Ischemic Postconditioning Protects Against Global Cerebral Ischemia/Reperfusion-Induced Injury in Rats

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Background and Purpose—Ischemic postconditioning has been found to decrease brain infarct area and spinal cord ischemic injury. In this study, we tested the hypothesis that ischemic postconditioning reduces global cerebral ischemia/reperfusion-induced structural and functional injury in rats.

Methods—Ten-minute global ischemia was induced by 4-vessel occlusion in male Sprague-Dawley rats. The animals underwent postconditioning consisting of 3 cycles of 15-second/15-second (Post-15/15), 30-second/30-second (Post-30/30), or 60-second/15-second (Post-60/15) reperfusion/reocclusion or 15-second/15-second reperfusion/reocclusion applied after a 45-second reperfusion (Post-45-15/15).

Results—Ten minutes of ischemia and 7 days of reperfusion destroyed 85.8% of CA1 hippocampal neurons and 64.1% of parietal cortical neurons. Three cycles of Post-15/15, Post-30/30, and Post-45-15/15 reperfusion/reocclusion markedly reduced neuronal loss after 7 days or 3 weeks of reperfusion and diminished the deficiency in spatial learning and memory. After reperfusion, a period of hyperperfusion followed by hypoperfusion was observed, both of which were blocked by postconditioning. The cytosolic level of cytochrome c increased significantly after 48 hours of reperfusion, and this was inhibited by Post-15/15, Post-30/30, and Post-45-15/15. However, 3 cycles of 60-second/15-second reperfusion/reocclusion failed to protect against neuronal damage, behavioral deficit, or cytochrome c translocation.

Conclusions—Our data provide the first evidence that an appropriate ischemic postconditioning strategy has neuroprotective effects against global cerebral ischemia/reperfusion injury and a consequent behavioral deficit and that these protective effects are associated with its ability to improve disturbed cerebral blood flow and prevent cytochrome c translocation. (Stroke. 2008;39:983-990.)

Key Words: brain ischemia ■ ischemic postconditioning ■ rats ■ reperfusion injury

Extensive research has been aimed at finding effective strategies and drugs to ameliorate or prevent brain ischemia and reperfusion (I/R) injury. However, few have been successfully applied in clinical practice, although several strategies and drugs have been shown to decrease ischemic damage in the brain in animal models. Ischemic preconditioning has been shown to provide powerful protection against I/R injury in both the heart and nervous system. However, its clinical application is only possible for cases in which the occurrence of stroke is predictable and controllable. Rapid initiation of reperfusion is the most effective treatment to reduce infarct area and the behavioral deficits caused by ischemia. However, reperfusion also has the potential to introduce additional injury; many studies have shown that overproduction of reactive oxygen species and overloading of calcium occur in the early reperfusion period and lead to reperfusion injury. Another endogenous protective strategy, termed “ischemic postconditioning,” has been recently reported, in which several repeated cycles of brief reperfusion and reocclusion of the coronary artery were applied at the onset of full reperfusion. Because ischemia cannot be predicted and happens suddenly, ischemic postconditioning, which can be applied after ischemia, is attracting considerable attention. Recently, it was reported that ischemic postconditioning is neuroprotective, can reduce infarct size after focal brain ischemia, and can reduce neuronal injury after spinal cord ischemia. In clinical emergencies, many accidents lead to global ischemia, such as drowning, cardiac arrest, and marked hypotension during cardiopulmonary surgery, and the brain is intrinsically more vulnerable to ischemia than other organs. Whether postconditioning has neuroprotective effects against global cerebral I/R injury is unknown.

In this study, we tested the hypothesis that ischemic postconditioning (interrupting reperfusion) protects against neuronal loss and behavioral deficits after global cerebral I/R.
Furthermore, to explore the mechanisms underlying the neurologic deficits and neuronal damage, we also determined whether the disturbance of cerebral blood flow (CBF) and the translocation of cytochrome c from the mitochondria to the cytosol, a known trigger for neuronal death, are involved.

