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ORIGINAL PAPER

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Immunocytochemical distribution of nitric oxide synthase in the human seminal vesicle: a light and electron microscopical study

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Abstract Although nitric oxide (NO) has been proven to be one of the most important non-adrenergic, non-cholinergic mediators in the control of human reproductive tract organs, to date information on the significance of NO-mediated signal transduction in the control of human seminal vesicle (SV) function is still sparse. Recent investigations have underlined the significance of NO in the maintenance of sperm capacitation and viscosity of the seminal plasma as well as in the control of mammalian seminal vesicle smooth muscle tone. In order to further investigate the functional impact of NO on the regulation of normal SV function, we examined the distribution of NADPH-diaphorase (NADPH-d), endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) in the cellular anatomy of human SV by means of light and electron microscopical immunocytochemistry (LM, EM) in combination with the tyramide signal amplification technique. Human SV were obtained from 15 patients who had undergone surgery for pelvic malignancies (carcinoma of the prostate or urinary bladder). SV specimens were fixed, sectioned and examined by LM and EM for the presence of NAPDH-d, eNOS and nNOS using specific antibodies and advanced staining procedures. LM revealed a dense NADPH-d reaction in glandular epithelial structures, whereas no substantial labeling was detected in the fibromuscular stroma. EM showed that the NADPH-d reaction product was abundantly detectable attached to membranes of the endoplasmic reticulum, mitochondria and the nuclei of glandular epithelial cells. nNOS staining was found in nerve fibers branching within the SV tissue. eNOS staining was present in small vessels but was only observed to a minor degree in glandular and subglandular structures and the smooth muscle stroma. Our results support the hypothesis that human SV is a site of NO production. The distribution of NADPH-d may give rise to the speculation that NO is mainly involved in the regulation of SV secretory activity. The sparse correlation between NADPH-d-, eNOS- and nNOS-staining might hint at the existence of a previously unidentified NOS isoform in human SV.

Keywords Seminal vesicles · Nitric oxide · Nitric oxide synthase · NADPH-diaphorase

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Introduction

The control of human genitourinary tract smooth musculature is a complex interaction between neurovascular and physiological events involving multiple neurotransmitters and vasoactive agents. Investigative work carried out by several groups over recent years has clearly implicated nitric oxide (NO) as the main non-adrenergic, noncholinergic (NANC) mediator in the control of the human lower urinary and reproductive tract [2, 17]. NO is generally considered to be involved in the regulation of smooth muscle tone, blood flow and secretory function in the lower urinary tract organs of mammals [1, 7]. Apart from its fundamental significance in the maintenance of male penile erection, a functional importance has been assumed with regard to the NO-cGMP cascade in the control of smooth muscle tension of the human ureter and urinary bladder as well as the myogenic components of the mammalian prostate gland [5, 25, 26]. Since the NO-cGMP pathway plays such a prominent physiological role in the genitourinary tract, therapeutic efforts for the treatment of lower urinary tract dysfunctions have been aimed towards preserving or enhancing this pathway [12, 27, 28]. NO is synthetized as a product of the

conversion of the amino acid L-arginine to L-citrulline by the enzyme nitric oxide synthase (NOS). Three major NOS isoenzymes have been described to date: the constitutive and Ca^{2+}-dependent neuronal and endothelial isoenzymes b/nNOS and eNOS, and the inducible, Ca^{2+}-independent iNOS which is typically associated with macrophages and other cells with immune competence. More recently, a novel nNOS isoform was found in the rat penis, urethra and prostate [19].

