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ORIGINAL ARTICLE

Low-Dose Ethanol Preconditioning Protects Against Oxygen-Glucose Deprivation/Reoxygenation-Induced Neuronal Injury By Activating Large Conductance, Ca^{2+}-Activated K^+ Channels *In Vitro*

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Abstract Increasing evidence suggests that low to moderate ethanol ingestion protects against the deleterious effects of subsequent ischemia/reperfusion; however, the underlying mechanism has not been elucidated. In the present study, we showed that expression of the neuronal large-conductance, Ca^{2+}-activated K^+ channel (BK_{Ca}) α-subunit was upregulated in cultured neurons exposed to oxygen-glucose deprivation/reoxygenation (OGD/R) compared with controls. Preconditioning with low-dose ethanol (10 mmol/L) increased cell survival rate in neurons subjected to OGD/R, attenuated the OGD/R-induced elevation of cytosolic Ca^{2+} levels, and reduced the number of apoptotic neurons. Western blots revealed that ethanol preconditioning upregulated expression of the anti-apoptotic protein Bcl-2 and downregulated the pro-apoptotic protein Bax. The protective effect of ethanol preconditioning was antagonized by a BK_{Ca} channel inhibitor, paxilline. Inside-out patches in primary neurons also demonstrated the direct activation of the BK_{Ca} channel by 10 mmol/L ethanol. The above results indicated that low-dose ethanol preconditioning exerts its neuroprotective effects by attenuating the elevation of cytosolic Ca^{2+} and preventing neuronal apoptosis, and this is mediated by BK_{Ca} channel activation.

Keywords Oxygen-glucose deprivation/reoxygenation · Ethanol preconditioning · BK_{Ca} channel · Neuroprotection · Apoptosis

Introduction

Ischemic stroke is one of the leading causes of mortality and is an important contributor to long-term disability [1]. To date, thrombolysis is the only approved treatment for ischemic stroke [2]. However, considering the high risk of bleeding and the small therapeutic time window for thrombolysis [3], researchers are investigating other, possibly more efficient methods of countering ischemic stroke. Recently, brain preconditioning has emerged as a novel neuroprotective strategy to counter ischemic stroke. Numerous stimuli, including ischemia [4], pharmacological agents [5–7], hypothermia [8], hypoxia [9], and other stimuli that cause cellular stress, may induce a preconditioning response [10]. Due to its ease of establishment, pharmacological preconditioning has been accepted as a promising paradigm to counter cerebral ischemic injury [11]. Ethanol is one of the pharmacological agents commonly used in this research, although heavy consumption of ethanol is associated with increased risk [12] and severity [13] of stroke. However, increasing evidence suggests that low to moderate ethanol ingestion (1–2 beverages per day or ~30 g ethanol) may protect against the harmful effects of subsequent ischemia/reperfusion (I/R)

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[14, 15], an effect known as ethanol preconditioning (EtOH-PC). For example, a meta-analysis indicated that light (<12 g/day) or moderate (12–24 g/day) ethanol consumption reduces the relative risk of ischemic stroke [16]. A prospective cohort study has also reported that low ethanol consumption has a modest beneficial association with functional outcome following stroke [17]. In a gerbil model, our previous study showed that moderate ethanol intake triggers a significant neuroprotective effect against I/R-induced injury [6]. The famous "French paradox" [18] also suggests cardioprotective and neuroprotective effects of ethanol in I/R injury. The prompt benefits of ethanol preconditioning last for about 2 h after ingestion. However, after the plasma ethanol level has returned to baseline, a delayed phase of further EtOH-PC emerges [14], leading researchers to postulate that ethanol ingestion may trigger a downstream signaling cascade which induces the protective effect against I/R injury. However, the underlying mechanisms of the neuroprotective effect of EtOH-PC remain to be elucidated. Sun *et al.* [19] recently showed that low-dose ethanol intake protects against transient focal cerebral ischemia through the peroxisome proliferator-activated receptor γ pathway. In addition, it has been suggested that ethanol improves endothelial-cell function by the activation of large-conductance, Ca^{2+}-activated K^+ channels (BK_{Ca}) [20].

The BK_{Ca} channel is a member of the K_{Ca} family, consisting of four α-subunits that form the ion-channel pore and four modulatory β subunits (β1-4) that are specific to tissue type, such as brain and lung [21, 22]. BK_{Ca} channels are broadly distributed in the plasma membranes and mitochondrial membranes of cells, and are activated by membrane depolarization and elevated levels of intracellular Ca^{2+}, resulting in a large K^+ conductance, which in turn re/hyperpolarizes the membrane [23] and closes voltage-dependent Ca^{2+} channels. These BK_{Ca} channel properties implement the negative-feedback regulation of neuronal excitability and Ca^{2+} signaling [24]. In the central nervous system (CNS), the BK_{Ca} channel is one of the intrinsic molecular determinants of the regulation of neuronal excitability and the release of neurotransmitters [25]. Some pathophysiological conditions, such as stroke, epilepsy, asthma, and hypertension, have been identified as potential targets for pharmacological intervention with BK_{Ca} channel openers [26]. Moreover, the use of pharmacological and knockout-mouse models has suggested a protective role of BK_{Ca} channels against I/R injury. For example, resveratrol attenuates oxygen-glucose deprivation (OGD)-induced neuronal impairment by enhancing the activation of BK_{Ca} channels [27], and the traditional Chinese medicine Baifuzi (*Typhonium giganteum* Engl.) antagonizes transient ischemic brain injury by interacting with the STREX (stress axis hormone-regulated exon) domain of the BK_{Ca} channel [28]. Wang *et al.* [29] reported that pretreatment with ethanol attenuates the brain leukocyte-endothelial adhesive interactions induced by I/R and that the effect may be associated with the BK_{Ca} channel. Recently, using organotypic hippocampal slice cultures exposed to glutamate, Piwonska *et al.* found that pre-incubation of slices with the BK_{Ca} channel opener NS1619 results in decreased neuronal cell death measured as reduced uptake of propidium iodide. This neuroprotective effect is reversed by pre-incubation with the BK_{Ca} channel inhibitors paxilline and iberiotoxin [30].

Although the neuroprotective effects of EtOH-PC and pharmacological activation of BK_{Ca} in I/R have been demostrated by several experiments, the possible mechanism requires investigation. First, the mechanisms underlying the neuroprotective effect of ethanol consumption remain unclear. Second, it is unknown whether the neuroprotective effects of EtOH-PC are mediated by BK_{Ca} channels. To address these questions, we designed experiments to elucidate the role of the BK_{Ca} channel in EtOH-PC, using an *in vitro* OGD/R model.

Materials and Methods

Ethics Statement

All the procedures were performed in accordance with the guidelines set by the Animal Care and Use Committee of Capital Medical University and were approved by the Ethics Committee of the Beijing Tiantan Hospital of Capital Medical University. Primary neurons were cultured from E16–18 Sprague-Dawley rat fetuses and the rats were obtained from Vital River Laboratory Animal Technology Co., Ltd., Beijing, China (Certification No: SCXK 2012-0001).

