

Developmental Expression and Endocrine Regulation of CYP1B1 in Rat Testis

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ABBREVIATIONS: CYP, cytochrome P450; FSH, follicle-stimulating hormone; GH, growth hormone; LH, luteinizing hormone; PCR, polymerase chain reaction; T₄, thyroxine; T₃, 3,5,3'-triiodothyronine.

ABSTRACT:

Mammalian testis expresses xenobiotic-metabolizing enzymes, including cytochrome P450 1B1 (CYP1B1), which catalyzes the bioactivation of procarcinogens and other chemicals. The factors that control testicular expression of CYP1B1 are largely not known. In the present study, we investigated the influence of age and pituitary, gonadal, and thyroid hormones on CYP1B1 expression in rat testis. Immunoblot analysis demonstrated that testicular CYP1B1 protein was expressed at a level of 5.9 ± 2.0 (mean \pm S.E.M.) pmol/mg microsomal protein in prepubertal 22-day-old rats, whereas it was 6.6-fold greater in pubertal rats (34 days old) and 9.6-fold greater in adult rats (84-91 days old). Hypophysectomy decreased testicular CYP1B1 protein levels by 69% in adult rats, when compared to intact rats of the same age. Intermittent subcutaneous administration of growth hormone to hypophysectomized adult rats further decreased it by 63%. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) increased CYP1B1 expression in hypophysectomized rats, but they did not restore protein levels to those in intact adult male rats. Prolactin treatment alone had no effect; however, it potentiated the increase in CYP1B1 mRNA and protein expression by LH. 3,5,3'-Triiodothyronine (T_3), but not thyroxine (T_4), resulted in a small increase in testicular CYP1B1 protein levels. Similarly, treatment of hypophysectomized rats with testosterone propionate elicited a small increase in CYP1B1 protein expression. In contrast, treatment of intact adult male rats with 17β -estradiol benzoate decreased it by 91%. Overall, our findings indicate that rat testicular CYP1B1 protein expression is subject to developmental and endocrine control, with multiple hormones playing a role.

Introduction

The testis contains two distinct sets of cytochrome P450 (CYP) enzymes. One set consists of steroidogenic CYP enzymes (e.g. CYP11A1 and CYP17A1), which are involved in testosterone biosynthesis (Shan et al., 1993). The other set comprises those CYP enzymes, such as CYP1B1 (Otto et al., 1992), that play a major role in the biotransformation of hydrophobic xenobiotic compounds to more water-soluble metabolites. The physiological role of xenobiotic-metabolizing CYP enzymes in testis is not definitively known, but it has been proposed that they catalyze the oxidative biotransformation of lipophilic xenobiotic or endogenous compounds within the testis, which has important implications for testicular toxicity (Schuppe et al., 2000).

Gene expression profiling of various CYP mRNA transcripts in mouse (Choudhary et al., 2003) and human (Bieche et al., 2007) tissues have shown that CYP1B1 is one of the most abundantly expressed CYP genes in extrahepatic tissues, such as testis and adrenal gland, with little or no CYP1B1 mRNA or protein detectable in human liver (Chang et al., 2003). An investigation of the tissue distribution of CYP1B1 in rats detected CYP1B1 mRNA only in the testis and adrenal gland of male rats (Walker et al., 1995).

Previous studies have shown that expression of several rat hepatic microsomal CYP enzymes is subject to developmental and complex hormonal regulation (Waxman and Chang, 2005). Growth hormone (GH), or more specifically, the pattern of GH secretion from the pituitary, gonadal steroids (testosterone and estradiol), and thyroid hormones are involved in the regulation of various rat hepatic CYP enzymes, including CYP2C11, CYP2C12, and CYP3A2. Much less is known about the hormonal regulation of CYP enzyme expression in rat extrahepatic tissues. In particular, there is little information on the developmental expression or hormonal regulation of testicular CYP1B1 protein. Jefcoate and co-workers reported that hypophysectomy

decreased testicular CYP1B1 protein levels by approximately 60% in adult rats (Otto et al., 1992). They also studied the hormonal regulation of CYP1B1 in rat adrenal gland (Bhattacharyya et al., 1995; Brake et al., 1999). The testis, like the adrenal gland, is a steroidogenic tissue, but in contrast to the adrenal gland, it is subject to different hormonal influences (Haider, 2004). Thus, regulation of CYP1B1 expression in rat testis is likely to be different from that in the adrenal gland or other tissues.

