Biochemical Significance of 19-Hydroxytestosterone in the Process of Aromatization in Human Corpus Luteum

SHINYA WATANABE, YOSHIRO TOHMA, HIROSHI CHIBA, YUKIKO SHIMIZU, HIROSHI SAITO AND TAKUMI YANAIHARA
Department of Obstetrics and Gynecology, Showa University, School of Medicine, Tokyo 142, Japan

Abstract. 19-Hydroxyandrogens are known to be an intermediary metabolite in the aromatizing reaction, though the physiological role of this compound has not yet been clarified. In this study, microsomes obtained from human corpus luteum were incubated with testosterone or 19-hydroxytestosterone (19-OHT) as the substrate to investigate the biochemical significance of 19-OHT in the process of aromatization in the ovary. The inhibitory effects of 4-hydroxyandrostenedione (4-OHA) on the formation of estradiol from testosterone and 19-OHT in human ovary were also investigated.

When testosterone was incubated with human ovarian microsomes, 19-OHT and estradiol were identified. When 19-OHT was used as the substrate, the formation of estradiol was demonstrated. To our knowledge, this is the first report to demonstrate the formation of estradiol from 19-OHT in human ovarian tissue. The Km value of aromatase for testosterone on human corpus luteum microsomes was 0.21 µM. 4-OHA exhibited inhibition with a Ki of 35 nM. With testosterone and 19-OHT as the substrate, the formation of estradiol was also equally inhibited by 4-OHA. A dose dependent inhibition of estradiol formation was observed, with no apparent accumulation of 19-OHT. These results suggest that 19-OHT may not only be an intermediary metabolite in the aromatization of testosterone by human ovary but could be a product of the microsomal enzyme.

Key words: Aromatization, Human ovary, 19-Hydroxy-testosterone (19-OHT).

THE CONVERSION of androgens to estrogens is catalyzed by a unique microsomal cytochrome P-450 dependent mono-oxygenase known as aromatase [1, 2]. From studies with human placental and ovarian aromatase it is known that the enzyme requires three equivalents of molecular oxygen and three equivalents of NADPH in the conversion of androgens to estrogens [3]. 19-Hydroxyandrogens are known to be an intermediary metabolite in the aromatizing reaction. Previously, we measured the concentration of 19-OHT in the ovarian vein by selected ion monitoring (SIM) [4], and suggested that 19-OHT is an endogenous steroid in human ovary.

In this study, microsomes obtained from human corpus luteum were incubated with testosterone or 19-OHT as the substrate to investigate the biochemical significance of 19-OHT in the process of aromatization in ovary. The inhibitory effects of aromatase inhibitor 4-hydroxyandrostenedione (4-OHA) on the conversion of testosterone and 19-OHT to estradiol were also studied.

Materials and Methods

Chemicals

Radiolabeled steroids, [4-14C]testosterone (SA,
57.3 mCi/mmol), [1, 2, 6, 7-3H]androstenedione (SA, 92.7 Ci/mmol), [6, 7-3H]19-OHA (SA, 46.3 mCi/mmol), [6, 7-3H]estradiol (SA, 52.0 Ci/mmol), [6, 7-3H]19-OHT (SA, 50.07 mCi/mmol) were obtained from New England Nuclear Corp. (Boston, MA) and purified by thin layer chromatography in a benzene-methanol (9:1, vol/vol) system before use. [6, 7-3H]19-OHT (SA, 50.07 mCi/mmol) was synthesized from [6, 7-3H]19-OHA by reduction with sodium borohydride, and was purified by thin layer chromatography. Unlabeled steroids were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) and Steraroids (Wilton, NH, USA). NADPH and 4-OHA were obtained from Sigma Chemical Co. (St. Louis, Mo, USA). Sodium borohydride was purchased from E. Merck (Darmstadt, Germany).

Subjects

The corpora lutea were collected from endocrinologically normal 5 women, ages 30 to 40 years, at hysterectomy due to cervical carcinoma. The stage (6 to 9 days after ovulation) of the menstrual cycle was assessed from their endometrial histology and the date of the last menstrual period. This study was approved by Showa University Ethical Committee and informed consent was obtained from all the subjects prior to the experiment. The ovarian tissues in ice-cold saline solution were brought to the laboratory and immediately prepared for incubation, as described below.

Tissue preparation

The corpus luteum was cut and carefully washed with SDP Buffer (0.25 M sucrose, 0.5 mM dithiothreitol in 67 mM phosphate buffer, pH 7.4). The tissues were then rinsed with SDP buffer and homogenized in 6 vol SDP buffer (grams of wet tissue per ml). The microsomal fraction was obtained by the method of Shiroshita et al. [5]. The microsomal pellet was diluted with SDP buffer and 20% glycerol, and stored at −80°C until analyzed. The protein concentration was measured by the method of Lowry et al. [6].

