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17β-Hydroxysteroid Dehydrogenase Activity in Endometrial Cancer Cells: Different Metabolic Pathways of Estradiol in Hormone-responsive and Non-responsive Intact Cells

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In this paper we report that two human long-term endometrial cancer cell lines, Ishikawa and HEC-1A, exhibit quite different abilities in metabolizing estrogens. As a matter of fact, incubation of Ishikawa cells with close-to-physiological concentrations of estradiol (E_2) as precursor resulted in: (1) elevated formation (up to 90%) of E_2-sulphate (E_2-S), using lower precursor concentrations; (2) very limited conversion to estrone (E_1) (< 10% at 24 h incubation), as either free or sulphate; and (3) low but consistent production of other estrogen derivatives, such as 2-hydroxy-estrogens and estriol. Conversely, scant amounts (if any) of E_2-S were found in HEC-1A cells, while no detectable formation of other estrogen metabolites could be observed after 24 h. On the other hand, E_1 production was significantly greater (nearly 60% at 24 h) than in Ishikawa cells, a large proportion of E_1 (over 50% of the total) being formed after only 6 h incubation using time-course experiments. The hypothesis that E_2 metabolism could be minor in Ishikawa cells as a consequence of the high rate of E_2-S formation encountered is contradicted by the evidence that conversion to E_1 also remains limited in the presence of much lower E_2-S amounts, seen using higher molar concentrations of precursor. Overall, we observe that 17β-hydroxysteroid dehydrogenase (17β-HSD) activity diverges significantly in intact Ishikawa and HEC-1A endometrial cancer cells. This difference could not merely be accounted for by the diverse amounts of substrate (E_2) available to the cells, nor may it be imputed to different levels of endogenous estrogens. It should rather be sought in different mechanisms controlling 17β-HSD activity or, alternatively, in the presence of distinct isoenzymes in the two different cell types.

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INTRODUCTION

The 17β-oxido-reductase is a complex system of isoenzymes that catalyzes two-way reactions on several steroid substrates, including estrogens, androgens and progestins. The estradiol (E_2) 17β-hydroxysteroid dehydrogenase (17β-HSD) nomenclature would only identify the oxidative component by which the substrate E_2 acts as a hydrogen donor at C17, being oxidized to estrone (E_1). To this oxidative enzyme component has been ascribed the unique function of regulating the response potential to steroid hormones by target tissues.

17β-HSD has been found to be mostly located in

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the secretory glandular epithelia and in proliferative endometrium [1]; it has also been reported to be induced by progestins, either *in vivo* or *in vitro*, in both normal and cancer tissues and cells [2–4]. The above studies, however, were mainly carried out using crude tissue extracts; only few reports or sparse information exist on 17β-HSD activity in pure epithelial endometrial cells. As an exception, some enzyme studies [5] have inspected 17β-HSD activity in estrogen-responsive Ishikawa endometrial cancer cells, which are commonly being used as a useful model system for *in vitro* hormonal studies; they, however, have never compared it to other non-responsive endometrial tumor cell lines. On the other hand, the emergence of different cell subclones, due to the selective pressures exerted by culture conditions expecially in lengthy cultures, may generate inconsistency of results. A case in point is represented by the Ishikawa-variant 1 cells, showing significant changes in both response to, and metabolism of, E_2 [6].

Armed with this body of evidence, we have investigated E_2 17β-HSD activity in intact responsive (Ishikawa) or non-responsive (HEC-1A) human endometrial cancer cells, using a novel approach to measure rates of both products' formation and precursor degradation.

EXPERIMENTAL

Cell culture

Ishikawa cells (passage 14) [7] were generously given by H. Rochefort (Université de Montpellier, France); HEC-1A cells (passage 126) [8] were obtained from the American Type Culture Collection (Rockville, MD). Cells were routinely grown in MEM medium with 15% fetal calf serum (FCS) (Ishikawa) or in RPMI-1640 medium with 10% FCS (HEC-1A), both supplemented with 2 mM L-glutamine and antibiotics (penicillin 100 UI/ml, streptomycin 100 μg/ml, 0.25 μg/ml amphotericin B), and periodically tested for mycoplasma contamination. Cells having a narrow range of passage number (Ishikawa, 18–24; HEC-1A, 129–134) were used for all experiments.

