

# Bone Morphogenic Protein-7 Contributes to Cerebral Ischemic Preconditioning Induced-Ischemic Tolerance by Activating p38 Mitogen-Activated Protein Kinase Signaling Pathway

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**Abstract**—Cerebral ischemic preconditioning (IPC), which refers to a transient and noninjurious ischemia is able to induce tolerance against the subsequent lethal ischemia, including ischemic stroke. We have previously reported that bone morphogenic protein-7 (BMP-7) contributes to the neuroprotective effects of IPC-induced ischemic tolerance, and thus ameliorates the following ischemia/reperfusion (I/R) injury in rats. Consequently, in the present study, we continued to explore the underlying regulatory mechanisms involved in BMP-7-mediated cerebral IPC in the rat model of ischemic tolerance. Male Wistar rats were preconditioned by 15-min middle cerebral artery occlusion (MCAO). After 2-day reperfusion, these animals were subjected to prolonged MCAO for 2 h. Our results showed that the phosphorylated p38 mitogen-activated protein kinase (MAPK) paralleling to BMP-7 was up-regulated by IPC in rat brain. Inactivation of p38 MAPK by pretreatment of SB203580, a p38 MAPK-specific suppressor, weakened the protective effect of IPC on CA1 neurons. Moreover, the enhanced phosphorylation of p38 MAPK induced by IPC was attenuated when the endogenous BMP-7 was inhibited by BMP-7 antagonist noggin. Besides, blockade of p38 MAPK signal transduction pathway *via* SB203580 abrogated the protective effects of exogenous BMP-7 against cerebral infarction. These present findings suggest that BMP-7 contributes to cerebral IPC-induced ischemic tolerance *via* activating p38 MAPK signaling pathway.

**KEY WORDS:** BMP-7; p38 MAPK; ischemic preconditioning; ischemic tolerance; cerebral ischemia/reperfusion.

## INTRODUCTION

Cerebral stroke continues to be a major cause of death and the leading cause of long-term disability worldwide [1, 2] and approximately 85 % of all the cerebral strokes are ischemic in nature [3]. Ischemic strokes, resulting from thrombus or embolism-induced middle cerebral artery occlusion (MCAO), usually lead to severe brain lesions including neuronal degradation and cerebral infarction [4]. Although progress has been made in stroke prevention, which focuses on optimizing the treatment of modifiable

risk factors, such as hypertension, diabetes, and dyslipidemia [5, 6], the therapeutic effects of pharmacological treatments are relatively limited [7]. Emerging evidence has proven that the brain has a remarkable capacity for self-preservation [8]. A brief period of cerebral ischemia, also known as ischemic preconditioning (IPC), confers transient tolerance to a subsequent ischemic challenge in the brain [9]. The endogenous mechanisms related to cerebral IPC are thus being widely studied.

Bone morphogenic protein-7 (BMP-7) is a member of the transforming growth factor beta (TGF- $\beta$ ) superfamily [10], and has been reported to mimic the effects of IPC to protect against intestinal ischemia/reperfusion (I/R)-induced intestinal and hepatic injuries in rats [11]. In accordance with these findings, our prior results have demonstrated that BMP-7 mediates IPC-induced ischemic tolerance in rats suffered from cerebral ischemia [12]. However, the underlying mechanisms are required to be fully elucidated.

