Ventromedial hypothalamic nitric oxide production is necessary for hypoglycemia detection and counter-regulation

Running title: VMH NO production is required for hypoglycemia counter-regulation.

Xavier Fioramonti¹, Nicolas Marsollier², Zhentao Song¹, Kurt A. Fakira¹, Reema M Patel¹, Stacey Brown³, Thibaut Duparc⁴, Arnaldo Pica-Mendez¹, Nicole M. Sanders⁵, Claude Knauf⁴, Philippe Valet⁴, Rory J. McCrimmon⁴, Annie Beuve¹, Christophe Magnan² and Vanessa H. Routh¹

From the ¹Department of Pharmacology and Physiology, New Jersey Medical School, Newark, New Jersey, USA; the ²CNRS-University Paris Diderot, Paris, France; the ³Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut, USA; the ⁴INSERM U858, Institut de Medicine Moleculaire de Rangueil, IFR150, Université Paul Sabatier, Toulouse, France and ⁵Division of Endocrinology/Metabolism, Veterans Affairs Puget Sound Health Care System, Seattle, WA.

Corresponding author:
Vanessa H Routh, PhD,
E-mail: routhvh@umdnj.edu

Submitted 20 March 2009 and accepted 8 November 2009.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org

This is an uncopyedited electronic version of an article accepted for publication in Diabetes. The American Diabetes Association, publisher of Diabetes, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of Diabetes in print and online at http://diabetes.diabetesjournals.org.
Objective-The response of ventromedial hypothalamic (VMH) glucose-inhibited (GI) neurons to decreased glucose is impaired under conditions where the counter-regulatory response (CRR) to hypoglycemia is impaired (e.g., recurrent hypoglycemia). This suggests a role for GI neurons in the CRR. We recently showed that decreased glucose increases nitric oxide (NO) production in cultured VMH GI neurons. These in vitro data lead us to hypothesize that NO release from VMH GI neurons is critical for the CRR.

Research Design And Methods-The CRR was evaluated in rats and mice in response to acute insulin-induced hypoglycemia and hypoglycemic clamps after modulation of brain NO signaling. The glucose sensitivity of VMN GI neurons was also assessed.

Results-Hypoglycemia increased hypothalamic constitutive NO synthase (NOS) activity and nNOS but not eNOS phosphorylation in rats. Intracerebroventricular (ICV) and VMH injection of the non-selective NOS inhibitor N\(^\text{G}\)-Nitro-L-arginine (LNMMA) slowed the recovery to euglycemia following hypoglycemia. VMH LNMMA injection also increased the glucose infusion rate (GIR) and decreased epinephrine secretion during hyperinsulinemic/hypoglycemic clamp in rats. The GIR required to maintain the hypoglycemic plateau was higher in nNOS knockout (KO) than wildtype (WT) or eNOS KO mice. Finally, VMH GI neurons were virtually absent in nNOS KO mice.

Conclusion-We conclude that VMH NO production is necessary for glucose sensing in GI neurons and full generation of the CRR to hypoglycemia. These data suggest that potentiating NO signaling may improve the defective CRR resulting from recurrent hypoglycemia in patients using intensive insulin therapy.
Intensive insulin therapy significantly reduces the onset and progression of hyperglycemia related complications in patients with Type 1 and advanced Type 2 Diabetes Mellitus. However, intensive insulin therapy also causes a clinically adverse effect: hypoglycemia (1). Powerful neuroendocrine and autonomic counter-regulatory mechanisms protect the brain from hypoglycemia (2; 3). These protective mechanisms, known as the counter-regulatory response (CRR) to hypoglycemia, involve the release of hormones (e.g. glucagon, epinephrine) which restore euglycemia by stimulating hepatic glucose production and inhibiting peripheral glucose uptake (3). Although the physiology of the CRR is well understood, the underlying cellular mechanisms by which the brain senses hypoglycemia and initiates the CRR remain elusive.

