Ghrelin-immunoreactive cells in the gastrointestinal tract of hypertensive rats

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Abstract
Introduction. Ghrelin, an appetite-stimulating hormone secreted by the endocrine cells of the gastrointestinal (GI) tract, has recently been shown to affect the function of the cardiovascular system. This study aimed to assess the number and morphology of ghrelin-immunopositive (GhrIP) cells in the gastrointestinal tract of rats at different developmental phases of experimentally evoked renovascular hypertension.

Material and methods. The study involved 40 rats divided into two groups: control (C; n = 20) and rats with experimentally induced hypertension (EH; n = 20). The Goldblatt model of two-kidneys, one clip (2K1C) was used to induce hypertension. Renovascular hypertension was maintained for either 3 (EH1 group; n = 10) or 42 (EH2 group; n = 10) days. Paraffin sections from the cardia, corpus and pylorus of the stomach, as well as from the duodenum, jejunum, ileum and colon were processed for peroxidase immunohistochemistry.

Results. The number of GhrIP cells was significantly higher in the cardia and corpus of the stomach as well as the duodenum and jejunum of hypertensive rats compared to that found in the control animals.

Conclusions. The increased number of GhrIP cells in the rat gastrointestinal tract after partial unilateral ligation of the renal artery suggests that renovascular hypertension may affect ghrelin secretion, which can contribute to the development of cardiovascular complications. (Folia Histochemica et Cytobiologica 2016, Vol. 54, No. 4, 181–185)

Key words: hypertensive rat; gastrointestinal (GI) tract; ghrelin; immunohistochemistry

Introduction
Keeping arterial blood pressure (BP) at a normal level to ensure proper perfusion of internal organs is one of key mechanisms of systemic homeostasis. Multiple factors interacting with one another are involved in the continuous adjusting of pressure to dynamically changing demands of organs and tissues. In recent years much attention has been focused on peptide hormones with a broad range of biological (endo-, auto- and paracrine) activities synthesized in various tissues including those of the heart and blood vessels (BVs) [1]. Ghrelin has been recently added to the list of substances which exert their action on the cardiovascular system.

Ghrelin is a peptide hormone discovered in 1999 as an endogenous ligand of the growth hormone secretagogue receptor (GHS-R) [2]. In humans, two thirds of the circulating ghrelin originate in the X/A-like cells of the oxyntic mucosa of the stomach and the rest is produced by intestinal endocrine cells [3]. Accordingly, in rat and human, the highest concen-
trations of the hormone are found in the mucosa of the fundus and body of the stomach [3]. The mucosa in other parts of the stomach and intestines contains distinctly lower ghrelin concentrations with the lowest being observed in the colon [3]. Another area participating in ghrelin synthesis is the central nervous system with the neurons of the arcuate nucleus in the hypothalamus being the most active [4] as well as thyroid gland [5].

Ghrelin acts via specific receptors present in many systemic tissues including the hypothalamus, pituitary gland, gastrointestinal (GI) tract, cardiomiocytes and BVs, and demonstrates a multimodal biological activity [1].

Being mainly involved in appetite and energetic homeostasis-regulating pathways ghrelin also participates in the control of the cardiovascular function [6].

Ghrelin and its major circulating form, des-acyl ghrelin, have been found to induce vasodilatation, ameliorate endothelial dysfunction and suppress the activity of the sympathetic nervous system [7]. The latter function can be important in the control of BP; however, neither the concentration of ghrelin nor the number of ghrelin-producing cells within the GI tract in an experimental rat model of unilateral renal artery stenosis have been determined.

To broaden the knowledge concerning the pathogenesis of digestive system disorders and in the absence of reports on the behavior of cells containing ghrelin subjected to the conditions of hypertension, it seemed worth investigating the location, number, or changes in the morphology of these cells in the GI tract of rats with experimentally-induced renovascular hypertension.

