Diphenyleneiodonium sulfate, an NADPH oxidase inhibitor, prevents early alcohol-induced liver injury in the rat

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Diphenyleneiodonium sulfate, an NADPH oxidase inhibitor, prevents early alcohol-induced liver injury in the rat. Am J Physiol Gastrointest Liver Physiol 280: G1005–G1012, 2001.—The oxidant source in alcohol-induced liver disease remains unclear. NADPH oxidase (mainly in liver Kupffer cells and infiltrating neutrophils) could be a potential free radical source. We aimed to determine if NADPH oxidase inhibitor diphenyleneiodonium sulfate (DPI) affects nuclear factor-κB (NF-κB) activation, liver tumor necrosis factor-α (TNF-α) mRNA expression, and early alcohol-induced liver injury in rats. Male Wistar rats were fed high-fat liquid diets with or without ethanol (10–16 g·kg⁻¹·day⁻¹) continuously for up to 4 wk, using the Tsukamoto-French enteral feeding protocol. DPI or saline vehicle was administered by subcutaneous injection for 4 wk. Mean urine ethanol concentrations were similar between the ethanol- and ethanol plus DPI-treated groups. Enteral ethanol feeding caused severe fat accumulation, mild inflammation, and necrosis in the liver (pathology score, 4.3 ± 0.3). In contrast, DPI significantly blunted these changes (pathology score, 0.8 ± 0.4). Enteral ethanol administration for 4 wk also significantly increased free radical adduct formation, NF-κB activity, and TNF-α expression in the liver. DPI almost completely blunted these parameters. These results indicate that DPI prevents early alcohol-induced liver injury, most likely by inhibiting free radical formation via NADPH oxidase, thereby preventing NF-κB activation and TNF-α mRNA expression in the liver.

MATERIALS AND METHODS

Animals and treatments. Male Wistar rats were fed a high-fat liquid diet with or without ethanol (10–16 g·kg⁻¹·day⁻¹, n = 5) continuously for up to 4 wk, using the intragastric enteral feeding protocol developed by Tsukamoto and French (39). Either DPI (1 mg·kg⁻¹·day⁻¹; Toronto Research Chemicals, Toronto, ON, Canada) or vehicle (5% glucose, 0.2 ml/day) was administered by subcutaneous injection for 4 wk. This dose of DPI was shown to be well tolerated and effective in long-term studies (8, 13). Rats were housed in a pathogen-free facility accredited by the American Association for Accreditation of Laboratory Animal Care, and surgical procedures used in this study were approved by the institutional animal care and use committee.

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A liquid diet described first by Thompson and Reitz (36) supplemented with lipotropes as described by Morimoto et al. (31) was used. It contained corn oil as fat (37% of total calories), protein (23%), carbohydrate (5%), minerals and vitamins, plus either ethanol (35–40% of total calories) or isocaloric maltose-dextrin (control diet) as described previously (38).

**Urine collection and ethanol assay.** Ethanol concentration in urine, which is representative of blood alcohol levels (3), was measured daily. Rats were housed in metabolic cages that separated urine from feces, and urine was collected over 24 h in bottles containing mineral oil to prevent evaporation. Each day at 9 AM, urine collection bottles were changed and a 1-ml sample was stored at -20°C for later analysis. Ethanol concentration was determined by measuring absorbance at 366 nm resulting from the reduction of NAD$^+$ to NADH by alcohol dehydrogenase (7).

**Blood collection and transaminase determinations.** Blood was collected from the aorta after 4 wk of enteral feeding and centrifuged. Serum was stored at -20°C until it was assayed for alanine aminotransferase (ALT) by standard enzymatic procedures (7).

**Pathological evaluation.** After 4 wk of ethanol treatment, livers were formalin fixed, embedded in paraffin, and stained with hematoxylin and eosin to assess steatosis, inflammation, and necrosis. Liver pathology was scored in a blinded manner by one of the authors and by an outside expert as described previously by Nanji et al. (32) as follows: for steatosis (the % of liver cells containing fat), 0% = 1+; 1–25% = 2+; 26–50% = 3+; 51–75% = 4+; and for inflammation and necrosis: 1 focus per low-power field = 1+; and 2 or more foci = 2+.

