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Cyanidin-3-Glucoside Ameliorates Ethanol Neurotoxicity in the Developing Brain

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Abstract

Ethanol exposure induces neurodegeneration in the developing central nervous system (CNS). Fetal Alcohol Spectrum Disorders (FASD) are caused by ethanol exposure during pregnancy and are the most common nonhereditary cause of mental retardation. It is important to identify agents that provide neuroprotection against ethanol neurotoxicity. Multiple mechanisms have been proposed for ethanol-induced neurodegeneration, and oxidative stress is one of the most important mechanisms. Recent evidence indicates that glycogen synthase kinase 3 β (GSK3 β) is a potential mediator of ethanol-mediated neuronal death (Luo, 2009). Cyanidin-3-glucoside (C3G), a member of the anthocyanin family, is a potent natural antioxidant. Our previous study suggested that C3G inhibited GSK3 β activity in neurons (Chen et al., 2009). Using a third trimester equivalent mouse model of ethanol exposure, we tested the hypothesis that C3G can ameliorate ethanol-induced neuronal death in the developing brain. Intraperitoneal injection of C3G reduced ethanol-mediated caspase-3 activation, neurodegeneration and microglial activation in the cerebral cortex of seven-day-old mice. C3G blocked ethanol-mediated GSK3 β activation by inducing the phosphorylation at serine 9 while reducing the phosphorylation at tyrosine 216. C3G also inhibited ethanol-stimulated expression of malondialdehyde (MDA) and p47phox, indicating that C3G alleviated ethanol-induced oxidative stress. These results provide important insight into the therapeutic potential of C3G.

Keywords

Apoptosis; development; fetal alcohol exposure; neuroprotection; oxidative stress

Introduction

Fetal Alcohol Spectrum Disorders (FASD) are caused by maternal alcohol consumption during pregnancy (Riley and McGee, 2005). The most devastating consequences of fetal

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alcohol exposure are malformations of the central nervous system (CNS) and mental retardation. In experimental FASD models, ethanol exposure produces profound cognitive impairment and social behavior deficits in adult animals (Riley, 1990; Barbaccia et al., 2007). Ethanol exposure disrupts a variety of developmental events; these include neurogenesis, neuron survival, cell migration, cell adhesion, axon outgrowth, synapse formation and neurotransmitter function (Miller, 1993, 1996; Swanson et al., 1995; Luo and Miller, 1998; Minana et al., 2000; Yanni et al., 2000; Bearer, 2001; Ikonomidou et al., 2001; Olney, 2004; Goodlett et al., 2005; Soscia et al., 2006; Kumada et al., 2007; Hoffman et al., 2008). These ethanol-induced structural and biochemical alterations in the brain may underlie many of the behavioral deficits observed in FASD. Despite attempts to increase public awareness of the risks involved, the number of women drinking during pregnancy in the USA has not declined (Ebrahim et al., 1999). Therefore, it is important to develop strategies that can prevent or ameliorate alcohol-induced damages to the CNS.

Ethanol increases the production of reactive oxygen species (ROS) in the brain and oxidative stress has been proposed as a potential mechanism for ethanol neurotoxicity (Goodlett and Horn, 2001; Chen and Luo, 2010). Glycogen synthase kinase 3 β (GSK3 β), a serine/threonine kinase, is a key mediator of neuronal apoptosis. Recent studies suggest that GSK3 β is a potential mediator of ethanol neurotoxicity (Luo, 2009). We have isolated a natural antioxidant from blackberries, cyanidin-3-glucoside (C3G), which can scavenge ethanol-induced ROS and also effectively inhibit GSK3 β activity *in vitro* (Ding et al., 2006; Chen et al., 2009). C3G is a member the anthocyanin family and is present in various vegetables and fruits, especially edible berries. C3G offers neuroprotection against cerebral ischemia in a mouse model (Kang et al., 2006). C3G and its metabolites protect neuronal cells against beta-amyloid peptide- and H₂O₂-induced mitochondria damage and DNA fragmentation *in vitro* (Tarozzi et al., 2007, 2008). We hypothesized that C3G may ameliorate ethanol-induced damage to the developing CNS. Rodent models of FASD have been extensively used to investigate the mechanisms of ethanol-induced damage to the CNS. Administration of ethanol to rodents during the synaptogenesis period (also known as the brain growth spurt period), which occurs in early postnatal days, is equivalent to ethanol exposure during the third trimester of pregnancy in humans (Maier et al., 1999). We used a well-established mouse model of developmental ethanol exposure in which ethanol was administered subcutaneously at a total dose of 5 g/kg to 7-day-old mice (Onley et al., 2002). Acute ethanol exposure in this study models binge ethanol intake in humans. Previous studies demonstrated that ethanol exposure caused a widespread neuroapoptosis which was accompanied by microglial activation (Saito et al., 2010). We showed here that an intraperitoneal (ip) injection of C3G reduced ethanol-mediated neurodegeneration and microglial activation in the cerebral cortex. C3G also inhibited ethanol-induced GSK3 β activation and oxidative stress.

