Preservation of Hepatocyte Nuclear Factor-4α Is Associated with Zinc Protection Against TNF-α Hepatotoxicity in Mice

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Hepatocyte nuclear factor-4α (HNF-4α), a zinc finger protein, is the most abundant transcription factor in the liver. HNF-4α regulates a large number of genes involved in most aspects of hepatocyte functions. The present study was undertaken to determine the role of HNF-4α in zinc protection against tumor necrosis factor-α (TNF-α) hepatotoxicity. Mice were treated with murine TNF-α *via* intravenous injection at 20 μg/kg body wt 30 mins after D-galactosamine (D-Gal) sensitization (800 mg/kg body wt). Two doses of zinc sulfate (5 mg elemental zinc/kg body wt) were administered at 36 and 12 hrs before TNF-α treatment *via* subcutaneous injection. TNF-α treatment after sensitization induced liver injury as detected by plasma alanine aminotransferase activity and apoptotic cell death in the liver. Zinc pretreatment attenuated TNF-α–induced liver injury. Furthermore, TNF-α–induced activations of caspase 3 and caspase 8 in the liver were significantly inhibited by zinc pretreatment. The mRNA and protein levels of HNF-4α in the liver were remarkably decreased by TNF-α treatment, which was suppressed by zinc. To determine if HNF-4α depletion is involved in D-Gal sensitization to TNF-α toxicity, mice were administered either D-Gal or TNF-α. Immunohistochemistry demonstrated that HNF-4α depletion in the liver is associated with D-Gal sensitization but not TNF-α treatment. To define the link between HNF-4α depletion and TNF-α–induced cell death, the effect of silencing the HNF-4α gene by siRNA transfection on TNF-α cytotoxicity was determined in HepG2 cells. A lactate dehydrogenase cytotoxicity assay showed that neither TNF-α nor HNF-4α siRNA transfection had a toxic effect, but TNF-α treatment after HNF-4α siRNA transfection caused HepG2 cell death. These results suggest that zinc protects against TNF-α hepatotoxicity, at least partially, through preservation of the zinc finger protein HNF-4α.
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Key words: TNF-α; hepatocyte nuclear factor-4α; apoptosis; zinc; liver

Introduction

Zinc is the second most abundant trace element in the body. Zinc is particularly important in cellular function because it is an integral component of numerous proteins, including metalloenzymes, structure proteins, and transcriptional factors. Previous reports have shown that zinc has hepatoprotective effects under a variety of toxic conditions (1–5). Although the antioxidant action of zinc has been documented as one of the protective mechanisms, increasing evidence suggests that regulation of transcription factors may be an important mechanism of zinc function.

Hepatocyte nuclear factor-4α (HNF-4α), a zinc finger protein, is the most abundant transcription factor in the liver. HNF-4α critically regulates a large number of genes involved in most aspects of hepatocyte functions (6, 7). Previous studies have shown that a decrease in the level of HNF-4α is associated with liver injury or liver failure. Depletion of HNF-4α in the liver has been reported after lipopolysaccharide challenge (8). Both human and animal data have demonstrated that a decrease in HNF-4α correlates significantly with severity of liver disease (9, 10). Conditional knockout of HNF-4α in the liver causes >70% mortality by 8 weeks of age in mice, and HNF-4α null mice die during embryogenesis (11, 12). Therefore, HNF-4α may be a critical cell survival factor in hepatocytes under disease conditions.

Our recent studies have demonstrated that zinc prevents alcohol-induced apoptosis and liver injury through preservation of gut barrier function and inhibition of endotoxin-induced tumor necrosis factor-α (TNF-α) production in the

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liver (4, 13, 14). TNF-α is a causal factor in development of alcoholic hepatitis. Hepatocyte apoptosis has been a pathologic feature of alcoholic hepatitis in both clinical and animal studies (15–17). Many studies have demonstrated that TNF-α is a critical mediator in alcohol-induced apoptotic cell death in the liver (15). Therefore, inhibition of TNF-α–induced hepatocyte apoptosis could be a therapeutic target in controlling alcoholic hepatitis. The present study was undertaken to determine the specific role of HNF-4α in zinc protection against TNF-α hepatotoxicity.

