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European Journal of Pharmacology 398 (2000) 323–331

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Ethanol alters angiotensin II stimulated mitogen activated protein kinase in hepatocytes: agonist selectivity and ethanol metabolic independence

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Received 27 December 1999; received in revised form 10 April 2000; accepted 12 April 2000

Abstract

Angiotensin II activated mitogen-activated protein kinase (MAPK) (p42 and p44) in rat hepatocytes exposed to ethanol and the relevance of ethanol metabolism on this activation was investigated. Hepatocytes, isolated from rat liver, were treated with or without ethanol for 24 h. Angiotensin II, vasopressin, insulin, serum and epinephrine significantly increased hepatocyte MAPK activity. Platelet activating factor (PAF), tumor necrosis factor-α (TNF-α), and insulin-like growth factor-1 (IGF-1) had little effect on MAPK activation. Interestingly, among the above agonists, which activated hepatocyte MAPK, ethanol exposure potentiated only angiotensin II and epinephrine-stimulated MAPK. Thus, potentiation of MAPK by ethanol exhibited agonist selectivity. In contrast to several other cells, there was prevalence of p42 over p44 MAPK band in hepatocytes. Angiotensin II treatment caused a rapid activation (peak 5 min) of MAPK followed by a decrease to basal levels in 30 min. Exposure with 100 mM ethanol potentiated the angiotensin II stimulated MAPK activity. This potentiation was partially blocked by pertussis toxin suggesting it to be a G-protein-dependent event. Treatment of the hepatocytes with pyrazole (an inhibitor of ethanol metabolism) or acetaldehyde (an ethanol metabolite) had no effect on potentiation. Thus, ethanol potentiation of hepatocyte MAPK is agonist-selective and independent of ethanol metabolism. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Ethanol; Acetaldehyde; Hepatocyte; MAPK (mitogen-activated protein kinase); Angiotensin II

1. Introduction

Chronic ethanol consumption causes liver damage such as alcoholic hepatitis, fibrosis and cirrhosis. Several factors are involved in the development of alcoholic liver disease including intracellular accumulation of acetaldehyde, alternations of extrahepatic matrix, formation of reactive oxygen species and accumulation of inflammatory lymphocytes (Lieber, 1985, 1988; Bailey and Cunningham, 1998). Modifications in signal transduction pathways also occur. Several lines of evidence indicate that ethanol modulates the activity of protein kinase C (Kruger et al., 1993; Depetrillo and Liou, 1993; Roivainen et al., 1994), phospholipase C (Hoek et al., 1992), phospholipase D (Bocckino et al., 1987), adenylate cyclase (Hoffman and Tabakoff, 1990), protein tyrosine kinases (Thurston and Shukla, 1992a,b; Resnicoff et al., 1993), c-*Jun* N-terminal kinase (JNK) (Pandey and Alling, 1996), p42/p44 mitogen-activated protein kinase (MAPK) (Reddy and Shukla, 1996; Tomber et al., 1998) and transcription factors such as signal-transducing activators of transcription (STAT) (Chen et al., 1997) and nuclear factor-κB (NF-κB) (Yang et al., 1998). In liver, acute exposure to ethanol increases the amount of cAMP, activates phospholipase C and increases intracellular calcium (Hoek et al., 1987). The chronic effects of ethanol are often opposite to the acute effects. Ethanol also modulates G-proteins function and inhibits cAMP dependent signaling in regenerating rat liver (Diehl et al., 1992). However, the correlation between these effects and the pathogenesis of ethanol in the liver has not been fully established.

Angiotensin II has a major role in vasoconstriction and regulation of salt and liquid homeostasis. In addition, it has been shown to be a growth factor for vascular smooth muscle cells (Zahradka and Saward, 1997), fibroblasts (Sadoshima and Izumo 1993), cardiac myocytes (Sadoshima et al., 1997) and hepatocytes (Dajani et al., 1996). Angiotensin II stimulates glycogenolysis in the liver

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PII: S0014-2999(00)00313-7

(Hothi et al., 1988). In hepatocytes, angiotensin II binds to at least two high-affinity receptors, namely angiotensin AT1 and angiotensin AT2 receptors (Dudley et al., 1990). The biological effects of angiotensin II are mainly mediated by the angiotensin AT1 receptors, which are coupled to G-proteins of both the Gq and Gi families (Wong et al., 1992). Binding of angiotensin II to angiotensin AT1 receptor activates phospholipase C through the activation of Gq family protein, release of inositol (1, 4, 5)-trisphosphate (IP_3) and 1, 2-diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2), leading to intracellular calcium release, activation of protein kinase C, MAPK, and ribosomal S-6 kinase (Tsuda and Alexander, 1990; Berk and Corson, 1997). It has been shown that angiotensin II induces the expression of proto-oncogenes such as c-*fos*, c-*Jun*, and c-*myc* (Gonzalez-Espinosa and Garcia-Sainz 1992). These results suggest that angiotensin II promotes cell growth.

