Cytochrome P450 2E1 inhibition prevents hepatic carcinogenesis induced by diethylnitrosamine in alcohol-fed rats

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Abstract: Chronic alcohol ingestion increases hepatic cytochrome P450 2E1 (CYP2E1), which is associated with hepatocarcinogenesis. We investigated whether treatment with chlormethiazole (CMZ), a CYP2E1 inhibitor, protects against alcohol-associated hepatic carcinogenesis in rats. Rats were fed either an ethanol liquid diet or a non-ethanol liquid diet, with or without CMZ for one and ten months. A single intraperitoneal injection of diethylnitrosamine (DEN, 20 mg/kg) was given to initiate hepatic carcinogenesis. CYP2E1 expression, inflammatory proteins, cell proliferation, protein-bound 4-HNE, etheno-DNA adducts, 8-hydroxy-2'-deoxyguanosine (8-OHdG), retinoid concentrations, and hepatic carcinogenesis were examined. Ethanol feeding for 1 month with DEN resulted in significantly increased hepatic CYP2E1 levels and increased nuclear accumulation of NF-κB protein and TNF-α expression, which were associated with increased cyclin D1 expression and p-GST positive altered hepatic foci. All of these changes induced by ethanol feeding were significantly inhibited by the one month CMZ treatment. At 10-months of treatment, hepatocellular adenomas were detected in ethanol-fed rats only, but neither in control rats nor in animals receiving ethanol and CMZ. The 8-OHdG formation was found to be significantly increased in ethanol fed animals and normalized with CMZ treatment. In addition, alcohol-reduced hepatic retinol and retinoic acid concentrations were restored by CMZ treatment to normal levels in the rats at 10 months of treatment. These data demonstrate that the inhibition of ethanol-induced CYP2E1 as a key pathogenic factor can counteract the tumor-promoting action of ethanol by decreasing TNF-α expression, NF-κB activation, and oxidative DNA damage as well as restoring normal hepatic levels of retinoic acid in DEN-treated rats.

Key Words: Ethanol; hepatic carcinogenesis; cytochrome P450 2E1; NF-κB; cell proliferation

Introduction

Chronic alcohol abuse is a major risk factor for hepatocellular carcinoma in the United States and it has been estimated that 32-45% of all cases of hepatocellular carcinoma are due to alcohol consumption (1). Carcinogenesis is a process comprised of three main stages: initiation, promotion, and progression. Initiation is rapid and occurs with high frequency in the acute setting (with exposure to a carcinogen). Promotion is a long-term process and requires chronic exposure to a tumor-promoting environment, such as alcohol-induced oxidative stress, inflammation and/or nutritional imbalances. This injury

can elicit a wide spectrum of cellular responses ranging from proliferation to growth arrest. Aside from oxidative stress, the causal relationship between inflammation and tumorigenesis has also been implicated in a variety of chronic inflammatory diseases. The pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) is reported to be significantly increased in alcoholic patients (2,3). A previous study demonstrated reduced rates of preneoplastic lesions and liver tumor formation in TNF-α receptor knock-out mice (4), suggesting an important role of TNF-α signaling in hepatocarcinogenesis. In addition, nuclear factor-κB (NF-κB), an important transcriptional factor regulated by TNF-α in hepatocytes, is shown to be closely associated with liver neoplastic progression, mediating transcriptional regulation of multiple genes involved in cellular transformation, proliferation, and survival (5). Recently, we have shown that massive hepatic steatosis and alcoholic foamy degeneration with a mixed infiltrate of inflammatory cells and hepatocellular adenoma were detected in ethanol-fed rats after 10 months of treatment (6); but not in the control rats given the same dose of the DEN carcinogen. This clearly indicates the promoting effect of alcohol consumption on hepatic carcinogenesis (6).

Cytochrome P4502E1 (CYP2E1)-dependent microsomal ethanol oxidizing system, an important pathway of ethanol metabolism in the liver, is induced by chronic ethanol consumption (7). The induction of CYP2E1 by chronic alcohol intake results in an enhanced activation of procarcinogens to carcinogens (8), induction of oxidative stress (9), and release of inflammatory cytokines TNF-α, IL-6, and IL-1 (10-13), which may stimulate carcinogenesis (9). CYP2E1 knockout mice are shown to develop less oxidative stress (14), less oxidized DNA adducts (15) and less diethylnitrosamine (DEN)-induced hepatic tumors (16), as compared with wild-type mice. It has been shown that treatment with CYP2E1 inhibitors, such as chlormethiazole (CMZ) (17,18), protects against ethanol-induced liver injury (12,19-22). CMZ treatment significantly reduces ethanol-induced hepatic cytokine expression in ethanol-treated rats (12), decreases macrovesicular fat accumulation, and inhibits oxidative stress in mice fed ethanol (21). Furthermore, CMZ was shown to block the generation of ethanol-DNA adducts by 70%~90% in ethanol-treated cells overexpressing human CYP2E1 (22). However, direct evidence that CYP2E1 inhibition plays a role in ethanol-promoted hepatocarcinogenesis in vivo is still lacking.

In the present study, using CMZ as an inhibitor of CYP2E1, we examined the action of CYP2E1 in carcinogen-initiated and ethanol-promoted hepatocarcinogenesis in short- and long-term ethanol-fed rats. We also investigated potential biological mechanisms by which CMZ could affect hepatic carcinogenesis by measuring liver tissue levels of molecular markers including key pro-inflammatory cytokine TNF-α, transcription factor NF-κB, lipidperoxidation products 4-hydroxynonenal (4-HNE), exocyclic etheno-DNA adducts, oxidative DNA damage marker 8-hydroxy-2'-deoxyguanosine (8-OHdG), and retinoid concentrations.

Methods

Animals, diet, and study groups

Male Sprague-Dawley rats (130-140 g; from Charles River Laboratories, Wilmington, MA) were used in this study. For the short-term (1 month) study, rats were distributed into three groups (n=6) by weight-matching: (I) control group (C); (II) ethanol-fed group (E); and (III) ethanol-fed and CMZ (100 mg/kg BW) supplemented group (E + CMZ). In the long-term study (10 months), rats were divided into four groups (n=6): C, E, E + Low CMZ (10 mg/kg BW), and E + high CMZ (100 mg/kg BW). All ethanol-fed groups were fed the Lieber-Decarli liquid diet (Dyets Inc., Bethlehem, PA) containing 36% of total calories as ethanol, yielding a concentration of 6.2% (vol/vol). Ethanol was gradually introduced into experimental diets over a 7-day period before providing animals with the final concentration. In the control diet, ethanol was replaced by an isocaloric amount of maltodextrin (Purina Test diets, Richmond, IN, USA). Both diets contained 18% of total calories as protein and 35% as fat; 47% of total calories were provided from carbohydrates in the control diet, whereas 11% of total calories were from carbohydrates in the ethanol diet. For CMZ supplementation, CMZ was dissolved in 95% ethanol and added directly into the liquid diet. Since the liquid diet provides physiological amounts of fluid, extra water was not given.

