Sphingosine Kinase Modulates Microvascular Tone and Myogenic Responses Through Activation of RhoA/Rho Kinase

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Background—RhoA and Rho kinase are important modulators of microvascular tone.

Methods and Results—We tested whether sphingosine kinase (Sphk1) that generates the endogenous sphingolipid mediator sphingosine-1-phosphate (S1P) is part of a signaling cascade to activate the RhoA/Rho kinase pathway. Using a new transfection model, we report that resting tone and myogenic responses of isolated resistance arteries increased with forced expression of Sphk1 in smooth muscle cells of these arteries. Overexpression of a dominant negative Sphk1 mutant or coexpression of dominant negative mutants of RhoA or Rho kinase together with Sphk1 completely inhibited development of tone and myogenic responses.

Conclusions—The tone-increasing effects of a Sphk1 overexpression suggest that Sphk1 may play an important role in the control of peripheral resistance. (Circulation. 2003;108:342-347.)

Key Words: microcirculation • muscle, smooth • arteries • genetics • vasoconstriction

Recent evidence suggests that the modulation of vascular smooth muscle Ca²⁺ sensitivity by RhoA is a major determinant of vascular tone in resistance arteries (RA) and hence an important factor in the control of systemic blood pressure. Increases in Ca²⁺ sensitivity result from a RhoA-mediated and Rho kinase–mediated inhibition of myosin light chain phosphatase (MLCP). The reduced MLCP activity leads to increased regulatory myosin light chain (MLC₂₀) phosphorylation at unchanged levels of intracellular Ca²⁺ and myosin light chain kinase activity. Different vasoconstrictors have been shown to exert their effects at least in part through activation of the RhoA signaling cascade. Furthermore, pharmacological inhibition of Rho kinase normalized blood pressure in three genetically different rat models of hypertension. For the RAs used in this study, we have previously demonstrated that Ca²⁺-sensitizing effects of exogenously applied oxidized LDLs (oxLDL) were also mediated by activation of RhoA and Rho kinase. The emerging importance of RhoA as a modulator of microvascular tone raises the question how the activity of the RhoA/Rho kinase pathway is regulated under physiological conditions, which mediators are involved, and whether dysfunction of this signaling pathway might contribute to enhanced contractile responses in RAs, thereby contributing to hypertension.

Because the mitogenic effects of oxLDL on pulmonary vascular smooth muscle cells (VSMC) were mediated by the sphingolipid metabolite sphingosine-1-phosphate (S1P), we speculated that S1P might also be involved in the RhoA-dependent vasoconstricting effects of oxLDL in RAs. S1P, generated by the enzyme sphingosine kinase (Sphk1), plays an important role in cell growth and survival, angiogenesis, and many other biological processes, particularly those related to cytoskeletal rearrangements regulated by activation of the Rho family. Most effects of S1P are mediated by a family of 5 highly specific G-protein–coupled receptors named S1PRs. S1P may also function as an intracellular second messenger to mobilize Ca²⁺ from intracellular stores independent of IP₃ formation by an as-yet unknown mechanism.

In skeletal muscle RAs used in this study, exogenous S1P potently induced concentration-dependent constrictions at constant Ca²⁺. Those were abolished after transfection of the RhoA-inhibiting protein C3 transferase into VSMCs of intact RAs, suggesting that they primarily resulted from an RhoA-dependent Ca²⁺ sensitization.

We studied whether S1P functions as an upstream modulator of the RhoA pathway within the microcirculation, with potential impact on the control of resting tone and the pressure-induced myogenic response (MR) that is of major importance for autoregulation. Since the MR significantly amplifies systemic vasoconstrictor effects and contributes up

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to two thirds of the increase in total peripheral resistance on increases in pressure,\textsuperscript{12} it directly affects systemic blood pressure.

