Ethanol withdrawal provokes opening of the mitochondrial membrane permeability transition pore in an estrogen preventable manner

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Abstract

We have reported that the major endogenous estrogen, 17β-estradiol (E2), protects against oxidative injury during ethanol withdrawal (EW) in a cultured hippocampal cell line (HT22). Here, we investigated whether the prooxidant nature of EW mediates opening of the mitochondrial membrane permeability transition pore (PTP) in a manner protected by E2. Excess PTP opening provokes mitochondrial membrane swelling (MMS) and the collapse of membrane potential (ΔΨm). HT22 cells were collected at the end of ethanol exposure (100 mM) for 24 hours or at 4 hours of EW to assess MMS by monitoring absorbance decline at 540 nm and to assess ΔΨm using flow cytometry. Protective effects of E2 on PTP were compared with an antioxidant butylated hydroxytoluene (BHT) and an E2 analogue, ZYC26 with higher antioxidant potency than E2. To assess cellular consequences of PTP opening, effects of a PTP inhibitor (cyclosporin A) on EW-induced cell death were assessed using the Calcein assay. Major findings were that 1) EW resulted in rapid MMS and ΔΨm collapse, 2) cyclosporin A attenuated EW-induced cell death, and 3) E2 treatment restricted to the EW phase protected against the PTP opening more prominently than BHT and to a similar degree to ZYC26. These findings suggest that EW provokes PTP opening partly but not entirely through the prooxidant nature and that E2 counteracts EW-associated factors to protect against the PTP opening.
Introduction

Ethanol withdrawal (EW) refers abrupt termination of long-term ethanol abuse. EW motivates alcoholics to relapse into ethanol abuse due to its discomfort and disorders, but there is little mechanistic insight into this important clinical problem. This study was undertaken to gain a better understanding of the mechanisms by which EW damages mitochondrial membranes in a cellular model of ethanol/EW. We used the immortalized hippocampal cell line (HT22 cells) because this cell line is advantageous to access an oxidative mechanism. HT22 cells lack ionotropic glutamate receptors and thus, the cellular injury is unlikely through excitotoxicity involving glutamate receptors (Zaulyanov et al., 1999). Instead, HT22 cells contain the glutamate/cystine antiporter, which is required for the delivery of cystine into neuronal cells for the synthesis of an endogenous antioxidant glutathione. Therefore, HT22 cellular injury is often associated with a reduction in endogenous antioxidant capacity (Tan et al., 1998). Using this cell line, we have demonstrated that EW is prooxidant by provoking protein oxidation and lipid peroxidation to a greater degree than ethanol per se and that 17β-estradiol (E2) treatment protects against the oxidative stress (Jung et al., 2006). In the current study, we tested whether EW damages mitochondrial membranes through its prooxidant nature in E2-preventable manner.

We focused on mitochondrion because this organelle is one of the major subcellular targets of ethanol intoxication and withdrawal (Mansouri et al., 2001; Minana et al., 2002). Reactive oxygen species (ROS) produced during ethanol metabolism altered mitochondrial function (Mansouri et al., 2001; Minana et al., 2002), and EW increased mitochondrial permeability in response to a superoxide generator (phenazine methosulfate) and oxidative stress (French and Todoroff, 1971; Hosein et al., 1980). In addition, acute administration of ethanol (5 g/kg, IP) to mice depleted mitochondrial DNA in heart, liver, and brain (Mansouri et al., 2001). The mitochondrial susceptibility appears to be in part...
due to compromised endogenous antioxidant capacity based on a study in which ethanol-treated hepatocytes showed depletion of glutathione (Fernandez-Checa et al., 1998).

Mitochondrial membranes contain a group of proteins that forms mitochondrial permeability transition pores (PTP). PTP regulate permeability to electrolytes, nucleotides and metabolic substrates, all of which are essential for ATP production. Prolonged opening of PTP perturbs the electrochemical gradient for $H^+$ ($\Delta \Psi_m$), driving force for oxidative phosphorylation, resulting in disrupted ATP production. Oxidative or apoptotic stress causes excess opening of PTP, which in turn permits diffusion of water and electrolytes across mitochondrial membranes. Consequently, $\Delta \Psi_m$ collapses and oxidative phosphorylation of ATP fails (Stuart, 2002). Due to such an intimate relationship between PTP and $\Delta \Psi_m$, $\Delta \Psi_m$ is often measured as a typical marker of PTP. Mitotoxic effects of ethanol have been reported in a study where the inhibition of PTP opening by cyclosporin A prevented ethanol-induced cell death (Minana et al., 2002). However, most studies have not systematically differentiated effects of ethanol exposure and EW on mitochondria; it is not clear whether mitochondrial damages were due to ethanol per se, or EW, or both. The differentiation is important because toxic effects of EW are not necessarily identical to those of ethanol and can cause more brain damage than ethanol per se (Phillips and Cragg, 1983; Jung et al., 2004).

