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Changes in the peptidergic innervation in the carotid body of rats chronically exposed to hypercapnic hypoxia: an effect of arterial CO_2 tension

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Summary. The abundance of neuropeptide Y (NPY)-, vasoactive intestinal polypeptide (VIP)-, substance P (SP)-, and calcitonin gene-related peptide (CGRP)-immunoreactive nerve fibers in the carotid body was examined in chronically hypercapnic hypoxic rats (10% O_2 and 6-7% CO_2 for 3 months), and the distribution and abundance of these four peptidergic fibers were compared with those of previously reported hypocapnic- and isocapnic hypoxic carotid bodies to evaluate the effect of arterial CO_2 tension. The vasculature in the carotid body of chronically hypercapnic hypoxic rats was found to be enlarged in comparison with that of normoxic control rats, but the rate of vascular enlargement was smaller than that in the previously reported hypocapnic- and isocapnic hypoxic carotid bodies. In the chronically hypercapnic hypoxic carotid body, the density per unit area of parenchymal NPY fibers was significantly increased, and that of VIP fibers was unchanged, although the density of NPY and VIP fibers in the previously reportetd chronically hypocapnic and isocapnic hypoxic carotid bodies was opposite to that in hypercapnic hypoxia as observed in this study. The density of SP and CGRP fibers was decreased. These results along with previous reports suggest that different levels of arterial CO_2 tension change the peptidergic innervation in the carotid body during chronically hypoxic exposure, and altered peptidergic innervation of the chronically hypercapnic hypoxic carotid body is one feature of hypoxic adaptation.

Key words: Carotid body, Systemic hypoxia (Hyper-, Iso-, and Hypocapnic hypoxia), Regulatory neuropeptides, Autonomic nervous system, Immunohistochemistry, Rat

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Introduction

The carotid body is enlarged by several folds in rats exposed to chronic hypoxia (Heath et al., 1973; Laidler and Kay, 1975a,b; Barer et al., 1976; Kusakabe et al., 1993, 1998a,b, 2000), and the glomus cells (type I cells and chief cells), which are thought to be chemoreceptor cells, also increase in number and volume (Bleesing and Wolff, 1973; Moller et al., 1974; Laidler and Kay, 1978; Peqiugnot and Hellström, 1983; Dhillon et al., 1984; McGregor et al., 1984; Pequignot et al., 1984; Pallot et al., 1990). As a result of enlargement, the carotid bodies show a spongy appearance with increased vascularization, bearing remarkable similarity to the carotid body of the normal amphibian (Kusakabe et al., 1993), whose arterial O_2 tension (PaO_2) is generally low (Toews and Heisler, 1982; West et al., 1987).

In various vertebrates, many kinds of regulatory neuropeptide-containing nerve fibers have been demonstrated in the parenchyma of the carotid body (Lundberg et al., 1979; Wharton et al., 1980; Jacobowitz and Helke, 1980; Chen et al., 1986; Kondo et al., 1986; Kondo and Yamamoto, 1988; Kusakabe et al., 1991, 1994, 1995, 1998b). In our recent studies of rat carotid bodies exposed to chronically isocapnic and hypocapnic hypoxia (Kusakabe et al., 1998a, 2000), the density of vasoactive intestinal polypeptide (VIP) immunoreactive fibers increased significantly, the density of substance P (SP) and calcitonin gene-related peptide (CGRP) immunoreactive fibers decreased significantly to under 50%, and the density of neuropeptide Y (NPY) immunoreactive fibers was unchanged in comparison with the normoxic controls (Kusakabe et al., 1998a,b, 2000). At least in these studies of ours, there were no conspicuous changes in the distribution and abundance of peptidergic fibers in the carotid body between hypocapnic and isocapnic hypoxia.

In addition to the isocapnic and hypocapnic hypoxia, systemic hypoxia includes hypercapnic hypoxia with

high arterial CO_2 ($PaCO_2$) levels (Hirakawa et al., 1997). Koehler et al. (1980) previously reported the modulating effect of CO_2 on the circulatory response to chronic hypoxia in sinoaortic denervated dogs to assess the arterial chemoreflex contribution. Elevating $PaCO_2$ attenuated tachycardia during hypoxia. This makes us suspect some interaction between the modulating effects of increased CO_2 level on cardiovascular responses and the carotid body chemoreceptor reflex.

