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Ethanol Prevents from Acetaminophen Inducible Hepatic Necrosis by Inhibiting its Metabolic Activation in Mice

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Concomitant administration of a single acute dose of ethanol (4 g/kg) protected mice from the hepatocellular injury observed upon administration of a large dose of acetaminophen (400 mg/kg). This was evidenced by the normal histological appearances of liver sections and by the lowered serum aminotransferase activities in mice treated with ethanol and acetaminophen together. In the mice treated with acetaminophen alone, along with the hepatic necrosis, the hepatic microsomal aminopyrine N-demethylase activity was decreased. However, co-administration of ethanol prevented this acetaminophen dependent inhibition on the microsomal mixed function oxidase activity. Pharmacokinetic studies indicated that the concentration of un-metabolized drug in the blood was increased in the ethanol treated mice. Furthermore, upon co-administration of ethanol, although the biliary levels of acetaminophen metabolites (glucuronide, sulfate and cysteine conjugates) were decreased, the level of unmetabolized acetaminophen was increased. Our findings suggest that co-administration of an acute dose of ethanol reduces the degree of hepatocellular necrosis produced by a large dose of acetaminophen and this ethanol dependent protection is, in major part, afforded by suppression of the hepatic microsomal mixed function oxidase activity catalyzing the metabolic activation of acetaminophen.

Key Words: Acetaminophen, Ethanol, Microsomal Mixed Function Oxidase, Conjugation

INTRODUCTION

Acetaminophen (paracetamol) is used widely as an analgesic and antipyretic agent. However, upon an overdose, the drug has been shown to produce hepatic injury in man and in experimental animals (Hinson, 1982; Nelson, 1990). Acetaminophen and many other clinically useful drugs are known to undergo hepatic biotransformation and form chemically reactive metabolites that bind covalently to macromolecules within the liver cell and produce hepatocellular necrosis (Prasad et al, 1990).

Under normal conditions, major portion of the administered acetaminophen is conjugated directly with UDP-glucuronic acid and activated sulfate in liver cells and excreted as glucuronide and sulfate conjugates in urine and bile. However, a small portion of the administered dose is oxidized by the hepatic microsomal mixed function oxidases and generates reactive and toxic metabolites which, under normal circumstances, could be detoxified by combining preferentially with glutathione within liver cells and excreted eventually as cysteine conjugates. One of the reactive metabolites arising from the administered acetaminophen has been identified as the N-acetyl-p-benzoquinoneimine (NABQI), and this electrophilic intermediary metabolite is believed to react with critical macromolecules within liver cells and to cause the hepatotoxicity (Powis et al, 1984).

Among the many cytochrome P-450 (CYP) containing hepatic microsomal mixed function oxidases,

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the CYP2E1 has been identified to be the principal enzyme responsible for the metabolic activation of acetaminophen (Lee et al, 1996). CYP2E1 is also the major component of the microsomal ethanol-oxidizing enzyme system (Coon et al, 1991) and is known to be involved in the oxidation of ethanol together with the cytosolic alcohol dehydrogenase. Upon chronic administration of alcohol, although the cytosolic alcohol dehydrogenase activity is not increased, the microsomal ethanol-oxidizing enzyme (CYP2E1) activity is known to increase (Sato et al, 1981). In connection with this, recent studies have indicated that chronic alcohol administration enhances the acute hepatic toxicity of acetaminophen and suggested that this is due to accelerated metabolism of acetaminophen produced by the enhanced level of CYP2E1 (Snawder et al, 1994). On the other hand, the administration of an acute dose of ethanol together with acetaminophen has been shown to attenuate the hepatotoxicity normally observed with acetaminophen (Thummel et al, 1989). This inhibitory effect of ethanol on the acetaminophen derived hepatic toxicity is considered to arise by the decreased production of NABQI presumably by a direct competitive inhibition on the CYP2E1 dependent oxidation of acetaminophen. Although the protective effect of ethanol on acetaminophen derived hepatotoxicity has been studied extensively, the mechanisms involved in this protective effect are still unclear.

