Tumor-promoting Phorbol Ester Down-Regulates the Androgen Induction of Prostate-specific Antigen in a Human Prostatic Adenocarcinoma Cell Line

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ABSTRACT

Prostate-specific antigen (PSA) is the most sensitive marker available for monitoring the progression of prostate cancer and response to therapy. In a previous study, we demonstrated tissue-specific expression of PSA glycoprotein and mRNA and its regulation through the androgen receptor. In this study, we examined the effects of protein kinase A (PKA) and protein kinase C (PKC) on the androgen regulation of PSA in a human adenocarcinoma cell line, LNCaP. Northern blot analysis demonstrated that forskolin, an activator of PKA, had no effect on the androgen regulation of PSA. However, the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a direct activator of PKC, showed a time- and dose-dependent repression of the androgen regulation of PSA glycoprotein and mRNA. The biologically inactive phorbol ester, 4α-phorbol-12,13-didecanoate, had no effect. Staurosporine, a PKC inhibitor, blocked the TPA-mediated repression of the androgenic stimulation of PSA glycoprotein. In addition, the calcium ionophore, A21387, was able to simulate the actions of TPA, presumably through activation of PKC via calcium mobilization. In summary, the androgen regulation of PSA protein and mRNA is repressed by tumor-promoting phorbol esters through the PKC pathway. This indicates that the effects of TPA may be secondary to repressed gene transcription or altered mRNA stability. In addition, this study emphasizes that the androgenic regulation of PSA is complex and may involve other extracellular transduction signals.

INTRODUCTION

PSA was first discovered in 1971 as a component of human seminal plasma and referred to as γ-seminoprotein (1). Subsequent investigations have demonstrated that PSA is a 240-kDa glycoprotein with a molecular weight of 34,000 (2-5). Functionally, PSA is a kallikrein-like, serine protease that is produced exclusively by the epithelial cells lining the acini and ducts of the prostate gland (6). In the seminal fluid, PSA is involved in the liquefaction of the seminal coagulum (7-8) and, therefore, may play an important role in male fertility.

Several studies have demonstrated the tissue-specific expression of PSA in prostatic tissue (6-10). PSA has been detected in the epithelial cells of normal prostate, benign prostatic hyperplasia, primary prostate cancer, and metastatic prostate cancer tissues (9-12). Recent investigations have shown that the expression of PSA is not uniform (12, 13). In addition, we have demonstrated recently that the PSA glycoprotein and mRNA are significantly decreased in malignant epithelium when compared to benign prostatic hyperplasia (9). Measurement of serum PSA is the most sensitive marker available for monitoring the progression of prostate cancer and the response to therapy (14-18). Tremendous interest has been generated recently in the use of PSA for the screening and staging of prostate cancer (19).

Expression of PSA protein has been shown to be correlated with fluctuating androgen levels during male development (20). In addition, we have demonstrated that the expression of the PSA glycoprotein and mRNA in a human adenocarcinoma cell line, LNCaP, are regulated by androgens via the androgen receptor (10). The evaluation of patients with benign prostatic hyperplasia and treated with antiandrogens has shown a direct correlation between serum testosterone and serum PSA (21). However, it is well known that prostate carcinoma is heterogeneous in its responsiveness to antiandrogen therapy (22). Thus, a complete understanding of the regulation of PSA expression is important because of its potential usefulness as a model and its clinical relevance to understanding the heterogeneous responsiveness of prostate cancer to antiandrogen therapy.

The regulatory mechanism of PSA is most likely a complex, multifaceted system. It has been demonstrated (23, 24) that both PKA and PKC are involved in biological activities in the prostate. Previous studies (25) have suggested that androgenic induction of glycolytic enzymes is mediated by cAMP-dependent kinases. Recently, it has been shown (26) that mitochondrial aspartate aminotransferase mRNA can be stimulated by the phorbol ester TPA in the prostate, suggesting the involvement of the PKC pathway in gene regulation. In the present study, we have examined the effects of both the PKA activator, forskolin, and the PKC activator, TPA, on the androgen induction of PSA expression in a prostatic adenocarcinoma cell line, LNCaP.

