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Ethanol preconditioning protects against ischemia/reperfusion-induced brain damage: Role of NADPH oxidase-derived ROS

Qun Wang[a], **Albert Y. Sun**[a], **Agnes Simonyi**[c], **Theodore J. Kalogeris**[a], **Dennis K. Miller**[d], **Grace Y. Sun**[c], and **Ronald J. Korthuis**[a,b,*]

a *Department of Medical Pharmacology and Physiology, University of Missouri School of Medicine, Columbia, MO 65212, USA*

b *Dalton Cardiovascular Research Center, University of Missouri School of Medicine, Columbia, MO 65212, USA*

c *Department of Biochemistry, University of Missouri School of Medicine, Columbia, MO 65212, USA*

d *Department of Psychological Sciences, University of Missouri School of Medicine, Columbia, MO 65212, USA*

Abstract

Ethanol preconditioning (EtOH-PC) refers to a phenomenon in which tissues are protected from the deleterious effects of ischemia/reperfusion (I/R) by prior ingestion of ethanol at low to moderate levels. In this study, we tested whether prior (24 hrs) administration of ethanol as a single bolus that produced a peak plasma concentration of 42-46 mg/dl in gerbils would offer protective effects on neuronal damage due to cerebral I/R. In addition, we also tested whether reactive oxygen species (ROS)-derived from NADPH oxidase played a role as an initiator of these putative protective effects. Groups of gerbils were administered either ethanol or the same volume of water by gavage 24 hrs prior to transient global cerebral ischemia induced by occlusion of both common carotid arteries (CCA) for 5 min. In some experiments, apocynin, a specific inhibitor of NADPH oxidase, was administered (5 mg/kg body wt, i.p.) 10 min before ethanol administration. EtOH-PC ameliorated behavioral deficit induced by cerebral I/R and protected the brain against I/R-induced delayed neuronal death (DND), neuronal and dendritic degeneration, oxidative DNA damage, and glial cell activation. These beneficial effects were attenuated by apocynin treatment coincident with ethanol administration. Ethanol ingestion was associated with translocation of the NADPH oxidase subunit, $p67^{phox}$, from hippocampal cytosol fraction to membrane, increased NADPH oxidase activity in hippocampus within the first hour after gavage, and increased lipid peroxidation (4-hydroxy-2-nonenal, HNE) in plasma and hippocampus within the first 2 hrs after gavage. These effects were also inhibited by concomitant apocynin treatment. Our data are consistent with the hypothesis that antecedent ethanol ingestion at socially-relevant levels induces neuroprotective effects in I/R by a mechanism that is triggered by ROS produced through NADPH oxidase. Our results further suggest the possibility that preconditioning with other pharmacological agents that induce a mild oxidative stress may have similar therapeutic value for suppressing stroke-mediated damage in brain.

*__Corresponding author:__ Ronald J. Korthuis, Ph.D., Department of Medical Pharmacology and Physiology, School of Medicine, One Hospital Drive, MA415, University of Missouri-Columbia, Columbia, MO 65212, Phone: (573) 882-8059, Fax: (573) 884-4276, E-mail: KorthuisR@health.missouri.edu.

Keywords

Ethanol preconditioning (EtOH-PC); neuroprotection; NADPH oxidase; reactive oxygen species; apocynin; cerebral ischemia/reperfusion; delayed neuronal death

INTRODUCTION

The results of a large number of epidemiologic studies indicate that consumption of red wine at low to moderate levels is cardioprotective, reducing the incidence and severity of myocardial infarction and stroke (1-3). Studies conducted in cultured cell and intact animal models attributed these beneficial effects to the polyphenolic antioxidants present in red wine (4-7). However, there is evidence that moderate consumption of ethanol alone also exerts protective effects against injury due to cerebral and myocardial ischemia (8-12). Furthermore, ethanol ingestion 24 hrs prior to ischemia/reperfusion (I/R) (ethanol preconditioning or EtOH-PC) completely prevented postischemic leukocyte-endothelial cell adhesive interactions by a mechanism that involves the ability for ethanol to activate an oxidant-dependent signaling pathway (13).

NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase) is an important enzymatic source for the generation of reactive oxygen species (ROS) that damage cells in a variety of pathologic conditions, including I/R-induced injury in the brain and other tissues (14,15). However, it is becoming increasingly apparent that these reactive molecules can also participate in a number of normal physiologic phenomena (16,17). Indeed, we have demonstrated a role for ethanol-induced NADPH oxidase-derived oxidants in the appearance of anti-adhesive and anti-inflammatory phenotype in postcapillary venules that becomes apparent on subsequent exposure to I/R (13,18,19). However, whether ethanol also exerts neuroprotective effects in stroke is unknown.

In this study, we determined whether antecedent ethanol ingestion would confer protection against I/R in the gerbil brain and whether ROS derived from NADPH oxidase played a role as an initiator of these putative protective effects. Although NADPH oxidase has been identified in the cerebrovascular system (20-22) and in brain of the mouse and rat (23,24), the presence of this oxidant-producing enzyme in the gerbil brain has not been evaluated. Therefore, this study included characterization of the NADPH oxidase subunits in hippocampal region of the gerbil brain.

MATERIALS AND METHODS

Production of ethanol preconditioning

Adult male Mongolian gerbils (60-80 g body wt) (Charles River, Wilmington, MA) were housed in the Small Animal Facilities of the University of Missouri-Columbia that was maintained at 22 ± 2°C with constant humidity under a 12:12 hrs light:dark cycle, and were allowed free access to water and lab chow. Experiments were carried out in accordance to the guidelines set forth by the NIH Guide for the Care and Use of Laboratory Animals.

Ethanol preconditioning (EtOH-PC) was induced by gavaging animals with a moderate dose of ethanol (volume ethanol in µl calculated from the relation: body weight (in g) × 0.6] + 0.3) 24 hrs before ischemia, as we have previously described (13,18). This volume of ethanol (95% in µl) was mixed in 0.3 ml of sterile distilled water before gavage. Animals in the sham control group (no I/R) and the I/R alone group (no EtOH-PC) received similar volume of sterile distilled water by gavage. After administration, all animals were returned to their cages, and were provided free access to food and water.

Induction of global forebrain ischemia

Twenty-four hrs after ethanol or distilled water administration by gavage, 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. Transient global cerebral ischemia was induced by occlusion of both common carotid arteries (CCA) for 5 min and followed by reperfusion as described previously (5). Sham-operated animals underwent similar procedures except CCA were not occluded. To eliminate the complicating effects on cerebral blood flow changes that occur in the subset of gerbils with communicating arteries, regional cerebral blood flow (rCBF) was monitored before and after bilateral clamping the CCAs using a laser Doppler blood flow monitor (MBF3D, Moor Instruments, Devon, UK). After CCA occlusion, gerbils showing a decrease in rCBF of less than 80% were excluded from subsequent analyses (5). After surgical procedures, animals were allowed to regain consciousness and maintained in the Small Animal Facility with free access to food and water for four days. The animal protocol was approved by the University of Missouri-Columbia Animal Care and Use Committee (Protocol #1741).

Preparation of brain samples

Four days after ischemia, gerbils were anesthetized by inhaling 2.5% isoflurane and were transcardially perfused with heparinized saline followed by fixation with 4% (w/v) paraformaldehyde in 0.05 M phosphate buffered saline (PBS, pH 7.4). Brains were post-fixed for 3 days and embedded in paraffin. Six μm coronal sections were prepared from the dorsal hippocampus as described previously by Wang et al (5).

Identifying NADPH oxidase subunits in gerbil brain

p47^{phox} and p67^{phox}, two of the main cytosolic subunits of NADPH oxidase, were used as markers to identify NADPH oxidase in gerbil brain. p47^{phox} and p67^{phox} immunoreactivities were measured in coronal brain sections from the dorsal hippocampus in normal gerbils as previously described by Wang et al (5). Mouse anti-p47^{phox} and p67^{phox} antibodies (1;100 dilution, Upstate, Charlottesville, VA) were used as primary antibodies and Alexa Fluor 568 goat anti-mouse IgG was used as the secondary antibody (1:200 dilution, Molecular Probe, Eugene, OR). To assess specific staining, alternate sections were incubated with PBS instead of the primary antibody as the negative control.

Experimental protocols

In order to characterize the time course for changes in plasma ethanol concentration and lipid peroxidation in gerbils (n = 6 for each time point), fresh plasma and hippocampi were collected from gerbils prior to and at 30 min, 1 hr and 2 hrs after ethanol administration by gavages.

In the second study, apocynin, a selective inhibitor of NADPH oxidase, was tested for its ability to inhibit NADPH oxidase activation (cytosolic subunit translocation)(25), activity (NADPH consumption)(25,26), and lipid peroxidation (HNE production)(27) in plasma and brain during the first two hours after ethanol ingestion. Gerbils were randomly divided into 3 groups; namely, control, EtOH alone, and EtOH plus apocynin pretreatment. Apocynin (Sigma, St Louis, MO) was dissolved in normal saline and administered by i. p. injection (5 mg/kg body wt) 10 min before administering ethanol or distilled water by gavage. Plasma and hippocampal samples were collected at 1hr and 2hrs after administration of ethanol or water.

