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Original

Ethanol-induced Stress Leads to Apoptosis Via Endoplasmic Reticulum Stress in SK-Hep1 Cells

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Abstract: Alcoholic liver disease causes oxidative stress and induces apoptosis during alcohol metabolism. Ethanol causes endoplasmic reticulum (ER) stress in hepatocytes, stimulating the unfolded protein response (UPR) pathway and / or Ca^{2+}-dependent calpain and caspase-4 activities. However, it is poorly understood whether ethanol-induced oxidative stress directly leads to apoptosis promoted by ER stress-associated pathways. This study investigated this question in human liver adenocarcinoma (SK-Hep1) cells, which were treated with 200 mM ethanol for 5 hours in the presence or absence of the antioxidant N-acetyl-cysteine (NAC). We found that treatment with ethanol significantly increased ROS production and cellular apoptosis in the SK-Hep1 cells, and that this response was significantly suppressed by pretreatment with NAC. Furthermore, pretreatment with NAC significantly reduced the observed increases in the mRNA expressions of Bip, Chop, and sXbp-1, and the activity of caspase-3 in ethanol-induced apoptotic cells. However, pretreatment with NAC did not attenuate the transient rise in cytosolic Ca^{2+} nor the activities of caspase-4 and calpain induced by ethanol. Together, these results revealed that ethanol-induced stress promotes apoptosis not only through mitochondria-mediated pathways, but also via ER stress. The findings further suggested that ethanol-induced oxidative stress and non-oxidative stress both stimulate the pathway regulating ER stress-mediated apoptosis.

Key words: ethanol, apoptosis, oxidative stress, ER stress, SK-Hep1 cells

Introduction

Excessive alcohol intake is a known cause of hepatic disorders such as fatty liver, alcoholic hepatitis, hepatic fibrosis, cirrhoses, and liver cancer[1)]. An underlying mechanism for this damaging effect involves the production of reactive oxygen species (ROS) during alcohol metabolism. Such oxidative stress in turn induces apoptosis in hepatocytes. There are several pathways implicated in ethanol-induced apoptosis such as mitochondrial and

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Fas death receptor signaling. Glutathione (GSH) levels have also been associated with caspase-8 activation in ROS-related apoptotic cell death. Immediate depletion of GSH inhibited caspase-8 activation, whereas prolonged GSH depletion induced apoptotic cell death due to suppressed anti-apoptotic actions[2].

The endoplasmic reticulum (ER) stress pathway is also affected by oxidative stress[3]. Although ER stress is a cell protection system[4], the resulting apoptosis may contribute to neurodegenerative disease[5], diabetic[6], cancer[7], and cardiovascular disease[8]. However, the relationship between ER stress and ethanol-induced oxidative stress is not known.

The ER is an essential organelle for the production and post-translational modifications of secretory and membrane proteins[9]. The ER is also a storage site for Ca^{2+}, which can be released in a controlled fashion to propagate cellular signal transduction. Nascent proteins are folded with the assistance of molecular chaperones and folding enzymes in the ER. It has been estimated that as many as 30% of nascent proteins are unfolded or misfolded, retained in the ER, retrotranslocated to the cytoplasm via the ER-associated degradation (ERAD) mechanism, and then rapidly degraded by ubiquitination[9]. Accumulation of unfolded proteins occurs when there is an increase in the translation of secretory protein that exceeds the capacity of the folding apparatus and ERAD system or when there are perturbations in the ER environment, such as alterations in redox state or Ca^{2+} levels. To cope with ER stress, a series of signaling pathways referred to as the ER stress response or unfolded protein response (UPR) is activated. Accumulation of unfolded or misfolded proteins in sensed by three main resident transmembrane sensors in the ER: (1) inositol-requiring enzyme 1 (IRE-1α), (2) activating transcription factor 6 (ATF-6), and (3) RNA-activated protein kinase (PKR)-like ER kinase (PERK). These sensors are normally held in an inactive state by the binding of intraluminal ER chaperones, in particular glucose-regulated protein78 (BIP). PERK phosphorylates the α subunit of the eukaryotic initiation factor 2 (eIF2α), which attenuates the initiation of translation and selectively increases translation of mRNA such as ATF-4, and this upregulates chaperones in response to ER stress. Upregulated ATF-4 increases the expression of transcription factor C/EBP-homologous protein (Chop). CHOP is of particular interest to this study as its increased expression, along with GRP78, is a hallmark of the ER stress response. IRE-1α functions as a nuclease to splice X box-binding protein 1 (sXBP-1) mRNA. The resultant sXBP-1 activates transcription through UPR elements in gene promotors controling ERAD. ATF-6 translocates to the nucleus where it interacts with ER stress response element (ERSE) to upregulate chaperones such as BIP, GRP94, and IRE-1α. Severe or prolonged ER stress increases expression of CHOP and induces apoptosis[10].

