Showa Univ J Med Sci **24**(2), 155～167, June 2012

Original

Ethanol Increases NADPH Oxidase-derived Oxidative Stress and Induces Apoptosis in Human Liver Adenocarcinoma Cells (SK-HEP-1)

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Abstract: Alcohol-induced liver injury is linked to oxidative stress and increased production of reactive oxygen species (ROS). Oxidative stress is an early event in the process of apoptosis. However, it is not completely understood how ethanol-induced oxidative stress induces apoptosis. In contrast, nicotinamide adenine dinucleotide phosphate oxidase (NOX) is known to generate ROS in hepatocytes. The purpose of the present study was to determine whether or not ethanol-induced ROS generation stimulates the death receptor or mitochondrial pathways of apoptosis in alcohol dehydrogenase containing human liver adenocarcinoma (SK-HEP-1) cells. Treatment with ethanol increased the generation of ROS and expression of *NOX4* mRNA, and also induced mitochondrial dysfunction in SK-HEP-1 cells. Moreover, ethanol induced the activation of caspase-8 and -3 in hepatocytes. These activities were suppressed by pretreatment with N-acetyl-cysteine, an antioxidant, or apocynin, an inhibitor of NOX activity. These results suggested that ethanol induces an increase in NOX-derived ROS generation upstream of caspase-8 activation and in the mitochondria in SK-HEP-1 cells. In conclusion, this study demonstrated that ethanol increases the generation of ROS and subsequently induces apoptosis using a mechanism involving mitochondrial dysfunction and caspase activation in SK-HEP-1 cells.

Key words: NOX4, reactive oxygen species (ROS), SK-HEP-1, ethanol, apoptosis

Introduction

It is well established that chronic alcohol consumption can result in severe liver injuries such as fatty liver, alcoholic hepatitis and cirrhosis[1]. There are approximately 2.4 million heavy alcohol drinkers in Japan[2]. Recently, alcoholic liver disease (ALD) has been associated with an increase in cellular death, particularly by an apoptotic or programmed

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mechanism, and this mode of cellular injury may play a significant role in the progression of alcohol-induced liver damage. In general, it has been shown that the apoptotic cascade in ALD can be triggered by signaling pathways that involve death receptor-mediated interactions and / or mitochondrial stress signals[3, 4]. These events result in the activation of caspases, which execute proteolytic cleavage and the ultimate demise of the cell. In the case of ethanol-related cell death, several studies have demonstrated that the induction of caspases is linked to the involvement of oxidative stress mechanisms[5].

Previous studies have used various animal models or cell lines to investigate ethanol-induced apoptosis in hepatic cells[3, 6, 7]. However, the results of these experiments were inconsistent because some hepatic cells lost most of their ability to express ethanol-metabolizing enzymes such as alcohol dehydrogenase (ADH)[8] and cytochrome P4502E1[9]. Therefore, the human liver adenocarcinoma cell line SK-HEP-1 was used for this study because these cells express higher levels of ADH, the main enzyme involved in alcohol metabolism in the liver, than other hepatocytes such as HepG2 cells. SK-HEP-1 cells are therefore the most suitable cells to use for experiments investigating the association between ethanol administration and hepatotoxicity.

Nicotinamide adenine dinucleotide phosphate oxidase (NOX) is known to generate ROS in hepatocytes[10, 11]. Recently, six NOX homologs were described : NOX1, NOX2, NOX3, NOX4, DUOX1 and DUOX2[10]. NOX4 is located in liver cells, where it acts as an oxygen sensor and catalyzes the reduction of molecular oxygen to various ROS. The ROS generated by NOX4 have been implicated in numerous biological functions including signal transduction, cell differentiation and tumor cell growth. Activation of NOX4 requires $p22^{phox}$, which in turn regulates NOX4-dependent ROS generation[10]. NOX is also involved in Fas activation, leading to caspase-dependent apoptosis[12]. However, the mechanism underlying the relationship between NOX and Fas-mediated apoptosis in hepatocytes is poorly understood. The aim of the present study was to demonstrate an association between ethanol-induced ROS generation and apoptosis in ADH-containing human liver adenocarcinoma (SK-HEP-1) cells.

Materials and Methods

Drugs and reagents

Fetal bovine serum (FBS) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Ethanol, N-acetyl L-cysteine (NAC), CM-H_2DCFDA (5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester) and Eagle's minimum essential medium with Earle's salts (E-MEM) were purchased from Wako Co. (Osaka, Japan). Penicillin G sodium, streptomycin sulfate and amphotericin B were obtained from Invitrogen Corp. (Carlsbad, CA, USA). Apocynin was purchased from Calbiochem (Laeufelfingen, Switzerland). All other chemicals used in this study were of the purest grade commercially available.

