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Induction of Tissue Plasminogen Activator mRNA and Activity by Structurally Altered Estrogens

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The effect of structure of the estrogen ligand on the accumulation of tPA mRNA and the activity of extracellular fibrinolytic enzyme has been examined in cultures of MCF-7 cells. Estradiol(E_2)-stimulated fibrinolytic activity was preceded by an increase in actinomycin D sensitive tPA mRNA synthesis which peaked at 18 h. Ten A- and D-ring structural analogs of E_2 affected tPA mRNA accumulation and extracellular fibrinolytic activity. Only in the case of two A-ring isomers (2- and 4-hydroxyestratrien-17β-ol) was the decreased effect of the ligand's structural change on tPA mRNA accumulation and fibrinolysis not explained by a comparable decline in affinity of the ligand for estrogen receptor. Both of these analogs functioned as antiestrogens. The stimulatory capacity of androstanediols on the tPA gene required that the 3-hydroxyl group be positioned in the β-configuration. Absence of the 17β-hydroxy group was beneficial to the maximum accumulation of tPA mRNA. As has been reported for other estrogen responsive genes (progesterone receptor, cathepsin D and pS2), regulation by estrogens is not related directly to the affinity of the ligand for ER, but this activity may be determined by the location of the electronegative isopotential above the A-ring of estrogenic ligands.

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INTRODUCTION

Human tumors have been shown to produce elevated levels of fibrinolytic activity relative to the normal tissue of origin [1]. This activity has been attributed to a serine protease, plasminogen activator, which converts the serum zymogen plasminogen to the active non-specific protease plasmin [2, 3]. MCF-7 human breast cancer cells have been shown to secrete both the specific tissue plasminogen activator (tPA) and urokinase plasminogen activator [uPA; 4]. Only tPA activity was controlled by estrogen via a receptor mediated mechanism [5–8]. Although the factors controlling tPA mRNA in normal human breast epithelial cells are unknown, this gene has been shown to be under hormonal control in other normal tissues [rat granulosa cells; 9, 10]. The role of tPA in tumor cells remains unclear. Nevertheless, it has been reported that in some systems the tumorigenicity of cells *in vivo* is correlated with their ability to produce plasminogen activator *in vitro* [11–14].

Plasminogen activator activity is also modulated by a specific inhibitor (type 1 plasminogen activator inhibitor, PAI-1) which is synthesized in a highly regulated manner by many cell types [15–18]. Glucocorticoids have been shown to enhance the levels of PAI-1 in cultures of breast tumor epithelium [19]. Concurrently, this hormone was shown to decrease the synthesis of uPA while not affecting the concentration of tPA message [19]. In rat liver cells (but not human) a similar hormonal control is carried out via a glucocorticoid-dependent enhancer element in the PAI-1 promoter fragment [20–22].

The differential induction of tPA activity and message in breast cancer cells by estrogen analogs has not been studied. It has been demonstrated in this laboratory that discrete alterations in the structure of estradiol (E_2) can either diminish the effect of the estrogen receptor (ER) complex on gene transcription, or lead to differential stimulation of specific genes [23, 24]. It is of interest to ascertain whether the structure of the ligand can modulate tPA activity via distinctive effects

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on the transcription of tPA mRNA, alterations in translation and secretion of the enzyme, and/or changes in PAI-1 levels.

MATERIALS AND METHODS

Steroids

All estrogens used in these investigations were synthesized in this laboratory except estratriene, estrone, estradiol-16α, estradiol-17α, estriol and E_2 which were purchased from Research Plus, Inc. (Bayonne, NJ) prior to purification. 1-, 2- and 4-hydroxyestratrien-17β-ols were synthesized according to published procedures [25]. The synthesis of estratrien-17β-ol and 3-hydroxyestratriene have also been reported [26, 27]. Purity of each estrogen analog was guaranteed by thin layer chromatography and crystallization. The level of contaminating estrogen in each analog was determined to be less than 1 part in 10,000 [23].

The androstanediols (5-androsten-3β,17β-diol, 5α-androstan-3β,17β-diol and 5α-androstan-3α,17β-diol) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and purified as described by VanderKuur *et al.* [23].

The antiestrogen 4-hydroxytamoxifen was a gift from Stuart Pharmaceuticals (Division of ICI Americas, Inc., Wilmington, DE) and ICI-164,384 was kindly supplied by Dr A. E. Wakeling, Imperial Chemical Industries (Alderly Park, England).

Cell culture

Subclone E3 [28] derived from the MCF-7 human breast cancer cell line was maintained in Eagles modified MEM supplemented with 0.5 μg/ml gentamicin sulfate or penicillin/streptomycin, 0.01 μg/ml phenol-red and 5% donor-calf serum which had not been treated with dextran-coated charcoal (DCC). Cells were plated at approx. 1.5×10^6 cells/75 cm^2 tissue culture flask for Northern analysis and 2.5×10^5 cells/25 cm^2 tissue culture flask for plasminogen activator assay. The cells were allowed to grow for 4–5 days until approx. 90% confluent. Flasks were then washed with phosphate buffered-saline (PBS, Sigma, St Louis, MO) and switched to phenol-red free media which was supplemented with the aforementioned antibiotics and 5% calf serum that had been treated at 37°C with DCC [29]. Cultures were allowed to grow in this media for 48 h. During this estrogen withdrawal period, the media was changed and the cells were washed daily with PBS to minimize residual E_2 contamination. Cells used for Northern analysis were cultured in media containing DCC treated serum supplemented with the estrogens (10^{-11}–10^{-6} M, added in 20 μl ethanol per 20 ml media) for the times indicated. Control flasks received 20 μl ethanol only. The enzymatic activity of tPA was determined in cultures exposed to estrogens for 48 h. Experiments were carried out in duplicate.

