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Ligand structure influences autologous downregulation of estrogen receptor–alpha messenger RNA

M.D. Davis [1], J.A. VanderKuur [2], S.C. Brooks*

Department of Biochemistry and Molecular Biology and the Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, 540 E. Canfield, Detroit, MI 48201, USA

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Abstract

A series of A- and D-ring substituted estrogen analogues have been examined for their effect on estrogen receptor-alpha (ERα) mRNA downregulation. Recently it has been proposed that ERα autologous downregulation occurs via transcriptional repression exerted by the binding of the ERα–ligand complex to the 5′ region of the coding region of the ERα gene. Placement of the phenolic hydroxyl group on the various carbons of the aromatic A-ring of estratrien-17βol (carbons 1–3) produced ligands which diminished the steady state level of ERα mRNA in relation to their affinity for receptor. 4-Hydoxyestratrien-17βol, on the other hand, was inactive in the downregulation of ERα mRNA. Although this A-ring isomer brought about apparent processing of the nuclear receptor, the ERα reappeared in the cytosol within 24 h. Unlike the stimulation of genes regulated via estrogen response elements, maximum autologous negative regulation of the ERα gene required the presence of an hydroxyl group on carbon 17 of the D-ring. These results suggest that the conformational alterations elicited in the ERα molecule by various ligands create surfaces capable of interacting with other transcription factors in a manner which is different when the receptor functions via a response element mechanism relative to interactions during autologous negative regulation of the ERα gene.

1. Introduction

Cells of hormone-dependent breast tumors contain a specific protein member of the nuclear-receptor superfamily which binds estradiol-17β (E_2) with high affinity. Activation of this receptor by ligand binding results in conformational changes which enable the complex to interact with, and perform as, transcriptional regulation factor(s). Positive regulation of estrogen responsive genes has been documented by numerous laboratories [1–6]. Information pertaining to negative regulation of transcription by estrogens is limited. Estrogens are known to reduce the transcription of c-jun [7] and the β-subunits of glycoprotein hormones [8] as well as various other uncharacterized proteins in MCF-7 cells [9]. However, the mechanism for this negative regulation remains obscure. E_2 has been shown to decrease the steady state levels of both estrogen receptor-alpha (ERα) mRNA and protein in many estrogen responsive tissues and cell lines (chick oviduct, endometrial cancer cells and MCF-7 and other cancer cells) by many investigators [10–13].

ER 'processing' was initially defined as the loss of approximately 50% of ligand binding, or monoclonal antibody recognizable, ERα within 6 h of E_2 exposure, which is not explained by the return of these 'processed' receptors to the cytosol [14]. However, the cloning of the ERα cDNA allowed a more detailed examination of this phenomenon and processing recently has been associated with downregulation of ERα mRNA [13,15,16]. The observed decrease in the rate of ERα gene transcription was cycloheximide resistant and dependent on the concentration of E_2

* Corresponding author. Tel.: +1-313-577-1511; fax: +1-313-577-4112.

[1] Present address: Department of Internal Medicine, University of Michigan, Ann Arbor MI.

[2] Present address: Department of Physiology, University of Michigan, Ann Arbor MI.

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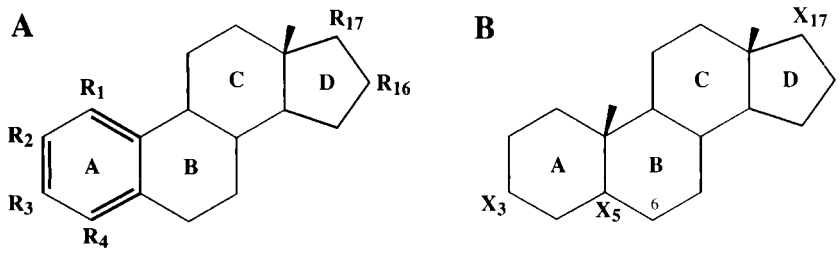

A: ESTROGENS	R_1	R_2	R_3	R_4	R_{16}	R_{17}
Estratriene	H_2	H_2	H_2	H_2	H_2	H_2
3-Hydroxyestratriene	H_2	H_2	OH	H_2	H_2	H_2
Estratriene-17β -ol	H_2	H_2	H_2	H_2	H_2	β-OH
Estradiol-17β (E_2)	H_2	H_2	OH	H_2	H_2	β-OH
Estradiol-17α	H_2	H_2	OH	H_2	H_2	α-OH
Estradiol-16α	H_2	H_2	OH	H_2	α-OH	H_2
1-Hydroxyestratrien-17β-ol	OH	H_2	H_2	H_2	H_2	β-OH
2-Hydroxyestratrien-17β-ol	H_2	OH	H_2	H_2	H_2	β-OH
4-Hydroxyestratrien-17β-ol	H_2	H_2	H_2	OH	H_2	β-OH
Estrone	H_2	H_2	OH	H_2	H_2	keto
Estriol	H_2	H_2	OH	H_2	α-OH	β-OH

B: ANDROGENS	X_3	X_{17}	X_5
5-Androstene-3β,17β-diol	β-OH	β-OH	C5-C6 double bond
5α-Androstane-3β,17β-diol	β-OH	β-OH	α-H
5α-Androstane-3α,17β-diol	α-OH	β-OH	α-H

Fig. 1. Structures of estogen analogues and androstanediols.

[15,16]. The absence of an estrogen response element (ERE) in the promoter of the gene suggests a unique ERα–DNA interaction in the regulation of the ERα gene. Recently, it has been proposed that ERα (or glucocorticoid receptor) autologous downregulation occurs via a transcriptional repression exerted by the estrogen (or glucocorticoid) receptor on their genes [17,18]. It is proposed that these receptors bind to specific DNA sequences within the gene. The rate at which functional ERα is synthesized and degraded (turnover) in estrogen responsive cells is a major factor in regulating the level of ERα and therefore cell responsiveness to E_2. Modulation of receptor level is quite possibly one mechanism which governs target gene transcription in response to estrogens.

Recent reports from this laboratory have demonstrated an influence of estrogen structure on the positive regulation of certain estrogen responsive genes (progesterone receptor [6], cathepsin D and pS2 [5] and tissue plasminogen activator [3]) in MCF-7 cells. As with positive regulation, the ligand-dependent downregulation of ERα may involve an estrogen structure-dependent mechanism. The studies described herein examined the effect of ERα downregulation of 14 estrogen ligands with modifications to the A- and D-rings.

2. Materials and methods

2.1. Steroids, antiestrogens and inhibitors (Fig. 1)

Estratriene, estrone, E_2, estradiol-16α and estradiol-17α were purchased from Research Plus (Bayonne, NJ). Other estrogens used in these investigations were synthesized in this laboratory. 1-, 2- and 4-hydroxyestratrien-17β-ol, estratrien-17β-ol and 3-hydroxyestratriene were synthesized according to published procedures [19–21]. The purity of each estrogen analogue was guaranteed by thin layer chromatography and crystallization. Chromatography was carried out on thin layer chromatography plates (silica gel), developed three times with hexane:ethylacetate (5:5), which resolved approximately one mg of the compounds (3–4 μmol). After exposure to iodine, spots were visualized under ultraviolet light (254 nm). This procedure enabled detection of 0.1 nmol of E_2. Thus, each analogue was determined to contain less than one part in 10,000 of contaminating estrogens [6].

