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17β-ESTRADIOL DEHYDROGENASE (E2DH) ACTIVITY IN T47D CELLS

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Summary—Activity of NAD-dependent 17β-hydroxysteroid dehydrogenase (E2DH), the enzyme which converts estradiol (E2) into its less active metabolite estrone (E1), has been previously characterized in normal human breast cells in culture and in benign and malignant breast tumors. E2DH activity is far greater in epithelial cells than in fibroblasts. Moreover, it is progesterone dependent in epithelial cells. It was therefore interesting to explore E2DH in the progesterone receptor (PR)-rich T47D cell line as a possible marker of hormone dependence in breast cancer cells. In T47D cells, transformation of [^{3}H]E2 to E1 is limited. The metabolism seems to be preferentially oriented in the way E1→E2 in these cells. However, in the presence of the cofactor NAD the conversion of E2 into E1 increases. Moreover, treatment of T47D cells in culture by the progestin R5020 stimulates E2 to E1 conversion 2- to 3-fold. Stimulation of E2DH (E2→E1) activity reflects both the presence and the operability of PR. This observation underlines the possible interest of E2DH assay in parallel to estradiol receptor and PR to evaluate hormone-dependence of breast cancer.

INTRODUCTION

Estradiol receptor (ER) and progesterone receptor (PR) are usually considered to be good markers of hormone-dependency in breast cancer. Tumors containing ER and PR (ER+, PR+) are much more likely to respond to endocrine therapy than ER−, PR− tumors [1, 2]. However, certain ER+, PR+ tumors do not respond to endocrine therapy and receptors are not totally reliable markers of tumor sensitivity [2]. Several other markers have therefore been proposed [3–11]. The NAD-dependent enzyme 17β-hydroxysteroid dehydrogenase (E2DH) appears to be a possibly more reliable marker of tumor hormone-dependency than ER and PR [8]. E2DH converts the potent estrogen estradiol (E2) into its less active metabolite, estrone (E1). In breast tissue [8, 12], as in the endometrium [13–16], it is stimulated by progesterone (P) and progestins and is one of the mechanisms by which P controls E2 action. Previous studies have demonstrated that E2DH activity was particularly high in normal human breast epithelial cells in culture, but low in fibroblasts of the same origin [17]; moreover, it was P-dependent only in the epithelial cells [17, 18]. E2DH may therefore be a marker of both epithelial cells and their P-dependency, since E2DH stimulation indicates that PR is not only present but also functional [18].

E2DH has been studied in human breast cancers from pre- and post-menopausal women [8, 19]. It was constantly low in PR− tumors and strikingly higher in ER+, PR+ tumors from either pre-menopausal patients operated during luteal phase or post-menopausal women receiving progestin treatment, except in some cases which raise the question of the functional character of PR. It was therefore relevant to study E2DH in the T47D breast cancer cell line. T47D cells are hormone-dependent. They are known to have high PR levels and they are a useful model for studies of hormone actions [20–22].

We examined the respective action of E2 and the progestin promegestone (R5020) on E2DH in parallel with cell growth in these T47D cells, in order to determine whether (1) E2DH is a possible control mechanism for E2 action by P in these cancer cells as it is in normal breast cells; (2) PR, which is observed at high levels, is in fact functional; and (3) E2DH can be used as a marker of P-dependency in these cells.

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MATERIALS AND METHODS

Chemicals and reagents

RPMI 1640 medium, foetal calf serum (FCS), $NaHCO_3$ solution (7.5%), L-glutamine, penicillin–streptomycin and sterile trypsin–EDTA solution, were provided by Biopro-Seromed (Strasbourg, France). Bovine pancreas crystalline insulin was obtained from Organon Corp. (Serifontaine, France).

Steroids

[6,7-^{3}H]E2 (sp.act 53 Ci/mmol), [6,7-^{3}H]E1 (sp.act. 56 Ci/mmol), [4-^{14}C]E2 (sp.act. 57 mCi/mmol), [4-^{14}C]E1 (sp.act. 57 mCi/mmol), [6,7-^{3}H]moexestrol ([^{3}H]R2858, sp.act. 71 Ci/mmol), [6,7-^{3}H]promegestone ([^{3}H]R5020, sp.act. 87 Ci/mmol) and unlabeled R2858 and R5020 were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.) and purified before use. Unlabeled E2 and E1 were obtained from Roussel-Uclaf Research Center (Romainville, France).