## Materials and Methods

### Animals and Surgical Procedures

Adult male Sprague-Dawley rats weighing 260 to 320 g, from Zhejiang University Animal Centre, were used. All procedures used in this study were approved by the ethics committee for the use of experimental animals at Zhejiang University. Transient cerebral ischemia was induced by the 4-vessel occlusion technique.\(^\text{10}\) Anesthesia was induced with 4% choral hydrate (350 mg/kg IP), and then the common carotid arteries were freed from surrounding tissues and the vertebral arteries were permanently electrocauterized. After the surgical incisions were closed, the rats were allowed to recover for 24 hours, and food was withheld for 8 hours before ischemia. On the following day, both common arteries were occluded with aneurysm clips to induce cerebral ischemia, and then the clips were removed for reperfusion. Rectal temperature was maintained at 36.5°C to 37.5°C throughout the procedures. Rats that had lost their righting reflex, had dilated pupils, and did not have seizures were selected for experiments.

### Postconditioning Protocols

Rats were assigned to 6 groups (see Figure 1). All groups were subjected to 10 minutes of global cerebral ischemia except the sham group. In the sham group, rats were subjected to the same procedures except for occlusion of the common carotid arteries. Control rats were subjected to 10 minutes of ischemia only, without any further interruption of reperfusion. Post-15/15 rats were subjected to 3 cycles of 15-second/15-second reperfusion/reocclusion after 10 minutes of ischemia. Post-30/30 animals were subjected to 3 cycles of 30-second/30-second reperfusion/reocclusion. Post-60/15 animals were subjected to 3 cycles of 60-second/15-second reperfusion/reocclusion. Post-45-15/15 animals were subjected to 3 cycles of 15-second/15-second reperfusion/reocclusion applied after 45-second reperfusion.

### Neuron Counts

For neuron counting, brains were removed after 7 days of reperfusion (Figure 1). Rats under anesthesia were perfused intracardially with normal saline, followed by 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer at 4°C (pH 7.4). Brains were removed quickly and further fixed overnight in 4% paraformaldehyde at 4°C. Postfixed brains were embedded in paraffin, and 5-μm coronal sections at the level of the bregma were cut on a microtome. The sections were stained with hematoxylin/eosin and examined under a light microscope. The numbers of surviving neurons in the hippocampal CA1 layer per 1-mm² area in the paretal cortex adjacent to both the hippocampus and striatum per 1 mm² were counted as neuron density.\(^\text{11}\)

### Behavioral Assessment

Seven days after ischemia, spatial learning and memory were tested in an open field water maze, a black, circular pool (diameter 150 cm, height 50 cm), surrounded by various extramaze visual cues.\(^\text{13}\) The maze was filled to a depth of 30 cm with water at 25±1°C. Chinese ink was added to make the water opaque. Six days after ischemia, the rats received a habituation trial (1 minute) in which there was no platform present. During a training trial, a rat was able to escape from the water only by locating and climbing onto a platform invisible to it (diameter 10 cm, 1.5 cm below the water surface). For all rats, the location of the hidden platform remained unchanged during the whole experiment. A trial was terminated as soon as the rat found the platform; if the rat did not succeed within 120 seconds, it was guided onto the platform with a stick. The rat was allowed to stay on the platform for 20 seconds before being removed. Training consisted of 2 daily sessions of 4 consecutive trials for 5 consecutive days. A minimum of 4 hours was allowed between sessions.\(^\text{13}\) The rats were released from the perimeter of the pool at north, west, south, or east in a predetermined pseudorandom order. Immediately after the final training trial, each rat was subjected to a probe trial (60 seconds) in which no platform was present. The time spent in the quadrant that formerly contained the platform was recorded as a measure of spatial bias. All trials were recorded on videotape for analysis with a computer-assisted image analyzer (Zhenghua Biologic Apparatus Facilities Co Ltd, Huaibei, China). Escape latency and swim speed in the training trials and time spent in the former platform quadrant in the probe trial were quantified. Each rat had 8 training trials per day, and the mean value from these trials was taken as the escape latency for this rat. Data from 8 rats in the same group were averaged to give a mean escape latency for that day. Brains of rats in the behavioral study were removed after 3 weeks of reperfusion and stained with hematoxylin/eosin by the same methods described earlier, and the surviving neurons were counted.

