Activation of Epsilon Protein Kinase C-Mediated Anti-Apoptosis Is Involved in Rapid Tolerance Induced by Electroacupuncture Pretreatment Through Cannabinoid Receptor Type 1

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Background and Purpose—Our previous study has demonstrated that the rapid tolerance to cerebral ischemia by electroacupuncture (EA) pretreatment was possibly mediated through an endocannabinoid system-related mechanism. The purpose of this study was to investigate whether activation of epsilon protein kinase C (εPKC) was involved in EA pretreatment-induced neuroprotection via cannabinoid receptor type 1 in a rat model of transient focal cerebral ischemia.

Methods—The activation of εPKC in the ipsilateral brain tissues after EA pretreatment was investigated in the presence or absence of cannabinoid receptor antagonists. At 2 hours after the end of EA pretreatment, focal cerebral ischemia was induced by middle cerebral artery occlusion for 120 minutes in rats. The neurobehavioral scores, infarction volumes, neuronal apoptosis, and the expression of Bcl-2 and Bax were evaluated after reperfusion in the presence or absence of εPKC-selective peptide inhibitor (TAT–εV1–2) or activator (TAT–εV1–2).

Results—EA pretreatment enhanced εPKC activation. Systemic delivery of TAT–εRACK conferred neuroprotection against a subsequent cerebral ischemic event when delivered 2 hours before ischemia. Pretreatment with EA reduced infarct volumes, improved neurological outcome, inhibited neuronal apoptosis, and increased the Bcl-l-to-Bax ratio after reperfusion, and the beneficial effects were attenuated by TAT–εV1–2. In addition, the blockade of cannabinoid receptor type 1, but not cannabinoid receptor type 2 receptor, reversed the increase in εPKC activation and neuroprotection induced by EA pretreatment.

Conclusion—EA pretreatment may activate endogenous εPKC-mediated anti-apoptosis to protect against ischemic damage after focal cerebral ischemia via cannabinoid receptor type 1, which represents a new mechanism of EA pretreatment-induced rapid tolerance to focal cerebral ischemia in rats. (Stroke. 2011;42:389-396.)

Key Words: apoptosis • cerebral ischemia • electroacupuncture • pretreatment • protein kinase C

Our previous studies have demonstrated that pretreatment with electroacupuncture (EA) induces rapid tolerance to cerebral ischemic insult, which appears at 2 hours after pretreatment, and that the rapid ischemic tolerance is possibly mediated through an endocannabinoid system-related mechanism in which EA pretreatment increases the production of the endocannabinoid 2-arachidonylglycerol and N-arachidonylethanolamine-anandamide, which elicit protective effects against transient cerebral ischemia via cannabinoid receptor type 1 (CB1) receptors. However, the factors linking EA pretreatment with the development of ischemic tolerance are complex and unclear.

The resultant activation of the CB1 receptor triggers signal transduction events that can influence compensatory responses. Cellular responses that elicit neuroprotection may involve CB1 receptors and their link to a variety of signaling elements, including the Gi/Go family of G-proteins, mitogen-activated protein kinase, and its substrate extracellular signal-regulated kinase. Previous studies have demonstrated that the protein kinase C (PKC) signaling pathway was implicated in cerebral ischemic preconditioning via the adenosine A1 receptor and adenosine-induced preconditioning in vitro. PKC is a widely expressed family of serine/threonine kinases. It has been demonstrated that the activation of PKC in the central nervous system may play a key role in mediating both rapid and delayed preconditioning. Interestingly, among the different PKC isozymes, multiple studies have now demonstrated that epsilon PKC (εPKC) was a key

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player in the induction of ischemic tolerance in various models of preconditioning.\textsuperscript{12–15} Furthermore, a recent study reported that $\epsilon$PKC confers acute tolerance to cerebral ischemic/reperfusion injury.\textsuperscript{10} Based on these findings, the present study was undertaken to test the hypothesis that an activation of $\epsilon$PKC was involved in EA pretreatment-induced neuroprotection via CB1 receptors in rat model of transient focal cerebral ischemia.