In addition, Ca^{2+}-dependent activation of calpain has been associated with ER-induced apoptosis[11]. Calpain is a calcium-dependent intracellular cysteine protease active at the cell membrane, which activates caspase-3 through caspase-4 and induces apoptosis.

This study sought to investigate this complex association between ethanol-induced oxi-

dative stress and ER stress in alcohol dehydrogenase (ADH)-containing human hepatoma cells (SK-Hep1 cells), with a focus on the UPR mechanism and Ca^{2+}-dependent activation of calpain pathway.

Materials and Methods

Materials

Ethanol, N-acetyl-L-cysteine (NAC), and Eagle's Minimum Essential Medium (E-MEM) were purchased from Wako (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Sigma-Aldrich (St.Louis, MO, USA). Penicillin G sodium, streptomycin sulfate, and amphotericin B were obtained from Invitrogen (Carlsbad, CA, USA), while 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) mixed isomers were purchased from Molecular Probes (Eugene, OR, USA). All other chemicals used in this experiment were of the purest grade commercially available.

Cell culture and drug treatment

SK-Hep1 cells (human liver adenocarcinoma, EC-91091816) were purchased from the European Collection of Cell Cultures (Salisbury, UK). SK-Hep1 cells were cultured in E-MEM containing 10% FBS and maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37℃. For studies on the effects of ethanol, SK-Hep1 cells were incubated in E-MEM with or without (control) ethanol (200 mM) for 5 hours. When we treated with 50–300 mM ethanol in preliminary experiments, 200 mM ethanol was a suitable concentration for inducing apoptosis (data not shown). Furthermore, acute alcohol intoxication in children appears at 10–100 mM of blood ethanol[11]. For studies under antioxidant, SK-Hep1 cells were pretreated with a NAC for 1 hour, followed by cotreatment with 200 mM ethanol and 10 mM NAC. All treatments were carried out under sterile conditions.

Detection of reactive oxygen species (ROS)

Diol groups can be oxidized by ROS to a fluorescent form, and diol-containing dyes have proved to be excellent probes for assaying ROS production[12]. SK-Hep1 cells were seeded in 96-well plates at 1×10^5 cells / ml and treated with 200 mM ethanol and 10 mM NAC for 5 hours. Free radical production was measured by incubating SK-Hep1 cells with CM-H_2DCFDA, which is a stable non-fluorescent molecule that passively diffuses into cells, where the acetate is acted on by intracellular esterases to produce a polar diol that is well retained within the cells. Redox state of cells was determined by exciting CM-H_2DCFDA-loaded cells at 488 nm and detecting emitted fluorescence at 530 nm using an LB 970T Fluorometer (Berthold Technologies GmbH & Co, KG, Bad Wildbad, Germany).

Detection of apoptosis

Single-stranded DNA (ssDNA)

Table 1. Sequences of forward and reverse oligonucleotides used for cDNA amplification, size of the PCR product, and the Genebank accession number for each cDNA.

	Sense	Antisense	PCR	Genebank
β-actin	5'-TCGTCACCAACTGGGACGACATGG-3'	5'-GATCTTGATCTTCATTGTGCTGGG-3'	764bp	X70351
Bip	5'-CGTGTTCAAGAACGGCCG-3	5'-CGTAGACAGTACGACAGCAACTGT-3	404bp	M19645
Chop	5'-GGCAGCTGAGTCATTGCC-3'	5'-GCAGATTCACCATTCGGTCA-3'	496bp	X71427
sXbp-1	5'-CCGCTCATGGTGCCAGCC-3'	5'-CACCTGCTGCGGACTCAG-3'	394bp	NM1079539

The presence of ssDNA provides specific evidence of the apoptotic process, and was determined in this study using a formamide-monoclonal antibody (mAb) against ssDNA as described in our previous report[13]. Formamide selectively denatures DNA in apoptotic cells, but not in necrotic cells nor in cells with DNA breaks in the absence of apoptosis[14].

Annexin V and Hoechst staining

SK-Hep1 cells cultured in 96-well plates and treated with 200 mM ethanol and 10 mM NAC for 5 hours were stained with a DNA dye, Hoechst33342 (Wako, Osaka, Japan) to visualize nuclear morphology. Stained cells were then washed in PBS, and specific binding of annexin V-cy3 (Annexin V-cy3 Apoptosis Detection Kit ; Medical & Biological Laboratories, Nagoya, Japan) was carried out by incubation of the cells for 5 min at room temperature in binding buffer containing annexin V. The staining was analyzed using Meta Xpress Image Acquisition (Molecular Devices, Tokyo, Japan).

