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Original article

Expression of NK1 receptor at the protein and mRNA level in the porcine female reproductive system

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Abstract

The presence and distribution of substance P (SP) receptor NK1 was studied in the ovary, the oviduct and the uterus (uterine horn and cervix) of the domestic pig using the methods of molecular biology (RT-PCR and immunoblot) and immunohistochemistry.

The expression of NK1 receptor at mRNA level was confirmed with RT-PCR in all the studied parts of the porcine female reproductive system by the presence of 525 bp PCR product and at the protein level by the detection of 46 kDa protein band in immunoblot. Immunohistochemical staining revealed the cellular distribution of NK1 receptor protein. In the ovary NKI receptor was present in the wall of arterial blood vessels, as well as in ovarian follicles of different stages of development. In the tubular organs the NK1 receptor immunohistochemical stainings were observed in the wall of the arterial blood vessels, in the muscular membrane, as well as in the mucosal epithelium.

The study confirmed the presence of NK1 receptor in the tissues of the porcine female reproductive tract which clearly points to the possibility that SP can influence porcine ovary, oviduct and uterus.

Key words: substance P, receptors, reproductive tract, pig

Introduction

The female reproductive system is innervated by autonomic afferent and efferent nerve fibers. Apart from classical neurotransmitters (noradrenaline for sympathetic and acetylcholine for parasympathetic nerve fibers) nerve fibers of the reproductive system contain non-classical neurotransmitters, being of peptide structure. Neuropeptides play a role of co-transmitters and neuromodulators co-acting with classical neurotransmitters in fine-tuning the nerve--effector interactions (Hokfelt et al. 1980). One of the key neuropeptides in the nervous system is substance P (SP) which is a neurotransmitter in sensory fibers (Ohtori et al. 2002). It is responsible for neurotransmission of pain stimuli, as mice devoid of substance P do not react to pain stimuli of medium and high intensity (Cao et al. 1998). It is also engaged in the neurotransmission in the autonomic afferent fibers, but it also exerts some specific functions in the reproductive organs. It affects functions of the oviduct, uterus and vagina influencing the contractility of the

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muscular membrane of the uterus (Bodelsson and Stjernquist 1992) and increasing the permeability of blood vessels (Lundberg et al. 1984).

Neurotransmitters, including neuropeptides, exert their action on target cells and tissues via receptors. SP, belonging to the class of tachykinins (together with neurokinin A and B) shows affinity to 3 classes of receptors called NK1, NK2 and NK3. SP shows the highest affinity to NK1 receptor, being bound by NK2 and NK3 with the affinity much lower. NK1 receptor was found in many tissues of many systems, including, alimentary (Frieling et al. 1999), immune (Hartung et al. 1986), bones (Goto and Tanaka 2002), nervous (Elde et al. 1990), and others.

The study of the receptors open new possibilities of developing novel methods of influencing functions of many tissues and organs. Today, approx. 50% of medicines are exerting their influence via binding to specific receptors (Drews 2000).

Not much is known about the distribution of NK1 receptor in the reproductive organs of the animals and man. It was found in the rat uterus (Villablanca and Hanley 1997) and nothing is known about its presence and distribution in the reproductive organs of farm animals. This is why we decided to study the presence and distribution of NK1 receptor in the porcine ovary, oviduct and uterus (uterine horn and uterine cervix).

Materials and Methods

The study was performed on 8 sexually immature gilts of the Large White Polish breed weighting ca. 25 kg. The animals were purchased from a commercial fattening farm. The animals were premedicated with azaperone (Stressnil, Janssen, Belgium; 8 mg/kg of body weight, i.m.) and xylazine (Xylavet, Scanvet, Poland; 2.8 mg/kg of body weight, i.m.) and then the lethal dose of Thiopental (Thiopental, Biochemie, Austria; 40 mg/kg of body weight, i.v.) was administered until the respiratory activity ceased. Animals for morphological studies were then transcardially perfused with 4% solution of paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion the abdominal cavity was opened and fragments of the ovary, oviduct, uterine horn and vagina were collected. After 1 hour of postfixation in the fixative as used for perfusion the tissues were rinsed in phosphate buffer overnight and then transferred to 18% sucrose in phosphate buffer (with 0.1% sodium azide) for storage.

