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Morphological characteristics and peptidergic innervation in the carotid body of spontaneously hypertensive rats

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Summary. We examined morphological characteristics of the carotid body of spontaneously hypertensive rats (SHR), those of age-matched normotensive Wistar rats (NWR), and age-matched genetically comparable Wistar Kyoto rats (WKY). We examined the distribution and abundance of four different regulatory neuropeptides: substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), and neuropeptide Y (NPY) in the carotid bodies of these three strains of rats. The carotid bodies of SHR were larger than those of NWR and WKY. The values of the long axis of the carotid bodies of SHR were significantly larger (1.3 times) than those of NWR and WKY. In the carotid bodies of SHR, the percentage of relatively large vessels was similar to that of the carotid bodies of WKY, although the carotid bodies themselves were significantly larger than in WKY. The density of VIP varicose fibers in the carotid bodies of SHR was lower than in the carotid bodies of WKY, although the density of SP, CGRP and NPY fibers was similar to that of the carotid bodies of NWR and WKY. These findings suggested that VIP was unrelated to enlargement of the carotid body of SHR, but it might modify the sensitivity of chemoreceptors in the carotid body.

Key words: SHR, Carotid body, VIP, Substance P, and CGRP

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Introduction

The mammalian carotid bodies are bilaterally located at the carotid bifurcation and are the primary organs for sensing changes in arterial blood gases (PaO_2 and $PaCO_2$) and hydrogen ion concentration. It is generally accepted that the carotid bodies of rats become enlarged in different conditions such as hypertension (Habeck et al., 1985, 1986, 1987) and chronic hypoxia (Heath et al., 1973; Laidler and Kay, 1975a,b; Barer et al., 1976; Kusakabe et al.,1993). The volume of the carotid bodies of spontaneously hypertensive rats (SHR) is greater than that of age-matched normotensive Wistar rats (NWR) as well as age-matched genetically comparable Wistar Kyoto rats (WKY) (Habeck et al., 1985, 1986, 1987).

Recently, the remarkable structural damage in the carotid bodies of SHR does exist, characterized by an extracellular matrix expansion which is highly correlated with the blood pressure level (Milei et al., 2004). The mechanism of the extracellular matrix expansion of SHR is still unclear.

On the other hand, we have focused on hypoxia, which is one condition affecting morphology of the carotid body, and have examined changes in general morphology and peptidergic innervations in the carotid bodies of rats exposed to three types of hypoxia with different levels of carbon dioxide (systemic hypoxia: hypocapnic, isocapnic, and hypercapnic hypoxia) to evaluate the effect of arterial CO_2 tension (Kusakabe et al., 1998a,b, 2000, 2002, 2004). The carotid bodies of chronically systemic hypoxic rats were found to be enlarged several fold. In addition, the peptidergic innervation in systemic hypoxic rats shows characteristic features. The density of vasoacive intestinal polypeptide

(VIP) immunoreactive fibers in the hypocapnic and isocapnic hypoxic carotid bodies significantly increases (Poncet et al., 1994), and that of neuropeptide Y (NPY) immunoreactive fibers remains unchanged, but the density of VIP and NPY fibers is preserved in the hypercapnic hypoxic carotid bodies. A number of previous findings on hypoxic adaptation of the rat carotid body have been summarized in a recent review (Kusakabe et al., 2005).

Exogenous conditions such as hypoxia affect the morphology and peptidergic innervation of the rat carotid bodies. Therefore, we wanted to learn how an endogenous condition such as hypertension affects the morphology of the rat carotid bodies. It is meaningful to compare the morphological characteristics of enlarged carotid bodies during different pathological conditions. As far as we are aware, however, there are no precise morphological studies especially focused on the vasculature of the carotid bodies of SHR.

In the present study, we compared the morphometric features of the carotid bodies in SHR, NWR and WKY, especially those in the vasculature. We also examined the distribution and abundance of four different regulatory neuropeptides: substance P (SP), calcitonin gene related peptide (CGRP), VIP, and NPY in the carotid bodies of SHR, NWR, and WKY. In addition, antisera against tyrosine hydrohylase (TH) was applied to demonstrate the glomus cells. Finally, we compared these morphological characteristics to findings in the carotid bodies of chronically hypoxic rats.

Material and methods

Tissue preparation

Male rats of a spontaneously hypertensive (SHR) strain (n=6) were used, and the Wistar-Kyoto (WKY) strain (n=6) and normotensive Wistar (NWR) rats (n=12) were also used as controls. 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 buffer 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 cut serially at 16 µm on a cryostat, and mounted in four alternate series on poly-L-lysine-coated slides.

