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Protective Effect of Ethanol against Acetaminophen-Induced Hepatotoxicity in Mice

ROLE OF NADH:QUINONE REDUCTASE

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ABSTRACT. The role of NAD(P)H:quinone reductase (QR; EC 1.6.99.2) in the alcohol-derived protective effect against hepatotoxicity caused by acetaminophen (APAP) was studied. In mice pretreated with dicoumarol (30 mg/kg), an inhibitor of QR, hepatic necrosis caused by APAP (400 mg/kg) was potentiated. Hepatocellular injuries induced by APAP, as assessed by liver histology, serum aminotransferase activities, hepatic glutathione (reduced and oxidized) contents, and liver microsomal aminopyrine *N*-demethylase activities, all were potentiated by pretreatment of mice with dicoumarol. Even in mice given APAP and ethanol (4 g/kg), in which APAP-inducible hepatic necrosis was abolished, the dicoumarol pretreatment again produced moderate hepatotoxicity and reversed the protective effect of ethanol. In mice pretreated with dicoumarol and ethanol, levels of APAP in blood and bile fluid between 90 and 240 min were higher than those in mice given ethanol. However, the biliary contents of sulfate and glucuronide conjugates of APAP were much lower than those in the ethanol group, particularly at early time points. In contrast, the biliary level of APAP–cysteine conjugate, which in the ethanol group was at its basal level, was increased maximally in the dicoumarol-pretreated mice. In the mice given dicoumarol and ethanol, the biliary APAP–cysteine conjugate level was increased moderately. These results suggest that ethanol inhibited not only the microsomal (CYP2E1 mediated) formation of a toxic quinone metabolite from APAP, but also accelerated the conversion of the toxic quinone metabolite produced back to APAP by stimulating cytoplasmic QR activity. In the presence of dicoumarol, however, QR activity was inhibited, and conversion of the toxic quinone metabolite back to APAP became inhibited and diminished the alcohol-dependent protective effect against APAP-induced hepatic injury. BIOCHEM PHARMACOL **58**;10: 1547–1555, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. acetaminophen; hepatotoxicity; ethanol; dicoumarol; NAD(P)H:quinone reductase

APAP‖ is used widely as an analgesic and antipyretic agent, but it can produce severe hepatic injury when an overdose occurs [1–3]. About 80% of ingested APAP is conjugated directly and forms sulfate and glucuronide esters before oxidation, and these conjugated esters are excreted in bile or urine [4]. Normally, less than 5% of the ingested APAP dose is oxidized by hepatic CYP2E1 to a highly reactive and toxic quinone intermediate, NABQI [5]. This electrophile is known to bind covalently to intracellular macromolecules, deplete glutathione, cause oxidative stress, and alter calcium and/or thiol status in liver cells, all leading to hepatocellular injury [6].

Cytotoxic effects of many quinone compounds, including the NABQI produced from oxidation of APAP, are thought to be mediated by semiquinone radicals, the one-electron reduction metabolites of quinones. Production of these toxic semiquinone radicals is catalyzed by the microsomal NADPH:cytochrome P450 reductase. These semiquinone radicals, in turn, can bind directly with cellular macromolecules to produce toxicity, or, alternatively, the radicals can be reoxidized back to their original quinones by donating one electron to molecular oxygen under aerobic conditions. This donation then generates reduced oxygen radical species such as superoxide anion and hydroxyl radical. Both semiquinone and oxygen radicals are known to be responsible for the cytotoxic effects observed with quinones [6, 7].

Alternatively to this toxic one-electron reduction pathway, quinone compounds also can be reduced by a direct two-electron reduction pathway to non-toxic hydroquinones, either enzymatically by QR (EC 1.6.99.2) or chemically by the oxidation of two molecules of GSH. Both of these direct two-electron reductions will occur without any production of the toxic semiquinone or oxygen radicals and, therefore, may provide a competitive protective path-

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‖ *Abbreviations*: APAP, acetaminophen; QR, NAD(P)H:quinone reductase; NAD^+, oxidized nicotinamide adenine dinucleotide; CYP2E1, cytochrome P450 2E1; NABQI, *N*-acetyl-*p*-benzoquinoneimine; ADH, alcohol dehydrogenase; and AST, aspartate aminotransferase.

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way against the toxicity caused by one-electron reduction of NABQI [8].

Clinical observations have shown that acute intake of alcohol immediately after the incidence of an APAP overdose can protect against APAP-inducible hepatocellular injury in humans [9, 10]. This alcohol-dependent protective effect against APAP-inducible hepatotoxicity has been demonstrated clearly in laboratory animals [11]. In support of this *in vivo* observation, *in vitro* studies using isolated liver microsomes have shown that ethanol provides competitive inhibition of CYP2E1-catalyzed oxidation of APAP to toxic NABQI [12]. This also has been observed in liver slices [13], hepatocytes [14], and perfused livers [15]. Thus, it was presumed that ethanol-dependent competitive inhibition of the CYP2E1-catalyzed production of NABQI provided the observed protective effect [16]. However, this competitive inhibition occurs only at extremely high ethanol concentrations and is not sufficient to account for the decreased formation of NABQI caused by ethanol. Although the protective effect of ethanol on APAP-induced hepatotoxicity has been studied extensively, the mechanisms involved in this protective effect still are unclear.

