Lipopolysaccharide-induced liver injury in rats treated with the CYP2E1 inducer pyrazole

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Submitted 8 February 2005; accepted in final form 13 April 2005

LPS IS A COMPONENT of the outer wall of gram-negative bacteria that normally inhabit the gut. LPS penetrates the gut epithelium only in trace amounts; however, LPS absorption can be elevated under pathophysiological conditions such as alcoholic liver disease (48). When LPS is released from gram-negative bacteria and enters the bloodstream, the liver tightly regulates the entry and processing of LPS by virtue of its ability to clear LPS and respond to LPS (56). In animals, LPS is cleared from the circulation within a few minutes after intravenous injection (37, 70). In addition to its ability to clear LPS, the liver also responds to LPS and produces cytokines. LPS directly causes liver injury by mechanisms involving inflammatory cells such as Kupffer cells and chemical mediators such as superoxide, nitric oxide, and TNF-α and other cytokines (6, 20, 26, 57, 62, 64). In addition, LPS potentiates liver damage induced by hepatotoxins including ethanol (31, 38). In experimental alcoholic liver disease, the combination of LPS and chronic ethanol produces hepatic necrosis and inflammation (3, 25). Ethanol alters gut microflora, the source of LPS, and ethanol increases the permeability of the gut, thus increasing the distribution of LPS from the gut into the portal circulation (endotoxemia). This causes activation of Kupffer cells, the resident macrophages in liver, resulting in the release of chemical mediators including cytokines and reactive oxygen species (ROS) and subsequently, alcoholic liver disease (17, 19, 48, 59, 65).

Oxidative stress plays an important role in alcoholic liver disease (29). There are many sources of ROS within the cell. Besides ethanol-induced increase in LPS with subsequent activation of Kupffer cells and production of ROS, cytochrome P-450 2E1 (CYP2E1), a form of P-450 that is induced by ethanol, is another important source. Whereas most ethanol is oxidized by alcohol dehydrogenase, CYP2E1 assumes a more important role in ethanol oxidation at elevated concentrations of ethanol and after chronic consumption of ethanol (34). Due to being poorly coupled with NADPH-cytochrome P-450 reductase, CYP2E1 exhibits enhanced NADPH oxidase activity and elevated rates of production of superoxide (O2•-) and hydrogen peroxide (H2O2) (5, 18, 49). CYP2E1-mediated oxidative stress is thought to play an important role in alcoholic liver disease (8, 9, 29).

Pyrazole is a potent inhibitor of alcohol dehydrogenase (32, 51, 58) and is frequently used to block the oxidation of ethanol or the metabolism of other alcohols such as methanol (41) or ethylene glycol (13). In addition, pyrazole has been shown to affect the metabolism of several drugs by the microsomal mixed-function oxidase system (35). Microsomes isolated from rats treated with pyrazole for ~2–3 days showed an approximately two- to fourfold increase in CYP2E1 (46, 66). After administration of pyrazole to rats, mRNA levels did not increase, suggesting that the mechanism of induction was at the level of protein stabilization (55, 66). Pyrazole has been used as an inducer of CYP2E1 to study the role of increased production of oxygen-free radicals mediated by CYP2E1 in cell death (47, 67, 68, 69).

Either LPS or CYP2E1 is considered an independent risk factors involved in alcoholic liver disease, but mutual relation-
ship or interactions between them are unknown. In the present study, we investigated the combined action of LPS and CYP2E1 in rats via administrating pyrazole to induce CYP2E1 followed by treatment with LPS. We found that pretreatment with pyrazole followed by LPS injection caused severe liver necrosis and oxidative stress, suggesting possible synergistic interactions between LPS and CYP2E1.

