Dynamics of calcitonin gene-related peptide-like cells changes in the lungs of two-kidney, one-clip rats

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Abstract

Taking into consideration renal hypertension-induced homeostatic disorders and the key role of calcitonin gene-related peptide (CGRP) in many, systemic functions regulating systems, a question arises as to what an extent arterial hypertension affects the morphology and dynamics of pulmonary CGRP-immunopositive cell changes. The aim of the present study was to examine the distribution, morphology and dynamics of changes of CGRP-containing cells in the lungs of rats in the *two-kidney*, one-clip (2K1C) renovascular hypertension model. The studies were carried out on the lungs of rats after 3, 14, 28, 42, and 91 days long period from the renal artery clipping procedure. In order to identify neuroendocrine cells, immunohistochemical reaction was performed with the use of a specific antibody against CGRP. It was revealed that renovascular hypertension caused changes in the neuroendocrine, CGRP-containing cells in the lungs of rats. The changes, observed in the neuroendocrine cells, depended on time periods from experimentally induced hypertension. The highest intensity of changes in the neuroendocrine cells was observed in the lungs of rats after 14 days from the surgery.

Introduction

Arterial hypertension results in ischemia of tissues and organic insufficiency, the process, occurring at both systemic and local levels. Hypertension leads to disorders of many systems and organs, causing cardiac hypertrophy, increased sclerosis development, metabolic disorders, anaemia and neurological disturbances, while local changes affect, first, vascular walls.[1,2] Endothelial dysfunction is observed, as well as activation of mitogenic factors, leading to hypertrophy of tunica intima and vascular media[3-5] and the production of aerobic radicals and prostaglandins is increased.[6]

The maintenance of arterial blood pressure within normal values is a significant link for the homeostatis controlling mechanisms,[7] involving the nervous system, the kidneys, the hormonal system and many other factors, significantly controlling the motor activity of vascular walls building smooth muscle cells.[8-10] Arterial hypertension develops from disorders in arterial blood pressure controlling mechanisms and from a disturbed relationship between vasodilative and vasoconstrictive factors.[11]

Regarding the pathogenesis of arterial hypertension, experimental studies prove a big role of the neuropeptides, secreted by neuroendocrine cells of the epithelium in the airways, the gastrointestinal tract and in the genitourinary tract.[12-18] The calcitonin gene-related peptide (CGRP) is one of the substances, fairly significant for hypertension aetiology.[19,20] CGRP participates in a number of physiological and pathological processes, including inflammatory processes, suppression of cell proliferation and cardiovascular control.[21] CGRP is one of the strongest vasodilators, increasing blood flow rates in cerebral and coronary vessels, as well as in cutaneous vessels,[22] exerting positive chronotropic and inotropic effects.[23] CGRP-containing, afferent, sensory nerve fibres innervate blood vessel walls.[24] CGRP is also localised in vascular endothelium.[25] Afferent pulsation causes CGRP release from nerve endings, contributing to vasodilative effects,[26] while both forms of CGRP, *i.e.*, alpha-CGRP and beta-CGRP are for this process responsible.[27]

It appears from reports of many authors that CGRP plays a significant role in the initiation and progression of hypertension.[22,27] It has been demonstrated that CGRP plays a protective function for endothelial cells and for smooth muscle cells.[28,29] It decreases muscular layer tension, binding with an appropriate receptor or enhancing nitrogen oxide synthesis.[30] Moreover, it exerts antagonistic effects on the renin-angiotensin-aldosterone (RAA) system and endothelins, demonstrating cardioprotective properties.[31] Therefore, it has been assumed that CGRP plays an important role in blood pressure modulation in physiological conditions, as well as in the pathophysiology of hypertension.

