Introduction

The pathogenesis of delayed and protracted arterial narrowing that characterizes cerebral vasospasm following subarachnoid hemorrhage (SAH) is the most critical complication, and the mechanism underlying the development and maintenance of vasospasm has not been fully elucidated. The causes of vasospasm have been thought to be related to a number of pathological processes, including smooth muscle contraction, endothelial damage, hyperviscosity, and inflammatory reactions (1–3). We reported that cerebral vasospasm did not result in severe damage to the intracellular mechanisms responsible for phosphorylation as mediated by protein kinases (4).

Various protein kinases, such as myosin light chain kinase (MLCK) (5), protein kinase C (PKC) (6), and Rho-kinase (7), have been reported to play a critical role in the signal transduction pathway of the development of cerebral vasospasm. The upregulation of the Rho/Rho-kinase pathway is observed not only in vessels but also in ischemic brain tissues and neutrophils when cerebral ischemia occurs (8, 9). Rho-kinase is thought to play a role in the mechanisms underlying the occurrence of secondary injury, for example, continuous hemodynamic dysfunction in cerebral circulation or inflammatory processes after acute ischemic stroke, including SAH (10).

Therapy with fasudil, a Rho-kinase inhibitor (RKI), is effective for the prevention of cerebral vasospasm and subsequent ischemic injury after surgery for aneurysmal SAH (11). Fasudil has been widely used in Japan for that indication since 1995 (12). Hydroxyfasudil, an active metabolite of fasudil, also inhibits Rho-kinase (13), and

Antivasospastic Effects of Hydroxyfasudil, a Rho-Kinase Inhibitor, After Subarachnoid Hemorrhage

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Abstract. We investigated the anti-vasospastic potential of fasudil’s active metabolite, hydroxyfasudil, a Rho-kinase inhibitor, after subarachnoid hemorrhage (SAH) and also its effect on hemorheological abnormalities following cerebral ischemia. Chronic cerebral vasospasm was produced using a two-hemorrhage canine model. On day 7, angiographic vasospasm was observed in all animals, and intravenous administration of hydroxyfasudil (3 mg·kg\(^{-1}\)·30 min\(^{-1}\)) significantly reversed the vasospasm (predose diameter of the basilar artery, 57.9% ± 2.0% of the baseline before the injection of blood; postdose diameter, 64.5% ± 1.9%). The viscosity of whole blood was significantly increased 24 h after 1 h middle cerebral artery occlusion in rats. Hydroxyfasudil (3 and 10 mg/kg, i.p.) significantly decreased blood viscosity. The specificity of hydroxyfasudil was examined against a panel of 17 protein kinases using ELISA analysis. Hydroxyfasudil inhibited Rho-kinase α and β at a concentration of 10 μM by 97.6% and 97.7%, respectively. No other protein kinase was inhibited with 10 μM hydroxyfasudil by over 40%. The present results indicate hydroxyfasudil is a selective inhibitor of Rho-kinase. The results also suggest that hydroxyfasudil contributes to the potency of fasudil to prevent cerebral vasospasm and hyperviscosity and suggest the potential utility of hydroxyfasudil as a therapeutic agent for patients with SAH.

Keywords: Rho-kinase, hydroxyfasudil, subarachnoid hemorrhage, vasospasm, hemodynamic dysfunction

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has a pharmacological profile similar to that of fasudil. Hydroxyfasudil improves hemodynamic function by increasing regional cerebral blood flow and ameliorating endothelial damage/dysfunction, and it prevents the inflammatory response by inhibiting neutrophil and monocyte infiltration (14–17). The area under the plasma concentration–time curve value of hydroxyfasudil was approximately 4.5 times higher than that of fasudil in patients with SAH receiving 30 mg of fasudil (18).

The aim of the present study was to observe whether hydroxyfasudil reversed angiographically demonstrable vasospasm in a two-hemorrhage canine model. Furthermore, we examined the effect of hydroxyfasudil on hyperviscosity following cerebral ischemia.

Materials and Methods

All animals were used in accordance with ethical procedures approved by The Japanese Pharmacological Society for the care and use of laboratory animals.

