INDUCIBILITY OF MALE-SPECIFIC ISOFORMS OF CYTOCHROME P450 BY SEX-DEPENDENT GROWTH HORMONE PROFILES IN HEPATOCYTE CULTURES FROM MALE BUT NOT FEMALE RATS

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Running Title: Sex-dependent induction of male P450s in hepatocyte culture

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ABSTRACT

Although in vivo expression levels of the male-specific hepatic isoforms of P450 (CYP2C11, CYP2C13, CYP2A2 and CYP3A2) are determined by the episodic growth hormone profile secreted by male rats, these isoforms have been completely refractory to growth hormone regulation in hepatocyte culture. By using species specific rat growth hormone, at subphysiologic in vivo concentrations administered in two daily episodic pulses, we successfully induced CYP2C11 and CYP2A2 to near normal concentrations. Whereas inductive levels of CYP2C13 were subnormal, CYP3A2 was unresponsive to all hormonal treatments; quickly declining to undetectable concentrations. In agreement with in vivo findings, we observed that induction levels of the isoforms were always greatest when the male hepatocytes were exposed to the masculine-like episodic growth hormone profile, and least stimulated by the continuous feminine-like hormone profile. When administered alone, dexamethasone consistently increased isoform levels. However, when administered with growth hormone, the glucocorticoid was always antagonistic, suppressing growth hormone-induction of CYP2C11, CYP2C13 and CYP2A2. Lastly, the P450 isoforms were completely unresponsive to all treatments when the hepatocytes were derived from female rats supporting earlier findings that expression levels of sexually dimorphic P450 isoforms are inherently irreversible between sexes.

Circulating growth hormone profiles in rats as well as other species have been shown to be sexually dimorphic (Shapiro et al., 1995). Male rats secrete growth hormone in episodic bursts (~200-300 ng/ml of plasma) every 3.5-4 h. Between the peaks, growth hormone 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 growth hormone are always measurable (Edén et al., 1987; Shapiro et al., 1995). These sex differences in the circulating growth hormone profiles, and not sexual differences in growth hormone concentrations, per se, are responsible for observed sexual dimorphisms ranging from body growth to the expression of hepatic enzymes (Legraverend et al., 1992; Shapiro et al., 1995). In this regard, rat, as well as murine liver each contain at least a dozen sex-dependent isoforms of cytochrome P450 (P450, CYP) that are regulated by the sex-dependent profiles of circulating growth hormone (Legraverend et al., 1992; Shapiro et al., 1995).

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 female-predominant P450s found in both sexes, but at higher levels in females. Essentially, there are four major male-specific isoforms in rat liver; CYP2C11, CYP2C13, CYP2A2 and CYP3A2. Expression of the major male-specific CYP2C11 comprising >50% of the total hepatic pool of P450 in male rats (Morgan et al., 1985) requires the episodic "on/off" masculine profile of growth hormone secretion. Although the feminine pattern of continuous hormone secretion blocks CYP2C11 expression, total

growth hormone depletion from the circulation allows CYP2C11 expression at ~30% of intact male levels (Morgan et al., 1985; Pampori and Shapiro, 1996; Agrawal and Shapiro, 2000). Although the expression levels of CYP2C11 are greatest when exposed to their sex-dependent growth hormone profiles, other isoforms are optimally expressed in the absence of growth hormone. Male-specific CYP2A2 and CYP3A2 are maximally expressed in the hypophysectomized rat, disappear when growth hormone is secreted constantly, but are only minimally suppressed under the influence of episodic growth hormone (Waxman et al., 1988; Pampori and Shapiro, 1996; Agrawal and Shapiro, 2000). Male-specific CYP2C13 is optimally expressed when exposed to the masculine hormone profile or under conditions of no growth hormone, whereas the feminine growth hormone profile completely suppresses CYP2C13 (Legraverend et al., 1992; Pampori and Shapiro, 1996; Agrawal and Shapiro, 2000)

Although the differential effects of the growth hormone profiles regulating male-specific P450s have all been determined in vivo, the system is complex making it an unattractive model in which to "dissect out" transduction factors mediating growth hormone regulation of P450 expression (Tannenbaum et al., 2001; Verma et al., 2005). Accordingly, investigators examining the mechanisms of growth hormone action have generally used cell cultures (Carter-Su et al., 1996; Herrington et al., 2000). Often non-hepatocytes such as COS and CHO cells that do not express the growth hormone receptor are cotransfected with truncated receptor chimeric fusion proteins and transcription factors (Yi et al.,

1996; Pircher et al., 1997). Not only do these models raise concerns about the physiologic relevance of the findings, but the cells can neither differentiate the sexually dimorphic growth hormone profiles nor express P450s. Other studies have used transformed hepatocyte cell lines (Schuetz et al., 1993; Tollet et al., 1995; Gebert et al., 1999). While these cells may or may not express the growth hormone receptor and some downstream signaling molecules, they express few (an occasional fetal form), if any P450s. In addition, when present, the signal transduction pathways may not behave as in vivo nor do the cells respond normally to typical inducing agents (Schuetz et al., 1993; Tollet et al., 1995; Gebert et al., 1999). For these reasons, studies examining mechanisms regulating expression of the inducible isoforms (eg., CYP1A1/2, CYP2B1/2) are conducted on primary hepatocytes (Schuetz et al., 1990; Davila et al., 1999). Unfortunately, the four male-specific isoforms of P450 are quickly lost in primary hepatocyte cultures, and attempts to induce their expression with growth hormone have failed (Guzelian et al., 1988; Legraverend et al., 1992; Liddle et al., 1992; Kocarek et al., 1993; Davila et al., 1999). However, these earlier studies did not use species specific growth hormone, physiologic concentrations or profiles in their experiments. Accordingly, in the present study, we have attempted to stimulate induction of the male-specific P450s in primary rat hepatocytes of both sexes by renaturalizing the physiologic-like growth hormone profiles using rat hormone in the culture media.

MATERIALS AND METHODS

Animals. Animals 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 [Crl: CD (SD)BR] were hypophysectomized by the vendor (Charles River Laboratories, Wilmington, MA) at 8 weeks of age and observed in our facility for 5 to 6 weeks. 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.

Hepatocyte Isolation and Cell Culture. Isolation of rat hepatocytes was performed with minor modifications (Thangavel et al., 2004) by in situ perfusion of collagenase through the portal vein of anesthetized rats (Seglen, 1976). In brief, initial perfusion with a calcium-free buffer was followed by a solution of collagenase (0.05% w/v). The "softened" liver was excised, and the hepatocytes were separated from connective tissue by filtering through 100-μm macroporous filters (Spectrum Co., Laguna Hills, CA) and from nonparenchymal cells by repeated low speed centrifugation in wash medium: high glucose DMEM (4.5 g/l) containing streptomycin (100 μg/ml), penicillin (100 U/ml), gentamycin (50 μg/ml), fungizone (0.25 μg/ml), Hepes (15 mM), sodium pyruvate (5 mM), and fetal bovine serum (5%). The cell pellet was suspended in wash medium and mixed with an equal volume of Percoll density media, buffered with phosphate-buffered

saline, and centrifuged (4°C) for 10 min at 50 x g. The pellet was washed three times with wash media before cell counting. The viability of the initial cell suspension of hepatocytes was typically between 80 and 90% (with trypan blue).