Recently, we observed that EW provoked oxidation of mitochondrial proteins in ovariectomized rats and E2 implantation markedly prevented the protein oxidation (Jung et al., 2008). Others also reported that ovariectomy caused an increase in peroxide production by mitochondria in a manner prevented by E2 (Borras et al., 2003), suggesting that estrogen plays a role in reducing oxidative burden in mitochondria (Simpkins et al., 2005). Despite abundant evidence of mitoprotection by estrogen, a mechanism by which E2 protects against mitotoxic EW remains uncertain. Therefore, in part I of this study, we assessed whether the prooxidant nature of EW mediates mitotoxicity. In part II
of this study, we examined mitoprotective effects of E2 and its potential antioxidant mechanisms by comparing with an antioxidant butylated hydroxytoluene (BHT) and an E2 analogue ZYC26 that previously showed higher antioxidant potency than E2 during EW (Jung et al., 2006).

Methods

Reagents

E2 was purchased from Steraloids (Wilton, NH). ZYC26 [(3-hydroxy-2-adamantyl(1)-4-methyl-estra-1,3,5(10)-17-one] was made in our laboratories using methods previously described (Perez et al., 2006). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO) unless indicated otherwise.

Cell culture

HT22 cells were obtained from David Schubert (Salk Institute, San Diego, CA). The HT22 cell line was originally selected from HT4 cells based on glutamate sensitivity. HT4 cells were immortalized from primary hippocampal neurons using a temperature-sensitive simian virus-40 T antigen (Morimoto and Koshland, 1990). HT22 cells were grown in DMEM medium supplemented with 10% charcoal-stripped fetal bovine serum (HyClone, Logan, UT) and gentamicin (50 μg/ml), at 37° C in an atmosphere containing 5% CO₂ and 95% air. Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin at concentrations ranging from 0.063 to 1 mg/ml as a standard curve.

Ethanol treatment

HT22 cells (4,000 cells/well) in 100 μl of cell culture medium were plated into 96-well culture plates or Petri-dishes. On the following day, the cells were exposed to control media dimethyl sulfoxide
(DMSO) or 100 mM of ethanol for 24 hours. The culture plates or Petri-dishes were tightly sealed with parafilm immediately after ethanol treatment to prevent ethanol evaporation. We have maintained an intended ethanol concentration for 24 hours using this method (Jung et al., 2006; see Result section). For the continuous ethanol exposure condition, cells were collected at the end of the 24 hour-ethanol exposure. In withdrawal experiments, the ethanol-containing medium was replaced with control media for 4 hours after the 24 hour-ethanol exposure (Mostallino et al., 2004). At the end of the 4 hours of EW, cells were collected to assess PTP opening and cell viability. An E2, BHT, or ZYC26 stock solution was prepared at a concentration of 0.1 μM or 1 μM in DMSO and the compounds were administered to cells either during the entire period of ethanol exposure/withdrawal or during the EW phase. There were no measurable effects of DMSO on PTP or cell viability under these conditions.

Assessment of PTP

1) Flow cytometric analysis of ΔΨm

HT22 cells (2x 10⁴ cells/ml) were cultured in 12-well culture plates. Cells were divided into four groups: control media, ethanol, EW, and EW + E2 groups. On the following day, cells were treated with ethanol (100 mM) as mentioned above and E2 (1 μM) was given when ethanol-containing media was replaced with control media to restrict E2 treatment to the EW phase. At the end of 24 hour-ethanol exposure or at 4 hours of EW, the cells were washed with phosphate-buffered saline (PBS) twice. 0.5 ml of JC-1 solution (2.5 μg/ml) was then transferred into each well. The cells were incubated at 37°C in a 5% CO₂ incubator for 20 minutes. Subsequently the cells were digested with trypsin to lyse cells. Precipitates obtained from the suspension after centrifugation (3 minutes, room temperature, 800 x g) were resuspend in 2 ml of PBS followed by centrifugation. The cell pellets were suspended in 0.5 ml of PBS, which was ready for flow cytometric analysis. JC-1 aggregates in healthy mitochondria emit red fluorescence at 590 nm. JC-1 monomers that were leaked from
stressed mitochondria emit green fluorescence at 530 nm. The red and green fluorescence were measured in the Green (FL-1) and Red (FL-2) channel of the flow cytometer, respectively. The cells were then immediately observed with a fluorescence microscope using a “dual-band pass” filter designed to simultaneously detect fluorescein and rhodamine or fluorescein and Texas Red™.

