Morphine pretreatment protects against cerebral ischemic injury via a cPKCγ-mediated anti-apoptosis pathway

XIAO-YAN ZHAO[1], JUN-FA LI[2], TIAN-ZUO LI[3], CHU-XIONG PAN[4], FU-SHAN XUE[5] and GU-YAN WANG[1]

[1]Department of Anesthesiology, Beijing Tongren Hospital, Capital Medical University, Beijing 100730; [2]Department of Neurobiology, Capital Medical University; Beijing Institute for Brain Disorders, Capital Medical University, Beijing 100069; [3]Department of Anesthesiology, Beijing Shijitan Hospital, Capital Medical University, Beijing 100038; [4]Department of Anesthesiology, Beijing Stomatological Hospital, Capital Medical University; [5]Department of Anesthesiology, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, P.R. China

Received January 14, 2021; Accepted June 21, 2021

DOI: 10.3892/etm.2021.10448

Abstract. It has been reported that morphine pretreatment (MP) can exert neuroprotective effects, and that protein kinase C (PKC) participates in the initiation and development of ischemic/hypoxic preconditioning in the brain. However, it remains unknown whether PKC is involved in MP-induced neuroprotection. The aim of the present study, which included *in vivo* and *in vitro* experiments, was to determine whether the conventional γ isoform of PKC (cPKCγ) was involved in the protective effects of MP against cerebral ischemic injury. The present study included an *in vivo* experiment using a mouse model of middle cerebral artery occlusion and an *in vitro* experiment using neuroblastoma N2a cells with oxygen-glucose deprivation (OGD). Furthermore, a cPKCγ antagonist, Go6983, was used to determine the involvement of cPKCγ in the protective effects of MP against cerebral ischemic injury. In the *in vivo* experiment, neurological deficits, ischemic infarct volume, neural cell damage, apoptosis and caspase-3 activation were evaluated. In the *in vitro* experiment, flow cytometry was used to determine the activation of caspase-3 in N2a cells with OGD. It was found that MP protected against cerebral ischemic injury. However, intracerebroventricular injection of the cPKCγ antagonist before MP attenuated the neuroprotective effect of MP and increased the activation of cleaved caspase-3. These findings suggested that MP may provide protection against cerebral ischemic injury via a cPKCγ-mediated anti-apoptosis pathway.

Correspondence to: Dr Gu-Yan Wang, Department of Anesthesiology, Beijing Tongren Hospital, Capital Medical University, 1 Dongjiaominxiang, Dong-Cheng, Beijing 100730, P.R. China
E-mail: guyanwang2006@163.com

Dr Fu-Shan Xue, Department of Anesthesiology, Beijing Friendship Hospital, Capital Medical University, 95 Yong-An Road, Xi-Cheng, Beijing 100050, P.R. China
E-mail: xuefushan@aliyun.com

Key words: middle cerebral artery occlusion, morphine, mice, neuroprotection, protein kinase C

Introduction

Stroke is a severe perioperative complication. Based on the cause of injury, strokes can be classified into hemorrhagic and ischemic, with ischemic stroke accounting for 87% of all perioperative strokes (1). Brain injury following cerebral ischemia includes the infarct core, which is enveloped by the ischemic penumbra. In the ischemic penumbra, mild inflammatory and excitotoxic mechanisms contribute to delayed cell death, which displays the biochemical characteristics of apoptosis. Moreover, brain cells stimulate innate protective programs that activate signaling cascades. Signaling cascades activated as part of innate protective programs not only determine cell survival, but also influence the neurological deficit and the mortality after stroke (2). It has been reported that ischemic or hypoxic pretreatment can provide significant protection against stroke-induced cerebral injury (3). However, this treatment is not suitable for elderly patients at high risk of stroke, as it is difficult to predict the outcomes of patients receiving ischemic pretreatment (4). Furthermore, there are significant individual differences in the therapeutic effects of ischemic pretreatment among patients (5). However, endogenous protection involves distinct molecular targets that form the rational basis for the development of neuroprotective drugs. Thus, current research aimed at reducing perioperative stroke has focused on the neuroprotective properties of anesthetics commonly used in the perioperative period (6).

Morphine (Mor), a potent opioid analgesic, is widely used for clinical anesthesia. Previous studies have reported that pretreatment with opioid drugs may preserve cellular integrity following acute cerebral hypoxia (7,8). Moreover, Mor pretreatment (MP) can significantly improve the early survival of animals after acute cerebral ischemia (9,10). It has also been shown that endogenous opioid peptides may protect against ischemic cerebral injury and have been considered as potential targets for stroke therapy (11). However, the post-receptor signaling mechanisms of MP-induced neuroprotection have yet to be fully elucidated. Therefore, further investigation into

the specific signaling mechanisms of neuroprotection induced by MP is necessary.

Protein kinase C (PKC) comprises a family of phospholipid-dependent serine/threonine kinases that are involved in a series of cellular functions, such as cell death and survival mechanisms (12,13). Ischemic tolerance induced by endogenous preconditioning agents is dependent on PKC activation, suggesting that PKCs serve as key regulators of ischemic preconditioning in the brain (14,15). Based on the activation requirements, PKCs are divided into the conventional (c)PKC (α, βI, βII and γ), novel (n)PKC (δ, ε, η and θ) and atypical (a) PKC (ι, λ and ζ) isoforms. The activation of cPKC requires Ca^{2+} and diacylglycerol, whereas the activation of nPKC and aPKC only requires diacylglycerol and lipid mediators, respectively. Accumulating evidence has indicated that PKC participates in the initiation and the development of ischemic/hypoxic preconditioning in the brain (16-19). Our previous work revealed that activation of cPKCγ was involved in the protective effect of hypoxic preconditioning against cerebral ischemic injury (20). Mor functions via three different types of opioid receptors, namely μ, κ and δ receptors. As the main opioid receptor, the signaling efficiency of μ receptors is tightly regulated and is ultimately limited by the coordinated phosphorylation of intracellular serine and threonine residues (21). The phosphorylation of μ receptors occurs primarily at T370, and phosphorylation of T370 stimulated by phorbol esters or heterologous activation of Gq-coupled receptors is mediated by PKC (22). Therefore, it was hypothesized that cPKCγ may be involved in the protective effect of MP against cerebral ischemic injury. Thus, a cPKCγ antagonist, Go6983, was used to determine whether cPKCγ participated in the protective effects of MP against cerebral ischemic injury in the *in vivo* and *in vitro* experiments.

Materials and methods

In vivo experiments

Ethics. The experimental protocol was designed in accordance with the Animal Protection Law of the People's Republic of China and was approved by the Animal Ethics Committee of Beijing Tongren Hospital Affiliated with Capital Medical University (approval nos. AEEI-2014-114, AEEI-2018-141 and AEEI-2019-115).

Animal housing and grouping. A total of 100 adult male C57BL/6 mice (age, 12-14 weeks; weight, 18-22 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were housed under standard conditions (temperature, 21±1˚C; relative humidity, 60±10%), with a 12-h light/dark cycle and free access to food and water. Using a random number table, the mice were divided into four groups in order to receive different treatments (n=25 per group) as follows: i) Sham; ii) ischemia; iii) Mor + ischemia (Mor + I); and iv) Mor + Go6983 + ischemia (Mor + Go6983 + I) groups. All the animals were included in the data analysis and assigned to different tests. Not all the animals in each group were included in each test. The specific animal numbers are indicated in the figure legends.

