Glucose, insulin, and leptin signaling pathways modulate nitric oxide synthesis in glucose-inhibited neurons in the ventromedial hypothalamus

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Canabal DD, Song Z, Potian JG, Beuve A, McArdle JJ, Routh VH. Glucose, insulin and leptin signaling pathways modulate nitric oxide synthesis in glucose-inhibited neurons in the ventromedial hypothalamus. Am J Physiol Regul Integr Comp Physiol 292: R1418–R1428, 2007. First published December 24, 2006; doi:10.1152/ajpregu.00216.2006.—Glucose-sensing neurons in the ventromedial hypothalamic (VMH) are involved in the regulation of glucose homeostasis. Glucose-sensing neurons alter their action potential frequency in response to physiological changes in extracellular glucose, insulin, and leptin. Glucose-excited neurons decrease, whereas glucose-inhibited (GI) neurons increase, their action potential frequency when extracellular glucose is reduced. Central nitric oxide (NO) synthesis is regulated by changes in local fuel availability, as well as insulin and leptin. NO is involved in the regulation of food intake and is altered in obesity and diabetes. Thus this study tests the hypothesis that NO synthesis is a site of convergence for glucose, leptin, and insulin signaling in VMH glucose-sensing neurons. With the use of the NO-sensitive dye 4-amino-5-methylamino-2'-7'-difluorofluorescein in conjunction with the membrane potential-sensitive dye fluorometric imaging plate reader, we found that glucose and leptin suppress, whereas insulin stimulates neuronal nitric oxide synthase (nNOS)-dependent NO production in cultured VMH GI neurons. The effects of glucose and leptin were mediated by suppression of AMP-activated protein kinase (AMPK). The AMPK activator 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR) increased both NO production and neuronal activity in GI neurons. In contrast, the effects of insulin on NO production were blocked by the phosphoinositide 3-kinase inhibitors wortmannin and LY-294002. Furthermore, decreased glucose, insulin, and AICAR increase the phosphorylation of VMH nNOS, whereas leptin decreases it. Finally, VMH neurons express soluble guanylyl cyclase, a downstream mediator of NO signaling. Thus NO may mediate, in part, glucose, leptin, and insulin signaling in VMH glucose-sensing neurons.

ventromedial hypothalamus; glucose-sensing neurons; leptin; insulin; nitric oxide; adenosine 5′-monophosphate-activated protein kinase

THE VENTROMEDIAL HYPOTHALAMUS (VMH), which contains the ventromedial (VMN) and arcuate (ARC) nuclei, is critical for the regulation of glucose and energy homeostasis (34). This region contains specialized neurons whose activity is regulated by physiologically relevant changes in extracellular glucose (36, 37, 42). Glucose-exited (GE) neurons decrease, whereas glucose-inhibited (GI) neurons increase, their action potential frequency (APF) when extracellular glucose is reduced (36). GE neurons activate ATP-sensitive K+ (KATP) channels in response to decreased glucose. GI neurons appear to close an ATP-activated Cl− channel, although the identity of this channel is unknown (36). Glucose-sensing neurons also possess insulin and leptin receptors (22, 39, 40, 42). Thus glucose-sensing neurons may integrate glucose, insulin, and leptin signaling in the VMH.

Nitric oxide (NO) synthesis is a novel candidate as a potential site of overlap for insulin, leptin, and glucose signaling in glucose-sensing neurons. Neuronal NO synthase (nNOS) produces NO as a byproduct of the conversion of L-arginine to L-citrulline. As a gas, NO readily diffuses from its site of synthesis and regulates adjacent cells. Many of NO’s effects are mediated by soluble guanylyl cyclase (sGC), which exists both in the soma and presynaptic terminals. sGC produces cGMP which, in turn, activates several downstream targets, including protein kinase G (PKG; see Ref. 6). Insulin injected in the nucleus tractus solitarius (NTS) stimulates nNOS (41), whereas inhibition of nNOS in the NTS prevents insulin’s ability to lower blood pressure (41). In contrast, leptin decreases nitric oxide synthase (NOS) activity in the brain (11, 30). Both activity and mRNA of NOS are elevated in the hypothalamus of leptin-deficient obese (ob/ob) mice (31). NO may also play a role in glucose sensing. NO released from neurons during glucose-oxygen deprivation increases glycolysis and decreases oxidative phosphorylation in astrocytes. This leads to increased lactate production in astrocytes, presumably to fuel neurons during energy deficit (2, 3, 18). On the other hand, hyperglycemia decreases endothelial NOS (eNOS) in glomerular endothelial cells (9). Finally, NO is involved in the regulation of food intake. Stimulation of NO synthesis by L-arginine increases food intake in mice (29). Furthermore, neuropeptide Y increases NO in the hypothalamus (30), and nNOS knockout mice are refractory to orexin-induced feeding (13).

