**Human Cytochrome P450 2E1 Mutations That Alter Mitochondrial Targeting Efficiency and Susceptibility to Ethanol-induced Toxicity in Cellular Models**

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**Background:** Induced expression of CYP2E1 is known to enhance alcohol liver toxicity. Novel mutations W23R/W30R and L32N in human CYP2E1 alter mitochondrial and microsomal targeting efficiency. Carriers of the novel W23R/W30R mutation in CYP2E1 are likely to be more susceptible to alcohol toxicity.

Human polymorphisms in the 5’-upstream regulatory regions and also protein coding regions of cytochrome P450 2E1 (CYP2E1) are known to be associated with several diseases, including cancer and alcohol liver toxicity. In this study, we report novel mutations in the N-terminal protein targeting regions of CYP2E1 that markedly affect subcellular localization of the protein. Variant W23R/W30R protein (termed W23/30R) is preferentially targeted to mitochondria but very poorly to the endoplasmic reticulum, whereas the L32N protein is preferentially targeted to the endoplasmic reticulum and poorly to mitochondria. These results explain the physiological significance of bimodal CYP targeting to the endoplasmic reticulum and mitochondria previously described. COS-7 cells and HepG2 cells stably expressing W23/30R mutations showed markedly increased alcohol toxicity in terms of increased production of reactive oxygen species, respiratory dysfunction, and loss of cytochrome c oxidase subunits and activity. Stable cells expressing the L32N variant, on the other hand, were relatively less responsive to alcohol-induced toxicity and mitochondrial dysfunction. These results further support our previous data, based on mutational studies involving altered targeting, indicating that mitochondria-targeted CYP2E1 plays an important role in alcohol liver toxicity. The results also provide an interesting new link to genetic variations affecting subcellular distribution of CYP2E1 with alcohol-induced toxicity.

Alcohol toxicity is considered as a major health problem worldwide and is associated with over 60 diseases, including hepatitis, liver damage, cirrhosis, insulin resistance (diabetes), liver cancer, etc., causing a multitude of financial losses. The metabolism of alcohol is known to generate reactive intermediates/metabolites that contribute to cell and tissue damage by altering various cellular signaling pathways and also oxidative stress (1–5). The metabolism of alcohol occurs mainly in the intestine and the liver by cytochrome P450 2E1 (CYP2E1) and alcohol dehydrogenase (ADH), which convert alcohol to toxic acetaldehyde. Acetaldehyde is then converted to acetate either by aldehyde dehydrogenase (ALDH) or by CYP2E1. Thus CYP2E1 is a major determinant of alcohol induced toxicity in the liver, intestine, brain, and other peripheral tissues where it is expressed (6–8).

CYP2E1 is of special interest because it is induced 5–15-fold by alcohol, isoniazid, and other small molecules in different tissues. In addition to ethanol, CYP2E1 catalyzes the metabolism of many low molecular weight endogenous and exogenous compounds (e.g. acetone), drugs (e.g. acetaminophen, chlorzoxazone, and isoniazid), and industrial solvents (e.g. benzene and styrene), many of the latter of which are carcinogenic (9). Induction of CYP2E1 is associated with pathophysiological conditions, such as diabetes, non-alcoholic liver steatosis, liver cirrhosis, starvation, and hepatic carcinogenesis (6, 10, 11). CYP2E1 is also thought to contribute to the production of reactive oxygen species (ROS) (12–15). It has also been proposed that elevated rates of superoxide (O2•−) and H2O2 production are mainly due to poor coupling of this enzyme with NADPH-cytochrome P450 reductase (16–18). Oxygen free radicals (ROS) generated by CYP2E1, in turn, could initiate membranous lipid peroxidation, contributing to tissue injury (6, 8, 19–21).

Recent studies in our laboratories as well as others have shown that CYP2E1 is also bimodally targeted to mitochondria by virtue of its N-terminal chimeric signal (22–26). In this new

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The abbreviations used are: CYP, cytochrome P450; Adx, adrenodoxin; Adr, adrenodoxin reductase; NPR, NADPH-cytochrome P450 reductase; DHFR, dihydrofolate reductase; CCCP, carbonyl cyanide m-chlorophenylhydrazone; mtTFA, mitochondrial transcription factor A; DNP, 2,4-dinitrophenol; CcO, cytochrome c oxidase; DAS, dialyl sulfoxide; SOD, superoxide dismutase; ROS, reactive oxygen species; MtO-Q, mitochondrial transcription factor A; OCR, oxygen consumption rate(s); ALD, alcholic liver disease; ER, endoplasmic reticulum; CFH, dihydroxyflurorescin diacetate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

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Human CYP2E1 Variants with Altered Targeting Signals

location, mitochondrial CYP2E1 interacts with and accepts electrons from the mitochondrial adenodoxin (Adx) and adenodoxin reductase (Adr) system and efficiently catalyzes the metabolism of an array of substrates. Using an in vitro mutagenesis approach, we showed that mitochondria-targeted CYP2E1 potentiated a higher level of alcohol-mediated ROS production and cell injury than the microsomal CYP2E1 in COS-7 and HepG2 cells (27, 28). A similar conclusion was reached by Robin’s group recently using a different targeting approach (29). We also demonstrated that mitochondria-targeted CYP2E1 caused excessive and direct damage to mitochondrial cytochrome c oxidase (CcO), which was nearly completely reversed by the mitochondria-targeted antioxidants Mito-CP and Mito-Q (27, 30), providing further confirmation that mitochondrial ROS in the presence of added alcohol is a key factor in inducing cellular toxicity.

The human CYP2E1 gene exhibits several polymorphisms (see the Human Cytochrome P450 Allele Nomenclature Database Web site), some of which affect gene expression at the transcriptional level, whereas others affect enzyme activity. The CYP2E1 gene has at least six polymorphic forms, of which one in intron 6 (Dral C/D) (mutant allele: 

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CYP2E1^*5B
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endoplasmic reticulum or mitochondria, and the variant L32N. All of these mutations affected targeting efficiency to the date. We report here three novel variants: W23R, W30R, and CYP2E1*5B. Intron 6 region and the ability of mitochondria-targeted CYP2E1 in

potentiating alcohol toxicity, we screened a bank of human liver

tein was also found to be associated with a decrease of about two-thirds in enzyme expression and activity in vitro (47).

In view of our recent findings on the altered targeting activity of CYP2E1 protein modified at the N-terminal signal sequence region and the ability of mitochondria-targeted CYP2E1 in potentiating alcohol toxicity, we screened a bank of human liver samples. We looked for mutations in the protein-targeting signal region of the CYP2E1 gene because this region was not examined in most other genetic screening studies carried out to date. We report here three novel variants: W23R, W30R, and L32N. All of these mutations affected targeting efficiency to the endoplasmic reticulum or mitochondria, and the variant proteins more preferentially targeted to mitochondria imparted susceptibility to alcohol-mediated toxicity in both COS-7 and HepG2 cells. Our results suggest a genetic factor linking mitochondrial targeting efficiency to alcohol liver toxicity.

EXPERIMENTAL PROCEDURES

Sources of Antibodies—Polyclonal antibodies to human CYP2E1 (anti-rabbit) were purchased from Oxford Biomedical Research (Oxford, MI). Antibodies to human cytochrome c oxidase subunit 1 (CCO 1) (anti-mouse), human cytochrome oxidase subunit Vb (CcO Vb) (anti-mouse), and human cytochrome oxidase IVh (CcO IV) (anti-rabbit) were from Mitosciences (Eugene, OR). Antibodies raised against human NADPH-cytochrome P450 reductase (NPR) (anti-mouse) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to porin and succinate dehydrogenase were from Abcam (Cambridge, MA). Antibodies to mitochondrial transcription factor A (mtTFA) were gift from Dr. David Clayton (Howard Hughes Medical Institute, Janelia Farm, Ashburn, VA).

