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*Gastrointestinal, Hepatobiliary, and Pancreatic Pathology*

# Zinc Deprivation Mediates Alcohol-Induced Hepatocyte IL-8 Analog Expression in Rodents via an Epigenetic Mechanism

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**Neutrophil infiltration caused by IL-8 production is a central mechanism in alcohol-induced hepatitis. This study was performed to examine if an epigenetic mechanism is involved in alcohol-induced IL-8 production. Mice were pair-fed an alcohol-containing liquid diet for 4 weeks. Alcohol exposure induced hepatitis as indicated by increased expression of keratinocyte-derived cytokine (mouse IL-8) and neutrophil infiltration. Alcohol exposure induced histone 3 hyperacetylation owing to inhibition of histone deacetylase (HDAC) in association with NF-κB activation. Cell culture studies showed that alcohol exposure induced IL-8 and cytokine-induced neutrophil chemoattractant-1 (CINC-1, rat IL-8) production in human VL-17A cells and rat H4IIEC3 cells, respectively, dependent on acetaldehyde production, oxidative stress, and zinc release. Zinc deprivation alone induced CINC-1 production and acted synergistically with lipopolysaccharide or tumor necrosis factor-α on CINC-1 production. Zinc deprivation induced histone 3 hyperacetylation at lysine 9 through suppression of HDAC activity. Zinc deprivation caused nuclear translocation of NF-κB, and reduced HDAC binding to NF-κB. Chromatin immunoprecipitation (ChIP) showed that zinc deprivation caused histone 3 hyperacetylation as well as increased NF-κB binding to the CINC-1 promoter. In conclusion, inactivation of HDAC caused by zinc deprivation is a novel mechanism underlying IL-8 up-regulation in alcoholic hepatitis.** ***(Am J Pathol 2011, 179:693–702; DOI: 10.1016/j.ajpath.2011.04.006)***

Neutrophil infiltration is well documented in patients with alcoholic hepatitis and experimental animals with alcohol-induced liver injury.[1–3] Neutrophils can cause liver tissue damage through releasing reactive oxygen species (ROS) and proteases.[3] CXC chemokines play a central role in chemoattraction of neutrophils, and IL-8 (CXCL-8) has been suggested as a major CXC chemokine in mediating alcohol-induced neutrophil infiltration in the liver.[4,5] Patients with alcoholic hepatitis showed an increased IL-8 level in both serum and the liver.[6–8] The serum IL-8 levels in alcoholic liver disease were correlated closely with severity of liver injury.[7,8] In addition, hepatic local IL-8 levels correlated with the degree of neutrophil infiltration.[7,8] Mouse keratinocyte-derived cytokine (KC) and rat cytokine-induced neutrophil chemoattractant-1 (CINC-1) are analogs of human IL-8. Chronic alcohol feeding has been shown to up-regulate KC/CINC in association with neutrophil infiltration in the liver.[1,9,10] A variety of cell populations in the liver are known to produce IL-8 in response to alcohol consumption. However, the mechanisms of IL-8 production in hepatocytes are poorly understood, although it has been shown repeatedly by both *in vivo* and *in vitro* studies.[8,11–16]

NF-κB is a key transcription factor in up-regulation of cytokine/chemokine genes in alcoholic liver disease.[4,5] Increasing evidence suggests that NF-κB–mediated

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facturer's instructions. The procedure involves the use of HDAC fluorimetric substrate (Fluor de Lys substrate), which comprises an acetylated lysine side chain and is incubated with a sample containing nuclear extract. Deacetylation sensitizes the substrate, which then generates a fluorophore. The fluorophore is excited with 360 nm light and emitted with 460 nm light.

### *Measurement of Acetaldehyde*

H4IIEC3 and VL-17A cells were seeded into flasks, respectively. After 24-hours of incubation, the medium was replaced with an equal volume of DMEM containing 25 mM HEPES and 50 mmol/L ethanol. The flasks were tightly sealed and incubated at 37°C for another 24 hours. Acetaldehyde concentrations in the media were measured with a kit (Rbiopharm, Germany) following manufacturer's instructions.

### *Measurement of ROS*

ROS accumulation in VL-17A/H4IIEC3 cells was examined by measuring the dihydroethidium fluorescence intensity. Nonfluorescent dihydroethidium is oxidized by ROS to yield the red fluorescent product, ethidium, which binds to nucleic acids, staining the nucleus a bright fluorescent red. VL-17A/H4IIEC3 cells were incubated with 5 $\mu$mol/L dihydroethidium (Molecular Probes, Eugene, OR) for 30 minutes at 37°C in the dark. The ROS-catalyzed ethidium red fluorescence was examined with an excitation wavelength of 544 nm and an emission wavelength of 590 nm.

### *Measurement of Cellular Free Zinc*

Cellular free zinc was detected by a green fluorescent zinc ion indicator (FluoZin-3; Molecular Probes). Cells were incubated with 5 $\mu$mol/L FluoZin-3 at 37°C for 30 minutes in the dark. The generated fluorescence signal was read by a fluorescence spectrophotometer (Synergy HT Multi-Mode Microplate Reader; BioTek, Winooski, VT) with an excitation wavelength of 494 nm and an emission wavelength of 516 nm.

