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Antecedent Ethanol Attenuates Cerebral Ischemia/Reperfusion-induced Leukocyte-Endothelial Adhesive Interactions and Delayed Neuronal Death: Role of Large Conductance, Ca^{2+} -activated K^+ Channels

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Abstract

Ethanol preconditioning (EtOH-PC) reduces postischemic neuronal injury in response to cerebral ischemia/reperfusion (I/R). We examined the mechanism underlying this protective effect by determining 1) whether it was associated with a decrease in I/R-induced leukocyte-endothelial adhesive interactions in postcapillary venules, and 2) whether the protective effects were mediated by activation of large conductance, calcium-activated potassium (BKCa) channels. Mice were administered ethanol by gavage or treated with the BKCa channel opener, NS1619, 24 hrs prior to I/R with or without prior treatment with the BKCa channel blocker, paxilline. Both common carotid arteries were occluded for 20 min followed by 2 and 3 hrs of reperfusion, and rolling (LR) and adherent (LA) leukocytes were quantified in pial venules using intravital microscopy. The extent of delayed neuronal death (DND), apoptosis and glial activation in hippocampus were assessed 4 days after I/R. Compared with sham, I/R elicited increases in LR and LA in pial venules and DND and apoptosis as well as glial activation in the hippocampus. These effects were attenuated by EtOH-PC or antecedent NS1619 administration, and this protection was reversed by prior treatment with paxilline. Our results support a role for BKCa channel activation in the neuroprotective effects of EtOH-PC in cerebral I/R.

Keywords

Large conductance; Ca^{2+} -activated K^+ channels; Ethanol preconditioning (EtOH-PC); cerebral ischemia/reperfusion (I/R); leukocyte rolling and adhesion; neuroinflammation; delayed neuronal death (DND); apoptosis

INTRODUCTION

Epidemiologic studies have demonstrated that consumption of moderate amounts of red wine is associated with significant reductions in cardiovascular and cerebrovascular morbidity and mortality [1–4], reducing both the incidence and severity of myocardial

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infarction and stroke [5,6]. Nonethanolic components of wine such as resveratrol, a polyphenolic nutrient from the grape skin, have been shown to be cardioprotective as well as neuroprotective in myocardial and cerebral ischemia/reperfusion (I/R) models by our laboratory and others [7–13]. Other reports indicate that alcohol alone limits postischemic injury and inflammation [14–16].

Ethanol preconditioning (EtOH-PC) refers to a phenomenon whereby tissues are protected from the deleterious effects of prolonged I/R by antecedent ingestion of the alcohol at low to moderate levels [16,17]. Doses of ethanol required for this effect produce a transient increase (i.e. 30–60 min) in plasma concentrations of ethanol to approximately 10 mM, levels similar to those measured in humans consuming 1–2 alcoholic beverages [16]. The beneficial actions of EtOH-PC become apparent within 2 hours of ingestion and remain effective for 2 hours before disappearing. However, a delayed or late phase of protection re-emerges 12–24 hours after consumption of moderate levels of ethanol [16]. Because preconditioning becomes apparent only after plasma levels of ethanol have returned to baseline, it has been postulated that ethanol consumption triggers a downstream signaling cascade which induces development of a protected phenotype that limits the detrimental effects of a subsequent I/R insult. Although we have recently demonstrated that ethanol ingestion 24 hours prior to induction of cerebral ischemia/reperfusion (I/R) reduces postischemic neuronal injury [18], the underlying mechanism for the protective effect of EtOH-PC is not clear.

Large conductance, Ca^{2+} -activated K^+ channels (BK_{Ca}) are localized to cell membranes throughout the body, including the central nervous system as well as mitochondrial membrane in cardiac myocytes [19,20]. These channels are activated by increase in cytosolic calcium largely in response to calcium influx via voltage-gated Ca^{2+} channels by cell membrane depolarization. BK_{Ca} channel opening allows cytosolic K^+ efflux, which promotes cell membrane repolarization. This, in turn, reduces Ca^{2+} entry by closing voltage-dependent Ca^{2+} channels [21]. The first study to show neuroprotection with BK_{Ca} channel opener was done by Busija's group [22]. Increases in BK_{Ca} channel activity cause the depression of neuronal excitability with potential beneficial effects on cell survival [19]. Indeed, it is being increasingly recognized that activation of BK_{Ca} channels is cytoprotective. For example, agonists that promote opening of BK_{Ca} channels reduce infarct size after I/R in heart and brain [23,24], and inhibit ROS production of isolated rat brain mitochondria [25], while administration of BK_{Ca} channel inhibitors increases pyramidal neuronal cell death in hippocampal slices subjected to oxygen/glucose deprivation, an *in vitro* model of ischemia [26]. Several other studies have demonstrated that BK_{Ca} channel inhibitors prevent the infarct-sparing effects induced by short bouts of ischemia (ischemic preconditioning) or pharmacological agents such as silfenadil and $\text{TNF}\alpha$ [27–31].

