Biochemical Pharmacology, Vol. 38, No. 3, pp. 535–538, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

EFFECTS OF SERUM TESTOSTERONE LEVEL WITH BUSERELIN ON THE ACTIVITIES OF DRUG AND TESTOSTERONE HYDROXYLASE AND ON THE CONTENT OF A MALE-SPECIFIC FORM OF CYTOCHROME P-450 IN MALE RATS

HIROAKI OHI,* MIYUKI IWASAKI, MASAYUKI KOMORI, TOSHIAKI MIURA, MITSUKAZU KITADA, SHORYO HAYASHI† and TETSUYA KAMATAKI

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, and †Pharma Research Laboratories, Hoechst Japan Limited, Saitama 350, Japan

(*Received* 17 *June* 1988; *accepted* 12 *September* 1988)

Abstract—The effects of administration of buserelin, a synthetic agonist of lutenizing hormone-releasing hormone (LH-RH), on the content of P-450-male, a male specific form of cytochrome P-450, and the activities of drug and testosterone hydroxylases were examined in liver microsomes of male rats. Administration of buserelin resulted in a decrease in the serum level of testosterone in a dose-dependent manner. Similar decreases were seen in the activities of aminopyrine *N*-demethylase, 7-propoxycoumarin *O*-depropylase and testosterone 2α- and 16α-hydroxylases, but not in the activity of aniline *p*-hydroxylase. Thus, correlations were observed between the serum level of testosterone and the activities of drug and testosterone hydroxylases except for aniline *p*-hydroxylase. These results indicate that the drug and testosterone hydroxylases are affected sensitively and sequentially by the changes in the testosterone levels in the body.

Cytochrome P-450 in liver microsomes plays central roles in metabolizing a wide variety of exogenous as well as endogenous substrates, including steroids and drugs, and constitutes a superfamily of enzymes, comprising some 20–30 families [1]. In view of the pharmacological significance of cytochrome P-450, forms of cytochrome P-450 have been purified and characterized to clarify the functions of each form of cytochrome P-450. Among forms of cytochrome P-450 purified so far, P-450-male‡ was found to exist specifically in liver microsomes of adult male rats [2–4]. Since sex-related differences in pharmacological and toxicological actions of drugs have been noted, the presence of the sex specific form of cytochrome P-450 has been considered one of the causes of such differences [2–4, 7].

Immunochemical quantitation of P-450-male showed that P-450-male appears in liver microsomes of male rats with sexual maturation [8, 10]. Thus, gonadectomy of newborn male rats completely prevented the appearance of P-450-male in mature life. The gonadectomy of four-week-old male rats clearly reduced the amounts of P-450-male in liver microsomes [4]. These results suggest that the testosterone level regulates the expression of P-450-male even in adult rats, while no evidence supporting the idea that there is a close correlation between the level of serum testosterone and the content of P-450-male has been reported.

Buserelin is a synthetic lutenizing hormone-releasing hormone (LH-RH) agonist currently being developed for clinical use. Daily administration of this drug produces a temporal sharp increase in the level of serum testosterone around one day after the first injection, then reduces the level remarkably [11].

Thus, this study was undertaken to know whether the control of testosterone level by buserelin administration reflects the content of P-450-male and subsequently the activities of drug and testosterone hydroxylases. We show herein that P-450-male and sex-dependent drug hydroxylases are controlled in close association with the serum level of testosterone.

MATERIALS AND METHODS

Materials. Testosterone was purchased from a commercial source and used after purification by recrystallization. The 6β- and 16α-hydroxytestosterone were provided by Steraloid and Sigma, respectively. The 2α-hydroxytestosterone was a generous gift from Dr. Kirk, Queen Mary College, University of London. NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were the products of Oriental Yeast (Tokyo, Japan). Goat anti-rabbit immunoglobulin G (IgG) and peroxidase-rabbit anti-peroxidase complex (PAP) were obtained from Cappel Laboratories (West Chester) and Daiichi Chemicals, respectively. Antibodies to P-450-male were prepared by immunization of female Japanese White rabbits according to the method reported elsewhere [4]. The IgG fraction from serum was partially purified by ammonium sulfate precipitation. Other reagents were from commercial sources but were of the highest grade available.

Treatment of animals and preparation of liver microsomes. Male Sprague–Dawley rats were div-

* To whom all correspondence should be sent.

