Delayed Hypoxic Postconditioning Protects Against Cerebral Ischemia in the Mouse

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Background and Purpose—Inspired from preconditioning studies, ischemic postconditioning, consisting of the application of intermittent interruptions of blood flow shortly after reperfusion, has been described in cardiac ischemia and recently in stroke. It is well known that ischemic tolerance can be achieved in the brain not only by ischemic preconditioning, but also by hypoxic preconditioning. However, the existence of hypoxic postconditioning has never been reported in cerebral ischemia.

Methods—Adult mice subjected to transient middle cerebral artery occlusion underwent chronic intermittent hypoxia starting either 1 or 5 days after ischemia and brain damage was assessed by T2-weighted MRI at 43 days. In addition, we investigated the potential neuroprotective effect of hypoxia applied after oxygen glucose deprivation in primary neuronal cultures.

Results—The present study shows for the first time that a late application of hypoxia (5 days) after ischemia reduced delayed thalamic atrophy. Furthermore, hypoxia performed 14 hours after oxygen glucose deprivation induced neuroprotection in primary neuronal cultures. We found that hypoxia-inducible factor-1α expression as well as those of its target genes erythropoietin and adrenomedullin is increased by hypoxic postconditioning. Further studies with pharmacological inhibitors or recombinant proteins for erythropoietin and adrenomedullin revealed that these molecules participate in this hypoxia postconditioning-induced neuroprotection.

Conclusions—Altogether, this study demonstrates for the first time the existence of a delayed hypoxic postconditioning in cerebral ischemia and in vitro studies highlight hypoxia-inducible factor-1α and its target genes, erythropoietin and adrenomedullin, as potential effectors of postconditioning. (Stroke. 2009;40:3349-3355.)

Key Words: adrenomedullin ■ erythropoietin ■ hypoxia ■ ischemia ■ postconditioning

Ischemic or hypoxic preconditioning is a sublethal stress able to reduce ischemia-induced injury when applied before ischemia, a phenomenon called ischemic tolerance. Understanding the mechanisms underlying this preconditioning-induced tolerance might allow identification of new therapeutic targets. However, its application is not clinically feasible because the occurrence of stroke is hardly predictable. By analogy with preconditioning, postconditioning represents another promising strategy to modulate brain ischemic damage. Based on studies in the heart, ischemic postconditioning was defined as a repetitive series of brief interruptions of reperfusion applied after ischemia that confers neuroprotection, probably by attenuating reperfusion injury. Inspired from these studies, the neuroprotective effect of ischemic postconditioning applied immediately after the ischemic insult has been reported in models of focal cerebral ischemia in the rat. More delayed ischemic postconditioning, starting 6 hours and 24 hours after focal and global cerebral ischemia, respectively, has been also recently described. Whereas the molecular mechanisms of cerebral ischemic preconditioning were addressed in many studies, those of postconditioning after focal ischemia were only suggested in a few studies. The protection has been associated with the activation of the protein kinase Akt. Besides ischemic postconditioning, the existence of hypoxic postconditioning has been described in the heart but not in the brain neither in vivo nor in vitro.

Therefore, the aim of this study was to search for the existence of hypoxic postconditioning against focal cerebral ischemia in the mouse. We show that a delayed application of chronic intermittent hypoxia after ischemia led to a significant neuroprotection assessed by T2-weighted MRI. To investigate the mechanisms potentially involved in hypoxic postconditioning, we used an in vitro model consisting of...
primary neuronal cultures submitted to oxygen glucose deprivation (OGD) followed by hypoxia 14 hours later. As we, and others, showed previously that adrenomedullin (AM) and erythropoietin (EPO) are major effectors of hypoxic preconditioning,\textsuperscript{8–12} we evaluated their putative involvement in hypoxic postconditioning.

**Materials and Methods**

**Focal Cerebral Ischemia and Hypoxic Postconditioning**

Male adult Swiss mice (approximately 30 g on Day 0; CERJ, Le Genest St. Isle, France) were used. Animal experiments were approved by the regional ethic committee (agreement number 05-028) and in agreement with the French national legislation. Transient focal ischemia was achieved by intraluminal occlusion of the right middle cerebral artery (MCAO) during 60 minutes under isoflurane anesthesia.\textsuperscript{13} Chronic intermittent hypoxia started either 1 day or 5 days after the onset of MCAO in 2 distinct experiments (MCAO hypoxia >24 hours and MCAO hypoxia >5 days, respectively) and lasted until 43 days after ischemia, at which time mice were killed under chloral hydrate anesthesia.

