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Morphological changes in the rat carotid body 1, 2, 4, and 8 weeks after the termination of chronically hypocapnic hypoxia

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Summary. Morphological changes in the rat carotid bodies 1, 2, 4, and 8 weeks after the termination of chronically hypocapnic hypoxia (10% O_2 for 8 weeks) were examined by means of morphometry and immunohistochemistry. The rat carotid bodies after 8 weeks of hypoxic exposure were enlarged several fold with vascular expansion. The carotid bodies 1 and 2 weeks after the termination of 8 weeks of hypoxic exposure were diminished in size, although their diameter remained larger than the normoxic controls. The expanded vasculature in chronically hypoxic carotid bodies returned to the normoxic control state. In the carotid bodies 1 week after the termination of chronic hypoxia, the density of NPY fibers was remarkably increased and that of VIP fibers was dramatically decreased in comparison with the density in chronically hypoxic carotid bodies. In the carotid bodies 2 and 4 weeks after the termination of hypoxia, the density of SP and CGRP fibers was gradually increased. In the carotid bodies 8 weeks after the termination of hypoxia, the appearance of the carotid body returned to a nearly normoxic state, and the density of SP, CGRP, VIP, and NPY fibers also recovered to that of normoxic controls. These results suggest that the morphological changes in the recovering carotid bodies start at a relatively early period after the termination of chronic hypoxia, and a part of these processes may be under the control of peptidergic innervation.

Key words: Recovery, Termination of Hypoxia, Carotid body, Regulatory neuropeptides, Immunohistochemistry, Rat

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Introduction

The carotid bodies, which are the primary organs for sensing changes in arterial blood gases (PaO_2 and $PaCO_2$) and hydrogen ion concentration, become enlarged in rats exposed to chronic hypoxia (Heath et al., 1973; Laidler and Kay, 1975a,b; Barer et al., 1976; Kusakabe et al., 1993). Although most previous studies on the hypoxic carotid bodies have not referred to the effect of carbon dioxide levels, recent studies on the carotid bodies of the rats exposed to three types of hypoxia with different levels of carbon dioxide (systemic hypoxia: hypocapnic, isocapnic, and hypercapnic hypoxia) evaluated different levels of arterial CO_2 tension (Kusakabe et al., 1998, 2000, 2002). The systemic hypoxic carotid bodies were found to be enlarged several fold, but the degree of enlargement was different for each. The characteristic morphological changes in the systemic hypoxic carotid bodies have been summarized in a recent review (Kusakabe et al., 2003).

On the other hand, the peptidergic innervation in the systemic hypoxic carotid bodies also changes with the progress of morphological changes (Kusakabe et al., 1998, 2000, 2002). In brief, the density of neuropeptide Y (NPY) immunoreactive fibers in the hypercapnic hypoxic carotid bodies significantly increases, and that of vasoactive intestinal polypeptide (VIP) immunoreactive fibers remains unchanged, but the density of NPY and VIP fibers is reversed in hypocapnic and isocapnic hypoxic carotid bodies. These characteristic findings in systemic hypoxia suggest that the different levels of arterial CO_2 tension also change the density of peptidergic innervation during chronic hypoxic exposure. In addition, the morphological changes in the vasculature and those in the peptidergic innervation in the carotid bodies of rats exposed to

hypocapnic hypoxia for 2, 4, and 8 weeks were examined to evaluate the different levels of hypoxic exposure (Kusakabe et al., 2003). Thus, most previous and recent studies on the hypoxic carotid bodies have referred to the changes after hypoxic exposure. Recently high altitude excercise has been adopted for an improvement of sport performance. Effect of high altitude excercise is depend on both the duration of stay in high altitude and the duration after the termination of high altitude excercise. As far as we are aware, however, there are no morphological studies on the carotid bodies after the termination of chronic hypoxia except in a few instances (Heath et al., 1973). It is meaningful for high altitude exercise to make clear the morphological changes of the chemoreceptor organs during deacclimatization after chronic hypoxia is terminated.

In the present study, we examined the morphological changes in the carotid bodies, especially those in the vasculatures, 1, 2, 4, and 8 weeks after the termination of chronically hypocapnic hypoxia. We also examined the changes in the distribution and abundance of four different regulatory neuropeptides, substance P (SP), calcitonin gene-related peptide (CGRP), VIP, and NPY in the carotid bodies in each of the four periods after the termination of chronic hypoxia to evaluate the different levels of deacclimatization. Some preliminary findings have been reported elsewhere (Kusakabe et al., 2000).

