Attenuated Expression of Episodic Growth Hormone-Induced CYP2C11 in Female Rats Associated with Suboptimal Activation of the Jak2/Stat5B and Other Modulating Signaling Pathways

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ABSTRACT:

Inherent sex differences in various parameters of growth, musculoskeletal function, metabolism, and cytochrome P450 (P450)-dependent drug metabolism have been reported in rats and humans administered typical intermittent/episodic growth hormone (GH) replacement therapy. Having infused and monitored the identical physiologic masculine (episodic) growth hormone profile to both hypophysectomized male and female rats, we observed that induction levels of hepatic CYP2C11 were 35 to 40% lower in females. Associated with the reduced expression of the P450 isoform in the episodic GH-treated females were dramatically lower activation levels of Janus kinase (Jak2), signal transducers and activators of transcription (Stat5A and 5B) as well as 50% less binding of Stat5B to the CYP2C11 promoter. Because the Jak2/Stat5B signaling pathway mediates the effects of the masculine GH profile on its target cells, we conclude that the lower induction level of CYP2C11 in females exposed to the masculine GH profile is probably due, at least in part, to the suboptimum activation of the Jak2/Stat5B pathway. In addition to the reduced activation of the Jak2/Stat5B pathway, we observed lower activational levels of mitogen-activated protein kinase (p44/p42) and, indirectly, nuclear factor-xB in the episodic GH-treated females that may be involved in attenuating the activity of the Jak2/Stat5B pathway diminishing CYP2C11 expression levels.

Whereas males and females secrete the same daily amounts of growth hormone (GH), the secretory patterns in various species examined, including rats, mice, and humans (Shapiro et al., 1995), are sexually dimorphic—characterized as “continuous” for females and “episodic” for males. In the case of rats, males secrete GH in episodic bursts (∼200–300 ng/ml plasma) every 3.5 to 4 h. Between the peaks, GH levels are undetectable. In females the hormone pulses are more frequent and irregular and are of lower magnitude than those in males, whereas the interpulse concentrations of GH are always measurable (Legraverend et al., 1992; Shapiro et al., 1995). These sex differences in the circulating GH profiles and not sexual differences in GH concentrations, per se, are responsible for observed sexual dimorphisms ranging from body growth to the expression of hepatic enzymes (Legraverend et al., 1992; Shapiro et al., 1995). In this regard, rat, as well as murine and human liver each contain sex-dependent isoforms of P450 that are regulated by the sex-dependent profiles of circulating GH (Legraverend et al., 1992; Shapiro et al., 1995; Dhir et al., 2006).

Sex-dependent, hepatic P450s in the rat are generally divided into three groups: male-specific isoforms only found in male liver, female-specific isoforms only expressed in female liver, and sex (generally female)-predominant P450s found in both sexes, but at higher levels in one sex. Essentially, there are four major male-specific isoforms in rat liver: CYP2C11, CYP2C13, CYP2A2, and CYP3A2. Expression of the major male-specific CYP2C11 comprising >50% of the total hepatic pool of P450 in male rats (Morgan et al., 1985) requires the episodic “on/off” masculine profile of GH secretion. Although the feminine pattern of continuous hormone secretion blocks CYP2C11 expression, total GH depletion from the circulation allows CYP2C11 expression at ∼25% of intact male levels (Morgan et al., 1985; Pampori and Shapiro, 1996; Agrawal and Shapiro, 2000). Although expression of CYP2C11 is greatest when exposed to its sex-dependent GH profile, other male-specific isoforms are optimally expressed in the absence of GH. CYP2A2 and CYP3A2 are maximally expressed in the hypophysectomized (HYPOX) rat and disappear when GH is secreted constantly but are only minimally suppressed under the influence of episodic GH (Waxman, 1992; Pampori and Shapiro, 1996; Agrawal and Shapiro, 2000). Expression of the major female-specific isoforms, e.g., CYP2C12, are solely dependent on the feminine profile of continuous GH secretion (Waxman, 1992; Legraverend et al., 1992). The sex-predominant isoforms, probably due, at least in part, to the suboptimum activation of the Jak2/Stat5B pathway diminishing CYP2C11 expression levels.

AABBREVIATIONS: GH, growth hormone; P450, cytochrome P450; HYPOX, hypophysectomized; Jak, Janus kinase; Stat, signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; IκB, inhibitory κB; rGH, rat growth hormone; PCR, polymerase chain reaction; ChIP, chromatin immunoprecipitation assay; Erk, extracellular signal-regulated kinase.
Al., 2000), which are examined here as the basis for the inherent absence of a pituitary gland or its fragments at necropsy. 

