Histol Histopathol (1999) 14: 735-741

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Changes in the distribution of the substance P and calcitonin gene-related peptide immunoreactive nerve fibers in the laryngeal mucosa of chronically hypoxic rats

T. Yoshida[1], H. Matsuda[1], Y. Hayashida[2], Y. Gono[3], T. Nagahara[1], T. Kawakami[4], T. Takenaka[5], M. Tsukuda[1] and T. Kusakabe[3]

[1]Department of Otorhinolaryngology, Yokohama City University School of Medicine, Yokohama, Japan,

[2]Department of Systems Physiology, University of Occupational and Environmental Health, Kitakyushu, Japan,

[3]Department of Anatomy, Yokohama City University School of Medicine, Yokohama, Japan,

[4]Department of Physiology, Kitasato University school of Medicine, Sagamihara, Japan and

[5]Department of Physiology, Yokohama City University School of Medicine, Yokohama, Japan

Summary. The distribution and abundance of substance P (SP) and calcitonin gene-related peptide (CGRP) immunoreactive nerve fibers in four different regions of the laryngeal mucosa were compared between normoxic and chronically hypoxic rats (10% O_2 and 3.0-4.0% CO_2 for 3 months). In the chronically hypoxic laryngeal mucosa, the number of SP and CGRP fibers within and just beneath the epithelium, and around the laryngeal gland was increased in comparison with those in the normoxic controls. Especially in the epiglottic and arytenoid regions, the number of intraepithelial SP fibers was increased remarkably. Most intraepithelial SP and CGRP fibers penetrated into the epithelium to extend to the luminal surface. There was no distinct difference in the distribution and abundance of these peptidergic fibers in the mucosa of the normoxic and chronically hypoxic vocal cord regions. These results suggest that the increased density of SP and CGRP fibers within the epithelium of the upper laryngeal mucosa is a predominant feature of hypoxic adaptation, and this may be involved in airway protection, swallowing, and other functions in the chronically hypoxic environment. In addition, the increased SP and CGRP fibers around the laryngeal gland suggest an enhanced mucous secretion, and this may participate in the airway defense mechanism in low O_2 conditions.

Key words: Larynx, Hypoxia, Neuropeptides, Immunohistochemistry, Rat

Offprint requests to: Dr. Tatsumi Kusakabe, Department of Anatomy, Yokohama City University, School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236, Japan. Fax: 81-45-787-2509. e-mail: tatsumik@med.yokohama-cu.ac.jp

Introduction

The larynx is a major interface between a potentially hostile air environment and the lung. The occurrence and distribution of substance P (SP) and calcitonin gene-related peptide (CGRP) immunoreactive nerve fibers in the airway mucosa have been reported in various mammals. In the airway system, intraepithelial SP- and CGRP-containing fibers are generally accepted to be involved in the sensory systems (Jancso et al., 1967; Lundberg et al., 1984; Uddman et al., 1985; Cadieux et al., 1986; Domeiji et al., 1991; Tanaka et al., 1993), and are considered to be related to local defense mechanisms concerned in vasomotion and glandular secretion (Lundblad, 1984). Chronic hypoxic exposure can cause various changes in the respiratory system. For example, ventilation is increased with chronic hypoxia, and this can be partly explained by changes in the afferent component of ventilatory chemoreflexes (Vizek et al., 1987).

Recently, Kusakabe et al. (1996) and Matsuda et al. (1998) reported that the density of SP and CGRP fibers significantly increases in the nasal and tracheal mucosa of chronically hypoxic rats. This indicates that increased peptidergic innervation in the upper and lower airway mucosa may be a predominant feature of sensory adaptation to chronic hypoxia. This leads us to suppose that a similar adaptation occurs in the laryngeal mucosa, because the larynx is a potent reflexogenic area of the upper airway which receives abundant sensory innervation.

In the present study, the distribution and relative abundance of SP and CGRP immunoreactive nerve fibers in the laryngeal mucosa were compared between normoxic and chronically hypoxic rats, and a possible

role of peptidergic innervation in a low O_2 environment is discussed.

