Ischemic Postconditioning Relieves Cerebral Ischemia and Reperfusion Injury Through Activating T-LAK Cell-Originated Protein Kinase/Protein Kinase B Pathway in Rats

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Background and Purpose—Ischemic postconditioning (IPostC) protects against ischemic brain injury. To date, no study has examined the role of T-LAK-cell–originated protein kinase (TOPK) in IPostC- affordned neuroprotection. We explored the molecular mechanism related with TOPK in antioxidant effect of IPostC against ischemia/reperfusion.

Methods—Focal ischemia was induced in rats by transient middle cerebral artery occlusion. Reactive oxygen species production in the peri-infarct cortex was detected using dihydroethidium. Malondialdehyde, as a marker of lipid peroxidation, and 3-nitrotyrosine, as a marker of protein oxidation, were detected by ELISA. The expression or location of antioxidant proteins and signal molecules TOPK, phosphatase, and tensin homolog, and Akt was analyzed by Western blotting and immunofluorescence.

Results—Our results revealed that IPostC relieved transient middle cerebral artery occlusion–induced oxidative damage by reducing reactive oxygen species, malondialdehyde, and 3-nitrotyrosine accumulation in the peri-infarct cortex and raised levels of antioxidants peroxiredoxin-1, peroxiredoxin-2, and thioredoxin-1. In addition, IPostC increased p-AKT and p-TOPK levels, which colocalized in neural cells. In vitro TOPK knockdown by small interfering RNA decreased the levels of antioxidants peroxiredoxin-1, thioredoxin, and manganese superoxide dismutase activity in PC12 cells. In vivo intracerebroventricular injection of TOPK small interfering RNA reversed IPostC-induced neuroprotection by increasing infarct volume and nitric oxide content and reducing manganese superoxide dismutase activity. Moreover, IPostC-evoked Akt activation was blocked by TOPK small interfering RNA in vivo, but the decreased phosphorylated phosphatase and tensin homolog level in ischemia/reperfusion was not influenced by IPostC or by TOPK small interfering RNA treatment.

Conclusions—Our results suggest that the antioxidative effects of TOPK/Akt might contribute to the neuroprotection of IPostC treatment against transient middle cerebral artery occlusion. (Stroke. 2014;45:2417-2424.)

Key Words: brain ischemia ■ ischemic postconditioning ■ oxidative stress ■ T-LAK cell–originated protein kinase pathways and downstream functional molecules to remove the oxidation products.

T-LAK-cell–originated protein kinase (TOPK) is a mitogen-activated protein kinase kinase–like serine-threonine kinase, which promotes tumor cell proliferation and prevents apoptosis.9 The prevention on ultraviolet radiation-B-induced apoptosis in RPMI7951 melanoma cells by TOPK was through regulation of peroxidase activity to block intracellular H$_2$O$_2$ accumulation.10 Furthermore, inhibition of TOPK disrupts the proliferation and self-renewal of neural progenitor cells, indicating its significance in nerve regeneration.11 In addition, previous studies have reported that the phosphatase and tensin homolog (PTEN)/Akt pathway is a downstream signal of TOPK in the proliferation and migration of tumor...
cells, and we found that TOPK was significantly activated by IPostC after 2-hour ischemia/1-hour reperfusion. We, therefore, hypothesized that (1) IPostC probably reverses the increases of oxidative stress within ischemic neural cells; (2) IPostC might activate TOPK that induce neural cells to express antioxidative molecules that would contribute to the neuroprotection of IPostC; and (3) the PTEN/Akt pathway is the downstream target of TOPK, all contributing to the neuroprotection of IPostC in vivo.

