Effects of Hypophysectomy and Growth Hormone Treatment on Sex-Specific Forms of Cytochrome P-450 in Relation to Drug and Steroid Metabolisms in Rat Liver Microsomes†

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Abstract—Hypophysectomy decreased the content of a male specific cytochrome P-450, P-450-male, in male rats, while it expressed P-450-male and completely depressed a female specific cytochrome P-450, P-450-female, in female rats. Intermittent injections of human growth hormone (GH), which mimic the secretion in males, restored P-450-male in hypophysectomized (Hypox) male rats and partially restored P-450-female in Hypox female rats. Continuous infusion of GH, which mimics the female secretion pattern, into Hypox male rats caused a further decrease in P-450-male content, and it caused the expression of P-450-female. In Hypox female rats, the same treatment depressed P-450-male and expressed P-450-female to the level of an intact female. These results indicate that the diurnal changes in the pattern of serum growth hormone level regulate the expression of P-450-male and P-450-female. The activities of testosterone 2α- and 16α-hydroxylases were closely correlated to P-450-male content with the correlation coefficients (r) of 0.955 and 0.929, respectively. Benzo(a)pyrene hydroxylation was also correlated to P-450-male content (r=0.850). Aminopyrine N-demethylation and propoxycoumarin O-depropylation were correlated to less extents (r=0.692 and r=0.720), while aniline hydroxylation and O-ethylresorufin O-deethylation were not correlated to P-450-male content. These results indicate that testosterone 2α- and 16α-hydroxylations are closely dependent, but the metabolism of a variety of drugs are dependent to different extents on P-450-male in rat liver microsomes.

Cytochrome P-450 in liver microsomes plays a major role in the oxidation of a wide variety of exogenous and endogenous compounds. Sex-related differences and androgenic regulation of cytochrome P-450 dependent oxidations of many drugs and steroid hormones in rat liver microsomes have been well-known (1-3). With the advances in the purification of cytochrome P-450, the presence of multiple forms of cytochrome P-450 has been demonstrated (4-6). We purified male-specific cytochrome P-450 from male rat liver and female-specific cytochrome P-450 from female rat liver and have designated them as P-450-male and P-450-female, respectively (7-9). We proved immunochemically that P-450-male and P-450-female exist specifically in liver microsomes from male and female rats, respectively, and their contents are about one-third of the total cytochrome P-450 (9, 10). P-450-male shows distinctly higher activities in the oxidation of various drugs and testosterone in comparison with those of P-450-female (9,
Moreover, Waxman (14) purified male specific cytochrome P-450, P-450 2c, which is responsible for the 16α-hydroxylation of testosterone in rat liver microsomes; and Ryan et al. (15) and MacGeoch et al. (16) purified female specific cytochrome P-450, designated as P-450i and P-45015β, respectively, which are responsible for 15β-hydroxylation of 5α-androstan-3α,17β-diol-3,17-disulfate in rat liver microsomes. On the basis of catalytic activities, N-terminal amino acid sequences and other properties, P-450 2c, and P-45015β and P-450i are probably identical with or have a close resemblance to P-450-male and P-450-female, respectively (12, 17).

On the other hand, the involvement of the pituitary gland in the occurrence of sex differences induced by gonadal hormones in drug and steroid metabolisms in rat liver microsomes has been demonstrated (3, 18–20). Growth hormone has been postulated to be the responsible factor in the pituitary gland.

More recently, P-45015β and steroid 16α-hydroxylase cytochrome P-450, designated as P-45016α, in rat liver has been reported to be regulated by the hypothalamo-pituitary system (16, 21).

In the present study, we have examined the roles of the pituitary gland and growth hormone in the regulation of the levels of P-450-male and P-450-female in relation to drug oxidation and testosterone hydroxylation in rat liver microsomes. Our results clearly indicate that the secretion pattern of growth hormone is the factor determining the expression and suppression of P-450-male and P-450-female, and it is the responsible for the sex-related differences in testosterone 2α- and 16α-hydroxylations, benzo(a)pyrene hydroxylation and aminopyrine N-demethylation in rat liver microsomes.

