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Different Conversion Metabolic Rates of Testosterone are Associated to Hormone-sensitive Status and -Response of Human Prostate Cancer Cells

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The main goal of the present work was to compare the ability of human prostate cancer (PCa) cells to metabolize testosterone (T) in living conditions. To this end we studied three different human PCa cell lines (LNCaP, DU145 and PC3) having different hormone-sensitive status and capability of response to androgens. We used an original approach which allows the evaluation of conversion metabolic rates in growing cells after administration of labeled steroid precursor (presently T), at physiological concentrations (1–10 nM). Analysis of both precursor degradation and formation of several products was carried out using reverse phase-high performance liquid chromatography (RP-HPLC) and "on line" radioactive detection. Comparison of the three human PCa cells revealed that their metabolic aptitude differed in many respects: (i) rates of precursor degradation, (ii) different products' formation, and (iii) extent of conjugate production. In detail, PC3 cells quickly degraded T and exhibited high formation rates of androstenedione (A-4-ene-Ad); both DU145 and LNCaP cells mostly retained high levels of unconverted T, with a limited production of A-4-ene-Ad and its 17-keto derivatives (if any). Either LNCaP or DU145 cells generated a relatively high amount of dihydrotestosterone (DHT). In contrast, neither DHT nor its main metabolites were detected in PC3 cells at both short and longer incubation times. As expected, T degradation and A-4-ene-Ad production were highly correlated ($r = 0.97$; $P < 0.03$); similarly, A-4-ene-Ad and DHT formation showed a negative, significant correlation. Negligible production of conjugates was noted in both PC3 and DU145 cells, whilst it was remarkable in LNCaP cells (ranging from 43 to 57%). Overall, our data indicate that human PCa cells degrade T quite differently, favoring alternatively reductive or oxidative patterns of androgen metabolism.

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INTRODUCTION

The human prostate is generally regarded as a prototype of androgen-dependent tissue [1]. In this respect, testosterone (T) conversion to its potent derivative dihydrotestosterone (DHT) through the 5α-reductase (5α-Red) enzyme activity represents a crucial step for androgen-mediated action. Several studies have addressed the question of whether androgens are potentially important in both the development and maintenance of benign and aberrant prostate growth. Previous work, comparing normal, benign and cancerous human prostate, mainly focused on measurements of either intratissue amounts of androgens or levels of key enzymes of androgen metabolism, such as 17β-hydroxysteroid-dehydrogenase (17βHSD) and 5α-Red. Unfortunately, results produced thus far are

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Trivial names: Testosterone, 4-androsten-17β-ol-3-one; dihydrotestosterone, 5α-androstan-17β-ol-3-one; 3α/3β-androstanediols, 5α-Androstan-3α/3β, 17β-diols; androstenedione, 4-androsten-3,17-dione; 5α-androstanedione, 5α-androstan-3,17-dione; androsterone, 5α-androstan-3α-ol-17-one; epiandrosterone, 5α-androstan-3β-ol-17-one; 5-ene-androstenediol, 5-androstene-3β, 17β-diol.

highly controversial, in that very often the intraprostatic amount of individual androgens, as estimated on tissue homogenates, is not in accordance with the extent of the relevant enzyme activities, as measured *in vitro* by the classical enzymology approach. The observation that DHT accumulation in prostate tumor tissues, is related to the subsequent response of patients to endocrine manoeuvres [2] suggests that hormone-responsive tissue might contain higher levels of the 5α-Red enzyme. This assumption, however, was contradicted by other studies indicating that this activity is even decreased in prostatic cancer with respect to both normal and hyperplastic tissues [3].

Whereas the classical enzymology approach fails to provide information on the direction of metabolism and is likely to represent the potential rather than the factual enzyme activity *in vivo*, we have employed an original approach to analyze patterns of steroid metabolism in living cells in log-phase growth [4].

