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A Free Radical Scavenger, Edaravone, Attenuates Steatosis and Cell Death via Reducing Inflammatory Cytokine Production in Rat Acute Liver Injury

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Background/Aims: Reactive oxygen radicals play an important role in various forms of liver injury. In this study, we evaluated the efficacy of edaravone, a newly synthesized free radical scavenger, in its clinical dosage on an experimental model of acute liver injury in rats.

Methods: The clinical dose of edaravone (3 mg/kg) was intravenously administered immediately and 3 h after intraperitoneal administration of carbon tetrachloride (CCl_4) in rats. Histological evaluation including apoptosis and cytokine profiles were examined.

Results: Fatty degeneration and necrosis with marked elevation of serum alanine aminotransferase and lactate dehydrogenase levels developed after CCl_4 administration were significantly reduced by edaravone. In addition, the apoptotic index assessed by TUNEL method was significantly lowered in the edaravone treated group. Serum and liver transcription levels of interleukin-6, tumor necrosis factor-α, interleukin-4, and interleukin-10 were increased following CCl_4 administration, and they were attenuated by edaravone treatment. The formation of malondialdehyde, 4-hydroxynonenal adduct and one of the markers for oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine, was also inhibited by edaravone treatment.

Conclusion: Edaravone has a remarkable protective effect on acute liver injury caused by oxygen radicals through not only attenuating the membrane lipid peroxidation, but also inhibiting the production of inflammatory cytokines. We theorize that edaravone may have a clinical benefit in the treatment of various liver injuries.

Keywords: Edaravone; Carbon tetrachloride; Acute liver injury; Free radicals; Inflammatory cytokine; Lipid peroxidation

INTRODUCTION

Oxidative stress is associated with a number of pathological conditions.[1,2] It is well known that many forms of liver injury are caused by oxidative stress and subsequent free radical formation.[3,4] Therefore, inhibition of radical formation may be important in the regulation of liver injury.

Carbon tetrachloride (CCl_4) is a widely accepted *in vivo* experimental toxin that induces acute hepatic injury and regeneration. CCl_4 induces pericentral necrosis, fatty change and also apoptosis in the liver.[5] CCl_4 is metabolized by cytochrome P-450 to trichloromethyl radical ($CCl_3^{\bullet}$), which reacts with O_2 to form trichloromethylperoxyl radical ($CCl_3O_2^{\bullet}$). These reactive free radicals initiate cell damage through membrane lipid peroxidation.[3,4,6] During this process, a variety of aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are generated as final products, and there are several reports showing an increase in the hepatic level of MDA and 4-HNE in rats treated with CCl_4.[7,8] Previous reports have shown that an increased level of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker for oxidative DNA damage, is found in various inflammatory related diseases,[9,10] and *in situ* detection of 8-OHdG was demonstrated in chronic liver diseases including chronic hepatitis C, autoimmune hepatitis, alcoholic hepatitis, and primary biliary cirrhosis.[11]

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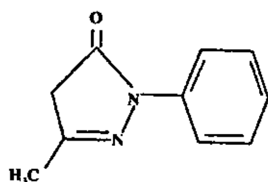

FIGURE 1 Chemical structure of edaravone, 3-methyl-1-phenyl-pyrazolin-5-one.

On the other hand, inflammatory cytokines such as TNF-α, IL-1β, and IL-6 also play an important role in pathophysiology of liver diseases.[12–15] These cytokines occur at elevated levels in various acute and chronic liver diseases. Recent investigations have shown that CCl_4 increases not only these pro-inflammatory cytokines but also anti-inflammatory cytokines such as IL-4 and IL-10.[16]

Hepatic antioxidants have been demonstrated to decrease the progression of liver injury induced by a single injection of CCl_4.[8,17,18] Endogenous vitamin E, a lipid-soluble antioxidant, increases at a progressive stage of liver injury, and it is important in protecting from oxidative damage. However, the lipophilic character of vitamin E causes slow incorporation into tissues and a non-realistic dosage is necessary for obtaining *in vivo* efficacy,[19,20] suggesting that vitamin E cannot be easily applied to acute hepatic injuries. Edaravone (3-methyl-1-phenyl-pyrazolin-5-one), a newly synthesized antioxidant (Fig. 1), inhibits both non-enzymatic peroxidation and lipoxygenase activity.[21,22] Furthermore, various animal models have suggested that edaravone has protective effects against cerebral and myocardial ischemia-reperfusion injuries.[23–25]

We therefore investigated the protective potential of edaravone during liver injury following CCl_4 administration in rats. To evaluate the oxidative stress we measured the level of MDA in liver homogenates and the levels of 4-HNE and 8-OHdG using an immunohistochemical technique, and to investigate the cytokines involved in the acute inflammatory response, we examined the gene expression of TNF-α, IL-4, IL-6, and IL-10 by RT-PCR.

MATERIALS AND METHODS

Animals and Drugs

Adult male Wistar rats (250 ± 20 g body weight) were housed in stainless steel cages with controlled temperature (22 ± 1°C) and a 12:12-h light–dark cycle. They were given standard laboratory diet and tap water during experiments.

Edaravone was a gift from Mitsubishi Wellpharma Incorporation (Tokyo, Japan). CCl_4 and olive oil was purchased from Kanto Kagaku Incorporation (Tokyo, Japan).

Treatment of Animals

This experiment was approved and performed under the guidelines of the animal experimentation committee of the school of medicine, Keio University. Acute liver injury was induced by intraperitoneal CCl_4 injection in the form of 50% CCl_4 solution in olive oil at a dose of 2 ml/kg. Rats were divided into three groups: a group with olive oil alone, a group injected with CCl_4 and saline, and a group injected with CCl_4 and edaravone. The edaravone (3 mg/kg) or saline was injected via the tail vein immediately after and 3 h after CCl_4 administration. The animals were sacrificed under ether anesthesia at each time point (6, 12, 24, and 48 h after CCl_4 injection), and blood was collected from the hearts and the liver was removed.

Blood Chemistry

Blood sample was obtained from the hearts at 6, 12, and 24 h after CCl_4 injection. Serum levels of alanine aminotranferase (ALT), total bilirubin (TB), and lactate dehydrogenase (LDH) were measured by using Dri-chem 3500i (Fuji Film Company, Tokyo, Japan).

Serum Cytokine Assays

Serum levels of TNF-α, IL-6, and IL-10 were measured by enzyme-linked immunoassay (ELISA) using a commercial available kit (Biosource International, Camarillo, CA, USA). Fifty microliter of test serum was applied to ELISA. The limits of sensitivity of TNF-α, IL-6, and IL-10 were 4, 8, and 5 pg/ml, respectively.

RNA Isolation and RT-PCR

The excised livers at 24 h after CCl_4 administration were frozen in liquid nitrogen and stored at −80°C until analysis. Total RNA was isolated from 10 mg samples using RNA isolation kit (Qiagen, Chatworth, CA). The quality of each RNA sample was tested by determining A 260/280 and the intactness of ribosomal bands. A total of 1.0 μg RNA was reverse-transcribed to complementary DNA (cDNA) as described previously.[26] Reaction mixtures were then subjected to the following; 1 min of denaturation at 94°C for 1 cycle, followed by 30–35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min using a PCR thermal cycler

TABLE I Oligonucleotide primer sequences for RT-PCR

TNF-α	Sense	5′-TACTGAACTTCGGGGTGATTGGTCC-3′
	Antisense	5′-CAGCCTTGTCCCTTGAAGAGAACC-3′
IL-4	Sense	5′-TCCATGCACCGAGATGTTTGTACC-3′
	Antisense	5′-GCAACTATTTCCCTCGTAGGATGC-3′
IL-6	Sense	5′-TTGCCGAGTAGACCTCATAGTGACC-3′
	Antisense	5′-CAAGAGACTTCCAGCCAGTTGC-3′
IL-10	Sense	5′-TGCCTTCAGTCAAGTGAAGAC-3′
	Antisense	5′-AAACTCATTCATGGCCTTGTA-3′
GAPDH	Sense	5′-CGGAGTCAACGGATTTGGTCGTAT-3′
	Antisense	5′-AGCCTTCTCCATGGTGGTGAAGAC-3′

(icycler, Biolad, Japan). Each of the primers was designed from a published mRNA sequence (Table I). The amplified PCR products (2 μl) were subjected to electrophoresis at 100 volts through 2% agarose gel. Primers for GAPDH, TNF-α, IL-4, IL-6, and IL-10 yielded products of 306, 295, 299, 614, and 346 bp. The relative amount of mRNA transcript was determined using Photoshop 5.0, and densitometric analysis of the captured image was performed using NIH image software. The content of each mRNA was semi-quantified using GAPDH content.

Hepatic MDA Measurement

Lipid peroxidation was determined by measuring MDA in liver homogenates using a commercially available kit (Lipid Peroxidation Assay Kit, Calbiochem, San Diego, CA). Hepatic tissues obtained 24 h after CCl_4 administration were used for the assay. Briefly, 0.2 ml of homogenate supernatant (1:10 w/v in 20 mM Tris–HCl, pH 7.4) was added to 0.65 ml of 10.3 mM *N*-methyl-2′-phenyl-indole in acetonitrile. After vortexing for 3–4 s and adding 0.15 ml HCl 37%, samples were mixed well and closed with a tight stopper and incubated at 45°C for 60 min. The samples were then cooled on ice and the absorbance was measured at 586 nm. The concentration of hepatic MDA was expressed as nmol/mg liver.

Histological Examination

The excised livers were immediately fixed with 10% neutral-buffered formalin, and they were cut into 5 μm sections after embedding in paraffin. Paraffin was extricated using xylene and ethanol, and subsequently stained with hematoxylin–eosin (H&E) and oil red O for morphological evaluation by light microscopy.

