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ESTRADIOL ESTERS CAN REPLACE 17β-ESTRADIOL IN THE STIMULATION OF DNA AND ESTERASE SYNTHESIS BY MCF-7 CELLS: A POSSIBLE ROLE FOR THE ESTROGEN-SENSITIVE MCF-7 CELL ESTERASE

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Summary—In this communication we extend our earlier observations on estrogen-sensitive carboxyl esterases in MCF-7 human breast cancer cells able to hydrolyze esters of estradiol. Using either estradiol acetate or *p*-nitrophenyl hexanoate as substrates, esterase activity was found to increase 2–3-fold in MCF-7 cells maintained in the presence of 10^{-8} M estradiol. Following sucrose density centrifugation, over 85% of total esterase activity was found in the cytoplasmic fraction. No esterase activity was found in spent media from growing cells. By size exclusion chromatography, estradiol acetate esterase activity exhibited a mol. wt of 45–50 kDa. Attempts to demonstrate incorporation of [^{3}H]estradiol into estradiol fatty acid esters by the above MCF-7 cell line (203P) were unsuccessful, although, such incorporation could be demonstrated in two other MCF-7 cell sublines. Incubation of the 203P cells with 10 nM [^{3}H]estradiol in the presence of 0.5 mM radioinert estradiol acetate resulted in the incorporation of 35 ± 12% of the label into the estradiol acetate in 10 min. In the absence of radioinert estradiol acetate, no incorporation was observed. When MCF-7 cells were incubated with [^{3}H]estradiol in the presence of a large excess of radioinert estradiol valerate, label was found only in estradiol valerate. Similarly, when the incubation was carried out in the presence of a mixture of radioinert estradiol acetate and estradiol valerate, label was incorporated into both esters. We conclude that the apparent formation of radiolabeled estradiol esters by MCF-7 cells incubated under the above conditions, results at least in part, from an esterase-catalyzed exchange reaction. Under conditions where no ester hydrolysis could be detected in the absence of cells, valerate and stearate esters of estradiol were found to be as effective as unesterified estradiol in stimulating esterase synthesis and the incorporation of [^{3}H]thymidine into DNA. These results are consistent with a model in which an intracellular esterase in MCF-7 cells can generate estradiol from an exogenous lipoidal steroid and elicit an estrogen response.

INTRODUCTION

Lipoidal ester derivatives of estradiol (E_2) have been shown to be present in a wide range of mammalian body fluids and tissues [1, 2]. These compounds show no physiologic activity presumably because of their inability to bind to the classical estrogen receptor [3]. It has been suggested that the lipoidal estrogens serve as storage forms of the active hormone in hormonally-sensitive tissues where the free steroid could be regenerated by hydrolysis [3]. Consistent with this hypothesis, we demonstrated the presence of intracellular carboxyl esterases in MCF-7 human breast cancer cells able to hydrolyze 17β esters of E_2 [4]. In these initial experiments, we focused our attention on an estrogen-sensitive enzyme able to cleave short chain fatty esters of E_2 with K_ms of approx. 10^{-7} M. Rates of 17β-E_2 acetate esterase levels activity increased 2–4-fold when MCF-7 cells were grown in the presence of 10^{-8} M E_2.

In the present study our aims were to establish a possible role for the estrogen-sensitive esterase in MCF-7 cells. We describe some of the properties of this enzyme including its subcellular distribution, apparent mol. wt and ability to catalyze *trans*-esterification reactions. We show that long and short chain fatty acid ester substrates of the MCF-7 cell esterase are able to replace E_2 in stimulating [^{3}H]thymidine incorporation into DNA and esterase activity in

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MCF-7 cell cytosol. Although, E_2 esters have been shown to be made by several tissues including breast cancer tissue *in vitro* [1, 2, 5], the particular cell type responsible for their synthesis *in vivo* has not been unequivocally identified. Adams *et al.* [5] have reported that E_2 esters can be synthesized by MCF-7 cells in culture. We show that the ability to synthesize E_2 esters varies among different MCF-7 cell sublines and does not appear to be related to levels of intracellular esterase activity.

MATERIALS AND METHODS

Materials

Cell culture materials were obtained from Gibco (Grand Island, N.Y.). Steroids were from Steraloids (Wilton, N.H.). [^{3}H]Acetic anhydride, [2,4,6,7-^{3}H]E_2 and [^{3}H]thymidine were from New England Nuclear (Boston, Mass). [^{3}H]E_2-acetate, [^{3}H]E_2-valerate and [^{3}H]E_2-stearate were prepared as previously described (4). Phenol Red-free, RPMI 1640 cell culture media, and *p*-nitrophenyl esters (esterase substrates) were obtained from Sigma (St Louis, Mo.). All other materials were of high purity and from commercial sources.

Cell culture

MCF-7 Cells (203rd passage, 203P) were obtained from the Michigan Cancer Foundation in September 1987. Most of the experiments described here were performed using these cells which were maintained in RPMI 1640 media supplemented with 10% heat-inactivated FBS, and 2 mM glutamine as described earlier [4]. The MCF-7 cell sublines MCF-7BK and MCF-7ML (generous gifts from Dr R. Hochberg), were maintained in MEM medium supplemented with 5% heat-inactivated calf serum, 2 mM glutamine and insulin (6 ng/ml) and DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM glutamine respectively. Cultures were maintained in humid air containing 5% CO_2. Media was changed every 2–3 days.

