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 5 α -androstane-3 α ,17 β -diol (3 α ,5 α -diol) and its 3 β epimer (3 β ,5 α -diol), the major conversion products of testosterone (48.3%), with 5 α -dihydrotestosterone as intermediary. The formation of 3 α ,5 α -diol and 3 β ,5 α -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 5 α -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 3 β ,5 α -diol and to a lesser extent 3 α ,5 α -diol bind with high relative affinity to hER α and hER β .

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, 3 β ,5 α -diol and to lesser extent 3 α ,5 α -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.

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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 & Brothrick 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

(Poulin *et al.* 1988, Jørgensen *et al.* 1997, Szelei *et al.* 1997, Adams 1998, Labrie *et al.* 2003, Greeve *et al.* 2004, Somboonporn & Davis 2004), though the absence of anti-proliferative effects on several human breast cancer cells has also been reported (Roy *et al.* 1992). Interestingly, several studies have shown that androgens may exhibit an initial inhibitory effect that shift to a late stimulatory effect on human breast cancer cell proliferation (Lippman *et al.* 1976, Hackenberg *et al.* 1993, Labrie *et al.* 1998, Aspinall *et al.* 2004).

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, Lønning 2004, Brueggemeier *et al.* 2005). These observations raise the important question as to whether androgens are locally bioconverted 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 5 α -steroid reductase type-1 gene has been reported in human breast cancer tumours (Suzuki *et al.* 2001).

To determine whether androgens are bioconverted to A-ring-reduced derivatives with intrinsic oestrogen-agonistic potency in breast cancer, we studied the metabolism of [14 C]-labelled testosterone and Δ^4 A 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 3 α ,5 α -diol and 3 β ,5 α -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- 14 C] Testosterone ([14 C]-T), specific activity (sp. act.) 45 mCi/mmol; [4- 14 C] androstenedione ([14 C]- Δ^4 A), sp. act. 57.5 mCi/mmol; [2,4,6,7,16,17- 3 H] oestradiol ([3 H]-E $_2$), sp. act. 148 Ci/mmol, [ring-3,5- 3 H] chloramphenicol, sp. act. 50 Ci/mmol and [3 H] 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 L-glutamine and supplemented with 5% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin and 10 nM oestradiol, in a 95% air:5% CO $_2$ 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 L-glutamine and supplemented with 10% stripped FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin in a 95% air:5% CO $_2$ 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 [14 C]-T and [14 C]- Δ^4 A 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 2×10^6 , at 37 °C, in a 95% air:5% CO $_2$ atmosphere, using the increasing substrate concentrations (1–10 μ M) of either

[^{14}C]-T or [^{14}C]- $\Delta^4\text{A}$, 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 cell homogenates at pH 7.4 and 5.2, in the presence of $2.5\text{ }\mu\text{M}$ 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 (4 $\times$) using three volumes of water-saturated ethyl acetate. The organic extracts were partitioned between petroleum ether and 10% aqueous methanol, and $2.5\text{ }\mu\text{g}$ 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 -androstane-3-one; $3\alpha,5\alpha$ -androstane- $3\alpha,17\beta$ -diol, 5α -androstane- $3\alpha,17\beta$ -diol; $3\beta,5\alpha$ -androstane- $3\beta,17\beta$ -diol; androstenedione ($\Delta^4\text{A}$) 4-androstene-3,17-dione and 5α -dihydroandrostenedione ($5\alpha\text{-A}$), 5α -androstane-3,17-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:acetone, 9:1 and benzene:ethyl acetate, 2:1) and recrystallisations to obtain a constant sp. act. Radioactive labelled [^{14}C] metabolites were located on chromatographic plates using a Packard instant imager (Downers Grove, IL, USA). Radioactivity was otherwise determined in a Packard Tri-Carb liquid scintillation spectrometer model 1900 TR (Packard Instrument Company, Inc.), using toluene containing 4 g/l 2,5-diphenyloxazole (PPO) and 100 mg/l dimethyl 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene as the counting solution. The counting efficiency for [^{14}C] was 86% and [^3H] was 65% and quenching was corrected in all samples by external standardisation. Non-radioactive steroid carriers were detected on chromatograms using the *p*-anisaldehyde-sulphuric/acetic acids reagent. The formation rates of the metabolic conversion products of androgens are expressed as pmol/mg of protein per hour. The enzyme-mediated bioconversion of androgens in MCF-7, MDA-MB 231 and HeLa cells is expressed as the percent conversion of testosterone and $\Delta^4\text{A}$ to their non-phenolic metabolites, as a function of time.