2) Spectrometric analysis of mitochondrial membrane swelling (MMS)

MMS is often measured as an indication of PTP opening by monitoring absorbance decline at 540 nm (Ruiz-Meana et al., 2006). Intact mitochondria scatter light at 540 nm wavelength; mitochondrial swelling and rupture due to prolonged or excessive PTP opening reduce mitochondrial light scattering and absorbance. Using a differential centrifugation method, mitochondria were isolated from HT22 cells that were collected at the end of 24 hour-ethanol exposure or at 4 hours of EW. The cells were homogenized in ice-cold isolation buffer (320 mM sucrose, 1 mM K₂EDTA, 10 mM Tris-HCl) and centrifuged at 1330 x g for 5 minutes at 4°C. The pellets were resuspended in 0.5 vol isolation buffer and re-centrifuged. The two supernatants were combined and centrifuged at 21,200 x g for 5 minutes. The resulting pellets were resuspended in 12% Percoll solution and centrifuged at 6,900 x g for 10 minutes. The resulting soft pellets were then washed once with mitochondrial isolation buffer and centrifuged again at 6,900 x g for 10 minutes. The precipitant mitochondrial pellets were suspended in medium containing phosphate which induces MMS and rupture more rapidly in vulnerable mitochondrial membranes than healthy mitochondrial membranes (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 suspension was measured at 540 nm in a Beckman DU 640 spectrophotometer.

Calcein-AM (calcein-acetoxymethylester) viability assay
Cell viability was quantitated using the membrane-permeant Calcein-AM dye (Molecular Probes, Eugene, OR). Calcein-AM is a fluorogenic esterase substrate that easily permeates live cells that have esterase activity and membranes. Once hydrolysis of Calcein-AM by intracellular esterases begins, it produces Calcein, a strongly fluorescent compound that is well retained in the cell cytoplasm, which enables us to measure relative fluorescent units (RFU). HT22 cells were treated with ethanol and withdrawn as described above. Cyclosporin A (0.2 μM) treatment was restricted to the EW phase such that cyclosporin A was treated when ethanol-containing media was replaced with control media to test whether EW-induced PTP opening mediates cell death. Calcein-AM was treated 30 minutes prior to the end of EW. Following the removal of the medium from the 96-well plates, the cells were rinsed once with PBS (pH 7.4) and incubated in a solution of 2.5 μM Calcein-AM in PBS. Twenty minutes later, fluorescence was determined using a Bio-Tek FL600 microplate reader (Winooski, VT) with an excitation/emission filter set at 485/530 nm. Cell culture wells treated with methanol served as blanks. The results, obtained in RFU, were expressed as the percentage of control media values. In HT22 cells, we have observed a linear relationship between the number of viable cells per well and measured Calcein-AM fluorescence when viable cell numbers were between 300 and 5,000 cells per well ($r^2 = 0.9991$). HT22 cell death induced by excitotoxin in the Calcein assay is consistent with previous reports utilizing alternative viability methods, such as the colorimetric methyl-thiazol-tetrazolium test (Tan et al., 1998).

**Ethanol concentrations in the HT22 cell culture**

Ethanol concentrations were measured to test whether E2, BHT, or ZYC26 *per se* alters ethanol kinetics. For ethanol exposure conditions, the cells were treated with control medium (DMSO) or 100 mM of ethanol for 24 hours. E2 (1 μM), BHT (1 μM), or ZYC26 (1 μM) was simultaneously treated with ethanol for 24 hours. Cells were then collected at the end of the ethanol exposure. For EW conditions, the compounds were readministered when ethanol containing media was replaced with
control media and the cells were collected at 4 hours of EW. The 10 µl of sample solution was added to 200 µl of ice-cold 0.55 M perchloric acid. The media was neutralized with 200 µl of 0.6 M KOH containing 50 mM acetic acid. This solution precipitates the perchlorate ion and buffers the solution to about pH 5. After the samples were centrifuged, the resulting supernatant was used to measure ethanol concentrations using an enzymatic assay (Smolen et al., 1986). Briefly, a 20 µl aliquot of the supernatant solution was pipetted into duplicate assay tubes and one blank tube. The assay solution consisted of 2.29 mM nicotinamide adenine, 30 units of yeast alcohol dehydrogenase, and 500 mM Tris-HCl (pH 8.8), in a total volume of 140 µl. The blank tube contained no alcohol dehydrogenase. The reaction mixture was incubated at room temperature for 30 minutes prior to measuring the absorbance of nicotinamide adenine dinucleotide formed at 340 nm in a Gilford 240 spectrophotometer. Ethanol concentrations were calculated from linear regression analysis of a standard curve of known ethanol concentrations.

