0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(9):3969–3976

doi: 10.1210/en.2002-0163

Effects of Chronic Ethanol on Hepatic and Renal CYP2C11 in the Male Rat: Interactions with the Janus-Kinase 2-Signal Transducer and Activators of Transcription Proteins 5b Pathway

T. M. BADGER, M. J. J. RONIS, S. J. FRANK, Y. CHEN, AND L. HE

Arkansas Children's Nutrition Center and Departments of Physiology/Biophysics (T.M.B., Y.C., L.H.) and Pharmacology/Toxicology (T.M.B., M.J.J.R.), University of Arkansas for Medical Sciences, Little Rock, Arkansas 72202; and Department of Medicine (S.J.F.), University of Alabama at Birmingham and Medical Service, Birmingham Veterans Affairs Medical Center, Birmingham, Alabama 35294

Chronic alcohol intake in male rats results in: 1) demasculinization of the GH pulse pattern; 2) reduced serum testosterone concentrations; and 3) decreased expression hepatic CYP2C11. Hepatic CYP2C11 expression is regulated by the male pattern of GH through the Janus-kinase/signal transducer and activators of transcription proteins (JAK/STAT) signal transduction pathway in the male rat. Renal CYP2C11 is regulated by testosterone, not GH. The involvement of the JAK/STAT5b signal transduction pathway in renal CYP2C11 signaling has not been studied. We tested the hypothesis that ethanol reduces CYP2C11 levels by interfering with the JAK/STAT5b pathway. Using a total enteral nutrition (TEN) model to feed rats a well-balanced diet, we have studied the effects of chronic ethanol intake (21 d) on hepatic and renal JAK/STAT pathway of adult male rats (8–10/group). We found decreased hepatic and renal expression of CYP2C11 in ethanol-fed rats with concomitant decreases in STAT5b and phospho-STAT5b, decreased *in vitro* hepatic STAT5b binding to a CYP2C11 promoter element and no effects on hepatic GHR levels. Ethanol caused tissue specific effects in phospho-JAK2 and JAK2, with increased levels in the liver, but decreased JAK2 expression in the kidney. We conclude that ethanol suppression of CYP2C11 expression is clearly associated with reductions in STAT5b levels, but not necessarily in reductions of JAK2 levels. The mechanisms underlying ethanol-induced suppression of STAT5b is yet to be determined, as is the question of whether this is secondary to hormonal effects or a direct ethanol effect. (*Endocrinology* 144: 3969–3976, 2003)

WE HAVE STUDIED the effects of diet on alcohol-induced tissue damage using a model system in which rats were fed exclusively by total enteral nutrition (TEN). TEN was used to avoid the nutritional deficiencies caused by lower food intake in rodents forced to drink ethanol-containing water or liquid diets (1–3). In the TEN model, control rats gain weight at essentially the same rates as rats fed standard commercial rat diets, and ethanol-fed rats have high blood ethanol concentrations (>300 mg/dl) (4, 5).

GH can have direct effects on gene transcription (6) or can act indirectly through other factors such as IGFs (7). GH can directly activate the JAK/STAT (Janus-kinase/signal transducer and activators of transcription proteins) pathway by binding to its cognate cell-surface GH receptor (GHR). Initial GH binding is followed by GHR dimerization and transphosphorylation of cytoplasmic tyrosine residues by the receptor-associated JAK tyrosine kinase. Phosphorylation activation of GHR-associated tyrosine kinase JAK2 leads to further phosphorylation of cytosolic STAT proteins that then form homo- or heterodimers and translocate to the nucleus, bind to specific promoter elements, and transactivate their target genes (8–11). This transactivation process is turned off by one of several mechanisms: ubiquitin-dependent degradation of these activator proteins, dephosphorylation inactivation, and protein inhibitors and inactivators (8).

GH secretion is pulsatile in humans. However, rats have greatly exaggerated serum GH pulses, and the secretory pattern of these pulses is sexually dimorphic, making them excellent models to study both GH regulation and pulsatile GH signaling at target cells. Importantly, it has been demonstrated that GH target cells are capable of differentially responding to male and female GH patterns in rats through the signal transducer and activators of transcription proteins, STAT5a and STAT5b. These proteins have been reported to have important roles in sexually dimorphic growth (and ultimate body size), deposition of body fat and immune system function (8, 12). STAT5b has been reported to be a key intracellular mediator of the effects of plasma GH pulses on several liver-expressed genes, including *CYP2C11* (8, 13). In the absence of the male GH pattern in rats, there is a down-regulation of hepatic STAT5b activity (13, 14), and STAT5b-deficient mice are resistant to GH pulse stimulation (15). Thus, based on these data, the sexually dimorphic effects of GH in rodents has been linked to male and female patterns of GH signaling and presumed to occur through STAT5b.

We have found that ethanol significantly reduces serum testosterone (T) and alters the typically low-frequency, high-amplitude GH pulse pattern of male rats to a higher frequency, lower amplitude pulse profile similar to that of a female rat, which is associated with reduced expression of

Abbreviations: DTT, Dithiothreitol; GHR, GH receptor; HRP, horseradish peroxidase; JAK/STAT, Janus-kinase/signal transducer and activators of transcription proteins; P, phospho; T, testosterone; TEN, total enteral nutrition.

the male-specific cytochrome P450, CYP2C11, in rat liver (4). CYP2C11 is a steroid 16α- and 2α-hydroxylase, and regulation of CYP2C11 production is tissue dependent in male rats (16). Under circumstances where body weight gains are normal and GH pulse patterns are typical of male rats, hepatic CYP2C11 is positively regulated by GH. Using a hypophysectomy model combined with hormonal restoration using pulsatile GH administration in a pattern that mimics the male rat GH pulse pattern, it has been demonstrated that GH positively regulates hepatic *CYP2C11* gene transcription (16). T did not restore hepatic CYP2C11 in hypophysectomized rats. In contrast, hormonal regulation of renal CYP2C11 is just the reverse of that in the liver, with GH not able to restore the hypophysectomy-induced decrease in CYP2C11 protein or mRNA, but T being able to completely reverse the decrease (16). Thus, the regulation of CYP2C11 expression is tissue dependent in the rat, with GH and T being the primary signals for liver and kidney, respectively.