Messenger RNA isolation

Whole cell RNA was extracted from MCF-7 cell homogenates in a guanididium isothiocyanate solution (100 μl per 10^6 cells) using a modified version of the one step procedure described by Chomczynski and Sacchi [30]. Upon acidification with 1/10 volume of 2 M sodium acetate, pH 4.0, the homogenate was extracted with phenol-chloroform (1/1, v/v), followed by a chloroform wash of the aqueous layer. Two volumes of absolute ethanol were added to the aqueous layer and the crude RNA precipitated for several hours at −78°C (dry ice/ethanol). Following centrifugation, the pellet was dissolved in a wash buffer (100 mM sodium acetate, 5.0 mM EDTA, pH 7.0) by heating in a 65°C water bath for 10 min with agitation. A 1/10 volume of sodium acetate (3 M, pH 5.2) was added and the RNA precipitated at −78°C with two volumes of isopropanol.

After centrifugation the pellet was next dissolved in a 0.5% sodium dodecyl sulfate (SDS) solution followed by the addition of 1/10 volume RNA extraction buffer (100 mM sodium acetate, 100 mM EDTA, pH 5.1). Phenol was added (1:1, v/v) and the mixture was allowed to equilibrate in a 65°C water bath for two 5 min periods, mixing the tubes after each period. After a 5 min cooling period at 25°C, chloroform (1:1, v/v with respect to the phenol) was added and the aqueous layer extracted. Samples were precipitated as stated above and the pellet resuspended in 500 μl diethyl pyrocarbonate (Sigma, treated 200 μl per liter water). To this solution 500 μl of 2 × binding buffer (1.0 M NaCl, 0.02 M Tris–HCl pH 7.5, 2 mM EDTA, 1% SDS) was added to raise the salt concentration to 0.5 M. The resulting solution was passed through a plastic syringe fitted with a 21 gauge needle to shear high molecular weight nucleic acids.

The sheared nucleic acid solution was then added to 25 mg of oligo-(dT)-cellulose which had been pre-equilibrated in 1 × binding buffer (0.5 M NaCl, 0.01 M Tris–HCl pH 7.5, 1 mM EDTA, 0.5% SDS). The resulting slurry was mixed for 35 min and centrifuged (3000 g) for 3 min. The pellet was then washed 3 times with 1 × binding buffer and resuspended in a final volume of 400 μl before being transferred to 0.45 μm centrifugal filtration tubes (Millipore) and centrifuged at 3000 g for 2 min to remove RNA bound oligo-(dT)-cellulose. The RNA bound oligo-(dT)-cellulose retained by the filter was washed a final time with 200 μl of 1 × binding buffer.

The filtration unit containing the RNA bound oligo-(dT)-cellulose was then transferred to a new sterile microcentrifuge tube and the poly(A) mRNA eluted by washing with 350 μl of elution buffer (0.01 M Tris–HCl pH 7.5, 1 mM EDTA, 0.05% SDS) and the mRNA precipitated in 0.15 mM sodium acetate in 80% ethanol solution.

Northern analysis

Messenger RNA was resolved by electrophoresis (3.0–6.0 μg per lane) on a 1.2% agarose gel containing 2.2 M formaldehyde and MOPS buffer (0.02 M morpholinopropane sulfonic acid, 5 mM sodium acetate, 1 mM EDTA pH 7.0). Denaturation of each mRNA sample was carried out at 70°C for 15 min in 25 μl of a solution containing 11.9 μl formamide, 8.5 μl formaldehyde, 4.6 μl MOPS buffer (10 × concentrated) and 3.0–6.0 μg mRNA in a total volume of 15 μl DEPC treated H_2O.

RNA was transferred to a charged nylon membrane (Schleicher and Schuell's Nytran, Keene, NH) via capillary action over 16 h. The membrane was then immersed in pre-hybridization solution consisting of 2 ml 50% dextran sulfate, 4 ml Northern prehybridization buffer (2 × concentrated, 5Prime-3Prime, Boulder Co. Inc.), 4 ml deionized formamide (Gibco/BRL, Gaithersburg, MD), denatured yeast RNA and sheared salmon sperm DNA (both at a final concentration of 40 μg/ml, 5Prime-3Prime) for 3–4 h at 42°C. Next, a hybridization buffer was added, with all the components and volumes as described above, except for the replacement of pre-hybridization buffer with 2 × hybridization buffer (5Prime-3Prime), the addition of dithiothreitol (10 mM) and the two radiolabeled probes (see below). This mixture was allowed to incubate for 18–20 h at 42°C, followed by several washes with SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0) in a 0.1% SDS solution at room temperature. The membrane was next stringently washed four more times at 55°C with SSC diluted 1 to 20 with 0.1% SDS and then dried at 60°C before being placed on film (Kodak Diagnostic Film X-OMAT, Rochester, NY) for 18 h.

