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$S1P_3$ receptors mediate the potent constriction of cerebral arteries by sphingosine-1-phosphate

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Abstract

We characterized the effect of Sphingosine-1-phosphate (S1P) on vascular tone. S1P selectively constricted isolated cerebral, but not peripheral arteries, despite ubiquitous expression of $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ receptor mRNA. *Clostridium* B and C3 toxins and the rho-kinase inhibitor Y27632 (trans-*N*-(4-pyridyl)-4-(l-aminoethyl)-cyclohexane carboxamide) reduced this vasoconstriction to S1P, indicating that the response was mediated through Rho. *Pertussis* toxin displayed only weak inhibition, suggesting minor involvement of $G_{i/o}$ protein. The S1P effect was specifically reduced by adenovirus bearing a *s1p*$_3$ but not *s1p*$_2$, antisense construct. Furthermore, suramin, which selectively blocks $S1P_3$ receptors, inhibited the vasoconstrictor effect of S1P, indicating that $S1P_3$ receptors account for at least part of S1P-mediated vasoconstriction in cerebral arteries. In vivo, intracarotid injection of S1P decreased cerebral blood flow, an effect prevented by suramin treatment. Because S1P constricts cerebral blood vessels and is released from platelets during clotting, the S1P/$S1P_3$ system constitutes a novel potential target for cerebrovascular disease therapy.

Keywords: Sphingosine-1-phosphate; S1P receptor; Cerebral vessel; Vasoconstriction; Suramin

1. Introduction

Lipid mediators, such as sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA), derived from membrane sphingolipids and glycerophospholipids, are released by activated platelets and affect the maturation and function of vascular constituents, including endothelial and smooth muscle cells (Eichholtz et al., 1993; Yatomi et al., 1995; Lee et al., 1999; English et al., 2000; Liu et al., 2000). They have gained increasing attention since the discovery of high affinity G-protein coupled receptors (Hla and Maciag, 1990; An, 2000). Although some S1P-mediated responses are attributed to an intracellular action, most of the effects of S1P are now thought to be receptor-mediated (Goetzl and An, 1998). These receptors were initially termed Endothelium Differentiation Gene (-related) receptors (EDG receptors), because *edg-1* transcripts were first detected after inducing differentiation of endothelial cells with phorbol ester (Hla and Maciag, 1990; An, 2000). A new nomenclature ($S1P_1$ to $S1P_5$; formerly $EDG_{1,5,3,6,8}$) has recently been adopted (Chun et al., 2002). S1P binding to $S1P_1$ regulates chemotaxis and in vitro angiogenesis of endothelial cells (Wang et al., 1999), whereas binding to $S1P_2$ and $S1P_3$ leads to cell rounding and neurite retractions (Postma et al., 1996; Van Brocklyn et al., 1999). S1P receptor expression has been documented in endothelial as well as in vascular smooth muscle cells (Hla and Maciag, 1990; Okazaki et al., 1993). Taken together with the capability of platelets to store and release S1P, their presence on blood vessels indicates a potential importance of S1P receptors in the regulation of vascular tone.

Bischoff et al. (2000a,b) indeed reported that S1P constricted renal and mesenteric microvessels in vitro and reduced renal and mesenteric blood flow in vivo. However, S1P was devoid of detectable in vitro vasoconstrictor effects at ≤ 1 μM, whereas it exerted only a modest vasoconstriction (15–20% of the norepinephrine effect) when added at 10 μM, i.e. two orders of magnitude higher than the S1P

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plasma concentration (Yatomi et al., 1997). S1P is a much more potent constrictor in both rodent (Salomone et al., 2000; Coussin et al., 2002) and canine cerebral arteries (Tosaka et al., 2001). The pharmacological profile of the receptor subtype(s) involved in these responses has not been characterized.

The aim of this study was to identify the S1P receptor subtype(s) mediating cerebral vasoconstriction. Our results suggest that S1P-induced vasoconstriction occurs mainly through $S1P_3$ receptor stimulation, because it is decreased after treating the arteries with an adenovirus bearing *s1p₃* antisense, or in the presence of the putative $S1P_3$ receptor antagonist suramin.

2. Materials and methods

2.1. Reagents

Sphingosine-1-phosphate and dihydrosphingosine-1-phosphate were purchased from Avanti Polar Lipids (Alabaster, AL); they were dissolved in methanol, dried under a stream of nitrogen and re-dissolved as millimolar solutions in 4 mg/ml fatty acid free bovine serum albumin for in vitro use. For in vivo use, 750 μg/ml solutions were prepared in 1 mg/ml bovine serum albumin–0.9% NaCl (75 μg/100 μl per rat were injected into the internal carotid artery). Lysophosphatidic acid (Biomol, Plymouth Meeting, PA) and sphingosylphosphorylcholine (Calbiochem, La Jolla, CA) were dissolved as millimolar solutions in 4 mg/ml fatty acid free bovine serum albumin. 5-Hydroxytryptamine creatinine sulfate complex (5-HT, serotonin) was from Sigma (St. Louis, MO); a 10-mM solution was prepared in water and further diluted as required. Suramin sodium salt was from Calbiochem, a 10-mM solution was prepared in water and further diluted as required; a 20 mg/ml solution was prepared in 0.9% NaCl for injection into the femoral vein (4 mg/200 μl per rat were injected). *Pertussis* toxin (Sigma) was dissolved as a 0.1 mg/ml stock solution in PBS; incubation of arterial segments was carried out in Dulbecco's modified minimum essential medium, containing 200 ng/ml *Pertussis* toxin, for 24 h at 37 °C, in a cell incubator (5% CO_2). *C. difficile* toxin B (List Biological Laboratories, Campbell, CA), was dissolved in water as a 20 μg/ml stock solution. *C. botulinum* C-3 exoenzyme (Biomol) was dissolved in water as a 0.5 mg/ml stock solution; 7.5 μg of C-3 exoenzyme were then mixed with 25 μg liposome (Transfectam, Promega, Madison, WI), resuspended in 0.5 ml physiological solution (for composition, see below) and directly applied onto two arterial preparations during 4 h, at room temperature; the same liposome suspension in physiological solution, but without C-3 exoenzyme, was applied onto two control preparations. Y 27632 (trans-*N*-(4-pyridyl)-4-(l-aminoethyl)-cyclohexane carboxamide) was from Biomol. Taq DNA polymerase (RedTaq™) was from Sigma. The kit used for producing recombinant adenoviruses (Adenoquest™) was from Quantum Biotechnologies (Montreal, Quebec, Canada). Dulbecco's modified minimum essential medium, fetal bovine serum, penicillin, and streptomycin were from Life Technologies (Grand Island, NY).

