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Limb remote-preconditioning protects against focal ischemia in rats and contradicts the dogma of therapeutic time windows for preconditioning

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Abstract

Remote ischemic preconditioning is an emerging concept for stroke treatment, but its protection against focal stroke has not been established. We tested whether remote preconditioning, performed in the ipsilateral hind limb, protects against focal stroke and explored its protective parameters. Stroke was generated by a permanent occlusion of the left distal middle cerebral artery (MCA) combined with a 30 minute occlusion of the bilateral common carotid arteries (CCA) in male rats. Limb preconditioning was generated by 5 or 15 minute occlusion followed with the same period of reperfusion of the left hind femoral artery, and repeated for 2 or 3 cycles. Infarct was measured 2 days later. The results showed that rapid preconditioning with 3 cycles of 15 minutes performed immediately before stroke reduced infarct size from $47.7 \pm 7.6\%$ of control ischemia to $9.8 \pm 8.6\%$; at 2 cycles of 15 minutes, infarct was reduced to $24.7 \pm 7.3\%$; at 2 cycles of 5 minutes, infarct was not reduced. Delayed preconditioning with 3 cycles of 15 minutes conducted 2 days before stroke also reduced infarct to $23.0 \pm 10.9\%$, but with 2 cycles of 15 minutes it offered no protection. The protective effects at these two therapeutic time windows of remote preconditioning are consistent with those of conventional preconditioning, in which the preconditioning ischemia is induced in the brain itself. Unexpectedly, intermediate preconditioning with 3 cycles of 15 minutes performed 12 hours before stroke also reduced infarct to $24.7 \pm 4.7\%$, which contradicts the current dogma for therapeutic time windows for the conventional preconditioning that has no protection at this time point. In conclusion, remote preconditioning performed in one limb protected against ischemic damage after focal cerebral ischemia.

Keywords

preconditioning; remote preconditioning; limb preconditioning; cerebral ischemia; focal ischemia

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Introduction

Ischemic preconditioning, a short period of sublethal ischemia conducted in the brain (Liu et al., 1992, Gidday, 2006, Perez-Pinzon, 2007) or in the heart (Murry et al., 1986), protects the brain or the heart from a subsequent prolonged ischemia. Such conventional preconditioning has two therapeutic time windows: rapid preconditioning performed about 1 to 3 hours (Murry et al., 1986, Perez-Pinzon et al., 1997), or delayed preconditioning conducted from 24 to 72 hours before the prolonged ischemia (Kitagawa et al., 1990, Kuzuya et al., 1993). However, an intermediate preconditioning conducted between these two time windows, for example at 12 hours, does not protect against ischemia (Kuzuya et al., 1993, Perez-Pinzon, 2004).

The concept of ischemic preconditioning has been extended to remote ischemic preconditioning, i.e. an ischemia performed in one organ protects against a subsequent prolonged ischemia in another distant organ (McClanahan et al., 1993). For example, remote preconditioning performed in limbs (Birnbaum et al., 1997, Oxman et al., 1997), in a kidney (McClanahan et al., 1993) or in mesentery (Liem et al., 2002) protects against a subsequent ischemia in the heart. Remote preconditioning has greater potential for clinical application than conventional preconditioning, since it can be performed in a non-vital organ, avoiding the high risk of inducing ischemia as preconditioning in the vital organ, such as the brain or the heart.

Although remote preconditioning has been studied for more than 14 years in the research field of myocardial ischemia (McClanahan et al., 1993, Przyklenk et al., 1993), it is currently an emerging concept for experimental stroke. Until now, few studies have reported that limb remote preconditioning reduces hippocampal neuronal injury after transient global ischemia or cardiac arrest in rats (Kakimoto et al., 2003, Zhao et al., 2004, Dave et al., 2006, Jin et al., 2006, Steiger and Hanggi, 2007, Zhao et al., 2007b). In these studies, limb preconditioning was induced by 3 cycles of 10 minute occlusion of the bilateral femoral arteries followed with 10 minutes reperfusion (Zhao et al., 2004, Jin et al., 2006), 30 minutes of a single occlusion (Vlasov et al., 2005), or 15 minutes or 30 minutes of a single occlusion (Dave et al., 2006). The global brain ischemia was conducted immediately after limb preconditioning (Zhao et al., 2004, Jin et al., 2006), or with an interval of 15 minutes or 48 hours (Vlasov et al., 2005). However, whether remote preconditioning protects against focal cerebral ischemia is not established. In addition, few have compared protection of remote preconditioning with different parameters. Furthermore, in all previous studies the preconditioning ischemia was induced in both limbs (Kakimoto et al., 2003, Zhao et al., 2004, Dave et al., 2006, Steiger and Hanggi, 2007), and it is not known whether preconditioning in a single limb offers protection. Moreover, few have addressed the therapeutic time windows for remote preconditioning. We hypothesize that remote preconditioning may have different characteristics from conventional preconditioning. To address the above issues, we examined whether remote preconditioning conducted in the ipsilateral hind limb reduces brain injury after focal ischemia in rats.

