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Ethanol Influences on Bax Translocation, Mitochondrial Membrane Potential, and Reactive Oxygen Species Generation are Modulated by Vitamin E and Brain-Derived Neurotrophic Factor

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Abstract

Background—This study investigated ethanol influences on intracellular events which predispose developing neurons toward apoptosis, and the capacity of the antioxidant α-tocopherol (vitamin E) and the neurotrophin brain-derived neurotrophic factor (BDNF) to modulate these effects. Assessments were made of the following: (1) ethanol-induced translocation of the pro-apoptotic Bax protein to the mitochondrial membrane, a key upstream event in the initiation of apoptotic cell death; (2) disruption of the mitochondrial membrane potential (MMP) as a result of ethanol exposure, an important process in triggering the apoptotic cascade; and (3) generation of damaging reactive oxygen species (ROS) as a function of ethanol exposure.

Methods—These interactions were investigated in cultured postnatal day 8 neonatal rat cerebellar granule cells, a population vulnerable to developmental ethanol exposure in vivo and in vitro. Bax mitochondrial translocation was analyzed via subcellular fractionation followed by Western blot, and mitochondrial membrane integrity was determined using the lipophilic dye, JC-1, which exhibits potential-dependent accumulation in the mitochondrial membrane as a function of the MMP.

Results—Brief ethanol exposure in these preparations precipitated Bax translocation, but both vitamin E and BDNF reduced this effect to control levels. Ethanol treatment also resulted in a disturbance of the MMP, and this effect was blunted by the antioxidant and the neurotrophin. ROS generation was enhanced by a short ethanol exposure in these cells, but the production of these harmful free radicals was diminished to control levels by co-treatment with either vitamin E or BDNF.

Conclusions—These results indicate that both antioxidants and neurotrophic factors have the potential to ameliorate ethanol neurotoxicity, and suggest possible interventions which could be implemented in preventing or lessening the severity of the damaging effects of ethanol in the developing central nervous system seen in the fetal alcohol syndrome (FAS).

Keywords

Ethanol; apoptosis; antioxidant; vitamin E; BDNF

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INTRODUCTION

Since the first characterization of the fetal alcohol syndrome (FAS; Jones and Smith, 1973), it has become clear that exposure to ethanol poses a strong threat to the proper execution of the normal developmental program. Within the developing central nervous system (CNS), such exposure can lead to a variety of anomalies, including disruption of neuronal proliferation, migration, and formation of appropriate patterns of connectivity, as well as enhancement and/or initiation of apoptotic cell death, leading to significant neuronal depletion (Miller, 1986; 1993; Miller and Al-Rabiai, 1994; Ikonomidou et al., 2000). These disruptions produce a range of abnormalities, ultimately resulting in impairments in cognitive, intellectual and behavioral functioning (West et al., 1994). These dysfunctions are accompanied by severe underlying neuropathology, as evidenced by examination of neuroanatomical data derived from both human autopsy materials, and from animal models of FAS (Clarren et al., 1978; Driscoll et al., 1990).

One area of research which has received attention in recent years is the potential of ethanol to produce alterations in the expression and/or functionality of apoptosis-related proteins. A number of aspects of the apoptotic process have been found to be influenced by early ethanol exposure. These include increased expression or activation of pro-apoptotic proteins of the Bcl-2 survival-regulatory gene family, along with concomitant decreases in expression of anti-apoptotic proteins of this family (Moore et al., 1999; Olney et al., 2001; 2002; Inoue et al., 2002; Heaton et al., 2003a;b;c; Ge et al., 2004; Nowoslawski et al., 2005; Lee et al., 2008). Activation of apoptosis effector Bax, a key component of the cell death program, results in the release of this protein from its cytosolic anchor, and subsequent translocation to the mitochondrial membrane (Gross et al., 1999; Polster and Fiskum, 2004; Tsuruta et al., 2004). This intracellular movement can lead to disruption of the mitochondrial membrane potential (MMP), release of mitochondrial cytochrome-C, and triggering of the execution phase of cell death (Kluck et al., 1997; Bras et al., 2005). In an earlier study, we found that ethanol exposure during periods of heightened sensitivity, in neonatal rat cerebellum, promotes such Bax translocation, along with enhanced cell loss (Siler-Marsiglio et al., 2005a).

There is a strong link between ethanol-mediated apoptosis during nervous system development and changes in oxidative processes. Ethanol enhances generation of destructive reactive oxygen species (ROS), often with concomitant reductions in the expression or activity of protective endogenous antioxidants (Uysal et al., 1989; Davis et al., 1990; Montoliu et al., 1994; Henderson et al., 1995; Kotch et al., 1995; Reddy et al., 1999; Heaton et al., 2002a; 2003c). Since the cellular redox status is closely associated with apoptosis (Kane et al., 1993; Fiers et al., 1999; Gomez-Lazaro et al., 2007), an attractive hypothesis is that alterations in this balance play a key role in ethanol-mediated neurotoxicity. A number of studies have demonstrated that co-administration of antioxidants with ethanol provide significant protection against deleterious ethanol effects, both in vitro and in vivo, lending support to this hypothesis (Kotch et al., 1995; Henderson et al., 1995; Agar et al., 1999; Mitchell et al., 1999a; b; Heaton et al., 2000a; Mansouri et al., 2001; Shirpoor et al., 2009). For purposes of the present investigation, it is notable that these antioxidants also have the capacity to alter expression of Bcl-2-related proteins in a manner favoring cell survival (Haendeler et al., 1996; Heaton et al., 2004a; Marsh et al., 2005).

In addition to modifications of oxidative processes, ethanol exposure during CNS development interferes with expression of supportive neurotrophic factors (NTFs) and/or their receptors (e.g., nerve growth factor [NGF; Vallés et al., 1994; Heaton et al., 1999]; brain-derived neurotrophic factor, neurorophin-3 [BDNF, NT-3; Heaton et al., 2004b]; glial-derived neurotrophic factor [GDNF; McAlhany et al., 1999;] neurotrophin receptors TrkA,

