The Xenoestrogen Bisphenol A Induces Inappropriate Androgen Receptor Activation and Mitogenesis in Prostatic Adenocarcinoma Cells[1]

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Abstract

Treatment for prostatic adenocarcinoma is reliant on the initial androgen dependence of this tumor type. The goal of therapy is to eliminate androgen receptor activity, either through direct inhibition of the receptor or through inhibition of androgen synthesis. Although this course of therapy is initially effective, androgen-refractory tumors ultimately arise and lead to patient morbidity. Factors contributing to the transition from a state of androgen dependence to the androgen-refractory state are poorly understood, but clinical evidence in androgen-refractory tumors suggests that the androgen receptor is inappropriately activated in these cells. Thus, the mechanisms that contribute to inappropriate (androgen-independent) activation of the androgen receptor (AR) is an area of intensive research. Here we demonstrate that bisphenol A (BPA), a polycarbonate plastic monomer and established xenoestrogen, initiates androgen-independent proliferation in human prostatic adenocarcinoma (LNCaP) cells. The mitogenic capacity of BPA occurred in the nanomolar range, indicating that little BPA is required to stimulate proliferation. We show that BPA stimulated nuclear translocation of the tumor-derived receptor (AR-T877A), albeit with delayed kinetics compared with dihydrotestosterone. This translocation event was followed by specific DNA binding at androgen response elements, as shown by electrophoretic mobility shift assays. Moreover, the ability of BPA to stimulate AR-T877A activity was demonstrated by reporter assays and by analysis of an endogenous AR target gene, prostate-specific antigen. Thus, BPA is able to activate AR-T877A in the absence of androgens. Lastly, full mitogenic function of BPA is dependent on activation of the tumor-derived AR-T877A. These data implicate BPA as an inappropriate mitogen for prostatic adenocarcinoma cells and provide the impetus to study the consequence of BPA exposure on prostate cancer.

Introduction

Prostatic adenocarcinoma is the second leading cause of cancer death among men in the United States (1). A major challenge in treatment of prostate cancer is the lack of effective therapeutic regimens for advanced disease. The most effective treatment regimens rely on the observation that prostatic adenocarcinomas are dependent on serum androgen for proliferation and survival (reviewed in Refs. 2 and 3); thus, androgen ablation is a first line of therapy (4–7). However, most tumor remissions are transient, as recurrent, androgen-refractory tumors ultimately arise, leading to a high rate of patient morbidity. The events that regulate or facilitate the transition from an androgen-dependent to an androgen-refractory state are poorly understood and are the focus of intensive research.

Androgen elicits its biological effect through activation of the AR,[3] a member of the nuclear receptor superfamily (8, 9). Prior to ligand binding, the AR exists in diffuse pools throughout the nucleus and cytoplasm and is held inactive through association with heat shock proteins (8, 10). In the prostate, the predominant ligand for the AR is DHT, which is reduced from testosterone through the action of 5-α-reductase (11). Interaction of DHT with the AR triggers dissociation of inhibitory heat shock proteins and rapid nuclear translocation (12, 13). Activated AR forms homodimers and stimulates gene transcription from AREs in target promoters (2). Expression of one such target, PSA is dependent on association of activated AR with the PSA promoter and enhancer regions (14, 15). Because AR activity is required for and reflective of prostatic adenocarcinoma proliferation, PSA expression is monitored clinically as a key indicator of prostatic adenocarcinoma progression (16).

The importance of AR activity in prostatic adenocarcinoma is apparent with regard to both early- and late-stage disease. First-line therapies for androgen-dependent tumors aim to inhibit AR activity. These therapies are designed to either block the synthesis of AR ligands (*e.g.,* luteinizing hormone releasing hormone agonists, 5-α reductase inhibitors, inhibitors of adrenal androgen synthesis, or bilateral orchiectomy) or directly inhibit AR transcriptional transactivation potential

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[3] The abbreviations used are: AR, androgen receptor; DHT, dihydrotestosterone; ARE, androgen response element; PSA, prostate-specific antigen; BPA, bisphenol A; FBS, fetal bovine serum; CDT, charcoal dextran treated; BrdUrd, bromodeoxyuridine; ETOH, ethanol; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FACS, fluorescence-activated cell sorter; PI, propidium iodide.

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(*e.g.,* Casodex or flutamide; Refs. 6, 17, and 18). Although these therapies are largely initially effective, the median time to relapse is 12–30 months, and formation of a recurrent tumor occurs with high frequency (4, 5, 19). Cells of these recurrent tumors are classified as androgen independent or androgen refractory; however, evidence suggests that these tumors harbor active ARs in the absence of androgen. For example, up to 80% of hormone-refractory tumors express high levels of nuclear AR, indicating that the receptor is activated even in the absence of natural ligand (2, 20). It has been shown recently that AR activity is essential, as specific AR disruption (via mRNA hammerhead ribozyme) inhibited proliferation of androgen-refractory prostate cancer cells (21). Moreover, amplification of the AR has been reported in between 22 and 30% of hormone-refractory prostate tumors, providing at least one explanation of how the receptor might be activated in the absence of testosterone and its derivatives (20, 22). Lastly, between 8 and 44% of androgen-refractory prostate tumors harbor AR gene mutations, many of which broaden ligand specificity of the receptor, allowing the receptor to bypass the requirement for androgen by using other steroids as ligands (23–28). For example, threonine to alanine mutation in the AR codon 877 (T877A) was found in up to 12.5% of hormone-refractory prostate cancers; this mutation permits usage of 17β-estradiol, progesterone, and specific antiandrogens as AR ligands, possibly contributing to therapy resistance (24). Collectively, these observations link inappropriate activation of the AR to the formation of androgen-refractory tumors.

