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Estrogen protects against brain lipid peroxidation in ethanol-withdrawn rats

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Abstract

This study examined whether 17β-estradiol (E2) administration protects against ethanol withdrawal (EW)-associated oxidative insults by assessing oxidative markers thiobarbituric-acid-reacting-substances (TBARS). Ovariectomized rats implanted with E2 (EW/E2) or oil pellets (EW/Oil) received chronic ethanol (7.5% wt./vol., 5 weeks) or control dextrin diet (Dextrin/Oil). At 24 or 48 h of EW, rats were tested for overt EW signs and the cerebellum, hippocampus, and cortex were prepared for TBARS assessment in the presence and absence of $FeCl_3$. For control experiments, we assessed E2 effects on blood ethanol concentrations and TBARS levels during ethanol exposure prior to EW. The EW/Oil group showed enhanced endogenous- and $FeCl_3$-stimulated membrane TBARS levels in the cerebellum and hippocampus in a manner inhibited by E2 treatment. There was a relationship between the severity of EW and elevation of TBARS levels, particularly in the cerebellum. The enhanced TBARS levels at 24 h of EW appeared to diminish at 48 h in the hippocampus, but persisted in the cerebellum. E2 treatment did not alter blood ethanol concentrations and ethanol exposure alone did not enhance TBARS levels. These data suggest that EW rather than ethanol enhances brain lipid peroxidation that is transient and brain-region specific. Estrogens protect against the brain lipid peroxidation in a manner independent of blood ethanol concentrations (Supported by NIH/NIAAA 013864).

Keywords: Ethanol withdrawal (EW); 17β-estradiol (E2); Oxidation; Thiobarbituric-acid-reacting-substances (TBARS); Rats

1. Introduction

Oxidative stress is believed to be an imbalance between the endogenous antioxidant defense system and free radical generation. Oxidative stress-induced neuronal damage has been implicated in a variety of neurodegenerative disorders. Chronic ethanol consumption and ethanol withdrawal (EW) generate oxidative free radicals and subsequent lipid peroxidation (Uysal et al., 1989; Nordmann et al., 1990; Montoliu et al., 1994), the latter of which reflects the interaction between oxygen and the polyunsaturated fatty acids of membrane lipids, generating deteriorating breakdown products (Esterbauer et al., 1991). The pro-oxidant nature of ethanol toxicity is particularly important in the central nervous system (CNS) because the CNS consists of a high content of unsaturated membrane lipid, and membranes are a preferred target of both reactive oxygen species and ethanol (Szelenyi and Brune, 1988; Terano et al., 1989; Kvietys et al., 1990; Hernandez-Munoz et al., 2000).

A previous study reports that enhanced reactive oxygen species are associated with EW-induced seizure activity (Vallett et al., 1997). In addition, we have reported estrogen protection against cerebellar neuronal damage in ethanol-withdrawn rats. Here, we attempted to characterize the role of estrogen in EW-related oxidative damage. The rationales behind our hypothesis are such that EW syndromes are attributed by an overexpression of excitotoxic brain systems (Rossetti and Carboni, 1995) and excitatory neurotransmitters correlate with oxidative markers during EW (Tsai et al., 1998). We focused on the withdrawal phase after chronic

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ethanol diet because withdrawal syndromes provide motivation to continue ethanol abuse despite the development of a tolerance to the drug's direct reinforcing effects (Barrett, 1985).

A variety of in vitro and in vivo studies documented oxidative stress during the ethanol intoxication and withdrawal phase. In in vivo studies, ethanol-fed rats displayed enhanced production of oxidative markers, such as thiobarbituric-acid-reacting-substances (TBARS), H_2O_2, and OH-like species (Dicker and Cederbaum, 1992). In the process of ethanol metabolism, acetaldehyde oxidation increased reactive oxygen species in chronically ethanol-fed cells and contributed to the occurrence of oxidative stress (Fernandez-Checa et al., 1998; Rodrigo et al., 1998). In a clinical study, a significant correlation was observed between excitatory neurotransmitters and oxidative markers in the cerebrospinal fluid of abstinent alcoholics (Tsai et al., 1998). Higher levels of lipid peroxide and lower levels of superoxide dismutase (antioxidant enzyme) activity were also seen in these studies (Tsai et al., 1998). These studies provide evidence that ethanol and/or EW are pro-oxidant.

17β-estradiol (E2), the most potent naturally occurring estrogen, has been reported to have a potent neuroprotective and antioxidant activity (Lacort et al., 1995; Moosmann and Behl, 1999; Sugioka et al., 1987). E2 can attenuate lipid peroxidation induced by β-amyloid (Green and Simpkins, 2000; Green et al., 2000), glutamate toxicity (Goodman et al., 1996), and $FeSO_4$ exposure (Goodman et al., 1996; Blum-Degen et al., 1998). E2 can further attenuate the increase in intracellular peroxides induced by haloperidol (Sagara, 1998) and H_2O_2 (Sawada et al., 1998). Recent studies have suggested a correlation between the neuroprotective and antioxidant effects of estrogens such that the antioxidant and neuroprotective effects are seen at identical concentrations (Behl et al., 1997; Sagara, 1998; Moosmann and Behl, 1999). Moreover, estrogens appear to diminish lipid peroxidation of redox cycling mechanisms coupled with glutathione (Green and Simpkins, 2000) and NADPH (a reduced form of Nicotinamide Adenine Dinucleotide Phosphate, an electron carrier; Prokai et al., 2003a,b).

Although there is evidence supporting pro-oxidant effects of ethanol and/or EW and antioxidant effects of estrogens, no studies have determined whether estrogens counteract oxidative damage during EW. Previously, we reported protective effects of E2 against neurobehavioral damage associated with EW in rats (Jung et al., 1999, 2000, 2002, 2003; Rewal et al., 2003). In this study, we employed the same rat model of EW to induce ethanol dependence and subsequent withdrawal, and examined whether EW increases oxidative markers in a manner that can be prevented by E2 treatment.

We employed a TBARS assay to detect levels of lipid peroxidation, a major indicator of oxidative stress (Armstrong and Browne, 1994; Lefevre et al., 1998).

This method measures several aldehydes derived from lipid hydroperoxides, has provided important information regarding free radical activity in normal and disease states, and has been used to measure the antioxidant activity of numerous compounds (Hunnisett et al., 1995; Villa-Caballero et al., 2000). Although there is controversy regarding the specificity of TBARS, it remains the most widely employed assay to determine lipid peroxidation (Armstrong and Browne, 1994; Hunnisett et al., 1995; Lefevre et al., 1998; Villa-Caballero et al., 2000). We chose the cerebellum, hippocampus, and cortex as target brain areas because of their susceptibility to the neurotoxic effects of ethanol/EW and oxidative insults (Allsop and Turner, 1966; Victor et al., 1971; Goldman et al., 1973; D'Mello et al., 1993; Kril and Halliday, 1999; Yu et al., 1999; Schweinsburg et al., 2001).

2. Methods

2.1. Subjects

Adult female Sprague–Dawley rats (Charles River, Wilmington, MA) were housed individually in a room with controlled temperature (22–25 °C) and humidity (55%). A 12-h light–dark cycle was maintained with lights on between 7 a.m. and 7 p.m. After animals were habituated, an ovariectomy was performed under isoflurane (2% vol./vol.) anesthesia such that a small incision was made in the abdominal cavity directly above the ovary. The ovaries were removed bilaterally, and the incisions were closed with stainless steel wound clips. Two weeks were allowed for recovery from the surgery and for ovarian hormone clearance before the ethanol diet began. Animal body weight was monitored until they were sacrificed. All housing and procedures were in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health (Institute of Laboratory Animal Resources, 1996) and were approved by the University of North Texas Health Science Center Animal Care and Use Committee.

