

Difluoromethylornithine Decreases Long-Lasting Protein Oxidation Induced by Neonatal Ethanol Exposure in the Hippocampus of Adolescent Rats

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Background: Ethanol exposure and withdrawal during central nervous system development can cause oxidative stress and produce severe and long-lasting behavioral and morphological alterations in which polyamines seem to play an important role. However, it is not known if early ethanol exposure causes long-lasting protein oxidative damage and if polyamines play a role in such a deleterious effect of ethanol.

Methods: In this study we investigated the effects of early ethanol exposure (6 g/kg/d, by gavage), from postnatal day (PND) 1 to 8, and of the administration of difluoromethylornithine (DFMO, 500 mg/kg, i.p., on PND 8), a polyamine biosynthesis inhibitor, on the extent of oxidative modification of proteins. Indices of oxidative modification of proteins included protein carbonyls, 3-nitrotyrosine (3-NT), and protein bound 4-hydroxynonenal (HNE) in the hippocampus, cerebellum, hypothalamus, striatum, and cerebral cortex of Sprague–Dawley rats at PND 40.

Results: Both ethanol and DFMO administration alone increased protein carbonyl immunoreactivity in the hippocampus at PND 40, but the combination of DFMO and ethanol resulted in no effect on protein carbonyl levels. No alterations in the content of protein-bound HNE, 3-NT, or carbonyl were found in any other cerebral structure.

Conclusions: These results suggest that the hippocampus is selectively affected by early ethanol exposure and by polyamine synthesis inhibition. In addition, the results suggest a role for polyamines in the long-lasting increase of protein carbonyls induced by ethanol exposure and withdrawal.

Key Words: Alcohol, Oxidative Stress, Polyamines, NMDA, Protein Carbonyl.

PRENATAL ETHANOL EXPOSURE disrupts central nervous system (CNS) development, leading to long-lasting cognitive and behavioral alterations in humans (Mattson and Riley, 1998; Roebuck et al., 1998) and rats (Barron et al., 1988). Ethanol most likely disrupts CNS development via a variety of mechanisms, including alterations in gene expression, cell adhesion molecule interactions, neurotrophic support, and free radical

production (Goodlett and Horn, 2001; West, 1994; West et al., 1994).

One possible mechanism that contributes to fetal alcohol effects is *N*-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity, which occurs during periods of ethanol withdrawal. Consistent with this hypothesis, blocking NMDA receptors with a noncompetitive antagonist, MK-801, during withdrawal attenuates some of the adverse effects of ethanol on behavioral and brain development in a rat model of early developmental ethanol exposure (Thomas et al., 1997) and in vitro (Prendergast et al., 2000). Moreover, the beneficial effects of MK-801 are time dependent (Thomas et al., 2001), indicating that NMDA receptor blockade is only effective during the withdrawal phase. *N*-methyl-D-aspartate receptors are comprised of an assembly of subunits (an NR1 subunit plus at least 1 type of NR2 subunit) with a number of modulatory sites, including a polyamine binding site, which is present in the NR2B subunit (Chenard and Menniti, 1999). The polyamine binding site of the NMDA receptor seems to be particularly relevant, from the pharmacological point of view, as it regulates important CNS functions, such as learning and memory (Berlese et al., 2005; Rubin et al., 2004) and alters the susceptibility to the neurotoxic effects of glutamate and ethanol

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Received for publication August 1, 2006; accepted January 22, 2007.

Financial support: CFM and MAR are recipients of CAPES (BEX0262/05-6) and CNPq (200016/2005-9) fellowships, respectively. Work supported by NIH grants to DAB (AG-10836; AG-05119), to SB (AA-014032), and to JML (AA-12600; AA-01388).

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DOI: 10.1111/j.1530-0277.2007.00369.x

(Ceberé et al., 2002; Ferrani-Kile et al., 2003; Gibson et al., 2003; Honse et al., 2003; Mayer et al., 2002; Mikolajczak et al., 2003). Interestingly, eliprodil, which inhibits NMDA receptor activity by interacting allosterically with the polyamine modulatory site on NMDA receptors, reduces the severity of learning deficits associated with developmental alcohol exposure (Thomas et al., 2004). In fact, *in vitro* studies have suggested a role for polyamines in ethanol withdrawal-induced neurotoxicity, as not only polyamine binding site antagonists, but also polyamine synthesis inhibitors protect against ethanol withdrawal-induced neurotoxicity (Gibson et al., 2003; Nagy et al., 2004). Accordingly, it has been demonstrated that polyamines and glutamate are released during ethanol withdrawal (Gibson et al., 2003), and that agmatine, a putative polyamine binding site ligand, reduces some effects of alcohol exposure on the developing brain (Littleton et al., 2001). Consistent with this view, eliprodil blocks ethanol withdrawal seizures (Kotlinska and Liljequist, 1996). Moreover, chronic ethanol exposure increases the activity of ornithine decarboxylase (ODC), the rate limiting enzyme for polyamine biosynthesis (Davidson and Wilce, 1998), further supporting a role for polyamines in ethanol-induced effects, and in its withdrawal.