Cell culture

SK-HEP-1 cells (human liver adenocarcinoma, EC-91091816) were obtained from "The European Collection of Cell Cultures" (ECACC, Wiltshire, UK). SK-HEP-1 cells were cultured in E-MEM containing 10% FBS and maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. For studies investigating the effects of ethanol, SK-HEP-1 cells were incubated in E-MEM with or without (used as the control) various concentrations of ethanol (25, 50, 100, 200, 300 and 500 mM) for 5 hours. Results from a preliminary experiment in which SK-HEP-1 cells were treated with various concentrations of ethanol (25, 50, 100, 200, 300 and 500 mM) showed that 200 mM ethanol was a suitable concentration for inducing apoptosis in SK-HEP-1 cells (data not shown). This result is consistent with results from a study reporting that 200 mM ethanol was equal to the physiological blood concentration of a heavy drinker[13]. SK-HEP-1 cells were pretreated with NAC (10 mM) or apocynin (300 μM) for 1 hour followed by co-treatment with 200 mM ethanol and 10 mM NAC or 300 μM apocynin for another 5 hours. All treatments were carried out under sterile conditions.

Detection of reactive oxygen species (ROS)

The ROS-sensitive probe 5-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) was used to investigate the effect of ethanol treatment on hydrogen peroxide production. CM-H_2DCFDA is a stable, non-fluorescent, cell-permeable indicator for the presence of ROS[14]. It passively diffuses into cells, where the acetate is acted on by intracellular esterases to produce a polar diol that is retained within the cells. SK-HEP-1 cells were seeded in 96-well plates at 1×10^5 cells / ml and pretreated with NAC (10 mM) or apocynin (300 μM) for 1 hour. After pretreatment, the cells were treated with 200 mM ethanol plus NAC (10 mM) or 200 mM ethanol plus apocynin (300 μM) for 5 hours. Free radical production was measured by incubating SK-HEP-1 cells with CM-H_2DCFDA. The Spectrafluor multiwell fluorescence reader (Berthold Technologies GmbH & Co., KG, Bad Wildbad, Germany) was used to measure the fluorescence intensity at excitation and emission wavelengths of 488 nm and 525 nm, respectively.

Detection of apoptosis

Single-stranded DNA (ssDNA)

SK-HEP-1 cells were pretreated with NAC (10 mM) or apocynin (300 μM) for 1 hour followed by co-treatment with 200 mM ethanol and 10 mM NAC or 300 μM apocynin for another 5 hours. The presence of single-stranded DNA (ssDNA), which provides specific evidence of the apoptotic process, was determined using a formamide-monoclonal antibody (mAb) against ssDNA as described in our previous report[15]. Formamide selectively denatures DNA in apoptotic cells, but not in necrotic cells nor in cells with DNA breaks in the absence of apoptosis[16].

Staining with annexin V-Cy3

SK-HEP-1 cells were pretreated with NAC (10 mM) for 1 hour followed by co-treatment with 200 mM ethanol and 10 mM NAC for another 5 hours. After treatment, detection of apoptotic cells was performed using the Cy3-labeled annexin V staining assay. Apoptotic hepatocytes stained with Cy3-labeled annexin V were observed as red fluorescence under a phase-contrast and fluorescence microscope (DIAPHOT TMD 300, Nikon Co. Ltd., Tokyo, Japan).

Measurement of caspase-8, -9 and -3 activities

SK-Hep-1 cells were pre-incubated with NAC (10 mM) or apocynin (300 μM) for 1 hour. After pre-incubation, the cells were incubated with 200 mM ethanol plus 10 mM NAC or 200 mM ethanol plus 300 μM apocynin for another 5 hours. Following incubation, the cells were rinsed with cold PBS and resuspended in chilled cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA), incubated for 10 min on ice and then centrifuged at 10,000 × g for 3 min. The supernatants were then added to reaction buffer containing 10 μM DTT (Medical & Biological Laboratories Co. Ltd., Aichi, Japan) and each peptide substrate and then incubated at 37°C for 2 hours. Substrates for caspase-8, caspase-9 and caspase-3 were IETD-7-amino-4-trifluoromethyl coumarin (AFC), LEHD-AFC and DEVD-AFC (Kamiya Biochemical Company, Seattle, WA, USA), respectively. The enzymatic release of AFC was measured spectrophotometrically at an excitation wavelength of 405 nm and an emission wavelength of 505 nm using an LB 970T Fluorometer (Berthold Technologies GmbH & Co., KG).

