Influence of Estrogen Structure on Nuclear Binding and Progesterone Receptor Induction by the Receptor Complex†

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ABSTRACT: The relationship between steroid structure, estrogen receptor (ER) binding affinity, nuclear binding of the ER complex, and induction of progesterone receptor (PgR) have been examined. The level of ER in membrane-free homogenates of MCF-7 cells was found to be 10.0 ± 0.5 fmol/μg of DNA by utilizing an enzyme immunoassay (EIA). However, only 2.5 ± 0.2 fmol of ER complex/μg of DNA was bound by nuclei during maximal stimulation of PgR synthesis (2.9 ± 0.2 fmol of PgR/μg of DNA; measured by EIA) following a pulse with 10^{-10} M E_2. Except at micromolar concentrations, estratriene was an ineffective estrogen. The addition of a hydroxyl group to either position 3 or position 17β of estratriene yielded ligands which were capable of causing nuclear binding and processing of ER as well as PgR induction. D-ring regioisomers of estradiol (E_2) had lower affinity for receptor than E_2. However, receptor complexed with these estrogens was fully capable of binding to nuclear material, undergoing processing, and inducing PgR. On the other hand, A-ring regioisomers of E_2 displayed significant differences in their ability to mediate nuclear binding of receptor complex and induction of PgR. Although 1-hydroxyestratrien-17β-ol was weakly bound by ER, this dihydroxyestrogen was capable of bringing about nuclear binding and processing of ER and the stimulation of PgR synthesis. In contrast, 2- and 4-hydroxyestratrien-17β-ol, which caused extensive nuclear binding of ER (5–7 fmol/μg of DNA), were incapable of significant PgR induction. Provided that the A-ring hydroxyl group was positioned correctly (3β) on 5α-androstanediols or 5-androstenediol, an aromatic ring was not required for nuclear binding of the ER complex and stimulation of PgR synthesis. With the exception of 2- and 4-hydroxyestratrien-17β-ol, induction of PgR by structurally altered estrogens correlated with the affinity of ligand for ER. Electrostatic models generated from this data were found to be useful in the characterization of electronegative isopotential regions of the estrogen (or androstanediol) molecules which were important in modulating the gene regulatory properties of ER.

Although there have been extensive investigations of the effect of structural alterations in the estrogen molecule on receptor affinity (Hahnel et al., 1973; Duax et al., 1981), there are only a few reports regarding the influence of specific changes in estrogen structure on the induction of the products of responsive genes. The estrogen metabolites estrone and estriol possess limited activity compared to E_2.[1] This attenuated activity, brought about by the addition of a 16α-hydroxyl in the case of estriol and the oxidation of the 17β-hydroxyl to a ketone in estrone, is thought to result from decreased receptor affinity and the attendant shorter nuclear half-life of the receptor complexes (Weichman & Notides, 1980). The range of estrogen responses in target cells appears unaffected for these metabolites; however, the degree of response for a given concentration of estrogen derivative is considerably less than that of the parent compound. The literature contains one report wherein moving the D-ring hydroxyl group from the 17β-position to the 16α-position was observed to result in a slower acting estrogen which was fully capable of receptor binding but which induced a more transient first phase of prolactin gene transcription in anterior pituitary cells (Schull & Gorski, 1985). Recently, it has been shown that the addition of certain 7α-alkyl carboxamide groups to E_2 yields a pure antagonist of estrogen activity (Wakeling & Bowler, 1987).

It is not unusual for structurally altered ligands to impart extremely different responses to the receptor complex. For nonsteroidal estrogens, the simple repositioning of the two *p*-hydroxyl groups on the estrogenic diethylstilbestrol ligand to the 3-position on each aromatic ring creates an estrogen which, when bound to receptor, blocked tumor growth while displaying greatly diminished uterotrophic activity (Schneider & Ball, 1986). In fact, triphenylethylenes, although less tightly bound, act as agonists or antagonists depending on their concentration, structural makeup, and/or conformation (Rochefort et al., 1983; Katzenellenbogen et al., 1984; Foster et al., 1985; McCaque et al., 1989). Furthermore, Johnson et al. (1989) have shown tamoxifen, 3-hydroxytamoxifen, 4-hydroxytamoxifen, and desmethyltamoxifen to have different inductive and antagonistic effects on each of the four estrogen-regulated genes.

Nuclear processing of the ER, defined as a 50% loss of estrogen binding capacity within 6 h of an E_2 pulse, was initially believed to be an essential element in the receptor-mediated activity of E_2 (Horwitz & McGuire, 1978; Gyling & Leclercq, 1988). More recently, it has been reported that processing of the ER is the result of a down regulation of ER mRNA by E_2 (Saceda et al., 1988, 1989; Berthois et al., 1990).

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[1] Abbreviations: E_2, estradiol-17β; ER, estrogen receptor; ER_n, nuclear estrogen receptor; PgR, progesterone receptor; MEM, minimal essential medium; ER-EIA, estrogen receptor enzyme immunoassay; PgR-EIA, progesterone receptor enzyme immunoassay; PCA, perchloric acid; RBA, relative binding affinity.

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Nevertheless, it has become evident that this phenomenon is related to certain ligand structures (Eckert & Katzenellenbogen, 1982), to selected estrogen responses (Eckert & Katzenellenbogen, 1982), and to kinetic alterations in the binding process (Strobl et al., 1984).

