Self-augmentation Effect of Male-specific Products on Sexually Differentiated Progesterone Metabolism in Adult Male Rat Liver Microsomes*

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It is well known that several 3-keto-4-ene steroids such as progesterone and testosterone are metabolized in a gender-specific or -predominant manner by adult rat liver microsomes. In the male, these steroids are primarily metabolized into two oxidized (16a-hydroxy and 6β-hydroxy) products mainly by the respective, male-specific cytochrome P450 subforms, CYP2C11 and CYP3A2. While they are primarily metabolized into the 5α-reduced products by female-predominant 5α-reductase activity in the female. These sexually differentiated enzyme activities are largely regulated at the transcription level under endocrine control. In the present study, we show that unlabeled 16α-hydroxyprogesterone and 6β-hydroxyprogesterone inhibited the 5α-reductive [3H]progesterone metabolism by adult rat liver microsomes without significantly inhibiting the CYP2C11 and CYP3A2 activities producing themselves, whereas 3α-hydroxy-5α-pregn-20-one and 5α-pregnane-3,20-dione not only stimulated the 5α-reductive metabolism producing themselves but also inhibited the male-specific oxidative metabolism. This finding compels us to propose a novel hypothesis that adult male rat liver microsomes may possess a self-augmentation system regulated by the male-specific products on sexually differentiated steroid metabolism, beside regulation by gene expressions of the related enzymes.

EXPERIMENTAL PROCEDURES

Materials—[1,2-3H]PROG (specific activity, 49.2 Ci/mmol) was obtained from PerkinElmer Life Sciences and purified by a paper chromatographic system of hexane, saturated with formamide. Unlabeled steroids were purchased from Sigma and Steraloids Inc. (Wilton, NH). Goat anti-rat NADPH P450 reductase antiserum and rat CYP3A2 supersomes were purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan), and Whatman No. 1 filter papers used for paper chromatographies were from Whatman Ltd. Other reagents were of analytical grade.

Preparation of Adult Male Rat Liver Microsomes—Male Wistar rats, originally provided by Japan Charles River K. K., were bred in our colony. They were castrated on the 70th day after birth and used 3–4 weeks later. The liver microsomes were prepared as described previously (11). The experiments were performed according to institutional guidelines for the care and use of laboratory animals.

[3H]PROG Metabolism by Rat Liver Microsomes—Effects of various unlabeled steroids on [3H]PROG metabolism by liver microsomes were examined, according to our previously described procedure (12). Briefly, the microsomal suspension (400–600 µg of protein/2.2 ml, total volume of the reaction mixture) was preincubated with [3H]PROG (20 nM) in the absence or presence of an unlabeled steroid (0.05–10 µM) at 36 °C for 30 min. Then NADPH (3.16 µM) was added, and the reaction mixture was incubated for a further 5 min. After the incubation, two
identical samples were mixed and extracted with toluene. In some cases, before the above described incubation procedure, microsomal suspension (250 μg of protein/1 ml, total volume of the reaction mixture) was preincubated with goat anti-rat NADPH P450 reductase (50 μl) at 25 °C for 30 min to inhibit P450-dependent oxidative 3H[PROG] metabolism. The toluidine-extractable 3H[PROG] metabolites (more than 98%) were isolated by various paper chromatographic systems and then identified by the recrystallization method (13). Because of the limited expense, the amounts of various 3H[PROG] metabolites were estimated, based on the mean values of purified efficiencies obtained from the recrystallization method in the first 10 and several important experimental batches. The mean ± S.D. values of purified efficiencies were as follows: unchanged 3H[PROG] (99.26 ± 2.59%), [3H]15α-OH-PROG (88.22 ± 4.14%), [3H]6β-OH-P450 reductase and 5α-reductase-dependent metabolisms in- 