In experiments where the effect of E_2 or E_2 esters was examined, cells were grown to confluence in Phenol Red-free RPMI 1640 media. On Day 1 of the experiment, cells were fed with RPMI 1640 containing 10% FBS which had been treated with charcoal-dextran [4] to remove endogenous steroids. On Days 2 and 3, the cells were again fed with media containing charcoal-dextran-treated FBS. On Day 4, media containing 10 nM E_2 were added. On Day 6, cells were harvested, washed twice and homogenized in 0.1 M phosphate, pH 7.4 and centrifuged. In initial experiments, the 100,000 *g* supernatant, which contained all detectable E_2 esterase activity was used. In later studies the 28,000 *g* supernatant was used as we could find no detectable difference between the amount of esterase activity in the supernatants after centrifugation at 28,000 *g* for 20 min or 100,000 *g* at 60 min.

Assay for esterase activity

Esterase activity in MCF-7 homogenates was determined using either E_2-17-[^{3}H]acetate or *p*-nitrophenyl hexanoate as the substrate. In the first case, a sample containing esterase activity was incubated with 0.5 μM E_2-[^{3}H]acetate (25 μCi/μmol) in 1 ml 0.1 M sodium phosphate, pH 7. At various intervals, 50 μl aliquots were withdrawn and partitioned between water and hexane. The increase in radioactivity in the aqueous phase, after correction for non-enzymatic hydrolysis, was used as a measure of esterase activity. Hydrolysis of nitrophenyl esters was measured under similar conditions from the change in absorbance at 405 nm.

The esterase-catalyzed exchange reaction between E_2-17-acetate and [^{3}H]E_2 was monitored by chromatography on Sephadex LH-20 essentially as described earlier for following the hydrolysis of [^{3}H]E_2-esters [4]. The lipid fraction in the sample was extracted with chloroform:methanol (2:1), taken to dryness and chromatographed on a 0.8 × 14 cm column of Sephadex LH-20 with isooctane:methanol:ethyl acetate (4:1:1) (Fig. 1). The percentage exchange was calculated from the amount of label in the E_2-ester fraction.

Discontinuous sucrose density gradient centrifugation

MCF-7 cells were fractionated by discontinuous sucrose density gradient centrifugation essentially as described previously [6]. Confluent cells from three 75 cm^2 flasks were washed and homogenized in a Dounce homogenizer (12 strokes) charged with 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4 and the homogenate centrifuged 40 min at 150,000 *g* in a Beckman SW 50.1 rotor. The supernatant containing the cytosol was saved. The pellet was resuspended in 4.25 ml of the same buffer and layered onto a discontinuous sucrose gradient consisting of 4.25 ml each of 60, 40 and 20% sucrose in 10 mM Tris–HCl, pH 7.4, and the gradient centrifuged 120 min at 175,000 *g* in a Beckman SW 41 rotor. The

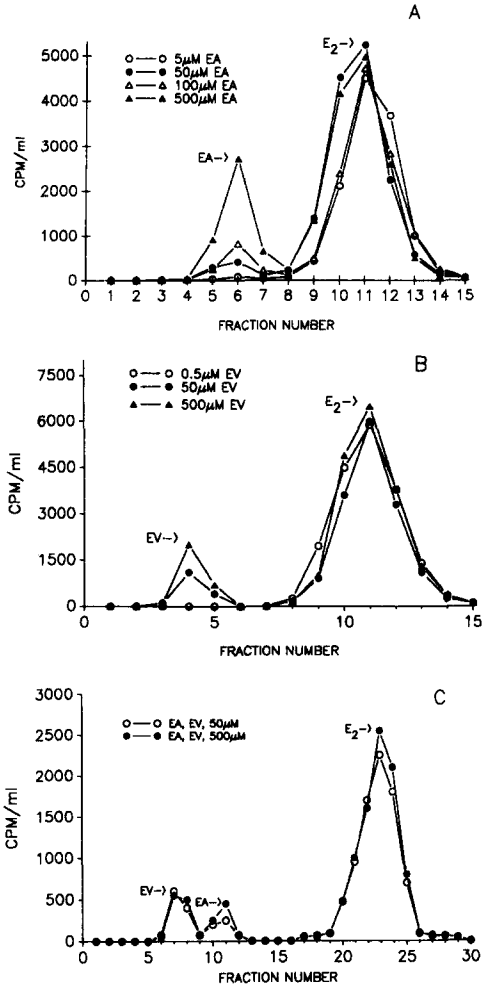

Fig. 1. Effect of radioinert E_2-acetate (EA) and E_2–valerate (EV) on the exchange of [^{3}H]E_2-esters. [^{3}H]E_2 (0.1 nmol, 2×10^5 cpm) and either EA and EV at the concentrations indicated, were added (in 50 μl DMSO) to growing cultures of MCF-7 cells. After 20 min, cells and media were harvested, sonicated and extracted with chloroform:methanol (2:1). E_2-esters were separated from free steroid by chromatography on LH-20 (0.8 × 14 cm) in the solvent system isooctane:methanol:ethyl acetate (4:1:1). Fractions of 1 ml were collected in (A) and (B) and 0.5 ml (to achieve better resolution) in (C). (A) Effect of EA concentration on exchange; (B) effect of EV concentration on exchange; (C). Exchange in the presence of EA and EV.

particulate fractions at the interface above the 20% sucrose band (Band A), above the 40% sucrose band (Band B), above the 60% sucrose band (Band C) and in the cell pellet were washed with buffer and collected by centrifugation at 150,000 *g*. Identity of the particulate fractions was established with: β-*N*-acetyl-glucosiminidase (Band A, lysosomes); 5′nucleotidase (Band B, plasma membranes), and cytochrome oxidase (Band C, mitochondria and endoplasmic reticulum). Esterase activity in the cell washes, homogenate, cytosol and membrane fractions was determined with nitrophenyl hexanoate.