The aim of the present study is the assessment of the number and morphological features of ghrelin-immunoreactive cells in the GI tract of rats at different stages of renovascular hypertension.

**Material and methods**

**Animals and the experimental hypertension model.** The study was carried out on 40 Wistar strain male rats which were 6 weeks old and had a body weight of 160–180 g (at the beginning of the experiment). All animals were housed and treated in accordance with the rules approved by the Senate Bioethical Committee of the Medical University in Bialystok, Poland (No. 49/2009).

After a one week period of acclimatization the systolic BP of each rat was measured and the animals of the experimental group were subjected to a surgical procedure to induce renovascular hypertension.

The rats were divided into the following groups: C1 — control group 1, 10 rats not submitted to any surgical procedures; C2 — control group 2, 10 rats submitted to sham operation (cutting of cutaneous integuments without ligation of the renal artery); EH — experimental hypertension group, 20 rats with renovascular hypertension induced by application of a standardized clip around the left renal artery (the internal clip diameter was 0.22 mm; according to the procedure known as “the two-kidney one-clip”, 2K1C) [8].

The animals of all groups were divided into further subgroups. C1/3 and 1/42 (each consisting of 5 rats) included animals sampled after 3 and 42 days, respectively. C2/3 (5 rats) included animals euthanized after 3 days and C2/42 (5 rats) after 42 days with both groups subjected only to the cutting of the cutaneous integument without ligation of the renal artery. EH1 and EH2 (10 animals each) involved rats euthanized 3 and 42 days, respectively, after clipping of the left renal artery.

**Material sampling and histological procedure.** After 3 and 42 days following the experimental surgical procedures, within the acute and transitional phase, the rats were euthanized with pentobarbital and through opening of the abdomen parts of their GI tract (cardia, corpus and pylorus of the stomach, as well as the duodenum, jejunum, ileum and colon) were dissected out and fixed in Bouin’s fluid for 48 hours at a temperature of 4°C. The tissue material was routinely transferred into paraffin blocks, sectioned (4 µm-thick), stained by hematoxylin and eosin (H&E) for general histological examination and processed for immunohistochemistry for ghrelin detection.

**Identification of endocrine cells by immunohistochemical method.** The EnVision method (DakoCytomation, Glostrup, Denmark) was used for immunohistochemical (IHC) investigations. The primary anti-ghrelin antibody (1:10,000; H-031-31, Phoenix Pharmaceuticals, Inc., Mountain View, CA, USA) was diluted in an Antibody Diluent (S 0809 Dako). Briefly, the paraffin-embedded sections were rehydrated and treated with a Peroxidase Blocking Reagent (S 2001 Dako) for 10 min to block endogenous peroxidase activity. They were then washed in distilled water and a Wash Buffer (S 3006 Dako) 3 times for 5 minutes and incubated with a ghrelin antibody for 24 h in a dark room at a temperature of 4°C. Next, the sections were again washed 3 times in a Wash Buffer. The antibody binding was visualized with the EnVision (+) HRP Kit (K-4011; Dako) containing Labeled Polymer-HRP. Vector QS (H-3404, Vector Laboratories, Inc., Burlingame, CA 94010, USA) hematoxylin was used (2–3 s) for cellular nuclei staining. Negative control was carried out by incubating the sections with the diluent and normal rabbit serum instead of the primary antiserum. All the control reactions gave negative results. Positive control, confirming the specificity of the staining obtained in the study, was performed with the specific tissue (fundus of the stomach) recommended by the manufacturer.
Quantitative analysis. Ghrelin-immunopositive (GhrIP) cells were counted in five randomly selected microscopic fields (Olympus BX51 microscope, Olympus, Tokyo, Japan), each field measuring 0.785 mm², at a total magnification of ×200. Three sections of a respective fragment of the GI tract from each animal were analyzed. The number of GhrIP cells in the mucosa of the stomach (cardia, corpus and pylorus), the small intestine (duodenum, jejunum and ileum) and the large intestine (colon) has been presented as mean values per 1 mm².

