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EFFECT OF ETHANOL ON HEPATOTOXICITY AND HEPATIC DNA-BINDING OF AFLATOXIN B_1 IN RATS

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Abstract—The hepatocarcinogen aflatoxin B_1 is converted to reactive metabolites that bind covalently to cellular macromolecules. These metabolites may also react with glutathione, resulting in the formation of glutathione conjugates and detoxication of the reactive metabolite. When rats were pretreated with ethanol by gastric intubation at a dose of 100 mmol/kg, 6 hr (the time of maximal GSH depletion) before the administration of aflatoxin B_1, the covalent binding of 8,9-epoxide-aflatoxin B_1 to DNA *in vivo* was increased by 47% and the hepatotoxicity was also potentiated. However, the covalent binding was not increased by pretreatment with ethanol 18 hr (time with approximately normal GSH levels) before administration of the toxin, and no potentiation of hepatotoxicity was observed. Pretreatment with a non-toxic dose of ethanol had no effects on the activity of glutathione *S*-transferase and glutathione peroxidase. These results suggest that the depletion of GSH and the increased formation of DNA-adduct from the liver constitute an important mechanism for the potentiation of aflatoxin B_1-induced hepatotoxicity by ethanol.

The aflatoxins are secondary fungal metabolites, and the most biologically potent of these compounds, AFB_1,§ is toxic, hepatocarcinogenic and mutagenic in a wide range of organisms [1]. The potentiation of AFB_1-induced hepatotoxicity by ethanol pretreatment has been reported [2]. However, the mechanism of this potentiation is not yet elucidated. It may be due to the induction of certain drug-metabolizing enzymes by ethanol pretreatment [3, 4] leading to an increase in the conversion of AFB_1 to active metabolite (8,9-epoxide derivative) [5]. Many situations have been described in which the different isoforms of cytochrome P450 can participate simultaneously in both metabolic activation and inactivation of procarcinogens [6], i.e. potentiation engendered by a phase I inducer, depending upon the metabolic balance between activation and detoxication. By contrast, phase II enzymes, such as glutathione *S*-transferase (GST), predominantly participate in the detoxication of xenobiotics, although there exist some notable exceptions to this premise [7].

The well-known pathways involved in the metabolic activation and detoxication of AFB_1 are summarized in Fig. 1. A major part of the reactive metabolites that are generated from toxic compounds, such as benzo[*a*]pyrene [8], AFB_1 [9], acetaminophen [10], and penicillic acid [11], can be detoxified by conjugating with glutathione (GSH) through the catalytic action of the GST before they can react with cellular macromolecules. There is considerable interest in factors which, by affecting the balance between intoxifying and detoxifying reactions, could affect the susceptibility of individuals to AFB_1. Of particular importance in this area is the evidence suggesting that the potentiation of the effects may arise from reducing the carcinogen inactivation through a reduction of the detoxification pathway [12, 13]. Ethanol has been reported to lower hepatic GSH levels [14–16]. Ethanol ingestion is known to potentiate the hepatotoxicity of AFB_1 [2]. This potentiation could be the consequence of enhanced microsomal activation of aflatoxin, exaggeration of the peroxidative rate, and/or interference with conjugation of aflatoxin and/or their metabolites with GSH.

In the present report, we describe the effects of ethanol on the reduction of the hepatic carcinogen detoxication pathway and the modification of aflatoxin–DNA interactions *in vivo*. The experimental results suggested that the modulation of the hepatic GSH level and related enzymes may be an important mechanism for potentiating AFB_1-induced hepatotoxicity.

MATERIALS AND METHODS

Chemicals. [^{3}H]AFB_1 (sp. act. 15 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, U.S.A.) and checked for purity by reversed-phase high-pressure liquid chromatography; it showed one major peak containing more than 98% of the AFB_1 in the sample. Nonradioactive AFB_1, GSH, Tris,

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§ Abbreviations: AFB_1, aflatoxin B_1; GSH, glutathione; GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 3,4-dichloronitrobenzene; ALT, alanine aminotransferase; AST, aspartate transaminase; GGT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; OPT, *o*-phthadialdehyde; COP, cumune hydroperoxide; TBA, 2-thiobarbituric acid; MDA, malondialdehyde; DEM, diethylmaleate; PB, phenobarbital; and BSA, bovine serum albumin.

