Regulation of prostate-specific antigen by activin A in prostate cancer LNCaP cells

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Fujii, Yasuhisa, Satoru Kawakami, Yohei Okada, Yukio Kageyama, and Kazunori Kihara. Regulation of prostate-specific antigen by activin A in prostate cancer LNCaP cells. Am J Physiol Endocrinol Metab 286: E927–E931, 2004. —Activins are multifunctional growth and differentiation factors and stimulate FSH-antigen by activin A in prostate cancer LNCaP cells. Activins increase cell growth of LNCaP cells in a dose-dependent manner, whereas DHT increased it in a biphasic manner. In contrast to their opposing actions on cell growth, both activin A and DHT upregulated PSA gene expression and increased PSA secretion by LNCaP cells. The effects of activin A and DHT to increase PSA production were synergistic or additive. Follistatin or bicalutamide was without effect on cell growth or PSA production. The effects of activin A on LNCaP cells were blocked by follistatin, not by bicalutamide, whereas effects of DHT were prevented by bicalutamide, not by follistatin. Activin A upregulates PSA production, and the effect is through an androgen receptor-independent pathway. The activin/follistatin system can be a physiological modulator of PSA gene transcription and secretion in the prostate tissue, and activins may cooperate with androgen to upregulate PSA in vivo.

ACTIVIN IS A MEMBER of the transforming growth factor-β (TGF-β) superfamily of growth factors. Activins are dimeric proteins composed of two inhibin-β proteins translated from at least five different gene transcripts, referred to as BA, BB, BC, BD, and BE. Activins are multifunctional growth and differentiation factors participating in the regulation of many biological systems and processes, including embryonic development, erythroid differentiation, and tumorigenesis (14, 17, 21, 22), by interactions with specific cell surface receptors that show considerable subtype diversity (12) and have been identified in many tissues (10), including the prostate (4, 20, 28) and prostate cancer cells (23). Activin A (the inhibin-βA dimer) and activin B (the inhibin-βB dimer) have profound effects on cell growth and function. Both stimulatory (8, 19, 30) and inhibitory (5, 27) effects have been documented depending on cell type. Follistatins are structurally unrelated proteins that are glycosylated monomeric proteins derived from either of two mRNA splice variants coding for 288 or 315 amino acid proteins after removal of a common signal peptide at the time of synthesis, and bind activin, resulting in the neutralization of activin bioactivity (3, 11).

Among the human prostate tumor cell lines LNCaP, DU145, and PC3, a strong growth-inhibitory effect in response to activin A is seen only after addition of exogenous activin A to the LNCaP cell line, which expresses functional activin receptors (2, 13, 24). This growth inhibition is dose dependent and, importantly, is androgen independent (13). Although activin was also found to inhibit proliferation of the DU145 cells to a lesser extent, no growth inhibition was seen in the PC3 cells (13).

Prostate-specific antigen (PSA) plays an important role in male reproductive physiology as well as being very important as a tumor marker for prostate cancer. PSA is a serine protease and member of the tissue kallikrein family of proteases, and it is secreted into seminal fluid at concentrations of 0.5–3 mg/ml (i.e., ~1 million times those in plasma) (25). In semen, PSA dissolves the gel forming after ejaculation by digesting semenogelin-1 and -2 and fibronectin, causing release of the sperm, which is essential for sperm function (9). Previous studies have shown that PSA is positively regulated by androgens via its androgen receptor (AR)-independent effect. Activins were first identified by their ability to regulate pituitary follicle-stimulating hormone (FSH). Activins stimulate FSH-β gene expression and FSH secretion by the pituitary gonadotropes. We hypothesized that, by analogy with FSH, activins regulate PSA in prostate cells. In the present study, using prostate cancer LNCaP cells, we show that activin A upregulates PSA gene expression and secretion and that the effect is through an androgen receptor (AR)-independent pathway.

MATERIALS AND METHODS

Cell culture. Human prostate cancer cell line LNCaP was obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C under 5% CO₂. For the treatment, LNCaP cells were plated at 2 × 10⁵ cells/well of 96-well assay plates with 100 µl of RPMI 1640 medium with 10% dextran-coated charcoal-stripped

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(DCC-)FBS, $2 \times 10^4$ cells/well of 24-well plates with 500 μl of medium, or $2 \times 10^5$ cells/well of 6-well plates with 2 ml of medium. After a 24-h incubation, cells were treated with 2% DCC-FBS with or without the test agents.