Ethanol is oxidized in the cytoplasm of liver cells primarily by NAD^+-dependent ADH (EC 1.1.1.1). Following acute intake of alcohol, continued oxidation of ethanol by ADH increases the level of NADH at the expense of NAD^+ and causes shifting of the intracellular redox state to a reduced state (decreased NAD^+ to NADH ratio). Under such conditions, due to the abundance of NADH, lactic acidosis occurs and alcohol and fatty acid oxidations are hindered, thus causing alcoholic fatty liver [17].

As mentioned above, quinone compounds can be reduced enzymatically by QR in hepatic cytoplasm directly to hydroquinones [18]. This QR reaction proceeds in an opposite direction from that of ADH. Therefore, when the quinone is reduced to hydroquinone by QR, NADH is oxidized to NAD^+, regenerating the oxidized cofactor needed for continued and accelerated alcohol oxidation. Thus, when QR and ADH are operating together in close proximity to each other (both enzymes are localized in the cytoplasm of liver cells in great abundance), quinones such as NABQI produced from APAP may become readily reduced back to APAP, thereby decreasing the level of NABQI and limiting the formation of the toxic NABQI semiquinone radical. With such cooperative interaction provided by QR oxidizing NADH back to NAD^+ in the presence of quinones, the alcohol oxidation rate will become enhanced due to an increased supply of NAD^+. This has been demonstrated in a recent series of studies conducted in this laboratory [19–21].

Therefore, the purpose of this study was to investigate the *in vivo* role of QR in ethanol-dependent protection against APAP-inducible hepatotoxicity. By using dicoumarol, an anticoagulant known to inhibit QR activity by competing with the binding of NADH [22, 23], and thus breaking the cooperative interaction between QR and ADH, functional involvement of QR in the observed ethanol-dependent protective effect against APAP-inducible hepatotoxicity was demonstrated.

MATERIALS AND METHODS

Chemicals

APAP was obtained from the Sigma Chemical Co. through the courtesy of the IL-Yang Pharm. Ind. Co. APAP metabolite standards used in HPLC analysis were donated by the McNeil Consumer Products Co. Dicoumarol was purchased from BDH Chemicals. Ethanol given to mice and other solvents used in HPLC were purchased from Merck-Darmstadt. The S.TA-test enzyme kit used for determination of serum AST was purchased from Wako Pure Chemical Ind., Ltd. All other chemicals used in this study were reagent grade and were commercially available locally.

Animals and Treatments

Male ICR mice weighing 23 ± 3 g were obtained from the Animal Breeding Laboratory of Yonsei Medical College and were acclimated to the laboratory conditions for at least 1 week. During this period, food (donated by Korea Purina, Inc.) and tap water were supplied *ad lib*. Mice were deprived of food for 24 hr and were given an i.p. injection of APAP (400 mg/kg in a pH 10.6 solution). For the group requiring *in vivo* inhibition of QR, an i.p. injection of dicoumarol (30 mg/kg in 1% gum tragacanth) was given 3.5 hr prior to the APAP injection. Ethanol (4 g/kg diluted to 20% in water) was given by oral intubation (p.o.) immediately after the APAP injection. Mice belonging to the respective control groups received only gum tragacanth (i.p.) vehicle.

Histology

At 6 and 18 hr after the APAP administration, a small piece of liver tissue from the anterior portion of the left lateral lobe was taken for light microscopy. Paraffin blocks were prepared after fixation in 10% neutral formalin and were sectioned and stained with hematoxylin and eosin. A quantitative analysis for the degree of hepatic necrosis in each experimental animal at the 6-hr time point was performed according to the method described by Mitchell *et al.* [24]. Numerical scores shown in Table 1 indicate the severity of liver injury and were based on the extent of damage present in microscopic fields: 0 = absent, 1 = less than 6%, 2 = 6–25%, 3 = 26–50% and 4 = greater than 50% of liver parenchymal cells showing evidence of centrilobular necrosis. Light microscopic observations obtained from livers of experimental mice at the 18-hr time point are shown in Fig. 1 for visual demonstration of hepatic injury caused by APAP, its potentiation by dicoumarol, and its prevention by ethanol.

TABLE 1. Quantitative summary of histological observations on ethanol-dependent protection and dicoumarol-dependent potentiation of APAP-induced hepatic necrosis

Treatment	No. of animals	Mortality (%)	Extent of necrosis in survivors (%)				
			0	+	++	+++	++++
APAP	8	0	37	13	25	25	0
APAP + ethanol	8	0	50	25	25	0	0
APA + dicoumarol	15	47	0	13	13	37	37
APAP + dicoumarol + ethanol	8	0	50	13	25	12	0

After a 24-hr fast, mice were injected witn APAP (400 mg/kg of body weight, i.p.) and killed 6 hr after the injection. The other treatment regimens are given in Materials and Methods. The extent of hepatic necrosis was scored in survivors according to the methods of Mitchell *et al.* [24]: 0 = absent, 1+ = necrosis in less than 6% of hepatocytes, 2+ = 6–25%, 3+ = 26–50%, and 4+ = greater than 50%.

Levels of APAP in Blood

At 15, 30, 60, 90, 120, 240, and 360 min after administering APAP, mice were decapitated and blood samples were collected. Serum levels of APAP were determined with HPLC using the procedure described by Black and Sprague [25]. Plasma proteins were precipitated in 50% acetonitrile, removed by centrifugation (15,000 *g* for 3 min), and discarded. Supernatants (4 μL) were injected into an HPLC system (Waters model 441) equipped with a C_{18} μ-Bondapak column and UV detector (254 nm). APAP was eluted isocratically with a mobile phase composed of 0.01 M acetate buffer (pH 4.0) and acetonitrile (93:7) at a flow rate of 2 mL/min.

