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Ethanol withdrawal hastens the aging of cytochrome c oxidase.

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Abstract

We investigated whether abrupt ethanol withdrawal (EW) age-specifically inhibits a key mitochondrial enzyme, cytochrome c oxidase (COX), and whether estrogen mitigates this problem. We also tested whether this possible effect of EW involves a substrate (cytochrome c) deficiency that is associated with proapoptotic BAX and mitochondrial membrane swelling. Ovariectomized young, middle-age, and older rats, with or without 17 β -estradiol (E2) implantation, underwent repeated EW. Cerebelli were collected to measure COX activity and the mitochondrial membrane swelling using spectrophotometry and the mitochondrial levels of cytochrome c and BAX using an immunoblot method. The loss of COX activity and the mitochondrial membrane swelling occurred only in older rats under control-diet conditions but occurred earlier, starting in the young rats under EW conditions. E2 treatment mitigated these EW effects. EW increased mitochondrial BAX particularly in middle-age rats but did not alter cytochrome c. Collectively EW hastens but E2 delays the age-associated loss of COX activity. This EW effect is independent of cytochrome c but may involve the mitochondrial overload of BAX and membrane vulnerability.

Keywords

age; brain-aging; female rats; BAX (Bcl2-Associated X protein); cytochrome c; cytochrome c oxidase; 17 β -estradiol; ethanol withdrawal; mitochondria; mitochondrial membrane swelling; mitochondrial respiration

1. Introduction

Mitochondria produce the majority of cellular energy through a series of enzyme complexes and electron carriers. Cytochrome c oxidase (COX) is the terminal enzyme (complex IV) that transfers electrons from cytochrome c to an oxygen molecule. In this process, a proton gradient is created across mitochondrial membranes, providing force to generate ATP. That

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Disclosure Statement

None of authors in this manuscript has any type of conflicts of interest including financial and personal matters.

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COX plays a critical role in mitochondrial integrity can be inferred from the fact that the deficiency of this enzyme is one of the most common defects found in mitochondrial diseases (Diaz, 2010). COX is also a target of oxidative stress. For instance, the genetic down-regulation of COX resulted in the excessive generation of mitochondrial reactive oxygen species (Ungvari et al., 2008). Although numerous studies have characterized COX, relatively little information is available regarding the COX deficit associated with alcoholism. An early study noted that ethanol-exposed rats lost COX activity in the liver (Thayer and Cummings, 1990). Not only ethanol but also abrupt termination from excessive ethanol consumption resulted in a loss of COX activity in our previous study (Jung et al., 2007). The loss of COX activity was more severe during ethanol withdrawal (EW) than during ethanol exposure, suggesting that the impact of EW on this enzyme is distinct from that of ethanol. In support of the significance of withdrawal effect, Sullivan (2007) stated “simple removal of the addicting substance can itself be dangerous and cause life-threatening withdrawal effects.”

The functions of mitochondria decay with age due to a variety of stressors (Ungvari et al., 2008; Liu et al., 2002). This view of mitochondrial aging is supported by our recent study in which EW-induced mitochondrial protein oxidation was more severe in middle-age rats than young rats (Jung et al., 2010). Others have also reported that the content of $\bullet\text{O}_2$ and H_2O_2 in mitochondrial fractions in the presence of glutamate or succinate was highest in middle-age rats compared to young or old rats (Guarnieri et al., 1992). Cumulative evidence indicates that COX deficits are involved in age-associated CNS disorders such as Alzheimer’s disease (Davis et al., 1997; Vitali et al., 2009). The role of COX in the aging process is further supported by an animal study in which mutant mice with COX deficiency showed a shorter lifespan than wild type mice (Fukui et al., 2007). Ungvari et al. (2008) demonstrated that in aged blood vessels, COX activity declined due to increased free radical production. Indeed, functional and quantitative changes in COX have been suggested as part of the characteristics of brain aging (Sen et al., 2007). These studies suggest that mitochondria, especially COX, play a significant role in the neurobiology of aging.

Estrogen is not only a female sex hormone but also a neuro- and mito-protectant. For instance, estrogen mitigated ATP depletion induced by H_2O_2 (Wang et al., 2006) or by a succinate dehydrogenase inhibitor that uncouples oxidative phosphorylation (Wang et al., 2001). Estrogen also mitigated H_2O_2 -induced apoptosis in endothelial cells (Lu et al., 2007) and enhanced mitochondrial respiratory functions (Irwin et al., 2008). In our rat and cell models of EW, 17β -estradiol (E2) protected against the swelling of mitochondrial membranes and the collapse of mitochondrial membrane potential upon EW insults (Jung et al., 2008, 2009). Moreover, E2 mitigated the age-specific effect of EW on mitochondrial protein oxidation (Jung et al., 2010). According to Jones and Brewer’s study (2009), estrogen protected against the age-related loss of COX cofactors and increased mitochondrial respiration in response to glutamate in both young and old rats. These studies indicate that estrogen has the ability to protect mitochondria in the face of a variety of stressors including EW. Given this information, the current study was intended to identify a protective role of estrogen in EW-induced mitochondrial aging at the level of COX.

COX catalyzes cytochrome c, which is normally located in the inner membrane space of mitochondria. However, apoptotic insults such as the excessive level of BAX (Bcl2-Associated X protein) in mitochondria provoke the leakage of cytochrome c to cytosol. Therefore, we tested whether the EW-induced inhibition of COX requires the depletion of its substrate (cytochrome c) upon the overexpression of BAX in mitochondria. Since, COX is located in mitochondrial membranes, the integrity of the membranes was assessed by testing mitochondrial membrane swelling. Finally, the consequence of COX damage to mitochondrial function was assessed by measuring mitochondrial respiration using XF respirometry (Seahorse Bioscience, MA) and an *in vitro* model of EW. We provide empirical evidence that repeated EW hastens whereas E2 delays the functional aging of COX, and we attempt to characterize the underlying mechanisms. .

