Synergistic Activation of the Androgen Receptor by Bombesin and Low-Dose Androgen

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ABSTRACT

Purpose: Neuropeptide growth factors such as bombesin are implicated in progression to androgen-independent prostate cancer (PC). We examined the impact of bombesin on androgen receptor (AR)-mediated gene expression.

Experimental Design: The AR together with the AR-responsive probasin ARR3k-luc or PSA-pPUE-ELB-luc promoter was cotransfected into Swiss 3T3 and PC-3 cells, both of which express high-affinity bombesin receptors; the cells were incubated with bombesin (0–50 nM) and dihydrotestosterone (DHT; 0–10 nM), and luciferase activities were measured. DHT increased transcription 40-fold at doses of 1 and 10 nM but had no effect at 10 pm. Bombesin alone, or with 1 or 10 nM DHT, did not further increase transcription. However, 5 nM bombesin and 10 pm DHT, doses that by themselves had no effect, resulted in a 20-fold increase in transcription (P < 0.005). This synergistic effect was blocked by bombesin receptor antagonists and recombinant neutral endopeptidase, which hydrolyzes bombesin. Bombesin and DHT together also increased binding of nuclear extracts from PC-3 cells transfected with AR to a consensus AR response element in mobility shift assays and increased the level of secreted prostate-specific antigen in LNCaP cell supernatant compared with DHT or bombesin alone. Immunoprecipitation of AR from 32P-labeled LNCaP cells revealed that 5 nM bombesin + 10 pm DHT induced AR phosphorylation comparable with 1 nM DHT, whereas bombesin or 10 pm DHT alone did not.

Conclusions: These data indicate that bombesin can synergize with low (castrate) levels of DHT to induce AR-mediated transcription and suggest that neuropeptides promote AR-mediated signaling in androgen-independent prostate cancer.

INTRODUCTION

The molecular events that contribute to the development and progression of androgen-independent PC continue to be defined. The majority of androgen-independent PCs express ARs and other androgen-regulated genes such as PSA. In animal models of PC, the gene expression profiles of PC cells before and after castration are nearly identical (1, 2). These data suggest that androgen-independent PCs maintain a functional AR signaling pathway despite the low levels of circulating androgen present after androgen withdrawal (3–5). One explanation for persistent AR signals in androgen-independent PC is that mutations occur in the AR that allow ligand-independent transcription (6) or enable other steroid hormones such as glucocorticoids to bind AR and initiate transcription (7). However, the incidence of AR mutations occurring in patients is low (8, 9).

Ligand-independent activation of the AR has been reported with polypeptide growth factors such as interleukin 6, keratinocyte growth factor, epidermal growth factor, and insulin growth factor 1 (10–13). Overexpression of the HER-2/neu receptor tyrosine kinase by PC cells results in ligand-independent growth and synergizes with low levels of androgen to activate AR signaling and PSA production (14, 15). The signaling pathways that mediate ligand-independent activation of AR signaling may vary for different receptors. HER-2/neu signaling to the AR appears to involve MAP kinase (15), but activation of PKA and PKC may also increase AR-mediated transcription, suggesting that there is cross-talk between these signal transduction pathways and AR activation that contribute to androgen-independent progression (16–19).

Numerous studies implicate neuropeptide growth factors such as bombesin, neurotensin, and endothelin-1 in the progression to androgen-independent PC (20, 21). Although neuropeptides appear to act as growth factors and survival factors, the manner by which neuropeptides facilitate androgen-independent progression is undefined. We considered whether neuropeptides synergistically interact with AR signaling pathways to induce ligand-independent transactivation of AR signaling. We report that bombesin does not confer ligand-independent transcrip-

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tional activation of AR; however, it synergizes with low levels of androgen to confer AR-mediated transcriptional activation comparable with that observed with physiological levels of DHT. These data demonstrate cross-talk between G-protein-coupled receptors and AR signaling pathways and provide additional evidence for the role of neuropeptide growth factors in the growth and progression of androgen-independent PC.