CGRP is one of the peptides, secreted by the, so-called- pulmonary neuroendocrine cells (PNEC), which occur individually in airway epithelium as neuroepithelial bodies (NEB).[32] It appears from a literature review that, in various pathological situations, both the number and morphology of these cells undergo certain changes, what lets assume their active participation in many processes.[32-35]

No data were found in the available literature, concerning CGRP-positive pulmonary cells in systemic hypertension of various aetiology. Taking into account that fact, it was decided to evaluate the dynamics of changes in the numbers of CGRP-positive pulmonary cells in rats with renovascular hypertension.

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Materials and Methods

Experimental animals

All the 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.[36]

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, Poland.

The study was performed on fifty (50) young male Wistar rats, their body weight at the beginning of the experiment remaining within 160-180 g (mean body weight: 170±10 g). The rats were housed in lighted and ventilated conditions with room temperature and regular day/night rhythm. The animals had a free access to standard granulated chow and drinking water was available *ad libitum*. All the experiments were performed at the same time of the day.

After 1 week of acclimatization, the systolic blood pressure (SBP) was measured in each rat was measured and the renovascular hypertension inducing procedure was performed.

Two-kidney, one-clip 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=30). Sham operated rats (n=20) had identical surgery except artery clipping.

Histology

Method of experimental material collection and fixation

After 3, 14, 28, 42 and 91 days from the renal artery clipping procedure, the rats (six examined and four controls) were anaesthetized with pentobarbital (50 mg/kg b.w.) and sacrificed by decapitation. Following thoracotomy, the lungs were collected in whole. Immediately after the preparation of the collected organs, Bouin's fluid was administered by syringe into the trachea to smooth the pulmonary pleura in the right lung. Following the trachea ligation, the lung was fixed in Bouin's fluid for 24 h at 4°C. Then, following lobectomy from circumference to the hilus, the anterior part of the superior pulmonary lobe was routinely placed in paraffin blocks and then sectioned by a Leica 2025 rotating microtome. The paraffin blocks were cut into 4 μm sections and attached to positively charged glass slides.

Identification of endocrine cells by immunohistochemical methods

In the immunohistochemical study, the EnVision method was used, according to Herman and Elfont.[37] An immunohistochemical reaction was run on the paraffin lung sections of the studied animals, searching for the calcitonin gene-related peptide in neuroendocrine cells. In those studies, a specific antibody against CGRP was applied (Cat. No C 8198, in 1:8000 dilution, Sigma-Aldrich, Saint Louis, MO, USA). The antiserum was diluted in an antibody diluent (S 0809, Dako Cytomation, Glostrup, Denmark).

Immunohistochemical reaction procedure

Paraffin-embedded sections were deparaffinized and hydrated in pure alcohols, then pretreated in a pressure chamber, heating for 1 min at 21 psi at 125ºC with S 2369 target retrieval solution citrate of pH 6.0 (Dako) for antigen retrieval. After cooling to room temperature, the sections were incubated with S 2001 peroxidase blocking reagent (Dako) for 10 min to block endogenous peroxidase activity. The sections were incubated overnight at 4ºC in a humidified chamber with the diluted antibody. The antibody binding was visualized with support of an EnVision Kit (K 4011, Dako) with labelled polymer-HRP anti-rabbit. The bound antibodies were visualized by 1-min incubation with liquid 3,3'-diaminobenzidine substrate chromogen. The sections were finally counterstained in Vector QS haematoxylin, mounted and evaluated under light microscope. Appropriate washing with Wash Buffer S 3006 (Dako) was performed between each step. The specificity tests, performed for the CGRP antibody, included: negative control, where the antibodies were replaced by normal rabbit serum (Vector Laboratories, Burlingame, CA, USA) at the respective dilution and positive control was obtained for specific tissue as recommended by the producer for CGRP is rat brain. 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.

