Overexpression of CYP2E1 in Mitochondria Sensitizes HepG2 Cells to the Toxicity Caused by Depletion of Glutathione*

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Induction of CYP2E1 by ethanol is one mechanism by which ethanol causes oxidative stress and alcohol liver disease. Although CYP2E1 is predominantly found in the endoplasmic reticulum, it is also located in rat hepatic mitochondria. In the current study, chronic alcohol consumption induced rat hepatic mitochondrial CYP2E1. To study the role of mitochondrial targeted CYP2E1 in generating oxidative stress and causing damage to mitochondria, HepG2 lines overexpressing CYP2E1 in mitochondria (mE10 and mE27 cells) were established by transfecting a plasmid containing human CYP2E1 DNA lacking the hydrophobic endoplasmic reticulum targeting signal sequence into HepG2 cells followed by G418 selection. A 40-kDa catalytically active NH₂-terminally truncated form of CYP2E1 (mtCYP2E1) was detected in the mitochondrial compartment in these cells by Western blot analysis. Cell death caused by depletion of GSH by buthionine sulfoximine (BSO) was increased in mE10 and mE27 cells as compared with cells transfected with empty vector (pCI-neo). Antioxidants were able to abolish the loss of cell viability. Increased levels of reactive oxygen species and mitochondrial 3-nitrotyrosine and 4-hydroxynonenal protein adducts and decreased mitochondrial aconitase activity and mitochondrial membrane potential were observed in mE10 and mE27 cells treated with BSO. The mitochondrial membrane stabilizer, cyclosporin A, was also able to protect these cells from BSO toxicity. These results revealed that CYP2E1 in the mitochondrial compartment could induce oxidative stress in the mitochondria, damage mitochondria membrane potential, and cause a loss of cell viability. The accumulation of CYP2E1 in hepatic mitochondria induced by ethanol consumption might play an important role in alcohol liver disease.

Generation of reactive oxidative species (ROS) is one of the mechanisms by which ethanol is toxic to the liver and other tissues. Induction of CYP2E1 by ethanol appears to be one of the central pathways by which ethanol is believed to generate a state of oxidative stress. CYP2E1 is active in oxidizing ethanol to acetaldehyde and in oxidizing many agents to reactive metabolites that are hepatotoxic (1). CYP2E1 is also reactive in the production of O₂⁻ and H₂O₂ during microsomal mixed function oxidase activity (2, 3). Correlations between induction of CYP2E1, lipid peroxidation, and ethanol-induced liver injury have been found in the intragastric infusion model of ethanol-induced liver injury (4–10), and inhibitors of CYP2E1 partially prevent the injury (6, 10). The addition of ethanol, iron, or arachidonic acid to HepG2 cell lines that overexpress human CYP2E1 (E9 cells, E47 cells) decreased cell viability and caused apoptosis (11–13).

Although CYP2E1 is predominantly located in the membrane of the ER, it has also been demonstrated to be present in lysosomes (14), peroxisomes (15), Golgi apparatus (16), and on the outer surface of the plasma membrane (17, 18). A recent study indicated that CYP2E1 is also present in mitochondria of rat liver and that it can be induced by pyrazole feeding (19). Molecular basis studies demonstrated that an NH₂-terminally truncated form of CYP2E1 was specifically targeted to the mitochondria (20). It was demonstrated that this mitochondrial-localized form of CYP2E1 was an NH₂-terminally truncated form of CYP2E1 of ~40 kDa, which was shown to be soluble, catalytically active, and localized in the mitochondrial matrix (21). In addition, this truncated short form of CYP2E1 was present in low levels in vivo in mitochondria isolated from rat liver (20).

In the present study, we found that chronic alcohol consumption could induce rat hepatic mitochondrial CYP2E1. To study the role of mitochondrial targeted CYP2E1 in generating oxidative stress and damage to mitochondria, HepG2 cell lines overexpressing CYP2E1 in mitochondria were established, and the role of mitochondrial targeted CYP2E1 in oxidative stress and damage to mitochondria and cellular toxicity was evaluated.

