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Histone H3 phosphorylation at serine 10 and serine 28 is mediated by p38 MAPK in rat hepatocytes exposed to ethanol and acetaldehyde*

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Abstract

Ethanol modulates mitogen-activated protein kinases (MAPKs). We have now investigated the influence of ethanol and its metabolite, acetaldehyde on histone H3 phosphorylation to ascertain downstream targets of MAPKs. In primary culture of rat hepatocytes, ethanol and acetaldehyde increased phosphorylation of nuclear histone H3 at serine 10 and serine 28. Specific inhibitors of p38 MAPK, SB203580, PD169316 and SB202190 blocked this phosphorylation. The inactive analogue, SB202474 had no effect. In contrast, c-Jun N-terminal kinase (JNK) inhibitor, SP600125 or MAP/ERK kinase (MEK) 1/2 inhibitor, PD98059 had no effect on the histone H3 phosphorylation. The p38 MAPK activation correlated with upstream activation of MAPK kinase (MKK) 3/6 but was independent of protein synthesis. In the nuclear fraction, the phosphorylation of p38 MAPK and its protein level increased with peak activation at 24 h by ethanol and at 30 min by acetaldehyde. These responses were ethanol and acetaldehyde dose dependent. Surprisingly, the phosphorylation of p38 MAPK was undetectable in the cytosolic fraction suggesting a subcellular selectivity of p38 MAPK signaling. The phosphorylation of JNK and p42/44 MAPK and their protein levels also increased in the nuclear fraction. Although ethanol caused translocation of all three major MAPKs (p42/44 MAPK, JNK, p38 MAPK) into the nucleus, histone H3 phosphorylation at serine 10 and serine 28 was mediated by p38 MAPK. This histone H3 phosphorylation had no influence on ethanol and acetaldehyde induced apoptosis. These studies demonstrate for the first time that ethanol and acetaldehyde stimulated phosphorylation of histone H3 at serine 10 and serine 28 are downstream nuclear response mediated by p38 MAPK in hepatocytes.

Keywords

Ethanol; Acetaldehyde; Hepatocytes; Histone; MAPK; Epigenetics

1. Introduction

The liver is a major target organ for the deleterious effects of ethanol. The ethanol effects can be elicited by ethanol itself or by its metabolites. Ethanol metabolic stress plays a major role

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(Shukla and Aroor, 2006). Acetaldehyde is a major product of oxidative metabolism of ethanol known to be involved in diverse effects of ethanol. Although multiple etiologic factors have been suggested as contributors in the development of alcoholic liver injury, the underlying mechanisms are not clear.

Ethanol affects the expression of hepatic genes known to be involved in a broad spectrum of liver functions including ethanol metabolism, cell proliferation, and lipid metabolism and this alteration in gene expression possibly leads to alcoholic liver injury. Histone modifications are considered as important mechanism in regulating gene expression and chromatin remodeling. Histone proteins can be modified, among others, predominantly by acetylation, phosphorylation and methylation. The acetylation of histone is most studied and has been reported to be closely related to regulation of transcriptional activity (Taunton et al., 1996; Strahl and Allis, 2000). Recently it was reported that ethanol increased acetylation of histone H3 at lysine 9 residue (H3-Lys 9). Furthermore, a correlation between the acetylation of histone H3-Lys9 and ADH-I gene expression in hepatocytes was demonstrated (Park et al., 2005). Such epigenetic alterations by ethanol may play important roles in cell injury (Shukla and Aroor, 2006).

The phosphorylation of histone H3 occurs at serine 10 and serine 28 residues during mitotic chromosome condensation (Wei et al., 1998; Goto et al., 1999). Histone H3 phosphorylation is associated with the expression of early genes, e.g. c-fos and c-myc (Strelkov and Davie, 2002; Chadee et al., 1999).

There are a few kinases identified as histone H3 kinase, i.e., mitogen- and stress-activated kinase-1 (MSK1), ribosomal S6 kinase 2 (RSK2), aurora B, and I κ B kinase α . Recent studies have shown that UVB irradiation induced the phosphorylation of histone H3 at serine 10 and at serine 28 mediated by mitogen-activated protein kinases (MAPKs) (Zhong et al., 2001; He et al., 2005).