Potential sources for ligand-independent AR activation include commercially produced compounds that contain substituted phenolic structures. One such compound, BPA, has been shown to have estrogenic activity in breast carcinoma cells at micromolar concentrations (29–31). Moreover, animal studies confirmed estrogenic activity of BPA *in vivo*, demonstrating its uterotrophic effect and an ability to stimulate precocious reproductive function (32–36). Human exposure to BPA can arise from the direct contact of food with BPA-containing plastics, certain canned foods (37), and from dental treatments (38). In addition, animal studies suggest that *in utero* exposure to BPA influences prostate function and is capable of altering differentiation patterns in the periductal stroma and changing epithelial secretory function (39). Moreover, a direct mitogenic effect of BPA on the fetal prostate has been demonstrated in explant studies (40). Although these results indicate that BPA exposure may affect prostate function, the specific effect of BPA on adult prostate tissue (normal or neoplastic) has not been determined. Given the importance of androgen dependence and abolition of AR activity in the treatment of prostatic adenocarcinomas, here we report the impact of BPA on prostate cancer cell proliferation. Using an androgen-dependent prostatic adenocarcinoma model system, we demonstrate that BPA stimulates inappropriate mitogenesis, which is dependent on activation of the tumor-derived AR variant. Our data suggest that exposure to BPA may contribute to the acquisition of androgen independence and therapeutic failure.

Materials and Methods

Cell Culture. LNCaP, CV-1, and MCF-7 cells were obtained from American Type Culture Collection (Rockville, MD). LNCaP cells were maintained in IMEM (Biofluids) containing 5% heat-inactivated FBS (Hyclone) supplemented with 100 units/ml penicillin-streptomycin and 2 mM L-glutamine at 37°C in a 5% CO_2 humidified incubator. LNCaP cells between passages 28 and 39 were used for the experiments shown. CV-1 and MCF-7 cells were maintained in DMEM (MediaTech) supplemented with 10% heat-inactivated FBS (Hyclone), 100 units/ml penicillin-streptomycin, and 2 mM L-glutamine at 37°C in a 5% CO_2 humidified incubator. For growth in steroid-depleted medium, cells were seeded in phenol-red free IMEM (LNCaP cells) or DMEM (CV-1 or MCF-7 cells) containing CDT FBS (Hyclone).

Plasmids. Plasmid encoding the wild-type AR (pSG5-AR) was a gift of Dr. C. Chang (University of Rochester, Rochester, NY). Plasmid encoding the LNCaP (tumor-derived) AR (T877A) was a gift of Dr. D. Feldman (Stanford University, Stanford, CA) and has been described previously (28). Plasmid encoding β-galactosidase was a gift of Dr. J. Y. J. Wang (University of California at San Diego, San Diego, CA). The PSA reporter plasmid, PSA61LUC, was a gift of Dr. K. Cleutjens (Erasmus University, Rotterdam, the Netherlands) and has been described previously (15). pcDNA3.1 was purchased from Invitrogen, Inc.

Chemicals and Reagents. BPA (4,4′-isopropylidenediphenol) was obtained from Aldrich (St. Louis, MO). Composition and purity of BPA was confirmed by both mass spectrometry and nuclear magnetic resonance analyses (data not shown). Bicalutamide (Casodex) was a generous gift of Astra-Zeneca Pharmaceuticals. 5α-DHT was kindly provided by Dr. James Fagin (University of Cincinnati College of Medicine, Department of Endocrinology). Before cell treatment, all reagents were solubilized in ethanol.

BrdUrd Incorporation and Cell Growth Assay. For BrdUrd labeling, 3.5×10^5 LNCaP cells were seeded onto poly-L-lysine-coated coverslips in six-well dishes. After 72 h of treatment, cells were labeled with Cell Proliferation Labeling Reagent (Amersham Pharmacia Biotech), according to the manufacturer's protocol. Pulse labeling continued for 14 h; cells were then processed to detect BrdUrd via indirect immunofluorescence as described previously (41). Experiments were performed at least in triplicate. Averages and SDs are shown. To assay cell growth, approximately 3×10^5 LNCaP cells were seeded in duplicate wells of a six-well dish in 5% CDT medium supplemented with 0.1 nM DHT, 1 nM BPA, or 0.1% ETOH vehicle. Cells were trypsinized and counted using trypan blue exclusion after 24-, 72-, and 168-h treatments. Data shown reflect the average and SDs.

Immunoblotting. Approximately 8×10^5 LNCaP cells were seeded in 10-cm dishes. After 96-h treatment with 0.1 nM DHT, 1 nM BPA, or 0.1% ETOH vehicle, whole-cell lysates were prepared as described previously (42). Equal concentrations (20 μg) of clarified, soluble protein were subjected to SDS-PAGE, and immunoblots were performed using the following antibodies: RB (851; gift of Dr. Jean Wang, University of California at San Diego); cyclin D1 (Ab-3; NeoMarkers); cyclin A (sc-751; Santa Cruz Biotechnology); and c-abl (8E9;

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PharMingen). Goat antimouse and goat antirabbit horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) were used to detect the antibody-antigen complex via enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

EMSA. LNCaP nuclear extracts were prepared from cells treated with 0.1 nM DHT, 1 nM BPA, or 0.1% ETOH vehicle for 4 h as described by Schreiber *et al.* (43). A total of 1 μg of nuclear extract was used in each of the DNA binding reactions. *In vitro* DNA binding was performed by incubating nuclear extracts in a total volume of 30 μl containing DNA binding buffer as described by Zhang *et al.* (44) with ~1 ng of double-stranded [^{32}P]ATP-labeled ARE oligonucleotide (sense strand, 5′-TTGCAGAACAGCAAGTGCTAGCTC-3′; Ref. 45). For competition experiments, nuclear extracts in DNA binding buffer were preincubated with or without unlabeled double-stranded AREs or double-stranded E2F (sense strand, 5′-CTAGAGCAATTTCGCGCCAAACTTG-3′) oligonucleotides in 100-fold molar excess for 15 min at room temperature before receiving a [^{32}P]ATP-labeled probe. Protein-DNA complexes were resolved under nondenaturing conditions as described by Sadar and Gleave (45). Gels were dried, and protein-DNA complexes were detected by autoradiography.