MATERIALS AND METHODS

Reagents—Rhodamine 123 and fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG were purchased from Molecular Probes, Inc. (Eugene, OR). Polyclonal antibody raised in rabbit against human CYP2E1 was obtained from Dr. Jerome M. Lasker (Institute for Biomedical Research, Hackensack, NJ) as a kind gift. Rabbit anti-human Mn-SOD antibody was obtained from Calbiochem. BSO, GSH, Trolox, 2′,7′-dichlorofluorescein diacetate, horseradish peroxidase-conjugated goat anti-rabbit IgG, minimal essential medium, and fetal bovine serum were purchased from Sigma.

Treatment of Animals and Isolation of Subcellular Fractions—Adult male Sprague-Dawley rats (150–170 g) were housed in a facility approved by The American Association for Accreditation of Laboratory Animal Care and were fed with commercially available ethanol (35%) and control diets (Bio-Serv, Frenchtown, NJ) that were isocaloric and had the same composition with respect to fat (42% of calories) and protein (16% of calories) for 2 months. Livers were perfused with ice-cold saline. Mitochondria were isolated as described (22). Briefly, 1 gram of liver tissue was suspended in 1 ml of cold isolation buffer (0.32 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) and homogenized using a glass homogenizer. The homogenate was centrifuged at 1330 × g for 5
min at 4 °C. The supernatants were centrifuged at 21,200 × g for 10 min at 4 °C. The pellet was suspended in a 15% Percoll solution, and aliquots were layered onto discontinuous Percoll gradients (23% over 40%) and then centrifuged for 15 min at 30,700 × g at 4 °C. The dense band of material at the interface between the 23 and 40% Percoll layers was collected, diluted 1:4 in fresh isolation buffer, and then centrifuged for 10 min at 16,700 × g at 4 °C. The pellet was resuspended in 2 ml of isolation buffer and centrifuged again for 10 min at 6900 × g. For rat liver mitochondrial CYP2E1 Western blot analysis, the mitochondria were further purified following the method as described (19). Briefly, the resultant mitochondria were washed twice with the isolation buffer, resuspended at a concentration of 10 mg/ml, and incubated at 4 °C for 2 min with digitonin (Sigma) at a final concentration of 75 μg/ml of protein. Mitoplasts were then washed three times with the homogenization buffer and resuspended in 50 mM potassium phosphate containing 0.1 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. For mitochondria isolated from cells in culture, the mitochondrial pellet was suspended in 100 μl of PBS after the Percoll purification and used for Western blot analysis or aconitate activity assay. Microsomes were isolated from the post-mitochondrial supernatant by centrifugation at 100,000 × g at 4 °C. The pellet was resuspended with PBS and used for Western blot analysis.

Plasmid Construction—The cDNA of CYP2E1 lacking the coding sequence for NH2 terminus amino acids 2–34 was generated by PCR using primers (forward, 5'-GGAGCAGATTCGGGACCATGGGTGTCCTTCCCGCTTCCATC-3' ; reverse, 5'-ATTAACCCCTCACACATTAAGGGA-3') and plasmid containing full-length human CYP2E1 cDNA (pCI-CYP2E1) as template. The resulting cDNA was digested with EcoRI and ligated in the EcoRI site of the restricted pCI-neo plasmid vectors pCI-neo and pCI-neo containing the human CYP2E1 cDNA lacking the coding sequence for amino acids 2–34 (pCI-mtCYP2E1) were transfected into HepG2 cells using the Lipofectamine™ 2000 transfection reagent (Invitrogen). Forty-eight hours after transfection, cells were trypsinized and seeded at low cell density onto 10-cm culture dishes in 10% fetal bovine serum plus minimal essential medium containing 10% fetal bovine serum in glass coverslips and were fixed at room temperature for 10 min with freshly prepared 2.5% paraformaldehyde in PBS. Cells were washed three times with 0.3M glycine prepared in PBS and permeabilized for 5 min in 0.5% Triton X-100 at room temperature followed by three washes in PBS. Cells were incubated for 1 h with a 1:500 dilution of a rabbit anti-human CYP2E1 polyclonal antibody and a 1:100 dilution of sheep anti-human Mn-SOD polyclonal antibody followed by three washes in PBS and incubated with a 1:160 dilution of a goat anti-rabbit antibody labeled with fluorescein isothiocyanate and a 1:100 dilution of a donkey anti-sheep antibody conjugated with Alexa Fluor 594. After three washes in PBS, cells were incubated with 0.1% Triton X-100 for 5 min followed by an additional three washes in PBS. The coverslips were mounted on slides and examined by confocal microscopy.

