British Journal of Pharmacology (2008) 153, 140–147

0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Analysis of sphingosine 1-phosphate receptors involved in constriction of isolated cerebral arteries with receptor null mice and pharmacological tools

S Salomone[1], EM Potts[1], S Tyndall[1], PC Ip[1], J Chun[2], V Brinkmann[3] and C Waeber[1]

[1]*Stroke and Neurovascular Regulation Laboratory, Department of Radiology, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA, USA;* [2]*Department of Molecular Biology, Helen L Dorris Child and Adolescent Neuropsychiatric Disorder Institute, The Scripps Research Institute, La Jolla, CA, USA and* [3]*Novartis Institutes for Biomedical Research, Auto Immunity and Transplantation, Basel, Switzerland*

Background and purpose: Sphingosine 1-phosphate (S1P) selectively and potently constricts isolated cerebral arteries, but this response has not been pharmacologically characterized.
Experimental approach: The receptor subtype(s) involved in S1P-induced cerebrovascular constriction were characterized using genetic ($S1P_2$ and $S1P_3$ receptor null mice) and pharmacological tools (phospho-FTY720, a $S1P_{1/3/4/5}$ receptor agonist; SEW2871, a $S1P_1$ receptor agonist, JTE-013, a $S1P_2$ receptor antagonist, VPC23019, a $S1P_{1/3}$ receptor antagonist). Isolated basilar or peripheral (femoral, mesenteric resistance) arteries, from either rat or mouse, were studied in a wire myograph.
Key results: S1P concentration-dependently constricted basilar artery in rat, wild-type (WT) and $S1P_2$ null mice, but barely affected vascular tone in $S1P_3$ null mice. Vasoconstriction to U46619 (a thromboxane analogue) or to endothelin-1 did not differ between WT, $S1P_2$ and $S1P_3$ null mice. JTE-013 inhibited not only S1P-induced vasoconstriction, but also KCl-, U46619- and endothelin-1-induced constriction. This effect was observed in WT as well as in $S1P_2$ null mice. VPC23019 increased the concentration-dependent vasoconstriction to S1P in both rat and mouse basilar arteries with intact endothelium, but not in rat basilar artery without endothelium. Phospho-FTY720 concentration-dependently constricted rat basilar arteries, but not femoral or mesenteric resistance arteries, while SEW2871 did not induce any response in the same arteries.
Conclusions and implications: S1P constricts cerebral arteries through $S1P_3$ receptors. The purported $S1P_2$ receptor antagonist JTE-013 does not appear to be selective, at least in rodents. Enhancement of S1P-induced contraction by VPC23019 might be related to blockade of $S1P_1$ receptors and NO generation.
British Journal of Pharmacology (2008) **153,** 140–147; doi:10.1038/sj.bjp.0707581; published online 19 November 2007

Keywords: S1P; basilar artery; JTE-013; FTY720; VPC23019; SEW2871

Abbreviations: E_{max}, maximal effect; ET-1, endothelin-1; eNOS, endothelial nitric oxide synthase; L-NAME, *N*-ω-nitro-L-arginine methyl ester hydrochloride; S1P, sphingosine 1-phosphate; WT, wild type

Introduction

Sphingosine 1-phosphate (S1P) is an important signalling molecule in the cardiovascular system (Waeber *et al*., 2004). S1P derived from membrane sphingolipids and glycerophospholipids and/or released by activated platelets binds to high-affinity G-protein-coupled receptors ($S1P_1$–$S1P_5$; formerly $EDG_{1,5,3,6,8}$; McGiffert *et al*., 2002; Anliker and Chun, 2004; Gardell *et al*., 2006). S1P receptor expression has been documented in endothelial cells as well as in vascular smooth muscle (Hla and Maciag, 1990; Okazaki *et al*., 1993).

Sphingosine 1-phosphate induces vasoconstriction in isolated arteries as well as *in vivo*. It constricts isolated cerebral arteries at submicromolar concentrations (Tosaka *et al*., 2001; Coussin *et al*., 2002; Salomone *et al*., 2003), but not peripheral arteries (Salomone *et al*., 2003). This effect on cerebral circulation has also been observed in *in vivo* experiments (Tosaka *et al*., 2001; Salomone *et al*., 2003). The fact that submicromolar S1P concentrations constrict cerebral arteries *in vitro* is likely to be physiologically relevant, because plasma S1P concentrations have been reported to be in this range (Yatomi *et al*., 1997; Murata *et al*., 2000). Moreover, the S1P concentration in the

Correspondence: Dr C Waeber, Department of Radiology, Massachusetts General Hospital, Harvard Medical School, CNY149 Room 6403, 149 13th Street, Charlestown, MA 02129, USA.
E-mail: waeber@helix.mgh.harvard.edu
Received 17 September 2007; revised 4 October 2007; accepted 10 October 2007; published online 19 November 2007

supernatant during clot formation has been shown to induce basilar artery spasm (Tosaka *et al*., 2001).

The identity of the receptor subtype mediating constriction to S1P is unclear. On the basis of the results of *in vitro* experiments using antisense gene delivery, we have previously proposed that the cerebrovascular constriction induced by S1P is mostly $S1P_3$ receptor mediated (Salomone *et al*., 2003). However, the involvement of $S1P_2$ in S1P-induced contraction of canine coronary smooth muscle cells has been suggested, based on the effect of JTE-013, an $S1P_2$ receptor antagonist (Ohmori *et al*., 2003). Furthermore, at least part of S1P-induced vasoconstriction seems to occur through a *Pertussis* toxin-sensitive mechanism (Bischoff *et al*., 2000; Salomone *et al*., 2003), suggesting that S1P receptors coupled to G_i/G_o proteins (possibly $S1P_1$; Lee *et al*., 1996) may play a role in S1P-induced vasoconstriction.