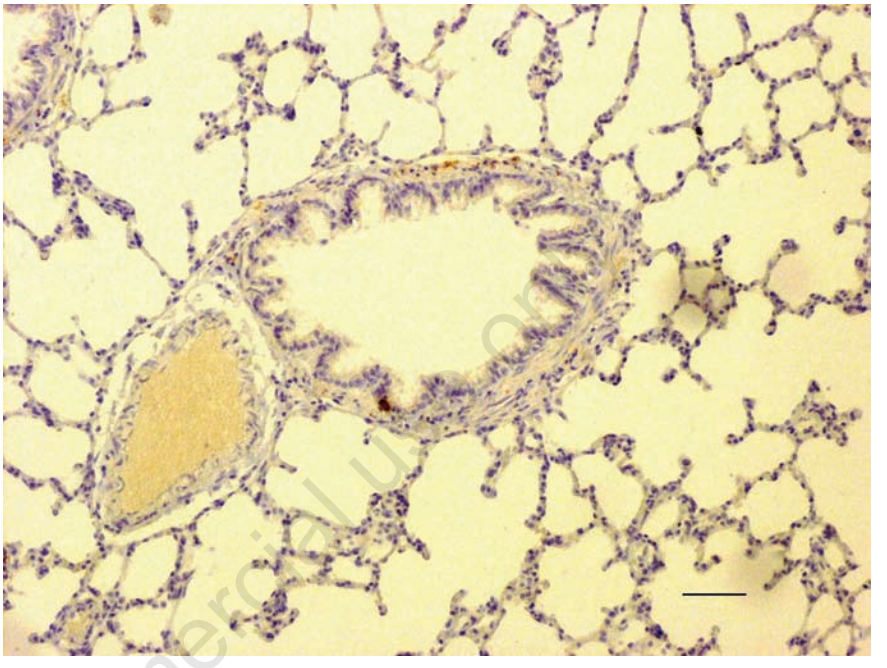

Figure 1. Single cell containing CGRP in the lung of control rat. Scale bar: 100 μm.

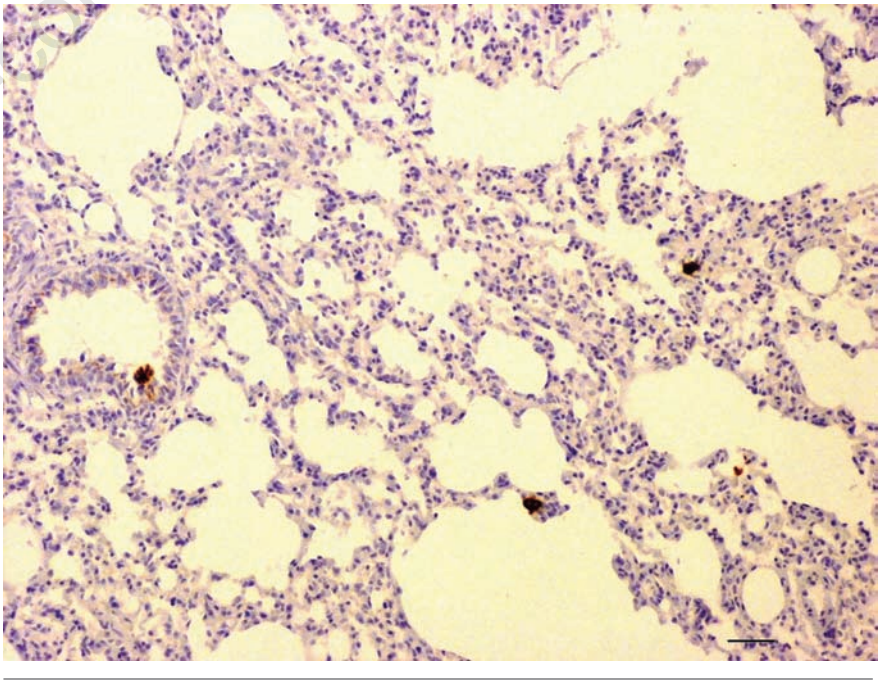

Figure 2. Small few-cell clusters of NE cells with CGRP expression in rat lungs after three days from left renal artery clipping. Scale bar: 100 μm.

