

NOX4/ROS mediate ethanol-induced apoptosis via MAPK signal pathway in L-02 cells

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Abstract. The aim of the present study was to assess the molecular mechanism of ethanol-induced oxidative stress-mediated apoptosis in L-02 liver cells in order to elucidate novel pathways associated with alcoholic liver disease. L-02 cells were treated with 400 mM ethanol with or without inhibitors. The cell viability was measured by an MTT assay. Cell apoptosis was assessed by flow cytometry and a single-stranded DNA (ssDNA) assay. Intracellular reactive oxygen species (ROS) production of L-02 cells was determined using the 2',7'-dichlorofluorescein-diacetate dye. The protein expression of c-Jun N-terminal kinase (JNK), phosphorylated (p)-JNK, P38, p-P38, NADPH oxidase (NOX)1, NOX4, p22^{phox}, B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein were measured by western blot analysis. The mRNA expression of NOX1, NOX4 and p22^{phox} was measured by reverse transcription polymerase chain reaction analysis. The results indicated that after treatment with various concentrations of ethanol for the indicated durations, L-02 cells were displayed a significant decrease in cell viability in a dose- and time-dependent manner. Ethanol-induced apoptosis and cell death of L-02 cells was accompanied by the generation of ROS, elevated expression of NOX, as well as phosphorylation of JNK and P-38. In addition, increased expression of Bcl-2 was induced by 400 mM ethanol. Furthermore, treatment with NOX inhibitor attenuated the ethanol-induced decrease in cell viability, and an increase in apoptosis and Bcl-2 expression. In conclusion, ethanol induced apoptosis in the L-02 hepatocyte cell line via generation of ROS and elevated expression of NOX4. This indicated that

activation of JNK and p38 in the mitogen-activated protein kinase pathway promotes apoptosis in L-02 cells.

Introduction

Excessive alcohol consumption may lead to various liver diseases, from simple steatosis to severe liver injury, including fatty liver, steatohepatitis, liver fibrosis, cirrhosis and even liver cancer (1). Alcoholic liver disease (ALD) is a major medical health issue, which not only places a heavy financial burden on individuals and society, but also causes losses to the world economy. ALD is the major cause of morbidity and mortality worldwide (2).

Although the pathogenesis of alcoholic liver disease has remained to be fully elucidated, mounting evidence indicates that oxidative stress has a key role in ALD. Ethanol-induced oxidative stress contributes to the elevated production of reactive oxygen species (ROS), increased lipid peroxidation and damage of the antioxidant system, which leads to cell apoptosis and necrosis (3). Hepatocyte cell death via apoptosis and necrosis may be the critical process in the exacerbation of ALD (4). Further studies noted that oxidative stress, excessive intracellular ROS production induced by ethanol and its metabolite have a critical role in ethanol-induced apoptosis (5,6), and put forward that apoptosis is mainly induced via the Fas-mediated and mitochondria-mediated pathways (7,8).

Mounting evidence indicates that six homologues of the transmembrane enzyme NADPH oxidase (NOX4) are highly expressed in hepatocytes and hepatic stellate cells, and it has therefore emerged as an important source of ROS in signal transduction, having roles in physiological and pathological processes of ALD (9). Based on this, it is a reasonable assumption that ROS derived from NOX4 on the membrane may be associated with Fas activation (10). It is well known that mitogen-activated protein kinases (MAPKs) determine the fate of various cells, and previous studies have demonstrated that p38 MAPK and c-Jun N-terminal kinase (JNK) promote the mitochondrial apoptotic pathway, leading to ethanol-induced death of SK-Hep1 or Hepg2 cells, suggesting an interaction between apoptosis and MAPK signalling systems (11,12).

L-02 cell is a new cell line established by the Shanghai Biochemical Institute of the Chinese Academy of Sciences, which originates from healthy human liver tissues. L-02 cells

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have been reported to possess alcohol dehydrogenase (ADH) activity (12). Therefore, the results may be more exact than those from the cancerous hepatocytes such as HepG2 or Hep3B. The L-02 cell line may be used as a model of regular liver cells to a certain degree, and it would be reasonable to assess ethanol-induced apoptosis in this established cell line. In the present study, the participation of mitochondrial apoptotic and MAPK pathways in the toxicity of ethanol to the L-02 cell line was reported for the first time, to the best of our knowledge.

Materials and methods

Drugs and reagents. JNK inhibitor (SP600125) and p38 MAPK inhibitor (SB202190) were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Fetal bovine serum (FBS), N-acetyl L-cysteine (NAC) and apocynin were purchased from Sangon Biotech, Inc. (Shanghai, China). Antibodies to B-cell lymphoma 2 (Bcl-2; ab59348), Bcl-2-associated X protein (Bax; ab53154), JNK (ab124956), phosphorylated (p)-JNK (ab207477), p38 MAPK (ab170099), p-p38 MAPK (ab4822), NOX1 (ab55831), NOX4 (ab13303) and p22^{phox} (ab75941) were purchased from Abcam (Cambridge, UK). Caspase-3 antibody (9662S) was obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). β -actin antibody (C640018) was purchased from Sangon Biotech, Inc. and oxidant-sensitive probe 2,7-dichlorodihydro fluorescein diacetate (DCFH-DA) was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). A single-stranded (ss) DNA Apoptosis ELISA kit was obtained from EMD Millipore (GIR7935; Billerica, MA, USA).

Cell culture. The L-02 hepatocyte line (human normal liver cells; no GDC079) was purchased from the China Center for Type Culture Collection of Wuhan University (Wuhan, China). L-02 hepatocytes were incubated with Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FBS, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. After 24 h, the medium was replaced with fresh medium. For subculturing purposes, cells were detached by 0.05% trypsin-EDTA treatment.

Viability assay. L-02 cells were cultured in 96-well plates (1.5x10⁴ cells/well). At ~80% confluence, L-02 cells were treated with or without (used as a control) various concentrations of ethanol (100, 200, 300, 400, 500, 600 and 700 mM) for 0, 3, 6, 12, 24 or 48 h. Cell viability was monitored by an MTT assay as previously described (13).

In order to assess the effects of ethanol, L-02 cells were cultured in DMEM with 10% FBS with or without (used as a control) ethanol for 0-48 h. Preliminary experiments had been performed, in which caspase-3 activity and cell viability were measured at a wide range concentrations of ethanol (100, 200, 300, 400, 500, 600 and 700 mM). These experiments indicated an obvious enhancement of caspase-3 activity at concentrations of no less than 400 mM ethanol (data not shown). Hence, 400 mM ethanol was used as a suitable concentration for triggering apoptosis in L-02 cells (11). To explore the effect of the antioxidants (NAC and apocynin) and the effect of MAPK signalling pathway inhibitors (SP600125

and SB202190) on ROS generation, L-02 cells were seeded in 96-well plates (1.5x10⁴ cells/well) and pre-treated with NAC (10 mM), apocynin (300 μ M), SP600125 (1 μ M) or SB202190 (1 μ M) for 1 h, followed by the addition of 400 mM ethanol and incubation for a further 5 h. Cells individually treated with the above agents were used as control groups.

