

Adrenal Androgens Stimulate the Proliferation of Breast Cancer Cells as Direct Activators of Estrogen Receptor α ¹

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ABSTRACT

Estrogens stimulate the proliferation of many breast tumors and cell lines derived from them. Antiestrogens have therefore become a powerful therapeutic agent to treat breast tumors that express estrogen receptor (ER) α . In addition, aromatase inhibitors are now used in postmenopausal women to block the *in situ* conversion of adrenal androgens to estrogens. This approach can only be successful if ER- α in a particular tumor is not directly stimulated by adrenal androgens. We have examined this possibility using several different cell lines as model systems: (a) wild-type MCF7 cells, an ER- α -dependent human breast cancer cell line; (b) MCF7SH cells, an estrogen-independent MCF7 variant; (c) Ishikawa cells, an ER- α -containing human uterine cell line; (d) ER-negative HeLa cells; and (e) budding yeast. Transactivation assays with transfected ER- α reporter genes reveal a direct activation of ER- α by dehydroepiandrosterone (DHEA), 5 α -androstene-3 β ,17 β -diol, testosterone, and the two nonaromatizable androgens, dihydrotestosterone and 5 α -androstane-3 β ,17 β -diol. The involvement of other steroid receptors could be ruled out with specific antihormones. Moreover, the same set of ligands stimulates the proliferation of the two breast cancer cell lines. At subsaturating and physiologically relevant concentrations of DHEA, DHEA stimulates the proliferation of MCF7SH cells, which correlates with a substantial, albeit submaximal, transcriptional response. Thus, adrenal androgens must also be considered as risk factors in postmenopausal women.

INTRODUCTION

Estrogens influence both the normal proliferation, differentiation, and physiology of breast tissue and the development and progression of breast cancer (reviewed in Refs. 1–3). The growth of many breast cancers is stimulated by estrogens, which has led to the application of antiestrogens such as tamoxifen in endocrine therapy of breast cancer (discussed in Refs. 4 and 5). The presence of the ER- α ³ is an important prognostic marker, which correlates with higher survival rates and lower risk of relapse. However, about half of ER- α -positive tumors fail to respond to antiestrogen therapy from the beginning, and most of the remaining ones eventually become resistant to the same treatment. The tumor progression from estrogen-stimulated and tamoxifen-sensitive growth to ER- α -independent and thus tamoxifen-resistant proliferation is well documented but remains essentially unexplained (for a discussion, see Ref. 6).

A large number of human breast cancer cell lines have been established, which seem to recapitulate many features of breast cancer progression. For this study, we have worked primarily with human breast cancer MCF7SH cells (7), a MCF7 variant that may represent

those tumor cells that are still ER- α positive and tamoxifen sensitive yet proliferate in a seemingly estrogen-independent manner. This phenotype is likely to be multifactorial and to involve alternative modes of activation of ER- α . ER- α could be activated by growth factors or by overexpressed cyclin D1 in the absence of estrogens (reviewed in Refs. 6 and 8). Alternatively, such ER- α -positive breast cancer cells could become estrogen hypersensitive; whereas this has been observed to occur upon long-term estrogen deprivation, the mechanism remains elusive (9, 10). In postmenopausal women, who have substantially reduced gonadal estrogen levels, adrenal androgens continue to be produced and can be a source for *in situ* conversion to estrogens (11). In addition, there are numerous reports suggesting that androgens might even act through ER- α by themselves (12–20). Using estrogen-independent MCF7SH cells as a model system, we have systematically examined a panel of gonadal and adrenal androgens for their ability to stimulate ER- α -dependent transactivation and cell proliferation without conversion to estrogens and without contributions from the AR, GR, or PRs.

MATERIALS AND METHODS

Reagents. E₂, DHEA, testosterone, ADIOL, and 3 β D were purchased from Sigma; DHT was obtained from Fluka (Buchs, Switzerland). Hydroxyflutamide and ZK98'299, hydroxytamoxifen, and letrozole were gifts from Schering (Berlin, Germany), Laboratoires Besins-Iscovesco (Paris, France), and Novartis (Basel, Switzerland), respectively.

