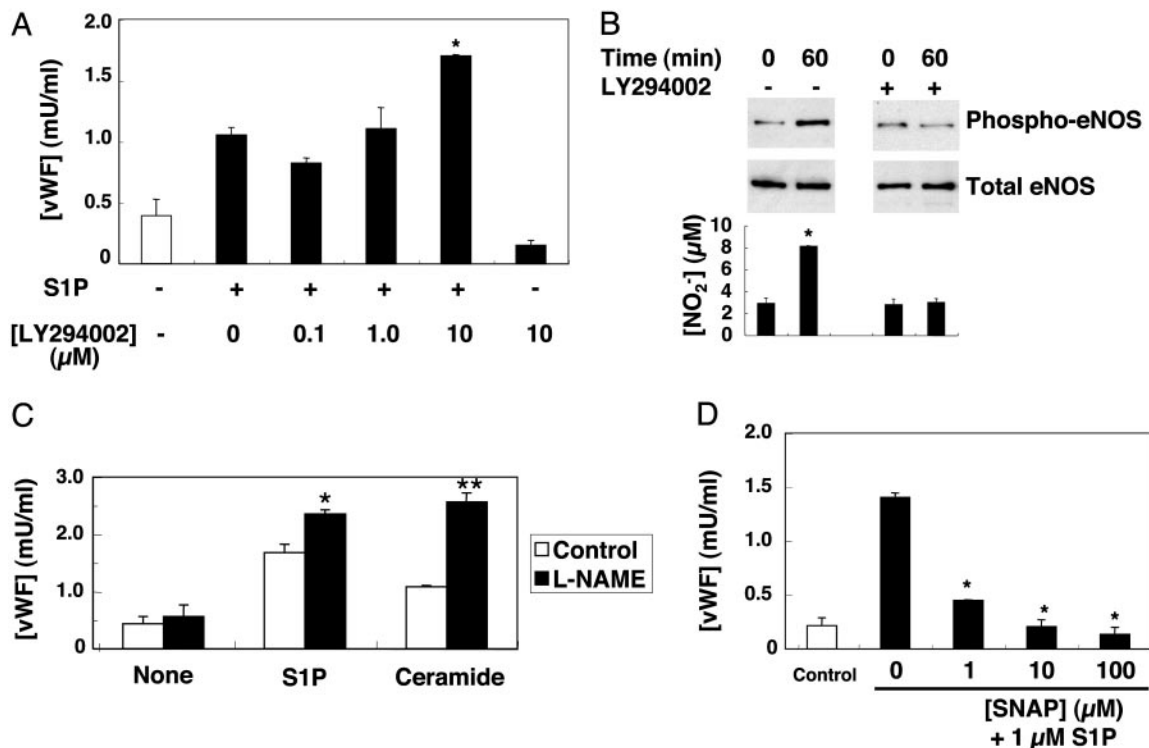
release of VWF. PTX pretreatment blocked S1P-induced VWF exocytosis (Fig. 3C). Furthermore, PTX also inhibited TNF- $\alpha$ -stimulated exocytosis (Fig. 3D). These results suggest that PTX-sensitive GPCRs mediate exogenous and endogenous S1P induction of Weibel–Palade body exocytosis.

**PLC- $\gamma$  and Calcium Mediate S1P-Activated Weibel–Palade Body Exocytosis.** Because S1P receptor signal transduction includes activation of PLC and increases in intracellular calcium, and because calcium can trigger exocytosis, we examined the effects of S1P on calcium signaling during Weibel–Palade body exocytosis. The PLC- $\gamma$  inhibitor U73122 decreased S1P-induced exocytosis (Fig. 4A). We then determined the role of intracellular and extracellular  $\text{Ca}^{2+}$  on VWF exocytosis. Exocytosis in  $\text{CaCl}_2$ -free media was significantly decreased (Fig. 4B). However, exocytosis from cells treated with 1,2-bis(2-aminophenoxy)ethane- $N,N,N',N'$ -tetraacetic acid was not decreased. These results suggest that extracellular  $\text{Ca}^{2+}$  but not intracellular  $\text{Ca}^{2+}$  mediates S1P triggered VWF exocytosis.

