Prevention of the Hypercontractile Response to Thrombin by Proteinase-Activated Receptor-1 Antagonist in Subarachnoid Hemorrhage

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Cerebral vasospasm is one of the major factors that determine the prognosis of subarachnoid hemorrhage (SAH). The prevention of posthemorrhagic vasospasm thus plays an essential role in the management of SAH patients. A Rho-kinase inhibitor (fasudil), a thromboxane A2 synthetase inhibitor (ozagrel sodium), calcium channel blockers, or papaverine are currently used to prevent and treat vasospasm.1–5 However, their therapeutic effects are still limited, and thus remain controversial.1–5 Elucidating the molecular mechanism underlying posthemorrhagic cerebral vasospasm is essential for developing effective therapeutic strategies for the prevention and treatment of vasospasm. Oxyhemoglobin, reactive oxygen species, platelet-derived substances such as thromboxane A2 and serotonin, endothelin-1, and histamine are among the candidates that contribute to the pathogenesis of such vasospasm.2,3,6–11 However, their precise involvement in vasospasm still remains controversial.2,3,6–11

Thrombin has been suggested to play a key role in the pathogenesis of posthemorrhagic vasospasm. The activity of thrombin in the cerebrospinal fluid correlated with the incidence and severity of the occurrence of cerebral vasospasm in SAH.12 The inhibition of the thrombin activity by antithrombin III or thrombin inhibitors,13 or the removal of clots in the subarachnoid space have been shown to be effective for preventing vasospasm.14–16 The vascular effects of thrombin are mainly mediated by proteinase-activated receptor 1 (PAR1), which belongs to a family of G protein-coupled receptors.17–19 However, precisely how thrombin and PAR1 contribute to the occurrence of vasospasm remains to be elucidated.

In the present study, we investigated the role of PAR1 in the enhancement of the contractility in SAH and the possibility that PAR1 antagonists may prevent the PAR1 expression in the isolated rings of basilar artery.

Key Words: antagonist receptors thrombin vasospasm
upregulation in SAH. A newly developed PAR1 antagonist of a 2-iminopyridine derivative, E5555,20,21 was used for this purpose.

Materials and Methods
Preparation of Subarachnoid Hemorrhage Rabbit Model
This study followed a protocol approved by the Animal Care and Committee of Graduate School of Medical Sciences, Kyushu University. Adult male Japanese white rabbits (2.5 to 3.0 kg) were anesthetized; 0.5 mL of cerebrospinal fluid was aspirated percutaneously from the cisterna magna, and then 3.0 mL of autologous arterial blood were injected (day 0). The animal was then positioned at a 30° angle with the tilting down for 30 minutes. On day 2, the second injection of autologous blood was similarly performed. The control animals received injections of the same volume of saline instead of the autologous blood, whereas the intact animals received no intervention. When the animals were treated with PAR1 antagonists, the indicated dosage of antagonist was injected into the cisterna magna twice on day 0 and day 2 together with autologous blood. At the time of euthanization, the clot formation was similarly observed over the region of the basilar artery of SAH, with or without E5555 administration (data not shown).

Tension Measurement in the Basilar Artery
On day 7, the rabbits were euthanized and the basilar artery was immediately excised, and the arterial rings measuring 500 μm in width were prepared. The endothelium was removed mechanically. The arterial rings were mounted onto 2 tungsten wires. One of the wires was fixed while the other was connected to a force transducer (U gauge; Minebea). The preparations were equilibrated in normal physiological salt solution (PSS) at 37°C for at least 60 minutes, and then the contractile response was measured at a 50-mg resting load, which was the minimal load to give the maximal tension development in response to 118 mmol/L K⁺. The data were expressed as a percentage, assigning the values obtained in normal PSS and 118 mmol/L K⁺ PSS to be 0% and 100%, respectively.

Fura-2 Fluorometry in the Basilar Artery
The arterial rings were loaded with Ca²⁺ indicator dye, fura-2, by the 4-hour incubation in Dulbecco modified Eagle medium containing 25 μmol/L fura-2 acetoxymethyl ester at 37°C, as previously reported.22 The ring preparations were mounted between 2 tungsten wires in a 2-mL organ bath, which was set on the stage of the inverted fluorescence microscope TMD56 (Nikon). The 500-nm fluorescence intensities at 340 nm and 380 nm excitation lights and their ratio were continuously recorded with a fluorometer CAM-220 (JASCO). The values of fluorescence ratio obtained in normal PSS and during the sustained phase of the 118 mmol/L K⁺-induced contraction at 5 minutes were assigned values of 0% and 100%, respectively.

