Communication

Estradiol Activates the Prostate Androgen Receptor and Prostate-specific Antigen Secretion through the Intermediacy of Sex Hormone-binding Globulin*

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These experiments were designed to examine the relationship between the effects of steroid hormones mediated by classic intracellular steroid hormone receptors and those mediated by a signaling system subserved at the plasma membrane by a receptor for sex hormone-binding globulin. It is known that unliganded sex hormone-binding globulin (SHBG) binds to a receptor (R_SHBG) on prostate membranes. The R_SHBG-SHBG complex is rapidly activated by estradiol to stimulate adenylylate cyclase, with a resultant increase in intracellular cAMP. In this paper we examine the effect of this system on a prostate gene product known to be activated by androgens, prostate-specific antigen. In serum-free organ culture of human prostate, dihydrotestosterone caused an increase in prostate specific antigen secretion. This event was blocked by the anti-androgens cyproterone acetate and hydroxyflutamide. In the absence of androgens, estradiol added to prostate tissue, whose R_SHBG was occupied by SHBG, reproduced the results seen with dihydrotestosterone. Neither estradiol alone nor SHBG alone duplicated these effects. The estradiol-SHBG-induced increase in prostate-specific antigen was not blocked by anti-estrogens, but was blocked both by anti-androgens and a steroid (2-methoxyestradiol) that prevents the binding of estradiol to SHBG. Furthermore, an inhibitor of protein kinase A prevented the estradiol-SHBG-induced increase in prostate-specific antigen but not that which followed dihydrotestosterone. These data indicate that there is a signaling system that amalgamates steroid-initiated intracellular events with steroid-dependent occurrences generated at the cell membrane and that the latter signaling system proceeds by a pathway that involves protein kinase A.

The standard model of estrogen action posits that hormonal effects are mediated via the intermediary of intracellular estrogen receptors (ER)1 (1–4). These receptors have nascent transcriptional activity that is unmasked by estrogens, thus allowing the transcription of specific genes. The exclusivity of this model in accounting for estrogen action is disputed by observations that estrogens may act by non-ER-mediated mechanisms (5–10). Furthermore, the plasticity of the model has had to be modified because of observations that the ER may initiate transcription in the absence of estrogens, so-called ligand-independent activation of transcription (2, 11–13). Although the data supporting the existence of ligand-independent activation of transcription is substantive, and also has been demonstrated for the progesterone receptor and vitamin D receptor (11, 14, 15), the physiological role of such activation and/or its relation to ligand-activated transcription remains to be clarified.

We have shown previously that estradiol (E2) participates in a signaling system that originates, not within the cell, but at the plasma membrane (5, 16). Through the intermediacy of the plasma protein, sex hormone-binding globulin (SHBG), it causes the generation of cAMP (17–19). In brief, unliganded SHBG binds to a receptor (R_SHBG) on certain cell surfaces and the R_SHBG-SHBG complex is rapidly activated by E2 to stimulate adenylate cyclase, with a resultant increase in intracellular cAMP. There is a paucity of information on events subsequent to the generation of cAMP by this system. In this paper we examine the effect of E2-SHBG-R_SHBG on an androgen-responsive gene.

The gene for prostate-specific antigen (PSA) contains an androgen response element. After binding its cognate ligand, the androgen receptor (AR) interacts with this response element to initiate PSA mRNA transcription (20, 21) and secretion (22, 23). We show that, in the absence of androgens, E2 in concert with SHBG-R_SHBG, acts at the cell membrane to cause secretion of PSA and that this effect is blocked by anti-androgens. This observation provides a first functional link between a classic steroid hormone receptor and a cell membrane-mediated steroidal effect.

EXPERIMENTAL PROCEDURES

Materials—Dihydrotestosterone (DHT), E2, and 2-methoxy-E2 were obtained from Steraloids, Wilton, MA. PKI, an inhibitor of protein kinase A (PKA) was purchased from Sigma. Other chemicals were obtained from Sigma or Boehringer Mannheim. Highly purified SHBG was prepared and evaluated for purity as described previously (16). It migrated as an uncontaminated doublet on SDS-polyacrylamide gel electrophoresis. Because SHBG is isolated with an equimolar concentration of DHT, it was stripped of steroids with dextran-coated charcoal before use (16, 24).