Therefore, the purpose of this study was to investigate the mechanisms involved in the hepatoprotective effect of ethanol against the hepatotoxicity caused by acetaminophen upon administration of an acute dose of alcohol together with acetaminiophen. To accomplish this purpose, we have utilized mice, an animal species whose patterns of acetaminophen metabolism is known to resemble that of human (Whitehouse et al, 1977), and examined comprehensively by correlating the histology, hepatic microsomal mixed function oxidase activity and the acetaminophen derived metabolites excreted in bile.

METHODS

Chemicals

Acetaminophen was donated by the Il-Yang Pharm. Ind. Co., LTD., Korea and its metabolite standards used in the analytical high performance liquid chromatography (HPLC) were donated by the McNeil Consumer Products Company of Fort Washington, PA, USA. Ethanol and other solvents used for the chromatography were purchased from Merck, Damstadt, Germany. The standard enzyme activity test kits used for determinations of serum aminotransferase activities (AST and ALT) were purchased from Wako Pure Chemicals Ind., LTD., Japan. All other chemicals were of the reagent grades commercially available locally.

Animals and treatments

Male ICR mice weighing about 23 ± 3 g were obtained from the Animal Breeding Laboratory of Yonsei Medical College and were acclimatized with the laboratory conditions for at least one week. During this period, food (donated by the Korea Purina, Inc.) and water were supplied *ad libitum*. Food was withdrawn for 24 hr before the beginning of each experiment. Animals were divided into 4 treatment groups; 2 control groups of mice were given an oral injection of either water or ethanol (4 g/kg in 20% water solution) and 2 other experimental groups were given an additional intraperitoneal injection of a basic solution of acetaminophen (400 mg/kg in pH 10.6 solution).

Histology

Acetaminophen alone or in combination with ethanol were given to groups of mice (8 per group) according to the regimen as described above. At 24 hr after the drug administration, liver samples were taken from the anterior portion of left lateral lobe for microscopy. Paraffin blocks were prepared after fixation with 10% neutral formalin and were sectioned and stained with hematoxylin and eoxin. A quantitative analysis of hepatic necrosis for each exprimental animal was performed initially according to the method described by Mitchell et al (1973).

Levels of acetaminophen in blood

At 5, 15, 30, 45, 90, 150 and 240 min after administering the acetaminophen, the mice were decapitated and blood samples were collected. Serum levels of the un-metabolized acetaminophen were determined with HPLC using the procedure described by Black and Sprague (1978). Plasma proteins were

removed and discarded before chromatography by precipitation with acetonitrile (50% final concentration) and centrifugation (15,600×g for 3 min). Supernatants (4 ul) were injected into an HPLC (Waters Model 441) equipped with a C_{18}-Bondapak column and a UV detector (254nm). Acetaminophen was eluted isocratically with the mobile phase composed of 0.01 M acetate buffer (pH 4.0) and acetonitrile (93 to 7 ratio) at a flow rate of 2 ml per min.

Acetaminophen and its metabolites in bile

Mice were sacrificed at 15, 30, 60, 90, 120, 240 and 360 min following the administration of acetaminophen. After the median laparotomy, cystic duct was ligated and the gall bladder was freed carefully from surrounding tissues. The levels of acetaminophen and its conjugated metabolites were determined in the bile samples collected from gall bladders according to the procedure described by Howie et al (1977). The bile samples were diluted with methanol and injected directly onto the μ-Bondapak C18 column of an HPLC. A mobile phase composed of 1% acetic acid, methanol and ethylacetate (90/15/0.1) flowing at 1.0 ml/min was used to elute the acet-aminophen and its conjugated metabolites. The UV detector was set at 254nm.

