Additive Effects of Mitochondrion-targeted Cytochrome CYP2E1 and Alcohol Toxicity on Cytochrome c Oxidase Function and Stability of Respirosome Complexes*

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Background: Alcohol toxicity affects mitochondrial function, which likely is a contributing factor in tissue injury.

Results: Cytochrome c oxidase is the primary target of CYP2E1-mediated alcohol toxicity and oxidative stress.

Conclusion: Damage to cytochrome oxidase affects respirosome complexes, which in turn may be the cause of increased ROS production.

Significance: Identification of targets of alcohol toxicity and reversing the damage by mitochondrion-targeted antioxidants.

Alcohol treatment induces oxidative stress by a combination of increased production of partially reduced oxygen species and decreased cellular antioxidant pool, including GSH. Recently, we showed that mitochondrion-targeted CYP2E1 augments alcohol-mediated toxicity, causing an increase in reactive oxygen species production and oxidative stress. Here, we show that cytochrome c oxidase (CcO), the terminal oxidase of the mitochondrial respiratory chain, is a critical target of CYP2E1-mediated alcohol toxicity. COS-7 and Hep G2 cell lines expressing predominantly mitochondrion-targeted (Mt†) CYP2E1 and livers from alcohol-treated rats showed loss of CcO activity and increased protein carbonylation, which was accompanied by a decline in the steady state levels of subunits I, IVI1, and Vb of the CcO complex. This was also accompanied by reduced mitochondrial DNA content and reduced mitochondrial mRNA. These changes were more prominent in Mt‡ cells in comparison with wild type (WT) CYP2E1-expressing or ER† (mostly microsome-targeted) cells. In addition, mitochondrion-specific antioxidants, ubiquinol conjugated to triphenyl phosphonium, triphenylphosphinum conjugated carboxyl proxyl, and the CYP2E1 inhibitor dialyl sulfide prevented the loss of CcO activity and the CcO subunits, most likely through reduced oxidative damage to the enzyme complex. Our results suggest that damage to CcO and dissociation of respirosome complexes are critical factors in alcohol-induced toxicity, which is augmented by mitochondrion-targeted CYP2E1. We propose that CcO is one of the direct and immediate targets of alcohol-induced toxicity causing respiratory dysfunction.

Excessive and chronic alcohol consumption leading to liver damage and alcohol liver disease is known to elicit a multitude of effects on the hepatic tissue as well as other tissues of the body. It is becoming increasingly apparent that the hepatic mitochondrial compartment is an important target of alcohol toxicity. Studies have linked alcohol-mediated effects to mitochondrial dysfunction, apoptosis, increase in ROS§ production, loss of cellular ATP, and eventually mitochondrial DNA (mtDNA) damage (1–8). mtDNA encodes 13 proteins associated with the mitochondrial OXPHOS complexes, and it is highly susceptible to oxidative damage (3, 4, 9). Inefficient mtDNA repair and the proximity of mtDNA to the mitochondrial respiratory chain, which is the main site of ROS formation, are thought to be important factors (10–14) in alcohol-induced toxicity.

A number of studies using both cellular and animal models have shown that mitochondrial electron transport chain activities are altered by alcohol treatment. Some studies suggest that complex I and complex II are altered in hepatocytes in various liver pathologies (15, 16), whereas the others consider complex II to be the most resistant complex, which is not damaged even in liver cirrhosis (3, 4, 17–19). Cytochrome c oxidase (CcO) is the terminal oxidase of the electron transport chain and contains three large catalytic subunits (I, II, and III) encoded in the mitochondrial genome and up to 10 smaller subunits encoded in the nuclear genome. Several studies over the past decade have reported different types of effects of alcohol on CcO activity in cell culture and animal models. In one of the earliest studies, Lieber and co-workers (20) showed that alcohol treat-

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3 The abbreviations used are: ROS, partially reduced O2 species; CcO, cytochrome c oxidase; Mito-Q, ubiquinol conjugated to triphenyl phosphonium; Mito-CP, triphenylphosphinum conjugated carboxyl proxyl; DAS, diallylsulfide; BNG, Blue Native gel; DNP, 2,4-dinitrophenol; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; OCR, oxygen consumption rate; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ER, endoplasmic reticulum; NAC, N-acetylcysteine; CYP2E1, cytochrome P4502E1.
ment in baboons resulted in markedly altered hepatic mitochondrial CcO activity. Similarly, exposure of mitochondrial membrane fractions with alcohol caused the structural perturbation of the a3-CuB site, affecting CcO activity (21). Another study showed that hepatic mitochondrial NO levels markedly increased under chronic alcohol treatment (22, 23), which covalently modified the heme moiety leading to reduced CcO activity. CcO gene expression and CcO activity were also impaired in chick embryonic cardiac myocytes (24) following alcohol treatment. However, the details of mechanisms of alcohol effects on the CcO complex and the precise subunits affected remain unclear.

It is known that some selective subunits of the CcO complex are degraded under oxidative stress conditions, experimental or chemical hypoxia, myocardial ischemia, or pathological conditions such as cancer (25–31). A previous study from our laboratory showed a steady and notable loss of subunits I, IV11, and Vb under chemical stress, hypoxia, and myocardial ischemia/reperfusion conditions (28, 31, 32). Others have shown lowering of subunits I, II, and VIc in addition to subunits IV11 and Vb (33) under different pathophysiological conditions.

