Objective: The mechanisms regulating spiral modiolar artery (SMA) tone are not known, yet their characterization is pivotal for understanding inner ear blood flow regulation. Sphingosine-1-phosphate (S1P), known to stimulate vasoconstriction in several vascular beds, is a candidate regulator of SMA tone with potential pathophysiological relevance.

Methods: Gerbil SMAs were isolated, cannulated and pressurized (30 mm Hg transmural) for experimentation under near-in vivo conditions. For functional experiments, vascular diameter and intracellular Ca2+ were simultaneously measured. Standard RT-PCR and immunohistochemical techniques were also employed.

Results: mRNA transcripts encoding sphingosine kinase, S1P phosphohydrolase and three S1P receptors (S1P1–3) were detected in the SMA. S1P induced dose-dependent vasoconstriction of the SMA (EC50 = 115 nmol/L), and enhanced the apparent Ca2+-sensitivity of the contractile apparatus. Noradrenaline did not elicit vasoconstriction. The Rho kinase inhibitor Y27632 (1 μmol/L) reversed S1P-induced vasoconstriction and the S1P-mediated enhancement of Ca2+-sensitivity. RhoA was observed to translocate to the plasma membrane in response to stimulation with 30 μmol/L S1P.

Conclusion: We conclude that all key signalling pathway constituents are present at the mRNA level for S1P to act as an endogenous regulator of SMA tone. S1P stimulates potent, RhoA/Rho kinase-dependent SMA vasoconstriction and Ca2+ sensitization. The high sensitivity to S1P suggests that SMA vasoconstriction is likely to occur under pathological conditions that increase intramural S1P concentrations (i.e., inflammation). From a clinical perspective, the present study identifies new potential therapeutic targets for the treatment of vascular-based, “stroke-like” inner ear pathologies: the enzymes responsible for S1P bioavailability and the S1P receptors.

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within hours to days. Inner ear pathologies share various risk factors in common with cardiovascular diseases [3] and often present clinically as “stroke-like”, fuelling the hypothesis that they are primarily vascular in origin.

Therapeutic strategies aiming to change the rheological blood properties often fail to improve the clinical condition. A subgroup of sudden sensorineural hearing loss (SSHL) patients was recently reported to have benefited from the removal of plasma low density lipoproteins (LDL) [4]. This interesting clinical observation could potentially advance a pathophysiological concept, where microvascular dysfunction ultimately leads to stroke-like inner ear pathologies. An important cardiovascular risk factor, LDLs, are rapidly oxidized into oxLDL once they enter the vascular wall, and gain pathophysiological relevance as activators of monocytes/macrophages. It is generally accepted that LDLs are a major substrate for atheroma formation, which is initiated by the direct uptake of LDLs by the macrophage scavenger receptor (SR) [5]. In this context, the expression of SR increases with age, and the density of SR on monocytes/macrophages correlates with increasing atheroma formation. The accumulation of oxLDLs within the atheroma is often referred to as atherogenic, as they are implicated in the initiation and progression of atheroma [5].

Our group has modified a previously described experimental model in order to investigate the mechanisms regulating SMA tone in pressurized arteries. Using this improved setup, we aimed to: (i) determine the mRNA expression of S1P-signalling elements (i.e., Sk1, S1P phosphohydrolase (SPP1) and S1P receptors 1–3); (ii) determine the vasomotor response to exogenous S1P; and (iii) measure Ca²⁺-sensitization and RhoA/Rho kinase activation in response to S1P.

2. Materials and methods

2.1. Materials

The Rho kinase inhibitor Y27632 [(+)-(R)-trans-4-(1-aminomethyl)-N-(4-pyridyl)] and Sphingosine-1-phosphate (S1P) were purchased from Biomol (Hamburg, Germany). The MOPS-buffered salt solution contained [mmol/L]: NaCl 145, KCl 4.7, CaCl₂ 3.0, MgSO₄·7H₂O 1.17, NaH₂PO₄·2H₂O 1.2, pyruvate 2.0, EDTA 0.02, MOPS (3-morpholinopropanesulfonic acid) 3.0, and glucose 5.0. Fura-2-AM and Fura Red were employed (2 µmol/L stock solutions at −80 °C). “Qiashredder” mini-spin columns (sample homogenization) and the “RNeasy Protect Mini Kit” (mRNA isolation) were purchased from Qiagen (Hilden, Germany); the “Titan One Tube System” (RT-PCR) was purchased from Roche (Penzberg, Germany). All other chemicals employed in this study were purchased from Sigma.