While there is evidence that the ability of an estrogen to elicit a certain response in a target tissue is not directly related to its affinity for receptor (Brooks et al., 1987; Zeelen, 1990), there is little information relating the nuclear binding of the estrogen receptor complex to gene induction by structurally altered ligands. In this study the effect of estrogen structure on nuclear binding, processing, and gene induction by the receptor complex is examined.

MATERIALS AND METHODS

Steroids. Estratriene, estrone, E_2, estriol, estradiol-16α, and estradiol-17α were purchased from Research Plus, Inc. (Bayonne, NJ). All of the A-ring-substituted estrogens utilized in these studies were synthesized in our laboratory according to published procedures. The syntheses of 1-, 2-, and 4-hydroxyestratrien-17β-ol have been reported (Palomino et al., 1990), as have the syntheses of estratrien-17β-ol and 3-hydroxyestratriene (Horwitz et al., 1986; Dannenberg & Kohler, 1964). Each of these analogues was purified by thin-layer chromatography and crystallized until contaminating estrogens (particularly E_2) were present at a level less than 1 part in 10 000. Chromatography was carried out on TLC plates (silica gel), which resolved approximately 1 mg of the compounds (3–4 μmol) in a system which was capable of separating E_2 (developed three times with hexane:ethylacetate, 8:2). Visualization of spots with UV light (254 nm), after exposure of the plates to I_2, enabled the detection of 0.1 nmol of E_2. The absence of a spot corresponding to E_2 on the TLC plate under these conditions was assurance that the chromatographed analogue contained less than 1 part E_2 in 10^4 parts of the compound.

5-Androstene-3β,17β-diol, 5α-androstane-3β,17β-diol, and 5α-androstane-3α,17β-diol were purchased from the Aldrich Chemical Co. (Milwaukee, WI) and purified by thin-layer chromatography (silica gel, as described above for estrogens).

Cell Culture. MCF-7 cells of human breast cancer origin were cultured in closed T_{75} and T_{25} flasks in Eagle's MEM with Hank's balanced salt solution, 2.5 mM L-glutamine, and 25 mM HEPES, supplemented with nonessential amino acids, 5 μg/mL gentamicin, and 5% of a donor calf serum which had been shown to be free of E_2 without DCC stripping (Wiese et al., 1992). For maintenance, cells were grown to confluence, and the attached cells were suspended with a syringe and passed at 2 million cells per T_{75} flask. For the experiments, cells were passed at 1 million cells per T_{25} flask. Nuclear-bound ERs were determined by ER-EIA (Abbott Laboratories, Chicago, IL) in confluent cultures following treatment with the estrogen analogues for the times indicated. Estrogen analogues were added to cultures in 5–50 μL of ethanol per 50 mL of media. Progesterone receptor induced by the estrogen analogues was determined by PgR-EIA (Abbott) in cultures treated at confluence with media containing the analogue; the media was changed daily, and the cells were harvested after 3 days.

ER-EIA. The treated cells were first washed with ice-cold saline and then removed in 5 mL of ice-cold MEM with a syringe, and the suspension was spun at 150g for 10 min at 4 °C. Nuclei were prepared as described by Kral et al. (1988). Briefly, the cells were lysed in buffer A (10 mM HEPES, pH 7.4 at 4 °C, 5 mM $MgCl_2$, 10 mM NaCl, and 5 mM monothioglycerol) containing 0.5% NP40; the lysate was spun at 150g, and the nuclear pellet was treated a second time with buffer A and NP40 before extraction with buffer A containing 400 mM KCl. Following centrifugation, the nuclear extract was assayed (EIA) for amount of nuclear estrogen receptor complex and the pellets were utilized for DNA determinations. The amount of ER present in the supernatant was determined by use of ER-EIA monoclonal kit according to the manufacturers instructions. Briefly, the supernatants were incubated with a polystyrene bead coated with an anti-ER monoclonal antibody (D547) for 18 h. The beads were then washed and incubated with a second antibody (H222) conjugated to horseradish peroxidase. The beads were again washed, and the amount of ERn bound by the two antibodies was quantitated colorimetrically using o-phenylenediamine as a substrate. The kit contains lyophilized ER standard from MCF-7 cells, from which a standard curve was constructed. The assay of ER utilizing beads coated with monoclonal antibodies (Abbott ER-EIA kit) has been shown to be nearly quantitative (95%, based on recovery of $^3HE_2$ following a 1-h pulse of MCF-7 cells) for nuclear receptor following the disruption of membranes with NP40, removal of the NP40 buffer, and then extraction of E_2R with 0.4M KCl for exposure of receptor to antibody (Kral et al., 1988).

PgR-EIA. The cells were harvested, after treatment for the indicated times with the various analogues, and ruptured in a Dounce homogenizer on ice in 2 mL of ice-cold immunoassay buffer (10 mM Tris, 1.5 mM EDTA, and 5.0 mM sodium molybdate, pH 7.4, with 1.15 μM monothioglycerol). Cytosol was obtained following centrifugation at 100000g, and the supernatant was assayed for PgR by utilizing the PgR-EIA monoclonal antibody kit (containing monoclonal antibodies KD68 and JZB39; Abbott). The nuclear pellet from each assay was stored at −20 °C for DNA determination.