APAP and Its Metabolites in Bile

Mice were killed 15, 30, 60, and 90 min and 2, 4, and 6 hr following the administration of APAP. After a median laparotomy, the cystic duct was ligated and the gallbladder was freed carefully from surrounding tissues. Bile fluid was collected from the gallbladder, and levels of APAP and its conjugated metabolites were determined according to the procedure described by Howie *et al.* [26]. Bile samples were diluted with methanol and injected directly onto the C_{18} μ-Bondapak column of an HPLC system. A mobile phase composed of 1% acetic acid, methanol, and ethyl acetate (90:15:0.1) flowing at 1.0 mL/min was used to elute APAP and its conjugated metabolites. The UV detector was set at 254 nm, and the concentrations of APAP and its metabolites were calculated from their respective standard curves.

Preparation of Liver Microsomes

Eighteen hours after the administration of APAP, a small piece of liver tissue was taken first for measurement of hepatic GSH content, and the remaining liver tissue was

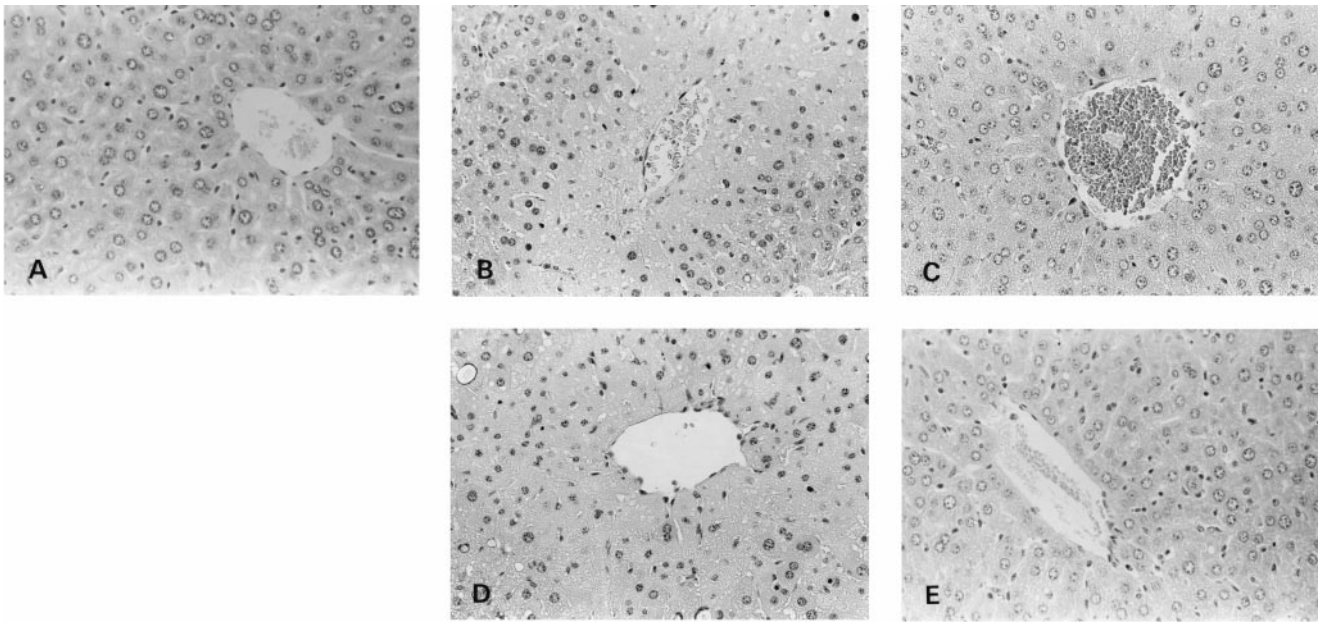

FIG. 1. Histological demonstration of ethanol-dependent protection and dicoumarol-dependent potentiation of APAP-induced hepatic necrosis. (A) Control, showing normal lobular architecture and cell structure. (B) APAP-treated, showing moderate to severe zone 3 necrosis with fatty changes containing many fat droplets. Nuclei of hepatocytes show karyolysis and pyknosis. (C) APAP with ethanol co-administration, showing mildly altered lobular structure and microvesicular fatty changes in hepatocytes. (D) Dicoumarol-pretreated and APAP-injected, showing severe zone 3 necrosis. Most hepatocytes in zones 2 and 3 showed moderate fatty changes, and their nuclei showed karyolysis and pyknosis. (E) Dicoumarol-pretreated and ethanol co-administered, showing moderate zone 3 necrosis and fatty changes in hepatocytes. All photographs are at 400x magnification.

used for preparation of liver microsomes. After taking a small fresh liver tissue sample from the anterior portion of the left lateral lobe for determination of hepatic GSH and GSSG contents, the remaining liver tissue was washed by infusing ice-cold saline into the hepatic vein. Washed liver tissue then was blotted, weighed, minced, and homogenized in 2 vol. of 150 mM KCl. After removing the mitochondrial fraction by an initial centrifugation (9000 *g* at 2°), the microsomal fraction was isolated by ultracentrifugation at 105,000 *g* at 2°, and the resulting microsomal pellet was resuspended in 100 mM phosphate buffer (pH 7.4).