Materials and Methods

Animal Care

The experimental protocol used in this study was approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University and was conducted according to the Guidelines for Animal Experimentation of the Fourth Military Medical University (Xi’an, China). Male Sprague-Dawley rats, weighing 280 to 320 grams, were provided by the Experimental Animal Center of the Fourth Military Medical University (Xi’an, China) and housed under controlled conditions with a 12-hour light/dark cycle, a temperature of 21°C to 2°C, and humidity of 60% to 70% for at least 1 week before drug treatment or surgery. The rats were allowed free access to standard rodent diet and tap water.

Peptide Preparation and Drugs

The $\phi$RACK (receptor for activated C kinase, an $\epsilon$PKC activator peptide, $\epsilon$PKC85–92, C-HDAPIGYD) or $\epsilon$V1–2 (an $\epsilon$PKC inhibitor peptide, $\epsilon$PKC14–21, C-EAVSLKPT) was synthesized as previously described.\textsuperscript{16} The protein transduction domain of the transactivator of transcription (TAT) protein (C-YGRKKRRQRRR), was conjugated to the $\delta$PKC peptide via a disulfide conjugation through free cysteines at the N-terminus.\textsuperscript{17} The new fusion protein was named TAT–$\phi$RACK or TAT–$\epsilon$V1–1, respectively. The TAT–$\beta$-galactosidase (TAT–$\beta$-Gal) fusion protein, as a control protein, was obtained as previously described.\textsuperscript{2,18} The $\epsilon$PKC isozyme-selective activity of TAT–$\phi$RACK (TAT–$\phi$RACK) and TAT–$\epsilon$V1–2 (TAT–$\epsilon$V1–2) previously has been demonstrated in both in vitro and in vivo models\textsuperscript{19,20} and, in particular, has been shown to alter $\epsilon$PKC activity in the brain after systemic intraperitoneal delivery.\textsuperscript{19} Peptide dose (0.2 mg/kg) was based on previous studies.\textsuperscript{19–21} The AM251 (a PKC activator particulate (membrane) fractions, as previously described.\textsuperscript{24} To compare $\epsilon$PKC concentration in each fraction, total protein concentration was assessed using Bradford reagent, and 20 $\mu$g of each fraction was subjected to gel electrophoresis (12% bisacrylamide gel) and transferred to nitrocellulose membrane. Blots from each fraction were probed with an anti-rabbit secondary antibody. 

Fourth Experiment

To test the regulatory effect of $\epsilon$PKC on neuronal apoptosis, rats were randomly divided into 5 groups: control, MCAO, EA + MCAO, TAT–$\epsilon$V1–2 + EA + MCAO, and TAT–$\phi$RACK + MCAO groups.

Fifth Experiment

To explore the role of cannabinoid receptors in neuroprotection induced by EA pretreatment, rats were randomly divided into 6 groups: MCAO, EA + MCAO, AM251 + EA + MCAO, AM251 + MCAO, AM630 + EA + MCAO, and AM630 + MCAO groups. To further investigate the regulatory effect of cannabinoid receptors on activation of $\epsilon$PKC after EA pretreatment, rats were randomly divided into 6 groups: sham, EA, AM251 + EA, AM251, AM630 + EA, and AM630 groups. Details of the experimental grouping and protocols are included in the online Supplementary Data (please see http://stroke.ahajournals.org).

EA Pretreatment

EA pretreatment was performed at the acupoint Baihui (GV 20) of rats under anesthesia with 40 mg/kg sodium pentobarbital (intraperitoneal), as described in our previous studies.\textsuperscript{1,2} The detailed methodology for EA pretreatment is described in the online Supplementary Data.

Transient Focal Cerebral Ischemia

Focal cerebral ischemia was induced by MCAO in rats using an intraluminal filament technique as described previously.\textsuperscript{2,18} Regional cerebral blood flow (rCBF) was monitored through a disposable microtip optic probe (diameter, 0.5 mm) connected through a Master Probe to a laser Doppler computerized main unit (PeriFlux 5000; Perimed AB). The MCAO was considered adequate if rCBF showed a sharp decrease to 20% of baseline (before ischemia level); otherwise, animals were excluded. Reperfusion was accomplished by withdrawing the suture after 120 minutes of ischemia, rCBF recovered up to >80% of baseline, and then wounds were sutured.

