Ischemic Postconditioning Inhibits Apoptosis After Focal Cerebral Ischemia/Reperfusion Injury in the Rat

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Background and Purpose—Ischemic postconditioning (Postcond) is defined as a series of intermittent interruptions of blood flow in the early phase of reperfusion that mechanically alters the hydrodynamics of reperfusion. A recent study showed that Postcond reduced infarct size in cerebral ischemia/reperfusion (I/R) injury. However, little is known about the mechanisms of Postcond in cerebral I/R injury. In the present study, we investigated the effects of Postcond in focal cerebral I/R injury in the rat middle cerebral artery occlusion model.

Methods—Adult male Sprague-Dawley rats were treated with Postcond after 60 minutes of occlusion (beginning of reperfusion). Neurologic scores and infarct volumes were assessed at 24 and 72 hours. Oxidative stress was evaluated by malondialdehyde assay, and apoptosis-related molecules were studied by Western blotting.

Results—Postcond treatment upregulated Bcl-2 and heat-shock protein 70 expression and downregulated cytochrome c release to the cytosol, Bax translocation to the mitochondria, and caspase-3 activity. Postcond treatment also reduced infarct volumes and oxidative stress levels and improved neurologic scores compared with the I/R-only group.

Conclusions—These findings indicate that Postcond inhibits focal cerebral I/R injury. This neuroprotective effect is likely achieved by antiapoptotic mechanisms. (Stroke. 2008;39:2362-2369.)

Key Words: ischemic postconditioning ■ ischemia/reperfusion ■ apoptosis ■ middle cerebral artery occlusion model

Stroke is one of the main causes of death and disability. Advances in intravascular techniques and thrombolytic agents have reduced functional deficits within an optimal time window in stroke patients. However, reperfusion itself generates an overproduction of reactive oxygen species (ROS) or free radicals, leading to reperfusion injury.1 Despite extensive research on reperfusion injury treatment in the past several decades; few neuroprotectants have been successfully from basic research into clinical application.

Apoptosis after cerebral ischemia/reperfusion (I/R) is one of the major pathways that leads to the process of cell death.2 In response to the oxidative load in mitochondria, the outer membrane of mitochondria becomes permeabilized,3 resulting in the translocation of Bax from the cytosol to the mitochondria and the release of cytochrome c, normally confined to the mitochondrial intermembrane space.4,5 This proapoptotic protein translocation is controlled by the family of Bcl-2 proteins.6 Release of cytochrome c into the cytosol leads to the formation of the apoptosome, a complex composed of apoptotic-protease activating factor-1, procaspase-9, and ATP.7 The apoptosome permits the autoactivation of procaspase-9, which is followed by the activation of procaspase-3.8 Active caspase-3 leads to DNA fragmentation.7 Some studies have demonstrated that apoptosis contributes to the development of ischemic infarction with DNA fragmentation.9 Thus, the ideal preventive or therapeutic approach would indeed target apoptosis after I/R.

Ischemic preconditioning is the phenomenon whereby prior ischemic stress renders the organ resistant to a subsequent ischemic insult.10 Although extensive research has demonstrated that ischemic preconditioning treatment reduces cerebral I/R damage,11 cerebral ischemic preconditioning is clinically feasible only when the occurrence of stroke is predictable. Compared with ischemia, the onset of reperfusion is more predictable.

Ischemic postconditioning (Postcond) is defined as a series of rapid intermittent interruptions of blood flow in the early phase of reperfusion that mechanically alters the hydrodynamics of reperfusion. Recent developments in cardiac physiology have indicated that Postcond significantly reduces infarct size and inhibits inflammation and apoptosis.12–19 In addition, a recent exciting clinical report demonstrated that
Postcond after coronary angioplasty and stenting protected the heart during acute myocardial infarction. Zhao et al. first documented that ischemic postconditioning reduced infarct size in cerebral I/R injury. However, whether the protective effect of Postcond is associated with inhibition of apoptosis in vivo has not been reported previously. In the current study, we tested the role of Postcond in apoptosis in rats with middle cerebral artery occlusion (MCAO).

Materials and Methods

Experimental Animals and MCAO Model

Adult male Sprague-Dawley rats (250 to 280 g) were cared for according to the Guide for the Care and Use of Laboratory Animals. The committee for experimental animals of Tongji Medical College approved all surgical procedures. Rats were anesthetized with chloral hydrate (350 mg/kg IP) and subjected to MCAO as described previously, with minor modifications.

