Iron primes hepatic macrophages for NF-κB activation in alcoholic liver injury

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Iron primes hepatic macrophages for NF-κB activation in alcoholic liver injury. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G1240–G1250, 1999.—NF-κB activation induced by lipopolysaccharide (LPS) in cultured hepatic macrophages (HM) may be abrogated by pretreatment of cells with a lipophilic iron chelator, 1,2-dimethyl-3-hydroxypyrid-4-one (L1, deferiprone), suggesting a role for iron in this molecular event [M. Lin, M., R. A. Rippe, O. Niemelä, G. Brittenham, and H. Tsukamoto, Am. J. Physiol. 272 (Gastrointest. Liver Physiol. 35): G1355–G1364, 1997]. To ascertain the relevance in vivo of this hypothesis, HM from an experimental model of alcoholic liver injury were examined for the relationship between nuclear factor (NF)-κB activation and iron storage. HM showed a significant increase in nonheme iron concentration (+70%), accompanied by enhanced generation of electron paramagnetic resonance-detected radicals (+200%), NF-κB activation (+100%), and tumor necrosis factor-α (+150%) and macrophage inflammatory protein-1 (+280%) mRNA induction. Treatment of the cells ex vivo with L1 normalized all these parameters. HM content of ferritin protein, ferritin L chain mRNA, and hemeoxygenase-1 mRNA and splenic content of nonheme iron were increased, suggesting enhanced heme turnover as a cause of the increased iron storage and NF-κB activation. To test this possibility, increased iron content in HM was reproduced in vitro by phagocytosis of heat-treated red blood cells. Treatment caused a 40% increase in nonheme iron concentration and accentuated LPS-induced NF-κB activation twofold. Both effects could be abolished by pretreatment of cells with zinc protoporphyrin, a hemeoxygenase inhibitor. To extend this observation, animals were splenectomized before 9-wk alcohol feeding. Splenectomy resulted in further increments in HM nonheme iron storage (+60%) and NF-κB activation (+90%) and mononuclear cell infiltration (+450%), particularly around the iron-loaded HM in alcohol-fed animals. These results support the pivotal role of heme-derived iron in priming HM for NF-κB activation and expression of proinflammatory genes in alcoholic liver injury. Kupffer cells; nuclear factor-κB; chemokines; inflammation; erythropagocytosis

A GROWING BODY of evidence exists for the effector role of hepatic macrophages (HM) in liver injury (see Ref. 37 for review). This role appears to be facilitated in part by the release of cytotoxic, proinflammatory, or fibrogenic mediators such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1, and transforming growth factor-β (9, 19, 24, 26). Inactivation of HM by treatment with gadolinium chloride (GdCl3) ameliorates liver injury induced by various hepatoxic agents or conditions, including alcohol intake (see Ref. 37 for review). TNF-α is believed to be a key mediator, mediating hepatocellular injury through its potential direct stimulation of oxidant stress in mitochondria (13) or initiation of inflammation via upregulation of chemokines and adhesion molecules (41). In fact, administration of antibodies against TNF-α or against soluble TNF-α receptor protects against liver injury in a manner similar to that of GdCl3 (8). Expression of TNF-α and many other proinflammatory mediators is regulated by a transcription factor, nuclear factor (NF)-κB (1, 6). For NF-κB to be translocated to the nucleus to bind to its target genes, the latent form has to be activated by the removal of the inhibitory protein (IκB) via phosphorylation and proteolysis of this protein (1). Oxidative stress is believed to be one of the critical events leading to the activation signaling of NF-κB (33).

Iron is essential to almost all known cell types (20). Iron not only participates in numerous vital biological processes but also plays a central role in oxidative stress as a major catalyst for hydroxyl radical (·OH) formation via the Fenton reaction (15). Hydroxyl radical is the most potent radical species that abstracts a hydrogen atom from biological molecules, leading to deleterious effects such as DNA damage, protein modification, and lipid peroxidation (3, 17). A lipophilic iron chelator, 1,2-dimethyl-3-hydroxypyrid-4-one (L1, deferiprone) has recently been shown to inhibit NF-κB activation in lipopolysaccharide (LPS)-stimulated cultured HM (24). In fact, the derivatives of dithiocarbamates, which have been used as specific inhibitors of NF-κB, function as antioxidants, partly through their metal chelating capacity (32). Thus these observations suggest that iron or iron-catalyzed oxidative stress is involved in NF-κB activation.

Both oxidative stress and iron are implicated in the pathogenesis of alcoholic liver injury (36, 39). Chronic alcohol consumption may cause an increase in hepatic iron (39), and iron supplementation results in exacerbation of alcoholic liver injury (36). Iron-mediated poten-
tiation of alcoholic liver injury is associated with enhanced NF-κB activation, upregulation of NF-κB-responsive chemokine gene expression, and mononuclear cell infiltration (38), suggesting a critical role for iron in the NF-κB-mediated inflammatory response in this experimental model.