Incubation

The microsomes (0.05 mg protein) were incubated with [4-14C]testosterone (2.0 nM, 0.1 µCi) as the substrate in the presence of NADPH (500 mg) in SDP buffer at pH 7.4, 37°C, O2: CO2 = 95 : 5, for 30 min. The final volume of the incubation mixture was 1.0 ml. Control incubations were also performed without tissue preparation. At the end of the incubation, ethyl acetate (5.0 ml) involving carrier steroids (non-radioactive steroid, 25 µg each; radioactive steroids, 30,000 dpm each); androstenedione, testosterone, 19-OHA, 19-OHT, estrone and estradiol, were added to the incubation mixture, and the mixture was shaken vigorously to terminate further enzymatic reaction and to extract steroids. When [6, 7-3H]19-OHT (2.0 nM, 0.1 µCi) was used as the substrate, radioactive carrier steroids were not added to the incubation mixture. The inhibitory effect of 4-OHA (0 − 10−4M) on estradiol formation from testosterone and 19-OHT was also investigated.

Separation, quantitation and identification of the metabolites

After removal of the ethyl acetate layer, the aqueous layer of the incubation mixture was extracted again with 5 ml ethyl acetate. Each extract was applied to a silica gel (Silica gel GF254 E. Merck) thin layer plate and then developed in a system of benzene-methanol (9:1, vol/vol) to separate androstenedione, 19-OHA, 19-OHT, estrone and estradiol. Spots of the carrier steroids on thin layer chromatograms were detected under an UV light (254 nm). Radioactive spots on the chromatograms were autoradiographically detected, and each spot was scraped out of the plate with a razor blade. Each radioactive metabolite was eluted from the scraped silica gel with 10 ml methanol, and an aliquot of the elute was taken to measure the radio-activity of individual metabolites in a liquid-scintillation spectrometer (Aloka LSC=651) in a toluene-PPO (2, 5-diphenyloxazole) (0.4%, wt/vol), POPOP (1, 4-bis-[2-(5-phenyloxazolyl)]benzene), (0.01%, wt/vol) system. The recovery in all cases was 35% – 80% of the initial radioactivity. Finally, after crystallization of a radioactive metabolite with the corresponding authentic steroid, the specific radioactivity of the crystal and the solid in the mother liquor was confirmed as constant within the permissible limits of experimental error.
Results

Identification of the products

When labeled testosterone was used as the substrate, radioactive spots which corresponded to authentic 19-OHT and estradiol were detected (Fig. 1). The Rf values for 19-OHT and estradiol were 0.14 and 0.34, respectively. Tracer amounts of the androstenedione, 19-OHA and estrone formed were noticed. Beside estradiol, the major reaction product, 19-OHT on TLC, was eluted and finally crystallized to constant specific activity. As shown in Table 1, the formation of 19-OHT from testosterone was established after three successive crystallizations. The formation of estradiol was also confirmed when 19-OHT was used as the substrate (Table 2). The rate of conversion to estradiol was approximately 7% and no other detectable amounts of metabolites were found.

Kinetic study of aromatase

Under the conditions of the assay, an almost linear relationship between the length of time of incubation and the rate of 19-OHT and estradiol formation from testosterone existed for at least 60 min. A linear relationship with the formation of 19-OHT and estradiol was observed for protein concentrations from 0 to 0.2 mg per flask. The formation of 19-OHT and estradiol from testosterone is shown in Fig. 2. The Michaelis constant (Km) and Vmax of aromatase for estradiol from testosterone were determined by the graphical method of Lineweaver & Burk (1934) as 0.21 µM and 104.7 pmol/min, respectively (Fig. 3).

Inhibiting effect of 4-OHA

As shown in Fig. 4, when testosterone was used as the substrate, the formation of estradiol was strongly inhibited by adding 4-OHA (10⁻⁴M). The same effect was observed when 19-OHT was incubated on the formation of estradiol. The rate of inhibition by 4-OHA of estradiol formation from testosterone (98.7%) was compatible with that from 19-OHT (97.3%). As shown in Table 3, dose dependent inhibition of estradiol formation was observed with no apparent accumulation of 19-OHT. The effect of varying the concentrations of 4-OHA on testosterone aromatization as analyzed by

Table 1. Radiochemical purity of 19-hydroxytestosterone

<table>
<thead>
<tr>
<th></th>
<th>Crystallization</th>
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<tbody>
<tr>
<td>Original</td>
<td>X-1 X-2 X-3 ML-3</td>
</tr>
<tr>
<td>SA</td>
<td>17.0 16.6 17.2 17.2</td>
</tr>
<tr>
<td>SA, specific radioactivities (³Hdpm/¹⁴Cdpm); X-1,2,3, crystallization step; ML-3, mother liquid; Solvent 1, Methanol, n-heptane; Solvent 2, Ethanol, n-heptane; Solvent 3, Ethanol, water.</td>
<td></td>
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</tbody>
</table>

Table 2. Radiochemical purity of estradiol

<table>
<thead>
<tr>
<th></th>
<th>Crystallization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>X-1 X-2 X-3 ML-3</td>
</tr>
<tr>
<td>SA</td>
<td>12.3 12.5 12.6 9.4</td>
</tr>
<tr>
<td>Legends are the same as those to Table 1.</td>
<td></td>
</tr>
</tbody>
</table>
Lineweaver-Burk plot is shown in Fig. 5 with the corresponding slopes of the reciprocal plots. 4-OHA exhibited an apparent Ki of 35 nM.

