Research Report

Therapeutic hypercapnia improves functional recovery and attenuates injury via antiapoptotic mechanisms in a rat focal cerebral ischemia/reperfusion model

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Abstract
Recent studies have demonstrated neuroprotective effects of therapeutic hypercapnia for different forms of brain injury. However, few studies have assessed the neuroprotective and neurobehavioral effects of hypercapnia in focal cerebral ischemia, and the underlying mechanisms are still unclear. Here, we investigated the effects of therapeutic hypercapnia in focal cerebral ischemia in the rat middle cerebral artery occlusion/reperfusion (MCAO/R) model. Adult male Sprague Dawley rats were subjected to 90 min of MCAO/R and subsequently exposed to increased carbon dioxide (CO\textsubscript{2}) levels to maintain arterial blood CO\textsubscript{2} tension (PaCO\textsubscript{2}) between 80 and 100 mmHg for 2 h. Neurological deficits were evaluated with the corner test at days 1, 7, 14, and 28. Infarction volume and apoptotic changes were assessed by 2, 3, 7-triphenyltetrazolium chloride (TTC) staining, and terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate-biotin nick end labeling (TUNEL) staining at 24 h after reperfusion. Apoptosis-related proteins (Bcl-2, Bax, cytochrome c, and caspase-3) were investigated by western blotting. The results of this study showed that therapeutic hypercapnia significantly reduced infarct volume and improved neurological scores after MCAO/R. Moreover, hypercapnia treatment increased the survival rate at 28 days after reperfusion. The TUNEL-positive neurons in the ipsilateral cortex were significantly decreased in the hypercapnia group. Mitochondrial Bcl-2 and Bax cortical expression levels were significantly higher and lower, respectively, in hypercapnia-treated rats. In addition, hypercapnia treatment decreased cytosolic cytochrome c and cleaved caspase-3 expression and increased cytosolic Bax expression. These findings indicate that therapeutic hypercapnia preserves brain tissue and promotes functional neurological recovery through antiapoptotic mechanisms.

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1. Introduction

Ischemic brain injury, including stroke, is increasingly recognized as an important disorder that threatens human health and life (Rosamond et al., 2008). In addition to high mortality, ischemic brain injury can also cause long-term disability (Hackett et al., 2000). Extensive research has been performed in the last several decades, but most of these works employed a relatively short observational period (usually 1–3 days) (Tsubokawa et al., 2007; Xing et al., 2008). Given that recovery from stroke in humans is usually evaluated in weeks and months, the evaluation for clinical improvement after an intervention should also be weeks or longer.

Therapeutic hypercapnia is induced by adding carbon dioxide (CO₂) to inspired gas and is regarded as a new treatment strategy for various lung injury models (Chonghaile et al., 2008; Ni Chonghaile et al., 2008). In addition to lung tissue, hypercapnia has also proved effective for myocardial, intestinal, and central nervous system ischemic injuries (Laffey et al., 2003; Nomura et al., 1994; Vannucci et al., 1995). We previously reported that therapeutic hypercapnia could attenuate hepatic ischemia-reperfusion (IR) injury in rats (Li et al., 2010). We also demonstrated that therapeutic hypercapnia (arterial blood CO₂ tension [PaCO₂] 80–100 mmHg) ameliorated neurological deficits and attenuated histological damage in a global cerebral ischemia-reperfusion injury model (Zhou et al., 2010). However, the effects of therapeutic hypercapnia have not been reported in a focal cerebral ischemia model. The present study is the first effort to assess the utility of therapeutic hypercapnia for the treatment of stroke in a rodent model of focal cerebral ischemia. We hypothesized that therapeutic hypercapnia exerted neuroprotective effects after middle cerebral artery occlusion/reperfusion (MCAO/R) in rats, and on the basis of our previous observations with caspase inhibition (Zhou et al., 2010), we further hypothesized that the hypercapnia-induced neuroprotection may be modulated through apoptosis-related proteins. The long-term functional benefit of hypercapnia treatment was evaluated 4 weeks after MCAO/R.

