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Acetaminophen Hepatotoxicity Precipitated by Short-Term Treatment of Rats with Ethanol and Isopentanol

PROTECTION BY TRIACETYLOLEANDOMYCIN

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ABSTRACT. Ethanol and isopentanol are the predominant alcohols in alcoholic beverages. We have reported previously that pretreatment of rats with a liquid diet containing 6.3% ethanol plus 0.5% isopentanol for 7 days results in a synergistic increase in acetaminophen hepatotoxicity, compared with rats treated with either alcohol alone. Here, we investigated the role of CYP3A in acetaminophen hepatotoxicity associated with the combined alcohol treatment. Triacetyloleandomycin, a specific inhibitor of CYP3A, protected rats pretreated with ethanol along with isopentanol from acetaminophen hepatotoxicity. At both 0.25 and 0.5 g acetaminophen/kg, triacetyloleandomycin partially prevented elevations in serum levels of alanine aminotransferase. At 0.25 g acetaminophen/kg, triacetyloleandomycin completely protected 6 of 8 rats from histologically observed liver damage, and partially protected the remaining 2 rats. At 0.5 g acetaminophen/kg, triacetyloleandomycin decreased histologically observed liver damage in 7 of 15 rats. In rats pretreated with ethanol plus isopentanol, CYP3A, measured immunohistochemically, was decreased by acetaminophen treatment. This effect was prevented by triacetyloleandomycin. These results suggest that CYP3A has a major role in acetaminophen hepatotoxicity in animals administered the combined alcohol treatment. We also found that exposure to ethanol along with 0.1% isopentanol for only 3 days resulted in maximal increases in acetaminophen hepatotoxicity by the combined alcohol treatment, suggesting that short-term consumption of alcoholic beverages rich in isopentanol may be a risk for developing liver damage from acetaminophen. BIOCHEM PHARMACOL **59**;4: 445–454, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. alcohols; acetaminophen hepatotoxicity; CYP3A; triacetyloleandomycin; isopentanol; ethanol and acetaminophen; short-term alcohols

The observation that alcoholics are susceptible to developing liver damage from therapeutic or otherwise non-toxic doses of APAP‡‡ was first reported in South Africa in 1977 [1]. Several other case reports were published during the late 1970s and early 1980s, including reports of alcoholics who experienced hepatotoxicity or even death from liver failure after receiving therapeutic doses of APAP in a hospital-based detoxification program [2–4]. In most experimental systems investigating the effect of consumption of alcoholic beverages on APAP hepatotoxicity, ethanol, the major alcohol in alcoholic beverages [5, 6], is the only alcohol administered. However, alcoholic beverages also contain higher chain alcohols, with isopentanol being the most abundant of the higher chain alcohols. The isopentanol content of beer, wine, whiskey, cognac, and bourbon ranges from 0.13 to 0.5% (w/v) [5, 6]. Although treatment of experimental animals with ethanol increases APAP hepatotoxicity [7, 8], we have found that combined treatment of rats with ethanol and isopentanol causes synergistic increases in APAP hepatotoxicity compared with pretreatment with ethanol alone [9].

Ethanol causes a large increase (4- to 10-fold) in hepatic CYP2E in rats [10–12], but only a small increase (less than 2-fold) in CYP3A [13, 14]. The combined alcohol treatment causes additive to synergistic increases in CYP3A

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‡‡ *Abbreviations*: ALT, alanine aminotransferase; APAP, acetaminophen; AST, aspartate aminotransferase; CYP, cytochrome P450; NAPQI, *N*-acetylbenzimidoquinone; and TAO, triacetyloleandomycin.

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with no further increase in CYP2E than that caused by ethanol alone [13]. Based mainly on inhibitor studies in animals, CYP2E is considered to be the major form of CYP responsible for increases in APAP hepatotoxicity associated with the consumption of alcoholic beverages [2, 8, 15–17]. However, many of the inhibitors of CYP2E used in such studies also inhibit CYP3A (for review, see Ref. 15). Using TAO, a specific inhibitor of CYP3A [18, 19], we have shown that TAO protects rats from ethanol-mediated increases in APAP hepatotoxicity [20, 21]. This TAO protection suggests that CYP3A has a major role in ethanol-mediated increases in APAP hepatotoxicity [15], consistent with the findings that CYP3A has a much greater affinity for APAP than does CYP2E [22, 23]. Using TAO as an inhibitor of CYP3A, we now have demonstrated that CYP3A plays a crucial role in APAP hepatotoxicity in rats pretreated with the combination of ethanol and isopentanol. We also found that APAP hepatotoxicity could be caused by pretreatment for only 3 days with as little as 0.1% isopentanol in combination with ethanol.

MATERIALS AND METHODS

Chemicals

APAP and TAO were purchased from the Sigma Chemical Co. Absolute ethanol (USP) was from PharmCo Products Inc. The Lieber–DeCarli diet was obtained from BioServ. Antibodies to CYP3A were prepared against purified human CYP3A4 and have been shown to detect rat CYP3A, but not CYP2E, in analyses of rat hepatic microsomes by immunoblots [20]. This antibody did not detect CYP2B1/2 or CYP1A1/2 in hepatic microsomes isolated from rats treated with phenobarbital or 3-methylcholanthrene, respectively (results not shown).