[^{3}H]Thymidine incorporation

MCF-7 cells maintained in Phenol Red-free RPMI 1640 media containing 5% dextran-charcoal-treated FBS were washed twice with Hank's balanced salt solution, trypsinized, pelleted and resuspended in fresh RPMI media. The resuspended cells were plated at a density of 2.5×10^5/1.1 ml using 24 well (16 mm) Costar plates. After a 2 h incubation to permit cell adherence, hormone or vehicle was added to each well and the plates were incubated for 60 h as described above. At the end of the incubation, test media was exchanged for fresh media containing 1 μCi [^{3}H]thymidine (84.1 Ci/mMol). After 2 h, the cells were washed and trypsinized. DNA was precipitated with 10% TCA, washed with 5% TCA, ethanol and ether and counted.

Zone electrophoresis

Zone electrophoresis was performed on 1% agarose gels (50 × 75 × 5 mm) in TBE (0.05 M Tris–HCl, 0.05 M boric acid, 1 mM EDTA, pH 8.0) buffer at 10 V/cm for 60–90 min. Staining of esterase activity on the gels after electrophoresis was accomplished by a modification of the procedure described by Schaller and von Deimling [7]. Gels were incubated for 45 min in 80 ml 0.07 M sodium phosphate, pH 6.5 containing 12 mg fast garnet and 1 ml of 6 mM α-napthyl acetate in DMSO (freshly prepared) and then rinsed with water.

RESULTS

Nitrophenyl ester hydrolysis by MCF-7 cell homogenates

In initial studies from this laboratory [4], [^{3}H]E_2 esters of acetate, valerate and stearate were used as substrates to monitor MCF-7 cell esterase activity. Because assay of the esterase with these compounds was somewhat cumbersome, a number of other esters were tested as potential substrates. The most convenient proved to be the fatty acid esters of nitrophenol (Table 1). The hexanoate ester showed the greatest activity and because it was a competitive inhibitor of E_2 acetate hydrolysis (data not shown) and a substrate for the estrogen-sensitive esterase, it was used for most assays. Under the conditions described in Table 1, no hydrolysis of myristate or stearate nitrophenyl esters by the MCF-7 cell homogenate was observed. Similarly, no hydrolysis of long chain fatty acid esters of

Table 1. Effect of fatty acid chain length on the hydrolysis of nitrophenyl fatty acid esters by MCF-7 cell homogenates

Fatty acid	Activity*
Acetate	0.333 ± 0.069
Butyrate	0.586 ± 0.066
Hexanoate	1.0
Decanoate	0.533 ± 0.047
Myristate	<0.001
Stearate	<0.001

MCF-7 homogenate (28,000 *g* supernatant, 224 μg protein) was incubated with 5×10^{-5} M substrate in 1 ml 0.1 M sodium phosphate pH 7. Esterase activity was determined from the change in absorbance at 405 nm. Reactions were linear for at least 10 min. Under these conditions the K_m for *p*-nitrophenyl hexanoate was determined to be 5.3×10^{-5} M.

*Relative activity, activity with *p*-nitrophenyl hexanoate as substrate considered to be 1.0. Values are means ± SE for 3 separate experiments.

Table 2. Localization of the esterase activity in MCF-7 cells

Fraction	*Total esterase activity** Exp. 1	Exp. 2
Cell wash	<0.001	<0.001
Cytoplasm	0.450	2.450
Band A	<0.001	<0.001
Band B	<0.001	0.075
Band C	<0.001	0.125
Pellet	0.006	0.075

Confluent cells were washed twice with buffer and homogenized in 0.25 M sucrose, 0.1 M Tris–Cl, pH 7.4 for 15 strokes in a Dounce homogenizer. The homogenate was centrifuged 40 min at 105,000 *g* and the supernatant (cytoplasmic fraction) was collected. The pellet was resuspended in this same buffer and centrifuged through a discontinuous sucrose gradient of 20, 40 and 60% sucrose at 175,000 *g* in a Beckman rotor. Particulate fractions at the interfaces between the sucrose phases were collected, washed, pelleted by centrifugation and resuspended in buffer before assay for esterase activity with *p*-nitrophenyl hexanoate. Identity of the particulate fractions was established with: β-*N*-acetylglucosiminidase (Band A, lysosomes); 5′ nucleotidase (Band B, plasma membranes), and cytochrome oxidase (Band C, mitochondria and endoplasmic reticulum).

*Total esterase activity as change in A_{405}/min/5 ml.

E_2 was observed except after long incubation with MCF-7 cells in culture. Consistent with our earlier findings, esterase levels in MCF-7 cells were higher when the cells were grown in the presence of E_2, whether esterase activity was determined with nitrophenyl hexanoate or E_2-acetate as a substrate.

