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

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CYP, cytochrome P450; EIP, combination of ethanol and isopentanol; GSH, glutathione; i.g., intragastric; i.p., intraperitoneal; MROD, methoxyresorufin *O*-demethylase; NAPQI, *N*-acetyl-*p*-benzoquinone imine; PNPH, *p*-nitrophenol *O*-hydroxylation; TAO, triacetyloleandomycin/ troleandomycin; TRZ, triazolam

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ABSTRACT

CYP2E1 is widely accepted as the sole form of cytochrome P450 (CYP) 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 to *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, while 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 cytochrome P450s (CYPs) 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 3 forms of rat and human CYPs active in the conversion of APAP to NAPQI, with CYPs 2E1 and 3A 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 due to 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 developing 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*., 2000b). In other animal studies, alcohol has been reported to also increase CYPs 3A and 1A1/2 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 Kawasaki, 1995; Niemela *et al*., 1998). CYP3A has been reported to have a role in APAP hepatotoxicity in rats by two different approaches. In one, 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*., 1995a). 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*., 1995a). In another, 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*., 2000a). Although administration of APAP to animals pretreated with inducers of CYP3A resulted in increased toxicity (Mitchell *et al*., 1973), a role of induced CYP3A in APAP hepatotoxicity in humans is less clear. The results of two crossover studies utilizing 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 pre-treated with phenobarbital (Mitchell *et al*., 1974), while 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 utilized TAO as an inhibitor of CYP3A (Pessayre *et al*., 1981; Loeper *et al*., 1994). 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.

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). Acetaminophen (APAP), *p*-nitrophenol, triacetyloleandomycin (TAO), isopentanol (a mixture of 70% 3-methylbutanol and 30% 2-methylbutanol; IP), triazolam (TRZ), NADP+, NADPH, Brij 58 (polyoxyethylene 20-cetyl ether), UDPGA (sodium salt), magnesium chloride, DL-isocitric acid, isocitric dehydrogenase, potassium phosphate buffer solutions, 2-acetamidophenol, 1-heptanesulfonic acid, perchloric acid, hydrochloric acid, and glutathione (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 glutathione metabolite of APAP (APAP-SG), used as a standard for HPLC analysis, was a generous gift from Dr. Mark W. Gemborys (Chem Design Corporation, Fitchburg, MA). 7-Methoxyresorufin 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 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 Company (Ann Arbor, MI). All other reagents were purchased from either Sigma-Aldrich or Fisher Scientific Co.

Animals. Wild-type and *Cyp2e1(-/-)* mice in a 129/Sv background (Lee *et al*., 1996) were maintained in a controlled environment with a 12 h light/dark cycle at the VA animal facility. Hepatic microsomes from each *Cyp2e1(-/-)* mouse were analyzed for CYP2E1 immunochemically, as described below, to confirm their phenotype. The Institutional Animal Care and Use Committees at the Department of Veterans Affairs Medical Center and Dartmouth College approved all animal protocols. At three months of age, male mice, weighing 18 to 30 g, were fed control liquid Lieber-DeCarli diet (F1259SP) for an initial 2-day adaptive period. In most investigations of alcohol-

mediated increases in APAP hepatotoxicity, animals are treated with ethanol alone. However, most alcoholic beverages contain higher chain alcohols, with isopentanol being the most abundant (for review, see Kostrubsky *et al*., 1995a). Therefore, in our studies on alcohol-mediated APAP hepatotoxicity, we use a combined treatment of ethanol plus isopentanol (EIP). Following 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 prior to the administration of APAP to allow for the elimination of EIP, since 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 previously described (Pessayre *et al*., 1981; Kostrubsky *et al*., 1997) and administered intraperitoneally (i.p.) 2 h prior to APAP administration. APAP, at the doses specified in the figure legends, was prepared as previously described (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 prior to euthanization by cervical dislocation. Liver slices were fixed in 10% buffered formalin for histological analysis. For analyses of CYPs 2E1, 3A and 1A2, separate groups of animals were either euthanized at the end of the 7 day EIP treatment or 11 h after withdrawal from 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 PMSF, and 22.7 µM BHT. The homogenates were centrifuged at 10,000 g for 10 min at 4^{o}C. The supernatant was centrifuged at 100,000 g for 1 h at 4^{o}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^{o}C.

Immunochemical Analyses of Cytochrome P450s. Hepatic microsomes were analyzed for CYP3A, CYP2E1 and CYP1A2 immunochemically. For CYP2E1 and CYP3A, microsomal proteins were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide) at 160 V for 1 h. A mixed anionic detergent was used during the 1 h electrophoresis of gels for CYP1A2, as previously described (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^{o}C. For all gels, proteins were then transferred to nitrocellulose electrophoretically and the cytochromes P450 detected immunochemically, as described (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 a pathologist who was blinded to the treatments. The numerical grading of damage was: 1, mild (in <30% of lobule); 2, moderate (in 30 to 60% of lobule); 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 (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 isocitrate dehydrogenase, and 5 mM $MgCl_2$. The reaction was initiated by the addition of APAP to a final concentration of 0.5 mM. After incubation at 37^{o}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) methanol, and 1% (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 (MI) Complex. To determine if the TAO dose and exposure time were sufficient to allow for a CYP3A-TAO MI complex to form, the cytochrome P450-TAO MI complex was measured in hepatic microsomes, as described (Wrighton *et al*., 1985). Since 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 16 α-carbonitrile (PCN), a prototypical inducer of CYP3A in mice, were used. These animals were administered control liquid Lieber-DeCarli diet, as described earlier, with or without daily injections of 50 mg PCN/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 assayed, as previously described (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 an MI complex with TAO.

Measurement of GSH in Liver Cytosolic and Mitochondrial Fractions. Mice were administered 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% w/v) 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 1,000 g for 10 min at 4^{o}C and the supernatants were re-centrifuged at 10,000 g for 10 min at 4^{o}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% (w/v) trichloroacetic acid (TCA), while 5% (w/v) TCA was added directly to the mitochondrial pellets. The resulting TCA extracts were centrifuged at 10,000 g for 10 min at 4o C and the supernatants used to analyze GSH as previously described (Sinclair *et al*., 2000a). 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 VA 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 previously described (Wolf *et al*., 2005). The microsomal formation of 6β-hydroxytestosterone was measured by HPLC analysis, as previously described

(Kostrubsky *et al*., 1999). Microsomal glucuronidation of APAP was measured as previously described (Court and Greenblatt, 1997). Triazolam (TRZ) 4-hydroxylation activity was assayed in hepatic microsomes, as previously described (Perloff *et al*., 2000). The hydroxylation of *p*-nitrophenol was assayed as previously described (Sinclair *et al*., 1989). The activity of CYP1A2 was measured fluorimetrically in hepatic microsomes as the formation of resorufin over time from the demethylation of methoxyresorufin, as previously described (Sinclair *et al*., 1997), except that the assay contained 2.5 µM methoxyresorufin as the substrate.