After developing the film, the resulting band densities were quantified using a Molecular Dynamics densitometer employing the Imagequant[tm] software (Sunnyvale, CA). Recorded density units were directly related to the amount of RNA applied to each lane and to the radioactivity in each band from hybridized cDNA.

Preparation of cDNA probes for Northern analysis

The cDNA for tPA (a generous gift of J. Sambrook) had been inserted into the pSVT7-tPA plasmid, whereas the estrogen insensitive gene, 36B4 (cDNA graciously supplied by P. Chambon), was inserted into pBR322. These plasmids were transfected into the competent *E. coli* strain DH5α, selected cells amplified and the plasmids extracted and purified using standard procedures. Restriction digests were performed (Xbal for tPA and Pstl for 36B4) and samples were run on a 1.2% agarose gel with ethidium bromide (0.4 μg/ml). The inserts were cut out and recovered via electroelution (Elutrap[tm], Schleicher and Schuell).

The radiolabeled probe of 36B4 cDNA was made using a commercially available random primed labeling kit (Boehringer Mannheim, Indianapolis, IN). A solution consisting of random hexanucleotide primers, purified water, dATP, dGTP, dTTP, and [^{35}S]dCTP was prepared and the denatured 36B4 cDNA (roughly 100 ng) was added to these reactants along with Klenow fragment and allowed to incubate at 37°C for 4 h. Double radiolabeled tPA cDNA was prepared using the same random primed labeling kit as above with the addition of [^{35}S]dATP in place of the nonlabeled dATP and an additional 2 h incubation at 37°C. Both reactions were stopped by the addition of 2 μl 0.5 M EDTA, pH 8.0. Unincorporated nucleotides were separated from the radiolabeled probes by passing the reaction mixture over a G-25 Select D mini-column (5Prime-3Prime). A 1.0 μl sample from each probe was counted in a Packard Tri-Carb 4530 scintillation counter to determine the radioactivity of each probe. Approximately 1.2×10^8 cpm of the tPA probe and 2×10^6 cpm of the 36B4 probe were added to the hybridization buffer after denaturation at 100°C for 2 min.

Western analysis of PAI-1

Medium collected from hormone treated cells was electrophoretically separated on 10% SDS–polyacrylamide gels. After electrophoresis, the gels were submerged in transfer buffer (192 mM glycine and 0.25 mM Trizma base pH 8.3) allowed to equilibrate for 30 min and electroeluted at 200 mA for 3.5 h to a polyvinylidene difluoride membrane (PDVF, United States Biochemical, Cleveland, OH). For Western blotting, the Protein Images Non-Isotopic Western Blotting Detection Kit was used according to the manufacturer's specifications (United States Biochemicals). The detection system used a chemiluminescent reagent which is cleaved by alkaline phosphatase (tethered to the secondary antibody) producing a signal detectable with standard X-ray film. All reagents, including the PDVF membrane, were supplied by United States Biochemicals. The membrane was subsequently incubated for 2 h at room temperature with primary antibody, murine monoclonal antibody against human PAI-1 (diluted 1 to 1000) in salt buffer plus 0.1% Tween 20 (American Diagnostica, Greenwich, CT). The membrane is washed and next incubated for 0.5 h with alkaline phosphatase conjugated goat antimouse antibody diluted 1 to 12,000 in salt buffer plus 0.1% Tween 20. After several washes the chemiluminescent reagent Lumi-Phos[tm] 480 was applied to the membrane according to the manufacturer's protocol. The membrane was then sealed in plastic and exposed to X-ray film.

Plasminogen activator assay

Media collected from hormone treated cells was assayed by measuring the release of tritiated peptide

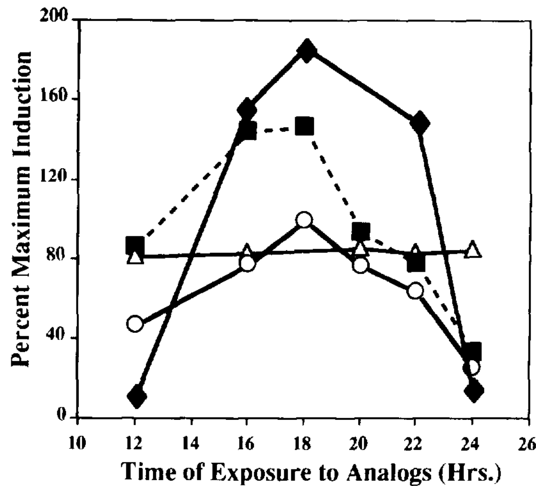

Fig. 1. Effect of time of exposure of MCF-7 cells to certain estrogen analogs on the accumulation of tPA mRNA. Cells were cultured in media supplemented with estrogens as described in Materials and Methods. Following incubation, the cells were harvested, mRNA isolated and Northerns performed as detailed in Materials and Methods. Estrogens added were: 10^{-8} M E_2, ○; 10^{-8} M 3-hydroxyestratriene, ◆; 10^{-7} M 2-hydroxyestratrien-17β-ol, △; 10^{-7} M 5-α-androstan-3β,17β-diol, ■. Maximum induction tPA mRNA is defined as the stimulation brought about by 10^{-8} M E_2 after an 18 h incubation (14-fold increase over the control value). Each point is the average of two experiments. Variations in duplicate experiments were: $< \pm 3\%$, E_2; $< \pm 5\%$, 2-hydroxyestratrien-17β-ol; $< \pm 7\%$, 5α-androstan-3β,17β-diol; $< \pm 7\%$, 3-hydroxyestratriene.

fragments from [^{3}H]fibrin coated wells of tissue culture plates [31]. The counts released were normalized to a urokinase standard curve which was run with each experiment and corrected for volume assayed and cell number [32].

