Fasudil and Ozagrel in Combination Show Neuroprotective Effects on Cerebral Infarction after Murine Middle Cerebral Artery Occlusion

Akihiro Koumura, Junya Hamanaka, Koh Kawasaki, Kazuhiro Tsuruma, Masamitsu Shimazawa, Isao Hozumi, Takashi Inuzuka, and Hideaki Hara

Molecular Pharmacology, Department of Biofunctional Evaluation, Gifu Pharmaceutical University, Gifu, Japan (A.K., J.H., K.T., M.S., H.H.); Department of Neurology and Geriatrics, Gifu University of Medicine, Gifu, Japan (A.K., I.H., T.I.); and Laboratory for Pharmacology, Asahi Kasei Pharma Co., Shizuoka, Japan (K.K.)

Received November 29, 2010; accepted April 13, 2011

ABSTRACT
Rho kinase (ROCK), one of the serine/threonine kinases, is involved in pathologic conditions, and its activation causes neuronal cell death. Fasudil, a selective ROCK inhibitor, has been reported to cause increased cerebral blood flow (CBF) in the ischemic brain and protect against neuronal cell death by inhibiting ROCK. Ozagrel, a thromboxane A2 synthase inhibitor, inhibits platelet aggregation and causes vasodilatation, thereby increasing CBF in cerebral thrombosis. The present study evaluates the combination therapy of fasudil and ozagrel on focal brain ischemia induced by middle cerebral artery occlusion (MCAO) in mice. Each monotherapy of fasudil at 10 mg/kg i.p. and ozagrel at 30 mg/kg i.p. significantly reduced cerebral infarction. The combination therapy of fasudil (3 mg/kg i.p.) and ozagrel (10 mg/kg i.p.), which are noneffective doses, resulted in reduction of cerebral infarction, and the protective effect was observed up to 5 min, but not 3 h, after reperfusion. Regional CBF after MCAO and phosphorylation of endothelial nitric-oxide synthase (NOS) significantly increased in response to the combination therapy, whereas these effects were not observed with monotherapy of either drug. The protective effect of combination treatment was antagonized by the treatment of a NOS inhibitor, nitro-L-arginine methyl ester hydrochloride. These findings indicate that the combination treatment of fasudil and ozagrel exhibits additive effects for neuroprotection after MCAO. These findings indicate that the combination treatment of fasudil and ozagrel may be useful as a potential therapeutic strategy for the treatment of stroke.

Introduction
Cerebral infarction is a life-threatening disease. Many patients who experience cerebral infarction suffer seriously from its after effects. Therefore, understanding its pathology and finding effective treatments have become very important. Currently, treatment of cerebral infarction is aimed primarily at improvement of cerebral blood flow (CBF). For example, tissue-plasminogen activator is a most effective therapeutic agent for acute cerebral infarction. However, tissue-plasminogen activator can sometimes cause fatal hemorrhagic complications (Wahlgren et al., 2007). Therefore, safer therapeutic agents for cerebral infarction are still needed.

Rho kinases (ROCKs) are serine/threonine kinases that exist as two isoforms, ROCK I and ROCK II (Riento and Ridley., 2003). ROCKs are activated by binding to the active GTP bound form of Rho (Amano et al., 2000). ROCKs are the most important elements involved in endothelial nitric-oxide synthase (eNOS) activity, cell migration, and hyperviscosity (Shibuya et al., 2005). Previous studies suggest that ROCKs play an important role in pathologic conditions such as coronary spasm (Masumoto et al., 2002), hypertension (Masumoto et al., 2001), vascular inflammation and remodeling (Kataoka et al., 2002), and arteriosclerosis (Miyata et al., 2000).

Fasudil, a Rho kinase inhibitor, is already in use in several countries as a treatment for cerebral vasospasms occurring after subarachnoid hemorrhage (SAH) (Sayama et al., 2006).