Plasmids. Firefly luciferase reporter plasmids used were XETL (21) for ER and GK1 (22) for the Gal4 fusion protein. The Renilla luciferase expression vector pRL-CMV (Promega) was used as a transfection standard. Plasmid GAL848.ER(G) was constructed by transferring the coding sequences for amino acids 1–848 of Gal4 and the wild-type HBD of human ER- α from plasmid pHCA/GAL4(848).ER(G) (23) into the mammalian expression vector pSCTEVgal93 (24). Plasmid pG/ER(G) (25), derived from expression vector pG-1 (26), was used as the yeast expression vector for ER- α , and pUC Δ SS-ERE (27) was used as its β -galactosidase reporter plasmid.

Cell Culture. Wild-type human breast cancer MCF7 cells were obtained from the European Collection of Cell Cultures (Salisbury, United Kingdom). They were maintained in DMEM without phenol red supplemented with 5% FCS and 100 nM E₂. The variant cell line MCF7SH (7) was maintained in DMEM without phenol red supplemented with 5% charcoal-stripped FCS, except for 1–2 days after thawing a new aliquot, when regular FCS was added. Human uterine Ishikawa cells were a gift from S. Mader (Universit  de Montr al, Montreal, Canada) and were maintained in DMEM without phenol red supplemented with 5% FCS. Note that all our cell lines contain exclusively ER- α and no ER- β as judged by reverse transcription-PCR (data not shown).

Transfections and Luciferase Assays. Cells were transferred into 24-well plates with 500 μ l of regular medium/well the day before transfection. On the day of transfection, the medium was replaced with medium supplemented with 5% charcoal-stripped FCS. Cells were transfected by calcium-phosphate coprecipitation with a mixture containing 1 μ g of reporter plasmid, 5 ng of pRL-CMV, and 1.5 μ g of denatured single-stranded carrier DNA/well; 0.2 μ g of GAL848.ER(G) was added where applicable. The following day, cells were washed with Tris-buffered saline and fed with DMEM lacking phenol red as well as serum. Ligands were added at this point, and cells were further incubated for about 24 h. One-tube assays for firefly and Renilla luciferases were performed using the Dual-Luciferase Reporter Assay System (Promega) as specified by the manufacturer.

Received 3/31/99; accepted 8/5/99.

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¹ Supported by grants from the Swiss National Science Foundation, the Krebsforschung Schweiz, and the Canton de Gen ve. M. M. was a visiting professor from and supported by the University of Calabria (Cosenza, Italy).

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³ The abbreviations used are: ER, estrogen receptor; 3 β D, 5 α -androstene-3 β ,17 β -diol; AR, androgen receptor; ADIOL, 5 α -androstene-3 β ,17 β -diol; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; E₂, 17 β -estradiol; HBD, hormone binding domain; PR, progesterone receptor; GR, glucocorticoid receptor.

Yeast Assays. The yeast strain RMY326 (a gift from R. Movva; Sandoz, Basel, Switzerland) was cotransformed with plasmids pG/ER(G) and pUCASS-ERE to assay the regulation of wild-type ER- α . Transformants were cultured overnight at 30°C in minimal synthetic medium, diluted 20-fold to obtain low-density cultures, and incubated for 12–18 h in the absence or presence of ligands. β -Galactosidase assays were performed as described previously (28) and corrected for cell density.

Proliferation Assays. For quantitative proliferation assays, 50,000 wild-type MCF7 cells were seeded in 30-mm plates in medium with 5% FCS, washed extensively once they had attached, and further incubated in medium without serum with or without ligands; on the second day, the medium was supplemented with 5% charcoal-stripped FCS. For qualitative long-term proliferation assays, both wild-type MCF7 and MCF7SH cells were switched to medium with 5% charcoal-stripped FCS 2 days before starting the experiment. On day 1, a small number of cells were seeded in 60-mm dishes in the same medium with or without hormones. Medium was changed every day. After 10 days, cells were fixed in the same dishes with methanol, stained with 0.1% (w/v) methylene blue in H₂O for 1 h, washed briefly with water, and air-dried.

Immunoprecipitation Assays. MCF7SH cells were grown in 60-mm dishes and exposed to hormones for 24 h before lysis in 1 ml of 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 0.5% NP40, 0.05% SDS, and a mixture of protease inhibitors. Equal amounts of total protein were incubated with the anti-ER- α mouse monoclonal antibody ER-17 (Ref. 29; a gift from D. F. Smith; University of Nebraska, Omaha, NE) for 3 h at 4°C. After binding to protein G-Sepharose (Pharmacia) for 30 min at 4°C, immunoprecipitates were washed, solubilized in sample buffer, and loaded onto a 10% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and probed with antibody ER-17. The peroxidase-conjugated secondary antimouse antibody (Cappel) was revealed by chemiluminescence.