Statistical Analyses. Values represent the means and SDs. 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* values <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 alanine aminotransferase (ALT). In the absence of EIP pretreatment, 300 mg APAP/kg was much more hepatotoxic in wild-type mice than in *Cyp2e1(-/-)* mice (Figure 1, $p<0.05$), confirming the results of Lee *et al*. (1996).

EIP pretreatment increased APAP hepatotoxicity in both wild-type and *Cyp2e1(-/-)* mice (Figure 1). However, the dose of APAP at which EIP increased toxicity was lower in wild-type mice. At 300 mg APAP/kg, EIP pretreatment increased APAP hepatotoxicity in wild-type mice, but not in *Cyp2e1(-/-)* mice (Figure 1). In *Cyp2e1(-/-)* mice, APAP hepatotoxicity was increased by EIP pretreatment at a dose of 600 mg APAP/kg (Figure 1). Thus, wild-type mice were more sensitive to EIP-mediated increases in APAP hepatotoxicity compared to *Cyp2e1(-/-)* mice, suggesting a role of CYP2E1.

Effect of Withdrawal from EIP on Hepatic Levels of CYPs 2E1, 3A, and 1A2.

Alcohol has been shown in rodents to increase hepatic levels of three CYPs involved in APAP activation, CYPs 2E1, 3A, and 1A2 (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, since blood ethanol levels as low as 5 mM protect animals from APAP hepatotoxicity (Thummel *et al*., 1988). Therefore, we measured hepatic levels of CYPs 2E1, 3A and 1A2, both before and after withdrawal from the diet, to assess the alcohol-mediated induction of these cytochrome P450s, as well as their status at the time of APAP administration.

In wild-type mice not withdrawn from the diet, EIP treatment increased CYPs 2E1, 3A and 1A2 (Figure 2A). The increase in CYP1A2 by EIP in wild-type mice was detected in 6 out of 8 mice tested, with a mean fold-increase for all 8 mice of 1.82 ± 0.26 ($p<0.001$). In *Cyp2e1(-/-)* mice, EIP increased CYP3A but not CYP1A2

(Figure 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 CYPs 2E1 and 1A2 decreased to constitutive levels in wild-type mice (Figure 2A). In contrast, EIP-induced levels of CYP3A remained elevated in both wild-type and *Cyp2e1(-/-)* mice (Figure 2 A and B).

Using microsomes from *Cyp2e1(-/-)* mice, we previously found 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*., 2000b). 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 in *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 to *Cyp2e1(-/-)* mice. The 6β-hydroxylation of testosterone, an activity attributed to CYP3A (Waxman *et al*., 1988), was also greater in wild-type mice compared to *Cyp2e1(-/-)* mice (3005 ± 384 vs. 1085 ± 293 pmole/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 Triacetyloleandomycin (TAO) on APAP Hepatotoxicity in EIP-Pretreated Mice. We investigated the role of CYP3A in EIP-mediated increases in APAP hepatotoxicity utilizing TAO, an inhibitor of CYP3A activity (Newton *et al*., 1995). APAP was administered at doses in which EIP treatment increased APAP hepatotoxicity in each mouse line (300 mg/kg for wild-type mice and 600 mg/kg for *Cyp2e1(-/-)* mice; Figure 1), and serum levels of ALT were measured. Administration of TAO, 2 h prior to APAP, protected both wild-type and *Cyp2e1(-/-)* mice from EIP-mediated increases in APAP hepatotoxicity (Figure 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). Pre-administration of TAO to EIP-pretreated animals 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 CYPs 1A2, 2E1, and 3A. 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 CYPs 1A2, 2E1, and 3A 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*., 2000c). As shown in Figure 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), since this activity is a specific measure of CYP2E1 in mice (Carlson, 2003; Wolf *et al*., 2004). TAO treatment did not affect microsomal PNPH activity (Figure 4). The activity of CYP3A was measured by the 4-hydroxylation of triazolam (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% (Figure 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 if 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 (Figure 5).

Comparison of Hepatic Glutathione (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 determine if differences in GSH levels could contribute to the different susceptibilities to APAP hepatotoxicity. In the absence of APAP administration, mitochondrial (Figure 6A) and cytosolic (Figure 6B) levels of GSH were similar in wild-type and *Cyp2e1(-/-)* mice. Treatment with EIP did not alter these levels in either mouse line (Figure 6 A and B). Since mitochondrial GSH levels have been reported to decrease early after APAP administration in rats (Vendemiale *et al*., 1996), we investigated the effect of a 1 h exposure to APAP, *in vivo*, on mitochondrial and cytosolic levels of GSH in both wild-type and *Cyp2e1(-/-)* mice. In these experiments, APAP was administered at doses in which EIP pretreatment increased APAP hepatotoxicity (300 mg/kg in wild-type mice and 600 mg/kg in *Cyp2e1(-/-)* mice; Figure 1). At 1 h after the administration of APAP to control mice, cytosolic GSH decreased in both wild-type and *Cyp2e1(-/-)* mice (Figure 6B). APAP alone caused a significant decrease in mitochondrial GSH in wild-type mice, but not in *Cyp2e1(-/-)* mice (Figure

6A), consistent with the greater APAP hepatotoxicity in wild-type mice (Figure 1). However, when mice were pretreated with EIP, hepatic levels of both mitochondrial and cytosolic GSH were decreased to similar extents in both mouse lines 1 h after the administration of APAP (Figure 6 A and B).

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*., 2000b). 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*., 1995b). 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*., 2000a). 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 to *Cyp2e1(-/-)* mice. In mice pretreated with EIP, hepatotoxicity occurred at a lower dose of APAP in wild-type mice (300 mg/kg) compared to *Cyp2e1(-/-)* mice (600 mg/kg) (Figure 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 (Figure 3; Table 2), suggesting a role for CYP3A. Since TAO, administered *in vivo*, inhibited the hepatic microsomal activity of CYP3A (Figure 4), but not the activities of CYP2E1 and CYP1A2 (Figure 4), the effect of TAO on APAP hepatotoxicity *in vivo* 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 vitro* in liver microsomes harvested 2 h after administration of TAO *in vivo* in an attempt to assess the activities at the time of APAP administration. TAO, administered *in vivo*, resulted in only a partial inhibition of CYP3A activity (40%), measured *in vitro* 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. Our overall findings suggest that both CYP3A and CYP2E1 contribute to alcohol-mediated increases in APAP hepatotoxicity.

