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Inhibition of PKCgamma membrane translocation mediated morphine preconditioning-induced neuroprotection against oxygen–glucose deprivation in the hippocampus slices of mice

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ABSTRACT

We previously reported that novel protein kinase C (nPKC) ε and *N*-methyl-D-aspartic acid (NMDA) receptors participated in morphine preconditioning (MP)-induced neuroprotection. In this study, we used Western blot analysis, 2,3,5-triphenyltetrazolium chloride (TTC) staining and lactate dehydrogenase (LDH) leakage assay to determine the involvement of conventional PKC isoforms (cPKC) in MP-induced neuroprotection against oxygen–glucose deprivation (OGD). Hippocampus slices (400-μm thickness) from healthy male BALB/c mice exposed to OGD for 5–45 min to mimic mild, moderate and severe ischemia in the presence of MP pretreatment. We found that OGD-induced damage in neuronal cell survival rate and LDH leakage could be improved by MP pretreatment (3 μM) within 20 min of OGD, which was abolished by concomitant incubation with non-selective opioid receptor antagonist naloxone (NaI, 50 μM). The results of Western blot analysis showed that only cPKCγ membrane translocation, not α, βI and βII, increased under the condition of OGD 10 min and 2 h reperfusion (OGD/2 h), and this increment of cPKCγ membrane translocation was inhibited by MP pretreatment. To further elucidate the role of cPKCγ in MP-induced neuroprotection, we found that cPKCγ membrane translocation inhibitor, Go6983 (6 nM) did not affect MP-induced neuroprotection while Go6983 alone exhibited a significant inhibition on OGD-induced increment in LDH leakage and decrease in cell survival rate. These phenomena were defined by the results that Go6983 could restore OGD-induced cPKCγ membrane translocation, but had no further effect on MP-induced inhibition of cPKCγ membrane translocation. These results demonstrated that MP can reduce OGD-induced neuronal injuries, and the down-regulation of cPKCγ membrane translocation might be involved in the neuroprotection.

Morphine, a non-selective opioid receptor agonist, is an analgetic agent used in clinical practice for a period of time. The opioid receptors including δ, κ and μ are widely expressed in the central nervous system [4,20]. It has been reported that exposure to morphine immediately or at 24 h before a 35 min oxygen–glucose deprivation (OGD, to simulate ischemia *in vitro*) reduced the OGD-induced neuronal death in the CA1 region of the rat hippocampal slice cultures, which was defined as morphine preconditioning (MP) [29]. Although the involvement of κ receptors has been indicated [8], most of the studies have suggested that MP can reduce myocardial or neuronal injury by activation of δ-opioid receptor [16,24,29]. However, the post-receptor signaling mechanism of MP-induced neuroprotection has not yet been fully delineated. This profound molecular mechanism underlying MP may provide a target for developing potential clinical therapeutic approach, such as the treatment of cerebral ischemic/hypoxic injuries and application of morphine before or during neurosurgical operation.

Protein kinase C (PKC) is a family of phospholipids-dependent serine/threonine kinase that participates in a serial of cellular functions [19]. According to their activation requirements, PKC are divided into conventional (cPKCα, βI, βII, γ), novel (nPKCδ, ε, η, θ) and atypical (aPKCι\λ, ζ) isoforms. cPKC require Ca^{2+} and diacylglycerol (DAG) for activation, whereas nPKC and aPKC are only responsive to DAG and lipid mediators for activation, respectively. Several studies have revealed that PKC participated in the initiation and development of ischemic/hypoxic preconditioning in brain, but the role of PKC in the cascade of events after the ischemic/hypoxic insult remains debate [14,21,23].

Opioid receptors are coupled to G protein, it can affect multiple intracellular signaling molecules including PKC once be activated [4]. Our previous study has shown that nPKCε and

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N-methyl-D-aspartate (NMDA) receptors were involved in MP-induced neuroprotection [9]. However, there is little consensus on whether individual PKC isoform play a damaging or beneficial role when cells are suffered to ischemic injury. In the present study, we try to determine which cPKC isoform mediate MP-induced neuroprotection in the hippocampus slices of mice.