Preparation of liver microsomes and measurement of aminopyrine N-demethylase activity

Ice-cold saline was infused into the removed liver tissue. The washed liver tissue was then blotted, weighed, minced and homogenized in 2 vol of 150 mM KCl. The whole homogenate was centrifuged using high-speed centrifuge and the collected supernatant was centrifuged again at 105,000×g at 2°C using an ultracentrifuge. The resulting microsomal pellet was resuspended in 100 mM phosphate buffer (pH 7.4). The aminopyrine N-demethylase activity present in hepatic microsomes isolated from the control and experimental groups of mice was measured *in vitro* according to the method described by Schenkman et al (1967). Briefly, the incubation medium (2 ml) contained; 7.5 μmol $MgCl_2$, 8 μmol glucose 6-phosphate, 0.75 μmol NADP, 2 units of glucose 6-phosphate dehydrogenase, 0.5 ml of microsomal suspension (4 mg/ml final protein concentration) and 0.1 M phosphate buffer (pH 7.4) making up the remaining final volume. Incubation was initiated by the addition of aminopyrine (1 mM) and continued for 10 min in 25 ml Erlen-meyer flask under air at 37°C. The demethylase activity was determined by measuring the generation of formaldehyde upon addition of the Nash reagent. The microsomal protein content was determined by the method of Lowry et al (1951) using the bovine serum albumin as the standard. The serum aminotransferase activities were determined using the standard spectrophotometric procedures employing the kit obtained form Wako Pure Chemicals Ind., LTD., Japan.

Statistical analysis

Significant changes in various parameters measured were determined using the one-way ANOVA. Differences between experimental groups were considered significant when the p value was less than 0.05. All results are presented as the mean±S.E.M.

Table 1. Hepatic necrosis caused by an overdose of acetaminophen in mice give co-administration of ethanol

Groups	Portal inflammation*	Centrilobular necrosis*	Kupffer cell hyperplasia*	Vacuole degradation*
Control	0.1±0.1	0.3±0.2	0.4±0.3	0.1±0.2
Ethanol	0.5±0.3	1.0±0.6	1.2±0.3	0.2±0.1
Acetaminophen	0.5±0.2	4.0±0.1**	2.0±0.1	3.0±0.2**
Acetaminophen+Ethanol	0.4±0.1	1.1±0.2 †	1.7±0.3	0.4±0.1 †

Each value is means±S.E.M. from 8 mice, **$p<0.01$ *vs* control. †$p<0.05$ *vs* acetaminophen.
*The histological scoring for the parameter indicated indicated are conducted according to the method described by Mitchell et al (1973).

RESULTS

Histology

Fig. 1 and Table 1 show the results of histological observations. In the livers of mice given the acetaminophen (400 mg/kg) at 24 hr earlier, there were extensive hepatocellular damages. This was evidenced by the presence of necrotic foci, hydropic changes and infiltration of inflammatory cells. This occurred in 85% of the centrilobular areas of liver tissue which have been examined. In contrast to this, in the livers of mice given the ethanol alone or the ethanol with acetaminophen, little necrotic changes were observed. Thus, administering an acute dose of ethanol together with acetaminophen protected the liver against the hepatocellular necrosis normally observed upon admi- nistration of acetaminophen in mice.

Serum aminotransferases

To support the histological results obtained by the concomitant administration of ethanol, activities of serum aminotransferases were determined from the collected bloods of mice whose livers were used for the histological examinations. The obtained results are shown in Table 2 and indicate that while the ethanol administration by itself did not produce any increases in the release of these cytosolic enzymes of

Table 2. Ethanol suppresses the increase of serum aminotransferase activities caused by administration of acetaminophen

Groups	Aminotransferase(IU/L)	
	AST	ALT
Control	100±5	50±4
Ethanol	89±7	44±4
Acetaminophen	823±30**	260±7**
Acetaminophen + Ethanol	252±7**††	119±4**††

Each value is means±S.E.M. from 8 mice, **$p < 0.01$ vs control. ††$p < 0.01$ vs acetaminophen.