Materials and Methods

Animals. Eight-week-old 129S mice were obtained from Taconic (Germantown, NY). The mice were housed in the animal quarters at the University of Louisville Research Resources Center. They were maintained at 22°C with a 12:12-hr light:dark cycle and had free access to rodent chow and tap water. The experimental procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association for the Accreditation of Laboratory Animal Care.

Mice (24–26 g body wt) were treated with recombinant human TNF-α (R&D Systems, Minneapolis, MN) *via* intravenous injection at 20 μg/kg body wt 30 mins after D-galactosamine (D-Gal) sensitization (800 mg/kg body wt) as described previously (18). Two doses of zinc sulfate at 5 mg elemental zinc/kg body wt were administered *via* subcutaneous injection at 36 and 12 hrs before TNF-α treatment. Saline was used for controls for both zinc and TNF-α treatments. Mice were also treated with D-Gal or TNF-α to determine their roles in HNF-4α depletion in the liver.

HepG2 Cell Culture. HepG2 cells obtained from the American Type Culture Collection (Rockville, MD) were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and penicillin (100 U/ml)/streptomycin sulfate (100 μg/ml) (Invitrogen). HepG2 cells were treated with TNF-α (20 ng/ml) with or without silencing the HNF-4α gene by siRNA transfection. HNF-4α siRNA transfection was conducted for 48 hrs with human HNF-4α siRNA (Ambion, Austin, TX) using the Lipofectamine 2000 transfection reagent (Invitrogen) following the manufacturer's instructions.

Alanine Aminotransferase (ALT) Activity. ALT activity in the plasma taken at 4 hrs after TNF-α treatment was measured following a colorimetric procedure (19).

Enzymatic Assay of Caspase 3 and Caspase 8 Activities. Enzymatic activities of caspase 3 and caspase 8 in the liver were measured as described previously (20). In brief, fresh liver tissue was homogenized in extraction buffer (25 m*M* HEPES buffer [pH 7.4] containing 5 m*M* EDTA, 2 m*M* dithiothreitol, 0.1% CHAPS, and 1% protease cocktail). The homogenate was centrifuged at 20,000 *g* for 30 mins. The resulting supernatants were diluted with assay buffer (50 m*M* HEPES, 10 m*M* dithiothreitol, 1.0 m*M* EDTA, 100 m*M* NaCl, 0.1% CHAPS, and 10% glycerol [pH 7.4]) and incubated at 37°C with caspase 3 substrate (Ac-DEVD-*p*NA) or caspase 8 substrate (Ac-IETD-*p*NA). *p*-Nitroaniline was used as the standard. Cleavage of the substrates was monitored at 405 nm, and the specific activities were expressed in pmol of the product, nitroaniline, per minute per mg of protein.

Terminal Deoxynucleotidyl Transferase (TdT) dUTP Nick-End–Labeling (TUNEL) Assay of Apoptotic Cell Death. Apoptotic cell death in the liver was assessed by detection of DNA fragmentation using the ApopTag Peroxidase *in situ* Apoptosis Detection Kit (Chemicon, Temecula, CA) as described previously (20). For detection of apoptosis in HepG2 cells, the ApopTag Fluorescein *in situ* Apoptosis Detection Kit (Chemicon) was used. HepG2 cells on eight-chamber culture slides were fixed with 1% paraformaldehyde for 15 mins at 4°C and post-fixed with precooled ethanol:acetic acid (2:1) for 5 mins at −20°C. The slides were incubated with the reaction mixture containing TdT and digoxigenin-conjugated dUTP for 1 hr at 37°C. The labeled DNA was visualized with fluorescein-conjugated antidigoxigenin antibody. The slides were counterstained with 4′,6-diamidino-2-phenylindole and observed with a fluorescent microscope.

