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SEXUAL DIMORPHIC REGULATION OF HEPATIC ISOFORMS OF HUMAN CYTOCHROME P450 BY GROWTH HORMONE

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ABSTRACT

Sex differences in drug metabolism have been reported in numerous species, including humans. In rats and mice, sex dependent differences in circulating growth hormone profiles are responsible for the differential expression of multiple sex-dependent isoforms of cytochrome P450 which is the basis for the sexual dimorphism in drug metabolism. In contrast, very little is known about sex differences in human isoforms of cytochrome P450 and their regulation by growth hormone. In this study, we have examined the effects of physiologic-like exposure doses to dexamethasone and/or pulsatile (masculine) or constant (feminine) human growth hormone on expression levels of CYP3A4, 1A2, 2D6, 2E1 and the glucocorticoid and growth hormone receptors in hepatocyte cultures obtained from men and women donors. We report that growth hormone can regulate expression of CYP3A4, 1A2 and 2D6. The masculine-like pulsatile growth hormone profile suppresses dexamethasone-induced CYP3A4, 1A2 and 2D6, whereas the feminine-like constant profile is permissive allowing isoform expression to be equal to or greater than glucocorticoid induction alone. There are intrinsic sexual differences in hepatocytes of men and women resulting in different levels of responsiveness of CYP3A4, 1A2 and hormone receptor expression to the same sexually dimorphic growth hormone profiles. Lastly, although real, the sexual dimorphic effects of growth hormone on human cytochrome P450 expression are not as dramatic as observed in rats and could easily be overlooked by the heterogeneous backgrounds of human populations.

The levels of drug metabolizing enzymes are influenced by a variety of factors, such as age, genetic background, disease, physiologic state, nutritional status, as well as exposure to drugs, hormones and environmental chemicals. One of the first factors known to affect drug metabolism and action was sex. It now has been more than 70 years since it was reported that adult male rats sleep a significantly shorter time than adult females when administered the same per kg body weight dose of hexobarbital (Nicholas and Barron, 1932). The subsequent discovery of 3 to 5 fold sex differences in hepatic drug metabolizing enzymes in the rat were found to result from sexual dimorphisms in individual isoforms of cytochrome P450 (P450, CYP) whose expression levels between sexes may vary by as little as 25% or exceed 20-fold (Legraverend et al., 1992; Shapiro et al., 1995). In contrast, much less is known about sex differences in drug metabolism in other species. Perhaps the unrealized expectation of finding similar exaggerated dimorphisms in other animals as in rats resulted in dismissing any significance to less dramatic sex differences in other species, including humans. Moreover, since drug metabolizing enzyme levels are regulated by innumerable factors, small sex differences might go unnoticed because of the contending heterologous background of most mammals. These confounding factors, easily contained in inbreed rodents maintained in a defined environment, can cloak inherent sexual dimorphisms in most species. Nevertheless, species as diverse as trout, chickens, ferrets, dogs, goats, pigs, hamsters cattle, mice (c.f. Shapiro et. al., 1995) and not surprisingly humans (O'Malley et al., 1971; Elfarra et al., 1998; Srinivas et al., 1997) have been shown

to exhibit sex differences in drug metabolism. In the case of humans, most attention has been directed to CYP3A4, the isoform responsible for the phase I metabolism of at least half of all consumed drugs and expressed in highest concentration of all isoforms; ie, 30 to 50% of the total pool of hepatic P450s (Shimada et al., 1994; Rendic, 2002). In general, in vivo (Gleiter and Gundert-Remy, 1996) and in vitro (Wolbold et al., 2003) studies have observed CYP3A4 to be female predominant with expression levels in women varying form ~25 to 200% above that in men.

The only endogenous factor known to maintain sexually dimorphic expression of hepatic P450s is growth hormone (GH) and the vast majority of studies have been conducted in the rat (Legraverend et al., 1992; Shapiro et al., 1995). More specifically, it is the sexually dimorphic ultradian rhythms in circulating GH that regulate sex-dependent isoforms of P450. Male rats secrete GH in episodic bursts about every 3 to 4 hr. Between the peaks, GH levels are undetectable. In female rats, the hormone pulses are more frequent and irregular, and are of lower magnitude than males, whereas the interpeak concentration of GH is always measurable. Exposure to the “continuous” or “constant” feminine secretory profile of GH produces the characteristic pattern of P450 isoforms expressed in females. Conversely, the “episodic” or “pulsatile” rhythm of GH secretion characterized as masculine is responsible for the expression of P450s observed in male rats (Pampori and Shapiro, 1996; Agrawal and Shapiro 2000). In addition to the rat, sexually dimorphic GH profiles have been reported in turkeys, sheep, horses, mice, chickens (c.f. Shapiro et al., 1995)

and humans (Hartman et al., 1993; Van den Berg et al., 1996; Engstrom et al., 1998). In the case of humans, numerous reports, generally using GH-deficient individuals, have shown that GH replacement can restore drug metabolizing enzymes to normal levels (Redmond et al., 1980; Cheung et al., 1996). One characteristic observed in the sex-dependent GH secretory profiles of all mammals is a more continuous secretion of the hormone in females. That is, the duration of the interpulse periods devoid of effective GH secretion is considerably longer in males, and this interpulse is the essential “signal” regulating induction and/or suppression of several predominant sex-dependent P450 isoforms in the rat (Waxman et al., 1991; Agrawal and Shapiro, 2001). In the present study, we have examined the effects of renaturalized sex-dependent GH profiles on the expression of human P450 isoforms in primary hepatocyte cultures obtained from donors of both sexes.

METHODS

Normal human hepatocytes isolated (Strom et al., 1996), suspended and plated on rat-tail collagen-coated flasks (T-25) in DMEM medium were obtained through the Liver Tissue Procurement and Distribution System (Pittsburg, PA). All the samples were obtained with donors consent and with approval of the appropriate hospital ethics committee. Male and female donors varied in age from 15 to 60 years. About 80% were Caucasian; the remainder African American and Hispanic. Alcohol consumption, smoking and drug history as well as causes of death varied from donor. About 60% of livers had some degree of steatosis (5-50%). Approximately 48 hr after isolation and plating, the primary hepatocyte cultures arrived at our laboratory. The medium was replaced as generally described earlier (Garcia et al., 2001; Thangavel et al., 2004) with serum free ice-cold DMEM/F-12 media supplemented with matrigel (233µg/ml) to enhance the substratum, streptomycin (100µg/ml), penicillin (100U/ml), glutamine (2mM), Hepes (15mM), insulin (10µg/ml), bovine transferrine (10µg/ml), Na_2SeO_3 (10ng/ml), aminolevulinic acid (2µg/ml), glucose (25mM), linoleic acid-albumin (0.5 mg/ml) and pyruvate (5mM). The cultures were also supplemented with the antimycotic fungizone (0.50µg/ml). Cultures were maintained in a humidified incubator at 37^{o}C under an atmosphere of 5% CO_2 and 95% air. The medium was changed every 12hr and cells were harvested after 5 days in culture.