Immunohistochemistry for Detecting 4-HNE and 8-OHdG

Immunohistochemical analysis was performed using an avidin–biotin–peroxidase complex technique. For analysis of 8-OHdG, sections (4 μm) were autoclaved for 10 min at 121°C. The sections were successively treated with blocking solution and 0.625 μg/ml anti-8-OHdG monoclonal antibody (Nikken Foods Company, Shizuoka, Japan) or normal mouse IgG (Biogenesis, England) overnight at 4°C. After rinsing in PBS three times, the sections were incubated with biotinylated rabbit anti-mouse immunoglobulins for 60 min at room temperature (RT), followed by treatment with streptavidin peroxidase (CSA system, DAKO, Denmark). For analysis of 4-HNE, 0.625 μg/ml anti-4HNE monoclonal antibody (Nikken Foods Company, Shizuoka, Japan) was used. The specificity of these monoclonal antibodies was confirmed with an absorption test by adding free antigens in the incubation of each primary antibody on glass slides. The number of hepatocytes positive for 8-OHdG was quantified by counting 500 hepatocyte nuclei in five randomly chosen non-overlapping areas of each section. The percentage of the positive hepatocytes was used as the labeling index for 8-OHdG.

TUNEL Method

Apoptotic cell was detected by TUNEL method as described before with some modifications.[27] The sections were first deparaffinized and treated with proteinase K in PBS (μg/ml) for 15 min at RT. They were then treated with 0.3% H_2O_2 for 20 min and then incubated in TdT buffer (200 mM Potassium Cacodylate, 25 mM Tris–HCL pH 6.5, 0.25 mg/ml bovine serum albumin, 1 mM $CoCl_2$, 0.01 mM Biotin-16-dUTP, 1120 U/ml TdT) for 60 min at RT. After washing with PBS, the sections were reacted with VECSATIN Elite ABC kit (Vectoe Laboratories, Burlingame, CA) and incubated for 30 min at RT. Then they were stained with 0.025% 3-3′-diaminobenzidine tetrahydrochloride (DAB) in Tris–HCl buffer. The number of TUNEL positive hepatocytes was quantified by counting 500 hepatocyte nuclei in five randomly chosen non-overlapping areas of each section. The percentage of TUNEL positive hepatocytes was used as the apoptotic index.

Statistical Analysis

All data are expressed as mean ± SE. The differences between the mean of two groups were evaluated by

TABLE II Serum ALT and LDH activities and TB levels after CCl_4 administration

		Hours after CCl_4 administration		
		6 h ($n = 4$)	12 h ($n = 4$)	24 h ($n = 6$)
ALT (IU/l)	CCl_4 + saline	501.0 ± 94.2	926.0 ± 116.3	1630.6 ± 271.4
	CCl_4 + edaravone	111.5 ± 35.8**	125.0 ± 17.6***	119.4 ± 42.9***
	Olive oil	20.3 ± 1.2***	21.9 ± 1.4***	21.0 ± 1.1***
TB (mg/dl)	CCl_4 + saline	0.4 ± 0.1	0.5 ± 0.1	0.9 ± 0.2
	CCl_4 + edaravone	0.3 ± 0.1**	0.3 ± 0.1*	0.3 ± 0.1*
	Olive oil	0.3 ± 0.1**	0.3 ± 0.1*	0.4 ± 0.1*
LDH (IU/l)	CCl_4 + saline	4935.0 ± 886.3	3977.5 ± 326.4	5068.0 ± 1322.2
	CCl_4 + edaravone	870.3 ± 271.9**	499.8 ± 29.0***	369.7 ± 108.9**
	Olive oil	197.5 ± 14.5***	207.6 ± 17.2***	203.7 ± 12.5**

Note: ALT; alanine aminotransferase, TB; total bilirubin, LDH; lactate dehydrogenase. Upper data indicates CCl_4 + saline treated group. The middle data represents CCl_4 + edaravone treated group. Lower data is of the olive oil injected group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. CCl_4 + saline group.

the Kruskal-Wallis test. P value less than 0.05 was considered significant.

RESULTS

Serum ALT, LDH, and TB Values

In the CCl_4 treated group, the serum ALT and LDH values at each time were significantly higher than those in the control group (olive oil alone). The increase in the serum ALT, LDH, and TB values was significantly reduced by administration of edaravone (Table II).

TNF-α, IL-6, and IL-10 Levels in Serum

Figure 2 shows serial changes in serum TNF-α, IL-6, and IL-10 levels. In the CCl_4 treated group, serum

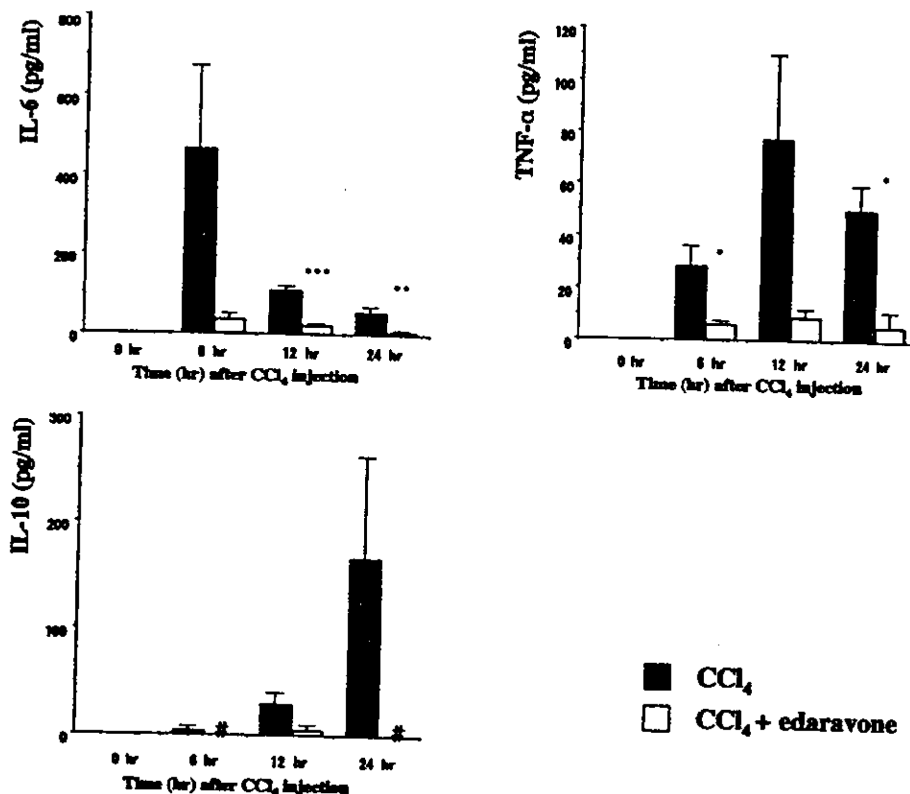

FIGURE 2 Sequential change of serum IL-6, TNF-α, and IL-10 levels after CCl_4, saline or edaravone treatment. Data represents means ± SE ($n = 3$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, compared with CCl_4 and saline treated group. #The result of the edaravone treated group was under the measurement limit.

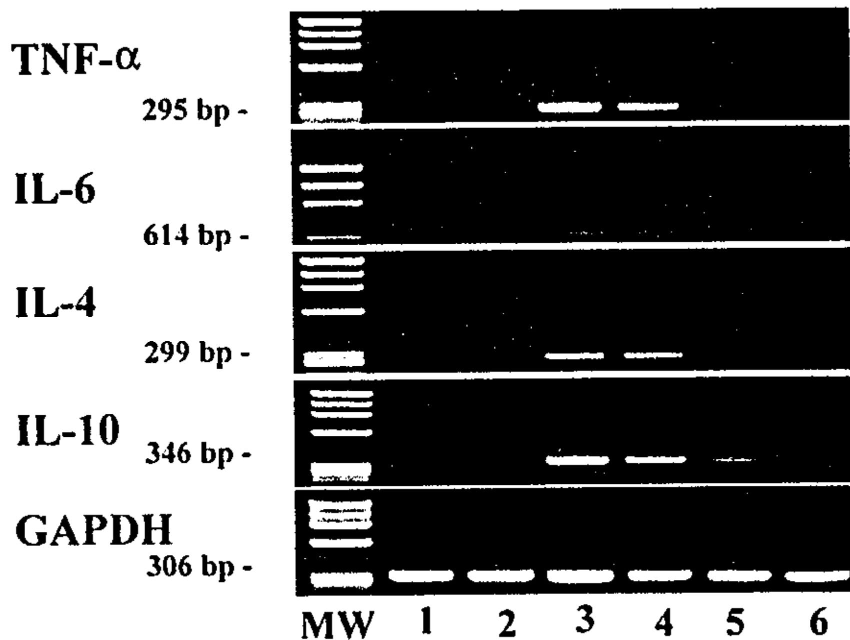

FIGURE 3 Detection of inflammatory cytokine transcripts in the liver by RT-PCR. Livers were excised 24 h after CCl_4 administration, and RNA was isolated as described in "Materials and Methods Section". MW; molecular weight markers (ϕ × 174/Hae III digest). Lane 1 and 2; control (olive oil i.p.) group, Lane 3 and 4; CCl_4 and saline treated group, Lane 5 and 6; CCl_4 and edaravone treated group. Samples shown are representative of four samples per group.

levels of IL-6 increased in its peak at 6 h after CCl_4 injection and declined at 12 and 24 h. TNF-α increased in its peak at 12 h. On the other hand, the level of IL-10 was below sensitivity limits at 6 h but markedly increased at 24 h. In the edaravone treated group, serum levels of TNF-α and IL-6 were almost attenuated at every time point. On the other hand, the attenuation of serum IL-10 level was not statistically significant, perhaps because the result of the edaravone treated group was under the measurement limit.

TNF-α, IL-6, IL-4 and IL-10 mRNA Expression in Liver Tissue

As shown in Fig. 3, mRNA expression of pro-inflammatory cytokines (IL-6 and TNF-α) and anti-inflammatory cytokines (IL-4 and IL-10) were significantly elevated in the CCl_4 treated rats in comparison with control rats 24 h after injection. Edaravone markedly suppressed these transcription levels. TNF-α was weakly expressed in control rats and its expression increased 24 h after CCl_4 administration. IL-4, IL-6, and IL-10 mRNA, however, was not transcribed in the control rats. Figure 4 shows the semi-quantitative analysis of these cytokines in the ratio to the densitometry of GAPDH expression determined in each four experiments. The ratio of TNF-α, IL-6, IL-4 and IL-10 mRNA transcription levels in the CCl_4 treated group was significantly increased compared with those in the control group, and edaravone significantly decreased these transcriptions.