Mitochondrial membrane potential change

A collapse in the mitochondrial membrane potential ($\Delta\psi$) is one of the earliest indications that induction of apoptosis has occurred. Changes in the mitochondrial $\Delta\psi$ in hepatocytes can be detected by the fluorescent mitochondrial probe 5,5',6,6'-tetrachloro-1,1'3,3'-tetraethylbenzimidazolcarbocyanine iodide, commonly known as JC-1[17]. If the hepatocytes are not undergoing apoptosis, the mitochondrial $\Delta\psi$ remains intact and the JC-1 reagent accumulates and aggregates in the mitochondria where it fluoresces red. At relatively low concentrations, JC-1 exists in a monomeric form that fluoresces at 527 nm, but when concentrated in mitochondria undergoing active respiration, JC-1 forms aggregates that fluoresce at 590 nm. The intensity of fluorescence at 590 nm is proportional to the mitochondrial $\Delta\psi$, and this is a measure of a closed permeability transition pore. When mitochondrial membrane permeability transition (MMPT) is induced, the $\Delta\psi$ is dissipated and the formation of JC-1 aggregates is prevented, resulting in diminished fluorescence at 590 nm. SK-HEP-1 cells were pretreated with NAC (10 mM) for 1 hour followed by co-treatment with 200 mM ethanol and 10 mM NAC for another 5 hours and then loaded with JC-1 (10 μg / ml) for 15 min at 37.0°C in a CO_2 incubator. After washing the hepatocytes, the change in mitochon-

drial $\Delta\psi$ was assessed by comparing the ratio of 590 nm (red) / 527 nm (green) emissions with an LB 970T Fluorometer. These cells were also examined by fluorescence microscopy.

Semiquantitative RT-PCR analysis

Semiquantitative RT-PCR analysis was undertaken following previously described methods[18]. To measure the mRNA expression levels of *NOX4, p22^{phox}* and *β-actin*, SK-HEP-1 cells were pretreated with 10 mM NAC for 1 hour and then treated with 200 mM ethanol plus 10 mM NAC for another 5 hours. After treatment, total RNA was extracted from SK-HEP-1 cells using the RNeasy RNA Mini kit (Qiagen K.K., Tokyo, Japan). Reverse transcription polymerase chain reaction (RT-PCR) analysis was carried out using the Omniscript RT Kit (Qiagen K.K.) according to the manufacturer's protocol. The sequences of the primers used for amplification were : *NOX4F* : 5'-CCACAGACTTG-GCTTTGGAT and NOX4R : 5'-GGCAGAATTTCGGAGTCTTG, *p22^{phox}*F : 5'-CTGGGCG-GCTGCTTGATGGT and *p22^{phox}*R 5'-GTGTTTGTGGCCTGCTGGAGT, and *β-actin*F : 5'-TCGTCACCAACTGGGACGACATGG and *β-actin*R : 5'-GATCTTGATCTTCATTGT-GCTGGG. Amplification by PCR for NOX4 was performed with 42 amplification cycles (one cycle comprised of 94℃ for 30 sec, 55℃ for 30 sec and 72℃ for 30 sec) and for p22phox was performed with 42 amplification cycles (one cycle comprised of 94℃ for 30 sec, 63℃ for 30sec and 72℃ for 30 sec), followed by a final extension at 72℃ for 7 min. The reaction (30 cycles) for *β-actin* was performed as follows : 95℃ for 1 min to 60℃ for 1 min followed by 72℃ for 2 min for each cycle. Each sample was tested in triplicate. The number of cycles for each product was determined on the basis of kinetic studies. Some samples were analyzed every two cycles, and the PCR products were electrophoresed on 2% agarose gels and visualized under ultraviolet transillumination (Atto Corp., Tokyo, Japan). Quantification was performed by densitometry using a scanner employing Scion Image version beta 4.0.2 software (NIH, USA). The amplification curves of each respective sample were plotted on graphs. The intensity of fluorescence was fitted to the data in the linear portion of the curves. The ratio of target cDNA to *β-actin* was used as a relative estimate of mRNA abundance.

Statistical analysis

The results are expressed as the mean ± standard error of the mean (S.E.M.). Comparison of the effects of various treatments with those of the untreated control cells was performed using one-way ANOVA and the Dunnett's test as the post hoc test. P values less than 0.05 were considered statistically significant.

