

Immunohistochemical Expression of No-synthase Constitutive Isoforms (cNOS) in Mammals Normal Prostate and in Human Prostatic Obstructive Hyperplasia

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Abstract: The aim of present study is to investigate the immunohistochemical identification of cNOS-containing prostatic structures in various species and in hyperplastic human prostate. We used specimens of rat, guinea pig and hamster. Human prostatic specimens were removed from patients suffering of prostatic hyperplasia. The study was processed by ABC method. Immunohistochemical staining with anti nNOS antibody revealed the existence of strong reactivity in numerous ganglionic cell bodies and varicose nerve fibers in the fibromuscular stroma, in smooth cells around acini and in blood vessels endothelium. Surprisingly nNOS immunoreactivity appears in the glandular epithelium. In the hyperplastic prostate the nitroxidergic innervation of stroma and glandular epithelium is almost absent. These data provide a morphological evidence for a segmental differentiation of NOS-containing prostatic structures and consequently suggest that in the prostate NO plays selective neural and paraneural roles in the control of blood flow, smooth-muscle tone (relaxation) and secretory function. Further immunohistochemical studies are carrying out to elucidate the prostatic role in aging, after hormonal perturbation and pathological processes.

Key words: Nitric oxide synthase, prostatic hyperplasia, fibromuscular stroma, smooth muscle cells, glandular epithelium

INTRODUCTION

An increasing volume of physiological, pharmacological and histochemical studies in animals and humans indicate that Nitric Oxide (NO), always considered in the atmosphere a simple noxious gas, represents, in the human body, a remarkable endogenous messenger involved in several processes of cell-communication, which plays a key role in the functions of several organs in physiological and pathological conditions (Bredt and Snyder, 1990, 1992; Burnett *et al.*, 1992, 1995, 1997; Dokita *et al.*, 1994; Jen *et al.*, 1996; Moncada *et al.*, 1991; Persson and Andersson, 1992; Pollock *et al.*, 1991; Smet *et al.*, 1994; Stuehr *et al.*, 1990; Takeda *et al.*, 1995; Tessitore, 1998). It is produced through the oxidation of L-arginine by a family of charming isoenzymes NO Synthase (NOS): Two of these isoforms (nNOS and eNOS) are constitutive (Bredt and Snyder, 1990; Pollock *et al.*, 1991) and produce *small quantities* (picomoles) of NO; the third (iNOS) expressed by induction by cytokine, TNF and bacterial endotoxins (LPS) produces *big quantities* (nanomoles) of NO (Stuehr *et al.*, 1990).

Our previous reports indicate, in agreement by other authors, that NO occurs in male and female genitourinary

organs in various animal species (Smet *et al.*, 1994; Tessitore *et al.*, 1996). Thus it widely appears that NO plays a crucial role in the neurogenic relaxation in the corpus cavernosum and consequently in penile erection (Burnett *et al.*, 1992; Tessitore *et al.*, 1994). Immunohistochemical studies in human prostatic tissues have received relatively little attention. Burnett *et al.* (1997) was the first to identify the presence of NOS in the human prostate. The aim of present study is to investigate the immunohistochemical identification of cNOS-containing prostatic structures in various species including humans, to explain the NO role in this gland. Furthermore the prostatic NOS immunohistochemical mapping provides morphohistochemical basic knowledge to understand prostatic clinical alteration and related potential therapeutical application.

MATERIALS AND METHODS

We have studied the immunohistochemical distribution of cNOS using rabbit polyclonal antibodies against bNOS and mouse monoclonal antibodies against eNOS. Twenty human prostatic specimens were collected during prostatectomy in patients suffering from prostatic

hyperplasia. Thirty mammalian (rat, guinea pig, hamster) were treated according to the Convention of Helsinki on the utilization of animals in biomedical research. They were sacrificed by means of terminal anaesthesia (50 mg kg^{-1} of endoperitoneal Nembutal) and after were removed a prostatic specimens. All the specimens were fixed in Bouin's mixture. After Bouin fixation the tissue was dehydrated in a graded series of alcohols, cleared in xylene and embedded in paraffin. Sections of 8μ were cut on to Leica microtome RM2145, dried overnight at 37°C and then stored at room temperature. On the day of the experiment, slides were dewaxed and rehydrated by sequential immersion in a graded series of alcohols. Slides were then transferred into water for 5 min; for the endogenous peroxidase inhibition slides were treated with 3% hydrogen peroxide in hydrated incubation enclosure at RT. After, the slides were transferred in PBS buffer (PBS: Na_2HPO_4 , KH_2PO_4 , KCl, NaCl pH 7.4 - 7.6) at RT.