It is still enigmatic how VSMC convert the mechanical stimulus stretch into intracellular signals, finally resulting in vasoconstriction.\textsuperscript{13} We hypothesized that S1P and RhoA/Rho kinase are integral parts of this process. Consistent with this hypothesis, increases in transmural pressure elicit rapid and sustained increases in resistance artery VSMC Ca\textsuperscript{2+} followed by vasoconstriction that progressively develops over several minutes, thereby reversing \textasciitilde50\% of the initial pressure-induced distension.\textsuperscript{14} As tone continuously increases without further increases in Ca\textsuperscript{2+}, Ca\textsuperscript{2+}-sensitizing mechanisms probably are involved. It is still unknown how these two mechanisms are orchestrated to allow for such a complex and highly reproducible vascular reaction as the MR. Here, endogenously released S1P, with its Ca\textsuperscript{2+}-increasing and RhoA-activating properties, could act as an integrating stimulus. The activity of the S1P-generating enzyme Sphk1 is increased by membrane depolarization and activation of voltage-gated Ca\textsuperscript{2+} channels.\textsuperscript{15} Both effects are known to be essential for MRs,\textsuperscript{11} leading us to hypothesize that Sphk1 is a part of the pressure-induced signaling cascade. To study a potential role of Sphk1 for this transduction pathway, we used a new transfection model to overexpress Sphk1\textsuperscript{16} and its dominant negative mutant hSK-G82D\textsuperscript{17} in VSMCs of isolated RAs. These experiments in genetically modified arteries demonstrated that Sphk1 is a major determinant of resting tone and an integral part of the signaling cascades regulating the pressure-induced myogenic constriction. Coexpression of dominant negative RhoA and Rho kinase mutants revealed that these actions are indeed mediated by activation of the RhoA/Rho kinase pathway.

Methods

Isolation of RAs and VSMC Transfection in Artery Culture

The preparation of the vessels, the technique of calcium and diameter measurements,\textsuperscript{18} and the artery culture and transfection method\textsuperscript{16} were previously described in more detail. Briefly, segments of small RAs (maximal diameter, 213±3 \textmu m; n=116) were excised from the gracilis muscle of hamsters, cannulated with micropipettes, and perfused with culture medium at a transmural pressure of 45 mm Hg. For selective transfection of VSMCs, 60 \mu g/mL transfection reagent (Effectene, Qiagen) and 5 \mu g of the respective DNA plasmid were added to the organ bath for 20 to 22 hours.

Transfection efficacy was assessed by using plasmids encoding for GFP or a Sphk-GFP fusion protein (Figure 1).

Plasmids

The plasmids encoding human Sphk1 and its dominant negative mutant hSK-G82D were described previously.\textsuperscript{17} The RhoA mutants N19RhoA and L63RhoA were a kind gift of Dr Alan Hall, University College London, UK; the plasmid coding for dominant negative Rho kinase was provided by Dr Shuh Narumiya, Department of Pharmacology, Kyoto University Faculty of Medicine, Sakyo, Kyoto, Japan.

Ca\textsuperscript{2+} and Diameter Measurements in Genetically Modified RAs

After termination of the culture period, RAs were washed with MOPS-buffered salt solution and transferred to an inverted microscope. Incubation with Fura-2 (2 hours at 37°C) from the abluminal side allowed selective determination of VSMC Ca\textsuperscript{2+} within the vascular wall. Simultaneous measurements of VSMC intracellular Ca\textsuperscript{2+} and diameter were performed as described previously.\textsuperscript{18} In all 116 arteries included in the study, VSMC and endothelial function was assessed by testing all RAs for their constrictor response to norepinephrine (NE, 0.3 \mu mol/L) and their dilator response to acetylcholine (ACH, 1 \mu mol/L). Arteries that did not show maximal dilation to ACh were excluded from the study (5 of 121 vessels). Measurements were performed at 37°C.

Calculation and Statistics

Tone was calculated as percentage of maximal diameter at 45 mm Hg, $\text{Tone} = \frac{(\text{Diameter}_{\text{rest}} - \text{Diameter}_{\text{max}})}{\text{Diameter}_{\text{rest}}}$, which was determined at the end of every experiment in Ca\textsuperscript{2+}-free solution under stimulation with 1 \mu mol/L ACh.

