

Abstract

The liver has a unique ability to regenerate itself after tissue damage or resection, while maintaining differentiated functions. The regeneration process is driven by a complex set of signals, involving multiple growth factors, cytokines, proteolytic enzymes and other proteins. A pituitary hormone prolactin (PRL) has been shown to accelerate liver regeneration after partial hepatectomy and trigger tropic/mitogenic responses in isolated rat hepatocytes. By contrast, acute and chronic alcohol treatment impairs liver regeneration, which may contribute to alcohol-induced liver damage. However, it has been reported that ethanol consumption increases circulating levels of PRL. The objective of this study was to compare the effects of acute ethanol exposure on PRL-mediated short-term signaling and induction of key transcription factors in normal hepatocytes and in rat liver cells, isolated after partial hepatectomy, using quantitative Western blotting method. Preincubation with a physiologically relevant dose of ethanol (50 mM) decreased PRL-induced tyrosine phosphorylation of Janus kinase 1 (JAK1) and Signal Transducers and Activators of Transcription (STAT) proteins in freshly isolated rat hepatocytes, but augmented the activation of Ras/mitogen-activated protein kinase (MAPK) cascade. In addition, ethanol treatment inhibited the PRL-induced increase in c-Fos, c-Jun and JunB expression levels. PRL signaling responses were greatly suppressed in rat hepatocytes derived from 24 hours regenerating liver as compared to normal cells. These findings suggest that ethanol treatment may interfere with the pro-regenerative effects of PRL through a differential effect on signaling processes downstream of the PRL receptor.

Introduction

The regeneration process after partial hepatectomy (PHx) or liver injury requires hepatocyte proliferation, which is facilitated by a simultaneous action of various growth factors and cytokines, released by hepatic non-parenchymal (Kupffer and stellate) cells and the cells from other organs as well as endocrine glands (Fig. 1).

Serum levels of pituitary gland-derived hormone prolactin (PLR), which reportedly stimulates cell cycle progression in liver, increase significantly at 5–15 min after PHx (1). PRL binding to its cognate class I cytokine family receptor (PRL-R) induces its homodimerization (2) and subsequent activation of non-receptor protein tyrosine kinases of the Janus (JAK) and Src families that are constitutively localized in lipid-rich fractions of the plasma membrane (3). The phosphorylation of PRL-R by JAKs and activation of Src family kinases initiate the stimulation of signal transducers and activators of transcription (STATs), phosphoinositide 3-kinase/Akt, the mitogen activated protein kinase (MAPK) cascades and other signaling pathways that control mitogenic, apoptotic, motogenic, differentiative and regenerative responses through the activation of transcription factors of AP-1 and other families and subsequent induction of the specific immediate-early genes (4-5).

Alcohol consumption is known to impair liver regeneration (6). However, despite recent advances in understanding of the molecular targets and the mechanisms for ethanol (EtOH) action, the effects of acute EtOH exposure on PRL-mediated signaling in hepatocytes derived from normal and regenerating rat liver have not been investigated.

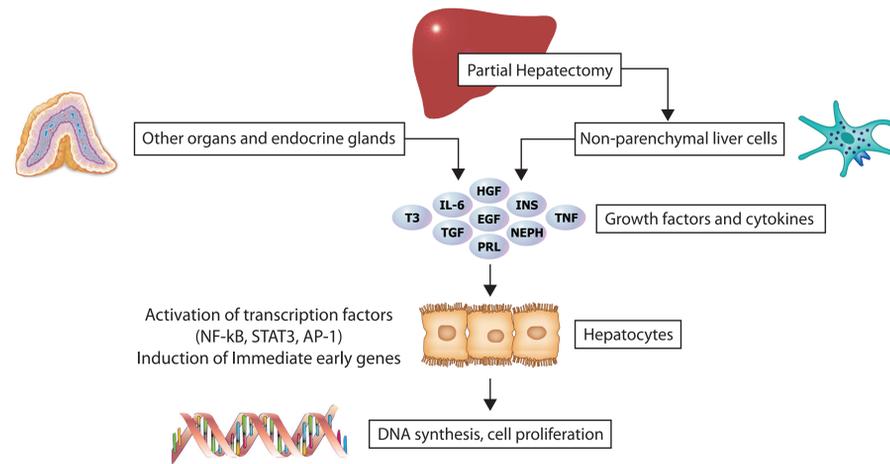


Fig. 1. Steps in liver regeneration.

