Noninvasive Delayed Limb Ischemic Preconditioning in Rats Increases Antioxidant Activities in Cerebral Tissue during Severe Ischemia-Reperfusion Injury

Heng-Jie Yuan, Ph.D., † Xue-Hui Zhu, Ph.D., † Qiong Luo, M.D., † Yan-Na Wu, Ph.D., † Yi Kang, B.M.S., † Jian-Jie Jiao, B.M.S., † Wei-Zhen Gao, M.D., † Yan-Xia Liu, Ph.D., † and Jian-Shi Lou, M.D. †, †

*Department of Pharmacy, General Hospital, Tianjin Medical University, Tianjin, China; and †Department of Pharmacology, College of Basic Medical Sciences, Tianjin Medical University, Tianjin, China

Originally submitted August 2, 2010; accepted for publication November 1, 2010

Background. To study the protection offered by noninvasive delayed limb ischemic preconditioning (NDLIP) against cerebral ischemia reperfusion (I/R) injury in rats.

Materials and Methods. Healthy male Wistar rats were randomly divided into four groups. The delayed protection offered by NDLIP was estimated in light of changes in the neural behavior marker and cerebral tissue antioxidative ability. Neurological functions were studied by observing neural behavior. Total superoxide dismutase (T-SOD), manganese-superoxide dismutase (Mn-SOD), glutathione peroxidase (GSH-PX), and xanthine oxidase (XOD) activity in cerebral tissue and malonaldehyde (MDA) content were detected using a spectrophotometer. Mn-SOD mRNA was measured by the reverse transcription polymerase chain reaction method.

Results. Cerebral infarct size was diminished in the early cerebral ischemia preconditioning (ECIP) D I/R and NDLIP D I/R groups compared with the I/R group (P < 0.05). The cortical and hippocampal antioxidant enzyme activity and Mn-SOD expression were increased in the ECIP D I/R and NDLIP D I/R groups. In contrast, the cortical and hippocampal XOD activity and MDA content decreased in the ECIP D I/R and NDLIP D I/R groups.

Conclusions. NDLIP decreased cerebral infarct size, increased cerebral antioxidative ability after I/R injury, and decreased peroxidative damage. The antioxidative protection offered by NDLIP was as effective as that offered by ECIP.

Key Words: noninvasive delayed limb ischemic preconditioning; delayed cerebral protection; cerebral infarction; antioxidative ability.

INTRODUCTION

The restoration of blood supply to organs after a certain period of no-flow ischemia that results in parenchymal damage is referred to as ischemia-reperfusion (I/R) injury [1]. Ischemic preconditioning (IPC) is an adaptive response to transient ischemia and serves to protect against subsequent prolonged ischemic insult and reperfusion injury. IPC was first identified in the heart by Murry et al. [2], at which time it received great attention. IPC was subsequently detected in the brain and a variety of organs, including the liver, intestine, kidney, and lung. The cerebral ischemic preconditioning (CIP) phenomenon received much attention when it was discovered, and it has since been demonstrated in vivo [3, 4], in brain slices [5], and in cultures [6].

The concept of IPC has evolved to remote ischemic preconditioning (RIPC), i.e., ischemia induced in one organ protecting against subsequent prolonged ischemia in another distant organ [7, 8]. RIPC is a novel method in which ischemia followed by reperfusion in one organ is believed to protect the remote organs either through the release of biochemical messengers in the circulation or by activation of nerve pathways, resulting in the release of messengers with protective effects. RIPC has greater potential for clinical application than conventional preconditioning since it can be performed in non-vital organs, avoiding the high risk of inducing ischemia for preconditioning in vital organs, such as the...
brain and heart. Kidneys and intestines are sensitive to ischemia, while skeletal muscles show some tolerance to ischemia; therefore, limb ischemic preconditioning (LIP) has been a focus of research.

Early studies on LIP have focused on myocardial ischemia [9]. Until now, few studies have reported that limb remote preconditioning reduces hippocampus neuronal injury after transient global ischemia or cardiac arrest in rats [10–17]. In these studies, limb preconditioning was induced by three cycles each of bilateral occlusion of the femoral arteries for 10 min followed by reperfusion for 10 min [11, 15] or a single occlusion for 30 min [17] or for 15 or 30 min [14]. Global brain ischemia was induced immediately after limb preconditioning [11, 15] or after an interval of 15 min or 48 h [17]. However, only one study [18] has addressed the protective effect of limb preconditioning against focal cerebral ischemia. It is impossible to predict most cerebral ischemic events; therefore, comparisons with noninvasive application of invasive LIP have been restricted.

