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Renovascular hypertensive decrease immunoreactivity of cells containing chromogranin A and pancreastatin in the pancreas of rats

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Summary. Hypertension significantly increases the risk of hyperglycemia in patients. It is known that chromogranin (CgA) and pancreastatin (PST) are involved in regulation of blood pressure and endocrine function of the pancreas. However, still little is known about the physiology of these hormones' secretion in hypertension.

The objective of the study was to examine the effects of hypertension on pancreatic islet cells containing CgA and PST in rats.

The studies were carried out on the pancreas of rats. After 6 week period of the renal artery clipping procedure, eight 2K1C rats developed stable hypertension. Cells containing CgA and PST were detected using immunohistochemical method. The hypertension significantly decreased the number of pancreatic endocrine cells immunoreactive to CgA and PST antisera. The differences between the hypertensive and normotensive rats concerned not only the number of endocrine cells but also intensity of reactions.

In conclusion, the research results indicate that hypertension causes the diminished biosynthesis of CgA and PST in the pancreas of rats and suggests the participation of those peptides in pancreatic disorders occurring in a state of elevated blood pressure.

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Introduction

Hypertension disrupts a number of processes essential for the maintainance of homeostasis in the body and significantly increases the risk of hyperglycemia in patients (Cheung and Li, 2012). Growing evidence from clinical and experimental data suggests that different types of biological substances produced by the cells of the diffuse neuroendocrine system (DNES) may participate in the pathogenesis of this metabolic disorder (Matsumura et al., 2003). Furthermore, recent data indicate that chromogranin A (CgA) may be one of the molecules connected with these disorders (Kim and Loh, 2005).

Chromogranin A is a member of the granin family of proteins which play a crucial role in the biogenesis of secretory granules in endocrine cells (Taupenot et al., 2003). It has been proven that CgA acts as prohormone giving rise to several biologically active substances. Pancreastatin (PST) was one of the first identified and to date is the best-known CgA-derived peptide (Iacangelo et al., 1988).

It is known that CgA and PST are co-stored and co-

Abbreviations. 2K1C, two kidney-one clip model of renovascular hypertension; CgA, chromogranin A; DNES, diffuse neuroendocrine system; IR, immunoreactive; NA, noradrenaline; NO, nitric oxide; PST, Pancreastatin; RAA, renin-angiotensin-aldosterone system.

released with catecholamines in the adrenal medulla and postganglionic sympathetic axons (Borges et al., 2013). CgA and PST were also detected in the neuroendocrine cells scattered among epithelial cells in the airways, gastrointestinal track and pancreas (Yoshida et al., 2009). Immunoexpression of CgA and PST has been observed in all types of pancreatic endocrine cells (Ravazzola et al., 1988; Kimura et al., 1995).

The results of experimental studies suggest the participation of CgA not only in the regulation of calcium and glucose metabolism, but also in gastrointestinal motility, tissue repair and inflammatory reactions (Scherübl and Grabowski, 2003). There is evidence that CgA and PST are involved in the regulation of pancreatic endocrine function. It has been demonstrated that CgA is necessary for the proper development of islet cells and that it impacts on the production of pancreatic hormones (Portela-Gomes et al., 2008). Experimental reports indicate that PST inhibits insulin and somatostatin secretion while glucagon promotes its biosynthesis (Ahrén et al., 1988).

Several studies indicate that CgA and PST are also required for the storage and release of catecholamines from adrenal chromaffin cells and postganglionic sympathetic axons (Sánchez-Margalet and Goberna, 1993; Kim and Loh, 2005). The results of research conducted by Mahapatra et al. (2005) showed that the deletion of the *Chga* gene causes a decrease in the size and number of chromaffin granules in adrenal glands, an increase in plasma catecholamines concentration and results in an increase in blood pressure in mice. Sanchez-Margelet et al. (1995a,b) noticed that patients with essential hypertension have elevated plasma pancreastatin levels, which significantly correlated with epinephrine and norepinephrine concentration in blood.
To date, there has only been one report regarding changes in the morphology of pancreatic islets under hypertensive conditions, demonstrating a modification in the number and activity of CART-, insulin-, glucagon-containing islet cells in renovascular hypertensive rats (Kasacka et al., 2015a).

