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Research Report

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.

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

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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-HT_{1A} 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-HT_{1A} 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

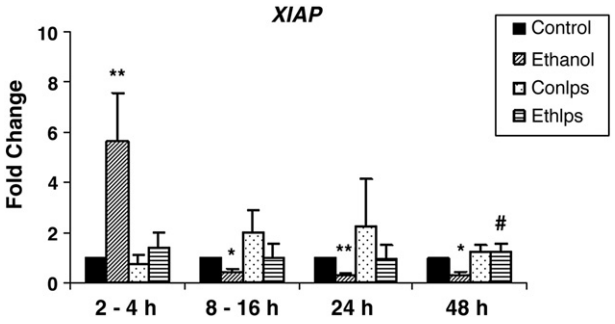

Fig. 1 – XIAP mRNA in fetal rhombencephalic neurons that were co-treated with 50 mM ethanol and 100 nM ipsapirone for periods from 2 to 48 h. Each value represents the mean ± the SEM of values obtained from three to nine separate experiments. Values are expressed as the fold change in mRNA as calculated 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 ($p<.05$).