ABBREVIATIONS: CBF, cerebral blood flow; rCBF, regional CBF; AUC, area under the plasma concentration-time curve; $C_{\text{max}}$, maximum concentration; NO, nitric oxide; eNOS, endothelial NO synthase; $P_{\text{eNOS}}$, phosphorylated eNOS; MLC, myosin light chain; MCAO, middle cerebral artery occlusion; PGJ2, prostacyclin; ROCK, Rho kinase; SAH, subarachnoid hemorrhage; $T_{1/2}$, elimination half-life; $T_{\text{max}}$, time to the maximum concentration; TTC, 2,3,5-triphenyltetrazolium chloride; TXA2, thromboxane A2.; L-NAME, nitro-L-arginine methyl ester hydrochloride.
In a previous report, fasudil showed neuroprotective effects after cerebral ischemia in animal models (Rikitake et al., 2005). In humans, treatment with fasudil within 48 h of onset of acute ischemic stroke significantly improved the patients’ clinical outcome (Shibuya et al., 2005). These findings indicate that fasudil is one of the most promising agents for treatment of cerebral infarction.

Ozagrel, a thromboxane A2 (TXA2) synthetase inhibitor, is presently used in several countries for the treatment of acute cerebral infarction or the prevention of cerebral vasospasms after SAH. TXA2 promotes platelet aggregation by increasing Ca2+ influx and facilitating the release of ADP (Aiken, 1984; Bosia et al., 1988). Ozagrel decreases TXA2 production and has antiplatelet and antithrombotic effects (Terashita et al., 1995). Ozagrel also causes a secondary increase in prostacyclin (PGI2) production, followed by an inhibition of TXA2 synthetase (Komatsu et al., 1986). Increased PGI2 causes a vasodilatation effect and increases CBF (Pickard et al., 1980). Ozagrel has been reported to improve motor coordination in rats after experimental stroke (Ichikawa et al., 1999).

In the present study, we hypothesized that a combination therapy of fasudil and ozagrel would show a greater neuroprotective effect after cerebral ischemia than would either drug administered as a monotherapy. The combined effect of fasudil and ozagrel has previously been evaluated in patients with delayed cerebral vasospasm after SAH (Suzuki et al., 2008). However, similar evaluation has not yet been documented with respect to cerebral infarction. Because the mechanism of action of ozagrel is different from that of fasudil, the purpose of the present study was to evaluate the neuroprotective effects of combined fasudil and ozagrel therapy against middle cerebral artery occlusion (MCAO)-induced ischemic damage in mice.

Materials and Methods

Animal Preparation. The experimental designs and all procedures were in accordance both with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Animal Care Guidelines issued by the Animal Experimental Committee of Gifu Pharmaceutical University. All experiments were performed using male ddY mice (5–8 weeks old; Japan SLC Ltd., Shizuoka, Japan). Every effort was made to minimize the number of animals used and their suffering.

Drugs. Fasudil and ozagrel, kindly donated by Asahi Kasei Co. Ltd. (Tokyo, Japan), were dissolved in saline and administered intraperitoneally. Other drugs, including 2,3,5-triphenyltetrazolium chloride (TTC), nitro-l-arginine methyl ester hydrochloride (l-NAME), pentobarbital sodium, and isoflurane, were purchased from Sigma-Aldrich (St. Louis, MO), Sigma-Aldrich, Nissan Kagaku (Tokyo, Japan), and Merck Hoei Ltd. (Osaka, Japan), respectively.

Plasma Concentration and Pharmacokinetic Parameters. Fasudil (3 and 10 mg/kg) was injected intraperitoneally. Blood samples were collected from the retro-orbital venous plexus 5, 15, 30, 60, and 180 min after dosing. Concentrations of fasudil and hydroxyfasudil in plasma were measured by LC-MS/MS (API 4000, LC-MS/MS System; Applied Biosystems, Foster City, CA). The area under the plasma concentration-time curve (AUC) was calculated using the linear trapezoidal rule with extrapolation. The maximum concentration (Cmax) and the time to the maximum concentration (Tmax) were derived directly from individual measurements taken. The elimination half-life (T1/2) was calculated by linear regression.

