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ORIGINAL ARTICLE

Modulating effects of preconditioning exercise in the expression of ET-1 and BNP via HIF-1α in ischemically injured brain

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Abstract

It is well-known that in ischemia-induced hypoxia, hypoxia-inducible factor -1α (HIF-1α) is critical in triggering expression of its downstream target genes to produce several products, such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), nitric oxide synthesis (NOS), glucose transportor-1 (GLUT-1), insulin-like growth factor (IGF), which further promote erythropoiesis, angiogenesis, vasodilation and capitalization of glucose to overcome hypoxia. Meanwhile, as the factors with opposite effects on blood vessels, endothelin-1 (ET-1) and brain natriuretic peptide (BNP) also stand out strikingly in ischemic pathophysiology. To this day, several preconditioning manners have been used to induce tolerance to ischemia. During our research, exercise preconditioning was applied and it was demonstrated that HIF-1α triggered expression of ET-1 and BNP, which confirmed their downstream target genes for HIF-1α. And ET-1 may influcence expression of BNP to some degree but not the only factor which regulates BNP expression. Therefore, our findings suggest exercise preconditioning may provide protection to the ischemic brain tissue via HIF-1α which in turn increases expression of BNP to cause vasodilation in cooperation with some other factors, such as VEGF and EPO, to increase the blood flow in the ischemic area and then relieve the injuries induced by ischemia.

Keywords Exercise preconditioning · Hypoxia-inducible factor -1α (HIF-1α) · ET-1 · BNP · Middle cerebral arterial occlusion (MCAO)

Introduction

Stroke, the commonly seen cerebral event, is per se ischemic hypoxic cerebral injury. Hypoxia-inducible factor-1 (HIF-1), serving as a transcription factor, plays a critical role in hypoxia pathophysiology process and growing attention has been drawn to the significance of HIF-1α (the active subunit of HIF-1) in this process. It is well-known that under normoxic conditions HIF-1α is unstable and degrades quickly whereas in the case of hypoxia, degradation of HIF-1α is inhibited so that it gradually accumulates in the cytoplasm and is subsequently transferred into the nuclei of cells where HIF-1a is bound to HIF-1β to form the complete active HIF-1 complex. Afterwards, this complex combines with HIF-1 responsive element (HRE) on DNA to modulate transcription of hundreds of target genes downstream (Semenza and Wang 1992; Zagorska and Dulak 2004). These target genes include genes for erythropoietin (EPO), vascular endothelial growth factor (VEGF), nitric oxide synthesis (NOS), glucose transportor-1 (GLUT-1), insulin-like growth factor (IGF) and via the effects of these products, HIF-1 stimulates erythropoiesis to enhance blood oxygen capacity, facilitates angiogenesis, limits production of oxidative stress (OS), promotes differentiation of neural stem cells (NSCs) and decreases neural apoptosis to provide protection for nervous tissue(Kaelin Jr. and Ratcliffe 2008). Moreover, some research has found out that ET-1 and BNP are also listed among the target genes under the control of HIF-1α at the level of transcription and they have the opposite effects on blood vessels: ET-1 has the strongest vasoconstrictive effects while BNP is a famous vasodilator. Three

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HIF-1α binding sites (HBSs) in the non-coding area of ET-1 gene in human umbilical vein endothelial cells (HUVECs) has been confirmed (Hu et al. 1998) and so is HIF-1 responsive element (HRE) in ET-1 gene in human microvascular endothelial cells (HMECs) (Minchenko and Caro 2000). HRE includes HBSs and some other functional sequences upstream and downstream of HBSs. Therefore, as mentioned above, ET-1 has been proved to be one of the target genes of HIF-1α. On the other hand, Wilhide ME (Wilhide and Jones 2006) has discovered that HIF-1α is also indispensible for activation of BNP gene, which indicates BNP's role in the network modulated by HIF-1α. ET-1 and BNP, are involved in many pathophysiological processes including ischemic injury. Hence, detection of the levels of HIF-1α, ET-1 and BNP and investigation of their intercorrelation are benefical to understand the pathophysiological processes of cerebral ischemic injury and will help seek prevention and treatment for cerebral ischemic injuries.

For the present, there are different preconditioning methods to induce tolerance to ischemia, such as hypoxia and exercise. Exercise preconditioning is a special neuronal ischemic preconditioning (NIPC), which can protect vital organs, such as heart and brain, against ischemic injuries. It is proved that 3-week exercise preconditioning before stroke improved the integrity of cerebral microvascular structure in rats (Ding et al. 2006a, b; Kang et al. 2011). Furthermore, study on exercise - preconditioned MCAO rats demonstrated that ET-1 was decreased by exercise preconditioning (Zhang et al. 2014). On the other hand, obvious shrinking of ischemic areas was accompanied by strikingly increased HIF-1α in the hypoxia-preconditioned rats' brains (Bernaudin et al. 2002) and concurrent relief of blood-brain barrier damages and cerebral edema (Masada et al. 2001). Hence, it is possible that ischemic preconditioning may maintain normal vascular endothelial functions, reduce damages on the blood-brain barrier and mitigate cerebral edema via inducement of HIF-1α and the subsequent expression of its target genes. Based on the proposed correlation between ET-1, BNP and HIF-1α, our research tried to prove the protective effects of exercise preconditioning on ischemic cerebral injury were produced by inducement of HIF-1α and subsequent expression of target genes, mainly ET-1 and BNP, and more importantly, to reveal the relationship among them.

Materials and methods

Animals

104 adult male Sprague-Dawley rats (250 g ± 10 g) were purchased from the Shanghai Slac Laboratory animal limited liability company. The rats were kept on an illumination cycle of 12 h with light and 12 h in darkness and housed at an ambient temperature of 24 ± 1 °C with food and water freely available. All surgical procedures were performed under anesthesia with pentobarbital sodium (400 mg/kg, i.p.). All animal procedures were approved by the Animals Care and Use Committees of Fudan University, Medical College, Shanghai, China.

Experimental design

All the rats were processed differently in 3 batches and received different treatments as shown in Fig. 1. The experimental design was displayed in Fig. 2:

Part I: As for the first batch of rats ($n = 54$), they were evenly randomly divided into Sham group, MCAO group and MCAO+exercise preconditioning group (i.e. MCAO+EP group) ($n = 18$). According to the method previously described by Longa, right middle cerebral artery occlusion was performed on MCAO group. As to Sham group, similar cervical operation was applied with the exception of occlusion of the right middle cerebral artery. As shown in Fig. 2, rats in MCAO+exercise preconditioning group were forced to have 2-day adaptive exercise prior to 3-week exercise preconditioning which was followed by MCAO. Just before the rats were put to death, the blood was drawn 24 h after the operation from the hearts of randomly selected 9 rats from each group to detect the blood levels of ET-1 and HIF-1a and then the rats were sacrificed to obtain cerebral tissue samples. The cerebral tissues from randomly selected 6 rats of each group were immediately used to detect infarct volume on the basis of TTC coloration. Another 6 cerebral samples from another 6 randomly chosen rats in the same group were used to measure the water content in the brain. And the corpus striata of the left 6 rats in the same group were specifically used to assay the striatum levels of HIF-1α, ET-1 and BNP via Western blotting.

