ABSTRACT:

Chronic ethanol consumption potentiates acetaminophen (APAP) hepatotoxicity through enhanced NAPQI formation via CYP2E1 induction and selective depletion of mitochondrial glutathione. Because the prevalence of the interaction is extremely low given the use of APAP and the incidence of alcohol abuse, we studied the effects of ethanol dose and ethanol withdrawal on selective mitochondrial glutathione (GSH) depletion and APAP toxicity in liver slices. Rats were fed the Lieber-DeCarli diet containing ethanol (0, 7, 18, 27, and 36% total energy) for 6 weeks. The highest ethanol-containing diet (36% energy as ethanol) was replaced by control diet for 2, 5, 12, and 17 h. Maximal CYP2E1 induction was caused by 36% energy as ethanol diet (2.2-fold, p < 0.05 versus control). The activity and liver protein content returned to the control level 17 h after ethanol withdrawal.

The 36% energy as ethanol diet caused maximal mitochondrial GSH depletion (51%, p < 0.05 versus control), which was restored 17 h after ethanol withdrawal (22.0 ± 4.9 versus 11.7 ± 1.7 nmol/mg protein of 0 h, p < 0.01). Elevated glutathione S-transferase-α release in liver slices (a measure of toxicity) was observed in rats fed 36% energy as ethanol diet (1 mM APAP: 69 ± 10 versus 3 ± 1% of control, p < 0.01). Enhanced toxicity disappeared when ethanol dose decreased and when ethanol was removed (7.2% ethanol: 3 ± 1% and 17 h: 2 ± 1%, p < 0.01 versus 0 h 36% energy as ethanol). In conclusion, high-dose ethanol potentiated APAP hepatotoxicity via CYP2E1 induction and selective mitochondrial GSH depletion. Mitochondrial GSH depletion quickly reversed when ethanol was withdrawn. The time window for both mechanisms to act in concert is narrow.

Acetaminophen (APAP) is a widely used analgesic and is considered to be safe when taken at therapeutic doses. However, more than 100 case reports have been published describing what is perceived as a “therapeutic misadventure”, in which alcoholics are claimed to be unusually susceptible to APAP hepatotoxicity at doses devoid of toxicity in nonalcoholics (Seeff et al., 1986; Zimmerman and Madrey, 1995). Given the ubiquity of ethanol abuse (~18 million Americans according to National Institute of Alcoholics and Alcohol Abuse) and APAP use (consumed by 23% Americans each week; Kaufman et al., 2000), the clinical observation of the APAP-ethanol interaction seems to be extremely rare.

APAP hepatotoxicity is mediated by its metabolite N-acetyl-p-benzoquinone imine (NAPQI), which is generated by liver cytochrome P450s and is detoxified by conjugation with hepatic glutathione (GSH; Mitchell et al., 1973; Nelson, 1990). The major P450 isofrom that is responsible for NAPQI formation, cytochrome P450 2E1 (CYP2E1), is induced by ethanol (Ronis et al., 1993; Takahashi et al., 1993; Roberts et al., 1995; Manyike et al., 2000). P450 induction by ethanol correlated with enhanced APAP hepatotoxicity in experimental animals (Sato et al., 1981; Zhao et al., 2002). NAPQI initiates its toxicity by first attacking mitochondria and the depletion of mitochondrial GSH is an early event in the development of toxicity (Tirmenstein and Nelson, 1989; Nelson, 1990; Burcham and Harman, 1991; Vendemiale et al., 1996). Chronic ethanol feeding in rats selectively depletes liver mitochondrial GSH without altering the cystolic GSH pool (Hirano et al., 1992). The likely mechanism is decreased mitochondrial inner membrane fluidity, which results in decreased importation of cytosolic GSH (Colell et al., 1997). Results from our laboratory have demonstrated that selective depletion of mitochondrial GSH caused by chronic ethanol feeding contributes to the enhanced APAP toxicity (Zhao et al., 2002). The combination of both CYP2E1 induction and selective depletion of mitochondrial GSH may largely explain the unusually high susceptibility to hepatic damage in alcohol abusers claimed by Zimmerman and Madrey (1995).

