Cyclooxygenase-2 Mediates Hyperbaric Oxygen Preconditioning in the Rat Model of Transient Global Cerebral Ischemia

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Background and Purpose—Hyperbaric oxygen (HBO) preconditioning (PC) allows brain protection against transient global ischemia. In the present study, we hypothesize that the mechanism of HBO-PC involves the induction of cyclooxygenase-2 (COX-2) in cerebral tissues before ischemia, which leads to a suppression of COX-2 and its downstream targets after global ischemic insult.

Methods—One hundred twenty-nine male Sprague Dawley rats (body weight 280–300 grams) were allocated to the naive control group and the sham operation group, and 3 groups of animals were subjected to 15-minute 4-vessel occlusion: untreated, preconditioned with HBO 2.5 atmospheres absolutes for 1 hour daily for 5 days, preconditioned as mentioned and administered with COX-2 inhibitor NS-398 (1 mg/kg body weight intraperitoneal) before each preconditioning session, and normal rats preconditioned with HBO without ischemia. The mortality, the incidence of seizures, and T-maze scores were recorded. The quantitative cell count in Nissl stain and TUNEL was conducted on day 7 after ischemia. The brain expression of COX-2 was analyzed with Western blotting and immunofluorescence staining.

Results—HBO-PC increased the number of surviving neurons in the Cornu Ammonis area 1, which was associated with the reduced COX-2 expression in the hippocampus and in the cerebral cortex at 1 and 3 days after ischemia. HBO-PC improved functional performance and tended to decrease mortality and the frequency of seizures. These beneficial effects of HBO-PC were abolished by the COX-2 selective inhibitor NS-398.

Conclusions—HBO-PC reduced COX-2 expression and provided brain protection after global ischemia. Administration of COX-2 inhibitor with HBO before ischemia abolished preconditioning effect, thereby implicating COX-2 as a mediator of HBO-PC in the ischemic brain. (Stroke. 2011;42:484-490.)

Key Words: global cerebral ischemia ■ hyperbaric oxygen preconditioning ■ ischemic tolerance

Hyperbaric oxygen (HBO) preconditioning (PC) has been shown to reduce neuronal injuries in animal models of neurological diseases. HBO-PC allowed neuroprotection against focal and global cerebral ischemia,1,2 spinal cord ischemia,3 traumatic and surgical brain injury,4,5 and neonatal hypoxia ischemia.6 It has been postulated that HBO-PC alleviates ischemic brain injury by the upregulation of hypoxia-inducible factor-1α and its downstream adaptive genes,7,8 inhibition of neuronal apoptotic pathways (blockage of caspase-3 and caspase-9 activity),6 reduction of early apoptosis,9 or the upregulation of antioxidant enzymes.10 However, investigations conducted thus far have not determined whether HBO-PC can provide brain protection through anti-inflammatory mechanisms in the setting of severe global cerebral ischemia.

Mounting evidence indicates that neuroinflammation contributes to brain injury developing after cerebral ischemia.11 The formation of arachidonic acid end products, catalyzed by cyclooxygenase-2 (COX-2), is a critical component of post-ischemic neuroinflammation.12,13 Pharmacological blockade of COX-2 with highly selective inhibitors (eg, rofecoxib) or genetic ablation of COX-2 confers robust neuroprotection in laboratory animals subjected to focal or global cerebral ischemia, whereas neuronal overexpression of COX-2 in transgenic mice potentiates neuronal injury after global ischemia (GI) insult.14–16 Therefore, we have conceptualized that the brain level of COX-2 expression may determine the outcome after transient global cerebral ischemia as either ischemic cell death or tolerance. We hypothesized that the mechanism of HBO-PC is mediated by a preischemic increase of COX-2 expression/activity followed by a suppression of COX-2 and its targets after ischemia, thereby producing brain-protective effects.

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Materials and Methods

Animal Groups and the Model of Global Cerebral Ischemia

One hundred twenty-nine male SD rats (Harlan, Indianapolis, IN) weighing 280 to 300 grams were randomly divided into 6 groups of rats: normal (norm; n=6); normal with HBO-PC (norm+HBO-PC; n=8); sham-operated (sham; n=20); GI (n=37); GI preconditioned with HBO (HBO-PC; n=31); and GI preconditioned with HBO and pretreated with NS-398 intraperitoneally (HBO-PCI; n=27).

