Suppression of Fas-Mediated Signaling Pathway Is Involved in Zinc Inhibition of Ethanol-Induced Liver Apoptosis

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Apoptosis is critically involved in hepatic pathogenesis induced by acute alcohol exposure. This study was undertaken to test the hypothesis that zinc interferes with an important Fas ligand-mediated pathway in the liver, leading to the inhibition of ethanol-induced apoptosis. Male 129/Sv^{PC}J mice were injected subcutaneously with $ZnSO_4$ (5 mg of Zn ion/kg) in 12-hr intervals for 24 hr before intragastric administration of ethanol (5 g/kg) in 12-hr intervals for 36 hr. Ethanol-induced apoptosis in the liver was detected by a terminal deoxynucleotidyl transferase nick-end labeling assay and was further confirmed by electron microscopy. The number of apoptotic cells in the livers pretreated with zinc was significantly decreased, being only 15% of that found in the animals treated with ethanol only. Characteristic apoptotic morphological changes observed by electron microscopy were also inhibited by zinc. Importantly, zinc inhibited ethanol-induced activation of caspase-3, the primary executioner protease responsible for alcohol-induced liver apoptosis, and caspase-8 as determined by enzymatic assay. Immunohistochemical analysis revealed that zinc inhibited ethanol-induced endogenous Fas ligand activation, which is a key component in signaling pathways leading to hepatic caspase-8 and subsequent caspase-3 activation and apoptosis. These results demonstrate that zinc is a potent inhibitor of acute ethanol-induced liver apoptosis, and this effect occurs primarily through zinc interference with Fas ligand pathway and the suppression of caspase-3. Exp Biol Med 228:406–412, 2003

Key words: zinc; acute ethanol; programmed cell death; caspase-3; Fas/Apo-1

Hepatocyte apoptosis was first observed in human liver disease caused by yellow fever virus (1). Since this observation, liver apoptosis has been described in chemical and viral hepatitis, liver allograft rejection, hepatocarcinomas, and alcoholic liver injury (1, 2–7). Histological analyses of clinical samples from patients with various stages of alcoholic liver disease have provided evidence that increased apoptosis occurs in hepatocytes (8, 9). Studies in mice and rats revealed that both acute and chronic alcohol administration resulted in significant increases in hepatocyte apoptosis, and this cell death occurred primarily in the perivenous regions of the liver (3, 5, 10). Potential mechanisms of acute ethanol-induced liver apoptosis include increased cytokine activity, Fas ligand (FasL) expression, and/or oxidative stress (11–13). These ethanol-induced changes present the liver with overwhelming challenges primarily because hepatocytes are both CD95/Fas/Apo-1 receptor positive and rich in high-affinity low-capacity tumor necrosis factor-α receptors (14, 15). Therefore, after acute ethanol consumption, there is an immense potential for the activation of both intrinsic and extrinsic apoptotic pathways in hepatocytes. However, recent studies by Zhou *et al.* (7) found that the Fas/FasL apoptotic pathway played a primary role in acute ethanol-induced liver apoptosis in mice.

Ethanol-induced liver apoptosis involves the activation of cysteine proteases or caspases (7, 16). These cell death proteases are responsible for transduction of apoptotic signals in both the initiation and execution phases of apoptosis (17). The systemic administration of specific caspase inhibitors has been used to examine the role of various caspases in liver apoptosis (18, 19). Of the 14 members of the caspase family examined, caspase-3 appears to play a critical role in apoptosis because it is activated in response to a myriad of stimuli, including alcohol (7, 20).

