Influence of renovascular hypertension on the distribution of vasoactive intestinal peptide in the stomach and heart of rats

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Abstract

Arterial hypertension is associated with serious dysfunction of the cardiovascular system and digestive system. Given the relevant role of vasoactive intestinal peptide (VIP) in the regulation of digestion process, control of blood pressure and heart rate as well as cardio- and gastro-protective character of the peptide, it appeared worthwhile to undertake the research aimed at immunohistochemical identification and evaluation of VIP-positive structures in the pylorus and heart of hypertensive rats. Up to now, this issue has not been investigated. The experimental model of hypertension in rats according to Goldblatt (two-kidney one clip model of hypertension) was used in the study. The experimental material (pylorus and heart) was collected in the sixth week of the study. VIP-containing structures were evaluated using immunohistochemical and morphometric methods. The analysis of the results showed a significant increase in the number of immunoreactive VIP structures and in the intensity of immunohistochemical staining in the stomach and in the heart of hypertensive rats. Our findings indicate that VIP is an important regulator of cardiovascular and digestive system in physiological and pathological conditions. However, to better understand the exact role of VIP in hypertension further studies need to be carried out.

Keywords: Vasoactive intestinal peptide, stomach, heart, rat, renovascular hypertension, immunohistochemistry

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Introduction

Besides the central nervous system and endocrine system, a crucial role in maintaining of organism homeostasis is attributed to neuropeptides secreted by the nerve terminals and cells belonging to diffuse neuroendocrine system (DNES).^{1,2} Therefore, in latest research much attention is paid to function of neuropeptides under physiological and pathological conditions. One of these neuropeptides is vasoactive intestinal peptide (VIP) that functions as a neurotransmitter and neuromodulator and exerts on function of many organ systems. It is known that VIP plays a very important role in the regulation of digestive system. It stimulates epithelial secretion and absorption in small intestine, whereas inhibits gastric secretion, directly affecting on parietal cells activity and by attenuating gastrin release from G cells.^{3–5} VIP also regulates smooth muscle activity and blood flow in the gastrointestinal tract.^{5–7}

Experimental research indicated that VIPs have capability to induce tachycardia and myocardial contraction, reduces the vascular tension, and lowers arterial pressure.^{8,9}

Documented literature data allow to suspect that peptide fulfills cardioprotective role in various pathological conditions.^{10,11}

VIP participates also in regulation of immune response and inflammatory processes. There are evidences that peptide inhibits secretion of proinflammatory cytokines and suppress phagocytic properties of macrophages.^{12,13}

Given the above and result of our previous observations, which demonstrate changes in activity of various types of DNES cells in several organs of two-kidney one clip (2K1C) rats^{14–16} as well as lack of literature data considering contribution of VIP in renovascular hypertension, it appeared worthwhile to undertake the research which aim is immunohistochemical identification and evaluation of VIP-positive structures in pylorus and heart of rats with renovascular hypertension.

Materials and methods

The hypothesis, the aim and the plan of the study, as well as the approach to animals were approved by the Senate Commission for the Supervision of Studies on Human and

Animal Subjects, Medical University in Białystok (Resolution no. 39/2013).

The study was performed on 20 ($n=20$) six-week-old male Wistar rats (body weight at the beginning of the experiment 160–180 g), divided in two control groups: first—five rats, did not undergo any surgical procedure, and second control group—five rats, underwent sham operation, and one experimental group—10 rats with induced hypertension.

After one-week period of acclimatization, the systolic blood pressure (BP) of each rat was measured, and the surgical procedure for induction of renovascular hypertension and sham operation was performed.

2K1C renovascular hypertension

Induction of experimental hypertension was performed according to procedure by Goldblatt *et al.*¹⁷ After the rats were anesthetized by exposure to pentobarbital (40 mg/kg, i.p.), a 3-cm retroperitoneal flank incision was performed under sterile conditions. The left kidney was exposed and the renal artery was carefully dissected free of the renal vein. The renal artery was then partially occluded by placing a silver clip with an internal diameter of 0.20 mm on the vessel. The wound was closed with a running 3-0 silk suture ($n=10$). Sham operated rats ($n=5$) underwent identical surgical procedures, except that a clip was not applied to the renal artery. After the surgery, the rats were kept in single cages till wound healing.

After six weeks after surgery, the systolic BP was measured by using a Student Oscillograph Rat Tail Blood Pressure Monitor, Harvard, England.¹⁸ The BP measurements were considered valid only when three consecutive readings did not differ by more than 5 mmHg. The average of the three measured values was then recorded. After this time, eight 2K1C rats developed stable hypertension (mean BP values 163 ± 3.2 mmHg).