For the long-term study, a low-dose diethylnitrosamine (DEN) (Sigma, St. Louis, MO) was given as an intraperitoneal injection of 20 mg/kg body weight one week before the ethanol feeding with or without CMZ began. While our recent study (6) has shown that this DEN treatment with chronic ethanol consumption over a ten-month period led to the development of hepatocellular adenoma in the DEN-initiated rats, we observed significant declines in preneoplastic placental form of glutathione S-transferase (p-GST) positive altered hepatocellular

foci (AHF) in rodent livers after one month (Ye, Q., Chavez, P., and Wang, X.D., unpublished data) and 6- and 10-months of alcohol-feeding (6). In order to investigate the inhibitory effect of CMZ on ethanol-induced CYP2E1 on p-GST foci formation and inflammation in the short-term study, we injected rats with DEN (20 mg/kg i.p.) three weeks after ethanol feeding with or without CMZ treatment. Body weights were recorded weekly. All rats were terminally exsanguinated at the end of the study under AErrane® (Fort Dodge Animal Health, Fort Dodge, IO, USA) anesthesia. Liver tissues were collected, frozen under liquid nitrogen, and stored at −80 ℃ for further analysis. All animals were maintained in an American Association for the Accreditation of Laboratory Animal Care accredited facility, in an environmentally controlled atmosphere (temperature, 23 ℃, 45% relative humidity) with 15 air changes of 100% fresh filtered air per hour and a 12/12 h light/dark cycle. All animals were observed daily for clinical signs of illness. This project was approved by the Jean Mayer USDA Human Nutrition Research Center on Aging Animal Care and Use Committee.

Immunohistochemistry

The altered hepatic foci, a marker for preneoplastic lesions (23,24), were measured by immunostaining of placental glutathione-s-transferase (p-GST) in the liver. Hepatocyte proliferation was quantified by immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) and Ki-67. Briefly, sections (5 μm thick) were cut from formalin-fixed and paraffin embedded liver samples. After a standard dehydration-rehydration procedure, liver sections were incubated with 0.3% H_2O_2 for 30 min to quench endogenous peroxidase activity. The sections were then heated using steamer for 20 min in 10 mM sodium citrate (pH 6.0) buffer to retrieve antigen. The routine biotin-streptavidin immunohistochemical method consisted of sequential incubations in goat or horse serum blocking solution, polyclonal anti-GST (Novocastra Laboratory, UK) monoclonal anti-PCNA (clone PC10, Dako Cooperation, Carpinteria, CA, USA) or monoclonal anti-Ki-67 (clone MIB-5, Dako Cooperation), biotinylated goat anti-rabbit or horse anti-mouse IgG, and streptavidin conjugated to a horseradish peroxidase label. The slides were developed with a diaminobenzidine substrate and counterstained with hematoxylin. The sections were examined under light microscopy by two blinded independent investigators. For PCNA staining, a total of 20 randomly selected fields were screened and PCNA positive cells with dark brown nuclear were expressed as PCNA (+) per 100 hepatocytes. For Ki-67 staining, we counted the number of Ki-67 positive cells with dark brown nuclear in the entire slide, and Ki-67 positive cells were expressed as Ki-67(+) per cm^2. For p-GST staining, we counted the number of animals that had p-GST-positive foci in each group.

Western blotting

Whole cell protein extraction for measuring CYP2E1, cyclin D1, and cleaved-caspase-3 protein levels, and nuclear extraction for measuring NF-κB p65 were prepared as previously described (25,26). Approximately 40 g protein extracts were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA). The membranes were blocked with 5% non-fat milk in TBST buffer and incubated with primary antibodies against CYP2E1 (Millipore Corp., Bedford, MA), NF-κB p65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), cyclinD1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), cleaved-caspase-3 (Cell Signaling Technology, Inc., Danvers, MA), or GAPDH (Millipore Corp., Bedford, MA). Membranes were then incubated with the secondary antibodies against rabbit or mouse (Bio-Rad Laboratory, Hercules, CA). The blots were then developed using a SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL). The protein levels were analyzed with an imaging densitometer (Bio-Rad, Hercules, CA) using GAPDH as a loading control.

Real-time PCR analysis for hepatic TNF-α mRNA

Total RNA was extracted using TriPure reagent (Roche Applied Science, Indianapolis, IN). cDNA was generated with M-MLV reverse transcriptase (Invitrogen) as indicated in the manufacturer's manual. Real-time PCR reactions were performed on an Applied Biosystems 7000 sequence detection system using Platinum SYBR Green qPCR Kit (Invitrogen) according to the manufacturer's instructions. Sequences for PCR primers are as follows: TNF-α, 5'-CCAGACCCTCACACTCAGATCA-3' and 5'-TCCGCTTGGTGGTTTGCTA-3', GAPDH, 5'-AGTGCCAGCCTCGTCTCATAG-3' and 5'-CCTTGACTGTGCCGTTGAACT-3'. Quantification of gene expression was normalized to the levels of GAPDH and then calculated by reference to the average values for the control group using the comparative Ct method. For

each sample and each gene, PCR reactions were carried out in duplicate and repeated twice.

Histopathology

Liver samples were fixed with 10% buffered formalin and embedded in paraffin wax. 5 μm sections were stained with hematoxylin and eosin (H&E). The slides were evaluated microscopically for the presence of tumors according to the criteria proposed by the World Health Organization (WHO)/International Agency for Research on Cancer (IARC) (27). The sections were photographed and examined by three independent investigators blinded to the treatment groups.

