

Sphingosine 1-phosphate and control of vascular tone

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Submitted 16 December 2002; accepted in final form 22 January 2003

Dantas, Ana Paula V., Junsuke Igarashi, and Thomas Michel. Sphingosine 1-phosphate and control of vascular tone. *Am J Physiol Heart Circ Physiol* 284: H2045–H2052, 2003; 10.1152/ajpheart.01089.2002.—Sphingosine-1-phosphate (S1P) is a platelet-derived lipid mediator that activates the endothelial isoform of nitric oxide synthase (eNOS) in endothelial cells. However, the role of S1P in endothelium-dependent vasodilation and the signaling pathways elicited by S1P in intact vessels are largely unknown. We found that S1P induces dose-dependent transient relaxation of isolated pressurized mesenteric arterioles (EC₅₀ 10 ± 3 nM); maximal vasodilation (55 ± 8%) is seen ~2 min after S1P addition and returns to baseline by 5 min. S1P promotes comparable responses in arterioles from wild-type but not eNOS^{null} mice. S1P-induced vasodilation is abrogated by removal of endothelium or by the addition of the NOS inhibitor N^ω-monomethyl-L-arginine but is not affected by the cyclooxygenase inhibitor indomethacin, nor by the blockade of K⁺ channels by using 4-aminopyridine. S1P-induced vasodilation is attenuated by pertussis toxin, by the phosphoinositide 3-kinase (PI3-kinase) inhibitor wortmannin, and by the calcium chelator BAPTA. With the use of high-sensitivity protein immunoblots in extracts from single pressurized vessels, we found that S1P, but not bradykinin, promotes the phosphorylation of eNOS at Ser¹¹⁷⁹. Maximum S1P-induced eNOS Ser¹¹⁷⁹ phosphorylation was reached at the time of maximum vasorelaxation, but enzyme phosphorylation persisted for several minutes after vasodilation had resolved. Thus regulatory pathways distinct from eNOS Ser¹¹⁷⁹ dephosphorylation serve to terminate agonist-promoted vasorelaxation. Taken together, our findings demonstrate that S1P, an important intercellular mediator of platelet-vessel wall interactions, is an effective arteriolar vasodilator that acts via G protein-dependent, calcium-sensitive, and PI3-kinase-modulated signaling pathways.

sphingolipids; endothelium-dependent relaxation; nitric oxide; endothelial nitric oxide synthase phosphorylation.

NITRIC OXIDE (NO) generated by endothelial NO synthase (eNOS) has a central role in the regulation of vascular tone and also modulates diverse vascular responses including inhibition of platelet aggregation, vascular remodeling, and angiogenesis (reviewed in Ref. 20). eNOS is activated by a variety of physiological and pathophysiological stimuli, including hormones, such as bradykinin (BK) (3) and estrogen (7), and

growth factors, such as vascular endothelial growth factor (12), and by mechanical stimuli (5). We recently established that sphingosine 1-phosphate (S1P), a bioactive sphingolipid released by activated platelets, is an important mediator of eNOS activation (14) in cultured endothelial cells. Subsequent studies (23, 28) explored the intracellular pathways by which S1P regulates eNOS in cultured cells, but the role of S1P in intact blood vessels remains largely unknown.

S1P is a platelet-derived lipid that can serve as an extracellular messenger molecule to elicit important biological responses in vascular tissues including angiogenesis, as well as endothelial cell migration, survival, and proliferation (4, 19, 25). The effects of S1P are mediated by its activation of a family of G protein-coupled cell surface EDG receptors (9, 11, 18). In the vascular endothelium, eNOS is implicated in at least some of the biological responses elicited by S1P-induced activation of EDG receptors (17, 28). However, the signaling pathways underlying the biological responses to S1P in intact vessels have not been characterized at the molecular level, and the role of S1P in modulation of vascular tone is poorly understood. In the present studies, we have explored the effects of S1P on the control of vascular tone in mesenteric microvessels and have correlated the physiological responses to S1P with EDG receptor-mediated activation of the phosphoinositide 3-kinase (PI3-kinase) signaling cascade in intact arteries.

METHODS

Arterial preparations. Experiments were conducted by using isolated mesenteric arterioles isolated either from male Sprague-Dawley rats (200–250 g) or from gene-targeted male mice with homozygous deficiency for the eNOS gene (eNOS^{null}), with age-matched wild-type male mice serving as positive control. All animal experimentation was performed according to protocols approved by the Harvard Medical School Committee on Use of Animals in Research.