The following protocol was used with the kit ULTRASTAIN POLYVALENT STREPT ABC-HRP YLEM code AFN600: After rinsing with PBS buffered, the tissue sections were blocked with Super Block reagent (normal goat serum in phosphate buffered saline containing carrier protein) for 10 min and then rinsed with PBS for 4 min.

Anti nNOS (Transduction laboratories code N31030), anti eNOS (Transduction laboratories code N30020) were then added at $10 \mu\text{g mL}^{-1}$ in PBS with BSA and incubated overnight at 6°C .

After the incubations any excess antibodies was removed by washing, the slides were rinsed 2X with PBS, 5 min each. Next we have added the Ultrastain Polyvalent Antiserum (biotinylated goat anti-mouse IgG and goat anti-rabbit IgG in PBS) for 10 min at RT, unbound antibody was removed by washing (2X with PBS, 5 min each) and subsequently we have applied Ultrastain Streptavidin for 20 min at RT. After incubation unbound enzyme was removed by rinse procedure (2X with PBS, 5 min each). The chromogenic development reagent, AEC chromogen in substrate buffer (3-amino-9-ethyl carbazole in N,N dimetilformamide) was then added for 5-15 min and we have stopped in DI water. We have removed slides from the water and we have applied one drop of aqueous mounting medium containing 15 mM sodium azide (DAKO Faramount) to the tissue and we have applied a coverslip.

Negative controls were performed by omission of primary antibody and by absorption control on the primary antibody with the purified antigen (Syntetic peptides eNOS (Transduction laboratories code N30020) and nNOS (Transduction laboratories code N31030).

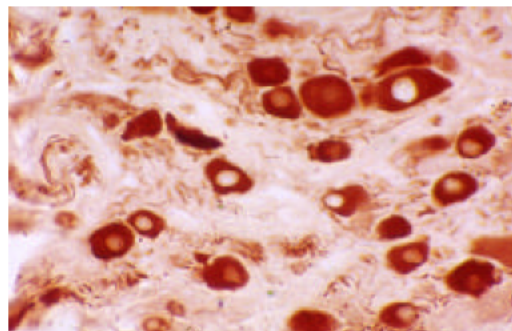


Fig. 1: Hamster prostate: Numerous ganglionic cell bodies and nerve fibers intensely nNOS immunoreactive 400x

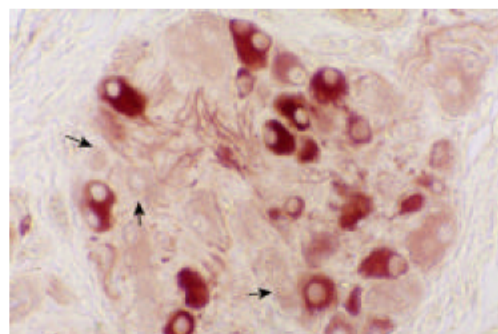


Fig. 2: Guinea pig prostate: Strong nNOS immunoreactivity in some ganglionic neurons; other neurons are negative 400x

RESULTS

Different of species have been observed with regard to the cNOS-immunoreactivity intensity, stronger in the guinea pig and hamster, in comparison to the rat and humans.

Immunohistochemical staining with anti-nNOS antibodies revealed the existence of strong nNOS-immunoreactivity in numerous ganglionic neurons of the pelvic plexus; about 70% of the nerve cell bodies of intramural ganglia were nNOS-immunoreactive (Fig. 1 and 2) nNOS-containing nerve fibers occur throughout the fibromuscular stroma; also many fine varicose nitroxidergic nerve terminals are located in the fibromuscular ring surrounding the acini. The smooth muscle cells express a discrete nNOS-immunoreactivity.

Vascular endothelium of small artery shows intense eNOS-immunoreactivity.