Animal model. The middle cerebral artery occlusion (MCAO)-induced permanent focal cerebral ischemia mouse model was established as previously described (20). For the MCAO surgery, mice were anesthetized with an intraperitoneal (IP) injection of 2% sodium pentobarbital (60 mg/kg). The left common artery and ipsilateral external carotid artery were exposed and ligated through a ventral midline neck incision. Following common carotid arteriotomy, a 4-0 surgical monofilament with a blunt tip (0.23 mm diameter; Guangzhou Jialing Biotechnology Co., Ltd.) was inserted into the common carotid artery until a mild resistance was felt, which was ~12 mm distal to the carotid bifurcation, thereby occluding the origin of the MCA. In the sham group, the common carotid artery was exposed, but not occluded. The operative time was 10-15 min. If more time was required, an additional 2% sodium pentobarbital dose (20-30% of the initial dose) was administered to the mice, if needed, to maintain the surgical plane of anesthesia. Throughout the procedure, the body temperature was maintained at 37˚C using a heating lamp and thermal blanket. After the surgery, the mice were kept warm in an undisturbed environment for a minimum of 2 h for observation, and cerebral blood flow was monitored using the laser Doppler Flowmetry (Perimed PeriFlux system 5000; Perimed AB).

Drugs. Mor (Sigma-Aldrich; Merck KGaA) was dissolved in normal saline (0.9% NaCl) and injected IP at concentration of 10 mg/1 ml to achieve doses of 10 mg/kg. The Mor dose was selected on the basis of previous research showing dose-related effects on changes in the behavior and central nervous system of mice (23-25). The drug dose used in mice was 10-20 fold that used in humans (26). Go6983 (Sigma-Aldrich; Merck KGaA) was dissolved in DMSO and mixed with normal saline (0.9% NaCl). The final concentration of DMSO was <0.1%. The total volume of Go6983 was 5 μl (6 nM), and the drug was introduced in advance into the polyethylene tube connecting the microinjection cannula and the microsyringe. The microinjection cannula was kept in the guide cannula for 5 min after drug administration to avoid a backflow of drug solution. The dose of the inhibitor used in the present study was appropriate for the microinjection method (27).

Drug administration. Mice in the Mor + I group were treated with 10 mg/kg Mor via intraperitoneal injection at 24 h prior to cerebral ischemia induction. Under anesthesia with sodium pentobarbital, mice in the Mor + Go6983 + I group were injected with 5 μl Go6983 (6 nM) or DMSO (Go6983 can only be dissolved in DMSO and it was necessary to exclude the extra effect of DMSO on the results) into the intracerebral ventricle. Go6983 treatment was performed in a blinded manner, in which statisticians and examiners were blinded to animal grouping and drug treatments. Go6983 is a general cPKC inhibitor at low concentrations, but more specifically inhibits cPKCγ activation at a concentration of 6 nM (28). Drug administration into the intracerebral ventricle was performed as previously described by Muñoz *et al* (29). Animals were positioned in a stereotaxic frame, and a cannula (28-gauge; stainless steel; inner diameter, 0.18 mm; outer diameter, 0.36 mm) was lowered stereotaxically into the left cerebral ventricle to a position defined by the following coordinates: 0.5 mm posterior and 1.0 mm lateral to the bregma, and 3.5 mm below the skull surface. To confirm that the solutions were administered exactly into the cerebral ventricle, some mice were injected with 5 ml of diluted 1:10 India ink to examine their brains macroscopically after sectioning.

Neurobehavioral tests. To evaluate the effect of MP on neurological recovery following MCAO-induced ischemia, a number of neurobehavioral tests were performed 6 h after MCAO surgery. At this time, the ischemic penumbra or peri-infarct region is more apparent, although the neurological deficits and infarct volume continue to progress (30). Using the neurological disability status scale (NDSS) reported by Rodriguez *et al* (31), the neurological deficits of mice were scored as follows: 0, no neurological dysfunction; 2, slight decrease in mobility and presence of passivity; 4, moderate neurological dysfunction and additional alterations, including moderate hypomobility, flattened posture, lateralized posture, hunched back, ataxic gait, decreased body tone and muscular strength, as well as slight motor incoordination; 6, disabled but able to walk, with more marked hypomobility, circling, tremor, jerks and/or convulsions, forelimb flection, and moderate motor incoordination; 8, respiration distress and total incapacity to move/coordinate; and 10, death. If the criteria for a precise grade given in the scoring list were not met, the nearest appropriate number was utilized: 1, 3, 5, 7 or 9. Before the experiment, the examiners were trained in NDSS evaluation and were proficient in the NDSS criteria.

Evaluation of ischemic infarct and edema. Immediately after the evaluation of the neurological deficits, a double-blind measurement of the infarct volume was conducted. Mice were anesthetized at 6 h via an IP injection of 2% sodium pentobarbital (60 mg/kg) after MCAO surgery, and the brain was quickly removed and cut into 1.5-mm coronal sections. The brain sections were incubated for 20 min in a solution of 0.5% 2,3,5-triphenyltetrazolium chloride (TTC) in 10 mM PBS at 37˚C, and then scanned into a computer. The infarct size was analyzed according to an evaluation procedure reported by Wexler *et al* (32). The edema ratio (E) was calculated using the following equation: $E = (\sum VL-\sum VR)/(\sum VL+\sum VR)$ x100%, where ∑VL and ∑VR are the volumes of the left (ischemic) and right (non-ischemic) hemispheres, respectively. The background (B) was calculated using the following equation: $B=\sum VS/\sum VT$ x100%, where ∑VS is the volume of the unstained white matter in the sham group and ∑VT is the total brain volume. To account for the effect of edema and background, the infarct size (I) was indirectly estimated and expressed as a percentage of the total brain using the following equation: $I = [\sum VI \times (1-E)/\sum VT \times (1-B)]$ x100%, where VI is the volume of the tissue of MCAO model mice not stained with TTC.

Nissl staining. Nissl staining was used to assess neuronal cell damage in brain sections. Mice were deeply anesthetized with an IP injection of 2% sodium pentobarbital (60 mg/kg) and were transcardially perfused with 100 mM PBS containing 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde at 4˚C for 24 h, followed by incubation in graded sucrose solutions (20% for 24 h and 30% for another 24 h) for dehydration at 4˚C. The brains were cut into 20-μm sections, washed in fresh PBS, stained with 0.04% cresyl violet (Sigma-Aldrich; Merck KGaA) and dissolved in acetate buffer for 1 h at room temperature. Images were captured under a Nikon 50i light microscope with a x40 objective (Nikon Corporation). Nissl-stained sections in the ipsilateral side to the MCAO were used for counting, and cell numbers in five random high-power fields were averaged in this area for each section. The images were analyzed using ImageJ software version 1.8.0 (National Institutes of Health).