Evidence obtained in our previous studies indicates that EtOH-PC prevents postischemic leukocyte-endothelial cell adhesive interactions and protects against delayed neuronal death (DND)-induced by I/R [16,18]. Since neutrophil infiltration plays an important role in the genesis of cerebral I/R injury [32–35] and BK_{Ca} channel activation has been implicated as an initiator for ischemic [23,28] and some forms of pharmacological preconditioning [21,23,24,31,35,36], we hypothesized that BK_{Ca} channel activation may play an important role in EtOH-PC, reducing I/R-induced leukocyte-endothelial adhesive interactions in pial postcapillary venules and delayed neuronal death in hippocampus. Our approach to this question was to use a mouse model of cerebral I/R to determine 1) whether selective pharmacologic activation of BK_{Ca} channels would reproduce the effects of EtOH-PC, and 2) whether treatment with BK_{Ca} channel inhibitors at the time of ethanol ingestion would abrogate the protective effects of EtOH-PC to attenuate cerebral microvascular dysfunction, neuroinflammation, and DND induced by cerebral I/R.

MATERIALS AND METHODS

Animal and groups

Wild-type (WT) male C57BL/6 mice (6–7 weeks) were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were given free access to water and lab chow and maintained at $22 \pm 2^\circ\text{C}$ with a constant humidity under a 12:12 hrs light:dark cycle. They were used at 8–10 weeks. The experimental procedures described were performed according to the criteria outlined in the National Institutes of Health guidelines and were approved by the University of Missouri Institutional Animal Care and Use Committee.

Experimental protocols

The mice were randomly divided into the following 8 treatment groups and all drug dosages were selected from previous reports in the literature:

Sham—These mice were surgically prepared (see below) in an identical manner as I/R mice, i.e. both common carotid arteries (CCA) exposed, installation of cranial window, however CCA were not occluded.

Ischemia/reperfusion (I/R)—After exposure of CCA, both arteries were occluded for 20 min, using aneurysm clips. Perfusion was re-established by removing the clips.

Ethanol preconditioning followed by I/R (EtOH-PC+I/R)—Ethanol was administered by gavage, 24 hrs prior to surgery for production of I/R.

NS1619 preconditioning followed by I/R (NS-PC+I/R)—To determine whether preconditioning with BK_{Ca} channel opener would mimic the effects of EtOH-PC, another group of mice were treated with the BK_{Ca} channel opener [37], NS1619 (NS, 0.1mg/kg, i.p.) 24 hrs prior to I/R.

Ethanol and NS-1619 preconditioning followed by I/R (EtOH-PC+NS-PC+I/R)—To determine whether there is a synergized action of enhanced BK_{Ca} channel activation under ethanol and NS1619 preconditioning paradigm, NS1619 i.p injection as well as EtOH-PC were administered at 24 hrs prior to I/R.

Pharmacological intervention with a BK_{Ca} channel inhibitor: To determine whether BK_{Ca} channels are involved in the putative neuroprotective effects of EtOH-PC and NS-PC, additional groups of mice were treated BK_{Ca} channel inhibitor, paxilline (PX, 2.5mg/kg, i.p.) [21,38], as described below.

Paxilline pretreatment in EtOH-PC (PX+EtOH-PC+I/R)—Paxilline was administered by i.p. injection, 10 min prior to administration of ethanol, and 24 hrs prior to I/R.

Paxilline pretreatment in NS-PC (PX+NS-PC+I/R)—Paxilline was administered 10 min prior to NS1619 preconditioning, and 24 hrs prior to I/R.

Paxilline alone followed by I/R (PX+I/R)—Paxilline was i.p. administered 24 hrs prior to I/R.

These treatment groups and experimental protocols are described in schematic form in Figure 1. Animals in Sham, I/R-alone, EtOH-PC+I/R and NS-PC+I/R groups received i.p. injections of saline (the vehicle for paxilline) at the same volume and time as the interventional groups.

Induction of ethanol preconditioning

EtOH-PC was induced by administering ethanol as a single intragastric bolus, 24 hrs before I/R. Using our dosing regimen, plasma ethanol increased to a peak value of ~45 mg/dl 30 min after gavage (equivalent to levels measured in humans consuming 1–2 alcoholic beverages) and returned to control levels within 60 min of alcohol administration [16]. The volume of 95% ethanol to be instilled (in μ l) was calculated as follows: [body weight (g) $\times$ 0.6] + 0.3. This volume of ethanol was mixed in 0.3 ml of sterile distilled water just before bolus administration to the animals by gavage. Animals in the sham control (no I/R) and I/R alone (no EtOH-PC) groups received sterile distilled water without ethanol by gavage.