Enzyme Assays and Analytical Procedures

Liver microsomal aminopyrine *N*-demethylase activity was measured *in vitro* according to the method described by Schenkman *et al.* [27]. The demethylase activity was determined by measuring formaldehyde after the addition of Nash reagent. Liver microsomal protein content was determined according to Lowry *et al.* [28] using bovine serum albumin as the standard. Hepatic contents of GSH and GSSG were determined according to the method of Tietz [29] as modified by Griffith [30]. Serum AST was determined using a standard spectrophotometric procedure employing the enzyme assay kit obtained from Wako Pure Chemical Ind., Ltd.

Statistical Analysis

The significance of changes in various parameters measured was determined using a one-way ANOVA test. Differences between experimental groups were considered significant at $P < 0.05$ using the Student-Newman-Keuls test for multiple comparisons. All results are presented as means ± SEM.

RESULTS

Histology

Table 1 and Fig. 1 show the results of histological observations. Whereas all mice survived, in the livers of mice given APAP there was extensive hepatocellular damage. This was evidenced by the presence of necrotic foci, hydropic changes, and infiltration of inflammatory cells. In contrast to this, in the livers of mice given ethanol with APAP, few necrotic changes were observed. In the mice pretreated with dicoumarol before the APAP injection, however, global centrilobular necrosis was observed. Furthermore, half of the mice in this group died, and additional mice had to be treated similarly to replace the missing ones. The co-administration of ethanol to mice pretreated with dicoumarol again produced moderate protection against the centrilobular necrosis.

Serum Aminotransferase

To support the results of histological observation (Table 1 and Fig. 1), serum AST activities were determined for 18 hr following the administration of APAP. As early as 2 hr after the drug administration, a marked increase of AST activity was observed in mice given APAP alone. In contrast, the AST activity was increased only slightly in the ethanol co-administered group. However, the levels of AST at 2 and 4 hr in the dicoumarol-pretreated groups were even higher than those of the APAP-treated control group. When ethanol was co-administered to mice given dicoumarol pretreatment, the serum AST level returned toward a normal level (Fig. 2).

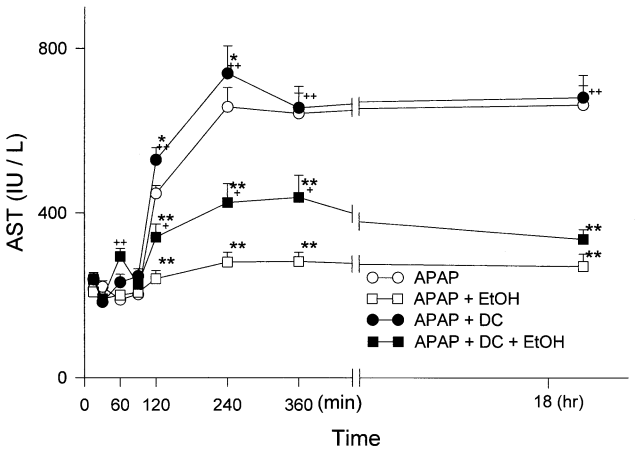

FIG. 2. Effect of ethanol, dicoumarol (DC), and their combination treatments on time-dependent increases of serum AST activity caused by APAP. Values indicate means ± SEM. Points with * and ** indicate that the mean serum AST levels (IU/L) obtained from 7 mice were significantly different from those observed in the APAP alone group at $P < 0.05$ and $P < 0.01$, respectively. Points with $^{+}$ and $^{++}$ indicate significant differences from the APAP and ethanol co-administered group at $P < 0.05$ and $P < 0.01$, respectively.

Hepatic Glutathione Concentration

As shown in Table 2, APAP administration by itself decreased the hepatic content of GSH by 26%. This decline of hepatic GSH content appeared to be accompanied by an insignificant increase of GSSG content, and thus the ratio of GSH to GSSG (an indicator of the hepatocellular redox state) declined by 49%. Whereas the ethanol administration by itself did not alter hepatic contents of GSH and GSSG (data not shown), in mice given ethanol together with APAP, the GSH content appeared to decrease (not significantly) when compared with that obtained in the control group. Hepatic GSSG content in these mice, however, was decreased significantly, and thus the cellular redox state indicated by the GSH to GSSG ratio was maintained within the normal range. As with ethanol, dicoumarol by itself did not decrease GSH or GSSG contents (data not shown). However, the hepatic content of GSH in the dicoumarol-pretreated and APAP-treated group appeared to decrease even more severely than that obtained in the APAP-treated control group. Hepatic GSSG content in this group

TABLE 2. Effects of ethanol co-administration and discoumarol pretreatment on hepatic glutathione concentration and microsomal aminopyrine *N*-demethylase activity in APAP-treated mice

Group	GSH (μmol/g liver)	GSSG (μmol/g liver)	GSH/GSSG (ratio)	Aminopyrine *N*-demethylase (nmol HCHO/mg protein)
Control	36.7 ± 2.5	2.7 ± 0.5	17.5 ± 4.8	14.5 ± 0.5
APAP	27.3 ± 2.5*	3.5 ± 0.5	8.5 ± 0.9*	8.9 ± 0.5†
APAP + ethanol	31.1 ± 0.4	1.6 ± 0.0*‡	19.3 ± 0.4‡	12.1 ± 0.4‡
APAP + dicoumarol	24.4 ± 3.3*	3.9 ± 0.5§	7.2 ± 1.4*§	5.1 ± 0.5†‖
APAP + dicoumarol + ethanol	20.3 ± 1.8†‖§	1.8 ± 0.3‖	12.8 ± 1.8§	8.3 ± 0.7†§

At 18 hr after administering APAP, an anterior portion of the left lateral lobe was taken for measurement of hepatic GSH and GSSG contents, and the remaining liver tissue was used for preparation and determination of microsomal aminopyrine *N*-demethylase activity.