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The p38 mitogen-activated protein kinase (MAPK) plays a pivotal role in regulating many cellular processes including inflammation, cell differentiation, cell growth, and death [13, 14]. Activation of p38 MAPK *via* extracellular stimuli such as bacterial pathogens and cytokines, mediates signal transduction into the nucleus to turn on the responsive genes [15]. Notably, plenty of researches have delineated BMP-7 as an upstream regulatory factor in p38 MAPK signaling transduction pathway. Exogenous BMP-7 is proven to induce the activation of p38 MAPK in murine renal epithelial cells [16] and human proximal tubule epithelial cells [17]. However, discrepant effects of BMP-7 on p38 MAPK signaling have been observed in several other cell lines. For instance, BMP-7 is observed to directly suppress the phosphorylation of p38 MAPK in human breast cancer cells [18] and articular chondrocytes [19]. These results indicate a multiple role of BMP-7 in regulating p38 MAPK signal at cellular level. Interestingly, a study from Nishimura *et al.* has illustrated that a 2-min global ischemia enhances the phosphorylation of p38 MAPK in the hippocampus of gerbils after I/R [20], implying the activation of p38 MAPK may be implicated in IPC-induced ischemic tolerance of the brain. Nevertheless, whether p38 MAPK is involved in IPC-induced ischemic tolerance related to BMP-7 is still unclear and requires to be further studied.

Therefore, in this study, a rat model of ischemic tolerance induced by short-term MCAO preconditioning was used to examine whether BMP-7 exerted its protective effects *via* p38 MAPK signaling pathway in cerebral ischemic injury.

## MATERIALS AND METHODS

### Animal Model and Experimental Grouping

Male Wistar rats, weighing from 280 to 320 g, were obtained from the Experimental Animal Center of China Medical University and were housed at a constant room temperature (20–22°C) and humidity (50–60%) with a 12-h light and 12-h dark cycle. The animal experimental protocol has been approved by the ethics committee of China Medical University which conforms to the provisions of the declaration of Helsinki in 1995 (as revised in Edinburgh 2000).

According to previous reported surgical procedures [21] with minor modifications, a rat model of ischemic tolerance was reproduced. Briefly, the right internal carotid artery (ICA) was carefully isolated and exposed, a 4–0

monofilament nylon suture with a rounded tip was introduced into the ICA lumen through the external carotid artery stump until faint resistance was felt. Cerebral IPC was induced by 15-min occlusion, and then the suture was gently withdrawn to permit blood perfusion. Sham-operated rats underwent the same procedure except the suture was not inserted. Forty-eight hours later, the suture was reinserted to induce ischemia by blocking the blood flow for 2 h, and then the suture was again slowly withdrawn to permit reperfusion. Rectal temperature was monitored and maintained between 36.5 and 37.5 °C using a temperature-regulated heating lamp throughout the experiment. Thereafter, rats were sacrificed at different time points for the following studies.

### *Part I. The Time-Course Expression of BMP-7, Total and Phosphorylated p38 MAPK after IPC*

Rats were divided into two groups: (1) Sham, the rats received sham surgery and (2) IPC, the rats were subjected to MCAO preconditioning for 15 min. Rats from group 2 were decapitated at 0, 1, 6, 12, 24, or 48 h after the sublethal ischemic treatment ( $n=5$  per time point) and their brain tissues were used to detect the protein expression of BMP-7 and total and phosphorylated p38 MAPK. The expression levels of these three proteins obtained from rats sacrificed immediately after the sham operation served as controls ( $n=5$ ).

### *Part II. The Effect of p38 MAPK Inhibitor SB203580 on IPC-Induced Neuroprotection in Rat Brain after I/R Injury*

To determine whether the activation of p38 MAPK could affect IPC-induced neuroprotection, 25  $\mu$ L 2% DMSO with or without 100- $\mu$ M SB203580 (Sigma-Aldrich, St. Louis, MO, USA), a pharmacologic p38 MAPK inhibitor [22], was injected into rat brain at 30 min before sham or IPC operation: (3) IS, the rats only received 2-h MCAO; (4) IPC + IS, the rats received 15-min MCAO, recovered for 48 h, and then underwent prolonged MCAO for 2 h; (5) IPC + IS + SB203580, the rats received intracranial injection of SB203580 before any ischemic treatment; (6) IPC + IS + DMSO, the rats were injected with DMSO before any ischemic treatment; and (7) Sham + SB203580, normal rats received SB203580 injection before sham operation. Rats from the above five groups along with the ones from groups 1 and 2 were sacrificed after 7-day reperfusion and their brain tissues were used for neuropathological evaluation ( $n=6$  per group).