Part II.: As for the second batch of rats ($n = 30$), they were evenly randomly assigned to 2 groups, i.e. MCAO group and MCAO+exercise preconditioning group (i.e. MCAO+EP group) ($n = 15$). 30 min prior to the operation of MCAO, NS, BQ123 (20 nmol; Sigma) or BQ788 (5.0 nmol; Sigma) was injected into the lateral cerebral ventricle of the rats, based on which different subgroups are named (i.e. NS group, BQ123 group and BQ788 group) ($n = 5$). 24 h after MCAO operation, corpus striatum was dissected to detect BNP levels in different subgroups via Western blotting. (BQ123: ET-1A receptor blocker; BQ788: ET-1B receptor blocker (Kreipke et al. 2010))

Part III: As for the third batch of rats ($n = 20$), they were evenly and randomly divided into MCAO group and MCAO + EP group ($n = 10$). 30 min prior to the operation of MCAO, 2ME2 dissolved in 1% DMSO and further diluted in phosphate buffer saline (PBS) (final<0.01% DMSO) (16 mg/kg;

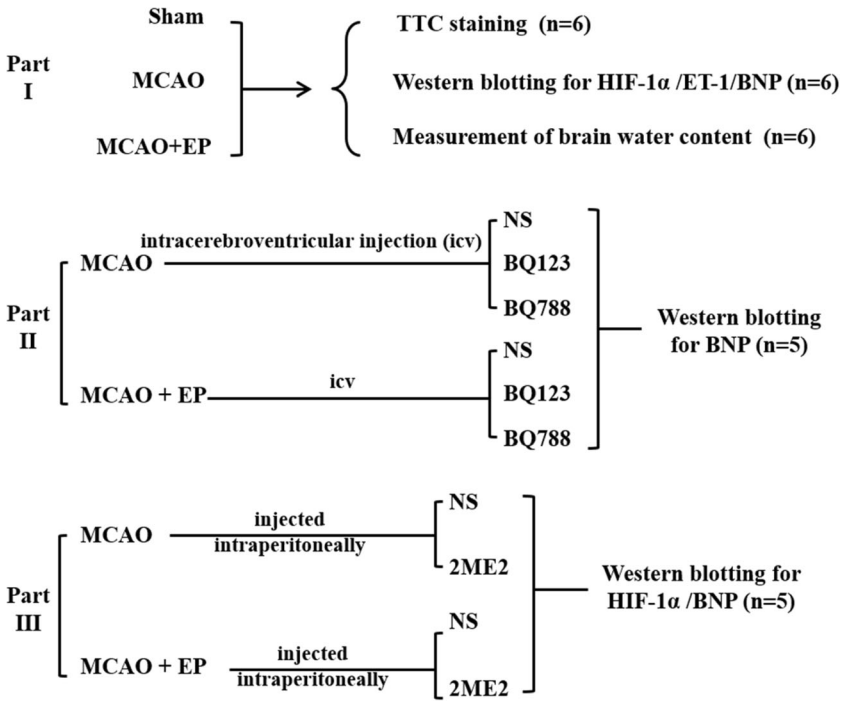

Fig. 1 The diagram to show grouping and treatments. The rats were processed and received treatments in 3 batches as detailed in Materials and Methods

Sigma) or the solution (DMSO diluted in PBS) of the same volume were injected intraperitoneally (i.p.) at the same time point to establish 2ME2 subgroup and vehicle subgroup, respectively ($n = 5$). 24 h after MCAO, corpus striatum was removed from the rats for the measurement of the levels of HIF-1α and BNP in the striatum. (2ME2: HIF-1α inhibitor (Li et al. 2011)).

Exercise preconditioning

In our research, exercise preconditioning was achieved by 3-week treadmill exercise with fixed frequency and duration. Immediately prior to the onset of 3-week treadmill exercise, an adaptive running exercise was enforced on the rats of MCAO + EP group in the manner of 5–8 m/min for 30 min/day for 2 days. After the adaptive running, the rats in MCAO + EP group started the formal training session on an electric treadmill machine (BW-YLS-15A Type 4-Lane Treadmill; Xinruan Biotechnology Co, Ltd., China), which was arranged as 20 m/min, 30 min/day for 5 days/week for 3 weeks. Whereas, the rats in sham and MCAO groups only ran freely in their cages for 3 weeks (Zhang et al. 2014).

Intracerebroventricular injection

30 min prior to ischemia / reperfusion surgery, saline(NS) / drugs (BQ123, BQ788) were injected into the lateral cerebral ventricle on the ischemic side (right side) of the rats of different subgroups, the process of which was as follows (Lu et al. 2015): after being anesthetized by intraperitoneal injection of 1% pentobarbital sodium, the rats were mounted in a stereotactic frame (RWD Biological technologies Co., LTD) and injected with 10μl solution containing drugs (BQ123 or BQ788) at the rate of 1μl/ min using the following stereotaxic coordinates: 0.8 mm posterior to the bregma, 1.5 mm right to the midline, and 4.5 mm ventral to the bregma. After the

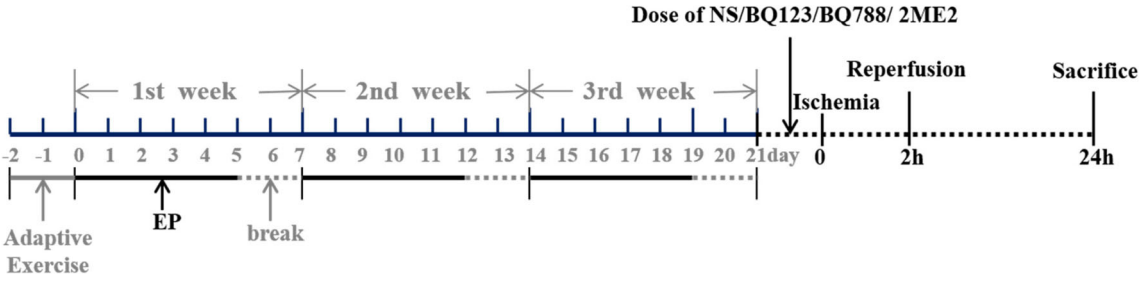

Fig. 2 Experimental flow chart. The treatments on the animals were administered in the following sequence as detailed in Materials and Methods part: exercise preconditioning, ischemia-reperfusion, being sacrificed under euthanasia, sampling

planned quantity of drugs or saline was injected, the needle was remained in the target location for 5 min more to avoid fluid reflux along the needle tract.