The androstanediols (5-androsten-3β,17-β-diol, 5α-androstan-3β,17-β-diol and 5α-androstan-3α,17-β-diol) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and purified by thin layer chromatography as described above [6]. The antiestrogen, 4-hydroxytamoxifen, was a gift from Stuart Pharmaceuticals (Division of ICI Americas, Wilmington, DE) and ICI-164,384 was kindly supplied by Dr. A.E. Wakeling, Imperial Chemical Industries (Alderly Park, UK). Cycloheximide and actinomycin-D were purchased from Sigma, St. Louis, MO.

2.2. Cell culture

A subclone of MCF-7 designated E3 [22] was maintained in a custom formulation of Eagles modified MEM (Gibco Formula 86-5126EF, Gaithersburg, MD) with Hank's balanced salt solution (HBSS), L-glutamine, 25 mM HEPES without phenol red and supplemented with nonessential amino acids (Sigma, St. Louis, MO), 5% donor calf serum (Flow Labs, Lot #9010763, McLean, VA), 0.5 μg/ml gentamicin sulfate (Sigma), and 0.01 μg/ml phenol red (Sigma). Cells were incubated at 37°C in closed flasks. All experiments were carried out on cells which had been passed between 170 to 200 times. For experiments, cells were passed at approximately 2×10^6 cells/75 cm^2 tissue culture flask (Corning, Corning, NY) and allowed to grow for 5–6 days (approximately 70% confluent) in MEM supplemented with 5% donor calf serum, 0.5 μg/ml gentamicin and phenol red. Cells were then transferred to a phenol red-free media that contained dextran-coated charcoal (DCC) treated [23] calf serum (Gibco Lot #38N1091). Cultures were allowed to grow for three days. After this estrogen withdrawal period, the cells were washed with phosphate buffered saline (PBS; 137 mM sodium chloride, 2.7 mM potassium chloride, 6.5 mM dibasic sodium phosphate, 1.5 mM monobasic potassium phosphate, pH 7.4). The cells were then exposed to the estrogens (10^{-11} to 10^{-6} M), added in 20 μl ethanol, for 18 h in the media containing DCC treated serum. Experiments were carried out in duplicate.

2.3. ER-EIA

The treated cells were washed with cold saline and removed from flasks in 5 ml MEM utilizing a wide bore syringe followed by centrifugation of the cell suspension at $150 \times g$ for 10 min at 4°C. For the determination of cytosolic receptor cells in the pellet were ruptured with a Dounce homogenizer in ice-cold immunoassay buffer (10 mM tris, 1.5 mM EDTA, 5.0 mM sodium molibdate pH 7.4 with 1.15 μM monothioglycerol). The homogenate was centrifuged for 1 h at $100{,}000 \times g$ at 47°C and the supernatant assayed for ERα using the enzyme immunoassay (ER-EIA) kit supplied by Abbot Laboratories (Chicago, IL). This kit utilized the sandwich technique employing the D547 and H222 ERα specific antibodies. The pellets were utilized for DNA determination by the method of Burton [24].

Determination of the nuclear ERα was carried out according to the protocol described by Kral et al. [25]. Briefly, the cellular pellet was resuspended in 1 ml of 0.5% NP_{40} cell lysis buffer (10 mM HEPES, pH 7.4 at 47°C, 10 mM NaCl, 5 mM monothioglycerol, 0.5% NP_{40} and 5 mM magnesium chloride) and kept cold for 10 min with occasional mixing. Following a second lysis, the nuclear pellet (150 g, 10 min) was extracted at 4°C with lysis buffer containing 0.4 M KCl (2 ml) by mixing every 2 min for 10 min. This mixture was centrifuged and the nuclear extract assayed (EIA) for the amount of nuclear estrogen receptor complex and the pellets were utilized for DNA determinations. The ER-EIA Kit contains lyophilized ERα standard from MCF-7 cells from which a standard curve was constructed. This method has been shown to recover 95% of the receptor in MCF-7 cells [25].

2.4. Messenger RNA isolation and Northern analysis

Messenger RNA was isolated from cell lysates according to a modified one-step procedure of Chomczynski and Sacchi [26] and the mRNA resolved by electrophoresis (3.0–6.0 μg per lane) on a 1.2% agarose gel as reported previously [3]. Northern analysis was performed [3] with radioactive probes prepared with the cDNAs for the human ERα and the 36B4 housekeeping gene. The washed and dried membranes were exposed to film (Kodak Diagnostic Film X-

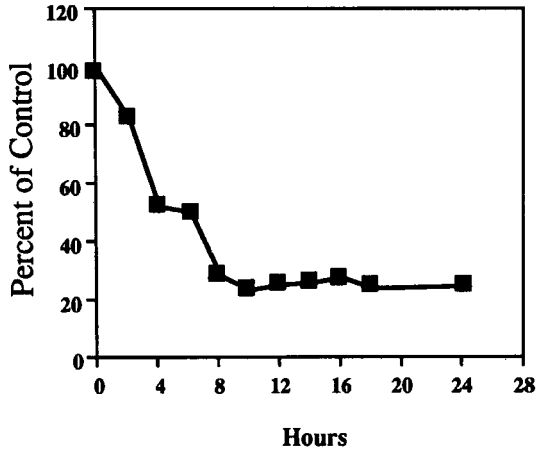

Fig. 2. Plot of the time-dependent downregulation of ERα mRNA in MCF-7 cells (clone E-3) by E_2. Confluent cultures maintained in estrogen free media 72 h were exposed to 10^{-8}M E_2 for the noted times and harvested. mRNA was collected as described in the Materials and methods. Desitometric analysis of the autoradiographs from Northern blots of the mRNA samples were normalized to the internal standard (36B4) and plotted as percentage of the normalized control (time = 0). Points are the average of duplicate flasks.

OMAT, Rochester, NY) for 18 h before developing and scanned with a Molecular Dynamics densitometer employing the Imagequent™ software (Sunnyvale, CA). Recorded density units were related to the amount of RNA applied to each lane and to the radioactivity in each band from the hybridized cDNA.

2.5. Preparation of DNA probes for Northern analysis

The 1.9 kb cDNA for ERα and the estrogen insensitive gene, 36B4 (both cDNAs graciously supplied by P. Chambon), were inserted into pBR322. These plasmids were transformed into competent cells of *E. coli* strain DH5alpha (Gibco/BRL), amplified, extracted and purified using procedures described previously [3]. Restriction digests were performed [3] utilizing Pst1 for 36B4; and *EcoR1* for ERα cDNA and fragments purified by agarose gel electrophoresis. The inserts were cut out and recovered via electroelution for 10–12 h using an Elutrap (Schleicher and Schuell).

Radiolabeled probes of each cDNA were made [3] using a commercially available random primed labeling kit (Boehringer Mennhein, Indianapolis, IN). A 1 μl sample from each probe was counted in a Packard Tri-Carb 4530 scintillation counter (Packard Instruments, Downers Grove, IL) to determine the radioactivity. Approximately 2.0×10^7 cpm of each probe was added to the hybridization buffer after denaturation at 100°C for 4 min.