Materials and Methods
Transient Focal Ischemia and Postconditioning in Rats
All animal experiments were approved by the Institutional Animal Care and Use Committee of Capital Medical University. Temporary focal ischemia was induced in male Sprague–Dawley rats weighing 280 to 300 g by intraluminal suture occlusion of the right middle cerebral artery (MCAO). To ensure the occurrence of ischemia by MCAO, regional cerebral blood flow was monitored using laser Doppler flowmetry (PeriFlux System 5000; Perimed, Stockholm, Sweden) at the following coordinates: 0.5 mm anterior and 5.0 mm lateral from bregma. The ipsilateral cerebral blood flow decreased to 15% to 25% of baseline. A total of 46 rats were used for experiments with 4 excluded, 2 in MCAO plus control small interfering RNA (siRNA) group, and 1 in MCAO plus control siRNA plus IPostC group because of early attrition, 2 in MCAO plus control siRNA group, and 1 in MCAO plus TOPK siRNA plus IPostC group because of failure to maintain cerebral blood flow reduction of <25% of baseline during study. Forty-two rats were assigned into 4 groups (n=12 per group, except for the sham group; n=6): sham operation; MCAO plus control siRNA (MCAO 2 hours followed by 24 hours of reperfusion); MCAO plus control siRNA plus IPostC (MCAO 2 hours followed by 5 episodes of reperfusion of the ipsilateral carotid artery for 10 seconds and reocclusion for 10 seconds and then reperfusion for 24 hours), MCAO plus TOPK siRNA plus IPostC (MCAO 2 hours followed by 5 episodes of reperfusion of the ipsilateral carotid artery for 10 seconds and reocclusion for 10 seconds and then reperfusion for 24 hours), TOPK siRNA (20 μmol/L; Santa Cruz Biotechnology, CA) was injected into the ipsilateral lateral ventricle after MCAO. The sequence of the siRNA-Topk-rat-745 was sense 5'-GGAGACAUAAA GUCUUCAATT-3' and antisense 5'-UGUGAGACUUUAUGUCUCCTT-3'.

Detection of Superoxide Generation
Superoxide generation was determined by fluorescent-labeled dihydroethidium (Vigorou Biotechnology Beijing Co, Ltd.) staining. Dihydroethidium is cell-permeable and reacts with superoxide to form ethidium, which in turn intercalates with DNA and produces nuclear fluorescence. Frozen brain sections were stained with 100 μmol/L dihydroethidium in phosphate buffered saline (PBS) for 90 minutes at room temperature. To determine the fluorescence intensity of oxidized dihydroethidium, 6 microscopic fields in the peri-infarct cortex were captured randomly by a fluorescence microscope (Carl Zeiss, Jena, Germany), with a setting of 20x magnification. The total number of cells in all images was counted and the percentage of nuclear dihydroethidium-positive cells was calculated.

Detection of Malondialdehyde and 3-Nitrotyrosine Levels and Manganese Superoxide Dismutase Activity
The peri-infarct cortex was defined as that used by Nagayama et al., as shown in Figure 1B. The ischemic core was detectable as an area of pallor, which was demarcated from the adjacent tissue. The peri-infarct cortex was dissected by an experienced technician mainly through the color and consistence of the parenchyma, which was handled carefully to minimize contamination by the infarct core to avoid an influence on the test results. Brain homogenates (10%; wt/vol) were prepared with cold PBS. Malondialdehyde (Usen Life Science Inc) and 3-nitrotyrosine (3-NT; Cusabio Biotech Co, Ltd) levels in brain homogenate supernatants were detected by ELISA. Manganese superoxide dismutase (MnSOD) activity was determined by Kit-WST (Beyotime Institute of Biotechnology, China) according to the manufacturer’s instructions. Absorbance was measured at 450 or 575 nm using a microplate reader. Malondialdehyde levels, 3-NT levels, and MnSOD activity were normalized to the total protein.

Western Blotting
Brain tissue from the peri-infarct cortex or PC12 cells were homogenized in a lysis buffer (1% Triton X-100, 50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl) containing protease inhibitors and phosphatase inhibitors (Sigma cocktail). Total protein (100 μg per sample) was resolved by 10% or 12% sodium dodecyl sulfate gel electrophoresis and transferred to polyvinylidene difluoride membranes. Blots were incubated with the following primary antibodies (1:1000) overnight at 4°C: p-TOPK Thr9, TOPK, p-PTEN (Ser380/Thr382/Thr383), Prx-2 (Epitomics), Prx-3 (EPITOMICS), and β-actin antibodies (Epitomics). Primary antibody was detected using horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology) for 60 minutes at room temperature and an enhanced luminiscence kit (Millipore, Germany).

Immunofluorescence
Rats were euthanized at 24 hours after reperfusion with intraperitoneal injections of chloral hydrate (300 mg/kg) and perfused transcardially with 4% wt/vol paraformaldehyde in PBS. After incubation for 2 hours in a blocking solution (0.3% Triton X-100, 2% normal goat serum, 1% bovine serum albumin, and 5% nonfat dry milk in PBS), frozen coronal sections were incubated with the primary antibodies p-AKT S473 and p-TOPK Thr9 (1:50). After washing with PBS, sections were incubated in a mixture of fluorescent secondary antibodies (Alexa 488/Alexa 594–conjugated antismouse/antirabbit immunoglobulin G). Images were captured with a fluorescence microscope.