Materials and Methods

Animals: Male and female Sprague-Dawley rats were purchased from Clea Japan, Tokyo, Japan. Some animals, which were hypophysectomized at 7 weeks of age and left to recover for a week, received a subcutaneous injection (0.2 IU/100 g body weight) twice a day or osmotic infusion (0.01 IU/hr or 0.002 IU/hr) of human growth hormone (GH) for 7 days. Two types of GH preparations, Crescormon® and a recombinant methionylated one, Somatonorm®, (both from Kabi Vitrum, Stockholm, Sweden), which were kindly donated from Sumitomo Pharmaceutical Co., were used. No significant difference in their activities was observed in the present study. For the infusion, an osmotic minipump, Alzet 2001 (Alza, Palo Alto CA), designed to release its contents at the rate of 1 μl/hr, was implanted on the back of the rats. A group of GH treated animals was given estradiol benzoate (500 μg/kg, dissolved in corn oil) on days 1, 3, 5 and 7 of the experiment. The animals were killed 8 days after the initial administration and were checked for the absence of a pituitary in Hypox rats. Hepatic microsomes were prepared as previously reported (22).

Immunochemical quantification of P-450-male and P-450-female: A peroxidase staining technique coupled with SDS-polyacrylamide gel electrophoresis (Western blot) was used to quantitate the content of P-450-male and P-450-female in hepatic microsomes (5, 22). The characteristics of P-450-male, P-450-female and monospecific antibodies to these cytochromes were described previously (9, 22).

Assays of the microsomal drug and steroid metabolizing activities: A typical incubation mixture (1 ml) consisted of NADP (0.8 mM), glucose 6-phosphate (8.0 mM), glucose 6-phosphate dehydrogenase (1 IU), magnesium chloride (6 mM), potassium, sodium phosphate buffer (pH 7.4, 100 mM), ethylenediaminetetraacetic acid (0.1 mM), liver microsomes (0.25 mg for the metabolisms of testosterone, benzo(a)pyrene and O-ethylresorufin, and 1.0 mg for 7-n-propoxyccoumarin, aminopyrine and aniline), and a substrate (2 mM testosterone, 0.1 mM benzo(a)pyrene, 2 μM O-ethylresorufin, 0.5 mM 7-n-propoxyccoumarin, 5 mM aminopyrine or 5 mM aniline). The incubation was started by addition of the NADPH generating system (NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and magnesium chloride), which was preincubated at 37°C for 3 min, and was reacted at 37°C for 15 min, except for O-ethylresorufin oxidation which was reacted for 5 min. The activities of
testosterone hydroxylases were quantitated by the method of van der Hoeven (23) with modifications. Testosterone metabolites were separated on a nucleosil 5C18 (Macherrey-Nagel) column. 17α-Methyltestosterone was used as the internal standard. For the metabolisms of 7-propoxycoumarin, benzo(a)-pyrene and O-ethylresorufin, fluorescent phenolic metabolites were determined by the methods of Aitio (24), Nebert and Gelboin (25) and Burke and Mayer (26), respectively. 3-Hydroxybenzo(a)pyrene was used as the standard for the assay of benzo(a)pyrene hydroxylation. Formaldehyde, detected by the method of Nash (27), was estimated for the activity of aminopyrine N-demethylation. For aniline hydroxylation, p-aminophenol was measured colorimetrically (28).

Microsomal protein was determined by the method of Lowry et al. (29) with bovine albumin as the standard. Total cytochrome P-450 content was estimated by the method of Omura and Sato (30), except that 20% glycerol and 0.2% Emulgen 913 were included.

Materials: O-Ethylresorufin and 7-n-propoxycoumarin were synthesized by the O-alkylation of resorufin (Eastman-Kodak, Rochester, NY) and umbelliferone (Nakarai Chemicals, Kyoto, Japan) with their respective alkyliodides in the presence of silver oxide and dimethylformamide (31). The products were purified on a silica gel column with diethylether and diethylether/chloroform as the eluants. Goat anti-rabbit Immunoglobulin G was purchased from Cappel Lab. (Cochranville, PA). Horseradish peroxidase-rabbit antiperoxidase complex was from Miles-Yeda (Rehovot, Israel). 3,3'-Diaminobenzidine, benzo(a)pyrene, testosterone, 17α-methyltestosterone, 16β-hydroxytestosterone were obtained from Sigma Chemicals, St. Louis, MO. Testosterone 2α-, 6β- and 16α-hydroxy derivatives of testosterone were supplied from Steraloids (Wilton, NH). Testosterone 7α- and 15α-hydroxy derivatives, and 3-hydroxybenzo(a)pyrene were the generous gift of Dr. Y. Nakamura, Shionogi and Co., Ltd., Osaka, Japan, and Dr. P. Fu, National Center for Toxicological Research, Jefferson, AR, respectively. Statistical analysis was made using Student’s t-test.