Hepatic MDA Analysis

A significant increase in hepatic MDA production was found in the CCl_4 treated group compared with the control group 24 h after the injection. In the edaravone treated group, however, a significant reduction of MDA production was found (Table III).

Histological Examination

CCl_4 administration induced marked fatty degeneration in the liver 24 h after intraperitoneal injection (Fig. 5A), and moderate necrosis in zone 3 (pericentral area) was present with a little inflammatory cell infiltration (Fig. 5B). The steatosis and necrosis induced by CCl_4 was markedly reduced by the intravenous administration of edaravone (Fig. 5C and D). Oil red O staining, used for the detection of fat deposits, revealed fatty degeneration throughout the rat liver in the group given only CCl_4 (Fig. 6A). On the other hand, these fatty deposits were significantly decreased and patchy when edaravone was administered (Fig. 6B). The patchy fat deposits seen in the edaravone-treated group were present mainly in zone 2 (between periportal and pericentral area)

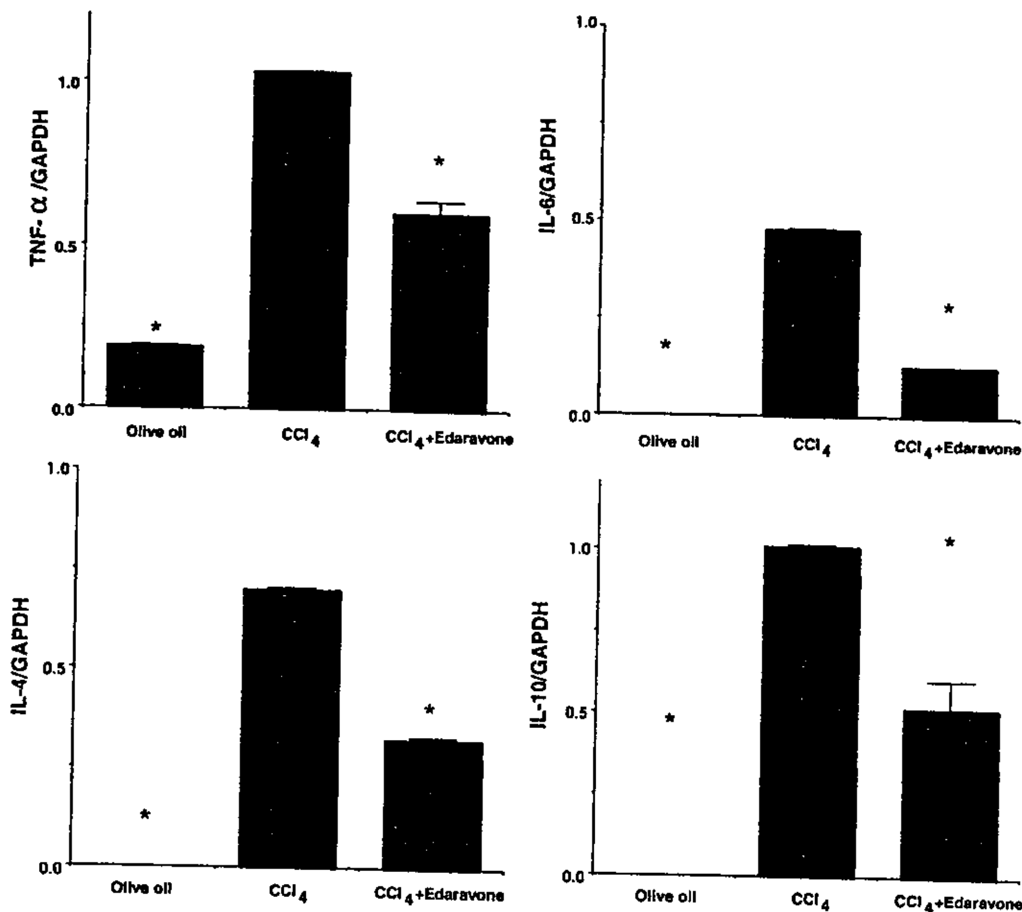

FIGURE 4 The semi-quantitative analysis of TNF-α, IL-6, IL-4, and IL-10 mRNA in the liver. Densitometric analysis of the bands between a cytokine mRNA and GAPDH is shown and the data shows the ratio from 6 different rats. Data represents means ± SE. $P < 0.001$, compared with CCl_4 and saline treated group.

and zone 3, and few were present in zone 1 (periportal area).

Immunohistochemical Study

Strong staining was detected around zones 2 and 3 with the anti-4-HNE monoclonal antibodies in the CCl_4 treated rats. The staining was detected mainly in the cytoplasm of hepatocytes and appeared in the form of small granules (Fig. 7A and B). In contrast, only slight staining was detected in the edaravone treated rats (Fig. 7C and D). In the control experiment, in which non-immune serum IgG was used instead of the anti-HNE antibody, no staining was observed. There were few hepatocytes with nuclear staining of 8-OHdG 24 h after CCl_4 administration (Fig. 8A), and the number of positive staining cells was not changed in the edaravone treated group (Fig. 8B). However, 48 h after CCl_4 administration, positive staining of 8-OHdG was found in the nuclei of hepatocytes (Fig. 8C), but few hepatocytes were stained in the edaravone treated group (Fig. 8D). The labeling index for 8-OHdG was significantly lower in the edaravone treated group than in the CCl_4 treated group at 48 h (CCl_4 + saline; 4.34 ± 0.51 vs. CCl_4 + edaravone; 0.12 ± 0.12, $P < 0.01$), but there was no significant difference at 24 h between two groups (Table IV).

TABLE III Hepatic MDA levels 24 h after drug administration

Treatment	Hepatic MDA (nmol/mg protein)
Control (saline) ($n = 6$)	0.18 ± 0.04
CCl_4 + saline ($n = 6$)	0.51 ± 0.16*
CCl_4 + edaravone ($n = 6$)	0.29 ± 0.09**

*$P < 0.01$ vs. control group, **$P < 0.05$ vs. CCl_4 + saline group.

TUNEL Method

Several TUNEL positive cells were detected in zones 2 and 3 at 24 h in the CCl_4 treated rats (Fig. 9A), and

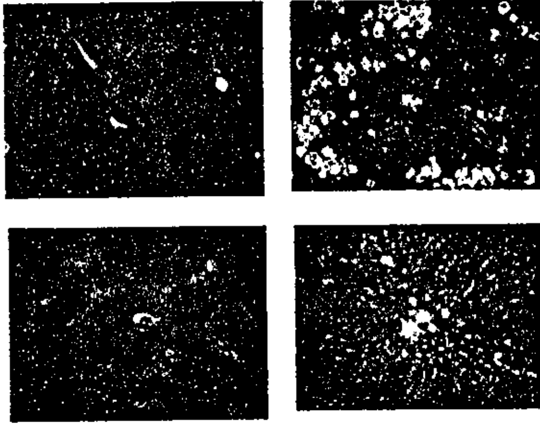

FIGURE 5 Histology of the rat liver 24 h after CCl_4 administration (H&E staining). (A, B) CCl_4 and saline treated group: (A) microvesicular fat deposits were observed, especially in zone 2; (B) moderate necrosis and inflammatory cell infiltration was detected in zone 3 (A × 40, B × 200). (C, D) CCl_4 and edaravone treated group; a dramatic decrease of fatty droplets and inflammation were seen (C × 40, D × 200).

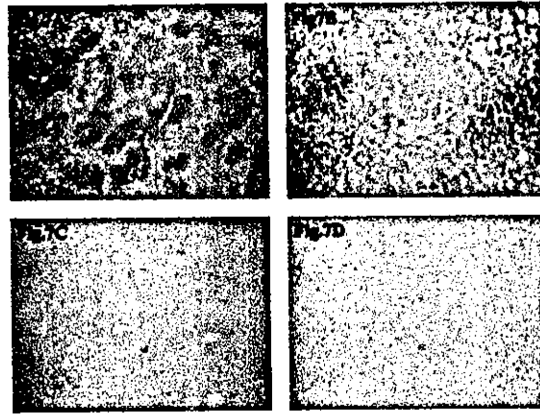

FIGURE 7 Immunohistochemical staining of 4-HNE in the liver sections of rats excised 24 h after CCl_4 administration. (A, B) CCl_4 and saline treated group; the staining was detected mainly in the cytoplasm of hepatocytes in the pericentral and zone 2 (A × 40, B × 200). (C, D) CCl_4 and edaravone treated group; only slight staining of 4-HNE was detected throughout the liver (C × 40, D × 200).

the number was increased in zone 3 at 48 h (Fig. 9B). On the other hand, a very few TUNEL-positive cells were observed in the edaravone treated group both at 24 (Fig. 9C) and at 48 h (Fig. 9D). The apoptosis index showed a significant difference between two groups (Table V).

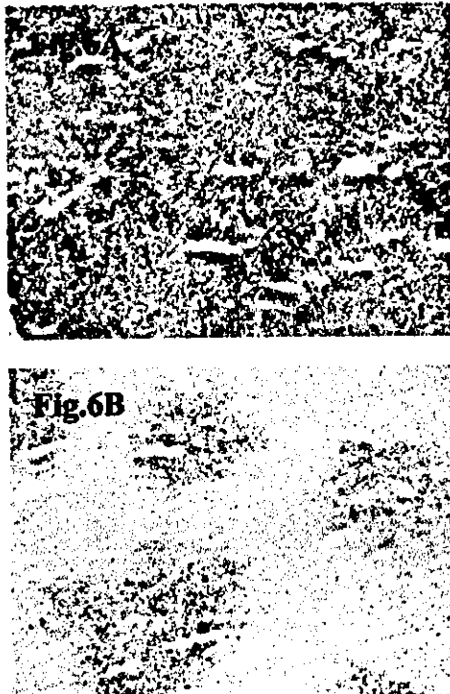

FIGURE 6 Histology of the rat liver 24 h after CCl_4 administration (Oil red O staining). (A) CCl_4 and saline treated group; diffuse fatty degeneration was observed throughout the liver. (B) CCl_4 and edaravone treated group; a significant reduction of fatty change was observed (A × 200, B × 100).