Animals were anesthetized with a subcutaneous injection of pentobarbital sodium (80 mg/kg). After an abdominal incision, the mesenteric vascular bed was removed and placed in a dish containing ice-cold PBS. Intact segments of dissected mesenteric arterioles (~250–300 μm from rat and ~65–75 μm from mice) were mounted between two glass cannulas in an arteriograph (Living Systems Instrumenta-

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tion; Burlington, VT) at room temperature (18–21°C) and pressurized to 60 mmHg under conditions of no luminal flow. Constant pressure was maintained via a pressure servo-control system (model PS200; Living Systems Instrumentation). Pressure transducers at both ends of the artery allowed continual monitoring of intraluminal pressure. Vessel diameter was quantitated by video microscopy by using a Nikon inverted microscope; a measurement of the external diameter was determined by using the Ionoptix SoftEdge system (Ionoptix; Milton, MA). The preparation was continually superfused with modified Krebs-Henseleit solution (in mM: 113 NaCl, 4.7 KCl, 2.5 CaCl₂, 25 NaHCO₃, 1.1 MgSO₄, 1.1 KH₂PO₄, 0.03 EDTA, 0.01 L-arginine, and 11 glucose, pH 7.4) and oxygenated with a mixture of 95% O₂-5% CO₂ at a rate of 10 ml/min. The superfusing solution was warmed to 37°C by passage through a heat-exchange coil before entering the monitoring apparatus. After a 60-min stabilization period, the sensitivity of smooth muscle cells to NO and endothelial integrity was confirmed by superfusion of sodium nitroprusside (SNP; 100 μM) and BK (1 μM), respectively. Only those vessels that developed a relaxation >80% to SNP and 70% to BK were used. After drug washout and a 30-min equilibration period, arteries were constricted with 5 μM norepinephrine before treatments with S1P. For dose-response curves, vessels were washed with Krebs-Henseleit for 30 min after the S1P-induced response returned to baseline. Vessel diameter measurements were quantitated and analyzed by using Ionoptix software (Ionoptix); vessel relaxation is normalized as the fractional reversal of norepinephrine-induced vessel contraction. In some experiments, endothelium was mechanically removed by perfusing the arteriole with air followed by a perfusion with distilled water, as previously described (8); removal of endothelium was confirmed by documenting that the treated vessel was unable to respond to BK but retained its responsiveness to SNP, analyzed as described above.

Immunoblot analyses in intact vessels. We performed high-sensitivity protein immunoblots in extracts from single, pressurized vessels. For these experiments, arterioles were analyzed in a pressure myograph specially designed for rapid freezing of a cannulated pressurized vessel (Living Systems Instrumentation). Within 1 s after the determination of the vascular response to a given agonist, the pressurized vessel was transferred to iced-PBS and immediately frozen in liquid nitrogen. Frozen tissues were homogenized by using a Polytron homogenizer in lysis buffer of 50 mM Tris, pH 7.4, 1 mM EDTA, 1% vol/vol NP-40, 150 mM NaCl, 2 mM Na₃VO₄, 0.25% sodium deoxycholate, 1 mM NaF, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.008% bromphenol blue, and a mixture of protease inhibitors (2 μg/ml leupeptin, antipain, soybean trypsin inhibitor, and lima trypsin inhibitor). After 30 min of incubation on ice, samples were centrifuged for 20 min at 14,000 *g* at 4°C to yield a solubilized preparation. Protein concentration was measured by using a protein assay (Bio-Rad; Richmond, CA). Equal quantities of protein from each sample (typically 10 μg) were resolved by SDS-PAGE on 9% gels and electroblotted onto nitrocellulose. After being blocked overnight with 5% bovine albumin in Tris-buffered saline with 0.1% (vol/vol) Tween 20 (TBST), membranes were incubated for 1 h in TBST containing 5% bovine albumin and the specified primary antibody (monoclonal anti-eNOS antibodies at a 1:10,000 dilution) (Transduction Laboratories) or polyclonal phospho-eNOS Ser¹¹⁷⁹ antibodies (1:5,000 dilution; Cell Signaling). After six washes with TBST (10 min each), the membranes were incubated for 1 h with a horseradish peroxidase-labeled goat anti-rabbit (for phospho-eNOS Ser¹¹⁷⁹) or anti-mouse (for eNOS) immunoglobulin secondary antibody (Pierce Chemical) at a 1:50,000 dilution

in TBST containing 5% bovine albumin. After being washed 10–15 additional times in TBST, the membranes were incubated with a chemiluminescent reagent according to the manufacturer's protocols (SuperSignal West Femto; Pierce Chemical) and exposed to X-ray film. Densitometric analyses of Western blots were performed by using a ChemImager 4000 (Alpha-Innotech).

Statistical analysis. Results are shown as means ± SE. Statistical analysis was performed by using one-way ANOVA, followed by the Tukey's test for multiple comparisons. Values were considered statistically significant at *P* < 0.05.

RESULTS

We first explored the effects of S1P on vascular tone in rat mesenteric arteries precontracted with norepinephrine. As shown in Fig. 1A, S1P treatment promoted rapid, dose-dependent, and reversible vascular relaxation, with an EC₅₀ of 10 ± 3 nM, well within the physiological range of S1P in plasma. Maximal S1P-dependent vasodilation (55 ± 8%) was observed within 2 min of drug addition and returned to baseline by 5 min. The magnitude of S1P vasorelaxation was less than that promoted by BK or SNP in this arterial preparation. S1P-mediated vasorelaxation is completely abrogated in endothelium-denuded arteries (Fig. 1B), in which SNP-mediated responses are retained. Comparable vasodilation responses to BK and S1P were seen in mesenteric arterioles prepared from wild-type but not gene-targeted eNOS^{null} mice (Fig. 2A). As shown in Fig. 2A, S1P promotes an entirely eNOS-dependent vasodilation; the magnitude of NO-dependent vasorelaxation induced by S1P is equivalent to that seen in response to classical NO agonists, such as BK.

