Original Research

Dynamics of cocaine- and amphetamine-regulated transcript containing cell changes in the adrenal glands of two kidney, one clip rats

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Abstract

Taking into consideration the homeostatic disorders resulting from renal hypertension and the essential role of cocaine- and amphetamine-regulated transcript (CART) in maintaining homeostasis by regulating many functions of the body, the question arises as to what extent the renovascular hypertension affects the morphology and dynamics of changes of CART-containing cells in the adrenal glands. The aim of the present study was to examine the distribution, morphology, and dynamics of changes of CART-containing cells in the adrenal glands of ''two kidney, one clip'' (2K1C) renovascular hypertension model in rats. The studies were carried out on the adrenal glands of rats after 3, 14, 28, 42, and 91 days from the renal artery clipping procedure. To identify neuroendocrine cells, immunohistochemical reaction was performed with the use of a specific antibody against CART. It was revealed that renovascular hypertension causes changes in the endocrine cells containing CART in the adrenal glands of rats. The changes observed in the endocrine cells depend on the time when the rats with experimentally induced hypertension were examined. In the first period of hypertension, the number and immunoreactivity of CART-containing cells were decreased, while from the 28-day test, it significantly increased, as compared to the control rats. CART is relevant to the regulation of homeostasis in the cardiovascular system and seems to be involved in renovascular hypertension. The results of the present work open the possibility of new therapeutic perspectives for the treatment of arterial hypertension, since CART function is involved in their pathophysiology.

Keywords: Cocaine- and amphetamine-regulated transcript, adrenal glands, renovascular hypertension, rat

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Introduction

During recent years, much attention has been paid to the role of cocaine- and amphetamine-regulated transcript (CART) in various processes of the organism. Extensive literature data indicate the involvement of peptide in central regulation of appetite and energy balance.[1] The presence of the peptide in neurons innervating the gastrointestinal tract prompted researchers to study CART participation in the control of digestive system functioning.[2] Further research confirmed that the peptide might act as an endogenous psychotropic substance and affect locomotor activity.[3]

There are also suggestions about participation of CART in the stress response and in control of the secretory function of the adrenal glands. Peptides may lead to intensive secretion of adrenaline by activating the sympathetic peripheral nervous system and may also provoke production of glucocorticoids through activation of the hypothalamus-pituitary-adrenal axis (HPA).[4] Stanley and coworkers have reported that injection of CART into the brain ventricles results in elevation of ACTH and thus corticosteroid serum levels.[5] Other experimental studies confirmed that intraventricular administration of the peptide causes increased release of adrenaline into peripheral blood.[6]

CART was found in regions of the central nervous system responsible for control of the cardiovascular system.[7] On this basis, it was supposed that peptide might participate in the central regulation of the circulatory system. This hypothesis was confirmed in several experimental studies. Hwang et al. demonstrated that administration of peptide into rat cisternal magna causes an increase in heart rate and elevation of blood pressure.[6] Matsumura and co-workers

obtained analogical results in studies performed on rabbits intracerebroventricularly injected with CART.[8]

Considering the co-occurrence of CART with biogenic amines and some hormones with vasotrophic and hemostatic properties and its essential role in the circulatory system, it is anticipated that CART is actively involved in the process of arterial hypertension.[6,8]

However, despite the enormous progress that has been made in recent years in terms of knowledge regarding the structure and physiological properties of CART peptide, its role in the pathogenesis of hypertension remains unexplained.

Renovascular hypertension causes a series of physiological changes that lead to organ dysfunction. Recent studies indicate increased activation of the sympathetic nervous system in its advanced stage.[9] As a consequence, it leads to increased secretion of main stress hormones, since the adrenal medulla is particularly sensitive to the activation of the sympathetic nervous system. Experimental studies showed that intraventricular administration of CART results in elevation of adrenaline and noradrenaline levels in blood serum, which indicates that peptide might be a possible mediator in central control of this mechanism.[8]

Knowledge about changes in the morphology and distribution of CART-positive structures in the adrenals of hypertensive rats might clarify the role of CART in physiological and pathological conditions.

