Доклади на Българската академия на науките
Comptes rendus de l'Académie bulgare des Sciences

Tome 77, No 7, 2024

MEDICINE

Neurology

GABA-IMMUNOREACTIVITY IN NEUROEPITHELIAL BODIES OF SPONTANEOUSLY HYPERTENSIVE RATS

Nikola Stamenov[1]✉, Dimitrinka Atanasova[2,3], Angel Dandov[1], Lazar Jelev[1], Nikolai Lazarov[1,2]

Received on November 16, 2023
Presented by B. Petrunov, Member of BAS, on November 28, 2023

Abstract

Neuroepithelial bodies (NEBs) are complex sensory receptors dispersed throughout the intrapulmonary airways. They are composed of pulmonary neuroendocrine cells (PNECs) which have a large amount of dense-core vesicles containing calcitonin gene-related peptide (CGRP), serotonin (5HT), substance P (SP) and γ-aminobutyric acid (GABA). NEBs are densely innervated by vagal sensory terminals, dorsal root afferents and intrinsic motor fibres. Their location, neurochemical composition and dense innervation make NEBs a key component for the regulation of lung homeostasis.

Pulmonary arterial hypertension is a rare disease characterized by morphological alterations in the microcirculation of the lungs causing a wide array of complications and high mortality. Spontaneously hypertensive rats (SHRs) are a common model for essential hypertension, that may develop secondary pulmonary hypertension and thus can be used as an animal model to study pulmonary pressure changes.

The present study focuses on the immunohistochemical demonstration of GABA in the NEBs and its potential role in the regulation of the pulmonary pressure. Two groups of SHRs were used and age-matched normotensive Wistar Kyoto (WKY) rats served as controls. We found single PNECs and clusters of GABA-immunoreactive cells protruding into the lumen of the intrapulmonary

This study was funded by the European Union-NextGenerationEU, through the National Recovery and Resilience Plan of the Republic of Bulgaria, project No. BG-RRP-2.004-0004-C01 "Strategic research and innovation programme for development of Medical University – Sofia".

https://doi.org/10.7546/CRABS.2024.07.14

airways in all examined groups of SHRs and in the normotensive WKY rats. However, the expressional levels statistically differed within the two age groups of SHRs and the controls. Specifically, the NEBs and PNECs in 1-month-old SHRs and particularly in 3-month-old SHRs showed more intense GABA-immunostaining compared to the WKY rats.

In conclusion, NEBs and PNECs react to the alterations of the pulmonary pressure homeostasis by an increased GABA expression as a preventive or reactive regulation mechanism against developing pulmonary hypertension.

Key words: neuroepithelial bodies, GABA, pulmonary hypertension, spontaneously hypertensive rats

Introduction. Neuroepithelial bodies (NEBs) are complex multifunctional sensory receptors, most commonly found at the bifurcations of smaller intrapulmonary airways [1]. They are composed of innervated clusters of pulmonary neuroendocrine cells (PNECs) which are covered by Clara-like cells (CLC) and together they form the so-called NEB microenvironment [2]. A striking feature of PNECs is the large number of dense-core vesicles in their cytoplasm that contain a wide array of vasoactive neurochemicals such as calcitonin gene-related peptide (CGRP), serotonin (5HT), substance P (SP) and γ-aminobutyric acid (GABA) [3]. Another key feature of NEBs is their extensive innervation which includes vagal sensory terminals, dorsal root afferents and intrinsic motor efferent fibres [1, 4].

The location, neurochemical composition and dense innervation make NEBs a strategic structure for sensing changes in the airway gas concentration, for the coordination of local blood flow to local aeration and also for many other functions in normal and pathological conditions. In particular, NEBs participate in the control of breathing, play a role in calcium homeostasis, and their dysfunction is implicated in pulmonary hypertension, bronchial asthma and cancer development [1,5,6]. Recent evidence also suggests their role as stem cell niche in the lungs [7].

Pulmonary arterial hypertension is a relatively rare disease with a wide array of complications and high mortality, affecting all vessels and cells of the lungs [8,9]. Literature data show that pulmonary hypertension is associated with systemic arterial hypertension because the higher blood pressure affects not only the left ventricle but also the right ventricle and the pulmonary circulation [10]. Indeed, a recent study has demonstrated an association of pulmonary hypertension with systemic arterial hypertension in patients with apparently normal left ventricular diastolic function [11].

