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17β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN CULTURED MYOMETRIAL CELLS: EFFECT OF SERIAL SUBCULTURES

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SUMMARY

17β-Hydroxysteroid dehydrogenase (17β-SDH) activity was studied in culture ovine myometrial cells. After primary culture, cells were routinely subcultured (every 7th day), seeded at $5 \cdot 10^5$ cells per dish and grown in a medium with 2% of serum. 17β-SDH activity was measured by incubating intact cell monolayers with [^{3}H]-estradiol ($5 \cdot 10^{-9}$ M) in serum-free medium. Metabolites were extracted from both cells and medium, and separated by thin-layer chromatography. 17β-SDH was expressed as total E_1 formed (cells + medium) in fmol/mg of protein as a function of time. 17β-SDH has an approximate K_m of $5 \cdot 10^{-6}$ M. After 3 min of incubation, all measurable E_1 is within the cells; it is progressively released but after 1 h only 40% of E_1 is found in the medium. 17β-SDH decreases from day 2 to day 8 of each subculture, whereas total proteins increase. Subculture partially restores 17β-SDH activity so that it is always higher on day 2 of any subculture than on day 8 of the previous one, however a progressive decline occurs with successive subcultures. This decline parallels the slowing of cell growth and overall protein synthesis and probably reflects cell ageing.

INTRODUCTION

Any factor that modifies the intracellular concentration of estradiol (E_2) in its target cells is relevant to the biological activity of this hormone. The enzyme 17β-hydroxysteroid dehydrogenase (17β-SDH) that can reversibly convert E_2 to estrone (E_1) [1] is one of these factors. As E_1 has weaker estrogenic activity relative to E_2 [2], this oxydation can be considered as an important inactivation metabolism in the regulation of E_2 action [3]. The presence of 17β-SDH has been documented in endometrium [4, 5], and myometrium [6, 7] of the human as well as other species such as rat [8] rabbit [9] and monkey [10]. This enzyme is hormone regulated. In human and monkey endometrium, it is clearly stimulated by progestins [11–13]; in the myometrium however, conflicting data exist about its regulation: Tseng and Gurpide[14] find no change in enzyme activity during the luteal phase or pregnancy, whereas Schmidt-Gollwitzer *et al.*[15] clearly show an increase at this period of the menstrual cycle.

We have previously described a culture system of ovine myometrial cells which retains its hormonal sensitivity [16]. In the present study we demonstrate and characterize, in these cells, 17β-SDH activity, a key enzyme in all estradiol sensitive cells. We also report its spontaneous evolution with time in culture and discuss the possible origin and significance of these changes.

MATERIALS AND METHODS

Material

Stock solutions of the following media were obtained under concentrated (10 times) form from Flow Laboratories: medium 199 (modified) with Earle's salts without sodium bicarbonate or glutamine (199); Dulbecco's modification of Eagle's medium without sodium bicarbonate or glutamine (DME). The media were diluted before use with sterile distilled water. Glutamine (200 mM), Pyruvate (100 mM) and sodium bicarbonate (7.5%) were obtained from the same source. Fetal calf serum, mycoplasma and virus screened and filter-sterilized (FCS) and Donor Horse serum mycoplasma and bacteriophage screened (DHS) were decomplemented at 56°C and filtered again prior to use on a 0.20 μ Nalgen filter unit. Trypsin solution (0.25%), lyophilized penicillin–streptomycin mixture and Fungizone were obtained from Gibco. The antibiotics were diluted in sterile distilled water to a concentration of 25.000 U/ml of penicillin, 25 mg/ml of streptomycin and 2.5 μg/ml of fungizone. Porcine insulin (40 IU/ml) was from Novo.

Steroids

[6,7-^{3}H]-Estradiol (50–60 Ci/mmol), [^{14}C]-estradiol (55 mCi/mmol) and [^{14}C]-estrone (52 mCi/mmol) were purchased from Amersham, and purified on thin-layer chromatography before use. Non radioac-

tive steroids (Sigma) were crystallized before use and stored at 5 mg/ml solutions in methanol.