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 (6×10^6) was extracted using TRIzol reagent and an aliquot ($5\text{ }\mu\text{g}$) from each sample was

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 MgCl_2 , 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 $52\text{--}64^\circ\text{C}$ 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'TGGGAGGAGGAAAGCCTATG (sense), 5'GCCACA-CCACTCCATGATTTC (anti-sense); human steroid 5α -reductase type-2 (SRD5A2; NM_000348): 5'CATACG-GTTTAGCTTGGGTGT (sense), 5'GCTTTCCGAGATTT-GGGGTAG (anti-sense); human 3β -hydroxysteroid dehydrogenase (AKR1C1; NM_001353) 5'GTAAAGCTTT-AGAGGCCAC (sense), 5'CACCCATGCTTATTATCGG (anti-sense); human 3α -hydroxysteroid dehydrogenase (AKR1C2; NM_001354) 5'GTAAA GCTCTAGAGGCCGT (sense), 5'CACCCATGGTTCTTCTCGA (anti-sense) and human GAPDH: 5'TTCGCTCTCTGCTCCTCCTG (sense), 5'ACCCGTTGACTCCGACCTTC (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 ER α and ER β genes (plasmid of the cytomegalovirus (pCMV) $_5$ -hER α and pCMV $_5$ -hER β) containing the coding sequence of hER α and hER β 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 3×10^5 cells/well in phenol red-free DMEM-HG supplemented with 5% stripped FBS, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cells were maintained in a 95% air:5% CO_2 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 hER α or hER β

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 dithiothreitol, 10 µg/ml leupeptine and 10 mM sodium molybdate) 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 [³H]-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 (*K_i*) 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 steroidal 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 1×10^6 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 (*R_F* = 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 and recrystallised to constant sp. act. (Table 1). Formation of 3α,5α-diol was higher than that of its 3β isomer. Zone 2 (*R_F* = 0.34) was identified as unchanged [¹⁴C]-T,

Figure 1 Representative chromatographic profiles of the methanolic extracts of MCF-7, MDA-MB 231 and HeLa cells incubated with 2 μ M [14 C]-labelled androgens. Extract aliquots were chromatographed on thin-layer plates. The radioactive zones detected are numbered according to their chromatographic mobility. The major metabolic conversion products of [14 C]-testosterone (T) in MCF-7 cells (A) were 3 α ,5 α - and 3 β ,5 α -androstane diols (diols), while in MDA-MB 231 cells (B) was 5 α -dihydrotestosterone (DHT) and in HeLa cells (C) was androstenedione (Δ^4 A). [14 C]- Δ^4 A incubated with MCF-7 cells (D), was extensively bioconverted to testosterone and diols and to a lesser extent to DHT, whereas [14 C]- Δ^4 A incubated with MDA-MB 231 cells (E) was extensively bioconverted to 5 α -A and DHT. The major metabolic conversion products of [14 C]- Δ^4 A in HeLa cells (F) were 5 α -A, testosterone and DHT. Only unchanged [14 C]-labelled substrates were detected in the extracts from yeast cells, cell-free and boiled inactivated cells, used as negative controls. For details see text.

while zones 3 ($R_F=0.46$) and 4 ($R_F=0.55$) corresponded to DHT and Δ^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 Δ^4 A. The metabolism of [14 C]-T in HeLa cells was characterised by large bioconversion to Δ^4 A and limited formation of DHT and 5 α -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]- Δ^4 A incubations with MCF-7 cells revealed the presence of five radioactive zones (Fig. 1D). Zone 1