The number of neutrophils in the liver sections was also determined after 4 wk by counting cells in three high-power fields (×400) per hematoxylin and eosin- and Giemsa-stained slide. Fat accumulation caused ballooning of hepatocytes and narrowing of the sinusoidal space. This could affect the number of hepatocytes and sinusoidal space in each field; therefore, the number of hepatocytes was also counted and the number of neutrophils was expressed per 100 hepatocytes (16).

**Collection of bile and detection of free radical adducts.** Ethanol concentration in the breath was analyzed by gas chromatography to verify that levels were similar between the groups when bile was collected (14). Rats were anesthetized with pentobarbital sodium (75 mg/kg), and the proximal bile duct was cannulated with PE-10 tubing. After the spin-trapping reagent α-(4-pyridyl-1-oxide)-N-t-butyl nitrotrone (POBN; Sigma Chemical, St. Louis, MO) was injected slowly into the tail vein, bile samples were collected at 30-min intervals for 3 h into 35 μl of 0.5 mM Desferal (defereroxamine mesylate, Sigma Chemical) to prevent ex vivo radical adduct formation. Samples were stored at -80°C until analysis of free radical adducts by electron spin resonance (ESR) spectroscopy (18). Samples were thawed and transferred to a quartz flat cell, and ESR spectra were obtained using a Bruker ESP 300 ESR spectrometer.
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Table 1. Effect of chronic enteral ethanol and DPI on routine parameters and pathology scores

<table>
<thead>
<tr>
<th></th>
<th>Vehicle High-fat control</th>
<th>Vehicle High-fat ethanol</th>
<th>DPI High-fat control</th>
<th>DPI High-fat ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT, U/l</td>
<td>37 ± 5</td>
<td>122 ± 4*</td>
<td>46 ± 4</td>
<td>41 ± 6†</td>
</tr>
<tr>
<td>Steatosis score</td>
<td>0.1 ± 0.1</td>
<td>2.6 ± 0.2‡</td>
<td>0.1 ± 0.1</td>
<td>0.3 ± 0.1§</td>
</tr>
<tr>
<td>Inflammation score</td>
<td>0</td>
<td>1.1 ± 0.1†</td>
<td>0</td>
<td>0.4 ± 0.2§</td>
</tr>
<tr>
<td>Necrosis score</td>
<td>0</td>
<td>0.6 ± 0.1</td>
<td>0</td>
<td>0.1 ± 0.1§</td>
</tr>
<tr>
<td>Total score</td>
<td>0.1 ± 0.1</td>
<td>4.4 ± 0.45‡</td>
<td>0.1 ± 0.1</td>
<td>0.7 ± 0.48‡</td>
</tr>
<tr>
<td>H&amp;E staining</td>
<td>2 ± 0.4</td>
<td>5.7 ± 0.6*</td>
<td>1.8 ± 0.5</td>
<td>1.8 ± 0.4†</td>
</tr>
<tr>
<td>Giemsa staining</td>
<td>0.8 ± 0.3</td>
<td>5.3 ± 0.4*</td>
<td>0.7 ± 0.3</td>
<td>0.8 ± 0.6†</td>
</tr>
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</table>

Values are means ± SE (n = 5). Diphenyleneiodonium sulfate (DPI; 1 mg·kg⁻¹·day⁻¹, sc injection) or vehicle (0.5 ml saline/day, sc injection) was given as described in MATERIALS AND METHODS. Serum alanine aminotransferase (ALT) and the no. of infiltrating neutrophils/100 hepatocytes. *P < 0.05 vs. control diet + vehicle (Bonferroni's post hoc test). †P < 0.05 vs. control diet + vehicle (Mann-Whitney rank sum test). ‡P < 0.05 vs. ethanol-containing diet + vehicle (Bonferroni's post hoc test). §P < 0.05 vs. control diet + vehicle (Mann-Whitney rank sum test).

