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Growth Hormone Regulation and Developmental Expression of Rat Hepatic CYP3A18, CYP3A9, and CYP3A2

*Masahiko Kawai, Stelvio M. Bandiera, Thomas K. H. Chang and Gail D. Bellward**

Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada

ABSTRACT. The present study investigated the role of growth hormone (GH) in hepatic CYP3A18 and CYP3A9 expression in prepubertal and adult male rats. For comparison, the effects of GH on CYP3A2 expression were also measured. Initial experiments demonstrated that CYP3A18 mRNA levels were greater during puberty and adulthood than during the prepubertal period, CYP3A9 mRNA was not expressed until puberty and its expression increased in adulthood, and CYP3A2 mRNA levels were relatively constant from prepuberty to adult life. Hypophysectomy, which results in the loss of multiple pituitary factors including GH, increased CYP3A2 and CYP3A18 mRNA expression 3- to 4-fold, but it did not affect CYP3A9 mRNA levels or CYP3A-mediated testosterone 2β- or 6β-hydroxylase activity in adult rats. GH administered as twice daily s.c. injections (0.12 μg/g body weight) to hypophysectomized or intact adult rats did not affect CYP3A18 or CYP3A9 mRNA expression. The same treatment decreased CYP3A2 mRNA and protein and testosterone 2β- and 6β-hydroxylase activity levels in intact but not hypophysectomized rats. However, in intact prepubertal rats, intermittent GH administration decreased CYP3A18 and CYP3A2 mRNA levels, but a higher dosage (3.6 μg/g) was required to suppress CYP3A2. Overall, the present study demonstrated that: (a) the constitutive expression of CYP3A18, CYP3A9, and CYP3A2 does not require the presence of GH, (b) CYP3A18 is more sensitive than CYP3A9 to GH modulation in adult rats; and (c) CYP3A2 is less sensitive to the suppressive influence of GH during the prepubertal period than during adult life. BIOCHEM PHARMACOL **59**;10:1277–1287, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. CYP3A2; CYP3A9; CYP3A18; growth hormone; testosterone 2β-hydroxylase; testosterone 6β-hydroxylase

Enzymes belonging to the CYP3A subfamily are major catalysts in the oxidative biotransformation of physiologic compounds such as steroids [1] and of antineoplastic, immmunosuppressive, and other therapeutic agents [2], as well as being involved in the bioactivation of various chemical procarcinogens [3]. CYP3A enzymes are found predominantly in liver [4] and are also present in extrahepatic tissues including the brain [5, 6], intestine [7, 8], kidney [9], and leukocytes [10]. Several laboratories have reported the existence of multiple rat CYP3A enzymes with similar substrate specificity and immunochemical reactivity [11–17]. Currently, CYP3A1, CYP3A2, CYP3A9, CYP3A18, and CYP3A23 are classified in the rat CYP3A subfamily [18], but recent evidence suggests that CYP3A1 and CYP3A23 are the same enzyme [19]. Most, if not all, hepatic CYP3A enzymes appear to be inducible to varying degrees by a diverse group of structurally unrelated compounds including glucocorticoids [17, 20–22], anticonvulsants [21, 23], and polychlorinated biphenyls [24]. There is evidence indicating that CYP3A1 is not expressed at appreciable levels in the liver of the untreated rat [9, 17, 25], but hepatic levels of other CYP3A enzymes have been measured [9, 17, 21, 26]. Of the CYP3A forms characterized to date, CYP3A2, CYP3A9, and CYP3A18 appear to exhibit age- and sex-dependent constitutive hepatic expression. The neuroendocrine mechanism for the sex- and age-related changes in CYP3A expression is not understood fully. The sexually dimorphic pattern of GH† secretion, which is not well defined until after puberty [27] and is influenced in part by estrogen and testosterone [28], may play a key role in the regulation of these enzymes.

CYP3A2 is expressed constitutively in prepubertal male and female rats and in adult male, but not in adult female rats [17, 29–31]. Despite being the best studied sexually regulated CYP3A enzyme in rat liver, the developmental expression of CYP3A2 is still not clear. Various studies have reported that CYP3A2 mRNA levels in prepubertal

* Corresponding author: Dr. G. D. Bellward, Division of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, The University of British Columbia, 2146 East Mall, Vancouver, B.C., V6T 1Z3, Canada. Tel. (604) 822-4103; FAX (604) 822-3035; E-mail: gailbe@unixg.ubc.ca
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† *Abbreviations*: GH, growth hormone; and RT–PCR, reverse transcription–polymerase chain reaction.

male rats were less than [32], greater than [31], or comparable to [17, 21, 30] those in adult male rats. Conflicting data also exist regarding the developmental and sexual expression of hepatic CYP3A18 [21, 26]. In the case of CYP3A9, however, there seems to be a consensus that CYP3A9 mRNA levels are greater in adult female rats than in adult male rats [21, 33, 34].

The pituitary regulation of hepatic CYP3A2 expression was examined in earlier studies, which showed that hypophysectomy of adult male rats increases hepatic CYP3A2 mRNA [35, 36] and protein levels [29, 37]. Administration of GH to hypophysectomized male rats by twice daily injections results in no change [37] or a decrease [29] in CYP3A2 expression. In contrast, neonatal administration of monosodium glutamate (MSG), which results in a selective loss of GH [38], suppresses expression of CYP3A2 protein in intact adult male rats [39], and intermittent GH injection stimulates CYP3A2 expression in these rats [40]. The basis for the differential effect of intermittent GH treatment on the expression of this CYP in hypophysectomized and MSG-treated adult male rats is not well understood. Possible contributing factors include the specificity of the antibody probe used in the different studies [29, 37, 40] and the age at which the rats were rendered GH-deficient.