Immunoblot Analysis of the Expression of PAR1
The isolated basilar arteries were homogenized in 50 mmol/L Tris-HCl, pH 7.2, 0.5 mol/L NaCl, 10 mmol/L MgCl₂, 0.5% sodium deoxycholate, 0.1% SDS, 1% TritonX-100, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 10 μmol/L 4-aminophenylmethane sulfonil fluoride. The 10 μg of total protein was separated by SDS-PAGE and then transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, Calif). The membranes were blocked with 5% nonfat dry milk in 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and

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Figure 1. Enhancement of the contractile response to thrombin and the upregulation of PAR1 in SAH. Representative recordings (a) showing the effect of 1 U/mL thrombin in the basilar artery of the control and SAH. In SAH, tension and [Ca²⁺]i were simultaneously monitored. The concentration-response curves (b) for the thrombin-induced contraction in intact, control, and SAH. The data are the mean±SEM (n=5 to 7). *P<0.05 vs the control. Immunoblot analysis (c) of the PAR1 expression in the basilar artery of SAH after the first injection of the autologous blood. Actin was detected by naphthol blue black staining. The level of the changes in the PAR1 expression was normalized by that of actin, and then was expressed as a percentage, assuming the intact value to be 100%. The data are the mean±SEM (n=3 to 4). *P<0.05 vs the intact.
0.05% Tween 20 at 4°C overnight. Then, the membrane was incubated for 1 hour at room temperature with anti-PAR1 antibody (sc-5605; Santa Cruz Biotechnology, Santa Cruz, Calif) diluted 200-fold in an immunoreaction enhancer solution named Can-Get-Signal (Toyobo, Osaka, Japan), followed by the 1-hour incubation with the secondary antibody conjugated with horseradish peroxidase (1000-fold dilution). The immune complex was detected with an ECL plus detection kit (Amersham Pharmacia Biotech). The luminescence signal was detected and analyzed with the ChemiDoc XRS-J image analysis system (Bio-Rad). After chemiluminescence detection, the band of actin was visualized by naphthol blue black staining to normalize the level of PAR1.

**Fura-2 Fluorometry in Cultured Human Coronary Artery Smooth Muscle Cells**

The cells were loaded with fura-2 by the 1-hour incubation in Dulbecco modified Eagle medium containing 10 mmol/L fura-2 acetoxyethyl ester at 37°C.23 After loading with fura-2, the cells were washed and equilibrated in HEPES-buffered saline (in mmol/L; Hepes 10, pH 7.4, NaCl 135, KCl 5, CaCl₂ 1.0, MgCl₂ 1.0, and D-glucose 5.5) for 30 minutes at room temperature before starting the measurements. The changes in 500-nm fluorescence intensities at 340 nm and 380 nm excitations and their ratio were continuously monitored with a front-surface fluorometer CAM-230.24 The fluorescence ratio data were expressed as a percentage, whereas the values at rest and at the peak [Ca²⁺] elevation induced by 10 µmol/L ionomycin were 0% and 100%, respectively.

**Quantification of E5555 in the Cerebrospinal Fluid**

The supernatant of the cerebrospinal fluid after a centrifugation at 12 000 rpm, 5 minutes on a microcentrifuge was subjected to an MS) analysis with a high-performance liquid chromatography system (Alliance 2795; Waters) equipped with the mass spectrometer (Quattro Premier; Waters). The detection limit of the analysis was 1 ng/mL (≈1.6 mmol/L).

**Drugs and Solution**

The composition of the normal PSS (in mmol/L) was NaCl 123, KCl 4.7, NaHCO₃ 15.5, KH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 1.25, and D-glucose 11.5. The high K⁺ PSS was prepared by substituting NaCl for an equimolar KCl. Thrombin (bovine plasma; 1880 NIH units/mg protein) was purchased from Sigma (St. Louis, Mo). TFLLR-NH₂ (PAR1-activating peptide), SLIGRL-NH₂ (PAR2-activating peptide), and AYPGKF-NH₂ (PAR4-activating peptide) were purchased from Bachem (Bubendorf, Switzerland). E5555 (C₂₉H₃₉BrFN₃O₅; MW=608.5; patent EP1391451A1) was kindly provided by the Eisai Co, Ltd (Tokyo, Japan).