The hepatocytes were plated in the wash media at a density of $2x10^6$ viable cells per well in 6 well plates 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 $\mu g/ml$), penicillin (100 U/ml), glutamine (2 mM), Hepes (15 mM), insulin (10 $\mu g/ml$), bovine transferrine (10 $\mu g/ml$), Na_2SeO_3 (10 ng/ml), aminolevulinic acid (2 $\mu g/ml$), glucose (25 mM), linoleic acid-albumin (0.5 mg/ml), and sodium pyruvate (5 mM). The cultures were also supplemented with the fungizone (0.25 $\mu g/ml$) 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. For reasons discussed below, the medium was changed twice a day and cells were harvested after 2, 4, 6 and/or 9 days in culture.

Hormonal Conditions. Whereas all cells were continually exposed to insulin, the dexamethasone and growth hormone treatments varied. Accordingly, some hepatocytes were exposed to neither dexamethasone nor growth hormone. Some wells were treated with only dexamethasone (10^{-8} M) at a concentration generally regarded as optimum for P450 expression (Iber et al., 1997). In other wells, rat growth hormone (2 ng/ml or 0.2 ng/ml) purchased from the National Hormone and Peptide Program (Torrance, CA) was continuously present. Moreover, hepatocytes exposed to continuous rat growth hormone were also

treated with either dexamethasone (10^{-8} M) or the steroid vehicle (0.004 μl ethanol/ml). Lastly, some cells were exposed to episodic rat growth hormone (2 ng/ml, 0.2 ng/ml, 0.02 ng/ml or 0.002ng/ml) for 30 min followed by two careful washings with growth hormone-free media that remained in the wells for 11.5 h. In addition, hepatocytes treated with episodic growth hormone were continuously exposed to either dexamethasone (10^{-8} M) or the steroid vehicle. To iterate, the media in all wells, whether or not they received episodic growth hormone, were changed every 12 h and all cells were continuously exposed to either dexamethasone or its diluent.

Isolation of Total Cellular RNA and Northern Blot Analysis. Hepatocyte cultures were washed with ice-cold phosphate-buffered saline containing 5 mM EDTA. Cells were then removed from the culture dishes with a cell scraper, transferred to tubes, and placed on ice for approximately 1 h to dissolve   the matrigel. After sedimentation and centrifugation (1,000 x g for 5 min at 4°C), the cell pellets were kept at -70°C until extraction of RNA. According to the vendor's protocol, cells were lysed in TRIzol (Life Technologies, Inc., Grand Island, NY) by several passages through a Pasteur pipette. Chloroform was added and mixed vigorously, followed by centrifugation.   RNA in the aqueous phase was precipitated with isopropanol and washed with 75% ethanol. RNA pellets were dissolved in Tris-EDTA buffer. RNA concentration and purity were assessed by absorbance at 260 and 260/280 nm, respectively. RNA samples (10 μg) were resolved on denaturing 1% agarose gels and transferred on to Nytran N filters from Schleicher and Schuell (Keene, NH). The northern blots were probed and

reprobed with γ-^{32}P-labeled oligonucleotide probes, using hybridization and high stringency washing conditions as described previously (Pampori and Shapiro, 1996). The sequence of oligonucleotide probes for CYP2A2, CYP2C11, CYP2C13 (Waxman, 1991) and CYP3A2 (Ram and Waxman, 1991) have been reported. 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 (Ramsden et al., 1993). The hybridized mRNA signals were quantified by scanning the autoradiographs with a FluorChem^{TM} IS-8800 Imager (Alpha Innotech, San Leandro, CA). The mRNA signals were normalized to the 18S rRNA signals in each lane and demonstrated a mean variation of only ± 6% indicating results were independent of loading errors.

Preparation of Whole Cell Lysate and Immunoblot Analysis. Whole cell lysate was extracted from cultured primary hepatocytes and the protein concentration of the cell lysate was measured by using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules,CA). Briefly, 25 μg or 75 μg of whole cell lysate protein was electrophoresed on 0.75-mm-thick sodium dodecyl sulfate-polyacrylamide (7.5%) gels and electroblotted on to nitrocellulose membranes. The blots were probed with monoclonal anti-rat CYP2C11 (Oxford Biomedical Research, Oxford, MI), monoclonal anti-rat CYP2C12/13 (kindly provided by Dr. Marika Rönnholm, Huddinge University Hospital, Huddinge, Sweden), polyclonal anti-rat CYP2A1/2 (kindly provided by Dr. Susumu Imaoka, Oska City University Medical School, Osaka, Japan), and polyclonal anti-rat

CYP3A1/2 (Gentest, Woburn, MA), and detected with SuperSignal® West Pico chemiluminescent substrate (Pierce, Rockford, IL,). Signals of the relative protein levels were quantitated by using a FluorChem™ IS-8800 Imager (Alpha Innotech, San Leandro, CA). Signals were normalized to a control sample repeatedly run on all blots.

The specificity of the antibodies has been discussed elsewhere (Pampori and Shapiro, 1996). Briefly, antibody against CYP2C11 has been found to be highly specific with no detectable cross-reactivities with known P450s. Antibody made against CYP2C12 strongly reacts with CYP2C13 protein. However, not only is CYP2C13 a male specific isoform (in contrast to the female-specificity of CYP2C12), but its location on the blot is easily distinguished from CYP2C12. The polyclonal anti-rat CYP2A1 strongly reacts with CYP2A2 protein, but its location on the blot is easily distinguished from CYP2A2. Although CYP3A1 and CYP3A2 proteins are recognized by the anti-rat CYP3A1/2, the former is basically an inducible, growth hormone-independent isoform only marginally expressed constitutively, whereas the latter is a major male-specific isoform.

Statistics. All data were subjected to ANOVA, and differences were determined with t statistics and the Bonferroni procedure for multiple comparison.

RESULTS

In contrast to other reports in which intact rats are the customary source of primary hepatocytes (e.g., Guzelian et al., 1988; Schuetz et al., 1990; Liddle et al., 1992; Kocarek et al., 1993; Iber et al., 1997, Davila et al., 1999), we chose to isolate hepatocytes from hypophysectomized rats. We wanted to avoid the often uncertain effects produced when exposure to hormones or inducing agents increase P450 levels when compared to their diluent-treated controls but nevertheless remain subnormal. (That is, below "zero time" P450 levels which are determined immediately following hepatocyte isolation.) Is the treatment actually increasing P450 transcription/translation or simply reducing turnover rates of the isoforms? In this regard, expression of all the constituent isoforms examined in the present study are regulated, to varying degrees, by growth hormone (Pampori and Shapiro, 1996; Agrawal and Shapiro, 2000, 2001). Thus at zero time each P450 is expressed at its baseline level and any hormone-induced changes are actually a result of altered transcription/translation.

Another consideration regards the use of cell culture to obtain information relevant to the in vivo condition. Although the use of primary hepatocytes offers the advantage of identifying individual factors regulating P450 expression in an isolated system, it is hardly physiologic. In response, we attempted to ameliorate this disadvantage by using physiologic-like concentrations of hormones in our media. We realized that because of radical differences in metabolism (eg, biotransformation, clearance, excretion), it is not possible to translate normal circulating hormone levels into equivalent in vitro doses, but we did base the

selected hormone concentrations in the hepatocyte cultures on physiologic levels. When comparing dexamethasone's biological activity (eg, gluconeogenic, glyconeolytic) to corticosterone, the predominant glucocorticoid in rodents, the present level (10 nM) falls within the range of resting plasma concentrations of the natural steroid in the rat (Loeb et al., 1999) and is the most effective in vitro concentration for maintaining rat P450s (Iber et al., 1997).