Because caspase-3 plays such a key role in alcohol-induced liver apoptosis, the inhibition of this cell-death protease by pharmacological means is of paramount importance in clinical applications. Studies have shown that systemic administration of caspase-3 inhibitors abrogated

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ethanol-induced caspase-3 activation and liver apoptosis in mice and rats (7, 21). However, the upstream signaling pathways leading to caspase-3 activation are also critical in alcohol-induced liver apoptosis, particularly in type I cells where caspase activation is primarily dependent upon death receptor stimulation (22). Therefore, any agent that has the capacity to interfere with multiple steps in apoptotic processes in both the initiation and execution phases of cell death would be of more clinical interest. Zinc may be an agent that has the capacity to interfere with multiple steps in apoptotic processes. Zinc is a trace element that has been shown to have unique protective qualities in many experimental systems (23, 24). Zinc has been shown to inhibit apoptotic DNA fragmentation and caspase-3 activity, implicating involvement in both nuclear and cytoplasmic compartments (25, 26). Furthermore, studies have shown that zinc inhibits the activation of caspase-3 by suppressing the cleavage of pro-caspase-3 (27). These studies together suggest that zinc would interfere with both upstream and downstream events of caspase-3 activation.

Previous studies have shown that neutralizing Fas ligand *in vivo* with a monoclonal antibody leads to inhibition of ethanol-induced caspase-3 activation and apoptosis in the liver. Therefore, the present study was undertaken to examine the inhibitory effects of zinc on Fas/FasL mediated caspase-3 activation and apoptotic cell death in acute ethanol-treated mouse liver.

Materials and Methods

Animals. Male 129/Sv^{PC}J mice (20–25 g, 8–10 weeks of age; Jackson Laboratory; Bar Harbor, ME) were used in this study. They were maintained at 22°C with a 12:12-hr light:dark cycle and had free access to rodent chow and water. The experimental procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care.

Acute Ethanol Challenge. A binge drinking model developed by Carson and Pruett (28) was followed for acute ethanol challenge, with minor modifications. This model was designed to achieve blood alcohol levels that would produce physiological effects comparable with human binge drinking. Animals were randomly assigned into four treatment groups (six mice per group): 1) isocaloric maltose water control, 2) ethanol, 3) zinc, and 4) zinc plus ethanol. We initially performed a dose-response study of zinc (0.5–10 mg/kg) injected subcutaneously in the form of $ZnSO_4 \cdot 7H_2O$ solution and found that doses greater than 5 mg/kg were injurious to the liver of mice. Therefore, we wished to maximize the amount of zinc delivered to the liver while avoiding toxicity. Accordingly, groups 3 and 4 mice were injected subcutaneously with 0.1 ml of a 2.2 mg/ml $ZnSO_4 \cdot 7H_2O$ solution per 10 g of body weight, which provided 5 mg zinc ion/kg body weight, in 12-hr intervals for 24 hr. Groups 1 and 2 mice were injected with the same volume of saline as control. Twelve hours after the last zinc dose and after 6 hr of fasting, groups 2 and 4 mice were administered 0.2 ml of 25% (w/v) ethanol per 10 g of body weight at a dose of 5 g/kg by three gavages in 12-hr intervals for 36 hr and groups 1 and 3 mice received isocaloric maltose water by gavage on the same schedule. Four hours after the final dose, the mice were anesthetized with Avertin (2,2,2 tribromoethanol), blood was drawn from the dorsal vena cava, and the livers were harvested for analysis.

Preparation of Liver Tissue for Light and Electron Microscopy. For light microscopy, liver tissues were fixed in 10% formalin in 10 m*M* phosphate-buffered saline (PBS), pH 7.4, and were embedded in paraplast. Tissue sections of 5-μm thickness were mounted on poly-L-lysine slides. The slides were then dried by incubation at 37°C for 48 hr before analysis. For electron microscopy, liver tissues were sliced into ~1 mm^3 and fixed in 2% freshly depolymerized paraformaldehyde with 0.5% glutaraldehyde in 100 m*M* sodium cacodylate buffer, pH 7.4, for 2 hr. After rinsing in sodium cacodylate buffer, the liver samples were dehydrated with ethanol and embedded in LR White resin. Ultrathin sections were cut and then observed with a transmission electron microscope (Philip; Eindhoven, The Netherlands).

