Sphingosine 1-Phosphate Induces Endothelial Nitric-Oxide Synthase Activation through Phosphorylation in Human Corpus Cavernosum

Roberta d’Emmanuele di Villa Bianca, Raffaella Sorrentino, Rosalinda Sorrentino, Ciro Imbimbo, Alessandro Palmieri, Ferdinando Fusco, Mario Maggi, Raffaele De Palma, Giuseppe Cirino, and Vincenzo Mirone

Dipartimento di Farmacologia Sperimentale, Napoli, Italy (R.d.V.B., Ra.S., Ro.S., G.C.); Centro Interdipartimentale di Ricerca Preclinica e Clinica di Medicina Sessuale (CIRMS), Napoli, Italy (Ra.S., C.I., F.F., G.C., A.P., V.M.); Andrology Unit, Department of Clinical Physiopathology, University of Florence, Florence, Italy (M.M.); and Dipartimento di Internistica Clinica e Sperimentale, Seconda Università di Napoli, Naples, Italy (R.D.P.)

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ABSTRACT

Sphingosine 1-phosphate (S1P) is the natural ligand for a specific G protein-coupled receptors. In endothelial cells, S1P has been shown to modulate the activity of the endothelial nitric-oxide synthase (eNOS) through phosphorylation operated by Akt. Nitric oxide (NO) produced by neuronal nitric-oxide synthase and eNOS plays a central role in triggering and maintaining penile erection. This study has assessed the possibility of a similar cross-talk between eNOS and S1P in human corpus cavernosum and whether this interaction is connected to penile vascular response. Quantitative reverse transcription-polymerase chain reaction demonstrated the presence of S1P1, S1P2, and S1P3 receptors in both the human corpus cavernosum (HCC) and the penile artery. S1P on its own did not relax or contract HCC strips, but on the other hand, incubation with S1P (0.1 μM) caused a 6-fold increase in relaxation induced by a subliminal dose of acetylcholine. This effect is dependent upon eNOS activation through an Akt-dependent phosphorylation, as demonstrated by pharmacological modulation with L-nitroarginine methyl ester and wortmannin and by Western blot studies. In human tissue, S1P seems to be the possible candidate for the activation of the eNOS calcium-independent pathway. This pathway may represent a new therapeutic area of intervention in erectile dysfunction (ED) to develop a way to selectively promote NO production at the endothelial level. This approach could also be used to enhance phosphodiesterase 5 therapy in patients with ED that are poor responders, such as in the case of diabetes.

Penile erection is one component of a complex series of integrated physiological processes and biochemical events coordinated at the level of the peripheral and central nervous system (Moreland et al., 2001). Erectile dysfunction (ED) can be thought of as a result of a number of hemodynamic events where change in the blood flow to the erectile tissue is the most common cause (Broderick, 1998). Basically, upon sexual stimulation, penile erection is significantly dependent on adequate blood flow to the erectile tissue. This requires coordinated arterial endothelium-dependent vasodilation and sinusoidal endothelium-dependent corporal smooth muscle relaxation. Nitric oxide (NO) is the principal peripheral proerectile neurotransmitter that is released by both nonadrenergic noncholinergic neurons and the sinusoidal endothelium to relax corporal smooth muscle through the cGMP pathway. This leads to corporal smooth muscle relaxation. NO synthase (NOS) catalyzes production of NO from L-arginine. Both isoforms, namely neuronal NOS (nNOS) and endothelial NOS (eNOS) expressed constitutively, mediate penile erection. The binding of a Ca2+/calmodulin complex is essential for the activation of the constitutive enzymes. Studies have shown that eNOS can be activated in a calcium-independent manner. The calcium-independent activation relies on the phosphorylation of the enzyme at serine 1177 operated by Akt (protein kinase B). It is known that blood flow-induced

