The Effects of Exercise Preconditioning on Cerebral Blood Flow Change and Endothelin-1 Expression after Cerebral Ischemia in Rats

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Stroke is an acute cerebrovascular disease with high incidence, morbidity, and mortality. Preischemic treadmill training has been shown to be effective in improving behavioral and neuropathologic indices after cerebral ischemia. However, the exact neuroprotective mechanism of preischemic treadmill training against ischemic injury has not been elucidated clearly. The present study investigated whether preischemic treadmill training could protect the brain from ischemic injury via regulating cerebral blood flow (CBF) and endothelin 1 (ET-1). We analyzed the CBF by laser speckle imaging and ET-1 expression by an enzyme-linked immunosorbent assay using an ischemic rat model with preischemic treadmill training. Generally speaking, ET-1 expression decreased and CBF increased significantly in the pretreadmill group. It is worth noting that ET-1 expression is increased at 24 hours of reperfusion in the pretreadmill group compared with the level of the time after middle cerebral artery occlusion. These changes were followed by significant changes in neurologic deficits and cerebral infarct volume. This study indicated that preconditioning exercise protected brain from ischemic injury through the improvement of CBF and regulation of ET-1 expression, which may be a novel component of the neuroprotective mechanism of preischemic treadmill training against brain injury. Key Words: Preischemic treadmill training—endothelin 1—cerebral blood flow—ischemic injury.

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Introduction

Stroke is an acute cerebrovascular disease with high incidence, morbidity, and mortality. A lot of basic and clinical research has been done on the pathogenesis of stroke, but because of the potential impairments and the limitation of treatments, finding out an effective prevention of stroke is particularly important.1 Among numerous preventive measures, exercise is a kind of safe and effective preventive measure and has been widely accepted. Evidence showed that training could elevate the cerebrovascular reactivity, which provided further support to the concept that exercise is a preventive measure in cerebrovascular and neurologic disease in the aged.2 Research proved that exercise could increase the number of small blood vessels in the brain of the healthy old people3 and reduce the abnormal rheological properties of blood.4
EXERCISE INDUCED CBF AND ET-1 CHANGES AFTER ISCHEMIA

A previous article demonstrated that at least 2 weeks of pretreadmill training (a kind of exercise way) was neuroprotective for cerebral ischemia rats.5,6 Likewise, other studies have shown that pretreadmill training was effective in improving behavioral and neuropathologic indices after cerebral ischemia,7,8 and maintaining neurovascular integrity,9 improving blood–brain barrier indices after cerebral ischemia,10-12 and maintaining effective in improving behavioral and neuropathologic studies have shown that pretreadmill training was neuroprotective for cerebral ischemia rats.5,6 Pretreadmill training can reduce brain inflammation, which is contributed to neuronal death following stroke after cerebral ischemic injury.13-15

There may be many ways to explain the neuroprotection mechanism of pretreadmill training for cerebral ischemia rats. Some studies4 have demonstrated that it is possibly related to the blood–brain barrier, cerebrovascular, nerve cell apoptosis, glutamic acid system, inflammatory reaction, and the neurogenesis. Among these reasons, the effect of pretreadmill training on a cerebral vascular system is particularly important.

Evidence showed that exercise can increase the number of small blood vessels of the healthy elderly, and rat brain capillary density and cortical blood volume.3,14 Studies on monkey found that new blood vessels appeared after 5 months of sports and then returned to baseline levels on monkey found that new blood vessels appeared after 12 weeks.15 Recent reports suggested that exercise could affect the vasomotor of cerebral vessels and maintain enough oxygen in the brain by regulating cerebral blood flow (CBF) under ischemic and hypoxic conditions.16 Meanwhile, studies have identified that hemodynamic shear stress was an important determinant of endothelial function and phenotype.17

Endothelin (ET), which is mainly generated by the vascular endothelial cells, is a polypeptide. It can cause vasoconstriction with a slow, long-lasting, and wide effect. ET is an important physiological and pathologic factor in the pathogenesis of cardiovascular and cerebrovascular diseases.18,19 A previous report showed that exercise training enhanced endothelium-dependent nitric oxide-mediated vasodilation in soleus and gastrocnemius muscle arterioles.19

In addition, researches have shown that CBF change could affect the synthesis and release of ET-1 and vice versa.20 On the basis of these findings, in this study we examined the influence of pretreadmill training on CBF change and ET-1 expression during ischemia and reperfusion of rat brain from injury induced by middle cerebral artery occlusion (MCAO).