2.2. E2 replacement

One-half of the rats were subcutaneously implanted with Silastic pellets containing E2, and the other half were implanted with Silastic pellets containing oil. Thirty-millimeter-long Silastic tubes (1.57 mm ID, 3.18 mm OD) were filled with E2 (4 mg/ml) or corn oil, and the tubes were closed with Medical Adhesive Silicone (Dow Corning, Midland, MI). We have used this method to achieve physiological E2 concentrations (29 to 34 pg/ml and 5.2 to 5.7 pg/ml in E2-pellets and oil-pellet implanted ovariectomized rats, respectively) using the Ultrasensitive estradiol radioimmunoassay (Diagnostic Systems Lab, Webster, TX; Yang et al., 2001; Jung et al., 2002). In brief, after anesthetized with isoflurane (2% vol./vol.) via inhalation, the rats were implanted with E2 or corn-oil-filled

Silastic capsules along the dorsal aspect, which provided a sustained release of hormone. Incisions were closed with Prolene, nonabsorbable and nonwicking sutures. For all experiments, E2 and oil pellets were replaced at the time of ovariectomy and every 3 weeks thereafter until the rats were sacrificed.

2.3. Chronic ethanol administration in liquid diet and EW

At the onset of ethanol administration, rats were 4 months old. The induction of ethanol dependence was accomplished by a liquid diet mode of administration, as modified by Dodd and Shorey-Kutschke (1987). The amount of dextrin and ethanol was calculated in combination to adjust the concentration of ethanol to 7.5% wt./vol. Control animals were fed a liquid diet with dextrin isocalorically substituted for ethanol (Lal et al., 1988). Saccharin was added to mask the ethanol taste. One hundred milliliters of the diet was placed in each home cage daily for 5 weeks. On the last day of the chronic diet, a 50-ml aliquot was administered and 12 h later, the diet tubes were removed. Twenty-four and 48 h after termination of the chronic diet, physical signs of EW were evaluated by an experimenter who was not aware of treatment group identity. Our laboratory has repeatedly employed a well-established method of evaluating such EW signs (Goldstein and Pal, 1971; Lal et al., 1988). The rating scale ranged from 0 to a maximum of 25 (Table 1). The amount of diet consumption was recorded daily. In all experiments, three groups of ovariectomized rats (5 rats per group) were employed as follows: (1) the control dextrin group was implanted with oil pellets (Dextrin/Oil), (2) the ethanol group was implanted with oil pellets (EW/Oil), and (3) the ethanol group was implanted with E2 pellets (EW/E2).

Table 1
Rating scores for ethanol withdrawal signs

Withdrawal signs	Score
Vocalization, urination, and defecation on handling	0–3
Caudal posture	0–3
0 pt. For limp or normal tail	
1 pt. For stiff, curls around finger,	
2 pts. For stiff, curls around finger, stays elevated after released	
3 pts. For spontaneous abnormal posture of tail such as severe deviation or lift above back, stiff, curls around finger, and stays elevated after released	
Tremor	0–3
0 pt. For no tremor	
1 pt. For mild tremor in one portion of body (i.e., face)	
2 pts. For generalized occasional tremor	
3 pts. For constant generalized tremor	
Startle	0–3
0 pt. For none	
1 pt. For twitch	
2 pts. For jump or freeze	
3 pts. For exaggerated jump or freeze	
Handler-induced convulsion	1
Spontaneous seizure	2
Death	10

2.4. The method of evaluating EW signs

The severity of EW signs was assessed by recording seven items for each rat. After each item was scored, the scores for seven items were summed, giving one total score for each animal. All tests were conducted in a specifically designated room in the vivarium in order to protect animals from environmental stimuli, such as noise or pollution.

The first item (vocalization, urination, and defecation on handling) was rated when the rater opened the home cage and picked up the rat. Most of ethanol-withdrawn rats showed all three signs immediately after the rater handled the rat. Each sign gave a score 1 and thus, score 3 when rats showed all three signs. The second item (caudal posture) was rated when the tail was drawn gently between the rater's fingers. If the tail was limp, a 0 was scored. A 1 was given when the rater felt normal tension of the tail. A 2 was given when the tail curled around finger with tension and stayed elevated after released from the finger. A 3 was scored when the tail showed an additional sign such as a very stiff and curled tail above back even before the rater touched the tail.

The third item (tremor) was rated during handling. A 0 was given when the rat was limp. A 1 was given when one portion of body showed a slight tremor. A 2 and a 3 were given when a whole body showed occasional tremor and constant generalized tremor, respectively. The fourth item (startle) was observed for 15 s. A 0 was rated when the rat showed the complete absence of the movement. A 1 was given when the rat showed wet-dog type shakes and a single jerk. A 2 was given when the rat showed a jumping or freezing movement. A 3 was given when a severe jumping or freezing movement occurred two to several times. Spontaneous seizure and handler induced convulsion were rated before and after handling, respectively. A 0 was rated when the rat showed no convulsion. A 1 was given when forelimb clonus occurred. A 2 was given when forelimb clonus and whole body shakes occurred. A 3 was given when tonic and clonic convulsions occurred.

2.5. Tissue preparation for TBARS assay

The first and the second sets of animals (Dextrin/Oil, EW/Oil, and EW/E2 groups) were decapitated 24 and 48 h after the termination of the chronic diet, respectively. The cerebellum, hippocampus, and cortex were collected and homogenized in an ice-cold buffer (20 mM HEPES, 2 mM EGTA, 1 mM PMSF, 2 mM DTE, and 20 μg/ml aprotinin). The homogenate was centrifuged at 100,000×g for 60 min at 4 °C. The supernatant was saved as the cytosol fraction.

The pellet was resuspended in a buffer containing 0.1% Triton X-100 and centrifuged at 50,000×*g* for 30 min at 4 °C, and the supernatant containing soluble membrane proteins was collected. All samples were adjusted by dilution to contain 00.05% Triton X-100. Samples to be assayed for TBARS assay were diluted to a protein concentration of 1 μg/μl.

2.6. TBARS assay

Membrane or cytosol fractions of brain samples (100 μl) were added to a solution containing 1% TBA, 12.5% TCA, and 0.8 N HCl and incubated at 55 °C for an hour. For an iron-stimulated TBARS assay, the iron chloride (50 μM $FeCl_3$) was added to the samples for 15 min at 37 °C to determine the interaction between pro-oxidant effects of iron and EW. Proteins were precipitated by centrifugation at 14,000 rpm for 2 min. Supernate was transferred to 96-well plates with calibration curves consisting of 1,1,3,3-tetramethoxypropane standards ranging from 0 to 20 μM. TBARS levels were determined using a BioTek FL600 plate reader at an excitation wavelength of 530 nm (bandwidth 25), emission wavelength of 590 nm (bandwidth 20), and a sensitivity of 100.

2.7. Control experiments

For all control experiments, separate groups of animals (Dextrin/Oil, EW/Oil, and EW/E2 groups) received the same hormone and ethanol diet treatment as described above. For the measurement of TBARS levels, the cerebellar membrane tissue was used.

2.7.1. E2 effects on ethanol consumption and blood ethanol concentration

A control experiment was conducted to determine whether E2 treatment alters blood ethanol concentration. During the third week on diet, whole blood (100 μl) was collected by cardiac puncture at five different time points, immediately after monitoring ethanol consumption: 2, 4, 8, 12, and 16 h after placing fresh diet at 5 p.m. Cardiac puncture was done using a heparin-coated 21-gauge syringe (Becton-Dickinson, Franklin Lakes, NJ, USA). The blood was then centrifuged with lymphocyte separation medium (Sigma, St. Louis, MO, USA) for 30 min at 1500 rpm (Lee et al., 2003). Blood ethanol concentration was measured using an enzymatic assay (Smolen et al., 1986).