To maintain the blind fashion of the study, we marked each animal with an identification number and kept the experimenters performing behavioral tests and cell count while unaware of group assignment. After the data were collected, another researcher classified the rats, computed original data, and then compared the results among groups. All surgical and euthanasia procedures were performed under anesthesia with ketamine (100 mg/kg intraperitoneal) and xylazine (10 mg/kg intraperitoneal) after atropine premedication (0.05 mg/kg subcutaneous). The animals were intubated and mechanically ventilated during surgery. The 4-vessel occlusion rat model of 15-minute global brain ischemia17 with our modifications was performed as described.9,18

NS-398 Treatment

NS-398 at a dose of 1 mg/kg suspended in 10% dimethyl sulfoxide in phosphate-buffered saline (n=31) was intraperitoneally injected 10 minutes before each HBO session. The rats in the HBO-PC group received vehicle by itself (10% dimethyl sulfoxide/phosphate-buffered saline) according to the same injection regimen.

Neurobehavioral Testing

Short-term memory deficits were tested in the T-maze. The evaluation was conducted at 3 days or 7 days after ischemia, as described.19 The results were expressed as percent of spontaneous alternations with respect to 50% reference.20

Nissl Staining and TUNEL

On days 1, 3, and 7 after GI, rats were perfused transcardially with 200 mL of ice-cold phosphate-buffered saline, followed by 300 mL of phosphate-buffered 10% formalin. Brains were postfixed and cryoprotected as described.21 Brain tissue blocks were cryosectioned into 10-μm-thick sections. A total of 14 tissue sections per brain were selected for Cornu Ammonis area 1 (CA1) neuronal count. To ascertain that the CA1 neurons were counted at the same rostrocaudal level, we first dissected the same regions from each brain using bregma as a reference point. Then, we collected 10-μm-thick coronal slices of the dorsal hippocampus (~3.3 mm to ~4.5 mm from bregma) at the same levels of sectioned specimens. To this end, we determined the distance between each analyzed brain section and the anterior face of the tissue block.

The frozen sections were dried at room temperature, rehydrated, and immersed in 0.5% cresyl violet (2 minutes) for Nissl staining.20 TUNEL was performed with an in situ cell death detection kit (Roche).19

Triple Immunofluorescence Staining

To determine neuron-specific colocalization of cleaved caspase-3 and COX-2 in CA1 and in the cerebral cortex 3 days after ischemia, we conducted triple fluorescence staining as described.22 Briefly, primary antibodies were rabbit anti-cleaved caspase-3 (Cell Signaling), goat anti-COX-2 (Santa Cruz), and mouse anti-NeuN (Millipore), all diluted 1:100. After blocking with donkey serum 5% for 2 hours at room temperature and incubation at 4°C overnight, the sections were treated with donkey secondary antibodies (diluted 1:200 at room temperature for 2 hours) raised against rabbit, goat, and mouse IgG conjugated with Texas Red, FITC, and AMCA, respectively.

Western Blot Analysis

The animals were euthanized under general anesthesia on days 1 and 3 after ischemia or at 24 hours after the last HBO session. The brains were collected and processed for Western blotting as described.23 Equal amounts (30 μg) of total protein were separated in 10% SDS-PAGE and blotted onto nitrocellulose membranes. The probing antibodies included rabbit anti-COX-2 polyclonal antibody (1:500;
Cayman Chemical Company), rabbit anti-heme oxygenase 1 (HO-1) polyclonal antibody (1:500; Enzo Life Sciences), rabbit anti-hypoxia-inducible factor 1α (HIF-1α) polyclonal antibody (1:500; Santa Cruz), goat anti-beta actin (1:2000; Santa Cruz), and the respective horseradish peroxidase-conjugated secondary antibodies diluted 1:2000 (Santa Cruz). Bands were detected by the chemiluminescence kit (Amersham Bioscience) and recorded on X-ray film (Kodak). Bands were quantified by optical density method (Image J), and densities were expressed relative to beta actin and sham (sham, n=6; GI, n=6; HBO-PC, n=6; HBO-PCI, n=5).

