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Inherent Sexually Dimorphic Expression of Hepatic CYP2C12 Correlated with Repressed Activation of Growth Hormone- Regulated Signal Transduction in Male Rats

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Abstract

Due to its myriad physiologic functions, it isn't surprising that the actions of growth hormone (GH) are mediated by recruiting/activating dozens of signaling molecules involved in numerous transduction pathways. The particular signal transduction pathway activated by the hormone is determined by the affected target cell, the sexually dimorphic secretory GH profile (masculine episodic or feminine continuous) to which the cell is exposed as well as the individual's sex. In this regard, expression of female-specific CYP2C12, the most abundant cytochrome P450 in female rat liver, is solely regulated by the feminine GH profile. Sex is a modulating factor in this response in that males are considerably less responsive than females to the CYP2C12-induction effects of continuous GH. Using primary hepatocytes derived from male and female hypophysectomized rats, we have identified several factors in a transduction pathway activated by the feminine GH regime and associated with the induction of hepatic CYP2C12. Elements in the proposed pathway, in their likely order of activation, are the growth hormone receptor, extracellular signal-regulated kinases (Erk 1 & 2), the CREB binding protein (CBP), and hepatocyte nuclear factors (HNF-4 α and HNF-6) which subsequently bind and activate the CYP2C12 promoter. Recruitment and/or activation levels of all the component factors in the pathway were highly suppressed in male hepatocytes, possibly explaining the dramatically lower induction levels of CYP2C12 in males exposed to the same continuous GH profile as females.

Whereas males and females secrete the same daily amount of growth hormone (GH), the secretory pattern 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 of plasma) every 3.5-4 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 (Jansson et al., 1985; 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 (Jansson et al., 1985; 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).

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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. While these isoforms may (e.g., CYP2C11) or may not (e.g., CYP2C13, CYP2A2 and CYP3A2) require exposure to the masculine episodic GH profile for maximal expression, they are most certainly completely suppressed by the feminine continuous GH profile (Waxman, 1992; Pampori and Shapiro, 1999; Agrawal and Shapiro, 2000). In contrast, expression of the major female-specific isoforms, e.g., CYP2C12, are solely dependent upon the feminine profile of continuous GH secretion but unresponsive to the masculine profile (Waxman, 1992; Legraverend et al., 1992; Agrawal and Shapiro, 2000). The sex-predominant isoforms are likely the most abundant, if not sole group in most species including mice and humans (Shapiro et al., 1995; Dhir et al., 2006). While generally responsive to both the continuous and episodic GH profiles, only one of the sexually dimorphic profiles can induce maximal expression of a sex-predominant isoform (Pampori and Shapiro, 1999; Agrawal and Shapiro, 2000).

Although 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 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 (i.e., physiologic, subphysiologic or supraphysiologic), female hepatocytes, either in vitro or in vivo (Legraverend et al., 1992; Shapiro et al., 1993; Waxman et al., 1995) cannot express male-like levels of male-specific P450s, nor can male hepatocytes, either in vitro (Thangavel et al., 2004) or in vivo (Pampori and Shapiro, 1999) be induced to express female-like levels of female-dependent P450s¹. The suboptimal response of hepatic P450s in females to the masculine GH profile can be explained by the muted response of the signal transduction pathways activated by the episodic GH profile. That is, activation of the Jak2/Stat5B signaling pathway normally mediating the cellular action (e.g., CYP2C11 induction) of episodic GH are highly suppressed in females (12) as a likely result of an inherent overexpression of Cis, a member of the suppressors of cytokine signaling family that normally down-regulate the Jak2/Stat5B pathway (13). In the present study, we have investigated the molecular basis for both the normal and suboptimum induction of female-specific CYP2C12 in female and male hepatocytes, respectively, exposed to the continuous GH regime.

MATERIALS AND METHODS

Animals

Rats 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 University's Institutional Animal Care and Use Committee. Male and female rats [CrI: CD (SD) BR] were hypophysectomized by the vendor (Charles River Laboratories, Wilmington, MA) at 8 weeks of age [by which time adult, sex-dependent patterns of plasma GH and cytochrome P450s are clearly established (Jansson et al., 1985)], maintained on commercial rat pellets and 5% sucrose drinking water, and observed in our facility for 5 to 6 weeks (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

¹Similar, although less dramatic intrinsic sex differences in expression levels of CYP3A4 and CYP1A2 regulated by the sex-dependent GH profiles have been reported in human hepatocytes (Dhir et al., 2006).

was verified by the lack of weight gain over this period and the absence of pituitaries or their fragments at necropsy shortly after euthanizing the rats.

Hepatocyte Isolation and Culture

Preparation of rat hepatocytes from long-term hypophysectomized rats free of any confounding effects of endogenous GH (Thangavel et al., 2004) was performed with minor modifications (Thangavel et al., 2006) by in situ perfusion of collagenase through the portal vein of anesthetized rats following standard protocol (Seglen, 1976). The viability of the initial cell suspension of hepatocytes was typically between 80 and 90% (with trypan blue). The hepatocytes from 5 or more rats per group were individually plated at a density of 5×10^6 viable cells per T-25 flask previously coated with matrigel ($274 \mu\text{g}/\text{cm}^2$). After allowing 3-4 h for cell attachment, serum-containing medium was removed and replaced by serum free DMEM/F-12 media supplemented with streptomycin (100 $\mu\text{g}/\text{ml}$), penicillin (100 U/ml), glutamine (2 mM), Hepes (15 mM), insulin (10 $\mu\text{g}/\text{ml}$), bovine transferrine (10 $\mu\text{g}/\text{ml}$), Na_2SeO_3 (10 $\mu\text{g}/\text{ml}$), aminolevulinic acid (2 $\mu\text{g}/\text{ml}$), glucose (25 mM), linoleic acid-albumin (0.5 mg/ml), and sodium pyruvate (5 mM). The cultures were also supplemented with fungizone (0.25 $\mu\text{g}/\text{ml}$) for the initial 48 h only. Cultures were maintained in a humidified incubator at 37°C under an atmosphere of 5% $\text{CO}_2/95\%$ air.