Terminal Deoxynucleotidyl Transferase (TdT) Nick-End Labeling (TUNEL) Assay. Apoptotic DNA fragmentation was examined by light microscopy utilizing DNA nick-end labeling according to the manufacturer's procedure (ApopTag kit; Intergen, Purchase, NY). Tissue sections were deparaffinized and treated with proteinase K (20 μg/ml). Endogenous peroxidases were quenched in 3% hydrogen peroxide in methanol. The slides were then incubated with TdT for 1.0 hr at 37°C in a humidified chamber. The tissue sections were covered with anti-digoxygenin horseradish peroxidase (HRP) for 30 min and TUNEL-positive cells were stained with diaminobenzidine-hydrogen peroxide solution under microscopic observation. The sections were then counterstained with 0.5% methyl green for 15 min. The sections were then washed in double-deionized water, dehydrated, and fixed. TUNEL staining of murine mammary tissue served as positive control. Negative controls were incubated in the absence of primary antibody. TUNEL results were semiquantified through examination of total hepatocytes in three separate fields from each stained liver section.

Caspase-3 and -8 Enzymatic Assay. Caspase-3 and -8 activity was examined by colorimetric assay using substrates containing the chromophore *p*-nitroanilide (*p*-NA) that can be detected at 405 nm when cleaved. Freshly harvested liver tissue was homogenized in extraction buffer (25 mM HEPES, 5 mM EDTA, 2 mM DTT, 0.1% CHAPS, and 1.0% [v/v] proteinase inhibitor cocktail, pH 7.4) using a glass mortar and Teflon-coated pestle. The homogenate was centrifuged at 20,000*g* for 30 min at 4°C. Aliquots of the supernatants were diluted with caspase activity assay buffer (100 m*M* NaCl, 50 m*M* HEPES, 10 m*M* DTT, 1.0 m*M* EDTA, 0.1% CHAPS, and 10% glycerol, pH 7.4) and

was incubated with 200 μM caspase-3 substrate Ac-DEVD-pNA or caspase-8 substrate Ac-IETD-pNA (Calbiochem, La Jolla, CA). *p*-Nitroanalide was used as the standard. Cleavage of the substrate was monitored spectrophotometrically at 405 nm in a microplate reader. Specific activity of each sample was expressed in picomoles of the cleavage product per milligram of protein.

Immunoperoxidase Staining of FasL. FasL in the liver sections was examined by light microscopic analysis of immunohistochemical staining with specific antibody. Endogenous peroxidases were quenched in 3% hydrogen peroxide in methanol. Nonspecific binding sites were blocked in 5% normal goat serum in 10 mM PBS. Tissue sections were then incubated overnight at 4°C with polyclonal rabbit anti-Fas ligand antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Sections were then incubated with biotinylated goat anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA) followed by incubation with streptavidin-horseradish peroxidase (Zymed, San Francisco, CA). The antibody binding was examined under light microscope using diaminobenzidine-hydrogen peroxide solution. Sections were then counterstained with 0.5% methyl green. Negative controls were incubated in the absence of primary antibody.

Statistical Analysis. Data are expressed as mean ± SD, and are analyzed according to a 2 × 2 (treatment: maltose water-fed versus ethanol-fed and saline-injected versus zinc-injected) factorial design. Differences were considered significant when $P < 0.05$.

