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

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Running title: Hepcidin and alcohol-induced Kupffer cell activation

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ABSTRACT:
Alcohol down-regulates 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-alpha 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 weeks and mice fed with 20 % ethanol in the drinking water for 1 week were used as experimental models. Interestingly, alcohol down-regulated 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-alpha levels and phosphorylation of NFKB subunit p65. The neutralization of TNF-alpha by specific antibodies inhibited p65 phosphorylation. However, neither the neutralization of TNF-alpha nor the lack of TNF-alpha 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. Conclusion: Our results demonstrate that alcohol-induced Kupffer cell activation and TNF-alpha 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.

Keywords: Alcoholic liver disease; Iron; Liver injury; Oxidative stress
INTRODUCTION:

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 down-regulate 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-alpha, mainly synthesized by Kupffer cells, play a critical role in ethanol-induced liver injury (10, 14, 30). TNF-alpha has been reported to down-regulate hepcidin expression in human hepatoma cells (17). However, LPS and the cytokines, IL-1 and IL-6 induce liver hepcidin expression (11, 17). Lou, D. et al. have demonstrated that the depletion of Kupffer cells by clodronate in mice does not abrogate the up-regulation of liver hepcidin expression by inflammation (13). On the other hand, Montosi et al. have reported that the inactivation of Kupffer cells by gadolinium chloride inhibits the LPS-induced elevation of hepcidin expression in mice (15). 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.
alpha (C/EBP alpha) in vivo (9). We investigated the involvement of Kupffer cell activation and TNF-alpha 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, Inc., 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 weeks. 129/Sv strain wildtype mice (Charles River Laboratories), and TNF-alpha receptor knockout mice, deficient in the expression of both TNF-alpha receptor isoforms, TNFR1 and TNFR2 (Jackson Laboratories) and control C57BL/6/129/Sv strain mice (Jackson Laboratories) were maintained on a rodent chow diet-7012 (Harland 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 b.w) or 129/Sv strain male mice (20µg/g b.w) via tailvein 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 week alcohol treatment. Rats were injected with gadolinium chloride or 0.9 % NaCl twice a week (days 1 and 4) during the 6 weeks L. De Carli diet treatment. Kupffer cell depletion: Mice were injected (tailvein) with 0.1 ml of liposomes containing clodronate (obtained from Vrije University, Amsterdam;
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 week. The liposome injections were repeated on day 5 of the 1 week 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 Corporation) 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 RNAase H- reverse transcriptase enzyme (Invitrogen Corporation). Gene expression was analyzed by real-time quantitative PCR, as described previously (8, 9). Primers (sense 5’-ACTCGGACCCAGGCTGC-3’; antisense 5’-AGATAGGTGGTGCTGCTCAGG-3’) and Taqman fluorescent probe (5’ 6-[FAM]-TGTCTCCTGCTTTCCCTCTGGCCA-3’ [TAMRA-Q]) flanking about 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 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 gene) was calculated using the comparative Ct method formula $2^{-\Delta\Delta C_t}$. 
TNF-alpha neutralization:

129/Sv male mice were injected (IP) daily either with a TNF-alpha-neutralizing antibody (250 µg/ mouse; Infliximab; Centocor Inc.) or control IgG (Jackson ImmunoResearch Laboratories) during the 1 week alcohol treatment.

Antibodies and Western blotting:

Western blots using total cell lysates were performed, as described previously (8, 9). In order 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, 1mM PMSF, Complete<sup>R</sup> protease inhibitor cocktail (Roche Diagnostics Corp.), phosphatase inhibitor cocktail A (Santa Cruz Biotechnology Inc., sc-45044), 1 % Triton-X-100] and incubated on ice for 20 min. Subsequently, the lysates were centrifuged (3000x g) for 5 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 Inc.)