The cytochrome CYP2E1 catalyzes the metabolism of numerous xenobiotics, industrial chemicals, and alcohol (34–35). Several studies implicate CYP2E1 in alcohol toxicity and alcohol liver disease, although the precise mechanism and subcellular target(s) remain unclear. Notably, CYP2E1 is induced in the liver and several extrahepatic tissues by small organic molecules such as ethanol, pyrazole, acetone, or isoniazide (36–39). Consequently, the tissue levels of this heme protein are significantly increased following alcohol consumption. The increased tissue level of CYP2E1 appears to have an additive effect on alcohol toxicity (34–37, 40). In a recent study, we showed that mitochondrial-targeted CYP2E1 markedly augmented ethanol-induced toxicity and oxidative stress in COS cells (40), whereas the microsome-targeted CYP2E1 had a marginal effect in mediating alcohol toxicity. In this study, we show that the catalytic function of CYP2E1 during alcohol treatment is a key factor in modulating the activities of mitochondrial electron transport chain complexes, in particular, the CcO complex and retention of electron transfer chain supercomplexes called respirosomes. Inhibitors of CYP2E1 or mitochondrial-targeted antioxidants alleviated the alcohol-induced effect providing a direct link between the metabolic activity of mitochondrial CYP2E1 and loss of CcO activity. Our results show a cumulative effect of mitochondrial CYP2E1 and alcohol on mitochondrial dysfunction that appears to represent a major part of alcohol toxicity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Alcohol Treatment—**COS-7 cells (ATCC, CRL, and 1651) and Hep G2 cells (ATCC CRL-10741) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (v/v) in the presence of added 1% penicillin/streptomycin. Preparation of COS cells stably expressing CYP2E1 constructs was described before (40). Transduction of Hep G2 cells with WT and Mt + + CYP2E1 cDNAs cloned in pBABE retroviral vector and selection of positive clones were as described for COS cells (40). COS cells were treated with ethyl alcohol, hereafter referred to as alcohol, at a concentration of 25–100 mM, and Hep G2 cells were treated with 300 mM alcohol. Cultures were fed each day for a maximum of 2 days (48 h) in the case of COS cells and 4 days (96 h) in the case of Hep G2 cells with alcohol containing fresh media to establish the peak alcohol concentration needed for inducing the toxic effects (40). We did not see significant cell death under these conditions.

**Animal Feeding Experiments—**Sprague-Dawley rats (about 150 g) were fed with alcohol for 2, 4, 6, 8, and 10 weeks, and pair-fed controls received isocaloric diet. The standard procedure for alcohol feeding was based on the Lieber De Carli protocol (40, 41). Animals were fed ad libitum a nutritionally balanced liquid diet containing 2–36% caloric equivalent of ethanol, 18% protein, 35% fat, and 11% carbohydrate as % of total calories (supplied by BioServe Corp., San Diego). The ethanol content was steadily increased from a caloric equivalent of 2–4% during the 1st week to 36% by the 4th week and then maintained at this level until 10 weeks. Control pair-fed animals received the same diet except that alcohol was isocalorically replaced by maltose dextrins. Feeding was carried out in the Animal Resource Facility of Thomas Jefferson University Medical College, Philadelphia, under their approved animal care protocol.

**Preparation of Mitochondrial Extracts—**Mitochondria from cultured cells and freshly extracted rat livers were prepared by differential centrifugation as described previously (32, 40). Cells were washed with cold PBS and homogenized with a Dounce glass homogenizer in H-medium (70 mM sucrose, 220 mM mannitol, 2.5 mM Hepes, pH 7.4, 2 mM EDTA, and Complete Protease Inhibitor Mixture). Livers were perfused with phosphate-buffered saline, and rinsed with the same buffer before use. Livers were sliced and homogenized in a Glass Col motor-driven glass-Teflon homogenizer as described before (32, 40). Mitochondria and microsomes were prepared by differential centrifugation and suspended in 20 mM K2HPO4 buffer containing 20% glycerol with added leupeptin, pepstatin, antipain, and PMSF. Protein concentration of cell fractions were determined by the method of Lowry et al. (42), and aliquots were stored at −80 °C until use.

**Analysis of Cellular O2 Consumption in a Seahorse XF24 Respirometer—**Oxygen consumption rates (OCR) were measured using the XF24 high sensitivity respirometer (Seahorse Bioscience) as described by Wu et al. (43) following the manufacturer’s instructions. Briefly, 20,000 cells were cultured in DMEM for 16 h and changed with XF assay medium, low buffered bicarbonate-free DMEM, pH 7.4, for 1 h before the measurement. The final concentrations of inhibitors used were 2 µg/ml oligomycin, 40 µM of the uncoupler 2,4-dinitrophenol (DNP) for ER − cells and 50 µM for WT and Mt + + cells, and 1 µM of the complex I inhibitor, rotenone. The plate along with the cartridge was loaded into the XF analyzer. OCR was measured under basal conditions and after sequential addition of oligomycin, DNP, and rotenone. All respiration rates were calculated as percentage of the rate. The difference in the cell density between wells is corrected by loading the cell lysate on 12% SDS-PAGE and probing with actin antibody by immunoblot analysis. The absolute rates of oxygen consumption were
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linearly related to cell numbers seeded within the measurement range. Respiration rates at each time point from three replicate wells were averaged.