Materials and methods

Chronic hypoxic exposure

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 flowmeter (MODEL-1203, KOFLOC, Japan) to deliver a hypoxic gas mixture (10% O_2 in N_2 and 3-4 % CO_2: total 10 L/min) into the chamber. 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 that measured during the control period. The flow of air, N_2, and CO_2 was regulated by the flowmeter and the O_2 and 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 isocapnic to the rats in our previous study (Hayashida et al. 1996). Nine rats were exposed chronically in this chamber for three months with food and water available *ad libitum*. Nine 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 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). Anesthetized animals were perfused through the heart with heparinized saline, followed by Zamboni's fixative solution, 0.2% picric acid and 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS) at pH 7.4. A laryngeal segment 1 cm long was dissected out, and postfixed in the same fixative solution for 24 h. After a brief washing in PBS, the specimens were transferred to 30% sucrose in PBS at 4 °C for 24 h. They were cut serially at 16 μm on a cryostat, and mounted in three 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 to inhibit endogenous peroxidase activity. After washing in 0.3% Triton X-100 in 0.1M PBS (PBST), sections were treated for 1 h with protein blocking agent (Immunon, Pittsburgh, USA) at room temperature to block nonspecific protein binding sites. Then they were incubated at 4 °C for 24 h with rabbit polyclonal antisera against SP (1:1500; Cambridge Research Biochemicals, Northwich, UK), and CGRP (1:1500; Cambridge Research Biochemicals, Northwich, UK). After rinsing in 3 changes of PBST, sections were transferred for 3 h to anti-rabbit IgG (Organo Technica, Durham, USA) diluted to 1:200 with PBST at room temperature. Then they were rinsed in 3 changes of PBS, and were transferred for 1.5 h to rabbit PAP complex (Jackson Immuno Research, West Grove, USA) diluted 1:200 with PBS at room temperature. After rinsing in 3 changes of PBS, preparations were transferred to 0.1M Tris-HCl buffer at pH 7.8 for 10 min, and then 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.

Controls

The reaction for SP and CGRP was verified by treating sections with primary antiserum which had been inactivated by overnight incubation with 50-100 μM of its respective peptide.

Results

To compare the immunoreactivity for SP and CGRP in the normoxic and chronically hypoxic rats, the segment of the larynx was classified into four regions: the laryngeal surface of the epiglottis (Fig. 1A); the arytenoid region (Fig. 1B); the dorsal wall of the ventricle; and the membranous part of the vocal cords (Fig. 1C). Immunoreactivity for SP and CGRP was recognized in the nerve fibers distributed in the mucosa of these four regions, although there were some differences in the distribution and abundance of immunoreactive fibers. These immunoreactive fibers had many varicosities. In the laryngeal mucosa of the normoxic control rats, many or a moderate number of SP and CGRP fibers formed a plexus just beneath the basement membrane in the epithelium of the epiglottis (Fig. 2A, 2C), arytenoid region (Fig. 3A, 3C), and dorsal wall of the ventricle (Fig. 4A, 4C), and some branches penetrated into the epithelium to extend to the luminal surface. In these regions, CGRP fibers within the epithelium were more numerous than SP fibers. Subepithelial SP and CGRP immunoreactive fibers were less numerous in the membranous part of the vocal cords (Fig. 5A, 5C) than in the other three regions, and there were no intraepithelial immunoreactive fibers in this region (Fig. 5A, 5C). Some SP and CGRP fibers were also observed around the laryngeal gland in the

Table 1. Distribution and relative abundance of SP and CGRP fibers in the normoxic and hypoxic larynx.

	SP		CGRP	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Epiglottis. Laryngeal side				
IE	+	+++	++	+++
SE	+++	++++	+++	++++
Arytenoid				
IE	+	++++	+++	++++
SE	++	+++	++	+++
Ventricle. Dorsal wall				
IE	±	+	+	++
SE	+	+++	++	+++
Vocal cord				
IE	–	–	–	–
SE	+	+	+	+
Laryngeal gland	+	+++	+	+++

Grading of frequency of immunoreactive nerve fibers: ± , very few; +, few; ++, moderate number; +++, many; ++++, abundant. IE: intraepithelium; SE: subepithelium.

