Effects of castration on the expression of the NGF and TrkA in the vas deferens and accessory male genital glands of the rat

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Nerve growth factor (NGF) is a member of the neurotrophin family. Neurotrophins exert their effects by binding to corresponding receptors, which are formed by the tyrosine protein kinases TrkA, TrkB, and TrkC, and the low affinity p75NTR receptor. The role of neurotrophins in the biology of male genital organs is far from clear. In particular, little is known about the influence of sex hormones on the expression of neurotrophins and their receptors. In the present study, using immunohistochemistry and real time RT-PCR, we investigated the expression of NGF and TrkA in the vas deferens and accessory male genital glands in normal and castrated rats.

In normal rats, both NGF- and TrkA-immunoreactivities (IR) were localized in the epithelial layer of the vas deferens. NGF-IR was also found in the stroma and epithelium of the vesicular gland and prostate. TrkA-IR was distributed in the epithelial cells of vesicular and prostate glands. The nerves were weakly immunoreactive in all the examined organs. After castration the immunoreactivities increased. Real-time RT-PCR experiments indicated that NGF and TrkA mRNA levels increased significantly after castration. These results suggest that NGF and TrkA are expressed in the internal male genital organs of the rat and that their expression is downregulated by androgen hormones. We hypothesize NGF and TrkA play a role in the processes that regulate the involution of these organs under conditions of androgen deprivation.

Key words: androgen hormones, stromal cells, immunohistochemistry, real-time RT-PCR, prostate.

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In the prostate, NGF and NGF precursor have been immunohistochemically localized in the glandular epithelium, suggesting that secretory epithelial cells are the site of production of this factor (Shikata et al., 1984; MacGrogan et al., 1991; Paul et al., 1996). Paracrine neurotrophin synthesis by stromal cells has also been postulated (Pflug et al., 1995; Dalal and Djakiew, 1997; Weeraratna et al., 2000). High- and low-affinity neurotrophin receptors have been recognized in the nerves and epithelial cells of the prostate (Weeraratna et al., 2000; Graham et al., 1992; MacGrogan et al., 1992; Paul and Habib, 1998; Guate et al., 1999), thus indicating that neurotrophins play a role as growth-regulating factors in this gland.

The exact role of neurotrophins in the biology of male genital organs, however, is far from clear. Recently, in the vas deferens and accessory male genital glands of the rat, the expression of the BDNF and its receptors (TrkB and p75NTR) has been reported to be regulated by androgen hormones (Mirabella et al., 2006; Mirabella et al., 2008). In castrated rats, moreover, BDNF has been hypothesized to regulate, via interacting p75NTR, the castration-induced regression of the sympathetic innervation (Mirabella et al., 2006).

The present study has, therefore, been undertaken to elucidate the presence and localization of NGF and TrkA in the vas deferens and accessory male genital glands of the rat. In addition, the expression of these proteins and their mRNAs have been determined after castration in order to evaluate whether this neurotrophin and its specific receptor are under the control of androgens.

### Materials and Methods

#### Rats, surgery and tissue removal

A total of 30 adult male Sprague-Dawley rats (13 weeks of age; body weight: 380±20 g; purchased from Harlan Italy) were used. They were housed in temperature- and light-controlled rooms and were given water ad libitum. Rats received humane care and the study protocol was in compliance with our institution’s ethical guidelines. All procedures were approved by Italian laws regarding animal use in research (art. 7 D.Lgs. 116/92). Fifteen rats were left intact and served as controls. Fifteen rats were castrated at 9 weeks of age (body weight: 300±20 g) and allowed to recover over the following 4 weeks. All surgical procedures were carried out aseptically under anaesthesia induced with urethane (1.2 g/kg). Vas deferens, vesicular and coagulating glands and dorsal and ventral prostate were removed from normal (control) and castrated rats after they had been anaesthetized and then killed. Specimens from five normal and five castrated rats were used for immunohistochemistry. Each specimen collected from the remaining ten castrated and ten intact rats was used for real-time reverse transcription/polymerase chain reaction (real-time RT/PCR) procedures.