To assess the neuroprotective effects of antecedent ethanol ingestion and the role of NADPH oxidase in these responses, gerbils were randomly divided into 4 groups: sham control (control, no I/R), I/R alone, antecedent ethanol ingestion 24 hrs prior to ischemia/reperfusion (EtOH-PC+I/R), and EtOH-PC coincident with apocynin treatment 24 hrs prior to induction of I/R

(Apo+EtOH-PC+I/R). Apocynin was administered by intraperitoneal injection (i.p., 5 mg/kg body wt) 10 min before ethanol ingestion. Gerbils in the control, I/R alone (no EtOH-PC) and EtOH-PC+I/R groups received i.p.injections of saline at the volume and time as in the Apo +EtOH-PC+I/R group.

Measurement of ethanol concentration in plasma

Ready-to-use Q.E.D. A150 Alcohol Test kit (OraSure Technologies, Bethlehem, PA) was used to quantify alcohol levels in plasma samples obtained from gerbils under control conditions and 30 min, 1hr and 2hrs after ethanol administration by gavage. Prior to the study, we verified the accuracy and linearity of the readout over a range of ethanol concentrations (10 to 145 mg/dl or 0.01-0.145 mg %).

Measurement of lipid peroxidation in plasma and hippocampus

Generation of 4-hydroxy-2-nonenal (HNE) in plasma and in hippocampus was used as an indication of lipid peroxidation (27). Briefly, at different time points after ethanol ingestion, blood was collected in EDTA vacutainer tubes and centrifuged briefly to remove blood cells. Hippocampi (~100mg) from both sides of the cerebrum were removed and homogenized in buffer (20 mM HEPES, pH 7.5, 0.25 M sucrose, 10 mM KCI, 1.5 mM $MgCl_2$ 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol). HNE in plasma and the hippocampus homogenates were detected using the Bioxytech HAE-586 spectrophotometric assay kit (OxisResearch, Portland, OR) as described by Wang et al. (15). Calculation of HNE in plasma or hippocampus was performed based on the standard curve generated by HNE standard and was expressed as μmol/dl in plasma or μmol/mg protein in hippocampus, respectively.

Western blotting to assess translocation of NADPH oxidase subunits

For preparation of membrane and cytosolic fractions, both sides of hippocampus were dissected and homogenized in 1 ml of homogenization buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 200 mM sucrose). The nuclei and cell debris were removed from the homogenate by centrifugation at 900 × g for 10 min at 4 °C. The resulting supernatant was centrifuged at 110,000 × g for 75 min at 4 °C. The resulting supernatant was collected as cytosolic fraction and the membrane pellet was solubilized in buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% Triton X-100) for 1 hr at 4 °C. Both of the membrane and cytosolic fractions were stored at −70 °C until use.

40 μg membrane or cytosolic proteins were subjected to SDS-PAGE. The separated proteins were transferred by electrophoresis to polyvinylidine difluoride membranes. The membranes were incubated with anti-$p67^{phox}$ antibody or anti-$gp91^{phox}$ antibodies (1:1,000 dilution; Upstate Biotechnology) at 4°C overnight and subsequently with a horseradish peroxidase-linked secondary antibody (1:5,000 dilution; Sigma) at room temperature for 90 min. The positive bands were revealed using Western blotting detection reagents (Pierce, Rochford, IL) and autoradiography film. The intensities of the immunoblot bands were quantified using the Quantity One software (Bio-Rad).

Measurement of NADPH oxidase activity in hippocampus

For measurement of NADPH oxidase activity, hippocampi from both side of the brain were homogenized together in Krebs-Ringer phosphate buffer at pH 7.4 (120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 2.2 mM $CaCl_2$, 0.1 M phospate buffer) with protease inhibitor cocktail (Sigma). Homogenates were centrifuged at 500 × *g* for 5 min at 4°C and the pellet discarded. Supernatants were spun at 100 000 × *g* for 1 hr at 4°C. Cytosolic (supernatant) and membrane (pellet) fractions were separated. The pellet was resuspended in 100 μL of buffer and kept at −70°C until analysis.

NADPH oxidase enzymatic activity was determined in membrane fraction using the protocol as described Wei and Whaley (25,26). Briefly, aliquots of the membrane fractions (30 μg proteins) were incubated with NADPH (100 μM) at 37°C. NADPH activity was determined by measuring the conversion of NADPH to NADH using a Radical Detector kit (Cayman Chemical) and a plate reader spectrophotometer (450 nm) at every 10 min. NADPH oxidase activity was normalized by the amount of protein and the increase in optical density between 10-20 min. Activity was calculated as mOD/μg protein/min.

Assessment of locomotor activity

Locomotor activity in gerbils was assessed using a procedure described previously (28,29). Locomotor activity was monitored automatically by the Med Associates Open Field Test Environments (ENV-515) (Georgia, VT). Each environment consisted of a 16 × 16 horizontal grid of infrared sensors and a bank of 16 vertical sensors. The sensor grids surrounded an acrylic cage (43.2 × 43.2 × 30.5 cm) and each sensor grid and cage was housed in a large sound-resistant cubicle. 24 hrs after ischemia, gerbils (n = 7 – 9 gerbils/group) were weighed and placed in the apparatus for 30 min. Data were collected in 5-min intervals by the Med Associates Open Field Activity Software (SOF-811), which recorded the number of sensor breaks and subsequently computed these data as distance traveled.

Histochemical and immunohistochemical staining for neurons and glial cells

Brain sections were stained with cresyl violet for neurons and immunohistochemical labeled with glial fibrillary acidic protein (GFAP) antibody for astrocytes according to the protocol described by Wang et al (5). Rabbit anti-human GFAP antibody (1:100 dilution, Sigma, St. Louis, MO) was used as primary antibody and Texas Red-conjugated goat-anti-rabbit IgG was used as secondary antibody (1:200 dilution; Jackson ImmunoResearch, West Grove, PA). Microglial cells were identified using FITC labeled isolectin B4 (20 μg/ml, Sigma, St. Louis, MO) according to the protocol outlined by (30) with modifications (5). The fluorescent dye, 4′, 6-diamidine-2′-phenylindole (DAPI) (0.1 μg/ml, Roche, Molecular Chemicals, Mannheim, Germany) was used to counter-stain the sections for identification of nuclear DNA in cells.

Fluoro-Jade B and MAP-2 staining for assessment of neuronal degeneration

Fluoro-Jade B is a 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 an excellent marker for detecting degenerating neurons. Fluoro-Jade B staining was performed according to the protocol described by Schmued et al (31).

MAP-2 is the major microtubule associated protein in brain tissue and MAP-2 is useful for identifying cytoskeletal structures in neurons. A mouse monoclonal antibody to MAP-2 (1:100, Sigma, St. Louis, MO) was used to mark the dendritic structures according to the protocol described by Monnerie et al (32). Alexa Fluor 568 goat anti-mouse IgG was used as the secondary antibody (1:200 dilution; Molecular Probe, Eugene, OR) and DAPI counter-staining was used to identify nuclear DNA damage in cells.

Detection of oxidized DNA by 8-OHdG immunohistochemistry

Formation of 8-OHdG (8-hydroxyl-deoxyguanosine) is regarded as a hallmark of oxidative DNA damage (33). A time course study by Hwang et al (34) showed an increase in immunoreactivity of 8-OHdG in the gerbil hippocampal CA1 region, with peaks at 12 hrs and 4 days after ischemic insult. In this study, determination of 8-OHdG was carried out with brain sections obtained at 4 days after I/R according to the protocol described previously by Wang et al (28). Briefly, brain sections were immunostained with mouse anti-8-OHdG antibody

(diluted at 5 μg/ml, OxisResearch, Portland, OR) followed by goat anti-mouse Ig G labeled with horseradish peroxidase (HRP) (dilution 1: 200, Sigma, St. Louis, MO).

Quantitative assessment and statistical analysis

Neuronal damage, degeneration, glial activation and oxidative DNA were quantified by counting the number of live neurons and fluorescent or immuno-reactive positive cells of Fluoro-Jade B, GFAP, Isolectin-B4, 8-OHdG in the middle of a defined CA1 area (0.17 μm × 0.54 μm) in both sides of the hippocampi per high magnification field (magnification 400x) using the Bioquant Image Analysis System (Bioquant True Color Windows 95 Software Version 2.50, Nashville, TN) and the Mata Imaging Serials (Version 6.1, Molecular Devices Corporation, Downingtown, PA). The average values of live neurons and the above positive stained cells were obtained from left and right hippocampus of each gerbil.

