Remote Limb Ischemic Conditioning during Cerebral Ischemia Reduces Infarct Size through Enhanced Collateral Circulation in Murine Focal Cerebral Ischemia

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Background: Remote ischemic conditioning (RIC) induces protection in focal cerebral ischemia. The conditioning is divided into pre-, per-, and postconditioning. However, the mechanisms of RIC remain unknown. *Objectives:* This study aimed to determine the most effective subtype of RIC. We also examined involvement of collateral circulation on RIC. *Methods:* Transient middle cerebral artery occlusion (MCAO) was performed with nylon sutures in adult C57BL/6 mice under the monitoring of cerebral blood flow (CBF). Fifty mice were divided into 5 groups: MCAO control group, delayed pre-RIC group (RIC 24 hours before MCAO), early pre-RIC group (RIC 5 minutes before MCAO), per-RIC group (RIC during MCAO), and post-RIC group (RIC 5 minutes after MCAO). In other middle cerebral artery (MCA) control and per-RIC groups, collateral circulation was visualized with latex compound perfusion. *Results:* After MCAO, CBF was reduced by 80% in all groups. At the end of MCAO, relative increase in CBF in per-RIC group was significantly greater than that in MCA control, whereas the infarct volume in per-RIC group was significantly smaller than that in other groups. The diameter of leptomeningeal anastomosis was larger in the per-RIC group than that in the control group. *Conclusions:* Among the 4 RIC procedures, only the per-RIC group showed clear brain protection. Enhancement of collateral circulation could play a role in the protective effect of per-RIC. **Key Words:** Brain protection—cerebral ischemia—collateral circulation—leptomeningeal anastomosis—remote ischemic conditioning.

Introduction

Endogenous neuroprotection induced by mild ischemic stress is a phenomenon known as ischemic tolerance or preconditioning.[1] However, clinical application of ischemic stress on the brain is not feasible.

With the induction of ischemic tolerance through several stimuli or drugs, known as cross-tolerance,[2] remote ischemic conditioning (RIC) has gained much attention.[3] RIC involves the repetitive inflation and deflation of a cuff above the systolic blood pressure (BP) on the limbs and induces endogenous protective pathways to distant organs such as the brain and the heart. In the field of cardiology, clinical trials have shown the utility of RIC on prevention of periprocedural myocardial infarction.[4]

RIC is classified into 4 types: remote delayed preconditioning (delayed pre-RIC), remote early preconditioning (early pre-RIC), remote per conditioning (per-RIC), and remote postconditioning (post-RIC).[3] In delayed pre-RIC, limbs are exposed to ischemia more than 24 hours

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Received June 6, 2017; revision received September 21, 2017; accepted September 24, 2017.

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1052-3057/$ - see front matter

https://doi.org/10.1016/j.jstrokecerebrovasdis.2017.09.068

before cerebral ischemia, and limb ischemia occurs immediately before cerebral ischemia in early pre-RIC. In per-RIC, limb ischemia occurs concurrently with cerebral ischemia, and in post-RIC, limb ischemia occurs immediately after the recirculation of cerebral ischemia. All 4 types of RIC have shown to be protective for cerebral ischemia,[5-10] but the most effective among these remains unclear. In terms of clinical utility, per-RIC and post-RIC are feasible and clinical trials of RIC for acute ischemic stroke are either promising[11] or in progress.[12] The mechanisms of RIC remain unknown, but both neural system and humoral factors are highly likely to be involved.[3]

We have developed model of pial collateral circulation enhancement in chronic cerebral ischemia in both rat and mouse models.[13-15] Unilateral carotid occlusion is employed as chronic cerebral ischemia, and it induces enlargement of leptomeningeal anastomosis after 14 days before markedly reducing in infarct size after permanent occlusion of the middle cerebral artery (MCA).

In this study, we aimed to clarify the protective effect of 4 RIC protocols in the same murine ischemia model, and to elucidate the involvement of collateral development during cerebral ischemia by RIC.

Materials and Methods

Animals

C57BL/6 strain mice were obtained from Japan SLC, Inc., (Shizuoka, Japan). All mice used in this experiment were mature males aged 8-10 weeks. Mice were maintained on a 12-hour light/dark cycle and provided free access to food and water before surgery. The experimental protocols involving laboratory animals have been approved by the Institutional Animal Care and Use Committee of Tokyo Women's Medical University. Reporting of this work complies with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Focal Cerebral Ischemia

General anesthesia was induced with 3.0% isoflurane and maintained with 1.0% isoflurane (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) with an open mask. A laser-Doppler flowmetry probe (Advance laser flowmetry, model ALF-21; Advance Co, Tokyo, Japan) was attached with dental cement to the intact skull 5 mm lateral and 1 mm posterior to the bregma. Left middle cerebral artery occlusion (MCAO) was induced by an intraluminal occlusion for 45minutes using a nylon filament suture as described previously.[16] In brief, the left common carotid artery was exposed and ligated, and a 7-0 medium MCAO suture (No. Re L45 PK5; Doccol Corporation, Sharon, MA) was introduced through the carotid bifurcation into the left internal carotid artery (ICA) and advanced by 10 mm until the cerebral blood flow (CBF) over the MCA territory dropped abruptly. After occlusion for 45 minutes, blood flow was restored by the removal of the nylon suture. During the operation, rectal temperature was monitored with a rectal thermometer and maintained at 37°C with a heat lamp.

Remote Ischemic Conditioning

Mice were assigned to 5 groups: MCAO control group and delayed pre-RIC, early pre-RIC, per-RIC, and post-RIC groups. Hind limb ischemia was accomplished by tightening a tourniquet (PE50; Natsume Seisakusho Co Ltd., Tokyo, Japan) around the upper thigh to achieve limb pallor for 4 cycles, with each occlusion or release phase lasting 5 minutes as described by Hahn et al.[8] Delayed pre-RIC was conducted 24 hours before MCAO surgery. Early pre-RIC was conducted immediately before the MCAO surgery. Per-RIC was performed during the 45-minute period of MCAO. Post-RIC was conducted immediately after the recirculation of MCAO. Individual experimental timelines are shown in Figure 1, A. In the MCAO control group, a tourniquet was loosely tied around the upper thigh during MCAO.

Neurobehavioral Tests

Twenty-four hours after transient MCAO, mice were evaluated for neurological deficits by a blind observer. The assignment of the neurological deficit score from 0 to 5 was based on the methods described previously by Yang et al[17]: 0, no neurological deficits; 1, failed to extend right forepaw while held by the tail; 2, circled to the right; 3, fell to the right; 4, unable to walk spontaneously; and 5, died.

Evaluation of Ischemic Damage

Twenty-four hours after transient MCAO, animals were euthanized with 4.0% isoflurane. Brain tissue was removed and sectioned coronally into seven 1-mm slices using a brain matrix (Muromachi Kikai Co., Ltd. Tokyo, Japan). Slices were placed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution for 30 minutes at 37°C. Infarct volume was measured using a computerized image analysis system.

Latex Perfusion

In another set of MCAO control and per-RIC groups, the leptomeningeal collaterals were visualized 45 minutes after MCA occlusion as described previously[14] (Fig 1, B). After completion of MCAO, the right atrium of the heart was incised under anesthesia with 4.0% isoflurane to allow for venous outflow, the left ventricle of the heart was cannulated, and 5 mL of saline was injected into it. After the saline injection, 1.0 mL of white latex compound (Product No. 563; Chicago Latex Products Inc, Crystal Lake, IL) mixed with 50 mL/mL carbon black was injected. The brains were removed carefully and immersed in Zamboni