Ras Protein Contributes to Cerebral Vasospasm in a Canine Double-Hemorrhage Model

Mitsuo Yamaguchi, MD; Changman Zhou, MD, PhD; Anil Nanda, MD, FACS; John H. Zhang, MD, PhD

Background and Purpose—Mitogen-activated protein kinase (MAPK) has been shown to be involved in the pathogenesis of cerebral vasospasm after subarachnoid hemorrhage (SAH). In the present study we examined the role of Ras protein, an upstream regulator of MAPK, and the effects of the inhibitors of Ras farnesyltransferase (FTase), FTI-277 and FTase inhibitor I, on angiographic vasospasm and clinical evaluations.

Methods—Twenty-five dogs were randomly divided into 5 groups: control, SAH, SAH+dimethyl sulfoxide, SAH+FTI-277, and SAH+FTase inhibitor I. An established canine double-hemorrhage model of SAH was used by injecting autologous arterial blood into the cisterna magna on days 0 and 2. Angiography was performed at days 0 and 7. Clinical behavior and the activation of Ras (GTP-Ras) and phosphorylated ERK1/2 of MAPK in the basilar arteries were examined.

Results—Severe vasospasm was obtained in the SAH and SAH+dimethyl sulfoxide dogs (42.5±2.5% and 38.9±2.4%, respectively). Enhanced GTP-Ras and phosphorylated ERK1/2 were observed in the spastic basilar arteries (P<0.05). Inhibitors of Ras FTase decreased GTP-Ras and phosphorylated ERK1/2, attenuated angiographic vasospasm, and improved appetite and activity scores.

Conclusions—Ras contributes to cerebral vasospasm, and inhibitors of Ras FTase may have potential in the management of cerebral vasospasm. (Stroke. 2004;35:1750-1755.)

Key Words: vasospasm, intracranial ■ MAP kinase ■ ras proteins ■ subarachnoid hemorrhage

The signaling pathways for cerebral vasospasm after subarachnoid hemorrhage (SAH) intrigue researchers. Multiple molecular factors have been identified for therapeutic targeting. After SAH, blood clots and their breakdown products, endothelins, and inflammatory responses all produce molecular signals. One of the most important pathways to relay these signals is the mitogen-activated protein kinase (MAPK) pathway, which is involved in cell differentiation, proliferation, contraction, and death.

The role of MAPK in cerebral vasospasm has been documented. Several upstream regulators of MAPK have been suggested to contribute to MAPK activation and the development of cerebral vasospasm. Among them, Src, Shc, and Raf-1 were activated in the spastic basilar arteries. While Shc is upstream of Src, Raf-1, an effector of Ras, activates MAPK. In addition, protein kinase C may phosphorylate Raf-1, either bypassing or activating Ras, to activate MAPK. Thus, as an important intermediate factor in the MAPK cascade, we evaluated the role of Ras, a downstream effector of Src but an upstream regulator of MAPK, in cerebral vasospasm after experimental SAH. Since multiple forms of Ras exist in the brain, this study targets H-Ras (FTase) as a first step.
femoral artery was injected into the cisterna magna at day 0 and then repeated at day 2. The dogs were tilted at a 20° angle for 10 minutes with their heads down, in a prone position, to permit pooling of blood around the basilar artery. The angiogram was repeated on day 7 before all dogs were killed. The basilar artery on angiogram was measured by a computer-based image analyzer (NIH Image, version 1.62).15

Inhibitor of Ras FTases
Two inhibitors of Ras FTase, FTI-277 and FTase inhibitor I, were purchased from Calbiochem. The dosages of the antagonists were individually calculated for each dog to reach similar drug levels in the dogs’ cerebrospinal fluid (CSF), with the assumption that canine CSF volume is 2 mL/kg.17 Inhibitors of Ras FTase were diluted in DMSO. One milliliter of CSF was withdrawn and mixed with inhibitors (≈200 μL), and the inhibitor containing CSF was intracisternally injected to obtain a final concentration of FTI-277 and FTase inhibitor I in the CSF of 10 and 50 μmol/L, respectively, within the range of other reports.18,19 In those reports, 10 μmol/L of FTI-277 and 50 μmol/L of FTase inhibitor I reduced Ras FTase activity sufficiently and were not toxic for cells. The same volume of DMSO (200 μL) was injected into the cisterna magna for SAH DMSO dogs. The first injection was conducted at 30 minutes after the first blood injection and was continued daily for an additional 3 days.