Values are means ± SEM obtained from 7 mice in each experimental group.

*,†Significant difference from the control group at $P < 0.01$, respectively.

‡Significant difference from the APAP group at $P < 0.01$.

§Significant difference from the APAP + ethanol group at $P < 0.01$.

‖Significant difference from the APAP group at $P < 0.05$.

appeared to increase, thus decreasing the GSH to GSSG ratio quite severely. In the mice pretreated with dicoumarol and given ethanol co-administration, both the GSH and GSSG contents were decreased significantly, and thus the GSH to GSSG ratio was not decreased as severely. Therefore, whereas the changes observed in the hepatic content of GSH by itself did not support the results of histological observation, the decreased ratio of GSH to GSSG (an indicator of oxidative stress) appeared to agree with the histological results obtained (Fig. 1).

Hepatic Microsomal Aminopyrine N-Demethylase Activity

Liver microsomal *N*-demethylation of aminopyrine is known to be catalyzed by CYP2A1/2 and CYP2B. Although these CYPs are not involved in APAP oxidation (catalyzed by CYP2E1), aminopyrine demethylase activity was measured to assess the overall degree of microsomal membrane destruction, presumably caused by the semiquinone metabolite of NABQI (a CYP2E1-generated metabolite of APAP). In mice given APAP 18 hr earlier, hepatic microsomal aminopyrine *N*-demethylase activity was decreased by 39% (Table 2). The microsomal enzyme activity of mice pretreated with dicoumarol prior to APAP injection was decreased even more severely (65%). Conversely, destruction of this general microsomal mixed-function oxidase activity in both groups of mice given ethanol co-administration was not as severe when compared with their respective control groups.

Blood Levels of APAP

As the results show in Fig. 3, blood levels of APAP in the ethanol co-administered mice were significantly higher (at 90 and 120 min) than those given APAP alone. Overall clearance of APAP was delayed as well. Blood levels of APAP in mice pretreated with dicoumarol were even higher than those obtained in the ethanol co-administered group. Blood levels of APAP observed in the dicoumarol-pretreated and then ethanol co-administered group appeared to be the highest for a longer duration (Fig. 3).

Levels of APAP and Its Metabolites in Bile

To determine the underlying reasons for these delayed APAP clearances, levels of the free APAP (Fig. 4) and the sulfate (Fig. 5) and glucuronide (Fig. 6) conjugates of APAP as well as the APAP–cysteine conjugate (Fig. 7) in the collected bile fluids at various time points were analyzed. As shown in Fig. 4, biliary APAP levels in the ethanol co-administered group were higher at all time points determined. APAP levels in the dicoumarol-pretreated mice were even higher than those obtained in the ethanol co-administered group. In mice pretreated with dicoumarol and then co-treated with ethanol, the biliary level of APAP at 240 min was significantly higher than the

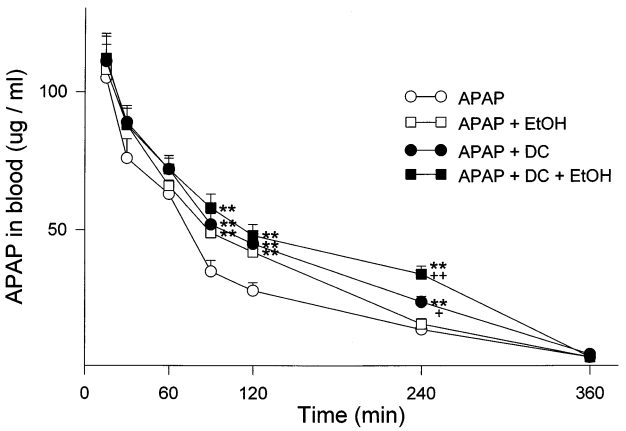

FIG. 3. Effect of ethanol, dicoumarol (DC), and their combination treatments on the time-dependent decrease of blood APAP levels. Values indicate means ± SEM. Points with ** indicate that the mean blood levels of APAP (μg/mL) obtained from 7 mice were significantly different from those observed in the APAP-alone group at $P < 0.01$. Points with $^{+}$ and $^{++}$ indicate significant differences from the APAP and ethanol co-administered group at $P < 0.05$ and $P < 0.01$, respectively.

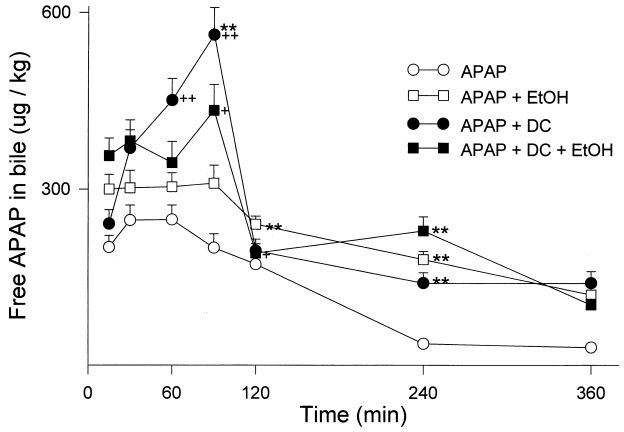

FIG. 4. Effect of ethanol, dicoumarol (DC), and their combined treatments on biliary levels of APAP. Values indicate means ± SEM. Points with ** indicate that the mean levels of APAP (μg/kg gallbladder) obtained from bile fluids of 6 mice were significantly different from those observed in the APAP alone group at $P < 0.01$. Points with $^{+}$ and $^{++}$ indicate significant differences from the APAP and ethanol co-administered group at $P < 0.05$ and $P < 0.01$, respectively.