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*., 2000a). During this withdrawal period, alcohol-induced levels of CYP2E1 decreased to

constitutive levels in mice (Figure 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 (Figure 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 to *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 to *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 to *Cyp2e1(-/-)* mice. Our findings that TAO treatment *in vivo* almost totally protected EIP-pretreated wild-type and *Cyp2e1(-/-)* mice from APAP hepatotoxicity (Table 2, Figure 3) and selectively inhibited CYP3A activity (Figure 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 to *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 phenobarbital, 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 end points, 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 6 out of 7 volunteers pretreated 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 associated 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 consumption on CYP2E1 (Oneta *et al*., 2002), CYP3A may have been induced. In that study, ingestion of 40 g ethanol per day as red wine resulted in a modest increase (31%) in CYP2E1, measured as the ratio of 6-hydroxychlorzoxazone to total chlorzoxazone 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 unexpected 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-hydroxylation of chlorzoxazone, possibly CYP3A (Gorski *et al*., 1997). However, in one uncontrolled study, moderate alcohol consumption did not increase CYP3A activity (Liangpunsakul *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 (Figure 6), as well as similar activities of glutathione reductase and glutathione peroxidase, two enzymes involved in GSH homeostasis and inactivation of lipid peroxides (results not shown). Therefore, GSH homeostasis is probably not responsible for the differences in APAP hepatotoxicity 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 due to a decreased ability to glucuronidate APAP. We found that the rates of APAP-Gluc formation were similar in the two mouse lines (Figure 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 occurred at lower APAP doses in wild-type mice compared to *Cyp2e1(-/-)* mice, suggesting that CYP2E1 has a role in the different susceptibilities. Since CYP2E1 decreased to constitutive levels at the time of APAP administration, EIP-induced levels of CYP2E1 cannot account for alcohol-mediated increases in APAP hepatotoxicity. 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 hepatotoxicity 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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FOOTNOTES

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FIGURE LEGENDS

Figure 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 in *Methods*. The diet was replaced with water for 11 h prior to the administration of APAP i.g., at the doses indicated, and 7 h later, the mice were euthanized, as described in *Methods*. The treatment groups consisted of the following: wild-type mice: control + 300 mg APAP/kg (n=4); EIP + 300 mg APAP/kg (n=10); and *Cyp2e1(-/-)* mice: control + 300 mg APAP/kg (n=5); EIP + 300 mg APAP/kg (n=6); control + 600 mg APAP/kg (n=9); EIP + 600 mg APAP/kg (n=12). Serum levels of ALT were measured, as described in *Methods*. Each value represents the mean with the SDs indicated by the vertical lines. ALT levels in untreated mice, both wild-type and *Cyp2e1(-/-)*, were 195 ± 16 U/L 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 WT mice treated with APAP alone; ***, $p<0.001$ versus *Cyp2e1(-/-)* mice treated with APAP alone.

Figure 2. Effect of withdrawal from EIP on hepatic CYPs 1A2, 2E1, and 3A 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 (No Withdrawal). For some animals, the diet was replaced with water for 11 h (Withdrawal).

At the end of these time periods, the mice were euthanized and hepatic microsomes were prepared. The microsomes were then analyzed for CYPs 1A2, 2E1, and 3A immunochemically, as described in *Methods*. **(A)** Wild-type mice. **(B)** *Cyp2e1(-/-)* mice. CYP1A2 positive control: sample from a C57BL/6 wild-type mouse treated with 3-methylcholanthrene (MC) to induce CYP1A1 and CYP1A2.

Figure 3. Effect of TAO on APAP hepatotoxicity in EIP-pretreated wild-type and *Cyp2e1(-/-)* mice. Wild-type and *Cyp2e1(-/-)* mice were fed EIP liquid diet as described in *Methods*. TAO (500 mg/kg) was administered i.p. at 9 h after withdrawal from the diet followed by administration of 300 mg APAP/kg or 600 mg APAP/kg i.g. to wild-type and *Cyp2e1(-/-)* mice, respectively. After a 7 h exposure to APAP, the animals were euthanized. The treatment groups consisted of the following: wild-type mice: no APAP (n=10); TAO (n=3); APAP (n=5); APAP + TAO (n=4); and *Cyp2e1(-/-)* mice: no APAP (n=5); TAO (n=4); APAP (n=4); APAP + TAO (n=5). WT mice from the EIP + APAP 300 mg/kg group and *Cyp2e1(-/-)* mice from the EIP + APAP 600 mg/kg group were also included in Figure 1. **(A)** Serum ALT levels were measured, as described in *Methods*. Each value represents the mean with the SDs indicated by the vertical lines. Data within each mouse line were analyzed by ANOVA. ***$p \leq 0.001$, comparing EIP + APAP with no APAP and EIP + APAP + TAO with EIP + APAP. **(B)** Histological examination of the liver.

Figure 4. Effect of triacetyloleandomycin (TAO) on enzymatic activities catalyzed by CYPs 1A2, 2E1, and 3A. Hepatic microsomes were prepared from wild-type mice

fed liquid diet containing EIP and withdrawn from diet for 11 h, as described in *Methods*. As indicated, TAO (500 mg/kg i.p.) was administered at 9 h after withdrawal from the diet. At 2 h after TAO administration, the animals were euthanized and hepatic microsomes were prepared and analyzed for methoxyresorufin *O*-demethylase (MROD) activity, *p*-nitrophenol *O*-hydroxylation (PNPH) activity and triazolam 4-hydroxylation activity (TRZ 4-OH), as described in *Methods*. The values are represented as the % activity by microsomes from animals administered EIP alone, which are as follows: MROD: 45 ± 5 pmoles/min/mg protein; PNPH: 3.1 ± 0.2 nmoles 4NC/ min/ mg protein; TRZ 4-OH: 0.12 ± 0.01 units/min/mg protein. Each value represents the mean of samples from 6 to 9 mice per treatment with the SDs indicated by the vertical lines. The data were analyzed by an unpaired Student's t test. ***, $p<0.001$ versus TRZ 4-OH in the absence of TAO.

Figure 5. Comparison of hepatic levels of APAP glucuronidation in wild-type and *Cyp2e1(-/-)* mice at the time of APAP administration. Hepatic microsomes were prepared from animals fed control or EIP liquid diet, as indicated, and withdrawn from diet, as described in the legend to Figure 2. Microsomal glucuronidation of APAP was measured as previously described (Court and Greenblatt, 1997). Each value represents the mean of samples from 4 mice per treatment with the SDs indicated by the vertical lines. Data were analyzed by ANOVA.