Accumulating evidence suggests that tyrosine phosphorylation may play an important role in this growth response. Angiotensin II was demonstrated to induce tyrosine phosphorylation in liver epithelial cells, glomerular mesangial cells (Force et al., 1991) and vascular smooth muscle cells (Molloy et al., 1993), but it is not known whether tyrosine phosphorylation has any role in the responses of angiotensin II in hepatocytes. Ethanol modulates serum-stimulated MAPK activation in mouse embryonic liver cells (BNLCL2) (Reddy and Shukla, 1996). We have now investigated whether acute ethanol-modulated p42/44 MAPK activity in rat hepatocytes stimulated by a variety of liver agonists and have addressed the relevance of ethanol metabolism to this process.

2. Materials and methods

2.1. Materials

Pertussis toxin, staurosporine, angiotensin II, benzamidine, β-glycerophosphate, protease inhibitors (aprotinin, leupeptin, and pepstatin A) were obtained from the Sigma Chemical (St. Louis, MO). The phospho-p44/42 MAPK kinase antibody and p44/42 MAPK kinase antibody were purchased from New England Biolabs, (Beverly, MA). The goat anti-rabbit immunoglobulin G (IgG) horseradish peroxidase conjugate was purchased from Bio-Rad Laboratories (Hercules, CA). (γ-^{32}P)ATP (3000 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Myelin basic protein (MBP) came from GIBCO BRL (Gaithersburg, MD).

2.2. Isolation and culture of hepatocytes

Hepatocytes were isolated by collagenase-perfusion protocol as described previously (Seglen, 1976). Briefly, male Spraque–Dawley rats (180–250 g) were anaesthetized with ether vapor, the abdomen was opened, and the portal vein was cannulated. The inferior vena cava was ligated just above the level of the renal veins to prevent the perfusion of the kidney. The chest was then opened and the right atrium portion was cannulated. The liver was perfused with Krebs–Ringer-bicarbonate buffer (KRB) containing 0.25 mM EGTA for 1–2 min to remove blood, and then switched to KRB containing 2 mM $CaCl_2$ and collagenase (35 mg/100 ml) for 10–15 min. After the digestion, the liver was carefully removed, transferred to a beaker containing 10–15 ml of medium. Scissors were used for gently cutting connective tissue and dispersing cells. The cell suspension was filtered through a nylon mesh and was separated from debris by centrifuging at $50 \times g$ for 30 s at 24°C. The isolated hepatocytes were washed twice with 50 ml KRB buffer containing 0.5% bovine serum albumin and 15 mM glucose by centrifugation as above and resuspended in the same buffer. Cell viability assessed by the exclusion of trypan blue was $90 \pm 5\%$. The hepatocytes (3×10^6 cells/60 mm dish) were plated on to collagen-coated culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. After 2 h, a time at which cells got attached, the medium was changed to DMEM containing 0.1% fetal bovine serum with or without ethanol. After 24 h, cells were stimulated with the desired agonists for various times. Cells were chilled immediately by placing the dishes onto ice and rinsed twice with ice-cold phosphate buffered saline (PBS). Next, 0.5 ml of ice-cold lysis buffer containing 20 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM benzamidine, 10 mM β-glycerophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin A was added. Cells were scraped into lysis buffer and transferred into test tubes at 4°C. After centrifugation at $12{,}000 \times g$ for 10 min at 4°C, the supernatant was collected and protein concentration was measured using the Bio-Rad DC protein assay kit.

2.3. MAPK assay

MAPK activity was determined by an in-gel kinase assay as described before (Gotoh et al., 1990). Briefly, equal amounts of extracts (20 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) containing MBP (0.5 mg/ml). SDS was removed by incubation in 50 mM Tris, pH 8.0, containing 20% isopropanol for 1 h at room temperature. Subsequently, proteins on the gel were denatured in 6 M guanidinium HCl and renatured overnight at 4°C in 50 mM Tris, pH 8.0, containing 50 mM β-mercaptoethanol and 0.1% Triton X-100. The gel was incubated in kinase buffer containing 40 mM HEPES pH 8.0, 1.5 mM EGTA, 40 μM ATP, 10 mM $MgCl_2$, 2 mM dithiothreitol and (γ-^{32}P)ATP

(5 μCi/ml, 3000 Ci/mmol). The gel was washed with 5% trichloroacetic acid containing 1% sodium pyrophosphate, dried, and exposed to X-ray (KODAK X-OMAT Blue) film. Relative kinase activities were determined by scanning each band with a laser densitometer. In some experiments, the gel bands corresponding to p42 and p44 MAPK were cut, and radioactivity counted to compare with densitometry data and were found to be in agreement.

2.4. Immunoblot analysis

The whole cell lysate protein (20 μg) was subjected to 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane (Bio-Rad) using Bio-Rad Trans-Blot apparatus. 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 3% non-fat dry milk for 2 h at room temperature. The membrane was next incubated with phospho-p44/42 MAPK antibody overnight at 4°C. After washing with TBST, the membrane was incubated with secondary antibody conjugated horseradish peroxidase for 1 h at room temperature. The horseradish peroxidase was detected by enhanced chemiluminescence (ECL; Pierce). The membrane treated with ECL reagent was exposed to X-ray film to detect the protein band. For repeated immunoblotting, membrane was stripped in 62.5 mM Tris–HCl, pH 6.7, 2% SDS, and 0.1 M 2-mercaptoethanol for 30–45 min at 50°C.