The aim of the present study was to assess the number and morphological features of CART-immunoreactive (CART-IR) cells in the adrenal glands of rats at different times of renovascular hypertension.

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. Study assumptions, aim, schedule and mode of animal treatment were approved by the Senate Committee for Supervision of Experiments on Humans and Animals, Medical University of Bialystok.

The study was carried out on 80 ($n = 80$) young male Wistar rats; their body weight at the beginning of the experiment was 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 *ad libitum*. All the experiments were carried out at the same time of the day.

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 (procedure according to Goldblatt et al.[10]).

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. In 40 rats ($n = 40$), 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. Twenty sham operated rats ($n = 20$) underwent identical surgical procedures, except that a clip was not applied to the renal artery. Twenty ($n = 20$) rats did not undergo any surgical procedure.

Histology

Method of experimental material collection and fixation. After 3, 14, 28, 42, and 91 days from the renal artery clipping procedure, the rats (eight operated and four from each control) were anaesthetized with pentobarbital (50 mg/kg b.w.) and sacrificed by intersection of the heart. The specimens of adrenal glands were collected, fixed in Bouin's fluid, routinely placed in paraffin blocks and then sectioned by a Leica 2025 rotating microtome. Sections were cut into 4-μm thick sections, and stained by hematoxylin-eosin (H + E) for general histological examination and processed by immunohistochemistry for CART detection.

Identification of CART-containing structures by immunohistochemical methods. In the immunohistochemical study, the EnVision method was used according to Herman GE, Elfont EA.[11] Immunostaining was performed by the following protocol: paraffin-embedded sections were deparaffinized and hydrated in pure 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 was provided by the United Kingdom National Physical Laboratory) at 125°C, using Target Retrieval Solution with pH of 9.0 (S 2367, Dako Denmark A/S, Produktionsvej 42, DK-2600, Glostrup, Denmark). After cooling to room temperature, the sections were incubated with Peroxidase Blocking Reagent (S 2001 Dako Denmark A/S, Produktionsvej 42, DK-2600, Glostrup, Denmark) for 10 minutes to block endogenous peroxidase activity.

The sections with the primary antibody for CART peptide (rabbit polyclonal CART antiserum, No H-003-61, purchased at the Phoenix Pharmaceuticals, Inc. Mountain View, CA, USA) were incubated overnight at 4°C in a humidified chamber. The antiserum had been previously diluted in Antibody Diluent (S 0809 Dako Denmark A/S, Produktionsvej 42, DK-2600, Glostrup, Denmark) in the ratio 1:10,000.

This procedure was followed by incubation with secondary rabbit antibody (conjugated to horseradish peroxidase-labeled polymer) (EnVision+ Kit HRP Rabbit K4011 Dako Denmark A/S, Produktionsvej 42, DK-2600, Glostrup, Denmark). The bound antibodies were visualized by 1-min incubation with liquid 3,3′-diaminobenzidine substrate chromogen. The sections were finally counterstained in Vector QS hematoxylin (H-3404; Vector Laboratories, Inc.,

Burlingame, CA, USA), mounted and evaluated under a light microscope. Appropriate washing with Wash Buffer (S 3006 Dako Denmark A/S, Produktionsvej 42, DK-2600, Glostrup, Denmark) was performed between each step.

Specificity tests, performed for the CART antibody included: negative control, where the antibodies were replaced by normal rabbit serum (Vector Laboratories; Burlingame, CA, USA) at respective dilution and a positive control was prepared with specific tissue (as recommended by the manufacturer, we used rat hypothalamus for our research concerning CART). The obtained results of immunohistochemical staining were submitted for evaluation using an Olympus Bx50 microscope. From each animal, three sections of each adrenal gland were studied. CART-containing structures were searched for and their topography was observed.

