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Effect of zinc intake on hepatic autophagy during acute alcohol intoxication

Juan P. Liuzzi¹, Vijaya Narayanan¹, Huong Doan¹, and Changwon Yoo²

¹Florida International University, Department of Dietetics and Nutrition

²Florida International University, Department of Biostatistics

Introduction

Autophagy is a conserved catabolic process used by eukaryotic cells for the degradation of damaged or superfluous proteins and organelles (Chen and Klionsky 2011). There are three forms of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (Chen and Klionsky 2011). Among these three types of autophagy, macroautophagy (hereafter referred as autophagy) is the focus of this study. Recent studies with mice and cell models have shown that acute ethanol ingestion activates autophagy in liver (Ding et al. 2010; Ni et al. 2013; Lin et al. 2013). However, chronic ethanol intoxication seems to suppress hepatic autophagy (Thomes et al. 2015; Cho et al 2014). The inhibition of autophagy has been shown to worsen ethanol driven steatosis and liver injury in mice (Ding et al. 2010; Ni et al. 2013; Lin et al. 2013). Conversely, pharmacological promotion of autophagy seems to alleviate ethanol-driven liver steatosis and liver injury (Lin et al. 2013). As a result, autophagy is regarded as a protective mechanism against the cytotoxic effects of ethanol and has emerged as an attractive target for the development of novel therapeutic agents for alcoholic liver disease (ALD). There is increasing evidence supporting the notion that zinc is critical for autophagy. It has been shown that zinc is essential for autophagy activated by tamoxifen in MCF-7 breast cancer cells and retinal cells, by hydrogen peroxide, hypoxia and re-oxygenation in astrocytes, and by ethanol in human hepatoma cells (Hwang et al. 2010; Hung et al 2013; Cho et al 2012; Pan et al. 2015; Lee et al 2009; Liuzzi et al. 2013). Given the potential role of zinc in autophagy, studies addressing the effect of zinc intake on autophagy during acute or chronic elevated ethanol intake in animal models could help underscore the importance of adequate zinc intake in individuals with alcohol use disorder. Moreover, these studies could help develop novel therapeutic approaches to treat ALD. Binge drinking episodes are an example of acute exposure to elevated ethanol intake in humans. Binge drinking is common among Americans with an estimated 23.1% of Americans participating in binge drinking once a month (Substance Abuse and Mental Health Services Administration 2011). Although chronic alcohol abuse is commonly associated with illness caused by alcohol, acute alcohol abuse is also detrimental and can cause liver injury and steatosis (Massey and Arteel 2012). Notably, zinc deficiency is one of the most common nutritional deficiencies in alcoholics (McClain and Su 1983; Bode et al.

1988). Poor zinc status in alcoholics is likely the consequence of the combined effect of inadequate zinc intake and altered metabolism of zinc (McClain and Su 1983; McClain et al. 1986; Dinsmore et al. 1985). It is significant that zinc supplementation has been observed to attenuate ethanol-induced liver injury in mice (Zhou et al. 2005; Kang et al. 2009).

In this study, we set out to examine the effect of zinc intake on hepatic autophagic flux mice exposed to acute ethanol intoxication. In addition, the role of zinc in autophagy during acute ethanol exposure was evaluated using an in vitro model of human hepatoma cells.

Methods

Animal Study

A mouse model of binge ethanol feeding was employed (2). C57BL/6 male mice were purchased from Jackson Laboratories (Bar Harbor, ME). Male C57BL/6 mice have been previously used to evaluate the role of ethanol in the regulation of autophagy (Ding et al. 2010; Ni et al. 2013; Cho et al 2014). Mice weighing 25 to 30g were employed in the study. Mice were housed individually in plastic cages with raised wire floors. AIN-93M semi-purified diets with casein replaced by 20% egg whites (Supplementary Table 1A). Egg whites were used as the source of protein instead of casein because of their lower zinc content (Stirn et al. 1935). Diets were purchased from LabDiet (St. Louis, MO). The zinc content of the diets was measured by inductively coupled plasma mass spectrometry (ICP-MS) using a Thermo 6500 dual view ICP instrument (Thermo Fisher Scientific Inc. Waltham, MA (Supplementary Table 1B). The dietary protocol utilized in this study to manipulate zinc status is similar to a previous study (Liuzzi et al. 2004). Conditions of zinc deficiency, adequacy and excess were generated in mice by feeding them diets containing three levels of elemental zinc for 28 days: low (1.0 mg/kg) (ZD), adequate (30 mg/kg) (AD), or supplemented (150 mg/kg) (SP). After this, mice were fasted for 4h and administered either water or ethanol (4.5g/kg body weight) by gavage (four gavages in 1 h) (Ding et al. 2010). There were six group of mice: 1) low zinc (ZD), 2) low zinc and ethanol (ZDE), 3) adequate zinc (AD), 4) adequate zinc and ethanol (ADE), 5) excess zinc (SP), and 6) excess zinc and ethanol (SPE). A group of mice was treated with either the autophagy inhibitor chloroquine (dissolved in saline) (CQ) (60 mg/kg body weight) or saline, 30 min prior ethanol or water treatment (Ding et al. 2010). Mice were sacrificed by anesthesia overdose (Isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane)) at 24h after ethanol treatment. This time point was selected because previous studies showed that ethanol stimulated autophagy in cells after 24h (Ding et al. 2010; Liuzzi et al 2014). The ethanol and CQ dosage proposed here were employed in a study in which the effect of acute ethanol exposure in autophagy was examined (Ding et al. 2010). The animal protocol was approved by the Institutional Laboratory Animal Care and Use Committee of Florida International University.