Although HYPOX female rats will respond to the masculine episodic GH profile with an induction of male-dependent P450 isoforms and suppression of female-dependent isoforms, and HYPOX male rats will respond to the feminine continuous GH profile with an induction of female-dependent P450 isoforms and concomitant suppression of male-dependent isoforms, the responses are inherently limited by the sex of the rat. That is, regardless of the restored GH profile, female hepatocytes, either in vitro or in vivo (Legraverend et al., 1992; Shapiro et al., 1993; Waxman et al., 1995; Thangavel et al., 2006) cannot express male-like levels of male-specific P450s, nor can male hepatocytes, either in vitro (Thangavel et al., 2004) or in vivo (Pam pori and Shapiro, 1999) be induced to express female-like levels of female-dependent P450s.

Accordingly, regulation of male-specific CYP2C11 by episodic GH appears to involve the activation of the Janus kinase (Jak2)/signal transducers and activators of transcription (Stat5B) pathway (Waxman and O’Connor, 2006), in addition to other possible signaling pathways, such as mitogen-activated protein kinase (MAPK) (Verma et al., 2005) and nuclear factor-κB (NF-κB) (Iber et al., 2000), which are examined here as the basis for the inherent sex-dependent response of CYP2C11 to episodic GH.

Materials and Methods

Antibodies and Chemicals. Antibodies were purchased against Stat5A, Stat5B, inhibitory κ-B (IκB-α), and β-actin; also purchased were horseradish peroxidase-conjugated anti-goat (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho-Jak2 (Chemicon International, Temecula, CA), anti-activated MAPK (Cell Signaling Technology Inc., Beverly, MA), antibodies against CYP2C11 (Oxford Biomedical Research, Oxford, MA), and CYP3A2 (BD Gentest, Woburn, MA), and, finally, horseradish peroxidase-conjugated anti-mouse and anti-rabbit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Recombinant rat GH (rGH) and materials used to assay plasma rGH were obtained from the National Hormone and Peptide Program and Dr. A. F. Parlow (Harbor-UCLA Medical Center, Torrance, CA). Other chemicals of molecular biology grade were purchased either from Sigma-Aldrich (St. Louis, MO) or from Roche Diagnostics (Indianapolis, IN).

Animals. Animals were housed in the University of Pennsylvania Laboratory Animal Resources Facility under the supervision of a certified laboratory animal medicine veterinarian. These animals were treated according to a protocol approved by the University’s Institutional Animal Care and Use Committee. HYPOX male and female [Crl:CD (SC) BR] Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Rats were HYPOX by the supplier at 8 weeks of age and maintained for 4 to 5 weeks on commercial rat pellets and 5% sucrose drinking water. These animals were treated according to a protocol approved by the University’s Institutional Animal Care and Use Committee. HYPOX male and female [Crl:CD (SC) BR] Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Rats were HYPOX by the supplier at 8 weeks of age and maintained for 4 to 5 weeks on commercial rat pellets and 5% sucrose drinking water. These animals were treated according to a protocol approved by the University’s Institutional Animal Care and Use Committee. HYPOX male and female [Crl:CD (SC) BR] Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Rats were HYPOX by the supplier at 8 weeks of age and maintained for 4 to 5 weeks on commercial rat pellets and 5% sucrose drinking water. These animals were treated according to a protocol approved by the University’s Institutional Animal Care and Use Committee.

Surgical Implantation of Catheter, GH Treatment, and Assay. Indwelling right atrial catheters were implanted by methods described previously (Pam pori et al., 1991). After 3 days, the unrestrained and unstressed, cathe terized HYPOX rats were infused with 40 μg of rGH/kg b.w. by an external syringe pump apparatus over a 3-min period with a frequency of 6 pulses/day (i.e., 1 pulse every 4 h) for a total of 14 pulses. Control rats were similarly infused with vehicle. After the sixth pulse, atrial blood samples were collected every 15 min for 6 to 7 h. GH patterns were determined by radioimmunoassay (Shapiro et al., 1989). Rats were decapitated at 5, 15, 30, 45, 60, and 100 min after the final rGH infusion. The 0-min rats were given rGH buffer and euthanized immediately. Livers were quickly removed and minced into small pieces on ice-chilled Petri dishes. A fraction of minced liver was stored in RNA-Later (Ambion, Austin, TX) at −70°C for RNA extraction. Other fractions were processed for the chromatin immunoprecipitation (ChiP) assay (see below) as well as for microsomal isolation as described previously (Shapiro et al., 1989).