#### Immunohistochemistry

The specimens were immediately fixed by immersion in Bouin’s fixative (6-24 h), processed for paraffin embedding in vacuum and cut at a thickness of 5-7 µm. The avidin-biotin-peroxidase complex (ABC) method was performed with the Vectastain ABC kit (Vector Laboratories, Burlingame, Calif., USA). Sections were deparaffinized in xylene and hydrated in a graded solution of ethanol. After the quenching of endogenous peroxidase activity in water containing 0.3% hydrogen peroxide for 30 min, nonspecific binding was blocked by treatment with 1.5% normal goat serum (Vector) in 0.01 M PBS (phosphate buffered saline; pH 7.2) for 30 min. Polyclonal rabbit anti-NGF (sc-549; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and -Trk A (sc-118; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were singularly applied to the sections at a dilution of 1:500 and each specimen was incubated in a moist chamber overnight at 4°C. After the sections had been washed three times in PBS, biotinyalted anti-rabbit IgG (Vector) was applied at a dilution of 1:200. The sections were again incubated for 30 min at room temperature. Freshly prepared ABC reagent (Vector) was applied and incubated for 30 min after three washes in PBS. The localization of NGF- and TrkA-immunoreactivities were visualized by incubating the sections for 5 min in freshly prepared diaminobenzidine-nickel solution (Vector). The specificity of the immunoreactions was tested by replacing the primary antibody with buffer. No immunoreaction was detected in control tests. The specificity of the primary antibody anti-NGF and anti-TrkA were tested by adsorption the primary
antibody with excess (up to 50 µg/mL in the final dilution) homologous antigen peptide (NGF peptide sc-549P; TrkA peptide sc-118P Santa Cruz Biotechnology). Five slides for each rat and for each control or castrated tissue were independently evaluated by two observers by using a Leica DMRA2 microscope.

RNA extraction and cDNA synthesis

RNA extraction was prepared from tissues collected from normal (n=10) and castrated (n=10) rats. Fresh tissues were immediately frozen on dry ice and stored at -80°C until extraction. Total RNA from tissues was prepared by using TRI-reagent (Sigma, St Louis, Mo., USA) according to the manufacturer’s protocol. Complementary DNA was synthesized with the cMaster RT kit (Eppendorf, Westbury, New York, USA) and random hexamers as primer.

Real-time RT-PCR

Primers specific for rat NGF and TrkA were designed from the published gene sequences (NGF gene, Genbank, XM227525; TrkA gene, Genbank, NM 021589) using the Primer Express™ software (PE Applied Biosystems). The sense and anti-sense NGF primers used were 5'-TGC ATA GCG TAA TGT CCA TGT TG-3' and 5'-CTG TGT CAA GGG AAT GCT GAA -3', respectively, which amplify a 148-bp fragment. The sense and anti-sense TrkA primers used were 5'-CAT GAC ACT GGG TGG CAG TT -3' and 5'-TCC CCT AGC TCC CAC TTG AGA -3', respectively, which amplify a 149-bp fragment. The sense and antisense Actin primers used were 5'- CGT GAC ATT AAA GAG AAG CTG TGC-3' and 5'-TAG TTT CAT GGA TGC CAC AGG AT -3', respectively, which amplify a 205-bp fragment (Genbank, NM 031144).

The PCR conditions were 50°C for 2 min and 94°C for 10 min, followed by 40 cycles of 94°C for 15 min and 60°C for 1 min. PCRs contained 1 µL cDNA (40 ng/well), 24 µL of Master Mix Sybr Green (Applied Biosystems) containing specific primers. A sample without cDNA template was used to verify that the master mix was free from contaminants. The samples were performed in triplicate. The β-Actin gene was also amplified in separate tubes under the same conditions as an active endogenous reference to normalize quantification of mRNA target. Real-time PCRs for target and reference genes were run in the same RT reaction.

Real-time detection was performed on an ABI-PRISM 7300 Sequence Detection System (Applied Biosystems) and data from SYBR Green I PCR amplicons were collected with ABI 7300 System SDS Software. The fluorescence signal baseline and threshold were set manually for each detector (NGF, TrkA, β-actin), generating a threshold cycle (Ct) for each sample. An amplification plot graphically displayed the fluorescence detected over the number of cycles that were performed. Standard curves for both targets and the endogenous reference gene, created on the basis of the linear relationship between the Ct value and the logarithm of the starting amount of cDNA, showed acceptable slope values (included between -3.8 and -3.3). Standard curves were obtained by using serial dilutions of sample cDNA (1:2, 1:4, 1:8, 1:16, 1:32).

