

Remote Ischemic Perconditioning Is Effective Alone and in Combination With Intravenous Tissue-Type Plasminogen Activator in Murine Model of Embolic Stroke

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Background and Purpose—Remote ischemic conditioning is cardioprotective in myocardial infarction and neuroprotective in mechanical occlusion models of stroke. However, there is no report on its therapeutic potential in a physiologically relevant embolic stroke model (embolic middle cerebral artery occlusion) in combination with intravenous tissue-type plasminogen activator (tPA).

Methods—We tested remote ischemic perconditioning therapy (RIPerC) at 2 hours after embolic middle cerebral artery occlusion in the mouse with and without intravenous tPA at 4 hours. We assessed cerebral blood flow up to 6 hours, neurological deficits, injury size, and phosphorylation of Akt (Serine⁴⁷³) as a prosurvival signal in the ischemic hemisphere at 48 hours poststroke.

Results—RIPerC therapy alone improved the cerebral blood flow and neurological outcomes. tPA alone at 4 hours did not significantly improve the neurological outcome even after successful thrombolysis. Individual treatments with RIPerC and intravenous tPA reduced the infarct size (25.7% and 23.8%, respectively). Combination therapy of RIPerC and tPA resulted in additive effects in further improving the neurological outcome and reducing the infarct size (50%). All the therapeutic treatments upregulated phosphorylation of Akt in the ischemic hemisphere.

Conclusions—RIPerC is effective alone after embolic middle cerebral artery occlusion and has additive effects in combination with intravenous tPA. RIPerC may be a simple, safe, and inexpensive combination therapy with intravenous tPA. (*Stroke*. 2012;43:2794-2799.)

Key Words: embolic stroke ■ IV tPA ■ remote ischemic conditioning

The Stroke Academic Industry Roundtable (STAIR) recommends therapeutic evaluation of neuroprotective agents in combination with intravenous (IV) tissue-type plasminogen activator (tPA) and to develop therapies to reduce reperfusion injury.¹ After so many failed pharmacological trials in stroke, harnessing endogenous pathways of protection provides another promising but untraveled avenue.² Murry et al first introduced the ischemic preconditioning therapy in a canine myocardial infarction model and follow-up studies have shown ischemic preconditioning to provide the most consistent and profound reduction in infarct size of all cardioprotective interventions.^{3,4} Subse-

quent studies have shown that ischemic conditioning can also be applied “at a distance,” so-called “remote ischemic conditioning”; brief episodes of ischemia–reperfusion in a distant organ such as the limb can provide protection to the ischemic heart or brain.⁵ Remote ischemic conditioning can also be applied during the ischemia and before reperfusion (perconditioning; RIPerC) or after reperfusion (postconditioning). RIPerC therapy can be simply performed using a blood pressure cuff on the arm and has been found protective in a prehospital clinical trial of myocardial infarction.⁶ A number of studies have now shown that limb RIPerC and postconditioning are protective in animal models of stroke.^{7,8}

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Cerebral blood flow (CBF) restoration is critical for neurovascular protection after stroke and requires stringent monitoring.^{9,10} Neurovascular protection conferred by ischemic conditioning is also mediated through vasodilatory factors and improvements in CBF.^{11,12} Embolic clot model of stroke (embolic middle cerebral artery occlusion [eMCAO]) better represents the majority of the human stroke cases and is more suitable to study thrombolytics such as tPA and related reperfusion injury.^{13–15}

To the best of our knowledge, none of these conditioning strategies has been ever reported in a physiologically relevant eMCAO model in combination with IV tPA. Because most of the stroke victims within a 4.5-hour treatment window will be candidates for IV tPA, it is necessary to know the effect of RPerC therapy and its additional benefits and safety with tPA treatment. We herein report that RPerC therapy is effective alone and provides further additional benefits in combination with IV tPA in a murine eMCAO model.

Materials and Methods

Animals and Experimental Groups

The Institutional Animal Care and Use Committee of Georgia Health Sciences University approved the experimental procedures as per the National Institute of Health guidelines. Ninety C57BL/6J wild-type male mice (20±1 weeks old; Jackson Laboratory, Bar Harbor, ME) housed in Georgia Health Sciences University's Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility were used in the following 2 experiments: Experiment I was performed to determine the effects of treatments on CBF using laser Doppler flowmeter (PeriFlux 5001, Perimed Inc), neurological outcome, and infarct size. The groups (N=10) were: eMCAO+Veh group, embolic stroke (eMCAO) with RPerC sham procedure followed by IV sterile phosphate-buffered saline treatment; eMCAO+RPerC group, eMCAO treated with RPerC therapy followed by IV phosphate-buffered saline treatment; eMCAO+tPA group, eMCAO with RPerC sham procedure followed by IV tPA (10 mg/kg) treatment; and eMCAO+RPerC+tPA group, eMCAO treated with RPerC therapy followed by IV tPA treatment. All the groups were euthanized at 48 hours post-eMCAO. Experiment II was performed in a separate set of animals with the same groups as described in Experiment I, but the tissue extracts were used for immunoblot analysis for phosphorylation of Akt (Serine⁴⁷³; p-Akt). Experiment II also included a sham group for stroke and RPerC sham procedures followed by phosphate-buffered saline infusion. Animals were also scanned for cerebral perfusion with a laser Doppler imaging system (PeriScan PIM3 System; Perimed Inc) by an investigator blinded to the groups.