Materials and Methods

Materials

Anti-active caspase-3, anti-GSK3 β , anti-phospho-GSK3 β (Ser9) and anti-phospho-GSK3 β (Tyr216) antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-p47phox antibody was obtained from Santa Cruz Biotech (Santa Cruz, CA). Anti-ionized calcium-binding adaptor molecule-1 (Iba-1) antibody was obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Fluoro-Jade C was a generous gift from Dr. Larry C. Schmued at the National Center for Toxicological Research/FDA (Jefferson, AR). Cyanidin-3-glucoside (C3G) was purified from blackberries using high performance liquid chromatography as previously described (Ding et al., 2006). The purity of C3G isolated by this method was 99%. Other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals and ethanol exposure paradigm

C57BL/6 mice were obtained from Harlan Laboratories (Indianapolis, IN) and maintained at the Animal Facility of the University of Kentucky Medical Center. All procedures were performed in accordance with the guidelines set by the NIH and the Animal Care and Use Committee of the University of Kentucky. An acute ethanol exposure paradigm, which had been shown to induce robust neurodegeneration in infant mice (Olney et al., 2002; Liu et al., 2009), was employed. Seven-day-old mice (PD7) were injected subcutaneously with saline or ethanol (2.5 g/kg, 20% solution in saline) at two different times: time 0 and 2 hours. C3G (30 mg/kg or 10 mg/kg, 5 mg/ml in saline) was injected intraperitoneally one day before the first ethanol injection and again 30 minutes before the first ethanol injection. At specified times after the ethanol injection, the brain was removed and processed for immunoblotting or immunohistochemical analysis. For the study of C3G distribution in the brain, there were ten animals (n = 10). For the remaining studies, there were five animals for each treatment group (n = 5).

Determination of C3G in brain tissues

Mice were injected intraperitoneally with C3G (30 mg/kg). At specified times after the injection, mice were anesthetized by an intraperitoneal injection of chloral hydrate (350 mg/kg), and then perfused with ice-cold physiological saline to remove the blood. The whole brain was quickly excised and weighed, the tissue samples were rapidly frozen in liquid nitrogen and kept at -80°C . Brains were crushed in 1% HCl aqueous solution (0.1g tissue/ml) and spiked with 50 ng/ml cyanidin 3,5-diglucoside as an internal standard. After centrifugation (3,500 g, 10 min) at 4°C , anthocyanins were purified with a Sep-Pak C₁₈ Vac solid-phase extraction cartridge (Waters, Milford, MA) and washed with 5% formic acid. C3G was eluted with methanol containing 5% formic acid. The eluent was carefully evaporated under N₂ flow at room temperature. The residue was dissolved in the mobile phase and applied to a LC-MS/MS system for analysis.

Preparation of brain tissue and immunoblotting

After treatment, the mice were anesthetized by an intraperitoneal injection of ketamine (20 mg/kg)/xylazine (3 mg/kg) (Sigma-Aldrich, Inc., St. Louis, MO), and the brain was immediately dissected. The tissues were frozen in liquid nitrogen and stored at -80°C . Proteins were extracted as previously described (Wang et al., 2007). Briefly, tissues were homogenized using an ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 0.5% NP-40, 0.25% SDS, 5 $\mu\text{g/ml}$ leupeptin and 5 $\mu\text{g/ml}$ aprotinin. Homogenates were centrifuged at 20,000 g for 30 min at 4°C and the supernatant fraction was collected.

