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ORIGINAL PAPER

The prostate response to prolactin modulation in adult castrated rats subjected to testosterone replacement

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Abstract Despite the androgenic dependence, other hormones, growth factors, and cytokines are necessary to support prostatic growth and maintain the glandular structure; among them, prolactin is a non-steroidal hormone secreted mainly by the pituitary gland. However, extra-pituitary expression of prolactin, such as in the prostate, has also been demonstrated, highlighting the paracrine and autocrine actions of prolactin within the prostate. Here, we investigated whether prolactin modulation alters ventral prostate (VP) morphophysiology in adult castrated rats. *Sprague Dawley* rats were castrated and after 21 days, divided into ten experimental groups (n = 6/group): castrated control: castrated animals that did not receive treatment; castrated+testosterone: castrated animals that received T (4 mg/kg/day); castrated+PRL (PRL): castrated animals receiving prolactin (0.3 mg/kg/day); castrated+T+PRL: castrated animals that received a combination of testosterone and prolactin; and castrated+bromocriptine (BR): castrated animals that received bromocriptine (0.4 mg/kg/day). The control group included intact animals. The animals were treated for 3 or 10 consecutive days. At the end of experimental period, the animals were euthanized, and the blood and VP lobes were collected and analyzed by different methods. The main findings were that the administration of prolactin to castrated rats did not exert anabolic effects on the VP. Although we observed activation of downstream prolactin signaling after prolactin administration, this was not enough to overcome the prostatic androgen deficiency. Likewise, there was no additional glandular involution in the castrated group treated with bromocriptine. We concluded that despite stimulating the downstream signaling pathway, exogenous prolactin does not act on VP in the absence or presence of high levels of testosterone.

Keywords Ventral prostate · Prolactin · Prolactin receptor · Castration · Bromocriptine

Introduction

The prostate is an accessory gland of the male genital system and its function is essential for reproductive success, since it secretes Zn^{2+}, enzymes, and citrate, which are essential for synchronizing the ejaculatory stimulus and reproductive success (Marker et al. 2003). In addition to its key role in reproduction, the prostate has caught medical-scientific attention worldwide owing to the high incidence of diseases, mainly benign prostatic hyperplasia (BPH) and prostate cancer (PCa) during aging (Dasgupta et al. 2012).

The prostate development and homeostasis are under androgenic control (Cunha et al. 1985; Gray et al. 2015; Eisenberg 2015). Despite the androgenic dependence, testosterone alone is not sufficient to fully stimulate normal prostate growth and function. Thus other hormones, growth factors, cytokines, and adhesion molecules are important to sustain prostatic growth, structure, and function (Timms et al. 1994; Prins and Putz 2008). In this sense, clinical and experimental studies have demonstrated the pleiotropic role of prolactin, a polypeptide hormone secreted mainly by the pituitary gland. It has been demonstrated that prolactin stimulates cellular proliferation and secretory activity in the

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prostate during adulthood, both under normal and pathological conditions (Ojo et al. 2015). Although the pituitary is the major source of prolactin, prolactin has extra-pituitary sources such as the breast, decidua, prostate, and the brain, highlighting the possible role of prolactin in paracrine/autocrine actions, regulatory functions, and influencing the development of disorders in these organs (Sethi et al. 2012).

Prolactin mediates its multiple functions through interaction of prolactin receptor (PRLR), a member of cytokine receptor superfamily. PRLR is expressed in a wide variety of cell types and tissues, such as in the brain, mammary and prostate epithlial cells. The prolactin attachment to the PRLR induces a downstream activation Janus kinase 2 (JAK 2), which in turn, phosphorylates multiple protein on tyrosine residues, mainly signal transducer and activator of transcription (STAT) proteins. The STAT family are the major effectors of PRL-dependent cell proliferation and activation of gene expression (Radhakrishnan et al. 2012). In addition to the activation of JAK/STAT pathways, it has been demonstrated that MAPK pathway is actvated by prolactin signaling. Although the precise role of this pathway is unknown, it seems to be associated with the induction of cell proliferation (Ahonen et al. 2002).

The prolactin signaling pathway has been explored in genetically engineered mouse models (Sackmann-Sala et al. 2014, 2015; Sackmann-Sala and Goffin 2015). Kindblom et al. (2003) developed a mouse model which expresses PRL transgene in the prostatic epithelial cells under the control of minimal probasin promoter (Kindblom et al. 2003). Probasin is an androgen-dependent secretory protein expressed abundantly in rodent prostatic lobes. It has been described as a marker of prostate differentiation and to explore androgen action in prostate biology. As the activation of probasin promoter is under androgen control, the increase of androgen levels during late prepuberty activates the prolactin synthesis and consequently increasing the intraprostatic prolactin.

Using this model, Sackmann-Sala et al. (2015) demonstrated that intraprostatic prolactin overexpression sustained activation of STAT5, leading to a increased proliferation of basal/stem cells through the paracrine activation pathway, stimulated by growth factors produced by stromal and luminal cells. Considering the involvement of basal/stem cells in prostate carcinogenesis, these authors related the result with initial stages of prolactin-induced prostate tumorigenesis. Herrera-Covarrubias and coworkers also described prostatic disorders in rats administered with prolactin. In this experiment, the administration of prolactin induced precancerous lesions in both dorsolateral and ventral lobes in intact (non-castrated) rats after 4 weeks of treatment, and the malignancy increased with a prolonged period (24 weeks) of treatment (Herrera-Covarrubias et al. 2015).

Experimental studies using rodents (Pérez-Villamil et al. 1992) and primates (Arunakaran et al. 1987) demonstrated that prolactin can act in synergism with androgens, increasing proliferation and secretory activity of prostatic cells (Reiter et al. 1999). In this context, androgen ablation in association with blockage of non-androgenic hormones or growth factors can be used to achieve a more efficient blockage of prostatic growth and function in both experimental or therapeutic conditions (Chandrasekar et al. 2015). Considering the involvement of prolactin in prostate morphophysiology, here we investigated the prostatic response to prolactin modulation in a model of testosterone ablation by surgical castration in adult rats.

Materials and methods

Animals and treatments

Sprague Dawley male rats weighing approximately 500 g (90 days of age, n = 6/group) were obtained from the Central stockbreeder at the State University of Campinas (CEMIB/UNICAMP). The animals were maintained under controlled light and temperature (photoperiod 12 h and 22–25 °C, respectively) and relative humidity (55%). The animals had free access to water and chow. The experimental protocol followed the Ethical Principles in Animal Research and the Brazilian legislation established by the Brazilian Council of Control in Animal Experimentation and was approved by the Ethics Committee for Animal Use from the Institute of Biosciences of Botucatu/UNESP (CEUA protocol 492).

