Alcohol Metabolism-mediated Oxidative Stress Down-regulates Hepcidin Transcription and Leads to Increased Duodenal Iron Transporter Expression*

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Duygu Dee Harrison-Findik†, Denise Schafer†, Elizabeth Klein†, Nikolai A. Timchenko‡, Hasan Kulaksiz‡, Dahn Clemens‡, Evelyn Fein‡, Billy Andriopoulos**, Kostas Pantopoulos**, and John Gollan†

From the †Department of Internal Medicine, University of Nebraska Medical Center, Omaha, Nebraska 68198-5820, ‡Department of Pathology and Huffington Center on Aging, Baylor College of Medicine, Houston, Texas 77030, §Department of Internal Medicine, Division of Gastroenterology, University of Heidelberg, Heidelberg, Germany, ¶Veterans Administration Medical Center, Omaha, Nebraska 68105, and **Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, Montreal, Quebec H3T 1E2, Canada

Patients with alcoholic liver disease frequently exhibit iron overload in association with increased hepatic fibrosis. Even moderate alcohol consumption elevates body iron stores; however, the underlying molecular mechanisms are unknown. Hepcidin, a circulatory peptide synthesized in the liver, is a key mediator of iron metabolism. Ethanol metabolism significantly down-regulated both in vitro and in vivo hepcidin mRNA and protein expression. 4-Methylpyrazole, a specific inhibitor of the alcohol-metabolizing enzymes, abolished the effects of ethanol on hepcidin. However, ethanol did not alter the expression of transferrin receptor1 and ferritin or the activation of iron regulatory RNA-binding proteins, IRP1 and IRP2. Mice maintained on 10–20% ethanol for 7 days displayed down-regulation of liver hepcidin expression without changes in liver triglycerides or histology. This was accompanied by elevated duodenal divalent metal transporter1 and ferroportin protein expression. Injection of hepcidin peptide negated the effect of ethanol on duodenal iron transporters. Ethanol down-regulated hepcidin promoter activity and the DNA binding activity of CCAAT/enhancer-binding protein α (C/EBPα) but not β. Interestingly, the antioxidants vitamin E and N-acetylcysteine abolished both the alcohol-mediated down-regulation of C/EBPα binding activity and hepcidin expression in the liver and the up-regulation of duodenal divalent metal transporter 1. Collectively, these findings indicate that alcohol metabolism-mediated oxidative stress regulates hepcidin transcription via C/EBPα, which in turn leads to increased duodenal iron transport.

Patients with alcoholic liver disease frequently exhibit increased body iron stores, as reflected by elevated serum iron indices (transferrin saturation, ferritin) and hepatic iron concentration (1–5). Even mild to moderate alcohol consumption has recently been shown to increase the prevalence of iron overload and to reduce the risk of iron deficiency and anemia (4). Moreover, increased hepatic iron content is associated with greater mortality from alcoholic cirrhosis, suggesting a pathogenic role of iron in alcoholic liver disease. Genetic hemochromatosis, a common iron overload disorder in the Caucasian population, in conjunction with excessive alcohol consumption exacerbates liver injury (6). Both iron and ethanol individually cause oxidative stress and lipid peroxidation, which result in cellular injury (7, 8). Despite these observations, the underlying mechanisms of iron accumulation observed in alcoholic liver disease remain unclear.

A series of novel iron-regulatory proteins, including iron transporters and soluble mediators have been identified, that have greatly enhanced our understanding of iron metabolism (9–11). Hepcidin (LEAP-1, Hamp) has recently been shown to play a central role in the regulation of iron metabolism (12–15). Hepcidin is an antimicrobial peptide that was first isolated from human urine and blood (16, 17). Hepcidin is synthesized in the liver as an 84-amino acid precursor protein that is subsequently cleaved into the 25-amino acid disulfide-bridged active peptide form (16–18). Hepcidin knock-out mice develop iron overload in the liver, pancreas, and heart, whereas mice overexpressing hepcidin display severe iron deficiency and anemia (13, 14). Iron overload increases and iron deficiency decreases the expression of hepcidin in the liver. Hepcidin is believed to act as a soluble mediator of iron metabolism by regulating intestinal iron absorption and the release of iron from macrophages (19, 20). Hepcidin is also regulated by inflammatory signals (15, 21–24). Patients with alcoholic liver disease have been observed to inappropriately absorb lipopolysaccharide from their intestine, leading to the release of proinflammatory cytokines and recruitment of inflammatory cells to the liver (25). However, the role of hepcidin in alcoholic liver disease is unknown.

