Regulation of liver hepcidin expression by alcohol in vivo does not involve Kupffer cell activation or TNF-α signaling

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Am J Physiol Gastrointest Liver Physiol 296: G112–G118, 2009. First published November 13, 2008; doi:10.1152/ajpgi.90550.2008.—Alcohol downregulates hepcidin expression in the liver leading to an increase in intestinal iron transport and liver iron storage. We have previously demonstrated that alcohol-mediated oxidative stress is involved in the inhibition of hepcidin transcription by alcohol in vivo. Kupffer cells and TNF-α play a key role in alcohol-induced liver injury. The aim of this study was to define their involvement in the regulation of hepcidin expression by alcohol. Kupffer cells were inactivated or depleted by employing gadolinium chloride and liposomes containing clodronate, respectively. Rats pair fed with the alcohol-Lieber-DeCarli diet for 6 wk and mice fed with 20% ethanol in the drinking water for 1 wk were used as experimental models. Interestingly, alcohol downregulated hepcidin expression in the livers of rats and mice independent of gadolinium chloride or clodronate treatment. One week of alcohol treatment was sufficient to induce a significant increase in TNF-α levels and phosphorylation of NF-κB p65. The neutralization of TNF-α by specific antibodies inhibited p65 phosphorylation. However, neither the neutralization of TNF-α nor the lack of TNF-α receptor expression reversed alcohol-induced suppression of liver hepcidin expression. The level of alcohol-induced ROS in the liver was also undiminished following Kupffer cell inactivation or depletion. Our results demonstrate that alcohol-induced Kupffer cell activation and TNF-α signaling are not involved in the suppression of liver hepcidin expression by alcohol-mediated oxidative stress in vivo. Therefore, these findings suggest that alcohol acts within hepatocytes to suppress hepcidin expression and thereby influences iron homeostasis.

alcoholic liver disease; iron; liver injury; oxidative stress

Hepcidin, a circulatory peptide synthesized by the liver, plays a central role in the regulation of iron metabolism by controlling the iron uptake from the intestine and iron release from macrophages (16, 18, 20). Both acute and chronic alcohol exposure downregulate hepcidin expression in the liver (3, 5, 7–9, 21). This results in an increase in the expression of iron transporters in the duodenum and the iron storage protein ferritin in the liver (8, 9).

The activation of Kupffer cells and inflammatory responses prime the liver for alcoholic liver disease (ALD) (1, 23, 25). The proinflammatory cytokine TNF-α, mainly synthesized by Kupffer cells, plays a critical role in ethanol-induced liver injury (10, 14, 30). TNF-α has been reported to downregulate hepcidin expression in human hepatoma cells (17). However, LPS and the cytokines IL-1 and IL-6 induce liver hepcidin expression (11, 17). Lou et al. (13) have demonstrated that the depletion of Kupffer cells by clodronate in mice does not abrogate the upregulation of liver hepcidin expression by inflammation. On the other hand, Montosi et al. (15) have reported that the inactivation of Kupffer cells by gadolinium chloride inhibits the LPS-induced elevation of hepcidin expression in mice. Kupffer cells have also been suggested to relay negative regulatory signals regarding hepcidin expression in the liver (24). However, the role of Kupffer cells in the regulation of liver hepcidin expression by alcohol is unknown.

Besides cytokines, Kupffer cells activated following alcohol exposure also release reactive oxygen species (2, 23, 28). We have demonstrated that alcohol-mediated oxidative stress suppresses liver hepcidin expression and promoter activity by inhibiting the DNA-binding activity of the transcription factor, CCAAT/enhancer-binding protein α (C/EBP α) in vivo (9). We investigated the involvement of Kupffer cell activation and TNF-α signaling in the regulation of liver hepcidin expression by alcohol in vivo. The findings of these studies will enable us to further understand the mechanisms involved in the regulation of liver hepcidin expression and iron metabolism by alcohol.