Procedures and Treatment Protocols

The mice were marked for identity and numbered. In Experiment I, the mice were randomized in a block size of 4 (4 animals from the same cage) to the different treatment groups after the stroke surgery. The surgeon performing the stroke and RPerC surgeries, and the investigators performing the RPerC sham procedure or therapy and intravenous treatments were blinded to the final identity of the groups and to the intravenous treatments (phosphate-buffered saline or tPA). In Experiment I, an investigator blindly scored the animals for neurological deficits before euthanasia. Mortality was monitored daily using a mortality chart. In Experiment II (5 mice per cage), one mouse from each cage was randomly picked for the sham group and performance of related procedures. The remaining 4 mice in each cage were randomized for the different treatments as discussed for Experiment I.

Stroke induction, CBF measurements and cerebral perfusion imaging, neurological score based on Bederson scale, infarct size

measurements after 2,3,5-triphenyltetrazolium chloride (TTC) staining, and immunoblot analysis were performed as described in our previous report with slight modifications.¹⁶ To induce stroke, a human fibrinogen-supplemented clot was prepared as reported earlier.¹⁶ Briefly, a modified catheter containing a 9.0±0.5-mm long clot was inserted into the right external carotid artery, advanced into the internal one, and the clot was gently delivered into the brain. Wherever required, either RPerC sham procedure or RPerC therapy was performed at 2 hours after eMCAO in the left hind limb as reported earlier (5 cycles/5-minute duration/5-minute interval between each cycle).⁸ Moreover, either tPA (10 mg/kg body weight) or phosphate-buffered saline was infused intravenously at 4 hours post-eMCAO as a 10% bolus and the balance over a period of 20 minutes in a volume of 0.1 mL/10 g body weight of the mouse.

Statistical Analyses

From our previous experience with adult male mice in an eMCAO model, we anticipated stroke injury size to be 50%±10% in the eMCAO+Veh group. Individual treatment with tPA or RPerC was hypothesized to reduce the infarct size by 20% to 40%±10% and the combined treatment by 50% to 25%±10%. A sample size of 7 in each group would provide 88% power at $\alpha=0.05$ to detect the main effects due to tPA and RPerC individual treatments. Also based on our experience, mortality up to 30% was anticipated for some groups over the survival period of 48 hours. Therefore, 10 mice were randomized to each of 4 groups to achieve a power of >80%. All the data are expressed as mean±SD. Statistical analyses were performed using SAS 9.3 (SAS Institute Inc, Cary, NC). A 2 RPerC (no versus yes) by 2 tPA (no versus yes) analysis of variance with interaction was used to analyze "within time CBF," neurological score, stroke injury, and p-Akt outcomes. In the absence of a significant interaction, the main effects are considered to be additive when combined. One-way analysis of variance was only used to compare p-Akt level for sham versus the other 4 groups using Dunnett test in Experiment II. Statistical significance was determined at $P<0.05$.

Results

RPerC Therapy Alone or in Combination With IV tPA Treatment Improved the CBF and Neurological Outcome

Figure 1A–B shows a significant effect of RPerC therapy in improving the CBF after eMCAO as compared with the RPerC sham-operated group (RPerC effect, $P=0.024$ at 4 hours post-eMCAO; $P=0.0035$ at 6 hours). Late tPA therapy also improved the CBF significantly (tPA effect $P<0.0001$ at 6 hours post-eMCAO). Late tPA infusion was not as efficient as early tPA treatment in improving the CBF (online-only Data Supplement Figure 1A). RPerC therapy showed an additive effect with tPA treatment in further improving the CBF. However, there was no interaction between the 2 treatments (interaction effect, $P=0.664$ at 6 hours post-eMCAO).

At 48 hours after eMCAO, the main effects of RPerC and tPA on the neurological outcome were significant ($P=0.0005$ and $P=0.05$, respectively; Figure 1C). No significant ($P=0.78$) interaction effect on the neurological outcome was found between the 2 treatments such that RPerC therapy provided a significant additive effect to the tPA treatment. RPerC significantly improved the neurological outcome in the eMCAO+RPerC group as compared with eMCAO+Veh (1.88±0.23 versus 3.0±0.22; $P=0.0084$). tPA treatment alone in the eMCAO+tPA group did not improve the neurological outcome signifi-

