

# Preconditioning with hyperbaric oxygen attenuates brain edema after experimental intracerebral hemorrhage

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**Object.** Preconditioning with hyperbaric oxygen (HBO<sub>2</sub>) reduces ischemic brain damage. Activation of p44/42 mitogen-activated protein kinases (p44/42 MAPK) has been associated with preconditioning-induced brain ischemic tolerance. This study investigated if preconditioning with HBO<sub>2</sub> protects against intracerebral hemorrhage (ICH)-induced brain edema formation and examined the role of p44/42 MAPK in such protection.

**Methods.** The study had three experimental groups. In Group 1, Sprague-Dawley rats received two, three, or five consecutive sessions of preconditioning with HBO<sub>2</sub> (3 ata, 100% oxygen, 1 hour daily). Twenty-four hours after preconditioning with HBO<sub>2</sub>, rats received an infusion of autologous blood into the caudate. They were killed 1 or 3 days later for brain edema measurement. Rats in Group 2 received either five sessions of preconditioning with HBO<sub>2</sub> or control pretreatment and were killed 24 hours later for Western blot and immunohistochemical analyses. In Group 3, rats received an intracaudate injection of PD098059 (an inhibitor of p44/42 MAPK activation) before the first of five sessions of preconditioning with HBO<sub>2</sub>. Twenty-four hours after the final preconditioning with HBO<sub>2</sub>, rats received an intracaudate blood infusion. Brain water content was measured 24 hours after ICH.

**Results.** Fewer than five sessions of preconditioning with HBO<sub>2</sub> did not significantly attenuate brain edema after ICH. Five sessions of preconditioning with HBO<sub>2</sub> reduced perihematomal edema 24 and 72 hours after ICH ( $p < 0.05$ ). Strong p44/42 MAPK immunoreactivity was detected in the basal ganglia 24 hours after preconditioning with HBO<sub>2</sub>. Intracaudate infusion of PD098059 abolished HBO<sub>2</sub> preconditioning-induced protection against ICH-induced brain edema formation.

**Conclusions.** Preconditioning with HBO<sub>2</sub> protects against brain edema formation following ICH. Activation of the p44/42 MAPK pathway contributes to that protection. Preconditioning with HBO<sub>2</sub> may be a way of limiting brain injury during invasive neurosurgical procedures that cause bleeding.

**KEY WORDS** • brain edema • hyperbaric oxygen • intracerebral hemorrhage • mitogen-activated protein kinase • preconditioning

**S**PONTANEOUS ICH, from a variety of sources, causes instantaneous mass effect, disruption of the surrounding brain, and often an early neurological death.<sup>15,20</sup> In patients that survive the initial ictus, the question of whether to remove the hematoma often arises. The recently reported Surgical Trial in Intracerebral Hemorrhage (STICH) failed to show a benefit of early surgery compared with conservative medical therapy for ICH,<sup>13</sup> and there is a great need for novel therapies.

The protective effect of ischemic preconditioning, in which a brief ischemic episode can increase tolerance to

subsequent severe ischemia, occurs in the brain.<sup>24</sup> Ischemic tolerance can be achieved not only by a brief period of global or focal ischemia, but also by a wide range of other preconditioning stimuli, including hypoxia, hyperthermia, cytokines, and intracerebral low-dose thrombin infusion.<sup>4,23</sup> Recent studies have demonstrated that preconditioning with HBO<sub>2</sub> can also reduce ischemic brain damage.<sup>14</sup> It is not clear, however, whether or not such preconditioning can reduce brain injury after ICH.