2. Materials and Methods

2.1. Subjects and experimental groups

Fisher 344 rats were housed individually in a room with controlled temperature (22–25°C), humidity (55%) and lights (7 AM –7 PM). Body weights were recorded twice a week. All housing and procedures were in accordance with the guidelines of the Institutional Care and Use Committee of the National Research Council (NIH publication no. 85–23, revised 1996) and were approved by the University of North Texas Health Science Center Animal Care and Use Committee.

All *in vivo* tests were done using a model of ovariectomy with or without E2 implantation except for the study of endogenous E2. Using this model enables us to avoid cyclic changes in ovarian steroids and the progesterone and thus to detect mainly effects of E2 on EW. After habituation, female rats were ovariectomized, implanted with an E2 or a control oil pellet, and recovered from the surgery for two weeks. They were then divided into 4 groups and subjected to a 25-day control dextrin or ethanol (6.5% w/v) diet and 5-day abrupt withdrawal. This cycle was repeated three times. The 4 groups were 1) dextrin + a control oil pellet (dextrin group), 2) dextrin + E2 pellet (dextrin + E2 group), 3) EW + oil pellet (EW group), and 4) EW + E2 pellet (EW + E2 group). Rats were fed regular chow pellets during withdrawal periods. They were sacrificed two weeks after the last dose of ethanol to test whether the effect of EW on COX persists even at two weeks of EW (Table 1 and Figure 1).

An additional group of rats was assigned to a continuous ethanol exposure group (ethanol group) to determine whether the effects of EW are merely the reflection of ethanol effects. They continuously received an ethanol-diet without withdrawal. At the end of the diet program, an ethanol-diet was gradually removed with step-down concentrations of ethanol to avoid withdrawal stress: 5% ethanol for 3 days, 3% ethanol for 2 days, and 2% ethanol-diet for 2 days. The ethanol-diet (2%) bottle was available until the next morning when they were sacrificed.

For the study of the effects of endogenous E2 on COX, gonadally intact young male and female rats and middle-age female rats were used in addition to ovariectomized young and middle-age rats. They were divided into a dextrin or an EW group and subjected to the

identical diet regimen described above. Dextrin-diet animals were not pair-fed with a regular chow diet or with a continuous dextrin diet. We have repeatedly observed no significant difference between a dextrin diet vs. a regular chow diet and intermittent vs. continuous dextrin diets in vital signs including body weights and in experimental outcomes such as oxidative markers (unpublished observations). Rats were 5- (young), 12- (middle-age), or 16-month-old (older) when they began a diet program and then approximately 8- (young), 15- (middle-age), or 19-month-old (older) when they were sacrificed after the completion of a diet program.

2.2. Ovariectomy and E2 implantation

Ovariectomy was performed 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. Immediately thereafter, Silastic pellets containing E2 or oil were subcutaneously implanted on the dorsal part of the rat. The E2 pellet releases physiological concentrations of E2 (29 to 34 pg/ml) for three weeks and thus was replaced every three weeks (Jung et al., 2002). We have previously demonstrated that EW does not alter the serum level of E2 in young female rats (Jung et al., 2002). Incisions were closed with Prolene, a non-absorbable and non-wicking suture. Two weeks were allowed for recovery from the surgery and for ovarian hormone clearance before an ethanol diet began.

2.3. Identify estrous cycle

The inclusion of ovary-intact groups was intended to test whether a natural decline in the level of endogenous E2 mediates the age-specific effect of EW. The Fischer344 female rats typically exhibit a 5-day estrous cycle: proestrus, estrus, metestrus, diestrus day 1, and diestrus day 2 (Page and Ben-Eliyahu, 1997). In the current study, Fischer344 female rats underwent daily vaginal cellularity smears (Markowska, 1999), and only those animals who consistently exhibited the 5-day estrous cycle were included in the study. We focused on estrus because in general, E2 levels are highest on estrus in the 5-day estrous cycle. Rats were identified as being on estrus by the homogeneous presence of cornified cells. Compared to young rats, fewer rats in the middle-age and older groups showed the homogeneous presence of cornified cells. A diet schedule that allows animals to be on estrus on the first day of EW was begun. Our rationale for this schedule was that the high level of E2 on estrus would protect animals from the severe stress of initial EW and a subsequent cascade of stressful molecular events. Exogenous estrogen treatment did not take place in these ovary-intact animals to mimic the naturally declining estrogen levels of aging women.

2.4. Chronic ethanol administration in a liquid diet and withdrawal

The induction of ethanol dependence was accomplished by a method that has been routinely employed in our laboratory (Jung et al., 2002). 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. Saccharin was added to mask the ethanol taste. One hundred ml of the diet was placed in each home cage daily. On the morning of the last day of each diet cycle, a 50 ml aliquot (6.5%) was administered and 12 hours later diet tubes were removed. The administration of the small

amount (50 ml) on the last day was to ensure that all rats in the EW groups finish the same amounts of ethanol by the time of diet removal.

