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Pro- and anti-apoptotic roles of c-Jun N-terminal kinase (JNK) in ethanol and acetaldehyde exposed rat hepatocytes

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Abstract

We have examined the significance of the activation of c-Jun N-terminal kinase (JNK) and p42/44 mitogen-activated protein kinase (MAPK) by ethanol and acetaldehyde in rat hepatocyte apoptosis. Acetaldehyde induced rapid and transient (15 min) activation of p42/44 MAPK followed by activation of JNK, which remained above control up to 1 h. Ethanol activated JNK for up to 4 h. Both ethanol and acetaldehyde caused apoptosis as determined by DNA fragmentation, caspase-3 activation and 2′ [4-ethoxyphenyl]-5-[4-methyl-piperazinyl]-2,5′ -bi-1*H*-benzimidazole (Hoechst 33342) staining. Ethanol-induced apoptosis was blocked by JNK inhibitor 1,9-pyrazoloanthrone (SP600125), indicating that JNK activation is pro-apoptotic. In contrast, acetaldehyde-induced apoptosis was not suppressed by this inhibitor. In fact, SP600125 potentiated acetaldehyde-induced apoptosis, suggesting that JNK activation is anti-apoptotic. Inhibition of p42/44 MAPK by MAPK kinase (MKK1) inhibitor, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126), potentiated apoptosis by acetaldehyde or ethanol, suggesting anti-apoptotic role of p42/44 MAPK. The activation of JNK by ethanol or acetaldehyde was insensitive to the genistein (tyrosine kinase inhibitor), GF109203X (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide, protein kinase C [PKC] inhibitor) and *N*-acetylcysteine (N-AC) (antioxidant), whereas p42/44 MAPK activation by acetaldehyde was inhibited by genistein and GF109203X. Furthermore, p42/44 MAPK activation is not necessary for the JNK activation. In summary, transient activation of JNK by acetaldehyde is anti-apoptotic, whereas sustained activation of JNK by ethanol is pro-apoptotic. The activation of p42/44 MAPK appears to be anti-apoptotic for both ethanol and acetaldehyde. Thus, JNK activation by ethanol and acetaldehyde can be both pro- and anti-apoptotic in hepatocytes.

Keywords: Ethanol; Acetaldehyde; Hepatocyte; p42/44 MAPK; JNK; Apoptosis

1. Introduction

Alcoholic liver damage evolves in stages and eventually leads to cirrhosis. Apoptosis is considered as one of the most important consequences during early alcoholic liver injury (Shimamatsu and Wanless, 1997; Natori et al., 2001). The pathogenesis of alcoholic liver injury is a complex process, which involves the adverse effects of multiple cellular injury mechanism and ethanol metabolism (Niemelä et al., 1995; Nanji and Zakim, 1996). In recent years, there has been increasing support for the role of acetaldehyde, an oxidative metabolite of ethanol, in the alcohol-induced liver damage (Peters and Ward, 1988; Lieber, 1998). However, the exact mechanisms for alteration in signaling by ethanol or acetaldehyde, responsible for apoptosis have not been well defined. Recently, it has been shown that many cellular responses of ethanol are mediated by modulation of mitogen-activated protein kinase (MAPK) signaling (Aroor and Shukla, 2004). MAPKs are involved in various cellular responses, e.g., proliferation, differentiation and apoptosis. Cellular responses mediated by MAPK signaling vary depending upon the intensity and kinetics of MAP kinases. In most cases, transient activation of MAPKs is involved in cell proliferation and DNA synthesis, whereas persistent activation of MAPKs correlates with cell apoptosis and inhibition of DNA synthesis (Chen et al., 1996; Tombes et al., 1998; Chang and Karin, 2001).

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The activation of MAPKs has been shown to be regulated by either activation of protein kinase cascades or inhibition of protein phosphatase. The activation of upstream kinase cascades can be triggered by the activation of tyrosine kinase, protein kinase C (PKC) or G-protein. The emerging evidences have suggested that reactive oxygen species (ROS), including H_2O_2, O_2^- and OH^-, are important signaling molecules regulating MAPKs activity and also important mediators in early alcoholic liver injury (Cardin et al., 2002; Hoek and Pastorino, 2002; Zhou et al., 2003). Moreover, it has been shown that oxidative stress increased JNK activity in hepatocytes (Matsumaru et al., 2003; Liu et al., 2003).

Ethanol has both modulatory and direct effects on MAPKs. It has been shown that ethanol modulates various agonist induced activation of MAPKs signaling (Reddy and Shukla, 1996; Chen et al., 1998; Weng and Shukla, 2000). Moreover, ethanol directly activated c-Jun N-terminal kinase (JNK) in hepatocytes (Ikeyama et al., 2001). We have shown that ethanol and its metabolite acetaldehyde activate p42/44 MAPK and JNK in rat hepatocytes in a temporally distinct manner (Lee et al., 2002). In liver cells, JNK pathway is essential for both cell proliferation and apoptosis. For example, JNK is involved in agonist-stimulated hepatocyte DNA synthesis and has antiapoptotic role in tumor necrosis factor (TNF)-α/cycloheximide-induced apoptosis in Huh7 hepatoma cells (Auer et al., 1998; Liedtke et al., 2002). JNK also acts as a critical mediator of hypoxia/reoxygenation-induced apoptosis (Crenesse et al., 2000) and also TNF-induced apoptosis by glutathione depletion (Matsumaru et al., 2003) in hepatocytes. However, direct correlations among p42/44 MAPK and JNK activation to either pro- or anti-apoptotic process, in relation to acetaldehyde have not been demonstrated. We have therefore conducted a systematic study to gain insight into the roles of ethanol- and acetaldehyde-induced activations of p42/44 MAPK and JNK in apoptosis of rat hepatocytes.

2. Materials and methods

2.1. Materials

The protease inhibitors (aprotinin, leupeptin and pepstatin A), *N*-acetylcysteine (N-AC), L-buthionine-[*S*,*R*]-sulfoximine (BSO) and diethyl maleic acid (DEM) were obtained from the Sigma-Aldrich (St. Louis, MO). The anti-phospho-p42/44 MAPK antibody, anti-phospho-p38 MAPK antibody and cleaved caspase-3 antibody were purchased from Cell Signaling (Beverly, MA). 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) and anti-active JNK antibody (phospho-JNK antibody) were from Promega (Madison, WI); [γ-^{32}P]ATP (3000 Ci/mmol) was from New England Nuclear (Boston, MA); 2′ [4-ethoxyphenyl]-5-[4-methyl-piperazinyl]-2,5′ -bi-1*H*-benzimidazole (Hoechst 33342) and 2′ ,7′ -dichlorofluorescein diacetate (DCFH-DA) were from Molecular probes (Eugene, OR). Glutathione *S*-transferase (GST)-c-Jun (1-79) and 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide (GF109203X) were purchased from BIOMOL (Plymouth Meeting, PA); genistein was from Sigma/RBI (Natick, MA), and 1,9-pyrazoloanthrone (SP600125) was from Calbiochem (San Diego, CA).

2.2. Isolation and treatment of hepatocytes

Hepatocytes were isolated from male Sprague–Dawley rats (200–250 g) using in situ collagenase perfusion method as previously described (Lee et al., 2002). Hepatocyte suspensions showed >90% viability as determined by trypan blue exclusion.

2.3. JNK assay

JNK activity was determined using recombinant substrate protein GST-c-Jun (1–79) (Hibi et al., 1993). Whole cell extracts (100 μg) were diluted by adding the equal volume of a diluting buffer (20 mM HEPES, pH 7.4 and 2.5 mM $MgCl_2$) and mixed with 10 μg of GST-c-Jun (1–79) and 20 μl of glutathione-sepharose beads (50% slurry). The mixtures were rotated at 4 °C for 3 h, and then the beads were collected by centrifugation at 12,000×*g* for 1 min at 4 °C. Beads were washed with 1 ml of cold HEPES binding buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA and 0.05% Triton-X) three times and with 1 ml of kinase assay buffer (20 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 2 mM dithiothreitol and 0.1 mM Na_3VO_4) one time. The pelleted beads were resuspended in 30 μl of kinase assay buffer containing 20 μM ATP and 5 μCi [γ-^{32}P]ATP. After incubation at 30 °C for 30 min, the kinase reaction was terminated by adding 1 ml of ice-cold HEPES binding buffer. The beads were resuspended in sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, and then analyzed on 10% SDS-polyacrylamide gel electrophoresis (PAGE) followed by autoradiography.