Neurobehavioral Evaluation and Infarct Assessment

At 72 hours after reperfusion, an 18-point scoring system reported by Garcia et al.\textsuperscript{22} with modifications was used for neurological assessment by a blinded observer. Then, animals ($n$=8 for each group) were decapitated and 2-mm-thick coronal sections from throughout the brain were stained with 2% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) to evaluate the infarct volume, as described in previous studies.\textsuperscript{2,18}

TUNEL Staining

Samples from 5 groups ($n$=5 for each group) in experiment 4 were used for experiments. At 24 hours after reperfusion, neuronal cell apoptosis in the ischemic penumbra was assessed in situ by TUNEL staining as described in our previous studies.\textsuperscript{2,18} The TUNEL staining was quantitatively evaluated with the method described by Wang et al.\textsuperscript{23} Briefly, 32 pixels of 0.10 mm$^2$ were placed by light microscope with 100× magnification, and then the total number of positively stained cells in these pixels was counted and expressed as cells/mm$^2$.

Western Blot Analysis

For translocation of $\epsilon$PKC, the brain tissues from identical ipsilateral area to ischemic penumbra were quickly isolated and snap-frozen. Tissue fractionation was performed to collect soluble (cytosolic) and particulate (membrane) fractions, as previously described.\textsuperscript{24} To compare $\epsilon$PKC concentration in each fraction, total protein concentration was assessed using Bradford reagent, and 20 $\mu$g of total lysate from each fraction was subjected to gel electrophoresis (12% bisacrylamide gel) and transfered to nitrocellulose membrane. Blots were blocked in 3% milk Tris-buffered saline Tween, probed with an anti-$\epsilon$PKC (C-15) rabbit polyclonal antibody (1:500 dilution; Santa Cruz Biotechnology) in 2% milk Tris-buffered saline Tween and probed with an anti-rabbit secondary antibody. For Bcl-2 and Bax, at 24 hours after reperfusion the ipsilateral ischemic penumbra ($n$=6 per group) was dissected and then homogenized and lysed on ice in RIPA buffer containing protease inhibitors. The supernatant was collected by centrifugation, and protein...
Physiological parameters and rCBF changes are summarized observed in the rCBF changes at different time points. No significance was observed. The rCBF was monitored in the period of transient remained in the normal range during the experimental period arterial pressure, body temperature, and plasma glucose against ischemic injury.

Statistical Analysis

Brain sections were examined by 2 independent and blinded investigators. The software (SPSS 13.0 for Windows; SPSS) was used to conduct statistical analyses. All values, except for neurological scores, are presented as mean±SEM and were analyzed by 1-way analysis of variance, and between-group differences were detected with post hoc Student-Newman-Keuls test. The neurological deficit scores were expressed as median (range) and were analyzed with Kruskal-Wallis test, followed by the Mann-Whitney U test with Bonferroni correction. Values of P<0.05 were considered as statistically significant.

Results

Physiological Parameters

Physiological parameters of animals in the period of EA pretreatment and transient focal cerebral ischemia and reperfusion were analyzed. There were no significant differences for the variables during EA pretreatment (at the onset of EA, 15 minutes after EA, and the end of EA) and surgery (at the onset of ischemia, 60 minutes after ischemia, and 30 minutes after reperfusion). Arterial blood gases (Po2, Pco2, pH), mean arterial pressure, body temperature, and plasma glucose remained in the normal range during the experimental period observed. The rCBF was monitored in the period of transient focal cerebral ischemia and reperfusion. No significance was observed in the rCBF changes at different time points. Physiological parameters and rCBF changes are summarized in the online Supplementary Data.

