Hypoglycemia impairs glucose sensing neurons

Recurrent hypoglycemia reduces the glucose sensitivity of glucose-inhibited neurons in the ventromedial hypothalamus nucleus (VMN)

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ABSTRACT

Recurrent hypoglycemia blunts the brain’s ability to sense and respond to subsequent hypoglycemic episodes. Glucose sensing neurons in the ventromedial hypothalamus nucleus (VMN) are well situated to play a role in hypoglycemia detection. VMN glucose-inhibited (GI) neurons, which decrease their firing rate as extracellular glucose increases, are extremely sensitive to decreased extracellular glucose. We hypothesize that recurrent hypoglycemia decreases the glucose sensitivity of VMN GI neurons. To test our hypothesis, 14-21 day old Sprague-Dawley rats were subcutaneously injected with regular human insulin (4 U/kg) or saline (control) for 3 consecutive days. Blood glucose levels 1 hour after insulin injection on day 3 were significantly lower than on day 1, reflecting an impaired ability to counteract hypoglycemia. On day 4, the glucose sensitivity of VMN GI neurons was measured using conventional whole cell current-clamp recording. Following recurrent insulin-induced hypoglycemia, VMN GI neurons only responded to a glucose decrease from 2.5 to 0.1, but not 0.5, mM. Additionally, lactate supplementation also decreased glucose sensitivity of VMN GI neurons. Thus, our findings suggest that decreases in glucose sensitivity of VMN GI neurons may contribute to the impairments in central glucose sensing mechanisms following recurrent hypoglycemia.

Key words: Insulin, lactate, counterregulatory response, hypoglycemia-associated autonomic failure, whole-cell current recording
INTRODUCTION

Hypoglycemia is a major side effect of intensive insulin therapy in diabetic patients. Recurrent hypoglycemia impairs the sympathoadrenal and neuroendocrine counterregulatory responses (CRR) to hypoglycemia that normally restore euglycemia. This results from a decreased ability of the brain to sense hypoglycemia (5). Severe hypoglycemia has potentially devastating effects on the brain, including impairment of memory and cognitive function (19). This is especially dangerous for the developing brain (13). However, the mechanisms by which the brain senses impending hypoglycemia and signals for the initiation of the CRR are unclear.

Local glucopenia in the ventromedial hypothalamus (VMH), caused by the non-metabolizable glucose analog, 2-deoxyglucose, triggers the release of the counterregulatory hormones, epinephrine and glucagons (2). Conversely, VMH glucose infusion suppresses their release during systemic hypoglycemia (3). The ventromedial hypothalamic nucleus (VMN) within the VMH contains glucose sensing neurons, which are exquisitely sensitive to decreases in extracellular glucose (16, 17). Thus, VMN glucose sensing neurons are well situated to play a key role in the detection of hypoglycemia and initiation of the CRR. Thus, we hypothesize that impaired central glucose sensitivity following recurrent hypoglycemia is associated with reduced glucose sensitivity of VMN glucose sensing neurons.
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Other fuel sources (e.g., lactate and ketone bodies) also support energy metabolism during systemic hypoglycemia (8, 18). For example, VMH lactate infusion suppresses the CRR during systemic hypoglycemia (4). Lactate regulates the activity of VMN glucose sensing neurons (17). Furthermore, if VMN glucose sensing neurons play a key role in hypoglycemia detection and initiation of the CRR, then their glucose sensitivity should be impaired in other situations where the CRR to hypoglycemia is suppressed. Thus, we also hypothesize that lactate supplementation will decrease the glucose sensitivity of VMN glucose sensing neurons.

