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# Ethanol rapidly inhibits IL-6-activated STAT3 and C/EBP mRNA expression in freshly isolated rat hepatocytes[1]

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**Abstract The ability of ethanol to inhibit regenerative processes in the liver is thought to play a key role in the development of alcoholic liver disease. To understand the underlying mechanisms, we investigated the effects of ethanol on the Janus kinase-signal transducer and activator transcription factor (JAK-STAT) signaling pathways in hepatocytes. Treatment of freshly isolated adult rat hepatocytes with 10–100 mM ethanol rapidly (< 3 min) inhibits interleukin-6 (IL-6)-induced STAT3 activation, tyrosine and serine phosphorylation and IL-6-induced CCAAT enhancer binding protein (C/EBP) α and β mRNA expression. Western analyses, in vitro kinase assays and in vivo cell labelling assays indicate that this inhibitory effect is not due to blocking the upstream-located JAK1, JAK2 or Tyk2 activation. On the contrary, acute ethanol exposure significantly potentiates IL-6-induced JAK1 autophosphorylation in vitro and in vivo. Pretreatment with sodium vanadate, a non-selective tyrosine phosphatase inhibitor, or with MG132 and lactacystin, proteasome inhibitors, does not abolish the ethanol inhibition of IL-6-induced STAT3 activation, suggesting that activation of protein tyrosine phosphatases or the ubiquitin-proteasome pathway is not involved. In view of the critical role of IL-6 signaling in liver regeneration, these findings suggest that the ability of biologically relevant concentrations of ethanol to markedly inhibit IL-6-induced STAT3 phosphorylation is one of the cellular mechanisms involved in the pathogenesis and progression of alcoholic liver diseases.**



*Key words:* Ethanol; Janus kinase-signal transducer and activator transcription factor; CCAAT enhancer binding protein

## 1. Introduction

The Janus kinase-signal transducer and activator transcription factor (JAK-STAT) signaling pathways were originally identified in the study of interferon signaling and are now believed to be the major pathways involved in signal transduction for many cytokines and growth factors. These pathways have been implicated in a variety of cellular functions in the hematopoietic, immunologic, neuronal and hepatic systems [1–6]. In the liver, JAK-STAT activated by interleukin-6 (IL-6) has been shown to play a critical role in hepatic regeneration and in the acute phase response [7–9]. It is well-established that the binding of IL-6 to the IL-6 receptor α chain induces homodimerization of the signal transducing β chain gp130, which is followed by activation of the receptor-associated tyrosine kinases, known as JAK1, JAK2 and Tyk2. This receptor-kinase complex interacts with and activates the SRC homology 2 (SH2)-containing cytoplasmic STAT3 transcription factor, which then translocates to the nucleus to activate the transcription of many target genes, such as c-jun, c-myc, Jun B, CCAAT enhancer binding protein (C/EBP), $p21^{WAF1/Cip1}$ [1–3,8,10].

It is well known that excessive ethanol consumption damages the liver and it is also well-established that the ability of ethanol to inhibit liver regeneration is a major factor in ethanol-induced liver damage [11–16]. We have previously shown that in vitro treatment of freshly isolated hepatocytes with biologically relevant concentrations of ethanol (50–100 mM) significantly inhibits IL-6-induced STAT3 activation [17]. In the present study, we demonstrate that ethanol inhibition of IL-6-induced STAT3 activation develops rapidly, is most likely due to inhibition of tyrosine and serine phosphorylation of STAT3 and it results in a suppression of IL-6-induced C/EBPα and C/EBPβ mRNA expression. The data further indicate that the ethanol inhibition on IL-6-induced STAT3 phosphorylation is not due to inhibition of JAK1, JAK2 or Tyk2 phosphorylation or due to activation of a tyrosine phosphatase.

## 2. Materials and methods

### 2.1. Materials

STAT3, JAK1, JAK2, Tyk2, anti-phosphotyrosine antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-phospho-STAT3 ($Tyr^{705}$) and anti-phospho-STAT3 ($Ser^{727}$) antibodies were from Bio-lab (Beverly, MA, USA). The following reagents were obtained from Sigma Chemicals (St. Louis, MO, USA): ethanol, collagenase type IV, sodium vanadate and Nonidet P-40. GF 109203X, MG132 and lactacystin were from Calbiochem (San Diego, CA, USA). IL-6 was a gift from Dr Tian Zhigang (Shandong Academy of Medical Sciences). Radiolabelled [$\gamma$-$^{32}$P]ATP and carrier-free [$^{32}$P]phosphate were from Dupont NEN (Boston, MA, USA).

### 2.2. Isolation of hepatocytes

Liver cells were isolated by a collagenase perfusion protocol as described earlier [17]. The isolated cells were washed twice and resuspended in Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 25 mM $NaHCO_3$ and 10 mM glucose) containing 1.5% gelatin and were further treated with ethanol or IL-6.