Figure 6. Comparison of hepatic glutathione (GSH) levels in wild-type and *Cyp2e1(-/-)* mice. Animals were fed control or EIP liquid diet, where indicated, as

described in the legend to Figure 1 and euthanized at the end of the 11 h withdrawal (No APAP) or 1 h after the administration of APAP i.g. (300 mg/kg to wild-type mice and 600 mg/kg to *Cyp2e1(-/-)* mice). Mitochondrial **(A)** and cytosolic **(B)** fractions were prepared from freshly harvested liver, as described in *Methods*, and GSH measured, as previously described (Sinclair *et al*., 2000b). Each value represents the mean of samples from 3 to 5 mice per treatment with the SDs indicated by the vertical lines. Data were analyzed by ANOVA. *, $p<0.05$ comparing No APAP with APAP; **, $p<0.001$, comparing No APAP with APAP.

TABLE 1

Effect of EIP on activation of APAP by hepatic microsomes from wild-type and Cyp2e1(-/-) mice

		APAP-SG Formation (nmoles/ min/ mg protein)	
Mouse Line	**Diet**	**No Withdrawal**	**Withdrawal**
Wild-type	Control	0.33 ± 0.10	0.50 ± 0.06
	EIP	0.71 ± 0.01	0.82 ± 0.02
Cyp2e1(-/-)	Control	0.23 ± 0.06	0.22 ± 0.02
	EIP	0.53 ± 0.12	0.43 ± 0.17

Wild-type Control vs. Wild-type EIP: ***
Wild-type Control vs. *Cyp2e1(-/-)* Control: **
Wild-type Control vs. Wild-type Withdrawal: ***
Wild-type EIP vs. *Cyp2e1(-/-)* EIP: ***
Wild-type Withdrawal vs. *Cyp2e1(-/-)* Withdrawal: **
Cyp2e1(-/-) Control vs. *Cyp2e1(-/-)* EIP: ***
Cyp2e1(-/-) Control vs. *Cyp2e1(-/-)* Withdrawal: ***
Cyp2e1(-/-) EIP vs. *Cyp2e1(-/-)* Withdrawal: *

Hepatic microsomes from wild-type and *Cyp2e1(-/-)* mice, treated as described in the legend to Figure 2, were analyzed for the activation of APAP to NAPQI, measured as the rate of formation of the glutathione adduct of APAP (APAP-SG), as described in *Methods*, using a concentration of 0.5 mM APAP. Each value represents the mean ± SD of microsomes from 4 to 7 animals. Data were analyzed by ANOVA with multiple comparisons testing. Group pairs that were found to be significantly different are indicated by connecting brackets: *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

TABLE 2

Effect of TAO on alcohol-mediated increases in APAP hepatotoxicity

Mouse Line	Treatment	# of Animals	Congestion					Necrosis				
			Normal	Mild	Moderate	Severe	*p* Value	Normal	Mild	Moderate	Severe	*p* Value
Wild-type	**EIP**	**5**	**5**	**0**	**0**	**0**	**NS**	**5**	**0**	**0**	**0**	**NS**
	EIP + TAO	**3**	**3**	**0**	**0**	**0**	**NS**	**3**	**0**	**0**	**0**	**NS**
	EIP + APAP	**5**	**0**	**0**	**5**	**0**	**<0.01**	**0**	**0**	**5**	**0**	**<0.01**
	EIP + TAO + APAP	**4**	**4**	**0**	**0**	**0**	**NS**	**0**	**4**	**0**	**0**	**<0.05**
Cyp2e1(-/-)	**EIP**	**2**	**2**	**0**	**0**	**0**	**NS**	**2**	**0**	**0**	**0**	**NS**
	EIP + TAO	**4**	**4**	**0**	**0**	**0**	**NS**	**4**	**0**	**0**	**0**	**NS**
	EIP + APAP	**4**	**0**	**1**	**0**	**3**	**<0.05**	**0**	**1**	**0**	**3**	**<0.05**
	EIP + TAO + APAP	**5**	**5**	**0**	**0**	**0**	**NS**	**3**	**2**	**0**	**0**	**NS**

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. The Fisher's Exact test was used to determine which numbers observed were significantly different. The results are displayed in the *p* value column.

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Figure 1.

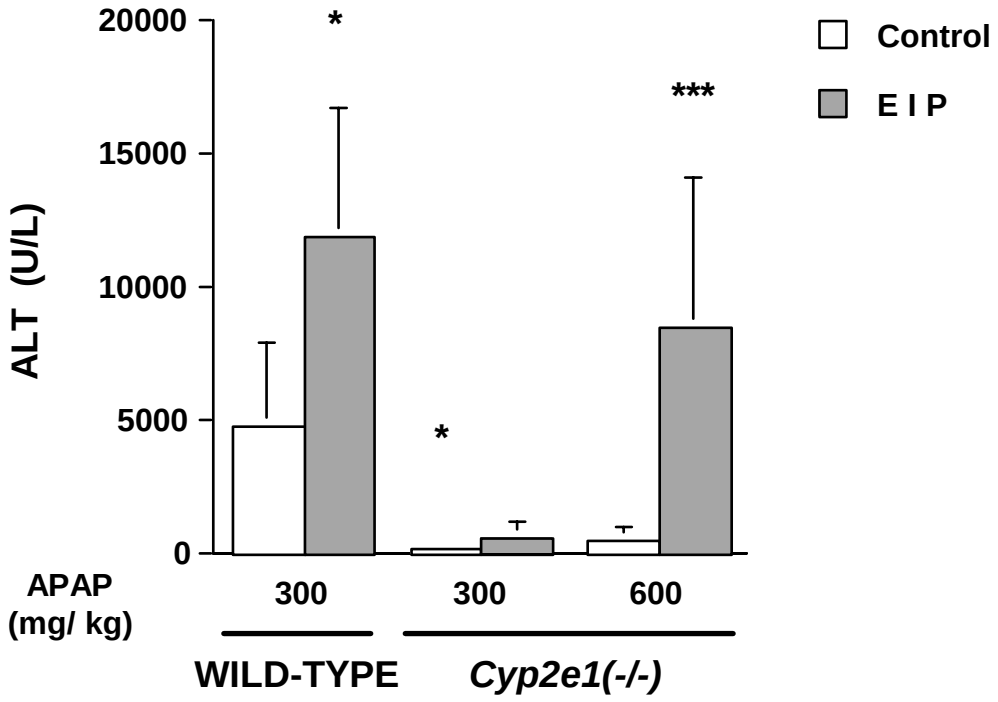

Figure 2.