A lack of reports regarding changes in CgA and PST biosynthesis in the pancreas under hypertensive condition and results of our previous studies demonstrating altered activity of different cell types of DNES in various organs of renovascular hypertensive rats (Sanchez-Margelet et al., 1995b) have prompted us to increase our knowledge about the pancreas in this metabolic disorder (Kasacka et al., 2014, 2015a,b).
The aim of the study was the assessment of cells containing CgA and PST in the pancreatic islets of rats with renovascular hypertension.

Materials and methods

Experimental animals

The assumptions, 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. 49/2009 on 30.09.2009, concerning application no. 2009/45).

The study was performed on twenty (n=20) young male Wistar rats, their body weight at the beginning of the experiment within 160-180 g (the mean body weight: 170±10 g). The rats were housed in polypropylene cages in groups of two or three rats per cage and received laboratory chow and water ad libitum. Light/dark cycle was 12 hours. After a one 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 in the experimental group.

In the experiment animals were divided into two control groups: first - 5 rats, did not undergo any surgical procedure, and second control group - 5 rats, underwent sham operation, and one study group - 10 rats with induced hypertension.

2K1C renovascular hypertension

Induction of experimental hypertension was performed according to procedure by Goldblatt et al. (1934). After the rats were anaesthetised 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.

6 week after the renal artery clipping procedure all rats were weighed and the systolic arterial pressure was measured by the tail-cuff method (Stewart et al., 1993). Arterial pressure was evaluated by using a Student Oscillograph Rat Tail Blood Pressure Monitor, (Harvard, England). 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=10) developed stable hypertension (mean blood pressure values 162.6±3.2 mmHg).

Method of experimental material collection and fixation

Six weeks after surgery, pancreas was collected under deep pentobarbital anesthesia (50 mg/kg b.w.). The same parts of the pancreas (body) were taken from the hypertensive and control animals, fixed in Bouin's fluid and embedded in paraffin in a routine manner. Sections were cut at 4 μm in thickness, and stained by haematoxylin-eosin (H&E) for general histological examination and processed by immunohistochemistry

for CgA and PST detection. Weight data of left and right kidney were additionally collected.

Detection of CgA and PST in rats pancreas by immunohistochemical methods

In the immunohistochemical study, the EnVision method was used according to Herman and Elfont (1991). 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 6.0 for CgA (S 236984, Dako; Glostrup, Denmark) and using Zytomed Systems HIER Citrate Buffer pH 6,0 for PST (ZUC028, Zytomed System, Berlin, Germany). After cooling down to room temperature, the sections were incubated with Peroxidase Blocking Reagent (S 2001 Dako; Glostrup, Denmark) for 10 minutes to block endogenous peroxidase activity.

Subsequently, sections were incubated with primary antibody for CgA (polyclonal rabbit anti-CgA, No 503-1521, purchased from the Zytomed System, Berlin) and for PST (polyclonal rabbit anti-PST, No H- 053-13 purchased from the Phoenix Pharmaceuticals, Inc., Mountain View, CA). PST-antibody was previously diluted in Antibody Diluent (S 0809 DakoCytomation, Glostrup, Denmark) in relation 1:2,000, CgA antibody was ready to use. Sections with CgA-antibody were incubated for 30 min in a humidified chamber at room temperature, whereas incubation with PST-antibody lasted 2 hours.

The procedure was followed by incubation with secondary antibody (conjugated to horseradish peroxidase-labelled polymer) (15 min for CgA and 30 min for PST). The bound antibodies were visualised by 1-min incubation with liquid 3,3'-diaminobenzidine substrate chromogen (DAB chromogen). The secondary antibody and DAB chromogen were included in DAKO EnVision™+ System (K4011, Dako, Glostrup, Denmark). 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 DakoCytomation; Glostrup, Denmark) was performed between each step.

