Role of c-Jun N-Terminal Kinase in Cerebral Vasospasm After Experimental Subarachnoid Hemorrhage

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Background and Purpose—Inflammation could play a role in cerebral vasospasm after subarachnoid hemorrhage (SAH). SP600125 a c-Jun N-terminal kinase (JNK) inhibitor reduces inflammation. The present study examined if SP600125 could reduce cerebral vasospasm.

Methods—Twenty-seven dogs were assigned to 5 groups: control, SAH, SAH plus dimethyl sulfoxide (DMSO), SAH plus SP600125 (10 μmol/L), and SAH plus SP600125 (30 μmol/L). SAH was induced by the injection of autologous blood into the cisterna magna on day 0 and day 2. Angiograms were evaluated on day 0 and day 7. The behavior of the dogs was evaluated daily. The activation of the JNK pathway, the infiltration of leukocytes, and the production of cytokines were also evaluated.

Results—Severe vasospasm was observed in the basilar artery of SAH and DMSO dogs. The JNK signaling pathway was activated in the basilar artery after SAH and SP600125 reduced angiographic and morphological vasospasm and improved behavior scores with a concomitant reduction of infiltrated leukocytes and IL-6 production.

Conclusions—These results demonstrate that SP600125 attenuated cerebral vasospasm through a suppressed inflammatory response, which may provide a novel therapeutic target for cerebral vasospasm. (Stroke. 2005;36:1538-1543.)

Key Words: cerebral vasospasm ■ inflammation ■ JNK ■ subarachnoid hemorrhage

Cerebral vasospasm is the major cause of morbidity and mortality in patients subjected to subarachnoid hemorrhage (SAH). The pathogenesis of cerebral vasospasm is unclear, but many studies indicate inflammation plays a putative role.1–3 C-Jun N-terminal kinase (JNK), a member of the mitogen-activated protein kinase group, has been shown to be involved in the response to a variety of extracellular stresses and has been implicated in numerous physiological processes, such as cell proliferation, cell survival, apoptosis, inflammation, and embryonic development. Experimentally, SP600125, an inhibitor of the JNK signaling pathway, has been applied to regulate the inappropriate activation of immune reactions in autoimmune and inflammatory diseases such as rheumatoid arthritis4 and asthma.5 Therefore, we hypothesized that the JNK signaling pathway might be a therapeutic target of cerebral vasospasm from the point of view of inflammation. The purpose of this study was to investigate if the JNK signaling pathway was activated by the stress of SAH and if inhibition of the JNK signaling pathway could attenuate cerebral vasospasm through a suppression of the inflammatory response.

Materials and Methods
All experiments were performed according to the protocol evaluated and approved by the Animal Care and Use Committees at the Louisiana State University Health Sciences Center and at the Loma Linda University, California.

Canine Double-Hemorrhage Model
Twenty-seven adult mongrel dogs (15.0 and 19.8 kg; Alder Ridge Farms, Inc, Lakewood, Pa) were randomly assigned to 5 groups: (1) control group (n=6); (2) SAH group (n=6); (3) DMSO group (n=6); SAH+dimethyl sulfoxide (DMSO); (4) SP10 group (n=6); SAH+SP600125 (10 μmol/L); and (5) SP30 group (n=6); SAH+SP600125 (30 μmol/L). The present study was semi-masked, meaning that the investigators knew the normal and SAH groups because no treatment was applied, but they did not know of the content of treatment in the 3 treated groups (DMSO, SP10, or SP30, prepared and recorded by a technician). The dogs of the control group were euthanized without injection of blood to harvest the basilar artery for analysis.

Procedures were performed as previously described.6–8 Briefly, after the dogs had received an intramuscular injection of acepromazine (0.1 to 0.5 mg/kg), atropine (0.05 mg/kg), and xylazine (1.1 mg/kg) as premedication, general anesthesia was maintained by endotracheal delivery of isoflurane and O2 with mechanical ventilation. The arterial blood pressure, end-tidal CO2, and SpO2 were monitored.
monitored by using a V60046 monitor (Surgi Vet). The flow of isoflurane was adjusted to control within normal ranges.

