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A Molecular Basis for the Sexually Dimorphic Response to Growth Hormone

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Once reserved solely for the treatment of short stature, the now readily available recombinant GH has expanded the use of the hormone to include the treatment of cardiovascular, renal, muscular, skeletal, immunological, psychosocial, and metabolic abnormalities associated with GH deficiency. There are also proposals for the widespread use of the hormone to ameliorate or reverse aging. However, this extensive use of GH has revealed intrinsic sexual dimorphisms in which females are considerably less responsive to the therapeutic regimen than are males. Dynamic changes in the Janus kinase-2 (Jak2)/signal transducers and activators of transcription (Stat5B) signaling pathway [as determined by transducer activation, Stat5B binding to the GH-responsive promoter of the *CYP2C11* gene, and expression levels of the suppressors of cytokine signaling family (Socs2, Socs3, and Cis)] were examined in male and female rat-derived primary hepatocyte cultures exposed to the masculine-like episodic GH profile. We report that the cellular actions of GH normally mediated by activation of the Jak2/Stat5B pathway are suppressed in female cells possibly due to an inherent overexpression of Cis, a member of the suppressors of cytokine signaling family that normally down-regulates the Jak2/Stat5B pathway. (*Endocrinology* 148: 2894–2903, 2007)

GH PLAYS AN IMPORTANT role in the development, growth, and maintenance of nearly every organ and tissue in the body by regulating protein, lipid, and carbohydrate metabolism in addition to synergistically enhancing the actions of other hormones (1). Whereas males and females secrete the same daily amount of GH, the secretory patterns are sexually dimorphic. In various species examined, including mice, rats, and humans (2), females secrete a so-called "continuous" GH profile comprised of numerous daily pulses interrupted by short-lived interpulses of usually low or undetectable hormone concentrations. In contrast, the "episodic" masculine GH profile is characterized by significantly fewer secretory bursts of the hormone separated by lengthy interpulses that are invariably devoid of GH. In fact, it is the difference between the continuous (female) and episodic (male) circulating GH profiles, and not plasma hormone levels *per se* that are responsible for phenotypic sexual dimorphisms ranging from growth patterns to expression levels of hepatic enzymes (2–4). In this regard, GH deficiency causes numerous abnormalities in growth rates, lean body mass; cardiovascular, bone, adipose, and muscle function; protein, carbohydrate, lipid, and electrolyte metabolism; (1, 5–9); and expression levels of hepatic IGF-1, IGF binding protein, GH-binding protein (3, 5, 6, 10–12), and cytochrome P450-dependent drug metabolizing enzymes (13, 14). However, hormone replacement therapy has clearly demonstrated an intrinsic, irreversible, sexually dimorphic response. Daily injections, evoking the masculine-like episodic GH profile, the most feasible, yet efficacious therapeutic approach, are significantly more effective correcting these GH deficiency abnormalities in men (or boys) than women (or girls) (6–15). Subsequent investigations in rats have reported similar intrinsic sex-dependent responses to GH replacement. For example, renaturalization of the masculine episodic GH profile cannot stimulate female hepatocytes, either *in vivo* or *in vitro* (16, 17) to express male-like levels of cytochrome P450 isoforms, indicating an irreversible sexually dimorphic responsiveness to GH regulation. Why then would bone, fat, muscle, and liver cells be less responsive to GH replacement in females?

GH signaling in liver by the episodic profile (in contrast to the continuous profile) is initiated by hormone binding and the resulting activation of GH receptors (GHR) on the surface of the target cells. This allows for the recruitment and/or activation of two molecules of Janus kinase-2 (Jak2), which then cross-phosphorylate each other as well as phosphorylating the receptor on key tyrosine residues. Signal transducers and activators of transcription (*i.e.* Stat5B), latent transcription factors, bind to these phosphorylated receptor docking sites, are in turn phosphorylated, homodimerize, and translocate to the nucleus where they bind to promoter sites initiating transcription of GH-regulated genes. An important negative regulatory mechanism of GH signaling, the suppressors of cytokine signaling (Socs/Cis) family of inhibitory proteins, which depending upon the protein, inhibit GH signaling by inactivating Jak2, by competing with Stat5B for common tyrosine-binding sites on the intracellular tail of the GHR, and/or by a proteasome-dependent mechanism that results in the degradation of the (GH-GHR-Jak2)·Cis complex (18–22). In this regard, Cis appears to be the dominant, if not sole, factor down-regulating the GH initiated

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Abbreviations: ChIP, Chromatin immunoprecipitation assay; GHR, GH receptor; IB, immunoblotting; IP, immunoprecipitated; Jak2, Janus kinase-2; rGH, rat GH; Stat, signal transducer and activator of transcription.

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Jak2/Stat5B signaling pathway in cells continuously exposed to the hormone (18, 21). In part, Cis exerts its inhibitory effect by competing with Stat5B for common tyrosine-binding sites in the cytoplasmic domain of the GHR (20, 21). The *Cis* gene is activated by a feedback loop regulated by Stat5B (19, 22). Accordingly, we have identified sex differences in this signaling pathway that could explain the sexually dimorphic responsiveness of target cells to the masculine episodic GH profile.

Materials and Methods

Animals

Rats, which uniquely express high levels of several sex-specific, GH-dependent hepatic isoforms of cytochrome P450 (2), were housed in the University of Pennsylvania Laboratory Animal Resources facility under the supervision of certified laboratory animal medicine veterinarians and were treated according to a research protocol approved by the Institutional Animal Care and Use Committee of the University. Male and female rats [Crl: CD (SD)BR] were hypophysectomized by the vendor (Charles River Laboratories, Wilmington, MA) at 8 wk of age [by which time adult, sex-dependent patterns of plasma GH and cytochrome P450s are clearly established (3)] and observed in our facility for 5–6 wk (12 h light/12 h dark; lights on 0800 h) to allow sufficient time for any residual pituitary tissue to regenerate. The effectiveness of the surgery was verified by the lack of weight gain over this period and the absence of pituitaries or fragments at necropsy shortly after euthanizing the rats (~1500 h).

Hepatocyte isolation and culture

Preparation of hepatocytes from long-term hypophysectomized rats free of any confounding effects of endogenous GH (23) was performed with minor modifications (17) by *in situ* perfusion of collagenase through the portal vein of anesthetized rats following standard protocol (24). The viability of the initial cell suspension of hepatocytes was typically between 80–90% (with trypan blue). The hepatocytes were plated at a density of 5×10^6 viable cells per T-25 flask previously coated with matrigel (274 $\mu g/cm^2$). After allowing 2–3 h for cell attachment, serum-containing medium was removed and replaced by serum-free DMEM/F-12 media supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), glutamine (2 mM), HEPES (15 mM), insulin (10 μg/ml), bovine transferrine (10 μg/ml), Na_2SeO_3 (10 ng/ml), aminolevulinic acid (2 μg/ml), glucose (25 mM), linoleic acid-albumin (0.5 mg/ml), and sodium pyruvate (5 mM). The cultures were also supplemented with fungizone (0.25 μg/ml) for the initial 48 h only. Cultures were maintained in a humidified incubator at 37 C under an atmosphere of 5% CO_2/95% air.

Hormonal conditions

To replicate the episodic GH profile, about 4 h after isolation, hepatocytes were exposed to recombinant rat GH (rGH) (0.2 ng/ml) (NHPP, Torrance, CA) for 30 min followed by two careful washings with GH-free media that remained in the wells for 11.5 h, at which time the cells were again washed and exposed to the next 30-min pulse of GH (17). On the 5th d, at which time CYP2C11 levels should have returned to normal (17), cells were harvested after 0, 7, 15, 30, 45, 75, 120, 180, and 240 min after the addition of the last GH pulse at 0700 h. To replicate the continuous GH profile, hepatocytes were constantly exposed to a 2-ng/ml concentration of rGH for 12 h, after which time the cells were washed and exposed for another continuous 12 h to the hormone (23). Cells were harvested on the 5th d after the final media change at the same time intervals as cells treated with the episodic profile.

