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Catecholestrogens are Agonists of Estrogen Receptor Dependent Gene Expression in MCF-7 Cells

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The catecholestrogens, namely 2-hydroxyestradiol (2-OH-E_2) and 4-hydroxyestradiol (4-OH-E_2) are important, naturally occuring metabolites of E_2. Here we studied their role on estrogen dependent processes. Using the MCF-7 cell line as a model system we analyzed the potency of 2- and 4-OH-E_2 on the synthesis of the 160 kDa secreted protein and on the transcription of the pS2 mRNA. Both processes are known to be E_2 inducible and are mediated by the estrogen receptor. Control incubations using E_2 and antiestrogens were performed to validate the assay procedure and to enable us to comparatively study the effects of the catecholestrogens. Stimulating MCF-7 cells for 2 days with 10^{-8} M 2- or 4-OH-E_2 resulted in an induction of the synthesis of the 160 kDa protein and in an increase in pS2 mRNA. Following hormonal stimulation with 2- or 4-OH-E_2 [^{35}S]methionine labeling of MCF-7 cells increased the level of newly synthesized and secreted 160 kDa protein 54 and 88% compared with the inductive potency of E_2 (100%). The pS2 mRNA in MCF-7 cells was increased by a 2 day treatment with 10^{-8} M 2- or 4-OH-E_2 by 48 and 79%, respectively, compared to E_2. Therefore, we conclude that the estrogen receptor is transcriptionally active in MCF-7 cells upon binding of catecholestrogens. The estrogen receptor *in vivo* may be active if the intracellular concentration of catecholestrogens generated is sufficient to allow occupation of the receptor. The possible action of these hormones *in vivo* is discussed.

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INTRODUCTION

Catecholestrogens (CE) are hydroxylated derivatives of estradiol (E_2) and estrone at C_2 or C_4 [1]. CE are generated *in vivo* by the action of hydroxylases [2, 3]. A number of different enzyme systems have been described [4, 5] and synthetases are present in a variety of tissues [6–8]. By the action of catechol *O*-methyltransferase (COMT, E.C. 2.1.1.6.), CE can be further metabolized to the corresponding methylether [2, 9].

A variety of biological effects of CE have been reported similar to the catecholamine system [2, 10], their involvement in hormonal carcinogenesis [11], on LH and FSH secretion [12, 13] and on uterine dry weight [2]. However, these results in part have been controversial, in particular those of the CE derived from estrone [14].

Various groups have shown that 2- and 4-OH-E_2 are able to bind the estrogen receptor (ER) with an affinity 20 to 40% of E_2, respectively [2, 15] or higher [16]. There is, however, no experimental evidence that the effects of 2- and 4-OH-E_2 really represent processes at the cellular level similar to the cascade of E_2 induced and ER mediated gene expression [17–19].

The induction of the progesterone receptor (PR) by 2,3 dibenzene derivatives of 2- and 4-OH-E_2 [20], the effects of 2- and 4-OH-E_2 on the progesterone biosynthesis in granulosa cells [21] and the influence of 2-OH-E_2 on the growth rate of MCF-7 cells [22] all provided evidence for agonistic actions in cell culture systems.

As a a model system to study the effects of CE on gene expression we have chosen the MCF-7 cell line [23] because it is one of the best characterized model systems for actions of E_2 on gene expression, protein biosynthesis and cell proliferation [24, 25]. As a prerequisite for estrogen dependent processes, these cells

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Abbreviations: 2-OH-diol, 2-hydroxyestradiol; 4-OH-diol, 4-hydroxyestradiol; CE, catecholestrogens; COMT, catechol *O*-methyltransferase; DCC, dextrane coated charcoal; ER, estrogen receptor; FCS, fetal calf serum; PR, progesterone receptor.

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contain a high amount of functional ER [26]. Among the ER dependent processes that have been described in MCF-7 cells are stimulation of proliferation rate [27] and the increase in PR concentrations [28]. More strikingly, secreted proteins have been described, the synthesis of which is regulated by E_2 [29, 30]. Finally the pS2 mRNA has been reported to be induced by treatment of MCF-7 cells with estrogens [31].

The experiments described were undertaken to establish whether CE are capable of inducing gene expression via the ER dependent pathway. We analyzed the inductive capacity of purified 2- and 4-OH-E_2 on the synthesis of the 160 kDa protein and pS2 mRNA in MCF-7 cells. We show here that both CE tested are able to stimulate the synthesis of these ER dependent gene products, suggesting that the ER of MCF-7 cells is functional upon binding of these CE.

MATERIALS AND METHODS

Hormones and reagents

E_2, tamoxifen and L-[^{35}S]methionine were obtained from Amersham (Braunschweig, Germany). Cell culture medium was from Sigma (Deisenhofen, Germany). All chemicals were of the highest purity available.

2- and 4-OH-E_2 were prepared from E_2 using the Fremy's salt technique as described previously [32]. The purity of our CE preparation is 99% [33]. In addition, the CE used for this study were purified by preparative HPLC using a ODS-Hypersil C 18 column (Latek GmbH, Eppelheim, Germany; 5 μm pore size, 150 × 30 mm). The peaks were detected using an UV detector (Schoeffel GmbH, Germany). The hormones were crystallized from ethylacetate/petrolether, dried and a 10^{-3} M stock solution in ethanol plus 0.1% ascorbic acid and 1% acetic acid was prepared. The CE were stored at 4°C in the dark. These procedures are necessary to stabilize solutions of CE [34]. Aliquots of the stock solutions containing 30 μg of hormone were analyzed for purity and integrity by an analytical HPLC procedure using the same mobile phase and a similar column (30 × 4.6 mm) as described above.