In the present study, we examined the distribution and abundance of nerve fibers immunoreactive for four different regulatory neuropeptides, NPY, VIP, SP, and CGRP, between the carotid body of normoxic and chronically hypercapnic hypoxic rats, and compared the present results with the previous ones in chronically hypocapnic- and isocapnic hypoxic carotid bodies to evaluate the effect of $PaCO_2$ level. In addition, antisera against tyrosine hydroxylase (TH) and p67phox were applied to demonstrate the glomus cells, although it has been stated by Kummer and Acker (1995) that the glomus cells are not the only cell type in the carotid body that contains p67phox.

Materials and methods

Chronically hypercapnic hypoxia

Male Wistar rats were placed in an air-tight acrylic chamber (50x50x60 cm) with two holes. One hole, located at the top of a side wall of the chamber, was connected to a multi-flowmeter (MODEL-1203, KOFLOC, Japan), and was used to deliver a hypoxic gas mixture (10% O_2 in N_2 and 6-7% CO_2: total 10 L/min) into the chamber. The CO_2 was added to the hypoxic gas mixture at the concentration of 6-7% because this much was necessary to maintain an arterial partial pressure of CO_2 close to hypercapnic which was measured during hypoxic exposure. The flow of air, N_2 and CO_2 was regulated by a multi-flowmeter and the O_2 and the CO_2 levels within the box were monitored with a gas analyzer (Respina 1H26, NEC San-ei, Japan). The second hole was located at the bottom of the opposite wall of the chamber and was used to flush out the gas mixture. The temperature within the chamber was maintained at 25 °C. This hypoxic condition has been confirmed to be hypercapnic to the rats in a previous study (Hayashida et al., 1996). Six rats were exposed chronically in this chamber for three months with food and water available ad libitum. Six control rats were housed for three months in the same chamber ventilated by air at the same flow rate. The chamber was opened for 10 min every 3 days for husbandry.

All experiments with animals were performed in accordance with "Principles of laboratory animal care" (NIH publ. no. 86-23, revised 1985) and with "Guiding Principles for the Care and Use of Animals in the Fields of Physiological Sciences" published by the Physiological Society of Japan.

Tissue preparation

The animals were intraperitoneally anesthetized with sodium pentobarbital (0.05 mg/g), and perfused through a thin nylon tube inserted into the ventricle with 0.1M heparinized phosphate buffered saline (PBS), followed by freshly prepared Zamboni's fixative solution (0.2% picric acid and 4% paraformaldehyde in 0.1M PBS) at a constant flow rate. The pair of carotid bodies were then removed under a dissecting microscope, and immersed in the same fixative for an additional 6-8 h at 4 °C. After a brief washing in PBS, the specimens were transferred to 30% sucrose in PBS at 4 °C for 24 h. The specimens were cut serially at 15 µm on a cryostat, and mounted in four series on poly-L-lysine coated slides.

Immunohistochemistry

The sections were processed for immunohistochemistry according to the peroxidase-antiperoxidase (PAP) method. Prior to PAP treatment, sections were dipped in a fresh 0.3% solution of hydrogen peroxide in methanol for 30 min at room temperature to inhibit endogenous peroxidase activity. After washing in several changes of 0.3% Triton-X in 0.1M PBS (PBST), the sections were treated for 30 min with a protein blocking agent (Immunon, Pittsburgh, USA) at room temperature to block nonspecific protein binding sites. Then they were incubated at 4 °C overnight with the primary rabbit antisera against the following neuropeptides: SP (1:1500; Cambridge Research Biochemicals, Northwich, UK), CGRP (1:1500; Cambridge Research Biochemicals, Northwich, UK), VIP (1:2000; Incstar, Stillwater, USA), and NPY (1:2000; Incstar, Stillwater, USA). In each experiment, some sections were incubated with antiserum for TH (1:200; Chemicon, Temecula, USA) and p67phox (1:100; Santa Cruz Biotechnol., USA) to demonstrate the total population of glomus cells. The antisera were diluted with 0.2% bovine serum albumin, 1% normal goat serum, and 0.2% sodium azide in PBST. After rinsing in several changes of PBST, the sections were transferred for 2 h to anti-rabbit IgG (Organo Technica, Durham, USA) diluted to 1:200 with 0.2% bovine serum albumin, 1% normal goat serum, and 0.2% sodium azide in PBST at room temperature. Then, the sections were rinsed with several changes of PBS, transferred for 2 h to rabbit PAP (Jackson Immuno Research, West Grove, USA) diluted to 1:200 with 0.2% bovine serum albumin, and rinsed in several changes of PBS. The peroxidase activity was demonstrated with 3,3'-diaminobenzidine. This immunostaining procedure has been detailed in a previous report (Kusakabe et al., 1991). Some sections were also stained with hematoxylin eosin for general histology.