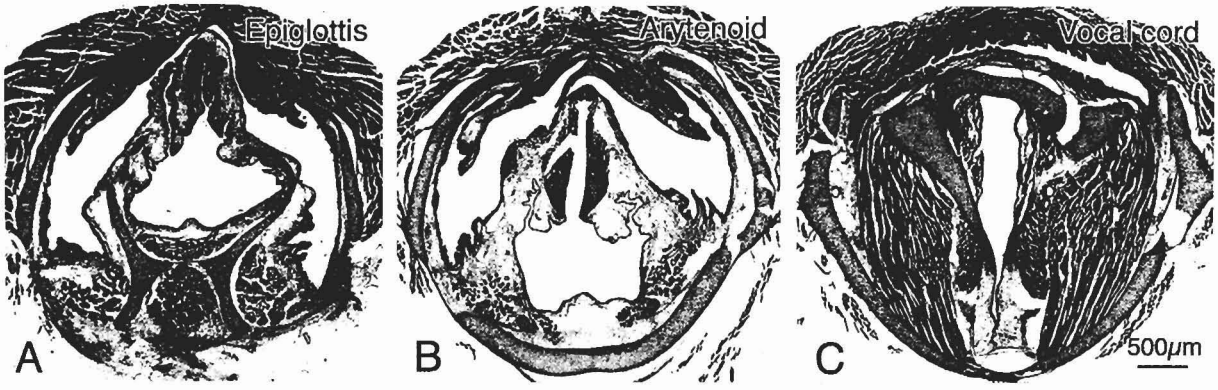

Fig. 1. Hematoxylin-eosin-stained sections showing three different regions, the epiglottis **(A)**, arytenoid **(B)**, and vocal cords **(C)**, of the normoxic larynx.

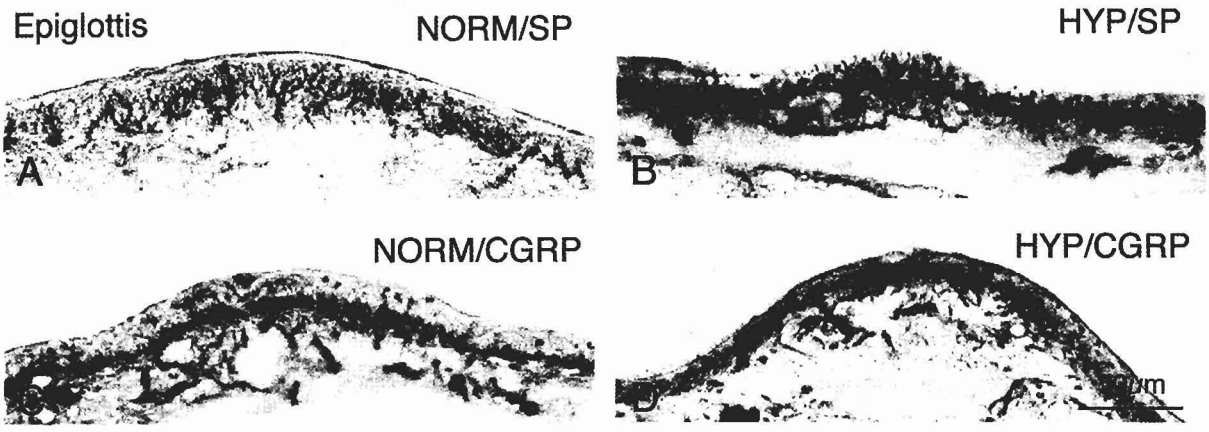

Fig. 2. SP and CGRP immunoreactive nerve fibers in the laryngeal surface of the epithelium of the normoxic (Norm, **A and C**) and chronically hypoxic (HYP, **B and D**) epiglottis. Note the remarkable increase in the number of intraepithelial SP fibers in the chronically hypoxic epiglottis.