Quantitative Cell Count
The brains of animals from day 7 were collected and processed for quantitative analysis of Nissl staining and TUNEL (sham, n=4; GI, n=4; HBO-PC, n=4; HBO-PCI, n=4). A total of 24 Nissl photomicrographs from the dorsal CA1 per brain were taken at 100× magnification for cell counts. Eight photomicrographs of CA1 were taken from each animal for counting TUNEL-positive cells.

Statistical Analysis
Data are expressed as mean± SEM. ANOVA supported by Sigma Stat (Systat Software) was used to verify the significance of intergroup differences for Western blot, Y-maze results, and cell counts. The repeated-measures ANOVA was applied to analyze differences in body weight. A χ² test was used for analyzing mortality and the incidence of seizures.

Results
Mortality, Neurological Scores, and the Incidence of Seizures
The body weight measured daily significantly decreased on days 1 through 3 and returned to the basal level on day 7 in all groups except HBO-PCI (Figure 1A). The body weight decrease was statistically equivalent regardless of the type of treatment. In the HBO-PC group, however, it showed a mild tendency toward earlier recovery. The mortality at 7 days was 0% (0/20 rats) in the sham group, 24.32% (9/37 rats) in the GI group, 12.90% (4/31 rats) in the HBO-PC group, and 25.92% (7/27 rats) in the HBO-PCI group. Most deaths occurred within the first 48 hours after the ischemic insult. Three rats died within 24 hours and 6 rats died between 24 and 48 hours in GI group. In the HBO-PC group, 1 rat died within first 24 hours and 3 rats died between 24 and 48 hours. In the HBO-PCI group, 3 rats died within 24 hours, 3 rats died between 24 and 48 hours, and 1 rat died between 48 and 72 hours. Although the mortality in the HBO-PC group tends to be lower than in the no treatment group, the differences between groups did not qualify as statistically significant in χ² analysis.
T-maze testing for spontaneous alternation demonstrated worse performance of the GI and HBO-PCI animals on days 3 and 7 compared to the HBO-PC animals (Figure 1C).

The incidence of seizure was 0% (0/25) in the sham group, 21.62% (8/37) in the GI group, 6.45% (2/31) in the HBO-PC group, and 25.92% (7/27) in the HBO-PCI group. The seizure incidence in each group was statistically equivalent in analysis (Figure 1D).

Nissl Stain
Nissl histology revealed delayed cell death characteristic for this model (Figure 2). The sham group did not exhibit dead cells in CA1 (Figure 2A, E, I). Cell loss and the presence of injured neurons with twisted axonal processes were noted in the CA1 at days 3 and 7 after ischemia (Figure 2F, J). In the HBO-PC group (Figure 2G, K), relatively less dead cells were observed compared to the GI group and HBO-PCI group (Figure 2H, L) on days 3 and 7. Dark neurons were seen throughout CA1, however. Quantitative cell count revealed that 14.94% of CA1 neurons survived GI until day 7 in the GI group (sham, 107.31 ± 18.66 neurons/mm; GI, 16.04 ± 16.07 neurons/mm; Figure 2 M, L). The number of surviving neurons increased 2.75-fold in the HBO-PC rats to 44.22 ± 28.07. However, pretreatment with COX-2 inhibitor before HBO-PC reduced the level of surviving neurons in CA1 after ischemia (22.25 ± 22.24).

TUNEL
There were no TUNEL-positive cells in the sham group (Figure 3A). The CA1 cells in the non-PC rats were almost entirely TUNEL-positive at 7 days after GI (Figure 3B). A remarkable decrease of TUNEL was observed in the HBO-PC group (Figure 3C) compared to the GI group. An increase of TUNEL positivity was observed in the HBO-PCI group (Figure 3D) compared to HBO-PC group. Cell counting (Figure 3E) showed that HBO-PC reduced number of TUNEL-positive cells by 37.10%. Pretreatment with COX-2 inhibitor increased the number of TUNEL-positive cells to 86.77% of the level in the GI group.

