Hyperglycemia impairs glucose and insulin regulation of nitric oxide production in glucose-inhibited neurons in the ventromedial hypothalamus

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Canabal DD, Potian JG, Duran RG, McArdle JJ, Routh VH. Hyperglycemia impairs glucose and insulin regulation of nitric oxide production in glucose-inhibited neurons in the ventromedial hypothalamus. Am J Physiol Regul Integr Comp Physiol 293: R592–R600, 2007. First published May 30, 2007; doi:10.1152/ajpregu.00207.2007.—Physiological changes in extracellular glucose, insulin, and leptin regulate glucose-excited (GE) and glucose-inhibited (GI) neurons in the ventromedial hypothalamus (VMH). Nitric oxide (NO) signaling, which is involved in the regulation of food intake and insulin signaling, is altered in obesity and diabetes. We previously showed that glucose and leptin inhibit NO production via the AMP-activated protein kinase (AMPK) pathway, while insulin stimulates NO production via the phosphatidylinositol-3-OH kinase (PI3K) pathway in VMH GI neurons. Hyperglycemia-induced inhibition of AMPK reduces PI3K signaling by activating the mammalian target of rapamycin (mTOR). We hypothesize that hyperglycemia impairs glucose and insulin-regulated NO production in VMH GI neurons. This hypothesis was tested in VMH neurons cultured in hyperglycemic conditions or from streptozotocin-induced type 1 diabetic rats using NO- and membrane potential-sensitive dyes. Neither decreased extracellular glucose from 2.5 to 0.5 mM, nor 5 nM insulin increased NO production in VMH neurons in either experimental condition. Glucose- and insulin-regulated NO production was restored in the presence of the AMPK activator, 5-aminimidazole-4-carboxamide-1-b-4-ribofuranoside or the mTOR inhibitor rapamycin. Finally, decreased glucose and insulin did not alter membrane potential in VMH neurons cultured in hyperglycemic conditions or from streptozotocin-induced rats. These data suggest that hyperglycemia impairs glucose and insulin regulation of NO production through AMPK inhibition. Furthermore, glucose and insulin signaling pathways interact via the mTOR pathway.

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Diabetes is characterized by dysregulation of glucose homeostasis (7). Strong evidence suggests that the hypothalamus, especially the ventromedial hypothalamus (VMH), plays a key role in glucose homeostasis (3, 7, 23, 46). Glucose regulates the action potential frequency of specialized glucose-sensing neurons (GSNs) in the VMH (41, 50). There are two major categories of GSNs; those which directly sense glucose [glucose-excited (GE) and glucose-inhibited (GI) neurons] and those which are presynaptically modulated by glucose (41, 50). GE neurons decrease, while GI neurons increase their action potential frequency (APF) when extracellular glucose is reduced (41). Critical signals of peripheral energy homeostasis (e.g., insulin, leptin) regulate the electrical activity of GSNs (19, 44, 45, 50). We have recently shown that glucose, insulin, and leptin also regulate nitric oxide (NO) production in VMH GI neurons (9).

Neuronal NO synthase (nNOS) produces NO as a byproduct of the conversion of l-arginine to l-citrulline. As a gas, NO regulates the neuron in which it is produced, as well as adjacent cells, by diffusion. Soluble guanylyl cyclase mediates many of NO’s effects (5). VMH NO signaling regulates food intake. Stimulation of NO synthesis by l-arginine increases food intake in mice, while inhibition of NO synthesis decreases food intake in food-deprived mice (29). NOS inhibition blocks orexin- and ghrelin-induced feeding changes (13, 15). Neuropeptide Y increases hypothalamic NOS activity (30), and nNOS knockout mice are refractory to neuropeptide Y- and orexin-induced feeding (31). Central NOS inhibition significantly reduces energy intake and body weight gain in diet-induced obese rats. This was associated with a reduced number of VMH nNOS-immunolabeled cells (37). NO signaling may also be involved in the VMH regulation of glucose homeostasis since NO signaling is dysfunctional in diabetes (35, 39, 53). We have shown that glucose and leptin inhibit NO production in VMH GI neurons via AMP-activated protein kinase (AMPK) inhibition. In contrast, insulin stimulates NO production in VMH GI neurons via the phosphoinositide-3 kinase (PI3K) pathway (9). Since soluble guanylyl cyclase is present in all VMH neurons (9), NO release from GI neurons has the potential to influence the activity of adjacent VMH neurons involved in food intake and glucose homeostasis (38). Thus, NO signaling may link the overall function of the VMH to energy status.