### Figure 1

Experimental groups and protocols for neuron counts and cytochrome c immunohistochemistry studies. Post-15/15 indicates 3 cycles of 15-second/15-second reperfusion/reocclusion; Post-30/30, 3 cycles of 30-second/30-second reperfusion/reocclusion; Post-60/15, 3 cycles of 60-second/15-second reperfusion/reocclusion; and Post-45-15/15, 3 cycles of 15-second/15-second reperfusion/reocclusion applied after 45-second reperfusion. Reperfusion lasted for 48 hours, 7 days, or 3 weeks.
CBF Measurement

Another 15 rats (3 rats per group, except the sham group) were used for CBF measurement with a laser Doppler flowmeter (PeriFlux System 5000, Perimed, Sweden). After the vertebral arteries were electrocauterized, the scalp was opened with a scalpel. The probe holder was attached to the right skull (from bregma: anterioposterior [AP] - 3.8 mm, mediolateral [ML] 2.0 mm) and protected with a plug. For CBF measurement, the probe was inserted to the probe holder. CBF was measured 2 minutes before both common arteries were occluded as the baseline value, at the time of ischemia, and at 10 seconds and 5, 10, 15, 20, 25, 30, 60, and 120 minutes after reperfusion. CBF values were expressed as percentages relative to baseline (100%).

Cytochrome C Western Blot

Animal brains (3 per group) subjected to ischemia or sham surgery were harvested 48 hours after ischemia. The brain was quickly removed, and the hippocampal CA1 region and parietal cortex were rapidly isolated. For mitochondrial and cytosolic subcellular fractionation, samples were prepared as described previously by a multiple centrifugation method.14,15 Protein concentration was determined by the Bradford method. Both cytosolic and mitochondrial fractions (50 μg protein/lane) were subjected to 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes for 1 hour. Membranes were incubated with rabbit anti-rat polyclonal cytochrome c antibody (3
μg/mL, BioVision) overnight, followed by horseradish peroxidase-labeled anti-rabbit IgG antibody (1:3000, BioVision) for 1 hour. Specific bands were detected by the enhanced chemiluminescence method. Films were scanned and the digitized images were analyzed quantitatively by a Kodak Digital Science EDAS 120 system and BandScanner 1D system (Eastman Kodak Co, Rochester, NY).

**Statistical Analysis**

All values were expressed as mean±SEM. Statistical significance was determined by 1-way ANOVA with a Newman-Keuls post test for neuron counting, Western blot analysis, and probe test for the behavioral study and by 2-way ANOVA with the Bonferroni post test for escape latency in the behavioral study. Values of $P<0.05$ were considered significant.

**Results**

**Global Ischemia–Induced Neuronal Death and Neuroprotective Effect of Ischemic Postconditioning**

All of the data for neuron counts and immunohistochemistry were collected from 2 specific regions in the CA1 layer of the hippocampus and in the parietal cortex. The surviving neurons in CA1 and in the cortex after 10 minutes of ischemia and different reperfusion times were counted in hematoxylin/eosin-stained sections. Normal neurons had round and pale stained nuclei, whereas dying neurons in the ischemic areas showed pyknotic nuclei (Figure 2A). After 3 weeks of reperfusion, the CA1 pyramidal cell layer contained a few neurons, the pyknotic nuclei had disappeared, and nonneural tissue (presumably neuroglia) occupied most of the layer (Figure 2C).

Ten minutes of ischemia and 7 days of reperfusion destroyed $\approx 85.8\%$ of CA1 neurons and $64.1\%$ of parietal cortical neurons. Three cycles of 15-second/15-second and 30-second/30-second reperfusion/reocclusion or 15-second/15-second reperfusion/reocclusion applied after 45-second reperfusion significantly decreased cell death in CA1 pyramidal and cortical neurons, whereas 3 cycles of 60-second/15-second reperfusion/reocclusion failed to provide neuroprotection (Figure 2B). For cortical neurons, there were no significant differences among the 3 postconditioning groups (Post-15/15, Post-30/30, and Post-45-15/15), whereas for the hippocampal CA1 neurons, the Post-45-15/15 regimen had the best neuroprotective effect in all postconditioning groups. Rats with 10 minutes of ischemia and 15-second/15-second or 30-second/30-second reperfusion/reocclusion or 15-second/15-second reperfusion/reocclusion applied after 45-second reperfusion followed by 3 weeks of reperfusion also showed reduced neuron death (Figures 2C and 2D), so the protective effect of postconditioning lasted for at least 3 weeks.