3. Results

3.1. The effect of E_2 and antiestrogen

Exposure of cultures of MCF-7 cells to 10^{-8}M E_2 for varying periods of time brought about a reduction in the expression of ERα mRNA (Fig. 2). After 2 h the level of ERα mRNA had decreased to 82% of that originally in the MCF-7 cells. This negative regulation continued, reaching a minimum value after 10 h (Fig. 2). The low level of ERα mRNA expression was maintained for the remaining 14 h of the experiment. Others have shown ERα mRNA expression to be repressed for at least 48 h after exposure of MCF-7 cells to E_2 [13,15]. E_2 downregulation of ERα mRNA was maximal at a concentration of 10^{-11} M, with higher concentrations being equally effective (Fig. 3(A), depicted as percent maximum downregulation).

Two vastly different antiestrogens effectively stimulated the downregulation of ERα mRNA after 18 h exposure. ICI164,384 a 'pure' antiestrogen reduced the cellular levels of ERα comparable to the effect of E_2 at a concentration of 10^{-10} M (Fig. 4). Likewise the partial agonist, 4-hydroxytamoxifen, decreased ERα to 50% of its initial level in untreated cells when present in culture media at 10^{-9} M. The apparent downregulation (following a 12 h exposure) of ERα mRNA by 4-hydroxytamoxifen has been reported [16]. The effect of ICI164,384 on ERα protein has been attributed to an increased rate of turnover of the receptor [27,28]. However, other antiestrogens such as LY117018 are ineffective [15]. Progesterone, which is often shown to possess antiestrogen activity blocks the E_2 autologous downregulation of ERα in these cells [15]. Yet in T47D cells, progestins are effective in the suppression of this receptor by blocking ERα synthesis [15].

3.2. Effect of structurally altered estrogens and androstanediols

The estratriene nucleus, devoid of hydroxyl groups, was capable of effecting a minimal downregulation of ERα mRNA over a concentration range of 10^{-9}–10^{-7} M (Fig. 3(B)). This repression of ERα mRNA accumulation occurred regardless of the immeasurably weak affinity of estratriene for ERα (relative binding affinity, RBA < 0.0005, relative to E_2, $K_a = 3.7 \times 10^9$ M^{-1}, RBA = 1.0). Repositioning the phenolic hydroxyl of E_2 to other A-ring carbons yielded estrogens with varying effects on ERα mRNA repression. 1-Hydroxyestratrien-17β-ol was 10,000-fold less active than E_2, decreasing ERα mRNA maximally at 10^{-7} M (Fig. 3(C)). This compound binds weakly to ERα, with a RBA of 0.005. 2-Hydroxyestratrien-17β-ol, which binds 150 times tighter (RBA = 0.71) to the receptor than 1-hydroxyestratrien-17β-ol was 100-fold more

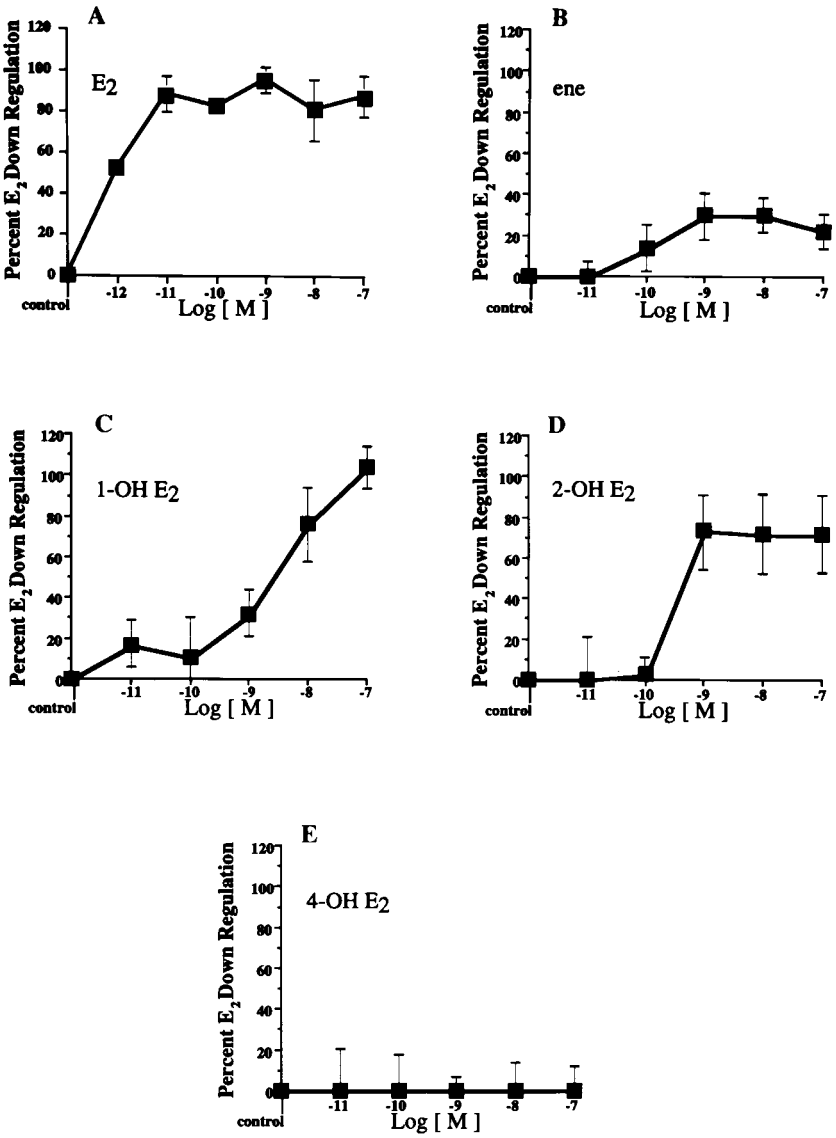

Fig. 3. Percent maximum downregulation of ERα mRNA in MCF-7 cells (clone E3) pulsed with different concentrations of estrogen analogs. Cultures were maintained and exposed to estrogens for 18 h as described in Materials and methods. mRNA was collected as described in Materials and methods. Densitometric analysis of the autoradiographs from Northern blots of the mRNA samples were normalized to the internal standard (36B4) and plotted as a percentage of maximum E_2 (10^{-8}M) downregulation. In these experiments, the normalized E_2 density ranged from 35–65% of control values. Cultures were treated with (A) E_2, (B) estratriene (ene), (C) 1-hydroxyestratrien-17β (1-OHE_2), (D) 2-hydroxyestratrien-17β-ol (2-OHE_2) and (E) 4-hydroxyestratrien-17β-ol (4-OHE_2). Standard deviations of several determinations are shown as brackets.

active, downregulating ER mRNA maximally at 10^{-9} M (Fig. 3(D)). Interestingly, 4-hydroxyestratrien-17β-ol, which bound ERα an order of magnitude better than 1-hydroxyestratrien-17β-ol (RBA = 0.07) was completely inactive in the downregulation of ERα mRNA, over the entire concentration range tested (Fig. 3(E)). This A-ring isomer, which has been shown to function as an antiestrogen [3], effectively blocked E_2 downregulation of ERα mRNA when it's concentration in the culture medium reached 10^{-7} M, a level at which 4-hydroxyestratrien-17βol competed with the added 10^{-8} M E_2 for receptor (the K_a of 4-hydroxyestratrien-17β-ol is 1/10th that of E_2, Fig. 5).

