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Activation of MEK 1/2 and p42/44 MAPK by Angiotensin II in Hepatocyte Nucleus and their Potentiation by Ethanol

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Abstract

Hepato-subcellular effect of Ang II and ethanol on the p42/44 MAP Kinase and MEK1/2 were investigated in the nucleus of rat hepatocytes. Hepatocytes were treated with ethanol (100 mM) for 24 hr and stimulated with angiotensin II (Ang II, 100 nM, 5 min). The levels of p42/44 MAPK and MEK1/2 were monitored in the nuclear fraction using antibodies. Ang II itself caused significant accumulation of phospho-p42/44 MAPK in the nucleus without any significant translocation of p42/44 MAPK protein there by suggesting activation of p42/44 MAPK in the nucleus. Ang II caused marked accumulation of phospho-MEK 1/2 in the nucleus without any significant accumulation of MEK1/2 protein. Ratio of phospho-MEK 1/2 to MEK 1/2 protein in the nucleus after Ang II treatment was 2.4 times greater than control suggesting phosphorylation of MEK 1/2 inside the nucleus. Ethanol had no effect on the protein level or the activation of p42/44 MAPK in the nucleus. Ethanol treatment potentiated nuclear activation of p42/44 MPAK by Ang II but not translocation of p42/44 MAPK protein. This was accompanied by potentiation of Ang II stimulated accumulation of phospho-MEK 1/2 in the nucleus by ethanol. MEK 1/2 inhibitor, U-0126 inhibited Ang II response or its potentiation by ethanol. These results suggest that Ang II mediated accumulation of phospho-p42/44 MAPK in the hepatocyte nucleus involves MEK 1/2 dependent activation and this effect is potentiated by ethanol.

Keywords

Angiotensin II; Ethanol; Mitogen activated protein kinase; MAP kinase kinase; Nuclear translocation; Hepatocytes

Introduction

Acute and chronic alcohol consumption results in a spectrum of altered hepatic functions including changes in redox state, alterations in lipid and carbohydrate metabolism, as well as protein and DNA synthesis (Lieber, 1988; Dey and Cederbaum, 2006). Although the biological effects of acute and chronic ethanol ingestion are well documented, the underlying mechanisms remain largely unknown. Among the key signaling pathways regulating mammalian cellular events is the p42/44 MAP kinase (p42/44 MAPK, ERK 1/2) cascade (Katz *et al.*, 2007; Zebisch

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et al., 2007). The activation of p42/44 MAPK signaling has been reported to mediate stimulation of DNA synthesis, suppression of DNA synthesis, induction of c-myc, and suppression of apoptosis in hepatocytes depending on the tone, duration and stimulus (Adachi *et al*, 1996; Tombes *et al*., 1998; Qiao *et al*, 2002). There is overwhelming evidence that different MAP kinases are modulated by ethanol in hepatocytes (Aroor and Shukla, 2004; Zima and Kalousová, 2005; Venugopal *et al.*, 2007). Ethanol potentiates serum stimulated p42/44 MAPK activation in embryonic liver cells (Reddy and Shukla, 1996) and enhances Gi-protein stimulated p42/44 MAPK activity in hepatic tumorigenic cells (McKillop, *et al*., 1999). Ethanol prolongs p42/44 MAPK activation induced by EGF, NGF, insulin and IGF in primary cultured hepatocytes (Chen *et al*., 1998; Tombes *et al*., 1998). Moreover, up regulation of p42/44 MAPK signaling has been reported in liver biopsy specimens from patients with alcoholic liver disease (Nguyen and Gao, 2002).

A key step in the p42/44 MAPK signaling cascade is its translocation into the nucleus where it phosphorylates transcription factors including c-myc, c-fos, Stat1/3 and Elk-1 involved in cell growth, differentiation and apoptosis (Chen *et al.*, 1992; Brunet *et al*, 1999; Turjanski, *et al.*, 2007; Whitmarsh *et al*., 2007). However, the nuclear translocation of p42/44 MAPK is stimulus specific (Traverse *et al.*, 1992) and cell type specific (Menice *et al.*, 1997). In addition, cytosolic and nuclear p42/44MAPKs can be differentially regulated (Wang *et al.*, 1996; Whitehurst *et al.*, 2004). Although nuclear activation of p42/44 MAPK was considered to be the consequence of nuclear translocation of p42/44 MAPK, recent studies indicate translocation of MEK1/2 and MEK1/2-dependent nuclear activation of p42/44 MAPK (Kim and Kahn, 1997; Tolwinski *et al*., 1999; Mizukami *et al*., 2000). Ethanol modulates serum activated p42/44 MAPK in the nucleus of BNLCL2 cells (Reddy and Shukla, 2000). Ethanol induces nuclear translocation of p42/44 MAPK and selective activation of p38 MAPK in hepatocyte nucleus (Lee and Shukla, 2008).