Semiquantitative RT-PCR analysis

To measure mRNA expressions of Bip, sXbp1, Chop, and β-actin, SK-Hep1 cells were treated with 200 mM ethanol and 10 mM NAC for 5 hours, and then subjected to total RNA extraction using the QIAamp RNAmini kit (QIAGEN K.K., Tokyo, Japan). Reverse-transcription polymerase chain reaction (RT-PCR) analysis was carried out using the Omniscript RT Kit (QIAGEN) according to the manufacturer's procedure. The sequences of the primers used for amplification are shown in Table 1.

Amplification by PCR for Bip (42 cycles), Chop (38 cycles), and sXbp1 (38 cycles) was performed (1 cycle comprised of 95℃ for 30 sec, 63℃ for 30 sec, and 72℃ for 30 sec), followed by a final extension at 72℃ for 7 min. The reaction (30 cycles) for β-actin comprised 94℃ for 30 sec to 60℃ for 30 sec followed by 72℃ for 1 min for each cycle. The PCR products were electrophoresed on 2% agarose gels and visualized by ultraviolet transillumination (Atto Corp, Tokyo, Japan). Bands were quantified by densitometry using a scanner employing Scion Image Version 4.02 software. The ratio of target cDNA to β-actin was used as a relative estimate of mRNA abundance.

Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) measurements by Meta Xpress Image Acquisition

Levels of $[Ca^{2+}]_i$ were analyzed using a calcium-sensitive fluorescence Fura-2 AM dye[15]. SK-Hep-1 cells were loaded with 3 μM Fura-2-AM diluted in Hepes-Tyrode buffer / 0.1% BSA [140 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.6 mM D-glucose, 0.49 mM $MgCl_2$, 0.37 mM NaH_2PO_4, 25 mM Hepes / NaOH (pH 7.4), 0.1% BSA] for 40 min at 37℃. After incubation, the cells were washed with Hepes-Tyrode buffer / 0.1% BSA. Changes in the $[Ca^{2+}]_i$ were measured by monitoring the ratio of the fluorescence signals of Fura-2 AM with the Meta Xpress Image Acquisition.

Measurement of caspase-3, -4, and calpain activities

SK-Hep1 cells were treated with 200 mM ethanol and 10 mM NAC for 5 hours. The medium was then discarded and adherent cells were harvested in PBS and sedimented by centrifugation at 500 × g. The pellets were resuspended in chilled cell lysis buffer (Medical & Biological Laboratories, Aichi, Japan), incubated for 10 min on ice, and then centrifuged for 3 min at 10,000 × g. The supernatants were added to reaction buffer containing 10 μM DTT (Medical & Biological Laboratories) and each peptide substrate and incubated at 37℃ for 2 hours. Substrates for caspase-3 and caspase-4 (Kamiya Biochemical Company, Seattle, WA, USA) were Asp-Glu-Val-Asp-7-amino-4-trifluoromethy coumain (AFC) and Leu-Glu-Ala-Asp-AFC, respectively. Calpain activity was determined in accordance with standard techniques using a commercially available Calpain Activity Assay Kit (Biovision, Mountain View, CA, USA). The substrate for calpain was Leu-Leu-Try-AFC. AFC released by the respective enzyme reactions was measured spectrofluorometrically with an excitation wavelength of 405 nm and an emission wavelength of 505 nm using the LB 970T Fluorometer.

Statistical analysis

Results are expressed as mean±standard error of the mean (S.E.M). Comparison of the effects of various treatments with those of the untreated normal cells was performed with one-way Dunnetts' as the post hoc test. Differences with *P*-values of less than 0.05 were considered statistically significant.

Results

The influence of ethanol on ROS production

To investigate the effect of ethanol treatment on hydrogen peroxide production, we used CM-H_2DCFDA, a ROS-sensitive dye. DCF fluorescence as an indicator of ROS formation is shown in Fig. 1. Treatment with 200 mM ethanol for 5 hours significantly increased the DCF fluorescence compared with that of normal cells. ROS production in hepatocytes treated with ethanol (50–300 mM) increased dose dependently compared with normal cells (data not shown). However, the increase in DCF fluorescence upon exposure to 200 mM ethanol was significantly eliminated by pretreatment with 10 mM NAC.

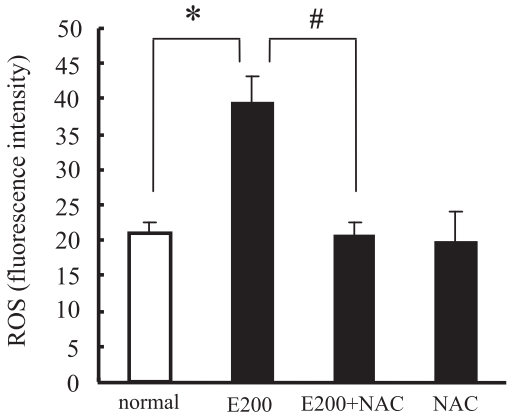

Fig. 1. Effect of ethanol on ROS generation in SK-Hep1 cells. Generation of ROS in SK-Hep1 cells treated with 200 mM ethanol or 10 mM NAC＋200 mM ethanol was analyzed spectrofluorometrically. Each value represents the mean ± s.e.m. of 6–12 samples. Significant statistical differences are shown for comparison with normal ($^{*}P < 0.05$) and comparison with treatment of ethanol ($^{\#}P < 0.05$).