TUNEL staining. Mice were anesthetized with an i.p. injection of 2% sodium pentobarbital (60 mg/kg) and were transcardially perfused with 100 mM PBS containing 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde at 4˚C for 24 h, followed by incubation in graded sucrose solutions (20% for 24 h and 30% for another 24 h) for dehydration at 4˚C. The brains (n=6) were cut into 10-μm sections for TUNEL staining. The TUNEL staining kit (DeadEnd Fluorometric TUNEL system; Promega Corporation) was used to assess neuronal apoptosis. According to the manufacturer's instructions, the brain sections were placed in equilibration buffer and incubated with nucleotide mix and rTdT enzyme at 37˚C for 1 h. The reaction was stopped using a termination buffer (300 mM NaCl; 30 mM sodium citrate) for 15 min at room temperature. Then, neuronal nuclei were stained with Hoechst 33258 and the slices were sealed by mounting medium (cat. no. S2100, Beijing Solarbio Science & Technology Co., Ltd.). The images were visualized by fluorescence microscopy with a x40 objective. The cell numbers in five random high-power fields were averaged in this area for each section (Leica DM4000B; Leica Microsystems GmbH).

Western blot analysis. Mice were deeply anesthetized by intraperitoneal injection of 2% sodium pentobarbital (60 mg/kg) and decapitated. The mouse brains were removed 6 h after MCAO surgery and immediately placed into ice-cold artificial cerebral spinal fluid (125.0 mM NaCl; 2.5 mM KCl; 2.0 mM $CaCl_2$; 26.0 mM $NaHCO_3$; 1.25 mM NaH_2PO_4; 1.0 mM $MgCl_2$; 5.0 mM glucose; pH 7.4) bubbled with 95% O_2/5% CO_2 and were then dissected, as described in a previous report (33). In brief, tissues 2 mm from the anterior tip of the frontal lobe were removed, and the remaining brain tissues were cut into four 2-mm sections. Each hemisphere was longitudinally cut 1 mm from the midline to remove the tissue supplied by the anterior cerebral artery. A 45˚ transverse diagonal cut was then made to separate the ischemic core and peri-infarct region. The corresponding regions from the non-ischemic hemisphere were dissected as the contralateral controls. All tissues were frozen in liquid nitrogen and kept at -70˚C for subsequent analysis.

To obtain whole cell lysates, the tissue samples were rapidly thawed, homogenized at 25˚C in buffer C (5 mM Tris-Cl; pH 7.5; containing 2 mM dithiothreitol, 2 mM EDTA, 1 mM EGTA, 5 μg/ml each of leupeptin, aprotinin, pepstatin A and chymostatin, 50 mM potassium fluoride, 50 μM okadaic acid, 5 mM sodium pyrophosphate and 2% sodium dodecyl sulfate); the homogenates were centrifuged at 30,000 x g for 30 min at 4˚C and the supernatants were collected . The protein concentration was determined using a BCA kit (Pierce; Thermo Fisher Scientific, Inc.) with albumin diluted in buffer C as the standard.

Samples loaded with equal amounts of protein (50 μg) were electrophoresed on 10% SDS-polyacrylamide gels and then transferred onto PVDF membranes (Cytiva) at 4˚C. After several rinses with TBS/Tween-20 (TBST; 20 mM Tris-Cl; pH 7.5; 0.15 M NaCl; 0.05% Tween-20), the PVDF membrane was blocked with 10% non-fat milk in TBST for 1 h at room temperature. The blocked PVDF membrane

was then incubated with rabbit anti-caspase-3 (1:1,000; cat. no. 9662; Cell Signaling Technology, Inc.) for 3 h at 25˚C. To verify equal loading of protein, the blots were reprobed with a primary monoclonal antibody targeting rabbit anti-β-actin (1:1,000; cat. no. 4970; Cell Signaling Technology, Inc.) for 3 h at room temperature, and with the corresponding secondary antibody AffiniPure mouse anti-rabbit IgG (H+L; 1:5,000, cat. no. 211-005-109; Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. After secondary antibody incubation, an ECL kit (Cytiva) was used to detect the signals. Images were digitized and analyzed by using ImageMaster 2D Platinum Software version 5.0 (Cytiva).

In vitro experiments

To further investigate the mechanisms underlying the involvement of cPKCγ in the MP-induced neuroprotection, mouse N2a neuroblastoma cells were exposed to conditions mimicking the *in vivo* ischemic-like state, and caspase-3-positive cells were analyzed via flow cytometry.

Cell treatment. Mouse N2a neuroblastoma cells (kindly gifted by Dr Yun Wang, Peking University), were grown to 50% confluence in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS in a 37˚C chamber (Thermo Electron LED GmbH; Thermo Fisher Scientific, Inc.) under normoxic conditions (5% CO_2, 95% O_2). To mimic the *in vivo* ischemic-like conditions, N2a cells were exposed to oxygen-glucose deprivation (OGD) treatment, in which the culture medium was replaced by glucose-free DMEM and cells were maintained in a hypoxic chamber (5% CO_2, 1% O_2, 94% N_2) for 3 h at 37˚C . After OGD exposure, cells were returned to glucose-containing DMEM under normoxic conditions for 24 h of reoxygenation at 37˚C. MP was performed by incubating cells with 3 μM Mor for 30 min followed by 30 min of washing before OGD. Furthermore, 6 nM Go6983 (a cPKCγ antagonist) were added 30 min before MP.

Flow cytometry. Activation of caspase-3 in N2a cells by OGD was analyzed using flow cytometry with FITC active caspase-3 Apoptosis kit (cat. no 550480; BD Pharmingen) according to the manufacturer's instructions. After 24 h of reoxygenation, cells were counted and the amount of antibody needed were calculated. According to the kit instructions, these reagents had been pre-diluted for use at the recommended volume per test and $1x10^6$ cells in a 100 μl experimental sample was recommended and with an antibody volume of 20 μl. The cells were resuspended in BD Cytofix/Cytoperm solution and incubated for 20 min on ice. The cells were washed with BD Perm/Wash buffer and incubated with anti-cleaved caspase-3 antibodies for 30 min at room temperature. The cells were then washed and resuspended in BD Perm/Wash buffer and analyzed via flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). A negative control was performed with no anti-cleaved caspase-3 antibody. The software used for analysis was BD CellQuest Pro version 6.0 (Becton-Dickinson and Company).

Statistical analysis. Statistical analysis was conducted using SPSS software (version 25; IBM Corp.). One-way ANOVA followed by pairwise multiple comparison procedures using the Bonferroni test were adopted. The number of experiments repeats was two. All data are presented as the mean ± SEM. $P<0.05$ was considered to indicate statistically significant differences.

Results

In vivo experiments

MP attenuates MCAO-induced neurological disability and cerebral ischemic injury. The neurological score was 8.1±0.5 and 5.0±0.4 in the ischemia and MP groups, respectively, with a statistically significant difference ($P<0.05$, n=10 per group; Fig. 1A). Furthermore, the cerebral infarct volume was significantly decreased in the MP group compared with that in the ischemia group (ischemia 38.3±0.2% vs. MP 28.1±0.5%, $P<0.05$; Fig. 1B). An illustrated sample of cerebral infarction is shown in Fig. 1C.