The role of the hypothalamic–pituitary axis in the regulation of CYP3A18 and CYP3A9 was explored in a recent study. Administration of GH by continuous infusion, as a means to mimic the continuous pattern of endogenous GH secretion in female rats, was shown to suppress CYP3A18 mRNA and increase CYP3A9 mRNA levels in intact adult male rats [34]. However, it remains to be determined if GH is necessary for the constitutive expression of CYP3A18 and CYP3A9 and if the intermittent pulsatile masculine pattern of GH secretion influences the expression of these enzymes in adulthood and during development. While previous studies have investigated the role of GH in the regulation of CYP enzymes in adulthood [41, 42], there is no information on the effects of GH on CYP enzyme expression during the prepubertal period, a time at which the pattern of GH secretion is not sexually differentiated [27]. The plasma GH profile is characterized by low trough levels interspersed with infrequent pulses of very small peak heights in prepubertal male and female rats. The biological effects of GH during the developmental period is of interest because of the increasing therapeutic use of this hormone in short-stature children, including those without GH deficiency [43].

The present report describes experiments designed to investigate the effect of hypophysectomy and intermittent GH administration on hepatic expression of CYP3A18, CYP3A9, and CYP3A2, and CYP3A-mediated testosterone 2β- and 6β-hydroxylase activities in prepubertal and adult male rats. The effect of sex and age on hepatic levels of these enzymes was also examined.

MATERIALS AND METHODS

Chemicals and Reagents

Rat recombinant GH was a gift from JCR Pharmaceuticals Co. Ltd. Testosterone was bought from the Sigma Chemical Co. Authentic 2β- and 6β-hydroxytestosterone metabolite standards were purchased from Steraloids, Inc. NADPH was obtained from Boehringer Mannheim. p-Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP) were bought from Pierce. Magnesium chloride, 10x PCR buffer II (100 mM Tris–HCl, pH 8.3, and 500 mM KCl), and AmpliTaq® DNA polymerase were purchased from Perkin-Elmer Canada Ltd. TriZol™, dithiothreitol, dNTP mix, oligo(dT)$_{12–16}$ primer, deoxyribonuclease I, and Superscript™ II reverse transcriptase were bought from Canadian Life Technologies. Forward and reverse primers for CYP3A2, CYP3A9, CYP3A18, cyclophilin, and β-actin were synthesized at the University of British Columbia Biotechnology Laboratory.

Animals

Intact 21-day-old male and 10-week-old male and female Sprague–Dawley rats were obtained from the Animal Care Center of the University of British Columbia. Hypophysectomized adult male rats were purchased from Charles River Breeding Laboratories. Rats were hypophysectomized by the supplier at the age of 8 weeks, and the animals were delivered at 10 weeks of age. Prior to performing the study, the effectiveness of hypophysectomy was confirmed by the absence of body weight gain for a week. The animals were allowed free access to water and food (Rodent Laboratory Diet, PMI Feeds, Inc.) and were housed in a temperature (23°) and light-controlled (7:00 a.m. on and 7:00 p.m. off) room. The drinking water for the hypophysectomized rats was replaced with 5% glucose solution [37].

Treatment of Animals

To characterize the developmental expression of CYP3A2, CYP3A9, and CYP3A18, intact male Sprague–Dawley rats were killed at 22, 34, 51, and 84–91 days of age. To determine the effect of intermittent administration of GH on hepatic CYP3A expression, intact and hypophysectomized adult rats were injected s.c. with rat GH (0.12 μg/g body weight at 8:00 a.m. and 5:00 p.m. for 7 consecutive days) or an equivalent volume of the vehicle containing 10 mM potassium phosphate (pH 8.3) and 0.9% sodium chloride [37]. To determine the effect of intermittent administration of GH on CYP3A expression in the prepubertal period, intact rats were injected s.c. with rat GH (0.12 or 3.6 μg/g body weight) at 8:00 a.m. and 5:00 p.m. on days 22–33 of age. Control rats received an equal volume of the vehicle. The lower dosage of GH has been reported to be effective in stimulating hepatic CYP expression [44]. The higher dosage is similar to the one used in an

earlier study that reported increased somatic growth caused by GH [45].

Preparation of Liver Microsomes

Rats were killed by decapitation 1 day after the last injection. One portion of the liver tissues was quick-frozen in liquid nitrogen and subsequently stored at −75° until used for RNA isolation. The remainder of the liver was used to prepare microsomes according to the method of Thomas *et al.* [46].

Total CYP and Microsomal Protein Assays

Total CYP content was determined from the sodium dithionite-reduced carbon monoxide difference spectrum, using a molar extinction coefficient of 91 cm^{-1} mM^{-1} [47]. Microsomal protein concentration was determined using the Bio-Rad Protein Assay Kit, with absorbance measured at 595 nm.

Testosterone Hydroxylation Assay

Microsomal testosterone 2β- and 6β-hydroxylase activities were measured by HPLC as described previously [48].

Antibodies

Mouse anti-rat CYP3A2 IgG (MAb L171), which is specific for CYP3A2 [17], was provided by Dr. Paul E. Thomas (Rutgers University). Control immunoblot experiments with this antibody showed the detection of one CYP3A band in hepatic microsomes isolated from untreated adult male rats, where *CYP3A2*, *CYP3A18*, and *CYP3A9* genes are expressed [21, 34]. In contrast, no CYP3A bands were detected in hepatic microsomes isolated from female rats, where *CYP3A9* and *CYP3A18* genes are expressed [21, 34]. Therefore, these findings indicate that the MAb L171 antibody is not likely to cross-react with CYP3A9 or CYP3A18.

Gel Electrophoresis and Immunoblotting

SDS–PAGE was performed according to the method of Laemmli [49] using a Hoefer SE 600 vertical slab gel unit as described previously [50]. Proteins resolved by SDS–PAGE were transferred electrophoretically to nitrocellulose membranes according to the method of Towbin *et al.* [51]. The membranes were incubated with mouse-anti rat CYP3A2 IgG (1 μg IgG/mL) at the concentration listed for 2 hr at 37° with shaking. The secondary antibody, alkaline phosphatase-conjugated goat anti-mouse $F(ab')_2$ (TAGO Immunologicals Inc.), was used at a 1:3000 dilution. Visualization of the protein bands was achieved with a substrate solution consisting of 0.01% NBT, 0.005% BCIP, in 0.1 M Tris–HCl buffer, pH 9.5, with 0.5 mM $MgCl_2$. Assay conditions for the reaction between alkaline phosphatase and substrate were optimized to ensure that color development did not proceed beyond the linear response range of the phosphatase reaction.