EA Pretreatment Enhances εPKC Activation

Using a time course of EA pretreatment, εPKC translocation was assessed in the brain tissues from identical ipsilateral area to ischemic penumbra. EA pretreatment produced an evident activation of εPKC as revealed by Western blot (Figure 1B). The semiquantitative analysis of Western blot indicated that at 30 minutes after the end of EA pretreatment, the proportion of εPKC in the membrane-bound fraction was significantly higher than that in the sham animals (96% increase; P<0.05). This translocation peaked at 60 minutes (134% increase; P<0.01) and was maintained at 120 minutes after the end of EA pretreatment (59% increase; P<0.05). However, there was no difference in εPKC translocation between the sham and naive control groups at 30, 60, and 120 minutes after the end of EA pretreatment (Figure 1C).

Activation of εPKC Conferred Neuroprotection Against Ischemic Injury

As noted in Figure 2, at 72 hours after reperfusion pretreatment with TAT–εRACK significantly improved the neurological scores and reduced the infarction volumes (33.5±1.8%) compared with those of the MCAO group (46.1±2.0%; P=0.000 and P=0.011, respectively). There were no statistical differences between MCAO and TAT–β-Gal+MCAO groups (45.5±2.1%; P=0.844 and P=0.971, respectively).

EA Pretreatment-Induced Neuroprotection Was Alleviated by TAT–εV1–2 Intervention

As shown in Figure 3B, EA + MCAO group showed a smaller brain infarct volume (24.1%±2.8%) compared with MCAO group (45.1%±2.9%; P=0.000). The infarct volume of the TAT–εV1–2+EA group (36.3%±2.7%) was smaller than that of the MCAO group (P=0.025) and was larger than that of the EA+MCAO group (P=0.003). However, the infarct volume of the TAT–β-Gal+EA group (26.6%±2.3%) was still significantly different from that of the MCAO group (P=0.000) and was similar to that of the EA+MCAO group (P=0.513). The result of the TAT–εV1–2+MCAO group (47.1±2.6%) was not significantly different from that of the MCAO group (P=0.612).

Similar changes were observed in neurological scores, except the neurological score of the TAT–εV1–2+EA group was similar to that of the MCAO group (P=0.579; Figure 3A).

EA Pretreatment-Induced Reduction of Neuronal Apoptosis Is Attenuated by TAT–εV1–2

No positive TUNEL staining (brown) was detected in the brain sections of control animals at 24 hours after reperfusion. How-
ever, a large number of TUNEL-positive cells in the ischemic penumbra of rat brain were seen in the MCAO, TAT–eV1–2+EA, and TAT–eV1–2+MCAO groups, whereas, in contrast, only small amounts of TUNEL-positive cells in the EA+MCAO and TAT–β-Gal+EA groups were observed (Figure 4A). The quantitative analysis of the number of TUNEL-positive cells in the ischemic penumbra of rats showed that the pretreatment with EA significantly reduced the number of TUNEL-positive cells (24.7 ± 2.0) at 24 hours after reperfusion compared to the MCAO (51.0 ± 2.3), TAT–eV1–2+EA (46.3 ± 1.6), and TAT–eV1–2+MCAO (21.7 ± 3.4) groups (P<0.05). There was no difference among the MCAO, TAT–eV1–2+EA, and TAT–eV1–2+MCAO groups (Figure 4B).

Expression of Bcl-2 and Bax Proteins in the Ischemic Penumbra
As shown in Figure 5, at 24 hours of reperfusion the levels of Bcl-2 proteins in the ischemic penumbra of rats were higher than in sham-operated animals (P<0.05; MCAO vs control). Compared with rats only subjected to MCAO, EA or TAT–ψRACK pretreatment markedly upregulated the Bcl-2 levels (P<0.05 vs MCAO) in the ischemic penumbra at 24 hours after reperfusion, whereas TAT–eV1–2 intervention before EA stimulus clearly suppressed the increase in Bcl-2 protein contents by EA pretreatment (P<0.05; TAT–eV1–2+EA vs EA+MCAO). Focal cerebral ischemia/reperfusion significantly increased the Bax content in ischemic penumbra at 24 hours after reperfusion (P<0.05; MCAO vs control). Interestingly, the upregulation of Bax in the ischemic penumbra was markedly reduced by EA and TAT–ψRACK pretreatment (P<0.05 vs MCAO). TAT–eV1–2 intervention before EA stimulus clearly reversed the reduction in Bax protein levels by EA pretreatment (P<0.05; TAT–eV1–2+EA vs EA+MCAO).

