J. Steroid Biochem. Molec. Biol. Vol. 38, No. 2, pp. 155–164, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

MULTIPLE STEROID METABOLIC PATHWAYS IN ZR-75-1 HUMAN BREAST CANCER CELLS

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(Received 2 July 1990)

Summary—In order to characterize the main enzymatic systems involved in androgen and estrogen formation as well as metabolism in ZR-75-1 human breast cancer cells, incubation of intact cells was performed for 12 or 24 h at 37°C with tritiated estradiol (E_2), estrone (E_1), androst-5-ene-3β,17β-diol (5-ene-diol), dehydroepiandrosterone (DHEA), testosterone (T), androstenedione (4-ene-dione), dihydrotestosterone (DHT) or androsterone (ADT). The extra- and intracellular steroids were extracted, separated into free steroids, sulfates and non-polar derivatives (FAE) and identified by HPLC coupled to a Berthold radioactivity monitor. Following incubation with E_2, 5-ene-diol or T, E_1, DHEA and 4-ene-dione were the main products, respectively, thus indicating high levels of 17β-hydroxysteroid dehydrogenase (17β-HSD). When 4-ene-dione was used, on the other hand, a high level of transformation into 5α-androstane-3,17-dione (A-dione), Epi-ADT and ADT was found, thus indicating the presence of high levels of 5α-reductase as well as 3α- and 3β-hydroxysteroid dehydrogenase. Moreover, some T was formed, due to oxidation by 17β-HSD. No estrogen was detected with the androgen precursors T or 4-ene-dione, thus indicating the absence of significant aromatase activity. Moreover, significant amounts of sulfates and non-polar derivatives were found with all the above-mentioned substrates. The present study shows that ZR-75-1 human breast cancer cells possess most of the enzymatic systems involved in androgen and estrogen formation and metabolism, thus offering an excellent model for studies of the control of sex steroid formation and action in breast cancer tissue.

INTRODUCTION

Breast cancer tissue is known to possess steroid metabolizing enzymes. Such data pertain to *in vitro* [1–8] as well as *in vivo* studies [8–10]. For example, the human breast cancer cell lines MCF-7 and ZR-75-1 metabolize 17β-estradiol (E_2) [11–15], androst-5-ene-3β,17β-diol (5-ene-diol) [11, 14–17], testosterone (T) [11, 14, 15], 4-androstene-3,17-dione (4-ene-dione) [14, 15, 18, 19] and dehydroepiandrosterone (DHEA) [14–17]. It becomes evident that the intracellular enzymatic systems are likely to play a major role in the control of intracellular levels of active steroids. In fact, the intracellular concentration of steroids is the most significant parameter of steroid action at the vicinity of the specific steroid receptors while circulating steroid concentrations only possess an indirect biological significance.

Among the enzymes involved in sex steroid biosynthesis and metabolism, 17β-hydroxysteroid dehydrogenase (17β-HSD) plays a cru-

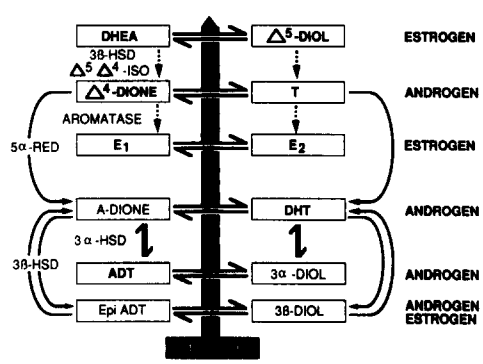

Fig. 1. Schematic representation of the enzymatic activities demonstrated in human breast cancer cell lines ZR-75-1 following incubation with the estrogenic and androgenic steroids and precursors, namely E_2, E_1, 5-ene-diol, DHEA, T, 4-ene-dione, DHT and ADT (bold characters). Note the presence of the four enzymes (———) and the absence of two enzymes (– – – –) in this model.

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Abbreviations: E_2, estradiol; E_1, estrone; 5-ene-diol, androst-5-ene-3β,17β-diol; DHEA, dehydroepiandrosterone; T, testosterone; 4-ene-dione, 4-androstene-3,17-dione; A-dione, 5α-androstane-3,17-dione; ADT, androsterone (5α-androstane-3α-ol-17-one); epi-ADT, epiandrosterone (5α-androstane-3β-ol-17-one); DHT, 5α-dihydrotestosterone; 3α-diol, 5α-androstane-3α,17β-diol; MeO, methoxy; FAE, fatty acid ester(s); RPMI medium, Roswell Park Memorial Institute medium; DCC-FBS, dextran-coated charcoal-treated fetal bovine serum.

cial role since it catalyzes the interconversion of E_2 and estrone (E_1), T and 4-ene-dione, 5-ene-diol and DHEA, 5α-androstane-3,17-dione (A-dione) and 5α-dihydrotestosterone (DHT), androsterone (ADT) and 5α-androstane-3α, 17β-diol (3α-diol) as well as epiandrosterone (Epi-ADT) and 5α-androstane-3β,17β-diol (3β-diol) (Fig. 1). The 17β-HSD enzyme complex is widely distributed in classical steroidogenic as well as in peripheral tissues [20–26].