All experimental protocols were approved by the Animal Care and Use Committee of Capital Medical University and were consistent with the NIH policy on the use of experimental animal; all efforts were made to minimize the number of animal used and their suffering. Morphine, naloxone, Go6983 and other reagents unless specified below were purchased from Sigma–Aldrich Company (St. Louis, MO, USA).

As previously described [9,12], hippocampal slices were prepared from male BALB/c mice at the age of 8–10 weeks (weighing 18–20 g). In brief, the hippocampus was rapidly isolated after decapitation, and placed in ice-cold modified artificial cerebrospinal fluid (mACSF in mM: NaCl 116.0, KCl 5.4, NaH_2PO_4 0.9, $MgCl_2$ 1.0, $NaHCO_3$ 26.2, and glucose 5.0 at pH 7.4) bubbled with 95%O_2/5%CO_2. The hippocampus was then sectioned with a McIlwain tissue chopper (Brinkman Instruments, Westbury, USA) into 400-μm thickness transverse slices. The hippocampal slices were placed into the mACSF at 0–4 °C for 30 min, and then transferred into oxygenated normal ACSF (mACSF + 2.0 mM $CaCl_2$) at room temperature for 1 h. Subsequently, the beaker containing hippocampal slices was immersed in a water bath to keep the temperature of ACSF at 37 °C for at least 30 min, the total duration before treatment are 2 h for recovery.

According to our previous reports [9,12], slices were washed three times with glucose-free ACSF, and then were transferred into an chamber filled with glucose-free ACSF bubbled with 95%N_2/5%CO_2 and contained 1 mM dithionite to absorb residual oxygen at 37 °C as the condition of OGD to mimic ischemia. Following OGD, the slices were returned to their original conditions in ACSF for 2 h reperfusion. Slices in the sham group were incubated in ACSF bubbled with 95%O_2/5%CO_2 at 37 °C for the same period of time. MP was performed by incubating hippocampal slices with 3 μM morphine for 30 min, and then 30 min wash-out time before OGD. For the treatment, non-selective opioid receptor antagonist naloxone (50 μM) or cPKCγ membrane translocation inhibitor Go6983 (6 nM) were added 30 min before MP.

For the lactate dehydrogenase assay, LDH activity in the culture medium was measured with an LDH kit (Beijing Bukt Clinical Reagent Co., Ltd., China) and Beckman DU-800 spectrophotometer system (Beckman Instruments, Fullerton, CA). The hippocampal slices were placed into the mACSF at 0–4 °C for 30 min, and then transferred into oxygenated normal ACSF (mACSF + 2.0 mM $CaCl_2$) for 1 h at room temperature and 30 min at 37 °C for recovery before the treatment. At the end of 2 h reperfusion after OGD, 100 μl of ACSF was added to react with reagent of LDH kit. After stabilization for 30 s, absorbance at 340 nm was recorded at 30 s intervals for 2 min. The changes in absorbance were expressed as concentration units (U) per liter, and then converted to the percentage of control levels as LDH leakage. To assess neuronal injury of hippocampus slices, slices were stained with 2,3,5-triphenyltetrazolium chloride (2%) for 60 min at 37 °C. After being washed, the slices were extracted with organic solvent (ethanol:dimethyl sulfoxide = 1:1, and 20 ml/g slices) for 24 h. The value of OD_{490} nm of the organic solvent with TTC formazan products was measured by Beckman DU-800 spectrophotometer system. The percentage of OD_{490} relative value to that of control group was expressed as the cell survival rate (%) [17]. To guarantee the accuracy of the results, the slices were weighed after TTC staining and the values of LDH release and cell survival rate were normalized by the slice mass.

