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Down-Regulation of Estrogen Receptors by Androgens in the ZR-75-1 Human Breast Cancer Cell Line*

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ABSTRACT. Much clinical evidence indicates that androgens have beneficial effects in the treatment of breast cancer in women. Physiological concentrations of androgens strongly inhibit both basal and estrogen-induced cell proliferation in the human breast cancer cell line ZR-75-1 through their interaction with the androgen receptor. The present study shows that androgens strongly suppress estrogen receptor (ER) and progesterone receptor contents in this model, as measured by radioligand binding and anti-ER monoclonal antibodies. Similar inhibitory effects are observed on the levels of ER messenger RNA (mRNA) measured by ribonuclease protection assay. The androgenic effect is observed at subnanomolar concentrations of the nonaromatizable androgen 5α-dihydrotestosterone, regardless of the presence of estrogens, and is competitively reversed by the antiandrogen hydroxyflutamide. Such data on ER expression provide an explanation for at least part of the antiestrogenic effects of androgens on breast cancer cell growth and moreover suggest that the specific inhibitory effects of androgen therapy could be additive to the standard treatment limited to blockade of estrogens by antiestrogens. (*Endocrinology* **125:** 392–399, 1989)

FROM 20–50% of women with advanced breast cancer respond to androgen therapy (1–3). The lack of fundamental studies illustrating the mechanisms responsible for the potent inhibitory effects of androgens on breast cancer cell growth probably explains the limited use of androgens in the clinical practice of breast cancer endocrine therapy, which is classically limited to antiestrogens. Pituitary suppression of gonadotropin secretion cannot solely explain the clinical efficacy of androgens since their benefits are unrelated to menopausal status (1). Despite the fact that androgens behave as estrogen agonists at supraphysiological concentrations (4–11), physiological concentrations of androgens (0.01–10 nM) have recently been found to strongly decrease growth rate and cell saturation density through their interaction with the androgen receptor (AR) in the estrogen-responsive ZR-75-1 human breast cancer cell line (12). Growth inhibition by androgens is observed in both steroid-deprived and estrogen-stimulated ZR-75-1 cells and is additive to that induced by antiestrogens (12).

A possible clue to the mechanism of action of dihydrotestosterone (DHT) in these breast cancer cells is provided by the observation that androgens and estrogens exert opposite effects on progesterone receptor (PgR) levels (13). Furthermore, we have also recently demonstrated the antagonism between androgens and estrogens on the accumulation of the gross cystic fluid disease protein-15 (GCDFP-15) and pro-cathepsin D messenger RNAs (mRNAs) (14). Considering the potential clinical usefulness of androgens in breast cancer, and to better understand the molecular mechanisms responsible for the antagonism between androgens and estrogens, we have investigated the effect of androgens on estrogen receptor (ER) expression in the ZR-75-1 human carcinoma cell line.

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Materials and Methods

Materials

Tissue culture reagents and media were purchased from Sigma (St. Louis, MO). [2,4,6,7-^{3}H]Estradiol (100–110 Ci/mmol) and [^{32}P]cytosine triphosphate (CTP) (800 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). [17α-methyl-^{3}H]R5020 (85–88 Ci/mmol) and unlabeled R5020 were obtained from New England Nuclear (Lachine, Québec, Can-

ada). Steroids were obtained from Steraloids (Wilton, NH). Hydroxyflutamide [2-methyl, 2-hydroxy-*N*-(4-nitro-3-[trifluoromethyl]phenyl)propanamide] was kindly provided by Drs. J. Nagabushin and R. Neri (Schering Corp., Kenilworth, NJ). All other reagents were of analytical grade.

Cell culture conditions

The human breast cancer cell line ZR-75-1 was obtained at its 83rd passage from the American Type Culture Collection (Rockville, MD). Cells were routinely grown in phenol red-free (15, 16) RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM Na pyruvate, 15 mM HEPES, 100 IU penicillin, and 10 μg streptomycin sulfate/ml, 10 nM 17β-estradiol (E_2), and 10% (vol/vol) fetal bovine serum (Flow Laboratories, Rockville, MD) at 37 C in a 95% air-5% CO_2 water-saturated atmosphere. For the study of steroid hormone effects, ZR-75-1 cells were harvested with trypsin/EDTA (0.05/0.02%, wt/vol) and resuspended in the medium described above but lacking E_2 and containing 5% (vol/vol) dextran-coated charcoal-treated fetal bovine serum as a serum supplement as well as 500 ng bovine insulin/ml (SD medium). Cells were plated at 4×10^3 cells/cm^2 in 75-cm^2 flasks or 100-mm dishes (for direct binding and immunological studies) or in Linbro 24-well plastic dishes (for the measurement of radioligand-specific uptake). Steroid additions were begun 48 h after plating and made from 1,000–10,000 × stock solutions in redistilled EtOH. Control cells received the ethanolic vehicle only (0.1%, vol/vol). Cell cultures were incubated for the indicated periods with medium being changed every other day. Cell number was determined with a Coulter counter (model ZM, Coulter Electronics, Inc., Hialeah, FL). DNA was assayed by the method of Burton (17).