We previously showed that in rats, noninvasive delayed limb ischemic preconditioning (NDLIP) induced by three cycles of 5-min occlusion and 5-min reperfusion of the left hind limb every day for 3 d confers the same cardioprotective effect as local IPC of the heart [19]. The aim of this project was to verify the hypotheses that NDLIP had neuroprotective effects during I/R injury. It is widely recognized that the generation of reactive oxygen species (ROS) greatly contributes to cerebral ischemic injury. We therefore determined whether NDLIP modulates the activities of oxidase and anti-oxidase enzymes during severe ischemia and reperfusion, without determining specific oxygen free radicals, in order to verify the neuroprotective effect of NDLIP.

MATERIALS AND METHODS

Experimental Procedures

Focal cerebral ischemia was induced in male Wistar rats weighing 250–280 g, as previously described [20]. All animal protocols in this study adhere to the guidelines of the Laboratory Animal Care and Use Committee of Tianjin Medical University and were approved by the Tianjin University Administrative Panel on Laboratory Animal Care. We made all efforts to minimize animal use and suffering in these experiments. Animals were housed in cages with normal room temperature and a 12-h light-dark cycle. Rats were anesthetized with chloral hydrate solution (10%). Core body temperatures were maintained at 36.2–37.2 °C using a heating light throughout the experiment until the animals regained full consciousness.

Focal Cerebral Ischemia

Focal ischemia was induced by occlusion of the left common carotid arteries (CCAs) for 30 min combined with permanent occlusion of the left distal middle cerebral artery (MCA) above the rhinal fissure. For MCA occlusion, a heat-blunted monofilament surgical suture (6-0) was inserted into the exposed external carotid artery, advanced into the internal carotid artery, and wedged into the circle of Willis to obstruct the origin of the MCA. The filament was left in place for 60 min and then withdrawn [20].

Induction of NDLIP

A modified blood pressure aerocyst was placed around the left thigh and inflated until the pulse of the dorsal pedal artery just disappeared; the pulse was monitored by a noninvasive blood pressure-measuring system (Taimeng Scientific and Technologic Co. Ltd., Chengdu, China). After 5 min, the aerocyst was deflated and the left hind limb was reperfused for 5 min. This inflation/deflation cycle was performed three times/d for 3 d [19].

Induction of Early Cerebral Ischemia Preconditioning

The left CCA was dissociated. Rats were subjected to three cycles of 5 min of ischemia and reperfusion of the left CCA to induce early cerebral ischemia preconditioning (ECIP).

Experimental Protocols

Healthy male Wistar rats were randomly divided into four groups: (1) sham group, left CCA dissociated but not occluded; (2) I/R group, rats subjected to 1-h occlusion of the MCA followed by 24-h reperfusion; (3) ECIP + I/R group, rats subjected to three cycles of 5-min ischemia and reperfusion of the CCA before 1-h ischemia and 24-h reperfusion; and (4) NDLIP + I/R group, rats subjected to three cycles of 5-min ischemia and reperfusion of the left hind limb for 3 d in order to induce NDLIP. On the fourth day, 1-h ischemia and 24-h reperfusion were induced.

Neurological Scores

We used the Garcia score modified for the evaluation of bilateral deficits to evaluate sensorimotor deficits [21]. In brief, symmetry in the movement of four limbs, body proprioception, spontaneous activity, and forepaw outstretching was evaluated. The score given to each rat at the completion of the evaluation is the summation of all four individual test scores. The minimum neurological score is 0 and the maximum is 11. The higher the score, the worse the neurological function.

Infarct Size Measurement

The rats were re-anesthetized with chloral hydrate solution (10%) and decapitated; their brains were rapidly removed and sectioned coronally at 2-mm intervals for a total of five sections. All slices were incubated in 2% 2,3,7-triphenyltetrazolium chloride (TTC) solution for 20 min at room temperature, fixed by immersion in 10% maldehyde solution overnight, and scanned. Using a computerized image analysis system (Image J, ver. 1.61, National Institutes of Health, Bethesda, MD), we measured the area of infarction in two sides of each section. Infarct size in the ischemic cortex was normalized to the non-ischemic cortex and expressed as a percentage, and an average value from five slices is presented.

