Ethanol withdrawal provokes mitochondrial injury in an estrogen preventable manner

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Abstract We investigated whether ethanol withdrawal (EW) oxidizes mitochondrial proteins and provokes mitochondrial membrane swelling and whether estrogen deprivation contributes to this problem. Ovariectomized female rats with or without 17β-estradiol (E2)-implantation received a control diet or a liquid ethanol diet (6.5%) for 5 weeks and were sacrificed during EW. Protein oxidation was assessed by measuring carbonyl contents and was visualized by immunochemistry. Mitochondrial membrane swelling as an indicator of mitochondrial membrane fragility was assessed by monitoring absorbance at 540 nm and was compared with that of male rats. Compared to the control diet group and ovariectomized rats with E2-implantation, ovariectomized rats without E2-implantation showed higher carbonylation of mitochondrial proteins and more rapid mitochondrial membrane swelling during EW. Such rapid mitochondrial membrane swelling was comparable to that of male rats undergoing EW. These findings demonstrate that EW provokes oxidative injury to mitochondrial membranes in a manner that is exacerbated by estrogen deprivation.

Keywords Ethanol withdrawal ·
Mitochondrial membrane swelling · Oxidation ·
Protein carbonyls · 17β-estradiol

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Abbreviations

CsA	cyclosporine A
DNPH	2,4-dinitrophenylhydrazine
EW	ethanol withdrawal
PTP	mitochondrial permeability transition pore
E2	17β-estradiol

Many ethanol-dependent individuals relapse into ethanol abuse to avoid discomfort and disorders associated with ethanol withdrawal (EW), but there is little mechanistic insight into this important clinical problem. This study was undertaken to gain a better understanding of the mechanisms involved in EW-attributed mitochondrial injury. We hypothesized that EW induces oxidative damage to mitochondrial proteins of female rats and that a loss of estrogen contributes to this problem. The brain is particularly susceptible to oxidative stress and generates more reactive oxygen species than other tissues because it processes large amounts of O_2 in a relatively small mass (Poon et al. 2004). Studies report that reactive oxygen species and free radicals oxidatively modify proteins (Nystrom et al. 2005). The oxidatively modified proteins are functionally inactive (Rivett 1989) and are accompanied by an increase in the number of carbonyl residues on the proteins (Amici et al. 1989). Therefore, augmented carbonyl contents have been measured as a cellular marker of oxidative damage to proteins in a variety of oxidative models (Levine et al. 1990; Forster et al. 2000). Numerous studies have demonstrated that ethanol can cause oxidative damage to proteins in different tissues and organs. Increased protein carbonyl formation has been observed in the blood of ethanol-dependent patients (Mutlu-Turkoglu et al. 2000) and in the liver of rats following ethanol exposure (Bailey et al. 2001).

We focused on mitochondrion because this organelle is one of the major subcellular targets of oxidative stress (Inoue et al. 2003) as well as ethanol and EW. Evidence is mounting in support of this idea, such that reactive oxygen species produced during ethanol metabolism altered mitochondrial function (Fernandez-Checa et al. 1998; Orellana et al. 1998; Mansouri et al. 2001; Minana et al. 2002), and EW dramatically increased the mitochondrial membrane permeability and oxidation (French and Todoroff 1971; Hosein et al. 1980). Mitochondrial inner membranes are well known for their impermeability; the passage of non-specific molecules can disrupt the fundamental function of the mitochondria. The inner mitochondrial membrane harbors a large number of proteins, including respiratory chain complexes and mitochondrial permeability transition pore (PTP) complexes, all of which are essential for mitochondrial integrity (Stuart 2002). While oxidative damage to mitochondrial proteins has been associated with pathological conditions, few studies have examined protein oxidation in conjunction with EW in this important organelle.

In contrast to the pro-oxidant effects of EW, estrogen has long been recognized for its antioxidant effect. Our recent findings extended the antioxidant effects of estrogen to rat and cellular models of EW; estrogen treatment prevented lipid peroxidation in ethanol-withdrawn female rats (Jung et al. 2004) and cells (Jung et al. 2006). In vitro treatment with estrogen protected against cell death induced by H_2O_2 in cultured neurons (Wang et al. 2003), suggesting that estrogen may play a role in decreasing the oxidative burden in mitochondria (Chen et al. 2004). In support of this idea, ovariectomy caused an increase in peroxide production in brain mitochondria in a manner prevented by 17β-estradiol (E2) (Borras et al. 2003). Collectively, these studies raise a possibility that a loss of estrogen exacerbates the pro-oxidant effects of EW at the mitochondrial levels.

In the present study, we tested whether EW creates oxidative damage to cellular and mitochondrial proteins and provokes mitochondrial membrane swelling. Whether or not a loss of estrogen contributes to the mitochondrial damage was tested by employing ovariectomized female rats with or without E2-implantation and by comparing with male rats. In all experiments except for the brain region test, we used the cerebellum because of its known susceptibility to ethanol toxicity, EW toxicity, and oxidative stress (Forster et al. 1996; Schweinsburg et al. 2001; Jung et al. 2004).

Materials and methods

Materials

E2 was purchased from Steraloids (Wilton, NH). All other chemicals including Coomassie Blue R-250, glycine,

dithiothreitol, 2,4-dinitrophenylhydrazine (DNPH), and rabbit anti-DNP antibodies were purchased from Sigma Chemical Co. (St Louis, MO).

Animal experimental protocols

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 and were approved by the University of North Texas Health Science Center Animal Care and Use Committee. Young-adult female and male Sprague–Dawley rats (Charles River, Wilmington, MA) were housed individually in a room with controlled temperature (22–25 °C) and humidity (55%). They were 5 months old at the onset of the ethanol diet. A 12-h light–dark cycle was maintained with lights on between 7 A.M. and 7 P.M.