DNA Quantification. The amount of DNA in each sample was measured by the method of Burton (1956). Briefly, the nuclear pellets were twice suspended in 3 mL of 10% PCA and spun down at 2500 rpm for 10 min. The washed pellets were then incubated at 70 °C for 20 min in 2 mL of 10% PCA and centrifuged. To 1 mL of the supernatant was added 1 mL of 10% PCA, 2 mL of diphenylamine, and 0.1 mL of acetaldehyde. The reaction was allowed to proceed overnight in the dark, after which the absorbance was determined by 600 nm. Sigma type 1 highly polymerized DNA (D-175) was dissolved (concentration of 1 μg/μL) and used for a standard curve.

Competitive Binding Assay. Cytosolic estrogen receptor preparation and assays were carried out at 4 °C according to classical Scatchard (1949) and competitive binding (Davies et al., 1975) methods utilizing dextran-coated charcoal.

RESULTS

Pulsing MCF-7 cells with E_2 brought about the classical tight nuclear binding of the cytosolic receptor complex, which reached a maximum within 3 h, leaving little receptor in the 100000g supernatant (data not shown). As previously reported, the total ER, represented by its nuclear form, was diminished by 50% after a 6-h exposure of the culture to E_2 (Figure 1A). This "processing" of receptor has been established in experiments which utilized tritiated E_2 (Horwitz & McGuire, 1978; Gyling & Leclercq, 1988) or in which receptor was assayed with monoclonal antibodies [Figure 1A; see also Gyling and Leclercq (1988), Saceda et al. (1988), and Berthois et al. (1990)]. The extent of nuclear binding and processing of ER was shown to vary with the concentration of E_2 to which the

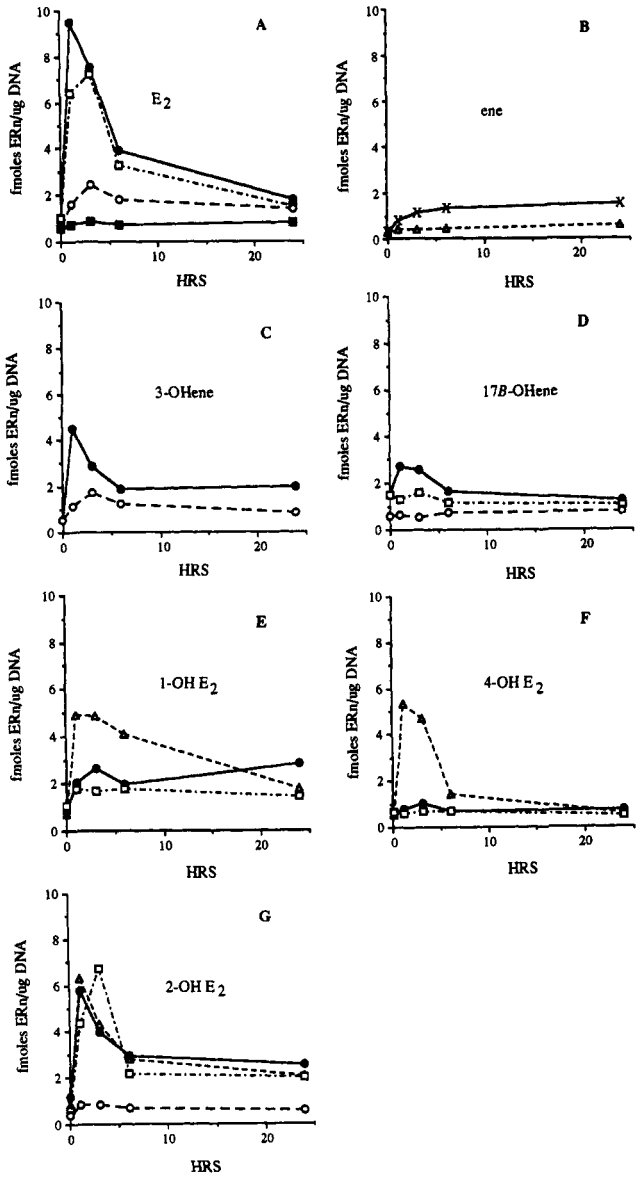

FIGURE 1: Nuclear binding of ER complex in MCF-7 cells pulsed with different concentrations of estrogen analogues. Cells were first exposed to estrogen analogues when the cultures were near confluence. The level of nuclear receptor (ER_n) in washed nuclei was measured with monoclonal antibodies (Abbott ER-EIA kit) following homogenization of the cells in buffer A containing 0.5% NP40 (see Materials and Methods). ER_n was determined 1, 3, 6, and 24 h after the cells were exposed to the various estrogens. (**A**) Cultures treated with the following concentrations of E_2: 10^{-11} M (■), 10^{-10} M (○), 10^{-9} M (□), and 10^{-8} M (●). (**B**) Cultures treated with estratriene (ene): 10^{-7} M (△), and 10^{-6} M (X). (**C**) Cultures treated with 3-hydroxyestratriene (3-OHene): 10^{-10} M (○) and 10^{-8} M (●). (**D**) Cultures treated with estratrien-17β-ol (17β-OHene): 10^{-10} M (○), 10^{-9} M (□), and 10^{-8} M (●). (**E**) Cultures treated with 1-hydroxyestratrien-17β-ol (1-OH E_2): 10^{-9} M (□), 10^{-8} M (●), and 10^{-7} M (△). (**F**) Cultures treated with 4-hydroxyestratrien-17β-ol (4-OH E_2): 10^{-9} M (□), 10^{-8} M (●), and 10^{-7} M (△) (**G**) Cultures treated with 2-hydroxyestratrien-17β-ol (2-OH E_2): 10^{-10} M (○), 10^{-9} M (□), 10^{-8} M (●), and 10^{-7} M (△). Points indicate averages of two determinations. The range of the determinations was <5%. Experiments were repeated two to five times, yielding similar patterns of ER_n.