2.4. Western blotting

Cell lysates containing equal amounts of protein were eletrophoresed on 10% SDS-PAGE gel. Following electrophoresis, proteins were transferred to nitrocellulose membrane (Bio-Rad). The membrane was washed with 25 mM Tris, pH 7.4, containing 0.1% Tween-20 and 137 mM NaCl, and then blocked with 5% non-fat dry milk for 1 h at room temperature. Blots were incubated with antibodies against phospho-p42/44 MAPK, phospho-JNK, phospho-p38 MAPK or cleaved caspase-3, overnight at 4°C. The blots were incubated with horseradish peroxidase conjugated anti-rabbit immunoglobulin G (IgG). After washing,

the blots were developed with enhanced chemiluminescence (ECL; Pierce) and exposed to X-ray film to detect the protein band.

2.5. DNA fragmentation

DNA fragmentation was measured as previously described (Webster and Anwer, 1998) with some modifications. Briefly, hepatocytes (3×10^6/60 mm dish) treated with ethanol or acetaldehyde for 24 h were washed with ice-cold phosphate buffered saline (PBS) and lysed in 10 mM Tris–HCl, pH 8.0, 10 mM EDTA and 0.5% SDS for 30 min at room temperature. The lysates were centrifuged at 13,000×*g* for 15 min at 4 °C. The supernatants were incubated with proteinase K (200 μg/ml) for 18 h at 37 °C. DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) (saturated with 10 mM Tris, pH 8.0 and 1 mM EDTA), and then precipitated with 2 volume of 95% of ethanol in the presence of 0.3 M sodium acetate and stored at −20 °C overnight. After DNA was collected by centrifugation, DNA pellet was washed twice with 70% ethanol and subsequently air dried. The DNA pellet was resuspended in Tris–EDTA (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA) and treated with RNase A (5 μg/ml) for 1 h at 37 °C. The DNA fragments (20 μg) were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and then visualized under Ultraviolet light.

2.6. Hoechst staining

The apoptotic hepatocytes were detected by staining with the DNA binding fluorescent dye Hoechst 33342 (Rauen et al., 1999). Cells grown on collagen coated glass coverslips (1×10^5/cm^2) were treated with ethanol or acetaldehyde for 24 h, and then washed with ice-cold PBS. Coverslips were fixed in ice cold methanol/acetic acid (3:1) for 5 min. After washing with cold PBS, coverslips were stained with Hoechst 33342 (5 μg/ml) in PBS for 10 min and washed in deionized water. Cells were mounted in 50% glycerol containing 20 mM citric acid and 50 mM disodium orthophosphate. Nuclear staining and morphology were evaluated using fluorescent microscope (Eclipse E600, Nikon). Apoptotic cells were identified as those with brightly staining condensed chromatin or nuclear fragmentation.

2.7. Fluorescence measurement of reactive oxygen species accumulation

To assess levels of accumulation of ROS in hepatocytes, DCFH-DA was used. Non-fluorescent DCFH-DA is oxidized to the fluorescent DCF in the presence of peroxides (Bass et al., 1983; Cathcart et al., 1983). Cultured hepatocytes (1×10^5/cm^2) on collagen coated coverslips were preincubated with 2 μM DCFH-DA for 15 min at 37 °C followed by treatment with ethanol or acetaldehyde. Cells were washed three times with PBS and formation of 2′,7′-dichlorofluorescein (DCF) was detected under the fluorescence microscope (Eclipse E600, Nikon). DCF fluorescence indicates the production of ROS. Phase contrast images of the cocultures were also observed with a transillumination light source for the microscope.

2.8. Statistical analysis

Data are expressed as mean±S.E.M. Differences between control and experimental groups were checked for statistical significance ($P<0.05$) by the Student's *t*-test (two-tailed, unpaired).

3. Results

In the present study, we monitored the pharmacological responses of JNK, p42/44 MAPK and caspase-3 to delineate the role of these components in apoptosis caused by ethanol and acetaldehyde. We have further established the correlation among these components as it relates to the apoptotic process.

3.1. Acetaldehyde- and ethanol-induced apoptosis in hepatocytes

Apoptosis plays a major role in the progression of alcohol-induced liver injury (Shimamatsu and Wanless, 1997). Acetaldehyde is also one of the critical mediators of ethanol induce apoptosis (Zimmerman et al., 1995; Holownia et al., 1999). We have shown earlier that acetaldehyde increases hepatocyte JNK activity in a dose (0.2–5 mM) dependent manner (Lee et al., 2002). Use of acetaldehyde at 1 or 5 mM can be viewed as very high concentration. However, the following evidences offer rationale that higher concentrations of acetaldehyde are patho-physiologically relevant. Chronic alcoholics exhibit as high as 30 μM acetaldehyde in peripheral vein (Salaspuro, 1991). The acetaldehyde concentration in hepatic vein is 10–30 times higher than that in peripheral vein in humans (Nuutinen et al., 1984). In animals also acetaldehyde concentration in portal vein is 10 times higher than in peripheral vein (Matysiak-Budnik et al., 1996). Thus, the estimated concentration in liver can be close to 1 mM. Furthermore, liver can be exposed to additional acetaldehyde in the portal vein absorbed from colon. In fact, the level of acetaldehyde in colon can reach upto 3 mM after 1.5 g/kg dose of ethanol in rats (Koivisto and Salaspuro, 1997). In vitro, serum has the capacity to bind more than 447 mM acetaldehyde and the detected concentration of acetaldehyde represents free rather than total acetaldehyde including its adducts (Brecher et al., 1997). Taken together, it is apparent that millimolar concentrations of acetaldehyde are rele-

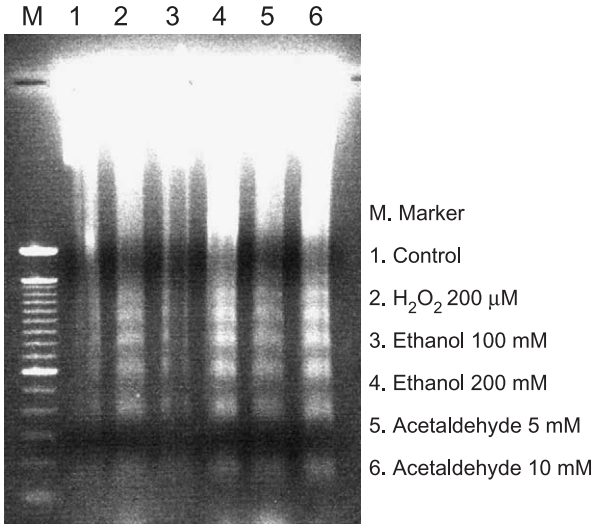


Fig. 1. Ethanol- and acetaldehyde-induced DNA fragmentation in hepatocytes. Hepatocytes were treated with vehicle control (lane 1), 200 μM H_2O_2 (lane 2), 100 mM and 200 mM ethanol (lanes 3 and 4), or 5 mM and 10 mM acetaldehyde (lanes 5 and 6) for 24 h. The DNA ladder marker size is 100 bp (lane M). DNA fragmentation assays were carried out as described under Materials and methods.

vant in vivo and justify the doses used in the present study.