Understanding the molecular mechanisms of ethanol and iron interaction is of considerable clinical importance because both alcoholic liver disease and genetic hemochromatosis are common diseases in which alcohol and iron appear to act synergistically to cause liver injury. In this study we determined the molecular mechanisms of iron overload mediated by alcohol metabolism.
**EXPERIMENTAL PROCEDURES**

**Animal Experiments**—Animal experiments were approved by the animal ethics committee at the University of Nebraska Medical Center. 129/Sv strain male and female mice (Charles River Laboratories) were housed individually and maintained on rodent chow diet-7012 (Harland Teklad Global Diets). 10–20% ethanol was added in the drinking water. For vitamin E experiments, mice were fed with either normal (control) (50 IU of vitamin E/kg) or high vitamin E (2000 IU of vitamin E/kg) diets (Research Diets, Inc.) for a week. Subsequently, these mice were either untreated or exposed to 20% ethanol for another week and then sacrificed. For N-acetylcysteine studies, untreated and ethanol-treated mice received a single daily injection (intraperitoneal) of either N-acetylcysteine (0.2 mg/g of body weight in 0.9% NaCl) or 0.9% NaCl alone (as control) for a week and then sacrificed. For hepcidin experiments, mice were either untreated or exposed to 20% ethanol for another week and then sacrificed. For vitamin E experiments, mice were fed with either normal (control) (50 IU of vitamin E/kg) or high vitamin E (2000 IU of vitamin E/kg) diets (Research Diets, Inc.) for a week. Subsequently, these mice were either untreated or exposed to 20% ethanol for another week and then sacrificed. For N-acetylcysteine studies, untreated and ethanol-treated mice received a single daily injection (intraperitoneal) of either N-acetylcysteine (0.2 mg/g of body weight in 0.9% NaCl) or 0.9% NaCl alone (as control) for a week and then sacrificed. For hepcidin experiments, mice were either untreated or exposed to 20% ethanol for another week and then sacrificed.

**Cell Culture and Alcohol and Acetaldehyde Treatments**—Parental HepG2 cells, a human hepatoma cell line, do not express alcohol-metabolizing enzymes nor do they metabolize alcohol. VL-17A cells, provided by Dr. D. Clemens, express both alcohol dehydrogenase and cytochrome P450 2E1 and metabolize alcohol (26). All cells were plated on poly-L-lysine (Sigma Aldrich)-coated flasks and maintained in high glucose Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with glutamine and 10% fetal calf serum (Hyclone). Antibiotics were routinely added to the media for the selection of alcohol dehydrogenase (G418, 0.5 mg/ml) and cytochrome P450 2E1 (zeocin, 0.4 mg/ml) expression. Cells (1 × 10^6) were plated in 25-cm^2 flasks the day before the experiment. The next day fresh culture medium was supplemented with 25 mM HEPE (pH 7.2) and alcohol or acetaldehyde, and the flask lids were tightened to avoid evaporation. For treatments of acetaldehyde, 75 μM cyanamide (Sigma Aldrich) was added to the media to eliminate the degradation of acetaldehyde. Cell viability was 80–95% (trypan blue exclusion) under these conditions.

**RNA Isolation and Northern Blotting**—Cells or organs washed with phosphate-buffered saline were lysed in Trizol® (Invitrogen), and total RNA was isolated according to the manufacturer's specifications. Northern blotting was performed with 5–10 μg of total RNA by using the Northern Max kit (Ambion, Inc.) according to the manufacturer’s instructions. Hybridization was performed with 32P-labeled mouse hepcidin, and 18 S ribosomal RNA transcripts were prepared by the MAXIscript T7 in vitro transcription kit (Ambion Inc.). Mouse hepcidin open reading frame sequences were amplified by using the following forward primer (5’-atgagcactcagcagcagc-3’) and reverse primer (5’-ctatgttttgaaacagataccagt-3’). PCR fragments were cloned into pGEM-T Easy Vector (Promega).