Although ethanol metabolism has been long known to cause acute oxidative cell damage by generating acetaldehyde or free radicals (Guerra et al., 1994; Nakano et al., 1995; Nakao et al., 2000) and to diminish antioxidant defense systems (Sun et al., 2001; Zima et al., 2001), given the above-mentioned role of NMDA receptor in ethanol withdrawal and the well-established role of these receptors in promoting oxidative stress, it is also possible that polyamines, through NMDA-mediated mechanisms, participate in the oxidative stress caused by alcohol withdrawal (Tsai et al., 1998). However, it is important to point out that polyamines have also been reported to act as antioxidants (von Deutsch et al., 2005; Yatin et al., 2001), stabilize the DNA (Bachrach et al., 2001), modulate calcium-activated potassium channels (Weiger et al., 2002), stimulate tyrosine and mitogen-activated protein kinases, and alter gene expression (Childs et al., 2003). The antioxidant activity and the stabilization of DNA induced by these compounds seem to confer a protective role for these aliphatic amines with a polycationic structure against apoptosis, for example (Huang et al., 2005; Liu et al., 2005), and might contribute for some of the effects of polyamines *in vivo*.

Ethanol increases protein carbonylation in the brain at postnatal day (PND) 9 when administered between PND 8 and 9 in a model of Fetal Alcohol Syndrome (Marino et al., 2004), and increases brain hydroxynonenal (HNE) production *in utero* (Ramachandran et al., 2001). However, it is not known if early ethanol treatment causes long-lasting alterations in the content of protein carbonyl, 3-nitrotyrosine (3-NT), and HNE content of the brain,

when chronic regimens are used. In addition, early ethanol exposure from PND 4 to 9 produces a long-lasting reduction in synaptic efficacy in the hippocampus of adolescent rats (Bellinger et al., 1999), but it is not known whether long-lasting oxidative protein modifications also occur in this model, and whether inhibiting polyamine biosynthesis protects rats from the long-term neurochemical consequences of alcohol exposure and withdrawal in neonatal life (PND 1–8). Therefore, in the present study we investigated whether early ethanol exposure from PND 1 to 8 alters the content of HNE, 3NT, and protein carbonyls in the cerebellum, striatum, hypothalamus, hippocampus, and cerebral cortex of adolescent (PND 40) rats, and whether inhibiting polyamine biosynthesis with the ODC inhibitor, difluoromethylornithine (DFMO), on PND 8 protects from the long-lasting early ethanol exposure-induced alterations in the contents of HNE, 3-NT, and protein carbonyls.

METHODS

All protocols involving animals were approved by the University of Kentucky Institutional Animal Care and Use Committee. Male Sprague–Dawley pups (at PND 1) were randomly assigned to each of the 4 treatment groups in such a way that each litter (8 pups each) contributed with no more than 1 animal for each treatment group. Although male and female rats were available, because females were reported to be less susceptible to the oxidative stress induced by ethanol withdrawal, only males were used in this study (Jung et al., 2005).

From PND 1 to 8, the animals were given 3 g/kg ethanol (in milk formula) or maltose (milk formula plus maltose, isocaloric control) by gavage (0.0278 mL/g bw, *b.i.d.*, at 10:00 AM and 2:00 PM) resulting in 6 g/kg/d with a plastic PE-10 tubing, as previously described (Gilbertson and Barron, 2005).

On PND 8, the animals were injected with DFMO [500 mg/kg, subcutaneous (*s.c.*)] or 0.9% NaCl (5 mL/kg, *s.c.*) immediately after the last gavage. The dose of DFMO used in this study effectively inhibits ODC and reduces the severity of withdrawal behaviors (Davidson and Wilce, 1998).

The animals (6 in each experimental group) were left undisturbed until PND 40, when they were killed under thionembutal anesthesia and their brains were removed and dissected on an inverted Petri dish placed on ice. The samples were frozen immediately and kept at -80°C until slot blot analysis.

Markers of Protein Oxidation

Protein Carbonyls. Protein carbonyls are markers of protein oxidation and were assessed by following the standard protocol described elsewhere (Butterfield and Stadtman, 1997). Samples (5 μL), 12% sodium dodecyl sulfate (SDS) and 10 times diluted 2,4-dinitrophenylhydrazine (DNPH) (10 μL) from a 200 mM stock solution were incubated at room temperature for 20 minutes. Samples were neutralized with 7.5 μL neutralization solution (2 M Tris in 30% glycerol). The resulting solution was loaded in each well on a nitrocellulose membrane under vacuum using a slot blot apparatus. The membrane was blocked in blocking buffer (3% bovine serum albumin) for 1 hour and incubated with a 1:100 dilution of anti-DNP polyclonal antibody in phosphate-buffered saline (PBS) containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 for 1 hour. The membrane was washed 3 times in PBS and was incubated for 1 hour with an antirabbit IgG alkaline phosphatase secondary antibody