Assay of steroid receptors by radioligand specific uptake or binding

Measurement of the specific uptake of [^{3}H]E_2 and [^{3}H]R5020 by intact cell monolayers was performed as described (18, 19). For the determination of cytosolic and nuclear ER and PgR in cell homogenates, cell cultures were washed twice with Ca^{2+}-free and Mg^{2+}-free PBS (pH 7.25) and harvested by repeated pipeting, after a 20-min incubation in Ca^{2+}- and Mg^{2+}-free PBS containing 1 mM EDTA. All subsequent steps were performed at 0–4 C unless otherwise indicated. After brief centrifugation (800 × g, 8 min), cells were resuspended in 400–500 μl TEMGM (10 mM Tris-HCl, pH 7.4, at 4 C; 1 mM EDTA; 10 mM monothioglycerol; 10% (vol/vol) glycerol; 10 mM Na_2MoO_4). Homogenization and preparation of the cytosolic and nuclear receptor extracts were performed as described (20, 21). Determination of ER binding activity was then made using a hydroxylapatite assay after incubation with 10 nM [^{3}H] E_2(± 2 μM diethylstilbestrol) for 18 h at 4 C or for 3.5 h at 30 C for cytosolic and nuclear receptors, respectively (20, 21). The apparent dissociation constant (K_d) and total number of estrogen binding sites were determined by Scatchard analysis (22) after incubation of aliquots of homogenate with 0.1–33 nM [^{3}H]E_2 ± a 200-fold excess of diethylstilbestrol. PgR binding activity was determined at 4 C by a hydroxylapatite assay after a 4-h incubation of aliquots of extract with 10 nM [^{3}H]R5020 (21) (± 2 μM radioinert R5020) in the presence of 0.3 μM dexamethasone.

Enzyme immunoassay (EIA) of ER and PgR

Total receptor extracts were prepared essentially as described above for cytosol and nuclear extracts except that TEMKM buffer (10 mM Tris-HCl, pH 8.5 at 4 C; 1 mM EDTA; 10 mM monothioglycerol-0.6 M KCl-10 mM Na_2MoO_4) was used instead for homogenization of cell pellets (900 μl/extract). The high speed supernatant (210,000 × g, 30 min at 4 C, Beckman TL-100 ultracentrifuge, Beckman Instruments, Fullerton, CA) was diluted with the same buffer to the equivalent of about 2×10^7 extracted cells/ml. Immunoreactive ER and PgR were then measured by monoclonal anti-ER and anti-PgR EIA using commercial kits (Abbott, North Chicago, IL) following instructions provided by the manufacturer. Values measured were found to lie within the linear range of ER and PgR concentrations measurable by the standard assay procedure (0–250 fmol/ml). The addition of high concentrations of KCl (up to 0.6 M final concentration) had no effect on the values measured for the human ER in the standard EIA (23).

Preparations of cRNA probes

The *Sac*II/*Pst*I fragment of the ER complementary DNA (cDNA) (24) was purified on a 5% (wt/vol) polyacrylamide gel, electroeluted (Bio-Rad, model 422, Bio-Rad Laboratories, Richmond, CA) and then cloned into the *Eco*RI/*Pst*1 sites of Bluescript M13KS vector (Stratagene Cloning System, San Diego, CA). The recombinant plasmid was linearized with *Eco*RI. Incubation of the linearized vector with [^{32}P]α-CTP (800 Ci/mmol) with T7 RNA polymerase (Amersham), produces a transcript of 441 nucleotides which includes 63 nucleotides from the vector and 378 nucleotides from the ER cDNA fragment. Complementary RNA (cRNA) probe was synthesized as described (25) in the presence of 10 μM unlabeled CTP. The quality of cRNA probe was ascertained on a 5% polyacrylamide gel, and the probe was then immediately used for ribonuclease protection assay.