2.7.2. Effects of an antioxidant butylated hydroxytoluene (lipid peroxidation inhibitors) on TBARS levels

A second control experiment was conducted to determine whether enhancement of TBARS levels reflects pro-oxidant nature of the EW stimulus or experimental artifacts from the reaction or tissue processing, e.g., the heating at 55 °C for 60 min. The same TBARS assay was used as above with the exception that butylated hydroxytoluene (2.5 μmol; Ko et al., 1995; Horakova et al., 2000) was added to the homogenization buffer before incubation at 55 °C.

2.7.3. Effects of chronic ethanol exposure on TBARS levels

A third control experiment was conducted to determine whether enhancement of TBARS levels reflects oxidative stress which occurred during the ethanol intake period or lipid peroxidation induced by the EW. The TBARS levels were measured at the time of sacrifice, prior to withdrawal using the same TBARS assay as above.

2.8. Analysis of data

All data were expressed as the mean±S.E.M. Data from all TBARS experiments were from at least two separate determinations. One-way analysis of variance (diet/hormone treatment) was conducted to determine a group difference in EW sign scores or TBARS levels followed by a post hoc Tukey test. Pearson correlation coefficients (Hines et al., 2000; Kuo et al., 2003) were calculated to assess the association between the magnitude of EW signs and TBARS levels. Pearson correlation efficient was suitable in this case because both *X* (EW sign scores) and *Y* (TBARS levels) variables were dependent variables. The significance level for all data analysis was set at $P<0.05$.

3. Results

3.1. Body weight and ethanol consumption

The body weights of all the rats before the chronic diet were 200 to 230 g. Twenty-four or 48 h after the termination of the chronic diet, the dextrin-diet rats weighed 240 to 270 g, and the ethanol-diet rats weighed 210 to 250 g. Although the range of body weight was different between control and ethanol animals, there was no statistically significant difference in body weight between two groups. Rats under the current ethanol diet regimen, do not gain much weight as was for the previous case (Lal et al., 1988) and remained healthy throughout the diet period. The diet consumptions in the Dextrin/Oil and the EW/Oil group were an average 70 and 60 ml/day, respectively. All ethanol-treated rats consumed a similar amount of ethanol (an average 15 g/kg/day). There was no significant difference in ethanol consumption between the EW/Oil group and the EW/E2 group.

3.2. EW stimulus and E2 effects on the overt physical signs

Fig. 1 illustrates the severity of EW signs which were scored from 0 to 25 scales. At 24 h of EW, the three groups of diet- and hormone-treated rats (Dextrin/Oil, EW/Oil, and EW/E2 groups) had different EW scores of overt physical signs [$F(2,12)=32.6$, $P<0.001$]. A post hoc Tukey

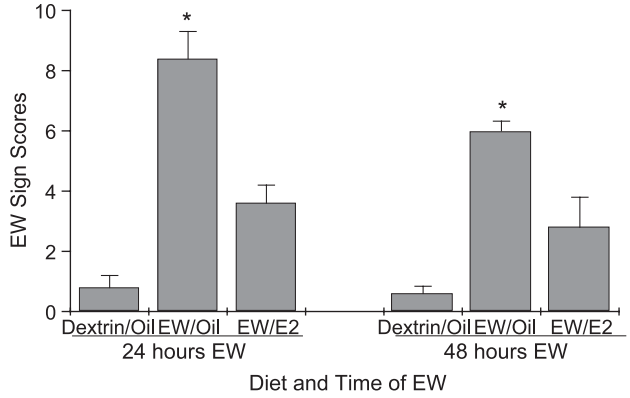

Fig. 1. Effects of ethanol withdrawal (EW) and estrogen on the overt withdrawal sign scores. 17β-estradiol (E2)- or oil-pellet-implanted ovariectomized rats received an ethanol diet (7.5% wt./vol., 5 weeks). For the control group, oil-pellet-implanted ovariectomized rats received a dextrin diet. At 24 and 48 h of EW, they were tested for withdrawal signs. At both 24 and 48 h of EW, withdrawal scores of the EW/Oil group were higher than those of the Dextrin/Oil groups ($P<0.001$) and the EW/E2 group ($P<0.05$). *Difference from the Dextrin/Oil group and the EW/E2 group. Data were collected from five rats per group.

comparison indicated that the EW/Oil group had higher withdrawal scores (8.4±0.9) than the Dextrin/Oil group (0.8±0.4, $P<0.001$), indicating the presence of EW. The EW/E2 (3.6±0.6) group had a lower withdrawal score than the EW/Oil group ($P=0.001$), but a higher score than the Dextrin/Oil group ($P=0.031$), thus demonstrating an attenuating effect of E2 on the EW signs. The Dextrin/Oil group showed no EW signs.

At 48 h of EW, the three groups of rats had different EW scores [$F(2,12)=17.0$, $P<0.001$]. Although somewhat decreased, the scores were still higher in the EW/Oil group (6±0.3) than the Dextrin/Oil group ($P<0.001$) and the EW/E2 group (2.8±1, $P=0.013$). There was no significant difference in the scores between the EW/E2 group and the Dextrin/Oil group (0.6±0.24).

3.3. Effects of E2 on TBARS levels during EW

For this experiment, TBARS levels were measured in the absence and presence of pro-oxidant $FeCl_3$ in order to determine whether lipid peroxidation induced by EW interacts with a redox cycle of iron.

3.3.1. Cerebellum

3.3.1.1. Membrane. Fig. 2A and B illustrates cerebellar membrane TBARS levels at 24 and 48 h of EW. At both time points, the EW/Oil group had higher levels of TBARS than the Dextrin/Oil group ($P<0.01$) and the EW/E2 group ($P<0.05$), in the presence [$F(2,12)=10$, $P=0.003$] and absence [$F(2,12)=22$, $P<0.001$] of $FeCl_3$ treatment. $FeCl_3$ treatment significantly increased TBARS levels in all three groups ($P<0.05$).

Time factor: Since we conducted assays at two different times, an exact comparison of TBARS levels at 24 and 48 h of EW was not possible. Nevertheless, there was a trend of the TBARS levels at 48 h of EW being decreased compared to those at 24 h. Although somewhat decreased at 48 h, the endogenous TBARS levels in the EW/Oil group appeared to remain high and similar to those at 24 h of EW.

3.3.1.2. Cytosol. Fig. 2C and D illustrates cytosol TBARS levels at 24 and 48 h of EW. At 24 h of EW, the EW/Oil group had higher levels of cytosol TBARS compared to the EW/E2 group ($P<0.05$) in the presence [$F(2,12)=4$, $P=0.043$] and absence [$F(2,12)=2$, $P=0.002$] of $FeCl_3$ treatment. There was no significant difference in cytosol TBARS levels between the Dextrin/Oil group and the EW/Oil group. $FeCl_3$ treatment significantly increased TBARS levels in all three groups ($P=0.013$).

At 48 h of EW, cytosol TBARS levels were not different among the treatment groups in the absence or presence of $FeCl_3$ treatment. However, $FeCl_3$ treatment significantly increased TBARS levels in all three groups ($P<0.001$).

3.3.2. Hippocampus

3.3.2.1. Membrane. Fig. 3A and B illustrates membrane TBARS levels at 24 and 48 h of EW in the hippocampus. At 24 h of EW, the three treatment groups had different membrane TBARS levels in the absence of $FeCl_3$ [$F(2,12)=17$, $P<0.001$], but these differences were abolished in the presence of $FeCl_3$ treatment. The EW/Oil group at 24 h of EW had higher TBARS levels than the Dextrin/Oil ($P=0.002$) and EW/E2 groups ($P=0.001$). $FeCl_3$ treatment significantly increased TBARS levels in all three groups ($P=0.003$).

At 48 h of EW, the membrane TBARS levels were not different among three groups in the absence or presence of $FeCl_3$ treatment. However, $FeCl_3$ treatment significantly increased TBARS levels in all three groups ($P<0.001$).

