Sphingosine 1-Phosphate Contracts Canine Basilar Arteries
In Vitro and In Vivo
Possible Role in Pathogenesis of Cerebral Vasospasm

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Background and Purpose—Sphingosine 1-phosphate (S1P) is a platelet-derived bioactive lipid that exerts a variety of biological responses, including vasocontraction. To understand the involvement of S1P in cerebral vasospasm, we investigated the effect of S1P on vasocontraction of the canine basilar artery in vitro and in vivo.

Methods—we recorded isometric tension in basilar arterial rings from dogs in vitro and estimated time-course changes in the diameter of canine basilar arteries and the S1P concentration in cerebrospinal fluid (CSF) by angiography and radioreceptor assays, respectively, after administering S1P into the cisterna magna. Changes in the supernatant S1P concentration during clot formation were monitored by using the in vitro subarachnoid hemorrhage model, in which blood is mixed with CSF.

Results—at concentrations ranging between $100 \text{nmol/L}$ and $10 \mu\text{mol/L}$, S1P induced a dose-dependent contraction of the basilar artery in vitro. This effect was significantly inhibited by Y-27632, a highly selective Rho-kinase inhibitor. The administration of S1P into the CSF induced a 60% to 70% decrease in the arterial diameter within 15 minutes, and vasocontraction continued for 2 days thereafter. The concentration of S1P in the supernatant during clot formation in vitro reached $\approx 300 \text{ nmol/L}$.

Conclusions—S1P induces vasocontraction in the canine basilar artery in vitro and in vivo, possibly through a mechanism involving activation of the Rho/Rho-kinase pathway. Thus, S1P might be considered as a novel spasmogenic substance involved in cerebral vasospasm after subarachnoid hemorrhage. (Stroke. 2001;32:2913-2919.)

Key Words: lipids ■ subarachnoid hemorrhage ■ vasospasm ■ dogs

The pathogenesis of cerebral vasospasm after subarachnoid hemorrhage (SAH) has been intensively investigated but is still not fully understood. Several spasmogenic candidates have been suggested on the basis of clinical and experimental approaches. These include oxyhemoglobin, endothelin-1, thrombin, serotonin, noradrenalin, and thromboxane $A_2$. Cerebral vasospasm may also be induced by the cooperative action of these vasocontractors rather than by a single factor. Cerebral vasospasm is associated with changes in the activity of several intracellular signal transduction pathways, including the activation of small GTPase Rho A and Rho-kinase. Activation of Rho-kinase by Rho A causes phosphorylation of the myosin-binding subunit of the myosin phosphatase complex, which leads to the inactivation of myosin phosphatase. Thus, the stimulation of small G-protein signaling results in the continuous contraction of cerebral arteries without changes in intracellular $\text{Ca}^{2+}$ concentration ([Ca$^{2+}$]), which may be responsible for the pathophysiology of cerebral vasospasm. However, the upstream regulatory mechanisms of the Rho/Rho-kinase system in cerebral vasospasm remain unknown.

Sphingosine 1-phosphate (S1P) has recently been paid much attention as a bioactive molecule that can be released from platelets and that stimulates several intracellular signaling events, including the Rho/Rho-kinase system. This lipid mediator stimulates these signaling events through a family of G-protein–coupled receptors called the endothelial differentiation gene, although it has also been reported that the lipid in some cases stimulates the cells through intracellular targets. S1P exerts the contractile effects on renal and mesenteric microvessels in vitro and in vivo. However, the effect on cerebral arteries has not been investigated. The present study investigated whether or not S1P induces vasocontraction in the canine basilar artery. The results showed that S1P potently contracted cerebral arteries possibly...

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through Rho/Rho-kinase activation. Thus, S1P might be considered as a novel spasmogenic substance involved in cerebral vasospasm after SAH.