### RESULTS

**Evaluation of the Present Assay System for 3H[PROG] Metabolism by Rat Liver Microsomes**—In the present study, the respective final concentrations of 3H[PROG] and NADPH were adjusted to be 20 nM and 3.16 μM, although these were approximately 2–4 orders of magnitude lower than those of customary enzyme assay systems (4, 5, 7, 9). The reasons are as follows. 1) When the final concentration of ethanol used for solubilizing 3H[PROG] and an unlabeled steroid) exceeded 2% (v/v), this induced aggregation of the microsomes, and Wiebel et al. (14) have shown that some P450-dependent enzyme activities could be affected by more than 1% (v/v) of ethanol. Therefore, ethanol concentration was fixed to be 0.68% (v/v) in the present study, by which some unlabeled steroids became insoluble in the reaction mixture at their final concentrations over 1.0 μM. 2) The 3H[PROG] concentration of 20 nM used seems physiological rather than those of the customary systems, since the plasma concentration is estimated to be about 10 nM in adult male rats (15, 16). 3) The yields of unidentifiable 3H[PROG] metabolites, included in both the water-soluble and toluene-extractable fractions, increased in a dose-dependent manner when either lower concentrations of 3H[PROG] or higher concentrations of NADPH were used.

### TABLE I

| Metabolite (formation) | Microsomes (μg protein) | NADPH (μM) | Unlabeled steroid (1 μM) |
|-----------------------|-------------------------|------------|-------------------------|
|                       | 414                     | 414        | PROG 16α-OH-P           |
|                       | 414                     | 414        | 11β-OH-P                |
|                       | 414                     | 414        | 3α-OH-5α-P              |
|                       | 414                     | 414        | 3α, 11β-(OH)-5α-P       |

|                       | % | % | % | % | % | % |
|-----------------------|---|---|---|---|---|---|
| Water-soluble         | 0.21 | 0.77 | 1.61 | 0.87 | 0.80 | 1.98 | 0.49 | 1.02 |
| Toluene-extractable   | 80.59 | 84.25 | 73.66 | 3.74 | 2.06 | 1.97 | 1.85 |
| 16α-OH-P              | 95.50 | 61.44 | 76.08 |
| 6β-OH-P               | 4.44 | 2.75 | 1.97 | 1.85 |
| 15α-OH-P              | 3.16 | 3.16 | 3.16 | 3.16 | 3.16 | 3.16 | 3.16 |
| 3α-OH-5α-P           | 1.51 | 1.58 | 6.91 | 6.08 | 4.17 |
| 5α-P                 | 8.53 |

a The yields of these oxidized (ox) and 5α-reduced (red) metabolites, formed under the experimental condition of which the microsomal suspension was incubated with 3H[progesterone in the presence of NADPH and absence of an unlabeled compound (the third column), were regarded as control throughout the present study.

2 A. Yamada, M. Yamada, Y. Fujita, T. Nishigami, K. Nakasho, and K. Uematsu, unpublished results.
dative metabolism, but the latter did not inhibit it. Most interestingly, 3α-OH-5α-P and 3α,11β-(OH)₂-5α-P, compared with PROG, not only showed stronger inhibitory effects on the oxidative metabolism but also conversely stimulated the 5α-reductive metabolism.

Classification of Various Steroids Based on Their Respective Effects on the Oxidative and 5α-reductive [3H]PROG Metabolisms by Rat Liver Microsomes—We found that various unlabeled steroids used could be divided into six groups, A, B, C, D, E, and F, based on their respective effects on the oxidative and 5α-reductive [3H]PROG metabolisms (Fig. 2 and Table I). The group A steroids such as 3α-OH-5α-P and 5α-P showed inhibitory effects on the oxidative metabolism, while having stimulatory effects on the 5α-reductive metabolism producing themselves. The group B steroids, PROG and TEST, inhibited both metabolisms as probably alternative substrates. The group C steroids, 5β-A-17β-ol and 3β-OH-P, showed inhibitory effects on the oxidative metabolism with no effect on the 5α-reductive metabolism, and conversely, the group D steroids, COR and 11β-OH-P, showed stimulatory effects on the 5α-reductive metabolism with no effect on the oxidative metabolism, despite possessing a 3-keto-4-ene structure that might be catalyzed by the 5α-reductase. Other 3-keto-4-ene steroids (group E), 16α-OH-P and 6β-OH-P, inhibited only the 5α-reductive metabolism without the product inhibition effects on the oxidative metabolism producing themselves. It is noteworthy that 16α-OH-P, as well as 20α-OH-P and 4-AN-CA already reported by other investigators (19, 20), were of the 3-keto-4-ene steroids showing the strongest inhibitory effect on the 5α-reductase activity. Finally, the group F steroids, 11α-OH-P and cholesterol, showed a slight effect or no effect on both of the metabolisms.