Location of the MCF-7 cell esterase

The location of the esterase activity in the MCF-7 cells was determined by discontinuous sucrose density gradient centrifugation (Table 2). In these experiments more than 85% of the total esterase activity was found in the cytoplasmic fraction and not with membrane associated proteins. It is significant that no esterase activity was found in spent media or cell washes. Barring artifacts introduced by the fractionation procedure, this suggests that the enzyme(s) is located entirely intracellularly and is not secreted by the MCF-7 cells.

Physical properties

Size exclusion chromatography on TSK-4000 (0.75 × 60 cm column) showed that E_2-acetate esterase activity eluted with proteins having a mol. wt of 45–50 kDa (Fig. 2). Zone electrophoresis of the MCF-7 cell homogenate (40 μg protein) on 1% agarose gels at pH 8.0 reproducibly showed the presence of two bands after staining for non-specific esterases with α-napthyl acetate and fast garnet (Fig. 3). Mobility of both bands was somewhat faster than that of the major esterase activity in normal human plasma and considerably slower than that of albumin. It is noteworthy that in the homogenate from cells incubated for 48 h in the presence of 10^{-7} M E_2 there is an apparent increase in the amount

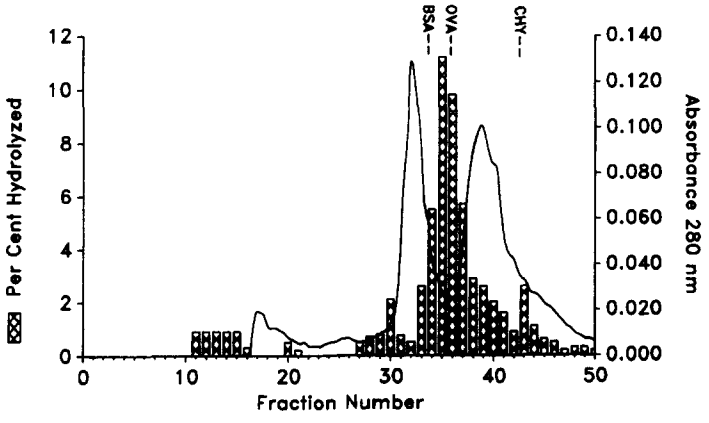

Fig. 2. Gel exclusion chromatography of the MCF-7 cell esterase activity. MCF-7 cell homogenate (0.5 ml, 180 μg protein) was chromatographed on a 0.75 × 60 cm TSK-4000 column in 0.1 M sodium phosphate, 0.05 M NaCl, pH 7.0 at a flow rate of 0.75 ml/min. The absorbance was monitored at 280 nm. Fractions (1 min) were collected and assayed for esterase activity with [^{3}H]E_2-acetate as described in the methods. The column was calibrated with bovine serum albumin (BSA), ovalbumin (OVA) and chymotrypsinogen (CHY).

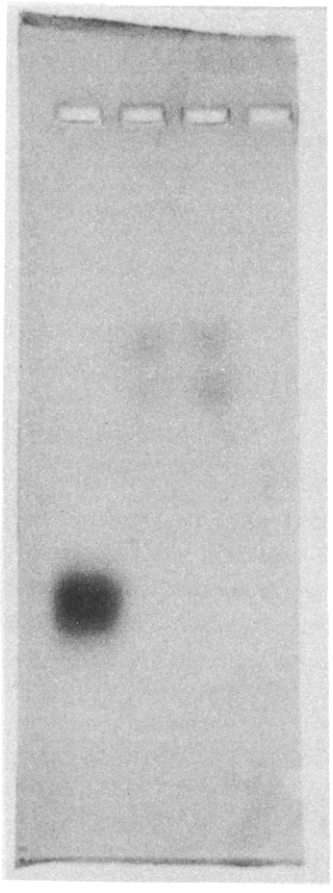

Fig. 3. Zone electrophoresis of MCF-7 homogenate (100,000 *g* supernatant) on 1% agarose gels (50 × 75 × 5 mm) in TBE buffer, pH 8.0, at 10 V/cm. Gels stained for nonspecific esterase activity with α-napthyl/fast garnet. Lane 1: purified mouse esterase, 2 μg; lane 2: untreated MCF-7 cell homogenate, 40 μg; lane 3: E_2-treated MCF-7 cell homogenate, 40 μg.

Table 3. Effect of fatty acid chain length and concentration on the exchange reaction catalyzed by the steroid esterase activity in MCF-7 cells

Substrate	Concentration (μM)	Exchange (%)
E_2-acetate	5	1.0 ± 0.1
	50	5.7 ± 0.8
	100	12.3 ± 0.8
	500	32.3 ± 9.4
E_2-valerate	50	7.2 ± 4.9
	500	15.7 ± 1.5
E_2-stearate	5	2.0
Corticosterone-stearate	5	2.2

[^{3}H]Estradiol (0.1 nmol, approx. 2 × 10^5 cpm) and radioinert steroid ester at the concentration indicated, were added to a 75 cm^3 flask of confluent MCF-7 cells in culture. After a 20 min incubation at 37°C, the cells were collected and sonicated and the lipid fraction extracted with chloroform:methanol (2:1). The extract was taken to dryness and chromatographed on a 0.8 × 14 cm column of LH-20 with isooctane:methanol:ethyl acetate (4:1:1) collecting 1 ml fractions as shown in Fig. 1.

Per cent exchange was calculated from the amount of label in the steroid ester peak. Values represent the mean ± SD for 3–4 separate experiments (E_2-acetate and -valerate). The values for the two stearate esters are single observations.

of esterase activity in the lower band. Although it is difficult to estimate the amount of activity in each band, a comparison with lane 1 (2 μg purified mouse esterase) suggests that it is appreciable.