Ribonuclease (RNase) protection analysis of ER mRNA

Total cellular RNA was prepared by the guanidinium isothiocyanate method as previously described (14, 26–28), and poly(A)$^+$ RNA was purified by chromatography on oligo-dT cellulose column (29). Five micrograms of poly(A)$^+$ RNA from each RNA sample were used per data point. Hybridization and RNase digestion were performed as described (25, 30) and as recommended by Promega Biotec (Bio/can Scientific Inc., Mississauga, Ontario, Canada). Briefly, poly(A)$^+$ RNA was resuspended in 30 μl hybridization buffer containing 40 mM 1,4-piperazine diethanesulfonic acid, pH 6.4, 0.4 M NaCl, 1 mM EDTA, 80% formamide) and 3×10^5 cpm ER cRNA probe, and hybridized at 45 C for 16 h. After hybridization, the samples were incubated at 30 C for 1 h in the presence of RNase A (40 μg/ml) and T1 (2 μg/ml). RNase-resistant hybrids were analyzed by electrophoresis on a 6% polyacrylamide-urea gel. The results were visualized by autoradiography and quantitated by

liquid scintillation spectrometry after cutting the corresponding radioactive bands.

Results

Using an exchange assay (20, 21), we first measured the specific binding of [^{3}H]E_2 in the cytosol and nuclear fractions of homogenates prepared from ZR-75-1 cells preincubated for 11 days with a range of DHT concentrations known to exclusively bind to the AR (7–11) (Fig. 1a). Detection of ER in the cytosol fraction likely arises from the artifactual leaking of unoccupied ER from the nucleus, which is the probable localization of both filled and unfilled receptor forms (31, 32). At the highest concentration used (10 nM), DHT pretreatment decreased cytosol and nuclear [^{3}H]E_2 specific binding by 96% and 50%, respectively, representing a 86% decrease in total estrogen binding activity. Likewise, the specific uptake of [^{3}H]E_2 in intact ZR-75-1 cell monolayers was decreased by as much as 88% after a 10-day preincubation with increasing concentrations of DHT (Fig. 1b). A half-maximal effect of DHT on [^{3}H]E_2 uptake was observed at 70 pM. Preincubation with dexamethasone and R5020 (100 nM each) had no effect on the specific uptake of [^{3}H]E_2 (data not shown). The addition of hydroxyflutamide, a nonsteroidal antiandrogen devoid of agonistic activity and with no significant affinity for receptors other than the AR (33, 34), competitively reversed the inhibition of [^{3}H]E_2 specific uptake by DHT. The inhibition constant (K_i) value for the reversal of DHT action by hydroxyflutamide was estimated (35) at 39 nM, in agreement with the affinity of the antagonist for the AR (36). Thus, the primary site of action of DHT on [^{3}H]E_2 specific binding was clearly consistent with a specific interaction with the AR, rather than a direct activation and processing of the ER by DHT (7–10, 37, 38).

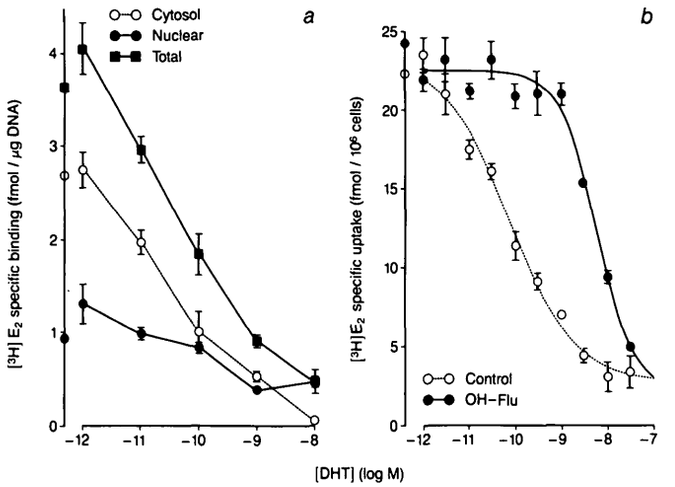

FIG. 1. Effect of preincubation with increasing concentrations of DHT on [^{3}H]E_2 specific binding in ZR-75-1 human breast cancer cells. a, Hydroxylapatite exchange assay of specific [^{3}H]E_2 specific binding of cytosol and nuclear (cytosol + nuclear = total) extracts obtained from ZR-75-1 cells preincubated for 11 days with the indicated concentrations of DHT. b, Specific uptake of [^{3}H]E_2 in intact ZR-75-1 cells preincubated for 10 days with the indicated concentrations of DHT alone (○, control) or in the presence of 3 μM antiandrogen hydroxyflutamide (●, OH-FLU). Values are given as means ± SE from triplicate determinations