**Sample size**

Three sample replicates (N = 3) were used for the mitochondrial swelling assay in each group for each test. Five sample replicates (N = 5) were used for Calcein-AM viability assay and ethanol concentrations. These experiments were repeated two to four times. Data are presented as average ± SEM obtained from the sample replicates of the most recent test. For the flow cytometric analysis of ΔΨm and the fluorescence microscopic imaging, two sample replicates (N = 2) were used in each group. The experiments were repeated twice. Data from the most recent experiments are presented.

**Statistical analysis**

For data illustrating absorbance decline (mitochondrial swelling), average absorbance values from three sample replicates were used for Repeated (by minutes) Measures one way ANOVA. For Calcein-AM cell viability assay, one way ANOVA (by treatment) was conducted using five sample
replicates in each group. When significance was found (P < 0.05), a post hoc Tukey’s multiple comparison was conducted to determine difference between treatment conditions. The significance level for all data analysis was set at P < 0.05.

Results

Part I: Effects of ethanol/EW on PTP

1. Effects of ethanol/EW on MMS

Unhealthy mitochondrial membranes that have prolonged opening of PTP lose ability to scatter lights, resulting in absorbance decline at 540 nm. Repeated (by minutes) Measures ANOVA indicated that MMS significantly differs between treatment conditions [F(2,38) = 19, P < 0.001] (Figure 1). MMS occurred more rapidly in ethanol-withdrawn mitochondria than mitochondria under ethanol exposure (P < 0.01) or control media (P < 0.001). No significant difference was observed between control media and ethanol exposure. These data suggest that EW renders mitochondrial membranes vulnerable to a greater degree than ethanol per se.

2. Fluorescence microscopic observation of ∆Ψm during ethanol/EW

To examine whether EW provokes PTP opening, we measured ∆Ψm using JC-1 dye fluorescence. Healthy mitochondria that have high ∆Ψm readily uptake JC-1, which results in the formation of JC-1 aggregates inside the mitochondria and the JC-1 aggregates emit red fluorescence at 590 nm. Damaged mitochondrial membranes that have low ∆Ψm cause JC-1 to leak to cytosol, which results in a monomeric form of JC-1, emitting green fluorescence at 530 nm. When cells were observed with a fluorescence microscope (Figure 2), EW-cells exhibited stronger green fluorescence and less red
fluorescence than control cells or ethanol exposure-cells, indicating that EW results in more depolarized mitochondria.

3. Cellular consequences of EW-induced PTP opening

Whether or not EW-induced excess PTP opening mediates cell death was tested (Figure 3). HT22 cells were treated with control media or 100 mM of ethanol for 24 hours and withdrawn for 4 hours. Cyclosporin A (0.2 μM), an inhibitor of PTP opening (Halestrap et al., 1997) was treated when ethanol-containing media was replaced with control media to determine whether EW-induced PTP, not ethanol-induced PTP is responsible for cell death during EW. Cell viability was assessed at the end of the ethanol exposure or at 4 hours of EW and reported as relative values to ethanol free-cells. As expected, cell viability significantly differed between treatments [F(3, 20) = 206, P < 0.001]. Ethanol withdrawn cells with control media treatment had lower cell survival than ethanol free-cells (100% dash line), ethanol exposure-cells (75% ± 2, †P < 0.001), or EW-cells + cyclosporin A (68% ± 1.4, ‡P = 0.014). These data indicate that EW-induced PTP opening at least in part accounts for cell death.

Part II: Estrogen protection against PTP opening

4. E2 protection against EW-induced MMS

Whether or not E2 protects against EW-induced MMS was assessed (Figure 4). E2 (0.1 or 1 μM) was treated during the entire period of ethanol exposure and EW. Repeated (by minutes) Measure One way ANOVA indicated that the rate of absorbance decline differs between treatment groups (rapid decline indicates more severe MMS) [F (3, 51) =17, P < 0.001]. At 4 hours of EW, EW-cells without E2 treatment showed the most rapid MMS (P < 0.01) among the treatment conditions. By
comparison, E2-treatment at 1 μM (P < 0.001) and 0.1 μM (P < 0.05) delayed the MMS toward a control condition in a dose-dependent manner (P < 0.05), indicating protective effects of E2 on MMS.