Materials and methods

Hypoxic exposure and recovery periods after the termination of hypoxic exposure

Eight-week-old Wistar rats were placed in an air-tight acrylic chamber 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: total 20 L/min) into the chamber. The flow of air and N_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 was confirmed to be hypocapnic to rats in our previous study (Hirakawa et al., 1997). The rats were returned to the normoxic atmosphere after 8 weeks of hypocapnic hypoxic exposure, and were housed for 1, 2, 4, and 8 weeks in the same chamber ventilated with air. At least three rats from each of the four recovery periods were examined. The chamber was opened for 10 min every 3 days for husbandry.

All experiments with animals were performed in accordance with the "Principles of Laboratory Animal Care" (NIH publ. no. 86-23, revised 1985) and with the "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 (4% paraformaldehyde and 0.2% picric acid in 0.1M PBS) at a constant flow rate. The pair of carotid bodies was 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 16 µm on a cryostat, and mounted in four series on poly-L-lysine-coated slides.

Immunohistochemistry

The sections were processed for immunohisto-chemistry according to the peroxidase-antiperoxidase (PAP) method. The immunostaining procedure has been detailed in a previous report (Kusakabe et al., 1991). In brief, the sections were incubated at 4 °C overnight with the primary 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). The peroxidase activity was demonstrated with 3,3'-diaminobenzidine. The reaction for neuropeptides was verified by treating sections with primary antibody which had been inactivated by overnight incubation with 50-100 µM of its peptide. Some sections were also stained with hematoxylin and eosin for general histology.

Data analysis

In hematoxylin and eosin-stained sections through the center of the carotid bodies, their short and long axes and the diameter of blood vessels were measured with an ARGUS 100 computer and image processor (Hamamatsu-Photonics, Japan). The measurement was performed on 6 sections taken from 6 carotid bodies of 3 rats returned to air for periods of 1, 2, 4, or 8 weeks. The values taken from experimental carotid bodies were expressed as means ± SD (n=6), and those from normoxic control ones and from the hypoxic ones exposed for 8 weeks were also expressed as means ± SD (n=6). The number of blood vessels of seven different ranges of diameter, less than 5 µm (~5), 6-10 µm (~10), 11-15 µm (~15), 16-20 µm (~20), 21-25 µm (~25), 26-30 µm (~30), and 31-35 µm (~35), in normoxic control and hypoxic carotid bodies was expressed as a percentage of the total number of blood vessels.

The density of immunoreactive fibers in the normoxic control and experimental carotid bodies was represented as the number of varicosities per unit area (10^4 μm^2) of parenchyma. The manner of measurement has been detailed in other reports (Kusakabe et al., 1998, 2000, 2002). The number of varicosities was counted on 6 sections of carotid bodies from each of four periods after the termination of hypoxic exposure.

The values were expressed as means ± S.D., and statistical comparisons between the control and experimental values were determined using Student's *t*-test. These data were additionally analyzed by multiple comparisons among the control and experimental groups using ANOVA.

Results

General histology of the carotid bodies 1, 2, 4, and 8 weeks after the termination of chronically hypocapnic hypoxia

As previously reported (Kusakabe et al., 2003), the carotid bodies of the rats exposed to hypocapnic hypoxia for 8 weeks were found to be enlarged several fold in comparison with those of normoxic control rats, and were composed of clusters of hypertrophied glomus cells and enlarged blood vessels (Fig. 1A,B). Throughout the experiments, there were no significant changes in the animals' weight in either normoxic or hypoxic rats,