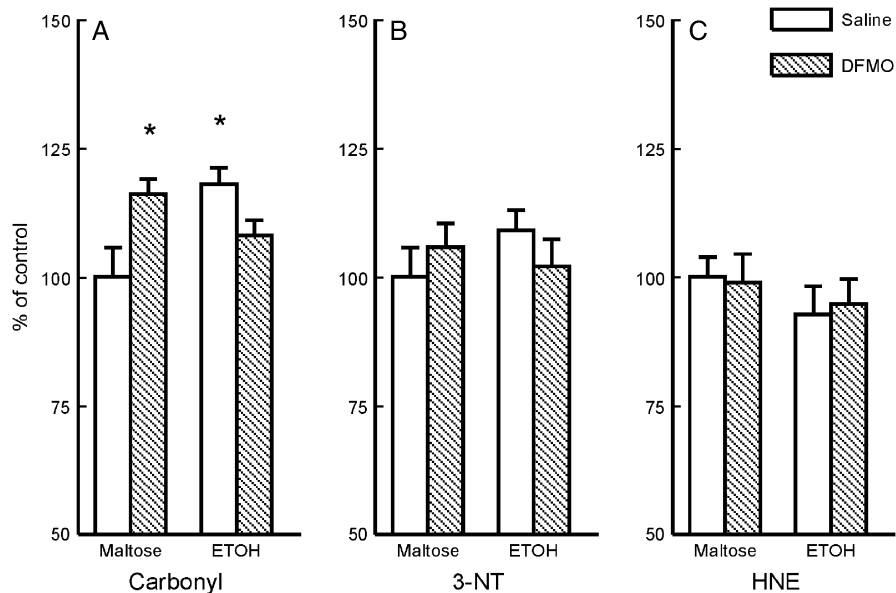


Fig. 1. Effect of early ethanol exposure [from postnatal days (PND) 1–8] and difluoromethylornithine (DFMO) (500 mg/kg, subcutaneous) administration in PND 8 on (A) protein carbonyl (carbonyl), (B) 3-nitrotyrosine (3-NT), and (C) 4-hydroxynonenal (HNE) immunoreactivity in the hippocampus at PND 40. * $p < 0.05$ compared with maltose–saline group (control) by the Student–Newman–Keuls test.

diluted in PBS in a 1:8,000 ratio. The membrane was washed 3 times in PBS for 5 minutes and developed in Sigma Fast tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe Photoshop, and quantified with Scion Image (PC version of Macintosh compatible NIH image).

4-Hydroxynonenal Bound to Proteins. Hydroxynonenal immunoreactivity was measured as previously described (Joshi et al., 2006). Samples (5 μ L) (normalized to 4 μ g/mL), 5 μ L of 12% SDS 5 μ L of modified Laemmli buffer containing 0.125 M Tris base pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol were incubated for 20 minutes at room temperature. Samples (250 ng of protein) were loaded in each well on a nitrocellulose membrane in a slot blot apparatus under vacuum. The membrane was blocked as above and incubated with a 1:5,000 dilution of anti-HNE Michael adduct polyclonal antibody in PBS for 1 hour 30 minutes. The membrane was developed as described above.

3-Nitrotyrosine. 3-Nitrotyrosine immunoreactivity was measured as previously described (Joshi et al., 2006). Sample (5 μ L) (normalized to 4 μ g/mL), 5 μ L of 12% SDS and 5 μ L of modified Laemmli buffer containing 0.125 M Tris base pH 6.8%, 4% (v/v) SDS, and 20% (v/v) glycerol were incubated for 20 minutes at room temperature, and the membranes were developed as described above except a 1:2,000 dilution of anti-3-NT polyclonal antibody was used. Previous studies demonstrated no nonspecific primary or secondary antibody binding to the membrane (Sultana et al., 2004). The HNE blot had a faint background that was corrected in image analysis.

Statistical Analyses. Carbonyl, 3-NT, and HNE immunoreactivities were analyzed by a MANOVA with treatment (maltose or ethanol) and drug (saline or DFMO) as fixed factors and each cerebral region (5 regions) and measures of oxidative damage (3 measures) as repeated measures. Post hoc analyses were carried out by follow-up ANOVAs, followed by the Student–Newman–Keuls test, by using the SPSS 12.0 program. $p < 0.05$ was considered significant.

RESULTS

Figure 1A–1C shows the effects of early ethanol exposure and the administration of DFMO on PND 8 on the

protein carbonyl, 3-NT, and HNE immunoreactivity in the hippocampus on PND 40, respectively. The multivariate analysis revealed a significant brain region by measure of oxidative damage interaction: Wilk's $\Lambda = 0.171$, $F(8, 13) = 7.82$, $p = 0.001$, indicating that the pattern of variation of the measures of oxidative damage varied among brain regions. Follow-up univariate ANOVAs revealed a significant effect of neonatal treatment (maltose or ethanol) by drug (saline or DFMO) interaction [$F(1, 20) = 11.57$; $p < 0.005$] for the content of protein carbonyl, only in the hippocampus. Post hoc analysis revealed that ethanol administration from PND 1 to 8 and DFMO administration on PND 8 increased carbonyl immunoreactivity on PND 40. When DFMO was administered with ETOH on PND 8, protein carbonyl levels were similar to control levels. A similar pattern of results was observed for 3-NT immunoreactivity in the hippocampus, but this trend was not statistically significant.

Early ethanol exposure from PND 1 to 8 and DFMO administration on PND 8 did not alter carbonyl, 3-NT, and HNE immunoreactivity in the cerebellum, striatum, hypothalamus, or cerebral cortex on PND 40 (Table 1).

DISCUSSION

This study shows, for the first time, that early ethanol exposure from PND 1 to 8 increases protein carbonylation in the hippocampus at PND 40, but not in other cerebral structures, including striatum, cerebellum, cerebral cortex, and hypothalamus. In addition, we showed that the administration of DFMO, an inhibitor of the synthesis of polyamines, on PND 8 increases protein carbonylation, but prevents ethanol-induced protein carbonylation at

Table 1. Early Ethanol Exposure (From PND 1–8) and DFMO (500 mg/kg, s.c.) Administration in PND 8 Have No Effect on Protein Carbonyl (Carbonyl), 3-Nitrotyrosine (3-NT), and 4-Hydroxynonenal (HNE) Immunoreactivity in the Striatum, Cerebellum, Hypothalamus, and Cerebral Cortex at PND 40

