

I. KASACKA

## QUANTITATIVE DISTRIBUTION AND LOCALIZATION OF CALCITONIN GENE-RELATED PEPTIDE-LIKE CELLS IN THE STOMACH OF TWO KIDNEY, ONE CLIP RATS

Department of Histology and Embryology, Medical University of Bialystok, Poland

The majority of research for the calcitonin gene-related peptide (CGRP) in the stomach in the hypertension has been devoted to the submucosal blood flow, and no attention has been paid to its quantitative distribution in the gastric neuroendocrine cells. The aim of the present study was to examine the number and distribution of CGRP-containing cells in the pylorus of "two kidney, one clip" (2K1C) renovascular hypertension model in rats. The studies were carried out on the stomach of rats. After 6 week period of the renal artery clipping procedure, eight 2K1C rats developed stable hypertension. The hypertension significantly increased the number of endocrine cells pylorus immunoreactive to calcitonin gene-related peptide (CGRP) antisera. The differences between the hypertensive rats and the control group concerned not only the number of endocrine cells but also their distribution. CGRP participates in the regulation of cardiovascular functions both in normal state and in the pathophysiology of hypertension through interactions with the prohypertensive systems. The changes induced by hypertension in the neuroendocrine cells containing CGRP of the rats are discussed.

**Key words:** *CGRP-positive cells, stomach, hypertension, rat*

**Abbreviations:** *CGRP – calcitonin gene-related peptide, 2K1C – two kidney, one clip, DNES – diffuse neuroendocrine system, BP – blood pressure, ROS – reactive oxygen species, IR – immunoreactive*

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### INTRODUCTION

Renovascular hypertension is the most common cause of secondary hypertension, with 1-2% prevalence among hypertensive patients. Despite the abundant literature devoted to the etiology, pathogenesis, diagnostics and treatment of arterial hypertension, many issues still remain to be explained (1, 2).

Arterial hypertension causes morphological and functional changes in myocardium, vascular smooth muscle or endothelium, and abnormalities in physiological regulatory system for blood pressure, including neurotransmitters and humoral factors (1, 2). In addition to elevated blood pressure, progressing renal failure and the CNS symptoms of varied intensity, also disturbances in other organs may predominate in the clinical picture. This particularly refers to the digestive tract, within which acute pancreatitis or intestinal ischemia may occur (3, 4).

It is well-known that the nervous, endocrine and immune systems have well-established and very closed related interrelations to regulate systemic homeostasis that involves the production and secretion of a variety of cellular mediators known as regulatory peptides. Peptide hormones, cytokines, chemokines, integrins, other related molecules regulate homeostasis in the tissue of external systems that facilitate restoration of local homeostasis (5, 6).

In arterial hypertension there is experimental evidence for involvement of neuropeptides in the pathogenesis (8-11). A

potentially interesting area is the immunoregulatory role of enteric nerves and neuroendocrine cells. Neuropeptide, like calcitonin gene-related peptide (CGRP), is the molecular mediator of neuroregulation in the gastric immune system, providing for interactions between nervous system and immunocytes (12).

Immunohistochemical localization of CGRP indicates that this peptide is present within the enteric nervous system being particularly abundant in gastric nerves of the myenteric and submucosal plexus, and is also found in neuroepithelial cells of the stomach (13). In terms of its physiological role, it has been reported that CGRP can inhibit gastric acid secretion and stimulate somatostatin release, which suggests that CGRP may act as a neuromodulator of gastric function (14). Furthermore, studies indicate that CGRP is capable of exciting myenteric plexus neurons and stimulating acetylcholine release from isolated myenteric neurons (15). In addition, it has been shown that reactivity to CGRP significantly enhanced in spontaneously hypertensive rats (16) and that CGRP has a significant role in eosinophilia in allergic inflammation (17).

The malfunction of calcitonin gene-related peptide which is a pleiotropic neuropeptide with potent vasodilatory properties which interferes with rennin release, may be involved in cardiovascular homeostasis (18).

The results of numerous studies (19-21) seem to indicate that in various pathological states the number and morphology of

endocrine cells in the stomach undergo some changes. This confirms the involvement of biologically active substances produced by endocrine cells in various pathological and adaptive processes in the body.

Apart from one report (16) indicating an increased number and staining intensity of CGRP immunoreactive gastric cells in spontaneous hypertension, no information on the expression of this neuropeptide in the cells of the stomach in the renal hypertension is available.

Taking into consideration the homeostatic disorders resulting from renal hypertension and the key role of CGRP in many systems which regulate the function of the organism, the question arises as to what extent the arterial hypertension affects the morphology and activity of CGRP-like cells in the stomach.

The aim of the present study was to examine the distribution and occurrence of gastric CGRP-like cells in renal (2K1C) hypertensive rats.