Treatment of Rats

Male Fischer 344 rats were purchased from Harlan Sprague-Dawley and maintained in a controlled environment with a 12-hr light/dark cycle. Rats weighed 160–210 g at the time of experimentation. Alcohols were administered continuously to the rats for 3–7 days as part of the Lieber–DeCarli diet, as indicated in the figure legends. The alcohols present in the diet were 6.3% (w/v) ethanol and 0.5% (w/v) isopentanol, unless indicated otherwise in the figure legends. Rats fed ethanol consumed less of the diet than the other animals; therefore, the groups were pair-fed so that there was no difference in weight gain, as was reported previously [9]. Although, with the Lieber–DeCarli diet, there is usually a period of adaptation before feeding the animals ethanol, we have found that no adaptation is needed with Fischer rats. The amounts of ethanol-containing diet consumed with and without the period of adaptation were identical (results not shown); therefore, the period of adaptation was eliminated. In contrast, with isopentanol in the diet, either with or without ethanol, rats consumed more diet than the control animals, and the amount of diet containing isopentanol and ethanol had to be decreased to pair-feed the animals to the control level. In rats not treated with the alcohols, the equivalent calories were provided by inclusion of maltose–dextrin in the liquid diet. Eleven hours before the administration of APAP, the liquid diet was replaced with water to ensure elimination of the alcohols. APAP was administered at the concentrations indicated in the legends by intragastric intubation, as described previously [9]. TAO was administered i.p. at a dose of 500 mg/kg, 2 hr prior to the administration of APAP (total time of exposure to TAO was 9 hr). The stock solution of TAO was dissolved in saline instead of DMSO, the solvent usually employed, since DMSO inhibits CYP2E [for review, see Ref. 15]. The TAO in saline was acidified to pH 4.0 with HCl [20]. Rats not receiving TAO were administered HCl–saline, pH 4, i.p. Each dose of APAP was prepared individually by adding solid APAP to saline in a syringe barrel and sonicating for 10 sec (Sonifier Cell Disruptor, model W140D) before administration. At 7 hr after administration of APAP, rats were anesthetized with CO_2, blood was collected by cardiac puncture, and the animals were decapitated. Slices of liver were stored in formalin for histological analysis.

To obtain livers containing high levels of CYP3A for use as a positive control in the immunohistochemical analysis of CYP3A, rats were treated with dexamethasone at 100 mg/kg/day for 4 days. The dexamethasone was dissolved in corn oil and injected i.p. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Veterans Administration Medical Center.

Histology

Paraffin sections were prepared after fixation with 10% neutral buffered formalin and stained with hematoxylin and eosin. CYP3A was determined immunohistochemically in paraffin-embedded sections by the following procedure: Paraffin was removed with xylene. The slides were rehydrated, followed by incubation with blocking buffer containing normal serum from the species in which the secondary antibody was made. An avidin and biotin block was also performed, as supplied and directed by the manufacturer (Vector Laboratories). The slides were then incubated with primary and secondary antibodies in blocking buffer, and the immunocomplex with CYP3A was detected using an alkaline-phosphatase kit (Vector Laboratories). The counterstain was hematoxylin. Included in each preparation were the following controls: i) lacking the primary antibody; ii) lacking the secondary antibody; and iii) a liver section from a rat treated with dexamethasone to induce CYP3A.

Additional Assays

Serum AST and ALT levels were assayed in the clinical laboratory at the VA Hospital by the automated DADE/Dimension Clinical Chemistry System.

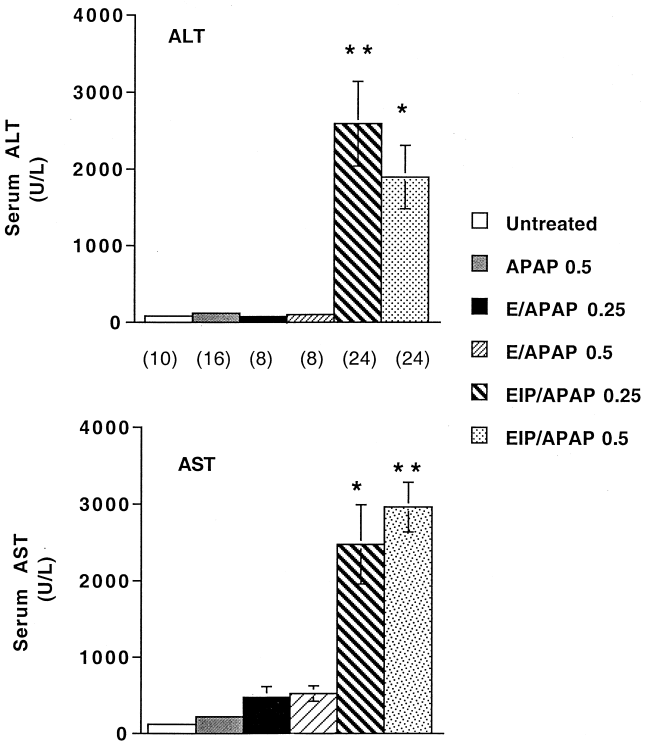

FIG. 1. APAP hepatotoxicity in rats pretreated with ethanol and isopentanol. Rats were fed the Lieber–DeCarli liquid diet containing 6.3% ethanol alone (E) or combined with 0.5% isopentanol (EIP) for 7 days. Rats not administered alcohols were given equivalent calories as maltose–dextrin in the liquid diet. Animals treated with the alcohols were administered APAP at doses of 0.25 or 0.5 g/kg, as described in Materials and Methods. Animals not pretreated with the alcohols were administered APAP at a dose of 0.5 g/kg. Serum levels of AST and ALT were measured as described in Materials and Methods. The numbers of animals per treatment group are presented in parentheses in the figure. Each value represents the mean, with the SEM indicated by the vertical line. Values were analyzed by ANOVA, followed by a Student–Newman–Keuls Multiple Comparisons Test. Key: (**) $P < 0.001$ and (*) $P < 0.01$, compared with E/APAP, APAP alone, and untreated.

