Role of CYP3A and CYP2E1 in Alcohol-Mediated Increases in Acetaminophen Hepatotoxicity: Comparison of Wild-Type and Cyp2e1(−/−) Mice


ABSTRACT:

CYP2E1 is widely accepted as the sole form of cytochrome P450 responsible for alcohol-mediated increases in acetaminophen (APAP) hepatotoxicity. However, we previously found that alcohol [ethanol and isopentanol (EIP)] causes increases in APAP hepatotoxicity in Cyp2e1(−/−) mice, indicating that CYP2E1 is not essential. Here, using wild-type and Cyp2e1(−/−) mice, we investigated the relative roles of CYP2E1 and CYP3A in EIP-mediated increases in APAP hepatotoxicity. We found that EIP-mediated increases in APAP hepatotoxicity occurred at lower APAP doses in wild-type mice (300 mg/kg) than in Cyp2e1(−/−) mice (600 mg/kg). Although this result suggests that CYP2E1 has a role in the different susceptibilities of these mouse lines, our findings that EIP-mediated increases in CYP3A activities were greater in wild-type mice compared with Cyp2e1(−/−) mice raises the possibility that differential increases in CYP3A may also contribute to the greater APAP sensitivity in EIP-pretreated wild-type mice. At the time of APAP administration, which followed an 11 h withdrawal from the alcohols, alcohol-induced levels of CYP3A were sustained in both mouse lines, whereas CYP2E1 was decreased to constitutive levels in wild-type mice. The CYP3A inhibitor triacetyloleandomycin (TAO) decreased APAP hepatotoxicity in EIP-pretreated wild-type and Cyp2e1(−/−) mice. TAO treatment in vivo resulted in inhibition of microsomal CYP3A-catalyzed activity, measured in vitro, with no inhibition of CYP1A2 and CYP2E1 activities. In conclusion, these findings suggest that both CYP3A and CYP2E1 contribute to APAP hepatotoxicity in alcohol-treated mice.

Acetaminophen (APAP) is considered to be a relatively safe drug and is widely prescribed and used for its analgesic and antipyretic properties. The first reports of hepatotoxicity in humans resulting from APAP overdose were in 1966 (Thomson and Prescott, 1966). Today, APAP is a frequent cause of liver failure and the leading cause of death due to accidental and deliberate overdose (for review, see Bromer and Black, 2003). APAP is mainly glucuronidated and sulfated in the liver by phase II metabolic pathways, leading to excretion of the drug. However, APAP is also metabolized by certain forms of cytochromes P450 (P450s) to N-acetyl-p-benzoquinone imine (NAPQI), a highly electrophilic metabolite that is considered to be responsible for triggering the ensuing liver damage. CYP1A2, CYP2E1, and CYP3A are three forms of rat and human P450s active in the conversion of APAP to NAPQI, with CYP2E1 and CYP3A having greater intrinsic activity than CYP1A2 (Patten et al., 1993; Thummel et al., 1993). Reduced glutathione (GSH) reacts with NAPQI, resulting in the inactivation of this reactive metabolite. When GSH levels become depleted, liver damage can occur as a result of the
binding of NAPQI to cellular proteins, generation of reactive oxygen species, or a combination of both processes (for review, see Bromer and Black, 2003). Consumption of alcoholic beverages is associated with an increased risk of development of liver damage from APAP (for review, see Bromer and Black, 2003). Alcohol consumption has been shown to increase CYP2E1 in experimental animals (Koop et al., 1982) and is associated with elevated levels of CYP2E1 in humans (Raucy et al., 1987; Wrighton et al., 1987). These alcohol-mediated increases in CYP2E1 are widely considered to have a major role in the association of chronic alcohol consumption and APAP hepatotoxicity (for review, see Bromer and Black, 2003). However, we have found that alcohol pretreatment increases APAP hepatotoxicity in Cyp2e1−/− mice, showing that CYP2E1 is not essential in the alcohol effect (Sinclair et al., 2000c). In other animal studies, alcohol has been reported to also increase CYP3A and CYP1A2 in the liver (Louis et al., 1994; Roberts et al., 1995; Gorman et al., 2004). Human consumption of alcoholic beverages is also associated with increases in CYP3A (Hoshino and Kawakatsu, 1995; Niemelä et al., 1998). CYP3A has been reported to have a role in alcohol-mediated increases in APAP hepatotoxicity in rats as shown by two different approaches. In one study, treatment with a combination of ethanol and isopentanol, the predominant alcohols in most alcoholic beverages, resulted in synergistic increases in CYP3A and APAP hepatotoxicity in rats (Kostrubsky et al., 1995b). The combined alcohol treatment resulted in no further increase in CYP2E1 than that caused by either alcohol alone, suggesting that CYP3A is responsible for the synergistic increase in APAP hepatotoxicity (Kostrubsky et al., 1995b). In another study, triacetyloleandomycin (TAO), an inhibitor of CYP3A (Pessayre et al., 1981; Newton et al., 1995), protected rats pretreated with ethanol, either alone or in combination with isopentanol, from alcohol-mediated increases in APAP hepatotoxicity, also supporting a role of CYP3A (Kostrubsky et al., 1997; Sinclair et al., 2000b).