In the present study, we characterized the developmental profile and investigated the influence of pituitary, gonadal, and thyroid hormones on the regulation of testicular CYP1B1 expression in rats. Our results indicate age-dependent expression of testicular CYP1B1 protein and suggest that although luteinizing hormone (LH) is the primary pituitary hormone controlling constitutive expression of CYP1B1, multiple hormones influence, either directly or indirectly, the regulation of this rat testicular enzyme.

Materials and Methods

Chemicals and Reagents. Rat recombinant GH was a gift from JCR Pharmaceuticals Co. Ltd. (Kobe, Japan). Testosterone propionate, 17β -estradiol 3-benzoate, 3,5,3'-triiodothyronine (T_3), thyroxine (T_4), follicle-stimulating hormone (FSH), LH, prolactin, and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). Rat CYP1B1-selective (anti-peptide) antibody and cDNA-expressed rat CYP1B1 protein were purchased from BD-GENTEST (Woburn, MA). Alkaline phosphatase-conjugated goat [Fab']₂ anti-rabbit IgG was bought from Biosource (Camarillo, CA). *p*-Nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate were obtained from Pierce (Rockford, IL). Platinum[®] *Taq* DNA polymerase, magnesium chloride, deoxynucleotide-5'-triphosphate, and SuperScript II[™] reverse transcriptase were bought from Invitrogen (Burlington, Ontario, Canada). PicoGreen[®] dsDNA Quantitation Kit was purchased from Molecular Probes, Inc. (Eugene, OR). QIAquick[®] Gel Extraction Kit was bought from QIAGEN Inc. (Mississauga, Ontario, Canada).

Animals. Intact and hypophysectomized adult male Sprague-Dawley rats (8 weeks old at the time of surgery) were obtained from Charles River, Inc. (St.-Constant, QC, Canada). Rats were housed on corn-cob bedding, with *ad libitum* access to food (Rodent Laboratory Diet #5002, PMI Feeds, Inc., Richmond, IN) and water. In the case of hypophysectomized rats, the drinking water was replaced with 5% glucose solution (Waxman et al., 1988). Animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

Treatment of Animals and Preparation of Microsomes. Hypophysectomized male rats were treated with GH, LH, FSH, prolactin, T_3 , T_4 , testosterone propionate, 17β -estradiol benzoate, or the vehicle, by s.c. injection, as indicated in each figure legend. For comparative

purposes, intact adult male rats were included as a reference group. All the animals were terminated on the morning after the last dose. Testes were immediately excised. A portion was snap-frozen in liquid nitrogen and then stored at -70°C for subsequent isolation of total RNA. The remainder of the testes was used immediately for preparation of testicular microsomes by differential ultracentrifugation (Thomas et al., 1981).

Microsomal Protein Assay and Determination of Total CYP Content. Microsomal protein concentrations were determined using the Bio-Rad Protein Assay Kit, with bovine serum albumin as the standard. The absorbance was measured at 595 nm. Total CYP content was determined from the sodium dithionite-reduced carbon monoxide difference spectrum, using a molar extinction coefficient of $91\text{ cm}^{-1}\text{ mM}^{-1}$ (Omura and Sato, 1964).

Gel Electrophoresis and Immunoblot Assay. CYP1B1 protein expression was determined by immunoblot analysis. Testicular microsomal proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrophoretically transferred onto nitrocellulose membranes, as described previously (Chang et al., 2003). Membranes were incubated with rabbit anti-rat CYP1B1 serum (1:500 dilution) for 2 h at 37°C , followed by a 2-h incubation with alkaline phosphatase-conjugated goat $[\text{Fab}']_2$ anti-rabbit IgG (Biosource, Camarillo, CA) at a dilution of 1:3000. Immunoreactive CYP1B1 protein bands were visualized by alkaline phosphatase-based colorimetric detection and quantified using a pdi 420oe scanning densitometer. Recombinant rat CYP1B1 (0.05-0.4 pmol/lane) was used as a calibration standard. Assay conditions were optimized to ensure that color development did not proceed beyond the linear range of the phosphatase reaction.