The possible contamination of the purified CE by E_2 was analyzed by adding a small amount of E_2 [150 ng = 0.5% (w/w)] to the sample. For 2-OH-E_2 no E_2 was detectable (detection limit below 0.02% of sample size = 6 ng), 4-OH-E_2 contained a trace amount of E_2 (<0.035%). The residual concentration of E_2 in our preparation of 4-OH-E_2 leads to a contamination of 3.5×10^{-12} M if used at 10^{-8} M during incubations with MCF-7 cells. This is well below the K_d for the ER in these cells (0.037 ± 0.028 nM, $n = 9$).

Cell culture and hormonal treatment of MCF-7 cells

MCF-7 cells originally derived from the Michigan Cancer Foundation were kindly provided by Professor M. Dietel (Kiel, Germany). The MCF-7 substrain used had been selected for high levels of ER and PR [35] and was termed MCF-7 M1. Cells were maintained in DMEM–F12 (1:1) medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal calf serum (FCS). Two weeks prior to experiments, cells were transferred to the same medium lacking phenol red, supplemented as described above but using FCS treated with dextrane coated charcoal (DCC-FCS) in order to remove steroids [27]. After filtration the serum was incubated with a sterylsulfatase (2 U/ml serum, type V; Sigma) for 2 h at 37°C followed by another treatment with DCC because sulfatated estrogens might be removed incompletely by the standard DCC procedure [36].

Cells were treated with various hormones (1000-fold stock solutions) for different time intervals up to 3 days. Control cells always received the respective amount (0.1%) of the solvent (ethanol plus 0.1% ascorbic acid and 1.0% acetic acid). For each parameter studied, the absence of effects of these additives was tested.

Analysis of labeled proteins

This procedure was carried out according to Westley and Rochefort [29] with modifications. MCF-7 cells were plated in 24 well plates (50,000–100,000 cells/well) and grown for 2–4 days in the steroid free medium as described above. After hormonal treatment newly synthesized proteins were then labeled for 6 h with 20 μCi L-[^{35}S]methionine in 100 μl steroid free medium with the methionine content reduced to 10% and supplemented with 3% DCC-FCS only. Thereafter the media were collected and cells and cellular debris precipitated by centrifugation at 1000 g for 5 min. Five aliquotes each of 2 μl of the supernatants were used to determine radiolabeled amino acid incorporation into proteins. For this purpose, proteins were precipitated with 10% tricloroacetic acid for 30 min on ice using bovine serum albumin (0.5 mm/ml) as a carrier and subsequently collected on GF-50 filters (Schleicher and Schüll, Dassel, Germany). After extensive washing with 5% trichloroacetic acid and ethanol, the filters were dried and the radioactivity was measured by liquid scintillation counting. The remaining supernatants containing radiolabeled proteins were stored in 2 aliquots of about 40 μl each at −30°C until use within 1–5 days. Equal amounts of labeled proteins from different incubations (20,000–60,000 cpm) were separated by electrophoresis through a 10% polyacrylamide gel. For estimation of the molecular weights ^{14}C radiolabeled calibration proteins (Gibco, Eggenstein, Germany) were run in parallel lanes. Following electrophoresis gels were soaked in 30% methanol and 10% acetic acid for 1 h, incubated for 1 h in Enhance (NEN, Bad Homburg, Germany), dried on a gel dryer at 80°C, and exposed to Kodak XAR film for 1–5 days using an intensifying screen. Densitometric scanning of the fluorographs were performed using a Hoefer GS 300 densitometer connected to a MAC II computer.

RNA preparation, electrophoresis and transfer to nylon membranes

MCF-7 cells were plated in 2–4 175 cm^2 cell culture flasks (Nunc GmbH, Wiesbaden, Germany) and treated with hormones as described. Cells were trypsinized prior to confluency and were homogenized by addition of a solution containing 4 M guanidinium thiocyanate, 5 mM sodium citrate, 0.1 M β-mercaptoethanol and 0.5% (v/v) sarcosyl. RNA was extracted by the guanidinium thiocyanate–CsCl procedure. The pellet resulting from ultracentrifugation (105,000 *g*, 17 h, 20°C) was dissolved in diethylpyrocarbonate-treated H_2O, extracted with a buffer containing 10 mM Tris–HCl (pH 7.4), 5 mM EDTA and 1% (w/v) SDS. Following centrifugation (4000 *g*, 10 min, 4°C) the RNA was precipitated with ethanol (−20°C, overnight). The pellet was washed with 70% ethanol and finally dissolved in a small amount of diethylpyrocarbonate-treated H_2O. The integrity of the RNA was checked by electrophoresis through an agarose gel (0.9%) using methylmercuric hydroxide as the denaturing agent. Equal amounts of total RNA (5–10 μg) from cells treated with different hormones were electrophoresed as described above and transferred to a nylon membrane (Hybond N, Amersham) by electroblotting. The blotting procedure was performed for 1 h at 25 V and 4–6 h at 40 V using a conventional electroblotting apparatus (Bio Rad, München, Germany). Thereafter the immobilized RNA was crosslinked to the filter by UV-illumination.