Cell cultures

Primary cell cultures were prepared from ewe myometrium as previously described [16] and frozen at −70°C, after one or two subcultures in 1 ml aliquots containing $2 \cdot 10^6$ cells in a medium consisting of 80% 199/DME (v/v), 10% FCS and 10% dimethylsulfoxide, in Nunc 2 ml plastic freezing vials.

Subsequent subcultures. Cells were rapidly thawed at 37°C and seeded at 5×10^5 cells per Falcon Petri Dish (diameter 100 mm) in 10 ml medium 199/DME (v/v) buffered with sodium bicarbonate (0.22%) and supplemented with glutamine (1.6 mM) pyruvate (1 mM), Insulin (160 U/l) and antibiotics (0.2%). For 24 h after thawing, cells were kept in medium containing 10% FCS. Thereafter, routine culture medium contained 2% of a mixture (v/v) of FCS and DHS. Cells were grown at 37°C in a humid atmosphere of 95% air and 5% CO_2. Medium was renewed every second day till cells reached confluency (approx. 7 days). They were then harvested by trypsinization, diluted, and seeded again at $5 \cdot 10^5$ cells per 100 mm dish or $2.5 \cdot 10^5$ cells per 60 mm dish. Cells were studied from the 2nd to the 13th subcultures included, and frozen thereafter for further studies.

17β-Hydroxysteroid dehydrogenase assay

Assay was performed in intact monolayers, each point representing one large (100 mm) culture dish. Before incubation, cells were washed with 5 ml of serum-free medium. They were then incubated for various periods of time with 2 ml of serum-free medium containing $5 \cdot 10^{-9}$ M [^{3}H]-estradiol ($\sim 10^6$ d.p.m.) in 0.5% ethanol. At the end of incubation time, dishes were put on ice to stop the reaction. After withdrawal of an aliquot for radioactivity counting, medium was extracted with 10 ml of cyclohexane–ethylacetate mixture (1/1) containing [^{14}C]-E_2 (6000 d.p.m.) and [^{14}C]-E_1 (1200 d.p.m.) to monitor recovery. After vigorous vortexing, the aqueous phase was rapidly frozen to allow easy separation from the organic phase which was evaporated to dryness. Cells were washed 3 times with phosphate buffered saline (PBS), harvested in PBS by scraping with a rubber policeman and centrifuged 5 min at 600 *g*. The cell pellet was suspended in 3 ml ethanol containing [^{14}C]-E_2 (1800 d.p.m.) and [^{14}C]-E_1 (360 d.p.m.) to monitor recovery. After 30 min standing at room temperature with vortexing every 5 min, cells were centrifuged at 5000 rev./min for 10 min. The supernatant was evaporated to dryness (cell extracts) and the pellet dissolved in 0.5 M perchloric acid and frozen for DNA or protein assay.

Two types of blanks were performed with each experiment: [^{3}H]-E_2 ($5 \cdot 10^{-9}$ M) was incubated in cell-free dishes with either fresh medium (2 ml) or medium removed from the cells at the beginning of the experiment. This last type of blank was to insure that no enzyme activity was released from the cells. After incubation, medium was extracted as above.

Thin-layer chromatography

Extracts (medium and cells) were dissolved in methanol and chromatographed on silica gel thin-layer plates (Merck HF 366-254-60) prepared to 200 μm thickness (Desaga apparatus). Plates were run in chloroform/ethylacetate (80/20). Unlabelled E_2 and E_1 were added to the extracts and visualized under u.v. light. E_1 has an R_f of 0.70 in this system and E_2 0.40. Area corresponding to these steroids were eluted into counting vials with 4 ml ethylacetate which was evaporated to dryness; each vial received 5 ml of scintillating solution (OCS, Amersham) and was counted in a Nuclear Chicago ISOCAP 300 with an efficiency of 45% for ^{3}H and 66% for ^{14}C; quenching was determined by the internal standard method; 17β-hydroxysteroid activity was expressed as total E_1 formed (cells + medium) in fmol/mg of protein as a function of time.

