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Aromatase-independent testosterone conversion into estrogenic steroids is inhibited by a 5α-reductase inhibitor

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Abstract

Estrogens are generated mainly by the action of aromatase, which converts testosterone to estradiol and androstenedione to estrone. However, in addition to estradiol and estrone, a variety of other steroids, whose synthesis is not dependent on aromatase, can stimulate the estrogen receptor. Here we show that testosterone is converted into such estrogenic steroids by aromatase-negative HeLa cells. This aromatase-independent generation of estrogenic steroids is seen in aromatase-positive MCF-7 cells as well. In both cell lines, the synthesis of estrogenic steroids was blocked by inhibition of testosterone conversion into dihydrotestosterone using a 5α-reductase inhibitor finasteride, suggesting that they are generated downstream of dihydrotestosterone. This finding raises the possibility that the combination of a 5α-reductase inhibitor and an aromatase inhibitor may reduce estrogenic steroids in vivo more completely than an aromatase inhibitor alone.
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Keywords: Breast cancer; Estrogen; Testosterone; Aromatase; 5α-reductase

1. Introduction

Aromatase inhibitors are effective for the treatment of breast cancer in postmenopausal women, and may be superior to tamoxifen [1]. They act by inhibiting the conversion of testosterone (T) into 17β-estradiol (E_2) (Fig. 1), thereby preventing E_2-induced activation of estrogen receptor (ER). However, ER is activated not only by estrogens but also by many other steroids, which may be generated aromatase-independently. Therefore, in patients with estrogen-dependent tumors, it may be desirable to inhibit the generation of such estrogenic steroids in addition to blocking the synthesis of traditional estrogens.

One such estrogenic steroid synthesized independently of aromatase is androstane-3β, 17β-diol (3βD), which is a non-aromatizable steroid [2]. Because 3βD is generated downstream of dihydrotestosterone (DHT) (Fig. 1), an aromatase inhibitor does not block its production, whereas a 5α-reductase inhibitor might impair its synthesis by reducing DHT, the precursor of 3βD.

In this report, we designed an in vitro model to assess the production of estrogenic steroids synthesized via an aromatase-independent pathway in non-breast cancer cells (HeLa) and in breast cancer cells (MCF-7). Using this model, we demonstrate that when estrogens are no longer produced due to the absence of aromatase action, the estrogenicity of other less estrogenic steroids synthesized from T by cultured cells is readily detected and that this estrogenicity is reduced by additional treatment of the cells with a 5α-reductase inhibitor finasteride (Fi), a drug currently used in the treatment of prostate cancer and hypertrophy [3,4].

2. Materials and methods

2.1. Chemicals

T, E_2, formestane (Fo) and aminoglutethimide (AG) were purchased from Sigma (St. Louis, MO), and Fi was obtained from Steraloids (Newport, RI). ICI182,780 (ICI) was a gift from Alan Wakeling. These compounds were added to

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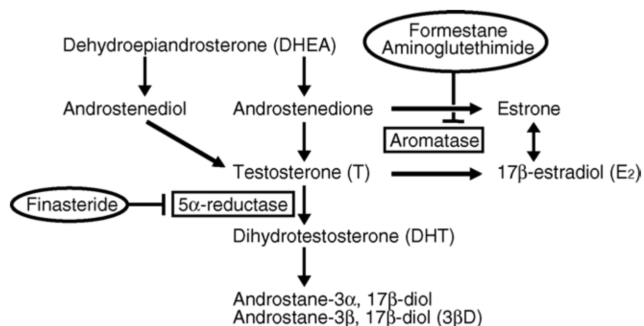

Fig. 1. Estrogen biosynthesis pathway [2]. Inhibitors used in this study and their target enzymes are shown in ovals and rectangles, respectively.

medium in 0.1% (v/v) ethanol vehicle in the reporter gene assays. Androst-4-ene-3,17-dione, [1β-^{3}H(N)] (9.25 MBq) was purchased from PerkinElmer Life Sciences (Boston, MA).

2.2. Cell culture

MCF-7, JEG-3 and HeLa cells were maintained in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 1:1 (DMEM/F12) with 8% (v/v) fetal bovine serum (FBS) and antibiotics. In the experiments, phenol red-free DMEM containing 8% dextran/charcoal-stripped FBS (Hyclone, Logan, UT) (DMEM-dcsFBS) was used to eliminate estrogenicity in the medium.