Angiotensin II (Ang II), the active component of renin-angiotensin system plays a major role in the regulation of blood pressure (Schulman *et al.*, 2007). However, recent studies have shown role for Ang II in growth, inflammatory, hemodynamic and metabolic responses in various tissues including liver (Brasier *et al.*, 2000; Bataller *et al.*, 2005; McAllister-Lucas *et al.*, 2007). Ang II increases glycogenolysis (Blackmore and Exton, 1985) and acts as a comitogen for hepatocyte DNA synthesis (Dajani *et al.*, 1996). Angiotensin II causes induction of early genes (González-Espinosa and Garcia-Sainz, 1992) and activates NF-kB in hepatocytes (Brasier *et al.*, 2000; McAllister-Lucas *et al.*, 2007). Increases in plasma Ang II levels are seen during both acute and chronic alcohol consumption in humans (Collins *et al.*, 1992).

Although Ang II, vasopressin, insulin and epinephrine significantly increased p42/44 MAPK activity in hepatocytes, ethanol exposure potentiated only Ang II and epinephrine stimulated p42/44 MAPK, indicating an agonist selective effect of ethanol on p42/44 MAPK signaling in hepatocytes (Weng and Shukla, 2000). We report here the effects of ethanol and/or Ang II on upstream MEK1/2 signaling in the nucleus of primary cultures of hepatocytes.

Materials and methods

Materials

Ang II, benzamidine, β-glycerophosphate, aprotinin, leupeptin, and pepstatin A were obtained from Sigma-Aldrich (St. Louis, MO). The antibodies for phospho-p42/44 MAPK, p42/44 MAPK, phospho-MEK 1/2, MEK 1/2, phospho- p38 MAPK, phospho- JNK, phospho- Elk-1 were purchased from Cell Signaling Technology, Inc.(Beverly, MA). Anti-acetyl histone H3 K9 antibody was from Upstate (Charlottesville, VA) The goat anti-rabbit IgG antibody conjugated with horseradish peroxidase was purchased from Bio Rad (Herculeus, CA). U-0126 (MEK1/2 inhibitor) was purchased from EMD biosciences (Madison, WI).

Isolation and culture of hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats (150–200g) by collagenase-perfusion method as described previously (Seglen, 1976; Weng and Shukla, 2000). The use of rats for these experiments was approved by the University of Missouri Animal Care & Use Committee. Hepatocytes were plated on to collagen coated culture dishes (7.5×10^6 cells/100 mm dishes) in Dulbecco's modified Eagles Medium (DMEM) containing 10% fetal bovine serum. Following an initial 2 h to allow the cells to attach, cells were washed with PBS and the media replaced with DMEM containing 0.1% FBS with or without ethanol. In order to avoid evaporation of ethanol, dishes were sealed with parafilm. After 24 hr, cells were subjected to desired treatments. We have shown earlier that after 24 hr treatment of cells with ethanol (100 mM) about 80 % of ethanol was still present in the medium (Weng and Shukla, 2000). Cell viability assessed by the exclusion of trypan blue was about 90 % before or after ethanol treatments (Weng and Shukla, 2000, 2002). Furthermore, hepatocytes isolated by this procedure showed Ang II activation of phosphorylase a/glycogenolysis, (Weng and Shukla, 2003) and elicited various receptor responses including Ca^{2+} mobilization, and MAP kinase, tyrosine kinase activations (Weng and Shukla, 2002). Hepatocytes used were therefore functionally viable.

Preparation of nuclear extracts

Following treatments, cells were washed with ice-cold PBS and then lysed using hypotonic lysis buffer (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 μg/ml each of aprotinin, leupeptin and pepstatin A) containing 10% glycerol. 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 g for 10 min at 4 °C and the resulting nuclear pellet was resuspended in hypotonic lysis buffer containing 0.2% NP-40 but without glycerol. The nuclear suspension was passed through a 26-gauge needle 6 times, followed by centrifugation at 500 X g for 10 min. This step successfully removed contaminated endoplasmic reticulum. The nuclear pellet was resuspended in 0.5 ml hypotonic lysis buffer and layered over 45% sucrose cushion. After centrifugation at 1600 X g for 30 min, the pellet containing nuclei was washed once with hypotonic buffer 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 hypotonic lysis buffer containing 1% SDS and boiling for 5 min. The nuclear preparations were sonicated for 5 sec to reduce viscosity. After centrifugation at 14000 X g for 10 min, the supernatant was used as nuclear fraction. Protein concentration was measured using a Bio-Rad DC protein assay kit.

