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ORIGINAL PAPER

Interaction of testosterone with inhibin α and βA subunits to regulate prostate gland growth

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Abstract Testosterone regulation of prostate gland growth has been shown to involve reciprocal interaction with inhibin and activin. This study was therefore conducted to correlate the effect of testosterone on prostate gland proliferation and differentiation with the level of expression of inhibin α and βA subunits. Immature dogs were treated with testosterone for 0, 3, 7, and 14 days and prostate gland growth was assessed by morphological and immunohistological localization of differentiation and proliferation markers. The results showed that testosterone treatment resulted in an initial significant increase in PCNA proliferation index by days 3 and 7, followed by a significant decrease by day 14 post-treatment. Interestingly, the decrease of cell proliferation was associated with structural and biochemical changes characteristic of glandular and stromal differentiation of the prostate gland. These changes include progressive glandular ductal canalization and interductal stroma differentiation which were apparent from a gradual shift from vimentin expression to vimentin and α-actin expression. Testosterone also had a differential effect on inhibin α and β subunits. Although testosterone treatment resulted in significant and constant inhibition of α subunit mRNA expression, it resulted in a significant increase of βA mRNA expression by day 3, followed by a decrease by days 7 and 14. These results indicated that testosterone acts first to drive proliferation of undifferentiated prostatic cells and then to maintain a low proliferation turnover of differentiated cells. Because it has been shown that activin is an antagonistic regulator of androgens, the attenuated stimulatory effect of testosterone on cell proliferation by day 14 might be mediated, at least in part, by interplay between testosterone and activin.

Keywords Prostate · Testosterone · Inhibin · Activin · Proliferation · Differentiation

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Introduction

We recently demonstrated that testosterone treatment guides the organization of the cellular components of the immature prostate gland into a fully mature-like structure [1]. In doing so, this androgen was shown to regulate the expression levels of locally produced signalling molecules and subsequently interact with them to finely tune prostate growth [2]. Some of these signalling molecules, for example inhibin and activin, are members of the transforming growth factor-β gene family. Functional inhibin and activin are dimeric proteins that share the same β subunit; homodimerisation of the β subunit forms activin and the heterodimerisation of α and β subunits forms inhibin. Because βA and βB are the most commonly recognized subunits, dimerisation of the α subunit with βA or βB leads to the formation of inhibin A and B, respectively. Dimerisation of different combinations of βA and βB result in the formation of activin A (βA, βA), activin AB (βA, βB), and activin B (βB, βB) [3]. Although activin induces its effect by interaction with its cell surface type I and II receptors [4], an inhibin signalling pathway has not been conclusively demonstrated [5].

Although activin and inhibin have been recognized primarily as gonad-derived regulators of pituitary gland secretion of follicle-stimulating hormone [6], a growing body of data suggests potential roles of activin and inhibin as local regulators of normal and abnormal prostate gland growth [7, 8]. Evidence supporting this proposal was the different expression and localization of inhibin α and β subunits by normal, malignant, and benign prostatic cells. Whereas inhibin βA subunit and dimeric activin have been shown to be expressed by normal [9, 10], malignant [11], benign [12] and prostate cancer cell lines [13], inhibin α has been shown to be expressed by normal differentiated epithelial cells only [9, 14] in benign prostate hyperplasia and in non-malignant regions of prostate carcinoma [15]. Expression of inhibin α by malignant prostatic cells or prostate cancer cell lines has not been detected [15]. The functional role of activin and inhibin in the regulation of prostate gland growth has been derived largely from in-vitro studies. These indicate that activin acts to inhibit the proliferation of primary cultured prostate cells [16] and prostate cancer cell lines [17, 18] in a dose and time-dependent manner. Although the role of inhibin in the regulation of prostate gland growth is not well defined, it has been shown that inhibin alone usually has very little effect; it serves to antagonize the action of activin [17]. Inhibin seems to interfere with activin signalling by competing to bind to activin receptor type II, consequently blocking activin action [19, 20]. On the other hand, activin regulation of prostate gland growth seemed to involve interaction with androgens. In this context it has been shown that activin expression by the epithelial cells of the prostate gland is suppressed by androgen withdrawal [10] and that activin inhibits androgen-stimulated prostate gland morphogenesis [14] and suppresses androgen-induced LNCap cell proliferation [21].