On the basis of these observations, we hypothesize that the effects of glucose, insulin, and leptin on glucose-sensing neurons are mediated, in part, by NO. We tested this hypothesis using membrane potential- and NO-sensitive dyes in cultured VMH neurons. Our data indicate that glucose, insulin, and leptin modulate NO signaling in VMH GI neurons via distinct mechanisms that overlap at the level of nNOS. Thus, via NO diffusion, GI neurons may link metabolism and neuronal activity in adjacent neurons.

MATERIALS AND METHODS

Preparation of brain slices and cultured neurons. Male 14- to 21-day-old Sprague-Dawley rats were obtained from colonies at the animal facility of New Jersey Medical School. Litters of eNOS and nNOS knockout mice were bred in-house. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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nNOS knockout mice were obtained from Jackson Laboratories, and male mice between 14 and 21 days old were studied. Neonatal rats/mice were housed with their dams at 22–23°C on a 12:12-h light-dark cycle and given purified diet (no. D03120101; Research Diets, New Brunswick, NJ) and water ad libitum. All experimental procedures were approved by the International Animal Care and Use Committee. On the day of experiment, rats or mice 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 mosmol/kgH₂O 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 (Vibrorslice; Camden Instruments) as described previously (37, 42). After being sectioned, slices were maintained at 34°C in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mmol/l): 127 NaCl, 1.9 KCl, 1.2 KH₂PO₄, 26 NaHCO₃, 2.5 glucose, 1.3 MgCl₂, and 2.4 CaCl₂ (osmolarity adjusted to ~300 mosmol/kgH₂O with sucrose, pH 7.4; see Ref. 36).

Cultured neurons. As described previously (42), brain slices were placed in Hibernate A/B27 (Brain Bits, Carlsbad, CA). The VMH (ARC + VMN) was dissected and digested in Hibernate A with papain. The tissue was incubated for 30 min in a 30°C water bath with a platform rotating at 100 revolutions/min and then rinsed with Hibernate A/B27 and subjected to gentle trituration. After the cell was triturated, suspension was centrifuged, and the pellet was resuspended with growth medium (Invitrogen, Springfield, IL). Neurons were plated in growth medium with fluoresbrite beads (Polysciences) for normalization of data and used within 3 days for evaluation of NO production.

Measurement of NO production in cultured neurons using 4-amino-5-methylamino-2',7'-difluorofluorescein. Neurons were visualized on an Olympus BX61 WI microscope with a ×10 objective for measurement of the NO-sensitive dye 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM; green filter, excitation 488 nm, emission 515–530 nm; see Ref. 24) and fluorometric imaging plate reader (FLIPR)-MPD (red filter, excitation 548 nm, emission 610–675 nm). DAF-FM fluorescence intensity increases when NO is produced (9, 20, 24). DAF-FM binds irreversibly so we cannot measure a decrease in fluorescence. Because 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 (in mM: 135 NaCl, 5 KCl, 1 CaCl₂, 2 MgCl₂, 10 HEPES, and 0.05 L-arginine, pH 7.4) with 5 or 2.5 mM glucose for 30 min or leptin (10 nM) in 2.5 mM glucose for 60 min. DAF-FM was then washed out, and glucose, insulin, or leptin levels were altered as described below. DAF-FM fluorescence intensity measurement began immediately after decreasing glucose concentration or leptin washout and was measured every min for 30 or 60 min, respectively. In contrast, because insulin is expected to increase NO production, DAF-FM fluorescence intensity was measured immediately after the addition of 5 nM insulin to experimental solution containing 2.5 mM glucose.

In a subset of experiments for glucose, leptin, and insulin, 0.25% FLIPR-MPD was added to the DAF-FM stain during the last 30 min of incubation. DAF-FM was then washed out, and glucose, insulin, and leptin levels were altered. FLIPR-MPD was reapplied and remained throughout experimental recordings. Control images were captured (Cool Snap HQ CCD camera; Photometrics) every 1 min for either 30 min (insulin and glucose) or 1 h (leptin) 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 from the time of solution change. DAF-FM and FLIPR-MPD are both toxic to cells when used in high concentrations. Thus a series of dye concentrations and incubation times were evaluated for each dye. The minimal combination that produced detectable labeling under control conditions (e.g., 2.5 mM glucose) for each dye was used for these studies.

