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Specific localization of manserin peptide in the rat carotid body

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ABSTRACT

The carotid body, located at the bifurcation of the common carotid artery, is a small sensory organ that detects changes in oxygen concentration and plays a vital role in controlling respiration. Although several molecules, such as neurotransmitters and neuropeptides, are involved in the regulation of the respiratory system, their detailed mechanisms have not been established yet. This study identifies that the presence of manserin, a neuropeptide, in the carotid body may play a crucial role in regulating respiration.

The carotid bodies of adult Wistar rats were perfused with paraformaldehyde, and the frozen sections were subjected to immunohistochemical analyses. The carotid body comprises two distinct types of cells, neuron-like glomus cells and glial-like sustentacular cells. We used specific antibodies to distinguish the specific location of manserin in the carotid body, which included a tyrosine hydroxylase-positive antibody for glomus cells and an S100 protein antibody for sustentacular cells. Immunofluorescence analysis revealed that while tiny, round signals were exclusively observed in the cytoplasm of glomus cells, no signals were observed in sustentacular cells.

Because manserin is believed to be secreted from precursor proteins by the endoproteolytic processing of a large precursor protein called secretogranin II, manserin secretion systems may exist in the carotid body, and thus, behave as potential regulators of respiration in the carotid body.

1. Introduction

Chemical receptors regulate the respiratory system by detecting mixtures or changes in blood oxygen and carbon dioxide concentrations (Gonzalez et al., 1994). These regulatory systems adjust the respiration status (such as ventilation rates) via the respiratory center in the medulla (Smith et al., 2013). The carotid body, located at the carotid bifurcation (anatomically between the internal and external carotid arteries), is a small cluster of chemoreceptors that detect changes in arterial oxygen concentrations and provide feedback to the respiratory center (see reviews by Gonzalez et al., 1994; Kumar and Prabhakar, 2012; Hall, 2015). In addition, these chemoreceptors sense changes in pH. Hence, their malfunction may lead to lethal respiratory failure. For instance, diseases such as abnormal breathing in sleep disorders, congestive heart failure, and certain forms of hypertension are supposedly included in this lethal respiratory group (Prabhakar and Peng, 2004). The carotid body mainly comprises two types of cells, glomus type I cells and sustentacular type II cells. Of these, the neuron-like glomus cell, the principal cell type, is considered to be the chemosensory cell of the organ and contains secretory granules packed with neurotransmitters. In contrast, glial-like type II cells, accounting for 15%–20% of all cells, are typically located at the periphery of the cell cluster and are considered to be supporting cells (see reviews by Atanasova et al., 2011; Gonzalez et al., 1994; Kumar and Prabhakar, 2012). Although the glossopharyngeal nerve, projecting into the carotid body, is considered to play a vital role in carotid body function, further mechanisms regarding these functions may still exist.

Manserin is a neuropeptide isolated from the rat brain (Yajima et al., 2004), and its distribution has been observed in the brain as well as the intestine (Yajima et al., 2008), pancreas (Tano et al., 2010), thyroid (Ohkawara et al., 2013), and adrenal glands (Kamada et al., 2010). Because manserin is proteolytically processed from its precursor protein secretogranin II, known as a neuroendocrine protein, manserin is also suggested to function as a neuroendocrine peptide. Recently, we demonstrated the stress-dependent expression of manserin in the rat adrenal glands (Kamada et al., 2010). In addition, we identified specific locations of manserin in the spiral ganglion and inner hair cells of the rat inner ear (Ida-Eto et al., 2012), suggesting manserin's *in vivo* potential role in response to various external environmental stimuli.

Apparently, various substances involved in the chemical control of breathing, such as substance P, opioids, and neurotransmitters including dopamine, noradrenaline, and acetylcholine, are localized in the carotid body (Ichikawa, 2002; Kumar and Prabhakar, 2012). This study aims to report the localization of manserin in glomus type I cells

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of the rat carotid body.

2. Materials and methods

2.1. Animals and tissue preparation

All animal experiments were approved by the Committee of Laboratory Animal Research Center of Mie University (Mie, Japan). Wistar rats were purchased from CLEA Japan, Inc. (Tokyo, Japan). Adult rats (12–16 weeks old) were anesthetized and transcardially perfused using saline, followed by 4% paraformaldehyde (PFA) in PBS. Further, the carotid arteries were dissected and immersed in 4% PFA in PBS overnight at 4 °C. After washing with PBS, tissues were cryoprotected in 30% sucrose at 4 °C, embedded in Tissue-Tek O.T.C. compound (Sakura Fineteck, Torrance, CA), and stored at −80 °C. After that, tissues were cryosectioned (12-μm thick sections), mounted on glass slides, dried for 30 min, and stored at −80 °C until use.

2.2. Antibodies

An affinity-purified rabbit anti-manserin antibody was prepared as previously described (Yajima et al., 2004; Kamada et al., 2010; Tano et al., 2010); its specificity has been previously verified using immunoblotting (Yajima et al., 2004) and further confirmed by absorption tests (Tano et al., 2010; Ohkawara et al., 2011; Ida-Eto et al., 2012; Ohkawara et al., 2013). The anti-tyrosine hydroxylase (TH) monoclonal antibody (clone 2/40/15; mouse monoclonal, Catalog no. MAB5280, Lot no. 2807054; Millipore, Temecula, CA) and anti-S100 monoclonal antibody (clone 4C4.9; mouse monoclonal, Catalog no. MA1-26621, Lot no. SF2403444A; Thermo Fisher Scientific, Rockford, IL) were used for immunohistochemistry. In addition, the secondary antibodies used were biotinylated goat anti-rabbit IgG (Millipore), Alexa Fluor 568–conjugated goat anti-rabbit IgG, and Alexa Fluor 488–conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA).