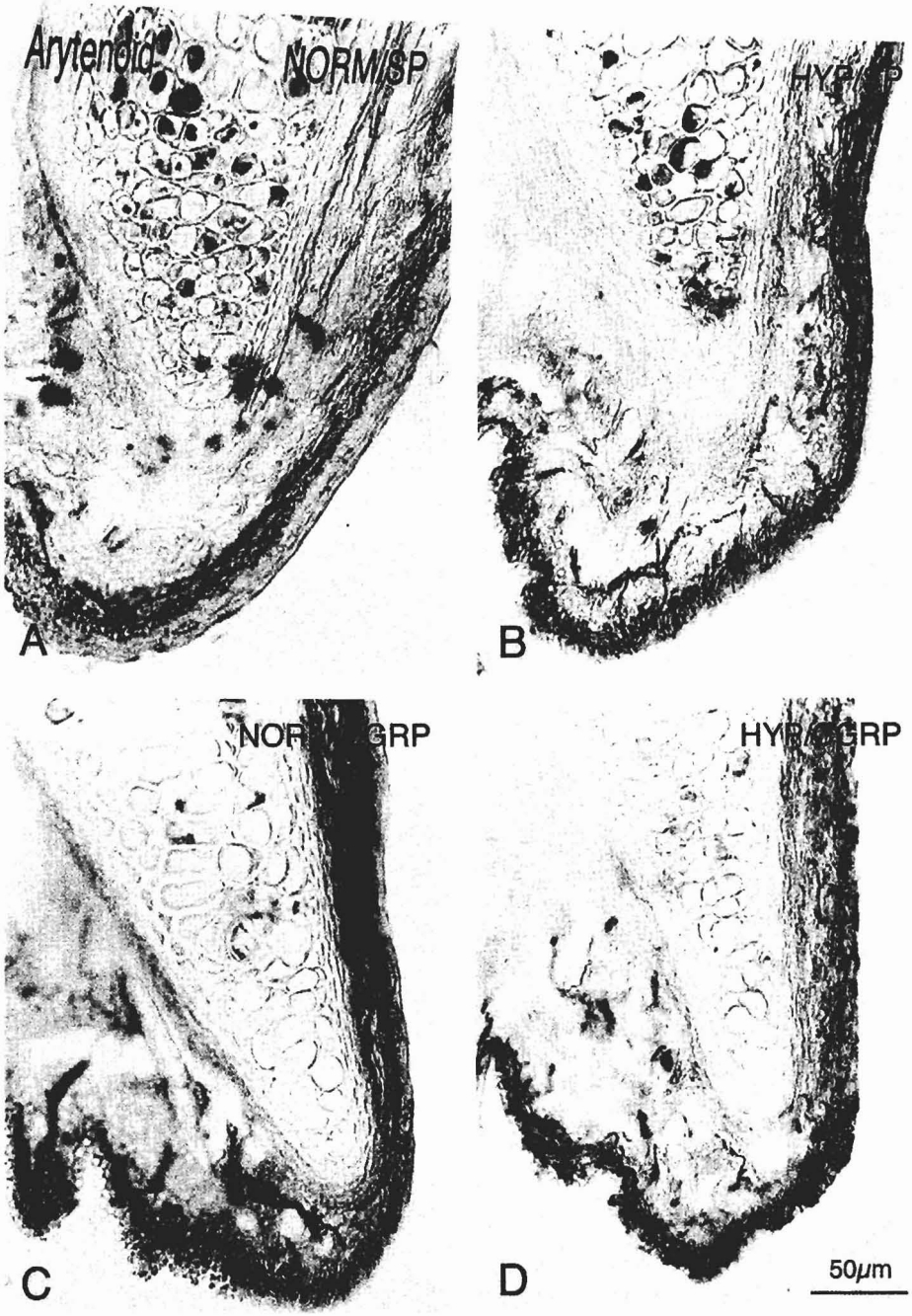

Fig. 3. SP and CGRP immunoreactive fibers in the arytenoid region of the normoxic (Norm, **A and C**) and chronically hypoxic (HYP, **B and D**) larynx. The number of SP and CGRP fibers within the epithelium of the chronically hypoxic arytenoid region is increased remarkably.

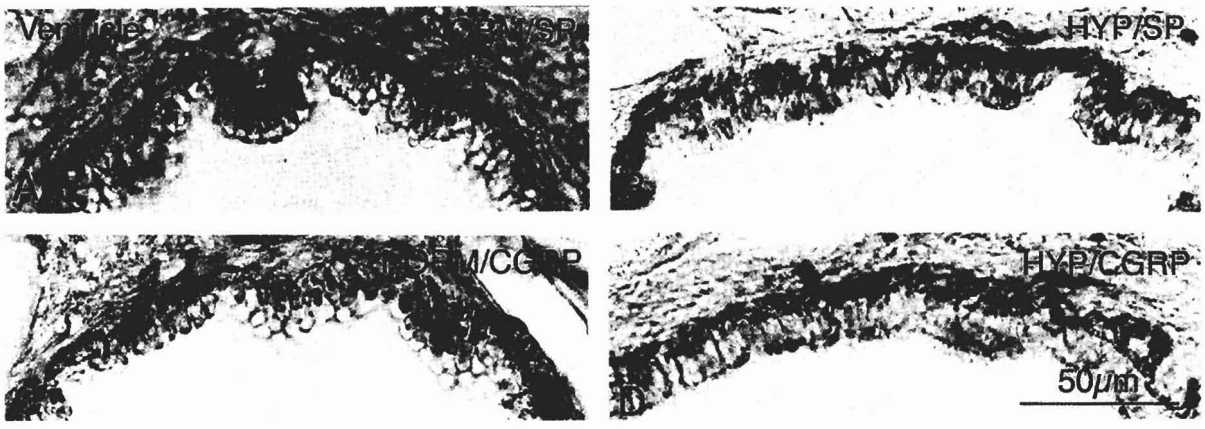

Fig. 4. SP and CGRP immuno-reactive fibers in the epithelial region of the normoxic (Norm, **A and C**) and chronically hypoxic (HYP, **B and D**) dorsal wall of the ventricle.