Immunohistochemistry:

Sections (10 µm) of formalin-fixed liver tissues were incubated with the polyclonal rat anti-mouse antibody, F4/80 (Serotec Inc.) and a goat anti-rat HRP-conjugated secondary antibody (STAR72; Serotec Inc.). 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-alpha 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 (15,000x g) for 15 min. (4°C) and the supernatants employed for ELISA assays. The results presented are expressed as pg protein per ml of serum and pg protein per mg of liver lysate protein.

**Dihydroethidium labeling of liver tissue:**

In situ reactive oxygen species (ROS) production was evaluated by staining with dihydroethidium (Invitrogen Corporation), 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 non-parametric 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 weeks, as described in Methods. Liver hepcidin expression was analyzed by real-time quantitative PCR (see Methods). Rats with chronic alcohol exposure exhibited a significant decrease in liver hepcidin expression, compared to control rats fed with regular Lieber-DeCarli diet (fig. 1). Gadolinium chloride treatment did not significantly alter basal liver hepcidin expression levels compared to control rats (fig. 1). Interestingly, alcohol-fed rats injected with gadolinium chloride also displayed significant down-regulation of hepcidin expression, compared to control rats (fig. 1).

In order 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 week (see Methods). We measured the TNF-alpha and IL-6 cytokine levels both in the liver and serum of these mice by ELISA. A significant 3.38 fold and a 2.78 fold increase in TNF-alpha protein expression was observed in the serum and liver respectively of alcohol-fed mice, compared to 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 to control mice (table 1). Furthermore, alcohol-fed mice exhibited a significant increase in the phosphorylation of NFκB subunit, p65 in the liver (fig. 2A & B). In order 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 week
Ethanol-fed mice displayed significantly reduced hepcidin expression in the liver compared to control mice fed with plain water (fig. 3). Gadolinium chloride treatment alone did not significantly alter basal hepcidin expression in the liver, compared to control mice (fig. 3). However, hepcidin expression was down-regulated in mice treated with gadolinium chloride and fed with ethanol compared to 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 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. 4A & B). These mice were fed with either plain water (control) or 20% ethanol for 1 week, as described in Methods. Mice injected with PBS liposomes and fed with ethanol displayed significantly reduced hepcidin expression compared to 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 down-regulated in mice injected with liposomes containing clodronate and fed with ethanol compared to 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-alpha 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). In order to determine the role of TNF-alpha signaling in the regulation of liver hepcidin expression by alcohol, we neutralized TNF-alpha by specific antibodies or employed TNF-alpha receptor knockout mice lacking the expression of both TNF-alpha receptor isoforms, as described in Methods. All mice were fed with ethanol for 1 week (see Methods). In order to confirm that TNF-alpha neutralization blocked TNF-alpha receptor signaling, we performed western blots with an antibody recognizing the phosphorylated form of p65 subunit of NFκB. Mice injected with control IgG and fed with 20% ethanol for 1 week displayed a significant increase in the phosphorylation of p65 protein in the liver, compared to mice injected with control IgG and fed with plain water (fig. 5A & B). TNF-alpha-neutralizing antibody injection did not significantly alter p65 phosphorylation (fig. 5A & B). However, mice treated with both TNF-alpha-neutralizing antibody and ethanol displayed a significant 2.6-fold decrease in the phosphorylation of p65 protein, compared to mice treated with control IgG and ethanol (fig. 5A & B). The level of liver hepcidin expression was significantly decreased in mice treated with control IgG and ethanol compared to control mice, which were treated with control IgG and plain water (fig. 5C). TNF-alpha-neutralizing antibody alone did not significantly alter basal hepcidin expression levels in the liver (fig. 5C). However, mice treated with TNF-alpha-neutralizing antibody and ethanol displayed reduced hepcidin expression in the liver compared to 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-alpha-neutralizing antibody and ethanol (fig. 5C). Similar results were obtained with alcohol-fed TNF-alpha 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 down-regulated hepcidin expression both in TNFR knockout mice and control C57BL/6/129/Sv mice, compared to control mice fed with water (fig. 6). There was no significant difference in liver hepcidin expression between wildtype 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 down-regulation 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. 7A, C, E & G). Alcohol-induced ROS production in the liver was not reduced following the inactivation or depletion of Kupffer cells (fig. 7B, D, F & 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-alpha 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 have therefore investigated whether Kupffer cells and TNF-alpha are involved in the down-regulation of hepcidin expression by alcohol in the liver.