Placement of a phenolic hydroxyl group on position-3 of estratriene yielded a monohydroxyestrogen capable of downregulating ER mRNA half maximally, at concentrations of 10^{-10}M and above (Fig. 6(A)). 3-Hydroxyestratriene was incapable of decreasing ER mRNA to the level brought about by 10^{-11}M E_2 when added to cultures of MCF-7 cells at concentrations up to 10^{-7}M. This compound, 3-hydroxyestratriene, has a high affinity for ERα, binding approximately 80% as

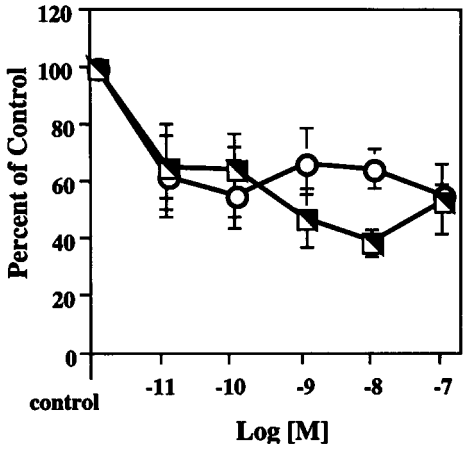

Fig. 4. Downregulation of ERα mRNA by antiestrogens. Confluent cultures were maintained in estrogen free media for 72 h then exposed to antiestrogens for 18 h and harvested. mRNA was collected as described in the Materials and methods. Downregulation of ERα mRNA by ICI 164,384 (○) and 4-hydroxytamoxifen (◥) is shown. Standard deviations are depicted as brackets for four to six experiments.

well as E_2 (RBA = 0.79). On the other hand, placing the D-ring hydroxyl group of E_2 on estratriene yielded a monohydroxyestrogen, estratrien-17β-ol (RBA = 0.11) with maximal negative regulation of ERα mRNA at 10^{-8}M (Fig. 6(B)). In fact, all ligands tested which possessed a 17-hydroxyl group (e.g. E_2, Fig. 3(A), estradiol-17β, RBA = 0.22 and estriol, RBA = 0.17, Fig. 6(C and E)) brought about maximal negative regulation of ERα mRNA. As with 3-hydroxyestratriene, a 16α-hydroxyl (estradiol-16α, RBA = 0.80, Fig. 6(D)) or a 17-keto substituent (estrone, RBA = 0.22, Fig. 6(F)) produced half-maximal downregulation of the ERα mRNA.

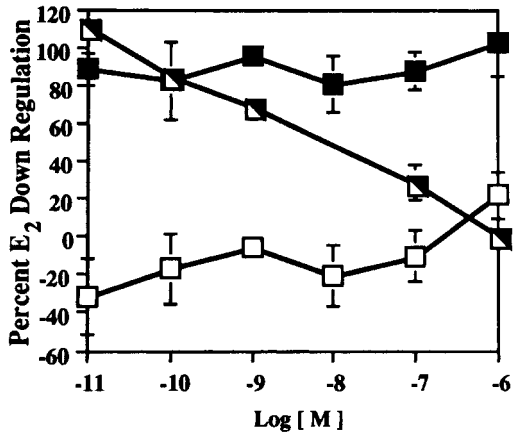

Fig. 5. Downregulation of ERα mRNA in MCF-7 cells (clone E-3) by E_2, 4-hydroxyestratrien-17β-ol and combinations. Confluent cultures were maintained in estrogen free media for 72 h then were supplemented with increasing concentrations of 4-hydroxyestratrien-17β-ol (□); increasing concentrations of 4-hydroxyestratrien-17β-ol + 10^{-8}M E_2 (◥) or increasing concentrations of E_2 (■) and harvested after 18 h. mRNA was collected as described in the Materials and methods. Densitometric analysis of the autoradiographs from Northern blots of the mRNA samples were normalized to the internal standard (36B4) and plotted as percentages of the normalized control sample (35–65%). Standard deviations of several experiments are shown as brackets.

Table 1
EC_{50} values of A- and D-ring substituted estrogens

Analogue	RBA[a]	EC_{50}[b]
(1) Estradiol-17β	1.0	1.1×10^{-11}
(2) Estradiol-16α	0.80	1.0×10^{-10}
(3) 3-Hydroxyestratriene	0.79	1.0×10^{-10}
(4) 2-Hydroxyestratrien-17β-ol	0.67	3.1×10^{-9}
(5) Estrone	0.27	7.9×10^{-10}
(6) Estradiol-17α	0.27	7.7×10^{-10}
(7) Estriol	0.17	1.4×10^{-9}
(8) Estratrien-17β-ol	0.11	4.5×10^{-10}
(9) 4-Hydroxyestratrien-17β-ol	0.071	1.0×10^{-6}
(10) 5-Androstene-3β, 17β-diol	0.0073	3.7×10^{-9}
(11) 5α-Androstane-3β,17β-diol	0.0049	1.0×10^{-8}
(12) 1-Hydroxyestratrien-17β-ol	0.0046	8.4×10^{-9}
(13) 5α-Androstane-3α,17β-diol	< 0.0005	6.1×10^{-7}
(14) Estratriene	< 0.0005	1.0×10^{-6}

[a] Relative binding affinities determined by the competitive binding assay as described in Vanderkuur et al. [6]. E_2, $K_a = 3.7 = 1.5 \times 10^9 M^{-1}$ is set at 1.0.

[b] EC_{50} is defined as the concentration of analogue which produces half maximal response. EC_{50} was calculated for each analogue as log [(% of maximal E2 response)/[100−(% of maximal E2 response)]. Compounds that did not reach 50% maximal response were given an arbitrary property value of 1×10^{-6} M (Sybyl Molecular Modeling Program — Theory Manual, 1992).

Without the aromatic character of the A-ring of E_2, steroids such as 5α-androstane-3β, 17β-diol (RBA = 0.005) or 5-androstene-3β, 17β-diol (RBA = 0.007) were capable of downregulating ER mRNA maximally at a 10^{-7} M concentration, yielding an EC_{50} between 10^{-8} and 10^{-9} M (Table 1). Repositioning the orien-

Table 2
Down-regulation of ERα mRNA in MCF-7 cells exposed to inhibitors of RNA and protein synthesis

Treatment[a]	Relative percentage of control[b]		
	$-E_2$	$+E_2$	Effect of added E_2 % Change[c]
None	100	34.2 ± 17.7	−65.8
Actinomycin-D	17.0 ± 15.5	18.4 ± 14.2	+8.2
Cycloheximide	41.0 ± 12.0	19.6 ± 12.0	−52.2

[a] MCF-7 cells were exposed to no inhibitor (none), actinomycin-D (4.0 μM), or cycloheximide (50 μM) in estrogen free media for 20 min prior to exposure ethanol vehicle ($-E_2$) or 10^{-8}M E_2 ($+E_2$). The cells were harvested after 18 h exposure.