Reporter Assays. CV-1 or MCF-7 cells were seeded 24 h before transfection in phenol red-free DMEM supplemented with 10% CDT. Transfections were performed using the *N,N*-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid-buffered saline/calcium phosphate procedure (46). Cells were cotransfected with plasmids expressing the AR (wild-type or T877A variant; 1.5 μg), the PSA61LUC reporter construct (1.0 μg), and cytomegalovirus-β-galactosidase (1.0 μg). Empty vector (pcDNA 3.1) was used to bring total transfection to 8 μg/6-cm dish. After transfection, cells were washed four times with PBS and replenished with DMEM supplemented with 10% CDT. After 6–8 h of recovery, cells were stimulated with either 1 nM BPA (Sigma) or 0.1% ETOH vehicle. After 16 h of treatment, cells were harvested by trypsinization, lysed, and processed to analyze luciferase activity (Promega), according to the manufacturer's protocol. To normalize for transfection efficiency, lysates were also analyzed for β-galactosidase activity using the GalactoStar system (Tropix), according to the manufacturer's protocol. Basal AR activity in vehicle-alone treated cells was set to 1, and relative activity in response to BPA is shown. Averages and SDs from experiments performed at least in triplicate are shown.

Bivariate Flow Cytometry. LNCaP cells were seeded at a density of 5×10^5 cells/6-cm dish in medium containing 5% CDT supplemented with either 0.1 nM DHT, 1 nM BPA, or 0.1% ETOH vehicle. After 48 h, the drugs were replenished, and treatment continued for another 48 h. Cells were then labeled with Cell Proliferation Labeling Reagent (Amersham Pharmacia Biotech) for 5 h and fixed with 95% ETOH. Cells were stained for BrdUrd incorporation and with propidium iodide as described (47). Quantitation was performed using Becton Dickinson FACSort. All experiments were performed at least in triplicate. Averages and SDs are shown.

RT-PCR Analysis. Total RNA was extracted from LNCaP cells treated with 0.1 nM DHT, 1 nM BPA, or 0.1% ETOH vehicle for 72 h using Trizol Reagent (Life Technologies, Inc.). RNA (5 μg) was reverse transcribed using Superscript II reverse transcriptase and random hexamers (Life Technologies, Inc.) according to the manufacturer's recommended protocol. Intron-spanning primers for PSA or GAPDH (internal standard) were made as follows: PSA, sense (5′-CTTGTAGCCTCTCGTGGCAG-3′) and antisense (5′-GACCTTCATAGCATCCGTGAG) with expected product size of 293 bp; GAPDH, sense (5′-CCACCCATGGCAAATTCCATGGCA-3′) and antisense (5′-TCTAGACGGCAGGTCAGGTCCACC-3′) with expected product size of 598 bp. Optimal PCR conditions for quantitative analyses were determined first, and PCR was performed using 2 μl of cDNA for 35 cycles (94°C for 30 s, 57°C for 30 s, and 72°C for 30 s). Products were separated on a 1.5% agarose gel stained with ethidium bromide, and the levels of PSA and GAPDH expression were quantitated using Metamorph software (Universal Imaging Corporation). PSA expression was normalized to GAPDH and set to 1 in ETOH (vehicle)-treated cells.

Confirmation of RT-PCR results was performed using quantitative real-time PCR. For these experiments, cDNA (generated as above) and primers generated against PSA (as above) and GAPDH (sense, 5′-TCACCAGGGCTGCTTTTAAC and antisense, 5′GGAGGCATTGCTGATGATCT; expected product size, 300 bp) were used. Reactions were performed in duplicate or triplicate for each condition, and PCR was carried out in an ABI Prism 7700 Sequence Detection System Cycler according to the manufacturer's instructions. Briefly, 1 μl of cDNA (or H_2O control) was placed into a 50-μl reaction volume containing 2 μl of each primer and 25 μl of Light Cycler SYBR Green (2× concentrate). Nucleotides, $MgCl_2$, Taq DNA polymerase, and buffer were included in the SYBR Green master mix (Applied Biosystems). The thermal cycling conditions comprised an initial denaturation step at 95°C (10 min), followed by 40 cycles of 95°C (15 s), 60°C (1 min). Cycle threshold values were obtained where fluorescent intensity was in the geometric phase of amplification, as determined via PE Biosystems analysis software. Products were verified on a 2% agarose gel. The ΔC_t values were determined for PSA and GAPDH in each condition. Relative PSA expression was normalized to that of steroid-depleted (ETOH-treated) cells using the established equation:[4] $2^{-\Delta\Delta CT}$, where C_T is the cycle number at the chosen amplification threshold, $\Delta C_T = C_{T\ PSA} - C_{T\ Reference\ (GAPDH)}$, and $\Delta\Delta C_T = \Delta C_{T\ sample} - \Delta C_{T\ Calibrator\ (ethanol)}$.

Nuclear Localization Studies. LNCaP cells were seeded onto poly-L-lysine-coated glass coverslips in six-well dishes at a density of 3.5×10^5 cells/well. The cells were depleted of steroids by culturing in 5% CDT for 72 h before steroid treatments (0.1% ETOH vehicle, 0.1 nM DHT, or 1 nM BPA) for 2 or 7 h. LNCaP cells were fixed in 3.7% formaldehyde after treatments and processed for AR immunofluorescence. Briefly, cells were permeabilized in 0.3% Triton X-100 for 15 min and then incubated with anti-AR (N-20; Santa Cruz Bio-

[4] K. Livak, PE ABI Sequence Detector User Bulletin 2.

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technology, Inc) at 1:60 dilution in PBS buffer containing 0.5% NP40 and 5 mg/ml BSA (Sigma) for 45 min at 37°C in a humidified chamber. AR was detected by indirect immunofluorescence with Rhodamine Red-conjugated antirabbit IgG (The Jackson Laboratory, Inc.) diluted 1:100 in the buffer described above. AR nuclear staining was quantitated by double-blind counting; at least 250 cells/experiment were analyzed from at least duplicate experiments.

Statistical Assessment. Quantitative results are expressed as mean ± SD of the mean. Statistical analysis was performed by using unpaired *t* test or one-way ANOVA. The criterion for statistical significance was $P < 0.05$.