## MATERIALS AND METHODS

### *Experimental animals*

Procedures involving the animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international law and with guidelines for the use of animals in biomedical research (22).

The study was performed on sixteen (16) young male Wistar rats, their body weight at the beginning of the experiment within 160-180 g (the mean body weight:  $170 \pm 10$  g). The rats were housed in polypropylene cages in groups of two or three rats per cage and received laboratory chow *ad libitum*. After 1 week period of acclimatization, the systolic blood pressure (BP) of each rat was measured, after which the surgical procedure for induction of renovascular hypertension was performed.

### *2K1C renovascular hypertension*

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 ( $n = 6$ ) underwent identical surgical procedures, except that a clip was not applied to the renal artery.

After 6 week period of the renal artery clipping procedure, the systolic arterial pressure was performed by the tail-cuff method (23). After this time, eight (8) 2K1C rats developed stable hypertension. 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, all 2K1C rats ( $n = 8$ ) developed stable hypertension (mean blood pressure values  $160.6 \pm 3.2$  mmHg).

### *Histology*

#### *Method of experimental material collection and fixation*

Six weeks after the renal artery clipping procedure, the animals were killed and the stomach immediately removed, opened, rinsed in saline. Tissue samples were collected from the pyloric regions of the stomach. They were fixed in Bouin's fixative for 24 hours at  $+4^\circ\text{C}$ , and processed routinely for embedding in paraffin. Sections were cut at  $4 \mu\text{m}$  in thickness,

and stained by hematoxylin-eosin (H+E) for general histological examination.

### *Identification of endocrine cells by immunohistochemical methods*

In the immunohistochemical study, the EnVision method was used according to Herman GE, Elfont EA (11). An immunohistochemical reaction to find calcitonin gene-related peptide in neuroendocrine cells was performed on paraffin stomach sections of the animals studied. In these studies, a specific antibody against CGRP was applied (Cat. No C 8198, in 1:8000 dilution, SIGMA-ALDRICH), purchased at the SIGMA-ALDRICH, Saint Louis, Missouri 63103, USA.

The antiserum was diluted in Antibody Diluent (S 0809 DakoCytomation, Denmark).

### *Immunohistochemical reaction procedure*

In short, the paraffin-embedded specimens were dewaxed, rehydrated and treated with Peroxidase Blocking Reagent (S 2001 DakoCytomation, Denmark) for 10 minutes to block endogenous peroxidase activity. Then, the sections were washed in distilled water and Wash Buffer (S 3006 DakoCytomation, Denmark), 3 times for 5 min, and incubated with a CGRP antibody for 1 hour, in dark-room at room temperature. Next, the sections were washed 3 times in Wash buffer. The antibody binding was visualized with the help of EnVision Kit (K 4011 DakoCytomation, Denmark) containing Labelled Polymer-HRP Anti-Rabbit. Vector QS hematoxylin was used (2 - 3 seconds) for cellular nuclei staining.

Negative control was carried out by incubating sections with the diluent and normal serum instead of the primary antiserum. All the performed control reactions gave negative results. Positive control was conducted on rat brain.

### *Quantitative analysis*

The obtained results of immunohistochemical staining were submitted for evaluation in an Olympus Bx50 microscope. Cells with CGRP expression were searched for and their topography was observed.

Immunopositive cells were counted in five (5) randomly selected microscopic fields, each field of  $0.785 \text{ mm}^2$ , in magnification of 200x (20x the lens and 10x the eyepiece) in the longitudinal sections of the stomach. Three (3) sections of each rat were analysed. The numbers of positively stained cells were presented as mean values per  $1 \text{ mm}^2$  of the analysed stomach section area. 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

As shown in *Table 1*, the partial occlusion of the left renal artery induced stable hypertension after 6 weeks following the clipping procedure. Throughout the 6 weeks study the sham-

*Table 1.* Mass of kidneys (gram), values of blood pressure (mmHg), and number of CGRP-IR cells normotensive and 2K1C of rats.

Group of rats	Mass of kidney (gram)		Values of BP (mmHg)	Number of CGRP-IR cells
	right	left		
Control	$1.37 \pm 0.19$	$1.35 \pm 0.13$	$120.2 \pm 5.89$	$48.40 \pm 6.27$
2K1C rats	$1.85 \pm 0.2$	$0.31 \pm 0.1$	$160.6 \pm 2.19$	$80.30 \pm 7.34$

operated animals had BP levels similar to those recorded before the surgical procedure.

Table 1 demonstrates the effects of chronic renal ischemia on kidneys mass, values of blood pressure, and number of CGRP-IR cells. As can be seen in Table 1, six weeks after the left renal artery clipping, the mass of the right unclipped kidneys were slightly increased, whereas the mass of the left ischemic kidneys were significantly reduced, as compared to the kidneys from sham-operated normotensive rats.

