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MOLECULAR AND FUNCTIONAL CHANGES IN GLUCOKINASE EXPRESSION IN THE BRAINSTEM DORSAL VAGAL COMPLEX IN A MURINE MODEL OF TYPE 1 DIABETES

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Abstract—Glucose concentration changes in the nucleus tractus solitarius (NTS) affect visceral function and metabolism by influencing central vagal circuits, especially inhibitory, GABAergic NTS neurons. Acutely elevated glucose can alter NTS neuron activity, and prolonged hyperglycemia and hypoinsulemia in animal models of type 1 diabetes results in plasticity of neural responses in the NTS. NTS neurons contributing to metabolic regulation therefore act as central glucose sensors and are functionally altered in type 1 diabetes. Glucokinase (GCK) mediates cellular utilization of glucose, linking increased glucose concentration to excitability changes mediated by ATP-sensitive K^+ channels (K_{ATP}). Using quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), Western blot, and *in vitro* electrophysiology, we tested the hypothesis that changes in GCK expression in the NTS accompany the development of diabetes symptoms in the streptozotocin (STZ)-treated mouse model of type 1 diabetes. After several days of hyperglycemia in STZ-treated mice, RNA expression of GCK, but not Kir6.2 or SUR1, was decreased versus controls in the dorsal vagal complex. Electrophysiological recordings *in vitro* indicated that neural responses to acute hyperglycemia, and synaptic responsiveness to blockade of GCK with glucosamine, were attenuated in GABAergic NTS neurons from STZ-treated mice, consistent with reduced molecular and functional expression of GCK in the vagal complex of hyperglycemic, STZ-treated mice. Altered autonomic responses to glucose in type 1 diabetes may therefore involve reduced functional GCK expression in the dorsal vagal complex.

Key words: GABA neuron, hyperglycemia, K_{ATP} channel, nucleus tractus solitarius, postsynaptic current, vagus.

INTRODUCTION

Diabetes mellitus, defined by unequivocally elevated blood glucose levels, affects over 29 million people in the United States (Centers for Disease Control and Prevention, 2014). Some of the serious complications of diabetes include heart disease, stroke, hypertension, blindness, nervous system damage, and gastrointestinal dysfunction. Treatments for the disease remain inadequate, despite substantial investment to reduce symptoms and complications of the disease. Multiple 'preautonomic' areas of the brain contribute to systemic glucose homeostasis (Zsombok and Smith, 2009; Kalsbeek et al., 2010; Yi et al., 2010) and are also affected by elevated blood glucose levels. In particular, neural circuits in the hindbrain play a critical role in regulating plasma glucose and insulin levels. More specifically, vagally-mediated parasympathetic output critically regulates visceral functions related to metabolic homeostasis, and abundant evidence indicates that the brainstem dorsal vagal complex plays a primary and critical role in glucose-sensitive modulation of plasma glucose and insulin levels, feeding, and energy balance (Ritter et al., 1981; Laughton and Powley, 1987; Ritter et al., 2000; Zsombok and Smith, 2009).

Neurons in the brainstem nucleus tractus solitarius (NTS) receive glutamatergic, primary vagal afferent synaptic input from the gut and other thoracic and abdominal viscera. Vagal afferents rapidly convey information about gastrointestinal distention and nutrient content to the NTS, where that information is processed, integrated with neuronal and humoral signals, and transmitted to other brain areas, including the vagal motor neurons of the dorsal motor nucleus of the vagus (DMV). Neurons in the NTS respond to acutely altered glucose concentration with either increases or decreases in neural excitability and altered synaptic input (Oomura et al., 1974; Balfour et al., 2006; Wan and Browning, 2008; Lamy et al., 2014; Boychuk et al., 2015a), which are glucokinase (GCK)-dependent. The depolarizing response is mediated by inactivation of ATP-sensitive K^+ (K_{ATP}) channels (Balfour et al., 2006; Boychuk et al., 2015a) and K_{ATP} channel modulation prevents the glucose-induced, GABA-mediated inhibition of vagal motor neurons (Ferreira et al., 2001). Type I

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Abbreviations: ACSF, artificial cerebrospinal fluid; CT, cycle threshold; DMV, dorsal motor nucleus of the vagus; EDTA, Ethylenediaminetetraacetic acid; EGFP, enhanced green fluorescent protein; EPSC, excitatory postsynaptic current; GA, glucosamine; GCK, glucokinase; IPSC, inhibitory postsynaptic current; K_{ATP}, ATP-sensitive K^+ channels; NTS, nucleus tractus solitarius; RT-PCR, reverse transcriptase-polymerase chain reaction; STZ, streptozotocin; TTX, tetrodotoxin.

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diabetes is characterized by uncontrolled hyperglycemia due to reduced insulin secretion from pancreatic beta cells. Synaptic and other cellular responses in the dorsal vagal complex are altered in models of type 1 diabetes, even after normalizing glucose concentration (Zsombok et al., 2011; Browning, 2013; Blake and Smith, 2014; Bach et al., 2015; Boychuk et al., 2015b). Vagal reflexes are often blunted during chronic hyperglycemia, and altered vagal function may contribute to diabetes-associated visceral dysfunction (Saltzman and McCallum, 1983; Undeland et al., 1998), suggesting that chronically-elevated glucose alters responsiveness of neurons in the dorsal vagal complex.

