Structural and functional differences of the carotid body between DBA/2J and A/J strains of mice

Shigeki Yamaguchi, Alexander Balbir, Brian Schofield, Judith Coram, Clarke G. Tankersley, Robert S. Fitzgerald, Christopher P. O’Donnell, and Machiko Shirahata

1Department of Environmental Health Sciences, The Johns Hopkins Bloomberg School of Public Health, and 2Departments of Medicine and 3Physiology, School of Medicine, The Johns Hopkins University, Baltimore, Maryland 21205

Submitted 9 August 2002; accepted in final form 23 October 2002

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The carotid body is a major player in the ventilatory responses to hypoxia (7, 11, 12). During hypoxia, the neural output from the carotid body increases and reflexly modifies several variables in the respiratory system. A prominent response is an increase in ventilation, but the hypoxic ventilatory response (HVR) among individuals varies widely (9, 38, 40). Studies with twins and longitudinal studies with the same individuals indicate that genetic factors significantly contribute to these differences in healthy humans (5, 19, 20, 25, 35). Recently, Tankersley et al. (33, 34) demonstrated differential ventilatory responses to hypoxia among several inbred strains of mice, which indicates that genetic determinants robustly influence HVR. They showed that the DBA/2J mice demonstrated the highest HVR and the A/J mice the lowest HVR (34). These data suggested that genetically regulated differences exist in the system controlling the HVR. The differences may be in the carotid body, chemoreceptor afferent, or the central integration network for hypoxia.

Previous studies have suggested some correlation between the size of the carotid body and its function. For example, small carotid bodies were found in victims of sudden infant death syndrome (4, 24) and congenital hypoventilation syndrome (6). In these patients, HVR appears to be blunted (16, 28). On the other hand, hypersensitivity of the carotid body has been reported in subjects with mild hypertension (17, 36, 37), and hypertrophy of the carotid body was noted in subjects with established hypertension (13, 15). Similarly, hypersensitivity of the carotid body to hypoxia and hyperoxia and enlargement of the carotid body have been shown in spontaneously hypertensive rats (13). Therefore, we hypothesized that morphological variations of the carotid body contributed to the differences in the function of the carotid body between the DBA/2J and A/J strains of mice.

To test this hypothesis, we employed several techniques and assessed the morphological and functional differences of the carotid body in these two strains of mice: morphometric measurements to determine the size of the carotid body, immunocytochemical detection of tyrosine hydroxylase (TH) to estimate the quantity of glomus cells, and microfluorometric measurements of intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) of glomus cells to assess functional responses to ACh. We report here that the size of the carotid body and the quantity of glomus cells were significantly less in the A/J mice than in the DBA/2J mice. The abnormal morphology may be correlated with the lower [Ca$^{2+}$]$_i$ response to ACh in the A/J mice. These results support our hypotheses...
esis and provide a model to investigate genetic determinants that influence the structure and function of the carotid body.

**METHODS**

**Experimental Animals**

We used the DBA/2J and A/J strains of mice with weights ranging from 23 to 33 g and ages ranging from 8 to 24 wk. Mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in a facility where the temperature (∼26°C) and the light cycle (12:12-h light-dark cycle) were controlled. Water and mouse chow (Agway Pro-Lab RMH 1000) were provided ad libitum before all experiments. All animal protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University.

**Morphology and Immunocytochemistry**

We used five mice from each strain for morphological and immunocytochemical studies. The age of mice (DBA/2J: 16.2 ± 1.9 wk; A/J: 15.3 ± 2.3 wk) and body weight were similar in both strains of mice (DBA/2J: 26.7 ± 2.0 g; A/J: 25.5 ± 0.7 g). Mice were deeply anesthetized with ketamine (100–150 mg/kg ip) and pentobarbital (100–150 mg/kg ip). The heart was removed to avoid bleeding in the neck, and both carotid bifurcations with carotid bodies were harvested immediately. One carotid body was used for morphological assessment, and the other was used for immunostaining for TH.

**Morphology.** The carotid bifurcation was immersed in buffered 4% formalin. The extra tissues were removed and the bifurcation was postfixed in buffered 4% formalin at room temperature for >12 h. Subsequently, tissues were embedded in plastic, sectioned at 3 μm, mounted on glass slides, and stained with toluidine blue.

