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Calbindin D-28k immunoreactive nerve fibers in the carotid body of normoxic and chronically hypoxic rats

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Summary. The distribution and ultrastructural characteristics of calbindin D-28k immunoreactive nerve fibers were examined in the carotid body of the normoxic control rats by light and electron microscopy, and the abundance of calbindin D-28k fibers in the carotid body was compared in normoxic and chronically hypoxic rats (10% O_2 and 3.0-4.0% CO_2 for 3 months). Calbindin D-28k immunoreactivity was recognized in nerve fibers within the carotid body. Calbindin D-28k immunoreactive nerve fibers appeared as thin processes with many varicosities. They were distributed around clusters of glomus cells, and around blood vessels. Immunoelectron microscopy revealed that the calbindin D-28k immunoreactive nerve terminals are in close apposition with the glomus cells, and membrane specialization is visible in some terminals. Some dense-cored vesicles in the glomus cells were aggregated in this contact region. The chronically hypoxic carotid bodies were found to be enlarged several fold, and a relative abundance of calbindin D-28k fibers was lesser than in the normoxic carotid bodies. When expressed by the density of varicosities per unit area of the parenchyma, the density of calbindin D-28k fibers associated with the glomus cells in chronically hypoxic carotid bodies was decreased by 70%. These immunohistochemical findings indicate a morphological basis for involvement of calcium binding protein in the neural pathway that modulates carotid body chemoreception.

Key words: Calbindin D-28k; Carotid body; Hypoxia; Chemoreception; Immunohistochemistry

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Introduction

The carotid bodies are the primary organs for sensing changes in arterial O_2 and CO_2 tension and hydrogen ion concentration (Biscoe, 1971; Eyzaguirre et al., 1983; Fidone and Gonzalez, 1986). In the rats exposed to chronic hypoxia, the volume of the carotid body increases by several fold with vascular expansion (Heath et al., 1973; Laidler and Kay, 1975a,b; Barer et al., 1976). The structural changes in the hypoxic carotid body may, in part, depend on the arterial O_2 tension (Kusakabe et al., 1993). This means that the chronically hypoxic carotid body can be used as a model to clarify the morphological and functional relationship in chemoreceptor mechanisms.

Recently, we reported changes in the distribution of neuropeptide-containing and nitric oxide synthase (NOS)-containing nerve fibers in the chronically hypoxic rat carotid body (Kusakabe et al., 1998a,b). The density of vasoactive intestinal polypeptide (VIP) fibers increased significantly 1.8 times, and the density of substance P (SP) and calcitonin gene-related peptide (CGRP) fibers in the chronically hypoxic carotid body decreased significantly to under 50%. The density of NOS fibers in the chronically hypoxic carotid body was also significantly decreased. These findings suggest that altered peptidergic and nitergic innervation of the chronically hypoxic carotid body are a feature of hypoxic adaptation, and the altered innervation may indicate changes in transmitter release at synaptic regions, whose processes are involved in changes in calcium concentrations in neurons.

It has been considered that the calcium binding proteins play important roles in the storage and transport of intracellular Ca^{2+} (Baimbridge and Miller, 1982). Calbindin D-28k, which was originally isolated from chick digestive tracts (Taylor, 1974), belongs to a family of calcium binding proteins (Andressen et al., 1993). The occurrence and distribution of calbindin D-28k has

been immunohistochemically demonstrated in central and peripheral neuron systems (Garcia-Segura et al., 1984; Heizmann and Hunziker, 1990; Ichikawa and Helke, 1996; Miyawaki et al., 1998). In the rat carotid body, the occurrence of calbindin D-28k immunoreactive nerve fibers has been only briefly reported (Ichikawa and Helke, 1995), and the physiological role of calbindin D-28k fibers in the carotid body has not yet been made clear.

In the present study, we examined the distribution and ultrastructural characteristics of calbindin D-28k immunoreactive nerve fibers in the carotid body of normoxic control rats, and abundance of calbindin D-28k fibers in the carotid body was compared in normoxic and chronically hypoxic rats. In particular, a possible correlation of calbindin D-28k and NOS fibers (Kusakabe et al., 1998a) in the chemoreceptor mechanism was discussed. In addition, an antiserum against tyrosine hydroxylase (TH), which is an effective immunohistochemical marker for glomus cells (Karasawa et al., 1982), was also used to demonstrate the interaction of calbindin D-28k immunoreactive fibers and glomus cells.