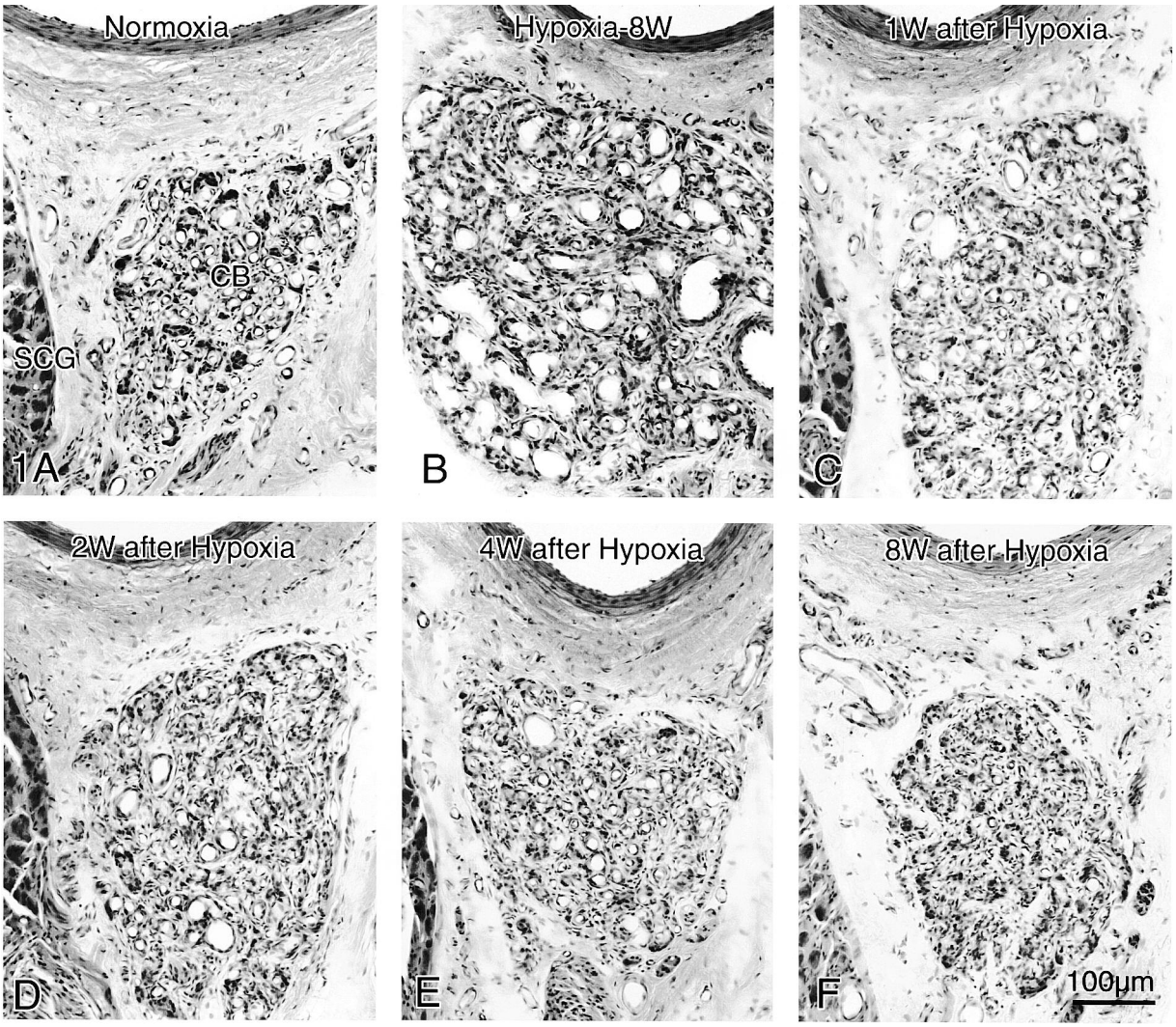


Fig. 1. Hematoxylin-eosin stained sections from the center of a control normoxic carotid body (CB) (**A**), a carotid body after 8 weeks of hypocapnic hypoxic exposure (**B**), and a carotid body 1 (**C**), 2 (**D**), 4 (**E**), and 8 (**F**) weeks after the termination of chronically hypocapnic hypoxia. The carotid bodies after the termination of chronic hypoxia were diminished in size. SCG, superior cervical ganglion.

although the weight in hypoxic animals were slightly lower than the normoxic controls. The mean short and long axes of the normoxic carotid bodies were 329.0±35.8 μm and 439.7±28.5 μm, and those of the carotid bodies of the rats exposed to hypoxia for 8 weeks were 485.2±16.7 μm and 760.9±29.9 μm, respectively, as recently reported (Fig. 2).

The carotid bodies 1, 2, 4, and 8 weeks after the termination of chronic hypoxia were diminished in size, although the rate of reduction was different for the carotid bodies from each of the four periods after the termination of hypoxia (Fig. 1C-F). The mean short and long axes of the carotid bodies 1, 2, 4, and 8 weeks after the termination of chronic hypoxia were 355.0±26.5 μm and 511.9±106.3 μm for 1 week, 344.0±25.1 μm and 502.6±104.4 μm for 2 weeks, 329.0±21.3 μm and 455.8±30.5 μm for 4 weeks, and 326.0±10.5 μm and 442.5±34.8 μm for 8 weeks (Fig. 2). The mean short axis of the carotid bodies 1, 2, 4, and 8 weeks after the termination of chronic hypoxia was 0.73 (355.0/485.2 μm), 0.71 (344.0/485.2 μm), 0.68 (329.0/485.2 μm), and 0.67 (326.0/485.2 μm) times smaller than in 8-week hypoxic carotid bodies (Fig. 2). The mean long axis was 0.67 (511.9/760.9 μm), 0.66 (502.6/760.9 μm), 0.60 (455.8/760.9 μm), and 0.58 (442.5/760.9 μm) times smaller than in 8-week hypoxic carotid bodies (Fig. 2). All values in the carotid bodies after the termination of chronic hypoxia were significantly ($p<0.01$ or $p<0.005$) smaller than those in 8-week hypoxic bodies (Fig. 2). There were no significant differences in the mean short and long axes of the carotid bodies between normoxic controls and those 1, 2, 4, and 8 weeks after the termination of chronic hypoxia (Fig. 2).

