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Original Article

Limb Ischemic Perconditioning Attenuates Blood-Brain Barrier Disruption by Inhibiting Activity of MMP-9 and Occludin Degradation after Focal Cerebral Ischemia

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ABSTRACT: Remote ischemic perconditioning (PerC) has been proved to have neuroprotective effects on cerebral ischemia, however, the effect of PerC on the BBB disruption and underlying mechanisms remains largely unknown. To address these issues, total 90 adult male Sprague Dawley (SD) rats were used. The rats underwent 90-min middle cerebral artery occlusion (MCAO), and the limb remote ischemic PerC was immediately applied after the onset of MCAO. We found that limb remote PerC protected BBB breakdown and brain edema, in parallel with reduced infarct volume and improved neurological deficits, after MCAO. Immunofluorescence studies revealed that MCAO resulted in disrupted continuity of claudin-5 staining in the cerebral endothelial cells with significant gap formation, which was significantly improved after PerC. Western blot analysis demonstrated that expression of tight junction (TJ) protein occludin was significantly increased, but other elements of TJ proteins, claudin-5 and ZO-1, in the BBB endothelial cells were not altered at 48 h after PerC, compared to MCAO group. The expression of matrix metalloproteinase (MMP-9), which was involved in TJ protein degradation, was decreased after PerC. Interestingly, phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2), an upstream of MMP-9 signaling, was significantly reduced in the PerC group. Our data suggest that PerC inhibits MMP-9-mediated occludin degradation, which could lead to decreased BBB disruption and brain edema after ischemic stroke.

Key words: ischemic stroke, remote ischemic perconditioning, blood-brain barrier, tight junction, matrix metalloproteinases

The blood-brain barrier (BBB) is a physical and metabolic interface that segregates the central nervous system (CNS) from the peripheral circulation in addition to regulating the CNS microenvironment [1]. Under physiological conditions, BBB integrity is maintained by endothelial cell through tight junctions (TJs) and the basal lamina, limiting paracellular movement of ions, solutes, and water [2]. Yet, TJs are key components of the BBB structure that seal the gaps between adjacent endothelial cells and restrict paracellular permeability [3]. The TJ proteins, occludin and claudins, are important molecules in forming this seal [4]. *In vivo*, cerebral ischemia induces claudin-5

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redistribution and occludin degradation [5], leading to a loss of junctional integrity and BBB disruption, which further cause an increased risk of brain vasogenic edema and hemorrhagic transformation [6-8]. Recent studies show that the members of the matrix metalloproteinase (MMP) family are activated during cerebral ischemic stroke, and can degrade the basal lamina proteins around the cerebral vasculature [2, 9, 10]. Yang et al reported that increased MMP-9 and MMP-2 resulted in fragmentation/relocalization of TJ proteins, occludin and claudin-5, after transient focal ischemia [9]. In addition, MMP-9 knockout mice significantly reduced infarct volume, edema and BBB disruption, which were associated with reduced expression of TJ protein, zonula occludens-1 (ZO-1) [11], suggesting that ischemia-induced disruption of tight junction proteins is mediated by MMPs. A very recent study also showed that increased BBB permeability is associated with activation of MEK/ERK1/2 in brain microvessels [12]. While ERK signaling regulates MMP-9 transcription after cerebral ischemia [13].

Limb remote ischemic PerC is a new therapeutic strategy for ischemic stroke [14-16]. A randomized clinical trial has found that PerC treatment has immediate neuroprotective effects in patients with acute ischemic stroke [17]. However, the underlying mechanisms are unclear. A recent study showed that limb remote preconditioning protects against brain edema and BBB permeability [18]. Similarly, our previous study showed that local ischemic postconditioning (applied after reperfusion) induced by occluding bilateral common carotid arteries could attenuate the expression of MMP-9 in the rat following focal ischemic stroke [19]. Because of the unpredictable property of the ischemic disease, the clinical application of ischemic preconditioning is limited. At the same time, local ischemic postconditioning may also be limited due to the characteristics and properties of inducing ischemic conditioning in vital organs [20].

Therefore, in this study, we investigated whether PerC could protect brain edema and BBB disruption during brain injury after focal ischemic stroke. In addition, the mechanisms underlying the protection of BBB dysfunction was also investigated. Our study suggested that PerC ameliorates brain edema and BBB disruption after ischemic stroke, and was associated with: 1) reduced phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) and MMP-9 expression, and 2) increased expression of TJ protein occludin.