2.2. Measurement of contractile tension in isolated arteries

Male Sprague–Dawley rats (250–300 g, Charles River, Wilmington, MA) or male C57Black/6 mice (18–24 g, Taconic, Germantown, NY) were sacrificed by decapitation. Male New Zealand white rabbits (2–3 kg, Charles River) were anesthetized with sodium pentobarbital and sacrificed by intravenous injection of KCl. Basilar, middle cerebral, carotid, femoral or coronary arteries were removed and immersed in physiological solution (composition, mM: NaCl, 118; KCl, 4.6; $NaHCO_3$, 25; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; glucose, 10; EDTA, 0.025; pH 7.4 at 37 °C). Only basilar artery was obtained from mice and rabbits. Arterial segments (1.5–2 mm long) were threaded onto 40 μm stainless steel wires and mounted in a isometric myograph (610M, Danish Myo Technology, Aarhus, Denmark). For mouse basilar artery 25 μm tungsten wires were used. After mounting, each preparation was equilibrated, unstretched, for 30 min, in physiological solution, maintained at 37 °C and aerated with a gas mixture of 95% O_2–5% CO_2. Then, the normalized passive resting force and the corresponding diameter were determined for each preparation from its own length–pressure curve, according to Mulvany and Halpern (1977). Contractile responses were recorded into a computer, by using a data acquisition and recording software (Myodaq and Myodata, Danish Myo Technology). After normalization and 30-min equilibration in physiological solution, the preparations were stimulated with a 100 mM KCl-depolarizing solution (in mM: NaCl, 22.6; KCl, 98.8; $NaHCO_3$, 25; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; glucose, 10; EDTA, 0.025, pH 7.4 at 37 °C). After washout and 30-min recovery, they were exposed to cumulative concentrations of 5-hydroxytryptamine (5-HT, 1 nM–10 μM). After washout and 30-min recovery, they were finally exposed to cumulative concentrations (10 nM–10 μM) of sphingosine-1-phosphate (S1P) or dihydrosphingosine-1-phosphate (DHS1P). Contractile responses to 5-HT, S1P or DHS1P were expressed in % of the 100 mM KCl-evoked contraction. When used, suramin was added to the organ bath 30 min prior to addition of 5-HT or S1P. When required, endothelium was removed as follows: rats were anesthetized, the descending aorta was clamped, the ascending aorta was cannulated and perfused with 50 ml phosphate buffered saline, then with 10 ml of 0.03% Triton-X100 in water, and finally again with 10 ml saline. The basilar artery was then removed and assayed for physiological responses. The absence of functional endothelium after this treatment was confirmed by the lack of relaxing response to 3 μM acetylcholine.

2.3. RNA extraction and RT-PCR

Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and perfused transcardially with saline (NaCl 0.9%), in order to remove blood cells from vessels. Basilar, coronary, carotid, femoral arteries and aorta were dissected and immediately frozen at − 80 °C. Total RNA was isolated from pools of 4 arteries, with an RNA extraction kit (RNeasy™, Qiagen) according to the manufacturer's instruction. After DNase treatment, RNA samples (1.0 μg) were reverse-transcribed using random hexamer-mixed primers and a reverse transcription kit (Omniscript™, Qiagen). Specific PCR primers for $s1p_1$, $s1p_2$, $s1p_3$ and $s1p_5$ were designed based on the sequences reported in Genbank (NM 017301 for rat $s1p_1$, U10699 for rat $s1p_2$, AF184914 for rat $s1p_3$, NM 021775 for rat $s1p_5$) as follows: 5′-ATG GTG TCC TCC ACC AGC ATC CC-3′ (sense) and 5′-TTA AGA AGA AGA ATT GAC GTT TCC-3′ (antisense) for rat $s1p_1$; 5′-ATG GGC GGT TTA TAC TCA GAG T-3′ (sense) and 5′-TCA GAC CAC TGT GTT GCC CTC-3′ (antisense) for rat $s1p_2$; 5′-CGG CAT AGC CTA CAA GGT CA-3′ (sense) and 5′-GAT CAC TAC GGT CCG CAG AA-3′ (antisense) for rat $s1p_3$; 5′-ATC TGT GCG CTC TAT GCA AG-3′ (sense) and 5′-TCT CGG TTG GTG AAG GTG TA-3′ (antisense) for rat $s1p_5$. The primers for the control rat *gapdh* (glyceraldehyde-3-phosphate dehydrogenase, AB 017801) were 5′-TAA AGG GCA TCC TGA GCT ACA CT-3′ (sense) and 5′-TTA CTC CTT GGA GGC CAT GTA GG-3′ (antisense). PCR was performed by incubating 1 μl of the RT product with 1.25 U RedTaq DNA polymerase (Sigma), 200 nM of each primer, 10× PCR buffer, 200 μM deoxynucleotides, and water to bring the final volume to 25 μl. The PCR mixture was incubated in a Peltier thermal cycler (PTC-200, MJ Research). The amplification program consisted of denaturation at 94 °C for 30 s, annealing at 55 °C (*gapdh*, $s1p_2$, $s1p_3$, $s1p_5$) or 57 °C ($s1p_1$) for 15 s and extension at 72 °C for 45 s. Amplification cycles were 38 for *s1p* and 36 for *gapdh* mRNAs. This yielded products of the expected size: 1151 bp for $s1p_1$, 1058 bp for $s1p_2$, 474 bp for $s1p_3$, 316 bp for $s1p_5$, 199 bp for *gapdh*, respectively. Appropriate negative controls were run by omitting the RT product. Amplified DNA fragments were electrophoresed on 1.0% agarose gel and visualized by ethidium bromide staining. All PCR products were checked with appropriate restriction enzymes. The relative density of bands was analyzed by an M4 image analysis system (Imaging Research, St. Catherines, Ontario, Canada).

2.4. Cloning and viral gene transfer of $s1p_2$ and $s1p_3$ antisense to basilar artery

Rat $s1p_2$ was amplified by RT-PCR from total RNA isolated from rat basilar arteries; human $s1p_3$ was amplified by PCR from genomic DNA. The primers were designed to add a *PmeI* site at both the 5′- and 3′-ends as follows; 5′-GGG GTT TAA ACA TGG GCG GTT TAT ACT CAG-3′ (sense) and 5′-GGG GTT TAA ACT CAG ACC ACT GTG TTG CCC-3′ (antisense) for $s1p_2$ and 5′-GGG GTT TAA ACA TGG CAA CTG CCC TCC C-3′ (sense) and 5′-GGG GTT TAA ACT CAG TTG CAG AAG ATC C-3′ (antisense) for $s1p_3$. PCR products were cloned into the *PmeI* site of the adenovirus shuttle vector (pQBI-AdCMV5-GFP, Quantum Biotechnologies). After transformation, colonies were picked up and the plasmids encoding antisense were selected, based on the sequencing and restriction analyses. These plasmids were transfected into 293A cells together with linearized adenoviral genome (Quantum Biotechnologies), using the calcium phosphate method; the cells and medium were harvested after observing plaque formation. The virus was then amplified on 293A cells for 3 days, harvested, purified by double cesium chloride gradient and stocked at − 80 °C. Viral titration was determined on 293A cells by the tissue culture infectious $dose_{50}$ ($TCID_{50}$) method (Mittereder et al., 1996). Basilar artery segments were incubated for 12–16 h at 37 °C in 5% CO_2 in Dulbecco's modified minimum essential medium (containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin) with or without 10^9 PFU/ml adenovirus (empty vector or adenovirus bearing $s1p_2$ or $s1p_3$ antisense). The segments were then put in fresh medium (without adenovirus) and further incubated for 48 h. After this treatment, the arterial segments were mounted into the wire myograph and tested for responses to KCl, 5-HT and S1P.

