Suicide inactivation of aromatase in human placenta and uterine leiomyoma by 5α-dihydronorethindrone, a metabolite of norethindrone, and its effect on steroid-producing enzymes

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Norethindrone (NET; 17α-ethynyl-19-nortestosterone), a progestogen component of the contraceptive pill, irreversibly inhibits aromatase activity in human placental microsomes. However, it is known also to be aromatized in vitro and in vivo to produce a biologically very active estrogen called ethynylestradiol (EE2). It is therefore inappropriate to administer a high dose of NET to estrogen-dependent cancer patients for a prolonged time period. In this study, we focused on 5α-dihydronorethindrone (5α-DHNET), a metabolite of NET that is not aromatizable, and the inhibitory effects of 5α-DHNET on human placental and uterine leiomyoma microsomal aromatase and other steroid synthetases. 5α-Dihydronorethindrone showed weak affinity for both estrogen and progesterone receptors. It inhibited significantly human placental aromatase activity in a dose-dependent manner (Kᵢ = 9.0 μmol/l; Kiact = 0.024/min), as well as that of uterine leiomyoma, but did not influence cholesterol side-chain cleavage or 17α-hydroxylase, 21-hydroxylase or 11β-hydroxylase activities. These results suggest that 5α-DHNET may be useful as an aromatase inhibitor, whose use in large doses is expected to reduce the size of estrogen-dependent tumors.

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The incidence of female genital cancer (e.g. breast cancer, uterine endometrial cancer and ovarian cancer) in Japan has shown a tendency to increase in recent years. The development of breast cancer (1) and uterine endometrial cancer (2) generally is known to be related closely to estrogen activity. The enhancement of estrogen activity in these local tumors contributes directly to cancer cell proliferation. These tumors usually have estrogen receptors similar to the corresponding normal tissue and usually exhibit estrogen synthetase (aromatase) activity. Estrogen produced in the tumor itself by autocrine or paracrine mechanisms supports the proliferation of tumor cells.

Anti-estrogens, which competitively inhibit the binding of estrogen to its receptor (ER), and medroxyprogesterone acetate (MPA), which reduces the ER content by binding to the progesterone receptor (PR), were developed as chemotherapeutic agents for patients with breast cancer or uterine endometrial cancer (3). Their clinical effectiveness has been demonstrated. In addition, various kinds of gonadotropin-releasing hormone (GnRH) analogs, which suppress endogenous estrogen production, and aromatase inhibitors, which suppress estrogen synthetase activity, are being developed.

Osawa et al. reported that norethindrone (NET, 17α-ethynyl-19-nortestosterone), a progestogen component of the contraceptive pill, irreversibly inhibits aromatase activity in human placental microsomes (4). We confirmed the fact that NET irreversibly inactivates human placental aromatase. However, it was later revealed that human adult liver (5, 6) and placental tissue (7) have the ability to aromatize NET to ethynylestradiol (EE2). It is inappropriate, therefore, to administer high doses of NET to patients with estrogen-dependent breast cancer for a prolonged period of time. Osawa’s group therefore focused their in vivo (8, 9) and in vitro (10) studies on 5α-dihydronorethindrone (5α-DHNET), a metabolite of NET that is not aromatized in humans.

In this study, we investigated whether or not 5α-DHNET inhibits aromatase activity in human placenta and uterine leiomyoma tissue. We studied also the affinity of 5α-DHNET for various sex steroid receptors and its effect on the activity of steroid synthetases other than aromatase.

Materials and methods

5α-Dihydronorethindrone was a gift from Mitsubishi
Kasei Co. (Tokyo Japan). [6,7-3H]Estradiol-17β ([3H]E2, 47.9 Ci/mmoll), [17α-methyl-3H]17α,21-dimethyl-19-norpregna-4,9-diene-3,20-dione ([3H]promegetestone, 86 Ci/mmoll), [1,3H]Androstenedione ([1,3H]AD, 27.8 Ci/mmoll), [4,5-3H]desoxy corticosterone (60.0 mCi/mmoll), [1,2-3H]progesterone (55.2 Ci/mmoll), [4-14C]androstenedione (52.0 mCi/mmoll), [7-3H]cholesterol (11.4 Ci/mmoll) and [4-14C]pregnenolone (57.2 mCi/mmoll) were all purchased from DuPont (NEN Research Products, Boston, MA). Non-labeled steroids and NADPH were purchased from Sigma Chemical Co. (St Louis, MO).