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 (Fig. 1A). 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 (Fig. 1B). This criterion was chosen because NOS inhibitors significantly reduced the number of neurons that increased DAF-FM fluorescence intensity during the first 10 min >5% (usually to <10%). 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. This criterion was chosen because the average percent change in FLIPR-MPD in the absence of treatment was 5.7 ± 2.3. Furthermore, the average percent change in FLIPR-MPD fluo-

![Image](http://ajpregu.physiology.org/Downloadedfrom.htm)
rescence intensity in cells defined as changing FLIPR-MPD fluorescence intensity in response to glucose was 16.8 ± 4.9. The percentage 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, in a subset of experiments, the cells were stained with 0.25% trypan blue (Sigma Chemicals, St. Louis, MO) for 5 min after fluorescent imaging to determine cell viability. This was necessary since increased NO production is sometimes associated with apoptosis (32). However, of the fluorescing cells, only 10% were stained with trypan blue. The neurons stained with trypan blue also showed a progressive increase in DAF-FM fluorescence intensity that did not plateau during the course of an experiment (Fig. 1C). Moreover, of the neurons that did not stain for trypan blue, none exhibited similarly progressive increases in DAF-FM fluorescence intensity. Thus the neurons that exhibited progressive increases in DAF-FM fluorescence intensity were judged to be unhealthy and excluded from all analyses.

**Electrophysiology in brain slices.** Viable neurons in hypothalamic slices containing the VMN were visualized and studied under infrared differential-interference contrast microscopy using a Leica DMLFS microscope equipped with a ×40 long-working distance water-immersion objective, as described previously (42). Current-clamp recordings (standard whole cell recording configuration) from 52 VMN neurons were performed using MultiClamp 700A (Axon Instruments, Foster City, CA) and analyzed using pCLAMP 9 software.

During recording, brain slices were perfused at 10 ml/min with normal oxygenated ACSF. Borosilicate pipettes (1.3–1.5 MΩ; Sutter Instruments, Novato, CA) were filled with an intracellular solution containing (in mM): 128 potassium gluconate, 10 KCl, 4 KOH, 10 HEPES, 4 MgCl₂, 0.5 CaCl₂, 5 EGTA, and 2 Na₂ATP; pH 7.2. Osmolarity was adjusted to 290–300 mosmol/kgH₂O with sucrose. Junction potential between the bath and the patch pipette was nullled before the formation of a gigaohm seal. Membrane potential and APF were allowed to stabilize for 10–15 min after the formation of the whole cell configuration. Neurons whose access resistance exceeded 20 MΩ after this time were rejected. Input resistance was calculated from the change in membrane potential measured during the last 1 min of a small 500-ms hyperpolarizing pulse (20 pA) given every 3 s. The membrane response was measured only after membrane potential and APF stabilized following a change in glucose concentration or the addition of 5-aminomimidazole-4-carboxamide-1-β-4-ribofuransoside (AICAR). All chemicals were obtained from Sigma Chemicals. Because of variation between neurons, data were analyzed using a paired Student’s t-test for comparison of input resistance changes within the same neuron with P < 0.05 considered statistically significant.

**Immunochemistry and confocal microscopy.** VMN neurons that had been in culture for 4 days were plated on coverslips, fixed in 4% paraformaldehyde, permeabilized with 100% acetone, and incubated in blocking solution (PBS containing 2% normal goat serum and 1% BSA) to prevent nonspecific binding of the primary antibodies. The primary antibodies against the β₁-subunit of sGC (1:50 dilution) were incubated for 2 h at room temperature. The specificity of the sGC antibody used here has been previously verified by Western blot analysis (25). For negative controls, a blocking peptide was applied (the COOH-terminal 15 amino acids for anti-β₁-subunit, data not shown). After incubation, the cells were again placed in a blocking solution. A solution containing the fluorescent secondary antibodies (Alexa green 488 for sGC diluted in PBS; 1:350 dilution) was applied for 1 h at room temperature in the dark. The cover slips were then mounted on labeled microscope slides using anti-fade mounting medium. Images were taken with a Nikon PCM 2000 microscope and Simple PCI software.

**Western blot analysis.** The VMH (ARC + VMN) was dissected out and placed in 2.5 mM glucose, 5 mM insulin, 0.5 mM glucose, or 10 mM leptin for 5 min. Each sample was pooled tissue from 2 rats for a total of 8 to 10 rats per experimental treatment (n = 4–5 samples).

The tissue was then homogenized and sonicated. Phosphorylation of nNOS was determined with a 10% Tris-HCl gel using an antibody against phospho-nNOS (Upstate, Lake Placid, NY). This antibody is phosphorylated by AMP-activated protein kinase (AMPK) on Ser¹⁴¹⁷, which is analogous to Ser¹⁴⁵¹ on the mu isoform of nNOS (skeletal muscle) as well as to Ser¹⁷⁷ on eNOS (7, 8). Finally, the data were normalized to β-actin (Sigma) and quantified using Scion Image. Each treatment was compared with 2.5 mM glucose using a two-tailed Students t-test with P < 0.05 considered to be statistically significant.