Control

The reaction for neuropeptides was verified by

treating sections with primary antibody which had been inactivated by overnight incubation with 50-100 μM of each respective peptide (Sigma, St Louis, USA).

Data analysis

The density of immunoreactive fibers in the carotid bodies of normoxic and chronically hypoxic rats was represented as the number of varicosities. In the sections through the center of the carotid body, the area of the parenchyma of the carotid body was measured with an ARGUS 100 computer and an image processor (Hamamatsu-Photonics, Japan) on 50 sections taken from 12 normoxic carotid bodies and 50 sections taken from 12 chronically hypoxic carotid bodies of each of the 6 animals examined, and the number of varicosities was counted. The value per unit area (10^4 μm^2) of parenchyma, excluding the area of vascular lumen, was expressed as mean ± S.D (n=50). Statistical comparisons between the control and experimental values were determined using the Student's *t*-test.

Results

General histology of the carotid bodies in normoxia and in chronically hypercapnic hypoxia

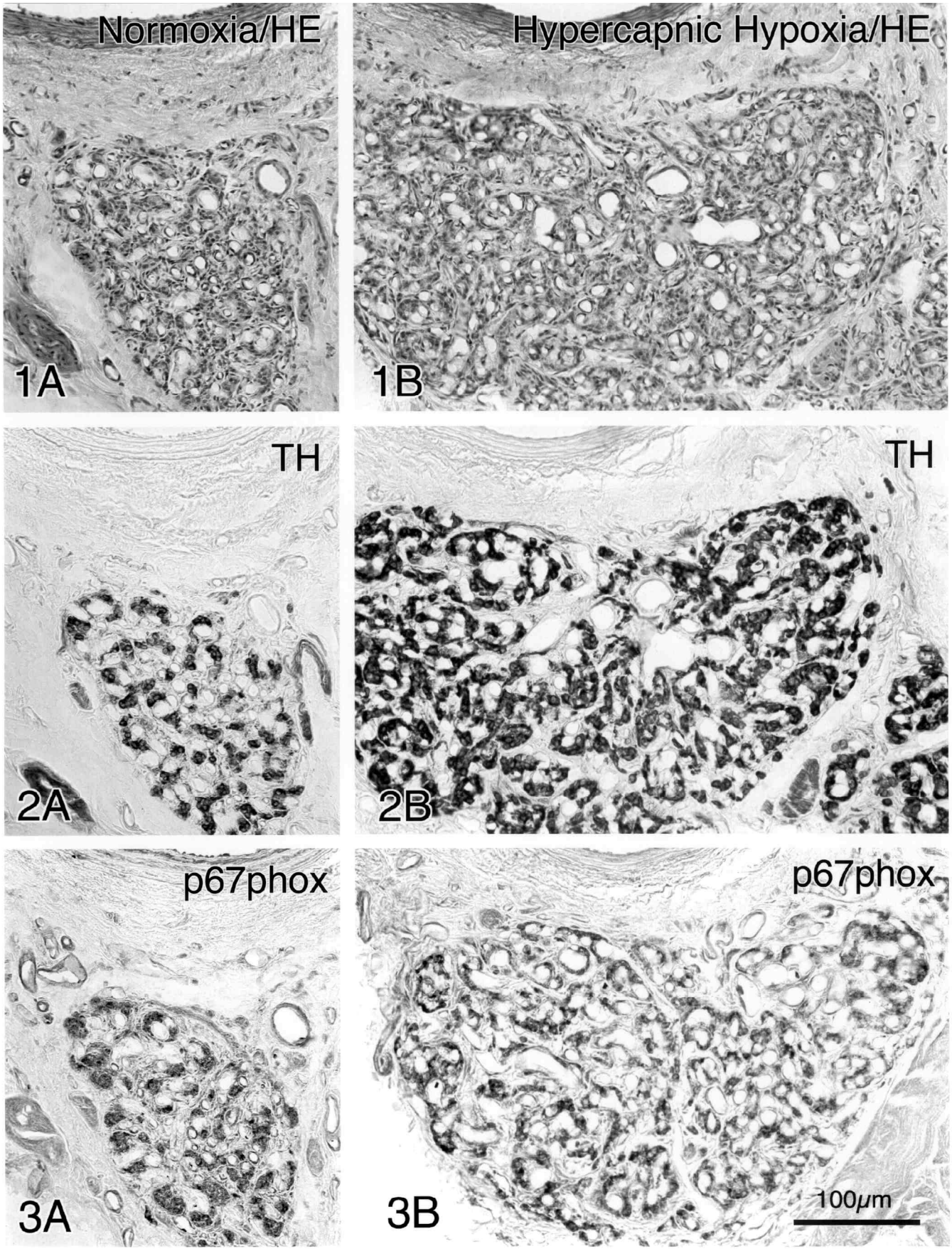
In the hematoxylin-eosin stained sections from the center of the carotid body of the control normoxic rats, the carotid body was an oval and highly vascular structure (Fig. 1A). In two serial sections, which were immunostained with TH and p67phox, a number of clusters of the immunoreactive glomus cells were located in the parenchyma between the blood vessels (Figs. 2A, 3A). The carotid body of the chronically hypercapnic hypoxic rats was found to be enlarged several fold in comparison with that of normoxic control rats (Fig. 1B). The enlarged hypoxic carotid bodies contained many blood vessels whose diameter was larger than those in the normoxic carotid bodies, and the percentage of vascular enlargement in the hypercapnic hypoxic carotid body was small. The mean area of parenchyma of the carotid body, excluding the area of expanded blood vessels, was about 2.3 times larger than in the controls ($8.71\pm0.93\times10^4$ m^2/$19.98\pm1.88\times10^4$ m^2). In two serial sections, the relative abundance of TH- and p67phox-immunoreactive glomus cells increased along with an increase in size of the carotid body (Figs. 2B, 3B), although the precise number of immunoreactive cells has not been counted.