2. Results

2.1. Physiological data

Experimental timelines are shown in Fig. 1. Rats were randomized to receive hypercapnia treatment (hypercapnia group) or 30% O₂ (ischemia-reperfusion [IR] group) for 2 h beginning at the reperfusion after 90 min of MCAO. A third group of rats underwent sham surgery (Sham group). Body weight, rectal temperature, and fasting blood–glucose (FBG) were not significantly different among the groups. Mean arterial pressure (MAP) and arterial blood gases parameters are presented in Table 1. After ischemia, there were no among-group differences in MAP, arterial oxygen tension (PaO₂), arterial blood CO₂ tension (PaCO₂), or pH. As intended, PaCO₂, PaO₂, and pH differed during reperfusion because of a different fraction of inspired gas in the hypercapnia group compared with the IR group (P < 0.05). MAP and PaO₂ were significantly increased in the hypercapnia group compared with the IR group (P < 0.05).

2.2. Therapeutic hypercapnia improved survival

In this study, rats in all groups were observed for 28 days. The survival rates were 27.3% and 40.0% in the IR group and hypercapnia group, respectively (Fig. 2). Hypercapnia conferred a better survival advantage compared with rats in the IR group (Fig. 2, P = 0.03). No rats in the sham group died.

2.3. Hypercapnia attenuated infarct volumes and improved neurological outcome

Hypercapnia resulted in a significant decrease in infarct ratio at 24 h after reperfusion (Hypercapnia group vs. IR group: 17.7% ± 1.0% vs. 24.8% ± 2.3%, P < 0.05, Fig. 3). Similar changes were observed in the corner test in that significant improvements were observed in the hypercapnia group (Fig. 4). Sham-operated rats did not exhibit significantly deficits. Compared with the IR group, the mean times of left turn was significantly improved in the hypercapnia group on days 1 (Hypercapnia group vs. IR group: 9.0 ± 1.3 vs. 9.9 ± 0.3, F = 6.27, P = 0.018), 7 (Hypercapnia group vs. IR group: 8.4 ± 1.6 vs. 9.5 ± 0.6, F = 4.40, P = 0.045), 14 (Hypercapnia group vs. IR group: 6.9 ± 1.6 vs. 9.3 ± 0.6, F = 14.65, P < 0.01), and 28 (Hypercapnia group vs. IR group: 6.3 ± 2.1 vs. 8.3 ± 1.2, F = 12.11, P < 0.01) after reperfusion.

2.4. Hypercapnia treatment reduced neuronal apoptosis

Neuronal cell death was quantitatively evaluated with the TUNEL assay and western blot assessment of apoptotic markers cytochrome c and caspase-3. The apoptosis-related proteins Bcl-2 and Bax were also estimated in the ischemic cortex area. Fig. 5A depicts TUNEL staining, and TUNEL-positive cell counts are shown in Fig. 5B. A large number of
Table 1 – Physiological variables in the experimental groups.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
<th>90 min after ischemia/sham surgery</th>
<th>After reperfusion (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
</tr>
<tr>
<td><strong>MAP (mmHg)</strong></td>
<td>Sham</td>
<td>116 ± 6</td>
<td>116 ± 7</td>
</tr>
<tr>
<td></td>
<td>IR</td>
<td>114 ± 6</td>
<td>120 ± 5†</td>
</tr>
<tr>
<td></td>
<td>Hypercapnia</td>
<td>115 ± 7</td>
<td>138 ± 3†</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>Sham</td>
<td>7.40 ± 0.04</td>
<td>7.34 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>IR</td>
<td>7.40 ± 0.06</td>
<td>7.33 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Hypercapnia</td>
<td>7.41 ± 0.06</td>
<td>7.08 ± 0.04†</td>
</tr>
<tr>
<td><strong>PaCO₂ (mmHg)</strong></td>
<td>Sham</td>
<td>32.6 ± 3.8</td>
<td>40.2 ± 8.1</td>
</tr>
<tr>
<td></td>
<td>IR</td>
<td>34.6 ± 3.2</td>
<td>38.2 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>Hypercapnia</td>
<td>33.1 ± 2.4</td>
<td>84.5 ± 5.0†</td>
</tr>
<tr>
<td><strong>PaO₂ (mmHg)</strong></td>
<td>Sham</td>
<td>223.6 ± 27.4</td>
<td>163.8 ± 33.7</td>
</tr>
<tr>
<td></td>
<td>IR</td>
<td>221.2 ± 21.1</td>
<td>148.8 ± 21.9</td>
</tr>
<tr>
<td></td>
<td>Hypercapnia</td>
<td>217.1 ± 19.2</td>
<td>199.2 ± 27.3†</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM.
MAP—mean arterial pressure; PaO₂—arterial oxygen tension; PaCO₂—arterial carbon dioxide tension; and IR—ischemia-reperfusion.
* P < 0.05 vs. Sham.
† P < 0.05 vs. IR.