Time factor: There was a trend of the endogenous and $FeCl_3$-stimulated TBARS levels being decreased at 48 h compared to the levels at 24 h of EW.

3.3.2.2. Cytosol. The three treatment groups did not have different cytosol TBARS levels in the absence and presence of $FeCl_3$ treatment. However, $FeCl_3$ treatment significantly increased TBARS levels in all three groups at both 24 and 48 h of EW ($P<0.001$). The cytosol TBARS levels are 34±2 in the Dextrin/Oil group, 38±3 in the EW/Oil group, and 39±2 (nmol/mg protein) in the EW/E2 group at 24 h of EW and in the absence of $FeCl_3$. At 48 h of EW in the absence of $FeCl_3$, the corresponding levels of the three groups are 38±1, 36±3, and 33±1 (nmol/mg protein). In the presence of $FeCl_3$, the corresponding TBARS levels of the three groups are 126±8, 123±9, and 124±16 (nmol/mg protein) at 24 h of EW. At 48 h of EW,

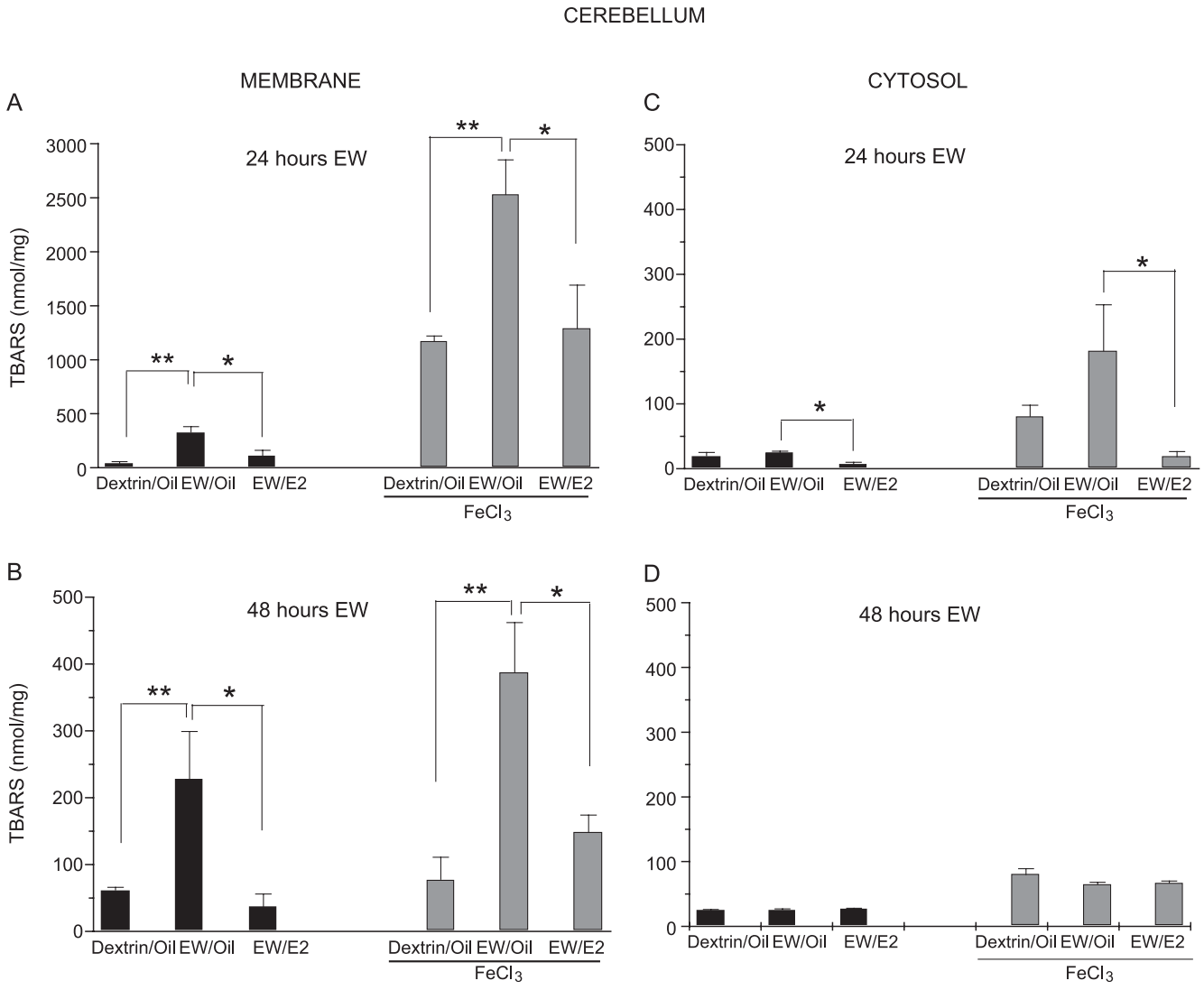

Fig. 2. Estrogen effects on thiobarbituric-acid-reacting-substances (TBARS) levels in the cerebellum of ethanol-withdrawn rats. 17β-estradiol (E2)- or oil-pellet-implanted ovariectomized rats received an ethanol diet (7.5% wt./vol., 5 weeks). For the control group, oil-pellet-implanted ovariectomized rats received a dextrin diet. At 24 and 48 h of ethanol withdrawal (EW), the cerebelli were collected for the TBARS assay. Data were collected from five rats per group. (A, B) At both time points (24 and 48 h) and under both conditions of $FeCl_3$, the EW/Oil group had higher levels of membrane TBARS than the Dextrin/Oil (**P<0.01) or the EW/E2 group (*P<0.05). (C, D) At 24 h of EW and under both conditions of $FeCl_3$, the EW/Oil group had higher levels of cytosol TBARS than the EW/E2 group (*P<0.05) but did not significantly differ from the Dextrin/Oil group. These differences disappeared at 48 h of EW in both the presence and absence of $FeCl_3$.

the corresponding TBARS levels are 110±8, 100±6, and 100±42 (nmol/mg protein).

Time factor: There was a trend of the TBARS levels being decreased at 48 h of EW compared to the levels at 24 h of EW.

3.3.3. Cortex

3.3.3.1. Membrane. Fig. 3C and D illustrates membrane TBARS levels in the cortex at 24 and 48 h of EW. Although there was a trend of higher TBARS levels in the EW/Oil, no significant group differences were found. $FeCl_3$ treatment significantly increased TBARS levels in all three groups (P< 0.001).

At 48 h of EW, the treatment groups did not have different membrane TBARS levels in the absence or presence of $FeCl_3$ treatment. $FeCl_3$ treatment significantly increased TBARS levels in all three groups (P<0.001).

Time factor: The TBARS levels did not appear to differ between the two time points of EW in the absence or presence of $FeCl_3$ treatment.

3.3.3.2. Cytosol. There were no group differences in the cytosol TBARS levels of the cortex at any time point of EW. However, $FeCl_3$ treatment (P< 0.001) significantly increased TBARS levels in all three groups at both 24 and 48 h of EW (P< 0.001).