2.2. Preparation of the spiral modiolar artery

Animal care and experimental protocols were conducted in accordance with German federal animal protection laws and approved by the Institutional Animal Care and Use Committee at the Ludwig-Maximilians University, Munich. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

The isolation of the spiral modiolar artery (SMA) has been previously described in detail [12]. Briefly, the gerbil cochlea was isolated, opened and the bone surrounding the modiolus was removed. The SMA was then cautiously dissected away from the eighth cranial nerve, taking care not to stretch the artery. We modified the existing model by cannulating the SMA segments (~1.5 mm in length) and pressurizing them hydrostatically (30 mm Hg) in order to better simulate in vivo conditions.

All functional experiments were performed at 37 °C in MOPS buffer. As previously described [13], SMA smooth muscle cells (SMCs) were loaded with Fura-2-AM (2 µmol/L, 75 min abluminal application) prior to experimentation in order to simultaneously measure changes in smooth muscle Ca²⁺ (Photomed; Seefeld, Germany) and outer diameter (custom-made software). For the purpose of obtaining high-quality digital images of SMC Fura loading, Fura Red was employed (2 µmol/L, 20 min abluminal application); vessels were digitally photographed using a Zeiss LSM410 confocal microscope equipped with a Kr/Ar laser and a 63×/1.2 W water immersion objective. SMA viability was confirmed by testing its vasoconstrictor response to 3 mmol/L Ca²⁺ under depolarizing conditions (125 mmol/L KCl).
Since DMSO was used as a solvent for Fura and Y27632, experimental controls for unspecific effects were performed. No effects of DMSO, in its final concentration, were observed with respect to vessel function.

2.3. Isolation of mRNA and RT-PCR

Samples of gerbil SMA, aorta, lung and heart tissue were frozen with liquid nitrogen and pulverized with a pestle. The pulverized samples were then suspended in 500 μl “RTL buffer” (RNeasy Protect Kit) containing 5 μl β-mercaptoethanol and homogenized with a “Qiashredder” mini-spin column. The mRNA was isolated from the resulting homogenate using the RNeasy Protect Kit, according to the manufacturer’s instructions. The resulting 50 μl sample was then split into 5–6 aliquots for subsequent RT-PCR.

One-step RT-PCR of the isolated mRNA samples was completed using the “Titan One Tube System”, according to the manufacturer’s instructions. Primers against rat GADPH, human sphingosine kinase 1 (Sk1), human sphingosine-1-phosphate phosphohydrolase 1 (SPP1) and three rat S1P receptors (S1P1–3) were employed for
amplification of cDNA (Table 1). The PCR was carried out as follows: initially, 10 cycles, each containing 10 s denaturation at 94 °C, 30 s annealing at 65 °C and 45 s primer extension at 68 °C; a further 25 cycles were then performed, with the primer extension time increasing 5 s per cycle. The PCR products were separated on a 1.5%

Fig. 2. mRNA expression of S1P signalling constituents. mRNA transcripts encoding sphingosine kinase 1 (Sk1), S1P phosphohydrolase 1 (SPP1) and three S1P receptors (S1P1–3 ) were detected in homogenates derived from gerbil SMAs. GAPDH was employed as a positive control for successful RT-PCR. “M” denotes the lanes used for the molecular weight marker. Each caption is representative of 5 separate experiments employing pooled homogenates of at least 10 SMAs each.