RESULTS

Androgens Activate a Transfected ER Reporter Gene. We began by assessing the ability of a transiently transfected ER reporter gene to respond to a variety of ligands. We chose the two adrenal androgens (DHEA and ADIOL) and the three gonadal androgens (testosterone, DHT, and β BD; Fig. 1). The reporter plasmid XETL carries firefly luciferase sequences under the control of an estrogen response element upstream of the thymidine kinase promoter. As an internal transfection control, we cotransfected a plasmid that expresses Renilla luciferase, which is enzymatically distinguishable from firefly luciferase, from the strong cytomegalovirus enhancer/promoter.

The MCF7SH cell line, a hormone-independent variant (7) of breast

cancer cell line MCF7, was transiently transfected and exposed to increasing concentrations of E₂, gonadal androgens, or adrenal androgens (Fig. 2A). XETL expression is induced more than 100-fold by 1 nM E₂, with a half-maximal response at about 0.1 nM. The latter is consistent with the reported hormone binding affinity ($K_D = 0.2$ nM) for ER from MCF7SH cells (7). Remarkably, all of the tested androgens are strong inducers of XETL expression, albeit at higher ligand concentrations and with somewhat reduced maximal efficacies (the fold induction). β BD and ADIOL are the strongest androgens, with half-maximal responses at about 10 nM, whereas DHEA requires about 100 nM for half-maximal response but still reaches about half of the maximal level of E₂ at higher concentrations.

For comparison, we performed the same transfection experiments with wild-type MCF7 cells and with uterine Ishikawa cells. The fold induction is more than 1 order of magnitude lower with these cell lines. This is due to the reduced maximal transcriptional activities in MCF7; however, the basal activities in the absence of hormones are very similar in MCF7 and MCF7SH cells (data not shown). The remarkable efficacies (the fold induction) of steroids in MCF7SH cells might be related to the increased ER- α content relative to wild-type MCF7 cells (7). Nevertheless, induction by androgens is readily observed in wild-type MCF7 cells. They are equally as sensitive to DHEA and β BD as MCF7SH cells, whereas the dose-response curve for E₂ is shifted by about 2 orders of magnitude to higher concentrations (Fig. 2B). In Ishikawa cells, all three tested ligands give a similar dose-response, with a half-maximal response around 10 nM (Fig. 2C). Note that the E₂ response of MCF7SH cells, unlike that of the two other cell lines, correlates more closely with the known E₂ dissociation constant of ER- α .

Although the use of an ER reporter plasmid strongly suggested that transcriptional activation is mediated by ER- α , this was ascertained by showing that the antiestrogen hydroxytamoxifen abolishes induction by both E₂ and the androgens. Similar results were obtained with MCF7SH (Fig. 3A) and Ishikawa (Fig. 3B) cells. By itself, hydroxytamoxifen did not have any agonist activity in this system (data not shown). We next wanted to determine whether the adrenal androgens act directly or only after being metabolized and aromatized to estrogens. Transactivation assays were performed in the presence of the aromatase inhibitor letrozole. Blocking aromatase had no effect (Fig. 3); this is consistent with our results obtained with androgens DHT and β BD, which cannot be aromatized (Fig. 1). At this point it was still possible that the androgens acted via another steroid receptor, albeit in an ER- α -dependent fashion. To rule out the involvement of AR, GR, and PR (see "Discussion"), we performed the assays in the presence of the antiandrogen hydroxyflutamide and the antiprogestin and anti-glucocorticoid ZK98'299 (Fig. 3). Neither antihormone is able to block activation by androgens, indicating that the androgens are direct activators of ER- α and do not work through AR, GR, or PR. This conclusion is consistent with the reported absence of PR in MCF7SH cells (7). Although we have detected low levels of AR mRNA by reverse transcription-PCR in MCF7SH cells, DHT and progesterone do not elicit an AR- or PR-mediated transcriptional response from an appropriate reporter plasmid (data not shown).