Significant depletion of mitochondrial GSH seems to occur only after more than 3 weeks of ethanol feeding (Hirano et al., 1992; Zhao et al., 2002). It is not known whether depletion of mitochondrial GSH requires a high ethanol dose, nor is it known how quickly the effect reverses after ethanol is removed from the diet. In this study, we took advantage of the rat as a model of both human alcoholic liver disease and APAP hepatotoxicity to evaluate the effect of ethanol dose and ethanol withdrawal on the depletion of mitochondrial GSH and APAP toxicity (Mitchell et al., 1973; Lieber et al., 1989).

Materials and Methods

Chemicals. Monobromobiamine (thiolate reagent) was purchased from Calbiochem (La Jolla, CA). Acetonitrile, ethyl ether, and methanol were purchased from...
obtained from Fisher Scientific (Pittsburgh, PA). Ketamine and xylazine were obtained from Phoenix Pharmaceutical, Inc. (St. Joseph, MO). Ethanol was purchased from Pharmaco Products, Inc. (Brookfield, CT). All other reagents were from Sigma-Aldrich (St. Louis, MO).

Animals. Male Sprague-Dawley rats (~180 g; Charles River Laboratories, Inc., Wilmington, MA) were fed a mixture of the Lieber-DeCarli liquid diet (Bio-Serv, Frenchtown, NJ; Lieber et al., 1989) and an isocaloric liquid diet such that 0, 7, 18, 27, and 36% of energy was contributed by ethanol. Rats received the assigned diet for 6 weeks before euthanization for the isolation of livers. For the ethanol-withdrawal study, the ethanol diet (36% energy) was replaced by an isocaloric liquid diet 2, 5, 12, and 17 h before rats were euthanized.

At the end of the 6-week pretreatment period (at which point rat body weight was ~350 g), rats were euthanized with ketamine/xylazine and livers were removed as described previously (Zhao et al., 2002). Livers to be used for hepatic necrosis in liver slices (measured by the percentage of glutathione-S-transferase-a (GST) released to the medium) were accomplished according to our previous study (Zhao et al., 2002).

Mitochondria Isolation and Assays. Liver mitochondria were isolated by homogenization and differential centrifugation in a buffer containing 200 mM mannitol, 50 mM sucrose, 10 mM KCl, 1 mM EDTA, and 10 mM HEPES-KOH, pH 7.4 (Graham, 1993). Liver was homogenized in 5x volume of mannitol-sucrose buffer and centrifuged at 1000g for 5 min. Part of the supernatant was used as homogenate for the measurement of CYP2E1 protein and chlorzoxazone oxidation. The remaining supernatant was centrifuged at 8000g for 10 min, and the resulting mitochondrial pellet was washed three times and reconstituted in the same buffer. The absence of cytosolic contamination of mitochondria was determined by the ratio of lactate dehydrogenase activities (Sigma-Aldrich) in the mitochondria and homogenates (<0.5%). CYP2E1 activity was measured by 6-hydroxy chlorzoxazone (6-OH CLZ) formation in liver homogenates. Incubations contained ~0.4 mg/ml homogenate protein, 1 mM chlorzoxazone, and 1 mM NADPH in 20 mM Tris/2 mM MgCl2 buffer, pH 7.4. The total volume was 1 ml. Substrate and protein were preincubated for 4 min at 37°C before NADPH was added. Reactions ran for 10 min (linear up to 30 min), and 6-OH CLZ formation was measured as described previously (Kharasch et al., 1993). Mitochondrial GSH was measured by high-performance liquid chromatography (Zhao et al., 2002).

Western Blot Analysis of CYP2E1. Homogenate protein (~10 µg) was resolved on a NuPAGE 12% SDS-polyacrylamide gel electrophoresis system (Invitrogen, Carlsbad, CA) and transferred to a nitrocellulose membrane. Western blot was carried out with a goat polyclonal antibody against rat CYP2E1 (BD Gentest, Woburn, MA). Dilutions of primary antibody and secondary antibodies (rabbit anti-goat IgG coupled with alkaline phosphatase; Sigma-Aldrich) were 1:1000 and 1:2000, respectively. Bands were visualized using BCIP/NBT substrate from Calbiochem. Quantitation of bands was done with a ChemiDoc imaging system from Bio-Rad (Hercules, CA) using rat liver CYP2E1 standard (BD Gentest).

