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Ethanol withdrawal posttranslationally decreases the activity of cytochrome *c* oxidase in an estrogen reversible manner

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Abstract

Cytochrome *c* oxidase (COX) is a key mitochondrial enzyme that catalyzes electron transfer at the terminal stage of respiratory chain and is composed of multisubunits. We hypothesize that ethanol withdrawal (EW) impairs the activity of COX and estrogen deprivation exacerbates this problem. Five-month-old ovariectomized rats with or without 17β-estradiol (E2) replacement received a control dextrin or a liquid ethanol diet (6.5%, 5 weeks). They were then sacrificed either during ethanol exposure or at 24 h of EW (EW group). Mitochondria of the cerebellum and cortex were processed to measure the activities of total COX, COX subunit I, and IV. The effects of EW and E2 on the protein levels of these subunits were also assessed using an immunoblotting method. As compared to the control dextrin and ethanol exposure, EW decreased the activities of total COX, COX I, and COX IV. E2 treatment prevented the effects of EW on the activities of total COX and COX IV but not COX I. Neither EW nor E2 altered the protein levels of the subunits. These findings suggest that a counteracting relationship exists between the effects of EW and E2 on the activity of COX in a subunit specific manner.

Keywords: Cytochrome *c* oxidase; 17β-Estradiol; Ethanol withdrawal; Mitochondria

Mitochondria produce cellular energy (ATP) through a series of mitochondrial enzyme complexes. Electrons are transferred across the enzyme complexes and create the electrochemical gradient between the mitochondrial membranes. Subsequently, the electrochemical gradient provides force to generate ATP. COX is a terminal enzyme complex among the series of enzymes and plays a key role in the mitochondrial function. Therefore, damage to this enzyme can cause serious clinical consequences. Indeed, the decreased activity of COX has been found in a variety of neurodegenerative illnesses such as Parkinson's disease and Alzheimer's disease [3,10,21].

Accumulated evidence indicates that mitochondria are vulnerable to ethanol/EW toxicity. Reactive oxygen species produced during ethanol metabolism altered mitochondrial function in rodents [26,24]. The mitochondrial membrane permeability and oxidation were dramatically increased during EW [32,14], suggesting that ethanol and/or EW preferentially target mitochondria. However, most of the studies did not differentiate between ethanol exposure and EW. Thus, it is not clear whether the observed damage is due to ethanol toxicity or EW. 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 [30,19]. In contrast to the deleterious effects of EW on mitochondria, estrogen appears to protect against mitochondrial integrity. Brain mitochondria in male rats produce over 80% more peroxides than those in females [4]. Furthermore, ovariectomy causes an increase in peroxide production by mitochondria in a manner prevented by E2 [4]. Given these findings, estrogen appears to play a role in reducing the oxidative burden in mitochondria [9].

The aim of this study is to elucidate how brain mitochondria respond to EW stress at the level of COX. COX is composed of 13 subunits with subunits I–III encoded by mitochondrial DNA and IV–XIII encoded by nuclear DNA [23]. The large number of subunits is a mystery because of no differences in spectral properties and proton-pumping activities between the 13-subunit enzyme [27,13]. To gain better understanding of the effects of EW on this enzyme, we measured (1) the activities of total COX, COX I, and COX IV and (2) mRNA expression and protein levels of the COX subunits in the presence and absence

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of E2. We chose cerebellum and cortex, that are vulnerable to ethanol both in humans and animals [12,28,29].

Young-adult-female Sprague-Dawley rats (Charles River, Wilmington, MA) were ovariectomized under anesthesia and were subcutaneously implanted with Silastic pellets (30 mm long, 1.57 mm ID, 3.18 mm OD) containing either E2 (4 mg/ml) or corn oil [20]. Pellets were replaced every 3 weeks. All housing and procedures were in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health (Institute of Laboratory Animal Resources [15] and were approved by the University of North Texas Health Science Center Animal Care and Use Committee.

Rat groups were (1) the non-ethanol diet (dextrin/oil), (2) the ethanol exposure (ethanol/oil), (3) the EW without (EW/oil), or (4) with E2 replacement (EW/E2) groups. The ethanol/oil group was sacrificed at the end of the ethanol diet before EW. All other groups were sacrificed at 24 h of EW. The ethanol dependence was induced by a liquid diet administration such that the amount of dextrin and ethanol was calculated in combination to adjust the concentration of ethanol to 6.5% w/v [11,22]. A fresh diet was (100 ml) placed in each home cage daily for 5 weeks. At 24 h of EW, physical signs of EW such as tremor, rigid tail curve, startle, and vocalization were evaluated by two experimenters who were not aware of a group identity [22]. Immediately thereafter, rats were deeply anesthetized with sodium pentobarbital (200 mg/kg, IP) and then sacrificed by decapitation. To extract proteins, brain tissue was rinsed to remove blood, hand ground in a glass homogenizer, and suspended in isolation buffer. The tissue was then centrifuged at $1330 \times g$. The pellets were resuspended and centrifuged at $3030 \times g$. The supernatants were pooled and a standard Biorad protein assay was performed to determine protein concentration.