Ovariectomy and E2-replacement

After habituation, female rats were ovariectomized under isoflurane (2% v/v) 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. At the time of ovariectomy, the rats were subcutaneously implanted with Silastic pellets containing corn oil or E2 (4 mg/ml) that yields physiological E2 concentrations (Jung et al. 2002): 5.2 to 5.7 pg/ml and 29 to 34 pg/ml in oil- and E2-implanted ovariectomized rats, respectively. The pellets were replaced every 3 weeks thereafter. Two weeks were allowed for recovery from the surgery before an ethanol diet began.

Chronic ethanol administration in liquid diet and EW

Female rats were divided into four groups based on diet (control dextrin or ethanol) and E2-implantation (control oil or E2 pellet). All groups were sacrificed 24 h after termination of the diet, except for the ethanol exposure group (Ethanol/Oil) that were sacrificed at the end of the ethanol diet while rats were under ethanol influence. Thus, the four groups were (1) Dextrin/Oil, (2) Ethanol/Oil, (3) EW/Oil, and (4) EW/E2 groups. Ethanol dependence was induced by a liquid diet administration, as modified by Dodd and Shorey-Kutschke (1987). Each liter of this diet contains an aqueous suspension of pulverized casein (42 g), L-methionine (0.6 g), vitamin mixture (2.1 g), mineral mixture (7.3 g), sucrose (25 g), xanthum gum (3 g), choline bitartrate (0.4 g), Celufil cellulose (1 g), corn oil (10.5 g), ethanol, and dextrin. The amount of dextrin and ethanol was calculated in combination to adjust the concentration of ethanol to 6.5% w/v. Control animals were fed a liquid diet with dextrin isocalorically substituted

for ethanol (Jung et al. 2004). One hundred milliliters of the diet was placed in each home cage daily for 5 weeks.

Physical signs of EW

The physical signs associated with the cessation of the ethanol diet were evaluated by two experimenters who were not aware of group identity 24 h after removal of the ethanol diet. For ethanol exposure groups, the diet tubes were kept until the morning of an EW sign test, allowing them to continue to consume the ethanol diet. Thirty minutes prior to the EW sign test, the ethanol exposure groups were given the last dose of ethanol by a gavage method (3 g/kg) and were sacrificed immediately after the EW sign test. The EW groups were sacrificed 24 h after removal of the diet. Those signs are vocalization, urination, and defecation on handling (0–3 score points); stiff, curled caudal posture: (0–3); tremor (0–3); startle (0–3); handler induced or spontaneous seizure (0–3); death (10, rare). The scores for each sign were summed to generate one total sign score in each rat for statistical analysis (Jung et al. 2004).

Isolation of mitochondria

Mitochondria were isolated by conventional differential centrifugation with slight modifications (Yan et al. 2007). In brief, rats were anesthetized with xylazine and decapitated for the collection of brain tissue. Immediately thereafter, cerebelli were dissected, rinsed, and rapidly transferred to a homogenizer containing ice-cold isolation buffer (320 mM sucrose, 1 mM K2EDTA, 10 mM Tris–Hcl). A homogenate was prepared and centrifuged at $1,330 \times g$ for 5 min at 4 °C and the supernatant was saved. The pellet was resuspended in 0.5 volume of the original isolation buffer and centrifuged again under the same conditions. The two supernatants were combined and centrifuged further at $21,200 \times g$ for 5 min. The resulting pellet was resuspended in 12% Percoll solution and centrifuged at $6,900 \times g$ for 10 min. The resulting soft pellet was washed once with mitochondrial isolation buffer and centrifuged again at $6,900 \times g$ for 10 min. The pellet, containing mitochondria, was used in this study. Purity of mitochondria was assessed as previously described (Lai and Clark 1976) by measuring the cytosolic marker enzyme lactate dehydrogenase and the synaptic marker enzyme acetylcholinesterase. Both enzymes showed a very low recovery in the mitochondrial fraction (data not shown), confirming the relative purity of the isolated mitochondria.

Measurement of protein carbonyl content

Carbonyl content was measured using DNPH, which detects aldehyde or ketone moiety of proteins by forming DNP-protein adducts. For this assay, we used cerebellum, cortex, and hippocampus to determine whether certain brain areas are more vulnerable to protein oxidation during EW. The brain tissues were homogenized in 50 mM HEPES buffer (pH 7.2) containing 10 mM KCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and a proteinase inhibitor cocktail. To 1.0 ml of homogenate, 0.2 ml of 10 mM DNPH in 2 N HCl was added, and 0.2 ml of 2 N HCl was added to another 1 ml homogenate aliquot that was used as a blank control. The mixture was incubated for 1 h at room temperature. The protein was precipitated with an equal volume of 20% trichloroacetic acid and was washed three times with ethanol/ethyl acetate (1:1 v/v). The final precipitate was dissolved in 2 ml of 6 M guanidine hydrochloride (pH 2.3), and insoluble debris was removed by centrifugation. The absorbance of the DNPH derivatives was measured at 360 nm. The protein carbonyl content was calculated by using an absorbance coefficient of 22 nM/cm and expressed as nmol carbonyl per mg of protein (Levine et al. 1990).