Analysis of CYP2C11 mRNA by Northern blotting

Total cellular RNA, isolated as previously reported (17, 23), was resolved on denaturing 1% agarose gels and transferred onto Nytran N filters from Schleicher and Schuell (Keene, NH). The Northern blot was probed and reprobed with γ-^{32}P-labeled oligonucleotide probes, using hybridization and high stringency washing conditions as described previously (25). The sequence of the oligonucleotide probe for CYP2C11 has been reported (26). The consistency of RNA loading between samples was confirmed by ethidium bromide staining of 18S and 28S ribosomal RNAs and was verified using an 18S oligonucleotide probe (27). The hybridized mRNA signals were quantified by scanning the autoradiographs using a FluorChem IS-8800 Imager (Alpha Innotech, San Leandro, CA). The mRNA signals were normalized to the 18S rRNA signals in each lane, which exhibited a mean variation of only ±5%, indicating results were independent of loading errors.

Semiquantitative RT-PCR analysis of Cis, Socs2, and Socs3 mRNAs

Cis, Socs2, and Socs3 mRNAs were analyzed by semiquantitative RT-PCR as previously reported (15, 28). The cDNA was made from total cellular RNA isolated from cultured hepatocytes. PCR primers used for Cis, Socs2, Socs3 (28), and rat β-actin (29) have been reported. The linear range of product amplification was established for each primer pair in pilot experiments. In all experiments, controls were carried out without cDNA (RT step) and yielded no products after PCR, indicating that there was no detectable amount of DNA in the RNA. The PCR products were analyzed on 2% agarose gel containing ethidium bromide with 1× TAE buffer. The mRNA signals were visualized and quantitated by using a FluorChem IS-8800 Imager (Alpha Innotech). The mRNA signals were normalized to the rat β-actin internal control.

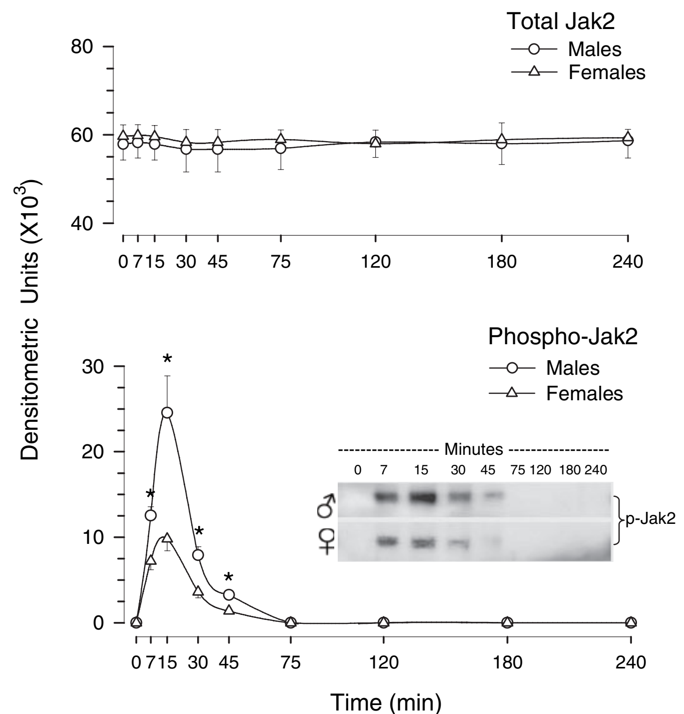

FIG. 1. Total Jak2, IB: anti-Jak2 (*top*), and phospho-Jak2, IP: anti-Jak2, IB: anti-pY (*bottom*) levels in primary hepatocytes derived from hypophysectomized male and female rats exposed to episodic rGH for 5 d in culture. Cells were harvested and analyzed at different time points between 0 and 240 min after the final hormone pulse. Sufficient viable cells were isolated from a single liver for all determinations at every time point presented in the figure. Each data point is a mean ± SD for cells from five or more rats. *, $P < 0.01$ compares females with males at the same time point. Absolute values should not be compared between panels. A representative Western blot of IP phospho-Jak2 (p-Jak2) is included in the *bottom panel*.

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Preparation of whole cell lysate and Western blot analysis

Whole cell lysate was extracted from cultured primary hepatocytes at various times after the last GH pulse, and the protein concentrations of the cell lysates were measured by using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Fifty micrograms of whole cell lysate protein were electrophoresed on 0.75-mm-thick sodium dodecyl sulfate-polyacrylamide (7.5%) gels and electroblotted onto nitrocellulose membranes for immunoblotting (IB). Accordingly, the blots were probed with antibodies against Jak2, phospho-Stat5 (Upstate, Lake Placid, NY), Stat5B (BD Biosciences, San Jose, CA), Stat5A, or Cis (Santa Cruz Biotechnology, Santa Cruz, CA). Additional fractions of the total cell lysates were immunoprecipitated (IP) with either Jak2 (Upstate) or Stat5B (BD Biosciences) antibodies. Next the immunoprecipitates were probed with anti-phosphotyrosine, anti-pY, (Upstate). This order allowed us first to concentrate the Jak2 and Stat5B proteins and then specifically measure the activated component. Signals of the relative protein levels were quantitated by using a FluorChem IS-8800 Imager (Alpha Innotech). Signals were normalized to a control sample that was repeatedly run on each blot and exhibited a concentration variant between blots of 2.8–6.1% for the different proteins. Last, blots were stripped and reprobed with actin antibody (Santa Cruz Biotechnology), normalized with actin loading control, and the resulting findings were comparable to those obtained with internal controls of the assayed protein were run repeatedly on all blots.

Chromatin immunoprecipitation assays (ChIP)

A ChIP assay was performed on harvested hepatocytes 0, 7, 15, 30, 45, 75, 120, and 240 min after the hormone pulse on the 5th d in culture. The ChIP assay was performed as described (30, 31) with slight modifications. Hepatocytes were treated with 1% formaldehyde for 10 min at room temperature. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M. The cells were harvested and washed three times with ice-cold Dulbecco's PBS buffer containing 5 mM EDTA. The nuclei were subsequently isolated and lysed. The lysate was sonicated to generate DNA fragments with an average length of 100-1000 bp. After removal of cell debris by centrifugation, the chromatin concentration was measured and about 10% of the chromatin was kept as an input, and 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 1 mg/ml BSA and 2 μg of sonicated salmon sperm DNA to reduce the nonspecific background. After removal of beads by centrifugation, 2 μg of Stat5B-specific antibody (BD Biosciences) was added and kept at 4 C overnight on a rotary platform. The immune complexes were collected by centrifugation after adding protein A agarose beads and kept at 4 C for 1 h. The immunoprecipitates were washed sequentially and eluted as described (30). Elutes were pooled and heated at 65 C for 6 h to reverse the formaldehyde cross-linking and also treated with DNase-free RNAase to remove RNA. The samples were treated with 40 μg/ml of proteinase K at 45 C for 1 h, followed by phenol/chloroform extraction and ethanol precipitation. The same process was also carried out for input chromatin. The IP DNAs and input DNAs were analyzed by semi-quantitative PCR using forward 5′-AAG GGG AAG CTT CCT AAG CA-3′ (−1280/−1260) and reverse 5′-GCC TCC ATG TAT GTC TGT GTG-3′ (−1010/−1031) primers of the CYP2C11 promoter made from the *CYP2C11* gene (GenBank X79081) to detect the promoter among the IP DNA. A negative control with a forward primer 5′-TGC ACA CCT TAA ATG TAG GC-3′ (−1640/−1621) and reverse 5′-TGG CTC CTC TTC AAG TGG TA-3′ (−1421/−1440) primer of a non-Stat5B binding region of the CYP2C11 promoter (GenBank X79081) was used to determine the specificity of Stat5B binding to its binding region.