Hormonal Conditions. Two concentrations (2 and 0.2 ng/ml) of recombinant human growth hormone (hGH) purchased from the National Hormone and Peptide Program (Torrance, CA) and one concentration of dexamethasone (4ng/ml) were used in the experiment. Some hepatocytes, however, were exposed to neither hormone. Cells from some flasks were only exposed to constant dexamethasone, other flasks contained constant hGH with or without constant dexamethasone, while remaining flasks were treated with pulsatile hGH with or without the glucocorticoid. Cells not exposed to dexamethasone were treated with its vehicle (4nl ethanol/ml). In the case of pulsatile administration, hGH was added for 30 min followed by two careful washings with GH-free media that remained in the flasks for 11.5 hr. Media were changed every 12 hr in all flasks whether or not they received pulsatile hGH.

Hepatocyte cultures were washed with ice-cold phosphate buffered saline containing 5mM EDTA. Cells were removed from the culture flasks with a cell scraper, transferred to two tubes (for protein and RNA, 2:1) and placed on ice for 1hr to dissolve the matrigel. Cells were centrifuged at 1000xg for 5min at 4^{o}C and the cell pellets were kept at -70^{o}C until extraction of RNA and protein analysis.

mRNA. Total RNA was isolated from hepatocytes by using TRIzol (Invitrogen, Carlsbad CA) extraction reagent. Cells were lysed in TRIzol by several passages through a pasteur pipette. Chloroform was added, mixed vigorously and centrifuged at 12,000g for 15 min. at 4^{o}C. The upper aqueous layer containing RNA was transferred to a fresh tube. RNA was precipitated by adding an equal

volume of isopropanol and washed with 75% ethanol. Purity of the RNA was assessed by absorbance at 260/280nm and quantitated by the absorbance at 260nm.

RNA from the hepatocytes was reverse transcribed into cDNA by using the following Promega reagents (Promega, Madison, WI). One µg of total RNA was incubated with a mixture of 200 units of M-MLV reverse transcriptase, 3mM $MgCl_2$, 50mM Tris-HCl buffer pH 8.3, 75mM KCl, 10mM DTT, 10nM of each dNTP, 40 unit RNasin ribonuclease inhibitor and 2.5µM oligo d(T)15 primer at 42^{o}C for 1hr followed by 5min heating at 95^{o}C and rapid cooling on ice. The cDNA was stored at -20^{o}C.

The PCR reaction was conducted in a small tube containing 5µl of reverse transcribed product which was amplified with 2.5 units of Tag DNA polymerase in a volume of 50µl containing 10mM Tris-HCl buffer pH 9.0, 1.5mM $MgCl_2$, 50mM KCl, 500µM of each dNTP and 0.2µM of each sequence specific primer. The PCR primer sequences, fragment sizes and annealing temperatures for CYP3A4, 1A2, 2D6, 2E1, β-actin (Rodríguez-Antona et al., 2000), human growth hormone receptor (hGHR) (Gebre-Medhin et al., 2001) and human glucocorticoid receptor (hGR) (Oakley et al., 1996) were reported elsewhere. A negative control was used for each P450 determination. β-Actin was used as an internal control. CYP3A4, 1A2, 2D6, 2E1, and β-actin were amplified for 4min at 94^{o}C followed by 40 PCR cycles of 40s at 94^{o}C, 45s at 60^{o}C, 50s at 72^{o}C and a final extension of 4min at 72^{o}C (Rodríguez-Antona et al., 2000). In the case of hGHR, after an initial incubation for 4 min at 95°C, samples were subjected to 40 cycles of 30s at

95°C, 30s at 55°C, 30s at 72°C followed by a final extension step at 72°C for 4min. The hGR was amplified at an annealing temperature of 60°C for 40 cycles. PCR was performed in a GeneAmp PCR system 2400 thermocycler (Perkin Elmer Life and Analytical Sciences, Boston, MA). The final PCR products were separated electrophoretically on 2% agarose gel containing ethidium bromide. Gels were run with 1xTAE buffer at 55V for 2hr. Five µl for each PCR reaction was removed at 2 cycle intervals, electrophoresed on agarose gels stained with ethidium bromide and found to be in the linear range.

PCR products for CYP3A4, 1A2, 2D6, 2E1, both receptors and β-actin were purified by Qiagen's QiAquick gel extraction kit and sequenced with a DNA sequencer Model 377 (Applied Biosystems, Foster City, CA) using specific primers for each. According to a Blast search (www.ncbi.nlm.nih.gov), the purified PCR products exhibited 100% sequence homology with each specific gene.

Western Blotting. Whole cell lysates were prepared from cultured hepatocytes as we previously described (Garcia et al., 2001). The estimated protein (20µg) was electrophoresed on 10% SDS-PAGE and electro-blotted onto a nitrocellulose membrane (Garcia et al., 2001; Thangavel et al., 2004). Individual blots for each isoform were probed with primary antibodies raised against recombinant human P450s: 3A4, 1A2, 2D6, and 2E1 (kindly provided by Dr. F. Peter Guengerich, Vanderbilt University School of Medicine, Nashville, TN). The primary antibody was located by using horseradish peroxidase conjugated to

anti-rabbit IgG (Amersham Bioscience, Little Calfont Buckinghamshire, UK) and detected with an enhanced chemiluminescence kit (SuperSignal West Pico, Pierce, Rockford, IL).

Relative mRNA and protein levels were quantified using a FluorChem™ 8800 gel documentation system (Alpha Innotech, San Leandro, CA). Integrated density values (IDV) were obtained by the software supplied with the gel documentation system for all the samples. PCR products were corrected by normalizing isoform levels to β-actin values and protein values were normalized to a control sample repeatedly run on each blot.