The structural differences between DBA/2J and A/J mice were examined with a standard bright field microscope (Olympus BH-2). The size of the carotid bodies was measured by using a computer imaging program (Scion Image Beta 2-4.02, Scion, Frederick, MD). The carotid body in each section was delineated, and the area of the carotid body was calculated. Subsequently, the volume of the whole carotid body (μm³) was estimated as: \( \Sigma (\text{the area of each section (μm}^2 \times 3 \text{ μm} \times \text{the thickness of each section}) \).

**Immunocytochemistry.** The quantity of glomus cells in the carotid body from both strains was estimated by the immunocytochemical signal of TH. The carotid body was dissected and stored in 4% formalin solution at room temperature. The thin tissue was embedded in parafilm and sectioned at 4–5 μm, and mounted on a poly-l-lysine-coated slide. Immediately before sections were immunocytochemically stained, they were paraffinized with xylene followed by ethanol. Subsequently, they were treated in boiling 0.01 M citric acid buffer (pH 6.0) for 5 min to retrieve antigens (29). Immunocytochemical procedures were similar to those described before (31). In short, endogenous peroxidase was quenched with 1% H2O2 in PBS for 15 min, and endogenous biotin was blocked with an avidin-biotin-blocking kit (Vector, Burlingame, CA). Other nonspecific binding was blocked with normal goat serum (1:75) and casein (CAS block, Zymed Laboratories, San Francisco, CA). The sections were incubated with a polyclonal antibody against TH (Chemicon International, Temecula, CA; dilution: 1:500 to 1:1,000; made in the rabbit) overnight at 4°C followed by an application of biotinylated anti-rabbit IgG made in the goat (1:2,000) for 1 h at room temperature. As negative control, normal rabbit serum was used instead of anti-TH antibody. Subsequently, standard avidin-biotin-peroxidase techniques were applied by using VECTASTAIN Elite ABC kits (Vector). As a chromogen, Vector SG (Vector) was used. Between each step, the slides were washed in 0.1 M PBS.

TH immunoreactivity was examined by using a light microscope (Olympus BH-2, Olympus, Japan). The largest section of each carotid body was selected. The area of the carotid body and the stained area for TH were measured by using computer imaging software (Scion Image Beta 4.02). TH-positive ratios were determined according to the following equation: TH-positive ratio (% emailAddress = (the stained area for TH/ the area of the carotid body) × 100. The analysis was also performed on a personal computer using Scion Image Beta 4.02.

**Microfluorometry**

We used 20 mice from each strain. There were no differences in age (DBA/2J: 9.6 ± 0.1 wk; A/J: 9.7 ± 0.3 wk) or weight (DBA/2J: 25.8 ± 0.7 g; A/J: 25.7 ± 0.6 g) between strains. The methods for culturing carotid body cells were similar to those used in cat carotid body cells (31). Mice were deeply anesthetized with ketamine (100–150 mg/kg ip) and pentobarbital (100–150 mg/kg ip). The heart was removed to avoid bleeding in the neck, and carotid bifurcations were harvested. Extra tissues were removed, and the carotid bodies were collected under a dissecting microscope. Carotid bodies from three or four mice were pooled together, and they were enzymatically (0.1–0.2% collagenase) and mechanically dispersed. Cells were seeded in wells made of a round glass coverslip (bottom: 25 mm in diameter) and a plastic cylinder (side: 5 mm in diameter). Cells were cultured in a defined medium in a CO2 incubator (5% CO2-air, 37°C) for up to 2 wk. The medium was changed twice a week. The basic nutrient solution was a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium supplemented with bovine serum albumin, bovine transferrin, bovine insulin, sodium selenite, 78-nerve growth factor and pyruvate (Sigma-Alrich, Saint Louis, MO).

