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

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A B S T R A C T
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 (Nal, 50 μM). The results of Western blot analysis showed that only cPKC 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 cPKC 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 cPKC membrane translocation, but had no further effect on MP-induced inhibition of cPKC membrane translocation. These results demonstrated that MP can reduce OGD-induced neuronal injuries, and the down-regulation of cPKC membrane translocation might be involved in the neuroprotection.

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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 (cPKCs, βI, βII, γ), novel (nPKCs, ε, η, θ) and atypical (αPKCs, λ, ρ) isoforms. cPKC require Ca2+ and diacylglycerol (DAG) for activation, whereas nPKC and αPKC 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...
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 hippocampal 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, NaH2PO4 0.9, MgCl2 1.0, NaHCO3 26.2, and glucose 5.0 at pH 7.4) bubbled with 95%O2/5%CO2 was sectioned into 200-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 CaCl2) 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%O2/5%CO2 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 for 30 min 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 CaCl2) 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 hippocampal 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 sulphoxide = 1:1, and 20 ml/g slices) for 24 h. The value of OD490 nm of the organic solvent with TTC formazan products was measured by Beckman DU-800 spectrophotometer system. The percentage of OD490 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 isofoms 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 isofoms 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
Fig. 1. Morphine preconditioning (MP) protects hippocampal slices against OGD and 2 h reperfusion-induced neuronal injury. Hippocampus slices (400-μm thickness) with or without MP pretreatment (3 μM, 30 min) were incubated in the condition of OGD for 0 min, 5 min, 10 min, 20 min and 45 min, and then returned to the oxygenated normal ACSF incubation for 2 h to mimic ischemia and reperfusion. (A) Slices were stained with 2% TTC for 60 min to assess neuronal injury. The percentage of OD490 relative value to that of control group was expressed as the cell survival rate (%). (B) LDH leakage in ACSF was determined as the OD340 absorbance and expressed as the percentage of control group. Compared with control group (100%): *p < 0.05; versus OGD/reperfusion alone: #p < 0.05, n = 8 for each group.

To further elucidate the role of cPKCγ in MP-induced neuroprotection, we used the LDH leakage and cell survival rate (TTC staining) to assess the effect of cPKCγ membrane translocation inhibitor, Go6983 on OGD and MP + OGD-induced cell injuries. We found that Go6983 (6 nM) had no effect on MP-induced neuroprotection (Go + MP, Fig. 3A and B) while the inhibitor alone (Go6983) exhibited a significant inhibition on OGD-induced increment of LDH leakage (Fig. 3A) and decrease in cell survival rate (Fig. 3B), respectively. These phenomena were defined by the following results. As shown in Fig. 4A and B, Go6983 could restore OGD-induced cPKCγ membrane translocation after OGD and 2 h reperfusion (Go + OGD). However, Go6983 had no further effect on MP-induced inhibition of cPKCγ membrane translocation after OGD or at the end of 2 h reperfusion (Go + MP + OGD) when compared that with MP + OGD group (Fig. 4A and B).

Hippocampal slice is an useful model to evaluate brain ischemic damage due to the susceptibility to ischemic/hypoxic conditions [5,22]. In this study, we found that cell viability decreased with prolonged OGD and a 10 min OGD is sufficient to induce neuronal loss, which is similar to the model used by Cao et al. [6]. Studies have suggested that MP induce acute and delayed neuroprotection, in which the activation of κ-, δ- or μ-opioid receptor were

OGD, OGD/2 h, MP + OGD and MP + OGD/2 h groups when we compared that with control group (data not shown).

Fig. 2. Effects of MP pretreatment on cPKCs membrane translocation in hippocampus slices under the condition of OGD 10 min and 2 h reperfusion. (A) Typical Western blot results showed the changes in cPKC isom-form-specific membrane translocation in hippocampus slices under the condition of OGD 10 min (OGD) or OGD 10 min plus 2 h reperfusion (OGD/2R) by using different primary rabbit polyclonal antibodies against cPKCs, β, βII, γ, and β-actin as an internal reference (20 μg total protein were separated by 10% SDS–PAGE). (B) Quantitative analysis indicated that OGD and OGD/2R caused a significant membrane translocation of cPKCγ, but not cPKCs, β, βII. However, the MP pretreatment (3 μM, 30 min) could inhibit the increased cPKCγ membrane translocation (*p < 0.05 vs control group; #p < 0.05 vs OGD or OGD/2R, n = 6 for each group).
involved [1,8,16]. This study also confirmed that the non-selective opioid receptor antagonist, naloxone, could block the MP-induced neuroprotection. However, we should keep in mind that this protective effect is limited to resist severe ischemic insult as MP was failed to improve OGD 45 min-induced neuronal injury. OGD 45 min is usually considered as severe ischemic stimulation [12,28]. Previous study have reported that OGD 40 min caused about 90% damage in CA1 sub-fields by quantification of propidium iodide (PI) fluorescence [27], and we also observed that the cell survival rate could go down 18.0 ± 1.6% after OGD 45 min.