Assay of Electron Transport Chain Activity—Assays of complex I–III were essentially as described by Birch-Machin and Turnbull (44) using a Cary 1E UV-visible spectrophotometer. Briefly, complex I activity (NADH:ubiquinone oxidoreductase) was measured by incubating 15 μg of freeze-thawed mitochondrial extract from control and alcohol-treated cells and tissues in 1 ml of assay medium (25 mM potassium phosphate, pH 7.4, 5 mM MgCl₂, 2 mM NaCN, 2.5 mg/ml bovine serum albumin, 13 mM NADH, 65 μM ubiquinone, and 2 μg/ml antimycin A) and measuring the decrease in absorbance at 340 nm because of NADH oxidation. Rotenone sensitive complex I activity was assayed by the addition of 40 μmol/liter rotenone. Complex II–III activity (succinate-cytochrome c reductase) was measured by incubating 20 μg of freeze-thawed mitochondrial extract in 1 ml of assay medium (25 mM potassium phosphate, pH 7.4, 2 mM NaCN, 20 mM succinate, 2 μg/ml rotenone, and 37.5 μM oxidized cytochrome c) and measuring the increase in absorbance at 550 nm because of cytochrome c reduction.

CcO activity was measured by incubating 2–10 μg of freeze-thawed mitochondrial extract prepared from control and alcohol-treated (100 mM for 48 h) cells, and tissues in 1 ml of assay medium (25 mM potassium phosphate, pH 7.4, and 0.45 mM dodecyl maltoside). Ferrocytochrome c (15 μM) was added, and the reaction rates were measured using Cary-1E spectrophotometer. First order rate constants were calculated based on regression analysis using the Cary-Win kinetics software. The ε of 21.1 was used for the conversion of OD to molar amounts of reduced cytochrome c oxidized. In some assays, cells were also treated with Mito-Q (1 mM), Mito-CP (2 μM), DAS (10 μM), or NAC (10 mM) as indicated.

Measurement of Heme aa₃ Content—Freeze-thawed mitochondrial extract (900 μg) form control and alcohol-treated rats were incubated on ice for 30 min in 2 ml of 25 mM phosphate buffer, pH 7.4, containing 2% dodecyl maltoside. Ferrocytochrome c (15 μM) was added, and the reaction rates were measured using Cary-1E spectrophotometer. First order rate constants were calculated based on regression analysis using the Cary-Win kinetics software. The ε of 21.1 was used for the conversion of OD to molar amounts of reduced cytochrome c oxidized. In some assays, cells were also treated with Mito-Q (1 mM), Mito-CP (2 μM), DAS (10 μM), or NAC (10 mM) as indicated.

Measurement of Extracellular H₂O₂ by Amplex Red—H₂O₂ in cells grown in 96-well plates (hereafter referred to as ROS) was measured using the Amplex Red hydrogen peroxide/peroxidase assay kit from Invitrogen. The method involves the horseradish peroxidase (HRP)-catalyzed oxidation of the colorless and nonfluorescent molecule, N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) to resorufin. The fluorescence was recorded at an excitation of 530 nm and emission at 590 nm in a MicroWin chameleon multilabel detection platform. 15,000 cells were plated in 96-well black bottom plate in phenol-free medium overnight. Alcohol treatment (300 mM) was carried out for 96 h as stated above. Mito-CP at a concentration of 2 μM was also added to the cells for a period of 12 h before measurement.

Immunoblot Analysis and Source of Antibodies—Antibody-specific ATPase subunit β (subunit of complex V), CcO IV, and CcO Vb (Mitosciences, Eugene, OR), and the 70-kDa subunit of complex II (Molecular Probes, Carlsbad, CA) were diluted 1:5000. Mouse monoclonal antibody to porin was from Abcam, Cambridge, MA. Polyclonal antibody to DNP moeity was from Millipore, Billerica, MA. Monoclonal antibody specific for CcO II was from Santa Cruz Biotechnology, Santa Cruz, CA. CYP2E1 antibody was from Oxford Biomedicals (Rochester Hills, MI). Blots were probed and developed using the SuperSignal West Femto System (Pierce) and imaged on a Bio-Rad VersaDoc Imaging System and Odyssey Licor (Rochester Hills, MI). Digital image analysis was performed using Quantity One Version 4.5 software from Bio-Rad.

Quantification of Mitochondrial DNA and mRNAs—Cells were washed extensively with 1× phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4), scraped from the culture dish, and collected by centrifugation at 2000 rpm for 10 min. Total cellular DNA was isolated using Qiagen DNA extraction kit. Total RNA was isolated using TRIzol reagent (Invitrogen) and stored at −80 °C. Total DNA
and RNA were isolated from freshly harvested rat livers essentially by the same procedure. For real time PCR analysis, RNA was digested with turbo DNase I (Ambion, Inc.), and cDNA was synthesized using the High Capacity cDNA Archive kit (Applied Biosystems). Relative mRNA levels of subunits CcO, Apase6, and CcoIV were determined by standard SYBR Green real time PCRs on an ABI 7300 real time PCR machine. The levels of the various CcO transcripts were normalized to β-actin/Gapdh, the housekeeping gene, and expressed as % change in relation to β-actin mRNA.