Immunohistochemical detection of etheno-DNA adducts

Staining on liver tissue was performed using the method developed in our laboratory (28,29). Formalin fixed slides were dipped in PBS for 10 minutes, then placed in 0.3% H_2O_2 in absolute methanol for 10 minutes to quench endogenous peroxidase. Slides were incubated with proteinase K (10 microg/mL) (Roche, Mannheim, Germany) in double distilled H_2O at room temperature for 10 minutes to remove histone and nonhistone proteins from DNA, increasing antibody accessibility. After washing with PBS, slides were treated with 20 microg/mL ribonuclease (RNAse; Roche, Mannheim, Germany) (heated for 10 minutes at 80 °C to inactivate DNAse) at 37 °C for 1 hour to prevent antibody binding to RNA adducts, and then washed in PBS. To denature DNA, 4 N HCl was used for 5 minutes at room temperature and subsequently rinsed in PBS and double distilled water. The pH was neutralized with 50 mM Trisbase buffer, pH 7.4 for 5 minutes at room temperature. Nonspecific binding sites were blocked with 8% bovine serum albumin, 2% horse serum, 0.05% Tween, and 0.05% Triton X-100 for 20 minutes at room temperature. A cross adsorbed horse anti-mouse antibody, conjugated to biotin (Vector Laboratories, Burlingame, CA) was employed to prevent unspecific binding to endogenous rat immunoglobulin. Slides were incubated with the primary monoclonal antibody EM-A-1 against edA (provided by Drs. P.Lorenz and M. Rajewsky, University of Essen, Essen, Germany, at a dilution of 1:20 at 4 °C overnight (29). After washing with PBS, the antibody detection was performed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Diaminobenzidine was used as a chromogen to visualize

the reaction. The reaction was stopped after 5 minutes with H_2O. Slides was counterstained with 4',6'-diamidino-2-phenylindole and mounted with Kaiser's glycerin-gelatin. All slides were subjected to the same standard conditions. Negative controls were performed by omitting the primary antibody.

Immunohistochemical staining for protein-bound 4-HNE

Formaldehyde fixed sections were treated with 0.5% H_2O_2 in absolute methanol for 10 minutes to quench endogenous peroxidase activity. Thereafter, sections were incubated for 2 hours at room temperature with the primary antibody (rabbit anti-4HNE, 1:500, Alexis, Loerrach, Germany) and 5% normal serum to block nonspecific binding. Vectastain Lite ABC kit (Vectastain Laboratories, Burlingame, CA) was used for detection according to the manufacturer's protocol. Staining was developed by incubating the sections for 5 minutes in diaminobenzidine. Sections were counterstained with hematoxylin and mounted in Aquatex mounting medium. Negative controls were performed by omitting the primary antibody.

Immunohistochemical staining for 8-OHdG

Formalin fixed liver sections were stained using the method developed by Yaborough *et al.* (30). After antigen retrieval, the slides were dipped in phosphate-buffered saline (PBS) for 10 minutes and then placed in 0.3% H_2O_2 in absolute methanol for 20 minutes to quench endogenous peroxidase. After washing with PBS, slides were treated with 10 μg/mL RNase A (EMD Chemicals, San Diego, CA92121, USA) (heated for 12 minutes at 80 °C to inactivate DNase) at 37 for 1 hour to prevent antibody binding to RNA adducts and then washed in PBS. To denature DNA, cells were treated with 4 N HCI for 5 minutes at room temperature and subsequently rinsed in double distilled water and PBS. The pH was neutralized with 50 mM Tribase buffer, pH 7.4, for 5 minutes at room temperature. Non-specific binding sites were blocked with 10% bovine serum albumin (BSA), 4% normal horse serum, 0.05% Tween 20 and 0.05% Triton X-100 for 20 minutes at 37 °C. Slides were incubated for 1 hour at room temperature with the primary monoclonal antibody mouse-anti-8-OH-guanosine (at a dilution of 1:8,000, Abcam, Cambridge, CB4 0WN, UK) and 2% normal horse serum to block nonspecific binding. After washing with PBS, the antibody detection was performed using the Vectastain Elite ABC

kit (Vector Laboratories, Burlingame, CA/USA) according to the manufacturer's protocol (incubation with secondary antibody: Horse anti-Mouse IgG (H + L) 1:400 for 40 minutes at room temperature). Diaminobenzidine (DAB) was used as a chromogen to visualize the reaction. The reaction was stopped after 5 minutes with PBS. Slides were counterstained with hematoxylin for 8 minutes at room temperature. After washed with running tap water for 5 minutes, differentiated with 1% HCl/70% Ethanol for 10 seconds and washed with tap water for 5 minutes. Turn blue with 1% sodium bicarbonate for 20 minutes. Dehydrated through 70% ethanol for 2×3 minutes, 95% ethanol for 2×3 minutes, 100% ethanol for 2×3 minutes and clear in xylene for 2×5 minutes. Mounted with mounting medium (Roti-Histokitt II). All slides were subjected to the same standardized conditions. Negative controls were performed by omitting the primary antibody.

Imaging and semiquantitative analysis of etheno-DNA adducts, 4-HNE, and 8-OHdG

Representative pictures were taken at a magnification of 200× with a Leica Image Manager 50 (Leica, Solms, Germany) and analyzed using Image J software (Toronto, Western Research Institure, UK). The frequency of etheno-DNA and 8-OHdG positive stained nuclei was expressed as percentage of stained nuclei over the total number of cells counted. Staining intensity for 4-HNE was assessed according to the scale devised by Tsutsumi *et al.* (31).

High performance liquid chromatography

Liver sample preparation and a gradient Reverse Phase-HPLC was performed to assess the retinoid levels in liver homogenates, as described previously (32,33). Briefly, 50 µL of each sample were injected onto the HPLC column. The gradient procedure at a flow rate of 1 mL/min was as follows: 100% solvent A (acetonitrile:tetrahydrofurane: water, 50:20:30, vol/vol/vol, with 0.35% acetic acid and 1% ammonium acetate in water) for 6 minutes, followed by a 6-minute linear gradient to 50% solvent A and 50% solvent B (acetonitrile:tetrahydrofurane:water, 50:44:6, vol/vol/vol, with 0.35% acetic acid and 1% ammonium acetate in water), an 11-minute hold at 50% solvent A/50% solvent B, a 2-minute gradient to 100% solvent B, and a 10 minute hold at 100% solvent B, then a 10-minute gradient back to solvent A, and finally a 7-minute hold at 100% solvent A. In this HPLC system, retinoic acid, retinol, and retinyl

acetate eluted at 2.5, 4.8, and 8.9 minutes, respectively. Individual retinoids were identified by co-elution with standards and absorption spectrum analysis. Retinoids were quantified relative to the internal standard (retinyl acetate) by determining the peak areas calibrated against known amounts of standards.

Statistical analysis

All results values are expressed as means ± standard error of mean (SEM). Group means were compared using one-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) post-hoc procedure. A difference between groups was considered significant if $P<0.05$. Groups not sharing the same letter are significantly different.