As a preliminary step to a detailed study of the mechanisms regulating androgen and estrogen biosynthesis and metabolism in breast cancer cells, we have identified and quantified the main metabolic pathways of androgens and estrogens in the well-known ZR-75-1 cell line, including the formation of steroid fatty acid esters (FAEs), glucuronides and sulfates.

EXPERIMENTAL

Chemicals

The following radioactive steroids were purchased from Amersham (Arlington, Heights, IL): [2,4,6,7-^{3}H]E_2 (94 Ci/mmol); [2,4,6,7-^{3}H]E_1 (108 Ci/mmol); [1,2,6,7-^{3}H]T (89 Ci/mmol); [1,2,6,7-^{3}H]4-ene-dione (91.8 Ci/mmol) and [1α,2α(*n*)-^{3}H]DHT (60 Ci/mmol). [1,2(*n*)-^{3}H]5-Ene-diol (60 Ci/mmol), [1,2(*n*)-^{3}H] DHEA (51.5 Ci/mmol) and [9,11-^{3}H]ADT (60 Ci/mmol) were obtained from New England Nuclear (Lachine, Qué., Canada). All radioinert steroids were purchased from Steraloids (Wilton, NH). Organic products were from BDH (Montreal, Qué., Canada). Culture media and supplements and all other chemical reagents were from Sigma (St Louis, MO).

Cell culture and incubation with ^{3}H-labeled steroids

Stock ZR-75-1 human breast cancer cells [27] were obtained from the American Type Culture Collection (Rockville, MD) and routinely grown in Phenol red-free [28, 29] RPMI supplemented as described [17, 30–33]. For studies of radiolabeled steroid incorporation, cells were plated in triplicate in 100 mm Petri dishes at 0.5–1 × 10^6 cells/dish and grown until confluence in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM Na pyruvate, 15 mM Hepes, 0.5 μg bovine insulin/ml, antibiotics and 5% (v/v) DCC-FBS (Hyclone, Logan, UT). The medium was changed every second day. Cells were then incubated for 12 h (E_1, T and 5-ene-diol) or 24 h (other steroids) (these time intervals were found in preliminary experiments to provide the desired spectrum of easily measurable metabolites) in fresh medium of identical composition (except for 12 h where no serum was added) containing the 10^{-10} to 10^{-5} M concentrations of ^{3}H-labeled steroid (added as an ethanolic stock solution to 0.1%, v/v, final concentration). After incubation, medium was removed and cells were quickly washed with 1 × 5 ml of RPMI alone (without serum and insulin) and harvested with 2 ml of 0.05% trypsin/0.02% EDTA (w/v) in Ca^{2+}, Mg^{2+}-free HBSS. The media and cell suspension were transferred separately into 20 × 150 mm glass tubes and frozen in an EtOH/solid CO_2 bath. The cell cultures used for the experiments herein described were between passages 93 and 107. Cell number was determined with a Coulter counter (model ZM).

The labeled steroids studied for metabolism in ZR-75-1 cells were: E_2, E_1, 5-ene-diol, DHEA, T, 4-ene-dione, ADT and DHT. Moreover, the amounts of free steroids, FAEs and sulfates recovered at the end of the isolation procedure were corrected for the losses due to the extraction, partition or solvolysis as calculated from these data.

Analysis of [^{3}H]metabolites

As illustrated in Fig. 2, free steroids can follow at least four general pathways of metabolism by conversion into: (1) another free steroid (see Fig. 1); (2) a FAE after action of steroid acyl COA transferase; (3) a glucuronide; or (4) a sulfate. These different classes of steroids and metabolites were separated by successive partitions from medium and cells treated separately. In previous experiments [15], we have demonstrated that following three extraction steps with diethyl ether, most of the steroids studied are extracted at an efficiency greater than 98%. The solvent partition procedure using hexane/aqueous MeOH 70% (v/v) [34]

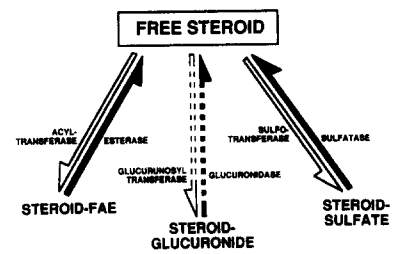

Fig. 2. General metabolic pathways of free steroids in ZR-75-1 cells. The acylation and sulfurylation routes (——) were observed at high levels while glucuronosylation was not detected (– – –).