Analysis of autophagy

Autophagic flux was inferred by combining several markers: LC3II/LC3I ratio, protein levels of p62/SQSTM1, Beclin1 and Atg7, and phosphorylation of 4EBP1 (Klionsky et al. 2016). Increased LC3II/LC3I ratio and LC3-puncta are indicative of activation of autophagy.

On the other hand, p62/SQSTM1 is known to be incorporated in autophagosomes and degraded in autolysosomes. Hence, a reduced p62/SQSTM1 protein level is considered as indicative of increased autophagy (Klionsky et al. 2016). Elevated expression of Beclin1 and Atg7 is indicative of autophagy activation (Klionsky et al. 2016). 4EBP1 is a target of mTOR, a serine/threonine kinase that is known to be a negative regulator of autophagy (Jung et al 2010). Thus, low levels of phosphorylated 4EBP1 could reflect activation of autophagy. The formation of autophagosomes and autolysosomes (AV and AL) in cultured cells was analyzed. Increased formation of AV and AL suggests stimulation of autophagy. Lastly, the degradation of the fusion protein GFP-LC3, a substrate of autophagy (Klionsky et al. 2016), was evaluated in VL17A cells. The autophagy inhibitor CQ was employed to determine whether the results observed for LC3II/I ratio and AV/AL formation were caused by impaired lysosomal function rather than induction of autophagy (Klionsky et al. 2016).

Measurement of tissue triglyceride levels

Total triglyceride content in liver was assayed using the Triglyceride Colorimetric Assay Kit from Cayman Chemical (Ann Arbor, MI).

Histological Analysis

10 micrometers frozen sections of liver (10 micrometers) were stained with Oil Red O to examine steatosis. Steatosis in tissue sections was determined by the number and size of lipid droplets.

Alanine Transaminase (ALT)

Liver injury was evaluated by measuring Alanine Transaminase (ALT) in serum. A colorimetric kit for Cayman Chemical was employed.

Zinc content in liver and diets

Zinc content in liver was analyzed by ICP-MS.

Cell culture and treatments

Experiments with human hepatoma VL17A cells were carried out to examine the effects of zinc deficiency, adequacy, and excess on autophagy. The VL17A cell line was generated from HepG2 cells (Donohue et al. 2006). VL17A cells were used because they have a close to normal expression levels of two key alcohol-metabolizing enzymes: alcohol dehydrogenase (ADH) and cytochrome P450 (CYP2E1) (Donohue et al. 2006). VL-17A cells were kindly donated by Dr. Dahn Clemens (Department of Veterans Affairs, Nebraska Western Iowa). Cells were grown in a 5% CO₂-enriched atmosphere in DMEM (GE Healthcare Life Sciences SH30285.01) medium containing 22 mM glucose supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 µg/ml streptomycin, and amphotericin B (Sigma, St. Louis, MO). Cells were seeded in 12 well plates (1.8×10^5 cells per well) and allowed to grow for 24h before they were treated. To generate zinc deficiency and adequacy, cells were grown in either low zinc (0 µM) or adequate zinc (5 µM) cell culture medium for 48h. To remove zinc from the medium, cell culture medium was prepared using fetal bovine serum (FBS) pretreated with the zinc chelating resin Chelex-100 (BioRad, Hercules, CA)