Results

Effect of Zinc on Ethanol-Induced Liver Apoptosis. Ethanol-induced hepatic apoptosis was examined by TUNEL assay. Acute ethanol exposure induced widespread apoptosis in mouse livers as seen in Figure 1C. TUNEL-positive nuclei of hepatocytes were found primarily in the perivenous regions of liver; however, the presence of positive nuclei extended as far as the 10th and 11th rows surrounding the venous tracts. The livers of mice treated with zinc before the administration of ethanol displayed a reduced apoptotic effect as seen in Figure 1D, where TUNEL-positive nuclei were found only in hepatocytes immediately surrounding venous tracts. As shown in Figure 2, semiquantitative analysis of the TUNEL assay revealed that mice pretreated with zinc exhibited significant inhibition to ethanol-induced liver apoptosis. Characteristic ultrastructural features of apoptosis such as mitochondrial swelling, cytoplasmic degeneration, and chromatin condensation can be examined readily by electron microscopy. Transmission electron microscopy revealed that the livers of acute ethanol-treated mice suffered extensive morphological changes indicative of apoptosis, including cytoplasmic degeneration, chromatin condensation, and nuclear collapse (Fig. 3C). Zinc pretreatment potently opposed these ethanol-induced changes in the liver (Fig. 3D).

Inhibition of Ethanol-Induced Caspase-3 and -8 activity by Zinc. Ethanol-induced apoptosis involves the activation of cysteine-dependent aspartate-specific proteases or caspases, therefore, the activities of caspase-3 and caspase-8 after acute ethanol treatment were determined by enzymatic assay. Acute ethanol exposure elicited a 5-fold increase in liver caspase-3 activity compared with that of control (Fig. 4). In the livers of mice pretreated with zinc, ethanol-induced caspase-3 activity was significantly decreased (Fig. 4). Caspase-8 is an upstream activator of caspase-3 in Fas/FasL-mediated apoptosis, therefore the activity of this initiation phase caspase was examined. Ethanol elicited a 4-fold increase in caspase-8 activity as compared with control (Fig. 5). However, mice pretreated with zinc had a significantly lower level of caspase-8 activity in the liver (Fig. 5).

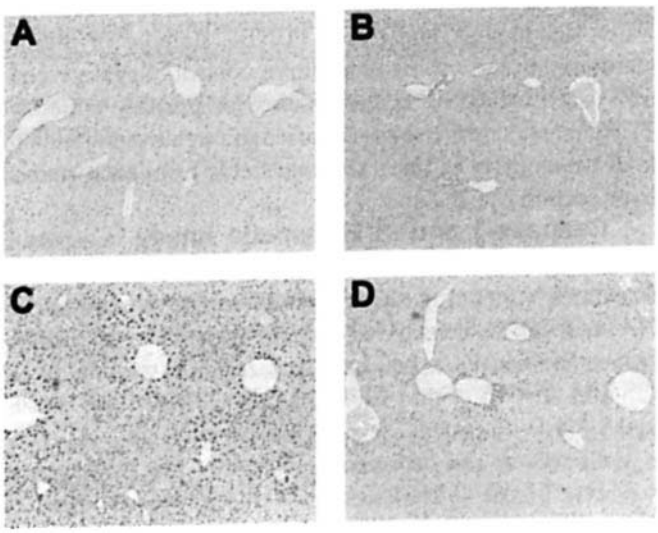

Figure 1. Effect of zinc on acute ethanol-induced apoptotic DNA fragmentation inn mouse liver detected by TUNEL assay. (A) control; (B) zinc; (C) ethanol; (D) zinc and ethanol. Magnification, ×130.

