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Immunohistochemical studies on brain nitric oxide synthase (bNOS) in the male genital accessory glands of the rat during postnatal development

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The aim of the study was to investigate the presence, localisation and function of brain nitric oxide synthase (bNOS) in the male genital accessory glands of rats in the course of their postnatal development. Localisation of the bNOS was immunocytochemically investigated in the epididymis, seminal vesicle and ventral prostate of male Wistar strain rats at 1, 5, 10, 20, 28, 35, 45 and 59 days of age. The method employed involved mouse monoclonal antibodies against rat bNOS in combination with tyramide signal amplification (CSA). The intensity of the reaction in the organs studied was determined using computer software to demonstrate the optical density of the reaction product obtained. In the epididymis a weak reaction was observed in the connective tissue/muscular sublayer on the 28th and 45th days of life. In the seminal vesicle and ventral prostate a positive reaction appeared in the epinuclear portions of glandular epithelial cells on the 20th day of life, reaching a maximum intensity on the 28th day and thus before the rats reached maturity. The results obtained allow the conclusion to be drawn that nitric oxide resulting from bNOS-activity participates in the processes of differentiation and of function in the epididymis, seminal vesicle and ventral prostate.

Key words: accessory gland, epididymis, seminal vesicle, prostate, nitric oxide synthase, NOS, postnatal development

INTRODUCTION

During the last 20 years of the last century numerous studies appeared which demonstrated the important role of nitric oxide (NO) in the control of several vital functions in the mammalian body.

In the endocrine system, NO is supposed to be responsible for the control of hormonal secretion by, for example, the adrenal cortex. Nitric oxide has been established as an important messenger molecule in various aspects of brain physiology, from development to synaptic plasticity, learning and memory [4]. In the peripheral nervous system it acts as a neurotransmitter. However, NO has also been viewed as a major agent of neuropathology [4].

Bredt and Snyder [2] are of the opinion that NO represents an unconventional new generation neurotransmitter. It is not present in synaptic vesicles, does not act through receptors and is secreted by neurons as if, on request, facilitating communication between neurons in the central nervous system.

As is generally recognised, NO is formed from L-arginine as a result of involvement of the enzyme

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nitric oxide synthase (NOS) in the presence of NADH as a co-factor. Three isoforms have been distinguished, depending on their origin: bNOS (nNOS, ncNOS, type I NOS), eNOS (ecNOS, type III NOS) and iNOS (mNOS, type II NOS). NOS isolated from the cerebellum, bNOS, and that from the vascular endothelium, eNOS, are considered constitutively expressed proteins. Constitutive NOS is regulated by Ca^{++} and releases NO within a short time. Inducible NOS is thought to be induced by cytokines, is independent of Ca^{++} and releases NO for prolonged periods of time. Such an isoform of NOS may be induced in macrophages [2, 14].

Nitric oxide has been proved to be one of the most important non-adrenergic, non-cholinergic mediators in the control of human reproductive organs [16]. In the reproductive tract, NO originates from the endothelium, glandular epithelium and from neurons. In the vascular system, nitric oxide acts on smooth muscles of the blood vessels and controls blood pressure and blood flow through the vessels. NO from nerve fibres inside the muscles and from neuronal subepithelial plexuses probably participates in the contractility of the smooth muscles in blood vessel walls. The subepithelial localisation of NOS is also supposed to affect secretory processes in a neuronal way [3, 6]. In the epithelium of the epididymis, NO may control its secretory activity and in this way affects sperm maturation and its displacement [18]. In the seminal vesicle, NO is known to cause smooth muscle relaxation by activating guanylate cyclase and increasing cGMP levels in the smooth muscle cells [2]. NO of the prostate epithelium probably influences autocrine or paracrine processes [6].

NOS is also detected in the organs of the male sexual system in various species. Activity of the enzyme was demonstrated in the rat epididymis [3, 7] and in the prostate of the rabbit [1], dog [5] and man [10]. Slight activity of the enzyme has also been described in the human seminal vesicle in the course of postnatal development [11].