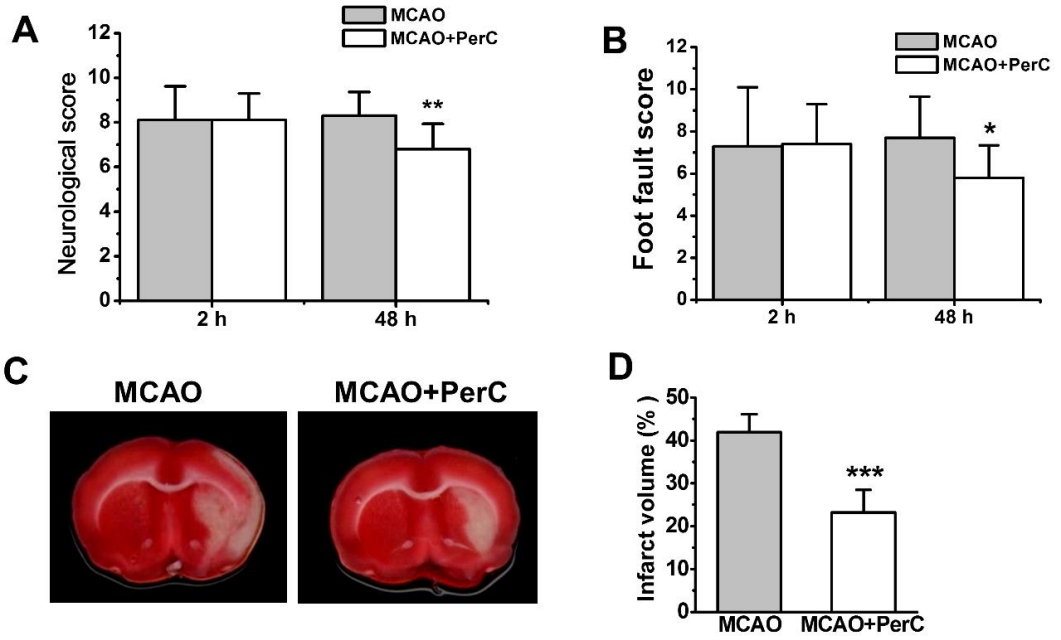

Figure 1. Limb remote ischemic PerC significantly reduced ischemic injury. (**A**) Neurological deficits were determined using neurobehavioral scoring system (higher scores correspond with more severe deficits) after MCAO. (**B**) Motor function was determined by ladder rung walking test (higher scores correspond with more severe deficits). **P*<0.05 and ***P*<0.01, versus MCAO group. N=10 per group. Error bars indicate SD. (**C**) Representative image of TTC-stained coronal brain slices at 48 h of reperfusion in MCAO group and MCAO + PerC group. (**D**) Quantitative analysis of infarct volume in MCAO group and MCAO + PerC group. ***P*<0.01, versus MCAO group. N=5 per group.

MATERIALS AND METHODS

Animals

All animal experiments were approved by Animal Care and Use Committee of Xuanwu Hospital, Capital Medical University, China, and conducted according to National Institutes of Health guidelines. Total 90 adult male Sprague–Dawley rats (280 to 320 g) were used in this study (Vital River Laboratories, Beijing, China). Animals were maintained on a 12-hour light/dark cycle with unlimited access to food and water.

Focal cerebral ischemia

1.5–3.5% enfluane in 70% nitrous oxide and 30% oxygen (Bickford veterinary anesthesia equipment model no. 61010; AM Bickford Inc., Wales Center, NY, USA) was used for induction and maintenance. Focal cerebral ischemia was generated using the intraluminal middle cerebral artery occlusion (MCAO) model as described previously [16, 21]. Briefly, the right common carotid artery and the right external carotid artery (ECA) were exposed. The ECA was then dissected distally, ligated, and coagulated. The MCA was occluded using a heparinized intraluminal filament (diameter 0.28 mm). After 90 min, the suture was withdrawn. During the operation, rectal temperature was monitored and maintained at 37±0.5°C with a thermostat-controlled heating blanket. Sham-operated rats underwent an identical surgery except that the MCA was not occluded.

Remote ischemic perconditioning

Rats were assigned to three groups: sham-operated group, MCAO group and MCAO + PerC group. PerC was initiated immediately after the onset of focal ischemia by occluding blood flow to the hind limbs bilaterally while under anesthesia [22]. Hind limb occlusion was accomplished by tightening a tourniquet (8 mm) around the upper thigh for 3 cycles, with each occlusion and release phase lasting 10 minutes. An observer blinded to the experimental conditions assigned the rats randomly into the MCAO group and MCAO + PerC group. Hind limb ischemia was monitored using laser doppler. The rats in the sham and MCAO groups were under anesthesia for an identical time as the PerC group except that the limbs were not tightened.

Neurobehavioral tests

Neurological deficits (N=10 per group) were determined by neurobehavioral scoring system, which was graded on a scale of 0 to 12 (minimal score, 0; maximal score, 12), based on one proposed by Belayev et al [23]. The tests included (1) postural reflex test to examine upper body posture, and (2) the forelimb placing test to examine sensorimotor integration. In addition, the ladder rung walking test was also used to assess deficits in coordination and integration of motor movement as previously described [24]. The number of errors and steps of the affected left forelimb in each trial was counted. Two trials were averaged to give a mean foot fault score, and testing was performed at 2 and 48 h after reperfusion by an investigator who was blinded to the experimental groups. Higher scores indicate worse deficits.

Infarct volume measurement

Rat brains (N=5 per group) were removed 48 h after reperfusion and sectioned coronally at 2-mm intervals to generate 6 sections. The sections were then stained with 2% solution of 2,3,4-triphenytetrazolium-chloride (TTC). The infarct area and the corresponding contralateral area were measured by a blinded observer using the Image-Pro Plus software 5.0 (Rockville, MD, USA). Infarct volume was calculated as a percentage of the volume of the contralateral hemisphere, as described previously [16].

Brain edema measurement

Brain edema was evaluated by measuring water content. The right and left hemispheres were separated and weighed to obtain the wet weight (WW). The tissue was then dried in an oven at 70°C for 72 h and weighed again to obtain the dry weight (DW). The water content was calculated using the formula [(WW-DW)/WW]×100% and is expressed as a percentage of the wet weight.

