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Ethanol Promotes Endoplasmic Reticulum Stress-Induced Neuronal Death: Involvement of Oxidative Stress

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Abstract

One of the most devastating effects of ethanol exposure during development is the loss of neurons in selected brain areas. The underlying cellular/molecular mechanisms remain unclear. The endoplasmic reticulum (ER) is involved in posttranslational protein processing and transport. The accumulation of unfolded or misfolded proteins in the ER lumen triggers ER stress, which is characterized by translational attenuation, synthesis of ER chaperone proteins such as GRP78, and activation of transcription factors such as ATF4, ATF6, and CHOP. Sustained ER stress ultimately leads to cell death. ER stress response can be induced experimentally by treatment with tunicamycin and thapsigargin. Using SH-SY5Y neuroblastoma cells and primary cerebellar granule neurons as in vitro models, we demonstrated that exposure to ethanol alone had little effect on the expression of markers for ER stress; however, ethanol drastically enhanced the expression of GRP78, CHOP, ATF4, ATF6, and phosphorylated PERK and eIF2 α when induced by tunicamycin and thapsigargin. Consistently, ethanol promoted tunicamycin- and thapsigargin-induced cell death. Ethanol rapidly caused oxidative stress in cultured neuronal cells; antioxidants blocked ethanol's potentiation of ER stress and cell death, suggesting that the ethanol-promoted ER stress response is mediated by oxidative stress. CHOP is a proapoptotic transcription factor. We further demonstrated that CHOP played an important role in ethanol-promoted cell death. Thus, the effect of ethanol may be mediated by the interaction between oxidative stress and ER stress.

Keywords

alcohol; apoptosis; cerebellum; development; fetal alcohol syndrome

Alcohol exposure alters the structure and physiology of the brain in many ways. During development, alcohol-induced structural and physiological alterations contribute to the brain dysfunction of children with fetal alcohol syndrome (FAS). FAS is the most common nonhereditary cause of mental retardation (May and Gossage, 2001). One of the most prominent alcohol-induced neuropathological changes in the developing brain is the loss of neurons (West et al., 1990; Luo and Miller, 1998; Ikonomidou et al., 2000; Goodlet and

Horn, 2001). In adults, heavy alcohol consumption also causes neuronal loss in selected brain regions (Kril et al., 1997; Gotz et al., 2001; Ikegami et al., 2003). The mechanisms underlying ethanol-induced neuronal loss, however, remain incompletely elucidated. Oxidative stress is caused by the disruption of intracellular redox homeostasis. The generation of reactive oxygen species (ROS) initiates this process and may cause neuronal death (Mattson et al., 2001; Watts et al., 2005; Loh et al., 2006). Alcohol exposure may induce oxidative stress in the central nervous system (Marino et al., 2004; Kumral et al., 2005; Chu et al., 2007), and the brain is particularly susceptible to ROS-induced damage (Annunziato et al., 2003). Oxidative stress has been proposed to be a potential mechanism for ethanol-induced damage (Sun et al., 2001; Chu et al., 2007).

The endoplasmic reticulum (ER) regulates post-translational protein processing and transport. Approximately one-third of all cellular proteins are translocated into the lumen of the ER, where posttranslational modification, folding, and oligomerization occur. The ER is also the site for the biosynthesis of steroids, cholesterol, and other lipids. A number of cellular stress conditions, such as perturbed calcium homeostasis or redox status, elevated secretory protein synthesis rates, altered glycosylation levels, and cholesterol overloading, can interfere with oxidative protein folding. This can subsequently lead to the accumulation of unfolded or misfolded proteins in the ER lumen and activate compensatory mechanism, which has been referred to as ER stress response or unfolded protein response (UPR; Ron, 2002; Xu et al., 2005). In mammals, several ER transmembrane proteins are identified as sensors of ER stress. These include pancreatic ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). PERK phosphorylates the α subunit of the eukaryotic initiation factor 2 (eIF2 α), which attenuates the initiation of translation in response to ER stress. The activation of IRE1 and ATF6 signaling promotes the expression of ER-localized chaperones, such as GRP78 and GRP94, which facilitate the restoration of proper protein folding within the ER, and the proapoptotic transcription factor CHOP (Ron, 2002; Xu et al., 2005). These protective responses result in an overall decrease in translation, enhanced protein degradation, and increased levels of ER chaperones, which consequently increase the protein folding capacity of the ER. However, sustained ER stress ultimately leads to the apoptotic death of the cell (Xu et al., 2005). ER stress has been implicated in various neurodegenerative processes, such as brain ischemia (Tajiri et al., 2004), Alzheimer's disease (AD; Katayama et al., 2004), Parkinson's disease (PD; Chen et al., 2004; Silva et al., 2005; Smith et al., 2005), Huntington's disease (HD; Hirabayashi et al., 2001), and amyotrophic lateral sclerosis (Turner and Atkin, 2006).