Spontaneously hypertensive rats (SHRs) are one of the most common experimental models for essential hypertension. However, SHRs could also be used as an animal model of pulmonary hypertension which they may develop secondary to essential hypertension and wherein all other respiratory pathologies can be excluded [12].

Materials and methods. For the present study two age groups of male SHRs – 1-month-old rats and 3-month-old rats ($n = 6$, per group) were used. Age-matched normotensive Wistar Kyoto (WKY) rats served as controls. All animals were provided by the vivarium to the Medical University of Sofia with the approval of the Research Ethics Commission of the Medical University of Sofia. The animals were fed on a standard diet and allowed free cage activities. All SHRs were monitored and their levels of blood pressure corresponded to the reference levels for hypertension (> 150/90 mmHg). WKY rats were also followed up and their blood pressure was measured as equal or less than 120/80 mmHg. The animals were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg). The chest was opened and a transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 was carried out. The lungs were additionally fixed by an intratracheal infusion of paraformaldehyde through a cannula. Afterwards, the lungs were quickly removed and proceeded to vacuum deaeration, postfixed in the same fixative overnight at 4 °C, embedded in paraffin, and cut into 8 µm thick sections.

The lung samples were then deparaffinized with xylene and ethanol and processed for avidin-biotin-horseradish peroxidase complex immunohistochemistry. All sections were passed through the immunohistochemical procedures simultaneously to avoid variations in staining intensity. The endogenous peroxidase was blocked with 1.2% hydrogen peroxide in absolute methanol. After washing in PBS, the samples were preincubated for 1 hour at room temperature in 5% normal goat serum. Thereafter, they were incubated in a humid chamber overnight at 4 °C with the following primary antibody provided by Invitrogen: rabbit anti-GABA (PA5-32241, working dilution 1:200). After rinsing in PBS, the sections were incubated for 1 h at room temperature with complementary secondary antibody, i.e. biotinylated goat anti-rabbit IgG (Dianova, Hamburg, Germany) at a dilution of 1:250, respectively. After rinsing the tissue sections, the ABC complex (Vectastain Elite Kit 6100, Vector Laboratories) was applied and colour development with diaminobenzidine as a chromogen was done for 3–5 min. Thereafter, the slides were washed in tap water, dehydrated in a graded series of alcohols, cleared in xylene and coverslipped with Entellan (Merck, Darmstadt, Germany).

The slides were observed and photographed using a Nikon Eclipse 80i (Japan) light microscope. Photomicrographs were analyzed using the ImageJ software by measuring the mean gray value of the NEBs which corresponds to their expressional level of GABA. We reversed the results in a such a way that 0 corresponds to white (lack of expression) and 255 corresponds to black (highest possible expression). The expressional levels of the GABA-immunoreactive NEBs were determined by GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). The data followed a standard Gaussian distribution, and the D'Agostino-Pearson omnibus normality test passed. The one-way analysis of variance ANOVA followed by post-hoc pairwise multiple comparison Tukey's Honest Significant Difference

(Tukey's HSD) tests were used. Differences were considered statistically significant if p-values were < 0.05.

Results. The light microscopy examination of the lungs revealed numerous GABA-immunostained NEBs of different caliber in the intrapulmonary airways, including larger bronchi, terminal and respiratory bronchioles and alveolar ducts. They appeared as clusters of cells protruding into the lumen of the airways in all examined groups of SHR and also in the normotensive WKY controls. We also observed a large number of individual PNECs dispersed throughout the bronchial tree. The NEBs and PNECs in 1-month-old SHRs and particularly in 3-month-old SHRs showed more intense GABA-immunostaining compared to the WKY rats (Fig. 1). We compared the mean gray value of the NEBs and PNECs using the ImageJ software and the gathered data was used for consecutive statistical analysis. A one-way ANOVA was performed to compare the expression of GABA in PNECs in WKY, 1-month-old SHRs and 3-month-old SHRs (Fig. 2). The test revealed that there was a statistically significant difference in mean value between groups [$F(2, 42) = 370.4$, $p < 0.0001$]. Tukey's HSD post hoc test showed that GABA expression in the NEBs of 3-month-old SHRs was statistically significantly higher compared to 1-month-old SHRs ($^{****}p < 0.0001$, 95% C.I. $= [-26.68, -20.44]$) and normotensive WKY animals ($^{****}p < 0.0001$, 95% C.I. $= [-37.25, -31.01]$). The grayscale intensity for GABA in 1-month-old SHRs was significantly higher than its expressional levels in normotensive WKY rats ($^{****}p < 0.0001$, 95% C.I. $= [-13.69, -7.453]$). The mean and standard deviation values in the groups were as follows: WKY (mean = 169.1, SD = 4.166), 1-month-old SHRs (mean = 179.6, SD = 3.281) and 3-month-old SHRs (mean = 203.2, SD = 2.995).