Experimental Procedures

Focal Cerebral Ischemia

Focal cerebral ischemia was generated in male Sprague–Dawley rats (270 to 330 g) as previously described (Zhao et al., 2006). Experimental protocols were approved by the Stanford University Administrative Panel on Laboratory Animal Care. Anesthesia was induced by 5% isoflurane and maintained with 1% to 2% isoflurane during surgery and early reperfusion. Core body temperatures were monitored with a rectal probe and maintained at 36.2–37.2°C using a heating pad and light during the whole experiment. Focal ischemia was induced by occluding the bilateral CCAs for 30 minutes combined with permanent occlusion of the left distal MCA above the rhinal fissure.

Limb remote preconditioning

Male Sprague–Dawley rats (60 rats) were randomly assigned into A, B, C & D groups (Fig. 1). Group A rats were subjected to control ischemia (n=6). For rats in groups B, C, D, the left femoral artery was separated below the left groin ligament for later induction of femoral artery occlusion. Group B was used to study the protective effect of rapid remote preconditioning. These rats were further assigned to 4 subgroups (n=6/each subgroup): 1) Isoflurane control: Isoflurane (1–2%) for 90 minutes without femoral artery occlusion; 2) 2 cycles of 5 minutes occlusion/reperfusion of the left femoral artery; 3) 2 cycles of 15 minutes occlusion/reperfusion; 4) 3 cycles of 15 minutes occlusion/reperfusion. After preconditioning or isoflurane treatment, the rats were immediately subjected to the brain ischemia as conducted in Group A. Group C was used to test whether remote preconditioning offers protection against brain ischemia induced 12 hours after preconditioning. Group C rats were assigned to 2 subgroups (n=6/each subgroup): 90 minutes of isoflurane without occlusion of the femoral artery, and 3 cycles of 15 minutes occlusion/reperfusion of the femoral artery. Group D was designed to examine the protective effect of delayed remote preconditioning. In this group, isoflurane treatment alone and preconditioning of 2 or 3 cycles of 15 minutes occlusion/reperfusion were conducted 2 days before the cerebral ischemia.

Infarct size measurement

The rats were reanesthetized with an overdose of isoflurane 48 hours after stroke, perfused intracardially with 100 ml of cold 10 mM sodium phosphate buffered saline (PBS; pH 7.4). The rats were then decapitated and the brains rapidly removed and sectioned coronally at 2-mm intervals, generating a total of 5 sections. All slices were incubated in 2% 2,3,7-triphenyltetrazolium chloride (TTC) solution for 20 minutes at room temperature, fixed by immersion in 4% paraformaldehyde solution overnight, and scanned. Using a computerized image analysis system (NIH image, version 1.61), the area of infarction of the two sides of each section was measured. Infarct size of the ischemic cortex was normalized to the non-ischemic cortex and expressed as a percentage, and an average value from the 5 slices was presented.

Statistical Analyses

One-way analysis of variance (ANOVA) was used to compare the protective effect of limb remote preconditioning on infarct size. Tests were considered statistically significant at P-values < 0.05. Data are presented as means±s.e.m.

Results

Rapid remote preconditioning reduced infarct size

The results of infarct size show that rapid limb-preconditioning with 2 or 3 cycles of 15 minutes occlusion/reperfusion of the femoral artery significantly reduced infarct size; preconditioning with 3 cycles of 15 minutes generated stronger protection (Fig. 2). However, preconditioning with 2 cycles of 5 minutes did not protect against ischemic injury. Isoflurane itself did not reduce infarct size either. From the representative infarcts, some islands of injuries were found in the spared ischemic tissues in the preconditioned rat brains (Fig. 2A).