In all procedures we followed the experimental animal care directives of our Institute (Yokohama City University) and of the national government (Japanese NIH).

Morphometry

In hematoxylin and eosin-stained sections from 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 each of SHR, WKY, and NWR. The values taken from carotid bodies were expressed as means±S.D. (n=6), and those from normoxic controls and from the hypoxic group (exposed for 8 weeks) were also expressed as means±S.D. (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 total number of blood vessels. The values were expressed as means±S.D, and statistical comparisons between the control and experimental values were determined using Student's *t*-test.

Immunohistochemistry

The sections were processed for immunohistochemistry according to the peroxidase-antiperoxidase (PAP) method. The immunostanding 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). In each experiment, some sections were incubated with antiserum for TH (1:200; Chemicon , Temecula, 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. Serial sections were also stained with hematoxylin and eosin for general histology.

The density of the 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 is detailed in other reports (Kusakabe et al., 1998b, 2000, 2002). The number of varicosities was counted on 6 sections of carotid bodies. The values were expressed as mean±S.D., and statistical comparisons between the control and experimental values were done using Student's t-test.

Results

General histology of the carotid bodies of NWR, WKY, and SHR

In hematoxylin and eosin-stained sections from the center of the carotid bodies, the bodies of NWR and

WKY were oval in shape and were mainly composed of clusters of glomus cells and a number of blood vessels with narrow lumens (Fig. 1). The mean short and long axes of the carotid bodies in NWR were 303.2±30.1 µm and 425.6±63.3 µm, and 311.5±31.3 µm and 444.1±43.9 µm in WKY (Fig.3). There was no significant difference in the mean short and long axes of the carotid bodies between NWR and WKY. The general histology of the carotid bodies of WKY was also similar to that of NWR (Fig. 1).

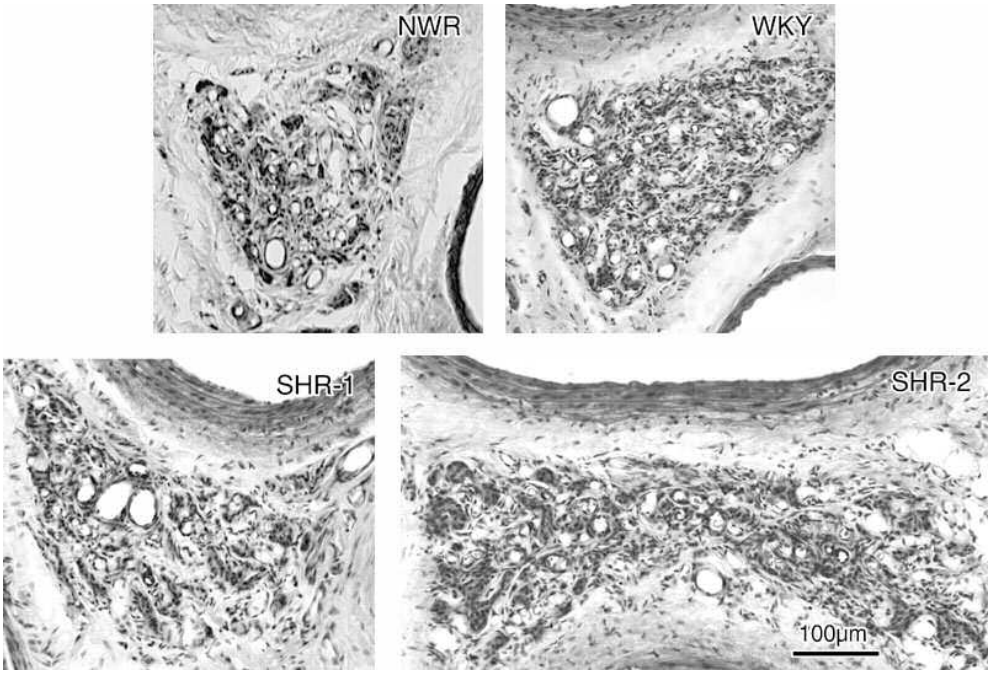

Fig. 1. Hematoxylin-eosin stained sections from the center of the carotid bodies of normotensive rats (NWR) and (WKY), and spontaneously hypertensive rats (SHR).