[b] Value represents the relative percentage of the corrected band density from Northern analysis of mRNA isolated from control cells which were not treated with inhibitor or exposed to estradiol.

[c] Difference in the level of ERα mRNA in control ($-E_2$) and E2 treated ($+E_2$) cells in control (none) or inhibitor treated cells.

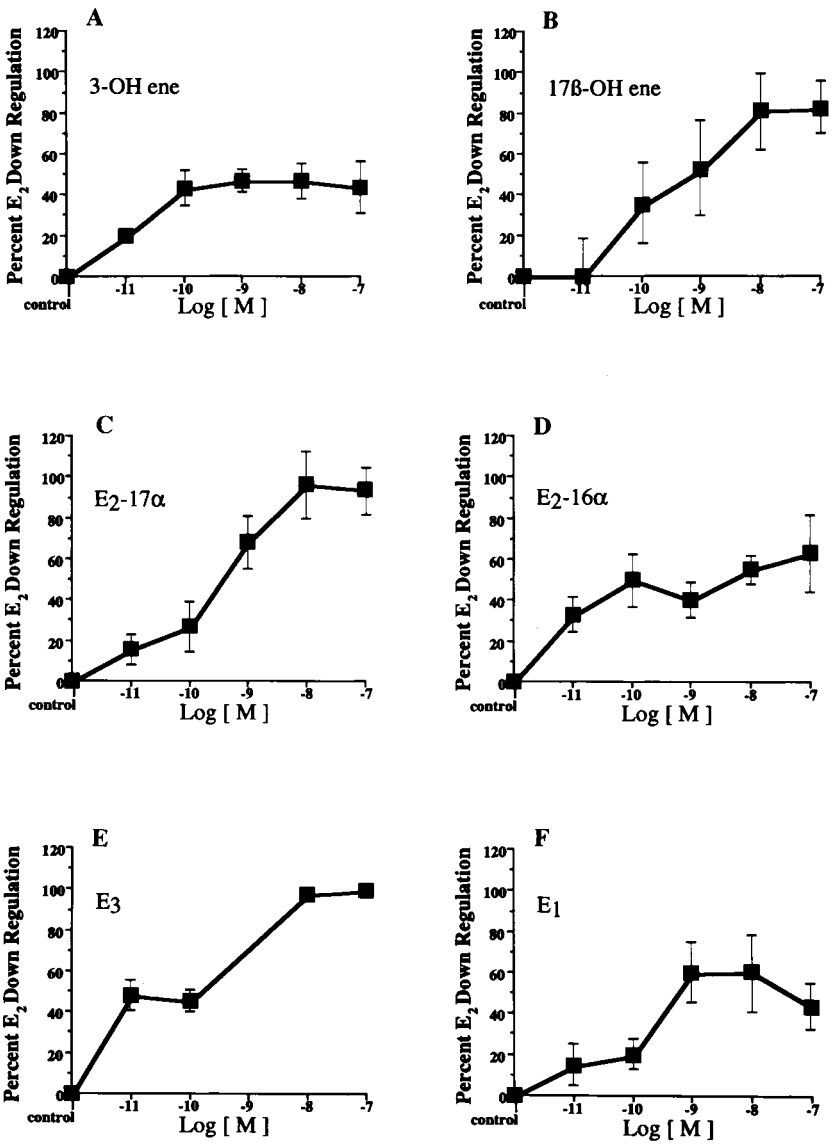

Fig. 6. Percent maximum downregulation of ERα mRNA in MCF-7 cells (clone E3) pulsed with different concentrations of estrogen analogues. Cultures were maintained and exposed to estrogens for 18 h and the mRNA was collected as described in Materials and methods. Densitometric analysis of the autoradiographs from Northern blots of the mRNA samples were normalized to the internal standard (36B4) and plotted as a percentage of maximum E_2 downregulation. In these experiments the normalized E_2 density ranged from 35–65% of control values. Cultures were treated with (A) 3-hydroxyestratriene (3-OHene), (B) estratrien-17β-ol (17β-OHene), (C) estradiol-17α(E_2-17α), (D) estradiol-16α(E_2-16α), estriol (E_3), (E) estrone (E_1). Standard deviations of several experiments are shown as brackets.

tation of the 3β-hydroxyl group of 5α-androstane-3β, 17β-diol to the 3α configuration, resulted in a compound, 5α-androstane-3α,17β-diol (RBA = < 0.0005), which brought about less than half maximal downregulation of ERα mRNA accumulation at the highest concentration tested 10^{-7} M (Table 1).

3.3. Effect of inhibitors

Actinomycin-D is known to reduce the E_2 induced downregulation of ERα mRNA in MCF-7 cells [14,29]. Confirming these observations, we found the estrogen-dependent reduction in the steady state level of ERα mRNA was totally blocked by 4.0 μM actinomycin-D (Table 2). Incubation of MCF-7 cells with cycloheximide has no apparent effect on the estrogen induced downregulation of ERα mRNA under the conditions of our experiments (Table 2 and Ref. [13]). However, both actinomycin-D and cycloheximide caused an apparent reduction in the overall yield of ERα mRNA in these experiments (Table 2).

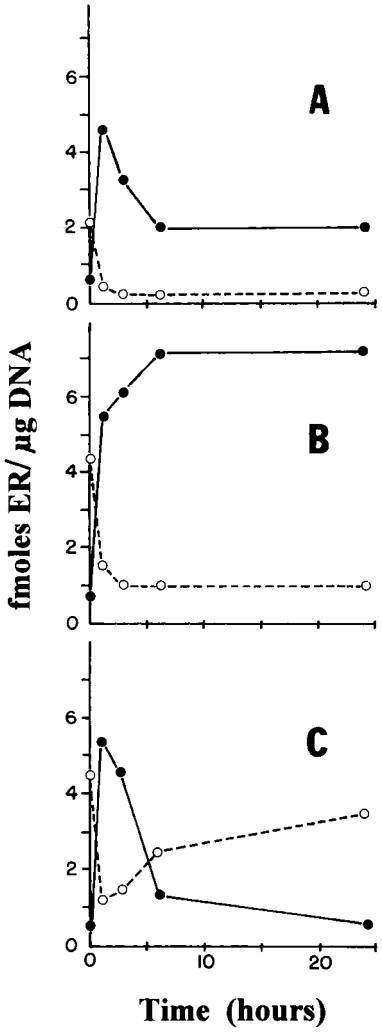

Fig. 7. Levels of cytosolic (○–○) and nuclear (●-●) ERα during a 24-h exposure of MCF-7 cells to: (A) E_2 (10^{-8}M), (B) 4-hydroxytamoxifen (10^{-8}M) and (C) 4-hydroxy-estratrien-17β-ol (10^{-7} M). EIA were carried out as described in Materials and methods. Points are the mean of several determinations with standard deviations of less than 10%. Patterns shown were independent of ligand concentration.