**Data Analysis**

The data are expressed as the mean±SEM. One basilar arterial ring obtained from 1 animal was used for each experiment; therefore, the numbers of experiments indicate the numbers of animals. Unpaired Student t test or ANOVA was used to evaluate any statistical significance. The normal distribution of data were confirmed by Kolmogorov-Smirnov test. In ANOVA, Dunnett post hoc test was applied. A value of P<0.05 was considered to be statistically significant.

**Results**

Enhancement of the PAR1-Mediated Contractile Response and the Upregulation of the PAR1 Expression in SAH

The contractile response to 118 mmol/L K⁺ in SAH (209.0±52.0 mg, n=5) did not differ from that seen in the control (219.6±22.4 mg, n=5). The level of 118 mmol/L K⁺-induced contraction was thus assigned to be 100%. The contraction induced by endothelin-1 (1, 10, 100 mmol/L) in SAH also did not significantly differ from that seen in the control (data not shown); 1 U/mL thrombin induced almost no contraction and 10 U/mL thrombin slightly contracted the artery in the intact rabbits (Figure 1b). The injection of saline (control) had no effect on the responsiveness to thrombin. However, 1 U/mL thrombin induced a significant sustained contraction and the contractile response to thrombin was markedly enhanced in SAH in comparison to the control.
intact rabbits (Figure 1a and 1b). The sustained contraction induced by 1 U/mL thrombin in SAH was associated with an increase in \[\text{Ca}^{2+}\]. TFLLR-NH₂, a PAR1-activating peptide, induced a significant contraction at 10 \(\mu\)mol/L in SAH, whereas it induced no contraction in the control (data not shown). Neither 100 \(\mu\)mol/L SLIGRL-NH₂ (PAR2-activating peptide) nor 100 \(\mu\)mol/L AYPGKF-NH₂ (PAR4-activating peptide) induced any contraction in SAH, although these peptides were confirmed to be effective in other cell types (data not shown). An immunoblot analysis revealed the upregulation of the expression of PAR1 in the basilar artery in SAH (Figure 1c). The levels of PAR1 on day 5 and day 7 were 218.2 ± 47.6% and 242.9 ± 48.1%, respectively, of that seen in the intact.

**Preserved Endothelium-Dependent Relaxing Response to Acetylcholine in SAH**

We also investigated the endothelium-dependent relaxation in SAH (Figure 2). Acetylcholine induced a transient relaxation in the control artery with an endothelium. The similar relaxant effect of acetylcholine was observed in SAH, whereas it induced no contraction in the control (data not shown). Neither 100 \(\mu\)mol/L SLIGRL-NH₂ (PAR2-activating peptide) nor 100 \(\mu\)mol/L AYPGKF-NH₂ (PAR4-activating peptide) induced any contraction in SAH, although these peptides were confirmed to be effective in other cell types (data not shown). An immunoblot analysis revealed the upregulation of the expression of PAR1 in the basilar artery in SAH (Figure 1c). The levels of PAR1 on day 5 and day 7 were 218.2 ± 47.6% and 242.9 ± 48.1%, respectively, of that seen in the intact.

**Preventive Effect of a PAR1 Antagonist, E5555, on the Enhancement of the Thrombin-Induced Contraction and Upregulation of PAR1 in SAH**

We treated the animals with PAR1 antagonists by injecting them twice into the cisterna magna together with autologous blood, and we examined the contractile responses and the PAR1 expression of the basilar artery in SAH (Figure 1c). The levels of PAR1 on day 5 and day 7 were 218.2 ± 47.6% and 242.9 ± 48.1%, respectively, of that seen in the intact.

Confined by a loss of the relaxant effect of acetylcholine (Figure 2c). In SAH, acetylcholine induced a delayed contraction in both presence and absence of endothelium. However, we did not further investigate the acetylcholine-induced contraction in the present study.