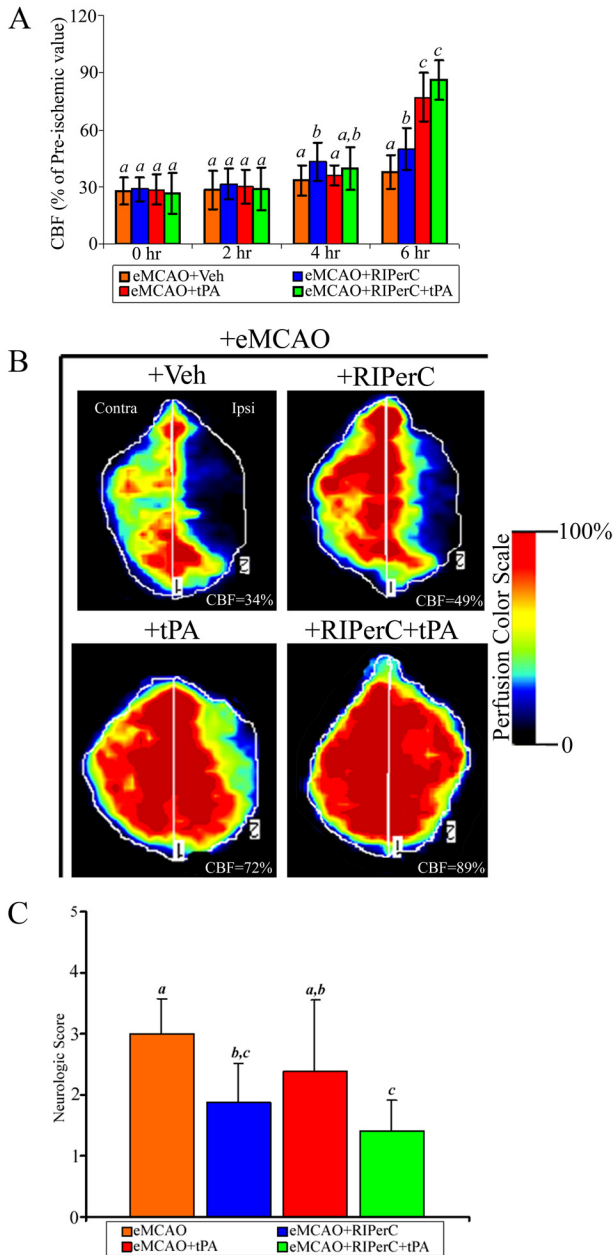


Figure 1. A, CBF measured with LDF and presented as percent of preischemic value (N=10). Comparisons between the groups were done “within the time point.” **B**, Representative images of cerebral perfusion detected with LDIS at 5 hours post-eMCAO. The CBF values in the panels are as compared with the contralateral sides. **C**, Neurological score as evaluated on Bederson scale at 48 hours post-eMCAO in Experiment I. eMCAO+Veh (N=7 survived out of 10), eMCAO+RIPerC (N=8 of 10), eMCAO+tPA (N=8 of 10), and eMCAO+RIPerC+tPA (N=10 of 10). Data presented as mean±SD. Pairs of means with different letters are significantly different, $P<0.05$. CBF indicates cerebral blood flow; LDF, laser Doppler flowmeter; LDIS, laser Doppler imaging system; eMCAO, embolic middle cerebral artery occlusion; RIPerC, remote ischemic preconditioning therapy; tPA, tissue-type plasminogen activator.

cantly as compared with eMCAO+Veh (2.38 ± 0.42 versus 3.0 ± 0.22 ; $P=0.13$). There were no significant differences found in the neurological outcomes between eMCAO+RIPerC and eMCAO+tPA groups ($P=0.20$). On the other hand, combination therapy in the eMCAO+RIPerC+tPA

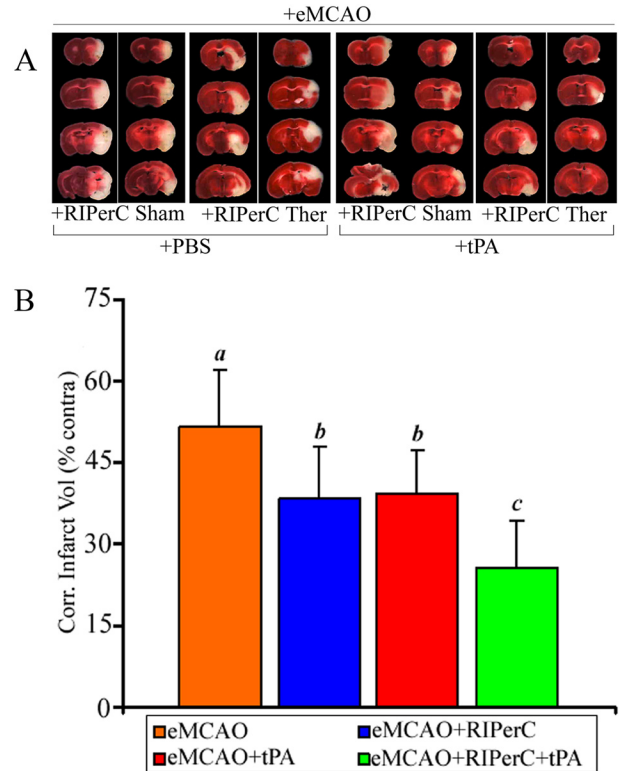


Figure 2. A, Representative TTC-stained coronal sections and **(B)** means of the corrected infarct volumes calculated as percent of their corresponding contralateral sides. Data are presented as mean±SD (N=as in Figure 1C). Pairs of means with different letters are significantly different, $P<0.05$. TTC indicates 2,3,5-triphenyltetrazolium chloride.

group significantly improved the neurological outcome as compared with eMCAO+Veh (1.4 ± 0.16 versus 3.0 ± 0.22 ; $P=0.0002$) and eMCAO+tPA ($P=0.012$).