The precise mechanisms involved in preconditioning-induced ischemic tolerance have not been fully determined. Many mechanisms, including improving the efficiency of energy metabolism, reducing excitotoxic injury, and limiting apoptosis, have been associated with ischemic toler-

Abbreviations used in this paper: DMSO = dimethyl sulfoxide; HBO<sub>2</sub> = hyperbaric O<sub>2</sub>; HSP = heat shock protein; ICH = intracerebral hemorrhage; MAPK = mitogen-activated protein kinase.

ance. Activation of p44/42 MAPK, which are cytoplasmic signaling molecules, may be an important step in preconditioning-induced brain tolerance.<sup>8,10,19</sup>

This study investigated whether or not preconditioning with HBO<sub>2</sub> can induce tolerance to brain edema formation after ICH and the role of p44/42 MAPK in HBO<sub>2</sub> preconditioning-induced protection.

## Materials and Methods

### *Animal Preparation and Intracerebral Infusion*

The animal study protocol was approved by the University of Michigan Committee on the Use and Care of Animals. Male Sprague-Dawley rats (Charles River Laboratories) weighing between 300 and 350 g were used in this study. Rats were allowed free access to food and water before and after the experimental procedure. Animals were anesthetized with an intraperitoneal injection of pentobarbital (45 mg/kg). The right femoral artery was catheterized to monitor arterial blood pressure, to obtain blood for intracerebral injection, and to analyze blood pH, PaO<sub>2</sub>, PaCO<sub>2</sub>, hematocrit, and glucose levels. Rectal temperature was maintained at 37 to 37.5°C using a feedback-controlled heating pad. Animals were then positioned in a stereotactic frame (Kopf Instruments), and a 1-mm cranial bur hole was drilled on the right coronal suture 3.5 mm lateral to the midline. Autologous whole blood, PD098059, or vehicle (4% DMSO) was infused into the right basal ganglia through a 26-gauge needle (0.2 mm anterior, 5.5 mm ventral, and 3.5 mm lateral to the bregma) using a microinfusion pump (Harvard Apparatus). The needle was removed, the bur hole was filled with bone wax, and the skin incision was closed using sutures.

### *Experimental Groups*

There were three groups of rats in the study. In Group 1, male Sprague-Dawley rats received two, three, or five consecutive sessions of preconditioning with HBO<sub>2</sub> (3 ata, 100% oxygen, 1 hour daily for 2, 3, or 5 days). Control animals received normobaric room air. Twenty-four hours after preconditioning with HBO<sub>2</sub>, the rats received an intracaudate injection of autologous whole blood (100 μl) and were killed 1 or 3 days later for brain edema measurement. The percentage of brain water content was then measured. In Group 2, rats received either five sessions of preconditioning with HBO<sub>2</sub> or control pretreatment and were killed for Western blot and immunohistochemical analyses of p44/42 MAPK 24 hours after the final HBO<sub>2</sub> preconditioning session. In Group 3, rats received a 5-nmol intracaudate injection of PD098095 (an inhibitor of p44/42 MAPK activation) or vehicle (4% DMSO) before the first session of preconditioning with HBO<sub>2</sub>, and then underwent five sessions of preconditioning with HBO<sub>2</sub>. Twenty-four hours after the final HBO<sub>2</sub> preconditioning session, rats received a 100-μl intracaudate injection of autologous whole blood. The percentage of brain water content was measured 24 hours after blood injection.

### *Hyperbaric Oxygen Procedure*

Animals in HBO<sub>2</sub> treatment groups were placed in a small rodent HBO<sub>2</sub> chamber (Marine Dynamics Corp.).

The chamber was pressurized for 15 minutes to a plateau pressure of 3 ata with 100% oxygen supplied continuously and maintained for 60 minutes. Decompression was then performed for 25 to 30 minutes. Control animals were also transferred into the HBO<sub>2</sub> chamber but received normobaric room air.

