Effects of ethanol on mitogen-activated protein kinase and stress-activated protein kinase cascades in normal and regenerating liver

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To understand the mechanisms by which ethanol inhibits hepatocyte proliferation, we studied the effects of ethanol on p42/44 mitogen-activated protein kinase (MAPK), p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK) in normal and regenerating rat liver. Treatment of rat hepatocytes with 100 mM ethanol *in vitro* for 16 h prolonged the activation of p42/44 MAPK and p38 MAPK induced by various agonists. Such treatment also increased basal JNK activity, but did not potentiate or prolong agonist-induced JNK activation. Ethanol potentiation of the activation of p42/44 MAPK was abolished by pertussis toxin. In contrast, chronic ethanol consumption *in vivo* inhibited the activation of p42/44 MAPK, p38 MAPK and JNK induced either by partial hepatectomy or by various agonists. However, both acute and chronic ethanol inhibited hepatocyte proliferation induced by insulin and epidermal growth factor. A selective inhibitor of p42/44 MAPK partially prevented the inhibition of hepatocyte proliferation caused by acute, but not by chronic, ethanol exposure, whereas a selective inhibitor of p38 MAPK further inhibited hepatocyte proliferation under both conditions. These data suggest that acute and chronic ethanol inhibit hepatocyte proliferation by different mechanisms. The effect of acute ethanol may be related to the prolongation of p42/44 MAPK activation, whereas inhibition of hepatocyte proliferation by chronic ethanol may be due to inhibition of p38 MAPK activation.

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INTRODUCTION

Alcoholic liver disease, a major cause of morbidity and mortality in Western societies, is a direct result of alcohol-induced hepatotoxicity coupled with impaired hepatic regenerative activity [1–4]. Direct liver injury by ethanol is due to several factors deleterious to the liver, including intracellular accumulation of protein and acetaldehyde, microsomal activation of hepatotoxins, alterations in hepatic redox state, and enhancement of lymphocyte cytotoxicity [1–4]. There is increasing evidence that the anti-regenerative effects of ethanol also contribute to the pathogenesis and progression of liver disease in alcoholic individuals [5–12], but the mechanisms by which ethanol inhibits liver regeneration and hepatocyte proliferation are still poorly understood.

Liver regeneration is controlled by multiple signalling pathways induced by a variety of hormones, growth factors and cytokines [13–15]. Recent evidence from knock-out mice deficient in interleukin-6 (IL-6) suggests that the following signalling sequence is critical for initiating liver regeneration [16,17]: tumour necrosis factor α (TNF α) $\rightarrow$ TNF α receptor $\rightarrow$ transcription factor NF- κ B $\rightarrow$ interleukin (IL)-6 $\rightarrow$ IL-6 receptor $\rightarrow$ transcription factor Stat3 $\rightarrow$ induction of critical target genes $\rightarrow$ liver regeneration. It has also been shown that various growth factors and hormones, as well as partial hepatectomy can activate p42/44 mitogen-activated protein kinase (p42/44 MAPK), p38 mitogen-activated protein kinase (p38 MAPK, also termed p38-RK or p38 stress-activated protein kinase) and c-Jun NH₂-terminal kinase (JNK, also termed p46/54 stress-activated protein kinase) in the rat liver [18]. These kinases have been shown

to play a pivotal role in cellular growth, transformation, differentiation and apoptosis in a variety of cell types [19–22], but their precise roles in hepatocyte proliferation have not been defined. We have recently reported a shift from p42/44 MAPK to p38 MAPK signalling in the post-hepatectomy regenerating liver and found that hepatocyte DNA synthesis can be blocked by an inhibitor of p38 MAPK, but not by an inhibitor of p42/44 MAPK [18]. These findings suggest that activation of p38 MAPK is a prerequisite for hepatocyte proliferation, whereas prolonged activation of MAPK may be antiproliferative in the liver [18]. JNK phosphorylates the activation domain of the c-Jun protein, but its precise role in hepatocyte proliferation is not clear. However, the recently reported failure of liver development in c-Jun knock-out mice [23,24] suggests that activation of this kinase is also involved in liver regeneration and hepatocyte proliferation. Ethanol has been shown to modulate the activity of several important signalling molecules, including the enzymes phospholipase C [24], adenylate cyclase [25], protein tyrosine kinase [26–29], PKC [30], JNK [30], p42/44 MAPK [31–33] and the transcription factors NF- κ B [34] and Stat3 [35]. However, the nature of the link between these effects and the well-documented antiproliferative effect of ethanol in the liver has not been established. Furthermore, despite the importance of p42/44 MAPK, p38 MAPK and JNK in hepatocyte proliferation, the effects of ethanol on these kinases in the liver have not yet been documented. Here we report that acute ethanol prolongs the activation of p42/44 MAPK and p38 MAPK induced by various agonists and increases the basal JNK activity in primary hepatocytes and that the effects of ethanol on p42/44 MAPK, but not