Nonetheless, the exact means whereby iron enhances NF-κB activation have not been determined. Increased NF-κB activation might be a direct effect of iron or an indirect consequence, perhaps as a result of iron-induced exacerbation of hepatocellular injury. Our previous studies of cultured HM treated with L1 provided evidence for a direct regulatory role of iron in NF-κB activation (24), but the pertinence of these findings in vitro to HM in vivo has not been investigated.

The present study has examined the role of increased iron stores within HM in enhancing NF-κB activation in experimental alcoholic liver injury. We found that increased iron stores were associated with intensified NF-κB activation in HM in the model and that both could be normalized by treatment of the cells ex vivo with L1. In addition, erythropagocytosis by cultured HM increased intracellular iron concentrations and promoted LPS-stimulated NF-κB. Finally, splenectomy of animals resulted in an incremental increase in HM iron and NF-κB activation following alcohol feeding.

METHODS

Animals. Male Wistar rats (~350–375 g body wt) were implanted with gastrectomy catheters under general anesthesia achieved with ketamine (35 mg) and xylazine (1.5 mg) (19). The animals received continuous intragastric infusion of a high-fat (35% calories as corn oil) or a low-fat (8% calories as corn oil) diet plus an increasing concentration of ethanol (HFD-alcohol or LFD-alcohol groups) for 9 wk, as previously described (19). Splenectomy was also performed on subsets of animals resulting in an incremental increase in HM iron and NF-κB activation.

Hepatic macrophage isolation. After a 9-wk intragastric infusion as described above, HM were isolated by sequential digestion of the liver with Pronase and type IV collagenase followed by arabinogalactan gradient centrifugation as previously described (19). The purity of HM was assessed by phase-contrast microscopy and latex bead (1.0 µm) phagocytosis (Sigma Chemical, St. Louis, MO) and always exceeded 85%. The cell viability examined by the trypan blue exclusion test was always >95%. By our method, the presence of sinusoidal endothelial cells and hepatic stellate cells in the HM fraction were <10% and 5%, respectively. The HM isolated from the animals were subjected to total RNA, nuclear protein, and cytoplasmic protein extraction, as well as measurement of iron and ferritin concentration. These cells were also used for an experiment ex vivo to test effects of L1 on NF-κB binding and TNF-α and macrophage inflammatory protein (MIP)-1 mRNA expression.

Primary cultures of HM from normal chow-fed rats were established using the adherence method and RPMI 1640 with 5% FCS and antibiotics as culture medium (19). The purity of these cells exceeded 95% as determined with latex beads. The cultured macrophages were used on the third day for experiments testing effects of erythropagocytosis.

Nuclear protein extraction and gel mobility shift assay. To examine the NF-κB binding, nuclear proteins of the HM were extracted using the method of Dignam and co-workers (10). The extract (5 µg) was incubated in a reaction mixture [20 mM HEPES, pH 7.6, 20% glycerol, 200 µg/ml poly(dI-dC)] on ice in the absence or presence of a 500-fold molar excess of unlabeled double-stranded oligonucleotide with the κB consensus sequence (top strand: 5′-GCAGAGGGGACTTTCCGGA-3′, bottom strand: 5′-GTCTGCAAAATCCTCCTG-3′) (2). One of the four NF-κB response elements in the TNF-α promoter was also used as a probe for the assay (6). After a 10-min incubation, 1–2 ng of 32P-labeled double-strand κB oligonucleotide were added and the incubation continued for an additional 20 min. The reaction mixture (a total volume of 20 µl) was then resolved on a 6% nondenaturing polyacrylamide gel using 0.4× Tris-borate-EDTA. The gel was dried and subjected to autoradiography at ~80°C. For the supershift assays, 1.5 µg (1.5 µl) of antibodies against p50 or p65 (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the reaction mixture for an additional 30-min incubation at room temperature subsequent to the 20-min incubation with 32P-labeled NF-κB probe. The competitive displacement of the shifted bands and the supershift assays were previously performed by us on HM nuclear extracts to support the specificity of NF-κB binding detected by this method (24).

Measurements of nonheme iron and ferritin concentrations. The nonheme iron concentration in HM and spleen was measured by a bathophenanthrolone sulfoxonic-thioglycolic acid chromogen assay (35). The ferritin content in HM was determined by a rat-specific ferritin ELISA assay (RAMCO Laboratories, Houston, TX).