Hormone response

Cell monolayers (60–70% confluent) were washed twice in phosphate buffered saline-A (PBS-A), harvested by trypsinization and seeded onto 6-well tissue culture plates at a density of 2×10^5 cells/well. After 24–48 h, medium was replaced with phenol red-free medium supplemented with 5% charcoal treated-FCS (CT-FCS), containing 0.01–100 nM E_2 in the absence or presence of 10^{-7} M ICI-182,780, a pure synthetic antiestrogen; control wells received vehicle (ethanol 0.1%) alone. After either 3 or 6 days incubation, medium was discarded and cells counted using a hemocytometer.

Ligand binding assay of estrogen receptors (ER)

Estrogen receptor (ER) content and status of both Ishikawa and HEC-1A cells was determined by means of radioligand binding assay (LBA), as extensively described elsewhere [9, 10]. Briefly, cells were pelleted by centrifugation at 120 *g* for 5 min at 4°C. The cell pellets were gently teflon–glass homogenized on a Potter S system homogenizer (B. Braun Biotech GmbH, Germany). Cell homogenates were spun at 800 *g* for 5 min at 4°C to separate the soluble (supernatant) from the nuclear (pellet) fraction. Aliquots (150 μl) of each cell fraction were incubated overnight at 4°C with increasing concentrations (0.1–5 nM) of [2,4,6,7-^{3}H]17β-E_2 as radioligand. For competition studies, a 100-fold excess of unlabeled diethylstilbestrol (DES) was used. After incubation, dextran-coated charcoal (DCC) absorption and filtration methods were used to separate bound from unbound ligand in soluble and nuclear fraction, respectively. Receptor data were analyzed using the Scatchard method and a modification (©OncoLog 2.2) of a least-square fit routine, yielding both dissociation constant (K_d) and concentration values; the latter were expressed either as fmol/mg protein or DNA for any cell compartment. Data were also analyzed using a model for one or two binding sites, depending on the best fit achieved. This allows identification of both type I (high affinity, limited capacity) and type II (lower affinity, greater capacity) sites of estrogen binding. Protein and DNA cell contents were determined using the method of Bradford and a modified Burton method, respectively.

Cytochemical assay of estrogen and progesterone receptors (PgR)

Presence of both ER and PgR was investigated using a modification of the commercially available Abbott ER-ICA and PgR-ICA kits [10]. Cells grown directly onto 2-well Lab-Tek® Tissue Culture Chamber Slides™ (Nunc, Naperville, IL) were fixed and exposed to goat serum for 20 min at room temperature to block non-specific binding. ER and PgR staining was revealed through a 24 h incubation at 4°C with primary rat monoclonal anti-ER or anti-PgR antibodies (0.1 mg/ml); parallel control wells received normal rat IgG. Cells were then incubated with a bridging antibody (goat anti-rat) and exposed to the rat PAP-complex for 30 min each step. Slides were incubated with solution of the chromogen substrate 3,3′ diaminobenzidine (DAB) for 15 min and finally counterstained for 10 min using 0.2% (w/v) ethyl green. Abbott ER-ICA and PgR-ICA control slides were simultaneously processed and used as positive controls. Estimation of the receptor staining was performed using the CAS™200 Image Analyzer (Becton-Dickinson Italia, Milan), which automatically measures the percentage of positively stained nuclei and the intensity of staining; the latter was defined as the summed optical density for the

positive receptor nuclear area over the summed total optical density of all the nuclei expressed as a percentage. Percentages ⩽30%, from 30 to 60% and >60%, respectively, identified weak, moderate and strong strain intensities.