RIPerC Therapy Alone or in Combination With IV tPA Treatment Reduced the Injury Size

Figures 2A and 2B show the representative images of TTC stained coronal sections of the brain and infarct volumes, respectively. The analysis of effects on the infarct volume reduction indicates that the main effects of RIPerC and tPA were significant ($P=0.0002$ and $P=0.0005$, respectively) but their interaction was not significant ($P=0.97$). RIPerC therapy alone in the eMCAO+RIPerC group significantly reduced the injury size as compared with eMCAO+Veh (25.7% relative reduction; 38.4 ± 9.5 versus 51.7 ± 10.4 ; $P=0.0082$). tPA treatment alone in the eMCAO+tPA group also reduced the injury size as compared with the eMCAO+Veh (23.8% relative reduction; 39.4 ± 7.8 versus 51.7 ± 10.4 ; $P=0.014$). There was no significant difference in the infarct volumes between eMCAO+RIPerC and eMCAO+tPA groups ($P=0.82$). Combination therapy in the eMCAO+RIPerC+tPA group significantly reduced the infarct size (50.0% relative reduction; 25.8 ± 8.6 versus 51.7 ± 10.4) as compared with the eMCAO+Veh ($P<0.001$) and also when compared with the individual treatment groups ($P=0.0037$ versus eMCAO+RIPerC and $P=0.0067$ versus eMCAO+tPA).

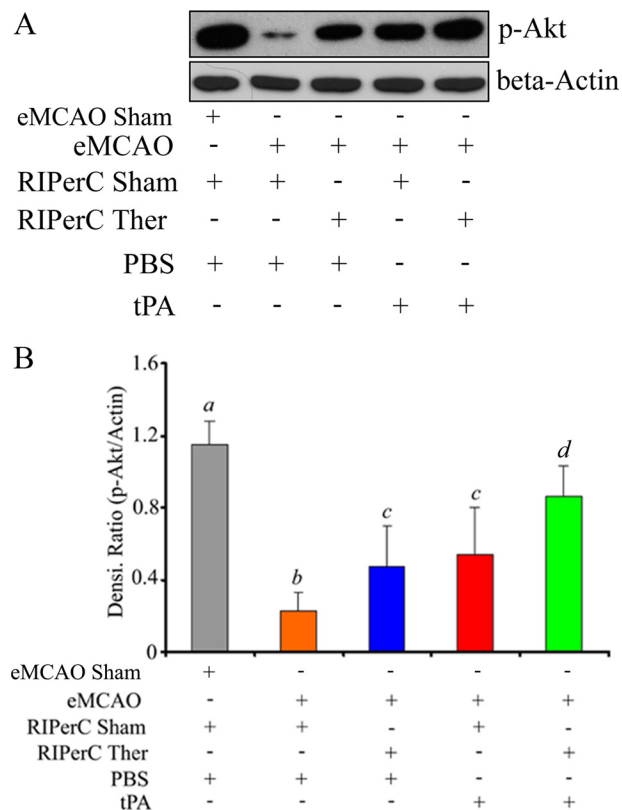


Figure 3. p-Akt (Ser⁴⁷³) level as detected by immunoblot analysis in the ischemic hemispheres at 48 hours post-eMCAO in Experiment II. **A**, Representative immunoblot image and **(B)** densitometric analysis of immunoreactive bands normalized to β -actin as loading control. Data are presented as mean \pm SD (N=6). Pairs of means with different letters are significantly different, $P < 0.05$. eMCAO indicates embolic middle cerebral artery occlusion.

RIPerC Therapy Prolonged the Akt Activation in the Ischemic Hemisphere

Figure 3A–B shows that p-Akt, a prosurvival signal after experimental stroke and ischemic conditioning therapy,^{17,18} was upregulated by both the individual treatments and their combination. All the eMCAO groups had significantly lower level of p-Akt when compared with sham at 48 hours. p-Akt level was significantly downregulated in the eMCAO+Veh group as compared with sham ($P < 0.0001$). The analysis of effects on the p-Akt level indicated that the main effects of RIPerC and tPA were significant ($P = 0.0026$ and $P = 0.0004$, respectively) but their interaction was not significant ($P = 0.67$). Both eMCAO+RIPerC and eMCAO+tPA groups had a significantly increased p-Akt level as compared with eMCAO+Veh ($P = 0.046$ and $P = 0.014$, respectively). There was no significant difference in p-Akt level between eMCAO+RIPerC and eMCAO+tPA groups ($P = 0.57$). Combination therapy in the eMCAO+RIPerC+tPA group significantly increased p-Akt as compared with eMCAO+Veh ($P < 0.001$) and also when compared with the individual treatments ($P = 0.0034$ versus eMCAO+RIPerC and $P = 0.013$ versus eMCAO+tPA).

Discussion

To the best of our knowledge, this is the first report demonstrating the efficacy of RIPerC therapy alone and in

combination with IV tPA in a physiological and clinically relevant embolic model. The key findings are that RIPerC alone increased the CBF and prosurvival signaling after stroke, resulting in improved neurological outcome and reduction in the size of injury. We also found additive beneficial effects of RIPerC to “late” IV tPA treatment leading to further improvements in the neurological outcome and reduction of injury size. Late IV tPA reduced the infarct size but did not improve the neurological outcome.