AM251, but not AM630, Inhibited Activation of ePKC by EA Pretreatment
As shown in Figure 6A, the EA+MCAO group (25.3 ± 2.3%) showed a smaller brain infarct volume compared with the MCAO group (45.6% ± 2.5%; P=0.000). The infarct volume of the AM251+EA+MCAO group (42.1% ± 2.7%) was
larger than that of the EA/H11001 MCAO group (P/H11005 0.003), whereas the infarct volume of the AM630/H11001 EA/H11001 MCAO group (26.6%/H11006 3.0%) was similar to that of the EA/H11001 MCAO group and smaller than that of the MCAO group. There was no difference among MCAO, AM251/H11001 MCAO (44.1%/H11006 2.9%), and the AM630/H11001 MCAO (43.6%/H11006 2.9%) groups. Similar changes were observed in neurological scores (Figure 6B).

Figure 4. Neuronal cell apoptosis at 24 hours after reperfusion in the rats with 120 minutes of middle cerebral artery occlusion (MCAO; n=5). A, Representative photomicrographs of TUNEL staining in the penumbral zone. The blue cells indicate viable cells and the brown cells indicate TUNEL-positive cells. Scale bars = 20 μm. B, Quantitative analysis of the number of TUNEL-positive cells in the ischemic penumbra of rats in 5 groups. *P<0.01 vs MCAO; #P<0.01 vs EA+MCAO.

Furthermore, there was no difference in translocation of εPKC among the sham, AM251, and AM630 groups. The proportion of εPKC in the membrane-bound fraction in the EA and AM630+EA groups was significantly higher than that of the MCAO group (P<0.01). However, AM251 inhibited the increase in εPKC activation induced by EA pretreatment (P<0.05; AM251+EA vs EA) and had no effect on the translocation of εPKC when administered alone (P>0.05; AM251 vs sham; Figure 6C).

Discussion
As one of the top killers of humans, cerebral ischemia claims hundreds of thousands of lives every year throughout the world. To fulfill an increasing need for an effective and practical intervention strategy, it is important to understand the mechanisms underlying a potent neuroprotection. Preconditioning, as a potent endogenous protective maneuver, activates several endogenous signaling pathways that result in protection against ischemia.27–28 Identification of these pathways and their targets will likely contribute to the development of novel therapeutic concepts.29 We recently reported that EA pretreatment produced rapid ischemic tolerance against lethal ischemia.1 However, the mechanisms responsible for ischemic tolerance are complex and remain to be further elucidated, but they appear to involve an early cellular response.30 The signaling mechanisms of rapid EA pretreatment remain obscure, except a potential involvement of adenosine A1 receptor1 and endocannabinoid system.2

The PKC family of serine/threonine kinases consists of at least 11 different isozymes. Importantly, PKC plays a potential role in mediating ischemic and reperfusion damages in the brain.30 However, individual PKC isozymes mediate different and sometimes opposing functions after activation by the same stimulus.17,31 On stimuli, PKC isoforms translocate from the cytosol to subcellular membrane regions, a process associated with their activation. Such translocation has been deemed as a hallmark of PKC activation.32 Activating...
We found that at 30 minutes after the end of EA stimulus, PKC translocation before ischemia protects the heart by mimicking preconditioning, whereas inhibition of δPKC during reperfusion protects the heart from reperfusion-induced damage. The role for δPKC in cerebral tolerance has been verified using various in vitro models in which δPKC was activated after a preconditioning stimulus, such as application of ischemia or adenosine or NMDA, and was required for preconditioning-induced protection. Upregulation of δPKC in cerebral tolerance has been verified using various in vitro models in which δPKC was activated after a preconditioning stimulus, such as application of ischemia or adenosine or NMDA, and was required for preconditioning-induced protection.12,15,20,34 Similar to these studies in vitro, using an in vivo animal model PKC isoform-specific membrane translocation and protein expression in brain regions in intact mice with hypoxic preconditioning was investigated. Those results demonstrated that the development of cerebral hypoxic preconditioning was accompanied by an obvious increase in membrane translocation of δPKC in the cortex and hippocampus, but no significant changes were noted in the membrane translocation of other PKC isoforms or in the whole protein expression of all 11 PKC isoforms, suggesting that activation of δPKC might be involved in the development of cerebral hypoxic preconditioning of mice.8 In addition, δPKC conferred acute tolerance to cerebral ischemic/reperfusion injury.10