### 2.3. DNA gel mobility shift assay (DMSA)

The DMSA was described previously [18]. The STAT3 binding site of oligo m67 (the high affinity serum-induced element m67) (5′-GTC

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*Abbreviations:* JAK-STAT, Janus kinase-signal transducer and activator transcription factor; IL-6, interleukin-6; C/EBP, CCAAT enhancer binding protein; PKC, protein kinase C; DMSA, DNA gel mobility shift assay


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GAC ATT TCC CGT AAA TCG TCG A-3′) [19,20] was used as a probe.

*2.4. Northern blotting analyses*

Northern analyses were done as described previously [21]. Briefly, total cellular RNA was extracted and purified by the guanidinium isothiocyanate method, using the RNAzol B kit (Cinna/Biotecx). The RNA was electrophoretically fractionated on a 1.0% agarose gel containing 6.5% formaldehyde and transferred onto a nylon membrane. The size of the bands was estimated by comparison with the migration of a 0.24–9.5 kb RNA ladder (Gibco). Blots were pre-hybridized for 2 h in a buffer containing 50% formamide, 5×Denhardt's solution, 100 μg/ml sheared salmon sperm DNA and 0.5% SDS. Hybridization was carried out at 42°C overnight in the above solution, using $^{32}$P-labelled C/EBPα or C/EBPβ cDNA probes. Blots were washed in 1×SSC, 0.1% SDS for 20 min at room temperature followed by two washes of 20 min each in 0.2×SSC, 0.1% SDS at 65°C. Bands were visualized and quantified by phosphorimaging, using a phosphorimager and the ImageQuant program (Molecular Dynamics).

*2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)*

The RT-PCR was described previously [18]. The following primer pairs were used: forward primer (5′-GAA TCT CCT AGT CCT GGC TC-3′) and reverse primer (5′-GAT GAG AAC AGC AAC GAG TAC-3′) for rat C/EBPα; forward primer (5′-GCC ACG GAC ACC TTC GAG G-3′) and reverse primer (5′-CGG CTC CGC CTT GAG CTG-3′) for rat C/EBPβ; forward primer (5′-TAC ATG GGT GGG GTG TTG AA-3′) and reverse primer (5′-AAG AGA GGC ATC CTC ACC CT-3′) for rat β actin.

*2.6. Cell extraction, immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*

Cells were lysed in lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM $Na_3VO_4$, 1% Nonidet P-40, 10% glycerol) on ice for 15 min, vortexed and spun at 16 000 rpm at 4°C for 10 min. The supernatants were incubated with antibodies against STAT3 or JAKs (Upstate Biotechnology) at 4°C for 2 h and then incubated with protein A agarose beads at 4°C for another 2 h. Immunoprecipitated complexes were then washed three times with lysis buffer and dissolved in Laemmli running buffer. Samples were boiled for 4 min and subjected to SDS-PAGE.

*2.7. Western blots*

After electrophoresis, proteins were transferred onto nitrocellulose membranes and blocked by incubation for 2 h at room temperature with 5% milk in TBS plus 0.05% Tween (TBST) when blotted with anti-phosphotyrosine antibody (4G10, Upstate Biotechnology) or blocked by incubation with 5% defatted milk in TBST when blotted with anti-STATs or anti-JAKs. The 4G10 antibody was used at a 1:4000 dilution for overnight incubations at 4°C, whereas anti-STATs or anti-JAKs were used at a 1:2500 dilution for 2 h at room temperature. Membranes were washed with TBST and incubated with a 1:4000 dilution of horseradish peroxidase-conjugated secondary antibodies for 45 min. Detection was by the Enhanced Chemiluminescence kit (Amersham).

*2.8. In vivo labelling of cells with [$^{32}$P]phosphate*

Hepatocytes (2–3×$10^7$ cells/ml) were incubated for 1 h in a phosphate-free buffer (118 mM NaCl, 4.75 mM KCl, 1.2 mM $MgCl_2$, 0.26 mM $CaCl_2$, 25 mM $NaHCO_3$) with ∼0.5–1 mCi/ml carrier-free [$^{32}$P]phosphate in the absence or presence of 0.1 M ethanol for 1 h. The cells were then stimulated with 20 ng/ml IL-6 for 5 min, followed by washing twice with phosphate-buffered saline (PBS) containing 1 mM $Na_3VO_4$. The cell pellets were lysed and immunoprecipitated with JAK2 antibody, the proteins fractionated by SDS-PAGE and analyzed by phosphorimaging.