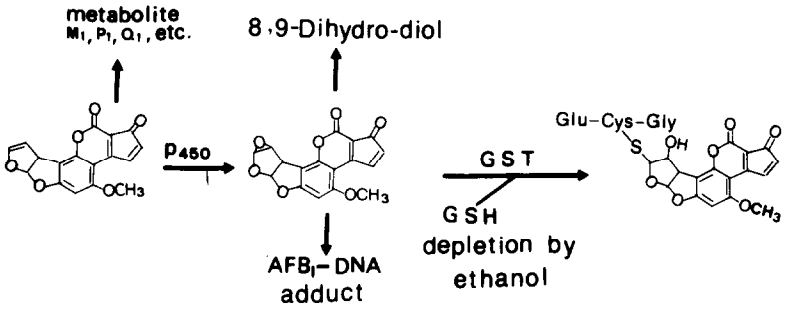

Fig. 1. Pathways involved in the metabolic activation of AFB_1 and depletion of GSH by ethanol.

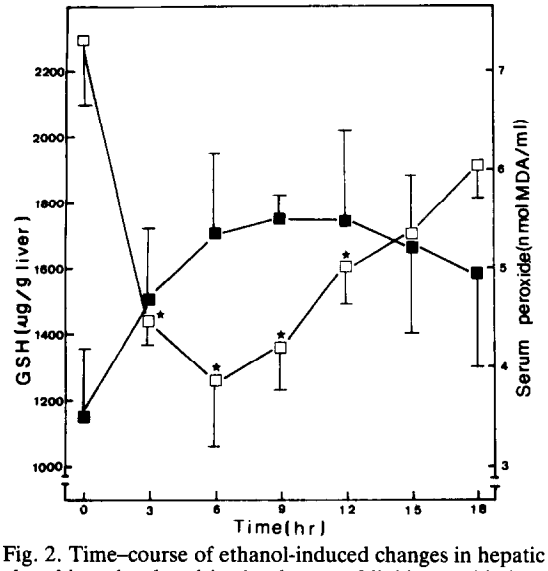

Fig. 2. Time–course of ethanol-induced changes in hepatic glutathione level and in the degree of lipid peroxidation. Rats were given 5 mmol/kg of ethanol by gastric tube and were killed at the selected times. Livers were removed for determination of glutathione (□—□), lipid peroxidation in serum was measured by the thiobarbituric acid test and is expressed as malondialdehyde production (■—■). Values are means ± SD, N = 3. Asterisks (*) indicate a significant difference from zero time, $P < 0.01$.

NADPH, GSH-reductase, CDNB, DCNB, calf thymus DNA, sodium azide, RNase, proteinase K, BSA, OPT and COP were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). TBA, tungstophosphoric acid, and reagent kits for AST, ALP, GGT and ALT were purchased from the E. Merck Co. (Darmstadt, F.R.G.). A reagent kit for GGT was also purchased from Boehringer, Mannheim GmbH Diagnostica, F.R.G. Aquasol-2 was from New England Nuclear (Boston, MA, U.S.A.).

Animals and treatment. Male Wistar rats (150–180 g body weight), purchased from the National Taiwan University Hospital Animal Center, were used. The animals were housed three per cage in an environmentally controlled animal room. Food (Purina Lab Chow) and water were provided *ad lib.* The time–course study was performed in fasted rats given 100 mmol/kg of ethanol as a 25% (w/v) solution in deionized water by gastric intubation and the rats were killed after 3, 6, 9, 12, 15, and 18 hr of treatment. Serum was collected and liver excised for the determination of AST, ALT, ALP, GGT, lipid peroxide, and hepatic GSH, and GST and GSH peroxidase activities.