Although administration of APAP to animals pretreated with inducers of CYP3A resulted in increased toxicity (Mitchell et al., 1973), the role of induced CYP3A in APAP hepatotoxicity in humans is less clear. The results of two crossover studies using prototypic inducers of CYP3A in human volunteers were contradictory as to the role of drug-induced CYP3A in NAPQI formation, measured as the glutathione adduct of APAP (APAP-SG). One study showed an increase in APAP-SG formation when subjects were pretreated with phenobarbital (Mitchell et al., 1974), whereas the other reported no effect with rifampin pretreatment (Manyike et al., 2000). Here, we investigated the relative roles of CYP2E1 and CYP3A in alcohol-mediated increases in APAP hepatotoxicity in mice. To assess the role of CYP2E1 in this toxicity, we compared alcohol-pretreated Cyp2e1−/− mice with wild-type mice. To assess the role of CYP3A, we used TAO as an inhibitor of CYP3A (Pessayre et al., 1981; Loeper et al., 1994; Newton et al., 1995). TAO protected alcohol-pretreated wild-type mice as well as Cyp2e1−/− mice from APAP hepatotoxicity. The administration of TAO in vivo inhibited CYP3A activity and had no effect on CYP2E1- and CYP1A2-catalyzed activities. Altogether, our findings suggest that CYP3A has a role in alcohol-mediated increases in APAP hepatotoxicity in mice.

Materials and Methods

Reagents. Lieber-DeCarli liquid diet and maltose-dextrin were purchased from Bio-Serv Inc. (Frenchtown, NJ). Absolute ethanol (USP) was from PharmCo Products Inc. (Brookfield, CT). APAP, p-nitrophenol, TAO, isopentanol (a mixture of 70% 3-methylbutanol and 30% 2-methylbutanol), triazolam (TRZ), NAD+, NADPH, Brij 58 (polyoxyethylene 20-cetyl ether), UDPGA (sodium salt), magnesium chloride, N-isocitric acid, isocitric dehydrogenase, potassium phosphate buffer solutions, 2-acetamidophenol, 1-heptanesulfonic acid, perchloric acid, hydrochloric acid, and GSH were obtained from Sigma-Aldrich (St. Louis, MO). Tris, methanol, sodium chloride, trichloroacetic acid, and acetic acid were purchased from Fisher Scientific Co. (Pittsburgh, PA). The Upjohn Company (Kalamazoo, MI) kindly provided 4-OH triazolam. The APAP-SG, used as a standard for HPLC analysis, was a generous gift from Dr. Mark W. Gennbrosy (Chem Design Corporation, Pittsburgh, MA). 7-Me-thoxyresorufin was from Molecular Probes (Eugene, OR). The polyclonal rabbit anti-human CYP2E1 antibody, which detected mouse CYP2E1, was obtained from Oxford Biomedical Research (Oxford, MI). The polyclonal goat antibody against rat CYP1A1/2 cross-reacted with mouse CYP1A1/2 but not with CYP3A or CYP2E1 (Sinclair et al., 1998). The polyclonal rabbit antibody prepared against human CYP3A4 detected mouse CYP3A, but not CYP1A2 or CYP2E1 (Sinclair et al., 1998). Electrophoresis and immunoblotting reagents were from Bio-Rad (Hercules, CA). Assay kits for glutathione reductase and glutathione peroxidase were purchased from Cayman Chemical (Ann Arbor, MI). All other reagents were purchased from either Sigma-Aldrich or Fisher Scientific Co.

Animals. Wild-type and Cyp2e1−/− mice in a 129Sv background (Lee et al., 1996) were maintained in a controlled environment with a 12-h light/ dark cycle at the Veterans Administration animal facility. Hepatic microsomes from each Cyp2e1−/− mouse were analyzed for CYP2E1 immunochromically, as described below, to confirm their phenotype. The Institutional Animal Care and Use Committees at the Veterans Administration Medical Center and Dartmouth College approved all animal protocols. At 3 months of age, male mice, weighing 18 to 30 g, were fed the control liquid Li ber-DeCarli diet (F1259SF) for an initial 2-day adaptive period. In most investigations of alcohol-mediated increases in APAP hepatotoxicity, animals were treated with ethanol alone. However, most alcoholic beverages contain higher chain alcohols, with isopentanol being the most abundant (for review, see Kostrubsky et al., 1995b). Therefore, in our studies on alcohol-mediated APAP hepatotoxicity, we use a combined treatment of ethanol plus isopentanol (EIP). After the 2-day adaptive period, EIP-treated animals were then administered 2.8% (w/v) ethanol and 0.4% (w/v) isopentanol in the ethanol liquid Lieber-DeCarli diet (F1258SP) for 7 days. The control diet was made isocaloric to the EIP-containing diet by the addition of maltose-dextrin, as directed by the manufacturer. For investigations of APAP hepatotoxicity and TAO protection, the liquid diets were replaced with water 11 h before the administration of APAP to allow for the elimination of EIP, because as little as 5 mM ethanol in the bloodstream was shown to be protective against APAP hepatotoxicity (Thummel et al., 1988). TAO (500 mg/kg) was prepared in acid-saline as described previously (Pessayre et al., 1981; Kostrubsky et al., 1997) and administered i.p. 2 h before APAP administration. APAP, at the doses specified in the figure legends, was prepared as described previously (Kostrubsky et al., 1997) and administered intragastrically (i.g.) to mimic the route of human ingestion. Seven hours later, the mice were anesthetized with carbon dioxide, and blood was collected by cardiac puncture before euthanization by cervical dislocation. Liver slices were fixed in 10% buffered formalin for histological analysis. For analyses of CYP2E1, CYP3A, and CYP1A2, separate groups of animals were either euthanized at the end of the 7-day EIP treatment or 11 h after withdrawal from the diet, as indicated in the figure legends, and hepatic microsomes were prepared as described below. Hepatic microsomes were also prepared from animals withdrawn from the diet for 11 h, but administered TAO 2 h before euthanization.