Immunochemical detection of carbonylated proteins

DNPH derivatization was carried out as described in the previous section, and samples prepared in the absence of DNPH were used as a control (Yan et al. 1997). DNP-derivatized protein samples were analyzed by SDS-PAGE (10% resolving gel). Proteins were then transferred to Immobilon-P membranes with Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) using transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) and constant 100 V for 1 h. The blots were incubated with 50 ml of nonfat dried milk (5% w/v) for at least 1 h, and then washed three times for 10 min with Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) containing 0.1% Tween-20 (TBST). Blots were incubated for 1 h at room temperature with anti-DNP antibodies (diluted 1:1,000 in TBST containing 0.2% BSA). The primary antibody was removed, and the blots were washed three times with TBST. The blots were then incubated in horseradish-peroxidase-labeled mouse anti-rat IgG (diluted 1:10,000 in TBST containing 0.2% BSA) for 1 h at room temperature. After the blots were washed three times with TBST, the oxidized proteins were visualized with an enhanced chemiluminescence detection kit.

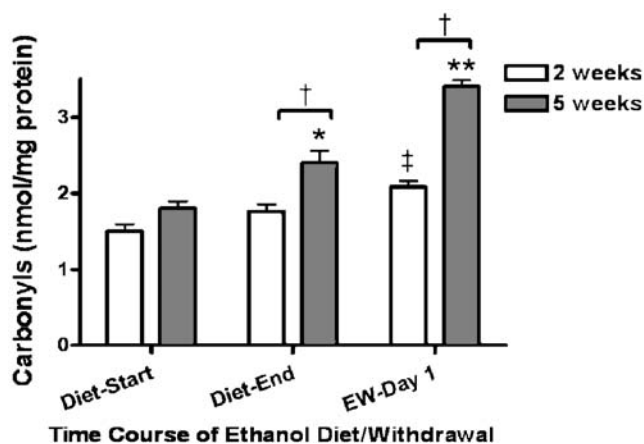## Assay of mitochondrial membrane swelling

Mitochondrial membrane swelling was assessed by suspending mitochondria (0.25 mg protein) in a medium containing phosphate, which induces swelling more rapidly in mitochondrial membranes under stress such as oxidative stress than control conditions (Yan et al. 2002; Menze et al.

2005). The medium contained 250 mM sucrose, 10 mM Tris–MOPS, 0.05 mM EGTA, 5 mM pyruvate, 5 mM malate, and 1 mM phosphate (pH 7.4). Absorbance by this mitochondrial suspension was measured at 540 nm using a Beckman DU 640 spectrophotometer (International MI-SS Inc, Corona CA). Some of the mitochondrial suspension was exposed to Ca^2 (250 μM), a promoter of PTP or with cyclosporine A (0.2 and 0.5 μM), an inhibitor of PTP. Intact mitochondria scatter light at 540 nm wavelength; mitochondrial swelling and rupture due to prolonged or excessive mitochondrial PTP opening reduces mitochondrial light scattering ability (Yan et al. 2002). The time required for absorbance to fall to the 50% of the difference between the initial and final absorbance values was determined to assess the latency to PTP opening in different treatment groups.
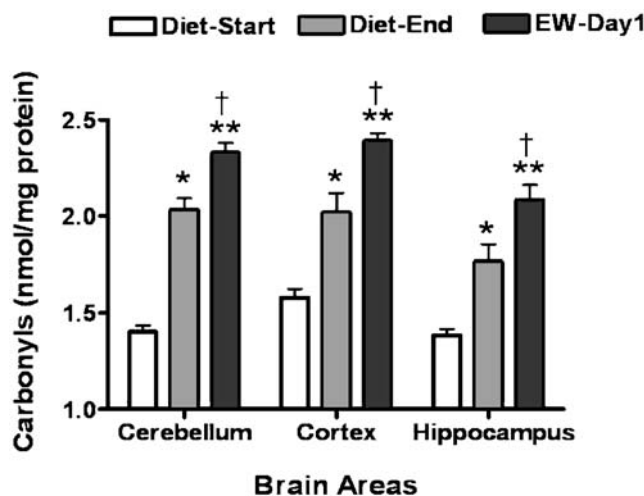
Statistical analysis

Numerical data were expressed as the mean±Standard Error of Mean (SEM). Data from all experiments were from two or three separate determinations using a minimum of five rats per group.

Two-way analysis of variance (ANOVA) was conducted to determine interaction between (1) diet length (2 or 5 weeks) and time course of an ethanol diet (Fig. 1), (2) brain region and time course of an ethanol diet (Fig. 2), and (3) whole cell or mitochondrial production of carbonyl and diet conditions (Fig. 3). For all other measurements, one

Fig. 2 Carbonyl production in three brain areas during ethanol/EW in whole cell lysates. Ovariectomized rats with oil-pellet-implantation received an ethanol diet (6.5%) for 5 weeks. They were then sacrificed at day 1 of the diet (Diet-Start), at the end of the 5-week diet (Diet-End), or at day 1 of EW (EW-Day 1). Whole-cell lysates of brain tissues were used for carbonyl assay. In all three brain areas, carbonyl contents were higher at day 1 of EW than those at the respective Diet-Start (**$p<0.001$) or Diet-End ($^{†}p<0.01$). Depicted are mean±SEM for five animals per group. *($p<0.01$) and **($p<0.001$) vs Diet-Start in respective brain area. †($p<0.01$) vs Diet-End in respective brain area

way ANOVA was conducted. After one way or two way ANOVA, a post hoc Tukey test was conducted for a pair wise comparison. The level of significance for all data analysis was set at $p<0.05$.
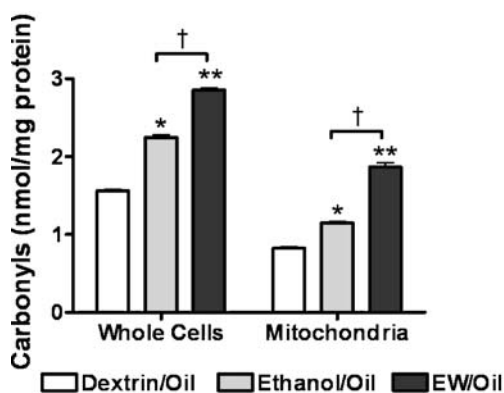
Results