Serum enzymes and peroxide assay. Serum AST and ALT activities were determined according to the method of Reitman and Frankel [17]. The serum was added to a buffer solution of α-ketoglutaric acid and aspartic acid or alanine, and the resulting oxaloacetic acid or pyruvic acid formed after incubation was measured colorimetrically at 546 nm by reaction with dinitrophenylhydrazine. For the estimation of ALP activity [18], 0.1 mL of serum was incubated with *p*-nitrophenylphosphate, and the reaction product, *p*-nitrophenol, thus formed was determined spectrophotometrically at 405 nm. The activity of GGT in serum was measured according to the method of Persijin and van der Slik [19]. Serum was incubated with L-γ-glutamyl-3-carboxy-4-nitroanilide and glycylglycine as substrate, and the resulting 5-amino-2-nitro-benzoate was determined spectrophotometrically at 405 nm. For the estimation of lipoperoxidation, a TBA assay for malondialdehyde (MDA) production [20] was performed in 20 μL serum supplemented with 10% tungstophosphoric acid, 1/12 N H_2SO_4 and precipitated with TBA aqueous solution by incubation for 60 min in boiling water. The precipitate was then extracted with *n*-butanol, and the absorbance of the butanol phase separated by centrifugation was measured at 553 and 515 nm.

GSH, GST and GSH peroxidase assays. Rats were killed by decapitation, and the liver was removed promptly. A small portion of the liver was used for GSH determination [21] and the remaining tissues were homogenized in 4 vol. (w/v) of a 50 mM Tris buffer (pH 7.5) containing 0.25 M sucrose. Homogenates were centrifugated at 105,000 *g*, and the resulting supernatant fractions were used for the estimation of GST (CDNB and DCNB as substrates) [22] and GSH-peroxidase (COP and H_2O_2 as substrates) [23]. Protein concentration was determined [24] using a standard commercial kit (Bio-Rad Laboratories Ltd., Watford, U.K.) with bovine serum albumin as standard.

Effect of GSH depletion on the hepatotoxicity of

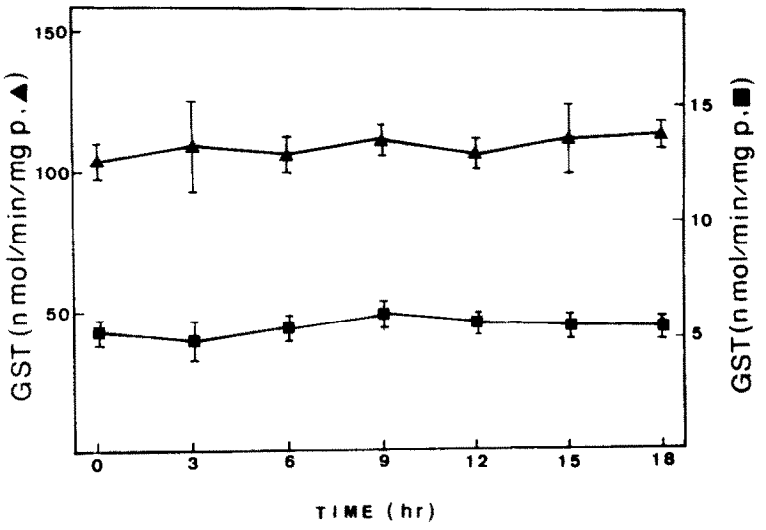

Fig. 3. Effect of ethanol on hepatic cytosol glutathione *S*-transferase activity. Rats were given 100 mmol/kg of ethanol and were killed at the selected times. Glutathione *S*-transferase activities in liver cytosols were assayed spectrophotometrically with CDNB (▲—▲) or DCNB (■—■) as substrate. Values are means ± SD, N = 3.