SDS-PAGE and immunoblotting

The nuclear fractions (40μg protein) were combined with equal volume of 2X Laemelli buffer and fractionated on 10% polyacrylamide gels. The proteins were electrophoretically transferred onto nitrocellulose membrane (Bio-Rad). The membrane was washed with 20 mM Tris, pH 7.5 containing 0.1 % Tween-20 and 150 mM NaCl (TBST) and incubated with TBST containing 5 % nonfat dry milk for 1 hour at room temperature. After washing, blots were incubated with specific primary antibodies in TBST buffer containing 3% BSA overnight at 4 °C. The blots were washed with TBST and incubated with 1:3000 diluted horse radish peroxidase conjugated sheep anti-rabbit secondary antibody for one hour at room temperature. The protein bands were detected by an enhanced chemiluminescence (ECL) reaction (Pierce) and exposed to X-ray film. Quantitative results were determined by laser densitometry. For repeat immunoblotting, membrane was stripped in 62.5 mM Tris-HCl, pH 6.7, 2 % SDS, and 0.1 M β-mercaptoethanol for 30–45 min at 50 °C.

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, paired).

Results

We have shown earlier that primary cultures of hepatocytes can be exposed to ethanol concentrations up to 200 mM for 24 h without affecting their viability (Weng and Shukla, 2000). It was also shown that at least 12 hr of treatment with ethanol is required to observe the potentiating effects of ethanol on Ang II stimulated phosphorylation of p42/44 MAPK. The maximum potentiation is noted at 24 hr. The potentiating effects are seen at 50–200 mM ethanol (Weng and Shukla, 2000). The concentration of Ang II (100 nM) and the time of stimulation (5 min) were thus selected based on observations from this laboratory (Weng and Shukla, 2000; 2002; 2003, Park et al; 2006) to investigate ethanol effects on nuclear translocation of the kinases. Accordingly, hepatocytes were treated with 100 mM ethanol for 24 h and were subsequently challenged with 100 nM Ang II for 5 min and the samples were then processed as needed. We have selected 100 mM ethanol to increase the sensitivity of the detection of nuclear translocation of p42/44 MAPK and MEK 1/2. In vivo concentrations of ethanol in chronic alcoholics have been observed as high as 300 mM (Deitrich and Harris 1996; Shukla *et al*., 2007).

Nuclear p42/44 MAPK after ethanol and/or Ang II treatment of hepatocytes

In this study, we first determined the levels of activated p42/44 MAPK in the nucleus of hepatocytes using polyclonal antibody specific for phospho-p42/44 MAPK that recognizes the activated form. Treatment of hepatocyte with ethanol (100 mM) for 24 h did not significantly affect phosphorylation of p42/p44 MAPK (Fig.1A). Next we determined p42/44 MAPK protein levels in the nuclear extracts. The p42/44 MAPK protein level was moderately increased (1.4 and 1.3 fold respectively) in ethanol treated cells compared to controls (Fig. 1B). However, the magnitude of increase in p42/44 MAPK protein accumulation observed by ethanol was not statistically significant. We next investigated whether ethanol modulated Ang II responses in the nucleus. Ang II (100 nM) treatment for 5 min increased phosphorylation of p42 and p44 MAPK by 2.7 fold and 3.2 fold, respectively in hepatocytes. Ang II increased p42 and p44 MAPK phosphorylation by 3.9 and 5.2 fold, respectively, in hepatocytes pretreated with ethanol for 24 h (Fig 1A) over the control. Thus, ethanol potentiated Ang II stimulated p42 and p44 MAPK activity by 1.4 and 1.5 fold respectively, over Ang II alone (no ethanol) stimulated phsopho-p42 and p 44 MAPK. Nuclear accumulation of phospho-p42/44 MAPK after ethanol pretreatment followed by Ang II stimulation was statistically significant for phospho-p42 MAPK and the values reached near significance for phospho-p44 MAPK (0.051) compared to nuclear accumulation of phospho-p42 MAPK and phospho-p44 MAPK stimulated by Ang II alone (no ethanol). Ang II stimulation (100 nM, 5 min) also caused a moderate increase in p42/44 MAPK protein (1.5 and 1.8 fold) in the nuclear fraction (Fig. 1B). After treatments with ethanol (100 mM, 24 h), further increase in the magnitude of p42/44 MAPK protein accumulation in the nucleus (2.2 and 2.2 fold, respectively) was seen when hepatocytes were stimulated with Ang II (Fig. 1B). Although the magnitude of increase in p42 MAPK protein observed with ethanol pretreatment followed by Ang II stimulation was statistically significant compared to controls, the nuclear accumulation of p44 MAPK protein after ethanol pretreatment followed by Ang II stimulation was not statistically significant (p=0.07) compared to controls. The nuclear accumulation of p42/44 MAPK protein after ethanol pretreatment followed by Ang II stimulation was not significant compared to stimulation by Ang II alone (no ethanol).