MATERIALS AND METHODS

Cell Lines and Reagents. PC cells were maintained in RPMI 1640 supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 units/ml streptomycin and penicillin, and 10% FCS, and Swiss 3T3 cells were maintained in DMEM containing 10% FCS. The reagents used were: DHT and bombesin (Sigma Chemical Co., St. Louis, MO); RO-31-7549 (Calbiochem, San Diego, CA); bicalutamide (Casodex; Zeneca Pharmaceutical, Wilmington, DE); rNEP (Arris Pharmaceutical, Inc., South San Francisco, CA); CGS24592, a competitive inhibitor of NEP, was supplied by Novartis Pharmaceutical (Summit, NJ; Ref. 22); Imx System PSA Assay kit (Abbott Laboratories, Abbott Park, IL); anti-AR monoclonal antibody (sc-7305; Santa-Cruz Biotechnology, Santa Cruz, CA); PKI, GF-109203X (PKC inhibitor), PD 98059 (MAP kinase inhibitor; Calbiochem, La Jolla, CA).

Transfection and Measurement of Luciferase Activities. For Swiss 3T3 cells, 20 μg of plasmid DNA were transfected with either probasin-ARR=tk-luc reporter plasmid DNA (kindly provided by R. Matusik, Vanderbilt University, Nashville, TN) or PSA-pPUE-ELB-luc (kindly provided by L. Freedman, Memorial Sloan-Kettering Cancer Center, New York, NY) with or without cotransfection of 2 μg of pSG5-AR expression vector DNA using Lipofectamine (Life Technologies, Inc.) according to the manufacturer’s recommendations. For PC-3 cells, transfections were performed using Gene Pulser (Bio-Rad, Richmond, CA) at 900 μFarads and 0.25 μV. After 24 h, cells were trypsinized, and equal numbers of cells were plated in the tissue culture plate (Falcon 3502 tissue culture plate). Cells grown in phenol-free medium containing charcoal-stripped serum for 24 h were treated by different agents for an additional 24 h. Cells were harvested, and cell lysates were prepared for performing luciferase assays using a luciferase enzyme assay system (Promega Corp., Madison, WI). Each transfection experiment was performed in duplicate or triplicate on at least three separate occasions. Results represent an average of independent experiments with data presented as relative luciferase activity using means of untreated controls as standards.

Resulting Shift Assays. Nuclear extracts were prepared as described (23). Binding reactions were carried out in binding buffer and 0.3–0.5 ng (2–3 × 10⁵ cpm) of end-labeled oligonucleotide, as described previously (23). Nuclear extracts (2–4 μg protein) were added, and the mixture was incubated at room temperature for 20 min and terminated by the addition of 2 μl of loading buffer [6.7 mM Tris-HCl (pH 7.9), 3.3 mM NaOAc, 50% (v/v) glycerol, 0.25% (w/v) bromphenol blue, and 0.25% (w/v) xylene cyanol]. The DNA/protein complexes were then resolved on nondenaturing polyacrylamide gels with the ion strength of 0.5× TBE (Tris-borate-EDTA buffer, pH 8.0). Oligonucleotides containing the sequence of the ARE derived from the rat prostate C3 gene were used in competition assays (sense, 5'-GGTTGAAACATAGTACGTATCTTTCAGATAG-3'; Ref. 24).

PSA Determination in LNCaP Cell Supernatant. LNCaP cells were cultured in phenol-free RPMI 1640 containing charcoal-stripped serum for 24 h, treated with the 10 nM of the specific NEP enzyme inhibitor CGS24592 for 1 h, followed by bombesin and/or DHT. After incubation for 72 h, the medium was removed and assayed by a PSA enzyme immunoassay per the manufacturer’s recommendations (Abbott). PSA levels were corrected for protein content in the supernatant. Experiments were performed on three separate occasions with similar results.