MRs were described as percentage of reversal of initial distension (RID), calculated as $\text{RID} = \frac{(\text{Diameter}_{\text{max}} - \text{Diameter}_{\text{rest}})}{\text{Diameter}_{\text{rest}}}$×100, with $\text{Diameter}_{\text{rest}}$ being the actual diameter of the artery at a given time point after increase in transmural pressure.

Graphic data displayed as changes in VSMC Ca\textsuperscript{2+} or diameter under pressure were described as percentage of change from resting Ca\textsuperscript{2+} or diameter, respectively. For Ca\textsuperscript{2+}: $\Delta\text{Ca}^{2+} = \frac{(\text{Ca}^{2+}_{\text{press}} - \text{Ca}^{2+}_{\text{rest}})}{\text{Ca}^{2+}_{\text{rest}}}\times100.$

The Student’s t test was used to compare steady-state values; differences were considered to be significant at error probabilities $<0.05$ ($P<0.05$).

To compare Ca\textsuperscript{2+} and diameter changes over time after increases in pressure, a nonlinear regression analysis was used. Briefly, the goodness of the fit to a Gompertz function was calculated for every
Results

Overexpression of Sphk1 Increases Resting Tone in RAs

Arteries transfected with green fluorescent protein (GFP, Figure 1c) or the fusion protein Sphk-GFP (Figure 1d) showed protein expression in virtually all VSMCs but not endothelial cells of the vascular wall (Figure 1). NE-induced (0.3 μmol/L) constriction by 51±3% and 49±4% of maximal diameter, respectively, and complete dilation after 1 μmol/L ACh revealed intact contractile and endothelial functions in these arteries.

However, RAs transfected with Sphk1 developed significantly higher resting tone (23±3%, diamax: 247±6 μm, n=12, Figure 1) than arteries transfected with GFP (10±1%, diamax: 228±7 μm, n=18, Figure 1), although levels of intracellular Ca2+ in both groups were not different.

To block endogenous generation of S1P, arteries were transfected with the dominant negative Sphk1 mutant hSK-G82D, which has previously been shown to inhibit agonist-stimulated S1P generation.17 hSK-G82D–transfected arteries developed no significant resting tone (2%, diamax: 247±5 μm, n=16, Figure 1), supporting the idea that Sphk1 plays a pivotal physiological role as a determinant of microvascular resting tone.

Figure 2. Kinetics of pressure-induced MRs of isolated resistance arteries transfected with GFP (n=8, a) or Sphk1 (n=12, b). Initial distensions elicited by increases in pressure from 45 to 110 mm Hg were partially reversed by a subsequent vasoconstriction that was significantly accelerated and augmented in arteries overexpressing Sphk1. Enforced expression of Sphk1 also augmented pressure-induced increases in VSMC Ca2+. Data were normalized to resting Ca2+ and diameter levels, respectively, and summarized every 10 seconds (symbols show mean±SEM).

Figure 3. Pressure-induced MRs in isolated resistance arteries after selective transfection of vascular VSMCs with mutants of Sphk1 (active: Sphk1, n=12; dominant-negative: hSK-G82D, n=18) alone or in combination with dominant negative mutants of RhoA (N19RhoA, n=8) or Rho kinase (KD1A, n=8). GFP-transfected arteries (n=8) served as controls, L63RhoA (n=8) genetically activated the RhoA/Rho kinase pathway. Bars depict steady-state levels of RID after 4 minutes (mean±SEM). *Significantly different (P<0.05) between different groups and GFP-transfected arteries; §differences to Sphk-transfected arteries.

RhoA/Rho Kinase Mediates Effects on Resting Microvascular Tone

To study a possible involvement of the RhoA/Rho kinase pathway, Sphk1 was coexpressed with dominant negative mutants of RhoA (N19RhoA) or Rho kinase (KD1A), respectively, to achieve highly specific inhibition of the RhoA pathway. Resting tone was almost abolished in RAs in which Sphk1 was coexpressed with N19RhoA (Sphk1+N19RhoA: 1.0±0.3%, diamax: 242±9 μm, n=8, Figure 1) or KD1A (Sphk1+KD1A: 2.8±1%, diamax: 239±8 μm, n=8, Figure 1).