TUNEL-positive cells were observed in the ipsilateral cortex of rats subjected to MCAO/R injury but not in sham-operated rats. Compared with the IR group, the hypercapnia group exhibited significantly fewer TUNEL-positive cells in the left cortex (Hypercapnia group vs. IR group: 74.1 ± 1.8 vs. 61.0 ± 1.9, P < 0.05, Fig. 5B).

Western blotting demonstrated that mitochondrial Bcl-2 was decreased in the IR group compared with the sham-operated group, and the hypercapnia group had higher levels compared with the IR group (Fig. 6B and E, P < 0.05). The mitochondrial and cytosolic expression levels of Bax were increased and decreased in the IR group, respectively, compared with the sham-operated group. Hypercapnia treatment significantly reversed this situation (Fig. 6A and D, P < 0.05). The level of cytosolic cytochrome c was significantly higher in the IR group compared with the sham-operated group, but hypercapnia treatment ameliorated this increase (Fig. 6A and G, P < 0.05).

The level of cleaved caspase-3 was noticeably increased in the IR group compared with the sham-operated group, which was significantly attenuated by hypercapnia treatment (Fig. 6A and F, P < 0.05).

3. Discussion

The present study shows that rats with hypercapnia (PaCO₂ 80–100 mmHg) had decreased infarct volumes and improved neurological outcomes after focal cerebral ischemic injury. Our findings support the hypothesis that moderate hypercapnia provided neuronal protection through an antiapoptotic mechanism, and this effect apparently involved regulating the expression of apoptosis-related molecules.

There are several reports of neuro- and cardioprotection with hypercapnia, and mechanisms have been proposed for these protective effects. For example, hypercapnia causes coronary vasodilatation, which may be beneficial during myocardial ischemia-reperfusion injury, and neuroprotective effects in the immature rat are achieved by reducing ambient levels of excitatory amino acids during hypoxia-ischemia (Nomura et al., 1994; Vannucci et al., 1995). Moreover, hypercapnia treatment could suppress enzymes involved in the generation of reactive oxygen species and subsequent oxidative damage (Shibata et al., 1998). It has also been demonstrated that hypercapnia treatment may inhibit inflammatory cytokines, such as interleukin-1, interleukin-8, tumor necrosis factor-α, and intracellular molecule-1 (Halbertsma et al., 2008; Lang et al., 2006; Ni Chonghaile et al., 2008). Further investigations are needed.

Fig. 2 – Hypercapnia increases survival rate 28 days after focal cerebral ischemia-reperfusion injury. There were no deaths in the sham group (n = 10). The survival rates of the IR and Hypercapnia groups were 27.3% (n = 11) and 40% (n = 10), respectively.
to precisely elucidate the protective mechanisms induced by hypercapnia.

Within minutes of MCAO, the cortex supplied by the occluded vessel is fatally damaged and subsequently undergoes necrotic cell death. This necrotic core is encircled by a zone, termed the "ischemic penumbra" that is rendered functionally silent but remains metabolically active (Ginsberg, 1997). Many studies have shown that neurons in the ischemic penumbra undergo apoptosis rather than necrosis, which means that they are potentially recoverable for a period after stroke onset (Ginsberg, 1997; Pulsinelli et al., 1997). Previous study have demonstrated that hypercapnia treatment might be protective because it induces antiapoptotic mechanisms (Zhou et al., 2010), but the exact pathways remain unclear. The TUNEL staining in our study demonstrated that hypercapnia significantly inhibited cortical neuronal apoptosis following MCAO/R. To further elucidate the exact mechanism, we investigated the mitochondrial pathway of apoptosis by performing western blots for apoptosis-related proteins. Our results show that hypercapnia treatment upregulated mitochondrial expression of the antiapoptotic Bcl-2 protein and apparently inhibited Bax translocation from the cytosol to the mitochondria. By regulating the balance of anti- and proapoptotic proteins, hypercapnia decreased the subsequent release of cytochrome c into the cytosol. Considerable evidence suggests that the release of mitochondrial cytochrome c plays a key role in the initiation of apoptosis (Li et al., 1997). Once released, cytochrome c binds apoptotic protein-activating factor-1 (Apaf-1) and procaspase-9 to form an "apoptosome," which subsequently activates caspase-3 and leads to apoptotic cell death (Love, 2003; Sugawara et al., 2004). As predicted, hypercapnia treatment attenuated caspase-3 activation and inhibited apoptosis. Our results suggest that hypercapnia protects neurons via an antiapoptotic mechanism through the mitochondrial pathway.