Prostate Tissue Explants—Human prostate tissue was obtained and handled as described previously (5). Prostate tissue was obtained at the time of transurethral or open resection for benign prostatic hyperplasia and immediately brought to the laboratory under sterile conditions. Discolored portions were removed and the remaining tissue was divided into approximately 5-mm cubes. The tissue was placed in 60-mm Primaria culture dishes (Becton Dickinson Labware) in RPMI 1640 (Life Technologies, Inc.) with 5% fetal bovine serum containing 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin, for 2–3 days. It then was minced into 1-mm³ portions and transferred to 16-mm wells in serum-free medium (0.5 ml of RPMI 1640) for about 18 h before beginning an experiment. All additions, e.g. SHBG, receptor; DHT, dihydrotestosterone; E2, estradiol; PKA, protein kinase A; PKI, protein kinase inhibitor; PSA, prostate-specific antigen; R_SHBG, sex hormone-binding globulin receptor; SHBG, sex hormone-binding globulin.
The secretion was 0.59 μg/mg tissue protein.

**Assay for Prostate-specific Antigen**—At the conclusion of all experiments, the medium was harvested and assayed for PSA by an immunoradiometric assay, Diagnostic Products Corp., Los Angeles, CA. The sensitivity of the assay is 0.1 ng/ml, and it has an intraassay coefficient of variation of ~4.0%.

**RESULTS AND DISCUSSION**

Although it is well known that the normal prostate secretes PSA, the experimental data *in vitro* are largely based on the prostate cancer cell line, LNCaP. As is the case for that cell line, DHT caused an increase in PSA secretion in benign prostatic hyperplasia explants, Fig. 1. In LNCaP cells, E₂ also caused increases in the secretion of PSA (23). That effect was due to the mutated AR in those cells which permits the binding of estrogens as well as androgens (25). The normal human prostate contains an ER (26), but unlike LNCaP cells, does not increase PSA secretion in response to E₂ (Fig. 1). However, in the presence of SHBG, E₂ caused a dose-dependent increase in PSA secretion that was as great as that observed in response to DHT (Fig. 1).

Because E₂-SHBG stimulates R\_SHBG to increase intracellular cAMP (5, 27), we examined the ability of increases in intracellular cAMP, brought about either by forskolin or the addition of cAMP to the medium, to affect PSA secretion. The effects of DHT and E₂-SHBG were mimicked both by forskolin (which increases intracellular cAMP) and a cell-permeant analog of cAMP, 8-(4-chlorophenylthio)-cAMP (Fig. 2). The response to 8-(4-chlorophenylthio)-cAMP was biphasic with a stimulation of PSA secretion at 1 and 10 nM that diminished at 100 nM and disappeared at 1000 nM. We have observed a similar biphasic effect of cAMP on the growth of a human prostate cancer cell line (ALVA-41) (28). cAMP begins its signaling cascade, by activating a protein kinase (PKA), the catalytic subunit of which then phosphorylates one or more proteins, which results in a change in their biological activity. Thus, if E₂-SHBG increases PSA secretion through this pathway and DHT does not, inhibition of the activation of PKA should prevent E₂-SHBG, but not DHT, motivated PSA secretion. That is precisely what occurs when PKA is inhibited with the PKA inhibitor, PKI (Fig. 3).

To evaluate the participation of the ER in the response of PSA to E₂-SHBG, anti-estrogens were added to prostate minces together with E₂ to determine if these agents altered the effects of E₂-SHBG. The increase in PSA caused by E₂-SHBG was blocked neither by tamoxifen nor by the pure anti-estrogen ICI 164,384 (Fig. 4). However, 2-methoxy-E₂, a hormonally inactive metabolite of E₂ that binds tightly to SHBG (29) and blocks R\_SHBG-mediated cAMP generation (27), blocked the effect of E₂-SHBG on PSA secretion (Fig. 4).

The promoter of the PSA gene has an androgen response element, and PSA secretion (22, 23) and the expression of PSA mRNA are androgen-regulated (20, 30). Hence, we examined the effect of hydroxyflutamide and cyproterone acetate, both potent anti-androgens, on the E₂-SHBG-mediated increase in PSA secretion. As expected (31, 32), both anti-androgens blocked the effect of DHT on PSA secretion (Fig. 5). Surprisingly, they also blocked the effect of E₂-SHBG on PSA secretion. Although estrogens can bind to and activate the mutated LNCaP androgen receptor (25, 33), there is no evidence that this is the case in nonmalignant prostate cells. Since E₂ is not exerting its effect by binding to the AR, e.g., it is not its cognate ligand, the E₂-induced secretion of PSA observed in this study is indicative of a ligand-independent activation of the AR.