MATERIALS AND METHODS

Animals and treatments. Sprague-Dawley rats (male, 160–180 g) were purchased from Charles River Breeding Laboratories (Boston, MA), housed in temperature- and light-controlled animal facilities, and permitted consumption of tap water and standard food ad libitum. Rats used in this study received humane care, and experiments were carried out according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals and with approval of the protocols of the Mount Sinai Animal Care and Use Committee. Rats were injected intraperitoneally with pyrazole (Sigma, St. Louis, MO), 200 mg/kg body wt, once per day for 2 days followed by an overnight fast to induce liver CYP2E1 or 0.9% saline as control. After the overnight fast, LPS (Sigma), serotype 055:B5, 10 mg/kg body wt, was injected via the tail vein or 0.9% saline as control. Eight hours after LPS or saline injection, blood was collected from the retroorbital venous sinus under anesthesia. The collected blood was centrifuged at 3,000 rpm for 5 min, and serum was separated. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed using diagnostic kits (Thermo Electron, Louisville, CO). Ten hours after the LPS or saline injection (2 h after blood collection), the rats were killed under anesthesia. Livers were removed, washed with cold saline, and excised into fragments; one aliquot of tissue was placed in 10% formalin solution for paraffin blocking, whereas the other aliquots were stored at 4°C for subsequent assays. All assays were finished within 3 mo.

Liver pathology and immunohistochemistry. Liver samples were fixed in 10% formalin solution and embedded in paraffin. Sections (5 μm thick) were stained with hematoxylin and eosin for pathological evaluation. Immunohistochemical staining for CYP2E1 was performed by using antibody against CYP2E1 (generous gift from Dr. Jerome Lasker, Hackensack Biomedical Research Institute, Hackensack, NJ) and a rabbit ABC staining system (Santa Cruz Biotechnology, Santa Cruz, CA). Samples were counterstained with hematoxylin.

Lipid peroxidation analysis. Hepatic tissues were placed in 0.15 M KCl supplemented with 50 μM Trolox and homogenized in a polytron homogenizer for 10 strokes. The resulting homogenate was centrifuged at 3,000 rpm for 12 min, and then 0.1 ml of the resulting supernatant fraction was incubated with 0.2 ml of trichloroacetic acid (TCA; 15% wt/vol)-thiobarbituric acid (TBA; 0.375% wt/vol)-HCl solution (0.25 N) in a boiling water bath for 10 min. After being cooled in an ice bath, 0.3 ml of 15:1 butanol-pyridine solution were added to the samples followed by centrifugation at 1,000 rpm for 5 min. The resulting supernatant was used to determine the formation of TBA-reactive components by evaluating absorbance at 535 nm. Varying concentrations of malondialdehyde (MDA) treated as above served as a standard.

Antioxidant enzymes and glutathione content in liver. Liver homogenate (5 μl), prepared as above, was added to a cuvette containing 100 μl of 20 mM H2O2 in 50 mM potassium phosphate buffer (pH 7.4), and the decrease in absorbance was measured at 240 nm for 1 min to determine catalase activity (1). The molar extinction coefficient of 43.6 M/cm was used to determine catalase activity. To measure glutathione peroxidase (GSH-Px), a mixture consisting of 650 μl of 50 mM potassium phosphate buffer (pH 7.4), 100 μl of 10 mM glutathione, 100 μl of 1.5 mM NADPH, and 100 μl of glutathione reductase (GR; 0.4 U/ml) were added to 50 μl of homogenate and incubated at 37°C for 10 min. Then, 50 μl of 12 mM t-butyl hydroperoxide were added to 450 μl of homogenate mixture to start the reaction, and the decrease in absorbance was monitored at 340 nm for 1 min. The molar extinction coefficient of 6.22 × 103/cm was used to determine GSH-Px activity (22). GR was measured by adding 5 μl of homogenate to a cuvette containing 5 μl of 20 mM GSSG, 5 μl of 2 mM NADPH, and 85 μl of 50 mM potassium phosphate buffer (pH 7.4) and measuring the decrease in absorbance at 340 nm for 1 min. The molar extinction coefficient of 6.22 × 103/cm was used to determine GR activity (7). Glutathione S-transferase (GST) was measured by adding 5 μl of homogenate to a cuvette containing 100 μl of 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM GSH in 0.1 M potassium phosphate buffer (pH 6.5) and measuring the increase in absorbance at 340 nm for 1 min. The content of heme oxygenase-1 (HO-1) and Cu/Zn-superoxide dismutase (Cu/Zn-SOD) was measured by Western blot analysis. GSH was assayed following the method of Tietze (61).