Catalytic Activity. Testosterone 6β-hydroxylase, reflective of the activity levels of CYP3A4 protein (Wolbold et al., 2003), was assayed according to our previously described method (Pampori and Shapiro, 1996; Agrawal and Shapiro 2000).

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

RESULTS

Mice whose genetic background may vary by as little as a few alleles can exhibit considerable variation in drug metabolism due to often profound differences in expression levels of individual isoforms of P450 (Shapiro et al., 1995). This divergence occurs in spite of the fact that the mice are all exposed to the same environment. Not surprisingly then, humans express huge inter-person variations in P450 levels. Depending upon the report, isoforms can vary up to 200-fold between individuals and usually >5 to 20-fold (Wrighton et al., 1996; Iver and Sinz, 1999). In addition to variations in genetic backgrounds responsible for polymorphisms and ethnic dichotomies, there are differences in the consumption of foods, alcohol, tobacco, therapeutic and recreational drugs, environmental chemicals as well as differences in life style, occupation, residency (eg. urban, rural), demography, sex and age that can all contribute to the large variations in P450 levels between individuals. Accordingly, it becomes understandable why an estimated 30% of hospitalized patients experience an adverse drug reaction; 0.31% of which are fatal (M:F, 4:1) (Kando et al., 1995). Considering the substantial influence these factors can have on the expression levels of human P450s, it becomes a challenge to identify those underlying, perhaps subtle physiologic factors regulating P450 expression. To limit the effects of these confounding factors, all donors in our study served as their own controls by normalizing P450 levels to values obtained from hepatocytes exposed to neither hormone (ie, hGH nor dexamethasone). In addition, we eliminated those few

donors whose results were dramatically contradictory to the norm. We had already pre-selected donors by omitting individuals that were prepubital or over 60 years of age. Nevertheless, there were always 1 or 2 donors whose hepatocytes' response to hormone treatment were so incongruous (eg. either completely unresponsive or totally inhibited by all treatments) that the inclusion of these finding would have skewed the results to statistical insignificance. Unfortunately, we were unable to identify a consistent single factor(s) from the limited information on the donors' charts that could explain their discrepant responses. Limitations in the use of human material includes an unknown number of untoward relevant factors in the donors' history as well as unknown postmortem effects influencing the harvesting, shipping and viability of tissue that could result in misleading observations. Thus, by eliminating results from those few donors whose hepatocytes' incongruous behavior may have unbeknownst to us a biological basis, reasonable and statistically significant hormone responses became clearly evident. Moreover, results of the Grubb's test for outliers as well as Dixon's test for extreme values agreed with our choice of outliers expunged from the data sets.

CYP3A4. The responses of the isoform to hormone treatment were in agreement at both the mRNA and protein levels (Fig.1). Exposure to dexamethasone, alone or in combination with GH (irrespective of profile or dose), was always highly inductive in hepatocytes from men and women. Treatment of hepatocytes with 2 daily pulses of GH suppressed CYP3A4 expression by >50% in men and a

statistically ($p<0.01$) smaller ~25% in women. That is, pulsatile GH was far more repressive than no GH. In all cases, addition of dexamethasone to cells exposed to pulsatile GH increased CYP3A4 expression to levels equal to that expressed in hepatocytes treated with neither hormone. In contrast, exposure to constant GH was not suppressive with CYP3A4 expression levels indistinguishable from hepatocytes treated with no hormone. However, the combination of constant GH and dexamethasone was considerably (>50 to 100%) more inductive than constant GH alone, inducing isoform concentrations equaling, and occasionally surpassing that induced by exposure to just the glucocorticoid. There were no significant GH dose effects when the 2.0 and 0.2 ng/ml concentrations were administered in the same profile.

The hormonal effects on CYP3A4 expression were examined at the catalytic level by measuring CYP3A4-dependent testosterone 6β-hydroxylase activity in hepatocytes derived from women (Fig. 2). The catalytic activity was in complete agreement with CYP3A4 protein and mRNA findings. Pulsatile GH dramatically suppressed hydroxylase activity which was elevated to control levels (cells receiving neither GH nor dexamethasone) when simultaneously treated with the synthetic glucocorticoid. The combined pulsatile GH and dexamethasone treatment was only half as inductive as dexamethasone alone. Constant GH was neutral having similar effects as no hormone treatment. The addition of dexamethasone to the constant GH regimen was equal to, or greater than dexamethasone alone. In descending order, the inductive effects of GH and dexamethasone on CYP3A4 mRNA, protein and catalytic activity are 1) constant

GH + dexamethasone, 2) dexamethasone alone, 3) constant GH alone, pulsatile GH + dexamethasone, or no hormonal treatment and 4) pulsatile GH alone.

CYP1A2. In general, mRNA and protein responses to GH and/or dexamethasone were identical (Fig.3). Dexamethasone alone had a small (~25%), but significant ($p<0.05$) inductive effect on CYP1A2 expression in both male and female hepatocytes. Whereas the isoform could not distinguish pulsatile from the constant GH profile, the response was sexually dimorphic; both GH profiles suppressing hepatic CYP1A2 expression to below control levels in men, while the same treatment to female hepatocytes had neither an inductive nor suppressive effect with subsequent CYP1A2 levels comparable to that observed in hepatocytes treated with neither hormone. The additive effect of dexamethasone only occurred when the glucocorticoid was concurrently administered with constant GH resulting in CYP1A2 levels often greater than any other treatment, or at least equal to that of dexamethasone alone.

CYP2D6. Again, mRNA and protein responses to hormone treatment were consistent (Fig.4). Like CYP3A4, 2 daily treatments with GH (pulsatile), at either dose, was suppressive whereas constant GH, at either dose, had no effect on CYP2D6 when compared to levels in hepatocytes treated with neither hormone. However, in contrast to CYP3A4 as well as 1A2 findings, GH exposure produced no sexually dimorphic effects. That is, pulsatile GH was equally suppressive in hepatocytes derived from both men and women while exposure to constant GH

resulted in similar levels of hepatic CYP2D6 protein and mRNA in both sexes. Lastly, and in contrast to both CYP3A4 and 1A2 responses; dexamethasone alone, or combined with either pulsatile or constant GH had no effect on CYP2D6 expression.