Body weight and ethanol consumption

The body weights of the rats before the chronic diet ranged from 200 to 230 g. We monitored all ethanol-treated rats and observed that they consumed a similar amount of ethanol (an average 15 g/kg/day). After a 5 week-diet, the dextrin-diet rats weighed 220 to 250 g, and the ethanol-diet rats weighed 230 to 250 g. There was no statistically significant difference in body weights between treatment conditions. Rats under this ethanol diet regimen remained healthy throughout the diet period. Furthermore, we have reported that E2-treatment does not affect ethanol kinetics in rats (Rewal et al. 2005).

Physical signs of EW

As we previously reported (Jung et al. 2002; Jung et al. 2005), all ethanol withdrawn rats showed signs of EW such as tremor, tail rigidity, or startle response (see the "Materials and methods" section) indicating that they were dependent on

Fig. 1 Timing effects of an ethanol diet on carbonyl production in whole cell lysates. Two groups of ovariectomized rats with oil-pellet-implantation received an ethanol diet (6.5%) for either 2 or 5 weeks. They were then sacrificed on day 1 of the diet (Diet-Start), at the end of the 2- or 5-week diet (Diet-End), or at day 1 of EW (EW-Day 1). Whole-cell lysates of cerebella tissues were used for carbonyl assay. Compared to initial carbonyl contents (Diet-Start), the carbonyl contents were increased at the end of the ethanol diet and further increased at day 1 of EW. Depicted are mean±SEM for five animals per group. *($p<0.05$) and **($p<0.01$) vs Diet-Start in 5 week-diet groups. ‡($p<0.05$) vs Diet-Start in 2 week-diet groups. †($p<0.01$) vs a 2-week diet groups

Fig. 3 Comparison of carbonyl contents between whole-cell lysates and mitochondrial fraction during EW. Three groups (Dextrin, Ethanol, and EW) of ovariectomized rats with oil-pellet replacement received a dextrin or an ethanol diet (6.5%) for 5 weeks. The rats were sacrificed at the end of the ethanol diet while they were under ethanol influence (Ethanol group) or 24 h after termination of the diet (EW group). A carbonyl assay was separately conducted using whole-cell lysates and mitochondrial fractions of cerebellum. Both whole cell lysates and mitochondrial fractions showed higher carbonyl contents in the EW group than the Dextrin or the Ethanol group. Depicted are mean±SEM for five animals per group. *($p<0.01$) and **($p<0.001$) vs respective Dextrin/Oil group. †($p<0.01$) vs respective Ethanol/Oil group

ethanol and experienced EW distress. Accordingly, scores for EW signs were higher ($p<0.001$) in EW rats (7.6±0.75) than dextrin control rats (1.7±0.2) [$F_{(2,16)}=37$, $p<0.001$]. As was in a previous case, rats that were implanted with E2 pellets (EW/E2) showed significantly lower EW-sign scores (2.8± 0.3) than oil pellet-implanted EW rats ($p<0.01$), indicating protective effects of E2 on the signs of EW.

Timing effects of an ethanol diet on carbonyl production in whole cell lysates

For this assay, ovariectomized female rats with oil-pellet-implantation received a 2-week or a 5-week ethanol diet (Fig. 1). They were divided into different groups based on the time of brain tissue collection; at the beginning of diet (Diet-Start), at the end of the ethanol diet (Diet-End), and at 24 h of EW (EW-Day 1). There was a significant difference in carbonyl content by factors of ethanol diet length [$F_{(1, 24)}=530$, $p<0.001$] and timing of the diet [$F_{(2, 24)}=226$, $p<0.001$], both of which showed interaction ($p=0.002$). For both the 2-week and 5-week ethanol diets, the carbonyl contents were increased at the end of the ethanol diet compared to those when the diet started and were peaked at 24 h of EW ($p<0.01$). At the end of the diet and at day 1 of EW, rats with a 5-week ethanol diet yielded higher carbonyl contents than a 2-week ethanol diet ($p<0.001$). These data indicate that longer ethanol diet produces more protein carbonyls and that a maximal level occurs during the most severe EW.

Carbonyl production in three brain areas during ethanol/ EW in whole-cell lysates

Figure 2 illustrates effects of ethanol/EW on carbonyl production in cerebellum, cortex, and hippocampus. In all three brain areas, carbonyl contents were higher at day 1 of EW than those at the respective Diet-Start (**$p<0.001$) or Diet-End (†$p<0.01$). When carbonyl contents at day 1 of EW were calculated relative to those of Diet-Start (100%), there was no statistically significant difference in carbonyl contents among cerebellum (166±5%), cortex (151±4%), and hippocampus (150±7%).

Comparison of carbonyl contents between whole-cell lysates and mitochondrial fraction during EW Since mitochondria are believed to be vulnerable to oxidative stress, we next tested whether EW creates greater carbonylation in mitochondrial proteins than in whole-cell proteins (Fig. 3). There was a significant difference in carbonyl content by factors of compartment (whole cell or mitochondria) [$F_{(1, 24)}=479$, $p<0.001$] and diet conditions [$F_{(2, 24)}=137$, $p<0.001$] that interacted with each other factor ($p<0.001$). In both whole cell lysates and mitochondrial fractions of cerebellum, carbonyl contents were higher during EW (day 1 of EW) than those of Dextrin or Ethanol exposure group. Such phenomenon appeared to be more prominent in mitochondria than in whole-cells ($p<0.01$) because compared to the Dextrin control, EW resulted in 2.3 fold and 1.8 fold increase in carbonyl contents in mitochondria and whole-cell lysates, respectively. These data suggest that mitochondria are susceptible to protein oxidation during EW.