Protein carbonyl assay. Liver protein oxidation was determined by measuring protein carbonyls using OxyBlot protein oxidation detection kit (Chemicon, Temecula, CA). Briefly, 5 μl of homogenate obtained as above were denatured by adding 5 μl of 12% SDS. The carbonyl groups were derivatized by adding 10 μl of 1 × 2,4-

Fig. 1. Effect of pyrazole, LPS, or LPS + pyrazole on levels of serum alanine aminotransferase (ALT; A) and aspartate aminotransferase (AST; B) in liver. Pyrazole (200 mg/kg body wt) or saline was injected ip daily for 2 days followed by LPS (10 mg/kg body wt) or saline injection via tail vein. Blood was collected 8 h after treatment with LPS, and transaminase levels were assayed as described in MATERIALS AND METHODS. Data are presented as means (SD) for 5 control, 5 pyrazole, 7 LPS, and 7 LPS + pyrazole rats. **P < 0.01, compared with control or pyrazole group or LPS group.
dinitrophenylhydrazine (DNPH). The negative control involved addition of derivatization-control solution instead of the DNPH solution. The resulting samples were then subjected to Western blot analysis. Proteins that have undergone oxidative modification were identified by appearing as a band only in the lane containing the derivatized sample but not in the lane containing the negative control.

**Content of nitrite+nitrate.** Production of endogenous nitric oxide was determined by measuring nitrite plus nitrate using a nitrate/nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). The homogenate was incubated in boiling water for 2 min followed by centrifugation at 3,000 rpm to exclude protein. Nitrate reductase and cofactors were added to reduce nitrate to nitrite. The Griess reagent was added, and absorbance at 540 nm was determined. Nitrate solution was used as standard.

**Determination of in situ generation of superoxide.** In situ production of superoxide was determined as described by Minamiyama et al. (42). Tissue sections were prepared from unfixed frozen liver tissues. Liver tissues were placed into 50 mM sodium phosphate containing 18% sucrose at 4°C for overnight, and then they were frozen and cut into 10-μm frozen sections. The oxidation-dependent fluorescent dye dihydroethidium (DHE; 40 μM, Molecular Probes, Eugene, OR) and NADPH (1 mM) were applied to the surface of each tissue section and were incubated in a light-protected humid chamber at 37°C for 30 min. After being rinsed, fluorescence of the section was detected using a fluorescence microscope. Control tissue sections were incubated with 40 μM DHE without NADPH.

**Western and slot blots.** Microsomal proteins (40 μg) were resolved by electrophoresis using 10% SDS-PAGE and were then transferred to nitrocellulose membranes for blotting with anti-CYP2E1 antibody (a gift from Dr. Jerome Lasker, Hackensack Biomedical Research Institute, Hackensack, NJ), followed by horseradish peroxidase-conjugated secondary antibody. Cytosol proteins (100 μg) were electrophoresed for inducible nitric oxide synthase (iNOS) detection using a rabbit anti-polyclonal antibody (Chemicon). Homogenate proteins (120 μg) were electrophoresed for HO-1 and SOD detection; anti-HO-1 monoclonal antibody was from StressGen (Victoria, BC, Canada), and sheep anti-SOD polyclonal antibodies were from Calbiochem (La Jolla, CA). Protein nitrotyrosine residues (3-NT) in liver homogenate were detected by a slot-blot technique. A total of 2 μg of homogenate protein was loaded onto a nitrocellulose membrane that was placed in a slot-blot microfiltration unit (Bio-Rad Laboratories, Hercules, CA). Then, polyclonal rabbit antisera against nitrotyrosine (3-NT; Upstate, Lake Placid, NY) followed by horseradish peroxidase-conjugated secondary antibody were added. Proteins were visualized by radiography using enhanced chemiluminescence Western detection reagent (Amersham Bioscience, Piscataway, NJ). Blots were quantified using the UN-SCAN-IT automated digitizing system version 5.1 (Silk Scientific), and results were expressed as arbitrary units.