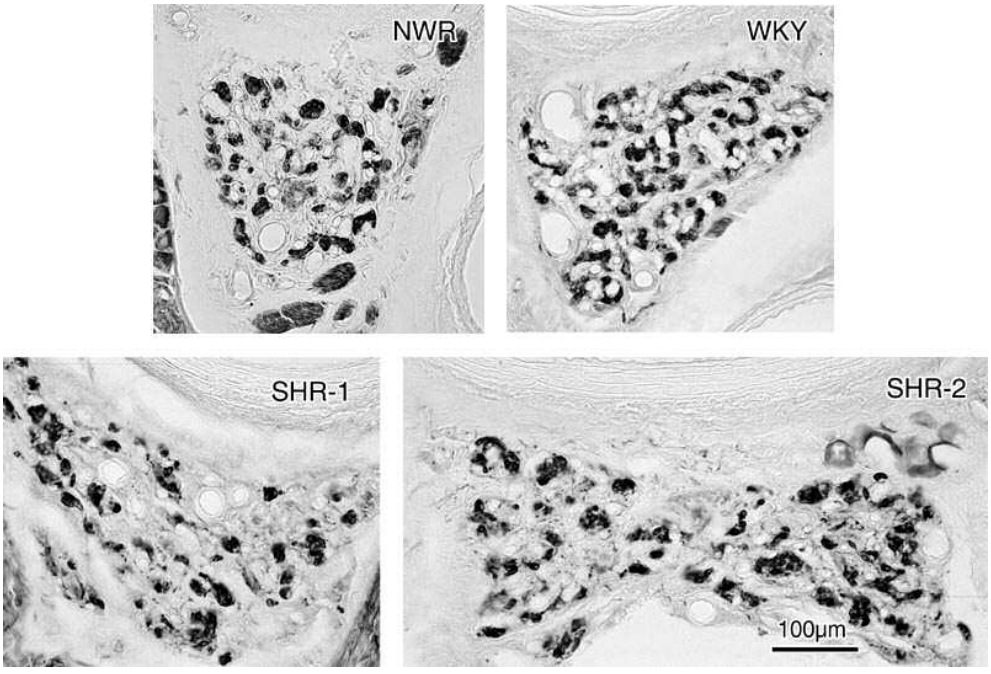

Fig. 2. TH immunoreactivity in the control normotensive carotid bodies of NWR, WKY, and a hypertensive carotid body of SHR.

The carotid bodies of SHR were larger than those of NWR and WKY (Fig. 1). Most carotid bodies were oblong in shape, and were situated the external caroid artery (Fig. 1). In the sections from the center of the carotid bodies of SHR, the mean short and long axes were 312.2±46.0 μm and 570.2±103.7 μm, respectively (Fig. 3). The values of the long axis of the carotid bodies of SHR were significantly (p<0.05) larger (1.3 times) than those of NWR and WKY, although there was no significance in the values of the short axis of the carotid bodies (Fig. 3).

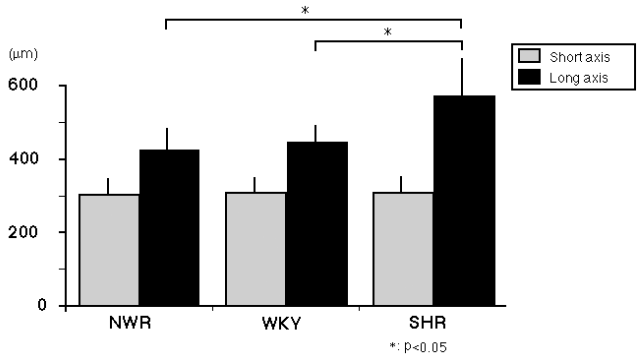

Fig. 3. Histograms comparing the short and long axes of normotensive (NWR, WKY) and hypertensive (SHR) carotid bodies. *p<0.05 in comparison with the normotensive control column.

When some serial sections were immunostained with TH antiserum, the clusters of glomus cells were immunoreactive against this antiserum. The TH immunoreactive glomus cell clusters in the carotid bodies in SHR were larger than in NWR and WKY, and the number of clusters was similar to the number in NWR and WKY (Fig. 2), although we have not yet performed a morphometric analysis.

In the carotid bodies of NWR and WKY, about 80-90% of the blood vessels were small, with diameters less than 10 μm (Fig. 4). The percentage of relatively large vessels with diameters greater than 16 μm was less than 10% in the carotid bodies of NWR and WKY (Fig 4). The percentage of vasculature in SHR was similar to the percentage in the control carotid bodies of NWR and WKY (Fig. 4).