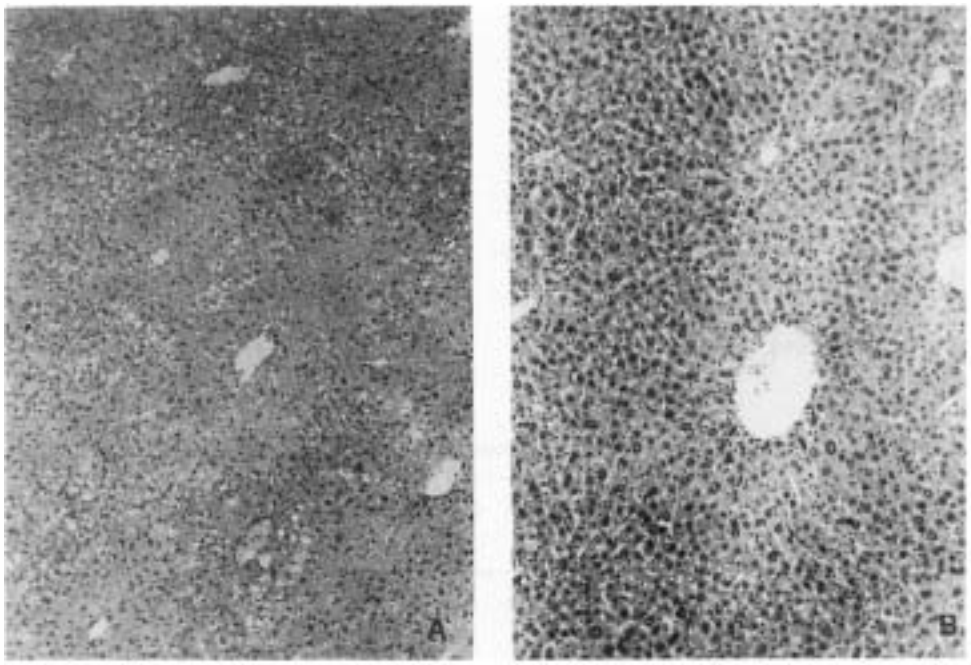

Fig. 1. Prevention of hepatic necrosis caused by an overdose of acetaminophen by co-administration of ethanol. Twenty four hour after the administration of acetaminophen (400 mg/kg, i.p.), extensive necrosis was observed around centrilobular vein (control, A). However, in the ethanol treated mice (B), necrotic changes were not observed. Hematoxylin and eosin stain, ×100.

Table 3. Ethanol protects aganist the decrease of aminopyrine N-demethylase activity in liver microsomes caused by administration of acetaminophen

Groups	Aminopyrine N-demethylase (HCHO nmole/mg protein)
Control	14.5±0.48
Ethanol	13.7±0.36
Acetaminophen	7.3±0.27**
Acetaminophen + Ethanol	12.1±0.40*††

Each value is means±S.E.M. from 8 mice, *$p<0.05$ *vs* control, **$p<0.01$ *vs* control, ††$p<0.01$ *vs* acetaminophen.

hepatocytes into blood, the administration of acetaminophen caused a marked increase of the serum enzyme activities. However, the co-administration of ethanol with acetaminophen inhibited the increase of these serum enzyme activities. Therefore, the protective effect of ethanol against the acetaminophen inducible hepatocellular necrosis, as evidenced by the histological observations, has been supported by these results.

Hepatic microsomal mixed function oxidase activity

As shown in Table 3, the hepatic microsomal aminopyrine N-demethylase activity, known to be catalyzed by CYP2A1/2 and CYP2B, is decreased significantly in mice given the acetaminophen injection 24 hr earlier. However, the microsomal enzyme activity in mice given ethanol and acetaminophen together was affected only slightly. Therefore, again, the co-administered ethanol appears to protect against the suicidal destruction of microsomal oxidases which is thought to occur upon production of reactive metabolites from the administered acetaminophen.