A variety of cellular effects of ethanol are due to its modulation of signaling pathways including MAPKs (Aroor and Shukla, 2004). MAPKs, proline directed serine/threonine protein kinases, play central roles in the integration of signal transduction processes leading to various cell responses including proliferation, differentiation, and apoptosis (Chang and Karin, 2001; Zhang and Liu, 2002). Three well characterized subfamilies of MAPKs, i.e. p42/44 MAPK, c-Jun N-terminal kinase (JNK), and p38 MAPK are regulated by phosphorylation cascade. Each MAPK is specifically phosphorylated by their specific upstream kinases, MAPK kinases (MKKs). p42/44 MAPK is phosphorylated by MKK1/2 (MEK1/2; MAP/ERK kinase 1/2). The phosphorylation of JNK occurs through MKK4 and MKK7. MKK3 and MKK6 have been implicated in p38 MAPK (Ahn et al., 1991; Moriguchi, 1997; Enslen et al., 2000). MAPKs localize to cytosol in unstimulated cells and translocate into the nucleus upon stimulation, e.g. in neural differentiation of PC12 cells (Traverse et al., 1992; Robinson et al., 1998) and mitogenesis of fibroblasts (Lenormand et al., 1993). However, the nuclear translocation of MAPK is stimulus specific (Traverse et al., 1992; Cavigelli et al., 1995; Robinson et al., 2001; Whitehurst et al., 2004) and cell type specific (Menice et al., 1997). In addition, cytosolic and nuclear MAPKs can be differentially regulated (Wang et al., 1996) and nuclear MAPK activity can be increased without translocation of cytosolic MAPK (Kim and Kahn, 1997). There is overwhelming evidence that different MAP kinases are modulated by ethanol and acetaldehyde (Aroor and Shukla, 2004) but the downstream targets of these kinases are poorly known. The present study deals with this issue focusing onto nuclear histone H3 as a downstream target in rat hepatocytes.

2. Materials and Methods

2.1. Materials

The protease inhibitors (aprotinin, leupeptin and pepstatin A) and anti β -actin antibody were obtained from the Sigma-Aldrich (St. Louis, MO). The antibodies for phospho-p38 MAPK, p38 MAPK, SAPK/JNK, phospho-p42/44 MAPK, p42/44 MAPK, phospho-MKK3/6 and cleaved caspase-3 were purchased from Cell Signaling (Beverly, MA). Anti-active JNK antibody (phospho-JNK antibody) was from Promega (Madison, WI), anti MKK3/6 and anti α -tubulin antibodies were from Santa Cruz (Santa Cruz, CA), antibodies for histone H3, phospho-H3 Ser10 and phospho-H3 Ser28 from Upstate (Charlottesville, VA), anti cytochrome C oxidase antibody from Molecular Probe (Eugene, OR), and MAPK inhibitors (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580), 4-(4-Fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole (PD169316), 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole (SB202190), Anthra[1,9-*cd*]pyrazol-6(2*H*)-one (SP600125), 2'-Amino-3'-methoxyflavone (PD98059), 4-Ethyl-2-(p-methoxyphenyl)-5-(4'-pyridyl)-1H-imidazole (SB202474)) were from EMD Biosciences (Madison, WI).

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. All protocols involving animals were approved by University of Missouri-Columbia Institutional Animal Care and Use Committee.

2.3. Subcellular fractionation

Subcellular fractionation was carried out as previously reported (Park et al., 2003) with minor modifications. Following treatments, cells were washed with ice-cold PBS, and then lysed in hypotonic lysis buffer (HLB) (20 mM HEPES, pH 7.4, 10 mM β -glycerophosphate, 1 mM EDTA, 1 mM Na-orthovanadate, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, and 10 μ g/ml each of aprotinin, leupeptin and pepstatin A). Cells were allowed to swell for 15 min followed by homogenization by passing through a 26 gauge needle 10 times. The homogenate was centrifuged at $500 \times g$ for 10 min at 4 °C. The postnuclear supernatant was further centrifuged at $14,000 \times g$ for 10 min and the supernatant was used as cytoplasmic fraction and the pellet was used as mitochondrial rich fraction. The nuclear pellet was resuspended in HLB containing 0.3 % NP-40 and vortexed for 10 s followed by centrifugation at $500 \times g$ for 10 min. The pellet was resuspended in 0.5 ml of HLB containing 0.05 % NP-40 and 10 % glycerol. The suspension was passed through a 26 gauge needle 3 times and layered over 1 ml of HLB supplemented with 45% sucrose cushion. After centrifugation at $1,600 \times g$ for 30 min, the pellet containing nuclei was washed once with HLB containing 10 % glycerol and examined under light microscope for purity of nuclei that are devoid of membrane contamination and other subcellular organelles. The isolated nuclei preparations were solubilized using HLB containing 1% SDS and boiling for 5 min and sonicated for 3 s. After centrifugation at $14,000 \times g$ for 10 min, the supernatant was used as nuclear fraction.

