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# Mechanism of ethanol enhancement of apoptosis and caspase activation in serum-deprived PC12 cells

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**Abstract**

Neuronal death is one of the most prominent consequences of alcohol exposure during development. Ethanol-induced neuronal death appears to involve apoptosis. The objective of the present study was to characterize the effect of ethanol on neuronal cell viability and to determine the mechanism by which ethanol enhances apoptosis in neural cells. For these studies the rat pheochromocytoma (PC12) cells were used. PC12 cells were incubated for 24 h in the presence or absence of 100 mM ethanol. Apoptosis was induced by serum withdrawal. Ethanol in the presence of serum-containing media did not alter cell viability, while incubation of PC12 cells in serum-free media resulted in a significant increase in cell death that was further significantly increased by 35% in cells exposed to ethanol. The temporal response of the PC12 cells to serum withdrawal was studied over a period of 22 h. At least 18 h of ethanol exposure was necessary to observe a significant increase in death for cells incubated in serum-free media. An increase in the caspase-3 activity in PC12 cells deprived of serum was observed that was further increased by ethanol exposure. This increase of caspase-3 activity was correlated with an enhancement of caspase-9 activity. Ethanol exposure increased the amount of cytosolic cytochrome *c* in PC12 cells incubated in serum-free media but did not alter the level of cytochrome *c* in cells incubated in serum. Finally, a 26% increase was observed in the number of cells with depolarized mitochondria due to ethanol treatment. The present study implicates an increase in the mitochondrial outer membrane permeability as a potential mechanism of enhancement of apoptosis in serum-deprived PC12 cells by ethanol.


*Keywords:* Fetal alcohol syndrome; Neuronal apoptosis; Ethanol; Caspases; Mitochondrial outer membrane permeability

## Introduction

Children born to mothers who consume alcohol during pregnancy are at greater risk of developing a set of defects and malformations. The most severe consequences of the fetal alcohol exposure involving craniofacial abnormalities, CNS dysfunction including mental retardation, and stunting of growth are referred to as the fetal alcohol syndrome (FAS) (Jones and Smith, 1973). In humans the fetal alcohol syndrome is typically associated with high levels of alcohol consumption. For example, Slavney and Grau (1978) reported a case in which the mother drank a fifth of whiskey per day through gestation, while Goldstein and Arulanantham (1978) reported a case in which the pregnant mother consumed the equivalent of 180 mL of absolute alcohol each day for the first two months of gestation. One mother whose child was diagnosed with the fetal alcohol syndrome had a blood alcohol concentration of ~81 mM at the time of delivery (Church and Gerkin, 1988).

The mechanism(s) responsible for the neurotoxic effects of ethanol is not completely defined. Non-invasive imaging techniques of individuals with FAS revealed a reduction in the size of several brain areas (Roebuck et al., 1998) that would appear to be attributed to a specific reduction in the number of neurons (West et al., 1986). Alcohol impairs neuronal migration (Miller, 1993; Liesi, 1997) and proliferation (Luo and Miller, 1997; Jacobs and Miller, 2001), but loss of the developing neurons exposed to ethanol cannot be solely attributed to inhibition of these processes. Thus early postnatal exposure to

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ethanol decreases the number of neurons in the principal sensory nucleus even though these neurons complete proliferation and migration prenatally (Miller, 1995).

Exposure to ethanol during the period of synaptogenesis, which occurs in the third trimester in humans but in the first postnatal week in rats, results in degeneration of neurons through apoptotic cell death (Ikonomidou et al., 2000; Light et al., 2002; Dikranian et al., 2005). Similarly, *in vitro* experiments using primary neuronal cultures and cultured neural cells also demonstrate that ethanol can induce apoptosis (Oberdoerster et al., 1998; Luo et al., 1999; Oberdoerster and Rabin, 1999a). The ethanol-induced apoptosis appears to involve activation of caspase-3, an effector cysteine protease. Thus, an increase in cleaved caspase-3 immunoreactivity was observed in the cerebella of mice administered ethanol (Olney et al., 2002; Nowoslawski et al., 2005), while an increase in caspase-3 activity was found in cerebellar granule cells treated with ethanol *in vitro* (Oberdoerster and Rabin, 1999a; Nowoslawski et al., 2005). An increase in caspase-3 can occur through either the intrinsic apoptotic pathway which involves the mitochondria, or the extrinsic apoptotic pathway which involves receptor-mediated activation of caspase-8. Ethanol was reported to increase the expression of suicide receptors such as Fas/Apo-1 in the developing fetal cerebral cortex which might lead to an enhancement of the extrinsic pathway of apoptosis (Cheema et al., 2000). Activated caspase-8 was not found in the forebrain of mice pups treated with ethanol (Young et al., 2003), but addition of ethanol did increase caspase-8 activity in cerebellar granule cells in culture (Vaudry et al., 2002).