Specificity tests, performed for the CgA- and PST-antibody included: negative control, where the antibodies were replaced by normal rabbit serum (Vector Laboratories; Burlingame, CA) at respective dilution and a positive control was prepared with specific tissue (as it was recommended by the manufacturer).

Five sections from each animal were prepared for immunohistochemistry with anti-CgA, and similarly five sections from each animal were stained immunohistochemically for PST.

CgA- and PST-positive structures were searched for and their topography was observed.

Quantitative analysis

Following immunostaining, morphometric evaluation was performed using an Olympus BX41 microscope with a digital camera (Olympus DP12) and standard morphometric program (NIS-Elements Advanced Research software of Nikon) installed on a computer. Ten randomly selected islets in each section were chosen, at a magnification 200x (20x the lens and 10x the eyepiece) for further morphometric analysis. The area of pancreatic islet was measured and the numbers of positively stained cells were counted in each analyzed islet. The number of CgA- and PST-IR cells were converted and presented as mean values per 0.1mm^2 surface of pancreatic islet. Then the intensity of immunohistochemical reactions for each antibody was analyzed. Intensity of immunohistochemical reaction was measured by using 0 to 256 grey scale level, where a completely black pixel got a value of 0, whereas one with a value of 256 is completely white or bright.

Statistical analysis

Results are expressed as means ± SD. The StatisticaVersion 10.0 program was used for the statistical analysis of the results. 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

The results concerning sham-operated animals were the only results taken into account since no significant differences between the two control groups of rats were found. Chronic renal ischemia significantly affected kidney weight and blood pressure (Table 1). Six weeks after the occlusion of the renal artery, the weight of the left ischemic kidney significantly decreased, whereas the mass of the right, unclipped kidney, slightly increased as compared to kidneys of normotensive rats.

In all pancreatic specimens, the routine histopathological examination did not show any identifiable pathological changes.

Table 1. Mass of kidneys (gram), body weight (gram) and values of blood pressure (mmHg) of normotensive and 2K1C of rats (mean ± SD). *p<0.05.

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

Immunohistochemical investigation revealed that CgA (Fig. 1) and PST (Fig. 2) were distributed mainly in endocrine cells of pancreatic islets. Moreover, positive reaction for CgA and PST was observed in the single exocrine cells and in the nerve fibres innervating pancreatic parenchyma (Fig. 1B). The presence of CgA and PST was demonstrated in the entire area of islets, although intensity of immunohistochemical reaction was not homogenous. Highly strong immunosignal for CgA and PST was observed at the periphery of the islets, presumably in alpha cells. However, in cells located at islet centers, most probably in beta cells, only weak immunoreaction was observed (Figs. 1A,C,D, 2).

CgA-containing islet cells were less numerous (1.7-fold) and immunostaining was significantly weaker in the pancreas of hypertensive rats (Fig. 1D) as compared to sham-operated animals (Fig. 1C, Table 2).

The population of PST-IR cells occurring in the pancreatic islets of hypertensive rats (Fig. 2B) was significantly smaller (1.25-fold) and their immunoreactivity was lower when compared to the control group (Fig. 2A, Table 2).

Discussion

Several reports indicate impaired secretion of islet hormones in hypertension, although the mechanism or mechanisms leading to the aforementioned pancreatic disorder are still not fully understood (Sechi et al., 1992; Cheung and Li, 2012). Given the role of chromogranin and pancreastatin in the regulation of blood pressure and pancreatic endocrine function, we felt it was worth