### *Brain Water Content Measurement*

Animals in Groups 1 and 3 were reanesthetized using an intraperitoneal injection of 60 mg/kg of pentobarbital and decapitated 24 hours after ICH to measure brain water, as described previously.<sup>21</sup> The rat brains were removed and a coronal tissue slice (about 3 mm thick) 4 mm from the frontal pole was cut using a blade. The brain tissue slice was divided into two hemispheres along the midline, and each hemisphere was dissected into cortex and basal ganglia. The cerebellum tissue served as a control. Five tissue samples from each brain were obtained: the ipsilateral and contralateral cortex, the ipsilateral and contralateral basal ganglia, and the cerebellum. Brain samples were immediately weighed on an electronic analytical balance (model AE 100, Mettler Instrument) to obtain wet weight. Dried weight of the samples was determined after the tissues were heated for 24 hours at 100°C in a gravity oven (Blue M. Electric Co). The percentage of tissue water content was then calculated according to the following formula:  $100 \times (\text{wet weight} - \text{dry weight})/\text{wet weight}$ .

### *Western Blot Analysis*

Animals in Group 2 were reanesthetized and underwent transcatheter perfusion using 0.1M phosphate-buffered saline until colorless perfusion fluid was obtained from the right atrium. A coronal brain section was then cut as described for brain water and ion content. Tissue samples of the ipsilateral and contralateral basal ganglia were obtained and immersed in 0.5 ml of Western blot sample buffer and then sonicated for Western blot analysis. Protein concentration of the sample was determined using a Bio-Rad protein assay kit. Western blot analysis was performed as described previously.<sup>22</sup> Fifty micrograms of protein for each sample was separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis after undergoing denaturation by boiling at 95°C for 5 minutes, and were then transferred to pure nitrocellulose membrane. The membranes were blocked in Carnation nonfat milk and probed with the primary antibody (polyclonal rabbit antiphospho-P44/42 MAPK, 1:1000 dilution, Cell Signaling Technology), and then immunoprobed by a second antibody (peroxidase-conjugated goat–antirabbit antibody, Bio-Rad). The antigen–antibody complexes were studied using a chemiluminescence system (Amersham Pharmacia) and exposure to photosensitive film (X-OMAT, Kodak). Relative densities of the band were analyzed using National Institutes of Health Image software (version 1.62).

### *Immunohistochemical Analysis*

Animals in Group 2 were reanesthetized and perfused intracardially with 4% paraformaldehyde in 0.1M phosphate-buffered saline (pH 7.4). Rat brains were removed

TABLE 1  
Physiological parameters recorded in each group of rats during intracerebral infusion\*

Group (no. rats)	MABP (mm Hg)	pH	PaO <sub>2</sub> (mm Hg)	PCO <sub>2</sub> (mm Hg)	Glucose (mg/dl)	Hematocrit (%)
control (40)	102 ± 6	7.40 ± 0.03	80 ± 6	47 ± 4	109 ± 14	40 ± 2
HBO <sub>2</sub> (60)	103 ± 7	7.41 ± 0.03	84 ± 8	45 ± 6	108 ± 7	39 ± 1

\* Values are expressed as mean ± SD. Measurements were taken at the time of ICH. Abbreviation: MABP = mean arterial blood pressure.

and suspended in 4% paraformaldehyde for 6 hours, then immersed in 25% sucrose for 3 to 4 days at 4°C. The brains were embedded in the mixture of 25% sucrose and optimal cutting temperature compound (OCT, Sakura Finetek) and sectioned on a cryostat 20- $\mu$ m thick. Sections were incubated according to the avidin-biotin complex technique. The primary antibody was polyclonal rabbit antiphospho-p44/42 MAPK (1:400 dilution, Cell Signaling Technology). The second antibody was goat anti-rabbit immunoglobulin G (1:500 dilution, Vector Laboratories). Normal rabbit immunoglobulin G (Vector Laboratories) was used as a negative control.<sup>19</sup>

#### Statistical Analysis

Data are expressed as means ± standard deviations. Statistical significance was analyzed using the two-tailed Student t-test or analysis of variance and a Scheffé multiple comparison test. A probability value of less than 0.05 was considered statistically significant.

