Estradiol causes the rapid accumulation of cAMP in human prostate

(androgens/sex hormone-binding globulin/dihydrotestosterone)

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ABSTRACT Androgens are widely acknowledged to be central to the pathogenesis of benign prostatic hypertrophy (BPH). However, BPH increases in prevalence as men age, at precisely the stage of life when plasma androgens are decreasing. The decrease in total plasma androgens is amplified by an age-related increase in plasma sex hormone-binding globulin (SHBG) that results in a relatively greater decrease in free androgens than in total androgens. In addition, estrogens have long been suspected to be important in BPH, but a direct effect on the human prostate has never been demonstrated. We present data that are consistent with a role for estradiol, and for a decrease in androgens and an increase in SHBG, in the pathogenesis of BPH. We show that estradiol, but not dihydrotestosterone, acts in concert with SHBG to produce an 8-fold increase in intracellular cAMP in human BPH tissue. This increase is not blocked by an antiestrogen and is not provoked by an estrogen (diethylstilbestrol) that does not bind to SHBG, thus excluding the classic estrogen receptor as being operative in these events. Conversely, dihydrotestosterone, which blocks the binding of estradiol to SHBG, completely negates the effect of estradiol. Finally, we demonstrate that the SHBG-steroid-responsive second-messenger system is primarily localized to the prostatic stromal cells and not to the prostatic epithelial cells. Thus, we have shown a cell-specific, powerful, nontranscriptional effect of estradiol on the human prostate.

Benign prostatic hypertrophy (BPH) is among the most common afflictions of aging men. Fifty percent of 60-year-old men have BPH, and this percentage increases to 90% by age 85 (1). Although there is an immense literature that deals with the endocrinology of the normal and abnormal prostate (2), there are great gaps in our understanding. For instance, although there is a general belief that estrogens are important in the pathogenesis of BPH (3, 4), there has never been a clear demonstration of a direct estrogenic effect on the prostate. Further, there is an apparent paradox involving two widely accepted endocrinologic facts. First, a basic tenet in the pathogenesis of BPH is that androgens are central to its development (2, 5, 6). Second, there is a decrease in total plasma androgens, and a concomitant increase in their binding protein, sex hormone-binding globulin (SHBG), with age (7, 8). The increase in SHBG amplifies the decrease in total androgens, so that the decline in the bioavailable (that is, free) plasma androgen pool is even greater than the decrease in the total. Hence, the paradox. Total and free androgens are decreasing as the incidence of BPH is increasing. In addition to its role in regulating the concentration of free androgens and estrogens in plasma (9), SHBG binds to a receptor on the cell membrane of the human prostate (10, 11). Because SHBG is a high-affinity binder of both estrogens and androgens (9, 12, 13), we examined BPH tissue to ascertain whether estrogens and androgens might affect BPH through

an effect mediated by SHBG. In these experiments we examined the ability of estradiol and dihydrotestosterone to activate a second messenger, cAMP, by a mechanism involving SHBG and its receptor in prostatic tissue obtained from men with BPH.

MATERIALS AND METHODS

SHBG. Highly purified SHBG was prepared as before (14–16). It migrated as the previously described doublet on SDS/PAGE. Overloaded gels were devoid of contaminants. Because SHBG is isolated with an equimolar concentration of dihydrotestosterone, it was stripped of steroids before use (16).

Prostatic Tissue. *Explants.* Prostatic tissue was obtained at the time of transuretheral resection for BPH and immediately brought to the laboratory under sterile conditions. Discolored portions were removed and the remaining tissue was divided into ≈5-mm cubes. The tissue was placed in 60-mm Primaria culture dishes (Becton Dickinson Labware) in RPMI 1640 (GIBCO) with 5% fetal bovine serum containing penicillin (100 units/ml), streptomycin sulfate (100 μg/ml), and amphotericin (0.25 μg/ml) and was incubated for 2–3 days. It was then minced into 1-mm³ portions and transferred to 16-mm wells in serum-free medium (0.5 ml of RPMI 1640) for about 18 hr before the start of an experiment. All additions (SHBG, steroids, controls) were made in serum free RPMI 1640.