The male pattern of GH activates the hepatic JAK/STAT pathway and this in turn is thought to maintain expression of male-specific proteins such as CYP2C11 (13, 16). Loss of the male GH pattern by hypophysectomy resulted in decreased phospho (P)-JAK2 and P-STAT5b, followed by diminished expression of male-specific proteins (13, 16). Furthermore, ethanol has been shown to inhibit 1) the prolactin-activated JAK/STAT pathway in cultured astrocytes (17); 2) IL-6 or interferon-activated JAK/STAT pathway in freshly isolated hepatocytes (18, 19); and 3) leptin-induced JAK/STAT pathway in Huh7 cells (20).

Despite the documented relationships between chronic alcohol and impaired endocrine and metabolic functions, the molecular mechanisms underlying the metabolic effects are less clear. Although several *in vitro* studies have demonstrated that ethanol inhibits activation of similar JAK/STAT signal transduction pathways (*i.e.* prolactin, leptin, cytokines, interferon), we are unaware of any studies documenting the effects of alcohol on the GHR-JAK2/STAT5b pathway following chronic *in vivo* alcohol treatment. In the current study, we tested the hypothesis that chronic ethanol intake as part of a TEN delivery system inhibits the JAK2/STAT5b signal transduction pathway that is thought to mediate GH signaling from the GHR to the CYP2C11 gene. Our thinking was influenced by previous results demonstrating that ethanol inhibited phosphorylation of JAK2, STAT1α, STAT5a, and STAT5b in cultured astrocytes, and that this was associated with decreased interferon regulatory factor-1 expression (17). These results suggested that ethanol inhibition of JAK2 and STAT5b phosphorylation may be one mechanism through which chronic ethanol alters gene expression.

Materials and Methods

Chemicals and reagents

All chemicals unless otherwise specified were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Radionucleotides were purchased from DuPont NEN Life Science Products (Boston, MA). T4 polynucleotide kinase was purchased from Promega (Madison, WI). A mouse monoclonal antibody against CYP2C11 was the kind gift of Dr. Paul Thomas (Rutgers, NJ) and horseradish peroxidase (HRP)-conjugated donkey antirabbit IgG was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antisera was purchased from the following companies: anti-STAT5b (Upstate Biotechnology, Lake Placid, NY); anti-P-STAT5 and HRP-linked antirabbit IgG (Cell Signaling Technology, Inc., Beverly, MA); and rabbit anti-JAK and anti-P-JAK2 (Biosource International, Inc., Camarillo, CA). A synthetic oligonucleotide against the published sequence in CYP2C11 (5′-GATATGTGAGTTAAAGCTAC-TATAGTTACC-3′) was purchased from Bio-Synthesis (Denton, TX) and was used for Northern analysis as described previously (4). A previously described anti-GHR serum (anti-$GHR_{cyt\text{-}AL47}$) was employed to assess hepatic GHR levels (21). A previously reported (22) second oligonucleotide corresponding to the STAT5b DNA binding site in the CYP2C11 promoter (−1159 to −1138) (5′-GCAAACATTTTCCATGAAAAAA-3′) and its complementary strand (5′-TTTTTTCATGGAAAATGTTTGC-3′) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

Animals and experimental protocol

Experiments conformed to ethical guidelines for animal research established by our institution and received prior approval by our animal welfare committee. Adult male Sprague Dawley rats were purchased from Harlan Industries (Indianapolis, IN). The rats were surgically cannulated with an intragastric tube, allowed to recover, and infused an ethanol-containing diet (13 g/kg·d) as described previously (4). The diet composition met the National Research Council minimal nutrient requirements for the rat (23). The control rats were infused the same diet except ethanol was isocalorically replaced with carbohydrate. The rats were killed following 21 d of continuous diet infusion and when their urine ethanol concentrations were high on the descending limb of an ethanol pulse, as previously described (24, 25), the liver and kidney were collected and stored at −90 C.

Northern blot analysis

Northern blot analysis of CYP2C11 was conducted as described previously using the oligonucleotide probe described in *Materials and Methods* (26). All filters were probed with the synthetic 18S rRNA oligonucleotide as an internal control. Bands were quantitated by densitometry of the autoradiogram and the ratio of CYP2C11 mRNA to 18S rRNA in the same sample was determined and expressed as relative RNA units or as percentage of that for the control.

Western immunoblot analysis

Liver and kidney microsomes, cytosol, and nuclear extracts were resolved on 8% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked overnight at 4 C with gentle shaking in TBST [50 mM Tris-buffered saline, 10 μM NaCl (pH 7.6), 0.05% (vol/vol) Tween 20] plus 5% (wt/vol) milk powder. Membranes were incubated with primary antibody diluted to 1:1000 for CYP2C11 and STAT5b and 1: 2500 for JAK2 in TBST plus 5% milk powder 1.5–3 h (1.5 h for CYP2C11 and JAK2; 3 h for STAT5b) at room temperature with shaking. After washing three times in TBST, the membranes were incubated for 1 h at room temperature in TBST plus 5% milk powder containing HRP-conjugated secondary IgG (1:1,500 for CYP2C11 and JAK2, and 1:20,000 for STAT5b). To detect P-STAT5 and P-JAK2, membranes were incubated with primary antibody diluted to 1:1000 for P-STAT5 and 1: 2500 for P-JAK2 in TBST plus 3% BSA 1.5–3 h (1.5 h for P-JAK2; 3 h for P-STAT5) at room temperature with shaking. After washing three times in TBST, the membranes were incubated for 1 h at room temperature in TBST plus 3% BSA containing HRP-conjugated secondary IgG (1: 1500 for P-JAK2 and 1:20,000 for P-STAT5). Membranes were washed three times in TBST and proteins visualized using SuperSignal West Pico chemiluminescence substrate (Pierce, Rockford, IL) and detected by autoradiography. Immunoquantitation was obtained by densitometric scanning of the resulting autoradiograms using a Bio-Rad (Richmond, CA) GS525 molecular imager. Similar procedures were employed for Western immunoblot analysis of GHR, with slight modifications as reported by Zhang *et al.* (21).

Preparation of rat liver and kidney nuclear extracts

The nuclear extracts were isolated from livers as previously reported (27). Briefly, liver tissue (stored at −70 C) was prepared using the nuclear extraction kit from Sigma Chemical Co. Liver (100 mg) was added into 1 ml 1× lysis buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$,

10 mM KCl, 1 mM dithiothreitol (DTT), and protease inhibitors. The tissue was homogenized for 40 sec and centrifuged for 20 min at 11,000 × *g*. The pellet was resuspended in 140 μl of extraction buffer containing 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol, 1 mM DTT, and protease inhibitors. Incubation occurred while shaking for 30 min followed by centrifugation at 20,000 × *g* for 5 min and snap-freezing of the supernatant.