Culture of Primary Cortical Neurons

Primary neurons were cultured from E16–18 Sprague-Dawley rat fetuses as previously described [31] with modification. The rats were anesthetized using 10% chloral hydrate. Cortical brain tissue was removed, pooled bilaterally, and treated with 0.125% trypsin-EDTA containing 0.5 mg/mL DNase for 15 min at 37 °C. The protease digestion was inhibited by Dulbecco's modified Eagle's medium (DMEM)/F12 medium with 10% fetal bovine serum (FBS) and 5% horse serum (HS). After centrifugation, the cells were re-suspended in plating medium and seeded into culture plates pre-coated with poly-L-lysine (0.1 g/L). Plating medium consisted of DMEM/F12, FBS (10%), HS (5%), penicillin/streptomycin (1%), and L-glutamine (0.5 mmol/L). Approximately 4 h later, once the

cells had attached, the plating medium was replaced with cell culture medium consisting of Neurobasal-R medium, B27 (2%), bovine serum albumin (1%), and penicillin/streptomycin (1%). The cultures were maintained in an incubator at 37 °C with 5% CO_2. Half of the culture medium was replaced once every other day.

Pharmacological Treatments

In these experiments, primary neurons were cultured for 7 days and subsequently exposed to different doses of ethanol (Sigma-Aldrich, St Louis, MO) for 24 h prior to OGD/R. The ethanol was added directly to the culture medium at a final concentration of 5, 10, 25, 50, or 75 mmol/L. The BK_{Ca} channel inhibitor paxilline (5 μmol/L) was added from stock solution in dimethyl sulfoxide (DMSO) to culture medium 10 min prior to the addition of ethanol. The BK_{Ca} channel activator NS11021 (0.1 μmol/L) was added to the culture medium 24 h before OGD/R.

Oxygen-Glucose Deprivation and Reoxygenation (OGD/R)

After 24 h of EtOH-PC, the culture medium containing ethanol was removed and replaced with glucose-free DMEM (Thermo Fisher Scientific, Inc.). The plate was exposed to a hypoxic environment of 5% CO_2, 2% O_2, and 93% N_2 at 37 °C for 2 h. Then the glucose-free DMEM was completely replaced with culture medium and the cells incubated under normoxic conditions with 5% CO_2 for 24 h (reoxygenation period). Control neurons were kept in normoxic conditions.

Immunofluorescence Analysis of Cultured Primary Cortical Cells for Neuron Identification

After 7 days in culture, the cells were washed with PBS and fixed in 4% paraformaldehyde for 30 min at room temperature. Then, the cells were rinsed three times with PBS and stored in PBS for later use. For immunofluorescence staining, the cells were permeabilized with 0.2% Triton-X-100 for 15 min and then blocked with goat serum (10%) diluted in 0.2% Triton-X-100 for 1 h. For neuron/glia discrimination, the cells were then incubated at 4 °C overnight with a mixture of primary antibodies as follows: (1) mouse polyclonal anti-β-tubulin (1:200; Beijing GuanXing Yun Science and Technology Co., Ltd., Beijing, China) and (2) rabbit polyclonal anti-glial fibrillary acidic protein (1:500; Beijing GuanXing Yun Science and Technology Co., Ltd.). The immunoreaction was visualized using Alexa-Fluor 488-conjugated goat anti-mouse antibody (1:500; Invitrogen; Thermo Fisher Scientific, Inc.) and Alexa-Fluor 568-conjugated goat anti-rabbit antibody (1:500; Invitrogen; Thermo Fisher Scientific, Inc.). The nuclear stain DAPI was applied as a counterstain.

Cell Viability Assays

Following the manufacturer's instructions, the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) reagent was added to the culture medium (1:10) and incubated at 37 °C for 4 h. The absorbance at 450 nm was measured (SpectraMax M5, Molecular Devices, Sunnyvale, CA).

Electrophysiology

For inside-out recording, the pipette was filled with a solution containing (in mmol/L): 140.0 KCl, 5.0 $CaCl_2$, 1.0 $MgCl_2$, 10.0 Hepes. The bathing solution for inside-out patch recordings contained (in mmol/L): 140.0 KCl, 1.0 $MgCl_2$, 0.9 $CaC1_2$, 10 EGTA, 10.0 Hepes. The pH was adjusted to 7.2 with KOH. Borosilicate glass patch-pipettes used for single-channel recordings had resistances between 3 and 5 MΩ when filled with the internal solution. Recordings were made with a patch-clamp amplifier and Patchmaster 2.73 amplifier (HEKA, Lambrecht, Pfalz, German). Single-channel recordings were filtered at 1 to 5 kHz and digitized at 20 kHz. All experiments were performed at room temperature (22–25 °C). Analysis of data was performed with Fitmaster software (HEKA, Lambrecht, Pfalz, German). Open probabilities were expressed as probability of channel opening (NPo), where N represents the number of single channels present in the patch, and Po is the open probability of a single channel. NPo was calculated using the following, equation: $NPo = (A_1+2\cdot A_2+3\cdot A_3+\ldots n\cdot An)/(A_0+A_1+A_2+\ldots A_n)$, where A_0 is the area under, the curve of the amplitude histogram corresponding to the closed state, and A_1–A_n represent the histogram areas reflecting the different open-state current levels for 1 to n channels present in the patch.

Cytosolic Ca^{2+} Level

The concentration of cytosolic Ca^{2+} was determined by loading the cells with fluo-4/AM. Following treatment, the 96-well plates were washed three times with Hanks' balanced salt solution (HBSS), and subsequently 1 μmol/L of fluo-4/AM from a 1-mmol/L stock solution in DMSO, diluted with HBSS, was added to each well. The plate was incubated in the dark at 37 °C for 30 min. The cells were then washed three times and incubated with HBSS for 30 min to ensure complete degradation of the fluo-4/AM. The fluo-4 fluorescence was excited at 488 nm and measured at 520 nm (SpectraMax M5).

Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP Nick End-Labeling (TUNEL) and DAPI Staining

To detect apoptosis, the *In Situ* Cell Death Detection kit for fluorescein (Roche, Shanghai, China) was used. Following the manufacturer's recommendations, cortical neurons were stained by TUNEL following reoxygenation and DAPI was used as a counterstain. Negative controls were treated in an identical manner; however, they were incubated without the TdT enzyme, while positive controls were treated with DNase. In the microscopic observations, TUNEL-positive samples were green and DAPI-positive samples were blue. Five fields of view were selected randomly to calculate the number of apoptotic cells. The counts of positively-stained cells from five fields were then averaged.

