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COMPARISON OF AROMATASE ACTIVITY IN HUMAN PROSTATIC, TESTICULAR AND PLACENTAL TISSUES

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The aromatase enzyme was quantified by the release of tritiated water from [1β-^{3}H] androstenedione. Tritiated water was released by the crude homogenates in 4 of 18 samples of benign prostatic hyperplasia tissue and one of 5 samples of prostate carcinoma tissue. However, this apparent aromatase activity was not inhibited by 4-hydroxyandrostenedione (0.5 and 5.0 μM), and none of the particulate fractions (100,000 g pellet) prepared from each of the prostatic tissues exhibited aromatase activity. Particulate fractions from rat ovary ($n = 3$) and human testes ($n = 6$) displayed significant aromatase activity (mean values of 9.9 and 0.033 nmol estrone formed/g protein/h, respectively). The testicular aromatase was inhibited by aminoglutethimide, 4-hydroxyandrostenedione and CGS 16949A with IC_{50} values of 6.4, 0.17 and 0.0017 μM, respectively. These are of a similar order to values obtained with the aromatase enzyme from human placental microsomes (14, 0.43 and 0.0075 μM, respectively).

KEY WORDS: Aromatase enzyme, aromatase inhibitors.

INTRODUCTION

Carcinoma of the human prostate gland is frequently androgen-dependent in newly diagnosed patients, approximately 80% of whom respond to first-line endocrine therapy.[1,2] The androgen dependency is mediated via testosterone and its active metabolite, 5α-dihydrotestosterone.[3] In contrast, there is some evidence to suggest that estrogens may be important in the development of both benign and malignant diseases of the prostate. Estrogen receptors have been detected in both types of tissue[4–6] and estrogens have been implicated in the induction of benign prostatic hyperplasia in the prostate of dogs.[7,8] It has also been suggested that oestrogens may

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be important in established disease since the aromatase inhibitor, aminoglutethimide when administered with replacement corticosteroids is of some benefit in advanced prostatic cancer patients.[9–11] Although this combination was originally used with the aim of suppressing adrenal androgen production, the overall effects on plasma androgen levels are minimal. It has therefore been suggested that any clinical benefit derived from this combination might be due to aromatase inhibiton.[12]

A possible explanation of these apparent estrogen-dependent effects was provided by the reports from some workers that androgens can be converted into estrogens by an aromatase enzyme within prostatic tissues.[13,14] However, other studies have failed to detect any prostatic aromatase activity.[15–18] The role of estrogens and the presence of the aromatase enzyme in human prostate thus remains controversial. In order to clarify the possible role of local estrogen production in the pathophysiology of prostatic disease we have examined subcellular fractions of benign and malignant prostatic tissues for aromatase activity by measuring the tritiated water generated from [1β-^{3}H]androstenedione during aromatization. The specificity of this reaction has been assured by the use of aromatase inhibitors, aminoglutethimide (AG), 4-hydroxy-androstenedione (4OHA) and CGS 16949A.

MATERIALS AND METHODS

Chemicals

[1β,2β-^{3}H]Androst-4-ene-3,17-dione (specific activity = 40–60 Ci/mmol) and [1β-^{3}H]androst-4-ene-3,17-dione (specific activity = 15–30 Ci/mmol) were purchased from New England Nuclear and purified before use by thin layer chromatography in dichloromethane:diethyl ether (9:1 v/v). Thin layer chromatography was performed on Kieselgel 60 F-254 precoated silica gel plates (5 × 20 cm, 0.25 mm thickness) obtained from BDH Chemicals Ltd., Poole, U.K. activated charcoal and unlabelled androstenedione were obtained from Sigma Chemical Co. Ltd., Poole, U.K. NADP, NADPH and glucose-6-phosphate dehydrogenase (yeast enzyme, grade II) were purchased from BCL, Boehringer Mannheim House, Lewes, Sussex, U.K. Aminoglutethimide (AG), 4-hydroxyandrostenedione (4OHA) and (CGS 16949A) (4-(5,6,7,8-tetrahydroimidazo [1,5α] pyridin-5-yl) benzonitrile hydrochloride) were kindly suplied by Ciba-Geigy Pharmaceuticals, Horsham, U.K.