For this reason, we examined the effect of EA pretreatment on δPKC translocation before ischemia insults in intact rats. We found that at 30 minutes after the end of EA stimulus, ~134% increase in translocation of δPKC to membrane fraction was observed. δPKC activation at early time points after the stimulus suggested that this isozyme was activated after even short periods of stress. Therefore, it was likely that δPKC was activated and may mediate the initial cellular response to exogenous stress, such as EA pretreatment in vivo. Sustained δPKC activation at 120 minutes after EA pretreatment also implied that δPKC activity contributed to the development of ischemic tolerance against more severe ischemic insults. These results were consistent with previous studies in which δPKC was activated within 1 hour after pretreatment stress.12

After our finding that δPKC was activated in response to EA stimuli, we further examined whether activation or inhibition of δPKC altered outcome after ischemic stroke. Systemic delivery of TAT-ψRACK (an εPKC-selective peptide activator) with direct activation of δPKC before ischemia conferred a significant reduction in infarct size and improvement of neurological function compared to control fusion protein (TAT–β-Gal). These results were consistent with previous studies in which activation of εPKC specifically mediated protective signaling before or early during ischemia; however, it may not be involved in promoting cell survival during the reperfusion period.10,12 Further support for the important role of δPKC in EA pretreatment emerged from this study showing that neuroprotection of EA pretreatment could be partly blocked with TAT-εV1–2, an εPKC-selective peptide antagonist. These results indicated that activation of εPKC before ischemia attack may act as an essential step to switch the cells to a tolerant state from ischemia insults, and failure of such translocation results in the loss of neuroprotection, as we observed in the presence of εPKC antagonist.

Compelling evidence indicates that apoptosis may occur in the ischemic penumbra after transient cerebral ischemia. In this study, we observed that the EA pretreatment significantly reduced the neuronal apoptosis in the ischemic penumbra, whereas TAT–εV1–2 intervention before EA stimulus clearly reversed the beneficial effect, suggesting EA pretreatment alleviated neuronal apoptosis via εPKC activation. Extensive evidence suggests that Bcl-2 family shows a complex network regulating apoptosis. Bcl-2 is an anti-apoptotic protein, whereas Bax is pro-apoptotic. The balance between these proteins is critical to turning on and off the cellular apoptotic machinery.35 Upregulation of Bcl-2 and attendant decrease of Bax-to-Bcl-2 ratio appear to have key roles in protective ischemic preconditioning.36 Our present study showed that ischemia significantly increased the ratio of the pro-apoptotic Bax to the anti-apoptotic Bcl-2, which was consistent with the previous studies.36 However, pretreatment with EA or TAT–ψRACK reduced the expression of Bax and increased the expression of Bcl-2, thereby ameliorating the ischemia-induced Bax-to-Bcl-2 ratio elevation in the ischemic penumbra. Moreover, TAT–εV1–2 intervention before EA stimulus clearly reversed the regulatory effect of EA pretreatment on Bax-to-Bcl-2 ratio. The results further support that the neuroprotective effect of EA pretreatment on ischemia-induced apoptosis might be, at least partly, mediated by regulating the expression of Bax and Bcl-2 through εPKC activation.