### *Histone Extraction*

For nuclear histone lysate preparation, after treatment cells were washed twice with ice-cold PBS containing 5 mmol/L sodium butyrate, scraped and lysed in hypotonic lysis buffer (20 mmol/L HEPES, 1 mmol/L EDTA, 10 mmol/L NaCl, 1 mmol/L dithiothreitol, 1 mmol/L sodium orthovanadate, 2 mmol/L $MgCl_2$, 1 mmol/L phenylmethylsulfonyl fluoride with 1% protease inhibitor cocktail, and 0.25% NP-40) on ice for 20 minutes followed by 15 passages through a 26-gauge syringe needle. The nuclei were pelleted by centrifugation at 12,000 $\times$ *g* for 1 minute and resuspended in 0.4 N HCl with 10% glycerol, rotated overnight at 4°C. The acid-soluble fractions were collected by centrifugation at 12,000 $\times$ *g* for 10 minutes, and precipitation with 20% trichloroacetic acid. The pellets were washed with acetone, dried, and dissolved in distilled water.

### *Immunoblot Analysis*

Liver or cell nuclear proteins were extracted using 10% Nonidet P-40 lysis buffer containing protease inhibitor cocktail (leupeptin, aprotinin, pepstatin, and phenylmethylsulfonyl fluoride). Protein concentrations were measured by a kit based on the Bradford method (Bio-Rad, Hercules, CA). Aliquots containing 15 to 60 $\mu$g proteins were loaded onto a 8% to 12% SDS-polyacrylamide gel, transblotted onto a polyvinylidene difluoride membrane (Bio-Rad), blocked with 10% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20, and then incubated with rabbit or mouse polyclonal antibody against NF-$\kappa$B, HDACs (HDAC1, HDAC2, HDAC3, HDAC4), total histone, acetyl-histone H3 (Lys9) (AcH3K9, Cell Signaling), p300, or $\beta$-actin (Santa Cruz Biotechnologies, Santa Cruz, CA). The membrane then was incubated with horseradish peroxidase–conjugated donkey anti-rabbit or goat anti-mouse IgG (Cell Signaling). The protein bands were visualized with an enhanced chemiluminescence system (GE Health Care, Piscataway, NJ), and quantified by densitometry analysis.

### *Immunofluorescence Microscopy*

H4IIEC3 cell chamber slides were fixed with cold methanol for 5 minutes at −20°C. The H4IIEC3 cells then were incubated with rabbit polyclonal anti–NF-$\kappa$B or anti-AcH3K9 antibody (Cell Signaling) overnight at 4°C, followed by incubation with a Cy3-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 minutes at room temperature. The cell nuclei were counterstained by DAPI.

### *Co-Immunoprecipitation*

The nuclear fraction of treated H4IIEC3 cells was isolated, and NF-$\kappa$B and HDAC1, HDAC2 complex were immunoprecipitated. NF-$\kappa$B antibody (1:1,000 dilution; Cell Signaling) was added to 400 $\mu$g of protein in a final volume of 500 $\mu$L and incubated at 4°C overnight. Protein A agarose beads (BD Biosciences, San Jose, CA) were added to each sample and rocked for 2 hours at 4°C. The samples then were centrifuged at 13,000 $\times$ *g* at 4°C for 5 minutes. The supernatant was discarded, and the beads were washed twice, pelleted by centrifugation, and resuspended by adding 20 $\mu$L of 5$\times$ sample buffer, boiled, and resolved by SDS-PAGE as described earlier. To show the interaction of NF-$\kappa$B protein with HDAC1 and HDAC2, immunoprecipitated NF-$\kappa$B was blotted against HDAC1 or HDAC2 (Cell Signaling).

### *ChIP Analysis*

H4IIEC3 cells were treated with 1 $\mu$mol/L TPEN with or without 50 $\mu$mol/L zinc for 3 days, and ChIP analysis was performed. Briefly, cell chromatins were prepared using an

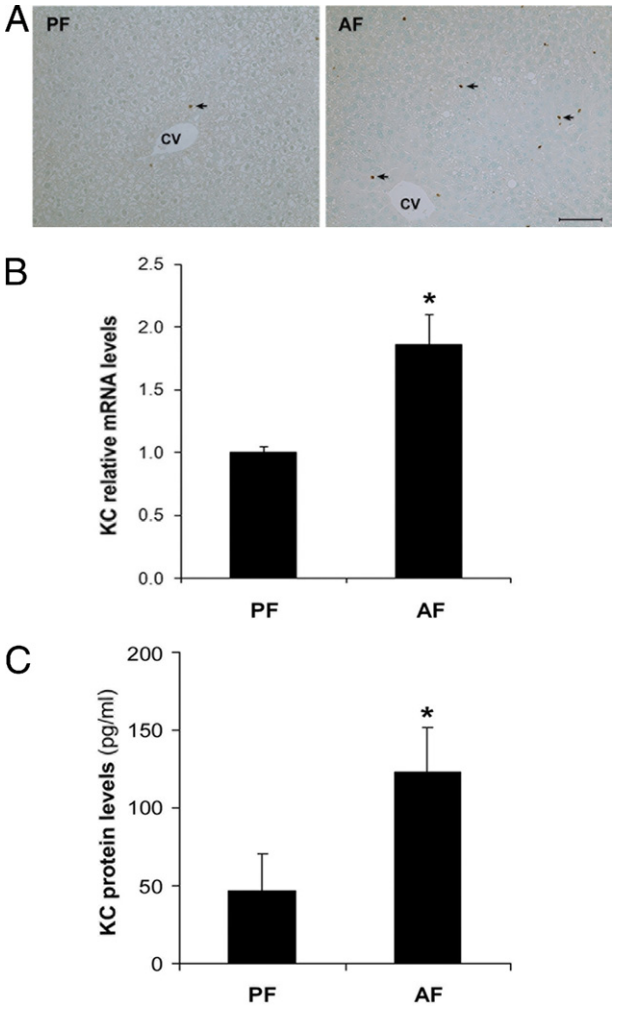


**Figure 1.** Hepatic neutrophil infiltration and IL-8 analog expression in mice fed alcohol for 4 weeks. **A:** Neutrophils (**arrows**) in the liver detected by IHC. Scale bar = 50 $\mu$m. **B:** Real-time RT-PCR analysis of mRNA levels of IL-8 analog, mice KC. **C:** ELISA analysis of KC protein levels. Results in B and C are means ± SD ($n$ = 6 to 8). Significant differences (*$P < 0.05$) between pair-fed (PF) and alcohol-fed (AF) mice were determined by $t$-test. CV, central vein.