The Ca2+ indicator indo-1 (Molecular Probes, Eugene, OR) was loaded into cultured cells by incubating the cells in culture medium including the dye (1 μM) for 1 h. A round coverslip with cultured cells was assembled to the recording chamber (30). The chamber was placed on an inverted microscope (Olympus IMT2), and cells were continuously perfused with Krebs equilibrated with 5% CO2 in air at 37°C. To measure [Ca2+], small clusters of cells were selected. ACh or tyrode (a vehicle control) was applied to the cluster via an electrically controlled puff pipette that was located close to the cluster. Indo-1 in the cells was repeatedly excited at a wavelength of 395 nm for 35 ms at every 2 s. Two wavelengths of emission light [405 nm (F405) and 495 nm (F495)] were recorded from a restricted microscope field with a dual-emission fluorometer (Biomedical Instrumentation Group). Background signals of F405 and F495 (F405bg and F495bg, respectively) were obtained from the field that did not contain cells. A PC-based computer and pCLAMP software (5.1) were used for the acquisition of the data. [Ca2+]i was estimated as a fluorescent ratio (F405/F405bg) and (F495/F495bg). The composition of Krebs was (in mM) 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4·7H2O, 1.2 KH2PO4, 0.0106 EDTA, 25 NaHCO3, and 10 glucose, pH 7.4 equilibrated with 5% CO2-air. A stock solution of ACh (100 mM in water) was kept at −20°C and diluted with Tyrode immediately before the ex
experiments. The composition of Tyrode was (in mM) 143.3 NaCl, 4.7 KCl, 1.8 CaCl$_2$, 1.2 MgSO$_4$•7H$_2$O, 1.2 KH$_2$PO$_4$, 0.0016 EDTA, and 10 HEPES, pH 7.4.

Statistical Analysis

All morphological and immunocytochemical measurements were performed by two trained investigators (S. Yamaguchi and A. Balbir). The mean value of measurements by each of the two investigators was used for analysis. When differences in the measurements between the two investigators were >10%, a third investigator reexamined these sections together with the two investigators. In the carotid body of the A/J mice, it was not always clear whether a part of the tissue was the carotid body. In such cases, we assumed the tissue under inspection to be the carotid body to avoid underestimating the size of the carotid body in the A/J mice. All data are presented as means ± SE. Mann-Whitney's U-test was used to evaluate the significance between the two strains of mice. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Morphology

We observed four major differences in morphology of the carotid body between the DBA/2J and A/J strains of mice. First, the carotid body of the DBA/2J mice was clearly distinguished from other tissues (Fig. 1A). The line of demarcation between the carotid body and other tissues, including connective tissues, bundles of nerves, and large vessels, was unequivocal. On the other hand, nerves and vessels were entangled with the carotid body of the A/J mice (Fig. 1B).

Second, the carotid body of the DBA/2J mice was approximately three times as large as that of the A/J mice ($6.3 \pm 0.5 \times 10^6 \mu m^3$ in the DBA/2J mice and $1.5 \pm 0.3 \times 10^6 \mu m^3$ in the A/J mice; Fig. 2). No overlap was observed in the distribution of the data points. Each section measured by the two investigators and the size reported by each were very similar in the DBA/2J mice (<5% differences). However, the two investigators occasionally disagreed with each other in the case of the carotid body of the A/J mice, and the ambiguous part was designated as the carotid body as described in METHODS.

Third, many glomeruli, which contained several glomus cells surrounded by sheath cells, were observed in the DBA/2J mice (Fig. 1C). However, in the carotid body of the A/J mice, glomeruli were not easily recognized (Fig. 1D).

Fourth, many glomus cells in the carotid body of the DBA/2J mice showed typical large and round nuclei.
and abundant cytoplasm (Fig. 1E), as described in other species (22). On the other hand, there were only a few typical glomus cells in the carotid body of the A/J mice (Fig. 1F). Light microscopic observation was inadequate for clearly establishing whether some cells with uncharacteristic cytoplasm and an irregular nucleus were also glomus cells.

**Immunocytochemistry**

In the DBA/2J mice, numerous cells in glomeruli were positively stained for TH (Fig. 3A). Some nerve fibers in the carotid body were also stained in the DBA/2J mice. In the A/J mice, however, fewer cells in the carotid body were stained for TH compared with the DBA/2J mice (Fig. 3B). The positive stained area for TH in the DBA/2J mice was 22.6 ± 1.6% of the carotid body (TH-positive ratio in Fig. 4). It was significantly larger than that in the A/J mice (5.2 ± 0.7%; P < 0.01). The individual data points for TH-positive ratio of the DBA/2J mice segregated from those of the A/J mice. In contrast to the carotid body, we did not observe any apparent differences in the staining for TH in neurons of the superior cervical ganglion between the DBA/2J and A/J mice (Fig. 3).

**[Ca^{2+}]_i Measurements**

The application of ACh (0.5 and 1 mM) dose dependently increased [Ca^{2+}]_i in most clusters of cultured carotid body cells in the DBA/2J mice (Fig. 5A). The response to 0.5 mM ACh was compared between the clusters of the DBA/2J mice and those of the A/J mice. In the DBA/2J mice, many more clusters of carotid body cells (81%; 21 clusters of 26) increased [Ca^{2+}]_i than those in the A/J mice (18%; 6 clusters of 34) (Fig. 5B).