Proteins and DNA assays. DNA was assayed after

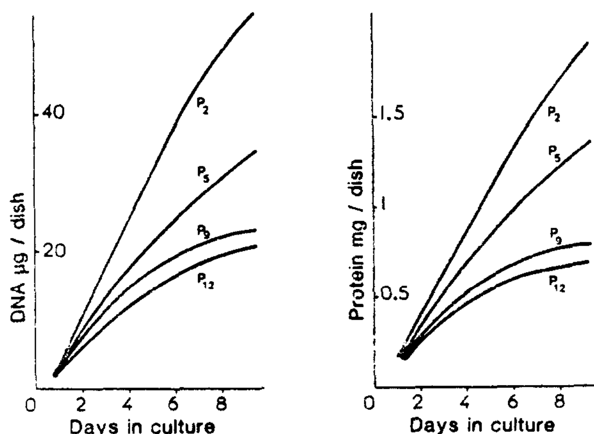

Fig. 1. Growth curves of cells from ovine myometrium in successive subcultures (P_2–P_{12}). Cells were collected in PBS every day from day 1 (24 h after subculture) to day 9. DNA and proteins were assayed on three pooled or separate dishes from 2 different subcultures. The doubling time was about 48 h.

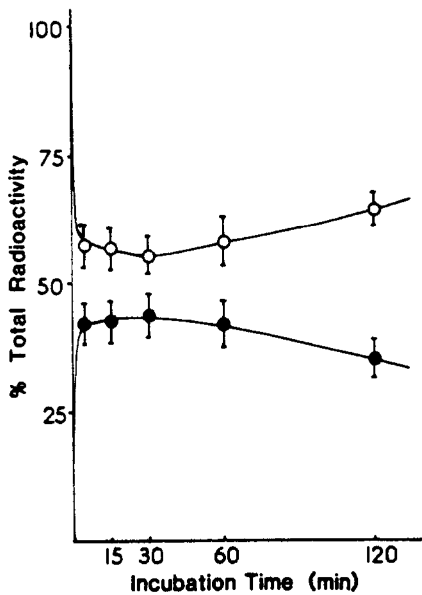

Fig. 2. Time course of estradiol uptake at day 5 of a subculture. Intact monolayers were incubated with [^{3}H]-E_2 for times ranging from 3 to 120 min. Results are expressed in percent of total radioactivity measured in medium (O——O) and cells (●——●). Each bar represents the mean ± SD of 3 dishes from different subcultures.

perchloric acid hydrolysis (70°C 30 min) either by the diphenylamine method of Burton[17] or by u.v. absorption at 265 nm. Proteins were assayed according to Lowry[18].

RESULTS

(1) *Cell growth in successive subcultures*

Figure 1 shows the evolution of the DNA cell-content as a function of time with successive subcultures. Cell division progressively slows down with each subculture, so that 5 days (d_5) after subculture there was approximately twice as many cells per dish after the second subculture (P_2) and compared to the 9th one (P_9). The difference was even greater at d_9. The same applies to the protein content of the cells although the differences are somewhat less important (Fig. 1).

(2) *Time course of E_2 uptake by the cells*

After 3 min of incubation 45% of the total radioactivity has disappeared from the medium (Fig. 2). The radioactivity of the medium remains stable throughout the following hour, and increases thereafter, indicating a progressive release from the cells. After 24 h (not shown) 80% of the total radioactivity is again found in the medium. In view of these data, cells have been routinely incubated 30 min for 17β-SDH assay unless otherwise stated.