During hypoglycemia, central and peripheral glucose sensors detect declining glucose levels (4). In the brain, the ventromedial hypothalamus (VMH), which includes the arcuate nucleus and the ventromedial nucleus (VMN), is important in the initiation of the CRR (5-7). This region contains specialized glucose sensing neurons (GSNs). VMH GSNs electrical activity is regulated by physiologically relevant changes in extracellular glucose levels (8-11). Glucose-excited (GE) neurons decrease, whereas glucose-inhibited (GI) neurons increase, their input resistance, membrane potential and action potential frequency when extracellular glucose is reduced (10). Many studies suggest that VMH GI neurons play a critical role in the control of the CRR (4). For example, the response of VMH GI neurons to decreased glucose is impaired under conditions where the CRR is impaired (e.g., recurrent hypoglycemia) (12; 13).

Nitric oxide (NO) is a gaseous messenger produced by NO synthase (NOS). Two classes of NOS have been identified in the brain: the inducible NOS (iNOS) and the constitutive NOS which includes the neuronal NOS (nNOS) and endothelial NOS (eNOS) isoforms (14). Hypothalamic NO is involved in the regulation of food intake and glucose homeostasis (15-18). In support of this, we have recently shown that VMH GI neurons produce NO via nNOS in response to decreased extracellular glucose levels (19; 20). Therefore, in this study, we test the hypothesis that NO production by VMH GI neurons is necessary for the CRR to hypoglycemia. We tested this hypothesis using a combination of in vivo and in vitro techniques in wildtype (WT) rats and mice as well as in transgenic nNOS and eNOS knockout (KO) mice.

**MATERIALS & METHODS**

**Animals:** All procedures were approved by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey. Adult male Sprague-Dawley rats were purchased from Charles Rivers. Adult 5-8 weeks old C57BL/6J wildtype (WT), nNOS KO (B6.129S4-Nos1tm1Plh/J) and eNOS KO (B6.129P2-Nos3tm1Unc/J) were purchased from Jackson laboratories (Bar Harbor, ME, USA). Animals were housed individually and maintained on a 12-12 hour light–dark schedule at 22-23°C with ad libitum access to food and water.

**In vivo experiments:**
Surgical procedures: Rats were anesthetized with sodium pentobarbital (50 mg/kg, IP, Ovation) and mice with ketamine/xylazine (80/8 mg/kg, IP; BionichePharma/Lloyd laboratories). Rats were surgically implanted with vascular catheters in the left carotid and/or the right jugular vein and mice were implanted with a vascular catheter in the right
jugular vein. The catheters were filled with heparin (10 U/ml) and flushed every other day. Additionally, rats were stereotaxically implanted with microinjection cannula guide positioned 1 mm dorsal to the VMH or in the right lateral ventricle according to stereotaxic coordinates (VMH cannulation; from bregma: -2.5 mm anterior-posterior, -2.8 mm medial-lateral, and -8.5 mm dorsal-ventral, at an angle of 20°; ICV cannulation; from bregma: -1.0 mm anterior-posterior, -1.4 mm medial-lateral, and -4.0 mm dorsal-ventral). Animals were allowed 5-7 days to recover from surgery and were handled every day. Animals that did not recover to their pre-surgery body weights were excluded from the study. Probe placement: At the end of each experiment, cannula placement was verified by methylblue (Sigma) injection.