Isolation of Total RNA, Reverse Transcription, and Real-Time Polymerase Chain Reaction (PCR). Isolation and quantification of total RNA, reverse transcription, quantification

of total cDNA, and real-time PCR analysis were performed as described previously (Chang et al., 2003). Sequences for the forward (5'-CGC-CCT-CTG-ACA-AGA-AAC-TC-3') and reverse (5'-GTC-TCT-GCT-GTC-TCC-CCA-AG-3') primers to amplify rat CYP1B1 cDNA were designed using the Primer 3 software program (version 0.2, www-genome.wi.mit.edu) and synthesized at the Nucleic Acid and Protein Service Unit at the University of British Columbia. Each 20- μ l PCR reaction contained 1 unit of Platinum *Taq* DNA polymerase in 1 \times PCR buffer [20 mM Tris-HCl (pH 8.4) and 50 mM KCl], 5 mM magnesium chloride, 1 ng of total cDNA, 200 μ M of deoxynucleotide 5'-triphosphate mix, 0.2 μ M of each of forward and reverse primers, 0.25 mg/ml bovine serum albumin, and 2 μ l of a 1:3000 solution of SYBR Green I. Real-time amplification of CYP1B1 cDNA was performed in a LightCycler (Roche Diagnostics, Mannheim, Germany). The cycling condition was 95°C for 1 s (denaturation), 58°C for 6 s (annealing), and 72°C for 8 s (extension). The initial denaturation was performed at 95°C for 5 min. Calibration curves were constructed as described previously (Chang et al., 2003). The level of CYP1B1 cDNA expression was normalized to the amount of total cDNA, as quantified by the PicoGreen dsDNA Quantitation Kit.

Statistical Analysis. The significance of the difference between the means of two groups was assessed by the two-tailed, independent student's t-test. When there were more than two groups in the experiment, the significance of the difference between the group means was assessed by one-way or two-way analysis of variance and, where appropriate, was followed by the Student Newman-Keuls multiple comparison test. All the statistical analyses were performed using the SigmaStat software program (SPSS Inc., Chicago, IL). The level of significance was set *a priori* at $p < 0.05$.

Results

Developmental Expression of Testicular CYP1B1. Testicular CYP1B1 protein levels were determined in prepubertal (22 days old), pubertal (34 days old), and adult (84-91 days old) intact rats. The mean (\pm S.E.M.) CYP1B1 protein content of testis isolated from 22-day old rats was 5.9 ± 2.0 pmol per mg microsomal protein, as determined by densitometric quantification of immunoblots, and it was 7-fold greater in 34-day-old rats and 10-fold greater in adult rats (Fig. 1). A mean testicular CYP1B1 protein content of 57 pmol per mg microsomal protein was determined for adult rats. This value represents almost 70% of the total CYP content of testicular microsomes, based on a value of 80 pmol of total CYP per mg testicular microsomal protein, as determined in the present study.

Effects of Hypophysectomy. To investigate the role of the pituitary-testis axis in the regulation of testicular CYP1B1 expression, we first determined the effect of hypophysectomy on CYP1B1 expression in rat testis. Hypophysectomy of adult rats decreased testicular CYP1B1 protein levels by approximately 70% (Fig. 2). Analysis of a larger set of samples ($n = 18$) indicated that hypophysectomy was associated with a reduction in body weight (431 ± 4 g vs. 194 ± 6 g, mean \pm S.E.M.), absolute testes weight (3.39 ± 0.05 g vs. 0.54 ± 0.06 g), and relative testes weight ($0.79 \pm 0.01\%$ of body weight vs. $0.28 \pm 0.03\%$ of body weight).

Effect of Treatment with Pituitary Hormones. Hypophysectomy disrupts the pituitary-testis axis by altering secretion of pituitary hormones, such as GH (Jansson et al., 1985), LH (Mendis-Handagama, 1997), FSH (Ghosh et al., 1992), and prolactin (Ghosh et al., 1992). To investigate the effect of GH on testicular CYP1B1 protein expression, intact and hypophysectomized adult male rats were treated with GH ($0.12 \mu\text{g/g}$ body weight) (Kawai et al.,

2000) or an equivalent volume of the vehicle by twice daily s.c. injections for 7 consecutive days to mimic the male pulsatile GH secretory profile (Waxman et al., 1991). As shown in Fig. 2, intermittent GH injections did not restore CYP1B1 protein levels in hypophysectomized adult rats, but suppressed CYP1B1 protein levels by 63%, when compared to vehicle-treated, hypophysectomized adult rats (i.e. CYP1B1 protein content was 11% of the levels in intact adult rats). In contrast, intermittent GH injections did not affect testicular CYP1B1 levels in intact rats. These findings indicate that one or more pituitary-determined factors, other than GH, are necessary for the basal expression of testicular CYP1B1 in adult rats.