Delayed remote preconditioning protected against focal ischemia

Delayed remote-preconditioning was conducted 2 days before the brain ischemia. The results show that delayed preconditioning with 3 cycles of 15 minutes occlusion/reperfusion reduced infarct size; but 2 cycles did not protect against cerebral ischemia. Isoflurane treatment alone did not affect infarct size (Fig. 3).

Intermediate remote preconditioning inhibited infarction

Intermediate remote-preconditioning performed 12 hours before cerebral ischemia also reduced infarct size, and isoflurane alone did not protect against ischemia (Fig. 4).

Discussion

We found that remote preconditioning conducted in the ipsilateral hind limb at three time windows protected against focal cerebral ischemia in rats and that the protective effects of remote preconditioning could be induced in the single hind limb ipsilateral to the ischemic hemisphere. In addition, we found that the protective effects depend on the cycle numbers of occlusion/reperfusion and time windows.

Although the protective effects of remote preconditioning have been studied extensively in the research field of myocardial ischemia (Przyklenk et al., 2003), they have received much less attention in the field of stroke. Until now only a few studies have demonstrated that limb ischemia reduces delayed neuronal death in the hippocampal CA 1 region in global ischemia (Kakimoto et al., 2003, Zhao et al., 2004, Dave et al., 2006, Jin et al., 2006, Steiger and Hanggi, 2007, Zhao et al., 2007b); few have addressed its protective effect against focal cerebral ischemia. In this study, we provided solid evidence that limb remote preconditioning reduced infarct size in a focal ischemia model in rats.

We found that remote preconditioning may have different therapeutic time windows from conventional preconditioning. Two “preconditioning windows” that are protective have been found in the past studies regarding conventional preconditioning (Murry et al., 1986, Perez-Pinzon et al., 1997). The protective effects of rapid preconditioning exist within 3h after preconditioning with a brief myocardial or cerebral ischemia, and it then disappears after 3h, and reappears between 24 to 72 h (Kitagawa et al., 1990, Kuzuya et al., 1993). Previous studies have confirmed that remote preconditioning also protects the ischemic heart and brain at these two time windows (Przyklenk et al., 2003, Dave et al., 2006, Jin et al., 2006); yet one study reports that remote preconditioning performed at 1h, 2h and 24h, but not at 48 h, reduced infarction (Zhao et al., 2007a). We found that remote preconditioning conducted at 48h also reduced infarction, though the protective mechanisms need further study. Nevertheless, few have studied whether remote preconditioning reduces ischemic injury in the time window between 3h to 24h. In the current study, we have shown that remote preconditioning conducted 12 h before the prolonged ischemia reduced infarct size, which contradicts the dogma of therapeutic time windows for conventional preconditioning. We do not know the underlying mechanisms by which remote preconditioning show different therapeutic time windows from conventional preconditioning. Nevertheless, remote preconditioning has different protective mechanisms from conventional preconditioning. For example, conventional preconditioning adapts the ischemic organ locally to a subsequent severe ischemia, but remote preconditioning conducted in a non-vital organ affects the heart through humoral factors or via nervous connections (Przyklenk et al., 2003). Similarly, remote preconditioning may protect against brain ischemia through different mechanisms compared with conventional preconditioning; thus, they may have different therapeutic time windows.

In conclusion, limb remote-preconditioning reduced infarct size in focal ischemia in rats when it was conducted at three time windows, including an intermediate time window where conventional preconditioning would have no effect.