The affinity of 5α-DHNET for ER and PR

Estradiol was administered to immature female rabbits (approx. 800 g body wt) (intramuscular 50 µg/day for 7 days) and 2 days after the final administration they were sacrificed.

The rabbit uterus was homogenized thoroughly with 5 vols/wt TEG buffer (10 mmol/l TRIS, 1.5 mmol/l EDTA and 10% glycerol, pH 7.4) and centrifuged at 800 g for 20 min. The supernatant fraction was then centrifuged at 150 400 g for 60 min and this supernatant fraction was used as the cytosol fraction after exposure (4°C, 1 h) to 0.1% dextran-coated charcoal (DCC: 0.001% dextran and 0.1% charcoal) solution suspended in TEG buffer. An aliquot of the cytosol fraction was incubated with [3H]promegetestone (0.2–5.0 mmol/l) at 20°C for 2 h with or without 5α-DHNET (20 nmol/l), NET (20 nmol/l) or progesterone (20 nmol/l) in triplicate. After the free steroids were removed by 0.25% DCC treatment (30 min at 4°C), the radioactivity of the labeled steroid–protein complex was measured by the Packard Tri-Carb 460 automatic scintillation system (Packard Instrument Company, Downers Grove, IL). The inhibition constants (K_i) of 5α-DHNET and NET were determined from the double-reciprocal plots.

Increasing concentrations (1–10⁴-fold excess) of various steroids (E2, diethylstilbestrol (DES), NET and 5α-DHNET) were added to a mixture of the cytosol and [3H]E2 (5 nmol/l), and the inhibitory effect of 5α-DHNET on [3H]E2–ER binding was compared with that of DES and E2.

Aromatase activity assay in human placenta and uterine leiomyoma tissue

The microsomal fractions (125 000 g for 60 min) of human term placenta were prepared as described previously (11). Uterine leiomyoma tissue was obtained with the patient’s consent from surgical specimens of a 38-year-old woman (height 160 cm, weight 58 kg) who had undergone a hysterectomy for a massive leiomyoma. The tissue was placed immediately on ice and transported to our laboratory. The tissue used was diagnosed as leiomyoma by subsequent histological examination. The microsomal fraction was prepared in the same manner as for the placentas.

5α-Dihydronor ethindrone (0–50 µmol/l) was incubated with human placental microsomes (4 g protein/l), with or without NADPH (0.5 g/l), in a total volume of 10 ml of phosphate buffer (pH 7.4, 67 mmol/l) at 37°C in air for 10–60 min. At specific time intervals, a 0.5-ml aliquot was removed and assayed for aromatase activity by the tritiated water method (11, 12). The aliquots were incubated with [1,3H]AD (100 pmol/l) and NADPH (0.5 g/l) in a total volume of 2.0 ml of 67 mmol/l phosphate buffer for 10 min at 37°C. 1.0 ml of 5% charcoal was added and the incubation continued for an additional 30 min. The mixture was centrifuged at 800 g for 5 min and the supernant was filtered through a cotton-plugged disposable pipette. The amount of tritiated water in the eluate derived from the substrate was assessed using the 1β-elimination mechanism. The blank incubations contained all reagents except NADPH and were subtracted from the data obtained from incubation with NADPH. The blank values generally equalled the theoretical zero time conversion.

The microsomes of uterine leiomyoma were preincubated with various concentrations (up to 80 µmol/l) of 5α-DHNET and NADPH (0.5 g/l) at 37°C for 15 min in air. This preincubated sample then was incubated at 37°C for 60 min with [1,3H]AD (100 pmol/l) and NADPH (0.5 g/l). The aromatase activity was determined as above. Protein concentrations were determined by the Bio-Rad protein kit (Bio-Rad, CA).

Various concentrations (up to 300 µmol/l) of 5α-DHNET were prepared as follows: 5α-DHNET dissolved in ethanol solution plus 0.5 ml of propylene glycol was evaporated completely and then displaced at various concentrations in buffer solution.