Peptidergic nerve fibers in the normoxic carotid body

As previously reported, immunoreactivity of NPY, VIP, SP, and CGRP was recognized in the nerve fibers distributed in the parenchyma of the carotid body (Kusakabe et al., 1998a, 2000). NPY-, VIP-, SP-, and CGRP fibers appeared as thin processes with many varicosities showing punctate structures (Figs. 4A-7A). NPY-immunoreactive varicose fibers were more numerous than VIP, SP, and CGRP fibers. These immunoreactive fibers were mainly associated with the vasculature and the clusters of glomus cells. The mean absolute number of varicosities in NPY, VIP, SP, and CGRP fibers per section was 407.4±71.7, 152.8±29.5, 72.3±11.8, and 157.2±22.6, respectively. The mean number of varicosities in these four peptidergic nerve fibers per unit area (10^4 μm^2) was 49.1±5.6, 17.6±3.4, 8.3±1.4, and 18.1±2.6, respectively. The mean absolute number and mean density of NPY fibers were the highest among the four peptidergic fibers examined. There were no glomus cells immunoreactive for these four neuropeptides.

Peptidergic nerve fibers in the chronically hypercapnic hypoxic carotid body

The distribution pattern of NPY-, VIP-, SP-, and CGRP-immunoreactive fibers in the chronically hypercapnic hypoxic carotid body was similar to that in the normoxic carotid body, and the absolute number of varicosities in these immunoreactive fibers increased along with the enlargement of the carotid body (Figs. 4B-7B). As shown in the normoxic control carotid body, these immunoreactive fibers were associated with the vasculature and the clusters of glomus cells. When the mean absolute number of varicosities in NPY, VIP, SP, and CGRP varicose fibers per section was compared between normoxic and chronically hypercapnic hypoxic carotid bodies, the values were significantly increased from 407.4±71.7 to 1276.9±139.9 ($p<0.005$), from 152.8±29.5 to 364.3±61.0 ($p<0.005$), 72.3±11.8 to 116.2±37.4 ($p<0.01$), and from 157.2±22.6 to 279.1±38.7 ($p<0.005$), respectively (Fig. 8A). Especially, the mean absolute number of varicosities of NPY fibers in the hypercapnic hypoxic carotid body was about 3.1 (1276.9/407.4) times higher than that of NPY fibers in the normoxic control carotid body (Fig. 8A). On the other hand, when the mean density of varicosities in NPY, VIP, SP, and CGRP fibers per unit area (10^4 μm^2) was compared in the same way, the density of varicosities in NPY fibers was significantly ($p<0.005$) increased from 49.1±5.6 to 64.8±7.1, although that of SP and CGRP fibers per unit area was significantly decreased from 8.3±1.4 to 5.9±1.9 ($p<0.01$), and from 18.1±2.6 to 14.1±2.0 ($p<0.005$), respectively (Fig. 8B). The density of VIP fibers was unchanged (Fig. 8B). The mean density of NPY fibers per unit area in the chronically hypercapnic hypoxic carotid body was about 1.3 (64.8/49.1) times higher than that of NPY fibers in the normoxic control carotid body. The mean density of SP and CGRP fibers per unit area was about 0.7 (5.9/8.3, 14.4/18.1) times higher than that of these fibers in the normoxic carotid body. No glomus cells with immunoreactivity for NPY, VIP, SP, and CGRP were found in the chronically hypercapnic hypoxic carotid