2.5. In vivo experiments

Male Sprague–Dawley rats (250–300 g, Charles River) were anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and supplemental doses were administered upon pain response to tail pinch. The animals were placed on a heating blanket and rectal temperature was maintained at 37.0 ± 0.5 °C. The left femoral vein and artery were exposed and cannulated with polyethylene tubing (PE 50, Becton Dickinson, Sparks, MD) to monitor mean arterial blood pressure (MABP), heart rate (HR) and arterial blood gases (Ciba Corning, Model 248). The left common carotid artery was exposed and permanently ligated. PE-10 tubing was then inserted and advanced 2 mm into the internal carotid artery for localized delivery of drugs under study. Animals were tracheotomized and mechanically ventilated to maintain arterial blood gases within physiological limits. The animals were placed in a stereotaxic frame. Relative cerebral blood flow (rCBF) was measured by a laser Doppler flow probe (Model PF 2B, Perimed, Sweden) affixed to the thinned bone of the skull corresponding to the vascular territory of the left middle cerebral artery (Bregma 2 mm posterior, 3 mm lateral to midline). Data (MABP, HR and rCBF) were continuously acquired and recorded on a MacLab system (AD Instruments, Milford, MA). Baseline rCBF was recorded for at least 20 min before drug administration and set as 100%. Changes in rCBF were expressed as a percentage of baseline and recorded for 20 min beginning at the onset of drug or vehicle infusion. S1P, DHS1P or vehicle was

infused (100 μl over 2 min, with a syringe infusion pump 22, Harvard Apparatus, Holliston, MA) into the left internal carotid artery. In an additional series of experiments, rats were pretreated with suramin via infusion (200 μl/2 min) into the left femoral vein. S1P was administered into the carotid artery 10 min after the suramin infusion.

2.6. Statistical analysis

Data are expressed as mean ± standard error of mean (S.E.M.). EC_{50} is the drug concentration producing an effect of 50% of the maximum, pD2 is the negative logarithm of EC_{50}; it was estimated by nonlinear regression from log concentration–effect curves. Statistical significance was determined by unpaired Student's *t*-test or, when comparing more than two groups, by two-way analysis of variance (ANOVA) followed by Tukey post hoc test. *p* values less than 0.05 were considered statistically significant.

3. Results

3.1. S1P is a selective vasoconstrictor of cerebral arteries, despite ubiquitous arterial S1P-receptor mRNA expression

Rat arteries (basilar, middle cerebral, carotid, femoral, coronary arteries) were mounted in wire myographs for measurement of vascular active wall tension. The contractile effect of KCl-induced depolarization and 5-hydroxytryptamine (5-HT) was assessed in each preparation, prior to testing the effect of S1P (Fig. 1A and B). Both KCl and 5-HT evoked contractile responses in all the arteries investigated. The maximum tone induced by 5-HT in most preparations reached or overcame the level of KCl-induced contraction (Table 1). The potency of 5-HT, however, varied, being higher in cerebral arteries (basilar and middle cerebral), intermediate in coronary and femoral arteries and lower in carotid artery (Fig. 1A and Table 1). In contrast, S1P evoked robust contractile effects only in isolated cerebral arteries, with a maximum effect comparable to that of 5-HT. Coronary arteries were weakly constricted by S1P (10–30% of the KCl- or 5-HT-mediated vasoconstriction), whereas carotid and femoral arteries were unresponsive (0–5% of the KCl- or 5-HT-mediated vasoconstriction; Fig. 1B and Table 1). Similarly, rat mesenteric resistance arteries were unresponsive to S1P concentrations as high as 3 μM; a weak contractile response was however observed at higher S1P concentrations (in % of KCl-evoked contraction: 9.9 ± 2.4% at 10 μM, $n = 3$; 21.7 ± 0.9% at 30 μM, $n = 3$). Dihydrosphingosine-1-phosphate (DHS1P) showed a similar pattern of specificity, evoking a vasoconstriction only in cerebral arteries, but it was less effective than S1P (Fig. 1C). Other sphingolipids, such as LPA and sphingosylphosphorylcholine (SPC), tested in isolated basilar and coronary arteries, were much less

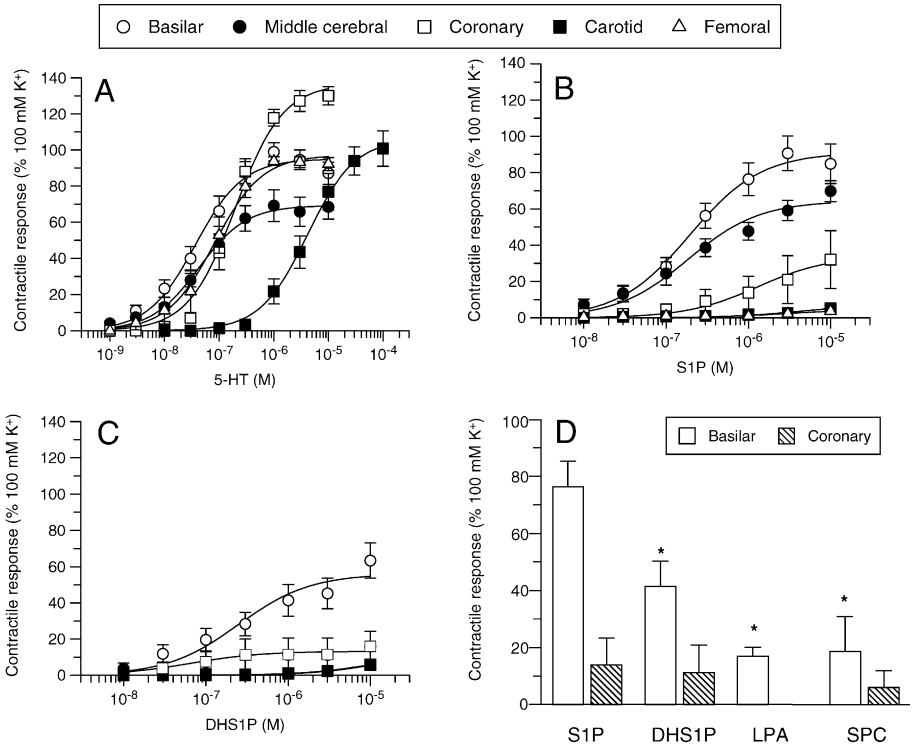

Fig. 1. Vasoconstriction to sphingolipids in isolated rat arteries. Concentration–contraction curves to 5-hydroxytryptamine (5-HT, A), sphingosine-1-phosphate (S1P, B) and dihydrosphingosine-1-phosphate (DHS1P, C). Points represent mean ± S.E.M. from *n* preparations as reported in Table 1. (D) Comparison of the contractile effects of different sphingolipids in basilar and coronary artery of rat; the effect of 1 μM S1P, DHS1P, lysophosphatidic (LPA) and sphingosylphosphorylcholine (SPC) are shown. *$p<0.05$ vs. S1P in basilar artery (two-way ANOVA followed by Tukey post hoc test).