Using this novel reverse phase-high performance liquid chromatography (RP-HPLC) method, with on-line radioactive (RA) detection, which allows the simultaneous sequential measurement of several enzyme activities in intact cultured cells, we have compared pathways of T metabolism in human prostate cancer cell lines (LNCaP, DU145 and PC3), having different androgen receptor (AR) contents and degree of hormone-responsiveness, growth rates and a likely distinct growth regulation [5]. In these model systems, we have been able to analyze, under controlled experimental conditions, rates and direction of T conversion to a range of either conjugate or unconjugated androgen metabolites. Results of these studies are reported herein.

EXPERIMENTAL

Cell culture

LNCaP.FGC (passage 19), DU145 (passage 59) and PC3 (passage 16) human prostate cancer cell lines were all purchased from the American Type Culture Collection (Rockville, MD). For routine maintenance, cells were grown on plastic dishes in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml, 0.25 μg/ml amphotericin B), all from GIBCO BRL (Uxbridge, Middlesex, England), at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were routinely tested for mycoplasma contamination. For all experiments cells having a narrow passage range were used (LNCaP = 21–24, DU145 = 61–63, PC3 = 18–20).

Testosterone metabolism

Methodological approach and procedures used to measure metabolic pathways of steroids in *in vitro* systems have been established previously and optimized [6–8]. Cells growing in log-phase were harvested by trypsinization, counted in a hemocytometer and plated onto 60 mm cell culture dishes at a density of $0.5–1 \times 10^6$ cells/dish. After 24–48 h, cells were washed twice with PBS-A and the medium substituted with FCS-free, phenol red-free RPMI medium. After a further 24 h medium was replaced with the same experimental medium containing $1–5 \times 10^{-9}$ M labeled T ([1,2,6,7-^{3}H(*N*)]T; sp. act.: 92.1 Ci/mmol; DuPont de Nemours Italiana SpA, Milan) as a precursor. Following either 24 or 72 h incubation, medium was transferred to sterile plastic tubes (Costar®, Cambridge, MA) and stored at −80°C until analysis; cells were washed three times using PBS-A and solubilized in 3 ml of 0.1% SDS at 37°C for 15–30 min. Aliquots (100 μl) of cell lysates were therefore used to estimate DNA content, as described elsewhere [9]. For time-course experiments, triplicate dishes of PC3 cells (5×10^5 cells/dish) were incubated in the presence of 1 nM labeled T for 30 min, 2, 8 and 24 h, under exactly the same experimental conditions. Medium and cells were therefore processed as described above.

Extraction procedures

Steroid extraction was carried out on the incubation medium, since it has been shown to contain a proportionally higher amount of radioactive steroids with respect to the cells themselves [10]. To prevent loss of radioactivity, all the glassware was precoated prior to sample manipulation with 4 μg of radioinert T. A 1 ml aliquot of medium was transferred to a scintillation vial to assess the total radioactivity (TR) processed per ml. Androgen extraction was performed at pH 10.0 in glass vials on 1 ml aliquots of incubation medium using 10 ml of diethyl ether. Samples were thoroughly mixed by gentle shaking in a water-bath at 4°C for 30 min and then left to stand for 5–10 min. The aqueous phase was transferred to separate glass tubes and freeze dried in a SVC100H Speed Vac® evaporator–concentrator (Savant Instruments Inc., Farmingdale, NY) for about 2 h. The dried extracts were resuspended using 970 μl of acetate buffer (0.75 M, pH 5.0), additioned with 30 μl of Glusulase enzyme mixture (DuPont Co., Wilmington, DE) and incubated at 37°C for 18 h to hydrolyse steroid conjugates (sulfates and glucuronides). Following incubation, samples were transferred to glass vials and extracted again using 10 ml diethyl ether as before: the resulting aqueous phase was read for non-extracted radioactivity (B), while the ether phase was processed as described below to extract hydrolysed steroids. The two ether phases (free and hydrolysed steroids) were evaporated to dryness under a gentle nitrogen stream at 42°C in a water-bath, resuspended three times with 2 ml acetone and transferred to separate tubes to be desiccated again as before; the empty vials from both phases were additioned with 10 ml of scintillation cocktail (Ready Gel™, Beckman) and counted for radioactivity adhered to walls (A1 and A2) in a β-counter (Beckman Instruments Italia,