Collection and Preparation of Tissue Samples

Malignant and benign prostatic tissues were obtained from patients by transurethral, cold punch and radical retropubic prostatectomy. Normal testicular tissue was obtained by bilateral orchidectomy. All samples were immediately snap-frozen and stored in liquid nitrogen. Rat ovaries and full term human placenta were obtained fresh and transported to the laboratory on ice for immediate processing. All procedures were carried out at around 4°C. Crude homogenates of prostatic tissues were prepared by homogenisation in 2 volumes of 0.1 M potassium phosphate buffer pH 7.4. A Polytron homogeniser (setting No. 5 for 2 × 10 sec bursts) followed by a Potter-Eljvehem homogeniser (2 passes at 2500 rpm) was used. The homogenate was centrifuged at 1500 g for 10 min to pellet nuclei and cell debris which were discarded. The supernatant was centrifuged at 100,000 g for 60 min to produce a particulate

pellet, which was resuspended in 0.1 M potassium phosphate buffer pH 7.4 and stored in liquid nitrogen. Aromatase assays were carried out within 24 h of preparation and the protein content was determined by a modification of Lowrys method.[19] Rat ovary and human testicular tissue were processed as described above, except that due to the less fibrous nature of these tissues only the Potter-Eljvehem was used to homogenise the tissue. Placental microsomes were prepared as described previously.[20]

Assay of Aromatase Activity in Placental Microsomes

The aromatase enzyme was monitored by quantifying the tritiated water released from radiolabelled androstenedione during aromatisation to estrone.[21,22] Each assay tube (1 ml) contained 50 mM potassium phosphate buffer pH 7.4, cofactors and the substrate solution in propanediol (2.5% v/v). Unlabelled androstenedione was combined with [1β,2β-^{3}H]androstenedione (0.5 μCi) to give a final substrate concentration of 0.38 μM. The cofactor solution consisted of 1 mM NADP, 10 mM glucose-6-phosphate and glucose-6-phosphate dehydrogenase (2 u/ml) and was preincubated for 2 min at room temperature before adding to the assay tubes.

The reaction was started by the addition of placental microsomes. Aliquots (0.2 ml) were removed after 5, 10 and 15 min of incubation and added to 1 ml of activated charcoal (1% w/v), 0.1% Tween 80 and 1 mM mercuric chloride on ice. The tubes were shaken and left to stand for 30 min at 4°C. Tritiated water was separated from the unreacted steroids by centrifugation (1500 g for 30 min at 4°C) and 0.5 ml portions of the supernatant were counted to determine enzyme activity. All assays were run in duplicate and the compounds to be tested were dissolved in methanol and added to the assay tubes to give 1% of the total volume. Methanol alone was added to the control tubes. The IC_{50} value was defined as the concentration of inhibitor required to reduce the enzyme activity to 50% of the control value at the final substrate concentration stated above.[23]

Assay of Aromatase Activity in Prostatic, Testicular and Other Tissues

Samples (0.5 ml) of homogenized tissues were transferred to a reaction vial containing 50 μl [1β-^{3}H]androstenedione (2 μCi, 72 pmol) in methanol. The incubation, at 37°C, was started by adding 0.5 ml of a NADPH regenerating system (6 mM NADPH, 30 mM glucose-6-phosphate, 8 mM magnesium chloride and 2 u/mol of glucose-6-phosphate dehydrogenase) to each sample. Aliquots (0.5 ml) were removed after 90 and 180 min of incubation and added to 5 ml of ethyl acetate containing 0.25 ml of 1 mM mercuric chloride to terminate the reaction. The tubes were allowed to stand for 30 min at room temperature and then centrifuged at 1500g for for 15 min. The supernatant was discarded and 0.5 ml of trichloroacetic acid (30% w/v) was added to the aqueous phase to precipitate the protein. After centrifugation at 1500 g for 15 mins, 1 ml of the supernatant was added to 1 ml of a 5% charcoal-0.5% dextran solution. The mixture was allowed to stand at 4°C for 30 min, then centrifuged at 1500 g for 30 min at 4°C. An aliquot (1 ml) of the clear supernatant was added to 10 ml of scintillation fluid and counted in a Packard 300C scintillation counter. Negative controls consisted of boiled tissue samples and blank tubes (only substrate and buffer). The mean values obtained from the negative controls were subtracted from all other samples. The results were expressed as picomoles of estrone formed per gram of protein per hour (pmol E_1/g protein/h).