The dendritic degeneration of neurons was assessed by quantifying the average intensity of the MAP-2 positive staining in the same defined area (0.17 μm × 0.54 μm) below the middle of CA1 in both sides of the hippocampi under the same magnification field (400x) using the Mata Imaging Serials (Version 6.1, Molecular Devices Corporation, Downingtown, PA.

Data (mean ± SEM) were subjected to a one-way ANOVA, followed by the Newman-Keuls post-hoc test using the GraphPad Prism program version 4.0. A p-value of less than 0.05 was considered to indicate significant difference.

In the assessment of locomotor activity, distance traveled data were analyzed via two-way repeated-measures ANOVA with treatment group as a between-groups factor and session time as a within-subjects factor. Tukey post-hoc tests were performed when appropriate ($p < 0.05$).

RESULTS

Plasma ethanol concentration

Using the same ethanol treatment regimen as described by Yamaguchi et al (18), we examined blood ethanol concentrations in gerbils. As shown in Fig. 1, ethanol administration resulted in an increase in blood ethanol concentration that reached a peak level of 42-46 mg/dl at 30-60 min after ingestion and returned to control levels 2 hrs after consumption. Thus, ethanol administration to gerbils by our dosing protocol produced an increase in plasma ethanol concentration similar to that noted in our earlier studies in mice (18).

Detection of p47^{phox} and p67^{phox} immunoreactivity in the gerbil brain

Neuronal immunopositive staining for p47^{phox} and p67^{phox} were observed in both the cerebral cortex and hippocampus (Fig. 2). Replacement of primary antibodies with PBS did not exhibit any immunoreactivity, confirming the specificity of the p47^{phox} and p67^{phox} antibodies used in this experiment. In the cortex, p47^{phox} and p67^{phox} immunoreactivities were distributed in all cortical layers but were especially prominent in layer V (Fig. 2A and 2E). In the hippocampus, p47^{phox} and p67^{phox} immunoreactivities were prominently observed in the pyramidal neurons in CA1 and CA3 regions (Fig. 2B, 2C, 2F and 2G) and also in the molecular and polymorphic layers of dentate gyrus (DG) (Fig. 2D and 2F). These observations provide the first description of immunoreactivity of NADPH oxidase subunits in the gerbil brain.

Apocynin inhibited lipid peroxidation after EtOH-PC

Administration of a moderate dose of ethanol resulted in a transient increase in plasma HNE levels and reached a peak at 1 hr (Fig. 3A). This time course is in agreement with previous studies showing lipid peroxidation in plasma at 1-1.5 hrs following ethanol exposure (35). Ethanol administration also caused an increase in HNE levels in the hippocampus but unlike

that in plasma, the levels of HNE in hippocampus remained elevated 2 hrs after ethanol administration (Fig. 3B). Apocynin pretreatment (i.p. 5 mg/kg body wt) 10 min before ethanol administration significantly attenuated the increases in both plasma (Fig. 3C) and hippocampal HNE (Fig. 3D) determined at 1 hr after ingestion, suggesting that increased ROS production due to ethanol administration was generated by NADPH oxidase. Pilot experiments using HPLC analysis demonstrated the ability of apocynin to cross the blood brain barrier (BBB) and reached the brain within 30 min after i.p. injection (data not shown).

Effects of apocynin on hippocampal NADPH oxidase subunit translocation and enzyme activity after EtOH-PC

NADPH oxidase activation requires translocation of cytosolic subunits to the plasma membrane (16,17). To further determine whether NADPH oxidase contributed to EtOH-PC-induced ROS production, Western blotting of $p67^{phox}$ and $gp91^{phox}$ was used and a significant increase in $p67^{phox}$ expression in the membrane fraction and a decrease in the cytosolic fraction at 1hr after ethanol administration (Fig. 4A and 4B). The ethanol effects were were inhibited by apocynin administration 10 min before ethanol administration. These results suggest that $p67^{phox}$ subunit translocation from cytosol to membrane occurred in response to ethanol administration, and that this translocation was inhibited by the NADPH oxidase inhibitor, apocynin. The plasma membrane $gp91^{phox}$ subunit was strongly expressed in hippocampus but was not significantly affected by ethanol and apocynin treatment (Fig. 4A and 4C).

Measurement of NADPH oxidase activity in hippocampal membrane fraction showed a significant increase at 1 hr after EtOH administration and this effect was also significantly reduced by apocynin pretreatment (Fig. 4D). The increases in $p67^{phox}$ translocation and NADPH oxidase activity after EtOH administration and their reduction by apocynin pretreatment are positive data supporting the notion that NADPH oxidase contributed to EtOH-PC-induced ROS in brain.

EtOH-PC ameliorated I/R-induced hyperactivity that was attenuated by coincident apocynin treatment

A widely used behavioral test in animal models of stroke involves assessment of locomotor hyperactivity (Fig. 5). Analysis of distance traveled data revealed a significant main effect of session time ($F(5,162) = 64.53$, $p < 0.001$) and treatment group ($F(3,162) = 9.07$, $p < 0.001$). The interaction of session time by treatment group was not significant ($F(15, 162) = 0.825$, $p = 0.65$). Post-hoc tests determined that there was greater activity for gerbils that received ischemia (mean = 10,624 cm, S.E.M. = ±2185 cm) than for gerbils in the sham condition (mean = 7922 cm, S.E.M. = ±644 cm). There was no significant difference in activity between the gerbils that received apocynin pretreatment (mean = 12,037 cm, S.E.M. = ±1648 cm) and gerbils that received ischemia. However, there was significantly less activity for gerbils that received ethanol (mean = 7771 cm, S.E.M. = ±929 cm) than for gerbils that received only ischemia. There were no differences between the gerbils that received ethanol and gerbils under sham conditions.

EtOH-PC induced neuroprotection in postischemic hippocampus was attenuated by coincident apocynin treatment

Cresyl violet staining revealed healthy neurons in the hippocampal CA1 area in the sham control group (Fig. 6A and 7B) and dead neurons 4 days after I/R (Fig. 6B and 1A). Fluoro-Jade B staining demonstrated very few degenerated neurons in the hippocampal CA1 region in sham control (Fig. 6E and 7B), but neuronal degeneration was markedly increased 4 days after I/R (Fig. 6F and 7B). Similar to description by Caceres et al. (36), we showed that the cytoskeletal protein MAP-2 is extensively localized in dendrites of hippocampal neurons. In the sham control group, immunoreactivity of MAP-2 was clearly visualized in the dendrites

of pyramidal neurons in the hippocampal CA1 area (Fig. 6I and 7C) but was decreased significantly after I/R (Fig. 6J and 7C). When ethanol was administered 24 hrs before CCA occlusion, I/R-induced delayed neuronal death (DND) and neuronal and dendritic degeneration in the hippocampal CA1 area were significantly reduced as compared to the I/R group without prior ethanol treatment (Fig. 6C vs. 6B, 6G vs. 6F, 6K vs. 6J and Fig. 7A,B,C). Apocynin administration 10 min prior to ethanol ingestion significantly attenuated the neuroprotective effects of EtOH-PC in I/R (Fig. 6D vs. 6C, 6H vs. 6G, 6L vs. 6K and Fig. 7A,B,C).

Apocynin inhibited EtOH-PC-induced neuroprotection against glial activation and DNA oxidation after I/R

Immunohistochemical staining of GFAP showed few GFAP-positive astrocytes in the sham control group (Fig. 8A and 9A) but a large increase was observed in the I/R group (Fig. 8B and 9A). When brain sections were stained with isolectin B4 to identify microglial cells, very few microglial cells were observed in the sham control group (Fig. 8E and Fig. 9B). However, there was a substantial increase in the number of microglial cells in both the hippocampal CA1 area and in the surrounding area after I/R (Fig. 8F and Fig. 9B). EtOH-PC 24 hrs prior to I/R significantly reduced the number of reactive astrocytes and microglial cells as compared to I/R group (Fig. 8C vs. 8B, 8G vs. 8F and Fig. 9A and 9B). Apocynin administration 10 min before EtOH-PC significantly attenuated the inhibitory effects of EtOH-PC on activation of astrocytes and microglial cells after I/R (Fig. 8D vs. 8C, 8H vs. 8G and Fig. 9A and 9B).

I/R-induced increases in DNA oxidation was assessed by immunohistochemical determination of 8-OHdG. In this study, weak 8-OHdG immunoreactivity was observed in the CA1 region of the sham control group (Fig. 8I and Fig. 9C) but was significantly increased by I/R (Fig. 8J). EtOH-PC significantly reduced the I/R-induced increase in 8-OHdG (Fig. 8K vs. 8J and Fig. 9C). Administration of apocynin prior to EtOH-PC significantly attenuated the ability of antecedent ethanol to reduce postischemic DNA oxidation (Fig. 8L vs. 8K and Fig. 9C).