Method of experimental material collection and fixation

Six weeks after surgery fragments of stomach (pylorus) and heart were collected under deep pentobarbital anesthesia (50 mg/kg b.w.). The specimens were fixed in 4% buffered formalin for 48 h at $+4^{\circ}\text{C}$ and processed routinely for embedding in paraffin. Sections were cut at $4\text{ }\mu\text{m}$ in thickness and stained by hematoxylin–eosin (H + E) for general histological examination and processed by immunohistochemistry for VIP detection. Weight data of left and right kidney were additionally collected.

Identification of VIP-containing structures by immunohistochemical methods

In the immunohistochemical study, the EnVision method was used according to Herman GE and Elfont EA.¹⁹ Immunostaining was performed by the following protocol: paraffin-embedded sections were deparaffined and hydrated in pure alcohols. For antigen retrieval, the sections were subjected to pretreatment in pressure chamber and heated for 1 min at 21 psi (one pound force per square inch [1 psi] equates to 6.895 kPa, the conversion factor has

been provided by the United Kingdom National Physical Laboratory) at 125°C , using Target Retrieval Solution with pH of 6.0 (S 2369 Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup). After cooling down to room temperature, the sections were incubated with Peroxidase Blocking Reagent (S 2001 Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup) for 10 min to block endogenous peroxidase activity.

The sections with the primary antibody for VIP peptide (rabbit polyclonal VIP antiserum, No H-064-16, purchased at the Phoenix Pharmaceuticals, Inc. [Mountain View, CA]) were incubated overnight at 4°C in a humidified chamber. The antiserum was previously diluted in Antibody Diluent (S 0809 Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup) in relation 1:10,000.

Procedure was followed by incubation with secondary antibody (conjugated to horseradish peroxidase-labeled polymer) (EnVision + Kit HRP Rabbit K4011 Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup). The bound antibodies were visualized by 1 min incubation with liquid 3,3'-diaminobenzidine substrate chromogen. The sections were finally counterstained in hematoxylin QS (H-3404, Vector Laboratories; Burlingame, CA), mounted and evaluated under light microscope. Appropriate washing with Wash Buffer (S 3006 Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup) was performed between each step.

Specificity tests, performed for the VIP antibody included: negative control where the antibodies were replaced by normal rabbit serum (Vector Laboratories; Burlingame, CA) at respective dilution (the performed control reactions gave negative results, no staining) and a positive control was prepared with specific tissue (for our research concerning VIP we used rat small intestine).

Microscopic and quantitative analysis

The results of immunohistochemical staining were submitted for evaluation in an Olympus BX41 microscope with Olympus DP12 camera. From each animal five sections of pylorus and heart were studied. VIP-containing structures were searched for and their topography was observed.

From all sections of heart and stomach, five randomly selected microscopic fields, each field of 0.785 mm^2 , magnification of 200x (20x the lens and 10x the eyepiece), were documented. Subsequently, images were submitted to morphometric evaluation by using NIS-Elements Advanced Research software of Nikon.

In each analyzed image, pyloric cells containing VIP were counted and presented as a mean value per 1 mm^2 of the stomach section area. In each of the analyzed images of the heart, the width of 25 randomly selected cardiomyocytes was measured and presented as mean values.

All data were statistically analyzed by means of software computer package Statistica Version 10.0. The corresponding mean values were computed automatically; significant differences were determined by Student's *t*-test; $P < 0.05$ was taken as the level of significance.

Results

Since no significant differences were found between the two control groups of rats, only results concerning sham-operated animals were taken into account.

Six weeks after placing the clip on renal artery, the values of BP were significantly increased, as compared to control animals (Table 1). Long-term restriction of blood supply resulted in the substantial atrophy of left kidney, while the mass of right unclipped kidney was slightly increased, when compared to kidneys of normotensive rats (Table 1).

Routine histopathological examination showed no noticeable changes in the morphology of the stomach section obtained from all study animals, whereas in the heart of hypertensive rats an increase was observed in the leukocyte count, compared to control rats. In the heart of 2K1C rats, cardiomyocytes varied in terms of intensity of cytoplasmic staining, in contrast to sham-operated rats, where eosin staining was weak and homogeneous in the majority of cardiac muscle cells (Figure 1(a) and (b)).

Positive immunohistochemical reaction was seen in all study of stomach and heart specimens; however, the density of immunoreactive VIP (VIP-IR) structures and intensity of their staining varied between experimental and control animals (Figure 2(a) to (d) and 3(a) and (b)).