EMSA

EMSAs were performed as described previously (27). Double-stranded oligonucleotides were prepared by combining and heating equimolar amounts of complementary single-stranded DNA to 95 C for 5 min in distilled water by cooling to room temperature overnight. The annealed oligonucleotides were diluted to a concentration of 10 μM and stored at −20 C. EMSA were carried out in 20 μl containing 100 mM KCl, 20 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 1.0 mM DTT, 0.3 μg BSA, 7% glycerol, and 1.5 μg of poly (deoxyinosine-deoxycytidine) (Roche Molecular Biochemicals, Indianapolis, IN). The nuclear extracts were pre-incubated with poly (deoxyinosine-deoxycytidine) for 15 min on ice and then 0.1 μg ^{32}P-end-labeled oligonucleotides were added to the reactions and incubated for another 15 min on ice after which 3 μl loading buffer was added, and the samples were loaded on a 5% nondenaturing polyacrylamide gel (acrylamid:bisacrylamide = 39:1) in low ionic strength Tris borate-EDTA. Serial amounts of nuclear extracts were tested in the experiment; in each reaction, the idea concentration was 10 μg. For the supershift experiment, 2 μl of rabbit antiserum was added to the reaction and an additional 30 min of incubation was carried out.

Urine ethanol concentrations

Urine ethanol concentrations were measured as previously described (25). Daily urine ethanol concentrations are excellent predictors of blood ethanol concentrations.

Statistics

Student's *t* test was used to determine whether group means differed at $P < 0.05$.

Results

Chronic feeding of the ethanol-containing diet using TEN resulted in the now well-characterized cyclic or pulsatile urine concentrations (data not shown) (5, 24, 25). Urine ethanol concentrations have been shown to be excellent predictors of blood ethanol concentrations (5). Daily urine ethanol concentrations were monitored over the course of the study, and ethanol pulses occurred about every 6 d with peak concentrations more than 400 mg/dl and nadir concentrations of near zero. There was a 69% reduction ($P < 0.005$) of mean levels of hepatic CYP2C11 mRNA and an 86% reduction ($P < 0.005$) in mean levels of hepatic microsomal CYP2C11 protein in ethanol-treated rats as compared with control rats (Fig. 1).

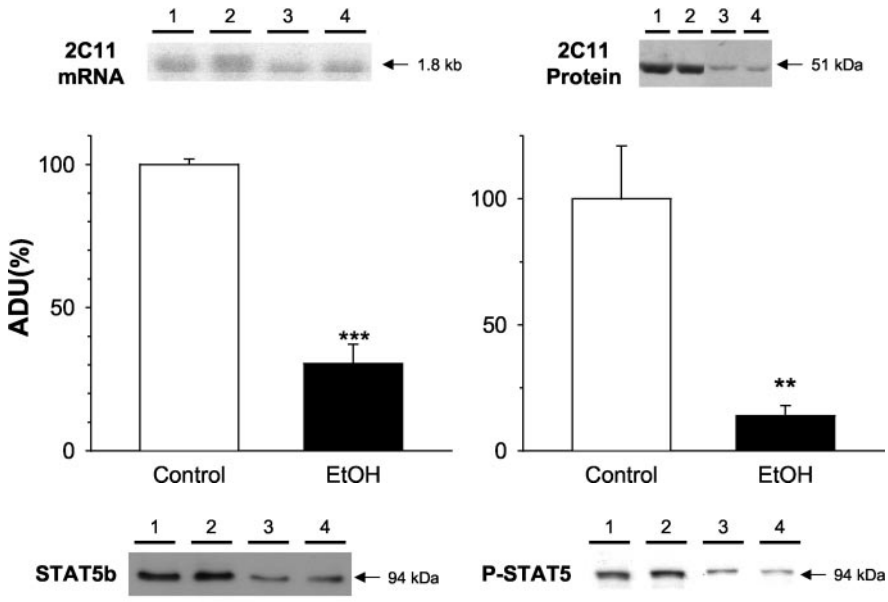

FIG. 1. Northern and Western analysis of hepatic microsomal CYP2C11: effects of ethanol. *Bar* values are mean (± SEM) of arbitrary densitometric units (ADU) expressed as percentages relative to controls (n = 6/group). The *upper portion* of each panel is a Northern blot (*left*) or a Western blot (*right*) of two representative rats per group. Control, Rats fed non-ethanol-containing diet; EtOH, rats fed ethanol-containing diet for 21 d. Lanes 1 and 2, Control rats; lanes 3 and 4, ethanol-fed rats. **, $P < 0.005$; ***, $P < 0.0005$.

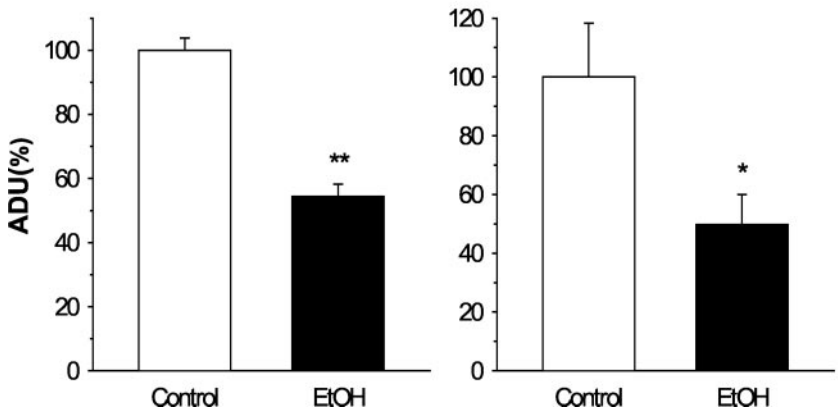

FIG. 2. Western analysis of hepatic cytosolic STAT5b and P-STAT5: effects of ethanol. *Bars* represent mean (± SEM) of arbitrary densitometric units (ADU) following Western blot analysis. Values are expressed as percentages relative to controls for six rats per group. *Upper portion* of each panel is a Western blot of two representative rats per group for STAT5b (*left side*) or P-STAT5 (*right side*). Control, Rats fed a non-ethanol-containing diet; EtOH, rats fed an ethanol-containing diet. Lanes 1 and 2, Control rats; lanes 3 and 4, ethanol-fed rats. *, $P < 0.05$; **, $P < 0.005$.