## Results

Physiological parameters in all groups were recorded during intracerebral infusion. All physiological variables, including mean arterial blood pressure, blood pH, PaO<sub>2</sub>, PCO<sub>2</sub>, hematocrit, and blood glucose level were controlled within normal ranges (Table 1).

Fewer than five sessions of preconditioning with HBO<sub>2</sub> did not significantly attenuate brain edema in the ipsilateral basal ganglia 24 hours after ICH ( $p > 0.05$ , Fig. 1). Five sessions of preconditioning with HBO<sub>2</sub> significantly reduced the percentage of brain water content in the ipsilateral basal ganglia 24 hours after ICH ( $79.5 \pm 0.9\%$  compared with  $81.2 \pm 1.1\%$  in the control group,  $p < 0.01$ , 9–10 rats per group; Fig. 1). Preconditioning using HBO<sub>2</sub> also attenuated perihematomal edema 72 hours after ICH ( $81.3 \pm 1.4\%$  compared with  $82.8 \pm 1.1\%$  in the control group,  $p < 0.05$ , 6 rats per group; Fig. 2). Brain water content in the contralateral hemisphere or cerebellum was not affected by preconditioning with HBO<sub>2</sub>.

To investigate whether or not preconditioning with HBO<sub>2</sub> affects activation of p44/42 MAPK, rats underwent five sessions of preconditioning with HBO<sub>2</sub> and were killed 24 hours later for Western blot analysis and immunohistochemical studies. Western blot analysis demonstrated that five sessions of preconditioning with HBO<sub>2</sub> significantly activated p44/42 MAPK in the basal ganglia ( $p < 0.01$ , activated p44 MAPK  $3116 \pm 972$  pixels compared with  $15 \pm 4$  pixels in the control group;  $p < 0.01$ , activated p42 MAPK  $10,750 \pm 2192$  pixels compared with  $173 \pm 33$  pixels in the control group; Fig. 3A). Few

p44/42 MAPK positive cells were detected in the basal ganglia of control rats (Fig. 3B), whereas strong p44/42 MAPK immunoreactivity was found in the basal ganglia 24 hours after five sessions of preconditioning with HBO<sub>2</sub> (Fig. 3C). Most activated p44/42 MAPK positive cells were neuronlike cells.

To examine the role of p44/42 MAPK activation in HBO<sub>2</sub> preconditioning-induced brain protection, rats received either an intracaudate injection of PD098059 (an inhibitor of MAPK kinase) or vehicle into the ipsilateral basal ganglia prior to the first session of preconditioning with HBO<sub>2</sub>. We found that PD098059 abolished HBO<sub>2</sub> preconditioning-induced protection against brain edema formation 24 hours after ICH. Edema was  $80.7 \pm 1.1\%$  in rats treated with preconditioning with HBO<sub>2</sub> in addition to PD098059, which was not significantly different from rats without preconditioning, whereas edema in rats with preconditioning with HBO<sub>2</sub> in addition to vehicle was identical to preconditioning with HBO<sub>2</sub> alone ( $p < 0.05$ ,  $79.5 \pm 0.5\%$ ; Fig. 4).

## Discussion

The major findings of this study are that preconditioning with HBO<sub>2</sub> can reduce perihematomal brain edema formation and that activation of p44/42 MAPK may play a key role in HBO<sub>2</sub> preconditioning-induced protection against hemorrhagic brain edema.