Fig. 3. S1P induces Rho kinase-dependent vasoconstriction of the spiral modiolar artery. (A) A representative tracing of vascular diameter and intracellular Ca2+ (based on the Fura-2 ratio F340 nm/F380 nm) following successive applications of S1P. Each application of S1P (0.3 nmol/L–30 nmol/L) stimulated a transient increase in Ca2+ with a transient constriction and an additional component of persistent vasoconstriction at near basal intracellular Ca2+-levels. The persistent constriction at 30 nmol/L S1P was reversed by inhibition of Rho kinase with 1 μmol/L Y27632. (B) The dose–response relationship for the persistent, steady state S1P-induced vasoconstriction possessed a calculated EC50 of 115 nmol/L (n =11). (C) Pooled data clearly show that the vasoconstriction induced by 30 μmol/L S1P was completely reversed following treatment with 1 μmol/L Y27632 (n =11). * Denotes significant difference from all other treatments. (D) There was no vasoconstriction observed in response to noradrenaline (n =2–12, as indicated for each concentration).
agarose gel, stained with ethidium bromide, and visualized/photographed with a BioRad Gel Doc 1000 imaging system. GAPDH acted as the positive control for successful RT-PCR.

2.4. Immunohistochemistry

Arteries were stimulated with S1P (30 μmol/L) for 30s and then fixed with 3.7% formaldehyde; control arteries received no stimulation. The fixed vessels were permeabilized with 0.3% Triton X-100, blocked with 1% BSA and incubated with a mouse monoclonal anti-human RhoA antibody overnight at 4°C (clone 26C4; 1:200 dilution; Santa-Cruz Biotechnology, USA). Subsequently, an Alexa 488-labelled rabbit anti-mouse IgG secondary antibody was used (1:200 dilution; 90 min at room temperature; Mobitec, Göttingen, Germany). Digital images were obtained by confocal microscopy.

2.5. Statistical analysis

All results are expressed as means±SEM of n experiments. Experimental groups, which represented paired observations, were analyzed with a one-way analysis of variance (ANOVA) followed by paired t-tests with Bonferroni correction. Differences were considered to be significant at error probabilities less than 0.05 (P<0.05).

3. Results

3.1. Architecture of the SMA vessel wall

The arteries employed in the study possessed an average diameter of $80±2\mu m$ ($n=37$). In terms of vessel architecture, the SMA is tortuous (Fig. 1a,b), with several “straight” regions (with corresponding regular VSMC alignment).

Fig. 4. S1P increases smooth muscle cell Ca$^{2+}$-sensitivity by a Rho kinase-dependent mechanism. The top graphs in (A) and (B) display measurements of intracellular Ca$^{2+}$ (based on the Fura-2 ratio $F_{340 \text{ nm}}/F_{380 \text{ nm}}$) during basal and increasing extracellular Ca$^{2+}$ concentrations (Ca$^{2+}_{\text{ex}}$; 0–10 mmol/L) under depolarizing (125 mmol/L K$^+$) conditions. The bottom graphs in (A) and (B) display the corresponding SMA tone measurements, calculated as percent vasoconstriction relative to maximal diameter measured at 0 mmol/L Ca$^{2+}_{\text{ex}}$ under depolarizing (125 mmol/L K$^+$) conditions. The graphs in (A) display experiments in vessels under control conditions, following treatment with 30 nmol/L S1P ($n=6$) and then subsequent treatment with S1P+Y27632 (30 nmol/L S1P/1 μmol/L Y27632; $n=5$). The graphs in (B) display experiments in vessels under control conditions and following treatment with 1 μmol/L Y27632 ($n=5$). The measured intracellular Ca$^{2+}$ levels were not different for any group at a given Ca$^{2+}_{\text{ex}}$. S1P induced a significant enhancement of the apparent Ca$^{2+}$-sensitivity, while a significant reduction in the apparent Ca$^{2+}$-sensitivity was observed in SMAs treated with Y27632 alone or S1P+Y27632. * Denotes significant increase compared to control; † denotes significant decrease compared to control; and ‡ denotes significant difference between S1P- and S1P+Y27632-treated vessels.
interspersed between rather “twisted or spiral” regions (with corresponding irregular/overlapping VSMC alignment). Because our experimental design required us to delicately straighten the vessel (although no longitudinal stretch was applied), we minimized the effect of VSMC geometry by always measuring diameter at one of the “straight” regions of the vessel, located at the distal end of its convoluted base. Using confocal imaging and Fura Red-AM, we confirmed that the Fura loading of these arteries was restricted to the smooth muscle cells when it was loaded from the adventitial side (Fig. 1d); this has been previously observed in other vessel preparations [13]. Fig. 1d also clearly shows that the SMA possesses only one layer of smooth muscle cells.

