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Ethanol Induces Cytotoxic Oxidative Stress in PC12 Cells: Protection by Reactive Oxygen Species Scavengers

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Abstract: Normal development of the Central Nervous System (CNS) requires a delicately timed sequence of neuronal proliferation, differentiation and remodeling. Ethanol exposure during gestation has been shown to perturb brain development. The mechanisms underlying this ethanol-induced toxicity are complex. Previous studies have indicated that ethanol exerts at least some of its neurotoxic effects via oxidative stress. Thus, we undertook a series of experiments to evaluate the role of oxidative stress in the toxic effects of ethanol on naive PC12 cells. In initial studies we evaluated whether Reactive Oxygen Species (ROS) were induced in PC12 cells exposed to increasing concentrations of ethanol. ROS levels in cells were quantitated using fluorescence microscopy and the superoxide sensitive dye, dihydroethidium. We then utilized adenovirus-mediated infection techniques to determine whether the over-expression of ROS scavengers, namely catalase, copper-zinc superoxide dismutatase (CuZnSOD) or manganese superoxide dismutatase (MnSOD), could protect PC12 cells from the cytotoxic effects of ethanol. The results obtained demonstrated a dose-dependent increase in ethidium positive cells in response to ethanol, indicating an increase in oxidative stress. In addition, PC12 cells over-expressing catalase, CuZnSOD and MnSOD were significantly protected from the cytotoxic effects of ethanol at concentrations greater than 50 mM as measured by cell viability assay. The level of protection afforded was greatest by MnSOD follwed by CuZnSOD and then catalase over-expression. We suggest that ethanol exerts its cytotoxic effects on PC12 cells by increasing mitochondrial superoxide production. Furthermore, we suggest that some of the neuronal cell death associated with Fetal Alcohol Syndrome (FAS) may be due to an increase in oxidative stress induced by ethanol metabolism.

Key words: Oxidative stress, antioxidant enzymes, alcohol, neuronal

Introduction

Normal development of the CNS requires a precisely timed sequence of neuronal proliferation and migration, neural process outgrowth, synapse formation and synaptic connection remodelling. As a result, early insults often are not isolated but have later ramifications, making the immature CNS uniquely vulnerable. As ethanol readily crosses the placental barrier, alcohol levels in the fetus and mother are essentially equivalent (Waltman and Iniquez, 1972). The fetus has only a limited ability to metabolize alcohol due to the absence of hepatic alcohol dehydrogenase and so it must rely on passive diffusion and maternal elimination to reduce alcohol levels (Dow and Riopelle, 1987).

Ethanol exposure during gestation has been shown to alter normal CNS development (Kotkoskie and Norton, 1988), particularly neuromorphogenesis (Miller, 1986) but also cognition. Ethanol

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therefore not only interferes with gross organ development but also with the formation of normal neural processes. Furthermore, the effect of ethanol appears to be different in discrete areas of the brain. Using the rat as a model, studies have determined that the cerebral cortex is particularly susceptible to the adverse effects of prenatal alcohol exposure (Miller, 1986, 1987), while the cerebellum appears to be relatively resistant to damage. The laminar organization of the cortex is disrupted with aberrantly distributed neurons throughout its layer following prenatal alcohol exposure, suggesting that normal patterns of neuronal migration are adversely affected. In contrast, postnatal alcohol exposure in the rat appears to have little effect on the cortex but has a disruptive effect on the cerebellum, where Purkinje cells appear to be the most at risk (Cragg and Philips, 1985; Pierce *et al*., 1993). Exposure to ethanol decreases dendritic branching and spine density in Purkinje cells and CA1 hippocampal neurons of adult rats and mice; it also inhibits dendrite growth and differentiation in pyramidal cells of developing neocortex and hippocampus (Hammer, 1986). However, in other regions of the brain, ethanol exposure actually enhances neural process outgrowth. Upon ethanol exposure during development, the length of terminal dendrite segments in Purkinje cells are increased (Tavares *et al*., 1986; Pentney and Quackenbush, 1990) and the number of dendritic spines in hippocampal dentate granule neurons (King *et al*., 1988) and somatosensory cortical pyramidal cells (Ferrer *et al*., 1989) are increased. Thus, the effects of ethanol on CNS are not only varied but are also timing dependent.