Table 1. HPLC elution profile of free steroids (estrogens and derivatives)

Steroids	Elution time (min)
2-OH E_2	7.0
4-OH E_2	7.5
2-OH E_1	10.2
4-OH E_1	11.8
E_2	13.0
4-MeO E_2	13.9
2-MeO E_2	15.8
E_1	20.4
4-MeO E_1	22.5
2-MeO E_1	24.9

HPLC was carried out by reverse phase on a Waters Radial-Pak Novapak C-18 column and the solvent system used was 0.05 M citric acid/acetonitrile (60:40, v/v) at a flow rate of 1 ml/min and a scintillation mixture at a flow rate of 2 ml/min.

Table 2. HPLC elution profile of free steroids (androgens and derivatives)

Steroids	Elution time (min)
T	9.6
5-ene-diol	10.1
4-ene-dione	11.0
3β-diol	12.5
DHEA	13.7
Epi-ADT	17.3
DHT	18.4
3α-diol	19.6
A-dione	21.3
ADT	26.1

HPLC was carried out by reverse phase on a Waters Radial-Pak Novapak C-18 column with a mobile phase H_2O/acetonitrile/tetrahydrofuran (65:23:12, v/v/v) at a 1.5 ml/min flow rate and a scintillation mixture at a flow rate of 3 ml/min.

also gave excellent results [15]. However, chemical solvolysis has an efficiency limited to 65% and appropriate correction was made to compensate for these losses. Finally, the composition of the lipoidal fractions of ZR-75-1 was not analyzed in detail since precise information is available in previous reports [14, 15, 35].

The procedure followed is as follows: after thawing, the cell suspension was extracted three times with 10 ml diethyl ether, and the pooled ether extracts were brought to dryness under N_2 prior to redissolution with 70% (v/v) aqueous MeOH, as indicated [15, 34]. Free steroids and their FAEs were then separated by two cycles of solvent partition using hexane/70% aqueous MeOH (1:1, v/v). The hexane extracts were then evaporated to dryness, redissolved in 1 ml hexane and an aliquot (900 μl) was directly counted for radioactivity by liquid scintillation spectrometry with correction for quenching using an external standard. The methanolic extracts containing intracellular free steroids were pooled, evaporated, redissolved in MeOH before analysis by HPLC, as detailed in the next section.

Media were similarly extracted with diethyl ether, and both the ether (mainly extracellular free steroids) and aqueous (mainly sulfates) fractions obtained at this step were evaporated to dryness and redissolved into MeOH. The other extracts were analyzed directly using HPLC (see the next section) while acid hydrolysis was used to release free steroids. Briefly, following evaporation of the aqueous phase and solubilization of the residue with 1 ml of 0.1 M phosphate buffer (pH 6.5), 0.1 ml 12 N HCl was added. After two extractions with 5 ml of diethyl ether saturated with HCl and H_2O, the mixture was left to stand at room temperature overnight. The organic phase was then evaporated and the residue solubilized with 1 ml of 0.2 M phosphate buffer (pH 7) and further extracted twice with 5 ml of diethyl ether/acetone (9:1, v/v). The organic phase was evaporated and analyzed by HPLC under the conditions as described below.

HPLC procedure

Radiolabeled metabolites (intracellular and extracellular free steroids and steroids hydrolyzed from sulfates) evaporated from the organic solvent and redissolved with MeOH/H_2O (1:1, v/v), were injected onto Radial-Pak NovaPak C_{18} reversed phase HPLC columns (8 mm × 10 cm) and eluted with 0.05 M citric acid/acetonitrile (60:40, v/v) at a flow rate of 1 ml/min for the separation of estrogenic compounds (Table 1). The metabolites of androgenic precursors were eluted with H_2O/acetonitrile/tetrahydrofuran (65:23:12, v/v/v) at a flow rate of 1.5 ml/min (Table 2). The HPLC system was a Waters Associates Unit including a model 510 pump, a U6K injector and a C_{18} pre-column. Radioactivity of the eluate was monitored using a Berthold LB506B HPLC Radioactivity Monitoring System with Formula 963 (Dupont–New England Nuclear) as scintillation mixture at flow rates of 2 and 3 ml/min for the estrogenic and androgenic systems, respectively. This HPLC system completely resolves the steroids with retention times as indicated in Tables 1 and 2.