Preparation of Hepatic Microsomes. Hepatic microsomes were prepared from 20% (w/v) liver homogenates made in buffer containing 0.1 M Tris-acetate (pH 7.4), 0.1 M KCl, 0.1 mM EDTA, 230 μM phenylmethlysulfonfluoride, and 22.7 μM butylated hydroxytoluene. The homogenates were centrifuged at 10,000g for 10 min at 4°C. The supernatant was centrifuged at 100,000g for 1 h at 4°C. The resulting pellet was washed with 0.15 M KCl to remove any contaminating hemoglobin. The final pellet was resuspended in 0.1 M phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 1 mM EDTA and stored at −80°C.

Immunochromical Analyses of Cytochromes P450. Hepatic microsomes were analyzed for CYP3A, CYP2E1, and CYP1A2 immunochromically. For CYP2E1 and CYP3A, microsomal proteins were separated by SDS-polyacryl-amide gel electrophoresis (10% acrylamide) at 160 V for 1 h. A mixed amionic detergent was used during the 1 h electrophoresis of gels for CYP1A2, as described previously (Sinclair et al., 1990a). To separate the CYP3A forms, the
following modifications were used (Wolf et al., 2005). The electrophoresis buffer contained 37.5 mM Tris base, 290 mM glycine, and 1.5% (w/v) SDS. The gels were electrophoresed at 160 V for 105 min, with the first 45 min at room temperature and the last 60 min at 4°C. For all gels, proteins were then transferred to nitrocellulose electrophoretically and the cytochromes P450 were detected immunchemically as described previously (Sinclair et al., 1990a). The secondary antibodies were either alkaline phosphatase conjugated goat anti-rabbit (CYP2E1 and CYP3A) or rabbit anti-goat (CYP1A2). For quantification of CYP1A2, the immunoblots were scanned into Adobe Photoshop (Adobe Systems, Mountain View, CA), using an HP Precision Scanner (Hewlett Packard, Palo Alto, CA), and OneDScan software (Scanalytics, Fairfax, VA) was used for quantification. Eight different samples were analyzed for each treatment; each sample was scanned in triplicate and normalized to an internal control. The intensities were expressed as relative arbitrary units.

**Histological Analysis of Liver Damage.** Formalin-fixed, paraffin-embedded liver sections, stained with hematoxylin and eosin, were graded histologically for congestion and necrosis by our pathologist (J.G.S.) who was blinded to the treatments. The numerical grading of damage was 1, mild (in <30% of lobule); 2, moderate (in 30–60% of lobule); and 3–4, severe (in >60% of lobule).

**Measurement of Microsomal Formation of APAP-SG.** The rates of microsomal NAPQI formation in the presence of APAP and GSH were measured from the rate of formation of APAP-SG as described previously (Sinclair et al., 1990b), with some modifications. The rate of APAP-SG formation was assayed in a total volume of 0.25 ml, containing 0.5 mg of hepatic microsomal protein, 37 mM Tris-HCl (pH 7.2), 2.5 mM glutathione, 2 mM NADPH, 20 mM isocitrate, 5 units of isocitrate dehydrogenase, and 5 mM MgCl₂. The reaction was initiated by the addition of APAP to a final concentration of 0.5 mM. After incubation at 37°C for 30 min, the reaction was terminated by the addition of 0.25 ml of 0.2% (v/v) perchloric acid. The supernatants were analyzed by HPLC, isocratically, using a Dynamax 5-µm C₁₈ Microsorb HPLC column (4.6 mm i.d. x 250 mm; Varian Inc., Palo Alto, CA), a mobile phase, containing 3.5 mM heptanesulfonic acid, 20% (v/v) acetic acid, and a flow rate of 0.9 ml/min. Absorbance was monitored at a wavelength of 254 nm over a 30-min time period. Purified APAP-SG was analyzed separately as a standard. APAP-SG eluted at 23 min with a 10% window.

**Measurement of the Cytochrome P450-TAO Metabolic Intermediate Complex.** To determine whether the TAO dose and exposure time were sufficient to allow for a CYP3A-TAO metabolic intermediate (MI) complex to form, the cytochrome P450-TAO MI complex was measured in hepatic microsomes as described previously (Wrighton et al., 1985). Because hepatic CYP3A levels are too low to measure the cytochrome P450-TAO MI complex in untreated and alcohol-pretreated animals (Kostrubsky et al., 1997), hepatic microsomes from wild-type mice pretreated with pregnenolone 16α-carbonitrile, a prototypical inducer of CYP3A in mice, were used. These animals were administered the control liquid Lieber-DeCarli diet as described earlier, with or without daily injections of 50 mg of pregnenolone 16α-carbonitrile/kg i.p. for the final 3 days. After a 2-h exposure to TAO (500 mg/kg i.p.), hepatic microsomes were prepared as described earlier, and the cytochrome P450-TAO MI complex was assayed, as described previously (Wrighton et al., 1985). The cytochrome P450-TAO MI complex accounted for 45% of the total hepatic cytochrome P450. These results indicate that the dose of TAO and exposure time were sufficient for CYP3A to form the MI complex with TAO.

