

Received 12 October 2002
Accepted 21 November 2002

Short Communication

EFFECT OF INTRAMUSCULAR APPLICATION OF SELECTED NEUROPEPTIDES ON MORPHOLOGY OF MUSCLE

HANNA GENDEK-KUBIAK*

Department of Cytophysiology, Histology and Embryology, Medical University
of Łódź, ul. Narutowicza 60, 90-136 Łódź, Poland

Abstract: The aim of the study was to examine a morphological picture of guinea pig skeletal muscles injected with neuropeptide Y (NPY), substance P (SP) or vasoactive intestinal peptide (VIP), to evaluate the influence of a single injection of the mentioned neuropeptides (NPS) on muscle morphology and mast cell, T lymphocyte and macrophage chemotaxis. There were different degrees of muscle fibre injuries as well as different intensities and compositions of infiltrations inside the muscle after the introduction of the NPS. The observed changes did not disappear, but increased after 24 hours, comparing to the 3-hour post-injection changes, suggesting that NPS are proinflammatory rather than antiinflammatory factors in skeletal muscles. The local, particularly delayed action of NPS *in vivo* requires further studies.

Key Words: Neuropeptides, Skeletal Muscles, Histology, Immunohistochemistry

INTRODUCTION

NPS are phylogenetically old protein compounds occurring in humans and in animals with a high degree of identity among vertebrate species [1]. They act as neurotransmitters or neuromodulators and also exert a number of functions within the immune system [2].

The SP innervation is mainly of primary sensory origin, the NPY innervation is chiefly derived from postganglionic noradrenergic sympathetic neurons, and VIP is present mainly in postganglionic cholinergic neurons [3, 4]. In neurogenic

* E-mail: h_kubiak@hotmail.com

inflammation, provoked by electric stimulation of primary afferent nerves, the sensory NPS (mainly SP) have been identified as potent mediators of inflammatory and immunologic reactions [5]. Both stimulatory and inhibitory effects on lymphocytes and macrophages have been demonstrated for different NPS *in vitro* at concentrations which are similar to those in the circulation and in tissues [6, 7]. SP mediates production of the stem cell factor in bone marrow [8] and NPY is an angiogenic and mitogenic factor [9].

Most of NPS-containing nerve fibres in skeletal muscles are located in blood vessel walls, and are able to induce vasodilation (SP, VIP) [10] and vasoconstriction (NPY) [11].

A quantitative image analysis showed that during both acute and persistent myositis, the area of SP immunoreactivity in spinal dorsal horn neurones was reduced [12]. This probably reflects an increased spinal release of SP from the terminals of primary sensory neurones activated by the inflammation.

The implicated questions concern the effects of NPS not only on the inflammatory processes in muscles, but also on the muscles themselves, e.g.: do NPS induce any histologically observable effects, when applied exogenously, intramuscularly, in a single dose? Such experiments have not been performed so far.

MATERIALS AND METHODS

12 male guinea pigs at the age of 5 - 6 months, weighing 350 - 400 G each, were used. 0.3 ml samples of 10^{-8} M of NPY (Tocris, GB), 3.3×10^{-6} M of SP (Sigma) and 10^{-6} M of guinea pig VIP (Tocris, GB) solutions were injected intramuscularly into the quadriceps muscle. Each of the studied NPS was injected into 4 animals. As a control, quadriceps muscles from the opposite side of the body of the same animals, injected with 0.9% NaCl, were used. The material (the central part of the muscle, ~ 1.5 cm long) was taken from animals subjected to sodium pentobarbital anaesthesia. Two animals of each treatment group were killed in the 3rd hour of the experiment, and the rest of them - in the 24th hour. One half of each excision was fixed in Bouin's solution, while the other one in Carnoy's solution; they were paraffin embedded and used for histology and immunohistochemistry.

Stainings of guinea pig pan T cells' antigen and of macrophages/monocytes were performed using a two-step indirect immunohistochemical method.

Macrophages were detected using monoclonal IgG1 mouse primary antibody, clone MAC387 (Serotec, GB) in dilution 1:100, and for T-cells monoclonal IgG1 mouse primary antibody clone CT5 in dilution 1:120 was used. Primary antibodies were incubated for 1 hour with tissue sections after blocking endogenous peroxidase with 2% hydrogen peroxide. Slides were washed with PBS and incubated for 1 hour with peroxidase-labeled rabbit F(ab')₂ anti-mouse IgG (Serotec, GB), in dilution 1:50. The negative control was obtained by

omitting the primary antibody. After thorough washing in PBS, they were detected using 3,3'-diaminobenzidine substrate solution (Sigma).

Additionally, Astra blue staining for mast cells was performed.

From each of the excisions, at least six non-consecutive slides were used for any of the staining methods. We established infiltration intensity rates in 10 fields of vision per slide using a magnification of 240x.