cultures were exposed (Figure 1A). At higher concentrations (10^{-8} M and above), ER_n reached a maximum level by 20 min and remained at the same level until after 1 h; thereafter, processing began (data not shown). At pulse concentrations of 10^{-9} M E_2 and lower, the ER_n did not peak until 3 h (Figure 1A). Elevated concentrations of E_2 (10^{-8} M, Figure 1A, and

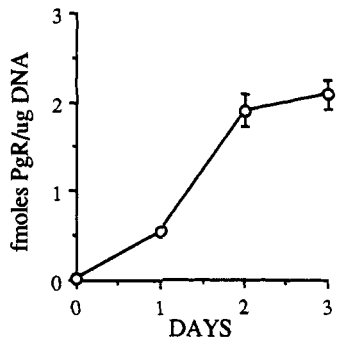

FIGURE 2: Time course of PgR induction in MCF-7 cells pulsed with 10^{-8} M E_2 for 1, 2, or 3 days. E_2-containing medium was renewed each 24 h. Cultures had reached confluence before exposure to E_2. PgR analyses were carried out concurrently, utilizing monoclonal antibodies (Abbott PR-EIA kit). Bars indicate standard deviations of three experiments.

higher, data not shown) brought about the bindng of 9.5 fmol (10.0 ± 0.5, $n = 7$) of ER_n by nuclear material 1 h following the administration of E_2. Seven femtomoles of ER_n bound to the nucleus of MCF-7 cells 3 h after the addition of 10^{-9} M E_2 to the culture. A peak of only 2.5 fmol of ER_n could be detected following a pulse of 10^{-10} M E_2, and little or no additional ER bound to the nucleus after the cells were exposed to 10^{-11} M E_2 (Figure 1A). Although processing of the nuclear ER was evident at the lower pulse concentrations of E_2, the extent was less than the 50% noted at 10^{-9} M E_2. The level of ER_n present in these cells at 24 h remained the same for an additional 48 h (data not shown).

In the absence of E_2, these cells did not contain levels of PgR which were detectable by monoclonal antibody. The induction of PgR was significant at 1 day and reached maximum levels following 2–3 days of exposure of MCF-7 cells to 10^{-8} M E_2 (Figure 2). The PgR induced by E_2 over a concentration range of 10^{-12}–10^{-7} M is shown in Figure 3A. Maximum PgR induced by E_2 within 3 days is 2.9 ± 0.2 fmol of PgR/μg of DNA ($n = 3$). Interestingly, this occurred after a 10^{-10} M E_2 pulse, indicating that the level of ER_n (3 h postpulse) required for maximal induction was only 2.5 ± 0.2 fmol of ER_n/μg of DNA ($n = 3$) (Figure 1A). At higher concentrations of E_2, the induction of PgR was shown to diminish (Figure 3A). Nevertheless, pulsing these cells with higher concentrations of E_2 resulted in greater nuclear binding of ER (as elevated as 9.5 fmol/μg of DNA; Figure 1A). The amount of PgR induction dropped off sharply to 0.6 fmol of PgR/μg of DNA when the cells were cultured in the presence of 10^{-11} M E_2 and to near 0 in the presence of 10^{-12} M E_2.

The basic ring structure of estrogens (estratriene) was incapable of forming receptor complexes which bound tightly to nuclear material except at a micromolar concentration (Figure 1B). The RBA of this compound was too low to measure with the competitive binding assay [<0.0005; by comparison, the E_2 RBA = 1.0, and the E_2 $K_a = (3.7 \pm 1.5) \times 10^9$ M^{-1}, $n = 7$]. Furthermore, synthesis of PgR was not induced following exposure of these cells to concentrations of estratriene between 10^{-9} and 10^{-8} M (Figure 3A). Only at the higher concentrations (up to 10^{-6} M estratriene) was slight nuclear binding of receptor complex apparent (approx 1.5 fmol/μg of DNA without processing; Figure 1B), which was accompanied by PgR induction (1.3 fmol/μg of DNA; data not shown). In the absence of an inducing agent such as a polycyclic aromatic hydrocarbon, MCF-7 cells are devoid of $CytP450_{1A1}$, the enzyme responsible for hydroxylation of the aromatic ring of estrogens (Vickers et al., 1989). Examination of these pulsed cultures with Northern blots showed that the

