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Diabetes induces neural degeneration in nucleus ambiguus (NA) and attenuates heart rate control in OVE26 mice

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ABSTRACT

Baroreflex sensitivity is impaired by diabetes mellitus. Previously, we found that diabetes induces a deficit of central mediation of baroreflex-mediated bradycardia. In this study, we assessed whether diabetes induces degeneration of the nucleus ambiguus (NA) and reduces heart rate (HR) responses to L-Glutamate (L-Glu) microinjection into the NA. FVB control and OVE26 diabetic mice (5–6 months) were anesthetized. Different doses of L-Glu (0.1–5 mM/l, 20 nl) were delivered into the left NA using a multi-channel injector. In other animals, the left vagus was electrically stimulated at 1–40 Hz (1 ms, 0.5 mA, 20 s). HR and mean arterial blood pressure (MAP) responses to L-Glu microinjections into the NA and to the electrical stimulation of the vagus were measured. The NA region was defined by tracer TMR-D injection into the ipsilateral nodose ganglion to retrogradely label vagal motoneurons in the NA. Brainstem slices at −600, −300, 0, +300, and +600 μm relative to the obex were processed using Nissl staining and the number of NA motoneurons was counted. Compared with FVB control, we found in OVE26 mice that: 1) HR responses to L-Glu injection into the NA at doses of 0.2–0.4 (mM/l, 20 nl) were attenuated ($p<0.05$), but MAP responses were unchanged ($p>0.05$). 2) HR responses to vagal stimulation were increased ($p<0.05$). 3) The total number of NA (left and right) motoneurons was reduced ($p<0.05$). Taken together, we concluded that diabetes reduces NA control of HR and induces degeneration of NA motoneurons. Degeneration of NA cardiac motoneurons may contribute to impairment of reflex-bradycardia in OVE26 diabetic mice.

Introduction

Baroreflex control of heart rate (HR) is impaired by diabetes mellitus in humans and experimental animal models (Lishner et al., 1987; Pagani et al., 1988; Dall'ago et al., 2007; Gu et al., 2008). However, the neural mechanisms underlying this baroreflex impairment are not well understood. Our ultimate goal is to systematically study diabetes-induced structural remodeling and functional alterations of multiple neural components [e.g. baroreceptor afferent terminals in the nucleus of the solitary tract (NTS), neurons in NTS, vagal motoneurons, cardiac ganglia, and their network connections] within the baroreflex circuitry. Previously, we demonstrated that diabetes induces a deficit in the central components mediating baroreflex-bradycardia in transgenic type 1 diabetic mice (OVE26) (Gu et al., 2008). In this study, we aimed to investigate diabetes-induced anatomical and functional changes of vagal cardiac motoneurons in the nucleus ambiguus (NA) which plays a key role in controlling HR (Cheng et al. 2004b).

It is well known that baroreflex control of HR is mediated through glutamatergic transmission in the NA (Sapru 2002). NA cardiac motoneurons express the ionotropic glutamate receptors α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and N-methyl-D-aspartate (NMDA) (Corbett et al. 2003). In addition, electrical stimulation of the nucleus of the solitary tract (NTS) evokes fast and slow postsynaptic potentials in the NA cardiac neurons that are mediated by both non-NMDA (AMPA or kainate) and NMDA receptors (Wang et al. 2001). Furthermore, microinjection of L-Glu, NMDA and AMPA into the NA induces large bradycardiac responses (Cheng et al. 2004b, Yan et al. 2008, 2009). Therefore, we hypothesized that diabetes attenuates the HR response to L-Glu microinjection into the NA of OVE26 diabetic mice, and that such attenuation would be accompanied by a significant loss of NA motoneurons. In searching the literature, we found that diabetes-induced remodeling of the NA and its pathophysiology of baroreflex impairment had not yet been studied.

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Materials and methods

Animals

OVE26 mice develop type 1 diabetes because of beta cell specific damage resulting in reduced insulin secretion and sustained high blood glucose levels well over 500 mg/dl by 30 days of age (Epstein et al., 1989, 1992; Zheng et al., 2004). Mice (5–6 months) were maintained on a 12-h light/dark cycle and received food and water *ad libitum*. Procedures were approved by the University of Central Florida Animal Care and Use Committee. Efforts were made to minimize animal suffering.

Surgical procedures

Mice were initially anesthetized with Nembutal (0.05 mg/g. i.p.) and ventilated with oxygen-enriched room air through the trachea. Hind paw pinch withdrawal reflexes were checked as indicators of the depth of anesthesia. Supplemental Nembutal (1/6 of initial dose, i.v.) was given as needed. Body temperature was maintained at 37 ± 1 °C with a homeostatic blanket (Harvard) and a rectal probe. Polyethylene catheters (PE-50) were placed into the left femoral artery to monitor arterial pressure (AP) and into the left vein to inject pharmacological agents (β1-blocker atenolol and M-blocker methyatropine), and into the right vein to inject supplemental doses of Nembutal. AP was measured using a pressure transducer (CB Sciences, BP100) connected to a Powerlab Data Acquisition System (PowerLab/8 SP). HR was calculated from pulse pressure using the ratemeter function of the Chart 5.2 software.