‡ P-450-male is identical with the following isozymes purified in other laboratories: UT-A [5], h [6], 2c [7], P-450$_{16\alpha}$ [8], and RLM$_5$ [9]. These isozymes belong to P-450 II family.

ided into five groups, namely, control group, low dose group, high dose group, recovery group and castration group. The rats in low and high dose groups received once-daily subcutaneous injection of buserelin in isotonic saline solution (1 μg or 100 μg per kg of body weight) for 2 weeks, starting at 10 weeks of age. The rats in the recovery group were given buserelin solution (100 μg per kg of body weight) for 2 weeks, starting at 8 weeks of age, then housed for 2 weeks without treatment. The rats in the castration group were castrated at 10 weeks of age. All rats were killed at 12 weeks of age by a blow on the head and decapitated. Livers were immediately removed and homogenized with 1.15% potassium chloride, and the microsomes were prepared at 4° by sequential centrifugations at 9000 *g* for 20 min and 105,000 *g* for 60 min, respectively. Testosterone level in serum was determined by specific radioimmunoassay [12]. The amount of microsomal protein and the content of cytochrome P-450 were measured by the methods of Lowry *et al.* [13] and Omura and Sato [14], respectively. Cytochrome b_5 was determined according to the method of Omura and Sato [15]. NADPH-cytochrome *c* reductase activity was measured by the method of Phillips and Langdon [16] with cytochrome *c* as an electron acceptor. The activity of NADPH-cytochrome *c* reductase was defined as a unit which reduced 1 μmol of cytochrome *c* per min.

Immunochemical quantification of P-450-male. Peroxidase staining technique coupled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to quantitate the amount of P-450-male in liver microsomes [5, 17]. SDS-PAGE was performed according to the method of Laemmli [18]. Proteins in the gel were transferred to a nitrocellulose sheet. Rabbit anti-P-450-male IgG were applied, and then goat anti-rabbit IgG and subsequently peroxidase–antiperoxidase complex added. Cytochrome P-450 was visualized with 3,3'-diaminobenzidine and hydrogen peroxide.

Assays of drug-metabolizing enzyme activities in liver microsomes. The incubation mixture (1 ml) for the assay of the activities of drug-metabolizing enzymes consisted of 100 mM sodium, potassium phosphate buffer (pH 7.4), 0.05 mM EDTA, 1 mg of microsomal protein, an NADPH-generating system (0.5 mM NADP, 5 mM glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase and 5 mM magnesium chloride), and a substrate (5 mM aminopyrine, 5 mM aniline or 0.5 mM 7-propoxycoumarin). The reaction was initiated by addition of the NADPH-generating system, and terminated by addition of trichloroacetic acid after 15 min incubations at 37°. The activities of aminopyrine *N*-demethylase, aniline *p*-hydroxylase and 7-propoxycoumarin *O*-depropylase were estimated by determination of formaldehyde [19], p-aminophenol [20] and 7-hydroxycoumarin [21], respectively. Testosterone hydroxylase activities were measured according to the method of Hayashi *et al.* [22] with slight modifications. Briefly, the reaction mixture (0.5 ml) consisted of 100 mM potassium, sodium phosphate buffer (pH 7.4), 0.05 mM EDTA, 0.5 mg of microsomal protein, the NADPH-generating system and 300 nmol of testosterone dissolved in 10 μl of methanol. The mixture was incubated for 15 min at 37° and extracted with 3 ml of benzene. The benzene layer was evaporated to dryness under reduced pressure. The residue was dissolved in 100 μl of methanol containing cortisone as an internal standard. The metabolites were determined using high-performance liquid chromatography.

RESULTS

Table 1 shows the effect of buserelin administration on the serum level of testosterone. Daily administration of buserelin for 2 weeks resulted in the decrease in the serum level of testosterone in a dose-dependent manner. A marked decrease in the testosterone level was also observed by castration of rats. Upon cessation of treatment of rats with the high dose of buserelin for 2 weeks, the serum testosterone increased to a level higher than the control.