Table 1. Relative retention time (RRT) of authentic androgen standard in RP-HPLC

Compound	RRT	Id. No.
16αOH-T	0.35	
19-OH-Ad	0.44	
5-Ene-diol	0.96	
T (i.s.)	1.00	(1)
A-4-Ene-Ad	1.18	(2)
3β, 17β-diol	1.28	
DHEA	1.36	
EpiA	1.58	(3)
DHT	1.66	(4)
3α, 17β-diol	1.75	(5)
5α-Ad	2.09	(6)
A	2.41	(7)

Conditions used for RP-HPLC analysis: Ultrasphere-ODS or Spherisorb ODS-II column (250 × 4.5 mm I.D.); mobile phase: acetonitrile–tetrahydrofurane–0.05 M Citric acid (31:9:60, by vol); flow rate: 1 ml/min. i.s.: internal standard; Id. No.: peak identification number. For abbreviations see text.

Milan). The dried extracts were finally stored at −20°C until chromatographic analysis. Extracts of either free or hydrolysed steroids were resuspended in 30 μl acetonitrile; 20 μl were used for HPLC analysis, while 5 μl were read in a β-counter to measure the radioactivity respectively extracted for each steroid fraction (C1 and C2). The extraction efficiency was finally calculated as follows:

$$\text{Extraction efficiency} = \frac{(C1 + C2) \times 6}{(C1 + C2) \times 6 + (A1 + A2 + B)}$$

Chromatographic analysis

Extracted steroids were chromatographically analyzed in RP-HPLC, using a Beckman model 324 HPLC system equipped with an UV detector (model 160), set at 214 nm, and with an "on-line" Flo-One/beta (model IC) three-channel radioactive detector (Radiomatic Instruments, High Wycombe, England). Steroids were separated under isocratic condition using an Ultrasphere ODS (Beckman) or Spherisorb ODS-II (Aldrich Chimica, Milan) column (250 × 4.6 I.D. mm), thermostated at 20°C. An optimized mobile phase, consisting of acetonitrile–tetrahydrofuran–0.05 M citric acid (31:9:60 by vol), at a flow rate of 1 ml/min was used to separate androgen metabolites in a total analysis time of approx. 30 min. Relative retention times (RRT) for authentic androgens are reported in Table 1. Radiometric detection was performed using a 1 ml flow cell and a Ready-Flow III (Beckman) as scintillation mixture at flow rate of 3 ml/min. Routine data integration was achieved by a Flo-One/beta F1B IC program (Radiomatic, Tampa, FL) and computed in net cpm, after correction for both sample residence time and background subtraction (40 cpm for ^{3}H).

RESULTS

Using physiological concentrations of tritium-labeled precursor administered to living cultured cells, growing in log phase, we were able to measure several enzyme activities of testosterone metabolism in different human prostate cancer cell lines.

After 24 h incubation, AR positive LNCaP and DU145 cells, on one hand and androgen unresponsive, AR-poor, PC3 cells, on the other, displayed divergent patterns of testosterone metabolism (see Table 2).

In fact, T remained mostly unconverted in either LNCaP as illustrated in Fig 1 and DU145 cells, the percent of labeled T being 82.3 ± 2.0 for LNCaP and 86.0 ± 5.7 for DU145 cells. A relatively high DHT formation was also revealed in either cell line, with a higher extent of the reaction being found in DU145 (meanly 9.2 ± 6.3%) with respect to LNCaP cells (meanly 1.8 ± 1.2). Conversely, the production of androstenedione (A-4-ene-Ad) and its 5α-reduced derivative 5α-androstanedione (5α-Ad) was greater in LNCaP (respectively, 11.6 and 3.7%) than in DU145 cells (respectively, 3.1 and 1.2%). In some cases, small amounts of both androsterone (A) and its epimer epiandrosterone (EpiA) were found in LNCaP cells.