Hyperglycemia inhibits AMPK activity. Therefore, we predict that diabetic hyperglycemia will impair glucose-regulated NO production in VMH GI neurons. Hyperglycemia causes peripheral insulin resistance by releasing AMPK-mediated inhibition of the mammalian target of rapamycin (mTOR). Increased mTOR activation prevents the insulin receptor from phosphorylating the insulin receptor subunit and activating PI3K (17, 24, 49). Thus, insulin-induced hypothalamic NO signaling may also be impaired in diabetes. In support of this, rats with streptozotocin (STZ)-induced type 1 diabetes have a significant reduction in nNOS-positive cells, NO release, nNOS mRNA expression, and nNOS protein levels in the paraventricular hypothalamic nucleus (53).

Impaired insulin-induced NO production may be associated with the development of insulin resistance. Several studies suggest that NO mediates many of the peripheral and central effects of insulin. The NOS inhibitor Nω-nitro-l-arginine methyl ester blocks insulin-stimulated glucose uptake in cul-
tured human vascular smooth muscle cells (2). nNOS knockout mice are insulin resistant (39). Insulin injected into the nucleus tractus solitarius stimulates nNOS, while inhibition of nNOS in the nucleus tractus solitarius prevents insulin’s ability to lower blood pressure (48). Thus, defective hypothalamic NO signaling may play a role in the dysfunctional glucose homeostasis during diabetes. In the current study, we hypothesize that hyperglycemia associated with type 1 diabetes decreases AMPK activity, thereby impairing glucose and insulin regulation of NO production in VMH GI neurons. We tested this hypothesis using membrane potential- and NO-sensitive dyes in cultured VMH neurons from STZ-induced diabetic rats.

MATERIALS AND METHODS

Preparation of cultured neurons. Male 14- to 21-day-old Sprague-Dawley rats were housed with their dams in the animal facility of New Jersey Medical School at 22–23°C on a 12:12-h light-dark cycle and given purified diet (prod. no. D03120101, Research Diets, New Brunswick, NJ) and water ad libitum. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the New Jersey Medical School. On the day of experiment, rats were anesthetized with ketamine/xylazine (80/10 mg/kg ip) and transcardially perfused with ice-cold oxygenated (95% O2, 5% CO2) perfusion solution containing (in mmol/l): 2.5 KCl, 7 MgCl2, 1.25 NaH2PO4, 28 NaHCO3, 0.5 CaCl2, 7 glucose, 1 ascorbate, and 3 pyruvate (osmolarity adjusted to ~300 mOsM with sucrose, pH 7.4). Brains were quickly removed and placed in an ice-cold (slushy) oxygenated perfusion solution. Sections (350 μm) were made through the hypothalamus using a vibratome (Vibrosei; Camden Instruments). Cultured neurons were obtained as described previously (9, 42, 50). Briefly, brain slices were placed in Hibernate A/B27 (Brain Bits, Carlsbad, CA). The VMH [arcuate + ventromedial hypothalamic nucleus (ARC + VMN)] was dissected and digested in Hibernate A with Papain. The tissue was incubated for 30 min and subjected to gentle trituration. After trituring, the cell suspension was centrifuged and the pellet resuspended with growth medium (Invitrogen, Springfield, IL). Neurons were plated in growth medium with fluorescent microspheres (Polysciences, Warrington, PA) for normalization of data and evaluated for NO production within 3 days (9). At the initiation of each experiment, neurons were placed in recording solution containing the following (in mM): 135 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES, and 0.05 l-arginine, pH 7.4. Glucose (0.5, 2.5, or 10 mM), insulin (5 nM), leptin (10 nM), the mTOR inhibitor rapamycin (50 nM) (49), and the AMPK activator, 5-aminomimidazole-4-carboxamide-1-b-4-ribosafuranoside (AICAR; 0.5 mM) (9), were added as described below.

STZ diabetic animal model. Juvenile rats were given a single intraperitoneal injection of STZ (Sigma, St. Louis, MO; 80 mg/kg in 0.1 mM citrate buffer [pH 4.5]) on neonatal day 5 (6, 33, 51). Controls were injected with citrate buffer only. Blood glucose was measured 2 days after injection and prior to death. Any animal that did not reach 15 mmol/l was eliminated from the study. Tissue was harvested 2 wk after injection, and cultured neurons were prepared as above.