Nuclear protein extraction and gel mobility shift assays. Binding conditions for NF-κB were characterized and electrophoretic mobility shift assays were performed as described previously (43). Briefly, nuclear extracts (40 µg) from liver tissues were preincubated for 10 min on ice with 1 µg poly(dI/dC) and 20 µg BSA (both from Pharmacia Biotech, Piscataway, NJ) in a buffer that contained 1 mM HEPES (pH 7.6), 40 mM MgCl₂, 0.1 M NaCl, 8% glycerol, 0.1 mM dithiothreitol, and 0.05 mM EDTA and 2 µl of a [³²P]-labeled DNA probe (10,000 counts·min⁻¹·µl⁻¹; Cerenkov) that contained 0.4 ng of double-stranded oligonucleotide. Mixtures were incubated for 20 min on ice and resolved on 5% polyacrylamide (29:1 cross linking) and 0.4× Tris-boric acid-EDTA gels. After electrophoresis, gels were dried and exposed to Kodak film. Specificity of NF-κB binding was verified by competition assays and ability of specific antibodies to supershift protein-DNA complexes. In the competition assay, a 200-fold excess of the unlabeled oligonucleotide was added 10 min before addition of the labeled probe. In the supershift experiment, 1 µg of rabbit antisera against p50 protein (Santa Cruz Biotech, Santa Cruz, CA) was added to the reaction mixture after incubation with labeled probe, which was further incubated at room temperature for 30 min. Labeled and unlabeled oligonucleotides contained the consensus sequence for NF-κB (top strand: 5'-GCAGAGGGGACTTTCCGGA-3'; bottom strand: 5'-GTCTGCCAAAGTCCCCTCTG-3') (4). Data were quantitated by scanning autoradiograms with GelScan XL (Pharmacia, Uppsala, Sweden).

RNA isolation and RT-PCR amplification. Immediately after the rats died, liver tissues were flash frozen in liquid nitrogen and stored at −80°C until analysis. Approximately 50 mg of liver tissue was collected, and total cellular RNA was extracted using the Qiagen RNeasy kit (Chatsworth, CA) according to the manufacturer’s instructions. The synthesis of cDNA and PCR amplification of TNF-α and glyceralde-
hyde-3-phosphate dehydrogenase was performed as described previously (24). When appropriate, the specificity of the PCR bands was confirmed by restriction site analysis of the amplified cDNA, which generates restriction fragments of the expected size (data not shown). The amplified PCR products were subjected to electrophoresis at 75 V through 2% agarose gel for 1 h. Gels were stained with 0.5 mg/ml ethidium bromide Tris-borate-EDTA buffer (ICN, Costa Mesa, CA) and photographed with type 55 Polaroid positive/negative film.

Statistics. ANOVA or Student’s t-test was used for the determination of statistical significance as appropriate. For comparison of pathological scores, the Mann-Whitney rank sum test was used. P < 0.05 was selected before the study as the level of significance.

RESULTS

Body weight. Diets were initiated after 1 wk to allow for full recovery from surgery. All animals survived 4 wk of the experimental period, and animals treated with DPI exhibited no complications. In spite of development of greater hepatic injury in ethanol groups, the rats grew steadily, making nutritional complications an unlikely explanation for these results. The mean body weight gains were 2.9 ± 0.4 g/day for the ethanol group and 2.8 ± 0.2 g/day for the ethanol plus DPI group (Fig. 1). There were no significant differences in body weight gains between the groups.

Ethanol concentrations in urine. As reported previously in several studies (1, 33, 37), alcohol levels in urine fluctuate in a cyclic pattern from 0 to 500 mg/dl. The urine alcohol cycle depends on an intact hypothalamic-pituitary-thyroid axis response to the ethanol-induced drop in body temperature by increasing the rate of ethanol elimination (27). DPI had no effect on cyclic patterns of ethanol. There were no significant differences in mean urine alcohol concentrations between rats given ethanol (Fig. 2A; 251 ± 38 mg/dl) and rats given ethanol plus DPI (Fig. 2B; 262 ± 33 mg/dl).

Serum transaminase levels. Serum ALT levels were 40 IU/l after 4 wk of high-fat control diet (Table 1). However, administration of enteral ethanol for 4 wk increased serum ALT levels significantly by about four-fold. DPI significantly blunted these values by ~70%.

Pathological evaluation. In control groups, there were no pathological changes after 4 wk (Fig. 3A and B). Administration of enteral ethanol for 4 wk caused severe fatty infiltration, mild inflammation, and necrosis (Fig. 3C), resulting in a total pathology score of 4.3 ± 0.3 (Table 1). Increases in pathology scores were blunted almost completely by DPI treatment (Fig. 3D; total pathology score: 0.8 ± 0.4).