We have previously reported persistent accumulation of phospho-p38 MAPK at 24 hr after ethanol treatment and transient accumulation of phospho-JNK at 15 min to 2 hr in hepatocyte nucleus (Lee and Shukla, 2008). However, Ang II alone did not cause significant activation of either p38 MAPK or JNK under the conditions employed for the activation of phospho p42/44 MAPK (100 nM Ang II, 5 min). Moreover, ethanol did not modulate Ang II induced activation of p38 MAPK and JNK in rat hepatocytes (data not shown).

Nuclear translocation of MEK ½ after ethanol and Ang II

MEK 1/2 is upstream kinase, which phosphorylates p42/44 MAPK. MEK1/2 itself is phosphorylated to be active. Phosphorylated MEK 1/2 gets translocated into the nucleus and can activate p42/44 MAPK (Tolwinski *et al*., 1999). Although MEK 1/2 is localized mainly in the cytosol because of the nuclear export signal in MEK (Fukuda *et al.*, 1997), there is also a transient or constitutive increase in the phospho MEK 1/2 in the nuclear compartment (Mizukami *et al*., 2000). Therefore, we monitored the nuclear activation and localization of phospho-MEK 1/2, using antiphospho-MEK 1/2 antibody that recognizes the phosphorylation site (ser-217/ser-222) necessary for the activation. Accumulation of phospho-MEK1/2 was not significant after exposure of hepatocytes to ethanol for 24 h (Fig. 2A). Ang II (100 nM) treatment caused significant increase (2.4 fold) in phospho-MEK1/2 in control hepatocytes. The accumulation of Ang II activated phospho-MEK1/2 was increased by 3.2 fold in hepatocytes pre-treated with ethanol. Thus, ethanol significantly ($p < 0.02$) potentiated Ang II stimulated nuclear accumulation of phospho-MEK 1/2 activity by 1.3 fold over its accumulation by Ang II (no ethanol) alone. The level of MEK 1/2 protein was not significantly different in the nuclear fraction after ethanol treatment. In contrast to significant accumulation of phospho- MEK1/2 by Ang II, the level of MEK1/2 protein was not increased in the hepatocyte nucleus after Ang II stimulation (Fig. 2A). Although nuclear accumulation of MEK1/2 protein was moderately increased (1.5 fold) by combined treatment of ethanol and Ang II. (Fig. 2A), the magnitude of increase was not statistically significant. It is striking that Ang II caused a large increase in phospho-MEK 1/2 in the nuclear fraction but a corresponding increase in MEK1/2 protein was not observed (Fig. 2A). Therefore, we compared the ratio of the increases in phosphorylated MEK 1/2 to MEK 1/2 protein. It became apparent that Ang II caused a substantial increase in the activation of MEK1/2 inside the nucleus (2.4 fold, Fig. 2B) These results suggest that increased levels of nuclear phospho-p42/44 MAPK, after Ang II stimulation of hepatocytes, may be due to nuclear p42/44 MAPK activation by phospho-MEK 1/2. The ratio of increase in phosphorylated MEK 1/2 to MEK protein is further increased after ethanol exposure (2.8 fold, Fig. 2B), but the magnitude of increase by ethanol plus Ang II was not statistically significant compared to Ang II alone. Although potentiation of nuclear accumulation of phospho-p42/44 MAPK by ethanol is through potentaition of Ang II mediated MEK 1/2 phosphorylation, nuclear translocation of p42/44 MAPK to nuclear compartment may be secondary to MEK1/2 activation. This is likely because moderate increase of p42/44 MAPK protein in the nucleus was observed after combined Ang II and ethanol treatment of hepatocytes. In order to examine the role of MEK1/2 activation in translocation of p42/44 MAPK protein, hepatocytes were treated with MEK 1/2 inhibitor U-0126 before the addition of ethanol and Ang II. Nuclear accumulation of phospho–p42/44 MAPK was significantly decreased after Ang II or ethanol treatment followed by Ang II stimulation but nuclear translocation of p42/44 MAPK protein was not affected after treatment with MEK 1/2 inhibitor (Fig.3).