Hypoxia was applied during 1 hour and repeated 3 times per week according to the following protocol (Supplemental Figure I, available online at http://stroke.ahajournals.org): for the MCAO hypoxia >24-hour group, hypoxia was performed on Days 1 to 3 through 5 to 8, and 10 to 12 through 15 to 17 through 19 to 21 through 24 to 26 through 29 to 31 through 33 to 36 through 38 to 40 through 43; and in the MCAO hypoxia >5-day group, on Days 5 to 7 through 9 to 12 through 14 to 16 through 19 to 21 through 23 to 26 through 28 to 30 through 33 to 35 through 37 to 40 through 42. Each session of hypoxia was performed in a hypoxic chamber (IN VIVO2 1000, 3 mol/L, France) fixed at 5% CO\textsubscript{2} and 37°C. After 9 days of culture in a humidified incubator, neuronal cells were submitted to an OGD or not for 1 hour.

In the first experiment, 4 groups were used: MCAO control (n=9), MCAO hypoxia >5 days (n=9), and 2 sham-operated groups were designed in the same way: sham control (n=7) and sham hypoxia >5 days (n=8). In the second experiment, 2 groups were used: MCAO control (n=10) and MCAO hypoxia >24 hours (n=7). All MCAO mice were divided into matched groups based either on their 48-hour infarct volume (hypoxia >5 days) or on their initial neurological deficit at 24 hours (hypoxia >24 hours). Lastly, 14 mice were added for weekly blood retro-orbital sampling to measure the effect of chronic intermittent hypoxia on hematocrit. Of note, we did not evidence any deleterious effects of chronic intermittent hypoxia on weight gain, MRI, histology, or behavior in sham animals (data not shown).

**Behavioral Tests**

Neurological score was performed by attributing a grade according to the severity of the deficit\textsuperscript{14} evaluated for several items: no circling toward the paretic side, resistance to push, and tactile stimulation of ears; other items were evaluated when the mouse was raised by the tail: no flexion of the body, the forelimbs, and hindlimbs. Hypoxic Postconditioning

Two sessions of MRI were performed after MCAO at 48 hours and 43 days. Images were acquired in a horizontal 7-T magnet (160-mm diameter; Pharmscan; Bruker). Fast T2-weighted imaging was performed (RARE sequence, acceleration factor: 8) using a linear volume coil (32 mm diameter) with the following parameters: TE/TR 46 ms/5 s, excitation number 6, field of view 20 mm\textsuperscript{2}, matrix: 256×192, 15 slices, 0.75 mm thick. Measurements of brain areas were carried out in a blind manner using ImageJ software. At 48 hours, the infarct volume corrected for edema was calculated as follows: infarct volume × contralateral hemisphere volume/total ipsilateral hemisphere volume (Figure 1A). At 43 days, the brain lesion volume was measured as the difference between the remaining healthy ipsilateral hemisphere and the contralateral one expressed as a percentage of the contralateral healthy hemispheric volume.\textsuperscript{14} The same procedure was applied to measure cortical, striatal lesion, and thalamic atrophy ([contralateral−ipsilateral]/[contralateral] volumes of each structure). Thalamic delineation is illustrated on thionine-stained histological and T2-weighted MRI slices from one animal in Figure 1D. Thalamic delineations from T2-weighted MRI slices are also illustrated on distribution maps of thalami (Figure 1E). These maps were obtained by coregistration of MRI slices from all animals followed by manual delineation of each thalamus to obtain a binary mask of these structures in each animal. Binary masks of thalami were then summed to give pixel values equal to the number of animals in which the thalamus is present at each pixel location. These distribution maps are then superimposed on averaged over all animals MRI slices.

**OGD, Hypoxia, and Pharmacological Treatments on Neuronal Cultures**

Neocortical cultures of neurons from mouse embryos (Swiss) at 14 to 15 days of embryonic development were prepared as previously described,\textsuperscript{11} and were maintained with DMEM (4.5 g/L glucose) supplemented with 2 mmol/L glutamine and 1% N2 defined-supplement (Invitrogen). Hypoxia and OGD experiments were performed in a hypoxic chamber (IN VIVO, 1000, 3 mol/L, France) fixed at 5% CO\textsubscript{2} and 37°C. After 9 days of culture in a humidified incubator, neuronal cells were submitted to an OGD or not for 1 hour as previously described.\textsuperscript{14} After 6, 14, or 24 hours of reoxygenation, neuronal cells were submitted to hypoxia (0.1%, 1%, or 2% O2), or not, for 1 hour.