(3) *Kinetics of E_1 formation*

Figure 3 shows the kinetics of E_1 formation after incubation of cells with [^{3}H]-E_2 for periods of time ranging from 2 to 120 min. The reaction was linear for the first 40 min; when incubations were continued for longer periods of time, E_1 formation kept increasing; after 24 h, E_1 represented 50% of the total radioactivity, however, a plateau was not yet reached at this time (not shown). In control dishes (blanks) E_1 formation was always non significant as compared to that obtained in the presence of cells.

(4) *Determination of apparent* K_m

Cells were incubated for 5, 10, 20 and 30 min with E_2 concentrations ranging from $5 \cdot 10^{-9}$ M to 10^{-5} M. Unlabelled E_2 was added to reach high substrate concentrations and specific activity was calculated for each point. Data were analyzed on a Lineweaver and Burk plot. The apparent K_m calculated was around $5 \cdot 10^{-6}$ M (Fig. 4). However cells were routinely incubated with $5 \cdot 10^{-9}$ M [^{3}H]-E_2 which is a physiological concentration; we have previously checked that with this substrate concentrations increasing enzyme concentration, in the range used, resulted in increased formation of E_1 suggesting that the substrate was not limiting.

(5) *Comparative activity as measured in cells and medium extracts*

Figure 5 shows the proportion of E_1 extracted from cells or medium as a function of time. After 3 min of incubation almost all measurable E_1 formed is in the

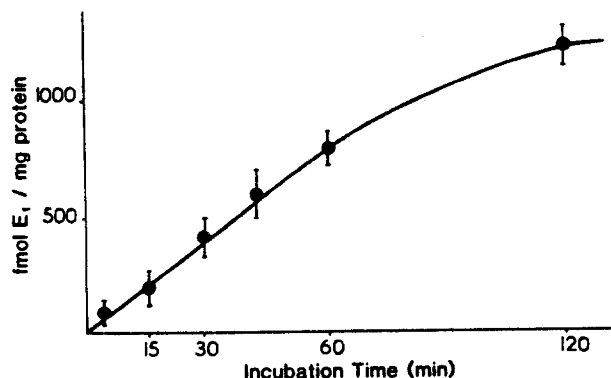

Fig. 3. Kinetics of E_1 formation in cultured myometrial cells on day 5 of a subculture. Each point is the mean of triplicate incubations of intact cell monolayers.

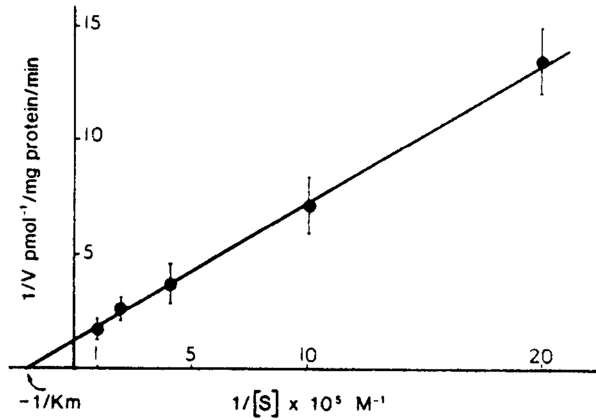

Fig. 4. Lineweaver–Burk plot of initial velocity of estradiol oxidation versus different concentrations of E_2 in cultured myometrial cells.