Autoregulation of ER- α . In the presence of E₂, the levels of ER- α molecules are known to be reduced. This is due to an increased turnover of the E₂-activated ER- α (30) and down-regulation of transcription of the ER- α gene itself (ref. 31 and the references therein). Thus, the reduction of ER- α levels can also be taken as an indication of ER- α activation. We therefore examined the levels of ER- α in MCF7SH cells after a 24-h treatment with androgens. The immunoprecipitation experiment shown in Fig. 4 reveals that those ligands that are efficient inducers of reporter gene expression are also the most efficient ones for autoregulation of ER- α .

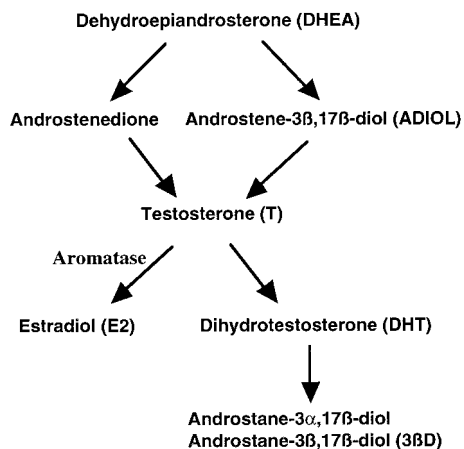


Fig. 1. Metabolic relationship between the steroids used in this study. Two other steroids were added for comprehensiveness: androstenedione, a major intermediate in androgen biosynthesis; and androstane-3 α ,17 β -diol, a major metabolite of DHT. The latter was not included in our analysis because it binds ER- α only poorly (13). Testosterone can be converted to estradiol by aromatase, whereas DHT and β BD cannot.

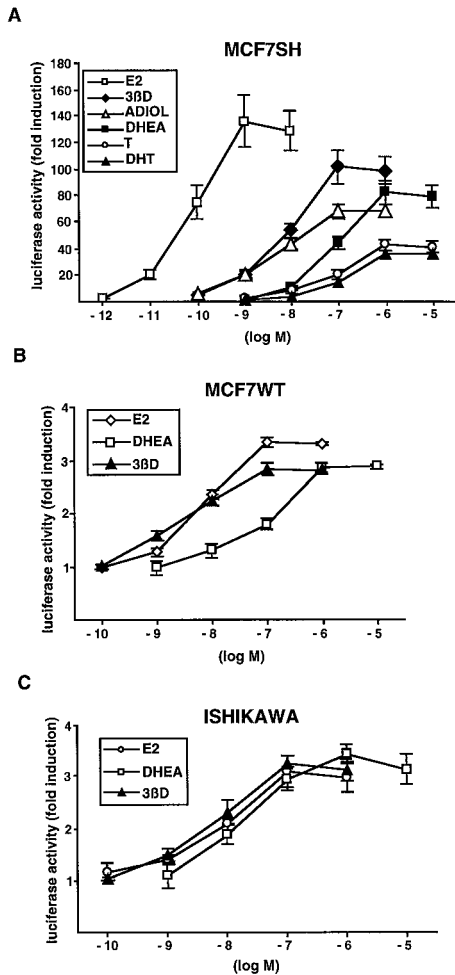


Fig. 2. Transcriptional responses of a transfected ER- α reporter plasmid to androgens. The indicated cell lines (A, MCF7SH; B, MCF7WT; C, Ishikawa) were transfected with the luciferase reporter plasmid XETL and treated with increasing concentrations of androgens (logarithmic scale). Luciferase activities were standardized to the internal transfection control, expressed as the ratio of induced activity:activity in the absence of ligand. Each data point represents the mean of triplicate samples of a representative experiment. *MCF7WT*, wild-type MCF7 cells.

Transcriptional Activation by Androgens in a Heterologous System. To provide further evidence that androgens can act directly through ER- α to activate transcription, we turned to completely heterologous systems. The budding yeast *Saccharomyces cerevisiae* has been a popular model organism for studies on estrogen signaling ever since the demonstration that human ER- α functions in yeast in a hormone-dependent fashion (32). As illustrated in Fig. 5A, all tested androgens are able to activate ER- α in yeast. The altered order of potencies of ligands in this system is not unusual and may be due to differential ligand uptake and/or export (33, 34). However, even the weakest androgen (testosterone) in yeast elicits a 44-fold induction at 10 μ M. To examine whether the ER- α HBD would be sufficient to respond to the androgens and in yet another heterologous system, we constructed a chimeric transcription factor expressed from plasmid GAL848.ER(G). This plasmid encodes a fusion protein consisting of the yeast transcription factor Gal4 fused to the HBD of ER- α . The Gal4 moiety, by itself a strong transcriptional activator in both yeast and mammalian cells, is subjected to hormonal control by the HBD. Fig. 5B shows that E₂ induces transcription mediated by this Gal4-ER fusion about 10-fold in human HeLa cells. All of the tested androgens are also able to induce transcription, with the two strongest androgens, ADIOL and 3 β D, reaching almost the same levels. As expected,