DISCUSSION

Results from the present study demonstrate for the first time that moderate ethanol ingestion 24 hrs prior to global cerebral I/R exerts preconditioning-like protective effects, ameliorating postischemic behavioral deficit and reducing postischemic DND, neuronal and dendrite degeneration, oxidative DNA damage, and glial cell activation in the hippocampus. We further demonstrate that this moderate dose of ethanol is sufficient to cause a mild oxidative stress during the period of ethanol exposure as indicated by the increases in lipid peroxidation (HNE levels) and NADPH oxidase activity in the hippocampus concomitant with plasma ethanol elevation after ingestion. Moreover, treatment with the NADPH oxidase inhibitor apocynin 10 min prior to ethanol ingestion not only suppressed the ethanol-induced ROS production with increased NADPH activity, but also attenuated the beneficial actions of EtOH-PC to reduce postischemic DND, neuronal and dendrite degeneration, oxidative DNA damage, glial cell activation and behavioral deficit. Thus, our results also provide the first evidence that antecedent ethanol ingestion initiates a protective response in the brain that attenuates postischemic neurodegenerative processes by an oxidant-dependent triggering mechanism. Although NADPH oxidase has been recently identified as a major source of ROS production in the cerebrovascular system (20-22) and in brain of mouse and rat (23,24), this study provides the first evidence for the presence of this oxidant-producing enzyme and action in the gerbil brain. Staining for NADPH oxidase subunits was observed in neurons in all regions of the gerbil brain, and particularly prominent in the hippocampus and cortex. This pattern of expression is similar to the distribution of NADPH oxidase reported in mouse and rat brain (23,24). The expression of NADPH oxidase subunits in neurons suggests the possibility that

superoxide produced by NADPH oxidase may play a role in normal as well as pathological function in the brain.

Several pharmacological inhibitors of NADPH oxidase are available including PR-39, diphenyliodonium, and apocynin. In prior studies, we have used PR-39 as a mechanistic probe for examining the role of NADPH oxidase-derived oxidants in the development of the anti-inflammatory phenotype induced by ischemic and ethanol preconditioning (13,37). However, this agent is now known to produce a variety of other effects, in addition to inhibition of NADPH oxidase, which relate to its ability to enter cells and bind to SH3 domain-containing proteins such as $p130^{Cas}$ (38), an adapter protein that plays a role in several signaling pathways involved in cell survival (39). More relevant to preconditioning is the fact that it is now known that PR-39 inactivates NF-κB (40), a transcription factor that plays an essential role in the infarct-sparing effects of delayed ischemic preconditioning by promoting the expression of inducible NOS (iNOS) (41-43). Diphenyliodonium has been employed as an NADPH oxidase inhibitor in a large number of studies, but this agent is not specific and known to interfere with activities of flavor-proteins including endothelial nitric oxide synthase (eNOS) and mitochondrial respiratory chain activity (44). The latter effect is important because we have obtained strong evidence supporting a role for eNOS-derived NO as a trigger for the development of the anti-inflammatory effects of ethanol preconditioning (18,45,46).

In light of these non-specific effects of PR-39 and diphenyliodonium, we considered the use of other agents that inhibit NADPH oxidase activity for investigating the role of this enzyme in late EtOH-PC. Apocynin is a naturally occurring compound that effectively inhibits NADPH oxidase by reacting with thiol groups required for enzyme subunit assembly (15). The suggested action is in agreement with our observation that apocynin inhibited the translocation of cytosolic $p67^{phox}$ subunit to membrane. Apocynin is derived from the rhizome of the Himilayan herb *Picrorhiza kurroa* and has been used as a treatment for many inflammatory conditions for centuries (47). In contrast to PR-39 and diphenyliodonium, apocynin does not scavenge xanthine oxidase-derived superoxide, inhibit eNOS, or prevent NF-κB activation (40,48-50). Coincident treatment with apocynin during the period of EtOH-PC attenuated the mild oxidative stress induced by ethanol ingestion, an effect that was associated with a concomitant reduction in NADPH activity that occurred during the time plasma ethanol rises and falls. Furthermore, apocynin treatment coincident with ethanol administration 24 hrs prior to induction of cerebral ischemia also markedly abrogated the postischemic neuroprotective action of EtOH-PC. These results suggest that moderate ethanol ingestion triggers entrance into this protected state through the formation of NADPH oxidase-derived oxidants during the period of ethanol exposure 24 hrs prior to I/R.

Some studies have correlated changes in cerebral I/R-induced biochemical indices of injury with neurological outcome in gerbils (51). Global cerebral ischemia in the gerbils produced a significant increase in locomotor activity which was correlated with a severe loss of hippocampal CA1 neurons after I/R (52). Our previous study demonstrated an increase in locomotor activity in gerbils after I/R, a behavorial effect of stroke that was reduced by curcumin treatment, a polyphenolic compound that also ameliorated hippocampal delayed neuronal death (29). We now extend these observations to the protective actions of EtOH-PC, which markedly inhibited the hyperlocomotion induced by I/R. Importantly, the reduction in the I/R-induced hyperactivity response by antecedent ethanol ingestion was abolished by coincident apocynin pretreatment.

It is important to note that while apocynin markedly decreased the oxidative stress induced by ethanol exposure and attenuated the neuroprotective effects that become apparent upon exposure to cerebral I/R, NADPH oxidase inhibition did not completely prevent these effects. It is possible that oxidative stress occurs secondary to ethanol metabolism by alcohol

dehydrogenase and cytochrome P4502E1 could also play a role in triggering the development of EtOH-PC. However, the fact that nitric oxide (NO) inhibits the catalytic activity of both alcohol dehydrogenase and cytochrome P4502E1 (53,54), when coupled with our observation that NO is formed during the period of ethanol preconditioning (18,45,46), argues against a role for either enzyme as additional sources of ROS in the EtOH-PC effects. Xanthine oxidase activity is increased by ethanol, although much higher ethanol doses were used to demonstrate this effect than were employed in the present study (13,55-57). Nevertheless, xanthine oxidase has been implicated as an important instigating event for the development of the postischemic anti-inflammatory phenotype noted in postcapillary venules 24 hrs after ethanol ingestion (13). This may relate to the fact that xanthine oxidase produces superoxide when metabolizing acetaldehyde, the major product of ethanol catabolism (13,57). It is also possible that production of mitochondrial oxidants at sites I and III contribute to the oxidative stress that precipitates the development of the neuroprotective phenotype in response to antecedent ethanol. This possibility is supported by the observation that ethanol stimulates the production of ROS in hepatocytes through mitochondria at complexes I and III (13,58,59), but again at ethanol concentrations higher than achieved in the present study. Although it is unclear whether this also occurs in the brain, especially at the concentrations achieved in our study, it is important to note that apocynin does not inhibit ischemia-induced mitochondrial oxidant production (13,60).

Excessive production of ROS is an important event underlying cerebral I/R injury (61,62) and the damaging effects can be ameliorated by administration of botanical antioxidants, including resveratrol from grape skin and curcumin from turmeric (5,29). We recently demonstrated that apocynin similarly attenuated postischemic neuronal damage in the same gerbil stroke model (15). These findings provide additional support for the notion that ROS contribute to the genesis of I/R injury and indicate that that NADPH oxidase may be an important source of cytotoxic oxidants in stroke (15). Chronic excessive ethanol ingestion is also known to cause a number of pathophysiological manifestations that arise secondary to oxidative damage (6,63). In light of this work, our demonstrated role for ethanol-induced oxidant formation by NADPH oxidase as an inaugural event that precipitates the development of a neuroprotective phenotype in I/R might be viewed as surprising. However, since the dose of ethanol used in this study raises plasma ethanol to a peak concentration that is only ~50% of that required for legal intoxication, it seems reasonable to hypothesize that this moderate dose of ethanol produces only slight fluctuations in redox status that allow NADPH oxidase-derived ROS to subserve a signaling function as opposed to the biomolecular damage induced by the much higher oxidant flux that is produced during I/R or after excessive ethanol exposure. Indeed, previous studies have demonstrated that short bouts of preconditioning ischemia and reperfusion (ie, ischemic preconditioning) protect against the deleterious effects induced by subsequent exposure to prolonged I/R in stroke models (64,65). More recent work has demonstrated that these protective effects are triggered by oxidants generated during ischemic preconditioning (66). In addition, we have previously demonstrated that ethanol preconditioning induces the development of an anti-inflammatory phenotype in postcapillary venules by a mechanism involved oxidants generated during the first hour after ethanol ingestion [13,19]. Taken together, our studies indicate that NADPH-oxidase derived oxidants can play dual and opposing roles in I/R, serving to produce neuronal injury in brains exposed to I/R alone and by acting as important initiators in the signaling cascade that is activated by antecedent ethanol ingestion to produce a neuroprotective phenotype.

