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CGRP- and VIP-Immunoreactivity in the Rat Carotid Body

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The carotid body (CB), the primary peripheral chemoreceptor in mammals, is a mass of vascular tissue located near the bifurcations of the carotid arteries. It registers changes in the oxygen concentration of arterial blood and helps to control respiratory activity. The most striking anatomical features of the CB are its rich vascularization and dense innervation. At a light microscopical level using immunohistochemistry we identified the localization and distribution of calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP)-containing nerve structures in the CB of rats. Both investigated vasoactive neuropeptides were expressed, although in a different manner, in periglomerular and intraglomerular nerve fibers which innervate blood vessels. Moreover, we observed strong VIP-like immunoreactivity not only in nerve fibers but also in the glomus cells. Our data provide immunohistochemical proof that the rat CB uses perivascular neuropeptides, which probably manage chemosensory activity through their actions on the vessels and neuron-like glomus cells.

Key words: carotid body, CGRP, VIP, immunohistochemistry, chemosensitivity.

Introduction

The carotid body (CB) is the main peripheral chemoreceptor responsible for monitoring changes in arterial blood levels of pO_2, pCO_2 and pH, and participates in the ventilatory responses to hypoxia, hypercapnia and acidosis [1, 4]. It is a neural crest-derived ovoid mass of tissue bilaterally located at the bifurcation of the common carotid artery, just before blood chemicals reach the brain, an organ that is quite sensitive to oxygen and glucose deficiency. The CB is composed of clusters of cells, surrounded by a dense meshwork of capillaries and penetrated by bundles of sensory nerve endings of the carotid sinus nerve, a branch of the glossopharyngeal nerve, and by sympathetic postganglionic nerve fibers from the superior cervical ganglion [4, 17, 23].

The cell clusters, also known as glomoids or glomeruli, are the essential morphofunctional units of the CB. The glomeruli consist of two juxtaposed cell types, neuron-like oxygen sensitive type I, or glomus cells, incompletely invested by glial-like type II or sustentacular cells [1, 4].

Besides classical neurotransmitters, possible involvement of various neuropeptides such as CGRP and VIP in the chemoreception has been proposed immunohisto-

chemically. Since the calcitonin gene-related peptide (CGRP), a 37-amino acid peptide, has been isolated the expression of CGRP was demonstrated to occur entirely in primary sensory neurons [8, 21]. Such a localization totally implies that CGRP is a specific marker for the identification of some sensory nerve endings in the peripheral nervous system [8].

Materials and Methods

The experiments were carried out on adult male Wistar rats, weighing 250-300 g. The study was conducted according to the European Communities Council Directive 2010/63/EU for the protection of animals used for scientific purposes. All experimental procedures have been approved by the Bioethical Commission of the Biomedical Research at the Institute of Neurobiology of the Bulgarian Academy of Sciences.

For the immunohistochemical experiments, the rats were deeply anesthetized and transcardially perfused first with 0.05 M phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde (PFA) in 0.01 M phosphate buffer (PB), pH 7.4. The carotid bifurcations were dissected out and postfixed in the same fixative overnight at 4 °C. Afterwards, the tissues were embedded in paraffin and cut into 7 μm thick sections. The samples were then deparaffinized with xylene and ethanol, and subsequently processed for avidin-biotin-horseradish peroxidase complex (ABC) immunohistochemistry. Briefly, the sections were treated with hydrogen peroxide (1.2% in absolute methanol; 30 min) to inactivate endogenous peroxidase and the background staining was blocked with 5% normal goat serum (NGS) in PBS for 1 hour. Between the separate steps, the sections were rinsed with cold PBS/Triton X-100. After that they were incubated for 24 hours with the corresponding primary antibodies, rabbit anti-CGRP (diluted 1:1000, PEPA 27, Serotec) and rabbit anti-VIP (1:500; Amersham International, Buckinghamshire, UK) overnight at 4 °C in a humid chamber, followed by biotinylated goat anti-rabbit IgG (Sigma, 1:250) for 2 h at room temperature, and finally the ABC complex (Vector Labs, Burlingame, CA, USA) was applied for 2 hours at room temperature. Lastly, the peroxidase activity was visualized using diaminobenzidine as a chromogen. After the immunoreaction, the sections were dehydrated in ethanols, cleared in xylene and coverslipped with Entellan (Merck, Darmstadt, Germany). The specimens were observed and photographed with a Nikon research microscope equipped with a DXM 1200c digital camera.

The specificity of the immunostaining was controlled by the omission of the primary antiserum from the incubation medium or its replacement by PBS. No immunoreactivity was detected in either case.

