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Ischemic Preconditioning Is Capable of Inducing Mitochondrial Tolerance in the Rat Brain

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Background: **Preconditioning to ischemia is a phenomenon whereby a brief episode of sublethal ischemia and other nonlethal stressors produce protection against a subsequent detrimental ischemic insult. As mitochondrial dysfunction is related to necrotic and apoptotic neuronal death after cerebral ischemia, the authors examined if ischemic preconditioning is capable of inducing mitochondrial tolerance.**

Methods: **Forebrain ischemia was induced by bilateral common carotid artery occlusion with simultaneous hypotension for 8 min in Wistar rats (275–300 g). A 3-min ischemic episode performed 48 h before the 8-min ischemia was used for preconditioning. The extents of hippocampal CA1 neuronal damage were evaluated 7 days after reperfusion by neuro-specific nuclear protein immunostaining. Brain mitochondria were isolated 48 h after animals were subjected to the sham operation or the 3-min conditioning ischemia. Loss of cytochrome c from mitochondria after cerebral ischemia *in vivo* and after exposure of brain mitochondria to calcium *in vitro* was used as an indication of mitochondrial dysfunction.**

Results: **Results showed that ischemic preconditioning induced by a 3-min ischemic episode dramatically reduced the loss of hippocampal CA1 neurons resulting from a subsequent 8-min ischemia 7 days after reperfusion, and this protection was associated with a preservation of mitochondrial cytochrome c as examined after early reperfusion. Exposure of isolated brain mitochondria to calcium produced a dose-dependent increase in cytochrome c release either at 30°C or at 37°C. Compared with those animals receiving only sham operation, cytochrome c release caused by 100 μM calcium was significantly reduced in conditioned animals.**

Conclusion: **Regarding the importance of mitochondrial dysfunction in mediating ischemic neuronal death, the above results indicate that mitochondria may serve as end-effecting organelles to ischemic preconditioning.**

ISCHEMIC preconditioning is a phenomenon whereby a brief episode of sublethal ischemia and other nonlethal stressors produce protection against a subsequent detrimental ischemic insult; this phenomenon was first described in the heart[1] and in the brain also.[2] Undoubtedly, an understanding of the mechanisms of ischemic tolerance would not only contribute to the development of new strategies for preventing and treating stroke but also would provide insight into what factors mediate ischemic neuronal damage.

To date, the mechanism by which ischemic tolerance is induced remains unclear. Mitochondrial dysfunction is known to occur after cerebral ischemia, and accumulating evidence indicates that mitochondrial dysfunction plays an important role in mediating ischemic neuronal death *via* either necrosis or apoptosis.[3,4] The release of cytochrome c from mitochondria after reperfusion has received particular attention for the following reasons. Cytochrome c is a small and water-soluble molecule, implying that cytochrome c is easier to be released from mitochondria than other mitochondrial proteins. Studies have shown that an early and persistent loss of cytochrome c selectively occurred in ischemic-vulnerable neurons after cerebral ischemia.[4–6] In addition, certain changes (*e.g.*, increases in intracellular calcium and alterations in Bcl-2 family protein expression) known to occur during and after cerebral ischemia[7–9] are able to alter cytochrome c release *in vitro*.[10,11] Finally, loss of cytochrome c can cause either necrosis or apoptosis.[12–14] Although the release of cytochrome c from mitochondria in cultured neurons has been shown to be a reversible event in response to apoptotic stimuli,[15] it is not in the case of cerebral ischemia, possibly because of the coexistence of irreversible protein synthesis inhibition.[4]

In the present study, we examined if ischemic preconditioning is able to induce mitochondrial tolerance by using loss of cytochrome c as an indication of mitochondrial dysfunction.

Materials and Methods

This study was approved by the Committee on the Guidelines for Animal Experiments of Niigata University.