### *Part III. The Phosphorylation Levels of p38 MAPK in Rat Brain at Different Ischemic Conditions*

To explore whether the phosphorylation of p38 MAPK is regulated by BMP-7 during IPC-induced ischemic tolerance, we measured the phosphorylation of p38 MAPK in the presence or absence of noggin (R&D systems, Minneapolis, MN, USA), a BMP-7 antagonist [23]. Twenty-five microliter normal saline with or without 2- $\mu$ g noggin was intracranially injected into rats 30 min before IPC operation: (8) IPC + IS + Noggin; (9) IPC + IS + Saline. The brain samples from the above two groups were subjected to Western blot analysis for total and phosphorylated p38 MAPK ( $n=5$  per group). Meanwhile, brain tissues from groups 1 to 4 were also used to detect the phosphorylation level of p38 MAPK.

### *Part IV. The Effect of p38 MAPK Inhibitor SB203580 on BMP-7-Induced Protective Effect against I/R Injury*

Recombinant BMP-7 (R&D systems; 30  $\mu$ M in 25- $\mu$ L normal saline) was given intracerebroventricularly in rats at 24 h before the 2-h MCAO. Next, these rats were administrated with intracranial injection of SB203580 (100  $\mu$ M in 25  $\mu$ L 2 % DMSO) at 30 min before the 2-h MCAO and allowed to recover for 24 h. Assessment of cerebral infarct volume was performed in rats from the following groups ( $n=6$  per group): (10) BMP-7 + IS; (11) BMP-7 + IS + SB203580; (12) BMP-7 + IS + DMSO. Rats from groups 3 to 4 were also subjected to detect the cerebral infarcted volume.

### **Western Blot analysis**

Protein samples from the ipsilateral ischemic cortex were extracted and subjected to Western blot analysis. Equivalent amounts of proteins were fractionated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking with 5 % ( $w/v$ ) skim milk, the membranes were immunoblotted overnight at 4°C with polyclonal antibodies against BMP-7 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p38 MAPK (Santa Cruz Biotechnology), and phosphorylated p38 MAPK (Santa Cruz Biotechnology). Thereafter, the membranes were incubated with appropriate secondary antibodies at room temperature for 45 min, and the protein blots on the membranes were visualized with an enhanced chemiluminescence (ECL) kit (Millipore) following the manufacturer's instructions.  $\beta$ -actin served as control.

### **Neuropathological Evaluation**

Animals were decapitated at 7 days after the last ischemic insult, and 3-mm thick brain samples, including the bilateral dorsal hippocampus, were excised coronally and fixed in 4 % paraformaldehyde for 6 h. Two days later, the brain tissues were embedded in paraffin and cut into 6- $\mu$ m thick sections. Next, these sections were dehydrated with alcohol, cleared with xylene, stained with thionine (Solarbio, Beijing, China), and then subjected to histological grade (HG) and neuronal density (ND) assessments [24, 25]. HG was detected under a light microscope: 0, no neuron death; I, scattered single neuron death; II, mass neuron death; and III, almost complete neuron death. Meanwhile, the average number of surviving and normal pyramidal neurons in six regions within 1-mm linear length of the hippocampal CA1 subfield was calculated as the ND value for each hippocampus.

### **Assessment of Cerebral Infarct Volume**

According to a previous reported protocol [26], brain tissue slices of rats from different experimental groups were subjected to infarction assessment. In brief, at 24 h after I/R, rats were decapitated and the brains were dissected, immersed in ice-cold saline for 5 min, and sliced into 2.0-mm thick sections for triphenyltetrazolium chloride (TTC; Sigma-Aldrich) assessment. The percentage of the infarct volume was determined by indirect measurement based on the noninfarcted cortex volume.