EZ-Zyme Chromatin Prep Kit (Upstate, Temecula, CA) according to the manufacturer's instructions. Immunoprecipitation was performed using 5 $\mu$g of anti-AcH3K9, or anti–NF-$\kappa$B antibody (Cell Signaling). CINC-1 promoter-specific primers with the product including NF-$\kappa$B–specific binding site were as follows: sense, 5′-GGGAATTTCCCTGGCCTG-GAG-3′ and antisense, 5′-GAAGCGAGCGGGTGGCT-GAG-3′. Relative fold changes in histone 3 acetylation or NF-$\kappa$B binding activity at CINC-1 promoter were determined by a semiquantitative assay using the ABI 7700 detection system (Applied Biosystems, Foster City, CA) as recommended by the manufacturer.

### *Real-Time RT-PCR*

The hepatic KC mRNA levels were assessed by real-time RT-PCR. In brief, the total RNA was isolated and reverse transcribed with the Moloney murine leukemia virus reverse transcriptase and oligo-dT primer. The forward (5′-AACCGAAGTCATAGCCACAC-3′) and reverse (5′-CA-GACGGTGCCATCAGAG-3′) primers were designed using Primer Express Software (Applied Biosystems). The SYBR green PCR Master Mix (Applied Biosystems) was used for real-time RT-PCR analysis. The relative differences of gene expression among groups were evaluated using cycle time values. The data were normalized to $\beta$-actin and expressed as relative changes, setting the values of control mice as one.

### *Statistics*

All data are expressed as mean ± SD ($n$ = 6 to 8). The data were analyzed by either analysis of variance and the Newman–Keuls multiple-comparison test for studies with more than 2 groups or the Student's $t$-test for studies with 2 groups. Differences between groups were considered significant at a $P$ value of $< 0.05$.

## *Results*

### *Effects of Chronic Alcohol Feeding on Hepatic IL-8 Analog Expression, NF-$\kappa$B Activation, and Histone 3 Acetylation*

As shown in Figure 1A, immunohistochemistry (IHC) showed neutrophil infiltration in the livers of mice after chronic alcohol feeding for 4 weeks. In accordance, chronic alcohol feeding up-regulated IL-8 analog, the mouse KC, gene expression (Figure 1B), and increased its protein levels (Figure 1C) in the liver. Associations of NF-$\kappa$B activation and histone acetylation with mouse KC expression were further determined. Chronic alcohol feeding activated NF-$\kappa$B, as indicated by increased nuclear translocation (Figure 2A). AcH3K9 was assessed by IHC, and chronic alcohol feeding caused histone 3

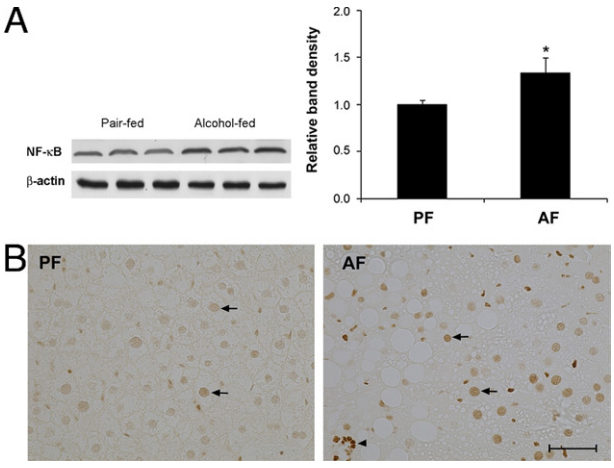


**Figure 2.** Hepatic NF-$\kappa$B activation and histone 3 acetylation in mice fed alcohol for 4 weeks. **A:** NF-$\kappa$B activation detected by immunoblot of nuclear NF-$\kappa$B protein. Significant differences (*$P < 0.05$) in band density between pair-fed (PF) and alcohol-fed (AF) mice were determined by $t$-test ($n$ = 3). **B:** Histone 3 acetylation at lysine 9 detected by IHC. Positive staining on hepatocytes and neutrophils are indicated by **arrows** and **arrowheads**, respectively. Scale bar = 100 $\mu$m.

