Inhibition of the p38 Mitogen-Activated Protein Kinase (MAPK) Pathway Attenuates Cerebral Vasospasm Following Experimental Subarachnoid Hemorrhage in Rabbits

Xing Zhang*, Xu Dong Zhao*, Ji Xin Shi, Hong Xia Yin

Abstract. The p38 mitogen-activated protein kinase (MAPK) plays an important role in apoptosis and is also involved in the development of cerebral vasospasm after subarachnoid hemorrhage (SAH). Here, we sought to examine whether inhibition of p38 MAPK could attenuate cerebral vasospasm and investigate the underlying mechanisms in a rabbit SAH model. SAH was established in rabbits (n=12/group) using the double-hemorrhage method. We observed apparent vasospasm in the basilar arteries of rabbits with SAH, which was significantly attenuated by SB203580, a selective p38MAPK inhibitor. Immunoblotting assays showed enhanced phosphorylation of p38 MAPK and ATF2 and increased caspase-3 cleavage following SAH, which were, however, markedly suppressed by SB203580. TUNEL staining further revealed significant apoptosis in the basilar arteries of rabbits with SAH, which was scantly present in rabbits treated with SB203580. Our results demonstrated that p38 MAPK was activated in cerebral vasospasm and associated with increased apoptosis in the basilar arteries and p38 MAPK inhibition suppressed apoptosis, suggesting that p38 MAPK could be a novel therapeutic target for cerebral vasospasm.

Keywords: subarachnoid hemorrhage, cerebral vasospasm, p38 mitogen-activated protein kinase, apoptosis

Introduction

Cerebral vasospasm is the most common cause of mortality and morbidity in patients who have survived the onset of subarachnoid hemorrhage (SAH) [1]. Prevention of cerebral vasospasm is considered as a major goal in the management of patients at risk for SAH. Even though many studies have implicated apoptosis in the pathogenesis of cerebral vasospasm, the exact underlying mechanisms for the disease still remain obscure [2,3]. The p38 mitogen-activated protein kinase 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 cellular proliferation, cellular survival, apoptosis, inflammation, and embryonic development [4]. The aim of the current study was to examine apoptosis in the basilar arterial wall following experimental SAH in rabbits and investigate whether the inhibition of the p38MAPK signaling pathway could attenuate cerebral vasospasm through the suppression of apoptosis.

Materials and Methods

Animal preparation. The experimental animal study protocol was approved by the Animal Care and Use Committee of Nanjing University and conformed to the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH Publications No. 80-23). All New Zealand white rabbits used in the present study were acclimated in a humidified room and maintained on standard pellet diet at the Animal Center of Jinling Hospital for 10 days before the experiment. The temperature in both the feeding room and the operation room was maintained at about 25°C.

Two-hemorrhage rabbit model. Experimental SAH was produced according to the two-hemorrhage method [5]. The rabbits were anesthetized with an intramuscular injection of a mixture of ketamine (25 mg/kg) and droperidol (1.0 mg/kg) on day 0. Under spontaneous breathing, a 23-gauge butterfly needle was inserted percutaneously into the cisterna magna. After withdrawal of 1.5 mL cerebrospinal fluid (CSF), an equal volume of non-heparinized fresh autologous auricular arterial blood was slowly injected into the cisterna magna for 1 min under aseptic condition. Then, the animals were
kept at 30°C in a head-down position for 30 min. After recovery from anesthesia, they were returned to the feeding room. Forty-eight hours after the first SAH, a second injection was performed in the same manner as in the first. In control animals, the same procedure was performed with injection of sterile saline instead of blood.

**Animal experimental design.** Forty-eight adult New Zealand white rabbits with weight from 2.4 to 2.8 kg were assigned randomly to four groups: the control group, the SAH group, the SAH + dimethylsulfoxide (DMSO) group, and the SAH + SB203580 group (n=12 in each group). The p38 MAPK inhibitor SB203580 (Tocris Bioscience, Ellisville, MO) was dissolved in DMSO. For preparation of SB203580, 1 mL of CSF was withdrawn and mixed with SB203580 to obtain a final concentration of 20 μmol/L, and the mixture was injected into the cisterna magna [6]. The procedure was performed every 24 hours beginning 30 min after injection of blood to the last day of the experiment. Rabbits in the SAH + DMSO group received equal volumes of DMSO at the corresponding time points. All the rabbits were sacrificed on day 7.

Six rabbits in each group were killed by the fixation-perfusion method. The basilar arteries were taken for hematoxylin and eosin (H&E), immunohistochemical and TUNEL staining. The other rabbits in each group were exsanguinated and decollated. The basilar arteries were removed and frozen in liquid nitrogen immediately for subsequent Western blotting analysis.

**Clinical evaluation.** Clinical scores of the experimental animals with SAH were recorded based on independent observations by a veterinarian who was blinded to the animal treatments. The scores of animals were recorded on day 3, 5, and 7 by using a scoring method (Table 1) modified from a previously described system [7].