We then used a series of pharmacological inhibitors of endothelium-derived relaxation to further characterize the response to S1P. Before analyzing agonist-mediated vasorelaxation responses, we treated arterioles for 30 min with various combinations of the NOS inhibitor N^ω-monomethyl-L-arginine (L-NMMA; 100 μmol/l); the cyclooxygenase inhibitor indomethacin (10 μM), which blocks prostacyclin-mediated vasorelaxation (29); or with the K⁺ channel blocker 4-aminopyridine (4-AP, 100 μM), which blocks the effects characteristic of endothelium-derived hyperpolarizing factor (EDHF) (32). As can be seen in Fig. 2B, none of these inhibitors block SNP-mediated vasorelaxation, a response mediated directly by action of this agonist on vascular smooth muscle cells (16). BK-mediated vasorelaxation was attenuated completely by the combination of L-NMMA and 4-AP, but the addition of L-NMMA plus indomethacin, or of indomethacin plus 4-AP, only partially inhibited the BK response. By contrast, the response to S1P was completely abrogated by either L-NMMA plus indomethacin or L-NMMA plus 4-AP, whereas the combination of 4-AP plus indomethacin had no substantive inhibitory effect. In a series of analogous experiments (*n* = 4), we found that L-NMMA alone completely blocks S1P-induced vasodilation (data not shown). Taken together, these findings suggest that the S1P response is mediated by an eNOS-