To determine whether this decrease in CYP2C11 was associated with alterations in STAT5b, we conducted Western immunoblot analyses of hepatic cytosols from the same rats. Chronic ethanol feeding reduced the mean levels of hepatic cytosolic STAT5b (Fig. 2, *left side*) by 45% ($P < 0.005$). A similar Western analysis of P-STAT5 revealed an approximate 50% reduction ($P < 0.05$) in nuclear extracts of ethanol-fed rats (Fig. 2, *right side*). There were no apparent effects on the ratio of STAT5b and P-STAT5, suggesting that the phosphorylation process was not affected by ethanol intake.

To verify specificity of the STAT5b results, we conducted an EMSA using rat hepatic nuclear extracts from control or ethanol-fed rats, labeled CYP2C11-oligo, and antibodies directed against the CYP2C11. The EMSA results demonstrated the appearance of a greatly reduced ($P < 0.05$) CYP2C11-STAT5b supershifted complex in ethanol-fed rats as compared with control rats (Fig. 3, *right side*), a finding consistent with reduced STAT5b levels. The nonsupershifted STAT5b DNA complex in lane 1 of Fig. 3 is a smear of multiple transcription factors bound to this element and this is consistent with EMSA analysis of the response element on the CYP2C11 promoter reported by Park and Waxman (22). The supershift in lane 2 (Fig. 3) very closely resembles the published supershifts of these investigators. Quantification of EMSA supershifted bands (from n = 6 controls or ethanol-fed rats) is shown on the *left side* of Fig. 3. Because phosphorylation activation of GHR-associated tyrosine kinase JAK2 leads to further phosphorylation of cytosolic STAT proteins, we conducted Western analyses to determine whether the reduction in STAT5b was positively associated with JAK2 and P-JAK2 levels. There was a 67% increase ($P < 0.05$) in the mean level of an approximate 130-kDa band corresponding to the molecular weight of JAK2 (Fig. 4, *left side*) and a 1.5-fold increase in the mean level of an approximate 130-kDa band corresponding to P-JAK2 (Fig. 4, *right side*) in hepatic microsomal fractions that contain the plasmalemma. Thus, the STAT5b decrease was not associated with reductions in either JAK2 or P-JAK2.

We studied immunoreactive GHR levels to determine whether the mechanism by which ethanol reduced STAT5b expression was related to lower GHR levels. Figure 5 demonstrates that no differences existed in GHR levels in ethanol-treated and control rats. The same antisera used to study GHR has been employed to study phosphorylation of the GHR in 3T3-F442A cells following acute GH administration where the levels are high (21). Our attempts to study phosphorylation of the GHR using these same techniques were unsuccessful, probably due to the extremely low P-GHR concentrations in liver as compared with acutely stimulated 3T3-F442A cells.

Because renal CYP2C11 is not regulated by GH in male rats

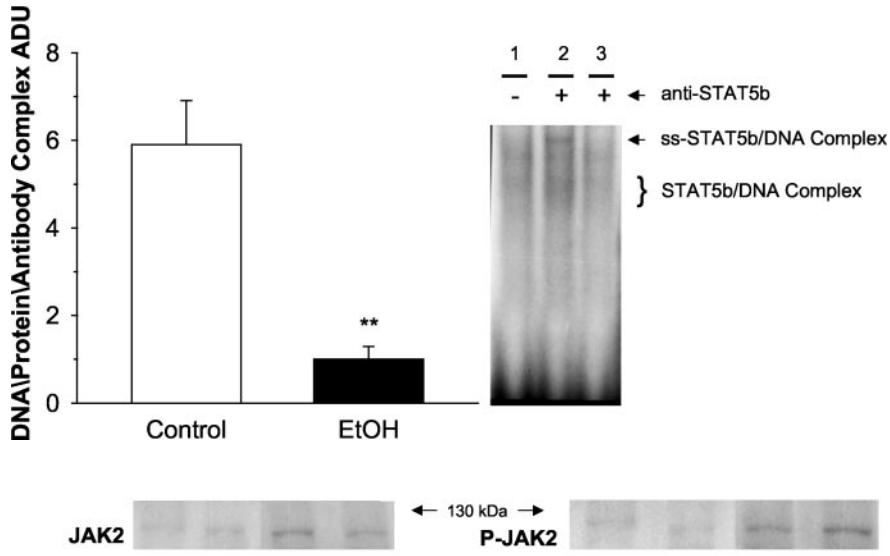

FIG. 3. Supershift of labeled CYP2C11 oligonucleotide with antibodies directed against the STAT5b. On the *right side* is the autoradiogram of EMSA performed using rat hepatic nuclear extracts from a control rat and the labeled CYP2C11-oligo in the absence of antibodies (lane 1) or the labeled CYP2C11-oligo in the presence of anti-STAT5b (lane 2). Lane 3 used rat hepatic nuclear extracts from an ethanol-fed rat with labeled CYP2C11-oligo in the presence of anti-STAT5b. On the *left side*, the *bars* represent the means (± SEM) of six rats per group. Control, Rats fed a non-ethanol-containing diet; EtOH, rats fed an ethanol-containing diet. **, $P < 0.005$.

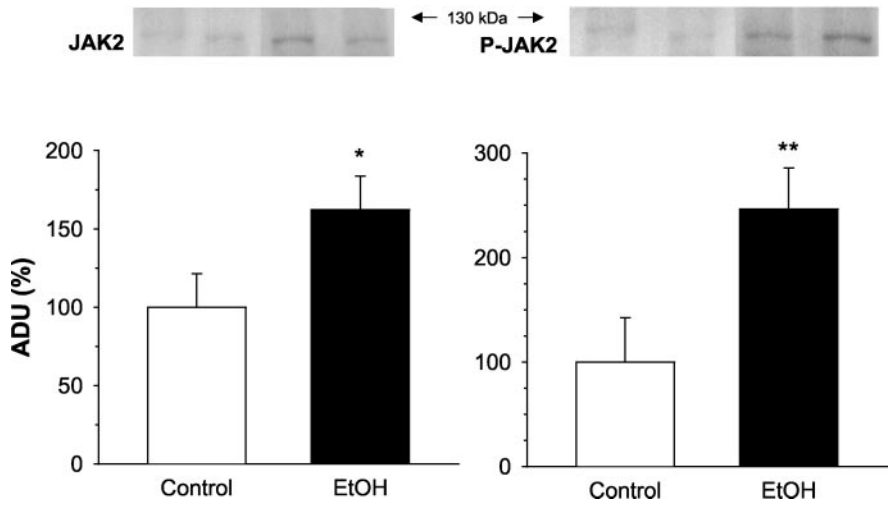

FIG. 4. Western analysis of hepatic cytosolic JAK2 and P-JAK2: effects of ethanol. *Bars* represent mean (± SEM) of arbitrary densitometric units (ADU) following Western blot analysis of hepatic microsomes. Values are expressed as percentages relative to controls for six rats per group. *Upper portion* of each graph is the Western blot of two representative rats per group for JAK2 (*left side*) and P-JAK2 (*right side*). Control, Rats fed non-ethanol-containing diet; EtOH, rats fed ethanol-containing diet. Lanes 1 and 2, Control rats; lanes 3 and 4, ethanol-fed rats. *, $P < 0.05$; **, $P < 0.005$.