Estrogen metabolism

Methodological approach and procedures used to assess metabolic pathways of steroids in cultured cells are extensively described elsewhere [11, 12]. Briefly, cells were harvested by trypsinization, seeded onto 60 mm cell culture dishes at a density of 0.5–1 × 10^6 cells/dish and left undisturbed for 24–48 h. The medium was then changed with FCS-free, phenol red-free RPMI medium containing 8–9.8 × 10^{-8} M tritiated estradiol ([6,7-^{3}H(N)]E_2; sp. act. 45 Ci/mmol; DuPont de Nemours Italiana SpA, Milan) as precursor. Following various incubation times (from 1.5 up to 96 h), medium was collected and stored at −80°C until analysis; cells were washed three times in PBS and solubilized using 0.1% sodium dodecyl sulphate (w/v) at 37°C. Aliquots (100 μl) of the cell lysates were used to measure DNA content using a fluorimetric Hoechst 33258 micromethod [13]. Extraction of both free and conjugate estrogens was carried out as previously reported [11] from the incubation medium, since it contains proportionally greater amounts of radioactive steroids than those found in the cells [9, 14]. The dried extracts of either free or hydrolyzed estrogens were stored at −20°C prior to chromatographic analysis. The latter was carried out using a Beckman 324 High Performance Liquid Chromatography (HPLC) model system in the reverse phase (RP-HPLC) mode, equipped with an UV detector (set at 280 nm) and an on-line Flo-One/beta (model IC) three-channel radioactive detector (Radiomatic Instruments, High Wycombe, U.K.). Estrogens were separated in isocratic conditions using a Spherisorb ODS-II (Aldrich Chimica, Milan) column (250 × 4.6 I.D. mm) at 20 ± 0.5°C and an optimized mobile phase, consisting of acetonitrile: 0.5 M citric acid (40:60, v/v), at a flow rate of 1 ml/min. Radiometric detection was performed using a 2.5 ml flow cell and a Ready-Flow III (Beckman) scintillation cocktail at a flow rate of 6 ml/min. Data integration routine was accomplished through a Flo-One/beta F1B IC program (Radiomatic, Tampa, FL) and computed in net cpm, after correction for both sample residence time and background (40 cpm for ^{3}H).

Table 1. Synopsis of human endometrial cancer cell lines

Cell line	Origin	Tumor grade	ER/PgR status	Hormone response
Ishikawa	Primary AdenoCa	G1	+/+	+
HEC-1A	Primary AdenoCa	G2	−/−	−

RESULTS

Table 1 illustrates the general features of the two human endometrial cancer cell lines, Ishikawa and HEC-1A, including their origin, ER status and response to hormones. Results of quantitative immunocytochemistry of both ER and PgR are shown in Table 2. Cytochemical assay clearly indicates that either receptor was under detection limits or at a very low level in HEC-1A cells. This parallels results obtained by measuring both soluble and nuclear ER using LBA (Table 3). In fact, HEC-1A cells displayed type II ER in both cell fractions, whilst limited amounts of type I ER were found in the nuclear fraction only. In contrast, Ishikawa cells showed fair amounts of ER by both immunostaining (Table 2) or LBA (as both type I and II binding, see Table 3); in particular, it is noteworthy that these cells expressed high levels of PgR.

Data concerning the growth response to E_2 by the two cell lines are shown in Fig. 1. Ishikawa cells exhibited a growth increase, at both 3 (60%, $P < 0.01$) and 6 (49% $P < 0.006$) days exposure to E_2, which is equivalent or greater than that previously observed by others using serum-free conditions [15]; conversely, the addition of E_2 did not affect the proliferative activity of HEC-1A cells at any incubation time. The

Table 2. Quantitative immunocytochemical assay of estrogen (ER-ICA) and progesterone (PgR-ICA) receptors in Ishikawa and HEC-1A cells

	ER-ICA			PgR-ICA		
Cell line	Positive nuclei*	Positive stain†	%CV‡	Positive nuclei	Positive stain	%CV
Ishikawa	19.97	37.09	37.74	41.47	73.75	30.31
HEC-1A	0.27	1.51	86.3	0.44	3.10	83.47

*Percentage of positively stained nuclei.

†Intensity of staining, defined as the ratio of positive to total nuclear area and expressed as percentage.

‡%Coefficient of variation measuring the heterogeneity of staining.

Values represent average percentages of three different experiments. For methodological details see Experimental.

Table 3. Radioligand binding assay of type I and II estrogen receptors in soluble and nuclear fractions of Ishikawa and HEC-1A cells

	Type I ER		Type II ER	
	K_d (nM)	fmol/DNA	K_d (nM)	fmol/DNA
Soluble				
Ishikawa	0.32	694.2	7.63	7923
HEC-1A	ND	ND	1.09	1635
Nuclear				
Ishikawa	0.11	245.5	3.52	1645
HEC-1A	0.18	229.7	5.30	1482

Values represent means of four different experiments. K_d, dissociation constant; ND, not detectable. For methodological details see Experimental.

evidence that the E_2-induced growth stimulation of Ishikawa cells is completely abolished by the simultaneous addition of the pure antiestrogen ICI-182,780 clearly suggests that, as expected, E_2 action is mediated via ER; furthermore, the exposure of these cells to ICI-182,780 alone resulted in a slight decrease of basal growth rate.