Blood levels of acetaminophen

Along with the competitive inhibition on the CYP2E1 catalyzed oxidation of acetaminophen by the administered ethanol, the overall metabolic clearance of acetaminophen from the blood is expected to be delayed. As the results shown in Fig. 2, the blood levels of acetaminophen in the ethanol treated group were higher than those given acetaminophen alone.

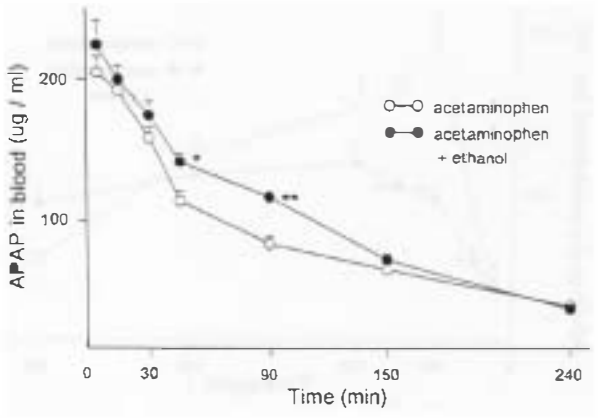

Fig. 2. Effect of ethanol co-administration on the levels of free acetaminophen in blood at various time points. *and ** indicate the values which are significantly different at $p<0.05$ and $p<0.01$, respectively, from the values obtained in mice given acetaminophen alone. Values indicate the mean±S.E.M. obtained from 6 mice per experimental group.

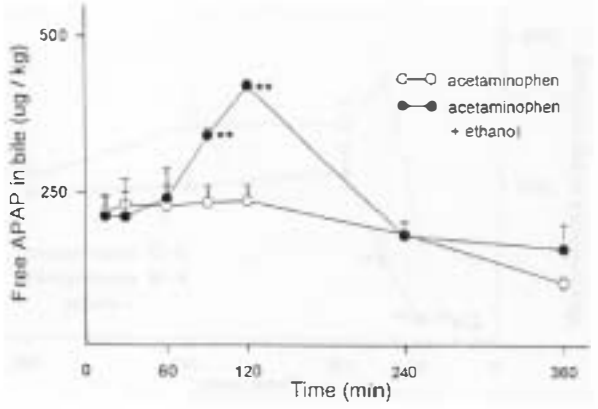

Fig. 3. Effect of ethanol co-administration on levels of free acetaminophen in bile fluid at various time points. **indicates the values which are significantly different ($p<0.01$) from the values obtained in mice given acetaminophen alone. Values indicate the mean±S.E.S. obtained from 7 mice per experental group.

This result suggested that the co-administered alcohol inhibited not only the oxidative metabolism of acetaminophen at the hepatic microsomal CYP2E1 level but perhaps also at the conjugation level and thus, delaying the overall clearance of the administered acetaminophen.

Levels of acetaminophen and its metabolites in bile

In an effort to make certain that ethanol inhibited

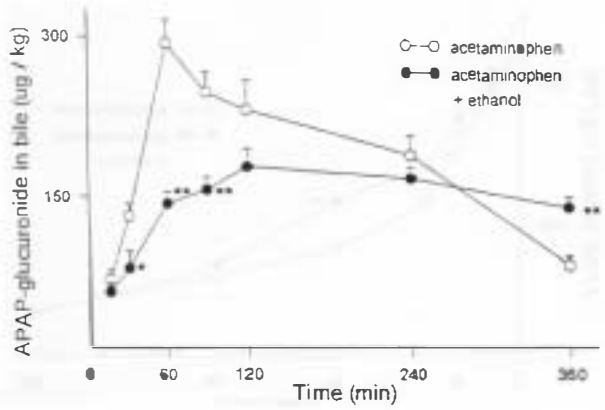

Fig. 4. Effect of ethanol co-administration on levels of glucuronide conjugate of acetaminophen in bile fluid at various time points. *and **indicate the values which are significantly different at $p<0.05$ and $p<0.01$, respectively, from the values obtained in mice given acetaminophen alone. Values indicate the mean ± S.E.M. obtained from 6 mice per experimental group.