**Caspase-3 and -9 activity.** Hepatic tissues were placed in 0.15 M KCl and homogenized in a polytron homogenizer for 10 strokes. The resulting homogenate was centrifuged at 3,000 rpm for 12 min, and the resulting supernatant fraction was used for caspase activity measurement. Caspase-3 and -9 activity was determined by measuring enzymatic cleavage of the substrate Ac-DEVD-AMC (Alexis Biochemicals, San Diego, CA) and Ac-LEHD-AFC (Calbiochem), respectively. Ac-DEVD-AMC (final concentration 0.2 mM) was dis-

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Fig. 2. Hematoxylin-eosin-stained liver sections after treatment with pyrazole, LPS, or combination of LPS plus pyrazole. Control group (A) showed normal liver histology; pyrazole group (B) had almost normal histology except for dilatation of sinusoid; LPS group (C) had congestion with blood cells; pyrazole + LPS group (D, E) had necrotic foci with inflammatory cells infiltration (arrow). D: intensive necrosis area (black arrow) with bleeding (white arrow) in the perportal area. E: local necrosis foci around the central vein (arrow).
solved in assay buffer containing 20 mM HEPES (pH 7.5), 10% glycerol, and 2 mM dithiothreitol. Homogenate (5 μl) was added to this assay buffer (1 ml) and incubated at 37°C for 1 h. Ac-LEHD-AFC (final concentration 10 μM) was dissolved in assay buffer containing 100 mM HEPES (pH 7.5), 10% sucrose, 10 mM dithiothreitol, and 0.5 mM EDTA. Homogenate (5 μl) was added to this assay buffer (0.5 ml) and incubated at 30°C for overnight. The fluorescence associated with the released AMC (caspase-3, excitation at 380 nm, emission at 460 nm) or AFC (caspase-9, excitation at 400 nm, emission at 505 nm) was assayed in a PerkinElmer spectrofluorometer (Wellesley, MA). The data were expressed as arbitrary fluorescence units per milligram of protein.

**CYP2E1 activity.** CYP2E1 activity was measured by the rate of oxidation of p-nitrophenol (PNP) to p-nitrophenol in the presence of NADPH according to the method of Reinke and Moyer (50). The reaction was performed with 500 μg of microsomal protein for 15 min at 37°C. The results are expressed as pimomoles per minute per milligram of protein.

**Statistics.** Results are expressed as means (SD). Statistical evaluation was carried out by one-way ANOVA followed by Student-Newman-Keuls post hoc test.

**Results.** Liver tissue necrosis. Both LPS or pyrazole treatment did not significantly increase serum ALT and AST levels over the saline control; however, the combination of LPS plus pyrazole increased AST and ALT levels about four times the levels in the pyrazole- or LPS-treated rats (Fig. 1, A and B). Pathological examination showed that pyrazole induced no obvious histological changes in liver (Fig. 2B). LPS induced tissue degeneration and congestion with blood, but no necrosis was observed (Fig. 2C). The combination of LPS plus pyrazole induced extensive necrosis of hepatocytes, mainly located both in the periportal and pericentral area, accompanied by marked infiltration of inflammatory cells (Fig. 2, D and E).

Liver tissue apoptosis. LPS has been shown to increase TUNEL-positive cells only 1 h after administration, with peak levels at 3–5 h, followed by a return to normal at 24 h after LPS administration (54). In situ TUNEL assay showed that no positive staining was detected in the nucleus of livers from pyrazole-treated rats (Fig. 3B). Positive TUNEL staining of the nucleus was detected in about 7% of the hepatocytes in LPS-treated rats (Fig. 3, C and D). Pyrazole treatment did not cause caspase-3 and -9 activation, consistent with the lack of increase in TUNEL (Fig. 4, A and B). LPS injection increased caspase-3 and -9 activity by about fourfold compared with the untreated control rats, consistent with the increase in TUNEL (Fig. 4C). However, the combination of LPS plus pyrazole did not further increase caspase-3 and -9 activity above the increase produced.
Thus the combination of LPS plus pyrazole did not increase apoptosis beyond the effects of LPS alone; however, the combination of LPS plus pyrazole further increased serum AST and ALT levels and caused obvious necrosis and inflammation.

Lipid peroxidation. Under conditions of oxidative stress, polyunsaturated fatty acids in membranes are subjected to ROS-induced oxidation, membrane integrity is lost, and there is leakage of enzymes, e.g., transaminase out of the cell. To assess whether oxidative stress occurs after the various treatments, the hepatic LPO end product MDA was analyzed. LPS or pyrazole did not significantly increase MDA levels (Fig. 5), but the combination of LPS plus pyrazole caused a significant increase in MDA levels of ~65% (*P < 0.05, compared with control group).