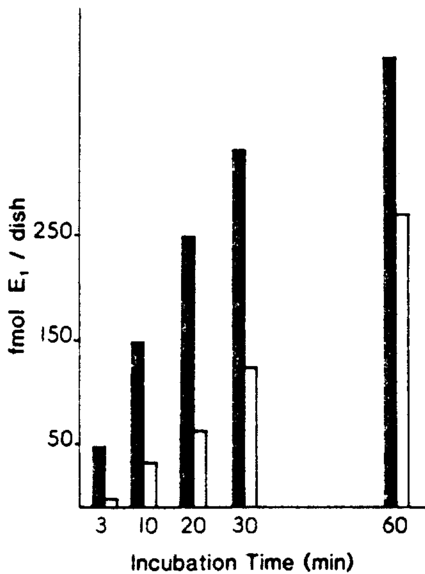

Fig. 5. Effect of incubation time on E_1 distribution. Results are expressed as fmol of E_1 per dish present in either the cells (■) or the medium (□).

cell extract; with increasing incubation times, E_1 is progressively released in the medium. However, E_1 amounts are always higher in the cell extracts than in the medium. This suggests a retention of E_1 in the cells.

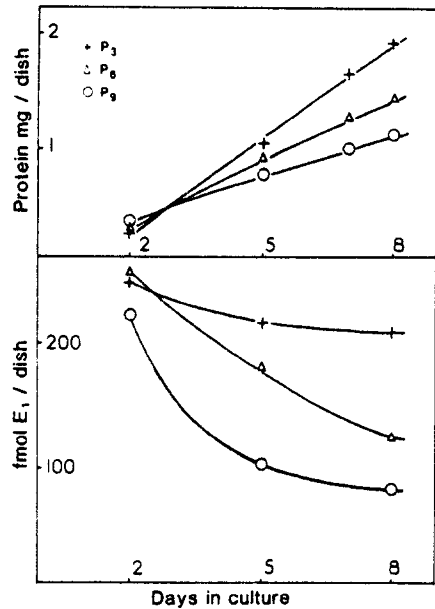

Fig. 6. Evolution of the 17β-SDH activity in cultured myometrial cells with time in a given subculture. Assays were performed on days 2, 5, 8 of the third (×) sixth (Δ) and ninth (O) subculture (P). The protein content of cells for each determination is represented above. Each point is the mean of triplicate dishes.

(6) *Evolution of 17β-SDH with time in culture*

(a) *During any given subculture.* 17β-SDH decreased with time (Fig. 6) whether expressed as total E_1 formed per dish or as fmol E_1/mg protein/min. In contrast, in the same cells, total proteins increased demonstrating the vitality of the cells and the integrity of the protein synthesis mechanisms.

(b) *During successive subcultures.* Cells have been studied from the 2nd through the 13th subculture; the following can be observed: (1) in all subcultures, 17β-SDH activity decreased from d_2 to d_8 (Fig. 6); (2) transfer apparently increased 17β-SDH activity so that it was always higher on d_2 than on d_8 of the previous subculture; (3) when 17β-SDH activity was

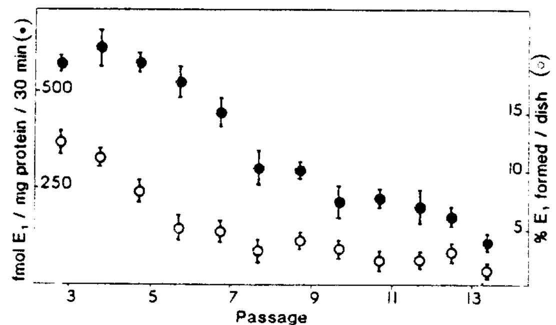

Fig. 7. Evolution of the 17β-SDH activity of myometrial cells over serial subcultures. Assays were performed on day 5 of each passage (P). Each point is the mean of six determinations (triplicate from two series of cultures).

Table 1. Evolution of 17β-SDH activity with time in a given subculture, as measured on cell suspensions containing the same amount of cells

Day	fmol E_1 formed in 30 min	% of total radioactivity from day 2
2	380 ± 12.5	100
5	200 ± 15..	53
8	172 ± 6	45

The number of dishes for each time point was adjusted to achieve the same number of cells and protein concentration.

compared on the same day (d_5) of successive subcultures, a progressive decline of enzyme activity was observed (Fig. 7).