Figure 2 illustrates radiometabolic profiles obtained in Ishikawa and HEC-1A cells after administration of labeled E_2 (in the range of 10^{-8}–10^{-7} M) for 24 h. As can be seen, HEC-1A cells revealed a high conversion rate to E_1 without any evidence of other radiometabolites; conversely, Ishikawa cells, under exactly the same experimental conditions, showed a very limited conversion to E_1 (less than 10% of all radioactivity detected), but also displayed formation of other radiometabolites, including estriol (E_3) and, though in tiny amounts, 2-hydroxy-estrogens. This suggests that other enzyme activities, specifically 16α- and 2-hydroxylases, other than catecholoxymethyltransferase (COMT) enzymes, are actively working at a comparatively high rate in Ishikawa cells, that is to say in the presence of low substrate concentrations, but never in the HEC-1A cell line, despite the much higher E_1 production observed.

Metabolic patterns of E_2 at 96 h (not shown) are consistent with those seen at 24 h for either cell line. In fact, behavior of Ishikawa and HEC-1A cells remained significantly different in metabolizing E_2. In HEC-1A cells, where the precursor E_2 is almost completely degraded, these longer incubation times did not give rise to any apparent formation of E_1 derivatives; in addition, both precursor degradation and E_1 formation showed a linear behavior in time-course experiments extended up to 24 h incubation (see Fig. 3). By contrast, Ishikawa cells showed a significantly different 17β-HSD activity, by which E_2 is mostly retained unconverted in either cells or experimental media.

It should also be stressed that 17β-HSD activity appears to be 6-fold higher in HEC-1A than in Ishikawa cells up to 12 h of incubation and that thereafter it declines but remains more than 3-fold greater up to 96 h (not shown).

DISCUSSION

The present work provides evidence for a significantly different 17β-HSD activity in two epithelial cancer cell lines from human endometrium, namely Ishikawa and HEC-1A cells. The use of an original approach based on RP-HPLC and radiodetection on line made allowance for simultaneous inspection of both precursor degradation and formation of several metabolic products, as previously reported [16].

More important, this approach eludes several methodological artefacts inherent in classical enzymology studies; they include addition of co-factors, use of

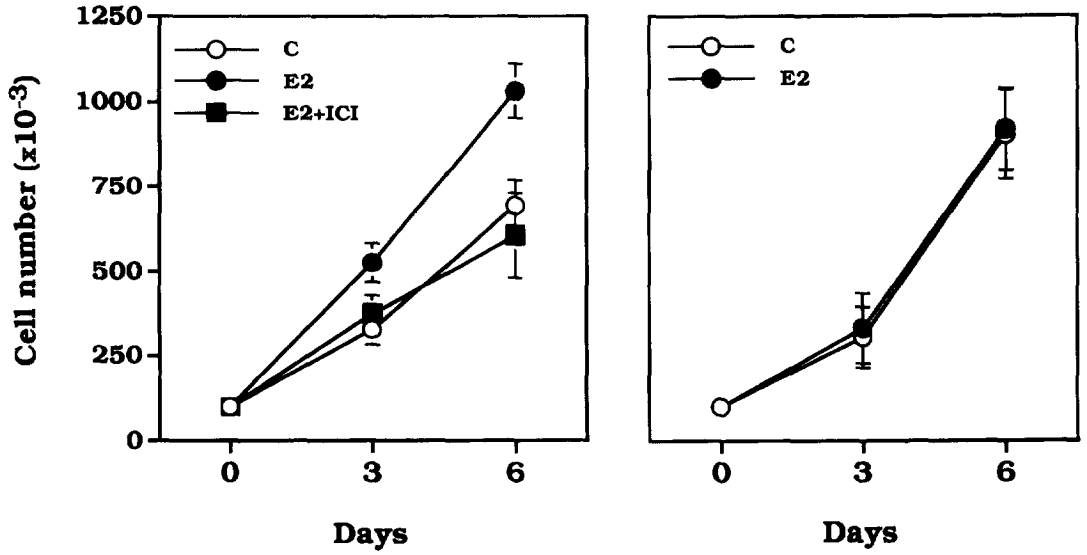

Fig. 1. Effects of estradiol (E_2) ±ICI-182,780 on growth of Ishikawa (left) and HEC-1A (right) cells. Cells were exposed for 3 and 6 days to 10^{-8} M E_2 in the absence or presence of 100 nM ICI-182,780 and cell proliferation measured by counting cells in a hemocytometer. For methodological details see text.