2.4. Extraction of acid-soluble proteins (histones)

Histones were extracted from nuclei as described by Park et al. (2005) with some modifications. Cells were washed with PBS two times and collected in HLB containing 10 % glycerol and kept on ice for 10 min. NP-40 was added to a final concentration of 0.2 % and the mixture was vigorously vortexed for 20 s and kept on ice for 5 min. After vortex for 3 s, the mixture was

centrifuged at $12,000 \times g$ for 30 s and the pellet was washed with HLB containing 10 % glycerol. The pellet was resuspended in 0.4 N HCl containing 10 % glycerol and the mixture was slowly rotated at 4 °C for 30 min. After centrifugation at $12,000 \times g$ for 10 min, acid-soluble proteins in supernatant were precipitated with a final concentration of 20 % trichloroacetic acid on ice for 1 h. After centrifugation at $12,000 \times g$ for 10 min, the pellet was washed once with acidic acetone (containing 0.02 N HCl) and once with pure acetone. Pellet was dried and dissolved in dH_2O .

2.5. Western blotting

Cell lysates were fractionated 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 137 mM NaCl and 0.1 % Tween-20 (TBST) and then blocked with TBST containing 5 % non-fat dry milk for 2 h at room temperature. Blots were incubated with primary antibodies overnight at 4°C. The blots were incubated with secondary antibodies (goat anti-rabbit or goat anti-mouse) conjugated to horseradish peroxidase. After washing, the blots were developed with enhanced chemiluminescence (ECL; Pierce) and exposed to X-ray film to detect the protein band. Quantitative analysis was performed by densitometry using Quantity One software (Bio-Rad). Our densitometric band quantification was always done in the linear range of X-ray film sensitivity by selecting appropriate exposure time. This was tested and validated using standard amount of protein and densitometric units. All the values presented were calculated within the linear range.

2.6. Statistical analysis

Data are expressed as mean $\pm$ S.E.M. Differences between control and experimental groups were checked for statistical significance ($P < .05$) by the Student's *t* test (two-tailed, unpaired).

3. Results

3.1. Phosphorylation of histone H3 at serine 10 and serine 28 by ethanol and acetaldehyde

We first examined whether histone H3 is phosphorylated at serine 10 and serine 28 by ethanol or acetaldehyde. Hepatocytes were treated with ethanol (100 mM) or acetaldehyde (5 mM) for different time (1 – 24 h) and acid-soluble nuclear proteins were subjected to Western blotting using anti-phosphohistone H3 at serine 10 (P-H3 Ser10) and anti-phosphohistone H3 at serine 28 (P-H3 Ser28) antibodies. It must be noted that the concentrations selected for ethanol and acetaldehyde are patho-physiologically relevant (Lee and Shukla, 2005). Changes in protein band intensities were normalized to β -actin, an endogenous protein whose levels did not change (see Fig. 1 legend for detail). The basal level of phosphorylation of H3 varied slightly over the time and therefore, the fold increase in phosphorylation of H3 was obtained by comparing to set of controls for each time point (see Fig. 1). Ethanol increased the phosphorylation of H3 both at serine 10 and serine 28. The phosphorylation of H3 at serine 10 increased by 1.2 fold at 1 h, 1.4 fold at 2 h and 2.2 fold at 24 h. Similarly, phosphorylation of H3 at serine 28 increased by 1.3 fold at 1 h, 1.2 fold at 2 h and 2.4 fold at 24 h (Fig 1A). Interestingly, both P-H3 Ser10 and P-H3 Ser28 at 4 h after ethanol treatment were consistently below control. The total histone H3 was not affected by ethanol. These results show that ethanol induced phosphorylations of H3 at serine 10 and serine 28 are biphasic and maximally increased at 24 h.

The effects of acetaldehyde on the phosphorylation of histone H3 at serine 10 and serine 28 were determined. Both P-H3 Ser10 and P-H3 Ser28 maximally increased by 1.6 fold and 4.3 fold, respectively at 1 h and this increase remained higher than the control at 2 h (Fig 1B). These results show that both ethanol and acetaldehyde increased phosphorylation of histone H3 at serine 10 and serine 28, but with noticeably different kinetics. The magnitudes of phosphorylation of H3 at serine 10 and at serine 28 by ethanol are similar. In contrast,

acetaldehyde induced phosphorylation of H3 at serine 28 is much greater than that of serine 10 (Fig 1A and 1B).