RESULTS AND DISCUSSION

Histological changes were not found in any control specimens. 3 and 24 hours after the NPS injections, at concentrations concordant with the orders of magnitude reported as biologically active [6,7], characteristic vascular reactions - extension and overfilling of blood vessels (SP, VIP) and constriction of arteries (NPY) were noted. There were infiltrations in the endomysium of affected areas composed primarily of mononuclears; neutrophils and other cells were rarely noticed (Figs 1-4). Mast cells were not numerous in the control and in the NPS-injected muscles, but they were never absent there. The specification of infiltrations is shown in Tab. 1.

Tab. 1. The maximal intensity and composition of infiltrations in quadriceps muscles after NPS introduction. We established an infiltration intensity rate in the field of vision, using a magnification of 240x: + - up to 20 cells of infiltration in the field of vision, ++ - over 20 to 50 cells of infiltration in the field of vision, +++ - over 50 to 100 cells of infiltration in the field of vision, ++++ - over 100 cells of infiltration in the field of vision.

NPS	time	intensity of infiltrations	% of macrophages	% of T cells
SP	3 h	++	above 50% (55-65%)	less than 50% (30-40%)
	24 h	++++	above 50% (55-65%)	less than 50% (35-45%)
VIP	3 h	++	about 70% (65-75%)	up to 25% (20-25%)
	24 h	+++	about 70% (65-75%)	up to 25% (20-25%)
NPY	3 h	+	about 10% (8-15%)	about 10% (8-10%)
	24 h	++	about 10% (8-12%)	about 10% (8-12%)

Muscle fibres in various stages of degradation were apparent; the effects seen earlier were amplified after 24 hours.

After SP introduction, the affected muscle fibres were numerous, many being fragmented, invaded by infiltrating cells and showing dissolution of their myofibrillar material (Figs 1,2). After guinea pig VIP injection, destruction of muscle fibres was less abundant - dense, mainly macrophagic infiltrations were located in the endomysium and inside scattered injured muscle fibres (Fig. 3).

After NPY injection, muscle fibre injuries were less pronounced than after SP and VIP. The affected muscle fibres were surrounded and invaded by infiltrating cells and, in the neighbourhood of injured fibres, there were many non-injured

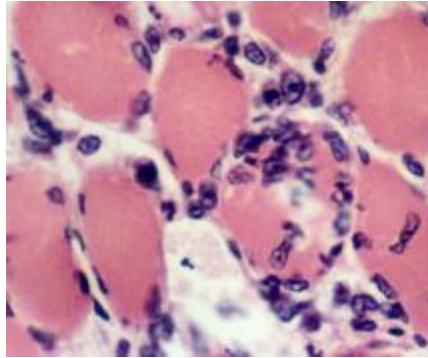


Fig. 1. Guinea pig quadriceps muscle 24 hours after SP injection. Some muscle fibres show massive mononuclear cell infiltration. Haematoxylin and eosin. x600.

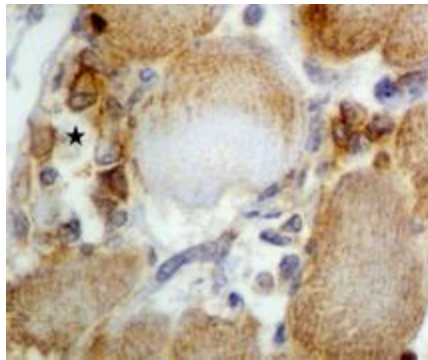


Fig. 2. Guinea pig quadriceps muscle 24 hours after SP injection. Immunostaining of T cells. Positive cells are present in infiltration among muscle fibres and inside completely damaged muscle fibres. x600.

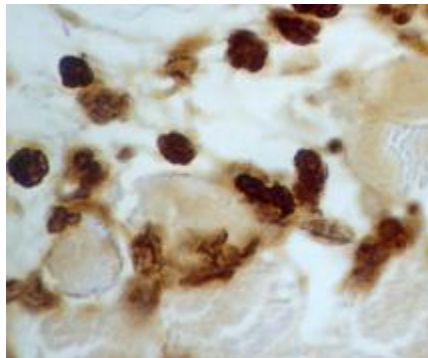


Fig. 3. Guinea pig quadriceps muscle 24 hours after VIP injection. Immunostaining of macrophages. Majority of infiltrating cells are positive. x600.