RNA extraction and RT-PCR. Total RNA from freshly isolated HM was extracted using a guanidinium-phenol-chloroform method (5). The integrity and equal loading of RNA samples were assessed by running a minigel of the samples and examining ethidium bromide staining of 18S and 28S rRNA. Because the numbers of HM isolated from the animal model were limited and multiple parameters needed to be examined on the cells, the use of a large number of the cells to extract sufficient RNA for Northern blot analysis or RNase protection assay was difficult. For this reason, semiquantitative RT-PCR analysis was performed on a small quantity of RNA to assess changes in the mRNA levels for cytokines, ferritin subunits, hemeoxygenase-1, and β-actin. Three micrograms of total RNA were reverse transcribed into cDNA with 600 units of Moloney murine leukemia virus reverse transcriptase and oligo(dT)15 (GIBCO BRL, Grand Island, NY) as a primer at 37°C for 60 min. To provide semiquantitative assessments on the mRNA level of NF-κB-responsive genes in the HM, a portion of the synthesized cDNA was amplified by using specific PCR primers for ferritin L chain, H chain, or hemeoxygenase-1 and β-actin. As a housekeeping gene, β-actin was amplified by PCR at the same time (29). The linear ranges of PCR amplification for all the above genes were assessed in preliminary experiments, and the appropriate cycles were determined.

RNA electrophoretic mobility shift assay. Binding activities of iron regulatory proteins (IRP), which mediate posttranscriptional regulation of ferritin expression, were examined by RNA electrophoretic mobility shift assay of HM cytoplasmic extracts. The RNA probe is a 108-base sense transcript
Table 1. Primers used for PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>CTTCTCATCAAGTC-</td>
<td>ATGGCCGAAAACGCCGTT-</td>
</tr>
<tr>
<td>MIP-1</td>
<td>GATGATCTTGGGAC-</td>
<td>TAGTGAGGTGCACCACTCCGTT-</td>
</tr>
<tr>
<td>Ferritin H chain</td>
<td>ATACCCGAGCATG-</td>
<td>CCGGGTGCGACCAACCTTGAG-</td>
</tr>
<tr>
<td>Ferritin L chain</td>
<td>CCCCCTGATGTG-</td>
<td>TACATTGCGGGATTGCT-</td>
</tr>
<tr>
<td>Hemexygenase-1</td>
<td>ATGCTGCGCATG-</td>
<td>ATGCTGCGCATG-</td>
</tr>
<tr>
<td>β-Actin</td>
<td>GGATCATGAGGCT-</td>
<td>AGCATGGCGGCTGCTGGC-</td>
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TNF-α, tumor necrosis factor-α; MIP-1, macrophage inflammatory protein 1.

Prepared with SP6 RNA polymerase from Smal-digested rat ferritin L-gene 66 (pseudogene), which was subcloned into the Sac I Hind III site of pGEM 2. The plasmid pGEM-iron-responsive element (IRE) was kindly provided by Dr. Elizabeth A. Lebold (Human Molecular Biology and Genetics, University of Utah) and contains the sequence corresponding to the IRE of rat ferritin L chain encompassing 38 bases of the 5′-flanking sequence and 70 bases of the 5′-UTR. A transcription reaction was performed in the presence of 1 µg of linearized template (Smal I digested), 60 µCi [α-32P]CTP (3,000 Ci/mmol; New Life Science Products, Boston, MA), 0.5 mM each NTP (except CTP), 10 units of SP6 RNA polymerase (Boehringer Mannheim, Indianapolos, IN), and 20 units of RNase inhibitor in a total volume of 20 µl. After 20 min of incubation at 37°C, the reaction was stopped by heating at 65°C for 15 min and incubated another 15 min at 37°C following addition of DNase 1 (20 units). The labeled RNA was separated from the nonincorporated ribonucleotide triphosphates on a 1-ml column (Nuctrap probe purification column, Stratagene, La Jolla, CA), and 45 µl of 1× STE (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were added to the eluent. The cytoplasmic extract was prepared by lysis in the ice-cold buffer of 10 mM HEPES (pH 7.6), 40 mM KCl, 3 mM MgCl2, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 2 µg/ml aprotinin, 5% glycerol, and 0.2% Nonidet P-40. After removal of the nuclei, the cytoplasmic fraction was centrifuged at 10,000 g, and the supernatant was stored in aliquots at −80°C. A binding reaction was carried out as previously described (23), using 20 µg of cytoplasmic extract and 104 cpm of 32P-labeled IRE probe in a buffer of 10 mM HEPES (pH 7.6), 3 mM MgCl2, 1 mM dithiothreitol, 40 mM KCl, and 5% glycerol in a total volume of 15–25 µl. After incubation at 25°C for 30 min, 1 unit of RNase T1 (Boehringer Mannheim) was added and incubated for 10 min followed by an additional 10-min incubation with heparin (5 mg/ml) (Sigma). This mixture was subjected to electrophoresis on 5% nondenaturing polyacrylamide gel (acylamide-to-methylene bisacrylamide ratio of 60:1). The gel was dried and analyzed by autoradiography at −80°C.