**Measurement of GSH in Liver Cytosolic and Mitochondrial Fractions.** Mice were administered the control or EIP diet as described earlier. After an 11-h withdrawal from the diets, the animals were euthanized immediately or 1 h after the administration of APAP. Liver homogenates (20% v/w) were prepared in buffer (pH 7.2) consisting of 0.25 M sucrose, 1 mM EDTA, 10 mM KCl, and 10 mM Tris HCl. The homogenates were centrifuged at 1000g for 10 min at 4°C, and the supernatants were recentrifuged at 10,000g for 10 min at 4°C. The resulting supernatant served as the cytosolic fraction and the pellet as the mitochondrial fraction. Cytosolic fractions were extracted with an equal volume of 10% (v/v) trichloroacetic acid (TCA), whereas 5% (v/v) TCA was added directly to the mitochondrial pellets. The resulting TCA extracts were centrifuged at 10,000g for 10 min at 4°C, and the supernatants were used to analyze GSH as described previously (Sinclair et al., 2000b). The TCA-extracted pellets were solubilized in 0.1 N NaOH/0.1% (w/v) SDS for protein determinations.

**Additional Assays.** Serum alanine aminotransferase (ALT) levels were analyzed in the Veterans Administration Medical Center’s clinical laboratory on an automated DADE Dimension AR Clinical Chemistry System (Dade Behring Inc., Deerfield, IL). Protein concentrations were assayed by the procedure of Lowry et al. (1951), using bovine serum albumin as the standard. Glutathione peroxidase and glutathione reductase activities were measured as described previously (Wolf et al., 2005). The microsomal formation of 6β-hydroxytestosterone was measured by HPLC analysis as described previously (Kostrubsky et al., 1999). Microsomal glucuronidation of APAP was measured as described previously (Court and Greenblatt, 1997). TRZ 4-hydroxylation activity was assayed in hepatic microsomes as described previously (Perloff et al., 2000). The hydroxylation of p-nitrophenol was assayed as described previously (Sinclair et al., 1989). The activity of CYP1A2 was measured fluorometrically in hepatic microsomes as the formation of resorufin over time from the demethylation of methoxyresorufin, as described previously (Sinclair et al., 1997), except that the assay contained 2.5 µM methoxyresorufin as the substrate.

**Statistical Analyses.** Values represent means ± S.D. Data analyses were performed by one-way analysis of variance (ANOVA) followed by the Student Newman-Keuls multiple comparisons test, two-way ANOVA, an unpaired Student’s t test, or a Fisher’s exact test, as indicated in the figure and table legends. Significance was indicated by p < 0.05.

**Results**

**Comparison of EIP-Mediated Increases in APAP Hepatotoxicity in Wild-Type and Cyp2e1(−/−) Mice.** To ascertain the contribution of CYP2E1 in EIP-mediated increases in APAP hepatotoxicity, we compared the responses of wild-type and Cyp2e1(−/−) mice. Liver damage was assessed by serum levels of ALT. In the absence of EIP pretreatment, 300 mg of APAP/kg was much more hepatotoxic in wild-type mice than in Cyp2e1(−/−) mice (Fig. 1) (p < 0.05), confirming the results of Lee et al. (1996).

EIP pretreatment increased APAP hepatotoxicity in both wild-type mice and Cyp2e1(−/−) mice. Animals were fed liquid Lieber-DeCarli diet alone or containing 2.8% (w/v) ethanol and 0.4% (w/v) isopentanol (EIP) for 7 days, as described under Materials and Methods. The diet was replaced with water for 11 h before the administration of APAP i.g., at the doses indicated, and 7 h later, the mice were euthanized, as described under Materials and Methods. The treatment groups consisted of the following: wild-type mice: control + 300 mg of APAP/kg (n = 4) and EIP + 300 mg of APAP/kg (n = 10); Cyp2e1(−/−) mice: control + 300 mg of APAP/kg (n = 5), EIP + 300 mg of APAP/kg (n = 6), control + 600 mg of APAP/kg (n = 9), and EIP + 600 mg of APAP/kg (n = 12). Serum levels of ALT were measured, as described under Materials and Methods. Each value represents the mean with the S.D. indicated by the vertical lines. ALT levels in untreated mice, both wild-type and Cyp2e1(−/−), were 195 ± 16 units/liter and treatment with EIP caused no elevation in serum ALT levels in either mouse line (results not shown). Data were analyzed by an unpaired Student’s t test, *p < 0.05 versus wild-type mice treated with APAP alone; ***, p < 0.001 versus Cyp2e1(−/−) mice treated with APAP alone.

**FIG. 1.** Effect of EIP pretreatment on APAP hepatotoxicity in wild-type and Cyp2e1(−/−) mice. Animals were fed liquid Lieber-DeCarli diet alone or containing 2.8% (w/v) ethanol and 0.4% (w/v) isopentanol (EIP) for 7 days, as described under Materials and Methods. The diet was replaced with water for 11 h before the administration of APAP i.g., at the doses indicated, and 7 h later, the mice were euthanized, as described under Materials and Methods. Each value represents the mean with the S.D. indicated by the vertical lines. ALT levels in untreated mice, both wild-type and Cyp2e1(−/−), were 195 ± 16 units/liter and treatment with EIP caused no elevation in serum ALT levels in either mouse line (results not shown). Data were analyzed by an unpaired Student’s t test, *p < 0.05 versus wild-type mice treated with APAP alone; ***, p < 0.001 versus Cyp2e1(−/−) mice treated with APAP alone.
and Cyp2e1(−/−) mice (Fig. 1). However, the dose of APAP at which EIP increased toxicity was lower in wild-type mice. At 300 mg of APAP/kg, EIP pretreatment increased APAP hepatotoxicity in wild-type mice, but not in Cyp2e1(−/−) mice (Fig. 1). In Cyp2e1(−/−) mice, APAP hepatotoxicity was increased by EIP pretreatment at a dose of 600 mg of APAP/kg (Fig. 1). Thus, wild-type mice were more sensitive to EIP-mediated increases in APAP hepatotoxicity compared with Cyp2e1(−/−) mice, suggesting a role of CYP2E1.