RESULTS

The expression of tPA message was found to be limited in comparison to the expression of the internal standard message, 36B4. Hence the tPA cDNA probe was double labeled to increase its specific activity. This narrowed the difference in specific radioactivity of the internal standard and the tPA cDNA to a range in which both mRNAs could be measured within the linear range of the film (0–4500 densitometric units). It was also determined that the measured density of the mRNA band (36B4) increased linearly with the amount of RNA added per lane (0–3.0 μg, data not shown).

E_2 actively stimulated the synthesis of tPA mRNA with maximum effect (16 ± 6-fold, $n = 4$) being elicited after 18 h when the cells had been exposed to a 10^{-8} M concentration of the hormone (Fig. 1). Continued exposure of the culture to E_2 resulted in loss of the mRNA (half life = 5 h). At peak stimulation by E_2, the synthesis of tPA mRNA was inhibited 80% by actinomycin D (5 μg/ml). Cyclohexamide (5×10^{-5} M) had no effect on transcription of the tPA gene.

The fibrinolytic activity of the intracellular enzyme (analysis of lysate) has been shown previously by this laboratory to follow a pattern similar to that shown in Fig. 1 for the mRNA [33]. In the presence of serum, the secreted zymogen displayed a linear increase in activation for up to 80 h [33, 34]. After 48 h of exposure to increasing concentrations of E_2, the secreted fibrinolytic activity rose from near the control level in cultures pulsed with 10^{-11} M E_2 to a maximum enzymatic activity in cultures exposed to 10^{-7} M E_2 [a 8.9 ± 2.5-fold increase, $n = 6$, Fig. 2(A)].

The inability of the estratriene nucleus to bind to receptor at physiologically relevant concentrations prohibits this ligand from inducing tPA transcription or the fibrinolytic activity [Fig. 2(B)]. However, the presence of either a 3- or 17β-hydroxyl group on estratriene creates an effective ligand. Although these monohydroxyestrogens displayed diminished affinity for ER relative to E_2 (RBA = 0.79 and 0.11, respectively), each is capable of stimulating a tPA message comparable to, or greater than, E_2 at a concentration of 10^{-8} M [Fig. 2 (C and D)]. Active fibrinolysis was also produced by these ligands. However, the enzyme activity which followed the 3-hydroxyestratriene pulse was considerably less than the enhanced effect of this estrogen on tPA mRNA (nearly 2-fold that of E_2).

(Fig. 2. Opposite)

Fig. 2. Induction of tPA mRNA (●) and the resultant extracellular fibrinolytic activity (○) in cultures of MCF-7 cells pulsed for 18 h with different concentrations of estrogen analogs. Cells were grown and treated with estrogens as described in Materials and Methods. Following incubation, the cells were harvested, mRNA isolated and Northerns performed as detailed in Materials and Methods. Analyses of conditioned media for tPA activity after a 48 h incubation is also described in Materials and Methods. The relative binding activity (RBA) of each analog is defined as its affinity for the estrogen receptor in relation to the affinity of E_2, which is set at a value of 1.0. These values were determined with the competitive binding assay and have been reported previously [23]. Cultures were treated with (A) E_2, RBA = 1.0; (B) estratriene, RBA = <0.0005; (C) 3-hydroxyestratriene (3-OHene), RBA = 0.79; (D) estratrien-17β-ol (17-OHene), RBA = 0.11; (E) 1-hydroxyestratrien-17β-ol (1-OHE$_2$), RBA = 0.005; (F) 2-hydroxyestratrien-17β-ol (2-OHE$_2$), RBA = 0.71; (G) 4-hydroxyestratrien-17β-ol (4-OHE$_2$), RBA = 0.07. In these experiments the maximum induction of tPA mRNA by 10^{-8} M E_2 ranged from 6–20-fold and the maximum increase in extracellular tPA activity in cultures supplemented with 10^{-7} M E_2 was 4–7-fold. These experiments which were carried out in duplicate were repeated 2–7 times. Points are averages of all experiments which examined each analog. Variations between duplicate experiments were $< \pm 10\%$.