Measurement of NO production in cultured neurons using 4-aminomethylamino-2',7'-difuorofluorescein. As described previously (9), neurons were visualized on an Olympus BX61 W1 microscope with a ×10 objective for measurement of the NO sensitive dye 4-aminomethylamino-2',7'-difluorofluorescein (DAF-FM; Invitrogen). Emission was detected with a fluorescent imaging plate reader system-membrane potential-sensitive dye (FLIPR-MPD; Molecular Devices, Sunnyvale, CA; red filter, excitation 548 nm, emission 515–530 nm) and fluorometric imaging plate reader system-membrane potential-sensitive dye (FLIPR-MPD; Molecular Devices, Sunnyvale, CA; red filter, excitation 548 nm, emission 610–675 nm). NO production increases DAF-FM fluorescence intensity (10, 18, 21). The oxidation of DAF-FM NO causes irreversible fluorescein release, so we cannot measure a decrease in fluorescence. Since glucose and leptin are expected to cause a decrease in NO production, we preincubated cultured neurons at 37°C in 1 μM DAF-FM in recording solution with 10, 5, 2.5, or 0.5 mM glucose for 30 min or leptin (10 nM) in 10, 5, 2.5, or 0.5 mM glucose for 60 min. The DAF-FM containing solution was then exchanged for one in which DAF was lacking, and either leptin was absent or glucose was decreased. DAF-FM fluorescence intensity was measured every minute for 30 or 60 min directly after solution change (9). Since insulin is expected to increase NO production, DAF-FM fluorescence intensity was measured immediately after DAF washout, and the addition of 5 nM insulin to experimental solution containing 10, 5, or 2.5 mM glucose. To determine whether the mTOR inhibitor rapamycin (50 nM) or the AMPK activator AICAR (0.5 mM) restored NO production, these compounds were included in the 30-min DAF-FM incubation. They were then reapplied following DAF washout, in the presence of either insulin addition or decreased glucose concentration.

In a subset of experiments, 0.25%, FLIPR-MPD was included during DAF-FM incubation (9). For the FLIPR-MPD experiments, DAF-FM was reapplied between treatments throughout experimental recordings. Control images were captured (Photometrics Cool Snap HQ charge-coupled device camera) every 1 min for 30 min after solution change. Images were acquired/analyzed with MetaMorph software (Universal Imaging). The fluorescence intensity was expressed as gray scale units per pixel. Data were normalized according to the intensity of the fluorescent bead standards. The percent change of DAF-FM or FLIPR-MPD fluorescence intensity for each neuron was calculated as a function of time after solution change (9).

As described previously (9), cells were considered to increase DAF-FM fluorescence intensity in response to treatment if the fluorescence intensity increased by at least 5% within 5–10 min and reached a plateau within 30 min. A lack of response was defined as either a slight decrease (photobleaching) or an increase in DAF-FM fluorescence intensity of <5% within the first 10 min (9). Cells were considered to increase FLIPR-MPD fluorescence intensity in response to treatment if the fluorescence intensity increased or decreased by >8% between 10 and 20 min after treatment (9). The percent of neurons that increased DAF-FM fluorescence intensity out of the total number of cells in each dish was compared between treatments using a two-tailed Student’s t-test with P < 0.05 considered to be statistically significant.

Finally, to test for cell viability in the STZ rats, a subset of cells was stained with 0.25% trypan blue (Sigma) for 5 min after fluorescence imaging (9). Only 2.5% (12 of 465) and 3.4% (10 of 286) of the total cells from control or STZ rats, respectively, were stained with trypan blue.

Western blot analysis. As described previously (9), the VMH (ARC + VMN) was dissected and placed in 2.5 mM glucose for 5 min. VMH were pooled from two rats for a total of six to eight rats per experimental treatment (n = 3–4 samples). The tissue was then homogenized and sonicated. Phosphorylation of nNOS was determined with a 10% Tris-HCl gel by using an antibody against phospho-nNOS (Upstate, Lake Placid, NY). The data were normalized to β-actin (Sigma) and quantified using Scion Image. Each treatment was compared with the control (2.5 mM glucose) by using a two-tailed Student’s t-test with P < 0.05 considered to be statistically significant.

Statistical analysis. The percentages of responsive cells for each dish within control and treatment groups were averaged (9). These averages were compared by using a two-tailed Student’s t-test with P < 0.05 considered to be statistically significant. All data are presented as means ± SE.