Effect of Withdrawal from EIP on Hepatic Levels of CYP2E1, CYP3A, and CYP1A2. Alcohol has been shown in rodents to increase hepatic levels of three P450s involved in APAP activation, CYP2E1, CYP3A, and CYP1A2 (Louis et al., 1994; Roberts et al., 1995; Gorman et al., 2004). In all studies investigating alcohol-mediated APAP hepatotoxicity, the alcohol-containing liquid diets are withdrawn 11 to 24 h before administration of APAP to ensure complete clearance of alcohol from the blood, because blood ethanol levels as low as 5 mM protect animals from APAP hepatotoxicity (Thummel et al., 1988). Therefore, we measured hepatic levels of cytochromes P450, as well as their status at the time of APAP administration.

In wild-type mice not withdrawn from the diet, EIP treatment increased CYP2E1, CYP3A, and CYP1A2 (Fig. 2A). The increase in CYP1A2 by EIP in wild-type mice was detected in six of eight mice tested, with a mean ± S.D. fold increase for all eight mice of 1.82 ± 0.26 (p < 0.001). In Cyp2e1(−/−) mice, EIP increased CYP3A but not CYP1A2 (Fig. 2B). The antibody to CYP3A detected at least two CYP3A proteins in both mouse lines. EIP treatment increased both proteins. However, by 11 h after withdrawal from the EIP diet, the levels of CYP2E1 and CYP1A2 decreased to constitutive levels in wild-type mice (Fig. 2A). In contrast, EIP-induced levels of CYP3A remained elevated in both wild-type and Cyp2e1(−/−) mice (Fig. 2, A and B).

Using microsomes from Cyp2e1(−/−) mice, we found previously that increases in APAP-SG formation at a substrate concentration of 0.5 mM APAP correlated with increases in immunoreactive CYP3A protein (Sinclair et al., 2000c). Therefore, we investigated whether this correlation also occurred in wild-type mice when CYP2E1 is present. In these experiments, NAPQI was measured by the formation of the glutathione conjugate APAP-SG, as described under Materials and Methods. In the current study, the activities in control mice not withdrawn from the diet were similar in wild-type and Cyp2e1(−/−) mice (Table 1), suggesting little to no contribution of CYP2E1 at a concentration of 0.5 mM APAP. EIP treatment increased the activities in both wild-type and Cyp2e1(−/−) mice (Table 1). After withdrawal from the diet, the EIP-mediated increases in activity were sustained in both mouse lines, and the activity was greater in wild-type mice (Table 1). These latter findings suggest that, at the time of APAP administration, after withdrawal of the diet, CYP3A activity was greater in wild-type mice compared with Cyp2e1(−/−) mice. The 6β-hydroxylation of testosterone, an activity attributed to CYP3A (Waxman et al., 1988), was also greater in wild-type mice compared with Cyp2e1(−/−) mice (3005 ± 384 versus 1085 ± 293 pmol/min/mg protein, respectively; p < 0.001). These results are consistent with our previous findings showing that EIP-mediated increases in CYP3A protein were greater in wild-type mice than in Cyp2e1(−/−) mice (Wolf et al., 2004).

Effect of TAO on APAP Hepatotoxicity in EIP-Pretreated Mice. We investigated the role of CYP3A in EIP-mediated increases in APAP hepatotoxicity using TAO, an inhibitor of CYP3A activity (Newton et al., 1995). APAP was administered at doses at which EIP treatment increased APAP hepatotoxicity, the alcohol-containing liquid diets are withdrawn 11 to 24 h before administration of APAP to ensure complete clearance of alcohol from the blood, because blood ethanol levels as low as 5 mM protect animals from APAP hepatotoxicity (Thummel et al., 1988). Therefore, we measured hepatic levels of cytochromes P450, as well as their status at the time of APAP administration.

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Effect of TAO on APAP Hepatotoxicity in EIP-Pretreated Mice. We investigated the role of CYP3A in EIP-mediated increases in APAP hepatotoxicity using TAO, an inhibitor of CYP3A activity (Newton et al., 1995). APAP was administered at doses at which EIP treatment increased APAP hepatotoxicity in each mouse line (300 mg/kg for wild-type mice and 600 mg/kg for Cyp2e1(−/−) mice) (Fig. 1), and serum levels of ALT were measured. Administration of TAO, 2 h before APAP, protected both wild-type and Cyp2e1(−/−) mice from EIP-mediated increases in APAP hepatotoxicity (Fig. 3). In addition, hepatotoxicity was measured in the livers of these animals by histological analysis (Table 2). Moderate to severe congestion and necrosis were observed in EIP-pretreated mice administered APAP (Table 2). Preadministration of TAO to EIP-pretreated animals re-

<table>
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<tr>
<th>Mouse Line</th>
<th>Diet</th>
<th>APAP-SG Formation</th>
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<tr>
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<tr>
<td>Wild-type</td>
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<td>Control</td>
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<td></td>
<td>EIP</td>
<td>0.53 ± 0.12</td>
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Group pairs that were found to be significantly different are indicated by connecting brackets: ***p < 0.001; **p < 0.01; *p < 0.05.
Receiving APAP prevented the development of centrilobular congestion and decreased necrosis in both wild-type and Cyp2e1(−/−) mice (Table 2). In the absence of APAP, no centrilobular congestion or necrosis was detected in EIP-pretreated wild-type and Cyp2e1(−/−) mice treated with or without TAO (Table 2). TAO also did not cause elevations in serum levels of ALT (results not shown), indicating that the dose of TAO administered was not hepatotoxic over this time period of exposure.