Identification of the downstream signaling elements that are activated by NADPH oxidase-derived ROS formed during the period of ethanol exposure and entrance into the preconditioned, neuroprotective state that becomes apparent when I/R is induced 24 hrs later are unknown. However, studies conducted by our group that have focused on the mechanisms whereby antecedent ethanol induces the development of an anti-inflammatory phenotype such

that postcapillary venules fail to support leukocyte adhesion on subsequent exposure to I/R suggest the role for NO/superoxide interactions, release of calcitonin gene-related peptide, and activation of specific protein kinase C isoforms (13,18,45,46,67,68). These observations, when coupled with the demonstration that leukocyte infiltration plays a critical role in the pathogenesis of cerebral I/R injury (69-72), suggest that these signaling elements may contribute to the protective actions of antecedent ethanol ingestion. In addition, mitochondrial dysfunction during cerebral I/R is well established as a contributor to stroke-induced injury (61,73,74), and amelioration of mitochondrial dysfunction reduces neuronal apoptosis and behavioral deficits after cerebral I/R (29). Clearly, much additional work will be required to identify the role of mitochondria and downstream mediators and ultimate effectors of the neuroprotective actions of antecedent ethanol in cerebral I/R.

The experimental design for the present study was based on earlier work by our group which demonstrated that ethanol ingestion 24 hrs prior to I/R completely prevented postischemic P-selectin expression and leukocyte/endothelial cell adhesion in postcapillary venules by an oxidant-dependent mechanism (13,18,75). The idea that moderate ethanol consumption induces a mild oxidative stress that provokes the development of a neuroprotective state in I/R is consistent with previous reports demonstrating that moderate ethanol consumption can limit the neurotoxicity and apoptosis induced by HIV-1 protein gp-120 and amyloid-β, respectively (76,77). These studies suggest that the neuroprotective effects of moderate ethanol consumption may be of therapeutic value in other neurological disorders. However, we are not advocating the use of ethanol as a neuroprotective intervention. Rather, we suggest that elucidation of the underlying mechanisms that account for its remarkable protective actions may provide a rationale for the development of new treatment modalities that mimic its protective actions but avoid the untoward side effects associated with even moderate ethanol consumption.

In summary, the present study provides the first anatomical evidence for the presence of NADPH oxidase in gerbil brain and that antecedent ethanol ingestion 24 hrs prior to induction of cerebral I/R attenuates postischemic neuronal injury and behavioral deficit. Moreover, our work suggests that ethanol-induces the formation of ROS derived from NADPH oxidase occurs during the period of ethanol exposure, which serve a critical signaling function to inaugurate entrance into a preconditioned state that affords significant neuroprotection on subsequent exposure to I/R 24 hrs after ethanol ingestion.

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Abbreviations

EtOH-PC
: Ethanol preconditioning

ROS
: reactive oxygen species

HNE
: 4-hydroxy-2-nonenal

NADPH oxidase
: nicotinamide adenine dinucleotide phosphate-oxidase

CCA common carotid arteries

rCBF regional cerebral blood flow

BBB blood brain barrier

I/R ischemia/reperfusion

DND delayed neuronal death

GFAP glial fibrillary acidic protein

MAP-2 microtubule associated protein-2

8-OHdG 8-hydroxyl-deoxyguanosine

DAPI 4′,6-diamidine-2′-phenylindole

eNOS endothelial nitric oxide synthase

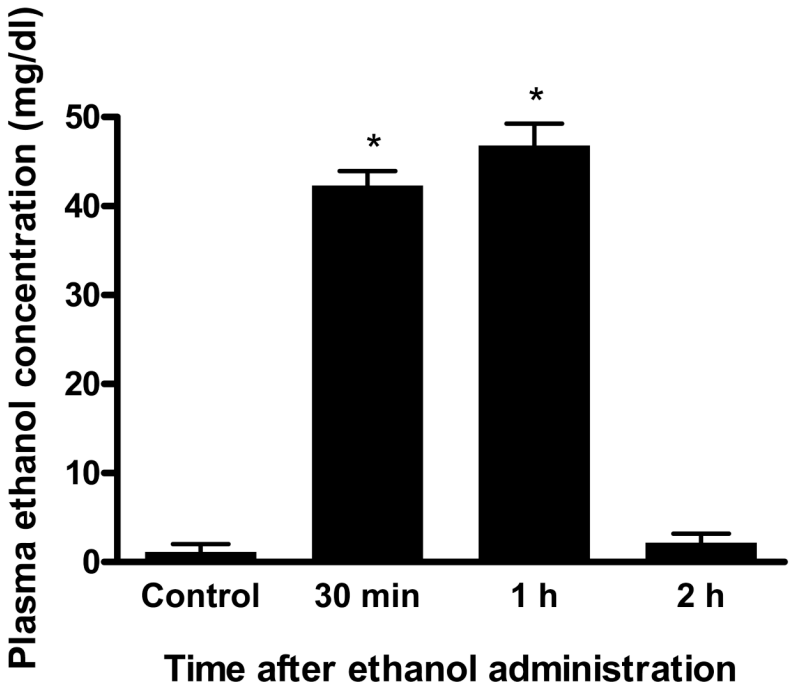

Figure 1.

Changes of plasma ethanol concentrations in gerbils after ethanol administration by gavage (n=6 for each time point). The volume (in µl) of 95% ethanol instilled in each animal was calculated as follows: [body weight (in g) × 0.6] + 0.3), and was mixed in 0.3 ml sterile distilled water just before administration as a single bolus by gavage. *Values that are statistically different from corresponding values obtained during the control period (before ethanol gavage) and 2 hrs after ethanol ingestion at $p < 0.05$.

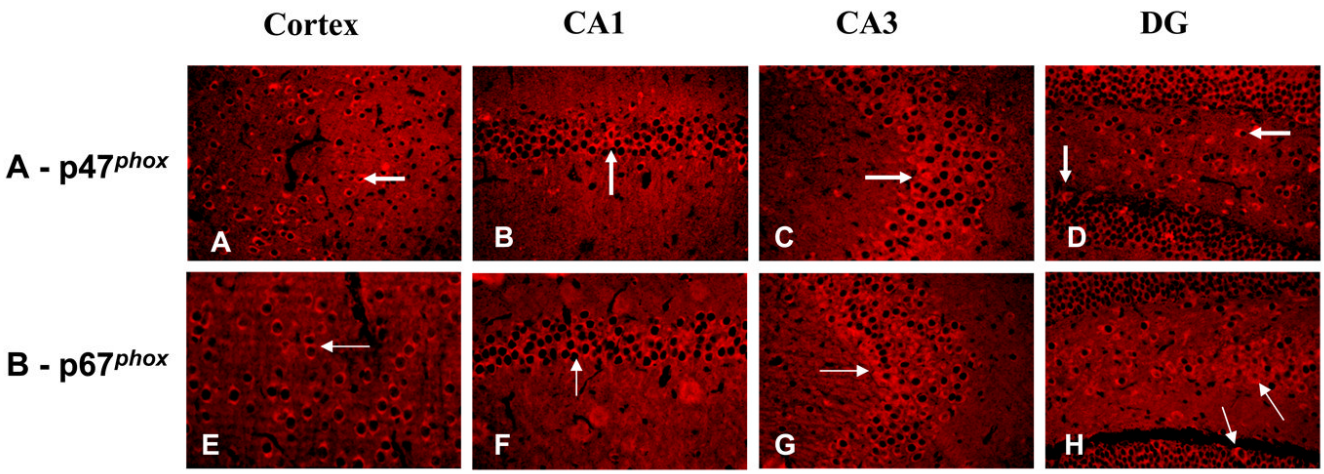

Figure 2.

Immunoreactivities (IR) of p47*phox* and p67*phox* in normal gerbil brain. In cerebral cortex, IR of p47phox and p67*phox* were observed in all layers, but were especially prominent in layer V (Panel A and E). In the hippocampus, p47phox and p67*phox* IR were prominently observed in the pyramidal cell layer of the CA1 (Panel B and F) and CA3 (Panel C and G) regions and in both the molecular and polymorphic layers of dentate gyrus (DG) (Panel D and F). Arrows highlight individual cells that are positive for p47phox and p67*phox* IR. (Magnification, 200x).