2.5. Measurement of ethanol levels in hepatocytes

To determine the level of ethanol in hepatocyte cultures, the media samples were taken at 0, 1, 2, 4, 8, 12 and 24 h after the addition of ethanol. Ethanol levels were determined using an ethanol dehydrogenase assay (Sigma). Samples (10 μl) were mixed with the assay cocktail in glycine buffer and incubated for 10 min at room temperature. Absorbance was measured at 340 nm to determine ethanol concentrations.

2.6. Data analysis

All results are expressed as means ± S.E.M. and were obtained by combining data from individual experiments. Statistical analyses were made using the Student's *t*-test (two-tailed, unpaired). Differences with a *P*-value of < 0.05 were considered statistically significant.

3. Results

3.1. Effect of ethanol on angiotensin II-stimulated MAPK activity in rat hepatocytes

Isolated hepatocytes were plated on to collagen-coated dishes in DMEM containing 10% fetal bovine serum. After 2 h, the medium was changed to DMEM containing 0.1% fetal bovine serum with or without 100 mM ethanol. After 24 h, cells were stimulated with varying concentrations of angiotensin II for 5 min. Whole-cell lysates were prepared and subjected to in-gel kinase assay for p42/44 MAPK.

In the initial experiments, hepatocytes were treated with 50, 100, 200 mM ethanol for 24 h, then stimulated with angiotensin II (100 nM) for 5 min, and extracts were prepared for kinase assay. At 100 mM ethanol, a large potentiation was observed and more than 90% of the hepatocytes were still intact and viable as measured by exclusion of trypan blue (data not shown). Hence, we used 100 mM ethanol in subsequent experiments. Hepatocytes were treated with 100 mM ethanol for 10 min, 1, 4, 8, 12, 20 and 24 h, and then stimulated with angiotensin II (100 nM) for 5 min, and cellular extracts were prepared for MAPK assay. Shorter period of exposure to ethanol (up to 12 h) did not affect angiotensin II-stimulated MAPK activity (data not shown). Maximal potentiation was observed at 24 h of ethanol treatment. Therefore, we used 24-h ethanol treatment in subsequent experiments. As shown in Fig.1, MAPK activation was dependent on the concentration of angiotensin II; activation was detectable at 10^{-9} M and maximal at 10^{-7} M. Pretreatment of hepatocytes with ethanol alone for 24 h had no significant effect on basal MAPK activity as monitored by laser densitometry. However, the angiotensin II-induced MAPK activity was potentiated by ethanol. In control hepatocytes, angiotensin II (10^{-7} M) stimulated p42 and p44 MAPK activity by 3.5- and 3.6-fold, respectively, and it increased to 8.3- and 6.3-fold, respectively, in hepatocytes pretreated with 100 mM ethanol. Thus, ethanol potentiated angiotensin II-stimulated p42 and p44 MAPK activity by 2.4 and 1.8 times over the control, respectively. There was an interesting and differential pattern of p42 and p44 MAPK activity by angiotensin II. The basal level of p42 was consistently seen to be about twice that of p44 in hepatocytes. This ratio remained after stimulation. This is in contrast with other cell lines. For example, in the BNLCL2 cells (Reddy and Shukla, 1996) and skeletal muscle cells (C2C12), the levels of p42 and p44 were the same in the basal level and after stimulation by serum. However, in the opossum kidney (OK) cells the amount of p42 was less than that of p44 in the basal and stimulated conditions (unpublished).

3.2. Time course for the activation of MAPK in control and ethanol-treated hepatocytes

We next examined the time course of MAPK activity stimulated by angiotensin II (100 nM). Control and ethanol-treated hepatocytes were stimulated with angiotensin II for different time periods, and MAPK activity was measured by using in-gel kinase assay. As shown in Fig. 2, angiotensin II-stimulated MAPK was highest at 5 min in control hepatocytes and declined rapidly to basal

