Enhanced formation of non-phenolic androgen metabolites with intrinsic oestrogen-like gene transactivation potency in human breast cancer cells: a distinctive metabolic pattern

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Abstract

Breast cancer is a sex steroid hormone-dependent malignant neoplasia. The role of oestradiol in this malignancy has been well documented; however, the involvement of androgens has remained controversial. To determine the role of non-phenolic androgen metabolites in human breast cancer, we studied the metabolism of [14C] testosterone and [14C] androstenedione in oestrogen-dependent MCF-7 cells and non-oestrogen-dependent MDA-MB 231 cells, at different substrate concentrations (1–10 μM) and time periods (30 min–48 h). Cultured non-oestrogen-dependent HeLa and yeast cells served as controls. Metabolites were identified and quantified by reverse isotope dilution. A distinctive pattern of androgen metabolism was identified in MCF-7 cells, being the 5α-androstane-3α,17β-diol (3α,5α-diol) and its 3β epimer (3β,5α-diol), the major conversion products of testosterone (48–3%), with 5α-dihydrotestosterone as intermediary. The formation of 3α,5α-diol and 3β,5α-diol (diols) was substrate concentration- and time-dependent, and abolished by finasteride. In contrast, very little of any diol formation was observed in MDA-MB 231, HeLa and yeast cell incubations. Additional enzyme gene expression studies revealed an overexpression of 5α-steroid reductase type-1 in MCF-7 cells, as compared with MDA-MB 231 cells. The oestrogen-like activities of diols were assessed in HeLa cells co-transfected with expression vectors for α or β subtypes of the human oestrogen receptor (hER) genes and for an oestrogen-responsive reporter gene. The results show that 3β, 5α-diol and to a lesser extent 3α,5α-diol bind with high relative affinity to hERα and hERβ.

Both diols induced hER-mediated reporter gene transactivation in a dose–response manner, similar to that induced by oestradiol, though with lower potency, an effect that was abolished by ICI-182 780. Furthermore, 3β,5α-diol and to lesser extent 3α,5α-diol induced MCF-7 cell proliferation. The overall results demonstrated that MCF-7 cells exhibit enhanced expression and activity of androgen-metabolising enzymes, leading to rapid and large diol formation, and provide evidence that these androgen metabolites exert a potent oestrogen-agonistic effect, at genomic level, in oestrogen-dependent breast cancer cells. The data suggest that diols may act as in situ intracrine factors in breast cancer and that its formation can be pharmacologically inhibited. Journal of Endocrinology (2006) 190, 805–818

Introduction

The key role of oestrogens for both the initiation and progression of most breast cancer tumours has been well established (Kirschner 1979, Nicholson et al. 1988, Pike & Spicer 1991, Pike et al. 1993, Musgrove & Sutherland 1994, Pasqualini & Chetrite 1996, Girdler & Brotherick 2000); however, the precise involvement of other sex steroid hormones on this malignancy has remained a controversial issue. Evidence has accumulated indicating that androgens may act as either stimulating or inhibiting hormonal agents in breast cancer, though the mechanisms by which they exert these effects have not yet been elucidated. Indeed, numerous studies in animal models have shown that androgens, at physiological concentrations, stimulate growth in mammary cancer (Smith & King 1972, Liao et al. 1998, Xie et al. 1999, Liao & Dickson 2002, Somboonporn & Davis 2004), while other reports have indicated that their administration results in tumour regression of chemically and hormone-induced breast cancers (Costlow et al. 1976, Zhou et al. 2000). The inhibitory capability of androgens in human breast cancer has been documented in experimental and clinical studies

The presence of androgens in normal and malignant breast tissue, as well as their enzyme-mediated conversion to oestrogens, has been well documented in pre- and post-menopausal women (reviewed in Pasqualini & Chetrite (1996)). Although the aromatisation of androgens in breast cancer cells has remained controversial, low, but reproducible aromatase activity has been detected in MCF-7 cells (Sonne-Hansen & Lykkesfeldt 2005). To avoid oestrogen formation, several aromatase inhibitors have been widely used in therapeutic schemes in breast cancer (Coombes et al. 1987, Santen et al. 1990, Demers 1994, Goss 1999, Ragaz 1999, Sasano et al. 1999), although the oestrogen-like effects of androgens are not completely suppressed by this treatment (Brodie et al. 1977, Brodie & Longcope 1980, Coombes et al. 1984, Wing et al. 1985, Lippman 1998, Buzdar 2002, Lonning 2004, Brueggemeier et al. 2005). These observations raise the important question as to whether androgens are locally biocverted to non-phenolic metabolites with intrinsic oestrogenic activities. Androgen-metabolising enzymes, other than aromatase, have been identified and characterised in human breast cancer tissue and cells (Bonney et al. 1983, Labrie et al. 1992, 1997, 2000, Sasano et al. 1996, Gingras et al. 1999, Ariga et al. 2000, Suzuki et al. 2000), and an over-expression of 5α-steroid reductase type-1 gene has been reported in human breast cancer tumours (Suzuki et al. 2001).