5. E2-treatment window for E2 protection

To determine whether E2 protection against EW results from E2 effects on the ethanol exposure phase or the EW phase, E2 (1 μM) treatment was restricted to the EW phase and compared with E2 treatment during the entire period of ethanol exposure (100 mM) and EW (Figure 5). When measured at 4 hours of EW, Repeated (by minutes) Measure One way ANOVA indicated that MMS significantly differs between treatment groups [F (3, 51) = 29, P < 0.001]. At 4 hours of EW, EW-cells without E2-treatment had a more rapid MMS than cells treated with control media (P < 0.001), E2-treatment during the ethanol + EW phase (P < 0.001), or E2-treatment during the EW phase (P < 0.01). There was no significant difference between the two treatment conditions of E2: E2 treatment during the combined period of ethanol exposure + EW or E2 treatment that was restricted to the EW phase. These data indicate that E2 counteracts EW-associated factors and that E2 treatment during the EW phase per se is sufficient to protect against EW.

6. Flow cytometric analysis of E2 protection against ΔΨm collapse during EW

We quantitated ΔΨm using flow cytometry that measures intensity of green and red fluorescence. The green and red fluorescence are emitted from a monomeric form of JC-1 in damaged mitochondria and emitted from JC-1 aggregates in healthy mitochondria, respectively. We computed a ratio of green to red (a higher ratio indicates greater ΔΨm collapse) at the end of ethanol exposure or at 4 hours of EW with or without E2 treatment that was restricted to the EW phase. EW resulted in a higher ratio of green/red (68%/32% = 2.1) than control (51%/44% = 1.1), ethanol exposure (53%/46% = 1.2), or EW+E2 (44%/56% = 0.8) conditions, indicating that EW depolarizes mitochondria in a manner that is protected by E2 treatment (Figure 6).
7. Effects of BHT or ZYC26 on MMS

To determine if the prooxidant stimuli of EW provoke MMS, we used an antioxidant BHT (1 μM) with which an inhibited PTP opening had already been shown (Colell et al., 2004). When measured at 4 hours of EW, there was a significant difference in MMS between treatment groups [F (3, 51) = 27, P < 0.0001] (Figure 7). As was the case for E2 treatment, BHT treatment that was restricted to the EW phase delayed MMS (P < 0.05) of EW-cells. Because ZYC26 previously exerted protection against EW-induced cell death and oxidation of HT22 cells, we tested effects of ZYC26 (1 μM) on MMS. ZYC26 that was treated during the EW phase per se also delayed the MMS of EW-cells (P < 0.001). We compared the degree of protection afforded by the three compounds (E2, BHT, and ZYC26) by computing how much these compounds delayed the absorbance decline (an indicator of MMS) of EW-cells (data not shown). E2 protection was greater than BHT (P < 0.01) and similar to ZYC26 [F (2, 36) = 4.1, P = 0.031]. Based on this and our previous observation that ZYC26 showed higher antioxidant potency than E2 during EW (Jung et al., 2006), these data indicate that pro/antioxidant effects of EW/E2 are part of, but not a sole mechanism of, EW/E2-induced mitochondrial injury/protection.

8. Ethanol concentrations

Finally, we measured ethanol concentrations to test whether the observed protection by E2, BHT, or ZYC26 results from altered ethanol kinetics. HT22 cells were exposed to ethanol (100 mM) for 24 hours and withdrawn for 4 hours. E2 (1 μM), BHT (1 μM), or ZYC26 (1 μM) was treated simultaneously with ethanol. Cells were collected at the end of the 24 hour-ethanol exposure or at 4 hours of EW. Cells treated with 100 mM of ethanol per se contained 4.5 ± 0.01 mg/ml of ethanol at 24 hour-ethanol exposure. Cells cotreated with E2, BHT, or ZYC26 had 4.48 - 4.54 mg/ml of ethanol when administered ethanol concentration was 100 mM. No measurable ethanol was detected at 4 hours of EW. Collectively, none of E2, BHT, or ZYC26 altered ethanol concentrations and cells
maintained intended ethanol concentrations for 24 hours that were eliminated at 4 hours EW, resembling \textit{in vivo} situations. These results indicate that the kinetics of ethanol does not account for protection by E2, BHT, or ZYC26 against the EW-induced PTP opening.

9. \textit{Effects of E2, BHT, or ZYC26 per se on PTP opening (data not shown)}

If treatment with E2, BHT, or ZYC26 per se alters mitochondrial membrane integrity, the observed effects of E2, BHT, or ZYC26 on ethanol withdrawn cells do not necessarily indicate protection against EW. This possibility was examined by treating ethanol free-cells with E2, BHT, or ZYC26 (0.1 an 1 \textmu M) for 24 hours and another 4 hours when media was replaced with fresh media. When measured at 4 hours of withdrawal from control media exposure, none of the three compounds significantly altered MMS. These data indicate that E2, BHT, or ZYC26 counteracts factors associated with EW, exerting protection against EW-induced PTP opening.