Besides histological changes, MCAO/R is also associated with a high incidence of sensorimotor dysfunction (Gharbawie et al., 2006; Modo et al., 2000). Some of the initial symptoms can spontaneously recover (Roof et al., 2001; Zausinger et al., 2000), making them less useful for long-term functional testing. Previous strategies have been shown to be effective in attenuating neurological functional injury after MCAO/R; however, animals were only tested 24 or 72 h after stroke (Tsubokawa et al., 2007; Xing et al., 2008). Thus, it is important for researchers to employ measures that assess long-term effects. A strong correlation between infarct volume and sensorimotor impairment has been shown by the corner test, even 90 days after MCAO/R (Zhang et al., 2002). The corner test requires integrated motor and sensory input that combine both cortical and subcortical functions, which are likely to be damaged by infarction in the MCA

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**Fig. 3** – Therapeutic hypercapnia attenuated infarct ratio 24 h after focal ischemia. (A) Representative TTC staining in comparable sections of rat brain from the IR (n=11) and Hypercapnia (n=11) groups 24 h after MCAO. (B) Quantification of infarct ratio 24 h after MCAO. Infarct ratio was significantly attenuated by hypercapnia treatment at 24 h. *P < 0.05 vs. IR.

**Fig. 4** – Evaluation of corner test at day 1, day 7, day 14, and day 28 after reperfusion. Values are presented as mean ± SEM. *P < 0.05 vs. Sham, #P < 0.05 vs. IR; n = 10–11/group.
This test has also been shown to be sensitive and objective for mice and rats (Li et al., 2004; Michalski et al., 2009). Although spontaneous recovery is known to occur in the rodent brain following ischemia stroke, the corner test was sensitive enough for neurological behavioral assessment 3 weeks after ischemic insult (Li et al., 2004).

In our study, the mean left turn percentage was significantly reduced in the hypercapnia group at days 1, 7, 14, and 28. These results support the hypothesis that hypercapnia could improve long-term neurological outcomes. MCAO/R also affects memory acquisition, which can be assessed by an avoidance task, maze task, or spatial memory test (Okada et al., 1995). Future studies should be designed to test cognitive function after focal ischemia and hypercapnia.

Our previous work demonstrated that mild to moderate hypercapnia (PaCO₂ 60–100 mmHg) is neuroprotective after transient global cerebral ischemia-reperfusion injury. Moreover, compared with rats with PaCO₂ 60–80 mmHg, neuroprotective effects were significantly increased in rats with PaCO₂ 80–100 mmHg (Zhou et al., 2010). Accordingly, we selected moderate hypercapnia (PaCO₂ 80–100 mmHg) to test the effects of hypercapnia in focal cerebral ischemia rats. In the current study, rats treated with PaCO₂ 80–100 mmHg exhibited decreased neurological deficit scores and less histological damage in a focal cerebral ischemia-reperfusion injury model. Similarly, Vannucci demonstrated that mild hypercapnia (PaCO₂ 50–70 mmHg) protects the immature brain subjected to hypoxic-ischemic brain damage (Vannucci et al., 1995). However, the same group also showed that hypercapnia in the range of 10.4–13.0 kPa (80–100 mmHg) neither protects nor aggravates perinatal hypoxic-ischemic brain injury (Vannucci et al., 2001).