($R_F=0.22$) and zone 2 ($R_F=0.34$), representing one the major metabolic conversion products of [14 C]- Δ^4 A, were identified as diols and testosterone respectively. Their radiochemical purity is shown in Table 1. Zone 3 ($R_F=0.46$) had a chromatographic behaviour identical to that of DHT. Zone 5 ($R_F=0.67$) had a chromatographic mobility corresponding to that of 5 α -A and zone 4 ($R_F=0.55$) corresponded to unconverted [14 C]- Δ^4 A. The radiochemical purity of A-ring-reduced metabolites of [14 C]- Δ^4 A is shown in Table 1. In contrast, the metabolic conversion pattern of [14 C]- Δ^4 A in MDA-MB 231 cells (Fig. 1E) was completely different from that observed in MCF-7 cells. The metabolism of [14 C]- Δ^4 A in HeLa cells was characterised by a limited

Table 1 Representative radiochemical purity of isolated metabolites after *in vitro* incubations of human breast and uterine cervical cancer with [¹⁴C]-labelled testosterone (T) and androstenedione (Δ⁴A)

Isolated metabolites from [¹⁴ C]-T incubations			Isolated metabolites from [¹⁴ C]-Δ ⁴ A incubations		
	Successive crystallizations specific activity ([¹⁴ C] dpm/mg)			Successive crystallizations specific activity ([¹⁴ C] dpm/mg)	
3α, 5α-Androstanediol	C ₁ ^a	797	5α-Androstanedione	C ₁	527
	C ₂	658		C ₂	518
	C ₃	597		C ₃	509
	ML ₃ ^b	504		ML ₃	562
3β, 5α-Androstanediol	C ₁	385	Testosterone	C ₁	1285
	C ₂	291		C ₂	1133
	C ₃	214		C ₃	987
	ML ₃	224		ML ₃	915
5α-Dihydrotestosterone	C ₁	741			
	C ₂	660			
	C ₃	697			
	ML ₃	515			
Androstenedione	C ₁	981			
	C ₂	763			
	C ₃	525			
	ML ₃	493			

^aCrystals.
^bMother 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 [¹⁴C]-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 [¹⁴C]-T, even at the highest substrate concentration studied (Fig. 2C–F). A large formation of Δ⁴A 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 [¹⁴C]-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 [¹⁴C]-Δ⁴A 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 Δ⁴A 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 [¹⁴C]-Δ⁴A 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 [¹⁴C]-Δ⁴A. On the contrary, the bioconversion pattern of [¹⁴C]-Δ⁴A 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 [¹⁴C]-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 [¹⁴C]-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 [¹⁴C]-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 [¹⁴C]-T or [¹⁴C]-Δ⁴A, 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 Δ⁴A 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 [¹⁴C]-T and [¹⁴C]-Δ⁴A.

Figure 2 Bioconversion of [^{14}C] testosterone to non-phenolic metabolites in MCF-7, MDA-MB 231 and HeLa cells, as a function of substrate concentration. Incubations were carried out for 2 h at 37 °C, using increasing substrate concentrations, at two different pH levels. The $3\alpha,5\alpha$ - and $3\beta,5\alpha$ -androstenediols (diols) were identified as the major metabolic conversion products in MCF-7 cells, even at the lowest substrate concentration. Diol formation was higher in incubations at pH 7.4 (A), as compared with those at pH 5.2 (B). Androstenedione ($\Delta^4\text{A}$) and 5α -dihydrotestosterone (DHT), in a smaller proportion, were also identified as testosterone metabolites at both pH levels (A) and (B). On the contrary, very little formation of metabolites occurred in incubations of [^{14}C]-testosterone with MDA-MB 231 cells (C) and (D), whereas a large bioconversion of [^{14}C]-testosterone to $\Delta^4\text{A}$ was observed in HeLa cells incubations, at both pH levels (E) and (F). Each point represents the mean $\pm$ S.E.M. of five experiments in triplicate.