The content of cytochrome P-450 in liver microsomes was not affected by the administration of buserelin, while it was decreased significantly by castration (Table 1). The content of cytochrome b_5 was slightly decreased by the low dose of buserelin and castration. In addition, the activity of NADPH-cytochrome *c* (P-450) reductase tended to decrease with an increasing dose of buserelin. Castration decreased the activity of the reductase remarkably. Such decrease in the reductase activity was restored to the control level 2 weeks after cessation of the administration of buserelin.

Aniline *p*-hydroxylase activity tended to increase with an increase in dose of buserelin. The activity had not returned to the control level 2 weeks after cessation of administration. There was no significant change in the activity by castration. On the other hand, the activities of aminopyrine *N*-demethylase and 7-propoxycoumarin *O*-depropylase decreased significantly by castration and by the administration of buserelin. Two weeks after the last injection of buserelin, aminopyrine *N*-demethylase activity returned to the control level, while 7-propoxycoumarin *O*-depropylase activity showed a higher activity than the control. The correlation coefficients between drug-metabolizing activities and serum testosterone level were determined to be −0.06, 0.65 and 0.72 for aniline *p*-hydroxylase, aminopyrine *N*-demethylase and 7-propoxycoumarin *O*-depropylase, respectively. These results suggest that buserelin treatment changed the population of forms of cytochrome P-450, since the treatment changed the activities of drug-metabolizing enzymes without affecting the specific content of total cytochrome P-450 spectrally determined in liver microsomes. The content of P-450-male in liver microsomes from busereline-treated rats is shown in Table 2. A dose-dependent decrease in the content was observed by the administration of buserelin. Castration of rats decreased the content significantly. The decrease of the content of P-450-male correlated with serum testosterone level ($r = 0.69$). The effects of buserelin treatment on the activities of testosterone 2α- and 16α-hydroxylases, which are characteristics of P-450-male [6–8, 23], were examined (Table 2). Both activities decreased dose-dependently by the administration of buserelin, and were restored by cessation

Table 1. Effects of buserelin on serum level of testosterone, content of cytochromes P-450 and b_5, the activity of NADPH-cytochrome *c* reductase, and drug-metabolizing enzyme activities in liver microsomes of rats

	Control	Treatment Low dose	High dose	Recovery	Castration
Serum testosterone level (ng/ml)	1.35 ± 0.42	0.86 ± 0.11	0.76 ± 0.08	2.04 ± 0.50	0.55 ± 0.04
Cytochrome P-450 (nmol/mg protein)	0.61 ± 0.03	0.60 ± 0.04	0.64 ± 0.04	0.64 ± 0.02	0.36 ± 0.08**
Cytochrome b_5 (nmol/mg protein)	0.41 ± 0.04	0.33 ± 0.06*	0.40 ± 0.01	0.45 ± 0.03	0.37 ± 0.04
NADPH-cytochrome *c* reductase (unit/mg protein)	0.21 ± 0.03	0.17 ± 0.01	0.15 ± 0.02**	0.19 ± 0.02	0.09 ± 0.03**
Aniline *p*-hydroxylase (nmol/min/mg protein)	0.41 ± 0.08	0.54 ± 0.11	0.60 ± 0.21	0.69 ± 0.07**	0.33 ± 0.17
Aminopyrine *N*-demethylase (nmol/min/mg protein)	7.83 ± 0.29	6.85 ± 0.45**	6.77 ± 0.33**	7.74 ± 0.61	3.68 ± 1.54**
7-Propoxycoumarin *O*-depropylase (nmol/min/mg protein)	0.92 ± 0.09	0.81 ± 0.10	0.62 ± 0.07**	1.33 ± 0.08**	0.27 ± 0.15**

Each value is shown as the mean ± SD of five animals. Experimental details are described in Materials and Methods.
*, ** significantly different from control group (*$P < 0.05$, **$P < 0.01$).