We have not encountered any data in the available literature on alterations in bNOS activity in the genital tract of the male rat in the course of postnatal development. Clarification of this matter is the aim of the present study.

MATERIAL AND METHODS

The studies were performed on male Wistar strain rats housed in stable conditions at a temperature of 20 ± 2°C with a light cycle of 10 L – 14 D and allowed free access to chow and water. On days 1, 5, 10, 20, 28, 35, 45 or 59 of life the rats were sacrificed by exsanguination under ether anaesthesia. The epididymis, ventral prostate and seminal vesicle were weighed, the fragments fixed in Bouin's solution and then embedded in paraffin. Each of the time points to be analysed in rat life was represented by 6 animals.

Immunocytochemical tests were performed on paraffin sections for the presence of brain nitric oxide synthase (bNOS). The method employed involved mouse monoclonal antibodies against rat bNOS (clone NOS-B1, Sigma-Aldrich), diluted 1:1000, in combination with tyramide signal amplification (CSA, Catalyzed Signal Amplification System, DAKO). A recombined fragment of NOS (a.a. 1–181) originating from a rat brain served as an immunogen to trigger production of the antibodies. In the protocol for bNOS the producer of the antibody (Sigma-Aldrich) excluded interaction of this antibody with NOS originating from macrophages (mNOS) or with NOS present in endothelial cells (eNOS). As a negative control for the immunocytochemical reactions performed, a procedure was used in which monoclonal IgG protein included in the CSA kit was substituted for specific anti-bNOS antibodies. In each age group the best 1–2 preparations were selected, on each of which 2 to 10 areas were marked, giving a total area of 0.096 to 0.48 mm^2.

The intensity of staining of bNOS-immunopositive areas as a reflection of the brown stain in the cytoplasm per cell (= mean optical density) was evaluated under × 350 magnification. Computer software was employed for automatic image analysis via a Image-Pro Plus and Nikon Eclipse E600 microscope equipped with a chromatic TV CCD camera. From the mean optical density of the bNOS-immunopositive areas was subtracted the mean optical density of the negative control, obtained with the use of IgG on the same microscope slide on which the immunocytochemical test for bNOS was performed. After the mean optical density of bNOS-immunoreactive areas had been measured, the slides with bNOS sections were stained with haematoxylin to demonstrate the cell nuclei, thereby facilitating analysis of the histological patterns.

The results obtained were statistically analysed using Student's *t* test.

RESULTS

The immunocytochemical studies of bNOS in the epididymis revealed a weak reaction, which appeared only on the 28th and 45th days of life in the nerve fibres of the connective tissue/smooth muscle sublayer (Fig. 1). The optical density values of bNOS were

low and were noted in the same compartment in the two groups of animals.

In the seminal vesicle a weak reaction developed in the epinuclear zone of cells of the glandular epithelium on the 20th day of life. Its mean optical density reached peak values on day 28 and remained almost unchanged until day 35. The minimal value of this parameter occurred in the 45-day-old animals. The level of mean optical density of bNOS-immunopositive areas reached its higher value later, on day 59 (Fig. 2).

In the prostate bNOS was observed in the epinuclear zone of the cells of the glandular epithelium beginning on the 20th day of life and the optical density was lowest at that time. The mean optical density in the prostate reached its maximum intensity on the 28th day. Later, the mean optical density of the immunopositive reaction for bNOS varied within a narrow range but always remained higher than that found on the 20th day of life (Fig. 3).

Changes in the mean optical density of the product of the immunocytochemical reaction for bNOS activity in the genital accessory glands of male rats during their postnatal development are illustrated in Figure 4.