Materials and Methods

Isolation of hepatocytes. Adult male Harlan Sprague Dawley rats were anaesthetized and subjected to two-thirds PHx by a ligation and resection of the liver median and left-lateral lobes, following the standard procedures. At 24 h post-PHx, the rats were sacrificed and remnant liver samples were harvested by a standard procedure (7). Briefly, liver was perfused for 10 min with a warm (37°C) carbogen-gassed (5% CO₂, 95% O₂) perfusion buffer (2 mM EDTA, 1 mM EGTA in Krebs-Ringer Bicarbonate (KRB) buffer, containing 0.1 M NaCl, 23.8 mM NaHCO₃, 5.5 mM Dextrose, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄ × 7H₂O, 10 mM HEPES in dH₂O, pH 7.4) at a flow rate of 20 ml/min or 30 ml/min for regenerating or normal liver, respectively. Perfusion medium was then switched into collagenase buffer (KRB, supplemented with 0.1 mM CaCl₂), containing 0.03 mg/ml of Liberase TM Reasearch Grade (Roche) (mixture of highly purified collagenase and neutral protease enzymes) and perfusion continued for another 10 min. Digested liver was minced to release the hepatocytes in collagenase buffer, filtered through a nylon mesh and centrifuged at 30×g for 3 min. Pellet was dissolved in 50/50 wash buffer (50% KRB, 50% KRB Suspension buffer (KRB with 1 mM CaCl₂). Suspension was centrifuged (50×g for 3 min) and subsequently washed twice. After removal of supernatant, the cells were kept in KRB Suspension buffer. Cell viability was determined by Trypan Blue staining and cell count in a hemacytometer chamber. Mean cell viability was 88% in regenerative (n=3) and 90% in normal (n=3) rat liver cells.

Cell stimulation. For study of EtOH effects on PRL signaling, the vials, containing normal or regenerating liver hepatocyte suspensions with viable cell density of 10 million cells/ml were preincubated in a humidified 5% CO₂ incubator for 30 min while gently shaking to optimize PRL-R presentation on the cell surface prior to treatment with EtOH (Acros Organics) or PBS-only solution (control) for 90 min and subsequent stimulation with PRL (PeproTech) for various time intervals in a shaking water bath at 37°C. Reactions were stopped by a 1:1 dilution of a sample of the incubation mixture with ice-cold lysis buffer (300 mM NaCl, 100 mM HEPES (pH 7.4), 2 mM EGTA, 2% Triton-X100, 20% glycerol in dH₂O). Detergent-insoluble materials were removed by centrifugation at 10,000×g for 10 min at 4°C. Equal amounts of solubilized proteins were dissolved in 4×NuPAGE LDS sample buffer (Invitrogen) supplemented with 50 mM DTT and heated for 5 min at 75°C. The protein separation by gel electrophoresis under reducing conditions and Multistrip Western blotting procedures were performed as described previously (8). Protein bands were detected by enhanced chemiluminescence system using SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology/Fisher Scientific). Bands were visualized and their signal net intensities were quantified via computer-assisted densitometry analysis by KODAK Image Station 440CF (Kodak Scientific Imaging Systems). The bar graphs were generated using SigmaPlot v.10 (Systat Software, Inc.).