GSH Measurement—The intracellular and mitochondrial GSH levels were evaluated as described in Ref. 23 with the fluorometric substrate o-phthalaldehyde (MP Biomedicals, LLC, Aurora, OH). Briefly, pCI-neo and mE27 cells were treated with or without 0.2 mM BSO. After 24 h of treatment, the total cellular extracts and mitochondrial compartments were prepared, and the protein concentrations were measured; 200 μl of sample containing 1 mg of protein was mixed with an equal volume of 10% (w/v) of trichloroacetic acid and were left at room temperature for 10 min followed by centrifugation at 10,000 rpm for 10 min. The supernatants were used for assaying GSH. The final assay mixture contained 0.1 ml of supernatant, 1.8 ml of phosphate-EDTA buffer, and 0.1 ml of the o-phthalaldehyde solution containing 0.1 mg of o-phthalaldehyde. The same volume of different concentrations of GSH solutions was used for preparing a standard curve. After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a cuvette. Fluorescence at 420 nm was determined with the activation at 350 nm. The GSH level was obtained by comparing with the GSH standards and represented as nmol/mg of protein. 

DCF Fluorescence as a Measure of Reactive Oxygen Production—After treatment of the cells with or without 0.2 mM BSO, an inhibitor of glutamate cysteine ligase that lowers cellular GSH levels, DCF-DA was added at a final concentration of 2 μM/ml, and plates were incubated for 30 min at 37 °C in the dark. Cells were washed twice with 1× PBS, trypsinized, and resuspended in 3 ml of 1× PBS, and fluorescence was immediately read in a PerkinElmer Life Sciences 650-105 fluorescence spectrometer at 503 nm for excitation and 529 nm for emission with a slit width of 5 nm for both excitation and emission monochromators.
Background readings from cells incubated without DCF-DA were subtracted. Results are expressed as arbitrary units of fluorescence per milligram of protein.

**MTT Assay**—Cytotoxicity of BSO to pCI-neo, mE10, and mE27 cells was determined by the MTT assay. Cells (1.5 × 10^5) per milliliter per well were plated onto a 24-well plate and incubated in 5% CO_2 at 37 °C. The MTT assay was performed using the Cell Titer 96 nonradioactive proliferation assay kit (Promega). Briefly, 15% volume of dye solution was added to each well after the appropriate incubation time. After 4 h of incubation at 37 °C, an equal volume of solubilization/stop solution was added to each well for an additional 1-h incubation. The absorbance of the reaction solution at 570 nm was recorded. The absorbance at 630 nm was used as a reference. The net A_570 - A_630 was taken as the index of cell viability. The net absorbance change taken from the wells of untreated cultured cells was used as the 100% viability value. The percentage of viability was calculated by the formula (A_570 - A_630)_sample / (A_570 - A_630)_control × 100.

**Aconitase Activity**—The mitochondrial aconitase activity was measured by using the Bioxytech Aconitase-340™ spectrophotometric assay kit (OxisResearch, Portland, OR) and following the instructions from the manufacturer. The aconitase activity is calculated according to the rate of the formation of NADPH, which is monitored by the increase in absorbance at 340 nm.

**Flow Cytometry Analysis of the Mitochondrial Membrane Potential**—Changes in the mitochondrial membrane potential were examined by monitoring the cells after staining with rhodamine 123 (24). Cells (5 × 10^5) were seeded onto 6-well plates. After treatment, the cells were then incubated with medium containing 5 μg/ml rhodamine 123 for 30 min. Cells were harvested by trypsinization and resuspended in 1 ml of minimal essential medium. The intensity of rhodamine 123 fluorescence was analyzed by flow cytometry.

**Statistics**—Results refer to mean ± standard deviation and are average values from 2–3 values/experiment; experiments were repeated at least twice. Groups were compared among themselves by using Student's t test for unpaired data. Differences at p < 0.05 were considered significant.

**RESULTS**

**Induction of Mitochondrial CYP2E1 by Ethanol in Rat Liver**—Ethanol has long been known to elevate levels of CYP2E1 in the ER (1). Initial experiments evaluated whether CYP2E1 is present in rat liver mitochondria and whether ethanol feeding elevates mitochondrial CYP2E1. Liver samples from rats fed with ethanol (Fig. 1, indicated by E) or isocaloric dextrose (indicated by C) were homogenized, and mitochondria and microsomes as well as total cell extracts were prepared. The liver mitochondrial preparations were routinely checked for purity and whether ethanol feeding elevates mitochondrial CYP2E1. These results validate the presence of CYP2E1 in the mitochondria (19–21) and indicate that mtCYP2E1 can be induced by ethanol.