Isolation of Mitochondria from Frozen Human Liver Samples—Human liver samples were obtained through Tennessee Donor Services (Nashville, TN) and used in accordance with Vanderbilt Institutional Board Guidelines. Mitochondria and microsomes were isolated from human liver samples using a differential centrifugation method described previously (48, 49). Briefly, livers were washed in ice-cold saline three times, minced with scissors, and homogenized in 10 volumes of sucrose-mannitol buffer (20 mM Na-HEPES, pH 7.4, containing 70 mM sucrose, 220 mM mannitol, 2 mM EDTA, and 0.5 mg/ml bovine serum albumin (BSA)) in a glass Potter-Elvejem homogenizer. Cell debris and nuclei were removed by centrifugation initially at 600 × g and later at 650 × g for 10 min each. Mitochondria were pelleted from centrifugation of the post-nuclear fraction at 7,000 × g for 15 min. Crude mitochondrial fractions were layered over 0.8 m sucrose. The fractions were centrifuged at 8,000 × g for 20 min, and the mitochondrial pellet was washed twice in the sucrose-mannitol buffer (see above). Mitoplasts were prepared by suspending the crude mitochondrial pellet in sucrose-mannitol buffer at a concentration of 50 mg of protein/ml and treating with digitonin (75 μg/ml of protein; Calbiochem) at 4 °C for 15 min. The resulting mitoplast pellet was washed twice in sucrose-mannitol buffer. Microsomes were isolated from the postmitochondrial supernatant by centrifugation at 100,000 × g for 1 h at 4 °C. All final subcellular membrane preparations were resuspended in 50 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol (v/v), 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF). Protein estimation was carried out using the method of Lowry et al. (50).

Cell Growth, Reagents, and Site-directed Mutagenesis—Control COS-7 and HepG2 stable cells transduced with retroviral pBABE-puro vector (28) expressing mock, wild type, and mutant CYP2E1 were cultured either in 96-well plates, 6-well plates, or 100-mm culture dishes, depending upon the requirement of the experiments (ROS, membrane potential, GSH, or F2-isoprostanes). These cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 25 mM glucose, supplemented with 10% fetal bovine serum (v/v) in the presence of added 1% penicillin/streptomycin in a 5% CO2, 95% air (v/v).
incubator at 37 °C. Various chemicals (ethanol, disulfiram, Mito-CP, Mito-Q, dialyl sulfide (DAS), 2,4-dintrophenol (DNP), oligomycin, rotenone, and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were added for a maximum period of 48 h unless specified otherwise. Concentrated stocks of DNP, rotenone, and oligomycin were prepared in DMSO. Stock concentrations were 500 mM DNP, 10 mM rotenone, and 10 mg/ml oligomycin. DNP was further diluted to 50 μM and adjusted to pH 7.4. Rotenone and oligomycin were diluted to 1 μM and 2 μg/ml, respectively. Puromycin (5 μg/ml) was added after every four passages to maintain the integrity of the cells expressing CYP2E1. All of the experiments were conducted in the stable cells cultured without puromycin for at least three passages to exclude the possibility of an effect of puromycin on mitochondrial functional integrity. Also, expression of CYP2E1 was tested in these cell lines as a function of time.

Mutant CYP2E1 cDNAs coding for various amino acid substitutions (W23R, L32N, and W23/30R) were generated using a Stratagene QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) using wild type cDNA (amino acids 1–160) subcloned into the vector PGEM-DHFR using an EcoRI linker for in vitro import studies and full-length in pBABE puro vector for stable expression in cells. The cells were harvested 48 h post-transfection, washed with phosphate-buffered saline (PBS), and used for isolating subcellular fractions as described previously (27, 28). The Met at position 22 was also mutated to Ala in the CYP2E1-DHFR fusion protein using the same strategy. This construct will be referred to as M22A CYP2E1.

Assay for ER Membrane Association—Membrane association/integration assay was carried out as described previously by Hegde et al. (51). Proteins were translated in the presence of increased units (0–2 units) of unwashed canine pancreatic microsome plus 50 μl of translation mix supplemented as described previously (52). Translation was stopped by adding 1 mM cycloheximide, and membranes were extracted with 0.1 M Na2CO3 (pH 11) for 1 h on ice by intermittent mixing and fractionated into soluble (200,000 × g supernatant) and insoluble (pellet) fractions as described before (51, 52). Both the membrane pellet and the soluble extract were resuspended in 40 μl of 10 mM Tris-HCl (pH 7.0) containing 2% SDS and subjected to SDS-PAGE. The other half of the reaction mix was used for assessing the input counts.

In Vitro Protein Import in Isolated Mitochondria—cDNAs encoding the N-terminal 160 amino acids (480 base pairs) of wild-type CYP2E1 and various mutated constructs (W23R, L32N, and W23/30R) cloned in the PGEM-DHFR cloning vector with restriction sites EcoRI/HindII were used as templates in SP6 polymerase-coupled rabbit reticulocyte transcription-translation systems (Promega, Madison, WI) as described before (48, 52). The translation mix was supplemented with 10 units of the catalytic subunit of PKA plus 50 μl of translation mix (Sigma). Translation was carried out in the presence of 0.1 mM ATP and [35S]Met (28, 53). Import of [35S]Met-labeled translation products in rabbit reticulocyte was carried out using the system initially described by Gasser et al. (54), subsequently modified by Addya et al. (48) and Bhat et al. (55), using freshly isolated rat liver mitochondria. Trypsin treatment of mitochondrial (150 μg/mg protein) was carried out for 30 min on ice as described previously (48, 55). The trypsin-treated samples were treated with soybean trypsin inhibitor (300 μg/ml) as described above. Mitochondria were resiolated from both trypsin-treated and untreated samples by sedimentation through 0.8 M sucrose and were washed twice with sucrose-mannitol buffer. Mitochondrial proteins were dissociated in Laemmli’s (56) sample buffer before electrophoresis and fluorography. The radioactive bands were visualized using the radiometric imager STORM (GE Healthcare) system and quantitated based on arbitrary units.

Protease Protection Assay—Mitochondria and microsomes were suspended at 10 mg of protein/ml in 50 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol and subjected to trypsin digestion (75 μg/ml of protein) for 30 min at room temperature. The reaction was stopped by adding 10-fold molar excess of trypsin inhibitor and an equal volume of 2× Laemmli sample buffer (56). The samples were incubated at 95 °C for 5 min and subjected to SDS-PAGE (12%, w/v) for immunoblot analysis.

Generation of Stable Cell Lines—COS-7 and HepG2 cells stably expressing various CYP2E1 mutants were established by cloning wild type (WT) and mutated CYP2E1 (L32N and W23/30R) cDNAs in the EcoRI and XhoI sites of a retroviral plasmid (pBABE-puro) as described previously (28). A “kill curve” was generated to determine the optimum concentration of puromycin to be used in subsequent cell culture experiments without affecting the growth parameters. For generating stable cell lines, these cDNAs (12 μg each) and the viral DNAs (Gag-Pol, VSV-G) were co-transduced into 293T cells using Fugene HD transfection reagent to obtain fully functional retroviral particles. The virus particles secreted in the cell culture medium were harvested 48 and 72 h post-transfection by centrifugation at 600 × g for 5 min and filtered through a 0.45-μm syringe filter. COS-7 and HepG2 cells were infected with the virus particles and added Polybrene (8 μg/ml), and the colonies were screened using puromycin (5 μg/ml) as the selection marker. Single colony clones were selected and subcultured in 100-mm plates, and the proteins were analyzed by immunoblotting to estimate levels of expression of CYP2E1.

