Dysregulation of hepatic zinc transporters in a mouse model of alcoholic liver disease

Qian Sun,1 Qiong Li,2 Wei Zhong,2 Jiayang Zhang,3 Xiuhua Sun,2 Xiaobing Tan,2 Xinmin Yin,3 Xinguo Sun,2 Xiang Zhang,3 and Zhanxiang Zhou1,2

1Department of Nutrition, University of North Carolina at Greensboro, North Carolina Research Campus, Kannapolis, North Carolina; 2Center for Translational Biomedical Research, University of North Carolina at Greensboro, North Carolina Research Campus, Kannapolis, North Carolina; and 3Department of Chemistry, University of Louisville, Louisville, Kentucky

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ALCOHOLIC LIVER DISEASE (ALD) is a major cause of morbidity and mortality among alcoholic populations (34). Zinc deficiency as a major nutritional defect has been well documented in ALD (29). Clinical studies showed that patients with advanced ALD had lower zinc levels in serum and liver but higher zinc level in urine (2, 16, 20, 33). Hepatic zinc levels in patients with hepatitis and cirrhosis were reduced to 49–54% of the healthy subjects (2). Chronic ethanol exposure significantly decreased hepatic zinc levels and induced production of reactive oxygen species (ROS) in mice (38). Dietary zinc supplementation to ethanol-fed mice improved hepatic zinc status, and attenuated oxidative stress and liver injury in association with increased antioxidant capacity (17, 38).

Zinc is an essential trace element that plays an important role in maintaining normal cellular functions (22). Zinc serves as a catalytic cofactor for hundreds of enzymes (4, 7). Zinc is also required for stabilizing the structure of thousands of proteins (4, 7, 21). Zinc deficiency is linked with impaired immune function, growth retardation, and dermatologic lesion (4, 35). On the other hand, a high level of free zinc is cytotoxic (7). Therefore, tight regulation of intracellular zinc concentrations is required, which is achieved mainly by two families of zinc transporters, Zn2+-regulated metal transporter (Zrt) and iron-regulated metal transporter (Irt)-like protein (ZIP), and zinc transporter (ZnT) (25).

The ZIP family has 14 members from ZIP1 to ZIP14, and they function in transporting zinc from extracellular space or intracellular organelles into cytosol. The ZnT family contains 10 members from ZnT1 to ZnT10, and they are responsible for exporting zinc from cytosol to extracellular space or intracellular organelles. The cytosolic zinc concentration is regulated positively by ZIPs and negatively by ZnTs, while the organelle zinc levels are regulated positively by ZnTs and negatively by ZIPs. Dysregulation of zinc transporters has been implicated in the pathogenesis of human diseases (6, 13, 26). Given the evidence that hepatic zinc deficiency is consistently observed in alcoholic patients and chronic ethanol-fed animals, and given the fact that ZIP and ZnT are two major zinc transporters in controlling extracellular and intracellular zinc trafficking, we postulated that dysregulation of zinc transporters would be responsible for alcohol-induced hepatic zinc deficiency. The present study was undertaken to determine whether or not chronic ethanol feeding alters hepatic expression of zinc transporters in mice. Because oxidative stress is a feature of ALD, the mechanistic link between oxidative stress and dysregulation of zinc transporters was also evaluated.

METHODS AND MATERIALS

Animal and alcohol feeding experiments. Male C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were treated according to the experimental procedures approved by the Institutional Animal Care and Use Committee. The mice were pair-fed a modified Lieber-DeCarli ethanol or control liquid diet for 2, 4, or 8 wk, respectively. The ethanol provided 28% of total calories in the diet. Urine samples were collected at 9:00 am 1 day before the animals were euthanized. The mice were anesthetized with isoflurane, and blood and liver tissues were harvested for assays.

Blood parameter assay. Blood glucose was determined by One-Touch Ultra2 blood glucose meter (Life Scan, Milpitas, CA). Plasma β-hydroxybutyrate was measured by Cayman Chemical
Liver weight, g 1.09

Effects of 2, 4, and 8 wk of alcohol exposure on body weight, liver weight, and blood parameters

Table 2.