Annexin V-PE and 7-AAD Double-Staining Assay for Apoptosis

Apoptosis was characterized using the PE Annexin V Apoptosis Detection kit I (BD Pharmingen^{TM}, San Diego, CA), detected by flow cytometry (BD FACSAria^{TM} II; BD Biosciences, Franklin Lakes, NJ). For flow cytometry, the cells were cultured in 6-well plates. After drug treatment and OGD/R, the cells were harvested and washed three times with PBS. Prior to flow cytometric analysis, 5 μL of Annexin V-PE and 5 μL of 7-AAD were used to co-stain in the dark for 15 min at room temperature. Early-stage apoptotic cells were PE-Annexin-V–positive and 7-AAD–negative, whereas late-stage apoptotic or dead cells were positive for both PE-Annexin V and 7-AAD.

Total Protein Extraction and Western Blot Analysis

Cells were washed three times with PBS and lysed in radioimmunoprecipitation buffer supplemented with a proteinase inhibitor cocktail for 30 min on ice. The cells were subsequently centrifuged at 1400 rpm at 4 °C for 15 min. The supernatants were collected for western blot analysis and determination of total protein concentration. Protein was assessed using a BCA Protein Assay kit (Applygen Technologies Inc., Beijing, China). Thirty micrograms of total protein per experimental condition were electrophoresed on 12% polyacrylamide gels, and then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in Tween-20 Tris-buffered saline (TBST) containing 5% non-fat milk for 1 h. The expression levels of the BK_{Ca} channel α-subunit, Bax, and Bcl-2 were determined using the following primary antibodies: rabbit polyclonal anti-BK_{Ca} α-subunit (1:1000; Abcam, Cambridge, MA), rabbit polyclonal anti-Bax (1:1000; Cell Signaling Technology, Inc., Danvers, MA), and rabbit polyclonal anti-Bcl-2 (1:1000; Cell Signaling Technology, Inc.). The primary antibodies were diluted in the blocking solution and incubated with the PVDF membranes overnight at 4 °C. After washing three times with TBST, membranes were incubated with the secondary antibodies for 1 h at room temperature. After washing three times, the blots were developed and analyzed using the FluorChem FC2 System (Cell Biosciences, Inc., Santa Clara, CA).

Statistical Analysis

All data are expressed as the mean ± SD. Statistical comparisons were made using Student's *t*-test or analysis of variance combined with Fisher *post hoc* testing, as appropriate. $P < 0.05$ was considered to be statistically significant.

Results

Immunofluorescence Analysis of Cultured Primary Cortical Neurons

We determined the percentage of neurons and astrocytes in the primary cortical cultures, and found that they consisted of ~95% β-tubulin-positive cells (Fig. 1).

Effects of Different Concentrations of Ethanol Preconditioning on OGD/R-Induced Neuronal Injury

The cells were pretreated with ethanol (5, 10, 25, 50, or 75 mmol/L) for 24 h, followed by OGD-2 h/R-24 h injury. At the end of reoxygenation, neurons exposed to OGD/R exhibited a decrease in cell survival rate compared with the control ($P < 0.01$). Pre-incubation with 10 mmol/L ethanol elevated the survival rate after OGD/R ($P < 0.01$), and the protective effect declined with increased ethanol dosage (Fig. 2A).

Effect of Ethanol Preconditioning on OGD/R-Induced Neuronal Apoptosis

The effect of 5, 10, and 25 mmol/L EtOH-PC on the neuronal apoptosis induced by OGD/R was subsequently assessed by flow cytometric analysis. The results revealed that the number of apoptotic neurons after OGD/R nearly doubled that of the control ($P < 0.01$), and that both 5 and 10 mmol/L EtOH-PC for 24 h caused a decreased ratio of apoptosis (Fig. 2B), providing further evidence for the neuroprotective effect of low-dose EtOH-PC.

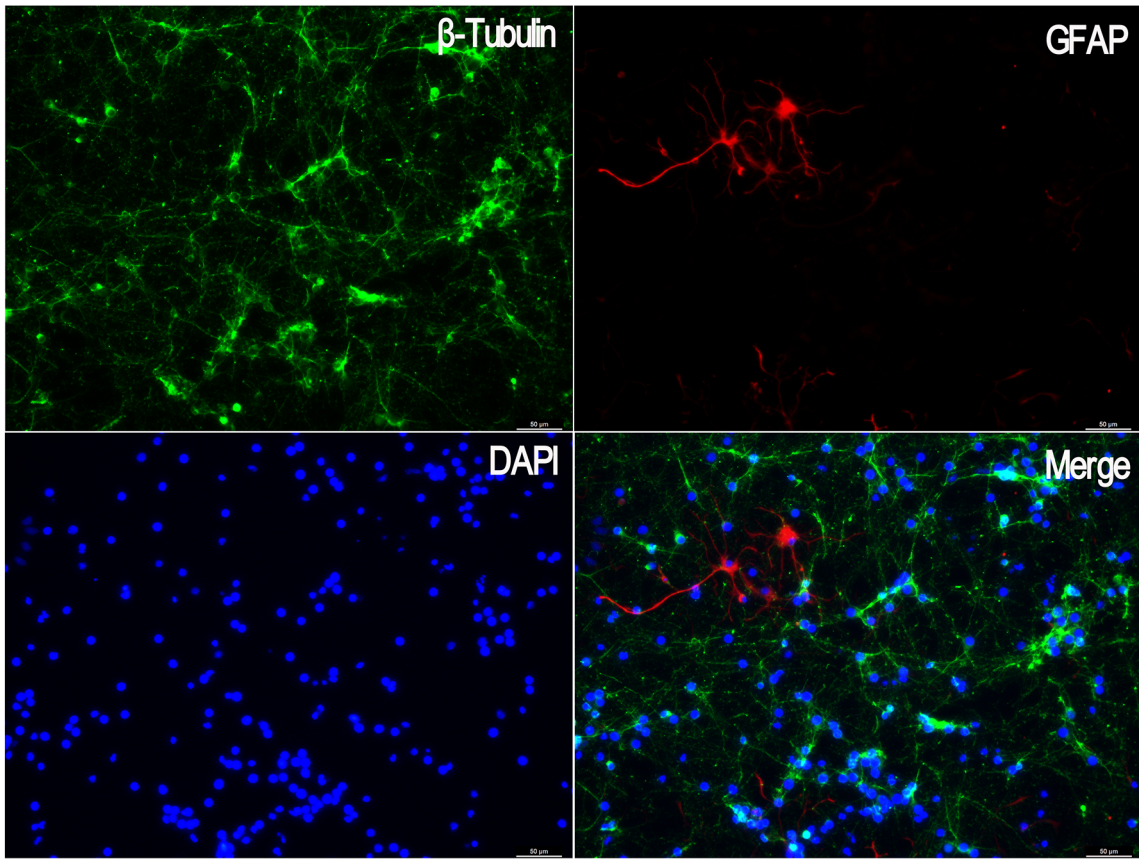

Fig. 1 Identification of primary cortical neurons. Double-labeling immunofluorescence staining with the neuron marker β-tubulin (green) and the astrocyte marker glial fibrillary acidic protein (GFAP, red); nuclei stained with DAPI; scale bars, 50 μm.