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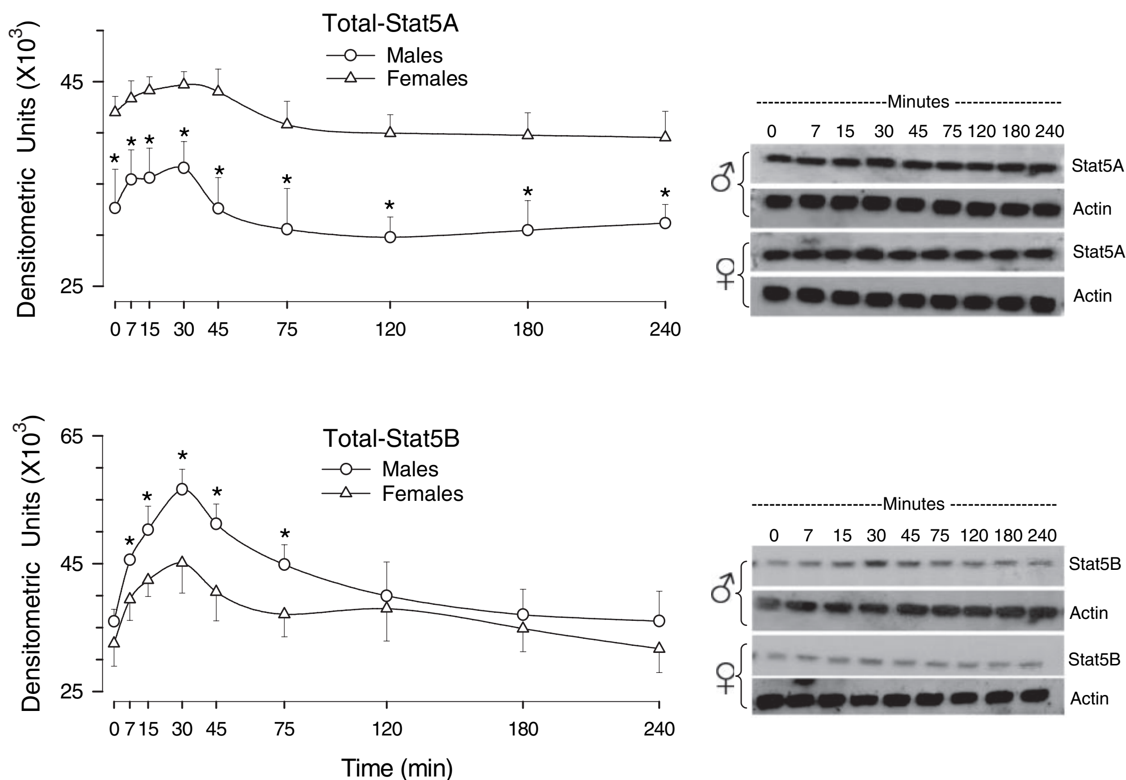

FIG. 2. Total Stat5A, IB: anti-Stat5A (*top*), and total Stat5B, IB: anti-Stat5B (*bottom*) levels in primary hepatocytes derived from hypophysectomized male and female rats exposed to episodic rGH for 5 d in culture. Cells were harvested and analyzed at different time points between 0 and 240 min after the final hormone pulse. Sufficient viable cells were isolated from a single liver for all determinations at every time point presented in the figure. Each data point is a mean ± SD for cells from five or more rats. *, $P < 0.01$ compares females with males at the same time point. Absolute values should not be compared between panels. Representative Western blots of Stat5A, Stat5B, and their actin loading controls are included on the *right*.

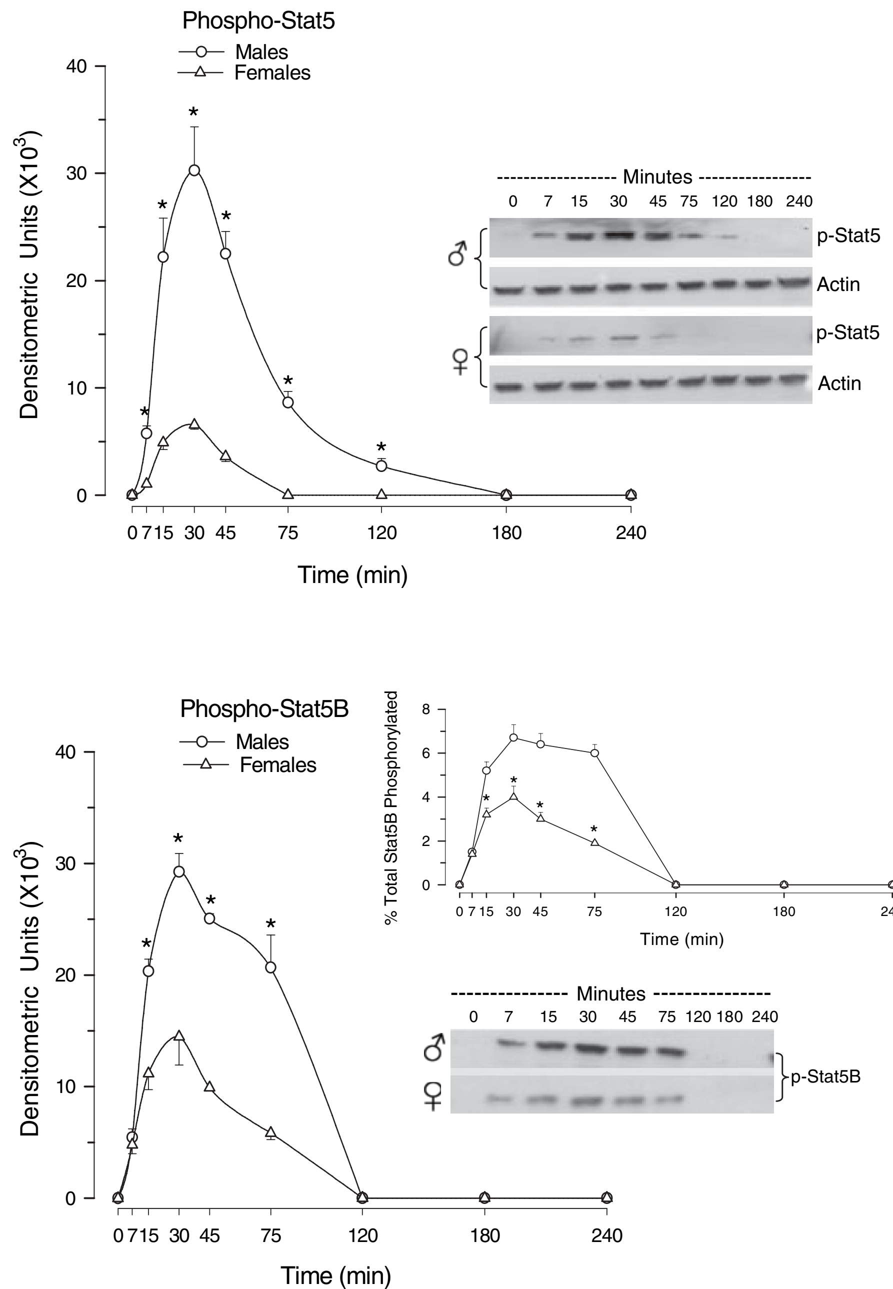

FIG. 3. Phospho-Stat5, IB: anti-p-Stat5 (*top*), and phospho-Stat5B, IP: anti-Stat5B, IB: anti-pY (*bottom*) and percent of the total p-Stat5B that is phosphorylated (*bottom inset*) in primary hepatocytes derived from hypophysectomized male and female rats exposed to episodic rGH for 5 d in culture. Cells were harvested and analyzed at different time points between 0 and 240 min after the final hormone pulse. Sufficient viable cells were isolated from a single liver for all determinations at every time point presented in the figure. Each data point is a mean ± SD for cells from five or more rats. *, $P < 0.01$ compares females with males at the same time point. Absolute values should not be compared between panels. Representative Western blots of p-Stat5 and their actin loading controls as well as p-Stat5B are included on the *right*.