3.2. mRNA expression of Sk1, SPP1 and S1P1–3

RT-PCR was used to amplify mRNA transcripts encoding proteins that control S1P bioavailability and signalling. These experiments detected the presence of the Sk1 and SPP1 transcripts in the gerbil SMA (Fig. 2), as well as other gerbil tissues (e.g., the aorta, lung and heart; data not shown). Further, we show that three sphingosine-1-phosphate (S1P) receptors (S1P1–3; formerly known as Edg1, Edg5 and Edg3), are expressed at the mRNA level in the SMA (Fig. 2). Because the SMA homogenates were derived from both endothelial and smooth muscle cells, it is not possible to specifically allocate the expression of the respective genes to one or both of the cell types.

3.3. Mechanisms underlying S1P-induced vasoconstriction

Representative tracings of SMA diameter and Ca2+ measurements following S1P application are displayed in Fig. 3a. S1P induced dose-dependent vasoconstriction, with an EC50 of 115 nmol/L (pEC50 = 6.94 ± 0.21, n = 11; Fig. 3b); after each application of S1P, a concomitant increase in intracellular Ca2+ was observed, however, these initial responses were transient in nature (Fig. 3a). The Rho kinase inhibitor Y27632 (1 μmol/L) completely reversed the vasoconstriction induced by the highest dose of S1P (30 μmol/L), indicating the mandatory involvement of the RhoA/Rho kinase signalling pathway (n = 11; Fig. 3a and c). Noradrenaline (0.1–300 μmol/L) failed to induce vasoconstriction (Fig. 3d) or changes in Ca2+ (n = 12).

Since the SMA is not perfused or innervated in our model, the release of endothelial autacoids and neuronal factors is likely absent. In vivo, these autacoids and neuronal factors are likely to modify the potency of S1P with respect to vasoconstriction. To substantiate this principle, we determined the vasoconstrictor response to S1P in the presence of acetylcholine (1 μmol/L), which is known to stimulate the release of endothelial autacoids (e.g., nitric oxide, prostacyclin, EDHF). Consistent with our hypothesis, the presence of acetylcholine significantly shifted the EC50 of S1P from 3.8 × 10^-7 mol/L to 6.0 × 10^-5 mol/L (n = 5).

Apparent Ca2+-sensitivity of the SMA contractile apparatus was assessed by increasing extracellular Ca2+ (from 0 to 10 mmol/L) under depolarizing conditions (125 mmol/L K+). This procedure leads to a new equilibrium for any given extracellular calcium concentration between forced calcium influx through L-type channels and defensive cellular mechanisms to reduce intracellular calcium (i.e., calcium pumps).

Time-matched control experiments (n = 6) indicated that the apparent Ca2+-sensitivity of the SMA was stable over the 30-min experimental period (data not shown). Stimulation of the SMA with 30 nmol/L S1P, a concentration that did not significantly pre-constrict the SMA, elicited a significant increase in Ca2+-sensitivity (n = 6; Fig. 4a). The Rho kinase

Fig. 5. Translocation of RhoA following stimulation with S1P. (A) Immunohistochemical staining of RhoA in a control, non-stimulated spiral modiolar artery. RhoA displayed a homogeneous, cytosolic localization within the smooth muscle cells, consistent with a non-activated state. (B) Following stimulation with S1P (30 μmol/L for 30 s), RhoA was localized to the plasma membrane (see arrows), indicating activation of the GTPase.
inhibitor Y27632 (1 μmol/L) not only prevented this leftward shift in sensitivity, it resulted in a substantial reduction in Ca\(^{2+}\)-sensitivity compared to the control (n = 5; Fig. 4a). All Ca\(^{2+}\)-sensitivity data represent paired observations; the measured maximal vessel diameter (dia\(_{max}\)), measured at 0 mmol/L Ca\(^{2+}\) under depolarizing conditions (125 mmol/L K\(^{+}\)), was not altered by any of the treatments (control: 84 ± 8, n = 6; S1P: 83 ± 8, n = 6; S1P + Y27632: 85 ± 9, n = 5). Based on the Fura-2 ratio determinations, intracellular Ca\(^{2+}\) levels were not different in control, S1P- and S1P + Y27632-treated SMAs at any of the applied extracellular Ca\(^{2+}\) concentrations (Fig. 4a). Y27632-treatment alone also profoundly reduced Ca\(^{2+}\)-sensitivity in the SMA (Fig. 4b).