Materials and Methods
Animal Preparation

Adult male Sprague–Dawley rats (weighing 250-300 g) were provided by Shanghai Laboratory Animal Center, Chinese Academy of Sciences. The rats were housed under a 12:12-hour light/dark cycle with food and water available ad libitum.

Treadmill Training

Rats were assigned randomly to 1 of the 3 groups (sham, ischemic, or pretreadmill); pretreadmill group rats were treated with 2 weeks of preischemic intervention. All rats received 2 days adaptive running exercise of 5-8 m/minute for 30 minutes/day before the formal treadmill training. After adaptive running, the rats in the pretreadmill group started training on an electric treadmill machine (DSPT-202 Type 5-Lane Treadmill; Litai Biotechnology Co, Ltd, China), and then they were scheduled to run on the treadmill for 2 weeks. The formal treadmill training was prescribed as 20 m/minute, 30 minutes/day for 5 days/week.3 The rats in the sham and ischemic groups ran freely in their cages for 2 weeks. The rats in all groups were bred under the same conditions. Parameters for treadmill exercise were set at a slope of 0° and belt speed of 20 m/minute.

Rat MCAO Model

Rats were anesthetized with 10% (vol/vol) chloral hydrate (.35 mg/kg, intraperitoneally) at the end of treadmill training, and using the modified Longa’s method21 the rat model of left MCAO was established. The left external carotid artery was occluded by the intraluminal suture technique. A surgical nylon monofilament with a blunt, silicone-coated tip (of .38 ± .02-mm diameter; Beijing Shadong Biotech Co, Ltd, Beijing, China) was immersed in heparin beforehand and inserted from the middle cerebral artery (MCA) into the lumen of the internal carotid artery. When the nylon monofilament arrived in the ostia of the MCA along the internal carotid artery, the MCAO model was successfully built. The rectal temperature of rats was maintained at 37°C by placing them on a heating pad during the whole surgical procedure. The brain ischemia was measured using a laser Doppler velocimeter (laser doppler perfusion monitor, Perimed, Jarfalla, Sweden). After 120 minutes of cerebral ischemia, the nylon monofilament was removed surgically to allow reperfusion. The same surgical procedure was performed on sham surgical rats but without occluding the MCA.

Evaluation of Behavioral Score

The rats in each group were evaluated at 24 hours after reperfusion based on a 5-point scale: 0, no neurologic symptoms; 1, unable to completely extend the front jaw to the other side; 2, rotating when crawling, and falling to the other side; 3, unable to walk without help; and 4, unconsciousness.

Determination of Brain Infarction Volume

At 24 hours after reperfusion, rats were anesthetized with chloral hydrate (10%). Brains were quickly removed and stored at −20°C for 10 minutes. Brain tissues were cut
into 6 sections at a coronal plane. The thickness of each section was 2 mm. The first cut was beginning at the midline between the anterior pole and the optic chiasma. The sections were rapidly put into 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution (37°C) for 30 minutes, and followed by fixation in 4% paraformaldehyde buffer. The sections were photographed 24 hours later with a digital camera (Kodak DC240; Eastman Kodak Co., Rochester, NY). Imaging software (Adobe Photoshop 7.0, Adobe Systems, Mountain View, CA) was used to trace the area of infarction.

Cresyl violet (CV) staining was also used to measure the infarct volume as previously described. In brief, frozen coronal brain sections were stained in .1% CV solution, followed by quick differentiation in 1% glacial acetic acid. The sections were dehydrated and clarified through ethanol and xylene, respectively, and mounted in neutral balsam. The infarct area was defined according to the published article.