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urine.

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Assay Kit (Ann Arbor, MI). Activity of alanine aminotransferase (ALT) was measured by Infinity ALT reagent (Thermo Scientific, Waltham, MA).

Determination of zinc concentrations in the liver, plasma, and urine. Zinc concentrations in the liver, plasma, and urine were determined by atomic absorption spectrophotometry (AAS). The frozen livers were air-dried in the hood overnight, and then each sample was digested with 1 ml concentrated nitric acid for ~12 h. Each test tube with digested sample was then incubated in water bath at 100°C for 1 h. After cooling, each sample was diluted with deionized water and measured with AAS. One hundred and twenty microliters of plasma or urine was directly measured with AAS. The urinary creatinine concentrations were measured with a urinary creatinine assay kit (Cayman Chemical, Ann Arbor, MI) to normalize urine zinc levels. The zinc concentrations in the liver, plasma, and urine were calculated as micrograms per gram dry liver weight, micrograms per deciliter, and micrograms per milligram creatinine, respectively.

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immunohistochemical detection of hepatic CYP2E1, 4-hydroxynonenal, Zn5P, Zn7P, and ZnT7. Hepatic CYP2E1, 4-hydroxynonenal (4-HNE), Zn5P, Zn7P, and ZnT7 levels were detected by immunohistochemical staining. Briefly, liver tissue paraffin sections were incubated with 3% hydrogen peroxide for 10 min to inactivate endogenous peroxidases. The endogenous mouse IgG was blocked by incubation with a

Antibody (Proteintech, Chicago, IL) at 4°C overnight, followed by incubation with EnVision+ Labeled Polymer-horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or anti-mouse IgG (DAKO, Carpinteria, CA) or HRP-conjugated goat anti-rabbit IgG (Thermo Scientific, Waltham, MA) at room temperature for 30 min. Di-
aminoenzidine (DAB) was used as HRP substrate for visualization.

Table 1. Primer sequence used for qPCR analysis

Table 2. Effects of 2, 4, and 8 wk of alcohol exposure on body weight, liver weight, and blood parameters

Data are means ± SD (n = 6–8). Mice were pair-fed control or ethanol liquid diets for 2, 4, or 8 wk. ALT, alanine aminotransferase. Significant differences (*P < 0.05) between control- and ethanol-fed mice determined by Student’s t-test. Ctrl, control; EtOH, ethanol.

References

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**RESULTS**

**Effects of ethanol exposure on body weight, liver weight, and plasma parameters.** As shown in Table 2, the body weight did not show significant difference between ethanol-fed mice and the controls at all three time points. The liver weight showed a remarkable increase at 4 wk and 8 wk in ethanol-fed mice compared with the controls. The ratio of liver weight to body weight was also significantly higher in ethanol-fed mice at all time points. The activity of plasma ALT and the level of plasma β-hydroxybutyrate were elevated, whereas the plasma glucose levels were reduced in ethanol-fed mice compared with the controls at 8 wk.

**Ethanol exposure impaired zinc homeostasis.** In order to investigate zinc status in mice after alcohol exposure, zinc levels in the liver, plasma, and urine were measured. As illustrated in Fig. 1, hepatic zinc levels were significantly lower at all three time points in ethanol-fed mice compared with the controls. However, zinc levels in plasma and urine were remarkably increased in ethanol-fed mice compared with the controls at all three time points.

**Ethanol exposure suppressed hepatic zinc proteins.** The major function of zinc is achieved through coordination to zinc proteins. To determine if decreased hepatic zinc levels were associated with inactivation of zinc binding proteins, the protein levels of HNF-4α, PPAR-α, and SOD1 were measured. As shown in Fig. 2, ethanol exposure significantly reduced the protein level of PPAR-α at 4 and 8 wk, and HNF-4α at all three time points. Although the protein level of cytosolic SOD1 was not affected by ethanol exposure, the activity of SOD1 was significantly higher at 4 and 8 wk, and HNF-4α were measured at all three time points. The protein bands were visualized by an Enhanced Chemiluminescence detection system (GE Healthcare, Piscataway, NJ) and quantified by densitometry analysis.