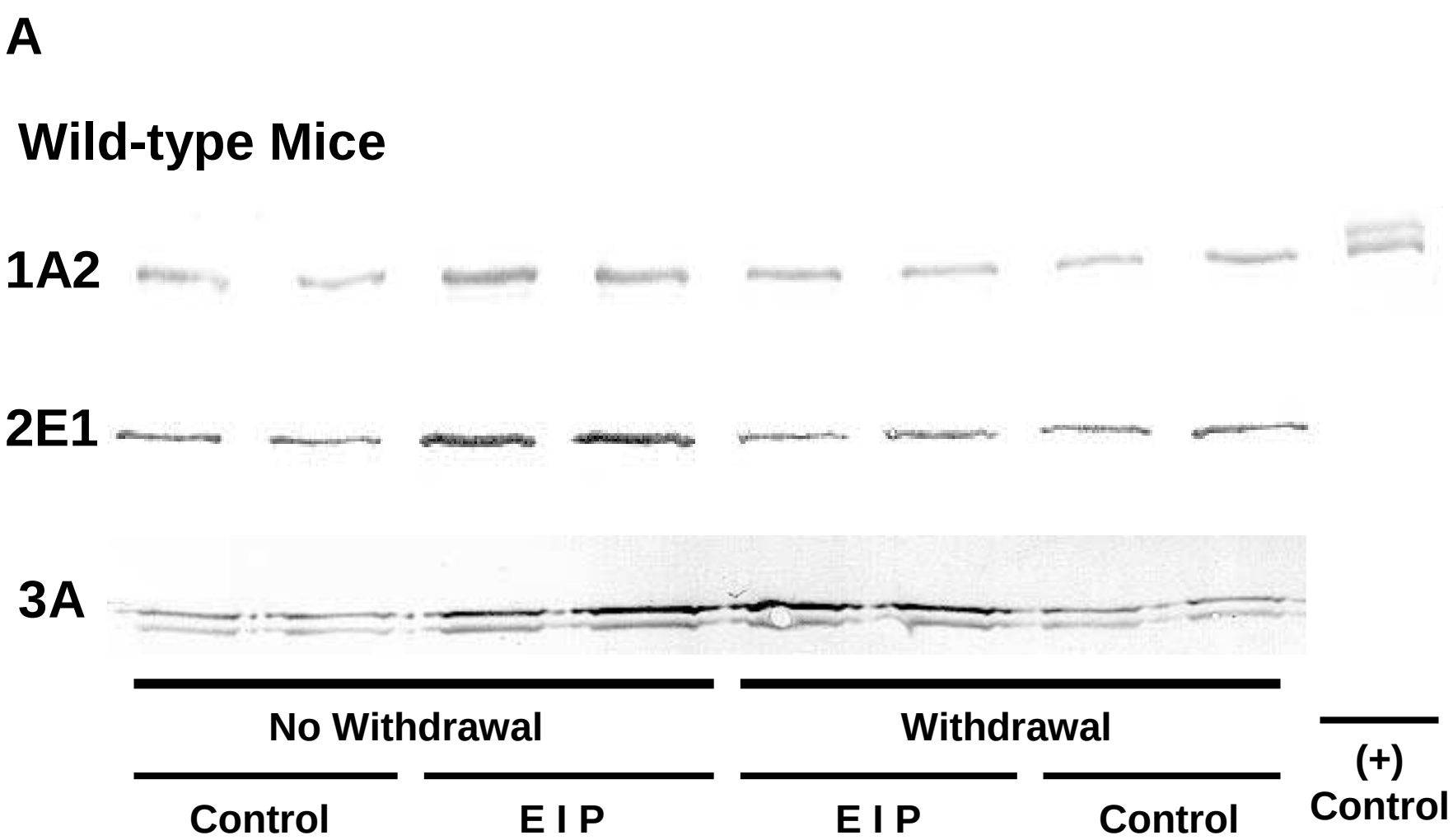

Figure 2.