Tissues for morphological studies were cut in a cryostat into 12 µm sections. Sections for immunohistochemistry were put on chrome alum-gelatin-coated slides, air-dried and then stored at -80 Centigrades in air-tight boxes with desiccant. Sections for in situ hybridization were put on slides coated with aminopropyl-trietoxy-silane (APES, Sigma, USA), air-dried and stored in boxes as described earlier.

Animals for molecular biology were dissected without prior perfusion with paraformaldehyde. Fragments of ovaries, oviducts, uterine horns and vagina were excised, weighted, wrapped in Parafilm and alufoil and then snap-frozen in liquid nitrogen. Tissues were then stored at -80 Centigrades until further processing.

Total RNA was isolated with TRIzol (GibcoBRL, USA) according to manufacturer instructions. Fragments of tissues weighting 100-200 mg were homogenized with UltraTurrax homogenizer (Janke & Kuhnel, Germany) in 10 ml of TRIzol. After the completed procedure the resulting precipitate of total RNA was dissolved in a nuclease-free water. The concentration of the preparate was determined spectrophotometrically at 260 nm with Ultrospec III UV/VIS spectrophotometer (LKB, Sweden). The purity of the preparate was determined by a A_{260}/A_{280} ratio which had to be in the range 1.6-2.0. The preparates of total RNA were stored at -80 Centigrades. The cDNA synthesis was performed as described elsewhere (Wasowicz 2003).

Since the sequence of porcine NK1 mRNA is unknown starters for PCR were designed on the basis of the analysis of homology of NK1 mRNA sequences of other species available in GenBank. The homology analysis was performed with ClustalW software. Sequences used for homology analysis were: human NK1 receptor (M74290), murine NK1 receptor (X62934), guinea pig SP receptor (X64323) and rat SP receptor (M31477). Transcript fragments with homologous sequences were identified and the starter sequences were picked up. The sequence of sense starter was CAGCCACGGCTACCAA, and the sequence of antisense starter was GCTTGAAGCCCAGACG. The melting temperature of both starters was 54 Centigrades. The expected length of PCR product was 544 base pairs (bp).

PCR reaction was performed as described elsewhere (Wasowicz 2003). 30 cycles were performed with a thermal profile of 95 Centigrades denaturation for 10 s, 54 Centigrades starter hybridization for 10 s and 72 Centigrades DNA synthesis for 30 s. PCR products were analysed in 0.8% agarose gel in TAE buffer. The size of PCR product was assessed with a DNA size marker (M1, DNA Gdansk, Poland). DNA fragments were visualized with ethidium bromide (EtBr, 10 mcg/ml of gel) and an UV transluminator with a wavelength of 302 nm. Gels were documented with a video camera, equipped with appropriate filters, coupled to PC computer equipped with an image grabber and image acquisition software.

Immunoblotting

Frozen fragments of the ovary, oviduct, uterine horn and cervix were homogenized in impact homogenizer (Kucharczyk TE, Poland). Pulverized tissues were transferred to the lysis buffer consisting of 2% SDS, 50 mM TRIS (pH 6.8), 100 mM di-thiotreitol (DTT, Sigma, USA), 10% glycerol (POCh, Poland) and 0.1% bromophenol blue (POCh, Poland). The volume of the buffer was adjusted to 100 mg tissue per 1 ml of the buffer. Tissues were homogenized with an UltraTurrax homogenizer (Janke & Kuhnel, Germany). Then the homogenate was heated to 95 Centigrades for 5 min, cooled to room temperature and centrifuged for 2 min at 12000g. The proteins were separated, blotted and detected as described elsewhere (Wasowicz 2003).