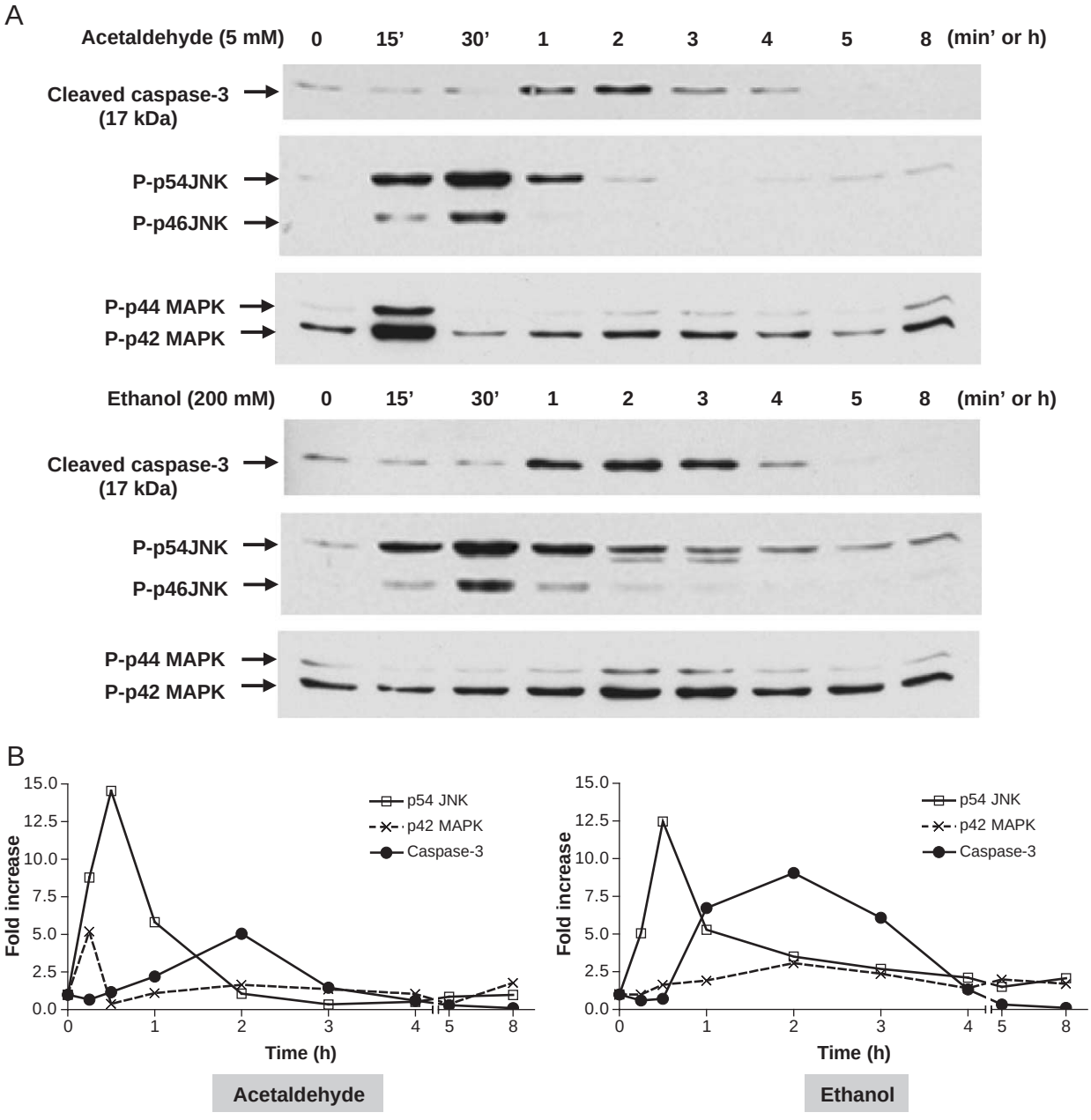
In the present study, the acetaldehyde- or ethanol-induced apoptosis was first examined in hepatocytes by detection of internucleosomal DNA cleavage. Cells were treated with acetaldehyde or ethanol for 24 h. For a positive control, cells were treated with H_2O_2 for 24 h. DNA was extracted from the cells and electrophoresed on the agarose gel. The results showed that treatment of cells with ethanol or acetaldehyde caused dose-dependent apoptosis in hepatocytes (Fig. 1). The extent of the DNA fragmentation by 200 mM ethanol was greater than that by 5 mM acetaldehyde. It may be noted that in the presence of the 200 mM ethanol about 0.35 mM acetaldehyde was produced in hepatocytes (Lee et al., 2002). We next determined caspase-3 activation, a marker of the apoptotic protease cascade, by immunoblotting with anti-cleaved caspase-3 antibody. Activation of caspase-3 requires proteolytic processing of its inactive enzyme. The antibody detects only the cleaved fragment (17/19 kDa) of activated caspase-3. Cells were treated with acetaldehyde or ethanol for different time periods (0–8 h) and the activation of caspase-3 was assayed (Fig. 2A). Both acetaldehyde and ethanol raised the levels of cleaved caspase-3 within 1 h with maximum increase at 2 h. Caspase-3 activity significantly decreased at 3 h after acetaldehyde treatment, but remained markedly elevated at 3 h after ethanol treatment, showing a longer activation of caspase-3 by ethanol (Fig. 2A and B). Ethanol increased caspase-3 activity about two times higher than that with acetaldehyde at 2 h; a profile consistent with DNA fragmentation observed by gel electrophoresis. This kinetic behavior of caspase-3 was compared with those of JNK and p42/44 MAPK. In acetaldehyde-treated cells, the transient activation of p42/44 MAPK occurred at 15 min (a ~5-fold increase) followed by maximal activation of JNK at 30 min and then caspase-3 activation. This demonstrated a unique time-dependent activation of p42/44 MAPK, JNK and caspase-3, in that order (Fig. 2A and B). In contrast, ethanol treatment first increased JNK activation, maximum activation at 30 min, followed by maximum activation of both p42/44 MAPK and caspase-3 at 2 h (Fig. 2A and B). Ethanol caused mild activation of p42/44 MAPK, ~2.5-fold at 2 h. Ethanol-induced JNK activation gradually decreased and the activity was observed elevated above basal level up to 4 h, which is longer activation than observed with acetaldehyde treatment. These results demonstrated different kinetic behavior of p42/44 MAPK, JNK and caspase-3 activation by acetaldehyde and ethanol. It may be mentioned here that treatment with acetaldehyde or ethanol under these (above) conditions do not alter the protein levels of the JNK or MAPK (Lee et al., 2002) and this also reflected that the loading of the gels was similar.

The effects of ethanol on p38 MAPK signaling in hepatocytes was also examined. The treatment of hepatocytes with ethanol (10 min–24 h) had no effect on activation of p38 MAPK (Fig. 2C). Thus, we did not pursue the role of p38 MAPK in the hepatocytes exposed to ethanol.

3.2. Role of JNK and p42/44 MAPK activation in acetaldehyde- and ethanol-induced apoptosis

Next, the roles of JNK and p42/44 MAPK activation in acetaldehyde- or ethanol-induced apoptosis were determined. JNK activation may cause caspase-3 activation (Ho et al., 2000) or may be a consequence of caspase-3 activation (Sawada et al., 2000). Comparison of the time course of JNK activation by acetaldehyde or ethanol and the time course of caspase-3 activation clearly showed that JNK activation preceded the caspase-3 activation (Fig. 2A and B). These results suggest that JNK activation may modulate caspase-3 activation. This was further examined by the use of JNK specific inhibitor, SP600125. Pretreatment of hepatocytes with 30 μM SP600125 for 3 h significantly decreased (~60%) acetaldehyde- and ethanol-induced JNK activation (Fig. 3A). Acetaldehyde and ethanol treatment for 2 h increased caspase-3 activation 3.5-fold and 6.9-fold,

Fig. 2. Ethanol- and acetaldehyde-induced MAPKs and caspase-3 activation in hepatocytes. (A) Hepatocytes were stimulated with 5 mM acetaldehyde or 200 mM ethanol for indicated time. Whole cell extracts were prepared and the activations of caspase-3, p46/p54 JNK and p42/44 MAPK were detected by Western blotting (see Materials and methods). (B) The data are representative of three independent experiments. Fold activation of p54 JNK, p42 MAPK and caspase-3 from Western blotting were quantitated by densitometric analysis. (C) Hepatocytes were stimulated with ethanol (0–200 mM) for indicated time (10 min–24 h). Whole cell extracts were prepared and the activations p38 MAPK was detected by Western blotting.


A
Acetaldehyde (5 mM) 0 15' 30' 1 2 3 4 5 8 (min' or h)
Cleaved caspase-3 (17 kDa)
P-p54JNK
P-p46JNK
P-p44 MAPK
P-p42 MAPK
Ethanol (200 mM) 0 15' 30' 1 2 3 4 5 8 (min' or h)
Cleaved caspase-3 (17 kDa)
P-p54JNK
P-p46JNK
P-p44 MAPK
P-p42 MAPK
B
Fold increase
p54 JNK
p42 MAPK
Caspase-3
Time (h)
Acetaldehyde
Ethanol
C
Ethanol (mM) 0 20 50 100 200
10 min
1 h
4 h
24 h
Phospho-p38 MAPK

respectively, in dimethylsulfoxide (DMSO) (vehicle for SP600125 and U0126)-treated cells (Fig. 3B). In the presence of SP600125, ethanol-induced caspase-3 activation was completely blocked, whereas, to our surprise, acetaldehyde-induced caspase-3 activation increased 10.7-fold. Thus, SP600125 treatment potentiated acetaldehyde increased caspase-3 activity by about three times over the DMSO control (Fig. 3B). These results indicated a differential role of JNK in hepatocyte apoptosis by ethanol and acetaldehyde. Fig. 2A shows that acetaldehyde treatment