Drug Treatments. Fasudil and ozagrel were diluted in saline. For the evaluation of dose dependence, mice were injected with fasudil (1, 3, or 10 mg/kg i.p.) and ozagrel (3, 10, or 30 mg/kg i.p.) at 10 min after MCAO. For the evaluation of the combination therapy, mice were injected with submaximal (noneffective for ischemic infarction) doses of fasudil (3 mg/kg i.p.) and ozagrel (10 mg/kg i.p.) concomitantly at 10 min after MCAO. For the evaluation of therapeutic time window of combination therapy, mice were injected with submaximal doses of fasudil (3 mg/kg i.p.) and ozagrel (10 mg/kg i.p.) at 5 min or 3 h after 2-h ischemia-reperfusion. For the evaluation of l-NAME with combined ozagrel and fasudil therapy, l-NAME (3 mg/kg i.v.) was treated immediately after the induction of MCAO, and then fasudil (3 mg/kg i.p.) and ozagrel (10 mg/kg i.p.) were treated concomitantly at 10 min after MCAO.

Surgery. Mice were anesthetized with 2 to 3% isoflurane (for induction) and maintained with 1.0 to 1.5% isoflurane in 70% N2O and 30% O2 via a facemask (Soft Lander; Sin-ei Industry, Saitama, Japan). Focal cerebral ischemia was induced (using an 8–0 nylon monofilament [Ethicon, Somerville, NJ]) coiled with silicone hardener mixture (Xantpre; Bayer Dental, Osaka, Japan) via the internal carotid artery, as described by Hara et al. (1996). In brief, a coated filament was introduced into the left internal carotid artery through the common carotid artery and then advanced up to the origin of the anterior cerebral artery via the internal carotid artery, so as to occlude the middle cerebral artery and posterior communicating artery. At the same time, the left common carotid artery was occluded. Anesthesia did not exceed 10 min. After 2 h of occlusion, the animal was reanesthetized briefly, and reperfusion was initiated via withdrawal of the monofilament. After surgery, the mice were kept for another 24 h in a cage under a heat lamp, which maintained the cage temperature at between 29 and 30°C. Thereafter, the mice were kept in the preoperative condition (24 ± 2°C) until sampling.

Physiological Monitoring. During surgery and ischemia, the body temperature of all animals was maintained between 37.0 and 37.5°C with the aid of a heat lamp and heating pad. In randomly selected animals, the left femoral artery was cannulated and blood pressure was measured during the preparation, with mean systemic arterial blood pressure (Power Laboratory; AD Instrument, Nagoya, Japan) measured for 3 min periods starting 10 min before and ending 30 min after MCAO. Arterial blood samples taken 30 min before and 30 min after the induction of ischemia were analyzed for pH and partial pressures of oxygen and carbon dioxide (i-STAT 3G; Abbott Point of Care Inc., East Windsor, NJ). Regional CBF was determined by laser-Doppler flowmetry (Omegaflow flo-N1; Owagawaye Inc., Tokyo, Japan) using a flexible 0.5-mm fiber optic extension to the master probe. The tip of the probe was fixed to the intact skull over the ischemic cortex (2 mm posterior and 6 mm lateral to bregma).

Analysis of Cerebral Infarction. At 24 h after MCAO, mice were given an overdose of pentobarbital sodium, then decapitated. The forebrain was divided into five coronal 2-mm sections using a mouse brain matrix (RBM-2000C; Activational Systems, Warren, MI). These slices were immersed for 20 min in a 2% solution of TTC in normal saline at 37°C, then fixed in 10% phosphate-buffered formalin at 4°C. The TTC reacts with intact mitochondrial respiratory enzymes to generate a bright red color that contrasts with the pale color of the infarction. The caudal face of each slice was photographed. The area of the infarction (unstained) in the left cerebral hemisphere was traced and measured using Image J (http://rsb.info.nih.gov/ij/download.html), and the infarction volume per brain (mm3) was calculated from the measured infarction area.