Presently, the mechanisms by which ethanol exerts its cytotoxic effects are, at best, poorly understood. This is due both to the heterogeneity of the brain and to the diverse effects of ethanol, making it difficult to determine cause and effect in whole animal models. The potential for generation of excess amounts of ROS during ethanol metabolism is particularly compelling. However, the extent to which this activity actually contributes to the overall toxicity of ethanol remains unclear. Studies have shown that ethanol exposure produced a decrease in reduced glutathione (GSH) levels and elevated rates of lipid peroxidation (Bondy, 1992). Most studies have evaluated the effects of ethanol on liver function; parallel studies in developing brain remain more equivocal (Rouach *et al*., 1997). Nonetheless, links have been found between ethanol and generation of increased ROS in neural crest cells (Davis *et al*., 1990), whole brain homogenates (Uysal *et al*., 1989a), neonatal brain (Ledig *et al*., 1981) and in PC12 cells (Sun *et al*., 1997).

A protective role for ROS scavengers in the context of ethanol toxicity has also been demonstrated. Antioxidants have been shown to prevent cerebral vascular damage after ethanol exposure (Altura and Gebrewold, 1996). In another study, ethanol-induced dysmorphogenesis in whole mouse embryos was decreased when ROS scavengers were added to the culture medium (Kotch *et al*., 1995). Similar results were shown with cultured neural crest cells (Davis *et al*. 1990; Chen *et al*., 1992) and cultured fetal hippocampal neurons (Mitchell *et al*., 1999).

Therefore, the present study was undertaken to investigate whether ethanol increased ROS levels and to further investigate whether the over-expression of ROS scavenging proteins could be utilized to reduce ethanol-mediated toxicity. We utilized the rat pheochromocytoma cell line, PC12, in our studies. Investigators have utilized this cell line as a model system in which to examine the molecular effects of ethanol on neuronal differentiation. PC12 cells are adrenergic chromaffin-like cells derived from the same population of neural crest cells that gives rise to sympathetic neurons during development and so they are particularly relevant (Greene and Tischler, 1976; Greene *et al*., 1987). The results obtained demonstrated a dose-dependent increase in ROS generation in naïve PC12 cells in response to ethanol indicating an increase in oxidative stress. In addition PC12 cells over-expressing catalase, CuZnSOD and MnSOD were significantly protected from the cytotoxic effects of ethanol at concentrations of greater than 50 mM. Results obtained indicated that the level of protection afforded was greatest with MnSOD followed by CuZnSOD and then catalase.

Materials and Methods

Cell Culture and Adenoviral Infections

PC12 cells were cultured and passaged as previously described (Sheehy *et al*., 1997). For adenoviral infections, cells (500,000) were plated onto collagen IV (BioCoat) coated 35 mm dishes. Cells were allowed to attach overnight before being infected. PC12 cells (500,000 cells) were plated onto 35 mm dishes, allowed to adhere overnight, then infected with either AdCuZnSOD, or AdMnSOD at an MOI of 100:1 in 1 mL of growth medium (preliminary experiments indicated that this MOI resulted in 50-60% of cells being infected). Cells were then incubated in 21% O_2 and 5% CO_2 at 37°C for 18 h when a further 2 mL of growth medium was added. Cells were then incubated for a further 24 h to allow expression of the gene of interest. Ethanol (0-200 mM) was then added. In all cases the dishes were sealed using paraffin to prevent evaporation.

Cell Proliferation Assays

Infected PC12 cells were seeded onto 96-well plates (Costar) at 25% confluence and allowed to adhere for at least 18 h. Cells were washed with PBS and incubated with complete medium containing ethanol (0-200 mM). After 24 h the number of viable cells were determined using the Cell Titer 96 AQueous One Solution kit (Promega) according to the manufacturer's instructions. Following a 4 h incubation period the absorbance at 492 nm was read using a Labsystems Multiskan EX plate reader (Fisher).