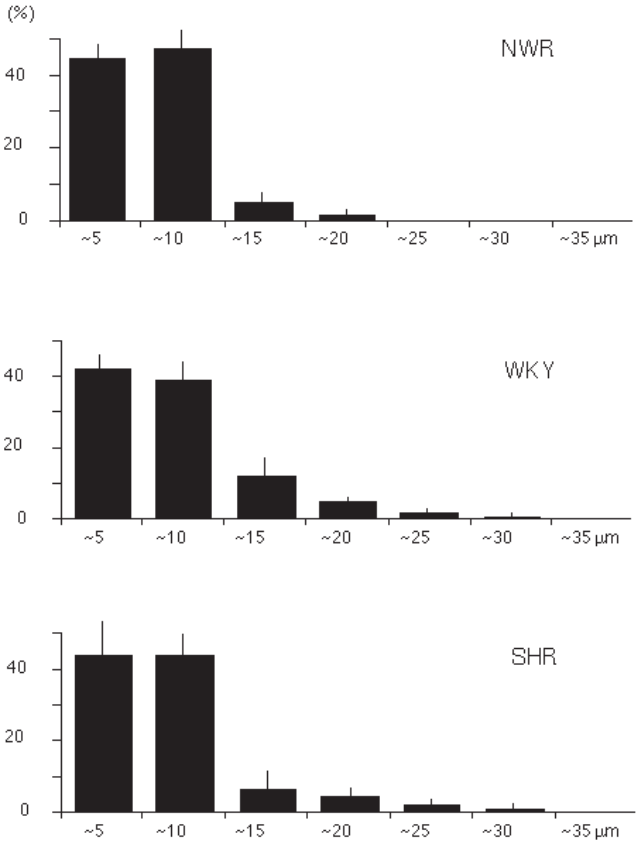

Fig. 4. Histogram representing the percentage of blood vessels of seven ranges of diameter in normotensive control carotid bodies (NWR, WKY), and t in hypertensive carotid bodies (SHR).

Peptidergic nerve fibers in the carotid bodies of NWR, WKY, and SHR

Immunoreactivity of SP, CGRP, VIP, and NPY was recognized in the nerve fibers distributed throughout the parenchyma of the control carotid bodies of NWR and WKY as previously reported by Kusakabe et al. (1998b, 2000, 2002, 2003, 2004). These immunoreactive fibers appeared as thin processes with many varicosities (Fig. 5). NPY-immunoreactive fibers were more numerous than the other three immunoreactive fibers. Most of the NPY were associated with the blood vessels within the carotid body. The mean density of varicosities of SP, CGRP, VIP, and NPY fibers per unit area (10^4 μm^2) in the carotid bodies of WKY was 5.4±0.5, 13.9±1.6, 11.3±1.8, and 44.6±4.4, respectively (Fig. 6). There was no significant difference in the density of these immunoreactive fibers in the carotid bodies between WKY and NWR.

In the carotid bodies of SHR, the mean density of varicosities of SP, CGRP, VIP, and NPY fibers per unit area (10^4 μm^2) was 4.1±0.8, 9.0±1.1, 2.4±0.8, and 29.4±2.3, respectively (Fig. 6). The density of SP (p<0.05), CGRP (p<0.05), VIP (p<0.05), and NPY fibers (p<0.05) was significantly decreased. In particular, the mean density of VIP fibers, which were mainly associated with the blood vessels, was about 0.2 (2.4/11.3) times lower than that in the control carotid bodies of WKY (Fig. 6). No glomus cells with the immunoreactivity of these four neuropeptides were observed in the carotid bodies of SHR, WKY, and NWR.