Relative quantification method 2-[Delta][Delta]Ct (2-ΔΔCt) was used for normalization of gene expression (Huggett et al., 2005; Livak and Schmittgen, 2001; Yuan et al., 2006) before using the ΔΔCt method for relative quantification (comparative method), a validation experiment was required in order to demonstrate that the efficiency of the target amplification and that of the reference amplification were almost equal. All PCR efficiencies were measured and found to be adequate (slope <0.1).

The difference between Ct values was calculated for each mRNA by taking the mean Ct of triplicate reactions and subtracting the mean Ct of triplicate reactions for the reference RNA measured on an aliquot from the same RT reaction (ΔCt = Ct(target gene) - Ct(reference gene)). All samples were then normalized to the ΔCt value of a calibrator sample to obtain a ΔΔCt value (ΔCt(target) - ΔCt(calibrator)).

The calibrator, defined as the sample used as the basis for comparative results, could represent an untreated control i.e. normal rats. Normal rats were chosen as calibrators. For the comparative method, relative quantifications were calculated in relation to the concentrations of the calibrator sample (2^-ΔΔCt), expressed in arbitrary units and normalized to the endogenous reference gene (β-actin).

Therefore, by using the 2^-ΔΔCt method, data were recorded as the fold-change in gene expression normalized with the endogenous reference gene and relative to the calibrator sample (Huggett et
Dissociation melting curves confirmed the specific amplification of the cDNA target and the absence of nonspecific products. The results are represented as mean (±SEM) of triplicate determinations for each tissue and from each experimental animal.

For statistical analysis, the data were expressed as the mean ± standard error. Significant differences in NGF and TrkA mRNA levels between the calibrator sample (normal rats) vs castrated rats were determined by using Student's t-test for independent samples. The level of statistical significance was set at p<0.05 for all.

**Results**

**Immunohistochemistry**

The results of NGF and TrkA immunoreactivities are summarized in Table 1.

**NGF- immunoreactivity**

In the vas deferens of normal rats (Figure 1a), NGF-IR was found in the ductal epithelium. After castration (Figure 1b), the immunoreactivity persisted in the epithelium in which it was confined to the apical border of epithelial cells. In the vesicular gland of normal rats (Figure 1c), NGF-IR was prevalently localized in the fibromuscular cells. After castration (Figure 1d), NGF-IR increased in the fibromuscular cells. In both the dorsal and ventral (Figure 1e, h) normal prostatic lobes, NGF-IR was found in the stroma surrounding the tubuloalveoli. In the stroma, immunoreactivity was localized in elongated spindle-shaped fibromuscular cells. In only few cases NGF-IR was found in epithelial cells (Figure 1f). After castration (Figure 1g, i), NGF-IR increased in the stromal tissue. The nerves were weakly immunoreactive in all the examined organs (data not shown).

**TrkA- immunoreactivity**

In the vas deferens of normal rats (Figure 2a), TrkA-IR was found in the columnar secretory cells of the ductal epithelium. After castration (Figure 2b), the immunoreactivity persisted in the epithelial cells. In the vesicular gland of normal rats (Figure 2c,d) TrkA-IR was located in secretory epithelial cells. After castration immunoreactivity increased in the secretory epithelial cells (Figure 2e). In both the dorsal (Figure 2f) and ventral (Figure 2h) prostatic lobes, TrkA-IR was observed in basal cells of the alveolar epithelium. Cells expressing TrkA were flat or cubical and located at the epithelial- stromal junction and did not reach the lumen of the alveoli. After castration (Figure 2g, h) immunoreactivity increased in the basal cells.

**Real-time RT-PCR**

Real-time RT-PCR experiments showed that the level of NGF mRNA expression increased significantly in the vesicular gland and dorsal and ven-

Table 1. Distribution of NGF (a) and TrkA (b) immunoreactivities in the vas deferens and accessory male sex glands of normal and castrated rats (b.e.c. basal epithelial cells, s.e.c. secretory epithelial cells, n. nerves, f.s.c. fibromuscular stromal cells + low density, ++ medium density, +++ high density).