To minimize the error introduced by edema, we used an indirect method to calculate the infarct volumes. Infarct volume percent = contralateral hemisphere region – noninfarcted region in the ipsilateral hemisphere/contralateral hemisphere area × 100%.

Laser Speckle Imaging

All procedures were performed using standard sterile precautions. The rat was anesthetized with intraperitoneal injection of 10% chloral hydrate at a dose of 360 mg/kg. The animal was placed in a stereotactic frame (David Kopf Instruments, Tujunga, CA) throughout the experiment. The scalp was shaved and disinfected with 70% ethanol and povidone–iodine solution. After a midline scalp incision, the galea and periosteum overlying the parietal bone bilaterally were swept and retracted laterally. The regions of interest, which were centered at 2.5 mm posterior and 2.5 mm lateral to the bregma over the left and right cortex with a 2.5 mm × 5 mm area each, were thinned using a high-speed dental drill (SDE-H37L; Marathon, Korea), until the inner cortical layer of a bone was encountered. Rectal temperature was maintained at 37°C during the experiment using a homeothermic blanket system (model TP-500T/Pump; Gaymar Industries, Inc, Orchard Park, NY). The laser speckle images (696 × 512 pixels) were acquired at 23 frames per second (exposure time T = 5 milliseconds) by laser speckle imaging (LSI) system (Dolphin BioTech Ltd Shanghai, China) with a laser diode (780 nm; Dolphin BioTech Ltd Shanghai) over the skull. The raw speckle images were processed by the random process estimator method after registration to obtain the contrast image. The blood flow speed V is related to contrast value according to the theory of laser speckle contrast imaging. Averaged data of the region of interest during MCAO surgery and 24 hours after reperfusion were used to quantify the CBF changes in fractional terms. $V = V/V_0 \times 100\%$.

Determination of ET-1 Levels in Plasma

Samples of blood were collected via jugular vein, respectively, before MCAO (Pre-MCAO), at 0 hours after MCAO (MCAO), and at 24 hours of reperfusion (Rep-24 hours). Venous blood (2 mL) was collected in a tube with ethylene diaminetetraacetic acid and aprotinin. All blood samples were centrifuged at 3000 rpm for 10 minutes at 4°C, and the resulting supernatants were stored at −80°C till further analysis. ET-1 in plasma was assayed using an enzyme-linked immunosorbent assay kit (Kit Xitang from Shanghai Biotechnology Co, Ltd, Shanghai, China) according to the manufacturer’s instructions.

Statistical Analysis

All values are expressed as the mean ± standard deviation. Statistical analyses were performed using SPSS 13.0 statistical software (SPSS Inc, Chicago, IL). Multiple comparisons between groups were analyzed using 1-way analysis of variance followed by the Tamhane multiple comparison post hoc test. A value of $P$ less than .05 was considered statistically significant.

Results

Neurologic Status and Cerebral Infarct Volume

To determine whether the observed changes in ET-1 expression and CBF accompanied by changes in behavioral recovery and lesion volume, we next evaluated sham, ischemic exercise, and pretreadmill groups at 24 hours after ischemia. A 5-point neurologic scale score
revealed that sham rats had no neurologic deficits. As shown in Figure 1, the rats in the pretreadmill group showed fewer deficits than those in the ischemic group (3.20 ± .8). The rats treated with 2 weeks of preischemia treadmill training demonstrated significantly better neurologic statuses (2.20 ± .20).

After behavioral tests, the rats were sacrificed to compare the ischemic area with the behavioral performances. Both TTC and CV staining methods were used to determine the infarct volume. Similar to the results of behavioral score evaluation, none of the sham rats had ischemic areas. As shown in Figure 2, the rats in the ischemia group had infarcts (47.44 ± 3.46% by TTC staining and 39.95 ± 2.5% by CV staining), whereas those in the pretreadmill group showed a significant reduction in the infarct volume (33.08 ± 2.53% by TTC staining and 27.76 ± 2.58% by CV staining). The results showed that exercise remarkably reduced the infarct volume.