Moreover, L63RhoA (dominant active RhoA, increase in tone by 22±2%, diamax: 223±8 μm, n=8, Figure 1) virtually mimicked the tone-increasing effect of Sphk1.

None of the genetic manipulations affected resting intracellular Ca2+ levels.

Role of RhoA/Rho Kinase for MR in RAs

The pronounced Ca2+-sensitizing effects we have previously demonstrated for RhoA/Rho kinase in RAs6 led us to hypothesize that activation of the RhoA pathway might also contribute to the initiation and development of the MR. In fact, MRs in GFP-transfected arteries (59±9% RID, n=8, Figures 2a and 3) that were comparable to those in freshly isolated or cultured arteries14 were virtually abolished in RAs overexpressing dominant negative mutants of RhoA (N19RhoA, 2±11% further distension, n=7) or Rho kinase (KD1A, 4±5% RID, n=6) or treated with the pharmacological Rho kinase inhibitor Y27632 (1±2% RID, n=10). Inhibition of the RhoA/Rho kinase pathway by these agents did not affect the pressure-induced increases in Ca2+ (N19RhoA: 16±3%, KD1A: 17±2%, Y27632: 19±3%). These pressure-induced increases in Ca2+ as well as subsequent constrictions in
control arteries were, however, completely inhibited by the L-type calcium channel blocker felodipine (1 nmol/L, n = 7).

Role of Sphk1 and S1P for MR
S1P (10 nmol/L) induced no significant constrictions but significantly augmented MRs in RAs (144 ± 11% RID, n = 8). This effect was blocked by the Rho kinase inhibitor Y27632 (3 ± 1% RID, n = 4) and absent in arteries transfected with the dominant negative RhoA mutant N19RhoA (2 ± 5% further distension, n = 3), suggesting that RhoA/Rho kinase activation mediated the modulation of the MR by exogenous S1P. This modulating effect was not confined to exogenous S1P, since forced expression of endogenous S1P-generating Sphk1 also significantly increased MRs (154 ± 14% RID, n = 12, versus 59 ± 9% for GFP, Figures 2b and 3). In contrast, MRs were only residual in arteries overexpressing the dominant negative Sphk1 mutant hSK-G82D (15 ± 5% RID, n = 16, P < 0.005). Augmented MRs in Sphk1-transfected arteries were associated with significantly higher initial pressure-induced increases in Ca\(^{2+}\) (maximal at 34 ± 3% after 40 seconds, n = 12, Figure 4) compared with GFP-transfected controls (20 ± 3% after 40 seconds, n = 8, P < 0.005, Figure 4). Genetic inhibition of Sphk1 with hSK-G82D significantly reduced initial increases in Ca\(^{2+}\) (9 ± 1% after 40 seconds, n = 16, P < 0.005, Figure 4), leaving a slow increase in intracellular Ca\(^{2+}\) that plateaued after 4 minutes. After 4 minutes, VSMC Ca\(^{2+}\) levels of all groups reached the same plateau level (19 ± 2% in Sphk1, 17 ± 2% in GFP and 17 ± 3% in hSK-G82D, all normalized to resting Ca\(^{2+}\) levels, Figure 4).

Stimulation of Sphk1-overexpressing arteries by repetitive increases in transmural pressure from 45 to 110 mm Hg over a period of 5 minutes decreased by 23 ± 3% of tone were prerelaxed with ACh (to 9 ± 3% RID, n = 8, Figure 3). Activation of the RhoA pathway alone, as achieved by transfection of the dominant active RhoA mutant L63RhoA, resulted in MRs of 92 ± 12% RID (n = 8, Figure 3), which, although greater than GFP-transfected (59 ± 9% RID, P < 0.005), were significantly smaller than in Sphk1-transfected (154 ± 14% RID, P < 0.005) arteries.