Hyperbaric O<sub>2</sub> preconditioning-induced ischemic tolerance was first reported by Wada and colleagues.<sup>17</sup> These

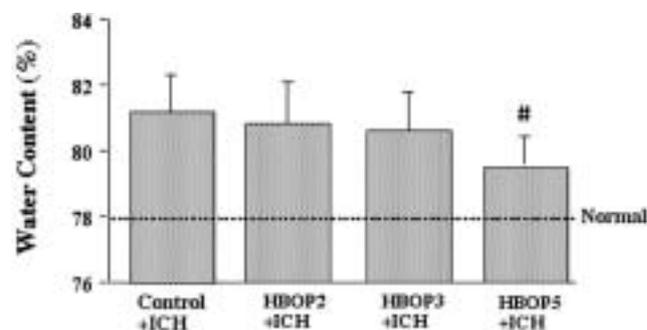


FIG. 1. Bar graph showing the percentage of brain water content in the ipsilateral basal ganglia 24 hours after ICH in rats with or without HBO<sub>2</sub> preconditioning (HBOP). Rats received two (HBOP2, 6 rats), three (HBOP3, 6 rats), or five sessions (HBOP5, 9 rats) of HBO<sub>2</sub> pretreatment. Control rats (10) had sham preconditioning. All rats had an ICH. The dashed line indicates normal (no ICH) brain water content (78%) in the basal ganglia. The symbol # above the HBOP5 + ICH group indicates a statistically significant difference ( $p < 0.01$ ) compared with control + ICH group, and compared with the HBOP2 group ( $p < 0.05$ ).

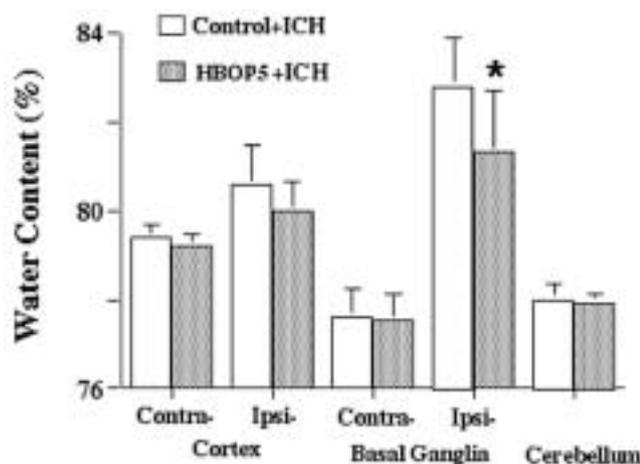


FIG. 2. Bar graph showing the percentage of water content in five different brain areas 72 hours after ICH in rats that received five sessions of HBO<sub>2</sub> pretreatment (HBOP5 + ICH, 6 rats) or sham pretreatment (control + ICH, 6 rats). The asterisk indicates a statistically significant difference ( $p < 0.05$ ) between the water content in the HBOP5 + ICH group in the ipsilateral basal ganglia compared with control + ICH group. Contra- = contralateral; Ipsi- = ipsilateral.

investigators found that preconditioning with HBO<sub>2</sub> (2 ata, 100% oxygen, 1 hour every other day for five sessions) induced ischemic tolerance to subsequent lethal global ischemia in gerbils. To determine what components (hyperoxia, hyperbaricity, or both) of HBO<sub>2</sub> are responsible for HBO<sub>2</sub> preconditioning-induced ischemic tolerance, Dong and coworkers<sup>3</sup> compared the effects of preconditioning with HBO<sub>2</sub> and hyperoxia in spinal cord ischemia in rabbits, and found that preconditioning with HBO<sub>2</sub> rather than simple hyperbaricity induces ischemic tolerance. Induced ischemic tolerance using preconditioning with HBO<sub>2</sub> may be strain-dependent because preconditioning with HBO<sub>2</sub> can induce ischemic tolerance in SV129 mice but not in C57BL/6 mice.<sup>14</sup> Investigators have also demonstrated that preconditioning with HBO<sub>2</sub> can induce ischemic tolerance in other organs, such as the liver.<sup>24</sup> In the present study, we demonstrated HBO<sub>2</sub> preconditioning-induced tolerance to hemorrhagic brain damage.