Induction of global forebrain ischemia

The C57BL/6 strain is highly susceptible to cerebral ischemia following bilateral common carotid occlusion [39]. At 24 hrs after gavage, transient global cerebral ischemia was induced by occlusion of both common carotid arteries (CCA) for 20 min followed by reperfusion [39]. For the morphological studies, animals were anesthetized with a mixture of 70% nitrous oxide, 30% oxygen and 2.5% isoflurane during preparation for surgical operation, after which isoflurane was reduced to 1% during surgery and ischemic insult. Although volatile anesthetics have been reported to induce preconditioning, any putative preconditioning effects would be similar in all groups. It is important to note that significant hippocampal injury occurred in our model, even in animals anesthetized as described above. Body temperature was kept at 37 °C by a warming pad. After exposure of both CCA, the arteries were clamped with aneurysm clips for 20 min and reperfusion was effected by removing the aneurysm clips. Sham-operated animals underwent similar procedures without occlusion of the CCA. For the intravital microcirculation studies, animals were anesthetized initially with a mixture of ketamine (150 mg/kg body wt i.p.) and xylazine (7.5 mg/kg body wt ip), then they were equipped with an infusion catheter (PE-10) installed in their right external jugular vein. Prior to making the cranial window, they were injected through their jugular cannula with Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) to fluorescently label circulating leukocytes.

Monitoring of regional cerebral blood flow by laser Doppler flowmetry

The presence of communicating arteries in mice was determined by monitoring the decrease in regional cerebral blood flow (rCBF) before and after clamping the bilateral CCA using a laser Doppler blood flow monitor (MBF3D, Moor Instruments, Devon, UK). Animals showing a decrease in rCBF of less than 80% of the initial value were excluded from subsequent analyses [12]. There is a significant decrease in rCBF after ischemia at less than 20% of the initial value of pre-ischemia ($p < 0.001$). However, there was no significant differences among the ischemia/reperfusion with or without intervention ($p > 0.05$) (See Table 1).

Cranial Window

The head of each animal was fixed in a plastic frame in the sphinx position. The left parietal bone was exposed by a midline skin incision, followed by a craniotomy (diameter: 2.5 mm), 1 mm posterior to the bregma and 4 mm lateral to the midline, using a drill. Artificial cerebrospinal fluid (ACSF composition in mM: NaCl 128, KCl 3.0, CaCl_2 1.3, MgCl_2 1.0, Na_2HPO_4 21.0, NaH_2PO_4 1.3) was used to suffuse the tissue exposed by the craniotomies. A 12-mm glass coverslip was placed over the exposed brain tissue, and the artificial CSF was superfused beneath this viewing window [32,34].

Intravital Fluorescence Microscopy and Video Analysis

The numbers of rolling (LR) and adherent (LA) leukocytes in pial postcapillary venules were quantified using intravital microscopy. Postcapillary venules (30 to 40 μm diameter) in the cerebral microcirculation were identified through the cranial window through a 20 $\times$ objective lens, using a Nikon-E600FN upright microscope. Intravital fluorescence images of the vessel with CFDA-SE-labeled leukocytes (excitation wavelength, 420–490 nm; emission wavelength, 520 nm) were detected with a charge-coupled device (CCD) camera (XC-77; Hamamatsu Photonics), a CCD camera control unit (C2400; Hamamatsu Photonics) and an intensifier head (M4314; Hamamatsu Photonics) attached to the camera. Microfluorographs were projected on a television monitor (PVM-1953MD; Sony) and recorded on digital video using a digital video recorder (DMR-E50; Panasonic) for offline quantification of measured variables during playback of the recorded image. A video time-date generator (WJ810; Panasonic) displayed the stopwatch function on the monitor.

The interactions between leukocytes and vessel walls were recorded in 5 randomly selected venular segments which were 30 to 40 μm in diameter and at least 100 μm in length. Rolling and adherent leukocytes were classified according to the duration of their immobility on the venular wall, as follows [34]: (1) rolling cells were adherent for >2 and <30 seconds, and (2) adherent cells were stationary for >30 seconds, and they were expressed as the number of cells per mm^2 of venular surface, calculated from diameter and length, with the assumption of cylindrical vessel shape.

Preparation of brain samples

Four days after ischemia, separate groups of mice, treated as described above for the intravital microscopy studies, were anesthetized with isoflurane, then transcardially perfused with 4% paraformaldehyde in 0.05 mol/L PBS. Brains were removed and post-fixed in the same fixative for 3 days. Brain tissues were embedded in paraffin and coronal sections (6 μm thicknesses) were cut from the dorsal hippocampal region.

Assessment of neuronal damage and apoptosis

Cresyl violet and Fluoro-Jade B histochemical staining were used to evaluate ischemic neuronal damage and neurodegeneration, respectively. Fluoro-Jade B is a novel polyanionic fluorescein derivative which sensitively and specifically binds to degenerating neurons (both apoptotic and necrotic). Its high affinity to degenerating neurons with green fluorescence has made it a definitive marker of neurodegeneration. Fluoro-Jade B staining to examine ongoing damage was performed according to the protocol described by Schmued et al [40].