As these data suggest a complex interplay between androgen, activin, and inhibin, they also raise the question of how these interactions are relevant to cell proliferation and differentiation within the course of prostate gland growth. This study was therefore conducted to evaluate the effect of testosterone treatment on the level of expression of inhibin α and βA subunits and their association with prostate gland differentiation and proliferation.

Results

Effect of testosterone on prostate gland differentiation

The effect of testosterone treatment on prostate gland differentiation was assessed by morphological and immunohistochemical localization of differentiation markers. Consistent with our previous investigations [1], the results of this study showed that testosterone treatment induced progressive structural and biochemical changes characteristic of glandular and stromal compartment differentiation. Glandular differentiation was characterized by ductal canalization and differentiation of its epithelial cells. The prostate glands of untreated dogs contain small cells with scant cytoplasm and large nuclei, which are the morphological characteristics of basal cells (Fig. 1A). As time passes after testosterone treatment there is a progressive increase in the size of some epithelial cells; these become cuboidal and columnar, which are the morphological characteristics of secretory epithelial cells (Figs. 1B–D). Differentiation of the glandular compartment was accompanied by parallel differentiation of the stromal compartment, which divided the gland into lobes and acini. Continuous and uniform immunostaining with vimentin, localized in the stroma between lobes and acini (Fig. 1A), was observed in the prostate gland of untreated dogs. Testosterone treatment resulted in no noticeable changes in vimentin immunolocalization—its expression pattern remained the same after 3, 7, and 14 days (Figs. 1B–D). In contrast with vimentin, α-actin was localized mainly in the stromal cells between lobes of the prostate glands of untreated dogs (Fig. 1E). Testosterone treatment resulted in a gradual development of α-actin immuostaining between acini (Figs. 1F–H). The shift of stromal cells from vimentin expression to vimentin and α-actin expression by testosterone treatment indicated that testosterone acts to drive the differentiation of fibroblasts to myofibroblasts.

Effect of testosterone on prostate gland proliferation

The effect of testosterone treatment on prostatic cell proliferation was assessed by immunolocalisation of PCNA. Although the results showed that most proliferating cells were confined to the glandular compartment, some proliferating cells were observed in the stromal compartment. The results are expressed as a PCNA proliferation index, which is the percentage of PCNA positive cells among 1,000 cells in randomly chosen fields. As shown in Fig. 2, the prostatic cells of untreated dogs retained the capacity to proliferate with a proliferation index of 20.56 ± 4.7. Testosterone treatment resulted in a significant ($P < 0.05$) increase in PCNA expressing cells by days 3 and 7 with PCNA proliferation indices of 53.32 ± 2.5 and 71.4 ± 1.5 respectively. The PCNA stained cells decreased significantly ($P < 0.05$) by day 14 post-treatment, however, reaching a level of 44.8 ± 1.4.

Effect of testosterone treatment on mRNA expression of inhibin α and βA subunits

The effect of testosterone treatment on the mRNA expression level of inhibin α and βA subunits was assessed

