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DIABETES ALTERS PROTEIN EXPRESSION OF HYPERPOLARIZATION-ACTIVATED CYCLIC NUCLEOTIDE-GATED CHANNEL SUBUNITS IN RAT NODOSE GANGLION CELLS

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Abstract—Vagal afferent neurons, serving as the primary afferent limb of the parasympathetic reflex, could be involved in diabetic autonomic neuropathy. Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are expressed in the vagal afferent neurons and play an important role in determining cell membrane excitation. In the present study, the protein expression and the electrophysiological characteristics of HCN channels were investigated in nodose ganglion (NG) afferent neurons (A-fiber and C-fiber neurons) from sham and streptozotocin (STZ)-induced diabetic rats. In the sham NG, HCN1, HCN3, and HCN4 were expressed in the A-fiber neurons; and HCN2, HCN3, and HCN4 were expressed in the C-fiber neurons. Compared to the sham NG neurons, diabetes induced the expression of HCN2 in the A-fiber neurons besides overexpression of HCN1 and HCN3; and enhanced the expression of HCN2 and HCN3 in C-fiber neurons. In addition, whole-cell patch-clamp data revealed diabetes also increased HCN currents in A-fiber and C-fiber neurons. However, we found that diabetes did not alter the total nodose afferent neuron number and the ratio of A-fiber/C-fiber neurons. These results indicate that diabetes induces the overexpression of HCN channels and the electrophysiological changes of HCN currents in the A- and C-fiber nodose neurons, which might contribute to the diabetes-induced alteration of cell excitability in the vagal afferent neurons.

Key words: autonomic nervous system, diabetes, hyperpolarization-activated cyclic nucleotide-gated current, immunofluorescence, nodose ganglion afferent neuron.

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Abbreviations: BLAST, basic local alignment search tool; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; cDNA, complementary DNA; C_m, membrane capacitance; DMEM, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglia; EGTA, ethylene glycol tetraacetic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCN, hyperpolarization-activated cyclic nucleotide-gated channel; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; IB4, isolectin B4; mRNA, messenger RNA; NG, nodose ganglia; PBS, phosphate buffered saline; PVDF, polyvinylidene fluoride; RT97, 200 kD neurofilament heavy antibody; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate; STZ, streptozotocin; TTX, tetrodotoxin; τ, time constant.

Diabetic autonomic neuropathy, a frequent and serious complication of diabetes, affects autonomic nerve functions (Guo et al., 2004; Vinik et al., 2003). Although its significant negative impact has been seriously considered on survival and quality of life in people with diabetes (Ewing et al., 1980; Vinik and Erbas, 2001), the mechanisms underlying the diabetic autonomic neuropathy are still poorly understood. The nodose ganglia (NG) contain the cell bodies of visceral afferent neurons, which serve as the primary afferent limb of the parasympathetic reflex including cardiovascular, respiratory and gastrointestinal reflex (Thoren et al., 1977; Kuo et al., 2005; Zhang et al., 2006; Browning, 2003). Dysfunction of the primary visceral afferent neurons is likely to be involved in the autonomic neuropathy observed in diabetic patients and animals. Therefore, understanding the cellular excitable mechanisms of vagal afferent neurons provide an important and new pharmacological target for the diabetic autonomic neuropathy.

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels were first identified in the heart (Noma and Irisawa, 1976; Brown et al., 1979) and CNS (Halliwell and Adams, 1982; Maccaferri et al., 1993). It is most widely known for the HCN channels to generate cardiac spontaneous pacemaker activity (DiFrancesco, 1993) and neuron oscillatory behavior (Pape, 1996) as the pacemaker channels. Besides in spontaneously active cells in the heart and CNS, the HCN channels are also found to express in the soma of viscero-sensory and somato-sensory neurons (Doan et al., 2004; Doan and Kunze, 1999). However, HCN channels play a different role in these sensory neurons and do not exhibit either pacemaker activity or oscillatory potentials (Doan and Kunze, 1999; Mayer and Westbrook, 1983) because viscero-sensory and somato-sensory neuron cell bodies are generally quiescent except that the cell bodies receive the signal of depolarizing stimulus from their peripheral sensory receptors (Doan et al., 2004). Previous studies (Doan et al., 2004; Li et al., 2008a) have already shown that inhibiting HCN channels by HCN channel blockers significantly enhances the cell membrane excitability via hyperpolarizing rest-membrane-potential and decreasing the stimulating threshold to evoke an action potential in NG afferent neurons.