Other enzyme assays

Cholesterol side-chain cleavage (CSCC) assay. For the assay of CSCC activity, each incubation mixture contained the mitochondrial fraction of bovine adrenal cortex (1.4 g protein/l). [7-3H] cholesterol (6.0 × 10³ dpm, 5 µmol/l), trilostane (20 µmol/l), NADPH (0.5 mg) and increasing amounts (0–160 µmol/l) of inhibitor in a total volume of 0.5 ml of PBS containing 10 mmol/l MgCl₂. Trilostane was added to block the subsequent conversion of pregnenolone to progesterone. After the mixture was prewarmed at 37°C for 2 min, the reaction was started by addition of NADPH (0.5 g in 0.05 l PBS). The reaction was linear for at least 45 min. The incubation was continued at 37°C for 30 min and stopped by the addition of 3 ml of dichloromethane containing [4-14C]pregnenolone (1.0 × 10⁴ dpm, 50 µg) as well as standard cholesterol (50 µg) and progesterone (10 µg). The metabolites were extracted twice with 3 ml of dichloromethane and the organic phase was concentrated and separated by thin-layer chromatography.
activity and pregnenolone

Fig. 1. Effect of 5α-dihydonorethindrone (5α-DHNET) and related unlabelled steroids (20 nmol/l each) on [3H]promegestone protein binding in immature rabbit uterine cytosol. Each sample was incubated with [3H]promegestone (0.2–5.0 nmol/l) at 20°C for 2 h with or without 5α-DHNET (20 nmol/l), norethindrone (NET, 20 nmol/l) or progesterone (20 nmol/l) in triplicate: (×) 5α-DHNET; (△) NET; (●) progesterone; (○) control.

( precoated TLC plates: silica gel 60F-254) using hexane–chloroform–acetone (65:5:30). The specific activity was calculated from the \(^{3}H/^{14}C\) ratio of pregnenolone formed.

11β-Hydroxylase and 18-hydroxylase assay. The 11β- and 18-hydroxylase activity was measured according to the method of Sato et al. (13) with some modifications. Incubation mixtures contained the mitochondrial fraction of bovine adrenal cortex (0.14 g protein/l), [4,14C]desoxycorticosterone (4.0 × 10\(^{4}\) dpm, 5.0 μmol/l), NADPH (0.25 g/l) and 5α-DHNET (0–300 μmol/l) in a total volume of 0.5 ml of PBS containing 10 mmol/l MgCl\(_2\). The incubation was carried out at 37°C for 5 min and stopped by the addition of 3 ml of dichloromethane containing 10 μg each of standard desoxycorticosterone, corticosterone, 18-hydroxydesoxycorticosterone, and 18-hydroxycorticosterone. The metabolites were extracted and separated by thin-layer chromatography (precoated TLC plates: silica gel 60F-254) using benzene–acetone (13:7).

Fig. 2. Double-reciprocal plots of the competitive inhibition of [3H]promegestone protein binding in immature rabbit uterine cytosol: (×) 5α-dihydonorethindrone; (△) norethindrone; (●) progesterone.

21-Hydroxylase assay. Incubation mixtures contained the microsomal fraction of bovine adrenal cortex (0.23 g protein/0.5 l), [1,2-3H]progesterone (2.0 × 10\(^{3}\) dpm, 10 μmol/l), NADPH (0.5 g/l) and 5α-DHNET (0–160 μmol/l) in a total volume of 0.5 ml of PBS containing 10 mmol/l MgCl\(_2\). Incubation was carried out at 37°C for 5 min in a manner similar to that for CSCC. The incubation was stopped by the addition of 3 ml of dichloromethane containing [4,14C]desoxycorticosterone (4 × 10\(^{3}\) dpm, 10 μg) plus 10 μg each of standard progesterone, 17α-hydroxyprogesterone and 11-desoxycortic. The metabolites were extracted and separated by thin-layer chromatography (precoated TLC plates: silica gel 60F-254) using chloroform–methanol (97:3).

Fig. 3. Effect of increasing concentrations of 5α-DHNET and related unlabelled steroids on [1H]estradiol-17β protein binding in immature rabbit uterine cytosol: (×) 5α-dihydonorethindrone; (△) norethindrone; (●) diethylstilbestrol; (○) estradiol-17β.
17α-Hydroxylase/C17-20 lyase assay. Incubation mixtures contained the microsomal fraction of bovine adrenal cortex (0.23 g protein/0.5 l), [1,2-3H]-progesterone (2.0 × 10^5 dpm, 10 μmol/l), NADPH (0.5 g/l) and 5α-DHNET (0–320 μmol/l) in a total volume of 0.5 ml of PBS containing 10 mmol/l MgCl₂. Incubation was carried out at 37°C for 20 min in a manner similar to that for CSCC. The incubation was stopped by the addition of 3 ml of dichloromethane containing [4-14C]AD (4 × 10^5 dpm, 10 μg) plus 10 μg each of standard progesterone and 17α-hydroxyprogesterone. The metabolites were extracted and separated by thin-layer chromatography (precoated TLC plates: silica gel 60F-254) using chloroform–methanol (97:3).