RESULTS

Acute hyperglycemia. DAF-FM-loaded cultured VMH neurons were visualized with bright-field and fluorescence microscopy. First, we investigated whether the effects of insulin and leptin are dependent on glucose concentration. Insulin in-
increased NO production in 51% of the neurons in 2.5, but in fewer than 7% in 0.5, 5, or 10 mM glucose (Fig. 1A). Adding insulin to 5 mM glucose in the presence of the mTOR inhibitor rapamycin (50 nM) or the AMP-activated protein kinase (AMPK) activator 5-aminoimidazole-4-carboxamide-1-b-4-ribofuranoside (AICAR; 0.5 mM) restored insulin-induced NO production in 5 mM glucose (Fig. 1B). In contrast, there was no significant difference in the percent of VMH neurons that increased NO production upon leptin washout in any glucose concentration (0.5, 2.5, 5, or 10 mM) (Fig. 1C).

Hyperglycemic culture (5 mM glucose). A subset of dishes of VMH neurons were cultured in growth media containing 5 mM glucose for 1–3 days. Thirty minutes prior to decreasing glucose, one group of these dishes of cells was placed in 2.5 mM glucose, while the other remained in 5 mM glucose. Extracellular glucose was then lowered from either 2.5 or 5 mM, for each group, respectively, to 0.5 mM. Similarly, VMH neurons cultured in 2.5 mM glucose were placed in either 2.5 or 5 mM glucose for 30 min prior to lowering glucose to 0.5 mM. While ~40% of VMH neurons cultured in 2.5 mM glucose increased NO production as extracellular glucose levels decreased from 2.5 to 0.5 mM or from 5 to 0.5 mM (Fig. 2A; bars marked 2.5 mM glucose), <2% of VMH neurons cultured in 5 mM increased NO production as extracellular glucose was lowered from 2.5 to 0.5 mM or 5 to 0.5 mM (Fig. 2A; bars marked 5 mM glucose).

Furthermore, the acute addition of AICAR to 2.5 mM glucose increased NO production in significantly fewer neurons cultured in 5 vs. 2.5 mM glucose (2 ± 1%; 6 dishes, 845 neurons cultured in 2.5 mM glucose were placed in either 2.5 or 5 mM glucose for 30 min prior to lowering glucose to 0.5 mM. While ~40% of VMH neurons cultured in 2.5 mM glucose increased NO production as extracellular glucose levels decreased from 2.5 to 0.5 mM or from 5 to 0.5 mM (Fig. 2A; bars marked 2.5 mM glucose), <2% of VMH neurons cultured in 5 mM increased NO production as extracellular glucose was lowered from 2.5 to 0.5 mM or 5 to 0.5 mM (Fig. 2A; bars marked 5 mM glucose).

Fig. 1. A: %ventromedial hypothalamus (VMH) neurons increased 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) fluorescence intensity [nitric oxide (NO) production] in response to the addition of insulin (5 nM) to 2.5, 0.5, 5, and 10 mM glucose (G). B: insulin addition in the presence of the mammalian target of rapamycin (mTOR) inhibitor rapamycin (Rap; 50 nM) or the AMP-activated protein kinase (AMPK) activator 5-aminoimidazole-4-carboxamide-1-b-4-ribofuranoside (AICAR; 0.5 mM) restored insulin-induced NO production in 5 mM glucose. C: %VMH neurons increased DAF-FM fluorescence intensity (NO production) in response to leptin (10 nM) washout from 2.5, 0.5, 5, and 10 mM glucose. Data are means ± SE; *P < 0.05. The number of dishes and total cells are at the top of each bar. At least 3 rats were used for each treatment.