Using complementary techniques of basic local alignment search tool (BLAST) searches of expressed sequence tag database, reverse transcription polymerase chain reaction (RT-PCR), and screening of cDNA libraries, the HCN channel has been divided into four isoforms (HCN1, HCN2, HCN3, and HCN4) (Ishii et al., 1999; Ludwig et al., 1998; Santoro et al., 1998; Vaccari et al., 1999). mRNA for HCN1–4 isoforms are also reported to express

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in rat NG neurons (Doan et al., 2004). In addition, the different NG afferent neurons express different HCN isoforms in different degrees (Doan et al., 2004; Li et al., 2008a). For example, HCN1 is presented in the neurons with myelinated A-fibers but not in the neurons with unmylinated C-fibers, whereas HCN2 is prominently expressed in the C-fiber nodose afferent neurons (Doan et al., 2004; Li et al., 2008a). Although we have reported that the expression of HCN1 and HCN2 channel protein is increased in diabetic A-fiber NG neurons (Li et al., 2008a), we do not know whether diabetes induces the changes of HCN3 and HCN4 channel protein in A-fiber NG neurons and all HCN channel isoforms in C-fiber neurons. In this study, therefore, we measured the following: (1) population and distribution of A-fiber and C-fiber neurons in the NG from sham and diabetic rats; (2) protein expression of HCN channel isoforms in A-fiber and C-fiber NG neurons from sham and diabetic rats; (3) HCN currents in A-fiber and C-fiber NG neurons from sham and diabetic rats.

EXPERIMENTAL PROCEDURES

Induction of diabetes

Male Sprague–Dawley rats (200–250 g) were housed two per cage under controlled temperature and humidity and a 12:12 h dark-light cycle, and were provided water and rat chow *ad libitum*. Experiments were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and were carried out in accordance with the National Institutes of Health (NIH Publication No. 85–23, revised 1996) and the American Physiological Society's Guides for the Care and Use of Laboratory Animals.

Rats were randomly assigned to sham (n=43) and diabetic rats (n=44). Diabetes was induced by a single i.p. injection of streptozotocin (STZ, 65 mg/kg, Sigma, St. Louis, MO, USA) in a 2% solution of 0.1 M cold citrate buffer. Sham rats received a similar injection of vehicle. Diabetes was identified by polydipsia, polyuria, and blood glucose >250 mg/dl (Accu-check Aviva, Boehringer Mannheim). Rats receiving STZ but with blood glucose <250 mg/dl (n=5) were excluded from study. Blood glucose and body weight in all rats were measured weekly. All experiments were taken at 6–8 weeks after single dose injection of STZ or vehicle.

Western blot analysis for HCN1, HCN2, HCN3, and HCN4 channel proteins

NGs from sham (n=20) and diabetic (n=16) rats were rapidly removed and immediately frozen in liquid nitrogen and stored at −80 °C until analyzed. The protein was extracted from NG homogenates with the lysing buffer (10 mM Tris, 1 mM EDTA, 1% SDS, pH 7.4) plus protease inhibitor cocktail (Sigma, 100 μl/ml). After a centrifugation at 12,000 *g* for 20 min at 4 °C, the protein concentration in the supernatant was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). The protein sample was mixed with the same volume of the loading buffer and heated at 100 °C for 5 min. Equal amounts of the protein samples were loaded and then separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The proteins of these samples were electrophoretically transferred at 300 mA for 1.5 h onto PVDF membrane. The membrane was probed with rabbit anti HCN1, HCN2, HCN3, or HCN4 channel antibodies (Alomone labs, Jerusalem, Israel) and a peroxidase-conjugated goat anti-rabbit IgG (Pierce Chemical, Rockford, IL, USA). The signal was detected using enhanced chemiluminescence substrate (Pierce Chemical, Rockford, IL, USA) and the bands were analyzed using UVP bioimaging system. The target protein was controlled by probing western blot with mouse anti-GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and normalizing target protein intensity to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Fluorescent immunohistochemistry on the fixed NG