Each value was expressed as the mean of four determinations in four experiments except for the controls, which were expressed as the mean of eight determinations.

Results

The affinity of 5α-DHNET for ER and PR

In order to examine the affinity of 5α-DHNET for PR, its inhibitory effect on [3H]promegestone–PR binding in rabbit uterine cytosol was compared with that of NET and progesterone. The affinity of 5α-DHNET for PR was weaker than that of NET and progesterone (Fig. 1).

Analysis by the double-reciprocal plot gave Kᵢ values of 99.4 nmol/l for NET and 4.0 nmol/l for 5α-DHNET (Fig. 2). The affinity of 5α-DHNET for ER was examined by comparing the effects of various steroids on [3H]E₂–ER binding in rabbit uterine cytosol. When 1–10⁴ μmol/l of 5α-DHNET, NET, DES or E₂ was added to the rabbit uterine cytosol, 5α-DHNET and NET showed little binding to ER, unlike E₂ and DES, which were stronger; 5α-DHNET showed almost no affinity for ER (Fig. 3).

The inhibitory effect of 5α-DHNET on human placental aromatase activity

Precipitation with 5α-DHNET inhibited significantly the aromatase activity as compared with the control. The degree of inhibition was proportional to the
The inhibitory effect of 5α-DHNET on aromatase activity in human uterine leiomyoma

As shown in Fig. 5, the aromatase activity of the microsomal fraction of uterine leiomyoma was inhibited dose dependently by the addition of 5α-DHNET (up to 80 μmol/l).

The effect of 5α-DHNET on the activity of other steroid synthetases

The formation of progesterone was reduced following the addition of trilostane, but the total sum of the formation of pregnenolone and progesterone was not altered, indicating that trilostane did not affect CSCC activity.

As shown in Fig. 6A, the CSCC enzyme activity of bovine adrenocortical mitochondria was not inhibited by the addition of 5α-DHNET (up to 160 μmol/l). The 11β-hydroxylase activity of bovine adrenocortical mitochondria was only inhibited slightly by the addition of 5α-DHNET (up to 300 μmol/l) (Fig. 6B).

The 21-hydroxylase activity of bovine adrenocortical microsomes was enhanced by about 20% by low concentrations (0–40 μmol/l) of 5α-DHNET but was not influenced by higher concentrations (40–160 μmol/l) of 5α-DHNET (Fig. 7A).

The 17α-hydroxylase activity of bovine adrenocortical microsomes was not affected by low concentrations (0–40 μmol/l) of 5α-DHNET but was inhibited dose dependently to some degree (20–40%) by more than 80 μmol/l 5α-DHNET (Fig. 7B).

The results show that 5α-DHNET slightly inhibits 17α-hydroxylase in higher concentrations but does not affect the activity of CSCC enzyme, 11β-hydroxylase or 21-hydroxylase.

Fig. 5. Inhibitory effect of 5α-dihydronorethindrone (5α-DHNET) on aromatase activity of uterine leiomyoma. The microsomes of uterine leiomyoma were preincubated with various concentrations of 5α-DHNET and NADPH (0.5 g/l) at 37°C for 15 min. The preincubated sample then was incubated at 37°C for 60 min with [1β-3H]androsterone and NADPH. Aromatase activity was determined by the 3H2O release method.

Preincubation time and was dependent on the 5α-DHNET concentration (Fig. 4). Preincubation of human placental microsomes with 5α-DHNET and NADPH showed significantly greater inhibition of aromatase activity than preincubation without NADPH. The results show that 5α-DHNET irreversibly inactivates human placental aromatase. The Kᵢ and Kᵣ values of 5α-DHNET were 9.0 μmol/l and 0.024/min, respectively, indicating a mild inhibitory effect.