DISCUSSION

In these studies on the immunocytochemical localisation of bNOS in the course of postnatal development, a weak reaction for the enzyme was observed in the epididymis. In 28- and 45-day-old rats the reaction was localised to the nerve fibres of the connective tissue/smooth muscle sublayer. No bNOS activity could be found in cells of the glandular epithelium. This specific localisation is consistent with the findings of other authors, who have observed NOS-activity in noradrenergic nerve fibres in the sublayer of the epididymis of rats [7] and humans [10]. These authors concluded that NOS and its product, nitric oxide, are responsible for the control of smooth muscle function in the epididymis of both species. Wiszniewska et al. [18] used immunocytochemical techniques to demonstrate iNOS activity in epinuclear portions of cells in the glandular epithelium in culture or in organ fragments. It has been suggested that the nitric oxide produced affects the sublayer of the rat epididymis in the course of sperm passage and provides nutrients for sperm. Ventura and Burnstock [17], using polyclonal antibody, observed enhanced expression of bNOS in the course of postnatal development in the rat epididymis. In rats both of 28–35 and of 150–180 days of age all sections of the cauda part of this organ showed virtually no NOS-immunoreactive nerve fibres in the smooth muscle layers. Only sections of the epididymis cauda taken from 24-month-old rats showed significantly increased bNOS-immunoreactivity. Burnett et al. [3] used rabbit antibodies to the whole rat cerebellar NOS protein to demonstrate nitric oxide synthase in nerve fibres of the sublayer and, in parallel, in endothelial cells of the epididymis as well as in the apical and perinuclear portion of the canalicular epithelium. NOS-activity in the rat epididymis was 7 times higher in the rat epididymis as compared to the seminal vesicle, while in humans the reverse was found, namely that NOS activity could be termed as negligible in the epididymis, while in the seminal vesicle it was high [3, 8].

Ventura and Burnstock [17] and Burnnett et al. [3] showed that NADPH-d localisation, except for small staining differences, yielded similar results to NOS-immunohistochemistry in the nerve fibres and epithelium of the epididymis. Uckert et al. [16] reported a poor correlation between NADPH-d and immunocytochemical staining of nNOS in the human seminal vesicle.

In our investigations on the rat seminal vesicle a positive reaction for bNOS appeared in the epinuclear zone of the glandular epithelium cells in the 20th day of life, while maximum optical density of the reaction was noted in the 28th and 35th days of life. In the adult rat Burnett et al. [3] detected only a weak reaction for NOS and did not indicate its localisation. Immunocytochemical studies on NOS in the seminal vesicle of 2-month-old to 3-year-old boys obtained at post-mortem examination detected weak bNOS-activity in the subepithelial nerve fibres and in noradrenergic nerve fibres linked to smooth muscles [11]. The data seem to suggest that nitric oxide participates in the autonomic control of organ function. Moreover, immunocytochemical studies on NOS in nerve fibres of the seminal vesicle in human foetuses detected an immunopositive reaction beginning at the 23rd week of pregnancy and the reaction was significant in the postnatal period [6].

In the prostate gland bNOS-activity appeared on the 20th day of life, while its localisation and day of maximal intensity resembled those observed in the seminal vesicle. According to Aikawa et al. [1], nitric oxide in the rabbit prostate was thought to induce relaxation of the smooth muscles.

Changes in NOS in the genito-urinary system have also been examined in human foetuses between the 13th and 30th weeks of intrauterine life [6]. A positive reaction in the prostate was detected as early as the

Figure 1. Epididymis of a 28-day-old rat. Weak reaction for brain nitric oxide synthase in the nerve fibres of the connective tissue/smooth muscle sublayer. Scale bar 24 μm.

Figure 2. Rat seminal vesicle: 28-day-old rat (**A)** and 35-day-old rat (**B**). Strong reaction for bNOS-immunoactivity in the glandular epithelium; **C.** 45-day-old rat. Weak reaction for brain nitric oxide synthase in the glandular epithelium. Scale bar 24 μm.