Table 2. Effects of buserelin on the specific content of P-450-male and testosterone hydroxylase activities in liver microsomes of rats

	Control	Treatment Low dose	High dose	Recovery	Castration
P-450 male (nmol/mg protein)	0.58 ± 0.11	0.45 ± 0.08	0.41 ± 0.04*	0.66 ± 0.06	0.39 ± 0.04**
Testosterone hydroxylase (nmol/min/mg protein)					
Androstenedione	0.80 ± 0.08	0.63 ± 0.02**	0.59 ± 0.05**	0.74 ± 0.05	0.37 ± 0.12**
2α-	0.74 ± 0.09	0.54 ± 0.09**	0.41 ± 0.05**	0.76 ± 0.12	0.14 ± 0.10**
6β-	0.10 ± 0.14	0.93 ± 0.19	0.77 ± 0.11**	1.15 ± 0.16	0.23 ± 0.16**
16α-	1.49 ± 0.13	0.99 ± 0.06**	0.79 ± 0.10**	1.21 ± 0.20*	0.33 ± 0.20**

Each value is as the mean ± SD of five animals.
*, ** Significantly different from control group (*$P < 0.05$, **$P < 0.01$).

of administration. In addition, the activity of testosterone 6β-hydroxylase, which was not catalyzed by P-450-male [6, 7, 24] but shows sex-related differences, was also decreased by buserelin administration. The correlation coefficients between the activities and serum testosterone level were 0.75, 0.81, 0.67 and 0.77 for androstenedione formation, 2α-hydroxylase, 6β-hydroxylase and 16α-hydroxylase, respectively.

DISCUSSION

In this study, we employed a new LH-RH agonist, buserelin, to control the serum level of testosterone. The mechanism of feed-back inhibition of pituitary and testis functions by the LH-RH agonist is not clear as yet, but it is assumed that buserelin diminishes the binding ability of LH-RH to the pituitary receptor [11]. The activities of testosterone hydroxylases and drug-metabolizing enzymes except for aniline *p*-hydroxylase were decreased by the administration of buserelin in a dose-dependent manner, suggesting that these enzyme activities respond well with the steady level of testosterone in serum but not with the tentative sharp increase in the level seen after the first injection. Thus, buserelin may be a useful tool when one wants to detect a possible involvement of testosterone in a certain mechanism of drug actions.

As shown in Tables 1 and 2, buserelin treatment caused appreciable decrease in the activities of drug and steroid hydroxylases without affecting the content of total cytochrome P-450 measurable by the carbon monoxide binding spectra. This result indicates that the population of cytochrome P-450 in liver microsomes is changed by buserelin. In accordance with that idea, we found that buserelin decreases the amount of P-450-male, a male specific

form of cytochrome P-450. The hydroxylation of testosterone at 6β-position is known to be catalyzed by the distinct form of cytochrome P-450 [7, 25]. The activity of the 6β-hydroxylase was decreased concomitantly with the activities of 2α- and 16α-hydroxylases, which are catalyzed by P-450-male. These results also imply that a form(s) of cytochrome P-450 other than these forms of cytochrome P-450 was increased by buserelin treatment, since the activity of aniline *p*-hydroxylase tended to increase by buserelin treatment. The form of cytochrome P-450 induced by buserelin treatment is not known at present.

Buserelin treatment caused the decrease in the activity of NADPH-cytochrome *c* reductase and the content of cytochrome b_5. The reductase and the hemoprotein are capable of being rate-limiting factors in the cytochrome P-450-mediated drug oxidations [26]. In this study, however, the decrease in the activities of drug-metabolizing enzymes is assumed to be caused by the decrease in the amount of certain forms of cytochrome P-450, since aniline *p*-hydroxylase activity was not decreased by the buserelin treatment.

Testosterone induces P-450-male by changing the secretion pattern of growth hormone from the pituitary gland [8, 24, 25, 27]. Buserelin decreased the serum testosterone level and the content of P-450-male. These results probably suggest that buserelin affects the secretion pattern of growth hormone. This possibility has not been examined yet.