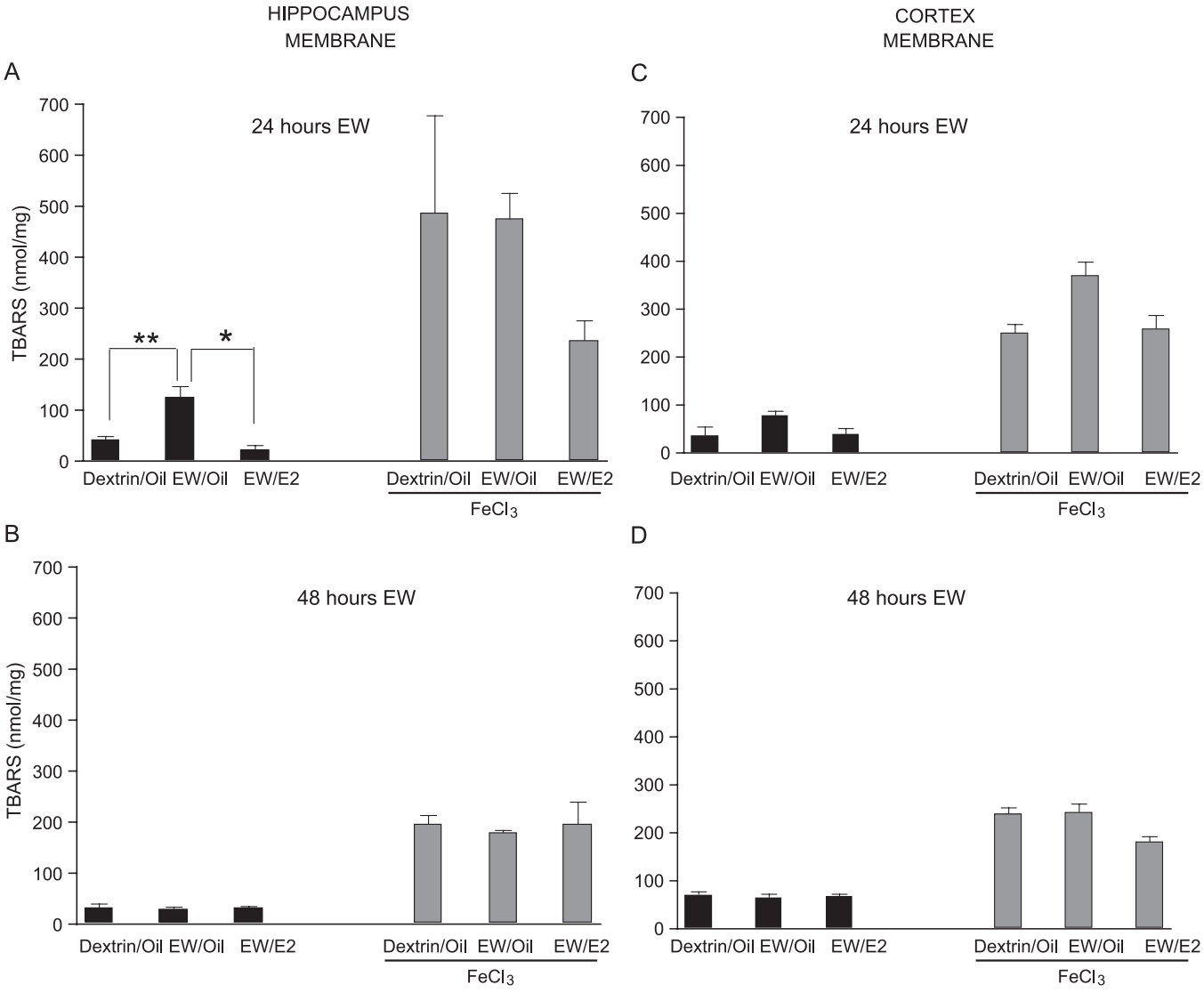

Fig. 3. Estrogen effects on thiobarbituric-acid-reacting-substances (TBARS) levels in the hippocampus and cortex of ethanol-withdrawn rats. 17β-estradiol (E2)- or oil-pellet-implanted ovariectomized rats received an ethanol diet (7.5% wt./vol., 5 weeks). For the control group, oil-pellet-implanted ovariectomized rats received a dextrin diet. At 24 and 48 h of ethanol withdrawal (EW), hippocampi were collected for the TBARS assay. Data were collected from five rats per group. (A, B) At 24 h of EW and in the absence of $FeCl_3$, the EW/Oil group had a higher level of membrane TBARS in the hippocampus than the Dextrin/Oil (**P=0.002) and the EW/E2 group (*P=0.001). These differences disappeared at 48 h of EW in both the presence and the absence of $FeCl_3$. (C, D) At 24 h of EW and under both conditions of $FeCl_3$, there was a trend of the EW/Oil group having a higher level of membrane TBARS in the cortex than the Dextrin/Oil and the EW/E2 group. This trend disappeared at 48 h of EW in the presence and the absence of $FeCl_3$.

The cytosol TBARS levels at 24 h of EW and in the absence of $FeCl_3$ are 55±4 in the Dextrin/Oil group, 53±2 in the EW/Oil group, and 56±3 (nmol/mg protein) in the EW/E2 group. At 48 h of EW and in the absence of $FeCl_3$, the corresponding levels of the three groups are 35±3, 39±2, and 38±2 (nmol/mg protein). In the presence of $FeCl_3$ at 24 h of EW, the corresponding TBARS levels of the three groups are 126±1, 134±12, and 116±27 (nmol/mg protein). At 48 h of EW, the corresponding TBARS levels are 128±5, 144±4, and 119±51 (nmol/mg protein).

Time factor: The TBARS levels did not appear to differ between the two time points of EW in the presence or absence of $FeCl_3$ treatment.

3.4. Correlation between EW sign scores and TBARS levels

Fig. 4 illustrates that TBARS levels covaried with EW sign scores. However, the correlation was found only in the membrane fractions and only at 24 h of EW, with the exception of cerebellum. In the cerebellar membrane, endogenous TBARS levels increased as EW sign scores increased at both 24 (Fig. 4A, Pearson r=0.73, P=0.002) and 48 h of EW (Fig. 4B, Pearson r=0.54, P=0.037). When stimulated by $FeCl_3$, a correlation was also found in the cytosol fractions of the cerebellum (24 h of EW, Pearson r=0.7, P=0.003, data not shown).

In the hippocampus (Fig. 4C, Pearson r=0.64, P=0.01) and cortex (Fig. 4D, r=0.54, P=0.037), a correlation

