Limb remote ischemic post-conditioning reduces brain reperfusion injury by reversing eNOS uncoupling

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Limb remote ischemic postconditioning (LRIP) can reduce ischemia-reperfusion injury (IRI), but its mechanisms are still unclear. We hypothesize that LRIP reduces IRI by reversing eNOS uncoupling. Focal ischemia was induced in Sprague-Dawley rats by middle cerebral artery occlusion for 2 h followed by a 24 h reperfusion. Before this surgery, folic acid (FA) was administered to the drug treatment group by gavage for 11 days. After a 24 h reperfusion, behavioural testing, vascular function, NO concentration and superoxide dismutase activity in the serum were determined. In addition, the infarct size of the brain was also detected. The mRNA of eNOS, nNOS, GTP cyclohydrolase I (GTPCH), P22phox and xanthine oxidase (XO) in the ischemic region were detected by RT-PCR, and nitrotyrosine (Tyr-NO2) was detected using Western blot analysis. The results showed that LRIP, FA and FA+LRIP all could improve behavioural score, and increase NO–mediated endothelium-dependent vasomotor responses, reduce infarction of rats subjected to IRI. Western blot and RT-PCR analyses showed that the Tyr-NO2 levels and the mRNA expression of NADPH oxidase catalytic subunit P22phox and XO were up-regulated in the ischemic brain, which was significantly inhibited by LRIP, FA and FA+LRIP. The mRNA expression of the rate-limiting enzyme in BH4 synthesis, GTPCH, was down-regulated in the ischemic brain, which could be significantly augmented by LRIP and FA+LRIP. It can be concluded that IRI induces eNOS uncoupling in the cerebral ischemic region and LRIP partially reverses the eNOS uncoupling induced by IRI.

Keywords: eNOS uncoupling, Ischemic stroke, Limb ischemic postconditioning, Nitrotyrosine, Reperfusion injury

Stroke affects 15 million people each year worldwide and is one of the world's leading causes of death and physical disability. From a global vision, stroke in developing countries accounts for nearly 70% of global stroke deaths, and 40% of those deaths occur in China. It is worth mentioning that the significant increase in incidence rates of ischemic stroke is also an important feature of epidemiological transition of stroke in China.

Thrombolytic therapy is widely used in the treatment of ischemic stroke, but sudden tissue reperfusion is deleterious, leading to brain–blood barrier disruption and hemorrhagic transformation or massive brain edema due to the ischemia-reperfusion injury (IRI). Limb remote ischemic postconditioning (LRIP) refers to an ischemia which is conducted in a distant organ that can increase the ischemic tolerance of another organ. LRIP can reduce IRI of heart, but less attention has been given to the effects of LRIP effects on IRI of brain, further its exact mechanisms are not satisfactorily explained. Nerve pathways and protein synthesis may play critical roles in the protection of LRIP to brain IRI.

IRI is recognized as a highly complex cascade of events that includes interactions between the vascular endothelium, interstitial compartments, circulating cells, and numerous biochemical entities. Acute ischemia leads to oxygen deprivation and adenosine triphosphate depletion, resulting in direct parenchymal damage through tissue necrosis. Upon restoration of blood flow to the ischemic tissue, a “no-reflow” phenomenon occurs. This kind of phenomenon refers to the clinical observation that blood flow to an ischemic organ is not fully restored following the release of a vascular occlusion.

eNOS may become “uncoupled” in the absence of the NOS substrate L-arginine or the cofactor tetrahydrobiopterin (BH4). In such uncoupled state,
of those proteins will be reduced or even lost. The active sites in some protein is nitrated, the functions of protein tyrosine nitration; when the tyrosine of the active sites in some protein is nitrated, the functions of those proteins will be reduced or even lost. The eNOS uncoupling can also lead to protein tyrosine nitration; when the tyrosine of the active sites in some protein is nitrated, the functions of those proteins will be reduced or even lost. The present study has been undertaken with an aim to clarify whether LRIP reduces brain IRI through ameliorate eNOS uncoupling.

Materials and Methods

Animals—Male Sprague-Dawley rats weighing 330–380 g were obtained from the Experimental Animal Center of China Pharmaceutical University (Nanjing, China). The animals housed under standard laboratory conditions, maintained on 12 h light/dark cycle, had free access to food and water. All animal experiments were strictly conducted under a protocol approved by the China Pharmaceutical University Administrative Panel on Laboratory Animal Care and conformed to internationally accepted ethical standards (Guide for the Care and Use of Laboratory Animals; NIH Publication 86-23, revised 1985).