Evaluation of blood-brain barrier integrity

BBB permeability was analyzed by measuring the amount of Evans blue extravasation from the ipsilateral and contralateral hemispheres [16]. Forty-six hours after reperfusion, Evans blue dye (2%, 4 mL/kg body weight) was administered intravenously and allowed to circulate for 2 h. Rats were then perfused with saline to wash away the remaining dye in the blood vessels. The Evans blue dye was extracted from the brains by homogenization and incubating in 2 mL N,N-Dimethylformamide at 54°C for 2 h thereafter. The solution was centrifuged at 12,000 g for 15 minutes. The absorption of the supernatant was measured at 620 nm with a spectrophotometer. The content of Evans blue from the two hemispheres was expressed as μg/g of brain tissue by using a standardized curve.

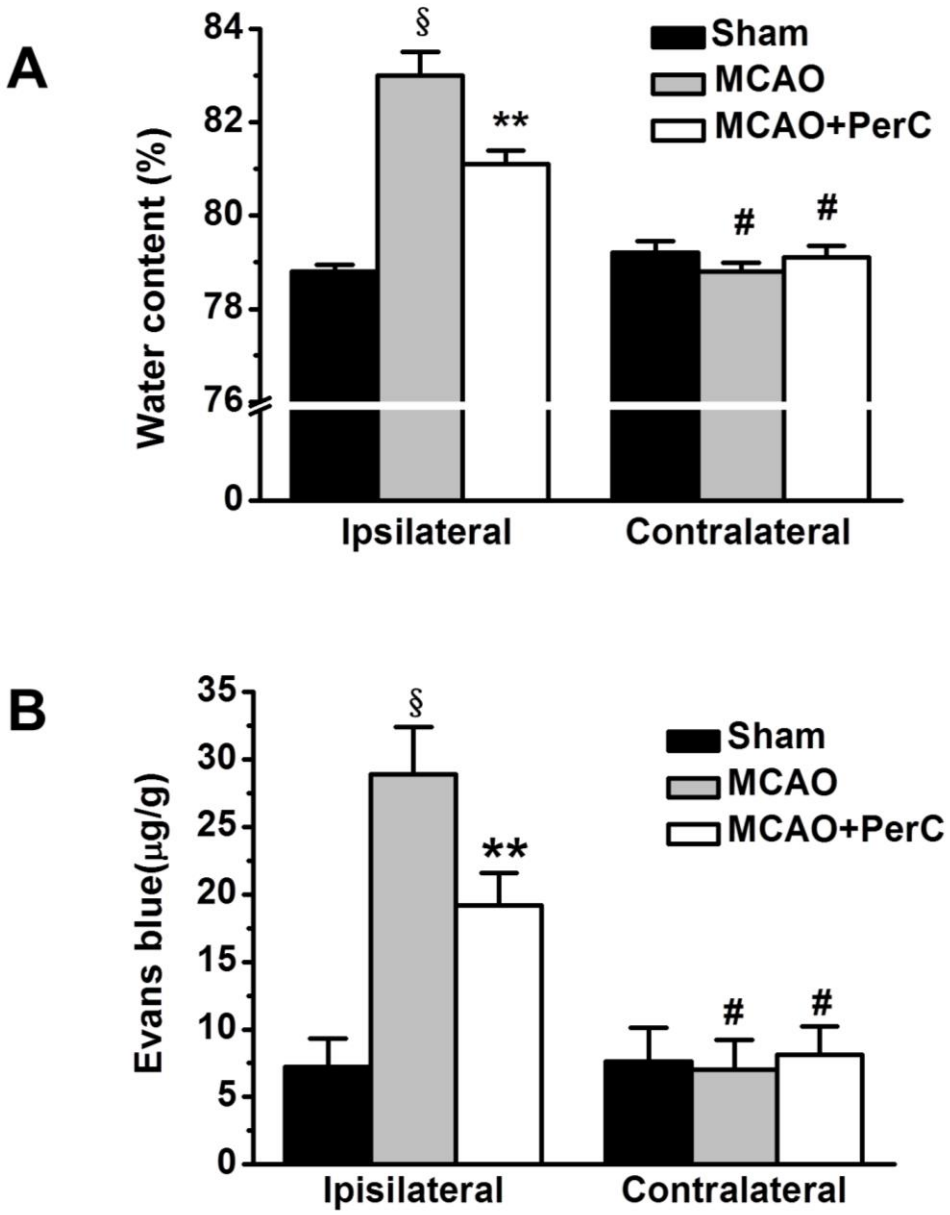

Figure 2. Limb remote ischemic PerC reduced brain edema and BBB leakage. (A) PerC treatment attenuated stroke-induced brain edema. ***P*<0.01, versus ipsilateral control groups; #*P*<0.001, versus corresponding ipsilateral ischemic hemisphere. N=5 per group. (**B**) PerC reduced BBB leakage. **P*<0.05, versus ipsilateral MCAO groups; #*P*<0.001, versus corresponding ipsilateral ischemic hemisphere. §*P*<0.01, versus ipsilateral sham group. N=5 per group. Error bars indicate SD.