Forebrain Ischemia Model

For induction of forebrain ischemia, male Wistar rats weighing 275–300 g (aged 8 or 9 weeks) were fasted but allowed to access water for 8–12 h. After animals were anesthetized with 4% halothane and tracheally intubated, they were mechanically ventilated with 1.5% halothane in 30% O_2/balance N_2O. Two digital thermistor probes (Multi-thermistor meter D321, Technol Seven, Yokohama, Japan) were placed in the rectum and under the left temporalis muscle to monitor core and pericranial temperatures. The left femoral artery and the right external jugular vein were cannulated for continuous monitoring of blood pressure, blood sampling, and blood withdrawing, respectively. Bilateral common carotid arteries were isolated from the carotid sheathes *via* a ventral midline incision. Fifteen minutes before

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occlusion of bilateral common carotid arteries, heparin (50 U) was injected intravenously, and an arterial blood sample (0.3 ml) was then taken for blood gas analysis and plasma glucose assay. Animals were acceptable if blood gas parameters and the levels of plasma glucose met the following criteria: P_{O_2}, 90–140 mmHg; P_{CO_2}, 35–45 mmHg; pH, 7.35–7.45; and glucose, 90–150 mg/dl. After mean artery blood pressure was lowered to 35–40 mmHg by injecting 0.5 mg of phentolamine *via* the vein catheter followed by withdrawing and injecting blood from the external jugular vein catheter *via* a heparinized syringe, both common carotid arteries were occluded with small vascular clips (60 g) for 8 min. The withdrawn blood was held at 37.5°C. Thereafter, the clips were removed, and the withdrawn blood was reinfused. Reperfusion in each artery was verified visually. During occlusion, the core temperature was controlled at 37.2 ± 0.2°C, whereas the pericranial temperature was allowed to decrease gradually from 36.8 ± 0.2°C at the beginning of occlusion to 36.0 ± 0.2°C at the end of occlusion. Before discontinuation of anesthesia, the vascular catheters were removed, and the wounds were sutured. The endotracheal catheter was removed after recovery of spontaneous respiration and righting reflex. The animals were then kept in a warm, humidified chamber (32 or 33°C) for another 3 h before being returned to their cages. For preconditioning, animals were subjected to a 3-min bilateral common carotid artery occlusion with simultaneous hypotension 48 h before being subjected to lethal ischemia.[4] Sham animals received the same operation without common carotid artery occlusion.

Tissue Preparation

At 24 h or 7 days after sham operation (n = 4 for each time point), single 3-min ischemia (n = 4 for each time point), 8-min ischemia plus the sham operation (n = 4 for each time point), or 8-min ischemia plus a 3-min conditioning ischemia (with an interval of 48 h, n = 4 for each time point), animals were perfused with 1× phosphate-buffered solution (PBS) containing heparin (4 U/ml), followed by 0.01 M periodate–0.075 M lysine–2% paraformaldehyde (PLP) in 0.0375 M PBS (pH 6.3). The whole brain was taken out from the skull, and the cerebellum and the underlying structures were removed. The remained tissue block containing the hippocampi was separated through the midline and postfixed in the same fixative for 4 h. The tissue pieces were then washed in gradually increasing concentrations of sucrose in 1× PBS (10% for 4 h, 15% and 20% for 12 h each) and thereafter rapidly frozen in 2-methylbutane chilled at −80°C. Consecutive coronal sections located around 3.5 mm posterior to bregma were prepared on a microtome and used for immunofluorescent stainings.

Immunofluorescent Stainings for Neuronal Nuclei-specific Protein and Cytochrome C

Ischemic neuronal damage was evaluated 7 days after reperfusion by neuro-specific nuclear protein (NeuN) immunostaining. Sections were washed four times with 1× PBS for 5 min each; incubated for 20 min in 1× PBS containing 0.2% gelatin, 1.5% bovine serum albumin (BSA), and 0.3% Triton X-100; blocked with 5% goat serum diluted in 1× PBS containing 0.3% Triton X-100 and 1.5% BSA for 120 min; incubated with a mouse NeuN monoclonal antibody (5 μg/ml) diluted in the blocking solution at 4°C over night; and washed six times (5 min each) with 1× PBS. The sections were then incubated with a Cy3-conjugated goat antimouse IgG (5 μg/ml) diluted in the blocking solution at 4°C for 4 h, washed 6 times with 1× PBS, mounted in 50% glycerol in 1× PBS, and visualized under a Nikon fluorescence microscope (Optiphot-2, Nikon, Tokyo, Japan). Both the mouse anti-NeuN monoclonal antibody and the Cy3-conjugated goat antimouse IgG were purchased from Chemicon International (Temecula, CA). After photographed, the number of NeuN-positive cells in the whole CA1 pyramidal cell layer was counted by an experimenter blind to the experimental groups and expressed as the number of cells per mm length of the CA1 pyramidal cell layer (neuronal density).