Neuronal apoptotic death staining was performed using a terminal deoxynucleotide transferase dUTP Nick End Labeling (TUNEL) assay, using an in situ cell death detection kit (Phoenix Flow Systems, San Diego, CA) according to the manufacturer's instructions. The deparaffinized and hydrated sections were covered with proteinase K (1:100 diluted in 10 mM Tris pH 8) and incubated for 20 min at room temperature, then washed with PBS, and incubated for 5 min with 3% H_2O_2 diluted in methanol. The slides were washed again with PBS and incubated with reaction buffer for 20 min at room temperature. After blotting the reaction buffer, the sections were incubated for 1.5 hours at 37°C with DNA Labeling Solution, then washed with PBS and incubated for 10 min with blocking buffer. The latter was blotted and the sections were incubated with antibody solution in the dark for 1.5 hours at room temperature, then washed with PBS and covered with blocking buffer for 10 min. After blotting the buffer, the sections were incubated at room temperature for 30 min with conjugate solution, then washed with PBS and incubated for 15 min with a DAB (3, 3'-Diaminobenzidine) solution, washed again with water and counterstained with methyl green for 3 min, then dipped in ethanol, blotted and mounted in Permount.

Assessment of neuroinflammatory activation

GFAP (Glial fibrillary acidic protein) and isolectin-B4 immunohistochemistry staining were used to demonstrate activation of astrocytes and microglial cells. Brain sections were stained with GFAP for astrocytes and isolectin-B4 for microglial cells. Rabbit anti-human GFAP (1:100 dilutions, Sigma, St. Louis, MO) antibody was used as primary antibody and microglial cells were identified using HRP (Horseradish peroxidase)-labeled isolectin B4 (20 µg/ml, Sigma, St. Louis, MO) as described previously [41] with modifications [12].

Quantitative assessment of hippocampal injury/inflammation

Neuronal damage, degeneration, apoptosis, and glial activation were quantified by counting the number of live neurons and fluorescent or immune-positive cells of Fluoro-Jade B, TUNEL, GFAP, Isolectin-B4 and in the middle of a defined CA1 area ($0.17 \mu\text{m} \times 0.54 \mu\text{m}$) in both left and right hippocampus per high magnification field (magnification 400x) using the Meta Imaging Serials (Version 6.1, Molecular Devices Corporation, Downingtown, PA). The average values of live neurons and above positive staining cells were obtained from left and right hippocampus of each animal.

Statistical analysis

Data (mean $\pm$ S.E.M.) were analyzed by one-way ANOVA followed by the Newman-Keuls post-hoc test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

BK_{Ca} channel inhibitor treatment attenuates the ability of EtOH-PC and NS-PC to decrease cerebral I/R-induced leukocyte-endothelial adhesive interactions

Twenty min of bilateral CCA occlusion followed by 2 and 3 hrs of reperfusion resulted in significant ($p < 0.001$) increases in LR and LA in pial venules (Fig. 2). In contrast to the increased postischemic adhesive events in venules, no LR or LA were observed in arterioles at any time during these experiments. Preconditioning with ethanol 24 hrs before I/R significantly decreased ($p < 0.001$) the postischemic increases in LR and LA, effects that were attenuated ($p < 0.001$ and $p < 0.01$ for LR and LA, respectively) by coincident treatment with paxilline (PX), a BK_{Ca} channel inhibitor (Fig. 2). Preconditioning with the BK_{Ca} channel opener, NS1619, was as effective as ethanol in reducing I/R-induced LR and LA (all $p < 0.001$). The anti-adhesive effects of NS-PC were also attenuated ($p < 0.001$ and $p < 0.01$ for LR and LA, respectively) by PX (Fig. 2). Preconditioning with both EtOH plus NS1619 did not showed any synergistic effect to further reduce I/R-induced LR and LA compared with EtOH-PC and NS1619 alone (Fig. 2) (all $p > 0.05$). Taken together, these data support the hypothesis that BK_{Ca} channel activation may play a critical role in initiating the development of an anti-inflammatory phenotype after EtOH-PC.

Paxilline reverses EtOH-PC and NS-PC-elicited decreases in cerebral I/R-induced neuronal injury and inflammation

Cresyl violet staining revealed healthy neurons in the hippocampal CA1 area in the sham control group (Fig. 3A) and largely dead neurons in I/R group (Fig. 3B). Ethanol or NS1619 administered 24 hrs before CCA occlusion both significantly ($p < 0.001$) decreased DND (based on neuron count) in the hippocampal CA1 area as compared with I/R group (without EtOH-PC and NS-PC (Fig. 3C, 3E vs. 3B). Similar to the findings with leukocyte-endothelial rolling and adhesion, paxilline administration 10 min prior to treatment with either ethanol or NS1619 significantly ($p < 0.05$ and $p < 0.01$) attenuated the neuroprotective effects of EtOH-PC and NS-PC in I/R (Fig. 3D vs. 3C, 3F vs. 3E and Fig. 4A).

Fluoro-Jade B staining was used as a marker of neurodegeneration. In the sham-operated group, very few degenerated neurons were observed in the hippocampal CA1 region (Fig. 3I). Positive staining of degenerated neurons significantly ($p < 0.001$) increased 4 days after I/R (Fig. 3J vs. 3I). Preconditioning with EtOH or NS1619 24 hrs before I/R significantly ($p < 0.001$) reduced the staining of degenerated neurons as compared with those in non-preconditioned, postischemic brain (Fig. 3K, 3M vs. 3J). Again, paxilline significantly reversed ($p < 0.05$ and $p < 0.01$ for EtOH-PC and NS-PC, respectively) the protective effects of EtOH-PC and NS-PC (Fig. 3L vs. 3K, 3N vs. 3M and Fig. 4B).