### **Statistical Analysis**

All results were presented as the mean  $\pm$  standard deviation (SD). The data were analyzed by one-way ANOVA, followed by Bonferroni post hoc test for multiple comparisons using SPSS 17.0 program. A  $P$  value less than 0.05 was considered as statistically significant.

## **RESULTS**

### **The Phosphorylation of p38 MAPK is Enhanced by Cerebral IPC in Rats**

Rats that underwent 15-min MCAO preconditioning were sacrificed at 0, 1, 6, 12, 24, and 48 h after reperfusion, and the brain tissues were subjected to determine the protein expression levels of BMP-7, p38 MAPK, and phosphorylated p38 MAPK. As compared with sham group, BMP-7 protein increased gradually after IPC treatment, with the highest value observed after 24-h

reperfusion (Fig. 1a). The expression pattern of the activated p38 MAPK was similar to that of BMP-7 during the whole process (Fig. 1b), whereas the total p38 MAPK expression levels were unchanged (Fig. 1b). The similar expression patterns of BMP-7 and phospho-p38 MAPK in rat brain indicated a potential interplay between them in IPC-induced tolerance.

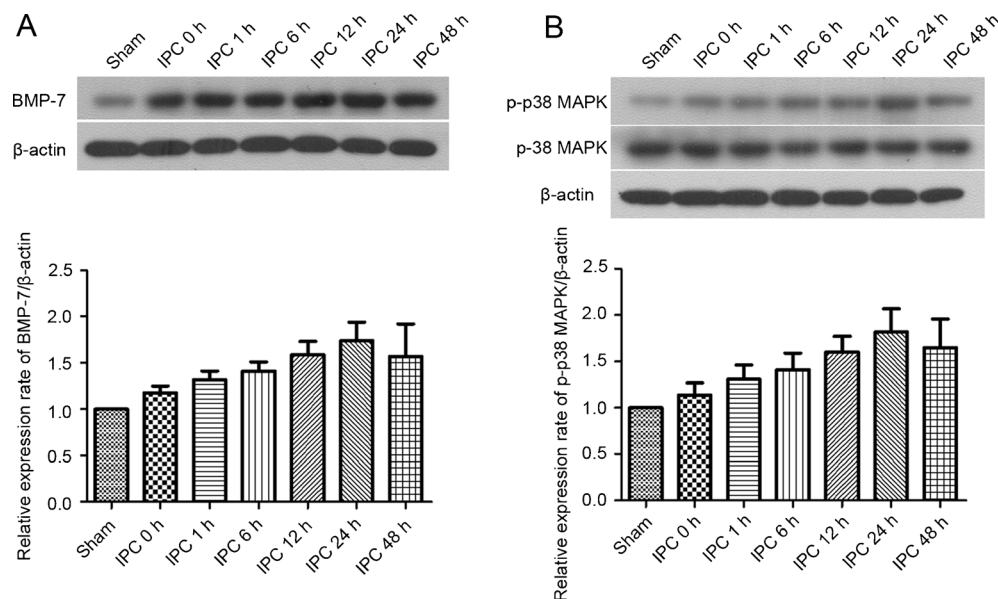
### SB203580 Attenuates IPC-Induced Improvement of Neuronal Morphology after Cerebral I/R Injury in Rats

To explore the role of p38 MAPK in IPC-induced ischemic tolerance in rat brain, the p38 MAPK-specific suppressor SB203580 [22] was used to inhibit the activation of p38 MAPK. Rats from different experimental groups were humanely killed at 7 days after the last treatment, and the morphologic changes of CA1 neurons were examined by thionine staining. We observed normal pyramidal neurons with clear cell outline and compact and abundant cytoplasm in the hippocampal CA1 subfield of sham-operated rat brains (Fig. 2a, b). No evident neuronal damage was found in the brain tissues from IPC group (Fig. 2c). Moreover, the severe neuronal degeneration induced by lethal cerebral ischemia (Fig. 2d) could be mitigated significantly if IPC was induced in the brain in advance (Fig. 2e). However, this neuroprotective effect of