In the current study, we found that brain protection against hemorrhagic edema formation can be induced by five sessions rather than two to three sessions of preconditioning with HBO<sub>2</sub>, suggesting the importance of stimulus intensity in preconditioning. It should be noted that there are no clear boundaries between tolerance and injury;<sup>2</sup> for example, in one study,<sup>18</sup> three or five sessions of preconditioning with HBO<sub>2</sub> induced ischemic tolerance, but 10 sessions of preconditioning with HBO<sub>2</sub> failed to induce ischemic tolerance and could cause brain injury.

A major advantage of preconditioning with HBO<sub>2</sub> compared with other preconditioning agents is that it is noninvasive and ready to use in patients if it is proved safe and effective in humans. Although ischemic preconditioning studies have been an excellent method for elucidating neuroprotective mechanisms, the potential medical importance of this phenomenon has been questioned because of

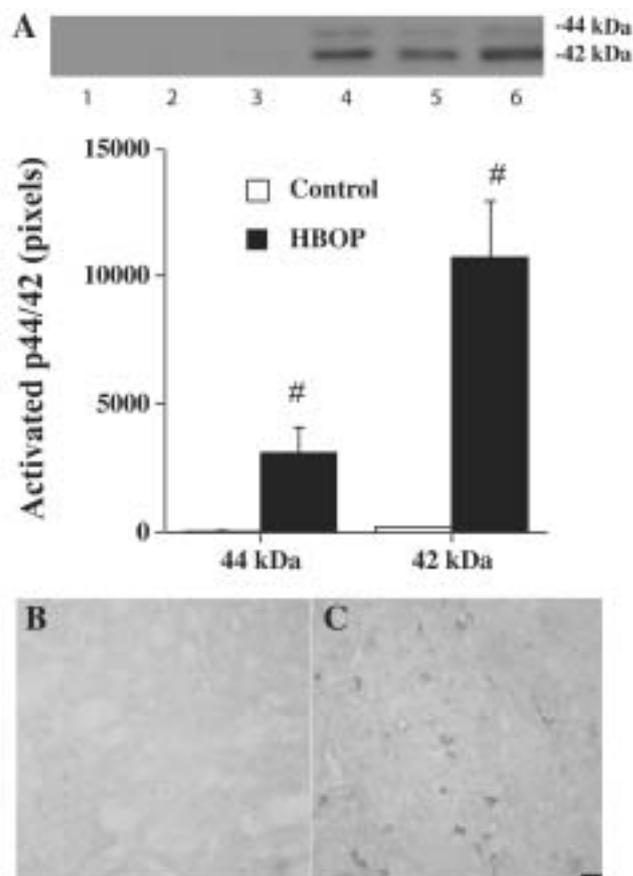


FIG. 3. Immunoreactivity of activated (phospho) p44/42 MAPK in the rat brain 24 hours after five consecutive sessions of HBO<sub>2</sub> pretreatment. A: Western blot analysis (upper) and bar graph (lower) showing activated p44/42 MAPK levels in the ipsilateral basal ganglia in rats pretreated with (upper, lanes 1–3) or without (upper, lanes 4–6) HBO<sub>2</sub> preconditioning (3 rats in each group). The symbol # represents a statistically significant difference ( $p < 0.01$ ) between the HBOP group compared with control group. B and C: Photomicrographs showing immunohistochemical analysis of activated p44/42 MAPK in the basal ganglia 24 hours after sham pretreatment (B) or preconditioning with HBO<sub>2</sub> (C). Note the number of positive cells after HBO<sub>2</sub> preconditioning treatment (C). Bar = 20  $\mu\text{m}$ .

the necessity for pretreatment. Yet neurosurgical procedures (or surgeries in other tissues) may result in edema, hemorrhage, and/or ischemia. Therefore, it is important to develop a safe way to precondition the brain before surgery. Preconditioning with HBO<sub>2</sub> may be such a treatment.

