ePKC May Contribute to the Protective Effect of Hypothermia in a Rat Focal Cerebral Ischemia Model

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**Background and Purpose**—Protein kinase C epsilon (εPKC) has been implicated as a neuroprotectant in vitro. We studied εPKC activation (by its localization and proteolysis) in a rodent stroke model and correlated the effects of hypothermia with εPKC activity after cerebral ischemia.

**Methods**—Rats were subjected to permanent distal middle cerebral artery occlusion plus 1 hour of bilateral common carotid artery occlusion. Body temperatures were maintained at 37°C or 30°C during common carotid artery occlusion. Brains were harvested at 10 minutes, 4 hours, and 24 hours after common carotid artery release, and the cortex corresponding to the ischemic core and penumbra was dissected. εPKC localization after stroke was assessed by Western blot and immunofluorescence microscopy. A caspase-3 inhibitor was used to test whether εPKC cleavage is caspase dependent.

**Results**—εPKC in the membrane fraction and whole-protein homogenates decreased moderately in the penumbra but decreased markedly in the ischemic core. Hypothermia blocked this decrease in both the ischemic core and penumbra. Confocal microscopy confirmed that neuronal εPKC expression decreased in the ischemic core at 4 hours after reperfusion, and this loss was prevented by hypothermia. Two carboxyl-terminal cleavage products of εPKC with molecular masses of 43 and 35 kDa were detected. Although the protein band of 43 kDa decreased after stroke, the 35-kDa band increased. Such changes were not dependent on caspase-3. However, hypothermia blocked changes in the cleavage form of 35 kDa but not 43 kDa after stroke.

**Conclusions**—Moderate hypothermia preserves εPKC activity after stroke. (Stroke. 2007;38:375-380.)

**Key Words:** εPKC ■ focal cerebral ischemia ■ hypothermia

**Protein kinase C epsilon (εPKC)** belongs to the novel subgroup of the PKC family and has been associated with antiapoptotic functions in a variety of cellular systems.1–3 Several studies have shown that εPKC is activated via subcellular translocation from the cytosol to the particulate membrane fraction.4–6 In addition, generation of a 43-kDa carboxyl-terminal catalytic fragment by caspase activity also results in the activation of εPKC in ischemia.1–9 (Figure 1A).

εPKC is an important component of the signal transduction pathways in ischemic preconditioning–induced neuroprotection.10 A recent study demonstrated that ischemic preconditioning neuroprotection could be emulated in vitro with an agonist of εPKC or blocked with its antagonist.6 Despite its importance, the spatial kinetics of εPKC activation after focal cerebral ischemia are unknown.

The neuroprotective effects of hypothermia are not understood completely. Recently, we have shown that proapoptotic δPKC activation via subcellular translocation and proteolytic cleavage is suppressed by hypothermia in a rat focal cerebral ischemia model.11 In this report we study εPKC activation in response to stroke and the effects of hypothermia on the kinetics of its activity.

**Methods**

**Focal Cerebral Ischemia and Hypothermia**

Experimental protocols were approved by the Stanford University Administrative Panel on Laboratory Animal Care. Focal ischemia was generated as described12 in male Sprague-Dawley rats (weight, 350 to 450 g). The distal middle cerebral artery was permanently cauterized, and the bilateral common carotid arteries (CCAs) were transiently occluded for 1 hour. Core temperature was maintained at 37°C throughout the surgery for normothermic animals; for hypothermia, the temperature was maintained at 30°C during ischemia by spraying 70% methanol onto the rat’s body (Figure 1B).12