Inhibition of cPKCγ activation abolishes MP-induced neuroprotection. The neurological score (8.0±0.4) and cerebral infarct volume (37.1±0.3%) in the Mor + I + Go6983 group were not significantly different from those in the ischemia group (8.1±0.5% and 38.3±0.2%, respectively) but were significantly increased compared with those in the MP group (5.0±0.4% and 28.1±0.5%, respectively; Fig. 1A-C), indicating that cerebral ventricular injection of the cPKCγ inhibitor Go6983 attenuated the MP-induced neuroprotection.

The number of Nissl-stained neural cells in the peri-ischemic region was significantly decreased in the ischemia group (149±10.0) compared with the sham group (209±10.4), but was not significantly different in the MP group (198±9.1). Furthermore, there was no significant difference in the number of Nissl-stained neural cells between the ischemia and Mor + I + Go6983 groups (149±10.0 vs. 138±8.9, respectively; Fig. 2A-C).

MP inhibits neuronal apoptosis and cleaved caspase-3 activation, but this inhibition is reversed by the cPKCγ antagonist. The number of TUNEL-positive neural cells in the peri-ischemic region was significantly increased in the ischemia group compared with that in the MP group (ischemia 8.6±1.2% vs. MP 5.6±1.2%), but was not significantly different between the ischemia and Mor + I + Go6983 groups (ischemia 8.6±1.2% vs. Mor + I + Go6983 9.2±1.3%; Fig. 3A and B).

Compared with the sham group, the protein expression level of cleaved caspase-3 in the peri-ischemic region was significantly increased in the ischemia group (ischemia 19.6±2.7% vs. sham 0.1±0.1%). Moreover, compared with the ischemia group, the protein expression level of cleaved caspase-3 in the peri-ischemic region was significantly decreased in the MP group (ischemia 19.6±2.7% vs. MP 1.4±0.3%). However, there was no significant difference in the cleaved caspase-3 protein expression between the ischemia and Mor + I + Go6983 groups (ischemia 19.6±2.7% vs. Mor + I + Go6983 10.4±2.1%; Fig. 3C and D).

In vitro experiment

MP reduces the number of cleaved caspase-3-positive cells after OGD, and the cPKCγ antagonist prevents this MP-induced reduction. In the flow cytometry analysis, the percentage of cleaved caspase-3-positive cells was significantly increased in OGD N2a cells compared with N2a cells under normoxic conditions (OGD 24.5±2.6% vs. normoxic 1.6±0.2%). Furthermore, the percentage of cleaved caspase-3-positive N2a cells was 3.7±0.7% in the MP treatment group and 18.4±1.5% in the Go6983 group. It was found that

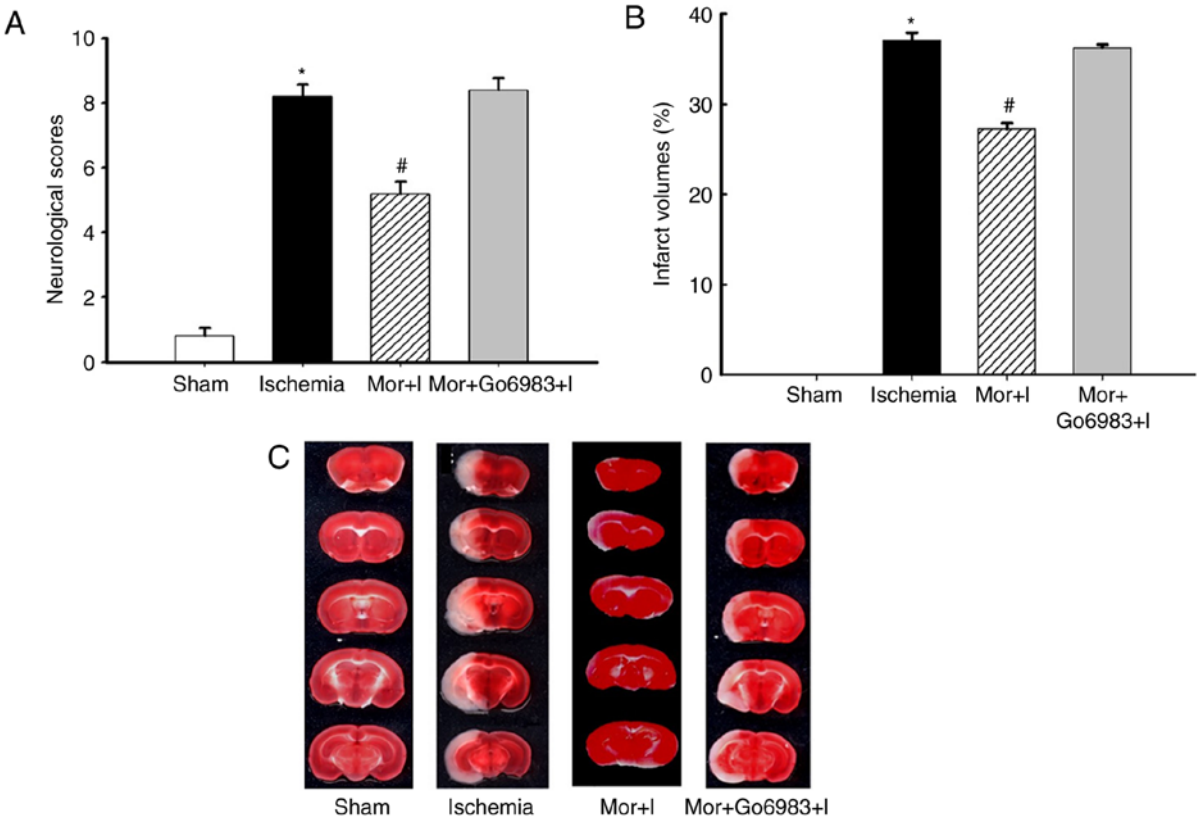


Figure 1. Effects of morphine pretreatment and cPKCγ antagonist Go6983 on neurological deficits and cerebral infarct volumes in middle cerebral artery occlusion mice. (A) Neurological scores in four groups. (B) Cerebral infarct volumes in four groups. (C) Representative images of 2,3,5-triphenyltetrazolium chloride-stained cerebral coronal sections; each set of brain sections is from one individual mouse brain. *P<0.05 vs. sham group; #P<0.05 vs. ischemia group, n=10 per group. cPKCγ, conventional γ isoform of protein kinase C; Mor, morphine; I, ischemia