Statistical analysis. Statistical analysis of the results was performed using the program Statistica version 10.0; significance of differences was determined by Student’s t test at p < 0.05.

Results

Effects of hypertension on kidney and body mass, and blood pressure

Three days after the procedure involving the clipping of the left renal artery in rats from C2/3 (sham-operated rats) and EH1 (induced hypertension rats) groups, no significant changes in the mass of the kidneys, body mass or blood pressure values were observed (data not shown). However, six weeks following the experiment, chronic renal ischemia significantly affected kidney mass and blood pressure of rats (Table 1). The mass of the right unclipped kidneys was slightly increased, whereas the mass of the left ischemic kidneys was significantly reduced, as compared to the kidneys of the sham-operated normotensive C2/42 rats (Table 1).

The body mass of C2/42 and EH2/42 rats did not differ; however, the mean value of arterial blood pressure in the EH2/42 group of rats was higher than in sham-operated animals.

The number of ghrelin-immunopositive cells in the GI tract of control and hypertensive rats

The staining of sections by the H&E method did not reveal any noticeable alterations or differences in the morphology of the GI tract of normo- and hypertensive rats (not shown).

Since no statistically significant differences in the number of GhrIP cells were found between the two control groups of rats (C1 and C2, data not shown), only the results obtained in sham-operated rats (C2/42 animals) were taken as control values against the EH groups.

In all rats GhrIP cells were present in the mucosa of the investigated parts of the GI tract. The GhrIP cells of the stomach were most often small and round, whereas those within the intestines were triangular or elongated. No significant differences were observed in the topography of GhrIP cells between the investigated parts of the GI tract of normo- and hypertensive rats. In the stomach of C2 and EH rats, 80% of GhrIP cells were found in the lower parts of glands while the other GhrIP cells were distributed in the superficial epithelium. In the duodenum, jejunum, ileum and colon, single GhrIP cells were scattered in the epithelia of crypts and villi among other non-endocrine epithelial cells.

In the acute phase of hypertension, i.e. on the 3rd day after the surgery, a significantly higher number of GhrIP cells in the cardia, corpus of the stomach and jejunum was found in hypertensive rats as compared to sham-operated animals (Table 2, Figure 1A, B). In the transitional phase of hypertension, i.e. 42 days after the surgery, the number of GhrIP cells in the corpus of the stomach was by 50% higher and in the duodenum four times higher than in control animals (Table 2).

In the duodenum of hypertensive rats (EH2) a significantly higher number of GhrIP cells was observed in the epithelium of the intestinal villi while in sham-operated animals (C2/42) presence of GhrIP cells was sporadic (Figure 1C, D).

Discussion

Ghrelin and its receptors are present throughout the heart and vasculature and there is no doubt that the

<table>
<thead>
<tr>
<th>Group of rats</th>
<th>Kidney mass [g]</th>
<th>Body mass [g]</th>
<th>Blood pressure [mm Hg]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right kidney</td>
<td>Left kidney</td>
<td></td>
</tr>
<tr>
<td>C2/3</td>
<td>0.95 ± 0.05</td>
<td>0.96 ± 1.59</td>
<td>166 ± 5.7</td>
</tr>
<tr>
<td>EH1</td>
<td>0.87 ± 0.05</td>
<td>0.92 ± 0.07</td>
<td>167 ± 7.0</td>
</tr>
<tr>
<td>C2/42</td>
<td>1.37 ± 0.19</td>
<td>1.30 ± 0.21</td>
<td>443 ± 44.6</td>
</tr>
<tr>
<td>EH2</td>
<td>1.85 ± 0.22*</td>
<td>0.80 ± 0.21*</td>
<td>437 ± 56.8</td>
</tr>
</tbody>
</table>

Hypertension was induced in rats by clipping left renal artery (EH group) and rats were sham-operated as described in Material and methods (C2 group). Animals were sampled after 3 days (C2/3 and EH1) and 42 days (C2/42 and EH2). Values represent mean ± SD. *Significantly higher vs. corresponding C2 group, p ≤ 0.05.
effects of ghrelin in the cardiovascular system are mediated not only via its growth-hormone-releasing effect but also by its direct effects on the heart [1].