Immunohistochemical Localization of HNF-4α. Localization of HNF-4α in the liver was performed by an immunohistochemical procedure as described previously (5). Briefly, liver tissue taken at 0, 2, 4, and 8 hrs after TNF-α treatment was fixed with 10% neutral formalin, and tissue sections of 5 μm were prepared. Sections were incubated overnight with a polyclonal rabbit anti–HNF-4α (Santa Cruz Biotechnologies, Santa Cruz, CA) followed by incubation with DAKO EnVision$^+$ System Labeled Polymer HRP Anti-Rabbit (DAKO, Carpinteria, CA) for 30 mins.

Western Blot Detection of HNF-4α Protein. Nuclear extracts of liver tissue and HepG2 cells were prepared as described previously (20). Aliquots containing 30 μg of protein were loaded on a 12% sodium dodecyl sulfate polyacrylamide gel. After electrophoresis, protein was transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% nonfat milk in Tris-buffered saline (pH 7.5) and probed with polyclonal rabbit anti–HNF-4α (Santa Cruz Biotechnologies). The membrane was then processed with HRP-conjugated goat anti-rabbit IgG (GE Healthcare, Piscataway, NJ). The protein bands were visualized by an enhanced chemiluminescent detection system (GE Healthcare) and quantified by densitometry analysis.

Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Assay of HNF-4α mRNA. The mRNA levels of HNF-4α in the liver and HepG2 cells were quantitatively measured by real-time RT-PCR as described previously (21). In brief, total RNA was isolated and reverse transcribed with Moloney murine leukemia virus reverse transcriptase and oligo(dT) primers. The forward and reverse primers of HNF-4α were designed