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TrkB and TrkC [Moore et al., 2004a;b). It has been speculated that reductions in this critical source of support may contribute significantly to ethanol-mediated neuronal loss (Heaton et al., 1993). In agreement with this hypothesis, a number of studies have found that supplementation of ethanol-treated cultured neurons with a range of NTFs, particularly those of the neurotrophin gene family, mitigate ethanol neurotoxicity (e.g., NGF, Brodie et al, 1991; Luo et al., 1997; BDNF, Mitchell et al., 1999c; Bhave et al., 1999]). Also, reductions in the availability of certain NTFs during CNS development (e.g., in BDNF gene-deleted animals) exacerbate ethanol toxicity in certain regions (e.g., cerebellum), while increased availability (via NGF transgenic overexpression) serves a protective function (Heaton et al., 2000b; 2002b). There are important interactions between NTFs and both apoptosis-related substances and oxidative processes, which may act to at least partially counter the deleterious effects of ethanol: Both NGF and BDNF, for example, have been shown to up-regulate expression of anti-apoptotic proteins, while suppressing expression or activation of pro-apoptotic molecules (e.g., Katoh et al., 1996; Muller et al., 1997; Aloyz et al., 1998; Liu et al, 1999; Rong et al., 1999]; Perez-Navarro et al., 2005]). These same substances also have the capacity to stabilize the cellular redox state, influencing activity of antioxidants and generation of ROS (Nistico et al., 1992; Sampath et al., 1994; Mattson et al., 1995; Fiers et al., 1999; Guegan et al., 1999). Thus, NTFs play a critical role in neuronal survival.

As noted above, Bax mitochondrial translocation and subsequent disruption of the integrity of the mitochondrial membrane are defining events in the apoptotic process (Polster and Fiskum, 2004; Tsuruta et al., 2004; Bras et al., 2005). These events have previously been shown to be induced by ethanol treatment (e.g., Siler-Marsiglio et al., 2005a). The objective of the present study was to determine whether either antioxidants or NTFs have the potential to modulate these destructive processes. Such modulation by either of these substances would represent important mechanisms underlying their neuroprotective effects against developmental ethanol exposure. For these determinations, we used cultured neonatal rat cerebellar granule cells, a population previously shown to be vulnerable to ethanol in vivo and in vitro (Hamre and West, 1993; Moore et al., 1999; Bhave and Hoffman, 1997; Saito et al., 1999; Heaton et al., 2004a). We examined (1) ethanol-induced Bax translocation to the mitochondria, and the potential of the powerful free radical scavenger α-tocopherol (vitamin E) and the neurotrophic factor BDNF to regulate this fundamental apoptotic process; (2) ethanol-mediated disruption of the MMP, and the capacity of vitamin E or BDNF to mitigate this disruption; and (3) ethanol effects on the generation of ROS, and the possible amelioration by vitamin E and/or BDNF. These two substances were chosen for these evaluations since both have been found to have protective properties in this developing neuronal population in the presence of ethanol (Bhave et al., 1999; Siler-Marsiglio et al., 2004a; 2005b). Thus, this study was designed to investigate molecular mechanisms involved in this protection.

MATERIALS AND METHODS

Cerebellar Granule Cell Cultures

All of the protocols used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Florida. Cerebellar granule cells cultures were prepared as previously described in detail (Siler-Marsiglio et al., 2004a). Briefly, postnatal day 8 (P8) pups from Charles River (Portage, MI) were sacrificed by Halothane overdose. Cerebellar tissue was dissected, minced, and placed in Hank's balanced salt solution (HBSS) containing trypsin and DNAse (Sigma; St. Louis, MO). The tissue was incubated at 37°C for 15–20 minutes, washed with Dulbecco's modified Eagle's medium (DMEM; Cellgro, from Fisher Scientific; Pittsburgh, PA) with 15% fetal bovine serum (Gibco/Invitrogen; Carlsbad, CA), penicillin/streptomycin (Fisher), fungizone (Gibco/Invitrogen), and trypsin inhibitor (Sigma), then triturated using a flame-narrowed borosilicate pipette to yield a homogeneous

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suspension. The cells were centrifuged, the supernatant removed, and fresh medium added to the pellet. After gentle aspiration, the cell suspension was filtered through a Nitrex filter (Sefar America; Kansas City, MO) to enrich the preparations for the small granule cells. Cells were plated in culture vessels appropriate for the different assays to be applied, all coated with poly-D-lysine (0.05 mg/ml; Sigma) at an average density of 35,000–39,000 neurons/cm^2, as determined by a hemocytometer. Cells were then incubated at 37°C, 3% CO_2/97% air, for 24 hours. Cells prepared in this manner were determined to be ~95% neuronal by immunochemical detection of glial fibrillary acidic protein (GFAP) and type III β-tubulin.

Application of Experimental Conditions

After an initial 24 hour plating period, the serum-containing medium was replaced with serum-free, modified N2 medium consisting of DMEM/F12 medium at a 3:1 ratio, 5 mM KCl, and 1% N2 supplement (Sigma). Addition of penicillin/streptomycin and fungizone was equivalent to that of the serum-containing medium. After a 24 hour interval, the following conditions were applied: (1) Control, in which the N2 medium was replaced without further supplementation; (2) Ethanol, in which 400 mg/dl ethanol was added to the N2 medium; (3); Vitamin E (50 μM; Sigma; in control N2 medium); (4) Ethanol plus vitamin E, with concentrations as above, in the N2 medium; (5) BDNF (50 ng/ml; Sigma; in control N2 medium); and (6) Ethanol plus BDNF, with concentrations as above, in the N2 medium. The vitamin E and BDNF concentrations used (50 μM and 50 ng/ml, respectively) were chosen based on previous studies, in which similar concentrations were found to afford protection against ethanol neurotoxicity (Heaton et al., 2004a; Mitchell et al., 1999c). The ethanol concentration chosen (400 mg/dl) has previously been found to elicit significant neuronal death in granule cell culture preparations (e.g., Heaton et al., 2000c; Heaton et al., 2004a). This concentration represents a relatively high physiological level, but is comparable to levels seen both in humans, and in animal studies of ethanol toxicity (e.g., Tran et al., 2005). It should be noted that much higher ethanol levels have been reported in awake human alcoholics (Lindblad and Olsson, 1976), and considerably higher levels have been applied in prior culture studies, as a means of defining toxicity limits with respect to the cellular populations under investigation (e.g., Siler-Marsiglio et al., 2004a;b). The culture dishes were wrapped in parafilm, to minimize ethanol evaporation. Ethanol concentrations were confirmed by the Diagnostic Chemical Limited (Oxford, CT) microenzymatic assay, and evaporation during the short exposure time (see below) was minimal.