To determine whether androgens are biocverted to A-ring-reduced derivatives with intrinsic oestrogen-agonistic potency in breast cancer, we studied the metabolism of [14C]-labelled testosterone and Δ⁴A in oestrogen-dependent (MCF-7) and non-oestrogen-dependent (MDA-MB 231) human breast cancer cells. Non-oestrogen-dependent human uterine cervical cancer (HeLa) cells and yeast cells served as experimental controls. The results disclose a distinctive androgen-metabolic pathway in MCF-7 cells, characterised by overexpression and enhanced activities of the androgen-metabolising enzymes, resulting in a large formation of 3β,5α-diol and 3β,5β-diol. In subsequent studies, the oestrogen-like activity of both the diols, was assessed by their binding affinity to the α and β subtypes of the human oestrogen receptor (hER), their capability to activate oestrogen response elements of a reporter gene in the construct assay employed, and their ability to induce cell proliferation in MCF-7 cells.

Further interest for the conduction of this study stemmed from the recent observations in our laboratory (Lemus et al. 2000, 2001, Larrea et al. 2001, Santillán et al. 2001, García-Becerra et al. 2002), demonstrating that the A-ring tetrahydro-reduced metabolites of norethisterone, levonorgestrel and gestodene, possess oestrogen-agonistic activities, suggesting that they could be involved in the activation of breast cancer cell proliferation induced by high doses of synthetic contraceptive progestins derived from 19-nor testosterone, as it has been previously reported (Catherino et al. 1993, Schoonen et al. 1995a, 1995b).

Materials and Methods

Steroids and chemicals

[4-14C] Testosterone ([14C]-T), specific activity (sp. act.) 45 mCi/mmol; [4-14C] androstenedione ([14C]-Δ⁴A), sp. act. 57.5 mCi/mmol; [2,4,6,7,16,17-3H] oestradiol ([3H]-E₂), sp. act. 148Ci/mmol, [ring-3,5-3H] chloramphenicol, sp. act. 50 Ci/mmol and [3H] thymidine, sp. act. 14 Ci/mmol were purchased from NEN Research Products (Boston, MA, USA) and non-radioactive steroids were supplied by Sigma. Cell culture media, enzymes, gene primers and reverse transcriptase (RT)-PCR kits and reagents were purchased from Invitrogen. Fetal bovine serum (FBS) was supplied by Hyclone Laboratories, Inc. (Logan, UT, USA). All reagents and solvents used were of analytical grade.

Cell lines and culture

Human breast cancer cells lines MCF-7 (hER dependent) and MDA-MB 231 (hER independent), obtained from Dr A Zentella (Instituto Nacional de Ciencias Médicas y Nutrición S. Zubirán, Mexico City, México) were cultured in T-45 flasks with phenol red Dulbecco’s modified Eagle medium-high glucose (DMEM-HG) containing t-glutamine and supplemented with 5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 nM oestradiol, in a 95% air:5% CO₂ atmosphere, at 37°C. Human uterine cervical cancer (HeLa) cells, kindly supplied by Dr A J Cooney ( Baylor College of Medicine, Houston, TX, USA), were cultured in T-45 flasks with phenol red DMEM-HG containing t-glutamine and supplemented with 10% stripped FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in a 95% air:5% CO₂ atmosphere, at 37°C. Yeast cells (Saccharomyces cerevisiae), were cultured in gold medium at 32°C by continuous shaking using air as the gas phase.