Pairfeeding 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 down-regulation 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 week was sufficient to activate the Kupffer cells, as demonstrated by our findings (i.e increases in liver and serum IL-6 and TNF-alpha levels, and activation of NFKB). However, as observed with chronic alcohol treatment, the inactivation of Kupffer cells by gadolinium chloride did not reverse the down-regulation 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 selectivity
depletes all Kupffer cells in the liver (26). Theurl et al. have reported that the basal hepcidin mRNA levels in the livers of mice are elevated 24 hours following clodronate injection (24). 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 down-regulation of hepcidin expression in the liver by acute or chronic alcohol exposure in vivo.

The proinflammatory cytokine, TNF-alpha 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-alpha (14, 23), our findings with the Kupffer cell experiments do not support a role for TNF-alpha in the regulation of liver hepcidin expression by alcohol. However, besides Kupffer cells, TNF-alpha may also originate from other sources in vivo. Most studies up to date confirming an increase in TNF-alpha levels have been performed with chronic alcohol exposure (14). Here, we demonstrate that feeding mice with ethanol for one week also induces a significant increase in both liver and serum TNF-alpha production. The transcription factor NFκB is a downstream target in TNF-alpha-mediated signaling and the activation of NFκB 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-alpha.
However, despite the inhibition of TNF-alpha signaling, alcohol suppressed liver hepcidin expression. Moreover, the experimental data with TNF-alpha receptor knockout mice also support these findings. In conclusion, the activation of Kupffer cells and TNF-alpha receptor-mediated signaling are not responsible for the down-regulation 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 alpha 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 down-regulation 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.
Acknowledgements:

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are not required for hepatic hepcidin activation during iron overload. *Hepatology* 41: 545-552, 2005.


FIGURE LEGENDS:

Fig. 1. Kupffer cell inactivation and hepcidin expression in rats with chronic alcohol exposure. Wistar male rats were pair-fed with regular (cont.) or alcohol (alc.)-Lieber-DeCarli diets and injected (tailvein) with gadolinium chloride (GdCl3) or 0.9 % NaCl (NaCl, as control for injection) for 6 weeks, as described in Methods. Hepcidin mRNA expression was determined by real-time PCR (see Methods). Hepcidin expression in treated rats was calculated as fold expression of that in the control rats (fed with regular diet and injected with NaCl) (mean ± S.E.M.; n=8 per group).

Fig. 2. Activation of NFkB. The phosphorylation of NFkB subunit protein, p65 (phospho-p65) in the liver lysates of 129/Sv male mice fed either with plain water (cont.) or 20 % ethanol in the drinking water (alc.) for 1 week was determined by western blotting employing an anti-phospho-p65 antibody, as described in 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 (mean ± S.E.M.; n=3 per group).

Fig. 3. Kupffer cell inactivation and hepcidin expression in mice with acute alcohol exposure. 129/Sv male mice were injected with 0.9 % NaCl (NaCl), as a control for injections or gadolinium chloride (GdCl3) and fed with either plain water (cont.) or 20 % ethanol in the drinking water (alc.) for 1 week, as described in Methods. Hepcidin mRNA expression was determined by real-time PCR (see Methods). Hepcidin
expression in treated mice was calculated as fold expression of that in the control mice (NaCl injected and water-fed) (mean ± S.E.M.; n=16 per group).

**Fig. 4. The depletion of Kupffer cells.** 129/Sv male mice were injected with 0.1 ml of liposomes containing either PBS, as control (A) or clodronate (B), as described in Methods. Fixed liver sections were immunostained with the macrophage-specific antibody, F4/80 (see Methods) and counterstained with hematoxylin. Arrows indicate the position of Kupffer cells.  