Treatment of HM with L1 ex vivo. To further test the association between NF-κB activity and TNF-α and MIP-1 mRNA expression by HM, isolated HM from HFD-alcohol and HFD-control rats were incubated in serum-free RPMI 1640 with or without L1 (100 µM) for 18 h at 37°C. The cells were then washed with cold PBS and subjected to RNA/nuclear protein extraction as described above.

Effects of erythrophagocytosis on NF-κB binding by cultured HM. This experiment was performed to examine whether an increased noneheme iron concentration produced by erythrophagocytosis affected NF-κB binding in cultured HM. Heat-denatured red blood cells (RBC) were used for erythrophagocytosis. Blood was collected from a male Wistar rat with a heparinized syringe, and RBC were washed three times with normal saline by centrifugation at 1,400 rpm for 4 min at 4°C. RBC were then suspended in 5 vol of ACD-B solution (Baxter Healthcare) and incubated at 40°C for 15 min. After incubation, heat-treated RBC were washed twice in normal saline, resuspended in normal saline, and kept on ice until they were used. Heat-treated RBC were added to cultured HM from normal male Wistar rats at the ratio of 10:1 (RBC to HM), and the cells were cultured in RPMI 1640 with 5% FCS for 2 h to allow phagocytosis of RBC. The nonadhering RBC were then removed by gentle washing with PBS, and noninternalized RBC were hypotonically lysed by exposure to cold water for 20 s. After another 18-h incubation, the cells were exposed to LPS (1 ng/ml) for 1 h and subjected to nuclear protein extraction for NF-κB gel shift assay. For inhibition of heme oxygense activity, zinc protoporphyrin (10 µM) was added to the culture 30 min before the phagocytosis period.

Electron paramagnetic resonance spectra of Kupffer cells. Freshly isolated Kupffer cells (107 cells/ml) from alcohol-fed and control animals were suspended in 5–10 mM glucose in PBS. To this mixture, 50 mM α-(4-pyridyl-1-oxide)-N-tort-butyl nitrotrine (POBN) and 0.1% (vol/vol) DMSO were added and the samples were immediately transferred to bottom-sealed Pasteur pipettes. The elecron paramagnetic resonance (EPR) spectra were recorded at room temperature in a Bruker ECS 106 spectrometer operating at 9.8 GHz. Instrument conditions were as follows: modulation frequency, 100 kHz; time constant, 1.3 s; sweep scan, 18 G/min; modulation amplitude, 0.9 G; and microwave power, 20 mW. The spectra were compared with simulated ones obtained by using the published hyperfine splitting constants and the simulation program from Oklahoma Research Center. Different simulation spectra were run with one species at a time until the best match was found in terms of line width, g values, number of lines, shifts, and peak heights.

Statistical analysis. Numerical results were presented as means ± SD. The significance of the difference between the groups was assessed by Student’s t-test.

RESULTS

NF-κB activation and cytokine expression by freshly isolated HM. NF-κB binding was examined in nuclear extracts of HM isolated from four groups of the rats intragastrically fed with a high-fat or low-fat diet plus ethanol (HFD-alcohol or LFD-alcohol) or isocaloric dextrose (HFD-control or LFD-control) as shown in Fig. 1. There was a twofold increase in NF-κB binding activity in HM from HFD-alcohol compared with the cells from the pair-fed control (HFD-control) rats. No increase was noted for NF-κB binding in HM from LFD-alcohol rats who received the identical amount of ethanol with the isocaloric low-fat diet. Because cirrhotic liver necrosis and inflammation were evident in HFD-alcohol but not in LFD-alcohol rats, these results indicate that NF-κB activation in HM in the model was not due to ethanol consumption per se but to conditions associated with progressive alcoholic liver injury. The specificity of NF-κB binding was supported by complete displacement of the shifted bands by competition with unlabeled probe in excess (data not shown) as previously reported by us (24).
Effects of iron chelator L1 on NF-κB binding activity and cytokine gene expression. To test the association between NF-κB activation and induction of TNF-α and MIP-1 mRNA expression, the isolated HM from HFD-alcohol and HFD-control groups were incubated overnight (18 h) in serum-free RPMI 1640 with or without L1 (100 µM), the iron-chelating agent, which has been previously shown to effectively block NF-κB activation in HM (24). Nuclear proteins and total RNA were extracted from these cells for the NF-κB gel mobility shift assay and cytokine RT-PCR. The treatment with L1 caused complete blockade of NF-κB activation by the HFD-alcohol HM, whereas the constitutive NF-κB binding in HFD-control HM was not affected by L1 (Fig. 1).