Androgen metabolism

To assess the in situ conversion of androgens to A-ring-reduced derivatives in human breast cancer, the metabolism of [14C]-T and [14C]-Δ⁴A in MCF-7 and MDA-MB 231 cells maintained in culture was studied using HeLa and yeast cells as experimental controls. Incubations were done at a cell density of 2X10⁶, at 37°C, in a 95% air:5% CO₂ atmosphere, using the increasing substrate concentrations (1–10 μM) of either
[14C]-T or [14C]-Δ4A, for different time periods (30 min–48 h), at pH 7·4 and 5·2, in the absence or presence of 1 mM finasteride (Merck). All incubations were carried out in phenol red-free and oestradiol-free, supplemented DMEM-HG culture medium. To determine the activities of the two types of 5α-steroid reductases (Russell & Wilson 1994), incubations were carried out at pH 7·4 and 5·2. In addition, similar incubations were done using homogenates at pH 7·4 and 5·2, in the presence of 2·5 μM NADPH. Final incubation volume was 3 ml. Cell-free and boiled inactivated cell incubations, carried out under identical conditions, were used as negative controls. Protein content was determined by a protein–dye-binding method (Bradford 1976) using BSA as standard. At the end of the incubation period, the reaction was stopped by the addition of ethyl acetate, and radiolabelled steroids were extracted (4X) using three volumes of water-saturated ethyl acetate. The organic extracts were partitioned between petroleum ether and 10% aqueous methanol, and 2·5 μg each of the following steroid carriers were added to the methanolic extracts: testosterone (T), 17β-hydroxy-4-androstene-3-one; 5α-dihydrotestosterone (DHT), 17β-hydroxy-5α-androstan-3-one; 3α,5α-androstanediol (3α,5α-diol), 5α-androstan-3α,17β-diol; 3β,5α-androstanediol (3β,5α-diol), 5α-androstan-3β,17β-diol; androstenedione (Δ4A) 4-androstene-3,17-dione and 5α-dihydroandrostanediol (5α,Δ4), 5α-androstan-3α,17β-dione. The identification and radiochemical purity of androgen metabolites were established by a reverse isotope dilution technique, which included identical behaviour to that of the steroid carriers in two different thin layer chromatographic systems (chloroform:acetic acid 85:15, petroleum ether:10% aqueous methanol, ratio 85:15). Samples were subjected to reverse transcription using a Superscript first strand cDNA synthesis kit (Invitrogen), according to the manufacturer’s protocol. Semi-quantitative PCR was performed using 2·5 U Platinum Taq DNA polymerase, 4 mM MgCl2, 0·4 mM dNTPs and 4 mM of each gene-specific steroid enzyme primer, using GAPDH as an internal standard. All PCRs were done up to 30 cycles, each cycle consisting of 5 min at 94°C, 30 s at 52–64°C and 7 min at 72°C. PCR products were electrophoresed on ethidium bromide-containing 1·2% agarose gels, and the bands were subjected to scanning densitometry using a gel analyzer (Kodak). Results are given as relative density (mRNA enzyme/mRNA GAPDH). Gene-specific primer sequences were as follows: human steroid 5α-reductase type-1 (SRD5A1; NM_001047): 5′TGGGAGGAGAAAGCCTATG (sense), 5′GCCACA-CCACTCCATGATTTC (anti-sense); human steroid 5α-reductase type-2 (SRD5A2; NM_000348): 5′CATACG-GTTTAGCTTGGGTGT (sense), 5′GCTTTCCGAGATTG-GGGGTAG (anti-sense); human 3β-hydroxysteroid dehydrogenase (AKR1C1; NM_001353) 5′GTAAAGCTTT-AGAGGCCAC (sense), 5′CACCCTATGTTCTTCTCGGA (anti-sense); human 5α-reductase type-1 (SRD5A1; NM_001354) 5′CTAAAGCCTTGGCCCT (sense), 5′CACCCTATGTTCTTCTCGGA (anti-sense) and human GAPDH: 5′TTCCGCTCTCGTCCCTCCTG (sense), 5′ACCCGTTTGACTCCGACCTTT (anti-sense).

Each PCR was run three times in duplicate according to the manufacturer’s recommendations and default settings.

**Plasmid constructs**

The expression vectors for human ERα and ERβ genes (plasmid of the cytomegalovirus (pCMV) 5α-ERα and pCMV5- hERβ) containing the coding sequence of hERα and hERβ were kindly provided by Dr A J Cooney. The oestrogen responsive reporter plasmid containing a fragment of the vitellogenin A2 gene promoter (positions −331 to −87) upstream of the adenovirus E1b promoter region fused to the chloramphenicol acetyltransferase (CAT) gene (ERE-E1b-CAT) was constructed according to the method described by Smith et al. (1993).

**Transfections**

The HeLa cells were plated the day before transfections in a six-well plate at a density of 3X10⁵ cells/well in phenol red-free DMEM-HG supplemented with 5% stripped FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in a 95% air:5% CO2 atmosphere at 37°C. The next day, cells were visualised in a microscope to assure that the cell density was 30–50% confluent. Transfections were performed in triplicate using SuperFect (Qiagen), according to the manufacturer’s protocol. Briefly, serum-free media (100 μl) was aliquoted and DNA added (1 μg reporter gene plasmid and 0·025 μg of either hERα or hERβ

**Steroid enzyme expression**

The gene expression of androgen-metabolising enzymes in MCF-7 and MDA-MB 231 cells was studied by RT-PCR. Total RNA from the cells (6X10⁶) was extracted using TRIzol reagent and an aliquot (5 μg) from each sample was

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gene expression vectors) and vortexed. SuperFect reagent (10 μl) was added and vortexed for additional 10 s. Following incubation at room temperature for 5–10 min, 600 μl supplemented DMEM-HG was added to each tube. The medium containing the transfection complexes was added to the cell monolayer previously rinsed with PBS. The plates were incubated for 3 h in a 95% air:5% CO₂ atmosphere, at 37 °C. After incubation, the plates were rinsed with PBS and 3 ml supplemented DMEM-HG was added to each well.