The activity of total COX was measured spectrophotometrically [8]. Briefly, reduced cytochrome *c* was prepared by mixing cytochrome *c* and ascorbic acid in potassium phosphate buffer. The oxidation of cytochrome *c* was recorded at 550 nm every 5 min for 20 measurements. The activities of COX subunits were measured according to a manufacturer's instruction (MitoSciences, Eugene, Oregon). A 96 well plate was blocked with dry-milk solution. One hour later, isolated mitochondrial samples were added to each well and incubated for 2 h. The subunit specific COX antibody and KH_2PO_4 were added to each well. Absorbance of each well was measured at 550 nm every 5 min for 20 measurements.

To conduct immunoblotting with monoclonal antibodies (Abcam, Cambridge, MA) for subunits (I and IV), protein samples were separated on SDS-polyacrylamide gel (12%) and transferred to a nitrocellulose membrane. Membranes were blocked by incubation with dry milk in phosphate-buffered saline and Tween 20 (PBST), followed by overnight incubation at 4 °C with the primary antibody in blocking buffer. The membrane was then incubated with the horseradish-peroxidase-conjugated secondary antibody, followed by wash in PBST. The membrane was then incubated and developed with an enhanced chemiluminescent kit (Pierce, Rockford, IL).

To extract RNA, the homogenized tissue was centrifuged and lysed in TriZol reagent (Life Technologies, Gaithersburg, MD). The RNA containing aqueous layer was separated by chloroform and incubation on ice for 5 min followed by centrifugation at $12{,}000 \times g$. Total RNA was precipitated from the resulting aqueous phase by mixing with an equal volume of isopropyl alcohol, followed by incubation on ice for 15 min and centrifugation at $12{,}000 \times g$. The precipitated total RNA was resuspended in 70 μl of water and stored at −80 °C until assayed.

To make cDNA using reagents from Promega (Madison, WI), total cellular RNA was incubated with random primers at 85 °C. RNasin, reverse transcriptase buffer, deoxyribonucleotides, and avian myeloblastosis virus reverse transcriptase were added to the reaction and were incubated at 42 °C and then at 94 °C. cDNA was stored at −20 °C until assayed. The primer sequences for COX I and COX IV were found using Primer 3 program available from MIT as follow; COX I sense primer: 5′GGAGCAGTATTCGCCATCAT3′, COX I antisense primer: 5′CGGCCGTAAGTGAGATGAAT3′, COX IV sense primer: 5′ACTACCCCTTGCCTGATGTG3′, COX IV antisense primer: 5′ACTCATTGGTGCCCTTGTTC3′. Negative controls containing all the reagents except the cDNA were included for each primer pair. Programmable temperature cycling (Mastercycler-Eppendorf-Scientific, Westbury, NY) was performed with the following cycle profile: (1) denaturation at 94 °C for 2 min and then at 92 °C for 2 min, (2) 40 cycles of annealing at 60 °C for 30 s and then at 72 °C for 90 s, and (3) denaturation at 92 °C for 45 s. Horizontal 1.5% agarose (Omnipure agarose, EMD) gel electrophoresis was performed using PCR reaction product and loading dye per lane with gel run in a Mini-Sub cell electrophoresis (Bio-Rad, Hercules, CA). A 100-base pair DNA ladder (Promega, Madison, WI) was used for molecular size standards [1].

All activity data were expressed as the mean ± S.E.M. A one-way ANOVA followed by Tukey post hoc was conducted to determine the main effects and group differences. All data were from at least two separate determinations using the tissues collected from the same five or six rats for the COX activity and three rats for the PCR and Western blot.

All ethanol withdrawn rats exhibited EW signs. Dextrin rats and ethanol exposure rats did not show any EW signs [19,18].

Fig. 1A illustrates that EW more than two-fold decreased the activity (nM/min/mg protein) of total COX ($p < 0.01$) in both cerebellum and cortex; whereas, ethanol exposure *per se* (ethanol/oil) did not significantly decrease the activity compared to the dextrin case (dextrin/oil). Fig. 1B illustrates that the activity of total COX did not significantly differ between the dextrin/oil and EW/E2 groups, indicating that E2 prevented the suppressing effects of EW on the total COX activity in both brain areas.

Fig. 2 illustrates that EW decreased the activity (nM/min/mg protein) of COX I ($p < 0.01$), and COX IV ($p < 0.01$) in both cerebellum and cortex, but E2 protection was observed only for COX IV ($p < 0.05$).

Fig. 3 illustrates mRNA (A) and protein (B) signals for cerebella COX IV that were detected at 188 base pairs and 17 KDa, respectively. When measured by Scion Image (NIH)