Expression of mRNA for NF-κB-responsive genes such as TNF-α and MIP-1. Semiquantitative analysis of the TNF-α mRNA level by RT-PCR indicated a 150% increase in HM from HFD-alcohol compared with the cells from the pair-fed control rats (Fig. 2), confirming our previous finding (19). MIP-1 mRNA expression was increased by ~280% in HFD-alcohol rats.

Fig. 1. Nuclear factor (NF)-κB binding of hepatic macrophage nuclear extracts from animals intragastrically infused with low-fat diet (LFD) or high-fat diet (HFD) plus ethanol (alcohol) or dextrose (control) for 9 wk (top). Bottom: relative changes in the scanning densitometry of NF-κB binding compared with the densitometry of respective controls (LFD-control and HFD-control). Note a 2-fold increase in NF-κB binding in HFD-alcohol compared with HFD-control animals (*P < 0.05). Numbers of animals studied for the 4 groups are listed in parentheses: HFD-control (5), HFD-alcohol (5), LFD-control (4), LFD-alcohol (4). mØ, Macrophage.

Fig. 2. Hepatic macrophage tumor necrosis factor (TNF)-α and macrophage inflammatory protein (MIP)-1 mRNA expression assessed semiquantitatively by RT-PCR in HFD-control and HFD-alcohol animals infused for 9 wk (top). Bottom: relative changes in the densitometric data normalized by β-actin compared with HFD-control. Note significant increases in both TNF-α and MIP mRNA levels in HFD-alcohol (n = 5) compared with HFD-control (n = 5) animals (*P < 0.05).
3). Coordinately, semiquantitative analysis of TNF-α and MIP-1 mRNA expression showed apparent suppression with L1 in HFD-alcohol but not in HFD-control rats (Fig. 4). These results support an association between NF-κB activation and TNF-α and MIP-1 mRNA induction by HM in experimental alcoholic liver injury and provide evidence for a critical role of iron in these molecular events.

Nonheme iron concentration and EPR spectrum. Nonheme iron concentration was determined in HM from HFD-alcohol and HFD-control groups before and after the L1 treatment. A significant 70% increase in the nonheme iron level was noted in HFD-alcohol compared with HFD-control rats (Table 2). The L1 treatment resulted in a significant reduction of nonheme iron concentration in the HFD-alcohol cells but not in the HFD-control cells (Table 2). There was no significant increase in the nonheme iron content in HM from LFD-alcohol compared with LFD-control or HFD-control groups. Because changes in intracellular iron are usually associated with the production of oxygen radicals, we evaluated the presence of oxygen radicals in these cells by EPR in conjunction with the spin trapping technique (Fig. 5). The EPR signals of HM from HFD-control were low, and the signal-to-noise ratio of this spectrum was far from desirable. However, the ratio increased with the number of the cells and decreased or was abolished if the cells were not viable (data not shown). The signals are low because the steady-state concentration of free radicals in normal living cells is low (below the nanomolar range). Nevertheless, the spectrum demonstrates a signal attributable to a composite of methyl, hydroxyl, and superoxide anion adduct signals in a ratio of 1:1.3:1, respectively. These signals, in addition to the signal of ethyl-POBN adduct (α-hydroxyethyl-POBN), were also observed in the spectrum obtained with HM from HFD-alcohol animals (Fig. 5, C and D), albeit of higher intensity (3-fold).

Ferritin and heme oxygenase-I expression. To assess a homeostatic response to the observed increase in the cellular iron levels, the ferritin protein content, ferritin L chain, and ferritin H chain mRNA levels were semiquantitatively examined in HM. The ferritin protein content was increased by 50% in HM from HFD-alcohol compared with HFD-control rats (Table 2). The ferritin L chain mRNA level was increased by twofold, whereas no change was noted for ferritin H chain mRNA expression (Fig. 6). Thus these results demonstrate that HM in alcoholic liver injury have the increased iron storage and consequent upregulation of the storage protein expression. Notably, mRNA expression of hemeoxygenase-1, a pivotal enzyme for the breakdown of heme, was increased by twofold in the HFD-alcohol HM (Fig. 7), suggesting that enhanced heme metabolism via increased erythrophagocytosis contributes to the increased iron storage in HM in alcoholic liver injury.