Results

Numerous intraglomerular and periglomerular nerve fibers immunoreactive for CGRP were found throughout the parenchyma of the CB (**Fig. 1B, C**). At a higher magnification, the immunoreactive nerve fibers appeared as fine and beaded fibers, and they were seen as enclosing small blood vessels and cell clusters of glomus and sustentacular cells (**Fig. 1C**). When omitting the specific primary antibodies, or applying PBS or non-immune serum at the same dilution as the primary antibody, no specific immunostaining was found (**Fig. 1A**).

On the other hand, the immunohistochemical experiments demonstrated VIP-like immunoreactivity in some of neuron-like glomus cells (**Fig. 1D, E**). The periglomerular

and intraglomerular nerve fibers appeared as thin varicosities and most of them were associated with blood vessels.

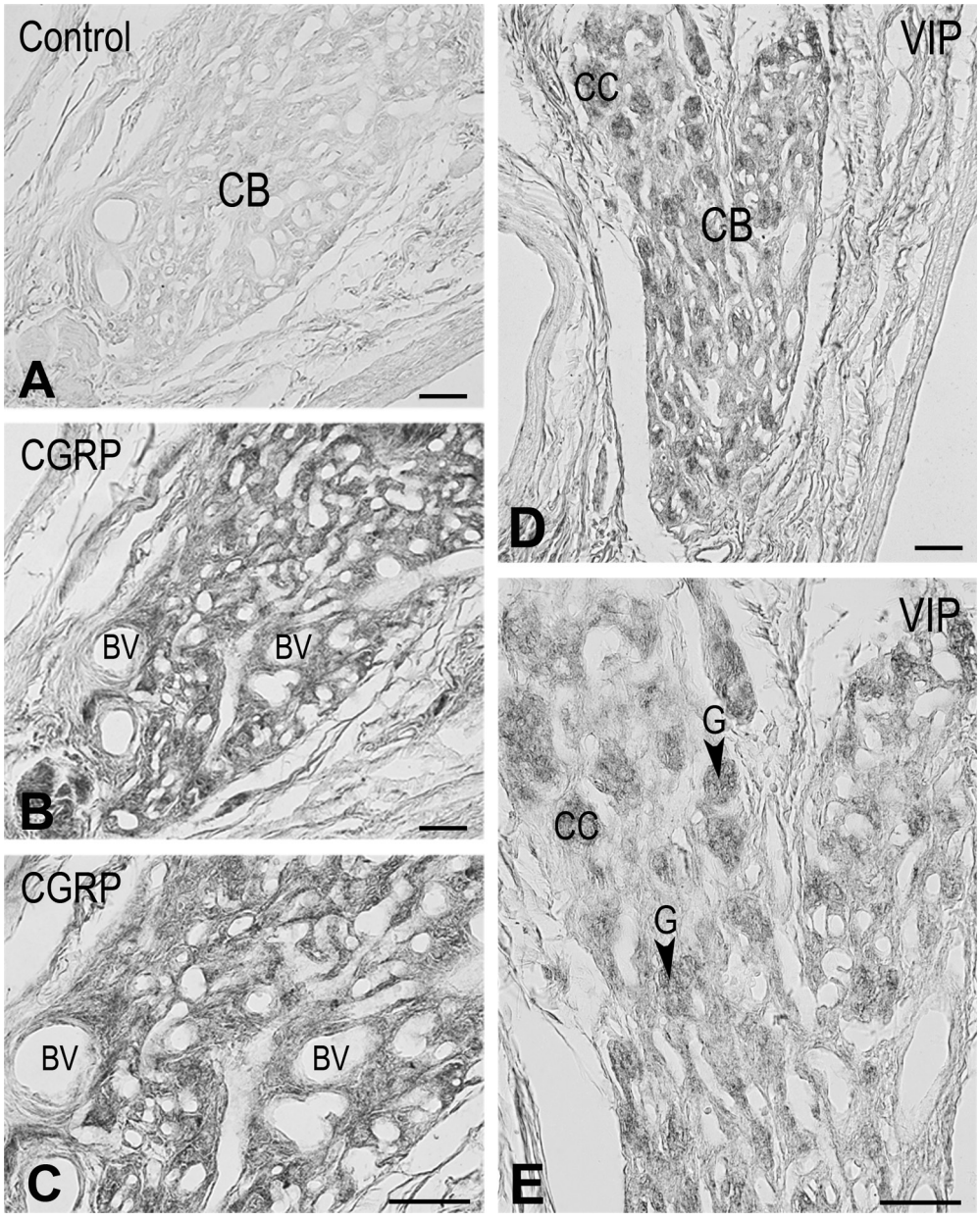

Fig. 1. Immunohistochemical demonstration of CGRP and VIP in the CB of adult rats. (**A**) No immunoreactivity is observed in the section incubated with PBS; (**B, C**) CGRP-immunoreactive intraglomerular and periglomerular nerve fibers in CB parenchyma; (**D, E**) Representative photomicrograph indicating the presence of VIP-immunoreactive perivascular nerve fibers and immunostained glomus cells (G) in cell clusters (CC). Blood vessels (BV). Scale bars = 50 μm