*2.9. Autophosphorylation*

Cells were washed twice with PBS containing 1 mM $Na_3VO_4$ and lysed in 0.5 ml of lysis buffer. Immunoprecipitated complexes were washed twice with lysis buffer and once with kinase buffer (50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 10 mM $MnCl_2$, 0.1 mM $Na_3VO_4$). Pellets were resuspended in 50 μl of kinase buffer containing 5 μCi of [γ-$^{32}$P]ATP and incubated at 30°C for 10 min. Beads were washed twice with 500 μl stop buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA) and then boiled in SDS sample buffer containing 2.5% 2-mercaptoethanol for 5 min. The solubilized proteins were resolved by SDS-PAGE and quantified by phosphorimaging.

## 3. Results

*3.1. Ethanol rapidly inhibits IL-6-induced STAT3 activation and IL-6-induced C/EBPα and C/EBPβ mRNA expression*

As shown in Fig. 1A, exposure of freshly isolated hepatocytes to 20 ng/ml IL-6 for 30 min activates STAT3 as indicated by STAT3 binding to its specific m67 oligonucleotide probe (lane 1 versus 2). Furthermore, this activation was dose-dependently inhibited by a 30 min pretreatment with ethanol, with inhibition already evident at 10 mM and complete at 100 mM ethanol. To determine the rate of onset of the inhibitory effect of ethanol, cells were exposed to 100 mM ethanol for various time periods before (B) or after IL-6 stimulation (C). Complete inhibition of STAT3 activation was evident already after a 2 min pre-incubation with ethanol and even a 1 min pretreatment caused 90% inhibition (Fig. 1B). Ethanol was also effective when added within 1–3 min

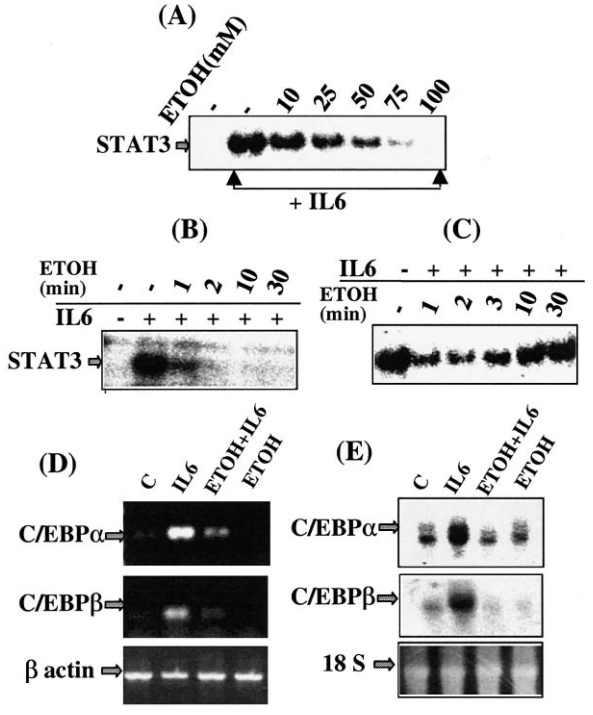


Fig. 1. Ethanol inhibits IL-6-induced STAT3 activation and C/EBP mRNA expression. (A) Freshly isolated hepatocytes were incubated with various concentrations of ethanol for 30 min, followed by stimulation with 20 ng/ml of IL-6 for 30 min. Whole cell extracts were prepared and DMSA was performed using the m67 probe, as described under Section 2. (B) Freshly isolated hepatocytes were treated with 100 mM ethanol for 1–30 min (lanes 3–6), followed by stimulation with 20 ng/ml of IL-6 for 30 min. DMSA was performed using the m67 probe. (C) 100 mM ethanol was added at various time points following 20 ng/ml of IL-6 stimulation (lanes 2–6). After IL-6 stimulation for 30 min, DMSA was performed using the m67 probe. (D and E) Freshly isolated hepatocytes were treated with 100 mM ethanol for 30 min and then stimulated with IL-6 for 1 h. The total RNA was isolated and used for RT-PCR (D) or Northern blotting (E) to determine C/EBPα and β mRNA expression, as described under Section 2. An autoradiogram representative of three independent experiments is shown in each panel.

