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Effects of neuropeptide S on the proliferation of splenic lymphocytes, phagocytosis, and proinflammatory cytokine production of pulmonary alveolar macrophages in the pig

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ABSTRACT

Neuropeptide S (NPS), a newly identified neuropeptide, is involved in many physiological and pathological activities through the NPS receptor (NPSR). Recently, the NPS and NPSR have been detected in peripheral systems of pigs including immune tissues, suggesting that NPS may play an important role in the regulation of immune function. The aim of this study was to demonstrate the presence and function of NPS and NPSR in splenic lymphocytes (SPLs) and pulmonary alveolar macrophages (PAMs) of pigs. By RT-PCR, the expression of NPS and NPSR mRNA was detected in the SPLs and PAMs. NPS immunoreactivity was observed in the membrane and cytoplasm of both SPLs and PAMs. We found that NPS could stimulate the proliferation of SPLs, when NPS was added at concentrations of 0.01, 0.1, 1, 10, 100 and 1000 nM alone or in combination with PHA/LPS *in vitro*. In macrophages from bronchoalveolar lavage (BAL) fluid of pigs, various doses of NPS (0.01, 0.1, 1, 10, 100 and 1000 nM) up-regulated the phagocytosis of PAMs in comparison to controls. In PAMs, NPS could induce the production of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. Taken together, all data suggest that NPS is capable of inducing phagocytosis of non-opsonized *E. coli*. NPS might act as potent neuroimmunomodulatory factors and affects the maintenance of immune homeostasis.

1. Introduction

Neuropeptide S (NPS) is the endogenous ligand of NPS receptor (NPSR), a previously named orphan G-protein-coupled receptor [2,9,18,20,23]. Distributions of NPS and NPSR have been reported in rats and pigs [23,24,25]. In our previous study, NPS and NPSR mRNAs were widely expressed in various tissues of the pig. NPS mRNA was highly expressed in CNS, while NPSR mRNA was widely expressed in many tissues, with high expression in the hypophysis, endocrine tissues and glands. In the pig brain, NPS immunoreactive cells are mainly found in the diencephalon, pons and hypophysis, while immunoreactive fibers are widely distributed in the hypothalamus and olfactory bulb. In the peripheral organs, NPS immunoreactive cells are observed in the respiratory tract, alimentary tract, endocrine organs, genitourinary tract, lymphatic organs, muscle tissue, skin and skin appendages [25]. NPS and NPSR constitute a novel neuropeptide system that plays important roles in regulating many physiological and pathological activities including arousal [23], anxiety [17,23], motor activity [15,18,23], food intake [1,5,14,21], stress and brain injury [3,21], drug reward [12], learning and memory [10], and antinociception [13]. Interestingly, few studies reported the effect of NPS or its receptor on the immune system. Pulkkinen et al. reported that NPS and its receptor modulate macrophage immune responses in mice, and that the macrophages which respond to NPS would reduce adhesion but increase phagocytosis and chemotaxis [16]. The evidences from genetic studies demonstrated the association of NPSR gene polymorphism with chronic inflammatory diseases of the respiratory [11] and gastrointestinal [6] systems. These findings indicate that the NPS–NPSR system may play critical role in modulating innate immunity and chronic inflammatory diseases of epithelial barrier organs. However, the role of NPS in immune system needs to be further investigated.

In our previous study, we successfully cloned the genes of NPS and NPSR, determined NPS and NPSR mRNA levels, and investigated the distribution of NPS in the pig [25]. It is worth noting that the NPSR has also been detected in immune tissues, including thymus, spleen, jejunal lymph node and soft palatine tonsil. However, the functions of NPS and NPSR in immune system are still unknown in the pig.

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In the present study, using specific primers, the expression of NPS and NPSR mRNAs in splenic lymphocytes (SPLs) and pulmonary alveolar macrophages (PAMs) of pigs was investigated by RT-PCR. Then the specificity for NPS immunolabeling was confirmed by immunohistochemistry in SPLs and PAMs. We further investigated the effects of NPS on the proliferation of splenic lymphocytes and macrophage phagocytosis. Moreover, we examined the effect of NPS on the production of pro-inflammatory cytokines in pig PAMs. Our results implicate a novel role for NPS in increasing the proliferation of SPLs or stimulating macrophage functions of PAMs. These findings provide the first evidence that NPS regulates pro-inflammatory immune responses. NPS might act as a potent immunomodulatory factor and affect the maintenance of immune homeostasis.

2. Materials and methods

2.1. Animals

Normal Meishan pigs aged 30 days and weighing 8 ± 2 kg were purchased from the breeding center of Jiangsu Polytechnic College of Agriculture and Forestry. All pigs were fed according to the breeding standards of Chinese Local Pigs and National Research Council (NRC). The procedures in this study were undertaken according to the guidelines of the regional Animal Ethics Committee and the rules for experimental animals of Nanjing Agricultural University.