After 1 week of acclimatization, the animals were anesthetized with intraperitoneal injection with Thiopentax (30 mg/kg body weight) followed by scrotal bilateral orchiectomy (Justulin et al. 2010). After 21 days of castration, the animals were divided into ten experimental groups: castrated control (CC): castrated rats that did not receive any treatment; castrated+testosterone (T): castrated rats that received testosterone cypionate (Sigma Co, St Louis, USA) diluted in corn oil (4 mg/kg, subcutaneously injected); castrated+prolactin (PRL): castrated rats that received prolactin diluted in saline (0.3 mg/kg) (Stoker et al. 1999); cas trated+testosterone+prolactin (TPRL): castrated rats that received a combination of testosterone and prolactin; and castrated+bromocriptine (BR): castrated rats that received bromocriptine diluted in saline (0.4 mg/kg) (Carón et al. 1994). In addition, a group of animals that did not undergo castration was used as control (CTR). All treatments were performed for 3 or 10 consecutive days.

After castration, the animals were housed in polyethylene cages (2/cage) until the end of the experiment and then, euthanized using sodium pentobarbital anesthesia (30 mg/kg, i.p.) followed by decapitation. Blood samples were collected from ruptured cervical vessels. The ventral prostate

(VP) lobes were dissected out, weighed, and processed for different analysis, as described below.

Hormone quantification

Blood samples from different experimental groups (n = 6/group) were collected at the time of euthanasia. Sera were obtained after centrifugation (20 min at 4000 rpm at 4 °C) and stored at − 20 °C. The serum concentrations of prolactin (PR063F-100, Calbiotech, CA, USA) and testosterone (Abcam, ab178663, CA, UK) were determined by colorimetric method (ELISA immunoenzymatic assay) following the protocol of the manufacturers.

Histological procedure

Samples of VP from different experimental groups (n = 6/group) were fixed for 04 h in metacarn (70% methanol + 20% chloroform + 10% acetic acid). The samples were then dehydrated in ethanol, diaphanized in xylene, and embedded in Paraplast (Sigma Co, Saint Louis, MO). Five-micrometer sections were produced in a rotative microtome, collected in silanized slides, and stored. The slides were stained with hematoxylin–eosin (HE) for morphological and stereological analyses.

Morphological, morphometric, and stereological analysis

The relative proportion of the VP components (epithelium, stroma, and lumen) was determined by stereological analysis (Weibel et al. 1966). Random measurements were performed in ten different fields (400×) and from five different individual prostatic lobe sections. The relative values were determined by counting the coincident points of the test grid and dividing them by the total number of points. The results were expressed as a percentage of each component and a proportion of the total area analyzed. According to (DeKlerk and Coffey 1978), 1 mg fresh prostate tissue has a volume of approximately 1 mm^3. Consequently, the VP weight (mg) can be considered as volume equivalent (mm^3). Thus, for the absolute volume calculation of each compartment, the relative volumes of lumen, epithelium, and stroma were multiplied by the mean of wet weight of the VP of the respective group (Justulin et al. 2006). All measurements were made using a Leica DMLB 80 microscope connected to a Leica DC300FX camera. The digitalized images were analyzed using Leica Q-win software Version 3 for Windows.

Immunohistochemistry

The immunohistochemical assays were performed as described previously by Colombelli et al. (2017). Briefly, the slides were incubated with a monoclonal anti-Ki-67 (1:150; ab16667-Abcam®) or polyclonal anti-androgen receptor (AR) antibody (1:100; sc816-Santa Cruz®). The sections were washed three times in PBS for 5 min and incubated with the specific secondary antibodies conjugated to a peroxidase at room temperature. The reactions were developed using diaminobenzidine (DAB) and counterstained with hematoxylin.

Determination of Ki-67 index

The Ki-67 index is expressed as percentages of positive cells from the total cells, counted in five histological sections of five VP lobes per group at 400× (Rinaldi et al. 2013; Santos et al. 2014). Approximately 8000 cells were counted per experimental group. The results are expressed as mean ± SD. All images and quantitative measurements were performed by the investigators blinded to both the animal identity and experimental condition.

Western blotting

Frozen samples of VP lobes (n = 6/group) from all experimental groups were homogenized in extraction buffer (50 mM Tris–HCl, 0.25% Triton-X 100) and centrifuged, with the total protein determined as proposed by Bradford (Bradford 1976). A total of 70 µg was analyzed by SDS–PAGE. Proteins were blotted onto the nitrocellulose membrane (Millipore, USA), blocked with 5% non-fat milk diluted in PBS, and incubated with the following primary antibodies: anti-PRLR (1:1000 AB2772-Abcam®, Cambridge, USA); anti-prostatein (1:1000, 7821-1009-Abd Serotec®, Raleigh, USA); AR (1:500; sc-7805 Santa Cruz, USA); STAT5 (1:1000, ab194898-Abcam®, Cambridge, USA) and STAT3 (1:1000, ab68153-Abcam®, Cambridge, USA). The membranes were washed with PBS and incubated with a specific secondary antibody for 1 h. After washing, the reactions were detected using an ECL kit (Amersham, USA) and the signals were captured using a CCD camera (ImageQuant LAS 4000 mini®; GE Healthcare™). The integrated optical densities (IODs) of the targeted protein bands were measured using Image J (http://rsb.info.nih.gov.ij/). The expression levels were normalized to that of β-actin (1:800; sc1615-Santa Cruz®) and the normalized results are expressed as the mean ± SD. For means of clarity, the relative proteins levels from CTR group (set at a value of one), were omitted from the graphics.

Statistical analysis

Statistical analyzes were performed using the GraphPadPrism® software (version 5.00; GraphPad, Inc., San Diego, CA). The results were submitted to analysis of

variance (ANOVA) followed by "Tukey–Kramer" test. The results are expressed as mean ± SD and were considered statistically significant differences when $p < 0.05$.

Results

Biometric parameters

The body weight did not change throughout the experimental period (Table 1). Castration resulted in an intense reduction of VP weight. In the T and TPRL groups, a progressive increase in VP weight was observed from day 3 to 10, with a statistical difference detected after 3 days of testosterone administration. There were no differences between the VP weight in the PRL and BR groups in both periods of treatment (Table 1). Moreover, prolactin administration in combination with testosterone did not induce an increment of the VP weight (Table 1).

Hormone quantification

Testosterone

The serum testosterone level was reduced to undetectable levels in the CC group. 3 days of androgen replacement resulted in supra-physiological levels of testosterone. After 10 days, the testosterone levels remained high, but without statistical difference compared to that in the CTR group. In TPRL 3 and 10, the serum testosterone concentration was similar to those observed in the T3 or T10 groups, respectively. Prolactin or bromocriptine administered alone had no effect on the testosterone concentration (Table 1).