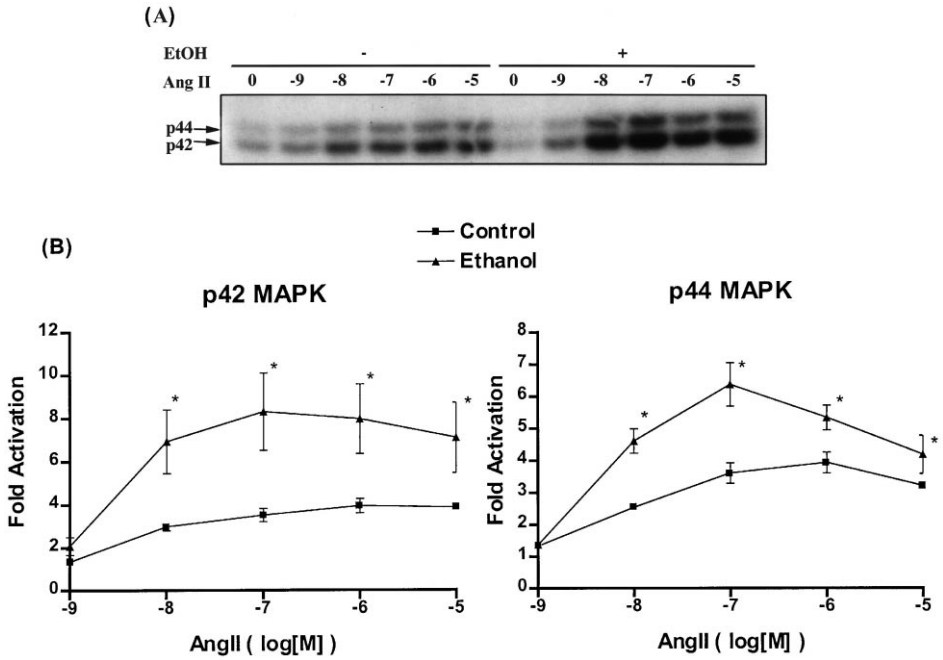

Fig. 1. Dose–response curves for the stimulation of hepatocyte MAPK activity by angiotensin II. (A) Freshly isolated hepatocytes were cultured for 24 h in the presence and absence of ethanol (100 mM) and stimulated with different concentration of angiotensin II for 5 min (see Section 2). Activities of MAPK were assayed by the in-gel kinase as described in Section 2. The positions of p42/44 MAPK are indicated by the arrows. (B) Relative kinase activities were determined by scanning each band with a densitometer. Results are presented as mean ± S.E.M. of three independent experiments. Values are expressed as fold changes over unstimulated control cells. $^{*}P < 0.05$, compared with corresponding samples not containing ethanol.

level in 30 min. Pretreatment with 100 mM ethanol for 24 h significantly potentiated the MAPK activity stimulated by angiotensin II, and the activity remained at elevated levels for 30 min. The above change in MAPK activity was not due to the change in protein expression level, since western-blotting data showed that ethanol treatment for 24 h did not affect the amount of p42/44 MAPK protein, although it potentiated MAPK activity as evi-

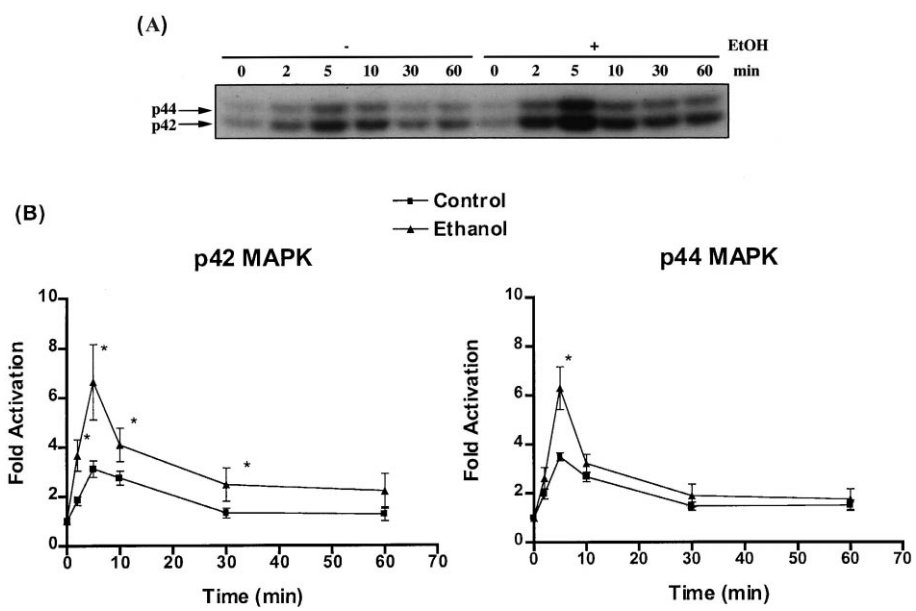

Fig. 2. Time course of MAPK activation by angiotensin II in hepatocytes. (A) Hepatocytes were cultured for 24 h in the presence and absence of ethanol (100 mM). These cells were stimulated with angiotensin II (100 nM) for the indicated times, and MAPK activity was determined by in-gel kinase assay as described in Section 2. The positions of p42/44 MAPK are indicated by the arrows. (B) Relative kinase activities were determined by scanning each band with a densitometer. Results are presented as mean ± S.E.M. (three experiments). Values are expressed as fold changes over unstimulated control cells (control value = 1).