In the carotid bodies exposed to hypocapnic hypoxia for 8 weeks, about 43.6% of the blood vessels were large, with diameters greater than 16 μm, and the percentage of small vessels with diameters less than 5 μm was under 20%, as reported by Kusakabe et al. (2003) (Fig. 3). In the carotid bodies 1, 2, and 4 weeks after the termination of chronic hypoxia, the percentage of large blood vessels with diameters greater than 16 μm decreased from 43.6 to 20-25%, and that of small vessels with diameters less than 10 μm increased to over 74.0% (Fig. 3). In the carotid bodies 8 weeks after the termination of hypoxia, the percentage of large vessels with diameters greater than 16 μm further decreased to 8.2%, and that of diameters less than 5 μm further increased to 89.6%, which was a condition similar to that in normoxic control carotid bodies (Fig. 3).

Peptidergic nerve fibers in the carotid bodies 1, 2, 4, and 8 weeks after the termination of chronic hypocapnic hypoxia

Immunoreactivity of SP, CGRP, VIP, and NPY was recognized in the nerve fibers distributed throughout the parenchyma of the normoxic control and enlarged hypoxic carotid bodies (Kusakabe et al., 1998). These immunoreactive fibers appeared as thin processes with a number of varicosities (Figs. 4A,B, 7A,B). In the carotid bodies of the rats exposed to hypocapnic hypoxia for 8 weeks, parenchymal NPY varicose fibers were more numerous than SP, CGRP, and VIP fibers. VIP fibers were mainly associated with the enlarged blood vessels (Fig. 6B). As Kusakabe et al. (2003) reported, the mean density of varicosities of SP, CGRP, VIP, and NPY fibers per unit area (104 μm2) was 4.6±0.6, 10.2±0.8, 22.7±4.6, and 45.7±4.8, respectively (Fig. 8). No glomus

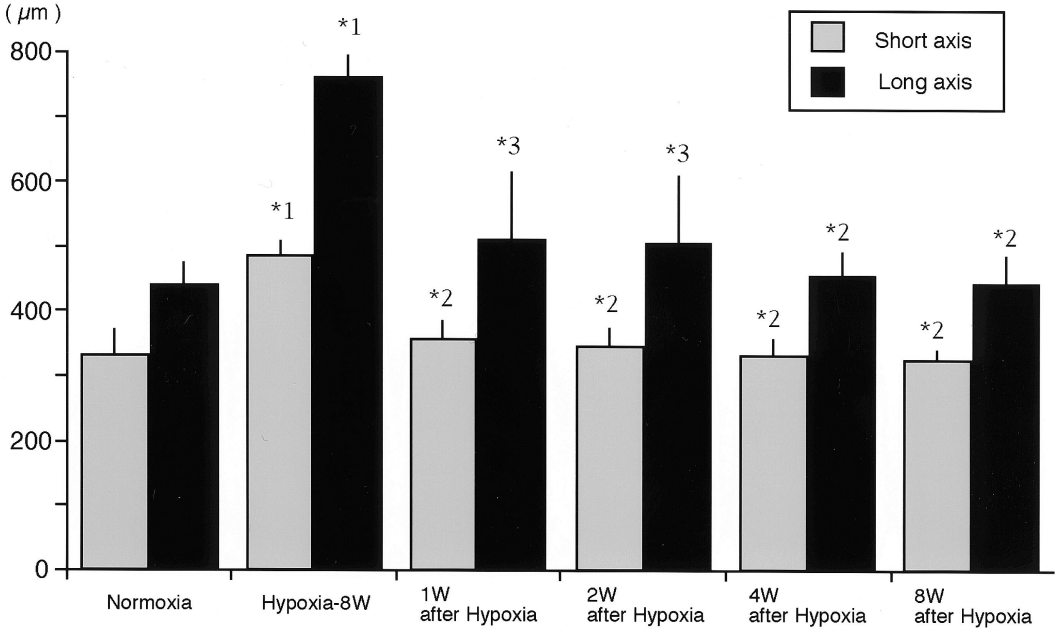


Fig. 2. Histograms comparing the short and long axes of normoxic control carotid bodies, those after 8 weeks of hypoxic exposure, and those 1, 2, 4, and 8 weeks after the termination of hypoxia. *1: $p<0.005$ in comparison with the normoxic control column. *2: $p<0.005$, and *3: $p<0.01$ in comparison with the Hypoxia-8W column.

cells with the immunoreactivity of these four neuropeptides were observed in the normoxic and hypoxic carotid bodies.