Statistical Analyses

For analyses of serum levels of AST and ALT, values represent means ± SEM. The data were analyzed by ANOVA, followed by a Student–Newman–Keuls Multiple Comparisons Test, or an unpaired Student's *t*-test, as indicated in the figure legends. P values of = 0.05 were taken to indicate significance.

RESULTS

APAP Hepatotoxicity in Rats Pretreated with Ethanol and Isopentanol: Effect of Duration of Pretreatment and Dose of Isopentanol

We have previously reported an increase in APAP hepatotoxicity in rats pretreated with 6.3% ethanol and 0.5% isopentanol compared with rats pretreated with ethanol alone [9]. In those studies, the lowest dose of APAP administered was 0.5 g/kg, and the alcohols were administered for 7 days in the Lieber–DeCarli liquid diet. Figure 1 shows that a dose of 0.25 g APAP/kg was as hepatotoxic as a dose of 0.5 g APAP/kg in rats pretreated with the combined alcohol treatment, as shown by similar elevations in serum levels of both AST and ALT (Fig. 1). The livers of rats pretreated with the alcohol combination followed by APAP at 0.25 or 0.5 g/kg had extensive histologically observed liver damage, with centrilobular congestion, steatosis, and necrosis. This damage was observed in all rats and was diffuse in 90% of the rats, indicating severe damage (Table 1). There was no significant difference in APAP hepatotoxicity whether rats were pretreated with the alcohol combination for 3 or 7 days. The dose of 0.25 g APAP/kg was equally hepatotoxic to animals pretreated with the alcohol combination for either 3 or 7 days, as indicated by serum levels of ALT (Fig. 2).

TABLE 1. Effect of pretreatment with ethanol and isopentanol on liver damage from APAP: Protection afforded by TAO

			Liver histology		
				Centrilobular congestion, steatosis, and necrosis	
Treatment	TAO	Total number of rats	Normal (# rats)	Focal (# rats)	Diffuse (# rats)
None	None	7	6	1	0
	+	7	7	0	0
EIP/0.25 g APAP	None	8	0	1	7
	+	8	6	2	0
EIP/0.5 g APAP	None	15	0	1	14
	+	15	3	5	7 (no necrosis)

Rats were fed the Lieber-DiCarli diet containing no alcohols or 6.3% ethanol and 0.5% isopentanol (EIP), as indicated. APAP was administered intragastrically, and rats were killed 7 hr after administration of APAP, as described in Materials and Methods. TAO (0.5 g/kg) was administered intraperitoneally, where indicated, 2 hr before APAP, as described in Materials and Methods. Slices of liver were fixed in formalin and examined histologically, as described in Materials and Methods.

Using a 3-day pretreatment with the alcohols, we investigated the concentration-dependent response of isopentanol [0.1, 0.25, and 0.5% (w/v)], in combination with a fixed dose of ethanol [6.3% (w/v)], on the hepatotoxicity of 0.25 g APAP/kg. Pretreatment with the alcohol combination alone caused no liver damage, as evidenced by no increase in serum levels of ALT or AST (Fig. 3) and no histologically observable liver damage (Table 2). At all doses of isopentanol administered in combination with ethanol, subsequent treatment with 0.25 g APAP/kg resulted in increased liver damage. Maximal toxicity was observed with the lowest concentration of isopentanol (0.1%) in the pretreatment (Fig. 3, Table 2). Such toxicity was manifested by increased serum levels of ALT and AST (Fig. 3) and centrilobular congestion and steatosis in almost all rats (Table 2). In this experiment, necrosis was observed in only 50–60% of the rats pretreated with the alcohols and subsequently exposed to APAP, regardless of the

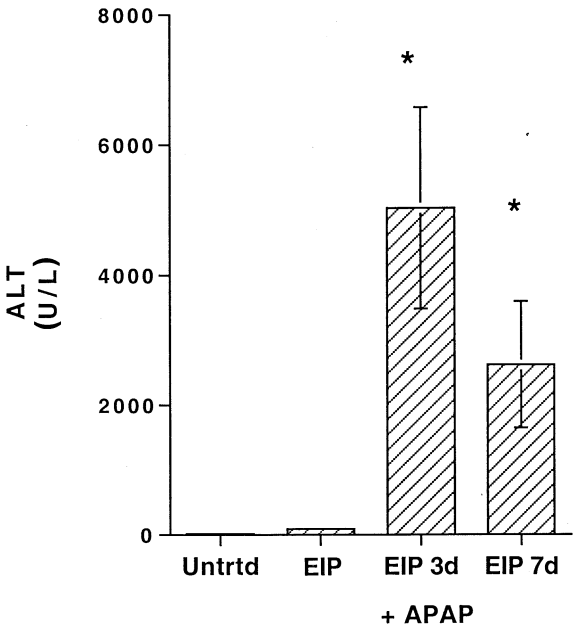

FIG. 2. Effect of length of pre-treatment with ethanol and isopentanol on APAP hepatotoxicity in rats. Rats were fed the Lieber–DeCarli liquid diet containing 6.3% ethanol and 0.5% isopentanol (EIP) for either 3 or 7 days, as indicated in the figure. Rats not administered alcohols were given equivalent calories as maltose–dextrin in the liquid diet. Animals treated with the combination of ethanol and isopentanol were administered APAP, as indicated, at a dose of 0.25 g/kg, as described in Materials and Methods. Serum levels of ALT were measured as described in Materials and Methods. Each value represents the mean, with the SEM indicated by the vertical line. For untreated and EIP-treated rats, there were 6 animals per treatment. For animals pretreated with EIP and exposed to APAP, there were 8 animals per treatment. Values were compared by an unpaired Student's *t*-test. Key: (*) significantly different from untreated and EIP, $P < 0.0001$.

isopentanol concentration (Table 2). There was also no significant difference in the elevated serum levels of ALT at all doses of isopentanol administered in the pretreatment (Fig. 3).