Cell proliferation assays. Cellular growth was assessed by total cell counts and by MTS assay. LNCaP cells were cultured with media with 10% FBS-DCC for 24 h, and then media were changed into fresh media with 2% FBS-DCC with or without test agents. After 48-h treatment, cells were counted in a hemocytometer, and the viability of cells was determined by trypan blue dye exclusion. MTS assay was also performed using the CellTiter 96 aqueous nonradioactive proliferation assay (Promega, Madison, WI), which is a colorimetric method for determining the number of viable cells in proliferation assays, as reported previously (7). For this assay, the MTS assay reagent was added to each well after 48-h treatment, and the absorbance was measured after 2-h incubation at 37°C using THERMOmax microplate reader (Molecular Devices, Sunnyvale, CA).

Total RNA extraction and cDNA synthesis. Total RNA was extracted using ISOGENE (Wako, Osaka, Japan), following the manufacturer’s instructions. The concentration and purity of RNA were determined spectrophotometrically. Two micrograms of total RNA were reverse-transcribed into first-strand cDNA by use of the ThermoScript RT system (Invitrogen, Carlsbad, CA) at the final volume of 20 μl.

Quantitative real-time RT-PCR. Real-time monitoring of PCR reactions was performed using the LightCycler system and the SYBR green I dye (Roche Molecular Systems, Indianapolis, IN), as previously described (29). The primers for PSA (accession no. X05332) were sense: 5'-TGACCAAGTTCTGCTGTGT-3' and antisense: 5'-GTCATTTCAAGTTCAAG-3', which are located in different exons. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and standard GAPDH cDNA were obtained from Search-LC (Heidelberg, Germany). Reactions included 0.5 μl of cDNA, 2 μl of LC DNA Master SYBR Green I mix, 1 μl of 10 μM primers, and for PSA PCR 1.6 μl of 25 mM MgCl₂. The final volume was adjusted with H₂O to 20 μl. The cycling conditions were as follows: for PSA, initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 10 s, and extension at 72°C for 10 s; for GAPDH, initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 68°C for 10 s, and extension at 72°C for 16 s. Fluorescent product was measured by a single acquisition mode after each cycle. The expressions of PSA and GAPDH in each sample were quantified in separate tubes. For distinguishing specific from nonspecific products and primer dimers, a melting curve was obtained as previously described (29).

PSA protein assay. LNCaP cells were cultured with media with 10% FBS-DCC for 24 h, and then media were changed into fresh media with 2% FBS-DCC with or without test agents. After a 48-h treatment, the conditioned media were collected, centrifuged to remove residual cells, and stored at −20°C. The Immulite third-generation PSA assay, which is a solid-phase, two-site sequential chemiluminescent enzyme immunoassay executed on the Immulite instrument (15, 26), was used to measure PSA levels of culture medium. MTS assay was performed at the same time, and results were
adjusted to nanograms per milliliter of PSA by the cell number of the control group.

**Hormones.** Recombinant human follistatin and activin A were obtained from Wako. The nonsteroidal anti-androgen bicalutamide was kindly provided by AstraZeneca Japan (Tokyo, Japan). 5α-Dihydrotestosterone (DHT) was purchased from Sigma (St. Louis, MO).

**Statistical analysis.** Statistical analysis was performed with JMP 5.0 software (SAS Institute, Cary, NC). Data are means ± SE of triplicate or quadruplicate wells. Differences in data among treatment groups were analyzed by the one-way analysis of variance (ANOVA) followed by a multiple comparison Dunnett’s test. All P values < 0.05 were considered statistically significant.

**RESULTS**

**Effect of activin A on cell growth of LNCaP cells.** LNCaP cells were treated with increasing concentrations of activin A (0.5 to 50 ng/ml) or DHT (0.1 nM to 1 μM) with or without their antagonists (follistatin or bicalutamide). Cell growth was assessed by total cell counts and by MTS assay, which demonstrated similar results. In agreement with previous studies (2, 13, 24), activin A treatment of LNCaP cells resulted in a significant inhibition of their growth in a dose-dependent manner (data not shown). LNCaP cells did not increase under 50 ng/ml activin A. Follistatin (125 ng/ml) or bicalutamide (5 μM) was without effect on LNCaP cell growth. The effect of activin A was blocked by follistatin, not by bicalutamide. In contrast, DHT stimulated cell growth in a biphasic manner with maximal effects observed at 1 to 10 nM, which was blocked by bicalutamide, not by follistatin. The effects of DHT returned toward basal levels at concentrations greater than the peak of maximal stimulation.

**Effect of activin A on PSA gene expression in LNCaP cells.** The effects of activin A and DHT with or without their antagonists (follistatin or bicalutamide) on the expression of PSA mRNA in LNCaP cell cultures were examined by quantitative real-time RT-PCR. The time course study showed that 50 ng/ml activin A upregulated PSA gene expression after 4 h, and the effect reached maximal at 8 h (data not shown). Figure 1A shows the PSA gene expression after increasing the concentration of activin A for 8 h. The effect of activin A to increase PSA mRNA levels was concentration dependent. As
shown in Fig. 1B, DHT also upregulated PSA gene expression, whereas follistatin or bicalutamide had no effects. Follistatin neutralized the effect of activin, whereas bicalutamide blocked the effect of DHT.