Experimental procedures: Animals undergoing hyperinsulinemic/hypoglycemic clamps were either fasted overnight (rats) or for 5 hours (9am to 2pm; mice). Two hours before the start of the study, catheters were externalized outside the cage to minimize investigator interaction and were connected to infusion pumps. Starting 30 minutes before insulin injection (see below), one group of rats was infused ICV (0.4 µl/min, 2 hours) where another group was injected in the VMH (0.1 µl/min, 10 minutes) with one of the following compounds in artificial cerebrospinal fluid (aCSF; containing in mM: 135 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, 10 Hepes, pH=7.4): N\textsuperscript{G}-Nitro-L-arginine (LNMMA, 50 mM in aCSF), 1H-[1,2,4]-Oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ, 0.1 mM in aCSF containing 0.1% DMSO). The control for LNMMA was injected with aCSF while the control for ODQ was injected with DMSO (0.1% in aCSF). Acute insulin infusion: Rats (100-150 g) were injected with an insulin bolus (1 U/kg; Regular human insulin, Eli-Lilly) through the jugular catheter 30 minutes after ICV or VMH infusion. Blood glucose was monitored every 15 minutes from -30 to 120 minutes post-insulin infusion via tail prick. Hyperinsulinemic/Hypoglycemic clamp: Starting 30 minutes after VMH or ICV infusion, rats (300-350 g) or mice (7-8 weeks old) were injected through the jugular catheter with an insulin bolus (rats: 0.4 U/kg; mice: 1 U/kg) in order to decrease glycemia to ~50 mg/dl within 30-40 minutes. This time course was used based on the results of Saberi et al. suggesting that brain \textit{vs} peripheral glucose sensors predominate in CRR initiation when blood glucose decreases rapidly (21). After this bolus, animals were perfused with insulin at 1.2 U/kg/h for 90 (rats) or 120 (mice) minutes. Glucose (20%) was co-perfused with insulin in order to maintain their plasma glucose level around 50 mg/dl. The concentration of blood glucose was measured every 10 minutes via tail prick. For clamps carried out in rats, arterial blood samples (500 µl) taken from the carotid catheter were collected at 0, 30, 60 and 90 minutes for subsequent measurement of plasma glucagon, epinephrine and norepinephrine. Glucocorticoid levels were not measured since they are not an essential aspect of the recovery from an acute hypoglycemic challenge (for review, see (22)). For glucagon, 250 µl of blood was collected in chilled tubes containing EGTA (1.6 mg/ml, Sigma) and aprotinin (250 KIU/ml, Sigma). For catecholamines, blood was collected in chilled tubes containing reduced glutathione (1.2 mg/ml, Sigma) and EDTA (1.8 mg/ml, Sigma). After removal of plasma, erythrocytes from experimental rats were resuspended in an equivalent volume of sterile NaCl 0.9% and reinfused after each blood sampling to prevent volume depletion. For mice clamp, trunk blood was collected at the end of the clamp in chilled tubes containing reduced glutathione (1.2 mg/ml, Sigma) and EDTA (1.8 mg/ml, Sigma) for plasma epinephrine and norepinephrine measurement.
Plasma glucagon and catecholamines determination: Plasma glucagon concentrations were determined using commercially available radio-immunoassay kits (Linco Research). Plasma epinephrine and norepinephrine concentrations were analyzed by high-performance liquid chromatography using electrochemical detection (ESA, Acton).

P-NOS Western blot, NOS activity: Rats (100-150 g) were injected with saline or insulin (2 U/kg, SC) and killed 60 minutes after by an overdose of sodium pentobarbital (Euthasol). The ventral hypothalamus was quickly harvested, snap frozen and stored at -80°C. P-NOS Western blot: Brain samples were lysed over ice in lysis buffer (150 mM NaCl, 0.02% sodium azide, 10 mM HEPES, 50 mM NaF, 0.1% SDS, 0.5% Deoxycholic Acid, 1% NP-40, 0.2 mM PMSF, 2 µg/ml Peptain-A, 2 µg/ml Leupeptin and 2 µg/ml Aprotinin). Cytosolic lysate supernatants are collected by centrifugation 14000 g for 10 minutes at 4°C. 15 µg of protein were electrophoresed and transferred to nitrocellulose membranes. Immuno-detection with 1° antibodies was performed for 12 hours at 4°C: P-nNOS (nNOS-Ser 1717) 1:5000 (Millipore), P-eNOS (eNOS-Ser 1177) 1:5000, nNOS and eNOS 1:2500 (Cell Signaling). After washing secondary antibody (donkey anti-rabbit, Jackson Immuno-Research) was added at 1:1000 1 hour at room temperature. Signals are visualized using ECL kit (Thermo) and quantified using Scion Image. Results are presented as percentage of control after normalization to total nNOS/eNOS. NOS activity: NOS activity was quantified using the radio-detection kit (Calbiochem) based on the biochemical conversion of [3H]-L-arginine to [3H]-L-citrulline by NOS. To distinguish Ca2+-dependent constitutive NOS activity (nNOS + eNOS), from Ca2+-independent iNOS activity, hypothalamic homogenates were prepared as above and divided into two sets of samples, one of which omitted calcium in the assay medium for measurement of iNOS activity.