Immunohistochemistry

Immunohistochemical stainings were performed on slides taken out from the freezer and then air-dried at RT for 20 min. Every section was outlined with a DAKOPEN marker (DAKO, Denmark). Then slides were processed as described elsewhere (Wasowicz 2003). The primary antibody was polyclonal rabbit anti-NK1 antibody (Cat. No. S83305; Sigma, USA) (1:200). The secondary antibody was biotinylated goat anti-rabbit antibody (Cat. No. E0432, DAKO, Denmark) (1:400).The secondary antibody was detected with Cy3-conjugated streptavidin (Cat. No. 016-160-084, Jackson Immunoresearch Labs, USA) (1:4000). Then slides were examined and with an Axiophot fluorescent microscope (Zeiss, Germany) with a filter set specific for Cy3. Sections were imaged using a laser scanning confocal microscope (MRA-2, Biorad, UK).

Staining specificity was verified by control stainings in which the primary antibody was either omitted, or replaced with a normal non-immune rabbit serum. In none of the control stainings any signal was detected.

Results

Expression of NK1 receptor at the level of mRNA and protein

RT-PCR performed with NK1-specific primers on cDNA synthesized with total RNA isolated from the ovary, oviduct, uterine horn and cervix gave in all parts of the reproductive system positive results. The obtained PCR product was estimated to be close to the expected size of 525 bp. The intensity of PCR product bands was of similar in all parts of the porcine reproductive system (Fig. 1).

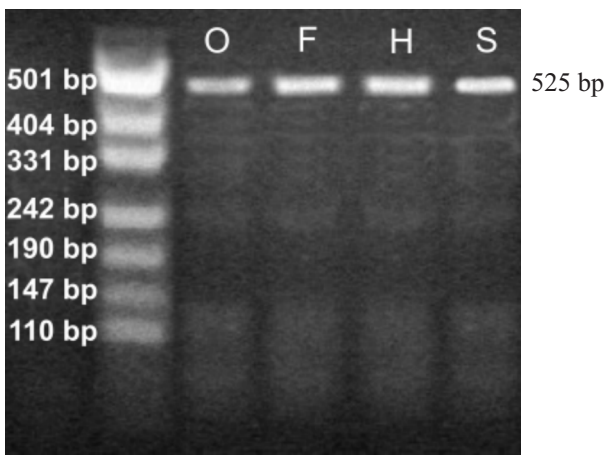

Fig. 1. Agarose electrophoresis of NK1 receptor PCR product (525 bp). O – ovary, F – oviduct, H – uterine horn, S – uterine cervix.

Immunoblot revealed in all studied parts of the porcine female reproductive system the presence of the band of the molecular weight of ca. 46 kDa. The intensity of bands in protein isolates from all studied parts of the reproductive system was comparable. In addition to the specific band some additional bands of lower intensity were also detected (Fig. 2).

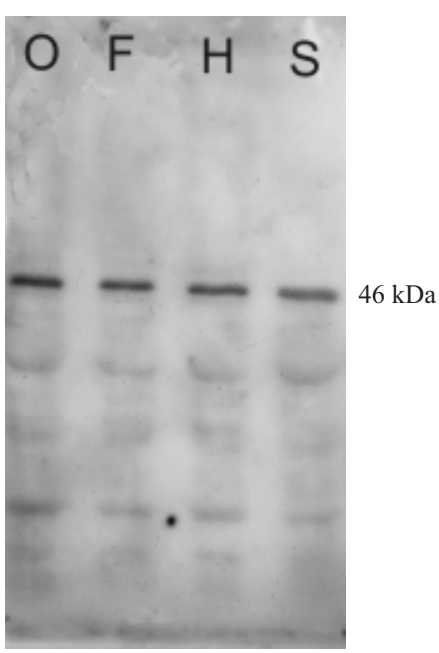

Fig. 2. Immunoblot of protein extracts from the porcine reproductive system. The antibody detected a protein with molecular mass of 52 kDa.). O – ovary, F – oviduct, H – uterine horn, S – uterine cervix