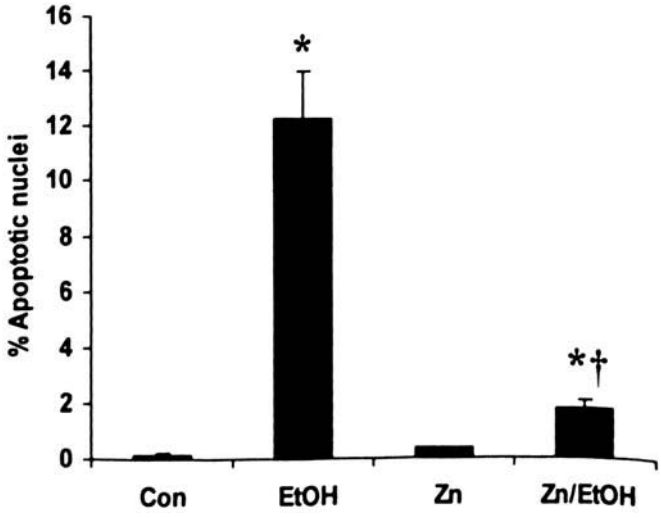

Figure 2. Semiquantitation of TUNEL assay. Con, control; EtOH, ethanol; Zn, zinc; Zn/EtOH, zinc and ethanol. An asterisk indicates significantly different from corresponding controls; † indicates significantly different from ethanol-treated animals. Values represent mean ± SD ($P < 0.05$).

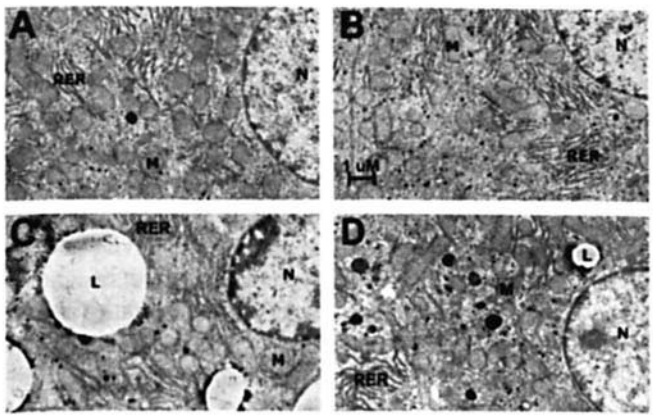

Figure 3. Effect of zinc on acute ethanol-induced ultrastructural changes in mouse hepatocytes. Representative transmission electron micrographs of ultrathin liver sections from control (A); zinc (B); ethanol (C); zinc- and ethanol-treated mice (D). N, nucleus; L, lipid droplets; M, mitochondria; RER, rough endoplasmic reticulum. Magnification, ×7500.

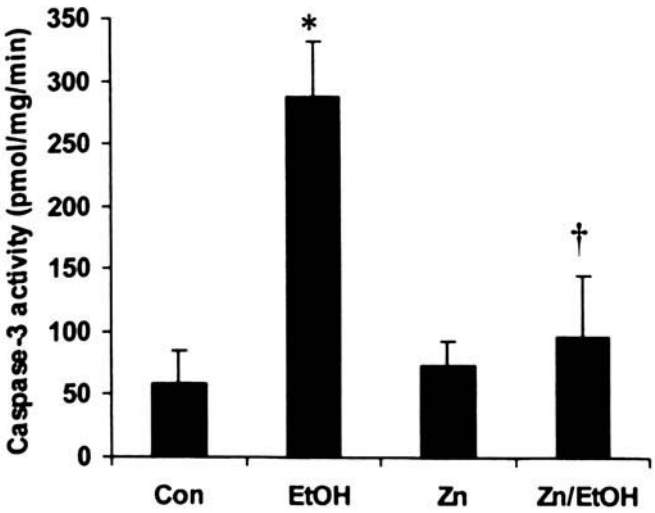

Figure 4. Enzymatic assay of caspase-3-like activity. Liver samples were obtained as explained under "Materials and Methods" and caspase-3 activity was measured using a colorimetric assay. Con, control; EtOH, ethanol; Zn, zinc; Zn/EtOH, zinc and ethanol. An asterisk indicates significantly different from corresponding controls; † indicates significantly different from ethanol-treated animals. Values represent mean ± SD ($P < 0.05$).

Effect of Zinc on Ethanol-Induced FasL Activation. The presence and location of FasL in liver tissue was examined by immunohistochemistry. The livers of acute ethanol-treated mice displayed drastic increases in FasL expression (Fig. 6C). The intensity of FasL immunohistochemical staining was strongest in the perivenous regions of ethanol-treated livers. Importantly, FasL was detected primarily in the intracellular space of hepatocytes, indicating upregulation of endogenous FasL expression. The livers of mice pretreated with zinc displayed a profound inhibition of FasL expression (Fig. 6D), where only those hepatocytes surrounding the venous tracts stained positive for FasL in zinc-pretreated livers.