In brief, we exposed the common carotid artery, internal carotid artery, and external carotid artery surgically. A 4-0 monofilament nylon suture (Beijing Sunbio Biotech Co Ltd) with a rounded tip was inserted into the internal carotid artery through the external carotid artery stump and gently advanced to occlude the MCA. After 60 minutes of MCAO, the suture was removed to restore blood flow (reperfusion confirmed by laser Doppler). Sham-operated rats were manipulated in the same way, but the MCA was not occluded. Regional cerebral blood flow (rCBF) was monitored by laser-Doppler flowmeter (Periflux System 5000, Perimed Inc) with the use of a flexible probe over the skull as described earlier. rCBF was measured before ischemia, during MCAO, and during reperfusion. Animals that did not show a CBF reduction of at least 70% were excluded from the experimental group, as well as animals that died after ischemia induction. Core body temperatures were monitored with a rectal probe and maintained at 37°C during the whole procedure. Mean arterial blood pressure (of the left femoral artery), pH, arterial blood gases, and blood glucose levels before, during, and after ischemia were measured. All surgical procedures were performed under an operating stereomicroscope.

Ischemic Postconditioning

Rats were divided randomly into 3 groups: (1) a sham-operated control group (n = 40), (2) an I/R group (n = 42) with 60 minutes of MCAO followed by 24 and 72 hours of reperfusion, and (3) an ischemic Postcond group (n = 42). (Postcond was performed after 60 minutes of MCAO, at the beginning of reperfusion.) For the Postcond study, reperfusion was established for 30 seconds, after which the MCA was occluded again for 30 seconds, followed by another 5 cycles of the same period of reperfusion and occlusion (Figure 1). Rats were killed at 24 or 72 hours. Furthermore, when there was evidence of subarachnoid hemorrhage on extraction of brain tissue, the animal was excluded from the study.

Figure 1. Experimental protocol used to determine the effect of ischemic Postcond after I/R. Sham indicates sham-operated rats; I/R, rats subjected to 60 minutes of MCAO followed by reperfusion; Postcond, rats treated with ischemic Postcond after 60 minutes of MCAO (beginning of reperfusion).

Neurologic Scoring

Neurologic scores were evaluated by a blinded observer at 24 and 72 hours with a scoring system as described previously. An 18-point scoring system was used to evaluate sensorimotor deficits (see supplemental Figure 1 and Table I, available online at http://stroke.ahajournals.org).

Measurement of Infarct Volume

At 24 and 72 hours after reperfusion, rats were decapitated and the brains were rapidly removed. Infarct volumes were measured as described previously. In brief, brains (n = 6 for each group) were cut into 2-mm-thick coronal sections in a cutting block and stained with 2% 2,3,5-triphenyltetrazolium chloride (Sigma) for 30 minutes at 37°C followed by overnight immersion in 10% formalin. The infarcted tissue remained unstained (white), whereas normal tissue was stained red. The infarct zone was demarcated and analyzed by Image J software (NIH Image, version 1.61). Infarct areas of all sections were added to derive the total infarct area, which was multiplied by the thickness of the brain sections to obtain the infarct volume. To compensate for the effect of brain edema, the corrected infarct volume was calculated as follows: corrected infarct area = measured infarct area × {1 −[(ipsilateral hemisphere area − contralateral hemisphere area)/contralateral hemisphere area]}.

Histologic Examination

At 24 hours after reperfusion, rats were deeply anesthetized with chloral hydrate and perfused with heparinized phosphate-buffered saline, followed by perfusion with 4% paraformaldehyde in phosphate-buffered saline. The brain was blocked and embedded in paraffin. Paraffin-embedded tissues were sectioned at 5 μm according to standard procedures. The sections were deparaffinized and hydrated sequentially and examined by immunohistochemistry and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) techniques, respectively.

Sample Processing

Rats were killed 24 hours after reperfusion and the brains were quickly removed to collect the cerebral cortex for oxidative stress, DNA fragmentation, and caspase-3 activity measurements; Western blotting; and reverse transcription–polymerase chain reaction (RT-PCR). A 3-mm coronal section was taken from the area perfused by the MCA starting 5 mm from the frontal pole. Fresh cortical tissue was collected from the MCA territory of the ischemic right hemisphere, frozen immediately in LN2, and stored at −70°C until needed for further processing.

TUNEL Assay

A TUNEL assay was used to assess DNA damage. The sections (n = 4 for each group) were treated as instructed with an in situ cell death detection kit (Roche, Basel, Switzerland). Diaminobenzidine (Sigma) was used as a chromogen. TUNEL-positive cells displayed brown staining within the nucleus of apoptotic cells. DNA fragmentation was quantified under high-power magnification (×400) by an
inhibitor which was blinded to the studies and was expressed as number per square millimeter.

