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

PhD Thesis

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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

Diabetes mellitus, defined by unequivocally elevated blood glucose levels, affects over 29 million people in the United States. 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 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.

Neurons in the brainstem nucleus of the solitary tract (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 to 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, which are

glucokinase-dependent. The depolarizing response is mediated by inactivation of K_{ATP} channels and K_{ATP} channel modulation prevents the glucose-induced, GABA mediated inhibition of vagal motor neurons. Type I Diabetes is characterized by uncontrolled hyperglycemia due to loss of 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. Vagal reflexes are often blunted during chronic hyperglycemia, and altered vagal function may contribute to diabetes-associated visceral dysfunction , 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 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 development of alternative therapies for the disease.

Juvenile and young adult (28–42 days) male CD-1 or transgenic 'GIN' mice (FVB-Tg (GadGFP) 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 EGFP in the somatostatinergic subset of GABA neurons in the NTS, which comprise a large proportion of NTS neurons.

On-cell and whole-cell voltage-clamp recordings were made using brain stem slices prepared from male and female GIN mice, 4-5 wk of age. Recording postsynaptic currents and cellular activity.

Brainstem punches including the NTS and DMV were used for QPCR to evaluate the Glucokinase (GCK) Kir 6.2 and SUR1 RNA expression level.

For Wetern blot, brainstem slices were used as same as for QPCR, to isolate protein and differenciate the expression of GCK.

Molecular expression of GCK and KATP channels. Punches of tissue containing the dorsal brainstem were collected from normoglycemic (n=8; glucose index 180 ± 4 mg/dl) and STZ-treated mice that were hyperglycemic for 3-4 days (n=8; 469 ± 5 mg/dl). All target transcript measurements were normalized to β-actin expression. Quantitative RT-PCR revealed a significant decrease in GCK expression in the dorsal vagal complex from STZ-treated hyperglycemic mice versus controls. No significant expression differences were detected for Kir6.2 or SUR1 transcripts between normoglycemic and hyperglycemic mice ($p>0.05$). Molecular expression of GCK, but not components of the K_{ATP} channel, was reduced in STZ-treated, hyperglycemic mice relative to control mice.

To determine if the decrease in mRNA transcription resulted in decreased protein expression, Western blots were performed on punches from an additional 8 control and 8 STZ-treated hyperglycemic mice. Western blot analysis indicated that GCK protein expression was significantly reduced in the dorsal vagal complex of STZ-treated hyperglycemic mice.

Effect of GCK inhibition on synaptic input to NTS neurons. Glucokinase inhibition prevents responses to acute hypoglycemia in a subset of GABAergic NTS neurons. Since GCK expression was reduced in the NTS of STZ-treated mice after several days of hyperglycemia, responses to the GCK inhibitor, GA (5 μM) were determined in GABAergic medial NTS (mNTS) neurons, identified by expression of EGFP in acute slices from normoglycemic control and STZ-treated GIN mice. In GABAergic mNTS neurons from normal mice, GA application

decreased the frequency of sEPSCs by ≥20% in 80% of neurons (12 of 15 neurons; 2.83 ± 0.34 Hz control ACSF; 1.72 ± 0.22 Hz in GA; n=15; $p<0.05$;). Glucosamine application resulted in either a decrease (n=12) or no change (n=3) in sEPSC frequency. No effect on synaptic current amplitude was detected ($p>0.05$). These findings are consistent with an effect of GCK in mediating excitatory synaptic responses to ambient glucose levels of GABAergic NTS neurons and indicated that GCK blockade inhibited excitatory, glutamatergic synaptic input to most GABA neurons.

In GABAergic mNTS neurons from STZ-treated mice after 3-5 days of hyperglycemia, effects of GA on sEPSC frequency were significantly less robust than in controls, being reduced (-22%) in only one of six neurons and unchanged in the remaining five cells. Overall, mean sEPSC frequency was unchanged in the presence of GA (7.63 ± 1.04 Hz in control ACSF; 7.04 ± 0.83 Hz in GA; n=6; $p>0.05$). Consistent with the decreased GCK expression in the vagal complex, modulation of excitatory synaptic activity by GA was reduced in GABAergic mNTS neurons from STZ-treated, hyperglycemic mice.

Effects of GA application on sIPSC frequency and amplitude were also determined. In GABAergic mNTS neurons from control mice, GA application was without effect on the overall population (1.22 ± 0.2 Hz control, ACSF; 1.25 ± 0.30 Hz, GA; n=9; $p>0.05$), but was either increased (n=3) or decreased (n=6) in individual neurons. The amplitude of sIPSCs was also unchanged by GA ($p>0.05$). Similar to results in control mice, there was no overall effect on sIPSC frequency in neurons from STZ-treated mice (1.04 ± 0.30 Hz control ACSF; 1.01 ± 0.31 Hz GA; n=7; $p>0.05$). sIPSC amplitude was also unchanged ($p>0.05$). In neurons from STZ-treated mice, GA application either increased (n=3), decreased (n-2) or was without effect (n=2) on sIPSC frequency. Although sIPSC frequency in GABAergic mNTS neurons was usually

altered by GA application, robust differences between responses in neurons from control and STZ-treated mice were not observed.