Quantitative analysis. The obtained results of immunohistochemical staining were submitted for evaluation by an Olympus Bx50 microscope with Olympus DP12 camera. Images from five randomly selected microscopic fields (each field of 0.785 mm^2, magnification of 200× (20× the lens and 10× the eyepiece)) were submitted to morphometric evaluation by using software to image analysis (NIS-Elements Advanced Research Nikon software).

The numbers of positively stained cells were counted in each analyzed image, and then the numbers of CART-containing cells were converted and presented as mean values per 1 mm^2 section area. CART-IR cells were also analyzed in terms of morphometric features such as length, width, area, diameter and circularity index, and intensity of immunohistochemical reaction. Intensity of immunohistochemical reaction was measured by using 0–256 gray scale level, where completely black pixel had a value of 0, whereas the one with a value of 256 is completely white or bright.

All presented 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 one-way ANOVA test, $p < 0.05$ was taken as the level of significance.

Results

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

Monitoring of blood pressure showed that at week 6 of the experiment the blood pressure in renovascular hypertensive rats stabilized at 162.6 ± 2.19 mmHg, while in control rats remained at 120.2 ± 5.89 mmHg. Hypertensive animals were gaining weight at a similar rate as normotensive rats.

Routine histopathological examination showed no microscopic significant pathological changes in the adrenal glands between normotensive and hypertensive rats.

Positive result of immunohistochemical reaction for CART was seen in the adrenal glands of each studied rat. Presence of the peptide was stated in endocrine cells in the adrenal medulla, and in sparse nerve fibers innervating cortex and capsule of the organ (Figure 1).

There were significant differences in the number, morphology, and staining intensity of CART-containing cells in different stages of development of hypertension.

After three days from renal artery clipping, the number of CART-positive cells and intensity of immunohistochemical reaction were significantly decreased compared to control rats (Table 1, Figure 2(a) and (b)). Morphometric analysis of CART –IR cells showed that values of parameters such as length and area were significantly lower, and cells were more round than in the adrenal of control animals (Figures 3 and 4).

Similarly, 14 days after clipping procedure, the population of CART-IR cells in rats with experimentally induced hypertension was decreased and the intensity of immunohistochemical reaction was weaker in comparison to normotensive animals (Table 1, Figure2(a) and (c)). However, it is worth highlighting that an observed decrease in number of CART-positive cells was stronger (3-fold decrease) than in the previous group (1.7-fold decrease) (Table 1). The studied cells were also significantly shorter, smaller, and closer to the circle-shape than cells observed in the adrenal glands of the control rats, but, on the other hand

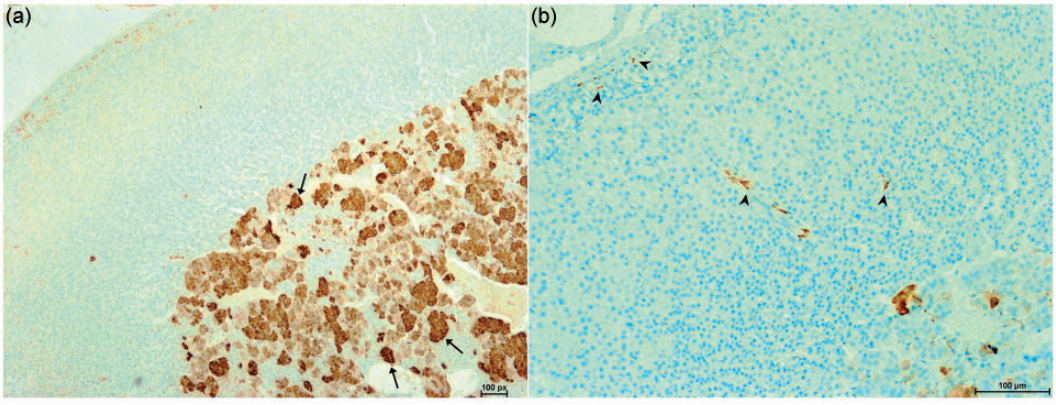

Figure 1 Positive result of immunohistochemical reaction for CART in rat adrenal gland. (a) numerous CART-containing cells in adrenal medulla (arrows) (91 days from artery clipping procedure). (b) CART-IR nerve fibers in the cortex and adrenal capsule (arrowheads) (control rat). (A color version of this figure is available in the online journal.)