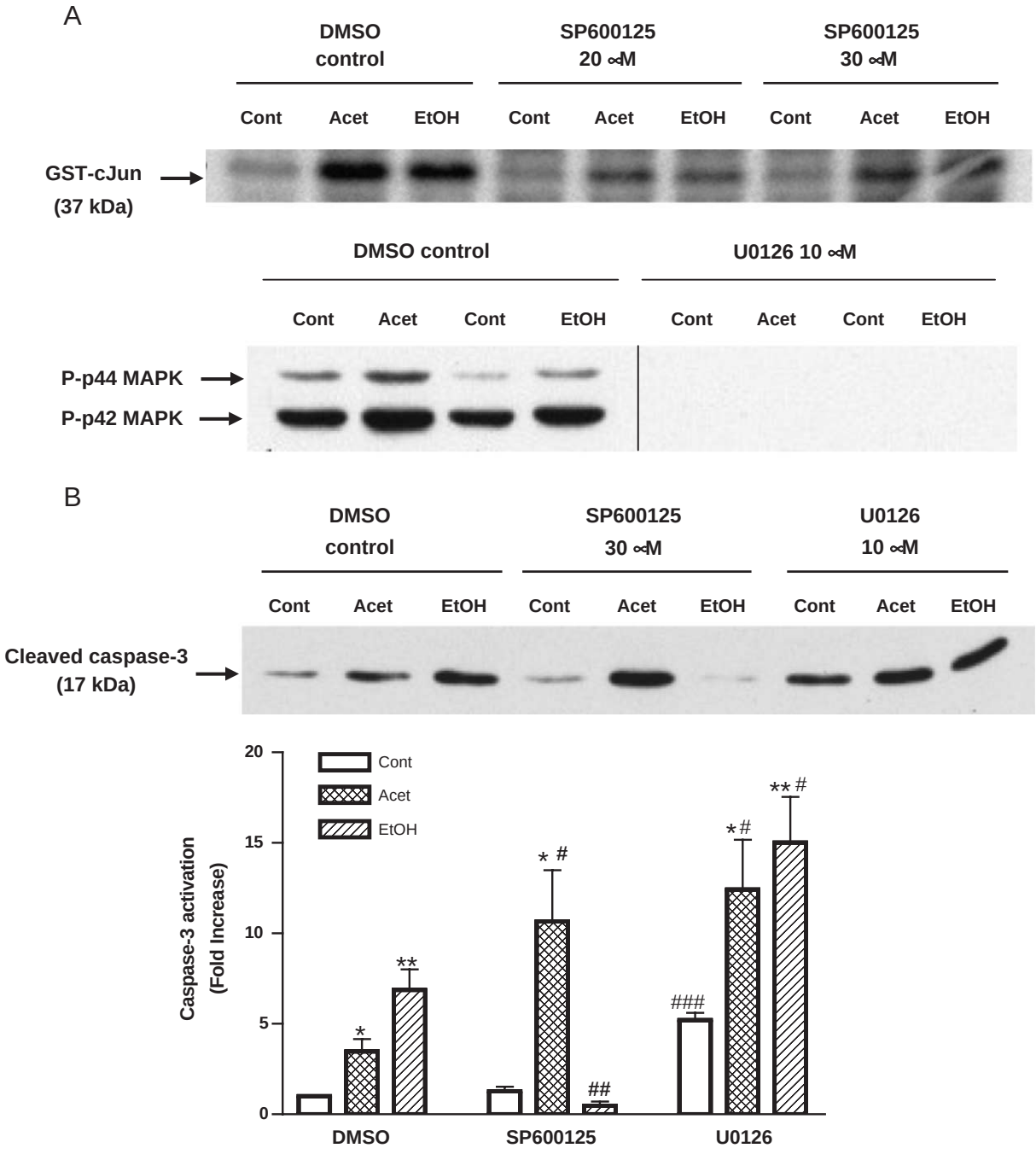


Fig. 3. Effects of SP600125 and U0126 on ethanol- and acetaldehyde-induced apoptosis in hepatocytes. (A) Hepatocytes were pretreated with SP600125 (20 or 30 μM) or U0126 (10 μM) for 3 h and 1 h, respectively. Then cells were stimulated with 5 mM acetaldehyde (1 h for JNK activity and 10 min for p42/44 MAPK activity) and 200 mM ethanol (1 h). Whole cell extracts were subjected to measurements of JNK activity using GST-c-Jun as a substrate and p42/44 MAPK activity by Western blot using antiphospho-p42/44 MAPK (P-p42/44 MAPK). (B) Hepatocytes were pretreated with 30 μM SP600125 for 3 h and 10 μM U0126 for 1 h, and then stimulated with 5 mM acetaldehyde or 200 mM ethanol for 2 h. The activation of caspase-3 was determined by Western blotting with anti-cleaved caspase-3 antibody. The fold increases in caspase-3 activity were quantitated by densitometry. Values represented are mean±S.E.M. (bars), n=4 (independent experiments). *P<0.05; **P<0.01 vs. control. $^{\#}P$<0.05; $^{\#\#}P$<0.01; $^{\#\#\#}P$<0.001 vs. corresponding DMSO-treated cells (Cont, control; Acet, acetaldehyde; EtOH, ethanol).

caused maximum activation of JNK at 30 min. The activity remained high for 1 h and decreased to basal level at 2 h. Ethanol-induced JNK activity also shows a maximum response at 30 min, but remained above control at least for 4 h. These differences in the sustenance of the JNK activation by acetaldehyde and ethanol may lead to different responses of cells to apoptotic change.

Next, we addressed the role of p42/44 MAPK in apoptosis by ethanol and acetaldehyde in hepatocytes by the use of MAPK kinase (MKK1) inhibitor, U0126. This inhibitor exerts its effects by suppressing the activation of MKK1 by Raf and not by blocking its activity (Davies et al., 2000). U0126 has been shown to inhibit other kinases including p38 MAPK, but only at ~1000-fold higher concentrations than those required for inhibition of MAPK cascade (Davies et al., 2000). DeSilva et al. (1998) have shown that p42/44 MAPK phosphorylation was blocked 96% in the presence of 10 μM U0126. In contrast, p38 MAPK phosphorylation was only slightly affected under the same condition, which did not result in a decrease in p38 kinase activity. In our study, 10 μM of U0126 was used, a concentration known to inhibit MKK1 but with negligible effect on p38 MAPK (DeSilva et al., 1998). Furthermore, 200 mM ethanol had little, if any, effect on the activation of p38MAPK as mentioned above. Therefore, any effect of U0126 on p38 MAPK is of no consequence in our studies. The inhibition of p42/44 MAPK by pretreatment of cells with U0126 for 1 h (Fig. 3A), increased both basal (5.2 fold) and acetaldehyde (12.4-fold)- or ethanol (15-fold)-induced caspase-3 activation, indicating that p42/44 MAPK inhibition had a pro-apoptotic effect (Fig. 3B).

In further studies, the morphological evaluation of apoptotic hepatocytes was performed by Hoechst 33342 staining, and then analyzed using a fluorescent microscope. This procedure provides bright fluorescence by which the apoptotic cells with condensed chromatin and with chromatin pieces of fragmented nuclei can be easily identified. Fig. 4A shows that the treatment of hepatocytes with both acetaldehyde and ethanol for 24 h caused apoptotic nuclear changes and an apparent reduction in the size of nuclei (Fig. 4Ab and Ac) compared with control cells (Fig. 4Aa). Acetaldehyde and ethanol treatment increased the amount of apoptosis by 7.7% and 19.2%, respectively (Fig. 4B). Ethanol increased apoptotic cells, observed by Hoechst 33342 staining, were about 2.5 times more than seen after acetaldehyde treatment. Pretreatment with SP600125 reduced the amount of apoptosis by ethanol to 13.4% albeit the sizes of nuclei still remain small (Fig. 4Af and B). However, acetaldehyde-induced apoptosis was enhanced by SP600125 to 12.7% (Fig. 4Ae and B). These observations are consistent with above results, i.e. ethanol (200 mM) caused more apoptosis than acetaldehyde (5 mM). The inhibition of ethanol-induced JNK had antiapoptotic effect, whereas the inhibition of acetaldehyde-induced JNK had pro-apoptotic effect.