**Binding affinity of androgen metabolites to hER**

After transfection, cells were harvested by centrifugation and washed with TEDLM buffer (20 mM Tris–HCl, pH 7.4 at 4 °C, 1:5 mM EDTA, 0:25 mM diethiothreitol, 10 μg/ml leupeptine and 10 mM sodium molibdate) in a ratio (w/v) 1:6. The cytosolic fraction was obtained by vortexing the cells with glass beads in TEDLM buffer followed by centrifugation at 180 000 g, for 1 h, at 2 °C, in an SW 50-1 rotor (Beckman Instruments, Palo Alto, CA, USA). To assess the binding affinity of 3α,5α-diol and 3β,5α-diol to hERα and hERβ, cytosol aliquots (0:5 mg protein/ml) of the co-transfected HeLa cells were incubated with 1 nM [³²P]-E₂ at 4 °C for 18 h, in the absence or presence of increasing concentrations (1–1000 nM) of radioinert oestradiol, 3α,5α-diol and 3β,5α-diol. Bound and free steroid fractions were separated by the addition of 800 μl Dextran-coated charcoal suspension (250 mg Norit-A and 25 mg Dextran T-70) in 100 ml TEDLM buffer and incubated for 10 min at 4 °C. Following centrifugation at 800 g, at 4 °C, for 15 min, aliquots (200 μl) of the supernatants were submitted to radioactive counting. Radioactive content in the aqueous samples was determined using Insta-Gel Plus (Packard, Downers Grove, IL, USA) as counting solution. The results are expressed as the relative binding affinities (RBA) and the inhibition constants (Kᵢ) of steroid competitors, as described by Reel et al. (1979) and Cheng & Prusoff (1973) respectively.

**Oestrogen-agonistic effect of androgen metabolites**

The oestrogen-agonistic actions of androgen metabolites were assessed in transiently co-transfected HeLa cells with the mammalian expression vector for hERα or hERβ genes and its cognate reporter vector ERE-E1b-CAT, using oestradiol as control. Twenty-four hours after co-transfection, cells were incubated in a complete medium containing the increasing concentrations (1 × 10⁻¹²–1 × 10⁻⁶ M) of 3α,5α-diol, 3β,5α-diol and oestradiol, using dimethyl sulfoxide as the steroid vehicle. Incubations were carried out in the absence or presence of 1 × 10⁻⁷ M ICI-182 780 (Zeneca Farma, Mexico City, Mexico), a potent steroid antioestrogen, in a 95% air:5% CO₂ atmosphere, for 24 h at 37 °C. At the end of the incubation period, cells were harvested and submitted to the liquid CAT assay as previously described (Lemus et al. 2000, García-Becerra et al. 2002). Results of the transactivation studies are expressed as the effective concentration values (EC₅₀) of 3α,5α-diol, 3β,5α-diol and oestradiol, obtained by a non-linear regression analysis, using a scientific graphic software (Origin 6.1; OriginLab, Northampton, MA, USA).

**Cell proliferation studies**

The MCF-7 cells were cultured in DMEM without phenol red, containing heat-inactivated FBS (5% v/v), 2 mM l-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated in six-well plates at a density of 1 × 10⁶ cell/well in a 5% CO₂ humidified incubator at 37 °C. After 24 h, the medium was replaced with DMEM containing dextran charcoal-stripped FBS (2:5%) and oestradiol (1 × 10⁻⁹ M) or 3α,5α-diol or 3β, 5α-diol (1 × 10⁻⁷ M) dissolved in ethanol and incubated for 48 h. Cells were quantified and scored to measure proliferation rate (Lopez-Diazguerrero et al. 2006). [³H]-Thymidine (1 μCi) was added to the culture medium and incubated for additional 24 h. Cells were washed with PBS and fixed for 15 min with 500 μl of 95% methanol in PBS. Subsequently, cells were gently washed twice with PBS and 500 μl 0.2 M NaOH were added. Alkaline extracts were submitted to radioactivity counting. Results were expressed as the cell proliferation (%) induced by diols, using oestradiol and vehicle as controls.

**Statistical analysis**

The comparisons of experimental groups with controls in metabolic studies were done by one-way ANOVA and statistical differences between groups were established by Student’s t-test, using the SigmaStat statistical software (Jandel Corporation, San Rafael, CA, USA). Group differences were considered significant when P < 0.001 was reached (two-tailed test). Variance analysis of enzyme expression and cell proliferation studies was performed using the statistical software Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL, USA) and group differences were considered significant when P < 0.05 was reached.