In all specimens examined, nNOS-immunoreactivity was found in the epithelium of the prostatic acini; the

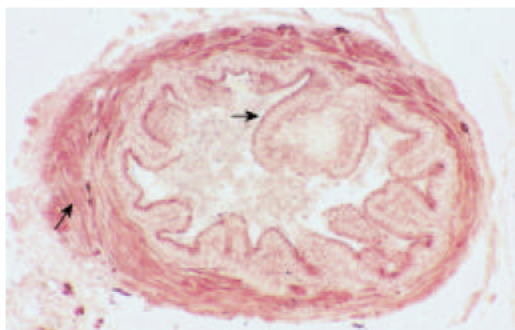


Fig. 3: Guinea pig prostate: nNOS immunoreactivity in glandular epithelium of working acinus more intense in the apical area of epithelial cells. nNOS immunoreactivity is present in smooth muscle cells surrounding the acinus 200x

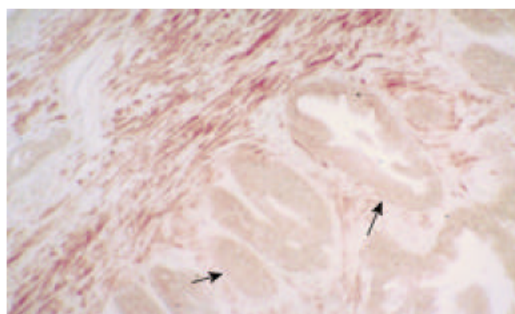


Fig. 4: Guinea pig prostate: Very weak nNOS immunoreactivity in scattered epithelial cells of not working acinus 200x

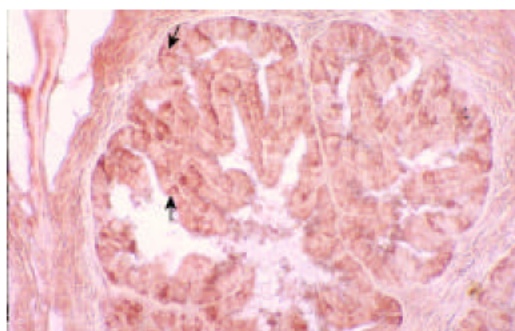


Fig. 5: Human prostate: Almost absent nNOS immunoreactivity in the glandular epithelium of acini. Non specific labelling in the fibrous stroma 100x

pattern of the glandular nNOS-immunoreactivity is not homogeneous because its intensity varied between the

acini: in detail, nNOS expression changed, depending on the functional acini activity :it is strong and more intense in the apical area of epithelial cells in working acini (Fig. 3) and weak in not working acini (Fig. 4).

In comparison to the immunohistochemical findings of the normal human prostate that show a dense nitroxidergic innervation in the fibromuscular stroma and nNOS reactivity in the secretory epithelium, sections of hyperplastic human prostatic tissue reveal only a poor nitroxidergic innervation of the fibromuscular stroma and an almost absent nNOS-immunoreactivity in the glandular epithelium (Fig. 5).

DISCUSSION

Our results provide histochemical evidence that nitric oxide may functions as important neurotransmitter in the prostate similarly to what evaluated in other male genitourinary organs.

Furthermore the present findings have shown that four different prostatic compartments (neurons, smooth muscle cells, blood vessels, glandular epithelium) appear to be the predominant targets of the nitric oxide. Consequently, this data suggest that in the prostate NO plays selective neural and paraneural roles.

The location of nNOS-immunoreactivity in autonomic ganglion cells and in nerve fibers in the stroma, among the smooth muscular cells, support the concept that NO may be mediate inhibition of prostatic smooth muscle tone. This role as relaxant neurotransmitter will have important consequence in prostatic pathophysiological conditions. A reduction of nitroxidergic innervation, as our findings show in human prostatic hyperplasia, may cause an increase of smooth muscular tone and consequently an obstructive micturition (Takeda *et al.*, 1995).

The presence of eNOS-immunoreactivity in endothelium of arterioles supports the hypothesis that NO is involved in the regional vascular regulation of the prostate.

The presence of nNOS-immunoreactivity in the glandular epithelial cells indicates their capacity to generate non neural nitric oxide and implies a novel paraneural role of NO for nitroxidergic signal trasduction in the autocrine or paracrine regulation of prostatic acini secretory activity, just like we have previously demonstrated histochemically in the other organs (Tessitore *et al.*, 1995; Tessitore and Bonaventura, 1996). Further histofunctional studies are carrying out to confirm these results and elucidate the prostatic role in aging, after hormonal experimental perturbation and in other pathophysiological processes.