ABBREVIATIONS: ED, erectile dysfunction; S1P, sphingosine 1-phosphate; NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; SNP, sodium nitroprusside; HCC, human corpus cavernosum; L-NAME, L-nitroarginine methyl ester; EDG, endothelial differentiation gene; PI3K, phosphatidylinositol 3-kinase; Akt, serine/threonine kinase or protein kinase B; nNOS, neuronal nitric-oxide synthase; ACh, acetylcholine; PE, phenylephrine; PGE, prostaglandin E; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride.
fluid shear stress in the penile vasculature stimulates phosphatidylinositol 3-kinase (PI3K) to phosphorylate protein kinase B, which in turn phosphorylates eNOS to generate NO. The importance of this pathway has also been demonstrated by in vivo studies in rats. Electrical stimulation of the cavernous nerve or intracavernous injection of a vasorelaxant agent has been shown to cause Akt phosphorylation and eNOS activation, giving an erectile response that had been abolished by Akt inhibitor pretreatment (Hurt et al., 2002). Thus, nNOS may initiate cavernosal tissue relaxation, although the shifting eNOS to a higher level of activation (Fulton et al., 2001) contributes to the attainment and the maintenance of full erection (Burnett, 2004). Recently, much attention has been focused on membrane phospholipids, in particular sphingosine 1-phosphate (SIP). SIP is a bioactive lipid produced by the metabolism of sphingomyelin (Pyne and Pyne, 2000; Watterson et al., 2003). SIP exhibits its action by binding to a family of G protein-coupled receptors named SIP 1 to 5, originally referred to as the endothelial differentiation gene (EDG) family. To date, five members, EDG-1/SIP1, EDG-5/SIP2, EDG-3/SIP3, EDG-6/SIP4, and EDG-8/SIP5, have been identified (Anliker and Chun, 2004). These receptors are coupled to different intracellular second messenger systems, including adenylate cyclase, phospholipase C, phosphatidylinositol 3-kinase/protein kinase Akt, and mitogen-activated protein kinases, as well as Rh-o- and Ras-dependent pathways (Hia, 2003; Waaber et al., 2004). SIP potently and robustly activates eNOS in cultured vascular endothelial cells in a pathway that involves G protein-dependent activation of Akt (Igarashi and Michel, 2001). Because the penile tissue vasculature is a perfect example of microcirculation, here we have evaluated whether in human corpus cavernosum exists a similar cross-talk between eNOS and SIP. Our study shows that 1) human corpus cavernosum possesses SIP receptors, 2) SIP strongly potentiates acetylcholine-induced relaxation of human corpus cavernosum strips, and 3) this effect is related to an increased phosphorylation of eNOS as demonstrated by pharmacological modulation studies.

Materials and Methods

Human Tissue. A male-to-female transsexual surgical procedure was performed by amputating the penis and testesicles and creating a neovagina to simulate female external genitalia. Patients were prescribed appropriate presurgery hormone treatment with antiandrogens and estrogens to facilitate a female appearance. The therapy was discontinued 2 months prior to surgery. The corpora cavernosa was carefully cut out of the amputated penis and placed in ice-cold oxygenated Krebs’ solution and washed thoroughly with heparinized Krebs’ solution. After the lavage, the corpora cavernosa was placed in an ice-cold oxygenated Krebs’ solution and kept on ice until experiment (Mirone et al., 2000). All patients were informed of procedures and signed their written consent. The protocol was approved by the Ethics Committee of the Medical School of the University of Naples.