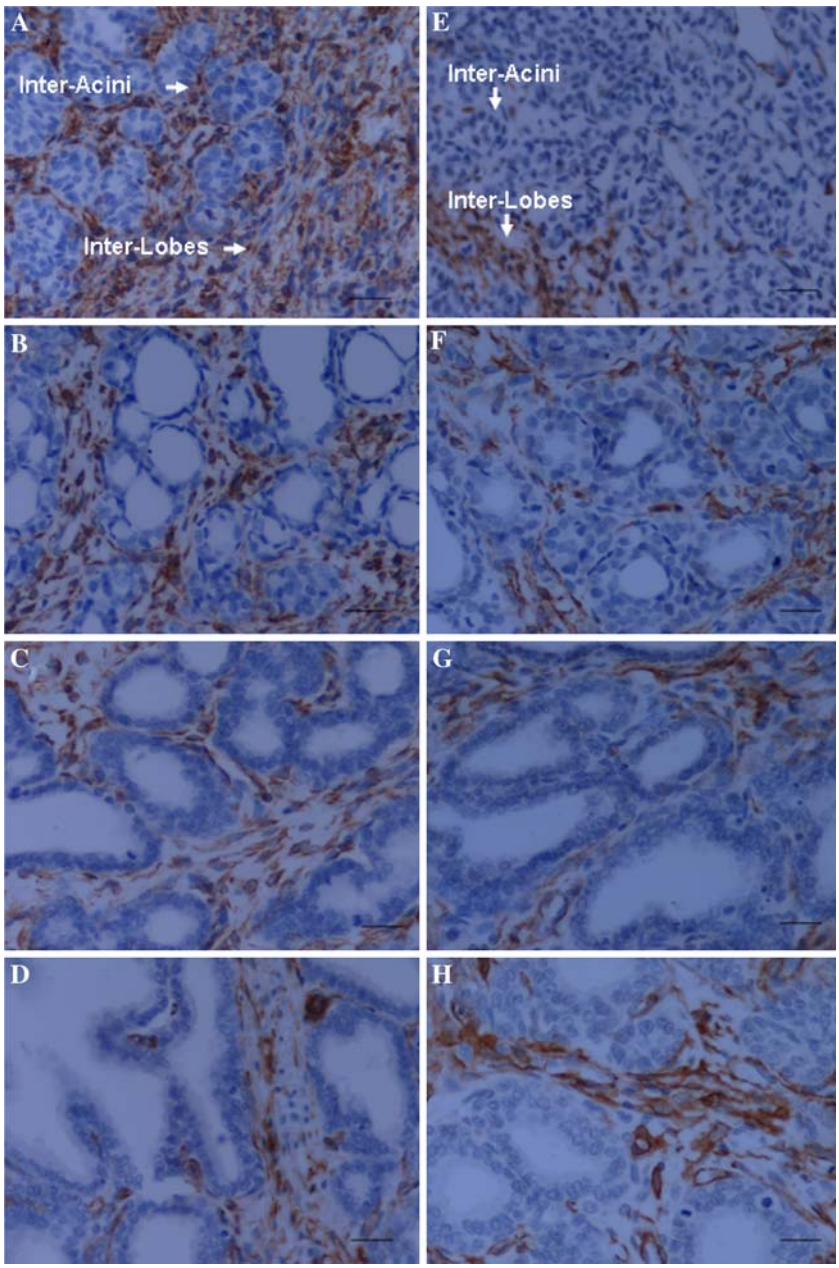

Fig. 1 Effect of testosterone on glandular and stromal cell differentiation of the prostate gland. Note the progressive ductal canalization of the glandular compartment after 3, 7, and 14 days of testosterone treatment (**B**, **C**, and **D**, respectively) compared with thecontrol (**A**). Whereas intense expression of vimentin localized in the stromal cells, between lobes and acini, was observed in prostate glands from untreated dogs (**A**), α-actin was localized mainly in the cells between lobes and no expression was observed between acini (**E**). Testosterone treatment had no effect on vimentin expression pattern, because it continued to be expressed by stromal cells between lobes and acini after 3, 7, and 14 days (**B**, **C**, **D**). Testosterone treatment, however, induced gradual development of α-actin expression by the stromal cells between acini as time passed after testosterone treatment (**F**, **G**, **H**). *Bars* = 20 μm

by quantitative real-time PCR. The results showed that testosterone had different effects on α and βA subunit mRNA expression. As shown in Fig. 3A, testosterone treatment resulted in initial up-regulation of the βA subunit, followed by down-regulation thereafter. The expression level of βA subunit mRNA increased significantly ($P < 0.05$) by day 3 (1.7 ± 0.6) post-treatment compared with the control (1.09 ± 0.26). Although there was a gradual decrease of βA mRNA expression by days 7 (1.31 ± 0.23) and 14 (0.55 ± 0.16), this decrease was only significant by day 14 compared with its expression level by day 3. The expression level of βA by day 14 was similar to that of the control, however, with no significant difference between them. In contrast with βA subunit mRNA expression, testosterone treatment resulted in significant ($P < 0.05$) and constant down-regulation of α subunit mRNA expression (Fig. 3B). The relative mRNA expression of inhibin α subunits by days 0, 3, 17, and 14 were 1.09 ± 0.28, 0.4 ± 0.05, 0.45 ± 0.13, and 0.19 ± 0.027, respectively. There were no significant differences among different treatment groups.