(Ho and Ames 2002). The levels of zinc, iron and, copper in the medium were analyzed using ICP-MS (Perkin Elmer Sciex, Model ELAN DRC-II). Zinc was not detectable in medium prepared with fetal bovine serum pretreated with Chelex-100. Chelex-100 pretreatment did not affect iron and copper levels in the medium. Chelex-100 can also bind calcium and magnesium (Blankenship and Heitman 2005). DMEM medium (SH30285.01) contains 200.00 mg/L CaCl_2 and 97.67 mg/L MgSO_4 . The average magnesium content in FBS is negligible as compared with the content in DMEM (Arigony et al. 2013). FBS is expected to contain 3 to 4 mM of calcium (Zoico et al. 2014). Thus, Chelex-100 treatment could lower calcium levels in the medium by 10%. However, this decrease in calcium levels in the medium should not affect the normal growth of cells. It has been shown that HepG2 cells can be cultured in either DMEM/F12 (50/50) or RPMI 1640 medium which have considerably lower calcium levels as compared to DMEM medium (Saliani et al. 2013; Joachimik et al. 2007). ZnSO_4 was added to zinc depleted medium to prepare medium with 5 μM zinc. Zinc deficient and zinc adequate cells were treated or not with ethanol for 24h in medium without added L-glutamine. L-glutamine was removed from medium because it can affect basal and induced autophagy (Sakiyama et al 2009). In some experiments, cells were exposed to the autophagy inhibitor CQ (30 μM) for 6 h. The effect of acute zinc treatment (24h) on autophagy during ethanol exposure was also tested. Excess zinc medium was prepared by adding ZnSO_4 to medium (prepared with FBS that was not pretreated with Chelex-100). The ethanol concentrations utilized in this study have been previously employed to evaluate the impact of ethanol exposure on hepatocytes (Ding et al. 2010; Ni et al. 2013; Liuzzi et al 2013).

Generation of *Atg7* knockout cell line

VL17-A cells were transfected with *ATG7*CRISPR/Cas9 KO (h) and *ATG7*HDR (h) plasmids from Santa Cruz Biotechnology. Stably transfected cells were selected by using puromycin (Santa Cruz Biotechnology, Dallas, TX). The use of the plasmids described above was approved by the Institutional Biosafety Committee of Florida International University.

Western Blot analysis

Liver samples and VL17-A cells were lysed in buffer (50 mM Tris and 2% Triton X-100) containing protease inhibitors (Halt protease inhibitor cocktail; Pierce, Rockford, IL). Cellular protein content was determined with a micro BCA protein assay kit (Pierce, Rockford, IL). Aliquots of homogenates were diluted with an equal volume of 5 $\times$ Laemmli sample buffer and resolved on 8–16% polyacrylamide gels (Genscript, Piscataway, NJ). Proteins were transferred onto nitrocellulose membranes using the eBlot® protein transfer system (Genscript, Piscataway, NJ). Anti-LC3B (MAP1LC3B) antibody was purchased from Novus Biologicals (cat. no. NB100-2220) (Littleton, CO), anti-p62/SQSTM1 (cat. no. 5114), anti-Becn1 (BECN1) (cat. no. 3495), anti-Atg7 (cat. no. D12B11), anti-p4EBP1 (cat. no. 2855) and anti-4EBP1 (cat. no. 4923) were purchased from Cell Signaling Technology (Danvers, MA). GFP tag monoclonal antibody (GF28R) (cat. no. MA5-15256) was purchased from ThermoFisher Scientific (Waltham, MA). Peroxidase conjugated goat anti-rabbit IgG and goat anti-mouse IgG antibodies (Santa Cruz Biotechnology) were used as secondary antibodies. Signal detection employed the SuperSignal West Pico

chemiluminescent substrate (Thermofisher). The membranes were also probed with an anti-Actin antibody (cat. no. A2066) (Sigma, St. Louis, MO) to control for sample loading. The specificity of anti-Atg7, anti-LC3B, and anti-p62/SQSTM1 was confirmed using VL17A cells lacking Atg7 (see below). These cells have no expression of Atg7 and LC3II band. In addition, these cells have overexpression of p62/SQSTM1 due to lack of autophagy. Two bands were detected with anti-p62/SQSTM1 at approximately 40 kDa and 60 kDa in VL17A cells lacking Atg7 and in liver samples. However, only the band at 40 kDa was consistently detected in cells expressing Atg7. The antibodies anti-Beclin1, anti-p4EBP1, and anti-4EBP1 exhibited the expected specific bands.

Assay of lactate dehydrogenase (LDH) activity

LDH release into the culture medium was assayed using the LDH cytotoxicity assay kit from Cayman Chemical.