Oxide and Anti-Oxide Assessments

After 24-h reperfusion, the rats were decapitated, and their brains were rapidly removed; the rhinencephalon, lower brain stem, and cerebellum were excised and discarded. The cerebral cortex and hippocampus were separated and stored at −80 °C for further assessments. Brain tissue homogenates were prepared as follows: 0.1 g of tissue sample was homogenized in 1 mL of ice-cold 0.9% potassium chloride buffer. Aliquots of homogenates were then used for analysis of lipid peroxides.
Activities of superoxide dismutase (SOD), manganese-SOD (Mn-SOD), and glutathione peroxidase (GSH-PX) were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). SOD and Mn-SOD were assayed by the xanthine/xanthine oxidase method according to the manufacturer’s instructions. In briefly, the determination of SOD activity was based on the xanthine oxidase method and expressed as nanomole of MDA per milligram protein (nmolMDA/mg protein). Xanthine oxidase (XOD) activity was measured by the modified method described by Sugawara et al. [22].

Reverse Transcription Polymerase Chain Reaction Amplification

Total RNA was extracted from the frozen tissues using Trizol Reagent kits (Invitrogen Life Technologies Inc., Gaithersburg, MD) according to the manufacturer’s instructions. RNA quantification and purity were ensured by the ratio of OD260 to OD280 as determined by a 751-GW ultraviolet spectrophotometer (Bio-Rad Laboratories, Milan, Italy); RNA samples with an OD260 to OD280 ratio between 1.8 and 2.0 were used for reverse transcription polymerase chain reaction (RT-PCR) with the Mastercycler Gradient Authorized Thermal Cycler PCR System (Eppendorf, Hamburg, Germany). The first-strand cDNA was generated from the total RNA using avian myeloblastosis virus reverse transcriptase and oligo(dT) primers (Dalian Bioengineering Ltd., Dalian, China). The cDNA products were amplified by PCR in a total volume of 40 μL with 1.25 U TaKaRa Ex Taq HS (TaKaRa, Tokyo, Japan) and 20 pmol each of the upstream and downstream primers. After pre-denaturation at 94°C for 5 min, 31 cycles were allowed to run for 45 s at 94°C, this was followed by 45 s at 64°C, 1 min at 72°C, and a final extension at 72°C for 10 min. The primers for Mn-SOD were sense 5'-GAC CTG CCT TAC GAC TAT GG-3' and antisense 3'-GAC CTT CCT CTT CTT TAT TGA AGC-5'. The primers for β-actin were sense 5'-TGA CGG GTG CAC CCA CAC TGT GCC CAT CTA-3' and antisense 5'-CTA GAA GCA TTG CGG TGG ACG ATG GAG GC-3' (Dalian Bioengineering Ltd., Dalian, China). The predicted sizes of the amplified Mn-SOD and β-actin DNA products were 666 and 358 bp, respectively. The amplified products (6 μL) were loaded onto 2% agarose gels that had been previously stained with 1 μg ethidium bromide; they were electrophoresed at 80 V for 20 min and then examined under a Universal Hood II gel imaging system (Bio-Rad Laboratories, Milan, Italy). The images were analyzed with Quality One software, and the semi-quantitative measure of mRNA expression was expressed as the ratio of integrated optical density (IOD) with Mn-SOD/β-actin.

Neurological Scores

The results of neurological scoring are presented in Figure 1 as median scores. Sham animals had no neurological deficit throughout the observation period.

Neurological scores in the I/R treatment group worsened significantly after ischemia. The reduction of neurological function was smaller, although significant, in the ECIP+/I/R and NDLIP+/I/R groups, with no significant differences between the two preconditioned groups.

Infarct Size Measurement

The results of infarct size are presented in Figure 2 as median percentage of infarct size against nonischemic areas. Sham animals had no infarct area. After ischemia and reperfusion, the percentage of infarct size increased to 17.34% in the I/R treatment group. Compared with the I/R group, the percentage of infarct size in the ECIP+/I/R and NDLIP+/I/R groups were much less, 10.82% and 10.72%, respectively (P < 0.05), with no significant differences between the two preconditioned groups.

Determination of T-SOD and Mn-SOD Activity

The results of T-SOD and Mn-SOD activity are presented in Figure 3. Compared with sham animals, the activities of T-SOD and Mn-SOD in the I/R groups were significantly lower. The decreased extent of T-SOD activity in the cerebral cortex of the I/R, ECIP+/I/R, and NDLIP+/I/R groups was 29.78%, 5.04%, and 10.24%, respectively, while the decreased extent of T-SOD activity in the hippocampus of the three groups was 29.90%, 2.47%, and 3.48%, respectively. The decreased extent of Mn-SOD activity in the cerebral cortex was much more significant in the hippocampus among the three groups. Compared with the I/R group, the increased extent of the activities of T-SOD and Mn-SOD in the cerebral cortex of the two preconditioning groups almost reached 30%, while the increased extent of the activities of T-SOD and Mn-SOD in the hippocampus almost reached 40%. There was no significant difference between the two preconditioning groups.