The effects of E2 on carbonyl contents during EW

Whether or not E2 protects against oxidative damage to mitochondrial proteins was tested. All ethanol withdrawn rats with (*$p<0.01$) or without E2 (**$p<0.001$) treatment had higher carbonyl contents than those of control dextrin rats. However, the carbonyl contents of the EW/E2 group were significantly lower than those of the EW/Oil group (†$p<0.01$), suggesting that E2 attenuates protein oxidation during EW (Fig. 4).

Immunochemical detection of protein carbonyls

DNPH-derivatized carbonylated proteins were separated by SDS-PAGE followed by immunochemical detection using an anti-DNP antibody. In Fig. 5, mitochondrial proteins with molecular weights equal to or less than 45 kDa were visualized by Coomassie blue staining (left panel). Carbonyl signal bands corresponding to the proteins were visualized in the right panel. For certain proteins as indicated by arrows, the EW/Oil group exhibited stronger

Fig. 4 The effects of E2 on carbonyl contents during EW. Rats were ovariectomized and implanted with either oil or E2 pellets (EW/Oil and EW/E2 groups). They received a dextrin (Dextrin/Oil group) or an ethanol diet (6.5%; EW/Oil and EW/E2 groups) for 5 weeks. They were then sacrificed at day 1 of EW, and mitochondrial fractions of cerebella tissues were prepared for carbonyl assay. Compared to the Dextrin/Oil group, the EW/Oil group had increased carbonyl content in a manner that was protected by E2. Depicted are mean±SEM for five animals per group. *($p<0.01$) and **($p<0.001$) vs the Dextrin/Oil group. †($p<0.01$) vs EW/E2 group

carbonyl signals than those of the Dextrin/Oil or the EW/E2 group. These data indicate that EW provokes oxidative damage to certain mitochondrial proteins in a manner that is protected by E2 treatment.

The effects of EW on mitochondrial membrane swelling

For this experiment, we used an additional control group: mitochondria were isolated from the cerebellum of the Dextrin/Oil group. The isolated mitochondria and Ca^2 at 250 μM were added to a mitochondrial swelling medium containing phosphate. Mitochondrial membrane swelling induced by phosphate should be further potentiated by a high Ca^2 concentration (Pepe 2000). We measured an absorbance rate based on the rationale that intact mitochondrial membrane scatters light that can be monitored at 540 nm, whereas the swelling of mitochondria due to excessive PTP opening impairs mitochondrial light scattering ability, hence a decreased reading at 540 nm (Yan et al. 2002). Thus, the lower absorbance reflects the more PTP opening. At most time points measured, the absorbance of the EW/Oil group was lower than that of the Dextrin/Oil, Ethanol/Oil, or EW/E2 group and mimicked that of the Ca^2-treated group (Fig. 6a). When calculated, the EW/Oil group had the shortest latency to 50% decrease in the absorbance among the treatment groups ($p<0.05$) except the Ca^2-treated group (Fig. 6b). The latency to the 50% decrease in absorbance was in the order of Dextrin/Oil>EW/E2>Ethanol/Oil>EW/Oil>Dextrin/Oil². These data indicate that EW provokes mitochondrial membrane swelling in a manner that is protected by E2 treatment.

The effects of a PTP inhibitor cyclosporine A on EW-induced mitochondrial membrane swelling

Cyclosporin A (0.2 and 0.5 μM) is a prototype inhibitor of PTP opening (Halestrap et al. 1997). We determined whether EW-induced mitochondrial membrane swelling is attenuated by this compound. Cyclosporin A (0.2 and 0.5 μM) was added to mitochondrial suspension 30 min before a medium containing phosphate (a promoter of PTP opening) was added. When absorbance at 540 nm was monitored for 12 min, cyclosporine A significantly delayed absorbance decline in a dose dependent manner and accordingly increased latency to 50% decrease in absorbance of ethanol withdrawn mitochondria. These data indicate that the absorbance decline during EW is sensitive to cyclosporine A (Fig. 7a,b).

The effects of EW on mitochondrial membrane swelling in male rats (control experiment)