**RESULTS**

**Glucose modulation of NO production in VMH neurons.** DAF-FM-loaded VMH cultured neurons were visualized with brightfield and fluorescent microscopy. Approximately one-half of the VMH neurons isolated from rats could be visually identified by DAF-FM fluorescence under control conditions (2.5 mM glucose; Fig. 2A). Approximately one-half of the neurons isolated from eNOS knockout mice also showed DAF-FM fluorescence in 2.5 mM glucose (Fig. 2B). However, no DAF-FM fluorescence was observed in nNOS knockout mice (Fig. 2C). Figure 2D shows that we were able to detect the NO receptor, sGC, in both cell bodies and neurites of all VMH cultured neurons.

Of the VMH neurons from rats that showed DAF-FM fluorescence in 2.5 mM glucose, ~80% (882 of 1,114 neurons) increased their DAF-FM fluorescence intensity (NO production) within 10–15 min after decreasing glucose from 2.5 to 2.5 mM (186 of 220 neurons), from 2.5 to 1 mM (240 of 263 neurons), or 0.5 (280 of 366 neurons) to 0.1 mM (176 of 265 neurons; Fig. 3). This corresponds to approximately one-half of all VMH neurons. The percentage of neurons that increased their NO production in response to decreased glucose was significantly reduced in the presence of 1 mM G-nitro-L-arginine methyl ester (LNAME) or 10 μM 7-nitroindazolone (Fig. 3). Like the rats, 49.8 ± 1.3% of VMH neurons isolated from eNOS knockout mice (7 dishes, 890 cells) increased DAF-FM fluorescence as glucose decreased from 2.5 to 0.5 mM. In contrast, decreased glucose did not result in detectable DAF-FM fluorescence in nNOS knockout mice (7 dishes, 907 cells).

The remaining VMH neurons that showed DAF-FM fluorescence in 2.5 mM glucose did not alter their DAF-FM fluorescence intensity in response to changes in glucose. Neurons that were not visually identified as fluorescent under control conditions did not show any change upon treatment. For this reason, data are expressed as the percentage of neurons that increase NO production in response to glucose (or insulin/leptin) out of the total number of VMH neurons. Thus the majority of neurons that produce NO under steady-state conditions also alter their NO production in response to changes in glucose. These data indicate that NO production, as shown by DAF-FM fluorescence intensity, may serve as a good marker for VMH glucose-sensing neurons.

To determine the mechanism by which decreased glucose increased NO production, extracellular glucose levels were then lowered from 2.5 to 0.5 mM in the presence of the AMPK inhibitor compound C (10 μM; see Ref. 23), the phosphatidylinositol 3-kinase (PI3-kinase) inhibitor wortmannin (10 nM; see Ref. 40), or in the absence of extracellular calcium. As seen with the NOS inhibitors, the presence of 10 μM compound C and 0 mM extracellular calcium significantly reduced the...
percentage of neurons, which increased NO production in response to decreasing glucose from 2.5 to 0.5 mM. In contrast, a similar percentage of VMH neurons increased NO production when glucose levels were reduced in the presence and absence of 10 nM wortmannin (Fig. 4). Because DAF-FM fluorescence in response to NO production is irreversible, we were unable to determine whether the effects of decreased glucose reversed upon washout. However, when the neurons were placed in 2.5 mM glucose after a 2-min exposure to 0.5 mM glucose, no increase in NO production was observed (leptin results discussed below; Fig. 5). This suggests that a return to 2.5 mM glucose arrests the NO production occurring in response to 0.5 mM glucose.

Relationship between NO production and membrane polarization in GE, GI, and non-glucose-sensing neurons. To determine the relationship between our previous electrophysiological characterization of GE and GI neurons (36, 42) and those neurons that increased NO production in response to decreased extracellular glucose, we simultaneously measured the fluorescence of a membrane potential dye (FLIPR-MPD) in a subset of cells exposed to DAF-FM. Interestingly, 75% (61 of 81) of the neurons that increased NO production in response to decreased glucose from 2.5 to 0.5 mM also increased FLIPR-MPD fluorescence intensity (Fig. 6A). An increase in FLIPR-MPD fluorescence intensity indicates depolarization. Thus 75% of the neurons that increase NO production in response to decreased glucose are GI neurons. This represents ~70% of the total VMH neurons that showed DAF-FM fluorescence in 2.5 mM glucose. In contrast, only 1 of the 81 neurons that increased NO production in response to decreased glucose decreased FLIPR-MPD fluorescence intensity, indicating that it is a GE neuron. The remaining neurons that increased NO
production in response to decreased glucose showed no change in FLIPR-MPD fluorescence intensity (19 of 81). The DAF-FM fluo-
rescing neurons that did not increase their DAF-FM fluorescence intensity in response to decreased glucose also showed no change in FLIPR-MPD fluorescence intensity (7 of 7). Finally, of the non-DAF-FM-fluorescing neurons, 57% (40 of 70) decreased FLIPR-MPD fluorescence intensity (hyperpolarized) as glucose decreased, whereas only 4% (3 of 70) increased FLIPR-MPD fluorescence intensity (depolarized). The remaining 39% (27 of 70) showed no change in FLIPR-MPD fluorescence intensity. These data suggest that GE neurons do not produce NO in 2.5 mM glucose (Fig. 6B). In contrast, 95% of GI neurons (61 of 64) increase NO production as glucose decreases, and 75% of neurons (61 of 81) that increase NO production as glucose decreases are GI neurons. Thus there is a very strong correlation between GI neurons and those neurons that produce NO as glucose decreases.