The mechanisms of εPKC activation induced by EA pretreatment may be associated with upstream activators or mediators. In the central nervous system, cannabinoids act mainly via stimulation of the central CB1 receptor that is highly localized in the basal ganglia, hippocampus, cortex,
and molecular layer of the cerebellum.37 Our previous study showed that EA pretreatment upregulated the neuronal expression of CB1 receptor in the rat brains. EA pretreatment-induced neuroprotective effects were attenuated by AM251 or CB1 knockdown. Those findings indicated that EA pretreatment elicited protective effects against transient cerebral ischemia through CB1 receptors.2 In the present study, AM251, a selective antagonist that blocks anandamide and 2-arachidonoylglycerol from binding the CB1 receptors, inhibited EA pretreatment-induced ePKC activation and neuroprotective effects, whereas AM630 (a selective CB2 antagonist) had no effect on the translocation of ePKC and beneficial effects on EA pretreatment when administered before EA pretreatment, indicating that the activation of ePKC involved in EA pretreatment-mediated neuroprotection is dependent on CB1 receptor.

Conclusion
Together with previous work, the current results strongly suggest that ePKC activation-mediated anti-apoptosis was involved in EA pretreatment through CB1 receptor. Although a further investigation is needed to elucidate the detailed signal cascades underlined in the CB1 receptor–PKC pathway of the EA pretreatment, the present findings may represent a novel mechanism for mechanism of pretreatment with EA-induced rapid tolerance to focal cerebral ischemia in rats and also provide considerable implication for other PKC-related anti-ischemia interventions.

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Disclosures
None.

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Supplemental Methods

1. Experimental protocols

   **Experiment I:** To assess effect of EA pretreatment on the εPKC activation prior to ischemia, rats were randomly divided into 3 groups: Naive, Sham and EA groups. The rats in Naive control group did not receive any treatment. The animals in EA group only received EA stimuli for 30 min without subjecting to MCAO. The rats in Sham group received an identical protocol as EA group without electrical stimulation. For εPKC translocation assays, rats were sacrificed at 30 min, 60 min or 120 min after the end of EA pretreatment. The brain tissues from identical ipsilateral area to ischemic penumbra were harvested and snap frozen for tissue homogenization.

   **Experiment II:** To determine whether activation of εPKC confer rapid tolerance against ischemic injury, the rats were randomly divided into 3 groups: MCAO, TAT–ΨεRACK+MCAO and TAT–β-Gal+MCAO groups. The rats were intraperitoneally injected with 1 ml saline, 0.2 mg/kg TAT–ΨεRACK, or 0.2 mg/kg TAT-β-Gal (all injections 0.2 mg/kg in 1 ml saline) at 2 h before MCAO. The neurological scores and infarct volumes were evaluated at 72 h after reperfusion.

   **Experiment III:** To evaluate the effect of TAT–εV1-2 on neuroprotection induced by EA pretreatment, male rats were randomly assigned to 5 groups: MCAO, EA+MCAO, TAT–εV1-2+EA, TAT–β-Gal+EA and TAT–εV1-2+MCAO groups. All rats were anesthetized with 1% sodium pentobarbital (40 mg/kg, i.p.) at 3 h before induction of focal cerebral ischemia. The animals in MCAO group only received MCAO and the rats in EA+MCAO group received EA pretreatment for 30 min at 2 h before induction of focal cerebral ischemia. Animals in TAT–β-Gal+EA group and TAT–εV1-2+EA group were pretreated with 0.2 mg/kg TAT–β-Gal and TAT–εV1-2 at 30 min prior to EA pretreatment respectively. Rats in TAT–εV1-2+MCAO group were intraperitoneally injected with 0.2 mg/kg TAT–εV1-2 at 3 h before MCAO. The neurological scores and infarct volumes were evaluated at 72 h after reperfusion.

   **Experiment IV:** To test the regulatory effect of εPKC activation on neuronal apoptosis, rats were randomly divided into 5 groups: Control, MCAO, EA+MCAO, TAT–εV1-2+EA and TAT–ΨεRACK+MCAO groups. The rats in Control group received an identical protocol as MCAO group without MCAO. The animals in other groups received the same procedure as described above. The neuronal apoptosis and the expression of Bcl-2 and Bax in the ischemic penumbra were assessed at 24 h after reperfusion.