3.2. The roles of MAPKs in histone H3 phosphorylation

The phosphorylation of histone H3 is regulated by various kinases including MAPKs. We therefore studied the role of MAPKs in histone H3 phosphorylation using pharmacological inhibitors of different MAPKs. Hepatocytes were pretreated with inhibitors of MAPKs, SP600125 (JNK inhibitor), SB203580 (p38 MAPK inhibitor), or PD98059 (MEK1/2 inhibitor) (Bennett et al., 2001; Davies et al., 2000) before ethanol or acetaldehyde treatment. As shown in Fig 2A, SB203580 inhibited both P-H3 Ser10 and P-H3 Ser28 induced by ethanol. However, no significant changes in P-H3 Ser10 and P-H3 Ser28 were observed by pretreatment with SP600125 and PD98059. Similarly, acetaldehyde induced P-H3 Ser10 and P-H3 Ser28 were decreased by SB203580, but not by SP600125 or PD98059. A group of pyridinyl imidazoles, exemplified by SB203580 have shown high specificity to p38 α and p38 β . These compounds do not inhibit p38 γ or p38 δ or a variety of other kinases including p42/44 MAPK and JNK at the concentration used in this study (Davies et al., 2000). These compounds competitively bind to ATP binding site and inhibit p38 kinase activity but not its activation by upstream kinase. SB202474, a structurally related but inactive compound, has no effect on either the kinase activity of p38 MAPK or its activation (Young et al., 1997) and was used as a negative control for p38 MAPK inhibitors. Fig 2B shows that other pyridinyl imidazoles, PD169316 and SB202190 also inhibited P-H3 Ser10 and P-H3 Ser28 by ethanol or acetaldehyde whereas SB202474 had little effect on P-H3 Ser10 and Ser28. These results showed that the phosphorylation of H3 at Ser10 and Ser28 by either ethanol or acetaldehyde was blocked by p38 MAPK inhibitor but not by inhibitors of JNK or MEK1/2.

3.3. The effects of ethanol and acetaldehyde on nuclear p38 MAPK

We have shown that in whole cell extracts, there is no significant change in p38 MAPK activity after ethanol treatment (0 – 24 h) (Lee and Shukla, 2005). Several lines of studies, however, have suggested that activation of nuclear MAPKs can occur through mechanisms independent of cytosolic MAPKs activation (Wang et al., 1996). We, therefore, investigated whether ethanol caused changes in nuclear p38 MAPK. Hepatocytes were treated with ethanol (100 mM) for different time (0 – 24h). Nuclear fractions were prepared for Western blotting to detect the level of phospho-p38 MAPK (activated form) and p38 MAPK protein. The purity of subcellular fractions was confirmed by probing the Western blot with α -tubulin as a cytosolic marker, histone H3 as a nuclear marker, and cytochrome C oxidase as a mitochondrial marker. This is shown in Fig 3. Surprisingly, ethanol treatment increased phosphorylation of p38 MAPK about 5 fold at 1 h. This increase remained up to 4 h and further increased by 17 fold at 24 h (Fig 4A and 4C), showing persistent activation of p38 MAPK by ethanol in the nucleus. The level of p38 MAPK protein also increased by 1.2 ± 0.34 fold at 1 h and 5.6 ± 0.73 fold at 24 h after ethanol treatment (Fig 4A and 4C), suggesting nuclear translocation of p38 MAPK protein.

Acetaldehyde also increased nuclear phospho-p38 MAPK and p38 MAPK protein by 4.6 ± 0.49 fold and 2.6 ± 0.50 fold, respectively, but at earlier time point i.e. at 30 min (Fig 4B and 4C). This subsequently decreased. These data show temporal differences in nuclear p38 MAPK activation by ethanol and acetaldehyde. The changes in phospho-p38 MAPK and p38 MAPK protein by ethanol and acetaldehyde were dose dependent (Fig 4D). Ethanol at 24 h increased phospho-p38 MAPK/p38 MAPK protein by 25.67/2.93 fold at 100 mM and 57.37/4.34 fold at 200 mM. Acetaldehyde at 30 min increased phospho-p38 MAPK/p38 MAPK protein by 1.92/1.72 at 1 mM and 4.35/3.27 fold at 5 mM.

To examine the subcellular location of p38 MAPK activation by ethanol or acetaldehyde, hepatocytes were treated with ethanol for 24 h or acetaldehyde for 30 min and nuclear and cytosolic fractions were prepared. The equal amount of protein (30 μ g) was used for Western blotting to detect phospho-p38 MAPK. Both ethanol and acetaldehyde increased phosphorylation of p38 MAPK in the nuclear fraction, consistent with above results (Fig 5). To our surprise, phospho-p38 MAPK signal was undetectable in cytosolic fraction under this condition. To determine the differences in the subcellular distribution of p38 MAPK protein, the membrane was reprobed with anti-p38 MAPK antibody. The immunoblot results showed that p38 MAPK protein levels were about 9 times higher in the cytosol than in the nucleus in control cells. Ethanol and acetaldehyde did not significantly change cytosolic p38 MAPK mass (Fig 5). This membrane was reprobed with anti- β -actin. More β -actin protein was detected in nuclear fraction than in cytosol although same amount of total protein was used. These results demonstrate a selective nuclear activation of p38 MAPK without changes in cytosolic p38 MAPK activity by ethanol and acetaldehyde.