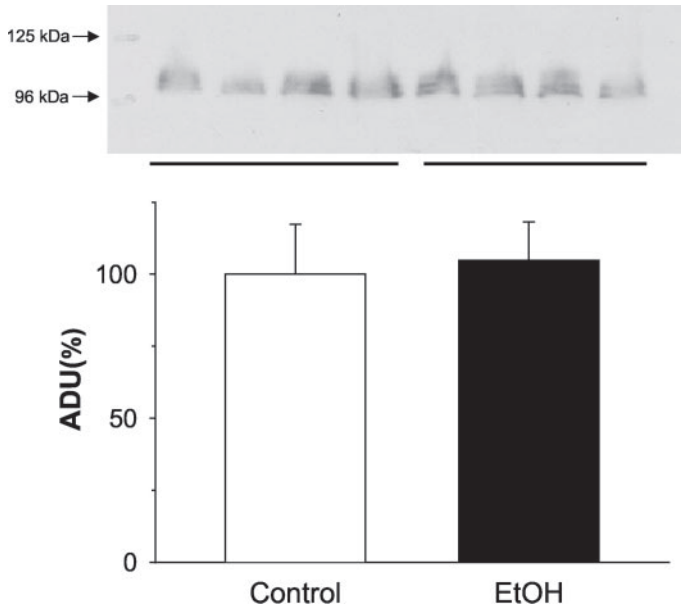

FIG. 5. Western analysis of hepatic GHR: effects of ethanol. *Upper portion* of the graph is the Western blot of four representative rats per group for GHR. Control, Rats fed a non-ethanol-containing diet; EtOH, rats fed an ethanol-containing diet. *Bars* represent mean (±SEM) of arbitrary densitometric units (ADU) is (n = 4) following Western blot analysis of hepatic microsomes. Values are expressed as percentages relative to controls for four rats per group.

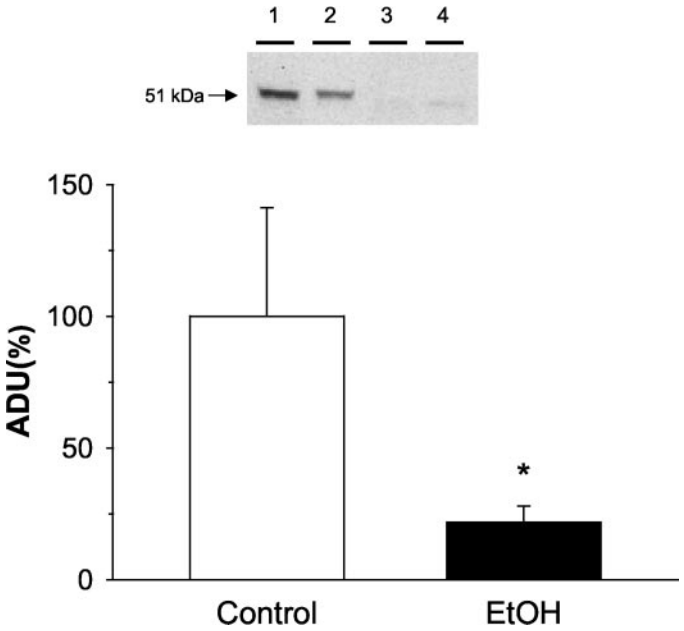

FIG. 6. Western analysis of renal microsomal CYP2C11: effects of ethanol. *Bar* represent means (±SEM) of arbitrary densitometric units (ADU) expressed as percentages relative to controls (n = 6/group). The *upper portion* is a Western blot of two representative rats per group. Control, Rats fed a non-ethanol-containing diet; EtOH, rats fed an ethanol-containing diet for 21 d. Lanes 1 and 2, Control rats; lanes 3 and 4, ethanol-fed rats. *, $P < 0.05$.

(16), but is regulated by T, the expression of kidney CYP2C11, STAT5b. P-STAT5b and JAK2 were also studied using the same techniques as described for the liver. Samples from rats fed ethanol had an approximate 80% reduction ($P < 0.05$) each in CYP2C11 (Fig. 6), STAT5b (Fig. 7), and P-STAT5b proteins (Fig. 8). Interestingly, however, renal JAK2 levels were reduced ($P < 0.05$) by approximately 50% (Fig. 9). These renal data are summarized in Table 1. Thus, there is a tissue-specific effect of ethanol on JAK2, with ethanol increasing JAK2/P-JAK2 in the liver and decreasing this protein in the kidney.

Discussion

Alcohol consumption may represent the proverbial two-edged sword, where on one edge there are health benefits associated with low levels of alcohol intake, and on the other edge are adverse health effects caused by chronic consumption of high alcohol levels. The mechanisms by which alcohol induces beneficial or adverse effects are essentially unknown, but they are clearly related to a variety of factors such as; alcohol dose, pattern of alcohol consumption, type of alcoholic beverage, diet, nutritional status, chronicity, underlying health conditions, exercise, and genetic predisposition. It is also clear that many, if not all, of the endocrine systems become involved at some point along the continuum of chronic alcohol effects.

It is likely that at least some health effects of alcohol are secondary to alcohol-induced changes in GH production or secretion, and other effects are downstream from the GHR. We have previously identified alterations in male rat GH secretion and GH-mediated actions by ethanol in the TEN model (4). We have also previously used hypophysectomy, anti-GH serum, and GH replacement to study the major ethanol inducible cytochrome P450, CYP2E1, and the GH-regulated male-specific protein, CYP2C11 (16, 28). Collectively, data from the above three studies demonstrate that chronic ethanol intake reduces serum T concentrations and alters GH secretion and that GH is important in regulating expression of hepatic CYP2C11 and CYP2E1. We have also learned that CYP2C11 regulation by hormones is tissue specific, with GH mediating some of the hepatic regulation on CYP2C11 expression, whereas T (rather than GH) was found to completely restore renal CYP2C11 to control levels (16, 28).