HCC Strips. Longitudinal strips (2 cm) of trabecular tissue were dissected from the penis and immediately placed in Krebs’ solution and kept at 4°C until use (Mirone et al., 2000). Krebs’ solution was composed of the following: 115.3 mM NaCl, 4.9 mM KCl, 1.46 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25.0 mM NaHCO3, and 11.1 mM glucose (Thermo Electron Corporation, Waltham, MA). HCC strips were mounted in a 2-ml organ bath containing oxygenated (95% O2 and 5% CO2) Krebs’ solution at 37°C. HCC strips were connected to isometric force transducers (model 7002; Ugo Basile, Comerio, Italy), and changes in tension were recorded continuously by using a polygraph linearencoder (WR3310; Graphitec, Yokohama, Japan). Tissues were preloaded with 2g tension and allowed to equilibrate for 90 min in Krebs’ solution that was replaced at 15-min intervals. After equilibration, tissues were standardized by using repeated phenylephrine (PE) (3 μM; Sigma-Aldrich, Milano, Italy), eliciting contractile responses until three equal responses were obtained. After standardization, endothelial integrity was assessed by testing the responses to acetylcholine (ACh; 0.01–10 μM; Sigma-Aldrich). Strips without endothelium were obtained by incubating with distilled water for 154. A curve concentration effect induced by SIP (0.01–10 μM; Tabu-bio Laboratories, le Perray en Yvelines, France) was performed in the presence or absence of endothelium using HCC strips precontracted with PE (3 μM). HCC strips were challenged with SIP (0. 01–10 μM) directly on basal tone to assess any contractile response. A curve-concentration effect was performed to evaluate the concentration of ACh needed to reach a 15% relaxation result. Once the subliminal dose was determined, the HCC strips were washed twice and contracted with PE (3 μM) and left to incubate in the organ bath with various doses of SIP (0.1–3 μM) for 10 min. They were then challenged with previously determined ACh doses (ranging between 10 and 300 nM depending on sensitivity of the human strips). The dose of SIP that gave the best potentiating effect was 0.1 μM, and this dose was used throughout the study. In another set of experiments, HCC strips were incubated with 1 NAME (100 μM; Sigma-Aldrich) or with wortmannin (2 μM; Tocris Cookson Inc., Bristol, UK). The inhibitors were added to the organ bath 30 min prior to SIP (0.1 μM). The optimal incubation time was previously determined in this study’s experimental conditions (data not shown). A curve concentration-effect of ACh (0.01–10 μM) was performed on HCC strips in the presence of wortmannin (2 μM). To demonstrate the specificity of SIP on ACh-induced relaxation, we used alprostadil (PGE1, 100 ng/ml, Caverjet; Pharmacia Italia S.p.A., Milan, Italy) or sodium nitroprusside (SNP; Sigma-Aldrich) to elicit the relaxant response.

Quantitative RT-PCR Analysis. The presence of SIP receptor was previously screened by PCR, and we determined that only SIP1, SIP2, and SIP3 were present (data not shown). Total mRNA from human corpus spongiosum or penile artery was extracted using TRIzol reagent (Invitrogen, Milan, Italy), according to the manufacturer’s recommendations. Reverse transcription was performed, and 100 ng of the above RNA samples were used for quantitative PCR. Samples were run in triplicate in 50-μl reactions using an ABI PRISM 5700 Sequence Detector System (Applied Biosystems, Foster City, CA). Samples were incubated at 50°C for 2 min and at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. SYBR Green oligonucleotides to detect human SIP1, SIP2, and SIP3 receptors were specifically designed using Primer Express Software (Applied Biosystem) and validated for their specificity. Oligonucleotides used were H-SIP1-F, 5′-gggccacacccaaagcaaa-3′; H-SIP1-R, 5′-gtcatggagggcctgaatcctg-3′; H-SIP2-F, 5′-ggccacacccaaagcaaa-3′; H-SIP2-R, 5′-cattgagggcctgaatcctg-3′; H-SIP3-F, 5′-ggtcatggagggcctgaatcctg-3′; and H-SIP3-R, 5′-ggagggcctgaatcctg-3′. Relative quantification of target cDNA was determined by arbitrarily setting the control value to 100, and changes in cDNA content of a sample were expressed as a-fold increase over this value. Differences in cDNA input were corrected by normalizing signals obtained with primers specific for glyceraldehyde-3-phosphate dehydrogenase. To exclude nonspecific amplification and/or the formation of primer dimers, control reactions were performed in the absence of target cDNA. All the experiments were run in triplicate.