Immunoquantification

Densitometric quantification of stained bands on nitrocellulose membranes was determined using a pdi 420 oe™ scanning densitometer (pdi Inc.) connected to an IBM-compatible personal computer and using pdi Quantity One® Version 3.0 software. A single concentration of the appropriate purified CYP protein was included on each blot as an internal standard. The amount of immunoreactive protein was determined from the ratio of the integrated intensity of the stained band. Values of integrated intensity were converted into picomole quantities using calibration curves generated by loading various concentrations of purified CYP standards on gels followed by immunoblotting and densitometric analysis as described above.

Isolation of Liver RNA

Total RNA was isolated with TriZol™ (Canadian Life Technologies), according to the manufacturer's protocol. The RNA pellet was dissolved in 10 mM Tris buffer (pH 8) containing 1 mM EDTA and stored at −70° until used. Total RNA concentration was determined spectrophotometrically at 260 nm. The integrity of the RNA preparation was assessed by agarose (1.7%) gel electrophoresis in the presence of 0.66 M formaldehyde.

RT–PCR Assay

Isolated liver RNA (2 μg) was incubated with 0.5 μg $oligo(dT)_{12–18}$ primer and diethylpyrocarbonate-treated water in a volume of 9 μL at 65° for 10 min. Then the mixture was placed on ice. After the addition of 2 μL of 10x PCR buffer II, 4 μL of 25 mM $MgCl_2$, 1 μL of 10 mM dNTP, 1 μL of 0.1 M dithiothreitol, and 2 U of deoxyribonuclease I, each tube was incubated at 37° for 30 min followed by 75° for 5 min and then cooled on ice. Reverse transcription was initiated by the addition of 200 U of SuperScript™ II reverse transcriptase. The mixture was then incubated at 42° for 20 min, and the reaction was stopped by heating at 95° for 5 min. The synthesized cDNA was stored at −20° until used.

PCR co-amplification of target and internal control cDNAs was performed based on a published method [21]. Sequences for the forward (5′-TTG-ATC-CGT-TGT-TCT-TGT-CA-3′) and reverse (5′-GGC-CAG-GAA-ATA-CAA-GAC-AA-3′) primers for CYP3A2, sequences for the forward (5′-GGA-CGA-TTC-TTG-CTT-ACA-GG-3′) and reverse (5′-ATG-CTG-GTG-GGC-TTG-CCT-TC-3′) primers for CYP3A9, sequences for the forward (5′-CAA-CTA-CGG-TGA-TGG-CAT-GT-3′) and reverse (5′-CAC-TCG-GTT-CTT-CTG-GTT-TG-3′) primers for CYP3A18, and sequences for the forward

(5′-TAT-GGA-GAA-GAT-TTG-GCA-CC-3′) and reverse (5′-CCA-CCA-ATC-CAC-ACA-GAG-TA-3′) primers for β-actin were from Mahnke *et al.* [21] and Zhang *et al.* [52]. Sequences for the forward (5′-CTT-CGA-CAT-CAC-GGC-TGA-TGG-3′) and reverse (5′-CAG-GAC-CTG-TAT-GCT-TCA-GG-3′) primers for cyclophilin were from Morris and Davila [53]. Each PCR reaction, in a total volume of 25 μL, contained 1x PCR buffer II [10 mM Tris (pH 8.3), 50 mM KCl], 2 mM $MgCl_2$, 5 μL cDNA, 400 μM dNTP mix, 25 pmol each of the forward and reverse primers for target gene and internal standard gene, and 4 U of AmpliTaq® DNA polymerase. PCR amplification was initiated by heating at 95° for 1 min. This was followed by 22 cycles (for CYP3A2 and cyclophilin), 25 cycles (for CYP3A18 and β-actin), or 27 cycles (for CYP3A9 and β-actin) of the following: 30 sec for denaturation at 94°, 1 min for annealing at 60°, and 1 or 2 min for extension at 72° (1 min for CYP3A2 and cyclophilin, and 2 min for the others). A final incubation was carried out for 10 min at 72°.

Statistics

The significance of difference between the group means was assessed by one-way ANOVA and, if applicable, was followed by the Student–Newman–Keuls test. The level of significance was set *a priori* at $P < 0.05$.

RESULTS

Sex-Dependent and Developmental Expression of Hepatic CYP3A2, CYP3A18, and CYP3A9

CYP3A2, *CYP3A18*, and *CYP3A9* gene expression was determined by RT–PCR analysis with primers specific for the individual genes and quantified relative to cyclophilin or β-actin as the internal standard (Fig. 1, A–C). To establish the reliability of the method, relative CYP3A2, CYP3A18, and CYP3A9 mRNA levels were measured in untreated adult male and female rats and compared with published results. Hepatic CYP3A18 mRNA levels were found to be approximately 7-fold greater in male than in female rats, whereas CYP3A9 mRNA expression was two times greater in female rat liver (data not shown). These findings together with the lack of detection of CYP3A2 mRNA in the liver of adult female rats are generally consistent with previous reports [17, 21, 34, 36].

The developmental expression of rat hepatic CYP3A2, CYP3A18, and CYP3A9 was investigated in prepubertal (22 days of age), early to late pubertal (34 and 51 days of age), and adult (84–91 days of age) intact male rats. As shown in Fig. 2A, relative hepatic CYP3A2 mRNA levels were constant in male rats between 22 and 84–91 days of age. Relative CYP3A18 mRNA levels were found to be low in male rats at 22 days of age, but increased approximately 3-fold by 34 days of age and thereafter remained elevated up to 84–91 days of age (Fig. 2B). By comparison, CYP3A9 mRNA was undetectable in livers from male rats at 22 and 34 days of age, but was readily detectable at 51 days of age and was increased further at 84–91 days of age (Fig. 2C).