2.3. RT-PCR

Total RNA was extracted from MCF-7 and HeLa cells and treated with DNase. Reverse transcription was performed using AMV reverse transcriptase (Promega, Madison, WI) in the presence of RNasin ribonuclease inhibitor (Promega). For detection of aromatase mRNA, an aromatase gene-specific primer (GTCTCATCTGGGTGCAAGGA) was used to synthesize cDNA, which was then used as a template for PCR using primers TTATGAGAGCATGCGGTACC and CGTCTCAGAAGTGTAACGA (anticipated product size: 291 bp). A 200-bp segment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was PCR-amplified (primers: TTCGACAGTCAGCCGCATCT and GAACATGTAAACCATGTAGT from cDNA that had been synthesized from the same RNA samples using random primers (Promega). All PCRs were done up to 40 cycles using KOD DNA polymerase (Novagen, Hartland, WI). PCR products were electrophoresed on the ethidium bromide-containing agarose gel. The bands apparently representing amplified products were purified from the gel and their sequences were verified.

2.4. Aromatase activity

Aromatase activity was measured, based on the method in a previous report [5]. MCF-7 cells (1.5×10^6 cells per dish) and JEG-3 cells (0.3×10^6 cells per dish) were seeded into 6-cm dishes (2 ml DMEM-dcsFBS per dish). Four days later, medium was replaced with 1 ml fresh medium containing 100 nM androst-4-ene-3,17-dione, [1β-^{3}H(N)]. Twenty-four hours later, the supernatant was retrieved and processed with 30% (w/v) trichloroacetic acid, chloroform, and activated charcoal to remove precipitated protein and residual steroids. The radioactivity in the aliquot of the supernatant was determined by a scintillation counter. The amount of [^{3}H] H_2O measured was corrected by subtracting the blank value, which was obtained from a dish without cells, from each sample's value. Data are shown as the mean ± standard deviation.

2.5. Preparation of HeLa cell conditioned medium

HeLa cells were seeded into 6-cm dishes ($0–10 \times 10^5$ cells in 3 ml DMEM-dcsFBS per dish). Twenty-four hours later, medium was replaced with 3 ml fresh medium to remove estrogenic impurities from the polystyrene dishes [6]. HeLa cells were treated as indicated ("addition to HeLa cells") for 48 h, and the conditioned medium was retrieved. The retrieved medium (400 μl per well) was added to MCF-7 cells transfected with either 3E-SV-FL or 3 mE-SV-FL.

2.6. Reporter gene assays

3E-SV-FL [6] and its negative control 3 mE-SV-FL are luciferase reporters constructed by inserting three estrogen response elements (EREs) (GTCAGGTCACAGTGACCTGA × 3) or three mutated EREs (GTCgtcgCACAGTGAtCaGA × 3), respectively, into the *Sma*I site of pGL3-Promoter Vector (Promega). The day before transfection, MCF-7 cells were seeded into 6-cm dishes (6×10^5 cells in 3 ml DMEM-dcsFBS per dish). After liposome-mediated transfection [7] of 3E-SV-FL or 3 mE-SV-FL (2.5 μg per dish), cells were trypsinized, mixed, and split equally into 24-well plates (unless mentioned otherwise, 1×10^5 cells in 500 μl DMEM-dcsFBS per well), such that the cell number and the transfection efficiency in each well were equal. In the experiments where HeLa cell conditioned medium was used, pretreatment of the transfected MCF-7 cells with an aromatase inhibitor (100 nM Fo or 50 μM AG) was started immediately after the transfected cells were split, in order to minimize T conversion into E_2. Twenty-four hours post-transfection, medium was replaced with the conditioned medium retrieved from HeLa cells, and treatment was started as indicated ("addition to MCF-7 cells"). Three wells were used for each treatment group. Twenty-four hours later, cells were harvested and luciferase activity was measured and normalized based on protein concentration of cell lysate. Multivariate analyses were performed by ANOVA followed by Fisher's PLSD, and $p < 0.05$ was considered as significant. Experiments were repeated at least three times, and representative results are shown. Data are presented as the mean ± standard deviation.

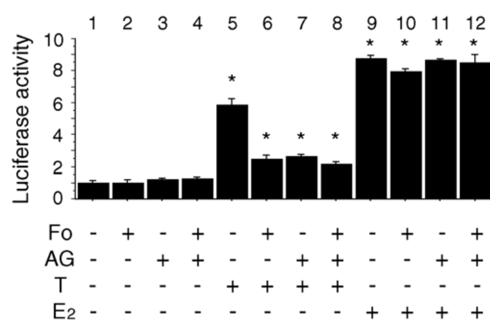

Fig. 2. Effects of aromatase inhibitors on T-induced ER activation in MCF-7 cells. 3E-SV-FL-transfected MCF-7 cells were split into 24-well plates (1×10^5 cells in 500 μl DMEM-dcsFBS per well). Twenty-four hours later, medium was replaced with fresh medium and cells were treated as indicated for 24 h. Asterisks indicate significantly higher luciferase activity than column 1, whose mean luciferase activity was defined as 1.0. Concentrations used were: Fo, 100 nM; AG, 50 μM; T, 100 nM; E_2, 1 nM.