Results

Effects of hypophysectomy and intermittent injections of GH on P-450-male and P-450-female contents: The intermittent injections of GH (0.2 IU/100 g, s.c., twice daily for 7 days) to intact male and female rats did not cause significant change in P-450-male, P-450-female and total P-450 contents (Fig. 1).

Hypophysectomy of male rats decreased P-450-male content, without change in total P-450 content and did not cause an appearance of P-450-female in male rats in
accordance with a previous report (17). On the other hand, hypophysectomy of female rats caused a disappearance of P-450-female and interestingly an expression of P-450-male. The intermittent injections of GH into the Hypox animals caused a complete recovery of P-450-male content in male rats and partially restored P-450-female and caused a further increase in P-450-male in female rats.

Effect of GH on P-450-male and P-450-female contents: The secretion patterns of growth hormone from the hypophysis are markedly different in male and female rats (32). The basal level of serum growth hormone is higher in female than in male rats, but surges in the serum level three or four times a day are observed in male rats by means of episodic releases.

Therefore, we have studied the effect of continuous infusion by the minipump on the contents of P-450-male and P-450-female in liver microsomes of both sexes.

In contrast to the effect of intermittent injection, the continuous infusion of GH into intact male rats caused a marked decrease in P-450-male content, a clear appearance of P-450-female and a slight decrease in total P-450 content (Fig. 2). Similar effects of the continuous infusion of GH were observed in Hypox male rats. On the other hand, in intact female rats, no significant change in the

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**Fig. 2.** Effects of hypophysectomy and GH treatment on the contents of P-450-male and P-450-female in rat livers. GH was given to adult control (Cont) and Hypox rats with an Alza minipump at the rate of 0.01 IU/hr for 7 consecutive days (GH(i, H)) or at the rate of 0.002 IU/hr (GH(i, L)). GH or estradiol benzoate was also injected intermittently into GH-treated rats. Other experimental details are the same as described in Fig. 1 and Methods. "a": statistically significant difference from the corresponding controls (P<0.05). "b": statistically significant difference from the corresponding Hypox rats. "c": statistically significant difference from the corresponding GH-infused Hypox rats. "d": statistically significant difference from the corresponding Hypox rats plus intermittent GH treated rats.
levels of P-450-female was observed by the continuous infusion. In Hypox female rats, the expression of P-450-male was depressed and that of P-450-female was stimulated by the continuous infusion.

The intermittent injections of GH into the Hypox male rats concomitantly receiving the continuous infusion caused a slight increase in P-450-male content, indicating that both episodic high level and low basal level of serum growth hormone may be needed for the full expression of P-450-male and P-450-female.

A concomitant treatment with estradiol and GH (intermittent injections) in Hypox female rats suppressed the expression of P-450-male and caused a partial restoration of P-450-female content. All the data presented in Fig. 2, indicate that the diurnal changes in the pattern of serum growth hormone level regulate the expression of P-450-male and P-450-female.

Effects of hypophysectomy and intermittent injections of GH on testosterone hydroxylation and drug oxidation: Hypophysectomy decreased testosterone 16α-hydroxylase activity in male rats, but increased it in female rats (Table 1). The intermittent injections of GH into the Hypox animals markedly enhanced the expression of 16α-hydroxylase activity in male and female rats. Similar results were also observed in testosterone 2α-hydroxylase activity. Therefore, these results indicate that the activities of testosterone 2α- and 16α-hydroxylases are closely associated with the level of P-450-male.

Similar profiles were noted between P-450-male content and the activities of propoxycoumarin O-depropylase, benzo(a)-