Cytosolic and particulate fractions were extracted as our report [15]. The frozen slices were homogenized at 0–4 °C in buffer A (50 mM Tris–Cl, pH 7.5, 1 mM dithiothreitol—DTT, 2 mM EDTA, 2 mM EGTA, 5 μg/ml each of leupeptin, aprotinin, pepstatin A, and chymostatin, 50 mM potassium fluoride—KF, 50 μM okadaic acid, and 5 mM sodium pyrophosphate). The homogenates were centrifuged at 30,000 × *g* for 30 min at 4 °C to yield the cytosolic fractions. The pellet was resuspended in buffer B (buffer A containing 0.5% Nonidet P-40) before being sonicated and centrifuged again, then the resulting supernatants were taken as particulate fractions. The protein concentrations were determined by BCA kit (Pierce, USA).

As our previous reports [15,21], 20 μg of total protein from cytosolic or particulate fraction was loaded per lane for 10% SDS–PAGE gel. After electrophoresis, proteins were transferred onto the nitrocellulose membrane (NC membrane, Schleicher and Schell, USA) at 4 °C. The transferred NC membrane was washed for 10 min with TTBS (20 mM Tris–Cl, pH 7.5, containing 0.15 M NaCl, and 0.05% Tween-20) followed by the blocking solution with 10% non-fat milk in TTBS. The blocked NC membrane was first incubated with primary rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., USA) of cPKCα, βI, βII and γ at 1:1000 dilutions for 3 h, respectively. And then, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham, USA) at a 1:5000 dilution for another 1 h. Finally, the ECL plus Kit (PerkinElmer Life Science, USA) was used to visualize the signals in X-ray film. As an internal control, the expression of β-actin (Sigma, USA) was detected in the same membrane.

Quantitative analysis for immunoblotting was done after scanning of the X-ray film with Gel Doc 2000 imaging system (Bio-Rad, USA). The relative optical density of each band was normalized against the corresponding β-actin, and the ratio of cPKC isoforms membrane translocation (band density in particulate/band densities both in particulate and cytosolic) was expressed as the percentage of that from control group. The presented values are expressed as mean ± S.E. Statistical analysis was conducted by one-way analysis of variance followed by all pairwise multiple comparison procedures using Bonferroni test, and the significance was regarded as $p < 0.05$.

Hippocampal slices presented a significant reduction in cell viability subjected to OGD following 2 h reperfusion. The cell survival rate by TTC staining decreased significantly at 5 (76.50 ± 1.84%), 10 (47.63 ± 4.31%), 20 (33.75 ± 4.27%) and 45 min (18.00 ± 1.58%). OGD following 2 h reperfusion when compared with control group (100%, $p < 0.05$, $n = 8$, Fig. 1A). Similarly, the LDH leakage increased significantly at the same time point of OGD following 2 h reperfusion ($p < 0.05$, $n = 8$, Fig. 1B). In addition, the cell survival rate and LDH leakage of the hippocampal slices could be improved by MP pretreatment (3 μM for 30 min before OGD) as shown in Fig. 1A and B. However, this protective effect of MP was abolished by concomitant incubation with non-selective opioid receptor antagonist, 50 μM naloxone (Nal, Fig. 3A and B).

To investigate which cPKC isoform participate in MP-induced neuroprotection, we selected OGD 10 min as the optimal ischemic stimulation. Slices from control, OGD 10 min alone and MP plus OGD 10 min groups immediately, and at the end of 2 h reperfusion after OGD were picked up for Western blot analysis. As shown in Fig. 2, the membrane translocation of cPKCγ, not α, βI and βII, increased to 121.2 ± 5.6% and 124.8 ± 2.3% (vs control: 100%, $p < 0.05$) after OGD and 2 h reperfusion (OGD/2 h), respectively. However, this increased cPKCγ membrane translocation could be inhibited by the pretreatment of MP (107.5 ± 4.3% in MP + OGD, and 113.6 ± 3.8% in MP + OGD/2 h groups, Fig. 2A and B). In addition, there were no significant changes in cPKCγ protein expression in