PC12 Cell Culture and Treatment with TOPK siRNA
Rat pheochromocytoma PC12 cells purchased from the Institute of Biochemistry and Cell Biology (Chinese Academy of Science, Shanghai) were plated in 6-well culture dishes (Corning Inc, Corning, NY) in Dulbecco Modified Eagle Medium containing 10% (v/v) heat-inactivated PBS, 5% horse serum, 1% penicillin, and 1% streptomycin. The cells were grown at 37°C in a humidified 5% CO2 environment. TOPK siRNA oligos (20 μmol/L) were transfected into PC12 cells.
for 48 hours using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer’s protocol.

**Statistical Analysis**

All data were reported as mean±SEM. Statistical analysis was performed using SPSS version 11.0 (SPSS, Chicago, IL). The significance of difference was assessed by Student t test (single comparisons) or by 1-way ANOVA with Tukey post hoc tests (multiple comparisons). The level of significance was set at $P<0.05$.

**Results**

**IPostC Alleviated I/R-Induced Reactive Oxygen Species Accumulation, Lipid and Protein Peroxidation Production**

To investigate the preventive effect of brain IPostC on oxidative stress in the peri-infarct cortex, intracellular $O_2^•−$ accumulation at 2-hour ischemia/24-hour reperfusion was detected with the fluorescent probe dihydroethidium; protein oxidation marker 3-NT was detected by immunofluorescence and ELISA; lipid oxidation marker malondialdehyde was detected by ELISA; and total SOD activity was determined with a kit. The immunofluorescence images showed that no dihydroethidium or 3-NT was detected in the sham-operated rats, but both were notably present in the peri-infarct cortex of transient MCAO (tMCAO) rats, which was decreased by IPostC, indicating that $O_2^•−$ accumulation after I/R was reduced significantly by IPostC treatment (Figures 1A; dihydroethidium, 1-way ANOV A $P<0.001$; post hoc test $P<0.05$). In brain homogenates from the peri-infarct cortex, 3-NT and malondialdehyde levels in the tMCAO rats were higher than that in the sham rats, which were remarkably decreased in the IPostC plus tMCAO rats (Figure 1C; 3-NT, 1-way ANOVA $P<0.001$; post hoc test $P<0.05$; malondialdehyde, 1-way ANOVA $P=0.012$; post hoc test $P<0.05$). By contrast, total SOD activity in tMCAO rats was higher than in sham rats, but was not decreased by IPostC treatment (Figure 1C; 1-way ANOVA $P=0.014$; post
Reperfusion can cause overproduction of reactive oxygen species. The change of cerebral blood flow during I/R was determined in rats from the I/R group and I/R plus IPostC group. The results showed that there were no significant differences in initial cerebral blood flow between the 2 groups, and the percentage of regional cerebral blood flow reductions was 78±6% and 74±5% for the I/R and I/R plus IPostC groups, respectively. Compared with the I/R group, IPostC with 5 cycles of 10-second occlusion and 10-second reperfusion prevented hyperperfusion at MCA territory, when measured at 30 minutes after reperfusion, which was not statistically significant (Figure 1D). Collectively, these data reveal the potent inhibitory effect of brain IPostC on oxidative stress induced by cerebral I/R.

IPostC Upregulated the Antioxidative Proteins After I/R

To investigate the preventive mechanism of IPostC on IR-induced oxidative stress in the peri-infarct cortex, we detected the expression of Prx-1, Prx-2, and Prx-SO3, a novel group of peroxidases containing high antioxidant efficiency, and thioredoxin, an electron donor for Prx-1, after 2-hour ischemia/1-, 4-, and 24-hour reperfusion. Western blot analysis showed that the levels of Prx-1, Prx-2, and Prx-SO3 in the IPostC plus tMCAO group were increased dramatically compared with the sham or tMCAO after 2-hour ischemia/1-, 4-, and 24-hour reperfusion (Figure 2A–2C; Prx-1, 1-way ANOVA $P=0.012$; post hoc test $P<0.05$; Prx-2, 1-way ANOVA $P=0.014$; post hoc test $P<0.05$; Prx-SO3, 1-way ANOVA $P<0.001$; post hoc test $P<0.05$). In addition, thioredoxin expression was decreased in the tMCAO group after 2-hour ischemia/4- and 24-hour reperfusion compared with the sham group and elevated by IPostC treatment after 2-hour ischemia/4-hour reperfusion (Figure 2D; 1-way ANOVA $P=0.029$; post hoc test $P<0.05$). The upregulation of antioxidants by IPostC treatment is a potential mechanism underlying its inhibition of oxidative stress in the ischemic brain.