In the carotid bodies 1 week after the termination of chronically hypocapnic hypoxia, the relative abundance of NPY fibers tended to be increased (Fig. 7C) and that of VIP fibers tended to be decreased (Fig. 6C) in comparison with that of the same two fibers in the chronically hypoxic carotid bodies. When the mean density of varicosities per unit area (10^4 μm^2) was compared between the chronically hypoxic carotid bodies and those 1 week after the termination of chronic hypoxic, the density of NPY fibers was remarkably increased (p<0.005) from 45.7±4.8 to 54.7±3.8, and that of VIP fibers was dramatically decreased (p<0.005) to 15.3±4.0, which is a control normoxic level (Fig. 8). The density of SP and CGRP fibers was also significantly increased (p<0.01) although the rate of increase of these two fibers was lower than that of NPY fibers (Fig. 8).

In the carotid bodies 2 and 4 weeks after the termination of chronically hypocapnic hypoxia, the density of NPY fibers (55.3±4.8, 53.5±9.5) remained significantly increased (p<0.005, p<0.05) (Fig. 7D), and in those 8 weeks after the termination of hypoxia, the density of NPY fibers (48.5±9.3) recovered to the normoxic control level (46.6±7.1) (Fig. 8). The density of VIP fibers (14.8±3.0, 14.6±2.1) also remained to be significantly decreased (p<0.005) (Fig. 6D) as in those 1 week after the termination of hypoxia (Fig. 8). The density of SP and CGRP fibers was gradually increased (Figs. 4C, 5C), and recovered to normoxic control level (SP: 8.5±1.1, CGRP: 20.7±2.0) in the carotid bodies 8 weeks after the termination of hypoxia (Fig. 8). Thus, the density of these four neuropeptide-containing fibers in the carotid bodies 8 weeks after the termination of hypoxia recovered to that of normoxic controls, although the changes in the density of these four peptidergic fibers in the carotid bodies varied in each of the four recovery periods after the termination of chronic hypoxia.