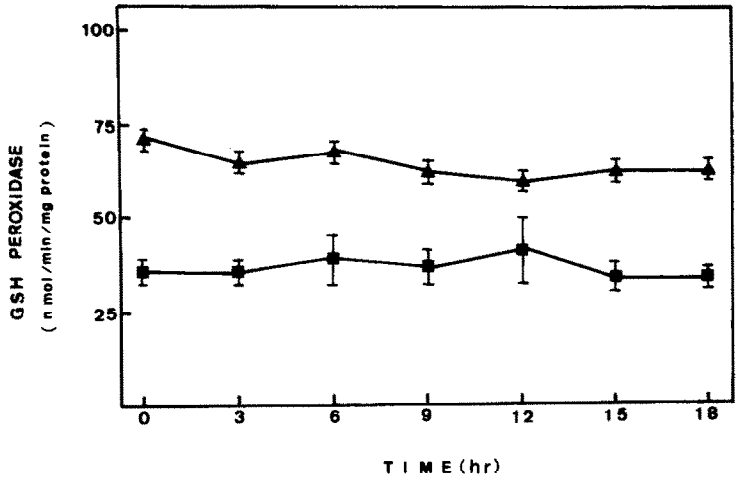

Fig. 4. Effect of ethanol on hepatic cytosol glutathione peroxidase activity. Rats were given 100 mmol/kg of ethanol and were killed at the selected times. Glutathione peroxidase activities in liver cytosol were assayed spectrophotometrically with COP (▲—▲) or H_2O_2 (■—■) as substrate. Values are means ± SD, N = 3.

Table 1. Effect of pretreatment of ethanol for 6 hr on the activities of serum AST, ALT, GGT and ALP measured 24 hr after administration of AFB_1 in rats

Treatment*	AST (I.U./L)	ALT (I.U./L)	GGT (I.U./L)	ALP (I.U./L)
Control	264 ± 48	44 ± 4	1.83 ± 1.67	230 ± 49
EtOH (6 hr)	211 ± 42	47 ± 7	1.17 ± 0.69	242 ± 53
AFB_1 (24 hr)	410 ± 65†	178 ± 108†	1.50 ± 0.96	246 ± 53
EtOH + AFB_1	753 ± 203‡	426 ± 222§	2.83 ± 0.67‖	267 ± 47

* All rats were killed 24 hr after the i.p. administration of AFB_1 (1 mg/kg). A single oral dose of ethanol (as a 25% solution in water) was given 6 hr prior to the i.p. administration of AFB_1.

Values are means ± SD, N = 6.

† $P < 0.02$, compared with the control group.

‡–‖ P values: ‡ $P < 0.001$, § $P < 0.05$ and ‖ $P < 0.02$, compared with the AFB_1-treated group.

Table 2. Effect of pretreatment of ethanol for 18 hr on the activities of serum AST, ALT, and ALP measured 24 hr after the administration of AFB_1 in rats

Treatment*	AST (I.U./L)	ALT (I.U./L)	ALP (I.U./L)
Control	242 ± 31	42 ± 5	250 ± 38
EtOH (18 hr)	236 ± 50	45 ± 6	285 ± 70
AFB_1 (24 hr)	384 ± 151†	162 ± 48‡	285 ± 38
EtOH + AFB_1	470 ± 240§	170 ± 28§	272 ± 12§

* All rats were killed 24 hr after the i.p. administration of AFB_1 (1 mg/kg). A single oral dose of ethanol (as a 25% solution in water) was given 18 hr prior to the i.p. administration of AFB_1. Values are means ± SD, N = 6.

† $P < 0.05$, compared with the control group.

‡ $P < 0.001$, compared with the control group.

§ Not significant, compared with the AFB_1-treated group.

AFB_1. From the time–course study, the maximal effect of GSH depletion by ethanol ingestion was attained after 6 hr. Therefore, for studying the potentiation effect of a non-toxic dose of ethanol on AFB_1-induced hepatotoxicity, ethanol was administered at a dose of 100 mmol/kg, 6 hr before the administration of AFB_1. AFB_1 was dissolved with dimethyl sulfoxide (DMSO) (10 mg/mL) and diluted with deionized water. Rats were killed by decapitation 24 hr after the administration of AFB_1 at a dose of 1 mg/kg. Blood was collected for the evaluation of hepatotoxicity by measuring the alteration of serum enzyme markers. The liver was removed, immediately frozen in ice, and stored at −70° for the determination of hepatic GSH, GST and GSH-peroxidase.