Detection of ROS. With investigation of the role of ethanol treatment on hydrogen peroxide generation, the oxidant-sensitive DCFH-DA, which is a molecular probe for detection of ROS, was used. The generation of ROS was measured by testing the fluorescence intensity of DCF, the reduction product of DCFH-DA, as described in a previous study (11). L-02 cells were cultured in petri dishes of 3 cm in diameter (1x10⁵ cells/plate) overnight and treated with or without (as a control) transforming growth factor (TGF)- β 1 (10 μ g/l; Sigma Aldrich; Merck KGaA) for 3, 4, 5, 6, 7 or 8 h at 37°C, followed by incubation with 10 μ M 2,7-DCFH-DA at 37°C and away from light for 30 min. Fluorescence images were captured under a fluorescence microscope. To assess the effect of the antioxidants (NAC and apocynin) and the effect of MAPK signalling pathway inhibitors (SP600125 and SB202190, respectively) on ROS generation, L-02 cells were treated as in the cell viability assay above. Subsequently, the fluorescence intensity was determined using a Tecan Infinite M200 PRO Microplate reader (Tecan Group Ltd., Männedorf, Switzerland) at an excitation wavelength of 502 nm and an emission wavelength of 523 nm.

Detection of apoptosis

Apoptosis analysis by flow cytometry (FCM). To further verify that ethanol induced cell apoptosis, an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (APT225; Sigma Aldrich; Merck KGaA) was applied with FCM analysis. L-02 cells were cultured in petri dishes of 6 cm in diameter (3x10⁵ cells/plate) overnight and treated with ethanol (400 mM) for 0, 3, 6, 9, 12 or 24 h at 37°C. The cells were then harvested by treatment with 0.05% trypsin without EDTA and washed with cold PBS three times. Subsequently, the cells were suspended with 400 μ l binding buffer and stained with 5 μ l Annexin V-FITC for 20 min away from light, followed by addition of 10 μ l propidium iodide on the ice for 5 min and analysis by FCM (BD Biosciences, San Jose, CA, USA).

Apoptosis analysis by ssDNA assay. ssDNA, which may be regarded as specific evidence of the apoptotic process, was measured using a formamide-monoclonal antibody against ssDNA using the ssDNA Apoptosis ELISA kit as described in a previous study (11). The assay is based on the principle that formamide denatures DNA selectively in apoptotic cells. L-02 cells were cultured in 400 mM ethanol for 5 h. The effect of NAC or MAPK inhibitors was detected by pre-treatment with NAC, apocynin or MAPK inhibitors followed by co-culture of cells with 400 mM ethanol as described above in cell viability assay.

Western blot analysis. L-02 cells were cultured in petri dishes of 10 cm in diameter (2x10⁶ cells/plate) overnight and treated with ethanol (400 mM) for 0, 3, 6, 9, 12 or 24 h. Cells were lysed in radioimmunoprecipitation lysis buffer (Beyotime

Table I. Primer sequences used for determination of NOX1, NOX4, p22^{phox} and β -actin by polymerase chain reaction.

Gene	Primer sequence	Annealing temperature (°C)	No. cycles	Product length (bp)
NOX1		56	35	106
F	5'-TTGTTTGGTTAGGGCTGAATGT-3			
R	5'-GCCAATGTTGACCCAAGGATTTT-3			
NOX4		58	35	136
F	5'-TGTGCCGAACACTCTTGGC-3'			
R	5'-ATATGCACGCCTGAGAAAATA-3'			
p22 ^{phox}		58	35	103
F	5'-TATTGTTGCAGGAGTGCTCA-3'			
R	5'-CACAGCGGTCAGGTACTTCT-3'			
Bax		53	35	155
F	5'-CCCGAGAGGTCTTTTTCCGAG-3'			
R	5'-CCAGCCCATGATGGTTCTGAT-3'			
Bcl-2		53	35	89
F	5'-GGTGGGGTCATGTGTGTGG-3'			
R	5'-CGGTTTCAGGTACTCAGTCATCC-3'			
β -actin		56	35	199
F	5'-GGACTCCTATGTGGGTGACGA-3'			
R	5'-ACGGTTGGCCTTAGGGTTCA-3'			

NOX, NADPH oxidase; F, forward; R, reverse; Bax, Bcl-2-associated X protein; Bcl, B-cell lymphoma.

Institute of Biotechnology, Shanghai, China) with protease and phosphatase inhibitor cocktail tablets (Sangon Biotech, Inc.). The lysates were centrifuged at 12,000 x g for 15 min at 4°C. The supernatant was harvested and the protein concentration was measured using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Equivalent amounts (50 μ g) of protein were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane and blocked for 1 h with 5% bovine serum albumin (BSA) (9048-46-8; Sangon Biotech, Inc.) in Tris-buffered saline containing 0.05% Tween 20 (TBST). For immune detection, the membrane was incubated overnight at 4°C with specific rabbit antibodies to Bcl, Bax, caspase-3, NOX1, NOX4, p22^{phox}, JNK, p-JNK, p38 MAPK or p-p38 (1:1,000 dilution in TBST containing 3% BSA), and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit antibody (D110058-0025, Cell Signaling Technology, Inc.; 1:5,000 dilution in TBST containing 3% BSA). Protein bands were visualized by enhanced chemiluminescence (E002-100; Sangon Biotech, Inc.) and quantified by densitometry using the ChemiDoc XRS imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). β -actin (1:1,000) was used as the internal control.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. The mRNA expression levels of NOX1, NOX4 and p22^{phox} were determined by RT-PCR. Total RNA was reverse-transcribed into complementary (c)DNA using reverse transcriptase (TIANScript cDNA; Tiangen, Beijing, China) according to the manufacturer's instructions. Using the MJ PTC-200 PCR System (MJ Research; Bio-Rad

Laboratories, Inc.), the cDNA was amplified with an RT-PCR kit (2X Taq PCR Master Mix; PC0902; Aidlab Co., Beijing China) according to the manufacturer's protocol. PCR was performed using specific primers (Table I) provided by Sangon Biotech Inc. The PCR products were identified using 1.5% agarose gel electrophoresis, and the optical density of the target gene bands in each sample was calculated using the ChemiDoc imaging system with adjustment through β -actin correction to finally obtain the relative expression of the target genes in each sample.

Transfection of small interfering (si)RNA. siRNA targeting NOX4 (NOX4-SiRNA) and the NC-siRNA expressing plasmid were designed and synthesized by GenePharma (Shanghai, China). L-02 cells were transfected with NOX4-siRNA or the NC-siRNA expressing plasmid using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. In brief, L-02 cells in the logarithmic growth phase were inoculated into 6-well plates at a density of 2x10⁵ cells/well in 2 ml antibiotic-free DMEM without serum. Gene transfection was performed when the cells had grown to 70-80% confluence, and 100 pmol of siRNA and 2 μ l of Lipofectamine were added to each well. Subsequently, serum- and antibiotic-free DMEM (500 μ l) was supplemented. The cells were incubated with the transfection mixture for 6 h. In the final phase of the incubation period, 1.5 ml of complete culture medium without antibiotics was added and the cells were cultured for another 18 h. Following ethanol treatment, the cells were collected and the expression of NOX4 was detected by RT-quantitative PCR and immunoblotting.