Experimental SAH and angiography

The method of inducing chronic cerebral vasospasm using a two-hemorrhage canine model was essentially the same as in previous reports (19, 20). Mongrel dogs of either sex, weighing 7–12 kg, were anesthetized with sodium pentobarbital (20 mg/kg, i.v.) on day 1. After endotracheal intubation, spontaneous respiration was permitted. A catheter was inserted directly into the vertebral artery, and a control angiogram was taken using 3 ml of 65% iothalamate meglumine. The cisterna magna was entered with a No. 22 spinal needle, and 4 ml of fresh autogenous blood was injected. On day 3, a second injection of blood (4 ml) to the cisterna magna was repeated following anesthesia with sodium thiamylal (15 mg/kg, i.v.).

On day 7, the dogs were anesthetized with sodium pentobarbital (20 mg/kg, i.v.), and a catheter for angiography was inserted into the vertebral artery. After the occurrence of chronic vasospasm was confirmed angiographically, each dog received 3 mg/kg of hydroxyfasudil dissolved in 30 ml of saline with an infusion pump over a period of 30 min. Angiography was performed at 30 min after the initiation of the infusion. The diameter of the basilar artery was measured on angiograms with a microdensitometer and was expressed as a percentage of the diameter of the arterial segment before SAH.

Experimental cerebral ischemia and blood viscosity

Male Wistar rats weighing 200–270 g were anesthetized with ether. The rats were placed in the supine position, and the right common carotid artery was exposed. After ligation of the common and external carotid arteries, and the right common carotid artery was exposed. Experimental cerebral ischemia and blood viscosity the diameter of the arterial segment before SAH. The diameter of the basilar artery was measured on angiograms with a microdensitometer and was expressed as a percentage of the diameter of the arterial segment before SAH. The method of inducing chronic cerebral vasospasm using a two-hemorrhage canine model was essentially the same as in previous reports (19, 20). Mongrel dogs of either sex, weighing 7–12 kg, were anesthetized with sodium pentobarbital (20 mg/kg, i.v.) on day 1. After endotracheal intubation, spontaneous respiration was permitted. A catheter was inserted directly into the vertebral artery, and a control angiogram was taken using 3 ml of 65% iothalamate meglumine. The cisterna magna was entered with a No. 22 spinal needle, and 4 ml of fresh autogenous blood was injected. On day 3, a second injection of blood (4 ml) to the cisterna magna was repeated following anesthesia with sodium thiamylal (15 mg/kg, i.v.).

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assayed in 10 μg/ml calmodulin, 1 mM CaCl₂, 100 μM ATP, 100 mM NaCl, 5 mM MgSO₄, 1 mM DTT, and 20 mM HEPES (pH 7.5). PKCα, βI, γ, and θ were assayed in 0.1 mg/ml phosphatidylserine, 0.02 mg/ml diacylglycerol, 0.01% Triton X-100, 1 mM CaCl₂, 100 μM ATP, 100 mM NaCl, 5 mM MgSO₄, 1 mM DTT, and 20 mM HEPES (pH 7.5). The final concentrations of fasudil and hydroxyfasudil were 10 μM. Primary antibodies used were as follows: rabbit anti–phospho-MBS antibody (Peptide Institute, Osaka), rabbit anti–phospho-MEK 1/2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti–phospho-MBS antibody conjugated with HRP (Upstate, Charlottesville, VA, USA), rabbit anti–phospho-MARCKS antibody (Santa Cruz), and anti–phospho-tyrosine antibody conjugated with HRP (Zymed, South San Francisco, CA, USA). In some cases, anti-rabbit IgG conjugated with HRP (Zymed) was used as a secondary antibody. Phosphorylation activities were assessed by measuring the absorbance at 490 nm in a color reaction with o-phenylenediamine.

Statistics

Values are expressed as means ± S.E.M. A statistical analysis of the data was done with Student’s t-test. P-values of 0.05 or less were considered to indicate a significant difference.