**cDNA Synthesis and Real-time Quantitative PCR Analysis**—cDNA was synthesized using 2–4 μg of isolated RNA, 2.5 μM random primers (Applied Biosystems), and 200 units of SuperScript II RNase H reverse transcriptase enzyme (Invitrogen). To exclude possible genomic DNA contamination, control samples were employed in which the reverse transcriptase enzyme was omitted from the cDNA synthesis reaction. Gene expression was analyzed by real-time quantitative PCR using an ABI Prism 7700 sequence detection system (Applied Biosystems). Species-specific primers and TaqMan fluorescent probes (5’-6-carboxyfluorescein; 3’-6-carboxytetramethylrhodamine) flanking about 70 base pairs of the open reading frame sequences were designed by the Primer Express 1.5 program (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)2 gene probes (Applied Biosystems) were used as the endogenous controls. Species-specific sequences of the TaqMan fluorescent probes and sense and antisense primers are listed in Table 1. cDNA was employed as a template in PCR reactions. After PCR (50 °C for 2 min, 95 °C for 10 min (1 cycle), 95 °C for 15 s, and 60 °C for 1 min (40 cycles)), the data were analyzed using Sequence Detection Systems software (Applied Biosystems), and 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^−ΔΔCt. The amplification efficiency of control and target genes was calculated from the kinetic curves as described (27).

**Antibodies**—The anti-DMT1 polyclonal antibody was produced by injecting rabbits with a mixture of rat glutathione S-transferase-rat-DMT1 (amino acids 1–66; 307–361; 374–412) fusion proteins (NCBI Entrez Protein accession number AAC53319), which share 96 and 88% overall homology with the identical regions of the mouse and human DMT1 proteins, respectively. The DH4 antisera was purified further with glutathione-agarose beads (Sigma Aldrich) to eliminate anti-glutathione S-transferase-specific IgG proteins. The anti-ferroportin polyclonal antibodies were produced by injecting mice with 2 keyhole limpet (KLH)-conjugated 23 amino acid peptides corresponding to amino acids 74–96 and 159–181 of rat ferroportin protein (NCBI Entrez protein accession number AAK77858). These regions of rat ferroportin protein share 100 and 93.5% homology with those of mouse and human ferropor-

### Table 1

| Gene                | Sense primer 5’-3’ | Antisense primer 5’-3’ | TaqMan probe 5’-3’ |
|---------------------|-------------------|------------------------|-------------------|
| Human hepcidin      | TGCCCATGTTTCAGAGGCA | CGCCAGCAGAAAATGCAGAATT | AGAGAGCAGCACACCATCCC |
| Mouse hepcidin1     | TCGAGAGGGAAGAAGGAGAGA | CCACTGACGATTGAGGACTTT | CAACTGCCGCTTCTGCTCCTT2T |
| Mouse hepcidin2     | CGCATTCCAGAAGAACTGCAA | TTATTACACGCTGACGAAGAATC | AGGAAGAGGAGACACATCACCCTC |
| Human TriR1         | AGACTGAGCAGACAGGATCTT | AAGCGACGTGCTGCAGGAAGATC | CAGTGAGGGAGGAGCCAGGAACT |
| Human H-ferritin     | AATTGCGTGACAGCCGAGACC | TCTCGGCAAGCCGATTC | TGTCACAGGAGGAGGCC |

2 The abbreviations used are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; C/EBP, CCAAT/enhancer-binding protein; ROS, reactive oxygen species; siRNA, small interfering RNA; IRP, iron regulatory RNA-binding protein; Ct, cycle number at the linear amplification threshold.
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tin proteins, respectively. Polyclonal IRP2 antibody was a gift from Dr. E. Leiboldt (Univ. of Utah), and ferritin heavy chain subunit antibody was obtained commercially (Novus Biologicals). N-terminal (EG2-Hep-N) and C-terminal (EG2-Hep-C) pro-hepcidin antibodies, which recognize the hepcidin precursor protein, were produced as described (18, 28). C/EBPα and C/EBPβ antibodies were purchased from Santa Cruz Biotechnology, Inc.

**Western Blotting**—Tissue lysates were prepared by homogenizing with a Dounce homogenizer and incubating (20 min, 4 °C) in lysis buffer (10 mM Tris/HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, Complete® protease inhibitor mixture (Roche Diagnostics), 1% Triton-X-100) and subsequent centrifugation at 3000 × g for 5 min. Supernatants were utilized for Western blots. To confirm equal protein loading, identical samples were blotted with the anti-GAPDH (Chemicon International) monoclonal antibody. Immune-reactive bands were detected by the ImmunStar™ anti-rabbit-AP or anti-mouse-HRP kits (Bio-Rad). The Western blots with pro-hepcidin antibodies were performed as described (18).