	Carbonyl	3-NT	HNE
<i>Striatum</i>			
Maltose+saline	100.0±5.6	100.0±7.8	100.0±9.8
Maltose+DFMO	104.1±5.0	96.8±9.9	96.9±9.1
Ethanol+saline	106.3±3.0	92.9±13.1	93.7±9.2
Ethanol+DFMO	100.0±6.3	91.8±7.7	95.7±5.9
<i>Cerebellum</i>			
Maltose+Saline	100.0±4.7	100.0±4.1	100.0±3.5
Maltose+DFMO	91.5±4.8	102.2±3.9	100.2±3.1
Ethanol+Saline	98.1±8.0	102.3±4.3	102.4±4.1
Ethanol+DFMO	99.6±8.0	94.7±3.8	100.7±3.4
<i>Hypothalamus</i>			
Maltose+Saline	100.0±5.6	100.0±7.8	100.0±12.1
Maltose+DFMO	106.5±8.2	104.1±5.3	103.1±5.3
Ethanol+Saline	100.6±6.1	100.0±7.0	94.1±5.1
Ethanol+DFMO	96.6±4.9	96.5±6.3	98.9±6.3
<i>Cerebral cortex</i>			
Maltose+Saline	100.0±4.7	100.0±6.5	100.0±2.7
Maltose+DFMO	92.8±4.1	103.0±4.1	100.5±3.1
Ethanol+Saline	98.6±8.0	107.9±3.2	104.7±3.2
Ethanol+DFMO	100.6±7.2	106.2±4.1	104.5±4.1

Data are expressed as mean percent of control (maltose+saline)±SEM, $n = 6$ for all groups. PND, postnatal day; DFMO, difluoromethylornithine; s.c., subcutaneous.

PND 40 in the hippocampus. Difluoromethylornithine did not alter the immunoreactivity to HNE or 3-NT in any of the other cerebral structures analyzed.

The bulk of evidence suggests a regional susceptibility to the toxic effects of alcohol (Light et al., 1989; Nixon et al., 2002), with well-defined temporal patterns of susceptibility (Heaton et al., 2002). It is interesting that acute ethanol exposure increases production of reactive oxygen species (ROS) in the cerebellum on PND 4, but decreases it on PND 7, suggesting that the ROS-producing effect of ethanol depends on developmental factors (Heaton et al., 2002), which may include the proteins Bax, Bcl-2, and Bad, and protective factors that are increased after PND 7 (Heaton et al., 1999, 2003, 2006). Within these, the Bax protein is particularly remarkable, as the loss of the bax gene can prevent the ROS production that occurs as a function of ethanol exposure (Heaton et al., 2006). Therefore, as the ethanol treatment in our study spanned both periods in which ethanol induces and inhibits ROS production, it is possible that the expression of a protective factor after PND 7 may have protected the cerebellum from long-lasting oxidative modifications. To our knowledge, it is not known whether the striatum, cerebral cortex, or hypothalamus show similar developmental alterations in protein expression that could render them more resistant to ethanol-induced oxidative damage, compared with the hippocampus. Consequently, this possibility cannot be ruled out at the moment, and merits further investigation.

The mechanisms by which early ethanol exposure caused a selective increase of protein carbonylation in the hippocampus at PND 40 are still unknown, but the recurrent association in the literature between chronic ethanol exposure, NMDA receptor activation, and reactive species

production (see the introduction) tempt us to speculate about the subject.

Some authors point out that some neurochemical features may render the hippocampus more sensitive to the effects of polyamines and of ethanol (Allgaier, 2002; Randoll et al., 1996; Sultana and Babu, 2003). One of these could be the increased expression of the NR2B subunit, which is the subunit to which polyamines bind to modulate NMDA receptor function. This view is supported by the findings that ethanol sensitivity to NMDA receptor correlates with its sensitivity to ifenprodil, a noncompetitive NMDA receptor antagonist preferential to the NR2B polyamine binding site (Allgaier, 2002). In fact, ethanol increases mRNA of the NR2B subunit in the hippocampus of adult rats (Follesa and Ticku, 1995), but results concerning the effect of early postnatal alcohol exposure on the expression of the NMDA receptor subunits are not conclusive. Ethanol administration from PND 4 to 9 increases NR2A subunit levels by 60% at PND 21 in the cerebral cortex, but not in the hippocampus (Nixon et al., 2002). The same study identified a 50% increase in subunit NR2B levels and a 70% increase in NR1 levels in the cerebral cortex, which did not achieve statistical significance, but no alterations on hippocampal NR2B levels were found. No differences in the expression of NMDA receptor subunits in the hippocampus or cerebral cortex at day 21 were noticed when ethanol exposure occurred during both the gestational (G12–G18) and early postnatal phases (PND 4–9) (Nixon et al., 2004). However, an increase in the polypeptide levels of NR1 and NR2B subunits of NMDA receptors was identified after ethanol exposure in vitro (Harris et al., 2003). Therefore, it is not established whether an increase in the NR2B expression in

the hippocampus contributes for the currently described selective increase in protein carbonylation in the hippocampus in this model. However, it is worth noting that no study has addressed the effect of early ethanol exposure from PND 1 to 8 on the expression of NMDA receptor subunits at day 40, the time at which we performed the neurochemical analysis.

One must also consider that changes in the concentration of modulators of the NMDA receptor, such as polyamines, may be related to the well-known increase of NMDA receptor activity due to ethanol exposure in the hippocampus (Grant et al., 1990; Sanna et al., 1993). It is particularly interesting that chronic ethanol exposure increases both the production of polyamines *in vitro* and *in vivo* and the activity of ODC, a key regulatory enzyme for polyamines biosynthesis (Davidson and Wilce, 1998), particularly immediately after ethanol withdrawal (Thadani et al., 1977). These results suggest that increased NMDA receptor sensitivity may be due to a transient increase in polyamine production and release during early ethanol exposure and its withdrawal. Our results support this view, as DFMO, at a dose previously reported as effective to inhibit ODC and reduce the severity of withdrawal behaviors (Davidson and Wilce, 1998), prevented the effect of ethanol on protein carbonylation at PND 40 in the hippocampus, in our model. However, although this dose of DFMO has been reported to inhibit ODC, it is possible that a wider range of doses of DFMO might alter the currently reported regional differences and/or the interaction between ethanol and DFMO.

