Role of Sphingosine-1-Phosphate Phosphohydrolase 1 in the Regulation of Resistance Artery Tone

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Abstract—Sphingosine-1-phosphate (S1P), which mediates pleiotropic actions within the vascular system, is a prominent regulator of microvascular tone. By virtue of its S1P-degrading function, we hypothesized that S1P-phosphohydrolase 1 (SPP1) is an important regulator of tone in resistance arteries. Hamster gracilis muscle resistance arteries express mRNA encoding SPP1. Overexpression of SPP1 (via transfection of a SPP1wt) reduced resting tone, Ca\textsuperscript{2+} sensitivity, and myogenic vasoconstriction, whereas reduced SPP1 expression (antisense oligonucleotides) yielded the opposite effects. Expression of a phosphatase-dead mutant of SPP1 (SPP1\textsuperscript{H208A}) had no effect on any parameter tested, suggesting that catalytic activity of SPP1 is critical. The enhanced myogenic tone that follows overexpression of S1P-generating enzyme sphingosine kinase 1 (Sk1\textsuperscript{wt}) was functionally antagonized by coexpression with SPP1\textsuperscript{wt} but not SPP1\textsuperscript{H208A}. SPP1 modulated vasoconstriction in response to 1 to 100 nmol/L exogenous S1P, a concentration range that was characterized as S1P\textsubscript{2}-dependent, based on the effect of S1P\textsubscript{2} inhibition by antisense oligonucleotides and 1 \textmu mol/L JTE013. Inhibition of the cystic fibrosis transmembrane regulator (CFTR) (1) restored S1P responses that were attenuated by SPP1\textsuperscript{wt} overexpression; (2) enhanced myogenic vasoconstriction; but (3) had no effect on noradrenaline responses. We conclude that SPP1 is an endogenous regulator of resistance artery tone that functionally antagonizes the vascular effects of both Sk1\textsuperscript{wt} and S1P\textsubscript{2} receptor activation. SPP1 accesses extracellular S1P pools in a manner dependent on a functional CFTR transport protein. Our study assigns important roles to both SPP1 and CFTR in the physiological regulation of vascular tone, which influences both tissue perfusion and systemic blood pressure. (Circ Res. 2008;103:315-324.)

Key Words: Ca\textsuperscript{2+} sensitization ■ signal transduction ■ transfection ■ vascular smooth muscle ■ myogenic response ■ cystic fibrosis transmembrane regulator

The bioactive sphingolipid metabolite sphingosine-1-phosphate (S1P) is a potent regulator of diverse biological processes, acting both as an intracellular second messenger and as an extracellular receptor ligand. Intracellular S1P (ie, second messenger) has been shown to mediate mitogenic/antiapoptotic\textsuperscript{1} and Ca\textsuperscript{2+}-mobilizing responses,\textsuperscript{2} although the mechanisms controlling the latter remain undefined. As an extracellular receptor ligand, S1P can activate small GTPases (eg, RhoA and Rac), phospholipase C and adenylate cyclase via five distinct, G protein–coupled S1P receptors (S1P\textsubscript{1-5}) (formerly known as EDG family receptors\textsuperscript{3}). Growing evidence supports an important role for S1P in the development and homeostasis of the vascular system,\textsuperscript{4-6} in the pathogenesis of vascular diseases,\textsuperscript{7} and in the regulation of acute vascular responses.\textsuperscript{8} Our laboratory has recently identified the S1P-generating enzyme sphingosine kinase 1 (Sk1) as a vascular smooth muscle cell (SMC) signaling component necessary for myogenic vasoconstriction in resistance arteries. Alterations in endogenous S1P formation, through transfection of resistance arteries with sphingosine kinase (Sk1\textsuperscript{wt}) or its dominant-negative mutant Sk1\textsuperscript{G232}, substantially affected resting tone, Ca\textsuperscript{2+} sensitivity, and Ca\textsuperscript{2+} handling.\textsuperscript{8} This suggests that S1P may be an important regulator of blood pressure and tissue perfusion, because these arteries originate from the primary site of pressure regulation.

Because of its pleiotropic effects,\textsuperscript{9} S1P bioavailability must be tightly regulated in a spatiotemporal manner. The formation and degradation of S1P comprises a dynamic equilibrium. Although the importance of S1P synthesis is evident, little is known about the biological significance of S1P degradation. Because perturbations in S1P metabolism result in prominent effects on microvascular tone and Ca\textsuperscript{2+} sensitivity,\textsuperscript{8} from a functional standpoint, S1P degradation may be as important as S1P synthesis.
We hypothesized that in isolated resistance vessels, the S1P-degrading enzyme SPP1 acts as a functional antagonist of Sk1. We focused on SPP1 for several reasons: (1) to be a "functional antagonist," the S1P-degrading enzyme should yield only the original substrate of Sk1 (ie, sphingosine) and not other metabolites that could have bioactive effects (such as those produced by S1P lyase); (2) the enzyme should not have broad substrate specificity, like lipid phosphohydrolases (LPPs), so that the effects are relatively limited to S1P metabolism; and (3) although both SPP1 and SPP2 fit these 2 metabolic criteria, only SPP1 has been localized to the plasma membrane, which would be potentially significant with respect to regulating the availability of S1P for ligation to its cell surface receptors.