MATERIALS AND METHODS

Animal Experiments

Animal experiments were approved by the animal ethics committee at the University of Nebraska Medical Center. Wistar male rats (Charles River Laboratories) were housed individually in metabolic cages and pair fed with either regular or ethanol-containing Lieber-DeCarli liquids diets (Dyets, cat. no. 710027, 710260, respectively), as described previously (8). The ethanol content of the diet was gradually increased over an 8-day period (no ethanol for days 1–2, 1/4 the amount for days 3–4, and 1/2 the amount for days 5–7) to the full amount (36% of total calories as ethanol). Rats were exposed to the full amount of ethanol for 6 wk. 129/Sv strain wild-type mice (Charles River Laboratories), TNF-α receptor knockout mice deficient in the expression of both TNF-α receptor isoforms TNFR1 and TNFR2 (Jackson Laboratories), and control C57BL/6J/129/Sv strain mice (Jackson Laboratories) were maintained on a rodent Chow diet-7012 (Harlan Teklad Global Diets). For alcohol experiments, mice were housed in individual cages and exposed to 20% ethanol in the drinking water or plain water (control) for 7 days, as described previously (8, 9).

Kupffer cell inactivation. Gadolinium chloride was administered to randomly selected rats (10 μg/g body wt) or 129/Sv strain male mice (20 μg/g body wt) via tail vein injection. Control animals were injected with 0.9% NaCl. The day after the injections, mice were fed with 20% ethanol in the drinking water or plain water (control), and the injections were repeated on days 2 and 5 of the 1-wk alcohol exposure period.

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treatment. Rats were injected with gadolinium chloride or 0.9% NaCl twice a week (days 1 and 4) during the 6 wk Lieber-DeCarli diet treatment.

Kupffer cell depletion. Mice were injected (tail vein) with 0.1 ml of liposomes containing clodronate (obtained from Vrije University, Amsterdam, The Netherlands; ClodronateLiposomes.org). Clodronate (a gift of Roche Diagnostics, Mannheim, Germany) was encapsulated in liposomes, as described previously (27). Liposomes containing PBS were employed as a control. The day after the injections, mice were exposed to 20% ethanol in the drinking water or plain water (control) for 1 wk. The liposome injections were repeated on day 5 of the 1 wk alcohol treatment. Kupffer cell depletion was evaluated by staining the liver sections from each mouse with a macrophage-specific antibody, F4/80 (see below).

RNA Isolation, cDNA Synthesis, and Real-Time Quantitative PCR Analysis

Tissues washed with PBS were lysed in TRIzol (Invitrogen) and total RNA was isolated according to the manufacturer’s instructions. cDNA was synthesized using 2–4 μg of isolated RNA, 2.5 μM random primers (Applied Biosystems), and 200 U Superscript II RNase H-reverse transcriptase enzyme (Invitrogen). Gene expression was analyzed by real-time quantitative PCR, as described previously (8, 9). Primers (sense 5′-ACTCGGACCCAGGCTGC-3′; antisense 5′-AGATTAGGTTGCTGTCAGG-3′) and Taqman fluorescent probe (5′-6-FAM-TTGCTCCTGTCTCTCCCTGCGA-3′ [TAMRA-Q]) flanking ~70 base pairs of mouse hepcidin gene open reading frame sequence were designed by the Primer Express 1.5 program (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (gapdh) gene probes (Applied Biosystems) were used as the endogenous controls. The data were analyzed by using Sequence Detection Systems software (Applied Biosystems), as described previously (9). The cycle number at the linear amplification threshold (Ct) of the endogenous control (gapdh) gene and the target gene was recorded. Relative gene expression (the amount of target, normalized to the endogenous control (gapdh) gene and the target gene was recorded. Relative gene expression was analyzed by real-time quantitative PCR (see MATERIALS AND METHODS). Liver hepcidin expression was analyzed by real-time quantitative PCR (see MATERIALS AND METHODS). Rats with chronic alcohol exposure exhibited a significant decrease in liver hepcidin expression, compared with control rats fed with regular Lieber-DeCarli diet (Fig. 1). Gadolinium chloride treatment did not significantly alter basal liver hepcidin expression levels compared with control rats (Fig. 1). Interestingly, alcohol-fed rats injected with gadolinium chloride also displayed significant