Leptin modulation of NO production in VMH neurons. To determine whether leptin suppresses NO production in VMH neurons, the cells were incubated in 10 nM leptin and 2.5 mM glucose for 1 h, and then leptin was washed out of the bath. Approximately 66% (119 of 181) of the fluorescent neurons, corresponding to ~30% of the total VMH neurons, increased their NO production when leptin was removed from the solution (Fig. 7A). The presence of L-NAME (1 mM), 7-NINA (10 μM), compound C (10 μM), and 0 mM extracellular calcium significantly reduced the percentage of neurons that increased NO production in response to leptin washout (Fig. 7A). In contrast, there was no significant difference in the number of neurons that increased their NO production in response to leptin washout in the presence and absence of 10 nM wortmannin. In experiments that simultaneously measured DAF-FM and FLIPR-MPD fluorescence, leptin washout increased the FLIPR-MPD fluorescence intensity in 64% (66 of 103) of the neurons that showed DAF-FM fluorescence in 2.5 mM glucose. This corresponds to 76% (66 of 87) of the neurons that increased NO production in response to leptin washout (Fig. 7B). Only 1 of these 87 neurons decreased FLIPR-MPD fluorescence intensity. Leptin washout changed FLIPR-MPD fluorescence intensity in only 9% (1 decrease, 6 increase) of the neurons that did not show DAF-FM fluorescence in 2.5 mM glucose. This indicates that leptin hyperpolarizes and suppresses NO production in the same neurons. Finally, when the neurons were placed in leptin after a brief 2-min exposure to 0.5 mM glucose, no increase in NO production was observed (Fig. 5). This suggests that 10 nM leptin arrests the NO production occurring in response to 0.5 mM glucose.

AMPK modulation of NO production and neuronal activity in GI neurons. Because inhibition of AMPK with compound C blocked the effects of decreased glucose and leptin, the AMPK activator AICAR (0.5 mM; see Ref. 23), was added to 2.5 mM glucose to determine whether AMPK activation increases NO production. The addition of 0.5 mM AICAR increased NO production in 40 ± 6.1% of all VMH neurons (6 dishes, 790 cells). In contrast, when AICAR was added in the presence of L-NAME, only 5 ± 1.6% (6 dishes, 432 cells; P = 0.0002) of VMH neurons increased their NO production. To further test the role of AMPK as a mediator of glucose sensing in GI neurons, we evaluated the effect of 0.5 mM AICAR on the APF of VMH GI neurons in brain slices. Of 52 VMN neurons evaluated (in 30 rats), 15 (29%) were GI, and, of these, 8 were recorded from for sufficient time to evaluate the effects of AICAR. In 2.5 mM glucose, AICAR mimicked the excitatory effect of low glucose on APF for all eight GI neurons (Fig. 8A). Input resistance was significantly lower in 2.5 mM glucose (680 ± 113 Ω) alone than in 0.5 mM glucose (771 ± 129 Ω; P = 0.03, Student’s paired t-test) or 2.5 mM glucose with 0.5 mM AICAR (748 ± 122 Ω; n = 7; P = 0.006, Student’s paired t-test; Fig. 8B). There was no significant difference in input resistance in 0.5 mM glucose and in 2.5 mM glucose with AICAR (P = 0.41, Student’s paired t-test; Fig. 8B). Thus AMPK activation mimics the effects of decreased glucose on NO production and neuronal activity in GI neurons.
Insulin modulation of NO production in VMH neurons.