Discussion

Although there is general agreement on the growth-promoting effect of androgens on the prostate gland, some studies have emphasized its proliferative effects whereas

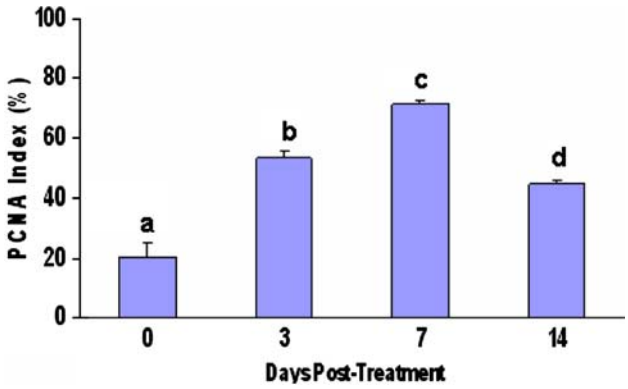

Fig. 2 Effect of testosterone treatment on PCNA proliferation index. The results are expressed as mean ± SEM for four dogs in each group. *Bars with different letters* are significantly different ($P < 0.05$)

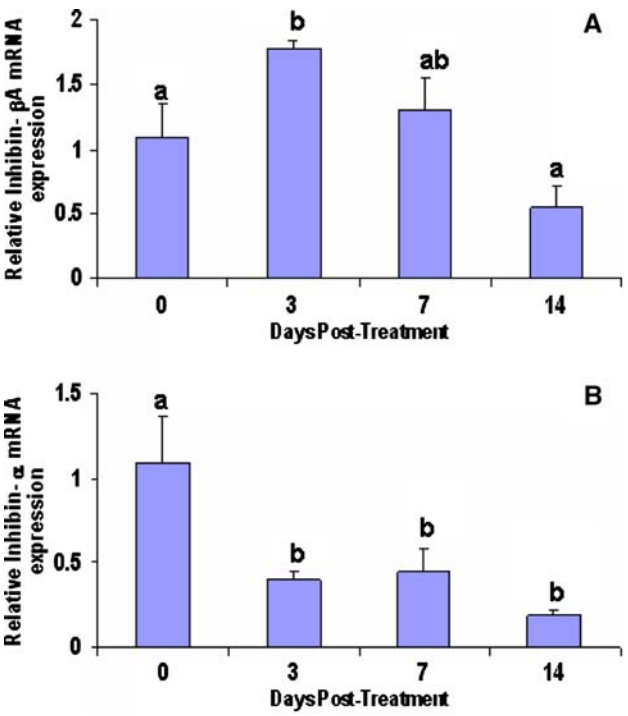

Fig. 3 Effect of testosterone treatment on relative mRNA expression of inhibin α and βA subunits. The results are expressed as mean ± SEM of for dogs in each group. *Bars with different letters* are significantly different ($P < 0.05$)