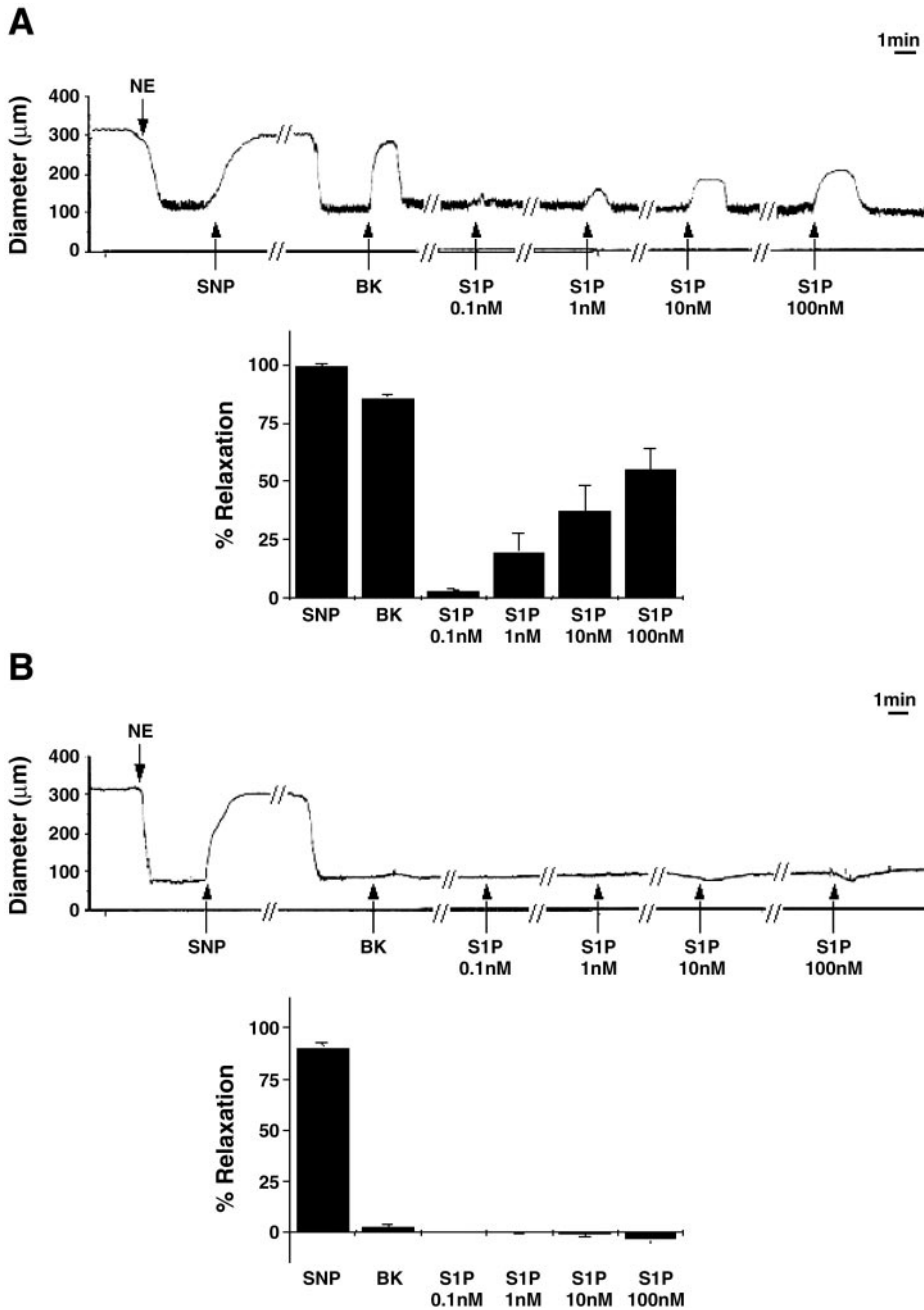


Fig. 1. Sphingosine 1-phosphate (S1P)-induced vasorelaxation. Shown are vascular responses determined in rat mesenteric arterioles, either with intact endothelium (A) or in endothelium-denuded vessels (B). A and B: representative tracings from a single experiment, as well as bar graphs showing pooled data from 8 experiments (means \pm SE). After precontraction of the vessel with norepinephrine (NE; 5 μ M), vessel relaxation was measured after the addition of sodium nitroprusside (SNP, 1 μ M), bradykinin (BK, 1 μ M), or S1P at the indicated concentrations. Data are expressed as percent relaxation of vessel contraction evoked by NE.

dependent pathway, whereas the BK-mediated vasorelaxation reflects both NO- and EDHF-dependent pathways; the lack of any effect of indomethacin suggests that neither S1P nor BK responses involve prostacyclin generation in this vessel preparation.

We then explored the proximal signaling pathways that mediate S1P-dependent vasodilation. As shown in Fig. 3, S1P-induced vasodilation was blocked in vessels pretreated with pertussis toxin, consistent with a role for a pertussis toxin-sensitive G protein pathway in mediating the S1P response. By contrast, the response to BK was not blocked by pertussis toxin, consistent with prior reports (27) documenting that BK-induced vasodilation is mediated by pertussis toxin-insensitive

signaling pathways. The cellular calcium chelator BAPTA suppressed by \sim 20% the maximal vasoconstriction induced by norepinephrine but completely blocked the vasodilation response to both BK and S1P. The PI3-kinase inhibitor wortmannin completely blocked S1P-induced vasodilation and had a small but statistically significant effect on the vasodilation response to BK.

Effects of S1P on eNOS phosphorylation have not previously been explored in intact vessels, but S1P-mediated eNOS activation of eNOS in cultured endothelial cells has been shown to involve the phosphorylation of the enzyme at Ser¹¹⁷⁹ by kinase Akt (13, 14, 23). To correlate S1P-mediated vasodilation with

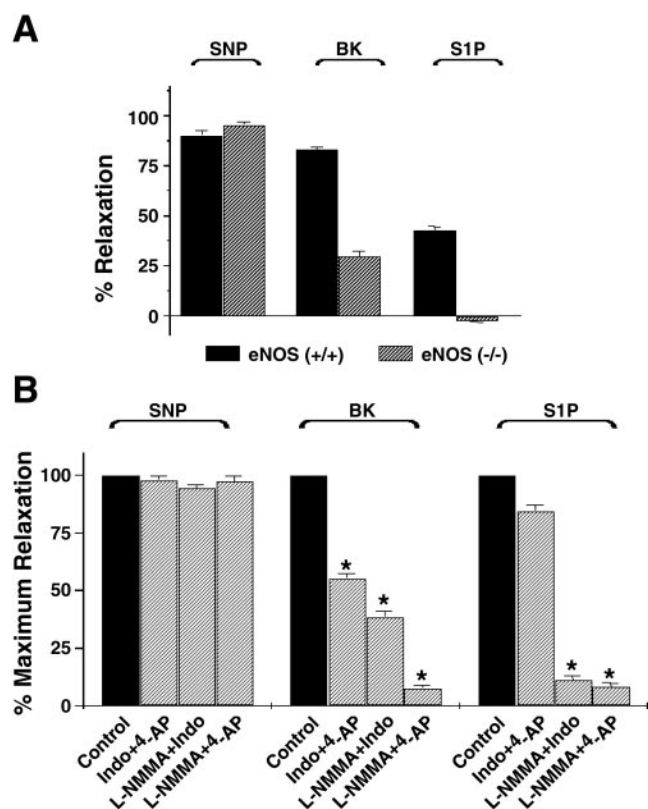


Fig. 2. S1P-induced endothelial nitric oxide synthase (eNOS)-mediated vasorelaxation. Agonist-induced vasodilation is determined in mesenteric arteries from mice [A, showing either wild-type (eNOS^{+/+}) or gene-targeted eNOS^{null} (eNOS^{-/-}) mice] or in mesenteric arteries of rats (B). Data are expressed as percentage of maximum vessel relaxation induced by SNP (100 μ M), BK (1 μ M), or S1P (100 nM) normalized in each case to the maximal response evoked by that agonist. Experiments in B were performed in the absence (control) or presence of pairwise combinations of the NOS inhibitor N^ω-monomethyl-L-arginine (L-NMMA, 100 μ M), the cyclooxygenase inhibitor indomethacin (Indo, 10 μ M), and/or the K⁺ channels blocker 4-aminopyridine (4-AP, 100 μ M). The data shown are pooled from 4–8 experiments and are presented as the means \pm SE; **P* < 0.05 compared with control.