**Dihydroethidium Labeling of Liver Tissue**—*In situ* reactive oxygen species (ROS) production was evaluated by staining with dihydroethidium (Invitrogen), which is freely permeable across cell membranes (29). In the presence of ROS, dihydroethidium is oxidized to ethidium bromide and stains nuclei bright red by intercalating with the DNA (29, 30). Fresh cross-sections (10 μm) of unfixed, frozen liver tissues were immediately incubated with 3 μM dihydroethidium (diluted in phosphate-buffered saline from 5 mM stock solution in Me2SO) at 37 °C for 15 min in a humidified chamber. The slides were subsequently washed twice with ice-cold phosphate-buffered saline and cover-slipped, and fluorescence was detected with a LS412 laser scanning confocal microscope at the University of Nebraska Confocal Laser Scanning Core Facility.

**Luciferase Reporter Assay**—The 624-bp (−633/−9) human hepcidin promoter region was amplified and cloned into the luciferase reporter vector, pGL3-basic (Promega), as described (31). VL-17A cells were transfected with the reporter constructs by using Lipofectamine 2000 (Invitrogen). 16 h after transfection, cells were either untreated or treated with 25 mM ethanol for 48 h. Reporter assays were performed with the luciferase assay system kit (Promega) according to the manufacturer’s instructions by using a luminometer (Luminoskan RS, Lab-systems). pGL3-basic and pGL3-promoter vectors were used as negative and positive controls, respectively.

**Electrophoretic Mobility Gel-shift Assay and Small Interfering RNAs (siRNA) Treatment**—Mouse liver nuclear lysate isolation and gel-shift assays were performed as described (32). Briefly, livers were homogenized in buffer A (20 mM Tris/HCl (pH 7.5), 50 mM KCl, 2 mM MgCl2, 1 mM EDTA, and 5 mM dithiothreitol). Nuclei were pelleted by centrifugation at 2000 × g for 10 min and washed with buffer A. High salt extraction of nuclear proteins was performed with buffer B (25 mM Tris-HCl (pH 7.5), 0.42 M NaCl, 1.5 mM MgCl2, 1 mM dithiothreitol, 0.5 mM EDTA, and 25% sucrose) for 30 min on ice. After centrifugation, the supernatant (nuclear extract) was frozen and kept at −80 °C. Gel-shift assays were performed by using the oligonucleotide 5’-CATGGATGTTTATGAAAT-CTG-3’ (C/EBP binding site is shown in bold) as a probe. 5–10 μg of nuclear extract protein was used for each binding reaction. pADtrack-CMV construct with siRNA against C/EBPα was created as described (33). VL-17A cells were transfected with siRNA by using Lipofectamine 2000 (Invitrogen). 48 h after transfection, green fluorescent protein-positive cells were counted by fluorescence-activated cell sorter Vantage SE/Divacell sorter (BD Biosciences) at the University of Nebraska Flow Cytometry Core Facility, and total RNA and cell lysates were prepared as described above.

**Statistical Analysis**—Each real-time quantitative PCR experiment was performed with duplicate samples for each tested gene. The results presented represent the mean (±S.E.) of several experiments (n = 3) performed with separate cDNA reactions. Statistical significance was calculated using the non-parametric Kruskal-Wallis test.

**RESULTS**

To investigate the effect of ethanol metabolism on *in vivo* hepcidin expression, we treated 129/Sv mice with 10–20% ethanol for 7 days. Mice consumed an average of 3.6 ± 0.38 ml of 10% and 2.55 ± 0.42 ml of 20% ethanol per day (Fig. 1A). Control (untreated) mice consumed 4.57 ± 0.21 ml of plain water using the oligonucleotide 5’-CATGGATGTTTATGAAAT-CTG-3’ (C/EBP binding site is shown in bold) as a probe. 5–10 μg of nuclear extract protein was used for each binding reaction. pADtrack-CMV construct with siRNA against C/EBPα was created as described (33). VL-17A cells were transfected with siRNA by using Lipofectamine 2000 (Invitrogen). 48 h after transfection, green fluorescent protein-positive cells were counted by fluorescence-activated cell sorter Vantage SE/Divacell sorter (BD Biosciences) at the University of Nebraska Flow Cytometry Core Facility, and total RNA and cell lysates were prepared as described above.