TOPK siRNA Decreased Expression of Antioxidative Proteins in PC12 Cells

To investigate the participation of TOPK in the upregulation of antioxidative proteins, we detected their expression after TOPK siRNA treatment for 48 hours in PC12 cells. Western blot analysis was conducted to verify the knockdown of TOPK (Figure 4A; $P<0.05$) and quantify changes in Prx-1, Prx-2, Prx-SO3, thioredoxin, and MnSOD levels; total SOD activity was detected with a kit. The expression of all antioxidative proteins and MnSOD activity, with the exception of Prx-2, was significantly decreased after TOPK siRNA compared with the control (Figure 4B–4D; $P<0.05$). These data demonstrated that TOPK activation is a potential kinase candidate for the irritative effect of IPostC on the increased expression of antioxidative proteins.
Protective Effect of IPostC on Brain Injury and Oxidative Stress After tMCAO Was Reversed by TOPK siRNA

To investigate the role of TOPK on neuroprotection afforded by IPostC against IR-induced brain injury and oxidative stress, TOPK siRNA was delivered by intracerebroventricular injection at the beginning of MCAO, and then the infarction volume, reactive oxygen species production, and MnSOD activity were detected after 2-hour ischemia/24-hour reperfusion. The infarction volume after tMCAO was decreased by IPostC, which was partially blocked by TOPK siRNA (Figure 77x333; 1-way ANOVA $P=0.003$; post hoc test $P<0.05$). In addition, nitric oxide levels were significantly increased after tMCAO, which was attenuated by IPostC treatment, but reversed by TOPK siRNA treatment (Figure 5B; 1-way ANOVA $P<0.001$; post hoc test $P<0.05$). MnSOD activity was increased after tMCAO, which was further elevated by IPostC treatment and was reversed by TOPK siRNA treatment (Figure 5C; 1-way ANOVA $P<0.001$; post hoc test $P<0.05$). These data suggested that the activation of TOPK was involved in the antioxidative and neuroprotective effect demonstrated by IPostC treatment.

Effect of IPostC on AKT Signaling After tMCAO Was Obstructed by TOPK siRNA

To investigate the crosstalk between TOPK and the PTEN/Akt signaling pathway in vivo, we detected the levels of p-PTEN and p-AKT after TOPK siRNA treatment by Western blotting. Compared with the sham group, the level of p-PTEN was significantly decreased in the tMCAO, tMCAO plus IPostC, and IPostC groups, while the level of p-AKT was significantly increased in the tMCAO, tMCAO plus IPostC, and IPostC groups (Figure 6; 1-way ANOVA $P<0.001$; post hoc test $P<0.05$). These data suggested that the activation of TOPK was involved in the antioxidative and neuroprotective effect demonstrated by IPostC treatment.
Stroke August 2014

and tMCAO plus IPostC plus TOPK siRNA treatment groups (Figure 6A; 1-way ANOVA $P=0.018$; post hoc test $P<0.05$). However, there was no significant difference between the tMCAO group and tMCAO plus IPostC treatment group or between the tMCAO plus IPostC treatment group and tMCAO plus IPostC plus TOPK siRNA treatment group. In contrast, the level of p-AKT was substantially increased after tMCAO compared with that of the sham group and was further elevated by IPostC treatment, but decreased by TOPK siRNA treatment (Figure 6B; 1-way ANOVA $P<0.001$; post hoc test $P<0.05$). These results demonstrated that activated TOPK/Akt pathway but not TOPK/PTEN/Akt is the downstream target of the irritative effect of IPostC treatment.

### Discussion

The present study showed that 5 cycles of 10-second reperfusion and 10-second ischemia was an effective IPostC procedure to suppress I/R-induced oxidative stress and brain injury in a rat MCAO model. Consistent with this, the expression and activity of major antioxidative enzymes in the peri-infarct cortex of rats were increased significantly by IPostC treatment. Further investigation showed that the phosphorylation of Akt and TOPK were also upregulated by IPostC treatment compared with MCAO, and they were colocalized in neural cells. Meanwhile, TOPK siRNA downregulated the expression of antioxidative proteins in PC12 cells, and TOPK siRNA treatment obviously inhibited the antioxidative and neuroprotective effect of IPostC in vivo. Interestingly, TOPK siRNA treatment also obstructed the IPostC-activated Akt pathway, showing greater crosstalk between TOPK and Akt pathways on IPostC treatment. Collectively, we demonstrated that the activated TOPK/Akt pathway–mediated antioxidative effect might contribute to neuroprotection of IPostC treatment against I/R injury.