The immunoblotting procedure has been previously described (Chen et al., 2004). Briefly, aliquots of the protein samples (30 μg) were separated on a SDS-polyacrylamide gel by electrophoresis. The separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with either 5% BSA or 5% nonfat milk in 0.01 M PBS (pH 7.4) and 0.05% Tween-20 (TPBS) at room temperature for 1 hour. Subsequently, the membranes were probed with primary antibodies directed against target proteins overnight at 4°C . After three quick washes in TPBS, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham, Arlington Hts., IL). The immune complexes were detected by the enhanced chemiluminescence method (Amersham). In some cases, the blots were stripped and re-probed with either an anti-tubulin or anti-actin antibody. The density of immunoblotting was quantified with Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Fluoro-Jade C staining

Fluoro-Jade C staining was performed as previously described (Schmued et al., 2005). Briefly, brain sections were mounted onto gelatin coated slides and dried overnight at room temperature. The slides were immersed in a solution of 1% sodium hydroxide and 80% ethanol for 5 min, then in 70% alcohol for 2 min followed by 2 min in distilled water. Slides were transferred in 0.06% potassium permanganate solution for 10 minutes. After rinsed in water for 2 minutes, slides were immersed in a solution of 0.1% acetic acid and 0.0001% Fluoro-Jade C for 10 min. Slides were washed 3 times in distilled water and dried. Fluoro-Jade C-positive cells were counted by the previous method (Ke et al., 2003). Briefly, five representative sections containing retrosplenial granular cortex and lateral parietal association cortex were photographed. For each section, Fluoro-Jade C-positive cells in on right retrosplenial granular cortex were counted with stereoInvestigator software (Version 8, MicroBrightField, San Diego, CA, USA). The mean of Fluoro-Jade C-positive cells on these sections were determined.

Immunohistochemistry

After treatments, the mice were deeply anesthetized with chloral hydrate (350 mg/kg), then perfused with saline followed by 4% paraformaldehyde in 0.1 M potassium phosphate buffer (pH 7.2). The brains were removed and post-fixed in 4% paraformaldehyde for an additional 24 hours and then transferred to 30% sucrose. The brain was sectioned at 40 μ m with a sliding microtome (Leica Microsystems, Wetzlar, Germany).

The immunohistochemical staining for active caspase-3 and Iba-1 was performed as previously described (Liu et al., 2009). Briefly, free-floating sections were incubated in 0.3% H_2O_2 in methanol for 30 min at room temperature and then treated with 0.1% TritonX-100 for 10 min in PBS. The sections were washed with PBS three times and then blocked with 1% BSA and 0.01% TritonX-100 for 1 hour at room temperature. The sections were incubated with primary antibodies: anti-cleaved caspase-3 antibody (1:10,000) or anti-Iba-1 antibody (1:1,000) overnight at 4°C. Negative controls were performed by omitting the primary antibody. After rinsing in PBS, sections were incubated with a biotinylated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA; 1:200) for 1 hour at room temperature. The sections were washed 3 times with PBS, then incubated in an avidin–biotin–peroxidase complex (Vector Laboratories Inc. 1:100 in PBS) for 1 hour and developed in 0.05% 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich, Inc.) containing 0.003% H_2O_2 in PBS. The number of Iba-1-positive cells was determined using a method similar to that for quantifying Fluoro-Jade C-positive cells.

Assessments of brain malondialdehyde (MDA) content

The MDA levels in the brain tissue were determined by a MDA detection kit (Nanjing Jiancheng Bioengineering Institute, China) which is based on the measurement of the interaction between thiobarbituric acid (TBA) and MDA. The principle of the method is based on measuring absorbance of the pink color produced by the interaction of TBA with MDA at 532 nm. Briefly, mice were anesthetized by an intraperitoneal injection of chloral hydrate (350 mg/kg). The cerebral cortex was immediately dissected and homogenized on ice in 9 volumes of saline. The homogenates were centrifuged at 4,500 g at 4°C for 10 min. The protein concentration in the supernatants was determined using a BCA protein assay kit (Pierce, Rockford, IL). The MDA concentration in the supernatant was determined with a spectrophotometer at 532 nm and expressed as nmol/mg protein according to the manufacturer's instructions.