Results

Effects of ethanol and CMZ on hepatic CYP2E1 expression

Ethanol feeding for 1 month significantly increased hepatic CYP2E1 protein levels by 2.6 fold, as compared with control ($P<0.05$); while CMZ supplementation inhibited ethanol-induced hepatic CYP2E1 protein expression by approximately 45% ($P<0.05$, E *vs.* E + CMZ) (*Figure 1A*). Ethanol feeding for 10 months also significantly increased CYP2E1 protein level, as compared with control ($P<0.05$). CMZ supplementation for 10 months at both doses (10 and 100 mg/kg BW) significantly blocked the ethanol-induced hepatic CYP2E1 protein levels (*Figure 1B*).

Effect of ethanol and CMZ on hepatocyte proliferation

Hepatocyte proliferation was assessed by immunohistochemical staining for PCNA (*Figure 2A*) and Ki-67 (*Figure 2B*) in rats fed ethanol for one month. Ethanol-feeding increased PCNA positive hepatocytes by 5.4 fold, while CMZ supplementation completely blocked ethanol-induced hepatocyte hyper-proliferation (*Figure 2A*). The result was confirmed by Ki-67 staining, another marker for cell proliferation. In the livers of rats fed a control diet, few hepatocytes were positively stained for Ki-67 (0.20 ± 0.12 cells/cm^2). The number of Ki-67 positive hepatocytes (2.25 ± 0.69 cells/cm^2) was dramatically increased by ethanol treatment ($P<0.05$, ethanol *vs.* control). CMZ supplementation significantly inhibited ethanol-induced hepatocyte proliferation, as no Ki-67 positive hepatocytes were detected in the livers of rats fed ethanol and
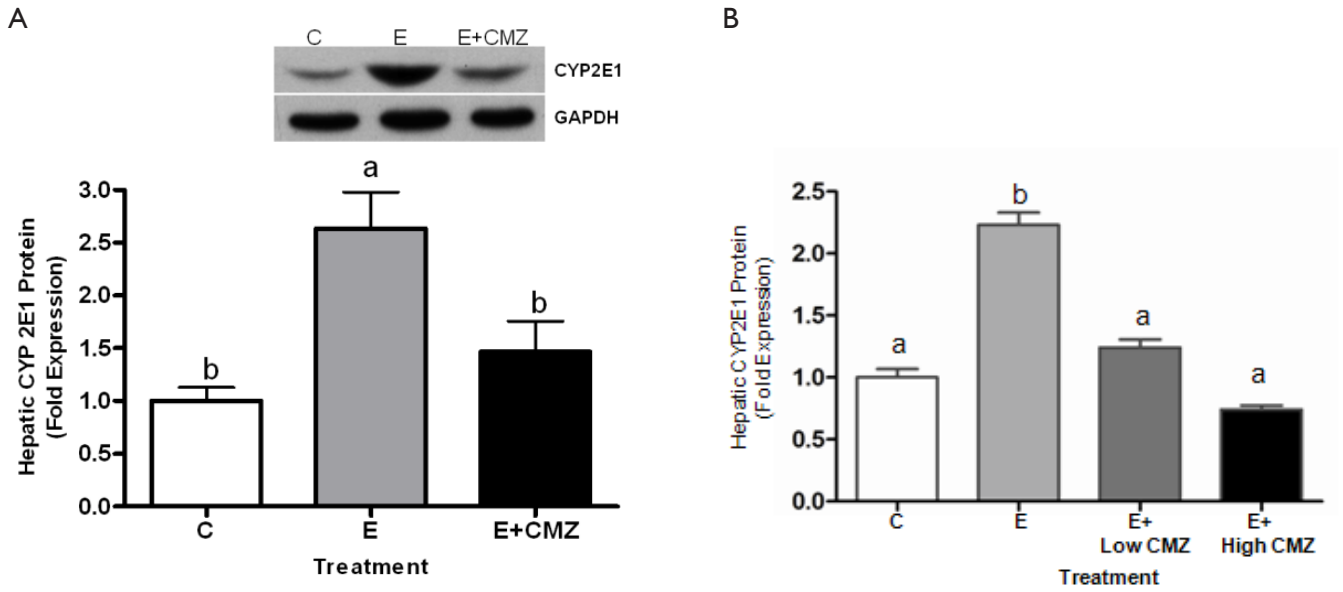
Figure 1 Effect of ethanol and CMZ on hepatic CYP2E1 expression. CYP2E1 expression were measured by western blot in liver tissues from rats. (A) CYP2E1 protein levels after 1-month treatment and a single i.p. injection of DEN at week 3. Upper panel is a representative western blot. (B) CYP2E1 protein levels after 10-month treatment. Values are means ± SEM. Means that do not share a letter differ, P<0.05. (C. control diet; E. ethanol-fed; and E + CMZ, ethanol fed plus CMZ supplementation)
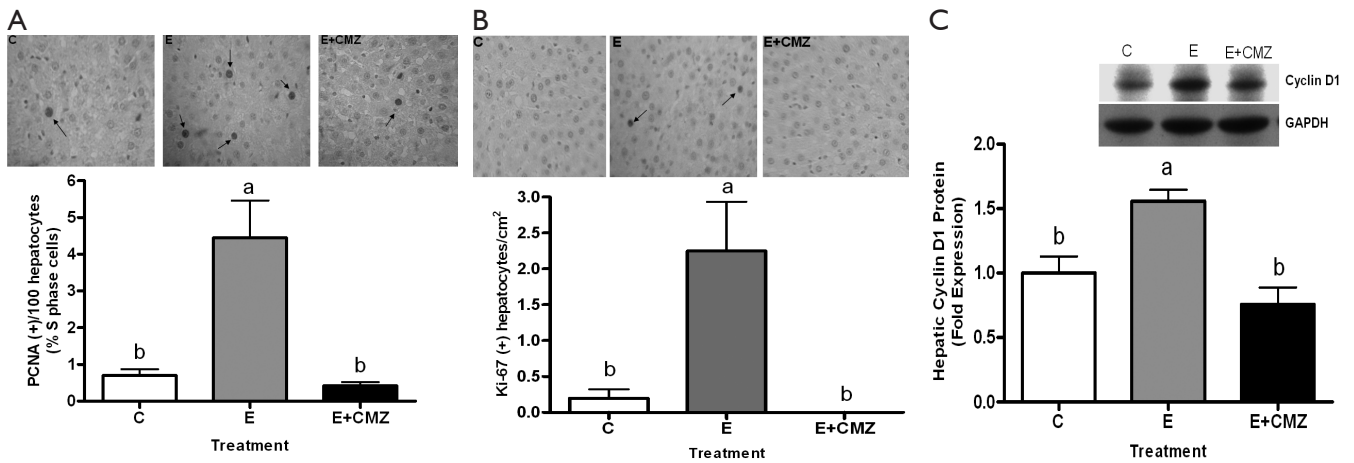