Metabolic Labeling with [32P]P and Immunoprecipitation. LNCaP cells were preincubated in phosphate-free RPMI 1640 overnight, incubated for 4 h with [32P]Pi, (0.2 μCi/ml), lysed in 200 μl of RIPA buffer, and incubated overnight with 1 μg of anti-AR antibody and for 1 h with 25 μl of antimouse IgG-agarose beads (Sigma) at 4°C. Immunoprecipitates were washed with RIPA buffer, resuspended in 2× SDS-PAGE sample buffer, resolved on 10% SDS-PAGE, dried, and exposed to film for 12 h. Immunoprecipitation experiments were performed on two separate occasions with similar results.

RESULTS

Bombesin Confers Androgen Inducibility in PC Cells. The effect of bombesin on transcriptional activity of the AR-responsive probasin promoter was examined using the reporter construct ARR=tk-luc, which contains the 5′-upstream DNA of the rat probasin gene, including three copies of the probasin ARR, ligated into a luciferase reporter vector (25). The ARRtk-luc reporter plasmid was cotransfected with an AR expression plasmid into PC-3 cells, which express high levels of high-affinity bombesin receptors (26–28). The cells were treated with increasing concentrations of DHT with a constant bombesin concentration of 5 nM (Fig. 1A) and with increasing concentrations of bombesin with a constant DHT concentration of 10 pm (Fig. 1B), and luciferase activities were measured. As illustrated in Fig. 1A, 5 nM bombesin had minimal transactivating activity on PC-3 cells in the absence of DHT or with physiological concentrations (1–10 nM) of DHT. However, luciferase activities were significantly increased at 0.01 and 0.1 nM concentrations of DHT. This effect was most pronounced at a concentration of 0.01 nM (10 pm), which is in the range of testosterone serum levels observed in patients who have undergone androgen withdrawal. PC-3 cells cultured in a constant concentration of 10 pm DHT demonstrated significantly increased luciferase activities at bombesin concentrations of 0.5, 5.0, and 50 nM (Fig. 1B). Deletion of the ARR from the tk-Luc vector resulted in no induction of transcription in response to 5 nM bombesin + 10 pm DHT (data not shown). To determine whether the interaction between bombesin and cestrin levels of DHT was not specific to the probasin ARR, we performed identical experiments using the AR-responsive PSA-pPUE-ELB-luc reporter construct, which contains the 5′ ARE of the PSA promoter. As illustrated in Fig. 1, C and D, luciferase...
activities were highest in PC-3 cells treated with 5 nM bombesin and 10 pm of DHT. At higher concentrations of DHT, bombesin did not increase reporter activity. Similar results were obtained using Swiss 3T3 cells, which express high levels of bombesin/GRP receptors (29) cotransfected with either ARR3 tk-luc or PSA-pPUE-ELb-Luc and AR expression reporter vectors (data not shown), indicating that the synergy between bombesin and DHT on AR signaling was not PC cell specific.

To further demonstrate the interaction between bombesin and DHT, we used mobility shift assays to examine the effects on binding of nuclear extracts from PC-3 cells to a consensus ARE in vitro. Fig. 2 shows that relative to the labeled ARE oligonucleotide in the presence of nuclear extracts from PC-3 cells transiently transfected with AR incubated in media alone (Lane 1) or media containing 5 nM bombesin (Lane 2) or 10 pm DHT (Lane 3), retarded mobility only occurred in the presence of nuclear extracts from PC-3 cells treated with both bombesin and DHT (Lane 4) or with 1 nm concentration of DHT (Lane 5). This shift, indicative of binding of AR protein to the oligonucleotide was eliminated by binding with 100-fold excess unlabeled ARE (data not shown), which shows that the complexes formed between DNA and nuclear proteins are sequence specific. Taken together, these data demonstrate that AR-mediated signaling can be activated in prostate cancer cells by physiological concentrations of bombesin in the presence of castrate concentrations of DHT, that bombesin by itself does not increase transcription at physiological concentrations of androgen, and that the magnitude of transcriptional activation resulting from bombesin and low levels of androgen is similar to the levels achieved with physiological levels of DHT alone.

