NADPH oxidase-derived oxidant stress is critical for neutrophil cytotoxicity during endotoxemia

Jaspreet S. Gujral,1,2 Jack A. Hinson,1 Anwar Farhood,3 and Hartmut Jaeschke1,2
1Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205; 2Liver Research Institute, University of Arizona, Tucson, Arizona 85724; and 3Department of Pathology, University of Texas Health Science Center, Houston, Texas 77030

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Gujral, Jaspreet S., Jack A. Hinson, Anwar Farhood, and Hartmut Jaeschke. NADPH oxidase-derived oxidant stress is critical for neutrophil cytotoxicity during endotoxemia. Am J Physiol Gastrointest Liver Physiol 287: G243–G252, 2004. First published March 25, 2004; 10.1152/ajpgi.00287.2003.—Neutrophils can cause liver injury during endotoxemia through generation of reactive oxygen species. However, the enzymatic source of the oxidant stress and the nature of the oxidants generated remain unclear. Therefore, we investigated the involvement of NADPH oxidase in the pathophysiology by using the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) in the galactosamine/endotoxin (700 mg/kg Gal:100 μg/kg ET) model of liver injury. In addition, we measured chlorotyrosine as an indicator for hypochlorous acid formation by myeloperoxidase. Gal/ET treatment of male C3HeB/FeJ mice resulted in sinusoidal neutrophil accumulation and parenchymal cell apoptosis (14 ± 3% of cells) at 6 h. At 7 h, 35% of neutrophils had transmigrated. The number of apoptotic cells increased to 25 ± 2%, and the overall number of dead cells was 48 ± 3%; many of them showed the characteristic morphology of necrosis. Hepatocytes, which colocalized with extravasated neutrophils, stained positive for chlorotyrosine and 4-hydroxynonenal (4-HNE) protein adducts. In contrast, animals pretreated with DPI (2.5 mg/kg) were protected against liver injury at 7 h (necrosis = 20 ± 2%). These livers showed little chlorotyrosine or 4-HNE staining, but apoptosis and neutrophil accumulation and extravasation remained unaffected. However, DPI-treated animals showed serious liver injury at 9 h due to sustained apoptosis. The results indicate that NADPH oxidase is responsible for the neutrophil-derived oxidant stress, which includes formation of hypochlorous acid by myeloperoxidase. Thus NADPH oxidase could be a promising therapeutic target to prevent neutrophil-mediated liver injury. However, the long-term benefit of this approach needs to be investigated in models relevant for human liver disease.

chlorotyrosine protein adducts; apoptotic cell death; diphenyleneiodonium chloride; neutrophil-induced liver injury; 4-hydroxynonenal

DURING AN ACUTE INFLAMMATORY response, polymorphonuclear leukocytes (neutrophils) can accumulate in different vascular beds of the liver, i.e., sinusoids, postsinusoidal venules, and portal venules (8, 16, 39, 64). Cytokines (TNF-α, IL-1), activated complement factors, and to some degree CXC chemoattractants are able to cause systemic neutrophil activation and can trigger their accumulation in the liver (1). Although the main function of the neutrophil response is to remove foreign particles (e.g., bacteria) and necrotic cells, under certain conditions neutrophils can severely aggravate liver injury. Neutrophil-induced liver cell dysfunction or injury has been documented during hepatic ischemia-reperfusion (38), endotoxemia (39), sepsis (58), alcoholic hepatitis (5), hemorrhagic shock (4), obstructive cholestasis (20), remote organ damage (28), and certain drug toxicities (11). However, the fact that neutrophils accumulate in the liver does not necessarily mean that they cause injury (8, 48). If the vascular endothelial cell layer is intact, neutrophil cytotoxicity requires transmigration and adherence to the hepatocytes (8). The transmigration process involves integrins on neutrophils and the corresponding counterreceptors ICAM-1 or VCAM-1 on endothelial cells (14, 15). For neutrophils to transmigrate, a signal from the parenchyma is necessary. This signal can be CXC chemokines (52) or can be other, as yet poorly characterized factors released from hepatocytes undergoing apoptotic (40, 49) or necrotic cell death (34, 51). Adhesion of neutrophils to hepatocytes is also dependent on β2-integrin-ICAM-1 interactions (7, 59), which trigger degranulation and the long-lasting formation of reactive oxygen species (ROS) (35, 63).