CYP2E1. We found no statistically significant effects of GH, pulsatile or constant, and/or dexamethasone on expression levels of CYP2E1 in hepatocytes from men and women (results not presented).

Growth Hormone Receptor. Although there was insufficient material to measure receptor at the protein level, we were able to determine transcript concentrations. In both sexes, dexamethasone alone, or in combination with GH at either profile, was highly inductive of the hGHR (Fig. 5, top panel). In contrast, GH alone at either dose tended to suppress (below control levels) hGHR mRNA in both sexes, although significantly less so in female hepatocytes exposed to constant GH. Concurrent exposure to dexamethasone with GH (irrespective of dose or profile) induced receptor transcript levels to concentrations considerably above that observed in hepatocytes exposed to neither hormone. However, the combined hormonal treatment resulted in a sexually dimorphic response inducing ~2x more receptor mRNA in hepatocytes from women than men.

Glucocorticoid Receptor. In contrast to its effects on the hGHR, dexamethasone alone, or when combined with GH (regardless of dose or profile)

dramatically suppressed accumulation of hGR transcript (Fig.5, bottom panel). The hGR mRNA response to GH or GH plus dexamethasone was sexually dimorphic. Whereas all GH treatments suppressed receptor transcript levels below control values in male hepatocytes, the same treatment was significantly less or not at all suppressive in female hepatocytes. Moreover, while addition of the glucocorticoid further reduced receptor mRNA in hepatocytes concurrently exposed to GH, the suppression was considerably greater in cells from male livers.

DISCUSSION

Two recent in vivo studies have reported the effects of GH therapy on CYP3A4 enzyme markers in GH-hormone deficient individuals. In one case, the effects of a daily subcutaneous GH injection on CYP3A4 activity were assessed separately in GH-deficient young boys and girls (Sinués et al., 2004). In a similar in vivo study, restoration of sex-dependent GH profiles (ie, pulsatile or constant) on CYP3A4 enzymatic activity was reported on a combined cohort of GH-deficient men and women (Jaffe et al., 2002). An in vitro study measured CYP3A4 mRNA, protein and specific catalytic activity in hepatocyte cultures, presumably from combined sexes, exposed to a constant pharmacologic GH dose (Liddle et al., 1998). While all of these studies have yielded important information regarding GH regulation of human P450s, we believe that ours is the first report to compare the differential effects of the masculine-like and feminine-like GH profiles in regulating expression of hepatic P450 isoforms in men and women.

Although the use of primary hepatocytes offers the advantage of dissecting out individual factors regulating P450 expression, it is hardly physiologic. In response, we attempted to ameliorated this disadvantage by using physiologic-like concentrations of hormones in our media. We realized that because of radical differences in metabolism, 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. Dexamethasone is a highly potent, synthetic glucocorticoid. However, when comparing its biological potency (eg, gluconeogenic, glycogenolytic) to cortisol, the present levels (10nM) would be comparable to resting plasma concentrations of the natural steroid in men and women (Haynes and Murad, 1985). In addition, our hGH dose of 2 ng/ml is physiologic (Murad and Haynes, 1985) while the lower 0.2 ng/ml dose is clearly subphysiologic.

Dexamethasone is a known inducer of CYP3A4, and the dose employed in the present study (10nM) has been reported, as we observed, to double expression levels of the isoform in human hepatocytes (Schuetz et al., 1993; Pascussi et al., 2001). Regarding GH, we found no dose effect of hGH on CYP3A4 as well as CYP2D6 and 1A2 expression levels in hepatocytes from men and women. The fact that a hGH dose ~10% of normal (0.2 ng/ml) is equally effective as the physiologic-like dose is not surprising in light of our earlier findings that replacement doses of rat GH at ~5% of physiologic are capable of maintaining normal expression levels, in vivo and in vitro, of female-specific CYP2C12 and male-specific CYP2C11 (Agrawal and Shapiro, 2000; Thangavel et al., 2004; Pampori and Shapiro, 1999). Thus, while physiologic levels of GH may be requisite for some functions, at least in the case of rats and possibly humans, they are apparently not required for maintaining normal hepatic P450 levels. In contrast, the sex-dependent profiles of hGH to which the cells were exposed had a significant effect on the expression levels of CYP3A4 and 2D6, but not so for CYP2A1. Two daily pulses of GH, previously shown to mimic the masculine profile in vivo and in vitro (Agrawal and Shapiro, 2001; Waxman et al.,

1991; Thangavel et al., 2004) had a dramatic suppressive effect on expression levels of CYP3A4, and to a lesser extent, but still a significant suppression of CYP2D6. On the other hand, exposure of the hepatocytes to the constant, feminine-like hGH profile was neutral, having neither an inductive nor suppressive effect. In contrast to our findings, human hepatocytes exposed to constant hGH for 4 to 6 days at levels 50 to 500-times higher than that used by us, induced CYP3A4 by ~12-fold (Liddle et al., 1998). Here we are probably seeing the difference between a physiologic and pharmacologic response to hGH.

Our results suggest that the sexually dimorphic secretory hGH profiles function to differentially modulate the clearly inductive effects of glucocorticoids on CYP3A4 and 1A2 expression. Whereas the masculine-like pulsatile or episodic release of hGH represses glucocorticoid induction of CYP3A4 and 1A2, the feminine-like constant or continuous release of the hormone is more permissive allowing expression levels of the isoforms to equal or even exceed that induced by the corticoid alone. Comparable GH dichotomies have been observed in the rat where the feminine GH profile allows for normal expression levels of female predominant CYP2C6 and 2A1 while the masculine profile is clearly suppressive (Pampori and Shapiro, 1996; Agrawal and Shapiro, 2000). In a sexually reversed response, the masculine GH profile allows for near normal levels of male-specific CYP2A2 and 3A2 while the feminine profile suppresses the rat isoforms (Pampori and Shapiro, 1996; Agrawal and Shapiro, 2000). A similar phenomen has been described in GH regulation of murine isoforms of

P450 (Shapiro et al., 1995). In vivo studies in GH-deficient humans (with presumably normal adrenal function), also found that compared to continuous hGH infusion, pulsatile replacement decreased CYP3A4 and 1A2 activities when assessed by the caffeine and erythromycin breath tests, respectively (Jaffe et al., 2002). In related studies, it has been demonstrated that the pattern in which GH is administered determines its effectiveness on lipid metabolism, growth rates and insulin-like growth hormone expression in men and women (Laursen et al., 1998; Achermann et al., 1999).