Table 1 Number of CART-IR cells in adrenal medulla of control and the 2K1C rats at different times of renovascular hypertension

			Intensity of immunohistochemical reaction in 0–256 gray scale		
Time after artery clipping procedure	**Group of rats**	**Number of CART-IR cells**	**Cells with strong reaction**	**Cells with medium reaction**	**Cells with weak reaction**
3 days	Control	765.8 ± 62.84	63.4 ± 12.87	133.4 ± 23.33	181.1 ± 21.49
	2K1C	448.9 ± 65.05	98.0 ± 19.22	165.3 ± 21.82	203.1 ± 10.32
14 days	Control	946.6 ± 76.13	63.4 ± 12.87	133.4 ± 23.33	181.1 ± 21.49
	2K1C	244.9 ± 46.45	99.3 ± 25.59	169.0 ± 20.72	207.8 ± 10.32
28 days	Control	978.16 ± 65.06	83.5 ± 16.33	139.1 ± 17.97	193.4 ± 8.89
	2K1C	1123.0 ± 57.74	71.5 ± 16.98	130.4 ± 16.70	188.5 ± 11.01
42 days	Control	947.4 ± 81.12	70.4 ± 12.95	141.4 ± 19.22	191.6 ± 14.86
	2K1C	1453.9 ± 10.25	65.8 ± 12.43	127.3 ± 20.43	178.8 ± 15.36
91 days	Control	343.7 ± 53.37	70.4 ± 15.49	153.3 ± 20.60	198.8 ± 11.68
	2K1C	926.8 ± 82.49	42.6 ± 18.92	112.1 ± 34.55	171.4 ± 23.12

Note: Intensity of immunohistochemical reaction in each category of CART-IR cells (mean ± SD); $P > 0.05$.