Prolactin

Castration resulted in a reduction of serum prolactin level compared to that in the CTR group. Exogenous prolactin administration to castrated rats had a biphasic effect on serum prolactin levels. In the PRL03 group, serum prolactin level increased, reaching levels of CTR. However, after 10 days, prolactin decreased to the levels of CC group. The same result was observed in T3 and T10 groups. The prolactin concentration in the TPRL groups increased after 3 and 10 days compared to that in the CC group and was similar to that in CTR. In the BR groups, serum prolactin level was not detectable, confirming the inhibitory effect of bromocriptine on prolactin secretion (Table 1).

Morphological and stereological analysis of the VP

The prostate of CTR animals presented a normal morphology with a large luminal compartment lined by columnar

Table 1 Biometric and hormonal data of the rats from different experimental groups

Parameters/groups	CTR	CC	T3	T10	PRL3	PRL10	TPRL3	TPRL10	BR3	BR10
Body weight (g)	526.7 ± 18.8	496.4 ± 67.5	491.1 ± 36.7	482.3 ± 63.0	497.9 ± 39.3	451.6 ± 50.8	490.8 ± 55.0	500.8 ± 43.5	491.9 ± 34.5	514.3 ± 45.1
VP weight (g)	0.72 ± 0.2[a]	0.09 ± 0.03[b]	0.18 ± 0.04[c]	0.84 ± 0.2[a]	0.08 ± 0.03[b]	0.07 ± 0.02[b]	0.13 ± 0.07[b]	0.65 ± 0.12[a]	0.09 ± 0.08[b]	0.07 ± 0.01[b]
Prolactin (ng/ml)	1.57 ± 0.24[a]	0.76 ± 0.48[b]	1.7 ± 0.34[a]	0.89 ± 0.64[b]	1.04 ± 0.12[a,c]	0.46 ± 0.95[b]	1.23 ± 0.16A[a]	1.37 ± 0.28[a]	nd	nd
Testosterone (ng/ml)	7.3 ± 0.16[a]	nd	13.5 ± 1.62	9.71 ± 1.4	nd	nd	11.32 ± 1.52	8.7 ± 1.38	nd	nd

Values are expressed as mean ± SD

Different letters indicate statistically difference among the groups ($p < 0.05$)

CTR Control, *CC* castrated control, *T3* castrated plus testosterone for 3 days, *T10* castrated plus testosterone for 10 days, *PRL3* castrated plus prolactin for 3 days, *PRL10* castrated plus prolactin for 10 days, *TPRL3* castrated plus testosterone and prolactin for 3 days, *TPRL10* castrated plus testosterone and prolactin for 10 days, *BR3* castrated plus bromocriptine for 3 days, *BR10* castrated plus bromocriptine for 10 days, *nd* not detected

epithelial cells and surrounded by a thin stromal layer. The prostatic morphology after 21 days of castration was characterized by reduction of the luminal compartment and epithelial height, associated with stromal thickening. 3 days of testosterone replacement elicited a glandular regrowth with an increase in epithelial height, acinar lumen, and protein secretion. After 10 days of testosterone administration, the prostate morphology was reestablished similar to those observed in the CTR group. The simultaneous administration of testosterone and prolactin did not cause additional prostatic regrowth compared to testosterone alone. The prolactin or bromocriptine, administered for 3 or 10 days had no effect on prostate regrowth or morphology in castrated rats (Fig. 1). Stereological data corroborated the morphological results described above (Table 2).

Immunohistochemistry

In the CTR group, there were few Ki-67 proliferative cells (Fig. 2a). Castration induced a decrease in the number of proliferating cells. On the other hand, in the T3 and TPRL3 groups, the number of proliferative cells was significantly increased. In the T or T+PRL groups treated for 10 days, the number of proliferative epithelial cells was still higher but