Subcellular Fractionation and Immunoblot Analysis—Cells, typically from ~80% confluent plates were collected after 48 h of treatment and washed twice with PBS (15 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl) before use. For preparing total cell lysates, cells were lysed with radiomunoprecipitation lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40 (v/v), 0.25% sodium deoxycholate (w/v), 150 mM NaCl, and 1 mM EDTA) containing protease inhibitors. Mitochondria from stable cells and transiently transfected cells were isolated as described previously (28, 49). Briefly, cells were washed with PBS and homogenized in 10 volumes of sucrose-mannitol buffer containing 2 mM EDTA and protease inhibitors (1 mM PMSF and 50 μg/ml each of leupeptin, pepstatin, aprotonin, and antipain). The homogenate was centrifuged twice at 600 × g for 10 min at 4 °C, and the supernatant was centrifuged at 8,000 × g for 20 min at 4 °C to obtain the mitochondrial pellet. The mitochondrial pellet was washed twice and suspended in the sucrose-mannitol buffer, passed through a 1.0 M
puromycin acetyltransferase gene of the vector construct in DNA integrated in the genome was determined by using the cells using a DNeasy Blood & Tissue kit in accordance with the Institutes of Health). Germany) and quantified using Image J software (National Institutes of Health).

Quantitative Real-time PCR—Total DNA was isolated from cells using a DNeasy Blood & Tissue kit in accordance with the manufacturer’s instructions (Qiagen, Valencia, CA). The vector DNA integrated in the genome was determined by using the puromycin acetyltransferase gene of the vector construct in relation to β-actin used as an internal control.

For real-time PCR analysis, 5 μg of total RNA was reverse-transcribed using a high capacity cDNA archive kit (Applied Biosystems, Carlsbad, CA), and 25 ng of the resulting cDNA was used in standard Power SYBR Green real-time PCRs on an ABI 7300 real-time PCR machine and analyzed using Primer Express version 3.0 (Applied Biosystems).

Immunofluorescence Confocal Microscopy—COS-7 stable cells were grown on coverslips in 6-well plates containing 3 ml of culture medium. After seeding (48 h), cells were washed with 1× PBS three times and then fixed with chilled methanol for 10 min, permeabilized with 0.1% Triton X-100 (w/v) for 1 min, and washed three times with PBS for 10 min each. The coverslips were incubated with 5% goat serum for 1 h at room temperature to minimize nonspecific interaction of antibodies. In order to label the ER, permeabilized cells were incubated with a 1:100 dilution of anti-NPR antibody, and to label the mitochondria, the cells were incubated with mouse anti-mitochondria-specific CcO I (1:100) followed by a 1:100 dilution of Alexa dye 594-conjugated anti-mouse IgG (Invitrogen) and a 1:100 dilution of Alexa dye 488-conjugated anti-rabbit donkey IgG. Incubations with primary and secondary antibodies were carried out at 37 °C for 1 h each. Coverslips were washed repeatedly with PBS to remove unbound antibodies and were mounted on slides using Permount mounting medium (Fisher). Finally, fluorescence confocal microscopy was carried out with a Leica TCS SP5 laser-scanning microscope, and the co-localization Pearson’s coefficients were calculated using Volocity version 5.3 software.

Measurement of Cytochrome P450 Content—The CYP contents of mitochondrial and microsomal membranes were measured by the difference spectra of carbon monoxide (CO)-treated and dithionite-reduced samples as described by Omura and Sato (57, 58) and as modified by Guengerich (59, 60), using a dual beam spectrophotometer (Cary 1E; Varian, Walnut Creek, CA). Mitochondrial or microsomal (0.4 mg) proteins were solubilized in potassium phosphate buffer (0.1 M, pH 7.4) containing 1 mM EDTA, 20% glycerol (v/v), sodium cholate (0.5%, w/v), and Triton N-101 (0.4%, w/v). Sodium hydrosulfite (dithionite) was added, and the base line was recorded. The solution in the sample cuvette was then bubbled gently with CO for 60 s. The spectrum was recorded in the range of 400–500 nm. The P450 contents were calculated as described (57, 58).

Assay of N,N-Dimethylnitrosamine N-Demethylation Activity—N-Demethylation of N,N-dimethylnitrosamine was assayed according to Eliasson et al. (61, 62) in the presence of added 0.2 nmol of Adx, 0.02 nmol of Adr, and 300 μg of mitochondrial protein/ml as enzyme source. In assays with the microsomal fractions, the microsome-associated NPR served as an electron donor for the reaction. Mitochondria were frozen and thawed four times in a hypotonic buffer (25 mM sodium phosphate buffer, pH 7.4) to permeabilize the membranes before the addition to the reaction mixtures. CYP2E1 antibody (50 μg), SKF 525-A (1 mM), and disulfiram (25 μM) were also used as controls to ascertain the role of CYP2E1. Details were essentially similar to those for the erythromycin N-demethylation assay published previously (28, 63, 64).

Measurement of ROS Production Using 2′,7′-Dichlorodihydrofluorescein Diacetate (DCFH-DA)—The level of intracellular ROS was estimated by the change in fluorescence resulting from oxidation of DCFH-DA (Molecular Probes). DCFH-DA can diffuse into cells and, after being cleaved by intracellular esterases, is trapped inside the cell generating 2′,7′-dichlorodihydrofluorescein. Within the cell, the probe reacts with intracellular ROS, oxidizes to 2′,7′-dichlorofluorescein (DCF), and becomes highly fluorescent, with excitation and emission wavelengths at 485 and 535 nm, respectively. Cells were seeded at a concentration of 15 × 10^4 cells/well of black 96-well plates in phenol-free medium and grown overnight with and without the inhibitor DAS and antioxidants (Mito-Q and N-acetylcysteine).

ROS generation in mitochondria was measured by the 2′,7′-dichlorodihydrofluorescein fluorescence method, modified from LeBel et al. (65). The modification involves the use of 5 μg of brain cytosolic fraction as source of esterase for assaying mitochondrial fractions as described before (27, 28). Stable non-fluorescent DCFH-DA (2.5 μM in methanol) was used in 200 μl of an assay mixture containing 135 μl of PBS, 20 μg of mitochondrial protein, 5 μg of brain cytosol, and 0.1 mM NADPH. Appropriate controls were used for normalizing the effects of cytosol alone on ROS production. In some cases, ROS was also measured in the presence of membrane-permeable SOD (30 units/ml) or membrane-permeable catalase (10 units/ml), both from Sigma. The fluorescence was recorded by using a LPS-220B spectrofluorometer (Photon Technology International, Birmingham, NJ) with an excitation wavelength of 485 nm and emission wavelength of 535 nm (for 20 min).

GSH Measurement—Intracellular GSH levels were measured as described by Tietze (66) with the colorimetric substrate 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), using an NWSS kit (Northwest Lifesciences, Vancouver, WA), following the manufacturer’s protocol. Briefly, the 200-μl reaction mixture, containing 100 μg of protein, 100 μl of buffer, and 25 μl of DTNB, was incubated at 37 °C for 3–5 min, and the reaction was started by the addition of 1 mM NADPH. After mixing, the absorbance
at 412 nm was measured using a Cary Win UV spectrophotometer, and the absorbance was recorded continuously for 3 min. The GSH levels (nmol/mg protein) were calculated using a GSH standard curve (28).