NOX4 overexpression. L-02 cells were electroporated with the P5 primary cell Nucleofector™ kit (Lonza, Basel, Switzerland). For each transfection, NOX4-cDNA (3 μ g) was dissolved in 82 μ l nucleofector solution, and 18 μ l P5-supplemented primary cell solution was then added. A total of 10^7 - 10^8 cells were suspended in 100 μ l cDNA plus P5 primary cell solution and immediately electroporated in a 4D-Nucleofector X kit L cuvette (Lonza). Electroporated cells were transferred to 6-well plates containing 2.5 ml warm complete medium and cultured in a humidified atmosphere of 5% CO₂ at 37°C. The medium was replaced with fresh medium after 24 h.

Statistical analysis. Values are expressed as the mean \pm standard deviation of three independent experiments. SPSS19.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Tukey's test was performed following one-way analysis of variance to evaluate significant differences between groups. $P < 0.05$ was considered to indicate a statistically significant difference between groups.

Results

Effects of ethanol the viability of L-02 cells. To assess the effect of ethanol on the viability of L-02 cells, an MTT assay was performed on L-02 cells in the absence or presence of ethanol. As presented in Fig. 1, ethanol treatment reduced the viability of L-02 cells in a dose- and time-dependent manner.

As presented in Fig. 1, addition of 400 mM ethanol led to a significant inhibition of cell viability compared with that in the control group after 12 h. The inhibition rate was $<10\%$ at 6 h but $>50\%$ at 24 h. These experiments indicated an obvious enhancement of caspase-3 activity at concentrations of no less than 400 mM ethanol (data not shown). Therefore, 400 mM ethanol was indicated to be a suitable concentration for the subsequent experiments in L-02 cells. The ethanol concentration of 400 mM for cell treatment was verified using phase-contrast microscopy, which indicated that no cell necrosis occurred when L-02 cells were cultured with 400 mM ethanol for 6 h (data not shown). However, if the L-02 cells were incubated with 400 mM ethanol for >6 h, cell damage occurred. Therefore, incubation for 6 h was selected to collect various experimental data on cell apoptosis in the present study.

Effect of ethanol on apoptosis of L-02 cells. Previous studies have demonstrated that ethanol induces apoptosis in HepG2 cells (1), SK-Hep1 cells (14) and the Chang human hepatocyte cell line (15). In order to clarify whether the decrease in L-02 cell viability observed in the present study is due to ethanol-induced apoptosis, a flow cytometric assay was used to measure cell apoptosis. The results indicated that apoptosis induced by ethanol in L-02 cells was time-dependent (Fig. 2A and B). It was therefore suggested that induction of apoptosis was, at least in part, the reason for inhibitory effect of ethanol on the viability of L-02 cells in present experiment (Fig. 1). Ethanol exposure at 400 mM for 24 h led to a marked increase of apoptosis in treated cells as measured by flow cytometry (Fig. 2A). The results indicated in the presence of 400 mM ethanol for 0-9 h, L-02 cells did not undergo apoptosis, but the apoptotic ratio of L-02 cells was $\sim 40\%$ at 12 h, which was

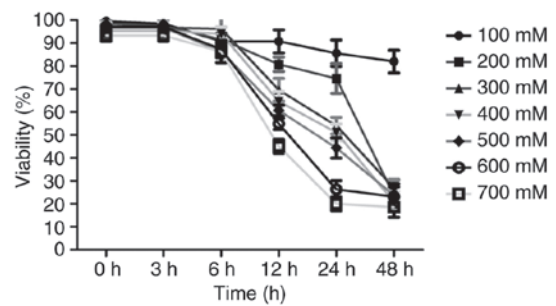


Figure 1. Effects of ethanol on the viability of L-02 cells. To study the effects of ethanol, L-02 cells were incubated with various concentrations of ethanol for 0-48 h. The viability of the L-02 cells was examined by an MTT assay and is expressed as the percentage of the untreated control ($n=3$). $^*P < 0.01$ vs. control group.

further increased to $\sim 50\%$ at 24 h. The apoptotic cells were mainly in the late phase of apoptosis (Fig. 2C). The apoptotic population in ethanol-treated L-02 cells was increased in a time-dependent manner.

In order to verify the occurrence of apoptosis, the effect of ethanol on apoptosis-associated proteins, Bax and Bcl-2, were also detected. Bax and Bcl-2 are involved in the maintenance of mitochondrial membrane stability (16). Western blot analysis demonstrated that the expression levels of Bax and Bcl-2 in L-02 cells were increased at 24 h after treatment with 400 mM ethanol, while the expression of Bcl-2 was lower than that of Bax at various time-points, which led to an increased Bax/Bcl-2 ratio with the relative density ratio reaching 2.37 at 24 h, indicating that apoptosis has an important role in ethanol-induced L-02 cell death (Fig. 2D and E).

JNK and p38 MAPK were demonstrated to be involved in ethanol-induced apoptosis, and may stimulate the activities of pro-apoptotic proteins, such as Bax, and promote apoptosis by inhibiting anti-apoptotic proteins, such as Bcl-2, to regulate the release of cytochrome *c* and cause apoptosis in ethanol-induced L-02 cells. The protein expression of the Bcl-2 family proteins Bax and Bcl-2, which control the release of cytochrome *c* from mitochondria, were measured in L-02 treated with or without 400 mM ethanol and optional pre-treatment with 10 mM NAC or 300 μ M apocynin or 1 μ M inhibitor of p38 JNK or MAPK. The results indicated that the mRNA expression levels of Bax were obviously increased in ethanol-treated group ($P < 0.01$) compared with those in the control group, and this increase was significantly blocked when cells were pre-treated with NAC, apocynin or inhibitors (SB202190, SP600125) of JNK and p38 MAPK. However, compared with those in the control group, after treatment with ethanol, the mRNA levels of Bcl-2 were significantly decreased ($P < 0.01$), while this reduction was distinctly inhibited by pre-treatment with NAC, apocynin or inhibitors of JNK and p38 MAPK. These results indicated that JNK and p38 MAPK promote apoptosis in ethanol-treated L-02 cells by regulating the expression of Bcl-2 and Bax (Fig. 2F and G).