submucosa (Fig. 6A, 6C). The relative abundance of the intra- and subepithelial SP and CGRP immunoreactive fibers of normoxic laryngeal mucosa is summarized in Table 1.

In the chronically hypoxic laryngeal mucosa, intra- and subepithelial SP and CGRP fibers were more numerous than in the normoxic mucosa (Figs. 2B-4B). Especially in the epiglottis and arytenoid region, the number of intraepithelial SP fibers was remarkably increased, and most intraepithelial SP and CGRP fibers extended almost to the luminal surface (Figs. 2B, 3B). There was no distinct difference in the abundance of SP and CGRP fibers in the membranous part of the vocal cords between the normoxic and chronically hypoxic larynx. The number of SP and CGRP fibers around the laryngeal gland in the chronically hypoxic mucosa was also increased in comparison with that in the normoxic mucosa (Fig. 6B, 6D). The relative abundance of SP and CGRP immunoreactive fibers of chronically hypoxic laryngeal mucosa is also summarized in Table 1.

Discussion

Recently, Kusakabe et al. (1996) and Matsuda et al.

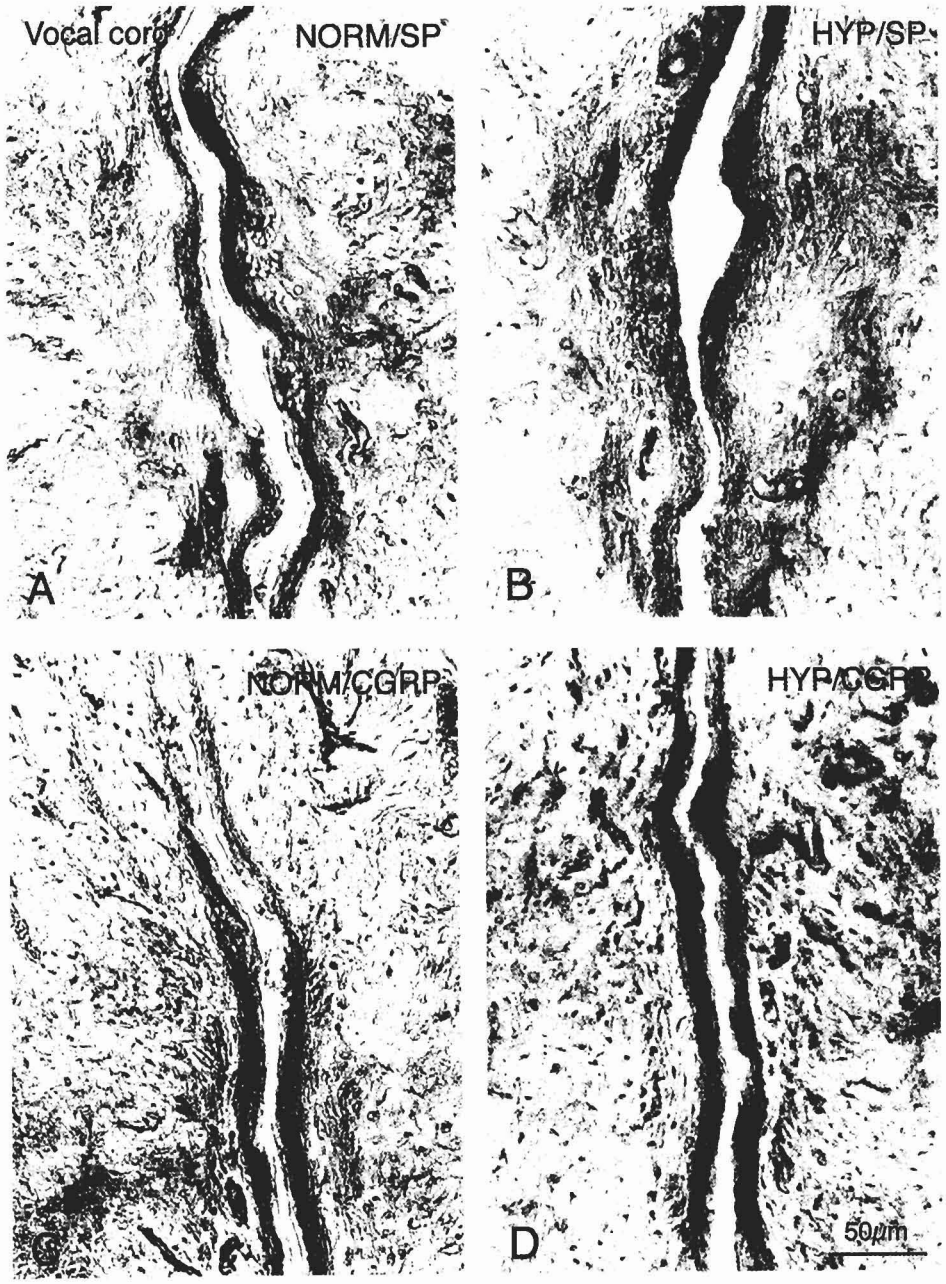

Fig. 5. SP and CGRP immunoreactive fibers in the membranous part of the normoxic (Norm, **A and C**) and chronically hypoxic (HYP, **B and D**) vocal cords. There are no conspicuous changes in the abundance of immunoreactive fibers.

(1998) reported that the density of intraepithelial SP- and CGRP-containing fibers in the chronically hypoxic rat tracheal and nasal mucosa was almost 1.5-3.0 times greater than in normoxic controls, and suggested that the increase in the intraepithelial SP and CGRP fibers may have resulted from increased branching of the nerve fibers just beneath the epithelium. In addition to the rat tracheal and nasal mucosa, the present study clearly showed the increase in the relative abundance of SP and CGRP fibers in the chronically hypoxic rat laryngeal mucosa. Especially in the laryngeal surface of the hypoxic epiglottis and arytenoid region, which are important for airway protection and respiration (Murano et al., 1993), the increase in the number of SP and CGRP fibers was conspicuous. Considered together with the previous findings in the chronically hypoxic rat tracheal (Kusakabe et al., 1996) and nasal mucosa (Matsuda et al., 1998), the increased density of intraepithelial SP- and CGRP-containing