Cultures procedures

The T47D human breast cancer cell line was kindly provided by Dr Chambon of Strasbourg. The cells were distributed into T75 flasks (Falcon-Becton Dickinson and Co., Grenoble, France) at a density of 5×10^6 per flask in 15 ml basal medium. The medium was RPMI 1640 supplemented with L-glutamine (2.8 mM), insulin (8 mIU/ml), penicillin–streptomycin 100 U/ml, 5% FCS and maintained at 37°C in a humidified atmosphere of 5% CO_2-air. The medium was changed every two days.

In studies on hormone actions, the T47D cells were subcultured in T25 flasks at a density of 5×10^5 cells per flask, and maintained in RPMI 1640 medium with 5% dextran coated charcoal (DCC)-stripped FCS 24 h before starting the various hormone treatments and during treatments.

Growth study

Growth study was evaluated on DNA assay carried out in triplicate flasks every day during the 7-day hormone treatments. DNA was assayed by the method of Burton [23].

Steroid receptor assay

ER and PR were measured using whole-cell binding assay on cultured cells [24, 25].

ER. Cells were incubated with [^{3}H]R2858 at concentrations ranging from 0.5 to 5 nM with or without a 200-fold excess of unlabeled R2858 for 1 h at 37°C. At the end of the incubation, the flasks were rapidly cooled on ice, medium was removed and the cells washed 3 times with cold phosphate-buffered saline (PBS). The cells were harvested with a rubber policeman in cold PBS and pelleted at 800 ***g***. The radioactivity contained in the cell pellet was extracted with 3 ml ethanol by vortexing every 10 min for 30 min, and counted in 10 ml OCS liquid scintillation (Amersham) in a Packard 300 C spectrometer. Specific binding was calculated from the difference between total and non-specific binding and expressed as pmol/mg DNA.

PR. The protocol was the same as for ER assay except that cells were incubated with [^{3}H]R5020 at concentrations ranging from 0.1 to 2 nM with or without a 200-fold excess of unlabeled R5020.

E2DH assay [17, 18]

Metabolism of E2 to E1. Assays were performed on intact monolayers of T47D cells in culture. Before incubation, the cells were twice-washed with 5 ml of serum-free RPMI 1640 medium. They were then incubated at 37°C with 5 ml of serum-free medium containing [^{3}H]E2 (2 nM). Five hundred microliter aliquots of the incubation mixture were drawn at various times from 15 min to 4 h. Steroids were extracted with 10 vol of ethyl acetate–cyclohexane (1:1) containing [^{14}C]E2 and [^{14}C]E1 (3000 dpm each) to monitor recovery. At the end of incubation, the remaining medium was subjected to steroid extraction, the cells twice washed and harvested for DNA assay. After extraction, the steroids were separated by thin-layer chromatography (TLC) in a chloroform–ethyl acetate system (80:20). Losses were estimated from the recovery of the ^{14}C-labeled tracers added to the samples. No radioactivity was recovered in the aqueous phase after ethyl acetate–cyclohexane extraction. From the radioactivity deposited onto TLC, >98% was always recovered in E1 and E2 migration areas in this solvent system. All experiments were performed in triplicate. Blanks consisting of cell-free fresh medium incubated for the same period of time and with the same concentrations of [^{3}H]E2 were included in each experiment. The amount of steroid inside the cells was always <1% of the total radioactivity. Therefore the kinetic study of E1 formation was performed on the incubation medium.

The amount of E1 formed from E2 was calculated by dividing the produced amount of [^{3}H]E1 by the specific activity of the [^{3}H]E2 substrate. The conversion rate of E2 to E1 was expressed as fmol E1/μg DNA/h.