hydroxytamoxifen abolishes this induction; it even represses transcription below the levels seen in the absence of ligand, perhaps by blocking the effect of residual steroids in the growth medium or by recruiting a transcriptional corepressor to the Gal4-ER fusion protein.

Androgens Stimulate Proliferation of Breast Cancer Cells. Having established with exogenous target genes that androgens are efficient activators of the transcriptional activity of ER- α , we wished to examine a physiological response. We assessed the stimulatory effect of androgens on the proliferation of both wild-type MCF7 and MCF7SH cells. The proliferation of the former is strictly hormone dependent, whereas the proliferation of the latter is independent of added estrogen but is stimulated by estrogen and can be blocked by antiestrogens (7). Representative results are shown in Figs. 6 and 7. Fig. 6 depicts the proliferative response of wild-type MCF7 cells in the first few days after the addition of E₂, testosterone, and DHEA. Note that there is no lag phase that might be indicative of a requirement for accumulating active metabolites of the aromatizable androgens testosterone and DHEA. The results of a long-term proliferation assay in which E₂-stimulated cells were allowed to reach confluence provide an alternative and pictorial representation of the stimulatory potential of androgens (Fig. 7). The effects are most striking with wild-type MCF7 cells because of their strict hormone dependence. MCF7SH cells proliferate, albeit to a lower density, even in the absence of ligands. High cell densities indicative of extensive proliferation are apparent in plates of wild-type MCF7 and MCF7SH cells that have been treated with E₂, testosterone, or 100 nM DHEA. Somewhat lower cell densities are reached in the presence of DHT. As

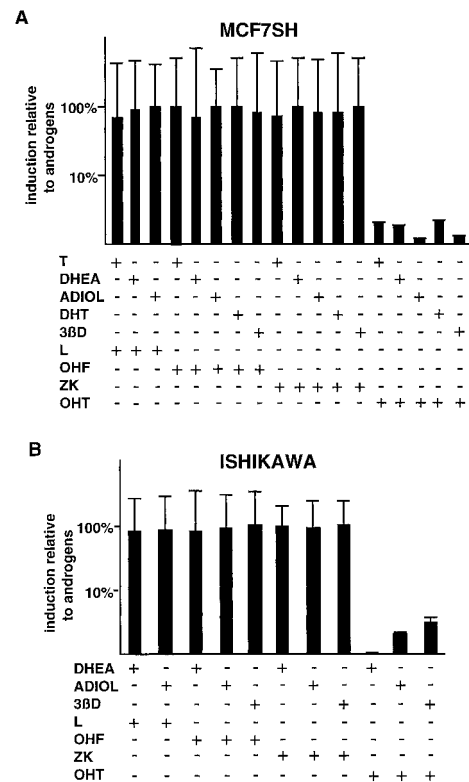


Fig. 3. Androgens activate the ER- α directly. MCF7SH (A) or Ishikawa (B) cells were transfected with the luciferase reporter plasmid XETL and treated with 100 nM androgens as indicated plus the following inhibitors at 10 μ M: L, aromatase inhibitor letrozole; OHF, antiandrogen hydroxyflutamide; and ZK, antiprogestin and antigluocorticoid ZK98'299. The antiestrogen hydroxytamoxifen (OHT) was added at 100 nM. Luciferase activities were standardized to the internal transfection control, expressed as the ratio of induced activity:activity in the absence of ligand. For each set of samples with a particular androgen, the obtained value (fold induction) in the absence of any inhibitor was set as 100%. Each data point represents the mean of triplicate samples of a representative experiment. Note that the large error bars are due to the logarithmic scale of the Y axis.