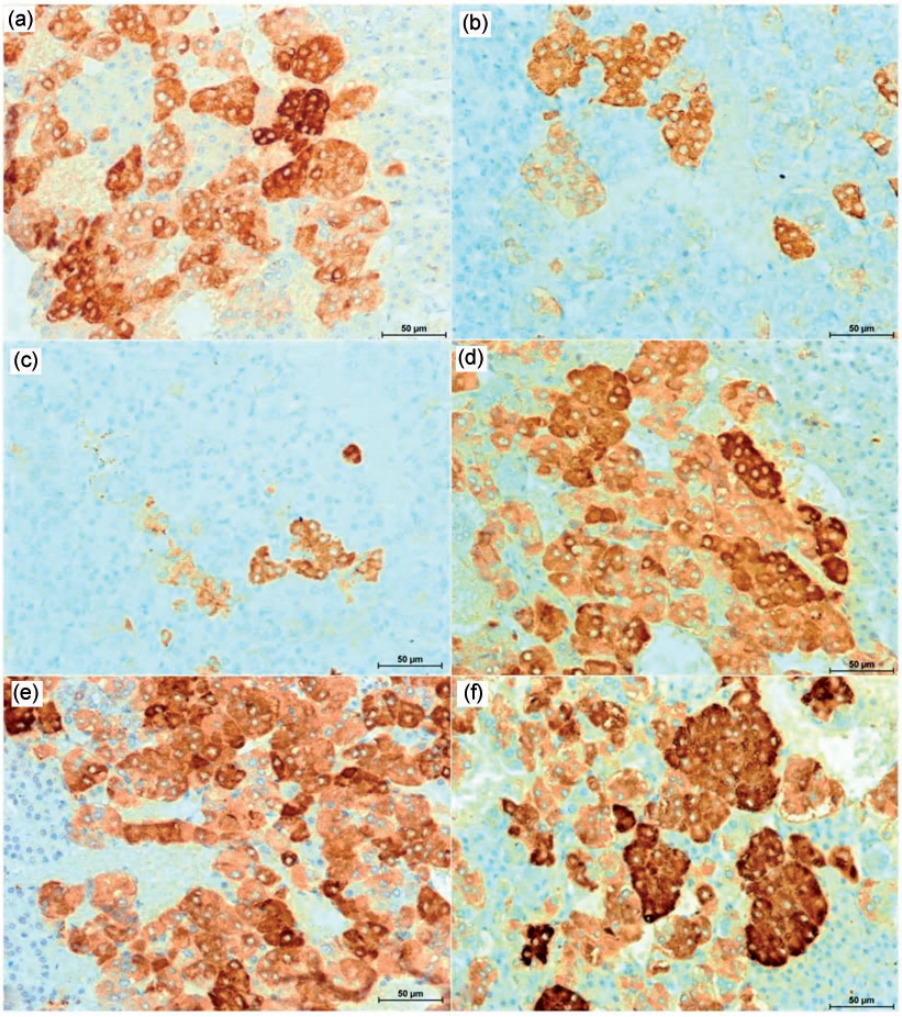

Figure 2 CART-immunoreactivity in adrenal medulla at different times of renovascular hypertension: normotensive rats (a), 3 days (b), 14 days (c), 28 days (d), 42 days (e), and 91 days (f) after induction of experimental hypertension. (A color version of this figure is available in the online journal.)

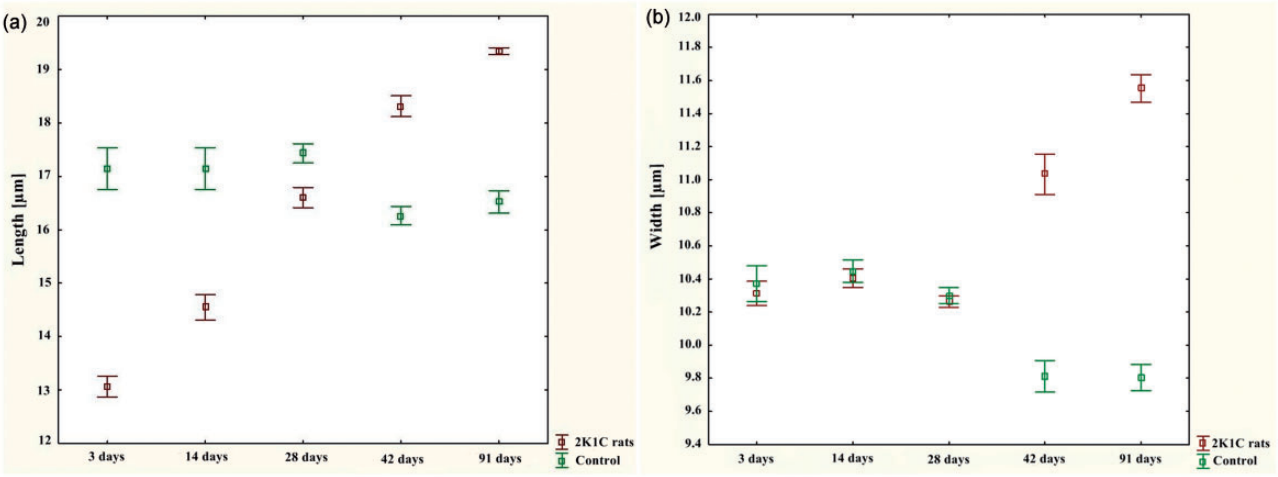

Figure 3 Comparative analysis of the length and width parameters of CART-containing cells in adrenals at different stages of renovascular hypertension (mean ± SD). (A color version of this figure is available in the online journal.)