Detection of ethanol-induced apoptosis

Presence of ssDNA was used to distinguish between apoptosis and necrosis in our treated cells. Primary monoclonal antibodies against ssDNA and HRP-labeled anti-mouse IgM were used to detect the formamide-denatured DNA. ssDNA levels in hepatocytes treated with ethanol (50–300 mM) was increased dose dependently compared with normal cells (data not shown). Fig. 2 (A) shows that ethanol-induced apoptotic cells generated significantly higher levels of ssDNA after 5 hours of incubation compared with normal cells, indicating that treatment with 200 mM ethanol induced apoptosis. Pretreatment of ethanol-induced apoptotic cells with NAC inhibited the increased ssDNA levels after 5 hours of incubation compared to cells not pretreated with NAC.

We further determined whether ethanol induces apoptosis in SK-Hep1 cells by annexinV-cy3 staining, and nuclear staining with Hoechst33342. In cells undergoing apoptosis, phosphatidylserine (PS) is exposed at the outer leaflet of the plasma membrane, and specific binding to PS by annexin V allows discrimination between viable and apoptotic cells[16]. The MetaXpress Image Acquisition analysis revealed a significantly increased proportion of apoptotic cells in 200 mM ethanol treated-SK-Hep1 cultures at 5 hours [Fig. 2 (B), (C)]. Pretreatment of the ethanol-induced apoptotic cells with NAC suppressed the ethanol-induced increase in apoptosis. This finding implicated increased cellular oxidative stress as the likely cause of ethanol-induced apoptosis in our cells.