2.5. Collection of brain tissues

Rats were humanely sacrificed under anesthesia [xylazine (20 mg/kg, i.p.) and ketamine (100 mg/kg, i.p.)] by decapitation. Cerebelli containing the vermis area were used for biochemical assays. We chose cerebellum because this brain area is vulnerable to both aging and EW. For instance, cerebellum from dog experiencing a neurodegenerative disease showed neuronal loss and apoptosis at an earlier age than healthy dog (Kondagari et al., 2010). Our recent study revealed that ethanol-withdrawn rats displayed impairment in cerebellar-related motor performance at an earlier age than healthy animals (Jung et al., 2010). In fact, Kraemer et al. (1997) found that people older than 60 were at increased risk for cognitive and motor impairment (cerebellar disorder) after EW. In this context, the current study may further strengthen the evidence supporting an age-associated vulnerability of cerebellum to EW.

2.6. Isolation of mitochondria

Mitochondria were isolated by conventional differential centrifugation with slight modifications (Yan et al. 2007). 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×g for 5 minutes 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×g for 5 minutes. The resulting pellet was resuspended in 12% Percoll solution and centrifuged at 6,900×g for 10 minutes. The resulting soft pellet was washed once with mitochondrial isolation buffer and centrifuged again at 6,900×g for 10 minutes. The pellet, containing mitochondria, was used in this study. Using isolated mitochondrial fraction, Bradford assay was conducted to analyze mitochondrial protein concentrations according to manufacture's instructions (Biorad, Hercules, CA).

2.7. Activity of COX

The activity of total COX was measured spectrophotometrically using a previously described method (Chen et al., 1999). Briefly, reduced cytochrome c was prepared by mixing 100 mg of cytochrome c and 4 mg of ascorbic acid in 10 ml of potassium phosphate buffer (10 mmol/L, pH 7.0). Mitochondria (20 µg protein) that were solubilized with 0.2% sodium deoxycholate were added to this solution to initiate an enzymatic activity. The excess ascorbic acid was removed by dialysis for 24 hours. The oxidation of cytochrome c was recorded at 550 nm every 5 minutes for 60 minutes and calculated with the molar extinction coefficient of 29.5 mM⁻¹·cm⁻¹.

2.8. Immunoblotting

A 30 µg of sample mitochondrial protein was electrophoresed on a 10% SDS-PAGE and then transferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked

with 5% fat free milk. The blot was washed in PBS containing 0.05% Tween-20 and probed overnight with a rabbit polyclonal antibody against cytochrome c at 1:200 dilution or BAX at 1:500 dilution (Cell Signaling, Danvers, MA). The blot was then incubated with horseradish peroxidase-conjugated secondary antibodies for one hour at room temperature. Bands were detected using the UVP western blotting luminescence system and quantified by an image densitometer. Immunoblottings for β -actin on the same gels that were used for the detection of cytochrome c or BAX were carried out as a positive and a loading control.

2.9. Mitochondrial membrane swelling

Since COX is located in mitochondrial membranes, the integrity of the membranes was assessed by testing mitochondrial membrane swelling. Mitochondria (0.25 mg protein) were suspended in medium containing 250 mM sucrose, 10 mM Tris-MOPS, 0.05 mM EGTA, 5 mM pyruvate, 5 mM malate, and 1 mM phosphate (pH 7.4). The absorbance by this suspension was measured at 540 nm using a Beckman DU 640 spectrophotometer. Phosphate in the medium induces swelling and rupture more rapidly in vulnerable mitochondrial membranes than healthy membranes (Menze et al., 2005). Intact mitochondria scatter light at 540 nm wavelength; mitochondrial swelling and rupture reduces mitochondrial light scattering and, thus, absorbance (Yan et al., 2002).

2.10. Mitochondrial respiration

For this assay, we employed an *in vitro* model of EW using HT22 cells (an immortalized mouse hippocampal cell line) because this cell line has been used to demonstrate that EW is mitotoxic, whereas E2 is mitoprotective (Jung et al., 2009). In addition, an *in vitro* system allows more focused mechanistic manipulation, such as treating cells with a COX inhibitor to test whether COX is directly related to mitochondrial functions. An *in vitro* model of EW has been used in others' studies in which EW induced the excitatory synaptic responses in cultured hippocampal cells (Thomas et al., 1998). Mitochondrial respiratory function was assessed by measuring the mitochondrial O₂ consumption rate according to a method provided by the XF respirometry manufacturer (Seahorse Bioscience, MA; Wu et al., 2007). Briefly, HT22 cells (600 cells/well) were seeded into 24-well microplates (Seahorse Bioscience, MA), cultured, and exposed to ethanol (100 mM) as described in our previous studies (Jung et al., 2006, 2009). The cell plate was then placed on an O₂ sensor cartridge and subsequently inserted to the XF respirometry. This step was done immediately after or 4 hours after a 3-day ethanol exposure to create an ethanol exposure or EW condition, respectively. E2's protection against EW-induced mitochondrial respiratory suppression was tested by treating cells with E2 (10 μ M) during the 4-hour EW period (Jung et al., 2009). The role of COX in mitochondrial respiration was tested by treating cells with a COX inhibitor (NaN₃, 1 mM). NaN₃ was preloaded in the reagent delivery chambers of the O₂ sensor cartridge and injected into the wells after the XF respirometry read the basal O₂ consumption rate. O₂ consumption rates (pmoles/minutes) were obtained approximately every 7 minutes.

2.11. Statistical analysis

All numerical data were expressed as mean $\pm$ standard error of mean (SEM). The results of COX activity, immunoblots, and O₂ consumption rate were analyzed by one-way (a diet or a

treatment condition) or two-way (age x diet) ANOVA. When significant differences were detected by ANOVA, *post hoc* Tukey's tests were performed to identify a specific difference between groups. For the membrane swelling assay, area-under-curve (AUC) was measured for a group comparison such that the smaller AUC due to the more rapid decline of a sigmoidal curve indicates the more severe swelling of mitochondrial membranes. The impact of EW relative to a control-diet was computed using a difference in AUC between dextrin and EW in which a bigger difference indicates greater membrane swelling induced by EW. *P* values < 0.05 were used to indicate statistical significance.