If estrogen deprivation contributes to higher vulnerability to EW, male rats with low circulating estrogen levels should be vulnerable to EW. We tested this hypothesis using male rats that received an identical ethanol diet regimen to that of female rats (Fig. 8a,b). When mitochondrial membrane swelling was tested at day 1 of EW in male rats, EW-mitochondria (*$p<0.001$) showed more rapid decline in the absorbance than Dextrin or ethanol exposure group as was the case for female rats. The ethanol withdrawn male rats
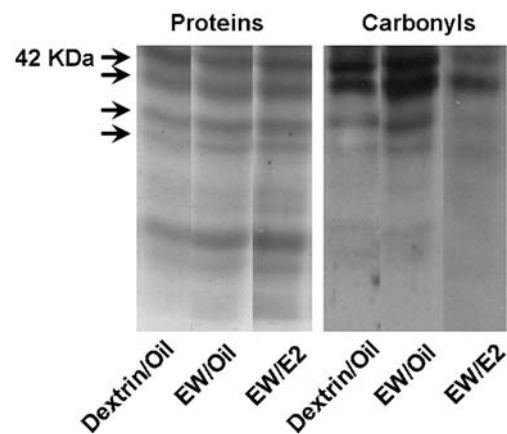
Fig. 5 Immunochemical detection of protein carbonyls. Ovariectomized rats with oil- or E2-pellet implantation received a dextrin (Dextrin/Oil group) or an ethanol diet (6.5%, EW/Oil or EW/E2 group) for 5 weeks. They were sacrificed at day 1 of EW and mitochondrial fractions of cerebella tissues were prepared for an immunochemical assay. Mitochondrial proteins with molecular weights equal to or less than 45 kDa were visualized by Coomassie blue staining (*left panel*). Carbonyl signals corresponding to the proteins are shown in the *right panel*. For certain proteins as indicated by *arrows*, the EW/Oil group exhibited stronger carbonyl signals than those of the Dextrin/Oil or the EW/E2 group. Cerebella mitochondria were pooled from five rats per group
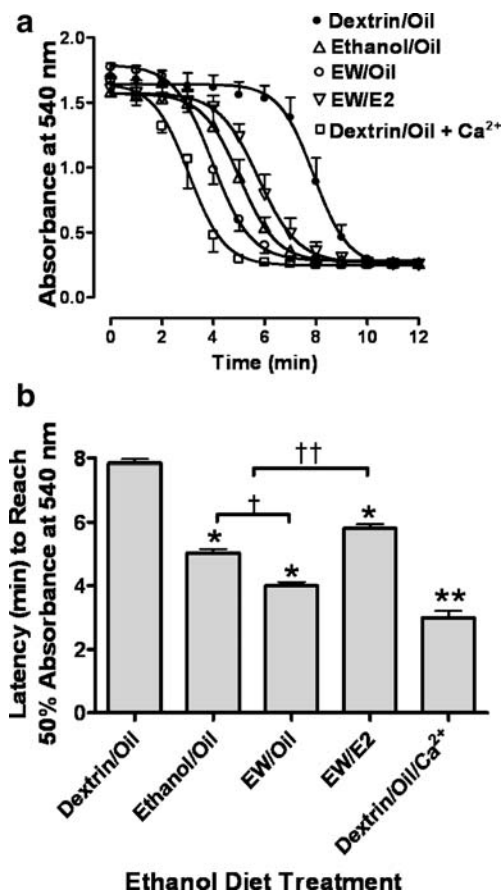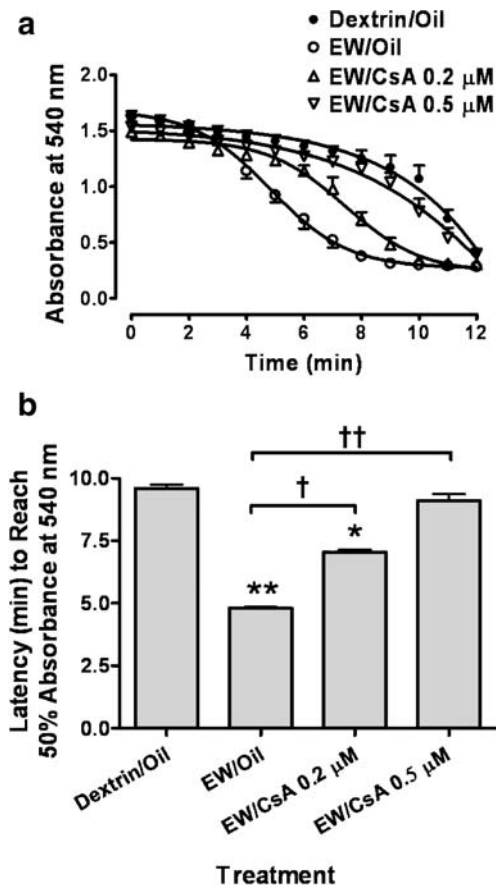
Fig. 6 The effects of EW on mitochondrial membrane swelling. Rats were ovariectomized and implanted with either oil or E2 pellets. They received a dextrin (Dextrin/Oil group) or an ethanol diet (6.5%, Ethanol/Oil, EW/Oil, and EW/E2 groups) for 5 weeks. They were sacrificed at the end of ethanol exposure (Ethanol/Oil group) or at day 1 of EW and mitochondrial fractions of cerebella tissues were prepared to assess mitochondrial membrane swelling. An additional control group was used: mitochondria were isolated from the Dextrin/Oil group and exposed to 250 μM Ca^2. The EW/Oil group had a lowest absorbance among all of the groups ($p<0.05$) except the Ca^2-treated group (**a**). Average latency to reach 50% decrease in absorbance was in the order of Dextrin/Oil>EW/E2>Ethanol/Oil>EW/Oil>Dextrin/Oil^2 (**b**). Depicted are mean±SEM for five animals per group. *$p<0.01$ and **$p<0.001$ vs the Dextrin/Oil group. $^\dagger p<0.05$ vs the Ethanol/Oil group. $^{\dagger\dagger}p<0.05$ vs Ethanol/Oil or the EW/Oil group

showed a comparable rate of decline in the absorbance to that of ovariectomized female rats without E2-implantation.