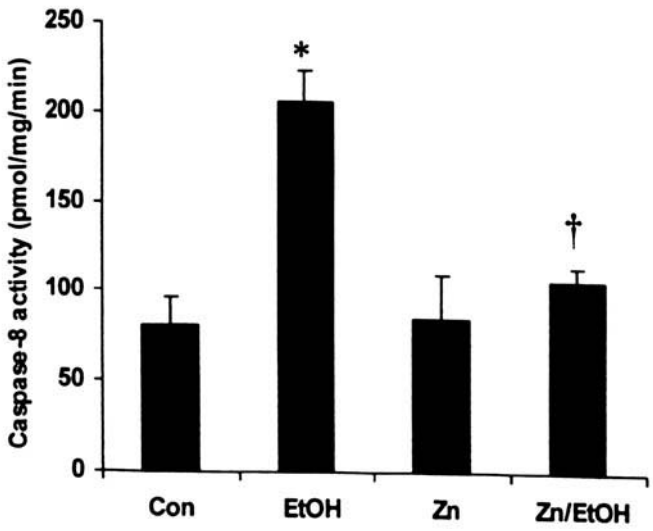

Figure 5. Enzymatic assay of caspase-8 activity. Con, control; EtOH, ethanol; Zn, zinc; Zn/EtOH, zinc and ethanol. AN asterisk indicates significantly different from corresponding controls; † indicates significantly different from ethanol-treated animals. Values represent mean ± SD ($P < 0.05$).

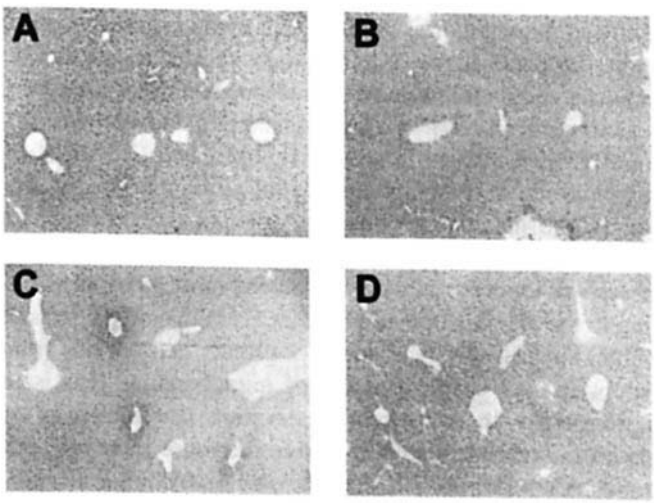

Figure 6. Effect of zinc on acute ethanol-induced FasL protein expression in mouse liver. Immunohistochemical staining of mouse liver sections using a mouse FasL-specific antibody. (A) control; (B) zinc; (C) ethanol; (D) zinc and ethanol. Magnification, ×130.

Discussion

The results obtained from this study demonstrate that zinc protects the liver from acute ethanol treatment-induced apoptosis. Zinc inhibited ethanol-induced apoptotic DNA damage in the liver as seen by a significant decrease in TUNEL-positive hepatocytes. This result was confirmed by electron microscopy where ethanol elicited morphological changes indicative of apoptosis; however, these changes were virtually absent in the livers of mice pretreated with zinc. This inhibition correlated with the suppression of caspase-3 and caspase-8 activation, indicating that zinc interferes with ethanol-induced apoptotic signaling pathways. Furthermore, immunohistochemistry results showed that zinc inhibited expression of FasL. Thus, an important inhibitory mechanism of zinc in acute ethanol-induced liver

apoptosis is the abrogation of Fas/FasL mediated caspase-8 activation and subsequent caspase-3 activation possibly via downregulation of FasL expression in hepatocytes.