Table 1
In vitro contractile parameters of arteries isolated from rat

Artery (n)[a]	Normalized diameter (μm)	Contractile response to KCl (mN/mm)	Contractile response to 5-HT		Contractile response to S1P		Contractile response to DHS1P	
			pD_2	E_{max} (% KCl)	pD_2	E_{max} (% KCl)	pD_2	E_{max} (% KCl)
Basilar E(+) (20,10,10)	319±10	1.70±0.22	7.42±0.11	99.5±5.1	6.60±0.06	96.7±8.4	6.61±0.19	58.8±9.4
Basilar E(−) (4, 4, 0)	299±4	0.97±0.14	7.09±0.12	97.1±9.2	6.65±0.03	85.5±4.0	n.d.	n.d.
Middle Cerebral (7, 7, 0)	199±7	1.05±0.26	7.36±0.02	73.2±7.3	6.53±0.11	77.6±9.2	n.d.	n.d.
Coronary (7, 4, 3)	378±13	1.87±0.27	6.70±0.10	119.3±12.4	n.d.	16.9±7.0	n.d.	15.9±8.4
Carotid (9, 6, 3)	1147±22	2.28±0.30	5.47±0.15	101.1±9.8	n.d.	5.2±3.4	n.d.	5.9±4.6
Femoral (7, 4, 3)	552±45	4.52±0.83	7.09±0.20	133.2±3.5	n.d.	3.6±1.9	n.d.	6.3±5.0

pD_2 is $-\log EC_{50}$, the concentration of drug producing 50% of the maximum effect. E_{max} is the maximum contractile response, expressed in % of the contraction evoked by 100 mM KCl in the same preparation. For basilar artery, E(+) indicates preparations with intact endothelium, E(−) preparations without endothelium. n.d.=not determined.

[a] The numbers in parenthesis indicate the number of preparations challenged with 5-HT, S1P or DHS1P, respectively.

effective vasoconstrictors than S1P (Fig. 1D). S1P also constricted basilar arteries in species other than rat, such as mouse and rabbit (10 μM S1P, in % of KCl-evoked contraction, mouse: 75.4 ± 21.7%, $n=3$; rabbit: 67.0 ± 8.4%, $n=3$), while it was ineffective in mouse aorta (not shown).

Vascular endothelium is known to release vasodilator and vasoconstrictor compounds upon appropriate stimuli. Furthermore, S1P has been shown to activate endothelial nitric oxide synthase (Igarashi and Michel, 2000; Igarashi et al., 2001), which, if occurring in our system, would decrease the effectiveness of vasoconstriction. Endothelium-denuded rat basilar arteries were therefore challenged with S1P. As shown in Table 1, removal of endothelium did not significantly modify the contractile response of rat basilar artery to S1P.

Because S1P was the most powerful vasoconstrictor among the lipids tested, we attempted to detect mRNA expression for S1P receptor subtypes in the arteries using RT-PCR. S1P is considered to be the endogenous agonist for the $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ receptor subtypes (Ancellin and Hla, 1999; Im et al., 2000). We did not investigate $S1P_4$ (formerly EDG-6), because, although capable of binding S1P, it is known to be almost exclusively localized to lymphatic tissues (Gräler et al., 1998). Using specific primers, transcripts for $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ were detected in all the arteries under investigation (Fig. 2).

3.2. Signal-transduction pathway of S1P-evoked vasoconstriction

To characterize the transduction pathway involved in S1P-induced vasoconstriction, segments of basilar artery were treated, in vitro, with bacterial toxins specifically

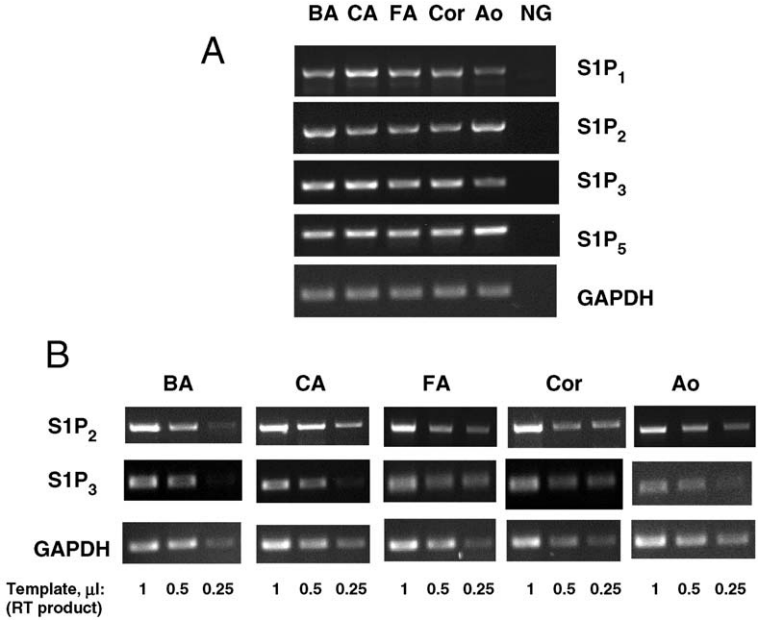

Fig. 2. (A) RT-PCR detection of $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ receptor mRNA in basilar (BA), carotid (CA), femoral (FA), coronary (Cor) arteries and aorta (Ao) of rat; negative controls (without RT product) were run in the last lane (NG). GAPDH represents glyceraldehyde-3-phosphate dehydrogenase (identical results have been obtained with three independent RNA extracts). (B) Effect of template dilution in PCR amplification of $S1P_2$, $S1P_3$ and GAPDH, showing a proportional decrease of the respective PCR products when the amount of template is reduced two- or fourfold.

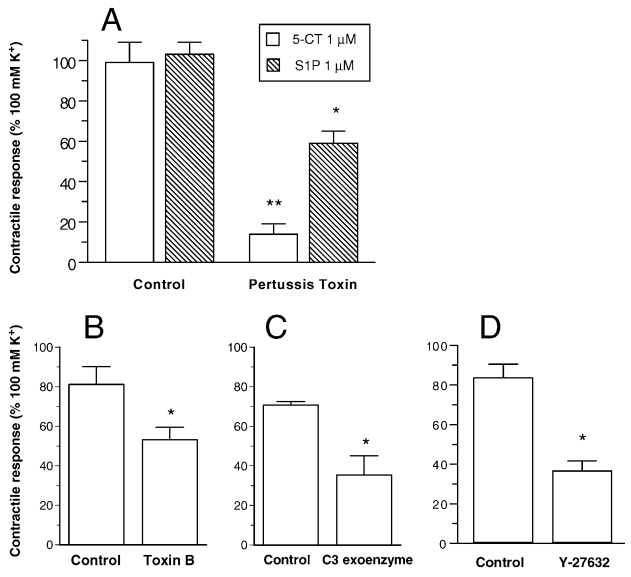

Fig. 3. Effect of toxins specific for different G protein subtypes and of the Rho-kinase inhibitor Y-27632 on basilar artery constriction induced by 1 μM 5-carboxamidotryptamine (5-CT) or 1 μM sphingosine-1-phosphate (S1P). (A) Effect of Pertussis toxin ($n=8$); (B) effect of toxin B ($n=8$); (C) effect of C3 exoenzyme ($n=4$); (D) ($n=8$) effect of Y-27632. *$p<0.05$, **$p<0.01$ vs. control; unpaired Student's t-test.

affecting $G_{i/o}$ (*B. pertussis* toxin) or Rho (*C. difficile* toxin B and *C. botulinum* C3 exoenzyme). As shown in Fig. 3, incubation with *Pertussis* toxin (200 ng/ml, 24 h, 37 °C) reduced by 80–90% the contractile effect of 5-carboxyamidotryptamine (5-CT, Fig. 3A), an agonist known to inhibit cyclic AMP accumulation in vascular smooth muscle cells in a pertussis toxin-sensitive manner (Ebersole et al., 1993). The contractile effect of S1P, although significantly reduced by *Pertussis* toxin, was less affected than 5-CT response. On the other hand, incubation with either toxin B (40 ng/ml, 4 h, 37 °C) or C3 exoenzyme (3.75 μg/preparation, 4 h, at room temperature) significantly reduced the S1P-induced vasoconstriction (Fig. 3B and C). To further ascertain the role of signal transduction through Rho in S1P-mediated vasoconstriction, some preparations were incubated in the presence of Y 27632 (5 μM), an inhibitor of Rho-kinase, 15 min before adding S1P. As shown in Fig. 3D, Y 27632 inhibited the contractile response to S1P by more than 50%. These results therefore indicate that signal transduction following S1P receptor mediated constriction was mostly via a small G protein of the Rho class, although $G_{i/o}$ proteins seemed also involved.