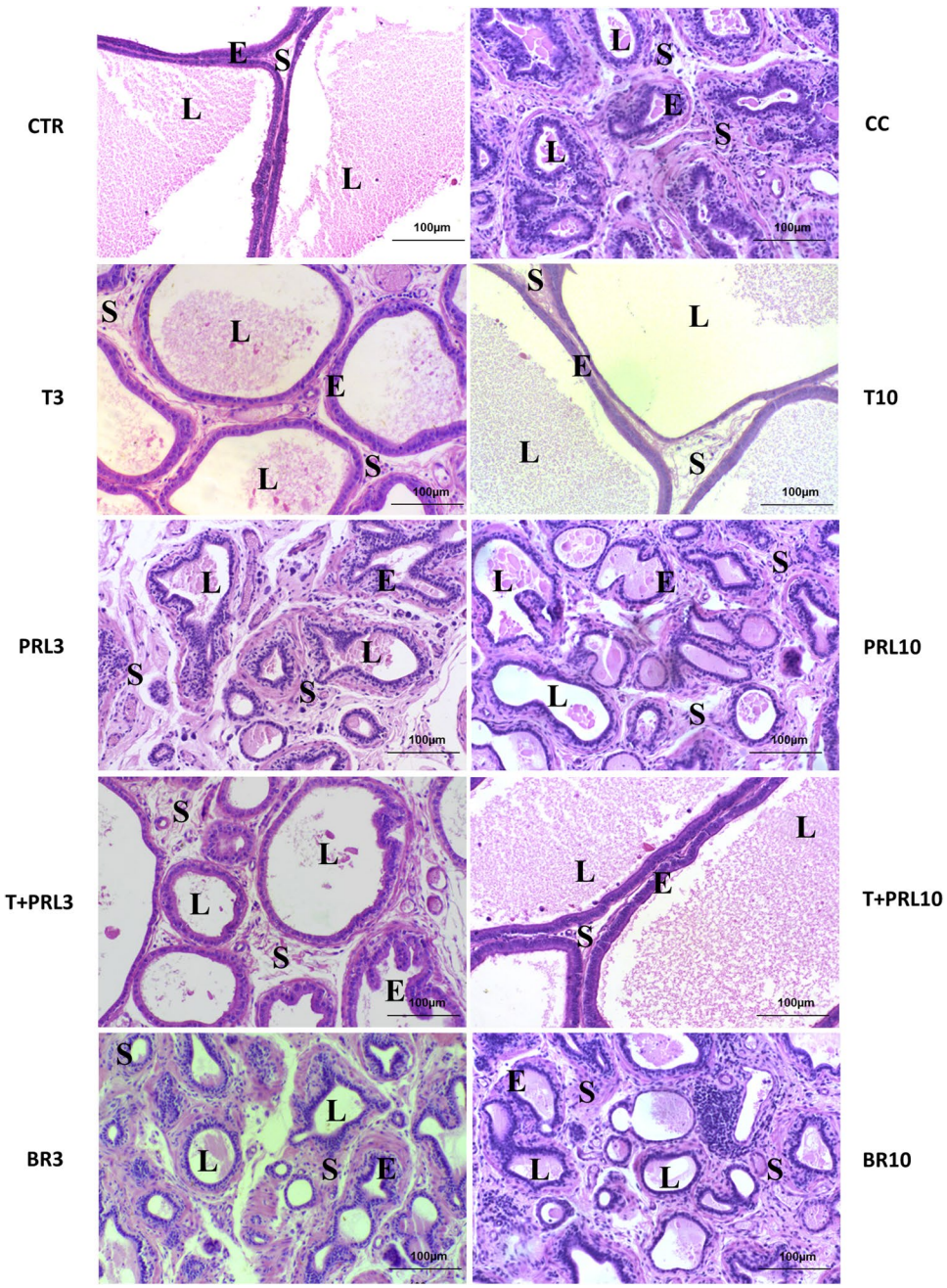

Fig. 1 Histological sections of the ventral prostate lobes from different experimental groups stained with hematoxylin-eosin. *CTR* control group, *CC* castrated control, *T3* 3 days testosterone treated group, *T10* 10 days testosterone treated group, *PRL3* 3 days prolactin treated group, *PRL10* 10 days prolactin treated group, *TPRL3* 3 days testosterone plus prolactin treated group, *TPRL10* 10 days testosterone plus prolactin treated group, *BR3* 3 days of bromocriptine treated group, *BR10* 10 days of bromocriptine treated group. *E* epithelium, *S* stroma, *L* lumen

Table 2 Morphological/stereological analysis of ventral prostate from different experimental groups

Parameters/groups	CTR	CC	T3	T10	PRL3	PRL10	TPRL3	TPRL10	BR3	BR10
Absolute volume (mm^3)	8.91 ± 5.4^a	1.96 ± 0.33^b	5.66 ± 0.88^c	15.10 ± 3.65^a	2.06 ± 0.15^b	2.00 ± 0.36^b	4.14 ± 0.63^c	15.00 ± 2.35^a	2.44 ± 0.32^b	1.72 ± 0.29^b
Epithelium	56.37 ± 8.9^a	1.43 ± 1.35^b	7.39 ± 1.41^b	61.81 ± 7.07^a	1.36 ± 1.03^b	1.26 ± 1.00^b	3.25 ± 1.20^b	44.02 ± 4.33^a	1.08 ± 0.65^b	0.65 ± 0.38^b
Lumen	5.18 ± 3	4.66 ± 1.07	5.66 ± 0.43	7.67 ± 4.30	4.69 ± 0.98	4.01 ± 0.74	5.14 ± 0.94	7.54 ± 0.80	6.05 ± 0.90	4.48 ± 0.60
Stroma	8.84 ± 0.53^a	3.99 ± 0.56^b	7.62 ± 0.39^a	9.11 ± 2.39^a	4.26 ± 0.54^b	4.33 ± 0.28^b	8.06 ± 1.73^a	11.31 ± 0.74^a	4.55 ± 0.51^b	4.16 ± 0.44^b
Epithelial height (μm)	8.84 ± 0.53^a	3.99 ± 0.56^b	7.62 ± 0.39^a	9.11 ± 2.39^a	4.26 ± 0.54^b	4.33 ± 0.28^b	8.06 ± 1.73^a	11.31 ± 0.74^a	4.55 ± 0.51^b	4.16 ± 0.44^b

Data are expressed as mean ± SD

Different letters indicate statistically difference with $p < 0.05$

CTR Control, *CC* castrated control, *T3* castrated plus testosterone for 3 days, *T10* castrated plus testosterone for 10 days, *PRL3* castrated plus prolactin for 3 days, *PRL10* castrated plus prolactin for 10 days, *TPRL3* castrated plus testosterone and prolactin for 3 days, *TPRL10* castrated plus testosterone and prolactin for 10 days, *BR3* castrated plus bromocriptine for 3 days, *BR10* castrated plus bromocriptine for 10 days

lower than that observed in the T and T+PRL groups treated for 3 days. Prolactin or bromocriptine did not induce changes in the number of proliferative epithelial cells in castrated rats (Fig. 2a). The Ki-67 index calculation confirmed the results of immunohistochemistry visualization (Fig. 2b).

Immunohistochemistry for AR demonstrated an intense reaction in the nuclei of epithelial cells in the CTR group. The number of stained cells and the intensity of reaction were reduced in castrated animals. Testosterone replacement progressively restored the AR staining in the VP in T3 and TPRL3 groups. In the T10 or TPRL10 groups, the AR staining was restored to the levels observed in the CTR group. The PRL or BR treatments did not affect AR detection by immunohistochemistry, which remained similar to that observed in the CC group (Fig. 3).

Western blotting

Western blot analysis demonstrated that castration increased intraprostatic prolactin receptor (PRLR) expression compared to that in the CTR group. Testosterone replacement for 3 or 10 days in castrated rats reduced the PRLR expression compared to that in the CC group. 3 days of prolactin administration to castrated rats decreased PRLR expression compared to that in the CC group. This value did not differ from those in the CC groups in the PRL10 group. There was a decrease in PRLR expression in castrated rats treated with the combination of prolactin and testosterone for both periods (TPRL03 or 10) compared to that in the CC group. Inhibition of pituitary prolactin release by bromocriptine led to a significant decrease in PRLR expression when compared to that in the CC group (Fig. 4a, b).

The STAT3 expression decreased in the CC group compared to that in the CTR group. The administration of testosterone alone or in combination with prolactin to castrated rats for 3 or 10 days increased the STAT3 expression compared to that in the CC groups. Prolactin treatment increased STAT3 expression compared to that in the CC group. Bromocriptine administered to castrated rats resulted in a transient increase of STAT3 expression compared to that in the CC group (Fig. 4a, b). The STAT5 expression was strongly reduced in the CC group compared to that in the CTR group. Treatment with T or T+PRL for 3 or 10 days increased the expression of STAT5 compared to that in the CC group. However, STAT5 was not affected by prolactin in castrated rats in both periods of treatment. In the same way, BR did not induce changes in STAT5 expression in castrated animals (Fig. 4a, c).