MATERIALS AND METHODS

Materials. [α-32P]dCTP (~3000 Ci/mmol) and mibolerone were obtained from Amersham Corporation (Arlington Heights, IL). TPA, 4α-phorbol-12,13-didecanoate, staurosporine, forskolin, and calcium ionophore (A23187) were purchased from Sigma Chemical Company (St. Louis, MO). These compounds were stored as single-use aliquots in anhydrous dimethyl sulfoxide at -100°C. Zeta probe blotting membrane was obtained from Bio-Rad (Richmond, CA). Trypan blue (0.4%) stain and guanidine isothiocyanate were purchased from Gibco (Grand Island, NY) and Fluka (Ronkowkoma, NY), respectively. PSA glycoprotein was measured with the Tandem-R PSA ImmunoradioMetric Assay from Hybritech (San Diego, CA).

Cell Culture. LNCaP cells (5 x 10⁴ cells/ml) were propagated in RPMI-1640 (GIBCO) with 5% fetal calf serum (GIBCO), 10 nM testosterone, and 2 mM glutamine until cultures reached 70% confluency. Cells were then cultured in RPMI-1640 with 2% charcoal-stripped fetal calf serum and 2 mM glutamine for 20 h, prior to any experiment.

Probe Preparation. The PSA complementary DNA probe was prepared as described previously (10). Briefly, an oligonucleotide (77 bases) corresponding to a region in exon 5 of the PSA gene was synthesized at the Mayo Molecular Biology Core Facility. A 20-base oligonucleotide corresponding to the 3' end of the 77-base oligonucleotide was also synthesized. The 77-mer and 2-mer oligonucleotides were mixed at a molar ratio of 1:30, and an asymmetrical polymerase chain reaction amplification (27) was used to produce antisense oligonucleotide probes. The amplification reaction (50 μl) contained 30 pmol of 20-mer oligonucleotides; 1 pmol of 77-mer oligonucleotide; 0.04 mM dATP, dGTP, dCTP; 1X GeneAmp PCR buffer (Perkin-Elmer); 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer); and 125 μCi [α-32P]dCTP (~3000 Ci/mmol). This reaction was performed with 12...
cycles of denaturation (1 min at 94°C), annealing (1 min at 56°C), and polymerization (3 min at 72°C). L-[α-32P]dCTP was incorporated into the newly synthesized probe by this PCR amplification reaction.

Northern Blot Analysis. Steroid-depleted LNCaP cells were cultured in RPMI 1640 with 2% charcoal-stripped fetal calf serum and 2 mM glutamine with the addition of the synthetic androgen, mibolerone, and test reagents. Cells were harvested at the indicated times, and total RNA was extracted by the guanidine isothiocyanate method (28). Equal amounts of RNA (about 5–15 μg/lane) were fractionated in the presence of ethidium bromide by denaturing gel electrophoresis and transferred to a Zeta Probe membrane (29). The amount of RNA used was quantified by both spectrophotometric assay at wavelength 260 μm in H2O and ethidium bromide staining of the gel and the Zeta Probe membranes. The Zeta Probe membranes were hybridized with the 32P-labeled PSA probe and washed sequentially at 50°C for 5 min with 50 mM NaH2PO4, pH 7.2, 5% sodium dodecyl sulfate, 1 mM EDTA, and 50 mM NaH2PO4, pH 7.2, 1% sodium dodecyl sulfate, 1 mM EDTA. In some experiments, rat glyceraldehyde-3-phosphate dehydrogenase complementary DNA (1.3 kilobase) labeled with 32P by random primer labeling was used for normalization of PSA mRNA. Autoradiographs were obtained by exposing the membrane for 20 h at -70°C.