**AR and Bombesin Receptor Antagonists Inhibit Transactivation.** DHT binds to the AR whereas bombesin induces its biological effect by binding to and activating its GPCR. To confirm that both receptors are required for bombesin/DHT transactivation, we incubated PC-3 cells, cotransfected with ARR3 tk-luc or PSA-pPUE-ELb-Luc, and AR expression reporter vectors with 5 nM bombesin and 10 pm DHT together with the AR competitive inhibitor bicalutamide and the
Bombesin and DHT Activation of AR

Fig. 2 Gel mobility shift assay of consensus AREs with nuclear extracts from PC-3 cells treated with DHT and bombesin. Radiolabeled consensus ARE oligonucleotides were incubated with nuclear extracts from bombesin- and/or DHT-treated PC-3 cells transfected with an AR expression vector and separated on a 4% polyacrylamide gel. Lane 1, no treatment; Lane 2, 10 pm DHT; Lane 3, 5 nm bombesin; Lane 4, 10 pm DHT + 5 nm bombesin; Lane 5, 1 nm DHT (positive control). Arrow, the complex formed between ARE and AR protein.

Bombesin/GRP receptor antagonists RC-3095 and RC 3940-II and measured luciferase activities. As illustrated in Fig. 3, bombesin-mediated augmentation of reporter gene activity in both the ARR3 tk-luc or PSA-pPUE-ELB-luc reporter vectors was significantly reduced by bicalutamide, RC-3095, and RC 3940-II. Furthermore, preincubation with recombinant neutral endopeptidase, a cell surface peptidase that inactivates bombesin through hydrolysis, also significantly inhibited the synergistic effect of bombesin and DHT on transcription.

Bombesin and DHT Stimulate PSA Secretion in LNCaP Cells. To verify that bombesin can stimulate the production of an androgen-responsive protein, we measured PSA levels in the supernatant of LNCaP cells treated with 5 nm bombesin and 10 pm DHT. LNCaP cells normally express high levels of cell surface neutral endopeptidase, which inactivates exogenous bombesin (30). Therefore, we first cultured LNCaP cells in medium containing charcoal-stripped serum for 24 h and pretreated the cells for 1 h with the NEP-specific enzyme inhibitor CGS24592. As illustrated in Fig. 4, bombesin without DHT had minimal effect on PSA production, whereas PSA production significantly increased in cells treated with bombesin and DHT. The increase in PSA production could be inhibited by bicalutamide, RC-3095, and RC 3940-II.

Bombesin-induced AR Phosphorylation. The phosphorylated form of the AR is the form that is transcriptionally active in gene regulation and may be necessary for DNA-binding activity (31, 32). Immunoprecipitation of AR protein from LNCaP cells labeled with 32P cultured with 5 nm bombesin ± 10 pm DHT revealed that AR was only phosphorylated after bombesin and DHT, similar to the degree of AR phosphorylation observed in cells treated with 1 nm DHT alone (Fig. 5). The mechanisms by which regulation of AR phosphorylation by androgen agonists occur are not well established but may involve PKA or other signaling pathways (31). The AR is directly activated by compounds that augment PKA, PKC, and MAP kinase signaling (5). We therefore examined the effects of a PKC, PKA, and a MAP kinase inhibitor on the synergistic effect of bombesin and DHT on AR transcription. As shown in Fig. 6, each inhibitor alone inhibited transcription of both the ARR3 tk-luc or PSA-pPUE-ELB-luc reporter vectors by 40–50%. A mixture of all three kinase inhibitors inhibited transcription by 80%.
coactivators including CBP/p300 and ARA70 (reviewed in Refs. 5) PKC, and MAP kinase pathways, as well as from a variety of growth factor, and Her2/neu) through stimulation of the PKA, (insulin-like growth factor I, keratinocyte growth factor, epidermal signaling occurs with polypeptide growth factor and their receptors factors (and/or coregulatory proteins) via cross-talk between the protein-protein interactions between the AR and other transcription progression to androgen-independent PC implicate the effects of pro-