In the present study, we took advantage of new pharmacological tools and, importantly, receptor knockout mice to identify the S1P receptor subtype(s) mediating cerebral vasoconstriction. On the basis of the lack of S1P-induced vasomotor tone in basilar artery isolated from $S1P_3$ receptor null mice, the lack of vasoconstriction following an $S1P_1$ receptor agonist (SEW2871) and the cerebrovascular selective vasoconstriction to phospho-FTY720 (an agonist for all S1P receptors but $S1P_2$), we conclude that S1P-induced vasoconstriction of rat and mouse basilar artery occurs through $S1P_3$ receptor stimulation.

Methods

Animals

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

$S1P_2$ and $S1P_3$ null mice (Ishii *et al*., 2001, 2002; Yang *et al*., 2002) were bred and housed in our animal facility. Animals had free access to water and food. All experiments reported here were performed using 12- to 16-week-old (20–30 g) male mice. Both wild-type (WT) littermates and commercial C57BL6/j were used as controls. The genotype of each mouse was confirmed by PCR. Rats were male Sprague–Dawley, weighing 250–350 g.

Myograph experiments

Mice or rats were killed by fluothane anaesthesia followed by decapitation, and their brains 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; $CaCl_2$, 1.2; glucose, 10; EDTA, 0.025; pH 7.4 at 37 °C). The basilar artery was dissected, cut into 1.5- to 2-mm long segments and threaded onto 40-μm stainless steel wires (rat) or 15-μm tungsten wires (mice). Each segment was mounted in one of the four organ chambers of an isometric myograph (610 M; Danish Myo Technology, Aarhus, Denmark). For mice, an entire basilar artery was mounted in each organ chamber. Rat femoral and mesenteric arteries, as well as mouse aorta or carotid artery were threaded onto 40-μm stainless steel wires. In experiments with rat basilar artery, aimed at comparing the effect of intact functional endothelium on S1P and VPC23019, a 25-μm stainless steel wire was used instead of the 40-μm wire. 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 and 5% CO_2. The normalized passive resting force and the corresponding diameter were then 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 isotonic depolarizing KCl solutions, in which part of NaCl had been replaced by an equimolar amount of KCl (composition (mM): NaCl, 22.6; KCl, 98.8; $NaHCO_3$, 25; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 1.2; glucose, 10; EDTA, 0.025, pH 7.4 at 37 °C). After washout and 30-min recovery, the preparations were exposed to agonists (for example, S1P, phospho-FTY720 or SEW2871). In some experiments, the vasoconstriction to S1P was tested in the presence of 10 μM JTE-013, 10 μM VPC23019 or 0.1 mM *N*-ω-nitro-L-arginine methyl ester hydrochloride (L-NAME; 30-min preincubation) in rat or mouse basilar artery. To study endothelium-dependent vasodilating effects, preparations were preconstricted with 1–10 μM phenylephrine (rat femoral and mesenteric resistance arteries), 0.3–1 μM 5-hydroxytryptamine (5-HT; rat basilar artery) or 0.3 μM U46619 (mouse basilar artery). Mouse basilar arteries were also challenged with cumulative U46619 (10 nM–3 μM), a synthetic prostanoid analogue of $PGF_{2\alpha}$, and with endothelin-1 (ET-1; 10 pM–30 nM). In some preparations, endothelium was removed by perfusion with 0.03% Triton X-100. To assess the effectiveness of endothelium removal, preparations preconstricted by 0.3 μM 5-HT were challenged with 1 μM acetylcholine (Ach).

Statistical analysis

The negative logarithm (pD_2) of the concentration producing 50% of the maximum effect (E_{max}) was calculated by linear regression from semi-logarithmic plots. Pharmacological parameters (pD_2 and E_{max}), calculated from different animals and treatments, were averaged by group and treatment and compared by one-way ANOVA followed by Tukey's *post hoc* analysis. Nonlinear fits of concentration–contraction curves were also compared by F-test, by using the PRISM software (Graph Pad Software, San Diego, CA, USA). *P*-values less than 0.05 were considered statistically significant.

Drugs

Sphingosine 1-phosphate (Avanti Polar Lipids Inc., Alabaster, AL, USA) was solubilized as a millimolar stock solution in a buffer containing 100 mM Tris, 145 mM NaCl and 4 mg ml^{-1} fatty acid-free BSA, pH 9.0. This buffer did not produce changes in the tone of basilar arteries, except when its total amount added to the bath was 1 and 3% of the total 5-ml bath volume. The transient increase in tone observed in this case lasted only about 1 min (see Figure 1b), possibly because