**Results**

**Androgen metabolism in MCF-7 and MDA-MB 231 cells**

After partition of the MCF-7 cells organic extracts, 96% of the incubated radioactive material was recovered in the methanolic fraction. When aliquots of methanolic extract from [¹⁴C]-T incubations were submitted to thin-layer chromatography, four radioactive zones were detected, as shown in Fig. 1A. Zone 1 (Rₛ = 0.22), representing the major metabolic conversion products of [¹⁴C]-T, had a chromatographic behaviour identical to those of the 3α,5α-diol and 3β,5α-diol carriers. After elution, representative aliquots were separately mixed with additional radioinert 3α,5α-diol and 3β,5α-diol carriers and recrystallised to constant sp. act. (Table 1). Formation of 3α,5α-diol was higher than that of its 3β isomer. Zone 2 (Rₛ = 0.34) was identified as unchanged [¹⁴C]-T.
while zones 3 \((R_e = 0.46)\) and 4 \((R_e = 0.55)\) corresponded to DHT and \(\Delta^4\)A respectively. The radiochemical purity of these \([^{14}C]\)-T metabolites is shown in Table 1. Chromatographic analysis of the MDA-MB 231 cells methanolic extracts revealed a completely different \([^{14}C]\)-T conversion pattern (Fig. 1B), with limited conversion to DHT and diols, and very little formation of \(\Delta^4\)A. The metabolism of \([^{14}C]\)-T in HeLa cells was characterised by large bioconversion to \(\Delta^4\)A and limited formation of DHT and \(5\alpha\)-A. No diols were detected in the HeLa cells methanolic extracts, as depicted in Fig. 1C.

Chromatographic analysis of the organic extracts from \([^{14}C]\)-\(\Delta^4\)A incubations with MCF-7 cells revealed the presence of five radioactive zones (Fig. 1D). Zone 1 \((R_e = 0.22)\) and zone 2 \((R_e = 0.34)\), representing one the major metabolic conversion products of \([^{14}C]\)-\(\Delta^4\)A, were identified as diols and testosterone respectively. Their radiochemical purity is shown in Table 1. Zone 3 \((R_e = 0.67)\) had a chromatographic mobility identical to that of DHT. Zone 5 \((R_e = 0.67)\) had a chromatographic mobility corresponding to that of \(5\alpha\)-A and zone 4 \((R_e = 0.55)\) corresponded to unconverted \([^{14}C]\)-\(\Delta^4\)A. The radiochemical purity of A-ring-reduced metabolites of \([^{14}C]\)-\(\Delta^4\)A in MDA-MB 231 cells (Fig. 1E) was completely different from that observed in MCF-7 cells. The metabolism of \([^{14}C]\)-\(\Delta^4\)A in HeLa cells was characterised by a limited
Isolated metabolites from \([^{14}\text{C}]\)-T incubations

<table>
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<tr>
<th>Isolated metabolites from ([^{14}\text{C}])-T incubations</th>
<th>Successive crystallizations specific activity ([^{14}\text{C}]) dpm/mg</th>
<th>Isolated metabolites from ([^{14}\text{C}])-(\Delta^4) A incubations</th>
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<td>ML(_3) 493</td>
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\(^a\)Crystals.
\(^b\)Mother liquors.

Biotransformation to testosterone and 5α-reduced metabolites, as depicted in Fig. 1F.

The metabolism of testosterone in MCF-7, MDA-MB 231 and HeLa cells, as a function of substrate concentration is shown in Fig. 2. Incubations were carried out at 37 °C, for 2 h, at pH 7·4 and 5·2. The metabolic pattern of \([^{14}\text{C}]\)-T in MCF-7 cells (Fig. 2A and B) was similar in incubations carried out at both pH levels, with the distinctive feature of a large formation of diols, noticed even at the lowest substrate concentration. Interestingly, a significantly larger formation of diols occurred in incubations at pH 7·4 as compared with those at pH 5·2. In contrast, very little, if any, diol formation was noticed in incubations of MDA-MB 231 and HeLa cells with \([^{14}\text{C}]\)-T, even at the highest substrate concentration studied (Fig. 2C–F). A large formation of \(\Delta^4\) A at both pH levels was observed in HeLa cells and a limited bioconversion of testosterone to DHT. When MCF-7 cell homogenates, added with NADPH, were incubated with \([^{14}\text{C}]\)-T for 2 h, the percent conversion of testosterone to diols was significantly higher at pH 7·4 (63·2%), as compared with those at pH 5·2 (37·4%).