Materials and methods

Chronically hypoxic exposure

Six rats (Wistar) were exposed to hypoxia for 3 months with food and water available ad libitum in an air-tight acrylic chamber to which was delivered a hypoxic gas mixture (10% O_2 in N_2 and 3-4% CO_2: total 10 L/min). The flow of air, N_2, and CO_2 was regulated by a multi-flowmeter (MODEL-1203, KOFLOC, Japan), 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 CO_2 was added to the hypoxic gas mixture at the concentration of 3-4% because this much was necessary to maintain an arterial partial pressure of CO_2 close to 34-36 mm Hg. The temperature within the chamber was maintained at 25 °C. This hypoxic condition (PO_2, 50.5±1.6 mm Hg; PCO_2, 35.4±1.3 mm Hg) was confirmed to be isocapnic to rats in a previous study (Hayashida et al., 1996). Six control rats (PO_2, 94.2±2.3 mm Hg; PCO_2, 34.3±0.8 mm Hg) were housed for three months in an identical chamber ventilated by air at the same flow rate. The chamber was opened for 10 min every 3 days for animal 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 0.2% picric acid and 4% paraformaldehyde in 0.1M PBS (Zamboni's fixative solution) 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 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-100 in 0.1M PBS (PBST), the sections were treated for 30 min with a protein blocking agent (Immunon, USA) at room temperature to block nonspecific protein binding sites. Then they were incubated at 4 °C overnight with rabbit polyclonal antiserum against calbindin D-28k (1:1500; Chemicon, USA). In each experiment, some sections were incubated with rabbit polyclonal antiserum against TH (1:200; Chemicon, USA) to demonstrate the 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, 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. Next the sections were rinsed with several changes of PBS, transferred for 2 h to rabbit PAP (Jackson Immuno Res., 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 (DAB). 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.

The protocol for immunoelectron microscopy was identical to that for the light microscopy as described above. After DAB reaction, the immunostained sections were postfixed with 1% OsO_4 buffered with 0.1M phosphate buffer for 1 h at room temperature. Following dehydration with ethanol, the sections were embedded in a mixture of Epon-Araldite. Ultrathin sections were serially cut and briefly stained with uranyl acetate. They were examined with a Hitachi H-7500 electron microscope.

Control

The reaction for calbindin D-28k was verified by treating sections with primary antibody which had been

inactivated by overnight incubation with an excess amount of recombinant calbindin D-28k (Sigma, USA).

Data analysis

As we performed previously (Kusakabe et al., 1998a,b), 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 image processor (Hamamatsu-Photonics, Japan) on 50 sections taken from fifteen normoxic carotid bodies and 50 sections taken from fifteen chronically hypoxic carotid bodies of each of the nine animals examined, and the number of varicosities was counted. The values per unit area (10^4 μm^2) of parenchyma, excluding the area of the vascular lumen, were expressed as mean±SD (n=50). Statistical comparisons between the control and experimental values were determined using Student's *t*-test.

Results

Normoxic carotid body

Immunoreactivity of TH was recognized in the glomus cell clusters distributed in the parenchyma of the carotid body (Fig. 1A). In the normoxic carotid bodies, adjacent sections immunostained with calbindin D-28k antiserum showed a large number of calbindin D-28k immunoreactive nerve fibers around and within the clusters of glomus cells and around the small arteries and arterioles (Fig. 1B). They appeared as thin processes with many punctate varicosities. This observation of two serial sections was useful to confirm the location of calbindin D-28k fibers and the glomus cell clusters. When expressed by the density per unit area (10^4 μm^2) of the parenchyma in the normoxic carotid bodies, the mean density of varicosities in calbindin D-28k fibers associated with glomus cells and with vasculature was 5.18±1.01 (n=50) and 2.71±0.63/10^4 μm^2 (n=50), respectively (Fig. 7). The density of calbindin D-28k