Antibodies and Western Blotting

Western blots using total cell lysates were performed, as described previously (8, 9). To prepare cell lysates, mouse livers were homogenized in lysis buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 10% glycerol, 1 mM PMSF, Complete protease inhibitor cocktail (Roche Diagnostics), phosphatase inhibitor cocktail A (Santa Cruz Biotechnology, sc-45044), 1% Triton-X-100] and incubated on ice for 20 min. Subsequently, the lysates were centrifuged (15,000 g) for 10 min at 4°C and supernatants were employed for Western blots. Anti-p65 and anti-phospho p65 monoclonal antibodies were obtained commercially (Cell Signaling Technology).

Immunohistochemistry

Sections (10 μm) of formalin-fixed liver tissues were incubated with the polyclonal rat anti-mouse antibody, F4/80 (Serotec), and a goat anti-rat horseradish peroxidase-conjugated secondary antibody (STAR72; Serotec). Immunostaining was visualized with 3,3′-diaminobenzidine (Vector Laboratories).

ELISA

ELISA assays were performed using a commercial kit (BioLegend), according to the manufacturer’s instructions with minor modifications. Briefly, to detect expression in the liver, the plates were first coated with anti-TNF-α or IL-6 antibody overnight (4°C) and then incubated with the liver lysates (30 μg protein/well). For lysate preparation, livers were homogenized in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, Complete protease inhibitor cocktail, 17.5 μg/ml aprotinin, 5 μg/ml bestatin, 10 μg/ml leupeptin, 1% Nonidet-P40] and incubated on ice for 15 min. The samples were centrifuged (15,000 g) for 15 min (4°C), and the supernatants were employed for ELISA assays. The results presented are expressed as picograms of protein per milliliter of serum and picograms of protein per milligram of liver lysate protein.

Dihydroethidium Labeling of Liver Tissue

In situ reactive oxygen species (ROS) production was evaluated by staining with dihydroethidium (Invitrogen), as described previously (9). Fluorescence was detected with a Zeiss 510 Meta laser scanning confocal microscope at the University of Nebraska Confocal Laser Scanning Core Facility.

Statistical Analysis

Statistical analysis of differences in the various treatment groups was performed by using the nonparametric Kruskal-Wallis test and Student’s t-test.

RESULTS

We inactivated Kupffer cells in rats to determine their involvement in the regulation of hepcidin expression by chronic alcohol exposure. For these experiments, rats were injected with gadolinium chloride or 0.9% NaCl (as control) and pair fed with regular or alcohol-Lieber-DeCarli diets for 6 wk, as described in MATERIALS AND METHODS. Liver hepcidin expression was analyzed by real-time quantitative PCR (see MATERIALS AND METHODS). Rats with chronic alcohol exposure exhibited a significant decrease in liver hepcidin expression, compared with control rats fed with regular Lieber-DeCarli diet (Fig. 1). Gadolinium chloride treatment did not significantly alter basal liver hepcidin expression levels compared with control rats (Fig. 1). Interestingly, alcohol-fed rats injected with gadolinium chloride also displayed significant
downregulation of hepcidin expression, compared with control rats (Fig. 1).