To date, the neuroprotective mechanism underlying IPostC has been investigated widely and has been found to be related to the suppression of oxidative stress, endoplasmic stress, cell apoptosis and autophagy, and the inflammatory response. Among them, oxidative stress is a major cause of necrotic and apoptotic cell death after I/R, and IPostC was reported to reduce protein oxidization by increasing the levels of antioxidants including SOD and catalase. We observed...
that brain IPostC treatment could reduce peroxidation accumulation including reactive oxygen species, lipid and protein oxidation after experimental tMCAO in rats. In addition, we found that IPostC increased the levels of the antioxidative proteins Prx-1, Prx-2, and thioredoxin in the peri-infarct cortex of a tMCAO rat model. These data support the notion that IPostC has a potent inhibitory effect on neural cell oxidative stress in the peri-infarct cortex after cerebral I/R. However, the mechanism for its interference with oxidative stress was so far unclear.

In the present study, we tried to indentify the early responsive kinases for IPostC treatment that might activate the antioxidative pathway and the downstream functional molecules that mop up the oxidation product. We found that TOPK was activated by cerebral I/R and further increased by IPostC treatment; hence we speculated that the rise of p-TOPK signaling may be a potential mechanism responsible for IPostC treatment to increase the expression and activity of antioxidants. Further in vitro study showed that TOPK siRNA treatment reduced the antioxidative enzymes levels including Prx-1, thioredoxin, MnSOD, and the activity of MnSOD in PC12 cells. In addition, an in vivo study demonstrated that TOPK siRNA treatment prevented the antioxidative and neuroprotective effects of IPostC treatment. Previous studies showed that TOPK is a key regulator for the neural progenitor, but its role in brain ischemia or I/R remains unclear. Here, we have proven that TOPK is a potential antioxidative stress regulator after cerebral I/R and is one of the molecular mechanisms involved in the neuroprotection of IPostC treatment.

The crosstalk between TOPK and the PTEN/Akt pathway on IPostC treatment was further investigated. Evidence supports that the PTEN/Akt pathway is an important downstream target of TOPK in promoting the proliferation and migration of tumor cells. One notion is that overexpression of TOPK decreases the expression of PTEN and increases the activation of Akt in lung cancer cell lines. Another idea is that TOPK phosphorylates PTEN at the S380 residue and inactivates PTEN, which in turn activates Akt that leads to proper G2/M progression. Although the modulation of PTEN by TOPK was different in these studies, both showed that TOPK could activate Akt. In the present study, the increase of both p-TOPK and p-AKT after I/R was enhanced by IPostC treatment, and p-TOPK was colocalized with p-AKT in same neural cells in the peri-infarct cortex, indicating the potential crosstalk between p-TOPK and p-AKT. Further studies showed that knockout down TOPK during tMCAO plus IPostC blocked the activation of Akt by IPostC treatment, suggesting greater crosstalk between TOPK and Akt on IPostC treatment. However, p-PTEN levels were decreased after 2-hour ischemia/24-hour reperfusion but not influenced by IPostC with or without TOPK siRNA treatment. Therefore, our study reveals that TOPK might be a potential mechanism by which IPostC treatment upregulates antioxidants to protect against I/R-induced oxidative stress, and that the Akt pathway might be a direct or indirect downstream target of TOPK involved in the antioxidative and neuroprotective functions of IPostC. In addition, TOPK functions upstream of some other important pathways, partly as a mitogen-activated protein kinase kinase by phosphorylation of p38 MAPK, JNK1, and ERK2. Whether these pathways interacted with TOPK on IPostC treatment needs further investigation.

In addition, IPostC with 5 cycles of 10-second occlusion and 10-second reperfusion decreased the level of hyperperfusion at MCA territory at 30 minutes after reperfusion; still there was no statistical significance compared with the MCAO group. It could prevent overproduction of reactive oxygen species and further reperfusion injury with hyperperfusion. Our results and others reports suggest that IPostC might be a feasible preventive method for reperfusion injury secondary to revascularization. IPostC may eventually be clinically applicable to certain clinical settings for patients with stroke or patients subjected to endovascular therapy associated with carotid artery or other cerebral vessel occlusion and revascularization. Challenges in the application of IPostC to pressing clinical cerebrovascular disease should be overcome through careful, well-designed, translational investigations.

Conclusions

Brain IPostC treatment increased TOPK activation, accompanied with the increased levels of antioxidants Prx-1, thioredoxin, as well as the activation of SOD. The relationship between TOPK activation and the increased levels of antioxidants was further established in vitro. This investigation is the first demonstration of the involvement of TOPK in the antioxidative and neuroprotective effects afforded by IPostC treatment against brain I/R injury. Furthermore, Akt pathway activation might initiate the irritative response of TOPK activation on IPostC treatment.

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Disclosures

None.

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