In contrast, PC3 cells exhibited high conversion rates of T, the percent of unconverted precursor being only 11.3 ± 2.6 after 24 h. Formation of metabolic

Table 2. Levels of free androgens, precursor ([^{3}H]T) and other products, at 24 h in human prostate cancer cells

	[^{3}H]T	DHT	5α-diol	A-4-ene-Ad	5α-Ad	A	EpiA
LNCaP	9.35 ± 0.55	0.15 ± 0.05	0.10 ± 0.02	1.45 ± 0.25	0.40 ± 0.10	*	*
DU145	10.56 ± 0.75	0.55 ± 0.15	0.09 ± 0.05	0.48 ± 0.10	0.16 ± 0.05	ND	ND
PC3	0.78 ± 0.01	ND	ND	6.14 ± 0.30	2.06 ± 0.16	0.29 ± 0.01	0.81 ± 0.07

Values (pmol/ml) are expressed as mean ± SD of triplicate experiments. Cells (0.5–1 × 10^6) were incubated with 1 × 10^{-8} M labeled T as precursor, in FCS, phenol red-free RPMI medium for 24 h and the incubation medium processed as described in the Experimental section. (*) low amount detected in the free fraction only, when using higher cell number or precursor concentration, namely A (7) = 12 and EpiA (3) = 17 fmol, as shown in Fig. 1.