**S1P Inhibits Exocytosis Through PI3-K Activation of eNOS.** We next examined the role of PI3-K in S1P induced VWF exocytosis. Inhibition of PI3-K with LY294002 enhanced S1P-induced VWF exocytosis (Fig. 5A). These data suggest that S1P activation of PI3-K inhibits exocytosis. Stimulation of HAEC with 1  $\mu\text{M}$  S1P for 60 min elicited an increase in phosphorylation of eNOS and NO production (Fig. 5B). Furthermore, pretreatment with 10  $\mu\text{M}$  LY294002 inhibited S1P-induced eNOS phosphorylation and NO production (Fig. 5B). These data confirm that S1P activates PI3-K, which in turn activates eNOS production of NO.

We next explored the effect of S1P activation of eNOS on exocytosis. We pretreated HAEC with L-NAME for 16 h before the addition of 1  $\mu\text{M}$  S1P. L-NAME increased S1P-induced VWF release by  $\approx 35\%$  (Fig. 5C). To confirm that NO can inhibit





**Fig. 5.** PI3-K activation of eNOS inhibits S1P-activated exocytosis. (A) PI3-K mediates S1P activation of exocytosis. HAEC were pretreated with the PI3-K inhibitor LY294002 for 10 min and then incubated with 1 μM S1P for 1 h. The amount of VWF released from cells into the media was measured by ELISA ( $n = 2 \pm \text{SD}$ ; \*,  $P < 0.05$  vs. 0 μM LY294002). (B) S1P activates eNOS. HAEC were treated with 1 μM S1P for 60 min in the absence or presence of 10 μM LY294002, and phospho-eNOS (S1177) levels were measured by immunoblot (Upper). The NO metabolite [ $\text{NO}_2^-$ ] in the media was measured by the Griess assay (below) ( $n = 3 \pm \text{SD}$ ; \*,  $P < 0.01$  for 0 vs. 10 μM LY294002). (C) Endogenous NO inhibits S1P induced exocytosis. HAEC were pretreated with 1 mM L-NAME for 16 h, stimulated with 1 μM S1P or 10 μM ceramide, and released VWF was measured as above ( $n = 3 \pm \text{SD}$ ; \*,  $P < 0.05$  vs. 0 μM L-NAME; \*\*,  $P < 0.01$  vs. 0 μM L-NAME). (D) Exogenous NO inhibits S1P-induced exocytosis. HAEC were pretreated with the NO donor SNAP for 4 h, then treated with 1 μM S1P, and released VWF was measured as above ( $n = 2 \pm \text{SD}$ ; \*,  $P < 0.01$  vs. 0 μM SNAP).

exocytosis, we added exogenous NO to HAEC for 4 h before treatment with S1P. NO inhibited S1P-activated VWF release in a dose-dependent manner (Fig. 5D). SNAP did not decrease VWF release due to cytotoxicity, because SNAP did not affect HAEC viability at these doses (Fig. 8, which is published as supporting information on the PNAS web site).

Finally, we explored the effect of S1P on exocytosis in mice. To monitor endothelial exocytosis *in vivo*, we measured blood levels of sP-selectin, which is released into the blood after endothelial exocytosis. We injected 10 pmol S1P or control into the tail veins of mice and then measured the levels of sP-selectin in the blood 1 h after treatment. S1P injection increased sP-selectin levels (Fig. 6). Furthermore, S1P injection led to a greater increase of sP-selectin levels in eNOS knockout than in wild-type mice (Fig. 6). These data suggest that endogenous NO inhibits exocytosis of Weibel–Palade bodies induced by S1P.

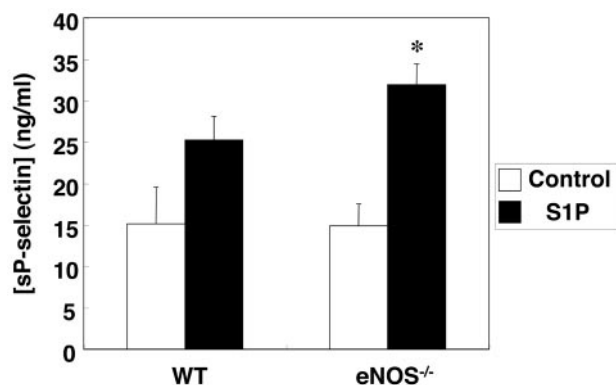
## Discussion

The major findings of these studies are that S1P has two opposing effects on endothelial cell exocytosis of Weibel–Palade bodies. S1P not only triggers exocytosis but also modulates exocytosis by distinct pathways (Fig. 6).