Immunodetection of the BK_{Ca} α-Subunit in Primary Cortical Neurons

Next, the expression of the BK_{Ca} α-subunit in the control and OGD/R groups was measured by western blot analysis. The results showed immunoreactive bands at the molecular weight of 134 kDa for BK_{Ca} channels (Fig. 3A). The protein expression of BK_{Ca} was normalized against that of β-actin, the loading control. The expression of the BK_{Ca} channel α-subunit was upregulated ($P < 0.01$) in the OGD/R group compared with the control group (Fig. 3B). We also assessed the expression of the BK_{Ca} α-subunit in the OGD/R and EtOH-PC+OGD/R groups; the results showed no significant difference between the two groups (Fig. S1).

Ethanol Activates BK_{Ca} Channels in Primary Cortical Neurons

To investigate whether 10 mmol/L ethanol directly activates the BK_{Ca} channel, ethanol was perfused onto the membrane after the inside-out patch clamp was established. Representative single-channel recordings of BK_{Ca} in the absence and presence of 10 mmol/L ethanol demonstrated that 10 mmol/L ethanol activated BK_{Ca} channels at all membrane voltages (Fig. 4A). The current-voltage curve of ethanol on BK_{Ca} channels was fitted using linear regression as in Fig. 4B, and the single-channel conductance was calculated. The conductance in ethanol group was 276 ± 60 pS, slightly higher than in control group (240 ± 70 pS). The NPo of BK_{Ca} was also increased in the ethanol group compared to the control group as shown in Fig. 4C. We also examined the effects of 5 and 25 mmol/L ethanol on BK_{Ca} channels using the inside-out patch, and the results showed that both concentrations of ethanol can activate BK_{Ca} channels (Fig. S2). In CCK-8 and flow-cytometry assays, we did not find a protective effect of 25 mmol/L ethanol preconditioning, suggesting that there may be other harmful effects of this concentration on neurons that antagonize the neuroprotecitve effect of activation of BK_{Ca} channels [32, 33].

Effect of BK_{Ca} Channel Activator NS11021 Pre-incubation on OGD/R-Induced Neuronal Injury

To determine whether activation of the BK_{Ca} channel has neuroprotective effects similar to EtOH-PC, cortical

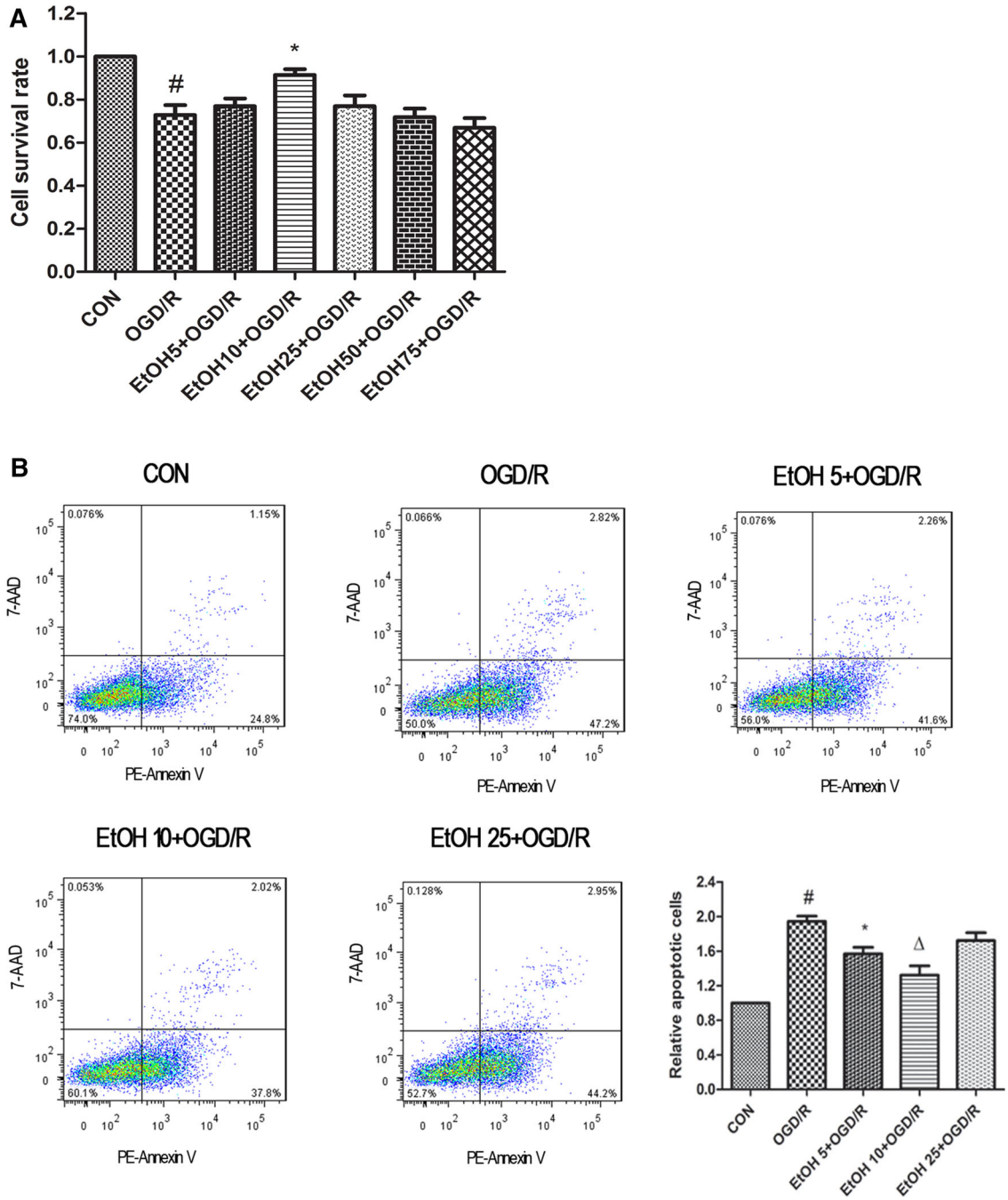

Fig. 2 EtOH-PC alleviates neuronal injury induced by OGD/R. **A** Effects of different concentrations of EtOH-PC on cell survival rate in neurons following OGD/R ($^{\#}P < 0.01$ *vs* control; $^{*}P < 0.01$ *vs* OGD/R; $n = 4$). **B** Detection of apoptotic cells by flow cytometry ($^{\#}P < 0.01$ *vs* control; $^{*}P < 0.05$ *vs* OGD/R; $^{\Delta}P < 0.05$ *vs* OGD/R; $n = 3$).

neurons were pretreated with NS11021, a selective BK_{Ca} channel activator, for 24 h prior to OGD/R and their viability was measured by CCK-8 assay. The results showed that pre-incubation with 0.1 μmol/L NS11021 elevated the cell survival rate decreased by OGD/R ($P < 0.01$, Fig. 5).