**SOD1 activity assay.** The liver tissues were homogenized with 200 mM HEPES buffer (pH 7.2, containing 1 mM EDTA, 210 mM mannitol, and 70 mM sucrose). Cytosolic fraction was isolated by centrifuging tissue homogenates at 1,500 g for 5 min at 4°C, and then the supernatant was centrifuged at 10,000 g for 15 min at 4°C. The supernatant was the crude cytosol of mouse liver tissues. Superoxide dismutase assay kit (Cayman Chemical Company, Ann Arbor, MI) was used to assess the activity of SOD1 by measuring the amount of superoxide radicals, which were generated from xanthine oxidase and hypoxanthine quenched by SOD1.

**Cell culture and treatment.** Murine FL-83B cells obtained from the American Type Culture Collection (Manassas, VA) were grown in Dulbecco’s modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and penicillin (100 U/ml) streptomycin sulfate (100 μg/ml) (Invitrogen, Carlsbad, CA). To evaluate the role of ROS on alterations of zinc transporters, 1 × 10⁵ cells were seeded to 10-cm dish to reached 70–80% confluence. The cells were treated with 5 μM 4-HNE or 100 μM hydrogen peroxide (H₂O₂) for 72 h. Cells were then washed with ice cold PBS and harvested with M-PER mammalian protein extraction (Thermo Scientific). Cell lysates were then stored at −80°C until immunoblotting analysis.

**Immunoblotting analysis.** Liver tissue proteins were extracted by T-PER tissue extraction reagent (Thermo Scientific) containing protease inhibitors (Sigma-Aldrich, St. Louis, MO). Aliquots containing 80 μg proteins were loaded onto an 8%-15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was probed with polyclonal antibodies against hepatocyte nuclear factor 4 (HNF-4α), superoxide dismutase 1 (SOD1), ZIP7, ZnT5, ZnT6, ZnT10 (Santa Cruz Biotechnologies), peroxisome proliferator-activated receptor alpha (PPAR-α), ZIP1, ZIP4, ZIP5, ZIP14, ZnT4 (Novus biological), ZIP8 or ZnT7 (Proteintech), respectively. The membrane was then incubated with HRP-conjugated goat anti-rabbit IgG, or goat anti-mouse IgG, or rabbit anti-goat IgG antibody. The protein bands were visualized by an Enhanced Chemiluminescence detection system (GE Healthcare, Piscataway, NJ) and quantified by densitometry analysis.
Ethanol exposure altered hepatic zinc transporters. To determine how ethanol exposure affects hepatic zinc transporters, the gene expression levels of the full panel of 14 ZIPs and 10 ZnTs were measured, and results are shown in Fig. 3. In the control mice, the expressions of ZIP1, ZIP4, ZIP5, ZIP7, ZIP8, ZIP9, ZIP13, ZIP14, ZnT1, ZnT4, ZnT5, ZnT6, ZnT7, and ZnT10 were relatively abundant at all three time points. The major effect of ethanol exposure on zinc transporter genes was downregulation. ZIP8, ZIP9, ZnT4, ZnT5, ZnT6, ZnT7, and ZnT10 genes were downregulated by ethanol exposure at 2 wk.