When insulin (5 nM; see Ref. 40) was added to 2.5 mM glucose, 51/11006 2.7% of VMH neurons increased their NO production. This corresponds to 94% (289 of 309) of the neurons that show DAF-FM fluorescence in 2.5 mM glucose. 7-NINA (10 nM) and the PI 3-kinase inhibitors wortmannin (10 nM; see Ref. 39) significantly reduced the percentage of VMH neurons that increased NO production (Fig. 9A). In contrast, there was no significant difference between the percentage of VMH neurons that increased NO production following insulin addition alone or in the presence of compound C or 0 mM extracellular calcium (Fig. 9A). In experiments that simultaneously measured DAF-FM and FLIPR-MPD fluorescence, insulin decreased the FLIPR-MPD fluorescence intensity in 79% (49 of 62) of the neurons that showed DAF-FM fluorescence in 2.5 mM glucose. This corresponds to 91% (49 of 54) of the neurons that increased NO production in response to insulin. Insulin also decreased FLIPR-MPD fluorescence intensity in 11% (6 of 51) of neurons that did not show DAF-FM fluorescence in 2.5 mM glucose. In contrast, insulin increased FLIPR-MPD fluorescence intensity in only 4 of 62 (6%) neurons showing DAF-FM fluorescence in 2.5 mM glucose and 2 of 51 (4%) neurons that did not. Those neurons that showed DAF-FM fluorescence in 2.5 mM glucose but did not increase it in response to insulin also did not change their FLIPR-MPD fluorescence intensity in response to insulin (8 of 8 neurons). Because 70% (61 of 88) of the neurons that showed DAF-FM fluorescence in 2.5 mM glucose were also GI neurons, it follows that insulin is increasing NO production and hyperpolarizing a large percentage of VMH GI neurons (Fig. 9B).

nNOS phosphorylation in the VMH. As shown above, the effects of decreased glucose were present in eNOS but not nNOS knockout mice (Fig. 2, B and C). Moreover, nNOS inhibitors blocked the effects of glucose, AICAR, insulin, and leptin. To further test the hypothesis that activation of nNOS was responsible for NO production in our studies, we evaluated nNOS phosphorylation in response to decreased glucose, AICAR, insulin, and leptin in pooled VMH from neonatal rats. As seen in Fig. 10, decreased glucose, AICAR, and insulin all increased the levels of phospho-nNOS as indicated by immunoblots for phospho-nNOS antibody. In contrast, leptin decreased phospho-nNOS levels.
DISCUSSION

In this study, we demonstrated that physiologically relevant decreases in extracellular glucose increase NO production in VMH GI neurons using NO- and membrane potential-sensitive dyes, that is, the majority (80%) of the cells that produce NO in 2.5 mM glucose also increase NO production as glucose decreases (Fig. 3). Using electrophysiological techniques, we have previously shown that the APF of GI (and GE) neurons is well correlated with transmembrane potential, implying that glucose-sensing neurons can be identified by changes in APF or in membrane potential (36, 37, 42). This opens the way for the use of a voltage-sensitive dye (e.g., FLIPR-MPD) to identify GI and GE neurons. Using FLIPR-MPD, we show here that almost all GI neurons (95%) increase NO production as glucose decreases (Fig. 6A). Moreover, 70% of the neurons that produce NO in 2.5 mM glucose and 75% of those that increase their NO production as glucose decreased were GI neurons. Thus decreased glucose increases NO production in GI neurons. In contrast, approximately one-half of the neurons that do not produce NO in 2.5 mM glucose were GE neurons (Fig. 6B). Because the majority of NO-producing neurons are GI neurons, the use of DAF-FM fluorescence will now significantly improve our ability to identify GI neurons before electrophysiological recording.

In this study, the number of VMH GI neurons estimated using membrane potential dye measurements in neuronal cultures was slightly higher than that estimated using electrophysiological techniques in brain slices (~40% vs. 29%, respectively). This may reflect differences between brain slices and isolated neurons. It may also reflect the comparative difficulty of the patch-clamp technique. It should be noted that we found a significantly higher percentage of GI neurons using electrophysiological techniques in the current study (29%), as well as our recently published data (23%; see Ref. 38), than we originally reported (3%) in 2001 (36). Since our original studies, we have consistently improved our brain harvesting.
slicing, and culturing skills. We have found glucose-sensing neurons to be extremely fragile. It is not surprising that, as we improve our skills, we observe a greater percentage of GI neurons. Thus, with improved slice techniques and, for the first time, the use of FLIPR-MPD to identify a population of GI neurons, our recent studies converge to show that GI neurons comprise ~30–40% of all neurons in the VMH.

Even with the limitation that changes in DAF-FM fluorescence intensity are irreversible, placing a constraint on the use of multiple ligands in each experiment, results in this study together with previous studies not only support the proposal that glucose, insulin, and leptin may act on the same cell but further suggest that the common site of convergence is NO production, that is, 75% of NO-producing cells in 2.5 mM glucose are GI neurons. Similarly, 94% of NO-producing cells in 2.5 mM glucose respond to insulin, whereas 66% respond to leptin. The correlation between the number of cells that produce NO in response to decreased glucose and insulin suggest strong overlap between insulin- and glucose-regulated neuronal populations. Furthermore, in spite of the limitations of DAF-FM, we were able to show that NO production in response to low glucose was halted by the addition of leptin, indicating that glucose- and leptin-responsive neuronal populations overlap (Fig. 5). Moreover, in this study, we used neurons that had been in culture for 1–3 days. At this time, they have not developed synaptic connections. They are also plated sparsely. Thus it is very unlikely that the effects of glucose, insulin, and leptin are mediated by synaptic input or diffusion of NO from other neurons in the culture dish. This is also apparent in Fig. 2, A and B, which shows that neurons quite close to fluorescent neurons do not necessarily fluoresce themselves. Finally, we have previously shown that a large percentage of VMH GE and GI neurons possess insulin and leptin receptors (22). These data strongly suggest that glucose, insulin, and leptin signaling converge at NO production in most VMH GI neurons.