|                | Vas deferens | Vesicular gland | Dorsal prostate | Ventral prostate |
|----------------|--------------|-----------------|-----------------|-----------------|
|                | normal       | castrated       | normal          | castrated       | normal          | castrated       |
| b.e.c.         | ++           | ++              | +               | ++              | +               | +               |
| s.e.c.         | ++           | ++              | ++              | +++             | ++              | +++             |
| f.s.c.         | +            | +               | +               | +               | +               | +               |
| n.             | +            | +               | +               | +               | +               | +               |

|                | Vas deferens | Vesicular gland | Dorsal prostate | Ventral prostate |
|----------------|--------------|-----------------|-----------------|-----------------|
|                | normal       | castrated       | normal          | castrated       | normal          | castrated       |
| b.e.c.         | ++           | ++              | ++              | +++             | +               | +++             |
| s.e.c.         | ++           | ++              | ++              | +++             | +               | +++             |
| f.s.c.         | +            | +               | +               | +               | +               | +               |
Figure 1. Distribution of NGF-immunoreactivity in the vas deferens (a, b), vesicular gland (c, d) and dorsal (e-g) and ventral (h, i) prostatic lobes of normal (a, c, e, f, h) and castrated rats (b, d, g, i). Ep epithelium, Mu tunica muscularis, Str stroma, Sm tela submucosa, arrow immunoreactive stromal cells, arrowheads immunoreactive epithelial cells. NGF-IR was found in the epithelium of the vas deferens (a) and glands (c, e, f, h) and in the stromal cells of the prostate (c, e, h). After castration, the immunoreactivity persisted in the epithelium and increased in the stromal cells (b, d, g, i). Scale bars 50 µm.
Figure 2. Distribution of TrkA-IR in the vas deferens (a, b), vesicular gland (c, d, e) and dorsal (f, g) and ventral (h, i) prostatic lobes of the normal (a, c, d, f, h) and castrated (b, e, g, i). Ep epithelium, Mu tunica muscularis, Str stroma, Sm tela submucosa, Av tubuloalveoli arrow immunoreactive epithelial cells, arrowheads nerves. TrkA-IR was found in the secretory epithelial cells of the vas deferens (a) and vesicular gland (c, d) and in the basal cells of the prostate (f, h). After castration the immunoreactivity persisted and it increased in the epithelium of the vas deferens (b) and glands (e, g, i). Scale bars 50 μm.
tral prostatic lobes of the castrated rats (Figure 3). The level of TrkA mRNA expression also increased significantly in the vesicular gland and dorsal and ventral prostatic lobes of the castrated rats (Figure 4).

In the vas deferens, the difference in the levels of NGF and TrkA mRNA expression between castrated and normal rats were not statistically significant (Figure 3, 4).

Discussion

The results of the present study demonstrate that NGF and TrkA are expressed in the vas deferens and accessory male genital glands of the rat. The presence of mRNAs of these proteins suggests in situ synthesis of the neurotrophic factor and its receptor.

NGF-IR was distributed in the epithelium of the vas deferens and genital glands and in the stromal cells of the vesicular gland and of the prostate. In particular NGF-IR was localized in secretory epithelial cells of the vas deferens and of glands and in the fibromuscular stromal tissue of the glands. The present results are in accordance with previous reports in which NGF-IR was observed in secretory epithelial cells of the adult male rat vesicular and prostate glands (Li et al., 2005), and in the Japanese monkey (Jin et al., 2006) and guinea pig prostate (MacGrogan et al., 1991; Shikata et al., 1984). These results suggest that secretory epithelial cells are the site of production of this factor (Harper et al., 1979; Shikata et al., 1984; MacGrogan et al., 1991; Paul et al., 1996). On the other hand, the localization of NGF-IR in the stromal cells of the prostate is consistent with previous findings reporting NGF-IR in cultured prostate stromal cells (Djakiew et al., 1991; Dalal and Djakiew, 1997). In addition, the localization of NGF-IR was reported in the human normal and adenocarcinoma prostate stromal tissues in vivo (Graham et al., 1992).

TrkA-IR was distributed in the epithelium of both vas deferens and glands. In particular TrkA-IR was found in the secretory cells of the vesicu-
lar gland and in the basal cells of the prostate. These results are in accordance with previous reports, in which immunoreactivity of TrkA was observed in secretory epithelial cells of the seminal vesicles of the adult male rat (Li et al., 2005) and in the basal epithelial cells of the human normal prostate (Guate et al., 1999). However, our findings disagree with those reported in the rat prostate where TrkA-IR was localized in secretory but not in basal epithelial cells (Li et al., 2005). The localization of TrkA in basal epithelial cells, i.e. the proliferating cells, suggests that TrkA specific ligands might regulate cell turnover in the epithelium of the rat prostate gland. NGF-IR was observed in the stromal fibromuscular cells of the prostate gland thus suggesting that NGF produced by muscular tissues interacts via a paracrine mechanism with TrkA receptor on the adjacent basal epithelial cells in regulating epithelial cellular growth. In the prostate gland, the presence of both NGF and TrkA immunoreactivities in epithelial cells supports the hypothesis that NGF of epithelial source influences in an autocrine/paracrine manner the cellular growth and secretory activity of the prostatic epithelium. NGF is one autocrine/paracrine factor regulating prostate function (Harper et al., 1979; Djakiew et al., 1991; Graham et al., 1992; Macgrogan et al., 1992; Paul et al., 1996). Prostatic growth and differentiation are regulated by androgens and other autocrine and paracrine factors secreted by both epithelial and stromal cells (Cunha, 1994). Alterations in these regulatory pathways may contribute to the initiation and progression of prostate cancer (Cunha, 1994; Djakiew et al., 1990; for reviews Geldof et al., 1998; Kruttgen et al., 2006).