**Extent of Protein Carbonylation of Electron Transfer Chain Complexes**—Mitochondrial membrane complexes solubilized in 0.2% lauryl maltoside containing buffer were resolved by Blue Native polyacrylamide gels as described above. Proteins were electrophoretically transferred to PVDF membrane, and the membrane strips were subjected to in-strip derivatization as described by Conrad et al. (47). The membranes were incubated in 10 mM 2,4-dinitrophenylhydrazine in 2 n HCl for 10 min at room temperature (48). The strips were washed twice with 2 mM Tris·HCl containing 30% glycerol for 15 min and equilibrated with PBS containing 1% Tween 20 and 5% w/v skim milk powder. The membranes were probed with polyclonal antibody to DNP moiety (1:150 dilution) and infrared dye-conjugated goat anti-rabbit IgG as described for immunoblot analysis.

**Cell Viability Assay**—The viability of cells treated with ethanol was determined using the Guava ViaCount assay. This assay distinguishes between viable and dead cells based on the differential permeability of DNA-binding dyes in the ViaCount reagent (Guava Technologies, Hayward, CA). The assay was carried out using the manufacturer’s suggested protocol. Cells treated with and without ethanol were trypsinized post-treatment and stained by mixing with ViaCount reagent at a 1:20 dilution, and the viable cell count was determined using Guava Personal Cytometer and CytoSoft 6.2 software.

**Statistical Analysis**—Statistical significance was determined by an unpaired two-tailed Student’s t test and paired wherever needed. Results for the cultured stable cells and isolated liver samples are presented as means ± S.D. of at least three data points from three different experiments. p values ≤0.05 were considered statistically significant, and p values ≤0.001 were considered highly significant.

**RESULTS**

**Effects of Alcohol on Respiratory Capacity of CYP2E1-expressing Cells**—In a recent study (40), we showed that mitochondrion-targeted CYP2E1 had a marked effect on alcohol-mediated toxicity. To evaluate the basis for CYP2E1-induced oxidative stress and cellular toxicity, we measured respiratory parameters in cells expressing WT, ER+ , and Mt++ CYP2E1 using Seahorse Extracellular flux analyzer. The construction of COS cell lines stably expressing WT, ER+ (predominantly ER-targeted), and Mt++ (predominantly mitochondrion-targeted) CYP2E1 was reported previously (40). The OCR in this experiment signifies ATP-coupled respiration, which was severely affected after alcohol treatment in all cells. Although not shown, DNP caused maximum uncoupled respiration, which was also affected by ethanol treatment. In all cases, the respiration was completely inhibited by rotenone, which inhibits complex I and prevents the entry of electrons from NADH into the complex (results not shown). The quantitation of respiration rate (%) presented in Fig. 1A shows that OCR was inhibited markedly in WT, ER+, and Mt++ cells by 100 mM alcohol, although the most severe inhibition was observed in Mt++ and ER+ cells. Fig. 1B shows the effects of 25 mM alcohol on the OCR. It is seen that 25 mM ethanol had a marginal effect on OCR in all cells. It is noteworthy that in Fig. 1, A and B, the basal OCR levels in Mt++ cells were significantly lower than in WT and ER+ cells. The results are consistent with our previous results showing induction of respiratory deficiency in yeast cells by mitochondrion-targeted CYP2E1 (40). Results also show that alcohol further augments respiratory deficiency in these cells.

**Effects of Alcohol on the Electron Transport Chain Complexes in CYP2E1-expressing Cells and Livers from Alcohol-treated Rats**—A number of studies have shown the effects of alcohol treatment on the activities of mitochondrial electron transport chain complexes. Here, we investigated the effects of overexpression of mitochondrion- and ER-targeted CYP2E1 on complex I activity (NADH:ubiquinone oxidoreductase), which is a major contributor of ROS, and complex IV activity (cyto-
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Cytochrome c oxidase (CcO), which is the terminal oxidase of the mitochondrial electron transport chain. Results (not presented) show that complex I activity was marginally inhibited in all three cell types expressing different forms of CYP2E1. Alcohol treatment had no significant inhibitory effect on cells expressing WT and ER⁺ CYP2E1. However, there was a significant (~20%) inhibition of complex I activity in Mt++ cells in response to added ethanol. Similar effect was also observed in the alcohol-treated rat livers at 6, 8, and 10 weeks of treatment (results not shown). There was no significant effect at earlier treatment regimens of 2 and 4 weeks. These results suggest a time delayed effect of ethanol treatment on complex I activity. A previous study showed a more pronounced inhibition of complex I activity in ethanol-fed rat livers (9). The difference in activities reported before and the present results likely reflects the methods used for assaying the complex I activity.