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The PCR products were resolved on 2% agarose gel containing ethidium bromide, and the band intensities at each time point were quantitated by using a FluorChem IS-8800 Imager (Alpha Innotech).

Confirmation of the Stat5B binding motif among the ChIP-PCR product by Southern blotting analysis

The PCR products (DNA) from the CHIP assays were denatured and transferred onto Nytran N filters from Schleicher and Schuell. Southern blotting was carried out (32, 33) to confirm the Stat5B-binding motif in the PCR product by using a γ-^{32}P-labeled nucleotide sequence of the Stat5B binding sites on the CYP2C11 promoter (34). The signals were scanned and quantitated by using a FluorChem IS-8800 Imager (Alpha Innotech). The signals were normalized with a positive control that was repeatedly run on each blot.

Statistics

All the data were subjected to ANOVA. Significant differences were determined with *t* statistics and the Bonferroni procedure for multiple comparisons.

Results

Sexually dimorphic response of Jak2 to episodic GH

For 5 d in culture, cells were exposed to an episodic GH profile shown in vivo and in vitro to be a highly effective inducer of numerous sex-dependent physiologic functions including body weight gain, hepatic IGF, and various isoforms of hepatic cytochrome P450 (3, 17, 35, 36). Cells were harvested at various time points after the final GH pulse.

Whereas total Jak2 concentrations were unaffected by GH or sex, activation (*i.e.* phosphorylation) of Jak2 was influenced by both factors (Fig. 1). Fifteen minutes after the GH pulse, activated Jak2 levels peaked and returned to pre-GH induction zero levels between 45–75 min in both sexes. However, concentrations of the activated tyrosine kinase were twice as great in male hepatocytes than in female cells.

Sexually dimorphic recruitment of Stat5A and B by episodic GH

After phosphorylation of Jak2, the next step in episodic GH-induced signal transduction is the recruitment of Stat5 molecules. Approximately 30 min after the final GH pulse on the 5th d in culture, we observed a small elevation ($P > 0.05$) in total Stat5A in both sexes. However, at all measured time points, total Stat5A concentrations were consistently approximately 35% greater in female hepatocytes (Fig. 2). Whereas baseline levels of total Stat5B exhibited no sex differences, 30 min after the GH pulse, the increase in the concentration of the transcription factor in female hepatocytes was only about half as much as in similarly treated male hepatocytes (Fig. 2). In fact, during the entire response period (7–75 min after GH) total Stat5B levels were always greater in the male cells. (The unexpected increase in Stat5B protein first observed at 7 min of GH exposure can be explained by the possible greater affinity of the antibody for the phosphorylated form of Stat5B than to the nonphosphorylated form.)

Sexually dimorphic activation of Stat5B by episodic GH

In agreement with the expected sequence of events, levels of phospho-Stat5 peaked 15 min after phospho-Jak2 peaked in cells from both sexes (Fig. 3). However, induction levels of the activated transcription factor were several fold greater in male hepatocytes. Stat5 antibodies recognize both the A and B forms, with the latter being implicated in episodic GH action (18, 19, 21) and the former exhibiting no such responsiveness (Fig. 2). By immunoprecipitation, we measured the levels of phospho-Stat5B. Although phospho-Stat5B peaked in both male and female cells 30 min after the GH pulse, more than twice as many molecules of the transcription factor were activated in the male hepatocytes (Fig. 3). When phospho-Stat5B levels (Fig. 3) were corrected to reflect the different protein concentration used to measure total Stat5B (Fig. 2), the normalized percent of the total Stat5B that was actually phosphorylated remained about twice as high in the male than female hepatocytes 15–75 min after the rGH episodic pulse (Fig. 3). When we exposed the male and female cells to a continuous GH profile, we observed minimal Stat5B phosphorylation (data not shown) reflecting about 10–15% of the levels induced by the episodic profile and in

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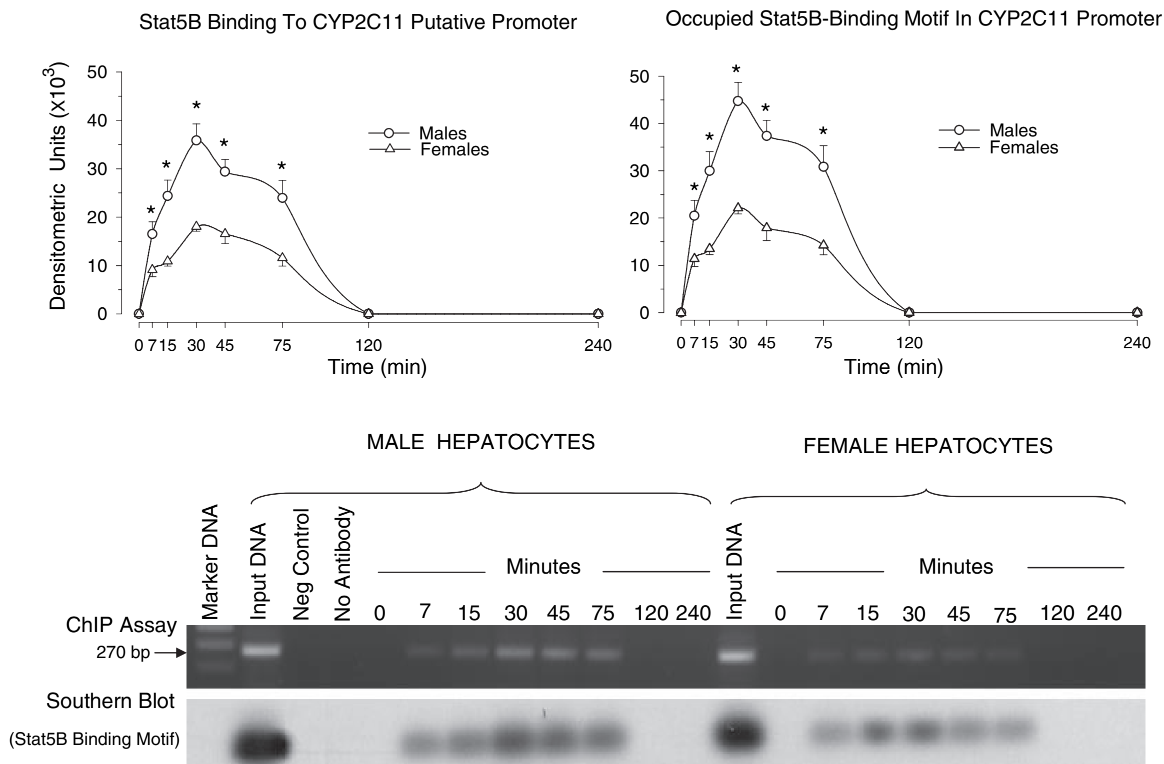

FIG. 4. Graphic quantitation of Stat5B binding to the CYP2C11 putative promoter (ChIP assay, *left*) and the occupied Stat5B-binding motif in the CYP2C11 promoter (Southern blot, *right*) as well as representative ChIP and Southern blots determined in primary hepatocytes, derived from hypophysectomized male and female rats, exposed to episodic rGH for 5 d in culture. Cells were harvested and analyzed at different time points between 0 and 240 min after the final hormone pulse. Sufficient viable cells were isolated from a single liver for both the ChIP assay and Southern blotting determinations for every time point presented in the figure. Each data point is a mean ± SD for cells from five or more rats. *, $P < 0.01$ compares females with males at the same time point. Absolute values should not be compared between panels.

agreement with findings using the rat liver cell line CWSV-1 (37) as well as intact rats (38).