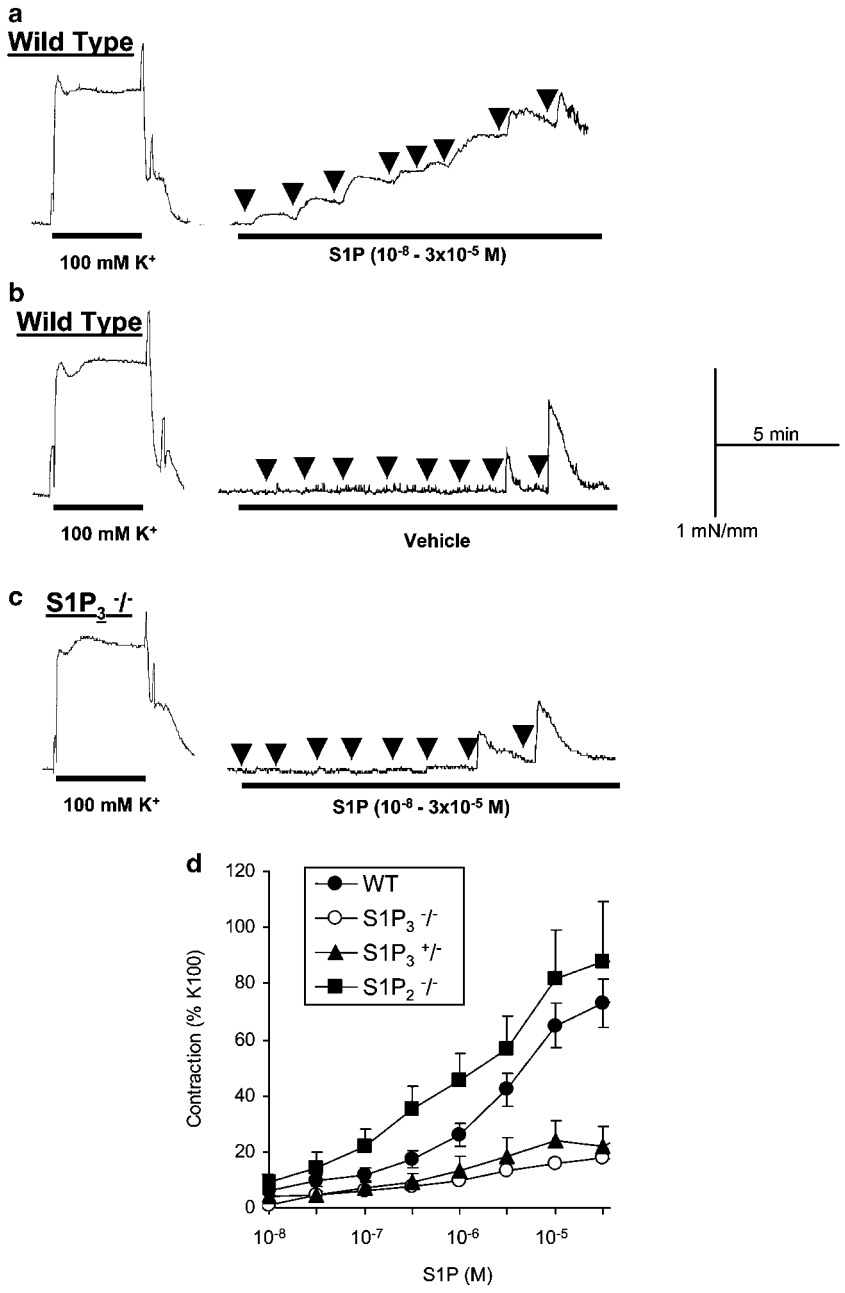

Figure 1 Vasoconstriction to sphingosine 1-phosphate (S1P) in isolated basilar artery from wild-type (WT) or $S1P_2$ or $S1P_3$ receptor null mice. (**a**) Typical recording showing the contractile responses to cumulative concentrations of S1P in basilar artery isolated from WT mice; arrows indicate the addition of each concentration of S1P, from 10^{-8} to 3×10^{-5} M, in half-log steps, to the organ bath; contractile response to high (100 mM) K^+-induced depolarization is shown for comparison. (**b**) Effect of the S1P solvent (transient vasoconstriction, when injecting 50 and 150 μl) in basilar artery isolated from WT mice. (**c**) Typical recording showing the contractile response to S1P in basilar artery isolated from $S1P_3^{-/-}$ mice. (**d**) Averaged contractile responses to cumulative concentrations of S1P in WT, $S1P_2^{-/-}$, $S1P_3^{-/-}$ and $S1P_3^{+/-}$ mice, expressed in % of high (100 mM) K^+-induced contractions in the same preparations: mean and s.e.mean. The corresponding pharmacological parameters are given in Table 1.

of a transient increase in the pH of the physiological bathing solution. Therefore, when measuring the effect of 10 and 30 μM S1P, we disregarded this transient peak and considered the ensuing steady-state tension. JTE-013 (Tocris, Ellisville, MO, USA) was dissolved in dimethyl sulphoxide as a 10-mM stock solution. VPC23019 (Avanti Polar Lipids) was dissolved in acidified dimethyl sulphoxide as a 10-mM stock solution. Phospho-FTY720 (Novartis Institutes of BioMedical Research, Basel, Switzerland) was dissolved in H_2O/dimethyl sulphoxide (50:50) as a 5-mM stock solution. SEW2871 (Cayman Chemical Company, Ann Arbor, MI, USA) was dissolved in ethanol as a 10-mM stock solution. U46619 (Sigma-Aldrich, St Louis, MO, USA) was dissolved in ethanol as a 30-mM stock solution. ET-1 (Sigma) was dissolved in H_2O as a 30-μM stock solution. FTY720 was from Cayman Chemical; PE, 5-HT, ACh, L-NAME were from Sigma; they were dissolved as 10-mM stock solution in H_2O.

Results

$S1P_2$ and $S1P_3$ receptor null mice displayed morphologically normal basilar arteries. Their normalized diameter and vasoconstrictor responses to 100 mM KCl, the thromboxane receptor agonist U46619 and ET-1 did not differ from those of WT basilar arteries (Table 1).

Sphingosine 1-phosphate concentration-dependently constricted basilar arteries isolated from either WT or $S1P_2^{-/-}$ mice (Figure 1). S1P did not constrict other mouse arteries (aorta, carotid artery; not shown). S1P was significantly more potent in basilar arteries from $S1P_2^{-/-}$ mice than in basilar arteries from WT mice (Table 1). In most basilar arteries from $S1P_3^{-/-}$ mice, S1P barely affected the vascular tone ($\leqslant$10% of 100 mM KCl-induced contraction). In two preparations, however, S1P induced a weak vasoconstriction (about 30% of 100 mM KCl-induced contraction). S1P-induced vasoconstriction was also significantly impaired in $S1P_3^{+/-}$ (heterozygous) mice (Figure 1 and Table 1).