Western blot

The brain tissues were lysed by ultrasonication in lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH = 7.5), 0.1% Sodium deoxycholate, 1% Triton-X-100, 0.1% SDS, 1% Protease Inhibitor Cocktail and/or Phosphatase Inhibitor (Roche)) and centrifuged. Protein concentrations were determined using the BCA method (Thermo Scientific, Rockford, IL, USA). Equal volumes (50 μl) of tissue extracts were separated by electrophoresis through 8-15% polyacrylamide gel and then transferred to PVDF membranes (Millipore). Membranes were blocked for 1 h

in 5% skim milk in TBS-T buffer and immersed overnight at 4°C with primary antibodies against occludin (1:1000; Santa Cruz), claudin-5 (1:500; Santa Cruz), ZO-1 (1:300; Santa Cruz), pERK1/2 (1:1000, Cell Signaling), MMP-9 (1:500, Abcam), MMP-2 (1:1000, Abcam), respectively. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used to verify equal protein loading. The specific reaction was visualized by the chemiluminescence substrate luminol reagent (GE Healthcare, UK). The optical density of protein was measured using Image-Pro Plus software 5.0 (Rockville, MD, USA) according to the manufacturer's instructions. The mean amount of protein expression from the control group was assigned a value of 1 to serve as reference [25].

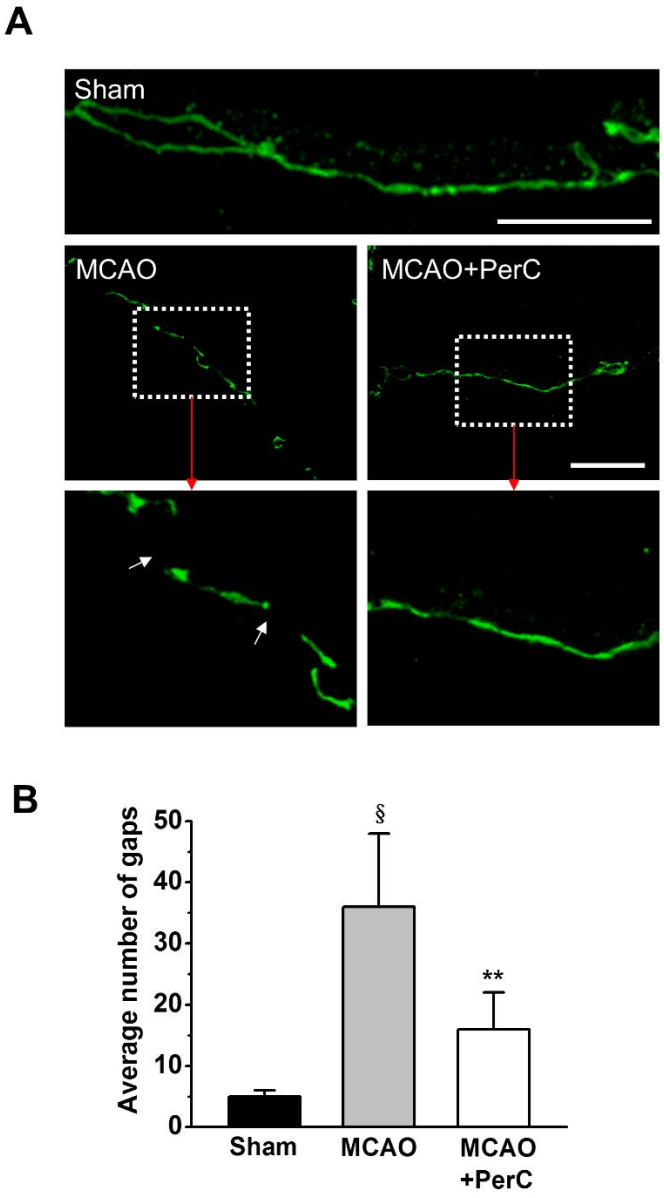

Figure 3. Remote ischemic PerC reduced TJ gap formation. (A) Representative images of claudin-5 positive vessels (green) in the penumbra of the rat brain. Vessels in the penumbra of sham-operated brain showed a continuous, linear labeling of claudin-5. In the MCAO group, a discontinuous, less regular distribution of claudin-5 in the vessels was noted (arrows), which was barely detectable in the PerC-treated group. (**B**) Quantification of gap formation in sham group, MCAO group and MCAO + PerC group. Values are mean ± SD. ***P*<0.01, versus MCAO group; §*P*<0.01, versus sham group. N=5 per group. Scale bar = 25 μm.

Gelatin gel zymography

To determine the enzymatic activity of MMP-9 and MMP-2, gel zymography was performed as described [26] with some modifications. Brain tissues (N=5 per group) were homogenized with lysis buffer (50 mmol/L Tris/HCl pH 7.5, 75 mmol/L NaCl, 1% Triton X-100 and 1mmol/L PMSF). Supernatants were incubated with 50% $(NH_4)_2SO_4$ for 15 min and then centrifuged. Pellets were resuspended in 250 µl Tris/HCl buffer (pH 7.5) and incubated with 100 µL Gelatine-Sepharose 4B beads (Amersham, GE healthcare, Pittsburgh, USA) at 4°C for 2 h. The collected beads were incubated with 200 µl of elution buffer (50mmol/L Tris/HCl, 1M NaCl, 7.5% DMSO, and 10mmol/L $CaCl_2$) for 1 h at 4°C with gentle shaking. The samples (20 µl per well) were electrophoretically separated on 7.5% SDS-PAGE gels under non-reducing conditions. Gel was washed in washing buffer (2.5% Triton X-100，50 mmol/L Tris – HCl, 5mmol/L $CaCl_2$, pH 7.6) and then incubated for 42 h in developing buffer (50 mmol/LTris/HCl, 10 mmol/L $CaCl_2$, 0.02% Brij-35, pH7.6) at 37°C. The gels was stained with Coomassie blue R-250 for 1 h, and then destained in destaining solution (5% acetic acid and 25% methanol) until clear bands are visible. Relative gelatinolytic activity was quantified via measurement of optical density and expressed as the ratio of loaded sham controls.