Sex differences in episodic GH-directed Stat5B binding to the CYP2C11 promoter

Because expression of the CYP2C11 gene is solely regulated by the episodic GH profile (2, 35, 36), we examined the binding kinetics of phospho-Stat5B to the CYP2C11 promoter by ChIP assay. In agreement with the expected sequence of signaling events, maximum binding of activated Stat5B to the promoter occurred between 30 and 45 min after the GH pulse in both sexes of hepatocytes (Fig. 4). However, almost twice the amount of the transcription factor was bound to the CYP2C11 promoter in male hepatocytes than in female hepatocytes. PCR amplification of a negative (Neg) control using primers flanking the CYP2C11 promoter at a genomic sequence not including the Stat5B binding site demonstrated no measurable nonspecific binding (Fig. 4). In conformation, using Southern blotting, we observed twice as much of the Stat5B-binding motif of the CYP2C11 promoter bound to the activated transcription factor in hepatocytes from male rats (Fig. 4).

Sex differences in Cis/Socs expression regulated by episodic GH

The Cis/Socs family of proteins function as negative regulators of episodic GH-induced Jak/Stat signal transduction. It would appear that the same GH pulse responsible for initiating the activation of the Jak2/Stat5B pathway subsequently induces expression of the Cis/Socs genes whose transcribed proteins, in turn, terminate the Jak2/Stat5B signal to prepare the target cell to respond to the next pulse of GH (18, 20–22). According to this proposed course of events in which maximum nuclear Stat5B binding occurs 30–45 min after the GH pulse; termination of the reaction as characterized by an elevated expression of the Cis/Socs genes should then follow, as we observed (Fig. 5), within 45–75 min of the hormone pulse. Disregarding Socs3, the expression levels of which were not sexually dimorphic and which is not involved in episodic GH-induced signal transduction (20, 39, 40), only hepatocytes from males exhibited a significant ($P < 0.001$) elevation in Cis and Socs2 mRNAs after the hormone pulse, with changes in Cis mRNA being far more dramatic (~100%) than Socs2 (~25%) (Fig. 5). Despite the poor responsiveness of these two inhibitory genes in female hepatocytes to the GH pulse, Cis and Socs2 levels were consistently higher in female hepatocytes at every measured time point. Cis protein levels in episodic GH-treated male and female hepatocytes were in agreement with transcript concentrations (Fig. 6).

Sex differences in Cis/Socs expression regulated by continuous GH

Female liver is normally exposed to a continuous GH profile that can only minimally activate the Jak2/Stat5B pathway (37, 38, 41). Accordingly, this feminine GH profile may result in a continuous overexpression of the Cis/Socs family of inhibitory proteins responsible for blocking the activation of the Jak/Stat signal transduction pathway in females. We tested this hypothesis by exposing hepatocytes from both sexes to constant GH at a highly effective concentration (2 ng/ml) shown to restore normal levels (mRNA and protein) of Jak/Stat-independent female isoforms of hepatic cytochrome P450 (23). Cells were harvested after 5 d in culture at various times after the last of 10 media changes. Despite the fact that the cells were continuously exposed to GH, they responded with an increased transcription of Cis/Socs in both sexes (male

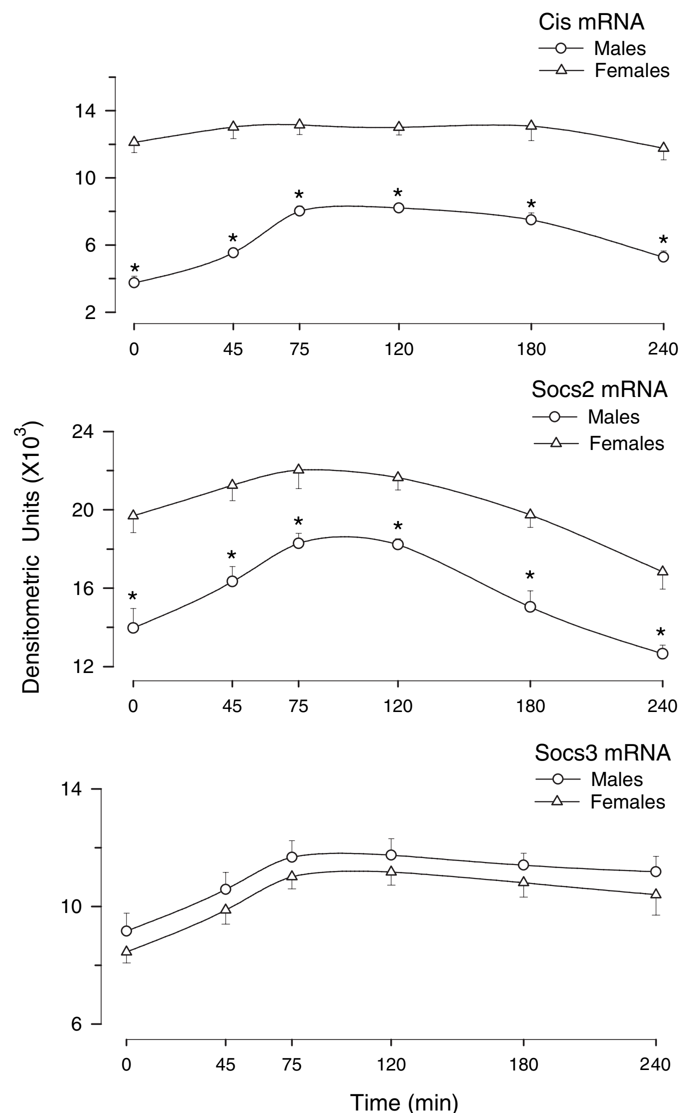

FIG. 5. Cis (*top*), Socs2 (*middle*), and Socs3 (*bottom*) mRNA levels in primary hepatocytes, derived from hypophysectomized male and female rats, exposed to episodic rGH for 5 d in culture. Cells were harvested and analyzed at different time points between 0 and 240 min after the final hormone pulse. Sufficient viable cells were isolated from a single liver for Cis, Socs2, and Socs3 mRNA determinations by RT-PCR for every time point presented in the figure. Each data point is a mean ± SD for cells from five or more rats. *, $P < 0.01$ compares females with males at the same time point. Absolute values should not be compared between panels.