<table>
<thead>
<tr>
<th>Metabolic activities (nmol/mg protein/min)</th>
<th>Control</th>
<th>Control+GH(s)</th>
<th>Hypox</th>
<th>Hypox+GH(s)</th>
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<tr>
<td><strong>Male</strong></td>
<td></td>
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<tr>
<td>Testosterone 16α-hydroxylation</td>
<td>1.870±0.510</td>
<td>1.520±0.470</td>
<td>0.565±0.140&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.220±0.445&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Testosterone 2α-hydroxylation</td>
<td>0.820±0.125</td>
<td>0.550±0.145&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.465±0.210&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.185±0.185&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Propoxycoumarin O-depropylation</td>
<td>1.299±0.157</td>
<td>0.743±0.129&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.846±0.309&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.200±0.068&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Benzo(a)pyrene hydroxylation</td>
<td>0.248±0.039</td>
<td>0.199±0.042&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.188±0.022&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.197±0.011&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Aminopyrine N-demethylation</td>
<td>7.33±0.75</td>
<td>5.19±0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.49±0.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.46±0.28&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td><strong>Female</strong></td>
<td></td>
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<tr>
<td>Testosterone 16α-hydroxylation</td>
<td>0.035±0.015</td>
<td>0.060±0.025</td>
<td>0.375±0.035&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.700±0.380&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<td>Testosterone 2α-hydroxylation</td>
<td>0.035±0.015</td>
<td>0.035±0.005</td>
<td>0.225±0.045&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.595±0.100&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<td>Propoxycoumarin O-depropylation</td>
<td>0.060±0.005</td>
<td>0.048±0.007&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.515±0.206&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.444±0.172&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Benzo(a)pyrene hydroxylation</td>
<td>0.039±0.006</td>
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<td>0.127±0.031&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.167±0.045&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<tr>
<td>Aminopyrine N-demethylation</td>
<td>2.00±0.21</td>
<td>2.04±0.30</td>
<td>2.00±0.38</td>
<td>2.83±0.45&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<sup>a</sup>: Statistically significant difference from the respective control (P<0.05). <sup>b</sup>: Statistically significant difference from the Hypox group (P<0.05). Intact (control) and hypophysectomized (Hypox) rats were given intermittent subcutaneous injections of growth hormone (GH(s)) twice a day for 7 days. Values shown were obtained from at least 5 different determinations (the mean±S.D.). Other experimental details are described in Methods.
pyrene hydroxylase and aminopyrine N-demethylase, with some deviations. For example, propoxycoumarin O-depropylase activity in male rats was slightly increased by hypophysectomy. The treatment of Hypox male rats with GH decreased the activity of propoxycoumarin O-depropylase and did not change the activities of benzo(a)pyrene hydroxylase and aminopyrine N-demethylase.

Effect of GH infusion on testosterone hydroxylation and drug oxidation: The continuous infusion of GH into intact male rats markedly decreased testosterone 2α- and 16α-hydroxylase activities (Fig. 3). The continuous infusion of GH caused a further decrease in 2α- and 16α-hydroxylase activities in Hypox male rats and depressed the elevated activities in Hypox female rats. The combined treatment with the intermittent injections and continuous infusion of GH caused a partial recovery of 2α- and 16α-hydroxylase activities.

The administration of estradiol to GH-treated Hypox female rats decreased the elevated 2α- and 16α-hydroxylase activities.

As shown in Fig. 4, we found similar results among the changes in P-450-male content and the activities of testosterone 2α- and 16α-hydroxylases, propoxycoumarin O-depropylase, benzo(a)pyrene hydroxylase and aminopyrine N-demethylase, although some dissimilarities were noted. For example, the activity of propoxycoumarin O-depropylation was slightly increased in Hypox male rats, while P-450-male was slightly decreased. In contrast, the changes in the activities of aniline hydroxylase and O-ethylresorufin O-deethylase were quite different from those in P-450-male content and benzo(a)pyrene hydroxylase activity (Fig. 5).

Discussion

The oxidation of drugs and steroid hormones by cytochrome P-450 is generally more efficient in liver microsomes from male rats than from females (2). Thus, sex-specific forms of cytochrome P-450, termed P-450-male and P-450-female, were isolated.
Fig. 4. Change in microsomal drug metabolizing activities on typical drugs showing sex-related difference by hypophysectomy and/or GH treatment. The activities were measured as described in Methods. Other experimental details are described in Fig. 3.

The oxidation of several drugs by the reconstituted system with P-450-male was higher than that found with P-450-female. P-450-male, therefore, seems to be the major form of cytochrome P-450 responsible for the sex-related metabolism of a variety of drugs. P-450-male has higher activity in testosterone 2α- and 16α-hydroxylations, while P-450-female does not show any significant activity for testosterone 2α- and 16α-hydroxylations (12, 13). Androgen and estrogen are apparently involved in the expression of sex-related differences in drug and steroid hydroxylations (1, 3, 33). Moreover, the pituitary gland also plays an essential role in the sex-related differences in drug and steroid hydroxylations (18, 19). Growth hormone has been postulated as a responsible factor (18, 20, 34, 35).

In the present paper, we have confirmed the involvement of the pituitary gland and the essential role of growth hormone in the regulation of P-450-male and P-450-female levels in hepatic microsomes of male and female rats.