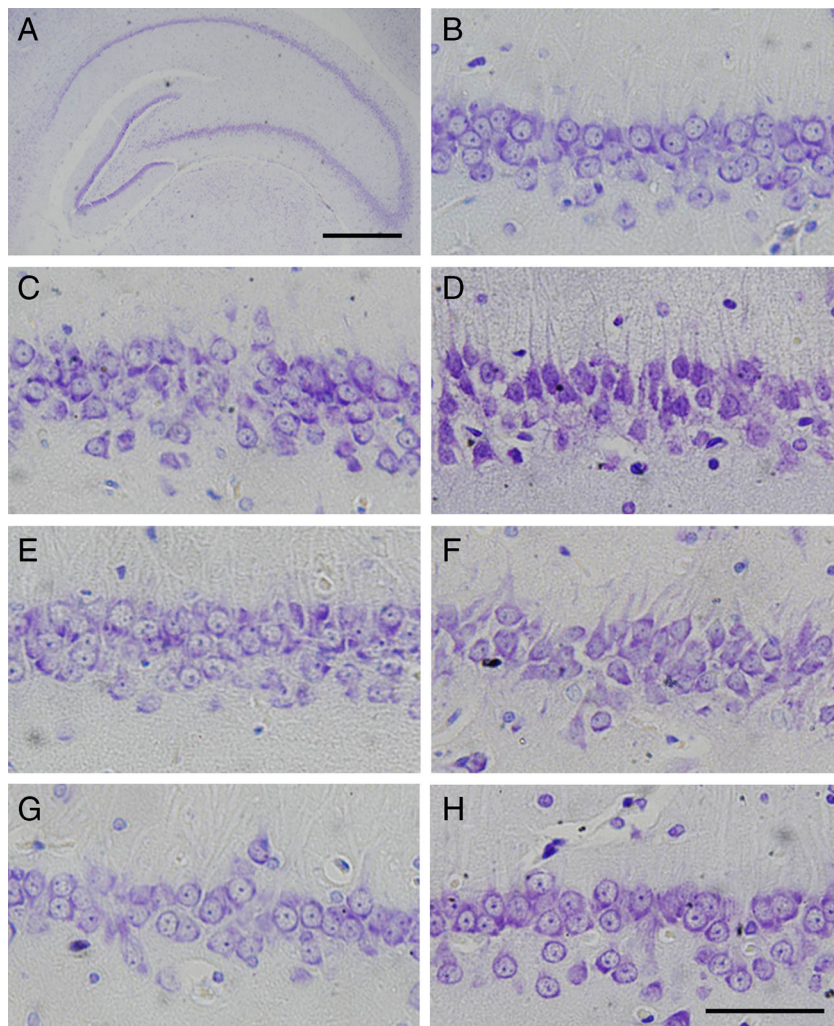
IPC in ischemic injury was abolished by SB203580 (Fig. 2f) as compared with DMSO-treated group (Fig. 2g). Additionally, the neurons of sham rats received DMSO injection remained untouched (Fig. 2h). The above observations were further confirmed by HG and ND analyses (Table 1). Collectively, the present morphological results demonstrated that inactivation of p38 MAPK could abolish the neuroprotection of IPC-induced ischemic tolerance against the subsequent detrimental ischemia.