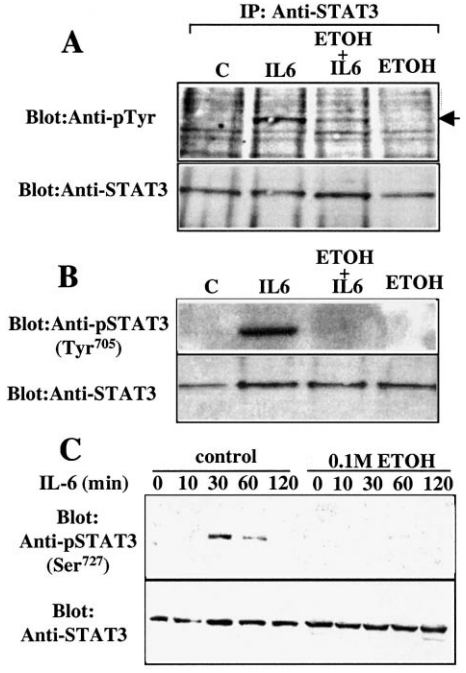


Fig. 2. Ethanol inhibits IL-6-induced STAT3 tyrosine and serine phosphorylation. (A) Freshly isolated rat hepatocytes were incubated with 100 mM ethanol or buffer for 30 min, then stimulated with 20 ng/ml of IL-6 for 30 min. The cell lysates were immunoprecipitated (IP) with an anti-STAT3 antibody, resolved by SDS-PAGE and blotted with anti-phosphotyrosine antibody (top panel). (B and C) Freshly isolated rat hepatocytes were incubated with 100 mM ethanol or buffer for 30 min, then stimulated with 20 ng/ml of IL-6 for 30 min. Cell lysates were resolved by SDS-PAGE and blotted with an anti-phospho-STAT3 ($Tyr^{705}$) antibody (top panel of B) or an anti-phospho-STAT3 ($Ser^{727}$) antibody (top panel of C). In A–C, the same blot in each top panel was stripped and re-probed with anti-STAT3 antibody (bottom panel). An autoradiogram representative of three independent experiments is shown in each panel.

following IL-6 treatment, causing approximately 60% inhibition of IL-6-induced STAT3 activation (Fig. 1C, lanes 2–4). These data indicate that ethanol inhibition of IL-6-induced STAT3 activation is rapid.

Next, we examined whether ethanol was able to inhibit IL-6-induced C/EBP gene expression. Freshly isolated hepatocytes were treated with 100 mM ethanol for 30 min, then stimulated with 20 ng/ml of IL-6 for 1 h. The total RNA was isolated and used for RT-PCR (Fig. 1D) or Northern analysis (Fig. 1E). It can be seen that IL-6 treatment significantly increased C/EBPα and C/EBPβ mRNA expression (lane 2 in Fig. 1D and E and these effects were inhibited by pre-incubation with 100 mM ethanol for 30 min (lane 3 in Fig. 1D and E). It is also evident that ethanol treatment alone did not affect the basal C/EBPα and β mRNA expression (lane 4 in Fig. 1D and E).

### 3.2. *Ethanol inhibits IL-6-induced STAT3 tyrosine and serine phosphorylation*

Rapid inhibition of IL-6-induced STAT3 activation by ethanol could either be due to inhibition of STAT3 translocation or inhibition of STAT3 tyrosine phosphorylation. To examine the latter possibility, Western analyses were performed by using an anti-phosphotyrosine antibody (Fig. 2A, upper panel) or an anti-phospho-STAT3 ($Tyr^{705}$) antibody (Fig. 2B, upper panel). It could be demonstrated by using either antibody that IL-6 induced a significant STAT3 tyrosine phosphorylation (lanes 2) and pretreatment of hepatocytes with 100 mM ethanol for 30 min abolished this activation (lanes 3). It is also evident that ethanol alone did not affect the basal STAT3 tyrosine phosphorylation (lane 4). As illustrated in the bottom panels of Fig. 2A and B, the same ethanol treatment did not significantly affect the cellular level of the STAT3 protein.

It has been reported that IL-6-induced serine phosphorylation is also critical for STAT3-promoter complexes [22,23]. Therefore, we wondered whether acute ethanol treatment also affects IL-6-induced serine phosphorylation. As shown in Fig. 2C, IL-6 treatment rapidly activates STAT3 serine phosphorylation (lanes 1–5), the peak effect occurring at 30 min and declining towards control levels after longer (120 min) exposures to IL-6. Pretreatment with 0.1 M ethanol for 30 min completely abolishes IL-6-induced serine phosphorylation (lanes 6–10). Ethanol alone does not affect the basal STAT3 serine phosphorylation (data not shown). The bottom panel in Fig. 2C indicated that the same ethanol treatment did not significantly affect the cellular level of the STAT3 protein. Taken together, these data suggest that etha-