Fluorescence Analysis

Cells were seeded onto 24-well plates (Costar) and allowed to adhere for at least 18 h. Cells were then washed in PBS and incubated in complete medium then ethanol (0-200 mM) was added for 6 h. Subsequently, dihydroethidium (20 μM, Molecular Probes) was added. Cells were washed with PBS and imaged using a Nikon TE300 inverted fluorescent microscope. Dihydroethidium-stained cells were observed using excitation at 518 nm and emission at 605 nm. Fluorescent images were captured using a Cool-Snap digital camera and quantified using Metamorph imaging software (Fryer). Statistical analyses between treatments were carried out as detailed below.

Assay for SOD activity

This was determined using the NBT in-gel-assay originally reported by Beauchamp and Fridovich (1971). Briefly, cell extracts were separated on non-denaturing 12% -polyacrylamide gels. The gels were then soaked in ice-cold Solution A (25 mg NBT, 10 mg riboflavin in 100 mL of doubly distilled water) for 20 min. Thereafter solution B (1% TEMED in 100 mL doubly distilled water) was added and the gel illuminated on a lightbox. The SOD bands appear colorless against a blue background and were quantitated as described below.

Assay for Catalase Activity

Catalase was measured by a manual method as described (Aebi, 1974). The method is based on the rate of degradation of hydrogen peroxide to form water and oxygen over time as a measure of catalase activity. Total protein extracts (40 μg) were diluted to 1000 μL in 50 mM phosphate buffer (pH 7.0). One milliliter of 10 mM H_2O_2 solution was added and the decomposition of the substrate recorded by the decrease in absorbance at 240 nm over a 30 sec period. Catalase activity was expressed as degradation of H_2O_2 μg protein/min.

Statistical Analysis

Quantitation of SOD in-gel assays results were performed by scanning (Microtek E6, Microtek, Compton, CA) the bands of interest into an image editing software program (Adobe Photoshop, Adobe Systems, Mt. View, CA). Band intensities were analyzed densitometrically on a Macintosh computer (model G4, Apple Computer, Inc., Cupertino, CA) using the public domain NIH Image Program (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). The means±standard deviation were calculated for the relative SOD activities and were compared by the unpaired t-test using the GB-STAT software program. A $p<0.05$ was considered statistically significant.

The relative fluorescence calculated for ethidium was expressed as mean±standard deviation. Comparisons between treatment groups were made by the unpaired t-test using the GB-STAT software program. A $p<0.05$ was considered statistically significant.

For cell viability assays the mean±standard deviation was calculated for all ethanol concentrations. Comparisons between treatment groups were made by the unpaired t-test using the GB-STAT software program. A $p<0.05$ was considered statistically significant.

Results

Ethanol Induced ROS Production

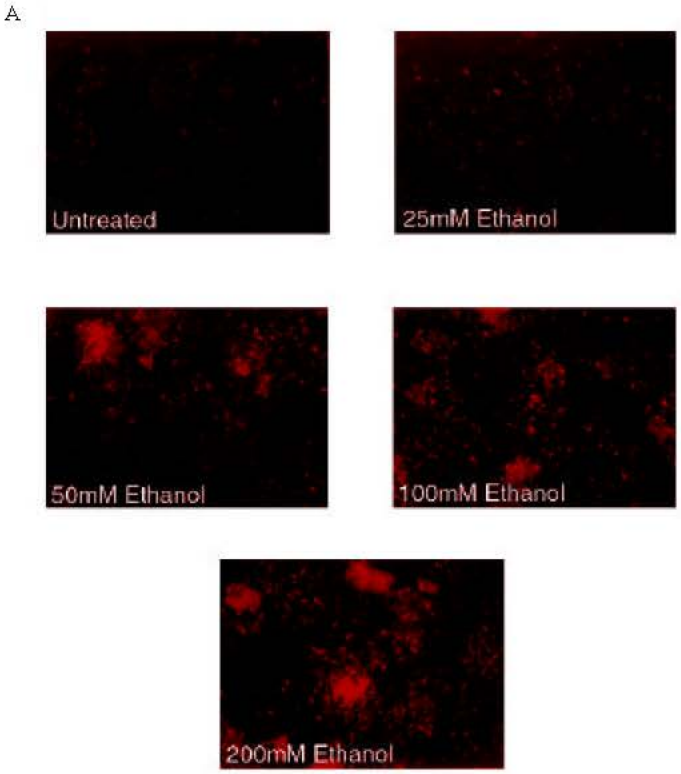
Initially we determined whether ethanol could stimulate ROS production in PC12 cells. Ethanol-mediated increased superoxide production by PC12 cells was determined using dihydroethidium, a superoxide-sensitive fluorescent dye and fluorescent microscopy. Results obtained demonstrated that exposure of PC12 cells to increasing concentrations of ethanol (0-200 mM) for 6 h resulted in a dose-dependent increase in superoxide production up to 100 mM then a plateau at 200 mM (Fig. 1).