The higher potency of S1P in arteries from $S1P_2^{-/-}$ may suggest that $S1P_2$ receptor activation inhibits S1P-induced constriction in WT mice. To test this hypothesis, we used JTE-013, a purported inhibitor of $S1P_2$ receptors (Ohmori *et al*., 2003). As shown in Figure 2, 10 μM JTE-013 strongly inhibited S1P-induced contraction in basilar arteries from WT. However, JTE-013 also inhibited the contraction induced by 100 mM KCl (by 64.0 ± 5.5%, $n = 5$, $P < 0.01$), U46619 and ET-1 (Figure 2). Furthermore, the inhibitory effects of JTE-013 on vascular contraction were also present in basilar arteries from $S1P_2^{-/-}$ (Figure 2), suggesting that the effects of JTE-013 are unrelated to $S1P_2$ receptor antagonism.

VPC23019 has been described as an $S1P_{1/3}$ receptor antagonist in stable transfectants expressing either human $S1P_1$ or $S1P_3$ receptors (Davis *et al*., 2005). In a guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate assay, their pK_b values (−log M) for $S1P_1$ and $S1P_3$ receptors were, respectively, 7.49 ± 0.15 and 5.98 ± 0.08. $S1P_1$ receptors have been described as stimulating endothelial release of nitric oxide, which produces vasodilatation (Igarashi *et al*., 2003). We therefore tested the effect of VPC23019 in rat basilar arteries either with intact endothelium or without functional endothelium. As shown in Figure 3a, S1P-induced contractile responses were significantly weaker ($P < 0.01$) in basilar arteries with intact endothelium (relaxation to ACh, 71.0 ± 4.6%, $n = 16$) than in basilar arteries without endothelium (relaxation to ACh, 9.2 ± 3.3%, $n = 16$). VPC23019 significantly increased the S1P-induced vasoconstriction in preparations with intact endothelium ($P < 0.01$), but did not change it in preparations without endothelium. In intact mouse basilar artery (relaxation to ACh, 45.6 ± 7.2%, $n = 16$), VPC23019 shifted to the left the concentration–contraction curve to S1P by a half log (Figure 3b); preincubation with 0.1 mM L-NAME produced a similar leftward shift (Figure 3b). VPC23019 did not modify the contractile responses to KCl, 5-HT or U46619 (not shown).

Phospho-FTY720 is the active form of the prodrug FTY720. It specifically interacts with $S1P_{1,3,4,5}$ but not with $S1P_2$ receptors (Mandala *et al*., 2002). FTY720 has also been shown to inhibit sphingosine phospholyase (the enzyme responsible for irreversible S1P degradation) *in vitro* in a dose-dependent fashion, with effects occurring at concentrations as low as 300 nM, while phospho-FTY720 is inhibitory only at 30 μM (Bandhuvula *et al*., 2005). In rat basilar artery, FTY720 (10 nM–0.1 mM) did not significantly affect the resting tone, or the tone induced by 5-HT or high K^+ (not shown). In contrast, phospho-FTY720 concentration-dependently induced a vasoconstriction (EC_{50} 0.6 ± 0.2 μM, E_{max} 2.04 ± 0.33 mN mm^{-1}, $n = 11$) comparable in amplitude to S1P-induced constriction (Figure 4). Phospho-FTY720 did not induce any significant contraction or relaxation in peripheral arteries such as femoral and mesenteric resistance arteries (not shown).

SEW2871, an $S1P_1$ receptor agonist (Jo *et al*., 2005), did not induce any contraction in rat basilar or mesenteric resistance arteries when tested at concentrations up to 10 μM (Figure 5). Because $S1P_1$ receptor activation has been linked to endothelial nitric oxide synthase (eNOS) activation (Igarashi and Michel, 2000), we tested the potential vasodilatory effect of SEW2871 on basilar, femoral and mesenteric resistance arteries preconstricted with either 1 μM 5-HT or 1–10 μM

Table 1 Morphological and pharmacological parameters of isolated mouse basilar arteries

	Wild type (n = 15)	*$S1P_3^{-/-}$* (n = 6)	*$S1P_3^{+/-}$* (n = 3)	*$S1P_2^{-/-}$* (n = 6)
Normalized diameter (μm)	173 ± 5	178 ± 5	181 ± 4	165 ± 17
Constriction to high (100 mM) K^+ (mN mm^{-1})	0.96 ± 0.08	0.69 ± 0.11	0.89 ± 0.25	0.80 ± 0.13
Constriction to U46619				
pD_2 (EC_{50}, μM)	7.01 ± 0.18 (0.18)	7.22 ± 0.14 (0.07)	6.73 ± 0.04 (0.19)	7.09 ± 0.30 (0.16)
E_{max} (% high K^+)	99 ± 4	124 ± 17	90 ± 3	125 ± 16
Constriction to ET-1				
pD_2 (EC_{50}, nM)	9.86 ± 0.26 (0.28)	9.87 ± 0.53 (0.32)	9.57 ± 0.23 (0.37)	10.12 ± 0.15 (0.09)
E_{max} (% high K^+)	78 ± 8	97 ± 22	53 ± 7	84 ± 19
Constriction to S1P				
pD_2 (EC_{50}, μM)	5.65 ± 0.08 (2.8)	ND (ND)	ND (ND)	6.16 ± 0.17** (1.0)
E_{max} (% high K^+)	78 ± 10	12 ± 4**	24 ± 7*	89 ± 21

Abbreviations: E_{max}, maximal effect; ET-1, endothelin-1; ND, not determined; pD_2, negative logarithm; SIP, sphingosine 1-phosphate.
*$P < 0.05$, **$P < 0.01$ versus wild type; one-way ANOVA and Tukey's HSD test.