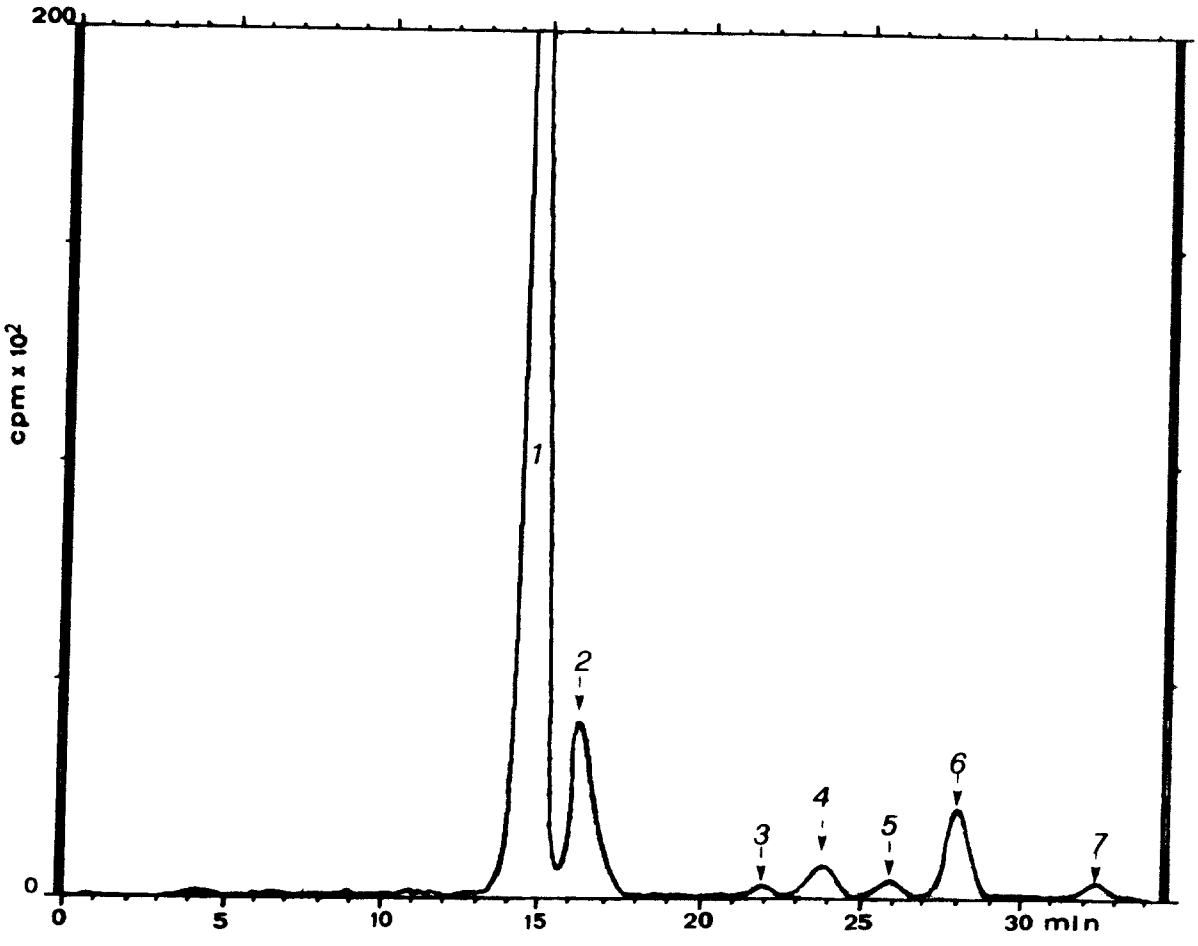

Fig. 2. Androgen metabolic profile in RP-HPLC and RA detection by LNCaP cells (4×10^5), 24 h after [^{3}H]T administration (2.0×10^{-8} M); crude cpm values are reported hereafter: (1) 309,983; (2) 48,608; (3) 1,971; (4) 4,298; (5) 2,592; (6) 13,686; (7) 2,006. For peak identification number see Table 1.

products preferentially followed the oxidative way, with a remarkable conversion of T to A-4-ene-Ad (nearly 70%) and a consequent formation of the 5α-reduced derivatives of the 17-keto series, 5α-Ad, A and EpiA. Overall, these three androgen metabolites accounted for more than 20% of total radioactivity detected by 24 h (see Fig. 2), with a proportional increase (up to 38%) seen after 72 h incubation (not shown). Most importantly, however, no detectable DHT formation was observed by 24 h in PC3 cells; as we have reported previously [11], this was also true at longer exposure intervals (72–96 h, not shown). In order to ascertain whether DHT formation may occur earlier and be masked by its rapid conversion to 5α-Ad, via 17βHSD, and to 3α/3β-androstanediols (3α/3β-diols), we set up time-course experiments using shorter exposures (30 min, 2, 8 and 24 h) in sequence (see Fig. 3). Again, we were unable to find measurable DHT or 3α/3β-diols production at any incubation time, while the prevalence of the oxidative pathway, leading to A-4-ene-Ad formation, was confirmed. In fact, T was increasingly converted to A-4-ene-Ad, an appreciable amount (over 8%) of this metabolite being found after only 30 min incubation. A proportional increase of 5α-Ad was also seen at 8 and 24 h. Formation of A-4-ene-Ad plus 5α-Ad was inversely and significantly related to the proportion of metabolized T ($r = 0.9706$, $P < 0.03$; Spearman correlation test).

Notably, however, a good proportion (range: 43 to 57%) of radioactive androgens was present under conjugate form (either sulfate or glucuronide) in LNCaP cells, mostly (over 90%) as unconverted T. By contrast, very low conjugate formation could be observed, as confirmed by percent values of extraction efficiency in both DU145 and PC3 cells.

DISCUSSION

In the present paper we report multiple sequence analysis of enzymes of T metabolism in LNCaP, DU145 and PC3 human prostate cancer cell lines using a novel chromatographic approach. The latter allows us to measure both precursor degradation and rates of product formation in intact cultured cells, using physiological concentrations of radioactive steroids. Two main distinct patterns of T metabolism have been

observed. AR positive LNCaP and DU145 cells exhibited little oxidation of T to A-4-ene-Ad through the 17βHSD enzyme and a relatively high 5α-Red (T → DHT) activity; by contrast, AR negative, hormone unresponsive PC3 cells displayed extensive 17βHSD oxidation to yield A-4-ene-Ad and subsequent production of the 5α-reduced derivatives of the 17-keto series, while no 5α-reduction of T to DHT could be detected. The latter finding is noteworthy. We have found that conversion of T by the 17βHSD activity in PC3 cells is remarkably shifted in favor of A-4-ene-Ad production (oxidative way), with a consequent formation of 5α-Ad. Although we could not exclude that DHT is quickly oxidized to 5α-Ad via 17βHSD, this seems unlikely in view of the fact that neither short nor longer incubation time revealed measurable DHT formation in this cell line. Additionally, any production of DHT from T is also doubtful because no evidence of DHT derivatives, such as 3α/3β-diols, could be obtained at any incubation time.