nNOS inhibitors significantly reduce the number of neurons that alter their NO production in response to glucose, insulin, and leptin. Furthermore, decreased glucose increased NO production in eNOS, but not nNOS, knockout mice. Others have shown that NOS inhibitors do not fully quench DAF-FM (19, 26). This is the most likely explanation for the few responsive cells observed in the presence of NOS inhibitors. Finally, decreased glucose and insulin increase, whereas leptin...
Phosphorylation of Ser1177 (8). AMPK phosphorylates the mu isoform of nNOS on Ser1451, which is comparable to Ser1177 on nNOS. Together these data indicate that glucose, insulin, and leptin modulate NO production via changes in nNOS activity.

Glucose, insulin, and leptin signaling in VMH neurons alter NO production using distinct signaling pathways (shown in Fig. 11), that is, glucose and leptin inhibit NO production, whereas insulin stimulates it. This is somewhat surprising since both insulin and leptin open the KATP channel on VMH GE neurons via the PI 3-kinase signaling cascade (39, 40, 42), whereas glucose closes the KATP channel on GE neurons (5, 36, 42). VMN GI neurons also possess a KATP channel (37). Furthermore, insulin and leptin hyperpolarized all VMH GI neurons in which they altered NO production (Figs. 7 and 9). However, there is precedence for opposing effects of insulin and leptin. Acute administration of insulin and leptin in the third ventricle exerts subadditive effects on food intake (i.e., the combined response is less than the sum of the individual responses; see Ref. 1). Insulin and leptin also exerted opposite effects on PI 3-kinase activity in hypothalamic agouti-related peptide neurons (43). Insulin stimulates nNOS in the NTS (41), whereas leptin-deficit obese ob/ob mice have elevated hypothalamic nNOS levels (31). Furthermore, oxygen-glucose deficit increases neuronal NO production (2, 3, 18), whereas hyperglycemia decreases the activity of eNOS (9). These data are consistent with the opposing effects of insulin and leptin on NO production in VMH GI neurons, whereas glucose and leptin act similarly.

Both glucose and leptin inhibit hypothalamic AMPK activity (27). AMPK is a cellular fuel sensor implicated in hypothalamic regulation of energy balance (21). AMPK increases the calcium sensitivity, and thus the activity of eNOS, through thalamic regulation of energy balance (21). AMPK increases glucose transport in muscle cells (15). Thus it is likely that AMPK also increases the activity of nNOS. Here, the AMPK activator, AICAR, increases NO production in VMH neurons (and mimics the effect of low glucose on GI neuronal activity). Although we used the nonspecific NOS blocker L-NAME to block the AMPK response, the effects of decreased glucose and leptin were blocked by the AMPK inhibitor compound C and the specific nNOS inhibitor 7-NINA. Furthermore, we show here that AICAR increased the phosphorylation of nNOS in the VMH above that seen in 2.5 mM glucose (Fig. 10). Finally, the effects of glucose and leptin on NO production were calcium dependent (Figs. 4, 6, and 7). This is consistent with the ability of AMPK to increase calcium sensitivity and thus activity of NOS (8). Because decreased glucose and leptin removal depolarized GI neurons (Figs. 6 and 7), we hypothesize that AMPK increases nNOS activity in these neurons by increasing the sensitivity of nNOS to calcium influx through voltage-gated channels.