COX-2 Protein Expression in the Brain After GI
Western blot analysis demonstrated an increase in COX-2 protein on days 1 and 3 after nonpreconditioned GI (Figure 4A, B). There was a statistically significant decrease in COX-2 in the HBO-PC group as compared to the GI and HBO-PCI groups at both time points.

Colocalization of COX-2, Cleaved Caspase-3, and NeuN
The results of triple immunofluorescence staining on day 3 after ischemia are shown in Figure 5. Normal (Figure 5A, G, M, S), sham-operated (Figure 5B, H, N, T), and normal HBO-PC (Figure 5F, L, R, X) rats did not show cleaved caspase-3 or COX-2 positivity in the CA1 or in the cerebral cortex. After non-PC GI, the majority of CA1 and cortical neurons showed cleaved caspase-3 and COX-2 colocalization (Figure 5C, I, O, U). In the PC group, the reduced COX-2 positivity and even more reduced cleaved caspase-3 also colocalized with the neuronal marker (Figure 5D, J, P, V). In this group, the NeuN immunoreactivity was noticeably stronger than after GI without PC. Pretreatment with COX-2 inhibitor resulted in the reappearance of strong immunoreactivity for cleaved caspase-3 and COX-2 in predominately neuronal cells (Figure 5E, K, Q, W).

HBO-PC Alone Increases HIF-1α Brain Level Without Affecting COX-2 or HO-1
We also measured COX-2, HO-1, and HIF-1α levels in brains of normal animals preconditioned with 5 HBO sessions (Figure 6A, B). There was no significant change in COX-2 or HO-1 levels after HBO-PC alone, either in the cerebral cortex (Figure 6A) or in the hippocampus (Figure 6B, C). In contrast, 5 HBO sessions induced HIF-1α in the hippocampus at 24 hours after the last session (P=0.002; Figure 6D).

Discussion
Our study shows that HBO-PC increased the number of surviving neurons, reduced apoptosis, and ameliorated cognitive deficit after global cerebral ischemia. We also observed that...
HBO-PC tended to decrease mortality and the incidence of seizures. In addition, HBO-PC reduced postischemic COX-2 upregulation. All these effects were significantly reduced by COX-2 inhibitor administered before HBO-PC sessions.

Consistent with reports by other authors, the damage of CA1 pyramidal neurons was detectable within 3 to 7 days after GI episode. Consequently, global cerebral ischemia resulted in the disorder of cognition and memory and the appearance of seizures. HBO-PC proved effective in conferring CA1 protection, lasting at least until day 7 after ischemia. Even though the duration of occlusion (15 minutes) was longer than in our previous study (10 minutes; 4-vessel occlusion used by Ostrowski et al.), we could still observe the beneficial effect of HBO-PC, which points toward a high efficacy of this modality.

We found a tremendous upregulation of COX-2 in the brain at 24 and 72 hours after GI. On the basis of colocalization of COX-2 with other markers, we hypothesized that HBO-PC might reduce the extent of neuronal damage by inhibiting COX-2 activity.

Figure 4. Western blot analysis of cyclooxygenase-2 (COX-2) levels in the hippocampus on day 1 (A) and day 3 (B) after global cerebral ischemia. *$P<0.05$ vs sham, †$P<0.05$ vs hyperbaric oxygen preconditioning (HBO-PC).

Figure 5. A–X, Triple immunofluorescence staining for cleaved caspase-3 (Texas Red), cyclooxygenase-2 (COX-2; FITC, green) and the neuronal marker NeuN (AMCA, blue) on day 3 after ischemia revealed a predominant colocalization of these epitopes (composite white color) in the CA1 (regular panels) and in the cerebral cortex (insets) affected by the ischemic insult. Hyperbaric oxygen preconditioning (HBO-PC) reduced immunoreactivity for cleaved caspase-3 and COX-2, although it tended to preserve the immunoreactivity of neuronal marker NeuN. Scale bars represent 30 μm.
cleaved caspase-3, COX-2, and NeuN, we infer that the majority of cells displaying COX-2 positivity were neurons dying through caspase-3-dependent apoptosis. More importantly, we found that the level of COX-2 in the hippocampus in the HBO-PC group is relatively lower than in the GI group at 2 different time points.