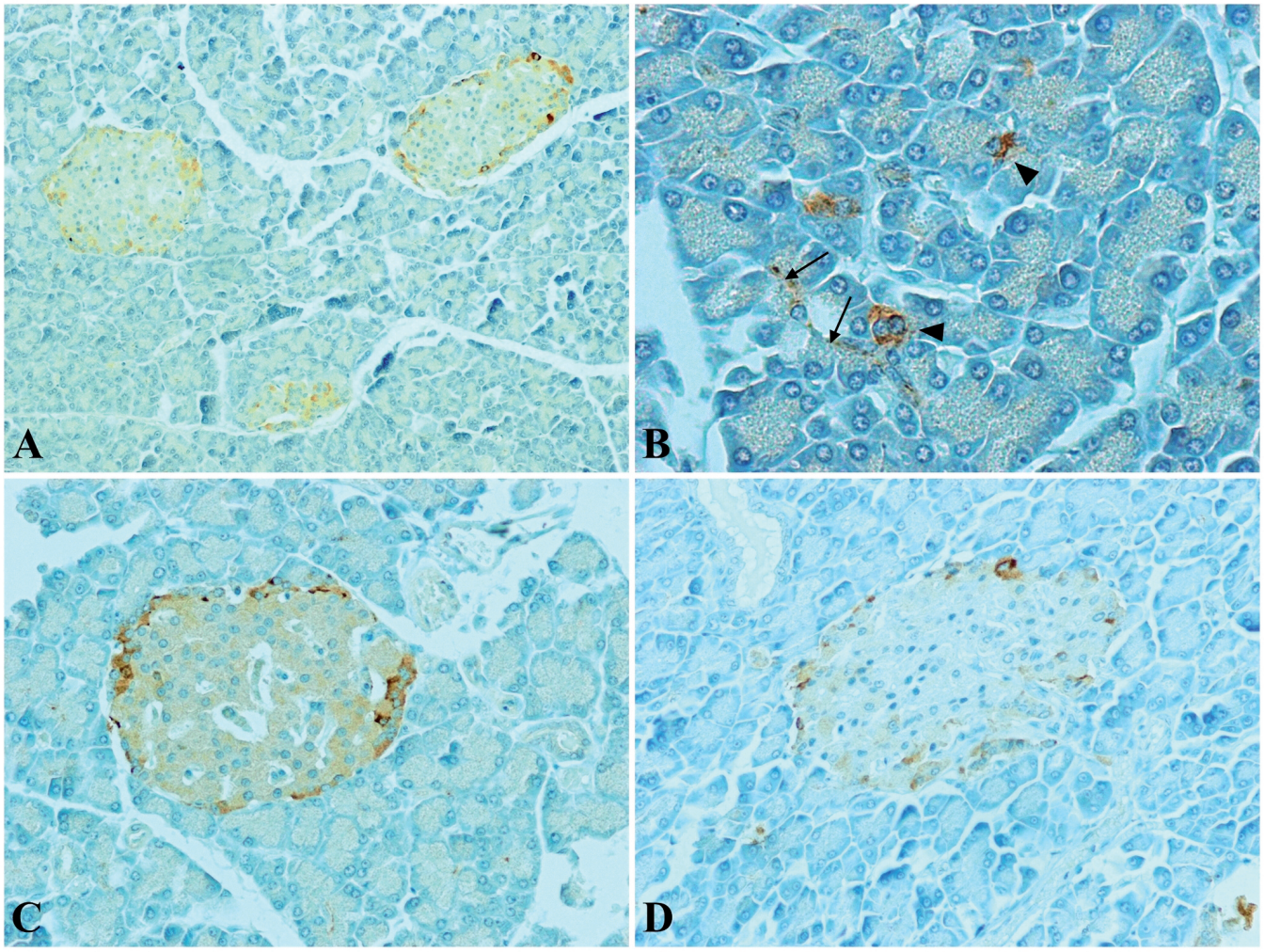

Fig. 1. Immunohistochemical reaction for CgA in pancreas of rats. **A.** control;. CgA-immunoreactivity in pancreatic islets. **B.** Control; positive CgA-reaction in a single exocrine cells (arrowheads) and nerve fibers innervating pancreatic parenchyma (arrows). **C.** control; strong CgA-IR in endocrine cells on islet edges **D.** 2K1C rat; less numerous cells, with weaker intensity of immunohistochemical reaction in pancreatic islet. A, x 100; B, x 400; C, D, x 200

investigating the potential changes in the distribution of CgA and PST in the pancreas of hypertensive rats.