**Assay of F2-isoprostanestates**—Levels of F2-isoprostanes in cells (with and without ethanol treatment) were determined using a gas chromatography-mass spectrometry-based method, as described previously (67).

**Membrane Potential**—Control and ethanol-treated cells (30,000) were plated in 96-well plates in phenol-free medium overnight. After 24 h of growth, the mitochondrial membrane potential was measured using the fluorescent dye tetramethylrhodamine methyl ester. Briefly, the dye was loaded onto cells at a concentration of 150 nM in assay buffer (25 mM Na-HEPES, pH 7.4, containing 80 mM NaCl, 75 mM KCl, and 25 mM D-glucose), incubated at 37 °C for 15 min, washed four times in PBS, and measured on a Chameleon microplate reader (excitation 535 nm, emission 590 nm). As a control, each assay was performed in parallel as above with the addition of 10 μM CCCP, which collapses the mitochondrial membrane potential. The CYP2E1 inhibitor disulfiram (63) was also added to observe the effect in rescuing the membrane depolarization.

**Assay of Cytochrome c Oxidase Activity**—CcO activity was measured by incubating 10 μg of freeze-thawed mitochondrial extract prepared from control or alcohol-treated (100 mM for 48 h) cells, in 1 ml of assay medium (25 mM potassium phosphate, pH 7.4, containing 0.45 mM dodecyl maltoside and 15 μM reduced cytochrome c) and measuring the decrease in absorbance at 550 nm due to cytochrome c oxidation (27, 68). In some assays, cells were also treated with Mito-CP (2 μM) or disulfiram (25 μM) as indicated.

**Analysis of Cellular Respiration (Seahorse XF24 System)**—Measurements of oxygen consumption rates (OCR) were made using a XF24 high sensitivity respirometer (Seahorse Bioscience, Billerica, MA) as described previously (27, 69) and following the manufacturer’s instructions. Briefly, 30,000 cells (with or without ethanol treatment (25 and 100 mM)) with or without DAS (10 μM) were cultured in DMEM for 16 h, and the XF assay medium (low buffered bicarbonate free DMEM, pH 7.4) was changed. The cells were incubated at 37 °C for 30 min to allow medium temperature and pH to reach equilibrium before the first rate measurement. Oligomycin (2 μg/ml), DNP (a mitochondrial uncoupler, 50 μM), or the complex I inhibitor, rotenone (1 μM), was injected through the cartridge, and oxygen consumption rates were measured under basal conditions as well as after sequential injections of oligomycin, DNP, and rotenone following the calibration procedure (30-min calibration, 10-min rest). Measurement cycles of 1-min sample mixing, 2-min waiting, and 3-min measurement of oxygen consumption rate were carried out. This procedure allowed determination of the basal level of oxygen consumption, the amount of oxygen consumption linked to ATP production, the level of non-ATP-linked oxygen consumption (proton leak), the maximal respiration capacity, and non-mitochondrial oxygen consumption. The cell numbers and concentrations of inhibitors used were optimized. All respiration rates were calculated as a percentage of the rate. The difference in the cell density between wells was corrected by loading the cell lysate on an SDS-polyacrylamide gel (12%, w/v) and probed with an actin antibody using immunoblot analysis. Absolute rates of oxygen consumption were linearly related to cell numbers seeded within the measurement range. Respiration rates at each time point from three replicate wells were determined and averaged.

**Statistical Analysis**—Mean ± S.D. values were calculated from 3–5 independent experiments. Statistical significance (p values) between control and experimental or paired experiments was calculated using Student’s t test. A p value of 0.05 (shown as an asterisk) was considered significant, and 0.001 (shown as double asterisks) was considered highly significant.

**RESULTS**

**Interindividual Variations in Human Liver Mitochondrial CYP2E1 Levels**—Digitonin-treated mitochondria (mitoplasts) and microsomes from 40 human liver samples were analyzed by immunoblot analysis using antibodies to CYP2E1, NPR, and mtTFA. The latter two proteins were used as markers for microsomes and mitochondria, respectively. Blots for 15 representative samples are shown in Fig. 1A, and the subcellular distribution of CYP2E1 in mitochondria and microsomes based on relative band intensities is presented in Fig. 1B. Seven human liver samples (HL108, HL109, HL127, HL129, HL130, HL132, and HL134) contained high microsomal CYP2E1 contents, ranging from ~70 to almost 100%. Samples HL111, HL113, HL114, HL123, HL126, and HL131 contained moderate levels of microsomal CYP2E1 (~25–70%). Samples HL112 and HL128, on the other hand, contained very low microsomal CYP2E1 (3–10%) but very high mitochondrial CYP2E1 (~90–98%). Samples HL111, HL126, and HL131 also contained high mitochondrial CYP2E1, in the range of ~65–90% of the tissue pool. These results indicated marked interindividual variations in the distribution of hepatic CYP2E1 in the mitochondrial and microsomal fractions. Notably, in some samples, mitochondrial CYP2E1 predominated over the microsomal content. It should be noted that the mitochondrial fractions contained high mtTFA and very low NPR, whereas the microsomal fractions contained very low mtTFA and high NPR, indicating the relative purity of subcellular fractions isolated by the procedure.

A protease protection assay was used for establishing the intramitochondrial localization of CYP2E1 for two of the samples, selected at random (Fig. 1C). The results show that the microsomal CYP2E1 was readily degraded by added trypsin, whereas ~70% of the mitochondrial CYP2E1 was protected against trypsin treatment. Notably, low digitonin treatment (which strips the mitochondrial outer membrane) also protected ~70% of the mitochondrial CYP2E1, which was also protected against limited trypsin treatment. These results confirm that the CYP2E1 isolated with the mitochondrial fraction is localized within the mitochondrial inner membrane.

**Polymorphism within the Signal Sequence Domain of Human CYP2E1**—Most of the genetic screening studies on human CYP2E1 have been focused on mutations in the catalytic sites of the protein, which can alter the enzyme activity, or at the 5’-proximal promoter region, which can affect transcription rates (32). Previously, we showed that point mutations that alter the hydrophobicity of the N-terminal signal domain or the putative PKA phosphorylation site at Ser-128 affected mito-
chondrial and microsomal distribution of rat CYP2E1 (24, 26, 70–73). We also showed that the N-terminal 160-amino acid region contained signal activity because it could predictably target reporter proteins to mitochondria and microsomes (24). We therefore investigated if the marked variation in the mitochondrial contents of CYP2E1 in the human liver samples was possibly caused by the result of mutations affecting the N-terminal signal activity by amplifying the amino acid 1–160 coding regions (480 bp) of human liver mRNAs by PCR and sequencing the resulting DNA fragments (mutations were also confirmed by amplifying the chromosomal DNA region and nucleotide sequencing). We observed the variants W23R in sample HL114, W30R in sample HL123, and L32N in two samples (HL127 and HL134). Three of the samples (HL128, HL112, and HL131) also contained double mutations W23R and W30R (Fig. 1D and Table 1). The sequence variations clustered within or immediately flanking the mitochondrial targeting signal region that we identified in these samples are shown by the arrows.