Until recently, it was unclear whether neutrophils are able to kill hepatocytes by reactive oxygen. The results from in vitro coculture experiments suggested that neutrophils cause hepatic cellular injury only through proteases without the involvement of ROS (17, 24, 56). Because ROS were shown to be important in the pathophysiology under in vivo conditions (43), it was hypothesized that ROS may support a protease-mediated cell killing by inactivating antiproteases (43, 66). In contrast, more recently we provided evidence for an intracellular oxidant stress in hepatocytes during neutrophil cytotoxicity in vivo (39, 41) and showed that glutathione peroxidase-1 gene knockout mice are considerably more sensitive to neutrophil-mediated injury than wild-type animals (41). These findings suggested that neutrophils are able to kill hepatocytes in vivo by ROS formation. However, a limitation of this study (41) is the fact that the source of ROS formation was not defined. The intracellular oxidant stress as measured by increased formation of glutathione disulfide could have been the result of the diffusion of neutrophil-derived hydrogen peroxide into the cell. Alternatively, the oxidant stress could have been generated by an intracellular source, e.g., mitochondria, as a consequence of cell injury (42, 60). Therefore, we tested the hypothesis that neutrophils are the primary source of the oxidant stress in the galactosamine-endotoxin (Gal/ET) shock model by using diphenyleneiodonium chloride (DPI), an inhibitor of NADPH oxidase in neutrophils and macrophages (10, 23, 61). Previous studies (45) showed that treatment with

Address for reprint requests and other correspondence: H. Jaeschke, Liver Research Institute, Univ. of Arizona College of Medicine, 1501 N. Campbell Ave, Rm. 6309, Tucson, AZ 85724 (E-mail: Jaeschke@email.arizona.edu).

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DPI attenuated alcohol-induced liver injury. In addition, NADPH oxidase-deficient mice suffered less liver damage after hemorrhagic shock and resuscitation (50). To assess specifically the role of neutrophil NADPH oxidase in the Gal/ET model, we investigated the formation of chlorotyrosine protein adducts, which are a footprint for the generation of hypochlorous acid by the neutrophil-derived myeloperoxidase (13, 26, 44).

MATERIALS AND METHODS

Animals. Male C3HeB/Fej mice (20–30 g body wt) were purchased from Jackson Laboratories (Bar Harbor, ME). The animals had free access to food (certified rodent diet #5002C; PMI Feeds, Richmond, IN) and water. The experimental protocols followed the criteria of the University of Arkansas for Medical Sciences and the National Research Council for the care and use of laboratory animals in research. Animals received an intraperitoneal injection of 100 µg/kg Salmonella abortus equi ET alone or in combination with 700 mg/kg Gal (Sigma, St. Louis, MO). Some animals from this group were either treated intraperitoneally with the pancaspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk, 10 mg/kg; Enzyme Systems Products, Dublin, CA) or the vehicle (0.2 ml phosphate-buffered saline) at 3 and 4.5 h after Gal/ET treatment. The animals were killed by cervical dislocation at 6 or 7 h. In another experiment, some animals were either subcutaneously injected with two doses of DPI (2.5 mg/kg; Sigma) or the vehicle (0.2 ml of 5% glucose) 24 and 1 h before Gal/ET injection. These animals were killed 5, 7, or 9 h after Gal/ET treatment. Blood samples were taken from the inferior vena cava with a heparinized syringe. Plasma was used for measurement of TNF-α and alanine transaminase (ALT) levels. Samples of the liver were homogenized immediately for caspase-3 activity measurements, snap-frozen in liquid nitrogen, or fixed in phosphate-buffered formalin.

Methods. To assess liver cell injury, ALT activities were measured in plasma by using the test kit DG 159-UV (Sigma). TNF-α levels were measured in plasma by using the ELISA-based Quantikine Mouse TNF-α kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. ATP and hemoglobin levels were measured in liver homogenates by using the ATP bioluminescent assay kit (Sigma) and the hemoglobin reagent set (Biotron Diagnostics, Hemet, CA), respectively. Caspase-3 activities were determined as described in detail (40) with some modifications (22). Briefly, liver samples were homogenized in 25 mM HEPES buffer (pH 7.5) containing 5 mM EDTA, 2 mM DTT, and 0.1% CHAPS. The homogenized samples were centrifuged at 14,000 g, and the diluted supernatant was assayed for the Z-VAL-FMK-inhibitable portion of the caspase activity by using the synthetic fluorogenic caspase-3 (CPP32) substrate acetyl-Asp-Glu-Val-Asp-7-amide (Peptide Institute, Osaka, Japan) at concentrations of 50 µM. The samples were assayed in duplicate wells, with or without 10 µM Z-VAL-FMK, and the kinetics of the proteolytic cleavage of the substrate was monitored in a fluorescence microplate reader (Cytofluor 2350; Millipore, Bedford, MA) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Caspase-3 activity was calculated from the slope of the recorder trace and expressed in change in fluorescence per minute per milligram of protein. Protein concentrations in the supernatant were assayed by using the bichinonic acid kit (Sigma).