### 3.4. RhoA translocation in response to S1P

Immunohistochemical experiments demonstrated that RhoA was localized to the cytosol and possessed a relatively homogenous distribution in control vessels (representative image in Fig. 5a). Following stimulation with S1P, RhoA was clearly localized to the plasma membrane, indicative of translocation (n = 5; representative image in Fig. 5b). This observation strongly supports the conclusion that the RhoA signalling in the SMA is activated in response to S1P.

### 4. Discussion

When we developed our SMA model beyond the existing ones (i.e., cannulation and pressurization), it was immediately clear that defining the transmural pressure for the SMA would be a challenging task. From an anatomical perspective, the feeding vessel of the SMA (labyrinthine artery) should have a near-systemic pressure (measured by Hata et al. to be 85 mm Hg [14]). The SMA itself feeds, via small branches, into capillary beds. Therefore, the pressure needs to drop over the length of the SMA. Indeed, the structure of the SMA (i.e., its extensive length), confers an ability to exert considerable resistance to blood flow over its length. Therefore, we expect the in vivo pressure at the distal end of the artery (which we employ for experiments) to be relatively close to the capillary perfusion pressure.

To validate this reasoning, we conducted preliminary experiments over a range of transmural pressures (20–60 mm Hg). We found that when the SMA was pressurized beyond 30 mm Hg (the pressure we use for all experiments in the present study), most vessel segments were unresponsive to constricting stimuli (data not shown). It is tempting to speculate that because in vivo the SMA is contained within the modiolar bone, the relatively weak, single layer smooth muscle wall may be protected against damage at higher transmural pressures. Such physical support is not present in our model, perhaps rendering the vessel more susceptible to damage from higher pressurization.

In principle, any mediator that increases SMA tone, whether bloodborne or synthesized within the vascular wall, can potentially induce a “stroke-like” inner ear pathology. We proposed that sphingosine-1-phosphate (S1P) is such a mediator with pathological potential: (i) S1P is a bloodborne substance [10]; (ii) it is synthesized by endothelial [15] and smooth muscle cells [11]; (iii) it induces RhoA/Rho kinase-dependent vasoconstriction [6] by modulating the apparent Ca\(^{2+}\)-sensitivity of the contractile apparatus [6,11]; and (iv) its synthesis is induced by a number of pathologically relevant mediators (TNF-α, growth factors, oxidized lipoproteins, etc [5,16,17]).

The enzymes which generate and degrade S1P (Sk1 and SPPI) are expressed at the mRNA level in the SMA, as are three specific receptors, S1P\(_1\)–3. As a caveat, verification of their protein expression remains to be completed, due to the following limitations: (i) commercially available antibodies are mostly directed against human epitopes and are rather unreliable in non-human tissue; and (ii) the availability of specific inhibitors against these enzymes and receptors is still lacking.

While S1P induces vasoconstriction of mesenteric, cerebral [7] and skeletal muscle [6] microcirculatory beds, contractile responses to S1P are largely absent in conduit arteries (i.e., aorta, carotid and femoral arteries [7,18]). S1P-induced vasoconstriction correlates with the expression of S1P\(_2\) and S1P\(_3\) receptors [18], which both can activate the pro-constrictive RhoA/Rho kinase pathway [19].