Results

BPA Induces Androgen-independent LNCaP Cell Proliferation. It has been suggested that prenatal exposure to BPA may affect prostate growth and/or differentiation patterns (39, 40). However, the effect of BPA on adult prostate tissue or prostatic adenocarcinoma cells has not been explored. Hence, we investigated the effects of BPA on androgen-dependent prostatic adenocarcinoma cells (LNCaP; Ref. 48). LNCaP cells were derived from a human lymph node deposit of prostatic adenocarcinoma. These cells retain characteristics common to human prostate tumors, including AR (T877A allele, a tumor variant) and PSA expression (49). Moreover, LNCaP cells are exquisitely dependent on androgen for proliferation, because these cells arrest in the G_1 phase of the cell cycle upon androgen withdrawal (42). It is well established that these cells exhibit a biphasic growth response to androgens, wherein 0.1 nM DHT stimulates proliferation, whereas lower or higher levels initiate growth arrest (50, 51). Therefore, to test the effect of BPA on LNCaP cellular proliferation, a dose-response curve was determined (Fig. 1*A*). Asynchronously proliferating LNCaP cells were seeded into steroid-free medium containing CDT serum, because charcoal dextran treatment removes steroid hormones but retains peptide growth factors. Culture in CDT serum does not alter the proliferation rate of androgen-independent prostatic adenocarcinoma cells, such as PC-3 or DU145, as compared with growth in complete FBS (Ref. 52; data not shown). LNCaP cells were cultured in CDT serum medium supplemented with increasing doses of BPA (0.1–100 nM), 0.1 nM DHT, or 0.1% ETOH vehicle. After 72 h of treatment (1.5 generation times), cells were pulsed with BrdUrd, and BrdUrd incorporation was detected via indirect immunofluorescence. As shown in Fig. 1*A,* LNCaP cells cultured in CDT serum supplemented with vehicle (ETOH) alone failed to incorporate BrdUrd at significant levels (2.75%). By contrast, ~25% of cells supplemented with physiological levels of DHT (0.1 nM) incorporated BrdUrd. These observations are consistent with results published previously (42). Interestingly, BPA elicited a biphasic growth effect in LNCaP cells. Subtle BrdUrd incorporation was observed with 0.1 and 10 nM (4.95 and 6.5%, respectively), whereas maximal stimulation was observed with 1 nM BPA (10.3%). Higher doses of the xenoestrogen (100 nM) inhibited LNCaP proliferation (3.1%), similar to the effect observed with DHT at such concentrations (50). To confirm the mitogenic effects of 1 nM BPA on prostatic adenocarcinoma cells, bivariate flow cytometric analyses (FACS) were performed. For these experiments, asynchronous LNCaP cells were seeded in CDT serum medium containing 0.1% ETOH, 0.1 nM DHT, or 1 nM BPA for 96 h and pulse-labeled with BrdUrd for 5 h before fixation (Fig. 1*B*). To determine the percentage of cells actively undergoing DNA replication and to monitor the efficacy of DNA replication, BrdUrd incorporation and DNA content (as determined by PI staining) were quantified by FACS. As shown in Fig. 1*B,* only 1.95% of cells treated with vehicle alone (ETOH) incorporated BrdUrd (*left panel*). Treatment with 0.1 nM DHT caused an increase in LNCaP BrdUrd incorporation to 17.2%, as expected (*right panel*). Finally, populations treated with 1 nM BPA demonstrated significant BrdUrd incorporation (6.82%, *middle panel*). Therefore, both simple BrdUrd incorporation experiments and bivariate FACS analyses indicate that BPA functions as an inappropriate mitogen in prostatic adenocarcinoma cells.

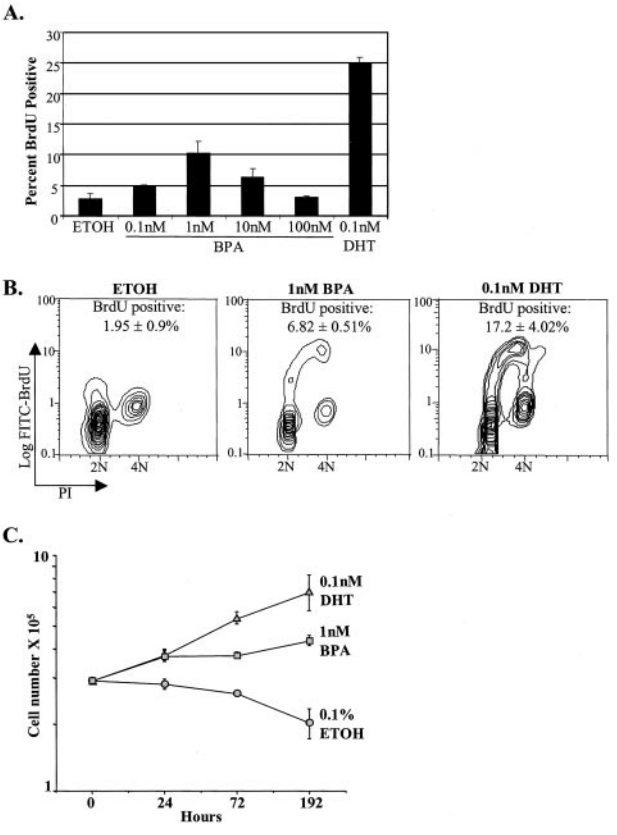

Fig. 1. BPA induces androgen-independent LNCaP cell proliferation. *A,* LNCaP cells were propagated for 72 h in 5% CDT supplemented either with 0.1% ETOH vehicle, increasing BPA concentrations (0.1–100 nM), or 0.1 nM DHT. Cells were then labeled with BrdUrd as described, and BrdUrd incorporation was detected via indirect immunofluorescence. Data shown are the average of at three independent experiments in which at least 250 cells/experiment were analyzed; *bars*, SD. *B,* LNCaP cells were cultured as indicated for 96 h, pulse-labeled with BrdUrd, and subsequently labeled with PI, as described. Shown are representative contour maps with the log FITC anti-BrdUrd staining *versus* PI. *C,* LNCaP cells were seeded into 5% CDT serum supplemented with either 0.1% ETOH, 0.1 nM DHT, or 1 nM BPA. Cell number was analyzed at times indicated by counting and trypan blue exclusion. Data shown are the average of two independent experiments; *bars,* SD.