More than 60% of preclinical studies for neuroprotection in stroke research are done using well-accepted mechanical vascular occlusion–reperfusion models.¹⁴ This closely resembles the clinical cases of reperfusion after thrombectomy. In a mechanical stroke model, postconditioning by interrupting sudden reperfusion attenuated the injury.¹⁹ On the other hand, opening of the blood vessels after eMCAO either by spontaneous recanalization or by thrombolysis is often gradual and/or partial, which leads to prolonged microcirculatory and reperfusion deficits. Therefore, eMCAO better models the majority of human stroke cases and may be more appropriate for further translational and mechanistic studies of the conditioning therapies.

CBF restoration is a promising target for the treatment of stroke.^{9,10,13} As evident from Figures 1 and 2, RIPerC therapy significantly increased the CBF, resulting in significant improvements in the neurological outcomes and infarct size reductions. Moreover, tPA, being a thrombolytic, also improved the CBF when infused alone at 4 hours post-eMCAO. tPA alone reduced the injury size but did not significantly improve the neurological outcome, possibly because of its unwanted effects in a few animals.^{20,21}

It should be noted that IV tPA was given at the “tail” of the protective window when it still had some effect on infarct size reduction. This effect of tPA was mainly due to opening of the major occluded arteries as detected with laser Doppler flowmeter (Figure 1A–B) and visualized by clot resolution (online-only Data Supplement Figure IC).²¹ In the absence of RIPerC therapy, 4 hours of untreated ischemia may have caused microvascular dysfunction, which prevented complete CBF restoration even after successful thrombolysis by late tPA infusion (Figure 1A–B; online-only Data Supplement Figure IA–C).^{21,22} This may be due to increased oxidative–nitritative stress after eMCAO (online-only Data Supplement Figure IIC–E), which impairs capillary reflow despite successful opening of an occluded artery.²² Increased nitritative stress also impairs clot lysis and correlated with the poor neurological outcomes after stroke in human.^{23–26} In fact, we observed more prominent and sudden increase in the nitritative stress level 3 hours post-eMCAO both in the plasma and brain (online-only Data Supplement Figure IIC–E), a critical time point after which the benefits and safety of tPA therapy start to decline. Mild to moderate hemorrhage after “tPA alone” treatment was found in a few animals at this tail of the protective window but it was attenuated when RIPerC therapy was performed 2 hours before tPA infusion (online-only Data Supplement Figure IC–D). Due to improvement in the CBF, RIPerC may have attenuated the neurovascular stress before tPA treatment,

resulting in a “preconditioned” window for safer late tPA therapy and additional benefits.

The molecular mechanisms of remote ischemic conditioning are still elusive and may be multifactorial.²⁷ The downstream effects of multiple mechanisms appear to converge on the upregulation of reperfusion injury salvage kinase pathway and blocking of opening of the mitochondrial transition permeability pore.²⁸ PI3K/Akt is a key prosurvival signal of the reperfusion injury salvage kinase pathway and may have important physiological effects. Pharmacological inhibition of PI3K/Akt activity abolished the protective effects of preconditioning on the infarct size reduction.^{17,18} We found that Akt activity after eMCAO in the untreated mice was “transient.” p-Akt level started declining after 3 hours and appeared to be correlated with the increasing nitrate stress of the plasma and brain (online-only Data Supplement Figure IIA–E). Postconditioning attenuated nitrotyrosine in the brain, a marker of increased nitrate stress.²⁹ Peroxynitrite also inhibits PI3K, a major upstream kinase and regulator of Akt activity.³⁰ Therefore, we tested RPerC therapy at 2 hours post-eMCAO. As evident from our online-only Data Supplement and Figure 3, RPerC preserved and prolonged the post-eMCAO Akt activation, which was sustained even up to 48 hours. In agreement with previous findings,^{17,18} prolonged Akt activation may have improved the post-eMCAO outcomes. Because of its recanalization effect, tPA alone also upregulated the p-Akt level at the end point, which was further increased due to additive effects of RPerC.

In this study, increased p-Akt level after the treatments may be related to both the vasculature and brain. Therefore, future studies in animals genetically mutated for different Akt isoforms are planned. In the endothelium, Akt activity increases nitric oxide production through improved endothelial nitric oxide synthase activity and helps to maintain vascular homeostasis.^{31,32} Because RPerC prolonged the Akt activity and improved the CBF, the beneficial “neuroprotective” effects of RPerC are likely to be partially related to improvements in the microvascular blood flow. Future studies on collateral and microvascular CBF modulation by RPerC are in progress, which will help us to better understand the mechanism of neurovascular protection by this therapy in eMCAO.

In summary, these findings could be helpful in translating the remote ischemic conditioning into stroke clinical trials. Further studies to test the therapeutic potential of RPerC in a window later than 4 hours and in aged animals of both sexes with and without comorbidities such as diabetes will better inform and facilitate a clinical trial. However, remote ischemic conditioning is promising and may open a new paradigm in stroke treatment, which may be safe, inexpensive, and generalizable to a wide variety of stroke systems of care.

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Disclosures

None.