Neurological Deficits. Mice were tested for neurological deficits at 24 or 72 h after MCAO. These were scored as described previously (Hara et al., 1996): 0, no observable neurological deficits (normal); 1, failure to extend the right forepaw (mild); 2, circling to the contralateral side (moderate); 3, loss of walking or righting reflex (severe); and 4, dead.

Western Blot Analysis. Mice were deeply anesthetized and decapitated at 6 h after MCAO. The brain was quickly removed, and an 8-mm coronal section was cut from the left hemisphere (between 2 and 10 mm from the frontal end of the forebrain). Samples were...
homogenized in RIPA buffer (Sigma-Aldrich) with 1% Triton X-100 and a protease/phosphatase inhibitor mixture. Homogenates were centrifuged at 14,000g for 40 min at 4°C. A 5-µg aliquot of protein was subjected to 5 to 20%-gradient SDS-polyacrylamide gel electrophoresis (SuperSep Ace; Wako Pure Chemicals, Osaka, Japan), and separated proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA). For immunoblotting, the following primary antibodies were used: polyclonal antibody to eNOS (1:1000 dilution; Funakoshi, Tokyo, Japan), phosphorylated eNOS (P-eNOS) (Ser1177) antibody (1:1000 dilution; Cell Signaling Technology, Danvers, MA), and monoclonal anti-β-actin (1:1000 dilution; Sigma-Aldrich). The secondary antibody was anti-rabbit horseradish peroxidase-conjugated IgG (1:2000 dilution) and anti-mouse horseradish peroxidase-conjugated IgG (1:2000 dilution). The immunoreactive bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA). The band intensity was measured using a Lumino imaging analyzer (LAS-4000; Fuji Film, Tokyo, Japan).

**Cell Culture.** Human aortic endothelial cells (Lonza Walkersville Inc., Walkersville, MA) were cultured in 12-well plates in human endothelial SFM basal medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum and growth factors (EGM-2; Lonza Walkersville Inc.). The medium was changed to human endothelial SFM basal medium with 0.5% fetal bovine serum and incubated with hydroxyfasudil and/or ozagrel for 1 h.

**Measurement of Phosphorylation of Myosin Light Chain.** Human aortic endothelial cells were lysed with SDS/urea lysis buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. Membranes were incubated with anti-myosin light chain (MLC) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), antiphospho-MLC antibody (Cell Signaling Technology), or antiactin antibody (Sigma-Aldrich). Rho-kinase activity was expressed as the ratio of the staining density of antiphospho-MLC antibody to that of anti-MLC antibody.

**Statistical Analysis.** Data are presented as the means ± S.E.M. Statistical comparisons were made using one-way analysis of variance followed by Student’s t test or Dunnett’s test using Statview version 5.0 (SAS Institute, Cary, NC), with P < 0.05 being considered as statistically significant.

**Results**

**Plasma Concentrations and Pharmacokinetic Parameters.** The plasma concentration-time curves and the pharmacokinetic parameters for fasudil and hydroxyfasudil are shown in Fig. 1 and Table 1. Fasudil, the parent drug, and hydroxyfasudil, its metabolite, were found after intraperitoneal administrations of fasudil (3 or 10 mg/kg). Plasma concentrations of fasudil at 60 and 180 min after administration at 3 mg/kg administration, and at 180 min after a 10 mg/kg administration, were below detectable limits. The Cmax value of hydroxyfasudil was approximately 100 and 134% of those of fasudil when fasudil was administered at 3 and 10 mg/kg, respectively. The AUC value of hydroxyfasudil was approximately four times higher than that of fasudil. As can be understood from T1/2 values, hydroxyfasudil was detectable in plasma for a longer period than that of fasudil.