Unlike CYP3A4 and 1A2, dexamethasone had no effect on CYP2D6 mRNA and protein levels. However, in agreement with CYP3A4 findings, there was a differential response of CYP2D6 to the sexually dimorphic hGH replacement profiles; only the masculine-like profile was suppressive. Although there are very few related studies in the literature, the report of somewhat higher in vivo CYP2D6 activities in women (Tamminga et al., 1999) agrees with our present in vitro findings.

While it would be speculative to correlate receptor transcript concentrations with hormonal regulation of P450 expression, the sexually dimorphic responsiveness of the receptors to hormonal regulation concurs with a similar response of the P450 isoforms. All hormone treatments caused significantly greater reductions of hepatocyte hGR mRNA in men than women. Similarly, all hGH treatments caused a greater reduction in the accumulation of hGHR mRNA in men than women. Just as dexamethasone, alone or combined with hGH, elevated hGHR transcript concentrations in hepatocytes from men, the

treatment was considerably more effective when hepatocytes were obtained from women. Basically, we have observed that receptor transcription is less responsive to the suppressive effects of hormones and more responsive to their inductive effects when hepatocytes are from women. This sexually dimorphic responsiveness of receptor mRNA to hormone treatment extends to P450 isoform responsiveness. Accordingly, while the masculine-like pulsatile profile of hGH suppressed CYP3A4 expression, the suppression was significantly greater in men, which may relate to the isoform's female-predominance. Moreover, whereas both sex-dependent profiles of GH suppressed CYP1A2 expression, the magnitude of suppression was greater in hepatocytes obtained from men. In this regard, the same GH replacement regimen was significantly more suppressive of CYP3A4 enzymatic activity in boys than girls (Sinués et al., 2004). This intrinsic sex difference in GH regulation of P450s also has been reported in rats in which restoration of the feminine circulating GH profile was considerably more effective, both in vivo and in vitro, in restoring expression levels of P450 isoforms in females than males (Thangavel et al., 2004; Pampori and Shapiro, 1999). Similar inherent sexually dimorphic responses to the same GH regimen have been reported for insulin-like growth factor 1, bone mineralization, lipid metabolism and growth hormone binding protein; men>women (Johansson et al., 1999; Span et al., 2001; Hubina et al., 2004).

In summary, the present results demonstrate that like other species examined 1) GH can regulate expression of human isoforms of P450, 2) the sex-dependent secretory profiles of GH can differentially regulate expression levels of

some isoforms, 3) there are intrinsic sexual differences in hepatocytes of men and women resulting in different levels of responsiveness to GH and 4) in agreement with most species but the rat, GH effects on P450 although real, can be subtle and easily concealed by the heterogenous background of human populations.

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FOOTNOTES

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LEGENDS

Fig.1 CYP3A4 mRNA and protein expression in primary human hepatocytes from both men and women. Cells were exposed either to pulsatile (pulse) or constant (const) human growth hormone (GH) alone (open bars) or dexamethasone alone or in combination with GH (striped bars) for 5 days in culture. Procedural details are described in the text. Sufficient viable cells were isolated from a single liver for protein and mRNA determinations for every treatment presented in the figure. Values are presented as a percentage of protein or mRNA obtained from hepatocytes exposed to neither growth hormone nor dexamethasone (first bar on the extreme left of each graph) arbitrarily designated 100%. Each data point is a mean ± SD of cells from ≥ 8 individuals. ∗ $p<0.01$ compared to cells exposed to no hormone treatment of the same sex. † $p<0.01$ compared to cells exposed to dexamethasone alone of the same sex. § $p<0.01$ compares the effects of the same dose of pulsatile to constant GH; dexamethasone treatment and sex the same. ¶ $p<0.01$ compares the additive effect of dexamethasone on growth hormone exposure of the same sex. # $p<0.01$ compares the effects of sex (men vs. women) under otherwise identical treatments.

Fig.2. CYP3A4-dependent testosterone 6β-hydroxylase expression in primary human hepatocytes from women. Cells were exposed either to pulsatile (pulse) or constant (const) human growth hormone (GH) alone (open bars) or dexamethasone alone or in combination with GH (striped bars) for 5 days in

culture. Procedural details are described in the text. Sufficient viable cells were isolated from a single liver to measure hydroxylase activity for every treatment presented in the figure. Values are presented as a percentage of enzyme activity obtained from hepatocytes exposed to neither growth hormone nor dexamethasone (first bar on the extreme left of each graph) arbitrarily designated 100%. Each data point is a mean ± SD of cells from ≥ 8 individuals. ∗ $p<0.01$ compared to cells exposed to no hormone treatment. † $p<0.01$ compared to cells exposed to dexamethasone alone. § $p<0.01$ compares the effects of the same dose of pulsatile to constant GH; dexamethasone treatment the same. ¶ $p<0.01$ compares the additive effect of dexamethasone on growth hormone exposure.

Fig. 3. CYP1A2 mRNA and protein expression in primary human hepatocytes from both men and women. Cells were exposed either to pulsatile (pulse) or constant (const) human growth hormone (GH) alone (open bars) or dexamethasone alone or in combination with GH (striped bars) for 5 days in culture. Procedural details are described in the text. Sufficient viable cells were isolated from a single liver for protein and mRNA determinations for every treatment presented in the figure. Values are presented as a percentage of protein or mRNA obtained from hepatocytes exposed to neither growth hormone nor dexamethasone (first bar on the extreme left of each graph) arbitrarily designated 100%. Each data point is a mean ± SD of cells from ≥ 8 individuals. ∗ $p<0.05$ compared to cells exposed to no hormone treatment of the same sex. † $p<0.05$ compared to cells exposed to dexamethasone alone of the same sex. ¶

p<0.05 compares the additive effect of dexamethasone on growth hormone exposure of the same sex. # p<0.05 compares the effects of sex (men vs. women) under otherwise identical treatment.