RESULTS

Using the assay conditions described for human placental microsomes, the aromatase enzyme has a mean K_m value of 0.038 μM $\pm$ 0.02 (n = 4) and a mean V_{max} of 6020 $\pm$ 1528 (n = 4) nmol E_1/g protein/h. With a substrate concentration of 0.38 μM, to ensure saturating conditions, enzyme activity was linear with time (up to 15 min) and protein concentration (up to 0.5 mg/ml). AG, 4OHA and CGS 16949A all inhibited this activity in a concentration dependent fashion giving the IC_{50} values in Table I. Experiments with placenta were conducted with [1β,2β-^{3}H]androstenedione. However, this is unsuitable for use in samples with low levels of the enzyme since release of the unstable tritium in position 2 of the steroid occurs giving high background values.[24] Therefore the modified procedure using [1β-^{3}H]androstenedione was utilised.[25] To be positive for aromatase activity, the samples had to display a time-dependent increase in tritiated water release and have values two-fold higher than the background. Background values consisted of the radioactivity in the negative controls (boiled tissue tube and a tube containing labelled substrate and buffer) which were 150 $\pm$ 50 dpm (mean of 6 expts, $\pm$ standard deviation). Crude homogenates of benign prostatic hyperplastic tissue, obtained by radical prostatectomy (n = 10), cold punch (n = 3) and transurethral prostatectomy (n = 5) were prepared and tested for aromatase activity. Four samples (all from radical prostatectomy) displayed time-dependent release of tritiated water, albeit at very low levels. These ranged from 0.04% to 0.25% conversion to ^3H_2O, which corresponds to 0.30 to 1.34 pmoles ^3H_2O released g/protein/h. With prostatic carcinoma tissue (obtained by transurethral prostatectomy), one of the five crude homogenates displayed tritiated water release (0.07% conversion, 0.41 pmol ^3H_2O released/g protein/h). However, no decrease in activity was observed on incubating all the positive samples with 4OHA at final concentrations of 0.5 and 5.0 μM. These concentrations were effective in inhibiting the human placental aromatase. In order to partially purify the enzyme and increase the sensitivity of the assay, the particulate fraction (100,000 g pellet) was prepared from all the above tissues and assayed, but none of the samples released tritiated water greater than that formed in the boiled tissue controls. To check the validity of the assay, particulate fractions from human testes and rat ovary were prepared. Both these tissues displayed aromatase activity (Table II). In addition, the aromatase activity from human testes could be inhibited by AG, 4OHA and CGS 16949A generating the IC_{50} values shown in Table I. The concentration dependent inhibition of the testicular aromatase by 4OHA is demonstrated in Figure 1.

Progsterone (5 μM) was added to prostatic particulate sample incubations (benign

TABLE I
Testicular and Placental Aromatase by AG, 4OHA and CGS 16949A

Compound	*Placental Aromatase*[a] IC_{50} (μM)	*Testicular Aromatase*[b] IC_{50} (μM)
AG	14.00 $\pm$ 1.5	6.40 $\pm$ 0.57
4OHA	0.43 $\pm$ 0.05	0.17 $\pm$ 0.02
CGS 16949A	0.0075 $\pm$ 0.003	0.0017 $\pm$ 0.001

[a]Substrate concentration = 0.38 μM
[b]Substrate concentration = 0.072 μM
Data shown is the mean of triplicate determinations $\pm$ the standard deviation.

TABLE II
Comparison of Aromatase Activity

Tissue	*Aromatase Activity** (n mol estrone formed/ g protein/h)
Human placental (n = 4)	6920 ± 1528
Human benign prostatic hyperplasia (n = 18)	none detected
Human prostatic carcinoma (n = 5)	none detected
Human testes (n = 6)	0.033 ± 0.022
Rat ovary (n = 3)	9.9 ± 1.2

*Except for the placenta, where the microsomal fraction was used, the particulate fraction was assayed. Data shown is the mean of the determinations ± standard deviation.