Effect of the BK_{Ca} Channel Blocker Paxilline on EtOH-PC-Induced Neuroprotection

The BK_{Ca} inhibitor paxilline was used to test whether the BK_{Ca} channel mediates the neuroprotective effect of EtOH-PC in OGD/R. The cells were divided into control,

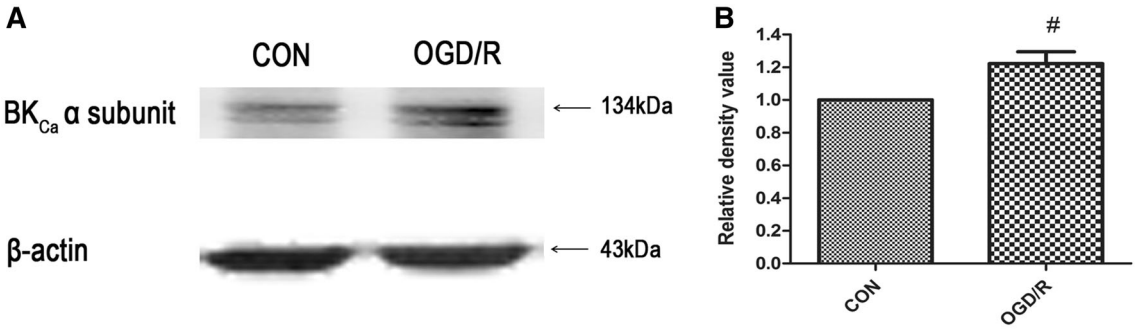

Fig. 3 Expression of the BK_{Ca} channel α-subunit. **A** Representative western blots for BK_{Ca}. **B** Expression of the BK_{Ca} channel α-subunit protein is significantly upregulated in the OGD/R group ($^{\#}P < 0.01$; $n = 3$).

OGD/R, EtOH-PC+OGD/R, paxilline+OGD/R, and paxilline+EtOH-PC+OGD/R groups. EtOH-PC elevated the survival rate decreased by OGD/R ($P < 0.05$, Fig. 6). When paxilline was used 10 min prior to EtOH-PC, the cell survival rate declined. However, paxilline alone did not affect the survival rate under OGD/R. This indicates that the effect of EtOH-PC in protecting against OGD/R injury is mediated by the BK_{Ca} channel.

Effect of EtOH-PC on OGD/R-Induced Dysregulation of Ca^{2+} Homeostasis and the Effect of the BK_{Ca} Channel

Dysregulation of Ca^{2+} homeostasis, especially Ca^{2+}-overload, is associated with neuronal death, so we evaluated the effect of 10 mmol/L EtOH-PC on cytoplasmic Ca^{2+} levels, as well as whether opening of the BK_{Ca} channels by EtOH-PC attenuated the elevation of cytosolic free Ca^{2+} level. Measurement of the cytosolic Ca^{2+} level by loading the cells with fluo-4/AM indicated that exposure to OGD/R caused an increase compared with control ($P < 0.05$, Fig. 7). Pre-treatment with 10 mmol/L ethanol decreased the increase of cytosolic free Ca^{2+} induced by OGD/R, while the BK_{Ca} channel inhibitor paxilline reversed the effect of EtOH-PC ($P < 0.05$). These results indicate that 10 mmol/L EtOH-PC decreases the elevation of cytosolic Ca^{2+} induced by OGD/R, and that paxilline reverses this protective effect, suggesting that activation of the BK_{Ca} channel by ethanol has a protective effect by maintaining intracellular Ca^{2+} homeostasis.

BK_{Ca} Channel Mediates the Neuroprotective Effect of EtOH-PC on Neuronal Apoptosis in OGD/R

We visualized one of the predominant features of neuronal apoptosis, DNA damage, by TUNEL assays and found that OGD/R exposure increased the prevalence of TUNEL-positive cells ($P < 0.01$, Fig. 8A). Preconditioning with 10 mmol/L ethanol significantly reduced the percentage of TUNEL-positive cells, while co-incubation with the BK_{Ca} channel inhibitor paxilline, added 10 min before ethanol, increased the TUNEL-positive cells by 48.86% ($P < 0.01$) compared with the EtOH+OGD/R group.

Effect of EtOH-PC on OGD/R-Induced Changes in the Expression of Apoptosis-Related Proteins and the Effect of the BK_{Ca} Channel

To investigate the mechanism by which EtOH-PC protects neurons from OGD/R injury, the expression levels of apoptosis-related proteins were determined by western blot. The results showed that the expression of anti-apoptotic Bcl-2 was reduced by OGD/R, but increased by EtOH-PC, while the BK_{Ca} channel inhibitor paxilline reversed this effect of EtOH-PC. In contrast, the pro-apoptotic Bax was upregulated by OGD/R, but down-regulated after EtOH-PC, and when co-incubated with paxilline, the expression of Bax increased (Fig. 8B). These results showed that EtOH-PC has neuroprotective effects against OGD/R-induced apoptosis by decreasing expression of the pro-apoptotic protein Bax and increasing expression of the anti-apoptotic protein Bcl-2 in cultured primary cortical neurons. In addition, the BK_{Ca} inhibitor paxilline diminished the protective effect of EtOH-PC.

Discussion

Is There an Optimal EtOH-PC Dose for Neuroprotection?

In Alzheimer's disease, moderate social drinkers have a reduced risk of Alzheimer's dementia compared to abstainers [34]. In traumatic brain injury, low to moderate blood ethanol concentrations are similarly associated with a survival benefit compared to the absence of ethanol ingestion [35]. In cerebrovascular disease, studies have