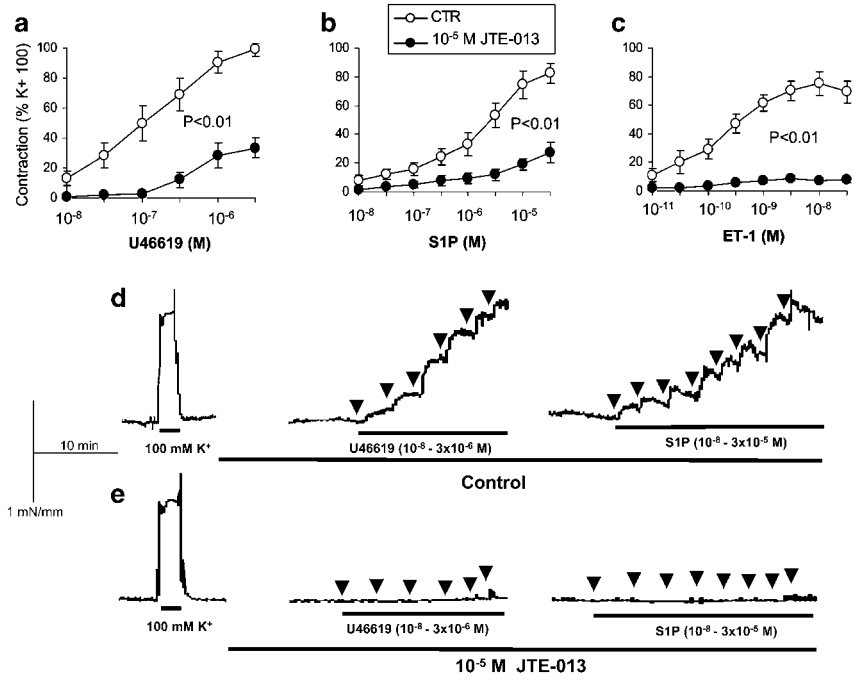

Figure 2 Effect of JTE-013 on the responsiveness of mouse basilar artery to sphingosine 1-phosphate (S1P) and other agonists. (**a**–**c**) Averaged contractile responses to cumulative concentrations of U46619, S1P or endothelin-1 (ET-1) in basilar arteries from wild-type (WT) mice, in the presence or absence of 10 μM JTE-013. Contractile responses are expressed in % of high (100 mM) K^+-induced contractions in the same preparations in the absence of JTE-013. Values are mean and s.e.mean; $n=5$. (**d**, **e**) Typical recordings (experiment repeated twice with similar results) showing the contractile responses to cumulative concentrations of U46619 and S1P, in the absence (**d**) or presence of 10 μM JTE-013 (**e**), in basilar artery isolated from S1P$_2^{-/-}$ mice; arrows indicate the addition of each concentration of U46619 (10^{-8} to 3×10^{-6} M) or S1P (10^{-8} to 3×10^{-5} M), in half-log steps, to the organ bath; contractile responses to high (100 mM) K^+-induced depolarization in the same preparations are shown for comparison. When used, JTE-013 was preincubated for 30 min before challenging with U46619, S1P or ET-1.

phenylephrine. In these preparations (Figure 5), SEW2871 did not elicit any significant vasodilatation. The functional integrity of endothelium was confirmed by challenging the preparations with ACh.

Discussion

This study used S1P receptor null mice (Ishii *et al*., 2001, 2002) to characterize unequivocally the S1P receptors responsible for cerebrovascular smooth muscle contraction. These data demonstrate that basilar arteries from S1P$_3^{-/-}$ mice show very weak or no vasoconstriction to S1P. Furthermore, S1P is a more potent vasoconstrictor in basilar arteries from S1P$_2^{-/-}$ mice than in basilar arteries from WT mice. On the basis of this evidence, we rule out the involvement of S1P$_2$ receptors and suggest that the S1P$_3$ subtype is the major contributor to S1P-induced vasoconstriction in cerebral circulation. However, some redundancy may exist in the signal-transduction pathways downstream of S1P receptor activation (for review see Waeber *et al*., 2004). For example, analysis of Rho activation in S1P$_3^{-/-}$ mouse embryonic fibroblasts showed that S1P activated Rho to an extent similar to that observed in WT cells (Ishii *et al*., 2001; Liu *et al*., 2003), an effect attributed to S1P$_2$ receptor stimulation, while PLC activation by S1P was clearly blunted in S1P$_3^{-/-}$ mouse embryonic fibroblasts. Considering that both Rho-Rho kinase and PLC are involved in the vasoconstriction induced by S1P in cerebral arteries (Salomone *et al*., 2003), an S1P$_2$/S1P$_3$ receptor redundancy may explain the weak contraction to S1P (about 30% of high K^+-induced tone) that we observed in two out of six S1P$_3^{-/-}$ mice. Furthermore, homeostatic compensatory mechanisms could occur in S1P$_3^{-/-}$ mice and may include receptor-independent effects of S1P, as recently implicated in smooth muscle contraction (Leiber *et al*., 2007).

The genetic evidence for a major role of S1P$_3$ receptors in S1P-induced vasoconstriction of cerebral arteries is reinforced by the observation that S1P-induced vasoconstriction in heterozygous S1P$_3^{+/-}$ mice was also blunted. It is worthy of note that basilar arteries from both S1P$_3^{-/-}$ and S1P$_3^{+/-}$ exhibited normal responses to other vasoconstrictors, such as high K^+-induced depolarization, U46619 and ET-1.

Because we observed a higher potency of S1P in arteries from S1P$_2^{-/-}$, we attempted to determine whether this receptor subtype inhibits vascular smooth muscle contraction by using JTE-013, a compound previously shown to inhibit contraction of coronary artery smooth muscle cells via S1P$_2$ receptor blockade (Ohmori *et al*., 2003). In our hands, however, JTE-013 not only inhibited S1P-induced contraction in basilar arteries but also inhibited the contraction induced by 100 mM KCl, U46619 and ET-1, suggesting that this effect was not specifically related to S1P receptor antagonism. Furthermore, the inhibitory effect of JTE-013 was also present in basilar arteries from S1P$_2^{-/-}$ mice, indicating that it was completely unrelated to antagonism of S1P$_2$ receptors. It is worth mentioning that the JTE-013 concentration used in our study (10 μM) is similar to the