PSA Glycoprotein Assay. Steroid-depleted LNCaP cells were cultured in RPMI 1640 with 2% charcoal-stripped serum and 2 mM glutamine (3 ml in 60 x 15 mm Corning tissue culture plates) in the absence or presence of mibolerone and test reagents. The above conditions were repeated in triplicate, and samples were collected at the indicated time intervals. PSA concentration of the spent media was measured using the Tandem-R PSA ImmunoRadioMetric Assay according to the manufacturer's recommendations. The cells were washed twice with 3 ml of 1x PBS (Ca2+,Mg2+-free), and a single cell suspension was obtained by trypsinization. Cells were stained with an equal volume of trypan blue (0.4%) stain, and viable cells were measured using a hemocytometer. Data were analyzed and plotted using Statview II software (Abacus Concepts) on a Macintosh IIX computer. PSA was expressed as ng/10^6 cells.

RESULTS

Phorbol Ester Down-Regulates the Androgen Induction of PSA mRNA. Our laboratory has demonstrated previously (10, 30, 31) that both PSA protein and mRNA are regulated by androgens through the androgen receptor in LNCaP cells. It was of interest to evaluate the effects of the cAMP activator, forskolin, and the phorbol ester, TPA, on the androgenic regulation of PSA expression in LNCaP cells. In order to address this question androgen-depleted LNCaP cells were stimulated with 3 nm mibolerone, a concentration which we had shown previously to induce maximal levels of PSA mRNA, in the presence of either 100 μM forskolin or 10 nM TPA. RNA was extracted at 9 h and fractionated on a formaldehyde agarose gel for Northern blot analysis. In the presence of forskolin, the androgen induction of PSA mRNA was repressed by phorbol ester, which is known to activate the protein kinase C pathway.

Dose Response of Phorbol Ester on Androgen Stimulation of PSA mRNA. To examine the dosage effect of TPA on androgen induction of PSA mRNA expression, LNCaP cells were subjected to various concentrations of TPA over 9 h in the presence of 3 nm mibolerone. As we have demonstrated previously (10), higher levels of PSA mRNA are observed after 9 h of mibolerone treatment when compared to no treatment (Fig. 2). The androgen induction of PSA mRNA expression was repressed with as little as 0.01 nM TPA (Fig. 2). A dose response can be seen, with a greater repression of androgen induction at higher concentrations. Moreover, the addition of cyclohexamide had little or no effect on the TPA repression, indicating that newly
TPA treatment, PSA mRNA levels remained between 18 and 32% that of untreated cells.

Next, we examined the effects of TPA on androgen-induced expression of PSA glycoprotein. Steroid-depleted LNCaP cells were grown under the following treatments: no treatment; 3 nM mibolerone; 0.1 nM TPA; and 0.1 nM TPA plus mibolerone over a 56-h period. The spent medium was removed at each time point, and PSA was measured with the Tandem-R ImmunoRadioMetric Assay. Cell counts were used to standardize for cell growth between plates. As we have demonstrated previously (30), mibolerone stimulates PSA production over the 56-h period (Fig. 4), which correlates with the expression of the transcript. Treatment with TPA alone or in combination with mibolerone showed an initial increase in PSA protein in the first 30 h. This elevation of PSA in the TPA-treated cells may be secondary to a stimulation of release or secretion of PSA from the cells, since it has been well documented (31) that phorbol esters can stimulate the release, secretion, and exocytosis of cellular constituents from a variety of endocrine and exocrine tissues. After 30 h, TPA reduced the androgen induction of PSA protein to approximately 50% of that of cells treated with mibolerone alone (Fig. 4). These data indicate that TPA represses both the PSA glycoprotein and mRNA stimulation by androgens. This suggests that decreased levels of PSA protein in the presence of TPA may be secondary to decreased gene transcription or mRNA stability.