Choosing a physiologic-like concentration of growth hormone to be applied in vitro was more complicated. Whereas the glucocorticoid is secreted in a circadian rhythm (Loeb et al., 1999), growth hormone secretion is ultradian (Shapiro et al., 1995). Thus, at any time within a 24 hr period, plasma growth hormone concentrations may vary from 5 to 10 ng/ml to 100 ng/ml in female rat and from undetectable to about 300 ng/ml in males (Pampori and Shapiro, 1996; Agrawal and Shapiro, 2000). In response, we chose a maximum in vitro concentration of 2 ng/ml. This concentration reflects the K_D for the growth hormone/growth hormone receptor complex (Fuh et al., 1992) and has effectively induced female-specific and –predominant P450 isoforms in primary rat hepatocyte cultures (Thangavel et al., 2004). Next, in order to replicate the sex-dependent growth hormone profiles in vitro, the hepatocytes were either continuously exposed to hormone (feminine-like profile) or exposed to growth hormone just twice per day (ie, 30 min every 11.5 hr) which has been shown to successfully restore the female- and male-dependent P450 isoforms, respectively, both in vitro and/or in vivo (Waxman et al., 1991; Shapiro et al., 1993; Agrawal and Shapiro, 2001; Thangavel et al., 2004).

CYP2C11. The most striking observation was the complete absence of any detectable levels of CYP2C11 mRNA (Fig.1) or protein (Fig. 2) in hepatocytes obtained from females after 2, 4, 6 and even 9 days in culture (data for the latter time period not presented) regardless of hormone treatment. As expected (Legraverend et al., 1992; Pampori and Shapiro, 1996; Agrawal and Shapiro, 2000), "zero time" hepatocytes from intact females expressed just trace concentrations of CYP2C11 mRNA and protein compared to similar cells from intact male rats. Also in agreement with our earlier in vivo findings (Shapiro et al., 1993, Pampori and Shapiro, 1996), zero time hepatocytes from hypophysectomized females expressed 41±7% and 62±9%[1] of CYP2C11 mRNA and protein, respectively, of comparable hepatocytes derived from hypophysectomized male rats. Whereas hepatocytes from hypophysectomized male rats were capable of retaining, in the absence of hormonal treatment, considerable levels of CYP2C11 (mRNA > protein) for at least 9 days in culture (Fig.3), isoform concentrations observed in zero time hepatocytes from female rats were quickly and irreversibly lost (ie, immeasurable) in cell culture.

In the case of male hepatocytes, CYP2C11 mRNA (Fig. 1) and protein (Fig. 2) were basically in agreement. As would be expected, changes in mRNA levels preceded changes in protein concentrations, and the magnitude of response to all regimens was always greater at the transcript level; in this study ~3 times greater. The length of time at culture generally produced only quantitative differences in CYP2C11 mRNA with maximal induction clearly occurring after 6 days. Lagging behind, protein expression began to exhibit a

hormone-induced pattern after 4 days in culture that required 6 days for full expression. In the absence of dexamethasone and growth hormone, CYP2C11 expression levels declined significantly below zero time concentrations, although still easily detectable. Exposure to dexamethasone for 4 to 6 days was clearly inductive, doubling (protein) and tripling (mRNA) expression levels of the isoform when compared to hepatocytes treated with no hormones. In contrast, when the glucocorticoid was administered with growth hormone, its effects were generally antagonistic, inhibiting any inductive effects of growth hormone. Expression levels of CYP2C11 in hepatocytes exposed to continuous growth hormone were basically similar to or even lower than that observed following no hormonal treatment. In contrast, episodic administration of growth hormone was highly inductive, with the lower dose (0.2 ng/ml) most effective, inducing transcript concentrations of CYP2C11 that were indistinguishable from that observed in hepatocytes isolated from intact males (308±29%). While 0.2 ng/ml of episodic growth hormone was the most inductive regimen, protein levels remained below that of cells from intact males (263±39%), but equal to that of zero time hepatocytes from hypophysectomized male rats. Although inductive when administered alone, as mentioned above, dexamethasone dramatically suppressed the inductive effects of episodic growth hormone on CYP2C11 expression.

In examining the dose effects of episodic growth hormone on CYP2C11 mRNA induction, we observed that 0.2 ng/ml was the most effective, 0.02 ng/ml significantly less so and 0.002 ng/ml the least inductive after 6 days in culture

(Fig. 3, top panel). Nevertheless, each concentration was more inductive than no hormone treatment, and the additional effect of dexamethasone resulted in a significant suppression of isoform transcript levels at all 3 episodic concentrations. When the 3 growth hormone concentrations were administered continuously, CYP2C11 mRNA levels in the male hepatocytes were all the same, but significantly ($p<0.01$) less than the lowest (0.002 ng/ml) episodic growth hormone concentration. After 9 days in culture (Fig. 3, bottom panel) the pattern of response to dexamethasone and/or pulsatile or continuous growth hormone at various concentrations was the same as after 6 days in culture with the exception that the magnitude of induction had declined after an additional 3 days in culture. Interestingly, CYP2C11 mRNA concentrations in the no hormone treatment cells basically remained the same (ie, ~50% of zero time) throughout the 9 days in culture.

CYP2A2. Like CYP2C11, the most striking observation was the complete absence of any detectable levels of CYP2A2 mRNA (Fig.4) or protein (Fig. 5) regardless of hormone treatment, in hepatocytes obtained from female rats after 2, 4 or 6 days in culture. This, in spite of the fact that at zero time, hepatocytes from hypophysectomized females expressed ~80% of the concentration of CYP2A2 as comparable hepatocytes from male rats. However, there were a number of dissimilarities between the responses of CYP2A2 and CYP2C11. In contrast to CYP2C11, but in agreement with our in vivo findings (Agrawal and Shapiro, 2000, 2001), the magnitude of response of CYP2A2 at the mRNA level was only slightly greater than at the protein level (≤ 1.5). In addition, expression

levels of the isoform increased only minimally during the 6 days in culture. Lastly, irrespective of treatment, CYP2A2 expression never obtained levels observed in zero time hepatocytes from hypophysectomized males. However, the most effective hormone regimen (episodic rat growth hormone without dexamethasone) induced expression of the isoform to concentrations that were indistinguishable from that of CYP2A2 mRNA (62±7%) and protein (61±4%) measured in hepatocytes derived from intact males at zero time.

Hormonal regulation of CYP2A2 was considerably muted when compared to CYP2C11. Exposure of the male cells to the continuous growth hormone profile, at either concentration, induced CYP2A2 mRNA by a small, but significant ~20% that was not translated into elevated protein levels. In contrast, the episodic profile was clearly inductive at both the mRNA and protein levels, with a dose effect only apparent at the transcript level (0.2 ng/ml > 2.0 ng/ml). Dexamethasone, alone, induced a small, often insignificant increase in CYP2A2 that suppressed expression of the isoform when concurrently administered with the episodic growth hormone profile.