Subcellular Fractionation

Bax translocation from the cytosol to the mitochondria was determined as in our previous studies (Siler-Marsiglio et al, 2005a). For this procedure, mitochondrial fractions were isolated using the BioVision kit (BioVision Inc., Mountain View, CA). Briefly, 40 minutes following exposure to the experimental conditions, granule cells were harvested and homogenized in a cocktail of protease and phosphatase inhibitors. This exposure time was chosen because alterations in apoptosis-related proteins have been found to occur rapidly following ethanol introduction (Ramachandran et al., 2003; Siler-Marsiglio et al., 2004a;b). The lysates were suspended in Cytosol Extraction Buffer containing the protease inhibitor cocktail and dithiothreitol (DTT). Following incubation on ice, the homogenates were centrifuged to remove nuclei, membranes, and whole cells. The supernatant was then centrifuged and the liquid phase containing the cytosolic fraction was removed from the pellet. For Western blots, the pellet was resuspended in mitochondrial extraction buffer with protease inhibitor and DTT. The suspensions were sonicated to release the mitochondrial contents. Protein content of the mitochondrial fractions was quantified using the bicinchoninic (BCA) assay kit (Pierce; Rockford, IL). Concentrations of fractions exposed

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to each condition were normalized for protein content for Western blot analyses, with 25 μg protein loaded per lane. To ensure a high quality separation of the mitochondrial fraction from the cytosolic fraction, preliminary Western blots were performed using a monoclonal mouse antibody against mitochondrially-specific cytochrome oxidase subunit Vb (COX IV; Molecular Probes, Carlsbad, CA). No COX IV was detected in the cytosolic fraction, while abundant levels were found in the mitochondrial fraction following fractionation. Representative Western blots illustrating the success of this fractionation are shown in Figure 1.

Western Blotting Procedures

Western blot analyses were performed according to procedures previously described (Heaton et al., 2003b). Briefly, cell homogenates were prepared, and protein concentrations determined as above. Homogenates were diluted in sample buffer and boiled for five minutes before loading onto SDS gels. Equal amounts of proteins were fractionated by SDS-gel electrophoresis using Ready Precast Gradient 8–16% SDS-polyacrylamide gels, and utilizing a BioRad electrophoresis unit, with 25 μg protein loaded per lane. Following fractionation, proteins were transferred to nitrocellulose membranes in a BioRad wet transfer unit. After transfer was completed, the blots were stained with Ponceau S and the gels with Coomassie Brilliant Blue, to verify successful transfer and protein separation. Membranes were blocked with TBS with Tween-20, and Blotto, then probed with a polyclonal antibody to Bax (Santa Cruz, Santa Cruz, CA; 1:500), followed by an anti-rabbit HRP-conjugated secondary antibody (also from Santa Cruz; 1:4000). The signal was detected by enhanced chemiluminescence using the Western blot detection kit RPN 2106 (Amersham Pharmacia Biotech, Piscataway, NJ). The film was developed by standard photographic procedures using Kodak X-OMAT film. A biotinylated-protein ladder was fractionated on each gel and HRP-conjugated anti-biotin was added to membranes to indicate molecular weights of the detected protein bands on film. Bands representing Bax were found to be 21 kD, using this biotinylated protein standard, a molecular weight which has been previously reported (Gao and Duo, 2000), confirming the specificity of the antibody detection. Quantification of signal strength was made by scanning the films with a UMAX Ultra scanner, and densitometric analyses were made using the Scion Image computer program.

JC-1 Procedure

The cultures were assessed for mitochondrial function using the JC-1 membrane potential detection kit (Molecular Probes, Eugene, OR). The loss of the MMP is a hallmark of apoptosis. The lipophilic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide) is a cationic dye which exhibits potential-dependent accumulation in the mitochondrial membrane as a function of the mitochondrial membrane potential (MMP). In healthy cells, the normal potential enables the dye to enter and accumulate in the membrane, where it produces a red signal. At the collapse of the membrane potential, as in apoptotic cells, the dye remains in the cytosol, and stains green. Thus, decreases in the red/green fluorescent intensity ratio can be quantified, reflecting membrane depolarization during the apoptosis process. This dye has been widely used to analyze mitochondrial status (White and Reynolds, 1996). For the JC-1 staining, granule cells were established for 24 hours in 96-well plates, with serum-containing culture media as described above. The media was then replaced with defined media, containing vitamin E (50 μM) or BDNF (50 ng/ml). Ethanol was then added to the appropriate wells as above. Exposure time for this assay was one hour. This time was allowed to increase the likelihood of detection of membrane dysfunction following possible Bax translocation. During this time, the plates were sealed and incubated at 37°C. The media was then removed and JC-1

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(4 μg/ml) was immediately added to the plates for a 10 minute incubation. Cells were then rinsed with PBS, and fresh PBS was added for the fluorescent evaluation.

In order to quantify the status of the MMP, the red/green intensity ratios of control and experimental cells were determined using a KC4 96-well fluorometric microplate reader (Red: excitation/emission 530 nm/590 nm; Green: excitation/emission 490 nm/530 nm). The JC-1 red/green intensity ratio is linearly proportional to mitochondrial membrane potential, and does not depend on mitochondrial shape, size, and density.

ROS Assays

ROS determinations were made as a means to confirm that both the antioxidant and the NTF, within our model system with the concentrations used, were able to offset the ethanol-induced free radicals generation, thus suggesting that an observed amelioration of ethanol effects may be related to these reductions. This procedure was as previously described in detail (Heaton et al., 2003a). Briefly, cells were established in 100 mm plates for 24 hours, as above, then exposed to the various conditions for 40 minutes. This time was chosen because it has previously been shown, both in our lab and in others, that alterations in ROS generation occur rapidly following ethanol introduction (Ramachandran et al., 2003; Siler-Marsiglio et al, 2005b). The cells were then harvested and homogenized in lysis buffer as described above. Protein concentrations in the lysates were normalized as above. For this procedure, homogenate samples were placed in 96-well plates and the assay reagents added. The samples were read on a microplate spectrophotometer at 595 nm. A standard curve was generated using BSA prepared in homogenization buffer and was used to calculate the protein concentration using linear regression.

Intracellular ROS levels were assessed by detecting the signal produced by the Fe^{2+}-amplification of the oxidation of the fluorescent compound 2',7'-dichlorofluorscin diacetate (DCF-DA; Sigma) to the fluorophore, 2',7'-dichlorofluorscein (DCF; Sigma), upon interaction with peroxides, especially hydrogen peroxide. The fluorescent signal correlates linearly with cellular oxidation. This technique is considered to be a reliable marker for cellular oxidation (Mattson et al., 1995). Lysates obtained from each condition, and DCF-DA were added to glass tubes containing buffer. Following a 10-minute incubation, deferroxamine and Fe^{2+} were added for an additional 30-minutes. The content of the tubes was transferred to glass cuvettes (200 μl cuvette). The fluorescent DCF emissions were measured (excitation/emission 488/510 nm) using a flurospectrophotometer (BioRad). Background fluorescence was corrected by using parallel blanks. DCF (4 mg in 1 ml dimethylsulfoxide) in Locke's buffer was used as the standard, diluted to measure from 0 to 1000 pmole concentrations.