**Discussion**

Concentrations of 19-hydroxyandrogens in human ovarian vein [4, 7] measured in our previous studies were 93 pg/ml for 19-OHT and 780 pg/ml for 19-OHA, suggesting that 19-hydroxyandrogens were produced by the human ovary. The conversion of androgens to 19-hydroxyandrogens and estrogens in the human ovary has been documented. Early studies by Sano et al. [8] showed that estradiol as well as 19-OHT were formed by preparations of human follicles and corpus luteum from radioactive testosterone. Moon et al. [9] identified significant amounts of 19-OHT from labeled testosterone in *in vitro* incubation.
19-HYDROXYTESTOSTERONE IN HUMAN OVARY

However, to our knowledge, no evidence of the formation of estrogens from 19-hydroxyandrogens has yet been reported.

In this present study, the formation of estradiol from 19-OHT was demonstrated in addition to the formation of 19-OHT from testosterone in microsomes of human corpus luteum. These results with the others suggest that 19-OHT is not only a product of testosterone, but also serves as a precursor of estradiol in the human ovary. However, from the data showing a linear relationship between the time of incubation and the rate of 19-OHT formation from testosterone, it is difficult to conclude that 19-OHT served as an intermediary metabolite from testosterone to estradiol. Under the condition used, formation of 19-OHT did not show so called bell-shaped curve which suggested the role as intermediary metabolites.

Efforts have therefore been made to study the kinetics of aromatase activity in microsomes in human corpus luteum. The $K_m$ value (0.21 µM) of aromatase for testosterone in the human ovary is consistent with the $K_m$ value on human placenta (0.039 µM - 0.21 µM [1, 2]) and animal ovaries (0.159 µM [10]). The effect on aromatase activity of the known aromatase inhibitor, 4-OHA, was also studied. When ovarian microsomes were incubated with 4-OHA, apparent competitive inhibition of the formation of estradiol from testosterone was observed. Using placental aromatase [11], 4-OHA exhibited an apparent $K_i$ of 15.0 nM which

Table 3. Inhibitory effect of 4-OHA on 19-OHT and estradiol formation in human ovary microsomes

<table>
<thead>
<tr>
<th>4-OHA added (M)</th>
<th>19-OHT</th>
<th>Estradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.4</td>
<td>128.5</td>
</tr>
<tr>
<td>$10^{-9}$</td>
<td>20.0</td>
<td>127.0</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>22.8</td>
<td>123.3</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>22.5</td>
<td>107.0</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>25.0</td>
<td>46.8</td>
</tr>
</tbody>
</table>

Results are expressed as n moles/mg protein. The experimental conditions are described in materials and methods.

Fig. 4. Effect of 4-OHA on estradiol (E2) formation from testosterone and 19-OHT. The inhibitor concentration is $10^{-4}$M. The figures are the averages for duplicate experiments.

Fig. 5. Lineweaver-Burk plot for 4-OHA. Inhibitor concentrations were 0M, $10^{-8}$M, $10^{-7}$M, $10^{-6}$M, and $10^{-5}$M. Incubation conditions were as described in Methods. The slope of the reciprocal plots vs. the inhibitor concentration.
was comparable with the values obtained in the present study. The formation of estradiol from 19-OHT was also strongly inhibited by 4-OHA. The inhibitory effect of 4-OHA on the formation of 19-OHT from testosterone was comparable with that of estradiol from testosterone. Bednarski et al. [12] reported an inhibitory effect of 4-OHA on the formation of 19-hydroxyandrogens and estrogens from androstenedione in human placental microsomes. Inhibitory effect of 4-OHA on the formation of estrone was reported to be higher than that of 19-OHA. If 19-hydroxyandrogens were freely dissociable, intermediary metabolites along a linear reaction pathway, then suicide inactivation of the first oxidative step would be expected to cause parallel inhibition of the formation of intermediary metabolites and products. This was not observed in their study. The mechanism by which 4-OHA inactivates the enzyme remains unknown. However, in previous investigations with testosterone as the substrate it was reported that 4-OHA inhibits the first oxidative step as a suicide aromatase inhibitor [13]. In this study, considering the inhibition of estradiol formation from 19-OHT as well as testosterone by 4-OHA, 4-OHA may be expected to inhibit at the first step in oxidation from each substrate.

In our experimental results it is interesting to note that this effect was also observed in the conversion from 19-OHT to estradiol with no accumulation of 19-OHT when testosterone was used as the substrate. These results suggest that 19-OHT is not only an intermediary metabolite in the aromatization process of human ovary, but also would be the product of its own autonomous enzyme activities in the human ovary. The presence of 19-hydroxy-androgens in the human ovarian vein supports this hypothesis. Non-aromatizing androgen 19-hydroxylase activity of P-450 has been characterized in adrenal tissue [14, 15]. The evidence presently available was insufficient to draw conclusions as to the enzyme multiplicity of P-450 which mediates the sequential obligatory reaction of aromatase in human ovary. Further study has to be undertaken to elucidate this.

References