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REFERENCES

- Bredt D.S. and S.H. Snyder, 1992. Nitric oxide, a novel neuronal messenger. *Neuron*, 8: 3-11.
- Bredt D.S. and S.H. Snyder, 1990. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. USA.*, 87: 682-685.
- Burnett, A.L. D.D. Ricker, S.L. Chamness, M.P. Maguire, J.K. Crone, D.S. Bredt, S.H. Snyder and T.S. Chang, 1995. Localization of nitric oxide synthase in the reproductive organs of the male rat. *Biol. Reprod.*, 52: 1-7.
- Burnett, A.L., C.L. Lowenstein, D.S. Bredt, T.S.K. Chang and S.H. Snyder, 1992. Nitric oxide: A physiological mediator of penile erection. *Science*, 257: 401-403.
- Burnett A.L., M. Takeda, M.P. Maguire, H. Lepor, S. Chamness, T.S.K. Chang and D.D. Ricker, 1997. Characterization and localization of Nitric Oxide Synthase in the human prostate. *Urology*, 43: 435-439.
- Dokita S., S.D. Smith, T. Nishimoto, M.A. Wheeler and R.M. Weiss, 1994. Involvement of nitric oxide and cyclic GMP in rabbit urethral relaxation. *Eur. J. Pharmacol.*, 266: 269.
- Jen Phillip P.Y.P., J.S. Dixon, J.P. Gearhart and J.A. Gosling, 1996. Nitric oxide synthase and tyrosine hydroxylase are colocalized in nerves supplying the postnatal human male genitourinary organs. *J. Urol.*, 155: 1117-1121.
- Moncada, S., R.M.J. Palmer and E.A. Higgs, 1991. Nitric oxide, physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, 43: 109.
- Persson K. and K.E. andersson, 1992. Nitric oxide and relaxation of pig lower urinary tract. *Br. J. Pharmacol.* 106: 416.
- Pollock, J.S., U. Fostermann, J.A. Mitchell, T.D. Warner, H.H.H.W. Schmidt, M. Nakane and F. Murad, 1991. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. USA.*, 88: 10480-10484.
- Smet P.J. K.A. Edyvane, J. Jonavicius and V.R. Marshall, 1994. Colocalization of nitric oxide synthase with vasoactive intestinal peptide, neuropeptide Y and tyrosine hydroxylase in nerves supplying the human ureter. *J. Urol.*, 152: 1292.
- Stuehr, D.J., H.J. Cho, N.S. Kwon, M.F. Weise and C.F. Nathan, 1990. Purification and characterization of the cytokine inducible macrophage nitric oxide synthase, an FAD- and FMN-containing flavoprotein. *Proc. Natl. Acad. Sci. USA.*, 88: 7773-7777.
- Takeda, M., R. Tang, E. Shapiro, A.L. Burnett and H. Lepor 1995. Effects of nitric oxide on human and canine prostates. *Urology*, 45: 440-445.
- Tessitore, V., 1998. The nitroxidergic neuron in central and peripheral nervous system and related paraneural structures. *Ital.J. Anat. Embriol.*, 103: 13-52.
- Tessitore, V., G. Bonaventura, M.L. Uzzo and A. Gerbino, 2002. Immunohistochemical distribution of nitric oxide synthases (nNOS-eNOS) in reproductive organs of some female mammals. *Nitric Oxide*, 6: 476-477.
- Tessitore, V., G. Bonaventura, M. Buscemi, A. Gerbino and G.M. Ippolito, 1995. The immunohistochemical distribution of NOS-containing structures in some mammals gallbladder and biliary tract in physiological and pathological conditions. *Ital. J. Anat. Embriol.*, 100: 97.
- Tessitore, V. and G. Bonaventura, 1996. Immunohistochemical mapping of NOS-containing structures in some mammals digestive tract. *Ital. J. Anat. Embriol.*, 101: 211.
- Tessitore, V., M. Buscemi, A. Gerbino and G. Bonaventura, 1996. Histochemical correlation between NOS immunoreactivity and NADPH-diaphorase staining in some mammals bulbourethral glands. *Ital. J. Anat. Embriol.*, 101: 215.
- Tessitore, V., M. Buscemi, A. Gerbino, 1994. Rilievo istochimico del Nitrossido nel pene di alcuni mammiferi. *Ital. J. Anat. Embriol.*, 99: 63.