Our primary objectives were as follow: to (1) confirm mRNA expression of SPP1 in isolated resistance arteries; (2) assign a tone-regulating role to SPP1; (3) validate SPP1 as a functional antagonist of Sk1; and (4) explore the mechanism by which SPP1 accesses the separate intra- and extracellular S1P pools.

Materials and Methods

Isolation and Transfection of Resistance Arteries

Animal care and experimental protocols were conducted in accordance with German and Canadian federal animal protection laws. We have previously described the procedure for resistance artery isolation and culture. Briefly, hamster gracilis muscle resistance artery segments (181±7 μm in diameter, ~1 mm in length, n=60) were carefully dissected and cannulated under sterile conditions, adjusted to their in vivo lengths, set to a transmural pressure of 45 mm Hg and transfected under culture conditions (using Effectene, Qiagen, Hilden, Germany) for 20 hours. To monitor vessel transfection efficacy, routine spot tests using a green fluorescent protein (GFP) construct, RT-PCR analysis, and immunolabeling of the c-myc tag were conducted.

All experiments were conducted at a transmural pressure of 45 mm Hg; pressure-induced responses were induced by a step change to 100 mm Hg. Before experimentation, resistance artery SMCs were loaded with Fura-2/acetoxymethyl ester (2 μmol/L, 2 hours, Invitrogen; Karlsruhe, Germany). Vessel viability at the start and completion of experiments was assessed by initiating vasomotor responses with 0.3 μmol/L noradrenaline followed by 1 μmol/L acetylcholine. Vessels that did not robustly constrict to noradrenaline (>30%) or fully dilate to acetylcholine were deemed to be damaged and excluded. Maximum vessel diameter was determined under depolarizing conditions (125 mmol/L KCl) in Ca²⁺-free buffer solution. As previously detailed, the SMC intracellular Ca²⁺ concentration (Delta Scan, Photomed, Seefeld, Germany) and outside diameter were determined simultaneously.

Isolation of mRNA From Resistance Vessels, RT-PCR, and SPP1 Sequencing

The procedures for mRNA isolation, RT-PCR, and sequencing can be found in the online data supplement at http://circres.ahajournals.org. A list of primers used in the present study is also included in this supplement.

SPP1\textsuperscript{12,20,83} Plasmid Construction

The pcDNA3(c-myc)-SPP1wt vector has been previously described. The QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, Calif) was used to prepare a SPP1 construct with a histidine (H2O8) to alanine mutation (SPP1\textsuperscript{20,83}). Mutagenesis was verified by DNA sequencing. Catalytic inactivity of SPP1\textsuperscript{12,20,83} was confirmed by measuring S1P phosphohydrolase activity in membrane fractions isolated from transfected HEK293 cells as previously described.

Calculation of Tone and Reversal of Initial Distension

All values of tone represent acute diameter measurements that have been normalized; the values represent the magnitude of vessel constriction relative to maximal diameter. The computation of tone is as follows: tone (% of diamax) = (diamax – diamax
\textsubscript{resting})/diamax \times 100.

Myogenic vasoconstriction was initiated by a stepwise change in transmural pressure from 45 to 110 mm Hg. The immediate passive distension of the vessel from its resting diameter is reversed by a continuous, active vasoconstriction. The "reversal of initial distension" (ie, the magnitude of the myogenic response) is calculated as the percentage of constriction compared to the initial distension and is computed as follows: reversal of initial distension (%) = (diamax – diamax
\textsubscript{resting})/(diamax
\textsubscript{resting} – diamax
\textsubscript{resting}) \times 100, where diamax
\textsubscript{resting} is the diameter immediately preceding the pressure step, diamax is the distended diameter measured immediately following the pressure step, and diamax
\textsubscript{resting} is the diameter measured 7 minutes following the pressure step, an arbitrary time point where the constriction is normally stable.

Statistics

Functional experiments were conducted under blinded conditions (ie, the transfected construct was not known to the experimenter until after the measurement of functional parameters). Data represent means±SEM for n experiments. Experimental groups were compared using an unpaired Student t test; when multiple groups were analyzed, an ANOVA followed by a Bonferroni correction was used. Experiments involving JTE013 and CFTR(inh)-172 were analyzed as paired or repeated measures datasets. Differences were considered significant at an error probability of P<0.05.

Results

SPP1 Is Expressed in Resistance Arteries of the Hamster

The RT-PCR primers for SPP1 amplified a sequence from hamster gracilis muscle resistance artery homogenates with the expected size of 574 bp (online data supplement) and 92% homology with the comparable region of mouse SPP1 mRNA. Based on this amplified sequence, which is predicted to be 50 bp downstream of the translation initiation site, an antisense oligonucleotide was generated (5’-TGTGTCTCCTCGGGATGTG-3’). A Cy5-conjugated version of the oligonucleotide was observed to be effectively delivered into the SMCs of resistance arteries (data not shown).

SPP1\textsuperscript{wt} Modulates Resting Tone and Intracellular Ca²⁺ in Resistance Arteries

Transfection of the SMCs of resistance arteries (data not shown).