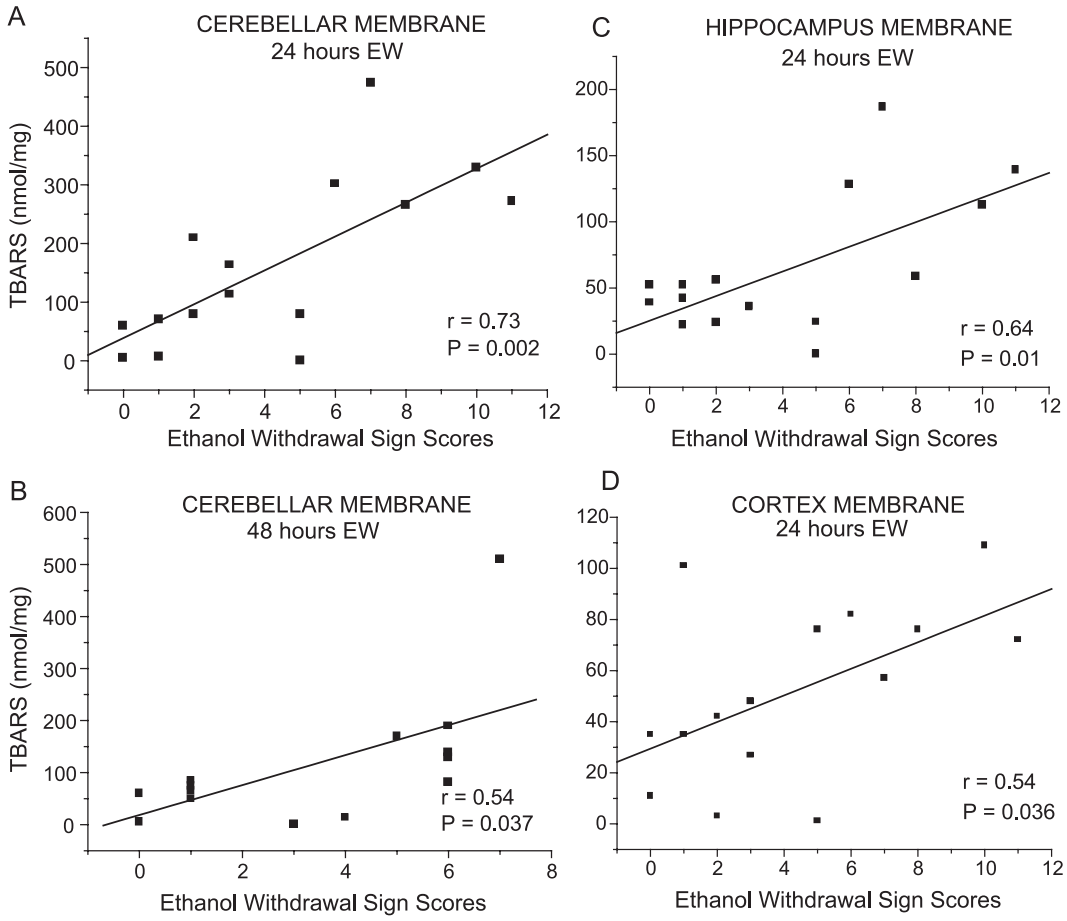

Fig. 4. Correlation between ethanol withdrawal (EW) sign scores and thiobarbituric-acid-reacting-substances (TBARS) levels. 17β-estradiol (E2)- or oil-pellet-implanted ovariectomized rats received an ethanol diet (7.5% wt./vol., 5 weeks). For the control group, oil-pellet-implanted ovariectomized rats received a dextrin diet. At 24 and 48 h of EW, rats were tested for EW signs, scoring from 0 to a maximum 25, and were immediately sacrificed for the TBARS assay. Data were collected from three groups of rats, and five rats per group. (A, B) The TBARS levels from the cerebellar membrane fractions covaried with EW sign scores at both 24 and 48 h of EW. This phenomenon appeared to be more robust at 24 h of EW (higher correlation coefficient) than at 48 h of EW. (C, D) The TBARS levels from the hippocampus and cortex membrane fractions covaried with EW sign scores at 24 h of EW.

between endogenous TBARS levels and EW sign scores was found only in the membrane fractions at 24 h of EW.

3.5. Control experiments

3.5.1. EW and E2 effects on ethanol consumption and blood ethanol concentration

Fig. 5A and B illustrates that ethanol consumption and blood ethanol concentration increased as a function of time elapsed after placing fresh dextrin or ethanol diet. The maximum ethanol consumptions were 24.8 g/kg/day in the EW/Oil group and 24.1 g/kg/day in the EW/E2 group, averaging approximately 15 g/kg/day over 5 weeks for both groups. The maximum blood ethanol concentrations were 3.2 mg/ml in the EW/Oil and 3.0 mg/ml in the EW/E2 group, which occurred at 12 h after placing fresh diet. Thereafter, blood ethanol concentrations appeared to decrease when observed 16 h after placing fresh diet. No statistically significant differences in ethanol consumption or blood ethanol concentration were observed between the EW/Oil and the EW/E2 groups. Ethanol consumption and blood ethanol concentration were 0 in the Dextrin/Oil group. At 24 h of EW, no blood ethanol was detected in any of the three treatment groups.

3.5.2. Effects of EW and E2 on TBARS levels after addition of an antioxidant

Fig. 6A illustrates cerebellar membrane TBARS levels at 24 h of EW in the presence and absence of an antioxidant butylated hydroxytoluene. The TBARS levels were somewhat decreased in all three treatment groups after inclusion of butylated hydroxytoluene but did not significantly differ from the TBARS levels prior to butylated hydroxytoluene addition. In addition, the TBARS levels in the presence of butylated hydroxytoluene were still higher in the EW/Oil group than the Dextrin/Oil group ($P < 0.001$) and the EW/E2 group ($P < 0.001$). The TBARS levels in the presence of butylated hydroxytoluene correlated with EW sign scores such that rats with higher EW signs scores showed higher TBARS levels (Fig. 6B, Pearson r=0.7, $P < 0.001$).

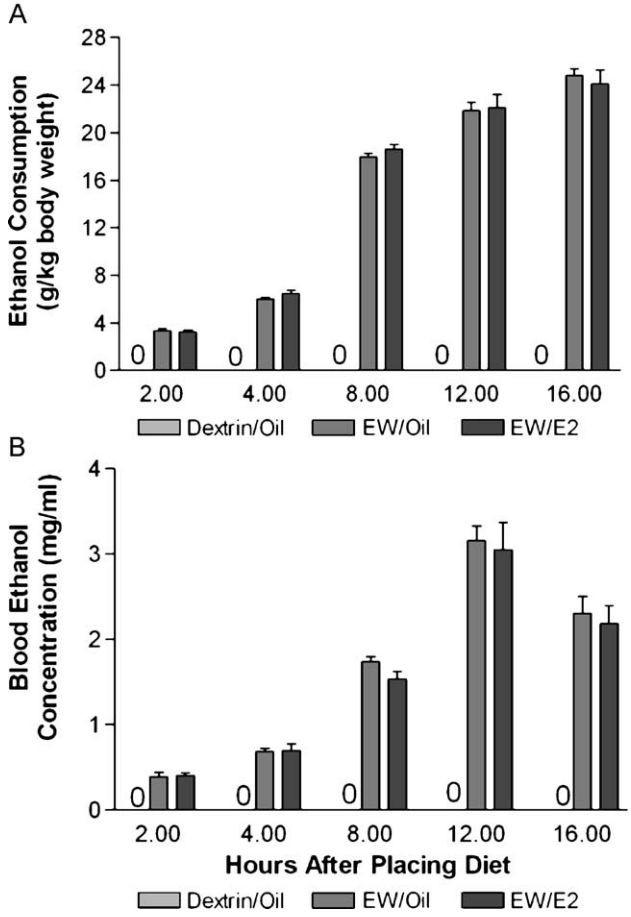

Fig. 5. Estrogen effects on ethanol consumption and blood ethanol concentrations. 17β-estradiol (E2)- or oil-pellet-implanted ovariectomized rats received an ethanol diet (7.5% wt./vol., 5 weeks). For the control group, oil-pellet-implanted ovariectomized rats received a dextrin diet. At the third week on diet, blood was obtained by cardiac puncture at 2, 4, 8, 12, and 16 h after placing fresh diet. There were no significant differences in ethanol consumption and blood ethanol concentrations between the EW/Oil and the EW/E2 groups. "0" indicates no ethanol consumption and 0 blood ethanol concentration in the Dextrin/Oil group. Data were collected from five rats per group.

3.5.3. Effects of ethanol exposure on TBARS levels

The cerebellar membrane tissue obtained from animals at the end of chronic ethanol exposure (5 weeks) prior to EW showed no significantly different TBARS levels as compared to the control dextrin or the EW/E2 groups under any of the three conditions used. $FeCl_3$ addition significantly increased the TBARS levels of all three groups of diet/hormone treatment [$F(1,16)=58$, $P<0.001$].

4. Discussion

This study demonstrated that ethanol-withdrawn rats have enhanced oxidative markers in a manner that is protected by estrogen treatment. The oxidative event appeared to be brain-region specific, EW time dependent, and reflection of EW toxicity rather than ethanol toxicity. Our findings provide a better knowledge of a counteracting interaction between antioxidant estrogen and poroxidant EW.

Growing evidence suggests that oxidative stress is associated with EW toxicity. Rats and humans during EW showed an increased level of the main component of TBARS malondialdehyde (Marotta et al., 1997; d'Ischia et al., 2000). The oxidative event may be transient, because the enhanced TBARS levels appeared to diminish as EW time elapsed. Likewise, levels of TBARS or lipid peroxidation are increased at 24 h of EW and return back toward the control level after 2–4 days of EW. Considering that oxidative stress and glutamatergic neurotransmission are mutually enhancing (Pellegrini-Giampietro et al., 1988; Coyle and Puttfarcken, 1993), relevant to our data is a report that excitotoxic glutamatergic neurotransmission is most robust during the first 48 h of EW (Snell et al., 1993).