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Literature

- Birnbaum Y, Hale SL, Kloner RA. Ischemic preconditioning at a distance: reduction of myocardial infarct size by partial reduction of blood supply combined with rapid stimulation of the gastrocnemius muscle in the rabbit. *Circulation* 1997;96:1641–1646. [PubMed: 9315559]
- Dave KR, Saul I, Prado R, Busto R, Perez-Pinzon MA. Remote organ ischemic preconditioning protect brain from ischemic damage following asphyxial cardiac arrest. *Neurosci Lett* 2006;404:170–175. [PubMed: 16781056]
- Gidday JM. Cerebral preconditioning and ischaemic tolerance. *Nat Rev Neurosci* 2006;7:437–448. [PubMed: 16715053]
- Jin RL, Li WB, Li QJ, Zhang M, Xian XH, Sun XC, Zhao HG, Qi J. The role of extracellular signal-regulated kinases in the neuroprotection of limb ischemic preconditioning. *Neurosci Res* 2006;55:65–73. [PubMed: 16530867]
- Kakimoto M, Kawaguchi M, Sakamoto T, Inoue S, Furuya H, Nakamura M, Konishi N. Evaluation of rapid ischemic preconditioning in a rabbit model of spinal cord ischemia. *Anesthesiology* 2003;99:1112–1117. [PubMed: 14576547]
- Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K, et al. 'Ischemic tolerance' phenomenon found in the brain. *Brain Res* 1990;528:21–24. [PubMed: 2245337]
- Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada M. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res* 1993;72:1293–1299. [PubMed: 8495557]
- Liem DA, Verdouw PD, Ploeg H, Kazim S, Duncker DJ. Sites of action of adenosine in interorgan preconditioning of the heart. *Am J Physiol Heart Circ Physiol* 2002;283:H29–37. [PubMed: 12063271]
- Liu Y, Kato H, Nakata N, Kogure K. Protection of rat hippocampus against ischemic neuronal damage by pretreatment with sublethal ischemia. *Brain Res* 1992;586:121–124. [PubMed: 1380876]
- McClanahan T, Nao B, Wolke L, Martin B, Metz T, Gallagher K. Brief renal occlusion and reperfusion reduces myocardial infarct size in rabbits. *FASEB J* 1993;7:A118.(abstract)
- Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986;74:1124–1136. [PubMed: 3769170]
- O'Duffy AE, Bordelon YM, McLaughlin B. Killer proteases and little strokes--how the things that do not kill you make you stronger. *J Cereb Blood Flow Metab* 2007;27:655–668. [PubMed: 16896349]
- Oxman T, Arad M, Klein R, Avazov N, Rabinowitz B. Limb ischemia preconditions the heart against reperfusion tachyarrhythmia. *Am J Physiol* 1997;273:H1707–1712. [PubMed: 9362234]
- Perez-Pinzon MA. Neuroprotective effects of ischemic preconditioning in brain mitochondria following cerebral ischemia. *J Bioenerg Biomembr* 2004;36:323–327. [PubMed: 15377866]
- Perez-Pinzon MA. Mechanisms of neuroprotection during ischemic preconditioning: lessons from anoxic tolerance. *Comp Biochem Physiol A Mol Integr Physiol* 2007;147:291–299. [PubMed: 17045830]
- Perez-Pinzon MA, Xu GP, Dietrich WD, Rosenthal M, Sick TJ. Rapid preconditioning protects rats against ischemic neuronal damage after 3 but not 7 days of reperfusion following global cerebral ischemia. *J Cereb Blood Flow Metab* 1997;17:175–182. [PubMed: 9040497]
- Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation* 1993;87:893–899. [PubMed: 7680290]
- Przyklenk K, Darling CE, Dickson EW, Whittaker P. Cardioprotection 'outside the box'--the evolving paradigm of remote preconditioning. *Basic Res Cardiol* 2003;98:149–157. [PubMed: 12883832]
- Steiger HJ, Hanggi D. Ischaemic preconditioning of the brain, mechanisms and applications. *Acta Neurochir (Wien)* 2007;149:1–10. [PubMed: 17151832]
- Vlasov TD, Korzhevskii DE, Polyakova EA. Ischemic preconditioning of the rat brain as a method of endothelial protection from ischemic/reperfusion injury. *Neurosci Behav Physiol* 2005;35:567–572. [PubMed: 16342612]
- Zhao H, Sapolsky RM, Steinberg GK. Interrupting reperfusion as a stroke therapy: ischemic postconditioning reduces infarct size after focal ischemia in rats. *J Cereb Blood Flow Metab* 2006;26:1114–1121. [PubMed: 16736038]