In cannulated and pressurized SMAs, S1P induced dose-dependent, Rho kinase-dependent vasoconstriction. The vasoconstriction was associated with a transient increase in smooth muscle Ca\(^{2+}\); this short-lived response, however, did not account for the persistent part of the constriction. According to the measured Ca\(^{2+}\)/diameter relationship (Fig. 4a), persistent elevation of intracellular Ca\(^{2+}\) (in depolarized arteries) resulted in rather limited vasoconstriction. However, the presence of S1P, even at a near-threshold concentration, substantially enhanced the apparent Ca\(^{2+}\)-sensitivity. Our suggestion that S1P targets RhoA/Rho kinase signalling to prominently regulate of SMA tone is supported by two key observations: (i) the translocation of smooth muscle cell RhoA from the cytosol to the plasma membrane following S1P stimulation, as demonstrated in the present study, indicates RhoA activation [20]; and (ii) inhibition of Rho kinase blocks S1P-mediated vasoconstriction and results in a drastically reduced apparent Ca\(^{2+}\)-sensitivity. Modulation of Ca\(^{2+}\)-sensitivity, therefore, might represent the dominant mechanism for regulating SMA tone. The prominent role of RhoA/Rho kinase signalling is further supported by our experiments indicating that basal Rho kinase activity in the SMA is high (Fig. 4b).

This observation provides a mechanistic rationale for the suggested clinical use of RhoA activity-reducing drugs, such as HMG-CoA reductase inhibitors [21], in the treatment of acute phase SSHL.

Interestingly, the prominent dependence of SMA tone on RhoA/Rho kinase signalling is in remarkable contrast to the...
tone-regulating mechanisms of most other vascular beds, where Ca²⁺-dependent mechanisms dominate [6]. In systemic resistance arteries, Ca²⁺-dependent mechanisms often mediate sympathetic activation. The lack of noradrenaline responses in the SMA segments studied (likely due to an absence of adrenergic receptors) further supports our hypothesis that a separate regulatory system controls SMA tone.

The EC₅₀ for S1P-induced vasoconstriction (115 nmol/L) corresponded well with the reported plasma concentration (191 ± 79 nmol/L [10]). Since the SMA endothelium provides a relatively impermeable barrier [22], it is unlikely that plasma S1P concentrations are reached within the SMC microenvironment under normal physiological conditions. Further, the release of endothelial autacoids in vivo, which can shift the dose–response relationship to S1P, could also serve as both a protecting and modulatory mechanism. The release of neuronal factors could serve a similar role as well.

However, an inflammatory state could compromise endothelial barrier function and autacoid production [23,24], allowing plasma S1P to reach the VSMCs. Blood-borne inflammatory mediators (e.g., TNF-α, oxLDL) could further exacerbate this situation by stimulating the intramural S1P synthesis [5,15,25], resulting in strong SMA vasoconstriction that compromises cochlear blood flow. Since S1P dramatically enhances the apparent Ca²⁺-sensitivity, even relatively low intramural S1P concentrations (i.e., ~10 fold lower than plasma concentrations) are likely sufficient to cause a detrimental increase in vascular resistance. In this context, it is interesting to hypothesize that alterations in SMA resistance do not primarily result from prominent diameter changes over the relatively short length of systemic resistance arteries. Rather, according to Poiseuille’s law, the relatively long, convoluted length of the SMA (Fig. 1) allows moderate diameter changes to substantially impact vascular resistance and hence, cochlear blood flow.

To possess clinical relevance, the intramural S1P concentration must increase in response to pathological stimuli. Such an event can reasonably occur by (i) passage of S1P from the blood into the vessel wall under circumstances of increased endothelial permeability [24] or (ii) increased synthesis of S1P from cells of the vessel wall (under the condition of bacterial [2] or viral inflammation [26,27], autoimmune responses [28] or increased transmural stretch [11]). However, measurement of intramural S1P levels and the (inflammatory) conditions that alter them were beyond the scope of the present study.

In summary, we report that S1P potently constricts the SMA by a RhoA/Rho kinase-dependent mechanism of Ca²⁺ sensitization. While the causal origin of several inner ear pathologies is unknown, many share a common link of vascular inflammation. The high sensitivity to S1P suggests that SMA vasoconstriction is likely to occur under inflammatory conditions that increase intramural S1P concentrations (from plasma or via stimulation of intramural S1P synthesis), resulting in inner ear stroke. From a clinical perspective, the present study identifies the mediators of S1P bioavailability and signalling as new potential therapeutic targets for the treatment of vascular-based inner ear pathologies.

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