Histology. Formalin-fixed tissue samples were embedded in paraffin, and 5-µm sections were cut. Liver sections were stained with hematoxylin and eosin (H&E) for evaluation of liver cell injury. In addition, sections of the livers were stained with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay as described in the manufacturer’s instructions (Roche Molecular Biochemicals, Indianapolis, IN). The number of apoptotic hepatocytes was quantified in these sections on the basis of positive TUNEL staining and morphological criteria, as described previously (19, 21, 22). The morphological criteria for apoptosis used to identify cells were cell shrinkage, chromatin condensation and/or margination, and the formation of apoptotic bodies. Apoptotic hepatocytes were counted individually in 10 high-power fields (HPF) and expressed as a percentage of the total number of parenchymal cells evaluated. Preliminary counts in control livers revealed that this area contains an average of 1,800 hepatocytes. Total cell death was evaluated in replicate sections stained with H&E. The percentage of total cell death, including both apoptosis and necrosis, was estimated by evaluating the number of microscopic fields, with cell death compared with the entire histological section (19, 21, 22). The following criteria were used for identifying a necrotic cell: increased eosinophilia, cell swelling, cell lysis, karyorrhexis, and karyolysis. All quantitations were done in a blinded fashion.

Immunohistochemistry for 4-hydroxynonenal adducts. Formalin- and paraffin-embedded liver sections were immunostained for 4-hydroxynonenal (4-HNE) adducts. After deparaffinization and rehydration, the endogenous peroxidase activity was quenched with the Immupure peroxidase suppressor (Pierce) for 1 h. This was followed by incubation with a protein block (Dako, Carpinteria, CA) for 7 h to block nonspecific binding. The sections were then incubated overnight with the primary antibody (rabbit anti-HNE-Michael adducts; Calbiochem, San Diego, CA) at a 1:100 dilution. Color development was done using the labeled streptavidin-biotin (LSAB) peroxidase kit specific for rabbit primary antibodies (Dako), according to the manufacturer’s instructions. AEC chromogen supplied in the kit was used as the substrate for the peroxidase. The sections were counterstained with Mayer’s hematoxylin (Sigma) and mounted with Faramount aqueous mounting medium (Dako).

Immunohistochemistry for chlorotyrosine-protein adducts. Protein adducts of chlorotyrosine were detected by immunohistochemistry. Paraffin-embedded liver sections were deparaffinized and rehydrated through a series of graded ethanol washes. The endogenous peroxidase activity was quenched, and then a protein block was added, as described above. The sections were then incubated for 60 min at room temperature with a rabbit anti-chlorotyrosine primary antibody. The antibody was generated with standard procedures as described in Immunization procedure using 3-chloro-4-hydroxybenzoic acid-keyhole limpet hemocyanin (KLH) as antigen. For color development, the suggested protocol described in the Dako LSAB peroxidase kit was followed. The slides were counterstained with Gills Hematoxylin II (Fisher Scientific, Houston, TX) and mounted. To ensure specificity for detection of chlorotyrosine, some sections were stained with the primary antibody, which had previously been incubated with the antigen (3-chloro-4-hydroxybenzoic acid-KLH) for 1 h at 37°C. To check whether the primary or secondary antibody bound nonspecifically to dead or injured areas in the tissue, liver sections from mice treated with 300 mg/kg acetaminophen for 6 h (22) were stained. No positive staining was seen in any of these control sections.

Quantitation of hepatic neutrophil infiltration. Liver sections were stained with the anti-Gr1 monoclonal antibody (Pharmingen, San Diego, CA) for evaluation of neutrophil infiltration into mouse livers. After the sections were deparaffinized and rehydrated, they were incubated with a protein block (Dako). This was followed by incubation with the rat anti-mouse Gr1 primary antibody (5 µg/ml) for 1 h at room temperature. The color was developed by using Vectastain, an avidin-biotin-conjugated alkaline phosphatase kit (Vector Laboratories, Burlingame, CA) and the Vector Red Substrate kit. Sections were counterstained using Gills Hematoxylin II and then mounted. Neutrophil accumulation in the liver was quantified by counting the total number of neutrophils in 10 HPF. The total number of neutrophils, i.e., cells localized in sinusoids as well as extravasated into the tissue, was counted. For colocalization of infiltrated neutrophils and chlorotyrosine-protein adducts, sections were immunostained sequentially for the presence of chlorotyrosine adducts and then for neutrophils by using the anti-chlorotyrosine and anti-Gr1 antibodies, respectively.
significantly increased plasma ALT levels. The total cell death be seen in liver sections stained with H&E. Gal/ET treatment for 7 h (Fig. 1).

RESULTS

Synthesis of immunogen and solid phase antigen. The immunogen 3-chloro-4-hydroxybenzoic acid-KLH was synthesized by a two-step method as described (12). Briefly, synthetic 3-chloro-4-hydroxybenzoic acid (27.5 mg) was dissolved in 2.5 ml of methanol and combined with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in 2.5 ml of 0.02 M potassium phosphate buffer (pH 8.0). This mixture was then added to a suspension of KLH (20 mg) in 8 ml of 0.2 M potassium phosphate buffer (pH 8.0) and was allowed to react at room temperature overnight. Subsequently, the incubation was dialyzed against 10 mM potassium phosphate-buffered saline (pH 7.2) with buffer changes at 2, 4, and 12 h to remove the resulting urea and unreacted 3-chloro-4-hydroxybenzoic acid. 3-Chloro-4-hydroxybenzoic acid was also attached to BSA by an identical procedure using BSA in place of KLH to produce a solid-phase antigen.