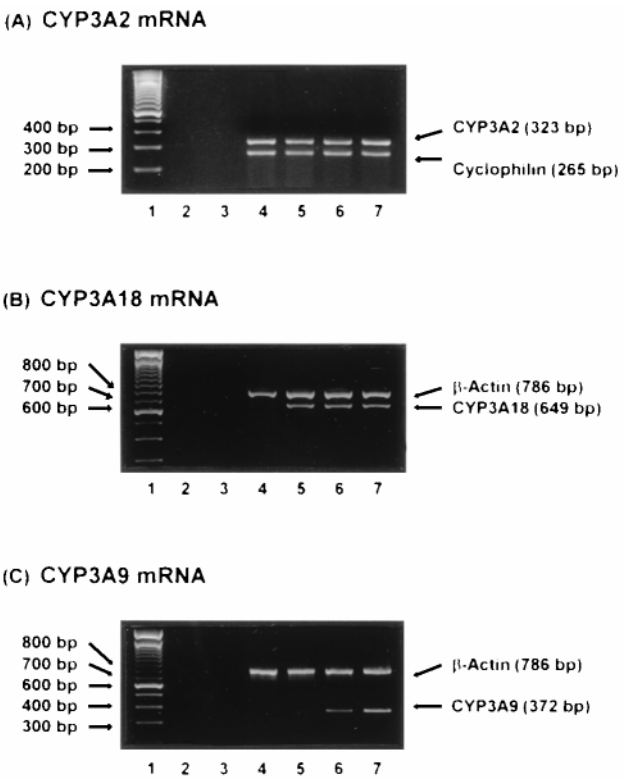

FIG. 1. RT–PCR analysis of hepatic CYP3A2, CYP3A18, and CYP3A9 mRNA expression in male rats of different ages. Total liver RNA was isolated from male rats at 22, 34, 51, and 84–91 days of age, and the RT–PCR assay was performed as described under Materials and Methods. Shown are photographs of ethidium bromide-stained agarose gels for the PCR co-amplification of CYP3A2 and cyclophilin (internal control) cDNA (panel A), CYP3A18 and β-actin (internal control) cDNA (panel B), and CYP3A9 and β-actin (internal control) cDNA (panel C). Lane 1: DNA ladder; lane 2; negative control (no primers); lane 3: negative control (no cDNA); lane 4, day 22 of age; lane 5: day 34 of age; lane 6: day 51 of age; and lane 7: days 84–91 of age.

RT–PCR data on relative CYP3A2 mRNA expression were compared with microsomal CYP3A2 protein content determined by immunoblot analysis. A monoclonal antibody, which is specific for CYP3A2 [17], detected a strongly reactive band in liver microsomes from male rats in the four age groups, although variation in CYP3A2 protein content was apparent among individual 84- to 91-day-old adult rats (Fig. 3A). Densitometric quantification of the immunoblots confirmed that expression of CYP3A2 protein was relatively unchanged between 22 and 84–91 days of age (Fig. 3B), in agreement with results obtained by densitometric analysis of the RT–PCR assay (Fig. 2A).

Considerable uncertainty exists with regard to the contribution of individual CYP3A enzymes to testosterone 2β- and 6β-hydroxylase activities because inhibitory antibodies for all CYP3A enzymes are not available. To investigate possible associations between CYP3A2, CYP3A18, and CYP3A9 and testosterone 2β- and 6β-hydroxylation, these two activities were measured in liver microsomes from intact male rats at 22, 34, 51, and 84–91 days of age. As

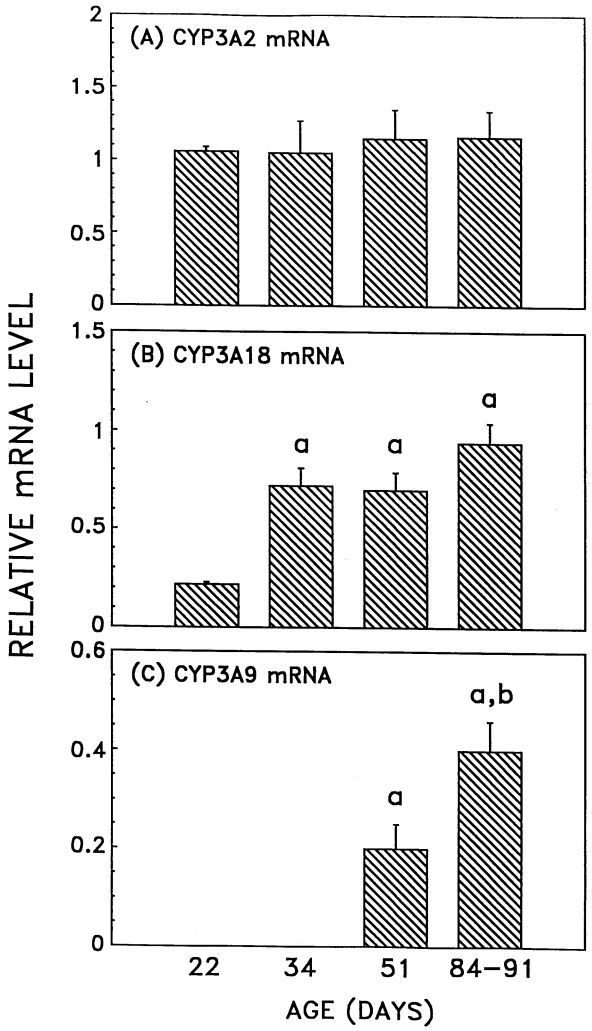

FIG. 2. Relative hepatic CYP3A2, CYP3A18, and CYP3A9 mRNA levels in male rats of different ages. Shown are results of the densitometric analysis of the RT–PCR assay (Fig. 1). The data are expressed as the mean (± SEM) ratio of the intensity of the CYP3A band to that of the internal control band (cyclophilin or β-actin). N = 4 individual rats per treatment group. Key: (a) significantly different from the 22-day-old group ($P < 0.05$); and (b) significantly different from the 51-day-old group ($P < 0.05$).