Effect of TAO on Alcohol-Mediated Increases in APAP Hepatotoxicity

In rats pretreated with 6.3% ethanol plus 0.5% isopentanol for 7 days, administration of TAO, 2 hr prior to APAP, prevented the increase in AST and ALT observed at both 0.25 and 0.5 g APAP/kg (Fig. 4). Treatment with TAO alone caused an elevation in serum AST with no increase in serum ALT (Fig. 4) and no histologically observed liver damage (Table 1), suggesting an extrahepatic source of AST.

Exposure to the alcohols alone (Fig. 5A) or APAP alone (results not shown) resulted in no liver damage, as observed histologically, similar to our previous findings [9]. Figure 5 also shows histological analyses of liver samples from rats pretreated with the alcohols, and then exposed to APAP with and without TAO, as described in Materials and Methods. Exposure to APAP at 0.25 g/kg (Fig. 5B) resulted in moderate centrilobular necrosis in the liver, as evidenced by the loss of nuclei, karyorrhexis, and karyolysis. There was mild to moderate centrilobular congestion and a notable absence of inflammation. Microvesicular steatosis was seen in several cells. As seen in Fig. 5D, exposure to 0.5 g APAP/kg also resulted in moderate centrilobular steatosis and congestion with mild necrosis, but no inflammation. There was loss of hepatocellular nuclei within the affected central lobule.

The degree of protection by TAO against histologically observed liver damage depended on the dose of APAP. At the lower dose of APAP (0.25 g/kg), 6 out of 8 rats pretreated with the alcohols were protected completely from liver damage (Table 1, Fig. 5C), and the remaining 2 rats showed only partial liver damage (Table 1). At a dose of 0.5 g APAP/kg, the livers of only 3 out of 15 rats pretreated with the alcohols showed normal histology (Table 1). In Fig. 5E, although the liver histology is normal, there are a few steatotic cells in the centrilobular region. Of the remaining rats treated with TAO, 5 showed focal damage, indicating considerable, yet incomplete protection, whereas the livers from 7 rats still had diffuse centrilobular congestion and steatosis, but no necrosis (Table 1). The TAO treatment did not decrease the serum levels of APAP (results not shown), indicating that TAO did not interfere with the uptake of APAP into the liver.

In rats pretreated with ethanol and isopentanol and exposed to TAO and APAP, CYP3A, measured immunohistochemically, was abundant in the centrilobular region (Fig. 6). In contrast, in the absence of TAO, there was little detectable CYP3A in this region. These results suggest that

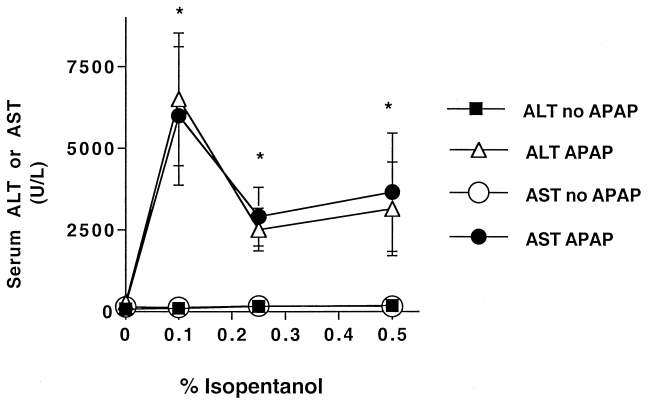

FIG. 3. Effect of increasing doses of isopentanol on APAP hepatotoxicity in ethanol-treated rats. Rats were fed the Lieber–DeCarli liquid diet containing 6.3% ethanol (E) plus 0.1, 0.25, or 0.5% isopentanol (IP), as indicated, for 3 days. APAP was administered, as described in Materials and Methods, at a dose of 0.25 g/kg. Serum levels of AST and ALT were measured as described in Materials and Methods. Each value represents the mean, with the SEM indicated by the vertical line; N = 6/group (E alone or EIP alone), N = 8/group (APAP + EIP). Values were compared by Student's *t*-test. Key: (*) significantly different from no APAP or from APAP alone, $P < 0.0001$.

TABLE 2. Effect of pretreatment with ethanol along with increasing amounts of isopentanol on liver damage from APAP

			Liver Histology		
				Centrilobular congestion, steatosis	
Pretreatment	APAP	Total number of rats	Normal (# rats)	Focal (# rats)	Diffuse (# rats)
None	None	4	4	0	0
	+	8	8	0	0
0.1% IP + E	None	5	5	0	0
	+	8	1	0	7 (5 with necrosis)
0.25% IP + E	None	5	5	0	0
	+	8	0	3	5 (4 with necrosis)
0.5% IP + E	None	5	5	0	0
	+	8	2	0	6 (4 with necrosis)

Rats were fed the Lieber-DiCarli diet containing 6.3% ethanol (E) and increasing concentrations of isopentanol (IP), as indicated, for 3 days. APAP (0.25 g/kg) was administered intragastrically, and rats were killed 7 hr after administration of APAP, as described in Materials and Methods. Slices of liver were fixed in formalin and examined histologically, as described in Materials and Methods.