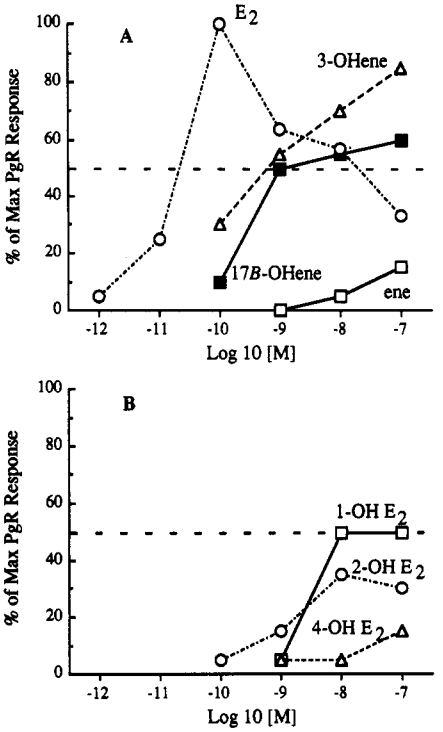

FIGURE 3: Induction of PgR in MCF-7 cells by E_2, estratriene, 3-hydroxyestratriene, and estratrien-17β-ol. Cultures were near confluence before exposure to the various estrogens. Medium with estrogens was renewed each 24 h. (A) PgR was determined in cultures treated for 3 days with the indicated concentrations of E_2 (○), estratriene (□), 3-hydroxyestratriene (△), and estratriene-17β-ol (■). (B) Induction of PgR in MCF-7 cells by 1-hydroxyestratrien-17β-ol (□), 2-hydroxyestratrien-17β-ol (○), and 4-hydroxyestratrien-17β-ol (△). Abbreviated compound names are defined in the caption to Figure 1. Points indicate averages of two determinations, with a range of <5%. Experiments were repeated two to four times, yielding similar patterns of PgR.

mRNA for this hydroxylase was absent and had not been induced by the analogues (R. N. Hines, Department of Pharmacology, Wayne State University, personal communication). Therefore, it is not likely that the estratrienes were hydroxylated by MCF-7 cells during the 72 h of PgR induction.

The addition of a hydroxyl group to position 3 or 17β of estratriene yielded, in each case, an analogue which was capable of binding to receptor (RBA = 0.8 for 3-hydroxyestratriene and 0.11 for estratrien-17β-ol) and, as well, of both nuclear binding and processing of the receptor complex (Figure 1C,D), followed by PgR induction in MCF-7 cells (Figure 3A). When added to cultures at a concentration of 10^{-8} M, both of these monohydroxyestrogens induced greater than half-maximal levels of PgR (which reached their highest levels by 24 h; data not shown). This induction of PgR required 4.8 fmol/μg of DNA of peak nuclear 3-hydroxyestratriene–receptor complex (Figure 1C) and 2.8 fmol/μg of DNA of the estratrien-17β-ol complex (Figure 1D).

A-ring regioisomers of E_2 showed significant differences in nuclear binding of the receptor complex and, ultimately, the induction of PgR. 1-Hydroxyestratrien-17β-ol (RBA = 0.005) exhibited the highest PgR stimulation after the cultures were exposed to a 10^{-8} M concentration (Figure 3B). Like E_2, only 2.5 fmol/μg of DNA of nuclear receptor complex was detected 3 h after pulsing these cells with a 10^{-8} M concentration of this estrogen analogue (Figure 1E). At this level, processing of ER_n was not apparent; again, a higher level (10^{-7} M) of the 1-hydroxyestrogen brought about greater nuclear binding and processing without greater PgR induction. In spite of similar nuclear binding of its receptor complex and induction of PgR (e.g., at 10^{-8} M), 1-hydroxyestratrien-17β-ol had less affinity for receptor than the A-ring-unsubstituted analogue (estratrien-17β-ol; Figures 1C,E and 3A,B).

Table I: Nuclear Binding of Receptor Complex and PgR Induction in MCF-7 Cells Pulsed with D-Ring Analogues of E_2

estrogen	RBA	concn (M)	peak[a] ER_n (fmol/μg of DNA)	EC50[b] (10^{-9} M)
$E_2$17α[c]	0.22	10^{-9}	4	2.9
$E_2$16α[d]	0.80	10^{-9}	7	0.32
estriol	0.17	10^{-8}	8	3.5
estrone	0.22	10^{-8}	6	6.1

[a] Highest level of ER_n during a 1–3-h pulse of estrogen. [b] Defined in the caption of Figure 3; EC50 for E_2 is 1.8×10^{-11} M. [c] Estradiol-17α. [d] Estradiol-16α.

Table II: Nuclear Binding of Receptor Complex and PgR Induction in MCF-7 Cells Pulsed with Two 5α-Androstanediols and One 5-Androstenediol

steroid	RBA	concn (M)	peak[a] ER_n (fmol/μg of DNA)	EC50[b] (10^{-9} M)
5-androstene-3β,17β-diol	0.007	10^{-7}	6	13
5α-androstane-3β,17β-diol	0.005	10^{-7}	6	10
5α-androstane-3α,17β-diol	<0.0005	10^{-7}	0	1000

[a] Highest level of ER_n during a 1–3-h pulse of steroid. [b] Defined in caption of Figure 3; EC50 for E_2 is 1.8×10^{-11} M.

Locating the phenolic hydroxyl at position 4 of estratrien-17β-ol created a dihydroxyestrogen (RBA = 0.07) which formed an ER complex with elevated nuclear binding (5 fmol/μg of DNA) and processing when cultures were pulsed with a 10^{-7} M concentration of this analogue (Figure 1F). Even at these levels, 4-hydroxyestratrien-17β-ol induced only small quantities of PgR (Figure 3B).