Thus, ethanol and acetaldehyde selectively modulated p38 MAPK activity in the nucleus. Acetaldehyde induced rapid and transient activation of p38 MAPK compared to gradual and persistent activation of p38 MAPK by ethanol. The increase in phospho-p38 MAPK and p38 MAPK protein in nucleus by ethanol is greater than that by acetaldehyde.

3.4. MKK3/6 activation by ethanol and acetaldehyde in the nucleus

To examine possible mechanisms responsible for selective activation of p38 MAPK by ethanol or acetaldehyde in the nucleus, we compared the amplitude of increase in phospho-p38 MAPK and p38 MAPK protein. The data in Fig 4C shows higher magnitude of increases in phospho-p38 MAPK than that in p38 MAPK protein by ethanol. It becomes more noticeable when represented as the ratio of the fold increase in phospho-p38 MAPK to p38 MAPK protein (Fig 6). The ratio of P-p38 MAPK/p38 MAPK increased above 2 at 30 min and reached about 4 at 4 h and was sustained until 24 h. Similarly, acetaldehyde treatment also induced higher fold increase in phospho-p38 MAPK than that in p38 MAPK (Fig 4C). The ratio of P-p38 MAPK/p38 MAPK after acetaldehyde treatment showed about 2 at 30 min and then declined to basal (ratio = 1) after 4 h (Fig 6) and this ratio is lower than that by ethanol. These results may suggest that phosphorylation of p38 MAPK in the nucleus can increase through mechanisms in addition to the translocation of cytosolic p38 MAPK.

We next examined whether nuclear p38 MAPK is modulated by upstream kinase MKK 3/6. Hepatocytes were treated with ethanol (100 mM) or acetaldehyde (5 mM) for different time (0–24 h). Nuclear fraction was prepared to detect phospho-MKK 3/6 by Western blot. Phosphorylation of MKK 3/6 by ethanol increased by ~ 2 fold at 30 min and ~ 3 fold at 2 h and then gradually decreased (Fig 7A and 7B). Acetaldehyde also increased phosphorylation of MKK 3/6 by ~ 1.5 fold at 30 min (Fig 7A and 7B), a time course similar to p38 MAPK activation. The amount of MKK 3/6 protein remained unchanged by ethanol or acetaldehyde. Taken together, these results suggested that there are two contributory mechanisms for increased phosphorylation of p38 MAPK in the nucleus after ethanol or acetaldehyde treatment: nuclear translocation of p38 MAPK from the cytosol and phosphorylation of nuclear p38 MAPK by MKK3/6.

3.5. Nuclear translocation of JNK and p42/44 MAPK by ethanol

In whole cell extracts of rat hepatocytes, it was shown that ethanol increased both JNK and p42/44 MAPK activation (Lee and Shukla, 2005). This raised the question whether the lack of effects of the inhibitors of these two kinases (SP600125 and PD98059) on histone H3 phosphorylation is due to the failure to translocation of JNK and p42/44 MAPK into the nucleus after ethanol treatment. We, therefore, investigated if ethanol treatment alters subcellular

localization of JNK and p42/44 MAPK. First, the time-dependent effect of ethanol on nuclear activation of JNK was examined. Hepatocytes were treated with ethanol (100 mM) for different time (0 – 24 h) and nuclear fraction was prepared for Western blot analysis using anti-phospho JNK antibody. Ethanol induced rapid activation of JNK at 15 min and peaked at 30 min (Fig 8A). The level of JNK protein also increased with similar kinetics to that of JNK phosphorylation (Fig 8A), suggesting nuclear translocation of JNK from cytosol after ethanol treatment. The β -actin level indicated that the differences in the level of phospho-JNK and JNK protein in the nucleus by ethanol are not due to the different protein loading amounts. These results showed that ethanol increased JNK activation with a concomitant increase in the level of its protein.