Since the androgen preferentially decreased cytosol [^{3}H]E_2 specific binding activity with a relatively small effect on nuclear binding, we performed saturation analysis (22) on the residual nuclear estrogen binding sites in order to obtain more precise information about the nature of the DHT-induced changes in this fraction. In addition to a limited number of saturable high affinity binding sites with ER-like properties, a large amount of competible, low-affinity [^{3}H]E_2 binding activity was present in high-KCl nuclear extracts obtained from steroid-deprived ZR-75-1 cells (Fig. 2a). On the other hand, a single class of estrogen-specific binding sites was detected in cytosol extracts, with an affinity ($K_d = 0.97 \times 10^{-10}$ M) and capacity (1.3 fmol μg^{-1} DNA) characteristic of the cytosolic ER present in this cell line (39) (data not shown). An 11-day preincubation with DHT suppressed only the ER-like, nuclear binding component. On the other hand, after E_2 treatment, the ER-like binding activity was elevated about 10-fold (Fig. 2b), due, at least in part, to the nuclear retention of E_2-ER complexes (37, 38). DHT, however, almost completely abolished high affinity [^{3}H]E_2 specific binding in E_2-treated cells.

The limitations of the ER exchange assay and possible interference by low affinity estrogen binding sites on ER detection were avoided by using an EIA with anti-human ER monoclonal antibodies (ER-EIA) recognizing both occupied and unoccupied receptor forms (23, 40, 41). Through this approach, complete suppression of immunoreactive ER could be demonstrated in ZR-75-1 cells preincubated for 11 days with DHT (Table 1). Incubation with E_2 increased intracellular amounts of its own receptor by about 3-fold. Coincubation of ZR-75-1 cells with E_2 and DHT, on the other hand, reduced immunoreactive ER to about 3% of control levels. Interestingly, competible [^{3}H]E_2 binding was recovered in soluble cell extracts after their immunoadsorption by optimal anti-ER antibody concentrations. This residual [^{3}H]E_2 binding accounts for a substantial portion of the quantitative difference observed between [^{3}H]E_2 binding assay and ER-EIA. These data indicate that the specific [^{3}H]E_2 binding component suppressed by the androgen is indeed the ER. The more DHT-resistant, low affinity [^{3}H]E_2 binding sites thus correspond to entities not recognized by anti-ER monoclonal antibodies. A strong indication that ER suppression by androgen leads to functional disruption of the estrogen regulation of specific gene expression was provided by the complete blockade by DHT of PgR induction by E_2 (Table 1).

