Effects of ethanol and ipsapirone on the expression of genes encoding anti-apoptotic proteins and an antioxidant enzyme in ethanol-treated neurons

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Abstract

Previously, this laboratory found that apoptosis was augmented significantly in fetal rhombencephalic neurons when they were treated with 50 mM ethanol for 24 h. These changes were associated temporally with a reduction in the phosphatidylinositol 3-kinase (PI3K) pro-survival pathway and in the downstream expression of several NF-κB dependent anti-apoptotic genes. The serotonin-1A agonist ipsapirone prevented ethanol-associated apoptosis; it also activated the PI3K→pAkt pro-survival pathway and the expression of specific NF-κB dependent anti-apoptotic genes in ethanol-treated neurons. The present study investigated the temporal effects of both ethanol and ipsapirone on the expression of three NF-κB dependent genes, XIAP, Bcl-xL, and catalase; these genes encode proteins that could potentially attenuate ethanol-induced apoptosis. Catalase activity was also measured. All three genes demonstrated an early activation by ethanol. After a brief treatment with 50 mM ethanol, i.e., 2 to 8 h depending on the gene, the expression of XIAP, Bcl-xL, and catalase was significantly increased, possibly as an initial attempt to survive. An ethanol-associated increase in catalase was followed by a rise in catalase activity. However, when ethanol treatment was continued for a longer time, there was a significant reduction in both XIAP and Bcl-xL. In addition, both catalase expression and activity returned to levels found in unstressed controls. Importantly, treatment with ipsapirone augmented the activity of catalase and the expression of Bcl-xL, XIAP, and catalase in ethanol-treated neurons at later time points. The latter effects are likely to contribute to the pro-survival effects of ipsapirone.

Keywords

Serotonin1A receptor; Apoptosis; XIAP; Bcl-xL; Catalase
1. Introduction

*In utero* ethanol exposure can cause serious functional and structural abnormalities. Included among the CNS disorders associated with Fetal Alcohol Syndrome (FAS) and/or Fetal Alcohol Spectrum Disorder (FASD) are problems with attention, behavior, cognition, memory, and executive function (Mattson et al., 1996, 1999; Riley et al., 2003; reviewed in Wattendorf, and Muenke, 2005). In addition, morphological abnormalities are found in several brain regions including the corpus callosum, cerebellum, and basal ganglia (Roebuck et al., 1998; Riley et al., 2004). Although the mechanism(s) by which alcohol exposure effects the deleterious changes in the developing brain have not been fully elucidated, both *in vivo* and *in vitro* animal studies suggest that apoptosis is likely to be involved (Castoldi et al., 1998; Cheema et al., 2000; Ikonomidou et al., 2000; Ramachandran et al., 2001).

Earlier studies from this (Tajuddin and Druse, 1999, 2001) and another laboratory (Sari and Zhou, 2004) showed that *in utero* ethanol exposure caused a significant reduction in serotonin (5-HT) neurons. Using a 24 hour treatment of fetal rhombencephalic neurons with 50 mM ethanol, *in vitro* studies established that this reduction was probably caused by ethanol-associated apoptosis (Druse et al., 2004, 2005, 2007), a decreased activity of the phosphatidylinositol 3-kinase (PI3K)→pAkt pro-survival pathway (Druse et al., 2005) and reduced downstream expression of several NF-κB dependent anti-apoptotic genes: *XIAP*, *cIAP1*, *cIAP2*, *Bcl-2* and *Bcl-XL* (Druse et al., 2006, 2007). Importantly, *in vivo* and *in vitro* treatment with the 5-HT1A receptor agonist ipsapirone prevented the ethanol-associated reduction of 5-HT and other fetal rhombencephalic neurons and the ethanol-associated decrease of pAkt (Tajuddin and Druse, 1999, 2001; Druse et al., 2004, 2005). Ipsapirone was able to increase expression of NF-κB dependent genes that encode XIAP and Bcl-XL in fetal rhombencephalic neurons treated with ethanol for 24 h prior to the addition of ipsapirone (Druse et al., 2006).

One way by which ethanol augments apoptosis is by increasing oxidative stress. In fact, several laboratories, including this one, show that ethanol increases reactive oxygen species (ROS) in developing neural tissue (Heaton et al., 2002; Ramachandran et al., 2003; Watts et al., 2005; Lee et al., 2007). The increased oxidative stress is associated with augmented apoptosis (Ramachandran et al., 2003; Lee et al., 2007), and co-treatment of fetal rhombencephalic neurons with specific antioxidants prevents ethanol-associated apoptosis (Antonio and Druse, 2008). Antioxidant treatment also prevents damage to ethanol-exposed cerebellar granule cells (Heaton et al., 2004; Siler-Marsiglio et al., 2005) and hippocampal neurons (Marino et al., 2004), although it has not proved neuroprotective in all studies (Grisel and Chen, 2005; Tran et al., 2005).