**C:** Liver hepcidin mRNA expression in mice injected with liposomes containing either PBS or clodronate and fed with plain water (cont.) or 20 % ethanol in the drinking water (alc.) for 1 week was determined by real-time PCR (see Methods). Hepcidin expression in treated mice was calculated as fold expression of that in the control mice (PBS-liposome injected and water-fed) (mean ± S.E.M.; n=8 per group).

**Fig. 5. TNF-alpha neutralization.** 129/Sv male mice were injected daily with either control IgG or TNF-alpha-neutralizing antibody and fed with plain water or 20% ethanol in the drinking water for 1 week, as described in Methods (mean ± S.E.M.; n=8 per group).  

**A:** p65 phosphorylation. The phosphorylation of NFKB subunit protein, p65 in the liver lysates of mice injected with control IgG (IgG) or TNF-alpha-neutralizing antibody (TNFα Ab.) and fed either with plain water (cont., TNFα Ab.) or 20 % ethanol (alc., TNFα Ab. & alc.) for 1 week was determined by western blotting employing an anti-phospho-p65 (p-p65) antibody, as described in Methods. An antibody recognizing the total levels of p65 protein was employed 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 treated mice was expressed as fold expression of that in control mice (*control IgG injected and water-fed*). **C:** Hepcidin mRNA expression was determined by real-time PCR (*see Methods*). Liver hepcidin expression in treated mice was expressed as fold expression of that in control mice (*control IgG injected and fed with water*).

**Fig. 6. TNF-alpha receptor knockout mice.** Age and genotype-matched control male mice (wildtype) and transgenic male mice (TNFR knockout) deficient in the expression of both TNF-alpha receptors were treated with plain water (cont.) or with 20 % ethanol in the drinking water (alc.) for 1 week, as described in Methods. Hepcidin mRNA expression was determined by real-time PCR (*see Methods*) and hepcidin expression in alcohol-treated mice was expressed as fold expression of that in control mice (*wildtype mice fed with water*) (*mean ± S.E.M.; n=8 per group*).

**Fig. 7: Kupffer cells and ROS production.** Frozen liver sections of untreated mice (A, B), mice injected with liposomes containing clodronate (C, D) or gadolinum chloride (E, F) and fed with plain water, as control (A, C, E) or 20 % ethanol (B, D, F) for 1 week were stained with dihydroethidium, as described in Methods (*mean ± S.E.M.; n=8 per group*). Red fluorescence was detected with Zeiss 510 Meta laser scanning confocal microscope at identical laser settings (original magnification 63X). **G:** Fluorescence intensities in randomly selected areas of multiple digital images corresponding to panels A-F were quantified by Zeiss LSM510 image analysis software.
Table 1: Cytokine expression in the serum and liver: The level of TNF-alpha and IL-6 protein expression in the sera and livers of mice fed with plain water (control) or 20% ethanol for 1 week was determined by ELISA assays, as described in Methods (mean ± S.E.M.; n=3 per group). There was a significant (p=0.001) difference in both serum and liver TNF-alpha and IL-6 levels between control and ethanol-fed mice.

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<th>Serum (pg/ml)</th>
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<td>control mice</td>
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<td>218.7 ± 53.6</td>
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<td>IL-6</td>
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FIGURES:

Fig. 1
Fig. 2
Fig. 3

Comparison of hepcidin mRNA expression (normalized to gapdh) in different conditions:
- **NaCl**:
  - Control (cont.): p = 0.001
  - Alcoholic (alc.): p = 0.8

- **GdCl₃**:
  - Control (cont.): p = 0.35
  - Alcoholic (alc.):
Fig. 4

A

PBS

B

Clodronate

C

fold hepcidin mRNA expression (normalized to gapdh)

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p = 0.236
p = 0.001
p = 0.0183
Fig. 5
Fig. 6

Fold hepcidin mRNA expression (normalized to gapdh)

- Wildtype: cont. = 0.5, alc. = 1.0, p = 0.255
- TNFR knockout: cont. = 0.012, alc. = 0.186

Cont. = control, Alc. = alcohol
Fig. 7