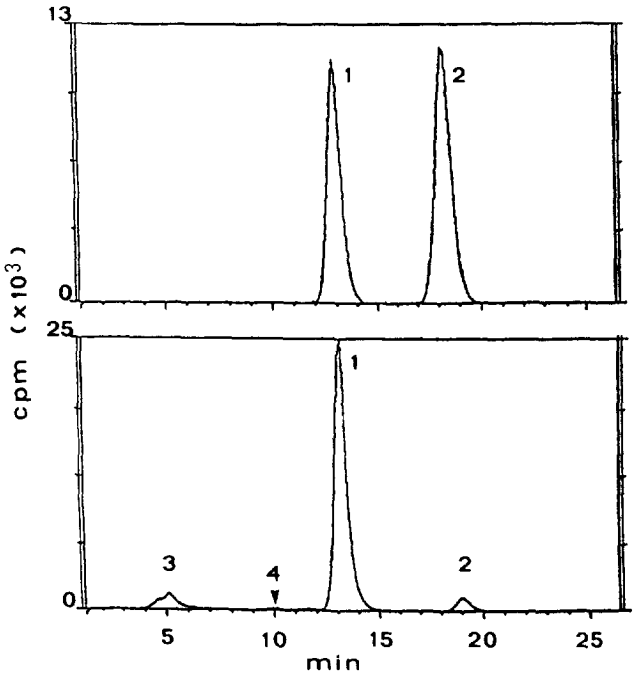

Fig. 2. Radiometabolic profiles of estrogen in HEC-1A cells (upper panel) and Ishikawa cells (lower panel). Cells (1×10^6) were incubated for 24 h in the presence of 9.8×10^{-8} M [^{3}H]E_2 as precursor. Peak identification numbers and crude cpm values are reported hereafter. HEC-1A cells (top): 1 = E_2, 109,740; 2 = E_1, 135,763. Ishikawa cells (bottom): 1 = E_2, 222,096; 2 = E_1, 11,402; 3 = E_3, 13,137; 4 = 2-hydroxy-E_1, 1239.

far-from-physiological substrate concentrations, as well as supernatural pH and temperature values. In particular, thanks to the radioactive detection on line to the chromatographic system, the present method attains very low sensitivity limits ($\leqslant$2.5 fmol) for a number of steroid metabolites; this allows inspection of sequential enzyme activities in intact cells in the log-phase of growth. This is noteworthy, since a given metabolic product may in turn serve as a substrate for a further enzyme activity and hence be rapidly transformed into a potentially important metabolite. It is therefore not surprising that the measurements of 17β-HSD activity using intact cells or enzymology approaches may often conflict.

It has been previously reported that endometrial 17β-HSD activity is substantially greater in the luteal phase, being significantly associated with levels of PgR [17]. Conversely, our results indicate that this enzyme activity, as measured by the formation rates of E_1 from E_2, is much higher (nearly 6-fold) in receptor-negative unresponsive HEC-1A than Ishikawa cells, which are endowed with both ER and PgR. This is true after both 24 and 96 h incubation with physiological concentrations of tritiated E_2 used as precursor.