Immunohistochemical detection of CgA and PST in the rat pancreas revealed their presence in the endocrine part of the organ, which is in accordance with research by Ravazzola et al. (1988) and Kimura et al. (1995). A positive reaction for CgA and PST was also observed in single exocrine cells and nerve fibres innervating the pancreatic parenchyma. Having performed immunohistochemical and morphometric tests, we found that the number and immunoreactivity of endocrine cells containing CgA and PST in the pancreatic islets of rats with renovascular hypertension was lower than in normotensive rats. It contradicts study results demonstrating elevated levels of CgA and PST in the sera of patients with hypertension (O'Connor, 1985; Takiyyuddin et al., 1995; Giampaolo et al., 2002). Similar findings regarding pancreastatin were documented by Sanchez-Margelet et al. (1995a,b).

Other experimental studies have demonstrated increased peptide levels in the blood and adrenal glands of animals in various models of hypertension including spontaneous hypertension (O'Connor et al., 1999), stroke-prone spontaneous hypertension (Schober et al., 1989) and renovascular hypertension (Takiyyuddin et al., 1993).

It is known that the disease being discussed is associated with heightened activity of the sympathetic nervous system (Johansson et al., 1999). Increased sympathetic neuronal traffic to the adrenal glands triggers the secretion of catecholamines. CgA is essential for the formation of chromaffin vesicles. The protein is co-stored and co-released with catecholamines, thus stimulating the sympathetic nervous system, which simultaneously results in the augmented biosynthesis of chromogranin in adrenal medulla (O'Connor et al., 1999). Since CgA is a precursor for PST, overactivity of the sympathetic nervous system also increases pancreastatin level in the gland (Jensen et al., 1994). On the other hand, the activation of the sympathetic nervous system has a negative effect on insulin secretion (Gilon and Henquin, 2001). Noradrenaline (NA) released from adrenergic nerves also impacts on glucagon-producing cells. It was documented that NA might influence α-islet cells in two ways, either by increasing cell activity via β adrenoceptors or decreasing glucagon production via α adrenoceptors (Iversen, 1973).

Immunohistochemical studies by Kasacka et al.

Table 2. Number of CgA- and pancreastain-IR cells per 0.1 mm^2 of islet area in pancreas of control and 2K1C rats and intensity of immunohistochemical reaction (small number shows higher immunoreactions). *p<0.05

Group of rats	Chromogranin (CgA)		Pancreastatin (PST)	
	Number of CgA-IR cells per 0.1 mm^2 area of pancreatic islet	Intensity of immunohistochemical reaction for CgA	Number of PST-IR cells per 0.1 mm^2 area of pancreatic islet	Intensity of immunohistochemical reaction for PST
Control	57.2±27.33	131.2±41.26	118.8±23.18	109.4±39.05
2K1C rats	33.7±11.96*	144.3±39.20*	95.81±18.95*	134.1±37.43*