Cell culture. Fibroblast-enriched cultures (17) were prepared from surgical specimens obtained as above. Fragments (1–3 mm³) were placed in 25 ml of digestion buffer (10 mM Hepes/142 mM NaCl/6.7 mM KCl, pH 7.4) and stirred for 30 min at 37°C in an atmosphere of 95% air/5% CO₂. After discarding the supernatant, fresh digestion buffer supplemented with 1% collagenase, 0.67 mM CaCl₂, 20 mM dextrose, and 0.05% DNase was added to the tissue for 30 min at 37°C. The supernatant was discarded again and the digestion was repeated twice. The cells released after the second and third digestion were collected, washed twice, and plated in fresh attachment medium (Ham's F12 supplemented with 5% fetal bovine serum). The medium was changed after 7 days and every 3–4 days thereafter. Three to four weeks after the original isolation, >95% of the cells had the typical morphology of fibroblasts. About 18 hr before the beginning of an experiment, cells were placed in serum-free medium (Ham's F12), and detached with a nonenzymatic cell dissociation solution (Sigma). They then were washed once and suspended (0.2–0.5 mg of protein per ml) in serum-free medium containing 50 nM SHBG. Prostatic epithelial cells were provided by D. M. Peehl (Stanford University) and were isolated as described by her (18). They underwent the same experimental protocol as the fibroblasts.
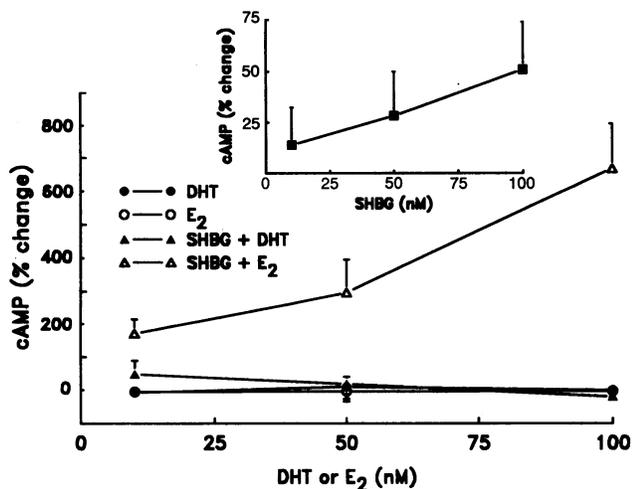
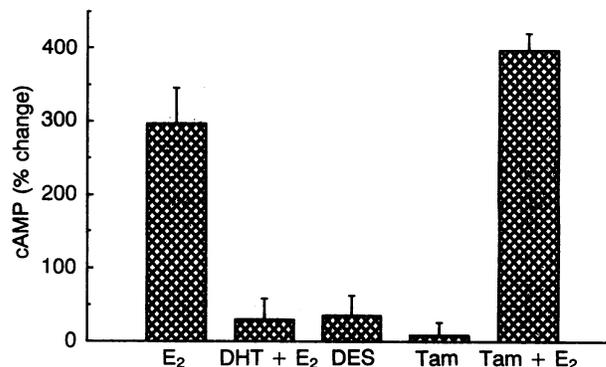Abbreviations: BPH, benign prostatic hypertrophy; SHBG, sex hormone-binding globulin.

5402

RESULTS

The overall experimental design was to add sufficient unliganded SHBG to minces of prostatic tissue in culture to saturate their SHBG receptors. After washing to remove excess SHBG, appropriate concentrations of dihydrotestosterone or estradiol were added, and 15 min later the experiments were terminated and intracellular cAMP was determined (Fig. 1). In the absence of SHBG, neither dihydrotestosterone nor estradiol effected a change in cAMP. SHBG without steroid caused a small increase in cAMP (48%, $P <$ 0.03, $n = 20$) compared to buffer. In contrast, in the presence of receptor-bound SHBG, estradiol caused a robust, dose-dependent increase in cAMP (Fig. 1). Surprisingly, dihydrotestosterone, which binds to SHBG with a 4.5-fold greater affinity than estradiol (14, 19), did not activate the SHBG–receptor–adenylate cyclase system.