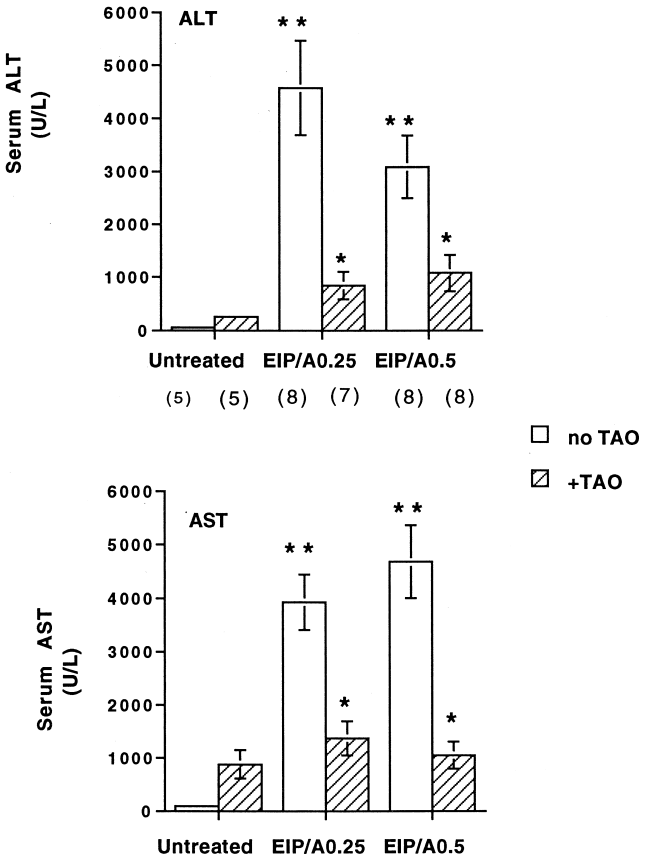

FIG. 4. Effect of TAO on APAP hepatotoxicity in rats pretreated with ethanol and isopentanol. Rats were fed the Lieber–DeCarli liquid diet containing 6.3% ethanol and 0.5% isopentanol (E + IP) for 7 days. Rats not administered alcohols were given equivalent calories as maltose–dextrin in the liquid diet. Animals treated with the combination of ethanol and isopentanol were administered APAP (A) at a dose of 0.25 or 0.5 g/kg, as described in Materials and Methods. TAO (500 mg/kg) was administered intragastrically 2 hr before APAP, as described in Materials and Methods. Serum levels of AST and ALT were measured as described in Materials and Methods. The number of animals per treatment group is given in parentheses in the figure. Each value represents the mean, with SEM indicated by the vertical line. Key: (*) significantly different from E + IP + APAP without TAO, $P < 0.01$; and (**) significantly different from no alcohol, $P < 0.001$.

the damage mediated by APAP was associated with destruction of alcohol-induced CYP3A, and that TAO prevented this destruction. A 9-hr exposure to TAO alone resulted in a small increase in CYP3A in the centrilobular region (Fig. 6, A vs B).

DISCUSSION

We had reported previously that TAO, a specific inhibitor of CYP3A [18, 19], protects rats pretreated with ethanol alone from APAP hepatotoxicity [20, 21], suggesting a major role of CYP3A. In that study, although TAO prevented histologically observable liver damage completely, it did not prevent the elevation in serum levels of AST. In the current study, TAO protected rats pretreated with ethanol plus isopentanol from APAP hepatotoxicity, decreasing both the elevation in serum ALT (Fig. 4) and the histologically observed liver damage (Table 1). In our previous study in rats pretreated with ethanol alone, a dose of APAP as high as 1 g APAP/kg was necessary to cause liver damage [9, 20, 21], whereas in rats pretreated with ethanol plus isopentanol liver damage was observed at a dose of 0.25 g APAP/kg (Fig. 1, Table 1). With the combined alcohol pretreatment, however, the degree of protection afforded by TAO, as observed histologically, was greater at an APAP dose of 0.25 g/kg than at 0.5 g/kg (Table 1). These results suggest that, at the lower dose of APAP, CYP3A is the major form of CYP responsible for alcohol-mediated increases in APAP hepatotoxicity. At the higher doses of APAP, another form of CYP, such as CYP2E, may also be involved, consistent with the lower affinity of CYP2E compared with CYP3A for APAP [22, 23].

The lack of complete protection by TAO may also result from incomplete inhibition of CYP3A or some other mechanism affected by the alcohols. One such mechanism could involve alcohol-mediated decreases in GSH. Alternatively, metabolism of the alcohols could lead to inhibition of GSH regeneration due to alterations in the redox status of the cells, thereby decreasing the cellular capacity to respond to APAP-induced oxidative damage. In rats, a 40% decrease in GSH does not of itself increase APAP