There is overall consensus that 5α-Red activity is distinctly decreased in human prostatic carcinoma with respect to both normal and hyperplastic tissues [12, 13]. Surprisingly, in spite of the decrease of 5α-Red, these studies have found unchanged or even higher DHT concentrations in malignant with respect to benign prostate tissues, as like as significantly higher intratumor T levels than in nonmalignant prostate. This finding has been interpreted as a consequence of DHT "trapping" in nuclei of prostate cancer cells through binding to intact AR [13]. This assumption, however, is rather unlikely since either the detected DHT concentrations (in the order of pmol/mg DNA) or the assessed 5α-Red levels (in the order of nmol/mg DNA) are far exceeding the AR binding capacity (in the order of fmol/mg DNA).

It ought to be emphasized that these enzymology studies use artificial environments (cell lysates, pH and temperature, excess of substrate concentration, cofactors addition) and are optimized to yield the maximum conversion rates for quantitation of a single enzyme activity. Therefore, this approach does not allow inspection of enzyme activity in intact cells and hence enzymology data are highly unlikely to resemble the *in vivo* condition. Using our novel "live analysis" system, which allows quantitation of both undegraded

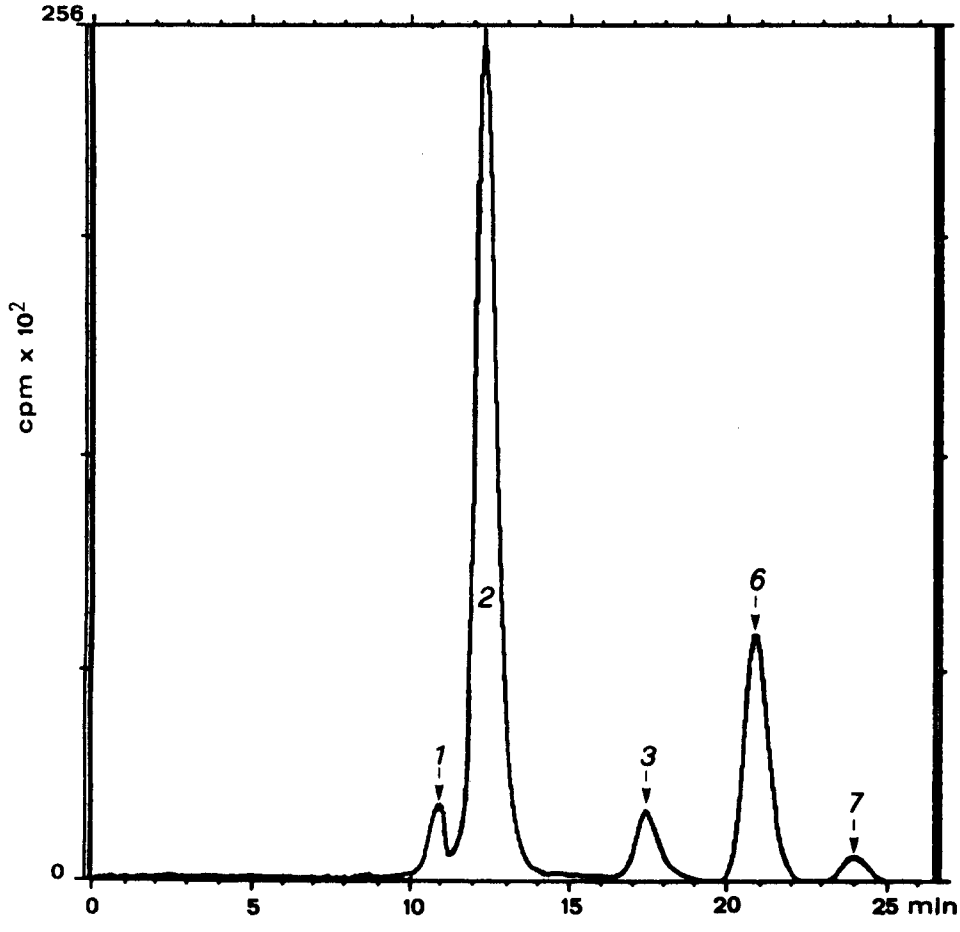

Fig. 2. Androgen metabolic profile in RP-HPLC and RA detection by PC3 cells (2×10^5), 24 h after [^{3}H]T administration (9.8×10^{-9} M); crude cpm values are reported hereafter: (1) 28,687; (2) 236,990; (3) 29,565; (6) 81,262; (7) 10,895. For peak identification number see Table 1.