Results

The influence of ethanol on ROS generation

Fig. 1 shows ROS formation as indicated by 2'7'-dichlorofluorescein fluorescence. The

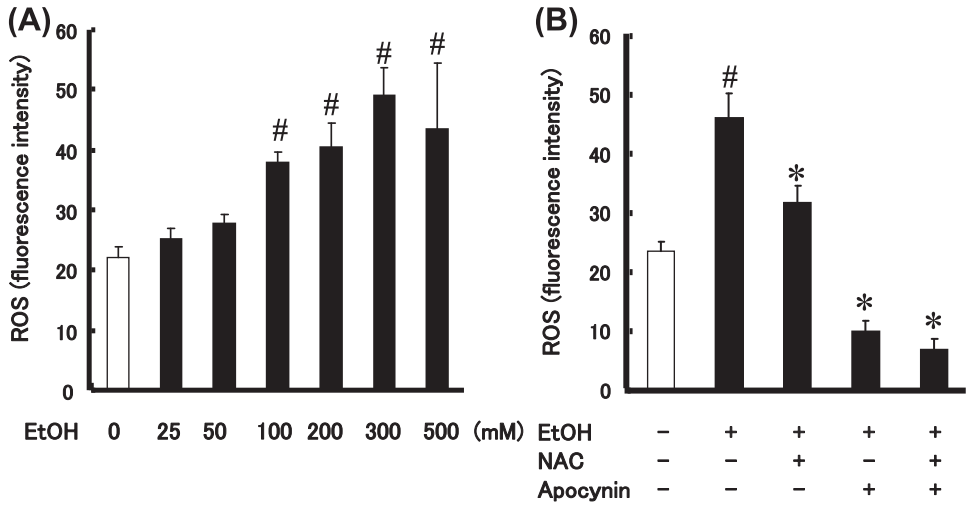

Fig. 1. The effect of ethanol on ROS generation in SK-HEP-1 cells.
SK-HEP-1 cells (1×10^5 cells / well) were treated with ethanol (25−500 mM), 200 mM ethanol plus NAC (10 mM) or 200 mM ethanol plus apocynin (300 μM) for 5 hours at 37°C. (A) The generation of ROS in SK-HEP-1 cells treated with ethanol (25−500 mM) was analyzed spectrofluorometrically. (B) The generation of ROS in SK-HEP-1 cells treated with ethanol (200 mM), ethanol (200 mM) plus NAC (10 mM) or ethanol (200 mM) plus apocynin (300 μM) were analyzed spectrofluorometrically. Each value represents the mean ± S.E.M. of 6−12 samples.
$^{\#}$: $P < 0.05$: significant difference from untreated SK-HEP-1 cells.
* : $P < 0.05$: significant difference from ethanol (200 mM)-induced apoptotic cells.

levels of ROS generated significantly increased following treatment with ethanol (100−500 mM) compared to untreated SK-HEP-1 cells (Fig. 1A), and the levels of ROS formed were dependent on the concentration of ethanol. Fig. 1B shows the effects of NAC and apocynin on ethanol-induced (200 mM) ROS generation in SK-HEP-1 cells. ROS generation in SK-HEP-1 cells treated with ethanol was significantly suppressed by NAC, an ROS scavenger, and apocynin, which blocks the $p47^{phox}$ subunit of NOX. This is consistent with the results of another study, which also showed that increases in ROS production could be abolished by NAC and apocynin[19].

Detection of ethanol-induced apoptosis

The presence of ssDNA was used to distinguish between apoptosis and necrosis in ethanol-treated SK-HEP-1 cells (Fig. 2). Fig. 2A shows that treatment of SK-HEP-1 cells with ≥200 mM ethanol induced apoptosis, and that significantly higher levels of ssDNA were generated in SK-HEP-1 cells treated with ethanol compared to untreated cells. Increases in the generation of ssDNA were dependent on the concentration of ethanol, and the number of apoptotic cells also increased with the concentration of ethanol.

The effects of NAC and apocynin on ethanol-induced ssDNA generation in SK-HEP-1 cells are shown in Fig. 2B. SK-HEP-1 cells pretreated with NAC or apocynin had sig-