Statistical analysis

Differences among treatment groups were tested using analysis of variance (ANOVA). Differences in which *p* was less than 0.05 were considered statistically significant. In cases where significant differences were detected, specific *post-hoc* comparisons between treatment groups were examined with Student-Newman-Keuls tests.

Results

C3G ameliorates ethanol-induced neurodegeneration

The distribution of C3G in the brain was evident 15 minutes after intraperitoneal injection (Fig. 1). The levels of C3G peaked 1 hour after administration (approximately 3.5 nmol/g). C3G was undetectable in the brain 6 hours following injection. Consistent with previously reported results (Olney et al., 2002; Liu et al., 2009), the ethanol injection caused the cleavage of caspase-3 (activation of caspase-3)(Fig. 2). Administration of C3G (10 mg/kg) significantly reduced the ethanol-mediated activation of caspase-3 (Fig. 2), suggesting that it decreased ethanol-induced apoptosis. The effect of a higher C3G dosage (30 mg/kg) was similar to that of 10 mg/kg. Therefore, 10 mg/kg was used for all our experiments. In control animals, No Fluoro-Jade C positive cell was detected. Ethanol exposure increased Fluoro-Jade C positive cells in the cerebral cortex, which was indicative of neurodegeneration (Fig. 3). Contrarily, C3G treatment diminished the number of Fluoro-Jade C positive cells, suggesting that C3G decreased neurodegeneration caused by ethanol exposure. We next determined the effect of C3G on ethanol-mediated microglial activation. As shown in Fig. 4, the resting microglia displayed multiple thin processes and an elongated cell body while active microglia had intensive Iba-1 immunostaining and a round cell body with retracted/thick processes (Fig. 4). Ethanol activated microglia and C3G treatment significantly reduced the amount of active microglia (Fig. 4).

C3G alleviates ethanol-induced GSK3 β activation and oxidative stress

Recent evidence indicated that GSK3 β is a potential mediator of ethanol neurotoxicity and excessive activation of GSK3 β may result in neuronal death (Luo, 2009). The activity of GSK3 β is positively regulated by its phosphorylation at tyrosine 216 and negatively by the phosphorylation of serine 9 (Luo, 2009). C3G blocked ethanol-induced phosphorylation of GSK3 β at tyrosine 216 and simultaneously enhanced the phosphorylation of serine 9, indicating that C3G inhibited ethanol-mediated activation of GSK3 β (Fig. 5). Furthermore, C3G blocked ethanol-induced MDA production, a product of lipid peroxidation (Fig. 6). Our recent data indicate that NADPH oxidase plays a critical role in ethanol-induced reactive oxygen species (ROS) in the brain (manuscript in preparation). p47phox is an essential regulatory protein of NADPH oxidase (Takeya and Sumimoto, 2003). Ethanol drastically increased the expression of p47phox. As shown in Fig. 7, ethanol caused an approximately 80 fold increase of p47phox in the developing brain and C3G significantly reduced ethanol-induced p47phox up-regulation.

Discussion

In this study, we demonstrate that C3G is able to ameliorate some of ethanol's neurotoxicity in the developing brain. C3G reduces ethanol-induced neurodegeneration and microglial activation in the brain of seven-day-old mice. It also inhibits ethanol-mediated activation of GSK3 β and oxidative stress.

It has been previously demonstrated that some anthocyanins including C3G are able to cross the blood-brain barrier (BBB) and localize in various brain regions important for motor, learning and memory functions (Chen and Luo, 2010). In a study where rats are fed a

blueberry supplement or a control diet for 8-10 weeks, several anthocyanins including C3G are found in the cerebellum, cortex, hippocampus or striatum of rats, but not in the controls (Andres-Lacueva et al., 2005). In another study where rats are fed a blackberry anthocyanin-enriched diet for 15 days, C3G is the predominant form of anthocyanins detected in the brain homogenate, and C3G content is higher in the brain than in the plasma (Talavéra et al., 2005).