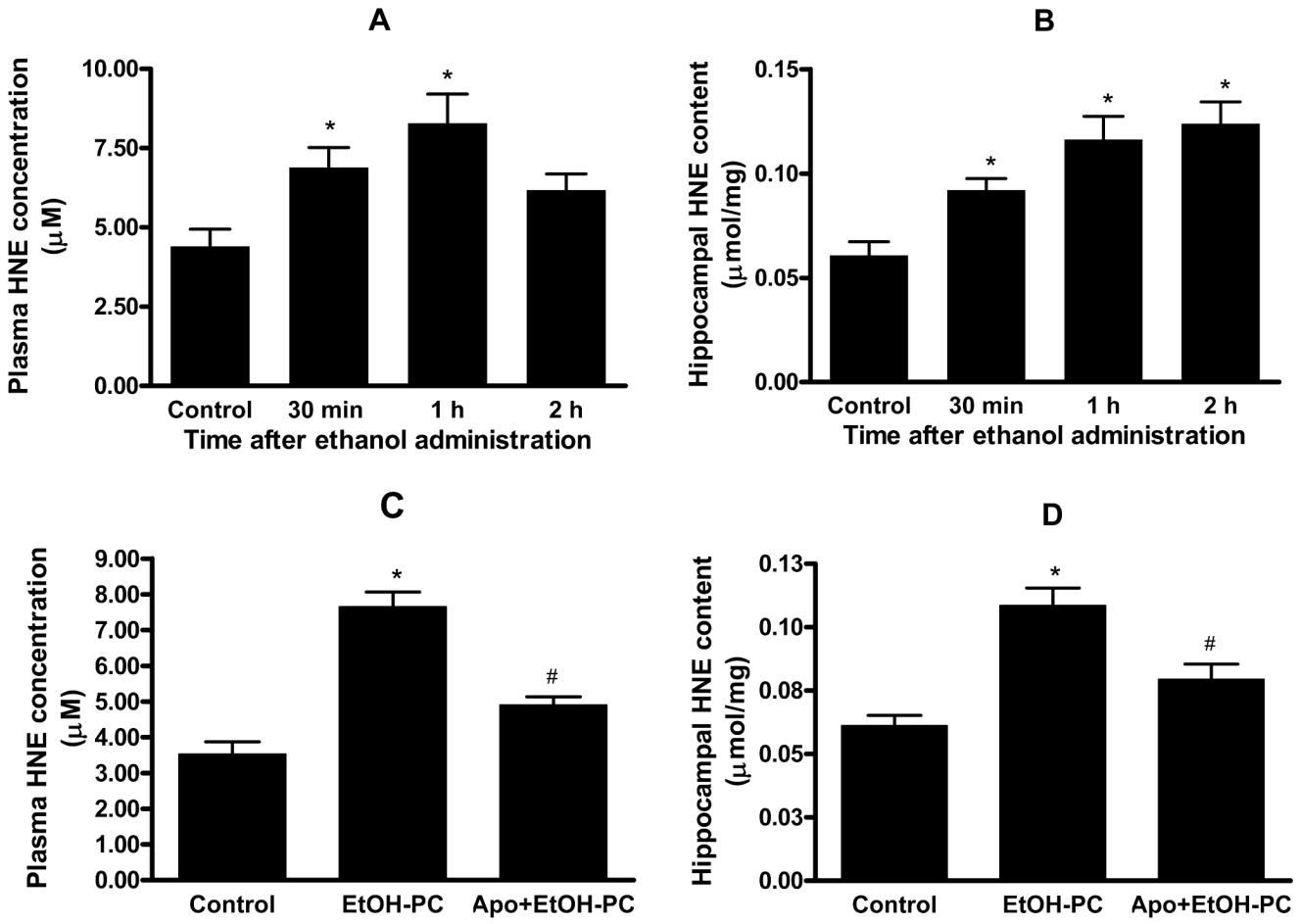

Figure 3.

Moderate ethanol administration by gavage increased lipid peroxidation in plasma (Panel A) and hippocampus (Panel B), as assessed by changes in 4-hydroxy-2-nonenal (HNE) levels (n=6 for each time point and each sample type), and effects of apocynin on plasma (Panel C) and hippocampal (Panel D) lipid peroxidation after ethanol administration by gavage. Data depicted in Panels C and D illustrate the HNE levels in plasma and hippocampus in water control, ethanol ingestion alone (EtOH-PC), and i.p. injection of apocynin 10 min prior to ethanol gavage (Apo+EtOH-PC). * and # indicate values that were statistically different from corresponding values in control and EtOH-PC groups at $p < 0.05$, respectively.

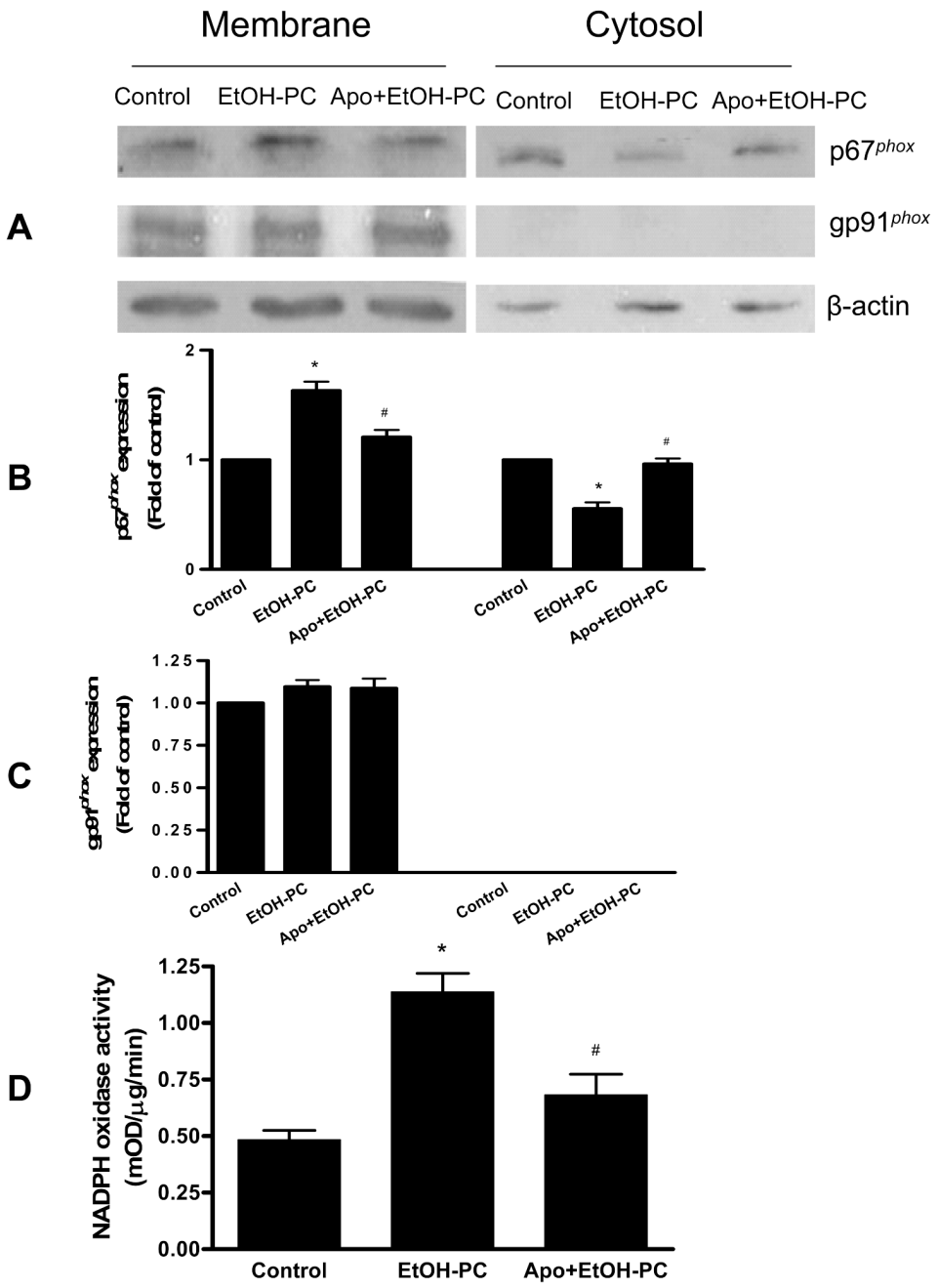

Figure 4.

Effects of apocynin on translocation of p67^{phox} (Panel A, B and C) and on NADPH oxidase activity in hippocampus (Panel D) after ethanol administration by gavage. Data in Panels A to C illustrate the effects of control, ethanol ingestion alone (EtOH-PC), and i.p. injection of apocynin 10 min prior to ethanol gavage (Apo+EtOH-PC) on p67^{phox} and gp91^{phox} expression in membrane and cytosolic fractions. Data in Panel D show NADPH oxidase activity in hippocampus. See details in Method for the assay protocols. Samples were collected 1h after administration of ethanol or distilled water vehicle. Western blotting results are expressed as fold of control for three independent experiments (Panel B and C). * and # indicate values that

are statistically different from corresponding values in control and EtOH alone groups at $p < 0.05$, respectively.