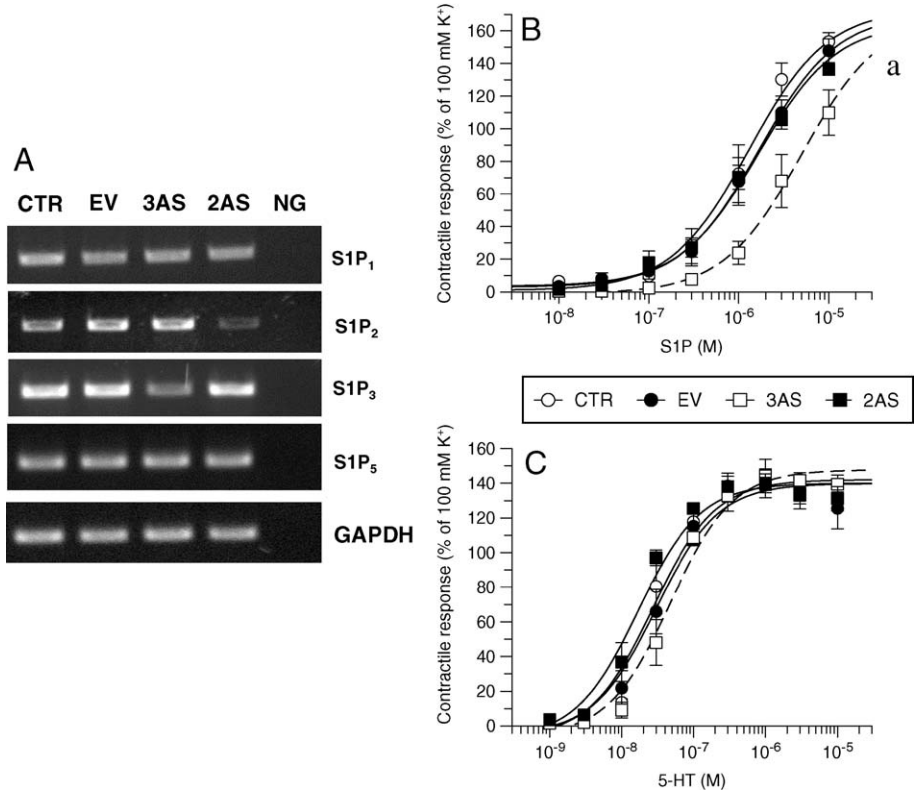

Fig. 4. Effect of adenoviral-mediated gene transfer of antisense sequence against *s1p₂* or *s1p₃* mRNA on sphingosine-1-phosphate-induced vasoconstriction. (A) RT-PCR of $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with RNA extracted from adenovirus-untreated (CTR) basilar arteries or from basilar arteries treated with the empty vector (EV), with the virus bearing *s1p₂* antisense (2AS) or the virus bearing *s1p₃* antisense (3AS). Negative controls (without RT product) were run in the last lane (NG). Concentration–contraction curves to sphingosine-1-phosphate (S1P, B) or 5-hydroxytryptamine (5-HT, C) were carried out in isolated basilar arteries, after the indicated treatments. Points represent mean ± S.E.M. from eight preparations. (a) Significantly different from all other treatment groups [$p<0.05$, two-way ANOVA followed by Tukey post hoc test].

We used a strategy of adenoviral-mediated antisense gene delivery to identify the relevant receptor(s). Recombinant adenoviral vectors were produced, bearing the antisense sequences of either rat *s1p*$_2$ or human *s1p*$_3$ open reading frames under the control of a *Cytomegalovirus* promoter. Basilar artery segments were incubated for 12–16 h in a medium containing 10^9 PFU/ml adenovirus, and incubated for another 48 h in an adenovirus-free medium. These conditions were chosen because, in preliminary experiments using adenovirus bearing the LacZ reporter gene, they resulted in intense expression of β-galactosidase activity in basilar arteries (not shown). After treatment, the preparations were mounted in the myograph and tested for responsiveness to 5-HT and S1P. We did not observe any run-down of the vascular responses to either KCl or 5-HT in preparations incubated for up to 64 h in DMEM (without or with adenovirus). Indeed, the active wall tension developed in response to KCl was not significantly different in the various groups: 1.70 ± 0.22 in freshly dissected preparations; 1.66 ± 0.21 in preparations incubated for 60 h without virus; 1.69 ± 0.24 in preparations incubated for 60 h with the empty vector; 1.56 ± 0.20 in preparations incubated for 60 h with 2AS; 1.99 ± 0.19 in preparations incubated for 60 h with 3AS. To verify that the antisense mechanism was operating effectively, total RNA from adenovirus-treated arteries was submitted to RT-PCR. Gene delivery of either *s1p*$_2$ or *s1p*$_3$ antisense specifically reduced the respective RT-PCR product by 80–90% (Fig. 4A), while it did not affect *s1p*$_1$, *s1p*$_5$ or *gapdh* RT products. The simplest interpretation for this observation is that the mRNA levels for *s1p*$_2$ and *s1p*$_3$ receptors were reduced, or that they became inefficient templates in the RT reaction (and presumably as templates for receptor protein synthesis, because of sense–antisense mRNA duplex formation). In preparations treated with *s1p*$_3$ antisense, the concentration–response curve to S1P was significantly shifted to the right ($p < 0.05$ vs. empty vector; Fig. 4B), whereas treatment with *s1p*$_2$ antisense did not modify the S1P contractile response. The empty vector and the adenoviruses bearing *s1p*$_2$ or *s1p*$_3$ antisense did not modify the contractile response to 5-HT (Fig. 4C). These results strongly suggest that the $S1P_3$ receptor mediates, at least in part, the vasoconstrictor response to S1P in cerebral blood vessels.