**Overexpression of Mitochondrial CYP2E1 in HepG2 Cells**—Two cell lines overexpressing mitochondrial CYP2E1 (mE10 and mE27) and one cell line transfected with empty vector (pCI-neo) were selected and used in the following experiments. Fig. 2 shows Western blot analysis for CYP2E1 in TCE, post-mitochondrial supernatant from TCE centrifuged at 21,000 g for 10 min (post Mito sup), and mitochondria (Mito) compartments in these HepG2 cell lines as well as E47 cells, which express wild type CYP2E1 in the ER (25). In TCE, full-length CYP2E1 was present in the E47 cells with a molecular mass of 52 kDa; no amino-terminal truncated form of CYP2E1 was detected. However, a 40-kDa band was present in mE10 and mE27 cells upon interaction with the CYP2E1 antibody, and no full-length 52-kDa form was detected. The 40-kDa band was not present in the E47 cells or in the pCI-neo transfected HepG2 cells. However, all of the samples showed equal amounts of β-actin expression (Fig. 2, TCE). The 40-kDa bands in mE10 and mE27 cells were very faint in the post-mitochondrial supernatant. It is likely that these faint 40-kDa bands were from the residual contamination of mitochondria in the post-mitochondrial supernatant and not mtCYP2E1, which is not translocated into the mitochondria, because similar faint Mn-SOD (a mitochondrial enzyme) bands were also detected in the post-mitochondrial supernatant of all of these four cells (Fig. 2, post Mito sup). These results indicate that the 40-kDa mtCYP2E1 was located almost exclusively in the mitochondrial, but not in the cytosolic, compartment. We did not detect any truncated precursor, ∆(1–34)CYP2E1, in these stable transfected cell lines, although it could be detected in transient transfected cells (data not shown). This might be due to increasing the rate of translocation of the precursor to mitochondria or an increase in its rate of degradation in these stable transfection cell lines as compared with the transient transfection cells and requires further study. Furthermore, mtCYP2E1 was overexpressed in the mitochondria isolated from mE10 and mE27 cells, but not in mitochondria from pCI-neo cells, although the mitochondrial marker enzyme Mn-SOD was present in all mitochondrial preparations (Fig. 2, Mito). The

**FIGURE 1.** Induction of mitochondrial CYP2E1 by ethanol in rat liver. Liver samples from rats fed with ethanol (E) or isocaloric dextrose (C) were homogenized, and mitochondria (Mito), microsomes (Micro), as well as TCE were prepared as described under “Materials and Methods.” Proteins from each compartment were resolved by electrophoresis on a SDS-polyacrylamide gel and electrobolted onto nitrocellulose membranes. The membrane was blotted with anti-CYP2E1, GRP78, cytochrome c, and Mn-SOD antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The figure is one representative experiment out of three.

**Cyto. C** and Mn-SOD. The results showed that GRP78 protein was detected in microsomes and TCE fractions, but there was no detectable GRP78 in the mitochondrial fraction, although cytochrome c and Mn-SOD were strongly detected in the mitochondrial fraction (Fig. 1). These results validate the presence of CYP2E1 in the mitochondria (19–21) and indicate that mtCYP2E1 can be induced by ethanol.
CYP2E1 protein in the mitochondrial compartment of mE10 or mE27 cells was about 50% of the CYP2E1 levels in the liver mitochondria of the control rats and 20% of that in the liver mitochondria of rats fed with ethanol (densitometric analyses of Western blots, data not shown).

CYP2E1 in isolated mitochondria from the mE27 cells was assayed for catalytic activity (Fig. 3). In the presence of Adx and AdR, disrupted mitochondria from mE27 cells had significant chlorzoxazone hydroxylation activity with greater activity than that of mitochondria from cells transfected with empty plasmid (pCI-neo). When incubated in the absence of Adx and AdR, no significant increase in catalytic activity was observed, indicating that the truncated mtCYP2E1 requires Adx and AdR as electron donors as expected for a mitochondrial localized P450.