The inhibition of transactivation of the estrogen and progesterone receptors by appropriate antihormones, in the absence of their cognate ligands, has been part of the evidence adduced to support the concept of ligand-independent activation of these
receptors (reviewed in Ref. 15). Our demonstration of the blockade of an AR-mediated effect by an anti-androgen, in the absence of androgens, is consonant with observations first made by Culig et al. (34). They used prostate cancer cell lines, cotransfected with an AR expression vector and an androgen-responsive reporter, to show that, in the absence of androgens, AR-mediated gene transcription could be driven by keratinocyte growth factor, epidermal growth factor, or insulin-like growth factor I. The transactivation induced by each of these was inhibited by anti-androgens. More recently, Nazareth and Weigel (35) showed that forskolin transactivated the human AR cotransfected into CV-1 cells with an appropriate reporter construct. Like the response to androgens, the response to forskolin was blocked by anti-androgens. Furthermore, PKI (an inhibitor of PKA) markedly diminished the response not only to forskolin but also to androgens. Thus, these data not only confirm that AR can undergo ligand-independent activation, but suggest a role for the PKA pathway in androgen initiated activation of the AR in this model. Although the data in that communication are generally consonant with our observations, the inhibition of androgen-induced reporter activity by PKI is at odds with the inability of PKI to inhibit DHT-induced PSA secretion (Fig. 3). It is reasonable to hypothesize that this important variance may reflect the difference between observations made on prostate explants and those made on transfected, non-prostatic cell lines. Ikonen et al. (36) elicited synergism between androgens and activators of PKA (in cells cotransfected with an AR expression plasmid and a reporter containing two androgen response elements); however, they could not demonstrate ligand-independent activation of the AR. The reasons for these discrepancies are undoubtedly several but probably include the fact that they transfected cells with an expression plasmid containing the rat AR, whereas the other studies were all human AR-based.

Although the existence of the RSHBG system has been apparent for a number of years, it is only recently that studies dealing with its biology have appeared. DHT, but not E₂, caused an increase in the rate of growth of ALVA-41 cells (a human prostate cancer cell line) in serum-containing media (37) but failed to do so in serum free media (28). However, the introduction of SHBG enabled both DHT and E₂ to enhance the growth of these cells in serum free media. Furthermore, the increase in growth was as great as that seen with DHT in serum-containing media (28). Working with a breast cancer cell line (MCF-7), Fortunati et al. (38) demonstrated that the RSHBG system antagonized the E₂-induced growth of these cells. The data supported their conclusion that this effect was not caused by the sequestration of E₂ by SHBG. Growth is a complex phenomenon, and the mechanisms underlying these observations on ALVA-41 and MCF-7 cells will undoubtedly be difficult to sort out. The observations in this communication deal with a simpler system and should be more amenable to a dissection of mechanisms.

There are a number of general ways in which E₂/ShBG-RSHBG-promoted increases in PSA secretion might be accomplished. PSA is synthesized and secreted by the prostate epithelial, but not the stromal, cell. However, although E₂/ShBG-RSHBG-mediated increases in cAMP occur in the epithelial cell, both normal (5) and cancerous (16, 38), the increases are most robust in the prostate stromal cell. Thus, although all the elements (RSHBG, AR, and PSA) needed to sort out the observations in this communication are generally consonant with our observations, it is only recently that studies dealing with its biology have appeared. DHT, but not E₂, caused an increase in the rate of growth of ALVA-41 cells (a human prostate cancer cell line) in serum-containing media (37) but failed to do so in serum free media (28). However, the introduction of SHBG enabled both DHT and E₂ to enhance the growth of these cells in serum free media. Furthermore, the increase in growth was as great as that seen with DHT in serum-containing media (28). Working with a breast cancer cell line (MCF-7), Fortunati et al. (38) demonstrated that the RSHBG system antagonized the E₂-induced growth of these cells. The data supported their conclusion that this effect was not caused by the sequestration of E₂ by SHBG. Growth is a complex phenomenon, and the mechanisms underlying these observations on ALVA-41 and MCF-7 cells will undoubtedly be difficult to sort out. The observations in this communication deal with a simpler system and should be more amenable to a dissection of mechanisms.

In summary, we have shown that E₂ can activate a typical AR-mediated event, PSA synthesis and secretion. It does so by activating SHBG-RSHBG and makes clear that there is cross-talk between a classic intracellular steroid hormone receptor and a steroid hormone-engendered event at the cell membrane.

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