In vitro experiments: Electrophysiology and cellular imaging: Coronal brain slices (250 µm) from WT and nNOS KO mice (5-7 weeks old) were prepared as previously described (8; 23). Electrophysiology: Briefly, viable neurons were visualized under infrared differential-interference contrast microscopy (Leica Microsystems, DM LFS microscope). Current clamp recordings (standard whole-cell configuration) from VMN neurons were performed using a MultiClamp 700A (Axon Instruments) and analyzed using pCLAMP9 software. During recording, brain slices were perfused at 10 ml/min with normal oxygenated artificial cerebrospinal fluid containing (in mM): 126 NaCl, 1.9 KCl, 1.2 KH2PO4, 26 NaHCO3, 2.4 CaCl2, 1.3 MgCl2, 2.5 glucose; 300-310 mOsM, pH 7.4). Borosilicate pipettes (3-5 MΩ; Sutter Instrument) were filled with an intracellular solution containing (in mM): 128 K-gluconate, 10 KCl, 4 KOH, 10 HEPES, 4 MgCl2, 0.5 CaCl2, 5 EGTA, and 2 Na2ATP (pH 7.2; 290-300 mOsM). Membrane potential, action potential frequency and input resistance in response to constant hyperpolarizing pulse (20pA) were monitored as extracellular glucose level was changed from 2.5 to 0.1 mM as described in figures. Cellular imaging: VMH neurons were prepared using a protocol modified from Murphy et al. (24; 25) (see supplementary data for detailed protocol). VMH neurons were perfused in a closed chamber at 0.6 ml/min with oxygenated extracellular solution containing (in mM): 132 NaCl, 5 KCl, 0.45 KH2PO4, 0.45 Na2HPO4, 1.2 CaCl2, 0.5 MgCl2, 0.4 MgSO4, 5 Hepes, 2.5 glucose (pH 7.3; osmolarity adjusted to 300-310 mOsM) in the presence of 0.5% membrane potential dye (FLIPR-MPD, Molecular Devices). After 10 minutes of equilibration, VMH neurons
were perfused with the same extracellular solution containing 0.1 mM glucose for 15 minutes followed by 15 minutes at 2.5 mM glucose. Images acquisition and analysis was performed as previously described (24; 25). Neurons were considered as GI neurons when their fluorescence intensity reversibly increased more than 25% in response to 0.1 mM glucose. Data are expressed in % of GI neurons detected per dish.

Hypothalamic NO real-time measurement: WT mice were killed by decapitation without anesthesia. The Hypothalamus was quickly harvested and maintained in 200µl Krebs-Ringer oxygenated solution containing 2.5 mM glucose at 37°C. A NO specific amperometric probe (ISO-NOPF100, WPI) was implanted directly in the tissue and NO release was monitored. The hypothalamus was exposed to the following sequence of glucose concentrations (15 minutes each): 2.5 mM, 0.1 mM, 2.5 mM. The concentration of NO gas in the tissue was measured in real-time with the data acquisition system LabTrax (WPI) connected to the free radical analyzer Apollo1000 (WPI). Data acquisition and analysis were performed with DataTrax2 software (WPI). The NO specific amperometric probe was calibrated as previously described (26).

Data analysis: All data are presented as mean ± SEM. Statistical analysis was performed using Graphpad Prism 4.0 by two-way ANOVA followed by Bonferoni post-hoc test, one-way ANOVA followed by Dunnett post-hoc test or by unpaired t-test as described in the figure legends. p<0.05 indicates statistical significance.

RESULTS

Hypoglycemia activates ventral hypothalamic nNOS. We have previously shown that decreased glucose concentration increases NO production in cultured VMH GI neurons in vitro using a membrane sensitive dye (20). To confirm that decreased glucose increases hypothalamic NO production, we performed amperometric measurement of NO release in hypothalamic chunks ex vivo using an NO sensitive electrode. As shown in Figure 1 decreased glucose from 2.5 to 0.1 mM significantly increases the amplitude (3.5 fold; p<0.05) and frequency (2.1 fold; p<0.05) of NO release. NO release returned to baseline when extracellular solution was subsequently raised to 2.5 mM glucose (Fig. 1).