CYP2C13. Because there were only minimal differences in CYP2C13 mRNA levels after 2, 4 and 6 days in culture (data not presented) and protein supplies for western blotting were nearly exhausted, we limited our data presentation to 6 days in culture. In spite of the fact that hepatocytes from hypophysectomized males and females expressed similar concentrations of CYP2C13 mRNA and protein at zero time, we were unable to detect the isoform, regardless of treatment conditions, in any of the cultured female-derived hepatocytes (Fig. 6).

Moreover, the magnitude of response of the male hepatocytes to all culture conditions was very poor. While hepatocytes from intact males at zero time expressed ~70% of CYP2C13 levels observed in comparable cells from hypophysectomized males, the most effective culture condition (0.2 ng/ml of episodic growth hormone without dexamethasone) was only capable of inducing 20% of zero time concentrations (Fig. 6). Although induction levels were low, the isoform still exhibited differential responses to the hormone treatments that were in agreement at the mRNA and protein levels. In the absence of dexamethasone and growth hormone from the culture media, CYP2C13 concentrations declined to ~5% of zero time levels. Dexamethasone, alone, increased CYP2C13 concentration several fold, but reduced expression of the isoform when administered with growth hormone at either profile or dose. Exposure to continuous growth hormone at the higher concentration (2 ng/ml) was equally ineffective as no hormone treatment. In contrast, the low concentration (0.2 ng/ml) of continuous growth hormone increased CYP2C13 levels several fold, but was less inductive than the episodic profile at the same dose.

CYP3A2. After just 2 days in culture, transcript concentrations declined to ~2% of zero time values. Dexamethasone, alone, increased CYP3A2 mRNA levels several fold above the no hormone treatment, but as previously seen, inhibited mRNA expression when combined with any of the growth hormone regimens. The low dose episodic growth hormone profile was the most inductive treatment after 2 days in culture. Because expression of CYP3A2 mRNA in hepatocytes

from both sexes was completely lost, under all conditions, after 4 and 6 days in culture, our findings have not been presented.

Interpretation of CYP3A2 protein levels was more problematic because the antibody could not differentiate CYP3A1 from CYP3A2. Since the former is highly responsive to the inductive effects of glucocorticoids, but much less so to growth hormone (Thangavel et al., 2004), and the latter isoform is highly responsive to growth hormone (Waxman et al., 1995; Pampori and Shapiro 1996), protein findings were not interpretable and have been omitted.

DISCUSSION

To our knowledge, this is the first report in which male-specific isoforms of P450 have been induced in rat primary hepatocyte cultures by growth hormone. Due to a paucity of information regarding earlier studies that failed to induce the male-specific isoforms in vitro (Guzelian et al., 1998; Liddle et al., 1992; Legraverend et al., 1992), we can only speculate as to the reasons for our success. First, we used species specific rat growth hormone which is a considerably more effective inducer of rat sex-dependent P450 isoforms than is human growth hormone (Pampori et al., 2001). Assuming dramatic differences in the in vivo and in vitro metabolism of growth hormone, we did not attempt to renaturalize the normally secreted six daily plasma pulses of the hormone, but rather administered two daily pulses of growth hormone shown to induce near physiologic levels of male-specific isoforms in hypophysectomized male rats (Waxman et a., 1991; Shapiro et al., 1993; Waxman et al., 1995). In this regard, we chose to isolate hepatocytes from long-term hypophysectomized rats that were clearly no longer under the competing influence of endogenous growth hormone. Lastly, also regarding differences in the in vivo and in vitro metabolism of growth hormone, we not only avoided pharmacologic levels of the hormone, but physiologic ones as well. If turnover of growth hormone was significantly reduced in cell cultures, then physiologic concentrations as well as the normal six daily pulses would result in a persistence of the hormone consequently restoring a near continuous growth hormone profile (feminine) rather than a masculine episodic profile.

Whereas the four hepatic male-specific isoforms (CYP2C11, CYP2C13, CYP2A2 and CYP3A2) are normally expressed by the masculine growth hormone profile and completely suppressed by the continuous feminine profile in vivo (Legraverend et al., 1992; Shapiro et al., 1995), the responses appeared somewhat different in culture. As expected, CYP2C11 induction was clearly greatest when the male hepatocytes were exposed to the episodic growth hormone profile, but were similar to no hormone treatment levels when exposed to the continuous hormone profile. Judging from in vivo findings, restoration of the feminine profile should have completely suppressed CYP2C11 expression. However, the cells were not exposed to normal feminine levels of growth hormone, but rather to clearly subphysiologic concentrations of continuous hormone, which in vivo allows for the same subnormal levels of CYP2C11 expression in male rats (Pampori and Shapiro, 1999) as observed in culture. Apparently, CYP2C11 regulators cannot discriminate between no growth hormone which allows 20 to 30% normal expression levels of CYP2C11 and very low continuous concentrations of the hormone.

In the case of the episodic profile, it might seem surprising that a concentration of growth hormone (0.2 ng/ml) equivalent to just 0.1% of the normal in vivo pulse amplitude (Agrawal and Shapiro, 2000) was the most effective inducer of CYP2C11. In fact, episodic concentrations of the hormone 100-times lower than 0.2ng/ml remained effective, elevating CYP2C11 mRNA to ~50% of maximum induction levels. Nor is it likely that higher hormone concentrations would have been more effective since the 2 ng/ml episodic dose

induced a significantly lower concentration of CYP2C11 mRNA and protein than the 0.2 ng/ml concentration. This incredible sensitivity of CYP regulation by episodic growth hormone can possibly be explained by known in vivo sensitivities of the isoform to growth hormone, the K_D of the hormone/receptor complex and differences between in vivo and in vitro hormone metabolism. Regarding the former, restoration of the masculine episodic growth hormone profile to hypophysectomized male rats with pulse amplitudes of only 2.5 to 10% of physiologic can induce above normal expression levels of CYP2C11 that are characterized by an induction ratio of mRNA and protein of ~3:1 (Pampori and Shapiro, 1994; Agrawal and Shapiro, 2000), which is similar to our findings in cell culture. Next, consider that the essential signal in the masculine secretory growth hormone profile regulating CYP2C11 expression is a minimum growth hormone devoid interpulse period of ~2.5 h which is equally effective when extended to >12 h. The actual pulse heights are of little consequence (Waxman et al., 1991; Shapiro et al., 1993; Agrawal and Shapiro, 2001). Accordingly, if the in vivo half-life of up to 20 min for growth hormone (Chapman et al., 1991) is considerably extended in cell culture, then it is possible that even a 0.5 h exposure to a hormone dose of > 2 ng/ml every 11.5 h could result in a persistence of the hormone reducing the requisite 2.5 h growth hormone devoid interpulse and suppressing CYP2C11 expression. Lastly, although the 0.2 ng/ml concentration of growth hormone translates into only 5% receptor occupancy (Fuh et al., 1992), the possibility of reduced hormone clearance in cell culture could result in

sufficient receptor binding to effectively induce CYP2C11 (Thangavel et al., 2004).