Isolation of DNA and analysis of AFB_1–DNA adducts. Ethanol was administered at a dose of 100 mmol/kg, 6 hr before the administration of [^{3}H]AFB_1 (10 μg/kg, 15 Ci/mmol) in methanol. Rats were killed by decapitation after the administration of AFB_1. Liver was excised and minced. For the isolation of DNA, the liver was ground into powder using a mortar and pestle maintained on dry ice. Lysing buffer (0.1 M NaCl; 0.025 M EDTA; 1% SDS; 0.5 M Tris, pH 7.4) was added followed by the addition of proteinase K (100 μg/kg). The resulting suspension was then incubated at 37° for overnight. Cell lysate was then extracted sequentially with phenol/chloroform/isoamylalcohol (24:24:1) and chloroform/phenol followed by precipitation of DNA by the addition of ethanol to the aqueous phase. DNA was dissolved in Tris–EDTA buffer (pH 8.0) and treated with RNase A (heat-inactivated at 100° for 15 min) for 1 hr at 65°. DNA was re-extracted sequentially with phenol/chloroform and chloroform followed by DNA precipitation with ethanol. Isolated DNA was dissolved with sodium citrate buffer (pH 7.0) and left to stand overnight. DNA was quantitated spectrophotometrically at 260 nm, and the purity was assessed by an absorbance ratio A_{260}/A_{280} of 1.80. Covalent binding was expressed as picomoles AFB_1 bound/milligram DNA. Radioactivity was determined by an Aloka LSC-900 liquid scintillation counter, and the specific activity of [^{3}H]AFB_1 used was 180,156 dpm/ng AFB_1.

RESULTS

Effect of ethanol on the levels of hepatic GSH, GST and GSH peroxidase. GSH plays a role in the protection of the liver against the toxic effects of several hepatotoxins [9–11]. Therefore, the effect of ethanol on hepatic GSH was studied. Ethanol-induced hepatic depletion of GSH in 3 hr was 63% ($P < 0.01$, Fig. 2), the maximum response being produced at 6 hr (56%; $P < 0.01$). The hepatic GSH level returned to approximately initial control values 12–18 hr after ethanol ingestion. The acceleration of hepatic peroxidation after ethanol ingestion (Fig. 2) was inversely related to the decrease in GSH concentration. The highest value for lipid peroxidation was also observed after 6 hr (163%, $P < 0.05$). During this state of GSH-depletion inducing lipid peroxidation, no significant alterations in the activities of hepatic GST and GSH-peroxidase were observed under the acute treatment with ethanol (Figs 3 and 4). This is in agreement with previous studies in which the effect of ethanol treatment on the peroxidative capacity of the rat liver was estimated by the same procedure [25]. Ethanol itself was not toxic for the liver, as demonstrated in serum AST, ALT, ALP and GGT activities which showed no deviation from those of normal rats (data not shown). Up to 18 hr after ethanol administration, there was no difference in the activities of these enzyme markers from zero time.

Effects of ethanol on the hepatotoxicity of AFB_1. For estimation of the effects of ethanol, we selected the times of maximal and minimal GSH depletion induced by ethanol pretreatment with a dose of 100 mmol/kg (6 and 18 hr, respectively) before the administration of AFB_1 (1 mg/kg). Rats were killed by decapitation 24 hr after the administration of AFB_1. Pretreatment with ethanol 6 hr before AFB_1 administration significantly potentiated the AFB_1-induced hepatotoxicity by an increase in the activities of serum AST ($P < 0.01$; Table 1) and ALT ($P < 0.05$). No significant increase in the AFB_1-induced hepatotoxicity was observed in the rats pretreated with same dose of ethanol 18 hr before AFB_1 administration (Table 2).