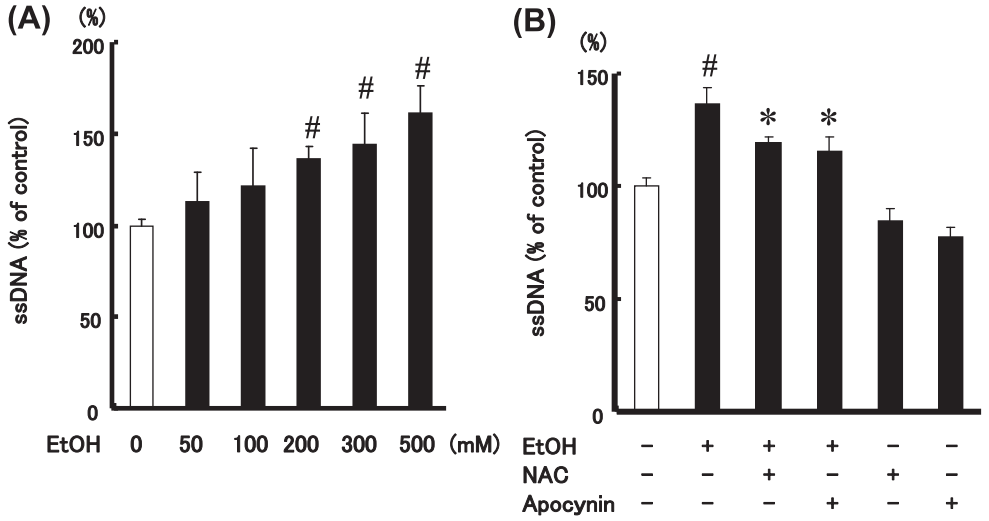

Fig. 2. The effect of ethanol on ssDNA in SK-HEP-1 cells.
Formamide-denaturable DNA was detected in apoptotic cells. (A) ssDNA in SK-HEP-1 cells treated with ethanol (50–500 mM) were measured. (B) ssDNA in SK-HEP-1 cells treated with ethanol (200 mM), ethanol (200 mM) plus NAC (10 mM) or ethanol plus apocynin (300 μM). Each value represents the mean ± S.E.M. of 6–12 samples.
$^{\#}$: $P < 0.05$: significant difference from untreated SK-HEP-1 cells.
* : $P < 0.05$: significant difference from ethanol (200 mM)-induced apoptotic cells.

nificantly lower levels of ssDNA compared to SK-HEP-1 cells treated with ethanol alone. However, after pretreatment with NAC or apocynin, levels of ssDNA significantly decreased in SK-HEP-1 cells, compared to the levels seen in ethanol-treated cells (Fig. 2B).

A well-established feature of an early event in apoptosis is the externalization of phosphatidyl serine (PS) from the inner to outer leaflet of the plasma membrane. The results obtained with the annexin V-cy3 binding assay of untreated and treated cells are represented in Fig. 3. Treatment of SK-HEP-1 cells with ethanol caused apoptosis during a 1-hour incubation. However, pretreatment with NAC reduced the number of ethanol-induced annexin V–positive cells compared to cells that were not pretreated with NAC.

Measurement of caspase-8, -9 and -3 activities

Caspases are critical mediators of apoptosis in SK-HEP-1 cells; thus, several caspase activities were measured using synthetic fluorometric substrates. The effects of ethanol on caspase-8, -9 and -3 activities in SK-HEP-1 cells are shown in Fig. 4. Caspase-8, -9 and -3 activities were significantly increased in SK-HEP-1 cells following ethanol treatment compared to those in untreated SK-HEP-1 cells. Furthermore, the activities of caspase-8, -9 and -3 were significantly suppressed when ethanol-treated SK-HEP-1 cells were pretreated with NAC or apocynin compared to cells treated with ethanol only.

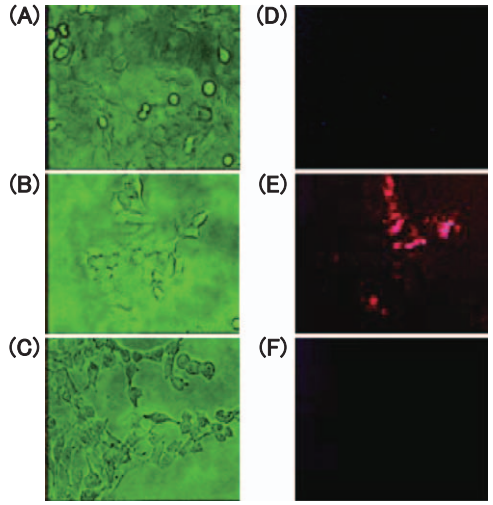

Fig. 3. The effect of ethanol on annexin V-Cy3 staining in SK-HEP-1 cells.
Cells were treated with 200 mM ethanol or 200 mM ethanol plus 10 mM NAC for 1 hour. SK-HEP-1 cells were observed under a phase-contrast microscope (A-C) and a fluorescence microscope (D-F). Images A and D : untreated SK-HEP-1 cells ; images B and E : SK-HEP-1 cells treated with 200 mM ethanol ; and images C and F : SK-HEP-1 cells treated with 200 mM ethanol plus 10 mM NAC.