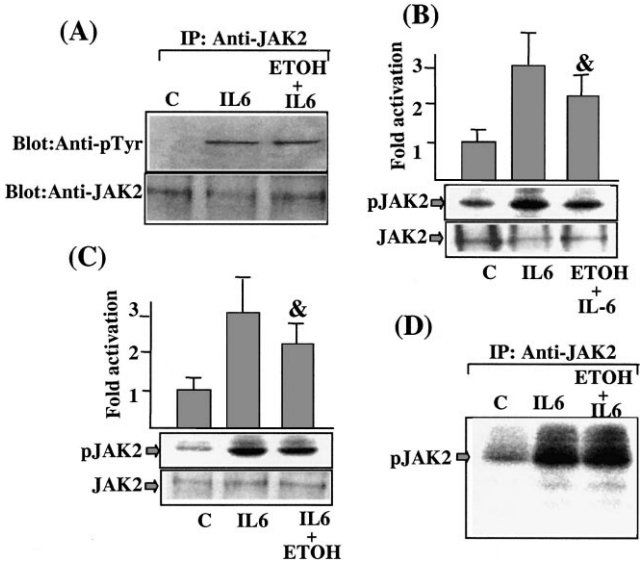


Fig. 3. Effects of acute ethanol on IL-6-induced JAK2 phosphorylation. (A and B) Freshly isolated rat hepatocytes were pretreated with 100 mM ethanol for 30 min and then stimulated with 20 ng/ml of IL-6 for 5 min. Cell extracts were immunoprecipitated (IP) with JAK2 antibody. The immunoprecipitated JAK2 proteins were subjected to Western blotting using anti-phosphotyrosine antibody (A) or in vitro autophosphorylation assays (B), as described in Section 2. (C) Cell extracts from freshly isolated rat hepatocytes following stimulation with 20 ng/ml of IL-6 for 5 min were immunoprecipitated with anti-JAK2 antibody. The immunoprecipitated JAK2 proteins were incubated with or without 100 mM ethanol for 30 min and then subjected to in vitro autophosphorylation assays. (D) Freshly isolated hepatocytes were labelled with [$^{32}$P]phosphate in the absence or presence of 100 mM ethanol and then stimulated with IL-6 for 5 min. The labelled phosphorylated JAK2 proteins were immunoprecipitated with anti-JAK2 antibody and fractionated by SDS-PAGE. The autoradiograms shown are representatives of three independent experiments. The radioactivities on the blots were quantified by phosphorimaging. Values shown are means ± S.E.M. from three independent experiments, expressed as fold changes over control. No significant difference from the IL-6-stimulated control is indicated by ampersands; i.e., & denotes $P > 0.05$.

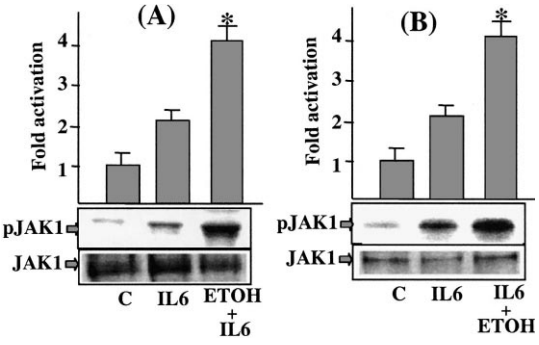


Fig. 4. Effects of acute ethanol on IL-6-induced JAK1 phosphorylation. (A) Freshly isolated rat hepatocytes were pretreated with 100 mM ethanol for 30 min and then stimulated with 20 ng/ml of IL-6 for 5 min. Cell extracts were immunoprecipitated (IP) with JAK1 antibody. The immunoprecipitated JAK1 proteins were subjected to in vitro autophosphorylation assays. (B) Cell extracts from freshly isolated rat hepatocytes following stimulation with 20 ng/ml of IL-6 for 5 min were immunoprecipitated with anti-JAK1 antibody. The immunoprecipitated JAK1 proteins were incubated without or with 100 mM ethanol for 30 min and then subjected to in vitro autophosphorylation assays. The autoradiograms shown are representatives of three independent experiments. The radioactivities on the blots were quantified by phosphorimaging. Values shown are means ± S.E.M. from three independent experiments, expressed as fold changes over control. Significant difference from the IL-6-stimulated control is indicated by asterisks; i.e., * denotes $P < 0.01$.

nol inhibition of IL-6-induced STAT3 activation is due to attenuation of STAT3 tyrosine and serine phosphorylation.

### 3.3. Acute ethanol does not inhibit IL-6-induced JAK2 phosphorylation in vivo or in vitro

To test whether IL-6-induced STAT3 tyrosine phosphorylation was inhibited by ethanol at an upstream site, JAK2 phosphorylation was analyzed by Western blotting, an in vitro kinase assay and in vivo cell labelling. Western analysis using an anti-phosphotyrosine antibody indicated that IL-6 induced the tyrosine phosphorylation of JAK2 (Fig. 3A, lane 2) and ethanol treatment did not inhibit this phosphorylation (lane 3). The bottom panel in Fig. 3A illustrates that similar amounts of protein were loaded in each lane. As illustrated in Fig. 3B, IL-6 treatment significantly induced the in vitro autophosphorylation of JAK2 (lane 2) and this effect was only slightly inhibited in extracts prepared from hepatocytes treated with 100 mM ethanol (lane 3). Ethanol alone did not affect the basal JAK2 activity (data not shown). In this experiment, as well as three replicate experiments with similar results, ethanol did not significantly inhibit IL-6-induced JAK2 phosphorylation.