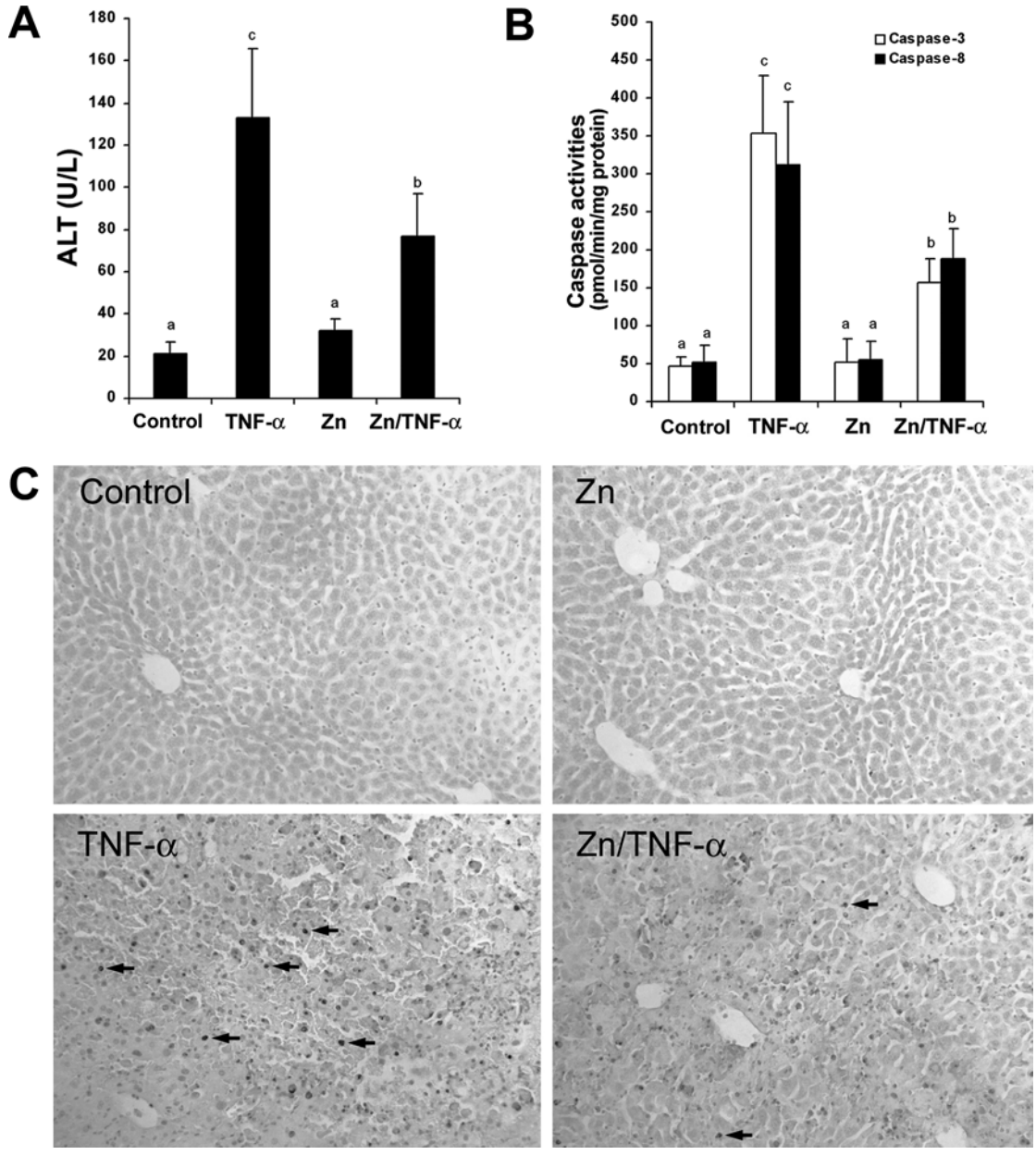

Figure 1. Effect of zinc on TNF-α–induced liver injury in mice. 129S mice were treated with recombinant murine TNF-α *via* intravenous injection at 20 μg/kg body wt 30 mins after D-Gal sensitization (800 mg/kg body wt). Two doses of zinc sulfate at 5 mg elemental zinc/kg body wt were administered *via* subcutaneous injection at 36 and 12 hrs before TNF-α treatment. Plasma and liver samples were taken at 4 hrs after TNF-α treatment. (A) Plasma ALT activities. (B) Enzymatic activities of caspase 3 and caspase 8 in the liver. (C) TUNEL assay of apoptosis in the liver. An ApoTag Peroxidase *in situ* Apoptosis Detection Kit was used for the TUNEL assay followed by counterstaining with methyl green. Arrows indicate apoptotic nuclei. Magnification: ×130. Results in A and B are means ± SD ($n = 6$). Significantly different ($P < 0.05$) among a, b, and c.

using Primer Express Software (Applied Biosystems, Foster City, CA): NM_008261–3203F: 5′-CGGAGCCCCTGCAAAGT-3′, NM_008261–3298R: 5′-CCAGTCTCACAGCCCATTCC-3′. The SYBR Green DNA PCR Kit (Applied Biosystems) was used for real-time RT-PCR analysis. The relative differences of gene expression among groups were evaluated using cycle time values and expressed as relative changes, setting the values of control mice as 100%.

Lactate Dehydrogenase (LDH) Cytotoxicity Assay. TNF-α–induced cell death in HepG2 cell culture was assessed by measuring LDH activity in the culture medium with the LDH Cytotoxicity Assay Kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions.

Statistics. All data are expressed as means ± SD and were analyzed by analysis of variance and Newman-Keuls' multiple-comparison test. Differences between groups were considered significant at $P < 0.05$.