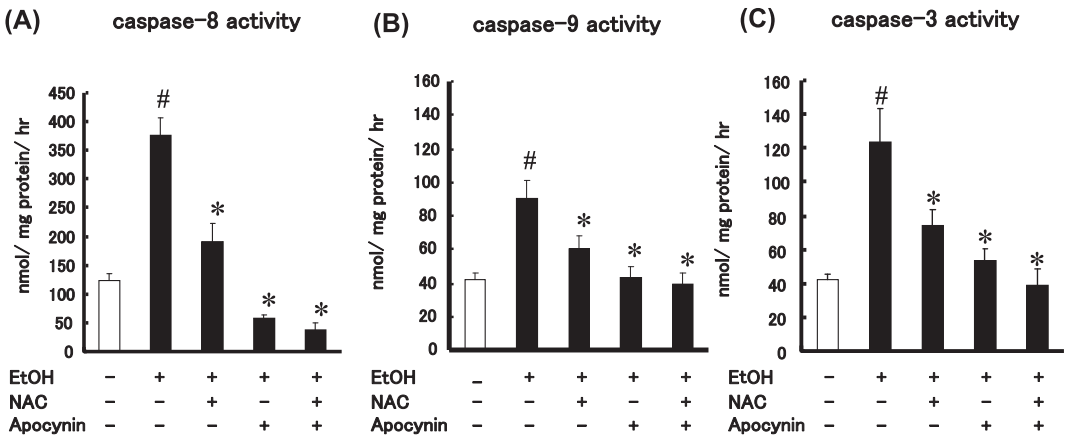

Fig. 4. The effect of ethanol on caspase activity in SK-HEP-1 cells.
(A) Caspase-8 activity in SK-HEP-1 cells was assayed using the substrate Ile-Glu-Thr-Asp-AFC. (B) Caspase-9 activity in SK-HEP-1 cells was assayed using the substrate Leu-Glu-His-Asp-AFC. (C) Caspase-3 activity in SK-HEP-1 cells was assayed using the substrate Asp-Glu-Val-Asp-AFC. Each value represents the mean ± S.E.M. of 6-12 samples.
: $P < 0.05$: significant difference from untreated SK-HEP-1 cells.
* : $P < 0.05$: significant difference from ethanol (200 mM)-induced apoptotic cells.

Mitochondrial membrane potential change

Mitochondrial function during exposure of SK-HEP-1 cells to ethanol was also assessed. The changes in mitochondrial $\Delta\psi$ were monitored by the fluorescent probe JC-1. We

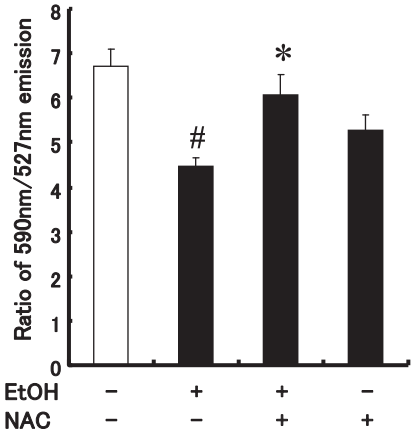

Fig. 5. Effect of ethanol on the change in mitochondrial membrane potential ($\Delta\psi$) in SK-HEP-1 cells. The changes in mitochondrial $\Delta\psi$ in hepatocytes treated with ethanol (200 mM) or ethanol (200 mM) plus NAC (10 mM) for 5 hours were measured by comparing the 590 nm / 527 nm emission ratios. Each value represents the mean ± S.E.M. of 6–12 samples. Other conditions were the same as described in Fig. 1.
: $P < 0.05$: significant difference from the untreated cells.
* : $P < 0.05$: significant difference from ethanol (200 mM)-induced apoptotic cells.

demonstrated a marked decline of mitochondrial $\Delta\psi$ in hepatocytes after a 5-hour exposure period to ethanol. However, mitochondrial $\Delta\psi$ was significantly increased when ethanol-treated SK-HEP-1 cells were pretreated with NAC compared to cells treated with ethanol only. The fluorescence ratios of hepatocytes incubated with ethanol alone or pretreated with NAC are shown in Fig. 5. Red fluorescence represented untreated hepatocytes and green fluorescence represented ethanol-treated hepatocytes, clearly demonstrating the significant decrease in mitochondrial $\Delta\psi$.

Semiquantitative RT-PCR analysis

The mRNA expression analyses of *NOX4* and *p22^{phox}* in treated SK-HEP-1 cells are shown in Fig. 6. The mRNA expression levels of *NOX4* and *p22^{phox}* in ethanol-treated SK-HEP-1 cells increased significantly compared to the levels measured in untreated cells. In contrast, the mRNA expression levels of *NOX4* and *p22^{phox}* were suppressed significantly in SK-HEP-1 cells treated with NAC plus ethanol compared to cells treated with ethanol only. These results demonstrated that NAC inhibits the increase in *NOX4* and *p22^{phox}* mRNA expression caused by ethanol-induced oxidative stress in SK-HEP-1 cells.