Abbreviations used: MAPK, mitogen-activated protein kinase; MEK1, MAPK kinase 1; JNK, c-Jun N-terminal kinase; PE, phenylephrine; EGF, epidermal growth factor; HGF, hepatocyte growth factor; TNF α , tumour necrosis factor α ; PKC, protein kinase C; MBP, myelin basic protein; IL, interleukin; NF- κ B, transcription factor NF- κ B; Stat3, transcription factor Stat3; PTX, pertussis toxin; GST, glutathione S-transferase; GG, glucagon; FCS, fetal-calf serum.

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on p38 MAPK or JNK activation, is blocked by pertussis toxin (PTX) pretreatment. However, feeding rats with an ethanol-containing diet for 60 days significantly inhibited the activation of p42/44 MAPK, p38 MAPK and JNK induced either by partial hepatectomy or by treatment with various agonists. Both acute ethanol and chronic ethanol significantly inhibit hepatocyte proliferation. A selective inhibitor of p42/44 MAPK partially prevented the inhibition of hepatocyte proliferation caused by acute *in vitro*, but not by chronic *in vivo*, exposure to ethanol, whereas a selective inhibitor of p38 MAPK further inhibited hepatocyte proliferation under both conditions. These findings suggest that inhibition of hepatocyte proliferation by acute ethanol *in vitro* may be related to the prolongation of the activation of p42/44 MAPK, whereas the antiproliferative effect of chronic ethanol consumption *in vivo* may be due to inhibition of p38 MAPK.

MATERIALS AND METHODS

Materials

P42/44 MAPK, p38 MAPK and JNK antibodies (in solution and/or conjugated to Protein A-agarose beads) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Hormones, growth factors, myelin basic protein (MBP) and collagenase were from Sigma Chemicals (St. Louis, MO, U.S.A.). PTX and calphostin C were from Calbiochem (San Diego, CA, U.S.A.). The MAPK kinase 1 (MEK1) inhibitor PD 98059 was from New England Biolabs (Beverly, MA, U.S.A.). The P38 MAPK inhibitor SB203850 and its inactive analogue, SKF106978, were kindly provided by SmithKline Beecham Pharmaceuticals (King of Prussia, PA, U.S.A.). Glutathione S-transferase (GST)-c-Jun (amino acids 1-169) was prepared with GST-Sepharose as described previously [18]. Radiolabelled [γ - 32 P]ATP was purchased from Dupont/NEN (Boston, MA, U.S.A.).

Isolation of hepatocytes and treatment of hepatocytes for kinase assays

Male Sprague-Dawley rats weighing 80-120 g were anaesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and the portal vein was cannulated under aseptic conditions. Liver cells were isolated by a collagenase-perfusion protocol as described previously [18]. The isolated cells were washed twice with hepatocyte medium (Dulbecco's modified Eagle's medium containing 10^{-8} M dexamethasone, 2.5 μ g/ml of amphotericin B, 50 μ g/ml of gentamicin, 67 μ g/ml of penicillin and 100 μ g/ml of streptomycin). The hepatocytes (2×10^5 cells/cm²) were plated on to polylysine-coated culture dishes in hepatocyte medium containing 2% fetal-bovine serum with or without 100 mM ethanol. After 1 h, the medium was changed to hepatocyte medium containing 10 ng/ml of EGF and 5 μ g/ml of insulin with or without 100 mM ethanol. After 16 h, cells were stimulated with various agonists for various times. Cells were homogenized in 400 μ l of ice-cold lysis buffer A [25 mM Hepes (pH 7.4)/5 mM EDTA/5 mM benzamidine/1 mM PMSF/1 mg/ml soybean trypsin inhibitor/40 μ g/ml pepstatin A/40 μ g/ml aprotinin/1 μ M Microcystin-LR/0.5 mM sodium orthovanadate/0.5 mM sodium pyrophosphate/1% (v/v) Triton X-100/0.1% (v/v) 2-mercaptoethanol]. The homogenate was incubated on ice for 5 min. After centrifugation at 14000 g for 10 min, the supernatant was collected and protein concentration was measured using the Bio-Rad protein assay reagent. The supernatant was subjected to the kinase assays, as described below.