E2DH activity measurement was carried out under various conditions of time, DNA concentration and cofactor (NAD) concentration in order to characterize the enzyme activity and determine standard conditions of E2DH assay.

The formation of E1 from E2 increased over time. It was linear up to 30 min and plateaued after 2 h; the plateau represents a E1 amount of 10–15% of incubated [^{3}H]E2. In subsequent studies, E2DH enzyme activity was calculated from the initial slope of E2 production as a function of time during the first 30 min and expressed as fmol/h as described previously [17].

E2DH activity increased as a function of DNA concentration. It was linear until about 15–20 μg DNA/flask and plateaued at higher concentrations. Most subsequent E2DH assays were therefore carried out on cultures of $< 15\ \mu$g DNA.

E2DH activity increased linearly with increasing concentrations of the cofactor NAD. However, for concentrations higher than 500 μM a plateau was observed. All subsequent studies were conducted in the presence of NAD in excess (1000 μM). This results in a 3–4-fold increase in E2DH activity which represents 35–40% of E1 produced from incubated [^{3}H]E2.

Metabolism of E1 to E2. The assay procedure was the same as for the E2 → E1 metabolic pathway except that tritiated E2 was replaced by [^{3}H]E1 (2 nM).

The conversion of E1 to E2 was linear up to 30 min and plateaued after 2 h. In subsequent studies, the enzyme (E1 → E2) activity was calculated from the initial slope obtained during the first 30 min, as for E2 → E1 activity.

Effects of steroids on cell growth and on E2DH activity

In order to study estrogen- and progestogen-dependency of cell growth as well as E2DH activity, cells were cultured in basal medium with 5% DCC-FCS in various hormonal conditions: (1) without any steroid addition; (2) supplemented with E2 (10^{-8} M); (3) with increasing R5020 concentrations (10^{-9} to 10^{-6} M); and (4) supplemented with both E2 (10^{-8} M) and R5020 (10^{-9} to 10^{-6} M).

The medium with or without hormone treatment was changed every 2 days. The hormonal treatment was carried out for 7 days for the cell growth study and 5 days for the E2DH study.

Data analysis

The influence of the various hormone culture conditions on cell growth evaluated by DNA assay, and on E2DH activity was compared within series of T47D cell cultures. Each measurement was carried out in parallel triplicate flasks and the results were expressed as the mean ± SD. Less than 10% intra-assay variation was observed. The hormonal effect obtained was always observed in the same proportions with < 15% inter-assay variations.

RESULTS

Growth study

The influence of E2 and R5020 was studied on T47D cell growth on the basis of DNA

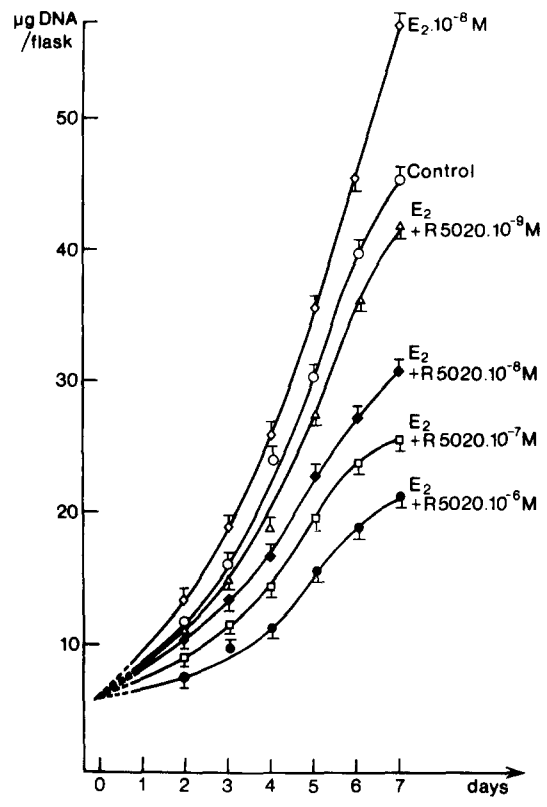

Fig. 1. Stimulatory effect of E2 and inhibitory effect of R5020 on the growth of T47D cells. T47D cells were plated at a density of $5 \cdot 10^5$ cells per T25 flask and grown in RPMI medium supplemented with 5% charcoal stripped FCS. Steroid treatments (10^{-8} M E2 ± 10^{-9} to 10^{-6} M R5020) started 24 h later. Control flasks had no hormone added. Cell growth was studied by daily assay of DNA. The results are expressed as μg of DNA per flask. Each point is the mean ± SD of determinations in parallel triplicate flasks.

values measured on every day of the steroid treatment.