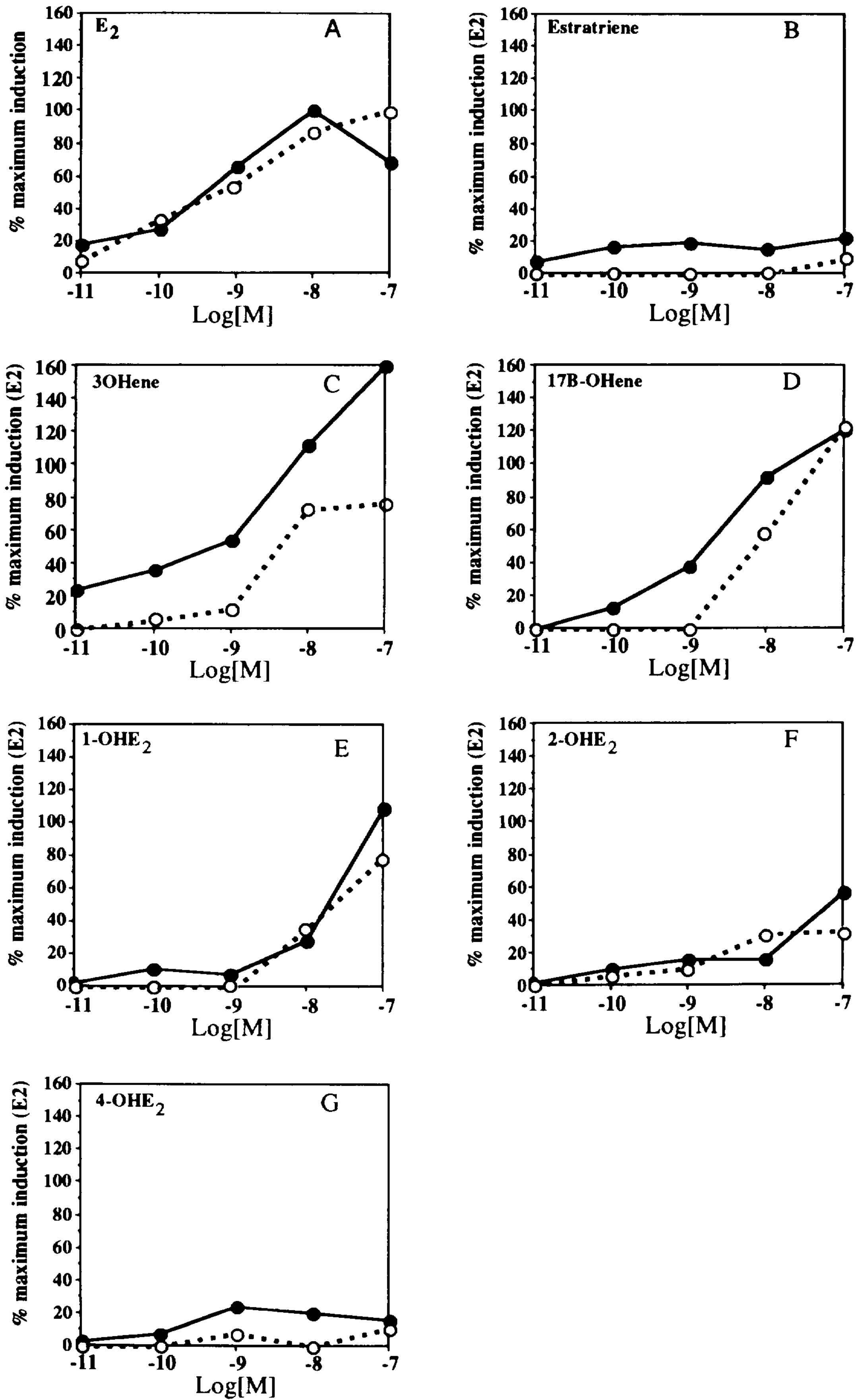

Fig. 2—Legend opposite

Table 1. Effect of D-ring functional groups on tPA mRNA induction and fibrinolytic activity in culture media

Estrogen	Concentration* (M)	RBA†	tPA mRNA‡ (% max. ind.)	Fibrinolysis§ (% max. ind.)
Estrone	10^{-8}	0.22	50	40
Estriol	10^{-8}	0.17	105	50
Estradiol-16α	10^{-7}	0.80	79	79
Estradiol-17α	10^{-7}	0.22	120	98

*Concentration producing maximum induction of tPA mRNA.
†Relative binding affinity, RBA $E_2 = 1.0$.
‡Percent of the maximum induction by 10^{-8} M E_2.
§Percent of the maximum induction by 10^{-7} M E_2.

In the presence of a 3-hydroxyl group, changes in the D-ring oxygen (17-keto, 17α-hydroxy, 16α-hydroxy or 17β,16α-dihydroxy) from the 17β-hydroxy in E_2 fashioned estrogens which stimulated both tPA message and activity. As in the case of the monohydroxyestrogens, the extent of transcription stimulation by each analog was not predicted by its affinity for ER (Table 1). For example, 3-hydroxyestratrien-16α-ol which binds ER with 80% the affinity of E_2 (Table 1), induced little tPA mRNA until a 10-fold excess (10^{-7} M) of this analog was added to the culture media. On the other hand, estriol with 1/5 the K_a of E_2 was equally active in the stimulation of tPA mRNA synthesis as E_2. Estradiol-17α induced tPA mRNA comparable to 3-hydroxyestratriene (or E_2) although the K_a of this analog was also 1/5 that of E_2.

A-ring isomers of E_2 (1-hydroxyestratrien-17β-ol, 2-hydroxyestratrien-17β-ol, 4-hydroxyestratrien-17β-ol) displayed a dramatic decrease in activity, both for the induction of tPA mRNA and the fibrinolytic activity of the secreted enzyme [Fig. 2(E, F and G)]. Whereas 1-hydroxyestratrien-17β-ol has a low affinity for ER (1/200th that of E_2), this analog was capable of maximum stimulation of tPA mRNA at a concentration of 10^{-7} M. On the other hand, 4-hydroxyestratrien-17β-ol, which possesses a higher affinity for ER (RBA = 0.07) was incapable of inducing the tPA mRNA or the resultant fibrinolytic activity at physiologically relevant concentrations. Furthermore, 2-hydroxyestratrien-17β-ol barely stimulated the tPA gene in spite of a high affinity for ER (2/3 that of E_2).