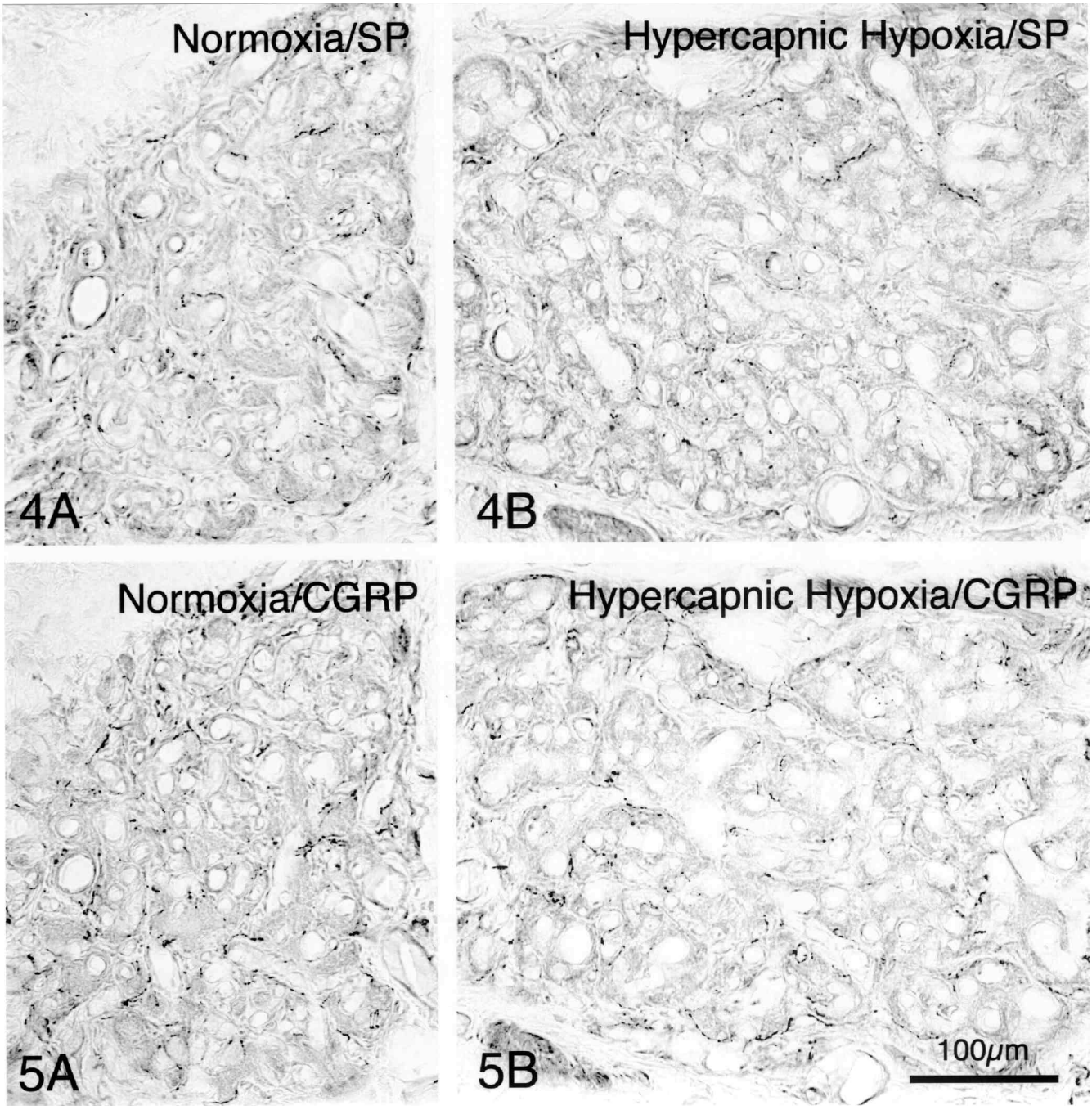


Figs. 1-3. Three semi-serial sections of a control normoxic **(A)** and chronically hypercapnic hypoxic **(B)** rat carotid bodies stained with hematoxylin-eosin (**Fig. 1)**, TH antiserum **(Fig. 2)**, and p67phox antiserum **(Fig. 3)**. Note the increase in volume of the carotid body with enlargement of vasculature and the increase in number of the cluster of glomus cells along with the enlargement of the carotid body.

body.