Fig. 2. A: VMH neurons cultured in 2.5 mM glucose for 1–3 days increased NO production when glucose levels were decreased from either 5 to 0.5 or from 2.5 to 0.5 mM glucose. In contrast, VMH neurons cultured in 5 mM glucose for 1–3 days did not increase NO production when glucose levels were decreased from 5 to 0.5 mM glucose. In addition, VMH neurons cultured in 5 mM glucose did not increase NO production when glucose was lowered to 0.5 mM following 30-min incubation in 2.5 mM glucose. The acute addition of AICAR (0.5 mM) to 2.5 mM glucose in VMH neurons cultured in 2.5, but not 5 mM glucose, increased NO production. B: VMH neurons cultured in 5 mM glucose increased NO production when glucose decreased from 2.5 to 0.5 in the presence of AICAR. C: significantly fewer VMH neurons were characterized as glucose-inhibited (GI) neurons by using fluorometric imaging plate reader system-membrane potential sensitive dye (FLIPR-MPD) fluorescence when cultured in 5 vs. 2.5 mM glucose. Data are means ± SE; *P < 0.05. The number of dishes and total cells are at the top of each bar. At least 3 rats were used for each treatment.
cells vs. 40 ± 6.1%; 6 dishes, 790 cells; \( P = 0.0001 \); Fig. 2A). However, when glucose was decreased from 2.5 to 0.5 mM in the presence of AICAR (0.5 mM), there was no significant difference between the percent of neurons cultured in 5 mM glucose, which increased NO (41 ± 2.7%; 6 dishes, 863 cells) vs. control 2.5 mM glucose cultures (55 ± 7.6%; 6 dishes, 489 cells; \( P = 0.1201 \); Fig. 2B). To determine whether impaired NO production in GI neurons in response to decreased glucose was correlated with loss of membrane depolarization, we simultaneously measured FLIPR-MPD fluorescence in a subset of cells exposed to DAF-FM. When glucose was decreased from 2.5 to 0.5 mM, 41% (186 of 453) of the neurons, which showed DAF-FM fluorescence in 2.5 mM glucose, increased FLIPR-MPD fluorescence (depolarized). This indicates that they are GI neurons. However, only 2.6% (6 of 223, \( P < 0.0001 \)) of VMH neurons that showed DAF-FM fluorescence cultured in 5 mM glucose increased FLIPR-MPD fluorescence when glucose was decreased (Fig. 2C). On the other hand, there was no significant difference between the percent of VMH neurons defined as GE neurons when glucose decreased from 2.5 to 0.5 mM in neurons cultured in 2.5 mM glucose (25 ± 3.3%; 40 of 158 neurons) and neurons cultured in 5 mM glucose (26 ± 0.8%; 59 of 223 neurons; \( P = 0.844 \)). Thus, hyperglycemia decreases the ability of GI neurons to increase their membrane potential and NO production in response to decreased glucose.

As previously shown, insulin (5 nM) increased NO production in ~40% of VMH neurons cultured in 2.5 mM glucose (9). In contrast, insulin only increased NO production in 1.3 ± 0.7% of VMH neurons cultured in 5 mM glucose (\( P < 0.0001 \); Fig. 3A). Since hyperglycemia increases mTOR activity as a result of AMPK inhibition (17, 49), we measured the number of VMH neurons cultured in 5 mM glucose, which increased NO production in response to insulin in the presence of rapamycin (50 nM) or AICAR (0.5 mM). Under these conditions, the percent of VMH neurons increasing NO production in response to insulin was no different than that in 2.5 mM glucose (Fig. 3A). When the PI3K inhibitor wortmannin (10 nM) was added in the presence of AICAR, it significantly reduced the number of NO producing cells to 4.6 ± 2.1%, (\( P < 0.0001 \); Fig. 3A). Finally, when insulin (5 nM) was added to 2.5 mM glucose, 43.7% (119 of 272) of the neurons cultured in 2.5 mM glucose decreased FLIPR-MPD fluorescence (hy- perpolarization). In contrast, insulin only hyperpolarized 14.6 ± 2.0% (26 of 178, \( P < 0.0001 \)) of VMH neurons cultured in 5 mM glucose (Fig. 3B).

NO production in STZ-induced diabetes. To test whether hyperglycemia associated with type 1 diabetes impairs glucose and insulin regulated NO production; we evaluated VMH neurons from STZ-induced diabetic rats that had been cultured for 1–3 days in 2.5 mM glucose. VMH neurons from STZ rats were then placed in either 2.5 or 5 mM glucose 30 min prior to decreasing glucose to 0.5 mM. In contrast to VMH neurons in control rats (Fig. 4A), VMH neurons from STZ rats did not produce NO in either 2.5 (Fig. 4B) or 5 mM glucose (not shown). Moreover, VMH neurons incubated for 30 min in 2.5 or 5 mM glucose did not increase NO production in response to decreasing glucose to 0.5 mM (Fig. 5A). In control rats, the acute addition of AICAR to 2.5 mM glucose increased NO production in 52 ± 0.7% of VMH neurons. In contrast, AICAR did not increase NO in VMH neurons from STZ rats (Fig. 5A).

However, when glucose was decreased from 2.5 to 0.5 mM in the presence of AICAR (0.5 mM), there was no significant difference between the percentage of VMH neurons from STZ rats that increased NO production compared with controls (51 ± 1.6; 5 dishes, 641 cells vs. 50 ± 2.7; 5 dishes, 588 cells, respectively, \( P = 0.8666 \); Fig. 5B). To determine whether impaired NO production in GI neurons in response to decreased glucose was correlated with loss of membrane depolarization, we simultaneously measured FLIPR-MPD and DAF-FM fluorescence. In control rats ~40% of VMH neurons were characterized as GI neurons. In contrast, only 2 ± 0.8% (\( P < 0.0001 \)) of VMH neurons from STZ rats were characterized as GI neurons using FLIPR-MPD fluorescence (Fig. 5C). On the other hand, there was no significant difference between the percent of VMH neurons defined as GE neurons when glucose decreased from 2.5 to 0.5 mM in control (25 ± 3.3%; 40 of 158 neurons) and STZ rats (28 ± 3.2%; 78 of 276 neurons; \( P = 0.828 \)). Thus, hyperglycemia associated with type 1 diabetes impairs the glucose sensitivity of VMH GI but not GE neurons.