To provide in vivo evidence that hypoglycemia increases hypothalamic NO production, constitutive (nNOS and eNOS) and inducible NOS (iNOS) activity was determined in ventral hypothalami from rats 60 minutes after insulin injection. Insulin-hypoglycemia significantly increased constitutive NOS activity by 1.45 ± 0.11 fold. Inducible NOS activity was not changed (Fig. 2A). Cortical constitutive NOS activity was not changed in insulin-induced hypoglycemia treated rats vs control (data not shown). To determine whether nNOS or eNOS is primarily responsible for hypoglycemia-induced hypothalamic NO production, western-blots against the phosphorylated nNOS and eNOS forms were performed. nNOS phosphorylation was significantly increased by 7.26 ± 0.36 fold whereas eNOS phosphorylation was not changed (Fig. 2B) suggesting that nNOS activation was responsible for increased VMH constitutive NOS activity during insulin-hypoglycemia. These data strongly suggest that insulin-hypoglycemia stimulates nNOS-derived VMH NO production.

Inhibition of VMH NO signaling impairs the CRR to hypoglycemia. We first evaluated the effect of brain NO on the counter-regulatory response to acute insulin-induced hypoglycemia. As shown in figure 3, rats infused with the non-selective NOS
VMH NO production is required for hypoglycemia counter-regulation.

Inhibitor LNMMMA either ICV or into the VMH showed significantly lower glycemia at 60 and 90 minutes post-insulin injection in comparison to control. Many of the effects of NO are mediated by its receptor, soluble guanylyl cyclase (sGC) (14). Inhibition of VMH sGC with ODQ decreased the glycemia at 45, 60, 90 and 120 minutes post-insulin injection (Fig. 3B).

To confirm that VMH NO production is involved in the CRR, we performed hyperinsulinemic/hypoglycemic clamps (5; 6; 21; 27). During the hypoglycemic clamp, blood glucose was decreased to similar levels in control (52 ± 1.1 mg/dl) and treated (54 ± 1.0 mg/dl) animals (Fig. 4). Administration of the non-selective NOS inhibitor LNMMMA in the VMH significantly increased the glucose infusion rate (GIR) necessary to maintain the hypoglycemia plateau (Fig. 4). Changes in GIR were associated with significant decreases in epinephrine levels at times 60 and 90 minutes in LNMMMA treated animals (Fig. 4). Glucagon (Fig. 4) and norepinephrine (data not shown) levels were not significantly reduced. Taken together, these data show that VMH NO-sGC signaling pathway is necessary for the full generation of the sympathoadrenal response to hypoglycemia.

VMH nNOS is involved in the CRR to hypoglycemia. Data from our laboratory and others suggest that VMN GSNs play a role in sensing hypoglycemia and initiating the CRR (9; 13; 27-32). Since we showed above that the CRR is impaired in nNOS KO mice, we wanted to determine whether the glucose sensitivity of GSNs is also impaired. We used whole-cell current clamp recording techniques to measure the membrane potential (MP), action potential frequency (APF) and input resistance (IR) of VMN neurons in response to decreased glucose levels from 2.5 to 0.1 mM in WT and nNOS KO mice. In WT mice, 3 neurons (3/36, 8%) were identified as GE neurons by a decrease in their MP, APF and IR in response to 2.5 to 0.1 mM glucose decrease whereas 11 neurons (11/36, 30%) increased MP, APF and IR in response to decreased glucose and were identified as GI neurons (Fig. 6A). In nNOS KO mice, 4 neurons (4/25, 16%) were identified as GE neurons (Fig. 6B). In contrast, no GI neurons (0/25) were found in nNOS KO mice VMN. Results are summarized in Figure 6C. We confirmed these electrophysiology data using a membrane potential sensitive dye in cultured VMH neurons. While 13.0 ± 1.2% of VMH
VMH NO production is required for hypoglycemia counter-regulation.

neurons were GI neurons in WT mice (14 dishes; 1352 neurons; 7 mice), only 2.4 ± 0.6% were GI neurons in nNOS KO mice (12 dishes; 961 neurons; 3 mice; p<0.05). These data suggest that VMH GI neurons glucose sensing is impaired in nNOS KO mice.