We have reported inhibition of ethanol induced histone H3 K9 acetylation by MEK 1/2 inhibitor in hepatocytes (Park *et al*., 2005). Although Ang II caused modest increase in histone H3 K9 acetylation, ethanol did not potentiate Ang II induced histone H3 acetylation (data not shown). We have also examined phosphorylation of nuclear p42/44 MAPK substrate Elk-1. Ang II caused increase in phosphorylation of Elk-1 and this was inhibited by U-0126. However,

phosphorylation of Elk-1 was lower in hepatocytes treated with ethanol followed by Ang II stimulation compared to Ang II alone treated cells (data not shown).

Discussion

This is the first report on activation of phospho MEK 1/2 and phospho-p42/44 MAPK induced by Ang II in hepatocyte nucleus and their potentiation by ethanol. The presence of phospho-p42/44 MAPK and p42/44 MAPK protein in the nucleus of unstimulated cell has been reported in several cells including hepatocytes (Kim and Kahn, 1997; Goetze, et al, 1999., Tolwinski *et al*., 1999; Mizukami *et al*., 2000., Rosseland et al., 2005). This may be due to a basal level of shuttling of p42/44 MAPK and MEK 1/2 between cytoplasm and nucleus (Fukuda *et al.*, 1997; Adachi *et al*., 2002). We have previously reported activation of p42/44 MAPK by ethanol and accumulation of phospho-p42/44 MAPK in hepatocyte nucleus between 1 and 3 h after exposure of hepatocytes to ethanol (Lee and Shukla, 2005; Lee and Shukla, 2008). However, accumulation of phosphorylated p42/44 MAPK was not significant at 24 hr after ethanol treatment. In this regard, phosphatases that were thought to inactivate p42/44 MAPK in the nuclear compartment were shown to be newly synthesized in response to p42/44 MAPK activation. It remains to be known whether the nuclear inactivation of p42/44 MAPK were mediated by the inducible nuclear MKPs encoded by dual specificity phosphatases such as MKP-1, MKP-2 and MKP-5 (Brunet *et al.*, 1999., Whitehurst *et al.*, 2004., Owens *et al.*, 2007). Ethanol has been shown to increase the expression of MKP-1 in gastric mucosal cells (Kawanaka *et al;* 2001) and human hepatoma cell line (Venugopal *et al*., 2007).

Notwithstanding ethanol potentiation of nuclear activation of p42/44 MAPK by Ang II by ethanol, nuclear accumulation of phospho-p42/44 MAPK by Ang II alone is intriguing. Nuclear accumulation of p42/44 MAPK can occur by nuclear translocation of phosphorylated p42/44 MAPK after its activation by MEK1/2 in the cytosol; or nuclear activation of through translocation of MEK 1/2; or nuclear activation of MEK 1/2 in the nuclear compartment by upstream kinase eg. PKC. Although, previous studies failed to show nuclear translocation of MEK1/2 in response to activation, these results were later modified demonstrating translocation of MEK1/2 to the nucleus independently of activation and these were rapidly removed from the nucleus by its nuclear export signal (Fukuda *et al*., 1997). Reactivation of inactive form of p42/44 MAPK retained in the nucleus by nuclear targeting of MEK1/2 has also been reported (Mandl *et al.*, 2005) Protein kinase C (PKC) has been reported to modulate both nuclear translocation of p42/44 MAPK and nuclear accumulation of MEK1/2 (Mizukami *et al*., 2000). Ang II has been shown to cause PKC ζ dependent activation and nuclear accumulation of p42/44 MAPK (Goetze *et al*., 1999). Moreover, src mediated p42/44 MAPK activation by Ang II was mainly localized in the cytosolic compartment where as G-protein and PKCζ mediated p42/44 MAPK by Ang II was localized to the nuclear compartment (Godney and Sayeski, 2006). In contrast to nuclear activation of p42/44 MAPK by Ang II alone, the nuclear accumulation of p42/44 MAPK protein by Ang II was not significant suggesting mechanisms independent of nuclear translocation of p42/44 MAPK are contributing to increased accumulation of phospho-p42/44 MAPK. On the other hand, accumulation of phospo-p42/44 MAPK mainly occurs by nuclear activation of MEK 1/2 without any significant nuclear translocation of MEK 1/2. It may be noted that MEK1/2 can be activated within the nucleus by protein kinase Cζ (Mizukami *et al.*, 2000). We have previously reported role of PKC in Ang II stimulation of p42/44 MAPK phosphorylation in hepatocytes (Weng and Shukla, 2002). Therefore a role of PKC in the activation of MEK1/2 in hepatocyte nucleus cannot be excluded at present.