SPP1\textsuperscript{wt} significantly reduced resting tone by 57%, compared to GFP-transfected arteries (n=6; Figure 1A). This decrease in tone was associated with a 25% decrease in resting Ca²⁺i (n=6; Figure 1B). In contrast, knockdown of endogenous SPP1 expression, via transfection of antisense oligonucleotides, enhanced resting tone by 34% compared to GFP-transfected vessel (n=6; Figure 1A); the resting Ca²⁺i concentration, however, was not significantly altered compared to GFP-expressing arteries (Figure 1B). Maximal vessel diameter (diamax
\textsubscript{resting}) was not different in any of the treatment groups (diamax
\textsubscript{resting,GFP}=191±15 μm, n=6; diamax
\textsubscript{resting,SPP1\textsuperscript{wt}}=196±6 μm, n=6; diamax
\textsubscript{resting,asSPP1}=190±8 μm, n=6; P>0.05). Modulation of SPP1 activity did not significantly affect vasomotor responses to 0.3 μmol/L noradrenaline (GFP: 52±3%; SPP1\textsuperscript{wt}: 47±5%, asSPP1: 49±6%) or subsequent
complete dilation in all three groups following 1 μmol/L acetylcholine.

Forced expression of SPP1\textsuperscript{wt} could have effects independent of its S1P-degrading actions. To examine this possibility, resistance arteries were transfected with a mutant form of SPP1 (SPP1\textsuperscript{H208A}), which is catalytically inactive (see measurements of phosphohydrolase activity in transfected HEK293 cells in Figure 4A).

Expression of SPP1\textsuperscript{H208A} had no significant effect on resting tone (Figure 4C; Con diam\textsubscript{max}=239±8 μm, SPP1\textsuperscript{H208A} diam\textsubscript{max}=253±14 μm; P>0.05), Ca\textsuperscript{2+}/i (control: 1.04±0.04; SPP1\textsuperscript{H208A}: 0.96±0.09; P>0.05), vasomotor responses to noradrenaline (control: 48±4; SPP1\textsuperscript{H208A}, 51±3% P>0.05), or subsequent complete dilation to acetylcholine.

### SPP1\textsuperscript{wt} Modulates Ca\textsuperscript{2+} Sensitivity and Myogenic Vasoconstriction in Resistance Arteries

Exogenous and endogenous S1P has been reported to enhance both VSMC contractile apparatus Ca\textsuperscript{2+} sensitivity and myogenic vasoconstriction.\textsuperscript{5,18} Consistent with these reports, a reduced diameter/Ca\textsuperscript{2+} relationship (constriction in response to increased extracellular Ca\textsuperscript{2+} [Ca\textsuperscript{2+}/ex]) was observed in resistance vessels transfected with S1P-degrading enzyme SPP1\textsuperscript{wt} (n=6; Figure 1C). For each Ca\textsuperscript{2+}/ex concentration, the measured Fura-2 fluorescence ratio (ie, correlate of Ca\textsuperscript{2+}/i) was not significantly different between GFP- and SPP1\textsuperscript{wt}-transfected arteries (data not shown). Transfection of resistance vessels with antisense oligonucleotides against SPP1 did not affect the diameter/Ca\textsuperscript{2+} relationship (n=6; Figure 1C).

Myogenic vasoconstriction in response to increased transmural pressure (from 45 to 110 mm Hg) was significantly reduced (by 69%) in resistance arteries transfected with SPP1\textsuperscript{wt} (n=6; Figure 2). Accordingly, myogenic vasoconstriction was enhanced (by 46%) following transfection of antisense oligonucleotides against SPP1 (n=6; Figure 2). Neither the amplitude nor the kinetics of the pressure-induced increase in intracellular Ca\textsuperscript{2+} associated with myogenic vasoconstriction was affected by either overexpression or downregulation of SPP1 (data not shown). Expression of the catalytically inactive mutant SPP1\textsuperscript{H208A} did not significantly affect myogenic responses (Figure 4B) or the diameter/Ca\textsuperscript{2+} relationship (apparent Ca\textsuperscript{2+} sensitivity; Figure 4C).

### S1P\textsubscript{2} Is the Predominant Receptor Mediating the Effects of Exogenous S1P

We have previously shown that exogenous S1P induces vasoconstriction that is primarily mediated through activation of the RhoA/Rho kinase pathway.\textsuperscript{14} RhoA is activated by at least 2 of the 5 identified S1P receptors, namely S1P\textsubscript{2} and S1P\textsubscript{3} (formerly known as Edg5 and Edg3).\textsuperscript{19} Hamster resistance artery homogenates possessed mRNA encoding S1P\textsubscript{2} but not S1P\textsubscript{3} (data not shown; primer efficacy was verified in hamster lung homogenates). Based on this observation, sense and antisense oligonucleotides against the S1P\textsubscript{2} receptor were generated (antisense: 5’-CTCTTCTAGCAGCAGATTATGT-3’; sense: 5’-ACATAATGCTTGGCGTAGAGAG-3’). Transfection of resistance arteries with S1P\textsubscript{2} antisense oligonucleotides had no effect on basal tone (diam\textsubscript{max}; S1P\textsubscript{2}=-