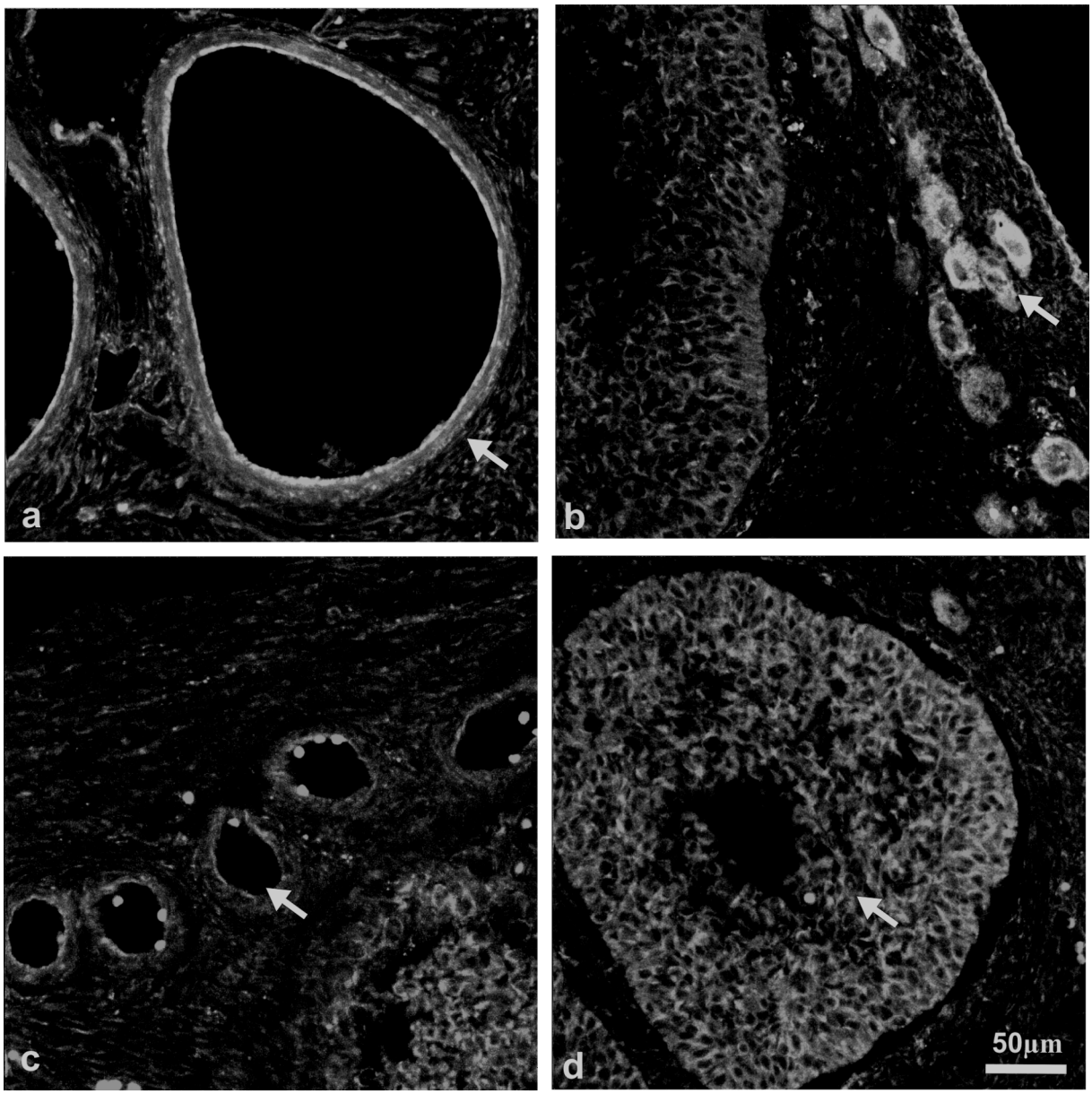

Fig. 3. a – Ovary. Immunostaining for NK1 receptor in the blood vessels (arrow); b – Ovary. Immunostaining for NK1 receptor in the primary ovarian follicles (arrow); c – Ovary. Immunostaining for NK1 receptor in the blood vessels of the ovarian core (arrow); d – Ovary. Immunostaining for NK1 receptor in the granulosa cells (arrow).