**Statistical Analysis**—Each real-time quantitative PCR experiment was performed with duplicate samples for each tested gene. The results presented represent the mean (±S.E.) of several experiments (n = 3) performed with separate cDNA reactions. Statistical significance was calculated using the non-parametric Kruskal-Wallis test.
Hepcidin has been shown to regulate duodenal iron absorption (34, 35). We, therefore, investigated the effects of ethanol for 7 days also displayed significant down-regulation of liver hepcidin 1 mRNA expression compared with untreated mice (data not shown). However, a significant down-regulation of liver hepcidin 1 expression was observed in livers of mice untreated (control) mice (Fig. 2). The median response difference in hepcidin expression between male and female mice was statistically significant (p = 0.005). E, Northern blot analysis of hepcidin expression in livers of mice untreated (control) or 20% ethanol treated for 7 days was performed as described under "Experimental Procedures." The expression of DMT1 (A and D), ferroportin (B and E), and GAPDH (control) (C and F) proteins in duodenum lysates (20 μg of protein) of untreated (control) or 20% ethanol-treated (alc.) 129 (A–C) and C57BL/6 (D–F) mice were determined by Western blotting. G–I, Hepcidin peptide injection. DMT1 (H), ferroportin (I), and GAPDH (control) expression in untreated (lane 1), 20% ethanol-treated (lane 2) 129 mice, untreated mice injected with soluble hepcidin peptide (lane 3), and ethanol-treated mice injected with soluble hepcidin peptide (1 μg/g of body weight) (lane 4) was determined by Western blotting with anti-rabbit-DMT1, antimouse-ferroportin, and anti-mouse-GAPDH antibodies as described under "Experimental Procedures."
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FIGURE 4. The effect of ethanol on in vitro hepcidin expression. A, hepcidin mRNA expression in untreated (lanes 1 and 7), 25 mM ethanol-treated (lanes 2 and 8), 50 mM isopropanol-treated (lane 3), 1 mM 4-methylpyrazole- and ethanol-treated (lane 4), 4-methylpyrazole-treated (lane 5), and 50 μM acetaldelyde-treated (lanes 6 and 9) VL-17A cells (lanes 1–6) and in HepG2 control cells (lanes 7–9) for 2 days was determined by real-time quantitative PCR. mRNA expression in treated cells was quantified as n-fold expression of that in untreated (control) cells. B–E, prohepcidin protein expression. Untreated (con.) and 25 mM ethanol-treated (alc.) lysates from VL-17A (B) and parental HepG2 (C) cells were blotted with N-terminal (EG2)-Hep-N) pro-hepcidin antibody as described (18, 28). −10-kDa pro-hepcidin protein is shown by a black arrow. Identical cell lysates from VL-17A (D) and parental HepG2 (E) cells were blotted with anti-GAPDH antibody to confirm equal protein loading.

2 (0.35 ± 0.01) compared with untreated cells (1 ± 0) (Fig. 4A, lanes 1 and 2). However, treatment with 50 mM isopropanol did not alter hepcidin expression (Fig. 4A, lane 3). 4-Methylpyrazole, a specific inhibitor of alcohol dehydrogenase and cytochrome P450 2E1 (36), blocked the down-regulation of hepcidin expression by ethanol (Fig. 4A, lanes 4 and 5). 50 μM acetaldelyde, the byproduct of ethanol metabolism, also down-regulated hepcidin expression (Fig. 4A, lane 6). Hepcidin expression was unaltered in 25 mM ethanol-treated parental HepG2 cells, which do not metabolize alcohol (Fig. 4A, lanes 7 and 8). However, 50 μM acetaldelyde down-regulated hepcidin expression in HepG2 cells (Fig. 4A, lane 9). In accordance with mRNA data, the expression of hepcidin precursor protein (prohepcidin) was also reduced in 25 mM ethanol-treated VL-17A cells but not parental HepG2 cells, as detected with anti-prohepcidin antibodies, as described under “Experimental Procedures” (Fig. 4, B–E).

Iron overload has been shown to up-regulate hepcidin expression in vivo but to down-regulate hepcidin expression in vitro (12, 13, and 37). We, therefore, determined the effect of ethanol on the expression of other iron-regulatory proteins, namely transferrin receptor 1, ferritin, and the RNA binding proteins, IRP1 and IRP2, which are regulated by changes in intracellular iron levels (11). Ethanol did not alter the expression of the transferrin receptor 1, ferritin, and IRP2 in VL-17A cells compared with untreated cells (Fig. 5, A–C). As confirmed by RNA gel-shift assays, exposure to 25 mM ethanol did not significantly (p < 0.245) alter IRP1 RNA binding activity in VL-17A cells (Fig. 5, D and E).