The model we proposed for this system (20, 21) has, among others, two necessary elements: (*i*) the classic intracellular estrogen receptor is not involved in the acute response to estradiol and (*ii*) for the system to be activated, SHBG that is bound to its receptor must have its steroid binding site available. The first element can be validated by showing that a strong estrogen that does not bind to SHBG does not affect cAMP and that the accumulation of cAMP is unaffected by a classic antiestrogen—i.e., one that blocks estrogenic effects by competing for binding to the estrogen receptor. The second element can be proven by blocking the effect of estradiol by preventing it from binding to SHBG. Dihydrotestosterone, which binds to SHBG more tightly than estradiol but does not result in the accumulation of cAMP (Fig. 1), is an ideal molecule to test this hypothesis. The data in Fig. 2 meet all these requirements. Diethylstilbestrol is a potent nonsteroidal estrogen that does not bind to SHBG (19) and does not cause cAMP accumulation (Fig. 2). The antiestrogen
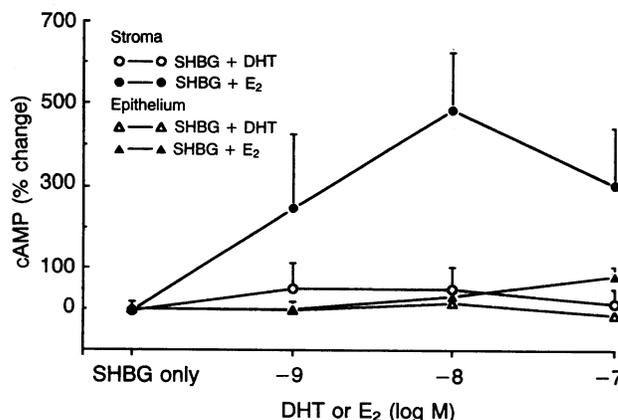

FIG. 2. Influence of antiestrogens, nonsteroidal estrogens, and dihydrotestosterone on the activation of the SHBG receptor by estradiol. This experiment illustrates the lack of participation of the intracellular estrogen receptor and the importance of SHBG in the accumulation of cAMP in prostatic minces. All experiments were done after preincubation of prostatic minces with 50 nM SHBG (as in the legend to Fig. 1). Estradiol (E_2), diethylstilbestrol (DES), or tamoxifen (Tam) was added at 100 nM for 15 min (as in the legend to Fig. 1). In the experiments in which DHT (50 nM) or tamoxifen (100 nM) were used together with estradiol, they were added 5 min before estradiol. Estradiol was then added for 15 min after which cAMP was evaluated as described in the legend to Fig. 1. The percent change is relative to that observed when vehicle was substituted for estradiol. The experiments were done in quadruplicate on tissue from a single patient. The results are representative of those obtained on tissue from two others. cAMP in the presence of SHBG alone was 15.0 ± 1.16 pmol/mg of protein (mean ± SEM).

tamoxifen does not bind to SHBG (19) and does not block the effect of estradiol. Finally, dihydrotestosterone negates the effect of the subsequent addition of estradiol on cAMP.

Human prostatic tissue consists primarily of epithelial and stromal cells. To localize the site of the response shown in Fig. 1, stromal and epithelial cells were examined separately in experiments similar to those in Fig. 1. As is apparent, the prostatic stromal cells (Fig. 3) appear to be responsible for the effects observed in whole prostate (Fig. 1). There is only a diminutive response in epithelial cells (Fig. 3). There is another difference between epithelial and stromal cells. In stromal cells, there was no significant response of cAMP to SHBG (50 nM) alone. In epithelial cells, SHBG caused a 68% increase in cAMP for SHBG compared with buffer ($P < 0.01$, $n = 6$).

FIG. 1. Effect of SHBG alone and of SHBG plus steroids on prostatic intracellular cAMP. *Inset* compares the effect on cAMP of increasing doses of unliganded SHBG with the effect of a buffer control. The main figure compares the effect on cAMP of increasing doses of dihydrotestosterone (DHT) or estradiol (E_2) alone, or these same steroids added after SHBG was bound to its receptor (SHBG + DHT and SHBG + E_2). Note the difference in scale on the ordinates of the *Inset* and main figure. To saturate prostatic SHBG receptors, highly purified SHBG (50 nM) was added to prostatic minces for 3 hr at 37°C. SHBG in the medium was then removed by a single wash, after which the minces were incubated for 15 min with vehicle or the indicated concentration of steroids and 0.1 mM 3-isobutyl-1-methylxanthine. At the end of this second incubation, cAMP was extracted and determined by a commercial ELISA (GIBCO/BRL). Data are the mean ± SEM of results from tissue obtained from three patients, each done in triplicate, and were corrected for protein concentration.