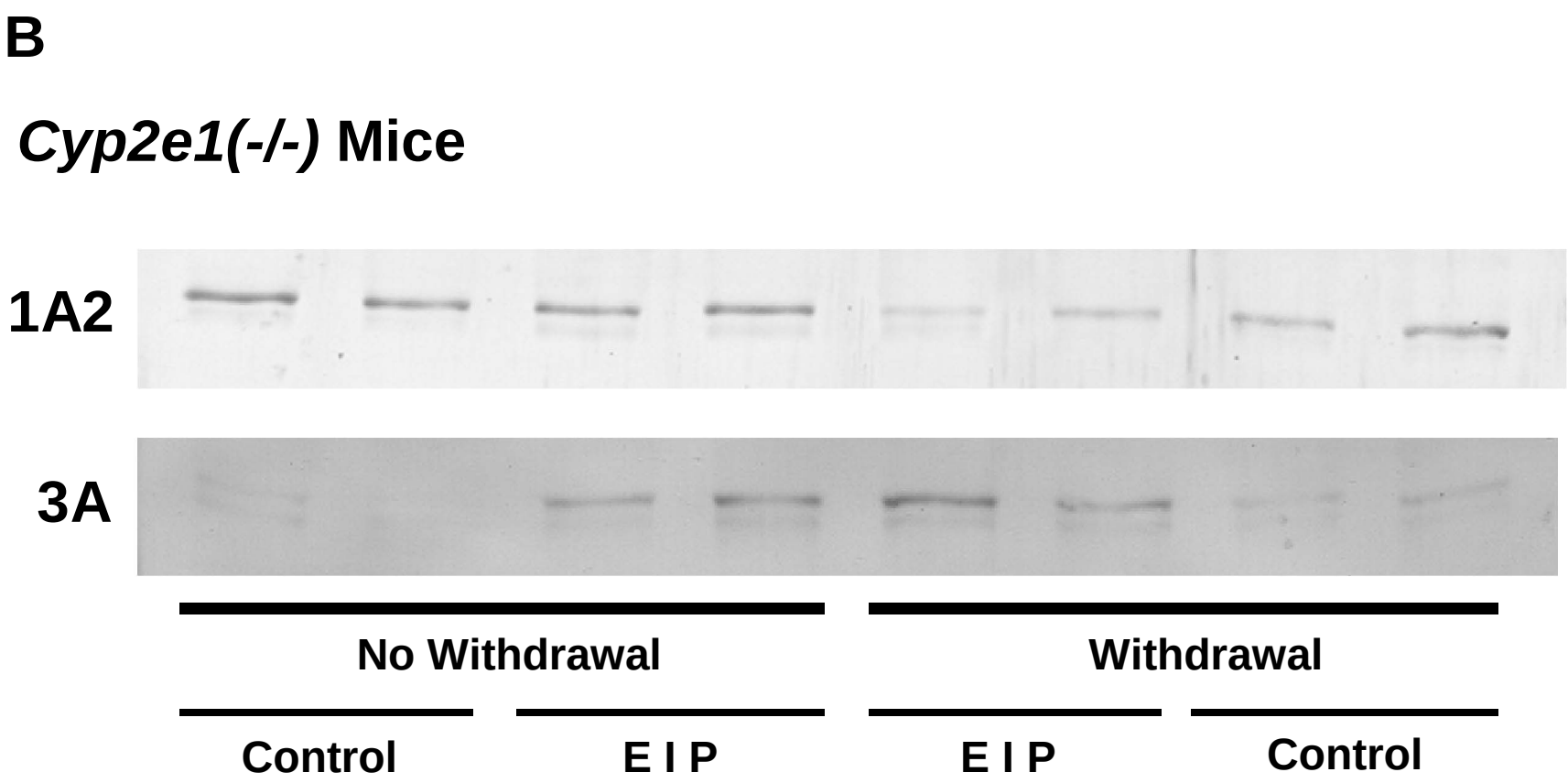

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Figure 3.

A

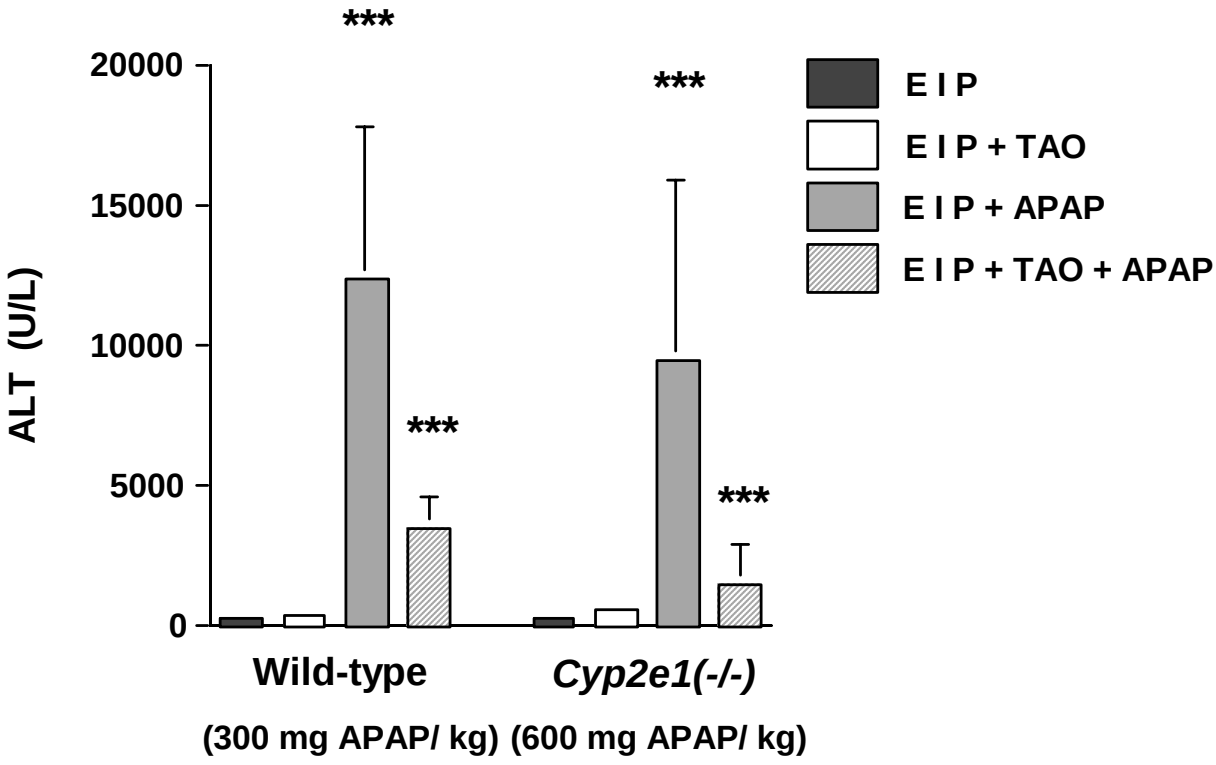
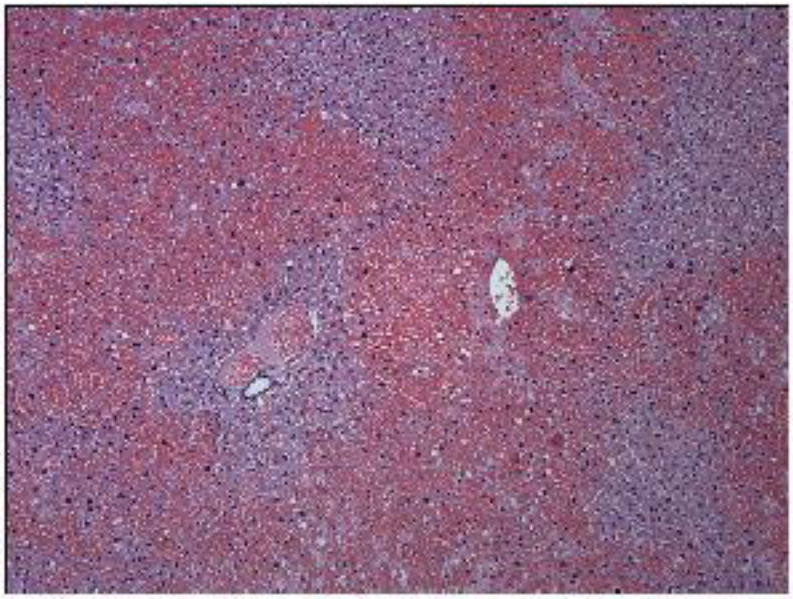


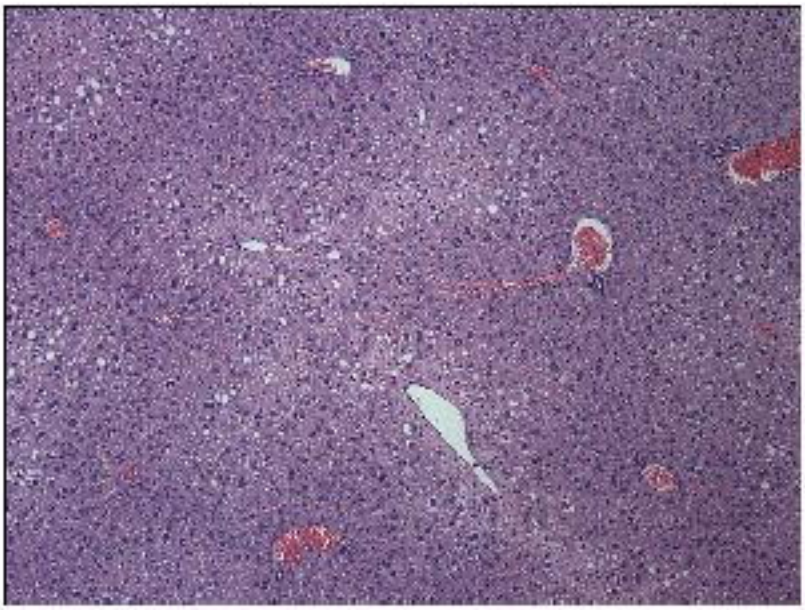
Figure 3.