The sections obtained 24 h after reperfusion were used for cytochrome c immunofluorescence staining. A mouse monoclonal antibody (6H2.B4, Pharmingen, San Diego, CA) was used against the native form of cytochrome c (mitochondrial cytochrome c) *in situ*. The protocol for cytochrome c immunofluorescence staining was described elsewhere.[4] Fluorescein (FITC)-conjugated rabbit antimouse IgG (Jakson ImmunoResearch Labs, West Grove, PA) was used as secondary antibody. Negative controls included the omission of the primary or the secondary antibody. Sections were examined with laser scanning con-

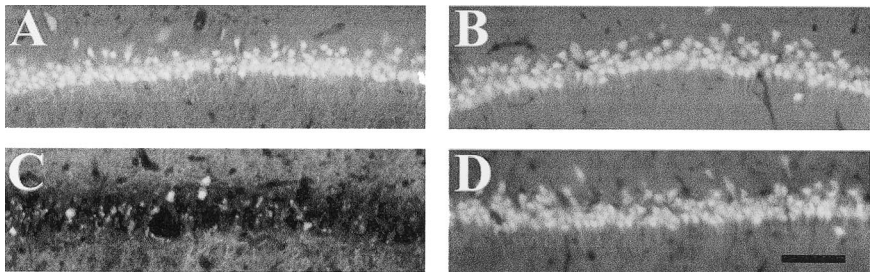

Fig. 1. Ischemic preconditioning reduces hippocampal CA1 neuronal damage in a rat forebrain ischemia model. Neuronal survival was examined 7 days after reperfusion by neuro-nuclear specific protein immunostaining. Bar = 100 μm. (*A*) Sham operation; (*B*) 3-min ischemia; (*C*) 8-min ischemia plus sham operation; (*D*) 8-min ischemia with preconditioning.

Table 1. Neuronal Density Evaluated 7 Days after Reperfusion in Four Groups of Animals

Groups	Number of Animals	Neuronal Density
Sham operation (SO)	4	174.5 ± 2.8
Conditioning ischemia (CI)	4	169.7 ± 7.9
8-min ischemia with SO	4	8.2 ± 0.9*
8 min ischemia with CI	4	129.6 ± 8.7†

Neuronal density represents the number of neuro–specific nuclear protein–positive cells per millimeter length of CA1 pyramidal cell layer. The duration of conditioning ischemia was 3 min. Values are mean ± standard deviation. Data were analyzed by using one-way analysis of variance followed by Tukey *post hoc* test for multiple comparisons.

* $P < 0.001$, compared with either the SO or the CI group. † $P < 0.001$, compared with 8-min ischemia with SO group.

focal microscopy (MRC 1024, Bio-Rad Laboratories, Hercules, CA), using a 60× oil-immersion lens.

Mitochondrial Isolation and Incubation

Brain mitochondria were isolated according to the method of Rosenthal *et al.*[16] At 48 h after the sham operation (n = 8) or the 3-min conditioning ischemia (n = 8), animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg) and then decapitated. The brains were removed and placed in ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, and 1 mg/ml of BSA (pH 7.2, adjusted with KOH). The cerebellum and the underlying structures were removed. The remaining brain tissue from single brain was finely minced and homogenized in a Dounce glass tissue homogenizer in 10 ml of isolation buffer containing 5 mg of bacterial protease Nagarse (*via* six strikes each with a loose-fitting pestle and then a tight-fitting pestle). Homogenate was brought to 30 ml, divided equally into three tubes, and centrifuged at 2,000 *g* for 3 min. Pellets were resuspended to 10 ml and centrifuged at 2,000 *g* for 3 min again. The supernatants were pooled, divided equally into three tubes, and centrifuged at 12,000 *g* for 8 min. The pellets were resuspended in two tubes to 10 ml each with isolation buffer containing 0.02% digitonin and centrifuged at 12,000 *g* for 10 min to further isolate mitochondria from synaptosomes. The brown mitochondrial pellets without the synaptosomal layer were then suspended again in 10 ml isolation buffer without EGTA and centrifuged at 12,000 *g* for 10 min. Finally, the mitochondrial pellets were suspended in 100 μl isolation buffer without EGTA and then combined. Protein concentrations were determined by using the method of Lowry with BSA as a standard.