Confirming immunohistochemistry data, western blotting showed reduction in AR expression after castration and a progressive increased in the T and TPRL groups. Bromicriptine or prolactin administration to castrated rats did not alter AR expression (Fig. 5a, b). The expression of prostatein

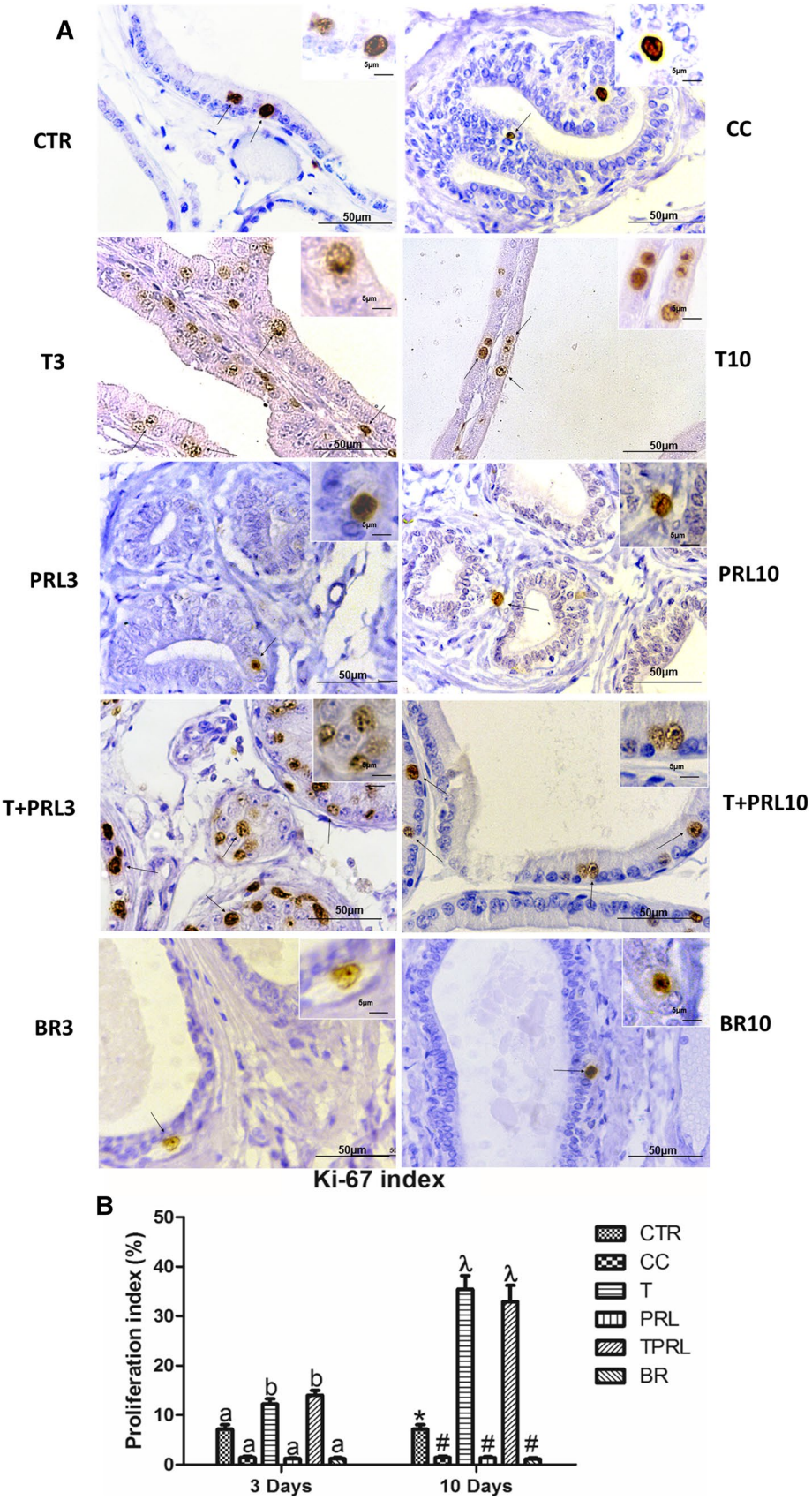

Fig. 2 **a** Representative immunohistochemistry of Ki-67 in the VP from different experimental groups. *Arrow* positive epithelial nuclei. **b** Graphics represent the percentage of positive cells for each marker in the VP from all experimental groups (n = 5/group). Data were expressed as mean ± SD. Different letters means statically differences with $p < 0.05$. *CTR* Control group, *CC* castrated control, *T3* 3 days testosterone treated group, *T10* 10 days testosterone treated group, *PRL3* 3 days prolactin treated group, *PRL10* 10 days prolactin treated group, *TPRL3* 3 days testosterone plus prolactin treated group, *TPRL10* 10 days testosterone plus prolactin treated group, *BR3* 3 days of bromocriptine treated group, *BR10* 10 days of bromocriptine treated group