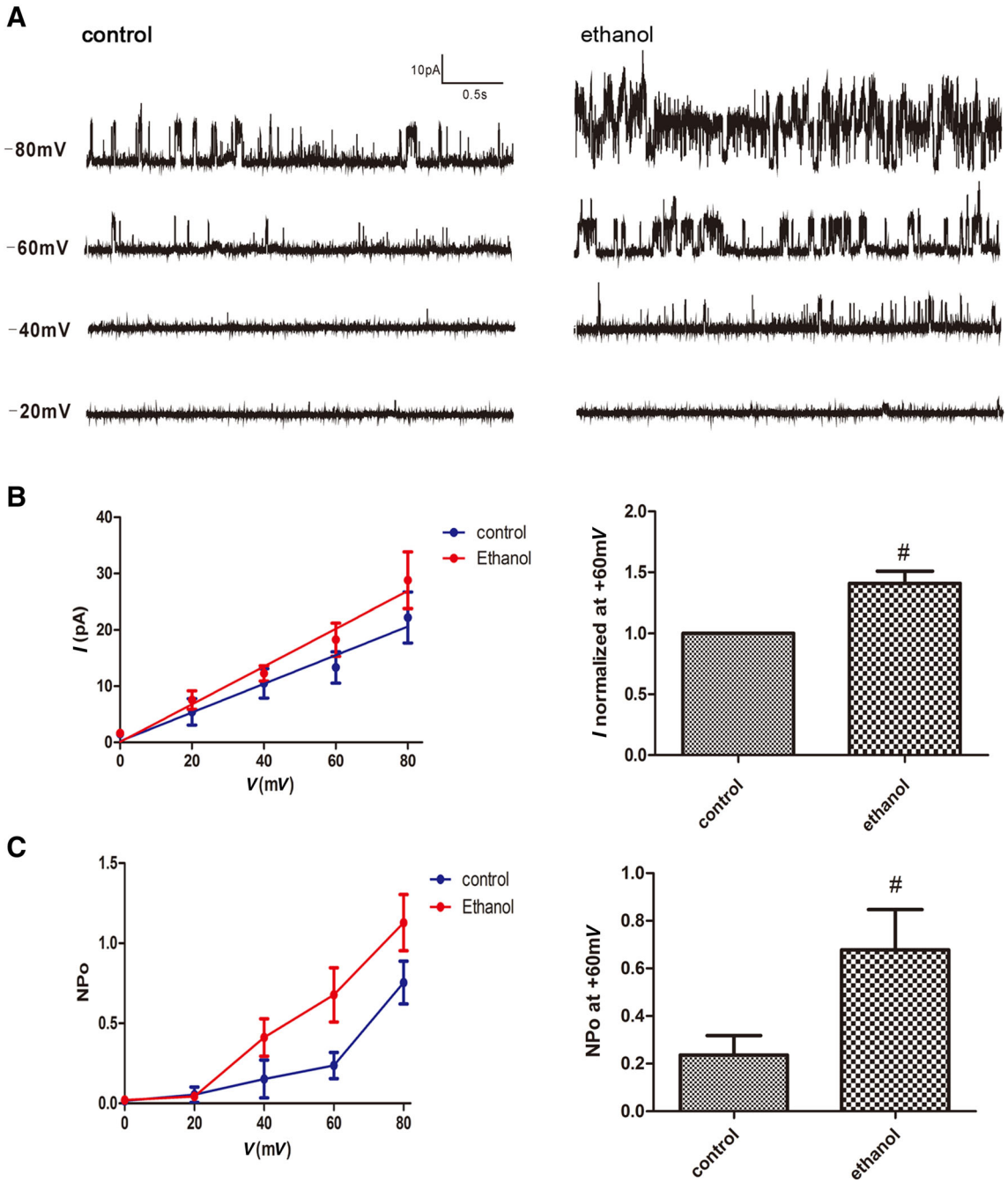

Fig. 4 Effect of 10 mmol/L EtOH on BK_{Ca} channels. **A** Representative single-channel recordings of BK_{Ca} channels in the inside-out configuration. **B** 10 mmol/L EtOH enhanced the BK_{Ca} channel current in a voltage-dependent manner ($^{\#}P < 0.05$ *vs* control; $n = 4$). **C** 10 mmol/L EtOH increased the NPo of single BK_{Ca} channels ($^{\#}P < 0.05$ *vs* control; $n = 4$).

shown that low-dose ethanol consumption protects against I/R injury in the rat brain, whereas chronic ingestion of high-dose ethanol has a harmful effect on cerebral I/R injury [36]. However, a few studies have shown harmful effects of moderate ethanol on the CNS. For example, moderate or high ethanol levels lead to morphological changes in cultured rat cortical neurons [32]. In addition, moderate ethanol consumption decreases neurogenesis in the adult hippocampus [33]. Therefore, it is not known whether moderate ethanol ingestion is good or bad for the brain.

This study revealed that both 5 and 10 mmol/L EtOH-PC had neuroprotective effects, providing further evidence for the protection by low dose. The protective effect was diminished at a higher EtOH dosage. It appears that a low, rather than moderate, concentration of ethanol protects

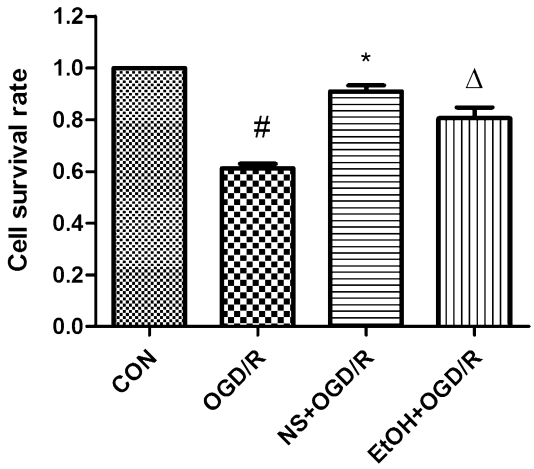

Fig. 5 BK_{Ca} channel activator NS11021 and ethanol preconditioning attenuate OGD/R-induced neuronal injury. CCK-8 assay revealed that 0.1 μmol/L NS11021 and 10 mmol/L ethanol preconditioning elevated cell survival rate decreased by OGD/R ($^{\#}P < 0.01$ *vs* control; $^{*}P < 0.01$ *vs* OGD/R; $^{\Delta}P < 0.01$ *vs* OGD/R; $n = 3$).

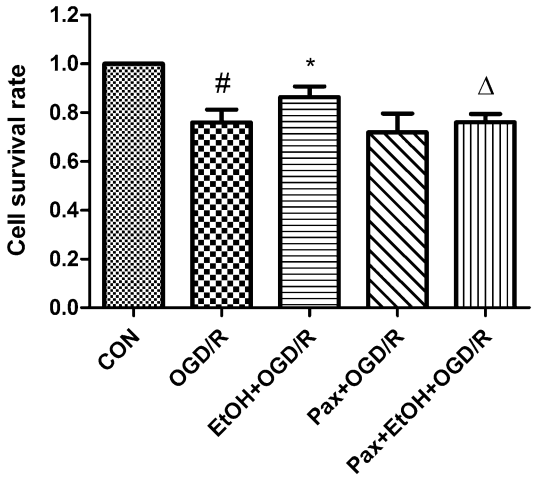

Fig. 6 The BK_{Ca} channel inhibitor paxilline diminishes the protective effect of EtOH-PC. CCK-8 assays revealed that paxilline diminished the protective effect of EtOH-PC on cell survival ($^{\#}P < 0.01$ *vs* control; $^{*}P < 0.05$ *vs* OGD/R; $^{\Delta}P < 0.05$ *vs* EtOH+OGD/R; $n = 4$).

primary cortical neurons against OGD/R injury. Possible explanations for the different protective concentrations of EtOH reported may be a result of the injury models and interventional environments.