Immunofluorescence staining

The brains were removed at 48 h after reperfusion. Brain cryosections (20 µm) were fixed in ice-cold acetone for 10 min, washed, blocked for 30 min in 1% Bovine Serum Albumin (BSA), and incubated at 4°C with primary antibodies against claudin-5 (1:100, Santa Cruz Biotechnology, Inc, USA) at 4°C overnight. After washing, the sections were subsequently incubated with immunofluorescence-conjugated IgG (1:200, Millipore Corporation, USA) for 1 h at room temperature (Molecular Probes, 1:500). Slides were mounted using Prolong Gold antifade reagent (Molecular Probes). Fluorescence signal was observed under a confocal laser-scanning microscope (Carl Zeiss, Germany). The quantitative analysis was expressed by the average gap number of twenty vessels. Three visual fields in the penumbra were analyzed.

Statistical Analysis

All the data were described as mean ± SD. Statistical analysis was performed with SPSS for Windows, version 17.0 (SPSS Inc.). For two groups, the differences were analyzed for statistical significance by Student's *t*-test. For three groups, the differences were analyzed for statistical significance by one-way ANOVA, following *Tukey post hoc test*. A P value of < 0.05 is considered significant.

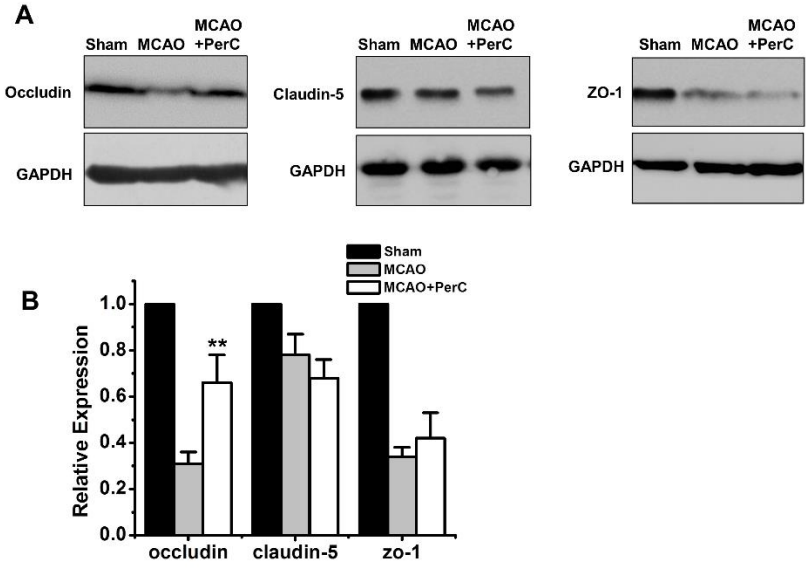

Figure 4. Remote ischemic PerC upregulated occludin expression. (**A**) Representative Western blots showing occludin, claudin-5 and ZO-1 expressions in MCAO group and MCAO + PerC group. (**B**) Quantification of occludin, claudin-5 and ZO-1 in sham group, MCAO group and MCAO + PerC group. Values are mean ± SD. ***P*<0.01, versus MCAO group; §*P*<0.01, versus sham group. N=5 per group.

RESULTS

PerC improved functional outcome and protected from ischemic injury

To determine whether limb remote ischemic PerC protects from brain damage, we first determined the effect of PerC on neurological functional outcome after ischemia. Neurological deficits (body posture and sensorimotor integration) significantly improved at 48 h in the PerC treatment group, compared with the MCAO group (P<0.01) (Fig. 1A). Motor deficits also improved at 48 h after PerC treatment based on the ladder rung walking test (P<0.05) (Fig. 1B).

We then asked whether PerC would affect infarct size at 48 h after reperfusion. Consistent with neurological behavioral results, infarct size was significantly reduced in the PerC group (23.1 ± 7.4%) compared with the MCAO group (42.3 ± 5.1%). This reduction by 45% proved to be significant (P<0.001) (Fig. 1C and D).