Labeling of RNA-probes and hybridizations

pS2 and 36B4 cDNAs subcloned in pBR 322 were kindly provided by Dr Pierre Chambon (Strasbourg, France). Pst 1 restriction fragments containing coding regions were subcloned into the vector pGem 4 (Promega, Heidelberg, Germany). Radiolabeled antisense RNA-probes were synthesized by SP6-transcription using α-[^{32}P]UTP (800 Ci/mmol) to a specific activity of 1×10^8 cpm/μg RNA. Filters were prehybridized at 65°C for 4 h in a solution containing 50% formamide, 5 × SSPE (1 × SSPE = 0.18 M NaCl, 10 mM $NaPO_4$ pH 7.7, 1 mM EDTA), 5 × Denhardt's solution (1 × Denhardt's = 0.02% of ficoll, polyvinylpyrrolidone and BSA each), 0.1% SDS and 150 μg/ml sonicated DNA from calf thymus. The hybridization was carried out using a small volume of the above buffer containing 6×10^6 cpm/ml pS2 antisense RNA probe overnight at 72°C. After hybridization, the filter was washed twice with a solution containing 1 × SSPE and 1% SDS at 65°C for 20 min each, once with a solution containing 0.1 × SSPE and 0.1% SDS for 15 min at 65°C and then was exposed to Kodak XAR film at −80°C using an intensifying screen. After removal of the pS2 probe (5 mM Tris–HCl, pH 8.0, 0.2 mM EDTA, 0.1 × Denhardt at 80°C) and a 1 h prehybridization, the filter was rehybridized with the 36B4 antisense RNA-probe under identical conditions as had been used for the hybridization with the pS2 probe. The 36B4 RNA level is not influenced by steroid hormones and is widely used as an internal control for studies involving pS2 mRNA levels in MCF-7 cells. For quantification of results, the exposed films were analyzed using a densitometer (see above). The results obtained for the pS2 hybridization were normalized to the results of the 36B4 hybridization in the same lane in order to minimize variations due to RNA application and blotting efficiency.

RESULTS

Influence of 2- and 4-OH-E_2 on the synthesis of the 160 kDa protein

The basal level of the 160 kDa protein in control lanes was 2.4 ± 0.9% (*n* = 10) of total labeled proteins as calculated from densitometric scanning of the fluorographs. This basal level of the 160 kDa protein was identical in MCF-7-cultures receiving ethanol + additives or ethanol only (compare lanes a1 and a2 in Fig. 1).

Cells treated with E_2 always responded with an induction of the synthesis of the 160 kDa protein (2.6 ± 0.8-fold, *n* = 10) after treatment for 2 days with 10^{-8} M as can be seen from Fig. 1, lane b and Fig. 2,

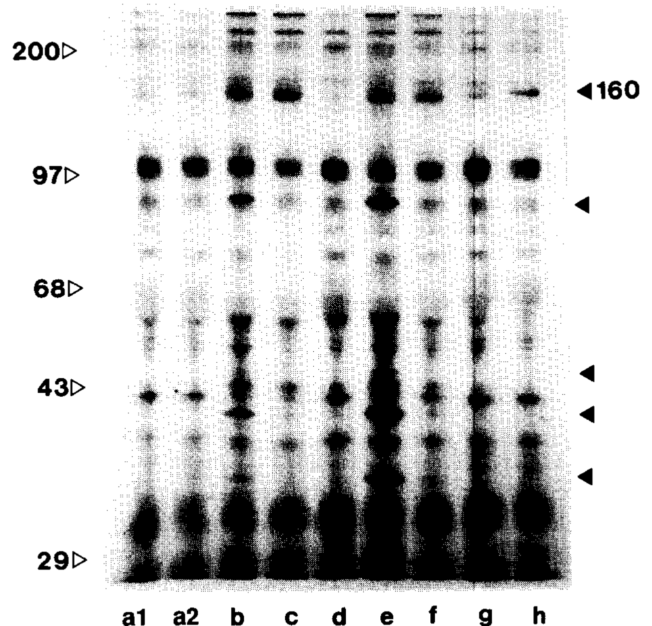

Fig. 1. Induction of the synthesis of secreted proteins of MCF-7 cells by 2-OH-E_2. Cells were seeded in wells of 24 well plates and were incubated with hormones for 2 days. Medium was changed and the hormonal concentrations thereby renewed every 12 h. Thereafter they were labeled with [^{35}S]methionine and equal amounts of the secreted proteins of the different incubations were analyzed by SDS gelelectrophoresis followed by fluorography as described in Materials and Methods. The positions of molecular weight standards electrophoresed in parallel and of the 160 kDa protein are indicated. The cells were incubated with E_2 in concentrations of 10^{-8} and 10^{-10} M (lanes b and c), with tamoxifen (10^{-6} M, lane d) or with 2-OH-E_2 at concentrations of 10^{-6}, 10^{-8}, 10^{-10} and 10^{-12} M (lanes e–h). Control cells received vehicle (ethanol + 0.1% ascorbic acid and 1.0% acetic acid (lane a1) or ethanol (lane a2) only.