The Effects of Ethanol on Prolactin Signaling in Normal and Regenerating Rat Liver

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Results

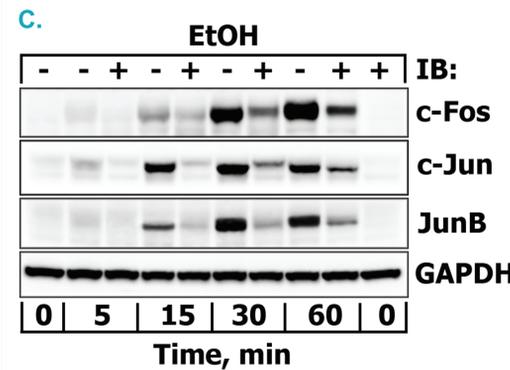
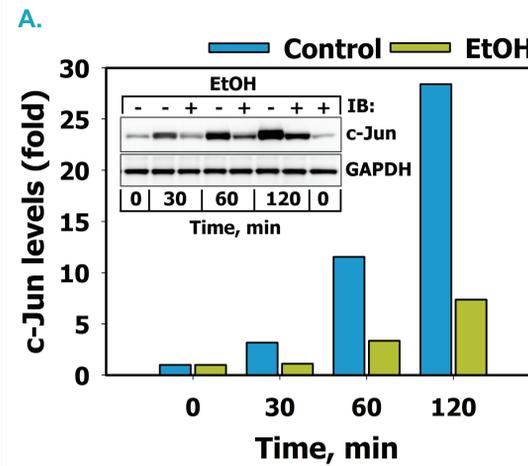


Fig. 2. Ethanol (EtOH) suppresses the prolactin-induced expression of AP-1 family transcription factors in freshly isolated hepatocytes from regenerating (A-B) and normal (C) rat liver. The cells were left untreated or were treated with 50 mM EtOH for 90 min and stimulated with 10 nM PRL for the indicated time intervals before lysis as described in "Materials and Methods". Resolved proteins were electro-transferred onto nitrocellulose membranes, which were blocked with 3% BSA at RT for 1 hr and immunoblotted (IB) with antibodies against the c-Jun, JunB, c-Fos and GAPDH (glyceraldehyde-3-phosphate dehydrogenase (loading control). Signal intensities of the c-Jun were normalized by the signal intensities of the GAPDH protein at each time point and then expressed as fold changes over the basal levels (in unstimulated cells). Bar graph (A) illustrates the quantitation of representative blot.

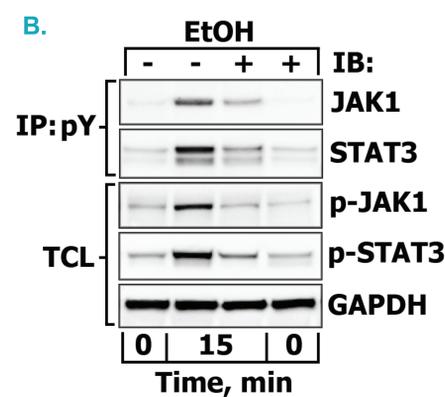
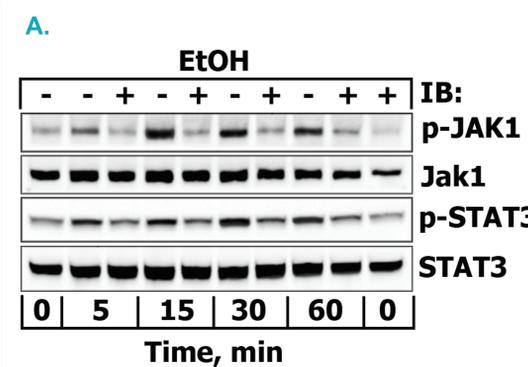
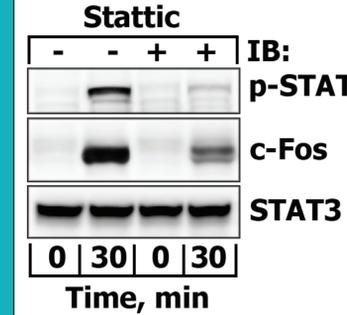


Fig. 3. Ethanol (EtOH) treatment alters the activating phosphorylation of JAK1 and STAT3 proteins that act downstream of the PRL receptor in freshly isolated hepatocytes from normal (A) and regenerating (B) rat liver. The cells were either left untreated or were pretreated with 50 mM EtOH for 90 min prior to stimulation with 10 nM PRL for the indicated time intervals, and lysed. Resolved proteins from total cell lysates (TCL) were electrotransferred onto nitrocellulose membranes, which were immunoblotted (IB) with antibodies against phosphorylated (p-) JAK1 (Tyr1022/1023), STAT3 (Tyr705) or total JAK1 and STAT3 proteins. Tyrosine phosphorylated proteins were immunoprecipitated (IP) from TCL of hepatocytes from 24 h regenerating rat liver (B) using the monoclonal agarose-conjugated anti-phosphotyrosine antibodies, pY-20, for 4 h at RT and three times washed with phosphate buffered saline solution. The blots were probed for JAK1 and STAT3.