Discussion

This study demonstrates that EW provokes protein oxidation and mitochondrial membrane fragility in female rats and that E2 deprivation contributes to this problem. Oxidative damage to proteins has been demonstrated in various models of ethanol toxicity. Chronic ethanol consumption increased protein carbonyl contents in the liver of adult rats (Bailey et al. 2001)

and mice (Zhou et al. 2005). Ethanol exposure during gestation also increased carbonyl contents in the liver and pancreas of weaning rats (Cano et al. 2001). Similarly, in a clinical situation, increased carbonyl contents were observed in the blood serum and plasma of human alcoholics (Mutlu-Turkoglu et al. 2000). However, most of the studies did not differentiate between ethanol exposure and EW, thus it is not clear whether the observed protein oxidation is due to ethanol toxicity, EW, or both. The differentiation is important because the toxic effects of EW are not necessarily identical to those of ethanol per se and can cause more brain damage (Phillips and Cragg 1983; Jung et al. 2004). In support of this idea, ethanol treatment followed by withdrawal resulted in a

Fig. 7 The effects of a PTP inhibitor cyclosporine A on EW-induced mitochondrial membrane swelling. Ovariectomized rats implanted with oil pellets received a dextrin (Dextrin/Oil group) or an ethanol diet (6.5%) for 5 weeks. They were sacrificed at day 1 of EW and mitochondrial fractions of cerebellum were prepared to assess mitochondrial membrane swelling. Mitochondrial suspension was exposed to a medium containing phosphate that provokes PTP opening. Some of mitochondrial suspension was exposed to cyclosporine A (CsA, 0.2 and 0.5 μM) prior to treatment with a phosphate-containing medium. Ethanol withdrawn mitochondria treated with cyclosporine A showed delayed-absorbance decline (**a**) and longer latency to 50% decrease in absorbance (**b**) as compared to a vehicle treated mitochondria during EW. Depicted are mean±SEM for five animals per group. *$p<0.01$ and **$p<0.001$ vs the Dextrin/Oil group. $^\dagger p<0.01$ and $^{\dagger\dagger}p<.0.001$ vs the EW/Oil group
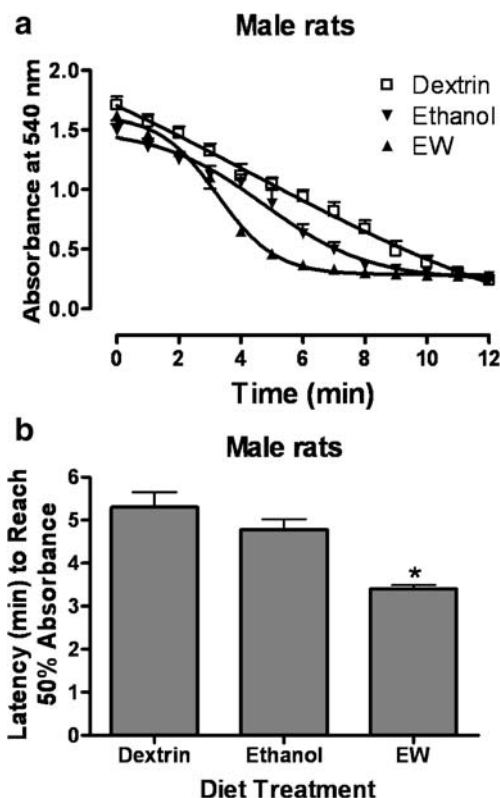
Fig. 8 The effects of EW on PTP opening in male rats. Male rats received a dextrin (Dextrin) or an ethanol (6.5%, EW) diet for 5 weeks. They were sacrificed at the end of ethanol exposure (Ethanol group) or at day 1 of EW and cerebella tissues were prepared to assess PTP opening. EW rats showed more rapid decline in the absorbance than the Dextrin or the Ethanol group as shown in the rate of absorbance (a) and latency to reach 50% decrease in absorbance (b). Depicted are mean±SEM for five or six animals per group. *$p < 0.01$ vs the Dextrin or the Ethanol group

neuronal loss in the mouse hippocampus and rat cerebellum (Phillips and Cragg 1983; Jung et al. 2002), whereas ethanol per se did not cause any neuronal loss (Phillips and Cragg 1983). In our previous findings, EW produced a greater lipid peroxidation than ethanol per se in rats (Jung et al. 2004). Our current findings are in agreement with these studies and suggest that the abrupt termination of a long term ethanol provokes protein oxidation that is distinct from and greater than that of ethanol exposure.

Controversially, some studies were unable to find protein oxidation in ethanol exposed rats (Koo-Ng et al. 2000; Reilly et al. 2000). Likewise, abstinent alcoholic patients did not show any increase in carbonyl contents in the cerebrospinal fluid (Tsai et al. 1998). Although the exact reason for this discrepancy is not clear at this time, there are a few possible explanations. They used peripheral tissues of animals or cerebrospinal fluid obtained from human alcoholics, giving rise to a possibility that peripheral tissues may respond to protein oxidation differently than brain tissues. In addition, the clinical study measured carbonyl

contents at a significantly delayed time after last drinking compared to 24 h after removal of an ethanol diet in our study. As time elapsed, the damaged proteins might have been replaced with newly synthesized proteins, decreasing carbonyl contents in that study. Our comparison between mitochondrial fractions and whole-cell lysates revealed that mitochondrial proteins are particularly susceptible to oxidative stress. The oxidative damage to mitochondria has been shown in previous studies in which H_2O_2 increased mitochondrial protein oxidation in conjunction with ATP depletion in cultured human fibroblasts (Miyoshi et al. 2006) and human lens epithelial cells (Wang et al. 2003). More relevant to the current study is an early study in which withdrawal from ethanol exposure (33 weeks) increased mitochondrial permeability to a superoxide generator (phenazine methosulfate) in male rats (French and Todoroff 1971). The authors suggested that the EW-induced mitochondrial permeability reflects hyperexcitability of the CNS during EW. Ethanol in the brain mainly acts as an inhibitory molecule that suppresses excitatory neurotransmitters such as glutamate. Consequently, rats undergoing EW suffer an abrupt transition-distress; from suppressed glutamate during ethanol exposure to excess glutamate during EW (Hoffman 1995; Evans et al. 2000). Such abrupt occurrence of excitotoxicity is behaviorally manifested as EW signs including tremor, tail rigidity, hyperactivity, and seizure, and in extreme cases, coma and death. Excessive glutamate provokes free radical-generation (Fujikawa 2005) that subsequently oxidize proteins (Sundari et al. 1997). Therefore, increased protein carbonylation during EW may result from sudden increase in the levels of excitatory molecules such as glutamate or Ca^2 (Fujikawa 2005) that directly or indirectly oxidize proteins. The vulnerability of mitochondria to oxidative stress can be serious in an ethanol environment because ethanol preferentially targets membrane and mitochondrion is one of the organelles that are especially rich in membranes. In a separate study, we observed that the activity of a redox-sensitive protein (dihydrolipoamide dehydrogenase) was unaltered by EW (data not shown), indicating that EW does not produce random, nonselective oxidation of all cellular proteins. In support of this, ethanol exposure decreased glutathione (an endogenous antioxidant) content in mouse heart mitochondria more so than in the cytosol (Kannan et al. 2004).