DISCUSSION

This study showed that edaravone attenuates CCl_4-induced acute liver injury in rats. In our study, rats were injected with 3 mg/kg edaravone. This dose was chosen on the basis of a clinical usage and previous reports showing a protective effect of edaravone on

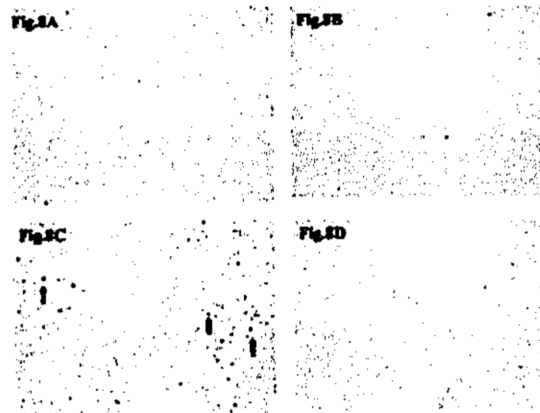

FIGURE 8 Immunohistochemical staining of 8-OHdG in the liver sections of rats excised after CCl_4 administration: (A) 24 h after CCl_4 and saline administration, (B) 24 h after CCl_4 and edaravone administration, (C) 48 h after CCl_4 and saline administration; positive staining was found in the nuclei of hepatocytes, black arrows indicate the nuclei stained with anti-8-OHdG, (D) 48 h after CCl_4 and edaravone administration; a few hepatocytes were stained with anti-8-OHdG, but the positive cell number was significantly decreased (A–D × 200).

TABLE IV Comparison of index of 8-OHdG between the saline and edaravone treated group

	Hours after CCl_4 administration	
	24 h	48 h
CCl_4 + saline	0.44 ± 0.19	4.34 ± 0.51
CCl_4 + edaravone	0.00 ± 0.00	0.12 ± 0.12**

**$P < 0.01$ vs. CCl_4 + saline group.

TABLE V Comparison of apoptotic index between the saline and edaravone treated group

	Hours after CCl_4 administration	
	24 h	48 h
CCl_4 + saline	4.06 ± 0.57	19.94 ± 1.58
CCl_4 + edaravone	1.14 ± 0.15*	2.68 ± 0.29**

*$P < 0.05$, **$P < 0.01$ vs. CCl_4 + saline group.

ischemia-induced brain damage.[23,24,28] In fact, edaravone has already been approved by the Japanese Ministry of Health, Labour and Welfare, and it is clinically used in stroke patients at the same dose as used in the present study and revealed great efficacy,[29] suggesting the possibility of expanding its indication to other diseases including acute liver injury.

Lipid peroxidation of the structural membrane is considered a critical target of the injury occurring after CCl_4-induced acute liver damage. CCl_4 is metabolized by CYP2E1 to form the trichloromethyl radical ($CCl_3^{\bullet}$), which readily reacts with oxygen to generate the trichloromethylperoxyl radical ($CCl_3O_2^{\bullet}$).[3,30] This reactive free radical is capable of removing hydrogen atoms from unsaturated lipids to form carbon-centered radicals. These lipid radicals inflict cell damage through the direct reaction with cell constituents or mitochondrial membrane phospholipids. Furthermore, hydroxyl radical ($OH^{\bullet}$) was also shown to participate in CCl_4 induced acute liver damage.[8] Previous reports showed the radical scavenging action such as hydroxyl radical ($OH^{\bullet}$) and peroxyl radical ($LOO^{\bullet}$) by edaravone *in vitro*.[31,32] A hypothetical radical scavenging pathway of edaravone is shown in Fig. 10. Edaravone exists in an anionic form (edaravone anion) at physiologic pH. An electron transfer from edaravone anion to free radicals yields edaravone radical, and this reaction breaks the chain oxidation of lipids. 2-oxo-3-(phenylhydrazone)-butanoic acid (OPB) is considered the final product of this pathway. By this radical scavenging action, edaravone may attenuate the CCl_4 induced acute liver injury. An alternative suggestion is that the mechanism of edaravone protection could be by decreasing the metabolic activation of CCl_4 by directly inhibiting P450. However, edaravone had no effect on this isozyme *in vivo*,[33] in which P-450 activity showed no significant change 24 h after repeated intravenous administration of edaravone at doses of 2 and 10 mg/kg/day for 7 or 21 days in rats.

During a process of the free radical formation, a variety of aldehydes such as MDA and HNE are generated.[6] Recently specific antibodies against HNE adducts have enabled a more direct and specific approach to detect these lipid peroxidation products.[34,35] 8-hydroxy-2′-deoxyguanosine (8-OHdG), a DNA modified product induced by reactive oxygen species is a mutation prone to alter G-C to T-A during DNA replication.[36] In the present study, we attempted to visualize the lipid peroxidation and oxidative DNA damage using the immunohistochemical analysis with a monoclonal antibody against HNE and 8-OHdG. In the rats 24 h after CCl_4 administration, strong staining was

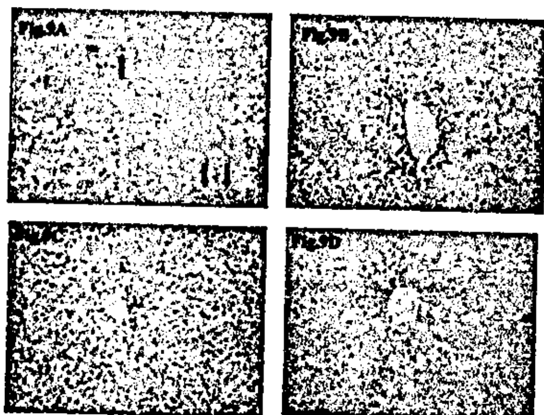

FIGURE 9 TUNEL staining of the liver after CCl_4 administration: (A) 24 h after CCl_4 and saline administration; several TUNEL-positive cells were detected in zones 2 and 3 (×200), black arrows indicate the TUNEL positive cell, (B) 48 h after CCl_4 and saline administration; the positive cell number increased and they were restricted in zone 3 (×200), (C) 24 h after CCl_4 and edaravone administration (×200), (D) 48 h after CCl_4 and edaravone administration (×200); very few TUNEL-positive cells were detected.

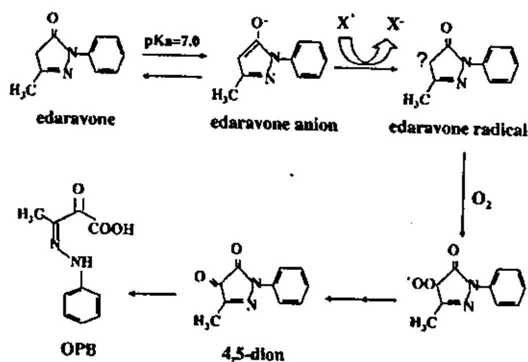

FIGURE 10 Radical scavenging mechanism of edaravone with free radicals.

detected with the anti-4-HNE antibody especially in pericentral areas. In contrast, only slight staining was detected in the edaravone-treated rats, lending credence to edaravone's role in suppressing free radical formation and subsequent lipid peroxidation. Furthermore hepatic MDA analysis quantitatively confirmed the result. The large amount of hepatic MDA produced by CCl_4 24 h after injection was significantly attenuated by edaravone treatment. Alternately, the number of hepatocytes with nuclei stained with 8-OHdG was significantly reduced by edaravone treatment at 48 h. Oxidative DNA damage in hepatocytes may be induced by chronic active inflammation through inflammatory cytokines, and continuous oxidative DNA damage may be one of the risk factors for hepatocarcinogenesis.[37,38] In this sense, edaravone may have a potential to suppress not only acute hepatic injury but also chronic injury and the development of liver cancer.

It is well known that pericentral necrosis is a major aspect of CCl_4-induced acute liver injury, and recent studies suggest that apoptosis, in addition to necrotic cell death, occurred after CCl_4 administration. In this study TUNEL assay was performed to detect apoptosis, and TUNEL positive cells were significantly reduced by edaravone administration compared with the CCl_4 treated group. Recently it is said that TUNEL positive cells can appear in both apoptosis and necrosis and they can be distinguished with each other,[5] on the other hand, this technique has been widely used to provide a sensitive and quantitative visualization of DNA fragmentation and apoptosis.[39–41] In the present study the degree of necrosis was not so severe after CCl_4 administration and most of the TUNEL positive cells were morphologically apoptotic, thus TUNEL assay was used as an index of apoptosis, although some portions of TUNEL positive cells might indicate necrosis. Anyway, it is obvious that edaravone can attenuate cell death including apoptosis and necrosis induced by CCl_4. The detailed mechanism of apoptosis induced by CCl_4 has not been clarified, mitochondrial damage caused by inhibition of cytochrome oxidase, Fas and Fas-ligand system, and the inflammatory cytokine release might be involved in the apoptotic process.

Numerous studies have demonstrated a close correlation between the levels of circulating pro-inflammatory cytokines and disease progression *in vivo* and *in vitro*.[8,15,16,42,43] Recent studies have shown increased levels of both pro- and anti-inflammatory cytokines in the liver of rats 24 h after CCl_4 intoxication,[16] suggesting these cytokines play a partial role in the acute phase liver damage. A characteristic feature of acute CCl_4 toxicity is a sudden increase in the number of non-parenchymal cells.[44] This increase is due to the recruitment of monocytes/macrophages from the bloodstream into the injured liver, resulting in the increased number of resident Kupffer cells. The cytokines produced by these newly recruited cells trigger a cascade of events leading to the onset of acute and fibrotic reactions.[45–47]

TNF-α and IL-6 have pleiotropic effects and are the major mediators of the acute phase response to inflammatory stimuli. Release of these cytokines might have been a component of the cell injury caused by chemicals such as CCl_4. The role of TNF-α in CCl_4-induced acute liver injury has been reported in studies showing a protective effect of soluble TNF-α antibodies.[14] On the other hand, the role of TNF-α and IL-6 as a modulator of cell proliferation and liver regeneration has been reported in other studies.[48,49] These various roles of the cytokine suggest the existence of very complicated cytokine cascades. IL-4 and IL-10 are potent Th2 cytokines, which inhibit the production of Th1 cytokines. The inhibitory effects of the Th2 cytokines on Th1 cytokine production and liver injury have been demonstrated in experimental models of acute and chronic liver injury.[50,51] In particular, IL-10 is a key cytokine in the inhibition of liver injury. Thompson *et al.*, concluded IL-10 was capable of down-regulating various aspects of pro-inflammatory macrophage function.[51] We investigated the levels of these cytokines in the serum and liver tissue. In the CCl_4 group, serum IL-6 levels were elevated in its peak at 6 h, serum TNF-α at 12 h, and serum IL-10 at 24 h, and mRNA levels of IL-6, TNF-α, IL-10, and IL-4 were increased in liver tissues after 24 h. Edaravone significantly attenuated the serum level of IL-6 and TNF-α, though the reduction of serum IL-10 was not statistically significant. In liver tissues, on the other hand, the expressions of all the cytokines investigated were significantly inhibited by edaravone. We cannot fully assess the reduction of anti-inflammatory cytokines such as IL-4 and IL-10, but one possibility is edaravone's direct anti-inflammatory effect. Secondary, the reason because anti-inflammatory cytokine levels decrease may be due to the inhibition of the liver injury caused by edaravone. Anyway, edaravone has a dramatic anti-inflammatory effect in the aspect of inflammatory cytokine profiles.