Immunofluorescence was carried out in permeabilized pCI-neo, E47, mE10, and mE27 cells incubated with rabbit anti-human CYP2E1 IgG and sheep anti-human Mn-SOD IgG followed by the addition of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and Alexa Fluor-conjugated donkey anti-sheep IgG. Slides were examined by confocal microscopy (Fig. 4). There is no CYP2E1-related green color in the image of pCI-neo cells as only Mn-SOD-related red color is observed. Although both CYP2E1 and Mn-SOD are present in E47 cells, there is no overlay between the CYP2E1 (green) and Mn-SOD (red), indicating the separate location of these enzymes, i.e. CYP2E1 present in the cytosol (endoplasmic reticulum) and Mn-SOD present in the mitochondria. However, CYP2E1 and Mn-SOD completely overlay each other in the mE10 and mE27 cells, indicating that the two enzymes co-localize in the mitochondrial compartment (Fig. 4). There was virtually no mtCYP2E1 (green) present that did not overlay with the Mn-SOD, indicating the absence of mtCYP2E1 from the cytosol.

Overexpressing Mitochondrial CYP2E1 Sensitizes HepG2 Cells to BSO-induced Toxicity—We have previously shown that depletion of GSH with BSO causes a greater loss of viability in E47 cells as compared with non-CYP2E1 expressing control HepG2 C34 cells (26). To determine whether overexpression of mitochondrial CYP2E1 increases the sensitivity of HepG2 cells to GSH depletion, pCI-neo, E47, mE10, and mE27 cells were treated with 0.2 mM BSO for 48 h, and cell viability was determined. Almost 80% of the mE10 and mE27 cells lost viability, whereas 40% of the pCI-neo cell survived at this time point (Fig. 5). To demonstrate that BSO-induced cytotoxicity is related to oxidant stress, the antioxidants, 2 mM GSH ethyl ester or 50 μM Trolox, were added into the culture medium at the same time with BSO, and cell viability was determined after incubation for 48 h. In mE10 and mE27 cells, toxicity induced by BSO can be prevented by administration of either GSH ester or Trolox, suggesting that enhanced oxidant stress plays a role in the toxicity. The morphology of pCI-neo, mE10, and mE27 cells without or with treatment with BSO alone or BSO plus GSH ethyl ester or Trolox for 48 h was recorded by visualizing cells under a light microscope. Most mE10 and mE27 cells lost normal morphology when treated with BSO, whereas pCI-neo cells treated with BSO as well as mE10 and mE27 cells treated with BSO plus GSH ethyl ester or Trolox retained their shape and structure (Fig. 6).
Treatment with BSO Equally Decreases Intracellular and Mitochondrial GSH Levels in mE27 Cells Overexpressing Mitochondrial CYP2E1 and Control pCI-neo Cells—To evaluate the effects of BSO on the depletion of intracellular and mitochondrial GSH, pCI-neo and mE27 cells were treated with or without 0.2 mM BSO for 24 h. GSH levels in total cell extracts or mitochondrial compartments were evaluated. Fig. 7 shows that both intracellular and mitochondrial GSH levels were significantly decreased (from 25 to 30 nmol/mg of protein to 10 nmol/mg of protein). No difference was found in both intracellular and mitochondrial GSH levels between pCI neo cells and mE27 cells. Thus, the greater sensitivity of mE27 cells to BSO-induced toxicity is not a result of differences in initial cellular or mitochondrial GSH levels or in the decline of cellular or mitochondrial GSH as a consequence of the BSO treatment.

Cells Overexpressing Mitochondrial CYP2E1 Produce ROS and Mitochondrial Protein Adducts—Intracellular ROS levels in pCI-neo, E47, mE10, and mE27 cells were determined by fluorescence spectrophotometry using the oxidant-sensitive dye 2′,7′-DCF-DA. ROS was elevated to a greater extent in mE10 and mE27 cells and E47 cells as compared with pCI-neo cells after treatment with 0.2 mM BSO for 24 h followed by incubation with 5 μM 2′,7′-DCF-DA for 30 min (Fig. 8). The comparable increases in DCF fluorescence found with E47 cells as compared with mE10 and mE27 cells suggests that the mtCYP2E1 is as effective as the microsomal CYP2E1 in generating ROS. To determine whether the mitochondria were subjected to oxidant stress, levels of protein nitrotyrosine adducts and HNE lipid peroxidation adducts were detected. pCI-neo, mE10, and mE27 cells were treated with or without 0.2 mM BSO for 24 h followed by incubation with 5 μM 2′,7′-DCF-DA for 30 min. ROS were measured as described under “Materials and Methods.” The increased 3-NT and 4-HNE protein adducts in mE10 and mE27 cells are pointed to by arrows. The figures are one representative experiment out of two.
Mitochondrial CYP2E1 and Cell Toxicity