TUNEL staining was used to determine apoptotic neuronal death. In the sham-operated group, very few TUNEL-positive cells were observed in the hippocampal CA1 region (Fig. 3Q). TUNEL-positive cells increased significantly ($p < 0.001$) 4 days after I/R (Fig. 3R). Preconditioning with EtOH and NS1619 24 hrs prior to I/R significantly ($p < 0.01$ and $p < 0.001$, respectively) reduced the number of TUNEL-positive cells as compared to those in non-preconditioned, postischemic brain (Fig. 3S, 3U vs. 3R). This protective effect was significantly attenuated by prior administration of paxilline just prior to EtOH-PC or NS-PC (all $p < 0.05$) (Fig. 3T vs. 3S, 3V vs. 3U and Fig. 4C).

Neuroinflammatory mechanisms play a critical role in the brain damage produced by cerebral ischemia, an effect found to be dependent upon recruitment of glial cells (astrocyte and microglia) [42,43]. Immunohistochemical staining with the glial cell-specific stain, GFAP, showed astrocytes with small cell bodies and fine cytoplasmic processes distributed around the hippocampal CA1 area. Few GFAP-positive astrocytes were found in the sham control group (Fig. 5A). However, animals subjected to I/R showed a large increase in reactive astrocytes, which were found in multiple hippocampal layers (Fig. 5B). Counting of GFAP-positive astrocytes within a fixed area in the hippocampus showed that I/R produced a significant ($p < 0.001$) increase in such cells (Fig. 5B vs. 5A and Fig. 6A). Preconditioning with either EtOH or NS1619 resulted in significantly fewer (all $p < 0.001$) reactive astrocytes as compared with the I/R group (Fig. 5C, 5E vs. 5B). Paxilline significantly attenuated the effects of EtOH-PC and NS-PC ($p < 0.05$ and $p < 0.01$, respectively) to reduce postischemic GFAP-positive cells (Fig. 5D vs. 5C, 5F vs. 5E and Fig. 6A).

When brain sections were stained with isolectin B4 to identify microglial cells, very few of these cells were observed in the sham control group (Fig. 5I). However, I/R resulted in a substantial increase ($p < 0.001$) in microglial cells in the CA1 area where pyramidal neuron death was apparent (Fig. 5J vs. 5I). EtOH-PC and NS-PC 24 hrs before I/R resulted in a significant ($p < 0.001$) reduction in isolectin B4-positive cells as compared to that noted after I/R (Fig. 5K, 5M vs. 5J). The decreases in the number of microglial cells in EtOH and NS1619 preconditioned mice were significantly ($p < 0.05$ and $p < 0.01$) attenuated by coincident treatment with paxilline (Fig. 5L vs. 5K, 5N vs. 5M and Fig. 6B).

As noted for the data depicted in Figure 2, there is no additive effect induced by coincident treatment with both EtOH and NS-1619 with regard to protection against postischemic neuronal damage and inflammation (Fig. 3–6).

DISCUSSION

The resistance of the brain and other tissues to ischemic injury can be transiently augmented by prior exposure to non-injurious preconditioning stimuli, most of which trigger two distinct time frames of increased tolerance to the subsequent ischemia [16,44]. The early or acute phase of protection (early EtOH-PC) is apparent 1–2 hrs after ethanol exposure, disappears 2 or 3 hrs later, and induces the development of a less powerful protected phenotype than the late or delayed phase of EtOH-PC. The latter phase re-emerges 24 hrs

after the preconditioning stimulus [16]. Since available evidence indicates that the delayed phase of preconditioning is characterized by a more profound protection than the acute phase [45,46], we chose to focus our present work on this late phase.

Our results clearly demonstrate that moderate ethanol ingestion 24 hrs prior to cerebral I/R exerts preconditioning-like protective effects on the brain as assessed by 1) indices of neuronal degeneration, inflammation, and damage, and 2) cerebral microvascular leukocyte-endothelial adhesive interactions. Preconditioning with the BK_{Ca} channel opener, NS1619, 24 hrs before I/R, mimicked these protective actions of EtOH-PC. The beneficial effects of EtOH-PC and NS-PC to reduce neuronal degeneration, inflammation, and damage were attenuated by prior treatment with paxilline, a BK_{Ca} channel inhibitor. These latter observations suggest that the neuronal and neurovascular protective effects of EtOH-PC may be initiated, in part, by BK_{Ca} channel activation.