3. Results

3.1. Ethanol consumption

Ethanol consumption was recorded to test if different amounts of ethanol consumption among age groups contribute to the study endpoints. The amount of ethanol consumption was normalized based on body weight and then average diet consumption during an entire diet period was obtained. Consistent with our previous reports (Jung et al., 2010), older rats drank an ethanol-diet (14 ± 0.5 g of ethanol/kg) less ($p < 0.05$) than young (16 ± 0.5 g of ethanol/kg) or middle-age (15 ± 0.4 g of ethanol/kg) rats. There was no difference in ethanol intake between young and middle-age rats. The consumption of a control dextrin diet was less in young rats than middle-age or older rats, but when the amount was normalized by body weights, it did not significantly differ between the age groups (data not shown).

3.2. Effects of age on COX activity

Before we imposed the stress associated with ethanol exposure or EW, we examined whether age *per se* alters COX activity in ovariectomized rats under a control-diet condition. Figure 2 illustrates the activity of COX relative to that of young dextrin diet rats. Older rats showed a lower COX activity than young rats [$F(2,90)=12.6$, $p < 0.001$]. The COX activity of middle-age rats did not significantly differ from that of young or older rats. E2 treatment did not alter COX activity at any age tested. These data indicate that COX activity decreases with age, and E2 treatment does not prevent this activity loss under a control-diet condition.

3.3. Effects of age-EW combination on COX activity

We have previously demonstrated that EW suppresses COX activity in young female rats. In the current study, we investigated the effect of age-EW combination on this enzymatic activity and intended to identify a vulnerable age. As mentioned earlier, while COX began to lose its activity at an older age (19 months) under a control-diet condition (Figure 2), a decrease in COX activity was observed as early as a young age (8 months) under the EW condition (Figure 3) [$F(3,48)=662$ by diet, $p < 0.001$; $F(2,48)=59$ by age, $p < 0.001$]. The EW-induced decrease in COX activity in young rats was amplified in middle-age rats ($p < 0.01$), and did not change further in older rats. E2 pretreatment prevented the activity loss at each age. Ethanol exposure also suppressed COX activity in middle-age ($p < 0.05$) and older rats ($p < 0.05$) compared to a control diet (at 100%), but the magnitude of suppression was smaller than ($p < 0.01$) that induced by EW. It should be mentioned that the purpose of including ethanol exposure groups was to differentiate between the effects of ethanol and EW, and the current study focused on EW stress rather than ethanol *per se*. Notice that

compared to young, ethanol-withdrawn rats, the onset of a deleterious age-EW interaction began at a middle-age in the absence of E2 but at an older age in the presence of E2. Collectively these findings show that EW hastens the suppressing effects of age on COX activity in a manner that is delayed by E2 treatment.

3.4. Effects of endogenous E2 on COX activity

Because E2 treatment mitigated the loss of COX activity, we hypothesized that the depletion of endogenous E2 contributes to the deleterious age-EW interaction. To test this hypothesis, we collected data (relative to a control-diet) from ethanol-withdrawn young male and female rats with or without ovariectomy and compared them with ethanol-withdrawn middle-age rats with or without ovariectomy (Figure 4). This selection of animal groups was based on the rationale that 1) the onset of deleterious age-EW interaction began at a middle-age (Figure 3) and 2) the level of endogenous E2 is lower in ovariectomized rats than in ovary-intact rats and lower in male rats than female rats. Therefore, if the age-dependent loss of COX activity is attributable to E2 depletion, COX activity may correlate with the level of E2. As described earlier, our diet schedule was designed to detect the effect of E2 among ovarian hormones such that ovary-intact rats underwent the most severe phase of EW on the day of estrus on which the overall E2 level is highest. Therefore, the comparison between ovary-intact rats and ovariectomized rats reflected the presence or the absence of E2, respectively.

One-way ANOVA revealed a significant difference in COX activity between groups [$F(4,31)=65$, $p < 0.001$]. COX activity during EW was lower in young male rats vs. young female rats, in ovariectomized rats vs. ovary-intact rats, and in middle-age female rats vs. young female rats, as shown in the rank order of COX activity: young male rats (lowest) $\approx$ ovariectomized middle-age rats $<$ ovariectomized young rats $<$ ovary-intact middle-age rats $<$ ovary-intact young rats (Figure 4). Together with the data showing that E2 replacement in ethanol-withdrawn, ovariectomized rats restored the activity of this enzyme (Figure 3), this observation suggests that a loss of endogenous E2 at least partly contributes to the suppressing effect of the age-EW interaction on this enzymatic activity.