eNOS phosphorylation, we developed a method to perform high-sensitivity immunoblot analyses in extracts isolated from single, pressurized mesenteric vessels processed immediately after physiological measurements assayed in the vessel perfusion chamber. Thus at specific times after drug treatment of the vessel, the vasodilation response is quantitated, and the pressurized vessel is immediately transferred into an iced buffer and frozen, homogenized in SDS-PAGE sample buffer, and the vessel extract is analyzed in immunoblots probed with phosphospecific antibodies. As shown in Fig. 4, the low basal level of eNOS Ser¹¹⁷⁹ phosphorylation in this arterial preparation was not altered by vasoconstriction with norepinephrine. S1P (100 nM), but not the classical eNOS agonist BK (1 μ M), promoted phosphorylation of eNOS at Ser¹¹⁷⁹. S1P-mediated eNOS phosphorylation was dose dependent with an apparent EC₅₀ value of \sim 10 nmol/l (Fig. 5), in good agreement with the EC₅₀ of vasodilator response elicited by S1P (Fig. 1).

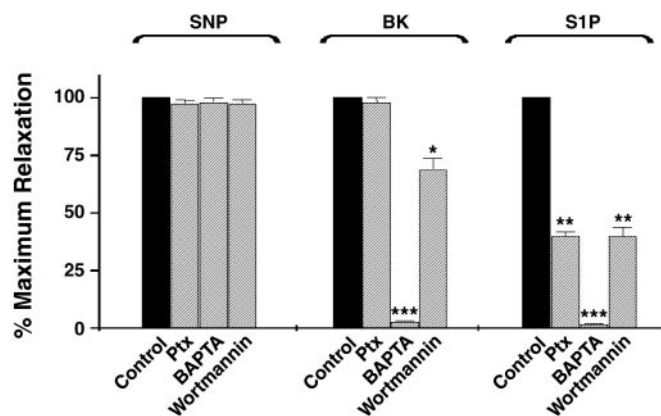


Fig. 3. Intracellular signaling pathways in S1P-, BK-, and SNP-induced vasorelaxation. Agonist-induced vasodilation is determined in rat mesenteric arteries treated with SNP (100 μ M), BK (1 μ M), or S1P (100 nM) plus various inhibitors of intracellular signaling pathways. Data are expressed as the percentage of maximum relaxation induced by each agonist, normalized in each case to the maximal response evoked by that agonist. Experiments were performed in the absence (control) or presence of combinations of pertussis toxin (PTX, 2 μ g/ml), the intracellular calcium chelator BAPTA (5 μ M), or the PI3-kinase inhibitor wortmannin (1 μ M). Data shown are pooled from 4–8 experiments, are presented as the means \pm SE; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared with control.

We then explored the time course of S1P-induced eNOS Ser¹¹⁷⁹ phosphorylation and correlated the temporal pattern of vasorelaxation with the level of phosphorylation determined in the same vessel. The addition of S1P (100 nM) led to a marked (\sim 3.5-fold) in-

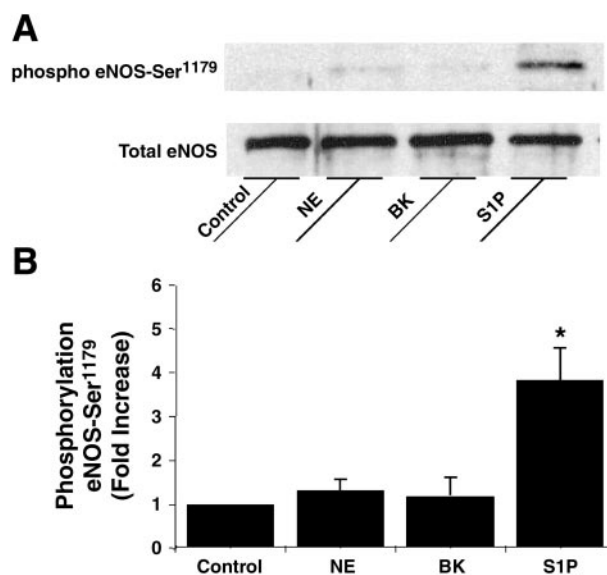


Fig. 4. Agonist-mediated Ser¹¹⁷⁹ eNOS phosphorylation in intact vessels. A: the results of immunoblots analyzed in single mesenteric arteries and probed with antibodies against eNOS phospho-Ser¹¹⁷⁹ or total eNOS, as indicated. Mesenteric arteries were perfused with buffer to a stable baseline pressure and then treated for 5 min with NE (1 μ M), BK (1 μ M), or S1P (100 nM). B: results of densitometric analyses from pooled data, plotted as the fold increase in eNOS Ser¹¹⁷⁹ phosphorylation relative to the signal obtained in the absence of drug (control). Each data point represents the means \pm SE derived from 4 independent experiments; **P* < 0.05 compared with control.