The metabolism of \([^{14}\text{C}]\)-\(\Delta^4\) A in MCF-7, MDA-MB 231 and HeLa cells, as a function of substrate concentration, at pH 7·4 and 5·2 is shown in Fig. 3. The bioconversion pattern of \(\Delta^4\) A in MCF-7 cells was similar in incubations carried out at both pH levels (Fig. 3A and B), and was characterised by a simultaneous large formation of testosterone and 5α-reduced metabolites (5α-A, diols and DHT). A moderately large formation of 5α-reduced \([^{14}\text{C}]\)-\(\Delta^4\) A metabolite was noticed in incubations at pH 7·4 as compared with those at pH 5·2. Similar results were obtained in incubations of MCF-7 cell homogenates with \([^{14}\text{C}]\)-\(\Delta^4\) A. On the contrary, the bioconversion pattern of \([^{14}\text{C}]\)-\(\Delta^4\) A in MDA-MB 231 and HeLa cells was characterised by very little formation of testosterone, noticed only at the highest substrate concentration used, and a relatively higher formation of 5α-A, particularly in incubations undertaken at pH 7·4 (Fig. 3C–F).

The enzyme-mediated formation of 5α-reduced metabolites of testosterone in MCF-7, MDA-MB 231 and HeLa cells, as a function of time, is depicted in Fig. 4. Cells were incubated with 2 μM \([^{14}\text{C}]\)-T at 37 °C, pH 7·4, at different time periods. The results demonstrated that radiolabelled testosterone incubated with MCF-7 cells was extensively bioconverted to diols, presumably through its prior conversion to DHT. Indeed, the maximal accumulation of DHT occurred at 30 min of incubation with a subsequent decline and a concomitant time-dependent increase on the formation of diols (Fig. 4A). At 48 h incubation, 48·3% of the \([^{14}\text{C}]\)-labelled testosterone was bioconverted to diols. According to recrystallisation data, the formation of 3α,5α-diol and 3β,5α-diol occurred in a ratio of 2:1. In sharp contrast, the absence of formation of diols was observed in incubations of MDA-MB 231 and HeLa cells with \([^{14}\text{C}]\)-T, with very small and delayed formation of DHT (Fig. 4B and C). The effect of a 5α-steroid reductases inhibitor on the metabolism of androgens in MCF-7 cells is shown in Fig. 5. Cells were incubated with 2 μM \([^{14}\text{C}]\)-T or \([^{14}\text{C}]\)-\(\Delta^4\) A, at 37 °C, for 48 h, at pH 7·4, in the absence or presence of 1 mM finasteride. As it can be seen, the addition of finasteride resulted in a significant diminution of the bioconversion of radiolabelled testosterone and \(\Delta^4\) A to their corresponding 5α-dihydro metabolites and in an abolishment of diols formation (Fig. 5, inserts). In all incubations with MCF-7, MDA-MB 231 and HeLa cells, neither oestradiol nor oestrone was detected as conversion products of \([^{14}\text{C}]\)-T and \([^{14}\text{C}]\)-\(\Delta^4\) A.
Only unmodified $[\text{14C}]$-labelled substrates were identified in incubations using yeast cells, boiled inactivated cells and cell-free preparations, used as negative controls.

**Enzymes gene expression profile in MCF-7 and MDA-MB 231 cells**

The results from androgen-metabolising enzymes gene expression in the two cell lines studied, as determined by semi-quantitative RT-PCR assays, are shown in Fig. 6. The level of expression of SRD5A1 was significantly ($P<0.05$) higher in MCF-7 cells, as compared with MDA-MB 231 cells. In contrast, the expression of SRD5A2 in both cell lines did not exhibit differences. Interestingly, the expression of SRD5A1 was significantly higher ($P<0.01$) in both cell lines, as compared with SRD5A2. The expression level of AKR1C1 and AKR1C2 was similar in MCF-7 and MDA-MB 231 cells, though the expression of AKR1C1 was significantly higher ($P<0.001$) than that of AKR1C2 in both cell lines studied.

**Binding affinity of androstanediols to hER**

The effect of increasing concentrations of non-radioactive diols upon the $[^3\text{H}]-\text{E}_2$ binding to hER in HeLa cells transfected with expression vectors for either hER$\alpha$ or hER$\beta$ is shown in Fig. 7. The $[^3\text{H}]-\text{E}_2$ bound to hER in the absence of steroid competitors was set at 100% in this radioligand competitive assay. Even though both diols were competitors for hER$\alpha$, the $3\beta,5\alpha$-diol exhibited higher affinity ($RBA=1.9\%$; $K_i=3.25\text{ nM}$) than its $3\alpha$-isomer ($RBA=0.009\%$; $K_i=625\text{ nM}$). The $3\beta,5\alpha$-diol was more efficient competitor for the $\beta$ subtype of hER ($RBA=0.92\%$; $K_i=6.5\text{ nM}$) than $3\alpha,5\alpha$-diol ($RBA=0.006\%$; $K_i=950\text{ nM}$). The $3\beta,5\alpha$-diol and to a lesser extent its $3\alpha$-isomer, bound to both subtypes
of hER, in a similar manner to that of oestradiol used as positive control, though with lower affinity.