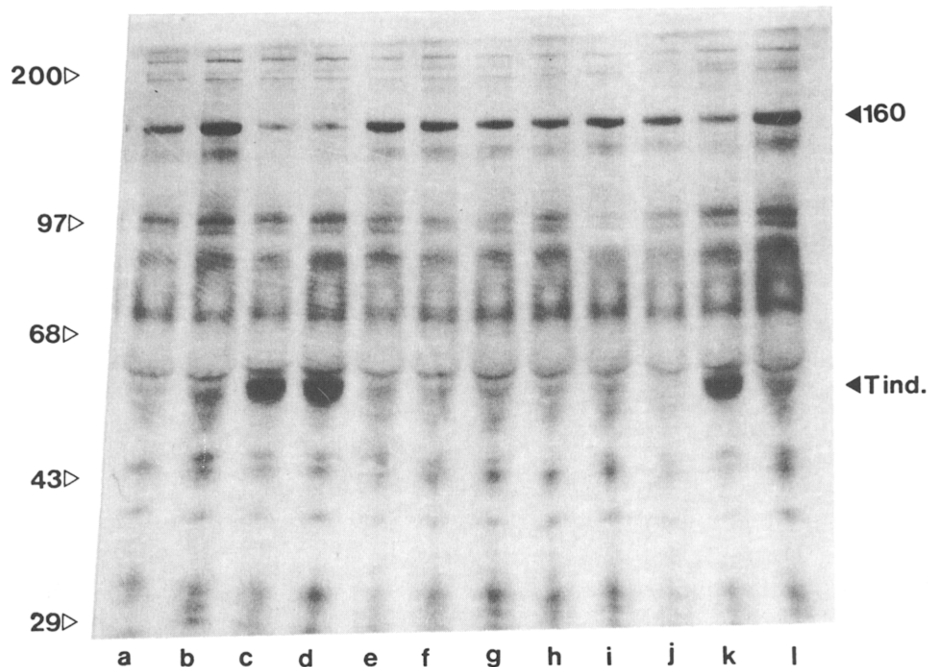

Fig. 2. Induction of the synthesis of secreted proteins of MCF-7 cells by 4-OH-E_2. For analysis we proceeded as described in Materials and Methods and in the legend of Fig. 1. The cells were incubated with E_2 in concentrations of 10^{-8} M (lane b), with tamoxifen or 4-OH-tamoxifen (10^{-6} M, lanes c and d) or with 4-OH-E_2 at concentrations of 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12} and 10^{-13} M (lanes e–j). Control cells received vehicle (ethanol + 0.1% ascorbic acid and 1.0% acetic acid, lane a) only. The cells of lane k received tamoxifen (10^{-6} M) and in lane l quinalizarin (10^{-6} M) was used in addition to 4-OH-E_2.

lane b. The 160 kDa protein synthesized and secreted from cells during antiestrogen treatment was reduced compared to control levels ($60 \pm 14\%$, $n = 8$) for tamoxifen as can be seen in Fig. 1, lane d and Fig. 2, lane c. Similarly 4-OH-tamoxifen had a reductive effect ($53 \pm 11\%$, $n = 6$) as shown in Fig. 2, lane d. One 53 kDa protein induced by the antiestrogen treatment was present as can be seen from Fig. 2, lanes c and d. The basal level of this protein was not quantitatively measurable in control cells, however, after incubations of MCF-7 cells with antiestrogens this protein increased to $6.2 \pm 2.5\%$ ($n = 8$ of 10 experiments, tamoxifen) and $6.9 \pm 1.7\%$ ($n = 6$ of 8 experiments, 4-OH-tamoxifen) of total secreted proteins.

2-OH-E_2 was able to stimulate the synthesis of the 160 kDa protein as is shown in Fig. 1. Qualitatively, the same induction of the 160 kDa protein synthesis was found with E_2 at concentrations of 10^{-8} and 10^{-10} M (induction 4.3- and 3.5-fold, Fig. 1, lanes b and c, respectively) compared to 10^{-6} and 10^{-8} M 2-OH-E_2 after stimulation with hormones for 2 days (Fig. 1, lanes e and f; induction 3.1- and 2.8-fold). However, at lower concentrations (10^{-10} and 10^{-12} M) 2-OH-E_2 had no effect compared to control cells (Fig. 1, lanes g and h). Some fluorographs revealed the presence of other induced proteins of molecular weights of 90, 50 and between 30 and 40 kDa as can be seen in Fig. 1.

4-OH-E_2 was also able to induce the synthesis of the 160 kDa protein (Fig. 2). If MCF-7 cells were incubated with 10^{-8} M for 2 days (lane e) 4-OH-E_2 led to a 2.0-fold induction vs 2.2-fold obtained from incubations with E_2 (10^{-8} M, lane b). This induction also occurred to a lesser extent at lower concentrations of 4-OH-E_2 (10^{-10} M) as can be estimated from densitometric scanning of the fluorograph (Fig. 2, lane g, 1.5-fold induction). The induction of the 160 kDa protein by 4-OH-E_2 at a concentration of 10^{-8} M was antagonized by a coincubation with 10^{-6} M tamoxifen (Fig. 2, lane k). Lane 1 of this figure corresponds to an incubation with quinalizarine, an inhibitor of COMT, at 10^{-6} M in addition to 4-OH-E_2 (10^{-8} M). Although the level of the 160 kDa protein seemed to increase compared to the 4-OH-E_2 treatment alone (lane f) densitometric scanning revealed no further induction. This behavior is probably due to a higher amount of radioactivity applied to this lane as calculated from densitometric scanning.

Further experiments were performed varying the time of hormonal treatment of MCF-7 cells with 2- and 4-OH-E_2. Densitometric scanning results are shown in Table 1. Due to reasons inherent to the methodology the absolute values of the 160 kDa protein on different gels vary, however the effects can be compared if the experiments are performed in parallel. Table 1 shows the scanning results of a representative experiment, used to study the potency of 2-OH-E_2, 4-OH-E_2 and E_2 on the synthesis of the 160 kDa protein. Parallel incubations with E_2 (when using CE 10^{-8} M for 2 days) and tamoxifen (10^{-6} M for 2 days) were performed. The effect of E_2 on the synthesis of the 160 kDa protein was already measurable after 12 h and the maximal effect of E_2 (induction 2.2 ± 0.2-fold, $n = 3$) was measured after an incubation period of 2 days. In the case of CE the effect was visible after 1.5 days of hormonal treatment and was maximal at 3 days thereby closely resembling the maximal effect of E_2 after an

incubation time of 2 days (1.8 vs 2.2-fold for 2-OH-E_2 and 1.7 vs 1.9-fold for 4-OH-E_2).

