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Evaluation of CART-, glucagon-, and insulin-immunoreactive cells in the pancreas of an experimental rat model of unilateral renal artery stenosis

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Summary. Hypertension is one of the most frequently occurring diseases worldwide. Approximately 10% of the population with hypertension reveal the secondary type of hypertension. The aim of this study was to evaluate the cells containing CART, insulin and glucagon in the pancreas of rats with renovascular hypertension.

An experimental model of hypertension in rats according to Goldblatt (2K1C model of hypertension) was used in the study. The experimental material (pancreas) was collected in the 6th week of the study. Cells containing CART, insulin and glucagon were evaluated using immunohistochemical and morphometric methods. Pancreatic islet cells were evaluated based on the number and intensity of staining.

The investigation showed an increase in the number and immunoreactivity of CART containing cells, 6 weeks after partial unilateral ligation of the renal artery. There was a significant decrease in the number of glucagon-IR cells. Although intensity of staining these cells did not change. No differences were observed in the number and staining affinity of insulin-containing cells.

On the basis of the study it can be stated that the endocrine system of pancreas undergoes changes in the course of renovascular hypertension. This may affect the production of hormones and contribute to the development of possible hypertension complications.

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Introduction

The pathogenesis of hypertension involves a variety of mechanisms which regulate vascular diameter, cardiac output and blood osmotic pressure in the organism. This applies especially to disorders in the synthesis and secretion of hormones, and other biologically active substances.

Dysfunctions in the secretion of islet hormones are common in patients with hypertension (Park et al., 2013). High blood pressure might evolve as an unwelcome consequence of hyperinsulinemia and insulin resistance. Insulin resistance is probably recruited as a mechanism that limits further weight gain in obese hypertensives as well as in non-obese hypertensives (Landsberg, 1989). Raised bloodstream insulin level causes stimulation of the sympathetic nervous system, increased sodium retention and vascular smooth muscle hypertrophy and consequently leads to elevation of blood pressure (Park et al., 2013). The link between insulin resistance, the resultant hyperinsulinemia and hypertension has been confirmed in several studies. However, this association has been found only in patients with essential hypertension and in spontaneously hypertensive rats (Reaven and Chang, 1991).

As shown by a literature review, little is known about the role of glucagon in the pathogenesis of hypertension. Glucagon is one of the insulin secretion stimulators and is supposedly involved in the development of insulin resistance in type 2 diabetes (Li

et al., 2008). Thus, glucagon might mediate a link between raised blood insulin level and hypertension. Furthermore, hyperglucagonemia purportedly activates the renal renin-angiotensin (RA) system, which is one of the main mechanisms leading to vasoconstriction of arterioles (Ueda et al., 1978; Li et al., 2006). It has been proven that long-term administration of glucagon causes a rise in systolic blood pressure (Li et al., 2008).

The results of previous research suggest that CART (cocaine and amphetamine regulated transcript) may also be involved in insulin-dependent pathogenesis of hypertension as its mediator. Wierup et al. (2004) have demonstrated that CART is expressed in several islet cell types during rat development. Thus, the authors suggest that the peptide promotes islet cell survival and plays a role in islet cell differentiation. In adult animals the presence of CART was found mainly in somatostatin-producing cells, therefore CART began to be considered a regulator of pancreas' endocrine function (Wierup et al., 2006; Arciszewski et al., 2008). CART has been shown to induce a decrease in glucagon level and a parallel fall in insulin secretion (Wierup and Sundler, 2006; Wierup et al., 2006). The peptide may affect the secretion of pancreatic hormones via activation of the autonomic nervous system (Kiba, 2004). CART is presumed to be a mediator of stress-induced sympathetic neural activity, which results in enhanced secretion of catecholamines and reduced insulin secretion (Kiba, 2004; Koylu et al., 2006). The peptide may also attenuate the activity of the parasympathetic nervous system and thus limit the activity of islet β cells (Vincentic and Jones, 2007).

Moreover, CART plays a role in the regulation of the cardiovascular system. Most likely, the peptide exerts an impact on the circulatory system through the activation of the hypothalamic-pituitary-adrenal axis or by stimulating the sympathetic nervous system (Koylu et al., 2006). Studies on rats and rabbits have proven that CART accelerates heart rate and increases peripheral blood pressure (Matsumura et al., 2001).