It has been reported that ethanol can directly inhibit IGF-1 receptor autophosphorylation in vitro [24]. To test whether ethanol may also directly affect JAK2 autophosphorylation in vitro, freshly isolated rat hepatocytes were stimulated with 20 ng/ml of IL-6 for 5 min, cell extracts were then prepared and immunoprecipitated with JAK2 antibody. Immunoprecipitated JAK2 proteins were incubated with or without 100 mM ethanol for 30 min and then subjected to in vitro autophosphorylation assays. As shown in Fig. 3C, ethanol did not significantly affect JAK2 autophosphorylation in vitro. In vitro treatment with ethanol alone did not affect the basal JAK2 autophosphorylation (data not shown).

Finally, the results of in vivo labelling experiments with [$^{32}$P]phosphate have also demonstrated that 100 mM ethanol treatment does not significantly inhibit IL-6-induced JAK2 phosphorylation. In this experiment, hepatocytes were incubated with [$^{32}$P]phosphate in the absence or presence of 100 mM ethanol and then stimulated with IL-6 for 5 min. The labelled phosphorylated JAK2 proteins were immunoprecipitated with anti-JAK2 antibody. As shown in Fig. 3D, IL-6 treatment significantly stimulated JAK2 phosphorylation in vivo (lane 2) and 100 mM ethanol treatment did not affect this activation (lane 3).

### 3.4. Acute ethanol treatment potentiates IL-6-induced JAK1 phosphorylation in vivo and in vitro

Next, we examined the effects of ethanol on IL-6-induced JAK1 phosphorylation. As shown in Fig. 4A, IL-6 stimulation slightly activated JAK1 phosphorylation (lane 2). Surprisingly, pretreatment with 100 mM ethanol for 30 min significantly potentiated this activation (lane 3). In a total of four replicate experiments, ethanol potentiated the IL-6-induced JAK1 phosphorylation by 2.2 ± 0.1-fold, as quantified with a phosphorimager (Fig. 4A). To test whether ethanol potentiation of IL-6-induced JAK1 phosphorylation is due to a direct effect of ethanol, the IL-6-activated JAK1 immunoprecipitated complex was incubated without or with 100 mM ethanol for 30 min and then subjected to in vitro autophosphorylation. As shown in Fig. 4B, ethanol significantly potentiated JAK2 in vitro autophosphorylation by 2.1 ± 0.2-fold. Treatment with ethanol alone did not affect the basal JAK1 autophosphorylation (data not shown). These findings suggest that

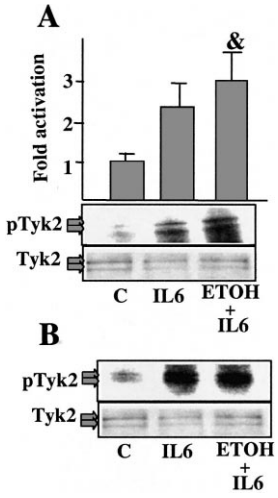


Fig. 5. Effects of acute ethanol on IL-6-induced Tyk2 phosphorylation. (A) Freshly isolated rat hepatocytes were pretreated with 100 mM ethanol for 30 min and then stimulated with 20 ng/ml of IL-6 for 5 min. Cell extracts were immunoprecipitated (IP) with Tyk2 antibody. The immunoprecipitated Tyk2 proteins were subjected to in vitro autophosphorylation assays. (B) Cell extracts from freshly isolated rat hepatocytes following stimulation with 20 ng/ml of IL-6 for 5 min were immunoprecipitated with Tyk2 antibody. The immunoprecipitated Tyk2 proteins were incubated without or with 100 mM ethanol for 30 min and then subjected to in vitro autophosphorylation assays. The autoradiograms shown are representatives of three independent experiments. The radioactivities on the blots were quantified by phosphorimaging. Values shown are means ± S.E.M. from three independent experiments, expressed as fold changes over control. No significant difference from IL-6-stimulated control is indicated by ampersands; i.e., $P > 0.05$.