**Ischemic Postconditioning Improved Behavioral Deficits**

Behavioral deficits are the major sequelae in patients after stroke, especially impairment of learning and memory after
global ischemia. To investigate whether the postconditioning strategies that reduced neuron death also led to functional improvement, we tested the rats’ learning and memory in the Morris water maze. All animals were able to swim normally and locate the hidden platform during the training trials. As expected, rats in the control and Post-60/15 groups required more time to find the platform than those in the sham group and the rest of the ischemic postconditioning groups (Post-15/15, Post-30/30, and Post-45-15/15; Figure 3A). Analysis of escape latency revealed significant differences between groups ($P<0.01$), but there were no significant differences in the swimming speed among these groups (data not shown). In the probe test, rats in the control and Post-60/15 groups spent significantly less time than the other groups in the quadrant where the platform had been, and there was no significant difference between the sham group and the postconditioning groups (Post-15/15, Post-30/30, and Post-45-15/15; Figures 3B and 3C). These data indicate that 10 minutes of ischemia and 7 days of reperfusion impaired learning and memory, but ischemic postconditioning, which rescued neurons in hippocampal CA1 and parietal cortex areas from degeneration, reduced the I/R-induced learning and memory deficit.

**Ischemic Postconditioning Improved the Disturbance in CBF After Ischemia**

During ischemia, CBF declined to an equivalent extent ($10.2\pm3.6\%$) in all groups. After reperfusion, a period of hyperperfusion followed by hypoperfusion was observed in all animals (Figure 4). After the clips on the common carotid arteries were removed, CBF acutely increased to $181.2\pm10.4\%$ in all groups. In the control group, hyperperfusion lasted $\sim30$ minutes and was then followed by several hours of hypoperfusion. The hyperperfusion time was shortened to 20 minutes, the hyperperfusion value was decreased, and the hypoperfusion value was increased by 3 cycles of reperfusion/reocclusion (except in the Post-60/15 group). These data suggest that ischemic postconditioning significantly improved the disturbance in CBF after ischemia.

**Ischemic Postconditioning Inhibited the Translocation of Cytochrome C**

In all mitochondrial fractions, cytochrome $c$ immunoreactivity was evident as a single band with a molecular mass of 12 kDa in both the cortex and hippocampal CA1 area (Figure 5A). In cytosolic fractions, the cytochrome $c$ immunoreactive band was detected after ischemia in the control and Post-60/15 group, and a weak band was detected in the Post-30/30 group, especially in CA1. However, in the sham, Post-15/15, and Post-45-15/15 groups, significant cytochrome $c$ immunoreactive bands were barely detected in the cytosolic fractions in both the cortex and CA1. On the contrary, a consistent amount of $\beta$-actin immunoreactivity was seen in all groups, suggesting that the amount of loaded protein was consistent. Statistical analysis showed that the amount of cytochrome $c$ translocation induced by I/R was significantly inhibited by the ischemic postconditioning strategies of Post-15/15, Post-30/30, and Post-45-15/15, whereas the Post-60/15 strategy failed (Figure 5B).

**Discussion**

We have demonstrated for the first time that ischemic postconditioning with repetitive cycles of briefly interrupted reperfusion in the rat (1) reduces neuronal cell death and a learning and memory deficit induced by transient global cerebral I/R and (2) the underlying protective mechanisms of postconditioning against global ischemic injury may involve reducing neuronal death by improving the disturbance in CBF
and decreasing the proapoptotic release of cytochrome c from mitochondria into the cytosol.

Many clinical events lead to global ischemia, and the brain, especially the hippocampal layer CA1 and cerebral cortex, is intrinsically vulnerable. We found that ischemic postconditioning reduced I/R-induced neuronal death in both hippocampal CA1 and parietal cortex, and this neuroprotective effect continued until at least 3 weeks of reperfusion, suggesting that postconditioning actually prevented, rather than just delayed, neuronal death after global ischemia.

In the present study, 3 cycles of 15-second/15-second reperfusion/reocclusion applied immediately after reperfusion or after 45-second reperfusion both had neuroprotective effects, whereas the protective effect was lost when the 15-second reocclusion was applied after 60 seconds of reperfusion every cycle, demonstrating that the timing of application of reocclusion is critical. It has been reported that ischemic postconditioning with 1 minute of reocclusion after 1 minute of reperfusion protects against spinal cord ischemic damage in rabbits, a larger species with a different neural structure than that of rats. In studies in the heart, it appears that smaller body size (and hence, heart size) requires shorter cycles, whereas models with larger body sizes (and lower myocardial metabolic rates) require cycles of longer duration. Therefore, we speculate that different animals need different strategies of reperfusion/reocclusion.