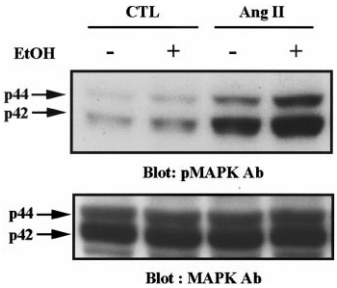

Fig.3. Angiotensin II does not affect protein content of MAPK. Hepatocytes were cultured for 24 h in the presence and absence of ethanol (100 mM). These cells were stimulated with angiotensin II (100 nM) for 5 min. After cell lysis, immunoblotting was performed with phospho-p44/42 MAPK kinase antibody and p44/42 MAPK kinase antibody. The positions of p42/44 MAPK are indicated by the arrows.

denced by increased phospho-MAPK reactivity (Fig. 3). In some experiments, the gel bands corresponding to p42 and p44 MAPK were cut, and radioactivity was counted. Basal MAPK activity in p42 was 74.5 ± 7.4 and 116 ± 38.9 cpm for control and ethanol-treated samples, respectively (mean $\pm$ S.E.M., $n \geq 3$). After angiotensin II stimulation (5 min), the values were 119.1 ± 18.0 and 337.2 ± 45.4 cpm, respectively. Likewise, for p44, the values for control and ethanol were 75.2 ± 5 and 103.5 ± 6.8 cpm before and 115.1 ± 35.3 and 262.4 ± 77.7 cpm after 5 min of angiotensin II stimulation. Thus, these results showed patterns similar to that observed with densitometry.

3.3. Effect of ethanol on MAPK activity stimulated by various agonists

The above observations indicated that ethanol potentiated the angiotensin II-stimulated p42/44 MAPK. To determine whether this effect of ethanol was elicited with other agonists, hepatocytes were stimulated with different agents, i.e. serum (fetal bovine serum, 10%), angiotensin II (100 nM), epinephrine (10 μM), platelet activating factor (PAF; 100 nM), vasopressin (100 nM), insulin (100 μM), tumor necrosis factor-α (TNF-α; 50 ng/ml), and insulin-like growth factor-1 (IGF-1; 50 ng/ml). The criteria for choosing the concentration of the various agents were based on literature values. As shown in Fig. 4, PAF, TNF-α, and IGF-1 had no effect on MAPK. Fetal bovine serum, vasopressin, insulin, angiotensin II and epinephrine

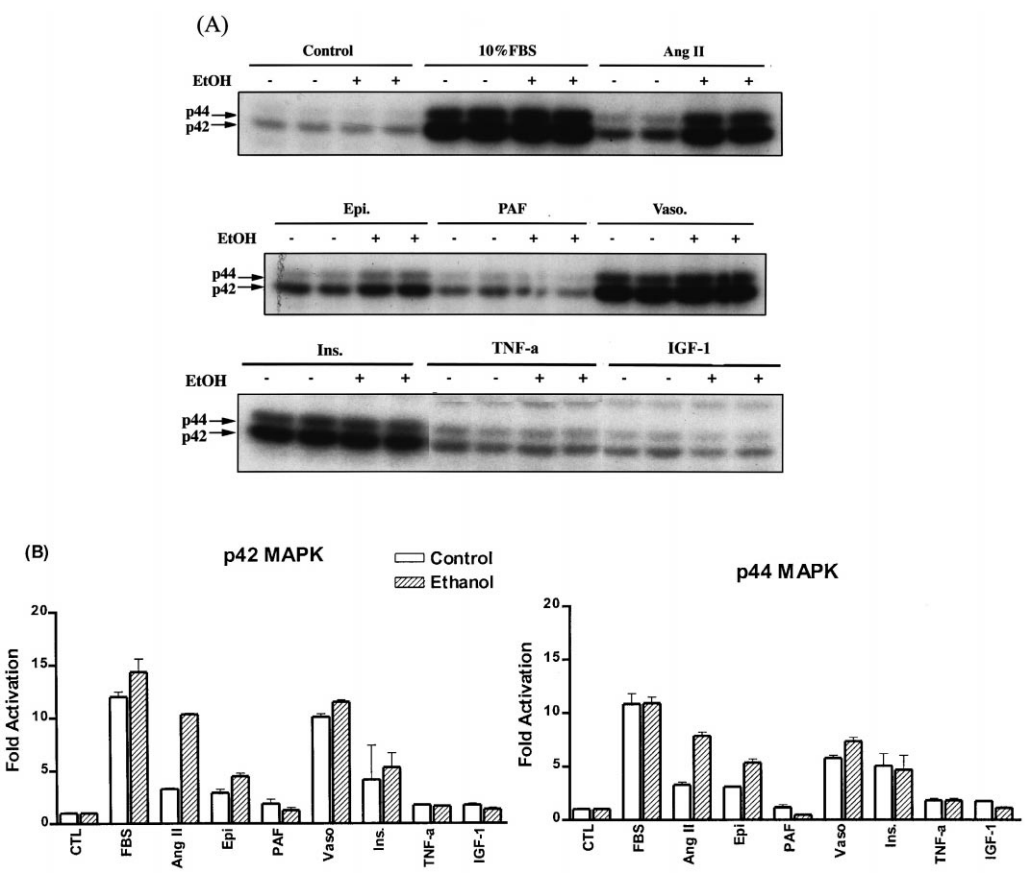

Fig. 4. Effect of ethanol on the stimulation of MAPK by various agonists. (A) Hepatocytes were incubated with (100 mM) or without ethanol for 24 h, and then stimulated with serum (fetal bovine serum, 10%), angiotensin II (100 nM), epinephrine (10 μM), PAF (100 nM), vasopressin (100 nM), insulin (100 μM), TNF-α (50 ng/ml) and IGF-1 (50 ng/ml) for 5 min. The MAPK activity was determined by in-gel kinase assay. Results are from one of three similar experiments. (B) MAPK activities were quantitated by scanning the bands with a laser densitometer. Results are presented as mean ± S.E.M. (three experiments).