**DISCUSSION**

In this study, we have demonstrated morphological differences in the carotid body between the DBA/2J and A/J inbred strains of mice. One prominent difference is that the carotid body of the DBA/2J mice is approximately four times larger in volume than that of the A/J mice (Fig. 2). Moreover, this difference might be underestimated, because difficulties in precisely delineating the carotid body of the A/J mice led us to make a conservative estimate of the size of the carotid body in the A/J mice. Despite this conservative approach, there was no overlap in the data points for the two strains of mice.

The size of the carotid body can be influenced by environmental factors. For example, when animals are exposed to chronic hypoxia, the carotid body is enlarged (12). This occurs after 4 wk of exposure to hypoxia in rats (1, 23). Animals living at high altitude are also known to have enlarged carotid bodies (12, 15, 21). In spontaneously hypertensive rats, the carotid body is enlarged before the development of high blood pressure (13, 15). In all these environmental conditions, hypertrophy of glomus cells and hyperplasia of sheath cells typically occur. In our study, both strains of mice were housed in the same room at sea level under normoxic conditions. They were supplied with the same mouse chow and water, and their age and A/J inbred strains of mice. One prominent difference is that the carotid body of the DBA/2J mice is approximately four times larger in volume than that of the A/J mice (Fig. 2). Moreover, this difference might be underestimated, because difficulties in precisely delineating the carotid body of the A/J mice led us to make a conservative estimate of the size of the carotid body in the A/J mice. Despite this conservative approach, there was no overlap in the data points for the two strains of mice.

The size of the carotid body can be influenced by environmental factors. For example, when animals are exposed to chronic hypoxia, the carotid body is enlarged (12). This occurs after 4 wk of exposure to hypoxia in rats (1, 23). Animals living at high altitude are also known to have enlarged carotid bodies (12, 15, 21). In spontaneously hypertensive rats, the carotid body is enlarged before the development of high blood pressure (13, 15). In all these environmental conditions, hypertrophy of glomus cells and hyperplasia of sheath cells typically occur. In our study, both strains of mice were housed in the same room at sea level under normoxic conditions. They were supplied with the same mouse chow and water, and their age and
weight were not different. Although we did not measure blood pressure, other investigators have shown that both DBA/2J and A/J mice are normotensive (14). Therefore, the difference in the carotid body size between these two strains of mice is more likely to be determined by genetic factors than by environmental factors. The fact that distribution of the carotid body size in each strain of mice was segregated also supports the view that genetic determinants influence the difference in the carotid body size of these two strains.

The cellular anatomy of the carotid body has been thoroughly investigated in the cat and the rat (for a review, see Ref. 22). Parenchymal cells of the carotid body are the glomus cells, the putative chemosensory cells, and the sheath cells that are glia-type. Glomus cells have a round nucleus and abundant cytoplasm. They group together and are surrounded by sheath cells. The groups of glomus cells and sheath cells, called glomeruli, are separated by connective tissue and blood vessels. These characteristics are also seen in other species, including rabbits, dogs, guinea pigs, and humans (12, 15, 22). The anatomical characteristics of the carotid body in the DBA/2J mice meet these classical and well-established criteria. However, in the A/J mice, the glomus cells were most frequently difficult to distinguish, and their shape appeared distorted. Although it is not clear from this study, glomus cells might degenerate prematurely or they might not fully differentiate during development. Abnormalities in glomus cells of the A/J mice were also apparent in immunocytochemistry for TH. The presence of TH in glomus cells is well known in several species (18, 26, 39) including mice (27), and, therefore, we used TH as a marker for glomus cells. We observed typical TH immunostaining in the carotid body of the DBA/2J mice. That is, the cytoplasm of the cell was well stained and the round nucleus was left unstained. Furthermore, the shape of the glomus cell was easily delineated. On the other hand, TH staining in the carotid body of the A/J mice was sporadic, and the shape of the glomus cell was often difficult to define. It is important to note here that the shape of neurons and the TH staining in the superior cervical sympathetic ganglion in the DBA/2J mice resembled those in the A/J mice (Fig. 3). Thus morphological changes did not occur globally in catecholamine-containing neurons in the A/J mice, but rather the differences were specific to glomus cells of the carotid body. The data suggest that genetic determinants affect the morphology of glomus cells.