Results

Location, morphology and immunohistochemical characteristics of neuroendocrine cells in the lung structures of control and *two-kidney, one-clip* rats

Following immunohistochemical reactions, the neuroendocrine (NE) cells were clearly distinguishable in structures of the analysed organs of all the studied rats. The NE cells, surrounded by other cells of the respiratory tract epithelium, were distinct by dye and shape, the latter ranging from pyramidal to polygonal, oval or columnar. In the lungs of the control rats, CGRP-immunopositive cells occurred in the respiratory tract as scattered, single or as intraepithelial, small groups (most often 2-3 cells only). The highest number of NE cells with positive reaction to the antibodies against CGRP was observed in larger bronchia, while the smallest PNE cell numbers occurred in alveolar ducts and alveoli. The intensity of immunohistochemical reaction to anti-CGRP antibodies was moderate in the PNEC of control rats (Figure 1).

After three days from artery clipping, the number and localization of CGRP-immunoreactive cells in the lungs of the rats were similar to their control counterparts. A higher number of NE cells, grouped in small, few-cell clusters of PNEC, was the only visible difference. The intensity of immunohistochemical reaction was also similar to that observed in the control rats (Figure 2).

Two weeks after the left renal artery clipping, clearly higher numbers of cells with positive reaction to anti-CGRP antibodies were found in the rats with experimentally induced hypertension vs. control animals. Single NE cells, as well as NEBs, were localised at various levels of the respiratory tracts of studied rats.

The most numerous NE cells, observed in the epithelium of bronchi, where CGRP-immunopositive cells, frequently grouped into large, multicellular clusters of PNE cells. Higher numbers of CGRP-immunopositive cells were also found in the lungs of rats in that group vs. the other groups. Some CGRP-immunopositive cells were also found at the level of the alveolar ducts and of the alveoli (Figure 3 A,B).

The strongest immunoreactivity of PNE cells, both single and in small clusters, as well as in large NEBs, was observed after 2 weeks from the clipping procedure. After 28 days from clipping, the number of NE cells with CGRP-immunopositive reaction was slightly smaller in the studied lungs, comparing to that in the previous group animals. Cells with a positive reaction to the anti-CGRP antibodies were found in the respiratory tract, most often as scattered, single NE cells or in small, several-cell clusters of NE cells (Figure 4). NEBs, with higher numbers of NE cells, were identified only sometimes and with a weaker expression of CGRP, when compared to the previous animal group.

The number and localisation of CGRP-immunoreactive cells in the lungs of the rats after 42 and 91 days from the left renal artery clipping were similar and comparable to those in the animals after three days from clipping. Most of the CGRP-immunopositive cells, which were observed in the respiratory tract, included single NE cells, while small, several-cell clusters of PNE cells were observed in the bronchia and bronchioli of rats in those experimental groups (Figure 5). The immunoreactivity of PNE cells, both single and clustered, was rather moderate.