High expression of growth hormone secretagogue receptor, the ghrelin receptor, in heart, kidney, and blood vessels suggests its possible involvement in the regulation of blood pressure and pathomechanisms of hypertension [4].

Despite numerous studies carried out during the last decade focusing on the influence of ghrelin on the cardiovascular system and its possible role in hypertension, no efforts were made to assess the effects of high blood pressure on the number and morphological features of GhrIP cells in the gastrointestinal system affected by renovascular hypertension. The present study showed that renovascular hypertension increases the number of cells containing ghrelin in gastric mucosa and intestine in rat. The effects of BP on the production and secretion of ghrelin in GI tract were studied by Hamada et al. [9] in the stomach of

Table 2. Number of gherlin-immunopositive (GhrIP) cells in the studied parts of the gastrointestinal tract in hypertensive and normotensive rats

<table>
<thead>
<tr>
<th>Group of rats</th>
<th>Number of GhrIP cells per 1 mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cardia</td>
</tr>
<tr>
<td>C2/3</td>
<td>5.3 ± 3.7</td>
</tr>
<tr>
<td>EH1</td>
<td>9.4 ± 13.2*</td>
</tr>
<tr>
<td>C2/42</td>
<td>6.85 ± 2.4</td>
</tr>
<tr>
<td>EH2</td>
<td>8.5 ± 4.3</td>
</tr>
</tbody>
</table>

Rats were sham-operated (C2 groups) or experimental hypertension (EH groups) was induced as described in Material and methods; rats were sampled either after 3 (C2/3 and EH1) and 42 days (C2/42 and EH2). Values represent mean ± SD. *Significantly higher number in EH groups vs. corresponding C2 groups, p ≤ 0.05.

**Figure 1.** The presence of ghrelin-immunopositive cells in the corpus of the stomach (A, B) or duodenum (C, D) after 3 (A, B) and 42 (C, D) days after induction of hypertension by clipping of the left renal artery. A and C — control, sham-operated rats; B and D — hypertensive animals. The immunohistochemical staining was performed as described in Material and methods. Original magnification ×200.
spontaneously hypertensive (SHR) rats. Similarly to our findings, they showed that the number of GhrIP cells was greater and the ghrelin content was higher in the stomachs and plasma of SHR as compared to normotensive rats [9]. The rise in the number of GhrIP cells in rats with renal hypertension observed in our study may indicate increased serum levels of ghrelin since previous studies have shown that ghrelin plasma concentrations were significantly higher in patients and animals with hypertension [10, 11].

Additionally, it has been shown that endogenous ghrelin acts as an anti-hypertensive hormone [12]. Moreover, experimental and clinical studies demonstrated that ghrelin decreased blood pressure in healthy individuals and animals with normal blood pressure as well as animals with hypertension [13]. It was suggested that the mechanisms responsible for the hypotensive effects of ghrelin include the suppression of sympathetic activity or direct vasodilatory activities [5, 14].

We have provided first evidence showing significant changes in the number of GhrIP cells in the duodenum of hypertensive rats compared to normotensive animals. We hypothesize that these changes are caused by increased proliferation rate of GhrIP cells as an adaptive response to homeostatic morphologic and functional changes in many organs and cells that occur as a result of hypertension.

Our findings suggest that ghrelin-secreting cells present in the stomach and other parts of the gastrointestinal tract constitute an important element of the homeostatic systems which can be affected by experimental renal hypertension. Further studies of the structure and function of ghrelin-producing cells in the GI tract will contribute to better understanding of the pathological processes in which they participate.

Conflict of interest

The authors report no conflict of interest.

References