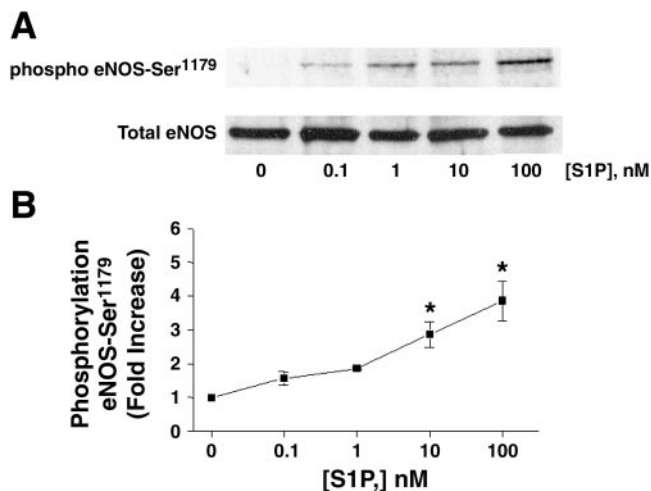


Fig. 5. Dose response for S1P-mediated Ser¹¹⁷⁹ eNOS phosphorylation. **A**: results of immunoblots analyzed in single mesenteric arteries and probed with antibodies against eNOS phospho-Ser¹¹⁷⁹ or total eNOS, as indicated. Mesenteric arteries were perfused with buffer to a stable baseline pressure and then treated for 5 min with the indicated concentrations of S1P. **B**: results of densitometric analyses from pooled data, plotted as the fold increase in eNOS Ser¹¹⁷⁹ phosphorylation relative to the signal obtained in the absence of S1P. Each data point represents the means \pm SE derived from three independent experiments; * $P < 0.05$ compared with control.

crease in eNOS Ser¹¹⁷⁹ phosphorylation, which reached a maximal level within 2 min, associated with the time of the maximum vasodilation response (Fig. 6). After the maximal S1P-induced response, vasodilation then returned rapidly to baseline, whereas eNOS Ser¹¹⁷⁹ phosphorylation remained elevated for >5 min after the return of vessel diameter to basal levels.

DISCUSSION

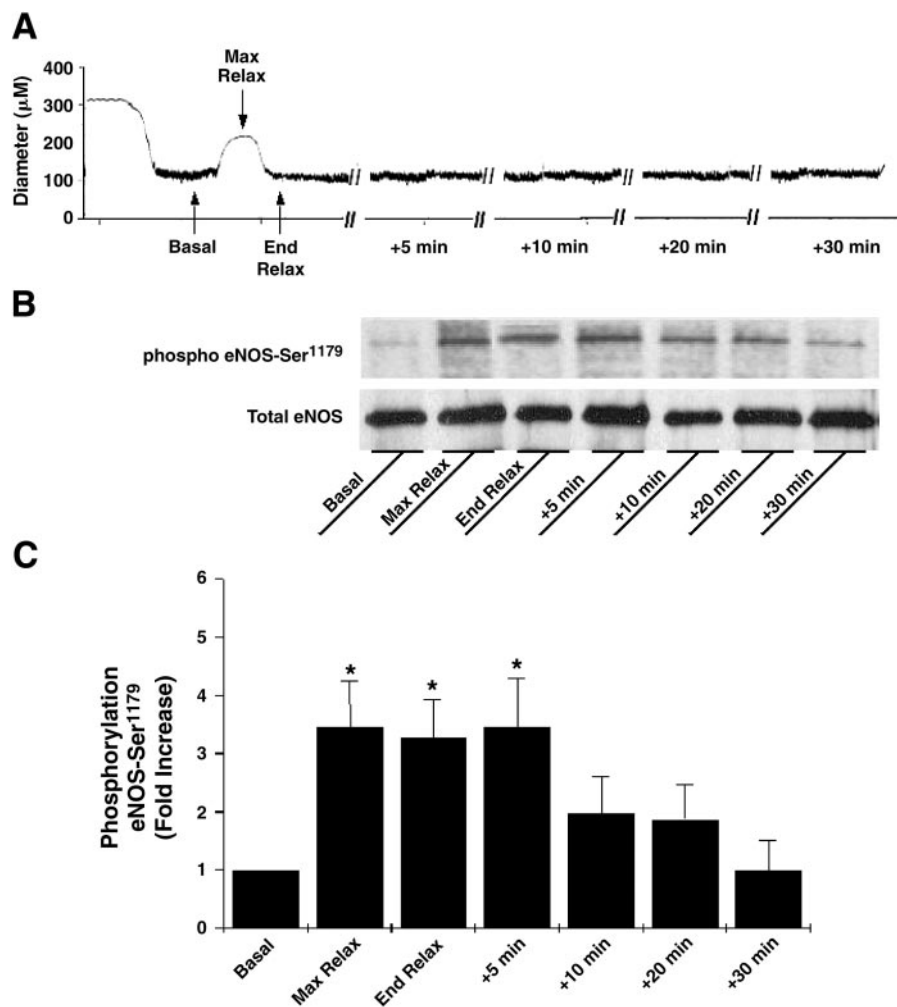
These studies have explored the effects of the platelet-derived sphingolipid S1P on endothelium-dependent arterial vasorelaxation in the mesenteric arterial tree. As shown in Fig. 1, the EC₅₀ for S1P-induced vasorelaxation (10 nM) is similar to the EC₅₀ previously established for S1P-induced eNOS activation in cultured endothelial cells (14). The magnitude of the vasodilatory response to S1P is about one-half the maximal vasodilation response seen in response to the classical agonist BK (Fig. 1). The NOS antagonist L-NMMA completely blocks S1P-dependent vasodilation (Fig. 2) but only partially attenuates the vasodilation elicited by BK. S1P-induced vasorelaxation is entirely abolished in mesenteric arterioles from eNOS^{null} mice; BK still produces some vasodilation in these gene-targeted mice, apparently by an eNOS-independent mechanism. Notably, the fraction of BK-induced vasodilation that is dependent on eNOS activation is equivalent in magnitude to the S1P-mediated vasodilation response, indicating that these two agonists activate eNOS to a similar extent. The remaining increment of the BK-dependent vasodilation response is sensitive to 4-AP, suggesting a quantitatively important role for endothelium-derived hyperpolarizing fac-