To investigate the effect of exogenous LH, FSH, and prolactin on testicular CYP1B1 expression, hypophysectomized adult male rats were treated with LH (0.12 unit/rat; equivalent to 100 μ g/rat), FSH (0.45 unit/rat; equivalent to 100 μ g/rat), or prolactin (4.6 I.U./rat; equivalent to 100 μ g/rat) by twice daily s.c. injections for 15 consecutive days, either individually or in various combinations. As shown in Fig. 3, when administered individually, LH and FSH, but not prolactin, increased testicular CYP1B1 protein expression in hypophysectomized rats, when compared to vehicle-treated, hypophysectomized rats, but the levels remained below that of intact, untreated adult male rats. Treatment with both LH and FSH was not more effective than either treatment alone. However, when LH was administered together with prolactin, CYP1B1 protein expression was increased above that produced by LH treatment alone to a level that was 72% of that in intact, untreated adult male rats. The combination of prolactin, LH, and FSH did not lead to a further increase, when compared to the group administered both prolactin and LH. Consistent with the protein data (Fig. 3), prolactin potentiated the effect of LH on CYP1B1 mRNA levels in hypophysectomized rats (Fig. 4).

Effect of Treatment with Gonadal Hormones. Hypophysectomy is known to disrupt androgen biosynthesis and to reduce circulating androgen levels (Ghosh et al., 1992). To determine if administration of exogenous androgen is capable of restoring testicular CYP1B1 expression, hypophysectomized adult rats were treated with testosterone propionate (0.5 or 5 mg/kg) or an equivalent volume of the vehicle by once daily s.c. injection for 14 consecutive days (Bandiera and Dworschak, 1992; Sundseth and Waxman, 1992). As shown in Fig. 5, treatment with testosterone propionate at both dosages increased testicular CYP1B1 protein levels to a similar extent (2-fold and 2.5-fold, respectively), when compared to the vehicle-treated group, but did not restore CYP1B1 expression to that of intact adult male rats.

The effect of estrogen on basal testicular CYP1B1 expression was assessed in intact adult rats. Treatment of intact adult male rats with estradiol benzoate (1.5 mg/kg) (Putz et al., 2001) by once daily s.c. injections for 14 consecutive days decreased testicular CYP1B1 protein expression by 91%, when compared to rats that received an equivalent volume of the vehicle (propylene glycol) (Fig. 6). Treatment with estradiol benzoate was as effective as hypophysectomy (Fig. 2) at suppressing testicular CYP1B1 expression.

Effect of Treatment with T₃ and T₄. To determine if thyroid hormones affected testicular CYP1B1 expression, hypophysectomized adult male rats were treated with T₃ (2 mg/kg/day), T₄ (50 µg/kg/day), or an equivalent volume of the vehicle by once daily s.c. injections for 7 consecutive days. The dosages of T₃ and T₄ are supraphysiological and receptor-saturating (Oppenheimer et al., 1977) and have been reported to render hypophysectomized rats euthyroid (Ram and Waxman, 1990). As shown in Fig. 7, treatment with T₃ increased testicular CYP1B1 protein expression by 3-fold, when compared to the vehicle-treated control group. Testicular CYP1B1 protein levels in T₃-treated rats were 13% of those in intact, untreated adult

male rats. In contrast, T_4 did not affect testicular CYP1B1 expression in hypophysectomized adult rats.

Discussion

CYP1B1 protein is expressed at a relatively high level in rat testis. At a protein content of 57 pmol/mg, CYP1B1 may be the most abundant CYP enzyme in testicular microsomes from adult rats. The reason why CYP1B1 is expressed at a high level in these tissues is not known. In an attempt to determine if CYP1B1 expression is dependent on hormonal factors, we investigated its ontogeny and the effect of hypophysectomy and administration of exogenous pituitary, gonadal, and thyroid hormones on testicular CYP1B1 levels.