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**Figure 1.** A. Enhanced expression of SPP1 in SMCs of resistance arteries (via transfection of SPP1\textsuperscript{wt}; n=6) significantly reduced resting tone. Accordingly, reduction of SPP1 expression (via antisense oligonucleotides; n=6) significantly increased resting tone. Maximal vessel diameters were not significantly different between any of the treatment groups. B. Transfection of resistance arteries with SPP1\textsuperscript{wt} significantly reduced basal SMC Ca\textsuperscript{2+} (Fura-2 method, ratio $F_{340 \text{~nm}}/F_{380 \text{~nm}}$). Treatment with antisense oligonucleotides directed against SPP1 had no effect on resting Ca\textsuperscript{2+}. C. Constriction of depolarized resistance arteries (120 mmol/L K\textsuperscript{+}) in response to increasing extracellular Ca\textsuperscript{2+} (Ca\textsuperscript{2+}/ex from 0 to 3 mmol/L) was significantly attenuated following transfection of SPP1\textsuperscript{wt} (n=6). Transfection of antisense oligonucleotides against SPP1 had no significant effect on the diameter/Ca\textsuperscript{2+}/ex relationship (asSPP1) (n=6). *P<0.05 compared to arteries transfected with GFP.
SPP1<sup>wt</sup> Modulates the Vasoconstricting Effects of Exogenously Applied S1P

To assess whether SPP1 accesses extracellular S1P pools, vasoconstriction in response to exogenous S1P (<0.1 nmol/L to 10 μmol/L) was monitored following modulation of SPP1 activity. Transfection of resistance arteries with SPP1<sup>wt</sup> significantly attenuated vasoconstriction in response to exogenous S1P; of note, complete abolition of vasoconstriction was observed at the lower, physiological concentration range of exogenous S1P (≤100 nmol/L; Figure 3C). Conversely, significantly enhanced S1P-mediated vasoconstriction was observed in resistance arteries transfected with SPP1 antisense oligonucleotides (Figure 3C). Responses to exogenously applied S1P were

205±8 μm, asS1P<sub>2</sub>=201±8 μm, n=6, *P<0.05; resting diameter: sS1P<sub>2</sub>=190±9 μm, asS1P<sub>2</sub>=196±9 μm, n=6, *P>0.05) or resting Ca<sup>2+</sup> (R<sub>440 nm/380 nm</sub>: sS1P<sub>2</sub>=0.80±0.04, asS1P<sub>2</sub>=0.73±0.03, n=6, *P>0.05). However, vasoconstriction to exogenous S1P (1 nmol/L to 10 μmol/L) was significantly reduced compared to the control group transfected with sense oligonucleotides (n=6; Figure 3A). Oligonucleotide treatment did not significantly affect vasmotor responses to noradrenaline or subsequent complete dilation to acetylcholine, compared to GFP controls (data not shown).

By using the chemical S1P<sub>2</sub> inhibitor JTE013 (1 μmol/L; 30 minutes), we also used a second, mechanistically distinct inhibitory strategy to substantiate our antisense oligonucleotide results. The inhibitory profile for JTE013 was similar to that observed for the S1P<sub>2</sub> antisense oligonucleotide treatment: attenuated vasoconstrictor responses to exogenous S1P, with no effect on resting tone (Figure 3B; diamax=241±16 μm). Taken together, the results suggest that S1P indeed acts as an extracellular ligand (S1P<sub>2</sub>-dependent) within the concentration range of 1 to 100 nmol/L.

Figure 3. A, Sphingosine-1-phosphate–induced constriction was significantly attenuated in resistance arteries treated with antisense oligonucleotides against S1P<sub>2</sub> (asS1P<sub>2</sub>, n=6) compared to arteries treated with sense oligonucleotides (sS1P<sub>2</sub>, n=6). *P<0.05. B, Dose-dependent responses to exogenous S1P were reduced following treatment with the S1P<sub>2</sub> blocker JTE013 (1 μmol/L, n=6). *P<0.05. C, Constriction induced by exogenous S1P was reduced in resistance arteries transfected with SPP1<sup>wt</sup> (n=6). Accordingly, treatment of resistance arteries with antisense oligonucleotides against SPP1 (asSPP1) (n=6) enhanced constriction to exogenous S1P. *P<0.05 compared to arteries transfected with GFP.
not affected by forced expression of the catalytically inactive mutant SPP1H208A (Figure 4D).

**SPP1**<sup>wt</sup> Antagonizes Enhanced Vascular Responsiveness in Sk1<sup>wt</sup>-Overexpressing Resistance Arteries

The vascular effects of SPP1<sup>wt</sup> in resistance arteries are opposite to those of Sk1<sup>wt</sup>, suggesting the existence of functional antagonism between these 2 enzymes that could tightly control S1P bioavailability within the microvascular wall. To prove that SPP1<sup>wt</sup> can effectively antagonize the tone-enhancing effects of Sk1, both enzymes were coexpressed in the SMCs of intact resistance arteries. Arteries expressing Sk1<sup>wt</sup> together with the phosphatase-dead SPP1 mutant (SPP1H208A) served as controls. Functional tests showed that the myogenic response in Sk1<sup>wt</sup>/SPP1<sup>wt</sup>-coexpressing arteries was (1) significantly weaker compared to arteries coexpressing Sk1<sup>wt</sup> and SPP1<sup>H208A</sup>, and (2) not statistically different compared to control arteries expressing GFP (Figure 5). Vasoconstriction in response to 0.3 μmol/L noradrenaline (Sk1<sup>wt</sup>/SPP1<sup>wt</sup>=49±1%, diamax=187±6 μm, n=5; Sk1<sup>wt</sup>/SPP1<sup>H208A</sup>=53±1%, diamax=184±4 μm, n=7; all values P>0.05 compared to GFP) or exogenous S1P (0.3 nmol/L to 30 μmol/L; Figure 5) was significantly reduced in Sk1<sup>wt</sup>/SPP1<sup>wt</sup>-coexpressing arteries, compared to Sk1<sup>wt</sup>/SPP1H208A expressing arteries. The diameter/Ca<sup>2+</sup> relationship (apparent Ca<sup>2+</sup> sensitivity) was largely the same in Sk1<sup>wt</sup>/SPP1<sup>wt</sup>- and Sk1<sup>wt</sup>/SPP1H208A-expressing arteries, except for 2 of the lower Ca<sup>2+</sup> concentrations tested (Figure 5). Initially, resting tone did not differ between the 2 groups (Figure 5B). Following depolarization, which activates Sk1,20 the new steady-state resting tone was significantly weaker in arteries coexpressing Sk1<sup>wt</sup>/SPP1<sup>wt</sup> compared to those coexpressing Sk1<sup>wt</sup>/SPP1<sup>H208A</sup> (Figure 5C).