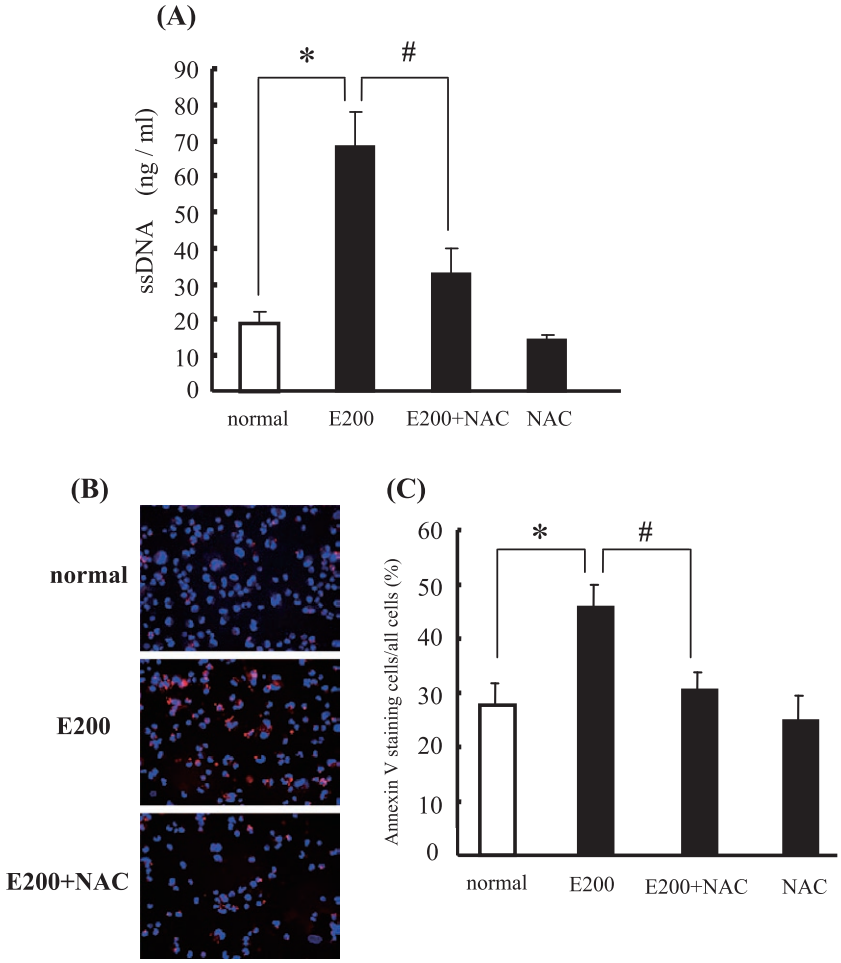

Fig. 2. Effects of ethanol on ssDNA and Annexin V staining in SK-Hep1 cells. SK-Hep1 cells (1×10^5 cells / ml) were incubated in E-MEM with 200 mM ethanol or 200 mM ethanol + 10 mM NAC for 5 hours at 37°C. (A) ssDNA assay. Formamide-denaturable DNA was detected in apoptotic cells. (B, C) Annexin V staining. After treatment with 200 mM ethanol for 5 hours, SK-Hep1 cells were stained with anti-annexin V-Cy3 and Hoechst33342 followed by MetaXpress Image Acquisition analysis. (C) The results of analysis revealed the proportion of apoptotic cells. Each value represents the mean±s.e.m. of 6–12 samples. For (A) and (C), significant statistical differences are shown for comparison with normal ($^*P < 0.05$) and comparison with treatment of ethanol ($^\#P < 0.05$).

Semiquantitative RT-PCR analysis

Fig. 3 (A)-(C) represent the mRNA expression analysis for Bip, Chop, and sXbp-1 as a ratio of target mRNA / β-actin mRNA in treated SK-Hep1 cells. The amount of target mRNA and β-actin expression increased with cycle numbers of PCR, and PCR products were semiquantified during the reaction by measuring the fluorescence intensity. The Bip and Chop mRNA expressions in SK-Hep1 cells treated with 200 mM ethanol for 5 hours

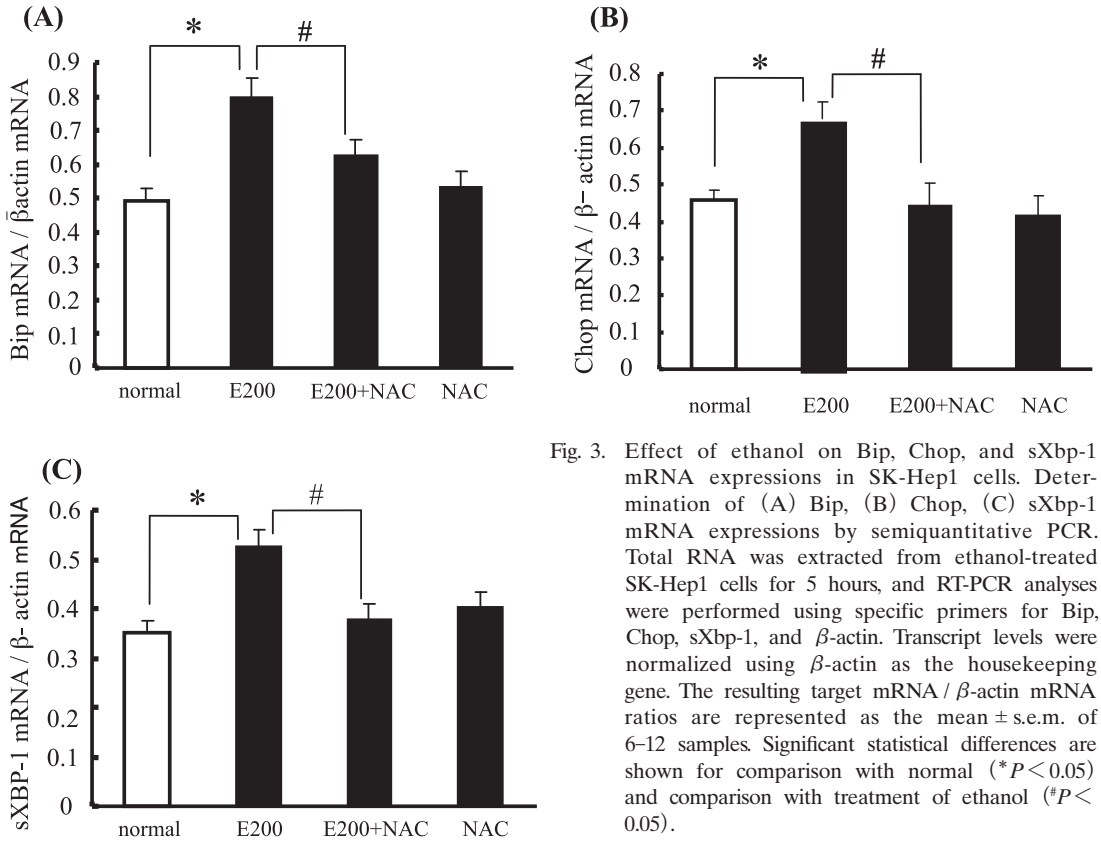

Fig. 3. Effect of ethanol on Bip, Chop, and sXbp-1 mRNA expressions in SK-Hep1 cells. Determination of (A) Bip, (B) Chop, (C) sXbp-1 mRNA expressions by semiquantitative PCR. Total RNA was extracted from ethanol-treated SK-Hep1 cells for 5 hours, and RT-PCR analyses were performed using specific primers for Bip, Chop, sXbp-1, and β-actin. Transcript levels were normalized using β-actin as the housekeeping gene. The resulting target mRNA / β-actin mRNA ratios are represented as the mean ± s.e.m. of 6–12 samples. Significant statistical differences are shown for comparison with normal ($^{*}P < 0.05$) and comparison with treatment of ethanol ($^{\#}P < 0.05$).