Further study is needed to clarify the mechanism between radical formation and inflammatory cytokine release and pathological change such as necrosis and apoptosis in the acute phase response to CCl_4 injection. Other factors such as chemokines may be involved in this liver injury. It is important to clarify in which phase and what signal edaravone suppresses. Moreover, since kupffer cells are activated after CCl_4 injection and toxic cytokines and free radicals are released,[52] further investigation on kupffer function in this model may shed light on the acute hepatic injury and its sequential regeneration.

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ETHANOL-INDUCED LIVER INJURY

Low-dose ethanol attenuates gut ischemia/reperfusion-induced liver injury in rats via nitric oxide production

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Abstract

Background and Aims: The acute administration of low-dose ethanol was demonstrated to attenuate liver injury elicited by gut ischemia/reperfusion (I/R). Nitric oxide (NO) has been found to be a modulator of adhesive interactions between leukocytes, platelets, and endothelial cells, but there has been much controversy about the effects of ethanol on NO regulation. The objective of this study was to investigate the role of NO in ethanol-reduced hepatic microvascular dysfunction elicited by gut I/R.

Methods: Male Wistar rats were exposed to 30 min of gut ischemia followed by 60 min of reperfusion. Intravital microscopy was used to monitor leukocyte recruitment and non-perfused sinusoids (NPS). Plasma alanine aminotransferase (ALT) activities were measured 6 h after the onset of reperfusion. In another set of experiments, ethanol (10%, 1 g/kg) was administered before ischemia.

Results: Gut I/R elicited increases in the number of stationary leukocytes, NPS, and plasma ALT activities; all of which were attenuated by pretreatment with ethanol or an NO donor. Gut I/R caused the apoptosis of hepatocytes, which was prevented by pretreatment with ethanol. Pretreatment with an NO synthase inhibitor diminished the protective effects of ethanol. The administration of ethanol increased plasma nitrite/nitrate levels.

Conclusion: These results suggest that low-dose ethanol attenuates the gut I/R-induced hepatic microvascular dysfunction and sequential liver injury by increasing sinusoidal NO levels.
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Key words: intravital microscope, nitric oxide synthase, tissue hypoxia.

INTRODUCTION

Nitric oxide (NO) has been found to be a modulator of adhesive interactions between leukocytes, platelets, and endothelial cells,[1–4] as well as an important modulator of tissue blood flow, arterial pressure, and neurotransmission.[3] These NO-dependent cell–cell interactions have been demonstrated in tissues exposed to ischemia and reperfusion (I/R), an injury process in which leukocyte–endothelial cell adhesion plays a critical role. A role of NO in the pathobiology of I/R injury is supported by observations that inhibition of NO biosynthesis elicits most of the microvascular alterations observed in tissues exposed to I/R.[2,5] Nitric oxide-donating compounds have been shown to provide significant protection against the microvascular dysfunction that is normally associated with I/R.[2] We developed a leukocyte-dependent model of the hepatocellular dysfunction that is elicited by gut I/R.[6,7] This murine model allows for the *in vivo* assessment of the effects of I/R on leukocyte sequestration in sinusoids of different regions of the liver lobule, leukocyte adherence in postsinusoidal venules, and the number of perfused sinusoids. We have recently used this model to demonstrate that either inhibition of NO synthase or supplementation with exogenous NO affects the leukocyte rolling, leukocyte adhesion, and sinusoidal perfusion elicited in the liver by gut I/R.[8,9]

Ethanol is known to have potentiality for causing hepatic microcirculatory disturbances.[10,11] It has a dual effect on vascular smooth muscle: in some blood vessels, it increases vascular tone, while in others, it relaxes

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vascular smooth muscle.[12] Ethanol has been reported to relax pulmonary arteries by releasing NO,[13] and in perfused liver, 25 mmol/L or more of ethanol has been reported to increase the production of endogenous NO, which was found to reduce ethanol elevated portal pressure.[11] This evidence supports the hypothesis that the ethanol-induced increase in NO levels reduces or compensates for the ethanol-induced hepatic microcirculatory disturbance. As NO can modulate leukocyte– and/or platelet–endothelial cell interactions,[1–4,8,9] it appears to have a more important role in *in vivo* inflammatory responses. Ethanol has also been reported to modulate I/R-induced tissue injury.[14,15] In a perfused liver model, ethanol enhanced I/R-induced hepatotoxicity (an increase in blood levels of liver enzymes) by enhanced production of reactive oxygen species.[14] In an *in vivo* gut I/R model, it also enhanced gut I/R-induced neutrophil accumulation in the intestinal wall.[16] In an *in vivo* cerebral I/R model, however, ethanol pretreatment was reported to reduce cerebral I/R injury,[15] and we have recently reported that low-dose ethanol attenuates the gut I/R-induced hepatic microvascular dysfunction in the midzonal region and sequential hepatocellular injury, whereas high-dose ethanol enhances hepatic microcirculatory disturbance in the pericentral region and sequential hepatocellular injury.[17]

Thus, while there has been controversy about the effect of ethanol on I/R-induced tissue injury, because there is a growing body of evidence that NO attenuates I/R injury,[1,2,5,8,9] the ethanol-induced increase in NO levels appears to affect I/R injury. Nonetheless, little is known about the effect of ethanol-increased NO levels on I/R injury *in vivo*.

In this study, we investigated the role of NO in gut I/R-induced hepatic microvascular dysfunction and sequential liver damage (hepatocellular injury) attenuated by low-dose ethanol, in the presence of altering the bioavailability of nitric oxide induced by inhibiting NO synthase.

METHODS

Animals and surgical procedure

Male Wistar rats (250–300 g) were fed standard rat chow and fasted for 18 h prior to the experiment. The rats were anesthetized with pentobarbital sodium (35 mg/kg) intraperitoneally. The left carotid artery was cannulated, and a catheter was positioned in the aortic arch to monitor the blood pressure. The left jugular vein was also cannulated for drug administration. All experiments were performed according to the criteria outlined in the USA National Research Council Guide.

Intravital microscopy

After laparotomy, one lobe of the liver was observed with an inverted intravital microscope (TMD-2S; Diaphoto, Nikon, Tokyo, Japan), assisted by a silicon intensified target camera (C-2400–08; Hamamatsu Photonicus, Shizuoka, Japan). The liver was placed on an adjustable Plexiglas microscope stage with a non-fluorescent coverslip that allowed for observation of a 2-cm^2 segment of tissue. The liver was carefully positioned to minimize the influence of respiratory movements, and the surface of the liver was moistened and covered with cotton gauze soaked with saline. Images of the microcirculation were observed on the surface of the liver through a ×20 fluorescent objective. Microfluorographs were recorded on videotape with a videocassette recorder (S-VHS-HQ; Victor, Tokyo, Japan).

Analysis of leukocyte accumulation and sinusoidal perfusion

Leukocytes were labeled *in vivo* with rhodamine-6G (1 mg dissolved in 5 mL of 0.9% saline) by using a previously described method[6] that was based on a method used in studies involving rat brains.[18,19] Rhodamine-6G has been shown to selectively stain white blood cells and platelets, but not endothelial cells.[19] Thus, the fluorochrome allows for differentiation between adherent leukocytes and endothelial cells. Rhodamine-6G (0.2 mL/100 g bodyweight) was injected prior to ethanol administration, with subsequent injections every 30 min, and rhodamine 6G-associated fluorescence was visualized by epi-illumination at 510–560 nm by using a 590-nm emission filter (Nikon). We selected one of the lobules that had well-perfused sinusoids and the fewest stationary leukocytes, and whenever all the conditions appeared to be equivalent, we chose the lobule farthest from the edge of the liver. Microfluorographs of the hepatic microcircula-tion, namely rhodamine-6G-labeled leukocytes in the sinusoids, were consecutively observed for 90 min after superior mesenteric artery occlusion and recorded on a digital video recorder (S-VHS-HQ) for 1 min at 0, 30, 60, and 90 min. The number of stationary leukocytes was determined off-line during playback of the videotape images. A leukocyte was considered stationary within the microcirculation (sinusoids) if it remained stationary for more than 10 s. The lobule that had the fewest stationary leukocytes was selected for observation as the baseline condition. Stationary leukocytes were quantified in both the midzonal and pericentral regions of the liver lobule and expressed as the number per field of view ($2.1 \times 10^5\ \mu m^2$). The percentage of non-perfused sinusoids was calculated as the ratio of the number of non-perfused sinusoids to the total number of sinusiods per field of view.

Alanine aminotransferase activity assay

Blood plasma samples were collected from the inferior vena cava 6 h after the onset of reperfusion. Alanine aminotransferase (ALT) activity was determined from the plasma samples by using a spectrophotometric assay obtained as a commercial kit (Sigma, St Louis, MO, USA).