Influence of ethanol pretreatment on GST and GSH-peroxidase activities in AFB_1-treated rats. As

Table 3. Effect of ethanol pretreatment on the hepatic GST and GSH-peroxidase activities induced by AFB_1 in rats

Treatment*	GST (nmol/min/mg protein)		GSH-peroxidase (nmol/min/mg protein)	
	CDNB	DCNB	COP	H_2O_2
Control	148 ± 16	11.7 ± 2.0	59.4 ± 8.4	43.1 ± 5.2
EtOH (6 hr)	143 ± 17	9.8 ± 1.5	69.2 ± 4.2	48.2 ± 3.7
EtOH (18 hr)	130 ± 10	9.4 ± 1.2	66.5 ± 5.8	49.7 ± 4.8
AFB_1	169 ± 16	11.6 ± 0.8	80.8 ± 3.3	63.2 ± 5.1
EtOH (6 hr) + AFB_1	152 ± 15	12.7 ± 2.4	68.1 ± 10.7	50.3 ± 4.3
EtOH (18 hr + AFB_1	153 ± 26	10.4 ± 0.3	68.2 ± 5.8	52.5 ± 3.5

* All rats were killed 2 hr after the i.p. administration of AFB_1 (10 μg/kg). A single oral dose of ethanol (as a 25% solution in water) was given 6 or 18 hr prior to the i.p. administration of AFB_1. Values are means ± SD, N = 3.

Table 4. Effect of ethanol pretreatment on the covalent binding to liver DNA induced by AFB_1 in rats

Treatment*	[^{3}H]Radioactivity bound (dpm/mg DNA)	AFB_1 bound (pmol/mg DNA)†	% of Control
AFB_1	111,512 ± 14,073	1.97 ± 0.25	100
EtOH (6 hr) + AFB_1	164,089 ± 10,854‡	2.90 ± 0.19‡	147
EtOH (18 hr) + AFB_1	125,942 ± 32,820§	2.22 ± 0.58§	113

* All rats were killed 2 hr after the i.p. administration of AFB_1 (10 μg/kg). A single oral dose of ethanol (as a 25% solution in water) was given 6 or 18 hr prior to i.p. administration of AFB_1. Values are means ± SD, N = 3.

+ The specific activity of the [^{3}H]AFB_1 used was 180,156 dpm/ng AFB_1.

‡ $P < 0.01$, compared with the AFB_1-treated group.

§ Not significant, compared with the AFB_1-treated group.

shown in Table 3, no significant alteration was found in the activity of the GSH-peroxidase 2 hr after the administration of AFB_1 (10 μg). Moreover, no effect of the GSH depletor (ethanol) on the GST was seen *in vivo* at the same period. Thus, the increased toxicity of AFB_1 by pretreatment with ethanol was less correlated with the activities of GST and GSH-peroxidase in our experimental conditions.

Effects of ethanol pretreatment on the hepatic DNA-binding of AFB_1 in rats. The covalent binding of AFB_1-metabolite (8,9-epoxide derivatives) to DNA was determined under two different time intervals: pretreatment with ethanol orally 6 hr (time of maximal GSH depletion, Fig. 2) or 18 hr (time of minimal GSH depletion) before the administration of [^{3}H]AFB_1 (10 μg/kg) and the rats killed 2 hr after the administration of [^{3}H]AFB_1. The formation of covalently bound adducts of 8,9-epoxide derivatives to rat liver DNA in rats pretreated with ethanol for 6 hr was increased by a concomitant increase in covalently bound [^{3}H]radioactivity. This was confirmed by analysis of the individual DNA adducts; the formation of the AFB_1 adducts was increased in rats pretreated with ethanol for 6 hr, while the formation of the AFB_1 adduct showed no change in rats pretreated with ethanol for 18 hr at the same dose (Table 4). Therefore, the increased formation of these adducts after ethanol pretreatment likely results from a depletion of GSH and also is consistent with potentiated AFB_1-induced hepatotoxicity.