Over-expression of ROS Scavenging Proteins

We over-expressed the ROS scavenging proteins CuZnSOD, MnSOD and catalase in PC12 cells utilizing adenoviral-mediated infections. Initial results utilizing an adenoviral vector that expresses human catalase indicated that the over-expression of catalase increased the amount of immunoreactive catalase protein (Fig. 2A). In addition cellular catalase activity, as measured by the metabolism of H_2O_2, was also shown to be increased, by 12.8-fold relative to uninfected cells ($p<0.05$, Fig. 2B). Results obtained also indicated that the over-expression of CuZnSOD increased the amount of immunoreactive CuZnSOD protein (Fig. 3A). SOD activity, as determined by the NBT in gel assay, was also increased, by 3.43-fold relative to uninfected cells ($p<0.05$, Fig. 3B and C). Similarly, the over-expression of MnSOD increased the amount of immunoreactive MnSOD protein (Fig. 4A). SOD activity, as determined by the NBT in gel assay, was also increased, by 3.65-fold relative to uninfected cells ($p<0.05$, Fig. 4B and C).

Effects of Ethanol on PC12 Cell Viability

Finally, we determined whether ethanol had a cytotoxic effect on naïve PC12 cells. In cell viablity assays, we demonstrated that ethanol produced an dose-dependent decrease in PC12 cell viability during a 24 h exposure (Fig. 5). This decrease was prevented in cells infected with the ROS scavenging proteins, CuZnSOD, MnSOD and catalase (Fig. 5). However, the level of protection afforded was not equal. The results obtained indicated that Mn-SOD gave a greater protection than CuZnSOD, which gave a greater protection than catalase (Fig. 5).



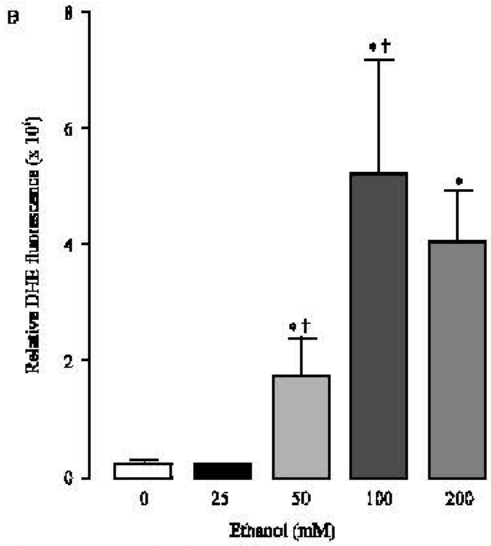


Fig. 1: *In situ* detection of superoxide in PC12 cells exposed to ethanol. Panel A. Representative fluorescent images of dihydroethydium (DHE)-treated PC12 cells in response to ethanol (0-200 mM)). Conversion of DHE by superoxide to ethidium results in red nuclear fluorescence. Under identical imaging conditions, fluorescence is increased in response to increasing ethanol concentrations. Panel B The fluorescent intensity of each image was quantified using Metamorph Imaging software and represented graphically. N = 8. Values are mean±SD. * $p<0.05$ vs untreated (UTD), † $p<0.05$ vs previous ethanol concentration