Fig. 3. The gene expression levels of zinc transporters in mouse livers. Mice were fed a liquid control diet or ethanol-containing diet for 2, 4, or 8 wk. The gene expression of zinc transporters was measured by qPCR. The expression levels of hepatic ZIP at 2 wk (A), 4 wk (B), and 8 wk (C). Hepatic ZnT mRNA levels at 2 wk (D), 4 wk (E), and 8 wk (F). Significant differences (*P < 0.05) between control- and ethanol-fed mice are determined by Student’s t-test.
ZIP1, ZIP5, ZIP7, ZIP8, ZIP9, ZIP13, ZnT1, ZnT4, ZnT5, ZnT6, and ZnT7 genes were downregulated by ethanol at 4 wk. Ethanol-downregulated genes at 8 wk include ZIP5, ZIP13, ZnT3, ZnT4, ZnT5, ZnT6, and ZnT7. However, ZIP14 gene was upregulated by ethanol exposure at 2 and 4 wk.

The protein abundance of zinc transporters with higher mRNA levels was then measured by immunoblotting, including ZIP1, ZIP4, ZIP5, ZIP7, ZIP8, ZIP13, ZIP14, ZnT1, ZnT4, ZnT5, ZnT6, ZnT7, and ZnT10. The protein levels of 7 ZIPs and 6 ZnTs are shown in Fig. 4 A and B, respectively. Ethanol exposure significantly reduced the protein levels of ZIP5 and ZIP14 at all three time points but significantly increased ZIP4 protein abundance at 4 wk and ZIP7 protein abundance at all three time points (Fig. 4A). Among the 6 ZnT proteins (Fig. 4B), ethanol exposure significantly reduced ZnT4 protein abundance at 8 wk, but significantly increased ZnT7 protein abundance at all three time points.

Distribution of ZIP5, ZIP7, and ZnT7 in the liver of mice fed ethanol for 8 wk was detected by immunohistochemistry. As shown in Fig. 5A, chronic ethanol exposure reduced the staining intensity of ZIP5 in the liver. On the contrary, the staining intensity of hepatic ZIP7 (Fig. 5B) and ZnT7 (Fig. 5C) was increased in chronic alcohol-fed, particularly in the area around the portal vein and central vein, compared with the controls.

Ethanol exposure induced hepatic CYP2E1 expression and caused lipid peroxidation. Hepatic CYP2E1 protein and lipid peroxidation product, 4-HNE, were detected by immunohistochemistry. As shown in Fig. 6A, the CYP2E1 staining was mostly found around central vein area, and the staining was weak in the liver of controls, although a slight increase was found at 4 and 8 wk compared with 2 wk. Ethanol exposure increased hepatic CYP2E1 as early as 2 wk and further increased at 4 and 8 wk. The immunohistochemical staining of 4-HNE is shown in Fig. 6B. While only weak staining was found in the control mice at 8 wk, ethanol exposure increased 4-HNE staining at all three time points, particularly at 4 and 8 wk, compared with the controls.

Treatment with 4-HNE or H2O2 altered expression of zinc transporters in murine FL-83B cells. Oxidant molecules, 4-HNE and H2O2, are generated in association with ethanol metabolism. In order to determine if these cytotoxic molecules mediate ethanol-induced alterations of zinc transporters, murine FL-83B hepatocytes were treated with these molecules for 72 h. As shown in Fig. 7A, the treatment with 5 μM 4-HNE significantly decreased the protein levels of ZIP5, whereas increased ZIP7 protein level compared with the controls. The treatment with 100 μM H2O2 significantly increased the protein levels of ZIP7 and ZnT7 but remarkably decreased ZIP14 protein level compared with the control (Fig. 7B).