![Figure 4](http://circres.ahajournals.org/)
CFTR(inh)-172 Restores S1P Responses in SPP1wt-Transfected Vessels

Our data strongly suggest that SPP1 degrades extracellular S1P. This would require either localization at the plasma membrane or a transport mechanism that shuttles S1P to the intracellular location of SPP1 (smooth endoplasmic reticulum)

(1). Our attempts to localize SPP1 to the plasma membrane, via confocal imaging and fractionation of cellular lysates, were inconclusive (data not shown). The alternate scenario (transport) is supported by recent reports that identify the cystic fibrosis transmembrane conductance regulator (CFTR) (also known as ATP-binding cassette subfamily C member 7 [ABCC7]) as a transporter of S1P.

Using primers targeting conserved regions in rat, mouse and human CFTR, we detected mRNA transcripts in hamster gracilis muscle resistance arteries (Figure 6A). To establish a link between CFTR and S1P transport in resistance arteries, we used the reversible and highly specific inhibitor, CFTR(inh)-172, at a concentration comparable to the reported K, for chloride transport (100 nmol/L).

Resting tone was unaffected by CFTR inhibition (con = 12 ± 2%, CFTR(inh)-172 = 11 ± 2%; n = 16, P > 0.05, diameter = 293 ± 13 μm). As previously shown in Figure 3C, vessels transfected with SPP1wt displayed reduced vasoconstriction at lower doses of exogenous S1P. Inhibition of CFTR in SPP1wt-transfected vessels restored S1P responses within the physiological concentration range (≤ 100 nmol/L; Figure 6B). Importantly, CFTR inhibition had no effect on vasomotor responses to noradrenaline (Figure 6C). CFTR(inh)-172 also reversibly enhanced myogenic vasoconstriction and the pressure-induced elevation of intracellular Ca2+ levels (Figure 7).

Discussion

There is accumulating evidence that the S1P signaling pathway is a prominent determinant of resting and pressure-induced myogenic microvascular tone and, hence, involved in several vascular pathologies. Although a link between Sk1-mediated synthesis of S1P in SMCs and the control of vascular tone has been established, potential antagonistic pathways that degrade S1P have yet to be investigated. In this regard, we are the first to characterize SPP1 as a negative regulator of resting and myogenic tone in resistance arteries.

We were able to confirm mRNA expression of SPP1 in hamster resistance arteries using RT-PCR and subsequent sequencing. The amplified transcript possessed a high degree of similarity to the published rat, human and mouse SPP1 coding sequences. We attempted to confirm SPP1 protein expression by using both commercial and custom-made antibodies against SPP1; however, both antibodies failed to detect the hamster epitope in protein lysates from arteries, lung, liver and heart (data not shown). We found that the same was true for a commercial antibody against the S1P2 receptor.

The inability to detect hamster SPP1/S1P2 prevents the direct assessment of (1) endogenous protein expression; (2) knockdown of the native protein by antisense oligonucleotides; and (3) in the case of SPP1, elevated protein expression following transfection with plasmids. Because the SPP1...
constructs include a c-myc label, we confirmed their expression via immunohistochemistry (online data supplement). We also confirmed that the antisense oligonucleotides were effectively delivered into the intracellular compartment by our transfection method (via fluorescent labels on the nucleotides). At this time, however, protein expression reductions following antisense oligonucleotide treatment can only be inferred by the functional effects (Figures 1 through 3). The fact that the antisense oligonucleotides displayed prominent functional effects, whereas 5 other oligonucleotides (S1P1 and S1P, antisense/sense and a S1P2 sense) had no effect, minimizes the concern that our results are attributable to nonspecific effects and supports the conclusion that the respective protein levels were indeed reduced.

Consistent with its proposed role in regulating intramural S1P levels, elevated expression of SPP1wt abolished vasoconstriction to exogenous S1P at concentrations of \(10^{-9}\) and \(10^{-8}\) mol/L and significantly attenuated vasoconstriction to \(10^{-7}\) mol/L S1P. Because S1P-mediated vasoconstriction is dependent on RhoA/Rho kinase in this type of artery,14 we propose that a cell surface receptor primarily mediates the response. In this regard, suppression of S1P2 expression (but not S1P1 and S1P3; data not shown) and blockade of the S1P2 receptor with the specific inhibitor JTE013 abolished vasoconstriction within the physiological concentration range of exogenously applied S1P (from \(10^{-9}\) to \(10^{-7}\) mol/L; Figure 3).