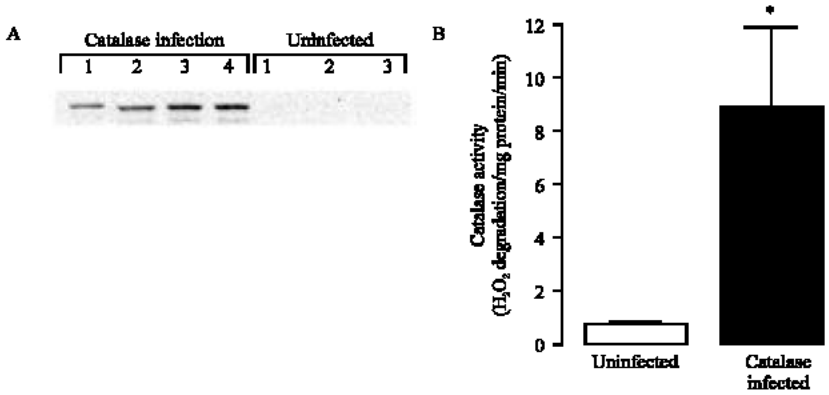


Fig. 2: Adenoviral mediated infection PC12 cells increases cellular CuZnSOD activity. Panel A: Protein extracts (20 μg), prepared from PC12 cells infected with AdCuZnSOD or uninfected, were separated on 12% denaturing polyacrylamide gels, electrophoretically transferred to Hybond membranes and analyzed using a specific antiserum raised against CuZnSOD. Panel B: Protein extracts (XX μg), prepared from PC12 cells infected with AdCuZnSOD, or uninfected, were separated on 12% non-denaturing polyacrylamide gels and subjected to the NBT in-gel-assay. Panel C: The values for relative SOD activity from 4 infections. Values are mean±standard deviation. * $p<0.05$ vs uninfected

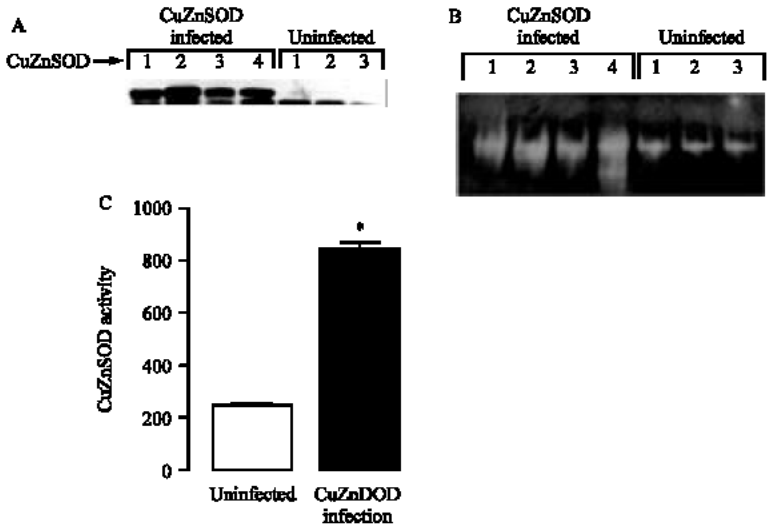


Fig. 3: Adenoviral mediated infection PC12 cells increases cellular MnSOD activity. Panel A: Protein extracts (20 μg), prepared from PC12 cells infected with AdMnSOD or uninfected, were separated on 12% denaturing polyacrylamide gels, electrophoretically transferred to Hybond membranes and analyzed using a specific antiserum raised against MnSOD. Panel B: Protein extracts (20 μg), prepared from PC12 cells infected with AdMnSOD, or uninfected, were separated on 12% non-denaturing polyacrylamide gels and subjected to the NBT in-gel-assay. Panel C. The values for relative SOD activity from 4 infections. Values are mean±standard deviation. * $p<0.05$ vs uninfected