3. Results

We initially tested the effects of exogenous T on ERE-mediated transcription in human mammary carcinoma MCF-7 cells. In cells transfected with an estrogen-responsive reporter (3E-SV-FL), ER was activated by T (Fig. 2, compare columns 1 and 5) and this activation was diminished by treatment with steroidal (Fo) and/or non-steroidal (AG) aromatase inhibitors (Fig. 2, compare columns 5 and 6–8), compatible with previously reported aromatase-dependent conversion of T into E_2 in MCF-7 cells [5,8–13]. There was no T stimulation of luciferase activity from a reporter containing mutant EREs (3 mE-SV-FL, data not shown), indicating that T stimulation occurs via the EREs. Of note, aromatase inhibitors did not completely reduce ERE-driven transcription to the basal level (Fig. 2, compare columns 6–8 and 1–4). Although aromatase mRNA was detected by RT-PCR in MCF-7 cells (Fig. 3), aromatase enzymatic activity was very low (2.9 ± 0.1 fmol [^{3}H] H_2O formed/24 h/10^6 cells) relative to placental JEG-3 cells (1886 ± 252 fmol [^{3}H]H_2O formed/24 h/10^6 cells). Thus, we considered the possibility that MCF-7 cells might metabolize T into estrogenic substances via an aromatase-independent pathway as well.

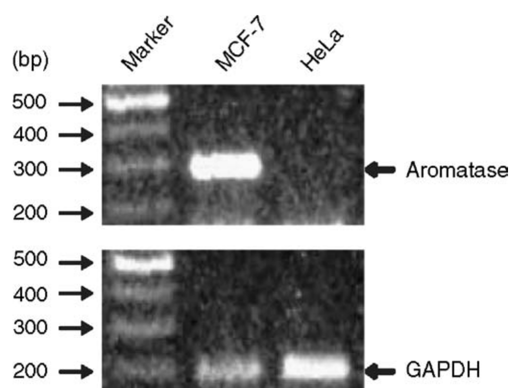

Fig. 3. Aromatase expression in MCF-7 cells but not in HeLa cells. The presence or absence of aromatase mRNA was demonstrated by RT-PCR. The same RNA samples were also tested for GAPDH mRNA to verify their qualities.

HeLa cells are known to be aromatase negative [14], and this was confirmed by RT-PCR (Fig. 3). The experimental protocol in HeLa cells is depicted in Fig. 4A. Note that after steroid addition to HeLa cells, the medium is transferred to ER-containing MCF-7 cells expressing 3E-SV-FL to assay for estrogenicity. When T was added to HeLa cells for 48 h, the estrogenicity of the medium was clearly enhanced (Fig. 4B, compare columns 1–3 and 4). Simultaneous addition of Fo (or AG, data not shown) did not change estrogenic activity in the medium (Fig. 4B, compare columns 4 and 5), consistent with the absence of aromatase in this cell line. A pure ER antagonist ICI inhibited the enhancement of luciferase activity (Fig. 4B, column 6), confirming that induction of luciferase activity was mediated via the ER. No change in luciferase activity was seen when HeLa cell conditioned media was added to MCF-7 cells transfected with the mutant reporter, 3mE-SV-FL (data not shown). This generation of estrogenic activity was dependent on the initial T concentration (Fig. 4C, compare columns 1–3 and 5), the incubation period (Fig. 4C, compare columns 4 and 5), and the number of HeLa cells per dish (data not shown). These results show that aromatase-negative HeLa cells metabolize T into an estrogenic compound(s).

Since HeLa cells express 5α-reductase [15], they can convert T into DHT. We considered the possibility that estrogenic steroids produced downstream of DHT, such as 3βD, might be involved in the estrogenicity observed in Fig. 4. Therefore, we tested the effects of a 5α-reductase inhibitor, Fi. One-micromolar Fi, which is sufficient to inactivate both types (types 1 and 2) of 5α-reductase [16], inhibited T conversion into estrogenic substances by HeLa cells (Fig. 5A) and MCF-7 cells (Fig. 5B), which also express 5α-reductase [17]. These observations indicate that T conversion into an estrogenic steroid(s) via DHT can be reduced by inhibition of 5α-reductase activity.