fibers suggests that this is a predominant feature of hypoxic adaptation throughout the upper and lower respiratory tracts. Because the intraepithelial SP- and CGRP-containing fibers in the respiratory tracts have been generally considered to be of a sensory nature (Jancso et al., 1967; Uddman et al., 1985; Cadieux et al., 1986), the sensory mechanisms in the laryngeal mucosa may be more sensitive during chronic hypoxia. Fluid and tactile stimuli of the larynx elicit many reflexes including apnea, swallowing, and cough (Widdicombe, 1986). Thus, the larynx is an important segment for several defense mechanisms. We believe that the increased sensitivity may take part in these physiological functions in a low O_2 environment.

Especially in the epithelium of the laryngeal surface of the epiglottis and the arytenoid region, the increase in number of SP fibers was greater than in CGRP fibers. This is also in agreement with the recent findings in the chronically hypoxic rat tracheal (Kusakabe et al., 1996) and nasal mucosa (Matsuda et al., 1998). It is generally thought that SP and CGRP coexist in the sensory nerve fibers in the epithelium of the respiratory tracts (Martling et al., 1988; Dey et al., 1990; Grunditz et al., 1994). On this basis, in the hypoxic rat nasal mucosa, we recently proposed that this difference in the rate of increase may depend on increased transcription of SP in the intraepithelial CGRP fibers, or may depend on increased translation of SP enhanced by chronic hypoxia (Matsuda et al., 1998). A similar mechanism may be present in the chronically hypoxic larynx.

In contrast, SP and CGRP fibers were less numerous in both normoxic and chronically hypoxic vocal cord mucosa, and there were no intraepithelial SP and CGRP fibers. These findings may be explained by two previous studies which reported that the membranous part of the vocal cords is a vibratory structure in which no delicate sensory innervation is necessary (Hisa et al., 1992), and that SP and CGRP fibers are not involved in phonation (Murano et al., 1993). Therefore, the changes in the peptidergic innervation of the chronically hypoxic larynx may be restricted to the upper area for airway protection, respiration, swallowing, etc. To make this clear, it is necessary to perform further physiological studies of the chronically hypoxic larynx.