Effects of 4α-Phorbol, Staurosporine, and Calcium Ionophore A23187. In order to study the mechanism by which phorbol esters repress the androgen induction of PSA, various agents which affect the protein kinase C pathway were tested for their effects on androgen induction of PSA. After LNCaP cells had been steroid depleted for 20 h, cells were grown in the presence of 3 nM mibolerone with the following reagents: 0.2 nM TPA; 10 nM 4α-phorbol [a biologically inactive phorbol ester (33)]; 100 μM A23187 [a calcium ionophore (33)]; and 5–40 nM staurosporine [a protein kinase C inhibitor (33)] plus 0.2 nM TPA. PSA was measured from the spent media at 48 h. As shown in Fig. 5, mibolerone markedly increased the PSA glycoprotein, which was about 4.5 times that of no treatment at

Figure 3. Time course effects of mibolerone and TPA on PSA mRNA in LNCaP cells. After 20 h of steroid depletion, cells were treated as follows: no treatment (control); 0.1 nM TPA; 3 nM mibolerone; and 3 nM mibolerone plus 0.1 nM TPA. The Tandem R ImmunoRadioMetric Assay (Hybritech) was used to measure PSA from the spent media at the indicated times. Cell counts were used to standardize cell growth between plates. Triplicate determinations for PSA were obtained from each of three separate plates of cells at each time point and are given as the mean value.

Figure 4. Effects of mibolerone and TPA on PSA glycoprotein secretion in LNCaP cells. After 20 h of steroid depletion, cells were treated as follows: no treatment (control); 0.1 nM TPA; 3 nM mibolerone; and 3 nM mibolerone plus 0.1 nM TPA. The Tandem R ImmunoRadioMetric Assay (Hybritech) was used to measure PSA from the spent media at the indicated times. Cell counts were used to standardize cell growth between plates. Triplicate determinations for PSA were obtained from each of three separate plates of cells at each time point and are given as the mean value.

Time Course of TPA Action on the Mibolerone Induction of PSA mRNA and Protein. To illustrate the time course over which TPA action takes place, steroid-depleted LNCaP cells were grown in the presence of 3 nM mibolerone in either the absence or presence of 0.1 nM TPA over 40 h. Total RNA was extracted at various time points. As can be seen in Fig. 3, TPA markedly repressed the androgen induction of PSA mRNA as early as 6 h and exhibited a maximum effect at 9 h. Inhibitory effects were observed as long as 40 h after exposure to TPA. Densitometric scanning of the autoradiograph showed that the PSA mRNA TPA-treated cells was reduced to 87% and 42% of TPA untreated cells at 3 h and 6 h, respectively. After 9 h of

![Densitometric scanning of the autoradiograph showing PSA mRNA levels relative to control.](cancerres.aacrjournals.org/content/cancerres/50/14/1527.f5)

Fig. 5. Effects of 4α-phorbol, A23187, and staurosporine on PSA glycoprotein secretion from LNCaP cells. After 20 h of steroid depletion, cells were grown in the absence (control, C) or the presence of 3 nM mibolerone alone or 3 nM mibolerone with the following compounds: 10 nM 4α-phorbol-12,13-didecanoate, 0.2 nM TPA; 5–40 nM staurosporine plus 0.1 nM TPA; or 100 μM A23187. The Tandem R ImmunoRadioMetric assay (Hybritech) was used to measure PSA at 48 h. Cell counts were used to standardize for cell growth between plates as described in "Materials and Methods." Triplicate determinations of PSA were obtained from three separate plates of cells for each treatment and are expressed as a mean value ± SEM.

synthesized protein factors are not required. These findings indicate that the phorbol ester inhibition of androgen induction of PSA mRNA is dose responsive, with effects discernible at a concentration as low as 10^{-11} M TPA (Fig. 2). In this experiment, glyceraldehyde-3-phosphate dehydrogenase was used not only to demonstrate the specificity of TPA induction of PSA mRNA but also to verify that 18S rRNA is suitable for normalizing PSA mRNA.

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the 48-h time point. The potent activator of the protein kinase C pathway, TPA, repressed the androgen induction of PSA. However, the inert analogue, 4a-phorbol, had no effect on the PSA expression. Staurosporine was able to block the effects of TPA on the androgen regulation of PSA, thus indicating specificity of the response to the protein kinase C pathway. The addition of A21387 demonstrated a marked repression of the mitobolone stimulation of PSA, indicating that calcium mobilization may be involved. These data suggest that TPA repression of the androgen induction of PSA is acting through the protein kinase C pathway.