Preparation of Subcellular Fractions of Liver for Signal Transduction Analysis. The bulk of each liver mince was processed by the method of Sierra et al. (1993) with minor modifications (Verma et al., 2005). In brief, minced livers were homogenized in buffer containing different protease and phosphatase inhibitors. The homogenized livers were centrifuged over a sucrose cushion for 60 min at 100,000g in a precooled rotor. The upper layer of cellular debris was discarded after centrifugation, and the remaining supernatant was designated as the cytoplasmic fraction. The nuclear pellet was washed with ice-cold normal saline to avoid contamination by other subcellular fractions. The nuclear pellet was lysed with nuclear lysis buffer also containing protease and phosphatase inhibitors. The resultant nuclear suspension was precipitated with ammonium sulfate and centrifuged at 100,000g for 60 min at 4°C. The tubes were removed immediately, and the supernatant was transferred into new tubes. Next, the supernatant was again incubated with a higher ammonium sulfate concentration and centrifuged at 100,000g for 20 min. The pellet was dissolved in a nuclear dialysis buffer containing protease and phosphatase inhibitors. The protein content of the subcellular fractions was quantified using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Western Blot. The different subcellular fractions of liver were electrophore sed under denaturing conditions on a SDS-polyacrylamide gel electrophores isis system. We used 1.5-mm, 10% SDS-polyacrylamide gels for CYP2C11, CYP3A2, IκB-α, Stat5A, Stat5B, and activated MAPK and 4 to 10% gradient gels (SDS-polyacrylamide gel electrophoresis) for phospho-Jak2. Western blot analyses were performed as reported previously (Verma et al., 2005). The blots were analyzed with an FluorChem 8800 gel documentation system (Alpha Innotech, San Leandro, CA) using a visible light source. Densitometric units were obtained as integrated density values as calculated by the software supplied with the gel documentation system. Equal loading of protein was confirmed by using Ponceau S staining and Western blot analysis for the expression of β-actin. Furthermore, protein values were normalized to two control samples repeatedly run on all blots.

Reverse Transcription-PCR. CYP2C11, CYP3A2, and CYP2A1 gene expressions were determined by reverse transcription-PCR. Total cellular RNA was extracted from liver tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). The concentration and purity of the isolated RNA were quantitated using spectrophotometry by measuring absorbance at 260 and the 260/280 nm ratio, respectively. One microgram of RNA from each sample was reverse-tran scribed in a 20-μl reaction volume. One microliter of cDNA was PCR-amplified according to our previous description (Verma et al., 2005). PCR primers for CYP2C11, CYP3A2 (Morris and Davila, 1996), and CYP2A1 (Geng and Strobel, 1993) were synthesized by Sigma-Genosys (The Wood lands, TX). The final PCR product was quantified with a FluorChem 8800 gel documentation system using a UV lamp. Densitometric units were obtained as integrated density values as calculated by the software supplied with the gel documentation system. The PCR products for the three P450s were purified and sequenced with DNA sequencer model 377 (Applied Biosystems, Foster City, CA) using the specific primers for each isoform. According to a Blast search (http://www.ncbi.nlm.nih.gov), the purified PCR products exhibited 100% sequence homology with their respective P450 genes (Rattus norvegi cus) (sequences not presented).

Catalytic Activity. Testosterone 7a-hydroxylase, reflective of the activity level of CYP2A1, was assayed according to our methods as described previously (Agrawal et al., 1995).