Discussion

The present results demonstrate that both examined dilatory neuropeptides are strongly expressed perivascularly in the rat CB, although in a different manner. In this study, we find that the vast majority of nerve fibers appear to contain certain sensory neuropeptides like CGRP. Furthermore, the most numerous CGRP-immunopositive sensory nerve fibers are associated with blood vessels and glomus cells, although some immunoreactive varicosities can be seen around the glomeruli too. We confirm results from previous immunohistochemical studies that also find CGRP-immunoreactivity in nerve endings close to blood vessels and glomic cell clusters in amphibians, birds and mammals [2, 5, 7, 8, 9, 11, 12, 13]. In addition, denervation experiments show that these nerve endings are sensory in nature, and most have their soma in the petrosal ganglion. In another study performed on the rat CB, it was observed that CGRP-immunopositive sensory fibers start to appear on the third day after birth, and increase continuously in number afterwards [8]. CGRP-immunoreactivity was not demonstrated in the glomus cells of chronically hypoxic or normoxic rat CBs [14, 20]. It was reported that in chronically hypoxic rat CB the density of CGRP-immunostained nerve fibers decreases significantly [14], and then increases several weeks after the termination of hypoxia [15, 16]. Various implications were described in different animal species, and it has been proposed that the action of CGRP is more likely to modulate the effects of neurotrophic factors than to impose a direct impact [3, 20]. Nevertheless, CGRP has been established in nerve fibers and not in glomic cells, it cannot be excluded that the release of CGRP by nervous terminations may exert some local trophic effect.

On the other hand, we have found VIP immunoreactivity in both the nerve fibers and glomus cells in the rat CB as opposed to studies performed by other authors who have been reported that VIP-immunoreactivity is confined to nerve terminations within the CB in different animal species [7, 10, 13, 19, 24]. Furthermore, immunohistochemical studies have also shown that weak VIP-immunoreactivity is located in type I cells of the human CB [22], although their data were not confirmed in the glomus cells of chronically hypoxic or normoxic rat CBs by another working group of researchers [14]. In the chronically hypoxic rat CB, the density of VIP-immunostained nerve fibers significantly increases [14, 19] and returns to a normoxic state in a few weeks after termination of hypoxia [16, 20]. Taken together with previous immunohistochemical and physiological [14] reports, our findings indicate that VIP most likely modulates the CB chemosensitivity and changes in spontaneous chemoreceptor discharges. VIP, a peptide abundant in autonomic nerve fibers, evokes vasodilation of blood vessels and it may be associated with chemosensory mechanisms by controlling local circulation [14, 20]. Furthermore, VIP may increase neuronal survival indirectly by the secretion of activity-dependent neurotrophic factors, cytokines, and chemokines [18, 20].

Conclusion

It can be inferred for this study that the two neuropeptides examined are strongly expressed in both sensory and autonomic nerve endings apposed to glomus cells, pericytes or vascular smooth muscle cells. The latter leads to the assumption that CGRP- and VIP-immunopositive nerve fibers may regulate the blood flow within the rat CB.