The same phenomena can be observed when incubations are performed on cell suspensions containing the same number of cells for each time point (Table 1).

DISCUSSION

We have demonstrated in this paper, a very active conversion of E_2 to E_1 by a 17β-hydroxysteroid dehydrogenase in cultured ovine myometrial cells. However, this activity progressively decreases with successive subcultures so that it is 10 times higher at early subcultures than at the 12th subcultures. The variations of 17β-SDH activity as a function of time were similar whether expressed as % E_1 formed relative to total radioactivity ($E_1 + E_2$) or as fmol E_1/mg P. Incubations were performed for 30 min because the reaction was linear during this time, and $E_1 + E_2$ represented more than 98% incubated E_2. However, this does not hold true for longer incubation time when other metabolites appear; these metabolites mainly chromatograph on thin-layer as 16-epiestriol or non polar metabolites; 16-epiestriol has also been identified as one of the main metabolites of estradiol in human myometrium [6].

We have expressed 17β-SDH activity as the total activity measured in both cells and medium. Indeed after 30 min of incubation, about 65% of E_1 formed is still retained in the cells and any measure of enzyme activity in medium alone would result in gross underestimation. This retention of E_1 within the cells is in agreement with the observation of Schmidt-Gollwitzer *et al.*[15] that E_1 is the main estrogenic hormone in the myometrium during the luteal phase.

17β-SDH has been studied in the uterus of many species including rat [8], rabbit [9], monkey [10] and human [4, 5] as well as in other tissues such as placenta [1], ovaries [19], prostate [20] and breast cancer [21]. However the highest enzyme activity is found in the endometrium where most studies have been performed [3–5, 10–13]. Enzyme activity has nevertheless been found in the rabbit [9] and human myometrium [7]. The apparent K_m values obtained in those tissues are around $7 \cdot 10^{-6}$ M and $1 \cdot 10^{-6}$ M respectively; this is very similar to the apparent K_m values obtained in ovine myometrial cells. However we should keep in mind that our studies have been performed on intact cells and not on purified enzymes: whereas this system realizes physiological conditions, it may lead to inaccuracy where enzyme kinetics are concerned.

Whereas cells continue to grow and multiply between d_2 and d_8 of a given subculture (as shown by DNA and protein content), 17β-SDH activity decreased progressively. This decrease was observed after monolayers incubation (where the number of cells incubated varied from day to day) or after cell suspensions incubations (where a constant number of cells was incubated whichever the day). After each transfer however, 17β-SDH increases again so that values obtained on d_2 of a given transfer are always higher than those obtained at d_8, though slightly lower than those obtained at d_2 of the previous subculture. For the moment we can only propose tentative explanations for this phenomenon: 17β-SDH is NAD(P) dependent; if, in course of cell growth another NAD(P) enzyme system develops which either increases faster or has a higher affinity than 17β-SDH for the cofactor, then NAD(P) could become limitant to 17β-SDH activity. Alternatively, some cell metabolite could accumulate and act as an inhibitor of 17β-SDH. In both cases, the transfer would result in dilution of either the competitor enzyme system of the inhibitor, and 17β-SDH activity is restored.

17β-SDH activity also decreased when compared at the same day of successive subcultures. This is in contrast with what has been described in other cell systems, in particular for the 5α-reductase activity of skin fibroblasts [22, 23]. It may be of interest to note that whereas skin fibroblasts are usually cultured with 10–20% fetal calf serum, we use 5–10 times lower serum concentrations. This may result in a more rapid ageing of the cells which could be responsible for the overall decline of enzyme activity observed with successive subcultures.

In conclusion, ovine myometrial cells in culture contain 17β-SDH activity as other estradiol target cells. However this activity decreases with time in culture. The decline observed in a given subculture is reversible and may reflect a change in cofactor or enzyme equilibrium; the decline observed in successive subcultures parallels the slowing of cell growth and overall protein synthesis and probably reflects cell ageing.

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