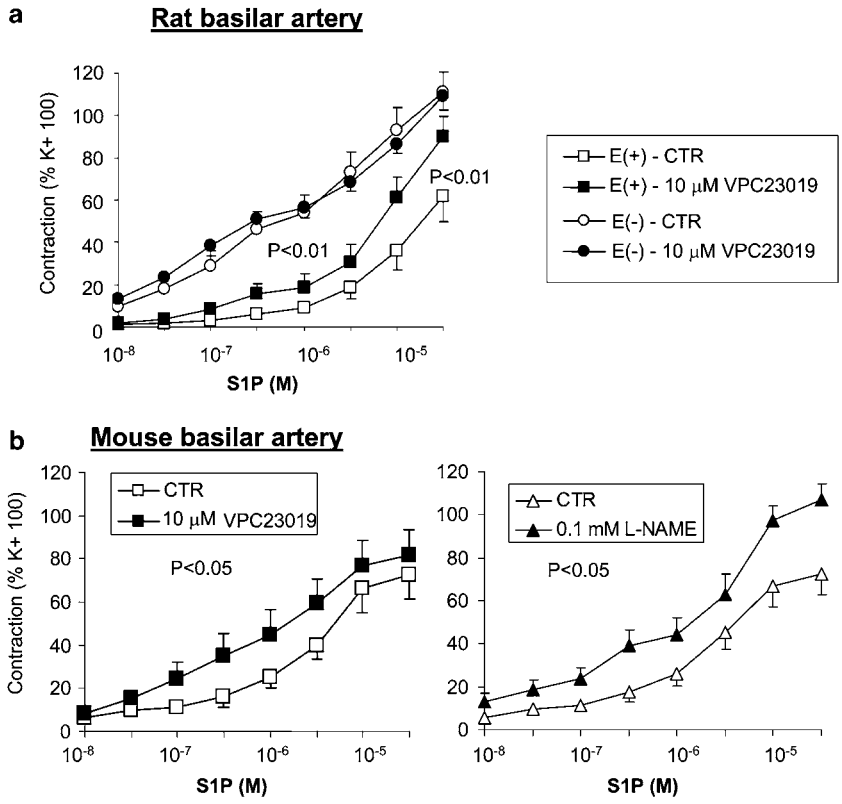

Figure 3 Effect of VPC23019 on the responsiveness of basilar artery from rat (**a**) and mouse (**b**) to sphingosine 1-phosphate (S1P). Contractile responses to cumulative concentrations of S1P, expressed in % of high (100 mM) K^+-induced contractions in the same preparations, are shown as mean and s.e.mean ($n=7$–9). Isolated basilar arteries were incubated for 30 min with VPC23019 (10 μM), vehicle (5 μl dimethyl sulphoxide; final concentration: 0.1%) or 0.1 mM *N*-ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) before being challenged with cumulative concentrations of S1P. Nonlinear fits for each individual data set were compared by F-test.

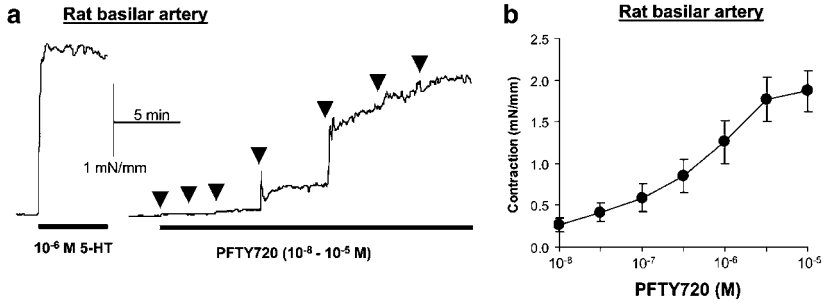

Figure 4 Vasoconstriction to phospho-FTY720 in isolated rat basilar artery. (**a**) Typical recording showing the contractile responses to cumulative concentrations of phospho-FTY720; arrows indicate the addition of each concentration of phospho-FTY720, from 10^{-8} to 10^{-5} M, in half-log steps, to the organ bath; the contractile response to 5-hydroxytryptamine (5-HT, 1 μM) is shown for comparison. (**b**) Averaged contractile responses to cumulative concentrations of phospho-FTY720: mean and s.e.mean ($n=12$). The corresponding pharmacological parameters are given in the text.

concentrations used in previous studies (Osada *et al*., 2002; Ohmori *et al*., 2003), and we found significant inhibition of KCl- and U46619-induced contraction even when using 1 μM JTE-013 (data not shown).

VPC23019 has been described as an S1P$_{1/3}$ receptor antagonist, exhibiting pK_b values of 7.5 and 6.0 for the S1P$_1$ and S1P$_3$ receptors, respectively (Davis *et al*., 2005); that is, the reported affinity of VPC23019 for S1P$_1$ receptor subtype is 30 times higher than that for S1P$_3$ receptors (Davis *et al*., 2005). S1P$_1$ receptors have been linked to activation of eNOS (Yatomi *et al*., 2000; Igarashi *et al*., 2001), which produces vasodilatation (Igarashi *et al*., 2003). Conceivably, S1P$_1$ receptor antagonism could increase S1P-induced vasoconstriction, by inhibiting a relaxing effect of S1P via eNOS activation. We observed that VPC23019 potentiated S1P-induced contractile response in both rat and mouse basilar arteries with intact endothelium; however, it failed to do so in preparations without endothelium. Our interpretation is therefore that VPC23019 might shift the S1P contraction curve to the left by inhibiting S1P$_1$ receptor-induced NO