**Effects of a Single Administration of Fasudil on Ischemic Damage.** We first evaluated the dose-dependent effects of a single treatment of fasudil on ischemic damage. At 24 h after MCAO, an ischemic zone was consistently identified in the left cerebral hemisphere. No mice died at 24 h after MCAO. The TTC staining results showed no clear differences between the fasudil (1 or 3 mg/kg i.p.) and vehicle treatment groups (Fig. 2, A–C). However, fasudil at 10 mg/kg i.p., administered at 10 min after MCAO, decreased the cerebral infarction at 24 h after MCAO (Fig. 2D). Fasudil at 10 mg/kg i.p., but not at 1 or 3 mg/kg, improved the neurological deficits (Fig. 2E). Based on infarction measurements, fasudil significantly reduced both the infarct area and volume in a dose-dependent manner, with significant effects seen at 10 mg/kg (Fig. 2, F and G).

**Effects of a Single Administration of Ozagrel on Ischemic Damage.** We next evaluated the effects of a single treatment of ozagrel on ischemic damage. No mice had died at 24 h after MCAO. The TTC staining results showed no clear difference between the ozagrel (3 or 10 mg/kg i.p., administered at 10 min after MCAO) and vehicle treatment groups (Fig. 3, A–C). However, ozagrel at 30 mg/kg i.p. decreased the cerebral infarction at 24 h after MCAO (Fig. 3D). Ozagrel at 30 mg/kg i.p., but not at 3 or 10 mg/kg, improved the neurological deficits (Fig. 3E). Based on infarction measurements, ozagrel reduced both the infarct area and volume in a dose-dependent manner (Fig. 3, F and G). Ozagrel at 30 mg/kg also improved the neurological deficits (Fig. 3F).
mg/kg i.p. was particularly effective at significantly reducing both the infarct area and volume.

**Effects of Combined Fasudil and Ozagrel Therapy on Ischemic Damage.** Next, we evaluated the effects of the combined fasudil and ozagrel therapy. Neither ozagrel at 10 mg/kg nor fasudil at 3 mg/kg caused any significant decrease in the infarct area and volume when supplied singly (Figs. 2 and 3). Thus, we administered ozagrel and fasudil together using each of these noneffective doses. No mice had died at 24 h after MCAO. The TTC staining results showed no clear difference between vehicle-, fasudil-, or ozagrel-treated groups (Fig. 4, A–C). On the other hand, the combination therapy decreased the cerebral infarction (Fig. 4D) and improved the neurological deficits (Fig. 4E). Based on infarction measurements, the combination therapy significantly reduced both the infarct area and volume (Fig. 4, F and G). Moreover, significant effects of the combination therapy on infarct area and volume could be seen in both the cortex and the subcortex (striatum) (Fig. 4, H and I).

**Effect of Combined Ozagrel and Fasudil on a Therapeutic Time Window.** We examined the therapeutic time window of combined ozagrel and fasudil therapy. When administered at 5 min after reperfusion, combined ozagrel and fasudil therapy significantly reduced the infarct area and volume at 24 h after MCAO (22-h reperfusion), but not at 3 h after reperfusion (Fig. 5).

**Physiological Parameters.** No significant differences were detected in mean arterial blood pressure, heart rate,
partial pressure of carbon dioxide, or partial pressure of oxygen among all of the groups (data not shown). We measured rCBF of mice by laser-Doppler flowmetry during MCAO and found that rCBF of all mice significantly decreased to approximately 30 to 40% of the previous value (Fig. 6). After reperfusion, rCBF of the combination group significantly increased compared with the value observed during MCAO (Fig. 6D), but this response was not seen in any of the other groups (Fig. 6, A–C).

Western Blotting of eNOS. Other studies have reported that eNOS is phosphorylated and activated in the ischemic brain hemisphere and P-eNOS exerts neuroprotective effects (Osuka et al., 2004). We measured eNOS and P-eNOS in penumbra lesions of the ischemic brain hemisphere. P-eNOS was more prominent in the combination therapy group than in the other groups, and there was no difference among the other groups (Fig. 7A). In a quantitative analysis, whereas fasudil or ozagrel did not increase P-eNOS, P-eNOS was significantly greater in the combination therapy group than in the control group (Fig. 7B). We also evaluated the ratio of P-eNOS/eNOS protein. The results were: control, 1.000 ± 0.093; vehicle, 1.119 ± 0.254; fasudil, 1.088 ± 0.184; ozagrel,
1.232 ± 0.227; combination, 1.506 ± 0.324 (each n = 5). It tended to increase the ratio, but did not reach the significant level (control versus combination; p = 0.193; Student’s t test).