The results of previous studies implicate insulin, glucagon, and CART in the regulation of blood pressure, whereas the role of these substances in the pathogenesis of secondary hypertension has not been fully explained.

The aim of this study was to evaluate the cells containing CART, insulin and glucagon in the pancreas 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 of Bialystok (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 (AnimaLab; Morawskiego 2/22; 60-239 Poznań, Poland.) their body weight at the beginning of the experiment was 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 (Maintenance diet #1320; AnimaLab; Morawskiego 2/22; 60-239 Poznań, Poland) 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.

The experimental 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.

After a 6 week period of 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, the pancreas was collected under deep pentobarbital anesthesia (50 mg/kg b.w.) (Brown and Vale, 1975). 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 (Leong et al., 2010). Sections were cut at 4 μm in thickness, and stained by haematoxylin-eosin (H&E) for general histological examination and processed by immunohistochemistry for CART, insulin and glucagon detection. Weight data of left and right kidney were additionally collected.

Detection of CART, insulin and glucagon in rat 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 in xylene and hydrated using several grades of alcohol. For antigen retrieval, the sections were subjected to pretreatment in a 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 9.0 for CART (S 2367, Dako; Glostrup, Denmark) and using Target Retrieval Solution with pH 7.2 for insulin and glucagon (S 1699, Dako; Glostrup, Denmark). 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 CART (rabbit polyclonal CART antiserum, No H-003-61, purchased at the Phoenix Pharmaceuticals, Inc., Mountain View, CA); for insulin (polyclonal guinea pig anti-insulin, No A 0564, purchased at the DakoCytomation, Glostrup, Denmark); and for glucagon (polyclonal rabbit anti-glucagon, No A 0565, purchased at the DakoCytomation, Glostrup, Denmark). All antibodies were previously diluted in Antibody Diluent (S 0809 DakoCytomation, Glostrup, Denmark) in relation 1:10,000 for CART-antibody; 1:1,500 for anti-insulin and 1:750 for anti-glucagon.

Sections with CART-antibody were incubated overnight at 4°C in a humidified chamber, whereas incubation with insulin- and glucagon-antibody lasted 15 minutes and was performed at room temperature.

The procedure was followed by incubation with secondary antibody (conjugated to horseradish peroxidase-labelled polymer). The bound antibodies were visualised 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, covered and evaluated under a light microscope. Appropriate washing with Wash Buffer (S 3006 DakoCytomation; Glostrup, Denmark) was performed between each step.

Specificity tests, performed for the CART-, insulin-, and glucagon- 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.

Five sections from each animal were prepared for immunohistochemistry with anti-CART, similarly five sections from each animal were stained immunohistochemically for insulin and glucagon. CART-, insulin- and glucagon-positive structures were searched for and their topography was observed.

Quantitative analysis

Following immunostaining, morphometric evaluation was performed using an Olympus Bx50 microscope with a digital camera (Olympus DP12) and standard morphometric program (NIS-Elements Advanced Research software of Nikon) installed on computer. In each section stained immunohistochemically for CART, ten islets were randomly selected at a magnification 200x (20x the lens and 10x the eyepiece) to 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 CART-containing cells, insulin-positive cells and glucagon-IR cells were counted, then 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 immunohisto-chemical 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 Mann Whitney test ; $p<0.05$ was taken as the level of significance.

Results

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

In all pancreatic specimens, the routine histopathological examination did not show any identifiable pathological changes. No significant

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*

differences were observed between the control and hypertensive rats in the morphology of the pancreases studied.

CART, glucagon and insulin immunoreactivity was found in the pancreas of each study animal, and it was concentrated in the endocrine part of the organ. Only CART- immunoreactivity was additionally noted within pancreatic ganglia and in nerve fibers innervating exocrine parenchyma (Fig. 1A,B). CART-containing cells and glucagon-positive cells were seen only on the outskirts of the islet (Figs. 2, 3) whereas insulin-IR cells occupied nearly the entire section of pancreatic islets (Fig. 4).

The density of CART-positive (Fig, 2A,B) and glucagon-IR islet cells (Fig. 3A,B) differed between rats with renovascular hypertension and control rats, but no changes were found in the density of insulin-containing cells (Fig. 4A,B). There were also noticeable differences in the intensity of CART-immunohistochemical reaction (Fig. 2A,B), whereas the intensity of immunohistochemical reactions for glucagon (Fig. 3A,B) and insulin remained unchanged (Fig. 4A,B) .