METHODS

Induction of hypoglycemia. Male 14-21 day old Sprague-Dawley rats were obtained from colonies at the New Jersey Medical School in Newark, New Jersey. Animals were housed with their dams on a 12-h light/dark cycle at 22-23 °C and fed with low-fat diet (Purina rat chow #5001) and water ad libitum. At 9:00 am, rats were subcutaneously injected with regular human insulin (4 U/Kg, Eli Lilly, Indianapolis, USA) or saline (1 ml/Kg) for 3 consecutive days. Pups were returned to their mothers after injection. Blood glucose was measured via tail blood with a blood glucose meter (OneTouch Ultra, LifeScan, Inc., Milpitas, USA) hourly, for 6 hours postinjection in a group of animals not used for electrophysiological study.
Preparation of brain slices. On the fourth day, a separate group of rats were anesthetized with ketamine/xylazine and transcardially perfused with ice cold oxygenated (95% O\textsubscript{2}/5% CO\textsubscript{2}) perfusion solution composed of the following (mM): 2.5 KCl, 7 MgCl\textsubscript{2}, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, 28 NaHCO\textsubscript{3}, 0.5 CaCl\textsubscript{2}, 7 Glucose, 1 Ascorbate, 3 Pyruvate; osmolarity adjusted to approximately 300 mOsm with sucrose; pH 7.4. Brains were rapidly removed and placed in ice cold (slushy) oxygenated perfusion solution. Sections (350 \(\mu\)m) through the hypothalamus were made on a vibratome (Vibroslice, Camden Instruments). The brain slices were maintained at 34 °C in oxygenated artificial cerebrospinal fluid (ACSF; mM: 126 NaCl, 1.9 KCl, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 26 NaHCO\textsubscript{3}, 10 glucose, 1.3 MgCl\textsubscript{2}, 2.4 CaCl\textsubscript{2}; osmolarity adjusted to approximately 300 mOsm with sucrose; pH 7.4) for 15 min, and allowed to come to room temperature. They were then transferred to normal oxygenated ACSF (2.5 mM glucose) for the remainder of the day.

Electrophysiology. Viable neurons were visualized and studied under infrared differential-interference contrast microscopy using a Leica DMLFS microscope equipped with a 40X long working-distance water-immersion objective. Current clamp recordings (standard whole cell recording configuration) from neurons in the VMN were made using a MultiClamp 700A and analyzed using pCLAMP 9 software. During recording brain slices were perfused at 10 ml/min with normal oxygenated ACSF. 1-3 M\(\Omega\) electrodes were filled with an intracellular solution containing (mM): 128 K-gluconate, 10 KCl, 4 KOH, 10 HEPES, 4 MgCl\textsubscript{2}, 0.5 CaCl\textsubscript{2}, 5 EGTA, and 2 Na\textsubscript{2}ATP; pH 7.2. Osmolarity was
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adjusted to 290 - 300 mOsm with sucrose. Input resistance (IR) was calculated from the change in membrane potential measured during the last 1 minute of a small 500 msec hyperpolarizing pulse (-20 pA) given every 3 seconds. Extracellular glucose levels were altered as described in the figures. Each data figure represents consecutive current clamp recordings from VMN neurons in brain slices using conventional whole cell patch clamp.

Changes in glucose sensitivity were quantitated using the percent change in IR. This is because action potential frequency (APF) can vary between brain slices due to variation in presynaptic inputs which remain intact as a result of the exact location of the slice. However, we have found that changes in IR in response to glucose are extremely consistent between slices as well as animals (17, 21).

Statistical analysis. All data were expressed as mean ± SE. Statistical analysis was performed using the Students t-test. P<0.05 was considered to be statistically significant.

RESULTS

14-21 day old Sprague-Dawley rats were subcutaneously injected with regular human insulin (4 U/kg;) or saline (control;) for 3 consecutive days. As shown in Table 1, blood glucose levels 1 hour after insulin injection on day 3 were significantly lower than on day 1 (1.4 ± 0.1 vs 2.3 ± 0.2 mM, respectively, n = 10 rats/group; p<0.05), reflecting an impaired ability to counteract
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Hypoglycemia. Blood glucose levels gradually recovered to normal levels 3 hrs after insulin injection. Saline injection had no effect on blood glucose levels at any of the times measured. These data indicate that the ability to counteract hypoglycemia was selectively impaired in the insulin-treated animals, and was not a result of injection stress.