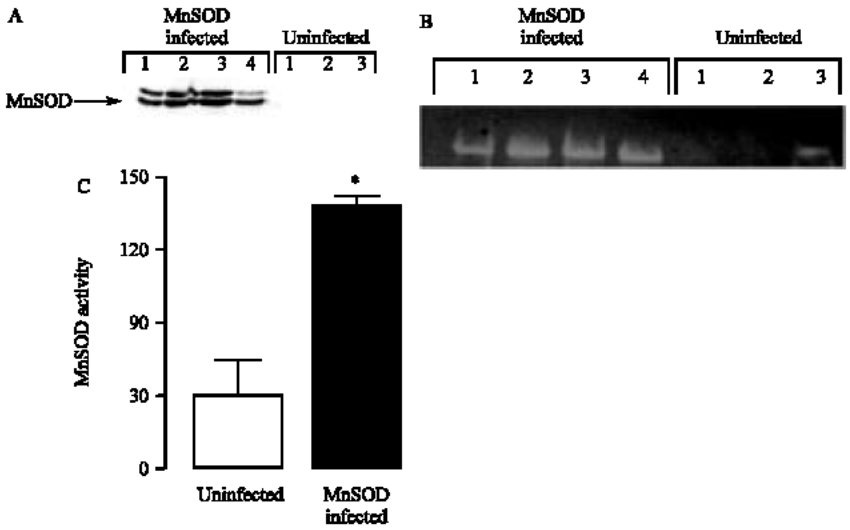


Fig. 4: Adenoviral mediated infection PC12 cells increases cellular catalase activity. Panel A: Protein extracts (20 μg), prepared from PC12 cells infected with Adcatalase or uninfected, were separated on 12% denaturing polyacrylamide gels, electrophoretically transferred to Hybond membranes and analyzed using a specific antiserum raised against catalase. Panel B: The values for relative catalase activity from 4 infections. Values are mean±standard deviation. * $p<0.05$ vs uninfected

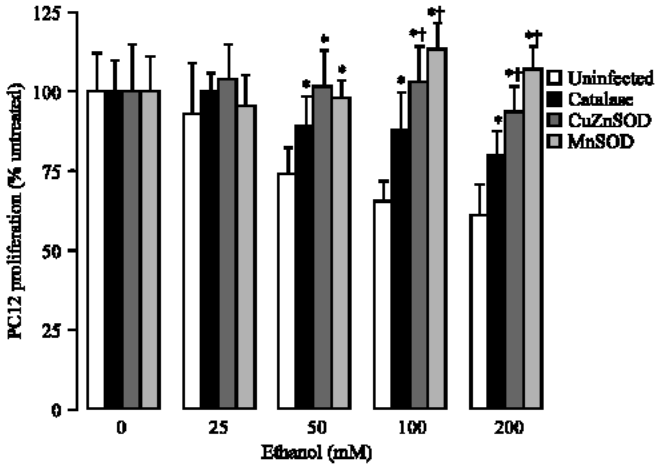


Fig. 5: Expression of ROS scavenging proteins protects PC12 cells from the cytotoxic effects of ethanol. Equal numbers of PC12 cells (3,000) were plated in a 96 well plate and allowed to adhere overnight. Ethanol (0- 200mM) was then added. After 24 h the number of viable cells was determined. Results are expressed relative to cells not exposed to ethanol and expressed as mean±SE from 3 different infections. * = $p<0.05$ vs untreated cells, † $p<0.05$ vs Catalase infected, ‡ = $p<0.05$ vs CuZnSOD infected

Discussion

The fetotoxic effects of maternal ethanol consumption have been documented for over three decades, yet the mechanisms underlying this devastating phenomenon remain uncertain. The wide

variety of cellular and biochemical effects of ethanol on fetal tissues is perplexing and is strongly suggestive of multiple mechanisms leading to the typical pathologic picture. Our results, utilizing the PC12 cell line as an *in vitro* model of neuronal cells, indicate that ROS generation, specifically the superoxide anion radical, is increased in a dose-dependent fashion in response to ethanol exposure. Concurrently, cell viability decreases in a dose-dependent manner. Cytotoxicity due to ethanol can be ameliorated by over-expressing ROS scavenging proteins, namely catalase, CuZnSOD and MnSOD. Therefore, ROS generation may be one important factor contributing to the ethanol effects that result in FAS. This contention is consistent with what is known about ROS. Oxygen free radicals are known to adversely affect a variety of cellular elements including proteins, DNA bases and sugars, polysaccharides and lipids. It is well documented that oxygen free radicals react with unsaturated membrane lipids, initiating a self-perpetuating process (Halliwell, 1992). This reaction can produce loss of membrane function and ultimately cell death given sufficiently severe damage. Ethanol itself has been shown to induce lipid peroxidation (Rouach *et al*., 1987). Indeed, the metabolism of ethanol produces reactive aldehydes then intermediate radicals formed in the peroxidation process; these in turn adversely affect a variety of cellular functions which are key for growth and development. Adverse events include cytoskeletal disruption (VanWinkle *et al*., 1994), mitochondrial dysfunction (Dicker and Cederbaum, 1988; Coleman and Cunningham, 1990) and alteration of membrane protein receptors and subsequent signal transduction (Hoek and Rubin, 1990).