CYP2E1 plays an important role in alcohol liver disease and in xenobiotic toxicity via producing ROS. CYP2E1 is mainly present in the endoplasmic reticulum membrane; however, it was also detected in mitochondria (19–21, 32–34). Robin et al. (19) showed that the mitochondrial CYP2E1 in rat liver was present at about 30% of the level of the microsomal CYP2E1 under basal conditions and at 40% of the level of the microsomal CYP2E1 after pyrazole treatment. In a similar manner, streptozotocin-induced diabetes elevated microsomal CYP2E1 2–3-fold and elevated mitochondrial CYP2E1 5–6-fold (34); mitochondrial CYP2E1 protein and catalytic activity was 25–35% that of microsomal CYP2E1 after treatment with streptozotocin. These experiments were carried out with highly purified mitochondria and indicate the presence of relatively high amounts of CYP2E1 in the mitochondria; moreover, this CYP2E1 is “inducible” by pyrazole and streptozotocin. In the current study, CYP2E1 was detected in mitochondria of rat liver, and it was induced 3-fold by chronic consumption of ethanol. It is important to validate that the presence of CYP2E1 in the mitochondria represents a real increase in mitochondrial CYP2E1 rather than an apparent increase due to contaminating microsomes. Fig. 1 shows that the mitochondria isolated from the chronic ethanol-fed rats and the pair-fed controls express CYP2E1, express mitochondrial markers such as cytochrome c and Mn-SOD, but do not express the microsomal marker GRP78. They also had very little activity of the microsomal enzyme NADPH-cytochrome P450 reductase. Conversely, microsomes isolated from these rats contained CYP2E1, expressed the microsomal marker GRP78, but had faint expression of cytochrome c and Mn-SOD (most likely due to mitochondrial contamination of the microsomes). Thus, the CYP2E1 in these mitochondria clearly represented mitochondrial CYP2E1 and not CYP2E1 derived from contaminating microsomes. Immuno-electron microscopy also showed that CYP2E1 was present in the matrix space of the mitochondria of the rat liver hepatocytes (data not shown), confirming results with Western blots.

The mechanism by which CYP2E1 protein is translocated to mitochondria is not quite clear. Essentially, two forms of CYP2E1 are reported being present in the mitochondria, a highly phosphorylated 52-kDa form mediated via cAMP-dependent protein kinase A (19, 33, 34) and a shortened 40-kDa NH₂-terminal-truncated form that lacks the mature CYP2E1 sequence and can be further truncated to produce a mature mitochondrial form of CYP2E1 lacking about 100 amino acids (20, 21, 32). The phosphorylation and the amino-terminal truncation are hypothesized to cause conformational changes and altered interactions with molecular chaperones and signal recognition particles and to divert the CYP2E1 to the mitochondria (21, 33). Both of these two forms of mitochondrial CYP2E1 are catalytically active with typical CYP2E1 substrates but require, as do other mitochondrial CYP2E1s, adrenodoxin plus adrenodoxin reductase (and NADPH) as electron donors and are inactive with NADPH-cytochrome P450 reductase (19, 21).

In the current study, HepG2 cell lines overexpressing mitochondrial CYP2E1 were established by transfection of plasmid containing human CYP2E1 cDNA lacking the NH₂-terminal hydrophobic ER targeting
Mitochondrial CYP2E1 and Cell Toxicity

The expression of a 40-kDa mitochondrial CYP2E1 was detected by Western blot analysis and verified by confocal microscopy. This truncated mitochondrial CYP2E1 showed catalytic activity in the presence of Adx and AdR plus NADPH. These results indicated that the hydrophobic NH$_2$ terminus of CYP2E1 is responsible for the cotranslational targeting of the protein to the membrane of the ER and indicated that deletion of the hydrophobic ER targeting signal results in targeting of this truncated protein to the mitochondria.