Effect of activin A on PSA secretion by LNCaP cells. As shown in Fig. 2, activin A increased PSA secretion by LNCaP cells in a dose-dependent manner and in parallel with the change in mRNA levels described above. Again, the effect was blocked by follistatin, not by bicalutamide. Neither follistatin nor bicalutamide stimulated PSA secretion by LNCaP cells. DHT also increased PSA secretion, which was partially blocked by bicalutamide. Follistatin had no effects on the effect of DHT.

Interactions of activin A and DHT to upregulate PSA production. The effects of activin A and DHT to increase PSA production were additive or synergistic in mRNA (Fig. 3A) or protein levels (Fig. 3B), respectively.

DISCUSSION

A number of investigations have clearly demonstrated hormonal regulation of the PSA gene expression primarily by androgens in the prostatic carcinoma cell line LNCaP (16). Over the last several years, a detailed understanding is evolving as to how AR functions as a transcriptional regulator via its binding to androgen response elements within promoter and enhancer regions of PSA (1). However, little progress has been made in identifying androgen-independent regulatory mechanisms involved in PSA gene regulation.

In the present study, we show that activin A upregulates PSA gene expression and increases PSA secretion by LNCaP cells. The effects of activin to increase PSA mRNA levels and PSA secretion were dose dependent (Figs. 1A and 2), both of which were neutralized by follistatin (Figs. 1B and 2). Previous studies have shown that the activin/follistatin system, which is present in the prostate tissue, plays a crucial role in the local regulation of human prostate cell growth (20, 28). In addition, our results suggest that activin and follistatin also play an important role in the regulation of PSA production and secretion by the prostatic cells.

To our knowledge, only one previous study (31) addressed the effect of activin on PSA expression. The investigators of that study reported that activin stimulated the expression of prostatic acid phosphatase, PSA, and AR in LNCaP cells (31). However, their experiments were not performed under androgen depletion conditions, which makes the results difficult to interpret. For example, we could consider that the result of activin to upregulate PSA expression is due to the AR upregulation. Our study is the first to demonstrate the upregulation of PSA by activins in the absence of androgens.

It is very likely that the effect of activin A on LNCaP cells is through an AR-independent pathway. First, activin A decreased cell growth of LNCaP cells, whereas DHT had the opposite effect. Second, the effects of activin A on both cell growth and PSA expression were blocked by follistatin, not by bicalutamide, whereas those of DHT were prevented by bicalutamide, not by follistatin.

Then we studied whether there are any interactions between activin A and DHT. The effects of activin A and DHT to increase PSA production by LNCaP cells are additive or synergistic in mRNA or protein levels (Fig. 3), respectively, providing further evidence that the activin effect is AR independent. Activin A enhanced the effect of DHT on PSA gene expression and secretion. Thus activins and androgens may work cooperatively to regulate PSA expression in the prostate cells.

Although a great deal is known regarding activin signaling in a variety of cells, very little is currently known about the specific signal transduction pathway leading to PSA expression by activin. As a member of the TGF-β superfamily of proteins, activin signals through a heteromeric receptor complex and intracellular signaling molecules known as Smad proteins. Recently, Kang et al. (6) has shown that Smad3 enhances PSA expression, which is induced by DTH, in LNCaP cells. Future researches are needed to study whether Smad proteins are involved in activin stimulation of PSA gene.

Furthermore, the fact that PSA expression is exclusive to prostate epithelial cells suggests the existence of the prostate-specific transcription factors necessary for activin- or androgen-dependent stimulation of PSA gene. Recently, Oettgen et al. (18) reported the characterization of a novel prostate epithelial-specific Ets transcription factor, prostate-derived Ets factor (PDEF), that is involved in PSA gene regulation and acts as a coregulator of AR. Importantly, PDEF acts as an androgen-independent transcriptional activator of the PSA promoter and also interacts directly with the DNA-binding domain of AR and enhances androgen-mediated activation of the PSA promoter. Interestingly, the LNCaP cell line expresses PDEF mRNA. Thus PDEF may be involved in the mechanisms by which activins regulate the PSA gene, and future studies to test this hypothesis are of considerable interest.

In conclusion, activin A upregulates PSA gene expression and increases PSA secretion by LNCaP cells, and the effect of activin is through an AR-independent pathway. In addition, activin enhances the effect of DHT on PSA production. The activin/follistatin system can be a physiological modulator of PSA gene transcription and secretion in the prostate tissue, and activins may cooperate with androgen to upregulate PSA in vivo.

GRANTS

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