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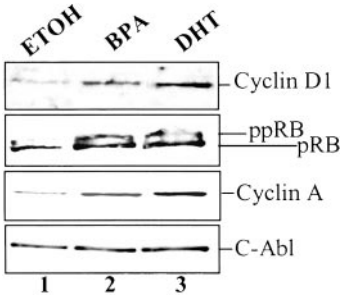

Fig. 2. BPA mimics DHT in the ordering of cell cycle events. LNCaP cells were propagated in 5% CDT supplemented with ETOH (*Lane 1*), 1 nM BPA (*Lane 2*), or 0.1 nM DHT (*Lane 3*) for 96 h. Cells were harvested, and the endogenous levels of cyclin D1, RB, cyclin A, and c-Abl were determined by immunoblotting, as described. *pRB,* underphosphorylated (active) RB; *ppRB,* hyperphosphorylated (inactive) RB.

To verify that BPA-mediated mitogenesis is associated with the completion of cellular proliferation, cell growth studies were performed. For these studies, asynchronously proliferating LNCaP cells were split into CDT serum medium in triplicate wells supplemented with either 1 nM BPA, 0.1 nM DHT, or 0.1% ETOH. Medium and the treatments were replenished every 72 h, and cell number was quantified after 24, 72, and 168 h of growth, using trypan blue exclusion and a hemacytometer. As shown in Fig. 1*C,* cells cultured in CDT serum medium supplemented with ETOH failed to proliferate, as expected. Cells treated either with 0.1 nM DHT or 1 nM BPA underwent cellular proliferation.

We have shown previously that DHT stimulates a distinct ordering of cell cycle events in LNCaP cells. Specifically, we have shown that in this model system, steroid induces cyclin D1 expression, which results in activation of the cyclin-dependent kinase 4 complex (42). Activation of cyclin-dependent kinase 4 is essential for phosphorylation/inactivation of the retinoblastoma tumor suppressor protein, RB (53). Inactivation of RB allows derepression of cyclin A expression and entry into the cell cycle (54, 55). Therefore, we characterized the response of these critical cell cycle regulatory proteins to BPA. As shown in Fig. 2, cells cultured in ETOH fail to express significant levels of cyclin D1, and RB remains in its active, underphosphorylated state (*Lane 1*). These observations underlie the diminished cyclin A expression and failure to enter S-phase and are consistent with previous reports (52, 54, 55). c-Abl tyrosine kinase is not regulated in a cell cycle-dependent manner (56), and immunoblots for c-Abl were provided as a loading control. By contrast, DHT addition (*Lane 3*) stimulates cyclin D1 expression, RB hyperphosphorylation/inactivation, and increased cyclin A expression. Similar results were observed upon BPA treatment (*Lane 2*). Taken together, these data demonstrate that BPA acts as a mitogen in prostatic adenocarcinoma cells, allowing them to bypass the requirement for androgen.

BPA Treatment Correlates with AR-T877A Activity. It is well established that androgen dependence of prostatic adenocarcinoma cells is exerted through the AR, as agents that antagonize AR activity block proliferation of prostate cancer cells (18, 21). Given that BPA proved able

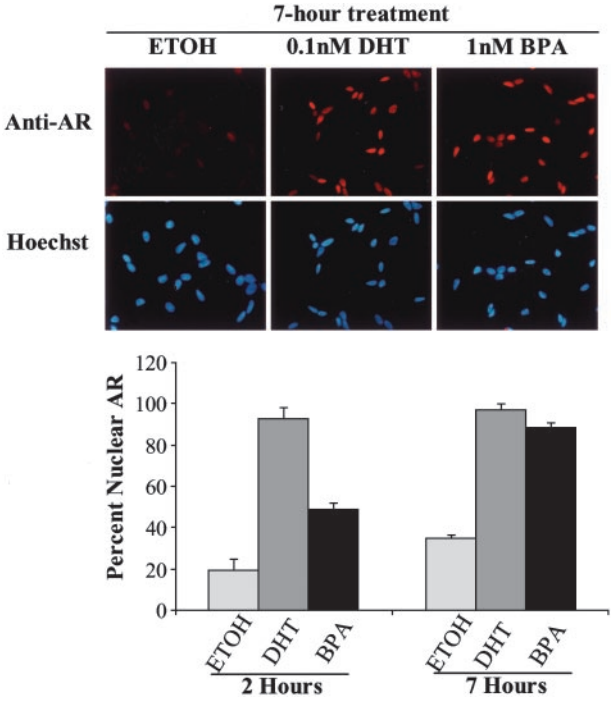

Fig. 3. BPA stimulates AR-T877A nuclear translocation. *A,* LNCaP cells were seeded onto coverslips and propagated in 5% CDT serum for 72 h before the addition of ETOH vehicle (*left panel*), 0.1 nM DHT (*middle panel*), or 1 nM BPA (*right panel*) for 2 or 7 h. Cells were fixed and processed for indirect immunofluorescence to detect nuclear AR-T877A, as described. Nuclei were stained with Hoechst. The levels of nuclear AR were determined by double-blind counting of at least 250 cells/experiment. Representative photomicrographs are shown (*upper panel*). Graphs reflect the average of three independent experiments (*lower panel*). Statistical analysis was performed using *t* test; $P < 0.001$ for all groups; *bars*, SD.

to bypass the androgen requirement in LNCaP cells, it could be postulated that this agent activates endogenous AR-T877A in this model system. Because the AR rapidly translocates to the nucleus upon activation (12, 13), the subcellular localization of the endogenous AR-T877A after BPA stimulation was examined. For these experiments, LNCaP cells were arrested by culture in CDT serum for 72 h and then stimulated for either 2 or 7 h with 1 nM BPA, 0.1 nM DHT, or ETOH vehicle. After stimulation, cells were fixed, and the subcellular localization of AR-T877A was detected via indirect immunofluorescence (Fig. 3). Since the AR exists in diffuse cytoplasmic and nuclear pools before activation, some background nuclear accumulation was observed in the vehicle-treated group (approximately 20–40%). Analysis of DHT-treated cells demonstrated a marked increase in AR-T877A nuclear accumulation; 90–95% of cells demonstrated positive nuclear staining after 2 h of stimulation. This level was static after 7 h of treatment. BPA initiated modest AR nuclear accumulation after 2 h of stimulation (45–50%), which increased after 7 h of exposure (85%). These data suggest that BPA-induced mitogenesis correlates with AR-T877A activation.