Formation of [^{3}H]E_2-acetate from [^{3}H]E_2 can occur by a trans-esterification reaction

In preliminary experiments, we found that incubation of MCF-7 (203P) cells in culture with [^{3}H]E_2 and an excess of radioinert E_2-17-acetate resulted in the incorporation of substantial amounts of the label into E_2-17-acetate in 20 min (Fig. 1A, Table 3). High levels of radioinert E_2 esters (0.5 mM) were required presumably to serve as competitive inhibitors of endogenous MCF-7 cell esterase activity. With this subline, no incorporation was observed in the absence of E_2-17-acetate or in the presence of low levels of unlabeled E_2-17-acetate (0.5 μM), either in growing cells or in cells homogenized under various conditions. Intact cells were necessary to demonstrate maximal exchange: no exchange was observed with the 100,000 *g* supernatant and very little exchange was observed in the crude homogenate from cells homogenized under mild conditions. For example: in the homogenate from cells homogenized 12 strokes in a Dounce homogenizer, maximal exchange in the presence of 0.5 mM E_2-acetate was 2.7% (as compared to 26% with intact cells). These results were consistent with either E_2 exchange catalyzed by a single esterase enzyme or else by separate synthetic and degradative reactions catalyzed by two different enzymes. In a cell-free system, exchange was completely blocked by diisopropylflurophosphate (DIFP), a potent inhibitor of serine esterases. However, the low initial level of exchange activity compromised the validity of the comparison. DIFP also blocked exchange activity in intact cells, however, because of the high concentration (10^{-2} M) required to completely inhibit exchange, these results were also considered to be equivocal. When MCF-7 (203P) cells were incubated with [^{3}H]E_2 in the presence of radioinert E_2 valerate, label was found only in E_2-valerate (Fig. 1B). Similarly, when the incubation was carried out in the presence of a mixture of radioinert E_2-acetate and E_2-valerate, label was incorporated into both esters (Fig. 1C). These results indicate that in the MCF-7 203P subline, an esterase-catalyzed *trans*-esterification reaction is responsible for part, if not all, of the incorporation of

[^{3}H]E_2 into E_2 esters. Although estradiol esters have limited solubility in aqueous systems, the experiments described above were conducted in culture media containing 10% FBS (approx. 5 mg/ml BSA). When 10 μM E_2-[^{3}H]-acetate is "suspended" in this media and centrifuged for 5 min in a microfuge at 8500 *g*, all of the counts remain in the supernatant. Whether or not this represents true solubility is unclear. It is clear, however, that trans-esterification is increased in the presence of increasing amounts of lipoidal steroid.

To further confirm the existence of an esterase-catalyzed *trans*-esterification reaction, the exchange of fatty acids between E_2 and the esters of other steroids was also examined. Under normal assay conditions, no hydrolysis of E_2- or nitrophenyl-stearate by MCF-7 cell homogenates could be detected (Table 1). However, when E_2-stearate was tested for its ability to undergo esterase-catalyzed exchange with [^{3}H]E_2, low levels of exchange were observed (Table 3). Similarly, corticosterone stearate was able to exchange its steroid moiety with exogenous [^{3}H]E_2.

Synthesis of E_2 esters by MCF-7 cells

Because net synthesis of fatty acid esters of E_2 by MCF-7 cells had been reported by others [5], and because MCF-7 sublines have been shown to demonstrate a great deal of variability, we examined MCF-7 cell sublines other than 203P for their ability to synthesize fatty acid esters of E_2 as measured by the incorporation of [^{3}H]E_2 into E_2 esters. Confluent flasks of MCF-7 cells were incubated with 0.3 nM [^{3}H]E_2 (59.2 Ci/mmol) for 72 h. Media and cells scraped from the flasks were extracted with chloroform:methanol (2:1) and the extracts chromatographed on LH-20 as described for the esterase activity assay in the methods and materials. With the ML subline, a small peak of radiolabeled material (3.1% of starting material) was found in the region where E_2 esters normally appear (Fig. 4A). The presumed E_2 ester was hydrolyzed (0.5 M KOH in benzene, 50°C, 120 min) and then rechromatographed on LH-20. The radioactivity now eluted with authentic E_2 (Fig. 4C). Similar results were obtained with the BK subline except that 5.0% of the original [^{3}H]E_2 was converted to the presumed ester (Table 4). Interestingly, levels of E_2-acetate esterase activity in these three cell lines were very similar (Table 4).

Table 4. Estradiol ester synthesis and esterase activity in various MCF-7 cell sublines

Subline	E_2 Ester synthesis[1] (fmol E_2 ester/mg DNA/2 h)	Esterase[2] (ΔA_{405}/min/mg protein)
203P	<0.01	0.130 ± 0.030
ML	40	0.153 ± 0.150
BK	65	0.173 ± 0.181

[1]Confluent T-75 flasks of the three MCF-7 cell sublines were incubated with 0.3 nM [^{3}H]E_2 for 72 h. Media and cells scraped from the flasks, were extracted with chloroform–methanol, and subjected to chromatography on LH-20 as shown in Fig. 4A. In the ML and BK sublines, small radioactive peaks were found in the area where estradiol esters elute. These peaks could be hydrolyzed with alkali to a compound which eluted with authentic E_2 as shown in Fig. 4C.