Discussion

Chronic alcohol consumption is an important risk factor for liver disease, and it leads to severe liver diseases such as acute / chronic hepatitis, steatosis, steatohepatitis, fibrosis, cirrhosis and / or cancer. Therefore, it is necessary to investigate the underlying mechanisms of alcohol-induced liver disorders. Nozaki *et al* reported that cytotoxic effects were observed after

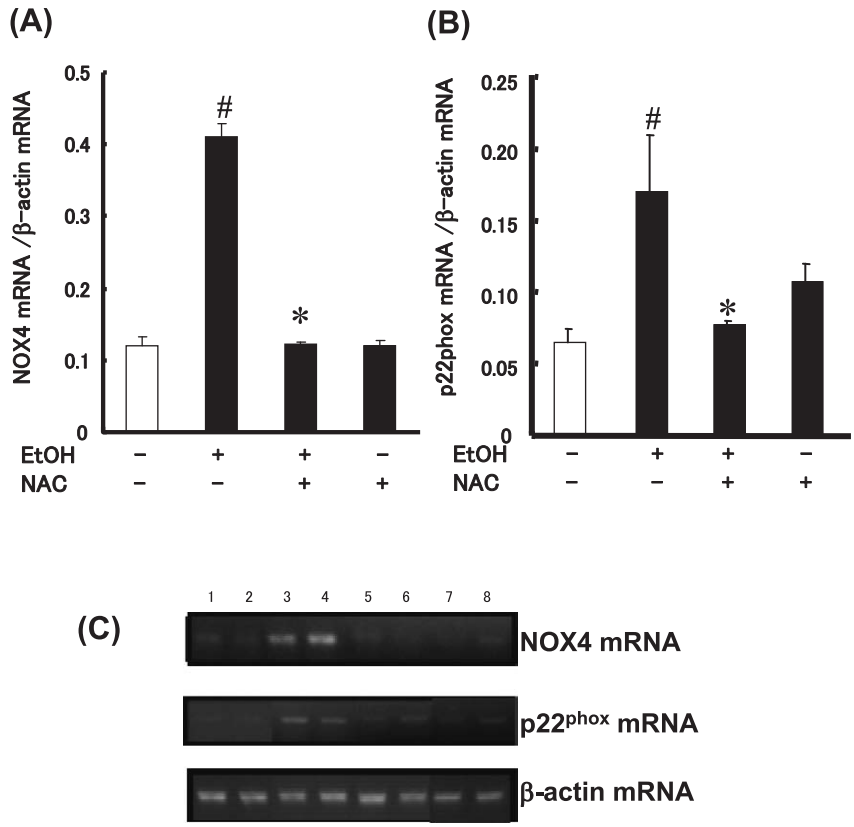

Fig. 6. The effect of ethanol on *NOX4* and *p22^{phox}* mRNA expression levels in SK-HEP-1 cells.
(A) The expression levels of *NOX4* mRNA in SK-HEP-1 cells treated with ethanol (200 mM), NAC (10 mM) and ethanol (200 mM) plus NAC (10 mM). (B) The expression levels of *p22^{phox}* mRNA in SK-HEP-1 cells treated with ethanol (200 mM), NAC (10 mM) and ethanol (200 mM) plus NAC (10 mM). *NOX4* and *p22^{phox}* transcript levels were normalized using *β-actin* as the housekeeping gene. The resulting *NOX4* or *p22^{phox}* mRNA / *β-actin* mRNA ratios are represented as the mean ± S.E.M. of 8–12 samples. (C) RT-PCR analysis of *NOX4*, *p22^{phox}* and *β-actin* expression after treatment with ethanol (200 mM) or ethanol (200 mM) plus NAC (10 mM). Lanes 1 and 2: untreated SK-HEP-1 cells; lanes 3 and 4: SK-HEP-1 cells treated with 200 mM ethanol; lanes 5 and 6: SK-HEP-1 cells treated with ethanol (200 mM) plus NAC (10 mM); lanes 7 and 8: SK-HEP-1 cells treated with 10 mM NAC.
$^{\#}$: $P < 0.05$: significant difference from the untreated cells.
*: $P < 0.05$: significant difference from the ethanol (200 mM)-induced apoptotic cells.

treating hepatocytes with high concentrations of ethanol (over 200 mM) for 48 hours[20]. The human hepatic cell line HepG2 is usually established *in vitro* when examining drug metabolism and hepatotoxicity because HepG2 cells express the human cytochrome P450 subtypes[21,22]. However, HepG2 cells express small levels of ADH, which is the main cytosolic enzyme of alcohol metabolism in hepatocytes. Kai *et al* reported that the concentration of ethanol required to induce apoptosis in HepG2 cells after a 24-hour incubation was very high at 860 mM[23]. The human liver adenocarcinoma cell line SK-HEP-1 has higher ADH expression levels than HepG2 cells[13]; therefore, SK-HEP-1 cells are more suited for

experiments investigating ethanol-induced hepatotoxicity.