References

1. **Atanasova, D. Y., M. E. Iliev, N. E. Lazarov.** Morphology of the rat carotid body. – Biomed. Rev., **22**, 2011, 41-55.
2. **Atanasova, D. Y., N. E. Lazarov.** Expression of some neuropeptides in the rat CB. – Acta Morphol. Anthropol., **19**, 2012, 15-19.
3. **Connat, J. L., V. Schnüriger, R. Zanone, C. Schaeffer, M. Gaillard, B. Faivre, L. Rochette.** The neuropeptide calcitonin gene-related peptide differently modulates proliferation and differentiation of smooth muscle cells in culture depending on the cell type. – Regul. Pept., **101**, 2001, 169-178.
4. **Gonzaez, C., L. Almaraz, A. Obeso, R. Rigual.** Carotid body chemoreceptors: from natural stimuli to sensory discharges. – Physiol. Rev., **74**, 1994, 829-898.
5. **Heath, D., M. Quinzanini, A. Rodella, A. Albertini, R. Ferrari, P. Harris.** Immunoreactivity to various peptides in the human carotid body. – Res. Commun. Chem. Pathol. Pharmacol., **62**, 1988, 289-293.
6. **Heym, C., W. Kummer.** Immunohistochemical distribution and colocalization of regulatory peptides in the carotid body. – J. Electron. Microsc. Tech., **12**, 1989, 331-342.
7. **Kameda, Y.** Distribution of CGRP-, somatostatin-, galanin-, VIP-, and substance P-immunoreactive nerve fibers in the chicken carotid body. – Cell Tissue Res., **257**, 1989, 623-629.
8. **Kondo, H., M. Yamamoto.** Occurrence, ontogeny, ultrastructure and some plasticity of CGRP (calcitonin gene-related peptide)-immunoreactive nerves in the carotid body of rats. – Brain Res., **473**, 1988, 283-293.
9. **Kummer, W.** Retrograde neuronal labelling and double-staining immunohistochemistry of tachykinin- and calcitonin gene-related peptide-immunoreactive pathways in the carotid sinus nerve of the guinea pig. – J. Auton. Nerv. Syst., **23**, 1988, 131-141.
10. **Kummer, W.** Three types of neurochemically defined autonomic fibres innervate the carotid baroreceptor and chemoreceptor regions in the guinea-pig. – Anat. Embryol., **181**, 1990, 477-489.
11. **Kummer, W., A. Fischer, C. Heym.** Ultrastructure of calcitonin gene-related peptide- and substance P-like immunoreactive nerve fibers in the carotid body and carotid sinus of the guinea pig. – Histochemistry, **92**, 1989, 433-439.
12. **Kummer, W., J. O. Habeck.** Substance P- and calcitonin gene-related peptide-like immunoreactivity in the human carotid body studied at light and electron microscopical level. – Brain Res., **554**, 1991, 286-292.
13. **Kusakabe, T.** Ontogeny of substance P-, CGRP-, and VIP-containing nerve fibers in the amphibian carotid labyrinth of the bullfrog, Rana catesbeiana. An immunohistochemical study. – Cell Tissue Res., **269**, 1992, 79-85.
14. **Kusakabe, T., Y. Hayashida, H. Matsuda, Y. Gono, F. L. Powell, M. H. Ellisman, T. Kawakami, T. Takenaka.** Hypoxic adaptation of the peptidergic innervation in the rat carotid body. – Brain Res., 806, 1998, 165-174.
15. **Kusakabe, T., H. Hirakawa, H. Matsuda, T. Kawakami, T. Takenaka, Y. Hayashida.** Peptidergic innervation in the rat carotid body after 2, 4, and 8 weeks of hypocapnic hypoxic exposure. – Histol. Histopathol., **18**, 2003, 409-418.
16. **Kusakabe, T., H. Hirakawa, S. Oikawa, H. Matsuda, T. Kawakami, T. Takenaka, Y. Hayashida.** Morphological changes in the rat carotid body 1, 2, 4, and 8 weeks after the termination of chronically hypocapnic hypoxia. – Histol. Histopathol., **19**, 2004, 1133-1140.
17. **McDonald, D.M.** Peripheral chemoreceptors. Structure-function relationships of the carotid body. In: Regulation of Breathing (Ed. T. F. Hornbein), New York, Dekker, 1981, 105-319.
18. **Moody, T. W., J. M. Hill, R. T. Jensen.** VIP as a trophic factor in the CNS and cancer cells. – Peptides, **24**, 2003, 163-177.
19. **Poncet, L., L. Denoroy, Y. Dalmaz, J. M. Pequignot, M. Jouvet.** Chronic hypoxia affects peripheral and central vasoactive intestinal peptide-like immunoreactivity in the rat. – Neurosci. Lett., **176**, 1994, 1-4.
20. **Porzionato, A., V. Macchi, A.R. Parenti, R. De Caro.** Trophic factors in the carotid body. – International Review of Cell and Molecular Biology, **269**, 2008, 1-58.

21. **Rodrigo, J., J.M. Polak, L. Fernandez, M.A. Ghatei, P. Mulderry, S.R. Bloom.** Calcitonin gene-related peptide immunoreactive sensory and motor nerves of the rat, cat, and monkey esophagus. – Gastroenterology, **88**, 1985, 444-451.
22. **Smith, P., J. Gosney, D. Heath, H. Burnett.** The occurrence and distribution of certain polypeptides within the human carotid body. – Cell Tissue Res., **261**, 1990, 565-571.
23. **Verna, A.** The mammalian carotid body: morphological data. – In: The carotid body chemoreceptors (Ed. C. Gonzalez), Texas, U.S.A, Landes Bioscience Austin, 1997, 1-29.
24. **Wharton, J., J. M. Polak, A. G. Pearse, G. P. McGregor, M. G. Bryant, S. R. Bloom, P. C. Emson, G. E. Bisgard, J. A. Will.** Enkephalin-, VIP- and substance P-like immunoreactivity in the carotid body. – Nature, **284**, 1980, 269-271.