[2]Supernatants following 28,000 *g* centrifugation of the homogenates from the three sublines were prepared and tested for esterase activity with *p*-nitrophenyl hexanoate as described in the methods and materials.

For the 203P subline, esterase activity is the mean ± SD for 7 separate experiments. For the ML and BK sublines, esterase activity represents the results of two separate experiments.

Effect of E_2 and E_2 esters on [^{3}H]thymidine incorporation and esterase activity

If the lipoidal-estrogens were to serve as storage forms of estrogens in target tissues, then they would be expected to replace estrogen in stimulating [^{3}H]thymidine incorporation and the activity of estrogen-sensitive enzymes. That this is the case is shown in Fig. 5 where the effects of E_2 and E_2 ester concentration on the behavior of MCF-7 cells are compared. E_2, E_2-valerate and E_2-stearate all served to stimulate [^{3}H]thymidine incorporation and esterase activity by MCF-7 cells. In these experiments, cell growth was monitored by the incorporation of [^{3}H]-thymidine (Fig. 5B) and E_2-dependent protein synthesis by the increase in esterase activity (Fig. 5A). This later assumption is based on the observation that when MCF-7 cells were grown in the presence of cycloheximide (4.5 μg/ml), E_2 was not able to stimulate esterase activity above the baseline level. It should be noted that the error bars in Fig. 5B, particularly in the case of 10^{-10} and 10^{-11} M E_2, are quite large. In other experiments with these cells, (data not shown), E_2 at these concentrations was found to have little or no effect on [^{3}H]thymidine incorporation.

Time-course for hydrolysis of E_2 esters by MCF-7 cells in culture

To verify that E_2 ester hydrolysis by MCF-7 cells occurred at a rate sufficient to generate physiologically effective levels of E_2, we examined the time-course for the appearance of [^{3}H]E_2 in the media after addition of [^{3}H]E_2 esters to MCF-7 cells in culture (Table 5). This experiment was particularly important in the case of E_2-stearate which was able to replace E_2

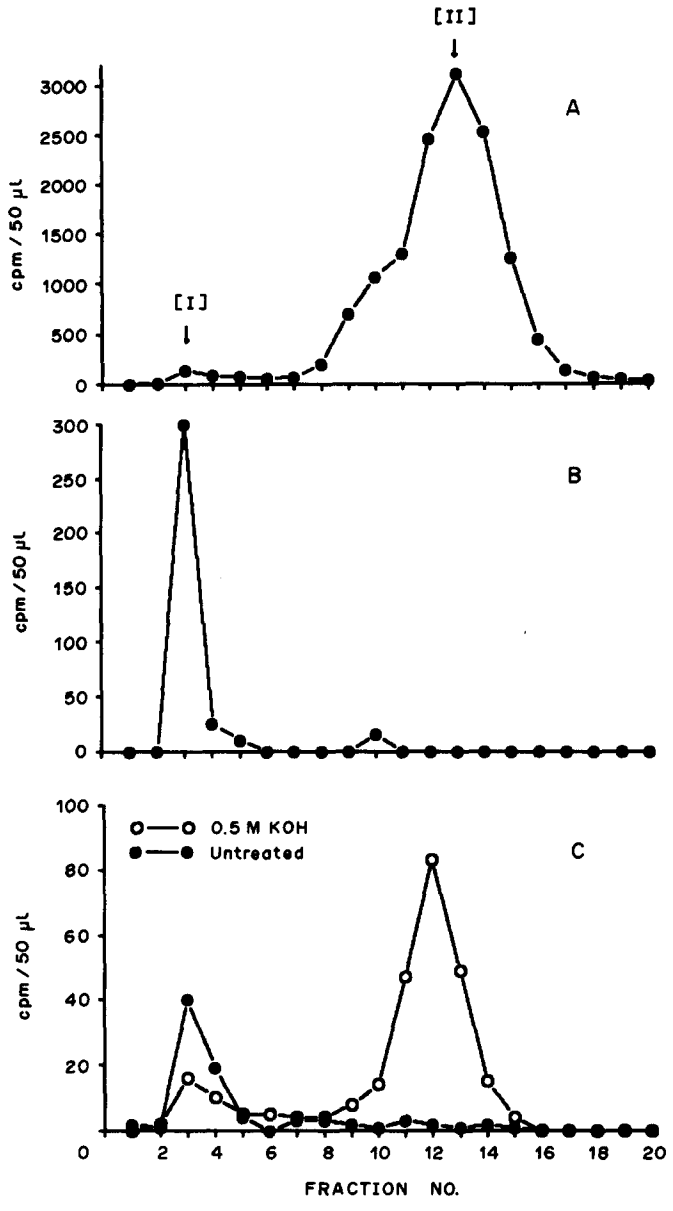

Fig. 4. Formation of lipoidal estrogens by MCF-7 cells in culture. [^{3}H]Estradiol, 59.2 ci/mmol, was added to confluent trays of MCF-7 cells in culture. After incubation for 72 h, cells scraped from the flask and media were extracted with $CHCl_3$:MEOH (2:1) and chromatographed on LH-20 as described in Fig. 1. (A) Chromatography of a chloroform–methanol extract following incubation of [^{3}H]E_2 with MCF-7 cells for 72 h. [I] Indicates the elution position of authentic E_2-valerate and E_2-stearate. [II] Indicates the elution position of authentic [^{3}H]E_2. (B) Re-chromatography of the [^{3}H]E_2 ester peak [I] [Fractions 3 and 4 from (A)]. (C) Rechromatography of fraction 3 in B after hydrolysis with 0.5 M KOH to cleave the lipoidal ester.