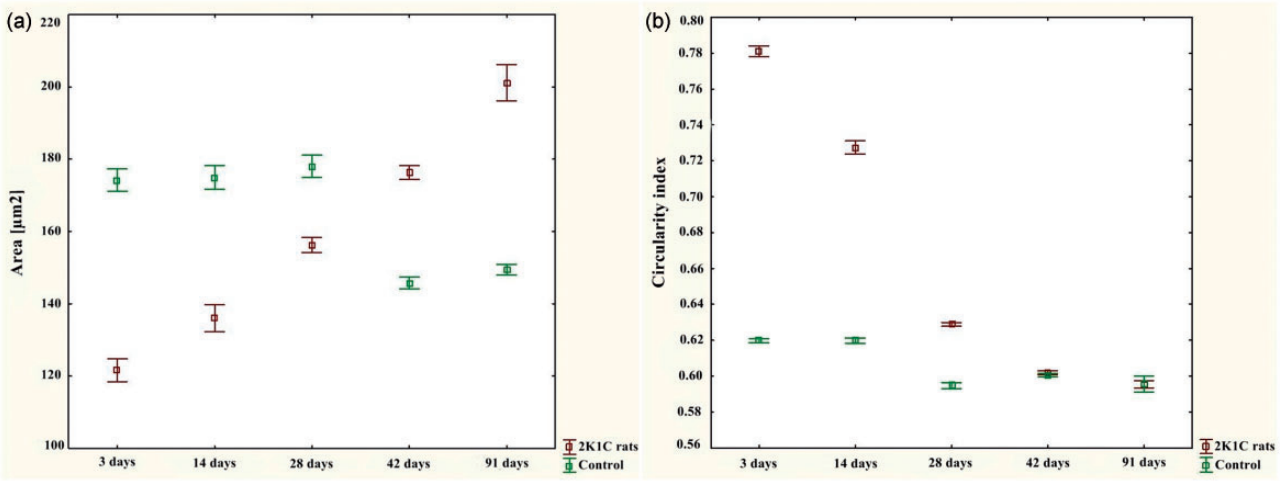

Figure 4 The area and circularity index parameters of CART-positive cells observed at various times after induction of experimental hypertension (mean ± SD). (A color version of this figure is available in the online journal.)

they were longer, larger and more oval than cells in rats after three days from induction of hypertension (Figures 3 and 4).

Twenty-eight days after surgery, CART-positive cells were more numerous, and the intensity of staining of those cells was stronger, when compared to both the control animals and rats of previously examined groups (Table 1, Figure 2 (a) to (e)). The values of morphological parameters of CART-IR cells, such as length and area, were slightly decreased, and their circulatory index pointed to the greater roundness of the cells compared to the cells in the adrenal glands of sham-operated rats (Figures 3 and 4). Values of morphological parameters such as elongation and surface area increased, while the roundness of the cells decreased as compared to those observed in the adrenal glands of rats with a shorter duration of hypertension (Figures 3 and 4).

Forty-two days after the left renal artery clipping, the number of cells with immunopositive reaction for CART in rats of this experimental group was the highest compared to all studied hypertensive and normotensive animals (Table 1, Figure 2 (a) (e)). The intensity of immunohistochemical reaction in the rats of this test group was stronger in comparison to the hypertensive rats of previous groups (Table 1, Figure 2(a) to (e)). The cells with positive reaction to anti-CART antibodies were longer, larger, and more oval than cells in rats with a shorter duration of hypertension (Figures 3 and 4). Unlike the previous experimental groups, except the circularity index, the values of all analyzed morphological parameters of CART-containing cells were significantly higher compared to the control rats (Figures 3 and 4).