Statistical Analyses

All data are expressed as means (standard deviation). Comparisons were carried out with paired or unpaired t-tests or one-way analysis of variance procedures as appropriate. Differences were considered significant at P < 0.05. All data summaries and statistical analyses were performed with SPSS 11.5 (SPSS Inc., Chicago, IL USA).

RESULTS

Neurological Scores

The results of neurological scoring are presented in Figure 1 as median scores. Sham animals had no neurological deficit throughout the observation period.

![Figure 1](image-url)  
**FIG. 1.** Neural behavior scores after cerebral 1 h of ischemia and 24 h of reperfusion in rats (n = 11, 14). *P < 0.01 versus I/R group.
**Determination of GSH-PX Activity**

The results of GSH-PX activity are presented in Figure 4. Compared with sham animals, GSH-PX activity in the I/R groups descended significantly. The decreased extent of GSH-PX activity in the cerebral cortex of the I/R, ECIP + I/R, and NDLIP + I/R groups was 27.02%, 13.92%, and 11.40%, respectively. Compared with the I/R group, the increasing extent of GSH-PX activity was 27.02% for the ECIP + I/R group and 13.92% for the NDLIP + I/R group. (Color version of figure is available online.)

**FIG. 2.** Cerebral infarct with TTC staining method after cerebral 1 h of ischemia and 24 h of reperfusion in rats. All brain slices were incubated in 2% 2,3,7-triphenyltetrazolium chloride (TTC) solution for 20 min at room temperature, fixed by immersion in 10% maldehyde solution overnight. (A) White area (arrow) means infarct, while red area means normal tissue. Sham: all are red, all are normal; I/R group: large areas are white; ECIP + I/R group and NDLIP + I/R group: smaller white areas. (B) *P < 0.05 versus sham group. (Color version of figure is available online.)

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**Figure 4:**

- **A**
  - Sham: All areas are red, indicating normal tissue.
  - I/R: Large areas are white, indicating infarct.
  - ECIP + I/R: Smaller white areas compared to the I/R group.
  - NDLIP + I/R: Smaller white areas compared to the I/R group.

- **B**
  - Bar graph showing cerebral infarct size (%):
    - Sham: 25%
    - I/R: 20%
    - ECIP + I/R: 15%
    - NDLIP + I/R: 10%
  - *P < 0.05 versus sham group.
activity in the cerebral cortex of the ECIP + I/R and NDLIP + I/R groups was 17.95% and 21.41%, respectively. There was no significant difference between the two preconditioning groups.

### Determination of XOD Activity

The results of XOD activity are presented in Figure 5. Compared with sham animals, XOD activity in the I/R, ECIP + I/R, and NDLIP + I/R groups increased significantly, 33.20%, 18.50%, and 9.83%, respectively. Compared with the I/R group, XOD activity in the ECIP + I/R and NDLIP + I/R groups decreased significantly, 11.04% and 17.55%, respectively.

### Determination of MDA Content

The results of MDA content are presented in Figure 6. MDA content in the cortical cortex and hippocampus of the three model groups was much higher than that of the sham group. Compared with sham animals, the increased extent of MDA content in the cortical cortex of the I/R, ECIP + I/R, and NDLIP + I/R groups was 42.03%, 30.56%, and 26.05%, while that in the hippocampus was 53.20%, 16.69%, and 19.58%, respectively. Compared with the I/R group, the decreased extent of MDA content in the cortical cortex of the ECIP + I/R and NDLIP + I/R groups was 8.08% and 11.26%, respectively, while that in the hippocampus was 23.83% and 21.95, respectively. There was no significant difference between the two preconditioning groups.