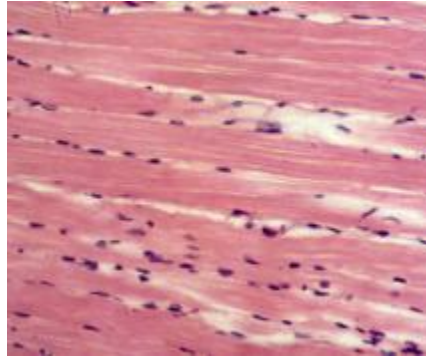


Fig. 4. Guinea pig quadriceps muscle 3 hours after NPY injection. Discrete infiltration of mononuclear cells without visible destruction of muscle fibres. HE. x240.

ones (Fig. 4). Up to 80% of mononuclear infiltrating cells were neither T-cells nor macrophages, and thus they might represent B-cells, which are mobilised in the blood by NPY [13]. The differences in infiltration components reported in this paper, probably result from diversified chemotactic action of the studied NPS on different kinds of cells.

Skeletal muscle damaging effects of NPS had not been reported earlier.

Since NPY, SP and VIP intramuscular injections induce muscle fibre damage and cellular infiltrations, endogenous NPS are probable factors potentiating muscle inflammatory processes. A large proportion of T-cells in infiltrations accompanying a pronounced muscle fibre injury may suggest cytokine-provoked changes [14]. The mechanism of the observed muscle damaging action of NPS is unknown and awaits elucidation. The local, particularly delayed action of NPS in skeletal muscles *in vivo* requires further studies.

Acknowledgements. This study was supported by Medical University of Łódź Grant No. 502-11-618.

REFERENCES

1. Hoyle, C.H.V. Neuropeptide families: evolutionary perspectives. **Reg. Peptides** 3 (1998) 1-33.
2. Pincelli, C., Fantini, F. and Giannetti, A. Neuropeptides and skin inflammation. **Dermatology** 187 (1993) 153-158.
3. Nohr, D. and Weihe, E. The neuroimmune link in the bronchus-associated lymphoid tissue (BALT) of cat and rat: peptides and neural markers. **Brain Behav. Immun.** 5 (1991) 84-101.
4. Karanth, S.S., Springall, D.R., Kuhn, D.M., Levene, M.M. and Polak, J.M. An immunocytochemical study of cutaneous innervation and the distribution

- of neuropeptides in man and commonly employed laboratory animals. **Am. J. Anat.** 2191 (1991) 369-383.
5. Payan, D.G. Neuropeptides and inflammation: the role of substance P. **Annu. Rev. Med.** 40 (1989) 341-352.
 6. Schratzberger, P., Reinisch, N., Prodinger, W.M., Kahler, Ch.M., Sitte, B.A., Bellmann, R., Fischer-Colbrie, R., Winkler, H. and Wiedemann, Ch.J. Differential chemotactic activities of sensory neuropeptides for human peripheral blood mononuclear cells. **J. Immunol.** 158 (1997) 3895- 3901.
 7. Song, C. and Leonard, B.E. Comparison between the effects of sigma receptor ligand JO 1784 and neuropeptide Y on immune functions. **Eur. J. Pharmacol.** 345 (1998) 79-87.
 8. Rameshwar P. Substance P: a regulatory neuropeptide for hematopoiesis and immune functions. **Clin. Immunol. Immunopathol.** 85 (1997) 129-133.
 9. Zukowska-Grojec, Z., Karwatowska-Prokopczuk, E., Rose, W., Rone, J., Movafagh, S., Ji, H., Yeh, Y., Chen, W., Kleinman, H.K., Grouzmann and Grant, D.S. Neuropeptide Y. A novel angiogenic factor from the sympathetic nerves and endothelium. **Circ. Res.** 83 (1998) 187-195.
 10. Morris, J.L., Grasby, D.J., Anderson, R.L. and Gibbins, I.L. Neurochemical distinction between skeletal muscle vasodilator neurons and pelvic vasodilator neurons in guinea-pigs. **J. Auton. Nerv. Syst.** 71 (1998) 64-68.
 11. Modin, A., Pernow, J. and Lundberg, J.M. Sympathetic regulation of skeletal muscle blood flow in the pig: a non-adrenergic component likely to be mediated by neuropeptide Y. **Acta Physiol. Scand.** 148 (1993) 1-11.
 12. Hoheisel, U., Kaske, A. and Mense, S. Relationship between neuronal activity and substance P-immunoreactivity in the rat spinal cord during acute and persistent myositis. **Neurosci. Lett.** 257 (1998) 21-24.
 13. Bedoui, S., Kuhlmann, S., Nave, H., Drube, J., Pabst, R. and von Horsten, S. Differential effects of neuropeptide Y (NPY) on leukocyte subsets in the blood: mobilization of B-1-like B-lymphocytes and activated monocytes. **J. Neuroimmunol.** 117 (2001) 125-132.
 14. Levite, M. Nerve-driven immunity. The direct effects of neurotransmitters on T-cell function. **Ann. N. Y. Acad. Sci.** 917 (2000) 307-321.