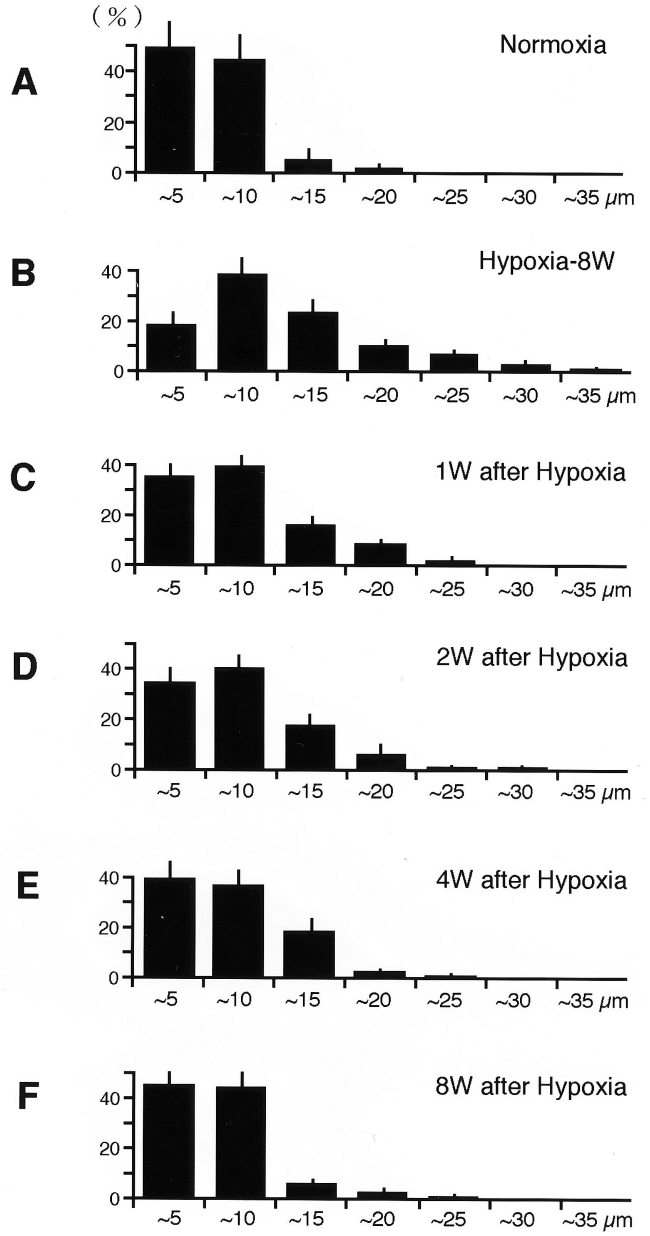


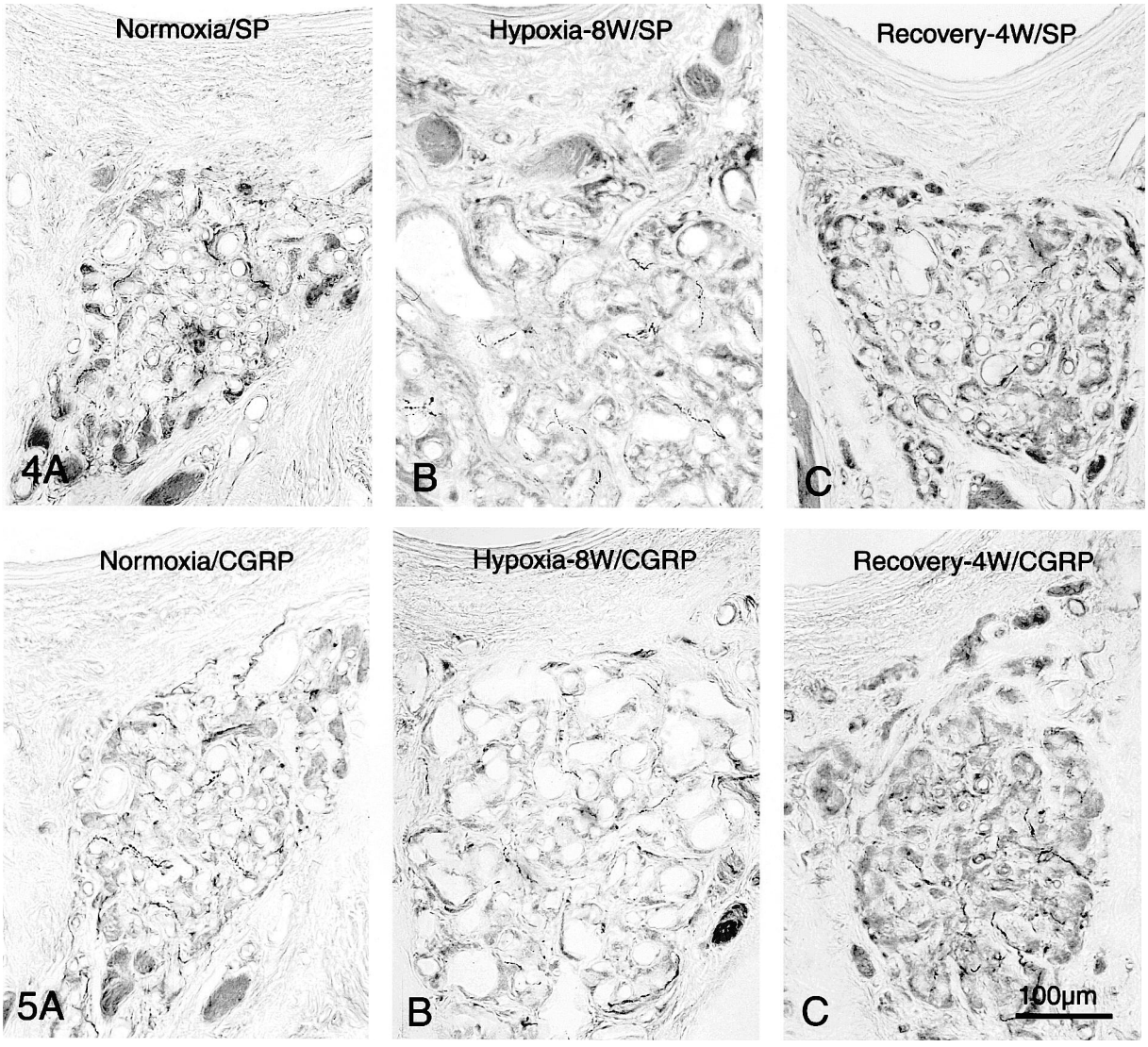
Fig. 3. Histograms representing the percentage of blood vessels of seven ranges of diameter in normoxic control carotid bodies (**A**), those after 8 weeks of hypoxic exposure (**B**), and those 1 (**C**), 2 (**D**), 4 (**E**), and 8 (**F**) weeks after the termination of hypoxia.