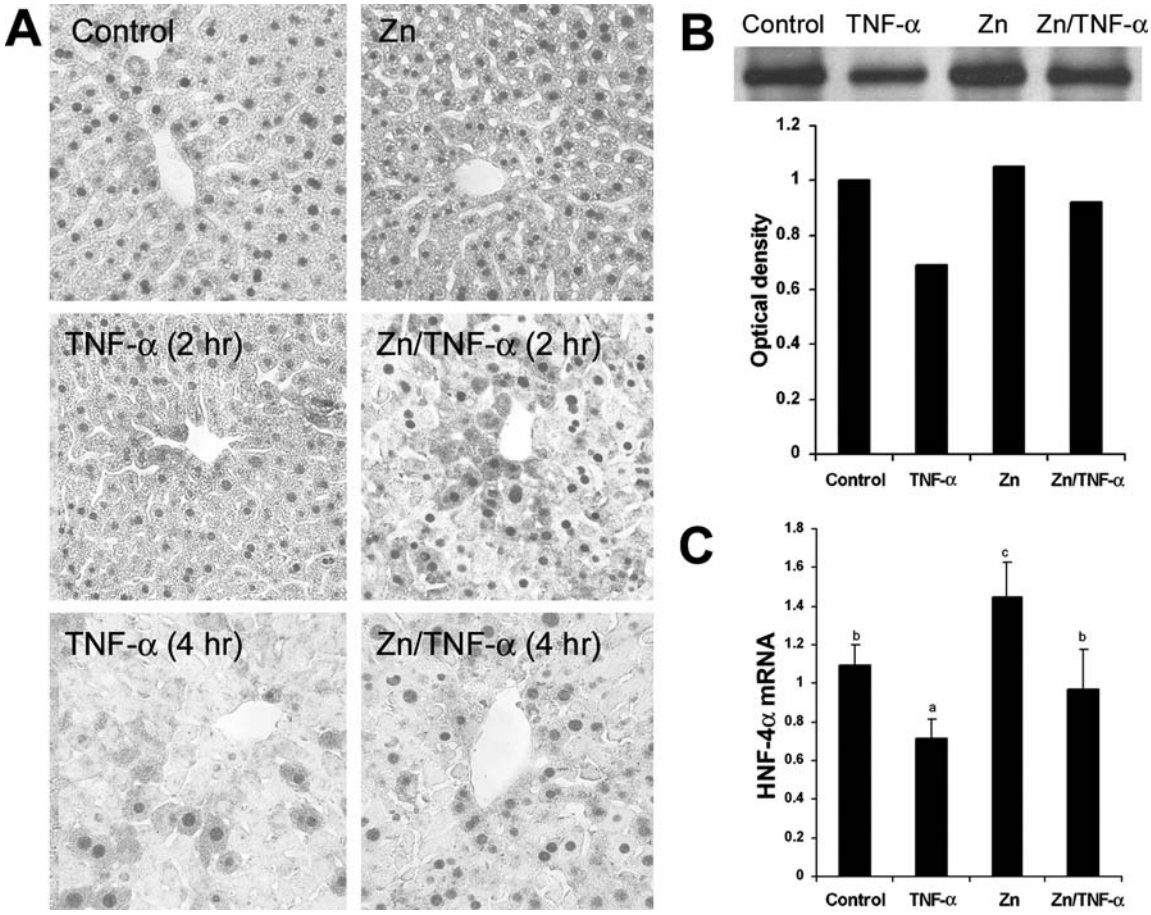

Figure 2. Effect of zinc pretreatment on HNF-4α in the liver of mice. 129S mice were treated with recombinant murine TNF-α *via* intravenous injection at 20 μg/kg body wt 30 mins after D-Gal sensitization (800 mg/kg body wt). Two doses of zinc sulfate at 5 mg elemental zinc/kg body wt were administered *via* subcutaneous injection at 36 and 12 hrs before TNF-α treatment. (A) Immunohistochemical staining of HNF-4α in the liver at 0, 2, and 4 hrs after TNF-α treatment. Positive staining of HNF-4α was found exclusively in hepatocytes with a major distribution in the nuclei. Magnification: ×260. (B) Western blot of HNF-4α protein in the liver 4 hrs after TNF-α treatment (pooled sample from four individuals). The bands were quantified by densitometry analysis. (C) Real-time RT-PCR assay of HNF-4α. Results in C are means ± SD ($n = 6$). Significantly different ($P < 0.05$) among a, b, and c.