The targeting efficiencies of the variant signal regions were analyzed using Psort II and WOLFPSORT algorithms (Table 1). The WT protein showed an endoplasmic reticulum-targeting efficiency of 67% and mitochondrial targeting efficiency of 11%. The mutations W23R and W30R (hereafter referred to as W23/30R) increased the mitochondrial targeting potential to 30–40%, compared with 11% for the WT CYP2E1. Surprisingly, the L32N mutation showed low mitochondria-targeting potential of ~11% but a higher endoplasmic reticulum targeting of ~67%. Interestingly, the mutational data correlated well with the human liver mitochondrial and microsomal contents of CYP2E1 in the samples. HL112, HL128, and HL131, showing high mitochondrial CYP2E1, also showed the W23/30R double mutation, and samples HL127 and HL134 (showing high microsomal CYP2E1 content) showed the L32N mutation. These results suggested the possibility that signal region mutations may be one reason for the marked variations in the subcellular distribution of CYP2E1. Because of the limited sample size, we were unable to determine the statistical significance of the mutational frequency in the human population.

The rate of signal recognition particle binding and ER association is a direct measure of the extent of nascent protein targeting to the ER. We tested the affinity of the WT and variant proteins for binding to unwashed canine pancreatic ER using a membrane integration assay. As predicted, we observed increasing membrane binding of WT nascent chains at increas-

![FIGURE 1. The interindividual variations in CYP2E1 content of human liver samples. A, immunoblot analysis of mitoplast (Mt) and microsomal (Mc) fractions isolated from human liver samples (50 μg of protein each). Blots were developed with polyclonal antibodies to CYP2E1 (1:1,000) and mtTFA (1:3,000) and monoclonal antibody to NPR (1:1,500). B, band intensities were quantitated using ImageJ software, and densitometric analysis was performed to determine the distribution of CYP2E1 in mitochondria and microsomes. C, immunoblot analysis of human liver mitochondrial, microsomal proteins from liver samples HL114 and HL134, subjected to limited trypsin digestion (150 μg/mg protein, 20 min on ice). The blot was developed with CYP2E1 antibody. D, N-terminal amino acid sequence of CYP2E1 protein depicting the ER targeting domain, mitochondrial targeting domain, and proline-rich domain. Polymorphisms within the putative signal sequence region are shown by the arrows.](image-url)
ing levels of ER (Fig. 2A). The L32N variant protein showed an even higher percentage of ER membrane binding. In the case of the W23/30R protein, however, the membrane association was substantially lower (Fig. 2, A and B). There was steady decrease in membrane binding at increasing levels of ER added. The reasons for this decrease remain unclear. These results confirm that ER targeting efficiency is significantly altered in the human variants. The double bands seen in both input and bound fractions are due to alternate translation start at Met residues at positions 1 and 22 (see Fig. 3D).

**Mitochondrial Import of WT and Variant Human CYP2E1 in Isolated Mitochondria**—The mitochondrial targeting potential of the WT and CYP2E1 mutants was tested by in vitro import in isolated mitochondria. For this purpose, we fused the N-terminal 160-amino acid regions of the CYP2E1 proteins with a cytosolic protein, dihydrofolate reductase (DHFR), which was used as a reporter (Fig. 3A). The rationale was that the ability to guide a cytosolic protein by an N-terminally fused CYP2E1 signal domain could be used as a test for the mitochondrial signal activity. The import reaction was carried out with 35S-labeled fusion proteins (48, 55), and mitochondria were resolated as described under “Experimental Procedures.” About 20% of input wild-type CYP2E1-DHFR was found to be associated with mitochondria (Fig. 3B), of which only 6% of the protein was rendered resistant to trypsin digestion, suggesting low import (Fig. 3B). The addition of PKA to the import mixture, on the other hand, increased the import of the WT fusion protein 2-fold. The W23R DHFR protein was imported at 50% higher levels than the WT-DHFR fusion protein. The PKA addition only marginally increased the import of these proteins. W23/30R-DHFR fusion was imported poorly, although the level of import was increased by over 21-fold by added PKA (Fig. 3B). The results (Fig. 3C) also show that L32N-DHFR fusion was not imported at a significant level even with added PKA. A canonical mitochondrial targeting signal of *Neurospora crassa*, ATPase 9/SU9 fused to the N terminus of DHFR, was used as a control and was imported efficiently and processed by the mitochondrial matrix protease (Fig. 3C, right), whereas DHFR alone was not imported into mitochondria at a detectable level. These results show that, as predicted by the sequence analysis using a computer algorithm, the W23/30R signal is an efficient mitochondrial import signal, comparable with the SU9 signal, whereas the L32N variant is a very poor signal for mitochondrial import. Further, unlike the SU9 N-terminal signal, the N terminus of imported CYP2E1 is not processed by the mitochondrial matrix protease.

**TABLE 1**

Human CYP2E1 variants showing altered mitochondria and microsome targeting potentials

Various polymorphisms within the putative mitochondrial targeting signal of CYP2E1 were identified by screening human liver samples. The subcellular targeting efficiency was predicted using a combinatorial approach utilizing computer-based analysis through Psort II and WOLFPSORT algorithms.

| Liver sample | Nucleotide change | Amino acid change | ER/mitochondria targeting | Cytosol |
|--------------|-------------------|------------------|---------------------------|---------|
| HL109        | None              | None/WT          | 67:11                     | 3.0     |
| HL114        | 100T→C            | W23R             | 30:26                     | 30.0    |
| HL123        | 121T→C            | W30R             | 30:26                     | 30.0    |
| HL127, HL134 | 127C→A            | L32N             | 67:11                     | 3.0     |
| HL112, HL128, HL131 | 100T→C  | W2R (W23/30R) | 13:44                     | 30.0    |

![FIGURE 2. The extent of ER membrane integration of nascent WT and mutant CYP2E1 proteins.](image)

The double bands observed with all CYP2E1 fusion constructs in Fig. 3, B and C, and the full-length mRNA translation products in Fig. 2A are due to translation start at two alternate positions of Met-1 and Met-22. This possibility was confirmed by using the M22A mutant construct for the in vitro import in Fig. 3D. The M22A mutant protein migrated as a single component on SDS gels, which was also efficiently imported as a single full-length protein in the presence of added PKA. Similar to the parent doublet protein, the import of this mutant protein was inhibited by the mitochondrial ionophore, CCCP.

**Mitochondrial Targeting of CYP2E1 in Stably Transfected COS-7 Cells**—To evaluate the extent of mitochondrial and microsomal targeting of human CYP2E1, we generated stably expressing WT, L32N, and W23/30R cDNAs in pBABE puro retroviral vector. Mitochondrial and microsomal fractions isolated from stable cells were analyzed by immuno-
blot analysis with anti-CYP2E1, co-developed with antibodies to NPR and succinate dehydrogenase, the endoplasmic reticulum and mitochondrial marker, respectively (Fig. 4A). The immunoblot (Fig. 4A) and quantitation based on band intensity (Fig. 4B) show that cells expressing WT CYP2E1 showed a 40/60% distribution in mitochondria and microsomes, whereas L32N showed a 20/80% distribution, respectively. The W23/30R variant, on the other hand showed a 30% endoplasmic reticulum/70% mitochondria distribution. The relative cellular distributions of these CYP2E1 variants are consistent with the computer-based analysis of the targeting signal properties (Table 1). The steady-state levels of CYP2E1 mRNA and puromycin acetyltransferase mRNA (Fig. 4, C and D) used as selection markers were very similar in all three cell lines, suggesting nearly identical integration of vector copies in these cells. The results also show that the isolated mitochondria did not contain significant microsomal contamination.