3.5. Effects of EW on the mitochondrial expression of cytochrome c and BAX

To characterize the mechanisms underlying the age-related deficit of COX upon EW insults, we tested whether COX loses its activity due to a substrate deficiency of this enzyme. To test this hypothesis, we measured the protein level of cytochrome c and BAX in mitochondria. The measurement of BAX was included due to reports that the excessive level of mitochondrial BAX depletes cytochrome c by promoting its release to cytosol (Kumarswamy and Chandna, 2009; Dejean et al., 2005; Kroemer et al., 2007). Therefore, we expected a down-regulation of cytochrome c and an up-regulation of BAX in mitochondria in response to EW stress. However, our results revealed that EW did not significantly alter the mitochondrial expression of cytochrome c at any age tested [Figure 5; $F(2,26)=0.4$, $p = 0.75$ by age; $F(1,26)=0.6$, $p = 0.5$ by diet]. In comparison, EW provoked the overexpression of mitochondrial BAX in ethanol-withdrawn middle-age rats ($p < 0.01$) compared to other diet and age groups [$F(2,12)=58$, $p < 0.0001$ by diet; $F(2,12)=5$, $p = 0.024$ by age] (Figure 6A). EW tended to increase the level of BAX in young and older rats, but it did not reach

statistical significance. Neither age *per se* nor ethanol exposure altered BAX. Our finding that age *per se* had no effect on BAX agrees with others' finding that BAX expression did not correlate with age in 177 specimens obtained from breast cancer patients (Yang et al., 1999). Since EW evoked the overexpression of BAX in middle-age rats, we selected this group and examined a potential protective effect of E2 on BAX. Unexpectedly, E2 failed to prevent the up-regulation of BAX induced by EW (Figure 6B). Collectively these results suggest that the age-dependent vulnerability of COX to EW is independent of a substrate level of COX, but it concurs with the mitochondrial overload of BAX.

3.6. Effects of age-EW combination on mitochondrial membrane swelling

Because COX is located in mitochondrial membranes, we assessed the integrity of mitochondrial membranes by measuring the membrane swelling. This was done by recording an absorbance decline at 540 nm, the more rapid decline of which indicates the more severe mitochondrial membrane swelling (Yan et al., 1998). For the purpose of group comparison and statistical analysis, area-under-curve (AUC) of each sigmoidal curve was measured in each rat; smaller AUC indicates more rapid mitochondrial membrane swelling. Compared to young dextrin-diet rats, more rapid mitochondrial membrane swelling began at an older age under a control-diet condition but it occurred as early as a young age under the EW condition [$F(2,48)=562$ by diet, $p < 0.001$; $F(2,48)=65$, by age, $p < 0.001$] (Figure 7). The magnitude of EW-induced mitochondrial membrane swelling relative to that of a dextrin condition was compared between age groups by obtaining a difference in AUC between dextrin and EW groups. The difference is indicated in Figure 7 as a grey area in the upper panel and as a vertical bar next to the dextrin bar in the lower panel. EW-induced mitochondrial membrane swelling was more severe in middle-age rats ($27 \pm 0.6\%$) ($p < 0.01$) than in young ($17 \pm 1.4\%$) or older ($15 \pm 1.2\%$) rats and it was more severe than ethanol exposure rats ($p < 0.01$) (Figure 7, lower panel). Compared to a control diet, ethanol also provoked membrane swelling in young ($p < 0.01$) and middle-age rats ($p < 0.01$). E2 pretreatment mitigated the EW-induced mitochondrial membrane swelling at all three ages tested ($p < 0.05$). These data indicate that the deleterious effect of EW injures the integrity of mitochondrial membranes more severely at a vulnerable age (middle-age) than at other ages.

3.7. Effects of EW on mitochondrial respiration

We determined whether a loss of COX activity during EW perturbs mitochondrial respiratory function by measuring the mitochondrial O_2 consumption rate. COX is the terminal enzyme among mitochondrial respiratory enzyme complexes and consumes most of mitochondrial O_2 by converting it to H_2O . Therefore, it is reasonable to hypothesize that the loss of COX activity impairs mitochondrial respiration. To test this hypothesis, we treated HT22 cells with a COX inhibitor (NaN3) in the presence or absence of E2 treatment and measured O_2 consumption rate using the XF respirometry. EW suppressed the basal O_2 consumption rate [$F(2,47)=6$, $p < 0.001$] more severely than ethanol exposure ($p < 0.01$) (Figure 8A). This effect of EW was mitigated by E2 treatment [$F(2,102)=45$, $p < 0.001$], suggesting that E2 protects ($p < 0.01$) against EW-induced mitochondrial respiratory suppression. The inhibition of COX by NaN3 mimicked EW by decreasing basal mitochondrial respiration [$F(1,102)=55$, $p < 0.001$] and blunted the protective effect of E2 (p

< 0.01) (Figure 8B). These data suggest that EW-induced damage to COX contributes to mitochondrial dysfunction in a manner that is protected by E2.

4. Discussion

We were interested in determining whether repeated EW promotes mitochondrial aging at the level of COX and whether estrogen protects against this problem. We did observe that female rats subjected to repeated EW suffered from a lower COX activity at an earlier age than control-diet rats (Figure 3). This effect of EW was prevented by estrogen pretreatment. Although numerous studies have reported the effects of alcohol or estrogen on COX, the current study is the first report that EW acts as stressor that provokes aging effects on this crucial mitochondrial enzyme in an estrogen-preventable manner.

We have previously demonstrated that the age-associated effects of EW on a stress protein kinase (P38) were not attributable to the different amounts of ethanol intake or blood ethanol concentrations between age groups (Jung et al., 2010). While young and middle-age groups consumed a similar amount of an ethanol diet, the activity of COX was lower in middle-age rats than young rats upon EW insults (Figure 3). Moreover, COX lost its activity more severely during EW than during ethanol exposure at all three ages. Given this, it seems clear that the age-dependent effects of EW on COX are not merely the result of a residual effect of ethanol. In regard to the effect of ethanol, its interaction with age differs between different parameters (e.g. mitochondrial membrane swelling and COX) or from EW. For example, the effect of ethanol on COX resembled that of EW in that its age-associated suppression began at a middle-age, although the magnitude was smaller than EW. In comparison, ethanol significantly provoked the swelling of mitochondrial membranes at a young age, but this effect was not exacerbated by age. Perhaps the effect of ethanol on the membrane swelling reached the maximum level at a young age, and thus no further membrane swelling occurred in older ages. A separate study may be required to fully explain the differences between the age-associated effects of ethanol and EW. At the very least, our data suggest that the mechanisms underlying the aging-like stimulus effects of ethanol and EW are not necessarily identical to each other.