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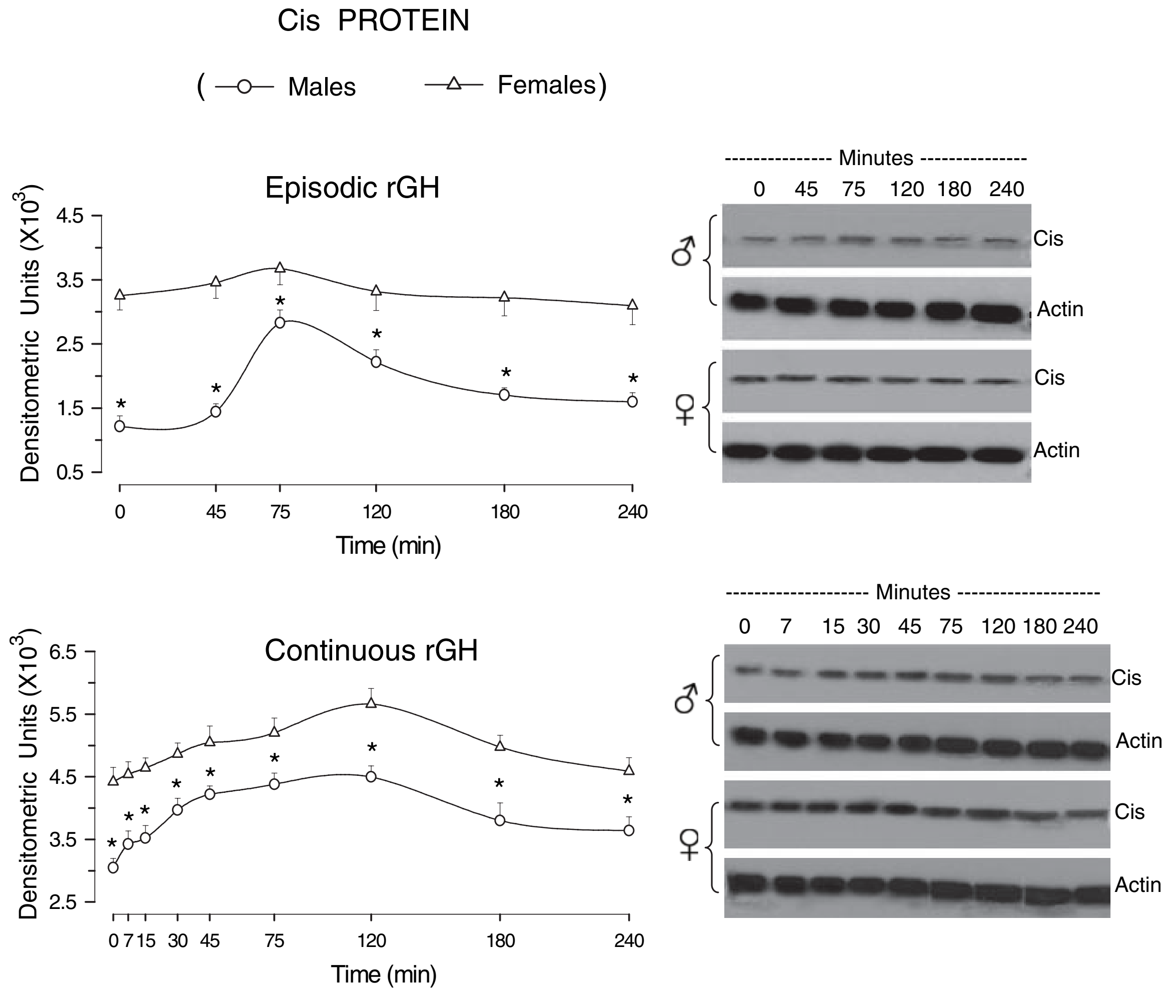

FIG. 6. Cis protein, IB: anti-Cis, levels in primary hepatocytes, derived from hypophysectomized male and female rats, exposed to episodic (*top*) or continuous (*bottom*) rGH, for 5 d in culture. Cells were harvested and analyzed at different time points between 0 and 240 min after either the final hormone pulse (episodic) or the last media change (continuous). Sufficient viable cells were isolated from a single liver for Cis determinations at every time point presented in the figure. Each data point is a mean ± SD for cells from five or more rats. *, $P < 0.01$ compares females with males at the same time point. Absolute values should not be compared between panels. Representative Western blots of Cis and their actin loading controls are included on the *right*.

~50%; females ~18%, $P < 0.01$ for all comparisons).[1] Similar to the effects of episodic GH (Fig. 5), female hepatocytes exposed to continuous GH expressed considerably greater concentrations of Cis and Socs2 mRNAs than male cells at every time point measured (Fig. 7). In fact, when the cells were first harvested from the hypophysectomized rats, Cis mRNA was nine times greater (5919 ± 206/645 ± 66; female/male) and Socs2 mRNA was 25% higher (8297 ± 206/6601 ± 244; female/male) in female hepatocytes; this despite an absence of GH for at least 5 wk and suggesting sex-dependent, irreversible imprinting. Cis protein levels in GH-continuously treated hepatocytes were in agreement with transcript concentrations, with Cis protein remaining at considerably higher levels in females at all time points, but male cells exhibiting a greater GH-induced increase in Cis (~50%, $P < 0.01$) than female cells (~25%, $P < 0.01$) (Fig. 6).

Irreversible sex-dependent responsiveness of hepatic CYP2C11 to episodic GH

Although the present results demonstrate a reduced responsiveness of the Jak2/Stat5B signal transduction pathway to episodic GH in female hepatocytes, the significance of this sexual dimorphism depends upon its biological consequences. Accordingly, we measured expression levels of CYP2C11, the male-specific isoform comprising more than 50% of the total cytochrome P450 in male rat liver (42). CYP2C11 expression is solely dependent upon exposure to the episodic GH profile; albeit its male specificity. The feminine profile of continuous GH secretion is completely suppressive. In the absence of GH (*e.g.* hypophysectomy), CYP2C11 is expressed at 20–

[1] Although it is possible that the concentration of GH declined between every 12 h of media change, it is unlikely that the hormone was ever eliminated (*e.g.* metabolized) from the culture. The GH regimen used in the experiment induces normal levels (mRNA and protein) of CYP2C12, an isoform whose expression is absolutely dependent upon continuous exposure to the hormone (3, 16).

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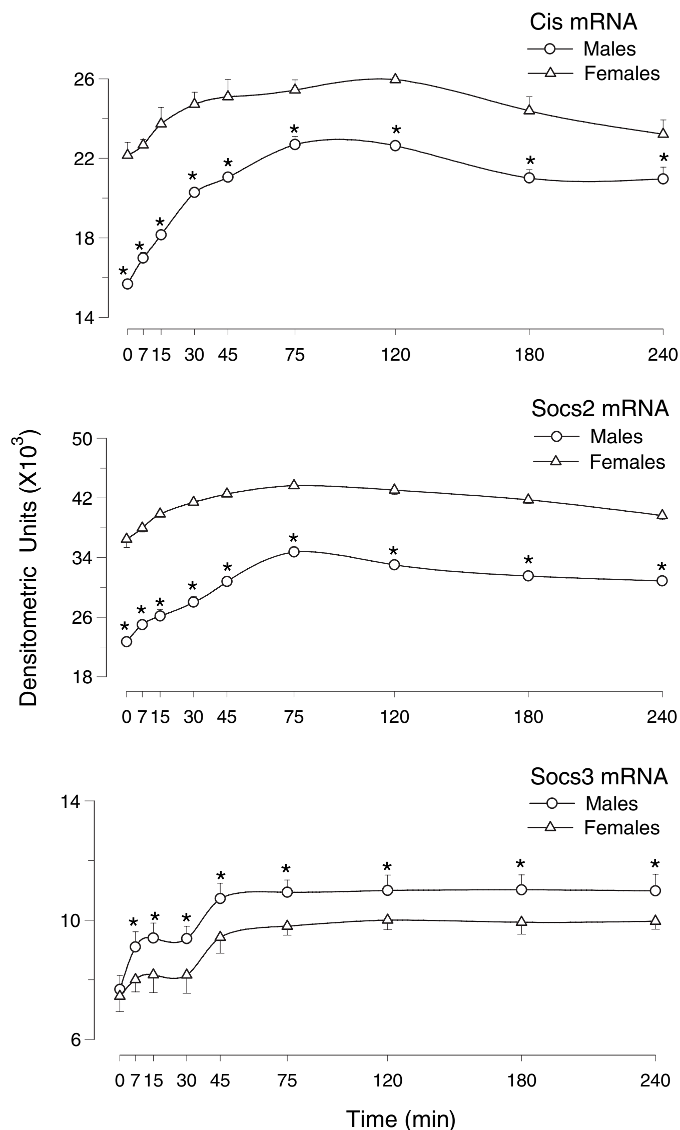

FIG. 7. Cis (*top*), Socs2 (*middle*), and Socs3 (*bottom*) mRNA levels in primary hepatocytes, derived from hypophysectomized male and female rats, exposed to continuous rGH for 5 d in culture. Cells were harvested and analyzed at different time points between 0 and 240 min after the last media change. Sufficient viable cells were isolated from a single liver for Cis, Socs2, and Socs3 mRNA determinations by RT-PCR for every time point presented in the figure. Each data point is a mean ± SD for cells from five or more rats. *, $P < 0.01$ compares females with males at the same time point. Absolute values should not be compared between panels.