CART-containing islet cells were more numerous in the pancreas of hypertensive rats, as compared to sham-operated rats (90.7±13.43; 63.0±15.30, respectively p=0.0001) (Figs. 2, 5). Likewise, the immunohistochemical reaction in CART-positive cells was more intense in the pancreas of 2K1C rats than in normotensive rats ($p<0.0001$) (Figs. 2, 6).

The population of glucagon-IR cells occurring in the

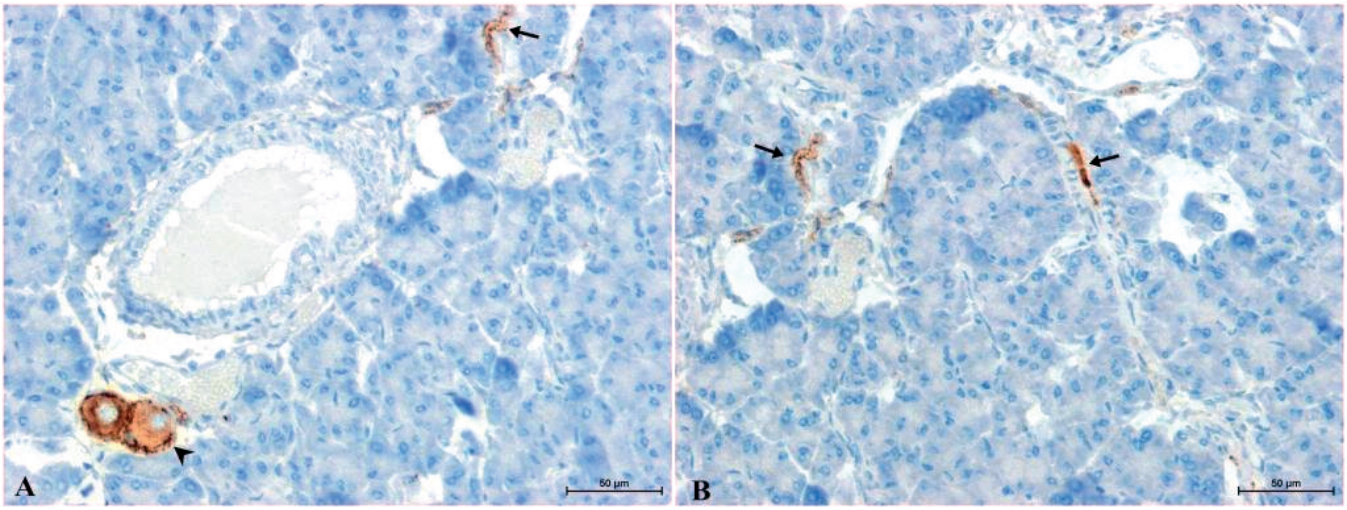

Fig. 1. CART-immunoreactivity in exocrine part of rats pancreas. **A.** Visible immunohistochemical reaction in nerve cell bodies within pancreatic ganglia (arrow head. **B.** Positive staining for CART in nerve fibers (arrows). Bars: 50 μm.