**Preventive Effect of a PAR1 Antagonist, E5555, on the Enhancement of the Thrombin-Induced Contraction and Upregulation of PAR1 in SAH**

We treated the animals with PAR1 antagonists by injecting them twice into the cisterna magna together with autologous blood, and we examined the contractile responses and the PAR1 expression of the basilar artery on day 7. The cerebrospinal fluid obtained from the rabbits treated with 2 \(\mu\)g/kg weight/injection contained no detectable E5555 (data not shown). In the basilar artery of SAH with the E5555 treatment, thrombin induced an attenuated contraction in comparison to that seen without the treatment (Figure 3a). E5555 dose-dependently inhibited the enhancement of the contractile response to thrombin (Figure 3b). Treatment with E5555 also significantly inhibited the upregulation of the PAR1 expression in SAH (Figure 3c). However, treatment with E5555 had no significant effect on the con-
tractile responses to high K⁺ depolarization and endothelin-1 (Figure 3d and 3e).

Ex Vivo Effect of E5555 on the Thrombin-Induced Contraction in the Basilar Artery and [Ca²⁺]i Elevation in Cultured Smooth Muscle Cells

We examined the specificity of E5555 as a PAR1 antagonist using both basilar arteries isolated from SAH (Figure 4a through 4c) and cultured smooth muscle cells (Figure 4d). In the basilar artery, E5555 concentration-dependently inhibited the 1 U/mL thrombin-induced contraction (Figure 4a). However, E5555, up to 10 μmol/L, had no significant effects on the 100 nmol/L endothelin-1–induced contraction (Figure 4b). The 118 mmol/L K⁺-induced contraction was significantly inhibited by 10 μmol/L E5555 (Figure 4c). Because the basilar arteries, even those of SAH, did not respond to the activating peptides of either PAR2 or PAR4, we used the cultured smooth muscle cells to examine nonspecific antagonistic effect of E5555 with the Ca²⁺ response as an indication (Figure 4d). In cultured smooth muscle cells, E5555 inhibited the thrombin-induced, but not trypsin-induced, [Ca²⁺]i elevation (Figure 4d). The IC₅₀ values of E5555 for the inhibition of the thrombin-induced contraction and [Ca²⁺]i elevation were estimated to be 1.0 μmol/L and 186 nmol/L, respectively. These values were thus 3- to 15-fold higher than those reported for the inhibition of the platelet aggregation induced by 1 U/mL thrombin (64 nmol/L).²⁰

Reversibility of Inhibitory Effect of E5555 on the Thrombin-Induced Contraction

In ex vivo experiments with the isolated basilar artery of SAH, the reversibility of the inhibitory effect of E5555 on the thrombin-induced contraction was investigated (Figure 5). Thrombin induced a sustained contraction in the basilar artery of SAH (Figure 5a), and this contraction was substantially inhibited in the presence of 1 μmol/L E5555 (Figure 5b). When the rings were treated with 1 μmol/L E5555 for 10 minutes, and then were extensively washed in PSS for 15 minutes, the subsequent stimulation with 1 U/mL thrombin failed to induce any significant contraction (Figure 5c).

Discussion

The present study demonstrated that the intracisternal injection of the PAR1 antagonists prevented the upregulation of PAR1 and the enhancement of the contractile response to thrombin in a rabbit double-SAH model. We also demonstrated the specificity of E5555 in antagonizing PAR1 in both rabbit basilar artery and cultured smooth muscle cells. These observations suggest that PAR1 plays a key role in the upregulation of the expression of itself and the enhancement of the contractile response to thrombin. Thrombin is the major agonist for PAR1 and it is produced after hemorrhage. Collectively, the activation of PAR1 by thrombin attributable to the hemorrhage is thus suggested to be a critical and initial step in the development of the hypercontractile state in SAH. We therefore propose PAR1 to be a new therapeutic target to prevent the development of posthemorrhagic vasospasm in SAH.

In SAH, we observed the expression of PAR1 to be upregulated, although the contractile response to thrombin and the PAR1-activating peptide was also enhanced in the basilar artery. The activating peptides for PAR2 and PAR4 failed to induce any contractile response in SAH. The contractile responses to high K⁺ depolarization and endothelin-1 in SAH did not differ from those seen in the control. Our observations with acetylcholine-induced relaxation do not support the major contribution of the endothelial dysfunction to the enhanced contractile response to thrombin. Furthermore, the prevention of the PAR1 upregulation by E5555 was associated with the prevention of the contractile...
response to thrombin. These observations thus suggested that the enhancement of the contractile response is specific to the PAR1-mediated contraction, and that such enhancement was mainly caused by the PAR1 upregulation.