Immunoprecipitations from hepatocyte homogenates

Protein A/G plus agarose slurry (25 μ l bead volume) was washed with 1 ml of PBS containing 0.1% (v/v) Tween 20, and resuspended in 0.4 ml of the same buffer. Antibodies (2 μ g in 20 μ l) were added to each tube and incubated (2 h, 4 °C) to allow for their conjugation to protein A/G plus agarose beads, followed by a subsequent wash to remove non-conjugated antibodies. In preliminary experiments the kinase activities were linear with protein concentration between 50 μ g and 2 mg. Therefore, 500 μ g of protein from the each homogenate was mixed with Protein A-agarose-conjugated antibody and rocked (2 h, 4 °C). The protein A-agarose was recovered by centrifugation, the supernatant discarded, and the immunoprecipitates were washed twice with lysis buffer A and once with washing buffer B [25 mM Hepes (pH 7.4)/15 mM MgCl₂/0.1 mM sodium orthovanadate/0.1% (v/v) 2-mercaptoethanol].

Kinase assays

The p42/44 MAPK and p38 MAPK assays were described previously [18]. Briefly, immunoprecipitates were incubated (final volume 50 μ l) with 50 μ l of washing buffer B containing 0.2 mM [γ - 32 P]ATP (5000 c.p.m./pmol), 1 μ M Microcystin-LR, and 1 mg/ml MBP. In preliminary experiments, the kinase activities were linear with respect to incubation time between 2 min and 40 min. Therefore, samples were incubated for 15 min, then 40 μ l of the reaction volume was spotted on to P81 paper (Whatman, Maidstone, Kent, U.K.) and immediately placed in 180 mM phosphoric acid. The papers were washed several times with phosphoric acid, followed by a final wash with acetone. The 32 P incorporation into MBP was quantified by liquid-scintillation spectrometry. Alternatively, the reactions were terminated with SDS protein loading buffer and prepared for SDS/PAGE (15% polyacrylamide gel) to quantify 32 P incorporation by phosphorimaging.

The JNK assay was described previously [18]. Briefly, immunoprecipitates were incubated (final volume 50 μ l) with 50 μ l of washing buffer B containing 0.2 mM [γ - 32 P]ATP (5000 c.p.m./pmol), 1 μ M Microcystin-LR, and 10 μ g GST-c-Jun (amino acids 1-169). After 30 min, the reactions were terminated with SDS protein loading buffer and the mixtures prepared for SDS/PAGE (10% polyacrylamide gel) to quantify 32 P incorporation by phosphorimaging.

Primary culturing of rat hepatocytes and [3 H]thymidine incorporation

Primary culturing of rat hepatocytes and measurement of [3 H]thymidine incorporation were described previously [18]. [3 H]-Thymidine uptake was measured as total d.p.m./mg of protein.

Chronic-ethanol-consumption model

Male Sprague-Dawley rats, initially weighing 200 g, were fed a nutritionally adequate ethanol-containing liquid diet (Bioserv, Frenchtown, NJ, U.S.A.) for 60 days, as described by DeCarli and Leiber [36]. Littermate controls of similar body weights were pair-fed on the same liquid diet, except that ethanol was replaced by carbohydrate. Ethanol consumption on this diet usually averages 14-16 g/day per kg body weight, and blood alcohol levels have been reported to be in the 25-50 mM range [36].