Experimental protocols

We observed the surface of the liver for 10 min before ligation of the superior mesenteric artery in order to ensure that all parameters measured online were in a steady state. The superior mesenteric artery was then ligated with a snare created from polyethylene tubing for 0 (sham) or 30 min. After the ischemic period, the ligation was gently removed. Leukocyte accumulation and the number of non-perfused sinusoids were measured before ischemia, immediately following reperfusion, and every 15 min for 1 h thereafter. In one set of experiments, the rats were divided into four different groups, and the following were administered 30 min before inducing ischemia: group 1, saline, intragastrically (as control); group 2, 10% ethanol (1 g/kg; 10 mL/kg, intragastrically); group 3, saline, p.o., and an NO donor (FK409; Fujisawa, Tokyo, Japan; 0.6 mg/kg vorus + 0.6 mg/kg per hour for 1 h after the onset of ischemia); group 4, saline, p.o., and an NO synthase (NOS) inhibitor, N^G-monomethyl-L-arginine (L-NMMA; Sigma; 0.5 mg/kg, i.v); and group 5, 10% ethanol (1 g/kg, intragastrically) and L-NMMA (0.5 mg/kg, i.v.). These experiments were performed in control rats (sham gut I/R), and after gut I/R, with five or six animals in each group.

Assessment of apoptosis

Apotolic hepatocytes were detected by using the terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labeling technique. Apoptosis was estimated by the number of apoptotic hepatocytes.

Nitrite and nitrate assay

Blood plasma samples were collected from the inferior vena cava 45 min after the administration of saline (as a control) or 10% ethanol (1 g/kg; 10 mL/kg, intragastrically). The combined levels of nitrite and nitrate in plasma were determined by using a previously reported method.[20] Five experiments were performed.

Statistical analysis

The data were analyzed by using standard statistical analyses, that is, one-way ANOVA and Scheffe's (post hoc) test. All values are reported as mean ± SEM, with five rats per group. Statistical significance was assumed at $P < 0.05$.

RESULTS

Table 1 compares leukostasis and sinusoidal perfusion responses (at 90 min) of rats to the administration of 0.5 mg/kg of L-NMMA, which was selected on the basis of studies in the literature.[8] The changes in leukocyte accumulation that occur in the sinusoids of the midzonal and pericentral (including the terminal hepatic venule (THV)) regions of the liver lobule, and in the entire liver lobule (sinusoids + THV) after exposure of the gut to ischemia and 60 min reperfusion are also shown. In control rats, gut I/R elicited significant increases in the number of stationary leukocytes and non-perfused sinusoids (NPS), when compared with basal values. While the 0.5 mg/kg dose of L-NMMA did not elicit significant changes in any of the measured variables, which is consistent with a previous report,[8] it aggravated the leukostasis elicited by gut I/R in the pericentral region (8.3 ± 1.2 I/R *vs* 13.6 ± 0.7 I/R + L-NMMA, per field). The NOS inhibitor also increased the total number of stationary leukocytes (20.8 ± 0.9 I/R *vs* 27.4 ± 1.4 I/R + L-NMMA) and the percentage of NPS (20.4 ± 1.1% I/R *vs* 33.0 ± 0.8%, I/R + L-NMMA). These results were also consistent with a previous report on a study in mice.[8]

Figure 1 illustrates the effects of ethanol (1 g/kg) and/or L-NMMA on the gut I/R-induced leukostasis in sinusoids of the midzonal and pericentral (including the THV, panel (a)) regions of the liver lobule, and the entire liver lobule (sinusoids + THV, panel (b)). The L-NMMA aggravated the leukostasis elicited by gut I/R in the pericentral region, as described previously. Pretreatment with the low-dose ethanol blunted the gut I/R-induced leukostasis in the midzonal region (I/R: 12.0 ± 0.7, I/R + low-dose ethanol: 6.0 ± 0.5 per field), and L-NMMA diminished the protective effects of low-

Table 1 Effects of gut ischemia/reperfusion and/or L-NMMA on stationary leukocytes and non-perfused sinusoids in rats

	Stationary leukocytes (per field) (%)	Non-perfused sinusoids	Midzonal pericentral	Total
Control	1.6 ± 0.5	1.0 ± 0.6	2.6 ± 0.8	3.7 ± 1.4
L-NMMA	2.0 ± 0.4	2.0 ± 0.3	4.0 ± 0.7	5.0 ± 1.3
I/R	12.5 ± 0.7*	8.3 ± 1.2*	20.8 ± 0.9*	20.4 ± 1.1*
I/R + L-NMMA	13.8 ± 0.8*	13.6 ± 0.7*,**	27.4 ± 1.4*,**	33.0 ± 0.8*,**

*$P < 0.05$ versus control, **$P < 0.05$ versus I/R. I/R, ischemia/reperfusion; L-NMMA, NG-monomethyl-L-arginine, a nitric oxide synthase inhibitor.

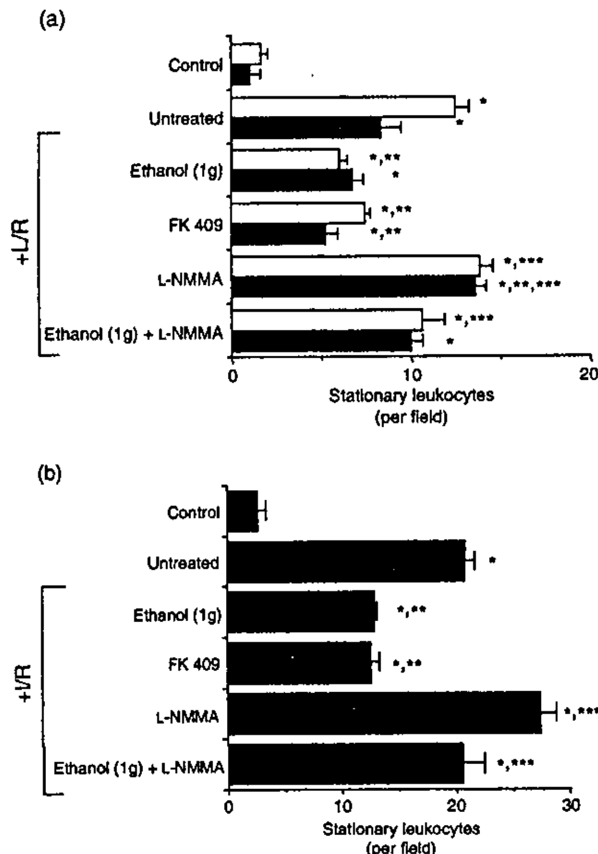

Figure 1 Effects of ethanol (1 g/kg) and/or an nitric oxide synthase inhibitor (L-NMMA) on the number of stationary leukocytes in each region (the (□) midzonal and (■) pericentral regions) (a) and the entire (combined) liver lobule (b) after 30 min of gut ischemia (I) and 60 min of reperfusion (R). The numbers of animals in each experimental group are controls = 6, I/R = 6, FK409 (an NO donor; Fujisawa, Tokyo, Japan) = 5, each group with ethanol and/or L-NMMA = 5. *$P<0.05$ versus control, **$P<0.05$ versus I/R group, ***$P<0.05$ versus ethanol group.

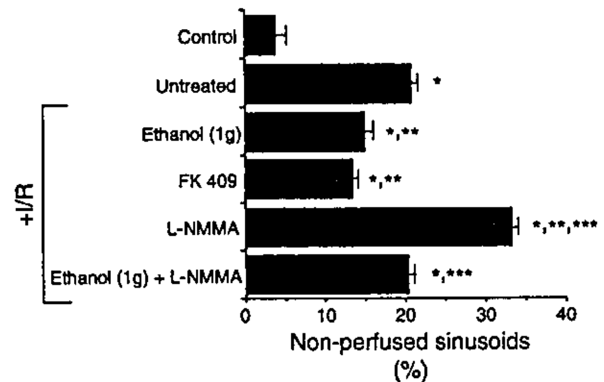

Figure 2 Effects of ethanol (1 g/kg) and/or an nitric oxide synthase inhibitor (L-NMMA) on the percentage of non-perfused sinusoids in a mouse liver at 60 min after gut ischemia/reperfusion (I/R). The numbers of animals in each experimental group are controls = 6, I/R = 6, FK409 = 5, each group with ethanol and/or L-NMMA = 5. *$P<0.05$ versus control, **$P<0.05$ versus I/R group, ***$P<0.05$ versus ethanol group.

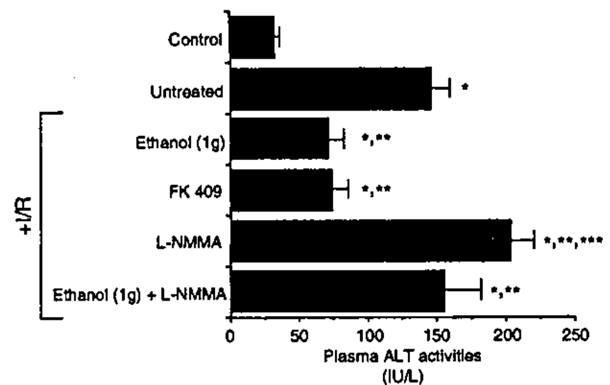

Figure 3 Effects of ethanol (1 g/kg) and/or an nitric oxide synthase inhibitor (L-NMMA) on plasma alanine aminotransferase (ALT) activities 6 h after gut ischemia/reperfusion (I/R). The numbers of animals in each experimental group are controls = 6, I/R = 6, FK409 = 5, each group with ethanol and/ or L-NMMA = 5. *$P<0.05$ versus control, **$P<0.05$ versus I/R group, ***$P<0.05$ versus ethanol (1 g/kg) group.

dose ethanol (leukostasis in the midzonal region: 10.6 ± 1.3 per field). Treatment with FK409 blunted the gut I/R-induced leukostasis in the liver.

Figure 2 shows the effects of ethanol (1 g/kg) and/or L-NMMA on the gut I/R-induced increase in the percentage of NPS. The L-NMMA aggravated the gut I/R-induced increase in percentage of NPS as described previously. Pretreatment with low-dose ethanol blunted the gut I/R-induced increases in NPS (I/R: 20.4 ± 1.1%, I/R + low-dose ethanol: 14.7 ± 1.1%). The L-NMMA diminished the protective effect of low-dose ethanol (NPS: 20.2 ± 0.8%). Treatment with FK409 blunted the gut I/R-induced increases in NPS.