The in vitro response of CYP2A2 in male hepatocytes to the sex-dependent growth hormone profiles was in complete agreement with in vivo reports. As we observed in cell culture, in vivo studies have found (Waxman et al., 1988; Pampori and Shapiro, 1999; Agrawal and Shapiro, 2000) that CYP2A2 mRNA, protein and catalytic activity is maximally expressed in the absence of growth hormone (ie, whole liver or zero time hepatocytes from hypophysectomized male rats), suppressed ~30% from the maximum in intact males (ie, 6 daily physiologic growth hormone pulses restored to hypophysectomized rats or 2 daily pulses administered to hepatocytes in culture), further suppressed by subnormal concentrations of continuous growth hormone (ie, ≤ 2 ng/ml of either circulating or media containing growth hormone) and completely suppressed by the daily physiologic feminine hormone profile (ie, ~40±25 ng/ml).

Although growth hormone regulation of male specific CYP3A2 is near identical to CYP2A2 (Waxman et al., 1988; Waxman et al., 1995; Agrawal and Shapiro, 2000), we, as well as others (Davila and Morris, 1999; Kocarek et al., 1993; Liddle et al., 1992) were unable to maintain detectable levels of the isoform in cell culture. Not only was episodic or continuous growth hormone ineffective, but dexamethasone, a highly potent in vivo inducing agent of CYP3A2 (Okey, 1990), was similarly ineffective in vitro. Although growth hormone regulation of CYP2A2 and CYP3A2 may be the same, the isoforms are very different proteins

sharing ~25% exact sequence identity (www.ncbi.nlm.nih.gov/blast) possibly resulting in different requirements for in vitro expression.

In vivo studies have demonstrated the same maximum expression levels of male-specific CYP2C13 in both the absence of growth hormone (ie, the hypophysectomized male rat) and in the presence of the normal episodic masculine profile of the intact male. In contrast, the continuous feminine growth hormone profile is completely suppressive (Legraverend et al., 1992; Pampori and Shapiro, 1996; Agrawal and Shapiro, 2000). In agreement, we did find that expression of CYP2C13 in male hepatocytes was most responsive to the low dose (0.2 ng/ml) of episodic growth hormone increasing protein concentration 10-times above controls, while the high continuous dose (2 ng/ml) of the hormone completely suppressed the protein. However, transcript and protein levels of the isoform could, at best, only be induced to a fraction (~20%) of normal. Whereas optimal expression of CYP2C13 can be maintained without growth hormone in vivo, we found that similar conditions in cell culture resulted in the near undetectability of the isoform. In this regard, under very different culture conditions, baseline (ie, no hormone treatment) levels of hepatocyte CYP2C13 mRNA were measured at ~50% of normal, although CYP3A2 expression still quickly disappeared (Legreverand et al., 1992; Liddle et al., 1992).

In agreement with earlier reports demonstrating physiologic-like levels of dexamethasone induced up-regulation of CYP2C11 in rat hepatocyte cultures (Liddle et al., 1992; Iber et al., 1997), we observed a similar inductive response of CYP2C11 as well as CYP2A2 and CYP2C13 to the glucocorticoid. However,

when administered concurrently with growth hormone, dexamethasone was antagonistic, suppressing the inductive effects of growth hormone on male-specific P450 expression. The inhibitory effects on growth hormone action can likely be explained by the fact that the glucocorticoid reduces tyrosyl phosphorylation of mitogen-activated protein kinases ERK-1 and ERK-2 as well as decreases the activation and number of growth hormone receptors (King and Carter-Su, 1995), requisite factors transducing episodic growth hormone induction of CYP2C11 expression (Verma et al., 2005).

Lastly, we have observed a dramatic sexually dimorphic response of the male-specific P450s to growth hormone regulation illustrated by the complete lack of induction in hepatocytes from female rats. [This, in spite of the fact that female derived hepatocytes are highly responsive to growth hormone induction of female-specific and –predominant isoforms (Guzelian et al., 1988; Legraverend et al., 1992; Thangavel et al., 2004).] These in vitro findings, in the absence of any confounding in vivo sexual factors, demonstrate intrinsic, apparently irreversible sex differences in the expression of male-specific P450s regulated by growth hormone. Whether the sexual dimorphism is inherent and dependent on genetic factors (ie, methylation status of P450 promoter regions) or imprinting by hormones is unknown. What is known, however, is that you cannot stimulate female hepatocytes, either in vitro or in vivo (Legraverend et al., 1992; Waxman et al., 1995; Shapiro et al., 1993) to express male-like levels of male-specific P450s nor can you stimulate male hepatocytes, either in vitro (Thangavel et al., 2004) or in vivo (Pampori and Shapiro, 1999) to express female-like levels of

female-dependent P450s. Accordingly, drug metabolism in males and females remains irrevocably different.

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FOOTNOTES

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[1] As stated in the figure legends, all P450 isoform values, including zero time values for hepatocytes isolated from intact males and females as well as hypophysectomized females are calculated as a percent of the isoform concentration in hepatocytes from male hypophysctomized rats at zero time arbitrarily designated 100%.

LEGENDS

Fig 1. CYP2C11 mRNA expression in primary hepatocytes derived from hypophysectomized male (M) and female (F) rats exposed continuously to dexamethasone (Dex), its diluent (-) and/or episodic or continuous rat growth hormone (rGH) for ether 2, 4 or 6 days in culture. Procedural details are described in the text. Sufficient viable cells were isolated from a single liver for mRNA determinations for every time point and treatment presented in the figure. Values are presented as a percent of CYP2C11 mRNA in hepatocytes from male hypophysectomized rats at "zero time" (ie, immediately following isolation and preceding plating) arbitrarily designated 100%. Each data point is a mean ± SD for cells from 4 rats (2 and 4 days in culture) or 8 rats (6 days in culture).

ND, not detectable.

∗, $p < 0.05$ and ∗∗, $p < 0.01$ compares all treatments to the no hormone regimen (first column on left) after the same number of days in culture.

††, $p < 0.01$ compares the effects of the rGH profile (episodic vs. continuous) on cells exposed to the same dose of rGH and Dex treatment after the same number of days in culture.

§§, $p < 0.01$ compares the dose effect of rGH (2 vs 0.2 ng/ml) on cells otherwise exposed to the same rGH profile and Dex treatment after the same number of days in culture.

¶, $p < 0.05$ and ¶¶, $p < 0.01$ compares the effects of Dex on cells exposed otherwise to the same treatment after the same number of days in culture.

Statistical comparisons between sexes were not performed due to a lack of quantitative values from female derived hepatocytes.

Fig 2. CYP2C11 protein expression in primary hepatocytes derived from hypophysectomized male (M) and female (F) rats exposed continuously to dexamethasone (Dex), its diluent (-) and/or episodic or continuous rat growth hormone (rGH) for ether 2, 4 or 6 days in culture. Procedural details are described in the text. Sufficient viable cells were isolated from a single liver for protein determinations for every time point and treatment presented in the figure. Values are presented as a percent of CYP2C11 protein in hepatocytes from male hypophysectomized rats at "zero time" (ie, immediately following isolation and preceding plating) arbitrarily designated 100%. Each data point is a mean ± SD for cells from 4 rats (2 and 4 days in culture) or 8 rats (6 days in culture).

ND, not detectable.

∗, $p < 0.05$ and ∗∗, $p < 0.01$ compares all treatments to the no hormone regimen (first column on left) after the same number of days in culture.