In order to compare the relative potencies of CE on the synthesis of the 160 kDa protein with E_2 we used the maximal effect of E_2 after an incubation with MCF-7 cells for 2 days as a reference. Consequently, the effects of CE were also calculated from incubations of only 2 days despite the fact that this time point does not represent the highest effect of CE measured after an incubation period of 3 days. Applying this criterion with the effect of E_2 (at 10^{-8} M for 2 days) set to 100%, 2-OH-E_2 is active at $54 \pm 12\%$ ($n = 3$) and 4-OH-E_2 at $88 \pm 20\%$ ($n = 3$).

Additional experiments were performed with renewal of hormonal conditions after 24 h (instead of the usual 12 h interval as used for the experiments described above) during hormonal treatment of MCF-7 cells: the inductive effect of 2-OH-E_2 on the synthesis of the 160 kDa protein was 48% and that of 4-OH-E_2 80% of E_2, respectively (data not shown).

Influence of 2- and 4-OH-E_2 on pS2 mRNA

RNA isolated from MCF-7 cells treated with E_2 at a concentration of 10^{-8} M for 2 days showed a marked increase in pS2 mRNA compared to RNA from control cells treated with vehicle only. The inductive capacity of E_2 was 10.4 ± 2.2-fold ($n = 6$). Control incubations of MCF-7 cells with tamoxifen (2 days at 10^{-6} M) revealed a smaller but measurable agonistic effect of 3.4 ± 1.1-fold ($n = 6$).

As can be deduced from Fig. 3, both 2- and 4-OH-E_2 were able to induce pS2 mRNA. Most strikingly, 4-OH-E_2 revealed an inductive potency comparable to E_2, whereas 2-OH-E_2 was less potent. For quantitative evaluations of the induction of the pS2 mRNA, densitometric scanning of the autoradiographs was performed and the units of pS2 signals in control and hormone treated lanes were compared. pS2 signals were normalized to 36B4 signals in each lane and the amount of pS2 mRNA in the control lane was set to 1.0. The resulting inductions of pS2 mRNA were calculated as x-fold inductions relative to control cells as shown in a representative experiment [Fig. 3(b)]. In this experiment the inductive effect of E_2 on pS2 mRNA was 12.2-fold, that of 2-OH-E_2 was 5.2-fold, and 4-OH-E_2 increased pS2 mRNA by 9.9-fold.

Table 1. Densitometric scanning of the time dependent induction of the 160 kDa protein by 2- and 4-OH-E_2

Treatment period (h)	2-OH-E_2	4-OH-E_2	E_2
12	0.9	2.6	3.0
24	1.9	3.3	2.9
36	2.5	3.7	3.5
48	2.8	4.4	4.2
72	3.1	4.7	2.3
Control incubations (48 h)			
Vehicle	1.6	2.7	1.8
Tamoxifen	1.1	1.8	1.5
E_2	3.4	5.2	

Cells were incubated with hormones for up to 3 days, labeled with [^{35}S]methionine and the secreted proteins were analyzed by SDS gelelectrophoresis followed by fluorographic treatment of the gels as described in Materials and Methods and the legends of Figs 1 and 2. Results are expressed as % 160 kDa protein of total proteins present in each lane. Cells were incubated with hormone (10^{-8} M) for 12, 24, 36, 48 and 72 h. Control cells received vehicle (ethanol + 0.1% ascorbic acid and 1.0% acetic acid) only. Additional control incubations were performed using tamoxifen (10^{-6} M) or E_2 (in experiments using CE, 10^{-8} M).