ChiP. A ChiP assay was performed on a portion of the freshly isolated minced liver that had been incubated in Dulbecco’s minimal essential medium containing 1% formaldehyde for 10 min at room temperature. This cross-linking reaction was stopped by addition of glycine to a final concentration of 0.125 M. The tissue was washed three times with ice-cold Dulbecco’s phosphate-buffered saline containing 5 mM EDTA. The nuclei were subsequently isolated and lysed, and the ChiP procedure was performed as described previously (Chia et al., 2006). Briefly, the lysate was sonicated to generate DNA fragments with an average length of 100 to 1000 base pairs. After removal of cell debris by centrifugation, the chromatin concentration was...
measured and about 10% of the chromatin was kept as an input; the rest of the chromatin was diluted 3-fold. Equal concentrations of chromatin from all time points were precleared with protein A agarose beads in the presence of salmon sperm DNA to reduce the nonspecific background. After removal of beads by centrifugation, 2 μg of Stat5B specific antibody (BD Biosciences, San Jose, CA) was added, and the mixture was kept at 4°C overnight on a rotary platform. The immunoprecipitates were washed sequentially, and elutes were heated to reverse the formaldehyde cross-linking and then treated with DNase-free RNase to remove RNA. After additional purifications, the immunoprecipitated DNAs and input DNAs were analyzed by semiquantitative PCR using forward 5'-AAG GGG AAG CTT CCT AAG CA-3' and reverse 5'-GCC TCC ATG TAT GTC TGT GTG-3' primers designed to detect that region of the CYP2C11 promoter (GenBank X79081) containing 40 to 60% GC and in which the Stat5 binding region is centrally located (Park and Waxman, 2001). A negative control with a forward primer 5'-TGC ACA CCT TAA ATG TAG GC-3' (−1280/−1260) and reverse 5'-GCC TCC ATG TAT GTC TGT GTG-3' (−1010/−1031) primers was used to determine the specificity of Stat5B binding to its binding region. The PCR products were resolved on 2% agarose gel containing ethidium bromide, and the band intensities in each time point were quantitated by using a FluorChem 8800 Imager. The signals were normalized with a positive control that was repeatedly run on each blot.

Confirmation of the Stat5B-Binding Motif among the ChIP-PCR Products by Southern Blotting Analysis. The 270-base pair DNA PCR products obtained from the ChIP assays at the various time points were denatured and transferred onto Nytran N filters from Schleicher & Schuell (Keene, NH). Southern blotting was performed (Hirokawa et al., 2003) to confirm the Stat5B-binding motif in the PCR products by using a γ-32P-labeled nucleotide sequence, 5'-gca-aac-att-TTC-CAT-GAA-aat-a-3' (−1150/−1142) (Stat5 consensus core sequence in capital letters) of the Stat5B-binding site on the CYP2C11 promoter (Park and Waxman, 2001). The signals were scanned and quantitated by using a FluorChem 8800 Imager. The signals were normalized with a positive control that was repeatedly run on each blot.

Results

Renaturalization of the Circulating Episodic rGH Profile. The pattern of circulating rGH observed from serial blood samples collected from control rats infused with rGH vehicle (Fig. 1) exhibited typical sexually dimorphic GH profiles (Shapiro et al., 1995). Intact males secreted GH in episodic bursts (~250 ng/ml plasma) about every 3.5 to 4 h. Between the peaks, GH levels were undetectable. In intact females the hormone pulses occurred more frequently and irregularly and were of lower magnitude than those in males, whereas the interpulse concentrations of GH were always measurable. Intra-atrial infusion of male and female HYPOX rats with 40 μg of rGH/kg b.wt. every 4 h produced an episodic circulating GH profile that was nearly identical to the hormone profile observed in intact male rats (Fig. 1).

Sexually Dimorphic Expression of P450 Isoforms by the Renaturalized Episodic GH Profile. Because restoration of the masculine GH profile to HYPOX rats is not, in itself, evidence of the treatment’s effectiveness, we examined expression levels of three GH-dependent P450 isoforms in the treated animals. We chose P450s that were each regulated differently by the episodic GH profile. As
Profile increased CYP2A1 concentrations in both sexes/H11011 of the masculine GH profile to HYPOX rats for mally suppressed under the influence of episodic GH. Administration is secreted continuously (i.e., the female pattern), but is only mini-
maximally expressed in the HYPOX rat, almost disappears when GH piro, 1996; Agrawal and Shapiro, 2000) that male-specific CYP3A2 is observed, as previously reported (Waxman, 1992; Pampori and Sha-
effects of the episodic profile in males than in females (Fig. 2). We demonstrated the inherent sexually dimorphic responsiveness of CYP2C11 in which the isoform is more responsive to the inductive
GH depletion from the circulation (e.g., HYPOX) allowed for mone secretion nearly completely blocked CYP2C11 expression, total secretion (Fig. 2). Although the feminine pattern of continuous hor-
migration of the masculine circulating GH profile, it does indicate that the rGH regimen was capable of activating signaling pathways responsible for expression of the P450 isoforms.