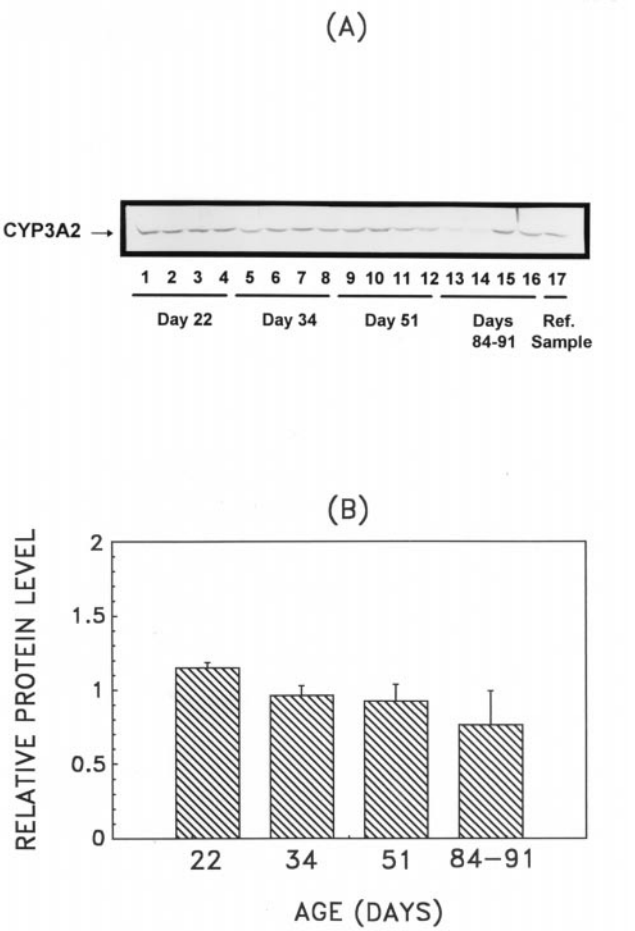

FIG. 3. Hepatic CYP3A2 protein expression in male rats of different ages. Hepatic microsomes were isolated from male rats at 22, 34, 51, and 84–91 days of age and subjected to SDS–PAGE (one individual microsome sample per lane) [50]. Shown in panel A is an immunoblot probed with mouse anti-rat CYP3A2 monoclonal antibody (MAb L171) at a final concentration of 1 μg/mL [17]. Lanes 1–4: day 22 of age; lanes 5–8, day 34 of age; lanes 9–12, day 51 of age; lanes 13–16, days 84–91 of age; and lane 17, reference sample (pooled microsomes from adult male rats). Shown in panel B is the result of the densitometric analysis of the CYP3A2 bands (panel A). The data are expressed as the mean (± SEM) ratio of the intensity of the CYP3A2 protein band to that of the reference band. N = 4 individual rats per treatment group.

shown in Fig. 4, testosterone 2β- and 6β-hydroxylase activities were similar at 22 and 84–91 days of age and were found to peak at 34 days of age. Comparison of the developmental profiles of these two CYP3A-mediated monooxygenase activities with those of CYP3A2 protein and mRNA levels indicates a lack of correlation with this CYP3A form. The same conclusion can also be drawn between testosterone 2β- and 6β-hydroxylase activities and CYP3A18 and CYP3A9 mRNA levels.

Effect of GH on Hepatic CYP3A2, CYP3A18, and CYP3A9 Expression in Adult Rats

The role of GH in the regulation of CYP3A2, CYP3A18, and CYP3A9 was investigated using intact and hypophysectomized adult rats. Administration of rat GH (0.12 μg/g body weight twice daily for 7 days) to intact 11-week-old male rats produced a 74% decrease in CYP3A2 mRNA levels (Fig. 5A) but did not alter the expression of CYP3A18 (Fig. 5B) or CYP3A9 (Fig. 5C). In contrast, hypophysectomy resulted in increases of 3- to 4-fold in hepatic CYP3A2 and CYP3A18 mRNA levels, but did not affect CYP3A9 mRNA levels. Treatment of hypophysectomized rats with GH had no effect on expression of CYP3A2, CYP3A18, or CYP3A9. The observed changes in CYP3A2 mRNA levels following hypophysectomy or GH treatment were reflected by similar changes in hepatic microsomal CYP3A2 protein content as determined by immunoblot analysis (Fig. 5D).

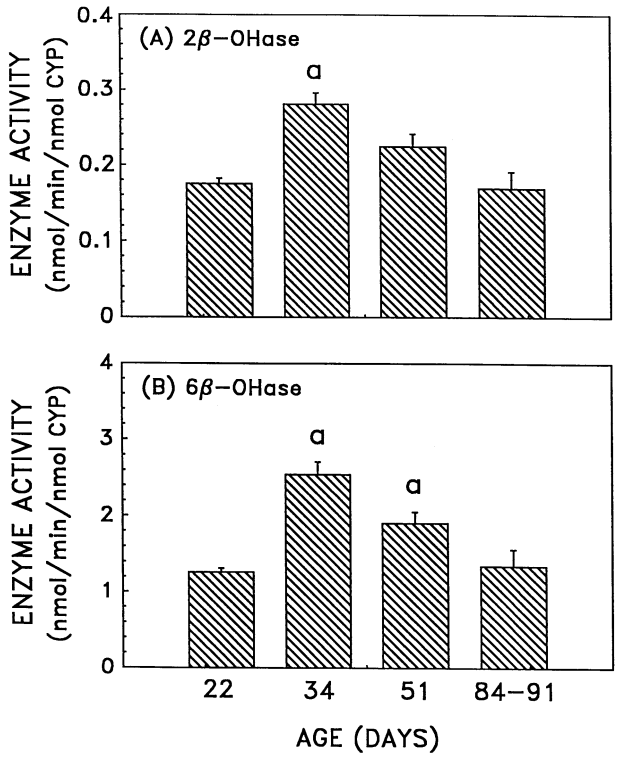

FIG. 4. Hepatic microsomal testosterone 2β- and 6β-hydroxylase activities in male rats of different ages. Hepatic microsomes were isolated from male rats at 22, 34, 51, and 84–91 days of age and testosterone 2β-hydroxylase (panel A) and 6β-hydroxylase (panel B) activities were determined by an HPLC assay [48]. Results are expressed as mean (± SEM) activity for 4 individual rats per treatment group. Key: (a) significantly different from the 22-day-old group ($P < 0.05$).

The effect of hypophysectomy and GH treatment on hepatic microsomal testosterone 2β- and 6β-hydroxylase activities reveals a somewhat different pattern (Fig. 5, E and F). Administration of GH to intact male rats resulted in decreases in testosterone 2β- and 6β-hydroxylase activities of approximately 80 and 60%, respectively. However, there was no change in either enzyme activity following hypophysectomy or GH treatment of hypophysectomized rats.