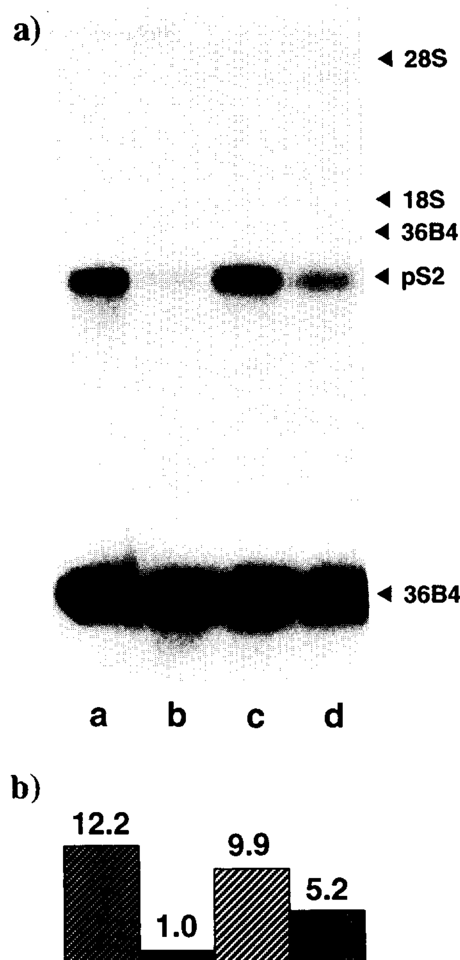

Fig. 3. Induction of the pS2 mRNA level by 2- and 4-OH-E_2. Cells of two 175 cm^2 cell culture flasks were incubated with hormones for 2 days. Medium was changed and the hormonal concentrations thereby renewed every 12 h. Thereafter RNA was isolated, electrophoresed, blotted to a nylon membrane and hybridized with a radiolabeled SP6 polymerase derived antisense RNA probe for detection of pS2 mRNA as described in Materials and Methods. Following autoradiography, the blot was stripped of radioactivity and a second hybridization was carried out using a similarly derived probe for 36B4 RNA, the level of which is not influenced by steroid hormones and consequently can be used for internal control purposes. The positions of pS2 and 36B4 mRNA and of 28 and 18 S ribosomal RNA are indicated. (a) The cells were incubated with 10^{-8} M concentrations of either E_2 (lane a), 4-OH-E_2 (lane c) or 2-OH-E_2 (lane d). Control cells received vehicle (ethanol + 0.1% ascorbic acid and 1.0% acetic acid, lane b) only. In (b) densitometric results are shown as quantitated using a GS 300 Hoefer scanning densitometer. The pS2 signal of each lane was quantitated relative to the 36B4 signals and pS2 mRNA in control cells was set to 1.0. The induction of pS2 mRNA by different hormones is expressed as n-fold induction relative to control cells.

Additional experiments were performed varying the incubation time of E_2 and 4-OH-E_2 following a single dose. As can be seen from Fig. 4(a and b) the time dependence for pS2 mRNA induction seemed to be similar for both hormones. Following the addition of E_2 for up to 24 h, a linear increase in pS2 mRNA is obvious. However, a control incubation (lane b) shows that upon renewal of hormonal conditions after 24 h pS2 mRNA was increased further. 4-OH-E_2 also showed a linear increase in pS2 mRNA. Under conditions as described in Materials and Methods (renewal of hormonal conditions after 12 h for longer incubations) a similar RNA level was reached after 2 days compared to a control incubation with E_2 performed under similar experimental conditions.

The densitometric data resulting from incubations of MCF-7 cells with E_2 at 10^{-8} M for 2 days were set to 100% and compared with the data from incubations with CE. 2-OH-E_2 is able to induce pS2 mRNA at 48% (5.5-fold, $n = 1$), whereas 4-OH-E_2 is inductive at $79 \pm 7\%$ (8.6 ± 1.8-fold, $n = 4$) of E_2.

An additional experiment performed with a change of medium and consequent renewal of hormonal stimulation every 24 h (instead of the 12 h procedure used for the experiments described above) revealed an almost equal potency of E_2 and 4-OH-E_2 (10^{-8} M each) on the induction of pS2 mRNA. In contrast, using these conditions pS2 mRNA in cells treated with 2-OH-E_2 at 10^{-8} M was not different from control cells (data not shown).

DISCUSSION

A number of proteins have been described that are under positive estrogenic control in MCF-7 cells. In addition to the 160 kDa protein analyzed here, Westley and Rochefort [29] described 46 and 19 kDa proteins. Using [^{14}C]cystein and [^{35}S]methionine labeling Sheen and Katzenellenbogen [30] detected a 32 kDa and a 52 kDa protein in addition to the 160 kDa protein. Iacobelli *et al.* [37] also described a 52 kDa protein. Using the MCF-M1 substrain of MCF-7 cells we detected only the 160 kDa protein, the synthesis of which was reproducible stimulated by E_2. Some fluorographs revealed additional proteins which are positively regulated by E_2 and 2-OH-E_2 (Fig. 1) or by E_2 and 4-OH-E_2 (data not shown). Except the 90 kDa band their molecular weights corresponded to glycosylated forms of cathepsin D as reported by Rochefort *et al.* [38]. However, because of it's highly reproducible appearance in our study we have focused on the 160 kDa protein only.