**DISCUSSION**

The present study demonstrated that chronic alcohol exposure disturbed zinc homeostasis as indicated by decreased hepatic zinc levels and increased plasma and urinary zinc levels. The alcohol-induced alteration in hepatic and urinary zinc levels from this study is consistent with previous reports (31, 38). However, controversial data on the effect of alcohol...
exposure on serum zinc levels have been reported from human studies. Decreased serum zinc levels have been shown in patients with alcoholic hepatitis and cirrhosis (31, 34, 37). Long-term alcohol exposure (16 wk) also reduces the plasma zinc levels in a mouse model of ALD. On the other hand, Hartoma et al. (10) reported that the serum zinc levels were elevated in alcoholic subjects with normal liver or fatty liver, while reduced serum zinc levels were found in patients with alcoholic hepatitis or cirrhosis. These clinical studies suggest that serum zinc levels could be elevated at an early stage of ALD, but decreased at advanced stage. In the mouse model of ALD showing reduced plasma zinc levels (17), the duration of alcohol exposure and dietary alcohol concentration were different from the present study. In that report, mice were exposed to ethanol for 16 wk, and the dietary ethanol content was increased to make up 30% of total calories. In the present study, the longest ethanol exposure time was 8 wk, and ethanol contributed to 28% of total calories, which generated a very early stage of alcoholic liver injury. These animal data also support the idea that plasma zinc levels may be increased at the early stage of alcoholic liver injury. Overall, all the observations suggest that zinc dyshomeostasis consistently exists in ALD, but the stage of ALD determines the redistribution pattern of zinc in body. In addition, plasma zinc level has been

Fig. 5. Immunohistochemical staining of ZIP5, ZIP7, and ZnT7 in mouse liver. Mice were chronically fed with ethanol or control diet for 8 wk. A: representative images of ZIP5 immunostaining. B: representative images of ZIP7 immunostaining. C: representative images of ZnT7 immunostaining. Scale bar = 50 μm.
used as an indicator for dietary zinc deficiency (22). Our mouse model of ALD used a liquid diet containing adequate zinc, but this zinc-adequate liquid diet differentially affected plasma and liver zinc levels. These results suggest that plasma zinc level is not always a good indicator for assessing dietary zinc status and organ zinc status at different pathophysiological conditions.

Zinc participates in diverse physiological activities via binding to proteins; therefore decreased availability of zinc leads to inactivation of zinc proteins. The present study demonstrated that zinc proteins including PPAR-α, HNF-4α, and SOD1 were inactivated, and previous reports have shown that the suppression of the three proteins correlates to the pathogenesis of ALD (3, 5, 11, 18, 19, 32). This study also shows that alcohol exposure differentially affects PPAR-α, HNF-4α, and SOD1. While alcohol exposure reduced PPAR-α and HNF-4α protein levels, it did not affect the protein level of SOD1. However, the activity of SOD1 was significantly decreased by alcohol expo-

Fig. 6. Immunohistochemical staining of cytochrome P-450 2E1 (CYP2E1) and hepatic 4-hydroxynonenal (4-HNE) in mouse liver. Mice were chronically fed with ethanol or control diet for 2, 4, or 8 wk. A: representative images of CYP2E1 immunostaining. Scale bar = 100 μm. B: representative images of 4-HNE immunostaining. Scale bar = 50 μm.
It is known that zinc does not coordinate to proteins with equal affinity (28, 30). Therefore, zinc proteins with lower zinc affinity will be affected by alcohol exposure at earlier time points. The data suggest that alcohol exposure-induced alteration of hepatic zinc levels may associate with zinc binding protein inactivation, which is manifested as decreased protein expressions or impaired protein activities.

Zinc transporters are major players in regulation of hepatic zinc homeostasis, but a whole picture of hepatic expression of zinc transporters is lacking. The present study first analyzed the expression of zinc transporter genes (14 ZIPs and 10 ZnTs) and found that 8 ZIP and 6 ZnT genes are relatively abundant in the liver. Then the protein levels of these abundant zinc transporters were further analyzed. Among the 14 zinc transporter proteins measured by immunoblotting, we detected 7 ZIP proteins and 5 ZnT proteins in the liver. However, ZIP9 and ZnT5 proteins were not detected, probably due to the limit of either antibody reactivity or the protein expression levels. Moreover, the results of mRNA and protein indicate that different zinc transporters might undergo various regulations. According to the results, we conclude that ZIP5 and ZnT1 might be regulated at the transcriptional level, but ZIP7, ZIP8, ZIP13, ZIP14, ZnT4, ZnT6, and ZnT7 might undergo a post-transcriptional regulation, among which, ZIP7, ZIP8, ZIP13, ZnT4, ZnT6 and ZnT7 might have a stabilized protein life, and ZIP14 might undergo degradation process.