No obvious tissue injury or lipid accumulation was detected in the liver of 129 mice treated with 20% ethanol, as determined by hematoxylin and eosin staining (Fig. 6, A and B). Moreover, we did not detect any changes in liver triglyceride levels in 129 mice treated with 20% ethanol compared with untreated mice (Fig. 6C). However, oxidative stress is considered to be one of the early events in the progression of alcoholic liver disease (38, 39). Thus, we studied the role of oxidative stress in ethanol metabolism-regulated hepcidin expression in vivo. Ethanol metabolism-mediated ROS production in mice liver was confirmed by dihydrothioldiamine staining, as described under “Experimental Procedures” (Fig. 7, A–D). The level of ethanol-mediated ROS was reduced when mice were maintained on the high vitamin E diet, as described under “Experimental Procedures” (Fig. 7, B and C). The ethanol-mediated down-regulation of in vivo hepcidin expression was significantly abolished in mice treated with high vitamin E (2000 IU/kg) compared
with mice treated with normal (control) vitamin E (50 IU/kg) (Fig. 8A, lanes 2 and 4). The median response differences in hepcidin expression between mice maintained on normal vitamin E and ethanol, high vitamin E, and high vitamin E and ethanol were statistically significant ($p = 0.024$). Similarly, injecting mice daily with the antioxidant N-acetylcysteine (0.2 mg/g of body weight) for 1 week also abolished the down-regulation of liver hepcidin expression observed with 20% ethanol (data not shown). Interestingly, the ethanol-mediated up-regulation of duodenal DMT1 protein was abolished by vitamin E treatment (Fig. 8B).

To study the effect of ethanol on the transcriptional regulation of hepcidin, reporter gene assays were performed. VL-17A cells were transfected with a 624 bp (−633/−9) hepcidin promoter region, as described under “Experimental Procedures” and treated with 25 mM ethanol for 2 days. Ethanol down-regulated the activity of 624-bp hepcidin promoter (Fig. 9A). 624-bp hepcidin promoter harbors binding sites for the transcription factor C/EBPα and -β, and C/EBPα has been suggested to be involved in the regulation of both human and mouse hepcidin promoters (31). To examine the effect of ethanol and ethanol-mediated oxidative stress on C/EBPα activity and expression, we performed gel-shift assays and Western blotting with liver lysates from untreated, ethanol-treated mice and mice simultaneously treated with ethanol and antioxidants. Ethanol exposure reduced the DNA binding activity of C/EBPα but not C/EBPβ as confirmed by antibody supershift (Fig. 9B, lanes 1–6). Interestingly, treatment with vitamin E abolished the effect of ethanol on binding activity of C/EBPα but not C/EBPβ (Fig. 9B, lanes 7–9). Similar results were observed with mice injected with the antioxidant N-acetylcysteine (data not shown). In accordance with the binding data, C/EBPα protein was reduced both in ethanol-treated mouse liver and VL-17A cells (Fig. 9, C and D, lanes 1 and 2). The effect of ethanol on C/EBPα protein expression was abolished by antioxidants (Fig. 9, C and D, lanes 2 and 3). C/EBPβ protein levels did not change significantly in ethanol-treated VL-17A cells (Fig. 9E, lanes 1 and 2). To examine a possible role for C/EBPα in the regulation of hepcidin promoter, we have inhibited C/EBPα expression in VL-17A cells by specific siRNA as described (33) and determined hepcidin transcription. The inhibition of C/EBPα by siRNA led to a dramatic reduction of transcription from the hepcidin promoter as shown by measuring the levels of hepcidin mRNA (Fig. 9F).

**DISCUSSION**

Alcoholic liver disease is a major cause of chronic disease in the United States and worldwide. The mechanisms of interaction of alcohol with molecular and cellular events that lead to liver injury are largely unknown. Iron is considered to be one of the secondary risk factors. We therefore were interested in studying the effects of alcohol on the expression of hepcidin, a central regulator of iron metabolism.