Ethanol potentiation of MEK 1/2 phosphorylation induced by Ang II suggests role of activation of MEK 1/2 activation for enhanced phosphorylation of p42/44 MAPK in the hepatocyte nucleus in cells pretreated with ethanol followed by Ang II stimulation. Although mean

increase of p42/44 MAPK protein by ethanol plus Ang II was significant compared to control, the magnitude of increase by ethanol pretreatment followed by Ang II stimulation was not significant compared to treatment with Ang II alone (no ethanol). These results suggest nuclear translocation of p42/44 MAPK does not play significant role in ethanol potentiation of Ang II stimulated nuclear accumulation of phospho-p42/44 MAPK.

MAPK pathways provide an important link between agonist induced cell stimulation and the nucleus through phosphorylation of nuclear substrates including transcription factors. A perplexing aspect of p42/44 MAPK signaling in general is the magnitude and duration of p42/44 MAPK signaling in the nuclear compartment in regulating different responses in the same cell. For example, proliferative response is characterized by robust but transient activation of p42/44 MAPK in the nucleus (Tolwinski *et al*., 1999). In contrast, cell cycle arrest and differentiation were shown to be associated with low but persistent activation of nuclear p42/44MAPK (Adachi *et al*., 2002). We have reported the antiapoptotic role of ethanol induced p42/44MAPK activation (Lee and Shukla, 2005). MAP kinases also modulate ethanol induced epigenetic histone modifications. We have recently shown dependence of ethanol induced histone H3 acetylation on p42/44 MAPK (Park *et al.*, 2005) and of histone H3 phosphorylation on p38 MAPK (Lee and Shukla, 2008). However, acetylation of histone H3 induced by Ang II alone was not marked and acetylation of histone H3 in cells pretreated with ethanol followed by Ang II was lower than sum of Ang II and ethanol induced acetylation. We have also examined the effects of ethanol and Ang II on phosphorylation of Elk-1 (a downstream target of p42/44 MAPK). Phosphorylation of Elk-1 was lower in cells pretreated with ethanol followed by Ang II stimulation compared to cells treated with Ang II alone. Dissociation of Elk-1 phosphorylation from p42/44 MAPK activation has recently been reported. In PC12 cells, Ndrg4 enhanced NGF-induced p42/44 MAPK activation but this was not coupled with Elk-1 activation (Hongo et al., 2006). Recently, we have reported dissociation of ethanol potentiation of Ang II stimulated p42/44 MAPK phosphorylation from p42/44 MAPK dependent phosphorylation of serine ^{727} of STAT3 in hepatocytes (Weng *et al*., 2008). It will be of interest to study the significance of ethanol induced persistent nuclear accumulation of p42/44 MAPK and ethanol potentiation of Ang II stimulated nuclear p42/44 MAPK activation through nuclear activation of MEK1/2 in the context of downstream targets, gene expression, metabolic function and survival of hepatocytes.