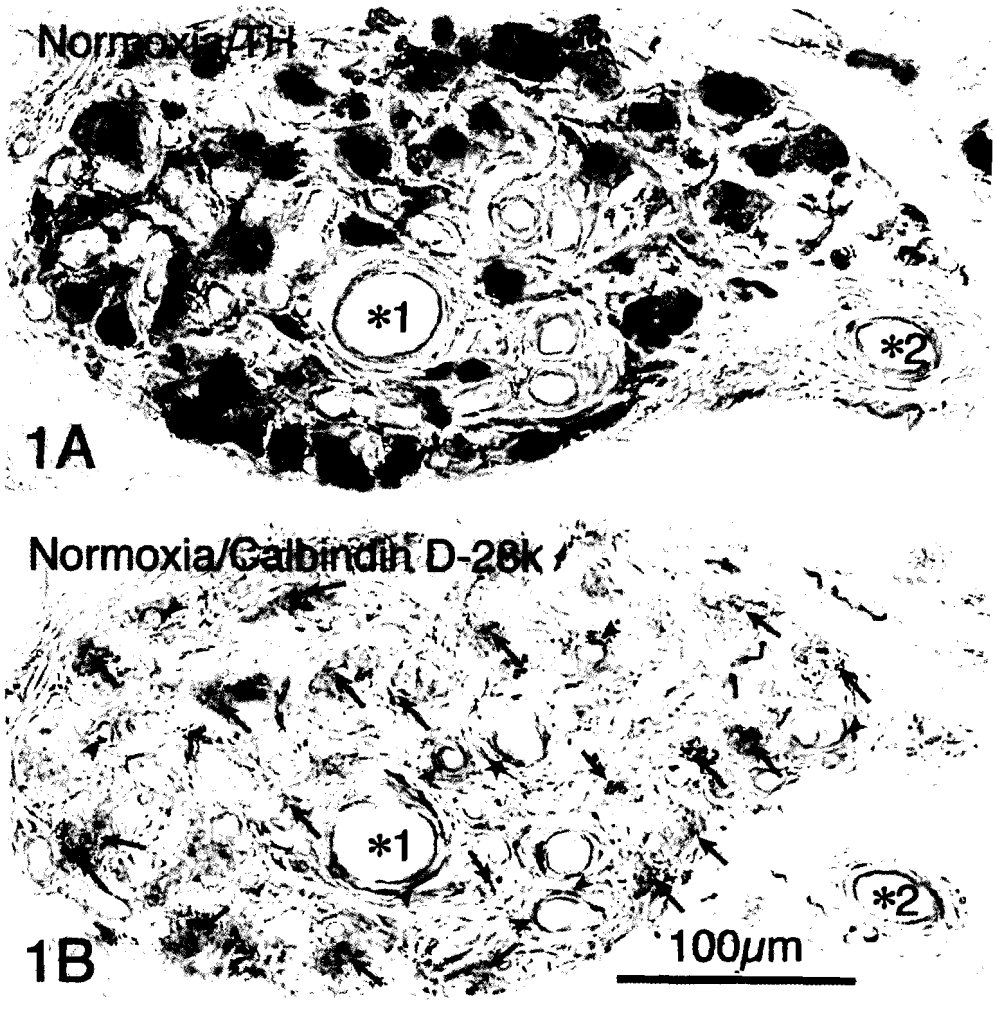