Calculations and statistical analysis

The results are expressed in femtomoles/10^6 cells. Triplicate determinations were made for each sample. In preliminary experiments, concentrations of [^{3}H] precursors ranging from 10^{-10} to 10^{-5} M were used to assess the kinetics of enzymatic activity. A linear relationship was obtained over the whole range of concentrations when the logarithms of the concentrations of precursor and corresponding product were used

Table 3. Extent of conversion of various substrates to free steroids, lipoidal derivatives and sulfates following 12 or 24 h incubation of ZR-75-1 cells

		Intracellular		Extracellular	
		Free steroid	Steroid FAE	Free steroid	Sulfate
Precursor	Metabolite	Relative quantity (%)		Relative quantity (%)	
		(26)		(74)	
E_2	E_2	22 ± 0.11	0.62 ± 0.03	32 ± 0.10	36 ± 0.05
	E_1	2.9 ± 0.03	—	3.4 ± 0.02	2.8 ± 0.005
		(14)		(86)	
E_1	E_1	7.7 ± 0.05	—	45 ± 0.08	11 ± 0.04
	E_2	6.0 ± 0.05	0.73 ± 0.006	24 ± 0.13	6.4 ± 0.03
		(12)		(88)	
5-ene-diol	5-ene-diol	5.3 ± 0.04	2.6 ± 0.02[a]	52 ± 0.07	5.7 ± 0.06
	DHEA	3.8 ± 0.02		30 ± 0.08	0.94 ± 0.01
		(23)		(77)	
DHEA	DHEA	18 ± 0.08	3.9 ± 0.01[a]	57 ± 0.06	13 ± 0.03
	5-ene-diol	1.4 ± 0.006		5.5 ± 0.04	1.2 ± 0.003
		(10)		(90)	
T	T	8.0 ± 0.04	1.3 ± 0.01	74 ± 0.07	1.6 ± 0.01
	4-ene-dione	1.1 ± 0.02	—	14 ± 0.03	—
		(37)		(63)	
4-ene-dione	4-ene-dione	12 ± 0.03	—	31 ± 0.19	—
	A-dione	8.8 ± 0.05	—	19 ± 0.05	—
	ADT	4.4 ± 0.03	ND	3.3 ± 0.02	ND
	Epi-ADT	9.5 ± 0.04	ND	8.2 ± 0.03	ND
	T	1.3 ± 0.01	ND	1.5 ± 0.004	ND
	DHT	1.2 ± 0.007	ND	0.33 ± 0.004	ND
		(27)		(73)	
DHT	DHT	2.4 ± 0.02	7.9 ± 0.03[a]	22 ± 0.06	ND
	3α-diol	6.5 ± 0.02		20 ± 0.05	ND
	3β-diol	3.0 ± 0.01		8.5 ± 0.01	4.1 ± 0.005
	A-dione	1.4 ± 0.01		7.3 ± 0.02	—
	ADT	2.4 ± 0.01		2.9 ± 0.005	ND
	Epi-ADT	3.7 ± 0.02		6.6 ± 0.005	1.2 ± 0.005
		(36)		(64)	
ADT	ADT	14 ± 0.04	13 ± 0.05[a]	21 ± 0.06	ND
	A-dione	1.2 ± 0.008		28 ± 0.24	—
	Epi-ADT	6.7 ± 0.13		7.2 ± 0.15	4.7 ± 0.05
	3α-diol	1.0 ± 0.008		2.8 ± 0.01	ND

[a]Total steroid FAE formed.
( ) Distribution between intracellular content and medium.
ND = not detectable.
Means of amounts formed expressed in percentage of quantity ± SEM (triplicate determinations).

with correlation coefficients between 0.97 and 1 ($P < 0.01$).*

We have chosen to describe metabolism of the substrates at the physiological concentration of 10^{-8} M, although similar results were obtained at all concentrations used. Moreover, the statistical standard deviation of Yh was calculated according to applied linear statistical models [36].

RESULTS

As mentioned above, eight different labeled steroid precursors were used to study the various metabolic pathways in ZR-75-1 cells and thus assess with precision their transformation into active and inactive compounds (Table 3).

(a) Estradiol and estrone

After 24 h of incubation with [^{3}H]E_2 (10 nM), 74% of the radioactivity was found in the medium while the remaining 26% was present in the cells under three forms corresponding to free E_2 (22%), free E_1 (2.9%) and E_2-17β-FAE_1 (0.62%) (Fig. 3A). Following incubation with E_2, esterification takes place mostly at the 17β-position, as reported for various human breast cancer cell lines [15, 37] and mammary tumor biopsies [38, 39]. In addition, among the precursors studied, E_2 was the steroid most extensively transformed into sulfate derivatives. In fact, 36% of total radioactivity was recovered as E_2-sulfate (E_2-S) and 2.8% as E_1-sulfate (E_1-S)

*Except for two products following ADT incubation (extracellular free steroids epi-ADT and epi-ADT sulfate where r^2 are 0.939 and 0.933, respectively and E_2-FAE-17 (from incubation of E_2) has an r^2 of 0.699.