The addition of E2 to the culture medium resulted in an obvious increase in cell growth (Fig. 1) On the contrary, addition of R5020 to E2 inhibited cell growth throughout the duration of the culture in a dose-dependent manner.

When R5020 was added alone without E2, it decreased cell growth but to a lesser extent than when added with E2 (data not shown).

Receptors assay

ER assay. Scatchard analysis of the specific binding on [^{3}H]R2858 to T47D cells provided a dissociation constant (K_d) of $0.92 \pm 0.17 \times 10^{-10}$ M and a maximal binding capacity (B_{max}) of 1.8 ± 0.6 pmol/μg DNA.

PR assay. Scatchard analysis of the specific binding of [^{3}H]R5020 to T47D cells resulted in K_d of $1.98 \pm 0.15 \times 10^{-9}$ M and a maximal binding capacity of 30.6 ± 4.7 pmol/μg DNA.

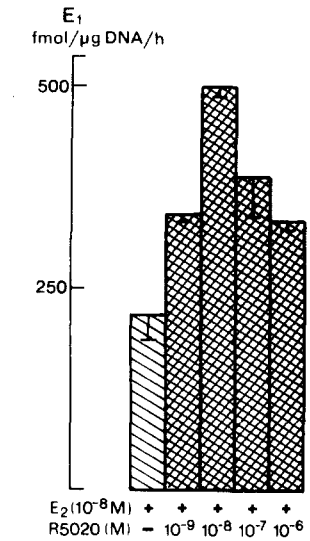

Fig. 3. Effect of increasing the concentration of R5020 (10^{-9} to 10^{-6} M) on E2DH activity (E2→E1) in T47D cells. For details, see legend Fig. 2 (*inset*) except that incubation was performed in the presence of NAD.

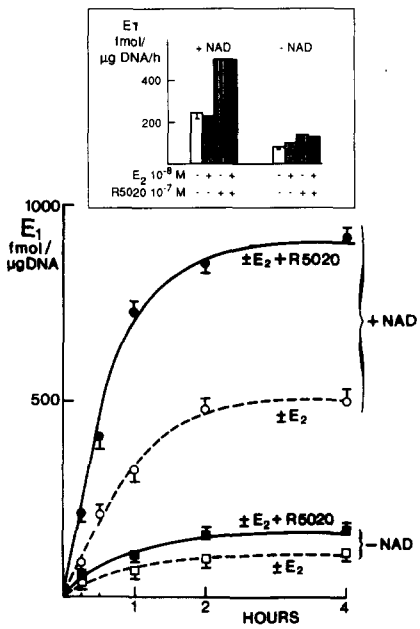

Fig. 2. Stimulatory effect of R5020 on E2DH activity (E2→E1) in the presence or absence of 1000 μM NAD. T47D cells were subcultured in RPMI medium supplemented with 5% charcoal-stripped FCS and the steroid treatments ($\pm 10^{-8}$ M E2, $\pm 10^{-7}$ M R5020) started 24 h later. E2DH activity was measured after 5 days of treatment. E1 produced was measured as a function of time and presence or absence of NAD (1000 μM) after incubation with [^{3}H]E2 (2 nM). The results are expressed as fmoles of E1 formed per μg of DNA. *Inset*: E1 produced was measured after 30 min incubation with [^{3}H]E2 (2 nM) in the presence or absence of NAD. The results are expressed as fmoles of E1 formed per μg of DNA and per hour. Each point represents the mean $\pm$ SD of three determinations. Presence of E2 in the culture medium did not alter E2DH activity whether or not the cofactor was present. In contrast, R52020 either alone or in the presence of E2 stimulated the conversion of E2 into E1.