4. Discussion

Estrogens, such as E_2 and estrone, are the most potent and abundant endogenous steroids with estrogenic activity. However, ER-dependent transcription can be enhanced by other steroids as well. In this study, we demonstrate that the estrogenicity of such steroids can be detected using an ERE reporter assay. We also show that a 5α-reductase inhibitor can further reduce estrogenic activity by inhibiting the production of DHT, which can be converted into weak estrogenic steroids. These findings raise the possibility that a substantial amount of such aromatase-independent estrogenic steroids might be synthesized in tissues (i.e., skin, liver, brain, prostate) that express 5α-reductase [18]. The concept

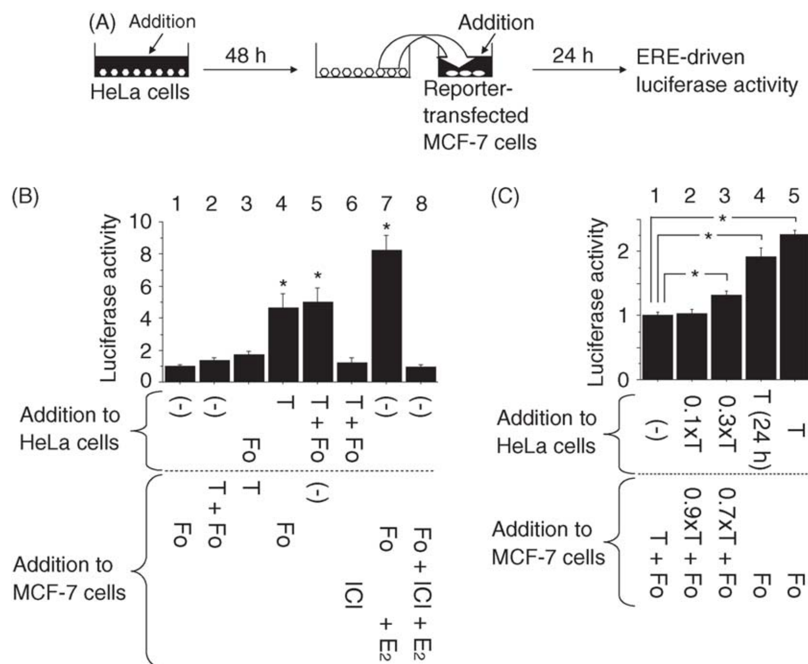

Fig. 4. Conversion of T into estrogenic substances by HeLa cells. (A) Experimental design. Transfected MCF-7 cells were pretreated with 100 nM Fo for 24 h, before stimulation with the HeLa cell conditioned medium was started. (B) Aromatase-independent conversion of T into estrogenic substances. Conditioned medium was retrieved from 6-cm dishes where 7×10^5 HeLa cells (per dish) had been initially seeded. Asterisks indicate significantly greater luciferase activity than column 1, the mean of which was defined as 1.0. The concentration used for T, Fo and ICI was 100 nM. The E_2 concentration is 1 nM. (C) Estrogenicity is dependent on T concentration and time. Initially, 7×10^5 HeLa cells were seeded into each 6-cm dish, and T concentration and time of HeLa cell incubation with T were varied, as indicated. The mean luciferase activity in column 1 was defined as 1.0. Asterisks denote statistically significant induction of ERE-mediated transcription by T. T and Fo indicate 100 nM T and 100 nM Fo, respectively.

of aromatase-independent estrogenic steroid generation is consistent with the observation that the aromatase-null mouse [19] has a less pronounced phenotype than the ER-deficient mouse [20].

One candidate for the estrogenic steroid(s) downstream of DHT is 3βD. It has been reported that 3βD elicits ERE-mediated transcription in MCF-7 cells at a concentration as low as 1 nM in medium [21] and that the serum concentration of 3βD in postmenopausal women is 5–10 nM [22]. Therefore, 3βD may be acting as an estrogenic steroid in vivo in postmenopausal women, although it is difficult to determine whether the concentration of biologically active, free 3βD in culture media is comparable to that in human serum. We performed gas chromatography–mass spectrometry analyses of the HeLa conditioned media but did not detect this steroid. However, we cannot exclude technical or assay sensitivity problems, and cannot deny the presence of undetectable, but biologically active protein-unbound 3βD in the conditioned media. Also, we cannot rule out the possibility that unidentified steroid(s) further downstream of 3βD, rather than 3βD itself, might be the main estrogenic steroid(s) in the media. Presently, therefore, the identity of the estrogenic steroid(s) that are generated in an aromatase-independent manner remains to be established.