Hormonal Conditions

To replicate the continuous GH profile, about 2-3 h after isolation, male and female hepatocytes were constantly exposed to a 2.0 ng/ml concentration of recombinant rat GH (NHPP, Torrance, CA) for 12 h after which time the cells were washed and exposed for another continuous 12 h to the hormone (Thangavel et al., 2004) resulting in complete media change every 12h. On the fifth day, at which time CYP2C12 levels should have returned to that normally expressed in liver of intact females (Thangavel et al., 2004), cells were harvested after 0,7,15,30,45,75,120, 180 and 240 minutes following the last change of media at 0700h. In some experiments, control cells were incubated in the presence of rGH diluent instead of the hormone, which were found to have no significant effect on the measured parameters (data generally not presented).

Preparation of Whole Cell and Nuclear Extracts

To isolate protein for immunoblots, cultured hepatocytes were harvested as described above. Following an initial centrifugation ($800 \times g$ for 10 min), cell pellets were resuspended in lysis buffer containing 50mM Tris-HCl (pH 7.5), 0.3 M NaCl, 1% Triton X-100, 5 mM EDTA, 0.5% Nonidet P-40, and 10 $\mu\text{g}/\text{ml}$ of leupeptin and aprotinin. The crude extract was passed through a 22-gauge needle 10 times. The solution was then gently mixed at 4°C for 20 min and centrifuged at $12,000g$ for 20 min. The supernatant (whole cell extract) was then removed and stored at -70°C until analyses. Briefly, nuclei were isolated according to Dignam et al., 1983, by a series of centrifugations of resuspended, homogenized and dialyzed crude nuclear extract originating from the low speed pellet. Protein concentration was determined by the Bio-Rad (Hercules, CA) protein assay with bovine serum albumin as standard. Sufficient material was isolated from each T-25 flask to permit the detection of all measured proteins by separate blotting and/or assay.

Western Blotting Analysis

Using standard protocol (Dhir et al., 2007; Thangavel and Shapiro, 2007) for those proteins identified by immunoblotting (IB) alone, 50 to 75 μg of whole cell extract was resolved on 10% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically onto Immuno-Blot™ PVDF Membrane (Bio-Rad, Hercules, CA) with a Bio-Rad transfer unit. The membranes were then blocked and incubated with antibodies against phospho-Erk1 and Erk2 (phospho-p42/p44 MAPK) (Cell Signaling Technology, Beverly, MA), HNF-4 α , HNF-6

(Santa Cruz Biotechnology, Santa Cruz, CA), rat growth hormone receptor (GHR_L and GHR_S) (a gift from Dr. G. Peter Frick, University of Massachusetts Medical School, Worcester, MA) or CYP2C12 (a gift from Dr. Marika Rönholm, Huddinge University Hospital, Huddinge, Sweden). Fifty µg of nuclear extract were used to detect nuclear HNF-4α and HNF-6 proteins. The protein signals were scanned and quantitated by using a FluorChem™ IS-8800 Imager (Alpha Innotech, San Leandro, CA). Lastly, blots were stripped and reprobed with actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), normalized, and the resulting findings presented as densitometric units.

Immunoprecipitation and Co-Immunoprecipitation Assays

Using Western blotting procedures described earlier (Thangavel and Shapiro, 2007), 1 mg of whole cell extract was immunoprecipitated (IP) with HNF-4α or HNF-6 antibodies and probed with acetyllysine antibody (Upstate, Lake Placid, NY). In another experiment, 1.5 mg of total cell extract was IP with anti-CBP (Santa Cruz Biotechnology, Santa Cruz, CA) and the immunoprecipitate was probed again with either anti-CBP (for the purpose of concentrating the protein), or with phosphotyrosine (AG10) antibody (Upstate, Lake Placid, NY). The blots were then stripped and reprobed for phospho-p42/p44 MAPK (Erk1/Erk2) antibody to identify co-immunoprecipitated phospho-p42/p44MAPK along with CBP. Finally, the blots were stripped and reprobed with CBP antibody to determine equal loading of protein in all lanes. The protein signals were scanned and quantitated by using a FluorChem™ IS-8800 Imager. Signals were normalized to known control samples processed along with the experimental samples to correct the data being presented as densitometric units.

Chromatin Immunoprecipitation Assay (ChIP)

ChIP assays were performed on harvested hepatocytes at 0,7,15,30,45,75,120 and 240 min following the GH exposure on the fifth day in culture. The ChIP assays were performed as described (Pierreux et al., 2004; Thangavel and Shapiro, 2007) with slight modifications. Basically, hepatocytes were treated with 1% formaldehyde for 10 min at room temperature. The cross-linking reaction was stopped by adding glycine to a final molarity of 0.125 M. The cells were harvested and washed thrice with ice-cold DBPS buffer containing 5 mM EDTA. The nuclei were subsequently isolated and lysed. The lysate was sonicated to generate DNA fragments with a range of 100 to 1000 bp. After removal of cell debris by centrifugation, 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 non-specific background. After removal of beads by centrifugation, 2 µg of HNF-4α or HNF-6 specific antibody 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 (Thangavel and Shapiro, 2007). 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 40µg/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 immunoprecipitated DNAs and input DNAs were analyzed by semiquantitative PCR using forward 5'-CTT CCA ACA AAA ATG ACA AAG TTA AAG GAG C-3' (-165/-135) and reverse 5'-CAG GGC TTG GTC TCC ATA GAT ACA GGA G-3' (+99/+72) primers of the CYP2C12 promoter made from the CYP2C12 gene (Endo et al., 2005) to detect the CYP2C12 promoter among the immunoprecipitated DNA. The -165 to +99 bp promoter fragment was chosen to obtain at least 40 to 60% GC in the primers and to keep the HNF-4α and HNF-6 binding sites in the middle of the product. A negative control with a forward primer 5'- CTG GGG AGA CTA AGG GAA TAC A-3' (-262/-241) and reverse 5'-GCT AAG CTT CGT