E2DH activity

Metabolism of E2 to E1: effect of E2 and R5020 on E2 and E1 metabolism. Presence of E2 in the culture medium did not alter E2DH activity whether or not the cofactor NAD was present (Fig. 2). In contrast, the addition of R5020 to the culture medium—either alone or in the presence of E2—stimulated the metabolism of E2 into E1 (Fig. 2). The stimulation of E2DH activity by R5020 was observed without NAD addition, but it was more marked under conditions of NAD excess (Fig. 2).

Metabolism of E2 to E1: dose-dependent stimulatory effect of R5020 on E2DH. R5020 10^{-8} M concentration stimulated E2DH activity more than did 10^{-9} M. It even appeared to be the optimal concentration since 10^{-7} M and 10^{-6} M stimulated E2DH less than did 10^{-8} M (Fig. 3).

Metabolism of E2 to E1: determination of the apparent Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) of E2DH (E2 → E1) in T47D cells cultured with or without E2 and R5020. Cells were incubated with E2 concentrations ranging from 10^{-8} to 10^{-5} M and data were analyzed from a Lineweaver and Burk plot. When the cells were treated in the presence or absence of E2 (10^{-8} M) alone, the apparent K_m was 15.4 ± 3.2 μM and V_{max} was 110.5 ± 5.2 nmol/mg DNA/h. The addition of

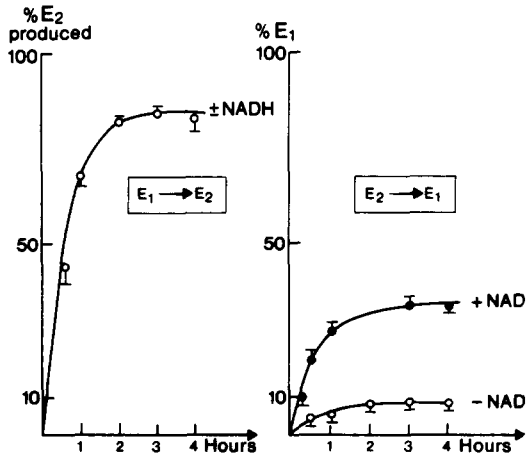

Fig. 4. Comparison between the two E2DH pathways (E2→E1 and E1→E2) in the presence or absence of cofactors. T47D were subcultured in RPMI medium supplemented with 5% charcoal stripped FCS. E1 produced from [^{3}H]E2 and E2 produced from [^{3}H]E1 were assayed in the presence or absence of cofactor. Results are expressed as percentage of E1 or E2 formed. Each point represents the mean ± SD of three determinations.

R5020 (10^{-7} M) to E2 resulted in a lower K_m (7.2 ± 2.1 μM) while V_{max} remained unchanged.

Metabolism of E1 to E2. When [^{3}H]E1 was used as a substrate, the conversion of E1 to E2 as a function of time in T47D cells was found to be linear up to 30 min. A plateau was reached by 2 h. At the plateau level, the conversion rate reached a markedly high value of 85% in contrast to the 10% conversion rate obtained when E2 was used as a substrate without any addition of cofactor (Fig. 4).

Addition of cofactor, either NADH or NAD, did not alter the kinetic of E2 production. Neither E2 nor R5020 treatment of the cells modified the transformation of E1 to E2 when compared with controls.

DISCUSSION

E2DH enzyme activity was first studied in the endometrium by Tseng *et al.* [14, 15] who demonstrated that it was P-dependent. E2DH activity was further observed in breast tissue [8, 12, 19] and normal human breast cells in culture [17, 18]. It is especially active and also P-dependent in epithelial cells, whereas its activity is very low and not stimulated by P in fibroblasts [17]. E2DH has therefore been suggested as a possible marker of both epithelial cell differentiation and PR functionality [8, 17, 18].

It was therefore interesting to check E2DH activity in breast cancer cell lines and particularly in T47D cells, which are rich in PR. In the present study, we showed that E2DH enzyme activity is controlled by P, and may participate in the mechanisms by which P regulates E2 action. It can therefore also be used as a marker of PR action and functionality.