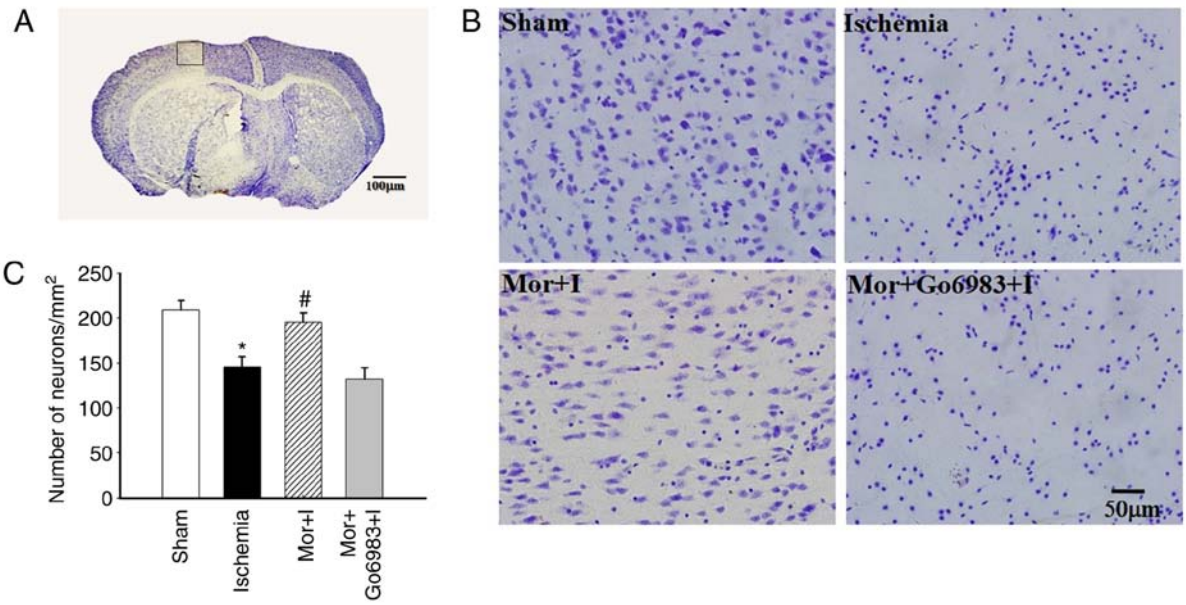


Figure 2. Effects of morphine pretreatment and cPKCγ antagonist Go6983 on neural cell loss in middle cerebral artery occlusion mice. (A) Representative images of Nissl-stained cerebral coronal sections (scale bar, 100 μm). (B) Representative images of Nissl-stained neural cells (scale bar, 50 μm). (C) Number of Nissl-stained neural cells in four groups. *P<0.05 vs. sham group; #P<0.05 vs. ischemia group, n=10 per group. cPKCγ, conventional γ isoform of protein kinase C; Mor, morphine; I, ischemia.

MP treatment significantly decreased the number of cleaved caspase-3-positive OGD N2a cells (OGD 24.5±2.6% vs. Mor + OGD 3.7±0.7%). However, pretreatment with Go6983 in OGD N2a cells before MP inhibited the reduction in the number of cleaved caspase-3-positive cells induced by MP (Mor + OGD 3.7±0.7% vs. Mor + Go6983 + OGD 18.4±1.5%; Fig. 4A-F).

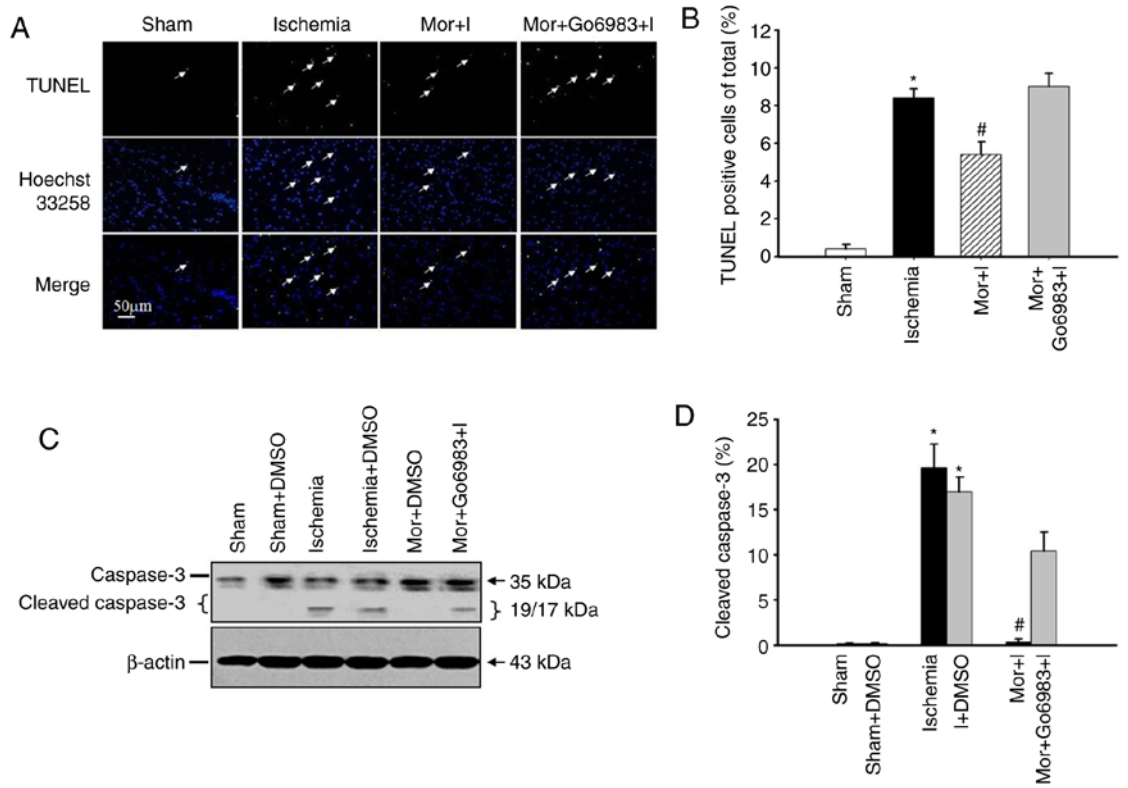


Figure 3. Effects of morphine pretreatment and cPKCγ antagonist Go6983 on neural cell apoptosis and cleaved caspase-3 activation by cerebral ischemia in middle cerebral artery occlusion mice. (A) Representative images of TUNEL stained neural cells (scale bar, 50 μm); (B) Number of TUNEL-positive neural cells in four groups. (C) Representative western blot images of cleaved caspase-3. (D) Protein levels of cleaved caspase-3 in four groups. *P<0.05 vs. sham group; $^{\#}$P<0.05 vs. ischemia group. n=6 per group. cPKCγ, conventional γ isoform of protein kinase C; Mor, morphine; I, ischemia.