On day 4, brain slices (350 \mu M) through the VMN from 38 saline treated and 19 insulin treated rats were perfused with artificial cerebrospinal fluid (ACSF) and evaluated for glucose sensitivity. Using conventional whole cell current clamp recording, we have previously established that VMN GI neurons increase their APF and IR in response to physiological decreases in extracellular glucose levels (17). Thus, GI neurons were initially defined in saline- and insulin-treated rats by an increase in APF and IR as glucose decreased from 2.5 to 0.1 mM. In order to be certain that this initial exposure of the brain slice to 0.1 mM glucose did not alter subsequent glucose sensitivity, a subset of neurons in saline-treated rats was exposed to a second decrease in glucose from 2.5 to 0.1 mM. There was no significant difference in the percent increase in IR between the two 0.1 mM glucose challenges (1st exposure: 53 +/- 11%; 2nd exposure: 52 +/- 9%; n = 8; P = 0.79, Students paired t test).

Similar percentages of GI neurons were observed in the saline- (15 out of 62 recorded neurons, 23%) vs insulin- (6 out of 26 recorded neurons, 24%) treated animals. There were no significant differences between membrane
potential (MP) and IR in 2.5 mM glucose for the saline (MP: -54 ± 2 mV; IR: 556 ± 39 Ω; n = 15) and insulin-treated rats (MP: -47 ± 4 mV; IR: 557 ± 12 Ω; n = 6). VMN GI neurons from saline-injected rats reversibly increased APF, IR and MP as extracellular glucose decreased (Fig. 1A). IR and MP increased significantly more as glucose levels decreased from 2.5 to 0.1 mM (55.6 ± 6.2%, n = 15, 17.0 ± 1.7%, n = 15, respectively) than from 2.5 to 0.5 mM (24.1 ± 8.0%, n = 9, p<0.05; 7.9 ± 2.4%, respectively, n=9, p<0.05). In contrast, following recurrent hypoglycemia VMN GI neurons failed to respond to a decrease in extracellular glucose from 2.5 to 0.5 mM (% change in IR: -0.7 ± 1.7%, n = 6 vs saline: 24.1 ± 7.9%, n = 9; p<0.001; Figs. 1B and 2A). However, IR increased to a similar degree in insulin-treated and control rats as glucose decreased to 0.1 mM (49.4 ± 20.5%, n = 6; vs 55.6 ± 6.3%, respectively, n = 9; p=0.7; Fig 2B).

Lactate addition also decreased the glucose sensitivity of VMN GI neurons. APF increased as glucose decreased from 2.5 to 0.1 but not to 0.5 mM (Fig. 3). IR of VMN GI neurons only increased by 8.5 ± 4.7% (n = 5) in the presence vs 36.9 ± 10% (n = 5) in the absence of added lactate (p<0.05; Fig. 4). Thus, recurrent hypoglycemia and lactate addition significantly reduced the glucose sensitivity of VMN GI neurons.

**DISCUSSION**

*In vivo* human and animal studies in adults clearly demonstrate that recurrent hypoglycemia blunts the CRR (6, 14). This results from an increased brain glucose threshold for the detection of hypoglycemia. Thus, brain glucose
levels are allowed to fall to dangerous or even lethal levels before the CRR is initiated (1, 5). We are the first to show that recurrent hypoglycemia also impairs the ability to counteract hypoglycemia in young and most importantly, suckling, rats as evidenced by lower blood glucose levels following recurrent hypoglycemia. \textit{In vivo} studies also show that the CRR is blunted when lactate is infused into the VMH during systemic hypoglycemia (4). We have previously shown that VMN glucose sensing neurons have the potential to sense impending hypoglycemia (17). Here, we found that recurrent hypoglycemia and lactate supplementation significantly reduced the glucose sensitivity of VMN GI neurons in suckling rats whose ability to counteract hypoglycemia was impaired. These data provide strong support for our hypothesis that VMN GI neurons play a key role in hypoglycemia detection and initiation of the CRR.