\textbf{Discussion}

We demonstrated for the first time that EW provokes PTP opening in HT22 cells partly through but not entirely through its prooxidant nature. E2 treatment restricted to the EW phase ameliorated the mitochondrial membrane injury, suggesting that E2 counteracts factors associated with EW, exerting the mitoprotection.

We previously observed that EW inactivated a key mitochondrial enzyme, cytochrome c oxidase and provoked oxidation of mitochondrial proteins in rats (Jung et al., 2007, 2008). In the current study, we used an \textit{in vitro} HT22 cell model because this cell line has advantage of assessing oxidative mechanisms. As mentioned earlier, HT22 cells lack ionotropic glutamate receptors (Zaulyanov et al.,
1999) but contain the glutamate/cystine antiporter, necessary for the synthesis of an endogenous antioxidant glutathione. Therefore, HT22 cellular injury reflects compromised endogenous antioxidant capacity (Tan et al., 1998). Accordingly, EW-induced PTP opening is unlikely due to excitotoxicity associated with glutamate receptors but may result from oxidative insults. This hypothesis is supported by the finding that an antioxidant BHT treatment restricted to the EW phase attenuated the EW-induced PTP opening (Figure 7) and cell death (unpublished observation). Vulnerability of mitochondrial membranes to ethanol has been reported in a previous study where ethanol treatment resulted in excess PTP opening in mice lacking superoxide dismutase (endogenous antioxidant enzyme) (Kessova and Cederbaum, 2007). The study suggests that loss of endogenous antioxidant capacity mediates PTP opening. In agreement, an early study (French and Todoroff, 1971) noted that EW increased mitochondrial permeability to a superoxide generator (phenazine methosulfate) in male rats. Ethanol dependent rats showed swollen mitochondria in liver, demonstrating the pathological consequence of PTP opening (Yan et al., 2007). Taken together, our findings agree with these studies and provide empirical evidence that prooxidant EW injures mitochondrial membranes, perturbs the regulatory function of PTP, and ultimately results in detrimental cellular consequences (Figure 3).

Our model of EW resembles alcoholism in which withdrawal plays a key role in neuronal and cellular damage. Using this model, we previously demonstrated that EW evoked greater lipid peroxidation and protein oxidation than ethanol per se in rats and cells (Jung et al., 2004, 2006). The prooxidant nature of EW also has been demonstrated in a clinical situation in which EW-induced hyperexcitability was associated with oxidative damages; cerebrospinal fluid of withdrawn alcoholics contained higher concentrations of -O$_2^-$ and excitatory amino acids than control subjects (Tsai et al., 1998). These studies suggest that deleterious interaction between pro-oxidants and excitatory neurotransmission accounts for EW-distress. As such, if prooxidants contribute to EW injury and to PTP opening
(Kessova and Cederbaum, 2007), one can speculate that EW renders more severe PTP opening than ethanol per se in part due to its greater prooxidant activity than ethanol exposure (Figure 1).

We next assessed whether PTP opening contributes to cell death during EW. PTP intimately affects cell survival/death because if PTP remains open, cells cannot maintain optimal ATP levels for respiration (Halestrap, 2006). If excess opening of PTP mediates cell death, preventing the pore opening should attenuate the cell death. This hypothesis was tested using a PTP inhibitor cyclosporin A. Cyclosporin A is a prototype inhibitor of PTP opening (Norenberg and Rao, 2007) by interfering with protein (cyclophilin D)-protein (adenine nucleotide translocator) interaction that is essential for PTP opening (Sullivan et al., 2005). Cyclosporin A protected against apoptotic and necrotic cell death associated with PTP (Ishida, 2004). In our results, while only 49% of cells survived EW toxicity, 68% of cells survived EW after treatment with cyclosporin A. Therefore, our data extend cytotoxic PTP opening to a cellular model of EW.

We and others have shown that estrogens have substantial effects on mitochondrial function (Vina et al., 2007), particularly in the face of stress. For instance, estrogen exerted protection against β-amyloid- or ROS-induced mitotoxicity (Vina et al., 2007). E2 protected against H₂O₂-induced decline in ATP synthesis in human neuroblastoma cells (Wang et al., 2006). Estrogens have been shown to exert their anti-apoptotic effects through maintenance of ΔΨm in the face of stresses (Nilsen and Brinton, 2003). Furthermore, non-feminizing estrogens shared this ability to protect ATP production (Wang et al., 2006). Collectively, these data indicate that estrogens potently stabilize bioenergetics functions of mitochondria during stress such as oxidative stress.