Iron response element binding by HM in alcoholic liver injury. The size of the intracellular pool of iron is regulated by the cytoplasmic proteins, iron regulatory proteins (IRP-1 and IRP-2). IRP bind to IRE within transferrin receptor and ferritin transcripts to coordinately mediate transferrin receptor mRNA stability and ferritin mRNA translational efficiency to control the intracellular "free" iron level. We analyzed the binding of IRP of HM cytoplasmic extracts to examine whether the observed increases in the iron and ferritin content in the HFD-alcohol HM were associated with suppressed IRE binding. Surprisingly, no changes in IRE binding were observed (Fig. 8).
Effects of erythrophagocytosis on NF-κB activation by cultured HM. Because the aforementioned data suggested erythrophagocytosis as a possible mechanism contributing to the increased iron storage in HM, we examined the effects of phagocytosis of heat-treated RBC by cultured HM to test whether the increase in storage iron produced by this technique affects NF-κB activation. To allow the recovery of the HM from phagocytosis-associated stimulation, the cells were incubated for an additional 18 h and examined for NF-κB binding with or without LPS stimulation. The erythrophagocytosis procedure resulted in a 40% increase in the nonheme iron content. Under basal conditions, these cells did not show a significant increase in NF-κB binding. In contrast, LPS-stimulated NF-κB activation was intensified by twofold (Fig. 9A) in the HM that had been iron loaded by phagocytosis of RBC. Furthermore, the pretreatment of the cells with zinc protoporphyrin before erythrophagocytosis completely abrogated the increase in LPS-mediated NF-κB binding (Fig. 9A), suggesting that the observed effect of erythrophagocytosis is dependent on heme oxygenase activity. The specificity of NF-κB binding was supported by the supershift assays with antibodies against p50 and p65 as shown in Fig. 9B.

### Table 2. Nonheme iron and ferritin content in hepatic macrophage and spleen

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<thead>
<tr>
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<th>Nonheme Iron</th>
<th>Ferritin</th>
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<tr>
<td><strong>Content in Freshly Isolated HM, µg/10^6</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HFD-control</td>
<td>0.82 ± 0.15</td>
<td>2.58 ± 0.37</td>
</tr>
<tr>
<td>HFD-alcohol</td>
<td>1.42 ± 0.09*</td>
<td>3.87 ± 0.51*</td>
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<tr>
<td><strong>Content in HM After Overnight Incubation, µg/10^6</strong></td>
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<tr>
<td>HFD-control</td>
<td>0.80 ± 0.27</td>
<td>ND</td>
</tr>
<tr>
<td>HFD-alcohol</td>
<td>1.40 ± 0.22</td>
<td>ND</td>
</tr>
<tr>
<td>L1</td>
<td>0.62 ± 0.19</td>
<td>ND</td>
</tr>
<tr>
<td>PBS</td>
<td>0.82 ± 0.22†</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Content in Spleen, µg/1g</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HFD-control</td>
<td>399.8 ± 102.9</td>
<td>ND</td>
</tr>
<tr>
<td>HFD-alcohol</td>
<td>893.3 ± 98.3*</td>
<td>ND</td>
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</table>

Values are means ± SE. Hepatic macrophages were isolated from rats intragastrically infused for 9 wk with a high-fat diet plus ethanol (HFD-alcohol, n = 5) or isocaloric dextrose (HFD-control, n = 5). Aliquots (10^6 cells) of these cells were immediately stored at −80°C until nonheme iron and ferritin content were analyzed as described in Methods. Cells (10^6 cells) were also incubated overnight with 100 µM 1,2-dimethyl-3-hydroxypyrid-4-one (L1) or PBS (solvent used for L1), centrifuged, and stored at −80°C for nonheme iron determination. Spleen was removed from HFD-alcohol and HFD-control groups at time of cell isolation and analyzed for nonheme iron content. ND, not determined. *P < 0.05 compared with HFD-control; †P < 0.05 compared with PBS treatment of HFD-alcohol.
Effects of splenectomy on HM iron storage and NF-κB activation in alcohol-fed animals. To further extend the above observation in vitro to conditions in vivo, we examined effects of splenectomy on HM iron storage and NF-κB activation in the HFD-alcohol and HFD-control rats. Our hypothesis was that splenectomy, by removing the iron storage site provided by splenic macrophages, would further increase the iron content in HM and consequently promote NF-κB activation in the cells, leading to enhanced inflammation in the livers of the HFD-alcohol rats. Our results demonstrate that HM from the splenectomized HFD-alcohol animal showed further increases in iron storage (1.98 ± 0.17 vs. 1.34 ± 0.21 µg/10⁷ cells, P < 0.05) and NF-κB binding (+90%) (Fig. 10, A and C) compared with HFD-alcohol without splenectomy. In addition, the morphometric analysis of liver histology demonstrated that the number of mononuclear inflammatory cells in the liver was increased fivefold in the splenectomized HFD-alcohol compared with the HFD-alcohol animal without splenectomy (Fig. 10B). In particular, the inflammatory cells were found in close association with HM with higher iron storage (Fig. 10D). HM from the splenectomized HFD-control animal also showed a similar increase in the nonheme iron content compared with HFD-control animals (1.48 ± 0.19 vs. 0.98 ± 0.13 µg/10⁷ cells, P < 0.05), and NF-κB binding was mildly increased in some of the former animals (data not shown). Nonetheless, the liver histology of these animals showed no apparent inflammatory response.