**Oestrogen-like transactivation effect of androstanediols**

The transactivation effect of \(3\alpha,5\alpha\)- and \(3\beta,5\alpha\)-androstanediols (diols) were identified as the major metabolic conversion products in MCF-7 cells (A) and (B). A larger formation of T and \(5\alpha\)-reduced \(\Delta^4\)A metabolites occurred at pH 7·4 (A), as compared with incubations at pH 5·2 (B). The major metabolic product of \([^{14}\text{C}]\Delta^4\text{A}\) in MDA-MB 231 and HeLa cells (C–F) was \(5\alpha\)-dihydroandrostanedione (\(5\alpha\)-A) with very little formation of T and DHT. Each point represents the mean ± S.E.M. of five experiments in triplicate.

**Effect of diols in cell proliferation**

To assess the effect of \(3\alpha,5\alpha\)- and \(3\beta,5\alpha\)-diols on cell proliferation in MCF-7 cells, a set of experiments using \([^{3}\text{H}]\)thymidine incorporation method was undertaken in triplicate. Oestradiol and vehicle served as experimental controls. The results indicated that after 48 h treatment, \(3\beta,5\alpha\)-diol (\(1 \times 10^{-7} \text{ M}\)) was capable of increasing the proliferation rate induced transactivation only at the highest concentration employed (\(1 \times 10^{-6} \text{ M}\)). The comparative potencies of both diols and oestradiol, to induce CAT gene transactivation mediated by the subtypes \(\alpha\) and \(\beta\) of hER, as judged by their EC\(_{50}\), are depicted in Table 2.
in MCF-7 cells significantly (150%) as compared with vehicle, in a similar fashion to that exerted by $1 \times 10^{-7} \text{M oestradiol (140%)}. Treatment with $1 \times 10^{-7} \text{M 3\alpha,5\alpha}$-diol also increased cell proliferation (50%) as compared with vehicle in MCF-7 cells. The effect of oestradiol, 3\alpha,5\alpha- and 3\beta,5\alpha-diols on MCF-7 cell proliferation was not seen in MDA-MB 231 cells.

**Figure 4** Formation of 5\alpha-reduced testosterone metabolites in MCF-7, MDA-MB 231 and HeLa cells, as a function of time. Incubations were carried out using 2 $\mu\text{M [14C] testosterone, at 37 ^\circ\text{C, pH 7-4, for different time periods. An early (30 min) bioconversion of testosterone to 5\alpha-dihydrotestosterone (DHT) and a subsequent large formation of 3\alpha,5\alpha- and 3\beta,5\alpha-androstanediols (diols) were observed in MCF-7 cells (A). After 48 h incubation, 48-3\% of the radiolabelled substrate was bioconverted to diols. A limited formation of diols was observed in MDA-MB 231 cells incubations (B), while a delayed formation of DHT without further conversion to diols occurred in HeLa cells incubations (C). Each point represents the mean $\pm$ S.E.M. in five experiments in triplicate.

**Discussion**

The results of this study clearly demonstrate that oestrogen-dependent human breast cancer cells efficiently biotransform androgens to A-ring tetrahydro-reduced metabolites, which possess the capability to transactivate oestrogen-regulated genes mediated by the $\alpha$ and $\beta$ subtypes of hER. Indeed, MCF-7 cells incubated with radiolabelled androgens exhibited a large and rapid formation of tetrahydro-reduced metabolites, thus indicating a great activity of 5\alpha-steroid reductases, types 1 and 2, and two enzymes of the aldo–keto reductases family, the 3\alpha- and 3\beta-hydroxysteroid dehydrogenase and the 3\beta-hydroxysteroid dehydrogenase. The large conversion of testosterone to 3\alpha,5\alpha- and 3\beta,5\alpha-diols in MCF-7 cells had DHT as an obligatory intermediary as it was demonstrated by its inhibition induced by finasteride. This observation supports the concept that formation of diols from testosterone requires enzyme-mediated 5\alpha-reduction (trans A/B ring junction) as the first metabolic step. In contrast, non-oestrogen-dependent MDA-MB 231 cells exhibited a limited bioconversion of androgens to 5\alpha-reduced metabolites and very little, if any, formation of diols. An absence of bioconversion of androgen to diols was observed in HeLa and yeast cell incubations used as controls. The finding of an enhanced enzyme 5\alpha-steroid reductase activity in MCF-7 cells, particularly in experiments carried out at pH 7-4, is in line with the report of Suzuki et al. (2001), who have demonstrated an overexpression of the 5\alpha-steroid reductase type-1 gene in human breast cancer tissues. Interestingly, our results revealed a selectively higher expression level of SRD5A1 in MCF-7 cells as compared with MDA-MB 231 cells, indicating a good agreement between enzyme expression levels and activities in human breast cancer cells. In contrast, no difference on the
expression levels of AKR1C1 and AKR1C2 in MCF-7 and MDA-MB 231 cells was noticed. These results confirm and extend the report of Wiebe et al. (2000), who, using radiolabelled progesterone as substrate, demonstrated an enhanced 5α-steroid reductase activity and also an over-expression of the 5α-steroid reductase type-1 gene in MCF-7 cells, as compared with oestrogen-resistant breast cells (MCF-10A). Furthermore, Wiebe & Lewis (2003) demonstrated the
lower expression levels of 3α- and 3β-hydroxysteroid dehydrogenase genes in breast cancer tissues as compared with paired breast normal tissue.