To see how temperature and different concentrations of calcium affect cytochrome c release from isolated mitochondria, mitochondrial samples (1 mg protein/ml) obtained from three normal animals were incubated in a buffer containing 125 mM KCl, 5 mM HEPES, 3 mM ATP, 4 mM $MgCl_2$, 5 mM succinate, and 5 mM glutamate (pH 7.0, adjusted with KOH) at 30°C for 60 min or at 37°C for 30 min in the absence and presence of calcium. For comparing the amount of cytochrome c release resulting from calcium application, samples obtained from sham-operated and conditioned animals were incubated at 30°C for 60 min with or without 100 μM calcium. After incubation, mitochondria were centrifuged at 12,000 *g* for 6 min. The supernatants (325 μl for each sample) were mixed throughout with 7.5 μl protease inhibitor cocktail (Sigma, St. Louis, MO) and stored at −80°C.

Western Blot Analysis of Cytochrome C

Protein (20 μl) from each sample was boiled in 40 μl sample buffer at 95°C for 4 min and electrophoresed in individual lanes of 15% SDS-PAGE gels as described previously.[4] Samples obtained from the same brain with or without calcium application (paired samples) were loaded adjacently into the same gel. Proteins were then transferred onto a nitrocellulose membrane (Bio-Rad) at 80 V for 75 min. Blots were probed with a mouse monoclonal antibody (7H8.2C12, Pharmingen, San Diego, CA) against

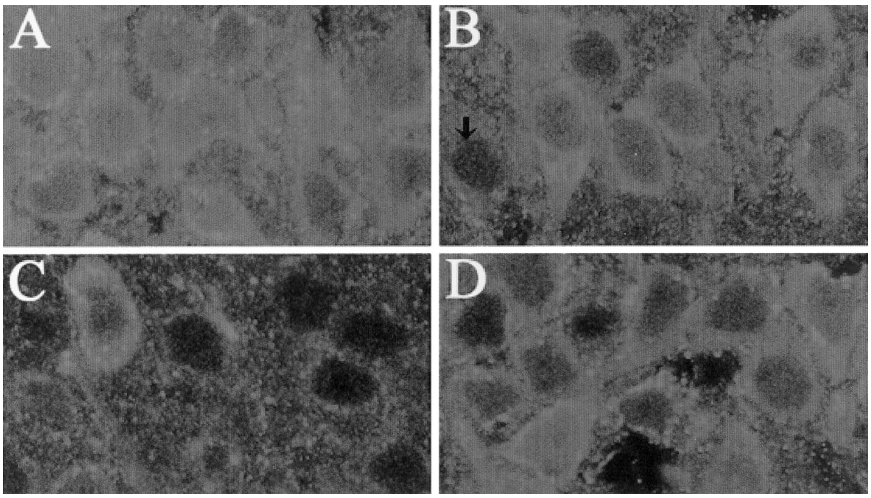

Fig. 2. Confocal images showing ischemic preconditioning attenuates lethal ischemia-induced cytochrome c loss in hippocampal CA1 pyramidal cell layer at 24 h after reperfusion. (*A*) Intensive cytochrome c staining with a punctate appearance in a sham-operated animal; (*B*) Most pyramidal CA1 cells, except the cell indicated by the arrow, show normal appearance of cytochrome c staining after a 3-min ischemia; (*C*) Almost all CA1 pyramidal cells show a lack of cytochrome c staining after an 8-min ischemia; (*D*) With a 3-min ischemia performed 48 h before the 8-min ischemia, mitochondrial cytochrome c is largely preserved.