Tissue distribution of NK1 receptor at the level of protein and mRNA studied with immunohistochemistry

In the ovary NK1 receptor staining was detected mainly in the walls of the blood vessels. The strongest staining was seen in the endothelium of the arteries located in the ovarian hilus. The staining was much weaker in the muscular layer of the blood vessels (Fig. 3a). In veins staining for NK1 receptor was seen only infrequently. In case of numerous veins the endothelium was lacking staining for NK1 receptor (Fig. 3c). Strong staining for NK1 receptor was seen in primary ovarian vesicles, located either singularly, or in cluster under the surface of the ovary (Fig. 3b). In the ovarian vesicles the staining was seen in the cells of zona granulosa, or in oocytes (Fig. 3d).

In the oviduct the distribution of NK1 receptor staining was similar in the isthmus and ampulla. NK1 receptor staining was seen in the endothelium of the arterial vessels in the mucosa, submucosa and muscularis (Fig. 4a), as well as in the smooth myocytes of the muscular layer (Fig. 4b). NK1 receptor staining was also seen in the epithelium of the mucosa (Fig. 4c).

In the uterine horn staining for NK1 receptor in the endothelium of the arterial vessels was very weak (Fig. 5a). In the muscular layer the staining was seen

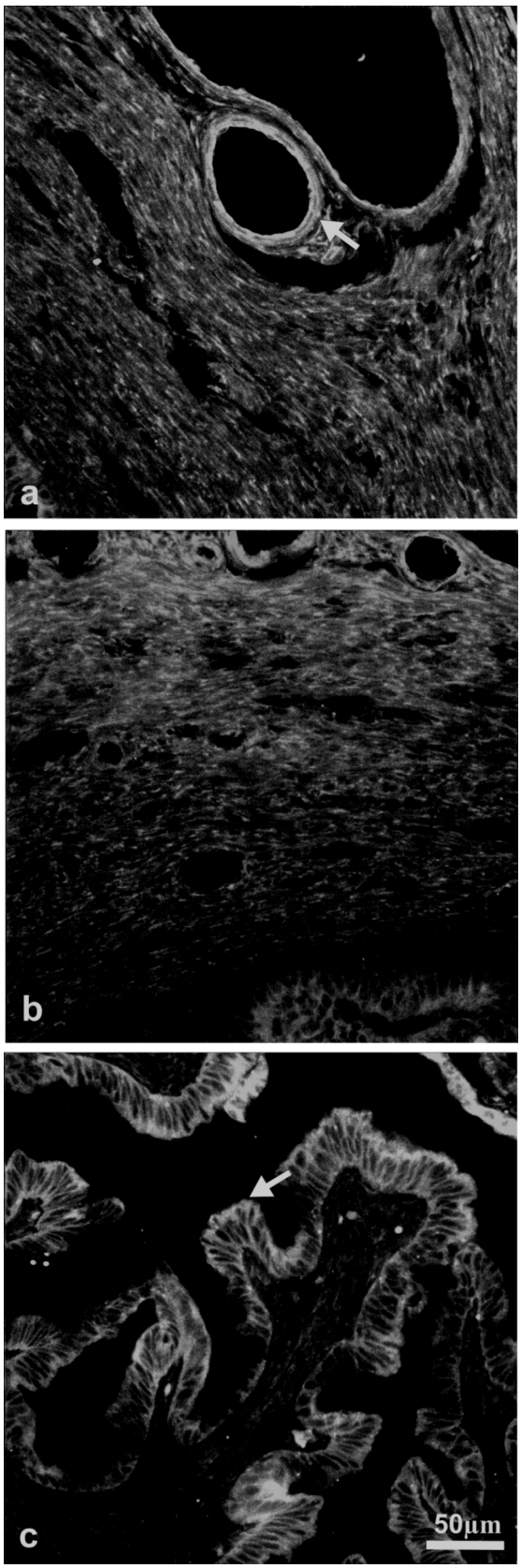

Fig. 4. a – Oviduct isthmus. Immunostaining for NK1 receptor in the blood vessels; b – Oviduct isthmus. Immunostaining for NK1 receptor in the muscular membrane; c – Oviduct isthmus. Immunostaining for NK1 receptor in the mucosal epithelium.