Figure 2 Effect of ethanol and CMZ on hepatic cell proliferation. A and B. Hepatocyte proliferation was measured by immunohistochemical staining for PCNA (A) and Ki-67 (B) in representative liver sections. Only hepatocytes with uniformly dark brown stained nuclei were counted as Ki-67 and PCNA positive cells. Upper portions of each panel are representative immnuostained sections in the same orders as in the bar graph (original magnification, ×200). C. Cyclin D1 expression was measured by western blot in liver tissues. Upper panel is representative western blot in the same orders as in the bar graph. The bar graph shows the intensity of the protein signal of cyclin D1. (C. control diet; E. ethanol-fed; and C + CMZ. ethanol fed plus CMZ supplementation. All rats were fed with ethanol diet for 1-month and were injected with DEN one week prior to killing. Values are Means ± SEM. Means that do not share a letter differ, P<0.05)
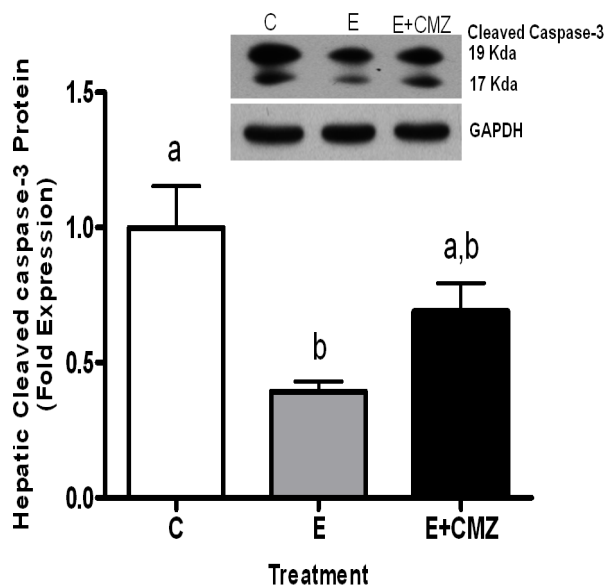
Figure 3 Effect of ethanol and CMZ on cleaved-caspase 3 protein levels in rat liver tissues. Cleaved caspase-3 protein expression was measured by western blotting analysis and followed by densitometric analysis. Values are Means±SEM (n=5 or 6). Means that do not share a letter differ, $P<0.05$. Upper panel is a representative Western blot. C. Control; E. Ethanol-fed; E+CMZ. Ethanol-fed plus CMZ supplementation

supplemented with CMZ (*Figure 2B*).

Hepatic cell proliferation was further evaluated by the expression of cyclin D1, a critical cell cycle regulator, using western blot. The cyclin D1 protein level was increased by about 50% in the liver tissues of ethanol-fed rats, as compared with rats fed a control diet ($P<0.05$); while no difference in liver cyclin D1 levels was found between control rats and rats fed ethanol and supplemented with CMZ (*Figure 2C*).

Effects of ethanol and CMZ on hepatocyte apoptosis in rat liver

Hepatic cell apoptosis was evaluated by the levels of cleaved-caspase-3 using western blot in rats fed ethanol for one month. Ethanol-feeding resulted in a 61% reduction in cleaved-caspase-3 protein levels in the rat livers ($P<0.05$, ethanol *vs.* control). CMZ supplementation in ethanol-fed rats increased the amount of cleaved-caspase-3 protein in liver tissues; however, the difference was not significant (*Figure 3*).

Effects of ethanol and CMZ on hepatic NF-κB activation and TNF-α expression

Hepatic NF-κB activation was assessed by nuclear distribution of NF-κB p65 subunit in rats fed ethanol for one month. Nuclear p65 protein levels were significantly increased in the liver of ethanol-fed rats (2.0-fold increase, as compared with control rats, $P<0.05$). In contrast, nuclear p65 levels in the livers of ethanol-fed and CMZ-supplemented rats were approximately 75% lower as compared with ethanol-fed rats ($P<0.05$) (*Figure 4A*).

Hepatic TNF-α mRNA expression was measured by quantitative PCR. Ethanol feeding significantly stimulated hepative TNF-α mRNA expression (75% increase as compared with control, $P<0.05$); while CMZ supplementation inhibited ethanol-increased TNF-α expression (53% inhibition, as compared with ethanol-fed group, $P<0.05$) (*Figure 4B*).

Effects of ethanol and CMZ on the development of hepatic p-GST positive foci and the incidence of hepatocellular adenoma

Development of hepatic pre-neoplastic lesion in DEN-treated rats after 1 month of ethanol exposure was evaluated by the presence of p-GST positive foci in the livers. While no p-GST positive foci were found in all 6 rats fed a control diet, 4 out of 5 ethanol-fed rats developed hepatic p-GST positive foci (*Figure 5*). In CMZ and ethanol-fed group, p-GST positive foci were only found in the livers of one out of six rats (*Figure 5*).

Development of hepatocellular adenoma in DEN-treated rats after 10 months of ethanol exposure with or without CMZ treatment was further examined. Without CMZ treatment, hepatocellular adenoma was detected in four out of five ethanol-fed rats after 10 months of treatment (*Figure 6*). None of ethanol-fed rats in groups treated with low- (10 mg/kg BW) or high- (100 mg/kg BW) doses of CMZ had hepatocellular adenoma after 10-month treatment period. Control rats given the same dose of DEN without ethanol did not develop hepatocellular adenoma at 10 months.

Effects of ethanol and CMZ on DNA adduct formation, 4-HNE and 8-OHdG positively stained nuclei in DEN-treated rats after 10 months of treatment