Effects of Initial Tone on the Strength of MRs
MRs were similar in GFP-transfected RA that either developed normal resting tone or that were preconstricted by 25 ± 2% (n = 5) to match the initial tone developed by Sphk1- and L63RhoA-overexpressing RA. Accordingly, MRs were not altered when Sphk1-overexpressing RA that normally develop 23 ± 3% of tone were prerelaxed with ACh (to 3 ± 1%, n = 5) to match resting tone of GFP-transfected RAs.

Effects of the Genetic Manipulations on Microvascular Contractility
To determine whether constrictions in transfected arteries were generally affected, constrictor responses to a single submaximal concentration of 0.3 μmol/L NE or to 120 nmol/L K\(^{+}\) were assessed in genetically altered RAs. Resulting NE-induced constrictions were similar in all groups.
(GFP: 51±3%, Sphk1: 51±2%, L63RhoA: 53±1%, Sphk1+KD1A: 51±2%, Sphk1+N19RhoA: 38±10%) except in those arteries that overexpressed hSK-G82D. They showed a marked reduction of NE-induced tone (27±6%, n=16, P<0.05).

Contractile responses to 120 mmol/L K+ were similar in GFP-, Sphk1-, L63RhoA-, and N19RhoA-overexpressing arteries (43±3%, 46±2%, 42±5%, and 45±3%).

Discussion
Early last century (1919), Bayliss described the intrinsic property of smooth muscle cells to react to stretch with an increase in tone. This effect is the basis for the autoregulation of blood flow by RAs that respond with a vasoconstriction to increases in transmural pressure (pressure-induced myogenic vasoconstriction), thereby providing “as far as possible for the maintenance of a constant flow of blood through the tissues supplied by them, whatever may be the height of the general blood pressure.”20 Furthermore, the MR directly affects systemic blood pressure and contributes up to two thirds of the increase in total peripheral resistance after increases in pressure.12

The results of this study suggest that activation of the RhoA pathway contributes to the initiation and development of the MR in RAs. Effects of RhoA/Rho kinase on the MR were independent of Ca2+, since pressure-induced increases in Ca2+ remained unaffected by any manipulation of the RhoA pathway.

However, the fact that the increases in Ca2+ and subsequent constrictions were completely inhibited by the L-type Ca2+ channel blocker felodipine suggested that both effects, the transmembrane influx of extracellular Ca2+ as well as the pressure-dependent increase in myofilament Ca2+ sensitivity, contribute to the initiation and development of myogenic vasoconstrictions.

It is still unclear how mechanisms that increase Ca2+ and those that sensitize the contractile apparatus to Ca2+ might interact, resulting in a highly reproducible reaction of arterial VSMCs to pressure.13 In this study, we introduce the concept that these two signaling pathways are not separately but rather simultaneously activated by S1P, allowing for their precise spatial-temporal interaction. Exogenous S1P significantly augmented MRs in RAs, an effect that was mimicked by overexpression of the S1P-generating enzyme Sphk1. Enhanced MRs in Sphk1-transfected arteries were associated with significantly higher initial pressure-induced increases in Ca2+ compared with GFP-transfected controls, whereas genetic inhibition of Sphk1 significantly reduced initial increases in Ca2+. These results suggest that Sphk1 has a modulatory effect on the initial increase in Ca2+, even under physiological conditions. Of note, VSMC Ca2+ levels of all groups plateaued on a similar level after 4 minutes. Corresponding myogenic vasoconstrictions, however, were stronger in Sphk1-overexpressing than in GFP-transfected RAs, and both were stronger than those in arteries that overexpressed the dominant negative Sphk1. This suggests that despite an effect on the early increase in Ca2+, a second calcium-sensitizing mechanism contributes to the overall response and that this is more active in Sphk1-expressing arteries than in GFP-transfected and inactive in hSK-G82D-transfected RAs.