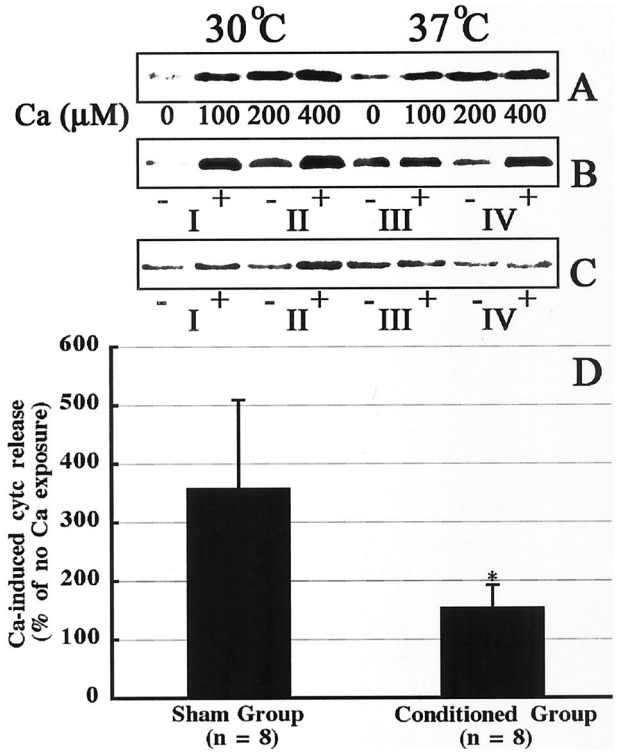

Fig. 3. Western blot analysis of cytochrome c showing conditioning ischemia reduces calcium-induced cytochrome c release from isolated mitochondria. Brain mitochondria (1 mg protein/ml) were incubated in a buffer with conditions indicated below. Calcium was added 2 min after incubation. (*A*) Mitochondria obtained from a normal rat were incubated at 30°C for 60 min and at 37°C for 30 min. (*B*) Mitochondria (representative four samples) isolated 48 h after sham operation were incubated at 30°C for 60 min in the absence (−) and presence of 100 μM of calcium (+). (*C*) Mitochondria (representative four samples) isolated 48 h after a 3-min ischemia were incubated at 30°C for 60 min in the absence (−) and presence of 100 μM of calcium (+). (*D*) A statistical comparison of calcium-induced cytochrome c (cytc) release from brain mitochondria isolated from rats received sham operation (sham group) or conditioning ischemia for 3 min (conditioned group). Cytochrome c release caused by calcium exposure was normalized to the paired one without calcium application in each sample. *$P < 0.01$, compared with the sham group.

the denatured form of cytochrome c at a dilution of 1:2,500 at 4°C for 60 min. After the primary antibody incubation, the membrane was washed and incubated with a horseradish peroxidase-conjugated goat antimouse IgG (1:4,000, American Qualex, San Clemente, CA) for 45 min at room temperature. The immunoreaction was visualized using Amersham enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Cytochrome c release resulting from calcium exposure was normalized to the paired one without calcium exposure by using the National Institutes of Health image program for Windows (v. 1.62, downloaded from http://www.scionimage.com).

Statistical Analysis

Quantitative data are expressed as mean ± SD and analyzed using one-way analysis of variance (ANOVA) or unpaired Student *t* test. After one-way ANOVA, we used Tukey *post hoc* test for multiple comparisons. A *P* value less than 0.05 is considered statistically different.

Results

As shown in figure 1, ischemic preconditioning provided strong protection against ischemic hippocampal CA1 neuronal damage in the rat forebrain ischemia model. In the section obtained from a sham-operated animal, NeuN immunoreactivity was mainly distributed in nuclei (fig. 1A). Although 3-min ischemia resulted in a loss of few hippocampal CA1 neurons (fig. 1B), an 8-min ischemia caused a nearly complete loss of hippocampal CA1 neurons 7 days after reperfusion (fig. 1C). A 3-min ischemia performed 48 h before the 8-min ischemia dramatically reduced the loss of hippocampal CA1 neurons (fig. 1D). Hippocampal CA1 neuronal density in the above-mentioned groups is shown in table 1.

Because loss of mitochondrial cytochrome c began soon after reperfusion and was almost completed 24 h after reperfusion,[4] we examined if ischemic preconditioning can attenuate loss of cytochrome c from mitochondria 24 h after reperfusion by means of confocal laser microscopic image. In the sections prepared from sham-operated animals, CA1 neurons have a bright, punctate cytoplasmic distribution of cytochrome c fluorescence (fig. 2A). Compared with sham-operated animals, mitochondrial cytochrome c was only occasionally changed by the 3-min ischemia (fig. 2B), but it was almost completely depleted by an 8-min ischemia (fig. 2C). With a 3-min ischemia performed 48 h before the 8-min ischemia, mitochondrial cytochrome c was largely preserved (fig. 2D). Regardless of how cytochrome c redistribution can cause neurons to die, the results indicate that mitochondrial dysfunction occurred after reperfusion and was attenuated by ischemic preconditioning.

As abnormal intramitochondrial calcium accumulation occurs after cerebral ischemia and is known to be important for mitochondrial dysfunction,[17] we tested whether ischemic preconditioning attenuated mitochondrial dysfunction independent of cytosolic factors by using isolated brain mitochondria. Exposure of isolated brain mitochondria to calcium resulted in a dose-dependent increase in cytochrome c release at 30°C and 37°C (fig. 3A). Similar to loss of cytochrome c from mitochondria in response to apoptotic stimuli,[18] cytochrome c release from isolated mitochondria in response to calcium application was largely temperature-independent. Cytochrome c release induced by 100 μM calcium was significantly less in the mitochondrial samples prepared from conditioned animals (n = 8) compared with those obtained from sham-operated animals (n = 8), as shown in figure 3B–D.