Besides of the different experimental model, this discrepancy suggests a difference in the sensitivity of mature and immature brains to pathological conditions. The immature brain is generally believed to be more resistant to the damaging effects of cerebrovascular...
However, there is considerable controversy in the literature regarding the correlation between brain damage and age. Yager et al. (1996) demonstrated that brain damage induced by hypoxia-ischemia was most severe in 1- and 3-week-old rats; the 6- and 9-week-old group had significantly less injury than the other groups. Not only are there differences between the newborn and the adult regarding sensitivity to damage, underlying mechanisms of damage differ as well. Biochemical investigation showed that the mitochondria were found to be oxidized in immature rats that were subjected to 3 h of cerebral hypoxia-ischemia (Yager, 1994). This upward shift in mitochondrial NAD\(^+\)/NADH during hypoxia-ischemia was an unexpected finding given the anticipated mitochondrial reduction consistently observed in ischemic adult brains (Duckrow et al., 1981; Ginsberg et al., 1976). Moreover, Nabetani et al. (1997) demonstrated that neural activity and intracellular Ca\(^{2+}\) homeostasis in the immature rat brain are more resistant to energy compromise (Duffy et al., 1975).
failure than in adult rats. These findings emphasize the need for greater understanding of the neuroprotective effects of hypercapnia.

Although hypercapnia dramatically reduces tissue damage after 90 min of MCAO/R, some of the most obvious but frequently ignored factors should be discussed here. The present study was done exclusively in adult male rats, but stroke is a disease that mainly affects the elderly (Rojas et al., 2008) and in ischemic stroke patients cannot be treated with it due to unusual neurological scores during ischemia. Another limitation of this study was the absence of intracranial pressure (ICP) measurements. ICP was not monitored because of considerations over mortality rate after MCAO/R. Moreover, our previous study demonstrated that 3 h after the onset of reperfusion, there were no statistically significant differences in ICP between hypercapnia and IR groups when using a transient global cerebral ischemia–reperfusion model (Zhou et al., 2010).

4. Experimental procedures

4.1. Experimental animals

Adult male Sprague Dawley rats were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, PR China). All surgical procedures and postoperative animal care were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996, USA) and was approved by the Animal Use and Care Committee of School of Medicine, Harbin Medical University.

4.2. Experimental protocol

Eighty-four rats were randomly allocated to one of three groups: Sham group, IR group (MCAO/R), and Hypercapnia group (PaCO2 80–100 mmHg). The rats in the Sham group (n=23) underwent left common carotid artery and external carotid exposure without MCA occlusion, followed by inhalation of 30% O2 for 2 h. In the IR group (n=31), under the same anesthetic conditions and surgical procedures as Sham group, the MCA was occluded for 90 min, followed by reperfusion and inhalation of 30% O2 for 2 h. In the Hypercapnia group (n=30), rats received the same IR protocol and the same fraction of inspired oxygen as described for the IR group, but hypercapnia was applied at the beginning of reperfusion. The experimental protocol is shown in Fig. 1. Animals were tested at 24 h, 7 days, 14 days, and 28 days to evaluate neurologic function. At 24 h after reperfusion, a subset of the rats was sacrificed, and the brains were quickly harvested for the measurement of infarction ratio and apoptosis.

4.3. Surgical preparations and MCAO/R model

Male Sprague Dawley rats, 250–280 g, were fasted for 12 h before the experiments but were allowed free access to water. They were weighed immediately before surgery and anesthetized with halothane followed by tracheal intubation and mechanical ventilation (Harvard small animal ventilator 683, Natick, MA, USA) with halothane in 21% O2/79% N2; tidal volume, 9 ml/kg; respiratory rate, 45 breaths/min; and inspiratory to respiratory ratio 1:1. All surgical incisions were infiltrated with 0.25% bupivacaine. A heating pad and lamp were used to maintain the rectal temperature at 36.5±0.5 °C. Rats were subjected to MCAO/R as described previously, with minor modifications (Xing et al., 2008). Briefly, the left
common carotid artery was exposed, and the external carotid artery was isolated and coagulated. A 4-0 silicone-coated nylon suture (Doccol Co., Albuquerque, NM, USA) was inserted into the internal carotid artery through the external carotid artery stump and gently advanced to occlude the MCA. Regional cerebral blood flow (rCBF) was monitored by laser Doppler flowmeter (PeriFlux system 5000, Preimed AB, China) with use of a flexible fiberoptic fixed to the intact skull above the territory of the left MCA. A reduction in rCBF is detected when the filament is appropriately inserted. Rats were excluded from the study if they did not show a rCBF reduction of at least 70%. After securing the filament and closing the incision, the rats were allowed to wake and were assessed for neurological damage as follows: 0=no deficit, 1=failure to extend forelimb, 2=circling, 3=unable to bear weight on affected side, 4=no spontaneous motor activity (Longa et al., 1989).