With in vitro neuronal models, the current study was designed to test the hypothesis that ethanol may induce ER stress or potentiate ER stress response in the presence of other stressors. Furthermore, we sought to determine whether oxidative stress is involved in the ethanol-induced ER stress response.

MATERIALS AND METHODS

Materials

Tunicamycin, thapsigargin, antiactin antibody, and antioxidant glutathione monoethyl ester (GSH) were purchased from Sigma (St Louis, MO). The antibodies directed against CHOP, GRP78, ATF4, ATF6 (N-terminal), eIF2 α and PERK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antiphospho-eIF2 α was obtained from Biosource International (Camarillo, CA), and phospho-PERK antibody was purchased from Cell Signaling Technology (Beverly, MA). Antioxidant N-acetyl-L-cysteine (NAC) was obtained from EMD Biosciences, Inc. (Darmstadt, Germany).

Cell Culture

Human neuroblastoma SH-SY5Y cells obtained from ATCC were grown in Eagle's MEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 25 µg/ml gentamicin, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C with 5% CO₂. CHOP^{+/+} and CHOP^{-/-} mouse embryonic fibroblasts (MEFs) were obtained from Dr. David Ron (Skirball Institute of Biomolecular Medicine, New York, NY) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and penicillin-streptomycin (100 U/ml-100 µg/ml) at 37°C with 5% CO₂. Cultures of rat cerebellum granule neurons (CGNs) were generated from 7-day-old rat pups as previously described (Chen et al., 2004). CGNs were maintained in Eagle's MEM containing the following supplements: 10% FBS, 25 mM KCl, 1 mM glutamine, 33 mM glucose, and penicillin (100 U/ml)/streptomycin (100 µg/ml). For the analysis of cell survival, 3×10^4 cells/well and 15×10^4 cells/well were plated into 96-well culture trays and 24-well culture trays, respectively. Cells were incubated at 37°C in a humidified environment containing 5% CO₂ for 24 hr. After this incubation period, cells were cultured in a serum-free medium overnight and then exposed to ethanol and other agents.

Ethanol Exposure Protocol

A method utilizing sealed containers was used to maintain ethanol concentrations in the culture medium. With this method, ethanol concentrations in the culture medium can be accurately maintained (Luo et al., 2001). A pharmacologically relevant concentration of 400 mg/dl was used in this study. In general, the concentration for in vitro studies is higher than that required to produce a similar effect in vivo (Luo et al., 2001).

Determination of Cell Number

Cell viability was determined by manual counting under a phase-contrast microscope as previously described (Luo et al., 1997) or by MTT assay as previously described (Chen et al., 2004). The MTT assay is based on the cleavage of yellow tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl tetrazolium bromide] to purple formazan crystals by metabolically active cells. Briefly, the cells cultured in 96-well microtiter plates were exposed to ethanol with/without tunicamycin or thapsigargin for 48 hr. Tunicamycin disrupts protein glycosylation and is the most commonly used pharmacological agent to induce ER stress experimentally (Nakagawa et al., 2000). Thapsigargin perturbs intracellular calcium homeostasis and is another commonly used ER stress inducer (Thastrup et al., 1990). After treatments, 10 µl of MTT labeling reagent was added to each well, and the plates were incubated at 37°C for 4 hr. The cultures were then solubilized, and spectrophotometric absorbance of the samples was detected by a microtiter plate reader. The wavelength to measure absorbance of formazan product is 570 nm, with a reference wavelength of 750 nm.