Evidence that CYP1B1 gene expression may be developmentally regulated was provided by an oligonucleotide-based microarray gene profiling study of rat Leydig cells at different stages of development (Ge et al., 2005). CYP mRNA levels, however, do not necessarily correlate with protein levels. In the present study, we showed that CYP1B1 protein was present at relatively low levels in the testis of prepubertal rats and the levels increased between 22 and 34 days of age and between 34 and 70 days of age. The developmental increase in testicular CYP1B1 protein expression (Fig. 1) did indeed reflect the changes in CYP1B1 mRNA levels determined in rat Leydig cells representing progenitor, immature, and adult Leydig cells (Ge et al., 2005). The transition from progenitor to immature Leydig cells occurs between 21 and 35 days postpartum and the transition to adult Leydig cells occurs by 56 days of age. Leydig cell proliferation and differentiation are associated with morphological and biochemical changes, including changes in gene expression, and are accompanied by increases in testicular and circulating testosterone levels in the rat. mRNA expression profiles of CYP17A1 and CYP11A1 genes, which encode

the major steroidogenic enzymes, exhibit developmental increases similar to that of CYP1B1 (Ge et al., 2005).

The role of pituitary and gonadal hormones in the regulation of testicular CYP1B1 expression was also explored. An intact pituitary is essential for constitutive expression of testicular CYP1B1 because hypophysectomy dramatically suppressed CYP1B1 expression in testicular microsomes. Of the pituitary hormones examined, LH and FSH, when administered alone, had the greatest effect in increasing testicular CYP1B1 protein levels in hypophysectomized rats. LH is the principal regulator of Leydig cell function and regulates CYP-dependent testosterone biosynthesis in Leydig cells (Ewing et al., 1983). In contrast, the primary action of FSH on the testis is not on Leydig cells. Rather, FSH stimulates Sertoli cells to synthesize androgen-binding protein with subsequent stimulation of spermatogenic cell differentiation (Sriraman et al., 2005). Testicular CYP1B1 is assumed to be localized predominantly in Leydig cells based on data showing CYP1B1 mRNA or protein expression in rat and mouse Leydig cells in culture (Mandal et al., 2001; Zheng et al., 2003; Ge et al., 2005), but the cellular localization of testicular CYP1B1 has not been thoroughly investigated. Hence, it is possible that CYP1B1 is expressed in both Sertoli cells and Leydig cells.

Similar effects of hypophysectomy and LH treatment have been reported previously for another testicular CYP enzyme. Hypophysectomy results in decreased CYP17A1 expression, but the levels were increased by treatment with exogenous LH but not FSH (Takeyama et al., 1986). The molecular basis by which LH induced CYP1B1 protein levels is not clear at this time, but it may involve LH receptor activation and increased intracellular levels of cAMP, which is the mechanism by which LH increases expression of genes encoding CYP enzymes involved in androgen synthesis (Eacker et al., 2008).

Prolactin had no effect when administered alone, but it increased testicular CYP1B1 protein expression when administered in combination with LH. Prolactin has been reported to increase the number of LH receptors on rat Leydig cells and to potentiate the effect of exogenous LH on testosterone synthesis (Takeyama et al., 1986). It is possible that increased LH receptor expression may be the basis for the increase in testicular CYP1B1 protein levels observed after the combination treatment of LH and prolactin. Such a mechanism has been proposed for the previous finding that prolactin potentiates the effect of LH on the testicular expression of steroid 5 α -reductase (Takeyama et al., 1986).

GH does not appear to play a positive role in testicular CYP1B1 expression because intermittent administration of GH to mimic the male pattern of endogenous GH secretion (Waxman et al., 1991) did not restore testicular CYP1B1 levels. We had anticipated that GH may be involved in the positive regulation of CYP1B1 because GH is involved in testicular development (Hull and Harvey, 2000) and because GH, especially the pattern of GH secretion from the pituitary, has been shown to play an important role in the regulation of several hepatic CYP enzymes (Waxman and Chang, 2005). For example, intermittent administration of exogenous GH to hypophysectomized rats completely restores hepatic CYP2C11 levels in male rats and suppresses CYP2C12 levels in female rats, whereas continuous GH infusion (female-type secretion) had the opposite effect. It is clear that regulation of testicular CYP1B1 is different from that of hepatic CYP2C11 and CYP2C12.