Statistical Analyses

Comparisons of the values obtained for the Western blot, JC-1 and ROS data were made via the Analysis of Variance (ANOVA), using the StatView program on a Macintosh G5 computer. This analysis was used to determine main effects of treatment, and was followed by the Fisher's Protected Least Significant Difference (PLSD) post-hoc test, where appropriate.

RESULTS

Bax Translocation in Ethanol and Vitamin E Cultures

Brief (40 minute) exposure of granule cells to 400 mg/dl ethanol resulted in a significant enhancement in mitochondrial localization of Bax protein, indicative of Bax translocation to the mitochondrial membrane. This effect was ameliorated, however, by co-treatment with

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vitamin E. The ANOVA applied to this data revealed a significant effect of condition ($F [3,17] = 15.49$, $p< 0.0001$). Post hoc comparisons showed an increase in mitochondrial Bax in the ethanol cultures compared to controls ($p=0.0020$), vitamin E ($p<0.0001$), and ethanol + vitamin E cultures ($p<0.0001$). Significant differences were also found between both vitamin E conditions and controls, with mitochondrial localization of Bax being somewhat lower in cultures treated with vitamin E ($p=0.0425$), or co-treated with ethanol + vitamin E ($p=0.0077$). The vitamin E alone and ethanol + vitamin E cultures did not differ. These results are depicted in Figure 2A. Representative Western blots are shown in Figure 2B.

Bax Translocation in Ethanol and BDNF Cultures

The inclusion of BDNF in the ethanol-exposed granule cells, like vitamin E, resulted in significantly diminished Bax translocation. The ANOVA applied to this data revealed a significant effect of condition ($F [3,4] = 12.58$, $p=0.0167$). Post hoc comparisons showed an increase in mitochondrial Bax in the ethanol cultures compared to controls ($p=0.0055$), BDNF ($p=0.0116$), and ethanol + BDNF cultures ($p=0.0076$). There were no differences between controls and BDNF alone, or controls and ethanol + BDNF conditions; and BDNF-alone and BDNF + ethanol values also did not differ. Thus, inclusion of BDNF in the culture medium along with ethanol provided a significant neuroprotective effect, since mitochonidrial Bax levels did not differ from controls. These results are depicted in Figure 3A. Representative Western blots are shown in Figure 3B.

JC-1 Analysis of Mitochondrial Membrane Integrity in Ethanol and Vitamin E Cultures

For the determinations of possible disruptions of the mitochondrial membrane potential, and the potential of vitamin E to mitigate these effects, the JC-1 procedure was used. As noted above, when this membrane is polarized, as in healthy cells, the dye fluoresces red, while in apoptotic cells, the membrane becomes depolarized, the monomeric JC-1 remains in the cytosol, and stains green. Thus, decreases in the red/green fluorescent intensity ratio are indicative of relative mitochondrial depolarization during apoptosis. The quantitative analyses of these intensity ratios in cultures with ethanol with and without vitamin E suggest that vitamin E had a significant protective effect. The ANOVA applied to these data shows a main effect of condition ($F [3,20] = 47.75$, $p< 0.0001$). Post hoc comparisons revealed significant reductions in the red/green ratio between ethanol-treated cells compared to (1) control cells, (2) vitamin E-only treated cells, and (3) ethanol + vitamin E-treated cells ($p <0.0001$ in all instances). The ratios measured in the ethanol + vitamin E cultures, however, differed from controls ($p=0.0012$), and vitamin E alone ($p=0.0003$). Thus, even in the presence of this antioxidant, the effects of ethanol were not completely blocked. The source of this residual disruption will be important to determine in future studies. It is possible that the antioxidant is less effective in modulating ethanol-induced alterations in other cellular elements or events capable of compromising or contributing to the compromise of mitochondrial membrane integrity, such as truncated Bid, Bak, or elevated intracellular calcium (Korsmeyer et al., 2000; Degenhardt et al., 2002; Ly et al., 2003). These results are presented in Figure 4.

JC-1 Analysis of Mitochondrial Membrane Integrity in Ethanol and BDNF Cultures

These analyses were conducted to determine the efficacy of the neurotrophin BDNF to protect against ethanol-mediated damage to the MMP. The ANOVA analyses of the JC-1 red/green ratios in cultures with ethanol, with and without BDNF, indicated a main effect of condition ($F [3,20] = 59.29$, $p< 0.0001$). Post hoc comparisons revealed significant ratio reductions in ethanol-treated cells compared to (1) controls, (2) BDNF-only treated cells, and (3) ethanol + BDNF-treated cells ($p <0.0001$ in all instances). In additional analyses of the cells grown in the ethanol + BDNF condition, ratio differences were found in comparisons with controls ($p=0.0285$), and BDNF alone ($p=0.0009$), with this measure

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being slightly but significantly lower in the ethanol + BDNF cells, indicating that, as with vitamin E, this NTF reduced but did not totally prevent the ethanol effect. As with the antioxidant, the source of this residual disruption will be important to determine in future studies. There were no differences in the intensity ratios between controls and BDNF-only cultures. These results are presented in Figure 5.

ROS Analyses with Ethanol and Vitamin E

This portion of the study was conducted to assess ROS generation as a function of ethanol exposure, and the potential of vitamin E to mitigate this effect. The ANOVA applied to these data revealed a significant main effect of condition ($F [3,36] = 11.52$, $p< 0.0001$). The individual post hoc comparisons showed that ethanol exposure resulted in significantly enhanced ROS levels compared to those measured in control, vitamin E, and ethanol + vitamin-treated cultures ($p <0.0001$ in each instance). ROS generation in control, vitamin E, and ethanol + vitamin E cells did not differ. Thus, vitamin E suppressed ROS generation in the presence of ethanol to control levels. These data are depicted in Figure 6.

ROS Analyses with Ethanol and BDNF

In cultures grown with ethanol, with and without BDNF, there was also a significant main effect of condition ($F [3,12] = 21.03$, $p< 0.0001$). The post hoc tests indicated that treatment with 400 mg/dl ethanol for 40 minutes increased ROS production compared to that measured in control ($p=0.0001$), BDNF ($p<0.0001$), and ethanol + BDNF cultures ($p<0.0001$). ROS generation in control, BDNF and ethanol + BDNF cells did not differ. Therefore, like vitamin E, this neurotrophin reduced ethanol-induced ROS to control levels. These data are presented in Figure 7.