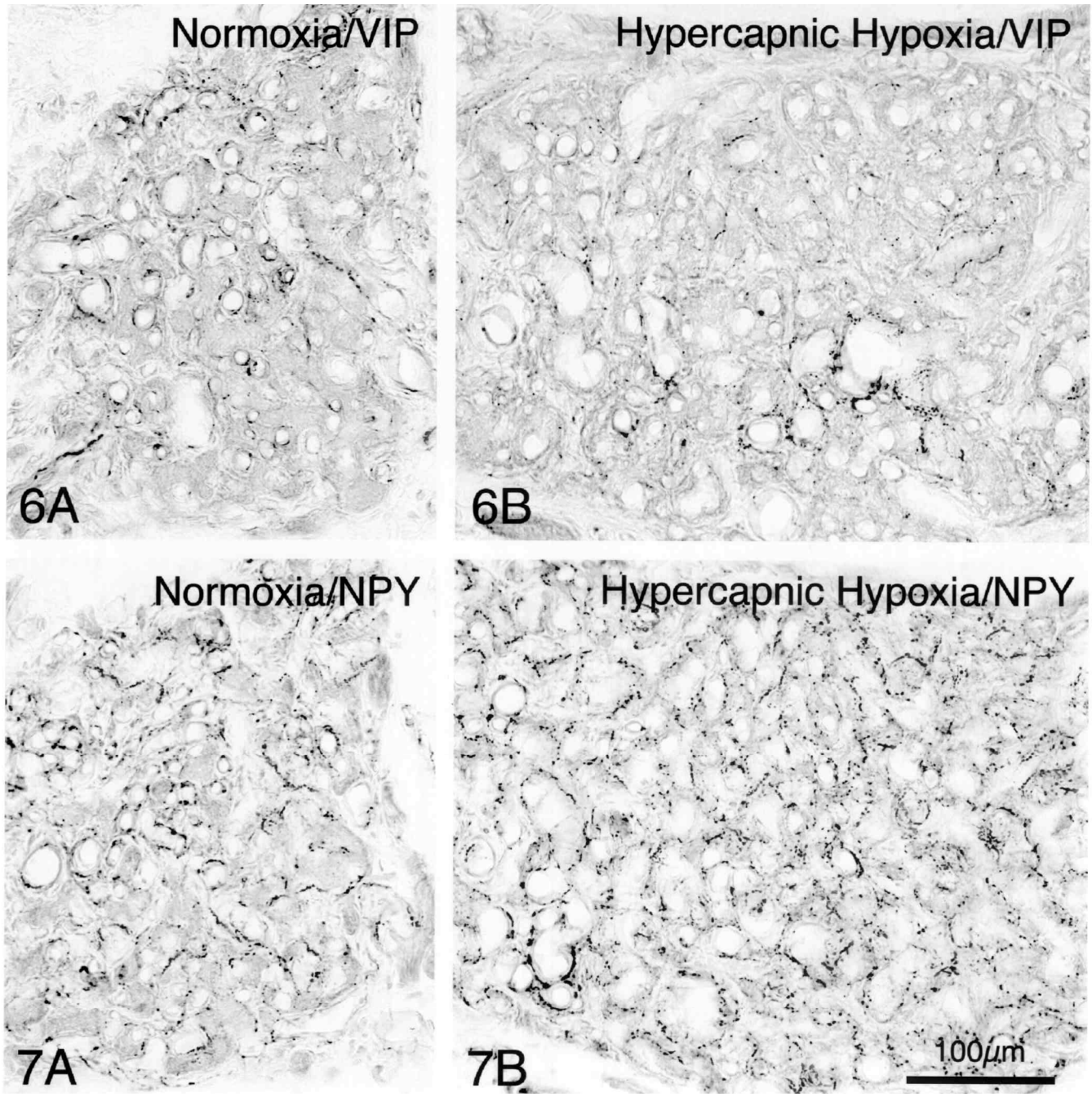
Discussion

In the present study of chronically hypercapnic hypoxic (10% O_2 in N_2, 6-7% CO_2 for 3 months) rat carotid bodies, the density per unit area of NPY immunoreactive nerve fibers was siginificantly increased, and that of VIP fibers was unchanged. However, in our previous studies of chronically hypocapnic (10% O_2 in N_2 for 3 months) and isocapnic hypoxic (10% O_2 in N_2, 3-4% CO_2 for 3 months) rat carotid bodies, the density per unit area of NPY and VIP fibers was reversed (Kusakabe et al., 1998a, 2000). That