30% of intact male-like concentrations in both sexes. In conformation (2, 16, 35), we have observed the expected sexual dimorphic expression and regulation of CYP2C11 by GH (Fig. 8). Because induction of CYP2C11 is reported to be mediated by episodic GH-activation of the Jak2/Stat5B pathway (39, 41), subnormal activation of the signaling pathway in female hepatocytes should reduce expression levels of the isoform. Indeed, whereas exposure of male hepatocytes to episodic GH restored CYP2C11 to intact male-like expression levels, female hepatocytes exposed to the identical episodic profile expressed no detectable concentrations of the isoform (Fig. 8).

Discussion

The episodic masculine-like GH profile produces its effects by initiating a specific series of signaling events at its target cell that sequentially include binding to its transmembrane receptor causing GHR activation, recruitment, and phosphorylation (*i.e.* activation) of Jak2, phosphorylation of the GHR, phosphorylation, homodimerization, and nuclear translocation of Stat5B, which initiates transcription of GH-responsive genes by binding to their promoters (18–22). In order for this signal transduction pathway to be effective, threshold levels of Stat5B (and possibly Jak2) have to be activated, translocated into the nucleus, and then bound to the promoter of GH-regulated genes (40, 43, 44). Because all of these signal transduction events were blunted by at least 50% in the female hepatocytes, and considering that as much as 70% of normal phospho-Stat5B translocated nuclear levels are insufficient to initiate episodic GH-transcription of the CYP2C11 gene (44), it seems reasonable to conclude that the sexually dimorphic responses to episodic GH are due to a failure by the hormone to stimulate an optimum cascade of signal transducers in the target cells of females. The reason for this insufficient responsiveness to GH in female cells may be due to the persistent overexpression of Cis, the sole member of the Socs/Cis family of inhibitory proteins responsible for terminating the episodic GH-activated Jak2/Stat5B pathway (18, 21, 39, 40). In this regard, compared with males, Cis levels are dramatically higher in the livers of intact females (18, 45). Moreover, we have found that female hepatocytes that were either GH-deficient (hypophysectomized), continuous, or episodic GH-treated expressed dramatically higher levels of Cis and it is the overexpression of the Cis protein in continuous GH-treated rats that is known to prevent Jak2/Stat5B-directed transcription of GH-dependent genes (18, 39). In contrast, male cells exposed to episodic GH express elevated levels of Cis for only a transient period, down-regulating the Jak2/Stat5B pathway after transcription initiation and allowing a subsequent reactivation of the pathway by the next GH pulse.

Under normal circumstances, the female secretes a continuous profile of GH suppressing the activation of the Jak2/Stat5B signal transduction pathway that otherwise could be responsible for the masculinization of numerous sexual dimorphisms ranging from musculoskeletal development to hepatic enzymes. Clearly, females were not intended to be exposed to the masculinizing episodic GH profile. However, it would seem that as an added precaution, females inherently overexpress Cis, guaranteeing that even independent of GH status, the Jak2/Stat5B pathway and dependent functions will be suppressed. Thus, we see a possible explanation for the subnormal responsiveness of females to the usual intermittent GH therapy.

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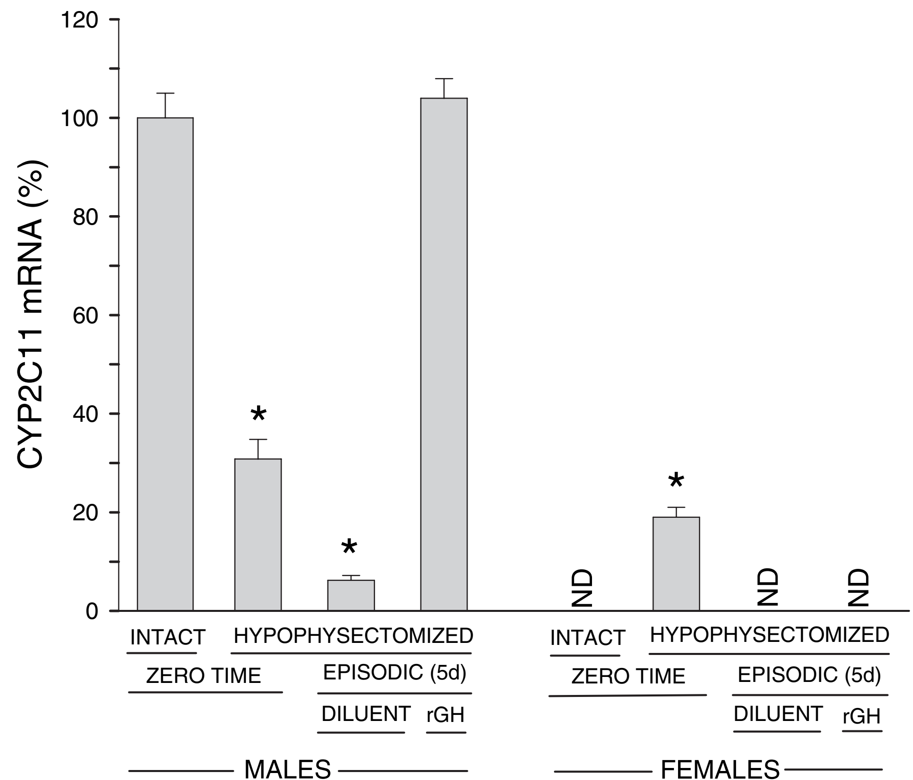

FIG. 8. CYP2C11 mRNA expression in "zero time" (*i.e.* immediately after isolation and before plating) hepatocytes derived from intact and hypophysectomized male and female rats as well as primary hepatocytes from hypophysectomized rats exposed to either episodic rGH or its diluent for 5 d (5d) in culture. Sufficient viable hepatocytes were isolated from each hypophysectomized rat for every treatment presented in the figure. Values are presented as a percentage of CYP2C11 mRNA in hepatocytes from intact male rats at zero time arbitrarily designated 100%. Each data point is a mean ± SD for cells from five or more rats. ND, Not detectable. *, $P < 0.01$ when compared with intact males at zero time.