The altered expression of hepatic zinc transporters by ethanol exposure may lead to zinc dyshomeostasis in liver. Beker Aydemir et al. (1) showed that ZIP14 was located on the plasma membrane of hepatocytes, and increased level of ZIP14 could sequester zinc from plasma into liver under infection.

**Fig. 7. Effects of 4-HNE and hydrogen peroxide (H$_2$O$_2$) on the expression of ZIP5, ZIP7, ZIP14, and ZnT7 in murine FL-83B cells.** FL-83B cells were treated with 5 μM 4-HNE or 100 μM H$_2$O$_2$ for 72 h. Four zinc transporters were analyzed by immunoblotting. The bands were quantified by densitometry analysis. A: immunoblotting of 4 zinc transporters by 4-HNE treatment. B: immunoblotting of 4 zinc transporters by H$_2$O$_2$ treatment. Results are expressed as means ± SD (n = 3). Significant differences (*P < 0.05) between control and treatment are determined by Student’s t-test. Ctrl, control.
of alcohol on zinc transporters may be mediated by the oxidative molecules, which are generated in association with alcohol metabolism. The results also indicate that different oxidative molecules may not affect zinc transporters in the same way. The ZIP7 protein was affected by both H$_2$O$_2$ and 4-HNE treatment, suggesting that ZIP7 was susceptible to oxidative stress. We assume that oxidative stress induced by ethanol metabolism might directly affect the zinc transporters at transcriptional levels or/and enhance its stabilization or degradation at protein levels, but we do not exclude that oxidative stress may indirectly cause alteration of the zinc transporters. Maret et al. (28) suggested that oxidants might induce zinc dissociation from zinc proteins and concurrently alter the availability of zinc ion. Consequently, altered labile zinc would affect the expression of zinc transporters by upregulation or downregulation of zinc sensing transcription factors, such as metal response element-binding transcription factor (MTF-1) (12, 24). However, the precise molecular mechanisms have not been defined. The current study provides solid evidence that oxidative stress could affect the expression of zinc transporters. Therefore, a combined dietary intervention with antioxidant and zinc might produce a better improvement in alcohol-induced zinc deficiency and liver injury.

In summary, ethanol-induced hepatic zinc reduction occurred as early as the second week of ethanol exposure in mice. Accompanying that, zinc proteins were affected either by decreased expression at protein levels (PAPα and HNF-4α) or impaired activity (SOD1). Zinc transporter screening detected 23 (except ZnT2) of 24 zinc transporters at mRNA levels in the mouse liver. Among the zinc transporters with relative abundant gene expression, 12 zinc transporters were detected at protein levels, for the first time, in the mouse liver. Alcohol exposure consistently affected the protein levels of 4 zinc transporters (ZIP5, ZIP7, ZIP14 and ZnT7) of 12 zinc transporters tested. The results from in vitro study demonstrated that the protein levels of ZIP5, ZIP7, ZIP14, and ZnT7 were affected by 4-HNE and H$_2$O$_2$. These results demonstrated that hepatic zinc transporters are remarkably altered by alcohol abuse via an oxidative stress-dependent mechanism, which might account for alcohol-induced hepatic zinc deficiency.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Q.S. and Z.Z. conception and design of research; Q.S., Q.L., W.Z., J.Z., Xiuhua Sun, X.T., X.Y., Xinguo Sun, and X.Z. performed experiments; Q.S. analyzed data; Q.S. and Z.Z. interpreted results of experiments; Q.S. prepared figures; Q.S. drafted manuscript; Q.S., Q.L., W.Z., Xiuhua Sun, X.T., and Z.Z. edited and revised manuscript; Q.S., Q.L., W.Z., J.Z., Xiuhua Sun, X.T., X.Y., Xinguo Sun, X.Z., and Z.Z. approved final version of manuscript.

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