Western Blot Analysis. HCC strips were placed in 24-well plates containing 1 ml of Krebs’ solution at 37°C and incubated for 60 min. After the stabilization of HCC strips incubated in the wells, ACh (3 μM) or SIP (0.1 μM) was added. SIP was also incubated with wortmannin (2 μM), which was added 30 min prior to the addition of SIP (0.1 μM), and left to incubate for an additional 10 min. At the
end of each specific treatment period, HCC strips were quickly frozen in liquid nitrogen and then used for Western blot studies. HCC strips were homogenized in lysis buffer (0.5 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 2 mM MgCl₂, 1 mM EGTA, and 1 mM DTT) and protease inhibitors by using an Ultra-Turrax homogenizer (Ika-labortechnik, Staufen, Germany). Protein concentrations were determined by using the Bradford assay (Bio-Rad, Milan, Italy). Protein samples (50 μg) were subjected to electrophoresis on an SDS 10% polyacrylamide gel and electrophoretically transferred onto a nitrocellulose transfer membrane (Protran; Whatman Schleicher and Schuell, Dassel, Germany). The immunoblots were developed with 1:500 dilutions of the indicated antibodies, and the signal was detected with the enhanced chemiluminescence system according to the manufacturer’s instructions (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Anti-eNOS and anti-phosphorylated eNOS-Ser1177 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and Alpha Diagnostic International Inc (San Antonio, TX). All salts used for Western blot analysis were purchased from MP Biomedicals (Irvine, CA).

Calculation and Statistical Analysis. Data are expressed as mean ± S.E.M. from nine separate human specimens, and at least nine strips were used for each experiment. The responses are expressed as relaxation percent of phenylephrine contraction (3 mM). For multiple comparisons, results were analyzed by analysis of variance followed by the Bonferroni’s post-test. For comparison between two values, the unpaired Student’s t test was used. A value of P < 0.05 was considered significant.

Results

PCR Analysis. Quantitative RT-PCR study showed that spongiosum tissue (Fig. 1a) and the penile artery (Fig. 1b) possess mRNA S1P receptors. In both tissues, S1P₃ mRNA was significantly (P < 0.001) higher than S1P₂ and S1P₁, respectively. S1P₃ was ~2000-fold more abundant than S1P₁ and 4-fold more abundant than S1P₂ in spongiosum tissue (Fig. 1a). In the penile artery, S1P₃ was 300- and 15-fold more abundant than S1P₂ and S1P₁, respectively (Fig. 1b).

In Vitro Study. S1P (0.01–10 μM) had no effect on precontracted HCC strips with or without endothelium (Fig. 2). Likewise, S1P (0.01–10 μM) did not contract HCC strips by itself (data not shown). The endothelial removal by distilled water did not modify the phenylephrine-induced contraction in HCC strips. Indeed, the contraction elicited by PE (3 μM) before treatment was 80 ± 7.1 dyne/mg tissue, whereas after treatment, contraction elicited by PE (3 μM) was 85 ± 6.2 dyne/mg tissue (n = 10; N.S.). Tests were performed to determine whether S1P could enhance submaximal ACh effect. The response to the concentration of ACh that gave 15% tissue relaxation was strongly enhanced by incubating the strips with S1P (0.1 μM) for 10 min (Fig. 3a) but not with the vehicle. During this experiment, S1P caused a powerful significant increase in ACh-induced relaxation (Fig. 3a, P < 0.01) that corresponded to a 6-fold increase in relaxation percent (P < 0.005). To assess the specificity of the action of S1P against ACh, we performed experiments with PGE₁ or SNP. Alprostadil (PGE₁, 100 ng/ml)-induced relaxation was not modified by either S1P (Fig. 3c) or its vehicle (Fig. 3b). The same effect was observed when SNP was used (data not shown).

Pharmacological Modulation. Two different agents capable of interfering in eNOS activity, either l-NAME, a false substrate, or wortmannin, as inhibitor of eNOS phosphorylation, were used to operate a pharmacological modulation of eNOS. Incubation of HCC strips with l-NAME reverted the response (Fig. 4a, P < 0.001), indicating that the potentiating effect observed is mainly driven through eNOS activation. To evaluate whether the effect observed was linked to an increased eNOS phosphorylation, we incubated the HCC strip with wortmannin. Wortmannin, similar to l-NAME, abolished the S1P-induced ACh potentiation (Fig. 4a, P < 0.001).