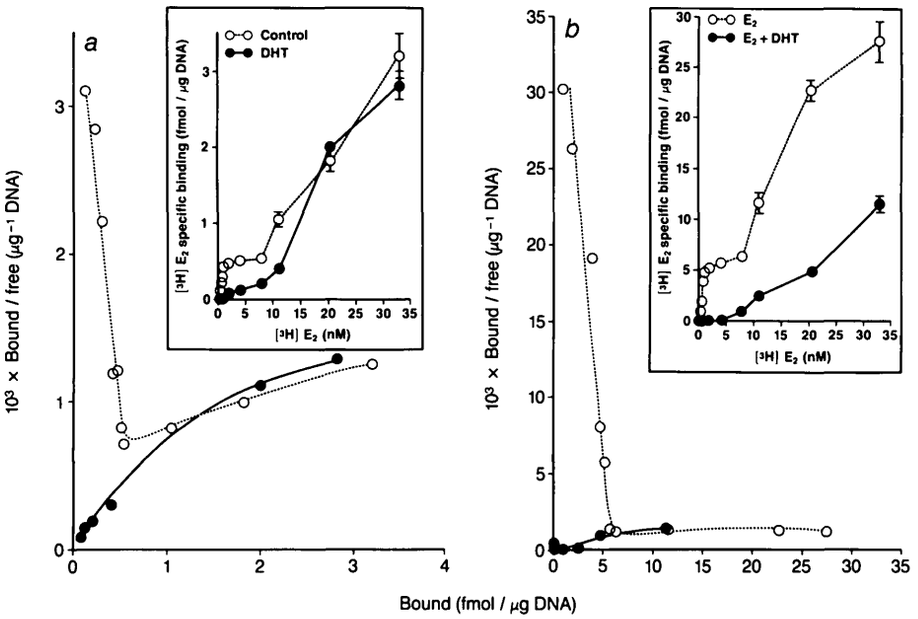

FIG. 2. Scatchard plots of [^{3}H]E_2 specific binding in nuclear receptor extracts obtained from ZR-75-1 cells pretreated for 11 days with 10 nM DHT in the absence (a) or in the presence (b) of 1 nM E_2. Control cells in panel a received the ethanol vehicle only (0.12% vol/vol). The hydroxylapatite exchange assay was performed after incubation of triplicate aliquots of nuclear extracts with increasing concentrations of [^{3}H]E_2 (from 0.16–33 nM) for 3.5 h at 37 C, ±200-fold excess of diethylstilbestrol to account for nonspecific binding. Additional details are given in *Materials and Methods*. *Insets*, Saturation analysis of the same binding data. Values are indicated as means ± SE (*bars*) of triplicate determinations.

TABLE 1. Effect of an 11-day incubation with DHT and/or E_2 on ER and PgR specific immunoreactivity and binding activity in ZR-75-1 cells

Assay	Receptor content (fmol/μg DNA)			
	Control	DHT	E_2	DHT + E_2
ER-EIA	2.02 ± 0.20	ND[a]	6.26 ± 0.29	0.06 ± 0.002
ER-BA				
Total	2.73 ± 0.16	0.71 ± 0.9	12.5 ± 1.10	2.65 ± 0.16
Mab-resistant	0.32 ± 0.01	0.37 ± 0.4	4.03 ± 0.63	1.72 ± 0.25
PgR-EIA	ND	ND	13.1 ± 1.45	ND
PgR-BA	0	0	12.1 ± 0.55	0.10 ± 0.04

Before the experiment, ZR-75-1 cells were grown in SD medium for 10 days in order to remove residual estrogenic effects. Forty-eight hours after plating, DHT (10 nM), E_2 (1 nM), or both steroids were added in fresh SD medium while control cells received the ethanol vehicle only. After 11 days of treatment, cultures were harvested, and total ER and PgR extracts were prepared as described in *Materials and Methods*. Immunoreactive ER and PgR were then measured by monoclonal anti-ER and anti-PgR EIA. ER and PgR binding activity (ER-BA and PgR-BA, respectively) was measured in parallel aliquots of extracts. In this experiment, ER-BA was also determined in receptor extracts after a 19-h incubation at 4 C with rat D457 monoclonal antibodies (MAb) immobilized on polystyrene beads (MAb-resistant; ref. 23, 40, 41). Values are means ± SEM of determinations from triplicate dishes.

[a] ND, Not detected; limits of detection were about 1 fmol and 2.5 fmol/ml of extract for ER and PgR immunoreactive proteins, respectively.

Immunoreactive ER content progressively increases (up to 2.3-fold) during growth until confluence is nearly reached (Fig. 3, a and b). In the presence of DHT, there was a gradual decline in ER content, which reached undetectable levels by day 11 of incubation with the androgen. The rather slow kinetics of ER suppression by DHT resemble those of the androgen-induced inhibition of cell growth (12) (Fig. 3a).

The autoregulatory effect of E_2 on ER levels shows a biphasic pattern. After an initial period (0–4 days) of ER depletion, E_2 increased total immunoreactive ER up to 3-fold over control levels (Fig. 3b). Likewise, prolonged treatment with DHT strongly decreased ER levels in ZR-75-1 cells coincubated with E_2 but did not affect the initial, transient processing of the ER. In agreement with the inability of DHT to block ER processing, there was still a transient induction of PgR by E_2 in the presence of DHT (Fig. 3c). Moreover, even in the presence of extensive ER depletion, growth inhibition by DHT was substantially relieved by the simultaneous addition of E_2 (Fig. 3a), suggesting more rapid kinetics of action of estrogens than androgens.