Discussion. Pulmonary hypertension is characterized by morphological changes in the microcirculation of the lungs [13]. It is believed that such changes and the maintenance of pulmonary pressure homeostasis are mediated through a wide array of neurotransmitters including GABA [14, 15]. The effects of GABA on the circulatory system are yet to be fully understood. Some authors suggest a role for GABA as a vasoconstrictor while others claim that it is a vasodilator [16, 17].

GABA is a known secretory product of NEBs and PNCEs and a potential factor for the regulation of pulmonary hypertension [3]. In addition, the NEBs are capable of registering mechanical and chemical stimuli and actively participate in the regulation of pulmonary homeostasis, including pulmonary pressure [14, 15]. Herewith, we studied the potential role of NEBs and GABA immunoreactivity in them for the regulation of pulmonary hypertension. It seems that SHR develop secondary pulmonary hypertension, and therefore other lung pathologies can be dismissed [12]. Nonetheless, pulmonary hypertension that develops as a consequence of essential hypertension should be at an early stage in the younger age groups of SHR [12]. Thus, it is likely that the alterations in the GABA expres-

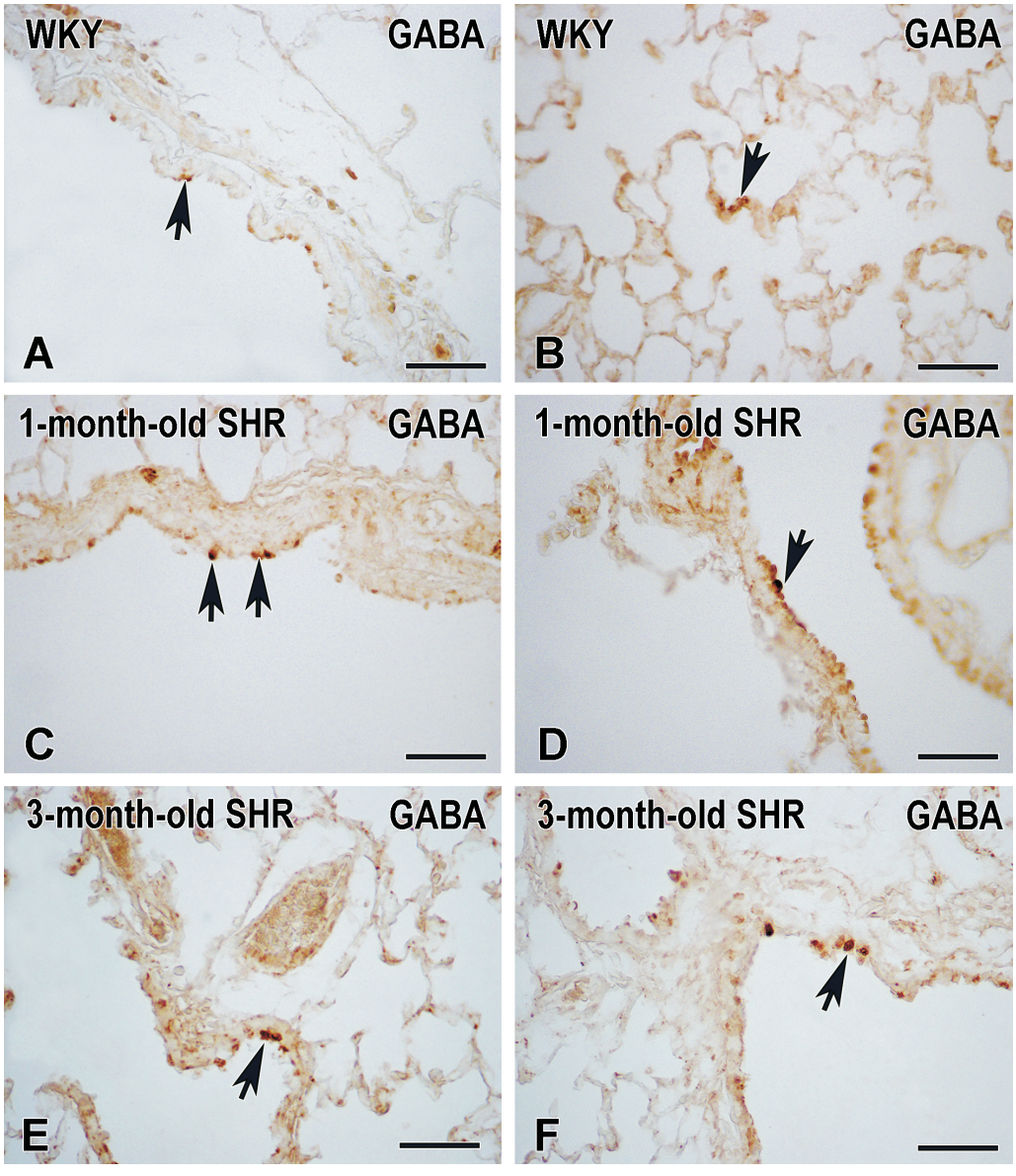


Fig. 1. Immunohistochemical staining for GABA in rat lungs. (A, B) NEB (arrows) in bronchus (A) and alveolar duct (B) in WKY rats showing relatively weak immunoreactivity. (C) Single PNCEs in terminal bronchiole (arrows) from a 1-month-old SHR. (D) NEB in respiratory bronchiole (arrow) from a 1-month-old SHR. (C and D) show more intense immunoreactivity compared to the WKY rats (A and B). (E) NEB in terminal bronchiole (arrow) from a 3-month-old SHR. (F) Several PNECs in terminal bronchiole of a 3-month-old SHR. Please note the highest immunoreactivity in a 3-month-old SHR (E, F) compared to a 1-month-old SHR (C, D) and WKY controls (A, B). Scale bar: 50 µm

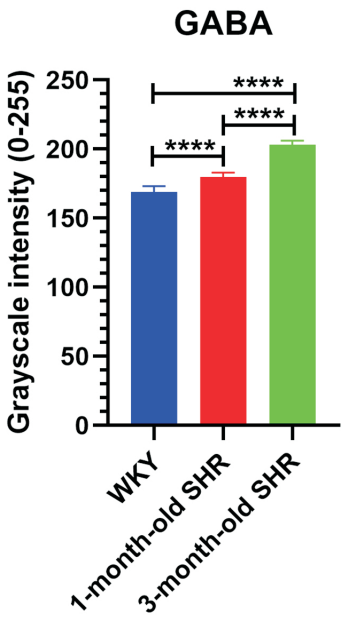