2.2. SPLs isolation, culture and treatment

Pigs were sacrificed according to the experimental protocol. The spleen was rapidly harvested and placed in a small culture dish, followed by an extraction to a cell suspension by squeezing through a cell strainer with the bended syringe needle of a 5-mL syringe plunger. The splenocytes were dissociated from the connective tissue capsule by gently pressing the organ through fine, sterile nytox mesh. Cells were centrifuged at $1700 \times g$ at 4 °C for 15 min, erythrocytes were lysed with red blood cell lysis buffer for 15 min, and the supernatant was discarded. The remaining cells were washed twice with 0.1 M PBS (pH 7.2). For the depletion of monocytes, spleen cells were cultured in RPMI 1640 complete medium containing 10% heat-inactivated fetal bovine serum (HIFBS) (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), 2 mM L-glutamine (Sigma, St. Louis, MO), 100 U/mL of penicillin, and 100 mg/mL of streptomycin (Invitrogen, Carlsbad, CA) and incubated for 1 h at 37 °C with 5% CO_2 to allow the monocytes to attach to the flask bed. The viability and concentration of cells were determined by Trypan blue exclusion in a hemacytometer. The final cell concentration was adjusted to 10^6 cells/mL. The cell suspension with 95% or higher viability was used for experiment assay.

2.3. Pulmonary alveolar macrophages (PAMs) isolation, culture and treatment

Bronchoalveolar lavage and collection were performed as previously described [4]. The cells were treated with red blood cell lysis buffer to remove erythrocytes and centrifuged at $1500 \times g$ at 4 °C for 10 min, and the supernatant was discarded. PAMs were washed with RPMI 1640 complete medium. The cell viability and concentration were evaluated by Trypan blue exclusion in a hemacytometer. Only those cells with viability above 95% were used.

2.4. Determination of NPS and NPSR mRNA

Total RNA was extracted from SPLs using TRIZOL (TaKaRa Biotechnology, Dalian, China) following the manufacturer's instructions. 2 μL of RNA (1 μg/μL) was reversely transcribed into cDNA with TaqMan reverse transcription reagents and oligo (dT)18 primers (Promega Biotechnology, Beijing, China). 2 μL of RT reaction mixture and 1.0 U Taq DNA polymerase (TaKaRa) were used for PCR in a final volume of 25 μL. The specific PCR primers for NPS and NPSR and 18S ribosomal RNA (18S rRNA) were designed using the primer premier 5.0 and based on the conserved region of pig NPS gene from GenBank (GenBank accession no. FJ024707), pig NPSR from GenBank (GenBank accession no. FJ444795) and pig 18S rRNA from GenBank (GenBank accession no. NR_002170). The primers, RT-PCR reaction system and amplification procedures were performed as described previously [25]. Different controls were used to monitor the possible contamination of genomic DNA and environmental DNA at both stages of RT and PCR. RT-PCR was performed in PCR system T-1 thermoblock (Biometra, Goettingen, Germany) and repeated for at least three times.

2.5. Immunohistochemistry

SPLs were centrifuged at 1000 rpm at 4 °C for 10 min, and the supernatant was discarded. The remaining cells were washed twice with 0.1 M PBS (pH 7.2). SPL suspensions were adjusted to a final concentration of 5×10^5 cells/mL in 0.1 M PBS. 30–50 μL cell suspensions were seeded onto microscopic slide and placed at room temperature for 10 min. The supernatant was carefully discarded. The slides were air-dried (<30 °C) for 15 min. 5×10^5 cells/mL PAMs were seeded onto cover slips in the six-well plates and incubated in RPMI 1640 for 44 h at 37 °C to allow the PAMs to attach to the coverslips. The coverslips were washed twice in 0.1 M PBS (pH 7.2) for 5 s and air-dried for 15 min. Slides were processed for immunohistochemistry using a previously validated method [25]. The cells on the slides or coverslips were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.2) for 10 min, then washed with PBS for 5 s. The anti-NPS antibody (ab9614, Millipore, Billerica, MA) was diluted in 0.1 M PBS (working dilution 1:800). Cells were counterstained with hematoxylin. In negative control, primary antibody was replaced with normal goat serum (Wuhan Boster Biological Technology Co. Ltd., Wuhan, China). Images were acquired using bright-field microscopy (Olympus BH-2, Olympus China Ltd. Corp., Beijing, China).

2.6. Proliferation assays

SPL proliferation was determined by a colorimetric technique using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution [22]. Briefly, SPLs (10^6 cells/mL) were harvested and reseeded in 96-well microplate supplemented with both NPS at desired concentrations (0.01, 0.1, 1, 10, 100, 1000 nM, AnaSpec, Inc., Fremont, CA) and 80 μg/mL phytohemagglutinin (PHA, Sigma) or 20 μg/mL lipopolysaccharide (LPS, Sigma). Additionally, cells were also stimulated with PHA/LPS alone or various concentrations of NPS alone. The experimental design is shown in Table 1. After incubation for 44 h at 37 °C with 5% CO_2, 0.5 mg/mL MTT (Amresco, Solon, OH) was added to each well, and incu-

Table 1
The method of sample application in proliferation experiment.

Group	Cells (μL)	NPS (μL)	PHA/LPS (μL)	RPMI1640 (μL)
NPS + PHA/LPS group	100	50	50	0
NPS group	100	50	0	50
PHA/LPS group	100	0	50	50
Control	100	0	0	100
Blank	0	0	0	200

NPS, PHA and LPS were diluted with RPMI-1640 complete medium.