Protein carbonyl. Carbonyl formation is an early marker for protein oxidation (16). The presence of protein carbonyls in the liver after the various treatments was determined by Western blot analysis using antibody against DNPH-protein adducts. In control rat livers, weak signals for DNPH-protein adducts were detected (Fig. 6). Treatment with pyrazole intensified protein carbonyls as did LPS treatment (Fig. 6). Highest levels of protein carbonyls were present in livers of the LPS plus pyrazole-treated rats (Fig. 6).

Production of superoxide. In situ detection of superoxide using the oxidation-dependent fluorescent dye DHE showed that the red fluorescence was strongest around central veins (Fig. 7E). The fluorescence was weakest in the saline control group and strongest in the LPS plus pyrazole group (Fig. 7, A, D, and E). The fluorescence intensity was slightly increased in either the pyrazole or LPS groups (Fig. 7, B and C) but by LPS alone (Fig. 4D). Thus the combination of LPS plus pyrazole did not increase apoptosis beyond the effects of LPS alone; however, the combination of LPS plus pyrazole further increased serum AST and ALT levels and caused obvious necrosis and inflammation.

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Fig. 4. Effect of pyrazole, LPS, or LPS + pyrazole on activities of caspase-3 (A) and caspase-9 (B) in liver. Pyrazole (200 mg/kg body wt) or saline was injected intraperitoneally daily for 2 days followed by LPS (10 mg/kg body wt) or saline injection via tail vein. Rats were killed 10 h after treatment with LPS, and activities of caspase-3 and -9 were determined as described in MATERIALS AND METHODS. Data are presented as means (SD) for 5 control, 5 pyrazole, 7 LPS, and 7 LPS + pyrazole rats. ***P < 0.005, compared with control group; ###P < 0.005, compared with pyrazole group.

Fig. 5. Effect of pyrazole, LPS, or LPS + pyrazole on levels of thiobarbituric acid reactive substrates in liver. Pyrazole (200 mg/kg BW) or saline was injected intraperitoneally daily for 2 days followed by LPS (10 mg/kg body wt) or saline injection via tail vein. Rats were killed 10 h after treatment with LPS, and lipid peroxidation was assayed by determining the levels of TBARS; results are expressed as malondialdehyde (MDA) equivalents. Data are presented as means (SD) for 5 control, 5 pyrazole, 7 LPS, and 7 LPS + pyrazole rats. *P < 0.05, compared with control group.

Fig. 6. Protein carbonyl formation after treatment with pyrazole (Pyr), LPS, and LPS + Pyr. Pyr(200 mg/kg body wt) or saline was injected intraperitoneally daily for 2 days followed by LPS (10 mg/kg body wt) or saline injection via tail vein. Rats were killed 10 h after treatment with LPS, and protein carbonyls were determined as described in MATERIALS AND METHODS. A lanes are negative controls; B lanes are derivatized samples, respectively. M is a standard molecular weight marker. Results are from 2 separate gels, each carried out with 1 rat from each group (n = 2).
remained lower than that in the combined LPS plus pyrazole group. Control sections incubated without NADPH had no fluorescence (Fig. 7F).

NO, iNOS, and 3-NT. We measured total nitrite plus nitrate to reflect the production of NO. Pyrazole had no effect on production of NO, whereas LPS increased strikingly the production of NO (Fig. 8A). Production of NO also increased in the LPS plus pyrazole group but was slightly lower than the LPS alone group (Fig. 8A). To validate the production of NO, the content of iNOS was measured by Western blot analysis. In either the control or the pyrazole group, iNOS was undetectable; however, iNOS was detected with similar intensity in the LPS and the LPS plus pyrazole groups (Fig. 8B).

3-NT protein adducts were detected by a slot-blot technique. 3-NT adducts were increased in the pyrazole group but surprisingly not in the LPS group, despite the induction of iNOS and elevated production of NO (Fig. 8C). 3-NT adduct levels were highest in the LPS plus pyrazole group (Fig. 8C).