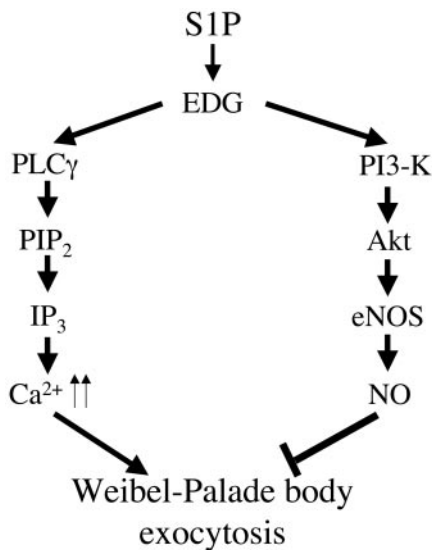
**S1P Activates Endothelial Exocytosis.** S1P activates exocytosis of Weibel–Palade bodies by triggering signaling pathways in endothelial cells that include the PLC-γ pathway (1–3). PLC-γ activation in turn leads to elevations in intracellular calcium, which plays a role in exocytosis (25–27). Increases in intracellular calcium are the final stimulus for membrane fusion in a variety of other cells. These data suggest the existence of undefined

molecules that can sense calcium and regulate endothelial exocytosis.

Endothelial exocytosis may play a role in some of the physiological effects of S1P. S1P affects many different cells, including lymphocytes, macrophages, smooth muscle cells, endothelial cells, and neuronal cells (1–3). S1P has a variety of effects on endothelial cells, inducing endothelial migration, proliferation, differentiation, and survival. S1P also promotes angiogenesis and evokes inflammation (4). One of the mechanisms by which



**Fig. 6.** S1P increases soluble P-selectin in wild-type and eNOS knockout mice. An ELISA was used to measure [sP-selectin] in plasma collected from wild-type (WT) or eNOS knockout mice 1 h after i.v. injection of 10 pmol S1P or control ( $n = 3-5 \pm \text{SD}$ ; \*,  $P < 0.05$  WT vs. eNOS<sup>-/-</sup>).



**Fig. 7.** Proposed scheme for S1P regulation of Weibel-Palade body exocytosis.

S1Ps induce vascular inflammation is through activation of NF- $\kappa$ B, a relatively slow pathway that depends on gene transcription (16). S1P activation of NF- $\kappa$ B depends in part on the S1P3 receptor subtype that is present in the endothelial cells we studied (Fig. 3) (16). Our findings suggest that S1P can also trigger rapid vascular inflammation by activating pathways leading to exocytosis.

**S1P Activates eNOS That Blocks Exocytosis.** S1P also modulates exocytosis by activating eNOS through a previously described PI3-K/Akt pathway (5–11, 33–35). Our results show that endogenous NO then inhibits Weibel-Palade body exocytosis triggered by S1P itself or by other agonists. Previously, we have shown that NO inhibits exocytosis by directly nitrosylating *N*-ethylmaleimide-sensitive factor, a key regulator of vesicle trafficking (20, 28). The present study shows that not only exogenous but also endogenous NO inhibits exocytosis. These results suggest that other agonists that increase eNOS expression or activity will also inhibit endothelial exocytosis. Furthermore, vascular expression of other NOS isoforms such as inducible NOS or neuronal NOS may also lead to regulation of endothelial exocytosis (20).

**S1P Has Opposing Effects on Exocytosis.** Paradoxically, S1P not only activates endothelial exocytosis through one set of pathways, but also inhibits exocytosis through a different set of pathways that include eNOS and NO (Fig. 7). Perhaps NO modulates the level of endothelial exocytosis and inflammation after vascular injury. High levels of active eNOS may limit vascular inflammation by decreasing exocytosis. However, low levels of eNOS or defects in pathways that activate eNOS may permit higher levels of exocytosis, leading to an increase in vascular inflammation. Our results may explain why patients with decreased eNOS activity are predisposed to increased vascular inflammation (36).

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