Jaatinen et al. (2003) employed a paradigm of intermittent ethanol exposure with repeated EW and observed a decreased activity of COX that was more severe in the Purkinje neurons of 24-month-old male rats than 4-month-old male rats. In that study, age *per se* did not alter COX in either the prefrontal or cerebellar cortex. Jones and Brewer (2009) have reported that the COX activity in hippocampus was decreased in the isolated neurons (*in situ*) of 24-month-old male rats compared to those of 9-month-old male rats. This discrepancy may be a result of the different brain areas studied or different models used such as an *in vivo* (Jones and Brewer, 2009) or an *in situ* (Jaatinen et al., 2003) model. Our findings that compared to young rats, COX activity became lower at an older age under a control-diet condition but at a middle age under EW stress agree with the age-associated vulnerability of COX. In addition, they suggest that EW hastens the effects of age on COX and even more so in the aging brain of females, especially when they are deprived of estrogen. Considering that mitochondria are the loci of the cell death or survival decision, this effect of EW on COX can be quite deleterious if it is related to mitochondrial dysfunctions. Mitochondria respire by

consuming O₂ to generate ATP, and COX is the terminal respiratory enzyme where O₂ is mainly consumed. Therefore, it is reasonable to speculate that COX deficit impairs mitochondrial respiratory function. Indeed, we observed that EW inhibited mitochondrial respiration, as did a COX inhibitor (NaN₃), and this effect of EW was exacerbated in the presence of NaN₃. Given this, the adverse effects of age-EW interaction on COX may result in destructive consequences to mitochondrial integrity.

Since aged females lose E2, we tested the hypothesis that the deficiency of endogenous E2 mediates the age-associated effects of EW on COX. This hypothesis is supported by two sets of our data. First, the suppressing effect of EW on COX activity corresponds to the low level of endogenous E2 status. Secondly, the loss of COX activity is prevented by E2 pretreatment in ovariectomized rats. These data suggest that an ovarian hormone, E2 in particular, plays a role in maintaining the homeostatic activity of COX. However, under a control-diet condition, E2 treatment did not affect COX activity. In comparison, under the EW condition, E2 treatment delayed the onset of the deleterious age-EW interaction. Given this, E2's protection appears to be more effective in the face of stress than in a normal condition. In support of this view, while E2 *per se* did not alter the generation of free radicals under a vehicle condition, it protected against it when neurons were challenged with excess glutamate (Yi et al., 2009). Perhaps more importantly, our findings suggest that E2 depletion contributes to but is not solely responsible for the age-EW interaction. Had E2 been the sole contributor, the age-related difference (Figures 2 and 3) would have been prevented by E2 treatment.

We next investigated whether the age-dependent inhibition of COX by EW is due to a substrate (cytochrome c) deficiency of this enzyme. To explore this possibility, we simultaneously assessed the expression of cytochrome c and a propapoptotic protein BAX, an important molecule in the depletion of mitochondrial cytochrome c. Studies report that apoptotic insults provoke the overexpression of BAX in mitochondria, which in turn promotes the leakage of cytochrome c from the intermembrane space of mitochondria to cytosol (Jia et al., 2001; Kumarswamy and Sudhir Chandna, 2009) or to the nucleus (Scovassi et al., 2009). For instance, BAX translocated from the cytosol to mitochondria when cells were transfected with BAX-overexpressing genes or in response to cytotoxic stimuli (Deng and Wu, 2000; Hsu et al., 1997; Jia et al., 1999; Rosse et al., 1998; Wolter et al., 1997). Subsequently, cytochrome c was released from mitochondria, which then triggered a cascade of apoptotic events (Galluzzi et al., 2009). A potential link between BAX and COX was suggested in a study in which a reduced COX activity was observed in BAX-stable transfectant cells that showed BAX overexpression (Jia et al., 2001). Moreover, the overexpressions of COX and BAX reciprocally counteracted each other in yeast cells (Eun et al., 2008; Manon et al., 2001). BAX overexpression also arrested cell growth in a manner that was related to a decrease in the amount of COX (Manon et al., 1997). Based on these studies, we expected that EW-induced suppression of COX would concur with the down-regulation of cytochrome c in response to the up-regulation of BAX in mitochondria.

Our results revealed that our hypothesis was only partly correct. BAX was overexpressed particularly in ethanol-withdrawn middle-age rats among the age and diet groups tested. These results are in agreement with the suppression of COX in terms of the vulnerability of

middle-age rats to EW stress. However, in spite of the up-regulation of BAX, neither age *per se* nor EW altered the protein level of cytochrome c (Figure 5). This is contradictory to others' findings (Jones and Brewer, 2009) in which the level of cytochrome c was decreased in 24-month-old male rats compared to 9-month-old male rats. This discrepancy may be due to a narrow age window (8–19 months) in our study compared to other study (9–24 months). Alternatively, there may be a sex difference in response of cytochrome c to certain stresses. At the very least, one can speculate that mechanisms involving cytochrome c and BAX and their impact on COX may be multifactorial. In addition, our data suggest that the age-related deficit of COX during EW does not necessarily require the depletion of cytochrome c.