   **Experiment V:** To explore the role of cannabinoid receptors in neuroprotection induced by EA pretreatment, rats were randomly divided into 6 groups: MCAO, EA+MCAO, AM251+EA+MCAO, AM251+MCAO, AM630+EA+MCAO and AM630+MCAO groups. All rats were anesthetized with 1% sodium pentobarbital (40 mg/kg, i.p.) at 3 h before induction of focal cerebral ischemia, received the same procedure as above experiment. The neurological scores and infarct volumes were
evaluated at 72 h after reperfusion.

To further investigate the regulatory effect of cannabinoid receptors on activation of εPKC following EA pretreatment, rats were randomly divided into 6 groups: Sham, EA, AM251+EA, AM251, AM630+EA and AM630 groups. The animals in Sham and EA groups received the same procedure as in experiment I. The rats in AM251+EA and AM630+EA groups were injected intraperitoneally 1 mg/kg AM251 or AM630 at 30 min prior to the beginning of EA pretreatment respectively. The animals in AM251 and AM630 groups were intraperitoneally injected with 1 mg/kg AM251 or AM630 at 2 h before tissue harvest respectively. The brain tissues from identical ipsilateral area to ischemic penumbra were harvested and snap froze for εPKC translocation assays at 60 min after the end of EA pretreatment.

2. Electroacupuncture Pretreatment

EA pretreatment was performed as described in our previous studies.1,2 Briefly, animals were anesthetized with 40 mg/kg sodium pentobarbital (i.p.), and inhaled oxygen by face mask at a flow rate of 1 L/min. The acupoint “Baihui (GV 20)”, which is located at the intersection of sagittal midline and the line linking two rat ears, was stimulated with the intensity of 1 mA and frequency of 2/15 Hz for 30 min by using the G6805-2 EA Instrument (Model No.227033, Qingdao Xinsheng Ltd., Qingdao, China). The core temperature of all the rats was maintained (Spacelabs Medical Inc., Redmond, WA) at 37.0°C±0.5°C during EA pretreatment by surface heating or cooling. The right femoral artery was cannulated for continuous monitoring of blood pressure and for arterial blood sampling. Arterial blood gases and plasma glucose were measured at the onset of EA, 15 min after EA and at the end of EA.

Supplemental Tables

S1. Physiologic variables during EA pretreatment. (n=5)

<table>
<thead>
<tr>
<th></th>
<th>MAP(mmHg)</th>
<th>Arterial blood gases</th>
<th>Glucose (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>PaO₂ (mmHg)</td>
<td>PaCO₂ (mmHg)</td>
</tr>
<tr>
<td>Onset of EA</td>
<td>102±3.1</td>
<td>7.40±0.02</td>
<td>132.6±4.8</td>
</tr>
<tr>
<td>15min after EA</td>
<td>106±3.8</td>
<td>7.39±0.03</td>
<td>135.2±5.1</td>
</tr>
<tr>
<td>End of EA</td>
<td>107±4.3</td>
<td>7.37±0.02</td>
<td>129.7±5.4</td>
</tr>
</tbody>
</table>

All the variables are presented in mean±SEM. MAP=mean arterial pressure; T=rectal temperature; PaO₂= arterial oxygen partial pressure; EA= electroacupuncture; PaCO₂= arterial CO₂ partial pressure;
S2. Regional cerebral blood flow of ischemic hemisphere during surgery. (n=10)

The initial rCBF before occlusion was recorded as 100% and subsequent flow changes are expressed relative to this value. During occlusion, the rCBF values were centralized and remained at <20% of baseline for all rats. At the onset of reperfusion, rCBF recovered up to >80% of baseline, and then returned to baseline within 30 min. As shown in S2, no significance was observed in the rCBF changes between MCAO group and EA+MCAO group. The animals in MCAO group only received MCAO and the rats in EA+MCAO group received EA pretreatment for 30 min at 2 h before induction of focal cerebral ischemia. Data represent mean ± SEM.

Supplemental References