For additional interesting information, 3α,11β-(OH)₂-5α-P, a
group A steroid, containing both a 3α-OH-5α-reduced structure and a C-11β-OH structure, showed an additively stimulatory effect on the 5α-reductive metabolism, as compared with its parental steroids, 3α-OH-5α-P and 11β-OH-P, and thus this steroid, although not actually produced in the liver, was the highest stimulator of the 5α-reductase activity.

By the way, one may envisage a possibility that such a stimulatory effect of group A steroids on the 5α-reductive metabolism may result from the increasing utilizations of free [3H]PROG and NADPH, left over by their inhibitory effects on the oxidative [3H]PROG metabolism and vice versa. However, this possibility may be largely refuted by the results of the following two experiments using the anti-rat NADPH P450 reductase antiserum and rat CYP3A2 supersomes.

**Inhibition of P450-dependent [3H]PROG Metabolism by Anti-rat NADPH P450 Reductase Antiserum**—We examined the direct effects of representative steroids on the 5α-reductive [3H]PROG metabolism, using the rat liver microsomes pretreated with goat anti-rat NADPH P450 reductase antiserum (Fig. 3). By this means, more than 85% of the P450-dependent, oxidative [3H]PROG metabolism was inhibited, irrespective of the absence or presence of an unlabeled steroid. Under such an experimental condition, PROG and 16α-OH-P inhibited the 5α-reductive metabolism, while 11β-OH-P, 3α-OH-5α-P, and 3α,11β-(OH)2-5α-P stimulated it, as the intact microsomes did (see Fig. 2). This result clearly shows that the effects of these steroids on the 5α-reductive metabolism could be brought about by their intrinsic properties, not affected by the co-existence of P450-dependent metabolism in intact rat liver microsomes.

For additional information, an addition of normal goat serum, as compared with the 130 mM KCl-based buffer (12), induced a tendency to decrease the oxidative metabolism and increase the 5α-reductive metabolism. Although the mechanism inducing such a tendency is wholly unclear at present, this may have been associated with lower stimulatory effects of 11β-OH-P, 3α-OH-5α-P, and 3α,11β-(OH)2-5α-P on the 5α-reductive metabolism by the antisera-treated microsomes, compared with the intact microsomes.

**[3H]PROG Metabolism by Rat CYP3A2 Supersomes**—In order to examine also the direct effects of representative steroids on the male-specific P450-dependent [3H]PROG metabolism, we used rat CYP3A2, but not CYP2C11, supersomes, which were composed of the microsomes of insect cells containing the cDNA-expressed rat CYP3A2, rat NADPH P450 reductase, and human cytochrome b5, since a recombinant CYP2C11 expression system has not come into the market, and we found that various unsubstituted steroids showed a similar inhibitory pattern on rat liver microsomal [3H]PROG 6β-oxidation and 16α-oxidation, mainly catalyzed by CYP3A2 and CYP2C11, respec-
Fig. 5. Effects of representative unlabeled steroids on the [3H]pregnesterone 6β-oxidizing activity by rat CYP3A2 supersomes. Rat CYP3A2 supersomes (82.5 μg of protein/1.1 ml, total volume of the reaction mixture) were used instead of rat liver microsomes, and the concentration of an unlabeled steroid was fixed to be 1.0 μM in this experiment. Other experimental conditions were the same as shown in Fig. 1. The data are means ± S.D. of at least three experiments. The percentage of formation of exclusively formed [3H]6β-OH-P was estimated to be 13.98 ± 1.80% (37.28 pmol/mg of protein/5 min) in the control experiments.