DISCUSSION

It has previously been shown that developmental ethanol exposure leads to loss of cerebellar granule cells, both in vivo and in vitro (Hamre and West, 1993; Bhave et al., 1999; Moore et al., 1999). The current investigation indicates that precursors to this apoptotic cell death include Bax mitochondrial translocation, disruption of the mitochondrial membrane potential, and elevated generation of reactive oxygen species. We further show that each of these early, potentially lethal events, can be significantly blunted by co-administering either the antioxidant vitamin E or the neurotrophic factor BDNF with ethanol, a finding consistent with prior demonstrations of protection against ethanol neurotoxicity afforded by such substances (Bhave and Hoffman, 1997; Mitchell et al., 1999a; b;c). These findings, then, reveal important mechanisms contributing to the neuroprotective potential of both NTFs and antioxidants with respect to ethanol neurotoxicity. In the following sections, we consider functional mechanisms of Bax-initiated apoptosis, processes involved in antioxidant and NTF neuroprotection, and also consider the present findings in the context of prior studies.

Ethanol Effects on Bax Translocation, the MMP, and ROS Generation

Bax, a pro-apoptotic member of the Bcl-2 survival-regulatory gene family, is normally a 21kd monomeric cytosolic protein, which plays a key role in the intrinsic cell death pathway (Young et al., 2003). When Bax is activated by a range of stimuli (Gavathiotis at al., 2008), it undergoes conformational changes and translocates to the mitochondrial membrane, where it homodimerizes, and heterodimerizes with apoptosis antagonists Bcl-2 and Bcl-xl, abrogating their protective functions. Through interactions with the mitochondrial permeability transition pore complex, Bax-induced mitochondrial dysfunction leads to the release of cytochrome-C, increased generation of ROS, activation of caspases, and initiation of the execution phase of apoptosis (Marzo et al., 1998; Gross et al., 1999; Gao and Duo, 2000). Thus, Bax translocation is a critical upstream event in the apoptosis cascade.

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The present study demonstrates that even brief ethanol exposure in neonatal cerebellar granule cells produces rapid translocation of Bax to the mitochondrial membrane, subsequent disruption of the mitochondrial membrane potential, and enhanced generation of harmful ROS. These observations of Bax activation in ethanol-mediated cell death in the developing CNS are consistent with several prior studies. Bax expression, for example, has been found to be elevated in response to ethanol exposure in a range of CNS regions, both in vivo and in vitro (e.g., Moore et al., 1999; Olney et al., 2001; Heaton et al., 2003a; b; Ge et al., 2004; Schindler et al., 2004; Nowoslawski et al., 2005; Siler-Marsiglio et al., 2005a; Lee et al., 2008). Bax has also been found to dimerize with Bcl-2 and Bcl-xl proteins following ethanol exposure, thus neutralizing their anti-apoptotic potential (Siler-Marsiglio et al., 2005a). In developing cerebellum, such pro-apoptotic protein-protein interactions in response to ethanol treatment are particularly marked at neonatal ages which are maximally sensitive to ethanol (e.g., postnatal day 4 [P4]), compared to later, ethanol-resistant ages (P7; Siler-Marsiglio et al., 2005a). In Bax gene-deleted animals, the early CNS appears less vulnerable to ethanol insult, and ROS production following ethanol treatment is also mitigated in these mutants (Young et al., 2003; Heaton et al., 2006).

The findings of ethanol-induced alterations in mitochondrial function are also in agreement with previous studies, in which this organelle has been shown to be a major target of ethanol, with some investigators suggesting that disturbance of mitochondrial membrane integrity is essential for ethanol-induced cell death (e.g., Ramachandran et al., 2001; Li et al., 2002; Adachi et al., 2004; Koch et al., 2004). Similarly, ethanol-related increases in ROS generation have been well-documented, and appear to be a prime factor in ethanol neurotoxicity, particularly since ethanol also downregulates protective cellular antioxidant content, thus seriously disturbing the cellular redox state (e.g., Ramachandran et al., 2003; Lee et al., 2005).

The Potential of Vitamin E to Ameliorate Ethanol-Induced Effects on Bax, the MMP, and ROS Formation

Vitamin E and other antioxidants are potent sources of protection against various forms of cellular trauma, including ischemic and traumatic brain injury, 1-methyl-4 phenylpyridinium (MPP+) toxicity, and ethanol-induced neurotoxicity and dysmorphogenesis. These beneficial effects have been found both in vitro and in vivo (e.g., Mattson et al., 1995; Kotch et al., 1995; Henderson et al., 1995; Agar et al., 1999; Mitchell et al., 1999a; b; Heaton et al., 2000a: Siler-Marsiglio et al., 2005b; Shirpoor et al., 2009). The present study suggests that important processes underlying this protective capacity with respect to ethanol include regulation of Bax mitochondrial translocation, stabilization of the critical MMP, and reduction in generation of cellular ROS. Both Bax translocation and ROS production in the presence of ethanol were restored to control levels when vitamin E was added to the culture medium, while MMP disruption was appreciably reduced.

There are a number of molecular mechanisms by which vitamin E can protect against neurotoxicity: In addition to its antioxidant/radical scavenging capacities, this molecule has other important, non-oxidant, properties. These include influences on gene expression, involving regulation of transcription, mRNA stability, protein translation, protein stability, and post-translational events (Ricciarelli et al., 2001; Azzi et al., 2003; Osakada et al., 2003). This antioxidant can also influence cellular oxidative homeostasis by upregulating endogenous antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH; Perumal et al., 1992; Agar et al., 1999; Osakada et al., 2003). Consistent with the present study, vitamin E has previously been demonstrated to affect expression and/or functionality of apoptosis-related proteins, including those of the Bcl-2 gene family, following exposure to ethanol and other adverse conditions. These effects have been seen in a variety of neuronal and non-neuronal cells, including PC12, endothelial and smooth

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muscle cells; neonatal myocytes; hippocampal and striatal neurons; and cerebellar granule cells. This modulation typically entails upregulation of pro-survival proteins (e.g., Bcl-2, Bcl-xl) and concurrent downregulation of proapoptotic substances (e.g., Bax, Bcl-xs, cytochrome-C, caspases; Haendeler et al., 1996; de Nigris et al., 2000; Qin et al., 2001; Post et al., 2002; Gonzalez-Polo et al., 2003; Heaton et al.,2004a; Siler-Marsiglio et al., 2004b). Our finding that vitamin E can mitigate ethanol-induced alterations in the MMP are also consistent with observations from prior investigations of antioxidant protection in the presence of toxic conditions. In an in vivo study of mouse skeletal muscle, for example, it was reported that vitamin E treatment prevented the mitochondrial dysfunction normally produced by imposition of hypoxic conditions (Magalhães et al., 2007).