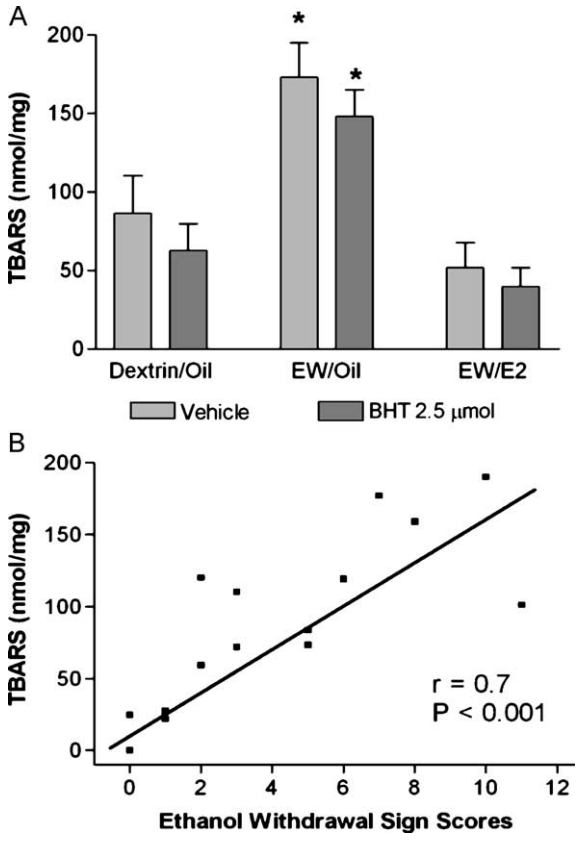

Fig. 6. Ethanol withdrawal (EW) and estrogen effects on thiobarbituric-acid-reacting-substances (TBARS) levels after addition of an antioxidant. 17β-estradiol (E2)- or oil-pellet-implanted ovariectomized rats received an ethanol diet (7.5% wt./vol., 5 weeks). For the control group, oil-pellet-implanted ovariectomized rats received a dextrin diet. At 24 h of EW, TBARS levels were measured after addition of an antioxidant butylated hydroxytoluen (2.5 μmol) to the cerebellar membrane fractions of ethanol-withdrawn rats. Data were collected from five rats per group. (A) Butylated hydroxytoluen did not significantly alter TBARS levels as compared to those in the absence of butylated hydroxytoluen. TBARS levels in the presence of butylated hydroxytoluen were higher in the EW/Oil group than the Dextrin/Oil (*$P<0.001$) or the EW/E2 group (*$P<0.001$). (B) The TBARS levels in the presence of butylated hydroxytoluen covaried with EW sign scores at 24 h of EW ($P<0.001$).

Indeed, Marotta et al. (1997) stated that the oxidative phenomena occur in the early phase of EW rather than the late phase. Such transient oxidative insults may not immediately result in neuronal death because no TUNEL positive cells were detected at 48 h of EW (data not shown). Ulrichsen et al. (1996) also showed no hippocampal neuronal loss during the early EW phase after multiple EW in rats. The observed lipid peroxidation at 24 and 48 h of EW may contribute to apoptosis and neuronal death which occur in the late phase of EW (Jung et al., 2000).

We chose the cerebellum, hippocampus, and cortex because of their susceptibility to ethanol toxicity and oxidative stress (Goldman et al., 1973; Kril and Halliday, 1999; Yu et al., 1999; Schweinsburg et al., 2001). The alcoholic brains exhibit a reduction in hippocampal volume (Agartz et al., 1999), neuronal loss in the frontal lobes of the cortex (Kril and Halliday, 1999), and cerebellar Purkinje cell loss in ethanol-withdrawn rats (Jung et al., 2000). However, brain response to ethanol appears to vary in different regions of brain. For instance, a putative ethanol-regulated gene is increased in the hippocampus and cortex of ethanol-withdrawn mice, but decreased in the cerebellum (Schafer et al., 2001). Ethanol enhances chloride uptake from cerebellar and cerebral cortical microsacs, but not from the hippocampal microsacs (Proctor et al., 1992). The synaptosomal membranes from the hippocampus show more drastic changes in calcium-binding activity than the cortex or cerebellum of ethanol-withdrawn rats (Virmani et al., 1985). Regional susceptibility to ethanol varies for such endpoints as genomic expression (Dave et al., 1990; Schafer et al., 2001), GABAergic neurotransmission (Charlton et al., 1997), the *N*-methyl-D-aspartate receptors (Follesa and Ticku, 1995), P-450 content (key enzymes of microsomal ethanol oxidation; Warner et al., 1988; Tindberg and Ingelman-Sundberg, 1996), and endogenous antioxidative systems (Tsai et al., 1998). In our study, the cerebellar membrane showed not only the maximum increase of TBARS levels, but also persisted longer than other areas tested, suggesting that the cerebellum has greater susceptibility to oxidative stress during EW. Although there is no consistent regionally specific response to ethanol toxicity, previous studies support our finding that the cerebellum is one of the brain areas most vulnerable to ethanol toxicity (Goldman et al., 1973). The cerebellar-related disorder ataxia is one of the most common syndromes in human alcoholics (Victor et al., 1971).

The strong correlation between the severity of EW signs and TBARS levels indicates that EW stimulus is associated with oxidative stress. In agreement, EW-induced seizure activity correlates with the production of hydroxy radicals in ethanol-withdrawn rats (Vallett et al., 1997). The highest correlation between cerebellar TBARS levels and the severity of EW signs is supported by a clinical study where cerebellar atrophy patients had markedly decreased levels of antioxidant Coenzyme Q10 with associated symptoms of seizures (Lamperti et al., 2003). The expression of the withdrawal syndrome is a behavioral manifestation of an excitotoxic event associated with multiple factors including increased glutamatergic neurotransmission, increased extracellular glutamate levels (Rossetti and Carboni, 1995), upregulation of calcium channels (Whittington et al., 1995) and an accumulation of intracellular calcium (Nagy, 2000). All these factors are involved in oxidative stress such that increased brain glutamate levels during EW correlates with the severity of EW signs (Rossetti and Carboni, 1995; Gonzales et al., 1996; Fadda and Rossetti, 1998), suggesting potential mechanisms underlying EW oxidation. Although withdrawal seizures may be responsible for lipid peroxidation, a few of withdrawn animals exhibited seizure in our study. Therefore, it appears that the enhanced TBARS levels are attributed by global excitotoxic effects of EW rather than seizure activity per se. Indeed, Coyle and Puttfarcken (1993) stated that persistent activation of glutamate-gated ion channels and oxidative stress are interacting processes that provide a final common pathway for cell vulnerability in the brain.

Our findings extend antioxidant properties of estrogen to the case for EW. Estrogens are potent lipid antioxidants and thus, block membrane oxidation (Green et al., 1996; Gridley et al., 1998). E2 treatment reduces the by-products of lipid peroxidation (Green et al., 2000) and reduces the oxidation of low-density lipoproteins (Mukai et al., 1990; Sacks et al., 1994). Estrogen protection against EW oxidation may also involve glutamate transmission because glutamate-induced oxidative stress is attenuated by E2 (Behl and Manthey, 2000) and by the quinol derived from E2 (Prokai et al., 2003a). Given these findings, the observed correlation between EW signs and TBARS may partly result from glutamatergic activation which is antagonized by estrogen.