Effect of GH on Hepatic CYP3A2 and CYP3A18 Expression in Prepubertal Rats

The effect of GH on CYP3A expression in prepubertal male rats, which have yet to develop a well-defined pulsatile GH secretion pattern [27], was examined using intact 34-day-old male rats that were treated from 22 to 33 days of age with either a high or low dosage of rat GH. The relative CYP3A2 mRNA level was unchanged following twice daily s.c. injection (0.12 μg/g body weight) of GH (Fig. 6A), whereas this dosage of GH resulted in a slight decrease in relative CYP3A18 mRNA expression in prepubertal rats (Fig. 6B). By comparison, mRNA levels of both CYP3A2 (Fig. 6A) and CYP3A18 (Fig. 6B) were decreased substantially following treatment with a higher dosage of GH (3.6 μg/g body weight twice daily). The lower dosage of GH had no effect on testosterone 2β-hydroxylase activity (Fig. 6C), but testosterone 6β-hydroxylase activity was reduced modestly (Fig. 6D). The higher dosage of GH substantially reduced both of these enzyme activities.

DISCUSSION

The present study examined the influence of GH on the developmental expression of the recently identified CYP3A18 and CYP3A9 as well as CYP3A2. The effect of GH on CYP3A expression during the prepubertal period was investigated because of the increasing clinical use of GH in short-stature children without GH deficiency [43] and the uncertainty about the hepatic metabolic consequences of exposure to pharmacological doses of GH during the developmental period.

Very little is known about the underlying neuroendocrine basis for the male-predominant expression of CYP3A18. The present study demonstrated that hypophysectomy of adult male rats resulted in a 3-fold increase in the relative mRNA level of this CYP. This stimulatory effect in the absence of the pituitary gland has also been observed with several male-specific CYP enzymes [35–37, 54, 55]. The finding that hypophysectomy increased CYP3A18 mRNA levels indicates that pituitary factor(s) has a negative influence on basal CYP3A18 expression. The identity of this suppressive factor(s) is not known. The results also show that intermittent GH administration did not modulate the elevated expression of CYP3A18 in hypophysectomized adult male rats. Similarly, intermittent GH administration did not alter CYP3A18 mRNA levels in intact adult male rats. By comparison, continuous GH infusion, which reflects to some extent the female pattern of endogenous GH secretion, has been reported to decrease CYP3A18 mRNA levels in intact adult male rats [34]. Therefore, the pattern of plasma GH levels appears to be a determinant of hepatic CYP3A18 expression.

Hepatic CYP3A9 mRNA expression appears to be greater in adult female rats than in adult male rats ([21, 33, 34], and the present study). In a recent study, estrogen was reported to be necessary for hepatic CYP3A9 expression [33]. Estrogen is thought to modulate CYP expression by its action on the hypothalamic–pituitary axis to influence the pattern of endogenous GH secretion [42]. To determine if the pituitary gland plays a role in hepatic CYP3A9 expression, the mRNA level of this CYP was compared between intact and hypophysectomized adult male rats. As shown herein, hypophysectomy did not change CYP3A9 mRNA levels. This result suggests that pituitary secretion of GH is not necessary for the constitutive expression of hepatic CYP3A9 in male rats. However, CYP3A9 expression can be stimulated by exogenous GH, depending on the mode of administration. Intermittent administration of GH did not affect CYP3A9 expression in either hypophysectomized or intact adult male rats. By comparison, treatment of intact adult male rats with continuous GH infusion resulted in