Oxidant defense. The content or the activities of the antioxidant enzymes Cu/Zn-SOD, HO-1, catalase, GSH-Px, GST, and GR were measured. LPS had no effect on catalase activity, whereas pyrazole treatment decreased catalase activity about eightfold compared with the saline control group. Catalase activity was further decreased another twofold in the LPS plus pyrazole group compared with pyrazole group (Fig. 9A). The level of Cu/Zn-SOD was decreased in the LPS or the pyrazole group and dramatically decreased in the LPS plus pyrazole group (Fig. 9D). HO-1 was comparably elevated in all treatment conditions.

Fig. 7. In situ detection of superoxide in livers after treatment with pyrazole, LPS, or LPS + pyrazole. In control group (A), fluorescence was weak but intensified in the LPS (C) and pyrazole (B) group. The strongest intensity was found in the LPS + pyrazole group (D). The fluorescence in the LPS + pyrazole group was maximal around the central vein (E). Control section (F) shows liver section of the LPS + pyrazole group incubated without NADPH; no fluorescence is observed.
groups compared with the saline controls (Fig. 9C). HO-1 is known to be transcriptionally upregulated as an adaptive response to oxidant stress (12). There were no differences in GSH-Px, GST, and GR between all groups (data not shown). Similar to HO-1, levels of GSH in the liver were increased in all treatment groups (Fig. 9B), suggesting some compensation or adaptive reaction occurred. Pyrazole treatment was previously shown to upregulate GSH synthesis (44).

CYP2E1 catalytic activity. Treatment with pyrazole increased CYP2E1 catalytic activity about two times, whereas LPS treatment slightly but not significantly decreased CYP2E1 activity (Fig. 10A). CYP2E1 activity was also increased by the combined treatment of pyrazole and LPS (Fig. 10A). CYP2E1 protein expression by Western blot analysis showed the same trends as activity among the different treatments, namely, increased CYP2E1.
content after pyrazole or LPS plus pyrazole treatment but not after LPS treatment (Fig. 10B). In addition, in situ distribution of CYP2E1 was evaluated by immunohistochemistry. CYP2E1 was distributed mainly around the central veins; interestingly, most necrotic foci were located in the same area as CYP2E1 (Fig. 11).

DISCUSSION

The mechanisms by which alcohol causes cell injury are still not certain. Oxidative stress has been suggested to play a central role in mechanisms of alcohol-induced damage. Many pathways have been suggested to play a key role in how oxidative stress is induced by ethanol; for example, redox state changes, acetaldehyde formation, mitochondrial damage, Kupffer cell activation by LPS, induction of CYP2E1, and others. Many of these pathways are not exclusive of one another, and it is likely that many systems contribute to the ability of ethanol to induce a state of oxidative stress (9, 29).

Among these pathways, possible synergistic effects of LPS and CYP2E1 on induction of oxidative stress and liver injury would be of interest to evaluate. CYP2E1 can be induced by ethanol, exhibits enhanced NADPH oxidase activity, and elevated rates of production of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) (5, 18, 49, 60) even in the absence of substrate. CYP2E1-mediated oxidative stress was suggested to play an important role in alcohol liver disease (8, 29), and inhibition of CYP2E1 may be a strategy for minimizing the hepatotoxicity of ethanol (39). LPS is produced by gut microflora; ethanol increases the permeability of the gut, thereby increasing the transfer of LPS from the gut into the portal circulation (endotoxemia). LPS activates Kupffer cells to produce cytokines such as TNF-\( \alpha \) and \( \text{O}_2^- \) via NADPH oxidase (65). In theory, it is reasonable to hypothesize that addition of LPS to organisms with elevated CYP2E1 causes an increase in oxidative stress and consequently enhanced liver injury. Indeed, the combination of LPS and chronic ethanol produce hepatic necrosis and inflammation (3, 25).