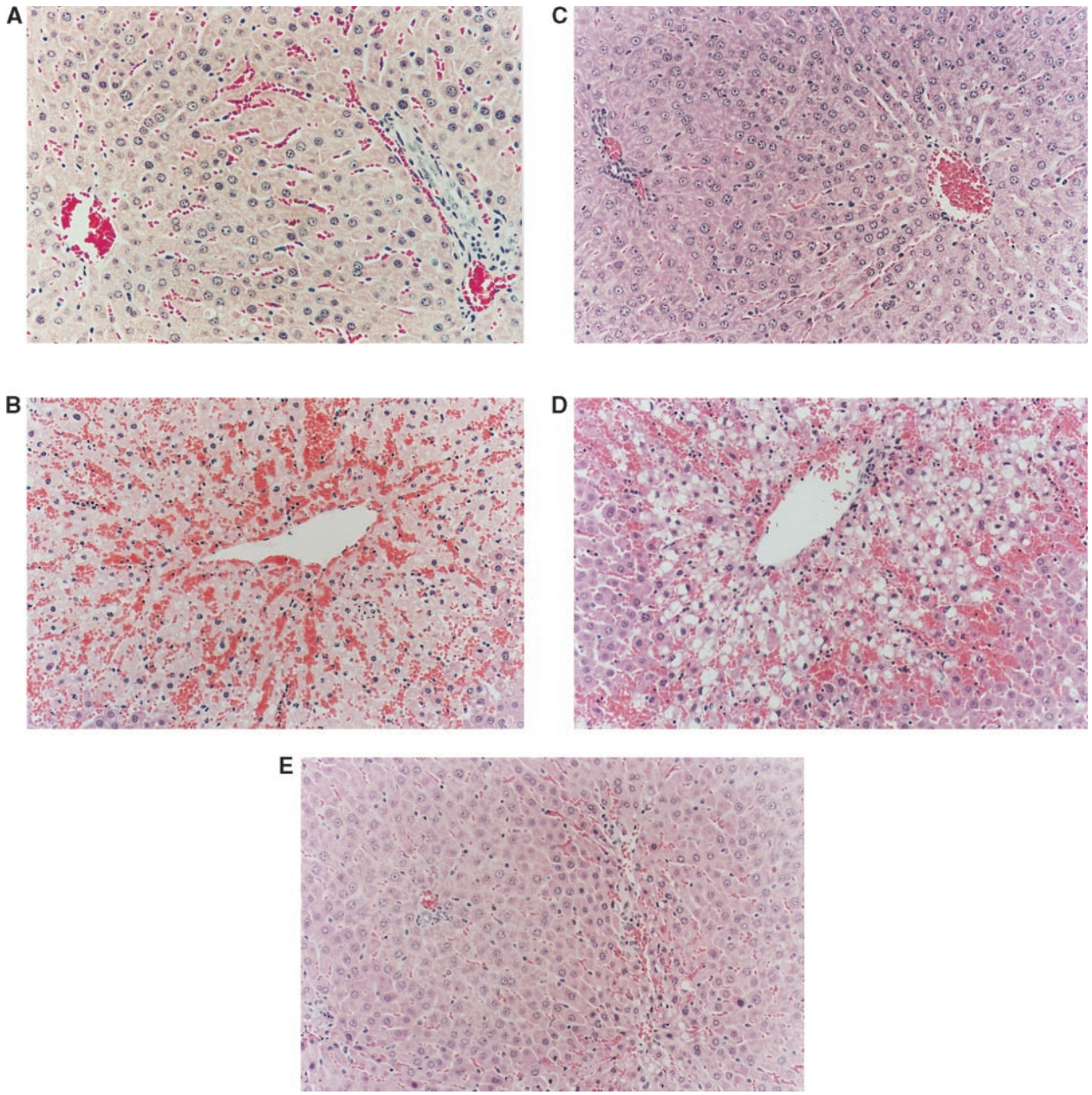

FIG. 5. Histological analysis of livers from rats treated with alcohols, APAP, and TAO. The magnification is 200x. (A) Representative section of liver from rats treated with ethanol plus isopentanol. (B) Section of liver from rats treated with ethanol plus isopentanol, followed by 0.25 g APAP/kg. (C) Section of liver from rats treated with ethanol plus isopentanol, followed by TAO and 0.25 g APAP/kg. (D) Section of liver from rats treated with ethanol plus isopentanol, followed by 0.5 g APAP/kg. (E) Section of liver from rats treated with ethanol plus isopentanol, followed by TAO and 0.5 g APAP/kg.

hepatotoxicity unless glutathione reductase is inhibited [26]. These findings suggest that regeneration of GSH is crucial for protection against APAP hepatotoxicity. However, we have found in cultured hepatocytes that, although a 30% decrease in GSH does not, of itself, increase APAP toxicity, this decrease, along with increases in CYPs activating APAP, does increase APAP toxicity [27].

Although consumption of alcohols has been associated with decreased levels of GSH in humans [28] and rats [24, 25, 29], in our prior studies, the 7-day treatment with ethanol alone or in combination with isopentanol did not result in decreases in hepatic levels of GSH [9]. Furthermore, a 7-hr exposure to APAP resulted in similar decreases in hepatic levels of GSH in rats administered the liquid diet alone or with the alcohols. Yet liver damage was only observed in the alcohol-pretreated rats [9]. Thus, in our studies, decreases in GSH do not appear to be a factor in the alcohol-mediated increases in APAP hepatotoxicity.

In humans consuming alcoholic beverages, increased APAP hepatotoxicity is associated with both necrosis and steatosis. The steatosis is attributed to the alcohol consumption, and the necrosis to damage from APAP, since