Immunization procedure. As previously described (30, 55), a rabbit was immunized with 380 μl of 3-chloro-4-hydroxybenzoic acid-KLH (500 μl) emulsified in 3 volumes of Freund’s complete adjuvant. The mixture was administered by subcutaneous injection at multiple sites along the back and by one intramuscular injection in each hindquarter. Five booster injections were administered at 4-wk intervals following the primary immunization. The booster injections consisted of 500 μg of 3-chloro-4-hydroxybenzoic acid-KLH immunogen in Freund’s incomplete adjuvant, using the original injection scheme. Seven to ten days after each injection, ~15 ml of arterial blood were collected from the rabbit ear and allowed to clot at room temperature. Serum was separated by centrifugation at 100 g for 20 min at 4°C. The single rabbit produced excellent antisera, and additional rabbits were not immunized. For characterization of the anti-chlorotryptosine antisera ELISAs were performed using 3-chloro-4-hydroxybenzoic acid as the solid antigen on Immunolog II 96-well plates as previously described (30, 55). These data indicated that dilution of the antisera at a concentration of 1:256,000 and greater gave absorbance using solid-phase antigens at concentrations of 3.125–50 μg/ml. At a lower dilution of antisera (1:1,024,000) absorbances were not increased over that of preimmune serum.

Statistics. Data are given as means ± SE. Comparisons between multiple groups were performed with one-way ANOVA followed by Bonferroni’s t-test. If the data were not normally distributed, the Kruskal-Wallis Test (nonparametric ANOVA) followed by Dunn’s multiple comparisons test was performed. P < 0.05 was considered significant.

RESULTS

Effect of DPI on Gal/ET-induced liver injury. Plasma ALT levels and total cell death were determined as indicators of liver cell injury after Gal/ET treatment for 7 h (Fig. 1). Untreated controls had low ALT levels, and no necrosis could be seen in liver sections stained with H&E. Gal/ET treatment significantly increased plasma ALT levels. The total cell death in these livers was 48 ± 3% of all hepatocytes. The pancaspase inhibitor Z-VAD-fmk effectively protected against liver cell injury as evidenced by >90% reduction in both plasma ALT levels and total cell death. Treatment with the NADPH oxidase inhibitor DPI prevented the increase of plasma ALT activities. However, total cell death was only reduced to 20 ± 2%, with apoptotic hepatocytes constituting most of the injured cells (Fig. 1).

Liver sections were stained with H&E to assess the mode of liver cell death. Controls had no necrosis in their livers, and apoptotic hepatocytes were rare. They had normal tissue architecture with no congestion in the sinusoids (Fig. 2A). After Gal/ET treatment numerous cells could be seen undergoing apoptosis (Fig. 2B). There were also large areas consisting of cells undergoing necrosis. A number of neutrophils were observed in the sinusoids as well as extravasated into the parenchymal tissue. Liver sections from mice treated with the pancaspase inhibitor Z-VAD-fmk showed very few cells undergoing apoptosis or necrosis (Fig. 2C). Neutrophils were observed in these livers too. However, most of them were seen inside sinusoids with very little extravasation. On the other hand, although DPI pretreatment reduced the extent of necrosis in the livers, it did not affect the extent of apoptosis or the neutrophil accumulation in the sinusoids or their extravasation into the tissue (Fig. 2D).

Effect of DPI on plasma TNF-α levels after Gal/ET treatment. Because endotoxemia is a model of TNF-induced hepatocellular apoptosis and TNF-dependent inflammation, plasma levels of this cytokine were measured. In untreated controls and in animals injected with DPI alone plasma TNF-α levels were low (7 ± 1 pg/ml). After Gal/ET treatment for 1.5 h, TNF-α concentrations increased to 1.696 ± 134 pg/ml. Mice treated with Gal/ET and DPI had similar peak plasma levels of TNF-α (1.339 ± 95 pg/ml; P > 0.05). Because the first dose of Z-VAD-fmk was injected at 3 h after Gal/ET, the pancaspase inhibitor was not able to affect cytokine formation. Effect of DPI on Gal/ET-induced apoptosis. With the use of a fluorogenic substrate, caspase-3 activities were measured in AJP-Gastrointest Liver Physiol • VOL 287 • JULY 2004 • www.ajpgi.org
liver homogenates and plasma as indicators of hepatocellular apoptosis. In addition, an increase in plasma caspase-3 activities signifies the release of cell contents into the blood when the cell undergoes lysis during secondary necrosis or neutrophil-induced cell injury (21). Controls had a low caspase-3 activity in the livers (Fig. 3A). No activity was detected in the plasma from these animals (Fig. 3B). However, at 7 h after Gal/ET, caspase-3 activities were dramatically increased in both the liver and plasma (Fig. 3). The pancaspase inhibitor Z-VAD-fmk reduced the activity by almost 100% in both livers and plasma. As expected, DPI pretreatment did not significantly affect hepatic caspase-3 activities. However, in parallel to reduced plasma ALT levels and necrotic cell death, it reduced the plasma caspase-3 activities by >90%. Some caspase-3 activities were still detectable in the plasma, probably owing to secondary necrosis.