as "patches" located on some smooth myocytes (Fig. 5b). The staining was present also in the apical parts of the epithelium of uterine glands (Fig. 5c) and of the mucosa (Fig. 5d). In the uterine cervix staining for NK1 receptor was seen in the muscular membrane of the arteries of the submucous membrane, although the small vessels were devoid of NK1 receptor staining. In the muscular membrane of the uterine cervix the staining was seen only in isolated clusters of smooth myocytes what has given the picture of "patches" dispersed in the muscular membrane. The staining was seen also in the cells of the epithelium of the mucosa.

Discussion

The discovery of neuropeptides and subsequent studies on their role in the nervous system and peripheral tissues sparked hopes for finding practical applications for this knowledge (Hokfelt et al. 2000, Hokfelt et al. 2003). Interest in Substance P was resulting not only from its involvement in sensory neurotransmission, but also from its role in regulation of the blood flow, blood vessels permeability (Brunsson et al. 1995) and the progress of the inflammation (Mantyh 1991). The action of neuropeptides through specific receptors directed also much of interest into this field, as receptors are natural targets for would-be drugs.

Despite that detailed distribution of SP-positive nerve fibers was described in the female reproductive system of many species, including pig and humans (Owman and Stjernquist 2003), the presence and distribution of receptors to this neuropeptide did not attract much attention. The presence of SP-positive nerve fibers in the tissues of the ovary, oviduct and uterus leads to the simple conclusion that this neuropeptide must influence the status of the female reproductive system through specific receptors located on smooth myocythes of the blood vessels and the oviduct and uterine wall, as well as on the glandular cells. Papers devoted to the distribution of neuropeptide receptors in the reproductive organs are scarce and usually studies were done either at mRNA level or with pharmacological methods and very rarely with morphological methods, like autoradiography, hybridocytochemistry, or immunohistochemistry.

The application of RT-PCR confirmed the presence of NK1 receptor in the tissues of the ovary, oviduct and the studied parts of the uterus. RT-PCR was used to detect the transcript of NK1 receptor in the central nervous system and peripheral tissues (Kaltreider et al. 1997, Togari et al. 1997, Rasley et al. 2002, Seybold et al. 2003). However, this method was rarely used for studies in the reproductive system and