The Potential of BDNF to Ameliorate Detrimental Ethanol Effects on Bax, the MMP, and ROS Formation

This investigation has shown that in addition to vitamin E, the neurotrophin BDNF protects cerebellar granule cells by mitigating ethanol-induced Bax translocation, decreasing the ethanol-mediated disruption of the MMP, and by diminishing levels of ROS. The protective effects of this neurotrophin paralleled those of vitamin E, with complete amelioration of ethanol-mediated Bax mitochondrial translocation and enhanced ROS generation, and significant reduction of the disruptive effects of ethanol on the MMP.

BDNF is a powerful survival factor for many neuronal phenotypes, including cerebellar granule cells, and this NTF and other members of the neurotrophin gene family are known to have multi-faceted roles in the developing CNS. Granule cells express the TrkB receptor, and BDNF suppresses apoptosis, at least in part by binding to this receptor and activating the PI3-kinase/Akt survival pathway (Li et al., 2004). BDNF and other NTFs have been found to protect against a number of toxic conditions, including low potassium levels, oxidative stress, glucose deprivation, and ethanol exposure (Kubo et al., 1995; Skaper et al., 1998; Tong and Perez-Polo, 1998; Mitchell et al., 1999c; de la Monte and Wands, 2002; Bonthius et al., 2003).

In some CNS regions, ethanol exposure during development reduces production and/or availability of BDNF and other neurotrophic proteins, thus potentially enhancing ethanol vulnerability (e.g., Vallés et al., 1994; Heaton et al., 1999; 2004b; McAlhany et al., 1999; Sakai et al., 2005). The importance of these trophic substances and their potential to modulate ethanol toxicity are underscored not only by observations of NTF rescue of cultured cells from a number of CNS regions from ethanol-induced cell death, but also by investigations of ethanol effects in NTF gene-altered animals. In BDNF knockouts, for example, the developing cerebellum exhibits increased ethanol-mediated cell loss, while overproduction of the related NTF, nerve growth factor (NGF), in transgenic animals, appears to blunt cell death in this region during early periods of maximal ethanol susceptibility (Heaton et al., 2000b). Basal levels of BDNF tend to be at appreciably higher during period of ethanol resistance in the early CNS, relative to earlier, ethanol sensitive periods (e.g., Heaton et al., 2003a; b).

As with vitamin E, NTFs such as BDNF appear to exert their protective functions through a variety of mechanisms, including regulating expression of elements of the apoptosis cascade, inhibiting oxidative stress, and enhancing antioxidant expression and/or activity (Lindholm et al., 1993; Courtney et al.1997; Perez-Navarro et al., 2005). Therefore, the mechanisms of BDNF protection against ethanol-mediated neurotoxicity seen in the present study are in agreement with a number of prior studies demonstrating the manner in which neurotrophic substances, particularly those of the neurotrophin gene family (e.g., BDNF, NGF, NT-3) act: In hippoampal CA1 neurons, for example, protective effects of BDNF are accompanied by upregulation of Bcl-xl at both the mRNA and protein levels, and protection

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of these neurons against glutamate-induced apoptosis is associated with enhanced Bcl-2 protein, and diminished caspase-3 activity (Almeida et al., 2005; Chao et al., 2010). Similarly, BDNF protects against excitotoxicity-induced neuronal damage in rat striatum at least in part by increasing expression of Bcl-2, Bcl-xl, decreasing Bax, and suppressing heterodimerization between Bax and pro-survival proteins (Perez-Navarro et al., 2005). In addition, BDNF protects cortical neurons and neuroblastoma cells against neurotoxic agents at least in part by modulating expression of the pro-apoptotic Bim protein (Li et al., 2007; Almeida et al., 2009). The critical relationship between Bax activation and BDNF-mediated neuronal survival was demonstrated in a study in which double Bax/BDNF null mutations were used. This investigation revealed that loss of Bax completely prevented the cell loss usually seen in the absence of BDNF. Thus, BDNF normally appears to promote neuronal survival by inhibiting activation of the Bax-dependent cell death pathways (Hellard et al., 2004).

NTFs have also been shown to have a strong influence on the cellular redox state, both by reducing ROS generation, and by modulating expression and/or stability of protective antioxidant enzymes. In a number of populations in vitro (e.g., hippocampal and auditory neurons, PC12 cells, the CATH catecholaminergic cell line) BDNF has been shown to increase activity levels of SOD, GSH (total, reductase and peroxidase) and CAT (Gabaizadeh et al., 1997; Gong et al., 1999; Mattson et al., 1995; Yamagata et al., 1999; Onyango et al., 2005). In the intact animal, chronic intrathecal infusion of BDNF following compression-induced spinal cord injury stabilizes SOD, enhances glial expression of myelin basic protein, and facilitates behavioral recovery (Ikeda et al., 2002). Conversely, ROS production can be elicited by decreased levels of NTFs, or by NTF withdrawal (Schulz et al., 1997; Skaper et al., 1998). Thus, the BDNF-mediated reductions of ethanol-induced ROS seen in the present study are entirely consistent with these earlier observations.

CONCLUSIONS

In summary, this investigation suggests critical mechanisms underlying ethanol neurotoxicity in developing neurons, and demonstrates the potential of exogenously applied antioxidants and neurotrophic factors to ameliorate these detrimental effects, thus considerably increasing the likelihood of neuronal survival under these toxic conditions. Such insights may eventually lead to strategies for minimizing ethanol-mediated cell death in the developing CNS, strategies which can be implemented in vivo. Future research in this regard should consider (1) aspects of the intracellular pathways involved in this neuroprotection, (2) points at which intervention might be most effectively applied, (3) the potential of additional NTFs or antioxidants to afford neuroprotection comparable to that seen with the substances chosen for the present study, and (4) the extent to which this neuroprotection translates to amelioration of behavioral and cognitive deficits seen following developmental ethanol exposure.

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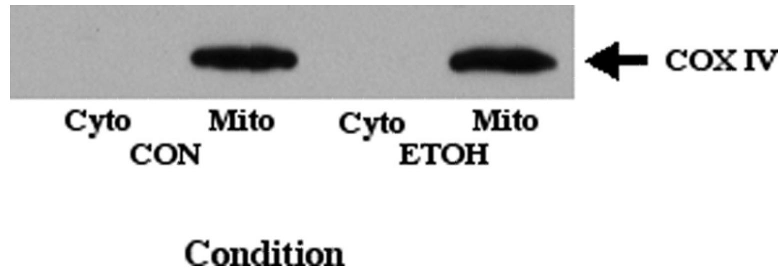

Figure 1.