DISCUSSION

This study confirms that decreased glucose increases VMH NO production in vivo. Moreover, this study supports our novel hypothesis that NO production is necessary for the full generation of the CRR and glucose sensing in VMH GI neurons. Pharmacological inhibition of VMH NO signaling decreases blood glucose recovery and impairs the CRR following hypoglycemia. Interestingly, the impaired CRR in mice lacking nNOS is associated with an almost complete loss of VMH GI neurons consistent with our recently published data showing that NO production is required for GI neurons to sense glucose (24). We have previously shown that VMH GI neurons are less sensitive to decreased glucose under conditions where the CRR is also impaired. These data suggested a role for VMH GI neurons in the CRR (12; 13; 28; 31; 33). Our current data strengthen the hypothesis that detection of hypoglycemia by VMH GI neurons is a necessary step in the full generation of the CRR.

We found previously, using in vitro cellular imaging, that among cultured VMH neurons only GI neurons produce NO in response to decreased glucose. nNOS but not eNOS, mediates NO production in VMH GI neurons (20). In the present study, we confirm this finding by showing that decreased glucose increases VMH NO release using a NO sensitive electrode. Moreover, insulin-induced hypoglycemia in vivo increases VMH NOS activity and nNOS phosphorylation. Since insulin increases nNOS-derived NO production in cultured VMH neurons (20), insulin injection may contribute to the increased VMH NO production during this clinically relevant form of hypoglycemia. These data strongly support our hypothesis that nNOS activation during insulin-induced hypoglycemia induces VMH NO production in vivo. Cabou et al. recently suggested that cerebral insulin injection during euglycemia increases hypothalamic NO production through eNOS (16). Insulin-induced hypoglycemia did not increase eNOS activity in our study. Moreover, since Cabou et al did not evaluate nNOS activity, they did not rule out a role for this NOS isoform in response to cerebral insulin injection. It is possible that prolonged hyperinsulinemia and/or recurrent episodes of insulin-induced hypoglycemia further increase VMH NO production through a combined increase in nNOS and eNOS activity.

What is the role of VMH NO production in energetic homeostasis during energy deficit? One putative function for VMH NO production is to increase cerebral blood flow leading to increased local nutrient availability. Human and animal studies show that insulin-induced hypoglycemia is associated with increased cerebral blood flow in many brain areas including the hypothalamus (34-36). For example, Page et al. recently showed that decreased blood glucose increased hypothalamic blood flow prior to the release of CRR hormones (37). One of the main physiological functions of NO is related to the vascular system. The role of eNOS mediated NO production in peripheral vasorelaxation is well established (38). One of the unique features of NO as a neurotransmitter is the ability to diffuse across cell membranes (14). Thus, although we did not see an increase in eNOS activity in our studies, NO produced in VMH GI neurons may diffuse to adjacent vascular smooth muscle cells lining cerebral vasculature and cause vasodilatation. However, we think that this is unlikely because Horinaka et al. showed that increased
cerebral blood flow in response to hypoglycemia was NO independent (39; 40). These data suggest that VMH nNOS mediated NO production does not play a role in blood flow regulation. This is consistent with other studies which suggest a role for the β-adrenergic receptor and/or the ATP-sensitive K channel (K<sub>ATP</sub>) in hypoglycemia-induced increases in cerebral blood flow (41; 42).

Another function of VMH NO production is through the CRR. We used 2 complementary approaches to show that VMH NO production is a physiologically required step in the full generation of the CRR. First, inhibition of VMH NO production slows down the recovery to euglycemia in response to acute insulin-induced hypoglycemia. While this is the most physiological evaluation of the CRR, it is difficult to reliably compare the levels of counter-regulatory hormones between treatments due to variation in the actual degree of hypoglycemia. Thus, we also employed the “gold standard” technique for studying the CRR: hyperinsulinemic/hypoglycemic clamps. Here we found that VMH NOS inhibition increases the GIR and decreases epinephrine production during hypoglycemic clamps. Moreover, the GIR is significantly greater and epinephrine production lower in nNOS KO vs WT mice. These data confirm our hypothesis that VMH NO plays an important role in the control of the CRR. However, it is also clear that the CRR was not completely abolished by either LNMMA injection or in the nNOS KO mice. These findings are consistent with parallel regulation of the CRR by other central or peripheral glucose sensors. Finally, both eNOS and nNOS KO mice exhibit insulin resistance (18; 43). In eNOS KO mice, there was a decrease in the GIR to maintain the hypoglycemic plateau which may reflect insulin resistance (18; 43). The milder insulin resistance in nNOS KO mice probably did not affect the GIR due to the high insulin concentration used for the hypoglycemic clamp.