Measurement of mitochondrial membrane swelling has been extensively employed in a variety of cellular and animal models as an indicator of PTP opening (Petronilli et al. 2001; Yan et al. 2002; Zhu et al. 2006). Although the composition of PTP remains uncertain, some researchers believe that PTP is minimally composed of adenine nucleotide translocase, mitochondrial creatine kinase, voltage dependent anion channel, and cyclophilin D (Dolder et al. 2001; Lipskaya 2001; Halestrap and Brennerb 2003).

These proteins intimately interact with each other to regulate the PTP opening and the passage of specific molecules across the mitochondrial membranes (Qin et al. 1997; Beutner et al. 1998; Dolder et al. 2001). The effective functional coupling is important because of fluctuating energy requirements, especially under the stressful conditions such as EW. Studies report that oxidative stress and high levels of mitochondrial Ca^2 can impede the regulatory function of the PTP proteins (Halestrap and Brennerb 2003), thereby inducing mitochondrial swelling and decreasing the efficiency of the respiratory chain (Pepe 2000; Yan et al. 2002; Halestrap and Brennerb 2003). We previously observed that EW suppressed activity of a key mitochondrial protein, cytochrome c oxidase, and estrogen treatment attenuated such insults of EW (Jung et al. 2007). Our current findings extend EW injury to the level of mitochondrial membranes in that ethanol withdrawn mitochondria showed mitochondrial swelling that mimicked the Ca^2-treated group. How EW targets mitochondrial proteins and membranes is uncertain but there are a few possible explanations. One possibility is that EW provokes excessive generation of free radicals, which can readily attack mitochondrial membrane proteins (Yan and Sohal 1998). The protein oxidative modification alters the stability, conformation, activity, and function of the PTP proteins, resulting in an opening of the PTP (Vyssokikh and Brdiczka 2003). Alternatively, during EW, excessive glutamate-induced neuronal excitation increases intracellular concentrations of Ca^2, which provoke PTP opening (Reynolds 1999; Brustovetsky et al. 2002; Yan et al. 2002; Halestrap and Brennerb 2003; Fiskum et al. 2004; Halestrap 2006). Supportive of this idea, a blockade of Ca^2 channels reduced EW signs (Kratzer and Schmidt 2003). Finally, if damaged or misfolded membrane proteins form PTP (He and Lemasters 2003), EW may trigger such pore forming events through free radicals or Ca^2.

Our findings suggest that estrogen is mitoprotective. That ovariectomized rats without E2-implantation exhibited much more rapid mitochondrial membrane swelling comparable to male mitochondria further strengthens the idea that estrogen depletion exacerbates mitochondrial vulnerability to EW. In our previous findings, in vitro estrogen protected against mitochondrial membrane potential collapse induced by H_2O_2 in a manner that correlated with its antioxidant effects (Wang et al. 2003). In vitro estrogen treatment to human neuroblastoma cells also protected against ATP depletion, the collapse of mitochondrial membrane potential, and the generation of reactive oxygen species induced by a mitochondrial toxin (Wang et al. 2001). In a rat model of oxidative stress, E2 prevented mitochondrial peroxidation induced by ovariectomy (Borras et al. 2003). Given these findings, estrogen seems to play a role in alleviating the oxidative burden in mitochondria, thus

increasing mitochondrial respiration efficiency (Chen and Yager 2004). As such, in spite of increasing evidence that estrogen is mitoprotective, little is known about the mitoprotection in the context of EW. Estrogens may activate a free-radical scavenging pathway (Dhandapani and Brann 2002) and thus attenuate the formation of carbonyl adducts with mitochondrial proteins during EW. The lipophilicity of estrogen may allow it to intercalate readily into the lipid membrane of mitochondria and modulate the structural configuration of the proteins so that they are more resistant to oxidation or excitotoxic EW insults. It is also possible that estrogen elevates the levels of endogenous antioxidants such as glutathione so that a favorable redox potential for an antioxidant environment is created (Prokai et al. 2003). Cyclosporin A is a prototype inhibitor of PTP opening (Kushnareva and Sokolove 2000; Di Lisa and Bernardi 2006; Norenberg and Rao 2007) by interfering a protein (cyclophilin D)-protein (adenine nucleotide translocator) interaction that is essential for PTP opening (Sullivan et al. 2005). Because cyclosporine A resulted in a similar degree of protection to E2, one cannot exclude a possibility that E2 may interfere with interactions among PTP proteins.

In conclusion, we have provided empirical evidence that EW provokes oxidative damage to mitochondrial proteins and membranes in a manner that is protected by E2. We did not assess whether estrogen protection is limited to brain, organ specific, or more effective in females than males. Nevertheless, a mitochondrial targeting strategy is worthy of further study for female alcoholics who are simultaneously undergoing EW and a loss of estrogen.

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