In spite of a plethora of evidence of mitoprotective estrogen, the mechanisms by which E2 protects mitochondria from EW insults remain uncertain. We first tested whether E2 protection against EW is
due to its residual effects on ethanol *per se*. However, this possibility is unlikely because E2 exerted protection even when E2 treatment was restricted to the EW phase, suggesting that E2 directly or indirectly regulates factors associated with EW, thereby exerting mitoprotection. Because BHT protected against EW-induced PTP opening, it is reasonable to speculate that antioxidant activity of E2 counteracts prooxidants EW, thereby inhibiting PTP opening. In support of this view, PTP opening was inhibited by vitamin E but was induced by H$_2$O$_2$ (Lee et al., 2005; Sokol et al., 2005). Unfortunately, it is challenging to differentiate an antioxidant mechanism from other mechanisms of E2 that protect PTP. In addition, we are not aware of an estrogen analogue that has physiologically and pharmacologically identical properties to E2 but lacking only antioxidant property. As an indirect strategy to test whether E2 protects PTP through an antioxidant mechanism, we compared the degree of protection against PTP opening among E2, an antioxidant BHT, and an E2 analogue ZYC26. ZYC26 previously showed a 10 fold higher antioxidant potency than E2 in the HT22 cell model of EW (Jung et al., 2006). ZYC26 contains an adamantyl group at the C2 position of estrone. This structural configuration allows ZYC26 to scavenge ROS more effectively than E2. The adamantyl group enhances the stability of the adjacent phenolic radical, which is an essential element of scavenging ROS (Dhandapani and Brann, 2002). We computed the magnitude by which these compounds prevent MMS of ethanol withdrawn cells at a given time point. Had antioxidant action, especially ROS-scavenging activity been a major mechanism of E2 protection against PTP opening, ZYC26 that has higher ROS-scavenging potency than E2 should have been more protective than E2. However, the magnitude of E2 protection against MMS was greater than BHT (P < 0.05) and did not differ from ZYC26 (data not shown). These observations indicate that other factors in addition to ROS-scavenging activity of E2 also mediate protection against PTP opening. For instance, E2 might have activated glutathione peroxidase that directly reduces membrane-bound lipid hydroperoxides (Ha and Smith, 2003; Lapointe et al., 2005). In addition, because HT22 cell injury is associated with compromised redox status, it is also possible that E2 might have maintained glutathione homeostasis...
in the face of EW insults, thereby reducing oxidative burden of mitochondrial membranes and subsequently attenuating PTP opening. Alternatively, because high levels of mitochondrial Ca\(^{2+}\) impede the regulatory function of the PTP proteins (Halestrap and Brennerb, 2003) and because E2 induces mitochondrial tolerance to Ca\(^{2+}\) load (Nilsen and Diaz Brinton, 2003), E2 might have increased a threshold of mitochondrial Ca\(^{2+}\) that activates PTP. At the very least, it appears that E2 orchestrates multiple mechanisms in addition to antioxidant action, effectively protecting PTP from EW.

Taken together, our findings permit a conclusion that EW perturbs mitochondrial membrane integrity partly but not entirely through its prooxidant nature and that E2 exerts mitoprotection through multiple factors perhaps, involving an antioxidant mechanism, independent of ethanol metabolism. We extended EW injury to mitochondrial membranes, attributing to PTP opening and cell death in the HT22 cell model of EW. Our findings may provide new insights into mitochondrial mechanistic pathways underlying counteraction between EW and E2.
References


Footnotes

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LEGENDS FOR FIGURES

Figure 1. Effects of ethanol/EW on MMS
HT22 cells were exposed to control media (DMSO) or ethanol (100 mM) for 24 hours. At the end of the 24 hours, ethanol-containing media was replaced with control media for 4 hours. Cells were collected at the end of the 24 hour-ethanol exposure (Ethanol condition) or at 4 hours of EW (EW condition). Absorbance at 540 nm was recorded for 12 minutes. More rapid absorbance decline indicates more severe MMS. EW provoked MMS more rapidly than control media- (P < 0.001) or ethanol exposure-cells (P < 0.01). Depicted are mean ± SEM for N = 3/group. Some of SEM were too small to be depicted.