Fig. 2A shows the CcO activity in CYP2E1-expressing cells (WT, ER⁺, Mt⁺, and Mt++). It is seen that CcO activity was inhibited in all four cell types compared with control cells, although the inhibition was more severe in Mt++ and Mt+++ cells, which express progressively higher levels of mitochondrial-targeted CYP2E1. Alcohol treatment further exacerbated the inhibitory effect with the highest level of inhibition (60–80%) observed in Mt++ cells. To study the possible role of CYP2E1 activity and mitochondrial ROS production in alcohol-mediated inhibition of CcO activity, we tested the effects of CYP2E1 inhibitor, DAS, and general as well as mitochondrion-targeted antioxidants, NAC, Mito-CP, and Mito-Q (49, 50), in attenuating the loss of CcO activity. Our results show that NAC prevented alcohol-induced inhibition of CcO activity. Furthermore, Mito-Q and Mito-CP, the two mitochondrion-targeted antioxidants also prevented alcohol-induced loss of CcO activity (Fig. 2B) suggesting that mitochondrially produced ROS may play a direct role in altering the structure/function of CcO enzyme. Interestingly, DAS, an inhibitor of CYP2E1, also rendered protection against alcohol-mediated loss of CcO activity suggesting the importance of the metabolic activity of CYP2E1 in inducing CcO dysfunction. These results are also consistent with the data showing the protective effects of DAS against alcohol-mediated liver injury and depletion of mitochondrial GSH pool when administered to rats and mice (51, 52). We also observed steady decreases in CcO activity in the livers of rats fed an alcohol-containing diet for 2–10 weeks. The decline in CcO activity correlated with increasing hepatic CYP2E1 content, and the most significant and linear decline in CcO activity occurred in rats treated with ethanol for 6, 8, and 10 weeks (Fig. 2C).

**Effects of Alcohol on the Steady State Levels of CcO Subunits**—To understand the molecular basis of decrease in CcO activity in response to alcohol treatment, we carried out immunoblot analysis of mitochondrial membrane fractions from treated and untreated cells and also liver mitochondria from alcohol-treated rats. As shown in Fig. 3A, levels of nucleolarly coded CcO IVl1 declined marginally in all CYP2E1-expressing cell types treated with ethanol. The cells transfected with an empty vector (mock-transfected) showed no change in subunit content after alcohol treatment. The decline was relatively more apparent in ER⁺ and Mt++ cells treated with alcohol compared with WT cells. The steady state levels of subunit Vb (Fig. 3B) declined more markedly in Mt++ cells, and moderately in ER⁺ cells following alcohol treatment. Results also show that pretreatment with DAS or Mito-Q prevented the alcohol-mediated decline of subunit VI11 and Vb levels in both WT and Mt++ cells (Fig. 3C). Although not shown, similar effects were observed in Mt+ cells. These results suggest that the alcohol-mediated decline in CcO activity in CYP2E1-expressing cells is associated with selective loss or degradation of some of the subunits of the complex and that inhibition of CYP2E1 activity or treatment with mitochondrion-targeted antioxidants effectively alleviated this loss. Immunoblot in Fig. 3D shows a similar loss of subunits IVl1 and Vb in liver mitochondria of rats treated with alcohol for 6, 8, and 10 weeks.
The loss of these subunits was minimal in rats from the 2- and 4-week treatment groups. These results suggest that the loss of specific CcO subunits also occurs under in vivo conditions. The multiple closely migrating bands IVI1 and Vb likely reflect multiple phosphorylated species.

Consistent with the reduced CcO activity and loss of selective subunits in livers of 6-week alcohol-treated rats, spectral analysis of cholate-solubilized mitochondrial membrane fraction shows a significant reduction in peak at 605 nm characteristic of heme a/a3, compared with pair-fed controls. The spectra in Fig. 4 also show that the peak at 548 nm remains unchanged in both alcohol-fed and control liver mitochondria suggesting no change in complex III level. Although not presented, we did not observe a change in complex III activity in CYP2E1-expressing cells as well as alcohol-treated livers.

**FIGURE 3. Effects of alcohol treatment on CcO IVI1 and Vb subunit levels.** A and B, immunoblot analysis of mitochondrial proteins from Mock, ER⁺, WT, Mt⁺, and Mt⁺⁺ cell lines treated with and without ethanol (100 mM, 48 h) and rat liver mitochondria from control and alcohol pair-fed rat livers. Proteins (50 μg each) were resolved by SDS-PAGE on a 12% gel and subjected to immunoblot analysis with anti-CcO IVI1 and anti-CcOVb antibodies. The blots were also probed with an antibody to the mitochondrion-specific marker succinate dehydrogenase (SDH) or porin as loading controls. C, effects of antioxidant Mito-Q (2 μM) and CYP2E1 inhibitor DAS (10 μM) on the steady state levels of proteins. D, rat liver mitochondria from control and pair-fed alcohol (50 μg each) were loaded. The blots were imaged through a Li-Cor Odyssey Infrared Imaging System, and the band densities were quantified using the Volume analysis software.