B

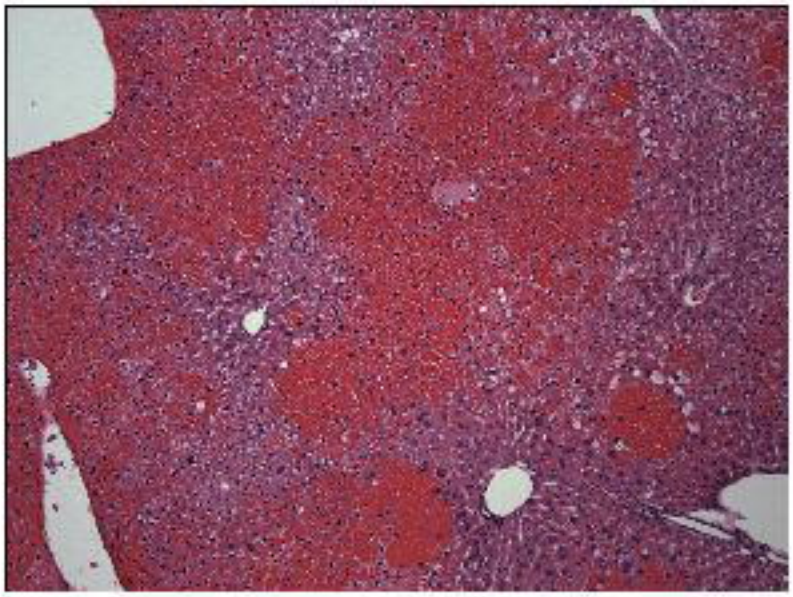
Wild-type: E I P + APAP



Wild-type: E I P + APAP + TAO



Cyp2e1(-/-): E I P + APAP



Cyp2e1(-/-): E I P + APAP + TAO

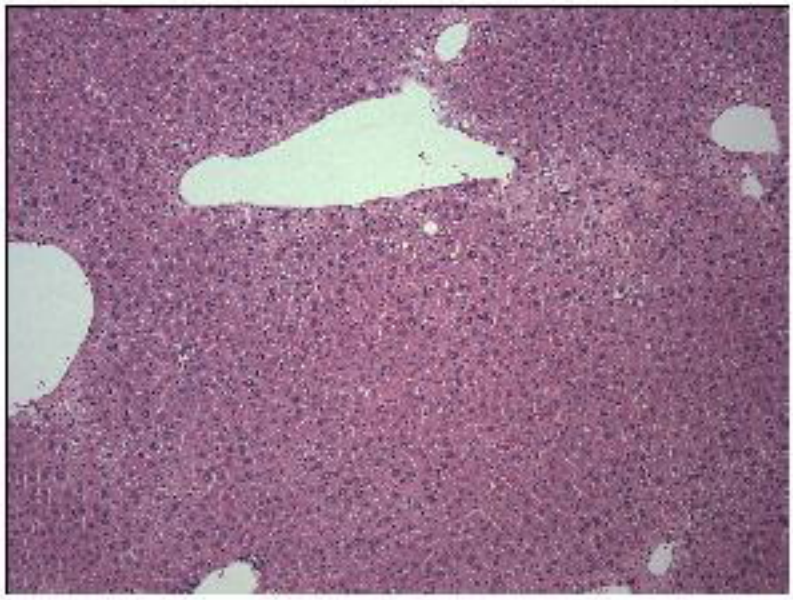


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Figure 4.

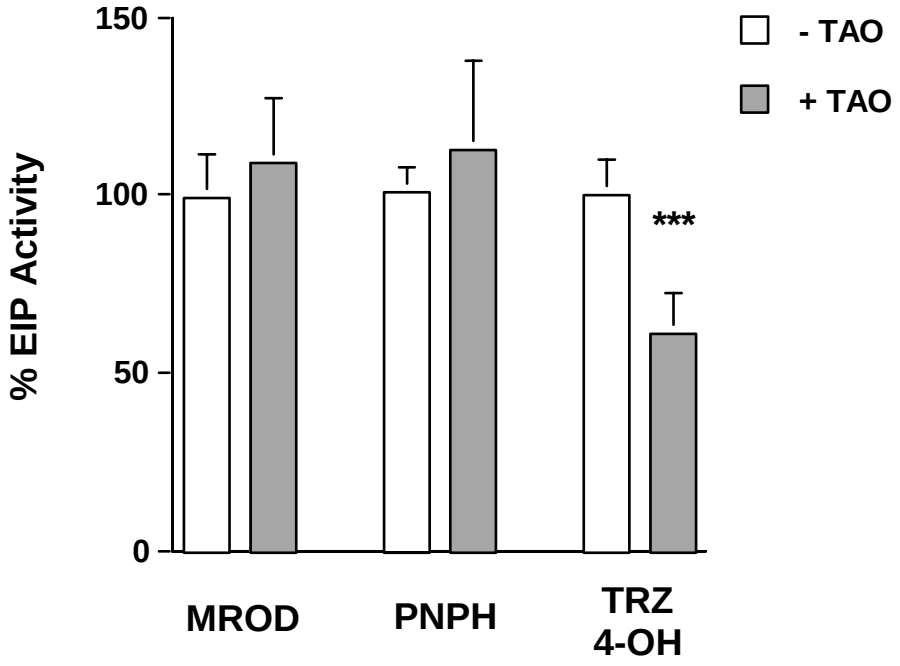

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Figure 5.