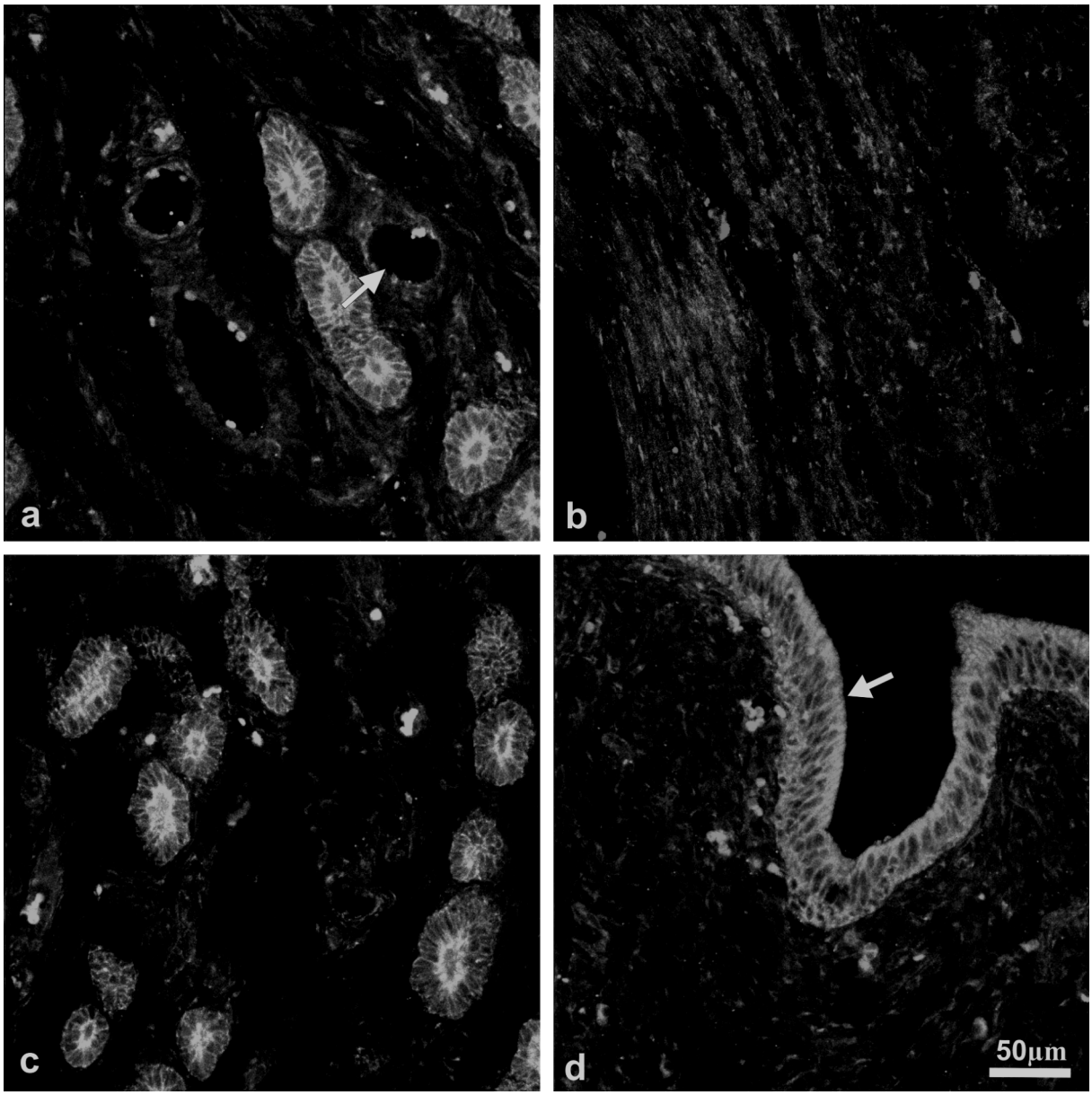

Fig. 5. a – Uterine horn. Immunostaining for NK1 receptor in the blood vessels (arrow); b – Uterine horn. Immunostaining for NK1 receptor in the muscular membrane (arrow); c – Uterine horn. Immunostaining for NK1 receptor in the submucosa (arrow); d – Uterine horn. Immunostaining for NK1 receptor in the mucosal epithelium (arrow).

papers dealing with this problem in the female reproductive organs are even more scarce (Garcia-Fernandez et al. 2003). NK1 receptor was detected in the rat uterus with RT-PCR (Candenas et al. 2001, Dangoor et al. 2005).

Application of the commercial antibody to NK1 in Western blot allowed to detect one specific protein band of the size identical with the expected size of the human NK1 receptor in all the studied parts of the porcine reproductive system – ovary, oviduct, uterine horn and cervix. The expected molecular weight was 46.25 kDa. In addition to the specific band some bands with molecular weight different from the expected one were detected. It is possible that the additional bands represent the receptor protein which underwent additional posttranslational modification, like glycosylation (Assil and Abou-Samra 2001). Human NK1 receptor protein contains two possible glycosylation sites. Coupling receptor protein to polysaccharide molecules may significantly alter the molecular mass and the position of the protein band in the gel. Another reason of the presence of additional bands may be the presence of different receptor protein forms resulting from the alternative primary transcript splicing (Pisarchik and Slominski 2002). The presence of NK1 receptor was detected with

immunoblot in tissues of laboratory animals (Rasley et al. 2002), but no such studies were done in the pig, nor in other species of domestic animals.

Morphological studies on the distribution of neuropeptide receptors in the peripheral tissues of animals are rather scarce and those dealing with the reproductive system are even scarcer (Rothstein et al. 1991).