3.3. Role of p42/44 MAPK in prolonged activation of JNK and apoptosis

Many studies have shown the existence of a crosstalk between MAPK cascades whereby the activity of one MAPK can be regulated by another (Surapisitchat et al., 2001; Shen et al., 2003). Ethanol and acetaldehyde caused different time dependent activation of JNK which is related to the opposite cellular response (Figs. 2A and 3B). Acetaldehyde increased JNK activation following p42/44 MAPK, whereas ethanol-induced prolonged activation of JNK occurs without the preceding activation of p42/44 MAPK. Therefore, the possibility that antiapoptotic (cytoprotective) effect of p42/44 MAPK was mediated through regulating the duration of JNK activation was investigated. Hepatocytes were pretreated with 10 μM U0126 for 1 h, and then stimulated with acetaldehyde or ethanol for different times (0–4 h). In DMSO (vehicle)-pretreated cells, acetaldehyde treatment caused no significant increase in JNK activation at 2–3 h, but it increased in the presence of U0126. Similarly, the inhibition of p42/44 MAPK also increased ethanol-induced JNK activation at 2–4 h (Fig. 5A and B). These results suggest that p42/44 MAPK inhibition prolonged JNK activation by acetaldehyde or ethanol treatment. Obviously, activation of p42/44 MAPK is not a prerequisite for JNK activation by ethanol or acetaldehyde.

3.4. Upstream regulation of MAPKs activation by ethanol and acetaldehydes

MAPKs activation can be regulated by many different upstream signaling pathway, including tyrosine kinase, PKC and Ras. In the previous study, however, genistein and GF109203X, which are selective tyrosine kinase and PKC inhibitors, respectively, failed to inhibit ethanol- or acetaldehyde-induced JNK activation (Lee et al., 2002). Also, treatment with manumycin, farnesyltransferase inhibitor which is known to inhibit Ras, failed to decrease JNK activation (unpublished data). It has been also suggested that the activities of p42/44 MAPK and JNK are regulated by distinct pathways (Yoshizumi et al., 2000; Wei et al., 2001; Lee and Esselman, 2002). To test this possibility, hepatocytes were pretreated with 10 μM GF109203X or 30 μM genistein and then stimulated with 5 mM acetaldehyde for 10 min. The activation of p42/44 MAPK was detected by Western blotting. Acetaldehyde-induced p42/44 MAPK was inhibited by genistein and GF109203X (Fig. 6), indicating the possible involvement of tyrosine kinase and PKC in the activation of p42/44 MAPK by acetaldehyde. It is therefore likely that distinct pathways for p42/44 MAPK and JNK activation by acetaldehyde exist in hepatocytes. It also favors the conclusion that activation of JNK by acetaldehyde is not dependent on p42/44 MAPK.

Since ROS are important signaling molecules regulating MAPKs activity, we investigated this possibility in our studies. We first measured the accumulation of ROS

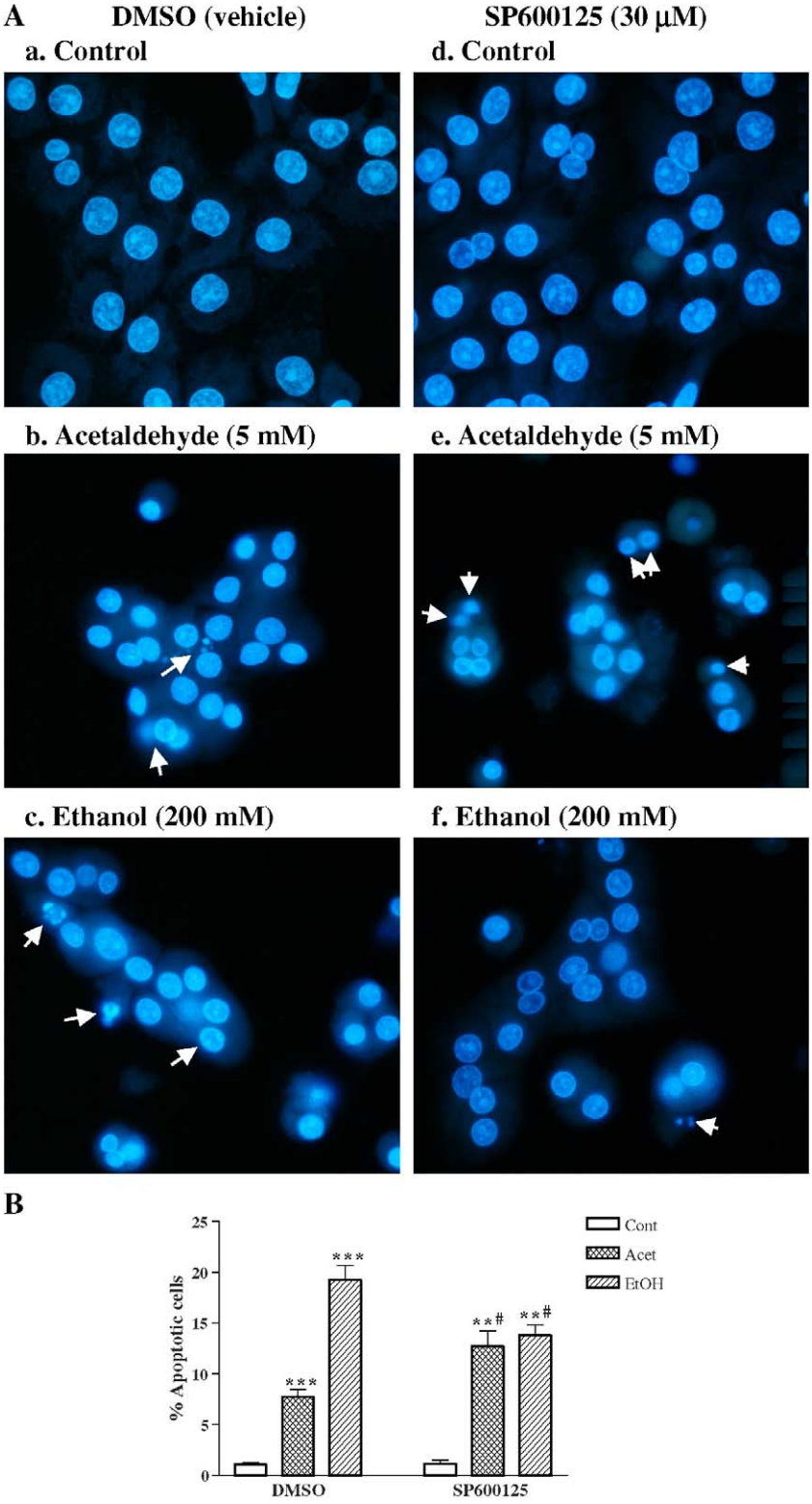


Fig. 4. Alterations in the nuclear morphology of hepatocytes by ethanol and acetaldehyde. (A) Hepatocytes were preincubated with DMSO vehicle control (a–c) or 30 μM SP600125 dissolved in DMSO (d–f) for 3 h, and these served as control (a, d) or were stimulated with 5 mM acetaldehyde (b, e) or 200 mM ethanol (c, f) for 24 h. Nuclear morphology was monitored by fluorescence microscopy (magnification, ×400) after staining the cells with membrane permeable DNA binding fluorescent dye Hoechst 33342 (5 μg/ml). Arrows indicate condensation and margination of the chromatin, nuclear condensation or nuclear fragmentation. (B) Five hundred cells were counted and the number of apoptotic cells expressed as a percentage of the total number of cells counted. Values represented are mean±S.E.M. (bars), n=3 (independent experiments). $^{**}P<0.01$; $^{***}P<0.001$ vs. control. $^{\#}P<0.05$ vs. corresponding DMSO-treated cells (Cont, control; Acet, acetaldehyde; EtOH, ethanol).