The activities of caspase-8 and caspase-3 in L-02 cells treated with or without 400 mM ethanol with optional pretreatment with 10 mM NAC or 300 μ M apocynin are presented in Fig. 2H and I. After culture with 400 mM ethanol for 6 h, cleaved-caspase-3 and -caspase-8 in L-02 cells were markedly

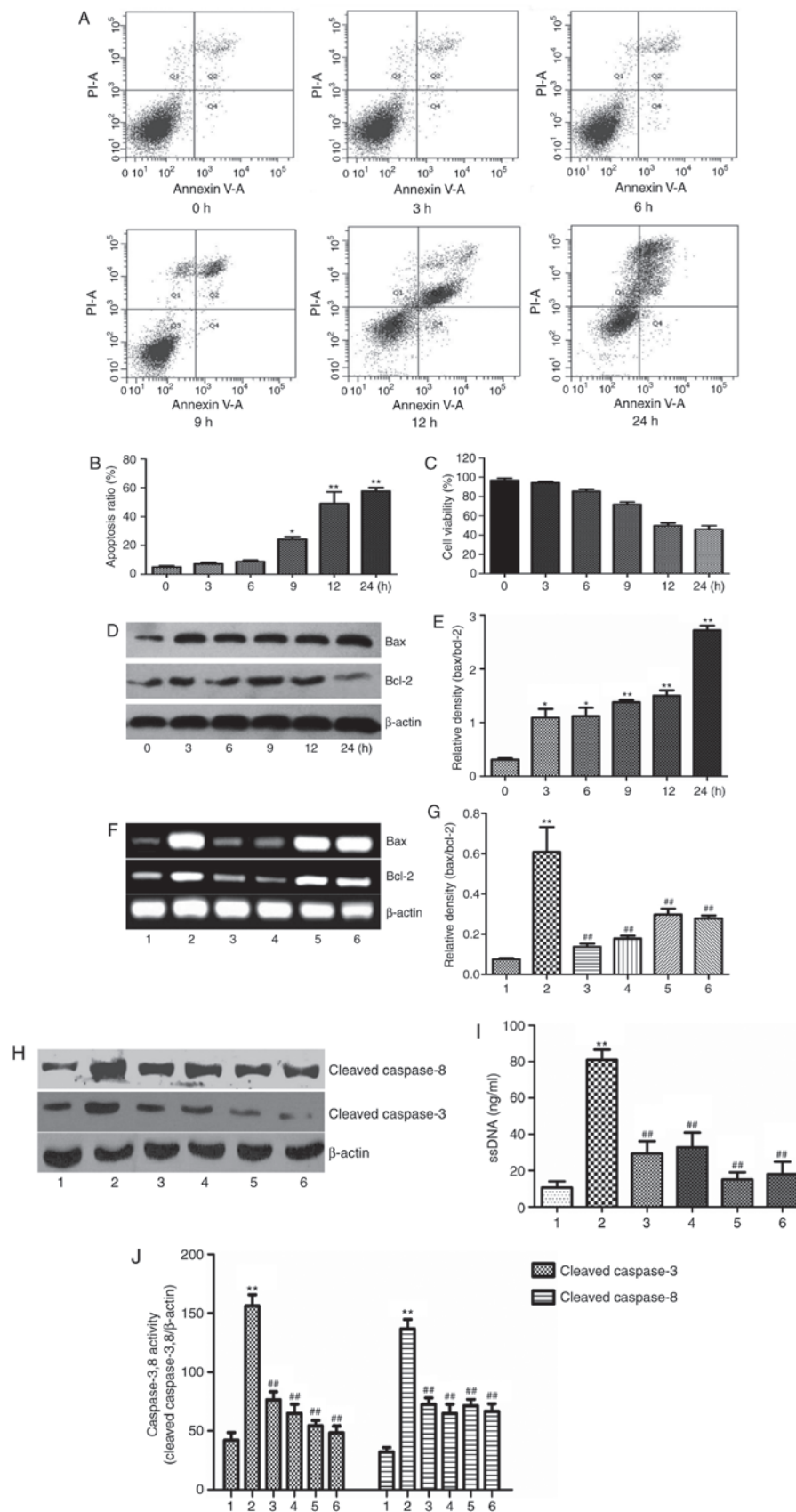


Figure 2. Effects of 400 mM ethanol on apoptosis in L-02 cells. After treatment with 400 mM ethanol for 0-24 h, (A and B) cell apoptosis and (C) viability were measured by a flow cytometric assay. (D) The protein expression of Bax and Bcl-2 was measured by western blot analysis and (E) the Bax/Bcl-2 ratio was determined. L-02 cells were treated with 400 mM ethanol with or without NAC (10 mM), Apocynin (300 μ M), SB202190 (1 μ M) or SP600125 (1 μ M) for 6 h, and (F) the gene expression by of Bax and Bcl-2 and (G) the Bax/Bcl-2 ratio were determined; in addition, the protein levels of (H) cleaved-caspase-8 and cleaved-caspase-3 were measured by western blot analysis and (I) densitometrically quantified. (J) Apoptosis of L-02 cells was determined using an ssDNA assay. Values are expressed as the mean \pm standard error of the mean (n=3). *P<0.05, **P<0.01 vs. the control group/0 h group; ##P<0.01 vs. the ethanol group. Lanes: 1, Control; 2, ethanol; 3, ethanol + NAC (10 mM); 4, ethanol + apocynin (300 μ M); 5, ethanol + SB202190 (1 μ M); 6, ethanol + SP600125 (1 μ M). NAC, N-acetyl L-cysteine; ssDNA, single-stranded DNA; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

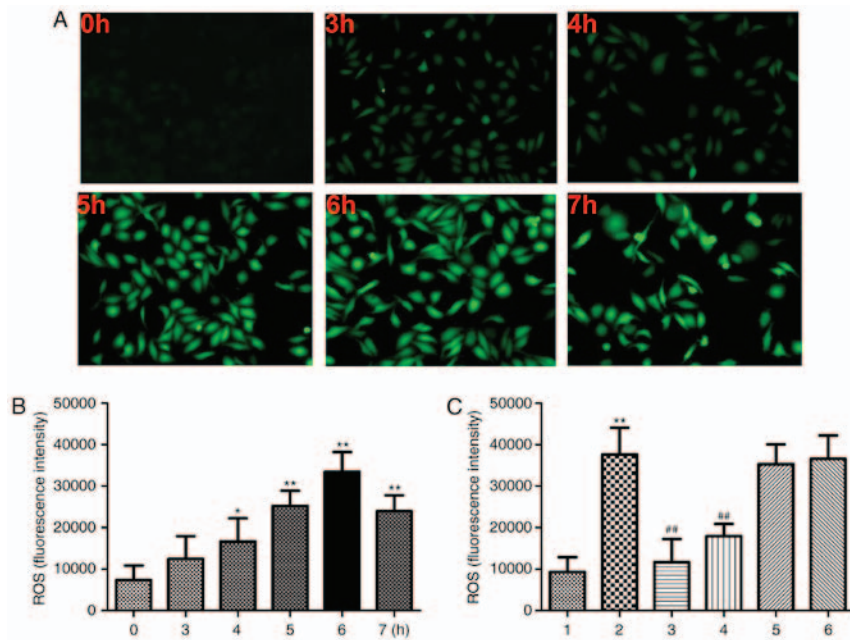


Figure 3. Effect of 400 mM ethanol on ROS generation. (A and B) The generation of ROS in L-02 cells (1×10^5 cells/plate) after treatment with 400 mM ethanol for 0-7 h at 37°C. Magnification, $\times 100$. (C) Generation of ROS in L-02 cells after treatment with 400 mM ethanol with or without NAC, Apocynin, SB202190 or SP600125 for 6 h at 37°C. Values are expressed as the mean \pm standard error of the mean ($n=3$). * $P<0.05$, ** $P<0.01$ vs. the control group/0 h group; ** $P<0.01$ vs. the ethanol group. Groups: 1, Control; 2, ethanol; 3, ethanol + NAC (10 mM); 4, ethanol + apocynin (300 μ M); 5, ethanol + SB202190 (1 μ M); 6, ethanol + SP600125 (1 μ M). NAC, N-acetyl L-cysteine; ROS, reactive oxygen species.