- Zhao H, Xiong LZ, Dong HL, Chen SY, Lu ZH, Sun YY, Sun J, Gao P. Protective effect of remote ischemic preconditioning against focal cerebral ischemia/reperfusion injury in rats. *Zhongguo Wei Zhong Bing Ji Jiu Yi Xue* 2007a;19:340–342. [PubMed: 17577439]
- Zhao HG, Li WB, Li QJ, Chen XL, Liu HQ, Feng RF, Ai J. Limb ischemic preconditioning attenuates apoptosis of pyramidal neurons in the CA1 hippocampus induced by cerebral ischemia-reperfusion in rats. *Sheng Li Xue Bao* 2004;56:407–412. [PubMed: 15224159]
- Zhao HG, Sun XC, Xian XH, Li WB, Zhang M, Li QJ. The Role of Nitric Oxide in the Neuroprotection of Limb Ischemic Preconditioning in Rats. *Neurochem Res* 2007b;32:1919–26. [PubMed: 17551830]

A. Control ischemia

B. Rapid

C. 12h

D. 2d

Fig. 1.

Protocols for surgery. A. Control ischemia was induced by 30 minute occlusion of the bilateral CCA combined with permanent occlusion of the left MCA. Rats were killed 2 days later for infarct size measurement. B. Rapid limb preconditioning. Rapid preconditioning was conducted in the left limb by 2 cycles of 5 minute occlusion/reperfusion, or 2 or 3 cycles of 15 minute occlusion/reperfusion. Brain ischemia was induced immediately after preconditioning. Rats in the isoflurane-control group were subjected to sham surgery by exposing the femoral artery without occlusion, but with 90 minutes of isoflurane. C. Intermediate limb preconditioning. Limb preconditioning of 3 cycles of 15 minutes was induced 12 hours before brain ischemia. A corresponding isoflurane group was used as a control. D. Delayed limb preconditioning. Preconditioning with 2 or 3 cycles of 15 minutes, and isoflurane control were conducted 2 days before brain ischemia.

Fig. 2.

Rapid limb-preconditioning reduced infarct size after stroke. Limb ischemia was induced immediately before brain ischemia. Rats were killed 2 days after stroke and rat brains were chopped into 5 slices. Infarcts were stained by TTC, and infarct cortex of each slice was measured and normalized to non-ischemic cortex as a percentage. Representative infarcts stained by TTC from each group are shown (A); arrows indicate islands of injury in the spared tissues in the spared tissues of ischemic brains treated with remote preconditioning. Note that isoflurane alone and preconditioning with 2 cycles of 5min did not reduce infarction compared with ischemic control. Bar graphs show the average infarct size of the 5 slices. Con-isc, control ischemia; Iso-isc, isoflurane of 90 minutes before ischemia onset; 2c-5 min, 2 cycles of 5

minutes occlusion/reperfusion of the femoral artery; 2c-15 min, 2 cycles of 15 minutes; 3c-15 min, 3 cycles of 15 minutes. * vs each of other groups, $P<0.001$.

Fig. 3. Delayed limb-preconditioning reduced infarct size. Limb preconditioning was induced 48 hours before brain ischemia. Representative infarcts stained by TTC are shown (A); arrows indicate islands of injury. For statistical results, infarct size of the control ischemia in Fig. 2 was represented in this figure for comparison with the control group of isoflurane. 48h iso-isc, 90 minutes of isoflurane was induced 48 hours before brain ischemia; 2c-15 min, 2 cycles of 15 minutes occlusion/reperfusion of the femoral artery; 3c-15 min, 3 cycles of 15 minutes. *, **, *** vs 2c-15 min (p=0.011), 48 h iso-isc (p=0.006), con isc (p<0.001), respectively.

Fig. 4.

Intermediate limb-preconditioning also protects against ischemic injury. To test whether limb preconditioning performed 12 hours before brain ischemia has protection, the strongest condition, 3 cycles of 15 minutes, was used. Representative TTC stained-infarcts are shown (A); similarly, islands of injury (arrow) in the spared tissues were also observed in ischemic brains treated with preconditioning. In the bar graph (B), infarct size of control ischemia was presented again for comparison. 12 h-iso-isc, 90 minutes of isoflurane was induced 12 hours before brain ischemia; 3c-15 min, 3 cycles of 15 minutes occlusion/reperfusion of the femoral artery. * vs each of the other groups, $P < 0.001$.