One of the hallmarks of apoptosis is the fragmentation of DNA (29). Endonucleases and DNA fragmentation factors are activated during apoptosis, resulting in degradation of chromatin DNA into internucleosomal units (25, 30). Ethanol-induced DNA fragmentation has been described in histological examination of ALD tissue samples as acidophilic bodies or Councilman bodies (8, 31). Acute ethanol treatment induces DNA fragmentation, and this phenomenon was assessed in this study using TUNEL assay and electron microscopy. Ethanol induced a significant increase in DNA fragmentation, as evidenced by the large population of TUNEL-positive hepatocytes in perivenous regions of the liver, and zinc pretreatment significantly inhibited this apoptotic DNA damage. Biava and Mukhlova-Montiel (32) were the first to describe intracellular morphological changes in liver tissue associated with alcohol damage. They used electron microscopic techniques where samples obtained from human patients with acute alcoholic hepatitis showed mitochondrial swelling and cytoplasmic vacuolization. To confirm the TUNEL assay results and to further illustrate zinc inhibition of ethanol-induced liver apoptosis, electron microscopy was used. Changes associated with apoptosis were evident in acute ethanol-treated livers such as cytoplasmic degradation and vacuolization, chromatin condensation, and nuclear collapse. Although zinc pretreatment inhibited these ethanol-induced morphological changes, there was evidence of alcohol-induced mitochondrial swelling in the animals treated with zinc and ethanol. This result might suggest that zinc does not protect hepatocytes from apoptotic cell death upstream of mitochondria. This is in agreement with our recent observation that inhibition of caspase-3 activation and apoptosis in mouse liver via neutralization of FasL did not decrease mitochondrial cytochrome c release (7). Thus, it appears that in this acute ethanol treatment model, the liver cell death may occur through a mechanism independent of mitochondrial changes. The same is true for the inhibitory effect of zinc on acute alcohol-induced liver cell death.

A large majority of apoptotic changes in both the nucleus and cytoplasm are mediated by caspase-3 via the proteolytic activation or inactivation of diverse sets of proteins, including other caspases (33). For this reason, caspase-3 is considered the primary executioner caspase of apoptosis, therefore, the inhibitory mechanism of zinc may be through the inhibition of caspase-3. Indeed, treatment of Molt4 cells with zinc inhibited etoposide-induced poly (ADP-ribose) polymerase (PARP) proteolysis, implicating zinc in the suppression of caspase-3 activity (34). Studies using HeLa cells have also demonstrated that zinc inhibited Adriamycin-induced caspase-3 activation by preventing pro-caspase-3 cleavage (27). Caspase-3 exists under normal physiological conditions in the cytoplasm in its inactive 32-kDa pro-caspase form, however during apoptosis. the proenzyme is proteolytically cleaved into 17- and 12-kDa fragments that then dimerize and subsequently form an active heterotetramer that carries out degradation of substrates containing a DXXD motif (17, 33, 35). Ethanol-induced liver apoptosis has been shown to involve caspase-3 activation in mice, rats, and humans (7, 21, 36). Previous studies found that the injection of a caspase-3-specific inhibitor abrogated acute ethanol-induced caspase-3 activity and apoptosis in mouse liver (7). Therefore, the effect of zinc on ethanol-induced caspase-3 activation was examined by enzymatic assay in this study. The result of the caspase-3 enzyme activity assay revealed that zinc did have an inhibitory effect on caspase-3 activity.