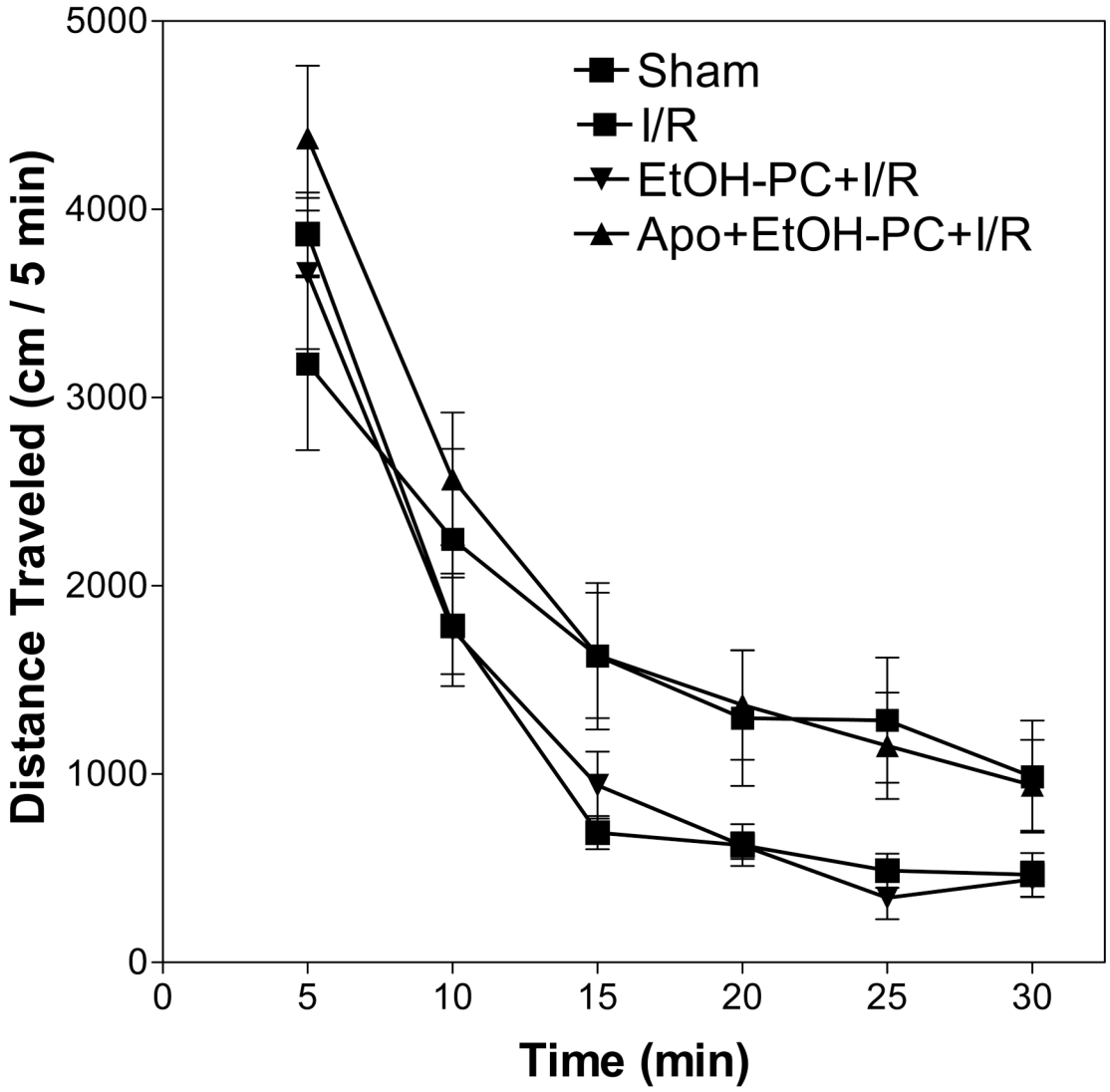

Figure 5.

Effects of ethanol preconditioning and apocynin pretreatment on the spontaneous locomotor activity after cerebral I/R. Data represent mean (± S.E.M.) distance traveled (in cm) (n = 7 – 9 gerbils/group). Gerbils were placed in an automated locomotor activity monitor 24 hrs after cerebral I/R and locomotor activity was measured for 30 min. Statistical analysis revealed a significant main effect of session time (p <0.001) and treatment group (p < 0.001), but the session time by treatment group interaction was not significant (p = 0.65).

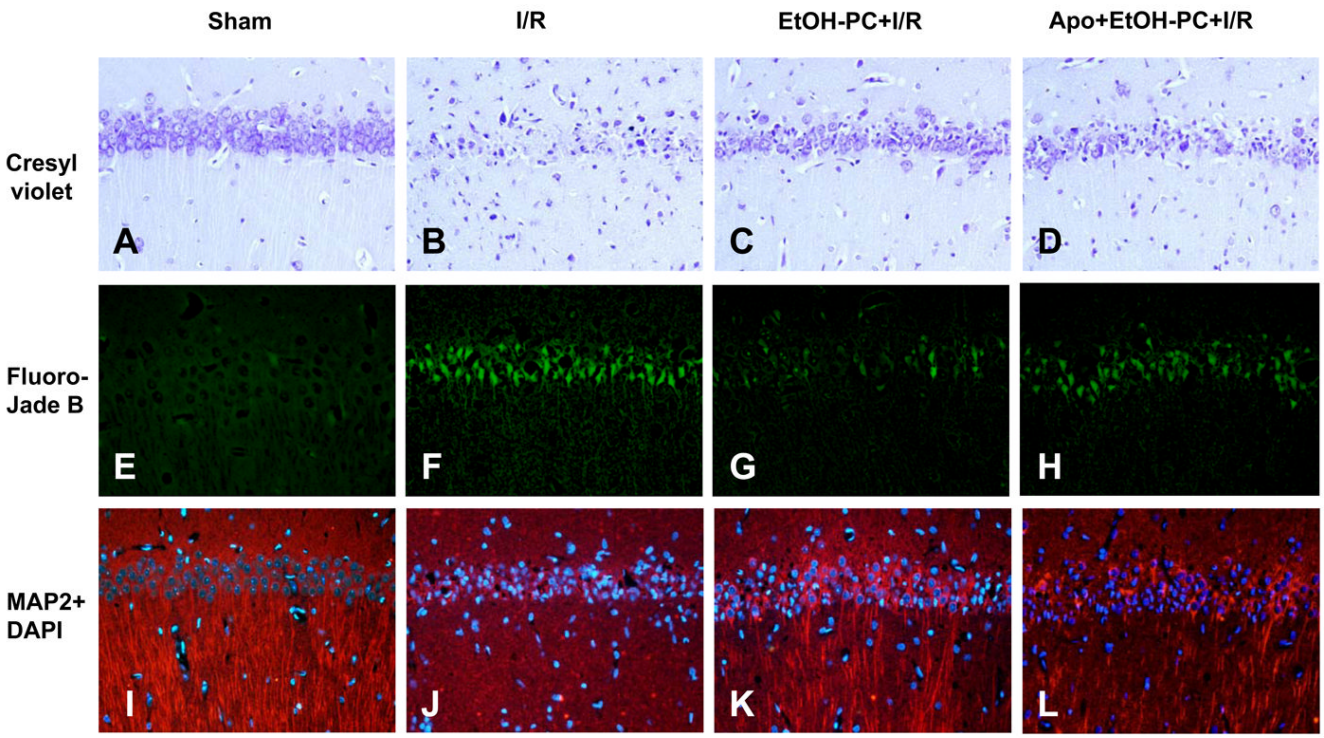

Figure 6.

Representative micrographs depicting I/R-induced delayed neuronal death (DND) (cresyl violet) and neuronal (Fluoro-Jade B) and dendritic degeneration (MAP-2) in the hippocampal CA1 subfield of gerbils subjected to sham operation (Panels A, E, and I, respectively), 5 min occlusion of the common carotid arteries (ischemia) followed by 4 days reperfusion (I/R) (Panels B, F, and J, respectively), EtOH-PC 24 hrs prior to I/R (EtOH-PC+I/R) (Panels C, G, and K, respectively), and apocynin treatment coincident with ethanol administration 24 hrs prior to I/R (Apo+EtOH-PC+I/R) (Panels D, H, and L, respectively). (Magnification, 200x).