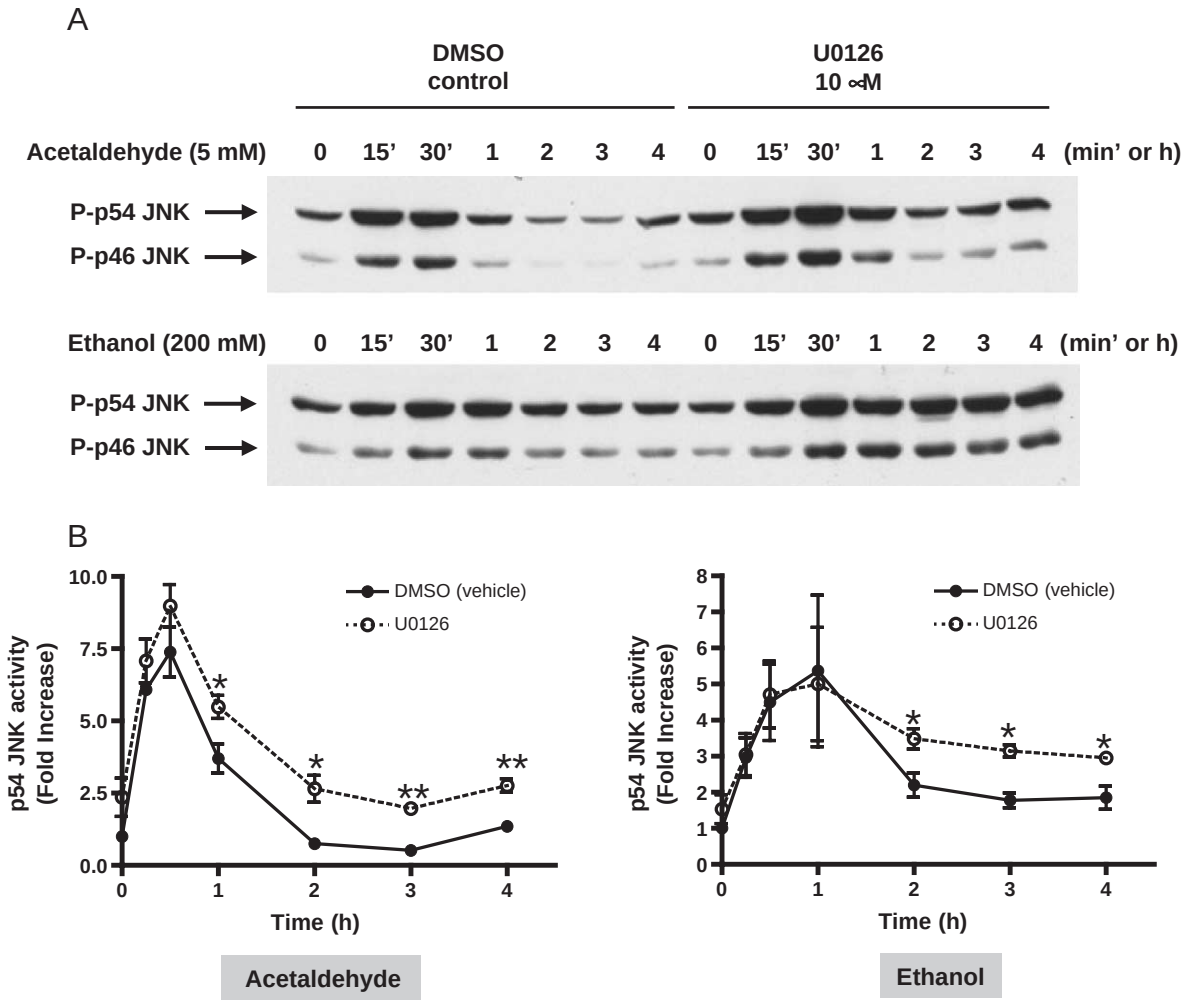


Fig. 5. The inhibition of p42/44 MAPK activation prolongs acetaldehyde- and ethanol-induced JNK activation. (A) Hepatocytes were pretreated with 10 μM U0126 for 1 h. Then, cells were stimulated with 5 mM acetaldehyde and 200 mM ethanol for the indicated times. The activation of JNK was monitored by Western blotting with anti-phospho JNK antibody. (B) The fold increases in p54 JNK activity were quantitated by densitometric analysis. The values represented are mean±S.E.M. (bars), n=3 (independent experiments). *P<0.05; **P<0.01 vs. corresponding DMSO-treated samples.

in ethanol- or acetaldehyde-treated cells using DCFH-DA (H_2O_2 or hydroxyl radical-sensitive probe) (Vanden Hoek et al., 1997; Duranteau et al., 1998). Cells were treated with DCFH-DA for 15 min followed by treatment with 5 mM acetaldehyde, 200 mM ethanol or 200 μM H_2O_2 for 1 h. DCF fluorescence increased in hepatocytes incubated with acetaldehyde, ethanol or H_2O_2 compared to control cells (Fig. 7A–D). N-AC is an antioxidant known to inhibit ROS production by increasing the level of intracellular glutathione (GSH) (Peristeris et al., 1992; Traber et al., 1992). Pretreatment of hepatocytes with N-AC for 24 h apparently diminished the increase in DCF fluorescence by acetaldehyde, ethanol, or H_2O_2 (Fig. 7E–H). To further examine the possible role of ROS in the activation of p42/44 MAPK and JNK, hepatocytes were treated with 1 mM H_2O_2 for different time periods (0–2

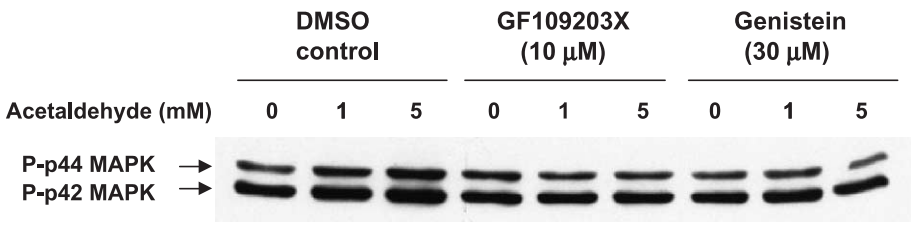


Fig. 6. Effects of genistein and GF109203X on the activation of p42/44 MAPK by acetaldehyde. Hepatocytes were incubated with or without 30 μM genistein and 10 μM GF109203X for 2 h and 30 min, respectively, then stimulated with 5 mM acetaldehyde for 10 min. Whole cell extracts were prepared and the activations of p42/44 MAPK was detected by Western blotting using antiphospho-p42/44 MAPK (P-p42/44 MAPK) antibody.

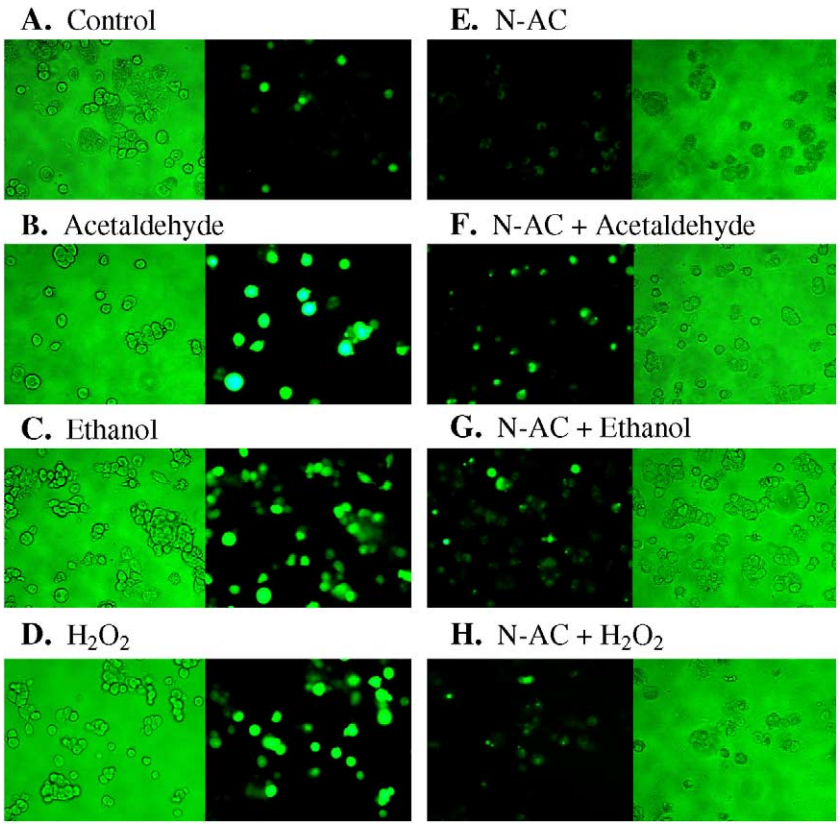


Fig. 7. ROS accumulation by ethanol or acetaldehyde treatment. Hepatocytes were preincubated with vehicle (A–D) or 10 mM N-AC (E–H) for 24 h. Then, these served as control (A and E) or stimulated with 5 mM acetaldehyde (B and F), 200 mM ethanol (C and G) and 200 μM H_2O_2 (D and H) for 1 h in the presence of 2 μM DCFH-DA. The formation of DCF (green fluorescence) was detected under the fluorescence microscope. Phase contrast images of the cocultures were also observed with a transillumination light source for the microscope.