**DISCUSSION**

The results of the present study support the pivotal role of increased intracellular iron content in NF-κB activation by HM in alcoholic liver injury. First, the increased intracellular iron concentration and NF-κB binding in HM were effectively and simultaneously normalized by iron chelation ex vivo. This ex vivo approach has obvious limitations in recapitulating the in vivo changes without introducing potentially confounding effects that may result from the isolation and incubation procedures. Nevertheless, the results obtained from the ex vivo experiment are still considered as the supporting evidence for the proposed role of iron. Second, the simultaneous increments in splenic iron concentration and in the expression of heme oxygenase-I in HM suggested that increased RBC uptake and enhanced heme catabolism contributed to the increased intracellular in HM of the alcohol-fed animals. In fact, the reproduction of this hypothetical mechanism in cultured HM by erythrophagocytosis (with or without the use of a heme oxygenase inhibitor) confirmed that increased intracellular iron promoted LPS-induced NF-κB activation in a heme oxygenase-dependent manner. To test this hypothesis in vivo, HM heme catabolism was further augmented in the alcohol-fed animals by splenectomy, a procedure that, by removing splenic macrophages, increases RBC uptake by HM. Indeed, the splenectomy resulted in an incremental increase in the nonheme iron content of HM, further promoted NF-κB binding, and caused a fivefold increase in the number of mononuclear inflammatory cells infiltrated into the livers of alcohol-fed animals.

Our results indicate that increases in intracellular iron prime HM for activation of NF-κB but do not identify the specific iron pool involved. It should be emphasized that, in our experiments, the HM were exposed to constant and relatively high concentrations...
of the iron chelator L1, conditions under which this bidentate chelator could bind iron effectively. With L1 in excess, three molecules of the chelator are available to occupy the six coordination sites of each atom of iron. In these experiments, we did not examine the effects of lower concentrations of the chelator. Other investigators have reported that L1 may potentiate iron-catalyzed oxidative damage in liver cells at low ratios of L1 to iron (7) and, in clinical use, may worsen hepatic fibrosis (30).

NF-κB activation by HM was found only in the HFD-alcohol group. Expression of TNF-α is under the transcriptional regulation of NF-κB (6). MIP-1, a C-C chemokine for neutrophils and mononuclear cells, is induced with LPS and TNF-α (11), presumably through NF-κB activation (14). In fact, both NF-κB activation and the induced TNF-α and MIP-1 mRNA expression by HM from HFD-alcohol animals were abrogated by ex vivo treatment with the iron chelator (L1), as previously demonstrated for NF-κB activation and TNF-α expression in cholestatic liver injury or under in vitro LPS stimulation (24). Thus these results support not only the role of iron in NF-κB activation but also the importance of NF-κB in TNF-α and MIP-1 expression by HM.

Our in vitro erythrophagocytosis experiment was crucial for demonstrating the link between the increment in intracellular iron in HM resulting from increased heme catabolism and accentuated NF-κB activation in response to LPS. After a series of preliminary experiments, the erythrophagocytosis technique described here was shown to achieve an ~30–40% increase in the nonheme iron concentration. This iron loading did not affect NF-κB under the basal condition but accentuated LPS-stimulated NF-κB activation (Fig. 9A). It was important to allow the cells to rest for a sufficient time after erythrophagocytosis, since the

Fig. 6. Relative changes in hepatic macrophage ferritin L chain and H chain mRNA levels in HFD-alcohol compared with HFD-control. Top: semiquantitative RT-PCR analysis demonstrated that ferritin L chain mRNA levels were significantly increased in HFD-alcohol compared with HFD-control, whereas ferritin H chain mRNA levels did not change. Bottom: densitometric analysis. *P < 0.05 compared with HFD-control (n = 4 for each group).

Fig. 7. Relative changes in hepatic macrophage hemeoxygenase-1 mRNA levels in HFD-alcohol rats. Top: hemeoxygenase-1 mRNA levels were semiquantitatively assessed by RT-PCR and were found to be significantly induced in HFD-alcohol compared with HFD-control animals. Bottom: desitometric analysis. *P < 0.05 compared with HFD-control (n = 4 for each group).

Fig. 8. Hepatic macrophage iron regulatory protein (IRP) binding activity in HFD-control and HFD-alcohol animals. No change in IRP binding was found in HFD-alcohol compared with HFD-control (n = 4 for each group).
The phagocytic process itself is known to be followed immediately by downregulation of macrophage functions (25). Another critical factor was the number of phagocytosed erythrocytes by one macrophage. Phagocytosis of more than 1.5 opsonized erythrocytes per Kupffer cell was shown to lead to cell cytotoxicity (21). Thus suppressed macrophage functions in patients with iron overload (40) or phagocytosis of a large number of erythrocytes (25) may be related to cell cytotoxicity. Our method of using the erythrocyte ratio of 10:1 (erythrocytes to Kupffer cells) and washing and lysing the erythrocytes after the 2-h incubation resulted in a phagocytosis ratio of 1:1.