Clinical Assessment
Three behavioral examinations (Table) were modified from a previous study15 and performed daily after SAH to record appetite, activity, and neurological deficits.

Morphological Assessment
After euthanasia with Beuthanasia-D, the dogs (n=1 for normal control and n=2 for SAH, SAH+DMSO, and SAH treated with FTI-277 or FTase I groups) were perfused via both common carotid arteries with 200 mL 0.1 mol/L PBS and then 500 mL 4% paraformaldehyde in 0.1 mol/L PBS at 4°C. The brain from each dog was removed and postfixed with 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4) at 4°C. The pons with the basilar artery was cut into sections 4 μm thick with a cryostat (Leica CM3050 S).

Behavior Scores

<table>
<thead>
<tr>
<th>Category</th>
<th>Behavior</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appetite</td>
<td>Finished meal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Left meal unfinished</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Scarcely ate</td>
<td>2</td>
</tr>
<tr>
<td>Activity</td>
<td>Active, barking, or standing</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Lying down, will stand and walk with some stimulation</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Almost always lying down</td>
<td>2</td>
</tr>
<tr>
<td>Deficits</td>
<td>No deficits</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Unstable walk due to ataxia or paresis</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Impossible to walk and stand because of ataxia or paresis</td>
<td>2</td>
</tr>
</tbody>
</table>

H&E Staining
Sections were stained in hematoxylin for 2 minutes and eosin for 1 minute. They were then dehydrated and mounted by Permount.

Immunohistochemistry Staining
The methods for immunohistochemistry staining have been described previously.20 The sections were incubated in rabbit polyclonal anti-phosphorylated ERK 1/2 (1:200) (Santa Cruz Biotechnology) overnight at 4°C. The sections were then incubated with goat anti-rabbit IgG as a secondary antibody (1:200) for 30 minutes, placed in avidin-peroxidase complex solution containing avidin-peroxidase conjugate for 30 minutes, and then mounted, air-dried, dehydrated, and coverslipped.

Phosphorylated ERK1/2
The basilar arteries for Western blotting analysis (n=2 from normal control; n=3 for SAH and SAH+DMSO; n=4 for SAH+FTI-277 and SAH+FTase I) were removed, frozen in liquid nitrogen, and stored at −80°C until use. The frozen arteries were homogenized at 4°C with ultrasonic waves in buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton-X 100, 10% glycerol, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium fluoride, 20 mmol/L

Figure 1. Representative angiograms of the basilar arteries. The angiograms on the left were obtained on day 0 before blood injection, and the one on the right was obtained on day 7 after 2 hemorrhages. In the SAH (n=5) and SAH+DMSO (n=5) dogs, severe vasospasm was shown on day 7. Moderate vasospasm was shown in the dogs treated with FTI-277 (n=6) and FTase inhibitor I (n=6). The residual diameters of the basilar arteries are summarized.
tetrosodium pyrophosphate, 2 mmol/L sodium orthovanadate, 0.1% sodium dodecyl sulfate, and 0.5% deoxycholate. Samples (20 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with 10% polyacrylamide gel. After electrophoretic transfer of the separated polypeptides to the nitrocellulose membrane, the membranes were then washed and incubated with the primary antibodies overnight at 4°C with 1% nonfat milk in Tween-TBS (TBST).

Rabbit polyclonal anti-ERK1, anti-ERK2, and anti-phosphorylated ERK1/2 (Santa Cruz Biotechnology) were used. After incubation with the primary antibodies, the nitrocellulose membranes were washed with TBST and incubated with the appropriate hors eradish peroxidase–labeled secondary antibodies (Santa Cruz Biotechnology). An enhanced chemiluminescence system (Amersham) was used to visualize the protein bands. The results were quantified by Quantity One Software (BioRad).

Activation of Ras (GTP-Ras)
Ras protein exists in 2 states: a GTP-bound active state (GTP-Ras) and a GDP-bound inactive state. Ras activity was assessed with the use of the Ras Activation Assay Kit (Upstate Biotechnology) according to the manufacture’s protocol. Briefly, beads conjugated with the Ras-binding domain of Raf were used to precipitate GTP-Ras. Western blotting was performed with anti-Ras antibody (Ras10; Upstate Biotechnology).

Data Analysis
Results were expressed as mean±SEM. The residual diameter, clinical score, and Western blotting analysis were analyzed by ANOVA, followed by the Schefe\'s post hoc test if significant variance was found. A probability value of \( P<0.05 \) was considered statistically significant.