The large body of experimental evidence emphasizes the significance of blocking COX-2 pathway in therapeutic strategy for global cerebral ischemia. The expression of COX-2 dramatically increased in the injured hippocampus after global cerebral ischemia in animal models\textsuperscript{14,15} and in autopsied brains obtained from patients who died of cardiac arrest.\textsuperscript{26} Conversely, the degree of hippocampal neuronal injury produced by GI in COX-2-deficient mice was lessened as compared to that in the wild-type mice, coincident with attenuation of DNA fragmentation in the hippocampus.\textsuperscript{15}

In the present study we used the selective COX-2 inhibitor NS-398 before each exposure to HBO to verify the role of COX-2 in the HBO-PC. We found that the neuroprotective effect of HBO-PC was abolished by administration of low-dose NS-398, as reflected by the reduced number of surviving neurons and decreased percentage of spontaneous alternations. The increased mortality and incidence of seizures in the HBO-PCI group further supports the notion that inhibition of COX-2 inactivates a major PC mechanism.

However, we need to point out that the detailed mechanism for how blockade of COX-2 with NS-398 removes the PC effect remains unclear. At first we hypothesized that NS-398 might reduce COX-2 downstream target, HO-1. In ischemic PC, COX-2 plays a mandatory role for induction of HO-1 that, once induced, may decrease heme-dependent enzymes, including COX-2.\textsuperscript{27,28} However, Western blot analysis showed that HBO-PC failed to affect HO-1 levels in the brain. Therefore, it is unlikely that the effect of NS398 on HO-1, if any, would be relevant in this setting. In contrast, our further investigation revealed that HBO-PC by itself increased the level of HIF-1.\textsuperscript{15} This finding allows the possibility that NS398 removes the PC effect by decreasing HIF-1 activation, because HIF-1 is an established mediator of ischemic tolerance. In addition, studies in other systems have shown that COX-2 product prostaglandin E2 may directly upregulate HIF-1 at both mRNA and protein levels.\textsuperscript{29} Therefore, the role of COX-2 in regulating HIF-1 pathway in HBO-PC deserves further study.

We also hypothesized that HBO-PC may trigger an increase in COX-2 expression in the brain and protect neurons from ischemia-induced damage by depleting COX-2 protein before the ischemic insult. In some experimental systems, PC effects have been reported to occur via an increase in COX-2 expression.\textsuperscript{30} Unfortunately, there was no significant difference in COX-2 protein levels in the cerebral cortex and in the hippocampus between normal preconditioned and normal nonpreconditioned rats.

Overall, the role of COX-2 in HBO-PC shows similarities to its involvement in the ischemic PC of the brain or in the noble...
gas PC of the heart. COX-2 was induced in rat brains subjected to PC by the middle cerebral artery occlusion for 10 minutes followed by different amounts of reperfusion time (1–24 hours). Pretreatment with the COX-2-selective inhibitor, rofecoxib, increased infarct size and abolished PC-induced COX-2 expression in vivo.26 Furthermore, the study led by Weber27 found that xenon-induced late myocardial PC did not cause increased expression of COX-2 mRNA and protein, although the ischemic tolerance was abolished by functional blockade of COX-2 before ischemia. A recent study also pointed out the role of COX-2 in the glutamine-induced PC of the heart.28 Collectively, these data may indicate that COX-2 is a common component of PC mechanisms in different modalities and types of injury.

Although the neuroprotective effects of HBO-PC in GI have been reported previously, the underlying mechanisms have not been to date established. Our recent study of surgical brain injury demonstrated the important role of COX-2 in HBO-PC; however, until now, it was not known if this paradigm can hold true in the setting of brain ischemia. Therefore, we believe that our present work is novel because it offers a new insight on the mechanism through which HBO-PC reduces brain damage in global cerebral ischemia.

### Conclusion

In summary, HBO-PC-induced brain protection after GI is associated with a reduction of COX-2 levels in cerebral tissues. Pretreatment with COX-2 inhibitor abolished neuroprotective effect, which indirectly confirmed the role of COX-2 activation in the mechanism of HBO-PC. However, the molecular nature of COX-2 pathway in this setting remains to be elucidated.

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### Disclosures

None.

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