Immunodetection of VIP in the rat pylorus showed its presence in ganglion cells of myenteric plexuses, in nerves innervating muscularis externa (Figure 2(a) and (b)) and in endocrine cells of gastric gland epithelium (Figure 2(c) and (d)). In the normotensive rat stomach, the reaction in intermuscular plexuses was delicate and only several VIP-positive nerve fibers were noticed in smooth muscle layers (Figure 2(a)). Much stronger VIP immunoreactivity and higher density structures containing the peptide were found in the stomach of hypertensive rats (Figure 2(b)). Similarly, the neuroendocrine cells containing VIP were more numerous in the stomach of 2K1C rats (Figure 2(d)), compared to control animals, where only single VIP-IR cells were observed (Figure 2(c)). The microscopic evaluation was confirmed by the morphometric analysis, which showed a threefold increase in the number of endocrine cells with a positive immunoreaction in hypertensive rats compared to sham-operated rats (2K1C rats— $30.3 \pm 2.14^*$, control rats— 10.1 ± 1.67 neuroendocrine cells/mm² of stomach section).

In the heart of rats, VIP-immunosignal occurred in nerve fibers innervating myocardium and in nerves reaching vessels. Positive reaction on VIP was also found in the endothelial cells of coronary vasculature (Figure 3(a) and (b)).

In the cardiac wall of control animals, VIP-IR nerve fibers were few in number and weakly stained (Figure 3(a)). In the heart of hypertensive rats, VIP-containing structures were more abundant and showed stronger immunostaining, in comparison to normotensive animals (Figure 3(b)).

The morphometric analysis showed an increase in the width of cardiomyocytes in rats with renovascular hypertension as compared to normotensive animals ($11.8 \pm 1.99^*$, 8.3 ± 1.73 , respectively).

Discussion

So far, few reports have appeared exploring the potential involvement of VIP in the process of increased BP. This is the first report undertaking the assessment of VIP in the heart and stomach in experimental renal vascular hypertension.

Our current results indicate that hypertension causes an increase in the number and immunoreactivity of VIP-positive structures in rat heart and stomach. We confirmed the presence of VIP in the nervous system of pylorus. In addition, VIP was also expressed in neuroendocrine cells in the rat pyloric mucosa.

The pathogenesis of essential hypertension is complex and, up to now, not well explained. Recent evidence has indicated an important role of inflammation in the pathogenesis of hypertension and organ dysfunction.²⁰ Many recent studies have pointed at VIP immunosuppressive properties. The VIP has been reported to modulate lymphocyte migration, inhibit mast cell degranulation, reduce production of various cytokines, and modulate humoral response.²¹ Literature data indicate that activation of immunologic processes in gastrointestinal track results in enhanced production of VIP. Independent scientific institutions have demonstrated increased density of VIP-IR nerve fibers in the colon and rectum of patients with inflammatory bowel disease.^{22–24} Gonzales-Rey and Delgado²⁵ found that injection of VIP to mice with experimental colitis reduced the disease symptoms, such as diarrhea, body weight loss, and also weakened intestinal inflammation, thereby improving survival of study animals. Thus, the increase in the number of VIP-containing structures in the pylorus rats with renovascular hypertension, demonstrated in our study, may be due to increased activity of the immune system induced by state hypertension. The major pathomechanism of renovascular hypertension involves the enhanced activity of the renin-angiotensin-aldosterone (RAA) system.²⁶ The VIP has a significant impact on biosynthesis and metabolism of the RAA system components, as the peptide inhibits the secretion of angiotensinogen from hepatocytes and suppresses the angiotensin-converting enzyme (ACE) activity.²⁷ Since VIP inhibits the production of angiotensin II (Ang II), the body seems to compensate for overactive RAA by increasing the peptide synthesis. This mechanism might explain the increased intensity of immunohistochemical reaction in the pylorus of 2K1C rats observed in the present study.

Currently, we evaluated and compared the expression of VIP in the heart of normotensive and 2K1C rats. Immunohistochemical studies revealed the presence of

Table 1 Mass of kidneys (g), body weight (g), and values of blood pressure (mmHg) of normotensive and 2K1C of rats (mean $\pm$ SD)

Group of rats	Mass of kidney (g)		Body weight (g)	Values of BP (mmHg)
	Right	Left		
Control	1.37 ± 0.19	1.35 ± 0.13	443 ± 44.6	120.2 ± 5.89
2K1C rats	$1.85 \pm 0.2^*$	$0.31 \pm 0.1^*$	437 ± 56.8	$162.6 \pm 2.19^*$

* $P < 0.05$.