Insulin added to 2.5 mM glucose increased NO production in VMH neurons from control rats. In contrast, VMH neurons
from STZ rats did not increase NO production in response to insulin (Fig. 6A). However, when insulin was added to VMH neurons from STZ rats in the presence of AICAR (0.5 mM) or rapamycin (50 nM) there was no significant difference between the percent of neurons that increased NO production in neurons from control or STZ rats (Fig. 6A). Finally, when the PI3K inhibitor wortmannin (10 nM) was added in the presence of AICAR, it significantly reduced the number of NO-producing cells to 3 ± 1.1% ($P < 0.0001$; Fig. 6A). In controls, when insulin (5 nM) was added to 2.5 mM glucose, 43.7% (119 of 272) of the neurons decreased FLIPR-MPD fluorescence (hyperpolarization). In contrast, insulin only hyperpolarized 14.4 ± 1.1%, $P < 0.0001$ of VMH neurons from STZ rats (Fig. 6B).

**DISCUSSION**

We previously showed that glucose and leptin inhibit NO production via AMPK inhibition, while insulin stimulates NO production through the PI3K pathway in VMH GI neurons (8). Here, we tested the hypothesis that hyperglycemia-induced AMPK inhibition impairs the ability of VMH GI neurons to sense decreased glucose using NO- and membrane potential-sensitive dyes. In addition, we hypothesized that hyperglycemia would also impair insulin regulation of VMH GI neurons. Our results show that hyperglycemia prevents both membrane depolarization and NO production in response to decreased glucose in VMH GI neurons (Fig. 2). Hyperglycemia also impairs insulin-induced membrane hyperpolarization and NO production in VMH neurons (Fig. 3). We observed the same response in neurons from STZ diabetic rats (Figs. 5 and 6) and in neurons from control rats cultured under hyperglycemic conditions (Figs. 2 and 3). This suggests that high
glucose itself is sufficient to prevent glucose and insulin-regulated NO production and membrane potential responses in VMH GI neurons. The fact that we observed similar impairments in glucose and insulin sensitivity in VMH GI neurons cultured in 5 mM glucose (Figs. 2 and 3) and in STZ rats (Figs. 5 and 6) strongly suggests that high glucose, and not STZ-induced neuronal damage, is responsible. Furthermore, restoration of glucose and insulin-modulated NO production by preincubation with AICAR or rapamycin is inconsistent with neuronal damage. In addition, there was no difference in the number of neurons stained by trypan blue in the control and STZ rats, indicating that VMH neurons from STZ rats were undamaged. Finally, the observation that culturing VMH neurons in 5 mM glucose is sufficient to induce changes in glucose and insulin sensitivity is physiologically relevant. Microdialysis studies indicate that VMH glucose levels in a healthy conscious rat are 1–2 mM (12). Measurement of VMH glucose using a glucose oxidase electrode in anesthetized rats indicates that VMH extracellular glucose levels are ~3 mM when blood glucose levels are ~8 mM (borderline hyperglycemia) and ~5 mM when blood glucose levels are 400 mg/dl (severe hyperglycemia) (38). Although cerebrospinal fluid glucose levels are slightly higher than VMH (~2–3 mM; Ref. 1), it is most likely that the glucose concentration in the extracellular milieu surrounding VMH neurons in STZ rats is close to 5 mM. Our data further suggest that impaired glucose and insulin sensitivity in the STZ rats is not due to low insulin or the STZ itself. STZ injection causes immediate destruction of the pancreatic β-cells (36). Injecting STZ on neonatal day 5 causes rats to become hyperglycemic and hypoinsulinemic. Thus, neonatal day 5 STZ rats at 2–3 wk of age serve as a model of hyperglycemia and hypoinsulinemia similar to type 1 diabetes (6, 33, 47, 51). Also genetic models of spontaneous type 1 diabetes do not develop hyperglycemia until at least 4 wk of age. It is difficult to isolate sufficiently healthy neurons from adult rats (>4 wk of age) for studies related to metabolic sensing. Thus, the STZ model is ideal for our studies. Use of the STZ-induced animal model raises the concern that effects observed are due to STZ-induced neuronal damage. However, several studies indicate that cen-