The number of infiltrating neutrophils in the liver was minimal and not different between the groups in the absence of ethanol. However, a significant increase was caused by 4 wk of enteral ethanol feeding (Table 1). This increase was prevented totally by DPI.

Effect of chronic ethanol and DPI on free radical adduct formation. Radical adducts were barely detectable in bile from rats fed an ethanol-free, high-fat control diet in both groups (data not shown). However, administration of enteral ethanol for 4 wk caused a significant increase in free radical adduct formation (Fig. 4). This increase was blunted significantly by DPI. ESR hyperfine coupling constants were AN = 15.70 G and AB = 2.72 G, characteristic of both the α-hydroxyethyl and lipid-derived POBN radical adduct. Studies using [13C]ethanol have demonstrated that both radical adducts are present in approximately equal amounts (18). ESR signal intensity was determined from the amplitude of the high-field member of the low-field doublet (second line from the left) of the ESR spectra (Fig. 5). The intensity of these signals was increased significantly by enteral ethanol but was blunted ~90% by DPI (Fig. 5).
In contrast, enteral ethanol significantly increased NF-κB binding by nearly threefold. DPI treatment significantly blunted this increase by ~50%.

To confirm that protein binding in nuclear extracts to labeled oligonucleotide probe was specific for the active form of NF-κB, gel shift assays were carried out either in the presence of an excess of unlabeled double-stranded oligonucleotide with a consensus sequence for NF-κB binding or with an antibody specific for the NF-κB p50 subunit (Fig. 6B). In the absence of nuclear proteins, no protein-DNA complex was detected (Fig. 6B, lane 1). Furthermore, unlabeled oligonucleotide that contained the NF-κB binding site could effectively compete for DNA binding with 32P-labeled probe (Fig. 6B, lane 3). Moreover, addition of anti-p50 serum reduced the intensity of the complex and supershifted the band to a higher molecular mass (Fig. 6B, lane 4).

**DISCUSSION**

Possible sources of oxidants in early alcohol-induced liver injury. Chronic enteral ethanol enhances reactive oxygen species in the liver. ESR spectroscopy of bile from animals treated with xenobiotics and given spin-trapping agents has been demonstrated (19) to be useful in monitoring hepatic free radical-adduct formation in vivo. Radical adducts detected in bile may be derived from both parenchymal and nonparenchymal cells. In the Tsukamoto-French intragastric ethanol infusion model, α-hydroxyethyl and lipid-derived free radicals have been detected (18). These radicals are most likely involved in alcohol-induced liver injury; however, the source of oxidants has remained unclear.

Fig. 5. Effect of chronic enteral ethanol and DPI on average radical adduct signal intensity. Conditions were the same as those given in Fig. 4 legend. ESR signal intensity was determined from the amplitude of the high-field member of the low-field doublet (second line from the left) of the ESR spectra and was averaged for rats treated as described in MATERIALS AND METHODS. Values are means ± SE (n = 4). *P < 0.05 compared with rats fed high-fat ethanol-containing diet + vehicle (VEH) (ANOVA and Bonferroni’s post hoc test).

Fig. 6. Effect of chronic enteral ethanol and DPI on hepatic nuclear factor-κB (NF-κB) activity. A: nuclear extracts (40 μg of total protein in each line) were prepared from frozen livers and used for gel shift assays as described in MATERIALS AND METHODS. Data shown are results of densitometric analysis of the NF-κB/DNA complex images. Density of the image in livers of rats fed high-fat control diet was set to 100%. Values are means ± SE (n = 5). *P < 0.05 compared with rats fed high-fat ethanol-containing diet + vehicle (ANOVA with Bonferroni’s post hoc test). B: protein binding to labeled oligonucleotide probe is specific for the active form of NF-κB. Lane 1, labeled probe with no nuclear extract added. Lane 2, nuclear extract from livers of rats fed high-fat control diet. Lane 3, a 200-fold excess of the unlabeled oligonucleotide was used in competition assays. Lane 4, p50 antibodies were used in supershift experiments as described in MATERIALS AND METHODS. Open arrow, native NF-κB/DNA complex; filled arrow, supershifted complex.
Two possible sources of radical adducts in alcoholic liver injury have been suggested. One possibility is that cytochrome P-450 (CYP)2E1, induced predominantly in the hepatocyte by ethanol, is the source of reactive oxygen species (2). In support of this hypothesis, Ronis and co-workers (35) have shown that a correlation exists between blood levels of alcohol and induction of CYP2E1 as alcohol cycles in the enteral model. Also, the level of CYP2E1 correlates with the degree of pathology, and inhibitors of CYP2E1 partially reduced hepatic pathology due to enteral ethanol (2). These results, while correlative, support the idea that oxidants from CYP2E1 may play a role in early alcohol-induced liver injury. On the other hand, it was reported (25) that CYP2E1 was induced to the same extent by ethanol or ethanol plus the Kupffer cell toxicant gadolinium chloride (GdCl3) treatment in the rat enteral model; however, liver pathology was prevented by GdCl3. Importantly, it was recently reported from our laboratory (20) that there were no differences in free radical formation and early alcohol-induced liver injury between wild-type and CYP2E1 knockout mice fed enteral ethanol chronically. These data suggest that oxidants from CYP2E1 either are not involved or play only a minor role in early alcohol-induced liver injury.