Results

Arterial Diameter on Angiography
All dogs in the SAH and SAH+DMSO groups developed severe vasospasm, as shown by angiography on day 7 after SAH (Figure 1A). The mean values of the residual diameter of the basilar artery on day 7, as a percentage of that on day 0, were 42.5±2.5% and 38.9±2.4%, respectively. In the FTI-277 and FTase inhibitor I groups, moderate vasospasm, 62.0±6.0% and 61.9±4.5%, respectively, was observed in all dogs (\( P<0.05 \) versus SAH or SAH+DMSO; Figure 1, bottom). No statistical difference was noted between the SAH and SAH+DMSO groups or between the 2 treatment groups (\( P>0.05 \), ANOVA).

Clinical Assessment
The behavior scores for appetite, activity, and neurological deficit are shown in Figure 2. The appetite score in treatment groups was better than that in the SAH group from days 2 to 6, even though statistical significance was achieved only at day 7 (\( P<0.05 \); Figure 2A). No statistical differences were found between the treatment groups or between the treatment groups and the SAH+DMSO group (\( P>0.05 \), ANOVA).

The activity of the dogs (Figure 2B) was significantly better in those treated with FTase inhibitor I for most days after SAH (\( P<0.05 \) versus SAH). In dogs treated with FTI-277, the activity score was significantly better at days 5 to 7 (\( P<0.05 \) versus SAH). The activity score for dogs in treatment groups was significantly better than that in the SAH+DMSO group (\( P<0.05 \)) only at day 7.

On the contrary, most dogs did not have neurological deficits, and no significant difference was observed among the groups (\( P>0.05 \); Figure 2C).

Morphological Study

H&E Staining
No vasospasm was noted in the control group (Figure 3A1). Morphological vasospasm was observed in all SAH dogs (Figure 3B1 to 3E1) and was characterized by corrugation of the internal elastic lamina and contraction of the smooth muscle cells.21 However, the thickness of the vessel wall was larger in the SAH and SAH+DMSO dogs (Figure 3B1 to 3C1), indicating severe vasospasm.21 Moderate vasospasm was obtained in dogs treated with FTI-277 and FTase inhibitor I (Figure 3D1 to 3E1).

Immunohistochemistry
Limited staining of phosphorylated ERK1/2 was visible in the control group (Figure 3A2). In the SAH and SAH+DMSO...
dogs, strong staining of phosphorylated ERK1/2 was observed in all layers of the basilar artery, especially in the adventitial layer and in the endothelial cells (Figure 3B2 to C2). In dogs treated with FTI-277 and FTase inhibitor I, limited staining of phosphorylated ERK1/2 was observed (Figure D2 to E2).

GTP-Ras and Phosphorylated ERK1/2
When the values in the normal basilar arteries were regarded as 100%, enhanced GTP-Ras and phosphorylated ERK1/2 (P<0.05 versus control, ANOVA) were observed in the spastic basilar arteries from the SAH and SAH+DMSO dogs killed at day 7 (Figure 4). Representative bands are shown at the top of Figure 4A and 4B. The values of Ras and ERK1/2 were calculated by dividing Ras-GTP by total Ras and dividing phosphorylated ERK1/2 by total ERK1/2.

Treatment with FTI-277 significantly suppressed the GTP-Ras compared with the SAH+DMSO dogs (P<0.05) and decreased phosphorylated ERK1/2 compared with the SAH and SAH+DMSO groups (P<0.05, ANOVA). Treatment with FTase inhibitor I significantly suppressed GTP-Ras and phosphorylated ERK1/2 compared with the SAH and SAH+DMSO groups (P<0.05, ANOVA). No significant differences were noted between the FTI-277 and FTase inhibitor I groups or between the treatment groups and the control dogs (P>0.05, ANOVA).

Discussion
The novel observations in this study are as follows: (1) GTP-Ras was increased in the spastic basilar arteries at day 7 after SAH, and (2) the 2 inhibitors of Ras FTase, FTI-277 and FTase inhibitor I, abolished GTP-Ras, attenuated angiographic vasospasm, and improved clinical scores. Increasing GTP-Ras proteins led to the phosphorylation of ERK1/2 in the spastic basilar artery,13 and, consequently, inhibition of Ras FTase by FTI-277 and FTase inhibitor I abolished the phosphorylated ERK1/2.