**Whole-Cell Homogenization, Subcellular Fractionation, and Western Blots**

At 10 minutes, 4 hours, and 24 hours after CCA release, rat brains were harvested, and tissue corresponding to the ischemic core and...
Penumbra was dissected as described\textsuperscript{12} (Figure 1C). Samples from sham-operated rats were also prepared. For whole-cell homogenates, tissues were homogenized in cell lysis buffer (Cell Signaling Technology) and centrifuged, and the supernatant was collected.\textsuperscript{12} Subcellular cytosolic (soluble) and membrane (particulate) fractions were also prepared as described.\textsuperscript{12} Proteins (10 μg) were loaded and separated by SDS-PAGE.\textsuperscript{12} A primary ePKC antibody (C-15, 1:10 000, Santa Cruz Biotechnologies) was

Figure 1. Hypothermia attenuates ePKC proteolytic cleavage. A, Proteolytic cleavage sites of ePKC by caspases. Arrows indicate cleavage sites, and an arrowhead indicates the recognition site of the ePKC (C-15) antibody. B, Experimental protocol. See details in the text. C, Tissue corresponding to the ischemic core (region II) and penumbra (region I) that was dissected for Western blots. D and E, Levels of ePKC and its cleavage products in the ischemic core (D) and the penumbra (E) from normothermic or hypothermic rats. Short exposure=3 seconds; long exposure=30 seconds (43 kDa=black arrowhead; 35 kDa=white arrowhead). F to H, Optical densitometric analyses for ePKC isoforms between normothermic and hypothermic rat brains in both the ischemic core and penumbra. Black bars indicate 37°C; gray bars, 30°C. *P<0.05 vs sham; #P<0.05 vs 30°C, 4 hours; †P<0.05 vs 30°C, 4 hours; n=5 per group.
probed, and signals were detected by enhanced chemiluminescence (Amersham). Densities of protein bands were analyzed with the use of ImageJ (NIH).

Caspase-3 Inhibitor Injection and ePKC Cleavage Assay
A cell-permeable caspase-3–specific inhibitor, Z-DQMD-FMK (Calbiochem), was dissolved in dimethyl sulfoxide and PBS (Z-DQMD-FMK, 0.3 μg/μL in 1% dimethyl sulfoxide in PBS; vehicle, 1% dimethyl sulfoxide in PBS). Rats were anesthetized and placed in stereotactic frames. The drug solution (1.5 μg) or the vehicle was injected into the ventricular space ipsilateral to the ischemia (5 μL; from bregma: 0.9 mm posterior, 1.5 mm lateral, 3.5 mm deep) at 2 hours after CCA release. At 24 hours after CCA release, brains were removed, and whole-cell extracts were prepared for full-length or cleaved ePKC detection by Western blots.

Figure 2. Effect of a caspase-3–specific inhibitor on ePKC cleavage. Representative protein bands of ePKC in the ischemic core and penumbra from rat brains treated with Z-DQMD-FMK or vehicle are shown (n=3 per group). Short exposure=3 seconds; long exposure=30 seconds (43 kDa=black arrowhead; 35 kDa=white arrowhead).

Figure 3. Brain tissues of sham-operated rats or operated rats (4 hours after CCA release) were stained for ePKC (green), MAP2 (red), and DNA (blue). ePKC decreased in the ischemic core at 4 hours after CCA release. Bar=10 μm.
Immunofluorescence Staining

Rat brains were collected and fixed in 4% paraformaldehyde as described, then cut on a vibratome into slices of 50 μm. Sections were stained with anti-ePKC (1:200) and anti-MAP2 (1:500, Sigma) antibodies 4°C overnight, then incubated with FITC or Cy3-conjugated secondary antibodies (1:200; Jackson ImmunoResearch Laboratories) for 2 hours. The slices were mounted with medium containing DAPI (Vector Laboratories) and examined under a laser-scanning confocal microscope (LSM510, Carl Zeiss).

Statistical Analysis

All data are presented as mean±SEM. All statistical analyses were performed with the use of ANOVA followed by the Tukey post hoc test.