††, $p < 0.01$ compares the effects of the rGH profile (episodic vs. continuous) on cells exposed to the same dose of rGH and Dex treatment after the same number of days in culture.

§, $p < 0.05$ and §§, $p < 0.01$ compares the dose effect of rGH (2 vs 0.2 ng/ml) on cells otherwise exposed to the same rGH profile and Dex treatment after the same number of days in culture.

¶, $p < 0.05$ and ¶¶, $p < 0.01$ compares the effects of DEX on cells exposed otherwise to the same treatment after the same number of days in culture.

Statistical comparisons between sexes were not performed due to a lack of quantitative values from female derived hepatocytes.

Fig 3. CYP2C11 mRNA expression in primary hepatocytes derived from hypophysectomized male rats exposed continuously to dexamethasone (Dex), its diluent (-) and/or episodic or continuous rat growth hormone (rGH) for either 6 or 9 days in culture. Procedural details are described in the text. Sufficient viable cells were isolated from a single liver for mRNA determinations for every time point and treatment presented in the figure. Values are presented as a percent of CYP2C11 mRNA in hepatocytes from male hypophysectomized rats at "zero time" (ie, immediately following isolation and preceding plating) arbitrarily designated 100%. Each data point is a mean ± SD for cells from 4 rats:.

∗, $p < 0.05$ and ∗∗, $p < 0.01$ compares all treatments to the no hormone regimen (first column on left) after the same number of days in culture.

†, $p < 0.05$ and ††, $p < 0.01$ compares the effects of the rGH profile (episodic vs. continuous) on cells exposed to the same dose of rGH and Dex treatment after the same number of days in culture.

§, $p < 0.05$ and §§, $p < 0.01$ compares the effects of the next highest dose of rGH (0.002 vs. 0.02, 0.02 vs. 0.2 ng/ml) on cells otherwise exposed to the same rGH profile and Dex treatment after the same number of days in culture.

¶, $p < 0.05$ and ¶¶, $p < 0.01$ compares the effects of DEX on cells exposed otherwise to the same treatment after the same number of days in culture.

Fig 4. CYP2A2 mRNA expression in primary hepatocytes derived from hypophysectomized male (M) and female (F) rats exposed continuously to dexamethasone (Dex), its diluent (-) and/or episodic or continuous rat growth hormone (rGH) for ether 2, 4 or 6 days in culture. Procedural details are described in the text. Sufficient viable cells were isolated from a single liver for mRNA determinations for every time point and treatment presented in the figure. Values are presented as a percent of CYP2A2 mRNA in hepatocytes from male hypophysectomized rats at "zero time" (ie, immediately following isolation and preceding plating) arbitrarily designated 100%. Each data point is a mean ± SD for cells from 4 rats (2 and 4 days in culture) or 8 rats (6 days in culture).

ND, not detectable.

∗, $p < 0.05$ and ∗∗, $p < 0.01$ compares all treatments to the no hormone regimen (first column on left) after the same number of days in culture.

†, $p < 0.05$ and ††, $p < 0.01$ compares the effects of the rGH profile (episodic vs. continuous) on cells exposed to the same dose of rGH and Dex treatment after the same number of days in culture.

§, $p < 0.05$ and §§, $p < 0.01$ compares the dose effect of rGH (2 vs 0.2 ng/ml) on cells otherwise exposed to the same rGH profile and Dex treatment after the same number of days in culture.

¶, $p < 0.05$ and ¶¶, $p < 0.01$ compares the effects of DEX on cells exposed otherwise to the same treatment after the same number of days in culture.

Statistical comparisons between sexes were not performed due to a lack of quantitative values from female derived hepatocytes.

Fig 5. CYP2A2 protein expression in primary hepatocytes derived from hypophysectomized male (M) and female (F) rats exposed continuously to dexamethasone (Dex), its diluent (-) and/or episodic or continuous rat growth hormone (rGH) for ether 2, 4 or 6 days in culture. Procedural details are described in the text. Sufficient viable cells were isolated from a single liver for protein determinations for every time point and treatment presented in the figure. Values are presented as a percent of CYP2A2 protein in hepatocytes from male hypophysectomized rats at "zero time" (ie, immediately following isolation and preceding plating) arbitrarily designated 100%. Each data point is a mean ± SD for cells from 4 rats (2 and 4 days in culture) or 8 rats (6 days in culture).

ND, not detectable.

∗, $p<0.05$ and ∗∗, $p<0.01$ compares all treatments to the no hormone regimen (first column on left) after the same number of days in culture.

†, $p<0.05$ and ††, $p<0.01$ compares the effects of the rGH profile (episodic vs. continuous) on cells exposed to the same dose of rGH and Dex treatment after the same number of days in culture.

§, $p<0.05$ compares the dose effect of rGH (2 vs 0.2 ng/ml) on cells otherwise exposed to the same rGH profile and Dex treatment after the same number of days in culture.

¶, $p<0.05$ and ¶¶, $p<0.01$ compares the effects of DEX on cells exposed otherwise to the same treatment after the same number of days in culture.

Statistical comparisons between sexes were not performed due to a lack of quantitative values from female derived hepatocytes.

Fig 6. CYP2C13 mRNA and protein expression in primary hepatocytes derived from hypophysectomized male (M) and female (F) rats exposed continuously to dexamethasone (Dex), its diluent (-) and/or episodic or continuous rat growth hormone (rGH) for 6 days in culture. Procedural details are described in the text. Sufficient viable cells were isolated from a single liver for mRNA and protein determinations for every treatment presented in the figure. Values are presented as a percent of CYP2C13 mRNA or protein in hepatocytes from male hypophysectomized rats at "zero time" (ie, immediately following isolation and preceding plating) arbitrarily designated 100%. Each data point is a mean ± SD for cells from 8 rats.

ND, not detectable.

∗∗, p<0.01 compares all treatments to the no hormone regimen (first column on left).

††, p<0.01 compares the effects of the rGH profile (episodic vs. continuous) on cells exposed to the same dose of rGH and Dex treatment after the same number of days in culture.

§, p<0.05 and §§, p<0.01 compares the dose effect of rGH (2 vs 0.2 ng/ml) on cells otherwise exposed to the same rGH profile and Dex treatment.

¶¶, p<0.01 compares the effects of DEX on cells exposed otherwise to the same treatment.