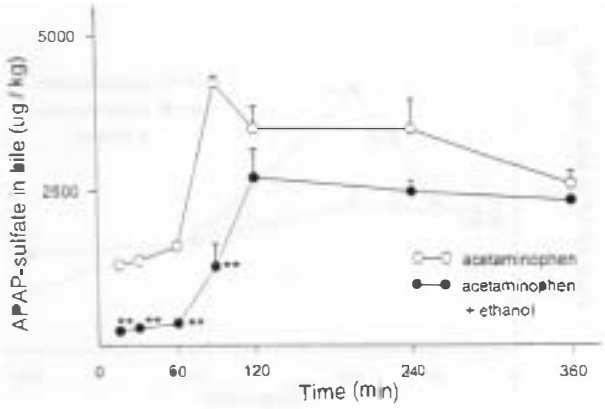

Fig. 5. Effect of ethanol co-administration on levels of sulfate ester conjugate of acetaminophen in bile fluid at various time points. **indicates the values which are significantly different ($p<0.01$) from the values obtained in mice given acetaminophen alone. Values indicate the mean ± S.E.S. obtained from 7 mice per experimental group.

not only the oxidative metabolism but also the conjugation and clearance of acetaminophen, levels of the un-metabolized drug as well as its glucuronide, sulfate and cysteine conjugates in bile have been determined. Results shown in Fig. 3 indicated that the biliary levels of free acetaminophen were significantly higher for the mice given an acute dose of ethanol together with acetaminophen. However, the biliary levels of glucuronide (Fig. 4) and sulfate (Fig. 5) conjugates of acetaminophen were signi-ficantly lower. Results shown in Fig. 6 indicate that the biliary levels of cysteine conjugate acetaminophen which could be produced only upon initial oxidation catalyzed by CYP2E1 were lower in the ethanol co-administered group. All together, the results shown in Fig. 3, 4, 5 and 6 supported and explained the higher blood levels of free acetaminophen in mice given an acute dose of ethanol concomitantly (Fig. 2). Thus, the co-administered ethanol appears to inhibit both the oxidation (Phase 1) and conjugation (Phase 2) steps of acetaminophen metabolism.

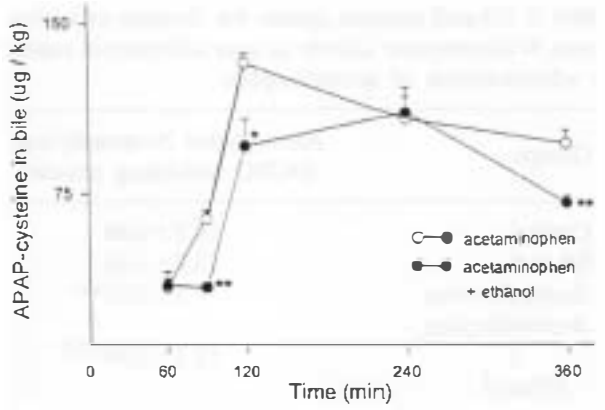

Fig. 6. Effect of ethanol co-administration on levels of cysteine conjugate of acetaminophen in bile fluid at various time points. *and ** indicate the values which are significantly different at $p<0.05$ and $p<0.01$, respectively, from the values obtained in mice given acetaminophen alone. Values indicate the mean ± S.E.M. obtained from 6 mice per experimental group.

DISCUSSION

In a clinical study, Rumack et al (1981) have observed that the hepatotoxicity resulting from an overdose of acetaminophen could be suppressed or prevented by administering an acute dose of ethanol quickly after the overdose accident is identified. This clinical finding has been confirmed in this study by the histological demonstration (Fig. 1). Thus, the administration of an acute dose of ethanol prevented the onset of hepatocellular necrosis normally produced by an over-dose of acetaminophen (Table 1). This histological observation was supported by the results of serum aminotransferase activities (Table 2). Thus, the co-administration of an acute dose of

ethanol has a dramatic protective effect against the hepatotoxicity casued by an overdose of acetaminophen.