Focal cerebral ischemia—Procedure for the middle cerebral artery occlusion (MCAO) model was performed as described previously. Sixty male Sprague-Dawley rats were randomly divided into following five groups of 12 each: sham group, MCAO group (control group), MCAO+LRIP group, MCAO+FA group and MCAO+LRIP+FA group. All invasive procedures were performed under general anesthesia. Anesthesia was induced with ip injection of 10% chloral hydrate (0.3 mL/100 g). Core body temperature was monitored using a rectal thermograph and was maintained at 36.5-37.5 ºC using a heating pad and lamp during the whole surgery. The right common carotid artery was exposed through a midline incision in the neck after standard sterile preparation of the skin over the neck. Approximately 19 mm of 3-0 nylon suture coated with poly-L-lysine was plugged into the right internal carotid artery through the carotid artery in order to occlude the middle cerebral artery for creating reversible focal ischemia. After ischemia for 2 h, the suture was withdrawn to allow reperfusion. The sham group just exposed the right common carotid artery but did not plug the nylon. Rats belonging to the MCAO+LRIP group and the MCAO+LRIP+FA group were treated with LRIP operation, wherein the left femoral artery was separated below the left groin ligament for later induction of femoral artery occlusion. LRIP was conducted in the left limb at the same time as reperfusion by occluding and releasing the femoral artery for 3 cycles and each occlusion or release lasted for 15 min. The skin was subsequently closed with sutures (Fig. 1A).

Behavioural testing was performed to each rat of the five groups after reperfusion for 24 h. All rats were sacrificed and perfused with cold phosphate-buffered saline (PBS, pH 7.4), and rats of each group were divided equally, as the brains of half of rats of each group were submitted for TTC staining by a person who was blind to the experimental conditions as described. The brains of the other rats were used for RNA or protein extraction.

Vibrissae-elicited forelimb placing test—Forelimb placing asymmetry was scored using the vibrissae-elicited forelimb placing test. Independent tests of each forelimb were induced by brushing the respective vibrissae on the edge of a table top once per trial for 10 trials. Intact rats responded reflexively by placing their forelimb on that side onto the countertop. Rats with unilateral damage, depending on the lesion site, would show varying degrees of impaired limb placing ability, while still placed the unimpaired limb reliably. The reflex was tested 10 times on each side per trial. The percentage of vibrissa stimulations in which a paw placement occurred was calculated.

Postural reflex test—Postural reflexes were assessed using a method modified from Bederson et al. Rats were placed on a table, and their tails were held by one hand; their shoulders were pushed gently and moved 20 cm laterally by the other hand. Then the operator repeated this test in the opposite direction, and repeated the process several times. The
use of the forelimbs to resist the lateral movement was scored. Normal rats gripped the table and braced frequently during the push, which was scored as 0. The contralateral forelimb of an ischemic rat may show less resistance and become stiff during the push, which was scored as 1. If the forelimb offered no resistance, that is, it did not brace and drag under the body during the push, this was scored as 2.

**Tail hang test**—Tail hang test was performed after 24 h of reperfusion as described by Borlongan et al. The rats were suspended at 5-10 cm above the table by its tail. An ischemia-damaged rat will immediately turn to the contralateral (left) side. This large left turns were counted when the angle reached 90° or more. Smaller turns were not counted. The rat was lifted no more than 5 sec on each trial and was released for a few seconds before the next trial. The test was repeated for 20 times in each test. The percentage of trials on which a left turn occurred was calculated.

**Drug injection**—FA was dissolved in 0.5% carboxymethylcellulose sodium to a final concentration of 5.0 mg/mL. Rats of the FA group and the LRIP + FA group received FA (50 mg/kg/d) or placebo by oral gavage for 11 days before the MCAO operation.

**Vasorelaxation assay**—The rats were killed by cervical vertebra dislocation after reperfusion for 24 h, and their hearts were perfused with 100 mL of cold PBS (pH 7.4). Their thoracic aortas were rapidly and carefully moved and then carefully dissected in ice-cold Krebs solution (mmol·L⁻¹): NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgCl₂ 1.2, NaHCO₃ 25, CaCl₂ 2.5, glucose 11, pH 7.4). The aortas were removed free of connective tissue and fat, and then made into rings of approximately 3 mm width. All dissecting procedures were done with extreme care to protect the endothelium from inadvertent damage. The rat aorta rings bath contained 2.5 mL Konstabil solution at 37 °C, and was bubbled with 95% O₂ and 5% CO₂ to give a pH of approximately 7.4. The artery segments of each group were equilibrated for 1.5 h, with a resting tension of 2 g prior to the experiments. The contractile capacity of each vessel segment was examined by being exposed to a K⁺-rich (60 mM) buffer solution, where NaCl was exchanged with an equimolar concentration of KCl. After the sustained tension to phenylephrine (PE, 1 µM) was obtained, acetylcholine (Ach) was added cumulatively (10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵ M) to induce endothelium-dependent vasorelaxation. The resting tension was taken as 100% vasorelaxation.