tor in the BK (but not the S1P) response in this arterial preparation. S1P-mediated vasodilation is abrogated by the intracellular calcium chelator BAPTA and is substantively inhibited by pertussis toxin and by the PI3-kinase inhibitor wortmannin (Fig. 3). These findings suggest that S1P-induced vasodilation, like S1P-dependent eNOS activation in cultured cells (14), is in each case mediated by pertussis toxin-sensitive G proteins in a calcium-dependent cellular pathway involving the activity of PI3-kinase.

The best characterized eNOS phosphorylation site is Ser¹¹⁷⁹ residue, a residue that is phosphorylated by protein kinase Akt, a downstream mediator of PI3-kinase. We have previously shown that S1P, but not BK, promotes the PI3-kinase/Akt-dependent phosphorylation of eNOS at Ser¹¹⁷⁹ in cultured bovine aortic endothelial cells (13, 14). However, other reports (10) have found that BK may lead to eNOS Ser¹¹⁷⁹ phosphorylation in these same cells (albeit independent of PI3-kinase), suggesting that subtle differences in experimental conditions may affect this pathway. By developing methods for high-sensitivity immunoblots analyzed in single, pressurized mesenteric artery preparations, we have been able for the first time in these studies to precisely and quantitatively correlate agonist-induced vasorelaxation with the state of eNOS phosphorylation. However, we were stymied in exploring the direct effects of S1P on Akt phosphorylation because of the cellular heterogeneity characteristic of these arterial preparations. Robust expression of kinase Akt in vascular smooth muscle cells as well as endothelial cells confounded our efforts to discern cell-specific Akt pathways in isolated arteries. We therefore focused on regulation of an endothelial cell-specific Akt substrate, eNOS. By using a phosphorylation state-specific antibody, we found that S1P but not BK promotes eNOS Ser¹¹⁷⁹ phosphorylation, as we previously observed in cultured endothelial cells. In untreated vessels, there is a very low level of basal eNOS phosphorylation at Ser¹¹⁷⁹, and vessel contraction with norepinephrine does not alter eNOS phosphorylation at this site (Fig. 4). Until the present report, there had been no previous studies of the effects of BK on eNOS Ser¹¹⁷⁹ phosphorylation in intact vessels. Importantly, although S1P and BK elicit a similar degree of eNOS-dependent vasodilation (Fig. 2), eNOS Ser¹¹⁷⁹ phosphorylation is stimulated by S1P but is unaffected by BK (Fig. 4). For both agonists, endothelium-dependent vasorelaxation is abrogated by the intracellular calcium chelator BAPTA, indicating a key role for receptor-mediated calcium mobilization in eNOS activation elicited by both S1P and BK. We (13, 14) have previously shown in cultured endothelial cells that there is excellent concordance between S1P-induced eNOS Ser¹¹⁷⁹ phosphorylation and S1P-induced eNOS activation. More detailed analyses of eNOS phosphorylation in these studies were hampered by the relatively weak phospho-eNOS signals detected in single mesenteric arterioles.