Fig.4. CYP2D6 mRNA and protein expression in primary human hepatocytes from both men and women. Cells were exposed either to pulsatile (pulse) or constant (const) human growth hormone (GH) alone (open bars) or dexamethasone alone or in combination with GH (striped bars) for 5 days in culture. Procedural details are described in the text. Sufficient viable cells were isolated from a single liver for protein and mRNA determinations for every treatment presented in the figure. Values are presented as a percentage of protein or mRNA obtained from hepatocytes exposed to neither growth hormone nor dexamethasone (first bar on the extreme left of each graph) arbitrarily designated 100%. Each data point is a mean ± SD of cells from ≥ 8 individuals. * p<0.05 compared to cells exposed to no hormone treatment of the same sex. § p<0.05 compares the effects of the same dose of pulsatile to constant GH; dexamethasone treatment and sex the same.

Fig.5. Human growth hormone and glucocorticoid receptor mRNA expression in primary human hepatocytes from both men and women. Cells were exposed either to pulsatile (pulse) or constant (const) human growth hormone (GH) alone (open bars) or dexamethasone alone or in combination with GH (striped bars) for 5 days in culture. Procedural details are described in the text. Sufficient viable

cells were isolated from a single liver for determinations of both receptors for every treatment presented in the figure. Values are presented as a percentage of mRNA obtained from hepatocytes exposed to neither growth hormone nor dexamethasone (first bar on the extreme left of each graph) arbitrarily designated 100%. Each data point is a mean ± SD of cells from ≥ 8 individuals. ∗ $p<0.05$, ∗∗ $p<0.01$ compared to cells exposed to no hormone treatment of the same sex. † $p<0.05$, †† $p<0.01$ compared to cells exposed to dexamethasone alone of the same sex. § $p<0.05$, §§ $p<0.01$ compares the effects of the same dose of pulsatile to constant GH; dexamethasone treatment and sex the same. ¶ $p<0.05$, ¶¶ $p<0.01$ compares the additive effect of dexamethasone on growth hormone exposure of the same sex. # $p<0.05$, ## $p<0.01$ compares the effects of sex (men vs. women) under otherwise identical treatment.