TGG CCC ATT T-3' (-1/-22) primer of a non-HNF-4 α and non-HNF-6 binding region of the rat G6PDH promoter (Rank et al., 1994) was used to determine the specificity of HNF-6 and HNF-4 α binding to their binding regions on the CYP2C12 promoter. The number of cycles used amplified the PCR products within the linear range and were resolved on 2% agarose gel containing ethidium bromide and the band intensities at each time point was quantitated by using a FluorChem™ IS-8800 Imager.

Confirmation of HNF-4 α and HNF-6 Binding Sites on the CYP2C12 Promoter by Southern Blotting

PCR amplified DNA (264 bp) products obtained from the ChIP assays were denatured and transferred onto Nytran N filters from Schleicher and Schuell (Keene, NH). Southern blotting was carried out (13) to confirm the HNF-4 α and HNF-6 binding motif in the PCR product by using a γ -³²P-labeled nucleotide sequence of the HNF-4 α (5'-GAG AGA TAA ACA GTG GCC AGA TGG CTG-3') and HNF-6 (5'-TAT AAA ATC TCT GGA GTG CCT GA-3') binding sites on the CYP2C12 promoter (17). The signals were scanned and quantitated by using a FluorChem™ IS-8800 Imager. The signals were normalized with a positive control which was repeatedly run on each blot and presented as densitometric units.

Statistics

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

RESULTS

Sex-Regulated Expression of Cultured Hepatocyte CYP2C12 Protein under Continuous GH

The feminine circulating GH profile (continuous) is the sole endogenous regulator of hepatic CYP2C12 expression in the rat (Legraverend et al., 1992; Pampori and Shapiro, 1999; Agrawal and Shapiro, 2000). Exposure to the masculine episodic GH profile as well as GH depletion (i.e., hypophysectomy) is completely ineffective in inducing the isoform. In agreement, we observed that freshly isolated hepatocytes from intact females expressed the expected high in vivo-like female levels of CYP2C12 (Fig. 1). In contrast, hepatocytes from intact males as well as hepatocytes from hypophysectomized rats of either sex, freshly isolated or cultured in GH-diluent, expressed no detectable protein concentration of CYP2C12. Whereas, female hepatocytes exposed to continuous GH in culture expressed indistinguishable levels of CYP2C12 from that observed in cells from intact females, the same GH regimen was only ~20% as effective an inducer of the isoform in hepatocytes derived from hypophysectomized males (Fig. 1). There were no detectable concentrations of male-specific CYP2C11 in any of the cultures treated with GH (data not presented).

Sex-Determined Expression Levels of Hepatic GHR under Continuous GH

GH initiates its signal at its target cells by binding/activating the GHR. Two forms of the GHR, the full-length receptor (GHR_L) and the short form (GHR_S) that lacks the transmembrane and intracellular domains of the GHR_L (Frick et al., 1998) were analyzed in male and female hepatocyte cultures exposed to 2ng/ml of continuous rGH (Fig. 2) previously shown to induce GHR mRNA expression in hepatoma cells (Nuoffer et al., 2000). Even in the absence of GH, the freshly isolated hepatocytes (pre-plating controls) from long-term hypophysectomized (5 to 6 weeks) female rats exhibited significantly higher concentrations of both forms of the GHR than similarly prepared male hepatocytes. While 5 days of exposure to continuous GH elevated baseline levels of the receptor in both sexes, the dimorphism (F>M) persisted at nearly all sampling times. So, while the 240 min response pattern of GHR_L and GHR_S (which were generally similar) to continuous GH were basically the same in both male and female

hepatocytes, concentrations of both receptor proteins were always (except at 180 min) significantly higher in liver cells from females.

Sex-Dependent Phosphorylation of Erk1 and Erk2 (p42/p44 MAPK) under Continuous GH

Numerous GH effects appear to be mediated by activating Erk1 and Erk2 signaling (Winston and Hunter, 1995; VanderKuur et al., 1997; Kelly et al., 2001) and hence, the present in vitro experiment examining the sexually dimorphic response of p42/p44 MAPK to continuous GH. Although the concentrations of activated (i.e., phosphorylated) Erk1 and Erk2 were very low in hepatocytes isolated from longterm hypophysectomized rats (i.e., GH depleted), there persisted a female predominance of ~2:1, F:M (Fig. 3). When the media, containing fresh GH was replaced on day 5 and the cells analyzed, there was a measured corresponding increase in the phosphorylated Erk proteins. The responses of Erk1 and Erk2 to GH were very similar. In contrast, sex had a dramatic effect on activation levels of the proteins induced by GH (Fig. 3). In both sexes, the levels of Erk phosphorylation peaked between 7 and 15 min after the media change, plateaued at these peak concentrations and declined towards pre-stimulation levels by 120 min. However, twice the amount of p42/p44 MAPK was activated in the female as compared to male hepatocytes. Although the differential was smaller, phospho-Erk1 and phospho-Erk2 concentrations remained significantly higher in female hepatocytes following the return to apparent baseline levels.

