Developmental Expression and Endocrine Regulation of CYP1B1 in Rat Testis

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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 showed that testicular CYP1B1 protein was expressed at a level of 5.9 ± 2.0 (mean ± 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 with 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 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, but not thyroxine, resulted in a small increase in testicular CYP1B1 protein levels. Likewise, 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 testicular CYP1B1 protein expression is subject to developmental and endocrine control, with multiple hormones playing a role.

The testis contains two distinct sets of cytochrome P450 (P450) enzymes. One set consists of steroidogenic P450 enzymes (e.g., CYP11A1 and CYP17A1), which are involved in testosterone biosynthesis (Shan et al., 1993). The other set comprises those P450 enzymes, such as CYP1B1 (Otto et al., 1992), which play a major role in the biotransformation of hydrophobic xenobiotic compounds to more water-soluble metabolites. The physiological role of xenobiotic-metabolizing P450 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 P450 mRNA transcripts in mouse (Choudhary et al., 2003) and human (Bièche et al., 2007) tissues have shown that CYP1B1 is one of the most abundantly expressed P450 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 P450 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 P450 enzymes, including CYP2C11, CYP2C12, and CYP3A2. Much less is known about the hormonal regulation of P450 enzyme expression in rat extrahepatic tissues. In particular, there is little information on the developmental expression or hormonal regulation of testicular CYP1B1 protein. Otto et al. (1992) reported that hypophysectomy decreased testicular CYP1B1 protein levels by approximately 60% in adult rats. They also studied the hormonal regulation of CYP1B1 in rat adrenal gland (Bhattacharya 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.
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 (T₃), thyroxine (T₄), follicle-stimulating hormone (FSH), LH, prolactin, and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). Rat CYP1B1-selective (antipeptide) 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 International (Camarillo, CA).

p-Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl 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 Canada Inc. (Burlington, ON, Canada). QIAGen dsDNA Quantitation Kit was purchased from Invitrogen (Carlsbad, CA). QiAquick Gel Extraction Kit was bought from QIAGEN Inc. (Mississauga, ON, 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 no. 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, T₃, T₄, testosterone propionate, 17β-estradiol benzoate, or the vehicle by subcutaneous 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 P450 Content. Microsomal protein concentrations were determined using the Bio-Rad (Hercules, CA) Protein Assay Kit, with bovine serum albumin as the standard. The absorbance was measured at 595 nm. Total P450 content was determined from the sodium dithionite-reduced carbon monoxide difference spectrum using a molar extinction coefficient of 91 cm⁻¹/mM⁻¹ (Omura and Sato, 1964).

Real-Time Polymerase Chain Reaction (PCR) Analysis. Total RNA was isolated from testes using QIAquick Gel Extraction Kit from Qiagen (Mississauga, ON, Canada). PicoGreen dsDNA Quantitation Kit was purchased from Invitrogen (Carlsbad, CA). 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 (Invitrogen).

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- or two-way analysis of variance and, where appropriate, was followed by the 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 (±S.E.M.) CYP1B1 protein content of testis isolated from 22-day-old rats was 5.9 ± 2.0 pmol/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/mg microsomal protein was determined for adult rats. This value represents almost 70% of the total P450 content of testicular microsomes based on a value of 80 pmol of total P450/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 testes. Hypophysectomy of adult rats decreased testicular CYP1B1 protein levels by approximately 70% (Fig. 2). Analysis of a

![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 (antipeptide) 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 ± S.E.M. for three to seven individual rats per group.*: Significantly different from 22-day-old rats. **: Significantly different from 22-day-old and 34-day-old rats.](http://www.dmd.aspetjournals.org/article/524/LEUNG.jpg)
larger set of samples ($n = 18$) indicated that hypophysectomy was associated with a reduction in body weight ($431 \pm 4$ versus $194 \pm 6$ g, mean $\pm$ S.E.M.), absolute testes weight ($3.39 \pm 0.05$ versus $0.54 \pm 0.06$ g), and relative testes weight ($0.79 \pm 0.01$ versus $0.28 \pm 0.03$% b.wt.).

**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 µg/g b.wt.) (Kawai et al., 2000) or an equivalent volume of the vehicle by twice-daily subcutaneous 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 with 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 µg/rat), FSH (0.45 unit/rat; equivalent to 100 µg/rat), or prolactin (4.6 IU/rat; equivalent to 100 µg/rat) by twice-daily subcutaneous 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 with vehicle-treated, hypophysectomized rats, but the levels remained below that of intact, untreated adult male rats. The combination of prolactin, LH, and FSH did not lead to a further increase when compared with 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 whether 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 subcutaneous 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- and 2.5-fold, respectively) when compared with 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 subcutaneous injections for 14 consecutive days decreased testicular CYP1B1 protein expression by 91% when compared with 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 whether 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 subcutaneous 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 with the vehicle-treated control group. Testicular CYP1B1 protein levels in T$_3$-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 P450 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 whether 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). However, P450 mRNA levels 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 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 P450-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 P450 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 P450 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 5α-reductase (Takeyama et al., 1986).

GH does not seem 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 P450 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 testes. 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 nonresponsive 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 P450 enzymes, except that T3 suppresses CYP19 mRNA expression and enzyme activity in Sertoli cells isolated from intact prepubertal rats (Andò et al., 2001). Our data show that T3 (which is the bioactive form of thyroid hormone) (Wagner et al., 2008), but not T4 (a precursor of T3), increased testicular CYP1B1 protein expression. However, T4 did not fully restore the levels to those in intact adult male rats. The reason for the lack of an effect by T4 is not clear. T3 is metabolized to T2 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 whether their expression is compromised after hypophysectomy. Overall, T3 seems 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 subcutaneous injections), whereas it is increased by LH, FSH, and, to a much lesser extent, testosterone and T3; 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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