Figs. 4-5. A relatively low number of SP-immunoreactive nerve fibers in the normoxic **(Fig. 4A)** and the chronically hypercapnic hypoxic carotid body **(Fig. 4B)**. There are many CGRP-immunoreactive fibers around the vasculature and the cluster of glomus cells in the normoxic **(Fig. 5A)** and the chronically hypoxic carotid body **(Fig. 5B)**.

is, the density of parenchymal VIP fibers was significantly increased, and that of NPY fibers was unchanged. The difference in the density of NPY and VIP fibers between the hypercapnic and the isocapnic hypoxic carotid bodies may suggest the involvement of a high level of arterial CO_2 tension, although there is no difference in the density of these fibers between the isocapnic and the hypocapnic carotid bodies. VIP is thought to have a long acting vasodilatory effect (Larson et al., 1976; Heistad et al., 1980; Wilson et al., 1981). On this basis, we suggested that at least part of the vascular expansion in the chronically hypocapnic and isocapnic hypoxic carotid bodies may be due to the vasodilatory effect of VIP. In addition, it has been considered that the



Figs. 6-7. A number of VIP-immunoreactive nerve fibers in the normoxic **(Fig. 6A)** and the chronically hypercapnic hypoxic carotid body **(Fig. 6B)**. Most immunoreactive fibers are associated with blood vessels of the enlarged hypoxic carotid body. There are numerous NPY-immunoreactive fibers around the vasculature and the cluster of glomus cells in the normoxic **(Fig. 7A)** and the chronically hypercapnic hypoxic carotid body **(Fig. 7B)**.

vascular enlargement is at least to great extent due to structural changes rather than functional states because there is a true angiogenesis in the hypoxic body. As a result of the vascular enlargement, the blood flow in the chronically hypoxic carotid body is increased. Accordingly we concluded in the previous studies that parenchymal VIP fibers, especially around the blood vessels, are indirectly involved in chemosensory mechanisms by controlling local carotid body circulation. In various mammalian vasculatures, NPY is thought to have a vasoconstrictory effect (Lundberg et al., 1982; Edvinsson et al., 1983; Brain et al., 1985). We speculate that a low percentage of vascular enlargement in the present chronically hypercapnic hypoxic carotid body may be due to the vasoconstrictory effect of the increased NPY resulting from hypercapnia. However, this speculation may be restricted to the chronically hypoxic rat carotid body. To make this clear, it is necessary to perform further morphological studies in vasculatures of various organs in hypercapnic hypoxic animals.

On the other hand, it has been suggested that arterial CO_2 pressure (PCO_2) affects the cerebral blood flow (Fensternacher and Rapoport, 1984). For instance, hypercapnia increases blood flow in both gray and white matter. Hypercapnia also increases the gastrointestinal blood flow, and the response of the ocular blood vessels to high CO_2 tension is similar to that of the cerebral blood vessels. Thus, CO_2 tension causes vasodilation in both central and peripheral vascular systems. In general histology of the chronically hypercapnic hypoxic carotid body, the percentage of vascular enlargement is smaller than in the previously reported chronically hypocapnic- and isocapnic hypoxic carotid bodies (Kusakabe et al., 1998a, 2000). This may indicate that high CO_2 tension causes the carotid body vasculature to constrict in the chronically hypoxic condition.

Recently we have reported the contribution of the autonomic nervous system to the cardiovascular response to hypoxia with different levels of CO_2 in conscious rats (Hirakawa et al., 1997). Hypercapnic hypoxia causes an increase in arterial blood pressure and renal sympathetic nerve activity, and a decrease in heart rate, whereas hypocapnic hypoxia causes a decrease in arterial blood pressure, and an increase in heart rate and renal sympathetic nerve activity. Sino-aortic denervation and atropine abolished the bradycardic response during hypercapnic hypoxic exposure. On this basis, we suggest that hypercapnic hypoxia causes sympathetic and parasympathetic activation, while hypocapnic hypoxia causes sympathetic activation and parasympathetic inhibition. We also reported that baroreflex function is affected by arterial CO_2 levels. From these results, we suggested that cardiovascular responses during hypoxia are modified by arterial PCO_2 levels, and mediated by the autonomic nervous system. Thus, the different level of arterial CO_2 tension affects the autonomic nervous system. In addition, Edvinsson et al. (1983) and Kondo et al. (1986) suggested a possible coexistence of noradrenaline and NPY in the sympathetic nerve. Considered together with these physiological and morphological analyses, the changes in the general histology and the peptidergic innervation, especially the increased density of NPY fibers, in the chronically hypercapnic hypoxic rat carotid body may be in part involved in the acclimatized changes in the autonomic nervous system.