Partial hepatectomy

Adult male Sprague-Dawley rats were subjected to two-thirds partial hepatectomy [37]. Rats were anaesthetized with sodium

Figure 1 Effects of acute ethanol (ETOH) treatment on p42/44 MAPK (A), p38 MAPK (B) and JNK (C) activation induced by EGF (5 ng/ml, A and B) or TNF α (20 ng/ml, C)

Hepatocytes were incubated with (○) or without (●) 100 mM ethanol for 16 h, then stimulated with EGF or TNF α for various periods of time. Whole-cell extracts were prepared, and 500 μ g of protein from each sample was subjected to kinase assays for p42/44 MAPK (A), p38 MAPK (B) and JNK (C) activities. Autoradiograms representative of three independent experiments for each panel are shown. The radioactivities on blots were quantified by

phosphorimaging. Significant difference from the corresponding agonist-stimulated control group is indicated by asterisks: * $P < 0.01$; ** $P < 0.05$. The basal levels of p42/44 MAPK and p38 MAPK were not significantly affected, whereas the basal level of JNK was significantly stimulated by ethanol treatment (control basal JNK: 19735 $\pm$ 2432 c.p.m.; 100 mM ethanol group basal JNK: 28824 $\pm$ 3527 c.p.m.).

Isolation of protein extracts from liver for kinase assays

The protein extracts isolated from liver for kinase assays were described previously [18]. Briefly, 50 mg of frozen tissue was homogenized in 1 ml of ice-cold lysis buffer A using a motor-driven Potter homogenizer, and then further homogenized with an all-glass Dounce homogenizer and incubated on ice for 10 min. After centrifugation at 14000 g for 30 min, the supernatant was collected and protein concentration was measured with the Bio-Rad protein assay. The supernatant was then subjected to kinase assays.

Data analysis

For multiple comparisons, one-factor analysis of variance followed by Tukey's *post hoc* test was used; a P value of less than 0.05 was taken to imply statistical significance.

RESULTS

Treatment of hepatocytes with 100 mM ethanol for 16 h prolongs the activation of p42/44 MAPK, p38 MAPK and JNK

The activation of p42/44 MAPK, p38 MAPK and JNK has been suggested to play an important role in liver regeneration and hepatocyte proliferation [18]. We tested whether ethanol can modulate the activation of these signalling pathways. Freshly isolated hepatocytes were plated on to rat-tail-collagen-coated culture dishes in washing medium containing 2% fetal-bovine serum with or without 100 mM ethanol. After 1 h, the medium was changed to serum-free medium and incubated for a further 16 h with or without 100 mM ethanol, after which EGF or TNF α was added for various periods of time. Whole-cell extracts were then prepared and subjected to kinase assays for p42/44 MAPK (Figure 1A), p38 MAPK (Figure 1B) and JNK (Figure 1C) activities.

As shown in Figure 1(A), EGF treatment caused a rapid activation of p42/44 MAPK that peaked at 5 min, and activity returned back to basal levels in 15–20 min (Figure 1A). Pre-incubation with 100 mM ethanol for 16 h did not significantly affect the maximal p42/44 MAPK activation by EGF, but significantly prolonged it, with the activity remaining elevated for 30 min. Figure 1(B) shows that EGF also rapidly activated p38 MAPK, and pretreatment with 100 mM ethanol also prolonged this activation (Figure 1B). The above changes in kinase activities were not due to the changes in protein expression level, since Western-blotting data showed that ethanol treatment for 16 h did not affect the p42/44 MAPK and p38 MAPK kinase protein expression (results not shown). Pretreatment of primary hepatocytes with 100 mM ethanol increased the basal JNK activity, but did not prolong or potentiate TNF α -induced fold activation of JNK (Figure 1C).

Figure 2 Effects of acute ethanol (ETOH) treatment on p42/44 MAPK (A), p38 MAPK (B) and JNK (C) activation induced by various agonists

Hepatocytes were incubated with or without 100 mM ethanol for 16 h, then stimulated with phenylephrine (PE, 10 μ M), EGF (5 ng/ml), insulin (Ins, 5 μ g/ml), HGF (20 ng/ml), TNF α (20 ng/ml) or GG (100 nM) for 15 min. Whole-cell extracts were then prepared and 500 μ g of protein from each sample was subjected to kinase assays for p42/44 MAPK (A), p38 MAPK (B) and JNK (C) activities. The autoradiograms shown are representative of three independent experiments. The radioactivities on blots were quantified by phosphorimaging. Values shown are means $\pm$ S.E.M. from three independent experiments, expressed as fold changes over control. Significant difference from corresponding agonist-stimulated control group is indicated