Of particular interest to the current study is the endogenous antioxidant enzyme catalase; along with other antioxidant enzymes, catalase participates in reactions that detoxify ROS. Catalase is of interest both because of its role as an antioxidant enzyme and because there is evidence that *catalase* is expressed in a NF-κB dependent manner (Zhou et al., 2001). Interestingly, catalase is also reportedly the key enzyme involved with ethanol metabolism in the brain of rodents (Zimatkin et al., 2006). Considering that the 5-HT1A agonist ipsapirone upregulates the NF-κB genes encoding the anti-apoptotic proteins Bcl-XL and XIAP (Kucharczak et al., 2003), this drug might also augment the expression of *catalase*; such an effect could be essential to reducing the ROS-mediated apoptosis caused by ethanol.

In order to better understand the contribution of potential neuroprotective effects of ipsapirone, this study evaluated the time course associated with the effects of ipsapirone and ethanol on two anti-apoptotic genes, *XIAP* and *Bcl-XL*, and on the gene that encodes the antioxidant enzyme catalase. This study also examined the effects of ethanol and ipsapirone on catalase activity.
2. Results

Treatment of fetal rhombencephalic neurons with 50 mM ethanol causes an early and transient increase in Bcl-xl, XIAP, and catalase. The increase in XIAP was nearly 6-fold (p<.01) at 2 to 4 h (Fig. 1), while that of Bcl-xl was 2-fold (p<.05) to 4-fold (p<.01) at 4 and 8 h, respectively (Fig. 2). There was a modest yet significant increase (p<.05) in catalase at 8 h (Fig. 3). The early elevation of all three genes was brief and transient. In fact, a prolonged (24 or 48 h) exposure to ethanol reduced the expression of Bcl-xl and XIAP below control levels (p<.05) and brought catalase levels down to those in unstressed controls.

The changes in catalase expression in ethanol-treated cultures were followed by similar changes in catalase activity (Fig. 4). That is, the ethanol-associated increase in catalase at 8 h preceded an elevation of catalase activity at 18 h (p<.05). In addition, a more prolonged ethanol exposure caused both catalase expression and activity to return to levels comparable to those in the unstressed control. Moreover, the ipsapirone-augmented expression of catalase in ethanol-treated cultures at 24 h was followed by increased enzyme activity at 48 h. In contrast, the elevation of catalase in control plus ipsapirone cultures did not appear to affect subsequent enzyme activity.

Importantly, co-treatment of ethanol-exposed cultures with ipsapirone (EthIps group) prevented the ethanol-associated reduction of Bcl-xl at 24 h and of XIAP at 24 and 48 h (Ethanol group); this treatment also increased expression of both genes in comparison with ethanol-treated neurons at 48 h (p<.05). Similarly, catalase was significantly elevated in ethanol plus ipsapirone-treated cultures (EthIps group versus Ethanol group) at 24 h (p<.05). Even when compared to control neurons, ipsapirone treatment caused a >6-fold increase in Bcl-xl in ethanol-treated neurons at 48 h and a significant increase in catalase at 24 h. It should be noted that addition of 100 nM ipsapirone to control neurons (ConIps group) significantly augmented the expression of catalase (Fig. 3) and Bcl-xl (Fig. 1) at 24 and/or 48 h (p<.05) when compared to control neurons. When the PI3K inhibitor LY294002 was included with ipsapirone, it blocked the ipsapirone-mediated increases in XIAP and Bcl-xl (data not shown), suggesting that the ipsapirone-mediated increased expression of these genes was mediated by the PI3K pathway. In contrast, LY294002 did not lower catalase in ipsapirone-treated cultures.