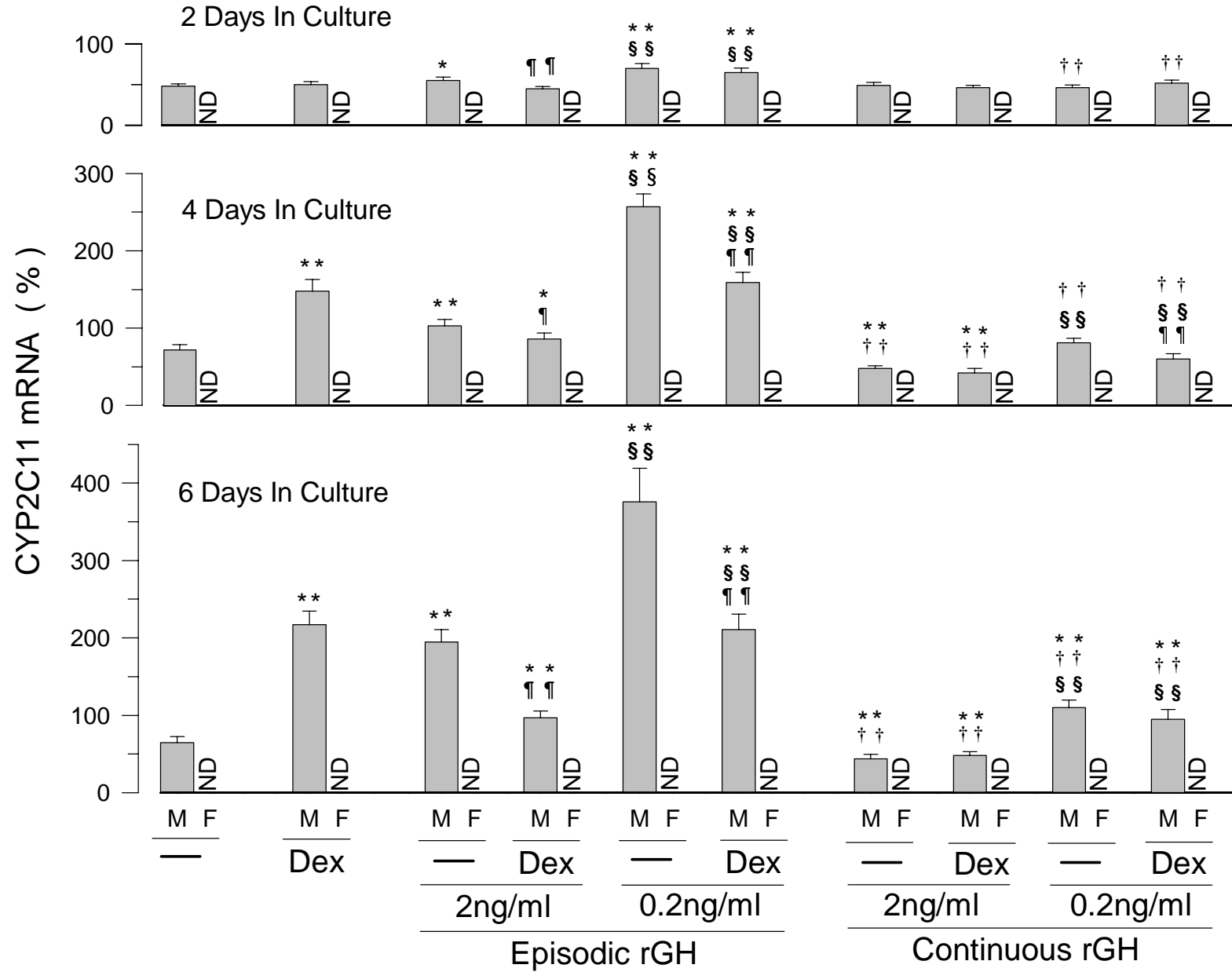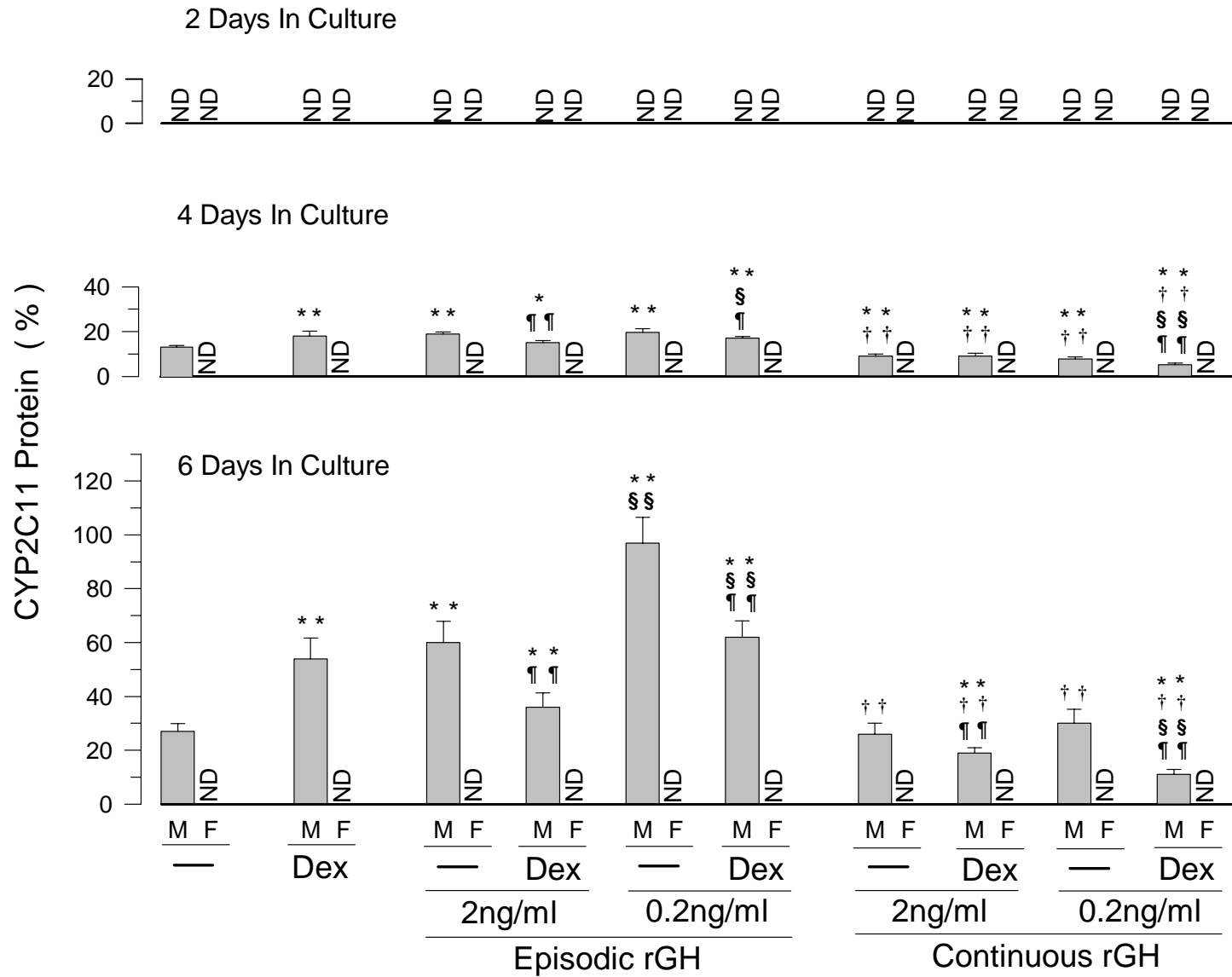Statistical comparisons between sexes were not performed due to a lack of quantitative values from female derived hepatocytes.