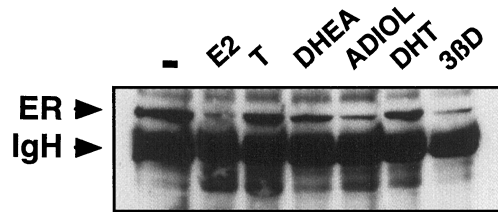


Fig. 4. Autoregulation of ER- α by androgens. Immunoblot of ER- α immunoprecipitated from MCF7SH cells treated with steroids as indicated for 24 h. The two arrowheads indicate the positions of ER- α (ER) and the antibody heavy chain (IgH).

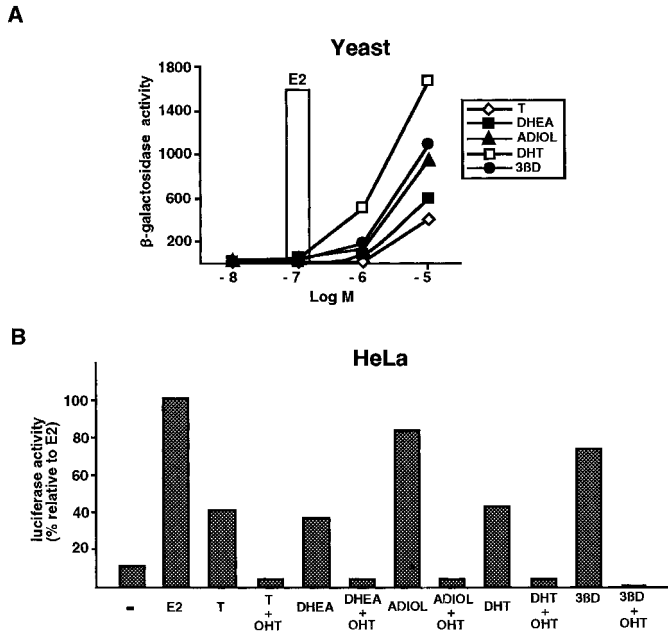


Fig. 5. Androgens activate ER- α in heterologous systems. *A*, activation of wild-type ER- α in yeast. For comparison, the open rectangle represents the activity obtained with 100 nM E₂. *B*, activation of a Gal4-HBD fusion protein in human HeLa cells. Cells were cotransfected with GAL84.ER(G) and the Gal4 reporter plasmid GK1 and treated with ligands as indicated. Luciferase activities were standardized to the internal transfection control and expressed as a percentage of the value obtained with E₂. OHT, the antiestrogen hydroxytamoxifen. The data points are the averages of duplicate samples of a representative experiment.

expected from the transactivation assays, the proliferative stimulus of androgens is blocked by an antiestrogen but not by the antiandrogen hydroxyflutamide. Conversion of aromatizable androgens (DHEA, ADIOL, and testosterone) to estrogens could also not be formally ruled out in this long-term assay system because the aromatase inhibitor letrozole was too toxic at 100 nM. However, we obtained similar results with the nonaromatizable androgen DHT. Interestingly, subsaturating concentrations of DHEA (10 nM) stimulate the proliferation of MCF7SH cells more efficiently than that of wild-type MCF7 cells (see "Discussion"). Thus, androgens are potent stimulators of proliferation of both wild-type and hormone-independent MCF7 cells.

DISCUSSION

The key conclusion from our data is that both gonadal and adrenal androgens are potent direct activators of ER- α . In the presence of androgens, ER- α transactivates a transfected reporter gene, down-regulates its own levels, and stimulates the proliferation of breast cancer cells. The latter finding suggests that androgens may be an important factor influencing breast cancer progression, notably in postmenopausal women.

Androgens have previously been shown to bind to ER- α directly by

hormone binding assays. With ER- α overexpressed in COS cells, the affinities for E₂, DHT, and testosterone have been determined to be 0.055 nM, 105 nM, and >10 μ M, respectively (12). According to another study with *in vitro* synthesized ER- α , DHT, DHEA, ADIOL, and 3 β D compete with half-maximal efficiency against E₂ at 221, 245, 3.6, and 6 nM, respectively (13). Direct binding to cell homogenates from ER-positive breast cancer cells has also been reported for ADIOL (14–16), DHT, and DHEA (16). Qualitatively, these values correlate well with the half-maximal doses that were required in our transactivation assays in breast cancer cells and with the order of potencies (apparent affinities) in the autoregulation and proliferation assays. In wild-type MCF7 and MCF7SH cells, the order of potency is E₂ \gg 3 β D, ADIOL > DHEA > testosterone and DHT. Additional evidence for a direct action of all tested androgens is provided by our findings that the aromatase inhibitor letrozole does not block activation by ADIOL and testosterone, the two androgens that could, in principle, be aromatized to E₂ (see Fig. 1), and that the androgens also activate ER- α in yeast. Although formally not impossible, it seems