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References

1. **Bengtsson BÅ** 1999 Growth hormone. Norwell, MA: Kluwer Academic Publisher; 1–359
2. **Shapiro BH, Agrawal AK, Pampori NA** 1995 Gender differences in drug metabolism regulated by growth hormone. Int J Biochem Cell Biol 27:9–20
3. **Jansson JO, Edén S, Isaksson O** 1985 Sexual dimorphism in the control of growth hormone secretion. Endocr Rev 6:128–150
4. **Laursen T, Lemming L, Jörgensen JOL, Klausen IC, Christiansen JS** 1998 Different effects of continuous and intermittent patterns of growth hormone administration on lipoprotein levels in growth hormone-deficient patients. Horm Res 50:284–291
5. **Jørgensen JOL, Christiansen JS** 2005 Clinical aspects of growth hormone deficiency in adults. Frontiers Horm Res 33:1–20
6. **Hubina E, Kovacs L, Szabolcs I, Szucs N, Toth M, Racz K, Czirjak S, Gorombey Z, Goth MI** 2004 The effect of gender and age on growth hormone replacement in growth hormone-deficient patients. Horm Metab Res 36:247–253
7. **Kuromaru R, Kohno H, Ueyama N, Hassan HMS, Honda S, Hara T** 1998 Long-term prospective study of body composition and lipid profiles during and after growth hormone (GH) treatment in children with GH deficiency: gender-specific metabolic effects. J Clin Endocrinol Metab 83:3890–3896
8. **Span JPT, Pieters GFFM, Sweep FGJ, Hermus ARMM, Smals AGH** 2001 Gender differences in rhGH-induced changes in body composition in GH-deficient adults. J Clin Endocrinol Metab 86:4161–4165
9. **Burman P, Johansson AG, Siegbahn A, Vessby B, Karlsson FA** 1997 Growth hormone (GH)-deficient men are more responsive to GH replacement therapy than women. J Clin Endocrinol Metab 82:550–555
10. **Soares DV, Conceição FL, Brasil RRLO, Spina LDC, Lobo PM, Silva EMC, Buescu A, Vaisman M** 2004 Insulin-like growth factor I levels during growth hormone (GH) replacement in GH-deficient adults: a gender difference. Growth Horm IGF Res 14:436–441
11. **Johansson AG** 1999 Gender difference in growth hormone response in adults. J Endocrinol Invest 22(Suppl 5):58–60
12. **Johansson AG, Engström BE, Ljunghall S, Karlsson FA, Burman P** 1999 Gender differences in the effects of long term growth hormone (GH) treatment on bone in adults with GH deficiency. J Clin Endocrinol Metab 84:2002–2007
13. **Berglund EG, Johannsson G, Beck O, Bengtsson BA, Rane A** 2002 Growth hormone replacement therapy induces codeine clearance. Eur J Clin Invest 32:507–512
14. **Sinués B, Mayayo E, Fanlo A, Mayayo Jr E, Bernal ML, Bocos P, Bello E, Labarto JI, Ferrández-Longás A** 2004 Effects of growth hormone deficiency and rhGH replacement therapy on the 6β-hydroxycortisol/free cortisol ratio, a marker of CYP3A activity, in growth hormone-deficient children. Eur J Clin Pharmacol 60:559–564
15. **Dhir RN, Dworakowski W, Thangavel C, Shapiro BH** 2006 Sexually dimorphic regulation of hepatic isoforms of human cytochrome P450 by growth hormone. J Pharmacol Exp Ther 316:87–94
16. **Shapiro BH, Pampori NA, Ram PA, Waxman DJ** 1993 Irreversible suppression of growth hormone-dependent cytochrome P450 2C11 in adult rats neonatally treated with monosodium glutamate. J Pharmacol Exp Ther 265:979–984
17. **Thangavel C, Dworakowski W, Shapiro BH** 2006 Inducibility of male-specific isoforms of cytochrome P450 by sex-dependent growth hormone profiles in hepatocyte cultures from male but not female rats. Drug Metab Dispos 34:410–419
18. **Karlsson H, Gustafsson JÅ, Mode A** 1999 Cis desensitizes GH induced Stat5 signaling in rat liver cells. Mol Cell Endocrinol 154:37–43
19. **Herrington J, Smit LS, Schwartz J, Carter-Su C** 2000 The role of STAT proteins in growth hormone signaling. Oncogene 19:2585–2597
20. **Greenhalgh CJ, Alexander WS** 2004 Suppressors of cytokine signalling and regulation of growth hormone action. Growth Horm IGF Res 14:200–206
21. **Landsman T, Waxman DJ** 2005 Role of the cytokine-induced SH2 domain-containing protein CIS in growth hormone receptor internalization. J Biol Chem 280:37471–37480
22. **LeRoith, D, Nissley P** 2005 Knock your SOCS off! J Clin Invest 115:223–236
23. **Thangavel C, Garcia MC, Shapiro BH** 2004 Intrinsic sex differences determine expression of growth hormone-regulated female cytochrome P450s. Mol Cell Endocrinol 220:31–39
24. **Strom SC, Pisarov LA, Dorko K, Thompson MT, Schuetz JD, Schuetz EG** 1996 Use of human hepatocytes to study P450 gene induction. Methods Enzymol 272:388–401
25. **Pampori NA, Shapiro BH** 1996 Feminization of hepatic cytochrome P450s by nominal levels of growth hormone in the feminine plasma profile. Mol Pharmacol 50:1148–1156
26. **Waxman DJ** 1991 P450-catalyzed steroid hydroxylation: assay and product identification by thin-layer chromatography. Methods Enzymol 206:249–267
27. **Ramsden R, Sommer KM, Omiecinski CJ** 1993 Phenobarbital induction and tissue-specific expression of the rat CYP2B2 gene in transgenic mice. J Biol Chem 286:21722–27126

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28. **Woelfle J, Rotwein P** 2004 In vivo regulation of growth hormone-stimulated gene transcription by STAT5b. Am J Physiol Endocrinol Metab 286:E393–E401
29. **Jiang HQ, Zhang XL, Liu L, Yang CC** 2004 Relationship between focal adhesion kinase and hepatic stellate cell proliferation during hepatic fibrogenesis. World J Gastroenterol 10:3001–3005
30. **Shang YF, Hu X, DiRenzo J, Lazar MA, Brown M** 2000 Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 103:843–852
31. **Woelfe J, Chia DJ, Rotwein P** 2003 In vivo regulation of growth hormone-stimulated gene transcription by STAT5b. J Biol Chem 278:51261–51266
32. **Boyd KE, Farnham PJ** 1999 Coexamination of site-specific transcription factor binding and promoter activity in living cells. Mol Cell Biol 19:8393–8399
33. **Hirokawa S, Sato H, Kato B, Kudo A** 2003 EBF-regulating Pax5 transcription is enhanced by STAT5 in the early stage of B cells. Eur J Immnunol 33:1824–1829
34. **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
35. **Legraverend C, Mode A, Westin S, Ström A, Eguchi H, Zaphiropoulos PG, Gustafsson JÅ** 1992 Transcriptional regulation of rat P-450 2C gene subfamily members by the sexually dimorphic pattern of growth hormone secretion. Mol Endocrinol 6:259–266
36. **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
37. **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
38. **Choi HK, Waxman DJ** 1999 Growth hormone, but not prolactin, maintains low-level activation of STAT5b in female rat liver. Endocrinology 140:5126–5135
39. **Ram PA, Waxman DJ** 2000 Role of the cytokine-inducible SH2 protein CIS in desensitization of STAT5b signaling by continuous growth hormone. J Biol Chem 275:39487–39496
40. **Miquet JG, Sotelo AI, Dominici FP, Bonkowski MS, Bartke A, Turyn D** 2005 Increased sensitivity to GH in liver of Ames dwarf (Prop1df/Prop1df) mice related to diminished CIS abundance. J Endocrinol 187:387–397
41. **Waxman DJ, Frank SJ** 2000 Growth hormone action: signaling via a Jak STAT-coupled receptor. In: Conn PM, Means AR, eds. Principles of molecular regulation. Totowa, NJ: Humana Press; 55–83
42. **Morgan ET, MacGeoch C, Gustafsson JÅ** 1985 Hormonal and developmental regulation of expression of the hepatic microsomal steroid 16α-hydroxylase cytochrome P-450 apoprotein in the rat. J Biol Chem 260: 11895–11898
43. **Choi HK, Waxman DJ** 2000 Plasma growth hormone pulse activation of hepatic JAK-STAT5 signaling: developmental regulation and role in male-specific liver gene expression. Endocrinology 141:3245–3255
44. **Verma AS, Dhir RN, Shapiro BH** 2005 Inadequacy of the Janus kinase 2/signal transducer and activator of transcription signal transduction pathway to mediate episodic growth hormone-dependent regulation of hepatic CYP2C11. Mol Pharmacol 67:891–901
45. **Tollet-Egnell P, Flores-Morales A, Stavréus-Evers A, Sahlin L, Norstedt G** 1999 Growth hormone regulation of SOCS-2, SOCS-3, and CIS messenger ribonucleic acid expression in the rat. Endocrinology 140:3693–3704

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