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ONLINE SUPPLEMENTAL

SUPPLEMENTAL METHODS

Preparation of clot

The modified method of clot preparation was adapted from our earlier report with slight modification.¹ To increase the strength and uniformity of the fibrin rich core of the clot and stability of the occlusion, mouse arterial blood was supplemented with human fibrinogen (2 mg/mL), and immediately clotted in PE-50 tube for 6 hours at room temperature followed by storage at 4 °C a day. Before the use, the clot (~5cm) was extruded and briefly washed with sterile deionized water into a petri dish. The fibrin rich core was obtained and transferred into another petri dish containing sterile phosphate-buffered saline (PBS) and left for further retraction at room temperature for 3 – 4 hours.

Embolic stroke model

It was performed as reported earlier by Hoda et al.¹ Mice were anesthetized with 3.5% isoflurane and maintained with 1.5 – 2.0% during the surgery. Body temperature was maintained at 37 °C by a thermo-regulated surgery pad. By a midline incision on the ventral side of the neck, the right common carotid artery (CCA), external carotid artery (ECA), and the internal carotid artery (ICA) were assessed. A temporary atraumatic clip was placed on the CCA to prevent loss of blood during catheter insertion. A modified PE-10 catheter containing a single fibrin rich clot (9 ±0.5mm length) was introduced into the ECA and advanced into the ICA. The clot was gently injected with 100 µL of PBS. Catheter was removed immediately after embolization and the arterial wound was secured to prevent blood loss. The temporary clip on the CCA was removed and the blood flow was restored therein. The site of surgery was closed.

Single point cortical laser-doppler flowmetry

Single point cortical laser doppler flowmetry was performed as reported earlier by us with slight modification.¹ The peripheral CBF pre/ and post occlusion, and also after thrombolysis/ reperfusion in the middle cerebral artery region was recorded semi-continuously up to 6 hours. For this purpose, a non-flexible laser doppler probe (Probe 407, Perimed Inc., Sweden) attached to a PeriFlux 5001 laser doppler flowmeter system (LDF; Perimed Inc.) was used. A shallow indentation was made in the parietal skull (A-P 0.5 mm, and M-L 4 mm with respect to the bregma) with a low-speed drill and the probe holder (PH 07-6; Perimed Inc.) was fixed with the glue at the above-mentioned coordinates. The LDF signal was recorded semi-continuously by placing the probe inside the holder. The absolute CBF value was obtained as an average of the values recorded over a period of 10-minutes at the required time points using PSW 2.5 software program (Perimed Inc.). The final data was calculated and presented as the percent of the pre-ischemic CBF value.

Cerebral perfusion imaging using laser doppler imaging system

For cerebral perfusion imaging, the mice from Experiment II were scanned after PBS/ tPA treatment and at 5 hours post eMCAO using laser doppler imaging system (PeriScan PIM 3 System, Perimed Inc.) as described previously by us.^{1,2} The scanner was positioned to scan a 1.5 × 1.5 cm area (1600 detection points), covering the cross-point of the coronal and sagittal sutures. A built-in photo detector assisted with LDPI win software (Perimed Inc) detected the reflected light from moving blood cells within 0.5 cm depth of the cortical surface. Color-coded images were acquired 3 times continuously, and the average CBF was calculated based on the concentration and mean velocity of the blood cells using the LDPI win software. The CBF for ipsilateral side was calculated

as percent of the corresponding contralateral side. The values are shown at the right side of the bottom of each image in Figure 1B.

Immunoblotting

Immunoblot experiments were performed as reported earlier by us with slight modification.¹ Animals were perfused under deep anesthesia via cardiac puncture and using chilled PBS at 48 hours post eMCAO or sham surgery. Ischemic hemispheres were collected, snap frozen in liquid nitrogen and homogenized in complete Lysis-M EDTA-free buffer (Roche Diagnostics, Indianapolis, IN). The amount of total protein in the samples was quantified using the EZQ[®] Protein Quantitation Kit (Invitrogen). Samples (25 µg of total protein) were subjected to SDS-PAGE using 10% NuPAGE[®] Novex[®] Bis-Tris ready to use gels (Invitrogen) and transferred to 0.2 µm PVDF membranes (Millipore, Billerica, MA). The membranes were blocked for non-specific binding and incubated with polyclonal primary anti p-Akt (Ser473) antibody (in Figure 3A, 1:1000 dilution; Cell Signaling Technology, Beverly MA; in Supplementary Figure S2A using anti p-Akt 1/2/3 (Ser473) antibody, 1:1000; Santa Cruz Biotechnology, Santa Cruz CA) at 4 °C overnight, followed by HRP-conjugated donkey anti-rabbit IgG antibody (1:10,000 dilution; Jackson ImmunoResearch, West Grove, PA). Membranes were re-probed with mouse monoclonal anti-β-actin antibody (Sigma-Aldrich Co.) as a loading control. Proteins were visualized with the ECL detection system (Pierce, Thermo Fisher Scientific) on the autoradiography film (Denville Scientific, Metuchen, NJ). The images obtained were scanned and processed for densitometric measurements using Image-J software. An investigator blinded to the treatment groups performed the analysis of p-Akt antigen. In the supplementary figures of immunoblots, self-prepared gels were used. For monomeric neuronal nitric oxide synthase (NOS), monoclonal primary anti nNOS antibody (1:1000; Cell Signaling Technology) was used.