Next, the time dependent effect of ethanol on nuclear p42/44 MAPK activation was examined. Ethanol induced phosphorylation of p42/44 MAPK at 30 min followed by decrease and then an increase at 24 h (Fig 8B). The level of p42/44 MAPK protein also increased at 30 min and this increase at 4 h and 24 h is higher than that of phospho-p42/44 MAPK. Although this increase in nuclear p42/44 MAPK activation was reproducible, the peak activation of p42/44 MAPK vary in different experiments. Mostly, nuclear p42/44 MAPK showed biphasic activation: between 30 min and 4 h for the early activation, and 24 h for the later activation. These results indicated that the activation of nuclear JNK and p42/44 MAPK increased by ethanol and these kinases may have nuclear targets other than histone H3.

3.6. p38 MAPK activation and histone H3 phosphorylation in relation to apoptosis

Histone H3 phosphorylation has been associated with cell apoptosis (Waring et al., 1997; Tikoo et al., 2001; Li et al., 2002). We have previously reported that ethanol and acetaldehyde increased caspase3 activation, a marker of apoptosis (Lee and Shukla, 2005). Therefore, the correlation between histone H3 phosphorylation and apoptosis was examined. Hepatocytes were treated with ethanol or acetaldehyde for 2 h, a peak time point of caspase3 activation by ethanol and acetaldehyde (Lee and Shukla, 2005), in the presence of SB203580 or DMSO (vehicle control). Both ethanol and acetaldehyde treatment increased the level of cleaved caspase-3 about 2 fold and this increase was not significantly affected by pretreatment with SB203580. In some instances, SB203580 treatment slightly increased the level of cleaved caspase-3 (Fig 9). These results indicate that p38 MAPK activation and histone H3 phosphorylation do not influence apoptosis induced by ethanol and acetaldehyde.

4. Discussion

In this study, we showed that both ethanol and acetaldehyde increased the phosphorylation of histone H3 at serine 10 and serine 28, which were blocked by SB203580, an inhibitor of p38 MAPK, but not by either SP600125, an inhibitor of JNK or PD98059, an inhibitor of MEK1/2. Thus, the phosphorylation of histone H3 at serine 10 and serine 28 by ethanol or acetaldehyde is mediated by p38 MAPK. The involvement of p38 MAPK was further supported by the use of two other inhibitors (PD169316 and SB202190) and also by lack of inhibition by the inactive analogue, SB202474. The phosphorylation of histone H3 decreased at 4 h after ethanol treatment. This decrease in H3 phosphorylation may be due to transient activation of protein kinase C (PKC) since PKC activates H3 phosphatase in human hepatoma cells (Huang et al., 2005) and ethanol has been shown to activate PKC in rat hepatocytes (Domenicotti et al., 1998). The phosphorylation of p38 MAPK and phosphorylation of histone H3 by acetaldehyde were transient compared to persistent activation by ethanol. The nuclear p38 MAPK activation and histone H3 phosphorylation induced by ethanol at 24 h may be caused by acetaldehyde independent pathways. Indeed, acetaldehyde independent effects of ethanol on signal transduction pathways have been shown in several cell types (Hoek and Kholodenko, 1998).

Ethanol and acetaldehyde treatment significantly increased the activation of p38 MAPK in the nucleus. Although mechanisms responsible for nuclear translocation of p42/44 MAPK have been extensively studied (Fukuda et al., 1997; Khokhlatchev et al., 1998; Whitehurst et al., 2004), the mechanisms of nuclear activation of p38 MAPK in response to stimuli is unclear. In contrast to marked activation of nuclear p38 MAPK, the phosphorylation of cytosolic p38 MAPK was undetectable under the same condition although p38 MAPK protein predominated in the cytosol. These results suggested selectivity of p38 MAPK activation by ethanol and acetaldehyde in the nucleus. It is intriguing that the activation of p38 MAPK (i.e., P-p38 MAPK) was detected only in the nucleus but not in the cytosol. Ethanol and acetaldehyde also increased p38 MAPK protein in nucleus with time course similar to phosphorylation of p38 MAPK. However, the magnitude of phosphorylation of p38 MAPK was much higher than that of p38 MAPK protein. On the other hand, MKK3/6 activation increased in nucleus by ethanol and acetaldehyde. These results indicated that the activation of p38 MAPK can be increased by upstream kinase in the nucleus. However, it is not clear whether nuclear translocation of p38 MAPK after ethanol or acetaldehyde treatment requires its activation. More recent observations have shown that the phosphorylation of MAPK is not prerequisite for nuclear translocation of MAPK. p42/44 MAPK translocated to nucleus without activation during ischemia and then is activated by MEK-2 (MKK2) in the nucleus during reperfusion (Mizukami and Yoshida, 1997). Moreover, MAPKs can be phosphorylated in the nucleus without translocation from cytosol (Kim and Kahn, 1997). Interestingly, cytosolic MKK3/6 was also activated by ethanol or acetaldehyde (data not shown). Therefore, it could not be ruled out that cytosolic p38 MAPK is activated and then instantly translocated to the nucleus. Lenormand et al. (1998) have shown that nuclear accumulation of p42/44 MAPK following serum stimulation requires protein synthesis of nuclear anchoring protein. However, our data showed that cycloheximide, an inhibitor of protein synthesis, did not inhibit nuclear activation of p38 MAPK by ethanol (data not shown), suggesting a mechanism independent of its neosynthesis.