In addition to the changes in the abundance of SP and CGRP fibers in the epithelial regions, their number around the laryngeal gland was also increased. The secretion from the mucous gland is an important defense

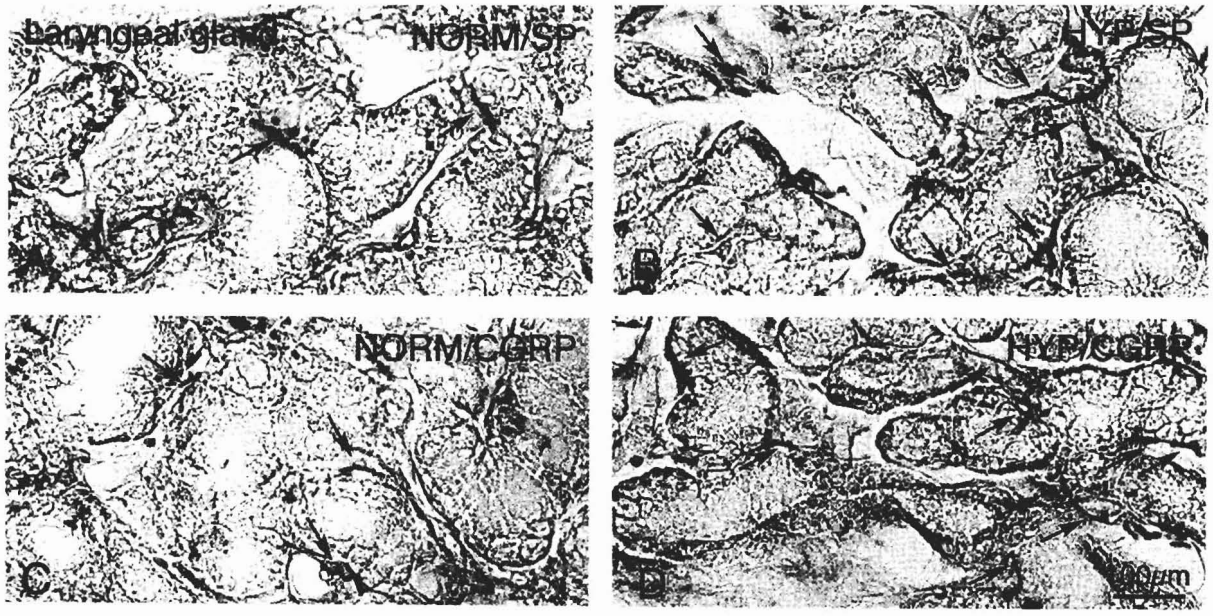

Fig. 6. SP and CGRP fibers around the laryngeal gland in the submucosa of the normoxic (Norm, **A and C)** and chronically hypoxic (HYP, **B and D**) larynx. Arrows indicate the immunoreactive fibers.

mechanism for respiratory tracts. The secretion is controlled by an axon reflex (Lundblad, 1984), and a systemic administration of SP causes an increase in the secretion from the laryngeal submucosal glands (Haxhiu et al., 1991). On this basis, the present increased density of SP fibers around the laryngeal gland in the chronically hypoxic larynx strongly suggests that the secretion from the mucous gland is elevated in low O_2 conditions, although it is not yet clear whether CGRP is involved in the mucous secretion or not.

In conclusion, the increased density of SP- and CGRP-containing nerve fibers within the epithelium of the laryngeal surface of the epiglottis, the arytenoid region, and the dorsal wall of the ventricle is a predominant feature of hypoxic adaptation, and the raised peptidergic innervation may be involved in airway protection, swallowing, and other functions in a chronically hypoxic environment. In addition, the increased SP and CGRP fibers around the mucous gland may participate mainly in the airway defense mechanism.

Acknowledgements. We wish to thank Ms. K. Haraguchi of the Department of Systems Physiology, University of Occupational and Environmental Health for hypoxic animal husbandry. We are also grateful to Dr. R.C. Goris of the Department of Anatomy, Yokohama City University School of Medicine, for his help in editing the manuscript. This study was supported by Grants-in aid 08670028 and 09670022 from the Ministry of Education, Science, Sports, and Culture, Japan.