Since BPA was able to induce nuclear translocation of endogenous AR-T877A in LNCaP cells, we questioned whether this translocation resulted in activation of this tumor-

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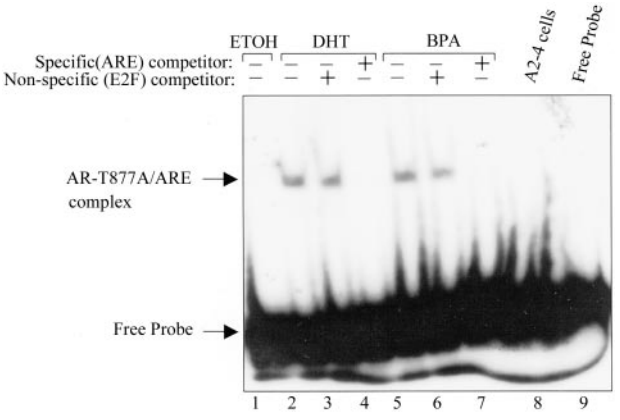

Fig. 4. BPA triggers AR-T877A DNA binding activity. EMSAs were performed using radiolabeled ARE oligonucleotides, and nuclear extracts were isolated from LNCaP cells incubated previously with the indicated compounds for 4 h, under conditions described in "Materials and Methods." Free probe is shown in *Lane 9,* and control (AR-negative) A2–4 rat fibroblast extracts were used in *Lane 8* to demonstrate specificity. Nuclear extracts from LNCaP cells treated with DHT (*Lanes 2–4*) or BPA (*Lanes 5–7*) were supplemented with 100× excess specific (*ARE*) or nonspecific (*E2F*) oligonucleotides where indicated. Protein-DNA complexes were detected by autoradiography.

derived AR. The AR, like other nuclear receptors, requires ligand for a sequence-specific DNA binding and subsequent transcriptional activation (9). Thus, DNA binding of AR-T877A to a specific ARE was examined in the presence or absence of BPA. LNCaP cells were steroid deprived for 72 h, at which point 1 nM BPA, 0.1 nM DHT, or 0.1% ETOH vehicle was added, and incubation continued for 4 h. LNCaP nuclear extracts were used in EMSA using a ^{32}P-labeled ARE to assess the DNA-binding activity of the endogenous AR-T877A (Fig. 4). Free probe is shown in *Lane 9*. As shown, crude cellular extracts from a rat fibroblast-derived cell line, A2-4 (57), demonstrate no mobility shift (*Lane 8*). As expected, nuclear extract from LNCaP cells treated with ETOH alone demonstrated little gel shift activity (*Lane 1*), whereas extracts from DHT-treated cells demonstrated a significant shift (*Lane 2*). This shifted band representing AR-T877A/ARE complex was not competed out by 100-fold molar excess of unlabeled nonspecific (E2F-binding site) oligonucleotide (*Lane 3*) but was significantly competed out by specific ARE unlabeled competitor (*Lane 4*) at the same concentration. This specific DNA binding was also observed in LNCaP cells treated with 1 nM BPA (*Lane 5*), which was competed out by unlabeled ARE oligonucleotides (*Lane 7*) and not by unlabeled E2F oligonucleotides (*Lane 6*). These data demonstrate that BPA can activate sequence-specific DNA binding of tumor-derived AR-T877A in the absence of other steroids in prostatic adenocarcinoma cells.

It is well documented that androgens stimulate PSA gene expression by regulating AR binding to specific AREs (9, 14, 15). Since we showed that BPA treatment correlates with AR-T877A nuclear translocation and DNA binding, we investigated whether this compound promotes AR-T877A transcriptional transactivation potential. Initially, these analyses were performed via transient transfection. As shown in Fig. 5*A,* CV-1 (*left panel*) and MCF-7 (*right panel*) cells were cotransfected in 10% CDT serum with plasmids encoding β-galactosidase, AR (wild-type or T877A), and the PSA61LUC reporter plasmid. After transfection, cells were stimulated with either ETOH control or 1 nM BPA for 16 h, at which time cells were harvested. After lysis, luciferase assays were performed to monitor PSA61LUC reporter activity, which was normalized for transfection efficiency using β-galactosidase activity as an internal control. Relative luciferase activity is shown. As shown in Fig. 5*A,* 1 nM BPA activated T877A-mediated PSA expression (~5.3-fold in CV-1 and 8-fold in MCF-7 cells). By contrast, the wild-type AR-mediated PSA expression was not affected by BPA exposure, indicating that the effect of BPA is specific to the tumor-derived AR variant.

To verify these observations in prostatic adenocarcinoma cells, endogenous PSA gene expression in LNCaP cells was examined. For these experiments, asynchronously proliferating LNCaP cells were propagated in CDT serum medium supplemented with 0.1 nM DHT, 1 nM BPA, or 0.1% ETOH for 72 h. RT-PCR reactions were performed using human-specific primers for PSA, which have been described previously (58). For each condition, PSA expression was normalized to GAPDH, and expression in vehicle-treated cells was set to 1. As expected, DHT treatment induced PSA mRNA levels ~4-fold, as compared with control (ETOH-treated cells), whereas GAPDH expression remained relatively stable throughout treatment (Fig. 5*B,* compare *Lanes 1* and *2*). Strikingly, LNCaP cells exposed to BPA demonstrated significant PSA mRNA induction, ~3-fold (compare *Lanes 1* and *4*). These data demonstrate that BPA induces endogenous AR-T877A targets. To provide evidence for the involvement of AR-T877A in the induction of PSA, we used a known nonsteroidal antiandrogen Casodex (bicalutamide). Casodex acts as a competitive inhibitor of the AR and AR-T877A by disrupting AR DNA binding activity (59). Minimal PSA expression was detected in ETOH-treated cells (Figs. 5*B, Lane 1*). As expected, Casodex significantly inhibited PSA mRNA expression induced by DHT but had no discernable effect on GAPDH expression (compare *Lanes 2* and *3*). Importantly, this specific antiandrogen also reduced PSA mRNA induction by BPA to approximately basal levels while having no effect on GAPDH expression (compare *Lanes 1, 4,* and *5*).