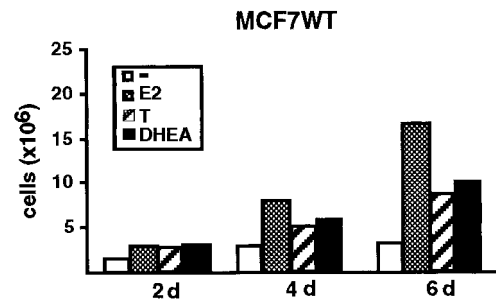


Fig. 6. Androgens stimulate the proliferation of wild-type MCF7 cells. A total of 50,000 wild-type MCF7 cells (MCF7WT) were seeded in duplicate plates, cultured with hormones as indicated, and counted after 2, 4, and 6 days. E₂, testosterone (T), and DHEA were added at 100 nM. The data points are the averages of duplicate samples.

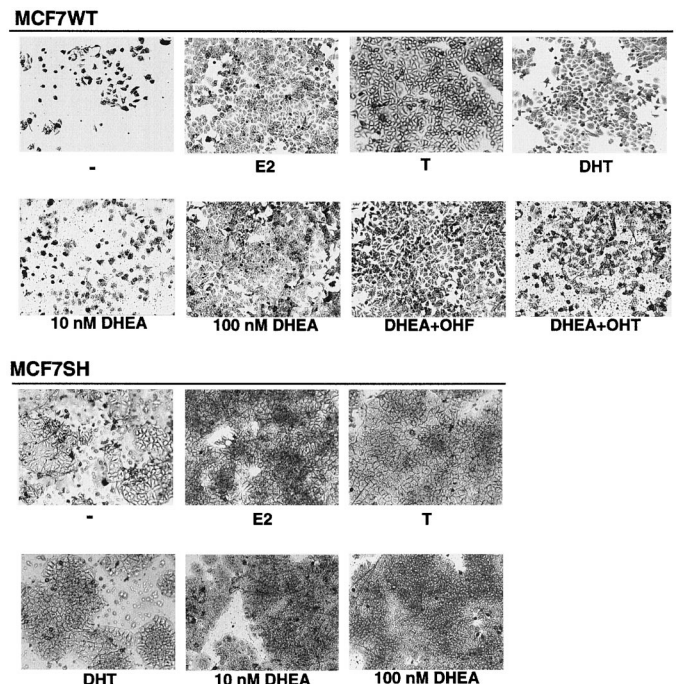


Fig. 7. Androgens stimulate the proliferation of breast cancer cell lines in a long-term assay. Wild-type (MCF7WT) and variant (MCF7SH) MCF7 cells were grown for 10 days in the presence or absence of the indicated ligands, fixed, and stained with methylene blue. E₂, OHT, and androgens were added at 100 nM unless indicated otherwise; OHF, the antiandrogen hydroxyflutamide; OHT, the antiestrogen hydroxytamoxifen.

extremely unlikely that these androgens could be converted to active metabolites by an aromatase-independent pathway that would also be active at 4°C in the above-mentioned *in vitro* systems as well as in yeast.

Given the potential importance of androgens as a source of estrogen precursors or direct ligands, their effects on ER- α function and on the proliferation of estrogen-dependent breast cancer cells have previously been examined. Initially, it was shown that androgens can induce the expression of endogenous estrogen-responsive target genes (14, 15). More recently, several androgens have been demonstrated to activate a stably integrated ER reporter gene in MCF7 cells (16, 17). A transiently transfected reporter gene has been found to respond to very high concentrations of DHT and testosterone in an ER-negative breast cancer cell line (12) and to DHEA, albeit very poorly, in a neuronal cell line (35). The proliferative response of ER-positive breast cancer cell lines to androgens has also been established (15, 18–20). In contrast to these earlier studies, we have now contributed a more comprehensive assessment of the transactivation and proliferative potency of a whole panel of gonadal and adrenal androgens in ER-positive breast cancer cell lines and provided formal proof that these responses are indeed directly ER- α mediated. This was possible by combining the androgens with the aromatase inhibitor letrozole, the antiestrogen hydroxytamoxifen, the antiandrogen hydroxyflutamide, or the antiprogesterin ZK98'299. It was particularly important to rule out an involvement of AR, GR, and PR because signaling cross-talk between different steroid receptors is conceivable at different levels. The nongenomic activation of mitogen-activated protein kinase by progesterone is an interesting example of such a cross-talk. Because it is mediated by a complex between liganded PR and unliganded ER- α , it extends the ligand specificity of ER- α by that of PR (36).