Contrast medium (7 mL; Visipaque) was injected to obtain the baseline angiogram of the basilar artery. The cisterna magna was punctured percutaneously, and then 0.5 mL/kg of blood withdrawn from the femoral artery was injected into this space on day 0 and repeated on day 2 without performing angiography. To minimize the parallax error, cerebral angiograms were performed by c-arm fluoroscopy with constant magnification and exposure factors. The cerebrospinal fluid (CSF) was collected before the first blood injection and after the angiography at day 7 for the measurement of cytokines. The collected CSF was centrifuged at 1000g for 20 minutes. The supernatants were passed through a 0.22-μm filter and stored at −80°C until use. The angiography was performed on day 7 again, and then all dogs were euthanized. The arterial diameters were measured at the following 3 portions: the distal portion (just before bifurcation of basilar tip), the proximal portion (just after vertebral-basilar union), and the mid portion (the center between previous 2 points) using the National Institutes of Health image analyzer ImageJ (version 1.32). The mean of these measurements was calculated and the mean diameter of the basilar artery on day 7 was quantified as a percentage of the mean on day 0.

**Pharmacological Inhibitor**

JNK inhibitor, SP600125, was purchased from Calbiochem. The dosage was calculated for each dog on the basis of previous reports. According to the manufacturer’s product data sheet, SP600125 is a potent, cell-permeable, selective, and reversible ATP competitive inhibitor. The final target concentrations of this inhibitor in CSF were determined on the basis of the first study about this inhibitor, which showed that SP600125 inhibited the phosphorylation of c-Jun with an IC₅₀ of 5 to 10 μmol/L on a cell-based assay, not a biochemical assay, and that no cell toxicity was observed within 50 μmol/L/L. The volume of dog CSF was estimated at ≈2 mL/kg. One milliliter of CSF was withdrawn and mixed with the appropriate volume of SP600125 diluted in DMSO to obtain final concentrations of 10 μmol/L and 30 μmol/L in CSF. The CSF containing the drug was injected into the cisterna magna percutaneously. The same volume of CSF containing DMSO without the inhibitor was injected for the dogs in the DMSO group. This injection was continued daily from day 0 to day 3.

**Clinical Evaluation**

The clinical behavior scores were recorded daily from day 0 to day 7, using the modified scoring table published previously. This scoring table had 3 categories: (1) appetite; (2) activity; and (3) neurological deficit. The points were given by following the scale: appetite: finished meal=2, left meal unfinished=1, scarcely ate=0; activity: active, barking, or standing=2, lying down, will stand and walk with some stimulation=1, almost always lying down=0; neurological deficit: no deficit=2, unstable walk because of ataxia or paresis=1, impossible to walk and stand because of ataxia or paresis=0.

**Western Blot**

After euthanasia with Beuthanasia-D, the dogs were perfused via both common carotid arteries with 200 mL of 0.1 mol/L phosphate-buffered saline, pH 7.4, and then 500 mL of 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline. The basilar artery with brain tissue were enucleated and postfixed in the same fixative. The tissues were placed in tissue freezing medium (Triangle Biomedical Sciences) and frozen. Ten-μm-thick sections were cut using a cryostat (Leica LM9260S) for morphological study.

The sections for hematoxylin and eosin (H&E) staining were fixed with hematoxylin for 3 minutes and eosin for 0.5 minutes. Immunohistochemistry was performed using ABC Staining System (Santa Cruz Biotechnology) as previously described. The primary antibodies used in this study, which were purchased from Santa Cruz Biotechnology, included phosphorylated c-Jun (1:200), anti-CD4 (1:200), anti-CD8 (1:200), anti-MPO (1:200), and anti-CD68 (1:200). For the negative control, the same procedure was performed with the exception that the primary antibody was omitted. Sections were air-dried, dehydrated, and covered with a glass coverslip.

**Morphological Evaluation**

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**Measurement of Inflammatory Cytokines**

The measurements of tumor necrosis factor (TNF)-α and IL-6 in CSF were performed using anti-human enzyme-linked immunosorbent assay kits (BioSource International, according to the manufacturer’s instructions. A previous report validated that there was cross-reactivity with TNF-α and IL-6 between humans and dogs.

**Statistical Analysis**

Results are presented as means±SEM. The clinical behavior score was analyzed by Kruskal-Wallis 1-way ANOVA on ranks, and then if significant differences were found, followed by Dunn multiple comparison procedure. The diameter of the basilar artery, Western blotting analysis, and enzyme-linked immunosorbent assay analysis were analyzed by 1-way ANOVA, then the Turkey–Kramer multiple comparison procedure if the significant differences were found by ANOVA. Significance was accepted at P<0.05.