Only unmodified [^{14}C]-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 androstenediols to hER

The effect of increasing concentrations of non-radioactive diols upon the [^3H]- E_2 binding to hER in HeLa cells transfected with expression vectors for either hER α or hER β is shown in Fig. 7. The [^3H]- 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 α , the $3\beta,5\alpha$ -diol exhibited higher affinity (RBA = 1.9%; $K_i = 3.25$ nM) than its 3α -isomer (RBA = 0.009%; $K_i = 625$ nM). The $3\beta,5\alpha$ -diol was more efficient competitor for the β subtype of hER (RBA = 0.92%; $K_i = 6.5$ nM) than $3\alpha,5\alpha$ -diol (RBA = 0.006%; $K_i = 950$ nM). The $3\beta,5\alpha$ -diol and to a lesser extent its 3α -isomer, bound to both subtypes

Figure 3 Bioconversion of [^{14}C]-labelled androstenedione ($\Delta^4\text{A}$) to non-phenolic metabolites in MCF-7, MDA-MB 231 and HeLa cells, as a function of substrate concentration. Incubations were carried out for 2 h at 37 °C, using increasing substrate concentrations, at two different pH levels. Testosterone (T) and $3\alpha,5\alpha$ - and $3\beta,5\alpha$ -androstenediols (diols) were identified as the major metabolic conversion products in MCF-7 cells (A) and (B). A larger formation of T and 5α -reduced $\Delta^4\text{A}$ metabolites occurred at pH 7.4 (A), as compared with incubations at pH 5.2 (B). The major metabolic product of [^{14}C]- $\Delta^4\text{A}$ in MDA-MB 231 and HeLa cells (C–F) was 5α - dihydroandrostenedione ($5\alpha\text{-A}$) with very little formation of T and DHT. Each point represents the mean $\pm$ S.E.M. of five experiments in triplicate.

of hER, in a similar manner to that of oestradiol used as positive control, though with lower affinity.

Oestrogen-like transactivation effect of androstenediols

The transactivation effect of $3\alpha,5\alpha$ -diol and its 3β -isomer in the co-transfected cell expression system employed is shown in Fig. 8. Both diols induced the transactivation of hER α -mediated CAT activity, in a dose–response manner, similar to that induced by oestradiol, yet with lower potency (Fig. 8A and B). At steroid concentrations that induced the highest CAT gene transactivation, the oestrogen-like effect of $3\beta,5\alpha$ -diol (1×10^{-7} M) was 100-fold less potent than oestradiol (1×10^{-9} M), whereas $3\alpha,5\alpha$ -diol (1×10^{-6} M) was ten-fold less potent than $3\beta,5\alpha$ -diol as depicted in Fig. 8A and B. The effect of both diols on hER β -mediated reporter gene transcription, as compared to that of oestradiol, is shown in Fig. 8C and D. The highest oestrogen-agonistic transactivation effect of $3\beta,5\alpha$ -diol (1×10^{-7} M) was 100-fold less potent than oestradiol (1×10^{-9} M), while $3\alpha,5\alpha$ -diol

induced transactivation only at the highest concentration employed (1×10^{-6} M). The comparative potencies of both diols and oestradiol, to induce CAT gene transactivation mediated by the subtypes α and β of hER, as judged by their EC_{50} , are depicted in Table 2.

The addition of ICI-182 780 to cell culture incubations, significantly inhibited ($P < 0.001$) the ability of $3\beta,5\alpha$ -diol, $3\alpha,5\alpha$ -diol and oestradiol to stimulate hER α - and hER β -mediated transcriptional activities as shown in Fig. 9. These results ensured the stimulatory activity of diols via both subtypes of hER.

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 [^3H]-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 (1×10^{-7} M) was capable of increasing the proliferation rate

Figure 4 Formation of 5 α -reduced testosterone metabolites in MCF-7, MDA-MB 231 and HeLa cells, as a function of time. Incubations were carried out using 2 μ M [14 C] testosterone, at 37 $^{\circ}$ C, pH 7.4, for different time periods. An early (30 min) bioconversion of testosterone to 5 α -dihydrotestosterone (DHT) and a subsequent large formation of 3 α ,5 α - and 3 β ,5 α -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.

in MCF-7 cells significantly (150%) as compared with vehicle, in a similar fashion to that exerted by 1×10^{-9} M oestradiol (140%). Treatment with 1×10^{-7} M 3 α ,5 α -diol also increased cell proliferation (50%) as compared with vehicle in MCF-7 cells. The effect of oestradiol, 3 α ,5 α - and 3 β ,5 α -diols on MCF-7 cell proliferation was not seen in MDA-MB 231 cells.