Based on its ability to regulate extracellular S1P levels, we propose that SPP1 possesses functional effects on vascular tone that are counteractive to those previously observed for Sk1wt.8,18 Consistent with this hypothesis, elevated expression of SPP1 (via transfection of the SPP1wt construct) induced similar functional effects to those previously associated with inhibition of Sk1, including reduced resting tone and basal Ca\(^{2+}\), attenuated myogenic vasoconstriction and decreased apparent Ca\(^{2+}\) sensitivity.8 Expression of a phosphatase-dead mutant of SPP1 (SPP1H208A) in resistance arteries did not affect any of these parameters, excluding proteomic effects of SPP1wt protein overexpression unrelated to its S1P-degrading function.

Although the effects of SPP1wt expression support the notion that SPP1 antagonizes Sk1 function, these results do not address whether endogenous SPP1 levels regulate S1P bioavailability under normal physiological conditions. We investigated this question by studying the effect of inhibited SPP1 protein expression/function. Notably, inhibition of SPP1 function via antisense oligonucleotides produced the opposite effect compared to SPP1wt expression: elevated resting tone, enhanced myogenic vasoconstriction and increased constriction to exogenous S1P. Together, these results strongly support the conclusion that SPP1 is a potent and physiologically relevant regulator of S1P bioavailability in hamster resistance arteries.

Decreased SPP1 expression would have been expected to increase Ca\(^{2+}\) sensitivity attributable to increased levels of extracellular S1P. Indeed, Figure 1A shows that basal tone is increased, despite unaltered resting Ca\(^{2+}\); however, no leftward shift in the diameter/Ca\(^{2+}\) relationship was observed (Figure 1C). This may represent a methodological limitation whereby measurement of S1P-induced increases in Ca\(^{2+}\)

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**Figure 6.** A, RT-PCR using homogenates of resistance arteries isolated from the gracilis muscle revealed vascular expression of CFTR mRNA. GAPDH acted as a control for PCR conditions. B, In SPP1wt-transfected vessels, dose-dependent responses to exogenous S1P were enhanced following treatment with the specific CFTR inhibitor CFTR(inh)-172 [100 nmol/L, 30 minutes; \(n=6\)]. C, In noncultured vessels, dose-dependent responses to noradrenaline were not affected CFTR inhibition [100 nmol/L CFTR(inh)-172, 30 minutes; \(n=4\)]. *\(P<0.05\) comparing pre- and post-CFTR inhibition.
sensitivity are compromised by the intrinsic sensitivity of Sk1 to depolarizing stimuli. The depolarizing conditions required to clamp intracellular Ca\(^{2+}\) (via extracellular Ca\(^{2+}\)) may activate Sk1\(^{20}\) and stimulate the release of S1P (or activate other Ca\(^{2+}\)-sensitizing mechanisms) such that measurement occurs under conditions of near-maximal Ca\(^{2+}\) sensitivity. This is in accordance with one of our previous observations, where exogenously applied S1P also failed to left shift the Ca\(^{2+}\)-sensitivity curve under these specific experimental conditions.\(^{18}\)

Although our data strongly suggest that SPP1 is a prominent “negative” regulator of S1P bioavailability within the microvascular wall, these data alone do not establish SPP1 as a functional antagonist of Sk1, because resolving the spatio-temporal characteristics of both enzyme activities (a prerequisite for functional antagonism) is required. We performed SPP1\(^{wt}\)/Sk1\(^{wt}\) cotransfection experiments to provide indirect functional evidence for spatiotemporal overlap. We expected that the coexpression of SPP1\(^{wt}\) would attenuate the documented proconstrictive effects of Sk1\(^{wt}\) overexpression.\(^{23}\) Indeed, SPP1\(^{wt}\) coexpression “normalized” myogenic vasoconstriction (ie, not different from vessels expressing GFP) in vessels where Sk1\(^{wt}\) overexpression alone would have augmented this physiological response.\(^{23}\)

Independent parameters rule out insufficient expression of one or both enzymes in this experimental series as a cause for observed “control-like” myogenic responses: (1) consistent with arteries expressing SPP1\(^{wt}\) alone (Figure 3C), arteries coexpressing SPP1\(^{wt}\) and Sk1\(^{wt}\) also displayed attenuated responsiveness to exogenous S1P; and (2) arteries coexpressing Sk1\(^{wt}\) and SPP1\(^{H208A}\) display an augmented myogenic response, similar to that observed in vessels expressing Sk1\(^{wt}\) alone.\(^{23}\) A possible inhibition of Sk1\(^{wt}\) activity or expression resulting from SPP1 coexpression is unlikely to cause the normalized myogenic response in Sk1\(^{wt}/\)SPP1\(^{wt}\) coexpressing arteries, because such a situation would resemble the single SPP1\(^{wt}\) expression (ie, reduced myogenic response).

Taken together, our functional data suggest that SPP1 effectively degrades newly synthesized S1P before it binds to cell surface receptors and promotes vasoconstriction. To do so, SPP1 would need to be either positioned close to the S1P receptors (ie, outer leaflet of plasma membrane), or in close proximity to the plasma membrane where transmembrane S1P transport occurs. With regard to our results (Figure 3C), plasma membrane localization is the most simplistic explanation for how SPP1 could regulate extracellular S1P. However, our attempts to localize transfected SPP1 in resistance artery vascular SMCs via immunolocalization (staining for the c-myc motif of transfected SPP1\(^{wt}\)) and fluorescence protein tagging did not provide convincing evidence for or against plasma membrane localization (data not shown).