Fig. 1. a, quantitative RT-PCR for S1P₁, S1P₂, and S1P₃ in spongiosum tissue. ***, P < 0.001 versus S1P₁ and S1P₂; ****, P < 0.001 versus S1P₂; b, penile artery. ***, P < 0.001 versus S1P₁ and S1P₂; ****, P < 0.001 versus S1P₂. The data represent the mean ± S.E.M. from three different human specimens.

Fig. 2. Effect of S1P (0.001–10 μM) on HCC strips precontracted with phenylephrine (3 μM) in the presence or absence of endothelium. ACh (0.01–10 μM) or SNP (0.01–10 μM) was used as positive control. The data represent the mean ± S.E.M. from nine separate human specimens (nine strips for each experiment).
Wortmannin by itself caused a significant reduction of ACh-induced relaxation ($P < 0.0001$; Fig. 4b). Subsequently, to confirm that the observed effect was the result of a modulation of eNOS phosphorylation, we performed a Western blot study. Incubation of HCC strips with S1P caused a significant increase in eNOS phosphorylation that was significantly higher than that caused by the vehicle ($P < 0.01$). Incubation with higher doses of S1P (1 μM) did not cause further increase in eNOS phosphorylation (data not shown). Incubation of HCC strips with wortmannin prevented this effect (Fig. 5), confirming that S1P selectively activates eNOS phosphorylation. Interestingly, in our experimental conditions, ACh (3 μM) by itself induced an increase in eNOS phosphorylation that was significantly lower than S1P.

**Discussion**

A substantial body of evidence implicates NO in normal erection function. NO from nerves and possibly endothelia plays a crucial role in initiating and maintaining intracavernous pressure increase that is necessary for penile erection. This hemodynamic event is dependent upon cyclic GMP synthesized by soluble guanylyl cyclase via NO in smooth muscle cells. The constitutive NOS isoforms, nNOS and eNOS, are regulated by cofactors such as calcium, calcium-binding protein calmodulin, oxygen, and reduced NADPH. However, eNOS can be shifted by phosphorylation to a higher level of activation through a calcium-independent pathway. This pathway of activation involves PI3K that activates the serine/threonine protein kinase (Akt), causing eNOS phosphorylation (Dimmeler et al., 1999; Michell et al., 1999; Morales-Ruiz et al., 2001). To evaluate whether a similar regulation exists in human tissue, we used corpus cavernosum obtained from patients that received an appropriate presurgery hormone treatment, with antiandrogens and estrogens, that was suspended two months before the surgery. A preliminary qualitative PCR study demonstrated the presence of mRNA for S1P1, S1P2, and S1P3 receptors in human corpus cavernosum and penile artery. Data from the quantitative RT-PCR indicate that the most abundant receptor is S1P3, both in corpus spongiosum and in the penile artery. S1P induces a dose-dependent vasorelaxation of isolated pressurized mesenteric arterioles in wild-type mice but not in eNOS$^{-/-}$ mice, and this effect is endothelium-dependent (Dantas et al., 2003). However, when we added S1P to strips contracted with PE, we did not observe any relaxant or contracting effect in the human tissue. No contracting effect was present on basal tone. On the other hand, S1P activates eNOS in cultured vascular endothelial cells by promoting eNOS phosphorylation (Igarashi and Michel, 2001). In addition, it has been demonstrated in vivo that the PI3K/Akt pathway physiologically mediates erection. Both electrical stimulation of the cavernous nerve and direct intracavernosal injection of papaverine cause an erectile response coupled to a rapid increase in phosphorylated Akt and eNOS (Hurt et al., 2002). Erection and phosphorylation are both diminished by wortmannin and LY294002, inhibitors of PI3K, the upstream activator of Akt (Hurt et al., 2002). Overall, these data indicate that a stimulus is necessary to

![Fig. 3. Potentiation of ACh response by S1P (0.1 μM) or vehicle added 10 min prior to ACh, **, $P < 0.01$ versus ACh and vehicle (a). Relaxant effect of PGE1 (100 ng/ml) in the presence of vehicle (b) or in the presence of S1P (0.1 μM) (c). The data represent the mean ± S.E.M. from nine separate human specimens (nine strips for each experiment).](image-url)