Fig. 2. A vertical bar graph showing a statistical comparison of the staining intensity for GABA in NEB of WKY rats (blue column), 1-month-old SHRs (red column) and 3-month-old SHRs (green column). Data are presented as Mean ± Standard deviation (SD) and compared by one-way analysis of variance ANOVA followed by post-hoc pairwise multiple comparison Tukey's HSD tests, where $^{****}p < 0.0001$

sional levels in NEBs and PNECs could be ascribed to a preventive mechanism.

Our present results show a statistically significant increase in the expressional levels of GABA between normotensive WKY rats and SHRs and also within the 1-month-old and 3-month-old SHR groups. Such a finding corresponds to the assumption that NEBs could react to the changes in the lung homeostasis with alterations in the systemic and pulmonary circulation. Our findings are also in agreement with the literature data stating that GABA is more commonly a vasodilator causing a decrease in the blood pressure, mainly by suppressing the sympathetic nervous system [15]. NEBs as complex and multimodal receptors are able to detect even the slightest changes in the lung homeostasis. This could be a plausible explanation of our findings on the increase in GABA expressional levels even in the younger group of SHRs, wherein the lung pressure alterations should be minimal and no morphological changes are as yet present. If so, then NEBs would react preemptively by increased GABA expression, which has already been reported in other pulmonary hypertension rat models [15].

In conclusion, NEBs and PNECs react to the alterations of the pulmonary pressure homeostasis by an increased GABA expression as a preventive or reactive regulation mechanism in developing pulmonary hypertension. Further ex-

periments could potentially reveal new diagnostic and therapeutic approaches referring to pulmonary hypertension and its relation to GABA.

REFERENCES

[1] Brouns I., D. Adriaensen, J. P. Timmermans (2023) The pulmonary neuroepithelial body microenvironment represents an underestimated multimodal component in airway sensory pathways, Anat. Rec. (Hoboken), https://doi.org/10.1002/ar.25171.

[2] Brouns I., L.Verckist, I. Pintelon et al. (2021) The pulmonary neuroepithelial body microenvironment. A multifunctional unit in the airway epithelium, Adv. Anat. Embryol. Cell Biol., **233**, 1–99.