Alternatively, oxidant-generating enzymes in Kupffer cells may contribute significantly to a dramatic increase in release of reactive oxygen species after ethanol administration. Indeed, destruction of Kupffer cells with GdCl3 diminishes free radical formation significantly (18) and prevents liver injury in the enteral alcohol model (1). Kupffer cells contain xanthine oxidase, NADPH oxidase, and other oxidant-generating enzymes. It has been shown recently (21) that allopurinol, a xanthine oxidase inhibitor and a free radical scavenger, prevented alcohol-induced liver injury in the enteral model. Moreover, DPI, an NADPH oxidase inhibitor, prevented free radical formation and early alcohol-induced liver injury almost completely in this study (Figs. 3–5). Thus oxidants from Kupffer cells could play a major role in early alcohol-induced liver injury (see Fig. 8). Indeed, release of superoxide anion leads to formation of both \( \alpha \)-hydroxyethyl and lipid-derived radicals, which could then be used as secondary or “marker” radicals because oxygen-derived radical adducts are generally too unstable to be detected in vivo (18). Importantly, the spin-trap POBN is rapidly absorbed and distributed throughout the body after intraperitoneal administration, and POBN radical adducts are relatively stable in vivo (29). Thus both \( \alpha \)-hydroxyethyl and lipid-derived adducts of POBN formed in the liver are excreted into the bile and detected by ESR.

Fig. 7. Effect of chronic enteral ethanol and DPI on tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) mRNA expression in the liver. TNF-\( \alpha \) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were determined using RT-PCR as described in MATERIALS AND METHODS. Data shown are representative of 4 samples/group.

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Fig. 8. Working hypothesis showing that Kupffer cells activated by gut-derived endotoxin produce free radicals during enteral ethanol administration. In this study, DPI, an NADPH oxidase inhibitor, prevented early alcohol-induced liver injury almost completely. These data are consistent with the hypothesis that oxidants from NADPH oxidase play an important role in early alcohol-induced liver injury. Kupffer cells then increase inflammatory cytokines such as TNF-\( \alpha \), because Kupffer cells are a major source of NADPH oxidase in the early phases of alcohol-induced liver injury.

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Possible mechanisms of effect of DPI in early alcohol-induced liver injury. A group of iodonium compounds, including DPI, represent a class of inhibitors of flavin enzymes that reduce activities of NADPH-dependent oxidase in the neutrophil and macrophage (34). DPI inhibited superoxide production by macrophages (15) and has been used to inhibit production of oxidants in several studies (9). Because it has been suggested that Kupffer cell NADPH oxidase plays a pivotal role in ethanol-induced superoxide production in the liver (6), this study was undertaken to determine if DPI could prevent liver injury by inhibition of free radical formation in the enteral feeding model. Indeed, DPI blunted increases in free radical formation (Figs. 4 and 5), NF-κB activity (Fig. 6), and TNF-α mRNA expression (Fig. 7) in the liver and prevented early alcohol-induced liver injury almost completely (Figs. 3 and Table 1).