The objective of the present study was to determine the mechanism by which ethanol enhances cell death and increases caspase-3 activity in neural cells. Accordingly, in this study the time course for ethanol-induced cell death was determined in PC12 pheochromocytoma cells in the absence and presence of serum deprivation as well as the effects of ethanol on the upstream components of caspase-3 activation.

## Materials and methods

### *Materials*

Cell culture medium and ethidium homodimer were purchased from Invitrogen (Carlsbad, CA). Fetal calf serum was purchased from HyClone Laboratories (Logan, UT) and horse serum from JRH Biosciences (Lenexa, KS). JC-1 cationic dye was purchased from Molecular Probes/Invitrogen (Eugene, OR). The Ac-DEVD-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was purchased from Bachem Biosciences (King of Prussia, PA), Ac-DEVD-aldehyde (Ac-DEVD-CHO) from Calbiochem (La Jolla, CA), Ac-LEHD-7-amino-4-methylcoumarin (Ac-LEHD-AMC), and Ac-LEHD-aldehyde (Ac-LEHD-CHO) from Biomol (Plymouth Meeting, PA). Anti-caspase-3 and anti-cytochrome *c* IgGs were obtained from Cell Signaling Technology (Danvers, MA). HRP-conjugated goat-anti rabbit antibody and the Western Blot Chemiluminescence Reagent Plus Kit were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Immobilon-P membranes were obtained from Millipore (Bedford, MA). The protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was purchased from Sigma-Aldrich (Saint Louis, MO). All other chemicals were purchased from common commercial suppliers.

### *Cell culture*

PC12 cells were maintained at 37 °C in DMEM containing 25 mM glucose, 4 mM glutamine, and supplemented with 15%, v/v, serum (5%, v/v, heat-inactivated horse serum and 10%, v/v, fetal calf serum). For experiments, cells were treated as described previously (Oberdoerster et al., 1998). Briefly, PC12 cells were removed from culture plates by trituration and centrifuged at 800 *g* for 3 min. The cell pellet was resuspended in DMEM, and the cells again centrifuged at 800 *g* for 3 min. This washing procedure was repeated, and the cells were plated onto collagen-coated tissue culture plates in DMEM in the absence or presence of serum. Ethanol exposure was carried out by including ethanol in the media and incubating the tissue culture plates at 37 °C in plastic desiccators containing an atmosphere of 95% air and 5% $CO_2$ that was saturated with the appropriate concentration of ethanol. This system has been shown to result in an approximate 20% loss of ethanol from the medium after 2 days (Rabin, 1988).

### *Evaluation of cell death*

Cell death was monitored using the fluorescent DNA-binding probe ethidium homodimer as described previously (Oberdoerster et al., 1998). For the ethidium binding assay, PC12 cells were plated onto collagen-coated 24-well plates ($1–2\times10^6$ cells/well), and were incubated for 1 h in phosphate-buffered saline (PBS) (137 mM NaCl, 10 mM $NaH_2PO_4$, 2.7 mM KCl, pH 7.4) containing 3 μg/mL ethidium homodimer. Ethidium fluorescence was measured using a fluorescent plate reader ($\lambda_{excitation}=485$ nm, $\lambda_{emission}=645$ nm) and expressed per mg of the total cell protein content.

### *Caspase activity assay*

PC12 cells ($2–3\times10^7$ cells) were removed from the culture plate in lysis buffer composed of 25 mM Hepes, 1 mM EGTA, 5 mM EDTA, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.01% w/v 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propansulfonate (CHAPS), 10 μg/mL pepstatin, 10 μg/mL leupeptin, 1 mM phenylmethylsulfonylfluoride, pH 7.4. After a 20 min incubation on ice, an aliquot was removed for determination of cellular protein, and the remaining lysate was centrifuged at 16,000 *g* for 30 min. The resulting supernatant was combined with 2 volumes of assay buffer composed of either 25 mM Hepes, 10 mM dithiothreitol, 15% w/v sucrose, 0.15% w/v CHAPS, pH 7.5, for the caspase-3 assay or 100 mM Hepes, 10 mM dithiothreitol, 20% w/v sucrose, 1% v/v Triton X-100, pH 7.5, for the caspase-9 assay. Caspase activity was determined by measuring the rate of coumarin liberation from the appropriate caspase substrate (100 μM; Ac-DEVD-AMC for caspase-3 and Ac-LEHD-AMC for caspase-9). Nonspecific substrate cleavage was measured in