To date, information on the significance of NO-mediated signal transduction in the functional control of mammalian seminal vesicles (SV) is still sparse. It has been assumed that NO might be involved in sperm capacitation and the maintenance of the viscosity of the seminal plasma [8]. More recently, we reported on the relaxing effects of nitric oxide donating drugs on adrenergic tension, as well as electrically evoked contraction of isolated human SV strip preparations [15, 18]. These findings prompted us to examine the occurrence of eNOS and nNOS in human SV by means of light and electron microscopical immunocytochemistry. We applied advanced fixation and staining protocols and the tyramide signal amplification (TSA) technique to enhance the sensitivity of the cytochemical staining procedure, especially for eNOS. The TSA method was first described by Bobrow et al. [3] and has been proven to be much more sensitive than conventional light microscopy (LM) immunoperoxidase staining [22]. In the present study, we modified this method and adapted it to electron microscopy (EM) in order to substantiate the distribution of constitutive eNOS alongside nNOS in human SV. The results were compared to those of the NADPH-d reaction.

Materials and Methods

Tissue source

Human SV were obtained from 15 males (aged 39–73 years) who had undergone pelvic surgery for carcinoma of the prostate or urinary bladder. None of the patients had received antihormonal therapy prior to surgery. Tissue specimens were stored in isopentan at −80°C or immediately frozen in isopentan chilled by liquid nitrogen.

Electron microscopical NADPH-d histochemistry

Free floating sections, sliced with a cryostat to 20 μM, were incubated for 90 min at 37°C in phosphate buffered saline (PBS) containing 0.2 mg/ml of the tetrazolium salt 2-(2′-benzothiazolyl)-5-styryl-3-(4′-phthalhydrazidyl)-tetrazolium chloride (BSPT), 1 mg/ml β-NADPH and 0.3% Triton X-100 (pH 7.4). The histochemical reaction was terminated by washing the tissue sections with PBS at room temperature. Sections were then postfixated with 1% osmium tetroxide, contrasted en bloc with 1% uranyl acetate, and flat embedded in Durcupan. Sections were examined with a Zeiss 900 electron microscope (Zeiss AG, Oberkochen, Germany).

eNOS and nNOS immunocytochemistry

Cryostat sections were mounted on glass slides and fixed with ice cold acetone for 10 min followed by a mixture of 4% paraformaldehyde and 0.4% glutaraldehyde in 0.1 PBS for 1–12 h. Assays were performed as previously described [23, 24]. Primary anti-eNOS and anti-nNOS antibodies were diluted 1:10,000 to incubate the sections (10–20 μM) overnight at 4°C. To visualize NOS-protein, the TSA Indirect Kit (DuPont NEN, Boston, USA) was used in combination with the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, USA) with 3.3′-diaminobenzidine (DAB), according to the instructions of the manufacturers.

Chemicals

Following thorough specificity tests (Western blot and immunocytochemistry using bovine aorta as a positive control), monoclonal eNOS- and nNOS-antibodies from Transduction Laboratories (Lexington, USA) were chosen. All other laboratory chemicals were either obtained from Sigma (St. Louis, USA), Merck (Darmstadt, Germany) or Mallinckrodt-Baker (Deventer, The Netherlands)

Results

LM and EM NADPH-d histochemistry

LM revealed an intense NADPH-d reaction related to the secretory epithelium of glandular structures. This reaction was most prominent in cryptic invaginations of the epithelial layer. More than 50% of the sections showed a positive reaction in the subepithelial areas, which was paralleled by significant nNOS staining. Although a certain degree of NADPH-d reactivity appeared in the smooth musculature surrounding the glandular structures, no labeling was observed in the majority of the SV smooth muscle stroma (Fig. 1). EM demonstrated that the NADPH-d reaction product BSPT-formazan was detectable as distinct electron dense precipitates at membranes of the endoplasmic reticulum and mitochondria, and the nuclear envelope (Fig. 2).