**Infarct size measurement**—The brains of the rats were rapidly removed and sectioned coronally at 2 mm intervals, generating a total of five sections as defined previously, with the slices incubated for 20 min in a 2% solution of 2,3,5-triphenyltetrazolium chloride at room temperature and fixed by immersion in 2% paraformaldehyde solution. The area of infarction on both 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 five slices was presented. The normal cortices in both the ipsilateral and contralateral hemispheres were measured from which the infarct was calculated according to the formula: [(area of the cortex in the non-ischemic hemisphere – area of the normal cortex in the ischemic hemisphere)/area of the cortex in the non-ischemic hemisphere] × 100%.

**NO concentration**—Generation of nitric oxide (NO) in the serum was determined by measuring nitrite accumulation in the serum using Griess reagent. Test solution (100 µL) was added to 96-well flat-bottomed plates containing 100 µL/well of Griess reagent (1% sulfanilamide, 0.1% naphthylethlenediamine dihydrochloride, and 5% phosphoric acid). After 10 min at room temperature, the absorbance of each well was measured at 540 nm and the NO₂⁻ concentration was determined from a sodium nitrite standard curve.

**SOD activity**—SOD activity was assayed using the xanthine and xanthine oxidase (XO) method. The xanthine and XO reaction system could produce O₂⁻, O₂ oxidized hydroxylamine to nitrite. SOD was able to reduce the production of nitrite. We used this system to detect the SOD activity in the serum of those experimental rats.

**Semi-quantitative RT-PCR**—Total messenger RNA was isolated from the ischemia area of rat brain using Trizol (Invitrogen) according to the manufacturer's protocol. Synthesis of first-stand cDNA was performed by reverse transcription (RT) for 30 min at 42 °C. PCR primers were eNOS, GTPCH (the rate-limiting enzyme for biosynthesis of tetrahydrobiopterin), p22(phox) subunit of NADPH oxidase) and XO, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used for the amplification of rat genes were as follows: eNOS, forward 5'-CTGCTGCCCAGATATCCTC-3' and
reverse 5'- AAGTAAGTGAGCCTGGCGCA-3';
XO, forward 5'- CGCAGAATACTGGATGACCGA
GTT-3' and reverse 5'- GCCGGTGGTTCTTCTTCTTGAA-3';
P22phox, forward 5'- GCTCATCTGTCTGCTGA-3'
and reverse 5'- TAACCCAGGAGACCCTCTCA-3';
P22phox, forward 5'- GCTCATCTGTCTGCTGA-3'
and reverse 5'- TAACCCAGGAGACCCTCTCA-3';
GTPCH, forward 5'- GGA TACCAGGAGACCATCTCA-3'
and reverse 5'- TACCATGGTGCTAGTGACAGT-3';
GAPDH, forward 5'- GCTGAGTACGTCGTGGAG-3'
and reverse 5'- ACACGGAAGGCCATGCC-3'.
The resulting PCR was electrophoresed on 1.2% agarose gels in
TAE buffer, and then photographed. The PCR
generated a DNA fragment for eNOS, GTPCH, XO,
p22phox of rat. Signal intensity of the products was
quantified by calculating the integrated volume of the
band and analyzed using ImageJ software. Obtained
values were expressed as percentages of the internal
controls.

Detection of NO_2-Tyr formation with Western
blot—After 24 h of reperfusion, the brain samples
were collected (n=4 to 5 in each group). Total
proteins in the ischemic region were extracted by
homogenizing in RIPA buffer. Those proteins were
mixed with sample loading buffer. Equal amounts of
proteins (50 µg) were loaded and the fraction was
separated on a 15% SDS-PAGE. The blots were
probed with anti-NO_2-Tyr antibody and were detected
using the ECL Western blot detection system.
GAPDH was also detected to determine the amount of
loaded protein.

Statistical analyses—Data were presented as
mean±SD. Statistical significance was estimated by
Student's t-test for unpaired observations between
two groups or by one-way analysis of variance
(ANOVA) followed by Fisher's least square
difference post hoc test for comparisons of multiple
groups. Tests were considered statistically significant
at P values < 0.05.

Results

Behavioural tests—Behavioural tests used include
the limb-use postural reflex test, vibrissa test, tail
hang test. Fig. 1(B, C, D) summarizes these
behaviours and the functions of those tests.