In addition to caspase activities, we quantified hepatocellular apoptosis in liver sections stained with the TUNEL assay. Apoptotic cells were identified by using morphological criteria in combination with positive TUNEL staining. In controls, 0.3 ± 0.1% of all hepatocytes were seen to be apoptotic (Fig. 4). Seven hours after Gal/ET treatment, ~25% of all hepatocytes were undergoing apoptosis. In concordance with the reduced caspase-3 activities, the pancaspase inhibitor Z-VAD-fmk reduced apoptotic cell death in the livers by 87% compared with Gal/ET alone. On the other hand, DPI did not have a significant affect on apoptotic cell death in these livers (Fig. 4).

Effect of DPI on hepatic neutrophil accumulation and oxidant stress after Gal/ET treatment. To cause liver cell injury, neutrophils have to extravasate into the parenchyma (8). We quantified the accumulation of neutrophils in the sinusoids and their extravasation in liver sections immunostained with the anti-Gr1 antibody (Fig. 5). The data are expressed as the total number of neutrophils in liver tissue and the number of extravasated neutrophils per 10 HPF. Very few neutrophils

Fig. 2. Histological assessment of liver injury in mice 7 h after G/ET treatment. Groups of animals were either injected with the pancaspase inhibitor Z-VAD-fmk (10 mg/kg) at 3 and 4.5 h or pretreated with DPI (2.5 mg/kg). Liver sections were stained with hematoxylin and eosin. Controls had normal tissue architecture with no congestion in the sinusoids (A). After G/ET treatment, numerous cells (arrows) could be seen undergoing apoptosis (B). There were also large areas consisting of cells undergoing necrosis (arrowheads). There were a number of neutrophils observed in the sinusoids as well as extravasated into the parenchymal tissue. Liver sections from mice treated with the pancaspase inhibitor Z-VAD-fmk showed very few cells undergoing apoptosis or necrosis (C). Neutrophils were observed in these livers. However, most of them were seen inside sinusoids with very little extravasation. On the other hand, although DPI pretreatment reduced the extent of necrosis in the livers, it did not affect the extent of apoptosis (arrows), the neutrophil accumulation in the sinusoids, or their extravasation into the tissue (D). Magnification for all photomicrographs, ×600.
were seen in sinusoids of controls, with virtually no extravasated cells (Fig. 5). After Gal/ET treatment for 7 h, the total number of neutrophils in these livers increased to 309 ± 40 per 10 HPF. Of these, 35% were observed to be extravasated into the tissue. In contrast, treatment with Z-VAD-fmk reduced the neutrophil extravasation to 5% (an 86% reduction from Gal/ET at 7 h). However, it did not significantly affect the total number of neutrophils accumulating in the liver. On the other hand, DPI did not affect either the extent of extravasation or the total number of neutrophils accumulating in the liver (Fig. 5).

To assess the neutrophil-induced oxidant stress, liver sections were immunostained with the anti-chlorotyrosine antibody to monitor the formation of chlorotyrosine-protein adducts in the liver as an indicator of neutrophil-derived hypochlorous acid. No positive staining was observed in control livers (Fig. 6A) or 6 h after Gal/ET (Fig. 6B), a time point at which most neutrophils are present in sinusoids (8, 40). After Gal/ET treatment for 7 h, the liver sections showed extensive positive staining for chlorotyrosine adducts inside hepatocytes, as well as along the sinusoids (Fig. 6C). To colocalize neutrophil infiltration and the intracellular oxidant stress induced by them, liver sections of mice treated with Gal/ET for 7 h were stained sequentially for both chlorotyrosine-protein adducts and for neutrophils. The neutrophils (stained red) were observed in large vessels, in sinusoids, and extravasated into the tissue (Fig. 6D). Extravasated neutrophils were found in areas that stained positive for chlorotyrosine-protein adducts (stained brown) inside hepatocytes. Livers treated with Z-VAD-fmk showed substantially reduced staining for chlorotyrosine inside hepatocytes. However, some positive staining was still observed in the sinusoids around the neutrophils (Fig. 6E). A similar effect was seen with DPI pretreatment (Fig. 6F).

As a further indicator of neutrophil-induced oxidant stress, liver sections were immunostained for 4-HNE adducts. Control livers did not show any positive staining (Fig. 7A). After Gal/ET treatment for 7 h, 4-HNE-positive staining was mainly observed inside hepatocytes (Fig. 7B). Similar to their effect on chlorotyrosine adduct formation, both Z-VAD-fmk and DPI reduced the formation of 4-HNE adducts as well (Fig. 7C and 7D).
in a few hepatocytes and slightly positive staining in the sinusoids (data not shown). No 4-HNE adducts were detectable in these livers (data not shown).