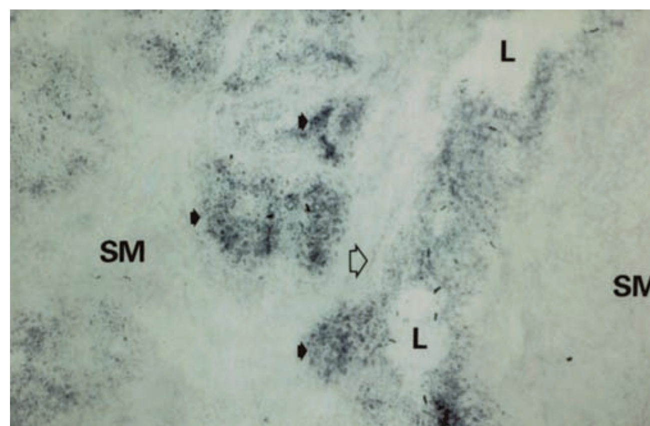

Fig. 1 Light micrograph displaying NADPH-d immunostaining in glandular epithelial (*dark arrows*) and subepithelial regions (*light arrows*) of human seminal vesicle (SV) tissue section. *SM* = smooth musculature, *L* = glandular lumen. Magnification 160×

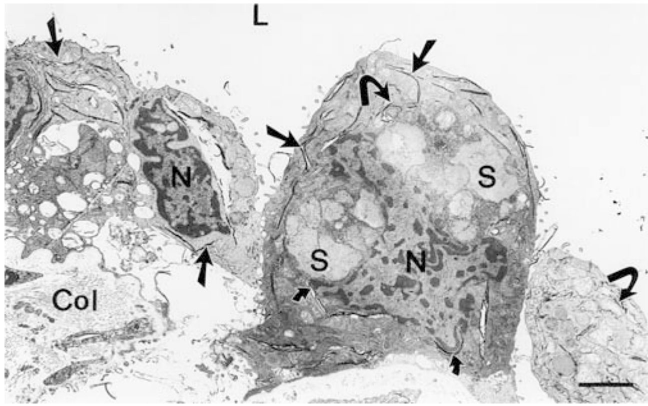

Fig. 2 Electron micrograph close-up illustrating NADPH-d localization in the glandular endothelium of human SV. BSPT-formazan deposits are located at membranes of the endoplasmic reticulum (*straight arrows*) and mitochondria (*angled arrows*). *Col* = collagen, *N* = nucleus, *L* = glandular lumen, *S* = secretory granula. *Scale bar* = 2.5 μM

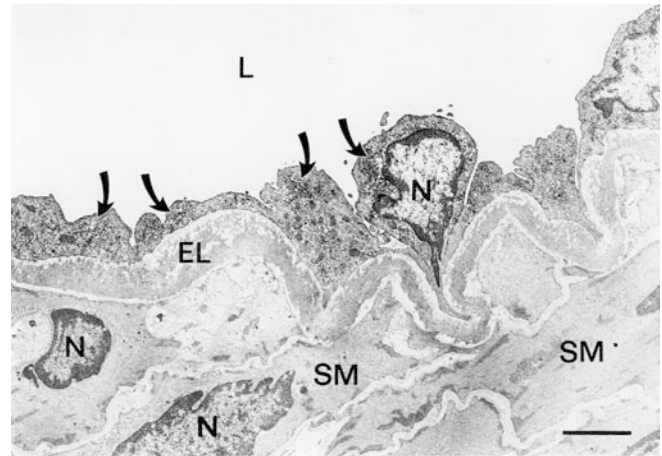

Fig. 4 Electron micrograph of a SV arterial section after eNOS immunostaining in combination with TSA. *Arrows* indicate endothelial cells labeled with the DAB reaction product throughout their cytoplasm. *EL* = lamina, *L* = vascular space, *N* = nucleus, *SM* = smooth musculature. *Scale bar* = 2 μM