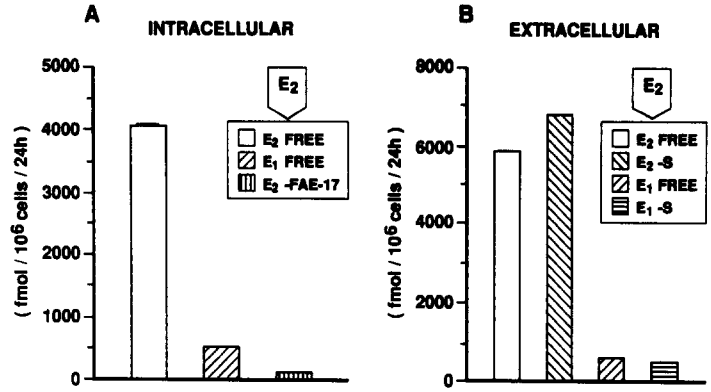

Fig. 3. Distribution of intracellular (A) and extracellular (B) steroids following 24 h of incubation of human breast carcinoma ZR-75-1 cells with 10 nM [^{3}H]E_2. Data are expressed as means ± SEM of triplicate determinations. When no bar is indicated, SEM is smaller than the symbol used.

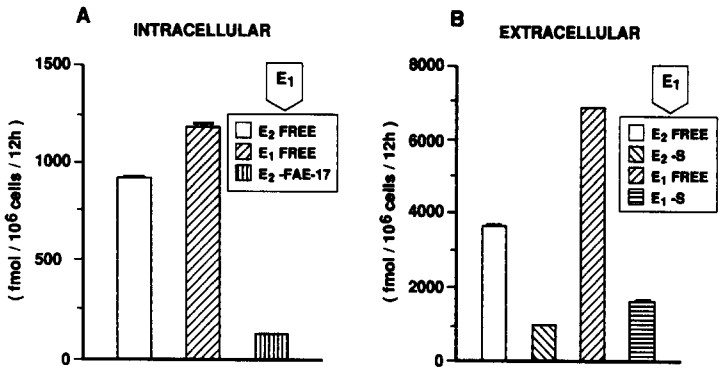

Fig. 4. Distribution of intracellular (A) and extracellular (B) steroids following 12 h of incubation of human breast carcinoma ZR-75-1 cells with 10 nM [^{3}H]E_1.

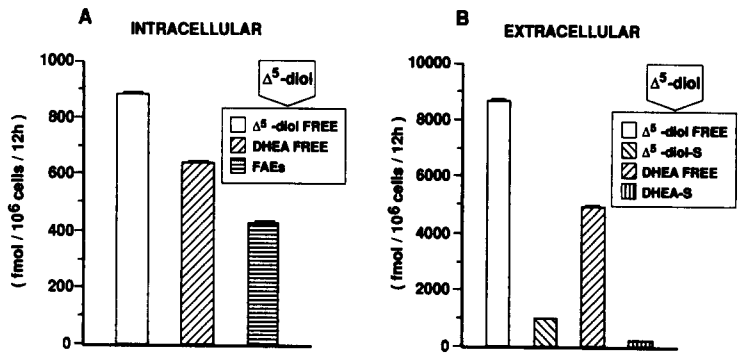

Fig. 5. Distribution of intracellular (A) and extracellular (B) steroids following 12 h of incubation of human breast carcinoma ZR-75-1 cells with 10 nM [^{3}H]5-ene-diol.

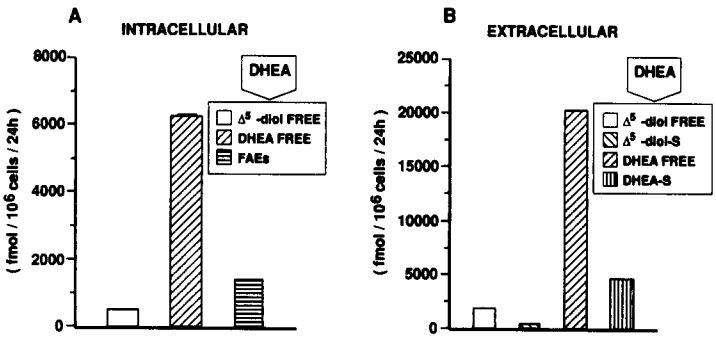

Fig. 6. Distribution of intracellular (A) and extracellular (B) steroids following 24 h of incubation of human breast carcinoma ZR-75-1 cells with 10 nM [^{3}H]DHEA.