Measurement of plasma peroxynitrite

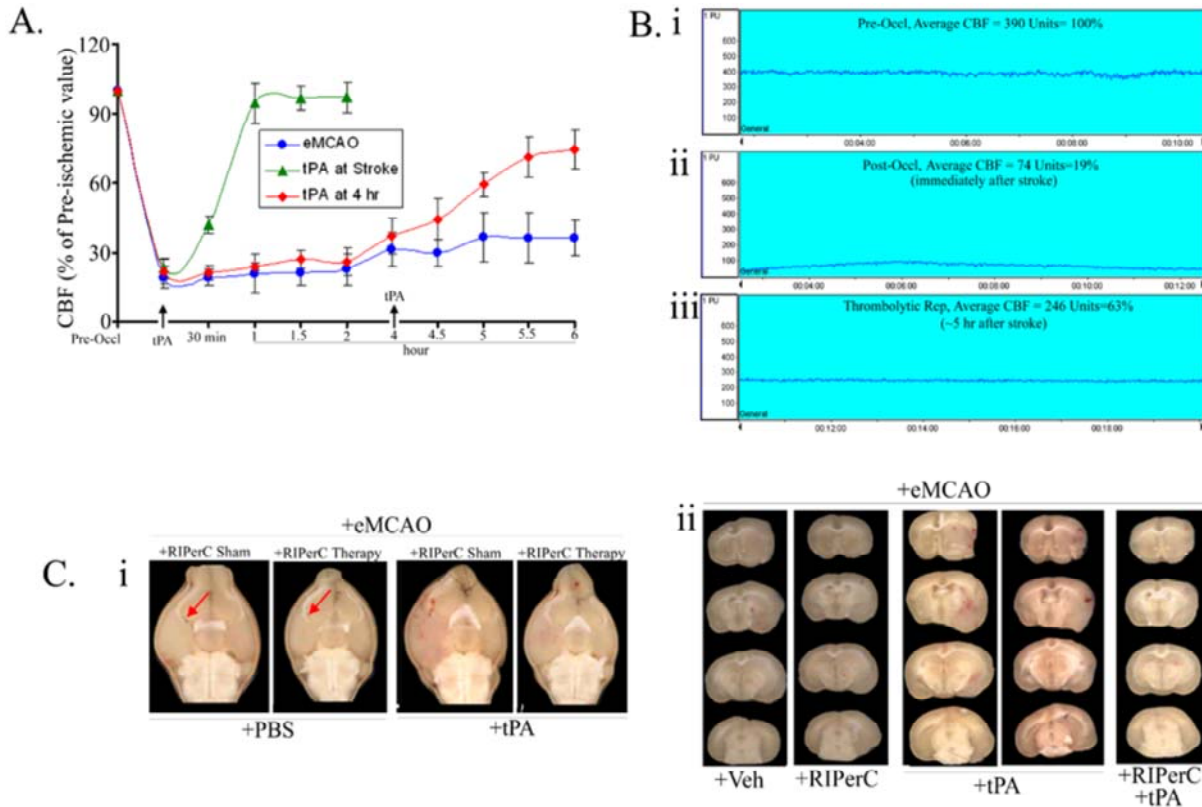
We measured plasma peroxynitrite by using a fluorescent probe dihydrorhodamine 123 (DHR123; Invitrogen) as reported earlier with certain modifications.^{3,4} DHR123 is oxidized to rhodamine 123 (Rhod123) in a peroxynitrite dependent manner. DHR123 was injected bolus (4 µmol/kg in 0.2 ml saline) after eMCAO and 3 hours before collecting the plasma. The plasma fluorescence was measured against the plasma of sham-operated animals treated with DHR123 similarly (λ_{ex} 500 nm, λ_{em} 536 nm).

Supplementary References

1. Hoda MN, Li W, Ahmad A, Ogbi S, Zemsanova MA, Johnson MH et al. Sex-independent neuroprotection with minocycline after experimental thromboembolic stroke. *Exp Transl Stroke Med.* 2011;3:16.
2. Li W, Prakash R, Kelly-Cobbs AI, Ogbi S, Kozak A, El-Remessy AB et al. Adaptive cerebral neovascularization in a model of type 2 diabetes: Relevance to focal cerebral ischemia. *Diabetes.* 2010;59:228-235.
3. Thiyagarajan M, Sharma SS. Neuroprotective effect of curcumin in middle cerebral artery occlusion induced focal cerebral ischemia in rats. *Life Sci.* 2004;74:969-985.
4. Szabo C, Salzman AL, Ischiropoulos H. Peroxynitrite-mediated oxidation of dihydrorhodamine 123 occurs in early stages of endotoxic and hemorrhagic shock and ischemia-reperfusion injury. *FEBS Lett.* 1995;372:229-232.