increased compared with those in the control group ($P<0.01$). The increases of caspase-3 and -8 activities triggered by ethanol were suppressed when L-02 cells were pretreated with 10 mM NAC or 300 μ M apocynin as well as MAPK inhibitors. These results indicated that the increase of caspase-3 and -8 is triggered by oxidative stress such as ethanol treatment in L-02 cells, as it was blocked by pre-treatment with the antioxidant NAC or apocynin and MAPK inhibitors. The presence of ssDNA was used to distinguish between apoptosis and necrosis in ethanol-treated L-02 cells. The results of this assay presented in Fig. 2J demonstrated that compared with the control, the levels of ssDNA were markedly raised following incubation with 400 mM ethanol ($P<0.01$), but this increase was significantly repressed when the cells were pre-treated with NAC and apocynin, as well as inhibitor of JNK or p38 MAPK ($P<0.01$), indicating that JNK and p38 MAPK have at least a partial role in the apoptotic process that was triggered by ethanol-induced oxidative stress.

In brief, ethanol significantly reduced L-02-cell viability in a dose- and time-dependent manner. It induced L-02-cell apoptosis in a time-dependent manner, and this apoptosis was significantly inhibited by NAC and apocynin, as well as the MAPK inhibitors. Furthermore, ethanol treatment induced the expression of Bax and the cleavage of caspase-8 and caspase-3, while reducing the expression of Bcl-2, which was inhibited by NAC and apocynin, as well as the MAPK inhibitors.

Effect of ethanol on ROS generation in L-02 cells. Previous studies have indicated that ethanol triggers ROS generation in various types of liver disease, including cancer (17,18). To investigate whether ethanol induces ROS generation in L-02 cells, the ROS expression levels in ethanol-treated L-02 cells were first evaluated by testing the oxidative conversion from

non-fluorescent DCFH-DA to fluorescent DCF. The results indicated that ethanol triggered ROS production in L-02 cells in a time-dependent manner (Fig. 3A and B). Ethanol treatment at 400 mM for 3, 4, 5, 6 and 7 h elevated the ROS levels by 0.9, 2.1, 3.2, 3.5, 3.0-fold, respectively, of the untreated control. An increase in ROS was also observed in SK-Hep1 cells subjected to the same ethanol treatment (data not shown). These results indicated that ethanol triggered ROS production in L-02 cells.

As demonstrated in Fig. 3C, treatment with ethanol (400 mM) for 24 h led to a marked enhancement of ROS generation in L-02 cells compared with that in the control group ($P<0.01$). Pre-treatment with 10 mM NAC or 300 μ M apocynin significantly suppressed the 400 mM ethanol-induced elevation of ROS production in L-02 cells compared with that in the group treated with ethanol only, as indicated in Fig. 3C ($P<0.01$). The ethanol-induced ROS production was also reduced by pre-treatment with the MAPK inhibitors SB202190 and SP600125; however, no statistical significance was reached.

In brief, ethanol induced intracellular ROS generation compared with that in the control group, which was dramatically suppressed by pre-treatment with antioxidant NAC or apocynin.

Effect of ethanol on NOX in L-02 hepatocytes. NOX1 and NOX4 are highly expressed in the liver and hepatocytes, and previous studies have indicated that NOX is a major producer of ROS (18,19). In the present study, the expression of NOX subunits was determined after ethanol treatment. A time-dependent kinetic study following ethanol stimulation was also performed. The L-02 cells were incubated with ethanol in DMEM containing 10% FBS for 0, 3, 6, 9, 12 or 24 h. The protein expression of NOX1, NOX4 and p22^{phox} was

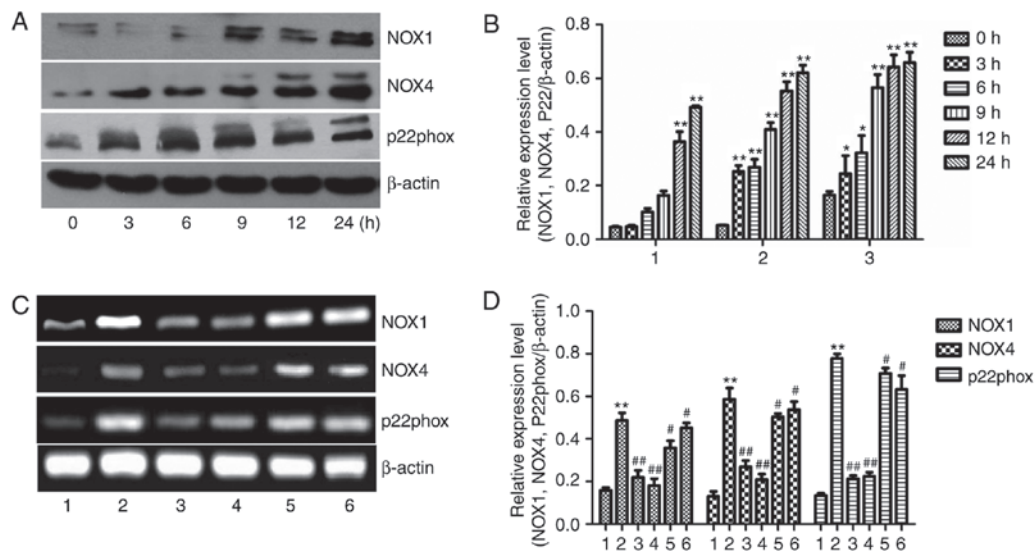


Figure 4. Effect of 400 mM ethanol on NOX1, NOX 4 and p-22^{phox} protein expression in the L-02 cell line. (A and B) Western blot analysis of the protein expression of NOX1, NOX4 and p-22 in L-02 cells after treatment with 400 mM ethanol for 0-24 h. (C and D) mRNA expression of NOX1, NOX4 and p22^{phox} in L-02 cells after the indicated treatments for 6 h. Values are expressed as the mean \pm standard error of the mean (n=3). *P<0.05, **P<0.01 vs. the control group/0 h group; #P<0.05, ##P<0.01 vs. the ethanol group. Groups: 1, Control; 2, ethanol (400 mM); 3, ethanol + NAC (10 mM); 4, ethanol + apocynin (300 μ M); 5, ethanol + SB202190 (1 μ M); 6, ethanol + SP600125 (1 μ M). NOX, NADPH oxidase; NAC, N-acetyl L-cysteine.

examined at the indicated time-points. The results suggested that ethanol induced an increase in the expression of NOX1 from 9 h onwards, with a significant increase from 12 h, increase in the expression of NOX4 and p22^{phox} in L-02 cells after 3 h and it increased in a time-dependent manner until 24 h (Fig. 4A and B). This demonstrated that ethanol exposure for 3 h is sufficient for inducing an increase of NOX1, NOX4 and p22^{phox} expression in L-02 cells and persists for at least 24 h.