Discussion

In conclusion, the present study clearly shows that the male-specific products, 16α-OH-P and 6β-OH-P, inhibited the female-predominant 5α-reductase activity without significantly inhibiting the male-specific CYP2C11 and CYP3A2 activities producing themselves. On the other hand, the female-predominant 5α-reductase in the female (1–10), and it is known that expressions of these sexually differentiated enzyme activities are largely regulated in transcription level under endocrine control of which GH plays a major role (6, 8–10).

In the present in vitro study using adult male rat liver microsomes (Tables I and II; Figs. 1–4) and rat CYP3A2 supersomes (Figs. 5 and 6; Table II), we showed for the first time that two major male-specific oxidized PROG metabolites, 6β-OH-P and especially 16α-OH-P, strongly inhibited the female-predominant 5α-reductase activity without significantly showing the inhibitory effects on the CYP3A2 and CYP2C11 activities producing themselves, and these events may be further...
ther enhanced by high levels of CYP2C11 and CYP3A2 gene expressions in the male (6, 8–10, 17). On the other hand, 5α-P and especially 3α-OH-5α-P not only inhibited both the CYP2C11 and CYP3A2 activities but also stimulated the 5α-reductase activity producing themselves. However, such adverse effects of the 5α-reduced products on the male pattern metabolism may be attenuated by a scanty expression of the 5α-reductase gene in the male (8, 10). Thus, our results compel us to propose a very interesting hypothesis, summarized in Fig. 7, that adult male rat liver microsomes may possess a self-augmentation system by the male-specific products on sexually differentiated steroid-metabolizing activities, coupled with the regulation system by gene expressions of the related enzymes under endocrine control. In other words, the results may also explain the reason why adult male rat liver should preserve not only much higher levels of CYP2C11 and CYP3A2 gene expressions but also lower 5α-reductase gene expression, as compared with the female.

Furthermore, it is of great interest and importance to investigate whether the female rat liver also possesses such a self-augmentation system, although the present results strongly suggest that at least female-predominant 5α-reductase activity (1, 3, 7, 8, 10, 18) may be further enhanced by its products, 5α-P and especially 3α-OH-5α-P. As regards these, an important question for future study is to elucidate the reason why adult male rat liver microsomes must metabolize PROG first into more hydrophilic products, 16α-OH-P and 6β-OH-P, while the female must metabolize it into more hydrophobic products, 5α-P, under the strictly regulated systems described above.

By the way, a similar scenario may occur on the androgen metabolism, since 3-keto-4-ene androgens such as TEST and 4-AN are also known to be catalyzed sex-dependently by the same enzyme systems (1–3, 5, 7–10, 18), and the effects of various 3-keto-4-ene and 5α-reduced androgens, especially TEST and 5α-A-3α,17β, on the [3H]PROG metabolism showed a similar pattern to those of various 4-pregnene and 5α-pregnane steroids described here (Fig. 2).²

As regards another interesting finding obtained from the present study, it has been reported that endogenous COR production in rat adrenal cortex is suppressed by exogenously administrated COR or cortisol in vivo and in cell culture systems and that this inhibition probably results from the various effects of these steroids, namely inhibiting ACTH secretion from the pituitary, decreasing ACTH sensitivity of adrenal cortex (23), and stimulating the adrenal 5α-reductase activity metabolizing COR into its 5α-reduced products (24). However, several recent studies have clearly shown that the two 11β-OH corticosteroids, COR and cortisol, are of the poorest substrate group for 5α-reductases of various organs probably including the adrenal cortex itself (19, 20, 25), and we showed in the present study that COR and 11β-OH-P, but not 11α-OH-P, rather stimulated [3H]PROG 5α-reductase activity of rat liver microsomes (Fig. 2). These results suggest that the C-11β-OH group of a steroid molecule may strongly disturb access of the steroid to the active site of the 5α-reductase, and we can propose another possibility that adrenal cortex may