It is generally accepted that PKC is an important cellular signaling molecule to participate in the initiation and development of different type of preconditioning in brain [14,15,23]. Studies have indicated that nPKCα was required for the induction of ischemia-induced tolerance and NMDA-mediated preconditioning in the organotypic hippocampal slice [23]; cPKCα, βI, βII and γ mediated the phorbol ester and estrogen-induced neuroprotection [7]; the activations of cPKCa and nPKCb were associated with ischemic preconditioning-induced tolerance in the rat hippocampus [14].

Our previous results also shown that the increased membrane translocation of cPKCβII, γ and nPKCε were involved in the early phase of cerebral hypoxic preconditioning in intact animals [15,21]. In this study, we only found an increased membrane translocation of cPKCγ. The differences in species, animal models, and the intensity and duration of ischemic/hypoxic stimuli might account for the discrepancy among the studies.

cPKCγ has attracted attention as a key molecule in ischemia and reperfusion events. For example, ischemic stimulus could trigger a detrimental signaling cascade and cause a profound translocation of cPKCγ to the cell membranes [13]; using middle common artery occlusion (MCAO) rat model, Matsumoto et al. [18] reported that cPKCγ translocated to cell membrane during and after ischemia, and drugs preventing PKCγ translocation are beneficial against ischemic cell death; hypothermia can provide significant neuroprotection concomitant with less PKC translocation during ischemia [11,26]. In this study, we also found an increase in membrane
translocation of cPKC\(^\gamma\) after OGD and 2 h reperfusion, which could be inhibited by MP pretreatment. All of these results raised a possibility that cPKC\(^\gamma\) activation mediates excitotoxic neuronal death and that reduced cPKC\(^\gamma\) activity may thereby reduce the risk of ischemic cell death. However, the mechanism underlying MP is still poorly understood. Here we proposed that MP may down-regulate cPKC\(^\gamma\) membrane translocation to prevent possible detrimental down-stream signal pathway, and then affect neural susceptibility to ischemic damage [25].

However, the role of cPKC\(^\gamma\) in ischemia/hypoxia-induced neuronal injury and protection is still in controversy. Aronowski et al. reported that the cPKC\(^\gamma\) translocation during permanent ischemia may be a deleterious factor [3], but the neuronal protective effect of cPKC\(^\gamma\) can also be found in reversible focal ischemia by using cPKC\(^\gamma\) knockout mice [2]. In addition, it was reported that the cPKC\(^\gamma\) activation was involved in insulin-mediated inhibition of hypoxia-induced neuronal necrosis [10]. In this study, the cPKC\(^\gamma\) membrane translocation level increased in MP pretreated groups (MP + OGD and MP + OGD/2 h), but was significant lower than that of OGD and OGD/2 h alone groups. In addition, we also found that the cPKC\(^\gamma\) inhibitor Go6983 could protect neurons against OGD 10 min caused cell injury, but no significant effect on MP-induced neuroprotection was observed. Go6983 had been used as broad PKC inhibitor [7], the non-specific property makes possibility of other isoforms involving in the process, and whether the Go6983-induced neuroprotection was mediated through other pathways also could not be ruled out from the present study.

There are some limitations in this study as in vitro models differ in many ways from intact brain. For example, the pharmacokinetics and pharmacodynamics of drugs, the changes in internal environment such as glutamate release and lactic acid accumulation, and the possible ischemic injury during the preparation of slices, all of these factors could potentially affect our observation. In conclusion, our results demonstrated that MP can reduce OGD-induced neuronal injuries, and the down-regulation of cPKC\(^\gamma\) membrane translocation might be involved in the neuroprotection.

Acknowledgments

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