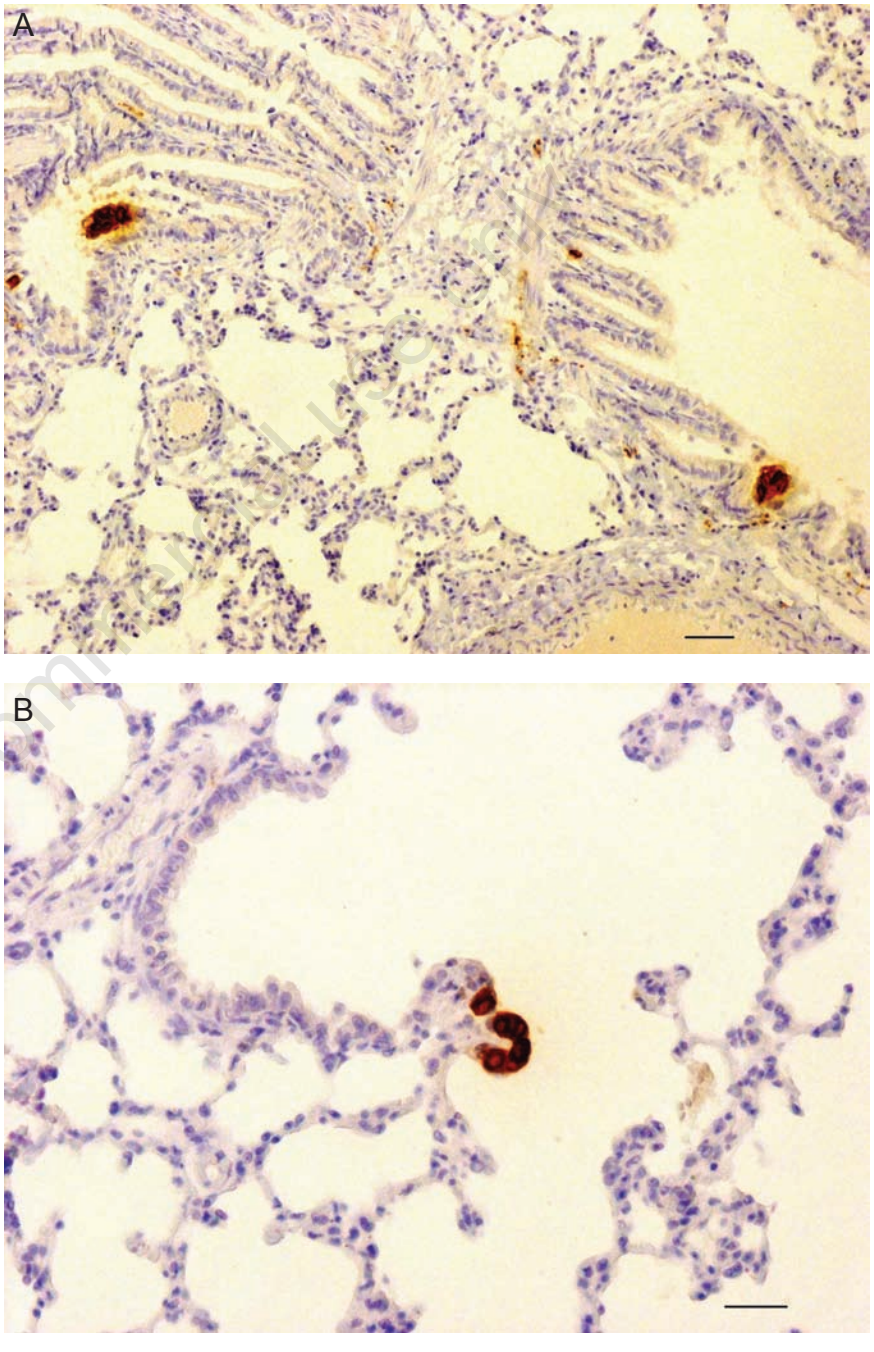

Figure 3. A) Multicellular clusters of PNE cells with strong CGRP expression in the cytoplasm; scale bar: 100 µm. B) Rat lungs after 14 days from the left renal artery clipping; scale bar: 50 µm.

Discussion

Topographic distribution and the occurrence of CGRP-immunoreactive endocrine cells were studied in lungs of hypertensive rats.

The pathogenesis of arterial hypertension is complex and multifactorial in character. 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, vasopressins and the nervous system.[4,10,38] There is strong interdependence and a mutual linkage among many mechanisms and blood pressure controlling factors. Despite the big progress, which, so far, can be perceived in the pathophysiology of arterial hypertension, it is still not known, which of these factors play a decisive role in hypertension formation and development.