The number of CART-IR cells in the adrenals of the hypertensive rats, 91 days following surgery was slightly decreased compared to the previous group, but significantly increased when compared to normotensive animals (Table 1, Figure 2(a) to (e)). The difference noticed in the

number of CART-containing cells between 2K1C rats and the control group was much greater (2.7-fold increase) than those observed after 42 days or 28 days from artery clipping (1.5- and 1.1-fold increase, respectively) (Table 1). After 13 weeks from induction of hypertension, the result of reaction with anti-CART antibodies was the strongest in comparison to the remaining experimental groups and sham-operated animals (Table 1, Figure 2(a) to (e)). As in the previous group with established renovascular hypertension, CART-IR cells were longer, wider and larger in comparison to cells in adrenal medulla of sham-operated rats, whereas there were no significant changes in the circularity index of those cells (Figures 3 and 4).

Discussion

Arterial blood pressure control is a very complex process, involving various organs and systems: the kidneys with the RAA system, the neurohormonal system with participation of catecholamines, vasopressin, and the nervous system.[9]

Results of tests carried out to date indicate that the CART, among other functions, contribute in the regulation of blood pressure.[6–8] However, no study has been carried out to define peptide contribution in the pathogenesis of hypertension. It prompted us to undertake a research aiming to identify, localize, and assess dynamics of changes in CART-immunopositive cells in adrenal glands in different time periods of experimentally-induced hypertension.

Literature data dedicated to functions of CART under pathological conditions are very limited.[12] This is the first study evaluating the adrenal CART-positive cells population in rats with renovascular hypertension, at different time-points of the disease.

Previous research concerning localization of the peptide in rat and porcine adrenal glands demonstrated that peptide is distributed particularly in the peripheral parts of medulla. The current study confirms that CART is synthesized in adrenal endocrine cells, although, in contrast to earlier investigations,[13] CART-positive cells were noticed in the whole adrenal medulla in all examined rats. These differences may be caused by the varying of methods and test conditions.

Our experiment demonstrates that the number, immunoreactivity and morphological features of CART-containing cells in the adrenal medulla underwent changes during the development of experimental hypertension.

The development of renovascular hypertension is characterized by the involvement of different mechanisms in particular phases of the disease.[9] Activation of the subsequent mechanism may differently affect the activity of adrenal cells, leading either to hyperfunction or hypofunction of endocrine cells, and may also differently affect the expression of the gene encoding CART in those cells. It has been reported that the production and release of neuropeptides could significantly be altered in several models of experimental hypertension.[14] However, no data were found in the available literature concerning the changing number of adrenal CART-containing endocrine cells in the conditions of experimental hypertension. Therefore, it is rather difficult to explain the cause of the changing quantity of adrenal CART-IR cells, as observed in hypertension.

After days 3 and 14 from renal artery clipping, the number and size of CART-IR cells were decreased and staining of those cells was weaker, compared to normotensive rats.

Arterial hypertension results in tissue ischemia and organic insufficiency, the process occurring at both systemic and local levels. In acute phase of renovascular hypertension, blood supply to the adrenal glands is limited.[15] In order to improve organ perfusion, there is an increase synthesis of nitric oxide (NO).[16] Nitric oxide modulates membrane permeability, and activates various signaling pathways in the cells. Ward et al.[17] showed that enhanced production of nitric oxide inhibits catecholamine secretion in bovine chromaffin cells. Moreover, experimental studies demonstrated that NO might lead to apoptosis of chromaffin cells through mitochondrial-dependent pathways.[18] Several reports confirm that disturbances in NO production might result in adrenal medulla dysfunction. It was found that NO may contribute to diabetic autonomic neuropathy, thereby affecting the functioning of chromaffin cells.[19] The results of previous research show a significant increase in NO production in the adrenals, in the degeneration of nerve fibers innervating adrenal medulla cells, as well as a substantial reduction in catecholamine secretion[20] in conditions of diabetes mellitus in rats. Most likely, NO might cause analogical anomalies in early stages of hypertension. Since CART is synthesized in adrenal medulla cells, using the same principle NO may modulate the viability and activity of CART-IR cells. As mentioned earlier, in the initial stage of hypertension development (3 and 14 days), CART-positive cells were less numerous, smaller, and less immunoreactive, which appears to support the above hypotheses.