The above observations indicate that ethanol is able to prolong the EGF-induced activation of p42/44 MAPK and p38 MAPK. To determine whether these effects of ethanol are limited to EGF or TNF α , primary hepatocytes pretreated with or without 100 mM ethanol for 16 h were stimulated with different agonists for 20 min. We chose the 20 min time-point to measure kinase activities, because elevated activities would then indicate prolonged activation. As shown in Figure 2(A), EGF, insulin and hepatocyte growth factor (HGF), but not phenylephrine (PE), tumour necrosis factor α (TNF α) or glucagon (GG) significantly increased p42/44 MAPK activity, and pretreatment with ethanol significantly prolonged activation by all three of the effective agonists. The data in Figure 2(B) illustrate that EGF, insulin, HGF, TNF α , and GG significantly increased p38 MAPK activation, and pretreatment with ethanol caused a marked increase in the activation of this kinase. Figure 2(C) shows that pretreatment with ethanol significantly increased the basal JNK activity, but did not increase the fold stimulation of JNK induced by various agonists. These data suggest that exposure of hepatocytes to 100 mM ethanol can markedly prolong the activation of p42/44 MAPK and p38 MAPK, and these effects of ethanol are not agonist-specific. The lack of activation of p42/44 MAPK, p38 MAPK and JNK by PE in Figure 2 is probably due to the decreased expression of α_{1B} adrenergic receptors in 16 h-cultured primary hepatocytes [18].

Ethanol prolongs the activation of p42/44 MAPK by a toxin-sensitive G-protein-dependent mechanism

It has been reported that ethanol potentiates p42/44 MAP kinase activation in embryonic liver cells by a PTX-sensitive G-protein-dependent mechanism [31] and in neural PC12 cells by a PKC-dependent mechanism [32]. We examined the involvement of these mechanisms in the ethanol prolongation of p42/44 MAPK, p38 MAPK activation and the ethanol potentiation of basal JNK activation in primary adult rat hepatocytes. Freshly isolated hepatocytes were treated with PTX or the PKC inhibitor calphostin C [38] for 30 min, and then incubated with or without 100 mM ethanol for 16 h. EGF or TNF α was then added for various periods of time before preparing cell lysates for the kinase assays. As shown in Figure 3(A), EGF rapidly activated p42/44 MAPK, with activity returning to basal levels in 20 min. Pretreatment of the hepatocytes with PTX or calphostin C alone had no effect on this activation. Preincubation with 100 mM ethanol prolonged EGF-induced p42/44 MAPK activation, and the effect of ethanol was abolished by PTX but not by calphostin C. These findings suggest that ethanol prolongs p42/44 MAPK activation by a PTX-sensitive G-protein-dependent mechanism. In contrast, prolongation of p38 MAPK activation and potentiation of the basal JNK activation by ethanol was not affected by either PTX or calphostin C (Figure 3).

Chronic *in vivo* treatment of rats with ethanol inhibits partial hepatectomy-induced activation of p42/44 MAPK, p38 MAPK and JNK

We tested whether the activation of p42/44 MAPK, p38 MAPK and JNK by partial hepatectomy is modulated by chronic ethanol consumption. To test this, rats were fed with an ethanol-

by asterisks: * $P < 0.01$; ** $P < 0.05$. The basal levels of p42/44 MAPK and p38 MAPK were not significantly affected, whereas the basal level of JNK was significantly stimulated by ethanol treatment (control basal JNK: 15521 $\pm$ 1489 c.p.m.; 100 mM ethanol group basal JNK: 29598 $\pm$ 2890 c.p.m.).