Sexually Dimorphic Response of Jak2 to the Episodic GH Profile. Renaturalization of the masculine circulating GH profile produced a robust response of Jak2 in HYPOX male rats (Fig. 3). Within 5 min after the last rGH pulse (i.e., the 14th pulse), cytoplas-
mic phospho-Jak2 levels rose from undetectable to peak values; returning to baseline 30 min after the pulse. Phospho-Jak2 in female HYPOX rats showed the same chronologic response to the final rGH pulse, i.e., peaking within 5 min and declining thereafter to baseline by 30 min. However, concentrations of the activated tyrosine kinase were 4 to 5 times greater in male liver than female liver exposed to the identical episodic rGH profile.

Sexually Dimorphic Recruitment of Cytoplasmic Stat5A and Stat5B by the Episodic rGH Profile. After phosphorylation of Jak2, the next step in episodic GH induced signal transduction is recruit-
ment of cytoplasmic Stat5 molecules for activation and nuclear trans-
location. In agreement with earlier reports (Waxman and O’Connor, 2006), after the last rGH pulse we observed that cytoplasmic concentra-
tions of total Stat5B and the much less abundant Stat5A declined precipitously (~80%) in males to a nadir level within 5 min of the pulse (Fig. 4). Thereafter, total Stat5B and total Stat5A returned to baseline 60 and 100 min, respectively. Although there was no statistically significant sex differences in cytoplasmic Stat5B concentra-
tions immediately preceding the rGH pulse, total Stat5A was somewhat (~25%, P < 0.01) higher in females at this time. The decrease in cytoplasmic Stat5A and Stat5B after the rGH pulse in females occurred as rapidly as in males but with a much smaller magnitude of decline (~30%, P < 0.01) than that observed in males. The concentrations of total Stat5A and total Stat5B in the hepatic
Sex Differences in Episodic GH-Directed Stat5B Binding to the CYP2C11 Promoter. Because the masculine episodic rGH profile is the sole regulator of CYP2C11 expression (Legraverend et al., 1992; Waxman, 1992; Shapiro et al., 1995) and is significantly more effective when administered to males than to females (Shapiro et al., 1993; Thangavel et al., 2006), we examined the binding kinetics of phospho-Stat5B to the CYP2C11 promoter by ChIP assay (Fig. 5). In agreement with the expected sequence of signaling events, the maximum binding of activated Stat5B to the hepatic promoter occurred about 45 min after the rGH pulse in both sexes. However, about twice the amount of the transcription factor was bound to the CYP2C11 promoter in males than in females. PCR amplification of a negative control using primers flanking the CYP2C11 promoter at a genomic sequence not including the Stat5B binding site demonstrated no measurable nonspecific binding (Fig. 5). In confirmation of the ChIP results, using Southern blotting, we observed that twice as much of the Stat5B-binding motif of the CYP2C11 promoter bound to the activated transcription factor in hepatocytes from male rats (Fig. 5).

Sexually Dimorphic Activation and Translocation of MAPK by the Episodic GH Profile. Because activation of the NF-κB pathway has been associated with the regulation of CYP2C11 (Iber et al., 2000) and CYP2C11 expression is dependent upon the episodic GH profile (Shapiro et al., 1995), we connected the “dots” and examined the sexually dimorphic response of IκB to the masculine GH profile. There was no sex difference in cytoplasmic IκB-α concentrations that were unaffected by episodic rGH, nuclear levels of the activated kinases were significantly (P < 0.01) increased 5 min after the rGH pulse. Thereafter, nuclear phospho-Erk1 and phospho-Erk2 returned to prestimulatory baseline levels within 25 min. Nevertheless, the amount of activated MAPK observed in the nucleus, at every time point, was 2- to 4-fold higher in the male hepatocytes.

Sex Differences in the Level of Cytoplasmic IκB-α by the Episodic rGH Profile. Because activation of the NF-κB pathway has been associated with the regulation of CYP2C11 (Iber et al., 2000) and CYP2C11 expression is dependent upon the episodic GH profile (Shapiro et al., 1995), we connected the “dots” and examined the sexually dimorphic response of IκB to the masculine GH profile. There was no sex difference in cytoplasmic IκB-α concentrations that were unaffected by episodic rGH, nuclear levels of the activated kinases were significantly (P < 0.01) increased 5 min after the rGH pulse. Thereafter, nuclear phospho-Erk1 and phospho-Erk2 returned to prestimulatory baseline levels within 25 min. Nevertheless, the amount of activated MAPK observed in the nucleus, at every time point, was 2- to 4-fold higher in the male hepatocytes.