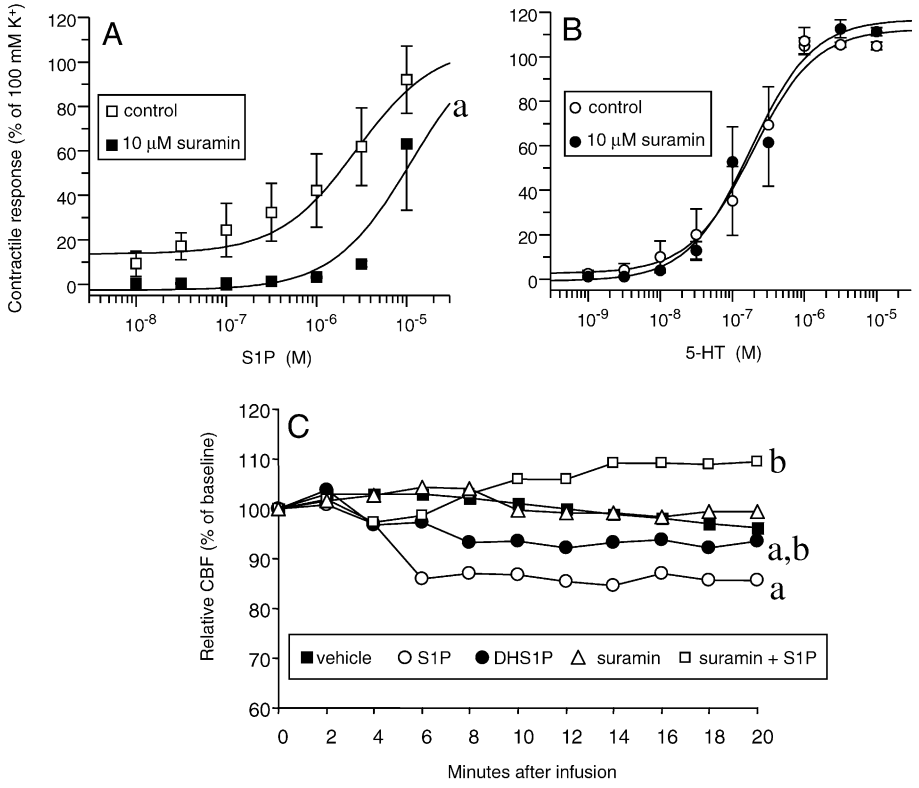

Fig. 5. In vitro and in vivo effects of suramin on sphingosine-1-phosphate-induced vasoconstriction. Concentration–contraction curves to sphingosine-1-phosphate (S1P, A) or 5-hydroxytryptamine (5-HT, B) were carried out in isolated basilar arteries in the absence (control) or in the presence of 10 μM suramin. When used, suramin was added to the organ bath 30 min prior to S1P. Points represent mean ± S.E.M. from four to six preparations. (C) Effects of S1P (0.3 mg/kg), dihydrosphingosine-1-phosphate (DHS1P, 0.3 mg/kg) or S1P (0.3 mg/kg) + suramin (16 mg/kg) on relative cerebral blood flow, as measured by laser Doppler flowmetry in anesthetized rats. Mean arterial blood pressure, heart rate and blood gases were not affected by the treatments. Points represent mean ± S.E.M. from five to seven animals. Significantly different from control/vehicle (a), or S1P (b) [$p < 0.05$, two-way ANOVA followed by Tukey post hoc test].

3.3. In vitro and in vivo antagonism of S1P-induced vasoconstriction

Suramin is reportedly a selective antagonist of $S1P_3$ receptor-mediated responses (Ancellin and Hla, 1999). We therefore examined whether the S1P-induced vasoconstriction of cerebral arteries was prevented by suramin. In vitro, preincubation of basilar artery in the presence of 10 μM suramin markedly depressed the contractile response to S1P (Fig. 5A), but did not modify the contractile response to 5-HT (Fig. 5B). In vivo, intracarotid injection of S1P (0.3 mg/kg in 100 μl over 2 min) produced a long lasting 15% decrease of cerebral blood flow, as measured by laser doppler flowmetry in anesthetized rats (Fig. 5C). DHS1P also reduced relative cerebral blood flow, although less potently than S1P. Pretreatment with suramin (16 mg/kg i.v.) abolished the S1P-induced reduction of cerebral blood flow (Fig. 5C). Intravenous suramin administration alone did not significantly affect relative cerebral blood flow or mean arterial blood pressure (not shown).

4. Discussion

These results show that S1P exerts a potent vasoconstrictor effect in cerebral arteries but not in peripheral arteries, confirming previous reports (Salomone et al., 2000; Coussin et al., 2002) and providing the first example of a vasoconstrictor acting selectively at the cerebrovascular level. The fact that the vasoconstrictor effect occurs, in vitro, in the submicromolar range (S1P's EC_{50} for rat basilar artery: 250 nM) is of particular relevance, because S1P plasma concentrations have been reported to be 200–350 nM (Yatomi et al., 1997; Murata et al., 2000a) and the S1P concentration in the supernatant during clot formation is sufficient to induce spasm of basilar artery (Tosaka et al., 2001).

Since S1P has been recently shown to activate endothelial nitric oxide synthase (Igarashi and Michel, 2000; Igarashi et al., 2001), we tested whether endothelium removal affected the contractile response of basilar artery. Removal of endothelium did not modify the contractile response to S1P, suggesting that S1P does not evoke a functionally significant activation of endothelial nitric oxide synthase in these preparations, at least not during the relatively short exposure to S1P in our assay (usually less than an hour).

S1P receptors are known to be activated by S1P and DHS1P at submicromolar concentrations (Ancellin and Hla, 1999; Im et al., 2000; Murata et al., 2000b), while SPC is two to three orders of magnitude less effective (and activates a distinct class of receptors; Xu et al., 2000; Zhu et al., 2001). Receptors for other phospholipids, such as LPA_1, LPA_2 and LPA_3 (formerly EDG-2, -4, and -7), are much less responsive to S1P than to LPA (Lynch and Im, 1999). The pharmacological profile of the basilar artery constriction to S1P, DHS1P, SPC and LPA is therefore fully compatible with a response mediated by one of the cloned S1P receptors (Chun et al., 2002).

A weak vasoconstrictor effect of sphingolipids on renal and mesenteric microvessels isolated from rat has been reported by Bischoff et al. (2000a). The sensitivity of this response to *Pertussis* toxin-treatment strongly suggests that the constriction is receptor-mediated. However sphingosylphosphorylcholine is more active than S1P in these vessels, which is not consistent with the pharmacology of the S1P receptors characterized up to now (Ancellin and Hla, 1999; Im et al., 2000; Murata et al., 2000b). In addition, this vasoconstricting response to sphingolipids is only observed at concentrations (10–100 μM) two orders of magnitude higher than those found to be effective in the present study (100 nM–1 μM), further suggesting that the constricting responses to S1P might involve different mechanisms in peripheral and central arteries.

S1P receptors signal through heterotrimeric G proteins such as G_i, G_o, G_q or $G_{12/13}$ (Ancellin and Hla, 1999; Im et al., 2000; Windh et al., 1999). It has been proposed that G_i is involved in a cell survival pathway through Ras, whereas G_q and G_{13} activation mediate S1P's effects on cytoskeleton, adherens junctions and cell shape (Lee et al., 1999) through activation of small G-proteins of the Rho family. In vascular smooth muscle cells, both G_i and Rho are involved in the mechanisms of contraction. A *Pertussis* toxin sensitive vasoconstriction is observed in some preparations following α-adrenoceptor stimulation (Spitzbarth-Regrigny et al., 2000), even though the effector stimulated by G_i remains to be determined. Rho activation increases the phosphorylation of myosin light chain through a Rho kinase-dependent inhibition of myosin light chain phosphatase (Gohla et al., 2000). The effect of S1P on cerebrovascular tone, being sensitive to *Pertussis* toxin, toxin B, C3 exoenzyme, and Y-27632, is therefore consistent with both G_i and Rho/Rho kinase signalling pathways. The partial inhibitory effect of Pertussis treatment on the response to S1P is noteworthy (Fig. 3A). It suggests that S1P might constrict basilar arteries via two independent mechanisms: a high affinity receptor signalling pathway through a Rho protein and a low affinity receptor, signalling through Pertussis sensitive G proteins. While the former pathway would be specific for cerebral blood vessels, the latter would be found in both central and peripheral vessels and might account for the response characterized by Bischoff et al. (2000a).