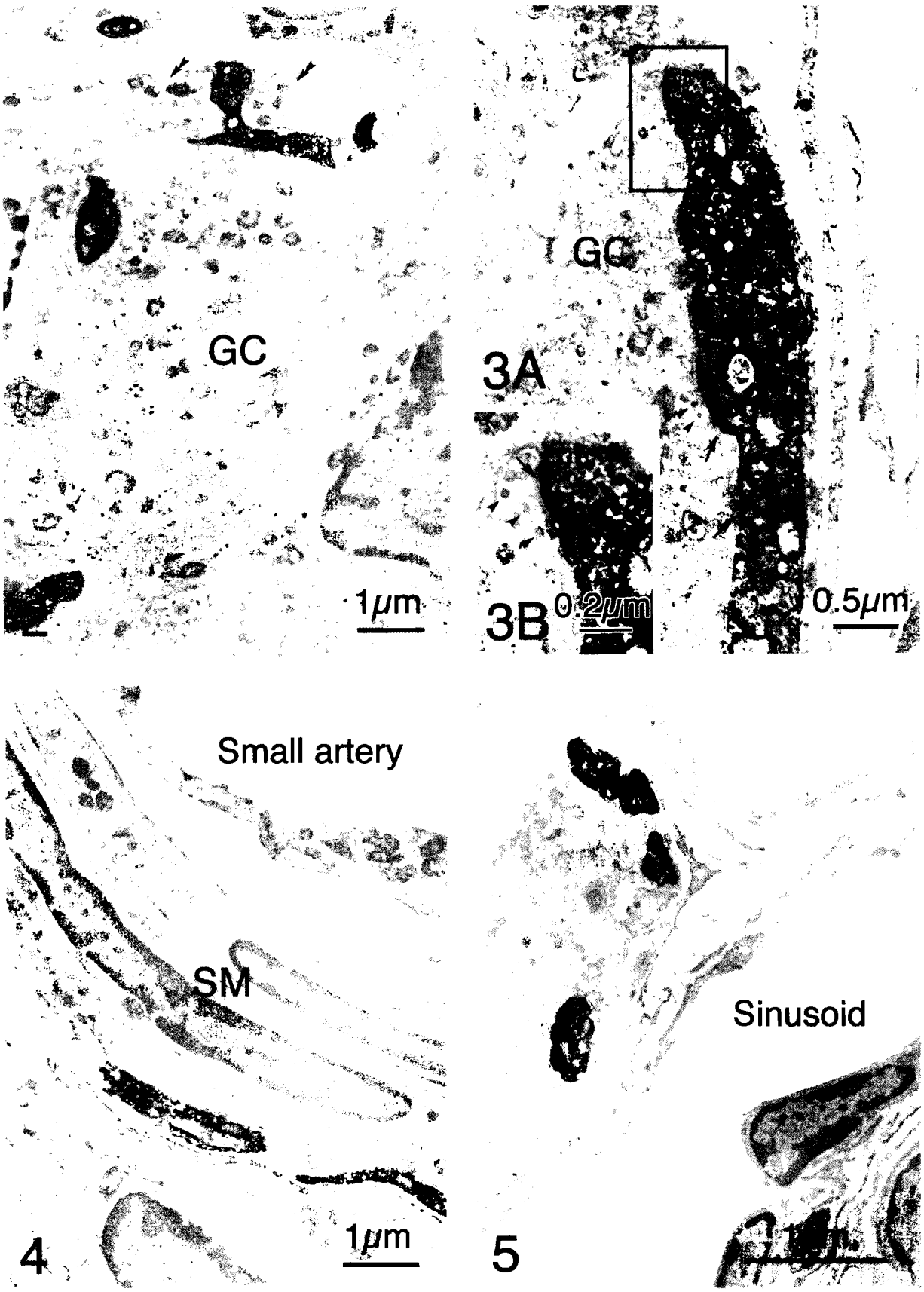