Furthermore, RT-PCR indicated that the mRNA expression of NOX1, NOX4 and p22^{phox} in the ethanol-treated group was significantly higher than that in the control group (P<0.01; Fig. 4C and D). By contrast, the ethanol-induced increases in the mRNA expression of NOX1, NOX4 and p22^{phox} in L-02 cells were significantly suppressed by pre-treatment with NAC and apocynin (P<0.01). NAC is an ROS scavenger and apocynin blocks the p47^{phox} subunit of NOX (20). As expected, SB202190 and SP600125 had no effect on the mRNA expression of the NOX subunits (P>0.05). These results are consistent with those of previous studies, which also indicated that increased ROS production may be abolished by NAC and apocynin (21,22).

In short, ethanol induced intracellular ROS generation in L-02 cells, which was markedly abolished by NAC and apocynin but not MAPK inhibitors. Furthermore, NAC or apocynin completely inhibited ethanol-induced activation of caspase-3 and caspase-8 via blocking the apoptosis of L-02 cells by activating the caspase-, MAPK- and ROS-dependent pathways. Apocynin alleviates oxidative stress and inhibits NADPH oxidase superoxide production. As NOX4 is one of the most important NADPH isoform in hepatocytes that contributes to ROS production, its role was then investigated. The protein and mRNA expression of total NOX4 was upregulated by ethanol treatment (Fig. 4A-D). The protein level and mRNA expression of NOX1 was also increased by ethanol treatment (Fig. 4A-D). In addition, the expression of p22^{phox}, an essential

adaptor protein for functional NOX4, was also upregulated by ethanol treatment. The ethanol-induced increase in the mRNA expression of total NOX1, NOX4 and p22^{phox} was reduced by NAC or apocynin treatment.

ROS mediates P-38 and JNK activation in ethanol-induced apoptosis of L-02 cells. Exposure of L-02 cells to ethanol induces the production of ROS and cell apoptosis. Previous studies have indicated that ROS production is a crucial step in the triggering of apoptotic signalling pathways (23-25).

Further experiments were performed to assess the association between ROS generation and apoptosis in L-02 cells exposed to ethanol. The L-02 cells were incubated with ethanol in DMEM containing 10% FBS for 0, 3, 6, 9, 12 or 24 h. The expression levels of JNK, p-JNK p38 MAPK and p-p38 MAPK were examined at the indicated time-points as presented in Fig. 5A-C. The expression levels of p-p38 MAPK and p-JNK were increased, but the levels of total p38 MAPK and JNK remained nearly unchanged after treatment with ethanol. At 12 and 24 h, the p-p38MAPK/p38MAPK ratio and the p-JNK/JNK ratio were significantly increased, indicating that both p38 and JNK activation contribute to L-02 cell apoptosis.

Since activation of p38 MAPK and JNK has been demonstrated to be associated with apoptosis, The above results indicated that ethanol induced apoptosis in L-02 cells is triggered, at least partially, by ROS production which acts upstream of the apoptotic signalling pathway, leads to an increased ratio of Bax/Bcl-2, followed by cells going into apoptosis. It points to that at least JNK and p38 MAPK have a partial role in the process of oxidative stress-mediated apoptosis, which was caused by ethanol.

NOX4 has an important role in L-02 apoptosis induced by ethanol. To further confirm the role of NOX4 in ethanol-induced L-02-cell apoptosis, NOX4 was silenced with

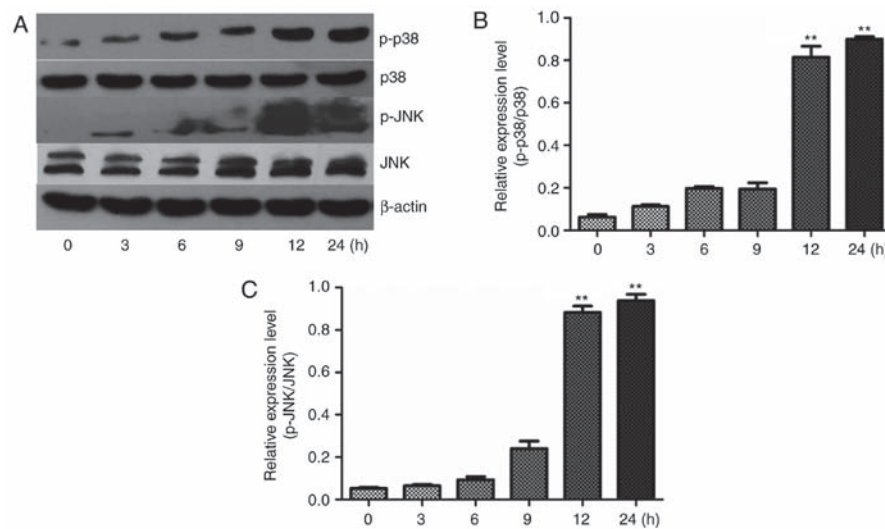


Figure 5. Effect of 400 mM ethanol on the activation of mitogen-activated protein kinase pathways in the L-02 cell line. (A) Western blot analysis of p38, p-p38, JNK and p-JNK protein levels after treatment with 400 mM ethanol for 0-24 h. Quantified (B) p-p38/p38 ratio and (C) p-JNK/JNK ratio. Values are expressed as the mean \pm standard error of the mean (n=3). **P<0.05 vs. the control group (0 h group) (n=3). p-JNK, phosphorylated c-Jun N-terminal kinase.