Figure 5 Effects of finasteride (F), a 5 α -steroid reductases inhibitor, on androgen metabolism in MCF-7 cells. Incubations were carried out with 2 μ M [14 C] testosterone ([14 C]-T) or 2 μ M [14 C] androstenedione ([14 C]- Δ^4 A), at 37 $^{\circ}$ C, pH 7.4, for 48 h, in the presence or absence of 1 mM finasteride. Addition of the enzyme inhibitor diminished the formation of 5 α -dihydrotestosterone (DHT) or 5 α -androstanedione (5 α -A) and precluded the formation of androstanediols (diols). The data represent the mean $\pm$ S.E.M. of five experiments in triplicate. Location of radiolabelled steroids in chromatographic plates is shown in the inserts.

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 α and β 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 α -steroid reductases, types 1 and 2, and two enzymes of the aldo-keto reductases family, the 3 α -hydroxysteroid dehydrogenase and the 3 β -hydroxysteroid dehydrogenase. The large conversion of testosterone to 3 α ,5 α - and 3 β ,5 α -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 α -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 α -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 α -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 α -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

Figure 6 5 α -Steroid reductases type-1 (SRD5A1) and type-2 (SRD5A2), 3 β -hydroxysteroid dehydrogenase (AKR1C1) and 3 α -hydroxysteroid dehydrogenase (AKR1C2) mRNA expression levels in MCF-7 and MDA-MB 231 cells. The expression of SRD5A1-mRNA is significantly higher in MCF-7 cells (1.45-fold) as compared with those of MDA-MB 231. No differences were observed in the SRD5A2 expression levels in both cell lines. Interestingly, the mRNA expression of SRD5A1 in MCF-7 and MDA-MB 231 cells were significantly higher than that of SRD5A2 expression (4.4- and 3.4-fold respectively). The AKR1C1 expression levels in MCF-7 and MDA-MB 231 cells did not exhibit significant differences, while the mRNA expression of AKR1C2 was higher in MCF-7 as compared with MDA-MB 231 cells. The expression of AKR1C1 in MCF-7 and MDA-MB 231 cells was significantly higher than that of AKR1C2 (2.4- and 3.6-fold respectively). Values are given as relative density ratios. The data represent the mean $\pm$ s.d. of four experiments performed in duplicate. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

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 5 α -steroid reductase activity and also an over-expression of the 5 α -steroid reductase type-1 gene in MCF-7 cells, as compared with oestrogen-resistant breast cells (MCF-10A). Furthermore, Wiebe & Lewis (2003) demonstrated the

Figure 7 Binding affinities of 3 α ,5 α - and 3 β ,5 α - androstenediols (diols) to human oestrogen receptors (hER). Cytosol aliquots of HeLa cells transfected with either hER α or hER β gene expression vectors were incubated with 1 nM [3 H] oestradiol at 4 °C, for 48 h, in the absence or presence of increasing concentrations of radioinert 3 α ,5 α -diol, 3 β ,5 α -diol and oestradiol (E₂). Bound and free steroid fractions were separated by the addition of dextran-coated charcoal. The 3 β ,5 α -diol efficiently bound to both subtypes of hER, though with higher affinity for hER α . The 3 α ,5 α -diol also bound to hER α and hER β , but only at the highest concentration used. E₂, used as the control, was the most potent competitor for α and β subtypes of hER. Results represent the mean $\pm$ s.d. of five experiments in triplicate.

Figure 8 Oestrogen-like transactivation effects of androstanediols. Increasing concentrations of 3 α ,5 α -androstanediol (3 α ,5 α -diol), 3 β ,5 α -androstanediol (3 β ,5 α -diol) and oestradiol (E₂) were incubated with HeLa cells transiently co-transfected with expression vectors for hER α or hER β and an ERE-E1b-CAT reporter gene, for 24 h, at 37 °C. Results are expressed as percentage of CAT activity. Concentrations of 1×10^{-8} M onwards of 3 β ,5 α -Diol efficiently induced hER α - and hER β -mediated transactivation, in a similar manner to that of E₂ (A) and (C). 3 α ,5 α -diol also induced hER α - and hER β -mediated transactivation, yet with lower potency (B) and (D). Values are the mean $\pm$ s.d. of five experiments performed in triplicate. For details see text.

lower expression levels of 3 α - and 3 β -hydroxysteroid dehydrogenase genes in breast cancer tissues as compared with paired breast normal tissue.