In the current study, we tested the hypothesis that chronic ethanol intake inhibits the JAK2/STAT5b signal transduction pathway that is thought to mediate GH signaling from the GHR to the CYP2C11 gene. The current results confirmed our earlier findings that alcohol-fed rats have significantly lower levels of hepatic CYP2C11 protein and mRNA, and consistent with our hypothesis, we demonstrated significant decreases in STAT5b. When combined with other published data (4, 12, 13, 16, 29), these results add support to the following scenario for the effects of ethanol on CYP2C11 in the liver. Chronic ethanol intake causes demasculinization of GH secretion. This leads to altered activation of GHR and decreased STAT5b because the normal male pattern of GH is necessary for activation of the GHR and subsequent maintenance of STAT5b levels. The low mean level of STAT5b observed in the male rats of this study is similar to that observed in female animals (30) and consistent with the altered GH pulse pattern from the typical male profile toward a female-like pulse pattern (4). The decreased STAT5b levels would be expected to lead to a reduced expression of

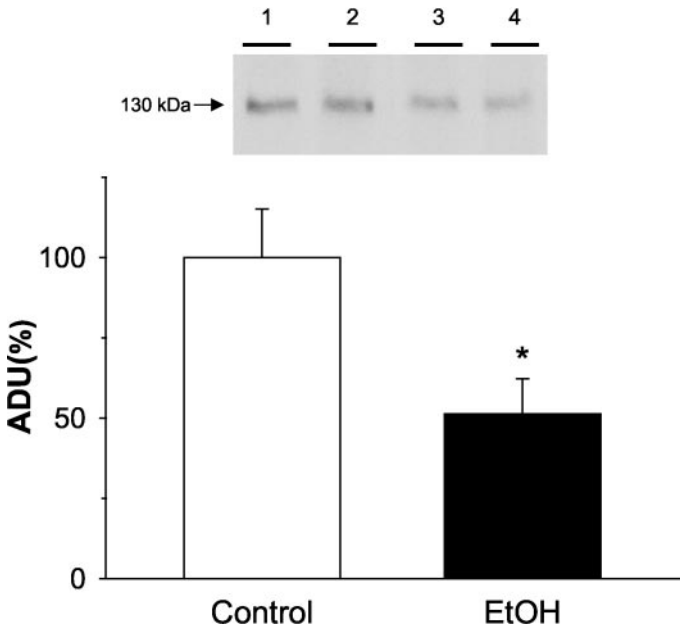

FIG. 7. Western analysis of renal cytosolic JAK2: effects of ethanol. *Bars* represent means (±SEM) of arbitrary densitometric units (ADU) expressed as percentages relative to controls (n = 6 rats/group). The *upper portion* is a Western blot of two representative rats per group. Control, Rats fed a non-ethanol-containing diet; EtOH, rats fed an ethanol-containing diet for 21 d. Lanes 1 and 2, Control rats; lanes 3 and 4, ethanol-fed rats. *, $P < 0.05$.

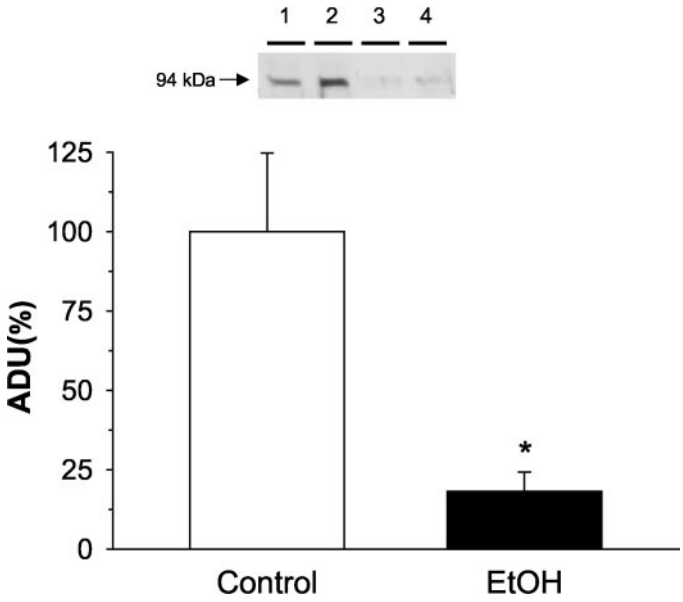

FIG. 8. Western analysis of renal cytosolic STAT5b: effects of ethanol. *Bars* represent means (± SEM) of arbitrary densitometric units (ADU) expressed as percentages relative to controls (n = 6 rats/group). The *upper portion* is a Western blot of two representative rats per group. Control, Rats fed a non-ethanol-containing diet; EtOH, rats fed an ethanol-containing diet for 21 d. Lanes 1 and 2, Control rats; lanes 3 and 4 are ethanol-fed rats. *, $P < 0.05$.

CYP2C11. Although these results suggest that ethanol is impairing CYP2C11 expression by altered pulsatile GH signal to the GHR, proof of this awaits the results from studies of CYP2C11 expression in hypophysectomized rats in which pulsatile GH replacement is given in the presence or absence of ethanol feeding.

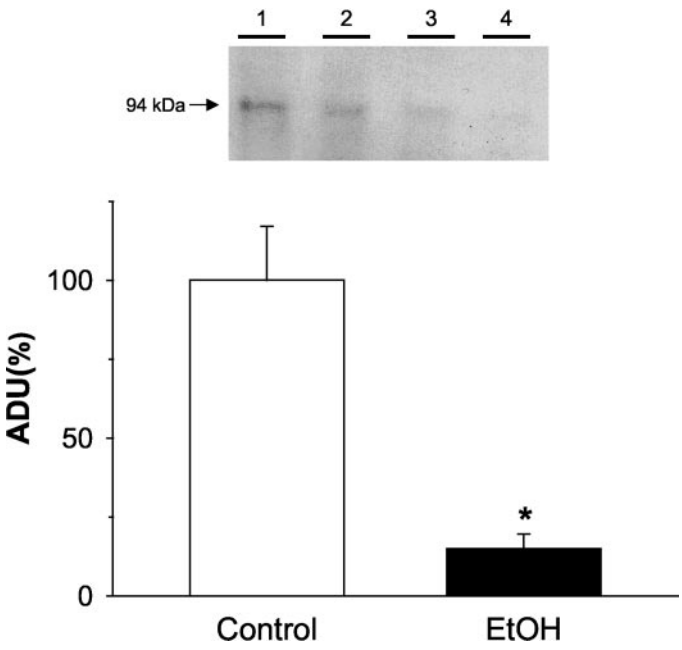

FIG. 9. Western analysis of renal cytosolic P-STAT5b: effects of ethanol. *Bars* represent means (±SEM) of arbitrary densitometric units (ADU) expressed as percentages relative to controls (n = 6 rats/group). The *upper portion* is a Western blot of two representative rats per group. Control, Rats fed a non-ethanol-containing diet; EtOH, rats fed an ethanol-containing diet for 21 d. Lanes 1 and 2 are control rats and lanes 3 and 4 are ethanol-fed rats. *, $P < 0.05$.