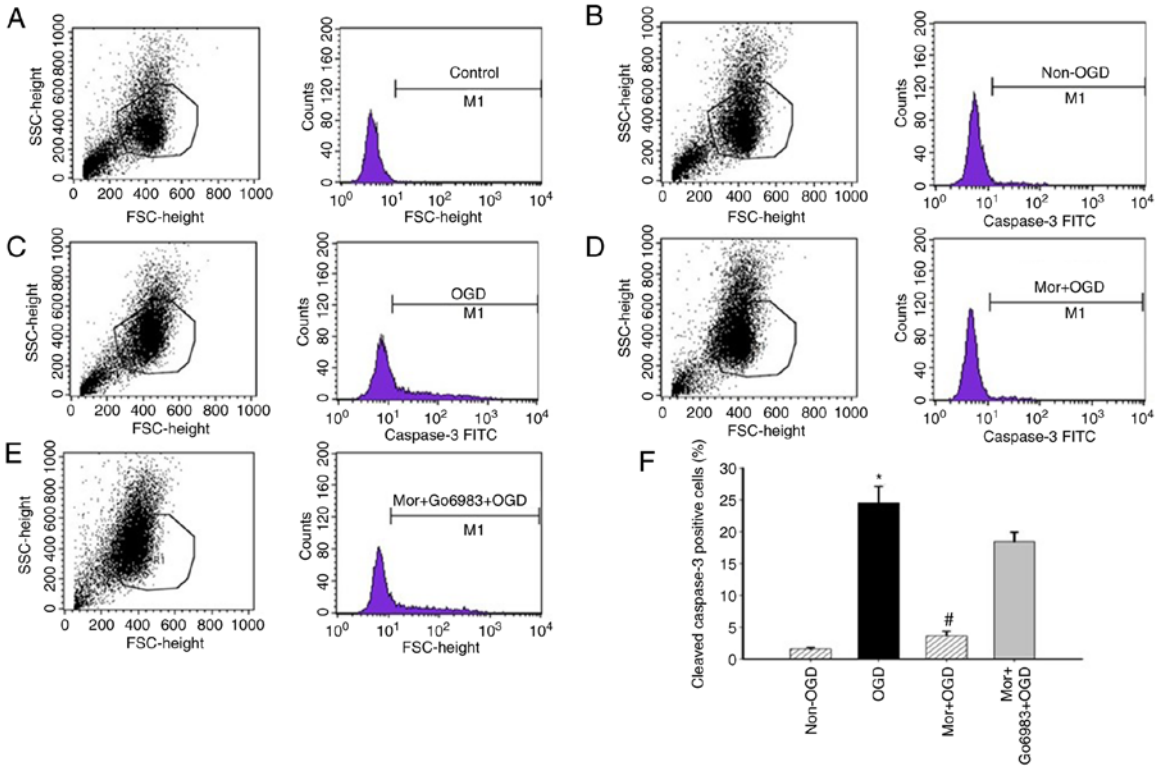


Figure 4. Effects of morphine pretreatment and cPKCγ antagonist Go6983 on the number of cleaved caspase-3-positive N2a cells under OGD conditions. (A-E) Representative images of flow cytometry from N2a cells treated with OGD, Mor + OGD and Mor + Go6983 + OGD. (F) Number of cleaved caspase-3-positive OGD cells in different groups. *P<0.05 vs. sham group, $^{\#}$P<0.05 vs. OGD group, n=6 per group. N2a cells were fixed and stained with anti-cleaved caspase-3 antibodies and analyzed by FCM. A negative control performed with an irrelevant antibody is shown in A. The percentage of cells exhibiting active caspase-3 is indicated on each histogram as the mean ± SEM. cPKCγ, conventional γ isoform of protein kinase C; Mor, morphine; OGD, oxygen-glucose deprivation.

Discussion

Stroke is a severe perioperative complication in elderly patients receiving surgery and represents a major socioeconomic burden. The traditional treatment methods, involving thrombolytic agents, tissue plasminogen activator, anticoagulation therapy and even carotid endarterectomy, can only be used for the therapy of ischemic strokes that have already occurred, and the time window is very narrow (30). Moreover, there is a lack of effective methods for the prevention and therapy of perioperative stroke. Sedative anesthetics, such as barbiturates, propofol and dexmedetomidine, have been reported to provide neuroprotection in the perioperative period (34). However, they are not routinely used prior to surgery due to their poor effect on decreasing patient anxiety, and their inhibitory effects on the circulation and cardiac function of the patients. Therefore, safe and more effective alternative strategies are urgently required for the prevention of stroke in the preoperative period.

As a potent opioid analgesic, Mor is used to ameliorate preoperative patient anxiety and perioperative pain (35). Previous studies have reported MP-induced protection in numerous organs (36,37), particularly in the brain (38). The main findings of the present study were that MP could significantly improve the neurological outcomes of MCAO model mice and that cPKCγ was involved in MP-induced neuroprotection.

At the onset of an acute ischemic stroke, lack of oxygen and other nutrients triggers a series of events causing electrophysiological, metabolic and molecular disorders, leading to irreversible brain tissue damage, which can manifest as neurological deficits with regards to behavior and cell loss with regards to morphology. In the MCAO model, animals develop neurological deficits following recovery from anesthesia (20). Thus, the degree of neurological deficits was evaluated in the present study, according to the NDSS classification described by Rodriguez *et al* (31), who suggest that the peri-infarct region (penumbra) becomes apparent and peak brain edema occurs at 6 h after MCAO. As this model has a high success rate of inducing cerebral ischemia in the cortex, it can make the results of experiment more uniform (32). In the present study, consistent MCAO-induced neurological deficits were observed, and application of MP at 24 h before MCAO significantly improved the neurological outcomes of MCAO model animals. This result was in line with the findings from a previous study by Zhao *et al* (39), in which application of Mor at 24 h before MCAO modeling decreased the cerebral infarct volume and improved neurological outcomes at 24 h after MCAO modeling in adult rats. Given that improved neurological outcomes were obtained at 6 and 24 h after MCAO modeling, in the present study as well by Zhao *et al* (39), it was suggested that MP-induced neuroprotection should last >1 day. In addition, our previous study reported that naloxone, a non-selective opioid receptor antagonist, could block MP-induced neuroprotection (40). Similarly, this finding was confirmed by Arabian *et al* (41). All these results indicate that opioid receptors are important for the protective effect of MP against cerebral ischemic injury.

It has been reported that the protective effects of acute and chronic opioid treatment in cerebral ischemic injury are mediated via different signaling pathways (42). For example, chronic MP is mediated by the signaling pathways involving protein kinase A (PKA) and Gs proteins, while acute MP is mediated via PKC and Gi proteins (41). Our previous study revealed that PKC activation was significantly inhibited in the OGD-treated hippocampal sections (20). Moreover, in a mouse model of MCAO-induced ischemic stroke, Zhang *et al* (20) confirmed that cPKCγ knockout significantly increased the cerebral infarct volume after 1 h MCAO/72 h reperfusion, as detected using TTC staining. It has also been shown that, in primary cultured cortical neurons, cPKCγ knockout can aggravate OGD-induced cell death and morphological damage of neurites, while cPKCγ restoration can alleviate ischemic injury (43). In the present experiment, when MCAO model mice were treated with intracerebroventricular injection of the cPKCγ antagonist prior to MP, the MP-induced neuroprotective effect was eliminated, as shown by the deteriorated neurological outcomes, expanded cerebral infarct volume and increased neural cell loss. Collectively, these findings suggest that PKC serves an important role in the protection of MP against cerebral ischemic injury in MCAO model mice; however, the involvement of other signaling molecules, such as PKA, cannot be excluded.