Recent studies have documented evidence of oxidant stress in fetal brain following short-term maternal ethanol consumption (Henderson *et al*., 1995). A binge model of ethanol exposure increased malondialdehyde (MDA) and conjugated diene levels in fetal brain (Henderson *et al*. 1995); both MDA and dienes are markers of oxidative stress. In addition, it was found that even a single exposure to ethanol *in utero* was sufficient to increased MDA levels in whole embryos at day 14 and day 17 of gestation (Henderson *et al*., 1999). Thus, short term *in utero* ethanol exposure can elicit oxidative stress in the fetus. There is also evidence that ethanol can deplete at least some important antioxidant defense systems. With respect to hydrophilic non-enzymatic antioxidants, there are reports that ascorbic acid excretion is stimulated by ethanol (Mochizuki and Yoshida, 1989) and that ethanol decreases tissue GSH (Ponsoda *et al*., 1999). Additionally, both acute and chronic ethanol consumption have been reported to decrease the alpha-tocopheral content of rat liver (Ponsoda *et al*., 1999) and of human plasma (Armstead *et al*., 1992). Thus, depressed non-enzymatic antioxidant levels could be a contributing factor to the increase in ROS we observed in our experiments following ethanol exposure. With respect to enzymatic antioxidants, studies have investigated the effects of ethanol exposure on activities of brain ROS scavengers (Devi *et al*., 1996) and no inhibitory effects were found on catalase, CuZnSOD and MnSOD activities, i.e., ethanol does not decrease enzymatic antioxidant activity. We thus conclude that the effects seen with our adenoviral infections were not due to replenishment of ROS scavenging potential.

The rapidly replicating and differentiating cells of the fetus place a high demand on metabolic energy production. In fact, these are the very processes that generate the electron fluxes yielding ROS. Yet fetal tissues appear to have both lower non-enzymatic and enzymatic detoxification capacity. Fetal brain appears to possess lower levels and activities of oxidative defenses compared to adult brain (Mariucci *et al*., 1990) and might thereby be inherently more sensitive to oxidative stress produced by ethanol. It is well documented that the fetus is very sensitive to oxidative stress; the generation of ROS can cause a spectrum of responses ranging from structural malformations to embryonic death (Jenkinson *et al*., 1986).

Finally, our studies showed that the greatest protection from ethanol toxicity was conferred by over-expression of MnSOD. This, too, is consistent with present knowledge, for ethanol has been shown to produce an adverse effect on mitochondrial morphology in fetal hepatocytes, in particular inner membrane damage (Uysal *et al*., 1989b; Henderson *et al*., 1999). MnSOD is exclusively localized

to the inner mitochondrial membrane. Although the effect of ethanol on mitochondrial integrity within neuronal cells is not clear, this evidence from hepatocytes is consistent with MnSOD rather than the cytoplasmic CuZnSOD conferring greater protection. It is unlikely that differences in protection by the ROS scavengers studied were due to differences in expression level as all protein levels were significantly increased as compared to uninfected cells. The relative elevation of SOD activity was approximately equal for CuZnSOD and MnSOD (3.43-and 3.65-fold increases respectively, Fig. 3 and 4) while catalase was even more dramatically increased (12.8-fold increase), yet catalase conferred the least amount of protection from ethanol cytotoxicity (Fig. 5).

In summary, we have shown that exposure of PC12 cells to ethanol induces a cytotoxic effect that is mediated at least in part by an increase in oxidative stress. In addition we have shown that the over-expression of the ROS scavenging proteins catalase, CuZnSOD and MnSOD can alleviate this cytotoxicity to different degrees. Given that the greatest protection from ROS was conferred via over-expression of MnSOD, we suggest that the mitochondria may be a major target organelle for the disruptive effects of ethanol. Causal links between ethanol-mediated oxidative stress, potential increases in membrane lipid peroxidation and any resultant effects on fetal development and viability have yet to be firmly established. However there exists an ever-growing body of suggestive data pointing to oxidant stress as one credible biologic mechanism contributing to the development of FAS. Antioxidants may well become a future therapeutic possibility for ameliorating some of the morbidity associated with maternal alcohol abuse.

Acknowledgments

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