Immunoblotting

Cells were washed with phosphate-buffered saline (PBS; pH 7.4) and lysed with RIPA buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 1% Nonidet P-40 (NP-40), 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid sodium, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 3% aprotinin] on ice for 10 min; solubilized cells were centrifuged, and the supernatant was collected. The immunoblotting procedure has been previously described (Chen et al., 2004). Briefly, after the protein concentrations were determined, aliquots of the protein samples (20–40 µg) were loaded into the lanes of an SDS-polyacrylamide gel. The protein samples were separated by electrophoresis, and the separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with either 5% BSA or 5% nonfat milk in 0.010 M PBS (pH 7.4) and 0.05%

Tween-20 (TPBS) at room temperature for 1 hr. Subsequently, the membranes were probed with primary antibodies directed against target proteins for 2 hr at room temperature or overnight at 4°C. After three quick washes in TPBS, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL) diluted at 1:2,000 in TPBS for 1 hr. The immune complexes were detected by the enhanced chemiluminescence method (Amersham). In some cases, the blots were stripped and reprobed with an antiactin antibody. The density of immunoblotting was quantified with the software Quantity One (Bio-Rad Laboratories, Hercules, CA). The expression of target proteins was normalized to the levels of actin.

Small Interfering RNA (siRNA) Transfection

CHOP siRNA was used to knock down the expression of CHOP as previously described (Chen et al., 2007). Briefly, CHOP siRNA (catalog Nos. 146320 and 146321; Ambion, Inc., Austin, TX) or Silencer Negative Control siRNA (catalog No. 4611; Ambion, Inc.) was transfected into SH-SY5Y cells by electroporation using Nucleofector (Amaxa Inc., Gaithersburg, MD). The transfection was performed according to the manufacturer's protocol. Forty-eight hours after the transfection, the cells were subjected to immunoblotting analysis for CHOP expression.

Detection of Intracellular ROS

Intracellular ROS levels were measured by using the fluorescent dye carboxy-H₂DCFDA staining method as previously described (Wang et al., 2007). Once incorporated into cells, H₂DCFDA is converted into a nonfluorescent polar derivative (H₂DCF) by cellular esterases. H₂DCF is rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) by intracellular ROS, mainly H₂O₂ (LeBel et al., 1992). Therefore, the intensity of the DCF signal reflects the quantity of intracellular ROS. SH-SY5Y cells were treated with ethanol and/or tunicamycin in the presence or absence of antioxidants for the indicated times. After treatment, cells were transferred to luminometer cuvettes containing 10 μM carboxy-H₂DCFDA (C400; Molecular Probes, Eugene, OR) in pre-warmed PBS for 30 min. Intracellular ROS levels (DCF signals) were measured with a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA) at an emission wavelength of 525 nm.

Statistical Analysis

Differences among treatment groups were tested by analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant. In cases in which significant differences were detected, specific post hoc comparisons between treatment groups were examined by Student-Newman-Keuls tests.

RESULTS

Ethanol Enhances Tunicamycin- and Thapsigargin-Induced ER Stress and Neuronal Death

First, we sought to determine whether ethanol induces ER stress in human neuroblastoma SH-SY5Y cells. We examined the effect of ethanol (400 mg/dl) on the expression of various markers of ER stress, including CHOP (GADD153), ATF4, ATF6, GRP78, and phosphorylated PERK and eIF2 α . As shown in Figure 1, ethanol (400 mg/dl) had little effect on the expression of these ER stress markers. Although exposure to ethanol alone did not initiate ER stress, it significantly potentiated tunicamycin-induced ER stress; ethanol synergized the effect of tunicamycin on the expression of ER stress markers in SH-SY5Y cells (Fig. 2A). Ethanol also enhanced thapsigargin-induced ER stress (Fig. 2B). A similar observation was obtained from primary cultures of cerebellar granule neurons (CGNs). Ethanol had a modest effect on the expression of ER stress markers (data not shown); it

potentiated ER stress caused by the treatment of tunicamycin or thapsigargin in CGNs (Fig. 2C,D). Consistent with its effect on ER stress, ethanol at 400 mg/dl did not affect the survival of SH-SY5Y cells and CGNs under this culture condition but potentiated tunicamycin- and thapsigargin-induced cell death (Fig. 3). Thus, ethanol worked in synergy with tunicamycin or thapsigargin to promote ER stress and enhance cell death.