It has been reported that SP- and CGRP-containing fibers in the mammalian carotid body are involved in chemoreceptor mechanisms (Helke et al., 1980; Jacobowitz and Helke, 1980; Wharton et al., 1980; Lundberg and Hökfelt, 1983) because SP and CGRP

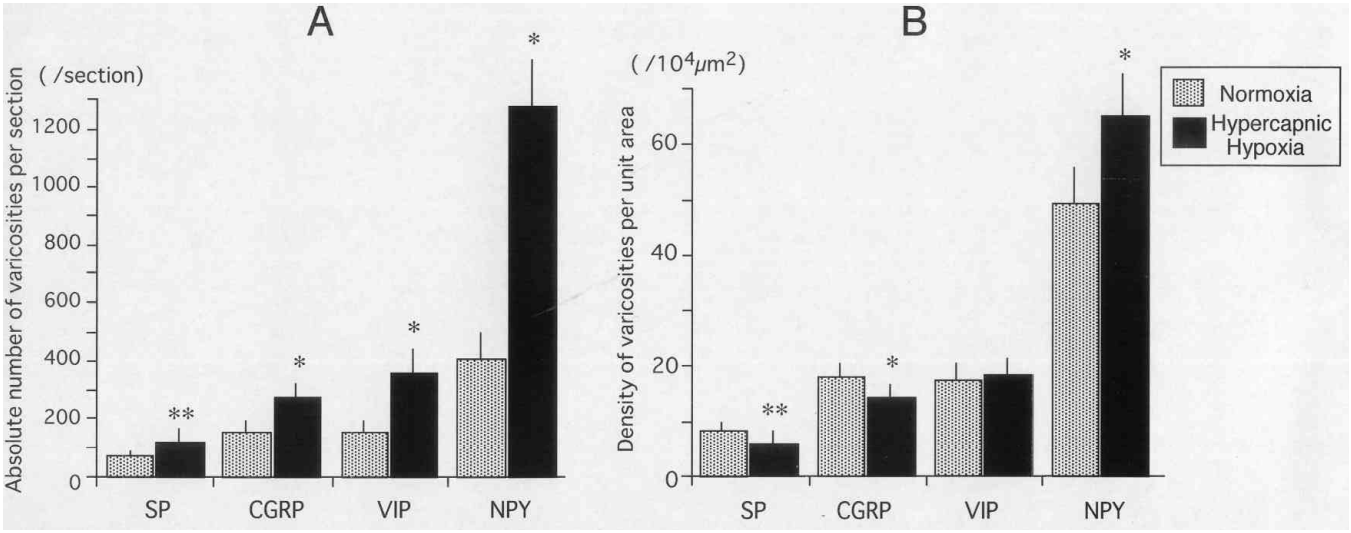


Fig. 8. Histograms comparing the absolute number of varicosities per section **(A)** and the density of varicosities per unit area **(B)** in normoxic and chronically hypercapnic hypoxic carotid body. *: $p<0.005$; **: $p<0.01$.

fibers in the carotid body originate from the sensory jugular and petrosal ganglia (Chen and Yates, 1986; Finley et al., 1992). On the other hand, some authors suggested a possibility that the efferent component of the glossopharyngeal nerve possesses vasodilatory SP and CGRP (Hallberg and Pernow, 1975; Samnegard et al., 1978; Edvinsson et al., 1981; Edvinsson and Uddman, 1982; Brain et al., 1985) in addition to a sensory role. In the present chronically hypercapnic hypoxic carotid body, the density per unit area of SP and CGRP immunoreactive fibers was significantly decreased, although that of SP and CGRP fibers in the chronically hypocapnic and isocapnic hypoxic carotid bodies was unchanged (Kusakabe et al., 1998a, 2000). This difference in the density of SP and CGRP may also be depend on the high level of arterial CO_2 tension in chronically hypercapnic hypoxia.

In conclusion, the high CO_2 tension affects the density of peptidergic nerve fibers in the carotid body during chronically hypoxic exposure. Altered peptidergic innervation in the chronically hypercapnic hypoxic carotid body is one of the features of hypoxic adaptation as in the chronically hypocapnic and isocapnic hypoxic carotid bodies.

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