Statistical Analysis. All data are reported as mean ± S.D. The difference between groups was compared by analysis of variance using Sidak method for pairwise comparison (Statsoft software; Stata Co., College Station, TX), p < 0.05 was used as the criterion for significance.

Results and Discussion

Effects of Ethanol Dose on APAP Hepatotoxicity. Fig. 1, A and B, show the relationships between CYP2E1 protein (Western blot) and activity (6-hydroxylation of chlorzoxazone) and the amount of ethanol in the diet. The magnitude of CYP2E1 induction increased with increasing ethanol dose, reaching a maximum of about 2-fold for both 6-OH CLZ formation and protein level when ethanol was 36% of energy. The result obtained when ethanol comprised 36% of dietary energy content was consistent with our previous findings (Zhao et al., 2002). Mitochondrial GSH was depleted by ethanol in a dose-dependent manner (Fig. 1C). Maximum depletion, 51%, was observed when ethanol was dosed at 36% of total energy (11.7 ± 1.7 versus 22.8 ± 2.5 nmol/mg protein of pair-fed, ethanol-free control, p < 0.01). At the lower ethanol doses (7.2 and 18% energy groups), mitochondrial GSH was not depleted (p = 0.00 and 0.16 versus control, p < 0.01 and 0.05 versus 36% energy diet group). APAP-induced necrosis (1 mM APAP, roughly the peak concentration caused by a 10-g single dose), reflected as GST release, in liver slice incubations is shown in Fig. 1D. Maximum GST release was seen in liver slices prepared from rats fed ethanol as 36% energy (69 ± 10%, p < 0.01 versus control). As ethanol content decreased, GST release decreased (21 ± 8, 6 ± 4, and 3 ± 1% for 27, 18, and 7.2% energy diet groups, respectively, p < 0.01 versus 36% energy diet group). At the lowest ethanol dose (7.2% energy) where mitochondrial GSH and CYP2E1 activity were comparable with the pair-fed control, APAP-induced GST release was not different from pair-fed control (3 ± 1 versus 3 ± 1%, p = 1.00).

Effect of Ethanol Withdrawal on APAP Toxicity. Figure 2, A and B, show the time courses of CYP2E1 protein and activity after ethanol withdrawal (36% energy). The increased protein level and activity returned to the value observed in rats receiving no ethanol by 17 h after ethanol was removed from the diet (p = 1.00 versus control for both protein and activity, and p < 0.01 versus 0 h for activity). During the ethanol-withdrawal phase, mitochondrial GSH returned to the pair-fed control value 17 h after the ethanol diet was replaced by the isocaloric ethanol-free diet (22.0 ± 4.9 versus 22.8 ± 2.5 nmol/mg protein, p = 1.00; p < 0.01 versus 11.7 ± 1.7 nmol/mg protein of 0 h; Fig. 2C). Figure 2D shows the results of ethanol withdrawal on APAP toxicity (1 mM APAP) in liver slices. GST release decreased as time after ethanol was removed from the diet increased (43 ± 20% at 5 h, p = 0.09; 23 ± 16 and 2 ± 1% at 12 and 17 h after ethanol withdrawal, p < 0.01 versus 0 h). Seventeen hours after ethanol withdrawal, liver slice GST release was not different from pair-fed control (2 ± 1 versus 3 ± 1% of pair-fed control, p = 1.00), at which time both CYP2E1 induction and mitochondrial GSH depletion were present.

In this study, we found that the selective depletion of mitochondrial GSH by chronic ethanol depended on ethanol content in the diet and quickly reverted upon ethanol withdrawal. The changes in mitochondrial GSH paralleled that of CYP2E1 induction. The enhanced APAP toxicity by chronic ethanol is closely associated with the magnitude of both CYP2E1 induction and mitochondrial GSH depletion; APAP toxicity disappeared when CYP2E1 and mitochondrial GSH pool returned to the levels of pair-fed controls.