It is important to emphasize that polyamines have other actions, as main inducers of eukaryotic cell growth and proliferation (Janne et al., 2005) and as putative antioxidant agents. In fact, while increasing polyamine synthesis protects against UV radiation-induced cell damage, the inhibition of polyamine synthesis increases cell damage *in vitro* (von Deutsch et al., 2005). Accordingly, free radical scavenging activity has been described for these compounds, although they may paradoxically increase oxidative damage induced by β -amyloid peptide (Yatin et al., 2001), one of whose mechanisms of neurotoxicity may involve activation of the NMDA receptor (Harkany et al., 1999). These conflicting data fit well with the view that polyamines may contribute to both the survival (through its antioxidant action) and death (through excessive NMDA receptor activation), and that the final result depends on the pathways activated in the target cell, as well as on the intensity of the cellular response elicited. If the target cell expresses NR2B subunits, for example, it may be a natural target for the neurotoxic effect of increased levels of polyamines. Conversely, the transient lack of effect of polyamines (presumably caused by DFMO) may also alter normal polyaminergic functions in those animals that did not receive ethanol, rendering these animals more susceptible to oxidative damage. Therefore, it is possible that the currently described

increased carbonylation at PND 40 in the hippocampus of maltose-treated and DFMO-treated animals is due to the inhibition of a basal polyaminergic activity, necessary for tissue proliferation and cytoprotection.

Finally, we would like to point out that the markers of oxidative modification of proteins assessed in the current study, namely protein carbonyls, 3-NT, and protein bound 4-HNE, are preferentially formed by different neurochemical mechanisms. Proteins or peptides containing reactive carbonyl groups can be generated by: (1) direct reactions of proteins with ROS; (2) secondary reactions of primary amino groups of lysine residues to proteins with reducing sugars or their oxidation products and Michael-addition reactions of histidine, lysine, or cysteine residues with α,β -unsaturated aldehydes formed during the peroxidation of polyunsaturated fatty acids. In view of the fact that the formation of protein carbonyl groups is orders of magnitude greater than other oxidative modifications, the level of protein carbonyl groups has become the most widely used marker of protein oxidation during oxidative stress, aging, and diseases (for reviews, see Butterfield and Stadtman, 1997; Stadtman and Levine, 2003). This is also probably one of the reasons why we identified a selective increase in protein carbonyls due to early ethanol exposure in this study.

Reaction of NO with O_2^- leads to formation of peroxynitrite ($ONOO^-$), which, following protonation, generates cytotoxic species that oxidize and nitrate proteins (Beckman, 1996). The more common amino acidic target of nitration is tyrosine (Souza et al., 2001). In particular, nitration of tyrosine residues is a formal oxidation (Butterfield and Stadtman, 1997), and the chemistry of nitration has been extensively studied (Greenacre and Ischiropoulos, 2001), showing that increased nitration is related to an increased production of NO.

4-Hydroxynonenal is an aldehyde product of lipid peroxidation that reacts with cysteine, lysine, and histidine residues of proteins, leading to protein oxidation and dysfunction. Accumulating evidence suggests that there is a direct correlation between the adduction of HNE to a glutamate transporter and loss of glutamate transporter function (Keller et al., 1997). In addition, formation of HNE adducts has been also associated to CNS dysfunction in SAMP8 mice (Poon et al., 2004), further supporting a deleterious role for HNE in neurodegenerative processes.

Therefore, one might conclude, based on the lack of effect of ethanol on the content of 3-NT and HNE adducts in the current study, that NO and HNE do not significantly contribute for ethanol-induced protein oxidation in this model. However, one also must be aware that, as we measured total 3-NT and HNE protein adducts, we cannot rule out that only selected 3-NT and HNE protein adducts, potentially relevant for the deleterious effects of early ethanol exposure, were formed. If this were the case, formation of 3-NT and HNE adducts would be important for the deleterious effects ethanol, but no increase in the

total nitration or HNE adducts would be found. In this context, it is worth remarking that, in some cases, such as Alzheimer's disease, only selected proteins are oxidized (Castegna et al., 2002a, 2002b, 2003).

In summary, this study reports a long-lasting effect of ethanol on a measure of oxidative damage. While the mechanisms by which ethanol causes such a long-lasting effect and how long it remains are not defined, the fact that the administration of the ODC inhibitor, DFMO, inhibited this effect of ethanol indicates that it may involve overproduction of endogenous polyamines. In addition, it may indicate that the currently reported increased carbonylation is related to the withdrawal of ethanol, as the animals received DFMO only in the day of alcohol withdrawal. If the increased carbonylation were due to the chronic exposition to ethanol, the administration of a single dose of DFMO would probably not revert the oxidative damage caused in the previous 7 days. However, further studies have to be conducted to clarify this point.

ACKNOWLEDGMENTS

The authors acknowledge Mr. B. O. Overgaauw for his technical assistance.