Fig. 1. Serial sections of a control normoxic rat carotid body stained with TH **(A)** and calbindin D-28k antisera **(B)**. The area of TH immunoreactive glomus cell clusters corresponds to that of calbindin D-28k immunoreactive varicose fibers (arrows in B). A number of calbindin D-28k fibers are associated with the glomus cells (arrows) and vasculature (arrow-heads). Two asterisks in A show a small artery and an arteriole corresponding to those in B.



Figs. 2-5. Immunoelectron micrographs of calbindin D-28k immunoreactive terminals associated with the glomus cells **(Figs. 2-3)**, and blood vessels **(Figs. 4-5)**. Two double arrowheads in Figure 2 indicate calbindin D-28k immunonegative terminals. In high magnification (Fig. 3), membrane specialization (arrow) and aggregation of dense-cored vesicles (arrowhead) are visible. Fig. 3B, enlargement of area in 3A. GC: glomus cell; SM: smooth muscle.

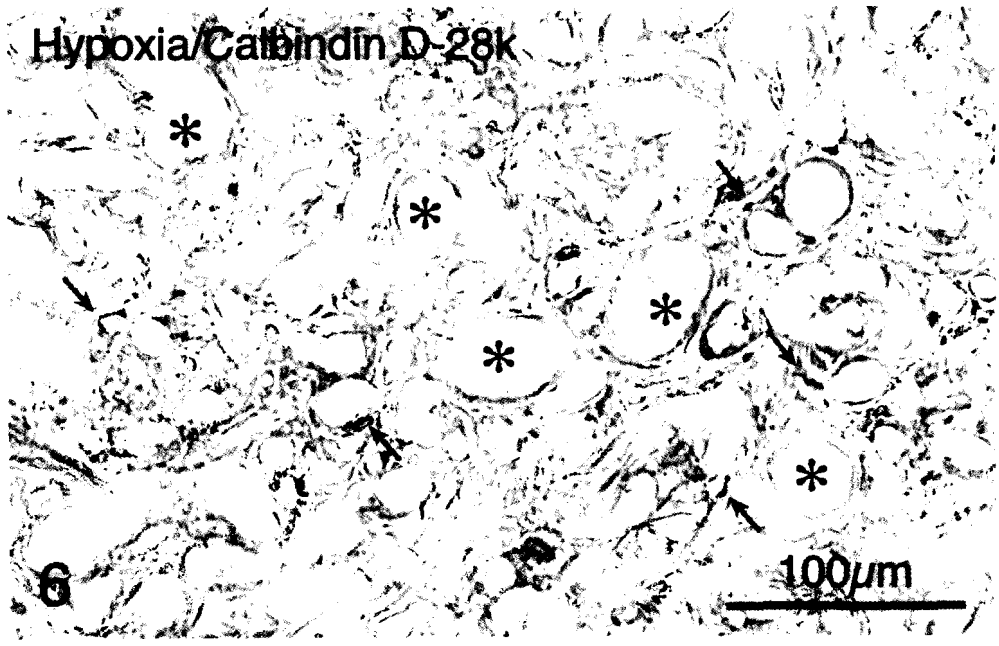


Fig. 6. Decreased density of calbindin D-28k immunoreactive fibers (arrows) in a chronically hypoxic carotid body with expanded blood vessels (*).

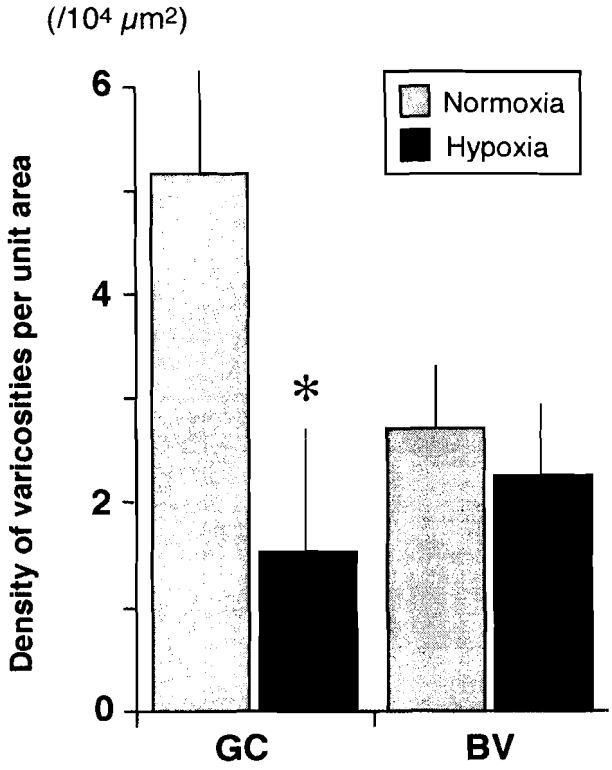


Fig. 7. Histogram comparing the density of varicosities per unit area associated with glomus cells (GC) and blood vessels (BV) in normoxic and chronically hypoxic carotid bodies. *: $p<0.005$.

fibers associated with the glomus cells was about 1.9 (5.18/2.71) times higher than that with the vasculature (Fig. 7).

At the ultrastructural level, immunoreactive products for calbindin D-28k were identified as electron-dense deposits. Immunoreactive nerve terminals containing mitochondria, vesicles, and others were observed around the glomus cells (Fig. 2) and small arteries and sinusoids (Figs. 4, 5). Between some immunoreactive terminals and the glomus cells, membrane thickenings were recognized in the terminal sides, and some dense-cored vesicles had accumulated near these synaptic regions of the glomus cell sides (Fig. 3A,B). Some calbindin D-28k immuno-negative nerve terminals associated with the glomus cells were also observed (Fig. 2).