**Immunohistochemistry**
Paraffin sections (n=4 for each group) were incubated with antibodies against cytochrome c and heat shock protein 70 (HSP70, Santa Cruz) after being blocked with bovine serum albumin. Then the samples were incubated with a biotinylated secondary antibody followed by avidin-biotin-peroxidase complex. Positive staining was visualized with diaminobenzidine. The images were captured by a digital camera connected to a microscope (Olympus IX71) and then analyzed with MagnaFire SP 2.1B software.

**Determination of Oxidative Stress**
Right cortical samples (n=4 for each group) were weighed. Malondialdehyde (MDA) level and superoxide dismutase (SOD) activity were measured as described previously. In brief, MDA level was measured by the thiobarbituric acid method. The amounts of lipid peroxides were measured as the production of MDA. Absorbance was measured at 532 nm by spectrometry. SOD activity was measured by the xanthine oxidase method. Absorbance was determined at 550 nm by spectrometry. MDA and SOD kits were purchased from Nanjing Jiancheng Bioengineering Institute. All protein concentrations of cortical tissue homogenate samples were determined with the Coomassie blue method (assay kit purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All protein concentrations of cortical tissue homogenate samples were determined with the Coomassie blue method ( assay kit was purchased from Nanjing Jiancheng Bioengineering Institute).

**DNA Fragmentation Analysis**
Genomic DNA was extracted from the right cortical samples (n=4 for each group) with use of the Easy DNA extraction kit (Fermentas Life Sciences). Ten micrograms of DNA was electrophoresed on a 2% agarose gel. Fragmented DNA was visualized by ethidium bromide under a UV light source.

**Caspase-3 Activity Assay**
Activities of caspase-3 were detected with a commercially available caspase-3 activity kit (Beyotime Institute of Biotechnology), with Ac-DEVD-pNA as the colorimetrically specific substrate. In brief, right cortical samples (n=4 for each group) were weighed and homogenized in lysis buffer containing 10 mmol/L HEPES/KOH (pH7.2), 2 mmol/L EDTA, 0.1% CHAPS, 5 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonylfluoride, 10 μg/mL aprotinin, and 20 μg/mL leupeptin. The lysate was centrifuged at 20 000 g for 10 minutes at 4°C, and supernatants were incubated for 1 hour at 37°C with 10 μL caspase-3 substrate (Ac-DEVDpNA, 2 mmol/L). Substrate cleavage was measured with a spectrofluorometer at 405 nm and was corrected as protein content in the lysate. The activity of caspase-3 was expressed as values of enzyme activity compared with control.

**Western Blot Analysis**
Western blot analysis was performed to measure apoptosis-related proteins. Right cortical samples (n=6 for each group) were weighed, and protein was extracted as previously described. In brief, whole-cell lysates were obtained by homogenizing the samples (n=6 for each group) with a homogenizer in 5 volumes 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 750g for 15 minutes at 4°C to separate the sample into supernatant A and pellet A. Supernatant A, containing the cytosolic/mitochondrial protein, was further centrifuged at 16 000g for 30 minutes 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. The protein samples were separated on 10% or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (20 to 50 μg/lane) and then transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% nonfat dry milk in TBST buffer and then incubated with primary antibodies overnight at 4°C. The primary antibodies and their dilutions were as follows: Bcl-2, cytochrome c, and Bax (Santa Cruz Inc, 1:200); caspase-3, caspase-9, poly(ADP-ribose) polymerase (PARP), and HSP70 (Santa Cruz Inc, 1:1000); and β-actin and OX IV (Abcam Inc, 1:5000). After being extensively rinsed with TBST buffer, the membranes were incubated with secondary antibodies (Santa Cruz Inc, 1:2000) for 1 hour at room
temperature and then developed with an enhanced chemiluminescence system (ECL kit, Pierce Biotechnology Inc).