Using both in vivo and in vitro models, we observed down-regulation of hepcidin after ethanol treatment. Mice express two duplicated hepcidin genes, hepcidin 1 and 2, and both genes are located on mouse chromosome 7 (12, 13). By utilizing specific probes, we demonstrated that hepcidin 1, but not hepcidin 2, is regulated by alcohol metabolism. Similarly, it has been suggested that hepcidin 1, but not hepcidin 2, plays a role in iron metabolism (40). Female mice have been reported to express more hepcidin than male mice (41), which may explain the sex-specific differences we observed in the ethanol-mediated down-regulation of hepcidin.

Alcoholic patients have been reported to exhibit enhanced intestinal iron absorption, but the underlying molecular
Iron transporters DMT1 and ferroportin play a key role in the absorption of dietary iron by duodenal enterocytes (45, 46). Alcohol-mediated down-regulation of hepcidin expression in the liver led to increased expression of DMT1 and ferroportin in the duodenum, which was abolished by the injection of hepcidin peptide. The fact that similar to 129, C57BL/6 mice also displayed alcohol-mediated down-regulation of hep- cidin in the absorption of dietary iron by duodenal enterocytes (34, 35) and ferroportin in the duodenum, which was abolished by the injection of hepcidin peptide. These findings strongly support a direct role for hepcidin in the alcohol-mediated increase in intestinal iron absorption. Hepcidin peptide has been reported to bind, internalize, and degrade ferroportin protein in vitro (47), and Yeh et al. (44) have reported a decrease in duodenal ferroportin expression in rats injected with recombinant hepcidin precursor protein. However, unlike ferroportin, which is expressed basolaterally, DMT1 is located on the apical surface of enterocytes and, hence, does not have direct contact with the circulation (9, 10). Rivera et al. (48) have documented hepcidin accumulation in the duodenum of mice injected with the radiolabeled hepcidin peptide. Moreover, a decrease in DMT1 expression has been reported in Caco2 cells (a human intestinal epithelial cell line) exposed to the hepcidin peptide (35). Thus, it is feasible that internalized hepcidin may interact with DMT1. However, it is highly likely that the iron depletion induced by increased ferroportin expression in enterocytes may lead to the up-regulation of DMT1.

Hepcidin is expressed mainly by the parenchymal cells of the liver. The VL-17A cells expressing both alcohol dehydrogenase and cytochrome P450 2E1 are a model for parenchymal cells, which metabolize alcohol. The down-regulation of hepcidin expression in VL-17A cells was specific for ethanol metabolism since 4-methylpyrazole, a specific inhibitor of alcohol metabolizing enzymes, abolished the effect of ethanol on hepcidin. Acetaldehyde, the byproduct of ethanol oxidation, also down-regulated hepcidin expression. It is of note that VL-17A cells express acetaldehyde dehydrogenase and are able to metabolize acetaldehyde (26). Unlike hepcidin, ethanol metabolism did not alter the expression of transferrin receptor 1, ferritin, IRP2, or the RNA binding activity of IRP1, suggesting that the decreased hepcidin expression in vitro is not an indirect effect of changes in intracellular iron levels but is specifically mediated by etha- nol metabolism. However, the fact that down-regulation of hepcidin by alcohol in vivo was more prominent compared with that in VL-17A cells suggests that non-parenchymal cells of the liver may play a role in the regulation of hepcidin by alcohol.

It was notable that no changes in triglyceride levels or histol- ogy of the liver of mice treated with ethanol was observed, sug-

FIGURE 7. ROS detection. Frozen liver sections of mice maintained on normal (control) (50 IU/kg) vitamin E diet (A), normal vitamin E diet and 20% ethanol (B), and high (2000 IU/kg vitamin E) diet and 20% ethanol (C) were stained with dihydroethidium as described under “Experimental Procedures.” Red fluorescence was detected with LS412 laser scanning confocal microscope at identical laser settings (original magnification 10×). D, fluorescence intensities in randomly selected areas of digital images corresponding to panels A (1), B (2), and C (3) were quantified by NIH image analysis software.