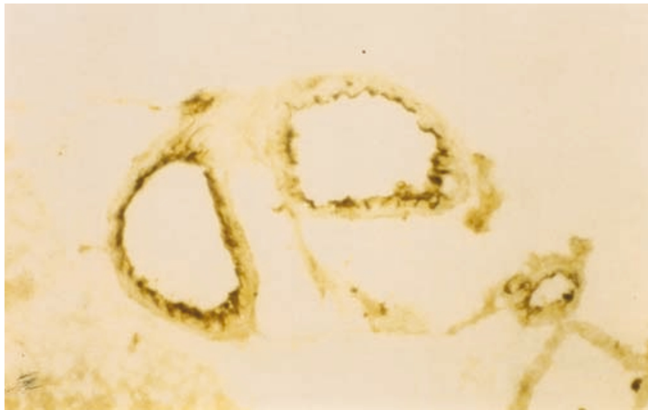

Fig. 3 eNOS immunohistochemistry using the TSA-method: light microscopy image presenting immunostaining in the endothelium of small arteries branching within the SV muscular stroma. Magnification 140×

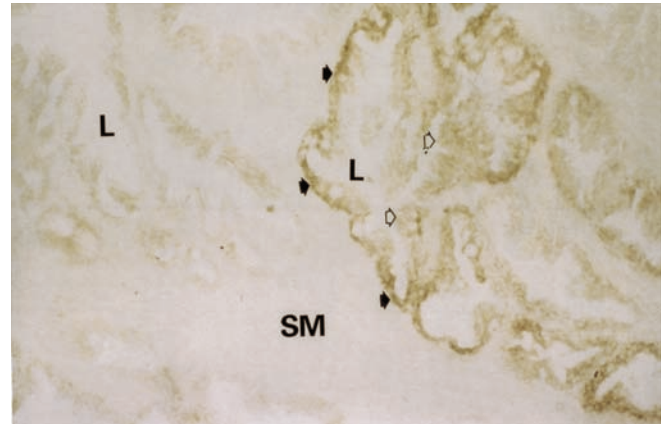

Fig. 5 Light microscopy image presenting dense nNOS immunostaining of human SV glandular epithelium (*dark arrows*) and subepithelial structures (*light arrows*). *L* = glandular lumen, *SM* = smooth musculature. Magnification 160×

LM and EM immunocytochemistry for eNOS and nNOS

eNOS staining was seen by LM and EM to be significantly present in endothelial cells covering the muscular tunica of arteries branching into the SV morphology (Fig. 3). Thin sections of human SV immunostained with the eNOS antibody in combination with TSA revealed DAB precipitates throughout the cytoplasm of the endothelial cells. No particular relation was shown with the nuclear envelope and the membranes of mitochondria, endoplasmic reticulum, and the Golgi apparatus (Fig. 4). No significant eNOS immunoreactivity was detectable in the epithelial or subepithelial layers of the glandular structures or in the smooth muscle stroma. In contrast, LM revealed intense nNOS staining related to subepithelial regions of the glandular structures, as well as in nerve fibers interspersing the fibromuscular stroma (Figs. 5, 6).

Discussion

The pioneer work of R. Furchgott, L. Ignarro and F. Murad has brought to light the obligatory role of inorganic NO in the regulation of smooth muscle tissue of the mammalian vascular system and urogenital tract [10, 11].

To date, the functional significance of NO-mediated pathways has been comprehensively evaluated in various organs of the lower urinary and reproductive tract of mammals. Nevertheless, knowledge on the impact of NO in the control of the human SV is still sparse. Since normal male sexual function requires the coordination of the processes of arousal, penile erection, orgasm and ejaculation, an intact function of the brain, penile erectile tissue, ductus deferens and seminal vesicles are the prerequisites for performing successful copulation and,

Fig. 6 Light microscopy image of SV tissue section: intramuscular bundle of nitrinergic nerves presenting nNOS immunolabeling indicated by *brownish colour*. Magnification 240×