Figure 3 A PTX-sensitive G-protein is involved in prolongation of p42/44 MAPK activation by ethanol (ETOH)

(A) Hepatocytes were treated with PTX (100 ng/ml) or calphostin C (Cal C; 0.5 μ M) for 30 min, then 100 mM ethanol or buffer was added for 16 h, following which cells were stimulated with EGF (5 ng/ml) or TNF α (20 ng/ml) for various periods of time. Cells incubated with calphostin C were exposed to light. Cell extracts were prepared and subjected to kinase assays for p42/44 MAPK, p38 MAPK and JNK activities. Autoradiograms shown are representative of three independent experiments. (B) The radioactivities for P42/44 MAPK (A) were measured by phosphorimaging. Values shown are means $\pm$ S.E.M. from three independent experiments, expressed as fold changes over control. Significant difference from corresponding agonist-stimulated control group is indicated by an asterisk: * $P < 0.01$. ●, Control (C); ○, control + ethanol; ▼, control + ethanol + PTX.

containing diet or a control diet for 60 days and then subjected to partial hepatectomy. Extracts of the remnant liver were assayed for p42/44 MAPK (Figure 4A), p38 MAPK (Figure 4B) and JNK (Figure 4C) activities. As shown in Figure 4(A), p42/44 MAPK was dramatically activated after partial hepatectomy in pair-fed rats, reached a peak at 5 h and returned to the basal level at 16 h. In ethanol-fed rats, this regenerative induction of p42/44 MAPK was significantly attenuated. Figures 4(B) and 4(C) show that both p38 MAPK and JNK are rapidly activated after partial hepatectomy in pair-fed rats, the peak activation occurring at 30 min, with the activity declining towards control levels at 16 h. In the chronic ethanol-fed rats, this regenerative induction of p38 MAPK and JNK is significantly inhibited. The inhibition of the regenerative activation of JNK by chronic ethanol intake is consistent with findings in a previous report [34].

Chronic ethanol treatment inhibits agonists-induced activation of p42/44 MAPK, p38 MAPK and JNK

The above data indicate that chronic ethanol consumption inhibits the activation of p42/44 MAPK, p38 MAPK and JNK following partial hepatectomy. Ethanol may reduce the concentration of endogenous hormones/growth factors mediating this effect or may decrease the sensitivity of the liver to these mediators. To test this latter possibility, hepatocytes were isolated from the livers of pair-fed or chronic-ethanol-fed rats and stimulated with various agonists for 10 min. Cell lysates were then prepared and assayed for p42/44 MAPK (Figure 5A), p38 MAPK (Figure 5B) and JNK (Figure 5C) activities.

Figure 5(A) illustrates that p42/44 MAPK is rapidly activated by PE, EGF, insulin and HGF, but not by TNF α or GG, in control hepatocytes, but this activation is significantly blunted in hepatocytes from ethanol-fed rats. Figures 5(B) and 5(C) show that both p38 MAPK and JNK are stimulated by PE, EGF, insulin, HGF, TNF α and GG in control hepatocytes and, again, this induction is significantly attenuated in hepatocytes from ethanol-fed rats. These data suggest that the decreased agonist-activation of p42/44 MAPK, p38 MAPK and JNK following

chronic ethanol treatment is due to a decrease in the sensitivity of the hepatocytes to the various agonists.

Inhibition of hepatocyte proliferation by acute and chronic ethanol is mediated by different mechanisms

It has been shown that acute exposure to ethanol can inhibit hepatocyte proliferation *in vitro* [39], but the mechanisms underlying this effect are unknown. Recent evidence suggests that prolonged activation of Raf-1 kinase and p42/44 MAPK can lead to cell-cycle arrest [19–22]. We wondered whether ethanol inhibition of hepatocyte proliferation may be also due to prolonged activation of p42/44 MAPK. To test this, freshly isolated hepatocytes in hepatocyte medium containing 2% fetal-calf serum (FCS) were treated for 30 min with PD98059, a specific MEK1 inhibitor, and were then incubated with or without 100 mM ethanol. After 2 h the medium was changed to hepatocyte medium containing 10 ng/ml EGF and 5 μ g/ml insulin with or without 100 mM ethanol. After 72 h, hepatocytes were harvested for measurement of [3 H]thymidine incorporation. As shown in Figure 6(A), 100 mM ethanol inhibited [3 H]thymidine incorporation by about 50%, which is consistent with the findings in previous reports [5,39]. Incubation of the cells with 20 μ M PD98059 resulted in a slight inhibition of basal [3 H]thymidine incorporation, but prevented in part the inhibition of hepatocyte proliferation caused by ethanol. In contrast, preincubation of the cells with SB203850, a highly specific p38 MAPK inhibitor, significantly decreased basal [3 H]thymidine incorporation, and enhanced the inhibition of hepatocyte proliferation caused by ethanol (Figure 6A). Incubation with SKF 106978, an inactive analogue of SB203850, had no significant effect on basal or ethanol-mediated inhibition of [3 H]thymidine incorporation (results not shown). These results suggest that prolongation of p42/44 MAPK is one of the mechanisms responsible for acute ethanol inhibition of hepatocyte proliferation *in vitro*.