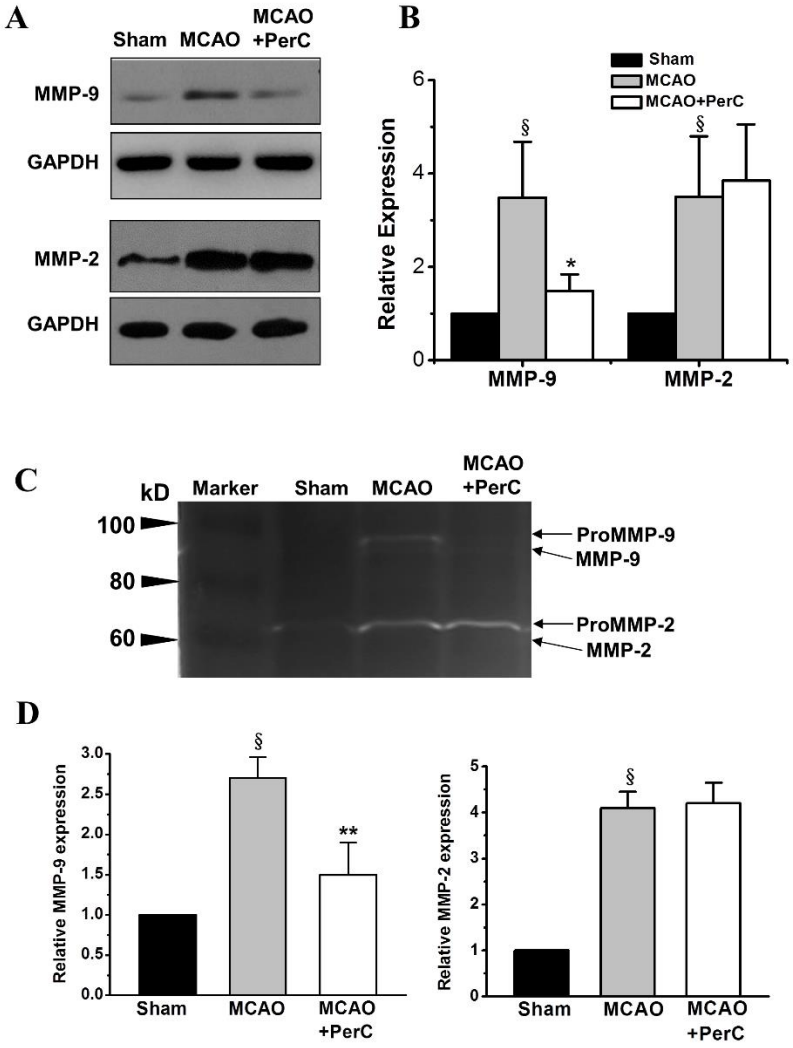

Figure 5. Remote ischemic PerC downregulated MMP-9 activity. (**A**) Representative Western blots showing MMP-9 and MMP-2 protein expressions after 48 h of reperfusion in MCAO group and MCAO + PerC group. (**B**) Densitometric quantification of MMP-9 expression in MCAO group and MCAO + PerC group. Values are mean±SD. **P<0.01, versus MCAO group; N=5 per group. (**C**) Representative gelatin zymography image of MMP-9 pro-form (proMMP-9) and pro-form of MMP-2 (proMMP-2) activities in each group. (D) Densitometric quantification of proMMP-9 (left panel) and proMMP-2 (right panel) in each group. Values are mean±SD. **P<0.01, versus MCAO group; §P<0.01, versus sham group. N=5 per group.