### Noggin Reduces IPC-Induced Phosphorylation Increase of p38 MAPK in Rat Brain

Since p38 MAPK is believed to be an effector of BMP-7 in various physiological processes in vertebrates [27], we determined whether the activation of p38 MAPK could be regulated by BMP-7 during IPC-induced ischemic tolerance. As compared with the sham group, the expression level of phosphorylated p38 MAPK significantly increased in IPC rats but markedly decreased in IS rats (Fig. 3). Furthermore, down-regulation of phospho-p38 MAPK caused by lethal ischemia could be partially reversed by cerebral IPC treatment (Fig. 3). However, this IPC-enhanced phosphorylation of p38 MAPK was abolished when BMP-7 was inhibited by noggin (Fig. 3). Such results indicated that the phosphorylation of p38 MAPK was regulated by BMP-7 during IPC-induced ischemic tolerance process in rat brain.



**Fig. 1.** Protein expression levels of BMP-7 and phospho-p38 MAPK are increased after IPC in rat brain. The protein expression levels of BMP-7 (a), total and phosphorylated p38 MAPK (b) determined by Western blot analysis at 0, 1, 6, 12, 24, and 48 h ( $n=5$  per time point) after 15-min middle cerebral artery occlusion (MCAO)-induced ischemic preconditioning (IPC).  $\beta$ -actin served as endogenous control. Data were presented as mean  $\pm$  standard deviation (SD).



**Fig. 2.** SB203580 abrogates the neuroprotective effect of cerebral IPC in rats after I/R injury. Representative thionine staining results of CA1 neurons in hippocampus ( $n=6$  per group) at 7 days after ischemia/reperfusion (I/R) injury. **a** and **b** Sham group; **c** ischemic preconditioning group (IPC); **d** ischemia group (IS); **e** IPC + IS group; **f** IPC + IS + SB203580 group; **g** IPC + IS + DMSO group; **h** Sham + DMSO group. Scale bar was 500  $\mu\text{m}$  for **a** and 50  $\mu\text{m}$  for **b-h**.

### Exogenous BMP-7 Fails to Reduce the Lethal Ischemia-Induced Cerebral Infarction when p38 MAPK Signaling Pathway is Blocked

To further elucidate whether BMP-7 provides protection against I/R injury *via* p38 MAPK transduction pathway, exogenous recombinant BMP-7 was given into rat brain at 24 h before the harmful ischemic treatment. Similar to the effect of IPC, exogenous BMP-7 markedly decreased the brain infarction caused by lethal ischemia after 24 h reperfusion (Fig. 4). In addition, pre-injection SB203580 at 30 min before the last ischemic insult diminished exogenous BMP-7-induced reduction of cerebral

infarction (Fig. 4). These data demonstrated that the exogenous BMP-7 induced ischemic tolerance in rat brain by activating p38 MAPK signaling pathway.

### DISCUSSION

Ischemic stroke, resulting either from global or focal decreases in perfusion, seriously threaten human health [9], and the endogenous self-protective mechanisms against this disease are currently being widely studied. We have prior demonstrated that BMP-7 contributes to the

**Table 1.** The Histological Grade (HG) and Pyramidal Neuronal Density (ND) of the Hippocampal CA1 Region in Each of the Groups

Group	Histological grade				ND <sup>a</sup>
	0~	I~	II~	III	
Sham	6				192.50±21.17
IPC	5	1			190.17±25.22
IS			2	4	32.33±7.67***
IPC + IS		4	2		117.50±13.67 <sup>s</sup>
IPC + IS + SB 203580		2	3	1	74.17±10.56 <sup>#</sup>
IPC + IS + DMSO	1	3	2		105.67±15.67
Sham + SB 203580	5	1			180.50±13.17

Histological grades: *Grade 0* no neuron death, *Grade I* scattered single neuron death, *Grade II* mass neuron death, *Grade III* almost complete neuron death