It has been estimated that 23% of the U.S. adult population consumes APAP in any given week (Kaufman et al., 2000). The National Institute for Alcoholism and Alcohol Abuse estimates that 10% of U.S. adults are alcohol abusers. Thus, approximately as many as 4 million U.S. adult alcohol abusers may consume APAP in any given week. Given the huge estimated incidence of concomitant use of these agents and the strong mechanistic evidence for ethanol to enhance APAP toxicity (CYP2E1 induction and mitochondrial GSH depletion), the interaction would be expected to be a common public health problem. However, the apparent incidence simply does not support such a conclusion.

The results from this study and others help to understand the apparently low prevalence with which the interaction is actually observed (Girre et al., 1994; Slattery et al., 1996; Chien et al., 1997; Dart et al., 2000; Thummel et al., 2000; Kuflner et al., 2001). Although ethanol induces CYP2E1 and depletes mitochondrial GSH, these effects require high doses of ethanol. Moreover, we and others have previously shown in humans that as long as ethanol is present in the circulation, CYP2E1 activity is actually inhibited, although levels of hemeprotein are increased (Chien et al., 1997). Enhanced CYP2E1
**Fig. 1.** Effect of dietary ethanol content (percentage of total dietary energy) on liver homogenate CYP2E1 protein (A), chlorzoxazone 6-hydroxylation in liver homogenate (B), liver mitochondrial GSH (C), and APAP hepatotoxicity in liver slices (D).

A, ethanol dose-dependent induction of liver homogenate CYP2E1 protein. Data are relative optical densities compared with rat liver CYP2E1 standard (1 pmol; BD Gentest), mean ± S.D. n = 3 rats. B, ethanol dose-dependent induction of liver homogenate CYP2E1 activity. Mean ± S.D. of three incubations from each group. C, ethanol dose-dependent changes of liver mitochondrial GSH levels. Mean ± S.D., n = 4 to 6 rats. D, relationship between ethanol dose and percentage of GST release in liver slices incubated with 1 mM APAP. GST released into the medium was expressed as a percentage of total enzyme activity (sum of medium + slice activity). Mean ± S.D., n = 4 to 6 rats. *, p < 0.05; **, p < 0.01, versus pair-fed control; †, p < 0.05; ††, p < 0.01 versus 36% energy ethanol. The difference of band migration between homogenate sample and CYP2E1 standard is due to a routinely observed matrix effect; the standard is expressed in the microsomal fraction of human lymphoblasts. The faint band below CYP2E1 in the rat liver homogenate lanes is most likely CYP2C11 (according to the antibody product information sheet, product 455115; BD Gentest).
activity requires the absence of ethanol in the circulation (Slattery et al., 1996; Thummel et al., 2000). Under these conditions liver mitochondrial GSH depletion will diminish. Because the increase in CYP2E1 activity in vivo requires removal of ethanol and the maximal effect of ethanol on hepatocyte mitochondrial GSH depletion is seen only while ethanol is continually administered, it is improbable that the two effects are observed simultaneously in an individual. Furthermore, both effects seem to decline quickly once ethanol is removed.

**Fig. 2.** Effect of ethanol withdrawal on CYP2E1 protein (A), chlorzoxazone 6-hydroxylation activity (B), hepatic and mitochondrial GSH (C), and APAP hepatotoxicity in liver slices from rats on the highest ethanol content diet (36% energy) for 6 weeks (D).

A, liver homogenate CYP2E1 protein (see comments in Fig. 1 regarding Western blot). Data are relative optical densities compared with rat liver CYP2E1 standard (BD Gentest), mean ± S.D., n = 3 rats. B, mean ± S.D. of three incubations from each group. C, mean ± S.D., n = 4 to 6 rats. D, percentage of GST release in liver slices incubated with 1 mM APAP. Mean ± S.D., n = 4 to 6 rats. *, *p < 0.05; **, p < 0.01, versus pair-fed control; †, p < 0.05; ††, p < 0.01 versus 36% energy ethanol at 0 h.
Taken together, the data explain the likely mechanisms by which ethanol can potentiate the toxicity of APAP while simultaneously explaining the low frequency with which the interaction is observed given the high incidence of concomitant use.

References