Insulin increases NO production in VMH GI neurons via PI 3-kinase (Fig. 9). We have previously shown that insulin activates the KATP channel on VMN GE neurons via PI 3-kinase (40). The exact mechanism mediating insulin activation of nNOS is unknown. Insulin activates eNOS via protein kinase B (PKB), the downstream mediator of PI 3-kinase (14, 16, 19). PKB phosphorylates eNOS on Ser1177 (16). However, unlike AMPK, PKB phosphorylation of Ser1177 results in a calcium-independent activation of eNOS. This apparent discrepancy is because of the intracellular localization of eNOS isoforms. Highly calcium-sensitive eNOS is targeted to the plasma membrane, whereas PKB activates a relatively calcium-insensitive eNOS isoform associated with the Golgi complex (16). NO synthesis from these isoforms plays different roles in cell function. The Golgi-targeted form may be involved in secretory functions, whereas the plasma membrane form is involved with extracellular signaling (16). Because eNOS and nNOS are conserved in the calcium-calmodulin binding region containing Ser1177 (8), AMPK vs. PKB phosphorylation may have similar effects on nNOS. Membrane- and Golgi-targeted nNOS isoforms are found in neurons (44). Our data showing that insulin hyperpolarized VMH cultured neurons and activated nNOS-mediated NO production via PI 3-kinase in a calcium-independent fashion are consistent with this hypothesis (Fig. 9). Finally, others have shown that insulin-induced PI 3-kinase signaling inhibits AMPK in the hypothalamus (33). Our data suggest that PI 3-kinase-induced nNOS phosphorylation outweighs decreased nNOS phosphorylation resulting from PI 3-kinase inhibition of AMPK. The relative contribution of direct PI 3-kinase-mediated nNOS phosphorylation vs. decreased nNOS phosphorylation resulting from PI 3-kinase-mediated AMPK inhibition may be dependent on nutrient status, that is, here insulin was added to 2.5 mM glucose, a level seen in the brain after a full meal (7.6 mM plasma glucose; see Ref. 35) when AMPK levels would be reduced (27). Thus, like insulin’s effect on GE neuron’s APF (42), insulin-mediated NO production in GI neurons may also be glucose dependent.

Importantly, small decreases in extracellular glucose increased NO production (Fig. 3). Previously, hypoxia and ischemia were shown to induce neuronal NO production (2, 3). Under these conditions, neuronal NO production increases lactate production and, presumably release, from glial cells as an energy source to local glucose-deprived neurons (2, 3, 18). We are the first to show that NO production may link neuronal activity to nutrient availability under conditions associated with meal-to-meal changes in brain glucose levels. VMH glucose levels vary from 1.5 mM in fed rats to 0.7 mM in overnight-fasted rats (12). These changes in extracellular glu-
cose are consistent with those used in the present study. One caveat is that brain glucose levels have only been measured in adult rats. It is difficult to isolate sufficiently healthy neurons from adult rats for studies related to metabolic sensing. This is especially germane for studies of NO because of its role in apoptosis. In fact, most studies of this type are performed on even younger rats than used here. However, care must be taken when extrapolating brain glucose levels from adults to neonates. Finally, NO signaling in the VMH regulates food intake and energy homeostasis (29, 30). We suggest that this is mediated by NO release from VMH GI neurons in response to changes in glucose, leptin, or insulin. NO then influences the activity of local neurons involved in energy homeostasis (34). This is supported by our observation that all VMH neurons express sGC, a key mediator in the downstream effects of NO (6). Thus VMH neurons with sGC near NO-producing GI neurons are positioned to detect alterations in NO. This would facilitate a form of cell-to-cell signaling not previously considered in studies of VMH energy homeostasis.

The mechanism by which NO might modulate the electrical activity of GI neurons, as well as adjacent GE and non-glucose-sensing neurons, is not known. However, there are several possibilities. First, both GI and GE neurons possess metabolically sensitive K\textsubscript{ATP} channels (37). NO inhibition of cytochrome c oxidase and the concomitant decrease in intracellular ATP may alter the activity of K\textsubscript{ATP} channels on these neurons (4). Alternatively, NO-induced changes in intracellular metabolism might alter the glucose sensitivity of GE and GI neurons independent of a direct effect on neuronal activity. Thus, although we have shown that glucose directly affects the APF of GE neurons, NO diffusing from GI neurons could indirectly modulate the glucose sensitivity of GE neurons. Finally, the downstream effectors of NO signaling (cGMP,PKG) could directly affect the activity of ion channels on GI and/or non-glucose-sensing neurons. In support of this, NO-induced depolarization of the optic nerve via hyperpolarization-activated cyclic nucleotide-gated channels is mediated by cGMP (17). Furthermore, PKG opens the mitochondrial K\textsubscript{ATP} channel on hippocampal neurons (10). Thus NO produced by VMN GI neurons has the potential to regulate the activity and/or metabolic sensitivity not only of GI neurons but also adjacent GE and/or non-glucose-sensing neurons.

In conclusion, glucose and leptin inhibit NO production via inhibition of AMPK, whereas insulin stimulates NO production via the PI 3-kinase signaling pathway (Fig. 11). The ability of NO to diffuse to adjacent neurons leads us to hypothesize that VMH GI neurons may link the overall function of the VMH to energy status. In fact, NO may mediate the presynaptic effects of glucose and leptin (36, 42, 43). Our data indicate that nNOS-dependent NO production is a site of convergence for glucose, insulin, and leptin on VMH neurons. Thus NO signaling may play an important role in the regulation of glucose and energy homeostasis.

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