Figure 7 (Continued). 110 mm Hg was reversibly enhanced following treatment with CFTR\((inh)-172 (n=6). C, The amplitude of the pressure-induced Ca\(^{2+}\) elevation following a pressure step from 45 to 110 mm Hg was reversibly enhanced following treatment with CFTR\((inh)-172 (n=5). *P<0.05 compared to arteries transfected with GFP.
In fact, the majority of reports in the literature strongly support an endoplasmic reticulum localization of SPP1, and only one study, where SPP1 was overexpressed in HEK293 cells, has reported a small proportion of SPP1 (less than 10%) localized to the plasma membrane.12 It is the close proximity of the smooth endoplasmic reticulum and plasma membrane in SMCs (e.g., the endoplasmic reticulum and plasma membrane can be separated by as little at 20 nm12) that makes the discrete subcellular localization of SPP1 in intact arteries technically challenging for optically based methods.

The close proximity of the smooth endoplasmic reticulum to the plasma membrane24 could serve as the structural basis for a putative S1P transport-degradation mechanism. The proteins that transport S1P across the plasma membrane are largely unknown. To date, only 2 transporters, which belong to the highly conserved ATP-binding cassette (ABC) family, have been shown to translocate S1P: ABCC125 and ABCC7 (CFTR).21 Because ABCC1 unidirectionally exports intracellular S1P,25 CFTR is the only known candidate that could shuttle extracellular S1P to intracellularly localized SPP1. Indeed, Boujaoude et al have demonstrated that CFTR transports extracellular S1P across the plasma membrane. Furthermore, in their model system, CFTR-mediated transport of S1P diverted it from interacting with cell surface receptors and consequently modulated S1P receptor-dependent effects.21

We therefore hypothesized that CFTR shuttles extracellular S1P to intracellularly localized SPP1 in our experimental model, thereby sequestering S1P from the S1P3 receptor and ultimately attenuating the proconstrictive effects of S1P. In accordance with this hypothesis, CFTR inhibition restored S1P responses in arteries expressing SPP1w26 (SPP1 overexpression). This effect appeared confined to the physiologically relevant S1P concentration range (Figure 6B) and was specific to S1P, because vasomotor responses to noradrenaline were unaffected (Figure 6C). Consistent with its tone-enhancing effect on exogenous S1P responses, CFTR inhibition enhanced myogenic vasoconstriction and the pressure-induced Ca2+ elevation (Figure 7). Intriguingly, these effects are remarkably similar to those observed for resistance arteries overexpressing Sk1,26 suggestive of increased S1P availability.

Taken together, our data support the hypothesis that CFTR transports S1P across the plasma membrane of resistance artery SMCs for degradation by SPP1. This represents the first proposed mechanistic explanation for recent observations that CFTR is involved in the regulation of vascular tone.26 It should be noted that CFTR is traditionally regarded as a chloride transporter (specifically, chloride secretion). CFTR inhibition, therefore, may have several effects, including a possible impact on membrane potential (a determinant of vascular tone). Because the inhibition of chloride export would result in hyperpolarization and consequently vasodilation, if there are membrane potential-related effects resulting from CFTR inhibition, this should cause us to underestimate the effects of CFTR inhibition with regard to S1P-stimulated and myogenic vasoconstriction.

In summary, we provide the first evidence that SPP1 contributes to the regulation of vascular tone in resistance arteries. Our data indicate that SPP1 can regulate extracellular S1P pools and therefore modulate extracellular S1P-mediated alterations in vascular tone. As an underlying mechanism, our data support a CFTR-dependent transport mechanism that shuttles extracellular S1P to the intracellular compartment, where it is degraded by SPP1. By acting as a functional antagonist to sphingosine kinase, SPP1 possesses a physiologically relevant role in the control of vascular and myogenic tone. We propose that, under normal physiological conditions, SPP1 contributes to the regulation of tissue perfusion, which also feeds back to systemic blood pressure.

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Disclosures

None.

References


Role of Sphingosine-1-Phosphate Phosphohydrolase 1 in the Regulation of Resistance Artery Tone
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Supplemental Materials and Methods

Materials

Sphingosine-1-phosphate (S1P) was purchased from Biomol (Hamburg, Germany); CFTR(inh)-172 from Sigma-Aldrich (Oakville, Canada) and JTE013 from Tocris Bioscience (Ellsville, USA). The MOPS-buffered salt solution contained [mmol/L]: NaCl 145, KCl 4.7, CaCl$_2$ 3.0, MgSO$_4$ $\cdot$ 7H$_2$O 1.17, NaH$_2$PO$_4$ $\cdot$ 2H$_2$O 1.2, pyruvate 2.0, EDTA 0.02, MOPS (3-morpholinopropanesulfonic acid) 3.0, and glucose 5.0. Culture medium was composed of Leibovitz L-15 base medium supplemented with 15% fetal calf serum (FCS), 0.4μmol/L L-glutamine, 20000U/L penicillin and 20mg/L streptomycin (all components from Sigma; Deisenhofen, Germany). The plasmids encoding the functional human SPP1 (SPP1$^{wt}$) 1,2 and Sk1 (Sk1$^{wt}$) 3,4 enzymes have been previously described.