The scores for the postural reflex test increased at day 2
after the stroke, but LRIP, FA and LRIP+FA could
not ameliorate this function. Placing of the forelimb
contralateral to the injured brain hemisphere was
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Fig. 1—Surgery protocols and behavioural testing [(A) Surgery
protocols, (B) postural reflex test. (C) vibrissa test. (D) tail hang
test, Values are mean±SD. P values: *<0.05, **<0.01 vs sham
control group; *<0.05, **<0.01 vs control group]
endothelium-dependent relaxation function of rat aorta incurring IRI was compared with normal, LRIP, FA and FA+LRIP, acetylcholine (Ach $10^{-8}$, $10^{-7}$, $10^{-6}$, $10^{-5}$ M) induced endothelium-dependent relaxation in a concentration-dependent manner. Rats treated with LRIP, FA, or FA+LRIP demonstrated a significant increase in Ach-induced vasorelaxation of aorta (Fig. 2). These results showed that LRIP, FA, or FA+LRIP could protect endothelial from ROS induced injury.

Both LRIP and FA had no significant impact on the NO concentration and SOD activity in serum. The results showed that when the brain of a rat was subjected to IRI, the NO concentration decreased. However, LRIP, FA, and FA+LRIP could not ameliorate it (Fig. 3A). It was worth mentioning that SOD activity in the rat serum between each group had no significant difference (Fig. 3B).

LRIP, FA, and LRIP+FA could reduce infarct size. Figure 4 demonstrated that LRIP could significantly reduce infarct volume compared with the control group (17.2 ± 5.0% and 32.3 ± 4.0% respectively) after 24 h of reperfusion.

To test if the BH₄ was crucial to limb ischemic postconditioning-mediated reduction in infarct volume, rats were pretreated with high concentrations of FA. FA could stabilize BH₄ by augmenting its binding affinity to eNOS and enhancing BH₄ regeneration from

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**Fig. 2**—ACh-induced vasorelaxation in various groups. (Rat abdominal aorta rings were precontracted with PE, then gradient concentration Ach was added to induce endothelium-dependent relaxation. The endothelium-dependent relaxation of (A) sham group, (B) LRIP group, (C) FA group, and (D) FA+LRIP group were compared with control group. Values are mean±SD. $P$ values: *< 0.05, **< 0.01 vs sham group; *< 0.05, **< 0.01 vs control group. (n = 4))

**Fig. 3**—NO content and SOD activity in the serum. [NO concentration (A) and SOD activity (B) in rat serum were detected. Values are mean±SD. $P$ values: ## < 0.01 vs sham group, (n = 10 or 12)]

**Fig. 4**—LRIP and/or FA reduced infarct size measured at 24 h after stroke. [(A) Representative infarcts stained by TTC from each group. (B) bar graphs show the average infarct size of the five slices. Values are mean±SD. $P$ values: *<0.05, **<0.01 vs sham group; *<0.05, **<0.01 vs control group, (n=6 or 8)]
oxidized and inactive BH$_2$<sup>18</sup>. Administration of FA could reduce infarct volume to 12.7 ± 6.2%. LRIP+FA could significantly reduce infarct volume to 19.2 ± 6.3%, LRIP could reduce infarct volume to 17.2 ± 5.0% (Fig. 4). From those results we presumed that LRIP and FA have no synergy.

**Expression of eNOS, nNOS, P22<sub>phox</sub>, GTPCH, Xanthine oxidase component in the ischemic area of brain**—To determine whether BH$_4$ participated in reperfusion caused injury, as shown Fig. 5, the expression of GTPCH was determined by semi-quantitative PCR. The results showed that LRIP, FA and LRIP+FA increased mRNA expression levels of GTPCH in the ischemic cortical areas. GTPCH is the rate-limiting enzyme of BH$_4$. From this it could be inferred that the expression of BH$_4$, one of the substrate of eNOS, was elevated by LRIP, FA and LRIP+FA. The results also showed that LRIP and LRIP+FA significantly increased eNOS mRNA expression and decreased XO and P22<sub>phox</sub> mRNA expression in the ischemic hemispheric brain as compared to the sham group.