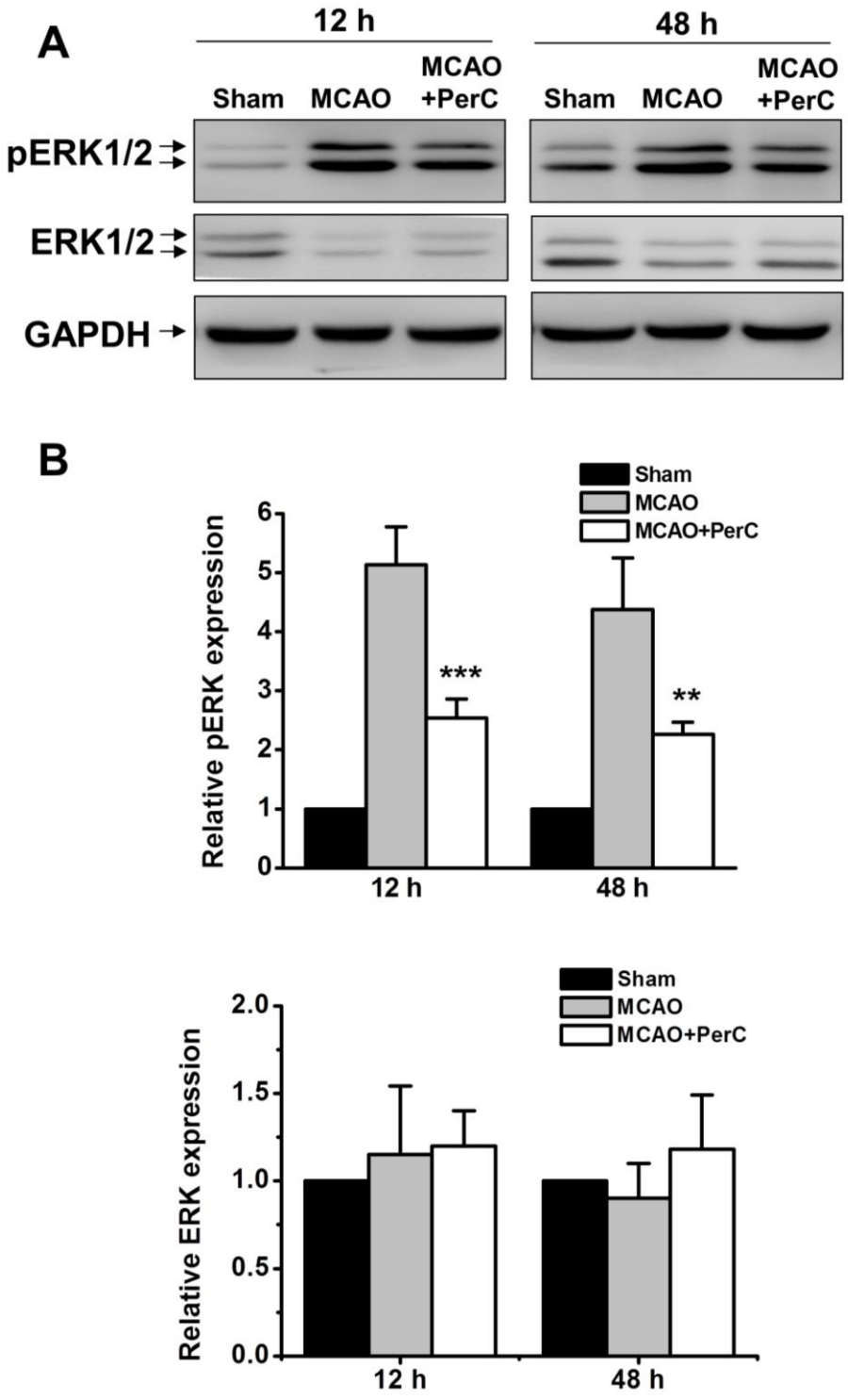

Figure 6. Remote ischemic PerC attenuated phosphorylated-ERK1/2 activity. (**A**) Representative Western blots of pERK1/2 and ERK1/2 protein levels in MCAO group and MCAO + PerC group. (**B**) Densitometric quantification of pERK1/2 (top panel) and ERK1/2 (bottom panel) in MCAO group and MCAO + PerC group. Values are mean ± SD. ***P*<0.01, ****P*<0.001, versus MCAO group; §*P*<0.01, versus sham group. N=5 per group.

PerC attenuated brain edema and BBB leakage

Brain edema continues to be a major cause of neurological function deficiency after ischemic stroke. As vasogenic edema usually occurs in the later stages after ischemia [7], we next determined whether PerC would affect brain edema at 48 h after reperfusion. As shown in Fig. 2A, the ipsilateral hemisphere in the MCAO group (83.2 ± 0.8%) contained more fluid than the corresponding contralateral hemisphere (78.7 ± 0.2%) (P<0.01), and the ipsilateral brain edema was significantly increased in the MCAO group compared with sham group (78.9 ± 0.2%) (P<0.01). However, water content in the ipsilateral hemisphere was significantly reduced after remote PerC (81.4 ± 0.4%) compared with the MCAO group (P<0.01).

BBB permeability determined by Evans blue dye extravasation in the ipsilateral hemisphere in the MCAO group (29.5 ± 4.1 μg/g) was increased compared with the sham group (7.5 ± 2.6 μg/g) (P<0.01). However, Evans blue dye was significantly reduced in the PerC group (18.2 ± 3.2 μg/g) compared with the MCAO group (P<0.01) (Fig. 2B), suggesting that PerC reduced BBB permeability.

PerC reduced gap formation and TJ protein degradation

TJ plays a critical role in the BBB integrity. Using confocal microscopy and three-dimensional construction of vascular structures, we found that focal ischemia induced a discontinuous staining of claudin-5 and showed considerable gap formation (Fig. 3). Quantitative analysis revealed that the average gap number was significantly reduced in the PerC group (16.2 ± 6.1) compared with ischemia MCAO group (36.1 ± 12.2). This reduction by 56% proved to be significant (P<0.01).

To further determine whether PerC treatment-induced attenuation of BBB disruption was due to TJ alterations, the expression of the TJ transmembrane proteins, occludin, claudin-5 and cytoplasmic protein ZO-1 was assessed by Western blot at 48 h after reperfusion. PerC treatment significantly increased occludin expression compared to the MCAO group (Fig. 4). This increment by 112% proved to be significant (P<0.05), whereas the expression levels of claudin-5 and ZO-1 were not significantly altered.

PerC inhibited MMP-9 expression

To further investigate whether PerC affected the upstream molecular pathway of occludin, the MMP-9/2 expressions were analyzed using Western blot at 48 h after reperfusion. As shown in Fig. 5A and B, the expressions of MMP-9 and MMP-2 were significantly increased in the MCAO group compared with the sham group (P<0.01). PerC significantly reduced the MMP-9, but not MMP-2 expression, compared with the MCAO group. This reduction by 57% proved to be significant (P<0.05). Using gel gelatin zymography, the pro-form of MMP-9/2 was increased in the MCAO group compared with the sham group (P<0.01). The pro-form of MMP-9 in the PerC group was significantly decreased compared with the MCAO group. This reduction by 44% proved to be significant (P<0.01) (Fig. 5C and D). However, there was no change in the pro-form levels of MMP-2 between the MCAO and MCAO + PerC groups.

Limb remote PerC modulated MEK/ERK1/2 signaling

Finally, we investigated the activation of MEK/ERK1/2 after PerC. Because previous documented that the MMP-9 activity are regulated by the MEK/ERK pathway [13]. The expression of phosphorylated ERK1/2 (pERK1/2) was analyzed by Western blot. We found increased pERK1/2 expression at 12 and 48 h after reperfusion in the MCAO group compared with the sham group (P<0.01) (Fig. 6). PerC significantly decreased the pERK1/2 protein levels, compared with the MCAO group at 12 h and 48 h after reperfusion, but no changes to ERK1/2 expression were seen (Fig. 6).

DISCUSSION

In this study, we found that limb remote PerC reduced functional neurological deficits, decreased brain edema and BBB breakdown after cerebral ischemia in the rat. In addition, gap formation in the endothelial cells of the ischemic brain was reduced and TJ protein occludin was significantly increased after PerC, along with reduced expression levels of proMMP-9. Interestingly, pERK1/2 was significantly reduced after PerC treatment. These findings suggest that PerC treatment attenuated BBB disruption and TJ, in part due to decreased ERK1/2 phosphorylation and MMP-9expression.