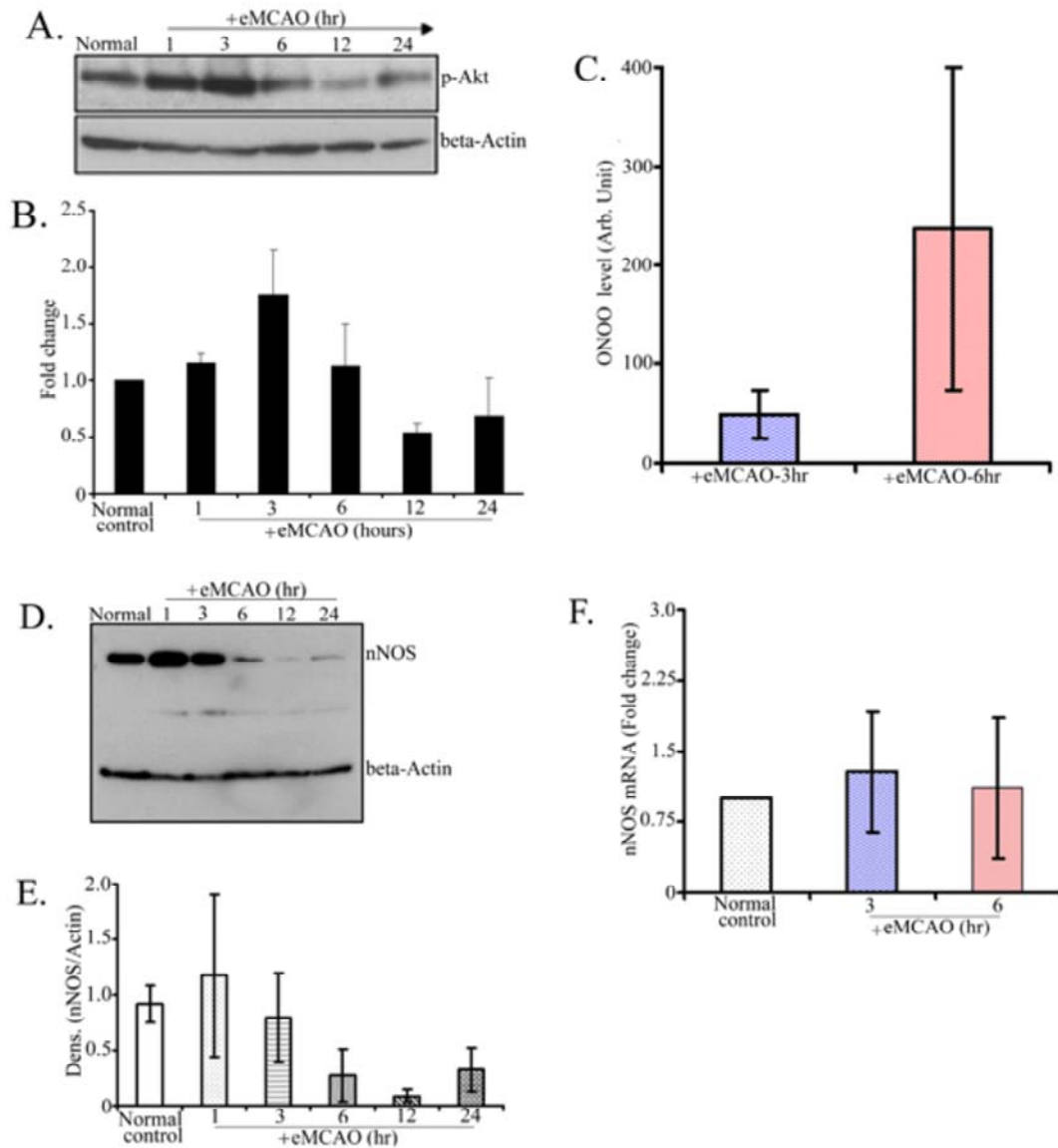
Supplementary Figure S1

FIGURE S1



Supplementary Figure S1: **A.** Comparison of CBF restoration after early and late intravenous tPA therapy (N=3; 10 mg/kg in 0.1 mL/10 g body weight of mouse). **B.** Representative images of real time LDF plots of one of the animals for approximately 10 minutes during semi continuous recording of CBF in eMCAO+tPA group in Experiment I; **i.** pre-occlusion, **ii.** immediately after occlusion, and **iii.** ~60 min after the initiation of tPA infusion, i.e., at 5 hours post eMCAO. **C.** Representative images of **i.** whole brain, showing tiny residual clot (indicated with red arrow) in the middle cerebral artery of non-tPA treated groups at 48 hours post eMCAO, and **ii.** 2 mm thick coronal sections of the brain of different animals from Experiment II at 48 hours post eMCAO showing mild to moderate hemorrhagic transformations (HT) in a few surviving animals (2/8) in eMCAO+tPA group.

Supplementary Figure S2



Supplementary Figure S2: Post eMCAO time dependent changes in the phosphorylation of Akt at (Serine473; p-Akt), and increase in nitrate stress in the plasma and brain. Decrease in the p-Akt level and the markers of nitrate stress appear to be correlated. **A.** Immunoblot images of p-Akt and β -actin, and **B.** Densitometric analysis of immunoreactive bands of p-Akt normalized to β -actin as loading control (N=3). **C.** Plasma peroxynitrite level. The higher variability in this figure can be attributed to lower sample size of “plasma” in this preliminary study. **D.** Immunoblot images of monomeric/ inactive form of nNOS (mono-nNOS) and β -actin, and **E.** Densitometric analysis of immunoreactive bands of mono-nNOS normalized to β -actin as loading control (N=3). **F.** No change in the mRNA expression level of nNOS normalized to GAPDH (N=3) between 3 and 6 hours time points showed that the disappearance of mono-nNOS is not at the transcriptional level. It is likely due to increased dimerization and further complexation with partner proteins, which increases nitrate stress in the brain after stroke.