significantly increased hepatocyte MAPK activity. However, ethanol potentiated MAPK stimulated only by angiotensin II and epinephrine and, to a small extent, by vasopressin. Fetal bovine serum and insulin-stimulated MAPK activity were not affected significantly by ethanol treatment. It was also observed that ethanol did not potentiate MAPK activated by lower concentrations of these agonists (fetal bovine serum, 5%; vasopressin, 1 and 10 nM; insulin, 10 and 100 nM) (data not shown). Thus, ethanol potentiation of MAPK showed agonist selectivity.

3.4. The effect of ethanol metabolism inhibitor pyrazole on MAPK activity stimulated by angiotensin II

Ethanol is oxidized in the liver to acetaldehyde by alcohol dehydrogenase (ADH) and then to acetate by aldehyde dehydrogenase (ALDH). Acetate is released into the circulation and is rapidly metabolized by extrahepatic tissues to CO_2 and water. Several lines of evidence indicate that both ethanol and its metabolite acetaldehyde are able to produce various pathologic effects. We therefore investigated whether MAPK potentiating effect of ethanol is due to ethanol or its metabolites, e.g. acetaldehyde. The direct addition of acetaldehyde can be one method for determining its effect on angiotensin II-stimulated MAPK activity. Alternatively, pyrazole, an ADH inhibitor, can be used to block ethanol metabolism and determine the involvement of acetaldehyde in angiotensin II-stimulated MAPK activity. We have utilized both approaches to address this possibility.

Hepatocytes were exposed to 100 mM ethanol in the presence and absence of pyrazole for 24 h and the samples were taken at 0, 1, 2, 4, 8, 12 and 24 h after the addition of ethanol to determine the remaining concentration of ethanol. Pretreatment of hepatocytes with pyrazole (2 mM) had no significant effect on ethanol metabolism up to 8 h. Concentrations of ethanol in without pyrazole and with pyrazole treatments were 91.4 ± 2.8 and 96.3 ± 2.9 mM. However, pyrazole significantly inhibited the ethanol metabolism at 24-h time point by 50% (without pyrazole: 78.8 ± 3.3 mM; and with pyrazole: 88.6 ± 1.3 mM). Hepatocytes were therefore exposed to ethanol plus or minus pyrazole for 24 h prior to stimulation with angiotensin II. The extracts from hepatocytes were analyzed for MAPK activity by the in-gel kinase assay. As shown in Fig. 5A, pyrazole increased the basal and angiotensin II-stimulated MAPK activity. However, there was no significant effect on the MAPK potentiation. Increasing the concentration of pyrazole to 10 or 20 mM also had no effect on the MAPK potentiation (data not shown). Pretreatment of hepatocytes with various concentrations (0–50 μM) of acetaldehyde had no significant effect on basal and angiotensin II-stimulated MAPK activity (Fig. 5C) suggesting that the ethanol metabolite acetaldehyde is also not involved.