A

No Withdrawal

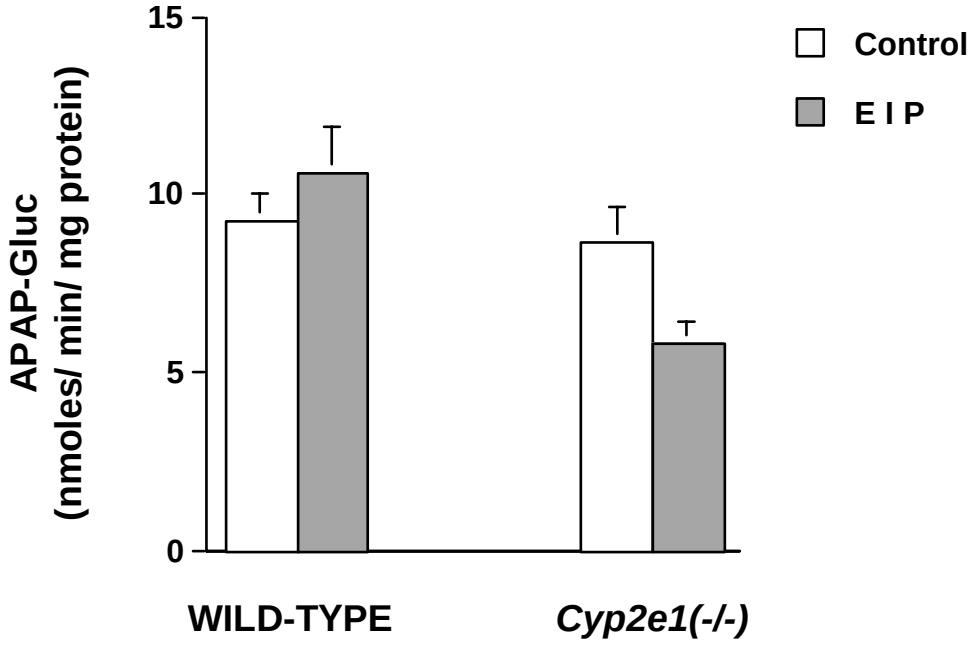

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Figure 5.

B

Withdrawal

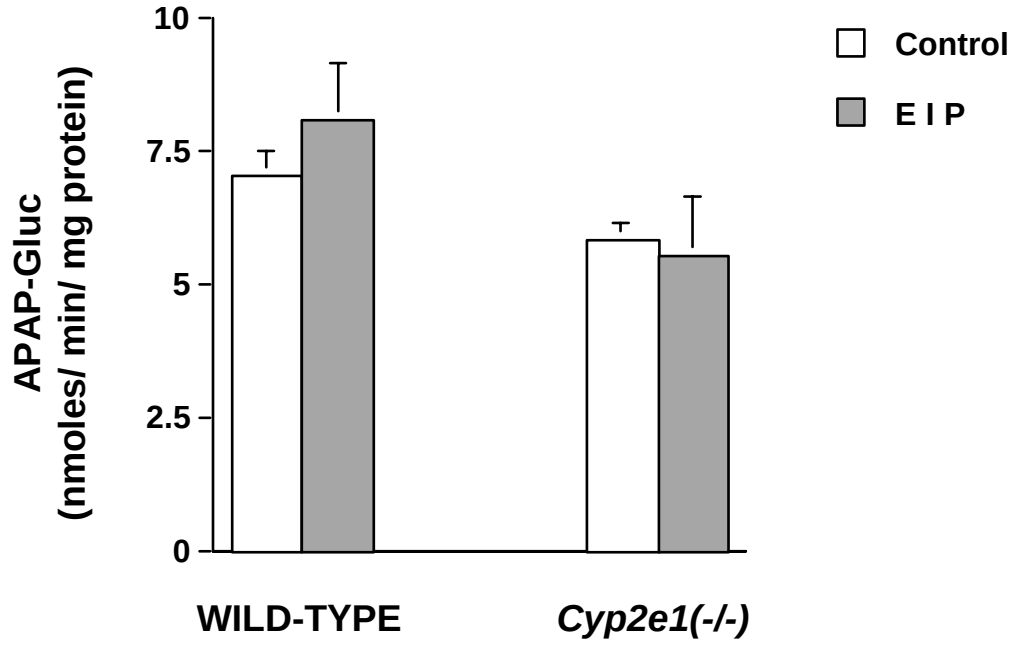

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Figure 6.

A

Mitochondrial

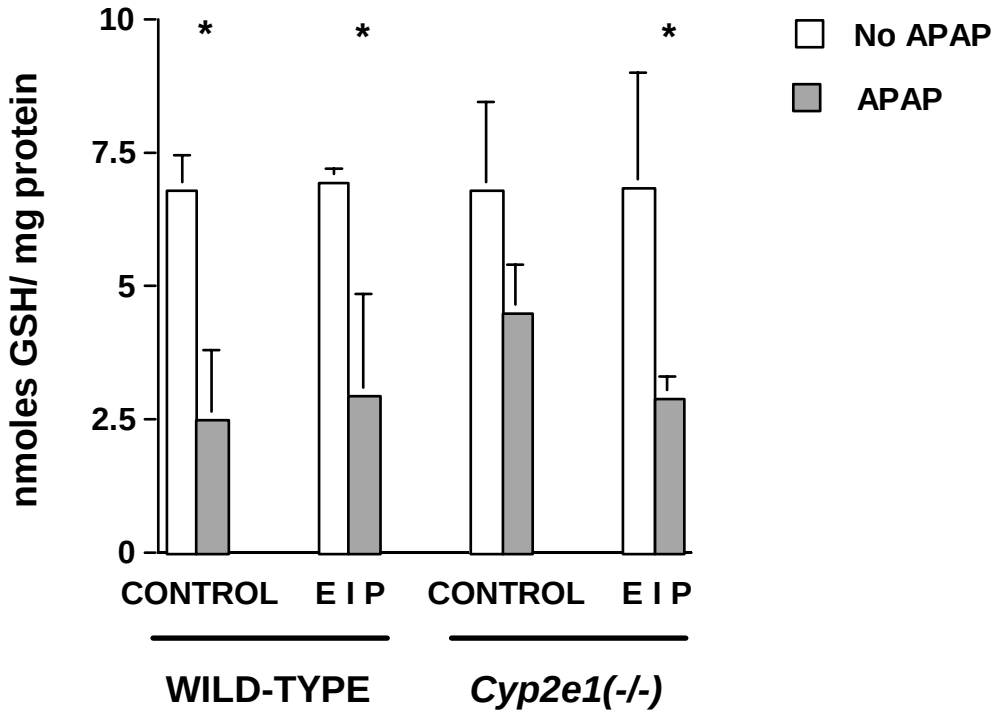

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Figure 6.

B

Cytosolic