RNA Isolation and RT-PCR
Total RNA (2 μg) from right cortical samples (n=4 for each group) was isolated by TRizol reagent (Invitrogen), and RT was performed with the Revert Aid H Minus M-mlV RT kit (Fermentas Life Sciences) according to the manufacturer’s instructions. PCR was performed with primers for HSP70 (forward, GGGTTTGGTTACTTTGGTTA; reverse, CCCATAAGTTGGGAAACAGT; 317 bp, GenBank accession No. NM_212504) and β-actin (forward, TCATGAAGTGTGACGTTGACATCCGT; reverse, CCTAGAAGCATTTGCGGTGCACGATG; 285 bp, GenBank accession No. NM031144). β-Actin was used as an internal standard for stable expression (housekeeping gene) in all experiments. PCR was performed with use of a Gene Cycler (Bio-Rad). Initial denaturation was done at 94°C for 5 minutes followed by 35 cycles of amplification. The amplification protocol was repeated cycles of denaturation (30 seconds, 94°C), annealing (30 seconds, 56°C), extension (1 minute, 72°C), and a final extension (7 minutes, 72°C). PCR products were electrophoresed through 2% agarose gels containing ethidium bromide (0.5 μg/mL). Gels were visualized under UV light and photographed, and optical densities of the bands were analyzed with Quantity One software (Bio-Rad).

Statistical Analyses
All data are expressed as mean±SEM. The Kolmogorov-Smirnov test was applied to test for a normal distribution. The means of the different groups were compared by a 1-way ANOVA Student-Newman-Keuls test. Significant differences were accepted when probability values were <0.05.

Results
Physiologic Data
We found no significant differences in physiologic parameters in the experimental groups (see supplemental Figure I and Table I). There was no significant change in rCBF.
between I/R and Postcond groups after occlusion (see supplemental Figure I and Table I).

### Postcond Attenuated Infarction Volumes and Neurologic Deficits
Postcond treatment significantly decreased infarct volumes 24 and 72 hours after reperfusion (Figures 2A and 2B). There was a significant improvement in neurologic score at both 24 and 72 hours after reperfusion with Postcond treatment (Figure 2C). Sham-operated rats did not have any deficits.

### Postcond Attenuated Oxidative Stress
The MDA level in the cortex, which is an index of lipid peroxidation, was significantly higher in the I/R group compared with the sham-operated group. There was significant reduction in MDA level in the Postcond group compared with the I/R group (Figure 3A). SOD activity in the cortex was decreased strongly in the I/R group compared with the sham-operated group, which was significantly restored by Postcond treatment (Figure 3B).

### Influence of Postcond on Apoptosis and Apoptosis-Related Molecules
A typical DNA laddering pattern was observed in the I/R group. Postcond treatment decreased cerebral I/R-induced DNA fragmentation (Figure 3D). In the TUNEL assay, a large number of TUNEL-positive cells was observed in the right cortex of rats subjected to I/R injury, whereas TUNEL-positive cells were not detected in the right cortex of sham-operated rats. The number of TUNEL-positive cells was significantly reduced in the right cortex in the Postcond group compared with the I/R group (Figure 3E).

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**Figure 4.** Cytochrome c, Bax, and Bcl-2 expression 24 hours after reperfusion. A, Representative blots showing the effects of Postcond treatment on cytochrome c and Bax expression in the cytoplasm. B, Representative blots showing the effects of Postcond treatment on cytochrome c, Bax, and Bcl-2 expressions in the mitochondria. C, Relative band densities of cytosolic cytochrome c to the mean value of control. D, Relative band densities of cytosolic Bax to the mean value of control. E, Relative band densities of mitochondrial Bcl-2 to the mean value of control. F, Relative band densities of mitochondrial cytochrome c to the mean value of control. Bars represent mean±SEM (n=6). *P<0.05 vs sham, #P<0.05 vs I/R.
Caspase-3 activity in the cortex was noticeably increased in the I/R group compared with the sham-operated group. In the Postcond group, there was a significant decrease in caspase-3 activity compared with that in the I/R group (Figure 3C). Western blot analysis showed a significant reduction of procaspase-9 and procaspase-3 in the I/R group compared with the sham group. In the Postcond group, the levels of procaspase-9 and procaspase-3 were significantly restored compared with those in the I/R group (Figure 4).

PARP is known to be a substrate of activated caspase. In our experiment, a significant amount of active 85-kDa PARP was detected in the I/R group, whereas the sham group showed 116-kDa intact PARP. In the Postcond group, the amount of active 85-kDa PARP was attenuated (data not shown).

Expression of cytochrome c in the mitochondria and cytosol was significantly decreased and increased, respectively, in the I/R group compared with the sham group. This cytochrome c release was significantly attenuated in the Postcond group compared with the I/R group (Figure 5). On the contrary, levels of Bax in the cytosol and mitochondria were decreased and increased in the I/R group, respectively, compared with the sham group. Administration of Postcond treatment reduced the I/R-induced rise in mitochondrial Bax levels and restored cytosolic Bax levels (Figure 5).
sion of mitochondrial Bcl-2 was reduced in the I/R group compared with the sham group. However, Bcl-2 was significantly increased in the Postcond group compared with I/R group (Figure 5).