Neuronal cell death was assessed 48 hours after OGD. Immediately at the end of OGD or hypoxia, nuclear samples were extracts from cells without reoxygenation using the published protocol.\textsuperscript{10} Total RNA was extracted from cells without reoxygenation, converted into single-strand cDNA, and amplified with primers for AM and \(\beta\)-actin as previously described\textsuperscript{15} and with primers for EPO: F 5’AGCTCAGAGGAATTGATGTCGC3’, R 5’AGGAAGTTGGCGTAGAGCC3’, 108 bp.

Analysis of hypoxia inducible factor-1\(\alpha\) (HIF-1\(\alpha\)) expression in nuclear extracts was assessed using an Immunoassay kit for mouse total HIF-1\(\alpha\) (R&D Systems) according to the manufacturer’s instructions. Protein levels in the supernatant were assessed using an enzyme immunoassay kit for rat AM (Phoenix Pharmaceuticals) and an immunoassay quantitative colorimetric sandwich kit for rat/mouse EPO (R&D Systems) according to the manufacturer’s instructions.

Pharmacological studies were performed with echinomycin (10 nmol/L; Sigma), human recombinant AM22-52 (hAM22-52, 10\textsuperscript{-7} mol/L; Sigma), mouse recombinant AM1-50 (mAM1-50; Phoenix Pharmaceuticals), human recombinant soluble EPO receptor (0.5 mg/L; R&D Systems), or recombinant EPO (rhEPO; Lundbeck).

**Statistical Analysis**

Values are presented as mean±SEM, except for neurological score (median±quartile). Results were analyzed by 2-way analyses of variance for repeated measurements or one-way analysis of variance followed by partial least squares difference of Fisher or Kruskal-Wallis test followed by Mann-Whitney U test for the neurological score.

**Results**

Delayed Hypoxic Postconditioning Reduces Cerebral Ischemia-Induced Thalamic Atrophy

Hypoxia applied 24 hours after ischemia did not modify the infarct volume assessed at 48 hours compared with the
MCAO control group (Figure 1B). When the brain lesion was examined 43 days after ischemia, hypoxia starting 24 hours or 5 days after MCAO, there was no effect on the final whole brain lesion. However, hypoxia starting 5 days after ischemia significantly reduced the thalamus atrophy (Figure 1C), as also illustrated by the distribution map of thalami (Figure 1E). This late hypoxic postconditioning led to a nonsignificant reduction of cortical (P=0.15) and striatal lesion (P=0.12; Figure 1C). Of note, chronic intermittent hypoxia did not have any effect on hematocrit (data not shown).

As already reported in this model, ischemia led to sensorimotor deficits in adhesive removal test and neurological impairments persisting up to 6 weeks post-MCAO. Hypoxia, whatever its time of onset, did not modify sensorimotor performances at the adhesive removal test (data not shown) nor the neurological score (Figure 2).

Hypoxic Postconditioning Reduces Neuronal Cell Death Induced by OGD

Hypoxic postconditioning performed with 0.1% O₂, 14 hours after OGD, significantly reduced neuronal death measured 48 hours after OGD compared with the sole OGD condition (Figure 3B). Similar results were obtained when hypoxia was performed with 1% O₂ but not with 2% O₂ (Figure 3D). Furthermore, when hypoxia (0.1% O₂) was applied earlier, that is, 6 hours or later, that is, 24 hours after OGD, it did not protect neurons from OGD (data not shown).

HIF-1 and Its Target Genes AM and EPO Are Involved in In Vitro Hypoxic Postconditioning-Induced Neuroprotection

Although OGD did not significantly change HIF-1α expression during or after OGD reoxygenation (Figure 4A), the
nuclear HIF-1α level was enhanced in neurons submitted to 0.1% O₂ hypoxia 14 hours after OGD compared with control or OGD conditions. This was, however, not the case when hypoxia was performed at 2% O₂. Echinomycin, a molecule able to inhibit HIF-1 DNA-binding activity, applied after hypoxia, abolished the neuroprotective effect of 0.1% O₂ hypoxic postconditioning against OGD (Figure 4B). This result suggests that HIF-1 and its target genes could be involved in hypoxic postconditioning.

Among them, we particularly focused on AM and EPO, 2 molecules already shown to be involved in neuronal tolerance induced by hypoxic preconditioning.1,9–12 Hypoxia performed at 0.1% O₂ induced a significant increase of AM mRNA expression in neurons compared with controls (Figure 5A). When applied after OGD, hypoxia at 0.1% O₂ but not at 2% O₂ increased AM mRNA expression (Figure 5A). In line with these results, AM release was significantly enhanced by hypoxic postconditioning compared with OGD (Figure 5B). Application of the AM receptor antagonist (AM22-52) during and after hypoxia reversed the neuroprotective effect of hypoxic postconditioning (Figure 5C). When recombinant AM was applied 15 hours after OGD, neuronal death was reduced to a level nonsignificantly different from control (P=0.09; Figure 5D).