2-Hydroxyestratrien-17β-ol (RBA = 0.71) formed a receptor complex which bound well to nuclear material (6.2 fmol of ER_n/μg of DNA) at pulse concentrations of 10^{-9} M or greater (Figure 1G). Nevertheless, this dihydroxyestrogen induced little PgR (Figure 3B).

Changes in the position of D-ring oxygen-containing groups on estrogens decreased the affinity of the ligand for receptor by 20% (estradiol-16α) to 83% (estriol) (Table I). Receptor complexed with these estrogens was fully capable of binding to nuclear material and undergoing processing. Furthermore, these D-ring analogues displayed equal or only slightly diminished capacity to induce PgR (Table I).

The estrogenic property of 5-androstene-3β,17β-diol in MCF-7 cells has been reported (Adams et al., 1981). This steroid was capable of binding to ER, bringing about high nuclear binding of the receptor complex followed by processing, as well as inducing the synthesis of PgR (Table II). The double bond in the B-ring was not essential for these activities since 5α-androstane-3β,17β-diol was equally stimulatory in MCF-7 cells. On the other hand, the configuration of the hydroxyl group on position 3 was critical to the estrogenic potential of this steroid. 5α-Androstane-3α,17β-diol did not compete with E_2 for receptor or activate the ER, nor did the α-hydroxyl group on the A-ring bring about the synthesis of PgR (Table II).

Using the K_a of each estrogen analogue and the androstanediols or 5-androstenediol, it is possible to determine the relationship between the affinity for receptor and the induction of PgR. In order to demonstrate this analogy, the ability of each estrogen to stimulate PgR synthesis was quantified by utilizing the EC50 (defined as the effective concentration of

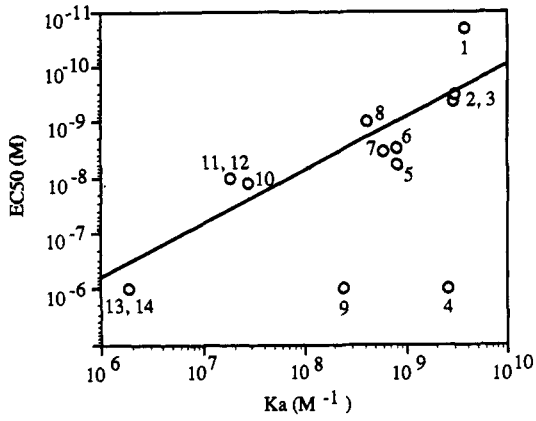

FIGURE 4: Relation of K_a of various estrogens, 5α-androstanediol, and 5-androstenediol to the induction of PgR. K_a's were determined by the competitive binding assay (see Materials and Methods). In order to relate the PgR induction potentency of each estrogen, the EC50 (defined as the effective concentration which produced half-maximal response) is utilized. EC50 was calculated for each analogue as log[(% of maximal E_2 response)/[100 − (% of maximal E_2 response)]]. Compounds that do not reach 50% maximum response have been given a very low, arbitrary property value (Cramer et al., 1988). R^2 of the line determined by all points except 4 and 9 is 0.83. Numbers refer to the following steroids: (**1**) E_2, (**2**) estradiol-16α, (**3**) 3-hydroxyestratriene, (**4**) 2-hydroxyestratrien-17β-ol, (**5**) estrone, (**6**) estradiol-17α, (**7**) estriol, (**8**) estratrien-17β-ol, (**9**) 4-hydroxyestratrien-17β-ol, (**10**) 5-androstene-3β,17β-diol, (**11**) 1-hydroxyestratrien-17β-ol, (**12**) 5α-androstane-3β,17β-diol, (**13**) 5α-androstane-3α,17β-diol, and (**14**) estratriene.

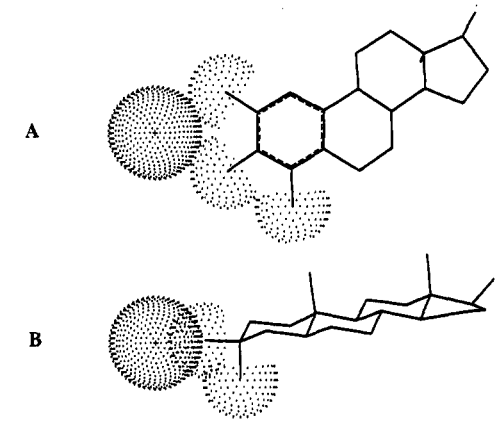

FIGURE 5: Molecular models of selected estrogens in relation to a possible H-bonding interaction site on the ER. (**A**) 2-Hydroxyestratrien-17β-ol, E_2, and 4-hydroxyestratrien-17β-ol superimposed at all A-, B-, C-, and D-ring atoms; only carbons and oxygens are shown. View is from above, normal to the A-ring plane. (**B**) 5α-Androstane-3α,17β-diol and 5α-androstane-3β,17β-diol superimposed at all A-, B-, C-, and D-ring atoms; only carbons and oxygens are shown. View is from slightly above the steroid rings, with carbons 4, 5, 6, and 7 in the foreground. Van der Waals radii of A-ring oxygens are highlighted by coarse dots. The proposed ER H-bonding interaction site is indicated by dense dots centered on the plane of the A-ring, 3.0 Å from the C2 and C3 oxygens of the estratrienes. Androstanes in B are oriented relative to estratrienes such that 5α-androstane-3β,17β-diol and E_2 are root-mean-square fit at the C3 and C17 oxygens and at C18. All molecular models represent PM3 optimizations (MOPAC 6.0, Program No. 455, The Quantum Chemistry Program Exchange, Indiana University, Bloomington, IN 47405) of X-ray structures (Palomino et al., 1990) or modified molecular fragments from SYBYL 5.5 (Tripos Associates, Inc., St. Louis, MO 63144). For each modeled structure shown, hydroxyl groups are in lowest energy orientation.