NGF and TrkA mRNAs was also present in all the examined tissues as demonstrated by results of the real-time RT-PCR experiments. Previous studies indicated the presence of NGF mRNA in the guinea pig prostate (MacGrogan et al., 1991, 1992).

One of the aims of the present study was to evaluate if and how castration influences the expression of the NGF and TrkA in the rat vas deferens and accessory male genital glands. Castration caused an increase of NGF-IR in stromal fibromuscular cells and of TrkA-IR in epithelial cells. Moreover, the results of real-time RT-PCR demonstrated that NGF and TrkA mRNA increased in both vesicular and prostate glands in castrated rats. These results suggest that androgen hormones negatively influence the expression of NGF and its receptor in male rat accessory male genital gland tissues. In the vas deferens, although the data were not statistically significant, castration produces a decrease of NGF and TrkA mRNA level expression. This discrepancy may reflect differences occurring among male genital tissues in the regulation of NGF and its receptor expression by androgen hormones.

These results are in contrast to those reported for NGF expression in several androgen target tissues. In the guinea pig prostate, castration has been reported to have no effect on NGF mRNA expression estimated by RNA blot hybridization analysis (MacGrogan et al., 1991). In addition, NGF has been reported to be markedly upregulated by androgens in mouse brain regions (Katoh-Semba et al., 1994) and submandibular salivary glands (Humpel et al., 1993). However, recently, we have observed that castration induced an increase of the expression of BDNF, another member of the neurotrophin`s family, in the accessory male genital glands of the rat (Mirabella et al., 2006). NGF- and TRKA-IRs were observed to be poorly distributed in the nerves, and, moreover, they were unaffected by castration. This result is in contrast to what reported for BDNF-IR (Mirabella et al., 2006) which strongly increased in the nerves after castration. The increase BDNF-IR after castration was addressed to a role played by this neurotrophin in regulating the castration-induced regression of sympathetic nerves. The increase of P75NTR-IR in the nerves after castration corroborates this hypothesis (Mirabella et al., 2008). In the present study, we have observed an increase in NGF and TrkA expression in stromal and epithelial cells but not in the nerves. These results suggest that NGF and its receptor are involved in regulating the apoptotic processes occurring in the sex gland epithelial tissue after castration rather than the regression of sympathetic nerves. This suggests that different neurotrophins play different roles in regulating the involutive processes which occur in male genital tissues after castration.

Several studies have reported that NGF induces apoptosis in some condition rather than trophic effects. NGF-mediated apoptosis is cell-cycle dependent and NGF/TrkA signal transduction...
pathway could activate apoptotic cell death programmes in CNS neuroepithelial progenitor cells and in childhood brain tumours (Muragaki et al., 1997). In human neuroblastoma, an elevated level of TrkA mRNA was associated with increased apoptosis in tumor cells (Nakagawara et al., 1993). In rat pheochromocytoma cell line PC12, NGF via TrkA induces apoptosis following serum deprivation (Ahn et al., et al., 2005). Although NGF and neurotrophins are primarily known for their ability to promote the proliferation, survival and maturation of target cells, these studies suggest that NGF can be cytotoxic depending on the culture condition or nature of cell type.

In conclusion, the results of the present study have shown that the expression of NGF and TrkA are expressed in the rat vas deferens and accessory male genital glands and that expression of these proteins are probably regulated by androgen hormones. In particular, expression of NGF and its receptor TrkA is increased in these organs after castration, thus suggesting a role for NGF in the apoptotic processes that regulate the involution of these organs under conditions of androgen deprivation.

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