Standard conditions for E2DH assay have been previously determined on normal human breast epithelial cells in culture [17] and further adapted for T47D cell studies. Measurements of E2DH activity have been carried out using a non-saturating concentration of substrate (2 nM). However, this concentration of substrate has been selected, as well as incubation time, DNA levels and NAD concentration, so that the amount of E1 produced from E2 was dependent on and proportional only to the amount of enzyme.

The results obtained clearly show that, in the T47D cell line, the cells are preferentially oriented toward production of E2 from E1, since transformation of [^{3}H]E1 to E2 is 85%, whereas there is only a 10 to 15% [^{3}H]E2 transformation into E1 in the absence of added cofactor. This differs somewhat from previous studies on breast cancer since some authors observed a predominant oxidative pathway *in vitro* [26] whereas others showed a greater reductive pathway through *in vivo* isotopic infusion studies [27]. Nevertheless, when an excess of NAD is added to the incubation of [^{3}H]E2, transformation to E1 attains 35%. In T47D cancer cells, the amount of cofactor—at least in the E2 → E1 pathway—seems to be a limiting factor. This might be due to the extensive and cofactor-consuming metabolic activities of these cancer cells. However, under excess NAD conditions, E2DH activity (E2 → E1) increases in T47D cells, and is markedly stimulated by the progestin promegestone (R5020): R5020 stimulates by 2- to 3-fold the activity of E2DH in the E2 → E1 pathway with maximal stimulatory effect at 10^{-8} M to 10^{-7} M concentration, but not in the E1 → E2 pathway which differs from the results obtained by Coldham and James [28] in the MCF 7 cell line.

This R5020 effect can be observed both in the presence and absence of E2. This was rather unexpected since the progestins are presumed to act principally through the PR, which is stimulated by E2. However, in T47D cells, PR level is constitutionally high and presence of E2 may not be a prerequisite for progestogen

action [22, 29]. In addition estrogen sulfates may have remained in DCC-FCS [30] and stimulate PR.

While noticeable, the stimulation of E2DH by R5020 in T47D cells, does not reach the stimulation levels observed in certain PR+ breast cancer tumors studied in patients operated under progestin therapy [8]. This suggests that PR though abundant may not be highly functional in T47D cells. This underlines the need for hormonal markers situated downstream from PR in the mechanism of action of P. For this purpose, E2DH appears to be a P-dependent protein in such cancer cell lines and a good candidate for identifying a genomic sequence with which P interacts.

Considering cell growth, in spite of the possible persistence of conjugated estrogens in the medium, addition of E2 to the culture medium has a stimulatory effect as shown previously on normal human breast epithelial cells in culture [18, 25]. By contrast, addition of increasing doses of R5020 antagonize this E2 stimulating effect and, at the highest concentrations, slows cell growth to lower levels than the control cells without E2 addition.

It should be noted that T47D cell growth is progressively inhibited by increased concentrations of R5020 (Fig. 1), whereas its effect on E2DH reaches a maximum at 10^{-8} M. This suggests that the progestin control of E2 action on cell growth is not mediated only through E2DH activity but may also involve other mechanisms such as the decrease in ER [31, 32], and possibly the inhibition by progestin of protooncogene expression such as the c-myc protooncogene [33].

In this T47D cancer cell line, the progestin R5020 behaves in the same way as in normal human breast epithelial cells: (1) it slows down cell growth as was previously reported by Vignon *et al.* [22] and Horwitz *et al.* [34, 35]; and (2) it stimulates cell differentiation in so far as E2DH may be considered as a marker of epithelial cell differentiation and P action [8, 17–19].

E2DH should now be considered as a possible hormonal marker downstream from PR in ER+, PR+ breast cancers in order to predict the response to hormonal therapy. Additional studies are now in progress with parallel assessment of E2DH, ER, PR, Scarff and Bloom grading [36, 37] and follow-up of a large cohort of patients with breast cancer, in order to evaluate E2DH as a marker for breast cancer hormone-dependency and prognosis.

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