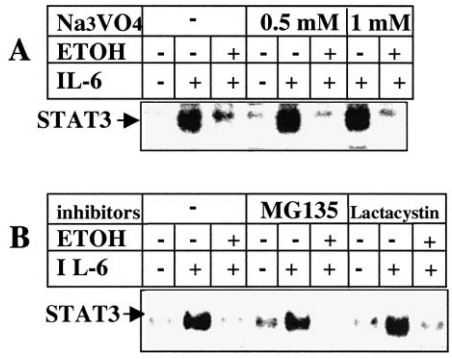


Fig. 6. Tyrosine phosphatases and the ubiquitin-proteasome pathway are not involved in ethanol inhibition of IL-6-induced STAT3 activation. Freshly isolated rat hepatocytes were pretreated with buffer, sodium vanadate (A) or proteasome inhibitors (50 μM MG135, 50 μM lactacystin) (B) for 30 min, followed by incubation with or without 100 mM ethanol for 30 min and then stimulated with 20 ng/ml of IL-6 for 30 min. DMSA was performed using the m67 probe. An autoradiogram representative of three independent experiments is shown in each panel.

ethanol potentiation of IL-6-induced JAK1 phosphorylation is due to a direct effect of ethanol on IL-6-activated JAK1.

### *3.5. Acute ethanol does not affect IL-6-induced Tyk2 phosphorylation in vivo or in vitro*

Effects of ethanol on IL-6-induced Tyk2 phosphorylation in vivo and in vitro were also examined. As shown in Fig. 5A, IL-6 significantly activated Tyk2 phosphorylation and treatment of hepatocytes with 100 mM ethanol slightly but not significantly increased this activation. In Fig. 5B, the Tyk2-immunoprecipitated complex was incubated with or without 100 mM ethanol for 30 min and then subjected to in vitro autophosphorylation. Ethanol only slightly increased the in vitro autophosphorylation of Tyk2. This suggests that ethanol does not significantly affect IL-6-induced Tyk2 phosphorylation.

### *3.6. Tyrosine phosphatases and the ubiquitin-proteasome pathway are not involved in ethanol inhibition of IL-6-induced STAT3 activation*

The ethanol inhibition of STAT3 tyrosine phosphorylation could be due to inactivation of kinases responsible for tyrosine phosphorylation of STAT3 or, alternatively, due to activation of a protein tyrosine phosphatase (PTPase) which dephosphorylates STAT3. To examine the latter possibility, hepatocytes were pretreated with the non-selective tyrosine phosphatase inhibitor sodium orthovanadate for 30 min, followed by IL-6 treatment for 30 min. Cell lysates were then subjected to a gel mobility shift assay to quantify STAT3 binding. As shown in Fig. 6, 0.5 or 1 mM sodium vanadate slightly enhanced IL-6-induced STAT3 activation (lanes 5 and 7) but did not prevent the ethanol inhibition of IL-6-induced STAT3 activation (lanes 6 and 8). This suggests that tyrosine phosphatases are not involved in ethanol inhibition of IL-6-induced STAT3 activation.

The ubiquitin-proteasome pathway has been implicated in the downregulation of activated STATs [25–27]. We wondered whether this pathway was also involved in ethanol-mediated inhibition of IL-6 activation of STAT3. As shown in Fig. 6, pretreatment of hepatocytes with the proteasome inhibitors, MG132 or lactacystin, did not significantly antagonize ethanol-mediated inhibition of IL-6 activation of STAT3, suggesting that the ubiquitin-proteasome pathway was not involved in ethanol inhibition of IL-6-induced STAT3 activation.

## 4. Discussion

In the present paper, we demonstrate that in freshly isolated rat hepatocytes, biologically relevant concentrations of ethanol rapidly and dose-dependently inhibit IL-6-induced activation of STAT3, as well as the expression of the downstream target genes C/EBPα and C/EBPβ. The results further indicate that this action of ethanol is not due to inhibition of upstream-located JAK1, JAK2 and Tyk2 kinases or activation of a tyrosine phosphatase, but probably involves direct inhibition of STAT3 tyrosine and serine phosphorylation. Ethanol inhibition of STAT3 phosphorylation develops rapidly and it is also evident when ethanol exposure follows the stimulation of IL-6. These observations also support the notion that ethanol directly interferes with STAT3 phosphorylation. However, the exact cellular mechanism by which ethanol inhibits STAT3 tyrosine phosphorylation remains unclear.