<sup>a</sup>Data are indicated as mean±SD

\*\*\* $P < 0.001$  compared with IPC + IS

<sup>s</sup> $P < 0.001$  compared with IPC + IS + SB 203580

<sup>#</sup> $P < 0.01$  compared with IPC + IS + DMSO

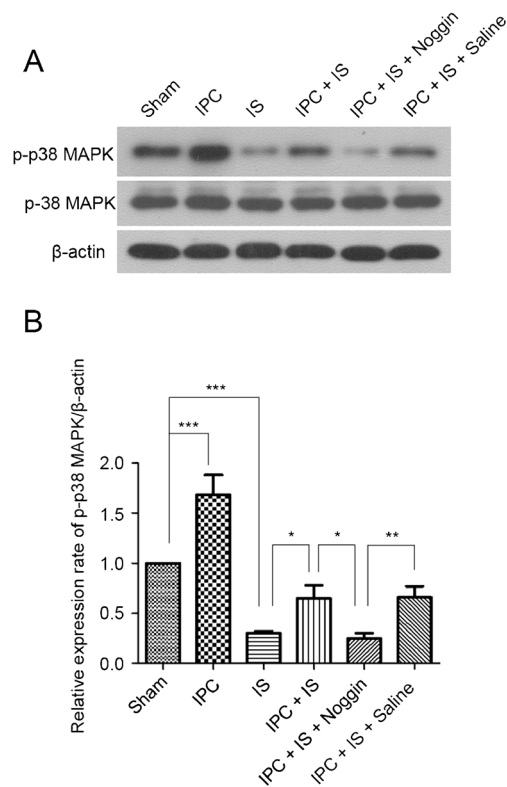
neuroprotective effects of IPC-induced tolerance in rat brain [12]. Nevertheless, how BMP-7 regulates ischemic tolerance in the brain needs further investigations.

Part of the cellular response to I/R injury involves activation of several members of the MAPKs, including the p38 MAPK [28]. An earlier study from Dreixler *et al.* has shown that the phosphorylated p38 MAPK protein is increased with retinal IPC in rats [29]. As a support to this previous research, we found that IPC occurred in the brain could activate p38 MAPK in rats. Similar stimulatory effect of IPC on p38 MAPK has also been reported in gerbils [20]. Besides, the expression pattern of phospho-p38 MAPK was similar to that of BMP-7, indicating the involvement of p38 MAPK in BMP-7-mediated IPC in rat brain.

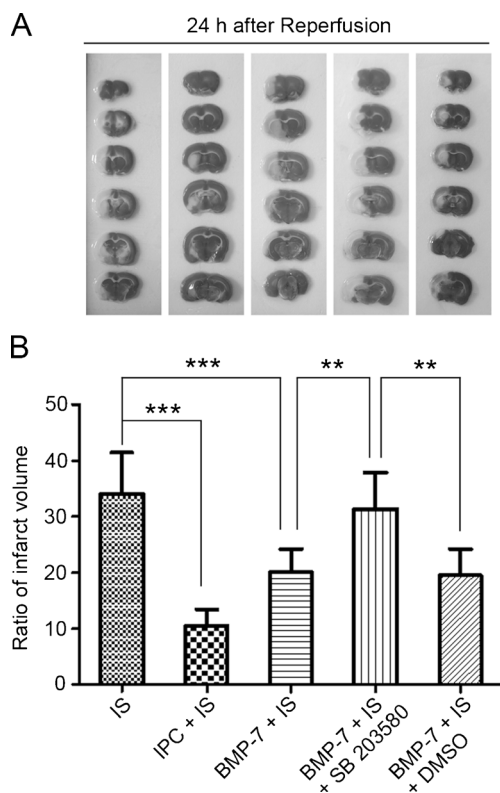
Whether the ischemia-induced activation of p38 MAPK is beneficial or deleterious in the brain still remains controversial. Some studies indicate that the activation of p38 MAPK during IPC exerts beneficial effect in rabbit heart [30] and gerbil hippocampus [20], respectively. On the contrary, it has been proven by several other researches that inhibition of p38 MAPK protects the heart [31] and liver [32] of rodents against ischemic damage. To determine the effect of p38 MAPK activation on cerebral IPC-induced protection, SB203580 was used to suppress p38 MAPK phosphorylation in rat brain. The morphological results of CA1 neurons showed that inhibition of p38 MAPK activation attenuated the protective activity of IPC to the following ischemic insult. Additionally, suppression of p38 MAPK activation *via* small interfering

RNA (siRNA) or the antagonist SB203580 is found to reduce the protective effects of IPC against retinal function after ischemia [29]. Such earlier research along with ours indicated that p38 MAPK activation contributed to the protective effect of cerebral IPC.