Materials and Methods

Drugs
SIP, purchased from Cayman Chemical Co., was dissolved in distilled water containing 10 mmol/L NaOH and stored at −20°C. (+)-R-trans-4-[(1-Aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632) was obtained from Welfide Corp.25,26 The Rho-kinase inhibitor was dissolved in distilled water and stored at 4°C. Iomeprol (containing 300 mg/mL of iodine), which was used for serial angiography, was obtained from Eisai Co, Ltd. All other reagents were of the highest grade.

In Vitro Assessment of Basilar Artery Contraction

All experiments were performed according to the rules governing animal experimentation and the Guidelines for the Care and Use of Laboratory Animals of Gunma University School of Medicine. Rings of canine basilar arteries were obtained from 14 adult mongrel dogs of both sexes (8 to 12 kg) after pentobarbital sodium anesthesia (30 mg/kg IV) and placed in HEPES-buffered Krebs solution at pH 7.4. The luminal surface of the vessels was perfused with 0.1% Triton X for 60 seconds to remove the endothelium, unless otherwise specified.27 In some experiments shown in Figure 1A and 1B, to evaluate the contribution of endothelium, Triton X treatment was not performed. The removal of endothelium was confirmed by the experiment in which the endothelium-lacking rings precontracted with prostaglandin F2α, failed to relax (>15%) to substance P (1 to 10 nmol/L).27 The 4-mm-long strips were placed in organ baths containing 5 mL normal Krebs-Ringer bicarbonate solution (mmol/L: NaCl 125, KCl 4.0, MgSO4 1.5, NaHCO3 14.0, NaH2PO4 1.2, CaCl2 1.9, and glucose 11.0) and continuously bubbled with 95% O2 and 5% CO2 (pH 7.4). The strips were set at 1.0 g of resting tension (T0) and the strips were routinely changed every 15 minutes. After equilibration, the strips were exposed to 40 mmol/L KCl until the contractile responses were stabilized. After another wash, cumulative dose-response curves for the contractile responses to SIP were examined. Control strips were exposed to 10 mmol/L NaOH (SIP solvent). (The contractions were not observed.) In another series of experiments, the rings were incubated with 10 μmol/mL SIP to obtain a stable plateau contraction, and then increasing doses of Y-27632 were applied, or the rings were first incubated with 10 μmol/L Y-27632 for 15 minutes, and then SIP- or KCl-induced contractions were measured. The tensions induced by 40 mmol/L KCl were 1.62 ± 0.16 g (n = 8) for endothelium-intact vessels and 2.02 ± 0.16 g (n = 13) for endothelium-lacking (Triton X–treated) vessels. Thus, Triton X treatment did not appreciably influence the contractile activity by KCl.

In Vivo Experimental Model and Angiographic Assessment of Basilar Artery Contraction

Eleven adult mongrel dogs of both sexes (9 to 12 kg) were anesthetized by pentobarbital sodium (30 mg/kg IV). Endotracheal intubation was performed, and respiration was mechanically controlled by use of a Harvard respirator (tidal volume 200 mL, respiratory rate 14 cycles/min). Each dog was placed in a supine position, the head was fixed, the left vertebral artery was catheterized via the right femoral artery, and control vertebral angiography was performed by using 5 mL of a contrast agent (iomeprol). The cisterna magna was atraumatically punctured by using a 21-gauge spinal needle. Cerebrospinal fluid (CSF, 4 mL) was collected and mixed with SIP or vehicle (1 mL in 10 mmol/L NaOH solution). The CSF/SIP mixture (total 5 mL) was then injected into the cisterna magna at a final concentration of 0 (vehicle), 25, or 50 nmol/kg SIP. Angiograms were obtained at 15, 30, 60, 120, and 180 minutes after the injection. Angiography performed at 24, 48, 96, and 144 hours after SIP injection was performed with the animals reassésthetized, intubated, and under controlled ventilation. During the experiments, the animals were maintained on a standard diet of pellets and water. The diameters of basilar arteries on films were measured 3 cm from the bifurcation of the basilar artery by using a ×10 surgical scope with a metric reticle graduated in 0.1-mm increments. The real diameter of the basilar artery on each angiogram was first expressed as mean±SEM, and then the relative diameter was plotted as percent change compared with the pretreatment mean (mean of pretreatment = 100%).1,4,5,8,12