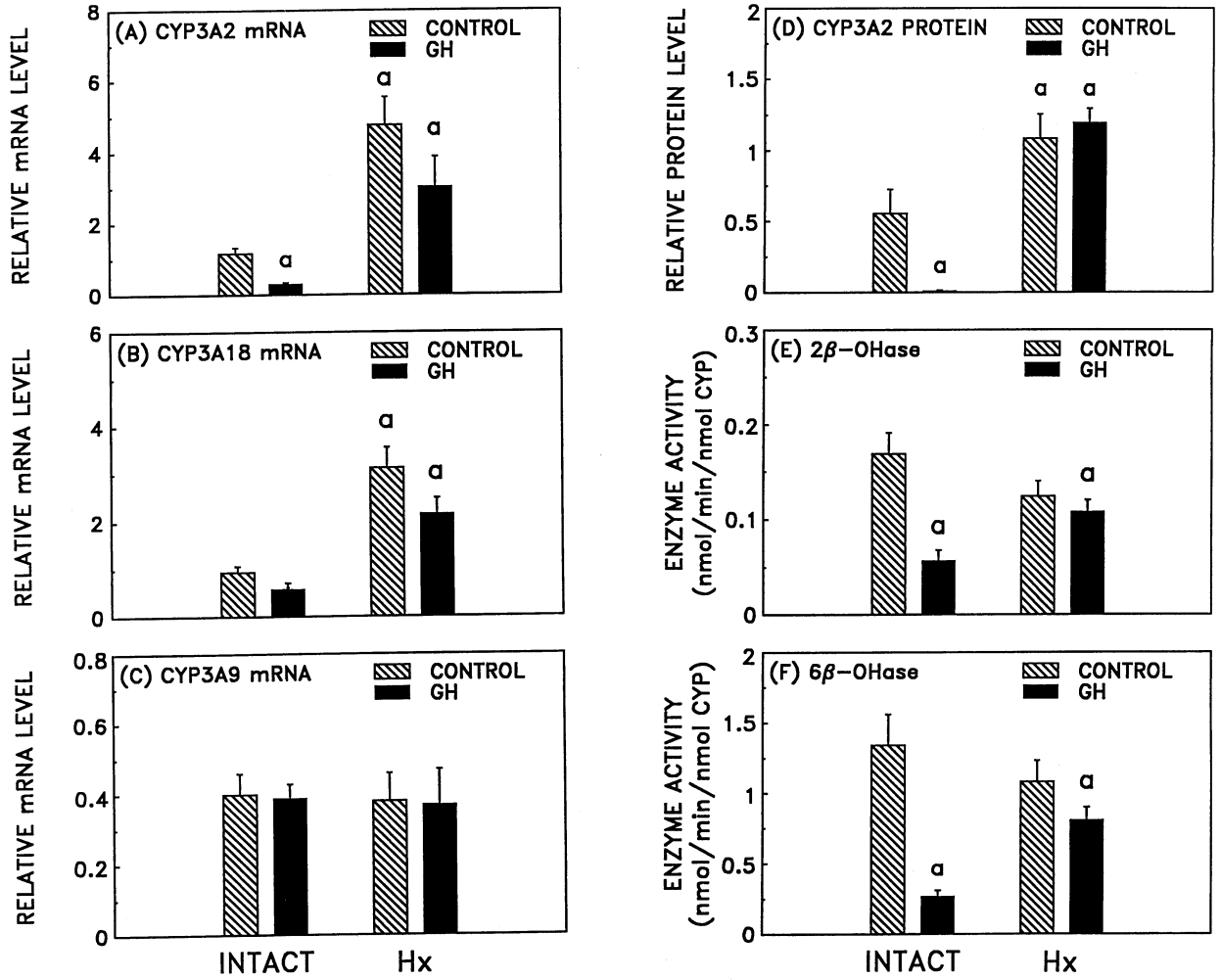

FIG. 5. Effect of hypophysectomy and intermittent GH administration on CYP3A mRNA, protein, and enzyme activity levels in adult male rats. Intact and hypophysectomized (Hx) adult male rats were administered s.c. injections of GH (0.12 μg/g body weight) or an equivalent volume of the vehicle (10 mM potassium phosphate, pH 8.3, containing 0.9% sodium chloride) twice daily (8:00 a.m. and 5:00 p.m.) for 7 consecutive days. All rats were killed 1 day after the last injection. Relative CYP3A2 mRNA (panel A), CYP3A18 mRNA (panel B), and CYP3A9 mRNA (panel C) levels were determined by RT–PCR as described under Materials and Methods. The relative CYP3A2 protein level (panel D) was determined by immunoblot assay with the mouse anti-rat CYP3A2 monoclonal antibody (MAb L171) [17]. Hepatic microsomal testosterone 2β-hydroxylase (panel E) and 6β-hydroxylase (panel F) activities were determined by an HPLC assay [48]. Results are expressed as means ± SEM for 4 individual rats per treatment group. Key: (a) significantly different from the intact control group ($P < 0.05$).

increased hepatic CYP3A9 mRNA expression [34], suggesting that the female-predominant expression of CYP3A9 could be a consequence of the continuous endogenous secretion of GH in female rats.

In agreement with earlier studies [29, 37], our results indicate that hypophysectomy increased CYP3A2 mRNA and protein levels. Whereas intermittent GH administration did not affect the elevated CYP3A2 expression in hypophysectomized adult male rats, it reduced the mRNA and protein levels of this CYP in intact adult male rats substantially. An explanation for the differential effect of exogenous GH in intact rats may relate to the route of GH administration. In the present study, GH was administered as twice daily s.c. injections; this dosage pattern results in relatively slow elimination so that low levels of plasma GH can be detected for several hours after each dose [40]. The s.c. administration of exogenous GH together with endogenous secretion of GH in the intact male rat may result in a plasma profile in which GH is present most of the time. Such a plasma GH profile may be sufficient to attenuate CYP3A2 expression in intact male rats because continuous GH infusion, even at a level that is 3% of the normal plasma GH concentration found in adult females, has been reported to suppress CYP3A2 [56].

GH at a dosage of 3.6 μg/g significantly decreased CYP3A2 and CYP3A18 mRNA levels in intact prepubertal male rats. The decrease in CYP3A18 expression by intermittent GH administration together with the findings that continuous GH infusion suppresses CYP3A18 mRNA expression in intact adult male rats [34] and that hypophy-