Figure 3 shows the effects of ethanol (1 g/kg) and/or L-NMMA on the gut I/R-induced elevation of plasma ALT activities. The L-NMMA aggravated the gut I/R-induced elevation of plasma ALT activities as described previously. Pretreatment with the low-dose ethanol blunted the gut I/R-induced elevation of plasma ALT activities, and L-NMMA diminished the protective effects of low-dose ethanol. Treatment with FK409 blunted the gut I/R-induced elevation of plasma ALT activities.

Figure 4 shows the gut I/R-induced apoptosis of hepatocytes. Gut I/R caused the apoptosis of hepatocytes (panel (a)), and pretreatment with low-dose ethanol reduced the number of apoptotic hepatocytes (panel (b)).

Table 2 demonstrates the effect of ethanol (1 g/kg) on plasma nitrite/nitrate levels. The administration of ethanol elevated the plasma nitrite/nitrate levels.

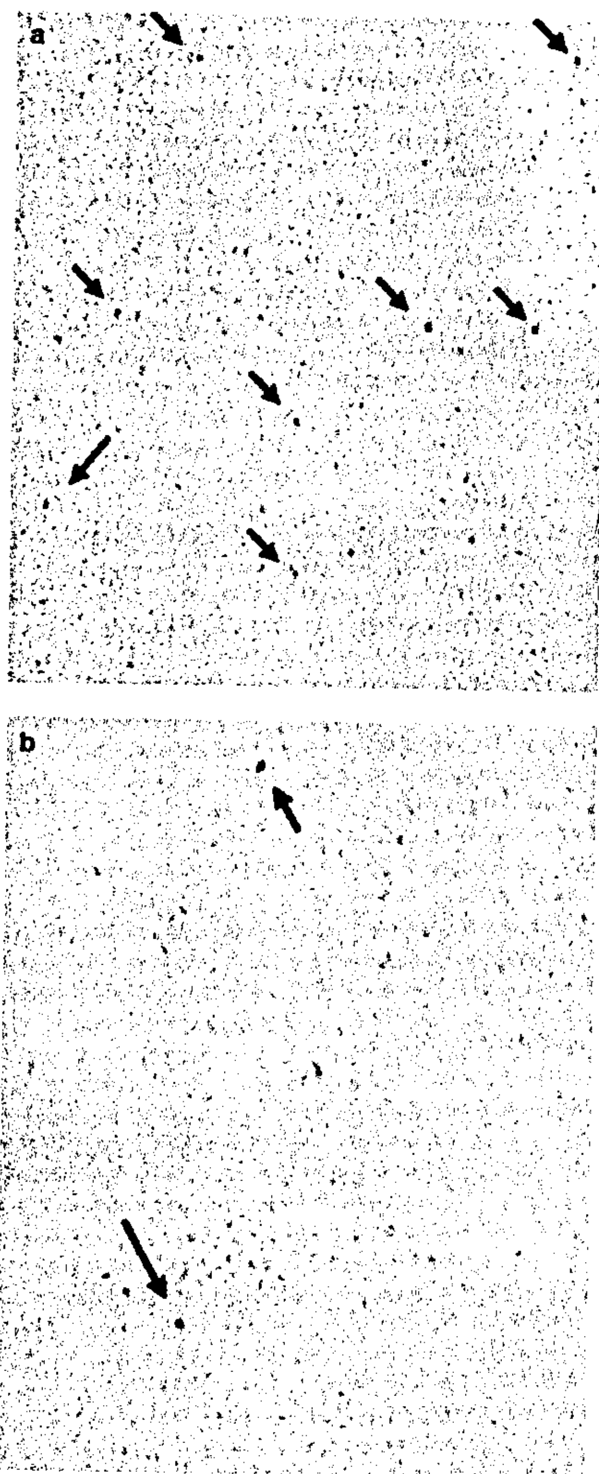

Figure 4 Histological assessment of apoptosis in the rat liver after gut ischemia/reperfuion by using the terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labeling technique. (a) Control (untreated) + I/R, (b) pretreated with low-dose ethanol. Arrows indicate DNA fragmentation in hepatocytes (indicating the apoptosis of hepatocytes).

Table 2 Effects of ethanol administrationon plasma nitrite/nitrate levels in rats

	Nitrite/nitrate levels (μM)
Control	17.1 ± 1.2
EtOH (1 g/kg)	27.0 ± 0.8*

*$P < 0.05$ versus control. EtOH, ethanol.

DISCUSSION

Previously published work has demonstrated that reperfusion of the ischemic small intestine elicits an acute inflammatory response both in the intestine and in distant organs, such as the liver[6,7,21] and lung.[21,22] The response in the liver is characterized by the leukocyte plugging of sinusoids, leukocyte adherence in postcapillary venules, a reduction in the number of perfused sinusoids, hepatocellular hypoxia, and leakage of enzymes (ALT) from hepatocytes.[6,7,21,23] The dependence of this hepatic microvascular dysfunction and hepatocellular hypoxia/injury on leukocyte sequestration is evidenced by the improved sinusoidal perfusion and blunted hepatocellular hypoxia/injury responses to gut I/R observed in mice that are genetically deficient in one of a variety of adhesion glycoproteins, including intercullular adhesion molecule-1, P-selectin, and CD11/CD18.[6,7] By using this model, we have recently demonstrated that low-dose ethanol attenuates the gut I/R-induced hepatic microvascular dysfunction in the midzonal region and subsequent hepatocellular injury, and that high-dose ethanol aggravates the hepatic microcirculatory disturbance in the pericentral region and sequential hepatocellular injury.[17]

Depletion and/or inactivation of NO has been implicated as a key event in the recruitment of leukocytes in tissues exposed to I/R.[24–26] Several types of cell are known to produce NO, including endothelial cells,[3] macrophages,[3] neurons,[27] and neutrophils.[3] In the liver, for example, three different resident cell populations have the ability to generate NO: Kupffer cells,[28] hepatocytes,[3] and endothelial cells.[3] Ethanol has been found to increase production of endogenous NO in perfused liver and the increase reduced ethanol-elevated portal pressure.[11] This evidence supports the hypothesis that the ethanol-elevated NO levels reduce or compensate for the ethanol-induced hepatic microcirculatory disturbance. Indeed, as NO can modulate leukocyte- and/or platelet-endothelial cell interactions,[1–4,8,9] ethanol-elevated NO levels in the liver appear to have a more important role in *in vivo* hepatic inflammatory responses. We therefore decided to investigate whether the manipulation of hepatic NO levels (NOS inhibition with L-NMMA) alters: (i) the leukostasis and leukocyte-dependent hepatic microvascular dysfunction observed in the liver after gut I/R; and (ii) the effect of ethanol on these responses.

There have been several reports on the effect of ethanol on NO production. In an *in vitro* study, ethanol

was reported to attenuate the LPS-induced expression of inducible NOS (iNOS) in Kupffer and hepatic endothelial cells.[29] By contrast, 25 mmol/L ethanol and higher concentrations, which reduces ethanol-elevated portal pressure, seem to increase the production of endogenous NO in perfused liver.[11] A likely explanation of the different effects of ethanol on NO production between *in vitro* and *ex vivo* studies is that ethanol has different effects on constitutive (endothelial) NOS (cNOS) and iNOS. The isoform of NOS that contributes to these responses in the liver remains unclear, however, because induction of NOS takes several hours,[3] cNOS appears to be a more likely candidate to explain the inflammatory responses observed in our *in vivo* model. Indeed, in the present study, administration of ethanol significantly elevated plasma nitrite/nitrate levels, but to less than twice their initial values. Although the significant increase in NO production by ethanol supports involvement of NO in the protective effects of ethanol, the small increase in NO production suggests the involvement of cNOS, not iNOS, in the responses.

From a microcirculatory stand point, the ethanol-elevated sinusoidal NO level appears to play a protective role in hepatic microcirculation. In the present study, treatment with L-NMMA resulted in exaggerated leukostasis and cellular injury in the rat liver after gut I/R. These results are consistent with our previous study in mice,[8] and support a protective role of NO in the rat liver. As ethanol increases NO production, as described previously, the ethanol-elevated NO levels are able to reduce or compensate for the gut I/R-induced hepatic microcirculatory disturbance. Our findings in the present study, that treatment with L-NMMA diminished the protective effect of ethanol on the gut I/R-induced leukostasis and microvascular dysfunction in the liver, support the hypothesis that ethanol-elevated sinusoidal NO levels play a protective role in hepatic microcirculation.

In the present study, pretreatment with L-NMMA resulted in exaggerated cellular injury (elevation of plasma ALT activities) in the rat liver after gut I/R, while pretreatment with an NO donor prevented the hepatocellular injury. These results are also consistent with our previous study in mice,[8] and support a protective role of NO in rat liver. Furthermore, pretreatment with L-NMMA diminished the protective effect of ethanol on gut I/R-induced hepatocellular injury (the elevation of plasma ALT levels).

These results indicate a protective effect of NO on gut I/R-induced liver injury in rats as well as in mice, and they suggest that ethanol prevents the gut I/R-induced liver injury in rats via NO production.

In the present study, gut I/R caused mild (not significant) accumulation of neutrophils in the liver after gut I/R. However, gut I/R caused apoptosis of hepatocytes, and pretreatment with low-dose ethanol reduced the gut I/R-induced apoptosis of hepatocytes. This result suggests that apoptosis, not necrosis, may be involved in the gut I/R-induced elevation of ALT activities. Nitric oxide is reported to act as a potent inhibitor of apoptosis in the liver.[30] This evidence supports our hypothesis that low-dose ethanol-elevated NO levels prevent the gut I/R-induced hepatocellular injury via an inhibition of hepatocyte apoptosis.