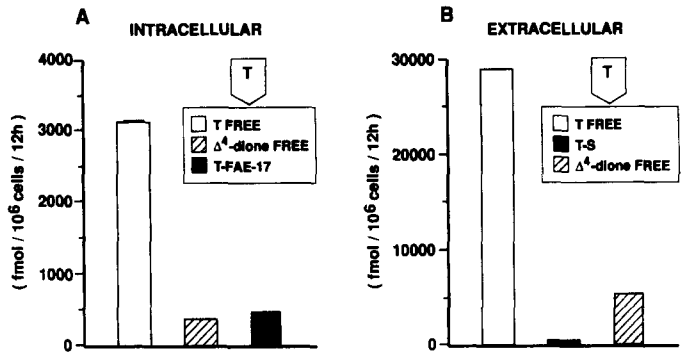

Fig. 7. Distribution of intracellular (A) and extracellular (B) steroids following 12 h of incubation of human breast carcinoma ZR-75-1 cells with 10 nM [^{3}H]T.

(Fig. 3B) at the end of the 24 h incubation, thus indicating the importance of estrogen sulfotransferase in these cells.

On the other hand, following a 12 h incubation period, E_1 was distributed as follows: 14% intracellularly and 86% in the medium (Table 3, Fig. 4). The metabolites measured were the same as those formed after E_2 incubation: E_1 was converted into FAE, sulfates and free E_2 accounting for 0.73, 17.4 and 30% of total radioactivity, respectively.

(b) Dehydroepiandrosterone and androst-5-ene-3β,17β-diol

As seen in Fig. 5, ZR-75-1 cells convert ^{3}H-labeled 5-ene-diol into DHEA, the formation of 5-ene-diol from [^{3}H]DHEA being also present, although at a lesser degree (Fig. 6). The only other detectable transformation is acylation (Figs 5A and 6A) and sulfurylation (Figs 5B and 6B) of the two steroids. As already reported [14], incubation with DHEA or 5-ene-diol led to the intracellular accumulation of DHEA-FAE, 5-ene-diol-FAE_1 (at the 3β- or 17β-hydroxy position) as well as 5-ene-diol-3β,17β-FAE_2. Such data indicate the presence of 17β-HSD [11, 14–17], steroid acyl CoA transferase [14, 15, 40] and steroid sulfotransferase [14, 16, 41] in this human breast cancer cell line.

(c) Testosterone and 4-androstene-3,17-dione

After 12 h of incubation with T, 10% of the steroid was found in the cell while the rest was present in the medium (Fig. 7). The FAE and sulfates identified were exclusively metabolites of T, since the following structural features must be present for esterification by steroid acyl CoA transferase, namely an hydroxyl group at C-3 or 17 in the β configuration [42]. T-FAE-17 accumulation was however low at 1.3% of total radioactivity while 4-ene-dione formation was also low in the medium at 14%, while T-S was only 1.6% of total radioactivity.

When [^{3}H]4-ene-dione was used as precursor (Fig. 8), the pattern of metabolites indicates the presence of particularly high levels of 5α-reductase activity [19] (Table 3). In fact, the 5α-androstane derivatives account for 64.2% of total intracellular radioactivity and 48.7% of extracellular radioactivity. Intracellular and extracellular T were 1.3 and 1.5% of total radioactivity, respectively, thus indicating the presence of 17β-HSD. No lipoidal or sulfate derivates were detected after the 24 h incubation with [^{3}H]4-ene-dione. A-dione, epi-ADT and ADT were the three principal 5α-reduced metabolites formed while 3α-diol and 3β-diol were undetectable after a 24 h incubation period (Fig. 8).

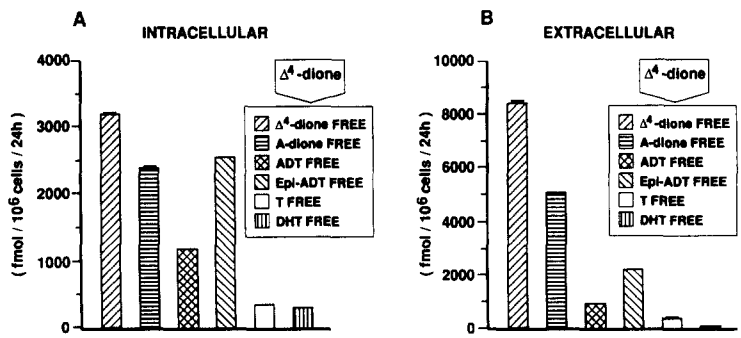

Fig. 8. Distribution of intracellular (A) and extracellular (B) steroids following 24 h of incubation of human breast carcinoma ZR-75-1 cells with 10 nM [^{3}H]4-ene-dione.