**Results**

**Arterial Diameters**

Whereas all dogs in the SAH and DMSO groups had severe vasospasm of the basilar artery develop, dogs in the treatment groups had reduced vasospasm on the basis of a dose-dependent manner (Figure 1A). The mean diameters of the basilar artery on day 7 (as the percentage of that on day 0) were 43±2% in SAH group, 39±2% in DMSO group, 49±4% in SP10 group (P<0.05 compared with DMSO, ANOVA), 65±5% in SP30 group (P<0.05 compared with SAH, DMSO, SP10, ANOVA). No statistical difference between SAH and DMSO group was observed (Figure 1B).

**Clinical Evaluation**

The clinical behavior scores are shown in Figure 2. The score of appetite in the SAH group slowly deteriorated, whereas the SP10 group showed a decrease up to 3 days but then improved. There was significant differences in these 2 groups on day 6 and day 7 (P<0.05; Figure 2A). Most of the dogs in the SAH and DMSO groups had loss of activity all the time, whereas some of the dogs in both treatment groups slightly decreased their activities. The activity of the dogs in both treatment groups was significantly better than that in SAH and DMSO group on day 1 and day 2 (P<0.05; Figure 2B). In contrast, most of the dogs did not have neurological deficits and there was no significant difference among the groups (P>0.05; Figure 2C).
Activated JNK and c-Jun
To confirm that the JNK signaling pathway was activated by SAH, the protein expression of phosphorylated JNK and total JNK in the basilar artery were examined by Western blot analysis. As shown in Figure 3A, the ratio of phosphorylated JNK to total JNK in all SAH groups significantly increased compared with the control group ($P < 0.05$). To determine whether SP600125 inhibited the JNK signal pathway, we investigated the expression of phosphorylated c-Jun and c-Jun, which are the downstream of JNK. The ratio of phosphorylated c-Jun to c-Jun in both the SAH and DMSO groups were significantly higher than the control group ($P < 0.05$) and those in both treatment groups were significantly lower compared with the SAH and DMSO groups ($P < 0.05$). No significant differences were observed between the SAH and DMSO group or between the SP10 and SP30 group (Figure 3B).

Morphological Study
No vasospasm was observed in the control group (Figure 4). Severe morphological vasospasm was observed in both the SAH and DMSO group, characterized by a corrugated internal elastic lamina, a thickened vessel wall, and contracted smooth muscle cell. There were mild morphological changes in dogs treated with SP600125.

In the control group, negative staining of p-c-Jun was seen. Strong positive staining was observed in all layers of vessel wall from the samples of the SAH and DMSO groups. Limited staining was visible in basilar artery from SP600125 treatment groups.

To evaluate the leukocyte infiltration, immunohistochemistry for several antigens, including CD4, CD8 (to detect T cell), MPO (to detect neutrophil), and CD68 (to detect macrophage) was performed (Figure 5). No positive cells were observed in the adventitia layer and the subarachnoid space in the control group. Some leukocyte infiltration to periadventitia and subarachnoid space were visible in the SAH and DMSO groups; however, extremely limited infiltration was observed in both treatment groups.

Measurement of Inflammatory Cytokines
The concentrations of IL-6 in the CSF on day 0 and day 7 are shown in Figure 6. The levels of IL-6 in the CSF on day 0 were low in all groups and there was no significant difference among the groups. On the contrary, the level in the SAH group on day 7 was significantly elevated compared with those in both treatment groups. TNF-$\alpha$ was not detected in any CSF samples.
Discussion
There were 3 novel observations in this study. First, the JNK signaling pathway was activated in the all layers of the arterial wall on day 7 after SAH. Second, the inhibition of the JNK signaling pathway by SP600125 led to an attenuation of both the angiographic and morphological vasospasm in the basilar artery. Third, the inflammatory responses such as the infiltration of leukocytes and the production of cytokines were suppressed by the inhibition of the JNK signaling pathway.

In this study, we have shown that there was a significant increase in the proportion of the protein expression of phosphorylated JNK responding to SAH on day 7 and the distribution of this activation in the basilar artery was exhibited in all layers, including endothelial cells, smooth muscle cells, and adventitial surface. JNK is activated by certain cytokines, mitogens, osmotic stress, and ultraviolet irradiation. This JNK activation causes the phosphorylation of c-Jun, other transcription factors, and cellular proteins, resulting in the regulation of altered gene expression, cellular survival, and proliferation. Although a previous study only showed that JNK activation in the cerebral vessel was observed 1 day after SAH in a rat model, the JNK activation on day 7 observed in this study indicates that the JNK signaling pathway could play a crucial role in cerebral vasospasm.