Fig.1

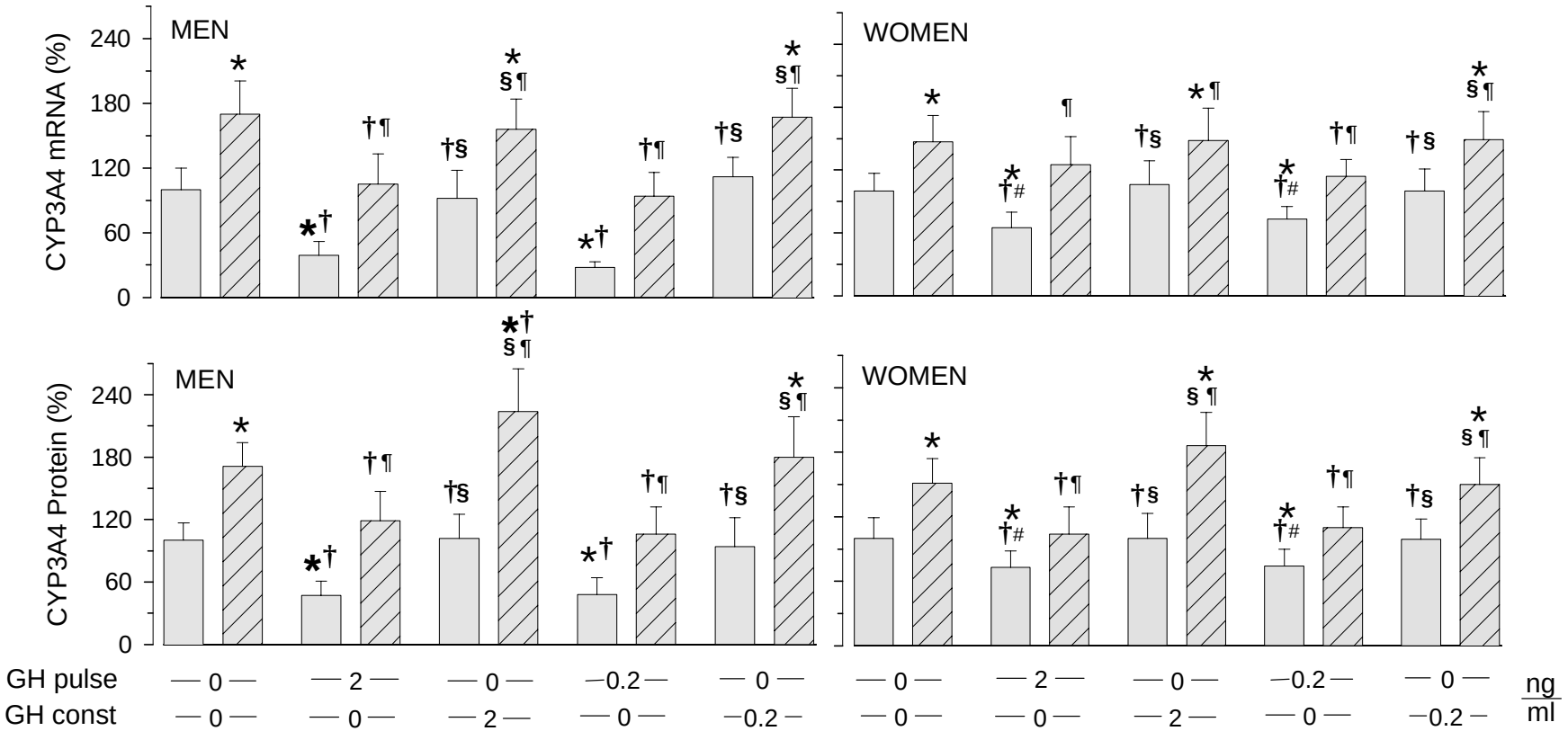

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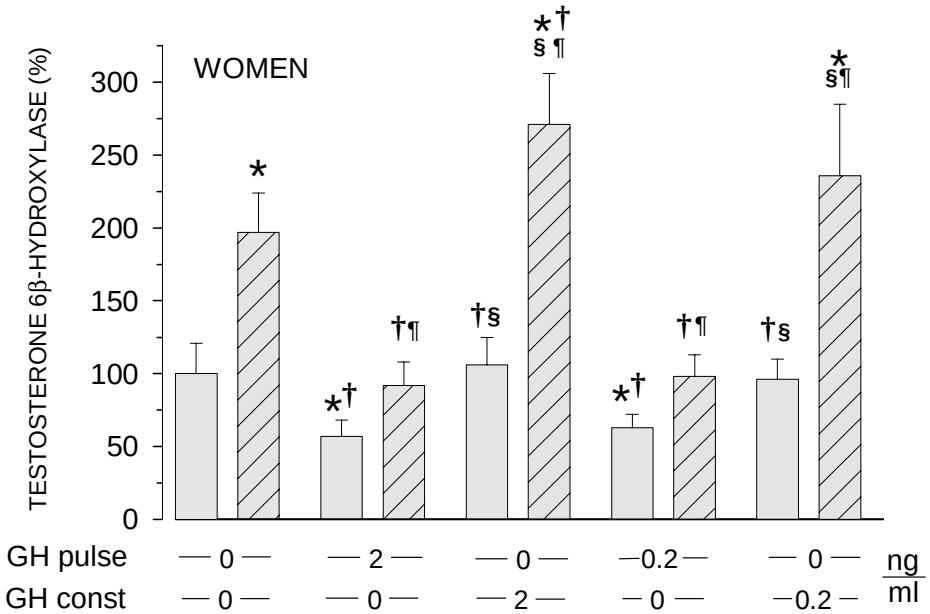
Fig.2



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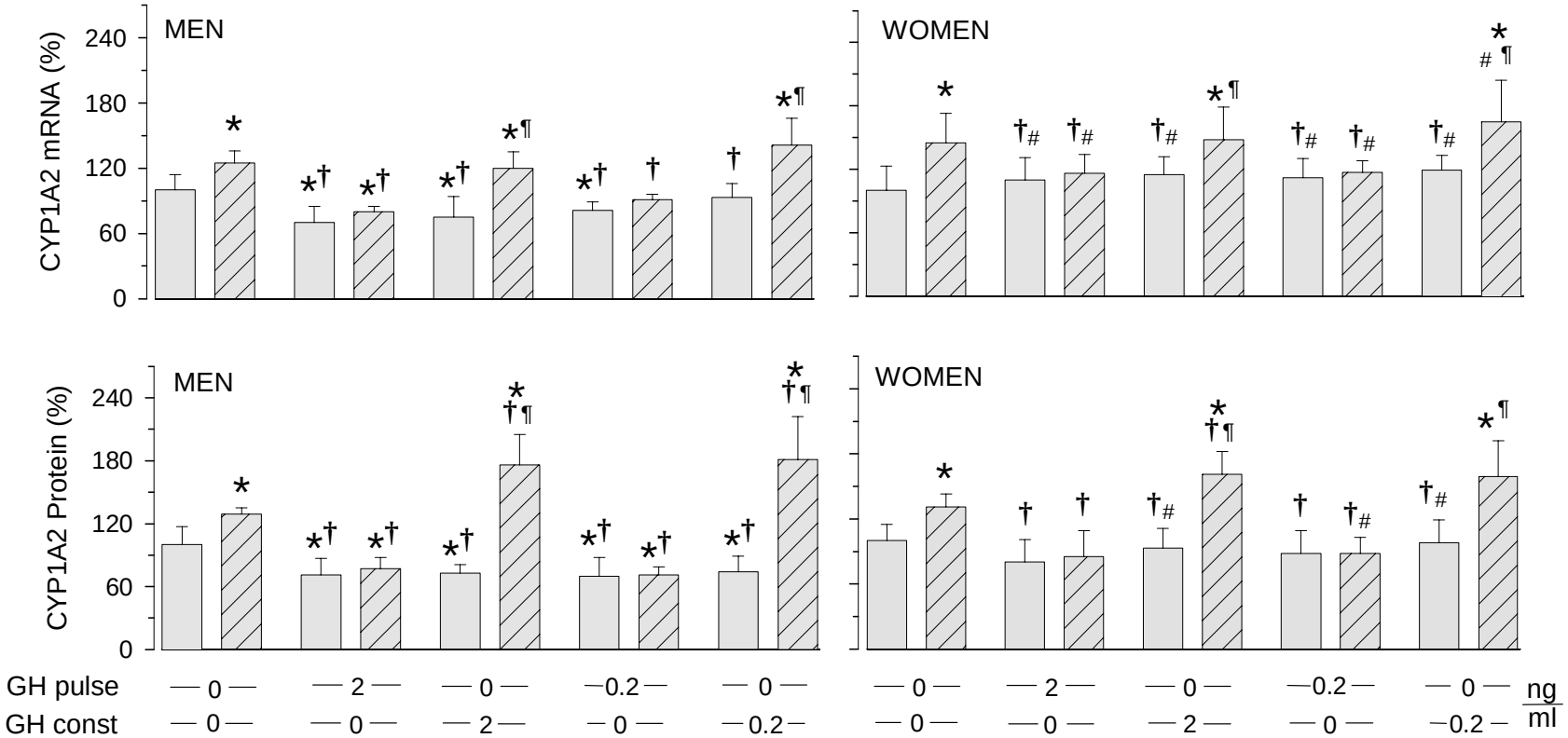