1 Because this is an in vivo study, and MAPK is known to mediate the activities of numerous factors in addition to GH, it is possible that the elevated nuclear phospho-Erk1 and phospho-Erk2 baselines in male liver were due to some normally occurring, but confounding, male-specific “signal” other than GH.
FIG. 5. Sex-dependent levels of Stat5B binding to the CYP2C11 promoter in HYPOX rats administered the masculine episodic rGH profile. The masculine circulating GH profile was renaturalized in HYPOX male and female rats by infusing, via an indwelling i.a. catheter, 14 pulses of rGH, 1 pulse every 4 h. Rats were euthanized at different time points between 0 and 100 min after the last pulse. Graphic quantitation of Stat5B binding to the CYP2C11 putative promoter (ChIP assay, left) and the occupied Stat5B-binding motif in the CYP2C11 promoter (Southern blot, right) as well as representative ChIP assay and Southern blots (bottom) were determined by procedures described under Materials and Methods. Values presented are the means ± S.D. of at least five animals at each time point. *, p < 0.01, comparing males with females at the same time point. Absolute values should not be compared between panels.

FIG. 6. Sex-dependent phospho-Erk1 and phospho-Erk2 levels in HYPOX rats after i.v. administration of the masculine episodic rGH profile. The masculine circulating GH profile was renaturalized in HYPOX male and female rats by infusing, via an indwelling i.a. catheter, 14 pulses of rGH, 1 pulse every 4 h. Rats were euthanized at different time points between 0 and 100 min after the last GH pulse. Phospho-Erk1 and phospho-Erk2 were measured in the cytoplasmic and nuclear fractions of livers using Western blot analysis as described under Materials and Methods. Values presented are the means ± S.D. of at least five animals at each time point. *, p < 0.01, comparing males with females at the same time point. Absolute values should not be compared between panels.
The masculine circulating GH profile was renaturated in HYPOX male and female rats by infusing, via an indwelling i.a. catheter, 14 pulses of rGH, 1 pulse every 4 h. Rats were euthanized at different time points (between 0 and 100 min after the last GH pulse). IkB-α was measured in the cytoplasmic fraction of livers using Western blot analysis as described under Materials and Methods. Values presented are the means ± S.D. of at least five animals at each time point. *, p < 0.01, comparing males with females at the same time points.

Discussion

Because the differential response of hepatic P450 isomers in male and female rats to the episodic GH profile is physiologic (Shapiro et al., 1993; Waxman et al., 1995), it seemed germane to investigate the possible mechanism(s) responsible for this sexual dimorphism under in vivo physiologic GH conditions. Moreover, the use of HYPOX rats allowed us to examine the selective role of GH in the absence of any possible confounding effects resulting from sex differences in other hormones, such as glucocorticoids and sex steroids. Accordingly, we successfully restored the typical as well as an indistinguishable masculine episodic circulating GH profile in both HYPOX male and female rats for ~2.5 days that effectively induced male-like levels of hepatic female predominant CYP2A1 as well as male-specific CYP2C11 and CYP3A2 in both sexes. Because, as expected from previous reports (Shapiro et al., 1993; Thangavel et al., 2006), the inductive response of CYP2C11 to the masculine GH profile was significantly greater in male than in female rats and the actions of the GH profile are mediated, at least in part, by the Jak2/Stat5B signal transduction pathway (Verma et al., 2005; Waxman and O’Connor, 2006), we examined episodic rGH-induced activation of the Jak2/Stat5B pathway in both sexes. GH signaling in liver by the episodic profile (in contrast to the continuous profile) is initiated by hormone binding to and the resulting activation of GH receptors on the surface of target cells. This allows for the recruitment and/or activation of two molecules of Jak2, which then cross-phosphorylate each other as well as phosphorylating the receptor on key tyrosine residues. Stat5B, a latent transcription factor, binds to these phosphorylated receptor docking sites, undergoes Jak2-catalyzed tyrosine phosphorylation, homodimerizes, and translocates to the nucleus where it binds to promoter sites initiating transcription of GH-regulated genes (Waxman and O’Connor, 2006). For this signal transduction pathway to be optimally effective, threshold levels of Stat5B (and possibly Jak2) have to be activated, translocated into the nucleus, and then bind to the promoter of GH-regulated genes (Choi and Waxman, 2000; Verma et al., 2005). Because all of these signal transduction events were blunted by at least 50% in the females, it seems reasonable to conclude that the sexually dimorphic response of hepatic CYP2C11 to episodic GH is due to a failure by the hormone to stimulate an optimum cascade of signal transducers in the target cells of females.