Acknowledgments

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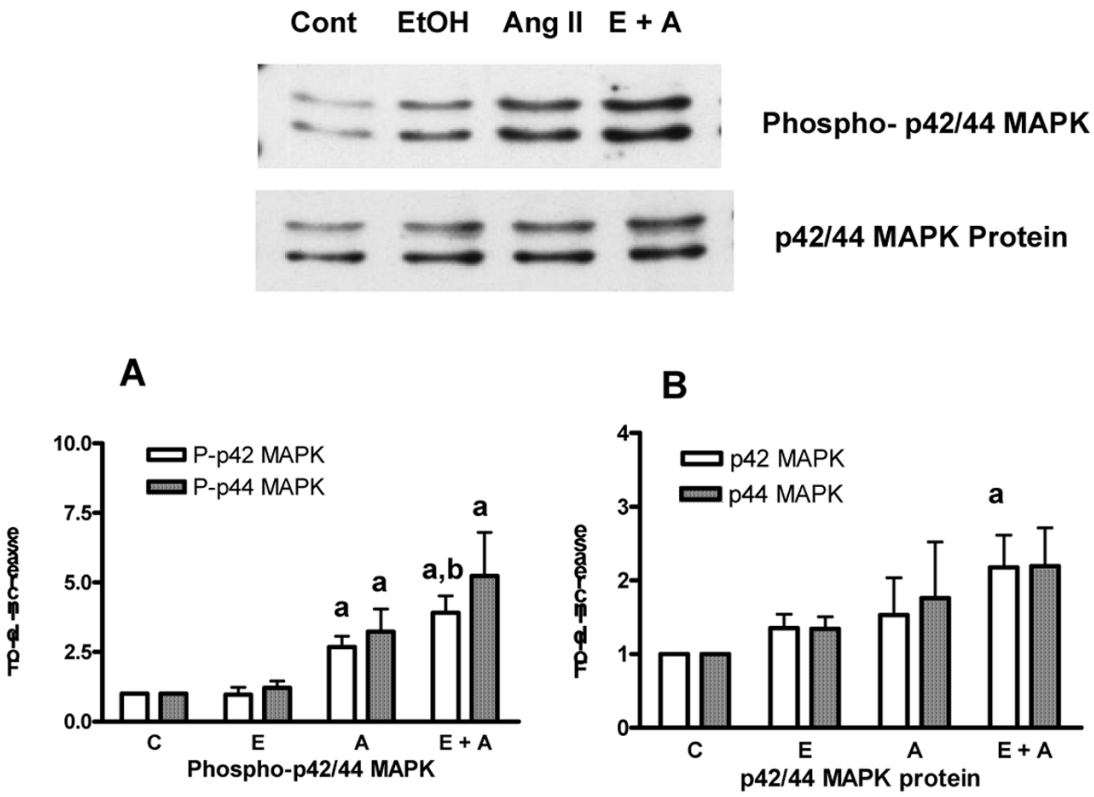

Fig. 1. Nuclear accumulation of phospho-p42/44 MAPK and p42/44 MAPK protein in response to ethanol and/or Ang II

Hepatocytes were exposed to 100 mM ethanol for 24 hr followed by stimulation with Ang II for 5 min. Nuclear extracts were prepared and aliquots were examined by Western blotting using phospho-p42/44 MAPK and p42/44 MAPK antibodies as described under “Methods”. Upper panel shows a representative western blot image. The lower panel is data compiled using densitometric analysis. A: Phospho-p42/44 MAPK; B: p42/44 MAPK protein. The Values represented are mean ± S.E.M. (bars), $n = 6$. **a**, $p < 0.05$ compared with corresponding unstimulated samples and **b**, $p < 0.05$ compared with Ang II stimulated samples (C; control, E; ethanol, and A; Ang II)