others emphasize its differentiation effects. The results of this study confirm and extend previous investigations by showing that testosterone is a growth-promoting factor that acts to coordinate prostate cell proliferation and differentiation. On the one hand, the results of this study indicate that testosterone treatment acts to drive progressive glandular ductal canalization and inter-lobular and inter-acinar stromal differentiation, apparent from the gradual shift from vimentin expression to vimentin and α-actin expression. These findings are consistent with the role of the androgen in driving [1] and maintaining [22] the differentiation status of the prostate gland. This effect of testosterone treatment on prostate gland differentiation was associated with a differential effect on prostatic cell proliferation. While most proliferating cells were found to be confined to the glandular epithelial cells, very few were observed in the stromal cells. The PCNA proliferation index indicated that cells from untreated immature dogs had a low level of proliferation which was increased significantly by testosterone treatment to reach its peak by day 7 and decline thereafter. Although following this decrease, cell proliferation by day 14 was significantly lower than that by day 7, it was still significantly higher than that of the control. These data indicate that the stimulatory effect of testosterone on cell proliferation was attenuated by day 14. Interestingly, the decrease in cell proliferation seemed to be concomitant with histological and biochemical changes characteristic of the glandular and stromal differentiation of the prostate. Consistent with our observations it has been shown that administration of testosterone to intact and castrated rats resulted in initial stimulation of cell proliferation then inhibition [23]. It has also been shown that inhibition by testosterone of epithelial [24] and stromal [25] cell line proliferation was associated with signs of their differentiation. In the light of these findings it seems that the androgen exerts a dual effect in directing the proliferation of undifferentiated cells, after which the stimulatory effect on cell proliferation was attenuated in association with cell differentiation.

The effect of testosterone treatment on prostate gland growth was associated with different expression of mRNA encoding the inhibin α and βA subunits—whereas testosterone had a significant and constant inhibitory effect on α subunit mRNA expression, it initially stimulated βA subunit mRNA expression and this was followed by gradual inhibition to reach a level not significantly different from its expression before treatment. Although the prostate gland seemed to express both α and βA mRNA, testosterone treatment led to predominant expression of βA mRNA rather then α mRNA. If the expression ratio of α and βA mRNA determines the formation of inhibin-A (α, βA dimer) or activin-A (βA, βA dimer), then testosterone treatment seems to promote activin formation. These changes in the pattern of expression of the βA subunit and its association with prostatic cell proliferation and differentiation seem to fit within the framework of activin interaction with testosterone to regulate prostate gland growth. In this context it has been shown that the pattern of expression of the βA subunit varies, depending on the stage of prostate gland development, and is relevant to androgen-regulation of these stages. immunolocalisation of βA subunits has been shown to be shifted from the epithelial buds and the surrounding undifferentiated mesenchyme of

the developing prostate to the differentiated epithelial cells of the mature gland [14]. Interestingly, this shift in βA immunolocalisation seemed to be related to the effect of androgen on prostate gland morphogenesis [14]. Similarly, previous finding from our laboratory indicate that castration-induced prostate gland regression is associated with βA subunit down-regulation [10]. Although these data suggest a regulatory role of testosterone on the level of expression of the βA subunit, other lines of evidence indicate that activin acts to inhibit testosterone-induced prostate gland morphogenesis [14] and testosterone-stimulated prostate cancer cell line proliferation [21]. This reciprocal interaction may, at least in part, account for the observed attenuation of the ability of testosterone to stimulate cell proliferation. In other words, testosterone acts to up-regulate the expression of activin, which might in turn interfere with the androgen-signalling pathway to maintain a low proliferation turnover of differentiated cells.

Materials and methods

Sixteen immature dogs of mixed breed were divided randomly into four groups. The age of the dogs ranged from 4 to 6 weeks and their weight from 2 to 2.2 kg. The dogs of the first group served as a control and therefore received no treatment. Dogs of the second, third and fourth groups were intramuscularly injected once with 45 mg per animal Primoteston containing testosterone enantate (Schering, Germany) and were euthanised after 3, 7, and 14 days, respectively, by intravenous injection of 10% thiopentone sodium. The controls were euthanised on day 0. The dogs were kept at the Veterinary Health Centre at the Faculty of Veterinary Medicine, and all animal-handling, treatment, and euthanasia procedures were approved by the Jordan University of Science and Technology Animal Care and Use Committee (JUST-ACUC). The prostate glands were then removed and divided into two symmetrical parts. The first part was stored in liquid nitrogen for mRNA isolation and further PCR analysis. The second part was washed with normal saline, fixed in 4% buffered formaldehyde for 4 h, and then routinely processed and embedded in paraffin for immunohistochemistry.