The reason(s) for this reduced responsiveness of the Jak2/Stat5B signaling pathway to episodic GH in females is far from clear. Because activation of the Jak2/Stat5B pathway, alone, is not sufficient to explain the actions of the episodic GH profile, other signaling factors have been considered. Previous studies using transfected cell lines (Waxman and O’Connor, 2006) and primary hepatocytes (Thangavel and Shapiro, 2007) have implicated hepatocyte nuclear factors and suppressors of cytokine signaling, respectively, in regulating the sexually dimorphic responsiveness of the Jak2/Stat5B pathway to the masculine GH profile. In an earlier in vivo study (Verma et al., 2005), HYPOX male rats were infused with single, but variable, doses of rGH whose resulting plasma pulse amplitudes varied from the physiologic (~250 ng/ml plasma) to the barely detectable (~3 ng/ml of plasma). Whereas all the doses of rGH stimulated Stat5B phosphorylation and its nuclear translocation (albeit, at considerably lower levels in males exposed to the smallest rGH pulses), only those rGH doses stimulating an activation and nuclear accumulation of MAPK also exhibited an induction of CYP2C11 transcription. The present findings demonstrate a similar correlation between episodic GH-induced expression of CYP2C11 and the activation and accompanying nuclear translocation of Erk1 and Erk2. That is, females who responded to the episodic GH profile with lower expression levels of CYP2C11 than males also exhibited lower levels of activated and nuclear translocated Erk1 and Erk2. Although the present finding supports earlier reports of GH stimulation of MAPK phosphorylation (Pircher et al., 1999; Piwien-Pilipuk et al., 2002), the involvement of Erk1 and Erk2 in episodic GH regulation of CYP2C11 expression remains circumstantial. Clearly, the masculine GH profile regulates the sex-dependent expression of numerous genes other than CYP2C11 (Ahluwalia et al., 2004) whose induction may be MAPK-dependent. Nevertheless, Erk activation/deactivation cycles are differentially regulated by the circulating profiles of hormones such as gonadotropin-releasing hormone (Kanasaki et al., 2005), and p42/p44 MAPK is a known down-regulator of the Jak/Stat signaling pathway (Valgeirs-döttir et al., 1999; Piwien-Pilipuk et al., 2002), suggesting the possibility that MAPK may be involved in the deactivation and subsequent activation cycling of Stat5B by the masculine GH profile requisite for inducing expression of CYP2C11 (Waxman and O’Connor, 2006). Whether the suboptimal activation and translocation of Erk1 and Erk2 by episodic GH in females is responsible for the reduced expression of CYP2C11 is not clear. But, were MAPK involved in CYP2C11 expression, it is possible that although the lower levels of nuclear Erk1 and Erk2 in the females permitted some cycling of Stat5B, it was insufficient for optimum CYP2C11 expression. Irrespective of any possible role for Erk1 and Erk2 in CYP2C11, the results do demonstrate an inherent sex difference in the response of the MAPK signaling pathway to the masculine GH profile.

Another signal transduction pathway implicated in CYP2C11 expression is inflammatory-induced NF-κB (Iber et al., 2000). There are, however, no reports examining a relationship between sex, GH, and NF-κB in regulating CYP2C11 expression. Accordingly, we conducted an initial experiment to probe a possible association. Members of the NF-κB family share a highly conserved homology domain that is responsible for DNA binding, dimerization, and interaction with IkB proteins. The activity of NF-κB is tightly regulated by its interaction with inhibitory IkB proteins. NF-κB is sequestered in the cytoplasm...
in an inactive form associated with inhibitory IκB proteins. This interaction blocks the ability of NF-κB to enter the nucleus and bind to DNA. After exposure to stimulatory, usually proinflammatory agents, the NF-κB signaling cascade is activated as a result of the phosphorylation and subsequent polyubiquitination-dependent degradation of IκB. This allows the translocation of unmasked NF-κB from the cytoplasm to the nucleus where it binds to NF-κB response elements in target genes and regulates their transcription. Functioning through a feedback loop, one of the target genes activated by NF-κB is that encoding IκB-α. Newly synthesized IκB-α can enter the nucleus, remove NF-κB from DNA, and export the complex back to the cytoplasm to restore its original latent state and prepare for another activation cycle (Liu and Malik, 2006).