3.4. Processing of ER in MCF-7 cells

The often reported 'classical' loss of ER in MCF-7 cells treated with E_2 is depicted in Fig. 7(A). Within 1 h of exposure to E_2, a maximum level of tightly bound receptor is found in the nuclear fraction, followed within 6 h by a diminished level (50%) of detectable ER which persists for 24 h. Concurrently, cytosolic ER remained low from 1 to 24 h. In these experiments 4-hydroxytamoxifen was capable of bringing about tight nuclear binding. However, processing of this nuclear receptor did not occur (Fig. 7(B)). On the other hand, 4-hydroxyestratrien-17βol, promptly brought about the binding of ER to DNA, which disappeared from the nuclear fraction by 6 h. However, in the case of this A-ring isomer the level of nuclear ER continued to diminish for 24 h (Fig. 7(C)). Unlike any of the other ligands examined in these experiments, immediately after the binding of ER to nuclei (1 h), receptor began to reappear in the cytosol following administration of the 4-hydroxy isomer of E_2. By 24 h, 3.5 fmol/µg DNA of cytosolic ER was detected, or 80% of the initial ER was present (cytosol and nuclear). All of the other estrogen analogues yielded a pattern of processing similar to that of E_2 (data not shown).

4. Discussion

ER processing has been documented in mammary carcinoma cells and many other experimental systems [12,14,30]. This phenomenon undoubtedly influences a target cell's response to estrogen stimulation. The purpose of this study was to examine the effects of structurally altered estrogens on the homologous downregulation ('processing') of ERα mRNA. The MCF-7 cell line chosen for these studies expresses ample levels of ERα mRNA and protein. ERβ protein has not been detected in these cells and only trace quantities of the mRNA for this receptor subtype has been detected by PCR [31].

Binding affinities for ERα of the various estrogens and androstanediols employed in these experiments have been calibrated [6], and shown, with two exceptions, to decrease as much as 200-fold less than the K_a of E_2. The exceptions being estratriene and 5α-androstan-3α,17β-diol which possessed affinities too low to measure. Nevertheless, the range of concentrations of these compounds added to cultures of MCF-7 cells was 10^4-fold above the level (10^{-11} M) at which E_2 displayed its maximum repression of ERα mRNA levels. Therefore each ligand (with the exception of estratriene and 5α-androstan-3α,17β-diol) examined would be expected to influence the 'processing' of ERα mRNA in this system, assuming in each case, the activity was related directly to the K_a for ERα. Overall, a plot which depicts the capacity of each compound to stimulate negative regulation of ERα mRNA shows excellent correlation ($R^2 = 0.82$) with the binding affinity for receptor of the estrogens and androstanediols (Fig. 8). The only anomaly was 4-hydroxyestratrien-17β-ol which bound receptor with an affinity comparable to, or greater than, a number of estrogens that were active in the downregulation of ERα mRNA. Yet, this dihydroxyestrogen was inactive. Previously, it had been demonstrated that 4-hydroxyestratrien-17β-ol was incapable of stimulating the induction of several mRNA's (cathepsin D and pS2, [5] and tissue plasminogen activator [3], also Fig. 8) whereas all other estrogen analogues tested induced these genes. In fact,

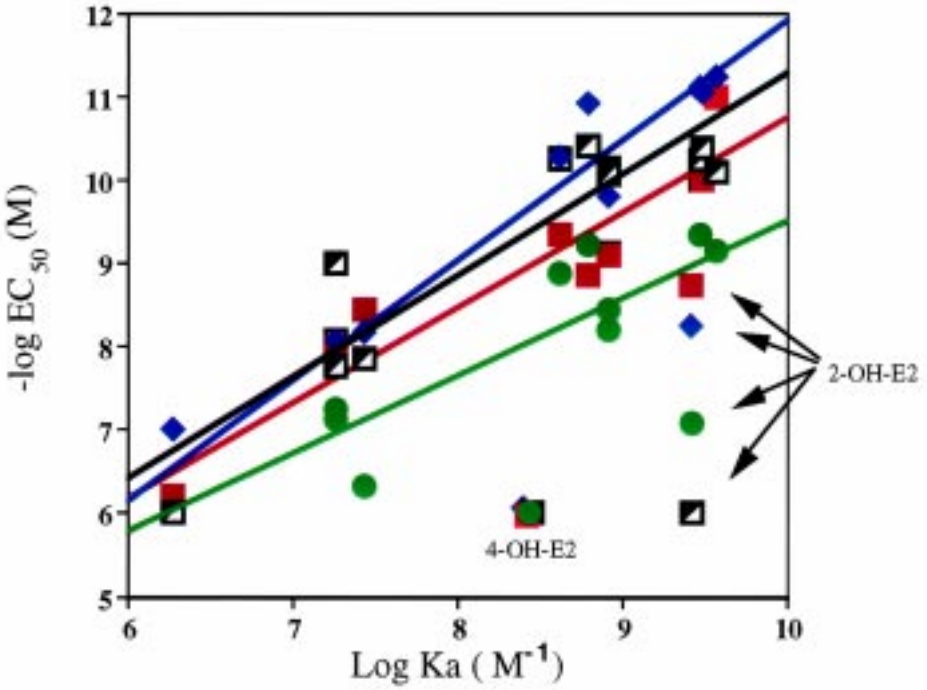

Fig. 8. Relation of K_a of selected estrogens and androstanediols to the induction of Cath D mRNA (◩); pS2 mRNA (◇), t-PA mRNA (○) and the downregulation of ER mRNA (□. EC_{50} is defined as the effective concentration which produced a half-maximal response. The EC_{50} of each analogue was calculated as log [% of maximal E_2 response]/[100−(% of maximal E_2 response)]. Analogs which had an inductive effect less than 50% maximal response were given a low and arbitrary property value of 10^{-6} [3,5]. K_a's were determined from the RBAs using the K_a of E_2 ($3.7 \times 10^9 M^{-1}$). R^2 values for the lines determined from the Cath D mRNA, pS2 mRNA, t-PA mRNA and ER mRNA results are 0.85, 0.97, 0.89 and 0.92, respectively, when the points for 2-hydroxyestratrien-17β-ol and 4-hydroxyestratrien-17β-ol are omitted. The ligands listed in Table 1 were used for these experiments.

there was a strong correlation between gene induction and receptor affinity in these previous experiments.

Although the estrogen analogues employed in these experiments have not, as yet, been examined by others, there exist abundant studies of ligand structure–estrogen receptor affinity relationships. These published data have recently been utilized to create an estrogen pharmacophore [32]. Regarding the K_a of estrogen analogues for receptor, these data demonstrated, as had Anstead et al. [32], that the C3- and 17β-hydroxyl groups are major contributors to ligand affinity, with the phenolic group contributing the greatest energy as an H-donor. The proximity of the 2-hydroxyl group apparently allows it to participate as an H-donor as well. A 3D QSAR study of 42 estrogen-like analogues (including those in this report) has been carried out in our laboratory [33] in which contour plots created for receptor affinity, as well as for a biologic response (growth) were compared. Most notable in these plots are the expanded steric and electropositive tolerance zones around the A-ring. Also, steric and electrostatic contours around the D-ring are depicted in this model which help explain the preference for 17β-hydroxysteroids over the less biologically active 17α and 16α-hydroxy compounds.

As we have suggested, endogenous gene regulation by estrogens is not related directly to the affinity of ligand for ERα, nor to the presence of either hydroxyl group, but this activity may be determined by the geographic location of the electronegative isopotential above the A-ring and the influence a variation in this structural characteristic may exert on the conformation of the receptor complex and its capacity to interact with the transcription apparatus [3,5,6,33].