Another striking finding of this study was that 3 α ,5 α - and 3 β ,5 α -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 that even the formation of 3 α ,5 α -diol from testosterone was almost double than that of its 3 β -epimer, according to recrystallisation data, the 3 β ,5 α -diol exhibited higher hER-binding affinity and higher oestrogen-like transactivation potency, as compared with its 3 α -epimer, as shown in Fig. 8 and Table 2. Further evidence that diols-induced gene transactivation in the construct assay is mediated via hER α and hER β , 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

hER α by 3 β ,5 α -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 hER α , but not through hER β , 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

	EC ₅₀	
	hER α (mol/l)	hER β (mol/l)
Steroids		
Oestradiol	6.74×10^{-11}	3.37×10^{-12}
3 β ,5 α -Androstanediol	1.61×10^{-9}	1.41×10^{-8}
3 α ,5 α -Androstanediol	9.10×10^{-8}	3.05×10^{-6}

Oestradiol served as control. Results are presented as the effective concentration values (EC₅₀). For details see text.

Figure 9 Effect of the antioestrogen ICI-182 780 on hER-mediated transactivation induced by androstenediols. HeLa cells transiently co-transfected with expression vectors for hER α or hER β and an ERE-E1b-CAT reporter gene were incubated with 1×10^{-7} M 3 β ,5 α -androstenediol (3 β ,5 α -diol), 3 α ,5 α -androstenediol (3 α ,5 α -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 androstenediols and E₂. Values are the mean $\pm$ s.d. of five experiments performed in triplicate. * $P < 0.001$ vs DMSO. For details see text.

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 intrinsic oestrogen-agonistic effects at the genomic level, is the result of enhanced local activities of 5 α -steroid reductases. This is mainly due to an overexpression of SRD5A1 and may have an important physiopathological significance, since these locally produced androgen metabolites are exerting their oestrogen-like intracrine effects in the same cells in which their synthesis take place, without significant diffusion into the circulation (Gingras *et al.* 1999, Labrie *et al.* 2003, Suzuki *et al.* 2005). Although diols could be inactivated via glucuroconjugation, the enzymes responsible for this metabolic process (glucuronosyl-transferases) have not been characterised in the human mammary gland (Labrie *et al.* 2003). This observation is in line with the results of the present study, in which no water-soluble androgen metabolites were found. Furthermore, the role of 3 β ,5 α -diol in breast cancer and other hormone-dependent neoplasias deserves further investigation because, in addition to its potent oestrogen-agonistic effects mediated via both subtypes of hER, its bioformation, in contrast to 3 α ,5 α -diol, is virtually irreversible (Steckelbroeck *et al.* 2004). In addition, 3 β ,5 α -diol may exert anti-proliferative and apoptotic effects in prostate epithelial cells through its interaction with hER β (Weihua *et al.* 2001, 2002). The preliminary results presented herein

demonstrating that 3 β ,5 α -diol and to a lesser extent its 3 α -epimer are capable of inducing cell proliferation in cultured MCF-7 cells, in a similar manner to that of oestradiol, yet with lower potency, suggest the involvement of diols in breast cancer progression, however, further studies are required to have a better understanding on the role of diols in this malignancy. The on-going studies in our laboratories indicate that diols bind with low affinity to the human androgen receptor (hAR) and induce very limited hAR-mediated transactivation, particularly the 3 α ,5 α -diol (A E Lemus, P Damian-Matsumura, R Garcia-Becerra, L Gonzalez, D Orolaz, F Larrea & G Perez, Unpublished observations). The finasteride-induced inhibition of DHT and diols formation in MCF-7 cells, as demonstrated in this study, may have additional interest, since various locally formed A-ring-reduced metabolites of progesterone (Wiebe *et al.* 2000, Wiebe & Muzia 2001, Wiebe & Lewis 2003) are capable of inducing cell proliferation in human breast cancer, through a novel, non-genomic mechanism (Weiler & Wiebe 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 5 α -steroid reductases 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.

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