To determine the role of acute alcohol exposure, we employed a mouse model, as described previously (9). Mice were fed with 20% ethanol in the drinking water or with plain water (control) for 1 wk (see MATERIALS AND METHODS). We measured the TNF-α and IL-6 cytokine levels both in the liver and serum of these mice by ELISA. A significant 3.38-fold and a 2.17-fold increase in TNF-α protein expression was observed in the serum and liver, respectively, of alcohol-fed mice, compared with control mice (Table 1). Similarly, alcohol-fed mice exhibited a significant 4.37-fold and a 2.17-fold increase in IL-6 protein expression in the serum and liver, respectively, compared with control mice (Table 1). Furthermore, alcohol-fed mice exhibited a significant increase in the phosphorylation of the TNF-α receptor p65 subunit of NF-κB in the liver (Fig. 2, B). To determine the role of Kupffer cells in the regulation of hepcidin expression, we inactivated Kupffer cells in mice by gadolinium chloride and fed them with either water (control) or 20% ethanol for 1 wk (see MATERIALS AND METHODS). Gadolinium chloride treatment alone did not significantly alter basal hepcidin expression in the liver, compared with control mice (Fig. 3). However, hepcidin expression was downregulated in mice treated with gadolinium chloride and fed with ethanol compared with control mice (Fig. 3). There was no significant difference in hepcidin expression between alcohol-fed mice and mice treated with gadolinium chloride and fed with alcohol (Fig. 3).

We have also performed experiments to deplete Kupffer cells in mice by clodronate. For these studies, mice were injected with liposomes containing either PBS (as control) or clodronate, as described in MATERIALS AND METHODS. Upon engulfment, clodronate is released and depletes macrophages by apoptosis (26). Kupffer cell depletion was confirmed by staining the liver sections of mice with an anti-mouse macrophage-specific antibody, F4/80. Unlike mice injected with PBS liposomes (control), mice injected with liposomes containing clodronate did not exhibit any macrophage-positive staining in their livers (Fig. 4, A and B). These mice were fed with either plain water (control) or 20% ethanol for 1 wk, as described in MATERIALS AND METHODS. Mice injected with PBS liposomes and fed with ethanol displayed significantly reduced hepcidin expression compared with control mice (Fig. 4C). The treatment with clodronate alone did not significantly alter hepcidin expression in the liver (Fig. 4C). Interestingly, hepcidin expression was also downregulated in mice injected with liposomes containing clodronate and fed with ethanol compared with control mice (Fig. 4C). No significant differences in liver hepcidin expression were detected between mice treated with ethanol and PBS liposomes or mice treated with ethanol and liposomes containing clodronate (Fig. 4C).

TNF-α plays a key role in the progression of alcoholic liver disease and has been reported to reduce hepcidin expression in hepatoma cells (10, 17, 30). To determine the role of TNF-α signaling in the regulation of liver hepcidin expression by alcohol, we neutralized TNF-α by specific antibodies or employed TNF-α receptor knockout mice lacking the expression of both TNF-α receptor isoforms, as described in MATERIALS AND METHODS. All mice were fed with ethanol for 1 wk (see MATERIALS AND METHODS). To confirm that TNF-α neutralization blocked TNF-α receptor signaling, we performed Western blots with an antibody recognizing the phosphorylated form of p65 subunit of NF-κB. Mice injected with control IgG and fed with 20% ethanol for 1 wk displayed a significant increase in the phosphorylation of p65 protein in the liver, compared with mice injected with control IgG and fed with plain water (Fig. 5, A and B). TNF-α-neutralizing antibody injection did not alter hepcidin expression in the liver (Fig. 4C). However, hepcidin expression was also downregulated in mice injected with liposomes containing clodronate and fed with ethanol compared with control mice (Fig. 4C). No significant differences in liver hepcidin expression were detected between mice treated with ethanol and PBS liposomes or mice treated with ethanol and liposomes containing clodronate (Fig. 4C).

Table 1. Cytokine expression in the serum and liver

<table>
<thead>
<tr>
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<th>Serum, pg/ml</th>
<th>Liver, pg/mg</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control mice</td>
<td>64.7±19.6</td>
<td>66.1±15.6</td>
</tr>
<tr>
<td>Ethanol-fed mice</td>
<td>218.7±53.6</td>
<td>183.8±38.2</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control mice</td>
<td>5.1±1.7</td>
<td>27±3.2</td>
</tr>
<tr>
<td>Ethanol-fed mice</td>
<td>22.3±3.5</td>
<td>58.7±9.9</td>
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The level of TNF-α and IL-6 protein expression in the sera and livers of mice fed with plain water (control) or 20% ethanol for 1 wk was determined by ELISA assays, as described in MATERIALS AND METHODS (means ± SE; n = 3 per group). There was a significant (P = 0.001) difference in both serum and liver TNF-α and IL-6 levels between control and ethanol-fed mice.