Oxidative Stress Is Involved in Ethanol's Potentiation of ER Stress

Ethanol induced a rapid increase in intracellular reactive oxygen species (ROS) levels; a significant increase was observed 30 min after ethanol exposure, and then a gradual increase occurred between 2 and 6 hr (Fig. 4A). Tunicamycin treatment neither stimulated ROS nor enhanced ethanol-induced production of ROS (Fig. 4B), indicating that ROS resulted primarily from ethanol treatment. Antioxidants N-acetyl-L-cysteine (NAC) or glutathione monoethyl ester (GSH) scavenged ethanol-induced production of ROS (Fig. 4B). These antioxidants eliminated ethanol-promoted expression of ER stress markers (Fig. 2). Consistently, these antioxidants blocked ethanol-enhanced cell death, but had little effect on tunicamycin-mediated cell death (Fig. 3). The results indicated that ROS played a critical role in mediating ethanol's action.

CHOP Mediates the Effect of Ethanol on Cell Survival

CHOP is a 29-kDa leucine zipper transcription factor that is ubiquitously expressed at a low level and robustly up-regulated in response to various stress conditions (Oyadomari and Mori, 2004). CHOP is proapoptotic and a key mediator of ER stress-induced cell death (Oyadomari and Mori, 2004; Szegezdi et al., 2006). Because the expression of CHOP was drastically up-regulated by ER stress inducers/ethanol, we sought to determine whether CHOP mediated ethanol-promoted cell death. We first examined the effect of ethanol and/or tunicamycin on CHOP knockout mouse embryonic fibroblasts (CHOP^{-/-} MEFs). Compared with wild-type CHOP^{+/+} MEFs, CHOP^{-/-} MEFs were more resistant to tunicamycin and ethanol-induced cell death (Fig. 5). Antioxidants blocked ethanol's potentiation of tunicamycin-induced death of CHOP^{+/+} MEFs (Fig. 5), verifying that ROS played an important role in ethanol's action. Using CHOP siRNA, we further confirmed the function of CHOP. As shown in Figure 6A, the CHOP siRNAs effectively knocked down the expression of CHOP in SH-SY5Y cells. Treatment of CHOP siRNA significantly reduced tunicamycin-induced cell death and eliminated ethanol's potentiation of cell death (Fig. 6B).

DISCUSSION

Several mechanisms have been proposed for ethanol-induced neuronal loss; these include: 1) inducing oxidative stress; 2) disrupting neurotrophic signaling and intracellular calcium homeostasis; 3) inhibiting the proliferation of neuronal precursors; and 4) altering neurotransmitter systems (Luo et al., 1997; Webb et al., 1997; Luo and Miller, 1998; Catlin et al., 1999; Ikonomidou et al., 2000; Goodlett and Horn, 2001; de la Monte and Wands, 2002; Davies, 2003; Kumral et al., 2005). The action of alcohol is complex. There is considerable interaction among these mechanisms.

Oxidative stress is caused by disrupted intracellular redox homeostasis. ROS induced under oxidative stress produce profound damages to the CNS, including apoptosis of neurons (Mattson et al., 2001; Watts et al., 2005; Loh et al., 2006). Because of its high metabolic rate, exposure to large amounts of oxygen, and low levels of the antioxidant enzyme catalyst, the brain is believed to be particularly susceptible to the damaging affects of ROS (Annunziato et al., 2003). Ethanol has been demonstrated to produce oxidative stress in the brain and neurons, and some damages are believed to be mediated by ROS production

(Bondy, 1992; Sun et al., 2001; Marino et al., 2004; Kumral et al., 2005). ER stress is caused by the accumulation of misfolded proteins and alterations in calcium homeostasis (Ron, 2002; Xu et al., 2005). There is considerable interaction between oxidative stress and ER stress (Gorlach et al., 2006). Although this is not entirely consistent, it has been shown that ROS can trigger ER stress response (Hayashi et al., 2003; Xue et al., 2005). Ethanol exposure disrupts intracellular calcium homeostasis in neurons (Webb et al., 1997), a condition that may elicit ER stress. It has been reported that ethanol causes ER stress in hepatocytes (Kaplowitz and Ji, 2006). Our results indicate that ethanol alone does not induce the expression of ER stress markers in cultured neuronal cells; however, it potentiates ER stress caused by the treatment of tunicamycin and thapsigargin.