A rabbit anti-human SPP1 polyclonal antibody (Exalpha Biologicals, Maynard, USA; cat#X1636P), a custom-made rabbit anti-mouse SPP1 polyclonal antibody (a generous gift from Dr. Richard Proia; NIH, Bethesda, USA) and a rabbit anti-human S1P$_2$ polyclonal antibody (Exalpha Biologicals; cat#X1636P) were tested using standard western blotting techniques and protein lysates from hamster resistance arteries, lung, liver and heart. The blots did not display immunoreactive bands at the expected molecular weights for SPP1/S1P$_2$. Coomassie staining confirmed adequate protein loading.

Isolation of mRNA from resistance vessels, RT-PCR and SPP1 Sequencing

To isolate mRNA, 2-3 vessel segments were pooled, frozen (liquid nitrogen), quickly pulverized with a metal pestle and homogenized with a “Qiashredder” mini spin column (Qiagen; Hilden, Germany). The mRNA was then isolated from the resulting homogenate using the RNeasy Protect Kit (Qiagen).

One-step RT-PCR was completed using the “Titan One Tube System” (Roche; Penzberg, Germany). Gene-specific primers were designed for the detection of SPP1, S1P$_2$, S1P$_3$ and GAPDH mRNA (Online Table I). PCR was carried out as follows: Initially, 10 cycles, each containing 10s denaturation at 94°C, 30s annealing (see Online Table I for appropriate annealing temperature) and 45s primer extension at 68°C; followed by 25 cycles with the extension time increasing 5s per cycle.

PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized/photographed with a BioRad Gel Doc 1000 imaging system. The housekeeping gene
GAPDH served as a positive control for successful RT-PCR amplification. The SPP1 amplicon was directly sequenced with the “BigDye Terminator” ready reaction kit on an ABI 377 Prism automated sequencer (both from PE Applied Biosystems, Weiterstadt, Germany).

**Immunofluorescence and digital imaging**

Arteries, either non-transfected or transfected with the (c-myc)SPP1wt fusion protein construct, were harvested after a 21h culture/transfection period and fixed with 3.7% formaldehyde, permeabilized with 0.3% Triton X-100, blocked with 1% BSA and incubated with a mouse monoclonal anti-human c-myc antibody overnight at 4°C (1:200 dilution; Santa-Cruz Biotechnology, USA). Subsequently, an Alexa 488-labelled rabbit anti-mouse IgG secondary antibody was used (1:200 dilution; 90min at room temperature; Mobitec, Göttingen, Germany,). Images were obtained using a Zeiss LSM410 confocal microscope equipped with a Kr/Ar laser and a 63x/1.2W water immersion objective.
Online Figure I:

RT-PCR using homogenates of resistance arteries isolated from the gracilis muscle and cultured hamster aortic SMCs revealed vascular expression of SPP1 mRNA (expected size: 574bp). GAPDH acted as a control for PCR conditions (expected size: 700bp).
Online Figure II:

(A) Confocal immunolocalization of SPP1\textsuperscript{wt} (via antibodies against c-myc) in arteries transfected with the c-myc / SPP1\textsuperscript{wt} fusion protein construct. The confocal plane was positioned within the smooth muscle cell layer, where the individual cells are orientated perpendicular to the vessel lumen. Fluorescence was detectable throughout the intracellular compartment and also at or very near to the plasma membrane.

(B) Non-transfected arteries did not display intracellular fluorescence observed in (A). The confocal plane was positioned within the smooth muscle cell layer, where the individual cells are orientated perpendicular to the vessel lumen.
**Online Table I:** Primers Used for RT-PCR

<table>
<thead>
<tr>
<th>gene</th>
<th>primer sequence</th>
<th>annealing temp.</th>
<th>amplicon length</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPP1 (human)</td>
<td>P1: 5’-CTACTGCCTGTCTGCTTTG-3’&lt;br&gt;P2: 5’-TGTGTCTCCTCGGGGATGTG-3’</td>
<td>62 °C</td>
<td>574 bp</td>
</tr>
<tr>
<td>S1P₂ (rat)</td>
<td>P1: 5’-CTCTCTACGCCAAGCATTATGGTGCT-3’&lt;br&gt;P2: 5’-ATCTAGACCTCAGACCCCGGTGTTGCCCT-3’</td>
<td>65 °C</td>
<td>728 bp</td>
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<tr>
<td>S1P₃ (rat)</td>
<td>P1: 5’-TCAGGGAGGGGCAGATGTTC-3’&lt;br&gt;P2: 5’-CTGACTCTTGAAGAGGATGG-3’</td>
<td>65 °C</td>
<td>503 bp</td>
</tr>
<tr>
<td>GAPDH (hamster)</td>
<td>P1: 5’-TCTACCAGGCAAGTTCAAGGC-3’&lt;br&gt;P2: 5’-CAGGAGACACCTGGTCCTCGG-3’</td>
<td>60 °C</td>
<td>700 bp</td>
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<tr>
<td>CFTR (mouse)</td>
<td>P1: 5’-AAAGAAAATATCATCTTTTGTTGT-3’&lt;br&gt;P2: 5’-TATACTGCTTTGCTAAAGAAAT-3’</td>
<td>47 °C</td>
<td>182 bp</td>
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Reference list for Supplement