In the present study, we evaluated whether LPS-induced liver damage is enhanced by pretreatment of rats with pyrazole, an inducer of CYP2E1. The results showed that LPS-induced liver damage in rats was enhanced by the pretreatment with pyrazole. On the basis of the increases in ALT and AST and the histology, the liver injury appears to be necrotic. Apoptosis in addition to necrosis appears to be occurring in the LPS plus pyrazole group, because TUNEL and caspase activities were also elevated. Usually, cells undergoing apoptosis do not show inflammation, because apoptotic cells are engulfed by phagocytes before they break down and induce inflammation. A transition from apoptosis to necrosis may happen when an imbalance between the rate of apoptosis and the rate of phagocytosis occurs. When the number of apoptotic cells in liver exceeds phagocytosis capacity, the apoptotic cells break down and induce inflammation (36, 45). In the current study, treatment with LPS alone induced apoptosis (positive TUNEL staining and elevated activity of caspase-3 and -9) but did not induce necrosis (no elevated serum levels of ALT and AST or focal necrosis and inflammatory cell infiltration). It is suggested that, under the current study conditions, the rate of apoptosis induced by LPS was not sufficient to cause necrosis. LPS injection following the pretreatment with pyrazole induced elevated serum levels of ALT and AST and focal necrosis and inflammatory cell infiltration but did not further increase the number of positive TUNEL-staining cells and activity of caspase-3 and -9. In other experiments, liver injury was evaluated in pyrazole rats treated for longer times than the 10 h after LPS addition shown in this report. The enhanced injury was necrotic not apoptotic in nature, and by 24 h after addition of LPS to pyrazole-treated rats, all the rats died. Therefore, it appears that the pretreatment with pyrazole enhanced LPS-induced liver injury through enhancing necrosis but not apoptosis.

Oxidative stress appears to be involved in alcoholic liver disease (2). Is oxidative stress involved in the enhanced LPS plus pyrazole-induced liver damage? Excessive formation and release of ROS and decreased activities of antioxidant enzymes represent the predominant components of oxidative stress, frequently resulting in LPO and protein carbonyl formation. Carbonyl formation is an early marker for protein oxidation (16). In the present study, when obvious liver damage was observed, increased LPO and protein oxidation (protein carbonyl) beyond the increases produced by the individual treatments were found; production of \( \text{O}_2^- \) also increased, and activity or content of major antioxidant enzymes, such as Cu/Zn-SOD and catalase, decreased dramatically. This suggests that oxidative stress may be involved in the enhancement of LPS hepatotoxicity by the pyrazole pretreatment. However, the effects on antioxidant status are complex, because certain antioxidants such as GSH and HO-1 were elevated by pyrazole or LPS alone and remained elevated in the combined LPS plus...
pyrazole group. Possible protection by antioxidants are a future goal of this work to further evaluate the role of oxidative stress in the LPS plus pyrazole injury.