Antagonistic Effect of L-NAME with Combined Ozagrel and Fasudil on Ischemic Damage. Because P-eNOS was increased in the combined ozagrel and fasudil therapy group (Fig. 7), we evaluated the effect of a NOS inhibitor, L-NAME, with combined ozagrel and fasudil treatment against MCAO-induced ischemic damage. There was no significant difference between L-NAME with the combined ozagrel and fasudil group and vehicle group in infarct area or volume at 24 h after MCAO (Fig. 8).

Effect of Hydroxyfasudil and Ozagrel on Myosin Light Chain Phosphorylation in Human Aortic Endothelial Cells. Hydroxyfasudil at 30 μM inhibited phosphorylation of MLC, whereas at 1 mM ozagrel had no effect on MLC phosphorylation, nor did it alter the inhibitory effect of hydroxyfasudil (Fig. 9).

Discussion

In the present study, we investigated the effects of fasudil and ozagrel, administered singly or in combination, in a murine model of transient MCAO. Fasudil and ozagrel both exhibited dose-dependent neuroprotective effects, with doses of 10 mg/kg i.p., for fasudil and 30 mg/kg i.p. for ozagrel significantly reducing both the infarct volume and the neurological deficits after MCAO. Neither fasudil at 3 mg/kg nor ozagrel at 10 mg/kg showed any neuroprotective effects, but when combined as a therapy at these doses, cerebral infarction was reduced. The combination therapy was able to prevent neuronal cell death even when it was injected at up to 5

Fig. 6. Changes in rCBF during MCAO and reperfusion. The rCBF value (percentage of before) was measured by laser-Doppler flowmetry. A, vehicle. B, fasudil at 3 mg/kg i.p. C, ozagrel at 10 mg/kg i.p. D, combination treatment of fasudil at 3 mg/kg i.p. and ozagrel at 10 mg/kg i.p. The rCBF significantly decreased after MCAO in all groups. The rCBF value significantly increased after reperfusion in the combination group, but not significantly in any other groups. ##, P < 0.01 versus before ischemia; *, P < 0.05 versus during ischemia (Student’s t test) (n = 4). n.s., not significant.

Fig. 7. Western blot analysis of P-eNOS. A, P-eNOS tended to increase after MCAO, but not significantly. It was more prominent in all therapeutic groups than it was in the control. No significant difference was seen for eNOS in any group. B, quantitative analysis of Western blotting of P-eNOS. P-eNOS in the combined treatment group was greater than in the control group. *, P < 0.05 versus control (Student’s t test) (n = 6).
min, but not at 2 h, after ischemia/reperfusion. The combination therapy also increased CBF after reperfusion and activated the phosphorylation of eNOS. Furthermore, the neuroprotective effect of the combination therapy was inhibited by an eNOS inhibitor, L-NAME.

Shimokawa (2002) has reported that abnormal ROCK function may contribute to the pathogenesis of ischemic stroke. ROCK activity has also been reported to increase in the ischemic brain hemisphere (Feske et al., 2009) and, in our previous study, fasudil inhibited ROCK and reduced ischemic damage after MCAO (Yamashita et al., 2007). eNOS is activated by the phosphatidylinositol 3-kinase/Akt pathway. Ming et al. (2002) reported that the Rho/ROCK pathway negatively regulated eNOS phosphorylation through inhibition of phosphatidylinositol 3-kinase/Akt. Rikitake et al. (2005) have reported that fasudil increased CBF by up-regulation of eNOS and decreased cerebral infarction in mice. On the other hand, ozagrel has been reported to decrease platelet aggregation and increase CBF by decreasing levels of TXA2 (Oishi et al., 1996). Thus, whereas fasudil seems to act primarily on vascular smooth muscle cells, ozagrel acts mainly on platelets.