Method to induce cerebral ischemia

Rats with physiological variables within normal range were subjected to transient focal cerebral ischemia induced by right middle cerebral artery occlusion (MCAO) as previously described by Longa et al. (1989; Zhang et al. 2006; Zhou et al. 2011) as the following: After anesthetized with 1% pentobarbital sodium (40 mg/ kg i.p.), the right common carotid, external carotid and internal carotid arteries of the rats were surgically exposed and then the distal end of the right external carotid artery was ligated and incised. Next, a 4–0 monofilament nylon suture (30 mm in length, 0.18 mm in diameter with a 0.24 mm-diameter round tip) was introduced into the right external carotid 20 mm up until its origin, only then this nylon suture was fixed to totally occlude the blood flow in the right middle cerebral artery. After 2 h, this suture was removed to restore the blood flow in the middle cerebral artery (MCA) to obtain reperfusion. After reperfusion, the animals were housed for 24 h at an ambient temperature of 24 ± 1 °C. The same cervical surgical procedures were performed on the animals in sham group except for MCAO.

Measurement of brain water content

The water content of the brain tissue was measured by the dry–wet weight method. Before measurement of water content, the rats were anesthetized for MCAO (Young et al. 1987) and sacrificed 24 h after MCAO as described above. After sacrifice, the brain was dissected and immediately weighed on an electronic analytical scale to obtain the wet weight. Then brain samples were dried at 100 °C in an electric blast drying oven for 24 h to obtain the dry weight and the brain water content was calculated via the following formula: (wet weight − dry weight)/ wet weight×100% (Jiang et al. 2009; Zhao et al. 2015).

Measurements of ischemic and infarct volume by TTC

To determine the volume of ischemia and infarction, TTC was applied to demonstrate the ischemic and infarct regions and used as the basis for density quantification to compare the ischemic and infarct volume between different groups. The brain slices were prepared as 2.0 mm sections and incubated in triphenyltetrazolium chloride (TTC, 20 g/L) for 30 min at 37 °C before being transferred into paraformaldehyde solution (40 g/L) for fixing the infarct area. The infarct region appears white or pale while the "normal" tissue appears red. Infarct volume was calculated by sampling each side of the coronal sections with a digital camera (Nikon). The infarct area was measured by image analysis software (Adobe Photoshop CS4). Relative infarct ratio was calculated by using the following formula: (infarct volume/ total size) × 100% (Bhalla et al. 2016).

Determination of HIF-1α and ET-1 levels in plasma by Elisa

Blood samples were collected from the heart 24 h after MCAO. All the blood samples were immediately centrifuged at 3000 rpm for 10 min to obtain plasma. Total HIF-1α and ET-1 were assayed by colorimetric using an enzyme-linked immunosorbent assay kit (Total HIF-1α ELISA Kit DYC1935–2 from R&D systems; ET-1 ELISA Kit ab133030 from Abcam, USA) according to the manufacturer's instructions (Chawla et al. 2014).

Determination of the expression of HIF-1α, ET-1 and BNP in corpus striatum by Western blotting

Rats in each group were sacrificed 24 h after MCAO to dissect striatum. Then all the corpus striata were quickly collected on ice, frozen immediately in dry ice and kept at −70 °C until use. Subsequently, the corpus striata were homogenized and 50 μg of the homogenate was loaded for SDS-PAGE. Proteins were separated on a 10% resolving gel, and transferred to poly cellulose acetate membrane (Millipore). Then these membranes were blocked with 5% non-fat dry milk powder in 0.1% Tween20 (TBS-T; 2 mmol/ L Tris-HCl, 50 mmol/ L NaCl, pH 7.4) for 2 h at room temperature and followed by overnight incubation of these membranes with the primary antibody (Rabbit polyclonal to HIF-1 alpha, ab2185; Mouse monoclonal to ET-1, ab2786; Rabbit polyclonal to BNP, ab19645; Abcam) at a 1:1000 dilution at 4 °C in the blocked buffer. After that, membranes were washed with 0.1% Tween20 and then treated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:5000) for 1 h at 37 °C. Peroxidase activity was visualized with an enhanced chemiluminescence substrate system (ECL, Santa Cruz Biotechnology). Stripping filters and re-probing for β-actin were carried out for normalization. Controls for nonspecific binding were determined by omission of the primary antibody. Films were scanned with a film scanner (Image Master VDS; Amersham Biosciences Inc., Piscataway, NJ) and subsequently analyzed by measuring optical densities of immunostained bands on the film using an image-processing and analysis system (Q570IW; Leica).

Data analysis

All results were designated as mean ± SE. The Kolmogorov Smirnov test was used to verify the normality of the distribution. Two-way analysis of variance test was used to compare the differences between the groups. The results between two

groups were analyzed by independent-sample T test. $P < 0.05$ was considered to be significant. All data were analyzed by using the SPSS software package.

Results

The protective effects of exercise preconditioning on cerebral ischemia-reperfusion injury in rats by determination of the effects of exercise preconditioning by TTC coloration, evaluation of ischemia and infarct volume and the assessment of water content in the brain via dry-wet weight method

As for all the rats in three groups (MCAO, MCAO+EP, Sham), 2 mm-thin sections through the brain were made 24 h after MCAO and used for TTC coloration. As shown in TTC coloration (Fig. 3a), ischemic and infarct region was pale in appearance while the normal region was red. The infarct volume was calculated on the basis of the surface area of the ischemic and infarct region and the thickness of the sections. As shown in Fig. 3b, the infarct volume in MCAO+ EP group was significantly smaller than that in MCAO group ($t = 4.47$, $p = 0.001$).

As shown in Fig. 3c, the water content in MCAO group and MCAO+ EP group were both obviously higher than that in Sham group (vs. MCAO group: $t = 16.52$, $p < 0.001$; vs. MCAO+ EP group: $t = 6.87$, $p < 0.001$) and MCAO+ EP group contained less water than MCAO group ($t = 8.43$, $p < 0.001$).

Effects of exercise preconditioning on plasma ET-1 and BNP by Elisa 24 h after cerebral ischemia-reperfusion injury in rats

As shown in Fig. 4a, Sham group expressed markedly lower ET-1 (vs. MCAO group: $t = 9.99$, $p < 0.001$; vs. MCAO + EP group: $t = 10.45$, $p < 0.001$). Furthermore, MCAO + EP group expressed lower ET-1 than MCAO group($t = 3.11$, $p = 0.007$).