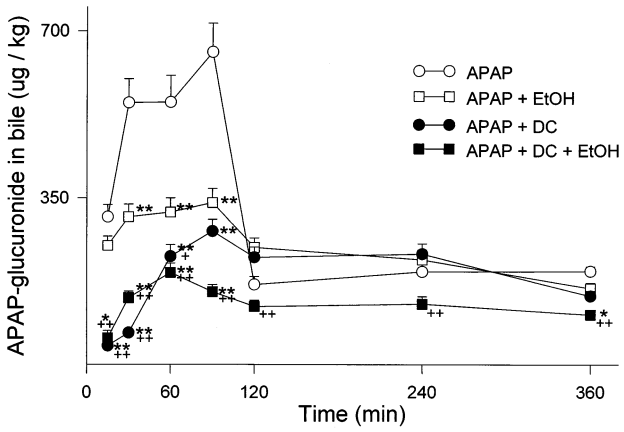

FIG. 6. Effect of ethanol, dicoumarol (DC), and their combined treatments on APAP–glucuronide conjugate in bile fluid. Values indicate means ± SEM. Points with * and ** indicate that the mean levels of APAP–glucuronide conjugate (μg/kg gallbladder) in bile fluids obtained from 6 mice were significantly different from those observed in the APAP alone group at $P < 0.05$ and $P < 0.01$, respectively. Points with $^{+}$ and $^{++}$ indicate significant differences from the APAP and ethanol co-administered group at $P < 0.05$ and $P < 0.01$, respectively.

level obtained in mice pretreated with dicoumarol only. Unexpectedly, the APAP levels at earlier time points in this group were, however, lower than that of the dicoumarol-pretreated group. In contrast to the free APAP level, biliary levels of the sulfate (Fig. 5) and glucuronide (Fig. 6) conjugates in the ethanol-administered group were much lower than those of its control group, particularly at early time points after APAP administration. Levels of these APAP conjugates in the dicoumarol-pretreated group were even lower than those of the ethanol co-administered mice. In mice pretreated with dicoumarol and co-administered ethanol, conjugated APAP levels at early time points were the lowest among all experimental groups. Biliary levels of the APAP–cysteine conjugate (Fig. 7), a presumed index of the electrophilic NABQI produced, in the ethanol co-administered group were decreased to the lowest level, and those in the dicoumarol-pretreated group were increased to the highest level. Administration of ethanol to these dicoumarol-pretreated mice again decreased the biliary level of the APAP–cysteine conjugate quite dramatically.

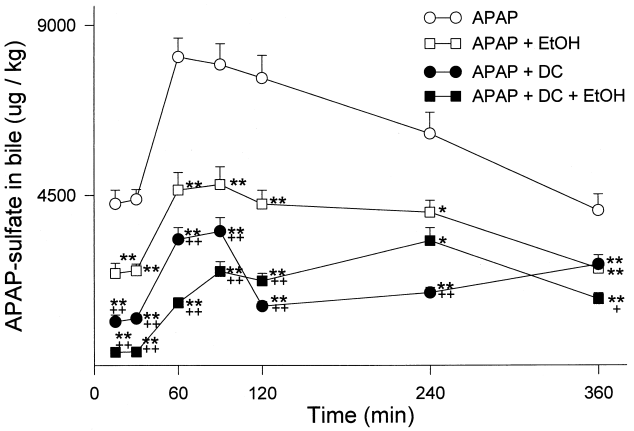

FIG. 5. Effect of ethanol, dicoumarol (DC), and their combined treatments on biliary levels of sulfate conjugate of APAP. Values indicate means ± SEM. Points with * and ** indicate that the mean levels of APAP–sulfate conjugate (μg/kg gallbladder) obtained from bile fluids of 6 mice were significantly different from those observed in the APAP-alone group at $P < 0.05$ and $P < 0.01$, respectively. Points with $^{+}$ and $^{++}$ indicate significant differences from the APAP and ethanol co-administered group at $P < 0.05$ and $P < 0.01$, respectively.

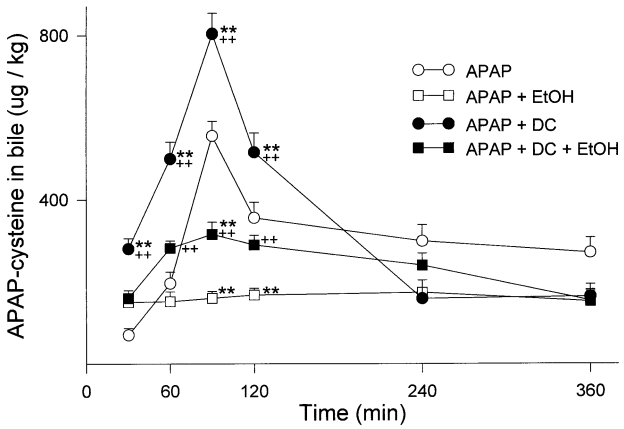

FIG. 7. Effect of ethanol, dicoumarol (DC), and their combined treatments on cysteine conjugate of APAP in bile fluid. Values indicate means ± SEM. Points with ** indicate that the mean levels of APAP–cysteine conjugate (μg/kg gallbladder) in bile fluids obtained from 6 mice in each experimental group were significantly different from those observed for the APAP-alone group at $P < 0.01$. Points with $^{++}$ indicate significant differences from the APAP and ethanol co-administered group at $P < 0.01$.