The next step was to explore the molecular and cellular mechanisms by which VMH NO production contributes to the CRR. Our previous studies suggested a role for VMN GI neurons in the generation of the CRR since their response to decreased glucose is impaired when the CRR is impaired (12; 13; 28; 31; 33). We have recently shown that NO production via nNOS is necessary for VMN GI neurons to depolarize in response to decreased glucose (24). In the present study, VMH GI neurons were not detected in nNOS KO mice in response to decreased extracellular glucose from 2.5 to 0.1 mM. This glucose concentration decrease, while supraphysiologic, was necessary because we have previously shown that recurrent episodes of hypoglycemia decrease the response of VMH GI neurons to decreased glucose. In fact, after recurrent hypoglycemia the response of VMH GI neurons to a glucose decrease from 2.5 to 0.5 mM was almost undetectable; however their response to a glucose decrease from 2.5 to 0.1 mM was intact (13). Thus, using a glucose decrease to 0.1 mM suggests that functional VMH GI neurons are almost absent in nNOS KO mice. The CRR was also impaired in nNOS KO mice. These data reinforce our hypothesis that activation of VMH GI neurons in response to decreased glucose is critical for the full generation of the CRR. Restoration of VMH NO expression in nNOS KO mice would lend to further strength—to this conclusion. However, the effects of NO are highly dependent on the localization of intracellular NO production which, in turn, is highly dependent on intracellular NOS localization (14). Overexpressing nNOS or injecting NO donors into the VMH of nNOS KO mice would not mimic physiological NO production and could lead to difficulties in data interpretation. Our data suggest also that
VMH NO production is required for hypoglycemia counter-regulation.

the NO receptor sGC mediates the effect of VMH NO on the CRR. sGC is expressed in all VMH neurons including GI neurons (20). Cyclic GMP produced by sGC has been shown to modulate neuronal activity (17; 44). Taken together, these data suggest that decreased glucose depolarizes VMH GI neurons through NO-sGC signaling and leads to full generation of the CRR.

On the other hand, our data suggest that VMH GI neurons are not the only mediator of the CRR because the CRR is still present, albeit impaired, in the absence of NO signaling. VMH GE neurons are normal in nNOS KO mice. Moreover, Miki et al. showed that the CRR was impaired and VMH GE neurons absent in K_{ATP} deficient mice (30). Therefore, it is likely that VMH GI and GE neurons as well as extra-hypothalamic glucose sensors are needed for the full generation of the CRR. Interestingly, glucagon but not epinephrine secretion in response to hypoglycemia was impaired in the K_{ATP} deficient mice (30). In contrast our data indicate that inhibition of VMH NO signaling impairs epinephrine but not glucagon or norepinephrine secretion in response to hypoglycemia. This suggests that different glucose sensors may control unique elements of the CRR.

In conclusion, the VMH NO-sGC signaling pathway is a key component in the generation of the CRR. Moreover, our data provide strong support for our hypothesis that VMH GI neurons play a crucial role in the central detection of hypoglycemia and generation of the CRR. These data also suggest that potentiating NO signaling may enhance epinephrine secretion and glucose recovery in diabetic patients exposed to recurrent hypoglycemia. The role of NO signaling in epinephrine secretion in response to hypoglycemia is extremely relevant for patients with Type 1 Diabetes who lack a glucagon response. Thus, the NO-sGC signaling pathway may offer new therapeutic targets to improve the treatment of patients with Type 1 and advanced Type 2 Diabetes Mellitus using intensive insulin therapy.

ACKNOWLEDGEMENT:

This work was supported in part be the Juvenile Diabetes Research Foundation (JDRF) (XF and VHR) and the National Health Institute (2RO1DK55619 and 1RO1DK64566; VHR). XF was supported in part by the Philippe Foundation.
VMH NO production is required for hypoglycemia counter-regulation.

REFERENCES
VMH NO production is required for hypoglycemia counter-regulation.