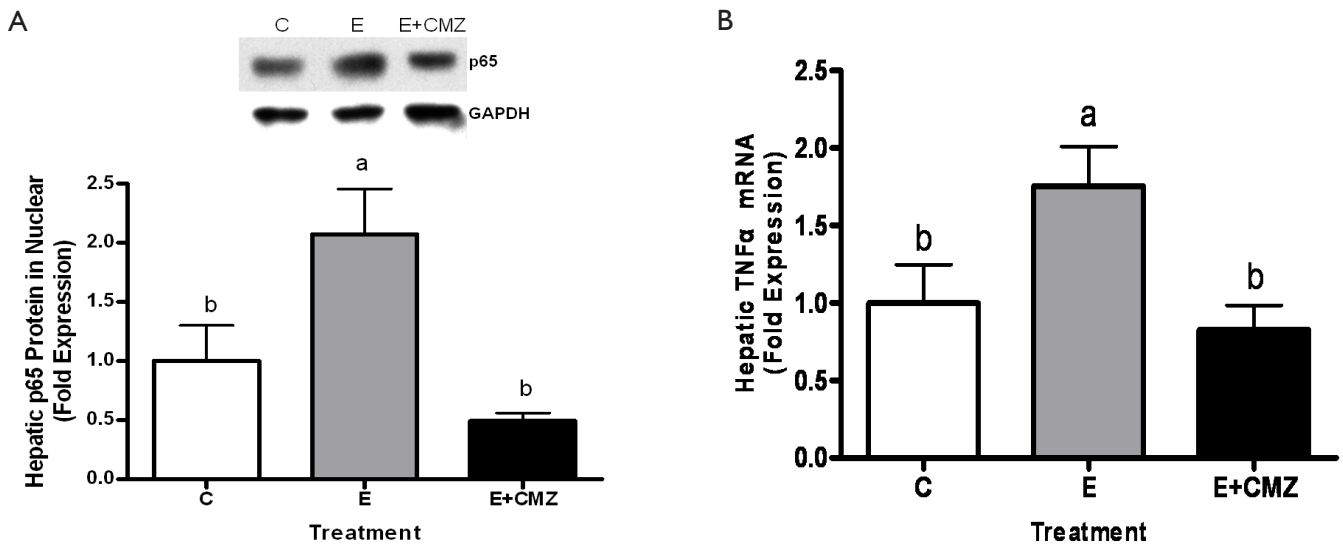Chronic ethanol feeding did not significantly affect the generation of exocyclic etheno-DNA adducts in the

A

B

Figure 4 Effect of ethanol and CMZ on hepatic levels of nuclear p65 protein (A) and TNF-α mRNA (B). A. p65 protein in nuclear extract was measured by western blot. Upper panel is representative western blot in the same orders as in the bar graph. The bar graph shows the intensity of the protein signal of p65. B. Hepatic TNF-α mRNA expression was measured by real-time PCR after reverse transcription of total RNA extracted from rat liver tissue. C. control diet; E. ethanol-fed; and C+CMZ, ethanol fed plus CMZ supplementation. Values are Means ± SEM. Means that do not share a letter differ, P<0.05

100× magnification
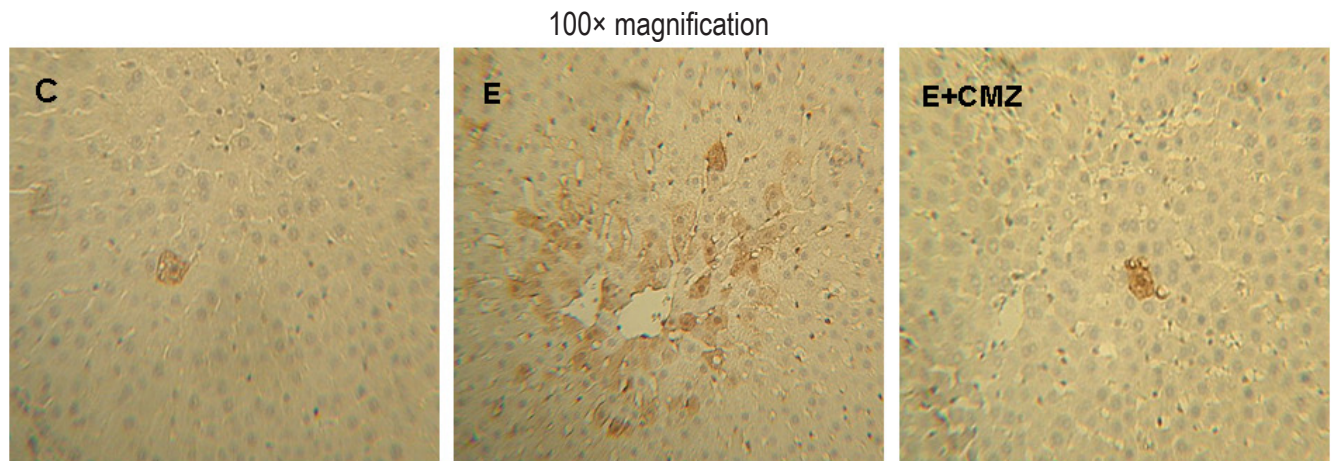
Group	C	E	E+CMZ
p-GST positive foci animals	0	4	1
Total animals	6	5	6

Figure 5 Effect of ethanol and CMZ on the development of p-GST-positive foci. p-GST was measured by immunohistochemical staining. Upper portions of each panel are representative immunostained sections in the same orders as in the bar graph (original magnification, ×100). C. control diet; E. ethanol-fed; and E + CMZ. ethanol fed plus CMZ supplementation

Ethanol

Ethanol + CMZ

X 40

X 400
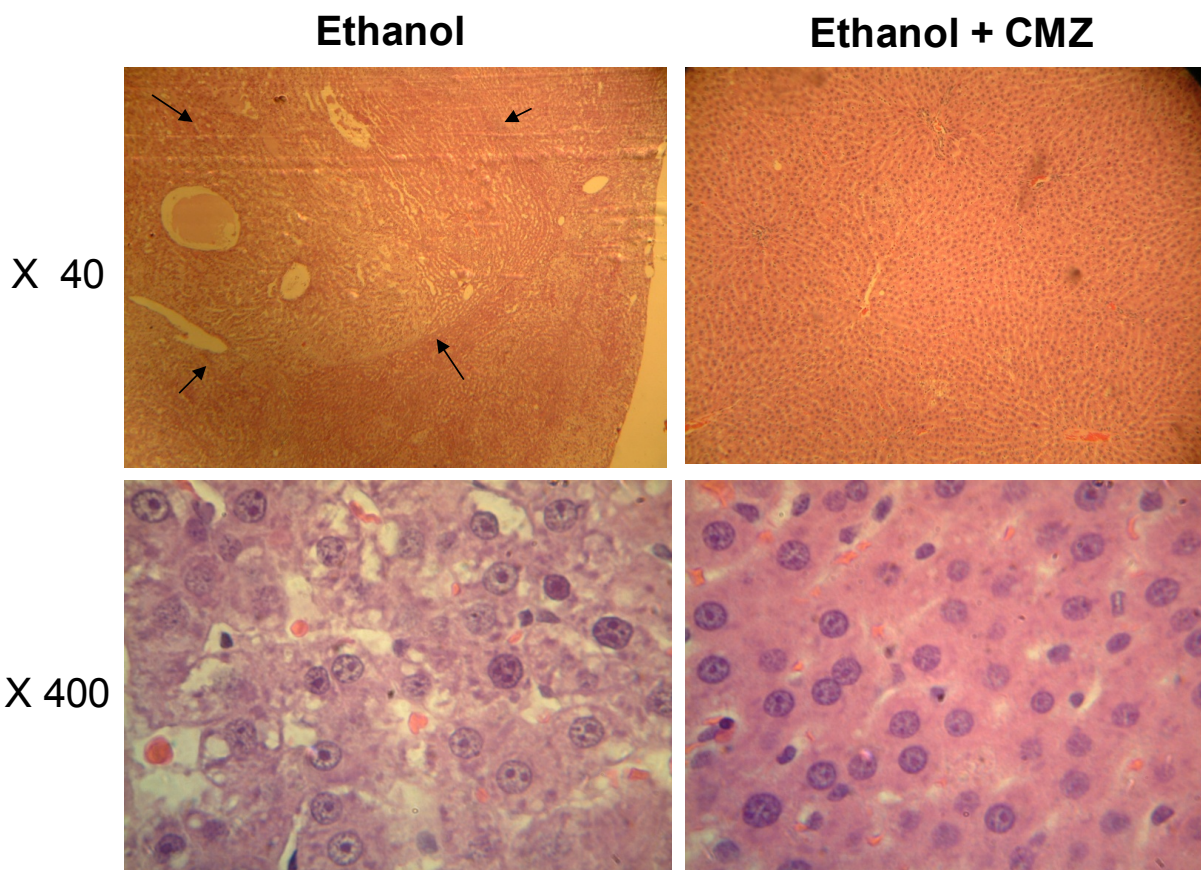
Group	C	E	E + low dose CMZ	E + high dose CMZ
Animals with adenoma	0	4	0	0
Total animals	6	5	6	6