Results

Zinc Pretreatment Attenuated TNF-α–Induced Liver Injury. Liver injury was determined by measuring plasma ALT activity and apoptotic cell death in the liver at 4 hrs after TNF-α challenge. As shown in Figure 1A, plasma ALT activity was elevated by TNF-α treatment, and zinc pretreatment markedly suppressed D-Gal–/TNF-α–elevated plasma ALT. TNF-α treatment significantly increased the enzymatic activities of caspase 3 and caspase 8 in the liver, which was attenuated by zinc pretreatment (Fig. 1B). The TUNEL assay demonstrated that TNF-α treatment induced massive cell death and zinc pretreatment significantly reduced TNF-α–induced hepatic cell death (Fig. 1C).

Zinc Pretreatment Preserves HNF-4α in the Liver. To understand the possible molecular mechanism by which zinc modulates TNF-α hepatotoxicity, the protein and mRNA levels of HNF-4α in the liver were determined. As shown in Figure 2A, immunohistochemistry demonstrated an exclusive localization of HNF-4α in hepatocytes with a major distribution in the nuclei. TNF-α treatment induced a marked decrease in HNF-4α staining as early as 2 hrs after HNF-4α challenge and caused a depletion of HNF-4α in most hepatocyte nuclei at 4 hrs after TNF-α challenge. Zinc pretreatment partially inhibited TNF-α–induced depletion of HNF-4α from the liver. Western blotting also demonstrated that TNF-α decreased the protein level of HNF-4α in the nuclear extract of liver and zinc pretreatment suppressed the effect of TNF-α on HNF-4α protein (Fig. 2B). The mRNA levels of HNF-4α in the liver were determined by real time RT-PCR. As shown in Figure 3C, zinc pretreatment prevented the TNF-α–induced decrease in the HNF-4α mRNA level in the liver.

To define whether HNF-4α depletion is involved in D-Gal sensitization to TNF-α toxicity, mice were administered either D-Gal or TNF-α. Immunohistochemistry demonstrated that treatment with D-Gal, but not TNF-α, caused a remarkable decrease in hepatic HNF-4α (Fig. 3). Plasma ALT activity was not affected by either D-Gal or TNF-α.

Silencing the HNF-4α Gene Sensitized TNF-α

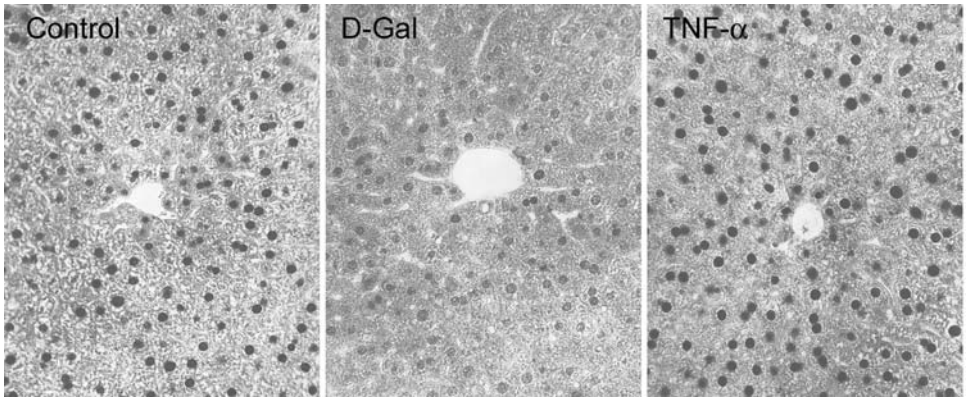

Figure 3. Effects of D-Gal on HNF-4α in the liver of mice. 129S mice were treated with either D-Gal (800 mg/kg body wt, intraperitoneal injection) or recombinant murine TNF-α (20 μg/kg body wt, intravenous injection). Immunohistochemical staining of HNF-4α in the liver 4 hrs after treatment showed that D-Gal caused depletion of HNF-4α in the nuclei of hepatocytes. Magnification: ×260.