**Effects of Alcohol Treatment on CcO Complex and Respirosome Complexes**—To further assess the nature of alcohol-induced changes in CcO complexes in WT and Mt⁺⁺ cells, we carried out BNG electrophoresis of detergent-solubilized mitochondrial membrane complexes (Fig. 5A) and probed the complexes with antibodies to ATPase β subunit (complex V) and subunit IVI1 of CcO (complex IV). Results of immunoblot (Fig. 5A, upper panel) and quantification of the band density (adjacent panel) show no decline in complex V levels but show a marked decline in complex IV following ethanol treatment. Complex IV levels were lower in Mt⁺⁺ cells compared with WT CYP2E1-expressing cells, and a further decline was observed in both cell types following ethanol treatment. Although not shown, there was no significant change in complex I–III levels. These results along with the results of Figs. 2 and 4 suggest that the alcohol toxicity selectively targets com-
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that the oxidative damage to complexes I and V were nearly similar in control and alcohol-fed livers. The CCO complex, from alcohol-treated livers, however, showed about 1.5-fold higher DNP antibody cross-reactivity suggesting a higher level of damage. Fig. 6B shows the patterns of oxidative damage in WT, ER\(^+\), and Mt\(^{++}\) cells with and without alcohol treatment. It is seen that none of the complexes were stained significantly in WT and ER\(^+\) cells even after ethanol treatment suggesting low or minimal oxidative damage to constituent protein subunits. In Mt\(^{++}\) cells, however, the CCO complex was prominently stained with DNP antibody suggesting a high level of oxidative damage. Notably, the CCO complex from ethanol-treated cells was more intensely stained compared with that from untreated cells. It is also seen that other complexes from Mt\(^{++}\) cells were not stained significantly suggesting a more preferential damage to the CCO complex. A companion blot stained with ATPase \(\beta\) subunit antibody as a loading control shows nearly same level of complex V. These results suggest that the loss of CCO subunits in response to alcohol treatment may be related to selective and direct oxidative damage to the complex.

Effects of Mitochondrion-targeted Antioxidants on mtDNA and Mitochondrial Steady State Transcript Levels—The levels of mtDNA were measured by PCR amplification of total DNA isolates using CcoI and Atpase6 genes as mitochondrial markers and CcoIV/I as the nuclear genomic marker. It is seen from Fig. 7A that the levels of mtDNA in 8 weeks of alcohol-fed rat livers is reduced by 30–40%, whereas there was no reduction in the nuclear CcoIV/I DNA level. Fig. 7, B and C, show the mtDNA contents assayed by PCR amplification of Atpase6 and CcoI genes in cells expressing WT, ER\(^+\), and Mt\(^{++}\) CYP2E1. It is seen that the mtDNA levels in WT and Mt\(^{++}\) cells were nearly similar, although there was a marginal increase in ER\(^+\) cells. The reason for this increase in mtDNA remains unclear. The mtDNA contents were significantly lower in all cells following alcohol treatment, although the loss was more severe in Mt\(^{++}\) cells. As expected, the level of nuclear CcoIV/I DNA did not change in response to alcohol treatment (Fig. 7D). Fig. 7, E and F, show that the steady state levels of CcoI and Atpase6 mRNAs were markedly lower in Mt\(^{++}\) cells compared with WT and ER\(^+\) cells. This is consistent with higher oxidative stress in these cells. Alcohol treatment further exacerbated this decline more markedly in Mt\(^{++}\) cells, although two other cell types also showed lower transcript levels. Remarkably, DAS, an inhibitor of CYP2E1, and also Mito-CP rendered protection against loss of mtDNA-encoded transcripts. The level of restoration in Mt\(^{++}\) cells was well above the untreated cell level suggesting a higher threshold of oxidative stress in these cells. Although not shown, the levels of CcoI and Atpase6 proteins were also restored following treatment with Mito-CP and DAS.

Alcohol-mediated Toxicity in Hep G2 Cells Expressing Mitochondrial CYP2E1—The physiological significance of these findings were further assessed in a panel of Hep G2 cells expressing WT and Mt\(^{++}\) CYP2E1 cDNAs. The rationale for the choice of Hep G2 cells was based on tissue origin and also known resistance of these cells to alcohol-induced toxicity. Fig. 8A shows the mitochondrial and microsomal levels of CYP2E1 from mock-transfected, WT, and Mt\(^{++}\) CYP2E1-expressing...
Hep G2 cells. Similar to what was shown in COS cells, the mitochondrial CYP2E1 content of WT cDNA-transduced cells was about 38%, whereas that of Mt+/H11001/H11001 cells was about 80%. Also, as shown previously (40), the mitochondrion-targeted CYP2E1 migrated as an 40-kDa protein possibly because of activation of a cryptic proteolytic site in this mutant protein. As shown previously (40), this N-terminal truncated protein exhibits catalytic activity and heme content similar to full-length CYP2E1 protein.

Fig. 8B shows that the CcO activities of WT and Mt++ cells were marginally reduced in comparison with the mock-transfected cells. Alcohol treatment significantly inhibited CcO activity in both mock-transfected and WT CYP2E1-expressing cells. The inhibition was more pronounced in Mt++ cells in response to ethanol treatment. As shown in Fig. 8C, the level of CcO subunit IV11 was significantly reduced in both WT and Mt++ CYP2E1-expressing cells following ethanol treatment. As observed in COS cells, the loss of subunit Vb in Mt++ cells treated with ethanol was drastic compared with the mock and WT CYP2E1-expressing cells. It should be noted that the levels of porin and ATPase β subunits used as loading controls did not significantly vary suggesting that reduced CcO subunit IV11 and Vb is not due to an aberrant derivative process due to cell death. Consistent with this conclusion, the % cell viability of ethanol-treated cells (Fig. 8D) did not vary markedly in response to ethanol treatment.