EPO mRNA expression was also increased when hypoxia was performed with 0.1% O₂ for 1 hour, whereas hypoxia with 2% O₂ did not change its expression (Figure 6A). Hypoxia (0.1% O₂), alone or after OGD, was also able to
increase EPO production 34 hours after reoxygenation compared with control or OGD conditions (Figure 6B). Soluble EPO receptor applied after hypoxia abolished the neuroprotection elicited by hypoxic postconditioning (Figure 6C). Recombinant EPO applied 15 hours after OGD limited neuronal death compared with the OGD condition (Figure 6D).

**Discussion**

The preconditioning stimulus can be applied several days or several hours before cerebral ischemia, referring thereby to, respectively, delayed and early preconditioning, the mechanisms of which differ considerably from each other. To date, early brain ischemic postconditioning has been first reported in focal ischemia, then delayed postconditioning against focal ischemia and in global ischemia. We have previously shown that hypoxic preconditioning (8% O2, 1 hour) performed 24 hours before focal cerebral ischemia in adult mice protects the brain. Furthermore, we reported in vitro a neuroprotective effect of hypoxic preconditioning (0.1% O2, 1 hour) when performed 24 hours before OGD on neurons. Based on these previous paradigms, we report here, for the first time, the existence of delayed neuroprotection induced by hypoxic postconditioning both in vivo, with a chronic intermittent hypoxia initiated 5 days after MCAO, and on cultured neurons with hypoxia performed 14 hours after OGD. Although the in vivo model offers a more integrated view of stroke pathophysiology and of this potential therapeutic strategy, the in vitro approach allows us to propose potential effectors of the hypoxic postconditioning-induced protection in neurons only.

A main finding of our study is that delayed chronic hypoxia is able to reduce thalamic atrophy, a delayed consequence of cerebral focal ischemia. This result is in accordance with previous work, showing that delayed chronic administration of a chemical inducer of HIF-1 is able to reduce the thalamic atrophy after ischemia. We can suppose that hypoxia, by reducing cortical and striatal damage, although not significantly, may have indirectly limited secondary thalamic degeneration or directly limited delayed apoptotic neuronal death in the thalamus. Hypoxic postconditioning did not alleviate ischemia-induced functional deficits, suggesting that the neuroprotection seen in the thalamus was not sufficient to improve functional recovery as measured with these tests. To assess the therapeutic time window of hypoxic postconditioning, we initiated hypoxia earlier (24 hours after ischemia) and did not find any evidence of a reduction of ischemia-induced brain damage. These results contrast with one study, which reported the existence of ischemic postconditioning 6 hours after ischemia. Nevertheless, in another experiment, we examined the effect of chronic hypoxia...
starting 6 hours after MCAO on ischemia-induced brain damage; hypoxia enhanced the mortality rate (41% significantly different compared to MCAO control: 9.1%), whereas a neuroprotective effect was observed in the remaining animals (∼38% of ischemic lesion at 48 hours, data not shown).

To date, in vitro hypoxic postconditioning has been only reported on cardiomyocytes. Similar to our results previously obtained for hypoxic preconditioning, hypoxia with 0.1% O2 applied 14 hours after OGD reduced neuronal death induced by OGD, whereas 2% O2 had no effect. It is well known that brain tolerance involves transcriptional factors such as the nuclear factor κB (NF-κB) and cAMP-responsive element-binding protein. We particularly focused on the role of HIF-1 and its target genes such as EPO and AM, which have also been reported as effectors of hypoxic postconditioning. Results obtained with pharmacological inhibitors of EPO or AM pathways or recombinant EPO or AM further support this hypothesis. These in vitro results are in line with other in vivo studies showing beneficial effects of delayed EPO and AM administration after ischemia.

In conclusion, delayed hypoxic postconditioning seems to be a beneficial stimulus to induce neuroprotection against ischemic damage. A postconditioning challenge, unlike preconditioning, is, at least theoretically, applicable to the clinical situation. Because ischemic tolerance models are useful paradigms to identify new therapeutic targets for stroke, a rational approach to study pre- and postconditioning mechanisms may be to search for molecules and pathways that are common to these paradigms. In this context, HIF-1 targets such as AM and EPO may be part of them.

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Disclosures

None.

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