Delayed neuronal death in the hippocampus following global ischemia/reperfusion has been attributed largely to apoptosis [47–49]. Our findings were consistent with this conclusion, since most of the CA1 pyramidal neurons after I/R demonstrated TUNEL-positive staining. These changes were inhibited in a paxilline-reversible manner by EtOH-PC and NS1619 administration, indicating that opening of BK_{Ca} channels may mediate the anti-apoptotic effects of EtOH-PC. The precise mechanism whereby BK_{Ca} channel opening may protect against delayed neuronal death is not clear. However, it has been shown that excitatory amino acid release, neuronal calcium overload and reactive oxygen species (ROS) production play a major role in delayed neuronal death following cerebral I/R [47,50,51]. BK_{Ca} channels are expressed in neurons throughout the vertebrate brain [52–54] and a coupling of BK_{Ca} channels with NMDA receptors has been demonstrated in neurons [55]. Most recently, it is reported that BK_{Ca} channel opening inhibits ROS production of isolated rat brain mitochondria and enhances neuronal survival [25]. The opening of neuronal BK_{Ca} channels after EtOH-PC may hyperpolarize the neurons and reduce NMDA receptors mediated Ca²⁺ overload and ROS production during I/R [19,52], thus BK_{Ca} channels act as a brake on the brain to prevent neuronal overexcitation and excessive calcium influx and ROS generation during cerebral I/R. In addition, BK_{Ca} channels have been identified in the inner mitochondrial membrane and the mitochondrial permeability transition pore (PTP) in the inner mitochondrial membrane is considered to trigger the pathway of apoptosis [56]. It was reported that the ischemic preconditioning opened brain mitochondrial BK_{Ca} channels which could close brain mitochondrial PTP [56]. Therefore, it is possible that the opening of neuronal mitochondrial BK_{Ca} channels after EtOH-PC may keep mitochondrial PTP closed thus protecting against DND from apoptosis after cerebral I/R. While this is an attractive hypothesis, the existence of mitochondrial BK_{Ca} channels is controversial. Thus, it is clear that much additional work will be necessary to determine the role of modulation of neuronal cellular calcium levels and mitochondrial PTP by BK_{Ca} channel activation in the protection against I/R-induced DND by EtOH-PC.

In addition to neuronal BK_{Ca} channels, endothelial BK_{Ca} channels in the cerebral microvasculature may be another cellular and molecular target of EtOH-PC that could contribute to neuronal protection. Recent findings have implicated leukocytes in propagating tissue damage after I/R in stroke [35,44]. Therefore, opening of microvascular endothelial BK_{Ca} channels by EtOH-PC, by limiting inflammatory responses in postcapillary venules, could play either a primary or contributory role in the protection against DND secondary to dysfunctional microcirculation after I/R.

Using intravital microscopy, we found that cerebral I/R induced large increases in leukocyte rolling and adhesion in pial venules compared with sham. The increased LR and LA were attenuated by EtOH-PC or antecedent NS1619 administration, and this protection was

reversed by prior treatment with paxilline. Since it has been shown that BK_{Ca} channels are expressed in vascular endothelial cells, but not leukocytes [57–59], our results suggest that activation of endothelial BK_{Ca} channels may play an important role in mediating the protective effect of EtOH-PC in limiting cerebral I/R-induced leukocyte-endothelial adhesive interactions. However, the presence of BK_{Ca} channels in native endothelial cells has recently been challenged [60]. In this regard, it is important to note that astrocytes and neurons, together with endothelial cells, form a functional neurovascular unit that regulates barrier function and vasomotor activity in brain microvessels. Since astrocytes and microglia also express BK_{Ca} channels [56,61], it is possible that they are a target for EtOH or NS1619.

Resident astrocytes and microglia, like neurons and vascular endothelial cells, also responded to EtOH-PC, and such responses were crucial to the foundation of the overall cerebroprotective phenotype. Moderate EtOH-PC interfered with various glial-mediated neurotoxic responses in the slices exposed to neurotoxic A-beta and HIV-1 protein gp120 [62–64]. BK_{Ca} channels have been identified in both astrocytes and microglial cells [56,61]. Astrocytes are one of the components of neurovascular unit and they have been considered as an integral part of the cellular mechanisms that regulate cerebral microcirculation and cerebral blood flow [65]. Therefore, it is possible that the opening of glial BK_{Ca} channels after EtOH-PC regulates cerebral microvascular function to further protect against neuronal damage induced by I/R.

A general consequence of brain inflammation is activation and proliferation of astrocytes and microglia and reactive gliosis [66] and accumulation of activated microglia and reactive astrocytes is observed around degenerating neurons in various inflammatory or degenerative disorders in the central nervous system [67]. The production of inflammatory cytokines in activated glial cells contributed to neuronal damage in the progression of neurodegeneration [68,69]. In our study we found that cerebral I/R produced extensive activation of astrocytes and microglial cells, abundant in the CA1 layer with prominent dying neurons; this was prevented by both EtOH-PC and NS1619, protective effects that were reversed by prior paxilline administration. It is thought that microglial cells are derived from the circulating monocytes through the disrupted blood brain barrier (BBB) in which the number of tight junctions has marked decreased [70]. Therefore, the observation that astrocyte and microglial activation occurs around degenerating neurons in CA1 after I/R is consonant with I/R-induced leukocyte-endothelial adhesive interactions in brain microcirculation and both support the anti-inflammatory property of EtOH-PC in ischemic brain.