Effect of DPI on Gal/ET-induced liver injury at 9 h. Because DPI reduced the inflammatory component of the injury at 7 h after Gal/ET, we investigated whether this translates into improved survival of the animals. Animals treated with Gal/ET alone died between 7 and 8 h. Surprisingly, although the neutrophil-induced cytotoxicity was inhibited almost completely by DPI at 7 h, the animals in this group died a few hours later, between 9 and 11 h. Therefore, DPI-treated groups were further analyzed at 7 vs. 9 h after Gal/ET (Table 1). Plasma ALT levels were 10-fold higher at 9 h compared with the DPI group at 7 h, and there was a more than threefold further increase of hepatic caspase-3 activities (Table 1). Histological assessment of liver sections confirmed extensive liver cell injury (Table 1). Although DPI prevented hemorrhage (hepatic Hb accumulation) and attenuated the decline of hepatic A TP levels at 7 h after Gal/ET, hemorrhage still occurred and ATP levels dropped at 9 h (Table 1). To confirm that DPI was still effective at these later time points, liver sections were stained for chlorotyrosine adducts. No positive staining was observed at 9 h after Gal/ET in the DPI-treated group (data not shown).

DISCUSSION

The objectives of this investigation were to test 1) whether inhibiting NADPH oxidase would protect against neutrophil-induced oxidant stress and liver injury and 2) whether chlorotyrosine and 4-HNE adduct formation in the liver could be used as markers for neutrophil-induced reactive oxygen formation and cytotoxicity in vivo. Our approach was to correlate immunostaining for chlorotyrosine and 4-HNE adducts with known neutrophil cytotoxicity in the well-characterized Gal/ET model of liver injury by using DPI (a NADPH oxidase inhibitor) on one hand and the pancaspase inhibitor Z-VAD-fmk on the other.

Administration of Gal/ET to mice induces the formation of TNF-α and other cytokines, which are responsible for systemic neutrophil activation (1, 15) and hepatic neutrophil sequestration between 1 and 4 h (15, 62). At 5–6 h, ~15% of parenchymal cells undergo apoptosis (40). At that point some of the neutrophils transmigrate, and the injury is drastically increased to 40–50% overall cell death by 7 h (Fig. 1) (40, 49). In contrast to the 6-h time point, a large number of cells appear to have necrotic morphology at 7 h, i.e., eosinophilia, cell swelling, vacuolation, and karyolysis. If parenchymal cell apoptosis is prevented by various caspase inhibitors (Figs. 3 and 4) (3, 36, 40), uridine (49), or injection of ET without Gal (8), transmigration of neutrophils does not occur and any injury is eliminated. The conclusion that the transmigrated neutrophils are responsible for the necrotic damage is based on a number of observations: 1) antibodies against the β2-integrin Mac-1 (CD11b) and the common β-subunit CD18 strongly attenuated the oxidant stress and injury (39), which is consistent with the known function of the Mac-1 receptor to trigger an adherence-dependent oxidant stress. 2) Antibodies against ICAM-1 and VCAM-1 prevented neutrophil transmigration and reduced the injury from 40–50% necrosis to 15–20% (14, 15); this means that the neutrophil-mediated damage is prevented without affecting the apoptotic cell death. 3) Mice deficient in gluta-
thione peroxidase-1, a major detoxifying enzyme for peroxidides, are selectively more susceptible to neutrophil-induced injury (41) but not the apoptotic cell death (2). Together, this is enough indirect evidence in this model to suggest that neutrophils attack and kill hepatocytes by generating ROS after they have transmigrated. Our present data are consistent with these findings and expand them by directly demonstrating the critical involvement of NADPH oxidase and myeloperoxidase in the injury process. NADPH oxidase has also been shown to mediate other forms of liver injury such as hemorrhagic shock (50) and alcohol-induced liver injury (45). However, these studies did not differentiate between Kupffer cells and neutrophils as the source of NADPH oxidase-derived superoxide.