Fig. 1

Fig. 2

Fig. 3

Fig. 4
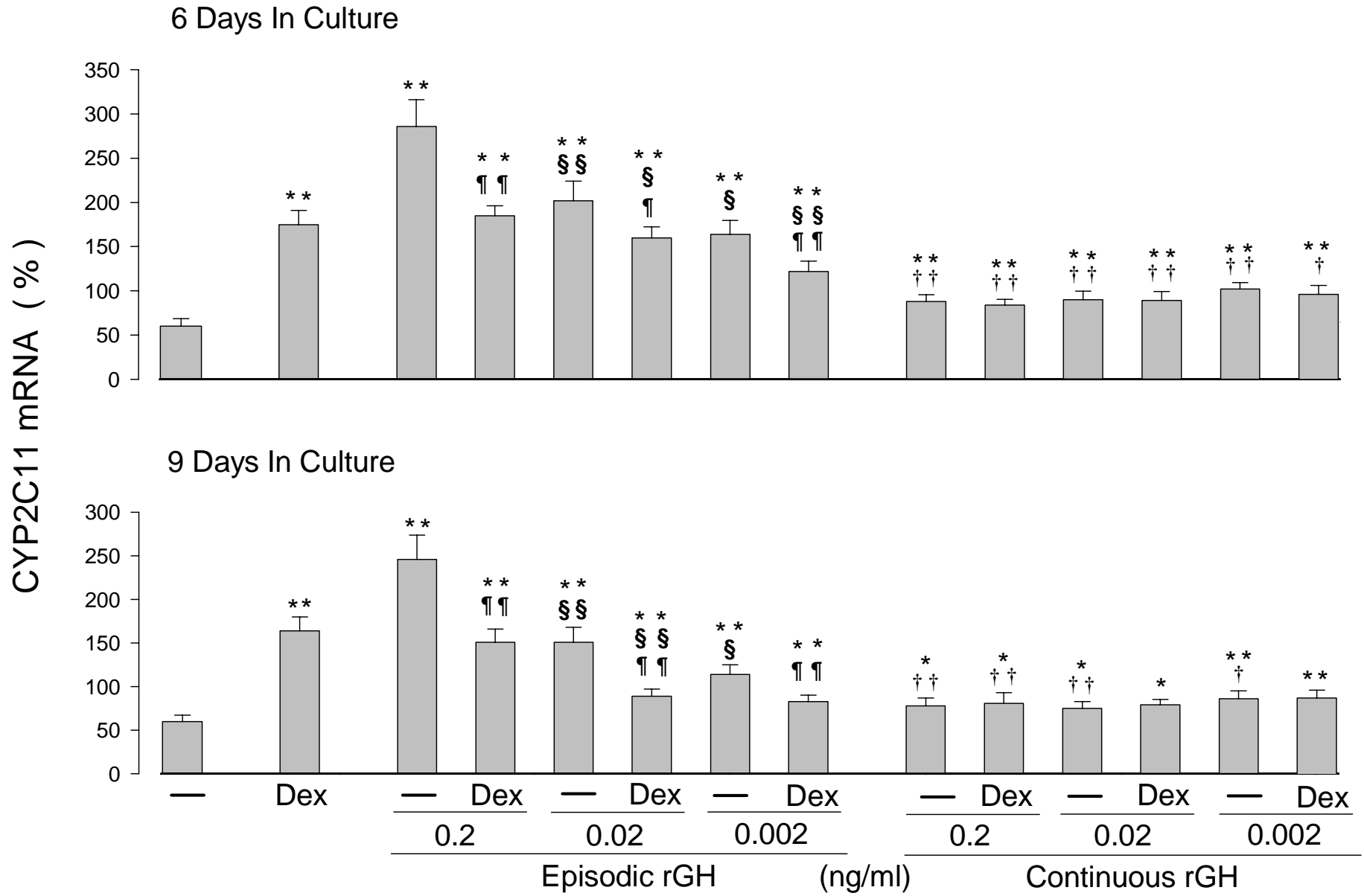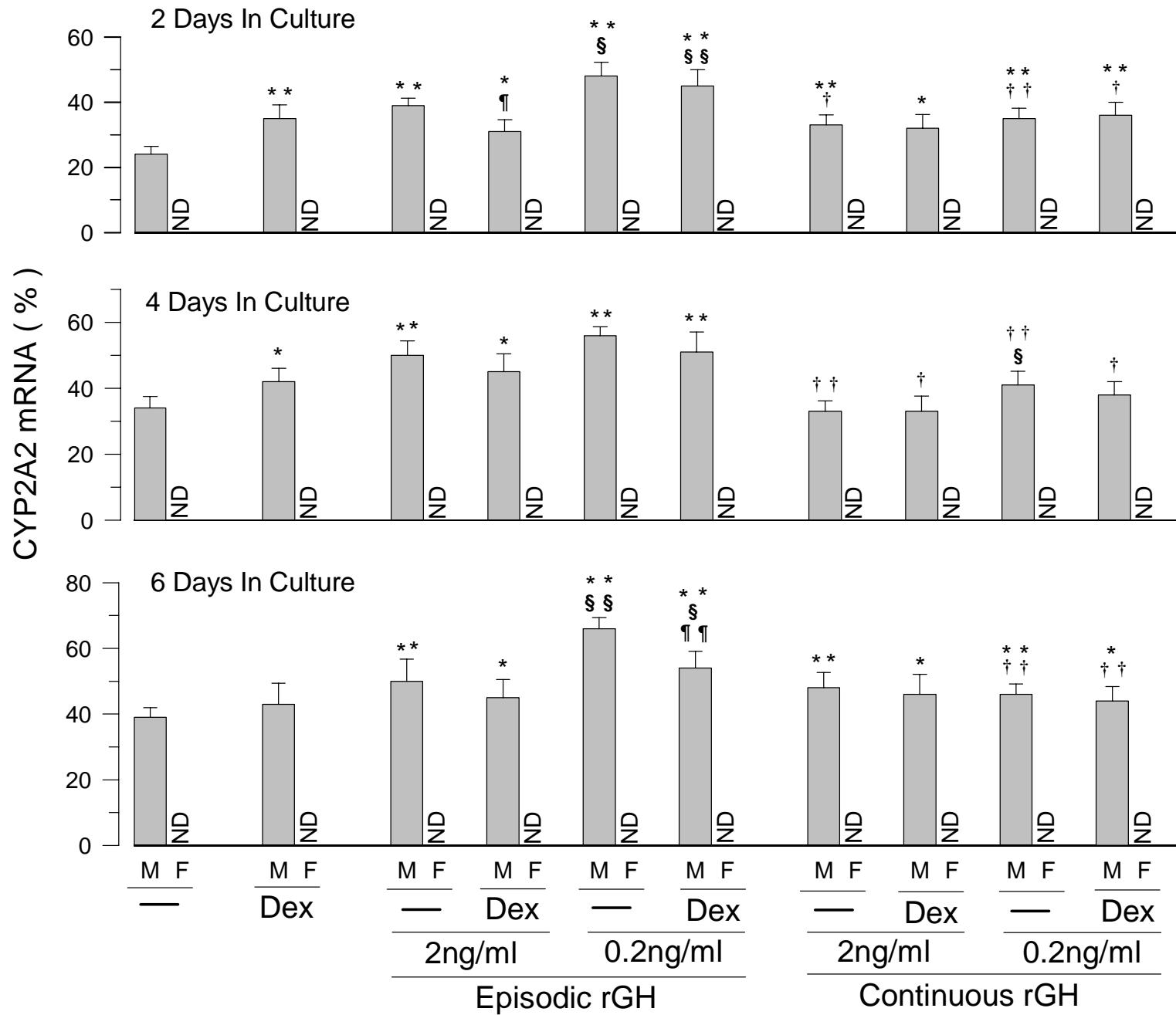
Fig. 5

Fig. 6