Quantitative Measurement of SIP in In Vivo Experimental Model

Four adult mongrel dogs of both sexes (10 to 11 kg) were anesthetized as described above. Approximately 0.5 mL of CSF was collected before 50 nmol/kg SIP was injected into the cisterna magna as described above. Thereafter, ~0.5 mL CSF was obtained at various intervals from 15 to 300 minutes after injection. Levels of SIP in these CSF samples were quantified by a recently established radioreceptor-binding assay as described.17,18

Analysis of SIP Content in Supernatant of Mixed Blood and CSF

To reproduce the situation in the subarachnoid space after SAH, CSF and autologous blood from anesthetized healthy dogs were coincubated at 37°C at a relative proportion of 2:1 for the indicated periods. All experiments were performed under sterile conditions. At the indicated times, the CSF/blood mixture was centrifuged at 3000 rpm for 15 minutes to collect the cell-free supernatant, which was stocked at ~80°C. The hemoglobin concentration and number of white blood cells in the CSF/blood mixture at this ratio in vitro were similar to those in the subarachnoid space of SAH patients.28 This procedure mimicked conditions in the subarachnoid space after SAH and, therefore, served as an in vitro model.29

Data Analysis

Results are expressed as mean±SEM. The pEC50 and pIC50 values in the in vitro vasocontraction study were calculated by fitting sigmoidal functions to the experimental data by using the GraphPad Software, because the SIP concentration-response curves did not reflect saturating maximal responses. Statistical evaluation of the results was performed by ANOVA, with the Bonferroni/Dunn test used for multiple comparisons. A value of P < 0.05 was considered statistically significant.

Results

SIP Contracts Canine Basilar Arteries, and Lipid Action Is Inhibited by Y-27632 In Vitro

Figure 1A shows a representative contraction tracing of a cerebral arterial ring with (top of panel A) or without (bottom of panel A) endothelium. In the endothelium-intact ring, the response to SIP was rapid compared with the ring without endothelium, but the peak response was followed by a prompt decrease and then a slow increase. On the other hand, in the endothelium-lacking ring, SIP induced a slow response, but the contraction was sustained. Thus, the contractile response patterns were totally different from each other. This may reflect the influence of endothelium-dependent NO production by SIP.29 However, there was no significant difference in
the peak response obtained by the respective dose of S1P, although the presence of endothelium tended to decrease the S1P-induced contractile response (Figure 1B). Thus, the secondary effect through the endothelium on the smooth muscle contractile activity seems to be very small, if not negligible, in our experimental conditions. Because of the unstable contraction pattern, we used arterial rings without endothelium in the following experiments.

To determine whether the Rho/Rho-kinase system is involved in the S1P-induced action, the rings were first contracted by 10 μmol/L S1P, and then cumulative amounts of Y-27632 were added to the incubation medium (Figure 1C). The inhibitory effect of the indicated concentrations of Y-27632 is expressed as percentage of the contractile response to 10 μmol/L S1P in the absence of inhibitor (D). Data are mean±SEM of 7 separate experiments. 