To further determine the detailed mechanisms underlying MP-mediated neuroprotection, neuronal apoptosis was also evaluated using TUNEL staining in the current study. The present results indicated that MP could significantly inhibit neuronal apoptosis and caspase-3 activation, but a cPKCγ antagonist could notably diminish the inhibitory effect of MP on MCAO-induced neuronal apoptosis and caspase-3 activation. Moreover, in the OGD-model N2a cells, the flow cytometry results revealed that MP decreased the number of OGD-induced caspase-3-positive cells, while the use of a cPKCγ antagonist eliminated the inhibitory effect of MP on the activation of OGD-induced caspase-3-positive cells. Thus, it was suggested that MP may protect against cerebral ischemic injury via a cPKCγ-dependent anti-apoptosis pathway.

The available evidence indicates that mitochondrial-related mechanisms may be involved in Mor treatment and pretreatment (39). However, oxidative stress can disrupt the mitochondrial membrane potential, which promotes apoptosis-inducing factor release and activates the caspase cascade. Furthermore, caspases serve as important drug targets in ischemic organ injury (44). In the activation of the caspase cascade, caspase-3 plays a key role by acting as the final executor of the apoptosis pathway (45). Scientific reports have shown the effect of μ-opioid agonists on the release of apoptosis-inducing factor and cytochrome *c*, as well as on caspase-3 activation, as the final executor of the apoptosis pathway, using immunoblotting in toxin- and drug-treated neuronal cells (45,46). The present study demonstrated that MP could significantly inhibit neuronal apoptosis and caspase-3 activation. Moreover, it was found that, in OGD-induced N2a cells, MP decreased the number of OGD-induced caspase-3-positive cells. Thus, it was suggested that such opioid effects may be mediated by anti-apoptotic activities via the reduction of the suppression of caspase-3 activation. Additional implicated mechanisms, such as apoptosis-inducing factor release and ion balance, must be further elucidated.

There were certain limitations to the present study. First, only one dose of Mor was tested. Thus, it remains unknown whether the protective effect of MP against cerebral ischemic injury was dose-dependent. Second, only the protective effect of MP at 6 h after cerebral ischemia was observed. Therefore, the time window of MP protection against cerebral ischemic injury must be determined. Third, this experiment was only focused on the involvement of the cPKCγ-mediated anti-apoptosis pathway in the protective role of MP against cerebral ischemic injury. The available evidence indicates that other mechanisms, such as mitochondrial ATP-sensitive potassium channels, PI3K and extracellular signal-regulated kinase pathways, autophagy, inflammation and oxidative stress, may contribute to opioid-induced preconditioning (11,47). The findings of the present study did not provide definitive evidence regarding the exact roles of these pathways and their interactions with the cPKCγ-mediated anti-apoptosis pathway in the protective effect of MP against cerebral ischemic injury. To address these aforementioned issues, further experiments are required.

In conclusion, the present study demonstrated that MP may protect against cerebral ischemic injury via a cPKCγ-mediated anti-apoptosis pathway.

Acknowledgements

The authors would like to thank Dr Yi-Qiong Liu (College of Life Sciences, PKU-IDG/McGovern Institute for Brain Research, Peking University, Beijing, China) for providing helpful suggestions.

Funding

The present study was supported by the National Science Foundation of China (grant nos. 81671044, 81970344, 314711475 and 31671205). The funders had no part in experimental design, data collection or analysis, decision to publish, or preparation of the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors participated in the design of the studies, analysis of the data and review of the manuscript. XYZ performed the immunohistochemistry, western blotting and behavioral tests. FSX performed statistical analysis and generated figures. XYZ and FSX wrote the manuscript. GYW and JFL interpreted the data and revised the manuscript. TZL and CXP designed the experiments. XYZ, FSX and GYW confirm the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocol was designed in accordance with the Animal Protection Law of the People's Republic of China and was approved by the Animal Ethics Committee of Beijing Tongren Hospital Affiliated with Capital Medical University (approval nos. AEEI-2014-114, AEEI-2018-141 and AEEI-2019-115).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Dabrowska S, Andrzejewska A, Lukomska B and Janowski M: Neuroinflammation as a target for treatment of stroke using mesenchymal stem cells and extracellular vesicles. J Neuroinflammation 16: 178, 2019.
2. Mergenthaler P, Dirnagl U and Meisel A: Pathophysiology of stroke: Lessons from animal models. Metab Brain Dis 19: 151-167, 2004.
3. Moncayo J, de Freitas GR, Bogousslavsky J, Altieri M and van Melle G: Do transient ischemic attacks have a neuroprotective effect? Neurology 54: 2089-2094, 2000.
4. de Lau LM, den Hertog HM, van den Herik EG and Koudstaal PJ: Predicting and preventing stroke after transient ischemic attack. Expert Rev Neurother 9: 1159-1170, 2009.
5. Rancurel G: Transient ischemic attacks in the elderly: New definition and diagnostic difficulties. Psychol Neuropsychiatr Vieil 3: 17-26, 2005 (In French).
6. Archer DP, Walker AM, McCann SK, Moser JJ and Appireddy RM: Anesthetic neuroprotection in experimental stroke in rodents: A systematic review and meta-analysis. Anesthesiology 126: 653-665, 2017.
7. Zhang J, Haddad GG and Xia Y: delta-, but not mu- and kappa-, opioid receptor activation protects neocortical neurons from glutamate-induced excitotoxic injury. Brain Res 885: 143-153, 2000.
8. Zhang J, Gibney GT, Zhao P and Xia Y: Neuroprotective role of delta-opioid receptors in cortical neurons. Am J Physiol Cell Physiol 282: C1225-C1234, 2002.
9. Endoh H, Taga K, Yamakura T, Sato K, Watanabe I, Fukuda S and Shimoji K: Effects of naloxone and morphine on acute hypoxic survival in mice. Crit Care Med 27: 1929-1933, 1999.
10. Endoh H, Honda T, Ohashi S and Shimoji K: Naloxone improves arterial blood pressure and hypoxic ventilatory depression, but not survival, of rats during acute hypoxia. Crit Care Med 29: 623-627, 2001.
11. Peart JN, Gross ER and Gross GJ: Opioid-induced preconditioning: Recent advances and future perspectives. Vascul Pharmacol 42: 211-218, 2005.
12. Mellor H and Parker PJ: The extended protein kinase C superfamily. Biochem J 332: 281-292, 1998.
13. Kaleli HN, Ozer E, Kaya VO and Kutlu O: Protein kinase C isozymes and autophagy during neurodegenerative disease progression. Cells 9: 553, 2020.
14. Bright R and Mochly-Rosen D: The role of protein kinase C in cerebral ischemic and reperfusion injury. Stroke 36: 2781-2790, 2005.
15. Perez-Pinzon MA, Dave KR and Raval AP: Role of reactive oxygen species and protein kinase C in ischemic tolerance in the brain. Antioxid Redox Signal 7: 1150-1157, 2005.
16. Kurkinen K, Busto R, Goldsteins G, Koistinaho J and Pérez-Pinzón MA: Isoform-specific membrane translocation of protein kinase C after ischemic preconditioning. Neurochem Res 26: 1139-1144, 2001.
17. Niu C, Li J, Cui X, Han S, Zu P, Li H and Xu Q: Changes in cPKC isoform-specific membrane translocation and protein expression in the brain of hypoxic preconditioned mice. Neurosci Lett 384: 1-6, 2005.
18. Sun MK, Hongpaisan J, Nelson TJ and Alkon DL: Poststroke neuronal rescue and synaptogenesis mediated in vivo by protein kinase C in adult brains. Proc Natl Acad Sci USA 105: 13620-13625, 2008.
19. Voronkov AV and Mamleev AV: Endothelial dysfunction and Protein kinase C activity development interrelation at ischemic injury of a brain. Patol Fiziol Eksp Ter 60: 134-142, 2016.