To verify the effect of BPA on endogenous PSA expression, quantitative real-time PCR was performed (Table 1). Cells were treated as above with either 0.1 nM DHT, 1 nM BPA, or 0.1% ETOH vehicle, and cDNAs were generated. Real-time PCR allows quantitation of the precise PCR cycle wherein amplification of the desired product reaches the geometric phase. Real-time PCR experiments were performed at least in duplicate, and relative expression of PSA to GAPDH was determined, as outlined in "Materials and Methods." The basal PSA expression in ETOH-treated cells was set to 1. As shown in Table 1, DHT stimulated PSA expression an average of 5.9-fold, whereas BPA induced a 5-fold induction of PSA expression, consistent with our previous experiments. Additionally, these results were con-

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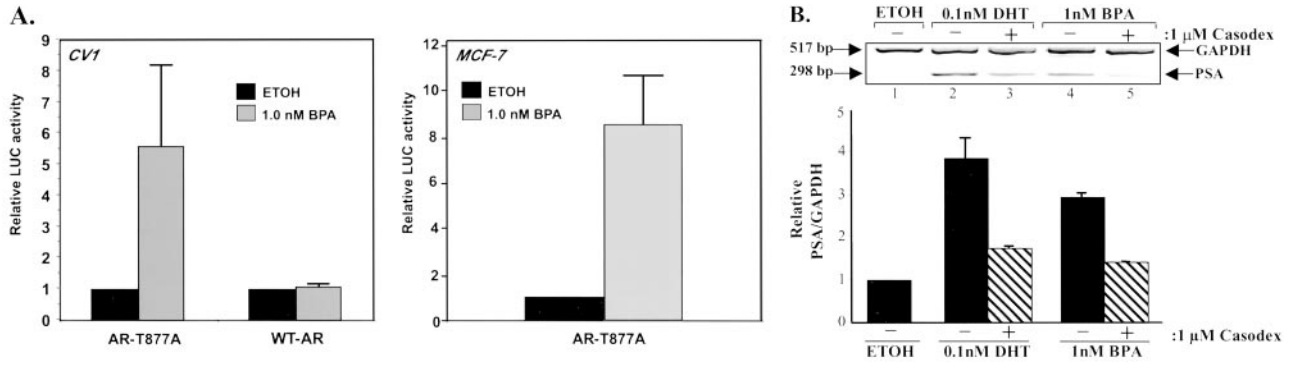

Fig. 5. BPA stimulates AR-T877A transcriptional transactivation potential. *A,* CV-1 (*left panel*) or MCF-7 (*right panel*) cells were transfected in the absence of androgen with plasmids encoding AR (wild-type or T877A), cytomegalovirus-β-galactosidase, and the PSA61LUC reporter. After transfection, cells were treated with either 0.1% ETOH or 1 nM BPA for 16–24 h. Cells were then harvested, lysed, and analyzed for luciferase activity (to monitor AR activity) and β-galactosidase activity (to normalize for transfection efficiency). Relative luciferase activity detected in ETOH-treated cells was set to 1. Experiments were performed at least in triplicate; *bars,* SD. *B,* PSA mRNA levels were determined by RT-PCR from LNCaP cells treated with ETOH (*Lane 1*), DHT (*Lane 2*), or BPA (*Lane 4*) for 72 h as described in "Materials and Methods." To determine the contribution of AR-T877A, cells were preincubated with 1 μM Casodex for 4 h before the addition of DHT (*Lane 3*) or BPA (*Lane 5*) and then incubated for an additional 72 h. PSA mRNA levels were quantified and normalized to GAPDH levels. $P < 0.005$ for all groups; *bars*, SD.

Table 1 Relative quantitation of PSA expression

Treatment	PSA Average C_T	GAPDH Average C_T	Δ C_T PSA-GAPDH	ΔΔ C_T $\Delta C_T - C_T$, ETOH	PSA relative to ETOH
ETOH	23.70 ± 0.70	20.91 ± 0.26	2.79 ± 0.75	0.00 ± 0.75	1.0 (0.6–1.7)
DHT	22.32 ± 0.26	22.08 ± 0.16	0.24 ± 0.31	−2.55 ± 0.31	5.9 (4.7–7.3)
BPA	23.18 ± 0.76	22.71 ± 0.25	0.47 ± 0.80	−2.32 ± 0.80	5.0 (2.9–8.7)

firmed via Northern analyses (data not shown). Thus, these data demonstrate that AR-T877A activation by BPA results in induction of endogenous AR target genes.

Mitogenic Activity of BPA Requires AR-T877A Activity. We have shown that BPA acts as an inappropriate mitogen in LNCaP cells, since BPA exposure bypasses the androgen requirement in these prostate cancer cells (Figs. 1 and 2). Moreover, we have shown that BPA treatment correlates with AR-T877A activation (Figs. 3–5). To establish a link between these events, the requirement of AR-T877A for mitogenesis was examined. For these experiments, LNCaP cells were cultured for 72 h in CDT supplemented with 0.1% ethanol, 0.1 nM DHT, or 1 nM BPA in the presence or absence of 1 μM Casodex. Cells were then pulsed with BrdUrd and processed to detect BrdUrd incorporation via indirect immunofluorescence. As shown in Fig. 6, cells treated with ETOH failed to enter S-phase (2.2–3% of cells incorporated BrdUrd), irrespective of Casodex addition. By contrast, BrdUrd incorporation rates upon Casodex treatment in the presence of DHT were reduced from 30 to ~9.5%. These observations are consistent with previous reports that AR activity is required for prostate cancer maintenance and growth (3, 8, 9, 21, 60). Remarkably, cells treated with BPA in the presence of 1 μM Casodex also demonstrated marked reduction in BrdUrd incorporation (5.2%, compared with 13.4%), indicating that AR-T877A activity is required for the mitogenic effect of BPA in this model system.