Cyto-ID autophagosomes and autolysosomes (AV and AL) measurement and visualization

Cells were incubated in HBSS containing CYTO-ID (Enzo Life Sciences, Farmingdale, NY) and Hoechst (0.1 µg/mL) (Immunochemistry Technologies) for 40 min. Thereafter, autophagosomes and autolysosomes (AV/AL) fluorescence was measured using a Biotek FLx800 plate reader (excitation 485 nm/emission 528 nm). Values were normalized to Hoechst fluorescence. Cyto-ID staining in cells was observed with a deconvolution microscopy system (Delta Vision imaging system, GE Healthcare Life-Sciences). The use of Cyto-ID to measure autophagy has been validated in previous reports (Guo et al 2015; Chan et al. 2012).

Transient transfection of GFP-LC3 and RFP-LC3

VL17-A cells were transiently transfected with either the DNA construct pEX-GFP-hLC3WT or pEX-HcRed-hLC3WT. PEX-GFP-hLC3WT encoding green fluorescent protein (GFP) fused to human LC3B and pEX-HcRed-hLC3WT encoding HcRed fluorescent protein (RFP) fused to human LC3B. The plasmids containing these constructs were a gift from Tanida et al. (2008) (Addgene plasmids #24987 and 24991) (Cambridge, MA). The reagent Effectene (QIAGEN, Foster City, CA) was utilized for the transfections. The use of pEX-GFP-hLC3WT and pEX-HcRed-hLC3WT plasmids was approved by the Institutional Biosafety Committee of Florida International University.

mRNA Quantitation by RT-PCR

Liver samples were homogenized in TRIzol (Life Technologies/Thermofisher, Grand Island, NY). Cells samples were lysed in TRIzol. Isolated RNA was treated with Turbo DNase (Life Technologies/Thermofisher). Quantitative PCR (qPCR) was performed using the iScript onestep RT-PCR kit with SYBR® Green (BioRad, Hercules, CA). The following set of primers were utilized for qPCR of human *p62/SQSTM1*: 5'-GCACCCCAATGTGATCTGC-3' and 5'-CGCTACACAAGTCGTAGTCTGG-3'; mouse *p62/SQSTM1*: 5'-AGGATGGGGACTTGGTTGC-3' and 5'-TCACAGATCACATTGGGGTGC-3'; human Actin 5'-CATGTAGGTTGCTATCCAGGC-3' and 5'-CTCCTTAATGTCACGCACGAT-3'; and

mouse *Actin*: 5'-TGTTACCAACTGGGACGACA-3' and 5'-GGGGTGTGTAAGGTCTCAAA-3'. These primers were purchased from Life Technologies/ThermoFisher. The sequences of primers were obtained from <http://pga.mgh.harvard.edu/primerbank/>. Predesigned primers (cat. no. QT02317819, Qiagen) were used to analyze *MT2A* (human *Metallothionein 2A*). Values were normalized to *Actin*.

Labile zinc and autophagosome/autolysosome (AV/AL) visualization

Cells were transfected with a plasmid encoding red fluorescence protein (RFP) and human *MAP1LC3B* gene. Transfected cells were treated with 20 μ M zinc for 48h and subsequently treated with 100 mM ethanol for 24h. Thereafter, cells were incubated with the cell-permeable zinc-fluorophore NBD-TPEA (Sigma-Aldrich). Labile zinc and AV/AL were observed using a deconvolution microscopy system (Delta Vision imaging system). Labile zinc was quantified using a Biotek FLx800 plate reader (excitation 485 nm/emission 528 nm). Values were normalized to Hoechst fluorescence.

Apoptosis measurement

Apoptosis was assayed using an Annexin V FITC apoptosis detection kit (BD Biosciences, San Jose, CA). Fluorescence was measured using a Biotek FLx800 plate reader (Winooski, VT). Values were normalized to Hoechst nuclear stain fluorescence (Immunocytochemistry Technologies, Bloomington, MN). Cells were observed with a deconvolution microscopy system (Delta Vision imaging system, GE Healthcare Life-Sciences, Pittsburgh, PA).

Statistical analysis

Data are presented as means $\pm$ standard error (SE). Two-way ANOVA was used followed by Tukey's post hoc test. Statistical significance was set at $p < 0.05$. Tests were performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com

Results

Food intake and weight gain

The dietary treatments did not affect food intake and body weight of mice (Supplementary Fig. 1, Supplementary Table 2).

Zinc liver content

The concentration of zinc in liver samples from zinc-deficient mice (ZD) was significantly lower than the concentration observed in samples from zinc-adequate (AD) ($p < 0.05$) and zinc-supplemented (SP) mice ($p < 0.01$) (Supplementary Fig. 2). However, the hepatic zinc concentration did not differ between AD and SP mice. Mice fed high zinc diet and ethanol (SPE) had significantly lower liver zinc content than SP mice.