Because of the involvement of GCK and K_{ATP} channel modulation in the neuronal response to glucose, and the altered responsiveness of NTS neurons in animal models of type 1 diabetes, we tested the hypothesis that GCK or K_{ATP} channel expression is altered after several days of chronic hyperglycemia/hypoinsulemia in the streptozotocin (STZ)-treated mouse. Understanding how glucose sensitivity in the dorsal brainstem is altered in diabetes may offer hypotheses to guide the development of alternative therapies for the disease.

EXPERIMENTAL PROCEDURES

Animals

Mice were treated and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the University of Kentucky Animal Care and Use Committee (Animal Welfare Assurance Number A3336–01). Euthanasia was accomplished by anesthesia with isoflurane to effect (IsoThesia; Henry Schein, Melville, NY, United States), followed by decapitation while anesthetized. Juvenile and young adult (28–42 days) male CD-1 (Harlan Laboratories, Indianapolis, IN, United States) or transgenic 'GIN' mice (FVB-Tg (GadGFP) 4570Swn/J; The Jackson Laboratory, Bar Harbor, ME, United States) were used for all experiments and housed under a standard 14-h light-10-h dark cycle, with food and water provided without restriction. The GIN mice express enhanced green fluorescent protein (EGFP) in a subset of GABA neurons in the NTS, which comprise a large proportion of NTS neurons (Oliva et al., 2000; Williams and Smith, 2006; Glatzer et al., 2007; Boychuk et al., 2015a).

STZ injection

Intra-peritoneal injection of STZ (200 mg/kg in 0.9% NaCl), which kills insulin-secreting pancreatic β cells, was used to induce chronic hyperglycemia in mice. Blood glucose concentration (non-fasted) was measured by tail puncture using a Nova Max PLUS glucometer from normal mice, which were then fasted for 4–6 h prior to STZ or vehicle injection. Control mice were either injected with saline (0.9% NaCl) or untreated. No differences in electrophysiological parameters were observed between normoglycemic saline-injected and untreated mice; they were therefore pooled and considered as a single control group. Blood glucose levels (non-fasted) were measured daily. Onset of hyperglycemia (i.e., blood glucose concentration above 300 mg/dl) varied between animals, but occurred between 1 and 3 days post-STZ injection and remained elevated until the day of the experiment. Animals were used for electrophysiological recordings and molecular analyses after 3–4 days of continuous hyperglycemia.

Brain slice preparation

On-cell and whole-cell voltage-clamp recordings were made using brain stem slices prepared from male GIN mice, 4–5 weeks of age. Animals were deeply anesthetized by isoflurane inhalation (IsoThesia; Henry Schein) and decapitated while anesthetized. The brain was rapidly removed and blocked to isolate the brainstem and then glued to a sectioning stage. Transverse (coronal) brain stem slices (300 μm) containing the caudal NTS (i.e., ±600 μm rostral and caudal to area postrema) were made in ice cold, oxygenated (95% O_2–5% CO_2) artificial cerebrospinal fluid (ACSF) using a vibrating microtome (Vibratome Series 1000; Technical Products, St. Louis, MO, United States). The ACSF contained (in mM): 124 NaCl, 3 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 1.4 NaH_2PO_4, 26 $NaHCO_3$, and 11 or 2.5 glucose (pH 7.15–7.3); osmolality was adjusted to 290–310 mOsm/kg with sucrose; equimolar ACSF was made by substitution of sucrose for glucose substitution experiments. Slices were incubated for an equilibration period for ⩾1 h in warmed (30–33 °C), oxygenated ACSF prior to recording.

Electrophysiology

A single brain slice was transferred to a recording chamber mounted on a fixed stage under an upright microscope (BX51WI; Olympus, Melville, NY, United States), where it was continually perfused by warmed (30–33 °C), oxygenated ACSF. EGFP-labeled NTS neurons were targeted for recording under a 40× water-immersion objective with fluorescence and infrared-differential interference contrast (IR-DIC) optics, as previously described (Williams and Smith, 2006; Glatzer et al., 2007; Gao et al., 2009; Boychuk et al., 2015a). For recordings from EGFP-labeled NTS neurons, initial visualization was made briefly under epifluorescence using a fluorescein isothiocyanate (FITC) filter set (excitation filter wavelengths: 450–490 nm).

On-cell and whole-cell patch-clamp recordings were obtained in the NTS using pipettes pulled from borosilicate glass (Garner Glass, Claremont, CA, United States; open tip resistance 4–6 MΩ) using a Sutter P-87 horizontal puller (Sutter Instrument Co., Novato, CA, United States). Pipettes were filled with a solution containing (in mM): 130 K^+-gluconate (or Cs^+-gluconate), 1 NaCl, 5 EGTA, 10 HEPES, 1 $MgCl_2$, 1 $CaCl_2$, 3 KOH (or CsOH), 2–4 ATP (pH 7.15–7.3). Electrophysiological signals were recorded using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA, United States), low-pass filtered at 3 kHz, and recorded onto a PC-style computer (Digidata 1440A, Molecular Devices) using pClamp 10.2 software