4.4. Therapeutic hypercapnia

Therapeutic hypercapnia was performed after 90 min of MCAO at the beginning of reperfusion. Rats with clear neurological deficits (neurological scores ≥1) were reanesthetized and mechanically ventilated with halothane for suture removal to restore blood flow after 90 min of occlusion (reperfusion). The tail vein was cannulated to monitor MAP and arterial blood gases. After reperfusion, the hypercapnia group inhaled CO2 to maintain PaCO2 between 80 and 100 mmHg. The sham and IR groups were maintained on mechanical ventilation without adding CO2 to the inspired gas. The physiologic variables were recorded at five time points: after 90 min of ischemia and every 30 min after the start of reperfusion (reperfusion 30, 60, 90, and 120 min). The end-tidal CO2 concentration and inspiratory O2 concentration were monitored continuously using a gas monitor (Mindray BeneView T8, Mindray Medical International Limited, China). After 2 h of reperfusion, the temperature probe and catheters were removed, incisions were closed, and the animals were extubated and placed in an oxygenated Plexiglass box until the animals were awake. They were given free access to food and water immediately after recovering from anesthesia.

4.5. Corner test

Neurological deficits were evaluated with the corner test (Michalski et al., 2009; Zhang et al., 2002). Rats were tested on days 1, 7, 14, and 28 depending on the length of survival. All behavioral assessments were made during the light phase of the circadian cycle. Animals were handled to reduce stress during behavioral testing. Equipment was cleaned and allowed to dry before testing. All behavior scores were evaluated by an observer who was blinded to rat experimental assignment.

The corner test was carried out as described previously with slight modifications (Michalski et al., 2009). The rat was placed between two boards with dimensions of 30 × 20 × 1 cm. The edges of the two boards were attached at a 30° angle with a small opening along the joint between the boards to encourage entry into the corner. When this occurred, both sides of the vibrissae were simultaneously stimulated, causing the rat to rear forward and upward before turning back to face the open end. The non-ischemic rat turns either left or right, but the ischemic rat preferentially turns toward the non-impaired, ipsilateral (left) side. Before the experimental, the corner test was trained for 7 days and the value was used as reference. Rats that turned preferably to one side were excluded. The number of left turns was recorded out of 10 trials for each test. Turning movements that were not part of a rearing movement were not scored. This test detects integrated sensorimotor function because it involves both stimulation of the vibrissae (sensory/neglect) and rearing (motor response).

4.6. Infarct volume measurement

At 24 h after surgery, the rats were deeply anesthetized with chloral hydrate (400 mg/kg, intraperitoneally) and then decapitated, after which the brains were rapidly removed. The brains were sliced into 2-mm-thick coronal sections by using a stainless steel mold (68709, RWD Life Science Co., Ltd., China) and stained with 2% 2,3,5-triphenyltetrazolium chloride (Sigma, St. Louis, MO, USA) for 30 min at 37 °C followed by overnight immersion in 10% formalin. The infarcted tissue remained white, whereas normal tissue was stained red. The infarction area and hemisphere area of each section were traced and measured using Image-Pro Plus software (Version 6.0, Media Cybernetics, Bethesda, MD, USA). Infarct areas of all sections were added to derive the total infarct area, which was multiplied by the thickness of the brain sections to determine the infarct volume. To compensate for the effect of brain edema, the corrected percentage of infarction was calculated as follows: percentage of infarction = [measured infarct area–(ipsilateral hemisphere area–contralateral hemisphere area)]/(ipsilateral hemisphere area+contralateral hemisphere area) (Lin et al., 1993; Xing et al., 2008).