Since these studies were carried out in juvenile animals, caution must be exercised in extrapolating these data to the adult human. However, we feel this animal model has important implications. First, the developing brain may be more sensitive to the damaging effects of hypoglycemia. Children under 10 years of age with type 1 diabetes mellitus show a broad degree of neurocognitive dysfunction, involving perceptual, motor, memory and attention tasks (13). Recurrent hypoglycemia impairs hippocampal long-term potentiation in young rats (22). Furthermore, infant (nursing) humans exhibit an impaired CRR to hypoglycemia (11). Finally, the CRR is initiated at lower glucose levels in young children (10).
On the other hand, young suckling animals exhibit higher levels of monocarboxylic acid transporters, suggesting that their brains may be using more lactate and ketone bodies than mature animals (20). This may reduce the effectiveness of our model of recurrent hypoglycemia. Furthermore, the pups may not all suckle equally when returned to their dams. This potentially increases the variability in this study. Therefore, this may not be the most robust model in which to study impaired glucose sensing mechanisms following recurrent hypoglycemia. However, despite these limitations, the data in Table 1 clearly demonstrate that even though the pups were returned to their dams to suckle after the insulin injection, they experienced a hypoglycemic challenge sufficient to impair glucose recovery during subsequent insulin-induced hypoglycemia. Since recurrent hypoglycemia (and VMH lactate perfusion) similarly impairs glucose recovery in adults, we believe that these results are germane to the deleterious effects of recurrent hypoglycemia observed clinically. It is possible that the injections elicited a stress response, independent of hypoglycemia. However, this does not seem likely since the saline-injected pups did not exhibit stress-induced hyperglycemia.

A final benefit of using young rats relates to the paucity of GI neurons in the VMN (12, 16). These neurons can not be identified prior to electrophysiological recording. Additionally, at least 5 to 10 min. are required to observe glucose effects (17). Due to variations in neuronal activity we are never
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absolutely confident of a glucose response unless we observe reversal upon washout. Therefore, we must maintain a giga-ohm seal in a healthy neuron for an hour in order to measure the response to at least 2 glucose concentrations. It is much easier to obtain healthy cells which can withstand extended recordings in younger animals. Moreover, we have published detailed glucose concentration-response relations for glucose sensing neurons in animals of this age using the whole cell patch clamp configuration (17, 21). Thus, we did not have to re-establish a half maximal concentration of glucose which would most likely reveal a difference in glucose sensitivity between saline and insulin-treated animals. Overall, the use of young, suckling animals facilitates the study of the mechanisms underlying the impaired CRR following recurrent hypoglycemia in a model which is extremely relevant to pediatric medicine.

The mechanism by which lactate reduces glucose sensitivity is not clear. One possibility is that it is being used simply as a fuel to support neuronal activity in both glucose sensing and non-glucose sensing neurons. In support of this, inhibition of lactate transport during hypoglycemia increases neuronal damage, which suggests that lactate fuels neurons during hypoglycemia (9). Increased lactate could supply ATP and replace glucose in the regulation of GI neurons. However, this is not consistent with our previous studies showing that lactate regulated the activity of VMN glucose sensing neurons in a manner distinct from that of glucose. For example, while GI neurons were inhibited by glucose, they were excited by lactate in both high and low glucose concentrations (17).
Another possibility is that lactate replaces glucose as a fuel source only for non-glucose sensing neurons, while also serving as a signal which regulates neuronal activity in glucose sensing neurons.