**Cerebral Blood Flow**

Figure 3 shows the LSI results before, during, and after MCAO. As compared, respectively, with the baseline CBF, which is before ischemia, the relative average velocity of capillary during reperfusion was determined at 24 hours. The relative average velocity of capillary was increased in the pretreadmill group than that in the ischemic group, which were both lower than that in the sham group. There were significant differences between the pretreadmill and ischemic groups (P < .05). Data indicated that preischemic treadmill training improved CBF during reperfusion.

**ET-1 Expression**

ET-1 regulates a diverse array of physiological processes including vasoconstriction, which is related to CBF. To find out how exercise alleviates CBF change to protect against ischemic injury, whether preischemic treadmill training may affect the expression of ET-1 was investigated. The concentration of ET-1 was determined at different time points of Pre-MCAO, MCAO, and Rep-24 hours, as shown in Figure 4. The concentration of ET-1 was 4.03 ± .65 pg/mL in the ischemic group and 1.95 ± .39 pg/mL in the pretreadmill group at 24 hours of reperfusion, and 5.26 ± .38 pg/mL in the ischemic group and 1.10 ± .25 pg/mL in the pretreadmill group after MCAO surgery.

**Discussion**

In our experiment, to explain the role of ET and CBF in the neuroprotective process induced by preischemic treadmill training after ischemic injury, we analyzed the ET-1 expression and changes in CBF using a rat MCAO model with preischemic treadmill training. In the pretreadmill group at 24 hours after reperfusion, CBF increased significantly and the expression of ET-1 was decreased than that in the ischemic group. But compared with the time point of MCAO, the concentration of ET-1...
was increased at 24 hours after reperfusion in the pretreadmill group. In addition, preischemic treadmill training also improved neurologic function and reduced lesion volume.

LSI is a novel technique that can provide information on perfused blood volume and real-time monitoring of CBF changes. The changes in CBF are very important during the cerebral ischemic injury process. The extent of the CBF change is positively correlated with the severity of brain injury. CBF was found to increase with exercise intensity in studies by Hollmann and DeMeirleir.26 Another study showed that dynamic exercise caused an increase in average global CBF.27 On the other hand, exercise has been shown to improve function in animal models and patients with cerebral ischemia, it seems that the reduced brain inflammation and induced brain ischemic tolerance after stroke may be attributable to the improvement of CBF during reperfusion caused by exercise. In our study, we found that CBF changed significantly after reperfusion in the pretreadmill group. Firstly, a substantial reduction in CBF occurred in rats of both the ischemic and pretreadmill groups. Interestingly, there was a substantial increase in CBF toward the baseline values in the rats of the pretreadmill group at 24 hours after reperfusion, and most of blood flow had been recovered compared with that of before MCAO. Meanwhile, there was an improvement in neurologic function and lesion volume. These results indicated that pretreadmill training could stabilize CBF and weaken CBF changes to reduce secondary damage induced by the changes in blood flow during the reperfusion and protect the brain from ischemic injury.

To find out how exercise alleviates CBF change, ET-1, a solely vasoconstrictor, was also studied. ET system plays a very important role in cardiovascular and neurovascular diseases, including chronic heart failure, hypertension, and atherosclerosis.28-30 Overexpression of ET-1 in endothelial cells results in more severe vascular permeability and blood–brain barrier breakdown after brain ischemic injury in rats following MCAO.31 Thus, the ET system is an effective target for the treatment of brain diseases, which are involved in neurovascular damage.20,32

Some studies have reported the effects of exercise on ET-1 expression. PreproET-1 messenger RNA expression in the kidneys was markedly higher in the exercise rats than that in the sedentary control rats.33 Some studies

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**Figure 3.** (A) Regional CBF images in pseudo color by laser speckle imaging. (B) Quantification of the relative CBF in the region of interest (compared, respectively, with the CBF baseline before ischemia [Pre-MCAO]). Data indicated that the sham group resulted in no ischemia after surgery and CBF velocity was maintained at a normal level, whereas the ischemic and pretreadmill groups exhibited a decreased CBF velocity before reperfusion. Twenty-four hours after reperfusion, the pretreadmill group demonstrated a higher CBF velocity than the ischemic group (*P < .05). Pre-MCAO: before MCAO surgery; MCAO: during MCAO surgery; Rep-24 hours: 24 hours after reperfusion. Data are expressed as the mean ± standard deviation. n = 12. Abbreviations: CBF, cerebral blood flow; MCAO, middle cerebral artery occlusion. (Color version of figure is available online.)