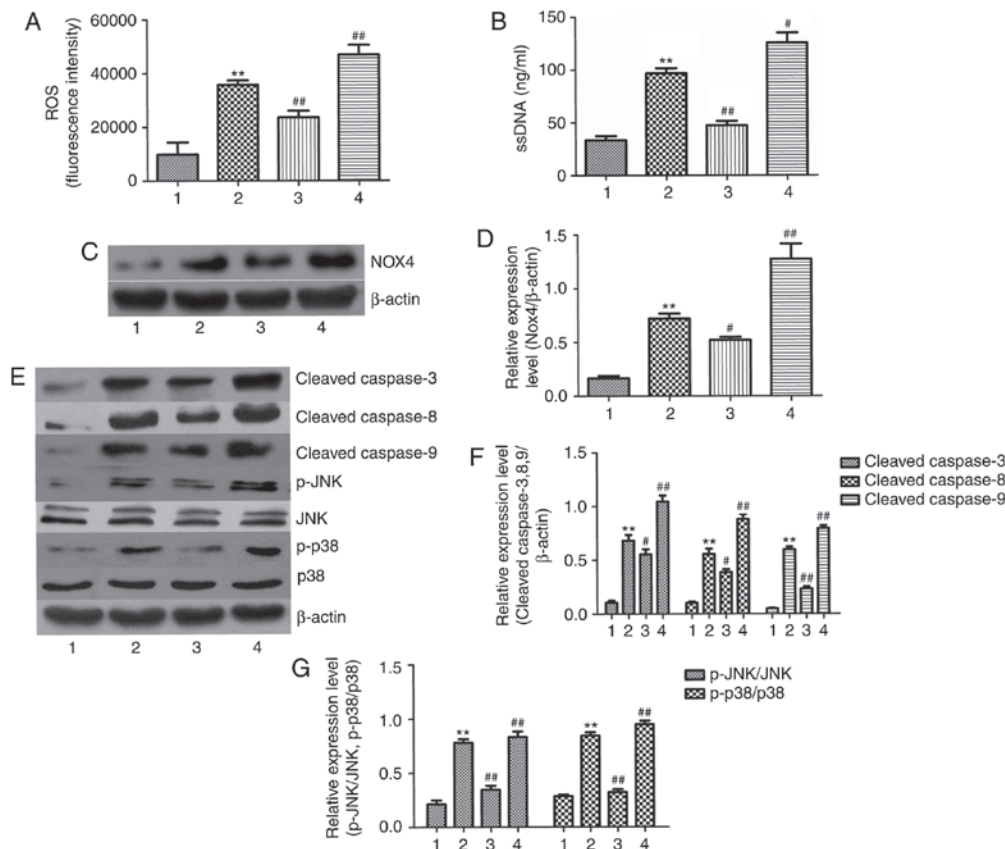


Figure 6. NOX4 has an important role in ethanol-induced apoptosis. Effects of ethanol (400 mM) treatment of L-02 cells with siRNA-mediated knockdown or vector-mediated overexpression of NOX4 for 24 h on the generation of (A) ROS and (B) ssDNA as well as the protein levels of (C and D) NOX-4, (E-G) caspase-3, -8 and -9, p38, p-p38, JNK and p-JNK. Values are expressed as the mean \pm standard error of the mean (n=3). **P<0.01 vs. the control group; *P<0.05, ##P<0.01 vs. the ethanol + NC-siRNA group. Groups: 1, NC-siRNA; 2, ethanol + NC-siRNA; 3, ethanol + NOX4-siRNA; 4, ethanol + NOX4 overexpression. NOX, NADPH oxidase; p-JNK, phosphorylated c-Jun N-terminal kinase; siRNA, small interfering RNA; NC, scrambled control siRNA; ssDNA, single-stranded DNA; ROS, reactive oxygen species.

NOX4-siRNA or NOX4 overexpressed using plasmid transfection technology. The results indicated that, compared with the ethanol + NC-siRNA group, the generation of ROS was significantly decreased in the ethanol + NOX4-siRNA-treated group,

while ROS was significantly increased in the NOX4 overexpression group (Fig. 6A). In correspondence with this, apoptosis was significantly decreased in the ethanol + NOX4-siRNA group, whereas apoptosis was significantly increased in the NOX4

overexpression group compared with the ethanol + NC-siRNA group (Fig. 6B). Furthermore, NOX4 protein expression in response to ethanol was significantly decreased in the NOX4-siRNA group but was markedly increased in the NOX4 overexpressed group (Fig. 6C). Simultaneously, silencing of NOX4 protected L-02 cells against ethanol-induced caspase activation (Fig. 6E and F). By contrast, NOX4 overexpression significantly increased ethanol-induced caspase activation. To further explore the underlying mechanisms, p38 MAPK- and JNK-dependent apoptotic pathways were investigated. Ethanol exerted an obvious effect on p38 MAPK- and JNK-phosphorylation in NC-siRNA cells and was further increased in NOX4-overexpressing cells (Fig. 6F and G). In addition, in the ethanol + NOX4-siRNA group, ethanol-induced increases in p38 MAPK and JNK phosphorylation were markedly inhibited. In summary, in NOX4-overexpressing L-02 cells, the effects of ethanol were significantly enhanced, while they were inhibited by NOX4 silencing.

Discussion

Chronic ethanol consumption is a high risk factor for hepatic disease and causes severe alcohol-associated liver disease, including hepatitis, steatosis, fatty liver, fibrosis, and eventually cirrhosis or even cancer, which carry a poor prognosis. Ethanol consumption elevates the production of ROS and increases the peroxidation of proteins, lipids and DNA (26). Prolonged alcohol abuse leads to severe pathologies that are associated with apoptotic cell death induced by ROS-mediated oxidative stress (17). It is important to select a cell line whose ethanol-metabolizing enzymes are expressed as in normal liver cells. For this purpose, the L-02 normal human hepatocyte cell line, which has been demonstrated to express an adequate amount of ADH, was utilized.

In the present study, the cytotoxic effects of ethanol on L-02 cells were assessed. The major findings include that i) ethanol induced L-02-cell apoptosis, which was dependent on the ethanol concentration, ii) NOX4 had an important role in ethanol-induced L-02-cell apoptosis, iii) ROS generation from NOX4 induction was involved in L-02-cell apoptosis in response to ethanol and iv) that JNK and P38 MAPK actively participated in ethanol-induced L-02-cell apoptosis.

The optimal ethanol concentration for treatment and suitable experimental time-points were determined using a cell viability assay and further preliminary experiments. The results indicate that the 400-mM concentration of ethanol and the treatment duration of 24 h were the optimal conditions for inducing apoptosis. ROS generation reached a peak following ethanol treatment at 400 mM for 6 h. Cells exposed to ethanol for 24 h exhibited a marked increase in apoptosis, as indicated by the Annexin V/PI assay. The apoptotic population in ethanol-treated L-02 cells increased in a time-dependent manner.

When L-02 cells were cultured with ethanol at the concentration of 400 mM for 0-8 h, ROS increased with the time of incubation. NOX4, which is one of six important homologues of the transmembrane protein NOX, is widely involved in ROS generation and has been identified to be highly expressed in the liver and hepatocytes (27). The results of the present

study indicated that when the L-02 cells were incubated with ethanol, the mRNA levels of NOX4 and p22^{phox}, which is the protein required to activate NOX4 on the membrane, were increased. From this, it was deduced that the generation of ROS in L-02 cells treated with ethanol is regulated at least partially by NOX4 on the membrane. It is well known that excessive ROS increases oxidative stress and causes apoptosis (22). The results of the present study indicated that in ethanol-treated L-02 cells, ROS generation induced by NOX4 on the liver cell membrane is one of the factors that leads to and promotes apoptosis.

Apoptosis induced via the mitochondria-dependent pathway may be triggered by stress, chemical agents and/or drugs (11); it is also regulated by numerous genes (28). Bax and Bcl-2 are important regulators; Bcl-2 has an anti-apoptotic role and Bax has an apoptosis-promoting role (29,30). Bax and Bcl-2 serve important roles in maintaining the stability of the mitochondrial membrane (16). The elevated expression of Bax leads to an increase in cell death stimulation and a shift in the mitochondrial membrane potential to trigger the release of cytochrome *c*. Bax in its cleaved form is an effector of apoptosis, while the anti-apoptotic protein Bcl-2 restrains the pro-apoptotic effect of Bax (30). In the present study, L-02 cells treated with 400 mM ethanol exhibited a slight increase in the expression of Bcl-2, while the expression of Bax was markedly elevated, and the ratio of Bax to Bcl-2 was greatly increased in a time-dependent manner and reached a maximum at 24 h. The increase of the Bax/Bcl-2 ratio is likely to change the cross-membrane potential of the mitochondria by triggering cytochrome *c* release, sensitizing caspase-9 and then activating caspase-3, inducing the occurrence of apoptosis (4,30). These results indicated that apoptosis has a key role in L-02-cell damage induced by ethanol and that ethanol induced mitochondrial apoptotic pathways. However, the exact mechanisms of how ethanol activates the mitochondrial apoptotic pathway remain to be fully elucidated.