Immunofluorescent staining was taken in sham (n=10) and diabetic (n=10) rats. Each rat was perfused transcardially with 100 ml heparinized saline followed by 500 ml of freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS). Both NGs in each rat were rapidly removed and postfixed in 4% paraformaldehyde in 0.1 M PBS for 12 h at 4 °C, followed by soaking the NG in 30% sucrose for 12 h at 4 °C for cryostat protection. The NG was serially cut into 10 μM-thick cross sections and then mounted on precoated glass slides for immunofluorescent staining.

Experiment 1: In order to determine the population and distribution of A-fiber and C-fiber neurons in the NG from sham and diabetic rats, the NG cross sections were incubated with 10% goat serum for 1 h followed by incubation with mouse anti-RT 97 antibodies (an A-fiber neuron marker; Perry et al., 1991, Abcam, Cambridge, MA, USA) overnight at 4 °C. Then the sections were washed with PBS and incubated with fluorescence-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Alexa FluorR 488 conjugated isolectin-B4 (IB4, a C-fiber neuron marker; Wang et al., 1994, Invitrogen, CA, USA), and DAPI (a nucleus marker, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 60 min at room temperature. After three washes with PBS, the sections were mounted on pre-cleaned microscope slides. Slides were observed under a Leica fluorescent microscope with corresponding filters. Pictures were captured by a digital camera system. No staining was seen when PBS was used instead of the primary antibody in the above procedure. Since the slices were 10 μM thick, and the NG neuron soma ranged about 20–60 μM, a same neuron could be double-counted. Therefore, we skipped seven slices every count to avoid this miscount.

Experiment 2: In order to identify the localization of HCN channels, the colocalization of HCN1, HCN2, HCN3, or HCN4 channel isoform and RT97 or IB4 were tested using the same procedure in experiment 1. Antibody control antigen for HCN1, HCN2, HCN3, or HCN4 (Alomone labs, Jerusalem, Israel) was used as negative control.

Expression of HCN channels were quantified using Adobe Photoshop CS3 (Photoshop Extended, Adobe Systems Incorporated, CA, USA). Cells colocalized with RT-97 or IB4 in each slice were selected in the image (red color) of HCN channels using the lasso tool. After a measurement scale was set (1 pixel=1 pixel), the integrated density (optical density, OD) of the HCN channel image was automatically measured by clicking record measurements in the analysis menu. Similarly, the total pixels (area) of all A-fiber cells (RT-97-labeled) or C-fiber cells (IB4-labeled) (with and without HCN channel image) were measured in each slice. The quantitative data were calculated by the integrated density of the HCN channel image/total pixels of all A-fiber or C-fiber cells and presented as OD/pixel.

Isolation of nodose sensory neurons

The region of the NG was excised 6–8 weeks after single dose STZ (n=13) or vehicle (n=13) injection. Both NGs in each rat were removed and placed in ice-cold Ringer's solution (mM): NaCl, 137; $NaHCO_3$, 25; KCl, 3; NaH_2PO_4, 1.25; $CaCl_2$, 1.2; $MgSO_4$, 1.2; glucose, 10. The NGs were dissected free and incubated for 30 min at 37 °C in an enzymatic Ringer's solution containing 0.1% collagenase/0.1% trypsin. The tissues were mechanically triturated and then transferred to a Ringer's solution containing 0.2% collagenase and 0.5% bovine serum albumin (BSA) incubated for 30 min at 37 °C. After digestion, dispersed NG cells were washed in Dulbecco's Modified Eagle's Medium