Ethanol, per se, is reported to increase oxygen consumption, and to enhance oxidative stress via the induction of cytochrome P450 2E1.[31] Moreover, it can produce a substantial amount of oxygen-free radicals in the hepatic sinusoids.[32] As NO can bind to oxygen-free radicals, it may act as a radical scavenger. Therefore, although NO has a protective effect on sinusoidal adhesive interactions, the inhibition of oxygen radical formation by NO may underlie the protective effects of low-dose ethanol on the gut I/R-induced leukostasis and microvascular dysfunction in the liver. Ethanol has some protective effects on the liver perfusion, such as an increase in red blood cell velocity in the sinusoids.[10] Thus, some agents other than NO elevation may be involved in the cytoprotective effects of ethanol. However, results in the present study demonstrated the role of NO in the cytoprotective effects of ethanol on gut I/R-induced liver injury, and represent the therapeutic usefulness of NO elevation for reperfusion injury.

ACKNOWLEDGMENTS

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Editorial

Common pathogenic mechanisms in ASH and NASH

Ludwig et al. [1] introduced the term nonalcoholic steatohepatitis (NASH) to describe a form of liver disease that is histologically indistinguishable from alcoholic hepatitis but occurs in persons who do not consume excessive alcohol. Nonalcoholic fatty liver disease (NAFLD) is an increasingly recognized condition that may progress to end-stage liver disease. Current best estimates make the prevalence of NAFLD (approximately 20%) and of NASH (2–3%) in the general population and NAFLD is perhaps the most common of all liver disorders [2]. The similar histological features and natural histories of alcoholic and nonalcoholic fatty liver suggest that common pathogenic mechanisms might be involved in these two conditions. In contrast to NASH, the pathogenesis of alcoholic steatohepatitis (ASH) have been well studied. Understanding the pathogenesis of alcoholic fatty liver disease (AFLD) should contribute to elucidating the pathogenesis of NAFLD.

It has been shown that the induction of cytochrome P4502E1 (CYP2E1) plays a key role in the pathogenesis of ASH because of the oxidative stress it generates [3]. The most significant role of CYP2E1 is its adaptive response to high blood ethanol levels with a corresponding acceleration of ethanol metabolism. The associated free radical production contributes to liver injury in alcoholics [3]. Furthermore, CYP2E1 activates a score of xenobiotics to highly hepatotoxic compounds. CYP2E1 induction also results in strikingly increased oxidation of ethanol to acetaldehyde, a highly reactive and toxic compound [4,5]. Thus, CYP2E1 is a major microsomal source of oxidative stress, and it has been explored as a candidate in the pathogenesis of NASH because CYP2E1 is also up-regulated in other clinical settings that are associated with NASH, such as diabetes mellitus and obesity [3]. Besides being highly inducible by ethanol, CYP2E1 is upregulated in the aberrant nutritional states of fasting, diabetes, and obesity as well as by a high-fat/low-carbohydrate diet. The increased circulating levels of ketone bodies and fatty acids observed in these seemingly disparate conditions may be directly involved in CYP2E1 induction [3,10]. In the review article of this issue [36], Lieber described clearly and comprehensively pathophysiological role of CYP2E1 in ASH and NASH.

Weltman et al. showed that rats fed with a methionine- and choline-deficient diet develop liver injury resembling NASH in which the extent and lobular distribution of CYP2E1 expression are closely related to the distribution of steatosis and inflammation [6]. They also reported that hepatic CYP2E1 was significantly increased in patients with NASH compared with controls. The increased expression and lobular distribution of hepatic CYP2E1 in NASH resembled that seen in ASH as well as in an animal nutritional model of steatohepatitis [7]. It has been recently shown that hepatic CYP2E1 activity is significantly higher in nondiabetic patients with NASH compared with age- and gender-based BMI-matched controls [8]. Morbid obesity is frequently associated with NAFLD. Hepatic CYP2E1 activity was up-regulated in morbidly obese subjects [9] with a positive association between the degree of steatosis and CYP2E1 activity. These results support the idea that CYP2E1 plays a role in the pathogenesis of NASH.

One of the key pathogenic factors for NASH may be insulin resistance, and insulin resistance is thought to be common in patients with NAFLD and NASH [11]. Insulin resistance could contribute to hepatic steatosis by favoring peripheral lipolysis with an increased hepatic uptake of fatty acids. It may also be the reason for an increased expression of CYP2E1, thereby contributing to the production of prooxidants in fatty liver.

The investigators surmised that the increased expression of CYP2E1 in patients with NASH may result in the production of free oxygen radicals capable of inducing lipid peroxidation of hepatocyte membranes. In a study of a mouse model of NASH, hepatic CYP2E1 was up-regulated and was associated with a dramatic increase in total lipid peroxide levels in the liver that were substantially inhibited by anti-CYP2E1 antibody [12]. Oxidative cellular damage has also been frequently detected in livers with NAFLD [13].

Although CYP2E1 plays an important role in pathophysiology of NASH, induction of CYP2E1 probably is not the only cause of lipid peroxidation and oxidative stress in NASH, and cannot also be the only factor required for the development of NASH because it is a common phenomenon during intake of drugs such as isoniazid that do not cause NASH [14]. Lipid peroxidation is probably a key pathogenic mechanism in NASH, but other factors may also contribute to the development of NASH, either by enhancing lipid peroxidation or by directly stimulating fibrogenesis and inflammatory response characteristic of NASH.

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Endotoxin has been implicated in the pathogenesis and progression of AFLD. Endotoxemia is often observed in patients with AFLD, and animal experiments have shown plasma endotoxin levels to be higher in rats fed with ethanol chronically than in control rats. Long-term ethanol feeding also increases intestinal permeability to and absorption of endotoxin, which can evoke Kupffer cell activation [15]. The level of gut-derived endotoxin in portal blood increases, the LPS then binds to LBP, and the LPS–LBP complex binds to CD14 on Kupffer cells. Toll-like receptor 4 (TLR4) associates with CD14 on the cell surface, mediating LPS-induced signal transduction, including NFκB activation and subsequent enhancement of proinflammatory agents such as TNFα and COX-2 in Kupffer cells, leading to a liver injury [16]. Furthermore, oral administration of antibiotics prevents ethanol-induced increases in plasma endotoxin levels and progression of liver injury [17].

Recently, an increased production of endogenous ethanol by the intestinal microflora has been demonstrated in genetically obese ob/ob mice, a model for NAFLD, even in the absence of ethanol ingestion [18]. Treatment with antibiotics has also been reported to improve NAFLD histology and reduce serum ALT levels in ob/ob mice [19]. Indeed, a subsequent pilot study of patients with NASH demonstrated increased breath ethanol concentrations among obese females with this condition, confirming the suspicion that increased intestinal production of ethanol occurs in some humans with NAFLD [20]. Surgical procedures to treat obesity such as jejunoileal bypass and gastroplasty have been suggested to be risk factors for NAFLD, associated with intestinal bacterial overgrowth [21]. Advanced alcoholic liver disease was much more frequently observed among individuals possessing the T allele at the −159 position of the promoter region of the CD14 gene, Kupffer cell receptor [22]. Homozygosity for the same allele was also significantly higher in NASH patients [23]. These results suggest some commonality with the pathogenesis of AFLD and NAFLD; bacterial overgrowth and increased endogenous production of ethanol in the intestinal flora, subsequently activated Kupffer cells, and hepatotoxic cytokines, such as TNFα, released from Kupffer cells [24].

Hepatic TNFα is known to play an important role in the pathogenesis of AFLD. Yin et al. reported that ethanol-induced liver injury was minimized in mice lacking TNF receptor 1 [25]. This study showed an essential role of TNFα in alcohol-induced liver injury. TNFα plays a central role in both proinflammatory and apoptotic responses to endotoxin and can transduce its signal by binding to one of the two cellular receptors: types I and II. Activation of TNF receptors by TNFα causes the release from mitochondria of reactive oxygen species, such as superoxide anion, as well as cytochrome c oxidase, which induce apoptosis and necrosis [26]. Treatment with anti-TNFα monoclonal antibody has been recently shown to be effective in patients with severe alcoholic hepatitis [27]. On the other hand, it is conceivable that inhibition of TNFα activity could improve "NASH" because anti-TNFα antibodies improved "NASH" in ob/ob mice [19]. Potential sources of TNFα are macrophages and activated lymphocytes infiltrating the liver tissue of NASH patients, whereas adipose tissue is a significant source of endogenous TNFα production [28]. The expression of TNFα is elevated in most rodent models of obesity and implicated in human obesity, and TNFα has been shown to induce insulin resistance [29]. Insulin resistance represents the most reproducible predisposing factor for NASH, and NAFLD has been appropriately considered as one of the components of the insulin resistance syndrome. Insulin resistance favors accumulation of free fatty acids in the liver and predisposes to oxidative stress by stimulating microsomal lipid peroxidases, and by the direct effect of high insulin levels in decreasing mitochondrial β-oxidation of fatty acids [30]. Recent reports have shown that overexpression of TNFα and TNF receptor1 mRNA is increased in the liver tissue of NASH patients [31]. Valenti et al. reported that TNF-α polymorphisms could represent a susceptibility genotype for NAFLD [32]. These findings support that TNFα is necessary for steatohepatitis in both alcoholic and nonalcoholic conditions.

The common pathogenic mechanisms in ASH and NASH might include other conditions such as hypoxia and impaired methionine metabolism [8,33]. As mentioned above, mechanisms other than CYP2E1 play roles in the pathogenesis of NASH. We agree that increased CYP2E1 per se is not sufficient to cause NASH; however, some reports on the strong causal relationship between CYP2E1 induction and occurrence of NASH have enabled the establishment of the important role of CYP2E1 in the pathogenesis of NASH.

The review by Lieber in this issue suggests the beneficial effects of CYP2E1 inhibitors in the treatment of ASH and NASH. They have recently reported that lycopene, the most abundant carotenoid in tomatoes, has a high anti-oxidant capacity and protects HepG2 cells expressing CYP2E1 against arachidonic acid toxicity [34]. Lieber and his group have also elucidated that lycopene opposes the ethanol-induced oxidative stress and apoptosis in HepG2 cells overexpressing CYP2E1 [35]. These results support that this carotenoid may be useful as a therapeutic agent for ASH and NASH and merit further evaluation.

In summary, the review by Lieber has taken us a step further in our understanding of the association of CYP2E1 and steatohepatitis. The forthcoming results of clinical trials evaluating new medications that specifically inhibit CYP2E1 activity will provide further insights into the relationship of CYP2E1 and steatohepatitis.

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