<table>
<thead>
<tr>
<th>Category</th>
<th>Behavior</th>
<th>Score</th>
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<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 upon 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>Unable to walk due to ataxia or paresis</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Unable to walk and stand due to ataxia and paresis</td>
<td>2</td>
</tr>
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**Western blotting assays.** Frozen basilar arteries were minced and homogenized ultrasonically (10 seconds, 3 times) in an extraction buffer. Lysates were then centrifuged at 13,000 g at 4°C for 10 min, and the supernatants were transferred to fresh tubes and stored at -80°C for further use. The samples (70 μg per lane) were separated by 8% SDS–PAGE and electro-transferred onto a polyvinylidene-difluoride (PVDF) membrane. The membrane was blocked with 5% skimmed milk for 2 hours at room temperature, incubated overnight at 4°C with primary rabbit antibodies against p38 MAPK, phosphorylated p38 MAPK, ATF2, phosphorylated ATF2 and cleaved caspase-3 (all from Cell Signaling Technology, Beverley, MA). The β-actin (Sigma, St. Luis, MO) was used as a loading control. After the membrane was washed for 10 min each for six times in PBS + Tween 20 (PBST), it was incubated with peroxidase-goat anti-rabbit immunoglobulin G. The blotted protein bands were visualized using enhanced chemiluminescence Western blot detection reagents (Amersham) and were exposed to X-ray film. Developed films were digitized using an Epson Perfection 2480 scanner (Seiko, Nagano, Japan). Optical densities were calculated using Glyko Bandscan software (Glyko, Novato, CA). The tissue of six animals was used for Western blot
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Analysis at each time point. All experiments were repeated at least three times independently.

**TUNEL staining.** Formalin-fixed tissue sections were embedded in paraffin and sectioned at 4 μm thickness with a microtome. The sections were detected for apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nicked labeling (TUNEL) method using the *in situ* Apoptosis Detection Kit as instructed by the manufacturer (Roche) [8]. TUNEL-positive cells contained dark-brown nuclei.

**Statistical analysis.** All data were presented as mean ± standard deviation. SPSS 12.0 was used for statistical analysis of the data (SPSS Inc., Chicago, IL). All data were subjected to one-way analysis of variance (ANOVA) combined with Tukey’s multiple comparison test. Statistical significance was defined as P < 0.05.

**Results**

**General observations.** No SAH-related death was observed in all the experimental groups. The body weight did not exhibit a significant difference among all the groups. The residual subarachnoid clots appeared around the basilar arteries of the rabbits in the SAH, SAH+DMSO and SAH+SB203580 group.

**p38 MAPK inhibition by SB203580 improves behavior scores of animals following subarachnoid hemorrhage.** We examined the behavior scores for appetite and activity of rabbits following SAH. We found that the appetite score for rabbits with SAH or vehicle controls was significantly higher than that of rabbits with SAH who were treated with the p38 MAPK inhibitor SB203580 at d 3, 5 and 7 post SAH (P < 0.05) (Figure 1A). The activity score in rabbits with SAH or vehicle controls was also markedly higher than that of those treated with SB203580 at d 3, 5 and 7 post SAH (P < 0.05) (Figure 1B). Furthermore, no statistically significant differences were found in appetite and activity scores between rabbits with SAH and vehicle controls (P > 0.05). Neurological deficit scores are not shown because most animals did not show any neurological deficits.

**p38 MAPK inhibition by SB203580 negates the reduction of the cross-sectional area of the basilar artery of rabbits with SAH.** We further examined the effect of SB203580 on vasospasm of the basilar arteries in rabbits following SAH. Our histological examination revealed the presence of profound vasospasm in the basilar arteries of rabbits with SAH or vehicle controls. By contrast, rabbits with SAH treated with SB203580 showed apparently reduced vasospasm (Figure 2A to 2D). In addition, corrugation of the internal elastic lamina and thickening of the media of the basilar arteries were severe in rabbits with SAH and vehicle controls than those treated with SB203580 group. In contrast, no corrugation of the internal elastic lamina and thickening of the media was found in the control group. Furthermore, rabbits with SAH showed a markedly reduced cross-sectional area of the basilar arteries (230,692 ± 48,667 μm²) compared with that of controls (520,113 ± 81,867 μm²) (P < 0.01) (Figure 2). Additionally, the cross sectional area of the basilar arteries in rabbits treated with SB203580 (410,552 ± 72,660 μm²) was significantly higher than that of vehicle controls (237,812 ± 37,507 μm²) (P < 0.01). No statistically significant difference in the cross sectional area of
the basilar arteries of rabbits with SAH and vehicle controls (P > 0.05).