The splenectomy experiment further extended the observation made in the in vitro phagocytosis experiment. Removal of the spleen indeed caused a further increase in HM iron concentration in alcohol-fed animals (A). This change was accompanied by further enhancement of hepatic macrophage NF-κB binding (C) and accentuated inflammatory response. The same procedure resulted in a similar magnitude of increase in the HM iron content of the pair-fed control animals (HFD-control). In fact, some

![Fig. 9. In vitro effects of erythrophagocytosis by hepatic macrophages. Hepatic macrophages isolated from normal male Wistar rats were cultured for 2 days and incubated with or without heat-treated red blood cells (RBC) for 2 h to allow phagocytosis. Undigested RBC were then removed by washing PBS and hypotonic lysis by adding cold water for 20 s. Macrophages were incubated for an additional 18 h with or without lipopolysaccharide (LPS) stimulation during the last 1 h, followed by nuclear protein extraction for NF-κB gel shift assay (A). Erythrophagocytosis promoted LPS-mediated NF-κB activation by cultured hepatic macrophages (lane 4). Addition of zinc protoporphyrin (PP; 10 µM) to the cultures 30 min before erythrophagocytosis abolished this effect (lane 5). B: representative supershift assays with anti-p50 and/or anti-p65 antibodies, demonstrating the p65/p50 and p50/p50 complexes.]

![Fig. 10. Effects of splenectomy on NF-κB activation by hepatic macrophages in alcoholic liver injury. Splenectomy resulted in a further increase in hepatic macrophage iron content in alcohol-fed animals (A). This change was accompanied by further enhancement of hepatic macrophage NF-κB binding (C) and accentuated inflammatory response in the liver (B). *P < 0.05 compared with HFD-alcohol group without splenectomy. Note inflammation is evident in the vicinity of iron-loaded hepatic macrophage stained by Perls' staining (D).]
but not all of these animals showed mildly increased NF-κB binding (data not shown). However, histologically, the livers were normal without any sign of inflammation. These results suggest that the increased iron concentration was not sufficient enough to induce inflammation in the control animals but primed the HM in the alcohol-fed animals for NF-κB activation, cytokine expression, and inflammation. These data corroborate with our in vitro finding that erythrophagocytosis did not enhance NF-κB activation by cultured HM under basal conditions but did so after LPS stimulation. An iron chelator blocks LPS-mediated NF-κB activation and TNF-α and IL-6 expression by cultured peritoneal macrophages (4) and Kupffer cells (24). Collectively, these results provide evidence for a regulatory role of iron or iron-catalyzed oxidative stress in NF-κB activation. Our in vivo and in vitro findings support this notion and a novel hypothesis that the increased intracellular iron in HM primes the cells for NF-κB activation.

The mechanism for the increase in intracellular iron stores in HM in the liver injury induced by alcohol has not been studied in detail. The effects of alcohol and alcohol-induced liver disease on RBC production and survival are complex. Overall, the observed increase in iron both within HM and in the spleen in the HFD-alcohol group (Table 2) is likely to reflect a combination of increased RBC uptake (erythropagocytosis) by macrophages associated with a decreased RBC life span and impaired mobilization of iron from macrophages. In particular, acetaldehyde, a metabolite of ethanol, may form adducts with hemoglobin in ethanol-consuming subjects, and the epitopes may be expressed on the surface of the RBC, which can be recognized by circulating antibodies against them (28). This facilitates Fc receptor-mediated phagocytosis of the opsonized cells by macrophages. In addition, modification of RBC membrane with malondialdehyde promotes phagocytosis of the cells by macrophages, which appears to involve both IgG-dependent and IgG-independent mechanisms (16). In alcoholic liver injury, many other reactive molecules are produced, including hydroxyethyl radicals (31) and lipid aldehydes produced via lipid peroxidation (18). Thus these reactive substances may readily modify erythrocytes, decreasing RBC survival and resulting in increased erythropagocytosis by macrophages. In addition, alcohol liver injury may cause the impairment of iron release by erythropagocytosed macrophages as shown for chronic inflammation models (21). If the increased iron content by erythropagocytosis is the underlying mechanism for the enhanced NF-κB activation in HM, then selective and targeted iron chelation in macrophages may provide a potential therapeutic or preventive modality for suppression of NF-κB-mediated cytotoxic or proinflammatory responses.

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