As shown in Fig. 4b, BNP in Sham group was evidently lower than that in MCAO group ($t = 18.17$, $p < 0.001$) and MCAO + EP group($t = 11.88$, $p < 0.001$). Moreover, MCAO + EP group expressed higher BNP in comparison with MCAO group ($t = 4.41$, $p < 0.001$).

Effects of exercise preconditioning on the levels of HIF-1 α, ET-1 and BNP in corpus striatum via Western blotting 24 h after cerebral ischemia-reperfusion injury (β-actin as the internal reference)

As displayed in Fig. 5a and b, MCAO+EP group expressed the highest HIF-1α in comparison with the other two groups (vs. Sham group ($t = 5.18$, $p < 0.001$); vs. MCAO group ($t = 12.46$, $p < 0.001$)) and HIF-1α in MCAO group was significantly higher than that in Sham group ($t = 6.74$, $p < 0.001$).

As for ET-1, MCAO group displayed the highest level of ET-1 in comparison with the other two groups (vs. Sham group ($t = 13.12$, $p < 0.001$); vs. MCAO + EP group ($t = 6.17$, $p < 0.001$)) and furtherly MCAO+ EP group expressed higher ET-1 than Sham group ($t = 9.56$, $p < 0.001$).

With regard to BNP, MCAO+ EP group also expressed the highest level in comparison with the other two groups (vs. Sham group ($t = 15.19$, $p < 0.001$); vs. MCAO group ($t = 5.41$, $p = 0.001$)) and furtherly MCAO group had the higher ET-1 compared with Sham group ($t = 15.95$, $p < 0.001$).

Exercise preconditioning increased the expression of BNP in the striatum 24 h after ischemia-reperfusion injury and respective administration of ET-1A blocker or ET-1B blocker decreased the expression of BNP to some extent

Measurement of BNP in corpus striatum via Western blotting after administration with NS, BQ123 or BQ788 (ET-A, ET-B receptor blocker, respectively) into the lateral cerebral ventricle on the ischemic side showed the following results:as displayed in Fig. 6a and b, as for the rats in MCAO groups administered with BQ123 or BQ788, expression of BNP in corpus striatum was significantly lower than that in NS group ($t = 5.69$, $p < 0.001$; $t = 5.45$, $p = 0.001$). It was same as in MCAO+EP groups: rats in MCAO+EP group applied with BQ123 or BQ788 expressed obviously lower BNP in striatum than those with only NS ($t = 7.27$, $p < 0.001$; $t = 11.72$, $p < 0.001$). Furthermore, in the case of NS administration, there was markedly higher BNP in MCAO+EP group compared with MCAO group ($t = 5.01$, $p = 0.001$). Meanwhile, in the case of BQ123 or BQ788 administration, MCAO+EP group expressed higher BNP than MCAO group ($t = 6.13$, $p < 0.001$; $t = 5.97$, $p < 0.001$) Fig. 7.

Exercise preconditioning increased the expression of HIF-1α and BNP 24 h after ischemia-reperfusion injury and 2ME2 significantly downregulated the expression of 1α and BNP

After intraperitoneal application with 2ME2 or vehicle of the same volume to the rats in MCAO group and MCAO+EP group the following results were shown: as suggested by Fig. 6a and b, it was found out that the expression of HIF-1α and BNP displayed the same tendency: MCAO+EP + vehicle group expressed the highest HIF-1α and BNP (vs. MCAO+ vehicle group ($t = 5.71$, $p < 0.001$; $t = 5.37$, $p = 0.001$); vs. MCAO+2ME2 group ($t = 33.01$, $p < 0.001$; $t = 18.42$, $p < 0.001$); vs. MCAO+EP + 2ME2 group ($t = 36.15$, $p < 0.001$; $t = 18.25$, $p < 0.001$)). Moreover, MCAO+vehicle

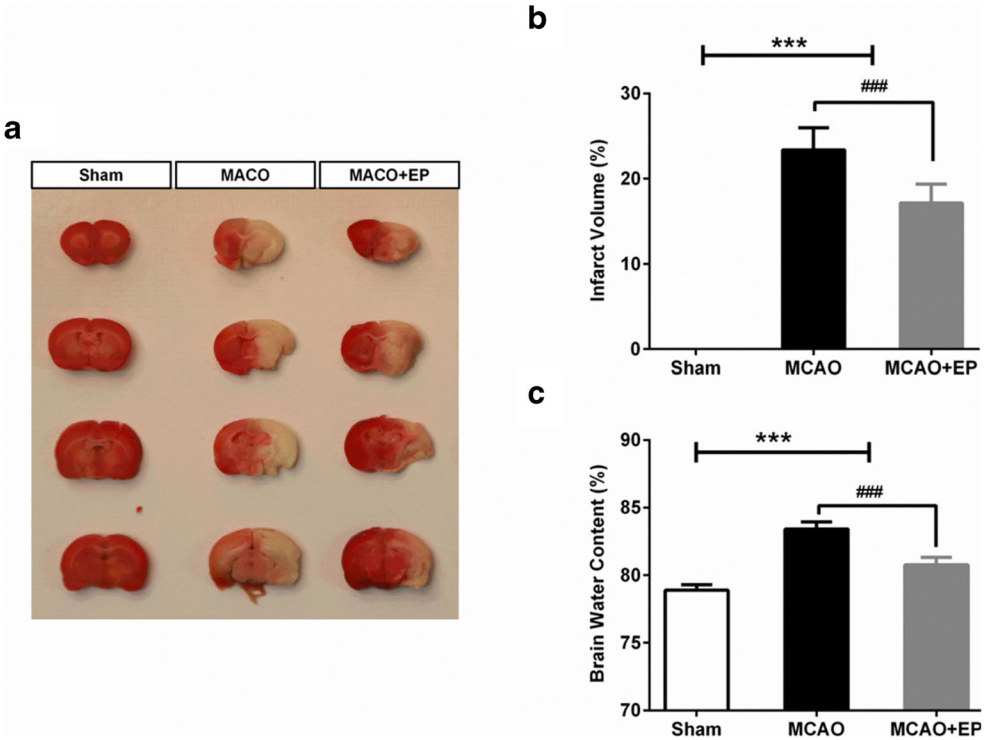

Fig. 3 The protective effects of exercise preconditioning on rat cerebral ischemia- reperfusion injury. a Display of the infarct region in the brain of different groups (Pale region represented the ischemia region while red region represented non- ischemia region). As shown in the images, the infarct region in MCAO+EP group was smaller than that in MCAO group. **b** Statistical comparison of the infarct volume in different groups. As shown in the diagram, the infarct volume in MCAO+ EP group is markedly less than that in MCAO group ($p < 0.001$). **c** Comparison of water content in the brain of different groups via the dry-wet weight method. Water content in MCAO and MCAO+ EP groups was significantly higher than that in Sham group ($p < 0.001$) and furthermore the water content in MCAO+ EP group was markedly lower than that in MCAO group ($p < 0.001$). *** $p < 0.001$ vs. Sham group; $^{###}p < 0.001$ vs. MCAO group

group expressed markedly lower levels of HIF-1α and BNP in comparison with MCAO+EP + vehicle group (t = 5.71, $p < 0.001$; t = 5.37, $p = 0.001$) but higher levels than MCAO+ 2ME2 group (t = 8.34, $p < 0.001$; t = 11.34, $p < 0.001$) and MCAO+EP+ 2ME2 group (t = 8.15, $p < 0.001$; t = 11.16, $p < 0.001$). And there was no significant difference between MCAO+2ME2 group and MCAO+EP + 2ME2 group (t = 1.14, $p = 0.30$; t = 0.75, $p = 0.48$).