#### *Morphological feature of endocrine cells in routine staining methods*

There were no microscopic significant pathological changes in the stomach from the hypertension (2K1C) rats compared with their normotensive controls.

The immunohistochemical studies revealed a positive reaction in the cytoplasm of endocrine cells of the stomach in all

the examined animals. (Fig. 1A and 1B). This shows that the antibody reacted against the antigen in CGRP-containing cells, indicating their site in the glandular epithelium of the stomach pylorus. In the control rats, these cells were mainly located in the basal portions of the glands (Fig. 1A), while in the hypertensive animals, they were diffused at various levels of the glands of the mucous membrane (the largest number was observed in the basal third of the gastric glands, while in the middle third their number diminished and in the top third only single cells were observed) (Fig. 1B). The differences, observed under the microscope between the hypertensive rats and the control group concerned not only the distribution of endocrine cells but also their number.

Computer analysis of CGRP-IR (CGRP-immunoreactive) cells demonstrated a statistically significant increase in the number of neuroendocrine cells in the stomachs of the rats six weeks after the left renal artery clipping ( $80.30 \pm 7.34$ ;  $p < 0.001$ ), when compared to the values in the control animals ( $48.40 \pm 6.27$ ).

CGRP-positive cells most frequently are round, flask or irregular in shape. The majority of these cells showed a strong immunostaining for CGRP (Fig. 1A and 1B).

## DISCUSSION

To the best of my knowledge, there are no reports in the literature that describe CGRP containing neuroendocrine cells in the mucosal epithelium of the stomach in hypertension.

The results of the present study demonstrate that the hypertensive state produced in rats by partial occlusion of the left renal artery influence the CGRP-producing cells in the stomach.

Hypertension-induced end organ damage is one of the most severe and common consequences of chronic increased blood pressure (BP). CGRP possesses a protective action against hypertension-induced organ damage. In light of several lines of indirect evidence suggesting that CGRP has such potent biological effects, defines it as an endogenous organ-protective agent. This activity could be mediated directly by CGRP or indirectly through subsequent neurohormonal or other physiological changes caused by CGRP gene deletion (24).

Renal hypertension cannot be perceived as a homogenous pathological entity but rather as a group of not well defined syndromes sharing increased arterial blood pressure. In addition to elevated blood pressure, progressing renal failure and the CNS symptoms of varied intensity, also disturbances in other organs may predominate in the clinical picture. A variety of diseases appear to be associated with either an excessive or a deficient adaptive process during the response to environmental stress. The gastric mucosa has been found to be an especially susceptible target to stress. The hypertension provides a useful vehicle for analyzing abnormal autonomic or hormonal responses to stress. Hypertension is a common complication of several disorders, such as haemorrhagic, red point lesions and inflammatory diseases, predominantly located in the gastric antrum (25). This also might influence the activity of endocrine cells, an important element of the intrinsic regulatory system in the digestive tract. All functions and processes of the digestive system are regulated by a complex network of regulatory molecules, i.e. diverse hormones, neurotransmitters, cytokines and growth factors (5).

The results of numerous studies show that CGRP plays important role in gastrointestinal functions, including motility and secretions from the stomach (can inhibit gastric acid secretion and stimulate somatostatin release) (26).

This study demonstrated the distribution and occurrence of CGRP-immunoreactive endocrine cells in the stomach of hypertensive rats. The results of the present study show that the number of CGRP-containing cells in the antral mucosa of hypertensive rats markedly increased compared with the