**p38 MAPK inhibition by SB203580 attenuates phosphorylation of p38 MAPK and ATF2 following SAH.** Our Western blotting analysis showed that there was a low level of phosphorylation of p38 MAPK and ATF2 in the basilar arteries of control rabbits. Severe vasospasm was evident in the SAH and SAH + DMSO group, but not in the control and SAH + SB203580 group. Densitometric analysis of Western blots showed that SAH caused markedly increased cleavage of caspase-3 in the basilar arteries compared with control rabbits (P <0.01) (Figure 4), suggesting the onset of apoptosis in these tissues. Treatment of rabbits with SAH with SB203580, however, significantly attenuated the increase in the cleavage of caspase-3 following SAH (P < 0.01). We further studied whether p38 MAPK inhibition affected the apoptosis of basilar artery tissues by TUNEL assays. We observed no apoptosis of the basilar artery tissues of control rabbits while noticeable TUNEL-positive cells were visualized in rabbits with SAH. Additionally, we observed only weakly TUNEL-positive cells of the basilar arteries of rabbits treated with SB203580 (Figure 5).

**Discussion**

A diagram for the p38MAPK activation and its relation to the caspase system is showed in Figure 6. The main findings of this study are as follows: (1) the p38MAPK pathway was activated after SAH and could be remarkably suppressed by SB203580, a p38MAPK inhibitor; (2) endothelial apoptotic cell death was ameliorated after intracisternal administration of SB203580; (3) SB203580 attenuated vasospasm and improved behavior scores obtained after SAH. These findings suggest that activation of the p38MAPK pathway plays a crucial role in aggravating the development of cerebral vasospasm in this rabbit SAH model, and may be through the facilitation of apoptosis.

Previous studies have documented that apoptosis, especially in the endothelial cells play an important role in the development and maintenance of cerebral vasospasm [9]. Apoptotic endothelial cell death may lead to destruction of the blood–brain barrier and expose smooth muscle cells to vasoconstrictors in the blood flow. Damage to endothelial cells decreases the generation and release of vasodilators such as nitric oxide and prostacyclin from these cells [10]. Endothelial apoptosis has also been associated with de-encryption of tissue fac-
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...tor activity that may lead to enhanced tissue factor procoagulant activity [11]. Dysfunction of cerebral endothelial cells has been demonstrated and is believed to contribute to the pathogenesis of cerebral vasospasm [12]. Endothelial cell death and detachment of the endothelium promote thrombosis and trigger cell migration and proliferation [13,14].

p38MAPK, which was reported to regulate cellular responses to a variety of cellular stresses, such as heat shock, hyperosmolarity, ultraviolet radiation, the endotoxin lipopolysaccharide (LPS), and hypoxia, is a member of MAPK family. Numerous genes regulated by the p38 MAPK pathway have been identified. These genes encompass a wide range of families including genes encoding cytokines, transcription factors and cell surface receptors. Thus, the p38MAPK pathway is involved in a variety of physiopathological process such as inflammation, cell differentiation, senescence and tumor suppression and apoptosis [15]. Abundant evidence for p38 MAPK involvement in apoptosis exists to date and is based on concomitant activation of p38 MAPK and apoptosis induced by a variety of agents [16,17]. Caspases are central to the apoptotic pathway and are expressed as inactive zymogens. Caspase inhibitors can block p38 MAPK activation through Fas cross linking, suggesting that p38 MAPK functions downstream of caspase activation. However, over expression of dominant active MKK6b, an upstream kinase...
that activates p38 MAPK, can also induce caspase activity and cell death, implying that p38 MAPK may function both upstream and downstream of caspases in apoptosis [18,19]. It must be mentioned that the role of p38 MAPK in apoptosis is cell type and stimulus dependent. While p38 MAPK signaling has been shown to promote cell death in some cell types, in some other cell types, p38 MAPK has been shown to enhance survival, cell growth, and differentiation.

Previous study has documented that p38MAPK is activated in the arterial wall after SAH and upregulates the production of inflammatory cytokines, leading to the development of vasospasm [20]. However, no work has focused on the effect of p38MAPK on endothelial apoptosis after SAH to date. In the present study, SB203580 was used as an inhibitor of p38MAPK. To determine the activity of p38MAPK, the level of its downstream kinase phosphorylated ATF2, the active state of ATF2, was examined. Additionally, the level of caspase-3, which plays a key role in the caspase dependent apoptotic pathway, was chosen to reveal the degree of apoptosis.

In summary, in the present study, we found that SAH could up-regulate the p38MAPK activity, and promote apoptosis of endothelial cells, which could be markedly modulated by SB203580 administration. The treatment of SB203580 in this SAH model resulted in attenuation of endothelial cell apoptosis and the degree of cerebral vasospasm following SAH. Moreover, the level of caspase-3 was down-regulated by administration of SB203580. These results suggest that SAH could induce endothelial apoptosis in the arterial wall, which might play a central role in the pathogenesis of cerebral vasospasm. The potential therapy targeted at suppression of the p38MAPK pathway might be beneficial due to its anti-apoptotic effect.

Acknowledgement

This work was supported by grants from the Jinling Hospital of Nanjing, Jiangsu province, China.

References