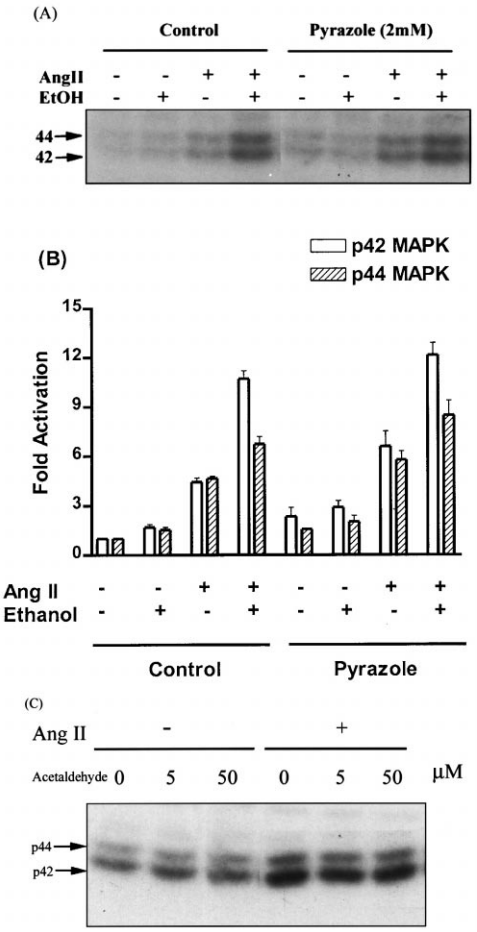

Fig. 5. Effect of pyrazole on MAPK activity stimulated by angiotensin II in hepatocytes. (A) Hepatocytes were exposed to ethanol ± pyrazole (2 mM) for 24 h prior to stimulation with angiotensin II (100 nM, 5 min). The extracts from hepatocytes were analyzed for MAPK activity by the in-gel kinase assay as described in Section 2. The positions of p42/44 MAPK are indicated by the arrows. Similar results were obtained in three separate experiments. (B) Relative kinase activities were determined by scanning each band with a densitometer. Results are presented as mean ± S.E.M. of three independent experiments. Values are expressed as fold changes over unstimulated control cells. (C) Hepatocytes were exposed to various concentrations (0–50 μM) of acetaldehyde for 1 h prior to stimulation with angiotensin II (100 nM). In this experiment, dishes were parafilmed to prevent acetaldehyde evaporation. The extracts from hepatocytes were analyzed for MAPK activity by the in-gel kinase assay.

3.5. Effect of pertussis toxin and staurosporine on MAPK activity potentiation by ethanol

It has been shown that potentiation of MAPK activity by ethanol is mediated via pertussis toxin-sensitive G-protein in BNLCL2 cells (Reddy and Shukla, 1996) and protein kinase C in PC12 cells (Roivainen et al., 1995). To address the involvement of these mechanisms, hepatocytes exposed to 100 mM ethanol for 24 h were treated with pertussis toxin (100 ng/ml) for 16 h or protein kinase C inhibitor, staurosporine (Sta) (5 nM) for 1 h prior to stimulation with angiotensin II. The extracts were prepared for the in-gel kinase assay. Pretreatment of hepatocytes with pertussis toxin alone had no effect on MAPK activity

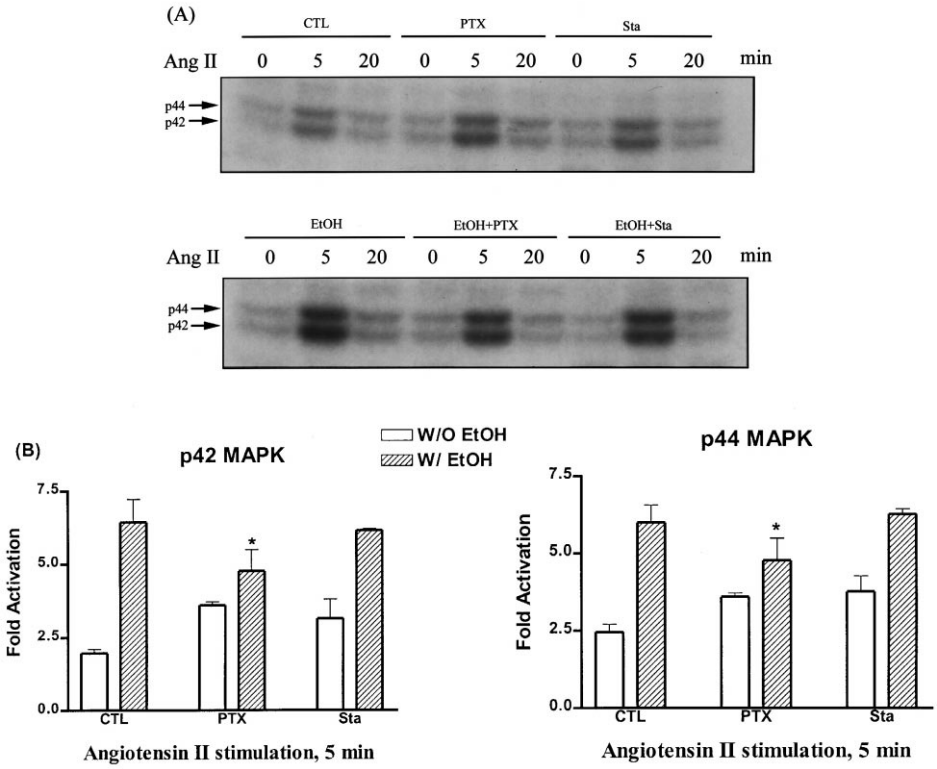

Fig. 6. Effect of pertussis toxin and staurosporine on ethanol potentiation of angiotensin II stimulated MAPK. (A) Hepatocytes were cultured for 24 h in the presence and absence of ethanol (100 mM). Cells were next treated with pertussis toxin (100 ng/ml) for 16 h or staurosporine (Sta; 5 nM) for 1 h prior to stimulation with angiotensin II (100 nM) for various periods of time. The extracts from hepatocytes were analyzed for MAPK activity by the in-gel kinase assay as described in Section 2. The positions of p42/44 MAPK are indicated by the arrows. Autoradiogram represents one of three similar experiments. (B) MAPK activities were quantitated by scanning the bands with a laser densitometer. Results are presented as mean ± S.E.M. (three experiments). Values are expressed as fold changes over unstimulated control cells. $^{*}P < 0.05$, compared with corresponding untreated samples.

stimulated by angiotensin II and staurosporine alone slightly increased basal MAPK activity (Fig. 6). Preincubation with 100 mM ethanol potentiated angiotensin II-stimulated MAPK activity, and this effect of ethanol was reduced by pertussis toxin to about 60% for p42 MAPK and 55% for p44 MAPK. Staurosporine treatment had no effect on potentiation. These results indicate that angiotensin II-stimulated MAPK is pertussis toxin insensitive but ethanol potentiation of MAPK stimulated by angiotensin II is pertussis toxin sensitive.