The atypical effect of 4-hydroxyestratrien-17β-ol on the conformation of full length ERα has been demonstrated in gel-shift assays [34]. In experiments wherein the concentrations of ligands were adjusted to assure receptor occupancy, 4-hydroxyestratrien-17β-ol did not induce a shift of the ERE–ER complex relative to that brought about by E_2. Nevertheless, certain other estrogens (3-hydroxyestratriene, estratrien-17β-ol and estradiol-17α) active in gene regulation experiments also did not induce a shift in the DNA–ER complex. It can be concluded that, indeed, the conformational changes generated in the ERE–ER complex by 4-hydroxyestratrien-17β-ol are different from those induced by E_2. However, the changes in conformation highlighted by gel-shift assays are not necessarily related to the gene regulatory activity.

The downregulation of ERα has been attributed to a diminished steady state level of the receptor's mRNA brought about by estrogenic ligands when bound to ERα [4,13–16]. In the absence of an estrogen response element in the promoter of this gene, the initial autologous negative regulation of ERα is the result of the binding of receptor complex to the 5′-end of the coding sequence of the ERα gene, thereby suppressing the transcription rate [17,18]. Recovery of transcription from this negative regulation within 3 h [13], suggests that the prolonged depression of ERα mRNA results from a second, posttranscriptional event [13,16,17]. Therefore, the typical picture of ERα processing involves the rapid tight binding of cellular ERα to the nuclear compartment following the interaction of receptor with an estrogen ligand. Within 6 h, 50–60% of this nuclear-bound protein is seen to become undetectable by antibody or tritiated ligand assays (Fig. 7, Refs. [4,6,13–15]). This diminished receptor status remains for at least 48 h [13,15].

The described pattern is typical of most estrogenic ligands including 9 of the 10 estrogen analogues studied in these experiments (Table 1). An unusual pattern was elicited by 4-hydroxyestratrien-17β-ol which did bring about the rapid tight nuclear binding of ERα. However, as the nuclear receptor decreased to levels as low as 15% of total Erα, cells treated with this A-ring isomer displayed a reappearance of ERα in the cytosol (Fig. 7(C)). Not only was this ligand unable to downregulate the early transcription of Erα mRNA (Fig. 3(E)), but the typical posttranscriptional depression of receptor was not apparent. Nevertheless, at a concentration commensurate with its binding affinity, this A-ring isomer was able to block the downregulation of ERα mRNA by E_2 (Fig. 4). Indicating that the ERα–4-hydroxyestratrien-17β-ol complex formed an initial tight association within the nuclear compartment. This analogue, however, did not facilitate the posttranscriptional suppression of ERα noted by others [13,17].

Antiestrogens, such as 4-hydroxytamoxifen and ICI164,384 display yet another effect on ERα synthesis and processing. These ligands downregulate ERα mRNA in a concentration-dependent fashion after an 18 h incubation (Fig. 4). The downregulation of ERα mRNA by 4-hydroxytamoxifen after a period of 12 h has been seen previously [16]. In our experiments this late decrease in ERα mRNA (to 50% the level in untreated cells) is not reflected in receptor protein as detected by monoclonal antibody over a period of 24 h (Fig. 7(B)). This lack of processing of ERα following a pulse of 4-hyroxytamoxifen has been reported by Kaneko et al. [17] in Rat1 + ER cells. Others have seen partial processing of ERα by tamoxifen [14]. Therefore, although this antiestrogen brought about a downregulation of ERα mRNA, it had no effect on the posttranscriptional process, resulting in its inability to decrease the levels of ERα in MCF-7 cells during 24 h of treatment (Figs. 4 and 7(B)).

The nature of the posttranscriptioal process involved in the downregulation of ERα is presently unknown. A recent publication [35] suggests that phosphorylation events directed to the transactivtion function-2 domain may play a role in receptor downregulation. In addition, a number of phosphorylation sites in the A/B region of ERα are known to be hormone sensitive. Phosphorylation of ERα potentiates its affinity for DNA [36] and treatment of the receptor with phosphotase diminished its DNA-binding activity [37]. Interestingly, 4-hydroxyestratrien-17β-ol was the only estrogen analogue which has been shown in unpublished investigations in this laboratory to block the phosphorylation of ERα.

Results from these studies of ERα mRNA downregulation by estrogen ligands reveal specific ligand structural requirements which have not been characteristic of gene regulation via the binding of receptor complex to the ERE. For example, the estratriene nucleus which possesses a low affinity for ERα and was inactive in target gene regulation [3,5,6], displayed autologous negative regulation of ER mRNA at concentrations as low as 10^{-10} M (Fig. 2(B)). In addition, unlike the regulation of the progestrone receptor [6], pS2 [5], cathepsin D [5] and tissue plasminogen activator [3] genes, the placement of the D-ring hydroxyl group was critical to maximal downregulation of ERα mRNA (Fig. 6). In the absence of a hydroxyl group on carbon-17, the ligand was only capable of generating half-maximal depletion of ERα mRNA (Fig. 5). Whereas these same D-ring variations in the estrogen molecule generated ligands which stimulated the above target genes to a degree proportional to their affinity [3,5,6]. This information suggests that the conformational alterations elicited in the ERα molecule by the various ligands, impinge upon other transcription factors differently when the receptor functions via the response element directed mechanisms than they may during autologous negative regulation of the ERα gene.

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References

[1] M. Beato, Gene regulation by steroid hormones, Cell 56 (1994) 335–344.
[2] A.M.C. Brown, J.-M. Jeltsch, M. Roberts, P. Chambon,