Figure 6 The incidence of hepatocellualr adenoma, detected under microscopy, in diethylnitrosamine-injected rats after 10 months of ethanol exposure with or without CMZ treatment. Hepatocellualr adenoma, under H&E staining (upper panel ×40, lower panel ×400). The tumor (indicated by arrow) is composed of sheets of hepatocytes that are larger than those of the surrounding liver, which shows compression. All rats were injected with DEN (20 mg/kg BW) and treated with either a non-ethanol (C) or an ethanol diet (E) with or without two doses (10- and 100-mg/kg BW) of CMZ supplementation for 10 months

liver [15.0±5.8% (C) *vs.* 16.0±8.9% (E) positively stained nuclei], while the administration of CMZ decreased the adduct formation, which, however was not statistically significant [16.0±8.9% (E) *vs.* 17.0±7.6% (E + Low CMZ) *vs.* 12.0±4.5% (E + High CMZ) positively stained nuclei]. This was paralleled by 4-HNE binding to protein. Again, no significant difference occurred between controls, ethanol fed rats and ethanol fed rats with CMZ [1.2±0.4 (C) *vs.*

1.2±0.5 (E) *vs.* 1.0±0.5 (E + High CMZ) score units]. In contrast, ethanol significantly increased 8-OHdG formation [11.7±4.1 (C) *vs.* 24±5.5 (E) positively stained nuclei, $P<0.05$] while the administration of CMZ significantly decreased 8-OHdG positively stained nuclei [14.3±5.4 (E + Low CMZ) and 14.0±5.0 (E + High CMZ), as compared with the E group, respectively, $P<0.05$].
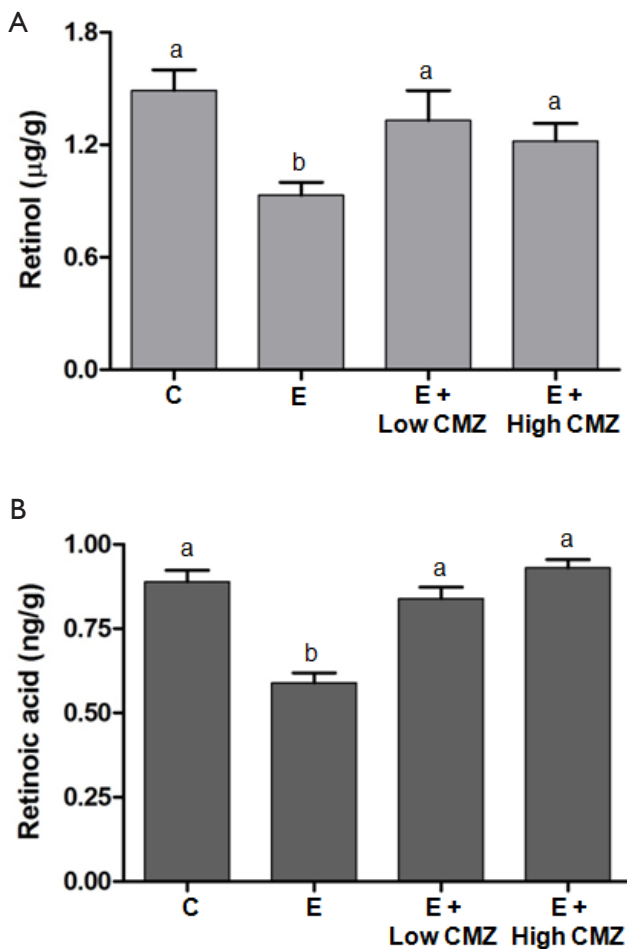
Figure 7 Effect of ethanol and CMZ on retinol and retinoic acid levels in rat liver tissues. Retinoids were measured by HPLC analysis. Values are Means ± SEM (n=5 or 6). Means that do not share a letter differ, P<0.05. C, Control; E, Ethanol-fed; E + Low CMZ, Ethanol-fed plus low dose of CMZ supplementation; E + High CMZ, Ethanol-fed plus high dose of CMZ supplementation

Effects of ethanol and CMZ on the hepatic retinoid concentrations in DEN-treated rats after 10 months of treatment

Ethanol feeding for 10 months significantly decreased the hepatic concentrations of retinol and retinoic acid, as compared to non-ethanol fed control rats (P<0.05) (*Figure* 7). Compared to ethanol-fed rats, CMZ treatment at two different doses (10- and 100-mg/kg bw) in ethanol-fed rats restored hepatic retinol concentrations. There were no significant differences in hepatic retinol concentrations among control rats *vs*. ethanol-fed rats with CMZ treatment (*Figure* 7).

Similarly, hepatic retinoic acid concentrations in low- and high-CMZ treatment groups were significantly increased as compared to ethanol-fed rats without CMZ treatment, and were not significantly different as compared to control rats.