increased significantly to 138.8% and 187.9%, respectively, relative to untreated control cells. Pretreatment with NAC prevented these ethanol-induced increases in Bip and Chop mRNA expressions. The ethanol treatment also significantly increased the mRNA expression of sXbp1, compared with normal cells [Fig. 3 (C)], and similarly, this effect was prevented by pretreatment with NAC, relative to apoptotic cells incubated with ethanol alone.

Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) alteration following ethanol exposure

We next investigated the effect of treatment with 200 mM ethanol and 200 mM ethanol +10 mM NAC on $[Ca^{2+}]_i$ homeostasis by MetaXpress Images Acquisition [Fig. 4 (A), (B)]. $[Ca^{2+}]_i$ levels in SK-Hep1 cells treated with 200 mM ethanol rose quickly and transiently within 3 sec, then returned to basal levels within 7 sec, and pretreatment with NAC had no influence on the elevated $[Ca^{2+}]_i$ levels.

Measurement of caspase-3, -4, and calpain activities

Caspases are critical mediators of apoptosis in SK-Hep1 cells; thus, we next measured several caspase activities using synthetic fluorometric substrates [Fig. 5 (A), (B)]. After

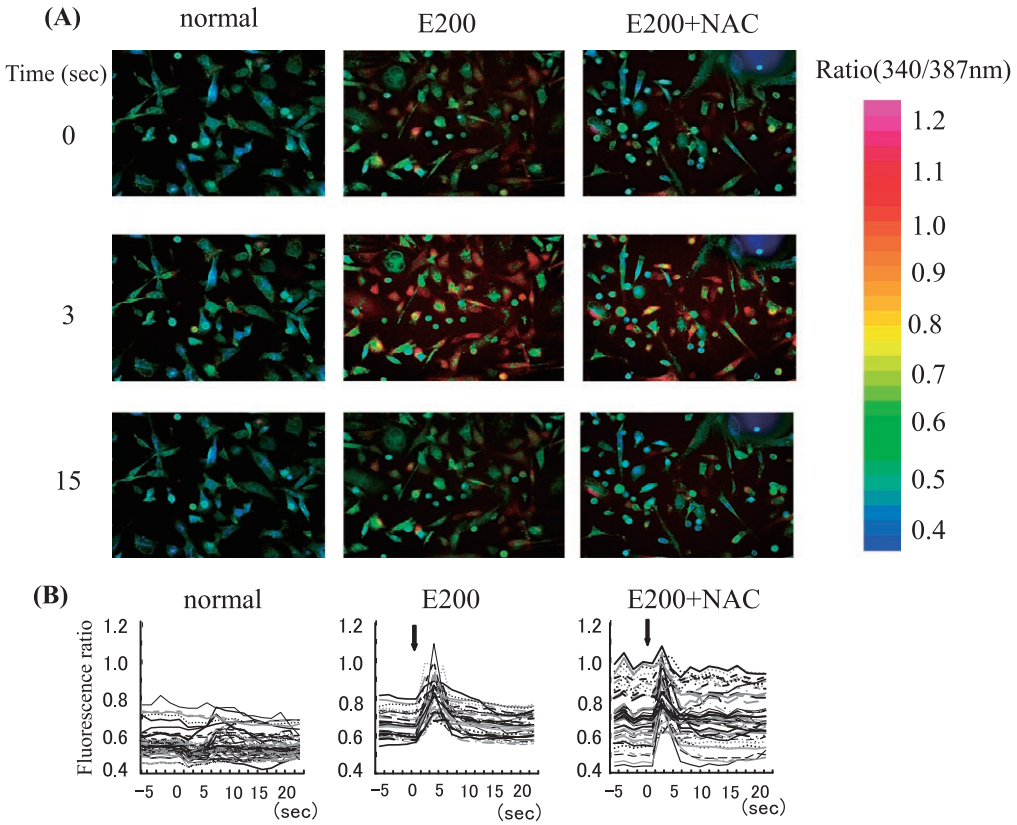

Fig. 4. Effect of ethanol on $[Ca^{2+}]_i$ levels in SK-Hep1 cells. (A) $[Ca^{2+}]_i$ levels of SK-Hep1 cells treated with 200 mM ethanol in the presence or absence of 10 mM NAC were analyzed by MetaXpress Image Acquisition. Cells were loaded with Fura-2 AM as described in the methods. (B) Alterations to $[Ca^{2+}]_i$ levels in SK-Hep1 cells treated with 200 mM ethanol or 200 mM ethanol + 10 mM NAC and in normal cells were measured. Ethanol was added to the SK-Hep1 cells at the times indicated by the arrows.

incubation with 200 mM ethanol for 5 hours, caspase-3 and caspase-4 activities significantly increased compared with those in normal cells. Pretreatment with NAC prevented the increase in caspase-3 activity, but not in caspase-4 activity.