The assay for estrogenicity using MCF-7 cells transfected with an ERE-dependent luciferase reporter is highly sensitive. This bioassay system can detect less than 1 pM E_2 (data not shown), as well as detecting the presence of other estrogenic substance(s) in the medium. We document that luciferase activity is unchanged in MCF-7 cells transfected with a mutant ERE reporter (3 mE-SV-FL). Such negative control experiments are important, because it is known that ER, along with an ER agonist, influences transcription mediated by DNA elements which bind other transcription factors such as AP1, Sp1, etc. [23–25]. By using both 3E-SV-FL and 3 mE-SV-FL, our strategy can specifically evaluate activation of ERE-mediated transcription, excluding the possibility that luciferase activity might be altered via some cryptic transcription factor-binding elements within the 3E-SV-FL plasmid sequence.

Recently, third-generation aromatase inhibitors such as anastrozole, letrozole and examestane have been used for the treatment of postmenopausal women with breast cancer. These agents act by inhibiting estrogen synthesis in peripheral tissues. It is possible that further reduction of estrogenic steroids might be achieved in vivo by a 5α-reductase inhibitor (Fi or a newer 5α-reductase inhibitor dutasteride [26]). The relative importance of 5α-reductase as a source of estrogenic steroids may depend on several factors including the T concentration, the abundance of 5α-reductase, and metabolism of estrogenic steroids. It is not possible, at present, to measure the actual concentrations

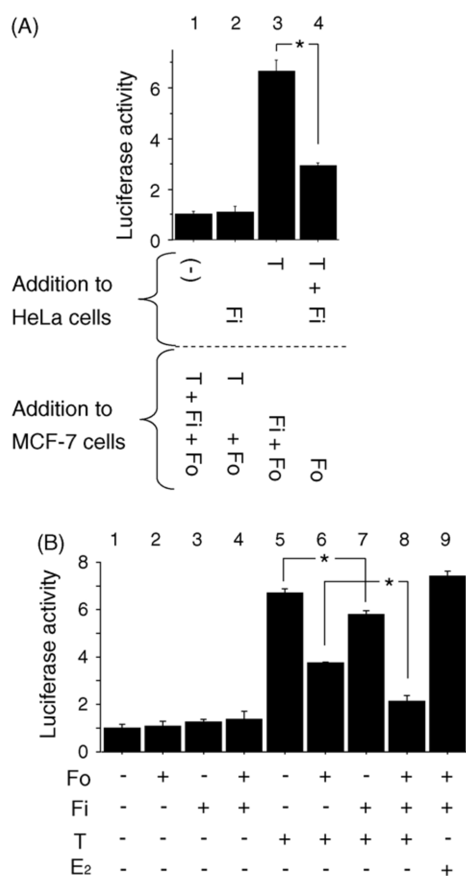

Fig. 5. Inhibition of T conversion into estrogenic steroids by Fi. (A) Effects of Fi on metabolism of T into an estrogenic compound(s) by HeLa cells. Conditioned medium was obtained from 6-cm dishes where 10×10^5 HeLa cells (per dish) had been seeded. Asterisks indicate significant reduction of luciferase activity by Fi. The mean luciferase activity in column 1 was defined as 1.0. T and Fi denote 100 nM T and 1 μM Fi, respectively. (B) Effects of Fi on T-induced ER activation in MCF-7 cells. Transfected MCF-7 cells were seeded into 24-well plates (4×10^5 cells in 500 μl DMEM-dcsFBS per well). Twenty-four hours later, medium was replaced with fresh medium and cells were treated as indicated for 24 h. Asterisks indicate significant reduction of luciferase activity by treatment with Fi. The mean luciferase activity in column 1 was defined as 1.0. The concentrations used were: Fo, 100 nM; Fi, 1 μM; T, 100 nM; E_2, 1 nM.

of all estrogenic steroids or their precursors in tumors or their surrounding tissues. It is also important to consider other estrogenic steroids, including environmental estrogens [27], whose synthesis is not reduced by either aromatase inhibitors or 5α-reductase inhibitors, as a source of ER stimulation.

In conclusion, we have demonstrated an aromatase-independent pathway of T conversion into estrogenic steroids. This pathway is inhibited by a 5α-reductase inhibitor, indicating that metabolites of DHT exert estrogenic activity. The results of our study suggest that 5α-reductase inhibitors, which can reduce estrogenic steroids downstream of DHT, might be useful to enhance the efficacy of aromatase inhibitors against estrogen-dependent conditions such as breast cancer.

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