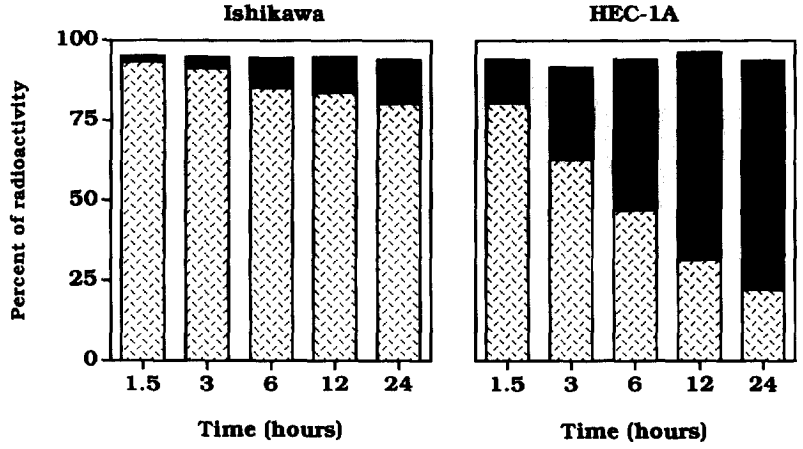

Fig. 3. Time-course experiments of E_2 metabolism in Ishikawa (left) and HEC-1A (right) cells. Cells were incubated in serum-free conditions with 9.8×10^{-8} M [^{3}H]E_2 as precursor for variables times up to 24 h. Percent values of both unconverted [^{3}H]E_2 (dashed bars) and formed E_1 (closed bars) are illustrated.

The discrepancy between *in vivo* classical enzymology and *in vitro* intact cell approaches cannot be accounted for by the presence of stromal compartment in the former, since 17β-HSD activity has been found to be much lower in stromal than in epithelial cells [18]. In our experience, the use of homogenized cells and much higher precursor concentrations, closer to those commonly encountered in enzymology studies, resulted in a reverse pattern, a greater 17β-HSD activity being found in Ishikawa than in HEC-1A cells (not shown). This hints at the assumption that the observed differences are merely methodological.

We report that Ishikawa cells, over 24 h of incubation, convert a limited amount of E_2 to E_1 and concurrently produce noticeable amounts of estrogen conjugates, mostly sulphates. This evidence combines with the presence in these cells of other enzyme activities, including 2- or 16α-hydroxylases and COMT. In contrast, these activities are negligible or even undetectable in HEC-1A cells, where the large amounts of E_1 formed are no further metabolized, at either short or longer incubation times. This model is cognate to that we have previously observed in human breast and prostate tumor cells for metabolic pathways of estradiol [12] and testosterone [11], respectively.

The significant differences observed in the metabolic behavior of these two cell lines appear to associate with both their ER status and response to hormones. The existence of a significant correlation between hormone-sensitivity of cells and their aptness in metabolizing steroids leads us to hypothesize a strict control of steroid enzymes, that eventually regulates levels of biologically active hormones available to target cells.

It has been proposed that these dissimilar metabolic patterns may be simply explained by the fact that the labeled precursor, i.e. E_2, binds its own receptors in Ishikawa cells, being hence limitedly available for metabolism; conversely, E_2 would be more freely accessible to enzyme activities in the ER-poor HEC-1A cells. This concept is however contradicted by the evidence that, using our experimental conditions, the most part of radioactivity (as both precursor and derivatives) is detected in the incubation medium, with recovery values consistently over 90%. In addition, the concentrations of labeled precursor used in our experiments are around 100-fold exceeding those of high affinity sites of estrogen binding in ER-positive hormone-responsive cell lines, including Ishikawa cells [9].

One could also speculate that metabolic rates would be much greater in HEC-1A than in Ishikawa cells due to the significantly different conjugate formation (very high in Ishikawa, little, if any, in HEC-1A cells) we have observed in the two cell lines. Nonetheless, a couple of evidences speak against this possibility. Firstly, sulphokinase activity has been reported to be inversely related to substrate concentration, being higher at 10^{-9} M but much lower at 10^{-7} M, i.e. in our experimental conditions; in addition, conjugate formation appears to be strictly dependent upon culture conditions, varying greatly according to the percent of FCS in the medium (Castagnetta *et al.*, in preparation). Despite this, Ishikawa cells maintain little E_1 formation regardless of both the above variables. Secondly, metabolic rates appear to be unrelated to the amounts of free estrogens (as either unconverted E_2 or produced E_1) in HEC-1A cells, where E_1, once formed, remains mostly as such.

Another conjecture is that diverse isoenzyme activities are sequestered in subcellular compartments, being differently regulated; this could also account for the striking differences observed in measuring enzyme activities in intact or solubilized cells. However, it remains to be clarified whether these activities are controlled by different mechanisms, strictly related to the hormone-sensitivity status of cells or, rather, distinct isoenzymes are operating in the two different cell types.

In this context, it appears essential to combine data gained using molecular biology approaches, which have recently identified five different 17β-HSD enzymes [19; Luu-The Van, personal communication], with the factual enzyme activity measured in living cells.

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