The mitochondrial and endoplasmic reticulum localization of the WT and variant forms of CYP2E1 was further investigated by immunofluorescence microscopy using antibodies to an ER-specific protein, NPR, and a mitochondria-specific protein, CcO1 (Fig. 5A and B). As expected, cells transduced with a mock vector showed no detectable CYP2E1-specific staining and insignificant co-localization with NPR antibody (Pearson’s coefficient of 0.24). In cells transduced with the WT CYP2E1 cDNA, anti-CYP2E1 antibody cross-reacted with membrane structures that co-localized with anti-CcO1 stained structures with a Pearson’s coefficient of 0.6, which is also considered insignificant (Fig. 5A). More intense staining with CYP2E1 antibody and a significant level of co-localization (Pearson’s...
coefficient of 0.81) was observed in W23/30R cells. In L32N cDNA-expressing cells, the CYP2E1 localization in mitochondria was below the level of significance (Pearson’s coefficient of 0.62). In L32N cells, the CYP2E1 antibodies mostly stained nuclear structures that were not stained by the CcO1 antibodies. The pattern of co-localization with the endoplasmic reticulum-specific NPR antibody was quite different (Fig. 5B). The CYP2E1 antibody-stained structures extensively co-localized with NPR-stained structures (Pearson’s coefficient of 0.8) in WT cells, which were markedly diminished in W23/30R cells. By contrast, in cells expressing L32N protein, co-localization of CYP2E1 antibody-stained structures with the NPR antibody-stained structures was very high (Pearson’s coefficient of 0.91). These results confirm the results of the biochemical analysis in Fig. 4.

**Enzyme Activities of Human CYP2E1 Variants**—Mitochondria and microsomes isolated from stable cell lines expressing the WT and human variant CYPs were analyzed for the P450 contents and assayed for N,N-dimethylnitrosamine N-demethylation activity, as described under “Experimental Procedures.” The microsomal activity was supported by the microsomal NPR. Mitochondria lose substantial amounts of soluble Adx (75). For this reason, the mitochondrial activity was reconstituted by adding purified Adx and Adr as described under “Experimental Procedures.” It was seen that the P450 contents of both mitochondria and microsomes from mock-transfected
cells were very low (Fig. 6A). In WT cells, both mitochondria and microsomes contained nearly similar levels of P450. In the L32N cells, the microsomal P450 content was 4–5-fold higher than the mitochondrial P450 content. The reverse was the case with cells expressing W23/30R variant protein. The N,N-dimethylnitrosamine N-demethylation activity was consistent with the level of protein expression (Fig. 4A) and the P450 content (Fig. 6A). The values in parenthesis (above the bar diagram in Fig. 6B) represent specific activity. These activities are generally low in mock-transfected cells and also mitochondria from L32N-expressing cells. All other fractions showed activities ranging from 0.09 to 0.16 (Fig. 6B). Results (Fig. 6B) also show that disulfiram (25 μM) inhibited the mitochondrial and microsomal activities of cells expressing WT, L32N, and W23/30R proteins by 60–80%, whereas the inhibitor had minimal effect on fractions from control, mock-transfected cells. The control experiments in Table 2 show that the enzyme activity of mitochondria from W23/30R-expressing cells is dependent on added NADPH plus Adx and ADR. Also, the activity was inhibited by a P450 inhibitor, pradifen hydrochloride (SKF525A), and CYP2E1 antibody. Although not shown, DAS (1 mM) also inhibited both the mitochondrial and microsomal activities. These results showed the specificity of the activity and also that mitochondria- and microsome-targeted CYP2E1 variants were catalytically functional.

**Roles of CYP2E1 Variants in Alcohol Toxicity**—COS-7 cells stably expressing WT, L32N, and W23/30R CYP2E1 were tested for ethanol-mediated ROS production and respiratory defects. The mitochondria-targeted antioxidant Mito-Q and Mito-CP were used to assess the relative levels of mitochondrial ROS. DAS and disulfiram were used as inhibitors of both mitochondrial and microsomal CYP2E1 activity. N-Acetylcysteine was used as a general antioxidant. The level of H₂O₂ production was assayed in whole cells (Fig. 7A) using a DCFH-DA method. The specificity of the fluorescent signal was confirmed using membrane-permeable catalase and SOD (Fig. 7D). In mock-transfected cells, the fluorescent signal remained nearly the same even after ethanol treatment. Mito-Q inhibited ROS production in control cells without treatment with ethanol. In WT CYP2E1-expressing cells with or without ethanol treatment, ROS production increased nearly 2-fold, which was effectively inhibited by Mito-Q (2 μM). In CYP2E1 L32N-expressing cells, ROS production was marginally higher than in the WT cells. Mito-Q marginally inhibited the ROS in both alcohol-treated and untreated cells. In the absence of added ethanol, W23/30R cells produced ROS at about the same level as in L32N cells. However, treatment with ethanol markedly increased ROS production, suggesting a role of mitochondria-targeted CYP2E1 in augmenting alcohol toxicity. Mito-Q effectively reduced ROS production in both treated and untreated cells, confirming that most of the ROS is mitochondrially located. Results also show that DAS and disulfiram, inhibitors of CYP2E1, inhibited ROS production effectively, indicating the role of metabolic activity of CYP2E1 in ROS production. The results show that mitochondrial CYP2E1 metabolic activity is critical in inducing ROS production.

The role of mitochondrial CYP2E1 in inducing oxidative stress was further confirmed by assaying ROS production in mitochondria and microsomes isolated from the three cell lines, regardless of whether the cells were treated with ethanol. Mitochondria from WT, L32N, and W23/30R cells produced progressively increasing levels of ROS as assayed by the DCFH-DA method. Treatment of cells with ethanol marginally increased mitochondrial ROS production in WT and L32N cells. However, ethanol treatment induced nearly 2-fold higher

### TABLE 2

**Specificity of the N-demethylation activity of mitochondrial CYP2E1**

| Electron transfer system | Inhibitor          | Activity |
|-------------------------|--------------------|----------|
| None                    | None               | 0.14 ± 0.02 |
| + Adx, + Adr, + NADPH  | None               | 3.5 ± 0.20  |
| + Adx, + Adr, + NADPH  | SKF 525-A          | 0.84 ± 0.05 |
| + Adx, + Adr, + NADPH  | Anti-P450 E1       | 0.98 ± 0.02 |
| (−Adx + Adr)            | None               | 1.50 ± 0.05 |
ROS production in cells expressing the W23/30R variant (Fig. 7B). Mito-CP treatment marginally reduced ROS production in mitochondria from ethanol-treated L32N cells, and the reduction was more pronounced in mitochondria from alcohol-treated W23/30R cells (Fig. 7C). The ROS production by the microsomal fractions from both cell lines was very low and was not altered by Mito-CP treatment. These results confirm that the mitochondrial compartment of these cells is a major contributor to ROS production. The control experiment in Fig. 7D shows the specificity of the ROS measurement and shows that membrane-permeable catalase markedly attenuated the signal, as expected.

In control reactions, added DCFH-DA alone (or with brain cytosolic extract without added mitochondria) yielded no sig-
significant fluorescence signal (and that has been subtracted from the fluorescence signal), suggesting the specificity of the reaction (results not shown). The microsomal membrane isolates from different cells showed lower ROS than the corresponding mitochondrial isolates.