In the present study, low doses of fasudil (3 mg/kg) and ozagrel (10 mg/kg) had no significant neuroprotective effects, whereas combined therapy at these doses decreased cerebral infarction, significantly increased CBF after reperfusion, and increased P-eNOS levels in the ischemic cerebral hemisphere. Xavier et al. (2010) reported that inhibition of TXA2 and PGI2 synthesis increased P-eNOS protein, but not eNOS release in mesenteric artery. The detailed mechanism is still unclear; however, ozagrel may activate phosphorylation of eNOS by inhibiting TXA2 synthase. Furthermore, PGI2 is known to collaborate with the system consisting of eNOS/NO (Gryglewski, 2008). Ozagrel may indirectly activate phosphorylation of eNOS. P-eNOS is related to the production of NO, whereas NO levels correlate with the extent of neuroprotection or vasodilatation. These findings indicate that the effect of ozagrel may be to modify the effect of fasudil on eNOS activation. However, further experiments will be needed to clarify the detailed mechanism.

Hydroxyfasudil, an active metabolite of fasudil, was found after administration of fasudil (10 mg/kg i.p.). The Cmax and AUC values of hydroxyfasudil were approximately 134 and 396% of the parent drug, respectively. Hydroxyfasudil and fasudil strongly inhibited Rho kinase with almost equally potency (Yano et al., 2008), and both can improve cerebral infarct in mice (Shin et al., 2007; Yamashita et al., 2007). Thus, hydroxyfasudil contributes to the potency of fasudil.

After an administration of fasudil (10 mg/kg i.p.), the AUC value of hydroxyfasudil was 1907.1 ng\(\cdot\)h/ml in mice. After intravenous infusion of fasudil (60 mg/60 min; approximately 1 mg/kg), the AUC value of fasudil plus hydroxyfasudil was 1319.8 ng\(\cdot\)h/ml in elderly healthy volunteers (Shibuya et al., 2005). The AUC value of fasudil (1 mg/kg, a dose that showed clinical effectiveness in patients with acute stroke) in humans was similar to that after an intraperitoneal administration of fasudil (10 mg/kg) in mice.

Rho kinase increases the phosphorylation of MLC through the inhibition of MLC phosphatase activity by phosphorylation of its myosin binding subunit (Sato et al., 2000). In the present study, hydroxyfasudil inhibited the phosphorylation of MLC in endothelial cells, whereas ozagrel showed no inhibition of MLC phosphorylation and did not alter the inhibitory effect of hydroxyfasudil. Ozagrel did not affect ROCK.
activity and did not alter the effect of fasudil. Suzuki et al. (2008) demonstrated that ozagrel did not inhibit ROCK activity in an enzyme immunoassay system. It is interesting that coadministration of fasudil and ozagrel increased phosphorylation of eNOS above that seen with single administrations in the mouse ischemia model. This suggests an as yet unknown in vivo mechanism by which ozagrel can enhance the phosphorylation of eNOS, although ozagrel does not have the potential to directly inhibit ROCK activity.

**Conclusion**

These findings indicate that a combination therapy of fasudil and ozagrel exhibits a greater neuroprotective effect on cerebral infarction after murine MCAO than does either drug administered as a monotherapy. The neuroprotective effect of combined therapy was expressed at least 5 min after ischemia/reperfusion. The effect apparently is achieved by increasing eNOS and consequently CBF. Hence, a combination therapy of fasudil and ozagrel may have potential as a novel therapy for the treatment of stroke in humans.

**Acknowledgments**

We thank Shino Bito, Masashi Ishiguro, and Yuji Ohtsuka (Asahi Kasei Pharma Co. Ltd.) for expert technical assistance with PK analysis and cell assays.

**Authorship Contributions**

*Conducted experiments:* Koumura, Hamaoka, and Kawasaki.

*Contributed new reagents or analytic tools:* Hozumi and Inuzuka.

*Performed data analysis:* Tsuruma and Shimazawa.

*Wrote or contributed to the writing of the manuscript:* Koumura, Hamaoka, and Hara.

**References**