Prolonged incubation with androgens also markedly decreases ER mRNA levels in ZR-75-1 cells. This effect is illustrated by a 80% reduction in the number of ER-specific transcripts by 10 nM DHT at the end of a 14-day incubation period as measured by RNase protection assay (25, 30) using a specific cRNA probe for ER mRNA (Fig. 4). The addition of E_2 (1 nM) alone consistently caused a significant, albeit smaller (43%) decrease in ER mRNA contents. Coincubation of ZR-75-1 cells with both DHT and E_2 led to a 65% decrease in ER mRNA levels. In addition, shorter incubation period (8 days) with DHT (10 nM), E_2 (1 nM), alone or in combination, decreased respective ER mRNA concentrations to 45 ± 6.3%, 70 ± 4.3%, and 50 ± 5.5% of control levels, respectively ($n = 4$; $P < 0.01$). The present data show that the suppressive effect of DHT on ER content may, at least in part, be attributed to a down-regulation of ER mRNA accumulation.

Discussion

The present data clearly support the hypothesis that at least part of the antagonism observed between the

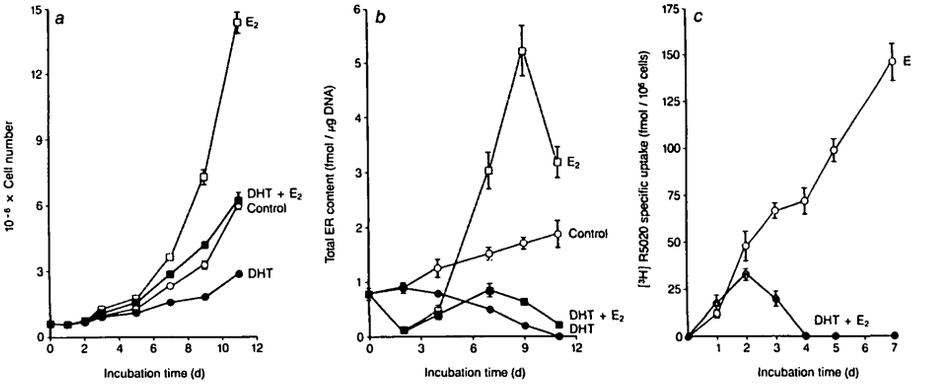

FIG. 3. Time course of growth (a), total immunoreactive ER content (b), and progesterone specific binding sites (c) in ZR-75-1 cells incubated in the absence of steroids or in the presence of 10 nM DHT, 1 nM E_2, or both steroids. Progesterone-specific binding sites were below detection limits in the absence of E_2 (results not shown). Values are indicated as means ± SE (*bars*) of triplicate measurements.

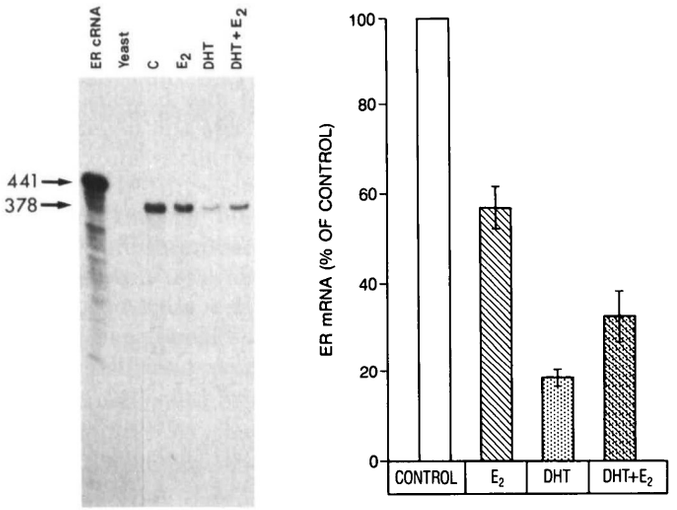

FIG. 4. RNase protection analysis of ER mRNA in ZR-75-1 cells treated in the presence or absence of DHT and/or E_2. *Left*, Typical autoradiogram of RNase protection assay. *Right*, Histogram derived from quantitation of 3 independent experiments. The *bars* indicate the SD. ER mRNA levels were evaluated by RNase protection assay as described in *Materials and Methods*. ZR-75-1 cells were grown for 14 days in the presence of medium alone or medium containing E_2 (1 nM), DHT (10 nM), or a combination of both. ER RNA represents the [^{32}P] CTP-labeled probe. It should be mentioned that the probe shows a single band after synthesis and thus during the hybridization assay period. No RNase-protected product is observed when 20 μg yeast RNA were used as control. The samples containing RNA from the ZR-75-1 cells after different treatments show (*arrow*) one major band of the expected size (378 nucleotides). The *uppermost arrow* indicates the total length of the probe itself (441 nucleotides, including 63 nucleotides from the vector and the 378 first nucleotides at the 5′ end of the ER cDNA fragment used for synthesis of the probe).