FIG. 3. Effect of SHBG plus steroids on intracellular cAMP in prostatic fibroblasts and epithelial cells. Experiments were done separately on prostatic epithelial cells and fibroblasts obtained as described in the text. The protocol was the same as that described in the legend to Fig. 1. The results represent quadruplicate experiments on fibroblasts obtained from prostatic tissue from three patients. DHT, dihydrotestosterone; E_2, estradiol.

DISCUSSION

The observations in this communication have implications in a number of distinct but related areas. They provide clear and convincing evidence that estradiol can act through mechanisms that are unrelated to the initiation of transcription (22). The lack of response of this system to diethylstilbestrol and the inability of tamoxifen to block the effect of estradiol show that the intracellular estrogen receptor does not participate in this acute response.

The magnitude of the increase in intracellular cAMP seen after activation of the SHBG–receptor–adenylate cyclase system in BPH tissue, ≈700%, is substantially greater than that observed in a human prostate cancer cell line (LNCaP), ≈80%, (16). This is consistent with the epithelial nature of LNCaP cells and the minor response of epithelial cells to this system (Fig. 3). There is a qualitative difference between these experiments and those with the LNCaP cells that is even more impressive than the aforementioned quantitative one. In LNCaP cells, both dihydrotestosterone and estradiol activated the system, whereas only estradiol was active in BPH tissue. Dihydrotestosterone, the active androgen in prostatic tissue (23), does not activate the SHBG receptor in BPH. Thus, the definition of the biological activity of steroids, previously defined in terms of their ability to activate transcription via cognate intracellular receptors, may or may not be applicable to this cell membrane-linked function. The system has constraints at three levels: (*i*) the cell must contain a specific SHBG receptor; (*ii*) it is necessary, but not sufficient, that a steroid bind to SHBG to activate the system; and (*iii*) the steroid must have biological activity, as defined by its ability to activate this system. The third level might be of substantial clinical significance. Properly chosen compounds—e.g., diethylstilbestrol—could initiate classic estrogen effects while bypassing activation of the SHBG receptor. It is possible to conceive of compounds in which the converse would be true.

Although speculation about a role for estrogens in human prostatic disease is abundant, neither a clear demonstration of their importance nor a direct biochemical effect on the prostate has been shown. Even the potential importance of prostatic estrogen receptors in pathological growth has been questioned because of their low concentration (24). The data in this communication show a clear-cut, direct biochemical effect of estrogens on the human prostate and provide a cellular mechanism by which estrogens may affect prostatic physiology. Substantial questions remain to be answered. The lowest concentration of estradiol we tested (1 nM) was effective in more than tripling cAMP. That concentration is about 6-fold greater than plasma estradiol in men. However, the concentration of steroids in plasma probably is not sufficient to explain many facets of BPH. As indicated in the Introduction, plasma androgens decrease with age at the same time that BPH is becoming manifest. Although plasma estradiol does not increase with age, there is a modest positive correlation between prostatic volume and plasma estradiol (25). Perhaps more interesting is the fact that there is almost a 3-fold greater concentration of estradiol in the stroma of BPH than in the stroma of normal prostatic tissue (26). Further, in normal prostates, stromal estradiol concentration is half that in epithelium, whereas in BPH, stromal estradiol concentration is almost double that of epithelium (26). These findings are consonant with earlier observations that showed a 2-fold increase in aromatization in BPH tissue as compared with normal (27). These local increases in estradiol, particularly because they are located in the stroma, the predominant site of the SHBG receptor, could play an important role in activating the system that we have described. Alternatively, or additionally, up-regulation of the

SHBG receptor, or SHBG, or both, could serve to amplify the effect of lower concentrations of estradiol (28).

Although we have no data that bear on the events distal to the generation of cAMP, it is tempting to speculate that phosphorylation and activation of the estrogen receptor might be the downstream consequences of this system. It is well established that all the steroid receptors, including the estrogen receptor, are phosphoproteins (29, 30). Further, *in vitro*, cAMP, or its analogues, can modify estrogen-induced growth (31, 32); regulation of estrogen receptor mRNA (33), and the concentration of the estrogen receptor itself (32). All of these observations were based on the external addition of cAMP to cells in culture. No one has elucidated a mechanism whereby the physiologic generation of cAMP *in vivo* might initiate these changes. The system we have described is a suitable, if unproved, candidate.

Finally, it should be recalled that there is extensive cross talk between prostatic epithelium and stroma (34). Hence, the fact that the SHBG receptor activity is predominantly stromal in location does not preclude either major or minor effects on the prostatic epithelium.

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