![Fig. 4. TH-positive ratio in the carotid body in the DBA/2J and A/J strains of mice. TH-positive ratio = (TH-positive area/the area of the carotid body) × 100. Mean ± SE ratios are presented next to each scattergram. The distributions of the data in the DBA/2J and A/J mice are distinctly different. The TH-positive ratio in the DBA/2J mice is significantly larger than that in the A/J mice (P < 0.01).](image)

![Fig. 5. Effect of ACh on intracellular Ca²⁺ concentration ([Ca²⁺]). A: most clusters of cultured carotid body cells from the DBA/2J mice responded to ACh (0.5 and 1 mM) by increasing [Ca²⁺]. ACh was applied to the cluster via a puff pipette for 15 s (indicated by a thick bar). B: differential responses of cultured carotid body cells to ACh (0.5 mM) in the DBA/2J (top) and A/J (bottom) mice.](image)
Glomus cells are considered chemosensory cells (12). Therefore, the quantity of glomus cells, at least up to some threshold level, may be related to chemosensory function. Because the cytoplasmic shape of glomus cells was irregular and the glomus cell was most often difficult to identify in A/J mice, the counting of glomus cells in plastic sections was not practical. Instead, we used the TH-positive area as an index estimating the quantity of glomus cells. We did not estimate the total volume of glomus cells by using TH immunostaining. Because glomus cells are small (10–15 μm in diameter), the thickness of the tissue section (4–5 μm) does not allow precise estimation of cell volume. Furthermore, the loss of tissue sections during immunostaining procedures, particularly at the edge of the carotid body, was unavoidable. Therefore, we used the largest part of the carotid body to measure the TH-positive area. The TH-positive area was expressed as percent of the carotid body area in the same section. This method provided us a semiquantitative estimation of the glomus cell volume, assuming that TH production in glomus cells was similar in both strains of mice. We found that the TH-positive area of the DBA/2J mice was approximately four times larger than that of the A/J mice. One might argue that glomus cells of the A/J mice do not contain TH, and, therefore, the measurement of the TH-stained area in the A/J mice does not reflect the quantity of glomus cells. Although we cannot completely exclude this possibility, the fact that TH immunostaining in the superior cervical ganglion was similar in both strains of mice suggests that the TH production itself in the A/J mice is not impaired. Furthermore, simple morphological observation showed that the number of typical glomus cells in the A/J mice was much lower than that in the DBA/2J mice. Taken together, the data suggest that the carotid body of the A/J mice contains considerably fewer glomus cells than that of the DBA/2J mice.

Fewer glomus cells in A/J mice may be, at least in part, the reason why most clusters of carotid body cells did not respond to ACh. ACh is known to excite chemoafferent nerves in several species (10). It also increases [Ca2+]i of rat (8) and cat glomus cells (30). Although hypoxia is also known to increase [Ca2+]i of glomus cells, significant heterogeneity in [Ca2+]i responses among cells and clusters were reported (3, 32). For this study, we used ACh as a stimulant, because the application of ACh is technically simple and a relatively homogenous [Ca2+]i response was observed in other species (8, 30). The increase in [Ca2+]i by ACh in the DBA/2J mice was similar to that found in these previous reports. The attenuated response to ACh in the A/J mice could also be due to some abnormalities of glomus cell function, such as the expression of cholinergic receptors. We did not test for the presence of cholinergic receptors on the glomus cell in this study. Possible differences in the presence, location, and density of these receptors need to be investigated in future studies.

In summary, we have shown that the size and morphology of the carotid body of the DBA/2J mice differed significantly from those of the A/J mice. The characteristics of the carotid body in the DBA/2J mice were similar to those in other species, but the carotid body of the A/J mice appeared to be abnormal. These differences may account for the reduced response of [Ca2+]i to ACh and the blunted HVR in the A/J mice. The segregation of the above data between the two strains suggests that genetic factors strongly influence the observed phenotypic differences between the DBA/2J and A/J mice. This study did not show underlying mechanisms of these phenotypic differences. Further studies are required to clarify when and how these phenotypic differences occur. Nevertheless, these two strains of mice could provide excellent models to study genetic regulation of the carotid body morphology and function as well as to study the role of carotid body in various pathological conditions.

This work was supported by National Institutes of Health Grants HL-66324, HL-61596, HL-50712, and ES-03819.

REFERENCES