Upstream events of caspase-3 activation are thought to generally involve two main pathways: mitochondrial disruption and/or death receptor activation. In acute ethanol-induced apoptosis in the liver, mice injected with a neutralizing FasL antibody showed inhibition to caspase-3 activation correlated with the suppression of apoptosis even though cytochrome c release still occurred (7). This would indicate that *in vivo*, the binding of FasL to Fas receptor is a primary mechanism of acute ethanol-induced hepatic caspase-3 activation and apoptosis. FasL (CD95L) is a type II transmembrane protein that, upon binding to Fas (CD95) receptor, initiates a cell death cascade involving the formation of a death inducing stimulatory complex or DISC (37). The DISC is a multimolecular complex of proteins that entails trimerized cytoplasmic death domains of Fas receptor, adaptor proteins such as FADD, and pro-caspase-8 (37, 38, 39). Pro-caspase-8 contains death domains that allow for association with the CD95 DISC and subsequent autocatalytic activation (38, 39). Activated caspase-8 then perpetuates the Fas/FasL death signal by proteolytically activating several other caspases, including caspase-3. In this study, we examined the activation of caspase-8 by enzymatic assay and found that acute ethanol treatment led to a significant increase in caspase-8 activity in mouse liver. Zinc pretreatment led to the inhibition of ethanol-induced caspase-8 activation. This result indicates that another site of zinc inhibition may be upstream of caspase-8 activation.

FasL is normally associated with cells involved in immune response or inflammatory conditions such as activated T lymphocytes or natural killer (NK) cells (40–42). Interestingly, hepatocytes express FasL endogenously in response to cellular stresses such as oxidative stress (43). The upregulation of endogenous FasL expression is extremely adverse in liver tissue because hepatocytes are Fas receptor positive (15). The presentation of Fas ligand by stressed hepatocytes can lead to killing of self or neighboring hepatocytes via an autocrine or paracrine mechanism (44). In this study, acute ethanol treatment led to an increase in FasL expression in mouse livers as seen in immunohistochemical data. Zinc pretreatment potently inhibited this acute ethanol-induced increase in FasL expression, suggesting that additional antiapoptotic effects of zinc are upstream of Fas death ligand synthesis. This result implies a synergistic pro-

tective mechanism of zinc where inhibition of FasL expression, presumably through suppression of oxidative stress, in the parenchyma translates into decreased stimulation of apoptosis in addition to the direct effects of zinc on apoptotic machinery. The mechanism of this zinc inhibition requires further examination in future studies.

The results presented herein implicate zinc inhibition of a particular apoptotic pathway, Fas/FasL, aftere acute ethanol intoxication of the liver. To say that zinc has preferential targets in apoptotic cascades *in vivo* has yet to be proven, although much data exist in favor of this hypothesis *in vitro*. In addition, although zinc inhibition of the Fas/FasL pathway of apoptosis is the focus of this research, the involvement of TNF/TNF-α in acute ethanol-induced liver damage cannot be excluded. Cell signaling induced by TNF-α has been well characterized and is known to induce both cell death and survival (45). However, TNF-α alone is virtually incapable of eliciting an apoptotic cell death response in cells that are not sensitized and, indeed, previous studies have shown that TNF-α-mediated liver cell death requires the mitochondrial permeability transition (MPT) (46). More importantly, mice injected with FasL neutralizing antibody after an acute ethanol challenge suffered no observable apoptotic-related liver damage (7).

Similar to most experimental animal studies involving zinc protection in pathophysiological processes, our model of zinc administration entailed treatment of animals before an injurious insult. Intuitively, the reason for pretreatment of zinc is to allow for deposition of this protective metal to tissues and organs of interest in advance of the toxic stimuli. More importantly, zinc is a strong inducer of metallothionein (MT) expression and this metal binding protein has been shown previously to possess potent antioxidant function in the liver (47, 48). Although we consistently use zinc pretreatment in studying the inhibition of ethanol-induced liver injury, simultaneous administration of zinc with ethanol has also been shown to be protective in experimental animals (49, 50).

Many questions remain pertaining to zinc inhibition of programmed cell death, especially *in vivo*; however, the data presented in this research provides valuable insight into the extent of zinc involvement in apoptotic mechanisms and lends support to nutritional intervention as a means of inhibiting liver damage incurred during an acute alcoholic binge.

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