Ethanol at 400 mg/dl causes oxidative stress but not ER stress in cultured SH-SY5Y cells and cerebellar granule neurons (Figs. 1, 4). This ethanol concentration has been used to investigate various effects of ethanol in vitro (Luo et al., 1997; Webb et al., 1997; Li et al., 2004; Ma et al., 2005). The results suggest that ethanol-induced ROS is not sufficient to trigger ER stress under this culture condition. It is noted that the phosphorylation of eIF2 α is consistently enhanced by ethanol (Fig. 1). In addition to ER-associated PERK, double stranded-RNA-activated protein kinase (PKR), heme-regulated inhibitor (HRI), and the GCN2 protein kinase participate in the regulation of eIF2 α phosphorylation (Williams, 2001; Ron, 2002). Among them, PERK and PKR respond to ER stress (Ron, 2002; Onuki et al., 2004). We have recently demonstrated that ethanol-induced PKR activation is mediated by promoting the interaction between PKR and RAX, a protein activator of PKR (Chen et al., 2006). Therefore, the increase in p-eIF2 α may result from ethanol-induced activation of PKR. Ethanol-stimulated ROS production precedes ER stress response. Antioxidants eliminate ethanol-mediated potentiation of ER stress. It has been shown that ROS may trigger the ER stress response (Hayashi et al., 2003; Xue et al., 2005). Taken together, although ethanol-stimulated ROS is not sufficient to cause ER stress response, it synergizes with ER stress inducers and produces much greater neuronal loss. The results suggest that stressed neurons are more susceptible to ethanol exposure.

The expression of CHOP is regulated mainly at the transcriptional level; three ER stress-responsive transcription factors, ATF4, ATF6, and x-box binding protein 1 (XBP1), participate in the regulation of CHOP (Okada et al., 2002). Our previous study indicates that tunicamycin does not activate XBP1 in SH-SY5Y cells (Chen et al., 2007). Ethanol enhances tunicamycin- and thapsigargin-induced expression of ATF4 and ATF6, suggesting that up-regulation of CHOP may be mediated by the activation of ATF4 and ATF6. CHOP is an important mediator of ER stress-induced cell death (Szegezdi et al., 2006). The mechanisms for CHOP regulation of cell death are not completely understood. Induction of CHOP is reported to perturb the cellular redox state by the depletion of cellular glutathione (McCullough et al., 2001). On the other hand, overexpression of CHOP leads to a decrease in antiapoptotic Bcl-2 protein and overexpression of Bcl-2 counteracts CHOP-induced apoptosis (McCullough et al., 2001). Overexpression of CHOP is also shown to induce translocation of proapoptotic Bax protein from the cytosol to the mitochondria (Gotoh et al., 2004). Thus, the CHOP-mediated death signal seems to be transmitted to the mitochondria, which functions as an integrator and amplifier of the death pathway. Recently, death receptor 5 (DR5) has been identified as a CHOP-responsive gene; the transcription of DR5 is directly regulated by CHOP (Yamaguchi and Wang, 2004). DR5 is a proapoptotic cell-surface death receptor; its overexpression sensitizes cells to apoptotic death (Yamaguchi and Wang, 2004). Regardless of how CHOP regulates cell death, our results showing that CHOP partially mediates ethanol-induced neuronal loss provide a potential therapeutic target for the treatment of neurotoxicity.

Abbreviations

ATF6	activating transcriptional factor 6
CHOP	C/EBP homologous protein
FAS	fetal alcohol syndrome
eIF2α	eukaryotic translation initiation factor 2 alpha
ER	endoplasmic reticulum
GRP78	glucose-regulated protein of 78 kDa
IRE1	inositol requiring enzyme 1
PKR	double-stranded RNA-activated protein kinase
PERK	pancreatic endoplasmic reticulum kinase
ROS	reactive oxygen species
UPR	unfolded protein response
XBP1	x-box binding protein 1

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Fig. 1.

Effect of ethanol on ER stress. SH-SY5Y cells were cultured in serum-free medium and treated with ethanol (Et; 0 or 400 mg/dl) for specified times. Cell lysates were collected, and the expression of markers for ER stress was examined with immunoblotting. To ensure equal loading, the blots were stripped and reprobed with an antiactin antibody. Tunicamycin was used as a positive control for inducing ER stress. The experiment was replicated three times.

Fig. 2.