Estrogenic androstanediols are ligands with low affinity for ER which nevertheless are capable of the regulation of transcription of estrogen responsive genes [23, 24]. The stearic configuration of the 3-hydroxyl group, however, is important for this activity. Active ligands for the induction of tPA mRNA are 5-androsten-3β,17β-diol and 5α-androstan -3β,17β-diol (Table 2). The stimulatory capacity of this steroid is greatly diminished by placing the 3-hydroxyl group in the α-configuration. The fibrinolytic activity of the secreted enzyme followed a similar pattern.

The "pure" antiestrogen, ICI-164,384, is inactive in this system, whereas 4-hydroxytamoxifen possessed a slight agnostic effect [Fig. 3(A)]. When MCF-7 cells were cultured with 10^{-8} M E_2 in the presence of increasing concentrations of 4-hydroxytamoxifen, inhibition of tPA mRNA accumulation did not occur until the concentration of the antiestrogen exceeded that of E_2 [Fig. 3(A)]. The A-ring isomers also acted as antiestrogens when these ligands were added to cultures with E_2 [Fig. 3(B and C)]. 4-Hydroxyestratrien-17β-ol blocked E_2 stimulation only when this analog reached the concentration of 10^{-7} M, a level at which it could compete with 10^{-8} M E_2 for receptor [K_a of 4-hydroxyestratrien-17β-ol is approx. 1/10 that of E_2, Fig. 2(F)]. 2-Hydroxyestratrien-17β-ol, which binds ER with an affinity near that of E_2, inhibits the induction of tPA mRNA by E_2 at concentrations two logs below the level at which this analog was capable of stimulating transcription of tPA [Fig. 3(C)]. Nevertheless, when competing with E_2, this A-ring

Table 2. Effect of various androstanediols on tPA and mRNA induction and fibrinolytic activity in culture media

Androstanediol	Concentration* (M)	RBA†	tPA mRNA‡ (% max. ind.)	Fibrinolysis§ (% max. ind.)
5-Androsten-3β,17β-diol	10^{-7}	0.007	45	49
5α-Androstan-3β,17β-diol	10^{-7}	0.005	70	40
5α-Androstan-3α,17β-diol	10^{-7}	<0.0005	8	22

*Concentration producing maximum induction of tPA mRNA.
†Relative binding affinity, RBA $E_2 = 1.0$.
‡Percent of the maximum induction by 10^{-8} M E_2.
§Percent of the maximum induction by 10^{-7} M E_2.

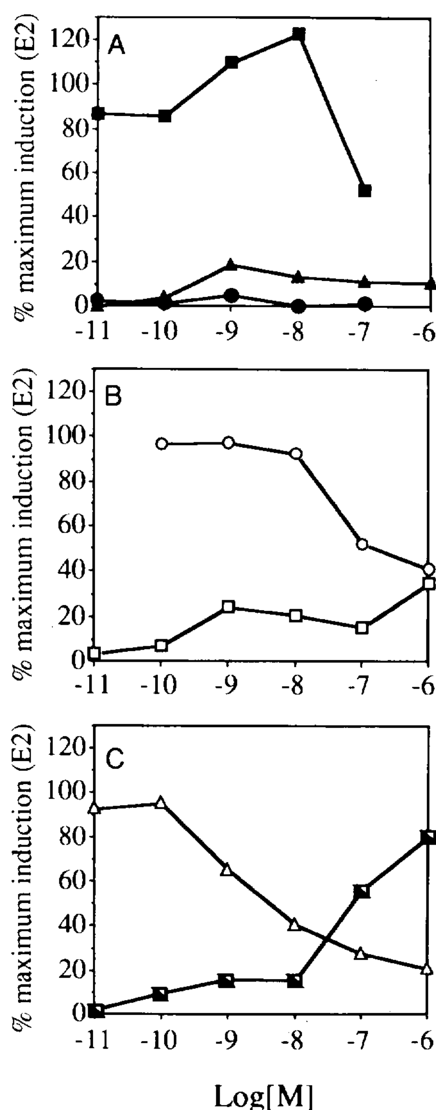

Fig. 3. Effect of antiestrogens on the induction of mRNA in MCF-7 cells treated with E_2. Cells were cultured, mRNA extracted and Northerns performed as described in Materials and Methods. (A) Culture flasks were supplemented with the indicated concentrations of: 4-hydroxytamoxifen, RBA = 2.3 (▲); ICI-164,384, RBA = 0.57 (●); 4-hydroxytamoxifen plus 10^{-8} M E_2 (■). (B) Culture flasks were supplemented with the indicated concentrations of: 4-hydroxyestratrien-17β-ol (□); 4-hydroxyestratrien-17β-ol plus 10^{-8} M E_2 (○). (C) Culture flasks were supplemented with the indicated concentrations of: 2-hydroxyestratrien-17β-ol (◪); 2-hydroxy-estratrien-17β-ol plus 10^{-8} M E_2 (△). The maximum fold induction of tPA mRNA elicited by 10^{-8} M E_2 ranged between 6.9 and 18.3 in the 14 experiments represented in panels (A), (B) and (C). Each point indicates the average of duplicate experiments. Variations between duplicate experiments were $< \pm 9\%$.