Cell death induced by oxidative stress is closely associated with the increase of ROS. It has been identified that the accumulation of ROS in the liver leads to functional disorders in the cell membrane, oxidative DNA damage and abnormal protein expression to finally cause liver cell injury. If the damage is irreversible and cannot be repaired, it eventually leads to cell apoptosis (31). Ethanol boosts oxidative stress by increasing the formation of ROS and the depletion of internal oxidative defences in cells (32). Oxidative stress is a vital apoptotic stimulant for cells, particularly in those that have a high-energy metabolism required for their rapid growth and proliferation. As a result, ROS are overly produced by the mitochondrial source and cause lipid peroxidation and DNA lesions, thereby leading to cell apoptosis (32). The present study demonstrated that ROS were gradually increased in L-02 cells from 0 to 6 h, where they reached a maximum and were then eventually reduced. NOX4 was also increased in line with the trends in ROS levels. Increased ROS generation in L-02 cells triggered apoptosis. Of note, ethanol-induced ROS formation in L-02 cells was inhibited by NAC and apocynin. NOX and mitochondria are two major sources of ROS in L-02 cells (4). Ethanol-induced caspase-3 and caspase-8 activation was almost completely inhibited by pre-treatment with NAC or apocynin, which are NOX inhibitors, suggesting

that ethanol-induced apoptosis was mediated by NOX-derived ROS. NOX4, one of the 7 isoforms of the NADPH oxidase, is considered to be the key enzyme of ROS production in HepG2 cell and has a vital role in regulating the fate of HepG2 and other cells (33,34). However, the biological roles of NOX4 in the L-02 cells still remain elusive. In the present study, ethanol induced NOX4 expression accompanied by the increased expression of p22^{phox}, an essential component for NOX4 activation. Silencing of NOX4 significantly inhibited ROS formation and the pro-apoptotic effects of ethanol, which was consistent with previous findings in SK-Hep1 cells and lung epithelial cells (11,35). To investigate the underlying mechanisms of this phenomenon, the roles of JNK and p38 MAPK were examined (36,37). In the present study, ethanol considerably increased the phosphorylation of JNK and p38, while inhibition of JNK and p38 inhibited ethanol-induced apoptosis in L-02 cells. In NOX4-overexpressing cells, JNK and p38 MAPK were activated and ethanol-induced L-02 apoptosis and death were promoted. Increased NOX4 expression, either by ethanol induction or NOX4 overexpression, therefore has a role in L-02-cell apoptosis.

The MAPK family is important for cells to regulate proliferation and death in response to different internal stressors (37). JNKs are known to be involved in stimulating apoptotic signalling by activating JNK via receptor-initiated extrinsic and mitochondrial intrinsic apoptotic pathways (37,38). JNK and ROS stimulate the activities of pro-apoptotic proteins (such as Bax) and promote apoptosis by inhibiting anti-apoptotic proteins (such as Bcl-2) (39). The present study indicated that ethanol activates the phosphorylation of JNK and increases the expression of Bax, suggesting that apoptosis of L-02 cells may be stimulated by the activation of JNK signalling.

p38 MAPK activation not only boosts the mitochondrial translocation of Bim and Bax and represses the function of Bcl-2 by increasing the phosphorylation of these factors, but also induces the activation of caspase-3 and caspase-9 (38). The present results demonstrated that increased activation of p38 MAPK, caspase-3 and -9 also increased the ratio of Bax to Bcl-2 in ethanol-treated L-02 cells. p38 MAPK activation induced by ethanol was suppressed by NOX4-SiRNA, providing further evidence for the involvement of MAPK in necrosis and apoptosis via NOX overexpression and ROS generation. Thus, p38 MAPK may act as a pro-death effector regardless of what type of cell death occurs in ethanol-treated L-02 cells.

The present study assessed the effects of MAPK inhibitors in L-02 cells exposed to ethanol and their impact on apoptosis. The mechanisms underlying ethanol-induced apoptosis in L-02 cells were indicated to include activation of the JNK and p38 MAPK pathways. Ethanol-induced apoptosis is likely to be mediated via multiple pathways. For instance, ethanol elevates the expression levels of p53, which boosts apoptosis in HepG2 cells via the apoptotic pathway mediated by p53 (40). In addition, ethanol augments the activities of caspase-8 and caspase-9 in HepG2 cells, thereby mediating apoptosis by means of extrinsic and intrinsic pathways (41). MAPK signalling is known to be closely associated with apoptosis (42). However, the effect of the activation of each component of the MAPK cascade is different and depends on its cellular specificity. It was reported that inhibition of the ERK pathway led to increased cell apoptosis and increased activity of caspase-9 and

caspase-3, while JNK inhibitor and p38 MAPK inhibitor markedly suppressed the apoptosis induced by ethanol and reduced caspase activity in SK-Hep1 cells (11). Thus, activation of p38 MAPK and JNK participates in ethanol-induced L-02-cell apoptosis. Therefore, it may be inferred that the apoptotic signal derived from ROS that was produced by NOX, was mainly transduced through the JNK and p38 MAPK pathways. In the present study, p38 MAPK and JNK inhibitors did not reduce the generation of ROS, rather they blocked the transduction of the apoptotic signal induced by ROS. Inhibition of p38 MAPK and JNK also resulted in a decrease in ethanol-induced cell death. This was further confirmed by downregulating NOX4. After NOX4 silencing, ROS generation was reduced followed by JNK and p38 MAPK phosphorylation and finally apoptosis was inhibited. By contrast, with NOX4 overexpression, ROS production was increased and the activation of JNK and p38 MAPK was enhanced, resulting in increased apoptosis of L-02 cells. Taken together, it may be postulated that activation of p38 MAPK and JNK signalling pathways to promote apoptosis were the underlying mechanisms of ethanol-induced toxicity on L-02 cells. In summary, the present study demonstrated that ethanol-induced apoptosis was caspase-dependent. ROS derived from NOX were revealed to have an important role in ethanol-induced apoptosis. L-02 cell viability and apoptosis were affected regulated MAPK inhibitors, suggesting that MAPK signalling pathways are involved in ethanol-induced apoptosis.

In conclusion, apoptosis induced by ethanol in L-02 cells was accompanied with the generation of ROS and elevated expression of NOX4. Subsequent activation of the JNK and p38 signalling pathways then promoted apoptosis in L-02 cells. MAPK inhibitors affected the viability and apoptosis of L-02 cells, indicating that MAPK signalling is involved in ethanol-induced cell apoptosis. These results opened up the possibility for exploring novel therapeutic target of organelle-based ROS to improve the treatment of hepatocyte apoptosis-associated liver diseases.

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Competing interests

The authors declare that they have no competing interests.

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