The concentration of S1P in plasma is ~ 100 nM (30), well above the EC₅₀ for S1P-induced vasorelaxation

Fig. 6. Temporal correlation between S1P-dependent vasorelaxation and S1P-mediated Ser¹¹⁷⁹ eNOS phosphorylation. **A**: tracings from a representative experiment analyzing the vasodilation response to S1P (100 nM) in a single vessel monitored in the myograph over the time period shown. **B**: representative immunoblot experiments in which individual pressurized vessels are treated with S1P and monitored for the vasodilation response; at the times indicated, the vessel is rapidly (<5 s) transferred while still pressurized from the physiological superfusion media in the myograph into ice-cold buffer and then into liquid nitrogen. The frozen vessel is then homogenized in SDS-PAGE sample buffer and analyzed in immunoblots probed with the Ser¹¹⁷⁹ phospho-eNOS antibody or with the antibody for total eNOS, as shown. **C**: results of densitometric analyses from pooled data from identically configured experiments, plotted as the fold increase in Ser¹¹⁷⁹ eNOS phosphorylation relative to the basal signal. Each data point represents the means \pm SE derived from three independent experiments.



(Fig. 1) or eNOS Ser¹¹⁷⁹ phosphorylation (Fig. 5). However, the fraction of free S1P is likely to be considerably lower, as this lipid is highly protein bound, both to albumin and HDL, and possibly other plasma proteins (24, 30). Most of the plasma S1P appears to derive from blood platelets, and local concentrations of S1P can increase in the vicinity of activated platelets (31). It is plausible that platelet-derived S1P functions in a feedback loop to suppress platelet aggregation by activating S1P receptors on endothelial cells, thereby leading to eNOS activation and the antiplatelet effects of NO synthesis. Our findings of S1P-induced vasodilation stand in striking contrast to previous studies (1, 2) that have reported S1P-induced vasoconstriction. It is important to note that these prior studies used S1P concentrations in the micromolar range, 100-fold greater than the EC₅₀ we observed for S1P-promoted vasorelaxation and >10-fold greater than the physiological concentration of S1P in plasma. It is possible that vasoconstrictor effects of S1P may be elicited by receptor-dependent as well as receptor-independent mechanisms at drug concentrations considerably higher than those found under physiological conditions. It is also plausible that different vascular beds will be differentially responsive to S1P, as is the case for many other

agonists at endothelial cell surface receptors in diverse locales (21, 22). The complexity of the S1P response is compounded by the fact that there are at least five different S1P receptor subtypes, several of which may be expressed in cultured smooth muscle cells and endothelial cells. In preliminary experiments, we found that the currently available subtype-specific S1P receptor antibodies are insufficiently sensitive to detect receptor expression in immunoblots analyzed in the arterial preparations used in the present study (data not shown). We (13) have previously shown that the S1P1 receptor subtype (formerly called EDG-1) can lead to eNOS activation, but the roles of the other S1P receptor subtypes in eNOS activation have not yet been clearly established. Although it would be interesting to determine the relative contribution of each S1P receptor subtype on S1P-induced vasomotion, studies in vascular preparations are hampered, because specific pharmacological agonists and antagonists for S1P receptor subtypes are not available.

The vasodilation response to S1P is transient, a characteristic feature of the temporal response to other agonists that promote vasodilation through the activation of calcium-mobilizing endothelial cell surface receptors. Transient receptor-mediated vasodilation re-

sponses may be terminated by receptor desensitization, by the return of intracellular calcium to basal levels by counterregulatory pathways between the receptor and the contractile apparatus, or some combination of the above. Current studies represent the first temporal correlation of receptor-mediated vasodilation with eNOS Ser¹¹⁷⁹ phosphorylation and serve to point out the complexities of eNOS regulation in intact vessels. It is intriguing to note that complete resolution of S1P-promoted vessel relaxation is seen, whereas eNOS Ser¹¹⁷⁹ phosphorylation remains at maximal levels (Fig. 6). Indeed, maximal eNOS Ser¹¹⁷⁹ phosphorylation persists for several minutes after the complete resolution of S1P-induced vasorelaxation. These observations suggest that other counterregulatory pathways aside from eNOS Ser¹¹⁷⁹ dephosphorylation serve to attenuate S1P-dependent eNOS activation. eNOS deactivation in the face of sustained Ser¹¹⁷⁹ phosphorylation may simply reflect the return of intracellular calcium concentration to baseline levels or may be consequent to phosphorylation of the enzyme at other residues (6, 15) or the intracellular translocation of the enzyme (27) leading to decreased extracellular NO generation.

Taken together, these studies have established that the platelet-derived sphingolipid S1P is a potent determinant of endothelium-dependent arterial vasorelaxation. The response to S1P is mediated by a pertussis toxin-sensitive G protein-coupled receptor pathway that involves the activation of PI3-kinase, yielding an eNOS-dependent vasodilation response similar in magnitude to that elicited by the classical agonist BK. Because plasma levels of S1P are dynamically mediated by platelet aggregation, we propose that S1P may be a key (patho)physiological determinant of NO-dependent signaling pathways in the vessel wall.

This work was supported, in part, by National Heart, Lung, and Blood Institute Grant HL-46457 (to T. Michel). A. P. Dantas was supported by a fellowship award from the Fundação de Amparo a Pesquisa do Estado de São Paulo.

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