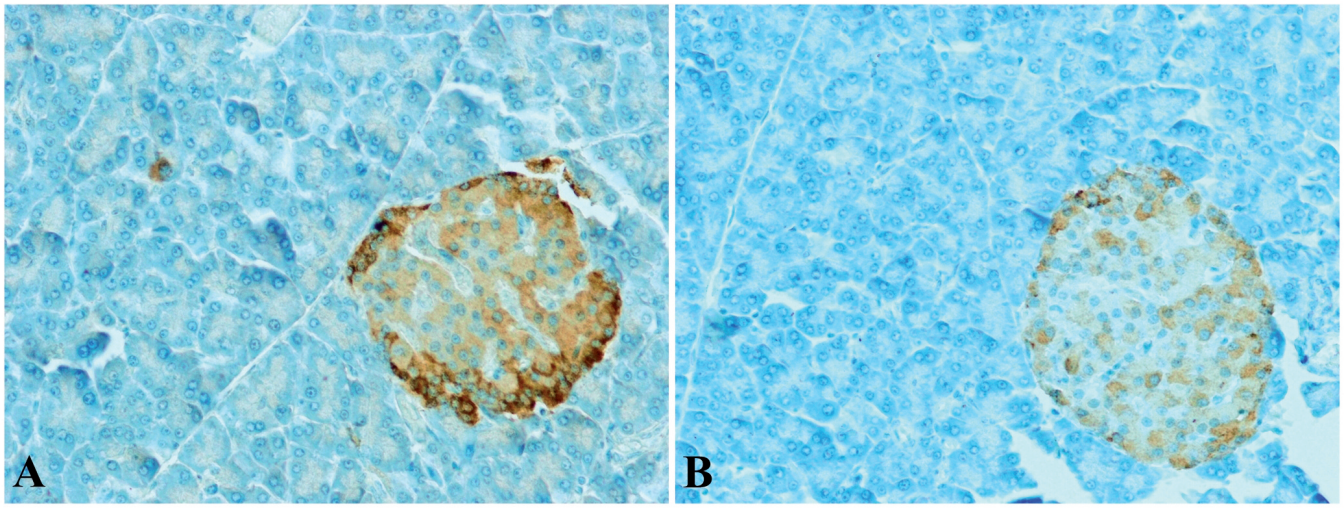

Fig. 2. PST-immunoreactivity in pancreas. **A.** Control rat; numerous endocrine cells with very strong (islet periphery) and strong (islet centre) intensity of reaction **B.** 2K1C rat. x 200

(2015a) revealed a decrease in the number and reaction intensity of the cells containing glucagon in the pancreas of 2K1C rats. This suggests that hypertension causes functional disorders in the alpha cells of pancreatic islets.

Since CgA and PST are present in β- and α islet cells and co-exist in granules with insulin and glucagon (Ravazzola et al., 1988), one can speculate that the decreased immunoreactivity for CgA and PST in rats with hypertension observed in the current study may be associated with the weakened secretory activity of islet cells.

Chromogranin and pancreastatin are involved in maintaining glucose homeostasis. It was found that the peptides reduced insulin secretion, stimulated glucagon release, inhibited glucose uptake by adipose cells and suppressed glycogenesis in hepatocytes (Ahrén et al., 1988; Portela-Gomes et al., 2008; Valicherla et al., 2013). Considering that hyperglycemia occurs 2.5 times more frequently in patients with hypertension than in individuals with normal blood pressure (Cheung and Li, 2012) it might be assumed that the reported reduction in the population of CgA- and PST-IR cells in the pancreas of 2K1C rats is an adaptive process preventing the development of diabetes in hypertension.

A major pathomechanism of renovascular hypertension is an enhanced activity of the renin-angiotensin-aldosterone (RAA) system (Martinez-Maldonado, 1991). Stimulation of the RAA-system causes systemic and local vasoconstriction and thus restricts blood flow to the pancreas. Hypoxic stress leads to enhanced production of nitric oxide and reactive forms of oxygen, which in turn contributes to the decreased viability of insulin-producing cells. Furthermore, it was documented that angiotensin II directly affects β-islet cells, promoting their apoptosis (Luther and Brown, 2011).

Experimental research indicates that CgA and PST play an important role in the regulation of cell proliferation and survival. Hooper and Pocock (2007), Kingham and co-workers (1999) indicated that the incubation of microglial cells with chromogranin increased the production of proapoptotic caspase-1, caspase-3 and nitric oxide (NO), induced mitochondrial depolarization, and as a consequence triggered cell death. Other studies demonstrated that PST increased NO production and inhibited DNA and protein synthesis in rat hepatoma cells (Sánchez-Margalet et al., 2001). Taking into consideration the above findings, the weakened immunohistochemical reaction for CgA and PST in the pancreas of 2K1C rats documented by the present study might be considered a mechanism counteracting a further loss of pancreatic endocrine function.

In the long term, the results presented in this study, showing differences in the number and intensity of immunostaining of cells containing CgA and PST in the pancreas of control and hypertensive rats, combined with the data obtained from previous studies may be of value for future clinical practice.

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