Plenty of studies have delineated BMP-7 as a mediator of p38 MAPK signaling pathway [17, 18]. In our study, we noted an interesting phenomenon that the expression pattern of phosphorylated p38 MAPK paralleled to that of BMP-7 after IPC in brain. Nonetheless, whether the activation of p38 MAPK is regulated by BMP-7 during cerebral IPC-induced tolerance is unclear. To address this question, the experimental rats were given intracranial administration of BMP-7 suppressor noggin before they were subjected to preconditioning or injurious ischemic



**Fig. 3.** Noggin attenuates IPC-induced increased p38 MAPK phosphorylation after I/R injury in rat brain. The protein expression levels of total and phosphorylated p38 MAPK were determined by Western blot analysis at 24 h after the last ischemic treatment. The phosphorylation of p38 MAPK was increased in ischemic preconditioning (IPC) group, but decreased in ischemia (IS) group. Inhibition of BMP-7 by noggin diminished IPC-induced up-regulation of phospho-p38 MAPK. The total p38 MAPK remained unchanged in all the experimental groups.  $\beta$ -actin served as endogenous control. Data were presented as mean±standard deviation (SD) ( $n = 5$  per group). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



**Fig. 4.** Exogenous BMP-7 reduces cerebral infarction caused by lethal ischemia *via* activating p38 MAPK signaling pathway. Representative 2,3,5-triphenyltetrazolium chloride (TTC)-stained 2-mm thick brain sections and quantitative evaluation of the infarction volume at 24 h after I/R injury ( $n=6$  per group). Exogenous BMP-7 failed to reduce noxious ischemia-caused cerebral infarction when p38 MAPK was inhibited by SB203580. Data were presented as mean $\pm$ standard deviation (SD). \*\*\* $P<0.01$ , \*\*\*\* $P<0.001$ .

treatment. The obtained data illustrated that the phospho-p38 MAPK dramatically decreased in non-preconditioned rats undergoing ischemia, whereas this reduction could be partially reversed by IPC. However, the up-regulation of phospho-p38 MAPK induced by IPC was abolished when the endogenous BMP-7 was inhibited by noggin. These results indicated that the activation of p38 MAPK was regulated by BMP-7 in IPC-induced ischemic tolerance in rats.

Nature IPC occurred in brain before ischemic stroke is a complex process [9], involving in numerous molecular signaling cascades-from stimuli and sensors to transducers and effectors [8]. Thus, the activated p38 MAPK signaling may respond to other endogenous stimuli induced by IPC besides BMP-7. To further elucidate whether BMP-7 mediated cerebral ischemic tolerance through p38 MAPK pathway, according to a previous reported protocol [33],

exogenous BMP-7 was intracranially injected into rats 24 h before noxious ischemic treatment to mimic IPC effects. Data showed that pretreatment of recombinant BMP-7 significantly mitigated injurious ischemia-caused cerebral infarction. However, this protective effect of exogenous BMP-7 was abrogated when p38 MAPK signaling pathway was blocked by SB203580. Collectively, these present results provided valid evidence to demonstrate that BMP-7 contributed to IPC-induced ischemic tolerance by activating p38 MAPK signaling pathway in the brain after I/R injury.

In summary, this study provides potent evidences to demonstrate that BMP-7 contributes to IPC-induced ischemic tolerance by activating p38 MAPK signaling pathway in rat brain.

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