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Aromatization of androstenedione and 16α-hydroxyandrostenedione in human placental microsomes
Kinetic analysis of inhibition by the 19-oxygenated and 3-deoxy analogs

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ABSTRACT

Inhibition of aromatase activity in human placental microsomes with androstenedione (AD) (**1a**) and its 19-oxygenated derivatives **1b** and **1c**, their 16α-hydroxy compounds **2** and **3**, and 3-deoxyandrost-4-ene compounds **5** and **6** was studied using [1β-^{3}H]AD as a substrate and compared to that with [1β-^{3}H]16α-hydroxyandrostenedione (16-OHAD). AD series of steroids, compounds **1**, inhibited competitively [1β-^{3}H]AD aromatization whereas other 16α-hydroxy steroids **2**, **3**, **5**, and **6** inhibited AD aromatization in a non-competitive manner. On the other hand, all of 16-OHAD series, compounds **2**, blocked the [1β-^{3}H]16-OHAD aromatization in a competitive manner whereas the AD series steroids **1** as well as the 3-deoxy-16α-hydroxy-17-one steroids **5** and 3-deoxy-16α,17β-diol steroids **6** inhibited 16-OHAD aromatization non-competitively. 3-Carbonyl and 16α-hydroxy functions of 16-OHAD play a critical role of selection of the 16-OHAD binding site. The results suggest that the AD derivatives **1** are kinetically aromatized at a different site from the 16-OHAD derivatives **2**. Physical and/or chemical environments around the aromatase protein in the microsomal membrane may play a significant role in the expression of the substrate specificity, and the present results do not exclude the idea that the placental microsomes have a single binding site.

1. Introduction

Aromatase is a cytochrome P-450 enzyme responsible for the synthesis of estrogens such as estrone, estradiol, and estriol from the corresponding androgens, androstenedione (AD), testosterone, 16α-hydroxyandrostenedione (16-OHAD), and 16α-hydroxytestosterone [1–3]. The principal estrogen secreted by the human placenta is estriol, whereas the ovary and adipose tissue secrete estradiol and estrone, respectively. Reaction sequences involved in the transformation of AD, testosterone, and 16-OHAD seem to be identical to each other in all the reactions involving three sequential oxygenations at C-19 followed by the eventual loss of the angular methyl group at C-19 and the elimination on the 1β- and

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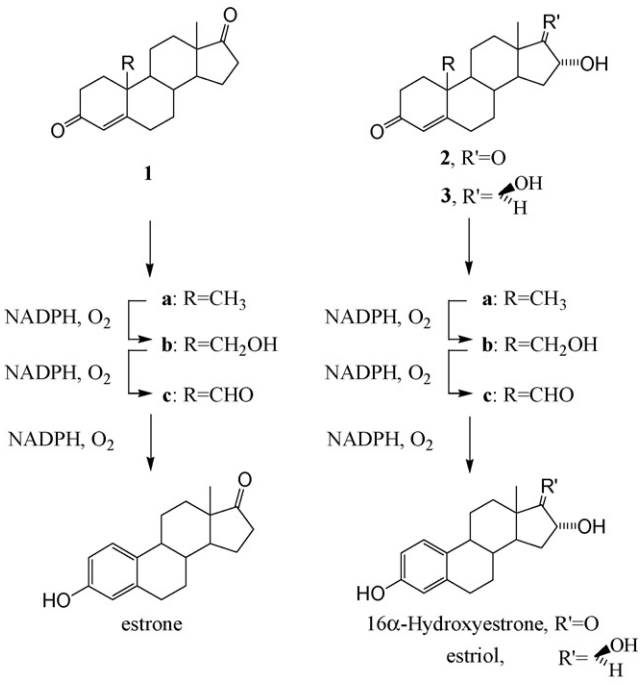

Fig. 1 – Aromatase reactions of AD and 16-OHAD with human placenta.

2β-hydrogens resulting in the production of estrogens (Fig. 1) [4–8].