One might expect that if ethanol inhibits GH-mediated hepatic gene expression through an inhibitory action on the JAK2/STAT5b pathway similar to that of the prolactin JAK/STAT pathway (17), P-JAK2 levels would be decreased in the current study. We found, however, that decreased P-STAT5b levels were not linked to lowered P-JAK2 in the liver but were linked to a decrease in STAT5b protein. Because the ratio of STAT5b to P-STAT5b in control and ethanol-fed rats did not differ, we conclude that chronic ethanol intake does not affect phosphorylation of STAT5b, but rather reduces the level of the STAT5b protein. This could occur through inhibition of STAT5b protein synthesis, increases in protein degradation, or decreased mRNA abundance; but it seems clear that is not due to reductions in JAK2. Because GH stimulates STAT5b phosphorylation and there were no effects of ethanol on the ratio of STAT5b/P-STAT5b, the ethanol effects on STAT5b may be due to mechanisms other than alterations in GH secretion. Studies are ongoing to determine the mechanisms underlying the reduced STAT5b levels.

Interestingly, renal CYP2C11, which has been shown to be regulated by T but not by GH (16), was reduced in rats fed chronic ethanol; this down-regulation was accompanied by decreases in JAK2, STAT5b, and P-STAT5b, as well as reduced serum T concentrations. We surmise that the mechanism by which ethanol reduces renal CYP2C11 is through T deficiency, perhaps via cross-talk with the JAK/STAT system. Alternatively, the changes in the renal JAK/STAT pathway relative to CYP2C11 expression may not be linked.

TABLE 1. Effects of ethanol on CYP2C11, JAK2, and STAT5b

	Control	Ethanol
CYP2C11	100 ± 29.0	18.9[a] ± 6.5
JAK2	100 ± 14.7	47.1[a] ± 10.6
P-JAK2	ND[b]	ND[b]
STAT5b	100 ± 17.3	20.0[a] ± 4.7
P-STAT5b	100 ± 1.7	17.5[a] ± 0.45

Data are means ± SEM of six per group for ADU and expressed as percent of control.

[a] $P < 0.05$.

[b] Not done.

Unraveling these complex interactions awaits future hormone replacement studies.

Because chronic ethanol intake alters concentrations of several hormones, cytokines, ILs, and other potential ligands for cell surface receptors that associate with one or more JAK2-mediated signal transduction pathways, it should not be surprising that hepatic JAK2 levels increased in the current study. But the increased P-JAK2 levels were not associated with increased phosphorylation of STAT5b in this study. This may indicate that phosphorylation of STAT5b is at a maximal level and cannot be induced further. On the other hand, because the liver samples studied in this experiment were composed of many cell types, the increased JAK2 levels could reflect increases in expression in cell types other than hepatocytes, and thus not be linked to the GHR-JAK2-STAT5b pathway in hepatocytes. Alternatively, GH-mediated phosphorylation of STAT5b may differ in the presence of ethanol, similar to that shown previously in studies on PRL in Nb2 cells where a clear dissociation between JAK2 and STAT5 activation has been demonstrated (31). Ethanol induced apparent uncoupling of the hepatic IL-6-activated JAK/STAT3 phosphorylation in freshly isolated hepatocytes (19). In fact, as compared with controls, ethanol-treated cells had greater JAK1 and Tyk2 levels in the presence of IL-6, whereas, ethanol treatment in the absence of IL-6 had no effect. Similarly, ethanol did not inhibit leptin-induced JAK2 phosphorylation in Huh7 cells cotransfected with the splice isoform of the leptin receptor gene (OBRb) cDNA and JAK2 cDNA (20). Thus, whereas some *in vitro* studies have shown ethanol inhibition of JAK2, others have not. Results from the current study suggest that *in vivo* ethanol intake reduces STAT5b levels and decreases CYP2C11 in the liver without inhibition of JAK2 signaling. Similarly, ethanol has been reported to reduce hepatic IGF-1 levels in mice that overexpress GH, suggesting ethanol inhibitory effects on GH signaling downstream from the GHR (32). This is important because it may point to possible signal transduction effects downstream from plasma receptors and may provide an explanation for the hepatic JAK2/STAT5b dissociation in the present study.

Lastly, our kidney data demonstrate that ethanol inhibits renal CYP2C11 expression. However, the relative roles of decreased T and down-regulation of JAK/STAT signaling in this process remains to be determined.

Acknowledgments

We would like to thank the following people for their technical assistance: Matt Ferguson, Kim Hale, Tammy Dallari, Terry Fletcher, Mark Robinette, Pam Treadaway, and Michele Perry. We also appreciate Drs. S. Nagarajan and F. Simmen for reviewing the paper.

Received December 17, 2002. Accepted June 3, 2003.

Address all correspondence and requests for reprints to: Thomas M. Badger, Ph.D., Arkansas Children's Nutrition Center, 1120 Marshall Street, Little Rock, Arkansas 72202. E-mail: badgerthomasm@uams.edu.

This work was supported in part by NIH Grants AA008645 (to T.M.B.) and DK46395 (to S.J.F.).