action of androgens and estrogens in breast cancer cells (11–14) may be explained by the heterologous down-regulation of the ER by an AR-mediated mechanism. The concentration of DHT needed to exert a half-maximal suppression of ER binding activity (0.07–0.1 nM) is 3 orders of magnitude lower than that known to induce binding and nuclear retention of the ER (7, 11). Moreover, the effect of DHT on ER content was competitively reversed by the antiandrogen hydroxyflutamide, a pure antagonist, which binds exclusively to the AR (33, 34, 36). In addition, prolonged incubation with E_2 induced an up-regulation of total ER intracellular binding activity as well as immunoreactivity, which would exclude an estrogenic action of DHT. Thus, the AR clearly mediates for the down-regulation of the ER by DHT observed in ZR-75-1 cells.

The suppression of ER expression induced by androgens is highly specific, since neither glucocorticoids nor progestins (in the absence of E_2) had any effect on ER content in ZR-75-1 cells despite the fact that under these conditions, glucocorticoids are as growth inhibitory as androgens (results not shown). Although the exact mechanism responsible for the androgen-induced down-regulation of the ER cannot yet be defined, the reduction in the number of ER gene transcripts to about 20% of control levels could be responsible, at least in part, for the results obtained.

A thorough assessment of the physiological importance of the down-regulation of ER by androgens would require further knowledge on the autoregulation of ER by estrogens themselves. The evidence obtained so far in MCF-7 cells, the most widely used model of human breast cancer, indicates that the down-regulation, or processing of the ER induced by estrogens mainly results from a sustained reduction in the number of ER transcripts (42, 43). However, the effect of estrogens on ER expression appears to differ between human breast cancer cell lines, since contrary to their effect in MCF-7 cells, estrogens up-regulate ER mRNA content in T47D cells (44). In the ZR-75-1 cells, a more complex situation is found where, after an early processing of the ER for the first 2–3 days of E_2 addition, ER immunoreactive protein and binding activity increases severalfold despite a sustained 45–55% reduction in ER mRNA content. Thus, in the present model, estrogens appear to markedly stimulate the rate of ER synthesis and/or decrease its degradation rate. This would be at departure from the MCF-7 cell system in which E_2 has been found to decrease the half-life of ER (45). In view of the fact that the down-

regulation of ER content by estrogens is only transient in ZR-75-1 cells, it is of interest that processing is not required for estrogen action in MCF-7 cells (38). Thus, transcriptional and turnover rates of the ER may be, at least in part, dissociated phenomena inZR-75-1 human breast cancer cells. Clearly, extensive comparative studies between different breast cancer cell lines are required in order to understand the mechanism and significance of the homologous regulation of ER by estrogens.

Down-regulation of the ER may contribute to the antagonism of estrogen action by androgens in ZR-75-1 cells, as suggested by the abolition of E_2-dependent induction of the PgR after treatment with DHT. This phenomenon had already been described in rodent estrogen target tissues (46, 47) as well as in the MCF-7 human breast cancer cell line (13). However, no effect of androgens on ER levels was reported in these previous studies. The present results do not eliminate the possibility that PgR expression is under direct control of the AR, *i.e.* that androgens suppress PgR and ER contents independently. However, since PgR expression is strictly dependent on estrogenic stimulation in ZR-75-1 (48) as well as in MCF-7 breast cancer cells (49), down-regulation of ER by androgens would at least potentiate, if not be responsible for, the virtual disappearance of the PgR in androgen-treated cells. Similarly, the partial inhibition by androgens of cell proliferation induced by E_2 observed in the present model (12, 14) is consistent with a suppressive effect of androgens on ER expression.