Limb remote PerC has several major advantages as a strategy for stroke management: it is widely accessible, noninvasive, simple, inexpensive, and can be initiated within minutes after stroke [27]. Of note, no cerebral hemorrhage and objective signs of neurovascular injury were observed in cerebral hemorrhage animal model and subarachnoid hemorrhage patients [28] [29]. These findings suggest that PerC is a practical acute-phase treatment to expand the thrombolytic therapeutic time window and protect reperfusion-induced irreversible damage of the ischemic penumbra. In this study, we demonstrated that PerC reduced infarct volume and improved neurological deficits after focal ischemia. Since previous studies have shown that ischemic preconditioning inhibited edema formation and BBB

permeability [18], and our data in this study indicated that brain edema was significantly reduced after PerC, we assumed that protection against ischemia-induced BBB damage may be one of mechanisms underlying PerC-mediated neuroprotection.

Two phases of BBB disruption have been described. The first phase of the biphasic permeability, which is closely associated with hemorrhagic transformation, is present at ~3-8 h post-reperfusion [2]. Vasogenic edema with the later phase of BBB permeability is present ~18-96 h after reperfusion, which is one of a leading cause of death subsequent to ischemia and the primary causes of clinical deterioration [2, 30, 31]. Belayev et al. reported that acute disruption of the BBB occurs at 3-5 h after focal ischemic stroke, and that increased regional BBB permeability is present at 48 h [32]. Therefore, in the present study, we analyzed the effect of PerC on the BBB permeability and brain edema at 48 h after reperfusion. Our results demonstrated that 90-min MCAO with 48 h reperfusion resulted in BBB disruption by Evan's blue extravasation, and PerC treatment significantly attenuated this disruption.

Tight junctions (TJs) are important structural components of the BBB and are composed of a combination of transmembrane occludin and claudin-5, which play important roles in regulating the integrity and proper functions of the BBB [1, 33]. It was reported that only disrupting occludin could cause functional changes of the TJs [34]. The disrupted occludin expression induced hypoxia-induced vascular leakage [26]. On the other hand, Claudin-5 is decreased in lesioned vessels with BBB breakdown in the ischemic brain [26, 35]. When claudin-5 expression is increased, BBB permeability is decreased [36]. ZO-1, which is the cytoplasmic protein of TJs, is decreased after ischemic stroke [37]. Our data showed that PerC significantly increased occludin expression, but not claudin-5 and ZO-1 expressions, suggesting that occludin is a specific PerC target. Studies have documented that occludin is a direct substrate of MMP-9/2 [38]. We found that PerC significantly reduced MMP-9 expression, whereas there was no change to MMP-2 expression, suggesting that inhibiting MMP-9-dependent occludin degradation may be an important mechanism underlying PerC-mediated BBB protection. Consistently, Asahi et al. reported that MMP-9 deficient mice showed reduced BBB leakage after focal cerebral ischemia [11], while no BBB leakage was seen in MMP-2 deficient mice [39]. Studies also showed that MMP-9 activity was associated with the later phase of BBB disruption and vasogenic edema formation [40] whereas MMP-2 activity is correlated with the early phase of BBB opening [9]. These reports support our study as we analyzed the effect of PerC on brain edema, BBB leakage, TJ proteins and MMP2/9 expressions at the later phase. It was shown that microvessel extracts containing activated MMP-9 did not affect claudin-5 protein degradation [41]. Additionally, MMP-9 inhibition reduced occludin degradation after hypoxia in mice [26], and normobaric hyperoxia attenuated BBB disruption by attenuating MMP-9 mediated occludin degradation in focal vertebral ischemia rat [41].

Studies evidence that MEK/ERK1/2 is upstream of MMP-9 [13]. In MCAO followed by 48 hours of reperfusion in rat, ERK1/2 inhibitor reduced expression of MMP-9 and BBB leakage [13]. Our results showed that PerC significantly decreased ERK1/2 activity at 12 h and 48 h after reperfusion. However, PerC did not affect the N-terminal kinases (JNK) and p38 expressions after ischemia (*data not shown*). In line with our results, ischemic preconditioning blocked ERK1/2 activity in the MCAO rat model [42]. However, Zhou et al. reported that limb ischemic postconditioning was no change to phosphorylated ERK1/2 expression [43], either because the involvements of signaling pathway are different between perconditioning and postconditioning, or because the ischemia model is different.

Our study has several limitations. First, ERK1/2 and MMP9 signaling may involve cellular mechanism, rather than a direct cellular mechanism of protection. Second, the PerC treatment was only performed one time after ischemia, and we only chose the episode (10min x 3 cycle). Studying dose dependent relationship can bring additional preclinical information. In the future, we foresee employing pharmacological agents and even transgenic technology to examine the direct and indirect mechanistic links between limb ischemia and the different functional, biological, and biochemical endpoints. E.g. MMP-9 levels in the plasma can be measured at a later time point since at 4 hours; we saw no differences between the MCAO and MCAO+PerC groups.

In conclusion, we determined that limb remote ischemic PerC decreased BBB leakage, and further protects the rat brain from ischemia/reperfusion injury. Although the mechanism(s) involved in limb ischemic PerC need to be further examined, the results from our study and the current literature supports the idea that the MMP-9 and occludin are involved in neuroprotection induced by PerC. The results demonstrated in this study may provide clues to help elucidate the protective capabilities of PerC. Moreover, this study could be important as it pertains to the translational aspect of stroke management in that PerC is inexpensive, convenient, and safe to administer. One clinical study in Europe found that PerC administration in the ambulance immediately after an episode of stroke could improve the functional outcome of patients three months later. The findings in our study indicate that PerC techniques could be maximized

and evaluated in depth to truly comprehend, understand, and appreciate the power of this treatment.

Acknowledgments

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Disclosure Statement

The authors have no conflict of interest to declare.

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