24. Murphy BA, Fakira KA, Song Z, Beuve A, Routh VH: AMP-activated Protein Kinase (AMPK) and Nitric Oxide (NO) regulate the glucose sensitivity of ventromedial hypothalamic (VMH) glucose-inhibited (GI) neurons. *Am J Physiol Cell Physiol*, 2009


VMH NO production is required for hypoglycemia counter-regulation.

**Figures Legends**

**Figure 1: Decreased glucose increases VMH NO release.** (A) Representative trace of ex vivo amperometric measurements of NO release from mouse hypothalamus in response to an extracellular glucose decrease from 2.5 to 0.1 mM. (B) Mean frequency and (C) mean amplitude of NO release calculated during the last 10 min recording for each glucose level (n = 4). *: p < 0.05 vs 2.5 mM glucose (One way ANOVA).

**Figure 2: Hypoglycemia increases ventral hypothalamic nNOS activity.** A) Ventral hypothalamic constitutive (e/nNOS) or inducible (iNOS) NOS activity from rats injected subcutaneously with saline (Control, n = 6) or insulin (2 U/kg; n = 6) 60 minutes post-injection. B) Representative western blot (left panel) of ventral hypothalamic total-nNOS, phosphorylated-nNOS (P-nNOS), total-eNOS and P-eNOS from control or insulin treated rats injected subcutaneously with saline (n = 5) or insulin (n = 5) 60 minutes post-injection. The right panel shows the quantification of the ratio between P-nNOS or P-eNOS and total nNOS or eNOS, respectively. Data are mean ± sem and represented as percentage of saline where the control group was considered to be 100%. *: p < 0.05 vs control (unpaired t-test).

**Figure 3: VMH NO signaling is necessary for recovery to euglycemia following insulin-hypoglycemia.** Blood glucose levels in response to insulin-induced hypoglycemia (1 U/kg, IV) in rats receiving A) ICV perfusion of ACSF (controls; n = 14), LNMMA (50 mM; n = 14) or B) unilateral VMH injection of aCSF (n = 7), LNMMA (50 mM, n = 7) or ODQ (0.1 mM, n = 5). *: p < 0.05 vs control (two-way ANOVA).

**Figure 4: VMH NOS inhibition impairs the CRR to hypoglycemia.** Blood glucose level (A); glucose infusion rate (GIR; B); plasma epinephrine (C) and glucagon levels (D) during hyperinsulinemic/hypoglycemic clamp (1.2 U/kg/h) of animals injected bilaterally in the VMH with aCSF (controls; n = 8) or LNMMA (50 mM; n = 6) animals. *: p < 0.05 vs controls (two-way ANOVA).

**Figure 5: nNOS is necessary for full initiation of the CRR.** Blood glucose concentration (A), glucose infusion rate (B) and plasma epinephrine taken at the end of the clamp (C) of WT (n = 14), eNOS (n = 6) and nNOS (n = 7) KO mice during hyperinsulinemic/hypoglycemic clamp (1.2 U/kg/h). *: p < 0.05 vs WT (two-way ANOVA).

**Figure 6: nNOS is necessary for glucose sensing by VMN GI neurons.** Representative whole cell current-clamp recordings of VMN glucose-excited (GE) and glucose-inhibited (GI) neurons in brain slices from WT (A) or nNOS KO mice (B). The dotted lines represent the resting membrane potential. Glucose concentration changes are schematically displayed below each recording. Downward deflections in whole-cell current-clamp recordings represent the membrane voltage responses to constant hyperpolarizing currents. (C) Table summarizing the number (and %) of VMN GE, GI or non-glucose sensitive (NG) neurons in WT or nNOS KO mice.
VMH NO production is required for hypoglycemia counter-regulation.

Figure 1

![Figure 1 diagram]

Figure 2

![Figure 2 diagram]
VMH NO production is required for hypoglycemia counter-regulation.

Figure 3

A  ICV infusion

B  VMH infusion

Figure 4
VMH NO production is required for hypoglycemia counter-regulation.

Figure 5

A Blood glucose

B Glucose infusion rate

C Plasma epinephrine

Figure 6

A Wild type mice

B nNOS KO mice

C Summary