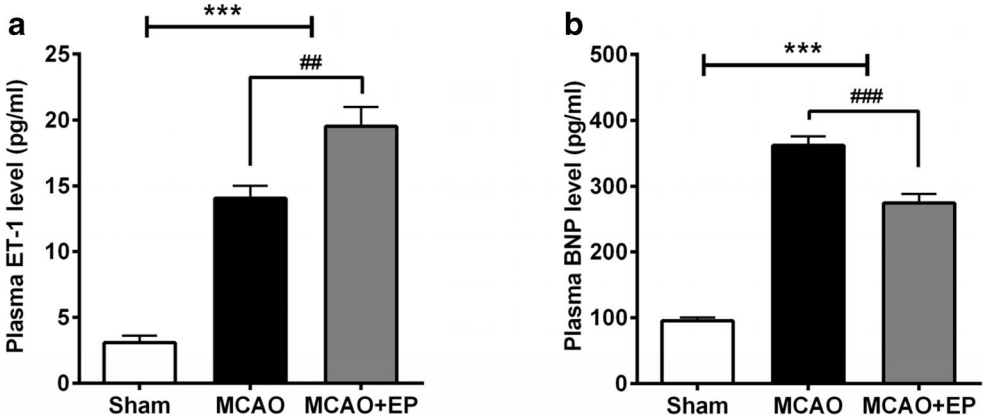

Fig. 4 Effects of exercise preconditioning on the levels of ET-1 and BNP 24 h after cerebral ischemia-reperfusion injury via Elisa. A:ET-1 level in MCAO and MCAO+ EP groups was significantly higher than that in Sham group ($p < 0.001$). Furthermore, ET-1 in MCAO+ EP group was obviously lower than that in MCAO group ($p < 0.01$). B: BNP level in MCAO and MCAO+ EP groups was strikingly higher than that in Sham group ($p < 0.001$) whereas BNP level was significantly higher in MCAO+ EP group than that in MCAO group ($p < 0.001$). *** $p < 0.001$ vs. Sham group; $^{##}p < 0.01$, $^{###}p < 0.001$ vs. MCAO group

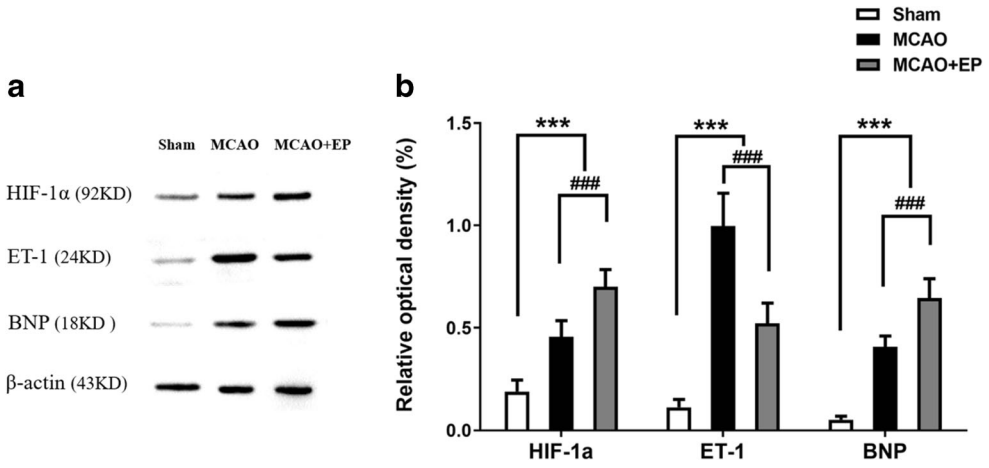

Fig. 5 Effects of exercise preconditioning on the expression levels of HIF-1 α, ET-1 and BNP in corpus striatum 24 h after cerebral ischemia-reperfusion injury. a Detection of HIF-1α, ET-1 and BNP in the corpus striatum 24 h after MCAO by Western blotting (β-actin as the internal reference). **b** Comparison in HIF-1α, ET-1 and BNP levels between different groups on the basis of density quantification. 24 h after MCAO, MCAO+ EP group and MCAO group both displayed obviously higher HIF-1α level than Sham group ($p < 0.001$) whereas between MCAO group and Sham group, MCAO group displayed significantly higher HIF-1α than Sham group ($p < 0.001$). On the other hand, 24 h after MCAO, the level of ET-1 in MCAO group was the highest among all the three groups ($p < 0.001$) and furthermore ET-1 in MCAO+EP group was significantly higher than that in Sham group ($p < 0.001$). As for the level of BNP, 24 h after MCAO, MCAO+ EP group expressed the highest BNP in comparison with Sham group and MCAO group ($p \leq 0.001$) and the expression of BNP in MCAO group was obviously higher than that in Sham group ($p < 0.001$). *** $p < 0.001$ vs. Sham group; $^{###}p \leq 0.001$ vs. MCAO group

Discussion

As described in previous studies, frequent exercise relieved abnormally increased blood pressure, obesity, metabolic anomalies of glucose and lipids and abnormal hemodynamic changes (Chrysohoou et al. 2005; Lee et al. 2003). Furthermore, exercise preconditioning may induce cerebral tolerance to ischemia, enhance neuroprotection and resist a cascade of subsequent cerebral injury caused by ischemia (Stetler et al. 2014; Zhang et al. 2011). Nahid (Aboutaleb et al. 2015) found out that exercise preconditioning reduced the infarct volume caused by ischemia/reperfusion and inferred that this shrinking was possibly caused by the inhibitory impact of exercise on the activity of caspase-3 in CA3 region of hippocampus, which in turn led to a down-regulation of expression of Bcl-2 and the subsequent inhibition of neuronal apoptosis. And the study carried out by Curry et al. (2010) displayed that 3-week treadmill exercise reduced inflammatory responses after ischemic injury in SD rats via inhibiting TNF-α. Moreover, Wang et al. (2015) proved that the reduced cerebral infarct volume induced by moderate exercise preconditioning may be related to the up-regulation of cerebral HIF-1α expression.