Sexually Dimorphic Expression of CBP under Continuous GH

CBP is a transcriptional co-activator possessing intrinsic acetyl transferase activity (Soutoglou et al., 2000) known to increase the stability and transcriptional activity of HNF-4 α and HNF-6 (Rausa et al., 2004; Soutoglou et al., 2000; Yoshida et al., 1997; Chen et al., 1999), two nuclear factors implicated in GH induction of CYP2C12 (Waxman and O'Connor, 2006). We observed a significant sexual dimorphism (1:1.5, M:F) in nuclear concentrations of CBP in freshly isolated hepatocytes from longterm hypophysectomized rats (Fig. 4). Because CBP expression was unresponsive to GH, the magnitude and sex ratio of the transcriptional co-activator remained unchanged after 5 days in culture.

Sex-Dependent Interaction of Activated Erk1 and Erk2 with CBP under Continuous GH

GH:GHR binding results in the phosphorylation of Erk1 and Erk2 via the Ras/Raf-1/MEK/ERK cascade (Winston and Hunter, 1995; VanderKuur et al., 1997; Kelly et al., 2001). Phosphorylated Erk proteins enter the nucleus and associate with CBP (Liu et al., 1999), which results in the activation (i.e., phosphorylation) and stimulation of the acetyl transferase activity of CBP (Gusterson et al., 2001; Ait-Si-Ali et al., 1999). We observed that exposure to continuous GH resulted in more than twice as much binding of phospho-Erk1 and phospho-Erk2 to CBP in female hepatocytes than identically treated male hepatocytes (Fig. 5). There was no significant difference in the binding profiles of phospho-Erk1 to CBP than that of phospho-Erk2 to CBP. Pre-plated hepatocytes lacking GH exposure for 5 to 6 weeks had extremely low levels of the protein complexes, but there still existed a statistical sex difference (F>M). Although the magnitude of the response of GH-induced activation of Erk proteins (Fig. 3) and Erk:CBP binding (Fig. 5) were sexually dimorphic, the response profile of the two events during 0 to 240 min were very similar in male and female hepatocytes.

Not surprisingly, the much greater binding of the Erk proteins to CBP in the GH-treated female hepatocytes resulted in greater activation (i.e., phosphorylation) of nuclear CBP (~3:1, F:M) (Fig. 5). The pre-plating controls confirmed that GH is needed for the phosphorylation of Erk1 and Erk2 (Fig. 3) and CBP (Fig. 5) as well as demonstrating the presence of intrinsic sexually dimorphic expression levels of the signaling proteins.

Sex-Dependent Expression Levels of Hepatocyte Nuclear Factors (HNF-4 α and HNF-6) under Continuous GH

HNF-4 α and HNF-6 are predominantly expressed in female rat liver and have been implicated in continuous GH regulation of CYP2C12 gene expression (Waxman and O'Connor, 2006; Lahuna et al., 1997; Endo et al., 2005). Although concentrations of both nuclear factors were dramatically reduced in the freshly isolated hepatocytes of longterm hypophysectomized rats, the sexual dimorphism persisted (F>M). Following 5 days of continuous GH exposure, HNF-4 α and HNF-6 measured in whole cell extracts were ~50% higher in female hepatocytes than male hepatocytes (Fig. 6). Whereas exposure to continuous GH elevated the baseline concentrations of both HNF-4 α and HNF-6 in the whole cell extracts of male and female hepatocytes, their levels remained constant following the last media change. In contrast, nuclear concentrations of HNF-4 α and HNF-6 exhibited a consistent increase for at least 3 hours following the final media change with “fresh” GH (Fig. 6). At all time points measured, nuclear HNF-4 α and HNF-6 levels were always greater ($P<0.01$) in female hepatocytes; though more so for HNF-6 (1.6:1, F:M) than HNF-4 α (1.2:1, F:M). Thus, the findings indicate that continuous GH is required for both high cellular expression of the nuclear factors as well as their activation and nuclear translocation; though with a significantly greater response in females than males.

Gender-Regulated Acetylation of HNF-4 α and HNF-6 under Continuous GH

The stability and transcriptional activity of HNF-4 α and HNF-6 is dependent upon their functional association with CBP whose acetyl transferase activity has been shown to acetylate (i.e., activate) lysine residues on human HNF-4 α and HNF-6 (Rausa et al., 2004; Soutoglou et al., 2000; Yoshida et al., 1997) upon GH treatment (Chen et al., 1999). [The near identical sequence homologies of HNF-4 α and HNF-6 in the human to that of the rat and mouse (Fig. 4) suggest that the activation of both hepatocyte nuclear factors by CBP acetylation of lysine residues reported in the human proteins is likely the case for rodents.] In the absence of GH (i.e., freshly isolated hepatocytes from longterm hypophysectomized rats), whole cell concentrations of acetyl-HNF-4 α and acetyl-HNF-6 were very low, though sexually dimorphic (F>M) (Fig. 7). The final change of media with “fresh” GH immediately stimulated an increased accumulation of both acetyl-HNF-4 α and acetyl-HNF-6 in males and females that peaked at 180 min ($P<0.05$ from time point to following time point) and declined thereafter. At all time points measured, GH-exposed female hepatocytes contained greater concentrations of both activated nuclear factors than male hepatocytes, with the magnitude of the dimorphism greater for acetyl-HNF-6. In fact, the response curves of cellular acetyl-HNF-4 α and HNF-6 (Fig. 7) were very similar to that of nuclear HNF-4 α and HNF-6 (Fig. 6), suggesting that the measured nuclear levels of the HNF proteins were likely acetylated.