Our results confirm that C3G is quickly distributed in the brain after ip injection; the brain C3G concentration reaches approximately 3.5 nmol/g 1 hour after administration. Similarly, Marczylo et al. (2009) demonstrate that the peak levels of C3G are achieved in the liver, kidneys, lungs, heart and prostate within 30 minutes after tail vein injection (1 mg/kg). The maximal concentrations of C3G in these organs range from 1-8 nmol/g. We have previously shown that an injection of C3G to mice (ip, 9.5 mg/kg, 3 times/week) significantly inhibits A549 tumor xenograft growth and metastasis in mice (Ding et al., 2006). Based on a simplistic approximation, the resulting concentration would be about 0.5 μ M in the plasma and near 1 nmol/g in the lungs and prostate (Marczylo et al., 2009). This tissue concentration is less than a tenth of the concentration shown to impair the growth and migration of A549 cells *in vitro* (Ding et al., 2006). We have previously shown that C3G at 5 μ M is able to reverse ethanol-induced inhibition of neuronal differentiation in culture. Therefore, the concentrations required for *in vivo* action of C3G could be lower than that of *in vitro*. We have not determined C3G concentration in the plasma. Compared to an anthocyanins-rich diet, administration of purified C3G may result in relatively higher C3G levels in the plasma. The estimated C3G in the plasma of mice would be around 0.5 μ M. However, the administration of an anthocyanins-rich diet such as elderberry extract result in only 0.045 μ M in human plasma (Prior and Wu, 2006).

C3G protection against ethanol-induced neuroapoptosis is evident by a decrease in caspase-3 activation and the number of Fluoro-Jade C positive cells. Ethanol-induced neurodegeneration is accompanied by microglial activation which may be a secondary response to ethanol-induced injury to the brain (Saito et al., 2010). C3G inhibition of microglial activation may result from an alleviation of neuronal damage. GSK3 β has been identified as a potential mediator of ethanol neurotoxicity (Luo, 2009). We have previously demonstrated that high expression/activity of GSK3 β sensitizes neurons to ethanol-induced apoptosis and inhibition of GSK3 β offers protection against ethanol-induced death of cultured neuronal cells (Liu et al., 2009); lithium, an inhibitor of GSK3, reduces ethanol-mediated neuroapoptosis in the developing brain (Luo, 2010). Full activity of GSK3 β generally requires phosphorylation at tyrosine 216, and conversely, phosphorylation at serine 9 inhibits GSK3 β activity. In the brain, C3G enhances the phosphorylation at serine 9 and inhibits ethanol-induced phosphorylation at tyrosine 216. Therefore, it is likely that C3G's neuroprotection is mediated by its inhibition of GSK3 β .

C3G is an antioxidant and scavenges ethanol-induced ROS production in cultured neuronal cells (Chen et al., 2009). The current study confirms the antioxidant property of C3G *in vivo* by showing it reduces ethanol-induced lipid peroxidation in the developing brain. Like us, Li et al. (2008) report that oral administration of C3G (4-8 mg/kg) significantly reduces ethanol-induced lipid peroxidation and free radicals in gastric tissues. Our recent results suggest that ethanol-induced ROS production in the developing brain is mainly mediated by the activation of NADPH oxidase through p47phox up-regulation (data not shown). We show here that C3G blocks ethanol-induced up-regulation of p47phox, suggesting that C3G may reduce ROS production by inhibiting the activation of NADPH oxidase.

C3G may undergo deglycosylation *in vivo*. We have not determined the levels of aglycone cyanidin in the brain. Tarozzi et al. (2007) demonstrate that aglycone cyanidin also offers

protection against oxidative stress-induced neuronal damage in culture. It is unknown whether aglycone cyanidin contributes to neuroprotection by peripheral administration of C3G. We have previously shown that C3G offers protection against ethanol neurotoxicity in culture. Regardless of the contribution of aglycone cyanidin, the importance of our finding is to demonstrate that peripheral administration of C3G is sufficient to ameliorate ethanol-induced neurodegeneration.

C3G exhibits diverse beneficial health properties. For example, it displays chemopreventive and chemotherapeutic properties in various *in vitro* and animal models of carcinogenesis and tumor development (Chen et al., 2005, 2006; Shih et al., 2005; Ding et al., 2006; Zhang et al., 2005, 2008). Other beneficial properties of C3G include anti-diabetic (Sasaki et al., 2007), anti-inflammation (Kim and Park, 2006; Xia et al., 2006), anti-atherogenic activity (Xia et al., 2006) and anti-obesity (Tsuda et al., 2003). C3G is a potent antioxidant and inhibits GSK3 β (Chen et al., 2009). Our finding that C3G can protect neurons against ethanol toxicity not only provides an insight into the therapeutic potential of this anthocyanins, but also help us to understand the mechanisms of ethanol-induced neurodegeneration.