Cardiovascular responses to microinjection of L-Glu into the NA

The procedure to expose the brainstem was identical to that previously described (Cheng and Powley, 2000; Cheng et al., 2004a,b; Ai et al., 2007a,b). Using a 7-channel pipette connected to a multi-injection system (MDI, PM8000, South Plainfield, NJ, USA), L-Glu was injected into the left NA at 7 different doses (0.1, 0.2, 0.3, 0.4, 0.5, 1, 5 mM/l, 20 nl) for each animal (FVB: $n = 7$; OVE26: $n = 9$) as monitored by the displacement of the meniscus observed with a microscope fitted with an eyepiece graticule. HR response (ΔHR) and mean AP response (ΔMAP) to L-Glu injection at each dose were measured and normalized by the ΔHR and ΔMAP to L-Glu at the maximal (saturation) dose. To evoke reliable HR and MAP responses, the injection sites were experimentally determined by injecting L-Glu to sites around the NA at the level of the area postrema. We selected the site of the largest HR and MAP responses to L-Glu at the saturation dose. Saline (20 nl) was injected at the same site to provide a vehicle control ($n = 3$/group). ΔHR and ΔMAP in response to saturation dose injection of L-Glu were measured before and after atenolol and methyatropine administration to measure the relative contribution of parasympathetic and sympathetic control ($n = 3$/group). In 3 additional animals (FVB: $n = 2$; OVE26: $n = 1$), we also injected the tracer DiI into the location of maximal HR response to confirm that the injection sites were similar to previous studies (Lin et al., 2008; Yan et al., 2008, 2009).

Cardiovascular responses to vagal stimulation

L-Glu injection into the NA may activate vagal cardiac motoneurons through glutamate receptors. In turn, vagal cardiac motoneurons control the heart through the vagal cardiac efferent innervation of cardiac ganglia. In order to evaluate the separate effects of vagal cardiac motoneurons and vagal cardiac efferent input to the heart, we electrically stimulated the left cervical vagus ($n = 6$/group). The vagal nerve was isolated from surrounding tissues, transected and the peripheral end of the vagal nerve was placed on a pair of bipolar platinum electrodes with the conventional arrangement (cathode near the heart). Square pulses were generated using a stimulator (S48K, Grass Instruments) at frequencies of 1–40 Hz (1 ms, 20 s) and delivered through a stimulus isolation unit (PSIU6, Grass Instruments) which provided the nerve with steady current (0.5 mA, 1 ms) during stimulation. Five minutes was allotted between stimulations, to allow HR and MAP to return to pre-stimulus baselines determined from the average values 60 s before stimulation. Peak HR and MAP responses (ΔHR and ΔMAP) relative to their baselines were calculated, and response curves were plotted against stimulus frequency.

Labeling of vagal motoneurons with tracer

As the name of nucleus ambiguus (NA) indicates, the NA does not have a clear-cut boundary. One concern is how to quantify NA motoneurons accurately. To obtain a relatively reliable count of Nissl-stained motoneurons in the NA, we labeled vagal motoneurons in the NA by injecting tetramethylrhodamine dextran (TMR-D, 7%, mw 3000, Molecular Probes D-3308) into the nodose ganglion label vagal motoneurons in the NA. Twelve animals ($n = 6$ for each side: left and right) of the same age and size were anesthetized with Nembutal (0.05 mg/g i.p.). A midline incision was made along the neck, and ventral neck muscles were gently separated by blunt dissection to expose the nodose ganglion medial to the internal carotid artery (Cheng et al., 2002). Multiple injections of TMR-D (100 nl) were made into the left or right nodose ganglion through a micropipette which was connected to an injector. After completion of all injections, the surgical wound was closed with sutures and the animal was returned to its cage. After a survival period of 5 days to allow for tracer transport to the brain stem, each animal was anesthetized by an overdose of Nembutal (0.1 mg/g) and perfused through the heart with phosphate-buffered (pH 7.4,) 0.9% saline and 10% formalin (100 ml). The brain stem and the nodose-petrosal ganglion complex were removed. Each brain stem containing the NA was stored in 15% sucrose formalin overnight and sectioned transversely at 40 μm by using a cryostat on the second day. All tissues were then dehydrated through a graded series of ethanol rinses (70%, 2 min; 90%, 2 min; and 2 times of 100%, 1.5 min each) and cleared in xylene (3 min). Finally, tissues were mounted and coverslipped with Cytoseal XYL. Serial brainstem sections were examined using epifluorescence and confocal microscopy. Brain stem sections at −600, −300, 0, +300, +600 μm were imaged. Using Neurolucida software, brainstems ($n = 6$/side) were scaled and superimposed on top of each other for each side of the brainstem. Boundaries of the tracer-labeled NA were superimposed to mark the boundary of the NA.

Nissl staining of the motoneurons in the NA region

Tissues were obtained as described above. Brainstem sections were processed for Nissl staining and examined with a Nikon 80i microscope. The number of motoneurons in the left and right NA was counted for each brainstem section ($n = 6$/group). Three criteria were used for counting NA motoneurons: (1) neurons located in the region defined by the NA reconstructed by the TMR-D labeling; (2) neurons with a maximum diameter of 10 μm or more; (3) neurons with a distinct nucleolus (Chen and Chuang, 1999; Hottinger et al., 2000; Xu et al., 2006; Yan et al., 2008). In this way, we could avoid counting non-motoneuron cells.

Statistical analysis

Group data were presented as means ± SE. Student's *t*-tests were used to compare HR and MAP responses to L-Glu injections and the number of NA motoneurons between groups. Effects of different frequencies of electrical stimulation on HR and MAP responses were analyzed using two-way repeated measures ANOVA followed by