References

1. **Rao GA, Sankaran H, Larkin EC** 1988 Rat models for chronic alcohol consumption. J Nutr 118:799–801
2. **Rao GA, Larkin EC** 1985 Inadequate intake by growing rats of essential nutrients from liquid diets used for chronic alcohol consumption. Nutr Res 5:789–796
3. **Rao GA, Larkin EC** 1987 Nutritional inadequacy of Lieber-DeCarli alcohol diet. Hepatology 7:416–417
4. **Badger TM, Ronis MJ, Lumpkin CK, Valentine CR, Shahare M, Irby D, Huang J, Mercado C, Thomas P, Ingelman-Sundberg M** 1993 Effects of chronic ethanol on growth hormone secretion and hepatic cytochrome P450 isozymes of the rat. J Pharmacol ExpTher 264:438–447
5. **Badger TM, Crouch J, Irby D, Hakkak R, Shahare M** 1993 Episodic excretion of ethanol during chronic intragastric ethanol infusion in the male rat: continuous vs. cyclic ethanol and nutrient infusions. J Pharmacol Exp Ther 264: 938–943
6. **Cunningham BC, Ultsch M, De Vos AM, Mulkerrin MG, Clauser KR, Wells JA** 1991 Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. Science 254:821–825
7. **Isaksson OG, Eden S, Jansson JO** 1985 Mode of action of pituitary growth hormone on target cells. Annu Rev Physiol 47:483–499
8. **Davey HW, Wilkins RJ, Waxman DJ** 1999 STAT5 signaling in sexually dimorphic gene expression and growth patterns. Am J Hum Genet 65:959–965
9. **Argetsinger LS, Carter-Su C** 1996 Mechanism of signaling by growth hormone receptor. Physiol Rev 76:1089–1107
10. **Argetsinger LS, Campbell GS, Yang X, Witthuhn BA, Silvennoinen O, Ihle JN, Carter-Su C** 1993 Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell 74:237–244
11. **Moutoussamy S, Kelly PA, Finidori J** 1998 Growth-hormone-receptor and cytokine-receptor-family signaling. Eur J Biochem 255:1–11
12. **Waxman DJ** 2000 Growth hormone pulse-activated STAT5 signalling: a unique regulatory mechanism governing sexual dimorphism of liver gene expression. Novartis Found Symp 227:61–74
13. **Waxman DJ, Ram PA, Park SH, Choi HK** 1995 Intermittent plasma growth hormone triggers tyrosine phosphorylation and nuclear translocation of a liver-expressed, Stat 5-related DNA binding protein. Proposed role as an intracellular regulator of male-specific liver gene transcription. J Biol Chem 270:13262–13270
14. **Gebert CA, Park SH, Waxman DJ** 1999 Down-regulation of liver JAK2-STAT5b signaling by the female plasma pattern of continuous growth hormone stimulation. Mol Endocrinol 13:213–227
15. **Davey HW, Park SH, Grattan DR, McLachlan MJ, Waxman DJ** 1999 STAT5b-deficient mice are growth hormone pulse-resistant. Role of STAT5b in sex-specific liver p450 expression. J Biol Chem 274:35331–35336
16. **Chen GF, Ronis MJ, Thomas PE, Flint DJ, Badger TM** 1997 Hormonal regulation of microsomal cytochrome P450 2C11 in rat liver and kidney. J Pharmacol Exp Ther 283:1486–1494
17. **DeVito WJ, Stone S** 1999 Ethanol inhibits prolactin-induced activation of the JAK/STAT pathway in cultured astrocytes. J Cell Biochem 74:278–291
18. **Chen J, Clemens DL, Cederbaum AI, Gao B** 2001 Ethanol inhibits the JAK-STAT signaling pathway in freshly isolated rat hepatocytes but not in cultured hepatocytes or HepG2 cells: evidence for a lack of involvement of ethanol metabolism. Clin Biochem 34:203–209
19. **Chen J, Kunos G, Gao B** 1999 Ethanol rapidly inhibits IL-6-activated STAT3 and C/EBP mRNA expression in freshly isolated rat hepatocytes. FEBS Lett 457:162–168
20. **Degawa-Yamauchi M, Uotani S, Yamaguchi Y, Takahashi R, Abe T, Kuwahara H, Yamasaki H, Eguchi K** 2002 Ethanol inhibits leptin-induced STAT3 activation in Huh7 cells. FEBS Lett 525:116–120
21. **Zhang Y, Jiang J, Kopchick JJ, Frank SJ** 1999 Disulfide linkage of growth hormone (GH) receptors (GHR) reflects GH-induced GHR dimerization. Association of JAK2 with the GHR is enhanced by receptor dimerization. J Biol Chem 274:33072–33084
22. **Park SH, Waxman DJ** 2001 Inhibitory cross-talk between STAT5b and liver nuclear factor HNF3β: impact on the regulation of growth hormone pulse-stimulated, male-specific liver cytochrome P-450 gene expression. J Biol Chem 276:43031–43039
23. **National Research Council** 1978 Nutrient requirements for laboratory animals. Washington, DC: National Research Council
24. **Badger TM, Ronis MJ, Ingelman-Sundberg M, Hakkak R** 1993 Pulsatile

blood alcohol and CYP2E1 induction during chronic alcohol infusions in rats. Alcohol 10:453–457
25. **Badger TM, Hoog JO, Svensson S, McGehee Jr RE, Fang C, Ronis MJ, Ingelman-Sundberg M** 2000 Cyclic expression of class I alcohol dehydrogenase in male rats treated with ethanol. Biochem Biophys Res Commun 274: 684–688
26. **Korourian S, Hakkak R, Ronis MJ, Shelnutt S, Waldron J, Ingelman-Sundberg M, Badger TM** 1999 Diet and risk of ethanol-induced hepatotoxicity: carbohydrate-fat relationships in rats. Toxicol Sci 47:110–117
27. **He L, Ronis MJJ, Badger TM** 2002 Ethanol induction of class I alcohol dehydrogenase expression in the rat occurs through alterations in CCAAT/enhancer binding proteins β and γ. J Biol Chem 277:43572–43577
28. **Chen GF, Ronis MJ, Ingelman-Sundberg M, Badger TM** 1999 Hormonal regulation of microsomal cytochrome P4502E1 and P450 reductase in rat liver and kidney. Xenobiotica 29:437–451
29. **Waxman DJ, Pampori NA, Ram PA, Agrawal AK, Shapiro BH** 1991 Interpulse interval in circulating growth hormone patterns regulates sexually dimorphic expression of hepatic cytochrome P450. Proc Natl Acad Sci USA 88:6868–6872
30. **Choi HK, Waxman DJ** 1999 Growth hormone, but not prolactin, maintains, low-level activation of STAT5a and STAT5b in female rat liver. Endocrinology 140:5126–5135
31. **Coss D, Kuo CB, Yang L, Ingleton P, Luben R, Walker AM** 1999 Dissociation of Janus kinase 2 and signal transducer and activator of transcription 5 activation after treatment of Nb2 cells with a molecular mimic of phosphorylated prolactin. Endocrinology 140:5087–5094
32. **Srivastava K, Dearth RK, Hiney JK, Chandrashekar V, Mattison JA, Bartke A, Dees WL** 2002 Alcohol suppresses insulin-like growth factor-I gene expression in prepubertal transgenic female mice overexpressing the bovine growth hormone gene. Alcohol Clin Exp Res 26:1697–1702