Discussion

The present study showed that the SHR carotid

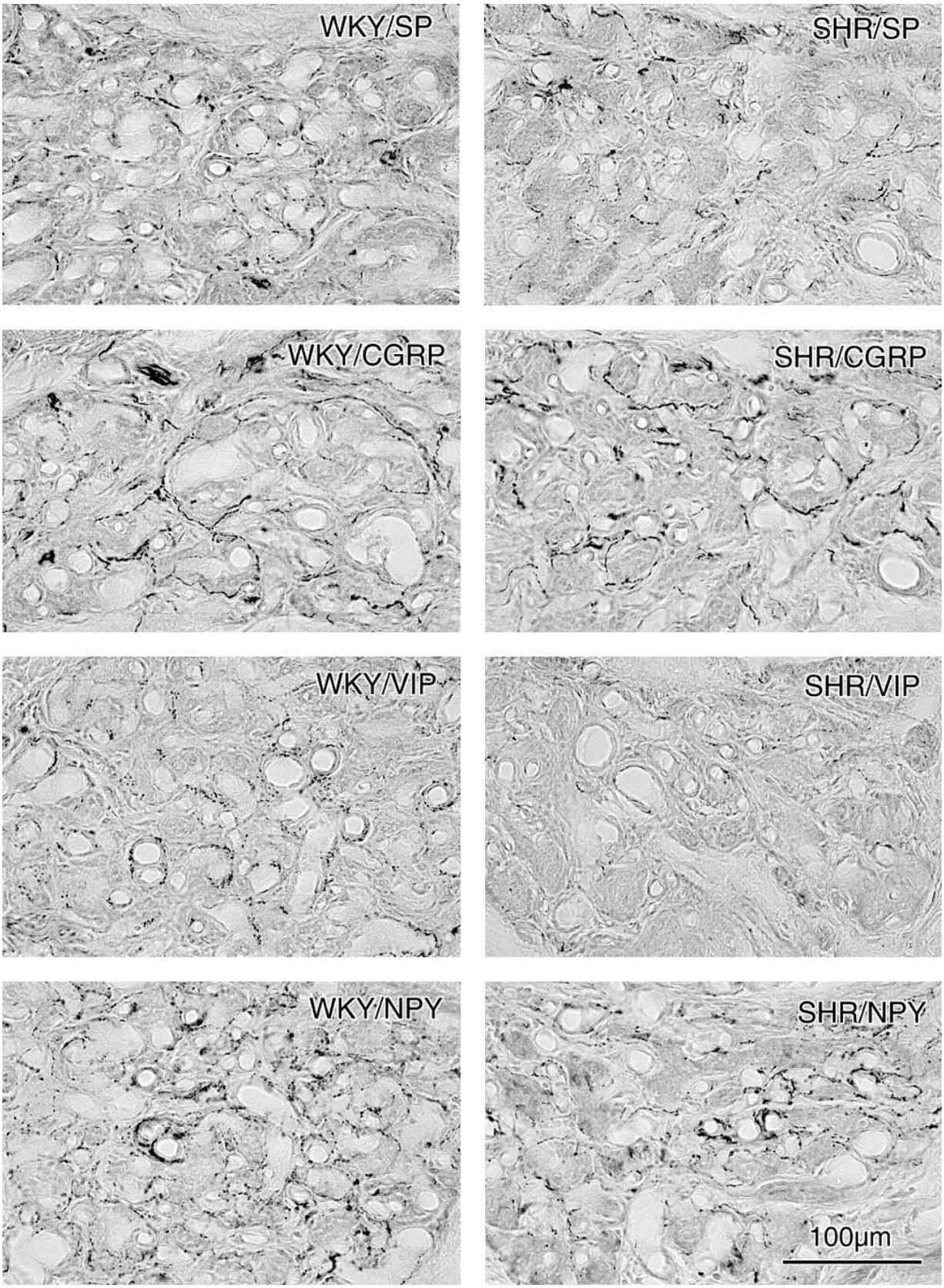

Fig. 5. SP, CGRP, VIP, and NPY immunoreactive nerve fibers in the carotid bodies of normotensive rats (WKY), and spontaneously hypertensive rats (SHR). The mean density of VIP fibers in the carotid bodies of SHR was lower than in the control carotid bodies of WKY.

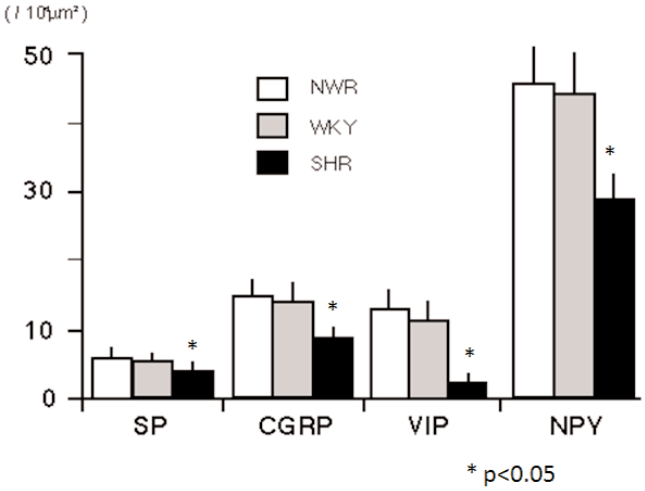

Fig. 6. Histogram comparing the density of varicosities per unit area in normotensive control carotid bodies (WKY), and those in hypertensive rats (SHR). *p<0.05 in comparison with the control column.