DISCUSSION
Recent studies exploring the molecular mechanisms of pro-
gression to androgen-independent PC implicate the effects of pro-
tein-protein interactions between the AR and other transcription factors (and/or coregulatory proteins) via cross-talk between the AR and other signal transduction pathways (32). Activation of AR signaling occurs with polypeptide growth factor and their receptors (insulin-like growth factor I, keratinocyte growth factor, epidermal growth factor, and Her2/neu) through stimulation of the PKA, PKC, and MAP kinase pathways, as well as from a variety of coactivators including CBP/p300 and ARA$_{rn}$ (reviewed in Refs. 5 and 32). We report here that signaling through the bombesin GPCR can also activate AR-regulated gene expression by synergizing with low levels of androgen to stimulate AR-mediated transcriptional activation. This conclusion is based on the following evidence: (a) treatment of PC-3 cells expressing AR with bombesin plus DHT increases binding of nuclear extracts to a consensus ARE; (b) bombesin significantly increases reporter activity of the probasin and PSA promoters in PC-3 cells transfected with AR and treated with low concentrations (10 pm) of DHT, whereas no bombesin effect on transcription is observed at physiological concentrations of DHT; (c) bombesin and 10 pm DHT can increase PSA production in LNCaP cells compared with either agent alone; and (d) the effect of bombesin on AR transcription is specific and can be blocked by recombinant neutral endopeptidase, which hydrolyzes bombesin, and bombesin receptor antagonists. In addition, bombesin and 10 pm DHT induce phosphorylation of AR, which has been shown previously to up-regulate AR-mediated gene expression (31).

Fig. 5 Bombesin and DHT induce phosphorylation of AR. Cell lysates obtained from PC-3/AR cells cultured in the presence of radioactive P$_{32}$, 10 pm DHT, 5 nm bombesin, DHT + bombesin, or 1 nm DHT for 4 h were immunoprecipitated with an antibody to AR, separated by SDS-PAGE, and exposed to film. Note phosphorylated AR protein in cells treated with 1 nm DHT or 10 pm DHT + 5 nm bombesin.

Signaling through the bombesin GPCR leads to second-messenger generation via heterodimeric G proteins and induces the activation of many signaling proteins, including phospholipase C leading to the formation of inositol 1,4,5-trisphosphate triggering the release of intracellular calcium and the release of diacylglycerol leading to activation of protein kinase C, p42$^{mapk}$/p44$^{mapk}$ (MAP kinase), Src kinase, focal adhesion kinase (p125$^{Fak}$), p70$^{s6k}$ kinase, and protein kinase D (33). Inhibition of PKC and MAP kinase signaling only partially blocked the synergistic effect of bombesin and DHT on AR-mediated transcription, suggesting that the interaction between bombesin and AR signaling pathways is complex and may involve multiple signals. Furthermore, we did not explore any potential effect of GPCR signaling on the activity of AR coactivators or corepressors, which may directly impact on AR transcription. Lee et al. (34) recently reported similar results to ours showing that bombesin and neurotensin can stimulate transactivation of androgen-responsive promoters. They reported that modification of AR signaling by neuropeptides is mediated in part by p125$^{Fak}$, Src, and Etk nonreceptor tyrosine kinases, and that activated MAP kinase may possibly also affect AR phosphorylation. Together these two independent studies suggest a complex series of events leading to neuropeptide-induced AR activation. Current studies are aimed at further defining the cross-talk between neuropeptide and AR signaling pathways.

Our results have major clinical implications. Neuroendocrine differentiation and neuropeptide growth factors have been implicated frequently in prostate cancer progression; however, the precise mechanisms by which neuropeptides contribute to androgen-independent PC were undefined. Our data suggest that
neuropeptides such as bombesin and endothelin-1, the expression of which increases after androgen withdrawal, can contribute to androgen-independent progression in some PCs by synergizing with castrate levels of DHT to signal through the AR. This understanding of the interaction between neuropeptide GPCR and AR signaling may lead to a better understanding of the development and progression of androgen-independent PC and provide a basis for developing new therapies aimed at inhibiting this interaction, leading to improved treatment of patients with advanced prostate cancer.

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