Administration of testosterone to hypophysectomized rats did not restore testicular CYP1B1 protein levels, suggesting that androgens are not involved in regulating constitutive CYP1B1 expression in rat testis. Testosterone acting through the androgen receptor is needed for normal development of the testis (Haider, 2004). Previous studies showed that treatment of

intact rats with high dosages of testosterone reduces LH secretion from the pituitary, represses transcription of genes involved in steroidogenesis, including CYP17A1 and CYP11A1, and inhibits spermatogenesis (Keeney and Ewing, 1990; Eacker et al., 2008). Treatment of adult hypophysectomized rats with testosterone can partially restore spermatogenesis, but it had little effect on Leydig cell proliferation or function (Keeney and Ewing, 1990). Our results indicate that testosterone has minimal effect on testicular CYP1B1 expression in the absence of LH.

It is now generally accepted that estrogens affect testicular development and function. Consistent with this view are the experimental findings indicating that estrogens can be synthesized in testis by CYP19-catalyzed aromatization of androgens (Abney, 1999) and that estrogen receptors (both the α and β isoforms) are expressed in testis (Fisher et al., 1997; van Pelt et al., 1999). In the present study, treatment of intact rats with exogenous estradiol had a profound negative effect on testicular CYP1B1 expression. A possible explanation for this finding is that estrogens disrupt the hypothalamic-pituitary axis, resulting in decreased serum levels of LH (Putz et al., 2001). As shown in the present study, LH is a positive regulator of testicular CYP1B1 expression. However, another possible explanation for our finding is that estradiol suppresses testicular CYP1B1 expression by a mechanism independent of LH. This proposal is consistent with the observation that administration of exogenous estradiol to intact adult rats leads to decreased testicular mRNA levels of steroidogenic enzymes, especially CYP17A1 and CYP11A1, but this occurs without altering serum LH levels (Sakaue et al., 2002).

The testis has been thought of as an organ that is non-responsive to thyroid hormones (Wagner et al., 2008). However, it is now recognized that thyroid hormones play a role in testicular development and function. There is no information on the role of thyroid hormone in testicular expression of CYP enzymes, except that that T_3 suppresses CYP19 mRNA expression

and enzyme activity in Sertoli cells isolated from intact prepubertal rats (Ando et al., 2001). Our data show that T_3 [which is the bioactive form of thyroid hormone (Wagner et al., 2008)], but not T_4 (a precursor of T_3), increased testicular CYP1B1 protein expression. However, T_3 did not fully restore the levels to those in intact adult male rats. The reason for the lack of an effect by T_4 is not clear. T_4 is metabolized to T_3 by iodothyronine deiodinase enzymes D1 and D2 (Wagner et al., 2008). Although these deiodinase enzymes are expressed in testis in intact rats (Bates et al., 1999), it is not known if their expression is compromised following hypophysectomy. Overall, T_3 appears to be a pituitary-dependent hormone that contributes minimally to the positive regulation of testicular CYP1B1 expression.

In summary, the major findings from the present study indicate that: 1) rat testicular CYP1B1 protein expression is developmentally regulated; 2) hypophysectomy of adult rats decreases testicular CYP1B1 expression; 3) the reduced CYP1B1 expression in hypophysectomized adult male rats is further decreased by intermittent GH pulses (i.e. twice daily s.c. injections), whereas it is increased by LH, FSH, and, to a much lesser extent, testosterone and T_3 ; 4) prolactin potentiates the increase in testicular CYP1B1 expression by LH in hypophysectomized adult rats; and 5) estrogen suppresses testicular CYP1B1 expression in intact male rats.

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Footnotes

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Figure Legends

FIG. 1. Influence of age on testicular CYP1B1 protein expression in intact male rats. Testicular microsomes were isolated from prepubertal (22 days old), pubertal (34 days old), and adult (84-91 days old) rats. CYP1B1 protein was detected with rat CYP1B1-selective (anti-peptide) antibody (1:500 dilution) followed by alkaline phosphatase-conjugated goat [Fab']₂ anti-rabbit IgG (1:3000 dilution). The intensity of the stained bands on the nitrocellulose membranes was quantified by densitometric analysis. Calibration curves were constructed with known amounts of cDNA-expressed rat CYP1B1 protein. Data are expressed as mean \pm S.E.M. for 3-7 individual rats per group. *Significantly different from 22-day old rats. **Significantly different from 22-day old and 34-day old rats.