Calpain is also activated by elevated $[Ca^{2+}]_i$ levels and ER stress. Treatment with ethanol for 5 hours significantly increased calpain activity in the SK-Hep1 cells compared with untreated cells [Fig. 5 (C)]. However, pretreatment with the NAC antioxidant had no influence on the increase in calpain activity. These results suggested that the increases in $[Ca^{2+}]_i$ levels as well as calpain and caspase-4 activities in ethanol-treated hepatocytes were not caused by the production of oxidative stress.

Discussion

Alcoholic liver disease (ALD) is the most common hepatic disease in Western countries.

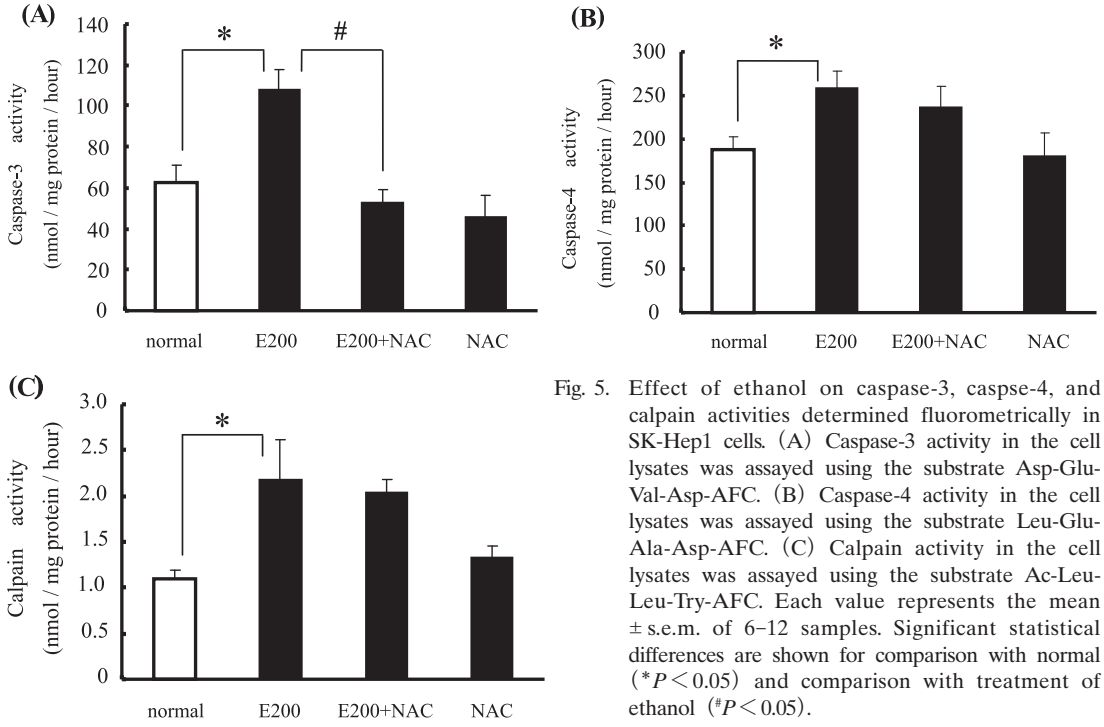

Fig. 5. Effect of ethanol on caspase-3, caspse-4, and calpain activities determined fluorometrically in SK-Hep1 cells. (A) Caspase-3 activity in the cell lysates was assayed using the substrate Asp-Glu-Val-Asp-AFC. (B) Caspase-4 activity in the cell lysates was assayed using the substrate Leu-Glu-Ala-Asp-AFC. (C) Calpain activity in the cell lysates was assayed using the substrate Ac-Leu-Leu-Try-AFC. Each value represents the mean ± s.e.m. of 6–12 samples. Significant statistical differences are shown for comparison with normal ($^{*}P < 0.05$) and comparison with treatment of ethanol ($^{\#}P < 0.05$).