Chronic ethanol consumption has also been shown to inhibit liver regeneration [7,11,12] and hepatocyte proliferation [40].

Figure 4 Chronic ethanol consumption inhibits p42/44 MAPK (A), p38 MAPK (B) and JNK (C) activation induced by partial hepatectomy

Rats fed with an ethanol-containing or a control liquid diet for 60 days were subjected to partial hepatectomy. Liver extracts were then prepared and assayed for p42/44 MAPK (A), p38 MAPK (B) and JNK (C) activities. Autoradiograms representative of three independent experiments for each panel are shown. The radioactivities on blots were quantified by phosphorimaging.

Figure 5 Chronic ethanol consumption inhibits p42/44 MAPK (A), p38 MAPK (B) and JNK (C) activation induced by various agonists

Hepatocytes isolated from pair-fed or ethanol-fed rats were stimulated with PE (10 μ M), EGF (5 ng/ml), insulin (Ins; 5 μ g/ml), HGF (20 ng/ml), TNF α (20 ng/ml) or GG (100 nM) for 10 min. Whole-cell extracts were then prepared and subjected to kinase assays for p42/44 MAPK (A), p38 MAPK (B) and JNK (C) activities. The autoradiograms shown are representative of three independent experiments. The radioactivities on blots were quantified by phosphorimaging. Values shown are means $\pm$ S.E.M. from three independent experiments, expressed as fold changes compared with control. Significant difference from corresponding agonist-stimulated control group: * P < 0.01; ** P < 0.05.

Figure 6 Acute and chronic ethanol (ETOH) inhibits hepatocyte proliferation via different mechanisms

Hepatocytes isolated from pair-fed (A) or ethanol-fed (B) rats were plated in minimal essential medium containing 10^{-8} dexamethasone and 2% FCS. The cells were incubated with or without PD98059, SB203850 and/or 100 mM ethanol for 2 h, and then stimulated with 5 ng/ml EGF and 50 ng/ml insulin. After 72 h, 2 μ Ci of [3 H]thymidine was added for an additional 2 h. Cells were then lysed for quantification of [3 H]thymidine incorporation. Values shown are means $\pm$ S.E.M. from three independent experiments, expressed as percentage [3 H]thymidine incorporation relative to the corresponding controls. Significant difference from corresponding control: * P < 0.01; significant difference from *in vitro* ethanol-treated group (# P < 0.05). [3 H]Thymidine incorporation in pair-fed and chronic-ethanol-fed controls was $11\,235 \pm 2653$ c.p.m. and 6589 ± 621 c.p.m. respectively.

Since activation of p38 MAPK plays a critical role in hepatocyte proliferation [18] and chronic ethanol consumption significantly inhibits activation of p38 MAPK in hepatocytes (see Figure 5B), it appeared plausible that the antiproliferative action of chronic ethanol is mediated by inhibition of p38 MAPK activity. To test this, hepatocytes isolated from pair-fed and chronic-ethanol-fed rats were assayed for [3 H]thymidine uptake. As shown in Figure 6(B), hepatocytes from the chronic ethanol-fed rats had significantly lower [3 H]thymidine incorporation than cells from pair-fed rats, and this reduced uptake was further inhibited by the p38 MAPK inhibitor SB203850, and slightly reduced by p42/44 MAPK inhibitor PD98059. These data suggest that chronic ethanol inhibits hepatocyte proliferation via inhibition of p38 MAPK activity.