In this study, JNK and p42/44 MAPK appear not to be involved in histone H3 phosphorylation although ethanol and acetaldehyde activated these MAPKs. Was it due to lack of their nuclear translocation? We therefore examined the nuclear localization of JNK and p42/44 MAPK after ethanol treatment. Ethanol increased phosphorylation of JNK in the nucleus with a concomitant increase in the amount of their proteins, suggesting nuclear translocation of JNK by ethanol. The kinetics of nuclear activation of JNK was similar to that found in whole cell (Lee and Shukla, 2005). Nuclear p42/44 MAPK activation and p42/44 MAPK protein were also increased by ethanol. These results indicated that among all three MAPKs in nucleus, histone phosphorylation was selectively affected by p38 MAPK.

During nonproliferating cell response, histone H3 phosphorylation has been associated with induction of immediate early genes (Strelkov and Davie, 2002; Chadee et al., 1999) and apoptosis (Li et al., 2002; Tikoo et al., 2001; Waring et al., 1997). In this study, we examined histone H3 phosphorylation in relation to apoptosis based on our previous study on ethanol and acetaldehyde induced apoptosis (Lee and Shukla, 2005). However, pretreatment of hepatocytes with SB203580 had no effect on caspase-3 activation induced by ethanol and acetaldehyde, indicating no correlation between p38 MAPK mediated histone H3 phosphorylation and hepatocyte apoptosis. Although p38 MAPK activation has been suggested to be involved in hepatocyte apoptosis (Pastorino et al., 2003; Suzuki and Tsukamoto, 2006), hepatocyte apoptosis also occurs through mechanism independent of p38 MAPK activation (Graf et al., 2002). Additional studies on the significance of ethanol and acetaldehyde induced histone H3 phosphorylation in relation to other cellular responses are warranted.

In summary, we have shown here conclusively for the first time that the serine 10 and serine 28 residues of the histone H3 protein in the nucleus of the rat hepatocytes are downstream targets mediated by p38 MAPK, but independent of p42/44 or JNK pathway.

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Fig. 1. Phosphorylation of histone H3 at serine 10 and serine 28 by ethanol and acetaldehyde
Hepatocytes were treated with 100 mM ethanol (EtOH) (A) or 5 mM acetaldehyde (Acet) (B) for indicated time. Acid soluble proteins were extracted from nuclear fraction. Western blotting was carried out to detect phospho-histone H3 at serine 10 (P-H3 Ser10), phospho-histone H3 at serine 28 (P-H3 Ser28) and histone H3. The membranes were re-probed with anti-β-actin antibody. Quantitative analysis of bands was performed by Quantity One software (Bio-Rad) which gave intensity values. The ratio of the intensity of P-H3 Ser10 or 28 to β-actin for control and treated samples were determined. The fold increase in ratio over control for each time point is presented in bar graph, where control value represents 1. Values are mean ± S.E.M. (bars), n = 3. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, greater than control; # $P < 0.05$ and ## $P < 0.01$, less than control.

Fig. 2. The effects of inhibitors of MAPKs on phosphorylation of histone H3 at serine 10 and serine 28 by ethanol and acetaldehyde

(A) Hepatocytes were pretreated with DMSO (vehicle control), 20 μ M SP600125, 10 μ M SB203580, 50 μ M PD98059 and then treated with 100 mM ethanol (EtOH) or 5 mM acetaldehyde (Acet) for 24 h or 1 h, respectively. The phosphorylation of histone H3 at serine 10 (P-H3 Ser10) and serine 28 (P-H3 Ser28) were monitored by Western blotting. (B) Hepatocytes were pretreated with DMSO (vehicle control), 10 μ M SB202474 (an inactive analogue of p38 MAPK inhibitors), 10 μ M PD169316, 10 μ M SB202190, 10 μ M SB203580 and then treated with 100 mM ethanol (EtOH) or 5 mM acetaldehyde (Acet) for 24 h or 1 h, respectively. The phosphorylation of histone H3 at serine 10 (P-H3 Ser10) and serine 28 (P-

H3 Ser28) were monitored by Western blotting. The data are representative of three independent experiments.