The intermittent subcutaneous injections of GH (0.2 IU/100 g, twice daily for 7 days) roughly mimic the episodic secretion pattern of growth hormone in male rats, and the continuous infusion by a minipump (0.002–0.01 IU/hr for 7 days) roughly mimics the secretion pattern of growth hormone in female rats (32, 35). The intermittent injections and continuous infusion of GH caused the observed changes in P-450-male and P-450-female contents, which were seen in male and female rats, respectively.

These results may be in accord with recent
Fig. 5. Effects of hypophysectomy and/or GH treatment on the activities of O-ethylresorufin deethylase and aniline hydroxylase. The procedures used for the measurement of the activities are described in Methods. Activities of intact (Cont) and Hypox rats with or without GH infusion (0.01 IU/hr) (GH(i, H)) are shown as the mean±S.D. Data are obtained from at least 5 different determinations.

reports on the regulation by growth hormone of male specific P-450\textsubscript{16α} and female specific P-450\textsubscript{15β} in rat liver (16, 21). However, the presences of multiple forms of sex-related cytochrome P-450 have been reported (36-38, Omura et al., personal communication), therefore, further studies will be needed for understanding the mechanism of the regulation of these forms of cytochrome P-450.

In the present studies, we demonstrated that the changes in the activities of testosterone 2α- and 16α-hydroxylases caused by hypophysectomy and the intermittent injections or continuous infusion of GH were closely correlated with those observed in P-450-male content (Fig. 6). The correlation coefficients between P-450-male content vs. 2α-hydroxylase activity and P-450-male content vs. 16α-hydroxylase activity were 0.955 and 0.929, respectively. In addition, we have found that anti-P-450-male immunoglobulin G inhibited testosterone 2α- and 16α-hydroxylations, but not 6β- and 7α-hydroxylations (Y. Yamazoe et al., unpublished observation).

Thus it is highly possible that P-450-male is the major form of cytochrome P-450 responsible for testosterone 2α- and 16α-hydroxylation in male rat liver microsomes. Our results on the regulation by growth hormone are in accordance with the previous report using androstenedione as a substrate (35).

Moreover, we observed that the activities of benzo(a)pyrene hydroxylase, propoxy-coumarin O-depropylase and aminopyrine N-demethylase were roughly correlated with P-450-male content, showing correlation coefficients of 0.850, 0.720 and 0.692, respectively (Fig. 6).

However, these activities were less correlated as compared with testosterone hydroxylations, suggesting the involvement of other forms of cytochrome P-450 in the oxidations of these drugs from the point of view of the multiplicity of cytochrome P-450 (39). The correlation coefficient between P-450-male content and benzo(a)pyrene hydroxylase activity was high enough, but showed the intercept in Fig. 6B at 0.057 pmoie/mg protein/min, suggesting that about one-fourth of the benzo(a)pyrene hydroxylating activity in male rat liver microsomes is
catalyzed by a cytochrome P-450 other than P-450-male. A similar high intercept value was also observed for aminopyrine N-demethylation. On the other hand, the observed intercept value for propoxycoumarin O-depropylation was low, but two good correlation lines could be drawn by dividing the data according to growth hormone depleted (Hypox rats) and growth hormone non-depleted rats (Fig. 7). These results suggest that propoxycoumarin O-depropylation is catalyzed by a form(s) of cytochrome P-450, which is readily suppressed by growth hormone, together with P-450-male. The patterns of change in the activities of aniline hydroxylase and O-ethylresorufin O-deethylase were quite different from that of P-450-male. All of these results indicate that both of the above activities are catalyzed by other form(s) of cytochrome P-450 in male rats and are in accord with the lack of clear sex difference found in aniline hydroxylation (40) and the presence of reversed sex differ-
ence (male<female) in O-ethylresorufin O-deethylation in rat liver microsomes (41). Together with the inhibition study using specific antibodies against P-450-male, the present studies may offer an excellent tool for classification of P-450-male dependency of drug metabolism in rat liver microsomes.

The mechanism of the effects of growth hormone on the contents of P-450-male and P-450-female is not known. It has been suggested that diverse effects of growth hormone on the liver and other tissues are mediated by producing somatomedin (42). In the present work, we used human growth hormone (GH), which has both somatogenic and lactogenic action (43). In a further study, therefore, we used ovine prolactin, a specific lactogen (44) but could not observe any significant effect (Y. Yamazoe et al., unpublished observation), indicating that the observed effect of human growth hormone is dependent on growth hormone receptors, but not on the prolactin receptors of rat liver.

Further studies will be needed to clarify the substrate specificity and to elucidate the regulation mechanism of sex-specific cytochrome P-450.

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