Determination of the Specificity of in Vivo TAO Treatment on Cytochrome P450-Catalyzed Enzyme Activities. TAO has been shown to be specific for inhibition of human CYP3A activity in vitro (Newton et al., 1995). Although TAO has been used in mice to identify a role of CYP3A in metabolism and toxicity studies (Pessayre et al., 1981; Loeper et al., 1994), its specificity for CYP3A after in vivo administration has not been demonstrated in mice. Here we investigated whether TAO administered in vivo inhibits the hepatic enzymatic activities of CYP1A2, CYP2E1, and CYP3A. In these studies, wild-type mice were pretreated with EIP and TAO as in the previous experimental protocols. Mice were euthanized 2 h after the administration of TAO, and the enzymatic activities of CYP1A2, CYP2E1, and CYP3A were measured in hepatic microsomes prepared from these animals. The activity of CYP1A2 was measured by the O-demethylation of methoxyresorufin (MROD). This activity is mainly catalyzed by CYP1A2 in the absence of CYP1A1 (Sinclair et al., 2000a). As shown in Fig. 4, microsomal MROD activities were not affected by short-term in vivo TAO treatment. Franklin et al. (2000) reported that daily administration of TAO for 1 month to mice also did not inhibit MROD activity, measured in vitro. The activity of CYP2E1 was measured by the O-hydroxylation of p-nitrophenol (PNPH), as this activity is a specific measure of CYP2E1 in mice (Carlson, 2003; Wolf et al., 2004). TAO treatment did not affect microsomal PNPH activity (Fig. 4). The activity of CYP3A was measured by the 4-hydroxylation of TRZ, a relatively specific measure of CYP3A enzymatic activity in mice (Perloff et al., 2000). In vivo administration of TAO to EIP-pretreated mice decreased TRZ 4-hydroxylation activity by 40% (Fig. 4).

Comparison of Hepatic Levels of APAP Glucuronidation in Wild-Type and Cyp2e1(−/−) Mice. In mice, the glucuronidation of APAP is a major pathway of detoxification (Wolf et al., 2005). To determine whether a difference in the glucuronidation of APAP could contribute to the differences in APAP hepatotoxicity observed in the two mouse lines, we compared the formation of the glucuronide-conjugated metabolite of APAP (APAP-Gluc) by hepatic microsomes. At 11 h after withdrawal from the diet, the rates of APAP-Gluc formation from both mouse lines were similar, regardless of EIP treatment (Fig. 5).
Effect of TAO on alcohol-mediated increases in APAP hepatotoxicity

Wild-type and Cyp2e1(−/−) mice were fed and administered APAP as described in the legend to Figure 3. Liver slices, fixed in formalin at the time of euthanasia, were stained with hematoxylin and eosin and examined histologically for congestion and necrosis. Grading for damage was as follows: normal, no damage; mild, damage in <30% of lobule; moderate, damage in 30 to 60% of lobule; severe, damage in >60% of lobule. The numbers in each column represent the number of mice with the noted degree of damage. Fisher's exact test was used to determine which numbers observed were significantly different. The results are displayed in the p value column.

<table>
<thead>
<tr>
<th>Mouse Line and Treatment</th>
<th>No. of Animals</th>
<th>Congestion</th>
<th>p</th>
<th>Necrosis</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
<td>Mild</td>
<td>Moderate</td>
<td>Severe</td>
</tr>
<tr>
<td>Wild-type</td>
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<td></td>
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<td>EIP</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EIP + TAO</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EIP + APAP</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>5</td>
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<tr>
<td>EIP + TAO + APAP</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cyp2e1(−/−)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIP</td>
<td>2</td>
<td>2</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EIP + TAO</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
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</table>

Comparison of Hepatic GSH Levels in Wild-Type and Cyp2e1(−/−) Mice. The hepatic level of GSH is a critical factor in APAP hepatotoxicity. GSH conjugates and deactivates NAPQI and decreases oxidative damage (for review, see Bromer and Black, 2003). Therefore, we compared GSH levels in the livers of wild-type and Cyp2e1(−/−) mice to ascertain the roles of CYP2E1 and CYP3A in alcohol-mediated increases in APAP hepatotoxicity. We found that EIP-treated wild-type and Cyp2e1(−/−) mice to ascertain the roles of CYP2E1 and CYP3A in alcohol-mediated increases in APAP hepatotoxicity. We found that EIP-mediated increases in APAP hepatotoxicity were greater in wild-type mice compared with Cyp2e1(−/−) mice. In mice pretreated with EIP, hepatic toxicity occurred at a lower dose of APAP in wild-type mice (300 mg/kg) compared with Cyp2e1(−/−) mice (600 mg/kg) (Fig. 1). Thus, one conclusion from these findings is that CYP2E1, when present, does have a role in EIP-mediated increases in APAP hepatotoxicity. We investigated whether EIP-mediated increases in CYP3A would also have a role. We found that TAO, an inhibitor reported to be relatively specific for CYP3A in vitro in human liver microsomes (Newton et al., 1995), protected both wild-type mice and Cyp2e1(−/−) mice from APAP hepatotoxicity (Fig. 3; Table 2), suggesting a role for CYP3A. Because TAO, administered in vivo, protected the hepatic microsomal activity of CYP3A (Fig. 4), but not the activities of CYP2E1 and CYP1A2 (Fig. 4), the effect of TAO on APAP hepatotoxicity was, thus, considered to be specific for CYP3A. In investigating the effect of TAO on the activities of the cytochromes P450, the cytochrome P450 activities were measured in liver microsomes harvested 2 h after