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**TABLE II**

| Unlabeled steroid     | Rat liver microsomes [3H]6α-OH (IC27,3) | Rat CYP3A2 supersomes [3H]6α-OH (IC27,3) | PROG     | 3α-OH-5α-P | 16α-OH-P | 5α-OH-P |
|-----------------------|----------------------------------------|-----------------------------------------|----------|------------|----------|---------|
|                       | ×10⁻⁶ M                                 | ×10⁻⁶ M                                 |          |            |          |         |
| PROG                  | 0.71                                   | 0.76                                    | 0.98     |            |          |         |
| 3α-OH-5α-P            | 0.08                                   | 0.06                                    | 0.12     |            |          |         |

* The IC27,3 and IC40 values, estimated from at least four experiments for separate rats, were defined as the molar concentrations (×10⁻⁶ M) of unlabeled PROG or 3α-OH-5α-P causing a 27.5% and a 40% inhibition against the respective [3H]PROG 6β-oxidizing (6β-[3H]6α-OH) and 16α-oxidizing (6α-[3H]16α-OH) activities, and these inhibitory percentages were conveniently created as showing half-maximal inhibitions by the concentrations up to 1.0 μM, a maximally solubilizable concentration, of unlabeled 3α-OH-5α-P.

* The K values are estimated from three experiments.

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**Fig. 7.** Supposed self-augmentation system on sexually differentiated progesterone metabolism in adult male rat liver. 16α-OH-P and 6β-OH-P are produced by male-specific microsomal P450s, CYP2C11 and CYP3A2, respectively. Both products inhibit PROG 5α-reduction without significant product-inhibition effects on the above P450 activities, and these events may be further enhanced by high levels of CYP2C11 and CYP3A2 gene expressions in the male. Actually, 16α-OH-P may make a higher contribution to the inhibitory effect on the 5α-reductase activity than 6β-OH-P, judging from the result shown in Fig. 2 and a higher expression of CYP2C11 gene than CYP3A2 gene (1, 9). On the other hand, 5α-P and 3α-OH-5α-P are produced by the 5α-reductase and subsequently cystolic (and, to a lesser degree, microsomal) 3α-hydroxysteroid dehydrogenase (26–28), respectively. Although these products, especially 3α-OH-5α-P, not only inhibit the CYP2C11 and CYP3A2 activities but also stimulate the 5α-reductase activity, such adverse effects of these 5α-reduced products on the male pattern metabolism may be actually attenuated by lower expressions of the 5α-reductase (1, 3, 7, 8, 10, 18) and 3α-hydroxysteroid dehydrogenase (27, 28) genes in the male, compared with the female. 16α-OH-P, 6β-OH-P, or 3α-OH-5α-P can be further metabolized into its glucuronide or sulfate and eventually excreted into urine and/or bile. It is most likely that the same self-augmentation system operates on the androgen metabolism. m, microsomal enzyme. c, cystolic enzyme. §, the magnitudes of sexually differentiated enzyme activities in adult male rat liver, compared with the female, are as follows: CYP2C11 (>30-fold higher) (1–3, 9), CYP3A2 (>20-fold higher) (1, 9), 5α-reductase (>10-fold lower) (2, 3, 7), 3α-hydroxysteroid dehydrogenase (2–3-fold lower) (27, 28), UDP-glucuronosyl transferase (≈1.5-fold higher) (29), and hydroxysteroid sulfotransferase (4–6-fold lower) (30, 31).
possess a short negative feedback system of which the excessively produced COR (and probably cortisol) inhibits its own production by stimulating the 5α-reduction of PROG (but not COR itself), the major precursor of COR.

In conclusion, we can propose two novel hypotheses on 1) the self-augmentation system on sexually differentiated steroid metabolism in adult male rat liver and 2) a short negative feedback system of COR production in adrenal glands. Although the action mechanisms operating these regulatory systems are largely unclear at present, an attempt to clarify them is currently under investigation in our laboratory.

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