Mechanism of Activation of the BK_{Ca} Channel by Which EtOH-PC Protects Against the Primary Neuronal Injury Induced by OGD/R

The α-subunits of the BK_{Ca} channel are broadly expressed in the CNS [37, 38], and are concentrated in the terminal areas of primary projection tracts. Confocal experiments in

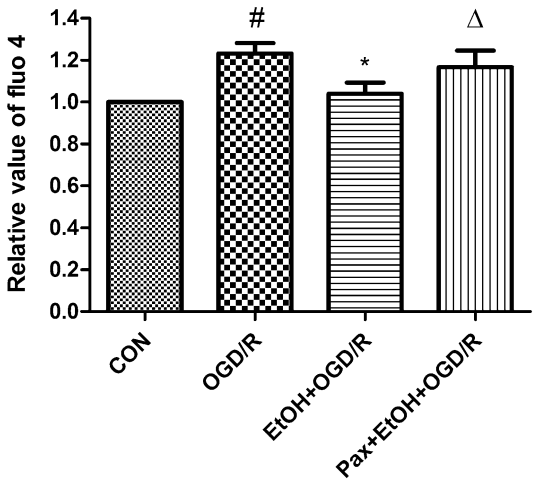

Fig. 7 Relative changes in fluo-4/AM, as measured by luminescence spectrophotometry. The level of cytosolic free Ca^{2+} was elevated in the OGD/R group and reduced in the EtOH-PC group. Paxilline diminished the effect of EtOH-PC ($^{\#}P < 0.05$ *vs* control; $^{*}P < 0.05$ *vs* OGD/R; $^{\Delta}P < 0.05$ *vs* EtOH+OGD/R; $n = 3$).

zones of hippocampal or cerebral cortical nerve termination have revealed that the α-subunit is more abundantly expressed in glutamatergic than in GABAergic terminals [39], indicating the relative importance of BK_{Ca} in regulating the release of various neurotransmitters. In cultured hippocampal neurons, the α-subunit of the BK_{Ca} channel is expressed in both the pre- and post-synaptic compartments [40], which may indicate an association with action potential repolarization. So far, attention has focused on functional alterations of the BK_{Ca} channel. Knowledge regarding the expression of BK_{Ca} is limited, particularly under pathological conditions in the brain. Ye *et al.* [41] found that expression of the BK_{Ca} α-subunit is increased in spinal cord injury. Notably, the gene and protein expression levels of the BK_{Ca} α-subunit are increased in adult Sprague-Dawley rats and in primary pulmonary artery smooth muscle cells under hypoxic conditions [42]. Here, we found higher expression of the BK_{Ca} channel α-subunit in the OGD/R group than in the control. This high expression may be induced by hypoxia-inducible factor [42], the expression of which is increased in ischemic stroke. In mouse knockout models, Liao *et al.* found that transient focal cerebral ischemia induces a larger infarct volume, more severe neurological deficits, and higher post-ischemic mortality in homozygous mice lacking the BK_{Ca} channel α-subunit than in wild-type littermates [43]. This indicates that neuronal BK_{Ca} channels play an important role in neuroprotection against ischemic brain damage. Further study is necessary to identify the mechanism by which OGD/R alters the expression of the BK_{Ca} channel subunit.

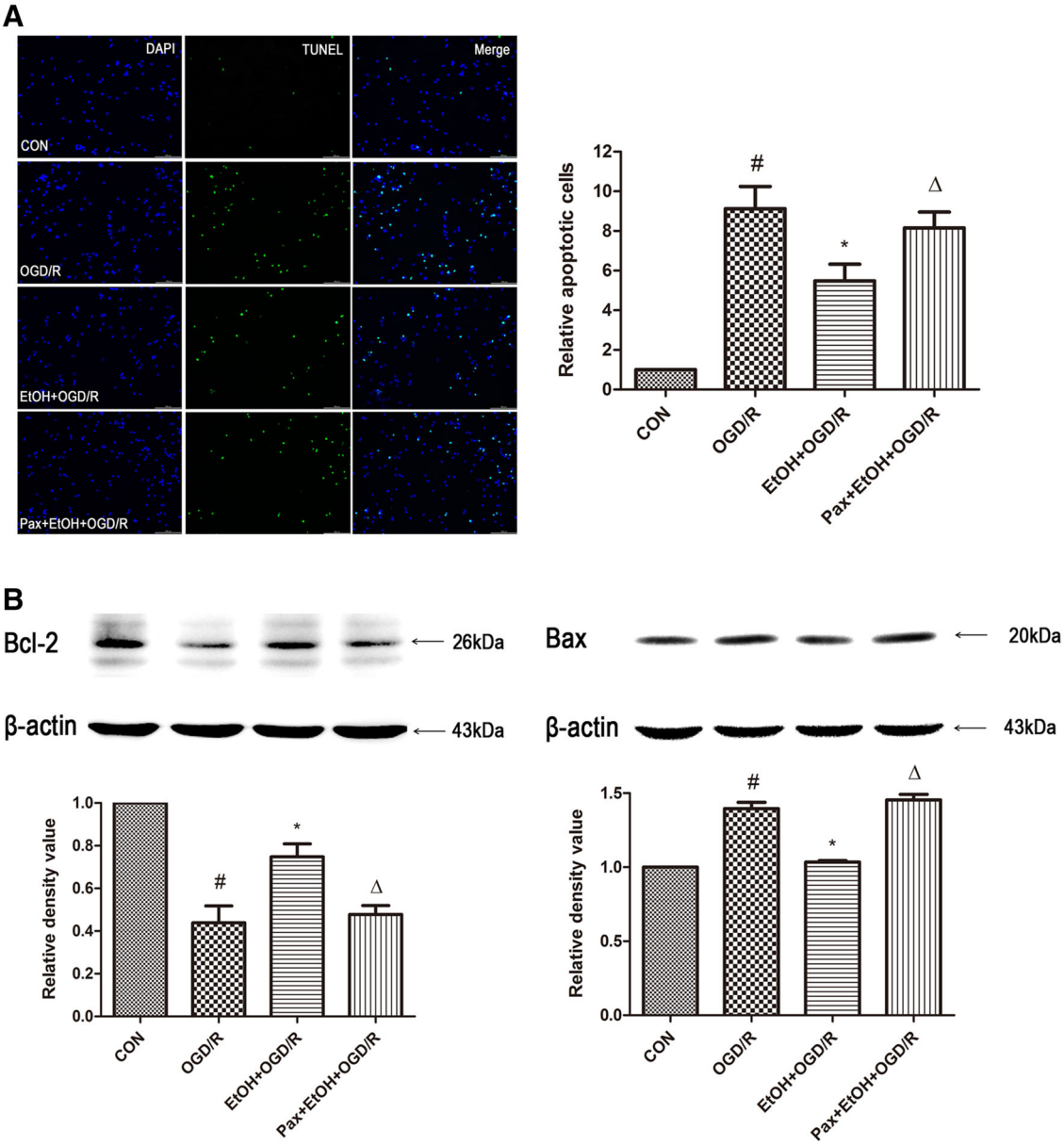

Fig. 8 TUNEL analysis and expression of Bcl-2 and Bax. **A** Representative photomicrographs of TUNEL staining (scale bar, 100 μm) and percentage of TUNEL-positive cells in each group. **B** Immunoblots and density values for Bcl-2 and Bax after various treatments. $^{\#}P < 0.01$ *vs* control; $^{*}P < 0.01$ *vs* OGD/R; $^{\Delta}P < 0.01$ *vs* EtOH+OGD/R; $n = 3$.