h). Whole cell extracts were prepared and subjected to immunoblot analysis with antiphospho-p42/44 MAPK and antiphospho-JNK antibody. H_2O_2 caused peak activation of p42/44 MAPK at 5–10 min, which returned to basal level at 30 min, showing a transient activation. On the other hand, JNK phosphorylation was significantly increased at 10–15 min followed by a decrease to the control level at 2 h (Fig. 8A). These results show that H_2O_2 increased activation of p42/44 MAPK followed by JNK activation, similar to that observed with acetaldehyde (Fig. 2B). The activation of p42/44 MAPK and JNK by H_2O_2 was concentration-dependent (Fig. 8B). The involvement of ROS in ethanol- or acetaldehyde-induced JNK activation was further examined using BSO, inhibitor of GSH biosynthesis, or DEM, glutathione depletor. Pretreatment of cells with 1 mM BSO for 16 h increased basal p46/54 JNK ~3.5/3-fold (Fig. 9A). Similarly, pretreatment of cells with DEM for 30 min also increased basal p46/54 JNK 4.4/5.3-fold at 200 μM and 10.4/12.8-fold at 500 μM (Fig. 9B). However, the fold increases in JNK activation by acetaldehyde and ethanol were not affected by BSO or DEM pretreatment (Fig. 9A and B). To examine the effect of antioxidant on JNK activation by ethanol and acetaldehyde, cells were pretreated with N-AC (10 mM) for 24 h, and then stimulated with acetaldehyde (5 mM) or ethanol (200 mM) for 1 h. Although pretreatment of cells with N-AC decreased basal JNK activation by ~50%, N-AC failed to decrease the fold increase in JNK activation by ethanol or acetaldehyde (Fig. 9C). Similarly, other antioxidants, α-tocopherol (vitamin E) and ascorbic acid (vitamin C) had no effect on JNK activation by ethanol and acetaldehyde (data not shown). These results suggest that the basal JNK activity is susceptible to antioxidant but ethanol- or acetaldehyde-induced JNK is not affected, indicating oxidative stress-independent pathway for JNK activation.

4. Discussion

We investigated the roles of ethanol- and acetaldehyde-induced activation of p42/44 MAPK and JNK in rat hepatocyte apoptosis. Our data demonstrated that both ethanol and acetaldehyde caused the activation of p42/44 MAPK and JNK and cell apoptosis. Ethanol caused prolonged activation of JNK and the inhibition of its activity decreased cell apoptosis. In contrast, acetaldehyde-induced activation of JNK was short-lived (transient) and its inhibition potentiated apoptosis. This suggested that JNK activated by ethanol and its metabolite acetaldehyde

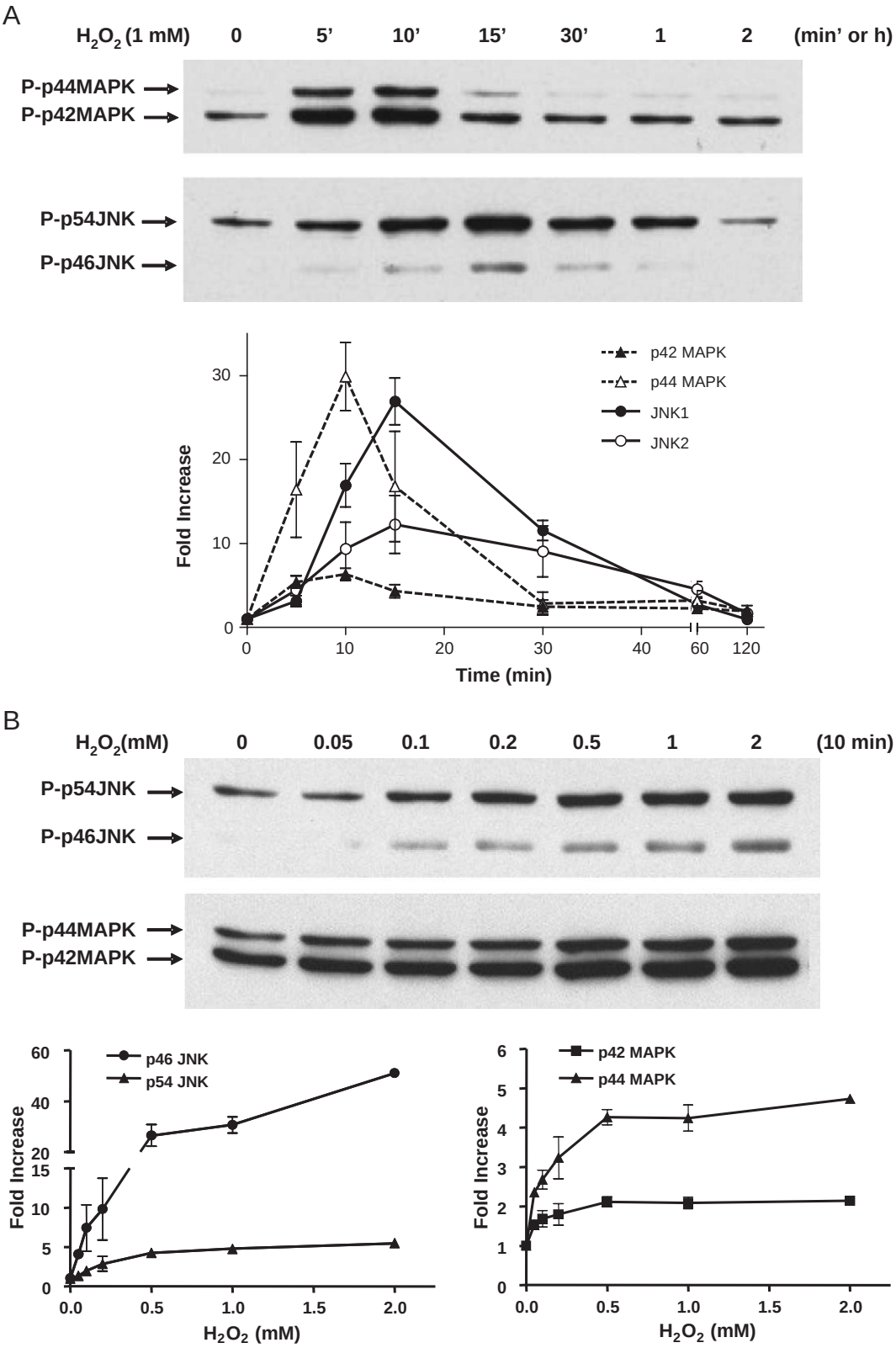


Fig. 8. Effects of H_2O_2 treatment on p42/44 MAPK and JNK activation. Hepatocytes were treated with 1 mM H_2O_2 for the indicated times (A) or stimulated with various concentrations of H_2O_2 (0–2 mM) for 10 min (B). Phosphorylation of p42/44 MAPK and p46/p54 JNK was monitored by Western blotting of whole cell extracts with antiphospho-p42/44 MAPK and antiphospho-JNK antibodies. The data are representative of three independent experiments.