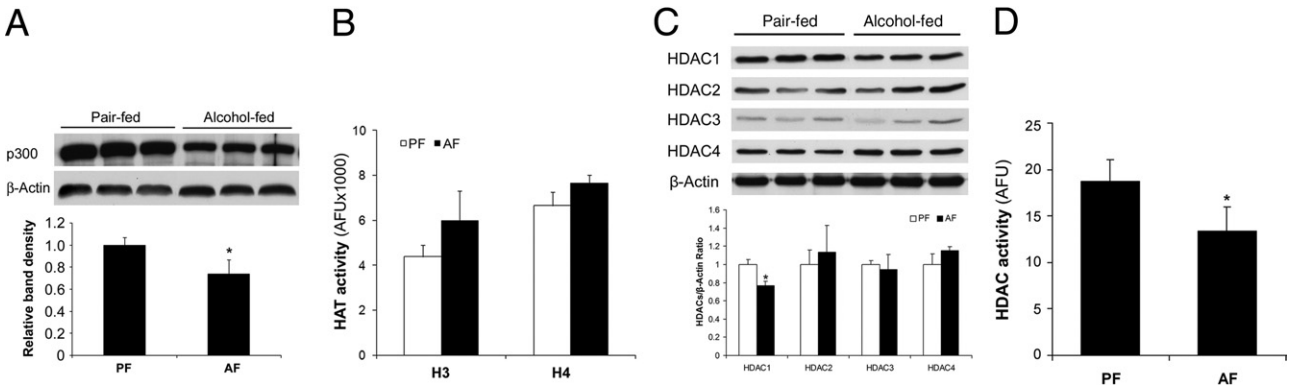


**Figure 3.** Hepatic HAT and HDAC activities in mice fed alcohol for 4 weeks. **A:** Immunoblot of p300 protein. **B:** HAT activity was measured using a fluorimetric assay kit with acetyl-CoA and either a histone H3 or histone H4 substrate peptide. **C:** Immunoblot of HDAC proteins. **D:** HDAC activity was measured with a fluorimetric assay kit. Significant differences ($^*P < 0.05$) between pair-fed (PF) and alcohol-fed (AF) mice in A, C, and D were determined by *t*-test (**A** and **B:** $n = 3$; **D:** $n = 6$ to 8). AFU, arbitrary fluorescence units.

hyperacetylation at lysine 9 in different cell types, including hepatocytes and neutrophils (Figure 2B).

## *Effects of Chronic Alcohol Feeding on Hepatic HATs and HDACs*

Protein levels and activities of histone acetylation–related enzymes were determined. As shown in Figure 3A, chronic alcohol feeding reduced the protein levels of p300, a histone acetyltransferase, but did not significantly affect the hepatic total acetyltransferase activities to either histone H3 or H4 (Figure 3B). Chronic alcohol feeding reduced protein level of HDAC1, but did not affect HDAC2, HDAC3, or HDAC4 (Figure 3C). Total hepatic HDAC activities were decreased by chronic alcohol feeding (Figure 3D).

## *Alcohol-Induced IL-8 Analog Production in Association with Accumulation of Acetaldehyde, ROS, and Free Zinc in Hepatoma Cells*

Alcohol exposure to VL-17A cells induced IL-8 production, which was normalized by either NAC or zinc (Figure 4A). Similarly, alcohol exposure also induced IL-8 analog, rat CINC-1, production in H4IIEC3 cells. Supplementation with NAC and/or zinc also normalized alcohol-induced CINC-1 production in H4IIEC3 cells. Because VL-17A cells produced a higher level of IL-8 in comparison with H4IIEC3 cells, acetaldehyde accumulation in the cell culture media was measured to define the role of alcohol metabolism in IL-8 production. In accordance with greater IL-8 production, acetaldehyde concentrations in the culture media were much higher in VL-17A cells (4383 ng/$10^6$ cells) than H4IIEC3 cells (975 ng/$10^6$ cells).

To determine whether zinc release is associated with alcohol-induced chemokine production, cellular ROS and free zinc were measured in H4IIEC3 cells. As shown in Figure 4C, alcohol exposure caused accumulation of ROS, which was normalized by NAC, and partially attenuated by zinc. Alcohol exposure also increased cellular free zinc levels as indicated by a fluorescence zinc probe, FluoZin-3 (Figure 4D). Both NAC and zinc supplementation attenuated the alcohol-increased cellular free zinc level.

## *Zinc Deprivation Induces IL-8 Analog Production in Hepatoma Cells*

To define the role of zinc deprivation in IL-8 analog production, H4IIEC3 cells were treated with TPEN for 3 days. Zinc deprivation by TPEN caused CINC-1 production,

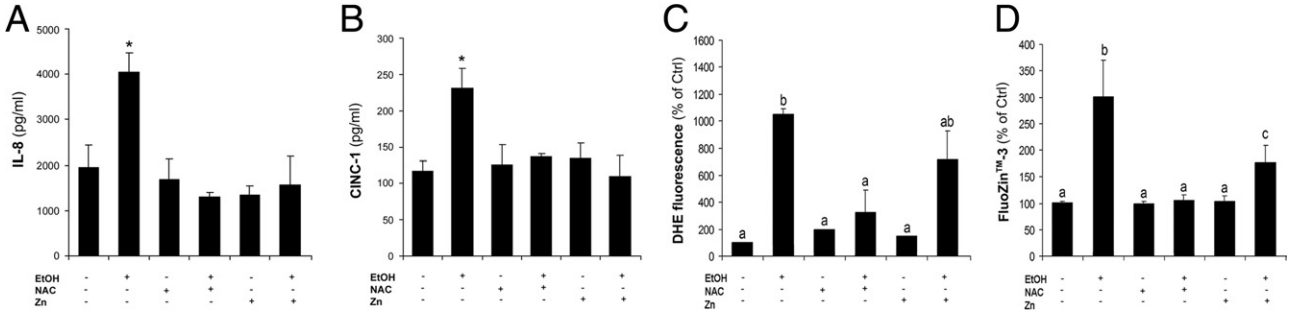


**Figure 4.** Alcohol-induced IL-8 analog production in hepatoma cells. **A:** ELISA analysis of IL-8 production from VL-17A cells. **B:** ELISA analysis of IL-8 analog, rat CINC-1, production in H4IIEC3 cells. **C:** Cellular ROS accumulation in H4IIEC3 cells as measured by DHE fluorescence probe. **D:** Free zinc levels in H4IIEC3 cells detected by FluoZin-3, a free zinc probe. Results are means ± SD ($n = 4$ to 6). Significant differences ($^*P < 0.05$) among a, b, and c were determined by analysis of variance and indicated by **asterisk** in **A** and **B** and letters in **C** and **D**.