**Figure 1.** S1P induces vasocontraction, and the lipid action is inhibited by Y-27632 in isolated canine basilar arterial rings. A, Typical tracings of KCl and dose-dependent contractile effects of S1P in the ring with (top) or without (bottom) endothelium. B, Contractile responses to the indicated concentration of S1P in the rings with (open circles) or without (solid circles) endothelium (E) expressed as percentage of the response induced by 40 mmol/L KCl alone. The pEC50 value in endothelium-lacking rings was calculated as 5.95±0.05. In the endothelium-intact rings, however, the pEC50 value could not be determined by curve fitting. Data are mean±SEM of 8 (with endothelium) and 13 (without endothelium) separate experiments. C and D, Effects of indicated concentrations of Y-27632 on 10 μmol/L S1P–induced contractile response in the endothelium-lacking basilar artery. Typical tracings of contractile response to S1P and of effects of Y-27632 on S1P-induced action are shown (C). The inhibitory effect of the indicated concentrations of Y-27632 is expressed as percentage of the contractile response to 10 μmol/L S1P in the absence of inhibitor (D). Data are mean±SEM of 7 separate experiments. E and F, Effects of pretreatment of Y-27632 on the S1P-induced contractile response in the endothelium-lacking basilar artery. Typical tracings of the effects of pretreatment with 10 μmol/L Y-27632 on the 40 mmol/L KCl and 10 μmol/L S1P–induced contraction are shown (E). The addition of Y-27632 caused a relaxation of the resting or basal tension; the magnitude corresponded to 14.5±1.1% of the tension obtained by 40 mmol/L KCl alone (n=8). KCl and S1P (10 μmol/L)–induced contractile response in the presence or absence of Y-27632 (Y, 10 μmol/L) is expressed as percentage of the control contraction obtained by KCl (40 mmol/L) without Y-27632 treatment (F). Data are mean±SEM of 4 separate experiments. **P<0.005, indicating that effect of Y-27632 is significant. For panels A, C, and E, vertical bar denotes tension (g); horizontal bar, time (min).
and 1D). The highly selective Rho-kinase inhibitor inhibited the S1P action in a concentration-dependent manner, with a pIC_{50} value of 5.80±0.04 (n=5). In another series of experiments, rings of canine basilar artery were incubated for 15 minutes with Y-27632 at 10 µmol/L, and then the contractile activities of S1P (10 µmol/L) and KCl (40 mmol/L) were monitored (Figure 1E). Under these conditions as well, the S1P-induced response was markedly inhibited, whereas the KCl-induced response was only slightly inhibited (Figure 1E and 1F).

S1P Induces Long-Lasting Vasocontraction of the Canine Basilar Artery In Vivo
S1P was injected into the cisterna magna, and angiographic vasoconstriction of the basilar artery was then monitored in vivo. In Figure 2A, representative angiograms from a dog injected with 50 nmol/kg S1P show vasoconstriction within 15 minutes, and a significant contractile activity persisted for 2 to 5 days after the S1P injection. The diameters of basilar arteries on angiograms from dogs treated with solvent (sham-operated dogs) or dogs treated with S1P at 50 nmol/kg or 25 nmol/kg are summarized in Figure 2B. In sham-operated dogs, the diameter of the artery did not significantly change. When 50 nmol/kg S1P was injected, the diameter of the artery was decreased to a maximum of 60% to 70% of the initial diameter at 15 minutes, and this persisted for 2 days. In the dogs injected with 25 nmol/kg S1P, the effect was smaller than that induced by 50 nmol/kg S1P, but the vasocontractile effect was significant from 15 minutes to 24 hours after the lipid injection.

Change in S1P Concentration in CSF After Lipid Injection
Under these experimental conditions in vivo, we monitored the S1P concentration in the CSF. Before the lipid injection, the S1P concentration in the CSF was below the limits of detection of our assay, i.e., <20 nmol/L. This value was significantly increased by an injection of S1P (50 nmol/kg). At 15 minutes, the S1P concentration reached 23.4±3.5 µmol/L (n=4) (Figure 2C). Although the S1P-induced vasocontraction was persistent (Figure 2B), the S1P concentration decreased to the basal level within 5 hours after the lipid injection (Figure 2C).

SIP Is Released in Supernatant of the Mixture of CSF and Blood in Association With Clotting
SIP is released by platelets during blood clotting. To confirm that blood clots formed in CSF actually release S1P, we mimicked the conditions in the subarachnoid space of patients with SAH in vitro by incubating CSF with autologous blood from healthy dogs. The S1P concentration in the supernatant in the mixture increased from 45±11 to 285±31 nmol/L (n=5). S1P in the initial sample may have been derived from plasma (Figure 3A). When clotting of the blood was suppressed by sodium citrate, the increase in the S1P concentration was distinctly suppressed (Figure 3B). Thus, the increase in S1P in the mixture of CSF and blood may be due to platelet activation.