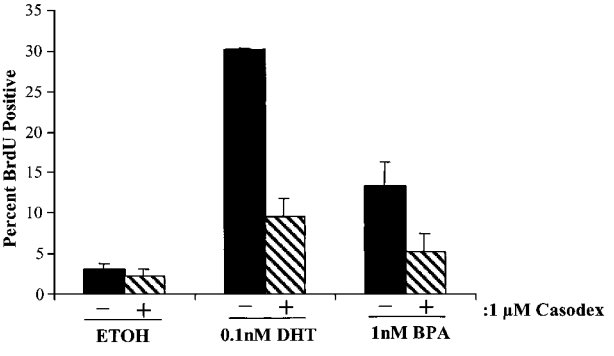

Fig. 6. Mitogenic activity of BPA requires AR-T877A activity. LNCaP cells were propagated in 5% CDT serum medium supplemented with ETOH, BPA, or DHT for 72 h with or without 4-h 1 μM Casodex pretreatment, as described. Cells were then labeled with BrdUrd, and incorporation was detected via indirect immunofluorescence. Data shown are the average of three independent experiments in which at least 250 cells/experiment were analyzed; $P < 0.001$ for all groups; *bars*, SD.

Discussion

This study demonstrates that the xenoestrogen BPA acts as an inappropriate mitogen in prostatic adenocarcinoma cells. We show that: (*a*) BPA stimulates cellular proliferation in the absence of other steroid hormones in LNCaP cells (Figs. 1 and 2); (*b*) BPA treatment results in activation of the endogenous AR-T877A receptor (Figs. 3–5; Table 1); and (*c*) AR-T877A activity is required for BPA-dependent mitogenesis in LNCaP cells (Fig. 6). These data implicate BPA exposure as a potential mechanism that could facilitate the transition of prostatic adenocarcinomas to androgen independence.

The LNCaP model was chosen for our investigation, because it is the only well-described androgen-dependent hu-

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man prostatic adenocarcinoma cell line. The tumor variant of the AR expressed in LNCaP cells is of obvious biological relevance, as it was reported in up to 12.5% of hormone-refractory prostate cancers (24). The role of the AR in prostate cancer progression is underscored by the observation that AR antagonists are effective initial therapy, which results in transient tumor regression (reviewed in Refs. 2 and 3). The data presented here provide the first evidence to our knowledge of AR activation by an environmental estrogen and suggest that BPA exposure may facilitate the transition to androgen independence, especially in the presence of tumor-derived AR mutations. Multiple tumor-derived AR variants have been described, many of which affect ligand specificity (23–27). For example, AR-K580R, AR-V715M, and AR-H874Y have all been reported to be induced by estradiol at 0.1 nM concentrations (27). The effect of BPA on other tumor-derived AR mutants is of obvious importance and is the focus of future investigation.

It should be noted that although BPA treatment certainly triggers AR-T877A activation, it remains to be solved whether this activation is direct. The kinetics of BPA-induced AR nuclear translocation was retarded as compared with DHT stimulation (Fig. 3), perhaps suggesting that BPA has a weak affinity for AR-T877A, or that its effect on AR-T877A is indirect. BPA has been shown to interact directly with both estrogen receptors (ER-α and ER-β; Ref. 61). Moreover, wild-type AR has been shown to potentially interact with ER-α and ER-β as part of a trimeric, Src-containing complex, contributing to rapid mitogen-activated protein kinase activation and subsequent mitogenesis (62). Therefore, it could be envisioned that BPA activates AR-T877A (and induces mitogenesis) indirectly through interaction with ER-β or other proteins. However, transfection of LNCaP cells with an estrogen-responsive reporter construct (ERE-LUC) failed to demonstrate significant reporter activity upon BPA treatment (data not shown). Although these data suggest that BPA-mediated AR-T877A activation and prostatic adenocarcinoma cell proliferation is not likely to occur through ER-β interaction, future studies will be directed at determining the precise mechanism by which BPA initiates these events.

As demonstrated herein, the outcome of BPA-mediated AR-T877A activation was androgen-independent prostatic adenocarcinoma cell proliferation. However, the impact of BPA on the normal prostate remains somewhat equivocal. Although in some rodent models prenatal exposure to low doses of BPA resulted in increased adult prostate size, enhanced AR expression, altered differentiation patterns of the prostate, and changes in secretory function of the gland (39, 40), these results are challenged by other studies that showed no significant effect upon BPA exposure in animal model systems (63, 64). In our investigation, the action of BPA was not confounded by effects of other hormone-secreting organs (*e.g.,* the neuroendocrine axis) and is in agreement with previous reports demonstrating that BPA exerts its estrogenic effect at relatively low concentrations (35, 40, 65, 66). Moreover, BPA was reported to induce fetal prostate growth in *in vitro* explants at 50 pg/ml or 0.22 nM dose (40). We observed a maximal mitogenic response in prostate cancer cells at 1 nM dose of BPA, and higher doses of BPA failed to induce androgen-independent cellular proliferation. This observation also indicates that minor exposure to estrogenic environmental compounds may result in significant biological consequences. The concentrations used herein are well within the range of expected human exposure, as microgram quantities of BPA were detected in liquid from canned foods (37) and in saliva from patients receiving dental sealants (38). Additionally, since higher concentrations of BPA were actually inert, careful dosing analysis is required to determine the functional consequence of xenoestrogen exposure, especially in animal models. Interestingly, a recent report demonstrates that serum BPA concentrations are higher in men and correlates with higher total testosterone, suggesting that androgen may regulate BPA metabolism (67). Moreover, this and another recent study (68) demonstrated that the serum dose of BPA in healthy men ranges from 1.4 to 6.5 nM, lending further importance to the dosages analyzed in our study.

In summary, the data presented provide evidence that the xenoestrogen BPA induces inappropriate tumor-derived AR activation and mitogenesis in prostatic adenocarcinoma cells. These findings implicate BPA as a putative contributing factor toward therapeutic relapse. In addition, our findings provide the impetus to further examine the effect of BPA on other AR tumor variants and monitor the influence of BPA and other xenoestrogens on prostate cancer progression and treatment.

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