Our findings indicate that the masculine GH profile induces a rapid decline in cytoplasmic IκB-α only in male rats. Cytoplasmic concentrations of the inhibitory protein in females were unaffected by the episodic GH profile. The decline in cytoplasmic IκB-α in males was presumably a result of its rapid degradation, leading to the activation and nuclear translocation of NF-κB. Although results are contradictory (Yi et al., 2006), there is evidence that GH can activate NF-κB (Jey et al., 2000). Persistent activation of the NF-κB pathway during inflammation can negatively regulate CYP2C11 expression by occupying a NF-κB-responsive element in the regulatory sequence of the CYP2C11 gene (Iber et al., 2000). However, activated Stat5B appears to reduce NF-κB activity (Han et al., 2007) by directly (Iber et al., 2000), or by indirectly competing for a nuclear factor(s) necessary for NF-κB-mediated activation of its target promoters (Luo and Yu-Lee, 2000), e.g., CYP2C11. CYP2C11 expression is absolutely dependent upon episodic or cycling events. Only the masculinized episodic GH profile can induce CYP2C11 expression. The episodic GH profile is responsible for the optimum, albeit episodic (on-off), Stat5B activation, nuclear translocation and binding to the CYP2C11 promoter. Although speculative, the possibility that the GH pulse in the episodic profile activates NF-κB by stimulating IκB-α degradation exists. NF-κB competes with Stat5B at the CYP2C11 promoter, where levels are already quickly declining from that induced by the previous pulse. Transcription of the CYP2C11 gene is temporarily interrupted. Subsequently, concentrations of activated Stat5B once again increase in the nucleus, competing and replacing NF-κB on the CYP2C11 promoter and again inducing CYP2C11 transcription. Thus, the requirement of episodic (on-off) stimulation for CYP2C11 expression is fulfilled. Why the episodic GH profile is presumably unable to activate the NF-κB pathway in females is not clear.

Our results suggest that reduced induction levels of CYP2C11 in female rats exposed to the masculine GH profile is probably due, at least in part, to suboptimum activation of the Jak2/Stat5B signaling pathway. Whether the reduced activation of the MAPK and/or NF-κB pathways, also observed in females exposed to the episodic GH profile, are directly involved in the reduced expression levels of CYP2C11 is suggestive. However, the findings do demonstrate inherent sex-dependent differences in the responsiveness of the Jak2/Stat5B, MAPK, and NF-κB signal transduction pathways to the circulating masculine GH profile. The effects of this sexual dimorphism response to GH is not limited to the expression of male- and female-specific P450 isoforms in rats (Shapiro et al., 1993; Waxman et al., 1995; Pampori and Shapiro, 1999; Thangavel et al., 2004). In the case of humans, GH deficiency causes numerous abnormalities in growth rates; lean body mass; cardiovascular, bone, adipose tissue, and muscle function; protein, carbohydrate, lipid, and electrolyte metabolism (Kuromaru et al., 1998; Span et al., 2001; Hubina et al., 2004; Jørgensen and Christiansen, 2005); and expression levels of hepatic insulin-like growth factor-1, insulin-like growth factor binding protein, growth hormone binding protein (Johansson et al., 1999; Hubina et al., 2004; Soares et al., 2004; Jørgensen and Christiansen, 2005), and cytochrome P450-dependent drug-metabolizing enzymes (Gil Berglund et al., 2002; Dhir et al., 2006). However, hormone replacement therapy has clearly demonstrated an intrinsic, irreversible sex-dependent response. Daily injections, evolving the masculine-like episodic GH profile, the most feasible, yet efficacious therapeutic approach, are significantly more effective in correcting these GH deficiency abnormalities in men (or boys) and women (or girls) (Kuromaru et al., 1998; Johansson et al., 1999; Span et al., 2001; Gil Berglund et al., 2002; Hubina et al., 2004; Soares et al., 2004; Dhir et al., 2006), suggesting that intrinsic sex differences in GH-activated signal transduction pathways in humans are similar to those in rats.

References
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dependent isoforms of hepatic P450 to the feminine plasma growth hormone profile. *Endocrinology* **140**:1245–1254.


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