As a safe preconditioning manner which can induce tolerance to ischemia, exercise preconditioning is supposed to be accepted as a feasible prevention or treatment for cerebral stroke. But its unclear protective mechanism limited its application in the aspect of prevention and rehabiliation of cerebral ischemic injury. Therefore, studies aiming at disclosing the protective mechanism of exercise preconditioning are drawing more and more attention, which is also the very objective of our research. In our study, we performed the same exercise paradigm of 3 weeks exercise preconditioning as previously depicted (Aboutaleb et al. 2015; Wang et al. 2015) and our results of decreased infarct volume in MCAO+ EP group in comparison with simply ischemia/reperfusion group (MCAO group) are in line with those past reports (Curry et al. 2010; Stetler et al. 2014; Zhang et al. 2011). Apart from this, we also determined water content in the brain to understand the state of cerebral edema and proved that milder cerebral edema was in MCAO+ EP group compared with MCAO group, which is also in accordance with previous research (Ding et al. 2006a, b).

Among all human organs, brain is the most sensitive one to the alteration of oxygen content and it is well-known that ischemia will definitely cause hypoxia, even though not vice versa. HIF-1α is the vital transcription factor, which senses oxygen content and modulates cellular adaption to hypoxia, which suggests that HIF-1α plays an important role in the pathogenesis of cerebral ischemic diseases (Yeh et al. 2011). In various cases of hypoxia, whether it is systemically or locally, there was confirmed activation of HIF-1α to induce its downstream transcription-related proteins so as to promote a series of responses to hypoxia (Lu and Kang 2010; Wacker et al. 2012). Matsuda et al. (2005) revealed that after spray of HIF-1α DNA on the surface of the rats' brain subjected to cerebral ischemia there was an

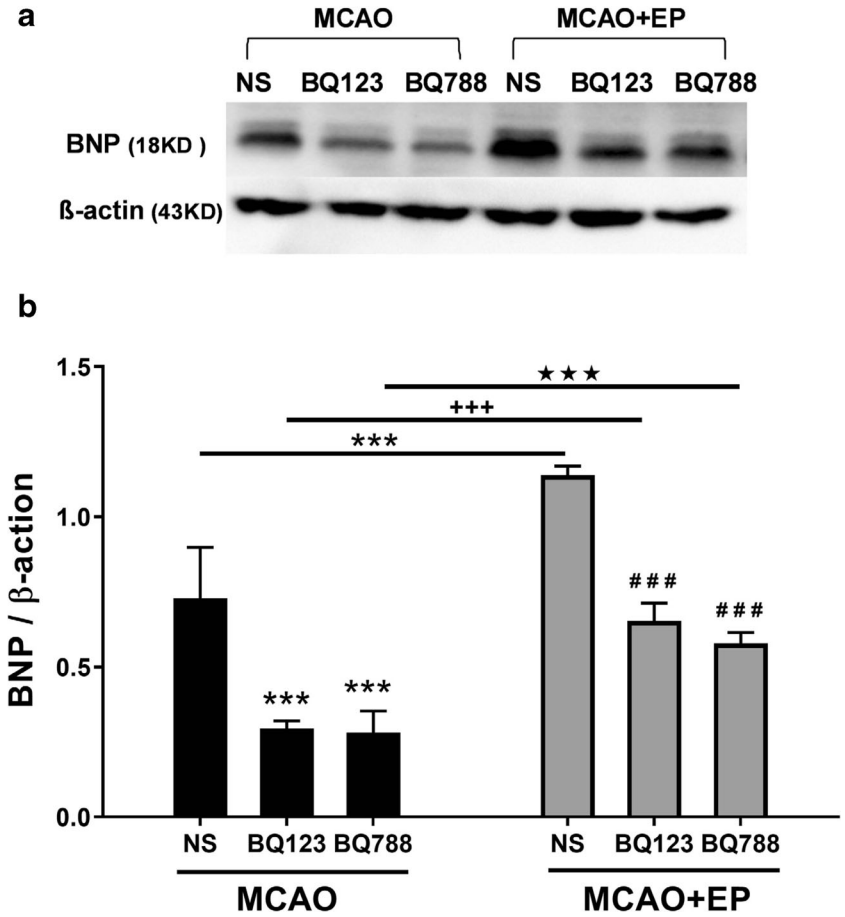

Fig. 6 Exercise preconditioning enhanced the expression of BNP 24 h after ischemia-reperfusion injury and respective administration of ET-1A receptor blocker and ET-1B receptor blocker down-regulated the expression of BNP to some degree. a Measurement of the level of BNP in corpus striatum by Western blotting after injection of NS, BQ123 or BQ788 into lateral cerebral ventricle of the rats of MCAO and MCAO+ EP groups 30 min before MCAO operation. **b** Comparison in the level of BNP between different groups on the basis of density quantification. MCAO+EP + NS group displayed higher BNP than MCAO+NS group ($p = 0.001$). MCAO+BQ123 group and MCAO+ BQ788 group displayed markedly lower level of BNP than the MCAO+NS group ($p \leq 0.001$), which was parallel with the comparison in BNP levels between MCAO+ EP groups applied with BQ123 or BQ788 and MCAO+EP + NS group (i.e. MCAO+ EP + BQ123 group or MCAO+EP + BQ788 group displayed lower BNP vs. MCAO+ EP + NS group) ($p < 0.001$). Moreover, MCAO+ EP groups applied with BQ123 or BQ788 expressed significantly higher BNP than MCAO groups applied with BQ123 or BQ788($p < 0.001$). $^{***}p \leq 0.001$ vs. NS group among MCAO groups; $^{\#\#\#}p \leq 0.001$ vs. NS group among MCAO+ EP groups; $^{+++}p < 0.001$ represents MCAO+EP + BQ123 group vs. MCAO+BQ123 group; $^{\star\star\star}p < 0.001$ represents MCAO+EP + BQ788 group vs. MCAO+BQ788 group

increase in HIF-1α and VEGF proteins and enhancement of collateral circulation, which in turn promoted angiogenesis and improved cerebral blood circulation to offer neuroprotection. Ogle et al. (2012) administered dimethyloxaloylglycine (DMOG), the inhibitor to prolyl hydroxylases (PHDs), on the local ischemic/reperfusion animal model and demonstrated obvious increase in expression of the downstream target genes of HIF-1α, such as VEGF, EPO and iNOS, which produced neuroprotection via enhancing blood flow to shrink the infarct area. In line with these findings, in HIF-1α knock-out models, neuroprotection caused by PHDs inhibitors was reduced markedly, which was represented by display of more neurons loss. Bemaudin et al. (2002) applied hypoxia preconditioning at normal pressure on rats 24 h before inducement of local ischemia and found that the cerebral infarct volume in the hypoxia preconditioning group was reduced by 30% in comparison with the control group along with increased expression of HIF-1α and its target genes, such as EPO and VEGF genes, which indicated the important role of HIF-1α in ischemia tolerance was possibly developed via modulating expression of its various downstream target genes.