Results

Evaluation of the efficacy of hydroxyfasudil in the two-hemorrhage dogs

On day 7, a vasospasm was observed in all animals. Intravenous infusion of saline solution (30 ml/30 min) led to no significant change in the diameter of the basilar artery. Representative angiograms from the dog with intravenous infusion of hydroxyfasudil (3 mg·kg⁻¹·30 min⁻¹) are shown in Fig. 1. The mean diameter of the basilar artery after repeated intracisternal injection of blood was 57.9% ± 2.0% compared to the baseline obtained prior to the intracisternal injection of blood, and intravenous infusion of hydroxyfasudil at a dose of 3 mg/kg reversed the vasospasm (Fig. 2). The diameter of the basilar artery at the end of administration of hydroxyfasudil was 64.5% ± 1.9% (P < 0.05 vs. predose diameter).

Fig. 1. Angiograms showing the effects of i.v. administration of hydroxyfasudil (3 mg·kg⁻¹·30 min⁻¹) on the basilar artery of a two-hemorrhage dog. Compared with the angiogram on day 1 before SAH (A), chronic vasospasm is observed on day 7, before administration of the drug (B). Reversal of vasospasm is demonstrated on the angiogram obtained 30 min after the start of the i.v. infusion of hydroxyfasudil (C).

Fig. 2. Effect of intravenous administration of hydroxyfasudil (3 mg·kg⁻¹·30 min⁻¹) on the diameter of the basilar artery in SAH dogs on day 7. The angiographic diameter of the basilar artery before the injection of arterial blood on day 1 is defined as 100%. Hydroxyfasudil significantly reversed the vasospasm in SAH. Each column represents the mean ± S.E.M. of 5 experiments. *P < 0.05 vs. predose control.
**Effect of hydroxyfasudil on blood viscosity** in a rat model of cerebral ischemia

The rats in the ischemic group showed significantly higher viscosity than sham-operated rats, which was concomitant with an increase in hematocrit (Fig. 3). Hydroxyfasudil decreased blood viscosity (Fig. 3). For example, whole blood viscosity at the high shear rate (150 s\(^{-1}\)) was 4.48 ± 0.05 cP in the ischemic group; the difference was significant compared with the blood viscosity of 4.18 ± 0.07 cP (\(P < 0.01\)) observed in the rats treated with 10 mg/kg of hydroxyfasudil (Fig. 3D). Hematocrit also increased in the ischemic group, while hydroxyfasudil decreased the hematocrit (Fig. 3A). For example, the hematocrit was 45.6% ± 0.4% in the ischemic group; the difference was significant compared with the hematocrit of 44.4% ± 0.2% observed in the hydroxyfasudil (10 mg/kg)-treated group (\(P < 0.05\)).

**Inhibition of protein kinases by hydroxyfasudil**

Hydroxyfasudil inhibited Rho-kinase \(\alpha\) and \(\beta\) at a concentration of 10 \(\mu\)M by 97.6% and 97.7%, respectively (Table 1). No other protein kinase was inhibited with 10 \(\mu\)M hydroxyfasudil by over 40%. Fasudil inhibited Rho-kinase \(\alpha\) and \(\beta\) at a concentration of 10 \(\mu\)M by 97.4% and 94.3%, respectively, and PKA by 59.2%. No other protein kinase was inhibited with 10 \(\mu\)M fasudil by over 30%.

**Discussion**

Fasudil inhibits various protein kinases, such as Rho-kinase \(\alpha\) (Ki = 0.091 \(\mu\)M), Rho-kinase \(\beta\) (Ki = 0.082 \(\mu\)M), PKC (Ki = 3.3 \(\mu\)M), and MLCK (Ki = 36 \(\mu\)M); and the Ki values of hydroxyfasudil for Rho-kinase \(\alpha\), Rho-kinase \(\beta\), PKC, and MLCK are 0.093, 0.039, 18, and 140 \(\mu\)M, respectively (21, 22). Fasudil is approximately 36 – 440 times and hydroxyfasudil is 190 – 3600 times more potent against Rho-kinase than PKC and MLCK. In the present study, a large panel of protein kinases was used to examine the specificities of hydroxyfasudil and