Discussion

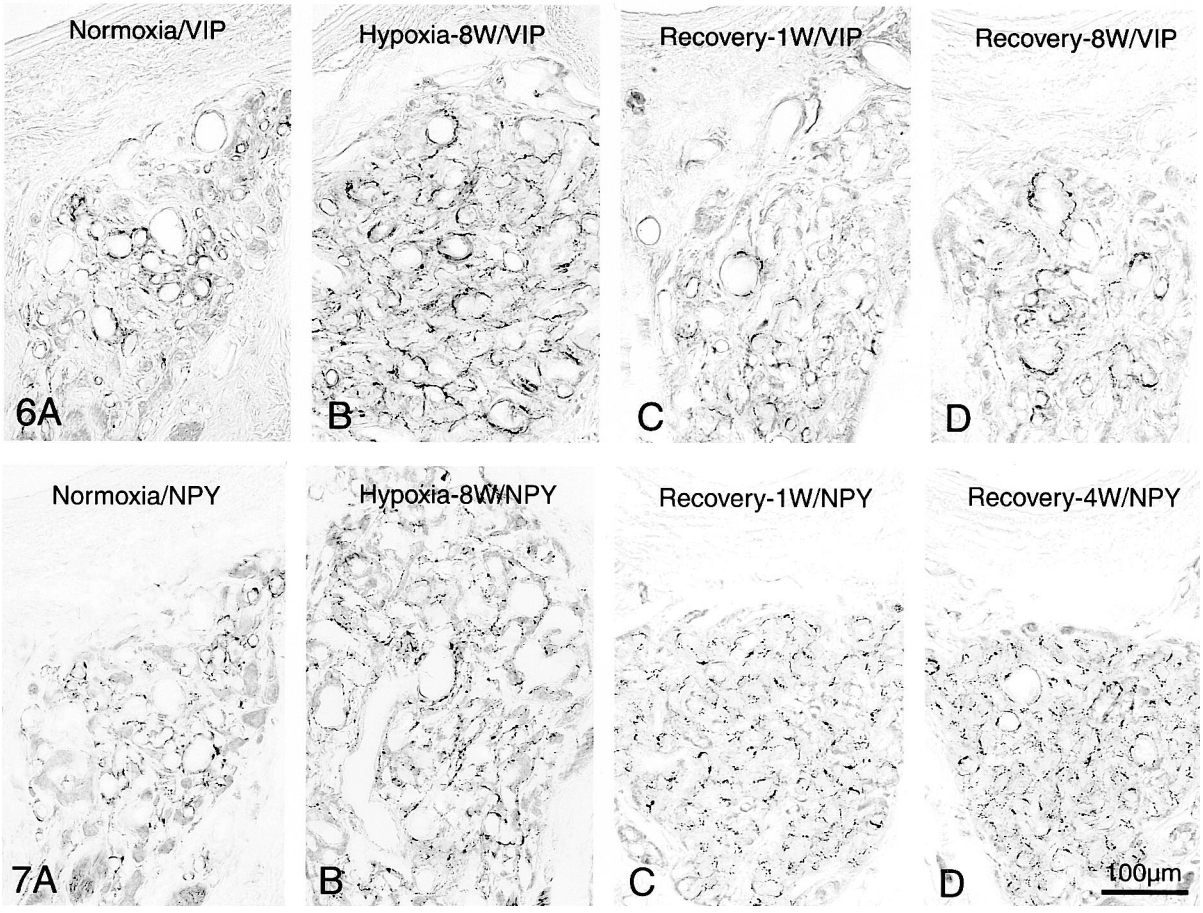
Heath et al. (1973) demonstrated that the morbid anatomical changes in the rat carotid bodies exposed for 5 weeks to a barometric pressure of 380 mmHg, equivalent to a simulated altitude of 5500 m above sea level, are almost totally reversible at 5 weeks after the termination of hypoxic exposure. From their report, we cannot estimate when the deacclimatized structural changes start. In the present study, we have shown the morphological changes in recovery stages at four different periods, 1, 2, 4, and 8 weeks after the termination of hypoxic exposure. The carotid bodies 1 week after the termination of chronic hypoxia were significantly diminished in size in comparison with the carotid bodies of the rats exposed to hypoxia for 8 weeks, and those 8 weeks after the termination of hypoxia were similar to the normoxic controls in size. In the present experimental conditions, this indicates that the recovery in the carotid bodies had already started at a relatively early period, 1 week after the termination of the chronic hypoxia, and recovered in size by 4-8 weeks after the termination of hypoxia. In the carotid bodies 1 week after the termination of the chronic hypoxia, the percentage of blood vessels with relatively wide lumens of greater than 26 μm decreased, and that of vessels with relatively narrow lumens of less than 5 μm increased.

These percentages of vasculature are similar to those in normoxic control carotid bodies. It has been suggested that the enlargement of the chronically hypoxic carotid bodies is mainly due to vascular dilation (Blessing and Wolff, 1973; Laidler and Kay, 1975a; Pequignot and Hellström, 1983). Pequignot and Hellström (1983) reported that vascular dilation is already evident in the carotid bodies after 1 week of exposure to hypoxia. Also in a study by us (Kusakabe et al., 2003), enlargement with vascular expansion was obvious in the carotid bodies after 2 weeks of hypoxic exposure. Naturally, the present study suggests that the diminution of the carotid bodies after the termination of hypoxia is due to vascular contraction. In a course of recovery, vascular contraction is also evident in the carotid bodies 1 week after the termination of hypoxia. The vasculatures in the carotid body may have high sensitivity to arterial O_2 tension. Thus, these findings, along with other recent ones, suggest that the enlargement of the carotid body with vascular dilation and its diminution with vascular contraction begin soon after the start and termination of hypoxic exposure, respectively.