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**Figure 4.** Venous plasma levels of ET-1. Blood samples were collected, respectively, for an enzyme-linked immunosorbent assay analysis. ET-1 concentration from the sham group, ischemic group, and pretreadmill group at various time points (*P < .05 relative to the sham group; #P < .05 relative to the ischemic group; and &P < .05 relative to MCAO of the pretreadmill group). Data are expressed as the mean ± standard deviation. n = 8. Abbreviations: ET-1, endothelin 1; MCAO, middle cerebral artery occlusion.
suggested that regular aerobic-endurance exercise reduces plasma ET-1 concentration in older humans and healthy young humans, and this reduction in plasma ET-1 concentration may have beneficial effects on the cardiovascular system. Some other reports showed that ET-1 expression increased after exercise. We considered that because of the various ways and intensity of training, ET-1 expression changed differently in each report. In this study, our results showed that ET-1 expression of ischemic rats has decreased with pretreadmill training. According to the increase in CBF observed before, we speculated that the downregulation of ET-1 resulted in vascular pressure release and alleviate the change in CBF. But ET-1 concentration was increased in the pretreadmill group from MCAO to 24 hours after reperfusion, which may be more beneficial to the injury repair process. It means that we cannot conclude exercise up- or downregulated ET-1 expression after ischemia simply, we can only say that exercise is helpful to protect brain from injury by regulating the ET-1 level. The results indicated that the increase in CBF was induced by exercise via regulating ET-1 expression, which could prevent the secondary damage phenomena after the reperfusion event, and improve neurologic status and histology injury. The exact mechanism behind this is still unknown, and it is a topic for future research as well.

In fact, we found that the protein levels of ET-1 in the pretreadmill group were lower than that in the ischemic group after MCAO injury. It is worth noting that our result is different compared with the results of most of the other studies, which indicated that ET-1 level was significantly higher after reperfusion. However, Zhang et al. reported that the level of ET in the ischemic group was lower than that in the sham group, and the contents then further reduced after reperfusion and reached the lowest level at 12 hours of reperfusion and at last became normal 6 days later. We thought that it may be because of the increase in the expression of vasoactive factors so that the ET-1 level was decreased after reperfusion, and the duration and area of ischemia also affected the results. Interestingly, ET-1 was expressed at the lowest level in the pretreadmill training group. On the basis of the results of CBF, behavior, and histology, we thought that the regulation of ET-1 is a mechanism of self-protection, and it was help for stabilizing CBF and weakening CBF changes to reduce the secondary damage. In a word, the effect of exercise on ET-1 expression after reperfusion is a complex and continuous process, which needs more improved studies to clarify.

This study has several limitations that should be noted. We just detected blood flow at 24 hours after reperfusion instead of continuous monitoring. Therefore, the blood flow changes at every time point were not captured, and continuous monitoring of CBF must be done in the future work. Cerebral blood analyzed here is overall blood, including venous and arterial blood of ischemic hemisphere; more work needs to be done to clarify the behavior of blood after ischemia in more detail.

In the present study, improvements in cerebral infarct volume and neurologic behavior have shown the neuroprotection of 2-week pres ischemic training after ischemia in rats, and exercise preconditioning is particularly beneficial to recovery after brain ischemia. In particular, the ET-1 expression was significantly changed and CBF was ameliorated at the same time, but the exact mechanism behind this is still unknown, and further study is needed to explore the exact effect and relationship between ET-1 and CBF on the pres ischemic treadmill training inducing neuroprotection. Clarifying these mechanisms may be a novel component of treating ischemia-induced brain damage.

References