Sexually Dimorphic Binding of HNF-4 α to the CYP2C12 Promoter under Continuous GH

Transcriptional activation of CYP2C12 appears to be mediated, at least in part, by continuous GH activation of several hepatocyte nuclear factors (Lahuna et al., 1997; Endo et al., 2005; Waxman and O'Connor, 2006). Accordingly, we examined the binding kinetics of HNF-4 α to the CYP2C12 promoter by ChIP assay. Although substantial levels of HNF-4 α were translocated to the nucleus within the first 45 min following the change in media (Fig. 6), we observed during this time no accompanying increase in HNF-4 α binding to the putative promoter (Fig. 8). Following this 45 min lag period, however, binding increased through 180 min, declining thereafter. At all measured time points, including the first 45 min of presumed baseline, HNF-4 α binding to the CYP2C12 promoter was significantly greater in hepatocytes derived from females. During the period of significant elevation in binding (i.e., post 45 min), HNF-4 α binding to the promoter was generally 50% greater in female cells. PCR amplification of a negative (Neg) control using primers of a non-HNF-4 α binding region of the rat G6PDH

promoter demonstrated no measurable nonspecific binding (Fig. 8). In confirmation, using Southern blotting, we observed ~50% more of the presumptive HNF-4 α binding motif of the CYP2C12 promoter bound to the hepatocyte nuclear factor in cells from female rats (Fig. 8). In fact, the response curves for the ChIP assay and the Southern blot were nearly superimposable.

Sexually Dimorphic Binding of HNF-6 to the CYP2C12 Promoter under Continuous GH

In addition to HNF-4 α , HNF-6 has been implicated in continuous GH regulation to CYP2C12 expression (Lahuna et al., 1997; Endo et al., 2005; Waxman and O'Connor, 2006). In this regard, we found a similar binding response of HNF-6 as that of HNF-4 α (Fig. 8), though to different sites on the CYP2C12 promoter, during exposure to continuous GH. An exception being that the magnitude of the sex difference was greater for HNF-6 binding (Fig. 9). Otherwise, like HNF-4 α , there were substantial levels of HNF-6 translocated to the nucleus within the first 45 min following the change in media (Fig. 6), but no accompanying increase in HNF-6 binding to the putative promoter (Fig. 9). Following this 45 min lag period, however, binding increased through 180 min, declining thereafter. At all measured time points, including the first 45 unresponsive minutes, HNF-6 binding to the CYP2C12 promoter was significantly greater in hepatocytes derived from females. In fact, during the period of maximum sex difference, ~180 min, HNF-6 binding to the promoter was nearly 100% greater in female cells. PCR amplification of a negative (Neg) control using primers of a non-HNF-6 binding region of the G6PDH promoter demonstrated no measurable nonspecific binding (Fig. 9). In confirmation using Southern blotting, we observed a maximum twice as much of the presumptive HNF-6-binding motif of the CYP2C12 promoter bound to the hepatocyte nuclear factor in cells from female rats (Fig. 9). In fact, the response curves for the ChIP assay and the Southern blot were nearly superimposable.

DISCUSSION

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 (Bengtsson, 1999). Not surprisingly then, GH actions (generally derived from in vitro, single pulse or brief GH exposure studies) are mediated at the cellular level by recruiting and/or activating a variety of signaling molecules, including MAPK (i.e., p42/p44 MAPK, Erk1 and Erk2), insulin receptor substrates, phosphatidylinositol 3'-phosphate kinase, HNF proteins, diacylglycerol, protein kinase C, Stat proteins, and intracellular calcium (Carter-Su et al., 1996; Waxman and O'Connor, 2006).

A possible artifact of in vitro studies examining prolonged GH exposure is seen in our observation that despite the continuous treatment of primary hepatocytes to rat GH, there occurred an enhanced, but transient activation of downstream responses (e.g., phosphorylation of Erk1, Erk2 and CBP, acetylation of HNF-4 α and HNF-6) shortly following the change in media containing fresh hormone. A similar transient activation event involving Ras, Raf and MEK was also reported in cells cultured in the continuous presence of GH (VanderKuur et al., 1997). Although our cultures contained what could be considered very low in vivo levels of GH [reflecting ~6% of the mean circulating concentration in female rats (Pampori and Shapiro, 1996)], it seems unlikely that the hormone was ever completely metabolized. The continuous exposure of liver cells to GH is obligatory for the expression of CYP2C12. The interruption of GH secretion in the continuous profile for periods of as little as 60 min or less completely prevents CYP2C12 expression (Agrawal and Shapiro, 2001). Accordingly, the induction of normal, in vivo-like levels of CYP2C12 in the female cell cultures indicates the continuous presence of biologically active GH. In this regard, the complete suppression of CYP2C11 (data not presented) is additional evidence that the cells were exposed to continuous GH levels

(Pampori and Shapiro, 1996). Most likely, as discussed previously (Thangavel et al., 2004), GH concentrations may have declined during 12h in culture, but were sufficient to maintain signaling pathways regulating CYP2C12 expression². However, the replacement of the media with fresh GH every 12 h may have increased its concentration gradient enough to boost the activation levels in signaling pathways.