REFERENCES

- Allgaier C (2002) Ethanol sensitivity of NMDA receptors. *Neurochem Int* 41:377–382.
- Bachrach U, Wang YC, Tabib A (2001) Polyamines: new cues in cellular signal transduction. *News Physiol Sci* 16:106–109.
- Barron S, Gagnon WA, Mattson SN, Kotch LE, Meyer LS, Riley EP (1988) The effects of prenatal alcohol exposure on odor associative learning in rats. *Neurotoxicol Teratol* 10:333–339.
- Beckman JS (1996) Oxidative damage and tyrosine nitration from peroxynitrite. *Chem Res Toxicol* 9:836–844.
- Bellinger FP, Bedi KS, Wilson P, Wilce PA (1999) Ethanol exposure during the third trimester equivalent results in long-lasting decreased synaptic efficacy but not plasticity in the CA1 region of the rat hippocampus. *Synapse* 31:51–58.
- Berlese DB, Sauzem PD, Carati MC, Guerra GP, Stiegemeier JA, Mello CF, Rubin MA (2005) Time-dependent modulation of inhibitory avoidance memory by spermidine in rats. *Neurobiol Learn Mem* 83:48–53.
- Butterfield DA, Stadtman ER (1997) Protein oxidation processes in aging brain. *Adv Cell Aging Gerontol* 2:161–191.
- Castegna A, Aksenov M, Aksenova M, Thongboonkerd V, Klein JB, Pierce WM, Booze R, Markesbery WR, Butterfield DA (2002a) Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part I: creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1. *Free Radic Biol Med* 33:562–571.
- Castegna A, Aksenov M, Thongboonkerd V, Klein JB, Pierce WM, Booze R, Markesbery WR, Butterfield DA (2002b) Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part II: dihydropyrimidinase-related protein 2, alpha-enolase and heat shock cognate 71. *J Neurochem* 82:1524–1532.
- Castegna A, Thongboonkerd V, Klein JB, Lynn B, Markesbery WR, Butterfield DA (2003) Proteomic identification of nitrated proteins in Alzheimer's disease brain. *J Neurochem* 85:1394–1401.
- Cebere A, Cebers G, Wagner A, Liljequist S (2002) Spermidine attenuates the inhibitory effect of ethanol on NMDA-induced neurotoxicity. *Naunyn Schmiedeberg Arch Pharmacol* 366:117–122.
- Chenard BL, Menniti FS (1999) Antagonists selective for NMDA receptors containing the NR2B subunit. *Curr Pharm Des* 5:381–404.
- Childs AC, Mehta DJ, Gerner EW (2003) Polyamine-dependent gene expression. *Cell Mol Life Sci* 60:1394–1406.
- Davidson M, Wilce P (1998) Chronic ethanol treatment leads to increased ornithine decarboxylase activity: implications for a role of polyamines in ethanol dependence and withdrawal. *Alcohol Clin Exp Res* 22:1205–1211.
- Ferrari-Kile K, Randall PK, Leslie SW (2003) Acute ethanol affects phosphorylation state of the NMDA receptor complex: implication of tyrosine phosphatases and protein kinase A. *Brain Res Mol Brain Res* 115:78–86.
- Follesa P, Ticku MK (1995) Chronic ethanol treatment differentially regulates NMDA receptor subunit mRNA expression in rat brain. *Brain Res Mol Brain Res* 29:99–106.
- Gibson DA, Harris BR, Prendergast MA, Hart SR, Blanchard JA II, Holley RC, Pedigo NW, Littleton JM (2003) Polyamines contribute to ethanol withdrawal-induced neurotoxicity in rat hippocampal slice cultures through interactions with the NMDA receptor. *Alcohol Clin Exp Res* 27:1099–1106.
- Gilbertson RJ, Barron S (2005) Neonatal ethanol and nicotine exposure causes locomotor activity changes in preweanling animals. *Pharmacol Biochem Behav* 81:54–64.
- Goodlett CR, Horn KH (2001) Mechanisms of alcohol-induced damage to the developing nervous system. *Alcohol Res Health* 25:175–184.
- Grant KA, Valverius P, Hudspeth M, Tabakoff B (1990) Ethanol withdrawal seizures and the NMDA receptor complex. *Eur J Pharmacol* 176:289–296.
- Greenacre SA, Ischiropoulos H (2001) Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. *Free Radic Res* 34:541–541.
- Guerri C, Montoliu C, Renau-Piqueras J (1994) Involvement of free radical mechanism in the toxic effects of alcohol: implications for fetal alcohol syndrome. *Adv Exp Med Biol* 366:291–305.
- Harkany T, Mulder J, Sasvari M, Abraham I, Konya C, Zarandi M, Penke B, Luiten PG, Nyakas C (1999) *N*-Methyl-D-aspartate receptor antagonist MK-801 and radical scavengers protect cholinergic nucleus basalis neurons against beta-amyloid neurotoxicity. *Neurobiol Dis* 6:109–121.
- Harris BR, Gibson DA, Prendergast MA, Blanchard JA, Holley RC, Hart SR, Scotland RL, Foster TC, Pedigo NW, Littleton JM (2003) The neurotoxicity induced by ethanol withdrawal in mature organotypic hippocampal slices might involve cross-talk between metabotropic glutamate type 5 receptors and *N*-methyl-D-aspartate receptors. *Alcohol Clin Exp Res* 27:1724–1735.
- Heaton MB, Moore DB, Paiva M, Gibbs T, Bernard O (1999) Bcl-2 overexpression protects the neonatal cerebellum from ethanol neurotoxicity. *Brain Res* 817:13–18.
- Heaton MB, Moore DB, Paiva M, Madorsky I, Mayer J, Shaw G (2003) The role of neurotrophic factors, apoptosis-related proteins, and endogenous antioxidants in the differential temporal vulnerability of neonatal cerebellum to ethanol. *Alcohol Clin Exp Res* 27:657–669.
- Heaton MB, Paiva M, Madorsky I, Siler-Marsiglio K, Shaw G (2006) Effect of bax deletion on ethanol sensitivity in the neonatal rat cerebellum. *J Neurobiol* 66:95–101.
- Heaton MB, Paiva M, Mayer J, Miller R (2002) Ethanol-mediated generation of reactive oxygen species in developing rat cerebellum. *Neurosci Lett* 334:83–86.
- Honse Y, Randall PK, Leslie SW (2003) Prenatal ethanol exposure modifies [3H]MK-801 binding to NMDA receptors: spermidine and ifenprodil. *Alcohol Clin Exp Res* 27:1993–2001.
- Huang CC, Hsu PC, Hung YC, Liao YF, Liu CC, Hour CT, Kao MC, Tsay GJ, Hung HC, Liu GY (2005) Ornithine decarboxylase prevents