### DISCUSSION

In earlier studies from our laboratory (9, 10), we demonstrated tissue-specific expression of PSA and the androgen induction of PSA mRNA working through the androgen receptor. The results of the current work show that a cyclic AMP-dependent protein kinase activator, forskolin, had little or no role in the androgen regulation of PSA. However, the phorbol ester, TPA, which is known to activate the PKC pathway, was shown to down-regulate the androgen induction of the PSA transcript and translation product. The potent phorbol ester, TPA, which binds and activates protein kinase C (34), showed a time- and dose-dependent repression of the androgen stimulation of PSA, whereas the biologically inactive phorbol ester (33), 4a-phorbol-12,13-didecanoate, had no effect. Staurosporine, a protein kinase C inhibitor (33), blocked the TPA-mediated repression of PSA mRNA and protein expression. These data indicate that the protein kinase C pathway may play a role in the regulation of androgen-dependent expression of PSA.

PSA has become the most important tumor marker for following patients with prostate carcinoma (14-19). The PSA glycoprotein is found in high concentrations in the epithelium of normal prostate and benign prostatic hyperplasia. The expression of PSA glycoprotein in prostate carcinomas is not uniform. Moreover, in a previous study we have demonstrated that PSA glycoprotein and mRNA are significantly reduced in malignant epithelium when compared to benign prostatic epithelium (9). Some studies have indicated that as many as 20-25% of all prostatic tumors exhibit either low levels of PSA or none at all (12, 13). These reports are in agreement with clinical data which have shown the unreliability of a low serum PSA after antiandrogen therapy (35, 36).

The heterogeneous expression of PSA indicates that the regulatory mechanism is complex and that other pathways may interact with the androgen stimulation of PSA. The regulation of citric acid in prostate tissue was initially thought to be regulated only by androgens (37). However, recent studies have shown that the regulation of prostate citrate is a complex multihormonal process involving the protein kinase C pathway. Also of great interest is the corollary that prostate citrate is regulated only by androgens (37). However, recent studies have demonstrated that phorbol esters decrease the binding of epidermal growth factor factor (44), insulin (45), β-adrenergic agonists (46), and α1-adrenergic agonists (47) to their specific receptors. The decrease in ligand binding to the epidermal growth factor receptor is associated with enhanced receptor phosphorylation (44), apparently mediated by the activation of protein kinase C. It is speculated that tumor-promoting phorbol esters acting through the protein kinase C pathway may alter the interaction between the androgen receptor and its cognate steroid response element and thereby affect PSA expression either directly or indirectly. There is substantial evidence that phosphorylation of the progesterone receptor in the transcription complex is one mechanism which modulates progesterone receptor-mediated transcription (48). It will be interesting to determine whether the PKC pathway has an effect on modifying (i.e., phosphorylating/dephosphorylating) the androgen receptor both structurally and functionally.

The promoter regions of several phorbol-inducible genes have a conserved cis-acting element known as a TPA response element, which is recognized by the transcription factor AP-1 (49). Examination of the 5′-flanking region of the PSA gene revealed a steroid response element-like sequence located approximately 160 base pairs from the cap site of the gene (10). In addition, a putative TPA response element has been identified upstream from this putative androgen response element. Further work will be needed to determine whether this TPA response element is involved in the suppression of the androgen stimulation of PSA. It will be important to determine whether there is any direct interaction between AP-1 proteins and the androgen receptor, as shown in the interaction between glucocorticoid receptors and Fos/Jun complex (50) for repression of gene expression.

In summary, the androgen regulation of PSA is repressed by tumor-promoting phorbol ester through the protein kinase C pathway. The androgen induction of PSA is suppressed by TPA at the levels of both mRNA and protein. This indicates that decreased PSA may be secondary to repressed gene transcription or altered mRNA stability. The effect of transmembrane signaling on PSA may provide a model system to elucidate the role that these extracellular signals might play on tumorigenesis.

Unpublished data.

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