steroid which produced half-maximal E_2 response). A plot of the EC50 of each estrogen versus the log K_a is shown in Figure 4. With the exception of the 2- and 4-hydroxyestratrien-17β-ol, the receptor affinity (K_a) of all of the hydroxyestrogens correlated well (R^2 = 0.83) with the induction of PgR (Figure 4). In addition, the K_a's of 5-androstene-3β,17β-diol and 5α-androstane-3β,17β-diol were directly related to the capacity of these steroids to induce PgR. Estratriene and 5α-androstane-3α,17β-diol bound poorly to ER and failed to induce significant levels of PgR at the concentrations examined.

DISCUSSION

All of the estrogen analogues examined in these investigations brought about tight nuclear binding and processing of appreciable levels of ER (2.5–7.0 fmol/μg of DNA) as detected by monoclonal antibodies. Results from these investigations, which utilized MCF-7 cells in culture, support the concept that intracellular ER requires ligand for tight nuclear binding and activation of transcription (McDonnell et al., 1991). Yet, certain estrogen analogues (2- or 4-hydroxyestratrien-17β-ol) induced little or no PgR (Figure 3B), although they brought about tight nuclear binding of the ER complex (Figure 1F,G). This phenomenon does not appear to be related to ligand affinity for ER.

Structural Requirements of Steroids Which Are Related to ER Affinity. Most important for high affinity of ligand for ER was the aromatic A-ring with a hydroxyl group on position 3 (cf. 3-hydroxyestratriene vs 5α-androstane-3β,-17β-diol and estratrien-17β-ol). Furthermore, the D-ring 17β-hydroxyl group had a function in receptor binding (e.g., estratrien-17β-ol) possibly by holding the ligand in the binding site via hydrogen bonding to receptor above the D-ring.

The 1-hydroxyl interfered with receptor binding (the K_a of 1-hydroxyestratrien-17β-ol is nearly 0.05× that of estratrien-17β-ol). The decreased affinity of this dihydroxyestrogen may be due to distortion of the four-ring structure brought about by the 1-hydroxyl group as indicated by X-ray crystallography (Palomino et al., 1990). When added to the A-ring of estratrien-17β-ol, the 4-hydroxyl group had little effect on the K_a of the parent estrogen, whereas a 2-hydroxyl group increased the K_a of estratrien-17β-ol to a value near the maximum displayed by the 3-phenolic group (E_2). It is conceivable that the aromatic hydroxyl group in E_2 and 2-hydroxyestratrien-17β-ol may share, via hydrogen bonding, a similar H-acceptor/donor on the receptor molecule (Figure 5A). This functional group(s) on the receptor may be accessible to the 1- or 4- hydroxyl group when added to estratrien-17β-ol. Likewise, only the 3β-hydroxyl of the androstanediols would be capable of interacting within this site on ER, with the 3α-hydroxyl being too remote (Figure 5B).

ER Affinity and Gene Regulation. The literature contains a number of reports that the regulation of genes by estrogens is not directly related to their affinity for receptor (Brooks et al., 1987; Guiochon-Mantel et al., 1988; El-Ashry et al., 1989; Inaba et al., 1989; Perlman et al., 1990; Zeelen, 1990). Utilizing discrete changes in the E_2 molecule, these experiments have demonstrated that very specific changes in the estrogen ligand could bring about great decreases in receptor affinity without affecting the ability of higher concentrations of the analogue to stimulate PgR synthesis (e.g., 1-hydroxyestratrien-17β-ol; Figure 3B). In contrast, other alterations in the estrogen molecule may have had a lesser effect on the K_a, yet the PgR regulation was diminished or nullified (e.g., 2- or 4-hydroxyestratrien-17β-ol; Figure 4). The position of the phenolic hydroxyl on the estrogen molecule appears to affect the induction of PgR, although its absence does not eliminate the stimulation of PgR synthesis (e.g., estratrien-17β-ol; Figure 3A). Alicyclic A-ring steroids show a lower affinity for