- methotrexate-induced apoptosis by reducing intracellular reactive oxygen species production. *Apoptosis* 10:895–907.
- Janne J, Alhonen L, Keinänen TA, Pietilä M, Uimari A, Pirinen E, Hyvönen MT, Jarvinen A (2005) Animal disease models generated by genetic engineering of polyamine metabolism. *J Cell Mol Med* 9: 865–882.
- Joshi G, Perluigi M, Sultana R, Agrippino R, Calabrese V, Butterfield DA (2006) In vivo protection of synaptosomes by ferulic acid ethyl ester (FAEE) from oxidative stress mediated by 2,2-azobis(2-amidino-propane)dihydrochloride (AAPH) or Fe(2+)/H(2)O(2): insight into mechanisms of neuroprotection and relevance to oxidative stress-related neurodegenerative disorders. *Neurochem Int* 48:318–327.
- Jung ME, Gatch MB, Simpkins JW (2005) Estrogen neuroprotection against the neurotoxic effects of ethanol withdrawal: potential mechanisms. *Exp Biol Med (Maywood)* 230:8–22.
- Keller JN, Mark RJ, Bruce AJ, Blanc E, Rothstein JD, Uchida K, Waeg G, Mattson MP (1997) 4-hydroxynonenal an aldehydic product of membrane lipid peroxidation, impairs glutamate transport and mitochondrial function in synaptosomes. *Neuroscience* 80:685–696.
- Kotlinska J, Liljequist S (1996) Oral administration of glycine and polyamine receptor antagonists blocks ethanol withdrawal seizures. *Psychopharmacology (Berlin)* 127:238–244.
- Light KE, Serbus DC, Santiago M (1989) Exposure of rats to ethanol from postnatal days 4 to 8: alterations of cholinergic neurochemistry in the cerebral cortex and corpus striatum at day 20. *Alcohol Clin Exp Res* 13:29–35.
- Littleton JM, Lovinger D, Liljequist S, Ticku R, Matsumoto I, Barron S (2001) Role of polyamines and NMDA receptors in ethanol dependence and withdrawal. *Alcohol Clin Exp Res* 25 (suppl ISBRA): 132S–136S.
- Liu GY, Hung YC, Hsu PC, Liao YF, Chang WH, Tsay GJ, Hung HC (2005) Ornithine decarboxylase prevents tumor necrosis factor alpha-induced apoptosis by decreasing intracellular reactive oxygen species. *Apoptosis* 10:569–581.
- Marino MD, Aksenov MY, Kelly SJ (2004) Vitamin E protects against alcohol-induced cell loss and oxidative stress in the neonatal rat hippocampus. *Int J Dev Neurosci* 22:363–377.
- Mattson SN, Riley EP (1998) A review of the neurobehavioral deficits in children with fetal alcohol syndrome or prenatal exposure to alcohol. *Alcohol Clin Exp Res* 22:279–294.
- Mayer S, Harris BR, Gibson DA, Blanchard JA, Prendergast MA, Holley RC, Littleton J (2002) Acamprosate, MK-801, and ifenprodil inhibit neurotoxicity and calcium entry induced by ethanol withdrawal in organotypic slice cultures from neonatal rat hippocampus. *Alcohol Clin Exp Res* 26:1468–1478.
- Mikolajczak P, Okulicz-Kozaryn I, Kaminska E, Szulc M, Dyr W, Kostowski W (2003) Lack of ifenprodil anxiolytic activity after its multiple treatment in chronically ethanol-treated rats. *Alcohol* 38:310–315.
- Nagy J, Horvath C, Farkas S, Kolok S, Szombathelyi Z (2004) NR2B subunit selective NMDA antagonists inhibit neurotoxic effect of alcohol-withdrawal in primary cultures of rat cortical neurones. *Neurochem Int* 44:17–23.
- Nakano M, Kikuyama M, Hasegawa T, Ito T, Sakurai K, Hiraishi K, Hashimura E, Adachi M (1995) The first observation of O₂⁻ generation at real time in vivo from non-Kupffer sinusoidal cells in perfused rat liver during acute ethanol intoxication. *FEBS Lett* 372:140–143.
- Nakao LS, Kadiiska MB, Mason RP, Grijalba MT, Augusto O (2000) Metabolism of acetaldehyde to methyl and acetyl radicals: in vitro and in vivo electron paramagnetic resonance spin-trapping studies. *Free Radic Biol Med* 29:721–729.
- Nixon K, Hughes PD, Amsel A, Leslie SW (2002) NMDA receptor subunit expression following early postnatal exposure to ethanol. *Brain Res Dev Brain Res* 139:295–299.
- Nixon K, Hughes PD, Amsel A, Leslie SW (2004) NMDA receptor subunit expression after combined prenatal and postnatal exposure to ethanol. *Alcohol Clin Exp Res* 28:105–112.
- Poon HF, Castegna A, Farr SA, Thongboonkerd V, Lynn BC, Banks WA, Morley JE, Klein JB, Butterfield DA (2004) Quantitative proteomics analysis of specific protein expression and oxidative modification in aged senescence-accelerated-prone 8 mice brain. *Neuroscience* 126:915–926.
- Prendergast MA, Harris BR, Blanchard JA II, Mayer S, Gibson DA, Littleton JM (2000) In vitro effects of ethanol withdrawal and spermidine on viability of hippocampus from male and female rat. *Alcohol Clin Exp Res* 24:1855–1861.