The present study also demonstrated the deacclimatized changes in the distribution of several immunoreactive peptidergic nerve fibers in the rat carotid bodies during the course of recovery. After the termination of hypoxia, the most striking feature of the peptidergic innervation is immediately increased density of NPY fibers and immediately decreased density of VIP fibers in the carotid body 1, 2, and 4 weeks after the



Figs. 4, 5. SP and CGRP immunoreactive nerve fibers in a normoxic carotid body (**Figs. 4A, 5B)**, those after 8 weeks of hypoxic exposure (**4B, 5B**), and those 4 weeks after the termination of hypoxia (**4C, 5C**). After the termination of hypoxia, the density of SP and CGRP fibers gradually increased.



Figs. 6, 7. VIP and NPY immunoreactive nerve fibers in a normoxic carotid body (**6A, 7A**), those after 8 weeks of hypoxic exposure **(6B, 7B)**, and those 1 (**6C, 7C**), 4 (**8D**), and 8 (**6C**) weeks after the termination of hypoxia.

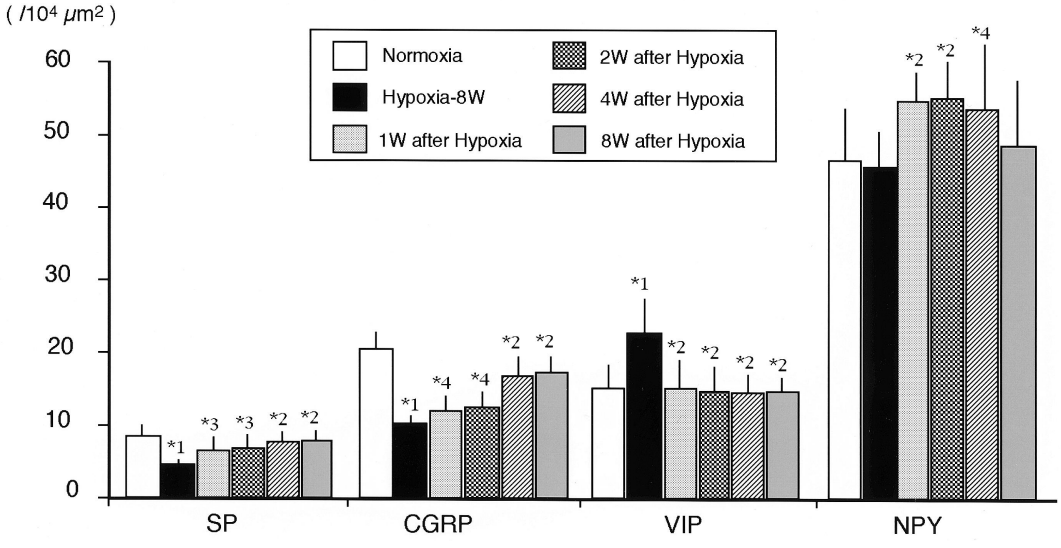


Fig. 8. Histogram comparing the density of varicosities per unit area in normoxic control carotid bodies (Normoxia), those after 8 weeks of hypoxic exposure, and those 1, 2, 4,and 8 weeks after the termination of hypoxia. *1: $p<0.005$ in comparison with the normoxic control column. *2: $p<0.005$, *: $p<0.01$, and *4: $p<0.05$ in comparison with the Hypoxia-8W column.

termination of chronic hypoxia. VIP is thought to have a long acting vasodilatory effect (Larson et al., 1976; Heistad et al., 1980; Wilson et al., 1981). As far as we are aware, there are no reports suggested that VIP is concerned in the chemoreceptor mechanisms. On this basis, we previously speculated that at least part of vascular dilation in the chronically hypoxic rat carotid bodies may depend on the vasodilatory effect of VIP, and concluded that VIP fibers are indirectly involved in chemosensory mechanisms by controlling local carotid body circulation (Kusakabe et al., 1998). Physiological significance of NPY on the chemosensory mechanisms is also not completely understood. In various mammalian vasculatures, NPY is thought to have a vasoconstrictory effect (Brain et al., 1985; Edvinsson et al., 1983; Lundberg et al., 1982). In addition to VIP fibers, NPY fibers are also involved in the regulation of local carotid body circulation (Kusakabe et al., 1998). As stated above, the percentage of blood vessels with relatively narrow lumens decreased in the carotid bodies 1 week after the termination of chronic hypoxia. It seems likely that the diminution of the carotid body with vascular contraction is caused by the increased density of vasoconstrictive NPY. At least part of the vascular constriction in the carotid bodies in recovery stages may depend on the vasoconstrictory effect of NPY.

In conclusion, the morphological changes during recovery stages begin soon after the termination of hypoxic exposure, and these processes may be under the control of peptidergic innervation.

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