physiologically but did not appear to be hydrolyzed by the MCF-7 cell homogenate under standard assay conditions. When MCF-7 cells in culture were incubated with 0.1 μM [^{3}H]E_2 stearate, 26.2% of the ester was hydrolyzed in 48 h, generating enough free E_2 to achieve a physiological response. With 0.1 μM [^{3}H]E_2-valerate under these conditions, 26% hydrolysis required approximately 4 h. In control experiments, identical except for the absence of the MCF-7 cells, no detectable hydrolysis of E_2-valerate or -stearate was observed in this time period.

DISCUSSION

An estrogen-sensitive esterase activity in MCF-7 cell cytoplasm has been identified. It appears to be located intracellularly and is not released from the cells into the media.

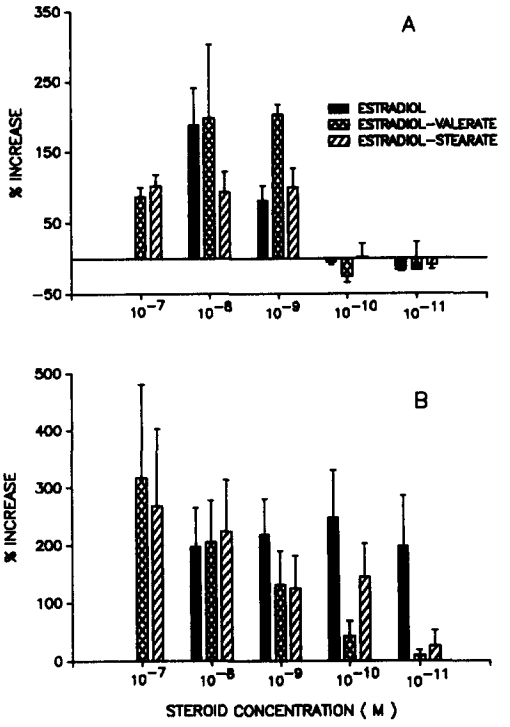

Fig. 5. Effect of E_2, E_2-valerate, and E_2-stearate on the stimulation of esterase activity and [^{3}H]thymidine incorporation by MCF-7 cells in culture. (A) Esterase activity: steroids, at the concentrations indicated, were added to confluent MCF-7 cells in 75 cm^2 flasks maintained in Phenol Red-free media containing 10% charcoal-stripped fetal bovine serum. After 60 h, cells were harvested in 0.1 M sodium phosphate, pH 7.0, homogenized and centrifuged at 28,000 *g* for 30 min. Esterase activity in the supernatant was determined with nitrophenyl hexanoate. The results, normalized for protein concentration, are the average of 3 separate experiments ± SD, and represent the increase in activity over untreated controls. (B) [^{3}H]Thymidine incorporation: steroids were added to cells plated in triplicate at a density of 2.5×10^5 in 16 mm wells in costar plates in 1 ml of media containing 5% charcoal-stripped fetal bovine serum. After 60 h, media was replaced with fresh media containing 1 μCi [^{3}H]thymidine (84.1 Ci/mmol). After 2 h, the cells were trypsinized, and the DNA precipitated with cold 10% TCA. The precipitate was washed and counted. Results are the average of at least four separate experiments ± SD and represent the increase in the incorporation of [^{3}H]thymidine over untreated controls.

Table 5. Hydrolysis of estradiol esters by MCF-7 cells in culture

	% Hydrolysis	
Time (h)	E_2-valerate	E_2-stearate
0	<0.01	<0.01
2	7.0 ± 5.1	1.3 ± 1.0
24	38.5 ± 22.8	9.4 ± 1.7
48	58.1 ± 13.5	24.1 ± 2.1

MCF-7 (P203) cells in 1 ml RPMI media containing 5% charcoal-dextran-stripped FBS were grown to confluence on 16 mm Costar plates. At $t = 0$, either [^{3}H] E_2-valerate or [^{3}H] E_2-stearate at a concentration of 0.1 μM was added. At the indicated times, 0.2 ml aliquots of the media was removed and subjected to chromatography on LH-20 as described in the materials to determine the amount of hydrolysis. Values are the average of three separate experiments ± SD. In the absence of cells under these conditions, no hydrolysis of either ester was detected after 60 h.

Fractionation by sucrose density centrifugation suggests that the esterase is not membrane bound. By gel exclusion chromatography, the esterase has an apparent mol wt. of 45–50 kDa. It is of some interest that Western blotting of the MCF-7 cell supernatant using a polyclonal, monospecific antibody to mouse esterase 1, the major esterase activity in mouse plasma [8] suggests that a protein in MCF-7 cells and mouse esterase 1 share common epitopes (T. H. Finlay and S. S. Kadner, unpublished results). Although the natural substrate of the MCF-7 cell esterase (or esterases) is unknown, it is able to cleave a variety of steroid and nitrophenyl esters with an apparent preference for esters containing short chain fatty acids. Hydrolysis of E_2-stearate and exchange of E_2 with corticosterone stearate was found to occur in intact MCF-7 cells and E_2-stearate was found to perform as well as E_2-valerate as a physiological replacement for E_2. These observations suggest that the apparent reduced rate of hydrolysis of long chain fatty acid esters by the esterase *in vitro* may be a consequence of their reduced solubility in aqueous media. Consistent with our findings, it has been reported that hydrolysis of long chain fatty acid esters of E_2 by a number of esterases including one from breast tumor homogenates does not occur except in the presence of detergents [9].