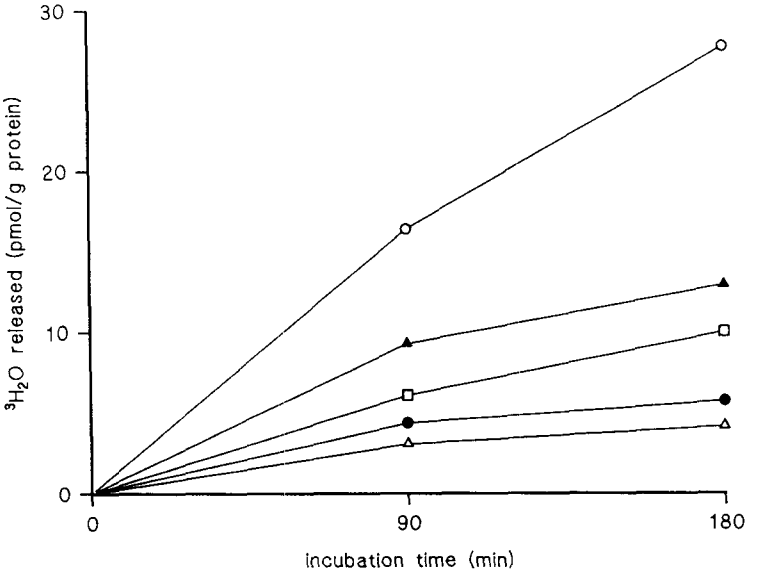

FIGURE 1 Inhibition of Human Testicular Aromatase by 4OHA. Assay details as described in the Experimental. Substrate concentration = 0.072 μM [1β-^{3}H]androstenedione. Control (0) and various concentrations of 4OHA; 0.125 μM (▲), 0.25 μM (□), 0.50 μM (●) and 1.00 μM (△). Each point is the average of duplicate values which were within ± 10%.

n = 3, carcinoma n = 3) in order to inhibit the 5α-reduction of androstenedione, thereby preventing the possible depletion of substrate for the aromatase enzyme. However, even under these conditions no aromatase activity was detected. Previous experiments confirmed that 5 μM progesterone had no effect on rat ovarian or human placental aromatase.[17]

DISCUSSION

We found the aromatase activity of human placental microsomes to display a K_m value of 0.038 μM with a maximum velocity of 6920 nmol E_1/g protein/h. These values agree with published data,[26,27] as do the IC_{50} values for inhibition by AG, 4OHA and CGS 16949A.[28-30] Previously, using [1β-^{3}H] androstenedione as substrate and the assay procedure described above, we have demonstrated significant aromatase activity in about half of the human primary breast carcinoma samples assayed. The activity ranged from 2 to 81 pmol E_1/g protein/h,[25] which was also consistent with previously published data.[31,32]

In this study, we used 2 μCi (72 pmol) of [1β-^{3}H]androstenedione in 1 ml of the assay mixture, as opposed to 1 μCi (36 pmol) for the breast tissue. Low levels of tritiated water release were present in 4 out of 18 crude homogenates of benign prostatic hyperplasia and one of 5 prostatic carcinoma crude homogenates. This would be equivalent to 0.30 to 1.34 pmol E_1/g protein/h, which are lower levels than we detected in breast tumours, however, the lack of suppression by 4OHA suggests that the tritiated water release is not mediated by an aromatase enzyme. The failure of particulate samples from each of the prostatic tissues to display aromatase activity implies that the enzyme is not present. Therefore our results support the findings of the majority of earlier workers[11-14] showing that local estrogen synthesis from androgens with benign or malignant prostatic tissues is either very low or absent. Using [1β-^{3}H]androstenedione, Brodie *et al.*,[18] recently demonstrated tritiated water release in crude homogenates of prostatic tissues. In addition, they found that 4OHA and N,N-diethyl-4-methyl-3-oxo-aza-5α-androstane-17β-carboxyamide, an established 5α-reductase inhibitor, reduced the formation of tritiated water. They concluded that androstenedione is not metabolized to estrogens and suggested that a metabolic process involving the 5α-reductase pathway may explain the loss of tritium from C-1 of androstenedione. Although we did not find any suppression of tritiated water formation by 4OHA, our preliminary studies show that 4OHA inhibits the 5α-reduction of testosterone and androstenedione in nuclear pellets from human prostatic tissues.[33]

Human testicular tissue contains aromatase activity[34,35] and such tissue was used to verify the ability of the assays to detect relatively low levels of aromatase activity. All the samples assayed exhibited time-dependent tritiated water release that corresponded to between 13 and 70 pmol E_1/g protein/h/ This is a similar level of activity to that found in the most active breast cancers. In addition, the enzyme was inhibited by AG, 4OHA and CGS 16949A. The degree of inhibition of the testicular activity was similar to that seen with the placental microsomes (Table I) suggesting that the aromatase enzyme from placenta has similar properties to those of the testes.

In conclusion, we have failed to detect any significant aromatase activity in either benign or malignant prostatic tissues. Thus aromatase inhibitors are unlikely to be effective in treating prostatic disease by virtue of any local effects on the aromatase enzyme. This does not exclude the possibility that estrogens in the circulation may influence prostatic tissue pathophysiology, or that the suppression of the estrogen levels by aromatase inhibitors may be a useful therapeutic manoeuvre. Human testes contain aromatase activity that is inhibited by established enzyme inhibitors. This may be important in the local control of testicular function. It should be recognized that the use of aromatase inhibitors in intact males may therefore have side effects on these organs.

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