Effect of zinc intake on hepatic autophagic flux in mice

Ethanol intake did not affect Atg7 expression (Fig. 1A and 1B). Low zinc intake was associated with decreased Atg7 expression in mice treated or not with ethanol (ZD and

ZDE) as compared to the other groups, except for ADE mice (Fig. 1A and B). Two bands were detected by anti-p62/SQSTM1 antibody in liver samples. One band at 60kDa and the other band at 40kDa. Both bands were significantly decreased by ethanol treatment in zinc supplemented mice. However, only the 40 kDa band was significantly downregulated by ethanol in zinc adequate mice. Interestingly, ethanol treatment did not change p62/SQSTM1 expression in mice fed low zinc diets. There was a significant interaction between ethanol and zinc on p62/SQSTM1 protein expression. Noteworthy, *p62/SQSTM1* mRNA levels were not affected by zinc status or ethanol treatment (Fig. 1C).

Beclin1 expression was significantly increased in SPE mice as compared to zinc deficient and zinc adequate mice exposed or not to ethanol. LC3II/I ratio was increased by ethanol in zinc adequate ($p<0.05$) and zinc supplemented mice ($p<0.0001$). Nevertheless, this marker was not affected by ethanol in zinc deficient mice. Moreover, zinc status did not alter LC3II/I ratio under basal condition. Two-way ANOVA analysis of LC3II/I ratio revealed a very significant ($p<0.0001$) interaction between zinc and ethanol. Lastly, the levels of phosphorylated 4EBP1 were significantly reduced in SPE mice as compared to ZD and ZDE mice.

Hepatic steatosis, triglycerides, and liver injury

Ethanol binge feeding increased Oil Red O staining in ZDE, ADE and SPE mice. However, ZDE mice exhibited the highest degree of steatosis and triglyceride accumulation (Fig. 2A and 2B). Ethanol treatment significantly increased the concentration of hepatic triglycerides in ZDE ($p<0.001$) and ADE ($p<0.01$) mice but not in SPE mice. However, hepatic lipid accumulation did not vary between ADE and SPE mice. A significant interaction between zinc and ethanol was found for hepatic triglycerides ($p<0.0001$). Interestingly, CQ treatment promoted ethanol-induced hepatic steatosis and lipid accumulation in ADE ($p<0.01$) and SPE ($p<0.05$) mice; however, it did not affect significantly hepatic triglyceride content in ZDE mice (Fig. 2A and 2C). Oil Red O staining showed that CQ decreased steatosis in ZDE mice. ALT levels in serum were increased by ethanol treatment in ZDE mice but not in ADE and SPE mice (Fig. 3A). CQ treatment did not increase ALT levels in mice (Fig. 3A and 3B).

Effect of zinc depletion on autophagy and in human hepatoma cells

Beclin1 and Atg7 protein expression was not significantly affected by zinc and ethanol treatment (Fig. 4A and 4B). The levels of p62/SQSTM1 (40 kDa band) were significantly decreased by ethanol in zinc adequate cells (cultured in medium with 5 μ M zinc) but not in zinc deficient cells (Fig. 4A and 4B). As expected, zinc depletion caused a drastic reduction in mRNA expression of the zinc regulated gene *MT2A* (Davis and Cousins 2000). However, *p62/SQSTM1* mRNA expression was not affected by zinc or ethanol (Fig. 4C). The effect of zinc depletion on LC3II/LC3I ratio and p62/SQSTM1 expression in cells exposed to different levels of ethanol and zinc was tested (Fig. 5A and 5B). LC3II/I ratio increased in zinc adequate cells exposed to 100 ($p<0.05$) and 200 mM ($p<0.01$) of ethanol. P62/SQSTM1 expression was reduced in zinc adequate cells treated with 50, 100 and 200 mM. There was a significant interaction ($p<0.05$) between zinc and ethanol for LC3II/I ratio and p62/SQSTM1. CQ treatment resulted in increased LC3II/LC3I ratio in zinc-deficient and zinc-

adequate cells exposed or not to ethanol (Fig. 6). To test whether the downregulation of p62/SQSTM1 in zinc adequate cells treated with ethanol is caused by stimulation of autophagy, we utilized VL17A cells lacking expression of the autophagy essential gene *Atg7* (*Atg7*^{-/-} cells). As expected, these cells exhibited elevated levels of p62/SQSTM1 as compared to wild type cells (*Atg7*^{+/+}) (Fig. 7). Furthermore, the expression of p62/SQSTM1 in these cells was not influenced by zinc in medium or ethanol treatment (Fig. 7). Consistent with the notion that zinc is critical for autophagy, zinc depletion caused suppression of degradation of the fusion protein GFP-LC3 in cells exposed to ethanol (Fig. 8). The formation of AV/AL (as measured using Cyto-ID) was negatively affected by zinc depletion (Fig. 9A, 9B and 9E). Moreover, the addition of CQ caused accumulation of AV/AL in zinc-deficient and zinc-adequate cells treated with ethanol (Fig. 9C and 9D). Interestingly, the formation of AV/AL was found to be increased by zinc supplementation in a dose-dependent manner in cells treated with ethanol (Fig. 9F).