DISCUSSION

Administration of 100 mmol/kg ethanol to rats reduced the hepatic GSH level and enhanced lipid peroxidation. These findings are in agreement with data in the literature [16, 26]. A close inverse relation between these two biochemical parameters in liver may be explained by the idea that ethanol-induced hepatic GSH depletion may occur through an increased utilization of GSH for the detoxication of peroxides, including lipid peroxide and hydroperoxide, during the period of ethanol intoxication [26]. However, Speisky *et al.* [27] proposed that the suppression of hepatic GSH synthesis by ethanol ingestion may deplete GSH, as related to enhancement of lipid peroxidation. No change in the activity of liver GST was found after an acute ethanol treatment. This is in agreement with previous studies that found non-inducibility of liver GST after a 2-week ethanol treatment by gavage at a 4.5 g/kg twice daily dose [28]. Previous studies indicated that GST was induced in rat and mouse after chronic ethanol intake [29, 30]. However, our present results show that GST was not changed significantly with hepatic GSH depletion induced by acute ethanol intoxication. Thus, the effect of ethanol-potentiated liver toxicity by AFB_1 may not be due to the suppression of GST. On the other hand, no change in the activity of GSH-peroxidase was observed. This result also clearly indicates that the depletion of hepatic GSH below a critical threshold-concentration allows the enhancement of lipid peroxidation evoked by exogenous promoters, while the GSH peroxidase remains unaffected [31].

The potentiation effect of AFB_1-induced hepatotoxicity by treatment with ethanol is well known [2].

The exact mechanism by which ethanol affects these changes in toxicity, however, is still not clear. The most attractive hypothesis is that ethanol, by inducing or activating the drug-metabolizing system, causes an accelerated biotransformation of the hepatotoxic agents to highly active metabolites [3–5], but the different isoforms of microsomal enzymes are responsive for the metabolic activation and inactivation of procarcinogens [6]. On the other hand, a depletion by ethanol of liver GSH levels may increase hepatotoxicity [12–16], since the conjugation with GSH is an important detoxifying pathway for AFB_1 [32]. Our results show that the marked potentiation by ethanol of AFB_1 hepatotoxicity occurred when AFB_1 was administered 6 hr after the ethanol, whereas this potentiation was not observed when ethanol was given 18 hr prior to i.p. administration of AFB_1. It appeared that the potentiation of AFB_1-induced hepatotoxicity was correlated with the maximal GSH-depletion period (6 hr) (Table 1). When the GSH level returned to the initial values 18 hr after ethanol ingestion, no significant effect on hepatotoxicity was observed. These results suggest that an increased hepatotoxicity of AFB_1 after ethanol pretreatment may be due to a depletion of hepatic GSH and a reduction of the detoxication pathway.

It has been well established that AFB_1 is activated by microsomal enzymes to yield 8,9-epoxide-AFB_1 [33], the ultimate electrophilic carcinogenic metabolite which covalently binds to DNA [34, 35]. Previous studies [36, 37] showed that AFB_1-DNA binding is modulated by the degree of epoxidation, GSH levels and GST activity. Hepatotoxicity of AFB_1 can be either decreased by PB pretreatment or increased by DEM treatment of rats [38], since PB stimulates activity of GST and decreases AFB_1-DNA binding; however, DEM treatment increases AFB_1-DNA binding with a depletion of GSH [37]. Our present data are similar to results with DEM treatment of rats with a corresponding increase in AFB_1-DNA binding. Therefore, the decreased formation of adducts after ethanol pretreatment most likely results from the depletion of GSH content and consequently provides more 8,9-epoxide-AFB_1 for DNA binding in rat liver. Consistent results were obtained on the time of maximal GSH depletion in an intoxicated liver, the elevation of serum AST and ALT indicating the enhanced toxicity by ethanol pretreatment 6 hr before the administration of AFB_1, while the constant serum AST and ALT activities indicated no potentiation effect in the 18-hr group. Similarly, the covalent binding to DNA was increased by 47% ($P < 0.01$) as compared with control when ethanol was given 6 hr prior to i.p. administration of AFB_1, whereas the formation of AFB_1-DNA adduct was not increased significantly by ethanol pretreatment in the 18-hr group.

In conclusion, our results indicate that GSH plays a vital role in the detoxication of reactive metabolites generated from the hepatocarcinogen, AFB_1, since GSH is very effective in competing with macromolecules for trapping of reactive metabolites of 8,9-epoxide-AFB_1. The study suggests that the level of GSH is an important modulator of AFB_1-DNA adduct formation. We have also demonstrated that ethanol can potentiate AFB_1-induced hepatic toxicity by a mechanism that lowers the hepatic GSH levels.

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