Results

In normothermic brains, Western blots indicated that ePKC in whole-cell homogenates decreased significantly
at 4 hours after CCA release in the penumbra, and greater decreases were detected in the ischemic core at 4 hours and 24 hours (Figure 1D to 1F). Such decreases in ePKC in both the ischemic core and penumbra at 4 hours (Figure 1D to 1F) were blocked by hypothermia. Carboxyl-terminal cleavage products of ePKC molecular masses of 43 and 35 kDa were detected (Figure 1D and 1E). The 35-kDa fragment increased at 10 minutes and 4 hours, returned to baseline at 24 hours after CCA release in the ischemic core (Figure 1D and 1H), and increased at 4 hours in the penumbra (Figure 1E and 1H). Hypothermia blocked all accumulation of the 35-kDa fragment. In contrast, the larger fragment (43 kDa) decreased in the ischemic core from 4 to 24 hours after stroke but did not change in the penumbra (Figure 1D and 1G). Hypothermia did not block such changes (Figure 1G).

To investigate whether ePKC cleavage after stroke is caused by caspase-3 activation, we examined the effect of a cell-permeable caspase-3–specific inhibitor, Z-DQMD-FMK, on generation of cleaved ePKC fragments (Figure 2). Caspase-3 inhibition did not suppress the decrease in full-length ePKC and the 43-kDa fragment in the ischemic core and penumbra after stroke.

Confocal microscopy indicated that ePKC is distributed in the cytoplasm and axons of neurons in nonischemic brains. Consistent with our results from Western blots, ePKC immunofluorescence decreased in the ischemic core at 4 hours after CCA release, and this decrease was suppressed by hypothermia (Figure 3).

Subcellular distribution of ePKC between the cytosolic and membrane fractions was also studied. In the ischemic core from normothermic brains, ePKC decreased at 4 hours after CCA release in both the cytosolic and membrane fractions compared with controls (Figure 4A and 4C). This decrease in both fractions was blocked by hypothermia. In the penumbra, cytosolic ePKC levels did not change after stroke, and hypothermia did not affect cytosolic ePKC levels. However, membrane-associated ePKC significantly decreased at 4 hours after stroke, and this decrease was blocked by hypothermia (Figure 4B and 4D).

Discussion

We report here that levels of full-length ePKC in cell lysates and membrane fractions decreased after stroke. This reduction was blocked by hypothermia. The manner by which hypothermia prevents a decrease in ePKC levels after stroke is not understood. It has been demonstrated by which hypothermia prevents a decrease in such changes (Figure 1G).

PKC activation and proteolysis in the normothermic and hypothermic stroke brain is consistent with functional studies showing that ePKC activity can be neuroprotective in an in vitro model. The fact that 2 disparate neuroprotective treatments, mild hypothermia and adenosine administration, both enhance ePKC activity indicates that this kinase lies at an important node of a neuroprotective cascade.

The present study has some limitations. First, because hypothermia protects ischemic brain through its multiple effects on various detrimental signals, we cannot exclude the possibility that preservation of ePKC levels is secondary to hypothermic protection. However, hypothermia also specifically modifies some gene expression or protein levels to reduce ischemic damage. For example, hypothermia inhibits Bax overexpression 4 hours after 30 minutes of incomplete cerebral ischemia but does not affect Bcl-2, p53, and Mdm-2 expression.14 Yenari et al15 demonstrated that hypothermia transiently attenuated cytochrome c release but did not alter Bcl-2 and Bax expression in a focal ischemia model. Most recently, we found that hypothermia generally maintains the Akt pathway signals but does not attenuate dephosphorylation of GSK-3β.12 In our present study, we found that hypothermia reduces ePKC cleavage and its subcellular translocation, suggesting, but not unequivocally proving, that preservation of ePKC protein level is an important component contributing to the protective effect of hypothermia. Another limitation of this study is that intras ischemic hypothermia was employed. The manner by which postischemic hypothermia, a paradigm more relevant to clinical application, blocks ePKC cleavage should be pursued in the future.

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Disclosures

None.

References


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