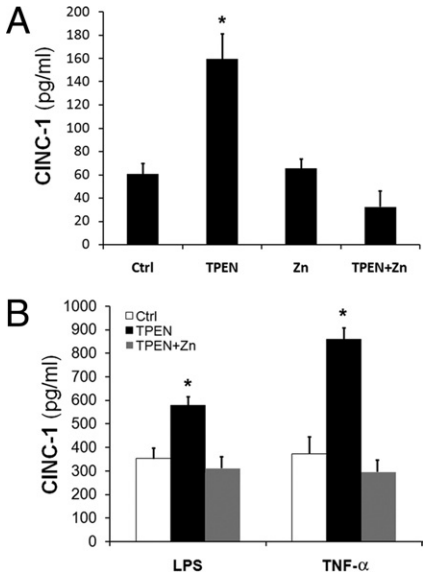


**Figure 5.** Zinc deprivation–induced IL-8 analog production in H4IIEC3 cells. **A:** TPEN-induced CINC-1 production (ELISA). **B:** TPEN-induced priming effect on LPS or TNF-α induction of IL-8 analog, rat CINC-1, production (ELISA). Significant differences (*$P < 0.05$) are determined by analysis of variance. Ctrl, control; LPS, lipopolysaccharide.

which was normalized by adding zinc back to the medium (Figure 5A). To determine whether zinc deprivation has a priming effect on CINC-1 production, H4IIEC3 cells first were treated with TPEN for 2 days, followed by LPS or TNF-α exposure for 24 hours. As shown in Figure 5B, zinc deprivation showed an additive effect on LPS-induced CINC-1 production, and a synergistic effect on TNF-α–induced CINC-1 production.

## *Effects of Zinc Deprivation on HDACs and Histone Acetylation in Hepatoma Cells*

Zinc deprivation by TPEN dramatically decreased HDAC activity, which was prevented by adding zinc back to the medium (Figure 6A). Immunoblot analysis showed that TPEN treatment increased protein levels of HDAC1, HDAC3, and HDAC4, but did not affect HDAC2 (Figure 6B). Zinc supplementation normalized the effects of TPEN on HDAC protein levels (Figure 6B). Histone 3 acetylation at lysine 9 was assessed by immunoblot analysis and immunocytochemistry. As shown in Figure 7A, TPEN treatment significantly increased histone 3 acetylation at lysine 9, without affecting the total protein level of histone 3. TPEN-induced histone 3 hyperacetylation was further confirmed by immunocytochemical staining, as indicated by intensified nuclear staining (Figure 7B).

## *Effects of Zinc Deprivation on NF-κB Activation and NF-κB–HDAC Interaction in Hepatoma Cells*

As shown by immunocytochemistry, NF-κB proteins mainly are located in the cytoplasm in H4IIEC3 cells under normal conditions (Figure 8A). Zinc deprivation by TPEN induced NF-κB activation as indicated by nuclear translocation, reduced cytoplasmic staining, and increased nuclear staining. Interactions between NF-κB and HDACs were assessed by co-immunoprecipitation. Zinc deprivation by TPEN increased nuclear NF-κB, although it decreased HDAC1 and HDAC2 binding to NF-κB (Figure 8B). Zinc supplementation prevented the TPEN effects on NF-κB and HDAC binding to NF-κB.

## *Effects of Zinc Deprivation on NF-κB Binding and Histone Acetylation at CINC-1 Promoter Region in Hepatoma Cells*

A ChIP assay was performed to determine whether zinc deprivation affects NF-κB binding to the CINC-1 promoter and modulates histone acetylation at the same time. A ChIP assay with AcH3K9 immunoprecipitation showed that TPEN increased histone 3 acetylation at lysine 9 at the CINC-1 promoter region in H4IIEC3 cells (Figure 9A). Similarly, a ChIP assay with NF-κB immunoprecipitation showed that zinc deprivation by TPEN dra-