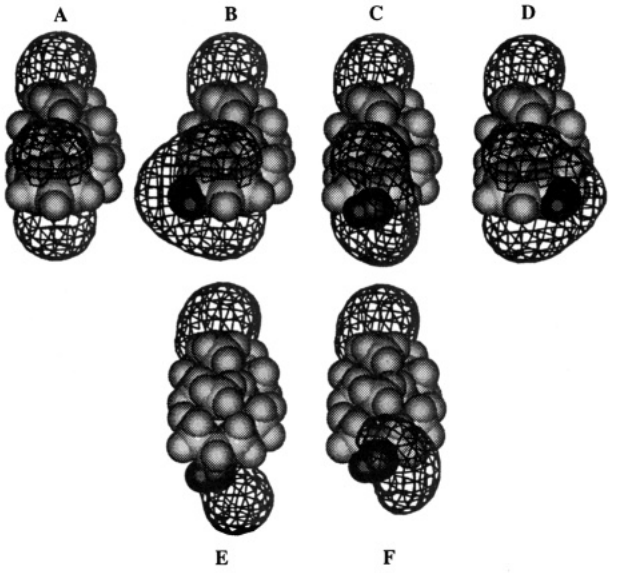

FIGURE 6: Space-filling models including negative isopotentials of selected estrogens: (**A**) estratrien-17β-ol, (**B**) 2-hydroxyestratrien-17β-diol, (**C**) E_2, (**D**) 4-hydroxyestratrien-17β-diol, (**E**) 5α-androstane-3α,17β-diol, and (**F**) 5α-androstane-3β,17β-diol. View is from the A-ring to the D-ring from slightly above the A-ring plane. A-ring hydroxyls are darkly shaded. Negative isopotentials from MOPAC charges are depicted by mesh enclosures (–1.0 kcal). Relative orientation of androstanes to estratrienes and molecular model geometry optimization are as in Figure 5.

receptor (Table II). Yet, compensation for decreased ER binding of these steroids by increasing the concentrations of the androstanediols to which the MCF-7 cells were exposed resulted in PgR induction, but only for steroids where the 3-hydroxyl group was in the β-configuration (Table II). ER binding, as well as PgR induction, did not occur with 5α-androstane-3α,17β-diol. Since the position of D-ring hydroxyls (or the ketone) had little or no effect on PgR induction (Table I), it appears that the most important requirements of the estrogen molecule for induction of PgR are the aromatic A-ring and the position of the phenolic hydroxyl. To some degree, the 3β-hydroxyl on the 5α-androstane-3β,17β-diol and 5-androstane-3β,17β-diol can mimic the requirement for estrogenicity.

Importance of Aromatic Character of the A-Ring. An interesting aspect of the data from these investigations is shown in Figure 4. Whereas nine estrogen analogues, 5-androstene-3β,17β-diol, and 5α-androstane-3β,17β-diol were able to induce PgR synthesis in direct relationship to their affinity for ER, two compounds (2- and 4-hydroxyestratrien-17β-ol) were either incapable (4-hydroxy) or weakly capable (2-hydroxy) of stimulating PgR synthesis, although these two dihydroxyestrogens bound relatively well to ER. This suggests that some constitutive property of these estrogens, other than their hydroxyl-related hydrogen bonding and the resultant K_a, is related to the regulation of the PgR gene. The estratriene molecule (which is devoid of hydroxyl groups) bound to ER, but with an affinity too low to measure. Yet, when MCF-7 cells were exposed to levels of estratriene at or near micromolar concentrations, minimal nuclear binding of the ER complex occurred and limited PgR synthesis was induced (Figures 1B and 3A). Furthermore, estratrien-17β-ol was an active stimulator of PgR synthesis. Clearly, hydrogen bonding involving a phenolic hydroxyl group is not essential for PgR induction. Therefore, it is conceivable that the aromatic character of the phenolic A-ring may contribute to the induction of PgR.

Computer-generated models of the important di- and monohydroxyestrogens are depicted in Figure 6. Added to these structures is the electronegative isopotential generated by the unpaired electrons on the phenolic oxygen and the π-electron cloud of the aromatic ring (also shown is the negative isopotential of the unpaired electrons on the 17β-oxygen). The geometry of the electronegative isopotentials surrounding the A-ring of E_2 and 2- and 4-hydroxyestratrien-17β-ol are quite different. Results from these investigations indicate that ER, bound to a ligand with the electronegative isopotential extending beyond the A-ring between carbons 3 and 4 (E_2), generates maximum PgR induction. Nevertheless, the ligand estratrien-17β-ol, with only the π-electron cloud (over and below the A-ring), stimulated PgR synthesis in relation to its diminished K_a. On the other hand, the ER complex did not induce PgR if the negative isopotential of the ligand's A-ring protruded over carbons 4 and 5 (4-hydroxyestratrien-17β-ol). 2-Hydroxyestratrien-17β-ol was a weak inducer of PgR, possibly due to the negative potential directed over carbons 1 and 2 of this estrogen. A model of 1-hydroxyestratrien-17β-ol showed the unpaired electrons on the phenolic oxygen to generate an extended negative isopotential beneath the A-ring which did not affect PgR induction (relative to estratrien-17β-ol) but did have a negative influence on the K_a. The unpaired electrons alone on the 3β-hydroxyl group of 5α-androstanediol or 5-androstenediol sufficed to promote binding and stimulated PgR synthesis (Figure 6 and Table II). In the absence of the electronegative isopotential above the nonaromatic A-ring, receptor binding and PgR induction were eliminated (cf. 5α-androstane-3α,17β-diol).

Biological Significance. An explanation for the mechanism by which the geometry of the electronegative isopotential surrounding the A-ring of the various dihydroxyestrogens affects the induction of PgR is unknown. At this point it can be speculated that the binding of certain estrogen analogues with ER could affect transcription of responsive genes (1) via ligand-induced alterations in the binding of the ER complex to the estrogen-responsive element (Carson-Jurica et al., 1990), (2) by interference with dimerization of the receptor complex (Lannigan & Notides, 1989), or, possibly, (3) by the ligand's influence upon transactivation function 2, which lies within the estrogen binding domain (Tora et al., 1989).

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