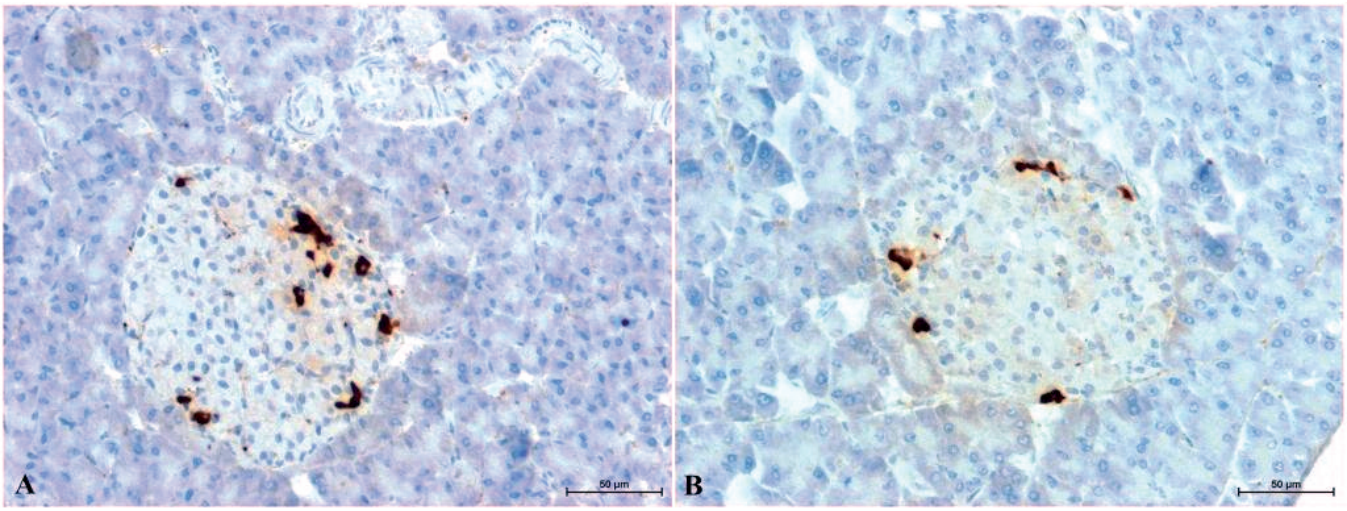

Fig. 2. Immunohistochemical reaction for CART peptide in pancreatic islet. **A.** 2K1C rat. **B.** Control rat. Bars: 50 μm.

pancreatic islets of hypertensive rats was significantly decreased in number when compared to the control group (275.6±42.02; 380.0±75.76, respectively p= 0.0317) (Figs. 3, 5). However, the immunohistochemical reaction for glucagon in the pancreas of rats with renovascular hypertension had similar intensity to that noted in normotensive rats (p=0.6494) (Figs. 3, 6).

No difference was observed in the number of insulin-containing cells (p=0.1431) (Figs. 4, 5) and in the intensity of immunohistochemical reaction for insulin (p=0.7461) (Figs. 4, 6).

Discussion

Homeostatic disorders are immediately manifested by abnormal secretion of hormones and other biologically active substances. This research concerns potential qualitative and quantitative changes in the insulin-, glucagon- and CART-containing pancreatic cells of rats after renal artery clipping.

Typical distribution of insulin and glucagon was observed in the pancreas of each rat studied. Insulin-positive cells were seen almost over the entire section of the islets, whereas glucagon-containing cells were seen only on the islet periphery (Lukens, 1959). CART-immunoreactive cells were located mainly on the outskirts of the pancreatic islets, which is in accordance with the results of previous studies on humans, rodents and sheep, but not with those on pig (Wierup et al., 2004, 2007; Arciszewski et al., 2008; Kasacka et al.,