Effect of ethanol on tunicamycin- and thapsigargin-induced ER stress. **A:** SH-SY5Y cells were pretreated with ethanol (Et; 0 or 400 mg/dl) with/without antioxidant NAC (10 mM) for 12 hr and then treated with tunicamycin (Tun; 0 or 3 μ g/ml) for specified times. Cell lysates were collected, and the expression of markers for ER stress was examined by immunoblotting. The blots were stripped and reprobed with an antiactin antibody. **B:** The notations are the same as in A except that cells were treated with thapsigargin (Tha; 0 or 1 μ M). **C:** Primary cultures of cerebellar granule neurons were pretreated with ethanol (0 or 400 mg/dl) for 12 hr and then exposed to tunicamycin (Tun; 0 or 3 μ g/ml) for specified times. The expression of ER stress markers was examined as described above. **D:** The

notations are the same as in C except that cells were treated with thapsigargin (Tha; 0 or 1 μ M). The experiment was replicated three times.

Fig. 3.

Effect of ethanol on tunicamycin- and thapsigargin-induced cell death. **A:** SH-SY5Y cells were pretreated with ethanol (Et; 0 or 400 mg/dl) with/without antioxidants (10 mM NAC or 5 mM GSH) for 12 hr and then exposed to tunicamycin (Tun; 0 or 3 μ g/ml) for 48 hr. The number of viable cells was determined by counting under the microscope and expressed as a percentage of the untreated control. Each data point ($\pm$ SEM; bar) is the mean of three experiments. **B:** The notations are the same as in A except that cells were treated with thapsigargin (Tha; 0 or 1 μ M). **C:** Primary cultures of cerebellar granule neurons were pretreated with ethanol (0 or 400 mg/dl) and/or NAC for 12 hr, then exposed to tunicamycin (Tun; 0 or 3 μ g/ml) for 48 hr. The number of viable cells was examined as described above. **D:** The notations are the same as in C except that cells were treated with thapsigargin (Tha; 0 or 1 μ M). *Significant difference from Tun- or Tha-treated groups.

Fig. 4.

Effect of ethanol on intracellular ROS concentrations. **A:** SH-SY5Y cells were exposed to ethanol (Et; 0 or 400 mg/dl) for specified times, the intracellular ROS concentrations were detected by flow cytometry as described in Materials and Methods. Each data point ($\pm$ SEM; bar) is the mean of three experiments. **B:** SH-SY5Y cells were exposed to ethanol (0 or 400 mg/dl) with/without NAC (10 mM) for 2 hr, then treated with tunicamycin (Tun; 0 or 3 μ g/ml) for 6 hr. The intracellular ROS concentrations were detected by flow cytometry.

*Significant difference from Ct or Tun-treated groups.

Fig. 5.

Effect of ethanol and tunicamycin on the survival of CHOP null mouse embryonic fibroblasts (CHOP^{-/-} MEFs). CHOP^{-/-} and CHOP^{+/+} MEFs were pretreated with ethanol (Et; 0 or 400 mg/dl) with/without NAC (10 mM) for 12 hr, then exposed to tunicamycin (Tun; 0 or 3 μ g/ml) for 48 hr. Cell viability was determined by MTT as described in Materials and Methods. The number of viable cells was expressed as a percentage of untreated controls. Each data point ($\pm$ SEM; bar) is the mean of three experiments.

*Significant difference from paired groups of CHOP^{+/+} MEFs.

Fig. 6. Effect of CHOP siRNA on ethanol- and tunicamycin-mediated cell death. **A:** SH-SY5Y cells were transfected with either CHOP siRNAs (320 and 321) or control siRNA (Ct-siRNA) for 24 hr. After that, cells were exposed to tunicamycin (Tun; 0 or 3 μ g/ml) for 6 hr. The expression of CHOP was examined with immunoblotting. **B:** After transfection with CHOP siRNA or a control siRNA (Ct-siRNA) for 48 hr, cells were pretreated with ethanol (0 or 400 mg/dl) with/without NAC (10 mM) for 12 hr and then exposed to tunicamycin (Tun; 0 or 3 μ g/ml) for 48 hr. Cell number was determined by counting under the microscope and expressed as a percentage of the untreated control. Each data point ($\pm$ SEM; bar) is the mean of three experiments. *Significant difference from paired untreated groups or groups treated with Ct-siRNA.