Fig. 3. Subcellular fractionation

Hepatocytes were treated with 100 mM ethanol for 24 h and then nuclear, cytosolic, and mitochondrial proteins were prepared. Same amount of proteins (30 μ g) from each fraction were used for Western blot analysis using antibodies against α -tubulin, histone H3 or cytochrome C oxidase. The data are representative of three independent experiments (C, control; E, ethanol; Nu, nucleus; Cyt, cytosol; Mito, mitochondria).

Fig. 4. Nuclear translocation of p38 MAPK by ethanol and acetaldehyde

Hepatocytes were treated with 100 mM ethanol (EtOH) (A) or 5 mM acetaldehyde (Acet) (B) for indicated time. Nuclear protein was subjected to Western blot to detect phospho-p38 MAPK (P-p38 MAPK) and p38 MAPK protein. The membranes were reprobed with anti β -actin antibody. The data are representative of three independent experiments. (C) The fold increase in P-p38 MAPK and p38 MAPK were quantitated by Quantity One software (Bio-Rad), where control value represents 1. Values represented are mean $\pm$ S.E.M. (bars), $n = 3$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with control (time = 0) of P-p38 MAPK; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ compared with control (time = 0) of p38 MAPK. (D) Hepatocytes were treated with different concentration of ethanol (EtOH) or acetaldehyde (Acet) for 24 h and 30 min, respectively. Nuclear protein was subjected to Western blot analysis to detect phospho-p38 MAPK (P-p38 MAPK) and p38 MAPK protein. The data are representative of three independent experiments. Values represented are mean $\pm$ S.E.M. (bars), $n = 3$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with control of P-p38 MAPK; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ compared with control of p38 MAPK.

Fig. 5. Selective activation of p38 MAPK by ethanol and acetaldehyde in nucleus

Hepatocytes were treated with 100 mM ethanol for 24 h or 5 mM acetaldehyde for 30 min. Nuclear and cytosolic proteins were prepared and same amount of proteins (30 μ g) were used for Western blot using anti-phospho-p38 MAPK antibody. The cell extract from C6 cell treated with UV was used as a positive control (+). The membrane was reprobbed with anti-p38 MAPK antibody and then reprobbed with anti β -actin antibody. The data are representative of three independent experiments (C, control; E, ethanol; A, acetaldehyde; Nu, nucleus; Cyt, cytosol).

Fig. 6. Ratio of phospho-p38 MAPK (P-p38 MAPK) to p38 MAPK in hepatocyte nucleus after ethanol or acetaldehyde treatment
Ratio of the fold increase in P-p38 MAPK to p38 MAPK after ethanol (EtOH) or acetaldehyde (Acet) for different times is presented.

Fig. 7. Nuclear activation of MKK3/6 by ethanol and acetaldehyde

(A) Hepatocytes were treated with 100 mM ethanol (EtOH) or 5 mM acetaldehyde (Acet) for indicated time. Nuclear protein was subjected to Western blot to detect phospho-MKK3/6 (P-MKK3/6) and MKK3/6 protein. (B) The fold increase in P-MKK3/6 was quantitated by Quantity One software (Bio-Rad), where control value represents 1. Values represented are mean $\pm$ S.E.M. (bars), $n = 3$. * $P < 0.05$ and ** $P < 0.01$ compared with control (time=0) by ethanol; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ compared with control (time=0) by acetaldehyde.

Fig. 8. Nuclear translocation of JNK and p42/44 MAPK by ethanol

Hepatocytes were treated with 100 mM ethanol (EtOH) for indicated time (0–24 h) and nuclear proteins were prepared. The phospho-JNK (P-p46/54 JNK), JNK protein (p46/54 JNK) (A), phospho-p42/44 MAPK (P-p42/44 MAPK) and p42/44 MAPK protein (p42/44 MAPK) (B) were detected by Western Blot. The data are representative of three independent experiments.

Fig. 9. The effect of SB203580 on caspase-3 activation by ethanol and acetaldehyde

Hepatocytes were pretreated with DMSO (vehicle control) or 10 μ M SB203580 for 1 h and then treated with 100 mM ethanol or 5 mM acetaldehyde for 2 h. (A) The activation of caspase-3 was determined by Western blotting with anti-cleaved caspase-3 antibody. (B) The fold increase in caspase-3 activation was quantitated by Quantity One software (Bio-Rad). The data are representative of four independent experiments. Values represented are mean $\pm$ S.E.M. (bars), $n = 6$ (C, control; E, ethanol; A, acetaldehyde).