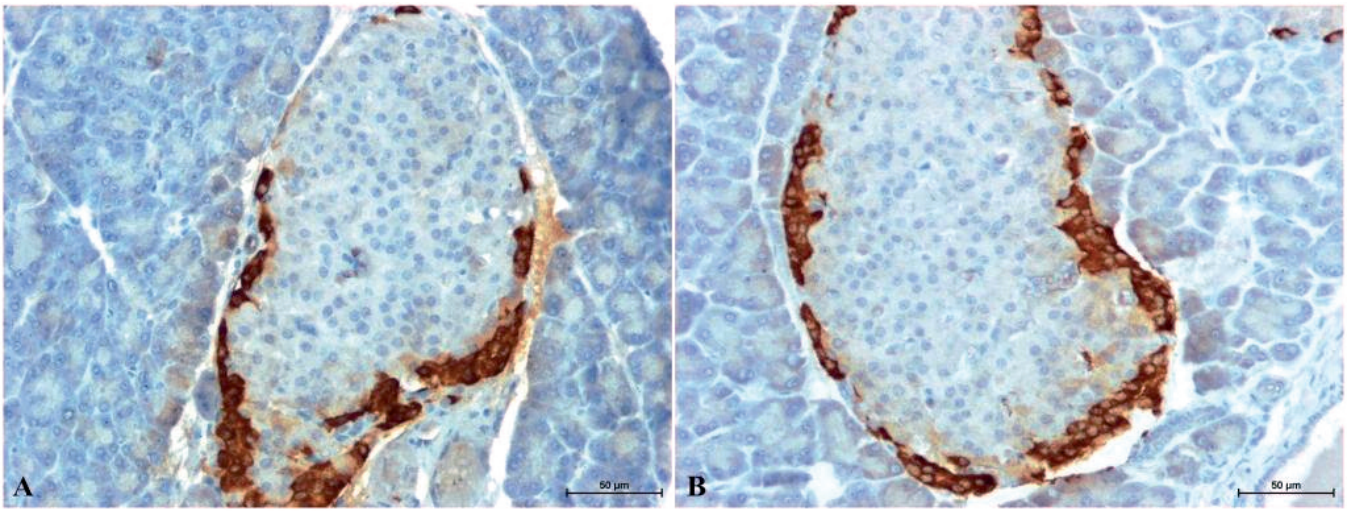

Fig. 3. Positive immunohistochemical reaction for glucagon. **A.** Rat with renovascular hypertension. **B.** Control rat. Bars: 50 μm.

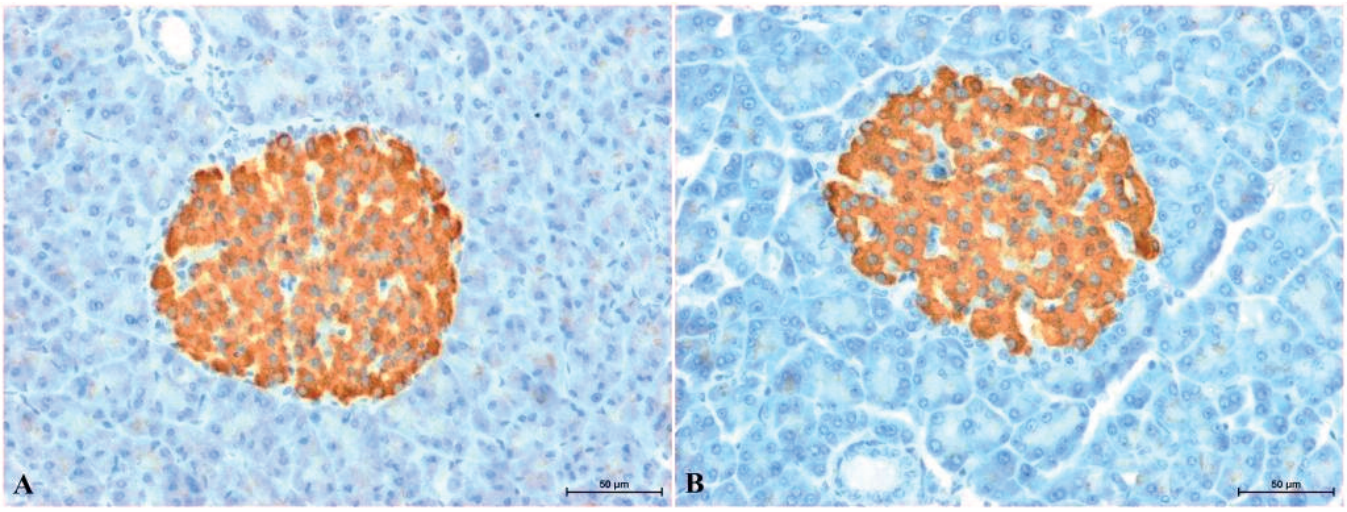

Fig. 4. Results of immunohistochemical staining for insulin. **A.** 2K1C rat. **B.** Control rat. Bars: 50 μm.

2012).

Our findings demonstrate changes in the density and immunoreactivity of CART-containing islet cells in conditions of renovascular hypertension. CART-IR cells were more numerous in the pancreas of rats with experimental hypertension, when compared to normotensive animals. Analogously, the immunohistochemical reaction for CART, which was seen in the pancreas of 2K1C rats, was stronger than the reaction observed in the control group. Wierup and colleagues (2006) made similar observations during research on rats with two different types of diabetes. The Authors noticed an elevation in the number of CART-positive islet cells and its immunoreactivity in the pancreas of diabetic rats as compared to control animals.

As far as we are aware, no previous literature data are available on the state of hypertension and distribution of CART- positive structures in the pancreas.

Iliff and co-workers (2005) have indicated a vasoactive role of CART by demonstrating that the peptide induces long-lasting constriction of cerebral arterioles. Thus, enhanced synthesis of CART in the pancreas of hypertensive rats might modulate blood flow to the organ. Sufficient pancreatic perfusion is critical to proper islet secretory function, since long-term restriction of blood flow to the pancreatic islets induces β cell apoptosis (Emsberger and Koletsky, 2007). However, the apparent increase in the number of CART-IR cells and lack of changes in the number of insulin-positive cells in the pancreas of 2K1C rats suggest a protective rather than destructive effect of CART peptide on β cells. Rats with renovascular hypertension have enhanced level of oxidative stress and CART, which exhibits antioxidant properties, may prevent β cell apoptosis caused by reactive oxygen species (Mao et al., 2012).