Fig. 5. VMH neurons cultured from control rats but not STZ rats increased NO production when glucose levels were decreased from 5 to 0.5 or from 2.5 to 0.5 mM glucose. The acute addition of AICAR (0.5 mM) to 2.5 mM glucose increased NO production in VMH neurons cultured from control, but not STZ, rats. B: VMH neurons cultured from STZ rats increased NO production when glucose decreased from 2.5 to 0.5 mM in the presence of AICAR. C: significantly fewer VMH neurons were characterized as GI neurons using FLIPR-MPD fluorescence when cultured from STZ rats vs. control rats. Data are means ± SE; *P < 0.05. The number of dishes and total cells are at the top of each bar. At least 2 rats were used for each treatment.

Fig. 6. A: insulin (5 nM) increased NO production in VMH neurons from control but not STZ rats. Insulin-induced NO production in 2.5 mM glucose was abolished in STZ rats. However, when insulin was added to 2.5 mM glucose in the presence of the mTOR inhibitor rapamycin (50 nM) or the AMPK activator AICAR (0.5 mM), insulin-induced NO production in VMH neurons from STZ rats was restored. The PI3K inhibitor wortmannin (10 μM) blocked the restoration of insulin-induced NO production in the presence of AICAR. B: insulin hyperpolarized significantly fewer VMH neurons cultured from STZ rats vs. control rats. Data are means ± SE; *P < 0.05. The number of dishes and total cells are at the top of each bar. At least 2 rats were used for each treatment.
tral injection of STZ does not alter peripheral glucose homeostasis (22, 32).

We previously showed that decreased glucose increases NO production via AMPK activation in VMH GI neurons (9). This is dependent on extracellular calcium (9). Depolarization and increased action potential frequency in VMH GI neurons in response to decreased glucose presumably increases calcium influx. nNOS would then be activated by calcium-calmodulin (4). Recent studies suggest that the cystic fibrosis transmembrane regulator (CFTR) chloride conductance mediates the effects of glucose on GI neurons (14). CFTR is inhibited by AMPK (16). Thus we hypothesize that low glucose increases AMPK activity, inhibiting the CFTR chloride conductance and depolarizing the cell. This allows calcium entry, which activates nNOS and increases NO production. Our data showing that hyperglycemia associated with type 1 diabetes blocks both depolarization and NO production in GI neurons support this hypothesis (Fig. 5).

Interestingly, a relatively brief episode of hyperglycemia is sufficient to inhibit insulin-induced NO production. That is, we found that a 30-min exposure to 5 or 10 mM glucose blocked insulin-induced NO production (Fig. 1A). Insulin-induced NO production was also absent in 0.5 mM glucose (Fig. 1A), but this is not likely to be due to an insulin-glucose interaction. We have previously shown that 0.5 mM glucose increases DAF-FM fluorescence (9). This effect appeared to saturate within 10 min (9). Therefore, it is likely that DAF-FM saturation prevented measurement of increased NO production in 0.5 mM glucose. On the other hand, DAF-FM fluorescence increases when glucose is lowered from 5 to 2.5 mM (9). Thus, the inability to measure increased NO production in 5 or 10 mM glucose was due to increased glucose and not DAF-FM saturation. In contrast to insulin, the effects of leptin were not glucose dependent (Fig. 1C).

Our results suggest that high glucose blocks insulin-induced NO production by activating mTOR. In peripheral insulin-sensitive tissues (e.g., skeletal muscle, white adipose tissue), hyperglycemia inhibits insulin signaling as a result of the following sequence of events. Increased glycolytic ATP reduces the AMP/ATP and inhibits AMPK activity (25). Reduced AMPK activity disinhibits the mTOR signaling pathway.
(52). Increased mTOR signaling prevents the insulin receptor from phosphorylating the insulin receptor subunit/PI3K (17, 49). Our data show that insulin-induced NO production in VMH GI neurons from STZ rats or 5 mM of culture is restored by the mTOR inhibitor rapamycin or the AMPK activator AICAR (Figs. 3A and 6A). Furthermore, the PI3K inhibitor wortmannin prevents restoration of insulin-induced NO signaling by AICAR (Figs. 3A and 6A). Together, these data strongly suggest that this mechanism for hyperglycemia-mediated inhibition of insulin signaling occurs in VMH GI neurons.