The effect of androgens on ER content has rather slow kinetics as compared with the early down-regulation, or processing (37, 38) of the ER resulting from addition of E_2 to ZR-75-1 cells. Thus, it is conceivable that estrogens and androgens, when present together, can simultaneously exert opposite effects on ZR-75-1 cell proliferation, consequently leading to a level of cell growth intermediate between that seen in the presence of either E_2 or DHT alone, as observed in Fig. 3a. The delayed inhibitory effect of androgens on ZR-75-1 cell growth, as compared with the mitogenic effect of E_2, was clearly illustrated in a previous study (12) by the complete lack of effect of androgens on E_2-induced cell proliferation when cultures were initiated at high densities.

The effect of androgens on ZR-75-1 cell proliferation, however, cannot be solely explained by the suppression of ER expression, since androgens still exert very potent inhibitory effects on growth in the absence of estrogens, even after prolonged periods of estrogen deprivation before exposure to steroids (12, 14). Moreover, the antiproliferative activity of androgens in estrogen-deprived ZR-75-1 cells is more pronounced and is additive to that exerted by antiestrogens (12, 50). If, as proposed for antiestrogens (50, 51), androgens act on cell proliferation in the absence of estrogens only by suppressing the constitutive activation of ER-controlled genes by unoccupied receptors, or by derepressing genes which are under negative estrogenic control (14, 52, 53), one would not predict significant additivity of the effects of androgens and antiestrogens. Therefore, AR probably controls the expression of positive and/or negative growth-regulatory activities independently of the ER.

Nevertheless, down-regulation of ER expression by androgens might be of crucial importance in their physiological mode of action, *i.e.* when estrogens are simultaneously present. In the specific case of human breast cancer, low concentrations of androgens might reduce the tumor cell sensitivity to estrogens by decreasing ER levels. This effect of androgens may be expected to leave the relative effectiveness of the competitive blockade of estrogen action by therapeutic antiestrogens unaffected, while decreasing the efficiency of any residual estrogenic stimulation of cell growth. Thus, the antagonism of estrogen action by androgens in the ZR-75-1 cell line is fundamentally different from that described for progestins in breast cancer (54–56) as well as other estrogen target tissues (57, 58). Progestins interfere acutely with ER dynamics by selectively and rapidly decreasing the nuclear retention of estrogen-ER complexes (54–58). However, with the exception of the T47D cell line (43, 56), progestins, unlike androgens, require estrogens for their action in breast cancer cells (48, 49, 55). Thus, their antagonistic effect on estrogen action is self-limiting and is abolished by the presence of an antiestrogen (48). The ability of androgens to down-regulate ER expression in the absence of estrogens indicates that they may be involved in the basal estrogen sensitivity of breast cancer cells. On the other hand, progestins may represent secondary modulators that can modulate ER gene expression only after the onset of estrogenic stimulation, inasmuch as estrogens control PgR expression.

The discrepancy observed between the amount of ER detected by radiological and immunological methods in ZR-75-1 cells can be explained, at least in part, by the presence of a second type of estrogen-specific binding site that is unrecognized by the D457 rat monoclonal anti-human ER antibody. The concentration of radioligand (10 nM $[^3H]E_2$) used to saturate the ER in this and other studies (21, 59, 60) was found to cause significant binding (30–35% of total) to low affinity binding sites with high total cellular capacity, since no evidence of saturability was obtained up to the highest concentration of $[^3H]E_2$ used (33 nM). Very similar estrogen binding properties have previously been observed in the ZR-75-1 cell line, but not in the other ER-positive breast cancer cell lines (61). With the knowledge of these characteristics, the quantitative estimation of ER content in ZR-75-1 cells requires Scatchard analysis using a well defined range of ligand concentration. Although many of the

features observed for the low affinity component of specific estrogen binding in ZR-75-1 cells are similar to those of the so-called type II nuclear estrogen binding sites described in human breast tumor tissues (62, 63) and normal rat uterus (64), further studies are required to characterize these non-ER binders. It is noteworthy that the abundance of these sites was strongly enhanced by E_2 treatment and partly antagonized by treatment with DHT (*cf.* MAb-resistant ER binding activity in Table 1). This observation would suggest that the expression of the low affinity E_2 binders is estrogen regulated, which would be consistent with their homology with rodent uterine nuclear type II binding sites (65).

In summary, the data presented in this study demonstrate for the first time that androgens exert a potent negative control on ER expression in estrogen-responsive human breast cancer cells. Whether this effect of androgens on ER can be extended to other cell lines and tissues deserves further study, in light of the fact that androgens specifically antagonize estrogenic action in several systems, in which no mechanism has yet been presented to account for their effect (6, 13, 46, 47, 66).

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