3. Discussion

An early (2 to 8 h) response of fetal rhombencephalic neurons to 50 mM ethanol is augmented expression of two pro-survival genes (i.e., XIAP, Bcl-xl) and the gene encoding the antioxidant enzyme catalase; catalase activity is increased at a later time point, e.g., allowing time for translation of the message into protein. The consequence of these effects could be an early attempt of fetal rhombencephalic neurons to defend themselves against the pro-apoptotic effects of ethanol, because XIAP inhibits the activity of caspase-3 (Salvesen and Duckett, 2002) and increased Bcl-xl stabilizes the mitochondria from releasing cytochrome C. Moreover, increased catalase would attenuate ethanol-associated oxidative stress and the associated apoptosis by catalyzing the conversion of hydrogen peroxide to water (Temple et al., 2005). Consistent with our findings, others noted that ethanol up-regulates the expression of catalase and the gene encoding another antioxidant enzyme, i.e. superoxide dismutase-2, in hepatic cells (Bardag-Gorce et al., 2006).

Although we cannot be certain about the mechanism by which ethanol exposure initially increased the expression of these genes, it could involve the rapid rise in ROS which accompanies ethanol exposure (Ramachandran et al., 2003; Watts et al., 2005; Lee et al., 2007). An increase in cellular ROS can activate several classes of genes, including those that are dependent on NF-κB (Zhou et al., 2001; Turapev, 2002; Ji et al., 2004) and Nrf2 (Zhu et
al., 2005), which binds to the antioxidant response element (ARE) (reviewed by Kensler et al., 2007). Because XIAP, Bcl-xL, and catalase can each be regulated by NF-κB dependent mechanisms (Kucharczak et al., 2003; Zhou et al., 2001), and because catalase also has an antioxidant response element that can bind to Nrf2 (Zhu et al., 2005), it is likely that this initial ethanol-associated augmentation of gene expression is mediated by the effects of increased ROS on such ROS-sensitive transcription factors. Microarray studies show that ethanol upregulates additional CNS genes that are known to be induced by oxidative stress (Treadwell and Singh, 2004).

Although the early ethanol-associated rise in catalase is followed by an increase in catalase activity at 18 h, this potentially protective effect is short-lived. Subsequently, both catalase expression and enzyme activity return to control levels. Although these levels are appropriate for unstressed control neurons, they could be too low to detoxify ROS in ethanol-treated neurons, which are undergoing oxidative stress. Similar to catalase, the initial ethanol-associated rise in XIAP and Bcl-xL is followed by a decline to subnormal levels; cIAP1, cIAP2, and Bcl-2 were also subnormal at 24 h (Druse et al., 2006). The timing of these changes correlates with augmented apoptosis (Druse et al., 2004, 2005, 2007) and with reduced activity of the PI3K→pAkt pro-survival pathway (Druse et al., 2005). Thus, the combined effects of subnormal levels of the anti-apoptotic genes and a return to catalase activity that is appropriate in unstressed cells are likely to contribute to the pro-apoptotic effects of ethanol.

Co-treatment of ethanol-exposed neurons with ipsapirone exerted potentially neuroprotective effects at later critical time points. In fact, at times when ethanol significantly reduced XIAP and/or Bcl-xL, i.e., (16–48 h), co-treatment with ipsapirone either brought the expression of these genes back to control levels or significantly increased expression above the levels in ethanol-treated or control neurons. Augmented expression of the NF-κB-dependent genes XIAP and Bcl-xL in ethanol-treated neuronal cultures appears to be related to the ability of ipsapirone to increase PI3K→pAkt (Druse et al., 2006), because the PI3K inhibitor LY 294002 blocked the ipsapirone-mediated increase (data not shown). That a similar effect of LY294002 on ipsapirone-mediated catalase expression was not observed suggests that changes in catalase might involve another signaling pathway. It should also be noted that the timing of the ipsapirone-mediated increases in XIAP, Bcl-xL, and catalase were distinct, suggesting that the possibility that multiple transcription factors participate in the regulation of these genes (Zhou et al., 2001; Turapev, 2002; Kucharczak et al., 2003; Ji et al., 2004; Zhu et al., 2005).

It is likely that ipsapirone's ability to block an ethanol-associated decrease in the expression of anti-apoptotic genes or augment such expression is meaningful biologically, because ipsapirone treatment also blocked ethanol-associated apoptosis at 24 h (Druse et al., 2004, 2005). In addition, the ipsapirone-mediated elevation of catalase at 24 h and catalase activity at 48 h could contribute to the neuroprotective effects that are observed when ipsapirone is co-administered with ethanol on a more prolonged basis (>24 h). For example, the catalase changes might contribute to ipsapirone-mediated neuroprotection found in vivo when ethanol-treated pregnant dams are treated with this or a related drug during the latter portion of gestation (Tajuddin and Druse, 1999, 2001).