The continuous exposure of female hepatocytes to GH maintained baseline concentrations of phospho-Erk1 and Erk2 at levels several fold higher than that found in female hepatocytes deprived of the hormone. In addition, the GH-treated cells experienced a media-replenished increase in the concentration of activated Erks that were sustained for at least 75 min. Although we did not determine whether the activation of p42/p44 MAPK by GH does (Winston and Hunter, 1995; Kelly et al., 2001) or does not (VanderKuur et al., 1997) require Jak2, it surely involves the GHR. In this regard, the continuous presence of GH was responsible for maintaining the relatively high cellular levels of both GHR_L and GHR_S [the latter being the immediate precursor of the secreted GH binding protein (Frick et al., 1998)]. In agreement, in vivo studies have reported very low levels of hepatic GHR mRNA in hypophysectomized rats that were increased up to 10-fold following continuous GH treatment, but were considerably less responsive to episodic GH treatment (Ahlgren et al., 1995). It may be that the constant exposure of the GHR to GH in females elevates receptor levels and its constant occupation by the hormone, signals the initial activation of p42/p44 MAPK and the subsequent transcription of CYP2C12. In contrast, the intermittent occupation of the membrane receptor by the masculine episodic GH profile activates the Jak2/Stat5B pathway leading to CYP2C11 expression (Waxman and O'Connor, 2006).

The next step in the proposed transduction pathway involves the nuclear coactivator CBP whose levels we found to be independent of GH status. In agreement with previous reports, though not involving GH (Liu et al., 1999; Gusterson et al., 2002), we found that activated Erk1 and Erk2 translocate to the nucleus where they bind to CBP, followed by a dramatic elevation in phospho-CBP. The phosphorylation of CBP activates its inherent acetyl transferase activity (Ait-Si-Ali et al., 1999). Activated CBP can acetylate lysine residues on HNF-4 (Yoshida et al., 1997; Soutoglou et al., 2000) and HNF-6 (Rausa et al., 2004). Acetylation of the HNF proteins is crucial for their proper nuclear retention as well as enhancing their DNA binding activity (Soutoglou et al., 2000; Rausa et al., 2004). From our studies, it is clear that exposure to continuous GH was responsible for inducing near constant elevated levels of HNF-4 α and HNF-6 as determined in whole cell extracts. In contrast, when measured in nuclei, replacement of the media was quickly followed by a transient increase in the HNF proteins characterized by broad peaks (~2 to 3 hr). In agreement with the known behavior of HNF proteins, our findings indicate that nuclear HNF-4 α and HNF-6 were acetylated. Once these proteins are activated (i.e., acetylated) in the nucleus, they can bind to DNA and participate in gene transcription. In fact, both HNF-4 α and HNF-6 have been reported to mediate continuous GH induction of CYP2C12 transcription by binding and subsequently activating the CYP2C12 promoter (Lahuna et al., 1997; Endo et al., 2005; Waxman and O'Connor, 2006). In agreement, we found under the influence of continuous GH, persistent, and occasionally elevated (due to media replacement) binding levels of HNF-4 α and HNF-6, each to a different site on the CYP2C12 promoter. Since there were barely detectable levels of activated HNF-4 α and HNF-6 in cells devoid of GH stimulation, it seems reasonable to assume that there was similarly inconsequential, if any, binding of the factors to the CYP2C12 promoter in these control cells.

In brief, our findings suggest that continuous GH induces CYP2C12 expression by activating Erk1 and Erk2 via the GHR, which in turn activates nuclear CBP, which acetylates HNF-4 α

²Continuous exposure of female rat primary hepatocytes to as little as 0.2 ng/ml of GH was sufficient to both induce and maintain CYP2C12 mRNA and protein at 40 to 60% of levels induced by a hormone concentration 10-times higher (Thangavel et al., 2004).

and HNF-6 which then bind to the CYP2C12 promoter, contributing to the gene's transcription. While this proposed pathway includes several elements not previously identified in continuous GH induction of CYP2C12 (e.g., Erk1, Erk2 and CBP), it certainly does not preclude other factors, some of which might be associated with different signaling pathways regulating CYP2C12 expression. The occurrence of multiple or redundant pathways is not uncommon in biological systems and could explain contradictory findings regarding mechanisms of CYP expression (Verma et al., 2005).

Lastly, we observed intrinsic sexually dimorphic responses to continuous GH exposure. In agreement with our earlier in vivo (Pampori and Shapiro, 1999) and in vitro (Thangavel et al., 2004) studies, we found that female hepatocytes were considerably (4 to 5 fold) more responsive to continuous GH induction of CYP2C12 than were male-derived hepatocytes. In fact, constituent levels of all the measured factors (i.e., GHR_L, GHR_S, phosphorylated Erk1 and Erk2, total nuclear CBP, phospho-Erk1:CBP, phospho Erk2:CBP, nuclear phospho-CBP, total and nuclear HNF-4 α and HNF-6 and acetylated HNF-4 α and HNF-6) in freshly isolated hepatocytes from hypophysectomized rats (i.e., deprived of GH exposure) as well as continuous hormone-treated hepatocytes, were all significantly higher in female cells suggesting an inherent or imprinted sexual dimorphism. Previous studies have also reported higher hepatic concentrations of GHR_L and GHR_S (Ahlgren et al., 1995; Frick et al., 1998), HNF-6 (Lahuna et al., 1997) and HNF-4 α (Wauthier et al., 2006) in females, but these sexual dimorphisms, measured in intact animals, were attributed to the reversible (and not intrinsic) effects of the circulating sex-dependent GH profiles.

For all measured parameters, we observed a striking sexually dimorphic response (F>M) immediately following the replacement of media with fresh GH. Although, the GH-stimulated "pattern of response" of GHR, Erk phosphorylation, Erk:CBP binding, CBP phosphorylation, HNF acetylation and nuclear translocation, and HNF binding to the CYP2C12 promoter was actually indistinguishable in male and female hepatocytes, the magnitude of the response was always greater in the female cells.