bodies are larger than those of NWR and WKY. According to our morphometry, the carotid bodies of SHR were significantly enlarged (1.3 times) in the mean long axis in comparison with those of NWR and WKY. In spite of the carotid body enlargement, there was no obvious vascular expansion in the carotid bodies of SHR. It has been generally accepted that the volume of the hypoxic rat carotid bodies increases several fold with vascular expansion (Heath et al., 1973; Laidler and Kay, 1975a,b; Barer et al., 1976). In our morphometry, the carotid bodies of the rats exposed to hypocapnic hypoxia for 8-12 weeks were significantly enlarged, 1.7 times in the long axis, and 1.5 times in the short axis, in comparison with normoxic control rats. The percentage of blood vessels with relatively wide lumens was increased and the percentage of relatively narrow lumens was decreased (Kusakabe et al., 1993b, 1998a,b, 2000, 2002, 2003, 2004). Thus, there was a distinct difference in the ratio of the carotid bodies, and in the ratio of vascular expansion in the carotid bodies between the SHR and chronically hypoxic rats. SHR showed a significant increase in extracellular matrix in the carotid body and autonomic ganglia compared with WKY. Recently, Milei et al. (2004) reported that severe structural damage was observed in carotid body and autonomic ganglia in relation to hypertension (Milei et al., 2004). This indicates that the mechanism of carotid body enlargement in SHR is different from that in hypoxic rats. The morphological difference of the carotid bodies between the SHR and chronically hypoxic rats may depend on the difference between endogenic and exogenic influences.

It has been reported that SP and CGRP nerve fibers in the carotid body originate from the carotid sinus nerve, whereas VIP and NPY fibers originate from the autonomic ganglia (Ichikawa, 2002). We have suggested that altered circulation caused by vascular expansion in the hypoxic carotid bodies is controlled by peptidergic innervation (Kusakabe et al., 1998b, 2000, 2002, 2003). In the hypocapnic hypoxic rat carotid bodies, the density per unit area of parenchymal VIP fibers increased significantly, whereas that of SP and CGRP fibers decreased significantly, and that of NPY fibers was unchanged in comparison with normoxic controls (Kusakabe et al., 1998, 2000, 2003). VIP is generally thought to have a long-acting, vasodilatory effect (Wilson et al., 1981). Therefore, it has been suggested that at least a part of the vascular expansion in the chronically hypoxic carotid body may depend on the vasodilatory effect of VIP. In the present study, the mean density per unit area of VIP, SP, and CGRP fibers in the carotid bodies of SHR was significantly lower than in the carotid bodies of NWR and WKY, although the degree was different for each. According to the enzyme immunoassays, the mean plasma VIP concentrations of SHR were significantly lower than that of WKY (Mori et al., 1993). Since severe damage was observed in the carotid body and in the autonomic ganglia in relation to hypertension, the amount of peptidergic innervation of the carotid body is undoubtedly changed.

The effects of VIP on carotid body chemoreception have been investigated (McQueen and Ribeiro, 1981). In their study, the spontaneous chemoreceptor discharge in the carotid body was decreased by a low dose injection of VIP. Therefore, VIP has an inhibitory effect on the chemoreception of the carotid body. In the present study, the density of VIP fibers in the carotid bodies of SHR was significantly lower than in the carotid bodies of NWR and WKY. Therefore, it seems that chemosensitivity in the carotid body of SHR is activated by the decrease of VIP fibers in the carotid body.

The carotid body chemoreceptor discharge in response to hypoxia and hypercapnia has been quantitatively compared between SHR and NWR as well as WKY (Fukuda et al., 1987). In their study, the chemoreceptor discharge in the carotid sinus nerve when exposed to hypoxia was significantly increased in SHR over NWR and WKY, while the discharge response to hypercapnea remained unchanged. They suggested that chemosensitivity to hypoxia in the carotid body of SHR was more sensitive than that of NWR and WKY. High sensitivity to hypoxia in the SHR carotid body may be caused by a decrease of VIP, which has an inhibitory effect on chemoreception in the carotid body. The present study suggests that a change of peptidergic innervation in the carotid body of SHR may modify sensitivity to hypoxia.

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