DPI belongs to a group of iodonium compounds that are inhibitors of flavin enzymes. Therefore, DPI reduces the activities of NADPH-dependent oxidase in neutrophils and macrophages (10, 23, 61). DPI pretreatment protected the Gal/ET-treated mice from neutrophil-induced liver injury and reduced markers of oxidant stress such as formation of chlorotyrosine and 4-HNE adducts in the liver. However, it did not affect plasma TNF-α levels, hepatocellular apoptosis, or the accumulation of neutrophils in sinusoids and their extravasation into the tissue. DPI may also inhibit several other hepatic radical-generating enzymes, such as nitric oxide synthase, NADPH-cytochrome P-450 reductase, and mitochondrial oxidases (46, 57, 65). However, the fact that with DPI treatment extravasated neutrophils could not cause an oxidant stress in hepatocytes strongly suggests that the protective mechanism of DPI was through the inhibition of NADPH oxidase. Moreover, there is no evidence in the literature that any of the other enzyme systems may contribute to the injury process in this model. In addition to neutrophils, Kupffer cells (the resident macrophages in the liver) also express NADPH oxidase (23, 45). However, in contrast to other inflammatory liver injury models, such as ischemia-reperfusion (32, 33), Kupffer cell-induced oxidant stress does not contribute to hepatocellular injury in...
our murine endotoxemia model (41). As we previously showed, a Kupffer-derived oxidant stress requires activation of the complement cascade with higher doses of ET than used in the present study (31). Moreover, the presence of chlorotyrosine adducts in endotoxemic livers, the colocalization of these adducts with necrotic areas, and their disappearance with DPI pretreatment strongly suggests a role of the neutrophil NADPH oxidase. The possibility that DPI could have inhibited the release of TNF-α from Kupffer cells in response to ET, and thus prevented the initial apoptosis and/or neutrophil accumulation and extravasation, is also ruled out by the fact that there was no change in plasma TNF-α levels, hepatic cellular apoptosis, and neutrophil accumulation with DPI pretreatment. Thus we conclude from these data that DPI acted by inhibition of the neutrophil NADPH oxidase, which protected against liver injury at 7 h after Gal/ET.

Neutrophils generate superoxide with NADPH oxidase. Superoxide dismutates and forms hydrogen peroxide and molecular oxygen. When fully activated, neutrophils also release the enzyme myeloperoxidase, which uses hydrogen peroxide and chloride to generate hypochlorous acid (13). This is a potent oxidant that can react with lipids, proteins, and nucleic acids. However, many of the reaction products are either unstable or formed in minute quantities. On the other hand, chlorotyrosine-protein adducts are stable and are relatively specific for hypochlorous acid (13, 26). Chlorotyrosine adducts were detected in a number of in vivo and in vitro systems of neutrophil-induced injury (18, 27, 29, 44). In most of these studies, gas chromatography and mass spectrometry were used to specifically detect these adducts. Immunohistochemistry, on the other hand, is an easier and faster method and has been used successfully to detect these adducts in the kidney and in atherosclerotic plaques (25, 53). Similar to chlorotyrosine protein adducts, which are markers for hypochlorous acid formation, detection of 4-HNE protein adducts indicate that lipid peroxidation occurred in these livers. Although the extent of lipid peroxidation is by far not severe enough to cause direct cell injury (54), 4-HNE adducts reflect an oxidant stress and Fenton-type reactions. Interestingly, in a previous paper from our laboratory (41) we measured lipid hydroperoxides, which are precursors of 4-HNE, and could not detect any evidence for lipid peroxidation in this model. Thus the measurement of 4-HNE protein adducts is a more sensitive parameter of lipid peroxidation due to its accumulation and slow turnover.

We detected chlorotyrosine and 4-HNE adducts only at 7 h after Gal/ET treatment, the time point when neutrophils induced an intracellular oxidant stress and cytotoxicity in hepatocytes. In contrast, at earlier time points after Gal/ET (6 h), after ET treatment alone (7 h), or in the presence of the pancaspase inhibitor Z-VA-D-fmk or the NAPDH oxidase inhibitor DPI, neutrophils did not cause cytotoxicity. Under all of these conditions, little or no chlorotyrosine and 4-HNE adducts were detected. Thus we can conclude that immunohistochemical detection of chlorotyrosine protein adducts are specific markers for activated and cytotoxically active neutrophils in liver tissue. 4-HNE protein adducts are useful markers for oxidant stress but can only be used for neutrophils when other sources of reactive oxygen formation have been excluded. As such, these parameters provide additional information about neutrophil activity compared with traditionally used methods to visualize neutrophils. Staining for chloroacetate esterase activity or immunohistochemical detection of integrins, myeloperoxidase, Gr1, or other neutrophil-specific proteins (8, 9, 37) are useful for neutrophil quantitation and localization but do not indicate whether the neutrophil is fully activated. Indeed, most neutrophils accumulated in the liver vasculature have upregulated expression of Mac-1 (67) and are primed for enhanced ROS formation (6, 32, 34) but are not cytotoxicially active. Only after the appropriate signaling from damaged cells do neutrophils extravasate and adhere to parenchymal cells (43). Transmigration and adherence to the target are necessary for the final activation of neutrophils (47), which leads to adherence-dependent oxidant stress and degranulation. Thus chlorotyrosine protein adducts and, under certain circumstances, 4-HNE adducts are cumulative indicators of neutrophil cytotoxicity.