Autophagosomes, autolysosomes (AV/AL) and free zinc in cells

The analysis of the intracellular localization of free zinc and AV/AL indicated a high degree of colocalization of labile zinc with these vesicles (Fig. 11A, 11B and 11C). Free zinc levels in cells were increased by zinc treatment. However, co-treatment with zinc and ethanol caused a further increase in free zinc levels in cells (Fig. 10D).

Effect of zinc depletion on apoptosis

Zinc-deficient cells exhibited increased ethanol induced apoptosis as compared to zinc-adequate cells (Figs. 11A and 11B). CQ treatment increased ethanol-induced apoptosis only in zinc-adequate cells (Figs. 11A and 11B). Of note, LDH leakage data in these cells indicated that the treatments did not cause noticeable increased cell death (Fig. 11C).

Discussion

In this study, we examined the effect of zinc deficiency, adequacy, and excess on autophagic flux under basal conditions and during acute ethanol exposure in the livers of mice and in human hepatoma cells. To our knowledge, this is the first study that demonstrates that zinc intake can affect autophagic flux in mice exposed to acute ethanol. The dietary treatments did not affect food intake or growth. Low zinc intake is known to cause anorexia in rats (Shay and Mangian 2000). However, mice did not seem to develop anorexia with mild to moderate zinc deficiency (Liuzzi et al 2004). Consistent with previous studies (Ding et al. 2010; Ni et al. 2013; Lin et al. 2013), in our study, ethanol binge feeding stimulated hepatic autophagy. However, our results suggest that adequate zinc intake is needed for proper activation of autophagy by acute ethanol. Zinc deficient mice that were fed ethanol exhibited lower protein expression of the autophagy gene *Atg7*, decreased LC3II/I ratio as well as increased p62/SQSTM1 expression. These results indicate that low zinc intake had an inhibitory effect on the stimulation of autophagy by ethanol. Additionally, ZDE mice had increased steatosis and liver damage. On the other hand, our results suggest that zinc supplementation enhances ethanol induced autophagy. SPE mice showed increased LC3 II/I ratio and increased Beclin1 expression as compared to ADE mice. P62/SQSTM1 is an ubiquitin-binding autophagic adaptor protein that participates in the selective degradation of