The most important conclusion from our comparative analysis with different cell lines relates to the question of how breast tumor cells that are estrogen independent yet ER- α positive arise. ER-positive breast cancer cells have been found to acquire reversible estrogen hypersensitivity upon long-term estrogen deprivation, a phenomenon that is not yet understood but apparently involves no increase in ER- α levels (9, 10). An alternative mechanism may be responsible for the apparent estrogen independence of MCF7SH cells. Whereas they do not require the addition of estrogens for proliferation, they do depend on ER- α activity, because antiestrogens inhibit their proliferation (7). The remarkable efficacy (the fold-induction) of the hormonal response of MCF7SH compared to wild-type MCF7 cells may be due to their 10-fold elevated levels of ER- α (7) and/or other alterations. It is likely that this renders them hypersensitive to estrogenic contaminants including both estrogens and androgens that may still be present in charcoal-stripped serum. At subsaturating hormone concentrations such as 10 nM DHEA, there is no transcriptional or proliferative response with wild-type MCF7 cells. With MCF7SH cells, the transcriptional response to 10 nM DHEA is only about 12% of the maximal response, but this corresponds to a >10-fold induction (Fig. 2). This robust transcriptional induction that reaches an absolute level above that of the maximal response in wild-type MCF7 cells appears to be sufficient for a partial proliferative response (Fig. 6). Thus, the hormone independence of certain ER-positive breast tumor cells may only be apparent. Their proliferation may be efficiently stimulated by relatively low and subsaturating levels of estrogens and androgens.

Despite the fact that the above-mentioned androgens are able to activate ER- α directly, *in situ* conversion to estrogens is likely to contribute to the estrogenic action of these steroids in certain cell lines and, most notably, in breast tumors. There is ample evidence for expression of aromatase and synthesis of estrogens in both epithelial and stromal cells (reviewed in Refs. 37 and 38). Thus, for breast

tumors, androgens have to be considered as precursors for the *in situ* production of estrogens and estrogenic ligands in their own right. Their physiological levels are very close to those that give partial or even full responses in MCF7SH cells. Plasma concentrations for ADIOL, 3 β D, and DHEA are 1–20 nM (for examples, see Ref. 16 and the references therein). The sulfated form of DHEA, DHEA sulfate, is the most abundant adrenal androgen with plasma levels in the micromolar range. Under the influence of steroidogenic enzymes and DHEA sulfatase, local concentrations of estrogenic ligands could even be higher than those in the blood.

Our results have implications for endocrine therapy of breast cancer. According to current clinical protocols, patients with a relapse after a first long-term treatment with tamoxifen are treated with an aromatase inhibitor to prevent the synthesis of genuine estrogens. For those tumors that remain estrogen dependent and tamoxifen sensitive, growth stimulation by adrenal androgens becomes a concern, because it would not be inhibited by aromatase inhibitors. Interestingly, a recent prospective study lends support to the role of adrenal androgens as risk factors for breast cancer in postmenopausal women (39). Perhaps a combined treatment with antiestrogens and aromatase inhibitors might be more appropriate. Whereas tamoxifen has undesired agonistic activity in other tissues, new and more selective antiestrogens such as raloxifen (4, 5) might render this therapeutic scheme more feasible.

ACKNOWLEDGMENTS

We are grateful to Drs. Sylvie Mader, Rao Movva, Maria-Luisa Panno, Walter Schaffner, David F. Smith, and Bart Van der Burg for providing reagents. We are indebted to the other members of the Picard laboratory and, notably, Bruno Cenni and Toufik Abbas-Terki for continuous support.

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Cancer Res 1999;59:4864-4869.

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