The presence of specific receptors may be deduced from the fact of the presence of nerve fibers containing the neuropeptide, as well as from the effect of the neuropeptide on the tissues (Brunsson et al. 1995).

The previously conducted studies showed that in sexually immature gilts SP-immunoreactive nerve fibers were comparatively scarce, located mainly under the capsulla and rarely seen around the ovarian follicles (Majewski 1997). No studies on the presence of SP-immunoreactive nerve fibers in the porcine oviduct and uterus were performed. However, in the laboratory animals the presence of SP-immunoreactive nerve fibers in the oviduct was described in the mucosa, both under the epithelium and around blood vessels, as well as in the muscular membrane of both organs (Owman and Stjernquist 2003). Such distribution suggests that the part of the SP-immunoreactive nerve fibers belongs to the sensory fibers, while the other ones influence the functions of the blood vessels and muscular membranes.

In the ovary NK1 receptor was detected immunohistochemically in endothelium and muscular membrane of the blood vessels, in primary ovarian follicles as well as in the granulosa of the bigger ovarian follicles. Results obtained with in situ hybridization gave similar results, although no NK1 receptor transcripts were not found in primary ovarian follicles. The presence of NK1 receptor concords with results of pharmacological studies where relaxatory effect of SP on ovarian blood vessels was found (Ottesen et al. 1983). NK1 receptor gene was found to be expressed in oocytes (Pintado et al. 2003), while SP was found to modulate the secretion of steroid hormones by ovarian interstitial cells (Angelova et al. 1991), what must be associated with the occurrence of NK1 receptor on these cells. In this study it was not definitely confirmed that NK1 receptors was present in ovarian structures other than blood vessels and ovarian follicles. The reason may be the insufficient sensitivity of immuno- and hybridohistochemistry, or the authentic absence of this receptor in the ovaries of immature gilts in which no sex steroids are present, what may affect NK1 receptor gene expression (Pinto et al. 1999).

In the uterus NK1 receptor was detected with immunohistochemistry and in situ hybridization in the endothelium of arterial vessels, muscular membrane as well as in the mucosal epithelium. Until now NK1 receptor was detected by autoradiography only in the blood vessels of the human oviduct (Nimmo et al. 1989). No studies dealing with the distribution of this receptor in the uterus exist. However, it is known that SP influences the contractility of the oviduct (Patak et al. 2003). NK1 receptor is responsible for the increase of vascular permeability in the uterine horn and cervix after the antydromic stimulation of the dorsal radices of lumbar nerves associated with the SP release form C-type nerve fibers (Pinter and Szolcsanyi 1995). Other effects of SP action in the uterus are contraction of muscular membrane, relaxation of blood vessels, disturbances of electrolyte transport across the endometrium (Gram and Ottesen 1982). The distribution of NK1 receptor in the oviduct and uterus resembles that described in other organs of laboratory animals. NK1 receptor was found in the vascular endothelium of the human urinary bladder (Burcher et al. 2000) and lungs (Mechiche et al. 2003). NK1 receptor was also found in the muscular membranes of the canine stomach and intestines (Mantyh et al. 1988), as well as human colon (Korman et al. 1989). The presence of NK1 receptor in the mucosal epithelium of the oviduct and ovary is amazing, however this receptor was also described earlier in mucosal epithelium of the guinea pig colon (Burcher et al. 1986) and rat trachea and bronchi (Ichikawa et al. 1995).

Our results clearly confirmed the expression of the NK1 receptor in all the organs of the porcine female reproductive tract at the level of mRNA and protein by the detection of the correct size PCR product and correct molecular weight protein band in immunoblot. The immunohistochemical stainings revealed the tissue distribution of the NK1 receptor protein. In addition to the expected localization of the receptor in the arterial epithelium and smooth muscles of the tubular organs, the receptor was found in ovarian follicles of various stages what may suggest the influence of SP on the maturation of the follicles, possibly the process of ovulation and the steroidogenesis. The described here presence of NK1 receptor in the mucosal epithelia suggest the influence of SP on the functions of the epithelia. However, the significance of these findings must be verified with physiological and pharmacological studies.

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