Chronic alcohol consumption causes liver damage leading to steatosis, alcoholic hepatitis, fibrosis, and cirrhosis, as well as the development of hepatocellular carcinoma in susceptible individuals[17, 18]. Acute liver injury induced by ethanol is also drawing increased attention because the incidence of acute alcoholism or binge drinking is on the rise worldwide[19]. ALD has wide-ranging biological effects including on the cellular redox state, oxidative stress, cytokine milieu and signaling, immune responses, and polymorphisms in the genes encoding SOD2, CD14 endotoxin receptor, TNF-α, TGF-α, and angiotensinogen inhibition[20].

Recent studies suggested an association between ER stress and ALD. Intragastric alcohol-fed mice revealed initially altered expression in a set of genes related to the UPR or ER stress response. The alcohol-fed mice also showed severe steatosis, scattered apoptosis, and necroinflammatory foci[21]. Moreover, alcohol-fed guinea pigs showed increased amounts of CYP2E1 and BIP mRNA, CYP2E and BIP protein, and activated caspase-12[22], in addition to liver steatosis and apoptosis. Finally, in a lipopolysaccharide (LPS)-induced injury rat model, cirrhotic livers exhibited partial UPR activation such as eIF2α activation in the basal state and full UPR such as activation of IRE1, ATF-6, and eIF2α after LPS challenge[23]. As stated above, it is conceivable that ALD involves ER stress.

In this study, ROS production in hepatocytes treated with ethanol was increased dose dependently compared with normal cells (data not shown). Oxidative stress is caused by disrupted intracellular redox homeostasis, and ROS production induced under oxidative

stress can cause profound cell damage including apoptosis of hepatocytes. A previous study showed that ethanol metabolism via alcohol dehydrogenase leads to increased ROS production, hepatocyte damage, and apoptosis in long-term ethanol-fed rats[24]. Such ethanol-induced oxidative stress in the hepatocytes is therefore believed to cause damage mediated by ROS production. When SK-Hep1 cells in this study were pretreated with an antioxidant for 1 hour before the ethanol treatment, ER stress was suppressed significantly. Ethanol has been directly associated with ER stress previously in hepatocytes[25], and considerable interactions have been documented between oxidative stress and ER stress. Although the results are not entirely consistent, ROS does seem to trigger the ER stress response[26].

ROS production in our cells was increased by treatment with ethanol and subsequently decreased to the level of normal cells by pretreatment with NAC. Furthermore, the ethanol-induced increase in ssDNA was also significantly inhibited by NAC, although not to the level in normal cells. This suggested that there are oxidative stress and non-oxidative stress pathways underlying ethanol-induced apoptosis.

ER stress is caused by the accumulation of misfolded proteins and alterations in $[Ca^{2+}]_i$ homeostasis, and several pathways have been proposed to link ER stress to apoptosis. The present study focused on UPR signaling and ethanol, by assessing changes in the mRNA expressions of Bip, Chop, and sXbp1 in ethanol-induced apoptotic cells. These expressions were increased in a time- and dose-dependent manner, and in turn suppressed by pretreatment with NAC. Our study therefore supported that ethanol-induced oxidative stress induces apoptosis through the UPR.

The expression of CHOP, an important mediator of ER stress-induced cell death[27], is regulated mainly at the transcriptional level, although the mechanisms by which CHOP contributes to regulating cell death are not completely understood. However a previous study showed that, increased CHOP expression may perturb the cellular redox state by the depletion of cellular glutathione[2]. And also overexpression of CHOP leads to a decrease in anti-apoptotic Bcl-2 protein and to the translocation of pro-apoptotic Bax protein from the cytosol to the mitochondria[27]. Thus, a CHOP-mediated death signal seems to be transmitted to the mitochondria.

Finally, we investigated the effect of ethanol on Ca^{2+}-dependent calpain activation, including $[Ca^{2+}]_i$, calpain activation, and caspase-3 and -4 activities. All these parameters were increased dose dependently by ethanol incubation ; however, pretreatment with NAC did not attenuate the transient rise in $[Ca^{2+}]_i$ nor the increase in caspase-4 and calpain activation induced by ethanol. These findings suggested that Ca^{2+}-dependent calpain activation is not associated with ethanol-induced oxidative stress, and these findings are consistent with a previous study in which oxidative stress does not lead to apoptosis through caspase-4 activity[28]. A recent study in PrP^c cells showed that H_2O_2 induces ROS generation, but not caspase-12 activation[28]. As mentioned above, the results consensus points to a role for ER stress in chronic alcohol-induced mitochondrial stress.

In conclusion, our study demonstrated that ethanol-induced oxidative and non-oxidative stress-induced apoptosis through ER stress in SK-Hep1 cells. Ethanol-induced oxidative stress activated the URP, but not Ca^{2+}-dependent calpain activation. Therefore, ethanol-induced oxidative stress promotes apoptosis through ER stress, but only via mitochondria-mediated and / or Fas receptor pathways. Our results on ER stress and ethanol-induced apoptosis could highlight a potential therapeutic target for the treatment of ALD.

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