Fig. 8E shows the level of H2O2 production measured by Amplex Red method in the three cell lines. Both WT and Mt++ CYP2E1-expressing cells generated a substantially higher level of ROS. Ethanol treatment increased the level, with Mt++ cells producing the highest level. Mito-CP treatment nearly completely abolished ROS production in all three cells. These results further support the role of mitochondrion-targeted CYP2E1 in augmenting alcohol-mediated toxicity and loss of CcO activity.

DISCUSSION

Despite some early contradictory reports (53), there is compelling evidence that CYP2E1 plays an important role in alco-
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![Diagram](https://via.placeholder.com/150)

**FIGURE 6. Extent of protein carbonylation of electron transfer complexes from ethanol-fed rats and cells expressing CYP2E1.** Mitochondrial proteins (150 μg each) solubilized in sodium maltoside containing buffer were subjected to BNG analysis as described in Fig. 5 and under “Experimental Procedures.” Proteins were transblotted to PVDF membrane and subjected to derivatization with 2,4-dinitrophenylhydrazine for the detection of side chain carbonylation by probing with antibody to DNP. A, levels of protein carbonylation of different complexes from control and alcohol-fed rat livers. Strips corresponding to indicated complexes were excised and probed with antibody to DNP domain. B, complexes from WT, ER⁺, and Mt⁺⁺ CYP2E1-expressing cells were resolved by BNG electrophoresis and probed with DNP antibody following derivatization (upper panel). The strip corresponding to complex V from an identically run companion blot and probed with ATPase β antibody without derivatization was used as loading control (lower panel).

Alcohol-induced toxicity and alcohol liver diseases (54–58). Morgan et al. (59) reported that in transgenic mice overexpressing CYP2E1, ethanol administration induced markers of liver injury. Other studies using various transgenic mouse models (60–65) also showed that CYP2E1 expression is a contributing factor in alcohol-mediated liver toxicity. For evaluating the roles of mitochondrial- and ER-targeted CYP2E1, recently we generated cells stably expressing mutant forms of proteins with altered targeting signal properties. CYP2E1 carrying the A2L/H11001/H11001 mutation was predominantly targeted to the ER, whereas CYP2E1 with the I8R/L11R/L17R mutation was predominantly targeted to mitochondria (40). Using these cell lines, we showed that Mt⁺⁺ cells expressing predominantly mitochondrion-targeted CYP2E1 were more vulnerable to alcohol-induced toxicity as observed by their ability to produce markedly higher ROS, increased lipid peroxides (F₂-isoprostanes), and induced mtDNA depletion in yeast cells. We also showed that the mitochondrion-targeted antioxidant MitoQ and CYP2E1 inhibitor DAS effectively reversed the alcohol-mediated toxicity in these cells. A parallel study by Knockaert et al. (66) using a different targeting strategy essentially came to the same conclusion. Using the targeting strategy that was used in our previous study (40) and using two different cell types for expression, we now show that mitochondrial CcO is likely to be the direct and immediate target of alcohol-induced toxicity in Mt⁺⁺ cells and also rat livers treated with alcohol for 6–10 weeks. Our results show selective depletion of CcO IVI and CcO Vb subunits, which caused disruption of CcO complex and respirosome super complexes. The loss of these subunits is most likely because of increased protein degradation because the CcO IVI and Vb mRNA levels remain the same in alcohol-treated rat livers and CYP2E1-expressing cells (Fig. 7A and data not shown).

The use of Seahorse micro-respiratory system in this study showed that OCR was markedly reduced in Mt⁺⁺ expressing COS cells as compared with ER⁺ and WT cells. Furthermore, alcohol treatment markedly reduced OCR in Mt⁺⁺ cells suggesting that mitochondrion-targeted CYP2E1 plays a role in alcohol-mediated mitochondrial dysfunction. In keeping with these results, both COS and Hep G2 cells expressing mitochondrion-targeted CYP2E1 showed significant loss of CcO activity, which was further reduced following alcohol treatment. These results further confirm and extend our previous studies that mitochondrion-targeted CYP2E1 augments alcohol-induced toxicity and mitochondrial dysfunction. One important observation in this study was the identification of CcO as a key mitochondrial target, which is probably the major cause of mitochondrial dysfunction.

Protein carbonylation is an important biomarker for oxidative stress-induced protein damage in various degenerative diseases. Carbonyl formation can occur by direct oxidation of amino acid side chains by ROS or they can be introduced indirectly through protein glycation and lipid peroxidation. We therefore assessed the relative levels of protein carbonylation by derivatization with DNP. Interestingly, both in alcohol-treated rat liver and Mt⁺⁺ cells treated with alcohol, we observed higher levels of protein carbonylation of the CcO complex. Notably, none of the other four complexes were carbonylated to any detectable level in Mt⁺⁺ cells even after alcohol treatment (Fig. 6B). These results provide direct and compelling evidence in support of our observation that CcO is a preferential target of CYP2E1-mediated alcohol toxicity.

It is increasingly realized that a major part of alcohol liver toxicity is elicited through oxidative stress. Metabolism of alcohol is known to involve formation of ROS, reactive nitrogen species, acetaldehyde, and other reactive metabolites (67–70). In addition, it is suggested that CYP2E1, which is one of the two major enzymes involved in alcohol metabolism, by itself generates ROS by inadvertent spillage of electrons because of its more open folded pattern (71, 72). Our results on complete or near complete reversal of CcO activity and oxidative stress conditions by mitochondrion-targeted antioxidants, Mit-Q and Mit-CP, indeed confirm that mitochondrially generated ROS (including NO) is a critical factor in alcohol-mediated mitochondrial dysfunction. Interestingly, DAS, a specific inhibitor of CYP2E1, also effectively reversed the loss of CcO activity in Mt⁺⁺ cells demonstrating that metabolic activity of CYP2E1 is essential for eliciting alcohol toxicity in the cell system.