finally, seminal emission. There are only a few studies documented in the literature which illustrate the mechanisms that may contribute to the regulation of mammalian SV function. Some of the data available strongly indicate that NO might be involved in the control of the secretory function and smooth muscle tension of the SV [8, 15, 21]. As yet, no data are available in the literature on the cellular and subcellular distribution of NOS isoforms in the human SV. This prompted us to investigate the localization of NADPH-d, eNOS and nNOS using advanced protocols for tissue fixation and staining. The present study is the first to provide evidence of NADPH-d, eNOS and nNOS expression in the human SV using LM and EM in combination with the TSA technique. We optimized the TSA method, screened the specificity of the immunolabeling for EM by omission of the primary NOS antibodies, as well as by using normal horse serum instead of the respective NOS antibody. Furthermore, a double-labeling procedure was applied to prevent cross-reactivity. The application of the TSA method allowed a reduction of the antibody concentration. Thus, the signal-to-noise ratio was greatly improved and hardly any background staining occurred. In a recent study, the comparative localization of NOS in the autonomic innervation to the human ductus deferens and SV was evaluated. The authors demonstrated dense NADPH-immunoreactivity in epithelial and subepithelial layers of traverse SV sections and concluded that this activity was attributable to the presence of a NOS isoform, probably eNOS. However, no specific antibodies to identify NOS isoforms were applied to the SV tissue, and even the authors stated that NADPH-d histochemistry alone cannot be considered a reliable marker for NOS [13].

Using LM and EM, we were able to demonstrate that the majority of the NADPH-d reaction in glandular structures of human SV tissue is not represented by eNOS. At the EM level, we discovered the NADPH-d reaction product, BSPT-formazan, in endothelial cells covering small arteries and SV glandular spaces as membrane-bound deposits attached to organelles. These findings are confirmed by the results of other authors who observed that diaphorase activity is partly attached to the Golgi apparatus, endoplasmatic membranes and the plasma membrane [9, 20]. While BSPT-formazan was seen to bind to endocellular membranes, distinct eNOS immunoreactivity, indicated by the DAB reaction product, was only present throughout the cytoplasm of arterial endothelial cells. This observation is supported by studies demonstrating considerable amounts of NOS in the cytosol of endothelial cells [14]. Our findings give rise to the speculation that eNOS might play a significant role in the regulation of SV hemodynamics. In contrast, eNOS staining was not seen in substantial amounts in SV glandular epithelial cells. This is in accordance with the results of a study by Burnett et al. demonstrating only poor eNOS staining and NADPH-d reaction in isolated rat SV [4]. Nevertheless, to our surprise, we observed significant amounts of NADPH-d reaction related to glandular epithelial layers and subepithelial regions of isolated human SV. Interestingly, this reaction was not associated with substantial eNOS immunostaining. Since the presence of NADPH-d activity is regarded as a general histochemical marker for the localization of NOS in cellular structures [6], the poor correlation between NADPH-d reaction and eNOS staining might hint at the existence of an unidentified NOS isoform in human SV which might be exclusively involved in the regulation of the secretory function. While no substantial amounts of NOS immunoreactivity were detected within the fibromuscular stroma of the organ, we observed the expression of nNOS in nitrinergic nerve fibers innervating the SV tissue. This staining was co-localized with a dense NADPH-d reaction. The abundant expression of nNOS in subepithelial structures and intramuscular nerve fibers is in accordance with the results from a study by Jen et al. on the presence of NOS in nerves supplying the postnatal human male vas deferens and seminal vesicle. In their study, Jen et al. demonstrated the co-localization of NOS, tyrosine hydroxylase, neuropeptide Y and vasoactive intestinal polypeptide in subepithelial nerve networks, as well as in nerve fibers branching within the smooth muscle layer of these organs [16]. Thus, our findings are in favour of the hypothesis of the importance of NO in the regulation of human SV secretory and smooth muscle function. This presumed pivotal role of NO in the control of SV contractility is also confirmed by the results from recent organ bath studies and measurements of cyclic nucleotides [15,18].

In conclusion, our results present further evidence for the hypothesis that the normal function of human SV is regulated by the generation and action of NO and, subsequently, cGMP. Our results underline the importance of NO in the control of human SV function and the contribution of nNOS to this process and suggest the existence of a possibly unidentified NOS isoform in human SV which might be exclusively involved in the regulation of the secretory function and sperm capacitation.

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