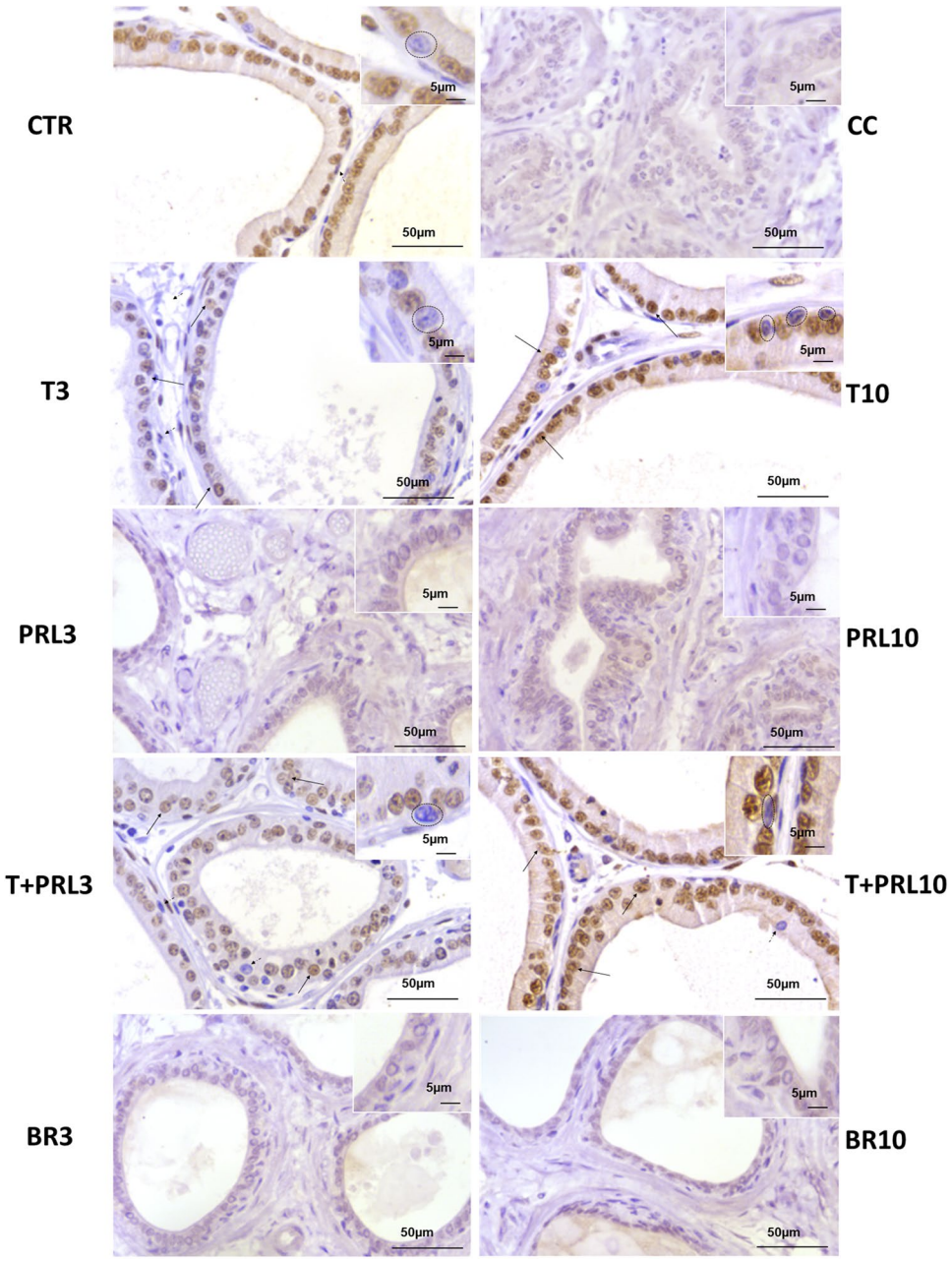

Fig. 3 Representative immunohistochemistry of AR in the VP from different experimental groups. *Arrows* positive epithelial nuclei, *arrowheads* positive stromal cells, *dotted circle* negative epithelial cell, *CTR* Control group, *CC* castrated control, *T3* 3 days testosterone treated group, *T10* 10 days testosterone treated group, *PRL3* 3 days prolactin treated group, *PRL10* 10 days prolactin treated group, *TPRL3* 3 days testosterone plus prolactin treated group, *TPRL10* 10 days testosterone plus prolactin treated group, *BR3* 3 days of bromocriptine treated group, *BR10* 10 days of bromocriptine treated group

(an androgen-induced secretory protein) was reduced after castration. Testosterone replacement for 03 days restarted prostatein synthesis, but only 10 days of testosterone administration alone or in combination with prolactin restored the prostatic secretory function. Prolactin or bromocriptine had no effect on prostatein expression (Fig. 5a, c).

Discussion

Prolactin is a peptide hormone secreted by the anterior pituitary. Although the pituitary is the major source of prolactin, it has been demonstrated that this hormone can be synthetized by extra-pituitary sources such as breast, decidua, prostate, and the brain, highlighting its involvement in paracrine and autocrine prolactin signaling, regulating functions, and influencing the development of disorders in these organs (Sethi et al. 2012; Sackmann-Sala et al. 2015). Prolactin has been linked to milk production and female infertility for a long time (Bernard et al. 2015), but this hormone also plays a key role in prostatic morphophysiology in both normal and disease conditions (Goffin et al. 2005). Prolactin acts via the prolactin receptor (PRLR) mainly at the epithelial cell membrane, regulating the uptake of zinc, citrate synthesis, and the expression

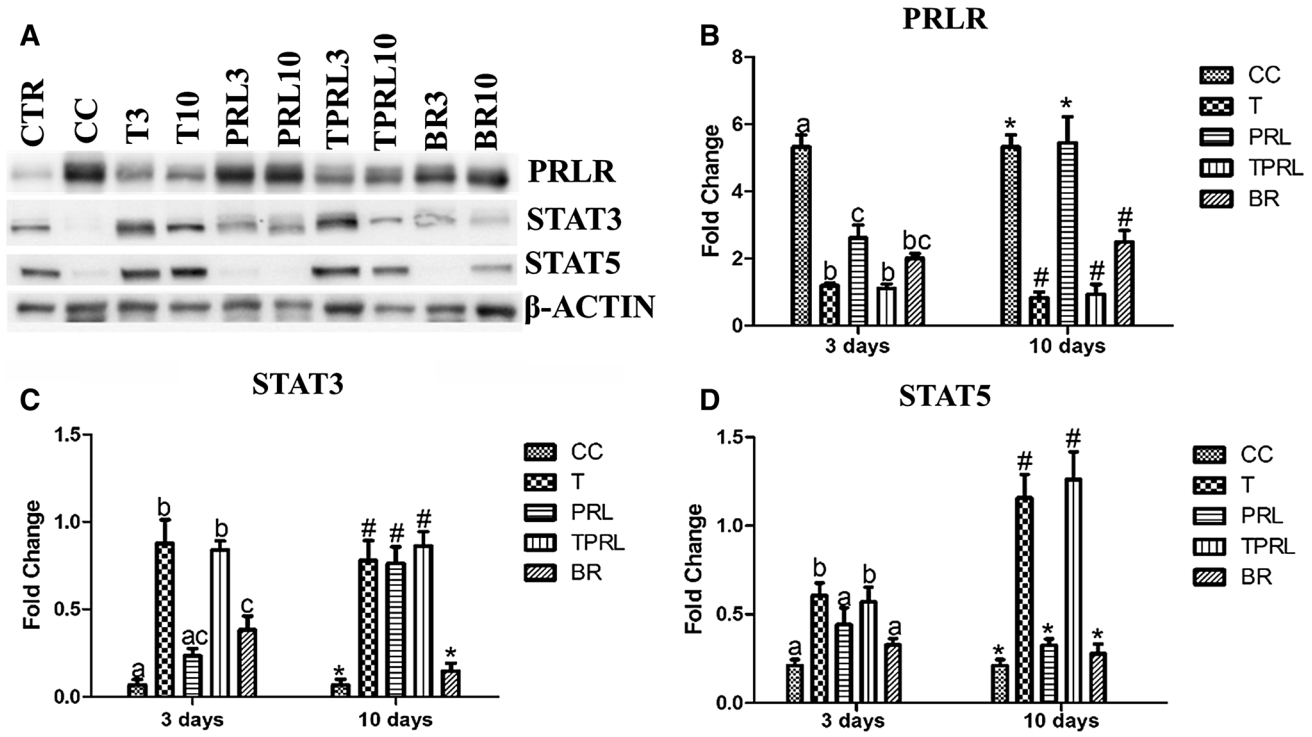

Fig. 4 **a** Representative western blotting analysis of PRLR, STAT3, and STAT5 in the rat ventral prostate from different experimental groups. **b, c** and **d** Graphics represent VP western blotting quantification (n = 6/group) of PRLR, STAT3 and STAT5, respectively. Data were expressed as mean ± SD. Different letters means statically differences among animals treated for 3 days and different symbols means statically differences among animals treated for 10 days, all with $p < 0.05$. Control group; *CC* castrated control, *T3* 3 days testosterone treated group, *T10* 10 days testosterone treated group, *PRL3* 3 days prolactin treated group, *PRL10* 10 days prolactin treated group, *TPRL3* 3 days testosterone plus prolactin treated group, *TPRL10* 10 days testosterone plus prolactin treated group, *BR3* 3 days of bromocriptine treated group, *BR10* 10 days of bromocriptine treated group

of the androgen receptor synergistically with androgens in the prostate (Nevalainen et al. 1996; Liu et al. 1997).