Activation of pS2 gene transcription is a primary response to estrogen in the human breast cancer cell line MCF-7, Proc. Natl. Acad. Sci. USA 81 (1984) 6344–6348.
[3] M.D. Davis, W.B. Butler, S.C. Brooks, Induction of tissue plasminogen activator mRNA and activity by structurally altered estrogens, J. Steroid Biochem. Mol. Biol. 52 (1995) 421–430.
[4] R.L. Eckert, B.S. Katzenellenbogen, Effects of estrogens and antiestrogens on estrogen receptor dynamics and the induction of progesterone receptor in MCF-7 human breast cancer cells, Cancer Res. 42 (1982) 139–144.
[5] M.J. Pilat, M.S. Hafner, L.G. Kral, S.C. Brooks, Differential induction of pS2 and cathepsin D mRNAs by structurally altered estrogens, Biochemistry 32 (1993) 7009–7015.
[6] J.A. VanderKuur, T. Wiese, S.C. Brooks, Influence of estrogen structure on nuclear binding and progesterone receptor induction by the receptor complex, Biochemistry 32 (1993) 7002–7008.
[7] C.K. Lu, M. Subramanian, K. Rasmussen, T.C. Spelsberg, Rapid inhibition of the c-jun proto-oncogene expression in avian oviduct by estrogen, Endocrinology 127 (1991) 2595–2597.
[8] M.A. Shupnik, S.D. Gharib, W.W. Chin, Estrogen suppresses rat gonadotrophin gene transcription in vivo, Endocrinology 122 (1990) 1842–1846.
[9] J.A. VanderKuur, S.C. Brooks, Effects of A-ring isomers of estradiol-17β on gene products in MCF-7 cells, Steroids 59 (1994) 548–554.
[10] R.B. Dickson, M.E. Lippman, Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma, Endocr. Rev. 8 (1987) 29–43.
[11] M.M.S. Gottardis, S.P. Robinson, V.C. Jordan, Estradiol-stimulated growth of MCF-7 tumors implanted in athymic mice: a model to study the tumoristatic action of tamoxifen, J. Steroid Biochem. 30 (1988) 311–314.
[12] B.I. Maxwell, D.P. McDonnell, O.M. Conneely, T.Z. Schulz, G.L. Greene, B.W. O'Malley, Structural organization and regulation of the chicken estrogen receptor, Mol. Endocrinol. 1 (1987) 25–35.
[13] M. Saceda, M.E. Lippman, P. Chambon, R.L. Lindsey, M. Ponglikitmongkol, M. Puente, M.B. Martin, Regulation of the estrogen receptor in MCF-7 cells by estradiol, Mol. Endocrinol. 2 (1988) 1157–1162.
[14] K.B. Horwitz, W.L. McGuire, Estrogen control of progestrone receptor in human breast cancer: correlation with nuclear processing of estrogen receptor, J. Biol. Chem. 253 (1978) 2223–2228.
[15] L.D. Read, G.L. Greene, B.S. Katzenellenbogen, Regulation of estrogen receptor messenger ribonucleic acid and protein levels in human breast cancer cell lines, by sex steroid hormones, their antagonists and growth factors, Mol. Endocrinol. 3 (1989) 295–304.
[16] M. Saceda, M.E. Lippman, R.K. Lindsey, M. Puente, M.B. Martin, Role of an estrogen receptor-dependent mechanism in the regulation of estrogen receptor mRNA in MCF-7 cells, Mol. Endocrinol. 3 (1989) 1782–1787.
[17] K.J. Kaneko, J.D. Furlow, J. Gorski, Involvement of the coding sequence for the estrogen receptor gene in autologous ligand-dependent downregulation, Mol. Endocrinol. 7 (1993) 879–888.
[18] S. Santagati, E. Gianazza, P. Agrati, E. Vegeto, C. Patrone, G. Pilliio, A. Maggi, Oligonucleotide squelching reveals the mechanism of estrogen receptor autologous downregulation, Mol. Endocrinol. 11 (1997) 938–949.
[19] E. Palomino, M.J. Heeg, J.P. Horwitz, S.C. Brooks, Binding, X-ray and NMR of the three A-ring isomers of natural estradiol, J. Steroid. Biochem. 35 (1990) 219–229.
[20] J.P. Horwitz, V.K. Iyer, H.B. Kardhan, J. Corombos, S.C. Brooks, In vitro inhibition of estrogen sulfoconjugation by some 2- and 4- substituted estra-1,3,5(10)-trien-17β-ols, J. Med. Chem. 29 (1986) 692–698.
[21] H. Dannenberg, T. Kohler, Uber die durch Friedel-Crafts-Acetylierung von 1,3,5(10)-Ostratrien-Verbindungen zuganglichen Amine, Chem. Ber. 97 (1964) 140–150.
[22] W.B. Butler, P.J. Berlinski, R.M. Hillman, W.H. Kelsey, M.M. Toeninges, Relation of in vitro properties to tumorigenicity for a series of sublines of the human breast cancer cell line MCF-7, Cancer 46 (1986) 6339–6348.
[23] T.E. Wiese, L.G. Kral, K.E. Dennis, W.B. Butler, S.C. Brooks, Optimization of estrogen growth response in MCF-7 cells, In Vitro Cell Dev. Biol. 28A (1992) 595–602.
[24] K. Burton, A study of the conditions and mechanism of the diphenylamine reaction for the calorimetric estimation of deoxyribonucleic acid, Biochem. J. 62 (1956) 315–322.
[25] L.G. Kral, L.M. Doherty, S.C. Brooks, Quantitative determination of nuclear estrogen receptors by and enzyme immunoassay with applicability and caveats, J. Steroid Biochem. 31 (1988) 459–466.
[26] P. Chomcynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[27] D.P. McDonnell, D.L. Clemm, T. Hermann, M.E. Goldman, J.W. Pike, Analysis of estrogen receptor function in vitro reveals three distinct classes of antiestrogens, Mol. Endocrinol. 9 (1995) 659–669.
[28] S. Dauvois, P.S. Danielian, R. White, M.G. Parker, Antiestrogen ICI164,384 reduces cellular estrogen receptor content by increasing its turnover, Proc. Natl. Acad. Sci. USA 89 (1992) 4037–4041.
[29] J.S. Strobl, A. Kasid, K.K. Huff, M.E. Lippmann, Kinetic alterations in estrogen receptors associated with estrogen receptor processing in human breast cancer cells, Endocrinology 115 (1984) 1116–1124.
[30] M. Borras, L. Hard, F. Lempeueur, A.M. el Khissiin, N. Legros, R. Gol-Winkler, G. Leclercq, Estradiol-induced down-regulation of estrogen receptor: effect of various modulators of protein synthesis and expression, J. Steroid Biochem. Mol. Biol. 48 (1994) 325–336.
[31] T. Watanabe, S. Inoue, S. Ogawa, Y. Ishii, H. Hiroi, K. Ikeda, A. Orimo, M. Muramatsu, Agonistic effect of tamoxifen is dependent on cell type, ERE-promoter context and estrogen receptor subtype: functional difference between estrogen receptors α and β, Biochem. Biophys. Res. Commun. 236 (1997) 140–145.
[32] G.M. Anstead, K.E. Carlson, J.A. Katzenellengogen, The estradiol pharmacophore: ligand structure–estrogen receptor binding affinity relationships and a model for the receptor binding site, Steroids 62 (1997) 268–303.
[33] T.E. Wiese, L.A. Polin, E. Palomino, S.C. Brooks, Induction of the estrogen specific mitogenic response of MCF-7 cells by selected analogues of estradiol – 17β: a 3D QSAR study, J. Med. Chem. 40 (1997) 3659–3669.
[34] J.K. Christman, S. Nehls, L. Polin, S.C. Brooks, Relationship between estrogen structure and conformational changes in estrogen receptor/DNA complexes, J. Steroid Biochem. Mol. Biol. 54 (1995) 201–210.
[35] S. Santagati, P. Ciana, C. Colli, G. Pallio, E. Vegeto, A. Maggi, Autologous down-regulation of estrogen receptor-α, in: Proceedings 80th Annual Meeting of The Endocrine Society, New Orleans, June 24–27, 1997, 1997, p. 233 (Pl-557).
[36] S.F. Arnold, J.D. Obourn, J. Jaffe, A.C. Notides, Serine 167 is the major estradiol-induced phosphorylation site on the human estrogen receptor, Mol. Endocrinol. 8 (1994) 1208–1214.
[37] R.R. Denton, N.J. Koszewski, A.C. Notides, Estrogen receptor phosphorylation: hormonal dependence and consequence on specific DNA binding, J. Biol. Chem. 267 (1992) 7263–7268.