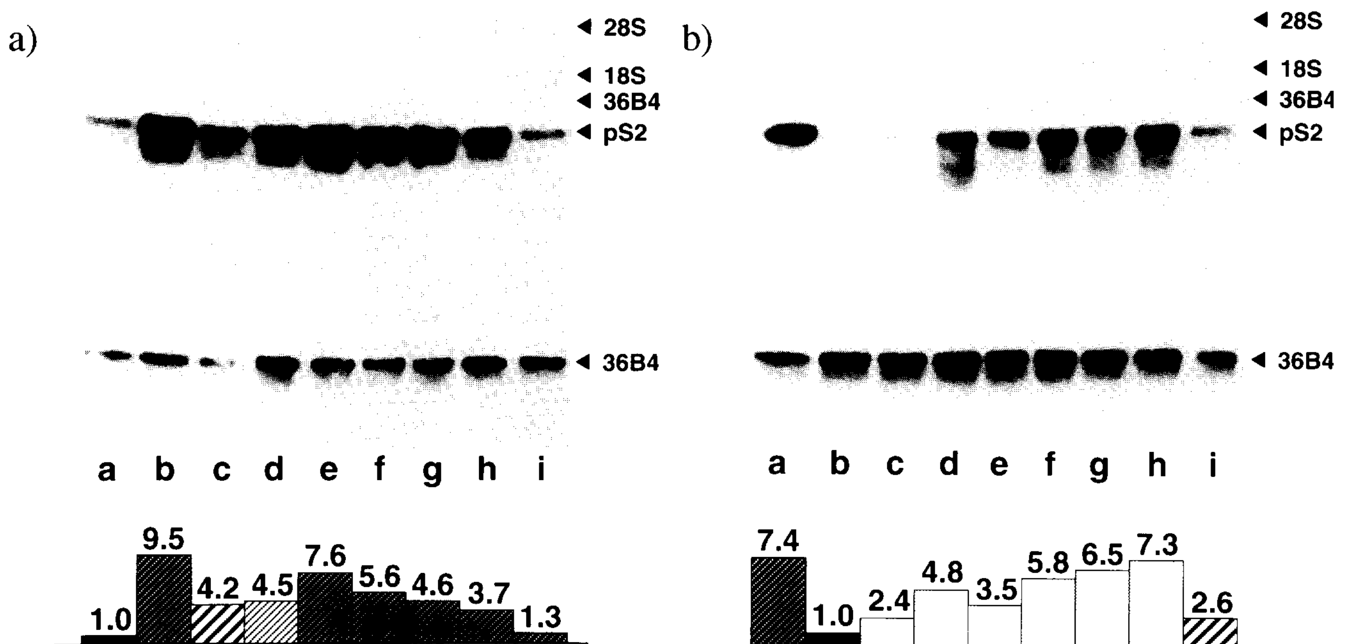

Fig. 4. Time dependence of the induction of pS2 mRNA by 4-OH-E_2 and E_2. For analysis we proceeded as described in Materials and Methods and in the legend of Fig. 3. In (a) cells received E_2 (10^{-8} M) for various time periods between 1 h and 2 days (lanes i–d). Control incubations with tamoxifen 10^{-6} M plus E_2 (10^{-8} M, lane c), and with vehicle (lane a) were carried out. The medium was not changed and the hormonal concentration was not renewed after every 12 h but every 24 h instead (for lane d). An additional control incubation is shown in lane b performed with E_2 (10^{-8} M) for 2 days with a change of medium and renewal of hormonal concentration every 12 h. In (b) the cells were incubated with a 10^{-8} M concentration of 4-OH-E_2 for 1, 2, 4, 8, 24 and 48 h (lanes c–h). Control incubations for 2 days using 10^{-8} M E_2 (lane a), 10^{-6} M tamoxifen plus 4-OH-E_2 (10^{-8} M, lane i) or vehicle (lane b) were carried out. Medium was changed and the hormonal concentrations thereby renewed every 12 h. Below a and b, densitometric results are shown as quantitated using a GS 300 Hoefer scanning densitometer. The pS2 signal of each lane was quantitated relative to the 36B4 signal and pS2 mRNA in control cells was set to 1.0. The induction of pS2 mRNA by different hormones is expressed as n-fold induction relative to control cells.

The control level of the 160 kDa protein ($2.4 \pm 0.9\%$ of total secreted proteins, $n = 10$) obtained in this study closely resembled data reported by Sheen and Katzenellenbogen [30], ($2.3 \pm 0.8\%$, $n = 11$). Their inductive effect of E_2 (2.5 to 3.0-fold) closely matches our data (2.6 ± 0.8-fold, $n = 10$). Westley *et al.* [39] reported a 3- to 4-fold induction of the synthesis of secreted 160 kDa protein by E_2. At that time, cell culture medium containing the dye phenol red was still in use, which is now known to contain lipophilic impurities leading to an estrogenic effect in control cells [40]. Sheen and Katzenellenbogen [30] described a 3-fold inductive effect measurable in phenol red free media that was reduced to 1.7-fold in medium containing phenol red. However, different results on the extent of induction of the 160 kDa protein might also depend on different substrains of MCF-7 cells used. Using low concentrations (10^{-12} M) of E_2 in our study, no effect on the synthesis of the 160 kDa protein was measurable. With E_2 at 10^{-11} M for 2 days the induction became detectable, however full induction was obtained using higher concentrations of E_2. These data are in concordance with results of Westley *et al.* [39] and Sheen and Katzenellenbogen [30]. In our study, antiestrogens used at 10^{-6} M were able to antagonize the effect of E_2 (10^{-8} M) on the synthesis of the 160 kDa protein. Tamoxifen and 4-OH-tamoxifen alone lead to a reduction of the 160 kDa protein level (60–70%) compared to control incubations. Additionally, an induction of the synthesis of a tamoxifen dependent secreted protein of 53 kDa became detectable. Sheen and Katzenellenbogen [30] also reported that antiestrogens are able to antagonize this stimulating effect of E_2 on the synthesis of the 160 kDa protein and to reduce the amount of the 160 kDa protein below control levels. In contrast to our results, they described a protein of 32 kDa that was increased by the antiestrogen treatment, the synthesis of which could be suppressed by E_2. This antagonizing effect of E_2 could also be demonstrated in our system for the 53 kDa protein. The nature of this protein and its relation to the antiestrogen induced protein described by Sheen and Katzenellenbogen remains to be determined.

Together these extensive control incubations demonstrated the functional hormonal regulation of the MCF-7 substrain used and thus enabled us to study the effects of CE on the synthesis of this protein. Both CE used strongly induced the synthesis of the 160 kDa protein after incubations of cells with hormones for 2 days at 10^{-8} M. The temporal pattern of induction of the synthesis of the 160 kDa protein was different when incubations with E_2 or CE were compared. E_2 induced the synthesis of the 160 kDa protein after an incubation of 12 h and reached a maximal effect after 2 days of incubations with MCF-7 cells as reported by Sheen and Katzenellenbogen [30] and Westley *et al.* [39]. In contrast, the effect of both CE became visible between 24–36 h and had reached a maximum after 3 days. Therefore, the kinetics of induction of the 160 kDa protein by CE appear to be slower. Another difference between E_2 and CE can be delineated from the results obtained by varying the concentrations of the hormones: At 10^{-8} M, 2-OH-E_2 had 50%, and 4-OH-E_2 had 88% of the inductive effect of E_2 on the newly synthesized and secreted 160 kDa protein. However, at 10^{-10} M, 2-OH-E_2 was inactive and 4-OH-E_2 exerted a reduced activity whereas E_2 still was strongly active at this concentration. Moreover, 2- and 4-OH-E_2, in contrast to E_2, were only partially able to suppress the inductive effect of tamoxifen on the 53 kDa protein, whereas E_2 fully antagonized the inductive effect of tamoxifen.