FIG. 2. Effect of hypophysectomy and intermittent GH administration on testicular CYP1B1 expression in adult male rats. Intact and hypophysectomized adult male rats were treated with GH (0.12 μ g/g body weight) or an equivalent volume (0.1 ml/100 g body weight) of the vehicle (10 mM potassium phosphate, pH 8.3, containing 0.9% NaCl), administered as twice daily s.c. injections for 7 consecutive days. All the animals were terminated on the morning after the last dose. Testicular microsomes were prepared and CYP1B1 protein levels were quantified by immunoblot analysis. Data are expressed as mean \pm S.E.M. for 3 or 4 individual rats per treatment group. *Significantly different from vehicle-treated and GH-treated intact rats. **Significantly different from vehicle-treated hypophysectomized rats and vehicle-treated intact rats.

FIG. 3. Effect of LH, FSH, and prolactin on rat testicular CYP1B1 protein expression in hypophysectomized adult male rats. Hypophysectomized adult male rats were treated with LH (0.12 unit/rat; equivalent to 100 μ g/rat), FSH (0.45 unit/rat; equivalent to 100 μ g/rat), prolactin (PRL; 4.6 I.U./rat; equivalent to 100 μ g/rat), or a combination of FSH and LH, PRL and LH, or PRL, FSH, and LH, administered as twice daily s.c. injections for 15 consecutive days. Control rats received an equal volume (0.1 ml) of the vehicle (0.9% NaCl). All the animals were terminated on the morning after the last dose. Untreated intact adult male rats were included for comparative purposes. Testicular microsomes were prepared and CYP1B1 protein levels were quantified by immunoblot analysis. Data are expressed as mean \pm S.E.M. for 6 individual rats per treatment group. *Significantly different from vehicle-treated hypophysectomized rats. **Significantly different from vehicle-treated hypophysectomized rats and untreated, intact rats.

FIG. 4. Effect of prolactin and LH on rat testicular CYP1B1 mRNA expression in hypophysectomized adult male rats. Real-time PCR analysis was performed to determine CYP1B1 mRNA expression in testis samples (Fig. 3) from intact adult rats and from hypophysectomized adult male rats treated with LH, prolactin (PRL), both LH and PRL, or an equal volume (0.1 ml) of the vehicle (0.9% NaCl). Data are expressed as mean \pm S.E.M. for 6 individual rats per treatment group. *Significantly different from vehicle-treated hypophysectomized rats. **Significantly different from all other groups.

FIG. 5. Effect of androgen administration on testicular CYP1B1 protein expression in hypophysectomized adult male rats. Hypophysectomized adult male rats were treated with testosterone propionate (0.5 or 5 mg/kg) or an equal volume (0.1 ml/100 g body weight) of the

vehicle (propylene glycol), administered as once daily s.c. injections for 14 consecutive days. Untreated intact adult male rats were included for comparative purposes. All the animals were terminated on the morning after the last dose. Testicular microsomes were prepared and CYP1B1 protein levels were quantified by immunoblot analysis. Data are expressed as mean \pm S.E.M. for 6 individual rats per treatment group. *Significantly different from vehicle-treated, hypophysectomized rats. **Significantly different from all other groups.

FIG. 6. Effect of estrogen administration on testicular CYP1B1 protein expression in intact adult male rats. Intact adult male rats were untreated or treated with estradiol benzoate (1.5 mg/kg) or an equal volume (0.1 ml/100 g body weight) of the vehicle (propylene glycol), administered as once daily s.c. injections for 14 consecutive days. All the animals were terminated on the morning after the last dose. Testicular microsomes were prepared and CYP1B1 protein levels were quantified by immunoblot analysis. Data are expressed as mean \pm S.E.M. for 5 individual rats per treatment group. *Significantly different from vehicle-treated rats.

FIG. 7. Effect of T₃ and T₄ administration on testicular CYP1B1 protein expression in hypophysectomized adult male rats. Hypophysectomized adult male rats were treated with T₃ (2 mg/kg), T₄ (50 μ g/kg), or an equal volume (0.1 ml/100 g body weight) of the vehicle (10 mM potassium phosphate, pH 8.3, containing 0.9% NaCl), administered as once daily s.c. injections for 7 consecutive days. Untreated intact adult male rats were included for comparative purposes. All the animals were terminated on the morning after the last dose. Testicular microsomes were prepared and CYP1B1 protein levels were quantified by immunoblot analysis. Data are

expressed as mean \pm S.E.M. for 6 individual rats per treatment group. *Significantly different from vehicle-treated hypophysectomized rats. **Significantly different from all other groups.