Although ipsapirone significantly increased catalase in control neurons, this change was not followed by increased enzyme activity. Similarly, ipsapirone-mediated increases in the expression of XIAP and Bcl-xL in control neurons did not augment survival in these neurons (Druse et al., 2004, 2005). Evidently, there are cellular compensatory mechanisms in control neurons which prevent overproduction of certain molecules.

This study shows that within hours after the addition of 50 mM ethanol, the expression of XIAP, Bcl-xL, and catalase and catalase activity were significantly increased in fetal
rhombencephalic neurons, possibly as an initial attempt to survive. However, when ethanol treatment was continued for a longer time, there was a significant reduction in both XIAP and Bcl-XL. Also, both catalase expression and activity returned to levels in unstressed control neurons; catalase activity which is appropriate for unstressed neurons is likely too low to detoxify ROS in ethanol-treated neurons undergoing oxidative stress. Consequently, the pro-apoptotic effects of ethanol might involve the reduction in anti-apoptotic genes and inappropriately low levels of catalase. Importantly, co-treatment with ipsapirone increases XIAP, Bcl-XL and catalase in ethanol-treated fetal rhombencephalic neurons at the later times, e.g., when cultures treated with ethanol alone exhibit augmented apoptosis. This treatment also increased catalase activity by 48 h. Thus, ipsapirone-mediated increases in XIAP, Bcl-XL and catalase and in catalase activity in ethanol-treated neurons at the later time points are likely to contribute to the neuroprotective effects of ipsapirone that are exerted either during a 24-hour in vitro treatment (Druse et al., 2004, 2005, 2007) or in a longer in vivo ethanol exposure (Tajuddin and Druse, 1999, 2001).

4. Experimental procedures

4.1. In vitro cell culture model

The methods used in this study have been described previously in several earlier publications from this laboratory (Druse et al., 2004–2007) All animal care and use procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Loyola University Chicago, Stritch School of Medicine. The brain region which contains the developing 5-HT neurons (Konig et al., 1989), i.e., the fetal rhombencephalon, was removed (Eriksen et al., 2002) from timed-pregnancy Sprague–Dawley rats at G14, where G = 0 corresponds to the day of insemination. Rhombencephalic tissue was mechanically disaggregated before seeding onto poly-d-lysine coated plates (Corning, Corning, New York) at a density of 8 × 10^6 to 10 × 10^6 cells/plate (55 cm^2). Cultures were maintained in a neuron-specific chemically defined media (CDM) that included Dulbecco’s Minimal Essential Media/F12 (DMEM/F12) media, hydrocortisone-21 sulfate, Basal Medium Eagle Vitamin Solution, antibacterial agent gentamicin sulfate (Honegger and Monnet-Tschudi, 1997), B27 serum-free medium supplement (Brewer et al., 1993), and .25% fetal bovine serum (FBS) (Druse et al., 2004). After 24 h, cytosine arabinoside (.4 μM) was added to arrest gliogenesis. Immunohistochemistry was performed using an antibody to glial fibrillary acidic protein (GFAP) to assess the contribution of astrocytes to the cultures; this method confirmed that astrocytes comprised <5% of the cells in these neuronally-enriched cultures. Cells were grown in control media (no ethanol) for 5 days, with media changes made on alternate days. Using an ethanol chamber system described by this laboratory (Druse et al., 2004), neuronal cultures were exposed to either 0 mM ethanol (control group) or 50 mM ethanol for periods of two to either 24 (all) or 48 h (Bcl-XL and XIAP) beginning on the fifth day in culture. Cultures were co-treated with 0 or 100 nM ipsapirone during the same period; this concentration of ipsapirone promotes the development of 5-HT neurons (Whitaker-Azmitia et al., 1990) and prevents apoptosis in fetal rhombencephalic neurons (Druse et al., 2004).