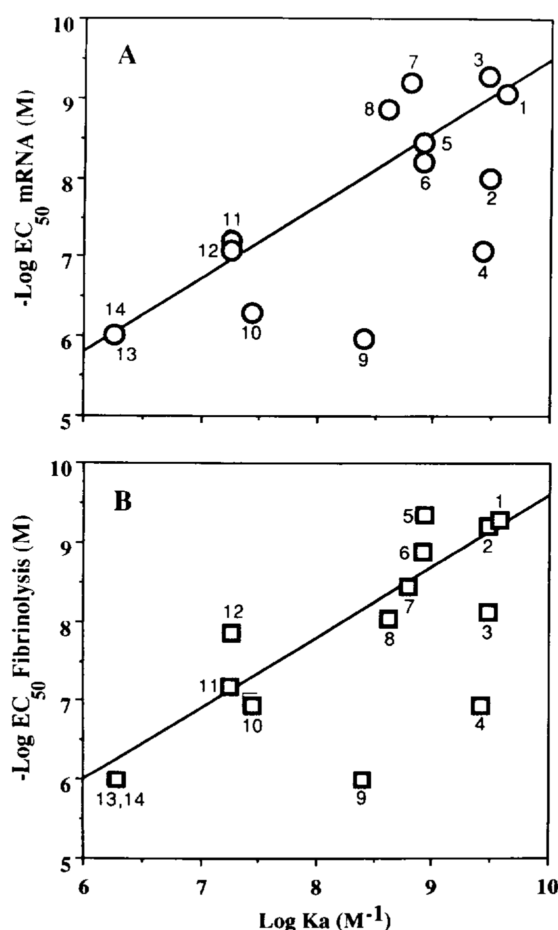

Fig. 4. Relation of K_a of various estrogen analogs and androstanediols to the induction of: (A) tPA mRNA and, (B) extracellular tPA activity. Affinity constants (K_a) were determined by competitive binding assay [23]. EC_{50} is defined as the effective concentration which produced a half-maximal response. The EC_{50} of each analog 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 [36]. R^2 values of lines determined by tPA mRNA induction data (○) and extracellular tPA activity (□) are 0.89 and 0.84, respectively, when points representing compounds 4 and 9 are omitted. 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, (10) 5-androstene-3β,17β-diol, (11) 1-hydroxyestradien-17β-ol, (12) 5α-androstane-3β,17β-diol, (13) 5α-androstane-3α,17β-diol, (14) estratriene.

isomer inhibited tPA mRNA accumulation, even at the higher concentrations.

Several of these analogs displayed a greater stimulatory effect on the synthesis of tPA mRNA than was reflected in the increase in secreted tPA enzymatic activity (Fig. 2, Table 2). This anomaly was examined by determining the effect of estrogens on the level of PAI-1 in MCF-7 culture media. Western analysis showed the amount of PAI-1 in media from experimental cultures to be the same as the level in control cultures which was comparable to the level of PAI-1 found in the serum containing media (data not shown). Glucocorticoid induction of PAI-1 in MDA-MB-231 cells [35] served as the positive control for these experiments. Steroids examined for their effect on PAI-1 were E_2, estriol, 3-hydroxyestratriene and

5α-androstan-3β,17β-diol, each over a concentration range of 10^{-11}–10^{-7} M.

DISCUSSION

E_2 stimulated the accumulation of tPA mRNA but had no effect on PAI-1

Steroid hormones have had a variety of effects on tPA in mammary carcinoma cells [34]. Glucocorticoids have been reported to decrease tPA synthesis in MDA-MB-231 cells [35], an effect presumed to be post-transcriptional since these investigations showed further that the glucocorticoid initiated decrease in tPA activity is related to the stimulation of PAI-1. On the other hand, this steroid increased tPA levels 2-fold in HBL-100 cells with an associated increase in the mRNA [19]. The level of PAI-1 was not affected in the glucocorticoid treated HBL-100 cells. We [8, 33] and others [5, 7] have demonstrated that E_2 is capable of increasing the amount of tPA related fibrinolytic activity in MCF-7 cells. As reported previously, the experiments reported herein have shown E_2 to increase the levels of tPA in cultures of MCF-7 cells [Fig. 2(A)]. Additionally, we have confirmed that this E_2 initiated elevation in fibrinolytic activity was accompanied by stimulated tPA mRNA synthesis [Figs 1 and 2(A)]. In this system, E_2 did not influence the levels of PAI-1.