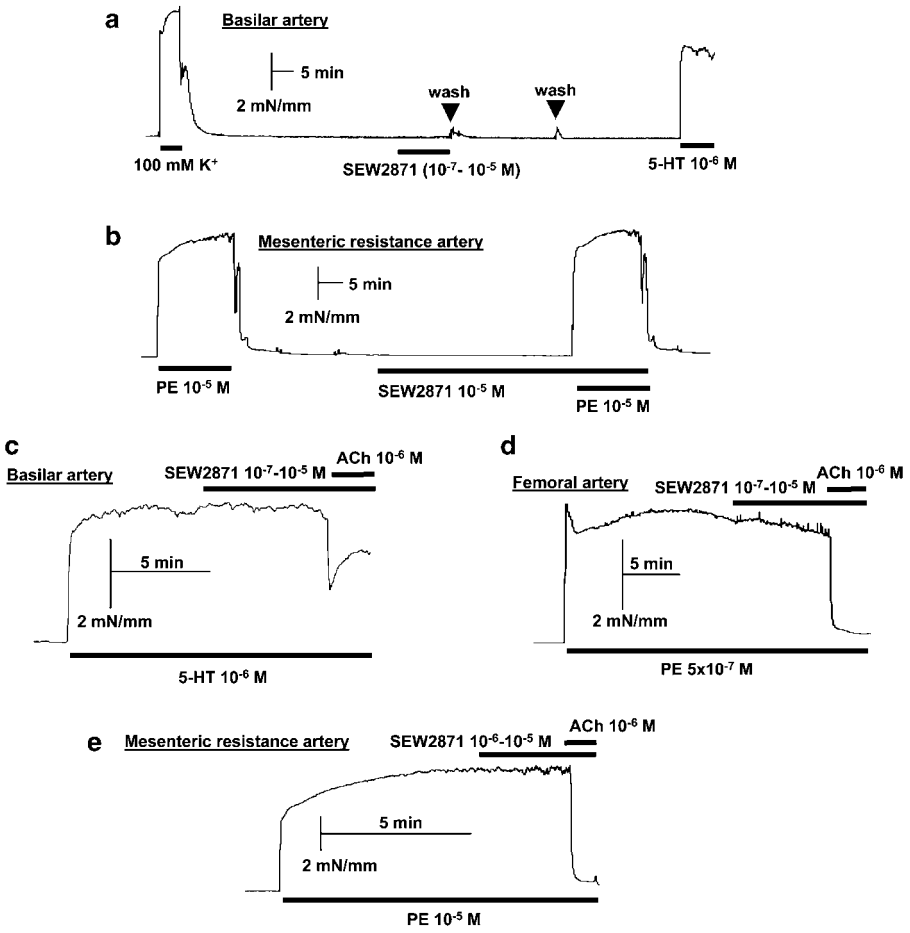

Figure 5 Absence of vasoconstriction (**a**, **b**) or vasodilatation (**c**–**e**) to SEW2871 in different arteries isolated from rat. Typical recordings show the lack of effect of SEW2871, either as single concentration or as cumulative concentrations (by half-log steps), in comparison with positive controls for vasoconstriction, such as high (100 mM) K^+-induced depolarization and 5-hydroxytryptamine (5-HT, 1 μM) in basilar artery (**a**) and phenylephrine (PE, 10 μM) in mesenteric resistance artery (**b**). For endothelium-dependent vasodilatation, acetylcholine (ACh; 1 μM) was used as a positive control in basilar (**c**), femoral (**d**) and mesenteric resistance arteries (**e**). Each preparation/condition was tested 3–4 times with similar results.

release, while it only weakly inhibited the S1P$_3$-mediated contraction, even at the highest concentration we tested. This is not unexpected, considering its published 30-fold lower affinity for the S1P$_3$ subtype. The observation that removing endothelium or incubating with the NOS inhibitor L-NAME shifts to the left the curve to S1P is consistent with an endothelial pathway of vasodilatation sensitive to S1P, presumably acting at S1P$_1$ receptor. It is also important to mention that VPC23019 binding to S1P receptors has been characterized in an HEK293T cell line overexpressing human S1P receptors (Davis *et al*., 2005), a system that may differ from our experimental system; for instance, minor species-specific differences in the primary sequence of human versus rodent S1P receptors may critically affect the drug-binding profile of S1P$_3$ receptors, such that the affinity of VPC23019 for rodent S1P$_3$ might be even lower than that for human S1P$_3$.

When using the S1P$_1$ receptor agonist SEW2871 (Jo *et al*., 2005; Wei *et al*., 2005), up to 10 μM, we did not observe any relaxation in rat basilar, femoral or mesenteric resistance arteries (Figure 5), where the presence of a functional endothelium was routinely confirmed by the relaxing effect of ACh. In our experimental system, SEW2871 appears therefore ineffective as a vasodilator in these vessels, suggesting that it does not have sufficient affinity and/or efficacy at the rodent endothelial S1P$_1$ receptors. In some vessels, however, S1P receptor subtypes other than S1P$_1$ may be involved in S1P-induced endothelium-dependent vasodilatation. For example, S1P$_3$ receptors have been shown to mediate vasodilatation in mouse aortas (Nofer *et al*., 2004; Tolle *et al*., 2005) when challenged with phospho-FTY720, an observation that we reproduced in our laboratory (result not shown), confirming previous reports.

In conclusion, because phospho-FTY720 is an agonist for all S1P receptors except S1P$_2$ (Brinkmann *et al*., 2002; Mandala *et al*., 2002), the fact that phospho-FTY720 potently constricted rat cerebral arteries, taken together with our observations in basilar arteries from S1P$_2^{-/-}$ mice, further reinforces the notion that S1P$_2$ receptors are not involved in cerebrovascular constriction. The purported S1P$_2$ receptor antagonist JTE-013 does not appear to be selective, at least in rodents. Our data show that S1P-induced vasoconstriction

in cerebral arteries is mediated by $S1P_3$ receptors. Enhancement of S1P-induced contraction by VPC23019 might be related to blockade of $S1P_1$ receptors and NO generation.

Acknowledgements

This study was supported by NIH Grants NS052195 and NS049263 (to CW) and NS048478, DA019674 and MH51699 (to JC).

Conflicts of interest

The authors state no conflict of interest.

References

Anliker B, Chun J (2004). Lysophospholipid G protein-coupled receptors. *J Biol Chem* **279**: 20555–20558.