Immunohistochemistry

The immunohistochemistry was performed as described elsewhere [22]. Serial sections 5 μm thick from each set of the experiment were mounted on microscope slides coated with Vectabond (Vector Laboratories). The sections were then deparaffinised in xylene and rehydrated in a descending series of alcohol concentrations. To expose the masked antigenic sites, sections were autoclaved at 121°C in citrate buffer (10 mmol L^{-1} sodium citrate pH 6.0) for 10 min then left to cool at room temperature for 20 min. To quench endogenous peroxidase activity, sections were incubated in 1% methanolic H_2O_2 for 20 min. Non-specific binding sites were blocked with Dako protein block for 30 min (Dako Protein Block, Serum-Free; Dako, Carpentaria, CA, USA). Sections were then incubated with specific monoclonal antibodies for vimentin (Dako), α-actin (R&D Systems, Minneapolis, USA), and PCNA (Dako) for 1 h at room temperature in a humidified chamber. The vimentin, α-actin, and PCNA were used at dilutions of 1:50, 1:300, and 1:50, respectively.

At the end of each primary antibody incubation, the sections were washed three times with phosphate-buffered saline (PBS) and sections were then incubated with universal biotinylated immunoglobulin (L.V.Dako LSAB+ Kit, HPR; Dako) for 30 min at room temperature in a humidified chamber. The sections were then washed three times with PBS and incubated with streptavidin (L.V.Dako LSAB+ Kit, HPR; Dako) for 30 min at room temperature in a humidified chamber. The immunoreactions were developed with a 1 mg mL^{-1} solution of diaminobenzidine tetrahydrochloride (DAB) (Dako). The reaction was terminated in distilled water and the sections were counterstained with Mayers haematoxylin, dehydrated, and mounted. The negative controls, in which the primary antibodies were omitted, were processed in parallel with the examined sections in each assay.

PCNA labelling index

The PCNA labelling index was calculated as described elsewhere [26]. The percentage of PCNA-positive cells was counted among 1,000 cells in randomly chosen fields at magnification of 400× from each dog in all groups. Statistical analysis was then performed and the data were presented as the mean ± SEM for each group.

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted from the frozen prostate using an SV Total RNA isolation kit (Promega, USA). The RNA concentration was determined by measuring the absorbance at 260 nm using a SmartSpec Plus spectrophotometer (Bio-Rad, USA). Samples were then stored at –20°C for subsequent RT-PCR analysis. A total of 0.5 μg of total RNA was used to synthesize complementary DNA (cDNA) using the reverse transcription kit (Promega). The RT reaction was conducted at 25°C for 5 min, then at 42°C for 60 min, and then at 95°C for 5 min. The samples were then placed on ice for 5 min and stored at –20°C for PCR amplification.

Real time PCR (Rotor-Gene 3000, Corbet Research, Australia) was used to quantitatively analyse the mRNA expression level of inhibin α and βA subunits. PCR was performed using a commercial PCR kit containing sybergreen florescent dye (QuantiTect SYBR Green; Qiagen, USA) in the presence of 2 μmol L^{-1} of specific primers. The primers were designed to be specific for the canine sequence using the web-based QuantiProb Design software (QuantiTec Custom Assays, www.qiagen.com). The forward sequence for the primer used for the α subunit was CCC TGT TCA TGG AGT CTT TG and the reverse primer was AAA AGG ATG GCC TGG GAG A. The forward primer sequence for the βA subunit was CAA GAA GCA CAT CCT CAA C and the reverse primer was TCA TCC TCT ATC TCC ACG AAC C. GAPDH was used as an endogenous reference gene. The forward sequence for the GAPDH primer was CTG GAG AAA GCT GCC AAA and the reverse primer was TGT TGA AGT CAC AGG AGA. The PCR amplification reactions were started with an initial denaturation at 95°C for 15 min, then 45 cycles each of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. At the end of the 45 cycles the melting curve for the reactions was obtained at temperatures ranging from 72 to 95°C. Relative mRNA gene expression was determined using the $2^{-\Delta\Delta C}$T method and normalized to GAPDH expression [27].

Statistical analysis

Statistical analysis of PCNA labelling index and mRNA expression was performed by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) multiple comparison test. Results are presented as mean ± SEM. A probability of less than 0.05 ($P < 0.05$) was regarded as statistically significant.

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