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**Fig. 3.** Effects of hydroxyfasudil on hematocrit (A) and hyperviscosity at shear rates 37.5 \(s^{-1}\) (B), 75 \(s^{-1}\) (C), and 150 \(s^{-1}\) (D) following cerebral ischemia. Each column represents the mean ± S.E.M. of 8 – 10 experiments. *\(P < 0.05\), **\(P < 0.01\) vs. ischemic group.
fasudil. Hydroxyfasudil and fasudil at 10 μM almost completely inhibited Rho-kinase α and β. Hydroxyfasudil (10 μM) inhibited other serine/threonine kinases by 0% – 40%. Fasudil (10 μM) inhibited PKA by 59.2%, but no other serine/threonine kinase was inhibited by over 30%. The tyrosine kinases tested were inhibited minimally or not at all by 10 μM hydroxyfasudil and fasudil. Hydroxyfasudil and fasudil revealed selectivity for Rho-kinase. The present and previous results indicate hydroxyfasudil is more selective than fasudil as an inhibitor of Rho-kinase.

In the present study, hydroxyfasudil reversed chronic vasospasm in a two-hemorrhage canine model. Experimental chronic cerebral vasospasm induced in the two-hemorrhage canine model is thought to mimic vasospasm seen in patients with SAH (1) because it is so refractory to pharmacological therapy such as treatment with calcium-entry blockers. We previously reported that fasudil, but not calcium-entry blockers, reversed chronic vasospasm, and fasudil was the first drug that angiographically dilated the spastic basilar artery on day 7 via intravenous administration in the two-hemorrhage canine model (19). The activation of Rho-kinase and the phosphorylation of the myosin binding subunit, which is a target of Rho-kinase, of myosin phosphatase occur concomitantly during vasospasm in the basilar artery of the two-hemorrhage dog (7).

Hydroxyfasudil was found following intravenous infusion of fasudil (30 mg/30 min) in patients with SAH (18). The elimination half-life of hydroxyfasudil is longer than that of fasudil, and the AUC value of hydroxyfasudil is approximately 4.5 times higher than that of fasudil. The present and previous studies demonstrate that the systemic administration of RKIs, such as fasudil and hydroxyfasudil, reverse chronic cerebral vasospasm, and that hydroxyfasudil contributes to the potency of the antispastic property of fasudil.

Vasospasm treatments are directed at preventing or reversing arterial narrowing, cerebral ischemia, or ischemic brain injury. Several treatments, such as triple-H (hemodilution, hypervolemia, and hypertension) therapy and vasodilator therapies, are in widespread clinical use (23). In general, cerebral blood flow falls as blood viscosity increases. The purpose of hemodilution is to reduce hematocrit, improve the rheology of blood flow, and therefore increase cerebral blood flow and perfusion; however, this effect is limited by decreasing cerebral oxygen-carrying capacity. We previously reported that the blood viscosity decreased by about 40% after blood samples taken from cerebral ischemic rats were diluted with saline and hematocrit was adjusted to the normal value (24). The remaining 60% of the component of hyperviscosity appeared to be refractory to hemodilution therapy and is thought to be related to several other factors including red blood cell deformability and aggregability, number and activation of white blood cells, and plasma fibrinogen and globulin concentration. A low dose (3 mg/kg) of hydroxyfasudil significantly decreased blood viscosity but there was no significant reduction in hematocrit. The reduction of viscosity by a low dose of hydroxyfasudil (3 mg/kg) is thought to be mainly related to mechanisms other than a reduction in hematocrit.

We previously reported that cerebral ischemia induced potent, systemic and long-lasting hyperviscosity in rats after MCA occlusion, and fasudil dose-dependently and significantly decreased blood viscosity (24). In the present study, the same animal model of cerebral ischemia was used to compare the efficacy of hydroxyfasudil on hyperviscosity with that of fasudil. Hydroxyfasudil (3 and 10 mg/kg) significantly decreased blood viscosity without lowering hematocrit levels below basal levels under normal conditions. Hydroxyfasudil’s efficacy in ameliorating blood viscosity was similarly potent to fasudil’s efficacy. These results suggest that hydroxyfasudil contributes to the hemorheological improvement observed after fasudil treatment due to a reversal of the hyperviscosity state.