References

Cadieux A., Springall D.R., Mulderry P.K., Rodrigo J., Ghatei M.A., Terenghi G., Bloom S.R. and Polak J.M. (1986). Occurrence, distribution and ontogeny of CGRP immunoreactivity in the rat lower respiratory tract: Effect of capsaicin treatment and surgical denervations. Neuroscience 19, 605-627.

Dey R.D., Altemus J.B., Zervos I. and Hoffpauir J. (1990). Origin and colocalization of CGRP- and SP-reactive nerves in cat airway epithelium. J. Appl. Physiol. 68, 770-778.

Domeiji S., Dahlqvist Å. and Forsgren S. (1991). Regional differences in the distribution of nerve fibers showing substance P- and calcitonin gene-related peptide-like immunoreactivity in the rat larynx. Anat. Embryol. 183, 49-56.

Grundizt T., Uddman R. and Sundler F. (1994). Origin and peptide content of nerve fibers in the nasal mucosa of rats. Anat. Embryol. 189, 327-337.

Haxhiu M.A. Cherniack N.S. and Strohl K.P. (1991). Reflex responses of laryngeal and pharyngeal submucosal glands in dogs. J. Appl. Physiol. 71, 1669-1673.

Hayashida Y., Hirakawa H., Nakamura T. and Maeda M. (1996). Chemoreceptors in autonomic responses to hypoxia in conscious rats. Adv. Exp. Med. Biol. 410, 439-442.

Hisa Y., Uno T., Tadaki N., Murakami Y., Okamura H. and Ibata Y. (1992). Distribution of calcitonin gene-related nerve fibers in the canine larynx. Eur. Arch. Otorhinolaryngol. 249, 52-55.

Jancso N., Jancso-Gabor A. and Szolcsanyl A. (1967). Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. Br. J. Clin. Pharmacol. 31, 138-151.

Kusakabe T., Anglade P. and Tsuji S. (1991). Localization of substance P, CGRP, VIP, neuropeptide Y, and somatostatin immunoreactive nerve fibers in the carotid labyrinths of some amphibian species. Histochemistry 96, 255-260.

Kusakabe T., Kawakami T., Powell F.L., Ellisman M.H. and Takenaka T. (1996). Distribution of substance P and calcitonin gene-related peptide immunoreactive nerve fibers in the trachea of chronically hypoxic rats. Brain Res. Bull. 39, 335-339.

Lundberg J.M., Hökfelt T., Martling C.-R., Saria A. and Cuello C. (1984). Substance P- immunoreactive sensory nerves in the lower respiratory tract of various mammals including man. Cell Tissue Res. 235, 251-261.

Lundblad L. (1984). Protective reflexes and vascular effects in the nasal mucosa elicited by activation of capsaicin-sensitive substance P-immunoreactive trigeminal neurons. Acta Physiol. Scand. Suppl. 529, 1-42.

Martling C.R., Saria A., Fisher J.A., Hökfelt T. and Lundberg J.M. (1988). Calcitonin gene-related peptide and the lung: neuronal coexistence with substance P, released by capsaicin and vasodilatory effect. Regul. Pept. 20, 125-139.

Matsuda H., Kusakabe T., Hayashida Y., Furukawa M., Kawakami T., Takenaka T. and Tsukuda M. (1998). Substance P- and calcitonin gene-related peptide-containing nerve fibers in the nasal mucosa of chronically hypoxic rats. Brain Res. Bull. 45, 563-569.

Murano K., Ngaotepprutaram P., Chung S.-K. and Furuta S. (1993). Distribution of the calcitonin gene-related peptide and substance P in the monkey larynx. Acta Otolaryngol. Suppl. (Stockh) 506, 75-79.

Tanaka Y., Yoshida Y., Hirano M., Morimoto M. and Kanaseki T. (1993). Distribution of SP- and CGRP-immunoreactivity in the cat's larynx. J. Laryngol. Otol. 107, 522-526.

Uddman R., Luts A. and Sundler F. (1985). Occurrence and distribution of calcitonin gene-related peptide in the mammalian respiratory tract and middle ear. Cell Tissue Res. 241, 551-555.

Vizek M., Pickett C.K. and Weil J. (1987). Increased carotid body hypoxic sensitivity during acclimatization to hypobaric hypoxia. J. Appl. Physiol. 63, 2403-2410.

Widdicombe J.G. (1986). Reflexes from the upper respiratory tract. In: Handbook of physiology. Volume II. Control of Breathing, Part 1. Fishman A.P. (ed). Waverly Press. Baltimore. pp 363-394.

Accepted November 24, 1998