4. Discussion

It was demonstrated in this paper that agonist activation of MAPK and its potentiation by ethanol exhibited characteristic behaviors in hepatocytes. Agents like angiotensin II, epinephrine, fetal bovine serum, vasopressin and insulin activate MAPK, albeit to different degrees. Interestingly, only angiotensin II and epinephrine-stimulated MAPK were potentiated by ethanol. Other agonists, i.e. PAF, TNF-α and IGF-1 had no effect on MAPK activity. The ethanol potentiation of MAPK stimulated by fetal bovine serum had been shown in BNLCL2 cells (Reddy and Shukla, 1996) but not in OK cells, astrocytes and skeletal muscle cells. These results indicated that ethanol potentiation of MAPK was cell- and agonist-selective. It also suggested that ethanol discriminately potentiates MAPK of certain liver stimuli and not others. This may have differential consequences on the responses of alcoholic liver to these stimuli. Activity of MAPKs may also be regulated by protein tyrosine phosphatase (Nebreda, 1994). It has been shown that ethanol potentiation of serum-stimulated MAPK was not changed in the presence or absence of sodium-orthovandate, a phosphatase inhibitor, in the BNLCL2 cells (unpublished data). Thus, ethanol potentiation of MAPK was due to an increase in the phosphorylation of MAPK and is unlikely due to the inhibition of phosphatases by ethanol. In fact, phospho-MAPK antibody data (Fig. 3), which showed increased p42/44 MAPK phosphorylated bands, further support this conclusion.

In the dose–response curves for the activation of MAPK, lower concentrations of angiotensin II increased the activity followed by a decline at higher concentrations. The decreases at higher concentrations may be due to receptor downregulation at high concentration of agonist or the appearance of angiotensin AT2 receptors. Angiotensin AT2 receptors can activate certain protein phosphatases in R3T3

fibroblast cells (Tsuzuki et al., 1996) and neuroblastoma cells (Bedecs et al., 1997), and activation of angiotensin AT2 receptors also can activate expression of MAPK phosphatase 1 (MKP-1) in rat ventricular myocytes (Fischer et al., 1998). Therefore, the angiotensin AT2 receptor-mediated dephosphorylation of MAPK can be predicted to contribute towards the decrease in angiotensin AT1 receptor-coupled MAPK activity at high concentrations of angiotensin II.

The mechanism of ethanol action in hepatocytes is distinct from the one recently described in PC12 cells. Our results demonstrate that pertussis toxin-sensitive G-protein is needed for ethanol potentiation of MAPK activity in hepatocytes. Treatment with ethanol (0–100 mM) for 24 h dose-dependently increased $Gi\alpha_3$-protein expression in rat hepatocytes (Mckillop et al., 1999). In erythrocytes, platelets and hepatocytes, acute ethanol exposure increased phospholipase C activation (Hoek et al., 1992). These results indicate that ethanol can affect both the level of expression and function of G-proteins. We found that exposure of hepatocytes to ethanol for shorter times (up to 12 h) had no effect on potentiation. The longer time needed for the effect of ethanol favors the speculation that changes in expression of G-proteins may be involved. It may also be relevant to note that angiotensin II receptors were slightly, but not significantly increased in ethanol consuming mice brain (Daubert et al., 1999). However, little is known about ethanol effect on density of angiotensin II receptor in cultured hepatocytes.

Treatment of cells with pyrazole, an inhibitor of ethanol metabolism, during exposure to ethanol did not alter the property of ethanol to potentiate angiotensin II-stimulated MAPK. Likewise, acetaldehyde exposure did not potentiate angiotensin II-stimulated MAPK. Therefore, these data do not favor involvement of ethanol metabolism or of acetaldehyde in this phenomenon and suggest a direct effect of ethanol on the potentiation.

In conclusion, it was demonstrated here for the first time that ethanol potentiation of MAPK activity exhibits remarkable selectivity to agonists. MAPK activation by several agonists was not affected by ethanol. The observation that MAPK, activated by certain agonists, is affected by ethanol and not by others may have important bearing upon the pathophysiological consequences of ethanol on hepatocytes and on its responses to different stimuli. This may also reflect differential mechanisms for ethanol modulation of MAPK. In this regard, studies with angiotensin II favor the conclusion that ethanol potentiation of MAPK in hepatocytes is independent of ethanol metabolism and staurosporine-sensitive protein kinase C but is mediated via a pertussis toxin-sensitive G-protein.

Acknowledgements

We are grateful to Professor Grace Sun for her advice and comments on this manuscript. We also thank Ms. Nancy Picht for assisting us in experiments shown in Fig. 5C, Ms. Pam Burgess for typing the manuscript and Mr. Thomas O'Donnell for technical assistance. This work is supported by grant from NIAAA (AA11962).

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