In a previous experiment, fasudil and hydroxyfasudil inhibited endothelial hyperpermeability (17). Endothelial

**Table 1.** Kinase selectivity profile of 10 μM fasudil and hydroxyfasudil

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Fasudil (% inhibition)</th>
<th>Hydroxyfasudil (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rho-kinase α</td>
<td>97.4</td>
<td>97.6</td>
</tr>
<tr>
<td>Rho-kinase β</td>
<td>94.3</td>
<td>97.7</td>
</tr>
<tr>
<td>Raf 1</td>
<td>2.0</td>
<td>-0.6</td>
</tr>
<tr>
<td>MEK 1</td>
<td>14.4</td>
<td>4.8</td>
</tr>
<tr>
<td>Erk 2</td>
<td>2.8</td>
<td>2.2</td>
</tr>
<tr>
<td>p38α</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>CaMK II</td>
<td>1.1</td>
<td>3.4</td>
</tr>
<tr>
<td>PKA</td>
<td>59.2</td>
<td>29.1</td>
</tr>
<tr>
<td>PKCα</td>
<td>8.1</td>
<td>3.3</td>
</tr>
<tr>
<td>PKCβ/γ</td>
<td>28.0</td>
<td>8.5</td>
</tr>
<tr>
<td>PKCγ</td>
<td>16.2</td>
<td>6.1</td>
</tr>
<tr>
<td>PKCθ</td>
<td>27.6</td>
<td>39.5</td>
</tr>
<tr>
<td>Abl</td>
<td>1.1</td>
<td>2.9</td>
</tr>
<tr>
<td>Lck</td>
<td>2.0</td>
<td>4.2</td>
</tr>
<tr>
<td>Src</td>
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<td>5.3</td>
</tr>
<tr>
<td>EGFR</td>
<td>0.5</td>
<td>2.6</td>
</tr>
<tr>
<td>PDGFR α</td>
<td>0.1</td>
<td>3.3</td>
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cells form a semipermeable barrier that regulates the exchange of fluid and solutes between blood and tissues. Rho-kinase mediates endothelial cytoskeleton constriction, and the resulting paracellular permeability is thought to contribute to barrier dysfunction (25). Excessive leakage across the vascular barrier would result in a net loss of fluid from the vascular space and an increase in hematocrit. Reduction of hematocrit by fasudil and hydroxyfasudil is thought to be—at least in part—due to an effect RKIs have on hyperpermeability in endothelial cells.

Several limitations can be raised in the present study. First, in the present study, the anti-vasospastic efficacy of hydroxyfasudil was evaluated using a two-hemorrhage canine model, and the effect of hydroxyfasudil on blood hyperviscosity following cerebral ischemia was evaluated using a rat MCA occlusion model. To define the relationship between the anti-spastic effect and the effect on hyperviscosity, an experimental SAH model should be used that concomitantly causes severe diffuse vasospasm and a stable hyperviscosity. However, to our knowledge, there is no SAH model that concomitantly causes severe diffuse vasospasm and stable hyperviscosity. We therefore used an animal model of cerebral ischemia instead of an SAH model to examine the effect on hyperviscosity. Development of an SAH model that concomitantly causes severe diffuse vasospasm and stable hyperviscosity and examination of the effect on hyperviscosity using this SAH model may be required to clearly establish the relationship between the anti-vasospastic effect and the effect on hyperviscosity by hydroxyfasudil. Second, the present study aimed to evaluate the improvement of hemodynamic dysfunction by hydroxyfasudil, and we did not measure Rho-kinase activity. Additional research may be required to define the role of Rho-kinase activation in the occurrence of vasospasm and hyperviscosity: for example, measurements of change in Rho-kinase activity in the spastic artery and blood cells after ischemia and determination of the inhibitory effect of hydroxyfasudil on Rho-kinase activation.

The present results indicate hydroxyfasudil is a selective inhibitor of Rho-kinase. The results also suggest that hydroxyfasudil contributes to the potency of fasudil to prevent cerebral vasospasm and hyperviscosity and further suggest the potential utility of hydroxyfasudil as a therapeutic agent for patients with SAH.

Acknowledgments

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