Expression of distinct receptor subtypes in different vascular beds can account for different responses to the same agonists (Kaiser et al., 1998). For example, expression of several 5-HT receptor mRNAs is known to vary among different arteries, consistently with the pharmacology of 5-HT-induced responses (Ullmer et al., 1995). Similarly, we hypothesized that a higher density of a specific S1P receptor subtype could account for the selective vasoconstriction by S1P in cerebral arteries. However, using RT-PCR, similar

levels of mRNA encoding four different S1P receptor subtypes were found in all arteries investigated. These results are at odds with recent results showing that the density of $S1P_2$ and $S1P_3$ receptor proteins was increased fourfold in rat cerebral artery compared with aorta (Coussin et al., 2002). Although it is likely that this difference in receptor levels accounts for part of the cerebro-vascular selectivity of the response to S1P, it is doubtful that this factor alone explains the striking functional differences we observe between vessels such as the basilar (S1P's $E_{max} = 100\%$ of KCl) and the femoral artery (virtually no detectable constriction) or mouse aorta (no constriction, not shown).

No truly specific antagonist for S1P receptor subtypes has been reported to date. We therefore used a strategy based on adenoviral-mediated antisense gene transfer to specifically decrease the density of $S1P_2$ and $S1P_3$ receptors and provide molecular evidence for a receptor-mediated vasoconstrictor effect of S1P. Our results indicate that the $S1P_3$ receptor subtype mediates, at least in part, the vasoconstrictor response to S1P in cerebral blood vessels, since *s1p₃* antisense specifically reduced both *s1p₃* RT-PCR product and the contractile response of basilar arteries to S1P. $S1P_2$ receptors are less likely to be involved, since the decrease of the *s1p₂* RT-PCR product was not accompanied by a modification of the S1P response. A very slow turnover of $S1P_2$ receptor proteins, however, could also account for the lack of effect of *s1p₂* antisense treatment on the response to S1P. A potential role for $S1P_1$ and $S1P_5$ receptor stimulation in S1P-evoked vasoconstriction of cerebral arteries remains to be addressed, as these receptors could account for the Pertussis toxin sensitivity of the response to S1P.

The fact that plasma levels of S1P are close to the concentrations constricting cerebral arteries in vitro suggests that local production by activated platelets can lead to a S1P release sufficient to evoke a vasospasm (Tosaka et al., 2001). Although this effect on vascular smooth muscle may be beneficial in physiological hemostasis, it would be detrimental in ischemic (thrombo-embolic) situations. S1P released from platelets could play a role in diminishing cerebral blood flow after thrombo-embolic stroke, as well as subarachnoid hemorrhage. Since hypotension is an important risk factor for stroke in humans (Bogousslavsky and Regli, 1986), and even brief periods of systemic hypotension significantly aggravate the histopathologic consequences of thromboembolic stroke in rats (Dietrich et al., 1999), the lack of effect of S1P on peripheral arteries suggests that interfering with S1P signaling (e.g. with $S1P_3$ receptor antagonists) could be beneficial in stroke therapy, since this would be likely to increase cerebral blood flow without lowering blood pressure. Indeed, intravenous administration of either S1P (Bischoff et al., 2000b) or suramin (this study, data not shown) did not significantly alter heart rate and mean arterial pressure. Unfortunately, specific S1P receptor antagonists are still unavailable. Suramin, a polianionic compound originally used in antiprotozoal and antihelmintic chemotherapy, does prevent S1P from acting at $S1P_3$ receptors in cell culture (Ancellin and Hla, 1999), but is far from being specific for S1P receptors (Voogd et al., 1993). Nevertheless, it did not alter the contractile response of basilar artery to 5-HT in vitro, and did not affect basal cerebral blood flow in vivo. Our in vitro and in vivo data showing that suramin decreased S1P-induced vasoconstriction, taken together with the selectivity of this agent for the $S1P_3$ subtype (Ancellin and Hla, 1999), corroborate the results obtained with our adenovirus antisense experiments. It should however be mentioned that in these in vivo experiments, it is difficult to estimate the S1P concentration near the receptors, because the pharmacokinetic properties of S1P are essentially unknown. Nevertheless, these data do suggest the therapeutic potential of S1P receptor antagonism. It is also worth mentioning that suramin has been shown to effectively antagonize intracellular calcium mobilization after human $S1P_5$ receptor stimulation, but not after rat $S1P_5$ receptor stimulation (IC_{50} 5800 μM; Niedernberg et al., 2002). This report therefore reinforces our view that, in rat, $S1P_5$ is not involved in S1P-induced vasoconstriction of cerebral arteries, since it is insensitive to suramin antagonism.

The discovery of pharmacological agents for the treatment of cardiovascular diseases was based on extensive research into the production and release of vasoactive agents, and their signaling mechanisms within vascular smooth muscle cells. The indication that the $S1P_3$ receptor subtype mediates, at least in part, S1P induced constriction of cerebral blood vessels provides a novel therapeutic target to design drugs for the treatment of stroke and other conditions associated with reduced cerebral blood flow.

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References

An, S., 2000. Molecular identification and characterization of G protein-coupled receptors for lysophosphatidic acid and sphingosine 1-phosphate. Ann. N. Y. Acad. Sci. 905, 25–33.

Ancellin, N., Hla, T., 1999. Differential pharmacological properties and signal transduction of the sphingosine-1-phosphate receptors EDG-1, EDG-3, and EDG-5. J. Biol. Chem. 274, 18997–19002.

Bischoff, A., Czyborra, P., Fetscher, C., Meyer Zu Heringdorf, D., Jakobs, K.H., Michel, M.C., 2000a. Sphingosine-1-phosphate and sphingosylphosphorylcholine constrict renal and mesenteric microvessels in vitro. Br. J. Pharmacol. 130, 1871–1877.

Bischoff, A., Czyborra, P., Meyer Zu Heringdorf, D., Jakobs, K.H., Michel, M.C., 2000b. Sphingosine-1-phosphate reduces rat renal and mesenteric blood flow in vivo in a pertussis toxin-sensitive manner. Br. J. Pharmacol. 130, 1878–1883.

Bogousslavsky, J., Regli, F., 1986. Unilateral watershed cerebral infarcts. Neurology 36, 373–377.

Chun, J., Goetzl, E.J., Hla, T., Igarashi, Y., Lynch, K.R., Moolenaar, W., Pyne, S., Tigyi, G., 2002. International union of pharmacology: XXXIV. Lysophospholipid receptor nomenclature. Pharmacol. Rev. 54, 265–269.

Coussin, F., Scott, R.H., Wise, A., Nixon, G.F., 2002. Comparison of sphingosine 1-phosphate-induced intracellular signaling pathways in vascular smooth muscles: differential role in vasoconstriction. Circ. Res. 91, 151–157.

Dietrich, W.D., Prado, R., Pravia, C., Zhao, W., Ginsberg, M.D., Watson, B.D., 1999. Delayed hypovolemic hypotension exacerbates the hemodynamic and histopathologic consequences of thromboembolic stroke in rats. J. Cereb. Blood Flow Metab. 19, 918–926.

Ebersole, B.J., Diglio, C.A., Kaufman, D.W., Berg, K.A., 1993. 5-Hydroxytryptamine1-like receptors linked to increases in intracellular calcium concentration and inhibition of cyclic AMP accumulation in cultured vascular smooth muscle cells derived from bovine basilar artery. J. Pharmacol. Exp. Ther. 266, 692–699.

Eichholtz, T., Jalink, K., Fahrenfort, I., Moolenaar, W.H., 1993. The bioactive phospholipid lysophosphatidic acid is released from activated platelets. Biochem. J. 291, 677–680.