Fig. 2. Activation of NF-κB. A: the phosphorylation of NF-κB subunit protein, p65 (phospho-p65) in the liver lysates of 129/Sv male mice fed either with plain water or 20% ethanol for 1 wk was determined by Western blotting employing an anti-phospho-p65 antibody, as described in MATERIALS AND METHODS. An antibody recognizing the total levels of p65 protein (p65) was employed as a control to confirm equal protein loading. B: quantification of p65 phosphorylation. Autoradiographs were scanned by a densitometer, and phospho-p65 protein (p-p65) expression in each sample was quantified by normalizing to the total levels of p65 protein expression. Normalized p-p65 expression in alcohol-treated mice was expressed as fold expression of that in water-fed (control) mice (means ± SE; n = 3 per group).
not significantly alter p65 phosphorylation (Fig. 5, A and B). However, mice treated with both TNF-α-neutralizing antibody and ethanol displayed a significant 2.6-fold decrease in the phosphorylation of p65 protein, compared with mice treated with control IgG and ethanol (Fig. 5, A and B). The level of liver hepcidin expression was significantly decreased in mice treated with control IgG and ethanol compared with control mice, which were treated with control IgG and plain water (Fig. 5C). TNF-α-neutralizing antibody alone did not significantly alter basal hepcidin expression levels in the liver (Fig. 5C). However, mice treated with TNF-α-neutralizing antibody and ethanol displayed reduced hepcidin expression in the liver compared with control mice (Fig. 5C). There was no significant difference in hepcidin expression between mice treated with control IgG and ethanol and mice treated with TNF-α-neutralizing antibody and ethanol (Fig. 5C). Similar results were obtained with alcohol-fed TNF-α receptor knockout mice, lacking both receptor isoforms, TNFR1 and TNFR2. The basal hepcidin expression in TNFR knockout mice was not significantly different from that in age and sex-matched control C57BL/6/129/Sv mice (Fig. 6). Alcohol exposure downregulated hepcidin expression both in TNFR knockout mice and control C57BL/6/129/Sv mice, compared with control mice fed with water (Fig. 6). There was no significant difference in liver hepcidin expression between wild-type control mice fed with ethanol and TNFR transgenic mice fed with ethanol. We have previously shown that alcohol-mediated oxidative stress is involved in the downregulation of liver hepcidin expression by alcohol (9). ROS production in the livers of untreated mice and mice treated with clodronate or gadolinium chloride was determined by dihydroethidium staining, as published previously (9). Similar to untreated (control) mice, no ROS production was observed in the livers of mice injected with clodronate or gadolinium chloride (Fig. 7, A, C, E, and G). Alcohol-induced ROS production in the liver was not reduced
following the inactivation or depletion of Kupffer cells (Fig. 7, B, D, F, and G).

DISCUSSION

Our previous studies demonstrated that both acute and chronic alcohol exposure suppress hepcidin expression in the liver, which leads to an increase in duodenal iron transport and liver iron storage (7–9). Kupffer cells and the proinflammatory cytokine TNF-α play a critical role in the progression of alcoholic liver disease (ALD) (1, 10, 23, 25, 30). They have also been suggested to negatively regulate hepcidin expression (17, 24). We therefore investigated whether Kupffer cells and TNF-α are involved in the downregulation of hepcidin expression by alcohol in the liver.