Previous studies demonstrated that HepG2 cells overexpressing 52-kDa CYP2E1 in the ER were more sensitive to ethanol, GSH depletion, arachidonic acid, and iron-induced toxicity than control HepG2 cells without CYP2E1, and antioxidants, such as catalase, SOD, GSH, and Trolox, protect these cells from toxicity (11, 13, 35–37). Cells overexpressing mitochondrial CYP2E1 also were considerably more sensitive to GSH depletion-induced toxicity than control cells, and antioxidants such as GSH ester and Trolox were able to protect these cells from toxicity. Like CYP2E1 in the ER, CYP2E1 in mitochondria was active in producing ROS especially when GSH was depleted by treatment with BSO.

Mitochondrial CYP2E1 and Cell Toxicity

FIGURE 11. Flow cytometry analysis of the mitochondrial membrane potential. pCI-neo, mE10, or mE27 cells were incubated in the absence of any addition or treated with 0.2 mM BSO, BSO plus 2 mM GSH ethyl ester, or BSO plus 50 μM Trolox for 36 h. Mitochondrial membrane potential was assayed by flow cytometry as described under “Materials and Methods.” M1 refers to a population of cells with low rhodamine 123 fluorescence intensity, which reflects a decrease in mitochondrial membrane potential. The percentage of cells with low rhodamine 123 fluorescence is shown in each panel. The figure is one representative experiment out of two.
Mitochondrial CYP2E1 and Cell Toxicity

bands may be due to mitochondrial contamination, and not to the actual presence of mtCYP2E1 in the cytosol of intact cells. Moreover, confocal microscopy showed that there was complete overlap between CYP2E1 in mE10 and mE27 cells with the mitochondrial Mn-SOD marker with virtually no CYP2E1 (green color) found outside the mitochondria (not overlaying with the Mn-SOD). As expected, in the E47 cells, none of the CYP2E1 overlaid with Mn-SOD. These results indicated that most, if not all, of the CYP2E1 is translocated into the mitochondria, with no or very little accumulation in the cytosol in the mE10 or mE27 cells.

Damage to mitochondria is thought to be a critical step for cellular toxicity by CYP2E1-derived reactive oxygen species (36, 37). Mitochondrial permeability transition and mitochondrial membrane potential are markers for mitochondrial damage and dysfunction (38–40). Mitochondrial dysfunction caused by ROS can lead not only to necrosis by depleting ATP but also to apoptosis by inducing the release of cytochrome c, which activates caspases together with other mitochondrial factors such as Apaf-1 (41, 42). We determined the mitochondrial membrane potential in cells treated with BSO. The decline in $\Delta \Psi$ was much less in pCl-neo cells than in mE10 and mE27 cells. GSH ester and Trolox were able to block the loss of mitochondrial membrane potential analogous to their maintaining cell viability. A mitochondrial membrane stabilizer, cyclosporine A, was effective in preventing the mitochondrial CYP2E1- and BSO-dependent toxicity. These results suggest that mitochondrial CYP2E1-dependent production of ROS, in the absence of GSH-dependent antioxidant defensive systems, may interact with the mitochondria to cause a decrease in the membrane potential followed by a decrease in ATP production, which subsequently leads to cell death. Future studies will evaluate mitochondrial function in these cells as well as the mode of cell death. The effect of ethanol on mE10 and mE27 cell viability will be determined.

GSH is the most abundant antioxidant in cells and plays a major role in the defense against oxidative stress-induced cell injury (43, 44). There is considerable interest in the effects of ethanol on GSH homeostasis and the role that GSH depletion plays in ethanol-induced liver injury. Although short term ethanol treatment lowers hepatic GSH levels, largely as a consequence of inhibiting GSH synthesis (45), the effects of long term ethanol treatment on GSH levels are less clear, with reports of decreased GSH levels (44, 46), unchanged levels (47, 48), or even increased levels (49). However, mitochondrial GSH levels are decreased after chronic ethanol treatment, and this decrease has been suggested to play a role in ethanol-induced liver injury (46, 50). Ethanol induction of mitochondrial CYP2E1 and depleting cellular GSH as modeled in this study may mimic or contribute to conditions by which ethanol causes liver damage in vivo.

In summary, the results in this study have revealed that CYP2E1 in the mitochondrial compartment could induce oxidative stress in mitochondria, damage mitochondrial membrane potential, and cause cell toxicity. Therefore, the accumulation of CYP2E1 in hepatic mitochondria induced by ethanol consumption might play an important role in alcohol liver damage.

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Mitochondrial CYP2E1 and Cell Toxicity

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