Discussion
The present study shows that S1P, a novel sphingolipid mediator, contracts the canine basilar artery in vitro and in vivo. The vasoconstricting activity of S1P has recently been identified in rat mesenteric and intrarenal microvessels. One key difference in vasoconstriction between different sources of vessels is the minimum effective dose of S1P. In the rat mesenteric and intrarenal microvessels, the minimum effective dose was ≈3 µmol/L, whereas it was 100 nmol/L in the canine basilar arteries. However, it remains unknown whether the higher sensitivity of the basilar artery to S1P is due to the differences in species or the location of the vessel.

Blood clotting surrounding the cerebral vessels after SAH may be an important process in terms of stopping bleeding and healing the wound, but it may also be responsible for cerebral vasospasm, which leads to cerebral ischemia. Actually, platelets isolated from the blood caused cerebral vasospasm in experimental animal models. Moreover, removal of subarachnoid clots is an effective means of preventing cerebral vasospasm. S1P is abundant in platelets and is released on stimulation with thrombin or phorbol ester. These results raised the possibility that S1P released from clots formed in the subarachnoid space might be involved in vasospasm after SAH. Homogenized samples of subarachnoid clots or spastic vessels obtained from in vivo experimental SAH models have been widely used to measure several substances that induce cerebral vasospasm. However, it is difficult technically to estimate S1P released into extracellular space by these methods, because the intracellular concentration of S1P is high. Thus, we coincubated autologous blood with CSF in vitro to mimic the conditions of blood clots around the cerebral arteries, and we measured the S1P concentration in the supernatant of mixed blood and CSF without homogenization. The study showed a significant amount of S1P in the plasma, and the lipid content in the supernatant after mixing CSF and blood in vitro increased to ≈300 nmol/L. The in vitro situation is assumed to reflect that of the subarachnoid space after SAH. This concentration of S1P exerted only a small but significant effect on vasoconstriction according to the in vitro experiments (Figure 1B). Because, in actual SAH, the cerebral arteries are surrounded by a large amount of blood and may directly contact platelets in the blood clots, we assume that the S1P concentration in the vicinity of the blood clots surrounding the cerebral vessels may be far higher than the experimental value. In addition, other spasmogenic substances in cooperation with S1P might also participate in vasospasm, as discussed later.

In the untreated basal CSF, the S1P content was <20 nmol/L, which was below the limits of detection of our assay. The administration of S1P at 50 nmol/kg into the cisterna magna caused a sharp increase in the S1P concentration in the subarachnoid space near the basilar artery to 23 µmol/L after the initial 15 minutes. This value rapidly decreased to <20 nmol/L within 5 hours after the S1P injection. The half-life of S1P was estimated to be ≈10 minutes (Figure 2C). On the other hand, the half-life of the injected oxyhemoglobin was estimated to be ≈4 hours, which may reflect the time required for the diffusion or...
washing out in CSF. Thus, the rapid decline of S1P from CSF suggests the rapid degradation or uptake into the surrounding cells or tissues, reflecting the amphipathic nature. In spite of the rapid decline of S1P in CSF, vasocontraction of the basilar artery induced by S1P was persistent. Vasospasm continued even 2 days after the injection. According to the in vitro experiments, >100 nmol/L S1P was required to exert a significant effect on vasocontraction. Thus, once the vessel was stimulated by S1P, the contractile activity seemed to continue independently of the extracellular S1P concentration. The intracellular or extracellular synergism might partly explain the long-lasting contraction by S1P, as discussed later. However, we cannot completely exclude the possibility that S1P absorbed or retained in the vascular wall stimulates smooth muscle cells persistently.