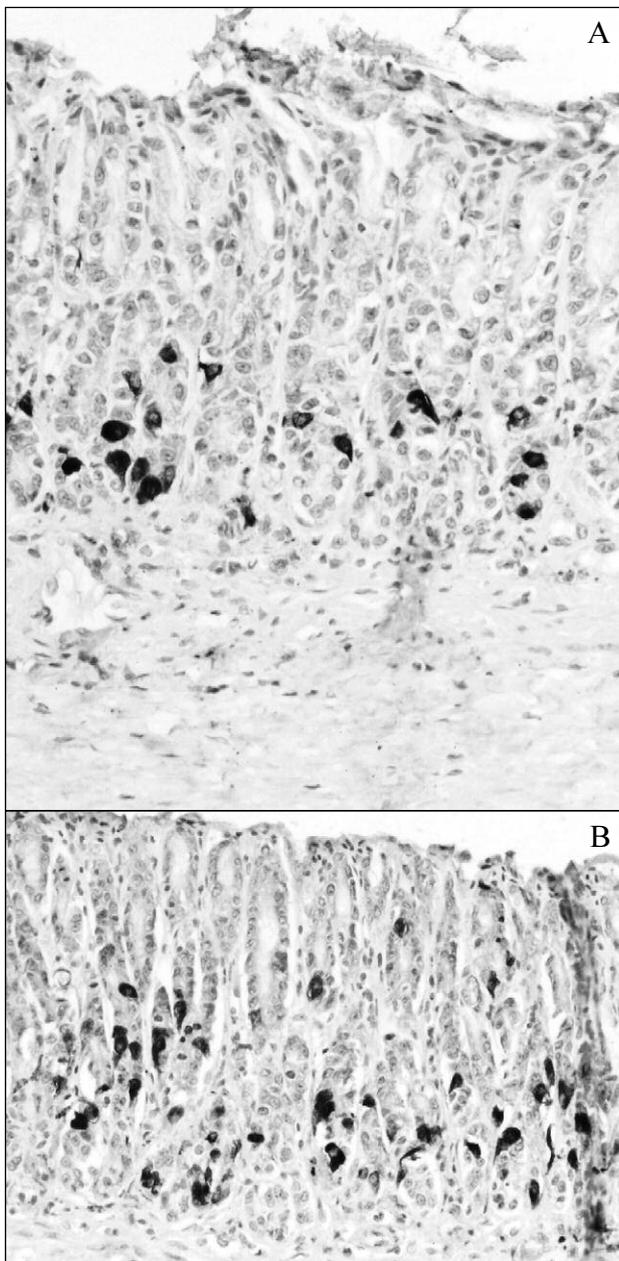


Fig. 1. CGRP- immunopositive cells of the pyloric mucosa: (A) control rat, (B) hypertensive (2K1C) rat, x200.

normotensive rats. The explanation of the pathophysiological mechanism of the CGRP-immunopositive cells number changes in the stomach of renovascular hypertensive may be a merely hypothetical consideration.

CGRP has pleiotropic and pathophysiological effects on various cells and organs. CGRP can stimulate somatostatin release and exerts stimulatory effects on the mucin biosynthesis in the surface epithelial cells of the gastric mucosa (27). Because stimulation of the gastric mucin synthesis is closely related to the mucosal protective activity, these finding suggest that the increase in the number of CGRP containing cells in the gastric pylorus could be a cause of the impaired mucosal mechanism in the hypertensive rats.

Similar processes have been also described by Supowit *et al.* (28). They suggest that CGRP possesses a protective action against hypertension-induced heart and kidney damage.

Neuroendocrine cells can release CGRP in response to local factors including nerve growth factor (NGF), vascular wall tension, bradykinin/prostaglandins, endothelin, and the sympathetic nervous system. It is possible that the factors altering acute release of CGRP can also modulate the long term production and release of this peptide (29). Thus, alterations in these factors, some of which are known to occur in hypertension, may mediate any changes seen in cellular CGRP expression. Several lines of evidence indicate that NGF can stimulate CGRP synthesis and release (12, 29, 30). The *in vivo* studies have shown that intraperitoneal treatment of spontaneously hypertensive rat with NGF enhances the synthesis and release of CGRP (31).

Taken together, although the mechanisms responsible for regulating the expression and release of CGRP are not known, the aforementioned studies suggest that several different classes of regulatory factors act to modulate CGRP synthesis and release by antagonizing or activating NGF-stimulated expression and release of CGRP (29, 30).

It is not clear what role CGRP plays in human hypertension. The reported levels of circulating immunoreactive CGRP in hypertensive humans have been conflicting (32).

Regarding the mechanism or mechanisms responsible for the increase number of endocrine cells containing CGRP in the pyloric mucosa of renovascular hypertension, CGRP could be stimulated directly by elevated BP, or hypertension could regulate CGRP expression through an indirect mechanism by either stimulating or inhibiting factors that directly modulated synthesis and/or release of CGRP.

The increased quantity of CGRP-positive cells, observed in the current study, rather indicates proliferation of the endocrine cells of gastric mucosa. This idea correlates well with the distribution pattern of CGRP containing cells, which is normally predominant in the basal third of the gastric glands and increases in the upper third in the hypertensive rats.

Date of present study suggest that CGRP may be involved in the pathogenesis of stomach mucosa by acting as a mediator.

The mechanism of increased number of CGRP-containing cells in the stomach, demonstrated in the present study, through the reduced stomach blood flow possible, leads to an increase in local tissue production of oxidative and inflammatory mediators or a direct protective effect of CGRP, among others.

This is the first demonstration that neuroendocrine cells producing CGRP play a significant role in renovascular hypertension in the rat.

It is supposed that more precise knowledge about neuroendocrine cells producing CGRP, their role and mechanisms of action, will not only allow to better explain clinical symptoms and make a sound prognosis but also may help in choosing the best possible treatment of hypertension.

Conflict of interests: None declared.

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Author's address: Dr hab. n. med. Irena Kasacka, Department of Histology & Embryology, Medical University of Białystok, ul. Kilinskiego 1, 15-089 Białystok, Poland; Phone./Fax: (+48 85) 748 54 55; e-mail: kasacka@umwb.edu.pl