Cytotoxicity in HepG2 Cell Culture. To define the link between HNF-4α depletion and TNF-α–induced cell death, the effect of silencing the HNF-4α gene by siRNA transfection on TNF-α cytotoxicity was determined in HepG2 cell culture. As shown in Figure 4A, Western blotting demonstrated that TNF-α treatment did not affect the HNF-4α protein level, but HNF-4α siRNA transfection did cause a dramatic decrease in the level of HNF-4α protein. The LDH assay of cell death showed that neither TNF-α treatment nor HNF-4α siRNA transfection had a toxic effect. However, TNF-α treatment after HNF-4α siRNA transfection induced remarkable cell death (Fig. 4B).

Discussion

The results in the present study demonstrated that zinc pretreatment significantly suppresses TNF-α–induced apoptotic cell death in the liver. The hepatoprotective effect of zinc was associated with preservation of HNF-4α in the liver. Using HNF-4α siRNA transfection, the *in vitro* study clearly showed that silencing HNF-4α in HepG2 cells sensitized TNF-α cytotoxicity. Our results are the first to demonstrate that HNF-4α depletion is a critical factor in TNF-α–induced hepatotoxicity and preservation of HNF-4α may provide a beneficial effect on TNF-α–related liver disease.

TNF-α is recognized as a primary trigger for alcoholic hepatitis. TNF-α signaling in the liver causes inflammation, oxidative stress, and eventually hepatocyte apoptosis (22, 23). Dysregulation of TNF-α metabolism in alcoholic hepatitis was first reported with the observation that cultured monocytes from alcoholic hepatitis patients spontaneously produced TNF-α and produced more TNF-α in response to endotoxin stimulus (24). Increased serum TNF-α concentrations in alcoholic hepatitis were then reported, and values correlated with disease severity and mortality (22, 23). Alcohol activates the TNF-α apoptotic pathway through at

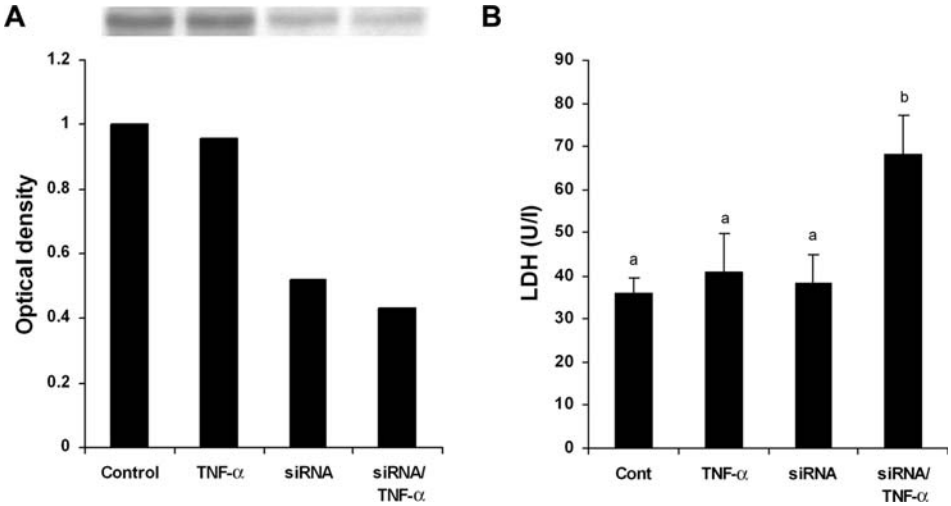

Figure 4. Effect of HNF-4α siRNA transfection on TNF-α cytotoxicity in HepG2 cells. HepG2 cells were transfected with HNF-4α siRNA and incubated for 48 hrs, followed by TNF-α treatment (20 ng/ml). (A) Western blot of HNF-4α protein (upper panel, pooled sample from six individuals). The bands were quantified by densitometry analysis (lower panel). (B) LDH activity in the medium. Results in B are means ± SD ($n = 6$). Significantly different ($P < 0.05$) between a and b.