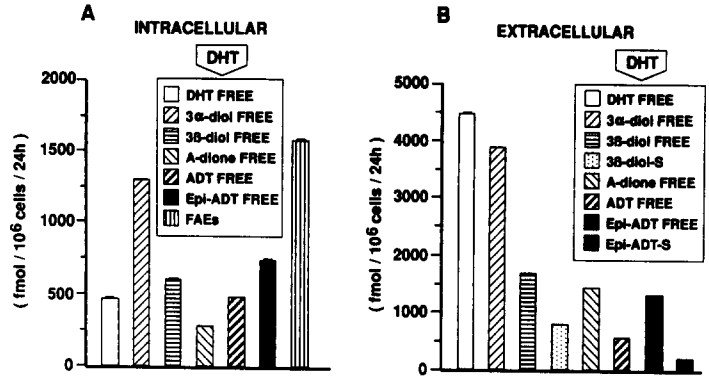

Fig. 9. Distribution of intracellular (A) and extracellular (B) steroid following 24 h of incubation of human breast carcinoma ZR-75-1 cells with 10 nM [^{3}H]DHT.

(d) 5α-dihydrotestosterone and androsterone

As illustrated in Fig. 9, DHT was extensively metabolized via 3β-reduction, 3α-reduction as well as 17β-HSD activity (Table 3). In fact, 62.4% of DHT was transformed into other free steroids while 7.9 and 5.3% of radioactivity was found in FAEs and sulfates, respectively. It should be noticed that transformation of DHT into other metabolites (ADT, epi-ADT) was required before acylation to fatty acids [15]. Only two steroids were substrates for sulfurylation, namely 3β-diol and epi-ADT, thus representing 5.3% of total radioactivity.

With 65% of transformation, ADT was the second best precursor for metabolization, 36% of radioactivity being found in the cells and 64% in the medium (Table 3, Fig. 10). As found following DHT incubation, ADT and its metabolites were esterified to long-chain fatty acids, the major components being ADT and epi-ADT FAEs (90%) [15]. On the other hand, epi-ADT-S accounted for 4.7% of total radioactivity after 24 h of incubation of the ZR-75-1 cells with ADT. The pattern of 5α-reduced metabolites formed is less complex than after DHT or 4-ene-dione incubation. In fact, only A-dione, epi-ADT and 3α-diol were formed in significant amounts. The presence of these metabolites indicates that 3α-hydroxysteroid and 3β-hydroxysteroid dehydrogenases as well as 17β-HSD activities are involved in the metabolism of ADT by ZR-75-1 human breast cancer cells.

DISCUSSION

While the activity of isolated steroid metabolizing enzymes has been described in human breast cancer cell lines [1–19], no detailed information was available on the simultaneous measurement of all main enzymatic activities. Such knowledge is a prerequisite for understanding of the critical steps controlling the intracellular level of active androgens and estrogens as well as the factors which regulate these various enzymatic systems. In fact, recent data strongly suggest that the intracellular concentration of active androgens and estrogens, plays a major role, not only for immediate regulation of cell growth and invasiveness but also for long-term maintenance of hormone sensitivity or development of hormone resistance [43].

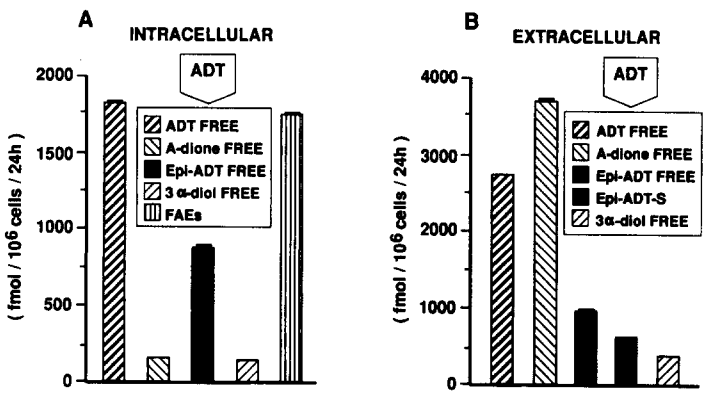

Fig. 10. Distribution of intracellular (A) and extracellular (B) steroids following 24 h of incubation of human breast carcinoma ZR-75-1 cells with 10 nM [^{3}H]ADT.

This study was intended to assess the extent of metabolism of several substrates and, accordingly, prolonged (12 or 24 h) incubation periods were chosen, during which some precursors tested were extensively metabolized, thus providing additional information about preferred metabolic pathways. In the present study, we have investigated the metabolites present in the medium and cells separately since fatty acylation represents only a minor fraction of the total steroids present at any time for a given substrate of extracellular origin. Moreover, lipoidal steroids are exclusively sequestrated inside the cells [14, 37, 40, 44] by virtue of their highly lipophilic character.