![Fig. 4. L-NAME (100 μM) or wortmannin (WT, 2 μM) reverts to S1P (0.1 μM)-induced potentiation of ACh-induced relaxation. ***, $P < 0.001$ versus ACh, L-NAME+SIP, and WT+SIP (a). WT (2 μM; ***, $P < 0.001$) reduces ACh-induced relaxation (b). The data represent the mean ± S.E.M. from nine separate human specimens (nine strips for each experiment).](image-url)
trigger the calcium-independent activation of eNOS; e.g., the phosphorylating mechanism. For this reason, we sought to investigate whether S1P could synergize with a physiological stimulus that uses eNOS activation to transduce the signal downstream receptor activation; e.g., acetylcholine. Tissue incubation with S1P caused a marked increase in the relaxant response of HCC strips to a subliminal dose of acetylcholine. Strikingly, S1P at a dose of 0.1 μM caused an increase of acetylcholine relaxant effect of approximately six times, indicating that S1P can give an important contribution to the peripheral machinery involved in supporting the smooth muscle relaxation necessary to achieve and maintain erection. The possibility that this mechanism is acting in vivo is supported by the fact that S1P concentration in serum is 0.5 μM (Yatomi et al., 2001). Interestingly, this potentiating effect was not present when we used other relaxant agents such as PGE1 or sodium nitroprusside, indicating that the effect observed in HCC is not linked to either the eicosanoid pathway or to a direct interaction with the endogenously generated NO. Pharmacological modulation of the response further demonstrated that the effect observed was mediated through eNOS activation. Indeed, eNOS inhibition by l-NAME reversed the S1P-potentiating action, and wortmannin, an inhibitor of the eNOS phosphorylation pathway, mimicked this effect. Indeed, wortmannin significantly reduced the relaxation elicited by acetylcholine, indicating that the calcium-independent pathway also plays a role in the dynamic physiological vasodilatory mechanisms. These functional data strongly suggest that the interaction responsible for the effect observed takes part at the eNOS level and that eNOS phosphorylation (e.g., the calcium-independent activation) is the main mechanism involved. This hypothesis is further strengthened by the Western blot studies where S1P caused a significant increase in the eNOS-phosphorylated protein within the time frame used, in the in vitro functional study, that was prevented by wortmannin.

In conclusion, we have demonstrated that human cavernous tissue as well as the penile artery has receptors for S1P and that activation by S1P does not directly cause either relaxation or contraction of HCC strips. Conversely, S1P strongly and selectively potentiates acetylcholine response, mostly by increasing NO release and promoting eNOS phosphorylation. Our finding suggests S1P as the possible endogenous mediator responsible for the calcium-independent eNOS activation through phosphorylation in human corpus cavernosum playing a “boosting role” in the hemodynamic events involved in erection. The mechanism described here fits with the physiological requirements for penile erection as a gradual hemodynamic phenomenon. It is feasible that NO released by nerve endings is sufficient to initiate the complex hemodynamic events involved in erection, but it is not enough to maintain and sustain the vasodilatation necessary to achieve a full erection. Both the eNOS phosphorylation pathway and the calcium-dependent pathway, essential for shifting eNOS from a basal state to a higher state of activation, contribute to the maintenance of the vasodilatory state (Fulton et al., 2001). Thus, the phosphorylated eNOS activation pathway may be explored as a new therapeutic area of intervention in ED to develop a way of promoting NO production at endothelial level. This approach could also be used to enhance phosphodiesterase 5 therapy in patients with ED who are poor responders, such as in the case of diabetes.

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References

Hurt KJ, Musicki B, Palese MA, Crone JK, Becker RE, Moriarity JL, Snyder SH, and...

Address correspondence to: Dr. Giuseppe Cirino, Dipartimento di Farmacologia Sperimentale, Via Domenico Montesano 49, 80131 Napoli, Italy. E-mail: cirino@unina.it