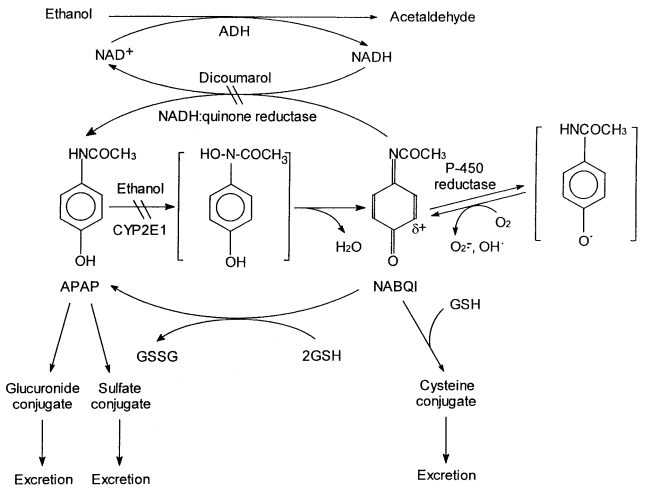

FIG. 8. Metabolic pathways of APAP and ethanol showing enzymes inhibited by dicoumarol and ethanol.

DISCUSSION

In clinical studies, Rumack *et al.* [31] and others [9, 10] observed that hepatic injury resulting from an overdose of APAP could be suppressed by administering an acute dose of ethanol. These clinical observations have been confirmed in animal studies conducted in this [16] and other [11] laboratories. A schematic view of APAP and ethanol metabolism is presented in Fig. 8, and the sites of biochemical interaction between the two metabolic pathways are indicated to explain the ethanol-dependent protective and the dicoumarol-dependent potentiative effects on APAP-inducible hepatic injury observed in clinical (alcohol) and experimental animal (ethanol and dicoumarol) studies.

Under normal conditions, the majority of the APAP administered is conjugated directly before oxidation to sulfate and glucuronide esters, and these conjugated esters are excreted mostly in bile without causing hepatocellular toxicity. Only a minor portion of the ingested APAP is thought to undergo *N*-hydroxylation by hepatic microsomal CYP2E1, and this intermediary metabolite is dehydrated quickly to produce NABQI, a more stable and measurable quinone metabolite [2]. The NABQI produced can be metabolized further by two pathways. First, the electrophilic NABQI may become conjugated with GSH and excreted as the cysteine conjugate [32] or become reduced back to APAP (hydroquinone) by taking two electrons from two molecules of GSH and producing GSSG, which can be excreted from liver cells [18]. This leads to eventual depletion of GSH [33]. Second, the NABQI may become reduced, either by one-electron reduction catalyzed by microsomal NADPH:cytochrome P450 reductase, producing a highly reactive semiquinone radical, or by the direct two-electron reduction catalyzed by cytoplasmic QR, thus regenerating APAP, a hydroquinone [34]. Once NABQI is reduced back to APAP by the two-electron reduction pathways, oxidizing both GSH and NADH in the cytoplasm of liver cells, it can easily be conjugated again to the sulfate and glucuronide esters and excreted into bile or urine as mentioned above. However, if NABQI is reduced to its semiquinone radical by the one-electron reduction catalyzed by P450 reductase, it can cause cellular toxicity either by direct covalent binding to cellular macromolecules or by indirect production of reduced oxygen radicals (O_2^-, $OH^\cdot$) under aerobic conditions [34, 35].

Ethanol is oxidized to acetaldehyde in the liver cell primarily by the NAD^+-dependent cytoplasmic ADH, and NADH is produced. When, however, a large amount of ethanol is administered acutely, in addition to the ADH-catalyzed alcohol oxidation, it is oxidized also by the NADPH-dependent microsomal CYP2E1 (microsomal ethanol-oxidizing system) [12]. Furthermore, it has been found that ethanol inhibits the metabolism of various drugs, and in the case of aminopyrine *N*-demethylase, ethanol inhibition is of a competitive nature only at high concentrations [12]. Under these conditions, ethanol competes with APAP for the CYP2E1-catalyzed oxidation and inhibits microsomal oxidation of APAP [11–15]. Thus, in previous studies, the ethanol-dependent protective effect against APAP-inducible hepatotoxicity has been explained by competitive inhibition of the CYP2E1-catalyzed production of the toxic quinone metabolite (NABQI) from APAP [36, 37]. While this may occur, it is possible only at extremely high and unphysiological ethanol concentrations [11, 16].

At a more physiological concentration, however, ethanol is oxidized mostly by ADH, and this ADH-catalyzed *in vivo* ethanol oxidation is thought to be rate-limited by the slow turnover of ADH [38]. The ADH reaction proceeds first by sequential binding of NAD^+ and ethanol to the enzyme, forming a ternary complex. In this complex, ethanol is oxidized to acetaldehyde, and NAD^+ is reduced to NADH. Next, acetaldehyde leaves the complex quickly, but NADH exits slowly, thus hindering another NAD^+ from binding to ADH and limiting rapid oxidation of ethanol [39]. Based on this mechanism, it was suggested by Cha and Heine [40] that co-operation of another cytoplasmic enzyme such as QR, which utilizes NADH and generates NAD^+ in the presence of quinone substrates (such as NABQI), would enhance the alcohol oxidation rate. The QR reaction proceeds in a directly opposite sequence from that of ADH. Thus, QR binds NADH and the quinone substrate in order, again forming a ternary complex. In this complex, quinone is reduced to hydroquinone, and NADH is oxidized to NAD^+. Whereas the hydroquinone is thought to leave QR quickly, NAD^+ departs slowly, constituting the rate-limiting step in the overall QR reaction [41]. This NADH-requiring and NAD^+-producing QR reaction may strip the residual NADH from the ADH–NADH complex and allow NAD^+ to bind and accelerate the ADH turnover rate, enhancing the alcohol oxidation rate. Thus, when QR and ADH are operating together in the cytoplasm of liver cells, quinones such as NABQI will become reduced to hydroquinones (APAP) more quickly in the presence of ethanol, and, in turn, ethanol will be oxidized faster in the presence