Figure 2. Fluorescence microscopic observation of ΔΨm during ethanol/EW
HT22 cells were exposed to control media or 100 mM ethanol for 24 hours and were withdrawn for 4 hours. JC-1 dye (2.5 μg/ml) was treated at 24 hours of ethanol exposure (ethanol exposure condition) or 4 hours of EW. Cells were then observed with a fluorescence microscope. EW resulted in more intense green fluorescence emitted from JC-1 monomers and less red fluorescence emitted from JC-1 aggregates, due to low ΔΨm than control or ethanol exposure-cells. 100 X magnification.

Figure 3. Cellular consequences of EW-induced PTP opening
HT22 cells were treated with control media or 100 mM of ethanol for 24 hours and withdrawn for 4 hours. Cyclosporin A (0.2 μM), an inhibitor of PTP opening was treated when ethanol-containing media was replaced with control media. Cell viability was assessed at the end of the ethanol exposure or at 4 hours of EW and reported as relative values to ethanol-free cells (100% dash line). EW-cells treated with control media had lower cell survival than ethanol exposure-cells (†P < 0.001) or EW-cells treated with cyclosporin A (‡P = 0.014). †P < 0.001 vs. ethanol-free cells. Depicted are mean ± SEM for N = 5/group.
**Figure 4. E2 protection against EW-induced MMS**

HT22 cells were exposed to control media or ethanol (100 mM) for 24 hours and withdrawn for 4 hours. E2 (0.1 or 1 μM) was simultaneously treated with ethanol and readministered when ethanol-containing media was replaced with control media at the end of the ethanol exposure. Cells were collected at 4 hours of EW (EW-cells) for assessment of MMS. EW-cells showed more rapid absorbance decline (more MMS) than cells treated with control (P < 0.001), 1 μM E2 (P < 0.001), or 0.1 μM E2 (P < 0.05). Depicted are mean ± SEM for N = 3/group. Some of SEM were too small to be depicted.

**Figure 5. E2-treatment window for E2 protection**

HT22 cells were exposed to control media or ethanol (100 mM) containing media. Ethanol treated cells were further divided into three groups; 1) ethanol + control media, 2) E2 treatment (1 μM) during the entire period of ethanol exposure and EW, and 3) E2 treatment that was restricted to the EW phase. Cells were collected at 4 hours of EW to assess MMS. EW-cells had the most rapid MMS (absorbance decline) among treatments (P<0.001). E2-treatment that was restricted to the EW phase significantly delayed (P < 0.05) MMS toward control-cells or toward EW-cells that were treated with E2 during ethanol exposure and EW. No difference was observed between E2 treatments during the two different time windows. Depicted are mean ± SEM for N = 3/group. Some of SEM were too small to be depicted.

**Figure 6. Flow cytometric analysis of E2 protection against ∆Ψm collapse during EW**

HT22 cells were treated with control media or ethanol (100 mM) for 24 hours and were withdrawn for 4 hours with or without E2 treatment that was restricted to the EW phase. JC-1 was treated at 24 hours of ethanol exposure or 4 hours of EW. Cells were then immediately analyzed with flow
cytometry in which a higher ratio of green (bottom right quadrant) to red (upper right quadrant) indicates more severe ΔΨm collapse. EW resulted in a higher ratio of green/red fluorescence (68%/32% = 2.1) than control (51%/44% = 1.1), ethanol exposure (53%/46% = 1.2), or EW+E2 (44%/56% = 0.8) conditions. The number in each quadrant indicates cell population (%) in the quadrant out of total cells (100%).

Figure 7. Effects of butylated hydroxytoluene (BHT) or ZYC26 on MMS

HT22 cells were exposed to control media or ethanol (100 mM) for 24 hours and withdrawn for 4 hours. BHT (1 μM) or ZYC26 (1 μM) treatment was restricted to the EW phase. Cells were collected at 4 hours of EW to assess MMS. BHT (P < 0.05) or ZYC26 (P < 0.001) treatment delayed EW-induced MMS. Depicted are mean ± SEM for N = 3/group. Some of SEM were too small to be depicted.
More severe mitochondrial membrane swelling

Figure 1

Absorbance at 540 nm vs. Latency (minutes)

- □ Non-ethanol control
- ○ Ethanol (100 mM)
- ● EW
<table>
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Figure 2
Figure 3
Figure 4

Absorbance at 540 nm vs. Latency (minutes)

- □ Non-ethanol control
- ● EW
- △ EW + E2 0.1 μM
- ▼ EW + E2 1 μM
Figure 5

Graph showing the absorbance at 540 nm over latency (minutes) for different conditions:

- **Non-ethanol control**
- **EW**
- **E2 treatment during**
- **Ethanol exposure & EW**
Figure 6

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Figure 7