Arterial hypertension rarely occurs as isolated condition. In many cases, hypertension is accompanied by medical conditions, which are, most often, its complications. It seems that, from the clinical point of view, the most significant complications are those that cause certain changes in the cardiovascular system. Neuropeptides, like CGRP, play an integral role in the pathophysiology of hypertension. CGRP participates in the regulation of vascular tone and regional organ blood flow, what was demonstrated in many experimental data, as well as observed in a number of clinical studies.[20,21,39-41]

CGRP-containing nervous fibers are localised on the borderline between the tunica adventitia and media myocytes. CGRP receptors are widely distributed in the cardiovascular system.[42] For this reason, CGRP receptors constitute a powerful effector system for the regulation of vascular tone and regional blood flow. It appears from studies of many authors that both CGRP and/or CGRP receptor expression increase in hypertension.[38,40] A blockade of CGRP receptors in various experimental models caused an evident increase of arterial hypertension.[38,43]

Potassium and calcium ions play a meaningful role in the pathology of hypertension. ATP (K ATP)-dependent potassium channels are observed in endothelial cells and smooth muscle cells of vessels, the channels being a component of tonic vasorelaxation in general and local circulation. Potassium channels are the main cell membrane potential determining elements and, in consequence, they take part in vascular wall tension control. Potassium channel opening by factors, released from the endothelium, *e.g.*, nitrogen oxide (NO), prostacyclin (PGI2), leads to flows of potassium ions outwards from the cell inside, resulting in hyperpolarisation, closure of potential-dependent membranous calcium channels and dilation of blood vessels. In turn, potassium channel closure with calcium channel opening, leads to depolarisation and contraction.[44] Disturbed functions of potassium channels are at the base of hypertension pathogenesis. The potassium channels, which selectively filter potassium ions, exert their effects on the membranous potential of smooth muscle cells. CGRP stimulates these channels, leading to hyperpolarisation of the membranes of vascular smooth muscle cells.[45]

The results of our earlier studies demonstrate that experimentally induced renovascular hypertension in rats exerts a huge effect on CGRP-positive cells in the lungs. A clear

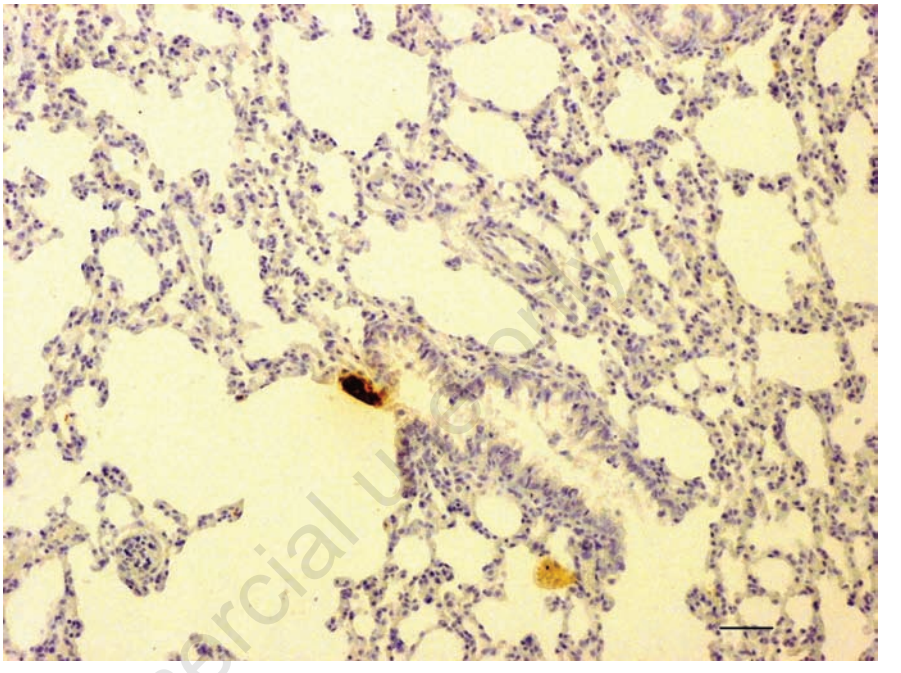

Figure 4. CGRP immunolabelling in the cytoplasm of NE cells (dark-colored granules) in the lung of 2K1C rat after 28 days from clipping. Scale bar: 100 µm.