Discussion

Consumption of alcoholic beverages has been identified as a risk factor in APAP hepatotoxicity. Although CYP2E1 is widely accepted to be the sole form of cytochrome P450 responsible for APAP hepatotoxicity (for review, see Bromer and Black, 2003), we previously found that EIP increased APAP hepatotoxicity in Cyp2e1(−/−) mice, showing that CYP2E1 is not essential in alcohol-mediated increases in APAP hepatotoxicity (Sinclair et al., 2000c). Alcohol has been shown to induce CYP3A along with CYP2E1 in rats (Louis et al., 1994; Roberts et al., 1995) and in primary cultures of human and rat hepatocytes (Sinclair et al., 1991; Kostrubsky et al., 1995a). TAO, an inhibitor of CYP3A, protected rats from alcohol-mediated increases in APAP hepatotoxicity, suggesting a role of CYP3A (Kostrubsky et al., 1997; Sinclair et al., 2000b). Here, we compared EIP-treated wild-type and Cyp2e1(−/−) mice to ascertain the roles of CYP2E1 and CYP3A in alcohol-mediated increases in APAP hepatotoxicity. We found that EIP-mediated increases in APAP hepatotoxicity were greater in wild-type mice compared with Cyp2e1(−/−) mice. In mice pretreated with EIP, hepatotoxicity occurred at a lower dose of APAP in wild-type mice (300 mg/kg) compared with Cyp2e1(−/−) mice (600 mg/kg) (Fig. 1). Thus, one conclusion from these findings is that CYP2E1, when present, does have a role in EIP-mediated increases in APAP hepatotoxicity. We investigated whether EIP-mediated increases in CYP3A would also have a role. We found that TAO, an inhibitor reported to be relatively specific for CYP3A in vitro in human liver microsomes (Newton et al., 1995), protected both wild-type mice and Cyp2e1(−/−) mice from APAP hepatotoxicity (Fig. 3; Table 2), suggesting a role for CYP3A. Because TAO, administered in vivo, protected the hepatic microsomal activity of CYP3A (Fig. 4), but not the activities of CYP2E1 and CYP1A2 (Fig. 4), the effect of TAO on APAP hepatotoxicity was, thus, considered to be specific for CYP3A. In investigating the effect of TAO on the activities of the cytochromes P450, the cytochrome P450 activities were measured in liver microsomes harvested 2 h after...
administration of TAO in an attempt to assess the activities at the time of APAP administration. TAO, administered in vivo, resulted in only partial inhibition of CYP3A activity (40%), measured as the hydroxylation of TRZ at the 4 position. This partial inhibition of CYP3A may have been sufficient to afford protection from APAP hepatotoxicity. Alternatively, either the CYP3A activity, measured in vitro, may underestimate the inhibition occurring in vivo or a longer exposure to TAO in vivo may have resulted in greater inhibition of APAP activation by CYP3A. However, TAO may conceivably have other effects on the liver, independent of CYP3A, that contribute to the protection.