Figure 1 Representative images of rat heart. (a) control rat, (b) hypertensive rat, noticeable inflammatory infiltrations and varying intensity of cytoplasmic staining. H & E staining. Magn. 200x. (A color version of this figure is available in the online journal.)

Figure 2 Immunolocalization of VIP in the stomach. (a) Control rat; visible only single nerve fibers (arrows) and neurons in the myenteric ganglia (arrowheads) with a very weak reaction to VIP. (b) Rat with hypertension; a significant increase in the density and intensity of the reaction in the VIP-containing structures. (c) Control rat; a single neuroendocrine cell containing VIP. (d) Rat with hypertension; more numerous VIP-immunoreactive cells. Magn. 200x. (A color version of this figure is available in the online journal.)

Figure 3 Immunohistochemical reaction determining VIP in the heart. (a) Control rat; visible a sparse nerve fibers (arrows) in the myocardium (b) hypertensive rat; numerous nerve fibers with VIP immunoreactivity (arrows) and endothelial cells with positive immunohistochemical reaction (arrowheads). Magn. 200x. (A color version of this figure is available in the online journal.)

the peptide in nerve fibers, innervating the myocardium and coronary arteries, and in the endothelial cells of intramural vessels. Other authors observed a similar location of the VIP expression in the heart of different animals.^{28,29} A significantly greater number of structures containing the peptide and stronger VIP immunoreactivity were observed in the heart of rats with renovascular hypertension, compared to normotensive rats.

Hypertension may be accompanied by left ventricular hypertrophy, which significantly increases the risk of heart failure and death.³⁰ The VIP might be a potential agent preventing the development of heart failure. Szema *et al.*^{31,32} demonstrated that deletion of the VIP gene in mice led to the alteration of genes related to heart hypertrophy and increased heart ventricular mass. Other studies have shown that the inhibition of VIP degradation limits the left ventricular hypertrophy in rats with hypertension.^{33–35}

It might be assumed that the increase in VIP immunoreactivity and in the number of structures containing the peptide, observed in the present work, is a mechanism that prevents ventricular hypertrophy caused by hypertension.

Our morphometric study showed an increase in the width of cardiomyocytes in the heart of renal hypertensive rats, which is consistent with other research.³⁶

It has been found that Ang II is involved in pathomechanism of cardiac hypertrophy.³⁷ Griffin *et al.*³⁸ demonstrated that infusion of Ang II to normotensive rats resulted in elevation of BP and heart hypertrophy. It was also shown that blockade of Ang II receptors or inhibition of ACE prevented the increase in left ventricular mass in a different model of hypertension.³⁷

In view of the above, enhanced generation of Ang II in renovascular hypertension may be a probable cause of the increased width of cardiac muscle cells in 2K1C rats observed in our study.

Results of *in vivo* and *in vitro* studies indicate that the peptide provides protection against cell destruction. The VIP suppresses intracellular production of apoptotic factors, such as caspase-3³⁹ and counteracts cell oxidative damage by inhibiting generation of reactive oxygen species.⁴⁰ The peptide improves cell survival also by modulating Bcl 2 and Bax gene expression.⁴¹ It has been shown that the inhibition of peptide degradation in rats with spontaneous hypertension lowers cardiomyocyte apoptosis. Therefore, it might be assumed that enhanced VIP biosynthesis in the heart of hypertensive rats aims to restrict the disease-associated loss of cardiomyocytes.

In the heart of hypertensive rats, we observed a varied intensity of cytoplasmic staining in cardiomyocytes. In response to enhanced energy demand, cardiomyocytes may also show an increased number of mitochondria. Changes in the proportion between cell organelles or protein content might explain more pronounced eosin staining in the cytoplasm of certain cardiomyocytes in the heart of 2K1C rats observed in the current study.

Data obtained in many research centers and in our study suggest that VIP present in various body organs exhibits a high neuroprotective and anti-inflammatory potential, and therefore should be taken into consideration in designing

novel therapeutic strategies for hypertension. The protective mechanism of VIP involves many intracellular pathways, e.g. metabolic, anti-inflammatory, which require further investigation.

Authors' contributions: IK, ZP participated in the study design; IK, ZP and IJ conducted the experiments, data collection, and analysis of the data; IK wrote the manuscript; IJ contributed to manuscript preparation and performed statistical analysis of those studies. All authors participated in interpretation of the studies and review and approved the final manuscript.

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