20. Zhang N, Yin Y, Han S, Jiang J, Yang W, Bu X and Li J: Hypoxic preconditioning induced neuroprotection against cerebral ischemic injuries and its cPKCγ-mediated molecular mechanism. Neurochem Int 58: 684-692, 2011.
21. Wolf R, Koch T, Schulz S, Klutzny M, Schröder H, Raulf E, Bühling F and Höllt V: Replacement of threonine 394 by alanine facilitates internalization and resensitization of the rat mu opioid receptor. Mol Pharmacol 55: 263-268, 1999.
22. Mann A, Illing S, Miess E and Schulz S: Different mechanisms of homologous and heterologous μ-opioid receptor phosphorylation. Br J Pharmacol 172: 311-316, 2015.
23. Vanderschuren LJ, De Vries TJ, Wardeh G, Hogenboom FA and Schoffelmeer AN: A single exposure to morphine induces long-lasting behavioural and neurochemical sensitization in rats. Eur J Neurosci 14: 1533-1538, 2001.
24. Ferguson SM, Thomas MJ, Robinson TE. Morphine-induced c-fos mRNA expression in striatofugal circuits: Modulation by dose, environmental context, and drug history. Neuropsychopharmacology 29: 1664-1674, 2004.
25. Cunningham CL, Bakner L, Schuette LM and Young EA: Morphine and ethanol pretreatment effects on expression and extinction of ethanol-induced conditioned place preference and aversion in mice. Psychopharmacology (Berl) 238: 55-66, 2021.
26. Blanchard OL and Smoliga JM: Translating dosages from animal models to human clinical trials--revisiting body surface area scaling. FASEB J 29: 1629-1634, 2015.
27. Sacchetti B and Bielavska E: Chelerythrine, a specific PKC inhibitor, blocks acquisition but not consolidation and retrieval of conditioned taste aversion in rat. Brain Res 799: 84-90, 1998.
28. Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ and Johannes FJ. Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes. FEBS Lett 392: 77-80, 1996.
29. Muñoz A, Nakazaki M, Goodman JC, Barrios R, Onetti CG, Bryan J and Aguilar-Bryan L: Ischemic preconditioning in the hippocampus of a knockout mouse lacking SUR1-based K(ATP) channels. Stroke 34: 164-170, 2003.
30. Rabinstein AA: Update on treatment of acute ischemic stroke. Continuum (Minneap Minn) 26: 268-286, 2020.
31. Rodriguez R, Santiago-Mejia J, Gomez C and San-Juan ER: A simplified procedure for the quantitative measurement of neurological deficits after forebrain ischemia in mice. J Neurosci Methods 147: 22-28, 2005. .
32. Wexler EJ, Peters EE, Gonzales A, Gonzales ML, Slee AM and Kerr JS: An objective procedure for ischemic area evaluation of the stroke intraluminal thread model in the mouse and rat. J Neurosci Methods 113: 51-58, 2002.
33. Ashwal S, Tone B, Tian HR, Cole DJ and Pearce WJ: Core and penumbral nitric oxide synthase activity during cerebral ischemia and reperfusion. Stroke 29: 1037-1047, 1998.
34. Zwerus R and Absalom A: Update on anesthetic neuroprotection. Curr Opin Anaesthesiol 28: 424-430, 2015.
35. Thomazeau J, Rouquette A, Martinez V, Rabuel C, Prince N, Laplanche JL, Nizard R, Bergmann JF, Perrot S and Lloret-Linares C: Acute pain factors predictive of post-operative pain and opioid requirement in multimodal analgesia following knee replacement. Eur J Pain 20: 822-832, 2016.
36. Murry CE, Richard VJ, Reimer KA and Jennings RB: Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. Circ Res 66: 913-931, 1990.
37. Schultz JE, Rose E, Yao Z and Gross GJ: Evidence for involvement of opioid receptors in ischemic preconditioning in rat hearts. Am J Physiol 268: H2157-H2161, 1995.
38. Gao CJ, Niu L, Ren PC, Wang W, Zhu C, Li YQ, Chai W and Sun XD: Hypoxic preconditioning attenuates global cerebral ischemic injury following asphyxial cardiac arrest through regulation of delta opioid receptor system. Neuroscience 202: 352-362, 2012.
39. Zhao P, Huang Y and Zuo Z: Opioid preconditioning induces opioid receptor-dependent delayed neuroprotection against ischemia in rats. J Neuropathol Exp Neurol 65: 945-952, 2006.
40. Liu Y, Li J, Yang J, Ji F, Bu X, Zhang N and Zhang B: Inhibition of PKCgamma membrane translocation mediated morphine preconditioning-induced neuroprotection against oxygen-glucose deprivation in the hippocampus slices of mice. Neurosci Lett 444: 87-91, 2008.
41. Arabian M, Aboutaleb N, Soleimani M, Mehrjerdi FZ, Ajami M and Pazoki-Toroudi H: Role of morphine preconditioning and nitric oxide following brain ischemia reperfusion injury in mice. Iran J Basic Med Sci 18: 14-21, 2015.
42. Peart JN and Gross GJ: Cardioprotective effects of acute and chronic opioid treatment are mediated via different signaling pathways. Am J Physiol Heart Circ Physiol 291: H1746-H1753, 2006.
43. Zhang N, Zhu H, Han S, Sui L and Li J: cPKCγ alleviates ischemic injury through modulating synapsin Ia/b phosphorylation in neurons of mice. Brain Res Bull 142: 156-162, 2018.
44. Faubel S and Edelstein CL: Caspases as drug targets in ischemic organ injury. Curr Drug Targets Immune Endocr Metabol Disord5: 269-287, 2005.
45. Eftekhar-Vaghefi S, Esmaeili-Mahani S, Elyasi L and Abbasnejad M: Involvement of Mu opioid receptor signaling in the protective effect of opioid against 6-hydroxydopamine-induced SH-SY5Y human neuroblastoma cells apoptosis. Basic Clin Neurosci 6: 171-178, 2015.
46. Arabian M, Aboutaleb N, Soleimani M, Ajami M, Habibey R, Rezaei Y and Pazoki-Toroudi H: Preconditioning with morphine protects hippocampal CA1 neurons from ischemia-reperfusion injury via activation of the mTOR pathway. Can J Physiol Pharmacol 96: 80-87, 2018.
47. Lu S, Liao L, Zhang B, Yan W, Chen L, Yan H, Guo L, Lu S, Xiong K and Yan J: Antioxidant cascades confer neuroprotection in ethanol, morphine, and methamphetamine preconditioning. Neurochem Int 131: 104540, 2019.