**Characterizing nitration of protein in ischemic region by Western blot**—When eNOS was uncoupling, it would produce O$_2$·-, and O$_2$·- could react with NO to produce ONOO<sup>-</sup>, proteins would be nitrated to nitrotyrosine (Tyr-NO<sub>2</sub>). The present result showed that Tyr-NO$_2$ level in the control group was higher than that in the sham group, and LRIP, FA and LRIP+FA could reduce the production of Tyr-NO$_2$ (Fig. 6), suggesting that LRIP, FA and LRIP+FA could reverse eNOS uncoupling to a certain extent.
Discussion

Thrombolytic therapy plays an important role in the clinical treatment of ischemic stroke. However, its impacts on subsequent IRI have brought new challenges to the clinical treatment of ischemic stroke. Researches have shown that the injury in liver or intestinal ischemia for 3 h then reperfusion for 1 h is more serious than ischemia for 4 h. Chen’s et al. showed that the cortical infarct volume is maximum when reperfusion lasts for 24 h. Thus, we decided to use 24-h reperfusion as the time point to study the effects and mechanisms of LRIP.

It is important to note that eNOS may become “uncoupled”, e.g., in absence of the NOS substrate L-arginine or the cofactor tetrahydrobiopterin (BH$_4$). In such state, electrons normally flowing from the reductase domain of one subunit to the oxygenase domain of the other subunit are diverted to molecular oxygen rather than to L-arginine, resulting in increased $\text{O}_2^-$ formation at the expense of NO formation. The results of RT-PCR showed that GTPCH expression was down-regulated in the ischemic cortex of control group. From this can be suggested that BH$_4$, the substrate of eNOS, was depleted. On the other hand, the expression of P22$^{phox}$ was up-regulated. P22$^{phox}$ is the subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The $\text{O}_2^-$ produced by the NADPH oxidase together with the limited availability of BH$_4$ may trigger eNOS uncoupling, resulting in production of $\text{O}_2^-$ rather than NO. It can be seen from the present results that P22$^{phox}$ could be down-regulated and GTPCH could be up-regulated by LRIP, FA, and LRIP+FA. From all these results it could be conjectured that LRIP, FA, and LRIP+FA could reverse eNOS uncoupling, thus the IRI would be reduced.

The present data also demonstrated that IRI induces upregulation of total mRNA of X O which in turn generates $\text{O}_2^-$. $\text{O}_2^-$ could react with ·NO to form peroxynitrite (ONOO$^-$), which could further form peroxynitrous acid (ONOOH), a very unstable and reactive oxygen species resulting in nitration of proteins to Tyr-NO$^2$. The results of western blot showed that the production of Tyr-NO$^2$ was increased. This was confirmed once again in the present study. It is worth mentioning that LRIP, FA, and LRIP+FA could also down-regulate the mRNA expression of XO, suggesting that they could reduce $\text{O}_2^-$ which was generated by xanthine oxidase.

It is well known that NO was mainly produced by eNOS, inducible nitric oxide synthase (iNOS), and neuronal nitric oxide synthase (nNOS) in vivo. From the RT-PCR results, it is clear that the expression of eNOS mRNA in the ischemic region did not change significantly after IRI. It is worth mentioning that the mRNA expression of nNOS in the ischemic regions has no significant difference between the five groups (Fig. 5). In addition, Wang’s et al. showed that the expression of iNOS mRNA increased in the ischemic region of the rat brain. On the other hand, the present results showed that the NO concentration in the serum of the control group was significantly reduced compared with that of the sham group (Fig. 3A). From the above experimental results, it can be inferred, to a certain extent, that eNOS in the ischemic region has produced $\text{O}_2^-$ other than NO.

BH$_4$ could be used to treat cardiovascular disease, because BH$_4$ could reverse eNOS uncoupling, and this has been proven to be a very effective strategy in animal models of cardiovascular disease. At the
same time, researchers have shown that high concentrations of FA can reduce cardiac IRI in rats. They concluded that FA can stabilize BH₄ by augmenting its binding affinity to eNOS and enhancing BH₄ regeneration from oxidized and inactive BH₂. Thus, FA can reduce IRI of heart by ameliorating eNOS uncoupling. Combined with the results of the present studies, it is speculated that both FA and LRIP can ameliorate eNOS uncoupling. In this study, we specifically added the LRIP+FA group to evaluate whether LRIP and FA have the synergistic effects and whether LRIP can be used to alternate BH₄ in the clinical treatment of ischemic stroke. The results showed that the combination of LRIP and FA have not superimposed the effects.

It is well known that IRI has the no-reflow phenomenon. To determine whether this phenomenon was caused by endothelial injury, we specially used rat thoracic aorta to evaluate endothelial damage. It was very interesting to find that the endothelium-dependent relaxation of the thoracic aorta declined after brain IRI, and LRIP, FA, and FA+LRIP could all improve the endothelium-dependent relaxation status of the thoracic aorta. These results may to some extent explain the no-reflow phenomenon caused by IRI. LRIP and FA could be used to treat the phenomenon.

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Conflict of interest
The Authors have no competing interests.

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