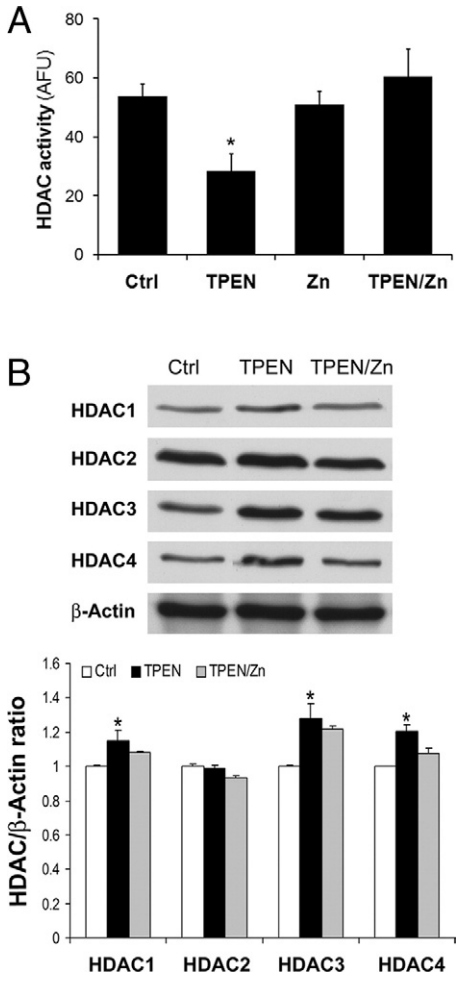


**Figure 6.** Inactivation of HDAC by zinc deprivation in H4IIEC3 cells. **A:** HDAC activity was measured with a fluorimetric assay kit. **B:** Immunoblot of HDAC proteins. Significant increases (*$P < 0.05$) by TPEN were determined by analysis of variance. AFU, arbitrary fluoresence units; Ctrl, control.

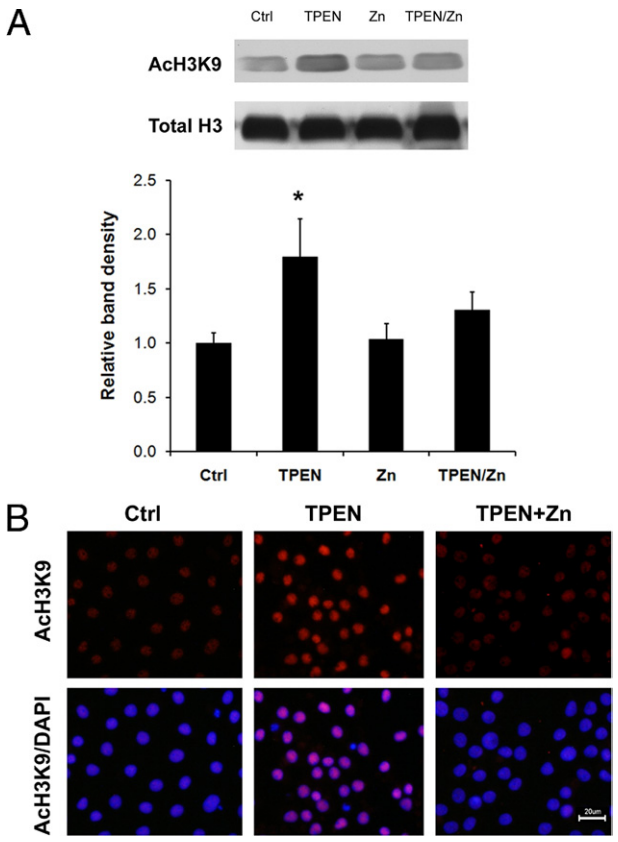


**Figure 7.** Histone 3 hyperacetylation by zinc deprivation in H4IIEC3 cells. **A:** Immunoblot of AcH3K9. **B:** Immunofluorescence microscopy of AcH3K9. Red: NF-κB; blue: DAPI counterstaining of nuclei. Significant increases (*$P <$ 0.05) by TPEN were determined by analysis of variance ($n = 3$). Ctrl, control.

matically increased NF-κB binding to the CINC-1 promoter (Figure 9B). Zinc supplementation prevented TPEN-induced NF-κB binding.

## *Discussion*

Results obtained in the present study showed that histone 3 acetylation at lysine 9 caused by HDAC inactivation was associated with NF-κB activation and mouse IL-8 expression in the liver after chronic alcohol exposure. Studies with VL-17A cells (ADH/CYP2E1-overexpressing HepG2 cells) and H4IIEC3 cells showed that alcohol-induced IL-8 expression in hepatocytes was dependent on oxidative stress and zinc deprivation. Experimental zinc deprivation not only induced IL-8 expression, but also primed LPS- and TNF-α–induced IL-8 expression in H4IIEC3 cells. The inhibitory effect of zinc deprivation on HDAC activity, rather than on protein levels, indicates a pivotal role for zinc in maintaining HDAC enzyme activity. Zinc deprivation caused NF-κB nuclear translocation, and reduced HDAC1/HDAC2 binding to NF-κB. Ultimately, zinc deprivation increased histone 3 hyperacetylation at the IL-8 promoter as well as NF-κB binding to IL-8 promoter. These data suggest that inactivation of HDAC owing to zinc deprivation is a novel mechanism of up-regulating IL-8 gene expression in alcoholic liver disease.

Chronic alcohol exposure to rodents has been shown to increase hepatic IL-8 analog (mouse KC or rat CINC-1) levels in association with neutrophil infiltration,[1,9,10] and the hepatocyte is one of the cell types responsible for IL-8 production.[37] Mechanistic studies have indicated that alcohol metabolism alone is able to trigger IL-8 production in hepatocytes, although results from different reports are controversial. Alcohol exposure to human and rat hepatocytes has been shown to induce chemotactic activity for neutrophils, which was attenuated by inhibition of alcohol metabolism with ADH inhibitor.[11,12] These results suggest that alcohol-induced IL-8 production in hepatocytes is dependent on acetaldehyde and/or oxidative stress. HepG2 cell culture showed that acetaldehyde and LPS, but not alcohol, induced oxidative stress and IL-8 production.[15] Another cell culture study with primary rat hepatocytes showed that rat IL-8 analog expression was stimulated by cytokines, in particular TNF-α, but not by LPS or alcohol.[38] Although there were no evidence to show that whether or not the primary hepatocytes maintained their alcohol metabolic capacity, HepG2 cells are known to lack ADH and CYP2E1.[36] Thus, HepG2 cells may not generate a significant amount of acetaldehyde and ROS, which could explain why alcohol exposure failed to induce oxidative stress and IL-8 production in HepG2 cells. The hepatoma cell lines of