Fig. 6. (A) Effect of 5α-dihydronorethindrone (5α-DHNET) on cholesterol side-chain cleavage (CSCC) enzyme activity in bovine adrenocortical mitochondria. The activity of CSCC enzyme was measured in bovine adrenocortical mitochondria. The mitochondrial fraction was incubated with [7-3H]cholesterol (5 μmol/l) and NADPH (0.5 g/l) at 37°C for 30 min in air. The [7-3H]pregnenolone was isolated by thin-layer chromatography. The CSCC enzyme activity was determined from the conversion rate of cholesterol to pregnenolone. (B) Effect of 5α-dihydronorethindrone (5α-DHNET) on 11β-hydroxylase activity in bovine adrenocortical mitochondria. The 11β-hydroxylase activity was measured in bovine adrenocortical mitochondria. The mitochondrial fraction was incubated with [4,14C]desoxycorticosterone (5.0 μmol/l), NADPH (0.5 g/l) and various concentrations of 5α-DHNET at 37°C for 5 min in air. The labeled corticosterone was isolated by thin-layer chromatography.
**Discussion**

Norethindrone is a synthetic steroid that is used widely as a progestogen component of the contraceptive pill. It has been shown to bind to PR and exhibit progestosterone activity.

However, it has been a controversial subject as to whether or not this synthetic steroid is aromatized in vivo. In the late 1960s, some investigators reported that EE2, which was suspected to be an aromatized metabolite of NET, was detected in the urine of women who took contraceptive pills containing NET at about 1% of the oral dose of NET (14, 15). Later, however, the EE2 was found to be an artifact of the phenolic extraction process used in urinary metabolite identification (16, 17). In humans, 19-norsteroids (e.g., 19-norandrostenone) are metabolized to 1/2-hydroxylated 19-norsteroids, which are aromatized by exposure to an acid or base during purification (13, 14). Thus, the question remained as to whether or not NET is aromatized in humans.

Osawa et al. (4) demonstrated that NET irreversibly inhibits estrogen synthetase (aromatase) activity of human placental preparations. They reported also that the irreversible inactivation of aromatase by NET was dependent on NADPH, incubation time and NET concentration. Analysis by Kitz and Wilson's method (18) showed that the K_i for the initial irreversible complex of NET was 1.7 µmol/l and the Kinact was 1.61 x 10^{-3}/s. Norethindrone was considered to be an ideal endocrinologic agent for estrogen-dependent cancer if it is not aromatized in vivo.

However, it was revealed later, when methods without exposure to acid or base were used, that NET is aromatized partially to EE2 in vitro (5–7). Using Bio-Rad AG1-X2 column chromatography, thin-layer chromatography and co-crystallization, we showed that human adult liver tissue (homogenates or hepatocytes) has the ability to aromatize NET. Barbieri et al. (7) showed that EE2 was formed when human placental microsomes were incubated with [6,7-^{3}H]NET and NADPH, using methods without exposure to acid or base. They found that NET aromatization with human placenta was linear with respect to placentar protein concentration and incubation time and that endogenous androstenedione inhibited the NET aromatization. They observed also that NET binds to the active site of human placental microsomal cytochrome P-450 with relatively low affinity (apparent K_i = 28 µmol/l). Reed et al. (19) reported that transfer constants for the conversion of NET to EE2 in two postmenopausal women with breast cancer were 2.26 and 2.34% in blood and 2.27 and 0.38% in urine. They precluded the use of acid or base to prevent possible artificial formation of phenolic metabolites of NET.

According to Barbieri et al. (7), the NET aromatizing activity of human placental microsomes is approximately 1 nmol.min^{-1}.kg^{-1} protein, which is about 1/100 of the aromatizing activity for androstenedione. However, there is a problem when using NET clinically as an aromatase inhibitor, because the EE2 formed by NET aromatization has a strong biological activity equivalent to E2. Unlike NET, 5a-DHNET is not aromatized in vivo. Also, 5a-DHNET inhibits human
placental aromatase activity, similarly to NET, and reduces the size of NMU-induced mammary tumors in rats (10).

In this study, we investigated the affinity of 5α-DHNET for various steroid receptors and the effect of 5α-DHNET on various cytochrome P-450 enzymes related to steroid biosynthesis and examined the inhibitory effect of 5α-DHNET on aromatase activity of human uterine leiomyoma and placental tissues. 5α-Dihydronestradiol was shown to have little affinity for rabbit ER or PR. It inhibited aromatase activity of human placenta and uterine leiomyoma, did not influence the CSCC, 21-hydroxylase and 11β-hydroxylase activities but slightly reduced 17α-hydroxylase activity at high dose. Also, low doses of 5α-DHNET may elevate slightly 17α-hydroxylase and 21-hydroxylase activities, but the reason for this is still unknown. These results suggest that 5α-DHNET is required to be administered in high doses as an aromatase inhibitor, and that it must be examined more from the views of other enzymes.

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