In order to determine the functional connection between STAT3 and c-Fos, we used 6-Nitrobenzo[b]thiophene 1,1-dioxide, also referred as Static, which inhibits binding of tyrosine-phosphorylated peptide motifs to STAT3 Src homology 2 (SH2) domain and thus inhibits STAT3 activation by upstream proteins, dimerization and nuclear translocation.

Fig. 4. STAT3 inhibition suppresses PRL-induced expression of c-Fos transcription factor in freshly isolated hepatocytes from normal rat liver. The cells were treated with DMSO-only solution (-) or 50 μM of STAT3 inhibitor Static (+) for 60 min and stimulated with 10 nM PRL for 30 min before cell lysis. Resolved proteins were electro-transferred onto nitrocellulose membranes, which were blocked and probed for p-STAT3 (Tyr705), c-Fos and total STAT3 (loading control).

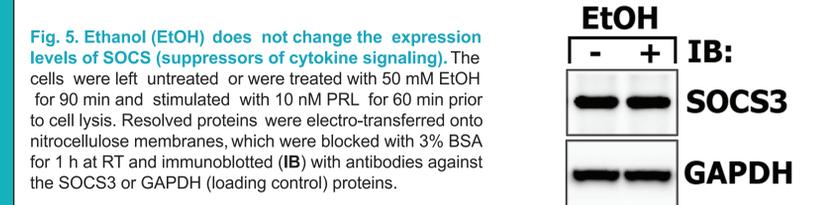


Fig. 5. Ethanol (EtOH) does not change the expression levels of SOCS (suppressors of cytokine signaling). The cells were left untreated or were treated with 50 mM EtOH for 90 min and stimulated with 10 nM PRL for 60 min prior to cell lysis. Resolved proteins were electro-transferred onto nitrocellulose membranes, which were blocked with 3% BSA at RT for 1 h and immunoblotted (IB) with antibodies against the SOCS3 or GAPDH (loading control) proteins.

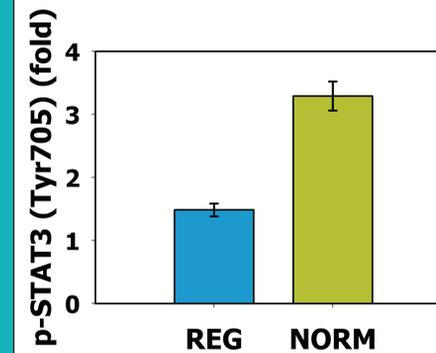


Fig. 6. The strength of responses to PRL stimulus differs between hepatocytes derived from normal (NORM) and regenerating (REG) rat liver.

The cells were stimulated with 10 nM PRL for 30 min. Resolved proteins were transferred on nitrocellulose membranes, which were blocked with 3% BSA at RT for 1 h. The blots were probed for various proteins, including p-STAT3 (Tyr705), PRL-R and β-actin. Bar graph illustrates the comparison of STAT3 activation, which is expressed as fold changes over basal levels (in respective unstimulated cells).

Conclusions

1. Acute exposure to ethanol caused a suppression of prolactin-stimulated c-Fos, c-Jun and JunB protein expression in hepatocytes derived from both normal and regenerating liver.
2. PRL receptor-stimulated gene expression is sensitive to acute ethanol exposure due to the ethanol-mediated negative effects on JAK/STAT signaling pathway possibly through the upregulation of protein tyrosine phosphatases (e.g. PTP1B) activity, but not the expression levels of SOCS3.

References

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Acknowledgements

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THANK YOU!