- Ramachandran V, Perez A, Chen J, Senthil D, Schenker S, Henderson GI (2001) In utero ethanol exposure causes mitochondrial dysfunction, which can result in apoptotic cell death in fetal brain: a potential role for 4-hydroxynonenal. *Alcohol Clin Exp Res* 25: 862–871.
- Randoll LA, Wilson WR, Weaver MS, Spuhler-Phillips K, Leslie SW (1996) *N*-methyl-D-aspartate-stimulated increases in intracellular calcium exhibit brain regional differences in sensitivity to inhibition by ethanol. *Alcohol Clin Exp Res* 20:197–200.
- Roebuck TM, Mattson SN, Riley EP (1998) A review of the neuroanatomical findings in children with fetal alcohol syndrome or prenatal exposure to alcohol. *Alcohol Clin Exp Res* 22:339–344.
- Rubin MA, Berlese DB, Stiegemeier JA, Volkweis MA, Oliveira DM, dos Santos TL, Fenili AC, Mello CF (2004) Intra-amygdala administration of polyamines modulates fear conditioning in rats. *J Neurosci* 24:2328–2334.
- Sanna E, Serra M, Cossu A, Colombo G, Follesa P, Cuccheddu T, Concas A, Biggio G (1993) Chronic ethanol intoxication induces differential effects on GABAA and NMDA receptor function in the rat brain. *Alcohol Clin Exp Res* 17:115–123.
- Souza JM, Chen QP, Blanchard-Fillion B, Lorch SA, Hertkorn C, Lightfoot R, Weisse M, Friel T, Paxinou E, Themistocleous M, Chov S, Ischiropoulos H (2001) Reactive nitrogen species and proteins: biological significance and clinical relevance. *Biol React Intermediates* 500:169–174.
- Stadtman ER, Levine RL (2003) Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* 25: 207–218.
- Sultana R, Babu PP (2003) Ethanol-induced alteration in *N*-methyl-D-aspartate receptor 2A C-terminus and protein kinase C activity in rat brain. *Neurosci Lett* 349:45–48.
- Sultana R, Newman S, Mohammad-Abdul H, Keller JN, Butterfield DA (2004) Protective effect of the xanthate, D609, on Alzheimer's amyloid beta-peptide (1–42)-induced oxidative stress in primary neuronal cells. *Free Radic Res* 38:449–458.
- Sun AY, Ingelman-Sundberg M, Neve E, Matsumoto H, Nishitani Y, Minowa Y, Fukui Y, Bailey SM, Patel VB, Cunningham CC, Zima T, Fialova L, Mikulikova L, Popov P, Malbohan I, Janebova M, Nespor K, Sun GY (2001) Ethanol and oxidative stress. *Alcohol Clin Exp Res* 25 (suppl ISBRA): 237S–243S.
- Thadani PV, Lau C, Slotkin TA, Schanberg SM (1977) Effect of maternal ethanol ingestion on neonatal rat brain and heart ornithine decarboxylase. *Biochem Pharmacol* 26:523–527.
- Thomas JD, Fleming SL, Riley EP (2001) MK-801 can exacerbate or attenuate behavioral alterations associated with neonatal alcohol exposure in the rat, depending on the timing of administration. *Alcohol Clin Exp Res* 25:764–773.
- Thomas JD, Garcia GG, Dominguez HD, Riley EP (2004) Administration of eliprodil during ethanol withdrawal in the neonatal rat attenuates ethanol-induced learning deficits. *Psychopharmacology (Berlin)* 175:189–195.
- Thomas JD, Weinert SP, Sharif S, Riley EP (1997) MK-801 administration during ethanol withdrawal in neonatal rat pups attenuates ethanol-induced behavioral deficits. *Alcohol Clin Exp Res* 21: 1218–1225.
- Tsai GE, Ragan P, Chang R, Chen S, Linnoila VM, Coyle JT (1998) Increased glutamatergic neurotransmission and oxidative stress after alcohol withdrawal. *Am J Psychiatry* 155:726–732.

- von Deutsch AW, Mitchell CD, Williams CE, Dutt K, Silvestrov NA, Klement BJ, Abukhalaf IK, von Deutsch DA (2005) Polyamines protect against radiation-induced oxidative stress. *Gravit Space Biol Bull* 18:109–110.
- Weiger TM, Hermann A, Levitan IB (2002) Modulation of calcium-activated potassium channels. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 188:79–87.
- West JR (1994) Recent findings on the mechanisms by which alcohol damages the developing nervous system. *Alcohol Alcohol* 2 (suppl): 395–399.
- West JR, Chen WJ, Pantazis NJ (1994) Fetal alcohol syndrome: the vulnerability of the developing brain and possible mechanisms of damage. *Metab Brain Dis* 9:291–322.
- Yatin SM, Yatin M, Varadarajan S, Ain KB, Butterfield DA (2001) Role of spermine in amyloid beta-peptide-associated free radical-induced neurotoxicity. *J Neurosci Res* 63:395–401.
- Zima T, Fialova L, Mestek O, Janebova M, Crkovska J, Malbohan I, Stipek S, Mikulikova L, Popov P (2001) Oxidative stress, metabolism of ethanol and alcohol-related diseases. *J Biomed Sci* 8: 59–70.