Under normal conditions, the reactive and toxic metabolite of acetaminophen (NABQI), known to be produced by the catalytic action of hepatic microsomal CYP2E1, undergoes conjugation with glutathione and is easily detoxified and excreted out of liver cells. However, when the rate of NABQI production becomes excessive (i.e., induction of CYP2E1) or the concentration of glutathione in liver cells becomes limiting and the produced NABQI cannot be eliminated effectively, the excess NABQI binds indiscriminately with microsomal and cytosolic enzyme proteins and membrane lipids, destroying their functions. In this connection, ethanol is known also to be a substrate of CYP2E1 and can inhibit the rate of drug oxidation catalyzed by CYP2E1 by competitive inhibition (Sato & Lieber, 1981). Therefore, upon co-administration of an acute dose of ethanol, the rate of NABQI production catalyzed by CYP2E1 may be inhibited and the small amount of NABQI produced under this condition could effectively be conjugated and detoxified by the glutathione normally present in liver cells. This may have been the underlying reason for the ethanol dependent protection against the acetaminophen derived hepatic necrosis (Fig. 1, Table 1 and 2).

As the foregoing discussions have stated, the intracellular site of producing the toxic reactive metabolite (NABQI) is in the endoplasmic reticulum (CYP2E1) and unless the NABQI is quickly detoxified by the cytosolic glutathione, it will attack the microsomal membranes causing lipid peroxidation and attack microsomal mixed function oxidase enzymes causing denaturation of cytochrome P-450 to cytochrome P-420. Thus, in the absence of added glutathione, the ability of isolated mouse liver microsomes to metabolize the acetaminophen becomes quickly self-limiting and also the speed of producing lipid peroxides and cytochrome P-420 was greatly accelerated (data not shown).

In this connection, Sato et al (1981) have already shown that the hepatic content of cytochrome P-450 is decreased after administration of acetaminophen and the co-administration of ethanol prevented this decrease. As the results shown in Table 3 indicate, the ability liver microsomes isolated from acetaminophen pretreated mice to carry out the N-demethylation of aminopyrine (CYP2A and CYP2B) was severely decreased and supported the observations of Sultatos et al (1978). In contrast, the ability of liver microsomes isolated from the ethanol- and acetaminophen-treated mice had virtually the normal N-demethylase activity (Table 3). This result provided additional support to the hypothesis that the co-administered ethanol protected the liver microsomes from the suicidal destruction by suppressing the production of reactive metabolite from acetaminophen. This hypothesis could be supported further by the increased levels of un-metabolized acetaminophen present in blood (Fig. 2) and in bile (Fig. 3) of the ethanol-treated group.

The glutathione conjugate of NABQI generated upon CYP2E1-dependent oxidation of acetaminophen undergoes further biotransformation and forms cysteine conjugates (Gregus et al, 1988) and is excreted into bile as well as into systemic circulation. The cysteine conjugate released into bile would eventually be reabsorbed back into systemic circulation via the enterohepatic recirculation system and, together with the conjugates released directly into systemic circulation, may undergo further metabolism in kidney to be released finally in the urine as the mercapturic acid form (Madhu et al, 1988). Thus, the amount of cysteine conjugate found in bile and blood or the mercapturic acid form found in urine would provide a direct estimation of the NABQI produced by the hepatic microsomal CYP2E1 activity. Studies using hamsters, mice, rats, rabbits and guinea pigs, in which the bile duct has been cannulated, have shown that major portion of the glutathione derived thioester conjugates of acetaminophen (cysteinyl glycine, cysteine and mercapturate forms) are excreted into bile and only a small fraction of the thioester conjugates of acetaminophen (mercapturate form) appear in urine (Madhu, 1988). Moreover, the animal species which are sensitive to the acetaminophen induced liver injury (i.e., mice and hamsters) excrete larger proportion of cysteine conjugates into bile than those which are resistant to the acetaminophen derived liver injury (i.e., rats, rabbits and guinea pigs) (Gregus et al, 1988). Our initial preliminary attempts to determine the levels of the thioester conjugates of acetaminophen in blood and urine were not successful and thus, we have determined the levels of acetaminophen cysteine conjugate in the bile. The amount of cysteine conjugate found in bile fluid of mice given co-administration of ethanol was lower than those given acetaminophen alone (Fig. 6). This result