[3] Brouns I., I. Pintelon, J. P. Timmermans, D. Adriaensen (2012) Novel insights in the neurochemistry and function of pulmonary sensory receptors, Adv. Anat. Embryol. Cell. Biol., **211**, 1-vii.

[4] Kim S. H., M. J. Patil, S. H. Hadley et al. (2022) Mapping of the sensory innervation of the mouse lung by specific vagal and dorsal root ganglion neuronal subsets, eNeuro, **9**(2), ENEURO.0026-22.2022.

[5] Freichel M., A. Zink-Lorenz, A. Holloschi et al. (1996) Expression of a calcium-sensing receptor in a human medullary thyroid carcinoma cell line and its contribution to calcitonin secretion, Endocrinology, **137**(9), 3842–3848.

[6] Linnoila R. I. (2006) Functional facets of the pulmonary neuroendocrine system, Lab. Invest., **86**(5), 425–444.

[7] Verckist L., R. Lembrechts, S. Thys et al. (2017) Selective gene expression analysis of the neuroepithelial body microenvironment in post-natal lungs with special interest for potential stem cell characteristics, Respir. Res., **18**(1), 87.

[8] Mak S. M., N. Strickland, D. Gopalan (2017) Complications of pulmonary hypertension: a pictorial review, Br. J. Radiol., **90**(1070), 20160745.

[9] Souza R., M. B. Amato, S. E. Demarzo et al. (2005) Pulmonary capillary pressure in pulmonary hypertension, Crit. Care, **9**(2), R132–138.

[10] Iliev A. A., G. N. Kotov, B. V. Landzhov et al. (2018) A comparative morphometric study of the myocardium during the postnatal development in normotensive and spontaneously hypertensive rats, Folia Morphol. Warsz., **77**(2), 253–265.

[11] Itelman E., M. J. Segel, R. Kuperstein et al. (2021) Pulmonary hypertension is associated with systemic arterial hypertension among patients with normal left ventricular diastolic function, J. Am. Heart. Assoc., **10**(24), e023603.

[12] Aharinejad S., D. E. Schraufnagel, P. Böck et al. (1996) Spontaneously hypertensive rats develop pulmonary hypertension and hypertrophy of pulmonary venous sphincters, Am. J. Pathol., **148**(1), 281–290.

[13] Lo C. C. W., S. M. Moosavi, K. J. Bubb (2018) The regulation of pulmonary vascular tone by neuropeptides and the implications for pulmonary hypertension, Front. Physiol., **23**, https://doi.org/10.3389/fphys.2018.01167.

[14] Lingeshwar P., G. Kaur, N. Singh et al. (2016) A study on the involvement of GABA-transaminase in MCT induced pulmonary hypertension, Pulm. Pharmacol. Ther., **36**, 10–21.

[15] Suzuki R., R. Maehara, S. Kobuchi et al. (2012) Beneficial effects of γ-aminobutyric acid on right ventricular pressure and pulmonary vascular remodelling in experimental pulmonary hypertension, Life Sci., **91**(13–14), 693–698.
[16] Fujimura S., H. Shimakage, H. Tanioka et al. (1999) Effects of GABA on noradrenaline release and vasoconstriction induced by renal nerve stimulation in isolated perfused rat kidney, Br. J. Pharmacol., **127**(1), 109–114.
[17] Lai F. M., T. Tanikella, P. Cervoni (1988) Effect of gamma-aminobutyric acid (GABA) on vasodilation in resistance-sized arteries isolated from the monkey, rabbit, and rat, J. Cardiovasc. Pharmacol., **12**(3), 372–376.

[1]*Department of Anatomy and Histology, Faculty of Medicine,*
Medical University of Sofia, 1 St. G. Sofiiski St, 1431 Sofia, Bulgaria
e-mails: nstamenov@medfac.mu-sofia.bg, adandov@medfac.mu-sofia.bg, ljelev@medfac.mu-sofia.bg, nlazarov@medfac.mu-sofia.bg

[2]*Institute of Neurobiology, Bulgarian Academy of Sciences,*
Akad. G. Bonchev St, Bl. 23, 1113 Sofia, Bulgaria
e-mails: d.atanasova@inb.bas.bg, nlazarov@bio.bas.bg

[3]*Department of Anatomy, Faculty of Medicine, Trakia University,*
11 Armejska St, 6000 Stara Zagora, Bulgaria
e-mail: dimitrinka.atanasova-dimitrova@trakia-uni.bg