Perhaps of greater importance than the transient response to the change in media, were the sex differences in the baseline levels of the signal transducers and nuclear factors maintained by continuous GH exposure. After all, these transient responses occurred for only a brief time during the 12 h period (the media was replaced twice per day). Whereas the induction of hepatic CYP2C12 is highly tolerant to the concentration of GH, i.e., normal, subphysiologic and even nominal levels are equally effective in vivo and in vitro (Pampori and Shapiro, 1996; 1999; Thangavel et al., 2004), continuous exposure to the hormone is obligatory (Legraverend et al., 1992; Shapiro et al., 1995). It is reasonable to assume then, that the continuous presence of the hormone is responsible for the constant activation of the signal transduction pathway(s) required for CYP2C12 expression. Not only were the concentrations of signal transducers and nuclear factors immediately following the media change much higher in female hepatocytes, but more significantly their prevailing baseline levels, even at its minimum after 12 h (zero time on the figures), were several-fold higher in females. In fact, while the baseline levels of signaling molecules in hormone-treated male cells approached the ineffectual concentrations found in control cells not exposed to GH, the baseline levels in GH-treated female cells remained several times higher than that found in female control cells. It is possible that male hepatocytes are unable to respond to the same levels of continuous GH as that presented to female cells with a persistent activation of the signaling pathway at levels sufficient for

optimum CYP2C12 expression³. Genetic or imprinting effects may have permanently reduced the ability of the male liver to respond to the feminine GH profile (Shapiro, 2004).

Our finding of intrinsic, irreversible sex differences in response to GH may have some clinical relevance. GH deficiency causes numerous abnormalities in growth rates; lean body mass; cardiovascular, bone, adipose and muscle function; protein, carbohydrate, lipid and electrolyte metabolism; and expression levels of hepatic IGF-1, IGF binding protein, GH-binding protein and cytochrome P450-dependent drug metabolizing enzymes. However, hormone replacement therapy has clearly demonstrated an intrinsic, irreversible, sexually dimorphic response in which the effectiveness of the same GH treatment differs in men and women (see Thangavel and Shapiro, 2007). The present finding offers one possible explanation for this clinical observation.

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Glossary

CBP, CREB binding protein
ChIP, chromatin immunoprecipitation
CREB, cAMP response element-binding protein
DMEM, Dulbecco's modified Eagle's medium
Erk, extracellular signal-regulated kinase
GH, growth hormone
GHR, growth hormone receptor
HNF, hepatocyte nuclear factor
IB, immunoblotting
IGF, insulin-like growth factor
IP, immunoprecipitation
Jak, Janus kinase
MAPK, mitogen-activated protein kinase
P450, cytochrome P450
Stat, signal transducer and activator of transcription

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³While it is possible that male hepatocytes metabolize GH more quickly than female hepatocytes, this is certainly not the case in vivo (Pampori and Shapiro, 1999). Moreover, increasing the concentration of GH in the media by 10-fold to 20ng/ml had no significant effect on the sexually dimorphic ratio of induced CYP2C12 (unpublished observation).

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Fig. 1.

Sex-dependent expression levels of CYP2C12 protein in hepatocyte cultures derived from male and female hypophysectomized rats exposed to continuous (femalelike) recombinant rat GH or its diluent for 5 days (5d). Control values were determined using freshly isolated (ZERO TIME) primary hepatocytes from male and female hypophysectomized and intact rats. Values are presented as a percentage of CYP2C12 in hepatocytes from intact female rats at zero time arbitrarily designated 100%. Each data point is a mean $\pm$ SD for cells from five or more rats. ND, not detectable. * $P < 0.01$ when compared to intact females at zero time.

Fig. 2.

Sex-regulated expression levels of GHR long form (IB: anti-GHR_L) and GHR short form (IB: anti-GHR_S) in whole cell extracts of cultured primary hepatocytes from hypophysectomized male and female rats exposed to continuous (female-like) rat GH for 5 days. Pre-induction values were determined using freshly isolated (Pre-Plating Control) hepatocytes from hypophysectomized male and female rats. Hepatocytes were harvested and analyzed at different time points between 0-240 min after the final media change. Sufficient viable cells were isolated from each of ≥ 5 livers for GHR_L and GHR_S protein determinations at every time point presented in the figure. Each data point is a mean $\pm$ SD for cells from five or more rats. *P<0.01 compares females to males at the same time point. Absolute values should not be

compared between panels. Representative immunoblots of GHR_L, GHR_S and their respective loading control (actin) are presented in the bottom panel.

Fig. 3.

Sex-dependent expression levels of phospho-Erk1 and phospho-Erk2 (IB: antiphospho-Erk) in whole cell extracts of cultured primary hepatocytes from hypophysectomized male and female rats exposed to continuous (female-like) rat GH for 5 days. Pre-induction levels were determined using freshly isolated hepatocytes (Pre-Plating Control) from hypophysectomized male and female rats. Cells were harvested and analyzed at different time points between 0-240 min after the last media change. Sufficient viable cells were isolated from each of ≥ 5 livers for all determinations at every time point presented in the figure. Each data point is a mean $\pm$ SD for cells from five or more rats. * $P < 0.01$ compares females to males at the same time point. Absolute values should not be compared between panels. Representative immunoblots of

phospho-Erk1, phospho-Erk2 and their respective loading control (actin) are presented in the bottom panel.

Presumptive Homology of HNF-4 α Residues Acetylated by CBP

94 121
VVDKDKRNQCRYCRLKKCFRAGMKKEAV Rat
VVDKDKRNQCRYCRLKKCFRAGMKKEAV Human
103 130
VVDKDKRNQCRYCRLKKCFRAGMKKEAV Mouse

Presumptive Homology of HNF-6 Residues Acetylated by CBP

321 339 350
CRSQGTLSDLLRNPKPWSKLKSGRETFRM Rat
CRSQGTLSDLLRNPKPWSKLKSGRETFRM Human
CRSQGTLSDLLRNPKPWSKLKSGRETFRM Mouse

Fig. 4.