Discussion

This is the first study which reports a significant inhibition of alcohol-associated hepatocarcinogenesis by CYP2E1 inhibition. CMZ is a potent CYP2E1 inhibitor and its concomitant administration with alcohol resulted in a significant reduction of hepatic CYP2E1 in ethanol-fed rats at 1 as well as at 10 months of treatment. At the same time, we observed that hepatic p-GST positive foci, a marker for liver preneoplastic lesions, was detected in 4 out of 5 ethanol-fed rats but only 1 out of 6 ethanol-fed rats with CMZ treatment after one month. The same dose of DEN was given in rats fed a control diet, and none of them developed hepatic p-GST positive foci. Similarly, when the same dose of DEN was given in rats one week before the alcohol-feeding, hepatic CYP2E1 induction and adenomas were detected in the ethanol-fed rats, but were completely inhibited by CMZ after 10-months of treatment. These data clearly indicate that the inhibition of ethanol-induced CYP2E1 by CMZ can block carcinogen-initiated and ethanol-promoted hepatic carcinogenesis regardless the DEN given before or after alcohol-feeding. This finding is in accordance with data from a recent study, which showed lower incidence of DEN-initiated liver tumors in CYP2E1 knockout mice as compared with wild-type mice (16). This further indicates CYP2E1 as a key pathogenic factor in ethanol associated hepatocarcinogenesis.

Hepatocarcinogenesis is a complex process (34) in which inflammatory cytokines and oxidative stress (35) are of considerable importance (36). For example, infectious agents, stress signaling, and pro-inflammatory cytokines (37-39) activate NF-κB transcription factor, which mediates the production of various inflammation cytokines, such as IL-1, IL-6, and TNF-α (40), therefore promoting hepatic carcinogenesis. In addition to mediating the inflammatory response, NF-κB also regulates the expression of genes involved in many important processes during the development and progression of cancer, including cell proliferation and apoptosis (41). The present study showed an increased nuclear protein level of NF-κB p65 subunit and a higher TNF-α mRNA expression in the livers of ethanol-fed rats after DEN-injection, as compared with rats received DEN-injection but remained in non-

ethanol diet. Since NF-κB promotes cell growth by regulating the expression of several genes in the cell cycle machinery, including cyclin D1 (42), it is important to note that the increased-nuclear accumulation of NF-κB protein and increased TNF-α expression by ethanol feeding was associated with increased hepatocyte proliferation markers and increased cyclin D1 protein expression in liver tissues. This association suggests that NF-κB activation may account for the increased hepatocyte proliferation. Consistent with our previous report (43), ethanol-feeding resulted in a significant reduction of apoptosis, as shown by decreased levels of cleaved caspase-3. The decreased apoptosis was associated with the activation of NF-κB in the livers of ethanol-fed rats. It is well known that NF-κB activation suppresses apoptosis, such that NF-κB targets various anti-apoptotic genes such as Bcl-X$_L$, c-IAP, and c-Flip (39,44,45). These data indicate that the activation of NF-κB, followed by induction of TNF-α, increased cell proliferation and decreased apoptosis in the ethanol pre-fed rats, thus contributing to DEN-initiated hepatic carcinogenesis.

The present study strongly supports that ethanol-induced over-expressed CYP2E1 is a critical mediator, possibly through oxidative stress, for ethanol-induced inflammatory response, cell proliferation, and reduced apoptosis related to hepatocarcinogenesis. This notion was supported by our observation that the CMZ treatment in ethanol-fed rats significantly blocked the ethanol-induced CYP2E1 expression, NF-κB activation, TNF-α mRNA expression, and cell proliferation. Although we observed a strong inhibition of the NF-κB activation by CMZ in the livers of ethanol-fed rats, the CMZ supplementation in ethanol-fed rats only partially restored the apoptosis to normal level as compared with the control group. This could be due to other mechanisms, such as the involvement of the JunD gene which is induced by CYP2E1 and ethanol and forms part of the transcription factor complex AP-1 (46) that may contribute to ethanol-decreased apoptosis (47). Importantly, we showed that CMZ supplementation not only inhibited the development of p-GST-positive hepatic foci in the short term but also decreased the incidence of hepatocellular adenoma that were induced by DEN in ethanol fed rats in a long term study.

It has been well documented that chronic alcohol consumption generates lipid peroxidation products such as malondialdehyde and 4-hydroxynonenal (4-HNE), and the latter binds to adenosine and cytosine forming highly carcinogenic exocyclic etheno DNA adducts (48,49).

These adducts have been identified in the liver of patients with alcoholic liver disease and other types of liver disease associated with inflammation and oxidative stress (22,50). In HepG2 cells overexpressing CYP2E1, we observed that these etheno-DNA adducts correlate significantly with CYP2E1 and 4-HNE and the adduct formation can be blocked by CMZ (22). Unexpectedly, in the present study, neither carcinogenic exocyclic etheno DNA adducts nor 4-HNE which is needed to form the adduct were changed among the treatment groups. In contrast, the oxidative DNA damage marker of 8-OHdG formation is clearly affected by ethanol and returned to almost normal with CMZ treatment. Since CYP2E1 induction forms reactive oxygen species (ROS) directly acting with DNA, it seems that the oxidative DNA damage reaction is favored compared to lipid peroxidation resulting in 4-HNE formation. The reason for this is not known, but may be related to the nitrosamine model used.

Retinoic acid is known to exert profound effects on cellular growth, differentiation, and apoptosis, thereby controlling alcohol-associated carcinogenesis (51). One of the observations in the present study is that the CMZ treatment not only prevented alcohol-promoted tumor formation but also restored reduced retinoic acid in the livers of rats after 10-months of treatment. These data were in agreement with our previous studies showing that alcohol-reduced levels of retinol and retinoic acid were prevented by CMZ, both *in vitro* and *in vivo*, indicating enhanced catabolism of vitamin A and retinoic acid by ethanol-induced CYP2E1 (19,20). This restoration of alcohol-reduced retinoic acid by CMZ provided further evidence supporting that the induction of hepatic CYP2E1 enzyme in chronic intermittent drinking is a factor in destroying retinol and retinoic acid. Further, the restoration of retinoic acid homeostasis by retinoic acid supplementation restored the normal status of both retinoid and MAPK signaling, thereby maintaining normal cell proliferation and apoptosis in the alcohol-fed animals (43,47). The observation that the CYP2E1 inhibitor can counteract the tumor-promoting action of ethanol by restoring normal hepatic levels of endogenous retinoic acid would have implications for the prevention of ethanol-promoted liver carcinogenesis, particularly regarding the detrimental effects of polar metabolites of vitamin A with alcohol feeding experiments (52). In summary, CYP2E1 inhibition may not only be an approach to prevent alcoholic liver disease, but also alcohol promoted hepatocarcinogenesis by restoring normal vitamin A levels.

Therefore, the use of a safe CYP2E1 inhibitor without side effects may provide complementary or synergistic protective effects against alcohol-related cancer risk.

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