Discussion

The major finding of the present study is that ischemic tolerance induced by sublethal ischemia is associated with mitochondrial protection as documented *in vivo* and *in vitro*.

Cerebral ischemic tolerance can be achieved not only by sublethal ischemia[19,20] but also by other stressors, including mild mechanical injury,[2] spreading depression[21,22] and pharmacologic inhibition of energy metabolism.[23] Although several mechanisms have been proposed to explain ischemic tolerance,[24] it is not yet resolved whether a common mechanism underlies preconditioning caused by different stresses.

Mitochondrial dysfunction is thought to be relative of ischemic neuronal death.[3,4] Evidence has emerged that ischemic tolerance in the myocardium and neurons induced by transient ischemia and increased intracellular calcium was associated with a preservation of mitochondrial function.[25–28] As the loss of cytochrome c occurs early and persistently after reperfusion and could lead to caspase activation, formation of free radicals, and disturbances of oxidative phosphorylation resulting in either apoptosis or necrosis, depending on intracellular circumstances (*e.g.*, intracellular ATP levels),[4] the loss of cytochrome c from mitochondria was used as an indication of mitochondrial dysfunction *in vivo* after cerebral ischemia and *in vitro* after calcium application. By immunofluorescently examining release of cytochrome c from mitochondria *in situ*, we demonstrated that ischemic tolerance was associated with an attenuation of cytochrome c loss. Regardless of which pathways participate in ischemic neuronal death caused by loss of cytochrome c, an attenuation of cytochrome c loss from mitochondria by ischemic preconditioning indicates that ischemic preconditioning can induce mitochondrial tolerance. The effect of preconditioning may be first on cytosolic factors, which then lead to mitochondrial tolerance that persists until the time at which mitochondria were isolated for testing. For elucidating if ischemic preconditioning is capable of inducing mitochondrial tolerance without ongoing involvement of cytosolic factors, we further examined if brain mitochondria prepared from conditioned animals are more resistant to calcium-induced cytochrome c release. As the levels of intracellular calcium in ischemic-vulnerable cells within 6–8 min of *in vivo* ischemia could increase as high as 300 μM,[8] we chose to use 100 μM calcium to stimulate cytochrome c release. Clearly, the conditioning ischemia was able to reduce calcium-induced cytochrome c release from mitochondria. Because the amounts of cytochrome c in most brain cells, including the hippocampal CA1 neurons, were almost unchanged after a 3-min ischemic episode, the reduction of calcium-induced cytochrome c release from isolated mitochondria by conditioning ischemia might not result from cytochrome c already having been released.

The mechanism by which ischemic preconditioning induces mitochondrial tolerance is not clear. Possible mechanisms are multiple. As shown in cardiac cells, ischemic preconditioning may activate mitochondrial ATP-dependent potassium channels.[29] Recent studies have shown that activation of mitochondrial ATP-dependent potassium channels reduces loss of mitochondrial cytochrome c because of oxidative stress and hypoxia in the myocardium.[27,30] Further studies should be taken to examine if there are certain changes in mitochondrial ionic channel function after the conditioning ischemia. Another possibility is the expression and redistribution of Bcl-2 family proteins. Bcl-2 family proteins are important endogenous factors in regulating cytochrome c release from mitochondria, in which antiapoptotic proteins (*e.g.*, Bcl-2, Bcl-XL, and others) inhibit, whereas proapoptotic proteins (*e.g.*, Bax, Bad, and others) enhance, cytochrome c release. There is evidence that ischemic preconditioning is able to induce antiapoptotic protein expression without changes in proapoptotic protein expression;[31] and, in addition, there is a redistribution of those proteins after ischemia.[32] Beyond the aforementioned pathways, ischemic preconditioning has been shown to enhance the immunoreactivity of manganese superoxide dismutase,[33,34] which may, in turn, play a role in limiting lethal ischemia-induced loss of cytochrome c.[35]

In conclusion, the present study demonstrates that ischemic preconditioning is capable of inducing mitochondrial tolerance, implying that mitochondria are the end-effecting organelles to ischemic preconditioning. Further studies are necessary to elucidate the mechanisms responsible for mitochondrial tolerance and to determine whether similar mitochondrial tolerance also occurs in other preconditioning paradigms.

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