Up-regulation of CART gene expression in conditions of secondary hypertension may affect other regulators of islet hormone secretion, such as somatostatin and glucocorticoids, and thus the peptide can modulate insulin and glucagon secretions also via indirect pathways (Koylu et al., 2006). Density of insulin- and glucagon-positive cells seen in the pancreas of the rats studied might result from mutual relationships between all regulative compounds.

Chronic renal ischemia is a stressor factor for the organism, therefore renovascular hypertension leads to enhanced activity of sympathetic nervous system and intensified secretion of stress hormones such as catecholamines (Buranakarl et al., 2000). Considering the results of research by Hunter et al. (2007) and Balkan et al. (2012), who demonstrated the impact of stress response on CART biosynthesis in various organs, it can be assumed that changes in the number and immunoreactivity of CART-IR cells in pancreas of 2K1C rats might also be associated with disease-related stress response.

Quantitative assessment demonstrated a decrease in the number of glucagon-IR cells in the pancreas of 2K1C rats. Our findings are contradictory to clinical data, indicating elevation of plasma glucagon level in human patients with essential hypertension (Sánchez-Margalet et al., 1995). Since glucagon has been shown to potentiate renin secretion, the elevated glucagon level may be a mechanism that leads to high activity of the RA system in primary hypertension (Li et al., 2006). Analogously, restoration of the proper plasma renin activity in the chronic phase of renovascular hypertension might be associated with a reduction in the population of glucagon-producing cells.

The decrease in the number of glucagon-positive cells observed in our study may be associated with the enhanced population of CART-synthetizing cells, as CART peptide has been shown to inhibit glucagon synthesis (Wierup et al., 2006; Wierup and Sundler,

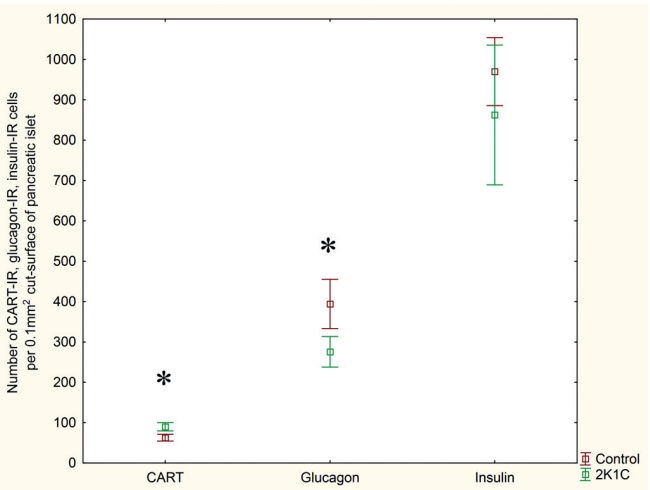

Fig. 5. The number of CART-IR, glucagon-IR, insulin-IR cells per 0.1 mm^2 cut-surface of pancreatic islet in 2K1C and control rats. *p<0.05.

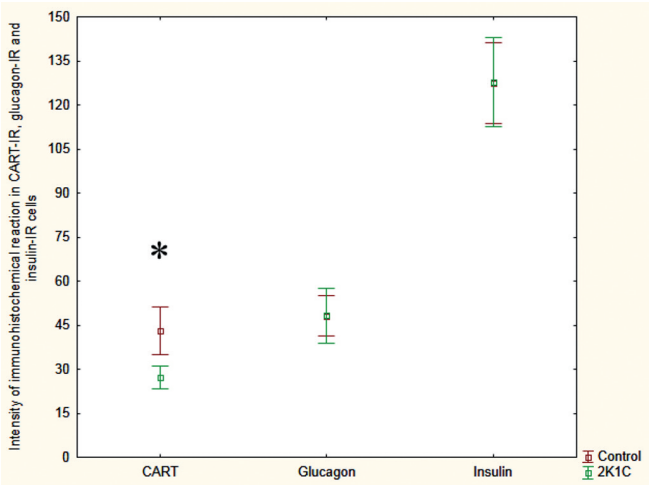

Fig. 6. Intensity of immunohistochemical reaction in CART-positive, glucagon-containing and insulin-producing cells in renovascular hypertensive and control rats. *p<0.05.