In either case, we speculate that hypoglycemia induces local neuroprotective mechanisms that impair the glucose sensitivity of VMN GI neurons. In support of this, hypoglycemia increases neuronal and blood brain barrier glucose and lactate transporter expression (7, 9, 15). This would protect the brain during an acute hypoglycemic event. However, recurrent hypoglycemia may result in sustained upregulation of nutrient availability/utilization to protect the brain. As a result, glucose sensing neurons would not perceive the intensity of the glucose deficit and signal for an appropriate CRR. Finally, these data indicate the importance of approximating the physiological milieu surrounding glucose sensing neurons in order to properly study them. Clearly, lactate which is a component of the extracellular fluid in the brain has a significant effect on the function of glucose sensing neurons. Thus, it is very important that studies, especially of isolated neurons, include lactate in the perfusion solution. This is less of a concern for studies of brain slices which contain glial cells, since these cells presumably provide a certain level of lactate to adjacent neurons.

In conclusion, our findings indicate that decreased glucose sensitivity of VMN GI neurons in young suckling rats may contribute to their impaired glucose
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ACKNOWLEDGEMENTS

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REFERENCES


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Table 1. Blood glucose levels in 14 – 21 day old Sprague-Dawley rats following s.c. insulin (4U/kg) or saline injections. Values are the mean +/- the standard error of the mean (SEM); n = 10 for both groups. Glucose levels reached their nadir 1 hour post insulin injection and recovered to control values by 3 hours. On day 3 blood glucose levels at 1 hour in the insulin treated rats were significantly lower than on days 1 and 2 (*P < 0.05). There was no significant effect of saline injections on blood glucose levels.
FIGURE LEGENDS

**Figure 1.** Consecutive whole cell current clamp recordings in a VMN GI neuron from a saline-injected rat. Resting membrane potential (RMP) is noted to the right of each trace in this and subsequent figures. The downward deflections represent the membrane voltage response to a constant hyperpolarizing pulse. **A.** Action potential frequency (APF), input resistance (IR) and membrane potential (MP) reversibly increased when glucose levels decreased from 2.5 to 0.1 (upper trace) or 0.5 mM (lower trace). **B.** Following 3 daily bouts of insulin-induced hypoglycemia, this VMN GI neuron responded to a glucose decrease from 2.5 to 0.1 mM (upper trace), but not from 2.5 to 0.5 mM (lower trace).

**Figure 2. A.** Three daily bouts of insulin-induced hypoglycemia significantly attenuated the increased IR in response to decreased glucose from 2.5 to 0.5 mM in VMN GI neurons. **B.** IR of VMN GI neurons was increased by a similar percent as glucose was decreased from 2.5 to 0.1 mM in saline- and insulin-injected rats.

**Figure 3.** Consecutive whole cell current clamp recordings of a VMN GI neuron. This VMN GI neuron did not respond to a decrease in glucose from 2.5 to 0.1 mM in the presence of 0.5 mM lactate.

**Figure 4.** The addition of lactate significantly attenuated the increased IR to a decrease in glucose from 2.5 to 0.1 mM.
FIGURE 1

A. 2.5 mM Glucose
    ↑  0.1 mM Glucose  2.5 mM Glucose

B. 2.5 mM Glucose
    ↑  0.1 mM Glucose  2.5 mM Glucose
FIGURE 2

A.

% change in IR

$n=9$

2.5 to 0.5 G
Saline

$n=6$

2.5 to 0.5 G
Insulin

B.

% change in IR

$n=9$

2.5 to 0.1 G
Saline

$n=6$

2.5 to 0.1 G
Insulin

*
FIGURE 3

2.5 mM Glucose

0.1 mM Glucose

2.5 mM Glucose

2.5 mM Glucose

+0.5 mM Lactate

0.1 mM Glucose

+0.5 mM Lactate

-55 mV

10 mV

1 min
FIGURE 4

[Diagram showing the effect of 0.1 mM Glucose and 0.1 mM Glucose + 0.5 mM Lactate on % change in IR. n=5 for both conditions.]