Recently, it was reported that NS1619 could induce immediate and delayed preconditioning in primary rat neurons exposed to oxygen-glucose deprivation, hydrogen peroxide, or glutamate excitotoxicity, but the preconditioning protection was not blocked by BK_{Ca} channel inhibitor paxilline [71,72]. This finding in cultured neurons differs from our present *in vivo* findings. In the central nervous system, BK_{Ca} channels are expressed, not only in neurons, but also in resident glial and vascular endothelial cells [56–59,61]. Thus, the apparent discrepancy between our results and those of Gaspar, et al [71,72] might be explained by BK_{Ca} channels in non-neuronal cells mediating the preconditioning effect. Other possible explanations could be differences due to injury model (I/R vs. simulated I/R), interventional environments (*in vivo* vs. cell culture), or species differences between mouse vs. rat. In addition, while it is clear that NS1619 activates BK_{Ca} channels, this compound also displays additional nonspecific effects at higher concentrations, such as inhibiting some Ca²⁺ currents and voltage-activated K⁺ and Na⁺ channels [37,73]. However, the dose of NS1619 we used (0.1 mg/kg body weight) was lower than that reported to induce these off-target effects. Moreover, the effects of NS1619 to induce preconditioning were blocked by treatment with paxilline, suggesting that the effects of NS1619 are due to activation of BK_{Ca} channels in our model.

The molecular mechanisms underlying the effect of ethanol to activate BK_{Ca} channels are not clearly understood. However, discrete binding pockets for ethanol have recently been identified in potassium channels, which may be involved in channel activation [74]. Other studies have found evidence for a modulatory effect of ethanol on certain BK_{Ca} channel subunits [75–78]. Finally, it has been suggested that ethanol may modify ion channel activity indirectly via alterations in membrane fluidity [79,80]. It is also unclear how BK_{Ca} channel activation 24 hrs prior to induction of I/R attenuates postischemic cerebral inflammation and injury. Rather than a direct effect, it seems likely that ethanol-induced BK_{Ca} channel activation stimulates as yet unidentified downstream effectors, which in turn, mediate the protective actions of EtOH-PC during I/R 24 hrs later. Although beyond the scope of the present study, likely candidates include iNOS, HO-1 and other downstream mediators implicated in other forms of preconditioning.

CONCLUSIONS

In summary, this study demonstrated that moderate ethanol consumption produces a protective phenotype in brain microvasculature and neurons against neurovascular inflammation and glial activation induced by cerebral I/R by a BK_{Ca} channel-dependent mechanism in either or both neurons and cerebral microvascular endothelial cells. Moderate consumption of ethanol may induce the development of a protected state wherein brain injury after stroke is minimized. Other pharmacological agents or physiologic stressors that activate BK channels may have therapeutic value for suppressing stroke-mediated damage in brain.

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ABBREVIATIONS

BK_{Ca}, Large conductance	Ca ²⁺ -activated K ⁺ channels
EtOH-PC	Ethanol preconditioning
I/R	ischemia/reperfusion
CCA	common carotid arteries
rCBF	regional cerebral blood flow
CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
LR	leukocyte rolling
LA	leukocyte adhesion
DND	delayed neuronal death
GFAP	glial fibrillary acidic protein
HRP	Horseradish peroxidase
DAB	3,3'-Diaminobenzidine
TUNEL	terminal dUTP nick-end labeling
PX	paxilline
NS	NS1619

CSF	cerebrospinal fluid
BBB	blood brain barrier
EEA	excitatory amino acids
ROS	reactive oxygen species
PTP	permeability transition pore

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Figure 1. Diagram of the experimental protocols assigned to each group. The numbers at *top* in minutes refer to the timeline of the protocol on *day 1*, 24 hrs before ischemia/reperfusion (I/R), *day 2*, the day of I/R, and *day 4*, the day of brain perfusion and fixation. Black triangles indicate administration of saline or drugs. White triangles indicate gavages of water or EtOH. Solid black bars indicate the 20-min period of ischemia during which both CCA had no blood flow. Short hatched bars indicate digital video recording (10 min) and long hatched bars indicate perfusion transcidentally. Two sets of experimental groups, each encompassing all treatments were used: one group was used for intravital microscopic studies of leukocyte rolling and adhesion in pial postcapillary venules at both 2 and 3 hrs of reperfusion. The other group was used for histological examination of hippocampal injury at 4 days of reperfusion. See text for further details and definition of groups.

Figure 2.

Role of BK_{Ca} channels in EtOH-PC 24 hrs prior to ischemia/reperfusion (I/R) on postischemic leukocyte rolling (upper panel) and adhesion (lower panel) determined after 2 and 3 hrs of reperfusion in wild-type C57BL/6J mice. BK_{Ca} channel opener, NS1619 was administrated i. p. 24 hours prior to I/R. BK_{Ca} channel inhibitor, paxilline was administered 10 minutes prior to EtOH-PC and NS1619 administration. Solid and open bars represent data obtained at 2 and 3 hrs of reperfusion, respectively. Values are mean $\pm$ S.E.M. for 6 mice per group. * denotes values statistically different from Sham ($P < 0.05$). # denotes values statistically different from I/R ($P < 0.05$). a denotes values statistically different from EtOH PC+I/R and b denotes values statistically different from NS-PC+I/R ($P < 0.05$).