PTPases [10–14] and the ubiquitin-proteasome pathway [25–27] have been implicated in the inhibition of JAK-STAT signaling pathways. The finding that the tyrosine phosphatase inhibitor sodium vanadate and the proteasome inhibitors MG132 and lactacystin do not antagonize the inhibitory effects of ethanol on STAT3 activation suggests that PTPases and the ubiquitin-proteasome pathway are not involved. On the other hand, our preliminary data showed that pretreatment of the cells with GF 109203X, a highly selective protein kinase C (PKC) inhibitor, largely prevents ethanol inhibition of IL-6-induced STAT3 activation (unpublished observations), suggesting that PKC is involved. It has been shown that acute exposure of hepatocytes to ethanol induces a rapid but transient activation of phosphoinositide-specific phospholipase C and a consequent rise in cytosolic $Ca^{2+}$ and diacylglycerol, which can further activate PKC [28]. However, the mechanism by which PKC inhibits IL-6-induced STAT3 activation is not clear. It has been proposed that the PKA-dependent inhibition of INFα/β-induced JAK-STAT activation is mediated through an interaction of PKA with the cytoplasmic domain of the α chain of the INFα/β receptor [29]. By analogy, it is possible that the ethanol-activated PKC interacts directly with the STAT3 protein to inhibit its activation. Interestingly, acute ethanol treatment also significantly inhibits INFγ-induced STAT1 activation and growth hormone-induced STAT5 activation (unpublished observations), suggesting that ethanol may interfere with the phosphorylation of STAT proteins in general and its molecular target may be conserved domains of STAT proteins. It is known that all STATs contain a highly conserved SH2 domain, which is critical for the recruitment of STATs to activated receptor complexes and for their interaction with JAKs [1–3]. Therefore, the general inhibitory effect of ethanol on STATs may be due to modulation of SH2 domains by ethanol-activated PKC. Indeed, it has been reported that several kinases containing SH2 domains can be affected by ethanol [30–34].

It has been reported that acute ethanol exposure directly inhibits IGF-1 receptor autophosphorylation in vitro [24] and chronic ethanol ingestion was found to impair EGF receptor autophosphorylation [35]. However, in the present experi-

ments, acute ethanol exposure did not inhibit JAK1, JAK2 and Tyk2 autophosphorylation, which eliminates these kinases as the possible cellular targets through which ethanol inhibits STAT3 phosphorylation. Instead, ethanol was found to markedly potentiate IL-6-induced JAK1 autophosphorylation. Ethanol alone did not stimulate JAK autophosphorylation but potentiated IL-6-induced JAK1 autophosphorylation. This suggests that ethanol acts on the JAK1 protein only once it has been phosphorylated and it probably modulates the phosphotyrosine residues, ATP binding domains or the conformation of the activated JAK1 protein. Regardless of the exact target site(s) of ethanol on the activated JAK1 protein, this effect is clearly insufficient to overcome the apparent inhibition of the JAK-STAT signaling pathway at a site downstream of JAK1.

IL-6 exemplifies a typical pleiotropic cytokine that modulates the transcription of many genes, including members of the C/EBP family of transcription factors [36]. It has been reported that IL-6 directly stimulates C/EBPβ gene expression by acting on STAT binding sites within its promoter region [37,38]. The effects of IL-6 on C/EBPα gene expression are less clear, there being only one report on IL-6-induced inhibition of hepatic C/EBPα gene expression in vivo [39] and no reports on the effect of IL-6 on C/EBPα gene expression in hepatocytes. The present study clearly demonstrates that IL-6 can directly stimulate C/EBPα gene expression, which suggests that the reported downregulation of C/EBPα gene expression by in vivo injection of IL-6 is an indirect effect. The parallel upregulation of C/EBPα and C/EBPβ gene expression by IL-6 is somewhat unexpected in view of the different and, in some ways, opposite functions of these two members of the C/EBP family of transcription factors. Like IL-6, C/EBPβ is believed to play a key role in hepatocyte proliferation. C/EBPβ is upregulated in the regenerating liver [40–42] and liver development and regeneration are severely compromised in C/EBPβ knockout mice [43]. In contrast, C/EBPα is most highly expressed in terminally differentiated hepatocytes and inhibits hepatocyte proliferation [36] and is thought to have a key role in the control of differentiated liver functions, such as the regulation of energy homeostasis [44]. The increase in the expression of both C/EBPα and C/EBPβ by IL-6 may serve to promote a tightly controlled process where hepatocyte proliferation is closely coupled to terminal differentiation to allow for uninterrupted metabolic functions in the regenerating liver. On the other hand, the demonstrated ability of ethanol to inhibit the IL-6-induced expression of C/EBPβ may be one of the mechanisms by which ethanol inhibits the regenerative response in the liver, whereas the concomitant suppression of IL-6-induced C/EBPα gene expression by ethanol could contribute to the well-documented disturbances in liver carbohydrate metabolism in chronic drinkers with alcoholic liver disease [45].

In summary, the present findings demonstrate that biologically relevant concentrations of ethanol markedly inhibit IL-6-induced STAT3 tyrosine and serine phosphorylation and IL-6-activated C/EBPα and β mRNA expression in freshly isolated hepatocytes and that STAT3 is likely a direct molecular target of ethanol. The inhibitory effects of ethanol on STAT proteins may also be involved in other alcohol-associated disorders, such as anemia, thrombocytopenia, immunosuppression, fetal alcohol syndrome, etc., since the STAT proteins have been implicated in a variety of cellular functions in the hematopoietic, immunologic, neuronal and hepatic system [1–8]. Therefore, the effects of ethanol on the JAK-STAT signaling pathways in other cells deserve further scrutiny.

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