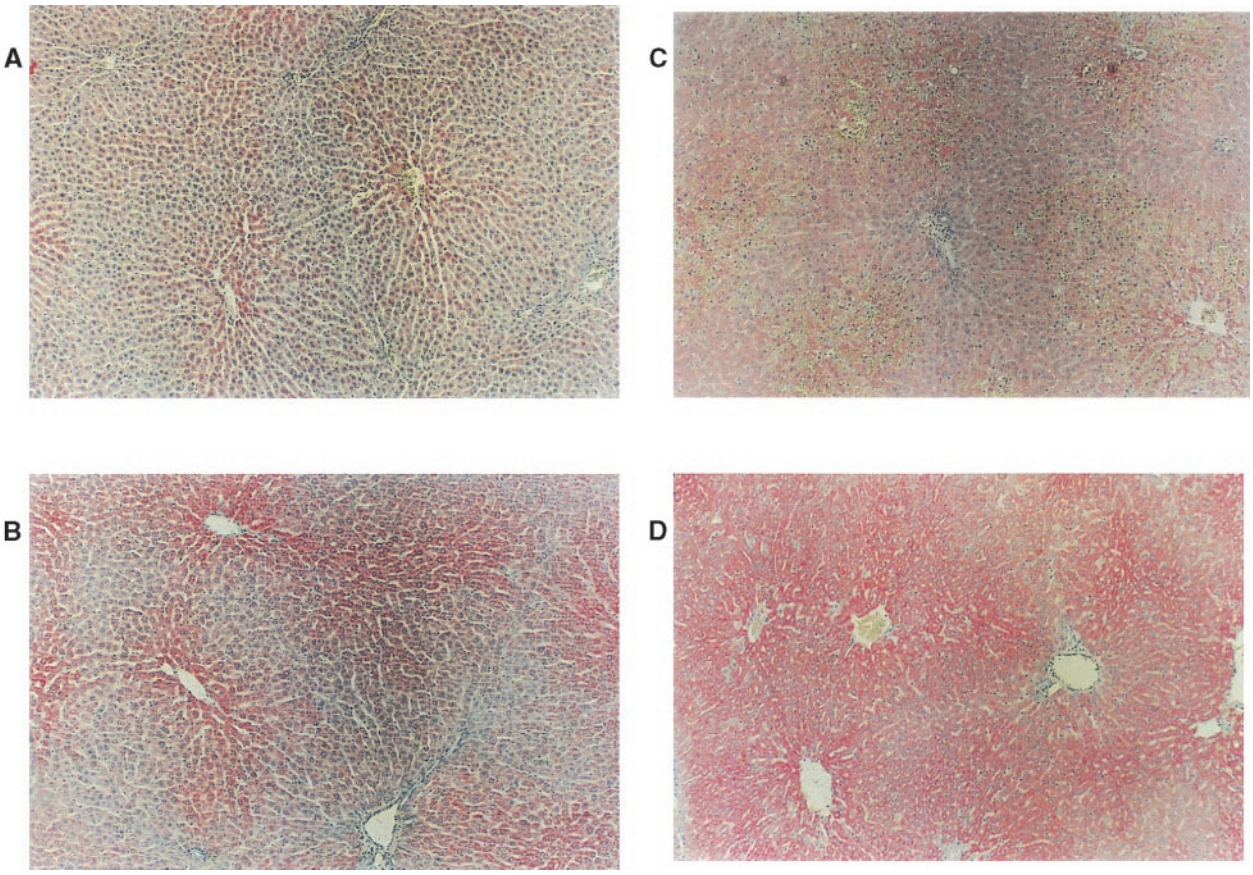

FIG. 6. Effect of TAO on immunohistochemical amounts of CYP3A. Rats were pretreated with ethanol (6.3%) and isopentanol (0.5%), as indicated, for 7 days. The diet was replaced with water for 11 hr and the rats administered APAP at 0.25 g/kg as described in Materials and Methods. TAO was administered 2 hr before administration of APAP, as described in Materials and Methods. CYP3A was analyzed immunohistochemically (red stain) in sections from liver slices fixed in formalin, as described in Materials and Methods. Treatments are labeled in the figure: (A) No alcohols, no APAP, and no TAO. (B) TAO alone for 9 hr. (C) Ethanol and isopentanol 7 days before a 7-hr exposure to APAP. (D) Ethanol, isopentanol, APAP, and TAO. The figure represents a 50x magnification.

only necrosis is observed in the livers of non-alcoholic individuals who have taken lethal doses of APAP for suicidal purposes [2]. In our studies in rats, there was no steatosis after a 7-day exposure to ethanol and isopentanol, and no detectable liver damage, as observed histochemically ([9], Table 2) and by the serum levels of ALT (Figs. 2 and 3). However, after exposure to APAP, the livers of these animals developed steatosis as well as necrosis (Tables 1 and 2, Figs. 5 and 6), similar to that observed in alcohol-mediated increases in APAP hepatotoxicity in humans [2]. At the time of APAP administration, it is unlikely that any ethanol or isopentanol was present in the liver, since the liquid diet was replaced with water 11 hr before the administration of APAP. The alcohols probably would have been removed completely by 6 hr, based on i) the serum levels of ethanol (40 mM) and isopentanol (0.017 mM) before removal of the alcohols [9], ii) the rate of elimination of ethanol in rats [25], and iii) the relative rate of metabolism of ethanol and isopentanol by alcohol dehydrogenase in the livers of these rats [30]. Exposure of rats to ethanol and a high-fat diet has been shown to increase the development of steatosis due to increased synthesis of fatty acids and decreased oxidation of lipids (for reviews, see Refs. 31 and 32). Some of these effects arise from the alteration in hepatic levels of NAD in the metabolism of ethanol [33]. In our studies, the ability of the liver to regenerate NAD may be impaired by the damage ensuing during exposure to APAP, resulting in steatosis.

CYP2E has been considered to be the major form of CYP responsible for alcohol-mediated increases in APAP hepatotoxicity in humans and experimental animals [2, 8, 15–17]. This presumption of the major role of CYP2E in APAP hepatotoxicity was supported by the following findings: i) in humans, consumption of alcoholic beverages is associated with elevated levels of CYP2E [34, 35]; ii) in experimental animals, ethanol, the major alcohol in alcoholic beverages [5, 6], increases hepatic levels of CYP2E [10, 11]; and iii) several inhibitors of CYP2E protect against ethanol-mediated increases in APAP hepatotoxicity (for review, see Ref. 15). However, many of the inhibitors of CYP2E used previously to evaluate the role of CYP2E in APAP hepatotoxicity are now proving to inhibit CYP3A [15]. Recently, two enzymatic assays used as indicators of CYP2E in inhibition studies, the hydroxylation of chlorzoxazone and *p*-nitrophenol, have been shown to be catalyzed by CYP3A [36–38].