Another striking finding of this study was that 3α,5α- and 3β,5α-diols, the major metabolic conversion products of testosterone in MCF-7 cells, interact with relatively high binding affinity with both subtypes of hER and are capable of transactivating an oestrogen-dependent reporter gene (CAT) in the transiently co-transfected cell expression system employed, resembling the effects of naturally occurring oestradiol, though with lower potency. It must be underlined than even the formation of 3α,5α-diol from testosterone was almost double than that of its 3β-epimer, according to recrystallisation data, the 3β,5α-diol exhibited higher hERα-binding affinity and higher oestrogen-like transactivation potency, as compared with its 3α-epimer, as shown in Fig. 8 and Table 2. Further evidence that diols-induced gene transactivation in the construct assay is mediated via hERα and hERβ, was derived from the observation that ICI-182 780 was able to preclude this effect. Our results confirm and extend previous reports on the activation of hERα by 3β,5α-diol. (Maggiolini et al. 1999, García-Becerra et al. 2002). These data are similar, though non-identical, with our previous observations with the tetrahydro-reduced metabolites of synthetic 19-nor progestins, which are also able to transactivate oestrogen-dependent genes mediated through hERα, but not through hERβ, behaving as selective hER modulators (Lemus et al. 2000, Larrea et al. 2001,

Table 2 Human oestrogen receptors (hER)-mediated gene transactivation induced by androstanediols in a co-transfected cell expression system

<table>
<thead>
<tr>
<th>Steroids</th>
<th>hERα (mol/l)</th>
<th>hERβ (mol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol</td>
<td>6·74×10⁻¹¹</td>
<td>3·37×10⁻¹²</td>
</tr>
<tr>
<td>3β,5α-Androstanediol</td>
<td>1·61×10⁻⁹</td>
<td>1·41×10⁻⁸</td>
</tr>
<tr>
<td>3α,5α-Androstanediol</td>
<td>9·10×10⁻⁸</td>
<td>3·05×10⁻⁶</td>
</tr>
</tbody>
</table>

Oestradiol served as control. Results are presented as the effective concentration values (EC₅₀). For details see text.
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García-Becerra et al. (2002). Even though the different structural characteristics for the α and β subtypes of hER displayed by the tetrahydro metabolites of naturally occurring androgens and those of 19-nor progestins have been studied (Kubli-Garfias et al. 1998, 2002), the understanding of their binding mechanisms still awaits further studies.

The study of enzyme gene expression disclosed that the large formation of diols in MCF-7 cells, with potent oestrogen-agonistic effects mediated via both subtypes of hER, its bioformation, in contrast to 3α,5α-diol, is virtually irreversible (Steckelbroeck et al. 2000). The overall results demonstrated that finasteride inhibits the formation of oestrogenic steroids (diols) generated downstream of DHT, as suggested recently by Ishikawa et al. (2006). In all, this study discloses a distinctive metabolic pathway of androgens in MCF-7 cells leading to a large formation of diols with oestrogen-agonistic activities, providing an insight into the controversial role of testosterone in human breast cancer. The data also demonstrated that pharmacological inhibition of 5α-reductase precludes the bioconversion of androgens to non-phenolic metabolites with oestrogen-like effects, opening a new avenue of approach in breast cancer research and treatment.

Figure 9 Effect of the antioestrogen ICI-182 780 on hER-mediated transactivation induced by androstanediols. HeLa cells transiently co-transfected with expression vectors for hERα or hERβ and an ERE-E1b-CAT reporter gene were incubated with 1 × 10⁻⁷ M 3β,5α-androstanediol (3β,5α-diol), 3α,5α-androstanediol (3α,5α-diol) and 1 × 10⁻⁸ M oestradiol (E2), in the presence or absence of 1 × 10⁻⁷ M ICI-182 780 (ICI). Incubations with ICI alone and with steroid vehicle (DMSO) served as controls. Results are expressed as percentage of CAT activity. Addition of the antioestrogen significantly inhibited (P < 0.001) the transactivation induced by androstanediols and E2, Values are the mean ± s.d. of five experiments performed in triplicate. *P < 0.001 vs DMSO. For details see text.
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