Pair feeding of rats with regular and alcohol Lieber-DeCarli liquid diets is an established chronic alcohol model (12). However, the inactivation of Kupffer cells by gadolinium chloride in these rats did not abrogate the alcohol-induced suppression of liver hepcidin expression. Of note, the activation of Kupffer cells, which primes the liver to alcoholic liver injury, is an early event in the progression of ALD (23, 25). Interestingly, the downregulation of hepcidin expression by alcohol occurs early (within 3–7 days) in the absence of any apparent histological changes or lipid accumulation in the liver (9). Hence, we have also investigated the role of Kupffer cells in an acute alcohol model. The treatment of mice with ethanol in the drinking water for 1 wk was sufficient to activate the Kupffer cells, as demonstrated by our findings (i.e., increases in liver and serum IL-6 and TNF-α levels and activation of NF-κB). However, as observed with chronic alcohol treatment, the inactivation of Kupffer cells by gadolinium chloride did not reverse the downregulation of hepcidin expression by acute alcohol exposure. Gadolinium chloride has been shown to function by blocking the phagocytosis and depleting the large, but not small, Kupffer cells (6). On the other hand, as confirmed by our immunostaining data (see Fig. 4), intravenous delivery of clodronate selectively depletes all Kupffer cells in the liver (26). Theurl et al. (24) have reported that the basal hepcidin mRNA levels in the livers of mice are elevated 24 h following clodronate injection. We did not observe this under our experimental conditions. In fact, mice treated with both alcohol and clodronate displayed a similar decrease in liver hepcidin expression as the mice treated with alcohol alone. Kupffer cells play an important role in iron recycling through...
erythrophagocytosis, and their depletion may alter iron homeostasis. It is known that iron regulates hepcidin synthesis in the liver (4, 19). However, we have previously demonstrated that alcohol exposure renders hepcidin insensitive to body iron levels (8). Collectively, our findings demonstrate that Kupffer cells are not involved in the downregulation of hepcidin expression in the liver by acute or chronic alcohol exposure in vivo.

The proinflammatory cytokine TNF-α is involved in the progression of ALD and reduces hepcidin expression in human hepatoma cells (10, 14, 17, 30). Since Kupffer cells activated by alcohol have been suggested to be the main source of TNF-α (14, 23), our findings with the Kupffer cell experiments do not support a role for TNF-α in the regulation of liver hepcidin expression by alcohol. However, besides Kupffer cells, TNF-α may also originate from other sources in vivo. Most studies up to date confirming an increase in TNF-α levels have been performed with chronic alcohol exposure (14). Here, we demonstrate that feeding mice with ethanol for 1 wk also induces a significant increase in both liver and serum TNF-α production. The transcription factor NF-κB is a downstream target in TNF-α-mediated signaling and the activation of NF-κB results in the phosphorylation of its subunit, p65 (29). Accordingly, these mice also displayed an alcohol-induced increase in p65 phosphorylation, which was inhibited following the neutralization of TNF-α. However, despite the inhibition of TNF-α signaling, alcohol suppressed liver hepcidin expression. Moreover, the experimental data with TNF-α receptor knockout mice also support these findings. In conclusion, the activation of Kupffer cells and TNF-α receptor-mediated signaling are not responsible for the downregulation of hepcidin expression by alcohol in the liver.

Besides cytokines, Kupffer cells activated by alcohol have also been reported to release ROS (2, 23, 28). We have previously demonstrated that alcohol-mediated oxidative stress suppresses hepcidin transcription and promoter activity by inhibiting the DNA-binding activity of C/EBP α in the liver (9). However, as we have shown here, Kupffer cell inactivation or depletion did not result in diminished levels of alcohol-induced ROS in the liver. It is possible that, despite their activation, Kupffer cells may not be the main source of ROS in this acute alcohol mouse model. Instead, hepatocytes could be the source of ROS due to ethanol metabolism occurring in these cells (hepcidin is primarily expressed in hepatocytes).
(22). We have previously shown that the treatment of alcohol-metabolizing human hepatoma cells with 4-methylpyrazole, an inhibitor of alcohol-metabolizing enzymes, abolishes the alcohol-mediated downregulation of hepcidin expression (9). Hence our findings suggest a role for the parenchymal cells of the liver in the regulation of hepcidin expression by alcohol-mediated oxidative stress. These findings help us to further understand the mechanisms involved in the regulation of iron metabolism in ALD.

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