After myocardial infarction and cancer, stroke is the third leading cause of morbidity and mortality in the developed world. Patients who recover from ischemia have neuropsychologic deficits such as anterograde amnesia or aphasia, as well as motor and sensory losses. Global cerebral ischemia, which leads to the most extensive neuronal damage in the CA1 layer of the hippocampus, results in a deficit in spatial learning and memory. The water maze, introduced by Morris in 1984, reveals an impairment in spatial learning and memory that is easily quantified. Thus, it is useful for investigating whether neuroprotective strategies that reduce neuronal damage also lead to functional improvements. It has been shown that 10 minutes of ischemia impairs rat learning and memory. Some neuroprotective strategies that decrease neuronal death also attenuate the learning and memory deficit. In this study, we also found that ischemic postconditioning, which decreased neuronal cell death, also reduced escape latency and increased the time spent during the probe test in the quadrant where the platform had been. Therefore, postconditioning improved learning and memory in rats subjected to global cerebral ischemia.

Previous studies have demonstrated that after focal or global ischemia, there is a period of hyperperfusion followed by a period of hypoperfusion. Both hyperperfusion and hypoperfusion do harm to recovery of the ischemic brain; in fact, there is a correlation between neuron count and CBF. In the early period of reperfusion, the resupply of O$_2$ is transformed to free radicals because the injured respiratory chain cannot fully use O$_2$. In a study of local brain ischemia, it was found that ischemic postconditioning decreased hyperemia and the production of superoxide radicals. In this study, we also found that postconditioning decreased hyperperfusion, perhaps helping to decrease the overproduction of free radicals. The overproduction of free radicals after ischemia and reperfusion is a key factor to brain I/R injury.

Neuron death after global ischemia occurs in a delayed manner. Neuron loss increases with reperfusion time, and terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling–positive neurons can be seen only 1 day after reperfusion. Some studies have demonstrated that this neuron death is, at least in part, caused by apoptosis. Release of cytochrome c from the mitochondria to the cytosol to activate caspases is a critical step for inducing apoptosis after cerebral ischemia, especially global ischemia.

Western blot analyses have shown that cytosolic cytochrome c from mitochondria.
c appears after the CA1 pyramidal cell layer as early as 2 hours after reperfusion and peaks between 12 and 48 hours.\(^3\)\(^0\) Another study also demonstrated that cytosolic cytochrome c levels increase in the cortex after 1 and 3 days after 10 minutes of ischemia.\(^3\)\(^1\) Some treatments that are neuroprotective also inhibit the release of cytochrome c, such as hypothermia and administration of an inhibitor of the mitochondrial permeability transition pore, bongkrekic acid.\(^3\)\(^0\),\(^3\)\(^1\) In our study, we found that the release of cytochrome c from the mitochondria to the cytosol was induced by 48 hours of reperfusion after 10 minutes of ischemia, whereas appropriate postconditioning markedly attenuated its release. Previous studies have demonstrated that ischemic postconditioning reduces the overproduction of reactive oxygen species and calcium overload, both of which contribute to mitochondrial dysfunction and cytochrome c release.\(^7\)\(^\)–\(^3\)\(^2\)\(^–\)\(^3\)\(^5\) It has been reported that neuron death in the penumbra after focal ischemia is mainly induced via apoptosis, and ischemic postconditioning decreases terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling–positive neurons in the penumbra.\(^7\) Therefore, we speculate that ischemic postconditioning inhibits the release of cytochrome c and the activation of caspases and then blocks I/R-induced neuronal apoptosis.

There are several limitations to the present study. First, our experimental design was based on only 10 minutes of ischemia and showed a subsequent protective effect of postconditioning. It is not known whether postconditioning can also be neuroprotective when animals are subjected to more prolonged ischemia. Second, the neuroprotective effect was demonstrated in a rat model of ischemia. Whether this can be transferred to other animal models or even humans (and what reocclusion/reperfusion strategy may be effective in humans) needs further study. Although these are limitations, ischemic postconditioning appears to be a simple and promising strategy to reduce or even prevent global cerebral I/R injury and has potential for future clinical application, because it induces endogenous protective mechanisms that may be components of a general protective strategy.

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Disclosures

None.

References


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