2006).

In turn, lack of changes in the intensity of immunohistochemical reaction in glucagon-positive cell, might be related with the mentioned increase of catecholamine secretion in the studied pathological condition. It has been shown that catecholamines stimulate glucagon secretion (Weir et al., 1974), thus, adrenal medulla hormones might contribute to intensive production of glucagon in fewer α cells.

Our research revealed no differences between 2K1C and control rats in terms of the number and immunoreactivity of insulin-positive cells.

Likewise, an unchanged systolic insulin level has been observed in humans with renovascular hypertension (Marigliano et al., 1990; Sechi et al., 1992). Our findings confirm the results of previous research on rats with experimental hypertension, which showed no changes in plasma insulin level and glucose tolerance (Buchanan et al., 1991).

On the other hand, the existing literature data have proved that essential hypertension is associated with hyperinsulinemia (Sechi et al., 1992; Sánchez-Margalet et al., 1995). This disparity between primary and secondary hypertension might be due to differences in the pathogenesis of each type of hypertension (Martinez-Maldonado, 1991; Higashiura et al., 1999). High insulin level and insulin-resistance in patients with essential hypertension is probably associated with increased angiotensin II systemic level (Higashiura et al., 1999). This hypothesis is supported by a study on mice, which showed an increase in plasma insulin concentration in animals treated with angiotensin II (Gletsu et al., 2005). Therefore, the lack of changes in insulin secretion in renovascular hypertensive rats may be explained by normal RA system activity in the chronic phase of secondary hypertension (Martinez-Maldonado, 1991).

Beyond the systemic RA system, fulfilling a crucial role in regulation of blood pressure, in the majority of organs Leung and Carlsson (2001) found the presence of tissue RA system, which is committed to maintenance of interorgan homeostasis. In renovascular hypertension, due to local and systemic vasoconstriction, the blood flow to pancreas is restricted. Literature data suggest that hypoxic stress leads to changes in the expression of tissue RA system components in various organs, including pancreas (Leung and Carlsson, 2001). Simultaneously, insufficient pancreatic perfusion might also cause enhanced production of proinflamatory factors and reactive oxygen species in the organ, destructively affecting pancreatic islet cells. Bearing in mind the impact of angiotensine II on the secretion of various growth and proliferative factors (Ferreri et al., 1997), it can be assumed that in conditions of renovascular hypertension, the tissue RA system is involved rather in adaptive processes limiting the loss of islet β cells, than in regulation of insulin-producing cells secretory function. Reported in the present paper, a lack of significant changes in the number and reactivity of insulin-IR cells between 2K1C and normotensive rats appears to confirm the above hypothesis.

The pathogenesis of experimental hypertension involves disorders in water and electrocyte balance. Numerous literature data indicate that renovascular hypertension frequently coexists with hiper-aldosteronism (Martinez-Maldonado, 1991). It was found that hiperinsulinemia, which occurs in hypertensive patients, is related to plasma aldosterone level. Colussi et al. (2007) demonstrated that patients with higher plasma aldosterone level had simultaneously higher plasma insulin level. Nonetheless, considering that our experiment did not reveal any quantitative or qualitative changes of insulin-IR cells in pancreas of renovascular hypertensive rats, it can be suspected that the effect of aldosterone on insulin secretion might be abolished by other factors inhibiting hormone production. For example, aldosterone-induced insulin secretion might be supressed by an increased level of CART, since the peptide was shown to inhibit insulin release. Considering the increase in the number of CART-producing cells in pancreas of 2K1C rats, demonstrated in present study, it can be assumed that peptide is involved in compensative processes restricting the development of hiperinsulinemia in renovascular hypertensive rats.

A decreased release of glucagon, which is a known activator of β cells' secretory function, might be another potential mechanism limiting the aldosterone-linked secretion of insulin. The result of the present study indicated reduced number of glucagon-IR cells in pancreas of hypertensive rats, which appears to support this hypothesis.

In conclusion, results of our research indicate the role of CART peptide in the internal regulation of pancreatic endocrine function during renovascular hypertension. It also suggests a potential involvement of glucagon in the development of experimental hypertension and confirms a lack of dependence between hyperinsulinemia and secondary hypertension.

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