provided an indirect evidence indicating that the administered ethanol inhibited the CYP2E1 and suppressed the production of NABQI and thereby prevented the hepatocellular necrosis.

In addition to inhibiting the production of NABQI at the CYP2E1 level via competitive inhibition, the co-administered ethanol may inhibit the accumulation of NABQI also by increasing the rate of its conversion back to the parent drug by accelerating the NADH: quinone reductase activity. This may occur by increased supply of NADH provided by ethanol oxidation. When the administered ethanol is oxidized by alcohol dehydrogenase in hepatic cytoplasm, much NAD is reduced to NADH and this excess NADH is utilized by the cytoplasmic NADH: quinone reductase to convert the NABQI back to parent acetaminophen. Thus, the rate of acetaminophen oxidation catalyzed by isolated liver microsomes could be suppressed when carried out in the presence of NADH and cytosol fraction (NADH:quinone reductase) (Dahlin et al, 1984; Powis et al, 1984). Although it is difficult to assess the importance of this recycling metabolism under the present *in vivo* experimental setting, this may explain at least a portion of the elevated levels of un-metabolized acetaminophen found in blood and bile of the ethanol treated mice (Fig. 1 and 2).

Under normal conditions, most of the administered acetaminophen is conjugated directly without oxidation and forms sulfate (major) and glucuronide (minor) esters (Price & Jollow, 1982). Only a small portion of the administered drug undergoes oxidation to form the reactive NABQI and its cysteine conjugates. This is readily apparent when one compares the units of these metabolites shown in Figs. 3, 4, 5 and 6. Thus, the administered acetaminophen is excreted into bile in the order (in absolute amounts) of sulfate ester, unconjugated drug, glucuronide ester and cysteine conjugate.

Ability of liver to form the sulfate ester conjugate was diminished upon co-administratioin of ethanol (Fig. 5). While the exact mechanism by which the administered ethanol inhibits sulfate conjugation remains unclear, several hypothesis can be entertained. One is that ethanol may cause depletion of inorganic sulfate pool (Levy & Yamada, 1971) and thus may limit the synthesis of 3-phosphoadenosine 5-phosphosulfate (PAPS), the cofactor required for sulfate conjugation. Another is that ethanol may inhibit the energy production (Cederbaum & Rubin, 1975) or may decrease the adenylate pool size (Aw & Jones, 1982) and again limit the synthesis of PAPS and suppress the sulfate conjugation.

Ethanol also inhibited the glucuronidation of acetaminophen (Fig. 4). This inhibition may have been caused by the ethanol dependent suppression on the synthesis of UDP-glucuronic acid (UDPGA), a cofactor required for glucuronidation. Biosynthesis of UDPGA occur upon oxidation of UDP-glucose and reduction of NAD which is catalyzed by UDP-glucose dehydrogenase. When the liver is engaged in oxidation of ethanol, intracellular concentration of NAD becomes limiting and UDPGA synthesis is inhibited (Minnigh & Zemaitis, 1982).

In conclusion, this study has shown that co-administration of an acute dose of ethanol has a dramatic protective effect against the hepatotoxicity caused by an overdose of acetaminophen. This protection is attributed, in major part, to an inhibitory effect of ethanol on the overall metabolism (both oxidation and conjugation) of acetaminophen. Due to this inhibition, the clearance of acetaminophen was delayed and produced a prolonged analgesic effect from a given dose of acetaminophen (Kim, 1988).

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