substrates by autophagy (Pankiv et al. 2007). Since p62/SQSTM1 is a substrate of autophagy, the levels of this protein inversely correlate with autophagic flux (Klionsky et al. 2016). Acute ethanol treatment decreased the levels of both bands of p62/SQSTM1 in ADE and SPE mice but not in ZDE mice. This suggests that zinc is likely a positive regulator of autophagic degradation of p62/SQSTM1. The fact that *p62/SQSTM1* mRNA expression was not responsive to dietary zinc intake indicates that the observed effects on the protein expression of this gene are not likely to be due to the result of regulation of transcription or mRNA stability by zinc. Overall, the results from the studies with VL17A cells support the idea that zinc is critical for ethanol-induced autophagy. Zinc-deficient cells that were exposed to ethanol exhibited lower LC3II/I ratio and AV/AL formation and had increased levels of p62/SQSTM1 and phosphorylated 4EBP1 as compared to zinc-adequate cells treated with ethanol. The fact that CQ treatment increased LC3II/I ratio and AV/L in cells demonstrates that the observed differences in these markers were not due to impaired lysosomal function. Moreover, the fact that p62/SQSTM1 expression is decreased concomitantly with an increase of LC3II/I ratio also suggests that there is no failure of late autophagy. It is important to mention that although p62/SQSTM1 is mainly degraded by autophagy, this protein can also be degraded by the proteasome (Wong and Cuervo 2010). Based on the result that the expression of p62/SQSTM1 in autophagy deficient cells (*Atg7^{-/-}* cells) was not affected by zinc or ethanol levels in the medium, it is unlikely that the observed results were caused by modulation of proteasomal degradation of p62/SQSTM1 by zinc. Consistent with the idea that zinc is a positive regulator of autophagy, the formation of AV/AL was negatively affected by zinc depletion and positively regulated by zinc in a dose-dependent manner. Furthermore, the inhibition of the degradation of the fusion protein GFP-LC3, a substrate of autophagy (Klionsky et al. 2016), by zinc depletion further supports the notion that zinc is critical for autophagy. The mechanisms by which ethanol induces autophagy are now beginning to be understood. Inhibition of mTOR and AKT, as well as activation of the transcription factors FOXO3A and TFEB, have been implicated in the activation of autophagy by acute ethanol (Ding et al. 2010; Ni et al. 2013; Thomes et al. 2015). Moreover, the oxidative stress generated by ethanol metabolism seems to be critical for the stimulation of autophagy by ethanol (Ding et al. 2010). The results of this study indicate that zinc depletion and zinc excess have a significant impact on autophagy during ethanol exposure. There was a consistent interaction between zinc and ethanol for LC3II/I ratio and p62/SQSTM1. In addition, ethanol caused a significant increase in free zinc levels in cells exposed to excess zinc. It is conceivable that the mobilization of zinc elicited by oxidative stress resulting from ethanol metabolism is an important component of the mechanism by which ethanol induces autophagy. Accumulating evidence from studies using “in vitro” models suggests that zinc modulates autophagy. It has been proposed that excess zinc could promote autophagy through phosphorylation of extracellular-signal-regulated kinases 1/2 (ERK1/2) and induction of the mRNA expression of autophagy genes (Hwang et al. 2010; Liuzzi et al. 2013; Wei et al. 2017). Moreover, zinc may play a role in AV/AL function. The colocalization of zinc with these vesicles found in this study and in a previous report (Lee et al. 2009) supports this hypothesis. Interestingly, a recent study showed that zinc depletion induced autophagy in yeast (Kawamata et al. 2017). Further studies are needed to determine what causes the differential response to zinc in yeast and mammalian cells. Reportedly, autophagy mitigates ethanol toxicity by promoting the

degradation of lipid droplets (lipophagy), and mitochondria (mitophagy) (Ding et al. 2010). Zinc is known to play a protective role against ethanol toxicity (Zhou et al. 2005; Kang et al. 2009). Hence, it is an attractive hypothesis that some of the protective effects of zinc could be mediated through its role in autophagy. This idea is supported by the fact that the protective effects of adequate or high zinc intake against ethanol driven steatosis and liver injury were completely blocked by the autophagy inhibitor CQ. Lastly, the results of the analysis of early apoptosis in the presence/absence of CQ also support the idea that the modulation of autophagy by zinc may play an important role in the protective action of zinc against ethanol toxicity. Nevertheless, it is important to consider that CQ can also have unspecific effects. More studies are needed with other autophagy inhibitors to determine whether autophagy is involved in the protective effects of zinc. A recent study indicated that CQ is a zinc ionophore (Xue et al. 2014). Given the results of our study (colocalization of AV/AL with free zinc), it cannot be ruled out that the increased free zinc levels in cells caused by CQ, observed in that study, could be the result of AV/AL accumulation.

Autophagy is a stress response mechanism that is believed to confer protection against ethanol driven cytotoxicity. However, inadequate zinc intake can interfere with the regulation of autophagy by ethanol. Zinc deficiency is commonly found in alcoholics (McClain and Su 1983; Bode et al. 1988); thus, poor zinc status is likely to be a contributing factor to faulty autophagy in alcoholics. Further studies are needed to establish the mechanisms by which zinc regulates autophagy. In addition, investigating the role of autophagy as a mediator of the protective effects of zinc could help develop novel therapeutic approaches to prevent alcoholic liver disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Hepatic autophagic flux. (A) Markers of autophagic flux; (B) Western blot densitometry values of markers of autophagic flux and mRNA expression of *p62/Sqstm1*. Values are average of 4 to 6 mice per group $\pm$ SE. Means without a common letter are significantly different ($p < 0.05$). LC3II/I ratio: interaction zinc vs ethanol $p < 0.0001$. P62/SQSTM1(60 kDa): interaction zinc vs ethanol $p < 0.0001$

Figure 2. Effect of zinc intake and autophagy on ethanol induced lipid accumulation in the liver. (A) Representative Oil Red O staining of the liver 20x; (B) and (C) concentration of hepatic triglycerides. Values are average of 4 mice per group $\pm$ SE. Means without a common letter are significantly different ($p < 0.05$). Interaction zinc vs ethanol $p < 0.01$. Interaction zinc vs CQ $p < 0.05$.

Figure 3. Effect of zinc intake and autophagy on ethanol induced liver damage. (A) and (B) serum alanine aminotransferase (ALT). Values are average of 3 to 4 mice per group $\pm$ SE. * Significantly different ($p < 0.05$).