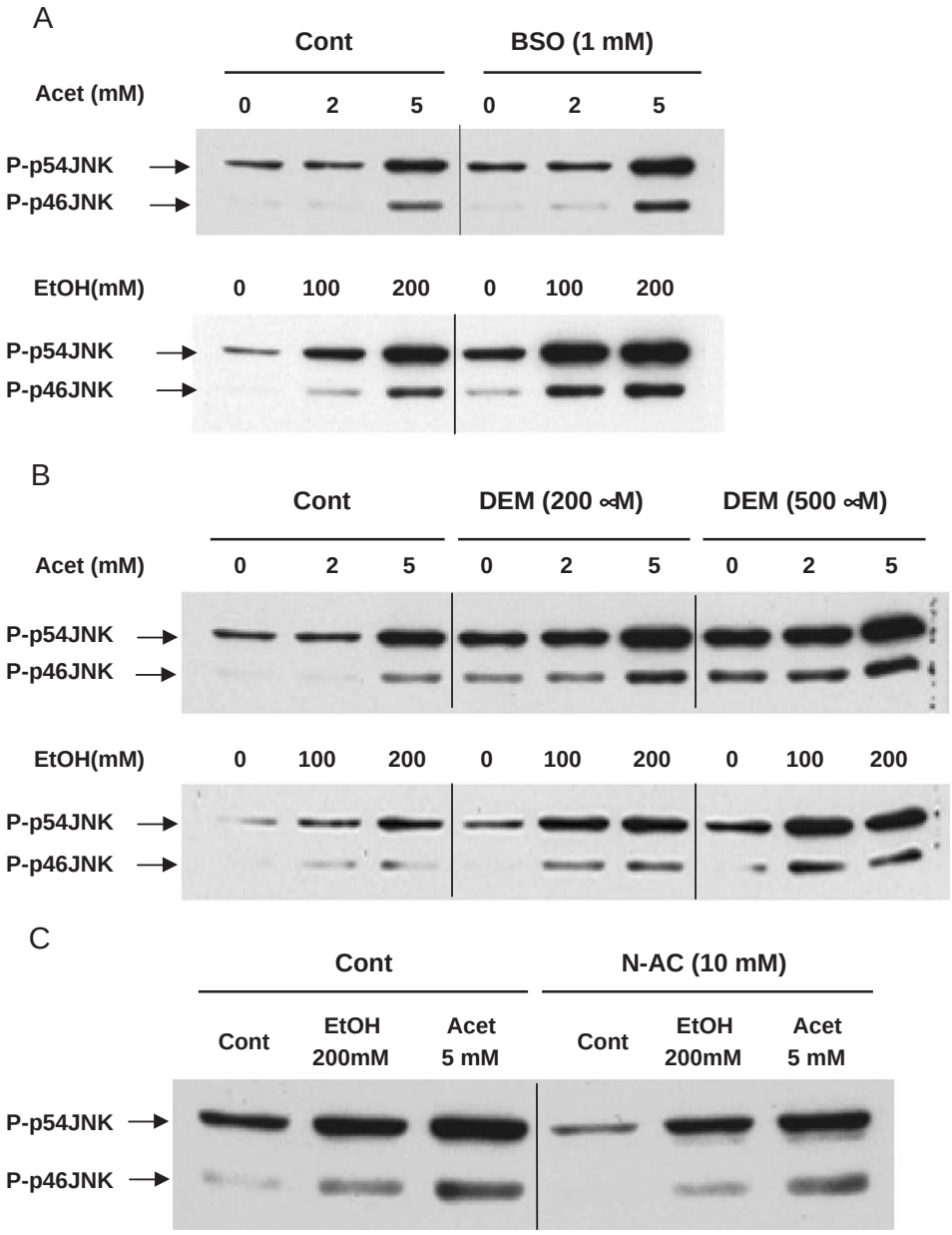


Fig. 9. Effects of BSO, DEM or N-AC on the activation of p46/54 JNK by ethanol or acetaldehyde. Hepatocytes were pretreated with BSO (1 mM) for 16 h (A), DEM (200 and 500 μM) for 30 min (B), N-AC (10 mM) for 24 h (C) or control vehicle (cont). Then, cells were stimulated with ethanol (EtOH) or acetaldehyde (Acet) for 1 h. Whole cell extracts were prepared and the same amount of protein from each sample was subjected to Western blotting with antiphospho-JNK antibody.

has opposite roles in apoptosis. In other words, ethanol- and acetaldehyde-induced apoptosis is JNK dependent and JNK independent, respectively. On the other hand, increase in p42/44 MAPK activity by both ethanol and acetaldehyde is involved in antiapoptotic role. Furthermore, p42/44 MAPK and JNK activity by acetaldehyde are regulated by distinct pathway, and activation of p42/44 MAPK is not necessary for JNK activation.

The activation of JNK has been shown to be involved in both cell survival and cell apoptosis in hepatocytes (Auer et al., 1998; Graf et al., 2002; Czaja, 2003; Schwabe et al., 2003). However, the mechanisms by which these reactions undergo are not well understood. Recent studies have suggested that the duration and magnitude of JNK activation are important for determining its biological effects on the cell (Chang and Karin, 2001). In agreement with our results, Liu et al. (2002) have shown that in TNF-treated hepatocytes, nuclear factor κB (NFκB) inactivation converted the transient activation of JNK to one of prolonged activation which initiated cell death. On the contrary, TNF/cycloheximide-induced JNK activation had antiapoptotic effects in Huh7 cells (Liedtke et al., 2002).

The activation of MAPKs has been shown to be regulated by phosphorylation cascades which are triggered

by tyrosine kinase, PKC or G-protein. The activation of p42/44 MAPK by acetaldehyde was inhibited by genistein (tyrosine kinase inhibitor) and GF109203X (PKC inhibitor), whereas these inhibitors have no effect on JNK activation (Lee et al., 2002). This implies that distinct pathways for p42/44 and JNK activation by acetaldehyde exist in hepatocytes and that ethanol- and acetaldehyde-induced JNK activation occur through pathways independent of tyrosine kinase and PKC. The treatment with manumycin, Ras inhibitor, also failed to block JNK activation. We have also examined the possible role of oxidative stress in ethanol- and acetaldehyde-induced JNK activation. Although pretreatment of cells with antioxidant, N-AC, decreased basal JNK activation, N-AC was ineffective in modifying acetaldehyde- and ethanol-induced JNK activation, indicating oxidative stress-independent pathway for JNK activation. It has been also reported that ethanol-induced activation of JNK in H9c2 cell line by a delay in JNK dephosphorylation without an increase in immediate upstream kinase SEK1 activity (Meriin et al., 1999). In other study, the activation of JNK in hepatocytes from rats chronically treated with ethanol was accompanied by both activation of SEK1 and the inhibition of JNK phosphatase (Chung et al., 2002). Therefore, it may be possible that the activation of JNK by ethanol or acetaldehyde increased by inhibition of JNK phosphatase in hepatocytes. This needs to be investigated in future. It would be interesting to delineate the mechanisms regulating the activation of JNK, which are ROS, tyrosine kinase and PKC-independent.

Although studies have shown that the activation of MAPKs are regulated by separate signal transduction cascade, recent evidences have suggested that in various cases cross talk among MAPK family members may regulate the activity of MAPKs differently (Surapisitchat et al., 2001; Shen et al., 2003). Our results show that the inhibition of p42/44 MAPK increased both basal and ethanol- or acetaldehyde-induced apoptosis, and did induce further sustained JNK activation by ethanol and acetaldehyde. These results suggest that p42/44 MAPK activation plays a role in the antiapoptotic effects by either activation of survival pathway or regulating the duration of JNK activation. In the absence of p42/44 MAPK, sustained activation of JNK is involved in hepatocyte apoptosis. It remains to be determined how p42/44 MAPK prolongs JNK activation in hepatocytes. One possibility is through MAPK phosphatases (MKPs). MKPs can be induced by MAPKs and may subsequently regulate their activity (Brondello et al., 1997; Hirsch and Stork, 1997; Keyse, 2000). There is evidence that the crosstalk between p42/44 MAPK and JNK is mediated by MKPs. In kidney epithelial cells, p42/44 MAPK suppressed JNK through MKP-2 induction (Paumelle et al., 2000).

Observations on the differential role of ethanol- and acetaldehyde-induced JNK activation in hepatocyte apoptosis are interesting. It has been suggested that many effects of ethanol including ethanol-induced cell apoptosis in astrocytes and Chinese hamster ovary cell line are mediated via ethanol metabolite acetaldehyde (Holownia et al., 1999; Zimmerman et al., 1995). Although acetaldehyde accumulation (0.35 mM) after ethanol (200 mM) treatment was detected in hepatocytes, JNK activation by 200 mM ethanol was much higher than that expected with 0.35 mM acetaldehyde generated under these conditions (Lee et al., 2002). This suggested that ethanol may activate JNK by both acetaldehyde-dependent and -independent pathways. Additionally, ethanol also activated JNK in HepG2 cell line (unpublished data) which does not express detectable Cyp2E1 (Dai et al., 1993) and has very low alcohol dehydrogenase activity (Carter and Wands, 1988). These results suggest that ethanol can activate JNK independent of ethanol metabolism. Differential roles of ethanol and acetaldehyde on signal transduction pathway have been described in the literature. In freshly isolated hepatocytes, ethanol inhibited JAK-STAT signaling pathway which did not involve ethanol metabolism (Chen et al., 2001). Different mechanisms by ethanol and acetaldehyde have also been shown to induce activation of AP-1 and NFκB in HepG2 cells (Roman et al., 1999). Thus, it is possible that ethanol and acetaldehyde activated JNK by distinct pathways leading to different cellular responses.

In summary, our data demonstrated that the treatment of hepatocytes with both ethanol and acetaldehyde induced JNK activation and apoptosis. However, the role of JNK activation in apoptosis by ethanol and acetaldehyde shows opposite effects; ethanol-induced JNK activation increased apoptosis, whereas acetaldehyde-induced JNK activation decreased apoptosis. Thus, ethanol metabolism could be one of the important factors determining the multiple roles of JNK in ethanol-induced apoptosis in vivo and may be relevant to our mechanistic understanding of the alcoholic liver damage.

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