Western blots of cytochrome oxidase subunit Vb (COX IV) protein in cytosolic (Cyto) and mitochondrial (Mito) subcelular fractions derived from granule cells cultured in control medium (CON) and in medium containing 400 mg/dl ethanol (ETOH).

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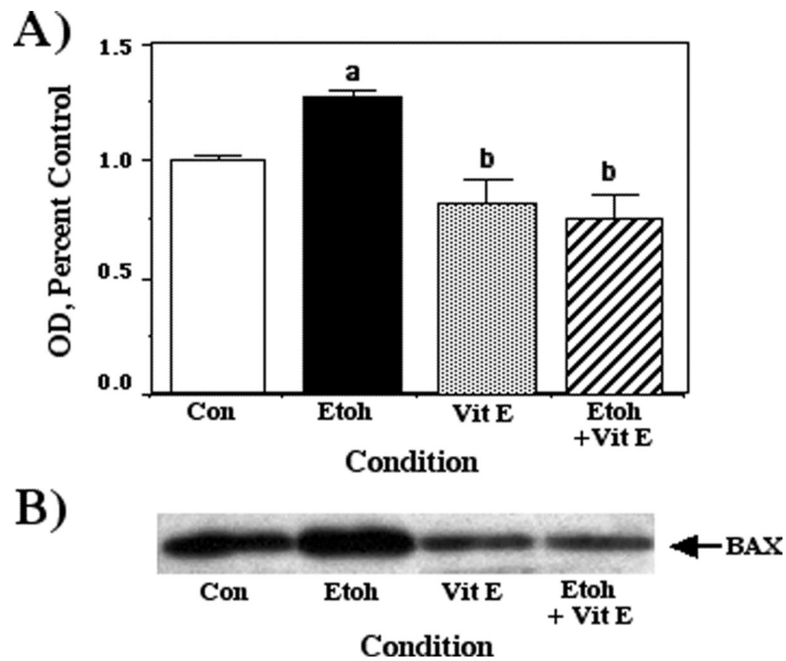

Figure 2.

(A) Bax mitochondrial translocation was measured by quantification of optical density (OD) levels of Western blots from isolated mitochondrial fractions derived from cerebellar granule cells cultured in control conditions (Con), and with 400 mg/dl ethanol (Etoh), 50 μM vitamin E (Vit E), and ethanol + vitamin E (Etoh+Vit E). The Cultures were established for 24 hours in serum-containing medium, then changed to defined medium. Experimental conditions were applied for 40 minutes. Error bars represent standard error of the mean (SEM). a=Significantly greater than Con, Vit E, and Etoh+Vit E, $p \leq 0.002$; b=significantly less then Con, $p < 0.05$. See text for exact p-values. (B) Representative Western blots of mitochondrial Bax protein in the four conditions.

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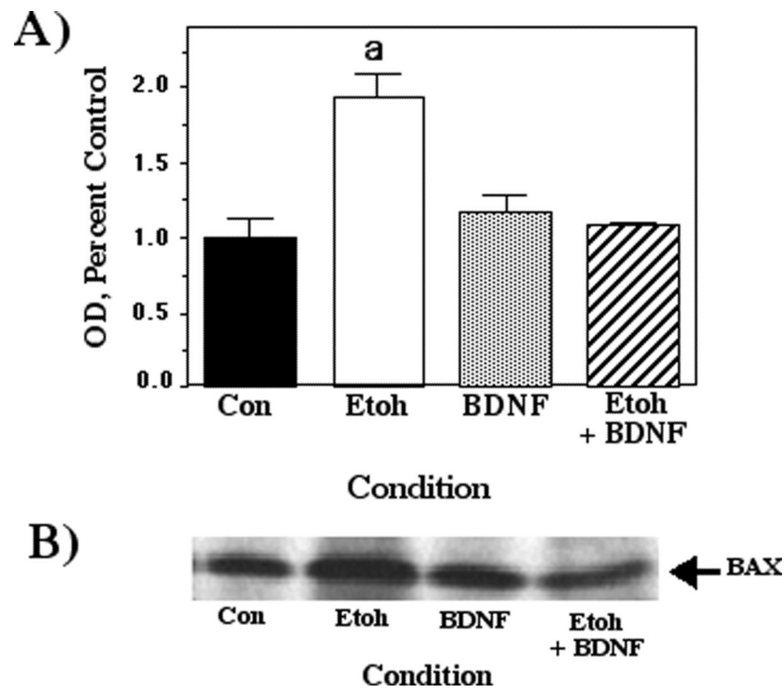

Figure 3.

(A) Bax mitochondrial translocation was measured by quantification of optical density (OD) levels of Western blots from isolated mitochondrial fractions derived from cerebellar granule cells cultured in control conditions (Con), and with 400 mg/dl ethanol (Etoh), 50 ng/ml BDNF, and ethanol + BDNF (Etoh+BDNF). The Cultures were established for 24 hours in serum-containing medium, then changed to defined medium. Experimental conditions were applied for 40 minutes Error bars represent standard error of the mean (SEM). a=Significantly greater than Con, BDNF, and Etoh+BDNF, $p<0.02$. See text for exact p-values. (B) Representative Western blots of mitochondrial Bax protein in the four conditions.

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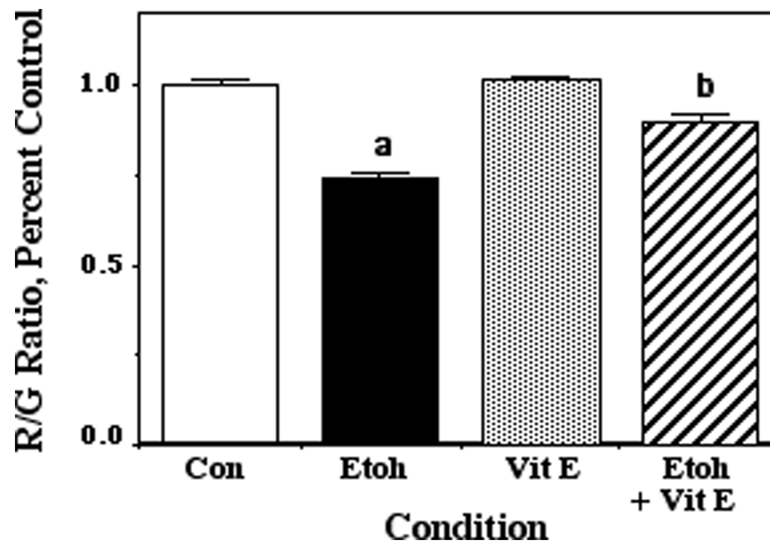

Figure 4.