16α-Hydroxylated androgens, 16-OHAD (**2a**) and 16α-hydroxytestosterone (**3a**) (Fig. 1), are non-competitive inhibitor of AD and testosterone aromatization, respectively, in human placental microsomes [9–11]. It was also reported that AD inhibits aromatization of 16-OHAD in a non-competitive manner [11]. AD, but not 16-OHAD, protected against the 4-hydroxyandrostenedione-mediated suicide inactivation of 16-OHAD aromatization [11]. In the inhibition study of the placental microsomes with 3-deoxy- and 6-oxo-steroids, the relative inhibitory activities obtained using [1β-^{3}H]16-OHAD as a substrate are different from those obtained using [1β-^{3}H]AD as a substrate [12]. We previously found that the known suicide substrates of AD aromatization such as 4-OHAD, 6-oxoandrostenedione and 17α-ethynyl-19-nortestosterone were ineffective at inactivating 16-OHAD aromatization by the placental microsomes [13] and they bind to the 16-OHAD binding site in a manner that does not cause suicide inactivation of 16-OHAD aromatization [14]. It was previously thought that there might be two species of aromatase in human placental micosomes, one of which is capable of aromatization of 16-deoxyandrogens but not 16α-hydroxyandrogens [9,10]. However, it is now believed that a single enzyme species catalyzes the aromatization independently of the difference in the D-ring substitution of androgens in all tissue [11,15,16].

We report here the inhibition study of aromatase activity by 19-oxygenated derivatives of AD, 16-OHAD, and 16α-hydroxytestosterone, compounds **1–3** (Fig. 1), which are thought to be intermediates in the aromatase reaction, and 3-deoxy-16α-hydroxy steroids **5** and **6** to understand the nature of the active site (binding site) of aromatase using [1β-^{3}H]AD or [1β-^{3}H]16-OHAD as a substrate and the placental microsomes as enzyme source.

2. Experimental

2.1. Chemicals

[1β-^{3}H]AD (25.4 Ci/mmol; ^{3}H-distribution, 1β/α = 74/26) was purchased from New England Nuclear (Boston, MA, U.S.A.) and NADPH from Kohjin Co. Ltd. (Tokyo, Japan). [1β-^{3}H]16-OHAD (715 mCi/mmol) was prepared by microbiological transformation of [1β-^{3}H]AD with *Streptomyces roseochromogenes* according to the method [17] reported previously, and its radiochemical purity was more than 98% by HPLC and dilution methods. 16α-Hydroxy-17-keto steroids [18–20], 16α,17β-dihydroxy steroids [18,19], 3-deoxy steroids [21–24] and 19-oxygenated ADs [25] used in this study were synthesized according to the known methods.

Melting points were measured on a Yanagimoto melting point apparatus (Kyoto, Japan) and are uncorrected. IR spectra were recorded in KBr pellets on a PerkinElmer FT-IR 1725X spectrophotometer (Norwalk, CT, U.S.A.), and ^{1}H NMR spectra were obtained in $CDCl_3$ solution with a JEOL JMS LA-400 spectrometer (400 MHz) (Tokyo, Japan) using tetramethylsilane as an internal standard. TLC was performed with Merk precoated silica gel TLC plates, with a layer thickness of 0.25 mm for analytical use and 0.50 mm for preparative use (Darmstadt, Germany). Silica gel column chromatography was conducted with Merck silica gel 60 (70–230 mesh).

2.2. Enzyme preparation

Human placental microsomes (sedimented after 60 min at 105,000 × *g*) were obtained as described by Ryan [26]. They were washed once with 0.05 mM dithiothreitol, lyophilized and stored at −80 °C. No significant loss of activity occurred during the period (6 months) of this study. The preparation of human placental microsomes was conducted under the approval of the ethical review committee of Tohoku Pharmaceutical University in accordance with the standard of the Helsinki Declaration.

2.3. Aromatase assay procedure

Aromatase activity was measured essentially according to the original procedure of Thompson and Siiteri [27]. This assay quantitates the production of tritiated water released from [1β-^{3}H]AD or [1β-^{3}H]16-OHAD by aromatization. All enzymatic studies, in duplicates, were carried out in 67 mM phosphate buffer, pH 7.5, at a final volume of 500 μl under initial velocity conditions [12,28]. The incubation mixture for the IC_{50} experiment contained 180 μM of NADPH, 300 nM of [1β-^{3}H]AD, or 400 nM of [1β-^{3}H]16-OHAD, 20 μg protein (for the [1β-^{3}H]AD aromatization experiment) or 40 μg protein (for the [1β-^{3}H]16-OHAD aromatization experiment) of the lyophilized microsomes, various concentrations of inhibitors, and 25 μl of MeOH, and the entire mixture was incubated at 37 °C for 20 min in the experiment with [1β-^{3}H]AD or for