In contrast to the early stages of the pathological state studied, a considerable increase in the number of CART-IR cells and intensity of immunohistochemical reaction was found after 28, 42, and 91 days of hypertension. In addition to quantitative changes, values of morphological parameters of CART-containing cells were increasing as the hypertension lasted longer, which also confirms their increased activity.

The transition phase of renovascular hypertension involves disorders in water and electrocyte balance.[9] Enhanced production of vasopressin and aldosterone leads to excessive sodium and water retention and could result in hypervolemia. Limited literature data show that an experimentally induced increase in fluid volume via infusion of 0.03 M NaCl intensifies expression of CART-gene in rat hypothalamus.[21] In the same experiment, researches noticed increased expression of corticotropin releasing factor (CRF) mRNA in paraventricular nucleus (PVN) neurons, suggesting activation of HPA axis.[21] Considering that expression of CART gene in hypothalamus and pituitary is regulated by CRF and adrenal cortical hormones, it is suggested that activation of HPA axis might be a possible mechanism leading to this up-regulation of CART gene in hypervolemia and hyperosmotic conditions.[21] Taking into account the results of studies by Krukoff and co-workers,[22] who demonstrated an increased number of neurons with

CRF mRNA in PVN of hypertensive rats, the same mechanisms might be also likely in the interpretation of the results obtained in our study.

Chronic renal ischemia is a stressor factor for the organism. Results of clinical and experimental research have demonstrated that in the advanced stage of renovascular hypertension, the activity of sympathetic nervous system is enhanced and secretion of stress hormones such as catecholamines and glucocorticoids is intensified.[9,23] It was found that stress might modify expression of CART gene, depending on its duration. Hunter and co-workers[24] show that acute stress does not influence CART biosynthesis, whereas long-lasting stress intensifies expression of CART-gene in hippocampus. In male rats which were forced to swim, stress caused increased CART synthesis in adrenal glands.[25] On the basis of the above findings, it can be assumed that the increased number of CART-positive cells in the advanced stage of renovascular hypertension in the experimental model applied in this study might also be associated with disease-related stress response.

Furthermore, the development of hypertension, besides activation of multiple mechanisms resulting in increased blood pressure, causes intensification of production of reactive oxygen species.[26] Based on the documented knowledge[27] concerning the protective ability of CART against oxidative damage, the increase in number of CART-containing cells found in our study may be associated with this phenomenon.

Moreover, hypertension is increasingly seen as an inflammatory process, leading to vascular and organ damage.[28] The latest reports indicate that CART is involved in regulation of immune response.[17,29] Chang and co-workers[29] demonstrated that injection of CART into mice with experimental stroke affects humoral and cellular immunity, and reduces inflammation-induced brain injury. It was also shown that stimulation of immune response by the injection of lipopolysaccharide affects the expression of CART gene in hypothalamus.[30] The above findings provide another possible explanation of the results obtained, and allow us to hypothesize that the observed changes in the number of adrenal cells with CART-positive reaction might be associated with inflammatory processes occurring in hypertension.

Our work confirms the presence of CART in endocrine cells in the whole adrenal medulla and in the nerve fibers of the cortex and capsule of gland. Furthermore, we have shown for the first time that the number and morphology of cells containing CART in the rat adrenal medulla change in different ways during the development of renovascular hypertension. We speculate that the changes in cell numbers and morphology may in fact be dynamic process. Initially, the density of CART-containing cells decreases, but may later increase in chronic hypertension. The pathophysiological character of these disorders and the mechanism responsible for cells containing CART changes in the adrenal glands are still unclear and require further studies.

CART is relevant to the regulation of homeostasis in the cardiovascular system and it seems to be involved in different stages of hypertension. Its decrease could be implicated in the first stage of hypertension, while its increase in release and/or response could be a mechanism to counteract high blood pressure.

Author contributions: IK, RZ participated in the study design; IK, ZP, and IJ conducted the experiments, data collection, and analysis of the data; IK wrote the manuscript; RZ 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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