Significantly inhibited myogenic vasoconstrictions in Sphk1-overexpressing arteries that coexpress dominant negative RhoA (N19RhoA) or Rho kinase (KD1A) mutants suggest that a Sphk1-dependent, S1P-mediated activation of the RhoA/Rho kinase pathway is an integral part of the MR. However, activation of the RhoA pathway alone, as achieved by transfection of the dominant active RhoA mutant L63RhoA, resulted in MRs which, although greater than GFP-transfected, were significantly smaller than in Sphk1-transfected arteries. This was different than under resting conditions in which Sphk1- and L63RhoA-overexpressing arteries showed virtually identical increases in tone, presumably due to sensitization of the contractile apparatus to Ca2+ at similar resting levels of Ca2+. In pressurized Sphk1-overexpressing vessels, however, the dual actions of endogenous S1P on RhoA activation and an augmented increase in VSMC Ca2+ synergize to further promote the resulting constriction. These immediate responses infer that Sphk1—or either directly or indirectly—reacts to changes in transmural pressure that translates to cell stretch in circularly orientated VSMCs. Because of the very small amount of tissue, endogenous S1P production of cannulated RAs is not directly accessible. However, we found that repetitive increases in transmural pressure progressively increased resting tone and accelerated and strengthened MRs. This effect probably resulted from an intramural accumulation of S1P produced at high pressure and can serve as an albeit indirect indicator of endogenous S1P production.

It remains unclear how the Sphk1 translates the physical signal stretch into higher activity. Several reports showed stretch-induced depolarization of VSMCs through activation of mechanosensitive nonselective cation channels, referred to as stretch-activated channels. A more recent study demonstrated that membrane depolarization and influx of extracellular Ca2+ increased cellular (PC12 and RINm5F cells) Sphk1-dependent formation of S1P.15 The current study links these two independent findings to a concept in which downstream the stretch-induced depolarization and subsequent influx of extracellular Ca2+ S1P (depolarization-dependently produced by Sphk1) translates these initial events into a release of Ca2+ from intracellular stores,10 combined with an activation of the Ca2+-sensitizing RhoA pathway. Consequently, genetic inhibition of Sphk1 by its dominant negative mutant resulted in significantly attenuated and delayed increases in Ca2+ and only residual myogenic vasoconstrictions.

Although all Sphk and RhoA/Rho kinase mutants affected resting tone, these alterations were not relevant for the strength of the MR. This was shown by pharmacological modulation of initial tone in the different groups that did not affect subsequent MRs.

Furthermore, genetic modulation of RhoA/Rho kinase might result in changes of cytoskeletal rearrangement that in turn can profoundly alter MRs.6 However, this was not likely to be the case in our experiments, since transfections neither affected pressure-induced Ca2+ increases that occur before myogenic constrictions within the signaling sequence and presume an intact cytoskeleton13 nor contractile responses to
NE and K+ that were similar in arteries overexpressing GFP, Sphk1, or RhoA mutants. Genetic inhibition of Sphk1 by hSK-G82D, however, reduced NE-induced tone, possibly indicating an indispensable role of the functional enzyme for cell homeostasis.

Our data show that resting tone and MRs in RAs are modulated by altering the expression and activity of Sphk1 and completely inhibited by dominant negative mutants of the RhoA/Rho kinase pathway. To our knowledge, this provides the first direct evidence that Sphk1 is a major determinant of microvascular tone and a leading candidate to orchestrate the two main components of the MR. Sphk1 appears to be an integral part of a pathway translating mechanical forces into intracellular signals and could be of general importance in all cell types that need to convert mechanical stimuli into specific biochemical signals. The proposed mechanism of an enhanced pressure-induced production of SIP offers an explanation for the old enigma how the myogenic constriction can be maintained although it abrogates its own stimulus. The effects of an enforced expression of Sphk1 on microvascular tone, which, in an intact organism would be reflected by increases in systemic blood pressure, make the enzyme an important candidate to cause forms of genetically determined hypertension. The pharmacological modulation of the enzyme’s activity could represent a promising tool for the treatment of hypertension.

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