With respect to nitrosative stress, the role of NO in LPS-induced liver injury remains controversial. Some studies suggest that excessive formation of NO can result in oxidative stress in the liver (53). iNOS may be required for the pathogenesis of early alcoholic liver disease via the production of NO-derived prooxidants (40). However, other reports showed that NO has a protective effect as the production of NO improved the microcirculation and oxygen metabolism (15, 52). Moreover, addition of the NO precursor arginine to the diet, protected against alcohol-induced liver injury (43). NO was reported to not be involved in LPS-induced cell damage (33). In the current study, LPS alone enhanced production of NO and induction of iNOS; whereas this may be important for the LPS-induced apoptosis, no obvious liver tissue necrosis was observed. After pretreatment with pyrazole, LPS induced liver tissue necrosis; however, there was no further increase in NO production or iNOS levels. LPS-induced liver damage may be dependent on the balance of the local production of NO and ROS such as $O_2^{-}$. Peroxynitrite (ONOO$^{-}$), formed by the rapid reaction between NO and $O_2^{-}$, has been shown to nitrate free and protein-associated tyrosine residues and produce nitrotyrosine (27). Nitrite is another major oxidation product derived from NO that can be oxidized by peroxidases to form reactive nitrogen intermediates that are capable of nitrating tyrosine (63). Because 3-NT adducts were not increased after LPS treatment, despite the increase in NO and iNOS, ONOO$^{-}$ does not appear to play a role in the LPS-induced apoptosis. Failure to detect enhanced 3-NT adducts after LPS alone treatment despite the increase in iNOS and NO production could reflect the small increase in $O_2^{-}$ production by LPS treatment (Fig. 7C), making the latter rate limiting for ONOO$^{-}$ formation. That tyrosine nitration might not be involved in LPS-induced liver damage was reported previously (4). Treatment with pyrazole alone did not induce obvious liver damage, although formation of 3-NT was increased, and the increased 3-NT formation may be due to a striking decrease in catalase, because catalase can prevent ONOO$^{-}$ formation (30). The highest levels of 3-NT adducts were found in the combined LPS plus pyrazole group; whether this plays a role in the oxidative stress and enhancement of liver damage in the LPS plus pyrazole group? Treatment with LPS suppresses CYP2E1 at
the transcription and translation level (11). Although the amount of CYP2E1 was slightly decreased by LPS alone, the levels of CYP2E1 in the LPS plus pyrazole group still remained higher than in the saline control group. Because CYP2E1 is an active producer of ROS, it is reasonable to propose that the elevated production of O$_2^-$ in the LPS plus pyrazole group is related, at least in part, to elevated CYP2E1. On LPS treatment, CYP2E1 levels were already elevated by the 2 days of prior pretreatment with pyrazole, so onset of LPS pathogenesis may occur earlier in the combined group than in the LPS alone group. Therefore, it is interesting to postulate that, when CYP2E1 is elevated, further treatment with LPS causes an additional increase in production of O$_2^-$ and other ROS (as well as NO and cytokines), which ultimately causes liver tissue necrosis. Pyrazole has long been known to inhibit catalase, via production of its metabolite 4-hydroxy-pyrazole, produced from the oxidation of pyrazole by cytochrome P-450s (21, 14). Does the pyrazole-induced decrease in activity of catalase alone sensitize the rats to LPS-induced oxidative stress, independent of CYP2E1? Catalase removes H$_2$O$_2$; however, GSH and GSH-Px are generally considered to be more important in H$_2$O$_2$ removal (28). GSH-Px activity was not shown). When we used LPS serotype 0128:B12 following pyrazole treatment, whereas in the 50-mg pyrazole group, only one of six rats died 24 h after LPS treatment, whereas in the 50-mg pyrazole group, only one of six rats died 24 h after LPS treatment. Therefore, the decrease in catalase activity may contribute but is not the major reason for the enhancement of LPS toxicity by the pyrazole treatment. However, further studies with CYP2E1 inhibitors or CYP2E1 knockout mice are needed to directly address the role of CYP2E1.

Pyrazole is an effective inducer of CYP2E1, and it has been used to study the role of CYP2E1 in oxidative stress-related cell toxicity. Pyrazole was reported to increase serum ALT levels in mice even at 50 mg/kg body wt, and ultrastructural changes in pyrazole-treated hepatocytes are indicative of oxidative injury (24). However, in the present study, pyrazole injected intraperitoneally to rats at 200 mg/kg body wt caused minimal increase in serum AST and ALT levels and did not produce significant pathology changes compared with saline-treated rats. CYP2E1 activity was increased about two times the same as in the LPS plus pyrazole group, which did exhibit liver injury. Clearly, the induction of CYP2E1 alone is not sufficient to promote liver injury but rather may prime or sensitize the liver to other insults such as LPS.

The serotype of LPS may be important in the enhanced liver damage caused by pretreatment with pyrazole. In initial experiments, we injected LPS serotype 0128:B12 following pyrazole treatment, but enhanced liver damage was not observed (data not shown). When we used LPS serotype 055:B5, enhanced liver damage was observed.

In conclusion, in the present study, a model to elicit enhanced LPS-induced liver damage in rats pretreated with pyrazole to elevate CYP2E1 was developed. The enhancement of liver damage reflected liver tissue necrosis and not elevated apoptosis. Injury appeared to be related to oxidative, and perhaps nitrosative, stress or a mixture of both. CYP2E1 may play a role in the enhancement of injury in this model; however, further studies are needed to clarify the enhancement mechanism. It is hoped that this model may provide information as to factors that contribute to the mechanisms which cause alcoholic liver disease.

ACKNOWLEDGMENTS

We thanks Dr. R. Ma for assistance with paraffin and frozen section preparation.

GRANTS

These studies were supported by United States Public Health Service Grant AA-03312 from the National Institute on Alcohol Abuse and Alcoholism.

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