2.3. Immunohistochemistry

Immunostaining with 3,3′-diaminobenzidine hydrochloride (DAB) was performed as previously described (Ida-Eto et al., 2011, 2014). Briefly, frozen sections were washed with PBS and treated with 3% hydrogen peroxide in PBS for quenching intrinsic peroxidase activity. After washing with PBS, the sections were blocked using 3% skimmed milk in PBS and incubated with anti-manserin antibody. Further, the sections were sequentially treated with biotinylated goat anti-rabbit IgG. Finally, the sections were immunostained using the ABC method (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA) and were visualized using DAB.

Furthermore, we performed immunofluorescence assays as previously described (Ida et al., 2006; Ida-Eto et al., 2014). Antigen retrieval was performed by heating slides placed in 0.01 M citrate buffer, pH 6.0, in a microwave oven for 10 min. After washing with PBS, the sections were blocked with 3% skimmed milk in PBS and incubated with anti-manserin antibody and either anti-TH or anti-S100 antibodies. Further, the sections were incubated with secondary antibodies conjugated with the fluorescent molecules as described earlier. After mounting with ProLong Gold Antifade reagent (Invitrogen), the sections were examined using a laser-scanning confocal microscope (FV1000, Olympus, Japan); confocal images were saved as TIFF files. Finally, images of manserin (magenta) were pseudocolored using Adobe Photoshop software (Adobe Systems, San Jose, CA).

3. Results

3.1. Localization of manserin in the carotid body

In adult rats, the carotid body is located in the region of the carotid bifurcation between the internal carotid artery and the external carotid artery. We conducted immunohistochemical experiments to determine the location of manserin in the rat carotid body. Manserin-immunopositive signals were detected along the oval shape of the carotid body (Fig. 1a). Under higher magnification, manserin was mainly detected in the round-shaped cells of the carotid body (Fig. 1b); however, manserin immunoreactivity was absent in the round-shaped nucleus of cells and seemed to be distributed in the cytoplasm (Fig. 1b). In addition, manserin was not detected in the superior cervical ganglion (Fig. 1a). Finally, no signals were detected in the absence of the anti-

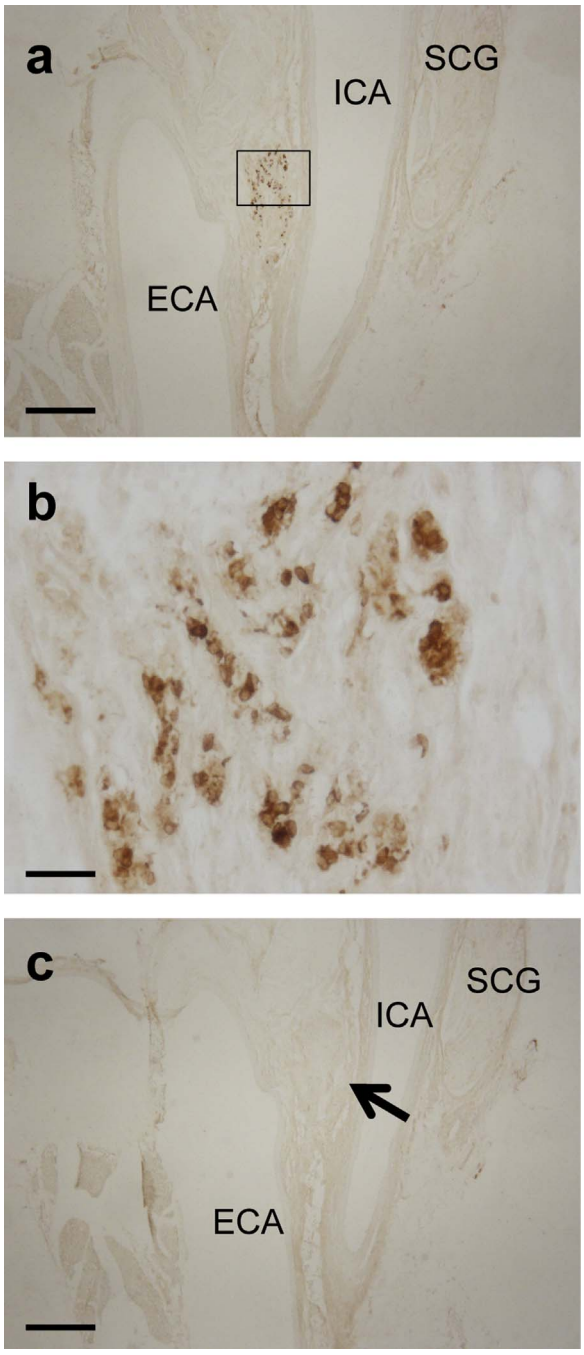

Fig. 1. Immunolocalization of manserin in the rat carotid body.
(a) DAB immunostaining of the carotid bifurcation longitudinal section with anti-manserin antibody. (b) A high-magnification view of (a). (c) Signals were not detected in the carotid body (arrow) without the anti-manserin antibody. ECA, external carotid artery; ICA, internal carotid artery; SCG, superior cervical ganglion. Scale bars, 400 μm (a and c) and 40 μm (b).