In our study, castration for 21 consecutive days induced a significant decrease in serum prolactin levels compared to that in the CTR group. There are conflicting data in the literature about the effects of castration on serum prolactin. Some studies demonstrated that serum prolactin level was increased in adult castrated rats (Euker et al. 1975). On the other hand, Shaar et al. observed a reduction in prolactin levels in both young and old castrated rats. Despite the divergent results, overall, our results pointed at a reduction of serum prolactin level after castration in adult rats (Shaar et al. 1975). On the other hand, 3 days of testosterone administration restored the serum prolactin levels to that observed in the CTR group. The prolactin administration alone or in combination with testosterone for 3 days also increased the serum prolactin level, but without statistical difference. As expected, treatment with bromocriptine decreased serum prolactin to undetectable levels. The administration of prolactin alone or in combination with testosterone did not seem to alter circulating levels of prolactin, although a tendency of a decrease compared to that in the group treated with testosterone alone. Our result can be explained by a short loop feedback, where prolactin itself acts in the brain to stimulate production of dopamine and thereby inhibit its own secretion (Rozenboim et al. 2004; Grattan 2015). In the group of animals treated for 10 days, no significant differences were observed between the experimental groups. Overall, our results demonstrated that exogenous prolactin administered alone down-regulates pituitary prolactin secretion, whereas testosterone appears to stimulate prolactin synthesis.

There was no change in the rat body weight among the experimental groups. Other studies from our group demonstrated no changes in body weights of castrated animals submitted to testosterone replacement (de Carvalho et al. 1997; Oliveira et al. 2007; Justulin et al. 2010). Administration of prolactin alone or in combination with testosterone or bromocriptine did not alter this parameter. Previous studies have reported a somatotrophic effect of exogenous prolactin administered to male rats during the period of 21–60 days of age, leading to body weight gain (Pérez-Villamil et al. 1992). These differences can be due to the short treatment period in our study, in addition to the different animal ages.

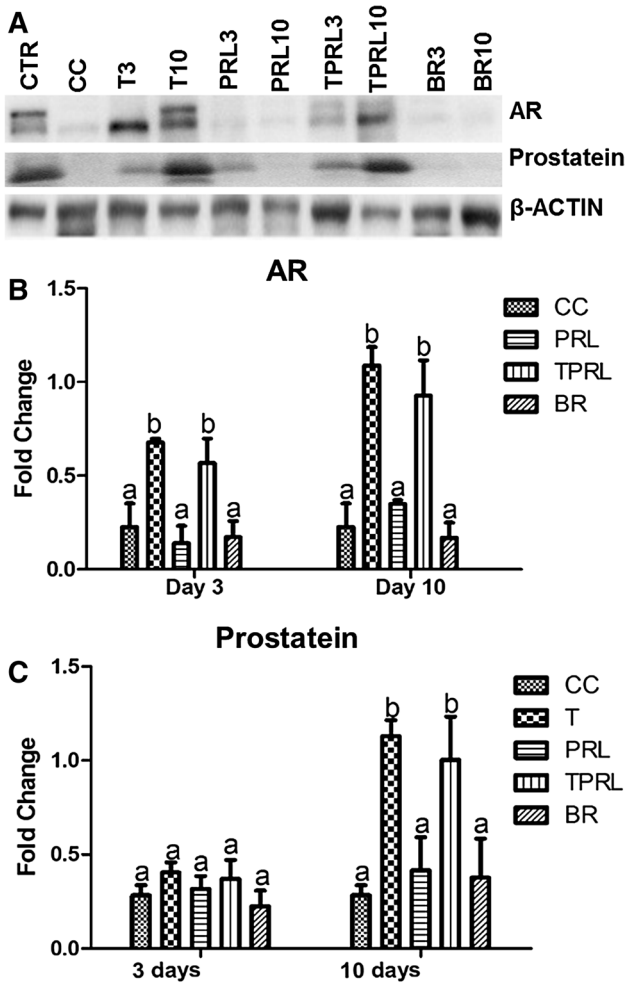

Fig. 5 **a** Representative western blotting analysis of AR and prolactin in the rat ventral prostate from different experimental groups. **b** and **c** Graphics represent VP western blotting quantification (n = 6/group) of AR and prostatein, respectively. Data were expressed as mean ± SD. Different letters means statically differences among animals treated for 3 days and different symbols means statically differences among animals treated for 10 days, all with $p < 0.05$. Control group; *CC* castrated control, *T3* 3 days testosterone treated group, *T10* 10 days testosterone treated group, *PRL3* 3 days prolactin treated group, *PRL10* 10 days prolactin treated group, *TPRL3* 3 days testosterone plus prolactin treated group, *TPRL10* 10 days testosterone plus prolactin treated group, *BR3* 3 days of bromocriptine treated group, *BR10* 10 days of bromocriptine treated group

The prostate is an accessory gland of the male genital system, whose development and secretory function are under androgenic control (Marker et al. 2003). The involution after castration and regrowth after testosterone replacement has been extensively used to study the kinetics of cell proliferation, apoptosis and stromal remodeling (Sandford et al. 1984; Marker et al. 2003; Justulin et al. 2006). Although the castration or testosterone replacement effects on prostate are well known (Sugimura et al. 1986; de Carvalho et al. 1997; Vilamaior et al. 2000; Justulin et al. 2010) in our study, these results were used to compare the effects of prolactin administration alone or in combination with testosterone on prostate morphophysiology. The prolactin administration to castrated rats for 3 or 10 days did not induce prostate regrowth or changes in glandular morphology, as promoted by androgen replacement. Other authors have demonstrated that chronic hyperprolactinemia induced in castrated rats did not affect VP regrowth, even after 30 or 60 days. Interestingly, Van Coppenolle et al. (2001) also described that intact (non-castrated) adult rats presented increased cell proliferation and VP growth after exposure to hyperprolactinemia for 30 or 60 days (Van Coppenolle et al. 2001). These results highlighted that, although prolactin is considered an anabolic hormone for prostate, in the absence of testosterone (castrated rats) prolactin administration is not sufficient to induce or sustain glandular regrowth.