Mitochondrial Oxidative Stress Parameters in Cells Treated with Ethanol—The GSH level in cells is an indicator of oxidative stress (2), and accordingly, we assessed the role of CYP2E1 variants in modulating cellular GSH levels. GSH levels were 27–28 nmol/mg protein in WT cells with or without treatment with ethanol (Fig. 8A). In L32N cells, the basal GSH level was higher, at 38 nmol/mg protein, a level that was not significantly altered by ethanol treatment. In W23/30R cells, the basal level was similar to that in WT cells (~26 nmol/mg protein). Treatment with ethanol, however, markedly reduced the GSH level by ~60%. Further, Mito-CP and DAS both restored the GSH level to the untreated control cell level, showing a marked protection against alcohol-induced oxidative stress. Lipid peroxidation is an immediate consequence of oxidative stress (76, 77), and the levels of F2-isoprostanes are probably the most reliable measure of oxidative stress (76–78). Consistent with this result, the cellular isoprostane levels were found to be markedly increased in W23/30R cells following treatment with ethanol (Fig. 8B).
Mitochondrial transmembrane potential ($\Delta \psi_{m}$) was also measured in these cells because it is a direct indicator of mitochondrial oxidative function. Fig. 8C shows that ethanol treatment in mock-transfected cells had no effect on $\Delta \psi_{m}$. In WT CYP2E1-expressing cells, however, ethanol treatment significantly reduced the transmembrane potential, which was in turn restored by disulfiram treatment. L32N cells showed a more robust $\Delta \psi_{m}$, which was not affected by alcohol treatment. W23/30R cells, on the other hand, showed significantly disrupted $\Delta \psi_{m}$, which was further reduced by treatment with alcohol. Although disulfiram markedly restored the $\Delta \psi_{m}$, the level of restoration was not as high as in WT cells. Treatment with CCCP, a known mitochondrial ionophore, disrupted $\Delta \psi_{m}$ by nearly 80% in all cells. These results together show that mitochondria-targeted CYP2E1 caused oxidative stress and disruption of transmembrane potential, which was restored substantially by inhibiting the metabolic activity of CYP2E1.

Alcohol-induced Changes in Mitochondrial Respiration and Energy-transducing Complexes in CYP2E1-expressing Cells—We examined the effects of expression of WT and mutant CYP2E1 constructs (L32N and W23/30R) on CcO activity. It is seen that CcO activities of WT and L32N variant cells were comparable with those of the mock-transfected cells. Mito-CP and disulfiram had modest effects on the CcO activity of alcohol-treated L32N cells (Fig. 9A). Cells expressing the W23/30R variant showed significantly lower CcO activity, and the decrease was further exacerbated by alcohol treatment. Mito-CP and disulfiram both rendered protection against alcohol-mediated inhibition of CcO activity. Notably, these agents reverted the level of activity to near that of mock-transfected cells (Fig. 9A), indicating that they also had a modulatory effect on the effects of mitochondria-targeted CYP2E1.

Previously, we showed that the loss of CcO activity in alcohol-treated cells was associated with reduced expression of CcO subunits I, IVI1, and Vb (27). Consistent with the loss of CcO activity, cells expressing the W23/30R variant showed markedly reduced CcO subunit IVI1 and Vb levels in response to alcohol treatment. CcO I subunit levels were reduced in response to alcohol treatment in WT- as well as W23/30R variant-expressing cells. In L32N-expressing cells, the loss of subunit I was minimal (Fig. 9B). These results show that predominantly mitochondria-targeted variant W23/30R augmented alcohol-mediated mitochondrial dysfunction.

A Seahorse Extralux analyzer was used to measure the rate of respiration and reserve respiratory capacity of cells treated with alcohol as well as cells without treatment. Basal OCR was significantly lower in WT- and L32N variant-expressing cells compared with W23/30R variant-expressing cells (Fig. 10, A–D). Low concentrations of alcohol (25 mM) had a marginal effect on basal OCR in all cells. After treatment with 100 mM alcohol, however, the basal OCR was markedly inhibited in W23/30R variant cells. This was also the case with maximal OCR measured after treating cells with DNP, the mitochondria-specific ionophore. The proton leak OCR was also highest in the W23/30R variant cells (Fig. 10E), which steadily increased following treatment with 25 and 100 mM alcohol. The extracellular acidification rate, which is indicative of metabolic shift to glycolysis, was also increased in W23/30R variant cells (Fig. 10F). Additionally, DAS attenuated the basal OCR as well as proton leak OCR significantly in W23/30R variant cells (Fig. 10, G and H). These results further confirm the role of mitochondria-targeted CYP2E1 in augmenting alcohol toxicity and indicate that W23/30R variant cells are more prone to alcohol-mediated respiratory deficiency/mitochondrial dysfunction.

Increased Susceptibility of HepG2 Cells Stably Expressing Human CYP2E1 Variants to Alcohol Toxicity—The responses of human CYP2E1 variants to cellular alcohol toxicity were further examined using HepG2 cells stably expressing WT CYP2E1 and two variants. An immunoblot (Fig. 11A) showed that the mitochondrial and endoplasmic reticulum CYP2E1 levels in stable HepG2 cell lines were nearly similar to those observed in COS-7 cells. In WT CYP2E1-expressing cells, the mitochondrial content was ~20%, which was increased to over 30% in W30R cells and 60% in W23/30R cells. In L32N cells, the mitochondrial content was ~18%, and the microsomal content was ~82% of total CYP2E1 (Fig. 11B).
The level of ROS production in five different cell lines, including the “mock”-transfected cell lines (as assayed by DCFH-DA method), did not change significantly without alcohol treatment (Fig. 11C). In WT CYP2E1-expressing cells, ROS production increased nearly 1.5-fold following alcohol treatment, and this was attenuated by Mito-CP or disulfiram. Treatment with membrane-permeable SOD increased the signal, suggesting that a high level of superoxide is formed. Treatment with catalase markedly reduced the signal, confirming the specificity of the assay. A similar pattern of ROS production was observed in cells expressing the W30R variant, except that the level of ROS following treatment with alcohol was significantly higher. Cells expressing the L32N variant also showed a >2-fold higher ROS level, which was not attenuated signifi-

FIGURE 10. Alcohol-mediated change in oxygen consumption rates in stable cells expressing CYP2E1. The OCR was measured using a Seahorse Bioscience XF24 Extracellular Flux Analyzer. Cells (30,000) from control and alcohol treatment (25 and 100 mM) were cultured for 16 h, followed by a change with XF medium (low buffered, bicarbonate-free, pH 7.4) for 1 h before the assay. The plate was incubated without CO2 for 1 h before recording the respiration rates. All parameters were analyzed using XF software and displayed as oxygen consumption rates (pmol O2/min). Oligomycin (2 μg/ml), DNP (50 μM), and rotenone (1 μM) were injected through Ports A, B, and C respectively. A, basal OCR, which accounts for base-line rates of oxygen consumption, was calculated as a percentage of WT control in 25 mM ethanol-treated cells. B, DNP-mediated uncoupling generated maximal OCR, which was also calculated as a percentage of WT control in 25 mM ethanol-treated cells. C and D, basal and maximal OCR calculated as a percentage of WT control with 100 mM ethanol. E, oligomycin-insensitive respiration or non-ATP-linked oxygen consumption (termed “proton leak”) was calculated as a percentage of WT control cells with added 25 and 100 mM ethanol. F, the extracellular acidification rate (ECAR), an indicator of glycolysis, was calculated using seahorse software with 25 and 100 mM ethanol. G, basal OCR in the cells with and without DAS for 48 h, calculated as a percentage of WT control cells. H, proton leak in the cells with and without DAS. Mean values ± S.D. (error bars) were calculated based on three separate measurements (*, p < 0.01; **, p < 0.001).
Human CYP2E1 Variants with Altered Targeting Signals

FIGURE 11. Subcellular distribution and alcohol-mediated ROS in HepG2 cells stably expressing WT or mutant CYP2E1. A, mitochondria and microsomes from HepG2 cells stably expressing CYP2E1 cDNAs were prepared as described under “Experimental Procedures.” Mitochondrial and microsomal fractions (50 μg of protein each) were subjected to immunoblot analysis using CYP2E1 antibody. The blot was also co-developed with antibody to NPR (a microsomal marker) and porin (a mitochondrial marker) to assess relative band intensity using LI-COR analysis tools. C, ROS levels in whole cells grown with or without ethanol (300 mM) were measured using DCFH-DA as the substrate. Cells (30,000) were plated overnight in phenol-free medium and treated with the mitochondria-targeted antioxidant Mito-CP (2.5 μM) and the CYP2E1 inhibitor disulfiram (25 μM). Cell-permeable SOD and catalase were used as controls. The fluorescence was recorded (excitation at 488 nm, emission at 525 nm) for 15 min. Values represent means ± S.D. (error bars) from three independent experiments. *, significant change (p < 0.05); **, highly significant change (p < 0.001).