The stimulation of mRNA synthesis was seen to be sensitive to actinomycin D and to peak after 18 h of exposure to E_2. This effect of an inhibitor of RNA synthesis suggests transcriptional control of the tPA gene by E_2. The absence of inhibition of tPA mRNA accumulation by cycloheximide indicates that the reported lowering of extracellular fibrinolytic activity and amino acid incorporation into tPA [5] was not the result of an effect on RNA synthesis. Further study is required to establish an estrogen effect on the initiation of RNA synthesis. Nevertheless, E_2 induced intracellular plasminogen activity has been reported to peak by 32 h [33]. Both the tPA mRNA (Fig. 1) and intracellular fibrinolytic activity [33] diminished with longer exposure to E_2. Apparently changes in tPA mRNA level and translation of tPA are slow processes [Fig. 1 and ref. 11]. On the other hand, secretion of the proenzyme is more rapid [11], resulting in an extracellular accumulation of the proenzyme which is activated by endogenous serum proteases. Extracellular fibrinolytic activity has been reported to increase linearly for 80 h following E_2 administration to the culture [33], an observation which conforms to the lack of induction of PAI-1 by E_2 in this system.

The effect of structurally altered estrogens on tPA induction was not directly related to their K_a for ER

Deviations in the structure of the estrogenic ligand did not alter the incubation time required for optimal tPA mRNA accumulation in MCF-7 cells (Fig. 1). Nevertheless, the effect of structural analogs of E_2 on tPA mRNA and the resultant extracellular fibrinolysis varied considerably (Fig. 2). In the case of certain analogs (2- and 4-hydroxyestratrien-17β-ol), the decreased effect on tPA mRNA accumulation and fibrinolysis is not explained by a comparable decrease in affinity of the ligand for ER (Fig. 4). Of the 14 steroids examined, only these two A-ring isomers of E_2 displayed a lower EC_{50} for both mRNA accumulation and plasmin formation than would be predicted by their K_as for E_2 (Fig. 4).

Antiestrogen activity of certain analogs

The partial agonist/antagonist 4-hydroxytamoxifen displayed a limited enhancement of tPA mRNA accumulation [Fig. 3(A)]. Although this antiestrogen bound ER with greater affinity than E_2 (RBA = 2.3), a 10-fold excess was required to block the effect of E_2 on the stimulation of tPA mRNA. An even greater excess of 4-hydroxytamoxifen is required to inhibit the induction of other genes in MCF-7 cells by E_2 [37]. 4-Hydroxytamoxifen may not perform efficiently in the inhibition of E_2 activity since this triphenylethylene derivative is suspected to bind ER at a site which is not identical to that occupied by E_2 [38]. On the other hand, two A-ring isomers of E_2 (2- and 4-hydroxyestratrien-17β-ol) acted as more effective antiestrogens in the inhibition of tPA mRNA induction by E_2 [Fig. 3(B and C)]. 4-Hydroxyestratrien-17β-ol, which possesses a K_a 1/10th that of E_2, influenced the induction of mRNA by 10^{-8} M E_2 at a concentration of 10^{-7} M [Fig. 3(B)]. 2-Hydroxyestratrien-17β-ol inhibited tPA mRNA stimulation by 10^{-8} M E_2 at a level (10^{-9} M) commensurate with its affinity for ER (RBA = 0.71). It would appear that these A-ring isomers of E_2 interact with ER much like E_2. Nevertheless, these two estrogen analogs displayed little or no stimulation of tPA mRNA in spite of K_as near that of E_2. The reason for the inability of 2- or 4-hydroxyestratrien-17β-ol to act as agonists at the concentrations which these analogs are antagonists may lie in post-receptor binding phenomena.

Structural characteristics of estrogens which influenced transcription regulation

As shown for three other estrogen responsive genes in MCF-7 cells (progesterone receptor, 23, cathepsin D and pS2, 24), the most important aspect of the structure of E_2 for the induction of tPA is the aromatic A-ring. Neither of the hydroxyl groups on E_2 was required to stimulate the accumulation of tPA mRNA, although the absence of both the 3- and 17β-hydroxyl group prevented receptor binding of the resultant estratriene nucleus. A 3-phenolic hydroxyl group augments the influence of the aromatic A-ring in tPA mRNA induction [Fig. 2(C)]. Positioning the phenolic hydroxyl group elsewhere on the A-ring of estratrien-17β-ol yielded dihydroxyestradiols with greatly diminished effects on the mRNA accumulation

in MCF-7 cells [1-, 2-, and 4-hydroxyestratrien-17β-ol, Fig. 2(E, F and G)]. Only one of these analogs (1-hydroxyestratrien-17β-ol) possessed a low affinity for ER which was commensurate with its decreased activity. As pointed out previously [23, 24, 39], endogenous gene regulation by estrogens is not related directly to the affinity of ligand for ER, 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. To some degree this requirement is met by the unpaired electrons on the 3β-hydroxyl group of the androstanediols and not by the 3α-hydroxyl group [23, 24, 39 and Table 2].

The effect of the electronegative isopotential above ring-A on transcription is envisioned to be carried out by interaction of the estrogen's A-ring with transactivation function-2 (TAF2) which is located in the steroid binding domain of ER [40–42]. This required sequence of 15 amino acids forms an α-helix near the A-ring of the estrogen ligand and is proposed to engage other regulatory factors in the promoter region of the endogenous E_2 responsive genes [42, 43]. Variations in the electronegative isopotential above the A-ring of estrogen analogs may affect TAF-2 in a fashion which allowed interaction with regulation factors found in the promoter region of certain endogenous genes [23, 24]. In the absence of regulatory factors, as in the case of reporter genes containing estrogen responsive elements and the minimal promoter, the estrogen–ER complex is effective regardless of the position of the A-ring hydroxyl group [37].

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