FIGURE 8. Role of antioxidants. Liver hepcidin mRNA (A), duodenal DMT1 (B), and GAPDH (control) (C) protein expression in mice maintained on normal (control) vitamin E diet (lane 1), normal vitamin E diet and 20% ethanol (lane 2), high vitamin E diet (lane 3), and high vitamin E diet and 20% ethanol (lane 4) was determined by real-time PCR or Western blotting with anti-DMT1 or GAPDH antibodies as described under “Experimental Procedures.” 5 mice were employed in each treatment group for every experiment (n = 2). mRNA expression in mice administered normal vitamin E and ethanol, high vitamin E only, and high vitamin E and ethanol were quantified as fold expression of that in mice maintained on normal vitamin E diet only. The median response differences in hepcidin expression between mice maintained on different diets was significant (p < 0.024). As an internal control for DMT1 antibody, lysates from HeLa cells transfected with pcDNA3.1 construct harboring DMT1 (IRE isoform) open reading frame (HeLa-DMT1) were analyzed in parallel.
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FIGURE 9. Effect of alcohol on hepcidin gene transcription and C/EBP binding activity. A, luciferase activity in untreated (lane 1) and 25 mM ethanol-treated (lane 2) VL-17A cells transfected with 624 bp of human hepcidin promoter construct were performed as described under “Experimental Procedures.” Reporter activity in treated cells is expressed as -fold light intensity of that in untreated cells. B, nuclear lysates from mice maintained on normal (control) vitamin E (lanes 1 – 3), normal vitamin E and 20% ethanol (lanes 4 – 6), high vitamin E diet (lanes 7 – 9), and high vitamin E diet (lanes 10 – 12) was determined by gel-shift assay. Antibodies (Ab) to C/EBPα (α) and C/EBPβ (β) were incorporated into the binding reaction before probe addition. C, C/EBPα (C) and GAPDH (F) protein expression in liver nuclear lysates from mice maintained on normal vitamin E (lane 1), normal vitamin E and 20% ethanol (lane 2), high vitamin E diet (lane 3), and high vitamin E diet (lane 4) and C/EBPα (D) and C/EBPβ (E), and GAPDH (G) protein expression in untreated (I) and 25 mM ethanol (lane 2)-treated and 1 mu N-acetylcysteine- and ethanol-treated (lane 3) VL-17A cells was determined by Western blotting. These experiments were performed (n = 3) with nuclear lysates prepared from different sets of mice. H, hepcidin expression in untreated (lane 1) and C/EBP siRNA-transfected (lane 2) VL-17A cells was determined by real-time PCR. The inset shows C/EBP protein expression. I, a representative (n = 3) dot plot of green fluorescent protein (GFP)-positive, siRNA-expressing VL-17A cells.

Suggesting that steatosis or liver injury was not involved in the regulation of hepcidin expression. However, the multiple oxidation steps in alcohol metabolism are accompanied by an altered cellular redox state (increased NADH/NAD redox ratio) and the formation of ROS (6). Both acute and chronic alcohol exposure are accompanied by elevated oxidative stress levels (39), and we detected ROS in the liver of mice treated with ethanol for a period of 7 days. Treatment with antioxidants abolished the effect of alcohol on liver hepcidin and duodenal DMT1 expression. Thus, oxidative stress may play a role in the regulation of hepcidin expression.

The role of ROS in transcriptional regulatory mechanisms is well described (49). Hence, it is feasible that redox changes accompanied by alcohol metabolism can regulate hepcidin gene transcription. Ethanol down-regulated both the hepcidin promoter activity and the DNA binding activity of C/EBPα transcription factor. The fact that alcohol altered the binding activity of C/EBPα but not C/EBPβ demonstrates that the effect of alcohol is specific for C/EBPα. The decrease in C/EBPα protein levels induced by alcohol also suggests that the decrease in DNA binding activity may be due to a decrease in protein expression. Our findings with siRNA experiments demonstrate that C/EBPα regulates hepcidin transcription. These findings are in agreement with Courcelaud et al. (31), who have reported that C/EBPα is a potent activator of both human and mouse hepcidin promoters. The fact that antioxidants abolished the effect of alcohol on C/EBPα binding activity and protein expression suggests a role for oxidative stress in the transcriptional regulation of hepcidin. We believe this is the first report showing a possible role for oxidative stress in the regulation of C/EBPα transcription factor.

Collectively, our data document that ethanol metabolism alters the expression and DNA binding activity of the transcription factor C/EBPα, leading to down-regulation of liver hepcidin gene transcription, and thereby increased duodenal iron transport. Oxidative stress appears to be at least one of the mechanisms responsible for the effect of alcohol metabolism on C/EBPα and hepcidin expression. These findings have profound implications for the elevated iron stores observed in patients with alcoholic liver disease in association with increased hepatic fibrosis and, hence, demonstrate...
a role for C/EBPα and hepcidin in the progression of alcoholic liver disease.

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