Sexually dimorphic expression of nuclear protein CBP, (IP: anti-CBP, IB: anti-CBP) in primary hepatocyte cultures derived from male and female hypophysectomized rats exposed to continuous (female-like) rat GH for 5 days. Pre-induction levels were determined using freshly isolated hepatocytes (Pre-Plating Control) from hypophysectomized male and female rats. Hepatocytes were harvested and analyzed at different time points between 0-240 min after the final media change. Sufficient viable cells were isolated from each of ≥ 5 livers for all determinations at every time point presented in the figure. Each data point is a mean $\pm$ SD for cells from five or more rats. * $P < 0.01$ compares females to males at the same time point. Absolute values should not be compared between panels. A representative immunoblot of

nuclear CBP is included in the middle panel. The presumptive homology of rat, mouse and human HNF-4 α and HNF-6 aligned at their lysine (K) residues known, in the human, to be acetylated by CBP (bottom panel).

Fig. 5.

Sex-dependent binding of phospho-Erk1 and phospho-Erk2 with CBP (IP: anti-CBP, IB: anti-phospho-Erk) and resulting levels of activated nuclear phospho-CBP (IP: anti-CBP, IB: anti-phospho-tyrosine) in the cultures of primary hepatocytes from male and female hypophysectomized rats exposed to continuous (female-like) rat GH for 5 days. Pre-induction levels were determined using freshly isolated hepatocytes (Pre-Plating Control) from male and female hypophysectomized rats. Hepatocytes were harvested and analyzed at different time points between 0-240 min after the final media change. Sufficient viable cells were isolated from each of ≥ 5 livers for all determinations at every time point presented in the figure. Each data point is a mean $\pm$ SD for cells from five or more rats. * $P < 0.01$ compares females to males at the same time point. Absolute values should not be compared between panels. A representative immunoblot of the phospho-Erk:CBP nuclear complexes and nuclear phospho-CBP are presented in the bottom panels.

Fig. 6.

Sexually dimorphic expression levels of whole cell and nuclear HNF-4 α (IB: anti-HNF-4 α) and HNF-6 (IB: anti-HNF-6) in cultures of primary hepatocytes from hypophysectomized male and female rats exposed to continuous (female-like) rat GH for 5 days in culture. Pre-induction levels were determined using freshly isolated hepatocytes (Pre-Plating Control) from hypophysectomized male and female rats. Cells were harvested and analyzed at different time points between 0-240 min after the last media change. Sufficient viable cells and nuclei were isolated from each of ≥ 5 livers for all determinations at every time point presented in the figure. Each data point is a mean $\pm$ SD for cells from five or more rats. * $P < 0.01$ compares females to males at the same time point. Absolute values should not be compared between panels.

Representative immunoblots of whole cell and nuclear HNF-4 α and HNF-6 and their respective loading control (actin) are presented in the bottom panel.

Fig. 7.

Sexually dimorphic expression levels of acetyl-HNF-4 α (IP: anti-HNF-4 α , IB: antiacetyl-lysine) and acetyl-HNF-6 (IP: anti-HNF-6, IB: anti-acetyl-lysine) in cultures of primary hepatocytes from hypophysectomized male and female rats exposed to continuous (female-like) rat GH for 5 days in culture. Pre-induction values were determined using freshly isolated hepatocytes (Pre-Plating Control) from male and female hypophysectomized rats. Hepatocytes were harvested and analyzed at different time points between 0-240 min after the last media change. Sufficient viable cells were isolated from each of ≥ 5 livers for all determinations at every time point presented in the figure. Each data point is a mean $\pm$ SD for cells from five or more rats. * $P < 0.01$ compares females to males at the same time point. Absolute values should

not be compared between panels. Representative immunoblots of acetyl-HNF-4 α and acetyl-HNF-6 are presented in the bottom panel.

Fig. 8.

Sexually dimorphic binding kinetics of HNF-4 α to the CYP2C12 putative promoter (PCR results for the ChIP assay, left) and the presumptive occupied HNF-4 α -binding motif in the CYP2C12 promoter (Southern blot, right) as well as representative ChIP and Southern blots determined in cultured primary hepatocytes from male and female hypophysectomized rats exposed to continuous (female-like) rat GH for 5 days. Hepatocytes were harvested and analyzed at different time points between 0-240 min after the last media change. Whereas negative (Neg) inputs (no DNA) and negative controls (G6PDH) were performed at every time point (never resulting in detectable bands), the figure includes only a representative finding. Sufficient viable cells were isolated from each of ≥ 5 livers for both the ChIP assay and Southern blotting determinations for every time point presented in the figure. Each data point is a mean $\pm$ SD for cells from five or more rats. * $P < 0.01$ compares females to males at the same time point. Absolute values should not be compared between panels.

Fig. 9.

Sexually dimorphic binding kinetics of HNF-6 to the CYP2C12 putative promoter (PCR results for the ChIP assay, left) and the presumptive occupied HNF-6-binding motif in the CYP2C12 promoter (Southern blot, right) as well as representative ChIP and Southern blots determined in cultured primary hepatocytes from male and female hypophysectomized rats exposed to continuous (female-like) rat GH for 5 days. Hepatocytes were harvested and analyzed at different time points between 0-240 min after the last media change. Whereas negative (Neg) inputs (no DNA) and negative controls (G6PDH) were performed at every time point (never resulting in detectable bands), the figure includes only a representative finding. Sufficient viable cells were isolated from each of ≥ 5 livers for both the ChIP assay and Southern blotting determinations for every time point presented in the figure. Each data point is a mean $\pm$ SD for cells from five or more rats. * $P < 0.01$ compares females to males at the same time point. Absolute values should not be compared between panels.