DISCUSSION

We have demonstrated that acute ethanol prolongs the activation of p42/44 MAPK, p38 MAPK and JNK by various agonists in primary cultured rat hepatocytes. Furthermore, a PTX-sensitive, G-protein-dependent mechanism was found to be involved in the prolongation of p42/44 MAPK, but not of p38 MAPK and JNK activation. In contrast, chronic ethanol consumption significantly inhibited the activation of p42/44 MAPK, p38 MAPK and JNK induced either by partial hepatectomy or by various agonists. Finally, a selective inhibitor of p42/44 MAPK was able to prevent the acute, but not the chronic-ethanol-induced inhibition of [3 H]thymidine incorporation into primary cultured rat hepatocytes, whereas a selective inhibitor of p38 MAPK caused further inhibition of [3 H]thymidine uptake under both conditions. These observations suggest that prolonged activation of p42/44 MAPK may be one of the mechanisms responsible for inhibition of hepatocyte proliferation by acute ethanol *in vitro*. In contrast, reduced activation of p38 MAPK may be responsible for

inhibition of hepatocyte proliferation by chronic ethanol consumption *in vivo*.

The first novel observation in the present study is the ability of a biologically relevant concentration of ethanol to acutely prolong the agonist-induced activation of p42/44 MAPK, p38 MAPK and the basal JNK activation in primary hepatocytes. Furthermore, the first effect, but not the last two effects, is mediated by a G_i -protein-dependent mechanism. These data are consistent with the documented involvement of a G_i protein in the activation of p42/44 MAPK [41], but not in the activation of p38 MAPK and JNK [42]. Ethanol activates a number of enzymes via a G_i -protein-dependent mechanism [36,43,44], although the nature of the effect of ethanol on G-protein function is not well understood. It is believed that ethanol can influence both the function and the gene expression of G-proteins [45,46]. We found that a brief (30 min) exposure of hepatocytes to ethanol prolonged the agonist activation of p42/44 MAPK (J. Chen, E. J. Ishac, P. Dent, G. Kunos and B. Gao, unpublished work), which suggests that ethanol may directly affect G-protein function in primary hepatocytes. The mechanisms responsible for prolonged and/or enhanced activation of p38 MAPK and JNK are not clear. Our data indicate that acute ethanol *in vitro* alone was able to activate JNK (Figure 1C and Figure 2C) in primary hepatocytes, which is consistent with findings in a previous report [34]. It has been shown that receptor clustering can activate p38 MAPK and JNK [47], and acute ethanol can induce disturbances in membrane order [48–50]. Whether ethanol can induce receptor clustering and activation of p38 MAPK and JNK by such a mechanism requires further study.

In contrast with the prolongation of p42/44 MAPK, p38 MAPK and JNK activation by acute ethanol, chronic ethanol intake *in vivo* significantly inhibited the partial-hepatectomy-induced activation of these kinases, and this inhibition could be attributed to a decreased sensitivity of hepatocytes to agonists. These findings suggest that chronic ethanol exposure interferes with the functioning of hepatic receptors and/or signal-transduction pathways. Indeed, chronic ethanol consumption has been shown to impair hepatic insulin-receptor function [51], and to suppress EGF-induced receptor autophosphorylation [52].

P42 MAPK, p38 MAPK and JNK represent a family of serine/threonine protein kinases whose activation is part of the early response to a variety of stimuli involved in cellular growth, transformation, differentiation, and apoptosis [19–22]. Recent evidence indicates that p38 MAPK plays a major role in hepatocyte proliferation [18], whereas chronic activation of p42/44 MAPK has been shown to lead to cellular growth arrest [20–22]. The precise role of JNK in hepatocyte proliferation is not clear, although there is evidence to suggest that activation of JNK leads to apoptosis [53]. Therefore one might speculate that activation of JNK in the liver might be involved in hepatocyte apoptosis. Although acute and chronic ethanol exposure have opposite effects on p42 MAPK, p38 MAPK and JNK activation, they both inhibit hepatocyte proliferation. An explanation of this paradox may be found in the observation that a specific inhibitor of p42/44 MAPK prevented the inhibition of [3 H]thymidine incorporation caused by acute, but not by chronic, ethanol treatment, whereas blocking p38 MAPK activation further enhanced both acute and chronic ethanol inhibition of hepatocyte proliferation. These data suggest that acute ethanol may inhibit hepatocyte proliferation via the prolongation of p42 MAPK activation, whereas the similar effect of chronic ethanol exposure is due to inhibition of p38 MAPK activation. Whether effects of JNK activation by acute and chronic ethanol also contribute to the inhibition of hepatocyte proliferation requires further studies.

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