Preconditioning with HBO<sub>2</sub> can activate p44/42 MAPK in the brain. The p44/42 MAPKs, also called extracellular signal-regulated kinases, are one of the subfamilies of MAPK. The p44/42 MAPKs are plentiful in the normal brain and are activated after various brain injuries such as cerebral ischemia and ischemic preconditioning.<sup>1,2</sup> Activation of p44/42 MAPK has been shown in neurons and astrocytes<sup>9</sup> and such activation has been linked to ischemic tolerance both in vitro<sup>5</sup> and in vivo.<sup>6,16</sup> Thus, Gonzalez-Zulueta et al.<sup>5</sup> found that activation of the p44/42 MAPK pathway is required for preconditioning

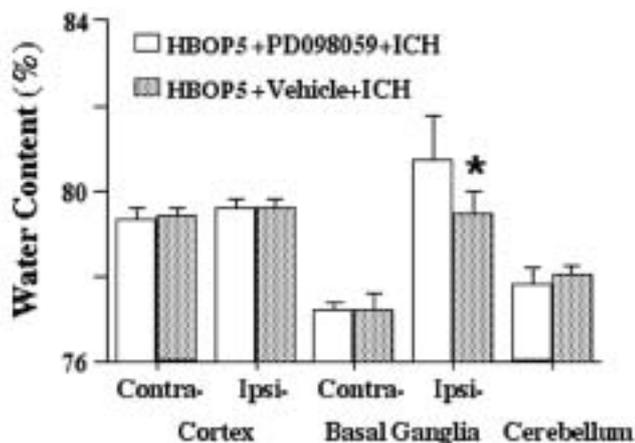


FIG. 4. Bar graph showing that PD098059 abolished HBOP-induced protection against brain edema formation 24 hours after ICH. Rats were pretreated with either an intracaudate injection of PD098059 (HBOP5 + PD098059 + ICH, 5 rats) or vehicle (4% DMSO, HBOP5 + vehicle + ICH, 5 rats), as well as with five sessions of HBO<sub>2</sub> pretreatment before ICH. The asterisk indicates a statistically significant decrease ( $p < 0.05$ ) of water content in the HBOP5 + vehicle + ICH group in the ipsilateral basal ganglia compared with the HBOP5 + PD098059 + ICH group.

induced by oxygen-glucose deprivation in primary cortical cell cultures.<sup>5</sup> In additional studies, the levels of activated p44/42 MAPK were increased after cerebral ischemic preconditioning<sup>6</sup> and were associated with ischemic tolerance.<sup>16</sup>

The p44/42 MAPK inhibitor PD098059 blocks HBO<sub>2</sub> preconditioning-induced brain tolerance, indicating a key role of p44/42 MAPK in preconditioning with HBO<sub>2</sub>. The p44/42 MAPK pathway may be associated with a preconditioning-related neuroprotective effect.<sup>7</sup> Activation of p44/42 MAPK, such as in cerebral ischemic preconditioning and thrombin preconditioning, induces brain tolerance.<sup>5,19</sup> Although the exact mechanisms by which p44/42 MAPK pathway activation results in brain protection are unknown, the protective effect may be caused partially through upregulation of HSPs.<sup>2</sup> Induction of HSPs may contribute to ischemia- and thrombin-induced brain tolerance.<sup>12,22</sup> Our previous studies have shown that thrombin-induced brain tolerance may be related to upregulation of HSPs, including HSP27 and HSP47.<sup>23</sup> Activation of MAPK is associated with upregulation of HSP27.<sup>11</sup>

### Conclusions

Five sessions of preconditioning with HBO<sub>2</sub> induce brain tolerance to edema formation following ICH. Activation of the p44/42 MAPK pathway contributes to this HBO<sub>2</sub> preconditioning-induced brain tolerance. Pretreatment with HBO<sub>2</sub> might be beneficial for neurosurgical patients undergoing operations that induce significant bleeding or ischemia.

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