For further characterization of oxidative mechanisms of EW, we tested a possibility that oxidative insults of EW interact with a pro-oxidant iron. Although iron has been reported to associate with alcoholic organ injury (Cederbaum, 2003), its pro-oxidant effect has not been established during EW. Iron is very capable of enhancing pro-oxidants' generation, lipid peroxidation (Halliwell and Gutteridge, 1984), and ethanol toxicity. Our results suggest that $FeCl_3$ plays a role in oxidative insults of EW and E2 acts as an effective inhibitor of iron-induced oxidation. Likewise, when myelin isolated from the rat brain was incubated with $FeCl_3$, significant levels of malondialdehyde were detected in a manner that is correlated with iron concentrations (Bongarzone et al., 1995), suggesting a pro-oxidant nature of iron. Furthermore, treatment with an antioxidant attenuated retinal neuron death induced by iron oxidation (Sohn and Yoon, 1998). In fact, iron levels were significantly increased in the cerebral cortex, striatum, and brainstem in neurodegenerative diseases (Kim et al., 2000) and in the microsomal fraction after ethanol treatment (Rouach et al., 1990).

Although the above studies indicate that iron contributes to oxidative stress, the exact mechanism underlying a higher

susceptibility of the ethanol-withdrawn tissue, especially cerebellum to iron-induced oxidation is not clear at this moment. Perhaps, ethanol-withdrawn rats have enhanced excitatory neurotransmission at baseline, such as glutamate (Fadda and Rossetti, 1998), which increases reactive oxygen species, such as hydrogen peroxide. The hydrogen peroxide is catalyzed to hydroxyl radical in the presence of iron (Crichton et al., 2000). If regional susceptibility to ethanol varies for endogenous antioxidative systems (Tsai et al., 1998) or P-450 content (Warner et al., 1988; Tindberg and Ingelman-Sundberg, 1996), the cerebellum during EW might have a lower level of antioxidant enzymes which decrease the amount of Fe^{2}+ available for Fenton reaction (production of hydroxyl radical catalyzed by iron; Sandstrom et al., 1997). It is also possible that the cerebellum has a higher P-450 content, generating reactive oxygen species in the presence of iron (Cederbaum, 2003).

Similar to our finding, E2 and 17α-estradiol, which has a low affinity for the estrogen receptor, attenuate oxidative neuronal death induced by iron in mouse cortical cultures (Bae et al., 2000) and inhibit reactive oxygen species in the human artery (Speir et al., 2000). E2 also decreases iron-induced lipid peroxidation in rat brain homogenate (Vedder et al., 1999). Findings that estrogen receptor antagonists failed to block the antioxidative effects of E2 (Behl et al., 1997; Gridley et al., 1998), and 17α-estradiol showed similar antioxidative effects to E2 (Bae et al., 2000), suggest receptor independent mechanisms of estrogen. Additionally, E2 may counteract pro-oxidant ethanol and iron through antioxidative enzymes because the enzyme activities were damaged by iron (Garcon et al., 2001) and downregulated by estrogen deficiency (Strehlow et al., 2003). The down-regulation of antioxidant enzymes is associated with increased production of free radicals and is prevented by estrogen replacement (Strehlow et al., 2003). As mentioned earlier, if antioxidant effects of E2 are in part mediated through glutamate neurotransmission, E2 probably suppresses membrane lipid peroxidation induced by iron through mechanisms involving the glutamate system (Keller et al., 1997).

Since a large portion of membrane consists of an unsaturated lipid moiety, membrane can be a preferred target of lipid peroxidation (Adachi, 2000; Lehotsky et al., 2002). This is an important issue because ethanol disturbs membrane fluidity, generates free radicals, and enhances lipid peroxidation, all of which further damage membrane integrity (Ahmad et al., 1988). In this context, our findings are significant in that membrane is also the major target of EW insults and the cytosol is minimally affected. Relevant to this issue, erythrocyte membrane fluidity of alcohol-dependent patients is altered during acute EW (Thompson, 1999), suggesting that a membrane-targeting treatment may be a useful research or clinical strategy.

The lipophilicity of E2 (octanol/water coefficient of 2.9) insures that at all estrogen concentrations, the vast majority (2999 of every 3000 molecules) will associate with lipid membranes. This intercalation of E2 into lipid membranes appears to be fundamental to its neuroprotective activity. We and others have demonstrated that the rank order of neuroprotective potencies of estrogens strongly correlates with their antioxidant potencies (Behl et al., 1997; Green et al., 1997; Green and Simpkins, 2000; and unpublished observations). Furthermore, we have demonstrated that physiologically relevant concentrations of estrogens interact synergistically with the antioxidant, glutathione in providing enhanced neuroprotective potency of the estrogens (Green et al., 1998; Gridley et al., 1998). This synergy with glutathione is likely a reflection of a novel estrogen redox cycle (Prokai et al., 2003a,b) that applied the reducing potential of abundant glutathione to the prevention of lipid peroxidation.

Finally, we examined the possibility that the altered TBARS levels are due to blood ethanol concentrations, an artifactual TBARS production from the tissue processing, or reflection of oxidative stress which had occurred during ethanol exposure. Our results do not support for any of such possibilities (Figs. 5–7). E2 treatment influenced neither ethanol consumption nor blood ethanol concentrations, indicating that the antioxidant effects of E2 were not due to differences in ethanol intake or blood ethanol levels. In addition, inclusion of an antioxidant, butylated hydroxytoluene (Ko et al., 1995; Horakova et al., 2000) to minimize an artifactual TBARS production did not suppress TBARS levels, suggesting that the enhanced TBARS levels are not from an artifactual TBARS production. Moreover, the TBARS levels after addition of butylated hydroxytoluene still correlated with the severity of EW signs. These results indicate that the enhanced TBARS levels result from the pro-oxidant nature of EW whereas suppressed TBARS

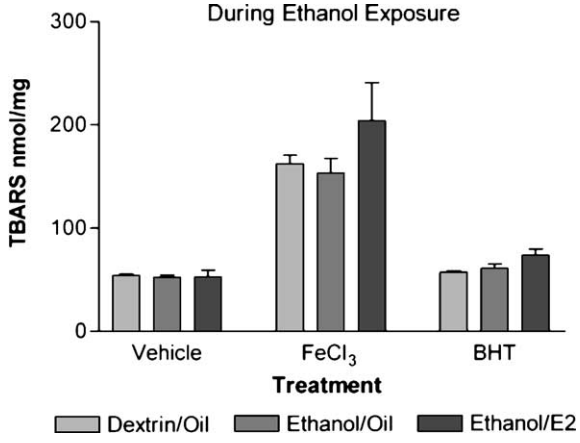

Fig. 7. Effects of chronic ethanol exposure on thiobarbituric-acid-reacting-substances (TBARS) levels. 17β-estradiol (E2)- or oil-pellet-implanted ovariectomized rats received an ethanol diet (7.5% wt./vol., 5 weeks). For the control group, oil-pellet-implanted ovariectomized rats received a dextrin diet. At the end of diet, TBARS levels were measured prior to EW in the presence of vehicle, $FeCl_3$ (50 μM) or butylated hydroxytoluene (BHT, 2.5 μM). There were no diet/hormone treatment group difference in TBARS levels under any of these three conditions.

levels after E2 treatment result from antioxidant nature of estrogen. Furthermore, the observed increase in the TBARS appears to be due to the withdrawal stimulus, since no increase in TBARS was detected at the end of the ethanol exposure period.

To summarize, we have used multiple approaches, such as different brain regions, membrane versus cytosol, and iron oxidation. The approaches allowed us to speculate that antioxidant estrogen counteracts pro-oxidant EW in the membrane lipid through a pathway involving iron oxidation. The cerebellar specific susceptibility is somewhat puzzling to comprehend. However, this phenomenon is commonly observed in human subjects in that impaired motor coordination (cerebellar deficit) occurs earlier than cognitive impairment (hippocampus or cortex deficit).

In conclusion, estrogen protects against brain lipid peroxidation associated with EW in a manner that is transient, brain-region specific, and independent of blood ethanol concentration. Therefore, estrogen can be an antioxidant research tool to discover more precise molecular mechanisms behind EW oxidation.

Acknowledgments

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