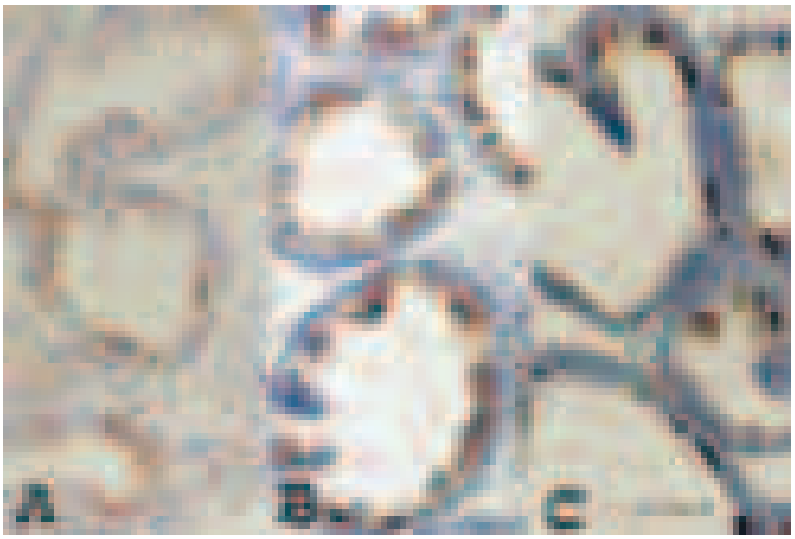

Figure 3. Rat prostate: **A.** 20-day-old rat. Weak reaction for brain NOS-immunoactivity in the glandular epithelium; **B.** 28-day-old rat; **C.** 35-day-old rat. Evident increase in bNOS-immunoactivity in the glandular epithelium. Scale bar 24 μm.

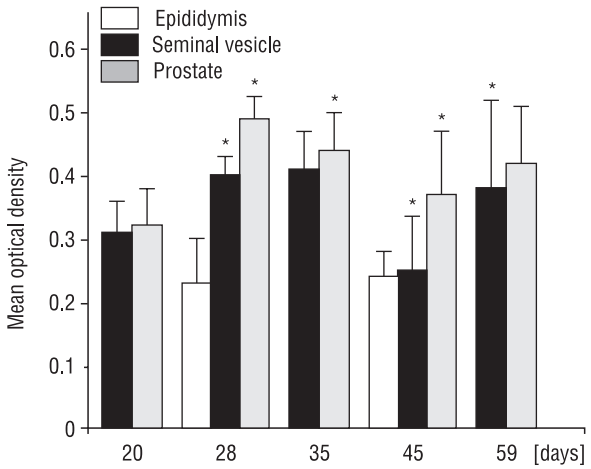

Figure 4. Optical density of the product of immunocytochemical reaction for brain nitric oxide synthase (bNSO) in the epididymis, seminal vesicle and prostate of rats at 20, 28, 35, 45 and 59 days of age. The results are means ± SD; *$p < 0.05$ significantly different from the younger group.

13th week of intrauterine life, while such a reaction only developed between the 23rd and 30th weeks of intrauterine life in the seminal vesicle and epididymis. The reaction was localised to nerve fibres supplying the smooth muscles and was also demonstrated in cells of the human glandular epithelium [6]. Constitutive NOSs (nNOS and eNOS) are expressed in both normal and hyperplastic human prostate and both are expressed in epithelial cells [9].

The appearance of a bNOS-specific reaction in the seminal vesicle and prostate in the 20th day of life and the development of maximum optical density of the reaction on day 28 found in the present study deserve attention. The reaction in the epididymis occurs slightly later, appearing in the 28th day of life. The maximum intensity of the reaction for bNOS activity markedly precedes the time when the rats reach sexual maturity.

In our previous morphometric studies on the rat male reproductive system during postnatal development we have shown that in their histological patterns and in morphometric indices rats reach a differentiation status similar to that observed in adult animals before they attain sexual maturity [15]. Manifestation of bNOS-activity in epinuclear parts of the glandular epithelium cells of the seminal vesicle and prostate corresponds to the localisation of cholecystokinin (CCK-8) and of bombesin observed in our earlier immunocytochemical studies [12, 13].

To sum up, the localisation and activity of bNOS prior to the rats reaching sexual maturity may suggest that synthesised NO affects not only the function of accessory genital glands and particularly their smooth muscles, but also the growth and differentiation of the epididymis, seminal vesicle and ventral prostate.

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