We next demonstrated that the cisternal administration of SP600125, a JNK inhibitor, significantly suppressed the phosphorylation of c-Jun in the basilar artery and attenuated the angiographic vasospasm in a dose-dependent manner. This suggests that SP600125 may be a potential therapeutic drug for cerebral vasospasm. In the present study, the inhibition of the JNK signaling pathway improved clinical behavior scores, including appetite and activity. Although the appetite scores were significantly different on day 6 and day 7, the activity scores were significantly different on day 1 and day 2. Even though the appetite score is consistent with the reduction of vasospasm, the activity score may not represent the effect of vasospasm because the timing of vasospasm in this canine model peaked at day 7. One possible explanation is that SP600125 may improve the activity score by reducing acute brain injury after SAH.

The inflammatory response could play a role in the development and maintenance of cerebral vasospasm. A recent review showed that in the case of SAH, the presence of a blood clot in the subarachnoid space activates the inflammatory response through a complex series of cellular and molecular events, which includes: (1) leukocyte recruitment, infiltration and activation; (2) cytokine production; (3) immunoglobulin and complement activation; and (4) transcription factor activation. Cerebral vasospasm might result from the interaction among these events. The present study
showed that the inhibition of the JNK signaling pathway suppressed the infiltration of leukocytes. Leukocytes such as lymphocyte, neutrophil, and macrophage infiltrate to the arachnoid space after SAH and could play a role in the pathogenesis of cerebral vasospasm. Leukocytes might release reactive oxygen metabolites, which initiate endothelial dysfunction and calcium influx. Furthermore, activated mononuclear leukocytes in the CSF after SAH produce endothelin-1, which is a possible mediator of cerebral vasospasm. In vitro, SP600125 was shown to suppress the expression of ICAM-1, which plays an important role in leukocyte recruitment into sites of active inflammation. Moreover, antibodies against ICAM-1 reduced cerebral vasospasm by inhibiting the infiltration of leukocytes. Therefore, our results indicate that the JNK inhibitor reduced cerebral vasospasm possibly by inhibiting infiltration of leukocytes.

Cytokines are recognized as low molecular proteins, which are pleiotropic and have multiple diverse biological activities. Previous findings showed that the level of inflammatory cytokines, including IL-1, IL-6, IL-8, and TNF-α were related to the severity of SAH, brain damage, and the occurrence of cerebral vasospasm. In this study, we investigated only IL-6 and TNF-α to evaluate the inflammatory response, because the enzyme-linked immunosorbent assay kits for IL-6 and TNF-α were available in respect to cross-reactivity between humans and dogs. The result that TNF-α in CSF was not detected is consistent with a previous study. In other reports, the concentration of TNF-α in CSF after SAH was ~100-times lower than that of IL-6. This matter may be settled if a highly sensitive kit for dog TNF-α was available. Our findings demonstrated that IL-6 levels in the CSF on day 7 were significantly higher than day 0 and the inhibition of the JNK pathway decreased the level of IL-6 in CSF on day 7. Evidence for the involvement of IL-6 in the pathophysiology of SAH has been reported. One is that the concentration of IL-6 in CSF was associated with abnormal blood flow velocity, which is related to cerebral vasospasm. Second, intracisternal injection of IL-6 induced vasoconstriction. Third, neutralizing antibody against IL-6 reduced posthemorrhagic vasospasm. Therefore, our results and these findings strongly suggested that the JNK inhibitor suppressed IL-6 production in CSF, thereby reducing cerebral vasospasm.

In conclusion, the JNK signaling pathway was activated after SAH and the inhibition of the JNK signaling pathway...
reduced cerebral vasospasm through a suppression of the inflammatory response.

Acknowledgments
This work was supported in part by a grant from the American Heart Association Bugher Foundation Award for Stroke Research and National Institutes of Health grants NS45694, HD43120, and NS43338 to J.H.Z. and by a fellowship from National Hospital Organization (Japan) to H.Y.

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Stroke. 2005;36:1538-1543; originally published online June 9, 2005;
doi: 10.1161/01.STR.0000170713.22011.c8
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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