Other authors demonstrated that administration of prolactin to intact (non-castrated) rats for a prolonged period (24 weeks) induced precancerous lesions in both dorsolateral and ventral (Herrera-Covarrubias et al. 2015). In a transgenic mouse model, which overexpress prolactin by epithelial cells, the sustained activation of STAT5 was associated with increased proliferation of prostatic basal/stem cells (Kindblom et al. 2003; Sackmann-Sala et al. 2015; Sackmann-Sala and Goffin 2015). Considering the involvement of stem cells in the prostate carcinogenesis, the authors related these results with initial stages of prolactin-induced prostate tumorigenesis.

It has been demonstrated that active sexual life produces a constant increase in serum prolactin levels, which is associated with the production of quantity and quality of prostatic semen to ensure fertilization (Rojas-Durán et al. 2015; Pascual-Mathey et al. 2016). In an experimental model of hyperprolactinemia induced by intraperitoneal prolactin injection or adenohypophysis transplantation under the renal capsule, Pascual-Mathey and coworkers demonstrated a sustained activation of the signaling pathway downstream to prolactin, involving its receptors, p-Stat3, and the MAPK pathway in VP of non-castrated rats, associating these results to the increase of synthesis and secretion of the prostatic fluid (Pascual-Mathey et al. 2016). In our experiment, castrated rats treated with prolactin for 3 or 10 days also demonstrated an increase of STAT3 expression. STAT5 also showed a tendency to be upregulated in both PRL groups, but without statistical difference, as observed by Pasqual-Mathey and coworkers (Pascual-Mathey et al. 2016). Overall, these results reinforce the important role of STAT3 in the response to prolactin in the rat VP.

Androgenic blockage followed by testosterone replacement has been used in order to elucidate the prostatic response to androgen and the relationship with the androgenic control of gene expression (Marker et al. 2003; Justulin et al. 2010). Moreover, other non-androgenic hormones and

growth factors also act in association with the androgenic signaling to sustain glandular morphophysiology; among them is prolactin (Ojo et al. 2015). Assimos and coworkers demonstrated a delay in the rate of prostatic regression in castrated rats immediately implanted with pituitary grafts under renal capsule and suggested that the prolactin response was not mediated through the androgen receptors in the prostate (Assimos et al. 1984). In this sense, the increase in prolactin levels observed in castrated animals can represent an adaptive response to the low levels of androgen and prolactin in order to reduce the prostatic involution in castrated rats. In spite of the increase in prolactin expression, there are no morphological evidences for a glandular response in castrated animals.

In our study, castration for 21 days led to a downregulation of both STAT3 and STAT5. This result is consistent with the reduction in the number of epithelial cells in the prostate after glandular involution in castrated rats. Although an increase in STAT signaling during PCa progression of androgen-independent tumors has been demonstrated (Wertz 2009; Zhou et al. 2015), we believe that, in contrast to androgen-independent cells, in the normal prostate, this effect does not occur, mostly because of the massive epithelial cell death after castration. In the T3 group, we observed the initiation of glandular recovery, however, without an increase of PRLR expression. The same result was obtained in the T10 group. Although we did not observe changes in PRLR expression in the T groups, the STAT3 and 5 protein expression was increased in both the T3 and T10 groups. Although there is evidence for prolactin acting synergistically with testosterone to stimulate prostate growth (Liu et al. 1997), our results demonstrate that testosterone does not directly regulate PRLR expression. Moreover, the combined administration of testosterone and prolactin did not affect PRLR expression, suggesting that androgenic signaling overlaps with the prolactin signaling pathway in prostate regrowth. These results are consistent with those of previous studies that demonstrated androgenic induction of STAT signaling in the renal cells (Liu et al. 2013). Overall, our data highlight that T-induced prostate regrowth involves pathways other than the canonical AR signaling.

Our results about AR expression in castrated prostate or after androgen replacement corroborate the previous published data (Vilamaior et al. 2000; Marker et al. 2003; Justulin et al. 2006). The AR expression in the VP from castrated rats was also not altered by prolactin administration. Moreover, there was no additional effect on AR expression by the combination of testosterone plus prolactin. Studies using VP organ cultures demonstrated that prolactin increased the nuclear uptake of [3H]DHT (Johansson 1976). Barañao and coworkers also observed an increase in nuclear androgen receptor content in the prostate of immature non-castrated rats receiving prolactin injections during early puberty (Baranão et al. 1981). On the other hand, Prins and coworkers demonstrated an increase in nuclear AR expression in the lateral prostate of castrated adult rats that underwent pituitary implants and testosterone replacement (Prins 1987). Thus these authors proposed that prolactin induced lateral prostatic growth by increasing nuclear AR levels optimizing prostatic response to circulating testosterone. Gomés and coworkers showed an increase in the number of AR-positive cells after treatment with prolactin in non-castrated rats (Gómez et al. 2009). Despite the divergent data in the literature, our results pointed out that in the absence of T, prolactin per se is not able to modulate AR expression. Additionally, there were no changes in prostatic AR expression in castrated rats treated with bromocriptine, corroborating that neither low nor high levels of prolactin interfered with the ventral prostate AR expression in the absence of detectable levels of circulating androgen.

In order to analyze whether prolactin interferes with the prostate secretory function in castrated animals, we evaluated the prostatein expression, an androgen-dependent protein secreted by VP (Santos et al. 2014). In our study, castration significantly reduced the prostatein expression, whereas the testosterone replacement restored the prostatein synthesis after 10 days of treatment. Similar results were observed by Fujimoto and coworkers in the VP of castrated rats submitted to testosterone replacement (Fujimoto et al. 2009). The prolactin administration to castrated rats had no effect on prostatein expression. This result demonstrates that prolactin does not exert stimulatory effects on prostate secretory functions in the absence of testosterone. On the other hand, Reiter and colleagues demonstrated that the administration of prolactin (10 µg) for 7 days to castrated and hypophysectomized adult rats induced prostatein expression in the VP (Reiter et al. 1995). These authors emphasize that prolactin regulates the gene expression of prostatic secretory proteins independently of androgen. In our study, we did not evaluate the gene expression of prostatein, however, our set of results demonstrate that prolactin administered to castrated rats has no effect on prostate secretory functions.

Conclusion

Prolactin administered at 0.3 mg/kg for 3 or 10 consecutive days to castrated rats did not exert anabolic effect on the VP. Although we observed an activation of downstream prolactin signaling, this was not enough to overcome the prostatic androgen deficiency. Likewise, there was no additional glandular involution in the castrated group treated with bromocriptine. Thus, we conclude that despite stimulating downstream signaling pathway, exogenous prolactin does not act on VP in the absence or presence of high levels of testosterone.

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