Although DPI protected against Gal/ET-induced liver injury at 7 h, it was unable to prevent progression of liver injury, hemorrhage, and death of the animals at later time points. A substantial further increase in hepatic caspase-3 activities at 9 h compared with 7 h indicated a higher level of apoptosis. Since apoptosis is an energy-dependent process, the low ATP levels at these later time points may have forced most of these apoptotic cells into secondary necrosis. The massive release of ALT at 9 h supports this conclusion. An additional factor contributing to the injury may have been ischemia due to microcirculatory disturbances and hemorrhage. Based on the lack of chlorotyrosine staining in the DPI-treated animals, it can be concluded that the injury did not involve neutrophils. These data are not consistent with the previously assumed killing of healthy hepatocytes by extravasating neutrophils (43). In contrast, these data suggest that there is an ongoing process of hepatocellular apoptosis beginning at 5 h after Gal/ET. This process can be interrupted by extravasating neutrophils, which induce rapid necrosis in these vulnerable cells. Thus many of the apparently healthy cells may actually be in the very early stages of apoptosis. In addition, the injury to endothelial cells leading to hemorrhage still occurred in DPI-treated animals. These observations suggest that endothe-

### Table 1. Effect of DPI treatment on Gal/ET-induced liver injury

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Gal/ET 7 h</th>
<th>Gal/ET 7 h + DPI</th>
<th>Gal/ET 9 h + DPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma ALT activities, IU/L</td>
<td>122 ± 50</td>
<td>2.884 ± 516*</td>
<td>427 ± 123†</td>
<td>4.422 ± 704‡†</td>
</tr>
<tr>
<td>Liver caspase-3, ΔF.min⁻¹.mg⁻¹</td>
<td>65 ± 15</td>
<td>852 ± 143*</td>
<td>970 ± 74*</td>
<td>2.755 ± 301‡‡</td>
</tr>
<tr>
<td>Total cell death, %</td>
<td>0 ± 0</td>
<td>50 ± 5*</td>
<td>23 ± 5*</td>
<td>60 ± 4‡</td>
</tr>
<tr>
<td>ATP, μmol/g liver</td>
<td>2.0 ± 0.1</td>
<td>0.3 ± 0.1*</td>
<td>1.0 ± 0.1†</td>
<td>0.4 ± 0.1‡</td>
</tr>
<tr>
<td>Hemoglobin, mg/liver</td>
<td>2.3 ± 1.4</td>
<td>12.7 ± 3.1*</td>
<td>4.0 ± 1.3†</td>
<td>7.5 ± 0.6‡‡</td>
</tr>
</tbody>
</table>

Animals were killed 7 or 9 h after treatment with 700 mg/kg galactosamine (Gal) and 100 μg/kg endotoxin (ET). Some animals received 2 doses of diphenyleneiodonium chloride (DPI; 2.5 mg/kg) 24 and 1 h before Gal/ET.

Alanine transaminase (ALT) activities were measured in plasma. Caspase-3 activities were determined in liver homogenates by using the fluorogenic substrate acetyl-Asp-Glu-Val-Asp-4-methylcoumaryl-7-amide. Total cell death was estimated in sections stained with hematoxylin and eosin. ATP and hemoglobin levels were determined in liver homogenates by using bioluminescence-based and colorimetric kits, respectively. Data represent means ± SE of 3–8 animals per group. *P < 0.05 vs. controls; †P < 0.05 vs. Gal/ET alone; ‡P < 0.05 vs. Gal/ET 7 h + DPI.
lial cell injury was not caused by neutrophils. Because Gal/ET does not induce caspase activation and apoptosis in sinusoidal lining cells (40), our data implicate an independent, third mechanism of injury that contributes to the overall pathophysiology in this model. Further studies are necessary to investigate this unknown injury mechanism for the sinusoidal lining cells.

In summary, our data showed that NADPH oxidase is the key enzyme responsible for neutrophil-derived oxidant stress during ET-induced liver injury. Furthermore, we showed that chlorotyrosine-protein adducts and 4-HNE adducts could be detected in the liver when neutrophils were extravasated, fully activated, and caused cell injury. Thus hypochlorous acid generated by myeloperoxidase is an important mediator of the neutrophil-induced oxidant stress. In addition, there is evidence that the oxidant stress also leads to lipid peroxidation in the tissue. Preventing neutrophil extravasation eliminated the neutrophil-induced oxidant stress and injury. In addition, inhibition of NADPH oxidase effectively prevented neutrophil-mediated liver injury despite extravasated neutrophils. Thus preventing neutrophil extravasation and inhibiting NADPH oxidase are promising therapeutic strategies to prevent neutrophil-mediated oxidant stress and liver injury. Although the selective inhibition of neutrophils did not affect the ultimate outcome after Gal/ET treatment, it has to be kept in mind that this is not a model for any specific human liver disease but an experimental system to study the effect of apoptotic cell death on the inflammatory response in vivo. As such, it demonstrates clearly the neutrophil attack on apoptotic cells and the killing mechanism involving NADPH oxidase-derived ROS. However, to make a judgment regarding the feasibility of using interventions against neutrophil functions as therapeutic targets, it is necessary to document a long-term benefit and improved survival in specific models of human liver disease, as has been shown for ischemia-reperfusion injury (35, 38) and obstructive cholestasis (20).

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REFERENCES