In our initial report on the properties of MCF-7 cell esterases we presented evidence for the existence of at least two different enzymes only one of which appeared to be estrogen-sensitive [4]. The zone electrophoresis experiments presented in Fig. 3 are in agreement with our earlier findings. Two distinct bands staining positively for non-specific esterase activity can be seen on these gels suggesting the presence of at least two esterases. Interestingly, the lower band appears to increase in intensity in cells treated with E_2. Whether this band represents the estrogen-stimulated esterase activity reported here and in earlier communications [4] remains to determined.

Incubation of the original MCF-7 subline (203P) with [^{3}H]E_2 resulted in no detectable incorporation of [^{3}H]E_2 into fatty acid esters. This observation is not surprising in view of the high levels of esterase activity. When the cells were incubated with [^{3}H]E_2 in the presence of an excess of unlabeled E_2-acetate (to act as a competitive inhibitor of the esterase), substantial amounts of the label were rapidly incorporated into the E_2-acetate (Fig. 1, Table 3). These results

were consistent with either esterase-catalyzed exchange or else the existence of separate degradative and synthetic enzymes. However, our results strongly suggest that the apparent synthesis of labeled E_2 esters observed in the presence of excess radioinert steroid ester results from an esterase-catalyzed exchange reaction. This finding is not unexpected as in the presence of acceptors other than water, carboxylesterases efficiently catalyze transacylation reactions [10]. Earlier we reported K_ms of approx. 0.1 μM for the hydrolysis of lipoidal estrogens by esterases present in MCF-7 cells [4]. These values were obtained under artificial conditions in aqueous solution and may be considerably greater than the K_m measured in the cytoplasm of a living cell. Under normal physiologic conditions $[S]/K_m$ ratios for most enzymes are typically between 0.01 and 1. Thus even if the physiological K_m for the hydrolysis of E_2-stearate were 0.1 μM, it would still be consistent with the concentrations reported for lipoidal steroids in MCF-7 cells [5]. However, because of the high E_2 ester concentration required for its observation, the transesterification reaction in MCF-7 cells is likely to be physiologically insignificant.

Recently Adams *et al.* reported that exposure of MCF-7 cells to low levels of [^{3}H]E_2 (1 nM) resulted in the rapid synthesis of lipoidal estrogens [5]. With higher levels of [^{3}H]E_2 (10 nM), substantial amounts of lipoidal estrogens (270 fmol/mg DNA/2 h) appeared to have been synthesized. Withdrawal of E_2 from the medium resulted in hydrolysis of the lipoidal estrogens. These results were attributed to a combination of esterification and deesterification reactions. Because of this report, we repeated our experiments with two additional cell lines. The 203P subline again did not demonstrate net estrogen ester synthesis, however, low levels of estrogen ester synthesis were found in the MCF-7ML and MCF-7BK sublines. In the light of our observation of similar esterase activities in each subline, it is likely that the differences in estradiol ester synthesis result from differences in the levels of acyl transferase. The MCF-7 cell line was originally derived from the pleural effusion of a patient with a malignant carcinoma of the breast in 1973 [11]. Unfortunately, this line has been independently passaged in different laboratories and there is considerable evidence that various MCF-7 cell sublines having marked heterogeneity now exist. Differences in antigenic phenotype [12], tumorigenicity in nude mice [13], estrogen responsiveness [13, 14], plasminogen activator activity [14] and amplification of the *N-ras* oncogene [15] have been found in MCF-7 clones from different laboratories. It is interesting that Adams *et al.* found the synthesis of lipoidal estrogens to be 3-fold higher (approx. 900 fmol/mg DNA/2 h) in 2 estrogen receptor-negative mammary cancer cell lines, an observation consistent with the existence of an estrogen-sensitive esterase activity in MCF-7 cells.

In this communication we show that in the human MCF-7 breast cancer cell line both long and short chain fatty esters of E_2 are as effective as unesterified E_2 in stimulating protein synthesis and the incorporation of [^{3}H]thymidine into DNA (Fig. 5). It should be noted, however, that [^{3}H]thymidine incorporation into TCA precipitable material may not necessarily reflect cell growth but rather perturbations in the intracellular thymidine pool [16]. This is not as remote a possibility as it seems as E_2 has been shown to increase dihydrofolate reductase levels in MCF-7 cells under certain circumstances [17]. We find the EC_{50} for the effect of the steroids on thymidine incorporation to be approximately 10-fold less than for their effect on the stimulation of esterase activity. May and Westley have found a similar range of E_2 concentrations necessary for the half-maximal stimulation of estrogen-regulated mRNAs in MCF-7 cells [18]. Although, long chain fatty acid esters of E_2 are poor substrates for the MCF-7 cell esterases, hydrolysis proceeds at a rate sufficient to provide enough free E_2 for activation of the estrogen receptor. It is possible that in normal (and malignant) breast epithelial cells a similar esterase could serve to generate E_2 from an exogenous lipoidal estrogen. If this were to be the case, then esterase levels might be of some value in predicting the outcome of certain hormonally-sensitive cancers.

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