Our experiments demonstrate that the inductive effect of E_2 on pS2 mRNA was already measurable after the shortest incubation period tested (1 h). Brown *et al.* [31] described an increase in pS2 mRNA after 15 min. The inductive effect of E_2 found in our study corresponded to the reported range [31, 41, 42]. The inductive effect of E_2 used at 10^{-8} M for 2 days on pS2 mRNA was reduced by the addition of tamoxifen at 10^{-6} M to about 30% of the induction of pS2 mRNA measured by E_2 alone. These results qualitatively correspond to those reported by May and Westley [43] for 4-OH-tamoxifen which is 1/10 as potent as E_2. An earlier report by Westley *et al.* [39] showed no effect of tamoxifen on pS2 mRNA, however in this latter study phenol red containing medium was used. Weaver *et al.* [41] reported a 2.5-fold induction of pS2 mRNA in MCF-7 cells (=20% of effect of E_2) using phenol red free media, this finding was not measurable using phenol red containing medium.

Our control incubations showed that the hormonal control mechanisms were functional in the MCF-7 cells used for this study and thus enabled us to comparatively study the effects of CE on pS2 mRNA: the effect on pS2 mRNA by 2-OH-E_2 was less prominent compared to 4-OH-E_2. If medium was changed with a consequent renewal of hormonal conditions once in 24 h instead of the 12 h period used in all other experiments shown, 2-OH-E_2 at a concentration of 10^{-8} M had not effect on pS2 mRNA whereas 4-OH-E_2 was qualitatively as active as E_2 (data not shown). The relative inductive capacities of the CE after 2 days of hormonal stimulation of MCF-7 cells at 10^{-8} M were in the same range as the inductive effect on the synthesis of the 160 kDa protein (48%, 2-OH-E_2; 79% 4-OH-E_2). The time dependence of the induction of pS2 mRNA by 4-OH-E_2 was similar compared to E_2.

The synthesis of the 160 kDa protein and pS2 mRNA are believed to be ER dependent [30, 31]. Therefore, it can be concluded that the effects of CE as shown in this report are mediated by the ER in MCF-7 cells.

Pilat *et al.* [44] recently also published effects of CE on pS2 mRNA in MCF-7 cells. Surprisingly in their study the compound with the higher ER binding affinity (4-OH-E_2 compared to 2-OH-E_2) had no effect whereas 2-OH-E_2 had some effect on the pS2 mRNA level. The reason for this discrepancy to our results is

not clear but may rely on different substrains of MCF-7 cells used. In our study both CE had effects on pS2 mRNA level and the synthesis of the 160 kDa protein. In addition the higher effects of 4-OH-E_2 compared to 2-OH-E_2 on these ER dependent processes reflect their different ER binding affinities.

Interestingly, a lower induction of the 160 kDa protein and pS2 mRNA by 2-OH-E_2 compared to 4-OH-E_2 was obtained, although both hormones were added at a concentration higher than the K_d of the hormones to the receptor. Also, by lowering the concentrations of the CE, the difference between 2- and 4-OH-E_2 on the synthesis of the 160 kDa protein increased. In addition, the relative potency of both CE compared to E_2 decreased at lower concentrations. The induction of pS2 mRNA by 2-OH-E_2 was only measurable if the hormonal conditions were reconstituted every 12 h and was not seen if reconstitution of the hormonal environment was performed at 24 h intervals.

The reasons for these findings might either be due to an inactivation of CE in the cell culture medium by artificial degradation or, more likely, by the action of COMT which is present in MCF-7 cells [45]. This enzyme converts the CE into the corresponding methylethers which have low receptor binding affinities [2]. The formation of CE-methylethers in MCF-7 cells has been demonstrated by Brueggemeier *et al.* [46] and occured also in our cells (unpublished results). Another reason for different potencies of 2-OH-E_2 compared to 4-OH-E_2 is the higher dissociation rate of 2-OH-E_2/ER compared to the dissociation kinetics of E_2/ER and 4-OH-E_2/ER [47]. Consequently this suggests that the potencies of CE on the processes described in this study would be higher if a steady state concentration of 2- or 4-OH-E_2 would have been applied.

The short half life of CE *in vivo* is due to the inactivation of CE by the action of COMT in the bloodstream [48]. However, as was first discussed by Fishman and Norton [6], they may act inside the cell where they have been formed. Recently an intracrine pathway of hormone action has been postulated [49]. In addition to the classical steroid hormone receptors an increasing number of orphan receptors with so far unknown ligands have been described [50, 51].

Unfortunately no non-toxic inhibitors of CE action or synthesis are available to date enabling studies on the intracellular concentrations of these hormones. However, synthetases present in different tissues have been described over the past years in brain [2, 8, 52], liver microsomes [5, 53], mouse uterus [3] and human placenta [4]. The presence of these hydroxylases and the effects of 2- and 4-OH-E_2 on the synthesis of the 160 kDa protein and pS2 mRNA in particular, as shown in this report, provide strong evidence for the activity of the ER on these processes.

In conclusion, in MCF-7 cells, CE stimulated processes known to be inducible by E_2 and to be ER dependent. Despite the presence of COMT which can inactivate the hormones by metabolizing the CE to the corresponding methylethers, the distinct effects on newly synthesized and secreted 160 kDa protein and on pS2 mRNA were measurable. Thus it can be concluded that the ER is transcriptionally active if occupied by CE in MCF-7 cells.

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