Figure 3.

Representative micrographs depicting I/R-induced DND (Cresyl violet) and neuronal degeneration (Fluoro-Jade B) and apoptosis (TUNEL) in the hippocampal CA1 subfield of mice subjected to sham operation (A, I and Q), 20 min occlusion of the CCA followed by 4 days reperfusion (I/R) (B, J and R), EtOH-PC 24 hrs prior to I/R (EtOH-PC+I/R) (C, K and S), NS1619 administration 24 hrs prior to I/R (NS-PC+I/R)(E, M and U), paxilline treatment prior to ethanol or NS1619 administration, 24 hrs prior to I/R (PX+EtOH-PC+I/R; PX+NS-PC+I/R) (D, L, T and F, N, V), paxilline alone 24 hrs prior to I/R (PX+I/R) (G, O, W), and both EtOH-PC and NS-PC 24 hrs prior to I/R(EtOH-PC+NS-PC+I/R) (H, P, X). (Magnification, 200x).

Figure 4.

Quantification of viable neurons (Cresyl violet, Panel A), neuronal degeneration (Fluoro-Jade B, Panel B) and apoptosis (TUNEL, Panel C) in the hippocampal CA1 region of mice subjected to sham control, I/R alone (I/R), ethanol preconditioning and NS1619 administration 24 hrs prior to I/R (EtOH-PC+I/R and NS-PC+I/R), and paxilline treatment coincident with ethanol and NS1619 administration 24 hrs prior to I/R (PX+EtOH-PC+I/R and PX+NS-PC+I/R), as well as paxilline alone 24 hrs prior to I/R (PX+I/R) and both EtOH-PC and NS-PC 24 hrs prior to I/R (EtOH-PC+NS-PC+I/R). Values are means $\pm$ S.E.M. for 10 mice per group. *, #, ^a and ^b = values statistically different from corresponding values in sham, I/R, and EtOH-PC+I/R groups, respectively, at $p < 0.05$.

Figure 5. Representative micrographs depicting I/R-induced activation of astrocytes (GFAP) and microglial cells (Isolectin-B4) in the hippocampal CA1 subfield of mice subjected to sham (A, I), I/R (B, J), ethanol and NS 1619 administration 24 hrs prior to I/R (EtOH-PC+I/R and NS-PC+I/R) (C, K and E, M), paxilline treatment coincident with ethanol and NS1619 administration, 24 hrs prior to I/R (PX+EtOH-PC+I/R and PX+NS-PC+I/R) (D, L and F, N), paxilline alone 24 hrs prior to I/R (PX+I/R) (G, O), and both EtOH-PC and NS-PC 24 hrs prior to I/R(EtOH-PC+NS-PC+I/R) (H, P). (Magnification, 200x).

Figure 6.

Quantification of astrocytic (GFAP, Panel A) and microglial activation (Isolectin-B4, Panel B) in the hippocampal CA1 region of mice subjected to sham control, I/R alone (I/R), ethanol and NS1619 administration 24 hrs prior to I/R (EtOH-PC+I/R and NS-PC+I/R), and paxilline treatment coincident with ethanol and NS1619 administration 24 hrs prior to I/R (PX+EtOH-PC+I/R and PX+NS-PC+I/R), as well as paxilline alone 24 hrs prior to I/R (PX+I/R) and both EtOH-PC and NS-PC 24 hrs prior to I/R (EtOH-PC+NS-PC+I/R). Values are means $\pm$ S.E.M. for 10 mice per group. *, #, ^a and ^b = values statistically different from corresponding values in sham, I/R, and EtOH-PC+I/R groups, respectively, at $p < 0.05$.

Forebrain rCBF by laser Doppler measurements immediately after global cerebral ischemia in mice (% value of pre-ischemia). There is a significant decrease in rCBF after ischemia at less than 20% of the initial value of pre-ischemia ($p<0.01$). However, there was no significant differences among the ischemia/reperfusion with or without intervention ($p>0.05$). (Data are expressed as mean $\pm$ S.E.M and $n = 10$).

Table 1

Sham	I/R	EIOH-PC+I/R	PX+EIOH-PC+I/R	NS-PC+I/R	PX+NS-PC+I/R	PX+I/R	EIOH-PC+NS-PC+I/R
100 $\pm$ 0.00	13.90 $\pm$ 1.81	14.80 $\pm$ 1.01	13.80 $\pm$ 1.44	12.50 $\pm$ 1.58	11.70 $\pm$ 2.02	11.40 $\pm$ 1.75	12.70 $\pm$ 1.58

Group	rCBF (% of baseline)
Sham	100
I/R	~14
EIOH-PC+I/R	~15
PX+EIOH-PC+I/R	~14
NS-PC+I/R	~13
PX+NS-PC+I/R	~12
PX+I/R	~11
EIOH-PC+NS-PC+I/R	~13