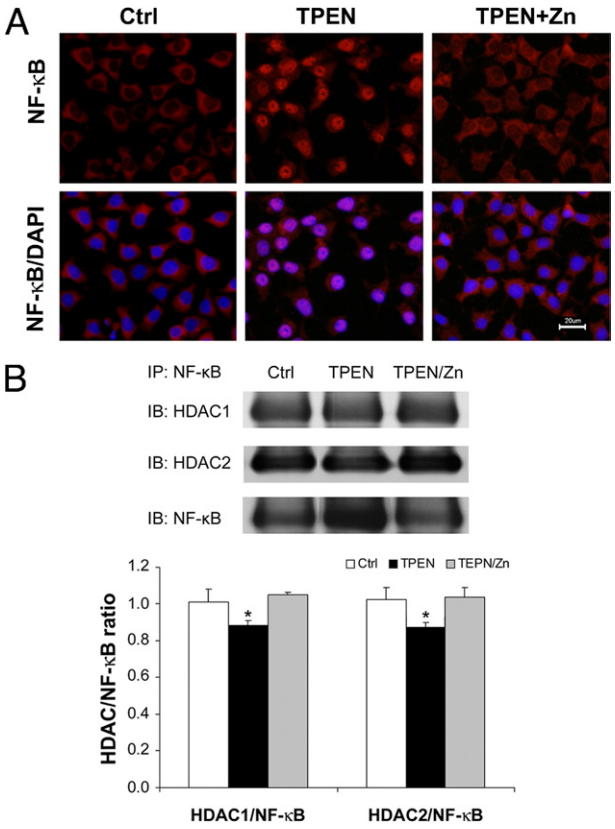


**Figure 8.** Zinc deprivation–induced NF-κB activation in H4IIEC3 cells. **A:** Immunocytochemistry of nuclear translocation of NF-κB after TPEN treatment at 1 μmol/L for 3 days. Red: NF-κB; blue: DAPI counterstaining of nuclei. **B:** Immunoprecipitation (IP) assay followed by immunoblotting (IB) of NF-κB binding with HDAC1 and HDAC2. Significant decreases (*$P <$ 0.05) by TPEN were determined by analysis of variance ($n = 3$). Ctrl, control.

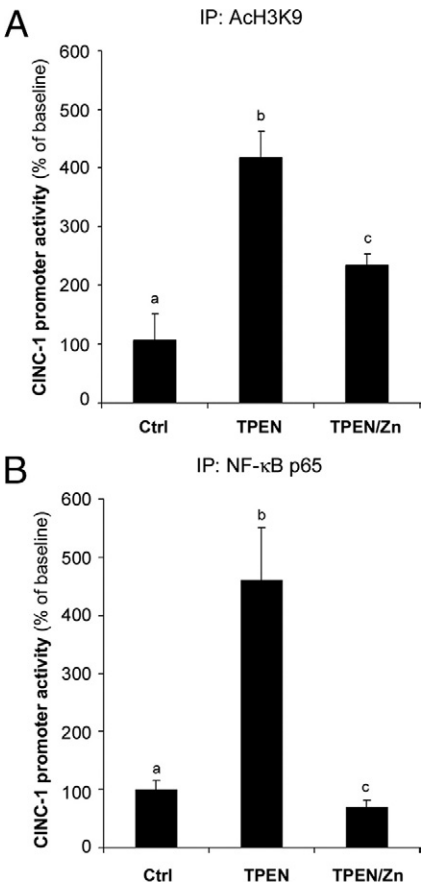


**Figure 9.** Effects of zinc deprivation on histone 3 acetylation and NF-κB binding at CINC-1 promoter in H4IIEC3 cells. **A:** Chromatin immunoprecipitation (ChIP) analysis of histone 3 acetylation at CINC-1 promoter. IP with anti-AcH3K9 antibody and real-time RT-PCR assay of CINC-1 expression. **B:** ChIP analysis of NF-κB binding to CINC-1 promoter. Immunoprecipitation (IP) with anti–NF-κB p65 antibody and real-time RT-PCR assay of CINC-1 expression. Significant increases ($^*P < 0.05$) among a, b, and c by TPEN were determined analysis of variance ($n = 4$). Ctrl, control.

VL-17A and H4IIEC3 used in the present study are known to express alcohol-metabolizing enzymes.[36,39] Alcohol metabolism generates acetaldehyde, which has been shown to contribute to ROS generation significantly.[40] The present study showed that VL-17A cells produced a greater amount of acetaldehyde in association with IL-8 analog production in comparison with H4IIEC3 cells as a result of overexpression of ADH and CYP2E1. These results suggest that alcohol exposure may stimulate IL-8 production in hepatocytes, at least partially, through generation of acetaldehyde and oxidative stress.