Among the notable features of the present work is the wide spectrum of steroids acting as substrates for the formation of lipoidal derivatives in ZR-75-1 cells. A description of the steroid composition of the lipoidal fraction was performed by our group [15], although, at that time, no effort was made to quantitate the various metabolic pathways other than the conversion of steroids into lipoidal derivatives. It has been proposed [42, 45–47] that this enzyme is not specific for E_2 and steroids bearing hydroxyl groups at the 3- and 17-positions are acetylated, thus suggesting a single enzyme with a wide substrate specificity. It should be mentioned that the accumulation of steroid lipoidal derivatives has been described in a large series of tissues, including plasma, breast cyst fluid, adrenals, uterus, corpus luteum and several breast cancer cell lines [14, 15, 35, 37, 48–50]. Although the exact function of liposteroids is not yet known, their potential role as a reservoir of steroids has received experimental support [51].

The present data on the metabolism of T and 4-ene-dione are of special interest. In fact, neither T nor its metabolites, nor 4-ene-dione or its metabolites were aromatized. Similar results for 4-ene-dione have been reported by d'Agata *et al.* [52] and Perel *et al.* [19] and for T, by Rademaker *et al.* [11]. It appears that the MCF-7 cell line, however, contains a low-activity aromatizing enzyme system [19]. Moreover, studies with normal breast tissue did not demonstrate the formation of E_2 from T [53].

As evidenced by the present data, 5α-reductase activity is present at a particularly high level in ZR-75-1 cells. In the present study, the best precursor for the production of the above-mentioned metabolites was DHT with 68% of total radioactivity transformed into A-dione, ADT, epi-ADT, DHT, 3α-diol and 3β-diol. Moreover, ADT and 4-ene-dione have led to almost equivalent amounts of 5α-androstane products (52 vs 55%).

The 5α-reduced steroids can exert either androgen receptor-mediated inhibition [30] or estrogen receptor-mediated stimulation of cell growth [54, 55] as well as strongly antagonize the expression of aromatase activity in human breast carcinoma cells [19, 56, 57]. It was thus found that local estrogen formation can be inhibited by 5α-reduced androgens via inhibition of aromatase activity in breast carcinoma cells at concentrations of 10^{-8} M. There might also be a competition between 5α-reductase and aromatase for substrates as suggested by Abul-Hajj and Kiang [58] in studies on mouse mammary tumors and studies of Berkovitz *et al.* [59] in human skin fibroblasts. Similar observations were reported in MCF-7 cells [19].

The experiments described above clearly show that the human mammary cancer cell line ZR-75-1 catalyzes the metabolism of E_2 to E_1 as well as to its conjugated forms (i.e. E_2-17- FAE_1, E_2-S and E_1-S), thus indicating the presence of 17β-HSD, estrogen sulfotransferase and acyl-CoA:$E_2$17β-hydroxy transferase activities. The major peak was unchanged E_2 (54%), while the second most important was identified as E_2-S (36%). Estrone derivatives (free E_1 and E_1-S) represent about 9.1% of total radioactivity. Such data suggest that sulfurylation may be a major route of estrogen inactivation in these cells [12]. Moreover, E_1 is a weak estrogen that competes poorly with E_2 for the binding to the estrogen nuclear receptor [60] and diffuse out of the target cells more easily than E_2 [61].

As observed in the present study, 5-ene-diol is metabolized more easily than DHEA by 17β-HSD. These data are in agreement with our demonstration of a more potent estrogenic effect of 5-ene-diol than DHEA or DHEA-S on the growth of the same human mammary cancer cell line [16, 17]. Dehydroepiandrosterone conversion into 5-ene-diol is in fact essential for its estrogenic action on cell proliferation since 5-ene-diol, and not DHEA, at physiological concentrations, acts as a genuine estrogen on the ZR-75-1 cells through its direct interaction with the estrogen receptor [17, 54, 62].

In summary, the present study shows that the human mammary cancer cells ZR-75-1 can synthesize androgens and estrogens very efficiently from inactive adrenal steroid precursors. This

area has recently been called intracrinology to describe the formation of hormones that are acting in the same cells where their action takes place [63]. The enzymatic systems involved not only synthesize the active steroids, but also metabolize them into inactive derivatives, thus controlling the level of intracellular active steroids. Moreover, the present data show that the ZR-75-1 cell line is devoid of significant 3β-hydroxysteroid dehydrogenase Δ^5–Δ^4 isomerase activity as well as aromatase activity (Fig. 1).

Acknowledgements—We are greatly indebted to Jean Couture for his technical assistance, to Diane Leroux for her statistical advice and to Mrs Elaine Leclerc and Josée Poulin for their help in the preparation of this manuscript.

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