Other evidence supporting a role of CYP2E in APAP hepatotoxicity are the findings that *Cyp2e1*(−/−) mice are more resistant to APAP hepatotoxicity than the wild-type mouse [39]. However, in one study, *Cyp1a2*(−/−) mice were found to be more resistant to APAP hepatotoxicity than the wild-type mouse [40], even though hepatic levels of CYP2E1 and CYP3A are not affected by the *Cyp1a2* deletion [41]. In the study by Genter *et al.* [40], no hepatotoxicity was observed in *Cyp1a2*(−/−) mice treated with a dose of 600 mg APAP/kg. In contrast, Tonge *et al.* [42] have reported that a dose of 250 mg APAP/kg is hepatotoxic to both *Cyp1a2*(−/−) and the wild-type strain. Since the route of APAP administration and time of exposure were similar in both studies, it is unclear why such disparate results were obtained. One problem may be the mixed genetic background of these mice, since they are a cross of C57BL6 and 129/olga [40], and the heterogeneity in the 129 mouse strains [43, 44]. Although the double knockout mouse, *Cyp2e1*(−/−)*Cyp1a2*(−/−), is also more resistant to APAP hepatotoxicity than the wild-type mouse, no comparisons to the individual knockout strains were made in that study [45]. The K_m and V_{max} values of expressed forms of CYP for APAP activation suggest that CYP1A2 would have a minor role in APAP hepatotoxicity compared with CYP2E1 or CYP3A [22, 23]. More than one form of CYP may contribute to APAP hepatotoxicity, depending on the dose of APAP and the relative amounts of each CYP in the liver.

We previously had found in rats that ethanol alone for 7 days caused a small increase in hepatic microsomal CYP3A, whereas combined treatment with ethanol and isopentanol caused greater increases in CYP3A than did ethanol alone [13]. In the current study, CYP3A, as measured immunohistochemically, was present throughout the liver in rats pretreated with ethanol plus 0.5% isopentanol, and exposed to TAO prior to treatment with 0.25 g APAP/kg (Fig. 6). In contrast, CYP3A was barely detected in alcohol-pretreated rats exposed to APAP in the absence of TAO (Fig. 6). These findings suggest that alcohol-induced CYP3A was destroyed by APAP and protected by TAO. CYP3A may be inactivated suicidally during the metabolism of APAP, or may be destroyed indirectly by the oxidative damage in the liver. Similarly, in mice, both CYP1A1 and CYP2E, as measured enzymatically, are decreased at a hepatotoxic dose of APAP (400 mg/kg) [46]. The extents of the decreases in these CYPs were similar whether the animals received no pretreatment or were pre-exposed to β-naphthoflavone or acetone to induce CYP1A1/2 or CYP2E, respectively. The CYPs may have been decreased by APAP either directly by suicidal inactivation or indirectly due to oxidative damage. In the same study [46], a lower dose of APAP (150 mg/kg) caused mild liver damage in animals pretreated to induce CYP2E or CYP1A1/2. Small decreases in an enzymatic activity associated with CYP2E were observed with no decreases in CYP1A1 [46]. Those results suggest that CYP2E but not CYP1A1 can be destroyed by low doses of APAP. Our findings in rats suggest that CYP3A can also be destroyed by low doses of APAP.

In humans, although chronic consumption of alcoholic beverages is associated with the development of liver damage from therapeutic doses of APAP [1–4], the risk associated with the amount of alcohol consumed or the length of time of consumption has not been identified unequivocally. Our results with isopentanol in combination with ethanol suggest that the isopentanol content of alcoholic beverages may be a risk factor for the development of liver damage from APAP. Homemade beers and wines can contain up to 0.5% isopentanol, and commercial wines, cognac, whisky, and rum can contain from 0.16 to 0.3% isopentanol [5, 6]. In rats treated with the combination of ethanol and 0.1% isopentanol in a liquid diet for only 3 days, we observed a dramatic increase in APAP hepatotoxicity at a dose of 0.25 g APAP/kg (Fig. 3, Table 2). Thus, the rat, a species previously considered insensitive to APAP hepatotoxicity, may become sensitized by treatment with the combination of ethanol and isopentanol. Since isopentanol is a potent and effective inducer of CYP3A and CYP2E in cultured human hepatocytes [47], our findings with rats warrant studies in humans to investigate whether short-term consumption of alcoholic beverages containing isopentanol is a risk factor for development of liver damage from APAP.

Consumption of alcoholic beverages has been associated with elevations in CYP3A in humans [48]. Our findings that TAO protects animals pretreated with ethanol alone [20, 21] or in combination with isopentanol (Fig. 4, Table 1) from APAP hepatotoxicity suggests that CYP3A plays a major role in the response. Many medications induce CYP3A4 in humans (for review, see Ref. 21), and, in experimental animals, weight loss induces CYP3A [49, 50]. Methylxanthines such as caffeine and theophylline increase the activity of CYP3A and increase APAP hepatotoxicity in rodents [51, 52]. Our findings suggest that factors in addition to alcohol consumption also may increase the risk of developing liver damage from APAP. We are investigating the contribution of some of these factors to APAP hepatotoxicity.

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