4.2. Quantitative real-time RT-PCR

Trizol reagent (Life Technology, Gaithersburg, MD) (Druse et al., 2006) was used to extract total RNA from cultured fetal rhombencephalic neurons. Glycogen (20 mg/ml) (Ambion, Austin, TX) was included to facilitate the precipitation of RNA and to maximize the yield of RNA. RNA was dissolved in DEPC-treated H2O, which was treated with DNA-free (Ambion) to remove contaminating genomic DNA. RNA was stored at –80° until use. Single strand cDNA was synthesized from 1 to 2 μg of total RNA (DNA-free) using the First Strand cDNA synthesis kit (Fermantas, Hanover, MD).
cDNA (DNA equivalent of 20 ng to 40 ng of total RNA) was diluted with Platinum Quantitative PCR Super Mix-UDG [1.5 U Platinum Taq DNA polymerase, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 3 mM MgCl₂, 200 mM dGTP, 200 mM dATP, 200 mM dCTP, 400 mM dUTP, 1 U UDG (Life Technology)], .25 mM Rox (Life Technology), 1/40,000 SYBR Green (Molecular Probes, Eugene, Oregon), and primers (Druse et al., 2006). A Perkin-Elmer Gene Amp 7300 Sequence Detector thermal cycler (Applied Biosystems, Foster City, CA) was used for PCR amplifications. RT-PCR data was analyzed using SDS software (Applied Biosystems). Sample inputs were normalized using expression of the GAPDH.

Plasmid containing target genes were used to generate standard curves for real-time quantitative RT-PCR assays (Druse et al., 2006), and a standard curve was generated from serial dilutions of known amounts of the input copy number of target genes. Standard curves for GAPDH and genes of interest (10–100,000 copies/μl) were performed in each experiment. Routinely we included a triplicate RT-PCR reaction lacking cDNA or known DNA template for each set of primers. Specific primary sequences for XIAP, Bcl-2, and catalase were selected using the Primer Express program (Applied Biosystems) and sequences available from the NCBI database. Primers were synthesized by Life Technology. The forward (f) and reverse (r) primer sequences for rat genes are included in Table 1. Typically, GAPDH, Bcl-xl, XIAP, and catalase were first detected at 22, 28, 30, or 28 cycles, respectively.

4.3. Catalase activity

The activity of catalase was determined using an enzymatic kit (Cayman, Ann Arbor, MI). This kit determines the ability of catalase to convert \( \text{H}_2\text{O}_2 \) and methanol to formaldehyde and 2 molecules of water. A positive control (bovine liver catalase) is included.

4.4. Statistical analyses

Data was analyzed using ANOVA and a post-hoc Dunnet’s procedure to test our hypotheses that ethanol treatment alters the expression of some NF-κB dependent survival genes and that ipsapirone restores expression in ethanol-treated neurons to levels found in untreated (control) cultures. A \( p \) value <.05 was considered to be significant.

Acknowledgments

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REFERENCES


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Fig. 1. XIAP mRNA in fetal rhombencephalic neurons that were co-treated with 50 mM ethanol and 100 nM ipsapirone for periods from 2 to 48 h. Each value represents the mean± the SEM of values obtained from three to nine separate experiments. Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The abbreviations Con, Eth, and Ips are used in place of Control, Ethanol, and Ipsapirone, respectively. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p<.05$ and $p<.01$ are represented, respectively by the * and **. The # identifies values in the EthIps group that are significantly different from those in the Ethanol group ($p<.05$).
Fig. 2.
Bcl-xL mRNA in fetal rhombencephalic neurons that were co-treated with 50 mM ethanol and 100 nM ipsapirone for periods from 2 to 48 h. Each value represents the mean±the SEM of values obtained from three to six separate experiments. Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The abbreviations Con, Eth, and Ips are used in place of Control, Ethanol, and Ipsapirone, respectively. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p<.05$ and $p<.01$ are represented, respectively by the * and **. The # and ## identify values in the EthIps group that are significantly different from those in the Ethanol group at $p<.05$ and $p>.01$, respectively.
Fig. 3.
Catalase mRNA in fetal rhombencephalic neurons that were co-treated with 50 mM ethanol and 100 nM ipsapirone for periods from 2 to 24 h. Each value represents the mean±the SEM of values obtained from three to eight separate experiments. Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The abbreviations Con, Eth, and Ips are used in place of Control, Ethanol, and Ipsapirone, respectively. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p<.05$ are represented by the *. The # identifies values in the EthIps group that are significantly different from those in the Ethanol group ($p<.05$).
Fig. 4. Catalase activity in fetal rhombencephalic neurons that were co-treated with 50 mM ethanol and 100 nM ipsapirone for periods from 18 to 48 h. Each value represents the mean±the SEM of values obtained from five to seven separate experiments. Values are expressed as a percentage of the time-matched controls. The abbreviations Con, Eth, and Ips are used in place of Control, Ethanol, and Ipsapirone, respectively. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p<.05$ are represented by the *.
The # identifies values in the EthIps group that are significantly different from those in the Ethanol group ($p<.05$).
### Table 1

Sequences of primers used

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<th>Rat gene</th>
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<td>Gapdh</td>
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