Our data suggesting that mTOR activation is responsible for the inhibition of insulin-induced NO production during hyperglycemia associated with type 1 diabetes mellitus (Fig. 6) are consistent with studies showing that mTOR signaling is sensitive to energy status. Liver and skeletal muscle mTOR is activated in high-fat fed obese rats leading to insulin resistance in these tissues (20). mTOR signaling is also linked to energy status in hypothalamic neurons. Cota D et al. (11) showed that a 48-h fast significantly reduced phosphorylation of mTOR at Ser2448 in ARC neurons. This indicates that when glucose is low, mTOR activity is low. Furthermore, intracerebroventricular administration of 1-leucine, which increases mTOR signaling (28), decreased food intake. Co-administration with mTOR, reversed this effect (11). This suggests that mTOR signaling in the hypothalamus regulates energy balance by responding to nutrient availability (11). Since mTOR signaling links glucose and insulin-regulated NO production in VMH GI neurons (Figs. 3, 6, and 8), it further suggests a role for GI neurons in the regulation of energy balance.

In conclusion, hyperglycemia associated with type 1 diabetes impairs glucose and insulin regulation of NO production in GI neurons in the VMH by inhibiting AMPK (Figs. 5 and 6). The fact that decreased glucose was unable to stimulate NO production in both STZ rats and neurons cultured in 5 mM glucose suggests that high glucose is sufficient to block NO production. On the other hand, increased mTOR activity during hyperglycemia blocks insulin-induced NO production. This is reversed by AMPK activation or mTOR inhibition (Figs. 3 and 6). Thus, AMPK inhibition is responsible for both the impaired glucose and insulin regulation of NO production during hyperglycemia (Fig. 8). Importantly, hyperglycemia also prevented membrane potential depolarization in VMH GI neurons (Figs. 3B). This is consistent with the impaired CRR observed during type 1 diabetes. Thus, our data shed light on the mechanisms underlying impaired central glucose sensitivity in type 1 diabetes mellitus.

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22. Lester-Coll N, Rivera EJ, Soscia SJ, Doiron K, Wands JR, de la Fuente AM, Beverly JL, De Vries MG, Arseneau LM, Shulman GI. Ventromedial hypothalamic neurons in the VMHGI by inhibiting AMPK (Figs. 3A and 6A). Furthermore, the PI3K inhibitor wortmannin prevents restoration of insulin-induced NO signaling by AICAR (Figs. 3A and 6A). Together, these data strongly suggest that this mechanism for hyperglycemia-mediated inhibition of insulin signaling occurs in VMH GI neurons.

Our data suggesting that mTOR activation is responsible for the inhibition of insulin-induced NO production during hyperglycemia associated with type 1 diabetes mellitus (Fig. 6) are consistent with studies showing that mTOR signaling is sensitive to energy status. Liver and skeletal muscle mTOR is activated in high-fat fed obese rats leading to insulin resistance in these tissues (20). mTOR signaling is also linked to energy status in hypothalamic neurons. Cota D et al. (11) showed that a 48-h fast significantly reduced phosphorylation of mTOR at Ser2448 in ARC neurons. This indicates that when glucose is low, mTOR activity is low. Furthermore, intracerebroventricular administration of 1-leucine, which increases mTOR signaling (28), decreased food intake. Co-administration with mTOR, reversed this effect (11). This suggests that mTOR signaling in the hypothalamus regulates energy balance by responding to nutrient availability (11). Since mTOR signaling links glucose and insulin-regulated NO production in VMH GI neurons (Figs. 3, 6, and 8), it further suggests a role for GI neurons in the regulation of energy balance.

In conclusion, hyperglycemia associated with type 1 diabetes impairs glucose and insulin regulation of NO production in GI neurons in the VMH by inhibiting AMPK (Figs. 5 and 6). The fact that decreased glucose was unable to stimulate NO production in both STZ rats and neurons cultured in 5 mM glucose suggests that high glucose is sufficient to block NO production. On the other hand, increased mTOR activity during hyperglycemia blocks insulin-induced NO production. This is reversed by AMPK activation or mTOR inhibition (Figs. 3 and 6). Thus, AMPK inhibition is responsible for both the impaired glucose and insulin regulation of NO production during hyperglycemia (Fig. 8). Importantly, hyperglycemia also prevented membrane potential depolarization in VMH GI neurons (Figs. 3B). This is consistent with the impaired CRR observed during type 1 diabetes. Thus, our data shed light on the mechanisms underlying impaired central glucose sensitivity in type 1 diabetes mellitus.

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GRANTS

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