of quinones. Such an *in vivo* cooperative interaction between QR and ADH, enhancing the ethanol oxidation rate, has been demonstrated in a recent series of studies conducted in this laboratory [19–21].

This hypothesis was supported by the decreased injury of liver cells (Table 1 and Fig. 1), loss of GSH content and destruction of microsomal aminopyrine *N*-demethylase activity (Table 2), elevation of serum aminotransferase activity (Fig. 2), and, most convincingly, by the decreased excretion of APAP–cysteine conjugate in bile (Fig. 7). In this connection, infusion of ethanol into rat liver that was being perfused with a quinone was shown to protect against quinone-inducible hepatic injury, and conversely, infusion of quinone into rat liver that was being perfused with ethanol was shown to enhance the rate of ethanol oxidation [42]. This previous observation agreed well with current results showing that co-administration of ethanol with APAP protected the liver from hepatocellular necrosis inducible with APAP alone. This may also explain the delayed clearance of APAP (Figs. 3 and 4).

In the present study, dicoumarol pretreatment has been used to inhibit QR and to assess the *in vivo* role of QR in the reduction or elimination of the toxic quinone metabolite (NABQI) back to the non-toxic hydroquinone (APAP). In a previous study conducted in this laboratory, the same dose of dicoumarol (30 mg/kg) was found to inhibit the *in vivo* QR activity by 75% [20]. Results obtained under the same conditions showed that the hepatic injury caused by APAP was potentiated (Table 1 and Fig. 1). This histological observation was supported by the markedly elevated serum transaminase activity (Fig. 2), by the decreased GSH:GSSG ratio and the destroyed microsomal aminopyrine demethylase activity (Table 2), and by the increased content of APAP–cysteine conjugate in bile fluid (Fig. 7). Thus, when the pre-existing QR activity was inhibited *in vivo* by the dicoumarol pretreatment, the NABQI produced could not be reduced back to APAP and remained to be conjugated with GSH (increased cysteine conjugate content in bile fluid, shown in Fig. 7) or to undergo the toxic one-electron redox cycling pathway for generation of toxic semiquinone and oxygen radicals, thus enhancing the APAP-dependent hepatocellular necrosis. Under these conditions, the blood and biliary concentration of APAP should have decreased, rather than being increased (Figs. 3 and 4). This unexpected discrepancy may have been caused by competitive blockade of APAP conjugation by dicoumarol (reduced APAP sulfate and glucuronide conjugates in bile fluid shown in Figs. 5 and 6), although we are not certain (sulfate and glucuronide conjugates of dicoumarol have not been determined).

However, when, mice were pretreated with dicoumarol and then given the APAP and ethanol treatment, due to inhibition of QR, the NADH $\leftrightarrow$ NAD^+ channeled cooperation with ADH did not occur. The results obtained showed that even under this QR-inhibited condition, a large dose of ethanol still provided moderate protection against hepatic necrosis (Table 1, Figs. 1 and 2). This moderate protection, observed together with increased levels of APAP in blood (Fig. 3) and in bile (Fig. 4), may have resulted in part from the dicoumarol-derived direct competitive inhibition of APAP conjugation [43] and also from the ethanol-derived indirect inhibition of the synthesis of cofactors such as PAPS and UDPGA, which are needed for conjugation of APAP to its respective sulfate (Fig. 5) and glucuronide (Fig. 6) esters [16]. Alternatively, ethanol provided direct competitive inhibition of CYP2E1-catalyzed production of NABQI, leading to decreased excretion of APAP–cysteine conjugate in bile (Fig. 7). These multiple actions of ethanol, even without cooperation from QR (severely inhibited by dicoumarol pretreatment), may have been responsible for the decrease of sulfate and glucuronide conjugates in bile and also the increase of APAP in blood and bile.

In summary, we have shown that inhibition of QR with dicoumarol enhanced the hepatic necrosis caused by APAP. The observed results suggested that, even in the absence of ethanol, QR plays a considerable role in the reduction of quinone and provides significant protection against hepatic injury caused by the toxic quinone metabolite (NABQI) arising from APAP. Furthermore, the turnover rate of QR was stimulated by the ethanol-driven potentiation of ADH activity, and the NABQI produced could be efficiently reduced back to APAP. Thus, this pre-existing role of QR may become magnified when ADH is activated by a physiological amount (small dose) of alcohol. By administering a large acute dose of ethanol, the CYP2E1-catalyzed production of the toxic NABQI is competitively inhibited, and this would provide additional protection. In the present study, attempts were made to assess the *in vivo* role of GSH in the back-reduction of NABQI to APAP by measuring the hepatic contents of GSH and GSSG. However, the results obtained were not conclusive. Thus, additional experiments employing inhibitors of GSH biosynthesis or depletors of GSH using a similar experimental model will be conducted in the near future.

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