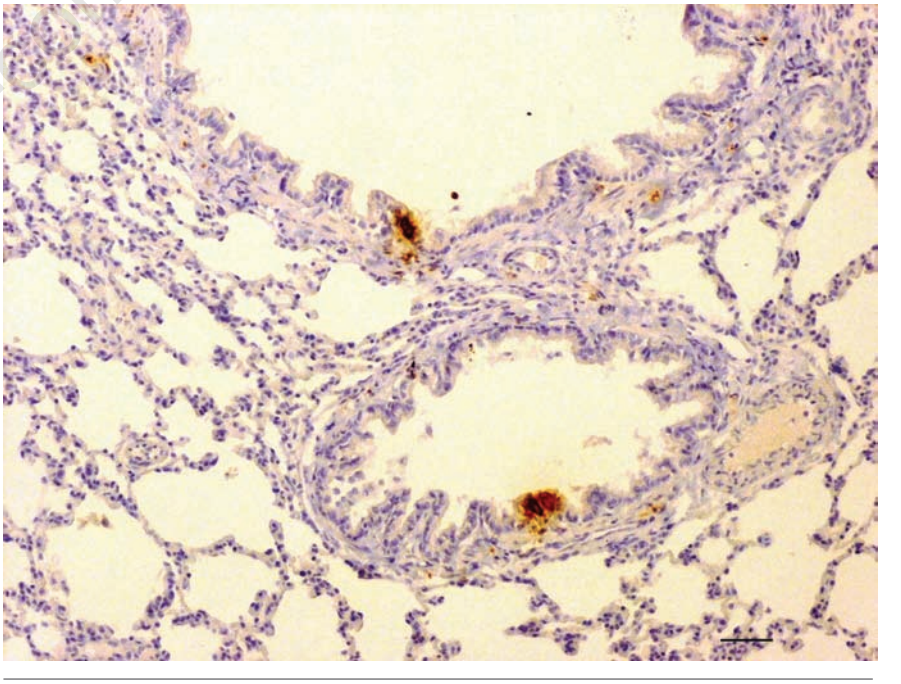

Figure 5. CGRP-immunopositive cells in the lung of 2K1C rat after 42 days from clipping. Scale bar: 100 µm.

increase in the number of CGRP-immunoreactive cells was observed in the studied animals, the cells being either single or clustered into neuroepithelial bodies in various sections of the airways. A considerable increase in the number of those cells was found after 14 days of hypertension. Then, after 28 subsequent days, the number of CGRP-positive cells was minimally lower than in the animals after the two-week duration of the experiment. While after 42 and 91 days from the study, the animals demonstrated decreased numbers of those cells, down to the level observed in the lungs of the animals on the third day of the hypertensive period.

Hyperplasia of CGRP-immunoreactive cells was noted in neonatal rats with congenital diaphragmatic hernia,[46] carcinoid tumours[32] and in cigarette smokers.[47] It was previously stated that the production and release of neuropeptides could significantly be altered in several models of experimental hypertension.[48-50] No data were found in the available literature concerning the increasing number of airway CGRP-containing neuroendocrine cells in the conditions of renovascular hypertension. Therefore, it is rather difficult to explain the cause of the increasing quantity of pulmonary, CGRP-immunoreactive cells, as observed in hypertension.

Stenosis of the renal artery mobilises the RAA system. An ischaemic kidney produces excessive renin volumes, that consequently results in an increased secretion of pressively and mitogenically acting angiotensin II.[5] An increased blood pressure causes reduced oxide supplementation to the lungs, while impairing the gas exchange. Additionally, hypoxia significantly leads to contraction of pulmonary blood vessels. Chronic oxygen deficiency increases the number of neuroendocrine cells in the airways.[15,51] It is confirmed by Keith's studies, who, in conditions of one-day hypoxia, observed hyperplasia of cells with 5-HT expression in rat foetuses. CGRP dilates pulmonary blood vessels, reducing the effect of chronic hypoxia.[52] The role of CGRP in hypertension is not clear. CGRP administration decreases blood pressure both in animals and in people.[23,50] Clinical studies have indicated that, in patients with systemic hypertension, the levels of circulating CGRP may be decreased, increased or unchanged,[22,53] while in hypertensive rats, the level of CGRP increases both *in vivo* and *in vitro* conditions.[50,54]