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Abbreviations

C3G	cyanidin-3-glucoside
FASD	Fetal Alcohol Spectrum Disorders
GSK3β	glycogen synthase kinase 3 β
ROS	reactive oxygen species

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Figure 1.

Determination of C3G in the developing brain. Seven-day-old (PD7) mice received an intraperitoneal injection with C3G (30 mg/kg). At 15 minutes, 30, minutes, 1 hour, 2 hours and 6 hours after C3G injection, the brain tissue was harvested and the concentration of C3G was determined with a LC-MS/MS system as described under the Materials and Methods. Each data point was the mean $\pm$ SEM of ten animals.

Figure 2. Effect of C3G on ethanol-induced caspase-3 activation in the developing brain. PD7 mice were pretreated with C3G (10 mg/kg) and injected with ethanol as described under the Materials and Methods. At specified times after the first ethanol injection, the brain was removed and the expression of cleaved caspase-3 was examined with immunohistochemistry (IHC) (**A**) or immunoblotting analysis (**B**). The immunoblotting analysis was replicated three times for each sample. Images were quantified and normalized to the expression of tubulin. Each data point was the mean $\pm$ SEM of five animals. The value on the Y axis was the fold increase over untreated controls (**C**). Bar = 200 μ m.

Figure 3.

Effect of C3G on ethanol-induced neurodegeneration in the cerebral cortex. PD7 mice were treated with C3G and ethanol as described above. At 8 hours after the first ethanol injection, the cerebral cortex was removed and sectioned. (A): The degenerating neurons were detected with Fluoro-Jade C staining (green) as described under the Materials and Methods. Bar = 200 μ m (B): The number of Fluoro-Jade C-positive cells was determined as described under the Materials and Methods. There were five animals for each treatment group. * denotes a statistically significant difference from ethanol-treated group.

Figure 4.

Effect of C3G on ethanol-induced microglial activation. **(A)**: PD7 mice were treated with C3G and ethanol as described above. The cerebral cortex was removed at 12 and 24 hours after ethanol injection, sectioned and processed for immunohistochemistry of Iba-1. Images of higher magnification are shown on the right panels. Left panels: Bar = 200 μ m. Right panels: Bar = 25 μ m. **(B)**: The number of Iba-1-positive cells following 24 hours of ethanol injection was determined as described under the Materials and Methods. There were five animals for each treatment group. * denotes a statistically significant difference from control and ethanol/C3G groups.

Figure 5.

Effect of C3G on ethanol-mediated phosphorylation of GSK3 β . PD7 mice were treated with C3G and ethanol as described above. At 8 hours after the first ethanol injection, the brain was removed and the expression of p-GSK3 β (Tyr216) and p-GSK3 β (Ser9) was examined by IHC (A) and immunoblotting analysis (B). Top panels: Bar = 200 μ m. Bottom panels: Bar = 50 μ m. The immunoblotting analysis was replicated three times for each sample. Images were quantified and normalized to the expression of actin. Each data point was the mean $\pm$ SEM of five animals. The value on the Y axis was the fold increase over untreated controls (C). * denotes a statistically significant difference from untreated controls. # denotes a significant difference from ethanol-treated groups.

Figure 6.

Effect of C3G on ethanol-induced lipid peroxidation. PD7 mice were treated with C3G and ethanol as described above. At 8 hours after the first ethanol injection, the brain was removed and the concentration of MDA, a product of lipid peroxidation, was determined as described under the Materials and Methods. Each data point was the mean $\pm$ SEM of five animals. The reading on the Y axis was a value relative to untreated controls. * denotes a statistically significant difference from all other groups.

Figure 7.

Effect of C3G on an ethanol-induced increase in p47phox. PD7 mice were treated with C3G and ethanol as described above. At 8 hours after the first ethanol injection, the brain was removed and the expression of p47phox was examined with immunoblotting analysis (**A**). The immunoblotting analysis was replicated three times for each sample. Images were quantified and normalized to the expression of actin. Each data point was the mean $\pm$ SEM of five animals. The value on the Y axis was the fold increase over untreated controls (**B**). * denotes a statistically significant difference from untreated controls. # denotes a significant difference from ethanol-treated groups.