Loss of CcO activity and attendant effects on mitochondrial function have been observed in a large number of diseases, including neurodegenerative diseases such as Alzheimer, myocardial ischemia, liver ischemia, cardiomyopathy, renal diseases, and cancer (29, 73–79). Doxorubicin, an anticancer drug,
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is known to induce cardiomyopathy as a secondary effect, which in turn is associated with mitochondrial dysfunction. It was shown that doxorubicin-induced cardiomyopathy involves direct association of the drug with heme a/a3, affecting CcO activity (80). In hypoxia and myocardial ischemia conditions, mitochondrial dysfunction was shown to be associated with loss of CcO activity (28, 31, 81), which was associated with selective loss of mitochondrion-encoded subunit I that houses the heme a/a3 and copper centers, and nuclear gene-coded subunits IV11 and Vb (82–84). Protein phosphorylation was implicated in these losses (28, 31, 85–87). Interestingly, our results show the loss of the same two nucleus-encoded subunits in alcohol-treated Mt++ cells and also in livers of rats fed with alcohol for <6 weeks (Fig. 5, B and C). It is likely that protein phosphorylation or oxidative modification of subunits by mitochondrially generated ROS is the cause of subunit degradation. Results presented here show that the CcO complex is subject to a higher level of oxidative damage in response to alcohol treatment. The molecular mechanism of degradation of CcO subunits is currently under investigation.

It is now widely accepted that mitochondrial electron transport complexes, which couple respiration to ATP production, are organized in supramolecular complexes called respirosomes (45). This organization is thought to facilitate efficient transfer of electrons extracted from substrates, thus increasing the efficiency of transfer between complexes. The respirosome assembly is also thought to reduce leakage of electrons that can form reactive free radicals. Different combinations of complex
I, III, and IV are known to exist as super- and subcomplexes. Super complexes containing 1–4 copies of CcO have been described in bovine mitochondria. In this study, we show that cells expressing mitochondrion-targeted CYP2E1 (Mt\(^{++}\) cells) contained markedly lower levels of super complexes than WT cells. Alcohol treatment caused a further depletion of super complexes. Treatment of rats with alcohol for 6 weeks had a similar effect on the hepatic mitochondrial content of respiration complexes. Interestingly, in case of rat liver mitochondria, in addition to a general decrease in these complexes, there appears to be a differential effect on individual super complexes with S1 and S2, the two largest complexes more susceptible to alcohol-mediated damage. The alcohol-mediated damage to super complexes observed here may result in OXPHOS dysfunction, in addition to increased ROS formation.

In line with previous results, our results with CYP2E1-expressing cells also show loss of mtDNA and markedly reduced mitochondrial genome-encoded mRNA levels in alcohol-treated cells. The alcohol effect is more severe in Mt\(^{++}\) cells compared with ER\(^{++}\) cells indicating that the extent of mtDNA loss is directly proportional to mitochondrial ROS production and oxidative stress. These DNA-damaging effects are reversibly less effective by DAS and a lot more effective by mitochondrion-targeted anti-oxidants, Mito-CP and Mito-Q (49, 50). We also see a similar loss of mtDNA and transcript levels in mitochondria from livers of alcohol-fed rats. We postulate that CcO is the immediate early target of CYP2E1 and alcohol-mediated toxicity. Loss of CcO activity further aggravates the oxidative stress conditions by increased ROS production by electron transport complexes either because of reverse electron flow or electron spillage as observed in CcO subunit Vb-depleted cells (28). The oxidative damage to mtDNA and other mitochondrial functional centers is possibly secondary to the CcO dysfunction.

Another noteworthy point is that despite a 30% reduction in mtDNA and mRNA contents in alcohol-fed rat livers, the levels...
of complex I and complex III, as well their activities are affected only marginally. The CcO content and activity, however, is markedly reduced under these conditions. In the case of Mt + + cells, a marked reduction of mtDNA and mitochondrially encoded mRNA levels are observed after alcohol treatment. Nevertheless, the complex I and complex III activities are not proportionately reduced. Increased stabilities of these complexes or altered turnover rates may be responsible for this effect. The CcO activity and complex levels, however, are selectively reduced by more than 70% suggesting more selective effects on this complex. We propose that an extreme sensitivity of CcO to mitochondrial ROS may be responsible for this selective effect.

In summary, our results on respirosome structures in alcohol-treated cells and livers imply a marked deficiency in mitochondrial OXPHOS, much more severe than previously envisioned. Our results also show that mitochondrial CYP2E1 plays a critical role in eliciting alcohol-mediated mitochondrial dysfunction, possibly by altering the CcO function. Damage to mitochondrial DNA and transcription may be secondary effects. Nearly complete recovery of these mitochondrial electron transfer complex and DNA damage by mitochondrion-targeted antioxidants and CYP2E1 inhibitors suggest possible ways to treat alcohol toxicity and tissue damage.

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