The mRNA and protein levels of HSP70 expression in the I/R group were greater than those in sham-operated rats. Moreover, this increase was much more prominent in the Postcond group (Figure 4). We localized cytochrome c and HSP70 by immunohistochemical techniques in the MCA territory of the ischemic cortex (Figure 6). Staining revealed that cytochrome c and HSP70 were expressed in neurons. Cytochrome c protein expression was reduced and HSP70 protein expression was increased significantly in the Postcond group compared with the I/R group.

Discussion

The present study showed that Postcond decreased infarct volumes and improved neurologic outcomes at different time points after focal cerebral I/R injury. Our findings support the speculation that Postcond provides neuronal protection by inhibiting apoptosis.

The early moments of reperfusion are important in the pathogenesis of postischemic injury. A previous study has demonstrated that ischemic postconditioning reduces cerebral I/R injury.21 The present study further supports that opinion. However, whether deleterious mechanisms were attenuated or whether beneficial mechanisms were triggered by Postcond is still unclear. In addition, the earlier study was performed after partial reperfusion in a model of permanent focal ischemia. Whether such a protective phenomenon is applicable to other I/R models needs to be elucidated. In the present study, we tested our hypothesis in a rat model of MCAO. Because cerebral cortex damage is the main event of brain ischemia in MCAO, the neurons in the cortex should be a target for this protection.

It is well known that generation of excessive ROS during reperfusion plays a major role in brain injury associated with stroke. Because of the brain’s low activities of antioxidative enzymes, it is very vulnerable to ROS induced by I/R injury, which causes oxidative damage to brain lipids, proteins, and DNA, leading to brain dysfunction and cell death.1 In our study, Postcond treatment decreased the level of MDA product and increased SOD activity, suggesting there was attenuated lipid peroxidation and reduced generation of superoxide anions in cerebral I/R. Our results are compatible with those of a previous report, which showed that Postcond inhibited oxidant generation and oxidant-mediated injury in myocardial I/R injury.17

Recent studies have demonstrated that Postcond exerts an ant apoptotic effect on the heart both in vivo and in vitro.18,19 Our study showed that Postcond significantly inhibited apoptosis of cortical neurons caused by I/R injury, which was proved by DNA fragmentation and activated caspase-3. To further clarify the mechanism of Postcond protection, we investigated the expressions of key apoptosis-related molecules. Our study showed that Postcond increased the level of antiapoptotic Bcl-2 protein in the mitochondria, inhibited Bax translocation to the mitochondria, and inhibited cytochrome c release from the mitochondria to the cytosol. Therefore, the aforementioned mechanism led to a decrease in the activation of caspase and PARP. It is known that cytochrome c is released from the mitochondria to the cytosol and plays a key role in the initiation of apoptosis through the activation of the caspase cascade.7 The 116-kDa nuclear protein substrate PARP is cleaved into the 85-kDa apoptotic fragment by activated caspase-3, which proceeds to the final apoptotic death.31 By regulating the Bcl-2 (antiapoptosis)/Bax (proapoptosis) balance, the Bcl-2 family maintains mitochondrial stabilization.32 Our study suggests that the mitochondrial pathway was an important target for Postcond.

Previous work has shown that overexpression of HSP70 is associated with a reduction of cytochrome c release from the mitochondria.33 Our study also revealed that Postcond increased the level of HSP70 in the cortex during cerebral I/R. Whether other endogenous protective molecules besides HSP70 are involved in the protection afforded by Postcond in cerebral ischemia requires further study.

In our study, we tested only 6 cycles of 30 seconds of reperfusion followed by 30 seconds of ischemia. An interval of 30 seconds was referred to in the study by Zhao et al.21 Whether Postcond played its role in an “on-off” or a “dose-dependent” manner was not fully elucidated in this study, and 30 seconds may not afford the maximal protective effect against cerebral I/R injury if Postcond acts in a “dose-
dependent” style. Thus, the exact number of optimal intervals and cycles may also need additional investigation.

In conclusion, our study demonstrated that Postcond attenuated apoptosis in focal cerebral I/R injury. This neuroprotective effect was associated with inhibiting apoptosis molecules of the mitochondrial pathway and activating endogenous protective molecules.

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Disclosures
None.

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