least three major steps: increased gut permeability and blood endotoxin elevation; Kupffer cell activation; and TNF-α production and action on hepatocytes (25, 26). Our previous studies demonstrated that zinc has inhibitory effects on alcohol-induced endotoxin elevation in the blood and TNF-α production in the liver in mice (13, 14). We also found that zinc inhibits endotoxin-induced Kupffer cell activation and TNF-α production in a lipopolysaccharide challenge mouse model (4). The present study further demonstrated that zinc also interferes with TNF-α signaling in hepatocytes, leading to suppression of hepatocyte apoptosis. These results suggest that zinc protects against alcohol-induced hepatocyte apoptosis through multiple actions.

Previous reports have shown that inhibition of oxidative stress is involved in zinc protective action (4, 5, 27, 28). Cellular zinc exits in only one redox state (II); thus, it cannot undergo redox reactions that are commonly responsible for the generation of reactive oxygen species, but zinc also has the ability to reduce $OH^{\bullet}$ formation and preserve cellular thiol pools (29, 30). Reactive oxygen species can directly affect the conformation and/or activities of all sulfhydryl-containing molecules, including transcription factors, by oxidation of their thiol moiety (31, 32). Thus, the antioxidant action of zinc could lead to protection against oxidative stress–induced alterations in transcription factors. Increasing evidence indicates that cellular zinc status is a critical regulator in gene expression and function of zinc finger transcription factors such as peroxisome proliferator-activated receptor α, Sp-1, Erg-1, and P53 (33–35). Zinc also has an important role in modulation of transcription factors that do not contain structural zinc, such as nuclear factor κB (NF-κB) and AP-1 (36, 37). Zinc treatment has been shown to attenuate TNF-α–induced interleukin 8 production by endothelial cells through inhibition of redox-sensitive transcription factors, NF-κB and AP-1 (36). Zinc treatment also has been shown to suppress generation of reactive oxygen species and activation of NF-κB and AP-1 in endothelial cells in response to linoleic acid or TNF-α treatment (37). Recent studies have demonstrated that zinc supplementation prevents spontaneous and experimentally induced diabetes through regulation of NF-κB and AP-1 in the pancreas of mice (38, 39). Therefore, regulation of transcription factors may be an important mechanism of zinc protective action.

HNF-4α is one of the most important transcription factors in the liver. Previous studies have shown that hepatic expression of HNF-4α correlates with liver disease. A significant decrease in hepatic HNF-4α mRNA level has been documented in cirrhotic patients (9). A hepatocellular carcinoma rat model also showed that impaired HNF-4α expression in the liver is associated with tumor progression (10). A marked decrease in hepatic HNF-4α was found in response to lipopolysaccharide treatment or experimental burn injury, which may contribute to acute-phase response–induced liver failure (8, 40). The present study demonstrated that HNF-4α depletion is associated with D-gal sensitization to TNF-α toxicity and preservation of HNF-4α is associated with zinc suppression of TNF-α–induced cell death in the liver. We also found that silencing the HNF-4α gene by siRNA transfection sensitized HepG2 cells to TNF-α killing. These results indicate that HNF-4α depletion is a critical factor in TNF-α–induced cell death.

In conclusion, the results of the present study demonstrated that zinc provides effective protection against TNF-α–induced hepatic cell death. The protective effect of zinc was associated with inhibition of HNF-4α depletion in the liver. HepG2 cell culture showed that silencing the HNF-4α gene sensitized TNF-α cytotoxicity. These results suggest that HNF-4α is a critical factor in TNF-α–induced hepatotoxicity and zinc may have a therapeutic role in the prevention and/or treatment of TNF-α–mediated liver injury through preservation of HNF-4α.

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