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Age-related changes in immunoreactivity for dopamine β-hydroxylase in carotid body glomus cells in spontaneously hypertensive rats

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ABSTRACT

The purpose of this study was to investigate immunoreactivity for dopamine β-hydroxylase (DBH) and tyrosine hydroxylase (TH) in carotid body (CB) glomus cells in spontaneously hypertensive rats (SHR/Izm) at 4 (prehypertensive stage), 8 (early stage of developmental hypertension), 12 (later stage of developmental hypertension), and 16 weeks of age (established hypertensive stage). Age-matched Wistar Kyoto rats (WKY/Izm) were used as controls. Staining properties for TH were similar between both strains at each age. Regarding DBH immunostaining, although some glomus cells showed intense DBH immunoreactivity at 4 weeks of age, these cells were rarely observed at 8, 12, and 16 weeks of age in WKY/Izm. In SHR/Izm, intense DBH immunoreactivity was observed in some glomus cells at 4 weeks of age, these cells were also observed at 8 and 12 weeks of age, and their number increased at 16 weeks of age. An image analysis showed that the percentage of DBH-immunopositive glomus cells in WKY/Izm was approximately 30% at 4 weeks of age and significantly decreased to approximately 10% at 8, 12, and 16 weeks of age ($p < 0.05$). This percentage in SHR/Izm was approximately 40% at each age. The gray scale intensity for DBH immunoreactivity in DBH-immunopositive glomus cells was similar in both strains at 4 weeks of age, but became significantly lower in WKY/Izm and higher in SHR/Izm with increase in age ($p < 0.05$). These results suggest that noradrenaline in glomus cells plays an important role in the regulation of neurotransmission between CB and afferent nerves during developmental hypertension.

1. Introduction

Essential hypertension is a heterogeneous disorder, genetic and environmental factors are associated with its pathogenesis, and it is a risk factor for cardiovascular disease and mortality. Carotid bodies (CB) are peripheral chemoreceptors that are responsible for monitoring changes in PO_2, PCO_2, and pH in arterial blood and play an important role in triggering respiratory and cardiovascular responses (Gonzalez et al., 1994; Nurse, 2005). Previous studies reported that CB was morphometrically enlarged in essential hypertensives (Smith et al., 1982; Habeck, 1986). Furthermore, hyperventilation was observed in essential hypertensive patients under resting conditions (Trzebski et al., 1982). Spontaneously hypertensive rat (SHR) strains are often used as an animal model for human essential hypertension in studies on the pathogenesis of essential hypertension. Similar to essential hypertensives, the enlargement of CB was reported in SHR (Heath et al., 1985; Habeck et al., 1987). SHR also showed hyperventilation under resting conditions (Habeck, 1991). Moreover, a stronger discharge in the afferent nerves (the carotid sinus nerve) of CB by hypoxic exposure was detected in anesthetized SHR (Fukuda et al., 1987). A recent study demonstrated that the development of hypertension in SHR was inhibited by denervation of the carotid sinus nerve at prehypertensive 4 weeks of age (Abdala et al., 2012). These findings suggest that functional alterations in CB are associated with the development of hypertension.

Glomus cells are regarded as chemoreceptor cells within CB, and many neurotransmitters including acetylcholine, ATP, and catecholamines have been detected in these cells (Gonzalez et al., 1994; Nurse, 2005). Regarding the expression of catecholamines in the CB of SHR, we previously investigated immunoreactivity for catecholamine-synthesizing enzymes in the glomus cells of established hypertensive 16-week-old SHR/Izm, which is one SHR strain, and age-matched Wistar Kyoto rats (WKY/Izm) (Kato et al., 2012). We reported that immunoreactivity for tyrosine hydroxylase (TH; EC1.14.16.2), the rate-limiting enzyme of catecholamine biosynthesis, was similar between the two rat strains, whereas that for dopamine β-hydroxylase (DBH; EC1.14.17.1), the enzyme catalyzing the synthesis of noradrenaline (NA) from dopamine, was stronger in the glomus cells of SHR/Izm than in those of WKY/Izm.

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Although our previous findings suggest that the expression of DBH is enhanced in glomus cells under hypertensive conditions, the comparison of immunoreactivity for DBH in glomus cells was only conducted on 16-week-old animals, and the relationship between increases in DBH in glomus cells and blood pressure changes during postnatal development currently remains unknown. Regarding age-related blood pressure changes in SHR, systolic blood pressure was found to gradually increase from 4 to 6 weeks, and reached a plateau 10 weeks or more after birth (Beierwaltes et al., 1982). Therefore, we hypothesized that increases in DBH in glomus cells are associated with the development of hypertension. In order to verify this hypothesis, changes in DBH immunoreactivity in glomus cells need to be examined using SHR/Izm at the prehypertensive stage and developmental hypertensive stage.

The present study was undertaken in order to clarify age-related changes in TH and DBH immunoreactivities in the glomus cells of SHR/Izm using immunohistochemistry. We investigated TH and DBH immunoreactivities in the glomus cells of SHR/Izm and WKY/Izm rat strains using an image analysis for immunostained sections.