Ethanol induces the formation of ROS in liver cells. A previous study was able to link the ethanol-mediated induction of oxidative stress to the observed increase in apoptosis[4]. In the present study, the increased generation of ROS and the induction of apoptosis in SK-HEP-1 cells were caused by treatment with ethanol. In linking the ethanol-induced oxidative stress with liver cell apoptosis, it was hypothesized that ROS cause damage to the mitochondria by altering the mitochondrial membrane potential and / or membrane permeability. The results from this study revealed that exposure to ethanol significantly decreased the mitochondrial $\Delta\psi$ in hepatocytes. However, pretreatment with the antioxidant NAC was able to inhibit ethanol-induced mitochondrial dysfunction. NAC, a precursor of the cytoprotective factor glutathione, scavenges ROS and protects cells against oxidative stress[24]. Mitochondrial dysfunction can initiate the release of proapoptotic factors such as cytochrome c, thus activating the caspase cascade[5]. In this study, ethanol treatment increased the activation of caspase-9 and -3, and these increased caspase activities were suppressed by pretreatment with NAC or apocynin, which is a NOX inhibitor. These results clearly demonstrated that ethanol administration caused enhanced oxidative stress and that apoptotic death is a possible consequence of these changes.

Activation of death-receptor pathways is one of the several mechanisms that have been proposed to explain alcohol-induced apoptosis in hepatocytes[25-27]. Fas (CD95 / Apo-1), a cell death receptor, is a type I membrane protein belonging to the tumor necrosis factor (TNF) receptor family, and is a receptor for the Fas ligand. Fas-mediated apoptosis is induced when the Fas ligand binds to Fas[28]. The Fas-mediated apoptosis pathway has been shown to occur in hepatocytes[12]. Activation of Fas, caused by binding to the Fas ligand, results in the formation of the death-inducing signaling complex (DISC) and leads to the activation of caspase-8, which initiates the caspase cascade. Caspase-8 induces the activation of caspase-3, which then finally results in apoptotic cell death[12]. The present study showed that ethanol induced the activation of caspase-8 in SK-HEP-1 cells. However, pretreatment with an antioxidant (NAC) or a NOX inhibitor (apocynin) suppressed ethanol-induced caspase-8 activity. These results suggest that ethanol may lead to an increase in the generation of ROS upstream of caspase-8 activity.

Furthermore, the results of the present study demonstrated that ethanol was able to upregulate the mRNA expression levels of genes involved in ROS production ($p22^{phox}$ and *NOX4*) in SK-HEP-1 cells. The production of ROS is affected by the mitochondrial electron transport chain and various intracellular oxidative enzymes such as xanthine oxidase, aldehyde oxidase, cyclooxygenase, monoamine oxidase and NOX[29]. The upregulation of *NOX4* mRNA expression levels by ethanol was suppressed by pretreatment with the antioxidant NAC. It has been suggested that the upregulation of NOX4 by ethanol leads to an increase in ROS production in hepatocytes, coincident with apoptosis. In addition, the Nox complex may be a major source of ROS production in hepatic cells. The hepatic expression

of *p22^{phox}* mRNA was also significantly upregulated in ethanol-treated cells. It is believed that p22^{phox} interacts with NOX1, NOX2, NOX3 and NOX4, and that these proteins are stable only as a heterodimer. *Fas* mRNA was also upregulated in ethanol-treated cells (data not shown). It is therefore possible that NOX4 and / or p22^{phox} increase Fas production, which in turn promotes apoptosis of ethanol-treated cells. Upregulation of *NOX4* mRNA by ethanol is required for its proapoptotic activity in SK-HEP-1 cells. Thus, it is believed that the sequence of events in SK-HEP-1 cell apoptosis occurs as follows: ethanol affects the expression of NOX on the cell's membrane, NOX then generates ROS, which then activate Fas on the hepatic cell membrane.

In conclusion, the results of this study demonstrated that ethanol induces an increase in the generation of ROS and apoptosis by mechanisms that involve mitochondrial dysfunction and caspase activation in SK-HEP-1 cells. It is proposed that ethanol-induced oxidative stress leads to severe liver disease, and that treatment with an antioxidant such as NAC or a NOX inhibitor such as apocynin could suppress the progression of liver disease caused by ethanol. Insights into the cellular mechanisms involved in the initiation and progression of apoptosis will significantly impact our understanding of alcohol-induced liver disease and may lead to the potential development of therapeutic intervention protocols.

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[Received February 13, 2012 : Accepted February 20, 2012]