Our conclusion that the receptor activation caused its upregulation is apparently inconsistent with the general idea that the receptor agonist causes the receptor desensitization. However, our conclusions are consistent with 2 previous reports that showed the thrombin-induced upregulation of PAR1.25,26 The apparent inconsistency may be related to the unique property of PAR1. In contrast to the general G protein-coupled receptors, PAR1 is irreversibly activated by the proteinase agonist. Therefore, the once-activated PAR1 is rapidly desensitized by either internalization or phosphorylation, and thereby the cells lose the responsiveness to the second stimulation of PAR1. We have reported such rapid desensitization and internalization of PAR1 after the PAR1 stimulation.27,28 The cellular responsiveness to the PAR1 stimulation is then recovered by either the recruitment of the intact receptor from the intracellular pool or the activation of de novo synthesis.29,30 As a result, the synthesis of PAR1 can be activated as a long-term consequence of the PAR1 activation. However, PAR1 antagonist did not completely prevent the PAR1 upregulation. Consequently, other factors, including inflammatory cytokines, platelet products, and reactive oxygen species, therefore may also contribute to the PAR1 upregulation in SAH. Such factors remain to be identified.

The observations with ex vivo treatment of the artery with E5555 and those seen with the intracisternal treatment ruled out the nonspecific inhibitory effect on the contractile mechanisms and the nonspecific receptor antagonistic effect. The observations seen with the cultured smooth muscle cells suggest the specificity over PAR2, whereas the specificity over PAR4 has been documented.20 Consequently, E5555 is suggested to specifically antagonize the effect of thrombin on PAR1, thereby preventing the PAR1 upregulation and the hypercontractile response to thrombin in the SAH model. However, our observation of the inhibitory effect of 10 μmol/L E5555 on the 118 mmol/L K⁺-induced contraction suggested some additional pharmacological effects of E5555, such as inhibition of the voltage-operated Ca²⁺ channels, especially at high concentrations.

The inhibitory effect of E5555 on the thrombin-induced contraction persisted after removing the antagonist from the bathing buffer. E5555 is highly hydrophobic, and they were thus dissolved in dimethylsulfoxide. The observed hydrophobic property may thus be related to the persistence of their cellular effect. However, there is no information regarding the metabolism of E5555 in the cerebrospinal fluid. The concentration of E5555 in the cerebrospinal fluid on days 2, 3, 5, and 7 in E5555-treated rabbits was under the detection limit (≈1.6 μmol/L). Our observation suggested 2 possibilities: (1) E5555 might be rapidly metabolized or removed from the cerebrospinal fluid; or (2) E5555 might be attached to and trapped in the clots formed by the injected autologous blood. However, it is possible that the residual amount of antagonists, if any, directly inhibited the contractile effect of thrombin in SAH.

PAR1 plays an important role in the platelet aggregation and smooth muscle proliferation and hypertrophy, as well as smooth muscle contraction.17,31 PAR1 antagonists are thus expected to prevent the acute coronary syndrome associated with the platelet activation and the development of the proliferative vascular lesions such as those seen associated with arteriosclerosis and post-angioplastic restenosis.32 The present study proposes a new therapeutic application of PAR1 antagonist for the prevention of posthemorrhagic vasospasm in SAH. The present study clearly demonstrated that PAR1 antagonist was able to prevent the upregulation of PAR1 while also inhibiting the enhanced contractile response to thrombin in the isolated basilar artery. Rho-kinase inhibitor, fasudil, has been used to directly inhibit the contractile mechanism for the treatment of cerebral vasospasm. In this respect, PAR1 antagonist could not only directly inhibit the thrombin-induced contraction but also could prevent the enhancement of the contractile responsiveness to thrombin. However, before establishing its clinical usefulness, further investigations are needed to determine the therapeutic effects
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on posthemorrhagic vasospasm, not only in an animal model but also in SAH patients.

In conclusion, the present study demonstrated, for the first time to our knowledge, that PAR1 antagonists can prevent both the upregulation of the PAR1 expression as well as the enhancement of the contractile response to thrombin in SAH. The inhibition of the hypercontractile response to thrombin by intracranial injection of PAR1 antagonists was considered to be mainly attributable to the prevention of the PAR1 upregulation. The observations of the present study suggest that PAR1 therefore plays a key role in PAR1 upregulation and the development of an enhanced contractile response in SAH. PAR1 therefore may be a new therapeutic target for the prevention and treatment of cerebral vasospasm in SAH. However, this possibility remains to be investigated in vivo.

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Disclosures

None.

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