Inferred from that hypoxia induces expression of HIF-1α and subsequent expression of its downstream target genes, which in turn provides neuroprotection against ischemic injury via several mechanisms, hypoxia has been tried to cause preadaptation to ischemia. To furtherly find out whether there is any relation between

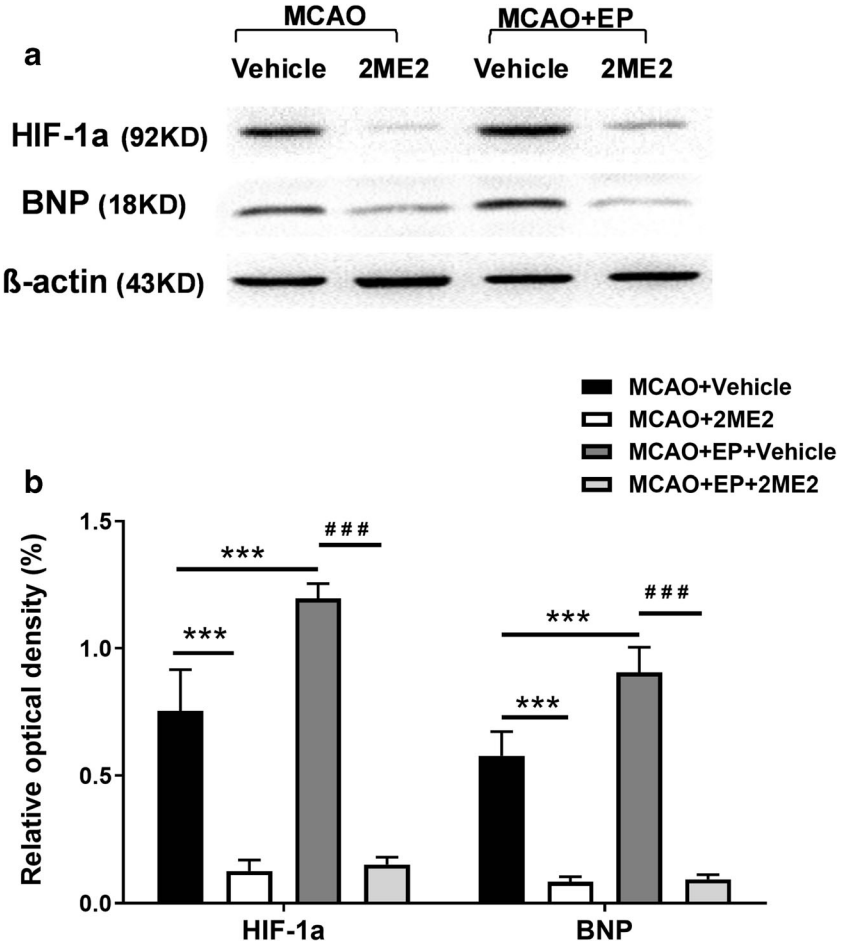

Fig. 7 Exercise preconditioning enhanced the expression of HIF-1α and BNP in corpus striatum 24 h after ischemia-reperfusion injury and administration of 2ME2 (HIF-1α inhibitor) significantly lowered the expression of HIF-1α and BNP. a Detection of the levels of HIF-1αand BNP in corpus striatum via Western blotting after administration of vehicle or 2ME2 (inhibitor of HIF-1α) 30 min previous to MCAO (β-actin served as the internal reference). **b** Comparison in the levels of HIF-1αand BNP between different groups on the basis of density quantification. As shown in Fig. 7B, the alteration in the expression of HIF-1αand BNP under the influence of different treatment was similar. MCAO +Vehicle group expressed higher HIF-1α and BNP than MCAO +2ME2 group($p < 0.001$), MCAO + EP + Vehicle group displayed higher HIF-1αand BNP than MCAO + EP + 2ME2 group($p < 0.001$) and MCAO + EP + Vehicle group displayed higher HIF-1α and BNP than MCAO +Vehicle group($p < 0.001$). $^{***}p \leq 0.001$ vs. MCAO +Vehicle group; $^{\#\#\#}p \leq 0.001$ vs. MCAO + EP + Vehicle group

exercise preconditioning and HIF-1α, exercise preconditioning was used in our study and consequently significantly higher expression of HIF-1α in corpus striatum on the ischemic/reperfusion side was observed in exercise preconditioning group, which is consistent with the study performed by Wang et al. (2015). Based on this result, we proposed the possible protective role of HIF-1α and its downstream target genes in the case of exercise preconditioning.

On the other hand, endothelin (ET) and its receptors are widely distributed in nervous system and ET-1 is the strongest vasoconstrictor among all ET subtypes. Hence, it was hypothesized that ET-1 could be one critical factor involved in cerebral ischemic injury. Barone et al. (1994) demonstrated higher ET expression in the cortex of systemic cerebral ischemia animal models compared to the control. Alioglu et al. (2002) measured the ET-1 content in 30 patients suffering from cerebral infarction and displayed that the content of ET-1 in patient group was significantly higher compared with that of the healthy control of the same age. And other previous studies also confirmed that ET was one important molecule to modulate vasoactivity and higher expression of ET-1 accompanied by strong vasoconstriction in cerebral blood vessels was the major pathological response to cerebral ischemia (Cheng et al. 2016; Jamison et al. 2011). Therefore, the research mentioned here all suggests a possible increase in ET-1 in the case of cerebral ischemia injury. Normally, it is known that ET-1 is secreted mainly by endothelial cells of the blood vessels whereas blood-brain barrier is impermeable to ET-1, hence, ET-1 detected in the brain tissue is produced

by the brain itself. Diverse areas in the brain, including the hypothalamus, express ETs and their receptors. Apart from hypothalamus, corpus striatum is also one of the major sites to express ET-1 in the brain. Likewise, BNP is found present both outside and inside of the brain. Therefore, Fig.4 was used to display the changes in the content of BNP and ET-1 in the peripheral blood after ischemia-reperfusion while Figs.5 and 6 were used to show the changes in the content of BNP and ET-1 in corpus striatum. Local contents of BNP and ET-1 have effects on the local vasoconstriction and vasodilation, therefore they may influence the developments of cerebral ischemia- reperfusion injuries. Furtherly, we administered HIF-1α blocker and ET-1 receptor blocker, respectively, to observe the changes in the content of BNP in corpus striatum after cerebral ischemia-reperfusion injury for the purpose of determining whether the expression of BNP was influenced by these two elements and whether exercise preconditioning produced its protective effects through modulating the expression of HIF-1α, ET-1 and BNP. In our research, higher ET-1 was observed both in the striatum and blood in MCAO group when compared with Sham group and moreover, MCAO + EP group displayed significantly less amount of ET-1 in comparison with that in MCAO group, which coincides with the previous reported results.