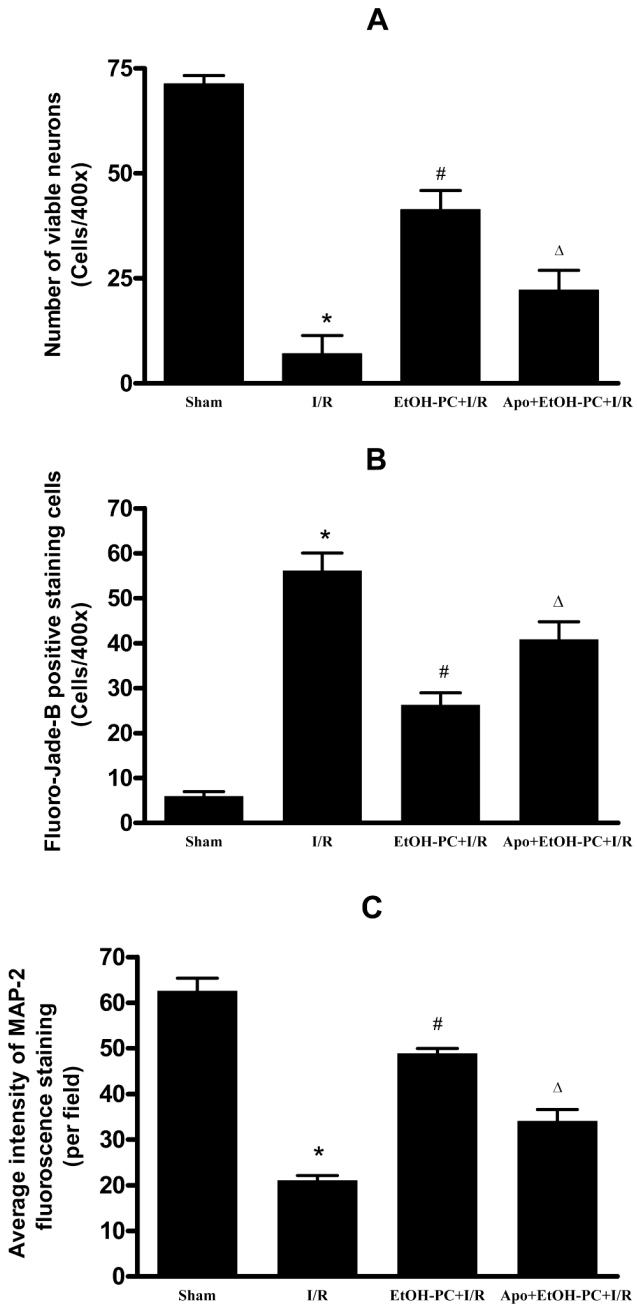

Figure 7.

Quantification of viable neurons (cresyl violet, Panel A) and neuronal (Fluoro-Jade B, Panel B) and dendritic (MAP-2, Panel 3) degeneration in the hippocampal CA1 region of gerbils subjected to sham, I/R alone, ethanol preconditioning 24 hrs prior to I/R (EtOH-PC+I/R), and apocynin treatment coincident with ethanol administration 24 hrs prior to I/R (Apo+EtOH-PC +I/R). *, #, and Δ = values statistically different from corresponding values in sham, I/R, and EtOH-PC+I/R groups, respectively, at $p < 0.05$.

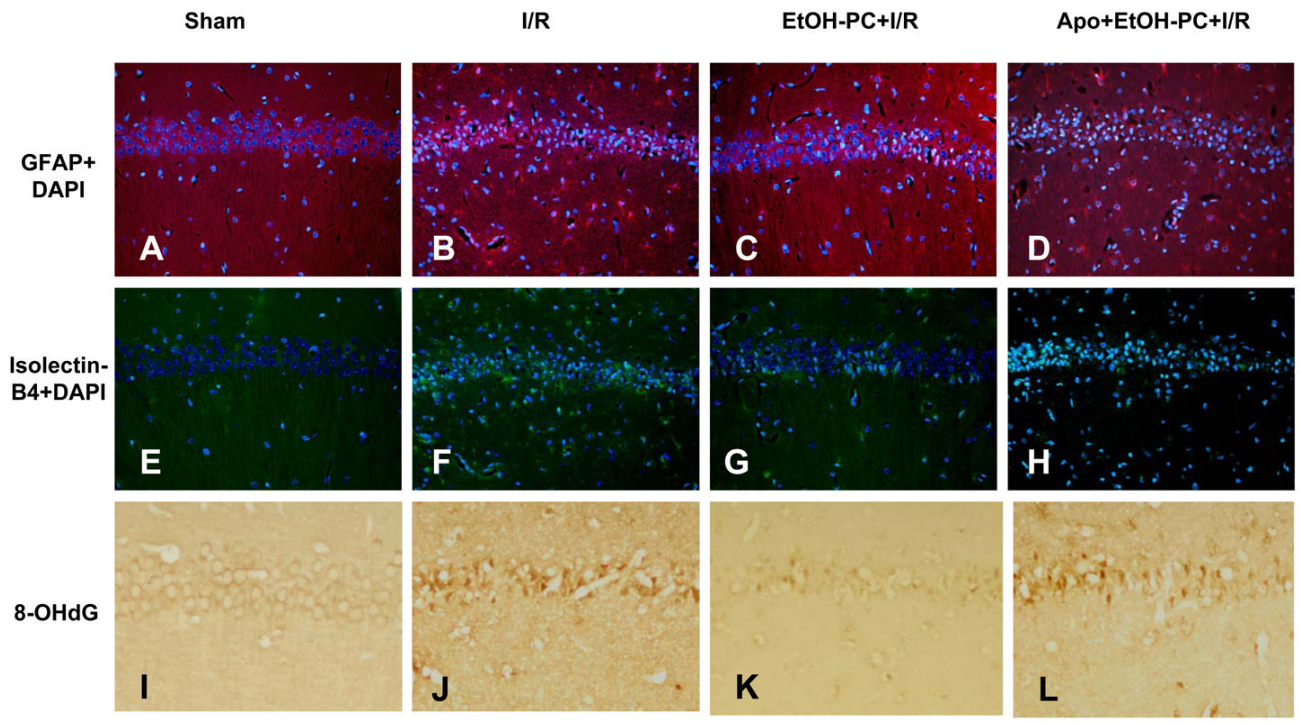

Figure 8.

Representative micrographs depicting I/R-induced activation of astrocytes (GFAP, Panel A), microglial cells (isolectin-B4, Panel B) and DNA oxidation (8-OHdG, Panel C) in the hippocampal CA1 subfield of gerbils subjected to sham (Panels A, E, and I, respectively), I/R (Panels B, F, and J, respectively), ethanol preconditioning 24 hrs prior to I/R (EtOH-PC+I/R) (Panels C, G, and K, respectively), and apocynin treatment coincident with ethanol administration prior to I/R (Apo+EtOH-PC+I/R) (Panels D, H, and L, respectively). (Magnification, 200x).

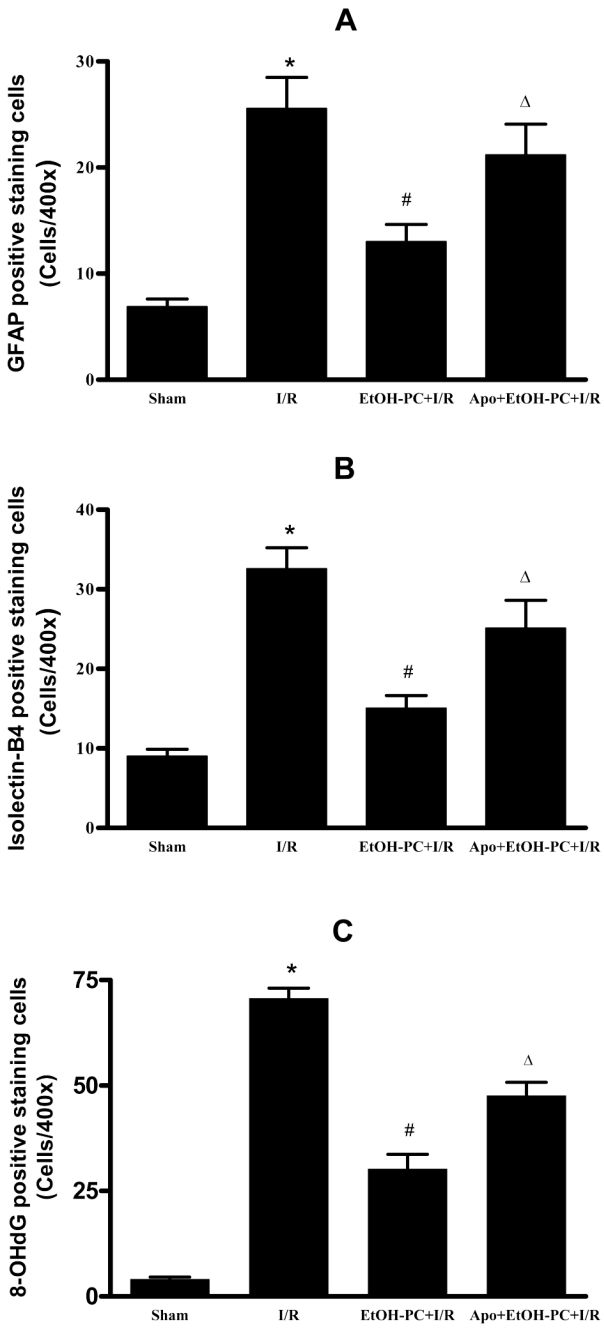

Figure 9.

Quantification of astrocytic (GFAP, Panel A) and microglial activation (Isolectin-B4, Panel B) and DNA oxidation (8-OhdG, Panel C) in gerbils subjected to sham, 5 min occlusion of the common carotid arteries followed by 4 days of reperfusion (I/R), ethanol preconditioning 24 hrs prior to I/R (EtOH-PC+I/R), and apocynin treatment coincident with ETOH-PC 24 hrs prior to I/R (Apo+EtOH-PC+I/R). *, #, and Δ = values statistically different from corresponding values in sham, I/R, and EtOH-PC + I/R groups, respectively, at $p < 0.05$.