DISCUSSION

A large volume of literature correlates CYP2E1 polymorphisms with a multitude of diseases, including ALD, diabetes, and several different cancers (31, 33, 35, 36, 38, 42, 43, 80, 81). A majority of the genetic screens so far have targeted either the 5′-promoter region, which regulates CYP2E1 transcription rates (CYP2E1*5B), or the catalytic domain (CYP2E1*6), which alters the catalytic rates of the enzyme (32). In this study, we report novel mutations that determine the extent of protein targeting to the endoplasmic reticulum and mitochondria. We showed that CYP2E1 variant W23/30R is preferentially targeted to mitochondria and that the L32N variant is more preferentially targeted to the endoplasmic reticulum. These observations lend further support to our hypothesis on bimodal protein targeting driven by the chimeric N-terminal signals and also provide evidence for a physiological significance of bimodal targeting and its role in diseases. Previously we showed, for family 1 and 2 CYPs, that either proteolytic processing or PKA/PKC-mediated phosphorylation at the N-terminal signal domain can activate the chimeric signals causing increased mitochondrial targeting of proteins (24, 48, 71, 72, 73). In this study, we conclude that an additional genetic factor affecting the signal recognition particle binding efficiency of nascent chains also affects targeting efficiency in a major way.

Previous studies have shown increased mRNA and protein levels in some human alcoholic and non-alcoholic liver tissues (6, 82–84). In most of these cases, increased steady-state levels were correlated with either increased transcription or altered stability of the protein. However, the consequence of these increases in terms of enzyme activity or alcohol toxicity remains unclear. The present study used a new strategy of analyzing the mitochondrial and microsomal isolates from frozen livers (which contained minimal cross-contaminating membranes) to evaluate the steady-state levels, followed by the sequence analysis of the of N-terminal 160-amino acid coding regions of cDNAs. We observed markedly variable levels of the mitochondrial and microsomal CYP2E1 contents in the sample pool (Fig. 1). In some cases, the mitochondrial CYP2E1 levels far exceeded the microsomal CYP2E1 levels, and vice-versa. Interestingly, the liver samples showing very high mitochondrial CYP2E1 showed a combination of W23R, W30R, and, in two cases, W23/30R double mutations. At least two samples showing very high microsomal CYP2E1 and very low mitochondrial CYP2E1 showed L32N mutations. Additionally, a computer algorithm analysis showed that W23/30R showed nearly 50% mitochondrial targeting potential, and the L32N variant showed less than 50% microsomal targeting potential, consistent with the extent of ER membrane integration. These results, for the first time, suggest that genetic variation affecting subcellular targeting of protein is an additional criterion to be considered in screens for genetic polymorphisms of CYPs. This information is of significant in view of our previous data showing that mitochondria-targeted rat CYP1A1 and CYP2B1 exhibit marked differences in substrate specificities compared with their microsomal counterparts. Similarly, mitochondria-tar-
targeted CYP2E1 showed a higher propensity for inducing oxidative stress and augmenting alcohol-induced toxicity.

CYP2E1 has often been considered to play an important role in alcohol-induced toxicity. Studies with CYP2E1-overexpressing mice have provided support for this hypothesis. Human ALD-like pathology is difficult to achieve by chronic alcohol feeding, and supplementation with cytokines (e.g. lipopolysaccharide (LPS)) is needed for this. However, studies with CYP2E1-overexpressing mice showed clear ALD-like pathology after 8 weeks of alcohol feeding (79). These results provide evidence for the role of CYP2E1 and its level of expression in alcohol toxicity, regardless of the mechanism. Our results, using cells stably expressing different variant forms, support these conclusions. More specifically, we showed that mitochondria-targeted CYP2E1 is more involved in toxicity, and therefore we hypothesize that carriers of W23/30R or other mutations that increase mitochondrial targeting of CYP2E1 are more prone to alcohol-induced liver disease.

Mitochondrial dysfunction has been implicated as an important factor in alcohol-mediated toxicity. Several studies, including our own, have shown oxidative damage to mitochondrial DNA and alterations in mitochondrial transcription in the livers of alcohol-treated rats and mice. Our results in both CYP2E1 stable expression cell lines and livers of alcohol-treated rats showed that CcO is a direct target for alcohol-induced toxicity. In support of this view, our present study using stably expressing cells showed that CYP2E1 variant W23/30R markedly induced alcohol-mediated mitochondrial damage. Specifically, our results showed increased basal as well as maximal OCR in CYP2E1 W23/30R-expressing cells, which was markedly reduced by alcohol treatment. However, both proton leak OCR and the extracellular acidification rate (which indicates lactate production) were increased in CYP2E1 W23/30R variant-expressing cells and were further increased by alcohol treatment. Alcohol had no effect on the proton leak and extracellular acidification rate in WT or L32N cells, suggesting the selective ability of the CYP2E1 W23/30R variant to augment alcohol toxicity.

Consistent with altered respiratory controls and proton leak OCR, the CcO activity in CYP2E1 W23/30R-expressing cells was reduced by ~25% in the absence of added alcohol and by > 50% in cells treated with alcohol, as compared with mock-transfected cells. This loss of activity was accompanied by markedly reduced levels of CcO subunits IV1, Vb, and CcO I. These same subunits were shown to be affected in cells subjected to hypoxia and myocardial tissues subjected to ischemia/reperfusion injury. Additionally, levels of the same CcO subunits were reduced in livers of rats treated with alcohol or cells expressing mitochondria-targeted (Mt+r) CYP2E1 treated with alcohol (27). Our results (Fig. 9) show that cells expressing W23/30R variant CYP2E1 show similar loss of CcO subunits in response to ethanol treatment. These results confirm our hypothesis that mitochondrial CcO is a primary target of alcohol-induced toxicity.

The metabolic activity of mitochondrial CYP2E1 appears to be a key requirement in amplifying alcohol toxicity because DAS and disulfiram, the CYP2E1 inhibitors used here, attenuated the alcohol-induced damage. At the concentrations used, both of these inhibitors did not cause any detectable cell death. It is possible that oxidation of alcohol and the accumulation of acetaldehyde may be an important cause of alcohol-induced toxicity. However, nearly complete reversal of mitochondrial damage by mitochondria-targeted Mito-CP and Mito-Q suggests that mitochondrial ROS also play an important role in the mitochondrial functional damage. Another possibility is that mitochondrial CYP2E1, in the presence of mitochondrial electron transfer proteins Adx and ADR, may also produce high levels of ROS in addition to producing toxic metabolites of alcohol. These possibilities are currently being investigated. However, the observation that the mitochondria-targeted antioxidants Mito-CP and Mito-Q not only rendered protection against but also reversed the toxic effects of alcohol suggests a novel therapeutic strategy for treating alcohol-mediated mitochondrial dysfunction and possibly tissue injury.

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