2. Materials and methods

2.1. Animals

Male SHR/Izm (Japan SLC, Hamamatsu, Japan) and male WKY/Izm (Japan SLC) were used in the present study. Rats were acclimatized to the animal facility for 1 week in advance, and then used for the study. The ages of the rats at the sampling time were 4, 8, 12, and 16 weeks, and five animals were used in each age group. In SHR/Izm, body weights at 4, 8, 12, and 16 weeks of age were 70–110 g, 210–230 g, 250–330 g, and 320–350 g, respectively. In WKY/Izm, body weights at 4, 8, 12, and 16 weeks of age were 80–110 g, 200–230 g, 310–340 g, and 350–380 g, respectively. The animal room was maintained at a room temperature of 24 ± 2 °C and relative humidity of 55 ± 5% with a regular light-dark cycle (14 h of light and 10 h of darkness per day). A commercial diet (CE-7; CLEA Japan, Inc., Tokyo, Japan) and ultrafiltrated water were given ad libitum. All experimental protocols were approved by the Animal Care and Use Committee of the National Defense Medical College (accession number: 15075) and all animal experiments were performed in accordance with the Regulations for Animal Experimentation of the National Defense Medical College.

2.2. Immunohistochemistry

Each rat was anesthetized by the intraperitoneal administration of medetomidine (0.6 mg/kg), midazolam (8 mg/kg), and butorphanol (10 mg/kg), and transcardially perfused through the ascending aorta with Ringer's solution (50–200 mL depending on body weight) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, 50–200 mL). The bifurcation of carotid arteries was removed and fixed with the same fixative at 4 °C overnight. Tissues were rinsed in phosphate-buffered saline (PBS; pH 7.4), soaked in PBS containing 30% sucrose, and frozen with O.C.T. compound medium (Sakura Finetek, Tokyo, Japan). Frozen tissues were serially sectioned at a thickness of 10 μm using a cryostat (HM 560; Microm, Walldorf, Germany) and mounted on silane-coated glass slides (Matsunami Glass, Osaka, Japan).

Cryostat sections were stained by double immunofluorescence using antibodies against TH or DBH with synaptophysin. In the rat CB, TH immunoreactivity has been observed in glomus cells (Kato et al., 2010, 2012), while DBH immunoreactivity has been detected in glomus cells and synaptic nerve fibers (Kato et al., 2012, 2013b). Synaptophysin is used as a marker protein for glomus cells (Kato et al., 2012, 2013b; Kato and Yamamoto, 2013a). Sections were rinsed with PBS, incubated with non-immune donkey serum (1:50 dilution; 017-000-001, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) diluted with dilution buffer (PBS containing 0.5% Triton-X) at room temperature for 30 min, and rinsed with PBS. Sections were then incubated at 4 °C overnight with either a monoclonal mouse antibody against TH (1:1000 dilution; MAB318, Millipore, Temecula, CA, USA) or monoclonal mouse antibody against DBH (1:4000 dilution; MAB308, Millipore) together with a polyclonal guinea pig antibody against synaptophysin (1:1000 dilution; Syn-GP-Af300, Frontier Institute, Hokkaido, Japan). Sections were rinsed with PBS and incubated at room temperature for 90 min with Alexa Fluor 488-labeled donkey anti-mouse IgG (1:200; 715-545-150, Jackson ImmunoResearch) together with Cy3-labeled donkey anti-guinea pig IgG (1:100; 706-165-148, Jackson ImmunoResearch). After rinsing with PBS, sections were coverslipped with mounting medium (Fluoromount; Diagnostic BioSystems, Pleasanton, CA, USA). Digital images of each section were acquired using a confocal laser scanning microscope (LSM 510 META; Carl Zeiss, Oberkochen, Germany) under the same conditions (e.g., pinhole, scan speed, and detection gain). These images were used to analyze TH and DBH immunoreactivities in either glomus cells or nerve fibers within CB as described below. PBS or non-immune serum was used as an immunohistochemical control instead of primary or secondary antisera, and the complete abolishment of specific labeling in the negative control was confirmed.

2.3. Image analysis of TH and DBH immunoreactivities

In order to analyze TH and DBH immunoreactivities in glomus cells, the gray scale intensities (GI) (range 0–255: black = 0, white = 255) of immunofluorescence for TH and DBH were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The methods used were described previously (Kato et al., 2012, 2013b). The cytoplasm of glomus cells was identified on micrographs of sections stained for synaptophysin. TH- or DBH-immunostained images were converted into gray scale images (256 shades of gray), and GI for TH or DBH immunofluorescence in the cytoplasm of glomus cells was measured. In order to exclude the effects of the background, GI values in connective tissues were measured in each TH- or DBH-immunostained image, and the GI of TH or DBH immunofluorescence in glomus cells was calculated by subtracting the background value from the raw GI value. In the analysis of GI values for TH immunofluorescence, at least 102 glomus cells were measured in each rat, and data from 100 glomus cells were randomly extracted. Similarly, in the analysis of GI values for DBH immunofluorescence, at least 164 glomus cells were measured in each rat, and data from 150 glomus cells were randomly extracted. Extracted data were provided for the statistical analysis. In the present study, glomus cells with GI > 0 were considered to be DBH-immunopositive.

In addition, we performed morphometrical analysis on DBH immunoreactive glomus cells. Using ImageJ software, we calculated the cross-sectional areas of isolated DBH immunoreactive glomus cells, cell counts and aggregated size in clusters of DBH immunoreactive glomus cells, and area densities of DBH-immunopositive glomus cells in CB. Also, we calculated the ratio of area occupied by DBH immunoreactive nerve fibers in CB as described previously (Kato et al., 2012).

2.4. Statistical analysis

Results were given as the mean ± standard deviation. As for the cross-sectional areas of isolated DBH immunoreactive glomus cells and cell counts or aggregated size in clusters of DBH immunoreactive glomus cells, the result were expressed as the maximum and minimum values. All statistical analyses were performed using R version 3.2.0. Statistical analyses were performed using a two-way analysis of variance, followed by Tukey's multiple comparison test or a two-sample *t*-test where applicable. Results were considered to be significant at $p < 0.05$.