Glucose effect on Action Potentials. Neurons in the NTS are glucose sensors, and this sensitivity may be especially prominent in GABAergic NTS neurons. Since GCK expression was reduced in the NTS of STZ-treated mice after several days of hyperglycemia, we examined action potential frequency of GABAergic NTS neurons in response to elevating glucose from 2.5 to 15 mM using on-cell recordings. In neurons from normoglycemic control mice, increasing glucose concentration resulted in a >20% change in action potential frequency in 78% of neurons (7 of 9 cells). Increasing glucose concentration resulted in an increase in action potential frequency in five of nine neurons (87 ± 30% increase; $p<0.05$), a decrease in two neurons (-26.5 ± 2% decrease), and no change in two neurons. In neurons from STZ-treated, hyperglycemic mice, elevating glucose resulted in a change in action potential frequency in only three of seven neurons (43%). The frequency of action potentials was increased in two neurons (115 ± 31%), decreased in one cell (-45%), and was unaffected in the remaining four cells. Whereas increasing glucose concentration resulted in a significant and large change in action potential frequency in the majority of neurons in normoglycemic mice, action potential frequency in most neurons from hyperglycemic mice was unaffected by increasing glucose concentration.

Neurons in the NTS receive direct input from the primary viscerosensory vagal afferents and comprise the initial response component of central parasympathetic regulatory circuits. Subsets of these neurons, and GABA neurons in particular, are known to be glucose-sensitive. Several physiological aspects of central vagal circuitry are altered functionally in diabetes, consistent with altered parasympathetic regulation of the viscera concurrent with the disease. Glucose sensing in NTS neurons involves GCK, which catalyzes the conversion of glucose to

glucose-6 phosphate in neurons and other cells, resulting in increased ATP/ADP ratio. In several neural systems, membrane responses after increased glucose concentration are caused by ATP binding to K_{ATP} channels to affect changes in membrane potential. Diabetes induces changes in GCK or KATP channel expression in the hypothalamus, which are consistent with altered neuronal responses to glucose. Altered electrophysiological responsiveness of NTS neurons in type 1 diabetes has been demonstrated, which could contribute to visceral dysregulation in diabetes, but the mechanisms of this plasticity are unknown. Here, we found that molecular and functional expression of GCK—but not components of the K_{ATP} channel—were diminished in the vagal complex of mice with type 1diabetes. Consistent with decreased mRNA transcription, GCK protein levels were reduced, neuronal and synaptic responses to GCK blockade were attenuated, and neuronal activity responses to increased glucose concentration were diminished. These findings suggest that responses of NTS neurons to increased glucose concentration may be altered as a consequence of chronic hyperglycemia in a GCK-dependent manner.

Neurons in the NTS normally respond to elevated or reduced glucose concentration with either increases or decreases in excitability, which are often GCK-dependent. Glucose-induced increases in excitation are mediated by inactivation of K_{ATP} channels in NTS neurons and K_{ATP} channel modulation prevents the glucose-induced, GABA mediated inhibition of vagal motor neurons. We tested the hypothesis that expression of molecular components of the K_{ATP} channel was altered after several days of hyperglycemia, as may occur in hypothalamic neurons. K_{ATP} channels in central neurons are mainly composed of SUR1 and Kir6.2 subunits and SUR1 is expressed by glucose-sensing NTS cells. We found that molecular expression of SUR1 and Kir6.2 was unchanged in the vagal complex of mice with type 1 diabetes.

Since K_{ATP} channel-mediated responses to glucose in the NTS require GCK, the decrease in GCK expression suggests a mechanism for blunted responsiveness to glucose that has been proposed to occur in chronically hyperglycemic mice. The decreased molecular and protein expression of GCK we observed was consistent with an attenuation of the electrophysiological response to GCK inhibition. Blockade of GCK activity resulted in altered synaptic excitability of most GABAergic NTS neurons from normoglycemic mice, consistent with tonic GCK-mediated activity in the slice. The effects of blocking GCK were reduced in neurons from mice with type 1 diabetes, especially on glutamate release, suggesting that GABAergic NTS neurons may be less responsive to excitatory synaptic input in mice with type 1diabetes. Moreover, responsiveness of GABAergic NTS neurons to increased glucose concentration was attenuated in STZ-treated, hyperglycemic mice. This further implied that synaptic activity in the NTS normally occurs in the context of glucose concentration, since GCK mediates glucose-responsiveness in these neurons.

Previous studies indicated that responses of NTS neurons to acute hypoglycemia required GCK activity. Responses to transient hypoglycemia were previously reported in NTS neurons that expressed glucose transporter 2 (GLUT2) and the expression of the transporter was colocalized in a subset of GABA neurons. We recently showed that GABA neurons were either depolarized or hyperpolarized by glucose, and the depolarization in particular was sensitive to blockade of GCK activity or by blocking K_{ATP} channels. Here, we found that attenuated neuronal responses to transient hyperglycemia in mice with type 1 diabetes coincided with reduced molecular, protein, and functional GCK expression in the vagal complex, suggesting that prolonged hyperglycemia affects glucose responsiveness in the vagal complex.

Elevated glucose concentration increases glutamatergic synaptic transmission from vagal afferents in rats and mice, and these effects were reported to be attenuated in a model of type 1 diabetes. Our results are consistent with this report, and offer a mechanistic explanation for the loss of response. The glucoregulatory response to nutritive substances applied in the intestine is prevented by blocking ionotropic glutamate receptors in the NTS, suggesting that glutamatergic, vagal afferent activation of NTS neurons may required for this response. Blockade of NMDA receptors in the vagal complex prevents the positive effect of bariatric surgery on systemic blood glucose levels, an effect hypothesized to occur by inhibiting a "gut-brainstem-liver" circuit in the vagal complex. Our results support the hypothesis that diminished GCK expression in the vagal complex of mice with type 1 diabetes results in reduced responsiveness of NTS neurons to glucose, including the response to synaptic glutamate release. It is likely that glutamatergic, visceral afferent synaptic input to the NTS, including inputs mediating mechano- and chemoreceptor activity in the gut, occurs in the context of glucose concentration. Restoring GCK expression may help restore physiological responsiveness of NTS neurons to glucose, thereby helping to normalize parasympathetic regulation of visceral function, including glucose homeostasis, in diabetes.

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