Bandhuvula P, Tam YY, Oskouian B, Saba JD (2005). The immune modulator FTY720 inhibits sphingosine-1-phosphate lyase activity. *J Biol Chem* **280**: 33697–33700.

Bischoff A, Czyborra P, Meyer Zu Heringdorf D, Jakobs KH, Michel MC (2000). Sphingosine-1-phosphate reduces rat renal and mesenteric blood flow *in vivo* in a pertussis toxin-sensitive manner. *Br J Pharmacol* **130**: 1878–1883.

Brinkmann V, Davis MD, Heise CE, Albert R, Cottens S, Hof R *et al*. (2002). The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J Biol Chem* **277**: 21453–21457.

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

Davis MD, Clemens JJ, Macdonald TL, Lynch KR (2005). Sphingosine 1-phosphate analogs as receptor antagonists. *J Biol Chem* **280**: 9833–9841.

Gardell SE, Dubin AE, Chun J (2006). Emerging medicinal roles for lysophospholipid signaling. *Trends Mol Med* **12**: 65–75.

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, Bernier SG, 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.

Igarashi J, Erwin PA, Dantas AP, Chen H, Michel T (2003). VEGF induces $S1P_1$ receptors in endothelial cells: implications for cross-talk between sphingolipid and growth factor receptors. *Proc Natl Acad Sci USA* **100**: 10664–10669.

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.

Ishii I, Friedman B, Ye X, Kawamura S, McGiffert C, Contos JJ *et al*. (2001). Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP(B3)/EDG-3. *J Biol Chem* **276**: 33697–33704.

Ishii I, Ye X, Friedman B, Kawamura S, Contos JJ, Kingsbury MA *et al*. (2002). Marked perinatal lethality and cellular signaling deficits in mice null for the two sphingosine 1-phosphate (S1P) receptors, S1P(2)/LP(B2)/EDG-5 and S1P(3)/LP(B3)/EDG-3. *J Biol Chem* **277**: 25152–25159.

Jo E, Sanna MG, Gonzalez-Cabrera PJ, Thangada S, Tigyi G, Osborne DA *et al*. (2005). S1P1-selective *in vivo*-active agonists from high-throughput screening: off-the-shelf chemical probes of receptor interactions, signaling, and fate. *Chem Biol* **12**: 703–715.

Lee MJ, Evans M, Hla T (1996). The inducible G protein-coupled receptor edg-1 signals via the G(i)/mitogen-activated protein kinase pathway. *J Biol Chem* **271**: 11272–11279.

Leiber D, Banno Y, Tanfin Z (2007). Exogenous sphingosine 1-phosphate and sphingosine kinase activated by endothelin-1 induced myometrial contraction through differential mechanisms. *Am J Physiol Cell Physiol* **292**: C240–C250.

Liu H, Toman RE, Goparaju SK, Maceyka M, Nava VE, Sankala H *et al*. (2003). Sphingosine kinase type 2 is a putative BH3-only protein that induces apoptosis. *J Biol Chem* **278**: 40330–40336.

Mandala S, Hajdu R, Bergstrom J, Quackenbush E, Xie J, Milligan J *et al*. (2002). Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* **296**: 346–349.

McGiffert C, Contos JJ, Friedman B, Chun J (2002). Embryonic brain expression analysis of lysophospholipid receptor genes suggests roles for s1p(1) in neurogenesis and s1p(1–3) in angiogenesis. *FEBS Lett* **531**: 103–108.

Mulvany MJ, 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 *et al*. (2000). Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions. *Biochem J* **352** (Part 3): 809–815.

Nofer JR, van der Giet M, Tolle M, Wolinska I, von Wnuck Lipinski K, Baba HA *et al*. (2004). HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. *J Clin Invest* **113**: 569–581.

Ohmori T, Yatomi Y, Osada M, Kazama F, Takafuta T, Ikeda H *et al*. (2003). Sphingosine 1-phosphate induces contraction of coronary artery smooth muscle cells via S1P2. *Cardiovasc Res* **58**: 170–177.

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

Osada M, Yatomi Y, Ohmori T, Ikeda H, Ozaki Y (2002). Enhancement of sphingosine 1-phosphate-induced migration of vascular endothelial cells and smooth muscle cells by an EDG-5 antagonist. *Biochem Biophys Res Commun* **299**: 483–487.

Salomone S, Yoshimura S, Reuter U, Foley M, Thomas SS, Moskowitz MA *et al*. (2003). S1P(3) receptors mediate the potent constriction of cerebral arteries by sphingosine-1-phosphate. *Eur J Pharmacol* **469**: 125–134.

Tolle M, Levkau B, Keul P, Brinkmann V, Giebing G, Schonfelder G *et al*. (2005). Immunomodulator FTY720 induces eNOS-dependent arterial vasodilatation via the lysophospholipid receptor S1P3. *Circ Res* **96**: 913–920.

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

Waeber C, Blondeau N, Salomone S (2004). Vascular sphingosine-1-phosphate S1P1 and S1P3 receptors. *Drug News Perspect* **17**: 365–382.

Wei SH, Rosen H, Matheu MP, Sanna MG, Wang SK, Jo E *et al*. (2005). Sphingosine 1-phosphate type 1 receptor agonism inhibits transendothelial migration of medullary T cells to lymphatic sinuses. *Nat Immunol* **6**: 1228–1235.

Yang AH, Ishii I, Chun J (2002). *In vivo* roles of lysophospholipid receptors revealed by gene targeting studies in mice. *Biochim Biophys Acta* **1582**: 197–203.

Yatomi Y, Igarashi Y, Yang L, Hisano N, Qi R, Asazuma N *et al*. (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.

Yatomi Y, Ohmori T, Rile G, Kazama F, Okamoto H, Sano T *et al*. (2000). Sphingosine 1-phosphate as a major bioactive lysophospholipid that is released from platelets and interacts with endothelial cells. *Blood* **96**: 3431–3438.