Hypoxic carotid body

The chronically hypoxic carotid bodies were found to be enlarged several fold in comparison with the normoxic carotid bodies as we previously reported (Kusakabe et al., 1993, 1998a,b). The enlarged carotid bodies contained many expanded blood vessels in a maze-like configuration (Fig. 6). Although the distribution pattern of calbindin D-28k fibers in the chronically hypoxic carotid bodies was similar to that in the normoxic carotid bodies, a relative abundance of immunoreactive fibers was smaller than in the normoxic carotid bodies. The mean density of varicosities in calbindin D-28k fibers associated with the glomus cells was significantly ($p<0.005$) smaller from 5.18±1.01 ($n=50$) to 1.53±0.63/10^4 μm^2 ($n=50$) (Fig. 7). There was no significant decrease in the density of calbindin D-28k fibers associated with the vasculature in the normoxic and chronically hypoxic carotid bodies. The ratio of calbindin D-28k fibers associated with the glomus cells in the chronically hypoxic carotid bodies vs. those in the normoxic control carotid bodies was about 0.3

(1.53/5.18) (Fig. 7).

Discussion

The occurrence of calbindin D-28k immunoreactive nerve fibers in the normoxic rat carotid bodies was consistent with the brief report of Ichikawa and Helke (1995). The present study provided a more precise distribution of calbindin D-28k fibers by using sets of two adjacent sections of the normoxic control carotid bodies, one immunostained with anti-TH serum and the other with anti-calbindin D-28k serum. The calbindin D-28k fibers were mainly found to be associated with the glomus cells and blood vessels, and the density of calbindin D-28k fibers associated with the glomus cells was about two times higher than that in the vasculature. We also examined the calbindin D-28k immunoreactivity by electron microscopy. Two ultrastructural characteristics, the membrane specialization between some calbindin D-28k immunoreactive nerve terminals and the glomus cells, and the aggregation of dense-cored vesicles in this synaptic region suggest an involvement of calbindin D-28k in the chemoreceptor mechanisms of the carotid body.

The present immunohistochemical study compared, for the first time, the distribution and abundance of calbindin D-28k immunoreactive fibers in the carotid body between normoxic control and chronically hypoxic rats. The density of calbindin D-28k immunoreactive nerve fibers associated with the glomus cells in chronically hypoxic rat carotid bodies was significantly lower (about 30%) than in the normoxic control carotid bodies. Altered density of calbindin D-28k-containing fibers in the chronically hypoxic carotid body may indicate a feature of acclimatization during chronic hypoxic exposure. Recently, in the same hypoxic carotid body, Kusakabe et al. (1998a) reported a similar decrease (about 23%) in the density per unit area of NOS immunoreactive nerve fibers, which originated in the glossopharyngeal petrosal ganglion (Wang et al., 1993). Many neurons in the petrosal ganglion showed the coexistence of calbindin D-28k and nicotinamide adenosine dinucleotide phosphate (NADPH) diaphorase, a histochemical marker for NOS (Ichikawa and Helke, 1996). This led us to suppose the coexistence of calbindin D-28k and NOS in the nerve fibers associated with the glomus cells in the carotid body. To clarify this, it is necessary to perform the double-labelling immunohistochemistry.

In the carotid body, it has been suggested that nitric oxide (NO) directly released from the axonal terminals of sensory C-fibers to adjacent glomus cells inhibits hypoxic activity in the normoxic carotid body through a cGMP second messenger system (Wang et al., 1993). On this basis, we recently suggested that the chemosensory mechanisms in the chronically hypoxic carotid body may be involved in 'disinhibition' resulting from reduced NO synthesis (Kusakabe et al., 1998a). The similar reduction in the density of calbindin D-28k and NOS-containing fibers in the chronically hypoxic carotid body and the possibility of coexistence of these two substances may indicate an interaction of these two substances during acclimatization to hypoxia. This shows that calbindin D-28k may be involved in NO release at terminals by modulating the calcium concentrations in neurons. Actually, it has been suggested that centrifugal nerve activity in the axonal terminals of sensory C-fibers in the carotid body activates NOS via peripheral axon reflexes, which are involved in a Ca^{2+}-dependent mechanism (Wang et al., 1995). Therefore, the present immunohistochemical findings of calbindin D-28k suggest an involvement of calcium binding protein for NOS activation in the axonal terminals of sensory C-fibers.

The present findings regarding the immunoelectron microscopical characteristics of calbindin D-28k immunoreactive terminals and the decreased density of calbindin D-28k fibers in the chronically hypoxic carotid body suggest a morphological basis for involvement of calcium binding protein in the neural pathway that modulates carotid body chemoreception.

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