REFERENCES

1. Nebert DW and Gonzalez FJ, P-450 genes: Structure, evolution, and regulation. *Annu Rev Biochem* **56**: 945–993, 1987.
2. Kamataki T, Maeda K, Yamazoe Y, Nagai T and Kato R, Partial purification and characterization of cytochrome P-450 responsible for the occurrence of sex difference in drug metabolism in the rat. *Biochem Biophys Res Commun* **103**: 1–7, 1981.
3. Kamataki T, Maeda K, Yamazoe Y, Nagai T and Kato R, Evidence for the involvement of multiple forms of cytochrome P-450 in the occurrence of sex-related differences of drug metabolism in the rat. *Life Sci* **31**: 2603–2610, 1982.
4. Kamataki T, Maeda K, Yamazoe Y, Nagai T and Kato R, Sex difference of cytochrome P-450 in the rat: Purification, characterization, and quantitation of constitutive forms of cytochrome P-450 from liver microsomes of male and female rats. *Arch Biochem Biophys* **225**: 758–770, 1983.
5. Guengerich FP, Dannan GA, Wright ST, Martin MV and Kaminsky LS, Purification and characterization of liver microsomal cytochromes P-450: Electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rat treated with phenobarbital or β-naphthoflavone. *Biochemistry* **21**: 6019–6030, 1982.
6. Ryan DE, Iida S, Wood AW, Thomas PE, Lieber CS and Levin W, Characterization of three highly purified cytochrome P-450 from hepatic microsomes of adult male rats. *J Biol Chem* **259**: 1239–1250, 1984.
7. Waxman DJ, Rat hepatic cytochrome P-450 isoenzyme 2c. Identification as a male-specific, developmentally induced steroid 16α-hydroxylase and comparison to a female-specific cytochrome P-450 isoenzyme. *J Biol Chem* **259**: 15481–15490, 1984.
8. Morgan ET, MacGeoch C and Gustafsson J-Ä, Hormonal and developmental regulation of expression of the hepatic microsomal steroid 16α-hydroxylase cytochrome P-450 apoprotein in the rat. *J Biol Chem* **260**: 11895–11898, 1985.
9. Cheng K-C and Schenkman JB, Purification and characterization of two constitutive forms of rat liver microsomal cytochrome P-450. *J Biol Chem* **257**, 2378–2385, 1982.
10. Maeda K, Kamataki T, Nagai T and Kato R, Postnatal development of constitutive forms of cytochrome P-450 in liver microsomes of male and female rats. *Biochem Pharmacol* **33**: 509–512, 1984.
11. Trachtenberg J, The effect of the chronic administration of a potent lutenizing hormone releasing hormone analog on the rat prostate. *J Urol* **128**: 1097–1100, 1982.
12. Furuyama S, Mayes DM and Nugent CA, A radioimmunoassay for plasma testosterone. *Steroids* **16**: 415–428, 1970.
13. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
14. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. *J Biol Chem* **239**: 2379–2385, 1964.
15. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239**: 2370–2378, 1964.
16. Phillips AH and Langdon RG, Hepatic triphosphopyridine nucleotide-cytochrome c reductase: Isolation, characterization, and kinetic studies. *J Biol Chem* **237**: 2652–2660, 1962.
17. Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354, 1979.
18. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond)* **227**: 680–685, 1970.
19. Nash T, The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem J* **55**: 416–421, 1953.
20. Imai Y, Ito A and Sato R, Evidence for biochemically different types of vesicles in the hepatic microsomal fraction. *J Biochem* **60**: 417–428, 1966.
21. Aitio A, A simple and sensitive assay of 7-ethoxycoumarin deethylation. *Anal Biochem* **85**: 488–491, 1978.
22. Hayashi S, Noshiro M and Okuda K, Isolation of a cytochrome P-450 that catalyzes the 25-hydroxylation of vitamin D_3 from rat liver microsomes. *J Biochem* **99**: 1753–1763, 1986.
23. Kamataki T, Maeda K, Shimada M and Kato R, Effects of phenobarbital, 3-methylcholanthrene and polychlorinated biphenyls on sex-specific forms of cytochrome P-450 in liver microsomes of rats. *J Biochem* **99**: 841–845, 1986.
24. Kato R, Yamazoe Y, Shimada M, Murayama N and Kamataki T, Effect of growth hormone and ectopic transplantation of pituitary gland on sex-specific forms of cytochrome P-450 and testosterone and drug oxidations in rat liver. *J Biochem* **100**: 895–902, 1986.
25. Yamazoe Y, Shimada M, Murayama N, Kawano S and Kato R, The regulation by growth hormone of microsomal testosterone 6β-hydroxylase in male rat livers. *J Biochem* **100**: 1095–1097, 1986.
26. Imai Y, Sato R and Iyanagi T, Rate-limiting step in the reconstituted microsomal drug hydroxylase system. *J Biochem* **82**: 1237–1246, 1977.
27. Gustafsson J-Å, Mode A,. Norstedt G and Skett P, Sex steroid induced changes in hepatic enzymes. *Annu Rev Physiol* **45**: 51–60, 1983.