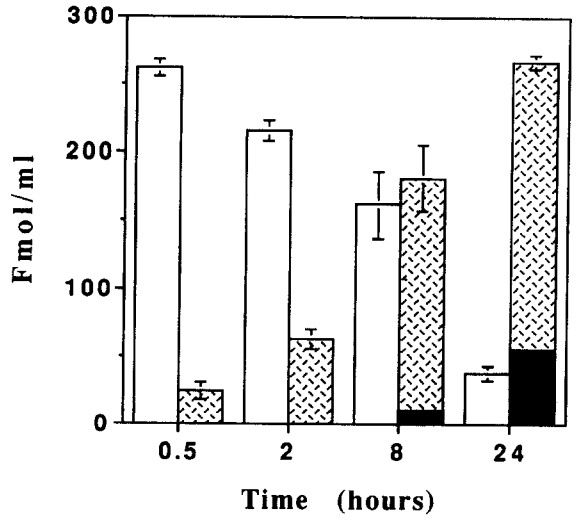

Fig. 3. Time course of T metabolism by PC3 cells (5×10^6) after [^{3}H]T (5×10^{-8} M) administration; open bars: unconverted [^{3}H]T, stippled bars: formed A-4-ene-Ad, closed bars (lower) formed 5α-Ad. Mean ± SD of experiments in triplicate.

substrate and formation of several metabolic products, we are able to encompass the artifacts of classically measured enzymic reactions but, above all, we may gain more information about the metabolic direction in individual tissues and cells. Our present data provide a likely explanation of the previously reported accumulation of both T and DHT in hormone-responsive prostate tumors [2]. In fact, in androgen-responsive LNCaP cells, activities of either 17βHSD or 5α-Red enzymes are directed in such a way that both T and DHT accumulate. Conversely, unresponsive PC3 cells favor oxidative degradation of T to A-4-ene-Ad and its 5α-reduced derivative 5α-Ad, consequently removing the proper 5α-Red substrate for DHT production. DU145 cells behave peculiarly. We previously found that this cell line, in spite of the presence of apparently intact AR machinery, fails to respond to either T or DHT. In the present study, we report that, like LNCaP, DU145 cells poorly metabolize T and form DHT but, unlike LNCaP, they do not produce conjugate androgens, as occurs in PC3 cells. These reported differences between LNCaP and DU145 concerning the amount of formed DHT, may be accounted for by very high levels of conjugate just formed by LNCaP.

The situation is further complicated by the fact that human prostate might contain several types of 5α-Red enzyme [14, 15]. More importantly, Martini *et al.* [16] demonstrated that the rat prostate contains two distinct 5α-Red isoenzymes, having differential sensitivity to both aging and 4-hydroxy-4-androstene-3,17-dione (4OH-A), an inhibitor of both 5α-Red and aromatase prostatic enzymes. The two 5α-reductases individually preside over formation of the 5α-reduced metabolites of the 17-OH series (DHT and 3α/3β-diols) and the 17-keto series. This evidence supports our data suggesting that these two activities may be differently expressed and/or regulated in human prostate cancer cell lines.

Overall, our results consistently indicate that human prostate cancer cell lines, having different biochemical and biological features, are endowed of distinct aptitudes to alternatively favor reductive or oxidative patterns of T metabolism. This different aptness could be relevant in relation to growth control fashion, either *in vitro* or *in vivo*, by PCa cells.

Further studies are on the way to investigate the potential weight of either stromal compartment or local paracrine factors in the regulation of enzyme activities which ultimately direct the metabolic fate of biologically active androgens in these systems.

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