Oxidative stress has been suggested as an important mechanism underlying LPS-induced cytokine production in Kupffer cells.[41] Activation of NF-κB is an important mechanism that links oxidative stress with cytokine gene expression. Hepatocytes are the major source of acetaldehyde and ROS generation after alcohol exposure, but the link between oxidative stress and IL-8 production in hepatocytes is poorly understood. Treatments with acetaldehyde and LPS induced IL-8 production in HepG2 cells; however, only acetaldehyde significantly increased lipid peroxidation.[14] Alcohol exposure to VL-17A cells and H4IIEC3 cells in the present study induced IL-8 analog production in association with oxidative stress and zinc release. Because supplementation with NAC or zinc prevented alcohol-induced IL-8 production, oxidative stress and zinc release are like key mediators. Although ROS are known to induce IL-8 production via activation of NF-κB, induction of IL-8 analog in H4IIEC3 cells by zinc deprivation suggests that zinc release could be a downstream event of ROS in IL-8 gene expression. Previous reports have shown that both ROS and acetaldehyde can release zinc from zinc proteins.[40,42,43] The present study showed that zinc deprivation inhibited HDAC activity without affecting protein levels, suggesting zinc release from the zinc figure. However, chronic alcohol exposure inhibited HDAC activity in association with a reduction of the HDAC1 protein level, suggesting that alcohol may inhibit HDAC activity at both the transcriptional level (protein expression) and the posttranscriptional level (zinc release).

Although deacetylation of histones is the major mechanism of transcriptional regulation by HDACs, recent studies have shown that deacetylation of NF-κB by HDACs affect NF-κB activity. The present study showed that zinc deprivation not only affected HDAC activity and NF-κB nuclear translocation, but also interfered with HDAC–NF-κB interaction. Because deacetylation of NF-κB by HDACs inhibits NF-κB activity, reduction of HDAC binding to NF-κB by zinc deprivation may impact NF-κB activity. These results suggest that HDAC inactivation as a result of zinc release may facilitate NF-κB–mediated transcription by directly affecting acetylation status of both histones and NF-κB.

Zinc deprivation not only activated NF-κB, but also inhibited HDAC activity in H4IIEC3 cells. Because HDACs play a critical role in histone acetylation and gene transcription, an epigenetic mechanism likely is involved in zinc regulation of IL-8 expression.

Increasing evidence suggests that inhibition of HDACs under oxidative stress conditions is a key epigenetic mechanism, even in IL-8 gene expression in lung inflammatory disease.[16–18] Oxidants ($H_2O_2$) or cytokines (TNF-$\alpha$ and IL-1$\beta$) have been shown to suppress HDAC activity associated with IL-8 production in alveolar epithelial cells.[23–25] Pharmaceutical inhibition of HDAC by trichostatin A not only stimulated IL-8 production via in-

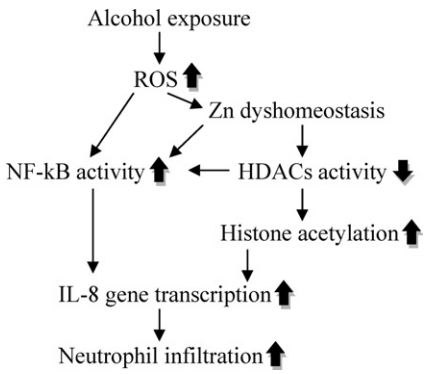


**Figure 10.** Working hypothesis on zinc deprivation and subsequent modulation of epigenetic control of IL-8 analog expression in alcoholic hepatitis.

duction of histone acetylation, but also had a synergistic effect on oxidant- or TNF-$\alpha$–induced IL-8 expression.[23–25] Because trichostatin A did not significantly affect NF-$\kappa$B activation, these studies suggest a causal role of HDACs in regulating IL-8 production. On the other hand, preservation of HDAC activity by glutathione compounds including NAC and glutathione monoethyl ester reversed oxidant- or cigarette smoke–induced IL-8 expression in alveolar epithelial cells and macrophages.[40,42] A clinical study showed that induction of HDAC activity is involved in the anti-inflammatory action of theophylline.[43] Although histone acetylation has been shown repeatedly in alcohol-induced liver injury,[26–30] the mechanistic link between HDACs and IL-8 expression has not been defined. The present study showed a reduction of HDAC activity, rather than increased HAT activity, was associated with chronic alcohol exposure–induced histone 3 hyperacetylation at lysine 9 in multiple cell populations including hepatocytes. Interestingly, alcohol only reduced the protein level of HDAC1, but not HDAC2, HDAC3, or HDAC4. Because our previous reports have shown that chronic alcohol exposure causes zinc deficiency in the liver, reduction of HDAC activity after alcohol exposure is most likely owing to reduced zinc coordination to HDAC proteins. Indeed, experimental zinc deprivation showed a significant inhibition of HDAC activity even with increased protein levels of HDACs, indicating a key role of zinc in maintenance of HDAC activity. HDAC inactivation owing to zinc deprivation not only caused histone 3 acetylation at lysine 9 at the CINC-1 promoter region, but also limited their binding to NF-$\kappa$B, an inhibitory mechanism of NF-$\kappa$B activation. These findings for the first time show that zinc deprivation up-regulates IL-8 analog expression via inactivation of HDACs.

In summary, the present study showed that alcohol exposure induces hepatocyte IL-8 expression in an epigenetic manner and zinc deprivation mediates alcohol action via inactivation of HDACs (Figure 10). Chronic alcohol exposure caused histone 3 acetylation at lysine 9 in association with IL-8 analog production in the liver, and inactivation of HDACs was responsible for histone 3 hyperacetylation. Cell culture studies showed that alcohol exposure induced IL-8 analog production in hepatocytes via an oxidative stress mechanism and zinc release is a downstream molecular event of oxidative stress. Inactivation of HDACs owing to zinc deprivation led to histone 3 acetylation at lysine 9, thereby facilitating NF-$\kappa$B–mediated IL-8 analog transcription. These results suggest that inactivation of HDACs as a result of zinc deprivation is a novel mechanism underlying alcohol-induced IL-8 analog expression in hepatocytes.

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