Figure 2. Effects of S1P injection into cisterna magna on vasocontraction of basilar artery and lipid concentration in CSF. S1P (25 nmol/L or 50 nmol/L) or vehicle was injected into the cisterna magna, and contractile effects on basilar arteries were examined as described in Materials and Methods. A, Typical angiograms of the basilar artery before (Pre) and after S1P (50 nmol/L) injection are shown. B, Diameter of basilar artery was measured at the indicated time, and the relative value is plotted as a percentage of the pretreated value. Pretreated diameter was 1.21±0.04 mm for the vehicle group (n=3), 1.20±0.03 mm for the 25 nmol/L S1P group (n=3), and 1.19±0.02 mm for the 50 nmol/L S1P group (n=5). Data are mean±SEM. *P<0.05 and **P<0.005 indicate significant differences from basal value. C, Concentration of S1P was measured after 50 nmol/L S1P was injected. When the level was marginal on this scale, actual values are shown on respective columns. Data are mean±SEM of 4 separate experiments.
Figure 3. Time-course change of S1P concentration in the mixture of CSF and blood incubated in vitro. A, CSF and blood were incubated at 37°C for the indicated periods at a ratio of 2:1. B, Experimental conditions were essentially the same as those for panel A, except the mixture was incubated at room temperature in the presence (+, solid triangles) or absence (–, solid circles) of sodium citrate. The CSF/blood mixture was then centrifuged to obtain supernatant, and S1P concentration was measured. Data are mean ± SEM of 5 separate experiments (A) and means of 2 separate experiments (B).

An increase in [Ca^{2+}]_{i} and subsequent myosin light chain kinase activation lead to the phosphorylation of the myosin light chain, a pivotal process for the contraction of smooth muscle cells. Recent studies have revealed that in addition to the Ca^{2+}/myosin light chain kinase pathway, Rho-dependent activation of Rho-kinase is involved in regulation of the phosphorylation of the myosin light chain. Rho-kinase causes the inhibition of myosin phosphatase by phosphorylating the myosin-binding subunit of the enzyme.12,25,26 Modification of the enzyme system results in an increase in the sensitivity of Ca^{2+} for contraction and thereby induces persistent vasoconstriction of smooth muscle cells. Several trimeric G-protein–coupled receptors, including S1P, lysophosphatidic acid, thrombin, and serotonin receptors, are linked to the Rho/Rho-kinase pathway.12,26 Small G-protein signaling is activated in vessels after experimental SAH.12,13 The present study showed that Y-27632, a potent inhibitor of Rho-kinase, significantly inhibited S1P-induced vasoconstriction regardless of the onset of treatment. These results suggest that the Rho/Rho-kinase pathway plays an important role in the contractile effect of S1P on canine basilar arterial rings.

In addition to activation of the Rho/Rho-kinase pathway, S1P increases [Ca^{2+}]_{i} in smooth muscle cells during the synthetic phase of the cells.20 Thus, a [Ca^{2+}]_{i} increase and activation of the Rho/Rho-kinase pathway as induced by S1P might synergistically induce persistent vasoconstriction. In addition, S1P has been shown to stimulate the production of cytokines and growth factors in a variety of cells.16,21,22 Thus, it might be possible that S1P stimulates directly or indirectly the production of spasmogenic substances that may secondarily induce the vasoconstriction. Furthermore, many types of spasmogenic substances might converge in the subarachnoid space after SAH.1–11 Thus, a synergy could also occur between S1P and these substances, which might lead to the persistent cerebral vasospasm after SAH. In any event, further effort is required to identify more important mechanisms involved in the persistent contraction. Finally, it should be noted that such a long-lasting vasospasm has also been observed not only with other spasmogenic substances, such as noradrenaline, 15-hydroperoxy arachidonic acid, and endothelin-1, but also with blood in the single-injection canine model of SAH in vivo.2–4,8,34 Thus, similar synergistic actions between spasmogenic substances might be involved in the persistent activation of Rho/Rho-kinase signaling in rats with chronic hypertension.26

In conclusion, SIP contracted canine basilar arteries under isometric tension in vitro as well as in an experimental model in vivo. SIP-induced vasoconstriction may be dependent on activation of the Rho/Rho-kinase pathway. Activated platelets in blood clots may be the source of SIP in the actual SAH. Thus, SIP might be listed as a novel mediator responsible for cerebral vasospasm and could be a therapeutic target.

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