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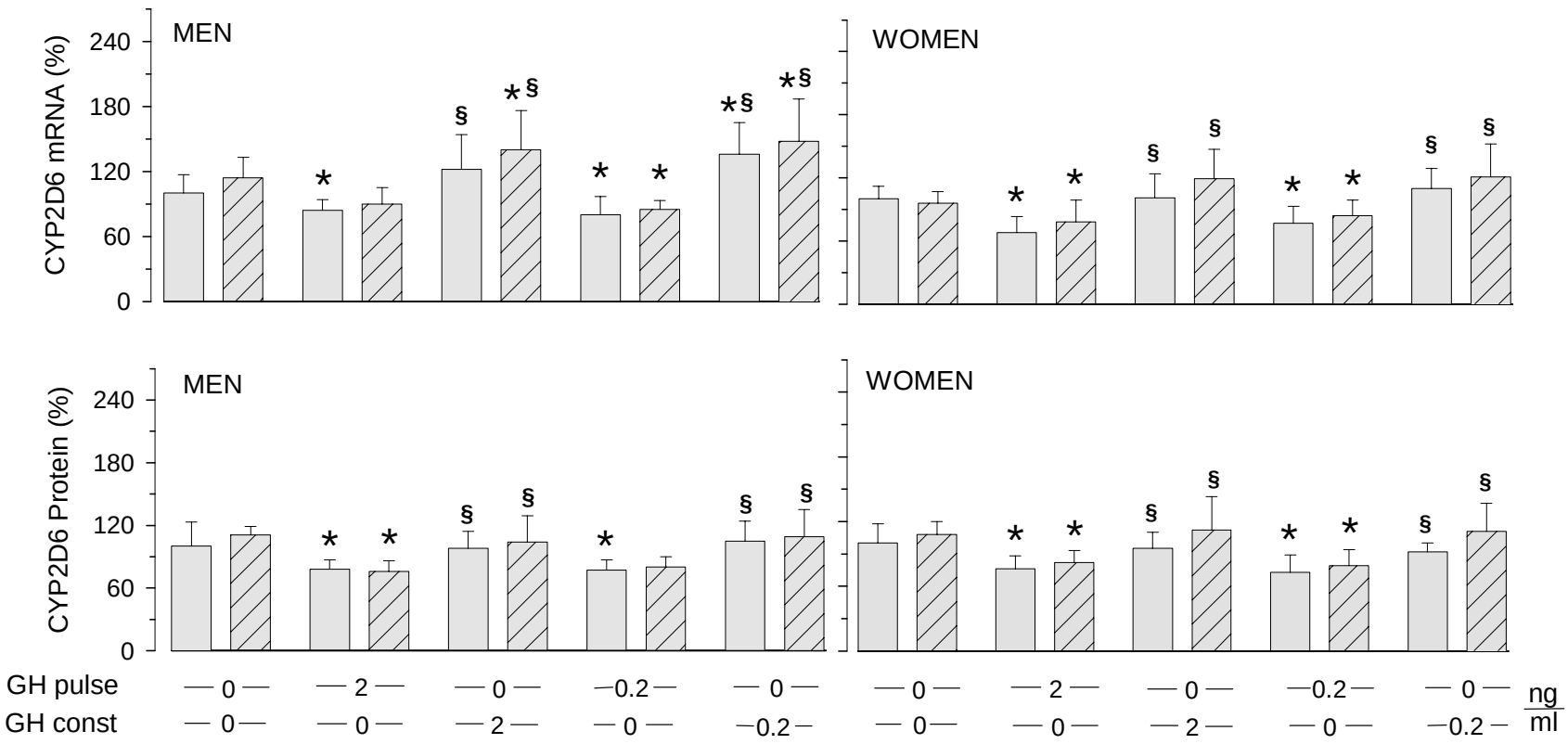

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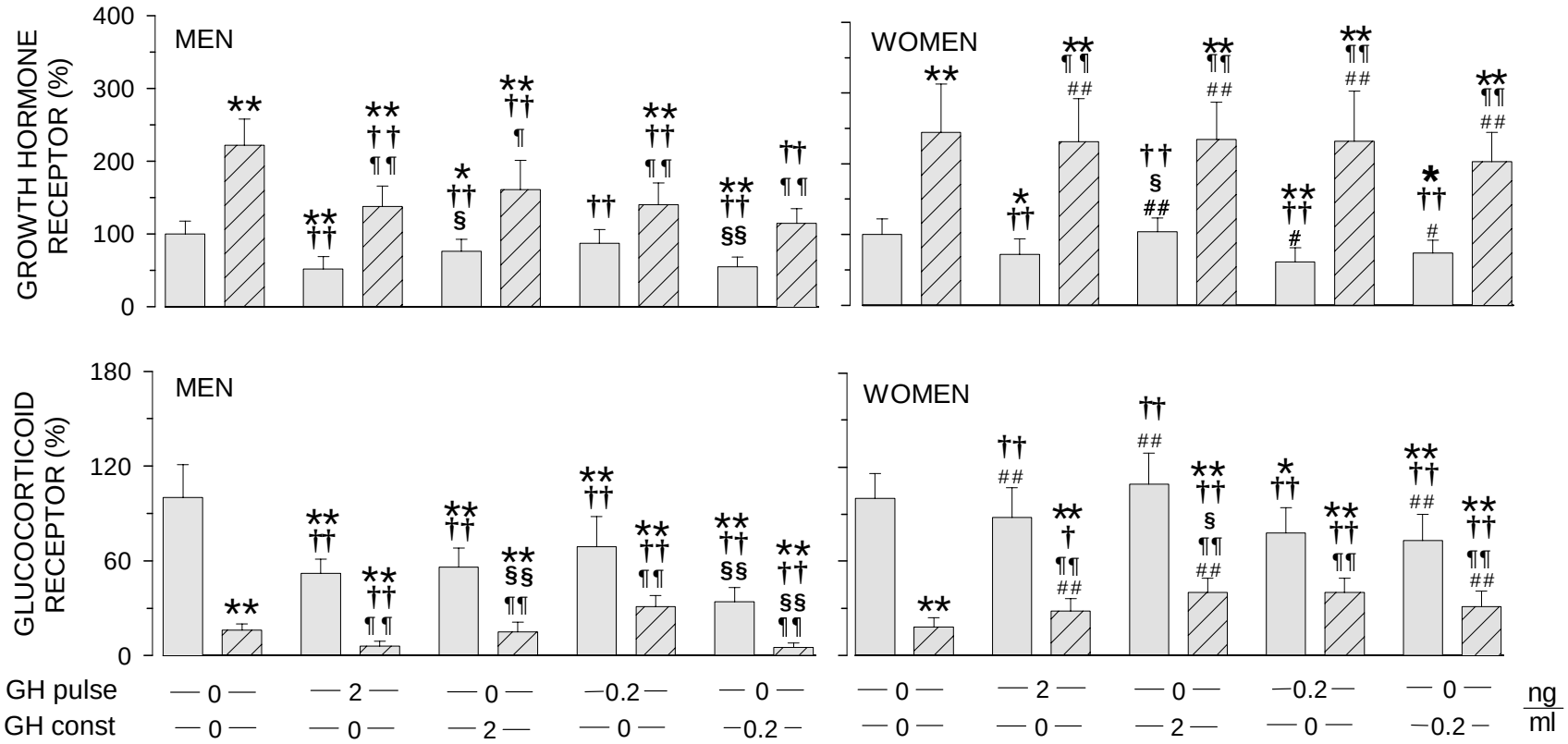
Fig.5



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