### Determination of Mn-SOD mRNA Expression Level by RT-PCR

The results of Mn-SOD expression are presented in Figure 7. Mn-SOD expression in the cortical cortex and hippocampus of the I/R group was much lower than that of the sham group. Compared with sham animals, the descended extent of Mn-SOD expression in the cortical cortex of the I/R group was 37.53% and 40.60%, respectively. Mn-SOD expression in the ECIP + I/R and NDLIP + I/R groups in both the cortex and the hippocampus was much higher than that of the I/R group. Compared with the I/R group, the increased extent of Mn-SOD expression in the cortical cortex of the ECIP + I/R and NDLIP + I/R groups was 61.23% and 53.85%, respectively, while that in the hippocampus was 61.28% and 55.21%, respectively. There was no significant difference between the two preconditioning groups.
Preconditioning is an endogenous strategy in which brief periods of hypoxia render a tissue more resistant to subsequent ischemic/hypoxic insult and a powerful sublethal treatment that induces neurons to become more resistant to subsequent ischemic insult. Some studies recently suggested that NDLIP reduced myocardial ischemic injury [19], and we hypothesized that NDLIP had protective effects toward cerebral tissue during ischemia-reperfusion injury.

Conventional preconditioning has two therapeutic time windows: rapid preconditioning performed at about 1–3 h or delayed preconditioning conducted 24–72 h before the prolonged ischemia. Studies have confirmed that remote preconditioning also protects the ischemic heart and brain during these two time windows [14, 15]. To utilize delayed preconditioning of RLIP, repeat noninvasive LIP was carried out to prevent stroke, which has the characteristics of abruptness and unpredictability in our project. Ren et al. [18] found that delayed preconditioning consisting of three cycles of 15 min for 1 d had enough protective effects against focal cerebral ischemia, yet rapid preconditioning consisting of two cycles of 5 min had insufficient protective effect. We found that continuously repeating three cycles of 5-min occlusion and 5-min reperfusion for three consecutive days provided as much protection as local preconditioning. Compared with the I/R group, cerebral infarct size was diminished in the ECIP+I/R and NDLIP+I/R groups. Perhaps preconditioning on several consecutive days can enhance the strength of preconditioning. Preconditioning with repetitive episodes of mild ischemia improves resistance of the organism to subsequent severe hypoxia including structural and functional resistance of brain neurons.

Ischemic preconditioning has been studied for many years, but the mechanism involved is not understood. One possible neuroprotective mechanism induced by hypoxic preconditioning is stated to involve up-regulating...
antioxidant enzymes to reduce oxidative stress associated with ischemia and reperfusion [23]. ROS are a significant factor in the higher tolerance level to a potentially lethal insult that has been induced by preconditioning from a sublethal stimulus. Although it is widely recognized that generation of ROS makes an important contribution to ischemic injury in the brain, the sources, mechanisms, and time course of ROS generation during ischemia and reperfusion are not clearly understood. We therefore investigated the activities of several kinds of oxidase to reflect the degree of ischemia reperfusion injury. Several major observations result from this study. Ischemic preconditioning increases the expression level of SOD, an enzyme that converts superoxide into hydrogen peroxide. Increased SOD activity results in a reduction in both superoxide and peroxynitrite toxicity. We examined the activities of the antioxidant enzymes SOD and GSH-PX, which can decrease the high level of hydrogen peroxide produced by the increased SOD activity, and found that the activities of T-SOD and GSH-PX in the NDLIP group increased. The increased extent of NDLIP exceeded that of ECIP, which was considered to offer maximum protection. SOD converts superoxide anion to hydrogen peroxide, which is then reduced to water by GSH-PX and catalase. The normal adaptive response to increased SOD activity is an accompanying increase in GSH-PX levels. This increase in GSH-PX detoxifies the high level of hydrogen peroxide produced by the increased SOD activity. Brain damage results when insufficient levels of GSH-PX are present to scavenge these superoxide radicals. In the present study, NDLIP increased the activities of SOD and GSH-PX, reduced the level of superoxide anion and hydrogen peroxide, and decreased ROS generation through two steps. It is well known that overexpression of Mn-SOD is neuroprotective and that changes in Mn-SOD expression under these conditions. Our results are theoretically consistent with those of a relevant study. In a word, NDLIP could increase the activity of anti-oxidase, enhance the expression of Mn-SOD, decrease the activity of oxidase, and reduce the level of oxygen free radicals at different links.

NDLIP is a simple, drug-free, noninvasive method capable of protecting important organs such as the heart or brain against I/R injury. NDLIP can be applied in the early stages of cerebral ischemia and may be extended to the infarct stage to limit any potential ongoing damage. This protective model may also have potential therapeutic applications for the prevention of ischemic cerebral diseases in clinical settings.

ACKNOWLEDGMENTS

This study was supported by the Natural Science Foundation of Tianjin (no. 09JCZDJC21100).

SUPPLEMENTARY DATA

Supplementary data associated with the article can be found in the online version, at doi:10.1016/j.jss.2010.11.001.

REFERENCES