Assessments of mitochondrial membrane potential using the JC-1 procedure. Decreases in the red/green fluorescent intensity ratio reflect mitochondrial membrane depolarization during the apoptosis process. Cells were cultured in control conditions (Con), and with 400 mg/dl ethanol (Etoh), 50 μM vitamin E (Vit E), and ethanol + vitamin E (Etoh+Vit E). The Cultures were established for 24 hours in serum-containing medium, then changed to defined medium. Experimental conditions were applied for 60 minutes. Error bars represent standard error of the mean (SEM). a=Significantly lower than Con, Vit E, and Etoh+Vit E, $p<0.0001$. b=significantly lower than Con and Vit E alone, $p<0.002$. See text for exact p-values.

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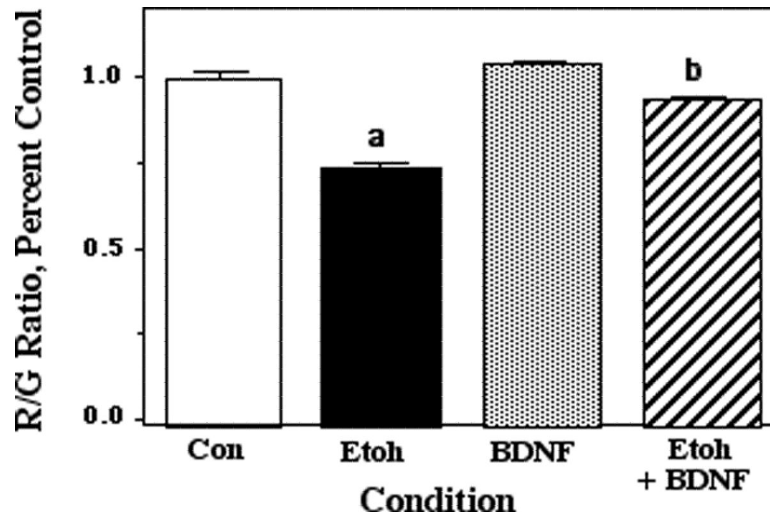

Figure 5.

Assessments of mitochondrial membrane potential using the JC-1 procedure. Decreases in the red/green fluorescent intensity ratio reflect mitochondrial membrane depolarization during the apoptosis process. Cells were cultured in control conditions (Con), and with 400 mg/dl ethanol (Etoh), 50 ng/ml BDNF, and ethanol + BDNF (Etoh+BDNF). The Cultures were established for 24 hours in serum-containing medium, then changed to defined medium. Experimental conditions were applied for 60 minutes. Error bars represent standard error of the mean (SEM). a=Significantly lower than Con, BDNF, and Etoh+BDNF, $p<0.0001$. b=Significantly lower than Con and BDNF alone, $p<0.03$. See text for exact p-values.

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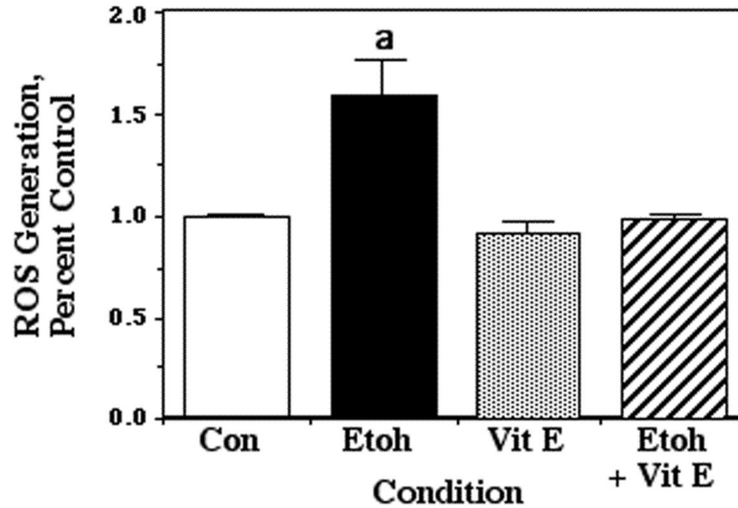

Figure 6.

Analyses of ROS generation in granule cells cultured in control conditions (Con), and with 400 mg/dl ethanol (Etoh), 50 μM vitamin E (Vit E), and ethanol + vitamin E (Etoh+Vit E). The Cultures were established for 24 hours in serum-containing medium, then changed to defined medium. Experimental conditions were applied for 40 minutes. Error bars represent standard error of the mean (SEM). a=Significantly increased compared to Con, Vit E, and Etoh+Vit E, $p<0.0001$.

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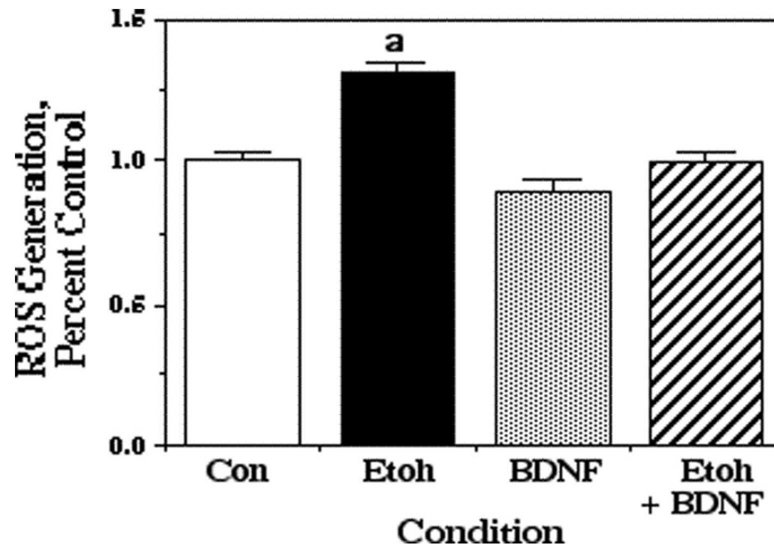

Figure 7.

Analyses of ROS generation in granule cells cultured in control conditions (Con), and with 400 mg/dl ethanol (Etoh), 50 ng/ml BDNF, and ethanol + BDNF (Etoh+BDNF). The Cultures were established for 24 hours in serum-containing medium, then changed to defined medium. Experimental conditions were applied for 40 minutes. Error bars represent standard error of the mean (SEM). a=Significantly increased compared to Con, BDNF, and Etoh+BDNF, $p<0.0001$.