The link between E2, BAX, and COX during EW remains obscure, and the effects of E2 on BAX are inconsistent among different studies. For instance, E2 treatment prevented the ovariectomy-induced up-regulation of BAX in young female rats (Sharma and Mehra, 2008). *In vitro* E2 treatment caused a decrease in the mRNA level of BAX in human adrenal cortex tissue (Tron'ko et al., 2009), suggesting that E2 inhibits BAX. In contrast, E2 treatment in ovariectomized rats enhanced the ratio of apoptotic BAX to antiapoptotic protein Bcl-2 and induced apoptosis (Zaldivar et al., 2009). Yang et al. (1999) also indicated that the expression of BAX does not correlate with estrogen receptor status in breast carcinoma cells. This discrepancy is puzzling. Perhaps E2 inhibits or promotes BAX expression depending upon a model of neuroprotection or cancer, respectively. While E2 significantly protected COX, it did not alter EW-induced BAX (Figure 6B) in our current study. This result prompted us to ask if perhaps the degree of EW-induced BAX was not sufficient to trigger E2's counteraction. If so, the failure of EW to alter cytochrome c might also have been due to an insufficient increase in the level of mitochondrial BAX, which might not have reached the threshold for cytochrome c release. The failure of E2 to protect against EW-induced BAX suggests a couple of possibilities: 1) BAX is not the sole mediator of EW's suppressing effect on COX and 2) although E2 does not directly act on EW-induced BAX, it may inhibit a pathway down-stream to BAX that may mediate EW suppression of COX. Alternatively, instead of acting directly on BAX, E2 may stabilize mitochondrial membranes, which may in turn provide homeostatic milieu to preserve COX activity. This possibility is supported by the result of mitochondrial membrane swelling such that E2 exerted robust protection against the membrane swelling that occurred more severely in ethanol-withdrawn, middle-age rats than in other age groups. Others have reported that inhibiting mitochondrial permeability transition pores with cyclosporine A preserved the expression of COX in failing cardiomyocytes (Sharov et al., 2005).

Our data show that a deleterious age-EW interaction begins at or targets middle age. In fact, the vulnerability of middle-age subjects is supported by numerous studies. The onset of memory impairment occurred at the age of 12 months in female rats (Markowska et al., 1999). The ability to simultaneously perform multiple tasks also declined during middle age (Finch, 2009; Li et al., 2001). As such, middle-age subjects may suffer from a variety of transition stressors while changing from young to old endogenous systems. Because mitochondria are believed to be a locus of cell death or survival decisions, the transition stressors may collectively perturb mitochondrial integrity, including COX. This view is supported by a study in which middle-age female rats displayed a low activity of COX and a low content of mitochondrial ATP upon estrogen withdrawal (Shi and Xu, 2008). In this

scenario, older subjects might have developed adaptations to the altered cellular and mitochondrial milieu, becoming more resistant to stress. On the other hand, age-associated alteration may depend on specific organelles or target molecules. In our previous study, while middle-age rats were most vulnerable to the EW-induced activation of P38 (stress-activated protein kinase), the generation of reactive oxygen species peaked in older-age rats (Jung et al., 2010). At the very least, the findings in the current study extend the age-associated EW stress to COX and strengthen the view that the age most vulnerable to EW insults is not necessarily the oldest age.

In conclusion, the current study provides empirical evidence that EW hastens the aging of mitochondria, especially at the level of COX, in a manner that is delayed by E2 treatment. While the age-specific inhibition of COX by EW is independent of its substrate level, it may involve excessive mitochondrial BAX and inflicted mitochondrial membranes. These observations may provide a new insight into the mechanisms involving COX by which EW acts as an age-provoking stressor to perturb the mitochondria of females, especially those suffering E2 depletion.

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Figure 1. Diet schedules.

All experimental groups underwent repeated withdrawals except for an ethanol exposure group. Some female rats were ovariectomized and recovered for two weeks from ovariectomy before a liquid diet began. All withdrawal groups received a 25-day control dextrin or ethanol (6.5% w/v) diet and 5-day abrupt withdrawal. This cycle was repeated three times. Rats were then sacrificed two weeks after the last dose of ethanol for brain collection. Rats in the ethanol exposure group continuously received an ethanol-diet and were gradually withdrawn from the diet for 7 days with step-down concentrations of ethanol at the end of the diet regimen. They were then sacrificed next morning.

Figure 2. Effects of age on COX activity.

Ovariectomized rats were implanted with oil or E2 pellets, received a 25-day control dextrin diet followed by a 5-day chow pellet diet. This cycle was repeated three times. Two weeks after the last dextrin diet, COX activity was measured in the cerebellar whole-cell lysates. Older rats had a lower activity of COX than young rats ($*p < 0.01$). The activity of middle-age rats did not differ from that of young or older rats. E2 *per se* did not alter COX activity. $*p < 0.01$ vs. young dextrin rats. $†p < 0.01$ vs. young dextrin + E2 rats. Data (%) are presented relative to a control-dextrin diet value in young rats. Depicted are mean $\pm$ SEM for 7–10 rats/group.

Figure 3. Effects of age-EW combination on COX activity.

Ovariectomized rats were implanted with oil or E2 pellets, received a 25-day control dextrin or ethanol (6.5% v/v) diet followed by 5-day abrupt withdrawal. This cycle was repeated three times. The ethanol exposure group continuously received an ethanol-diet without withdrawal. COX activity was measured in the cerebellum obtained two weeks after or at the end of the last dose of ethanol for the condition of EW or ethanol exposure, respectively. EW suppressed COX activity in young rats, further so in middle-age rats, and thereafter, did not significantly alter the activity in older rats. This effect of EW was prevented by E2. * $p < 0.01$ vs. young EW rats. † $p < 0.01$ vs. young EW + E2 rats. ‡ $p < 0.01$ vs. control diet rats (100%) at each age. Some statistical symbols are omitted for the clarity of a figure. Depicted are mean $\pm$ SEM for 7–10 rats/group.