4.7. Histological examination and TUNEL staining

At 24 h after reperfusion, rats were deeply anesthetized and transcardially perfused with heparinized phosphate-buffered saline (PBS) and 4% paraformaldehyde dissolved in 0.1 mol/L PBS (pH, 7.4). The rats were decapitated, and the brains were removed, postfixed in 4% paraformaldehyde for 24 h, and embedded in paraffin. Paraffin-embedded tissues were sectioned at 5 μm according to standard procedures. The sections were deparaffinized, hydrated sequentially, and processed for TUNEL staining, which was used to assess DNA damage as a marker of apoptosis (Nomura et al., 1994). The section (n=4 for each group) were treated as instructed with an in situ cell death detection kit (Roche, Indianapolis, IN, USA). Diaminobenzidine was used as a chromogen, and TUNEL-positive apoptotic cells exhibited brown nuclear or cytoplasmic staining. The number of apoptotic cells in the ipsilateral cortex (region of interest, Fig. 5A-d) was semiquantitatively assessed using light microscopy (five different random high-power fields per sector, 400x magnification). All TUNEL-positive cells were counted. In order to exclude
the false positive cells, cell counting were performed by a pathologist who was blinded to group assignment.

### 4.8. Western blot analysis

Apoptosis-related proteins were measured by western blot analysis. At 24 h after surgery, rats were sacrificed under deep anesthesia. Brains were stored at −80°C until analysis. Western blot and protein extraction were performed as described previously (Tsubokawa et al., 2007; Xing et al., 2008). Briefly, whole-cell lysates were obtained by homogenizing the left cerebral cortices (region of interest, Fig. 6H) with a homogenizer (Bullet Blender Homogenizer, Next Advance, Inc., Averill Park, NY, USA) in a 5-ml volume of buffer (20 mmol/L HEPES, 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 250 mmol/L sucrose, 0.1 mmol/L phenylmethylsulfonylfluoride, 1 mmol/L dithiothreitol, and proteinase inhibitor cocktail tablets; pH 7.9). Samples were further centrifuged at 800 g for 15 min at 4°C to separate the sample into supernatant A and pellet A. Supernatant A containing cytosolic/mitochondrial proteins was further centrifuged at 16,000 g for 30 min at 4°C to separate supernatant B from pellet B. Supernatant B was used as the cytosolic fraction, and pellet B was used as the mitochondrial fraction after resuspension in buffer. Protein concentration was determined by the Bradford protein assay. Equivalent amounts of protein (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline with Tween (TBST) buffer and then incubated with primary antibodies to cytochrome c, Bax, cleaved caspase-3 (all 1:1000, Cell Signaling Technology Inc., Danvers, MA, USA). After extensive rinsing in TBST buffer, the membranes were incubated with appropriate secondary antibodies (1:5000, Rockland Inc., Gilbertsville, PA, USA) for 1 h at room temperature and then developed with an enhanced chemiluminescence system (ECL Plus, Amersham Bioscience, Piscataway, NJ, USA). Blot bands were densitometrically quantified with ImageJ software (v1.33, NIH, Bethesda, MD, USA). β-actin (1:1000, ZSGB-Bio, Beijing, China) or COX IV (1:1000, Cell Signaling Technology Inc., Danvers, MA, USA) was used as the loading control for the cytosolic and mitochondrial fractions, respectively. The quantified values were expressed as a percentage of sham (100%).

### 4.9. Statistical analysis

Statistical analyses were performed using SAS (version 9.13, SAS Institute Inc., Cary, NC, USA). Dates are expressed as mean ± standard error of mean (SEM). Physiological parameters and arterial blood gases were analyzed using repeated measures analysis of variance (ANOVA) followed by a post-hoc test. TUNEL-positive cell counting: infarct volume; and protein levels of Bcl-2, Bax, cytochrome c, and cleaved caspase-3 were assessed with one-way ANOVA followed by a post-hoc test. Corner test results were assessed using a mixed effect model. Survival analysis was analyzed with log-rank tests. Values of P < 0.05 were considered statistically significant.

### 5. Conclusions

Our results demonstrate that hypercapnia treatment increased CO₂ levels (PaCO₂, 80–100 mmHg), decreased infarct volume, and improved neurological outcomes after focal cerebral ischemia/reperfusion injury. These neuroprotective effects were associated with the expression of apoptosis-related proteins and endogenous protection mechanisms.

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