DPI may also inhibit several other hepatic radical-generating enzymes, such as microsomal NADPH-CYP reductase, mitochondrial oxidases, and nitric oxide synthase (26, 30, 41). However, it was reported recently (22) that the free radical formation and liver injury observed in wild-type mice fed enteral ethanol were almost completely blunted in NADPH oxidase-deficient mice. These data are consistent with the hypothesis that NADPH oxidase is the predominant source of oxidants in alcohol-induced liver injury.

Infiltrating neutrophils are also a source of oxidants via NADPH oxidase. However, the number of infiltrating neutrophils increased significantly only after 3–4 wk of enteral feeding in this study (Table 1), suggesting that Kupffer cells are most likely a predominant source of oxidants, at least during the early phases of the pathological process. Therefore, it is concluded that DPI prevents liver injury, most likely by inhibiting free radical formation via Kupffer cell NADPH oxidase, preventing NF-κB activation and inflammatory cytokine production (see Fig. 8).

Role of NF-κB in early alcohol-induced liver injury. NF-κB is normally present in an inactive form bound to its inhibitor, IκB, in the cytoplasm (5). Typically, NF-κB is rapidly activated in response to immunologic stimuli such as lipopolysaccharide, cytokines such as TNF-α, and oxidants (5). Activation of NF-κB involves rapid phosphorylation and proteolytic cleavage of IκB from NF-κB, leading to translocation of NF-κB to the nucleus (5). Binding sites for NF-κB have been identified within the regulatory elements of genes for several proinflammatory cytokines such as TNF-α and interleukin-1. Thus NF-κB plays an important role in regulation of the innate immune system that participates in inflammation. Increases in inflammatory cytokines and adhesion molecules by activation of NF-κB could be one explanation for pathogenesis of early alcohol-induced liver injury. Indeed, NF-κB activity in the liver was increased significantly by about three- to fivefold over control values by alcohol (Fig. 6) (28). Moreover, DPI blunted this increase and prevented liver injury significantly in this study (Table 1 and Fig. 6). These results indicate that NF-κB activation plays an important role in early alcohol-induced liver injury (see Fig. 8).

TNF-α plays an important role in early alcohol-induced liver injury. Activation of NF-κB could increase TNF-α expression in the liver (40). TNF-α is a cytokine produced mainly by activated macrophages that stimulates endothelial cells to synthesize adhesion molecules [i.e., intercellular adhesion molecule-1 (ICAM-1)], leading to accumulation of leukocytes in the liver. Recent evidence from our laboratory (42) supports the hypothesis that TNF-α plays a pivotal role in early alcohol-induced liver injury. This conclusion is based on the observation that alcohol-induced liver injury is present in wild-type mice fed enteral ethanol chronically but is prevented in TNF receptor-1 knockout mice (42). Indeed, ethanol increases TNF-α mRNA and ICAM-1 expression in the liver in the enteral alcohol model (Fig. 7) (16, 17). Furthermore, anti-TNF-α antibody reduces inflammatory cell infiltration and necrosis in the Tsukamoto-French model (17). Moreover, TNF-α mRNA expression was blunted significantly by DPI in this study (Fig. 7).

The predominant pathological change observed in this study was steatosis (Table 1 and Fig. 3) (20, 42). Feingold and Grunfeld (12) reported that the synthesis of fatty acids in the liver is increased after TNF-α administration. TNF-α also stimulates peripheral lipidolysis, leading to an increase in circulating free fatty acids (11). Importantly, TNF-α is also involved in fat accumulation caused by enteral ethanol in the liver. Indeed, steatosis was observed in wild-type mice fed enteral ethanol but was significantly blocked in TNF receptor-1 knockout mice (42). In this study, DPI blunted increases in hepatic fat accumulation and TNF-α mRNA expression significantly (Figs. 3 and 7). Thus it is concluded that TNF-α plays an important role in early alcohol-induced liver injury (Fig. 8).

DPI prevented early alcohol-induced liver injury almost completely by inhibiting free radical formation in this study. These data are consistent with the hypothesis that oxidants from NADPH oxidase play a major role in early alcohol-induced liver injury (see Fig. 8). Kupffer cells then increase production of inflammatory cytokines such as TNF-α, because Kupffer cells are the predominant source of NADPH oxidase in liver during the early phases of alcohol-induced liver injury.

Portions of this work have been previously published in abstract form (23).

REFERENCES