English, D., Welch, Z., Kovala, A.T., Harvey, K., Volpert, O.V., Brindley, D.N., Garcia, J.G., 2000. Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. FASEB J. 14, 2255–2265.

Goetzl, E.J., An, S., 1998. Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. FASEB J. 12, 1589–1598.

Gohla, A., Schultz, G., Offermanns, S., 2000. Role for G(12)/G(13) in agonist-induced vascular smooth muscle cell contraction. Circ. Res. 87, 221–227.

Gräler, M.H., Bernhardt, G., Lipp, M., 1998. EDG6, a novel G-protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue. Genomics 53, 164–169.

Hla, T., Maciag, T., 1990. An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. J. Biol. Chem. 265, 9308–9313.

Igarashi, J., Michel, T., 2000. Agonist-modulated targeting of the EDG-1 receptor to plasmalemmal caveolae. eNOS activation by sphingosine 1-phosphate and the role of caveolin-1 in sphingolipid signal transduction. J. Biol. Chem. 275, 32363–32370.

Igarashi, J., Bernier, S.G., Michel, T., 2001. Sphingosine 1-phosphate and activation of endothelial nitric-oxide synthase. Differential regulation of Akt and MAP kinase pathways by EDG and bradykinin receptors in vascular endothelial cells. J. Biol. Chem. 276, 12420–12426.

Im, D.S., Heise, C.E., Ancellin, N., O'Dowd, B.F., Shei, G.J., Heavens, R.P., Rigby, M.R., Hla, T., Mandala, S., McAllister, G., George, S.R., Lynch, K.R., 2000. Characterization of a novel sphingosine 1-phosphate receptor, Edg-8. J. Biol. Chem. 275, 14281–14286.

Kaiser, J.R., Cox, B.E., Roy, T.A., Rosenfeld, C.R., 1998. Differential development of umbilical and systemic arteries. I. ANG II receptor subtype expression. Am. J. Physiol. 274, R797–R807.

Lee, M.J., Thangada, S., Claffey, K.P., Ancellin, N., Liu, C.H., Kluk, M., Volpi, M., Sha'afi, R.I., Hla, T., 1999. Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. Cell 99, 301–312.

Lynch, K.R., Im, D.S., 1999. Life on the edg. Trends Pharmacol. Sci. 20, 473–475.

Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C.X., Hobson, J.P., Rosenfeldt, H.M., Nava, V.E., Chae, S.S., Lee, M.J., Liu, C.H., Hla, T., Spiegel, S., Proia, R.L., 2000. Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. J. Clin. Invest. 106, 951–961.

Mittereder, N., March, K.L., Trapnell, B.C., 1996. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J. Virol. 70, 7498–7509.

Mulvany, M.J., Halpern, W., 1977. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. Circ. Res. 41, 19–26.

Murata, N., Sato, K., Kon, J., Tomura, H., Yanagita, M., Kuwabara, A., Ui, M., Okajima, F., 2000a. Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions. Biochem. J. 352, 809–815.

Murata, N., Sato, K., Kon, J., Tomura, H., Okajima, F., 2000b. Quantitative measurement of sphingosine 1-phosphate by radioreceptor-binding assay. Anal. Biochem. 282, 115–120.

Niedernberg, A., Scherer, C., Busch, A., Kostenis, E., 2002. Comparative analysis of human and rat S1P(5) (edg8): differential expression profiles and sensitivities to antagonists. Biochem. Pharmacol. 64, 1243–1250.

Okazaki, H., Ishizaka, N., Sakurai, T., Kurokawa, K., Goto, K., Kumada, M., Takuwa, Y., 1993. Molecular cloning of a novel putative G protein-coupled receptor expressed in the cardiovascular system. Biochem. Biophys. Res. Commun. 190, 1104–1109.

Postma, F.R., Jalink, K., Hengeveld, T., Moolenaar, W.H., 1996. Sphingosine-1-phosphate rapidly induces Rho-dependent neurite retraction: action through a specific cell surface receptor. EMBO J. 15, 2388–2392.

Salomone, S., Yoshimura, S., Reuter, U., Thomas, S., Cutrer, F.M., Waeber, C., 2000. Selective cerebral vasoconstriction by sphingosine-1-phosphate: mechanism of action. Soc. Neurosci. Abstr. 646, 11.

Spitzbarth-Regrigny, E., Petitcolin, M.A., Bueb, J.L., Tschirhart, E.J., Atkinson, J., Capdeville-Atkinson, C., 2000. Pertussis toxin-sensitive G(i)-proteins and intracellular calcium sensitivity of vasoconstriction in the intact rat tail artery. Br. J. Pharmacol. 131, 1337–1344.

Tosaka, M., Okajima, F., Hashiba, Y., Saito, N., Nagano, T., Watanabe, T., Kimura, T., Sasaki, T., 2001. Sphingosine 1-phosphate contracts canine basilar arteries in vitro and in vivo: possible role in pathogenesis of cerebral vasospasm. Stroke 32, 2913–2919.

Ullmer, C., Schmuck, K., Kalkman, H.O., Lubbert, H., 1995. Expression of serotonin receptor mRNAs in blood vessels. FEBS Lett. 370, 215–221.

Van Brocklyn, J.R., Tu, Z., Edsall, L.C., Schmidt, R.R., Spiegel, S., 1999. Sphingosine 1-phosphate-induced cell rounding and neurite retraction are mediated by the G protein-coupled receptor H218. J. Biol. Chem. 274, 4626–4632.

Voogd, T.E., Vansterkenburg, E.L., Wilting, J., Janssen, L.H., 1993. Recent research on the biological activity of suramin. Pharmacol. Rev. 45, 177–203.

Wang, F., Van Brocklyn, J.R., Hobson, J.P., Movafagh, S., Zukowska-Grojec, Z., Milstien, S., Spiegel, S., 1999. Sphingosine 1-phosphate stimulates cell migration through a Gi-coupled cell surface receptor. Potential involvement in angiogenesis. J. Biol. Chem. 274, 35343–35350.

Windh, R.T., Lee, M.J., Hla, T., An, S., Barr, A.J., Manning, D.R., 1999. Differential coupling of the sphingosine 1-phosphate receptors Edg-1, Edg-3, and H218/Edg-5 to the G(i), G(q), and G(12) families of heterotrimeric G proteins. J. Biol. Chem. 274, 27351–27358.

Xu, Y., Zhu, K., Hong, G., Wu, W., Baudhuin, L.M., Xiao, Y., Damron, D.S., 2000. Sphingosylphosphorylcholine is a ligand for ovarian cancer G-protein-coupled receptor 1. Nat. Cell. Biol. 2, 261–267.

Yatomi, Y., Ruan, F., Hakomori, S., Igarashi, Y., 1995. Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. Blood 86, 193–202.

Yatomi, Y., Igarashi, Y., Yang, L., Hisano, N., Qi, R., Asazuma, N., Satoh, K., Ozaki, Y., Kume, S., 1997. Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. J. Biochem. (Tokyo) 121, 969–973.

Zhu, K., Baudhuin, L.M., Hong, G., Williams, F.S., Cristina, K.L., Kabarowski, J.H., Witte, O.N., Xu, Y., 2001. Sphingosylphosphorylcholine and lysophosphatidylcholine are ligands for the G Protein-coupled Receptor GPR4. J. Biol. Chem. 276, 41325–41335.