In all studies on alcohol-mediated APAP hepatotoxicity in experimental animals, alcohol is withdrawn 11 to 24 h before APAP administration (for review, see Sinclair et al., 2000b). During this withdrawal period, alcohol-induced levels of CYP2E1 decreased to constitutive levels in mice (Fig. 2), similar to the findings in rats (Roberts et al., 1995; Kostrubsky et al., 1997). However, constitutive levels of CYP2E1 may still contribute to APAP toxicity in alcohol-pretreated animals. Our findings that EIP-induced levels of CYP3A were sustained in both wild-type and Cyp2e1(−/−) mice after the withdrawal period (Fig. 2), while increases in CYPs 2E1 and 1A2 were not sustained in wild-type mice, support the conclusion that CYP3A may also have a role in EIP-mediated increases in APAP hepatotoxicity. We previously reported that the amount of EIP-induced CYP3A protein was greater in wild-type mice compared with...
Cyp2e1(−/−) mice (Wolf et al., 2004). Here we found that, at the
time of APAP administration, CYP3A-catalyzed activity, measured as
the 6β-hydroxylation of testosterone, was also greater in wild-type
mice compared with Cyp2e1(−/−) mice, all pretreated with EIP.
These findings suggest that the higher level of CYP3A in wild-type
mice may also contribute to its greater sensitivity to EIP-mediated
increases in APAP hepatotoxicity compared with Cyp2e1(−/−) mice.
Our findings that TAO treatment in vivo almost totally protected
EIP-pretreated wild-type and Cyp2e1(−/−) mice from APAP hepa-
totoxicity (Table 2; Fig. 3) and selectively inhibited CYP3A activity
(Fig. 4) further support a role for CYP3A.
In our studies with untreated mice, there was no difference in the
rate of APAP-SG formation by hepatic microsomes from wild-type and
Cyp2e1(−/−) mice (Table 1), suggesting no contribution of
CYP2E1 at the concentration of APAP used in the assay (0.5 mM).
Surprisingly, in untreated mice withdrawn from the diet, the rate of
APAP-SG formation by hepatic microsomes was 2-fold greater in
wild-type mice compared with Cyp2e1(−/−) mice, suggesting that
the cytochrome P450 responsible for this activity, possibly CYP3A,
along with CYP2E1, could contribute to the greater sensitivity of
wild-type mice to APAP hepatotoxicity.
Our findings that CYP3A has a role in alcohol-mediated APAP
hepatotoxicity in rodents are consistent with other rodent studies
in which treatment with prototypic inducers of CYP3A, such as pheno-
barbital, which do not induce CYP2E1 (Johansson et al., 1988),
increase APAP hepatotoxicity (Mitchell et al., 1973). Retrospective
studies in humans suggest that treatment with inducers of CYP3A is
a risk factor in APAP hepatotoxicity (for review, see Bromer and
Black, 2003). In two separate crossover studies in humans, treating
with inducers of CYP3A and using APAP pharmacokinetics and
formation of APAP metabolites as endpoints, contradictory results
have been obtained on whether drug-induced CYP3A has a role in
APAP activation in humans. In one study by Mitchell et al. (1974),
APAP-SG formation was increased in six of seven volunteers pre-
treated with phenobarbital, an inducer of CYP3A in humans (Watkins
et al., 1985). However, another crossover study (Manyike et al., 2000)
failed to find increased APAP-SG formation in volunteers pretreated
with rifampin, another inducer of CYP3A in humans (Ged et al.,
1989). Thus, further investigation is needed to determine whether
induction of CYP3A in humans may be a potential risk factor for
APAP hepatotoxicity. TAO has been administered to humans to
ascertain the role of CYP3A in the metabolism of particular drugs in
vivo (Wanwimolruk et al., 2002). Perhaps TAO should be used to
ascertain the role of CYP3A in APAP activation in humans.
In two human studies, excessive alcohol consumption was associ-
cated with elevated hepatic levels of CYP3A (Hoshino and Kawasaki,
1995; Niemela et al., 1998). Thus, it is possible that CYP3A may
contribute to APAP hepatotoxicity in alcoholics. In a controlled
crossover study investigating the effect of moderate alcohol consump-
tion on CYP2E1 (Oneta et al., 2002), CYP3A may have been induced.
In that study, ingestion of 40 g of ethanol/day as red wine resulted in
a modest increase (31%) in CYP2E1, measured as the ratio of 6-hy-
droxychloroxazone to total chloroxazone in serum. This increase
persisted for 3 weeks and was concluded by the authors to be due to
stabilization of CYP2E1. By week 4, however, there was an un-
expected additional increase in activity (~200%) (Oneta et al., 2002). It
is possible that it may have taken 4 weeks of alcohol consumption to
induce other forms of cytochrome P450 that catalyze the 6-hydroxy-
lization of chloroxazone, possibly CYP3A (Gorski et al., 1997).
However, in one uncontrolled study, moderate alcohol consumption
did not increase CYP3A activity (Liagpunsakul et al., 2005).

In knockout mice, pathways of metabolism other than the targeted
gene deletion can also be altered (Leiter, 2002). Hepatic levels of
GSH are critical factors in APAP hepatotoxicity, conjugating and
deactivating NAPQI and decreasing oxidative damage (for review, see
Bromer and Black, 2003). We found similar amounts of mitochondrial
and cytosolic GSH in wild-type and Cyp2e1(−/−) mice (Fig. 6), as
well as similar activities of glutathione reductase and glutathione
peroxidase, two enzymes involved in GSH homeostasis and inactiva-
tion of lipid peroxides (results not shown). Therefore, GSH homeosta-
sis is probably not responsible for the differences in APAP hepatoto-
xicity observed between wild-type and Cyp2e1(−/−) mice.

Glucuronidation of APAP is a major pathway of detoxification (for
review, see Bromer and Black, 2003). Therefore, we compared the
rates of glucuronidation of APAP in hepatic microsomes from wild-
type and Cyp2e1(−/−) mice to ascertain whether wild-type mice are
more sensitive to APAP because of a decreased ability to gluco-
ronidate APAP. We found that the rates of APAP-Gluc formation
were similar in the two mouse lines (Fig. 5), suggesting that APAP
glucuronidation in vivo may be similar between the two mouse lines,
assuming that the rates measured in vitro reflect metabolism in vivo.

In summary, EIP-mediated increases in APAP hepatotoxicity oc-
curred at lower APAP doses in wild-type mice compared with
Cyp2e1(−/−) mice, suggesting that CYP2E1 has a role in the differ-
ent susceptibilities. Because CYP2E1 decreased to constitutive levels
at the time of APAP administration, EIP-induced levels of CYP2E1
cannot account for alcohol-mediated increases in APAP hepatotoxic-
ity. However, constitutively expressed amounts of CYP2E1 may still
contribute to the toxicity. Treatment with TAO in vivo protected
EIP-pretreated wild-type and Cyp2e1(−/−) mice from APAP hepa-
toxicity and specifically inhibited CYP3A activity. Our overall
findings suggest that both CYP3A and CYP2E1 contribute to EIP-
mediated increases in APAP toxicity in mice.

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