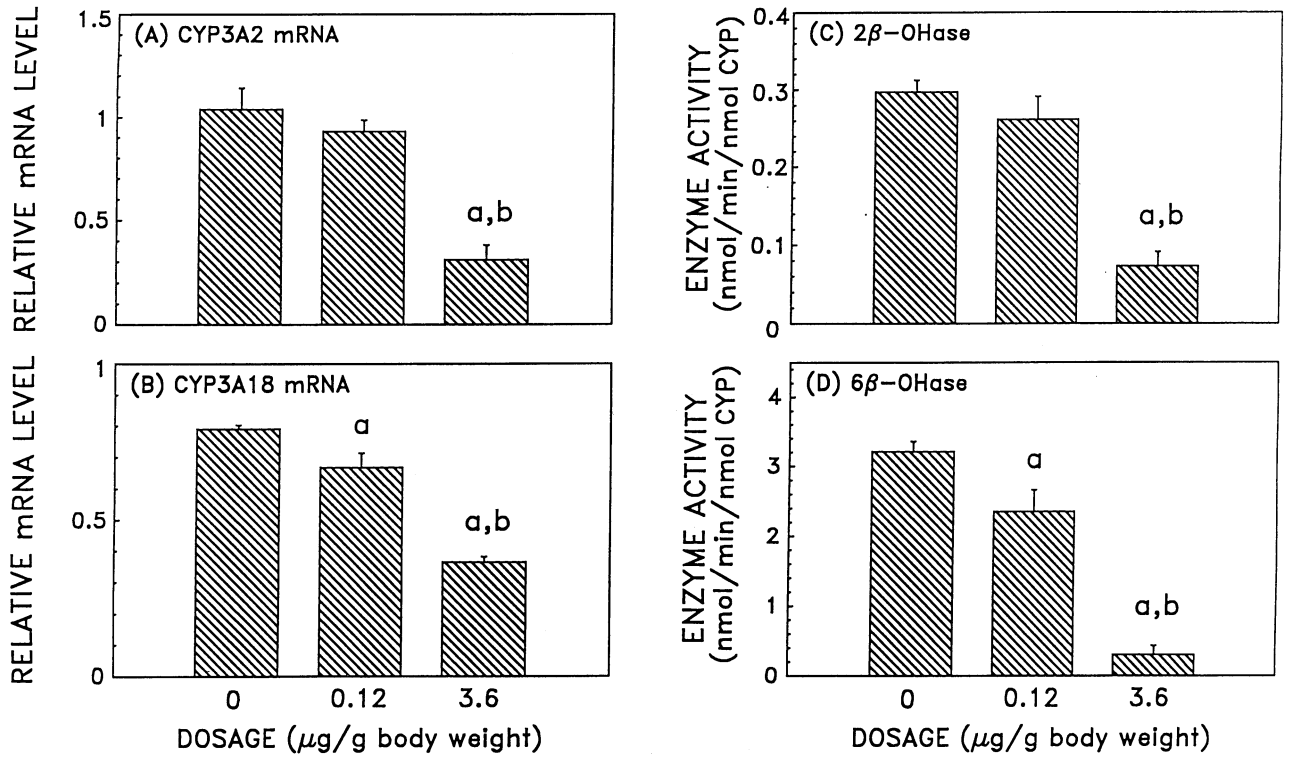

FIG. 6. Effect of intermittent GH injection on hepatic CYP3A2 and CYP3A18 mRNA expression and testosterone 2β- and 6β-hydroxylase activities in prepubertal intact male rats. Prepubertal (22-day-old) intact male rats were administered s.c. injections of GH (0.12 or 3.6 μg/g body weight) or an equivalent volume of the vehicle (10 mM potassium phosphate, pH 8.3, containing 0.9% sodium chloride) twice daily (8:00 a.m. and 5:00 p.m.) for 12 consecutive days. All rats were killed at day 34 of age. Relative CYP3A2 mRNA (panel A) and CYP3A18 mRNA (panel B) levels were determined by RT–PCR as described in Materials and Methods (N = 5 individual rats per group). Hepatic microsomal testosterone 2β-hydroxylase (panel C) and 6β-hydroxylase (panel D) activities were determined by an HPLC assay [48] (N = 12 individual rats for the control group and 6 for each of the GH-treated groups). Results are expressed as means ± SEM. Key: (a) significantly different from the control group ($P < 0.05$); and (b) significantly different from the group treated with the 0.12 μg/g dosage of GH ($P < 0.05$).

sectomy enhances CYP3A18 expression lead us to conclude that GH negatively regulates this male-predominant CYP. The same suppressive effects also have been observed with another male-predominant CYP enzyme, CYP3A2 ([36], and the present study). Interestingly, a larger dosage was required to suppress CYP3A2 expression in intact prepubertal rats when compared with intact adult male rats. The hormonal basis for the altered sensitivity of CYP3A2 to the suppressive influence of GH in prepubertal male rats is not known. However, the unresponsiveness of the intact prepubertal rats to the lower dosage (0.12 μg/g) of GH is unlikely to reflect age-dependent differences in the pattern of GH secretion or in the levels of CYP3A2-suppressive factors such as thyroid hormones [36]. As demonstrated herein and in three other studies [17, 21, 30], the magnitude of CYP3A2 expression is similar between prepubertal and adult rats.

The present study shows an age-dependent expression of CYP3A9 and CYP3A18 in the livers of male rats. CYP3A9 mRNA expression could not be detected until puberty, and the levels were greater at 84–91 days of age than at 51 days of age. The relative hepatic mRNA level of CYP3A18, as determined by RT–PCR, was found to be greater during puberty and adult life than during the prepubertal period. The female-predominant expression of CYP3A9 and the male-predominant expression of CYP3A18 in adult rats observed herein are consistent with previous reports [26, 34, 57], but the age-related expression of both proteins differs from recent studies. Mahnke *et al.* [21] reported that CYP3A9 mRNA levels are constant between 7 and 20 weeks of age in male rats and are undetectable in rats younger than 5 weeks old. However, the same study indicated that there were no sex or age differences in hepatic CYP3A18 expression. By comparison, Nagata *et al.* [26] showed that the CYP3A18 mRNA became detectable at 20 days of age, and the mRNA level increased steadily in male rats over the first 60 days of life.

CYP3A2 expression, both at the mRNA and protein levels, was found to be relatively constant in prepubertal, pubertal, and adult male rats. This finding is in agreement with the results of three previous studies [17, 21, 30], but differs from the report by Wright *et al.* [31], which indicated that CYP3A2 protein was expressed at a lower level in adult male rats than in prepubertal male rats. The discrepancy may be due to the specificity of the anti-peptide antibody used to detect CYP3A2 in that study or may be

attributed to the inter-animal variability in CYP3A2 expression reported previously [23, 48] (cf. Fig. 3A). By comparison, the level of P450PCN2 mRNA was determined to be greater in adult male rats than in prepubertal male rats [32]. P450PCN2 was thought to correspond to CYP3A2, although recent evidence suggests that it may be an allelic variant of CYP3A2 [58].

The developmental expression of testosterone 2β- and 6β-hydroxylase activities was not reflected in the developmental expression of CYP3A2, CYP3A18, or CYP3A9 in male rats. A lack of correlation between hepatic CYP3A2 protein and microsomal androstenedione 6β-hydroxylase activity was reported recently [31]. Experiments with immunologically purified or cDNA-expressed enzymes have shown that CYP3A2 and CYP3A18 are active in testosterone 2β- and 6β-hydroxylation [15, 19, 26], but it is not known whether CYP3A9 catalyzes these reactions. It is possible that CYP3A2, CYP3A18, and other CYP enzymes contribute to the two enzyme activities in liver microsomes from male rats. Consistent with this proposal, several purified but unidentified CYP enzymes have been shown to be catalysts of testosterone 2β- and 6β-hydroxylation [15, 19, 59].

In summary, the present study demonstrated that hypophysectomy increased hepatic expression of CYP3A18 and CYP3A2 but not CYP3A9 in adult male rats. Moreover, twice daily s.c. GH administration suppressed CYP3A18 in hypophysectomized but not in intact adult rats. The same treatment decreased CYP3A2 in both of these groups of rats, although the sensitivity of this CYP to the negative regulation by GH appears to be influenced by age. Finally, both CYP3A18 and CYP3A9 were expressed constitutively in an age- and sex-dependent manner.

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