In hypertension, regardless of its aetiology, it comes to remodelling of blood vessels; these changes include hyperplasia of the muscular coat, fibrosis involves the tunica intima and the tunica adventitia, what results in increased peripheral resistance.[55] Cogo *et al*. found muscularisation of pulmonary vessels and proliferation of fibroblasts in various oxygen conditions.[56] In hypertensive lungs of the experimental animals, thickened vascular media was observed, as well as big amounts of connective tissue, both in tunica intima and tunica adventitia. The observed morphological changes were accompanied by an increased number of single CGRP-positive cells and of neuroepithelial bodies, as well as by hyperplasia of NEBs, especially after two weeks of hypertension. However, it is still an open question why the cells increase in their numbers in the lungs of animals with renovascular hypertension.

The mean half-life of CGRP in plasma is relatively short, amounting to approximately 10 min, then it undergoes inactivation with the participation of tryptase and chymotrypsin.[57] Therefore, it should be supposed that PNEC hyperplasia, which was found in those studies, is a CGRP deficit compensation mechanism. The lungs in physiological and pathological conditions are subject of complex control processes, in which nervous and hormonal factors play a significant role, tailoring their functions to the actual load of the system. There are also local control mechanisms, which integrate the elements of the airway functions, including, by all means, the peptide, associated with calcitonin gene, i.e., CGRP. There are many factors responsible for the control of CGRP expression and release. Locally produced factors, such as bradykinin and prostaglandins,[58,59] endothelin and the sympathetic nervous system,[58] as well as the nerve growth factor (NGF) reveal high physiological significance in pulmonary flow control.[60,61] It has been shown that NGF is a stimulant of CGRP mRNA synthesis. NGF would decrease blood pressure through the stimulation of CGRP synthesis and release.[61] The study suggested that bradykinin effects resulted from the cyclo-oxygenase mediated increase in prostaglandin production.[59] It may be assumed that those relationships can be the cause or one of the causes of CGRP release.[6,62] Probably, these factors alter acute releases of CGRP and can also modulate long-term production of this peptide.

An increased number of CGRP immunopositive cells may be a consequence of disturbed secretion of the peptide, what has been confirmed by Gosney's studies.[46] He found an increased quantity of CGRP-positive cells in rats with nitrofen-induced diaphragmatic hernia, which was an effect of the suppressed release of the peptide; which, in turn, predisposes to hypertension development, characteristic for this disease.

A number of pathological pulmonary conditions are accompanied by PNEC hyperplasia, which may result either from their mitotic divisions or from post-mitotic differentiation of non-endocrine cells;[63,64] however, this problem has still been awaiting its final solution. Antihypertensive effects of CGRP may result from the activation of neuroendocrine cells to synthesis and release of this neuropeptide and/or from an increased sensitivity of the vessels to CGRP activity.[20,27]

Hypertension-induced end organ damage is one of the most severe and common consequences of increased blood pressure. Since CGRP has such potent biological effects on the lung suggesting that peptide is an endogenous organ-protective agent and has important role in the regulation of lung blood flows. The primary mechanism responsible for control pulmonary flow can be arterial dilation. CGRP can significantly weaken the pathological effects of chronic pulmonary hypertension. Another possible hypothesis is that the regulation of lung blood flows can be mediated by an up-regulation of CGRP synthesis and release, or through an enhanced sensitivity of the vascular to the dilator effects of this peptide.

In the future, it is hoped that dissection of the mechanisms that control CGRP production and its actions will provide novel therapeutic targets for hypertension and other pathologies.

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