In the present study we used a double-hemorrhage canine model of SAH and applied treatment via the cisterna magna, as described in our previous studies.15,22 Consistently, double blood injections produced severe angiographic and morphological vasospasm in dogs. The attenuation of angiographic vasospasm by inhibitors is always accompanied by the relief of morphological vasospasm in this animal model, which has been shown previously21 and was not repeated in detail in the present study (Figure 3). DMSO did not relieve vasospasm, which is consistent with our previous studies and probably is due to low concentration of DMSO. Thus, the results obtained from this study are comparable to those of previous studies and provide a further step in confirming the crucial role of MAPK, especially ERK1/2, in the pathogenesis of cerebral vasospasm.5,13

Ras proteins require several posttranslational modifications, including prenylation, proteolysis, carboxymethylation, and palmitiolation, to acquire full biological activity.23,24 In the first step of these modifications, prenylation is catalyzed by the enzymes farnesyltransferase (FTase) and geranylgeranyltransferase (GGTase).23,24 FTI-277 is a highly potent, selective inhibitor of FTase and is effective and selective in disrupting constitutive H-Ras–specific activation of MAPK.25 FTase inhibitor I is 37-fold more active against FTase than against GGTase18 (Calbiochem catalog). In the present study FTI-277 and FTase inhibitor I achieved similar degrees of relief in angiographic vasospasm, abolished GTP-Ras, and consequently abolished phosphorylated ERK1/2 in the spastic basilar arteries, indicating that FTI-277 seems more potent since a 5-fold lower dosage was used. In biological tissues, there are 3 different Ras genes: H-Ras, N-Ras, and K-Ras. They may differ in their relative abilities to activate effectors of Ras.26 K-Ras is resistant to FTase inhibitor27 and becomes geranylgeranylated in the presence of FTase inhibitors.28 Since N-Ras and K-Ras processing requires both FTase and GGTase inhibition, it is likely that H-Ras is inhibited by the FTase inhibitors FTI-277 and FTase inhibitor I. Since the
selectivity of these FTase inhibitors, especially at the concentrations used in the present study, remains debatable, in addition to the fact that FTase inhibitor I inhibits not only FTase but also GGTase, it is conceivable that nonspecific but beneficial effects of these compounds could include inhibition of other key proteins that participate in the signaling pathways related to smooth muscle contraction and cerebral vasospasm.

One of the difficulties in studying cerebral vasospasm is the lack of suitable animal models that demonstrate cerebral ischemia as seen in patients.29 In the present study we modified the behavior score15 and intended to obtain a detailed presentation of the clinical outcome of cerebral ischemia. We evaluated each dog daily and recorded the findings in 3 areas: appetite, activity, and neurological deficits. Even though no major neurological deficits were observed (Figure 2C), loss of appetite and especially the inactivity of the SAH dogs (Figure 2B) indicate a degree of behavioral disturbance, which may be the result of cerebral ischemia caused by vasospasm (Figure 1). Interestingly, Ras FTase inhibitors not only attenuated angiographic vasospasm (Figure 1) but also improved clinical behavioral scores (Figure 2B). This suggests that the disturbance of the behavioral scores was not caused by blood clot stimulation but rather was produced by cerebral ischemia caused by vasospasm. Another possibility is that FTase inhibitors may have an additional neuroprotective effect and may improve clinical scores.

Even though Ras inhibitors reduced angiographic vasospasm, the overall effect was mild, retaining the diameter of the basilar artery up to 60% of the normal value. It is unclear whether this 50% increase in diameter, from 40% to 60%, will be translated into any clinical value in the measure of cerebral blood flow or cerebral perfusion pressure, both of which are extremely important to the survival of brain tissues.30 The mild effect of Ras inhibitors may not be due to the potency of the inhibitors used, the half-life, or the timing of application since FTI-277 and FTase inhibitor I abolished GTP-Ras and phosphorylated ERK1/2 at day 7 (Figure 4), indicating the potency of these 2 inhibitors and their long-lasting effects. In addition, we used inhibitors selective for FTase but not for GGTase, and we are not certain whether R-Ras and N-Ras may contribute to cerebral vasospasm. Other factors that may contribute to cerebral vasospasm include mechanical impact, sympathetic and parasympathetic nervous stimulation, endothelial injury, vascular remodeling, membrane depolarization, and vasoactive agents that produce contraction not mediated by Ras-MAPK pathways.1 In conclusion, Ras-MAPK pathways play a significant but partial role in the development of vasospasm.

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