Figure 4. Effects of endogenous E2 on COX activity.

This experiment used gonadally intact young male and female rats and middle-age female rats and ovariectomized young or middle-age female rats. They received a 25-day control dextrin or ethanol (6.5% v/v) diet followed by 5-day abrupt withdrawal and repeated this cycle three times. Ovary-intact female rats were on estrus on the first day of EW when the most severe EW stress occurred. Two weeks after the last ethanol-diet, COX activity was measured in the cerebellar whole-cell lysates. COX activity during EW was lower in young male rats vs. young female rats, in ovariectomized rats vs. ovary-intact rats, and in middle-age female rats vs. young female rats. All data (%) in this figure are from ethanol-withdrawn groups and presented relative to a control-diet value at 100 %. Depicted are mean $\pm$ SEM for 5–10 rats/group. The number indicates a *p* value between two groups indicated with a horizontal line.

Figure 5. Effects of EW on the expression of mitochondrial cytochrome c.

Ovariectomized rats received a 25-day control dextrin or ethanol (6.5% v/v) diet followed by a 5-day abrupt withdrawal. This cycle was repeated three times. Two weeks after the last ethanol-diet, the protein level of cytochrome c was measured using an immunoblot method in the mitochondrial fraction of cerebellum. The bar graph indicates no significant difference between diet and age groups. β -actin was used as a loading control. A clear image was selected from 5–6 rats/group.

Figure 6. Effects of EW on the expression of mitochondrial BAX.

Ovariectomized rats received an identical diet regimen as described in Figure 3 legend. Two weeks after or at the end of the last dose of ethanol, the protein level of BAX was measured using an immune blot method in the mitochondrial fraction of cerebellum. EW provoked an increase in the expression of BAX in middle-age rats among diet or age groups ($*p < 0.01$) (Figure 6A). $*p < 0.01$ vs. all other groups. Figure 6B illustrates that E2 does not alter the mitochondria level of BAX in middle-age rats. $^{\dagger}p < 0.01$ vs. dextrin. β -actin was used as a loading control. A clear image was selected from 3 rats/group.

Figure 7. Effects of age-EW combination on mitochondrial membrane swelling.

Ovariectomized rats received an identical diet regimen as described in Figure 3 legend. Two weeks after or at the end of the last dose of ethanol, cerebelli were collected to measure mitochondrial membrane swelling by recording an absorbance decline at 540 nm (Figure 7, upper panel). In the lower panel of Figure 7, the smaller area-under-curve (AUC) indicates the more rapid swelling of mitochondrial membranes. The magnitude of EW-induced mitochondrial membrane swelling relative to a dextrin condition is illustrated as a grey area in the upper panel and a vertical bar (%) next to a dextrin in the lower panel. Compared to a control-diet, EW provoked more severe mitochondrial membrane swelling in middle-age (27%) ($^{\dagger}p < 0.01$) than young (17%) or older (15%) age groups in a manner mitigated by E2 treatment. $^*p < 0.01$ vs. a dextrin diet, ethanol exposure, or EW + E2 at each age. $^{\ddagger}p < 0.01$ vs. young dextrin group. $^{\dagger\dagger}p < 0.01$ vs. young or middle-age dextrin group. Depicted are mean $\pm$ SEM for 5–10 rats/group.

Figure 8. Effects of EW on mitochondrial respiration.

HT22 cells (600 cells/well) were plated in 24-well microplates, exposed to ethanol (100 mM) for 3 days, and withdrawn for 4 hours. The cells were treated with E2 (10 μ M) during the 4 hour-EW period. The microplate was then placed on the O₂ sensor cartridge and inserted to XF respirometry immediately or 4 hours after the 3-day ethanol exposure for an ethanol exposure condition or EW condition, respectively (Figure 8A). COX inhibitor (NaN₃, 1 mM) was applied to cells approximately 21 minutes after XF respirometry began to read basal O₂ consumption rate (Figure 8B). Compared to a control condition, EW suppressed mitochondrial respiration more severely than ethanol ($p^{\dagger} < 0.01$, Figure 8A) in a manner mitigated by E2 ($\ddagger p < 0.01$) treatment (Figure 8B). COX inhibitor suppressed the basal level of mitochondrial respiration ($p^* < 0.001$ vs. the basal level of Control) and blunted E2's protection ($p^{\ddagger} < 0.01$ vs. the basal level of EW+E2). N=4 wells/each condition.

Table 1.

Animal groups Ovariectomized rats were divided into 5 groups: dextrin, dextrin + E2, EW, EW + E2, and ethanol exposure groups. They were subjected to a diet program consisting of a dextrin or an ethanol-diet (6.5% w/v) for 25 days followed by abrupt withdrawal for 5 days. This cycle was repeated three times. The ethanol exposure group (Ethanol) continuously received an ethanol-diet until they were sacrificed. Separately, gonadally intact male and female rats were divided into Dextrin and EW groups. They received the identical diet program as described earlier. Rats were 5-month (young), 12-month (middle-age), or 16-month (older) old when they began a diet program and approximately 8-, 15-, or 19-month-old when brains were collected.

Sex	Initial age (month)	Diet	Final age (month)
Ovariectomized	5, 12, or 16	Dextrin	8, 15, or 19
Female		Dextrin + E2	
		EW	
		EW+E2	
		Ethanol	
Ovary-intact	5 or 12	Dextrin	8 or 15
Female		EW	
Gonadally	5	Dextrin	8
Intact Male		EW	