Figure 4.

Effect zinc depletion on Atg7, Beclin1 and p4EBP expression in VL17-A cells. (A) Western blot images; (B) Western blot densitometry values of markers of autophagic flux; (C) mRNA expression of *MT2A* and *p62/SQSTM1*. Cells were incubated in zinc depleted medium (prepared with FBS treated with Chelex-100), supplemented with 0 or 5 μ M zinc for 48h and subsequently exposed to 0 or 100 mM ethanol for 24h. Means without a common letter are significantly different ($p < 0.05$). * Significantly different ($p < 0.05$).

Figure 5.

Effect of zinc deficiency and ethanol dosage on LC3II/I ratio and p62/SQSTM1 expression. (A) Western blot images; (B) Western blot densitometry values of LC3II/I ratio and p62/SQSTM1 expression. Cells were incubated in zinc depleted medium (prepared with FBS treated with Chelex-100), supplemented with 0 or 5 μM zinc for 48h and subsequently exposed and exposed to various levels of ethanol. Values are average $\pm$ SE of three independent experiments. Means without a common letter are significantly different ($p < 0.05$). LC3II/I ratio interaction zinc vs ethanol $p < 0.05$. P62/SQSTM1 expression interaction zinc vs ethanol $p < 0.05$.

Figure 6.
Effect of Chloroquine (CQ) on LC3II/I ratio in zinc deficient and zinc adequate cells treated or not with ethanol. Cells were treated as described in figure 4 legend. Some cells were treated with 30 μM of CQ for 6h.

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Figure 7. Effect of zinc depletion on the protein expression of p62/SQSTM1 in autophagy deficient VL17A (cells lacking *Atg7* expression). Cells were treated as described in figure 4 legend. Representative of three independent experiments.

Figure 8. Degradation of GFP-LC3 fusion protein in VL17-A cells. Cells expressing GFP-LC3 fusion protein were treated as described in figure 4 legend. Representative of three independent experiments.

Figure 9.

Representative images of autophagosomes and autolysosomes (AV/AL). For A, B, C, D and E: cells were incubated in zinc depleted medium (prepared with FBS treated with Chelex-100), supplemented with 0 or 5 μ M zinc for 48h and subsequently exposed to 0 or 100 mM ethanol for 24h. (A) Zinc depleted cells treated with 100 mM ethanol for 24h (B); cells treated with 5 μ M and 100 mM ethanol for 24h; (C) zinc depleted cells treated with 100 mM ethanol (24h) and 30 μ M CQ (6h) and (D) cells treated with 5 μ M of zinc, 100 mM ethanol, and 30 μ M CQ (6h). (E) and (F) Fluorimetric quantification of AV/AL utilizing CytoID. (F) Cells were incubated simultaneously with ethanol (100 mM) and different levels of zinc for 24h (acute zinc treatment). Values are average $\pm$ SE of 3 to 4 independent experiments. Means without a common letter are significantly different ($p < 0.05$). Interaction zinc vs ethanol $p < 0.05$.

Figure 10.

Autophagosomes and autolysosomes (AV/AL) and free zinc in VL17A cells. A, B and C: Colocalization of free zinc with autophagosomes and autolysosomes. Cells were treated with 20 µM of zinc for 48h and subsequently treated with 100 mM of ethanol for 24h. (A) Composite image showing colocalization of autophagosomes and autolysosomes with free zinc, (B) Free zinc and (C) autophagosomes and autolysosomes. (D) Fluorimetric quantification of free zinc in VL17A cells. (F) Cells were incubated simultaneously with ethanol and zinc for 24h. Values are average $\pm$ SE of four independent experiments. Means without a common letter are significantly different ($p < 0.05$).

Figure 11.

Zinc depletion enhances ethanol induced apoptosis and cell death through inhibition of autophagy. (A) Annexin V levels in VL17-A cells. (B) Representative images of Annexin V and Hoechst staining in VL17A cells. (I) Zinc depleted cells treated with 100 mM ethanol; (II) cells treated with 5 μ M and 100 mM ethanol; (III) zinc depleted cells treated with 100 mM ethanol and CQ and (IV) cells treated with 5 μ M of zinc, 100 mM ethanol, and CQ. (C) LDH leakage (percentage of “control group” defined as of cells treated with 5 μ M zinc (no ethanol or CQ)). Cells were treated as described in figure 5. Some cells were treated with 30 μ M of CQ for 6h. Values are average $\pm$ SE.*Significantly different ($p < 0.05$). Annexin V: interaction zinc vs chloroquine $p < 0.0001$.