The effects of ethanol on the BK_{Ca} channel have been implicated in several conditions. For example, ethanol decreases the excitability of neurons in rat dorsal root ganglia by activating the BK_{Ca} channel, and this is associated with ethanol-related analgesia [44]. In rat nucleus accumbens neurons, ethanol accelerates the action potential repolarization by activating the BK_{Ca} channel [45]. In cultured human umbilical vein endothelial cells, 10 and 50 mmol/L ethanol directly activate BK_{Ca} channels, leading to increased endothelial proliferation, indicating a possible beneficial effect of ethanol on endothelial function [20]. Moreover, in ischemic stroke, our previous study revealed that EtOH-PC attenuates I/R-induced adhesive interactions between leukocytes and endothelial cells, and that this effect is reversed by the BK_{Ca} channel inhibitor paxilline [29].

In the CCK-8 assay, we found that both the BK_{Ca} channel activator NS11021 and EtOH-PC protected neurons against the OGD/R-induced decrease in viability. To test whether the BK_{Ca} channel mediates the neuroprotective effect of EtOH-PC in OGD/R, the BK_{Ca} channel inhibitor paxilline was used. When paxilline was applied 10 min prior to EtOH-PC, the cell survival rate elevation by EtOH-PC declined again (Fig. 6). However, paxilline

alone did not affect the survival rate under OGD/R. This indicated that the effect of EtOH-PC in protecting against OGD/R injury is mediated by the BK_{Ca} channel. This conclusion was further supported by inside-out patch recordings, showing that 10 mmol/L ethanol incubation directly enhanced the BK_{Ca} channel current in a voltage-dependent manner and increased the NPo of single BK_{Ca} channels. The mechanism by which ethanol activates the BK_{Ca} channel is not clearly understood. Liu *et al*. found that ethanol fails to gate the channel in the absence of activating Ca^{2+}, indicating that ethanol modulates the BK_{Ca} channel by acting as an adjuvant of Ca^{2+} [46]. In *Xenopus* oocytes, gintonin-mediated BK_{Ca} channel activation is blocked by a PKC inhibitor [47]. In cultured cerebellar granule cells [48] and PC12 cells [49], ethanol has been reported to cause an increase of PKC activity. Therefore, in the case of cortical neurons with OGD/R injury, activation of the BK_{Ca} channel by ethanol may be mediated by increased activity of PKC.

It is known that I/R injury is a result of a complex pathological cascade, including Ca^{2+} overload, oxidative stress, inflammation, and excitotoxicity. Accumulation of pathological levels of intracellular Ca^{2+} is a major cause of ischemic cell death. As activation of the BK_{Ca} channel depends on depolarization and an increased intracellular Ca^{2+} level, it appears well-suited for the negative-feedback regulation of Ca^{2+} influx through voltage-gated Ca^{2+} channels [50]. Therefore, we examined the effect of low-dose EtOH-PC on the cytosolic Ca^{2+} level under OGD/R conditions. Elevation of cytosolic Ca^{2+} occurred after OGD/R, as demonstrated by increased fluo-4/AM fluorescence. The 10 mmol/L EtOH-PC treatment attenuated the elevation of Ca^{2+}, while the effect of ethanol was antagonized by the BK_{Ca} channel inhibitor paxilline. Therefore, these results indicated that activation of BK_{Ca} by a low dose of EtOH-PC attenuates the OGD/R-induced elevation of cytosolic Ca^{2+} levels.

It has been reported that the BK_{Ca} channel α-subunit is expressed in the inner membrane of neuronal mitochondria [37]. Mitochondrial membrane ion channels are important for cell survival, while the mitochondrial permeability transition pore (MPTP) is considered to trigger the intrinsic apoptosis pathway. Cheng *et al.* [51] demonstrated that hypoxia inhibits the MPTP and increases the activity of mitochondrial BK_{Ca} channels in rat astrocytes, indicating that the activation of the BK_{Ca} channels maintains the MPTP in a closed position. Therefore, we postulated that EtOH-PC has an anti-apoptosis effect by activating BK_{Ca} channels and keeping the MPTP closed. We therefore investigated the effect of EtOH-PC on the neuronal apoptosis induced by OGD/R, and the probable effect of the BK_{Ca} channel. TUNEL staining demonstrated that EtOH-PC decreased the rate of apoptosis-positive cells induced by OGD/R, while paxilline attenuated this effect.

Furthermore, we assessed the expression levels of the apoptosis-related proteins Bcl-2 and Bax. The Bcl-2 protein family plays an important role in apoptotic signal transduction by regulating mitochondrial function [52]. The pro-apoptotic protein Bax forms a channel that is responsible for the release of proteins from the mitochondrial intermembrane space into the cytosol, facilitating the release of cytochrome-c. By contrast, the anti-apoptotic protein Bcl-2 functions to restore the membrane potential and inhibit cytochrome release. We found that ethanol increased the Bcl-2 levels and decreased the Bax levels in primary cortical neurons exposed to OGD/R. The BK_{Ca} channel inhibitor paxilline antagonized this effect. These findings imply that EtOH-PC prevents apoptosis by regulating the mitochondrial signaling pathway, and that the BK_{Ca} channel, presumably mitochondrial BK_{Ca} channel, mediates the effect.

Our previous study showed that the neuroprotection seen in cases of I/R preceded by socially relevant levels of ethanol intake is triggered by reactive oxygen species (ROS) [6]. A previous study of the heart also revealed that the protective effects of the BK_{Ca} channel opener NS1619 are effectively inhibited by MnTBAP, a synthetic dismutator of O_2^-, indicating that cardiac preconditioning with BK_{Ca} channel openers requires ROS generation [53]. Therefore, the present study has led to the hypothesis that the opening of BK_{Ca} channels by EtOH-PC in OGD/R also requires the generation of ROS, and this will be assessed in a future study.

Conclusions

(1) Preconditioning with low doses of ethanol protects primary cortical neurons against OGD/R-induced injury by decreasing the cytosolic Ca^{2+} levels and neuronal apoptosis. (2) Expression of the BK_{Ca} α-subunit is upregulated in cultured primary cortical neurons under OGD/R conditions, and this may contribute to the neuroprotective effect on OGD/R. (3) Neuronal BK_{Ca} channels mediate the neuroprotective effect of preconditioning with low doses of ethanol on the primary neuron injury induced by OGD/R. These results indicate potential neuroprotective mechanisms of low-dose EtOH-PC and provide support for the hypothesis that low-dose ethanol drinking protects the brain from the deleterious effects of a subsequent ischemic stroke.

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