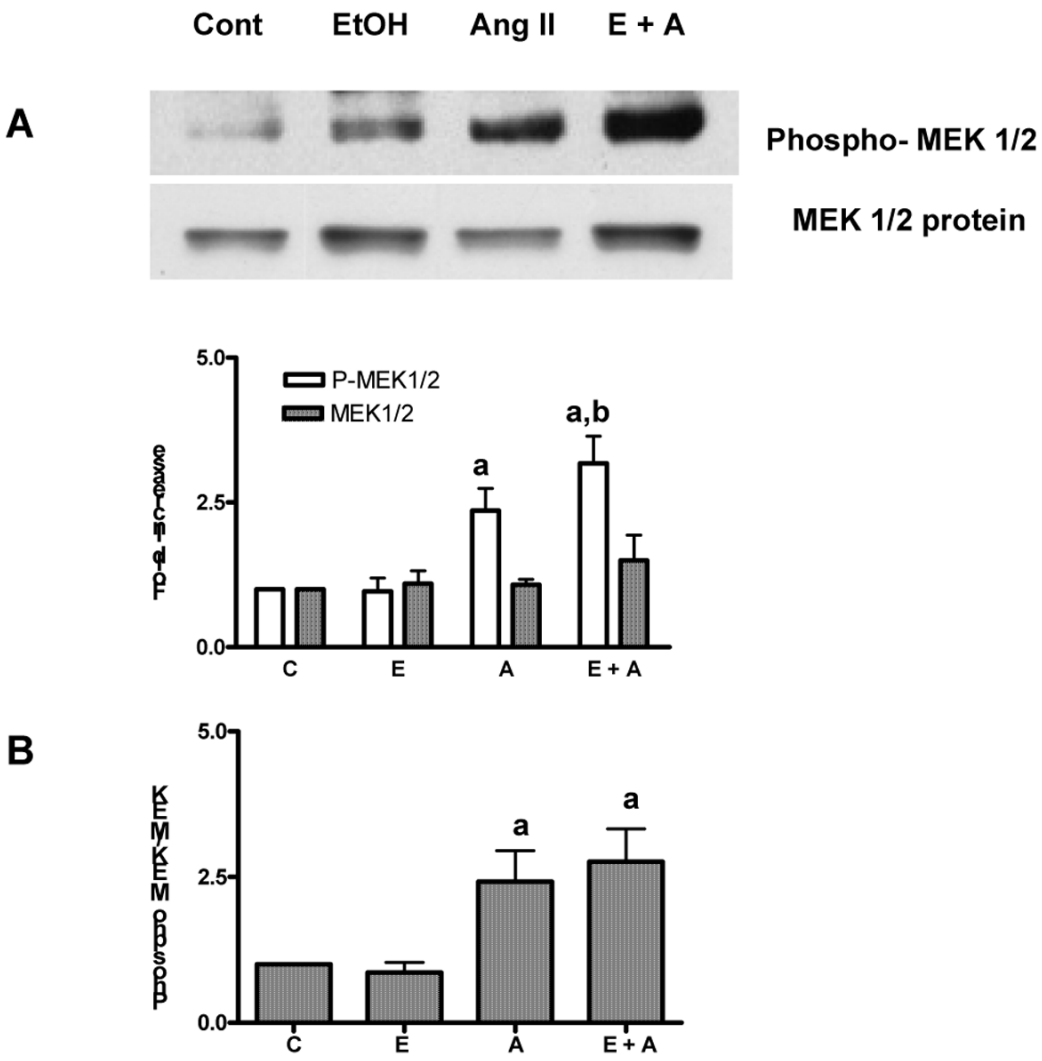

Fig. 2. Nuclear accumulation of phospho-MEK1/2 and MEK 1/2 in response to ethanol and/or Ang II

Hepatocytes were exposed to 100 mM ethanol for 24 hr followed by stimulation with Ang II for 5 min. Nuclear extracts were prepared and aliquots were examined by Western blotting using phospho-MEK1/2 or MEK 1/2 antibody as described under " Methods ". **A.** Upper panel shows a representative western blot image. The lower panel is data compiled using densitometric analysis. **B**. Ratio of the fold increases in phospho-MEK 1/2 to MEK 1/2 protein after different treatments are presented. Values represented are mean ± S.E.M. (bars), $n = 6$. **a**, $p < 0.05$ compared with corresponding unstimulated samples and **b**, $p < 0.05$ compared with Ang II stimulated samples (C; control, E; ethanol, and A; Ang II)

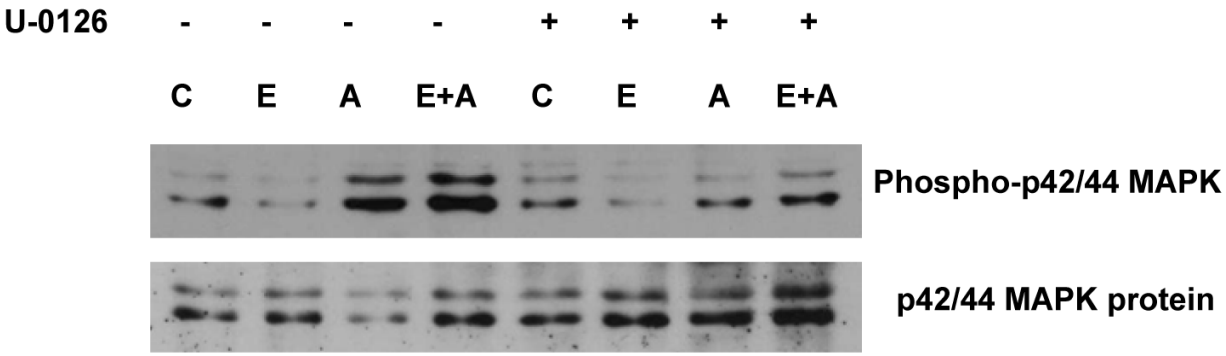

Fig. 3. Effect of MEK 1/2 inhibitor on nuclear accumulation of phospho-p42/44 MAPK and p42/44 MAPK protein in response to ethanol and/or Ang II

Hepatocytes were pretreated with 10 μM MEK 1/2 inhibitor U-0126 for 1 hr and exposed to 100 mM ethanol for 24 hr. After 24 treatment with ethanol, hepatocytes were stimulated with 100 nM Ang II for 5 min. Nuclear extracts were prepared and aliquots were examined by Western blotting using phospho-p42/44 MAPK and p42/44 MAPK antibodies as described under “Methods”. Upper panel shows a representative phospho-p42/44 MAPK western blot image and lower panel shows representative of p42/44 MAPK protein. Results are representative of three independent experiments from three different hepatocyte preparations. C; control, E; ethanol, and A; Ang II