On the basis of previous studies, ET-1 gene is regarded as one of HIF-1α downstream target genes modulated by HIF-1α at the transcription level, which is supported by several studies mentioned below. Hu et al. (1998) detected that there were three HIF-1α binding sites (HBSs) in the non-coding strand of ET-1 gene in human umbilical vein endothelial cells (HUVECs). Minchenko et al. (2000) found HIF-1α responsive element (HRE) in ET-1 in human microvascular endothelial cell (HMEC), which was indicative of ET-1 as the downstream target gene for HIF-1α. Furthermore, Gras et al. (2012) inferred from their experiment that inflammation and blood vessels reconstruction caused by chronic intermittent hypoxia were under the influence of HIF-1α and ET-1. Robitaille et al. (2004) proposed that activation of ET-1 by TGFG-β1 was actually via HIF-1. Kakinuma et al. (2002) displayed that HIF-1α enhanced the expression of ET-1 in the heart suffering from heart failure to adjust to overactivation of glycolysis due to metabolic dysfunction. In summary, all these studies stated clearly that the expression of ET-1 after ischemia and hypoxia may be modulated by HIF-1α. Our results that increased expression of HIF-1α and ET-1 in the corpus striatum on the ischemic/reperfusion side is also identical with the studies mentioned above.

BNP is a peptide hormone with various effects serving as the important modulator in somatic and local tissues. It promotes sodium excretion, diuresis and vasodilation to lower blood pressure and inhibits overaction of renin angiotensin system (RASS) and secretion of antidiuretic hormone (ADH) (Maack 2006; van der Zander et al. 2002). BNP is present not only in the heart but also in the brain and lung. With regard to central nervous system (CNS), BNP content is rich in supraoptic nucleus and paraventricular nucleus of hypothalamus and trigeminal ganglia but the highest in medulla and corpus striatum and lowest in hippocampus and hypophysis (Aburaya et al. 1991). Several research over the past years pointed out that not only ET but also BNP increased abnormally in acute cerebrovascular diseases and the degree of elevation was parallel with the severity of the diseases as well as related to prognosis of the diseases. Therefore, BNP and ET may be used as the indicator for assessing severity of acute cerebrovascular diseases and predicting prognosis of the diseases (McGirt et al. 2004). At present, great amount of clinical studies demonstrated marked increase in BNP content in the case of acute cerebral infarction (ACI) (Iltumur et al. 2006) and further it was stated that BNP amount was positively correlated with severity of cerebral ischemic injury so that it could be indicative of mortality rate in acute phase of ACI (Makikallio et al. 2005; Tomita et al. 2008). More interestingly, some studies also found out after intravenous push of BNP to the intracerebral hemorrhage rat model, there was obvious increase in cerebral blood flow and relief of inflammation, which aided in restoration of nervous functions and provided protection to the brain (James et al. 2009). Our present research proved the increase in BNP content both in the striatum and blood in MCAO group, which is in consistent with the previous studies. And it is noteworthy there was even greater significant elevation in BNP content in MCAO + EP group in comparison with MCAO group.

There were different views about the reason for elevated BNP. On one hand, several studies have stated that BNP was one of the downstream target genes for HIF-1α. Some researches discovered that BNP induced by hypoxia was dependent on HIF-1α and expression of human BNP gene could be activated by the formation of hybridized factor: HIF-1α/VP16 (Wilhide and Jones 2006). Hence, it is possible that hypoxia first induced expression of HIF-1α, which was followed by subsequent activation and expression of BNP mRNA. On the other hand, there were studies holding that ET-1 could also cause expression of BNP. Bruneau et al. (1997) revealed that in isolated rat atria, stretching and administration of ET-1 could both activate atrial myocytes to produce and secrete BNP. Likewise, ET-1 could also activate CNS to secrete BNP (Kuwahara et al. 1998).

To distinguish the impact of HIF-1α and ET-1 on BNP expression, our present research separately applied HIF-1α inhibitor and ET receptor blocker to observe alteration in BNP content in MCAO rats and MCAO + EP rats. For one thing, we administered ET-A receptor blocker, BQ123 and ET-B receptor blocker, BQ788, to blockade binding between ET-1 and its receptor (A and B) and found out there was general decrease in BNP content in striatum by 50% in comparison with NS control both in MCAO group and MCAO + EP group. And further statistical comparison showed that there was still significantly higher amount of BNP in MCAO + EP group when compared with MCAO group. Therefore, application of ET receptors blocker, BQ123 and BQ788 (Kreipke et al. 2010), can only partly blockade BNP expression in striatum. For another thing, we also applied HIF-1α inhibitor, 2ME2 [55] to observe alteration in HIF-1α and BNP levels since 2ME2, as a reliable HIF-1α inhibitor, can blockade production and expression of HIF-1α mRNA (Wu et al. 2013). And it was proved in our study there was a significant decrease in HIF-1α content to less than 10% of that produced in Solvent Control in the striatum of the ischemia/reperfusion rats, which is just another solid proof that 2ME2 can block expression of HIF-1α. Besides, there was no significant difference in the quantity of BNP protein between MCAO group and MCAO+EP group. Therefore, 2ME2 can produce an almost cut-off inhibition on HIF-1α expression and led to no significant difference between MCAO and MCAO+EP group. As a result, we hypothesize that BNP expression is predominantly controlled by HIF-1α.

In summary, from our present research, we propose that in the case of cerebral ischemia / reperfusion injury, exercise preconditioning possibly first up-regulates expression of HIF-1α and under the control of which, expression of BNP is induced to produce neuroprotection.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Abbreviations BNP, Brain natriuretic peptide; ET-1, Enthothelin-1; ELISA, Enzyme linked immunosorbent assay; EPO, Erythropoietin; EP, Exercise preconditioning; GLUT-1, glucose transportor-1; HMECs, Human microvascular endothelial cells; HUVECs, Human umbilical vein endothelial cells; HIF-1α, Hypoxia-inducible factor -1α; HBSs, HIF-1α binding sites; HRE, HIF-1 responsive element; IGF, Insulin-like growth factor; MCAO, Middle cerebral arterial occlusion; NSCs, Neural stem cells; NIPC, Neuronal ischemic preconditioning; NOS, Nitric oxide synthesis; OS, Oxidative stress; NS, Physiological Saline; TTC, Triphenyltetrazolium chloride; VEGF, Vascular endothelial growth factor

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