Brain Insulin Action Regulates Hypothalamic Glucose Sensing and the Counterregulatory Response to Hypoglycemia

Kelly A. Diggs-Andrews,1 Xuezhao Zhang,1 Zhentao Song,2 Dorit Daphna-Iken,1 Vanessa H. Routh,2 and Simon J. Fisher1,3

OBJECTIVE—An impaired ability to sense and appropriately respond to insulin-induced hypoglycemia is a common and serious complication faced by insulin-treated diabetic patients. This study tests the hypothesis that insulin acts directly in the brain to regulate critical glucose-sensing neurons in the hypothalamus to mediate the counterregulatory response to hypoglycemia.

RESEARCH DESIGN AND METHODS—To delineate insulin actions in the brain, neuron-specific insulin receptor knockout (NIRKO) mice and littermate controls were subjected to graded hypoglycemic (100, 70, 50, and 30 mg/dl) hyperinsulinemic (20 mU/kg/min) clamps and nonhypoglycemic stressors (e.g., restraint, heat). Subsequently, counterregulatory responses, hypothalamic neuronal activation (with transcriptional marker c-fos), and regional brain glucose uptake (via 14C-2deoxyglucose autoradiography) were measured. Additionally, electrophysiological activity of individual glucose-inhibited neurons and hypothalamic glucose sensing protein expression (GLUTs, glucokinase) were measured.

RESULTS—NIRKO mice revealed a glycemia-dependent impairment in the sympathoadrenal response to hypoglycemia and demonstrated markedly reduced (3-fold) hypothalamic c-fos activation in response to hypoglycemia but not other stressors. Glucose-inhibited neurons in the ventromedial hypothalamus of NIRKO mice displayed significantly blunted glucose responsiveness (membrane potential and input resistance responses were blunted 66% and 80%, respectively). Further, hypothalamic expression of the insulin-responsive GLUT 4, but not glucokinase, was reduced by 30% in NIRKO mice while regional brain glucose uptake remained unaltered.

CONCLUSIONS—Chronically, insulin acts in the brain to regulate the counterregulatory response to hypoglycemia by directly altering glucose sensing in hypothalamic neurons and shifting the glycemic levels necessary to elicit a normal sympathoadrenal response.

Diabetes 59:2271–2280, 2010

From the 1Division of Endocrinology, Metabolism and Lipid Research, Department of Internal Medicine, Washington University School of Medicine, Saint Louis, Missouri; the 2Department of Pharmacology and Physiology, New Jersey Medical School (UMDNJ), Newark, New Jersey; and the 3Department of Cell Biology and Physiology, Washington University School of Medicine, Saint Louis, Missouri.

Corresponding author: Simon J. Fisher, sfisher@dom.wustl.edu.

Received 22 March 2010 and accepted 4 June 2010. Published ahead of print at http://diabetes.diabetesjournals.org on 14 June 2010. DOI: 10.2337/db10-0401.

© 2010 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See http://creativecommons.org/licenses/by-nc-nd/3.0/ for details.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

diabetes.diabetesjournals.org

Intensive insulin therapy markedly increases the risk of severe hypoglycemia in people with type 1 (1) and type 2 (2) diabetes. Thus, hypoglycemia is the rate-limiting step for tight glycemic management in diabetic patients. In response to hypoglycemia, glucose sensors in the central and peripheral nervous system coordinate efferent autonomic responses resulting in the release of key counterregulatory hormones—glucagon, norepinephrine, epinephrine, and cortisol. This coordinated response stimulates hepatic glucose output and restricts glucose utilization to increase blood glucose levels. Patients with diabetes often have an impaired ability to sense and respond to hypoglycemia (3–5) because several components of the counterregulatory response have been shown to be either absent (i.e., failure in insulin, rise in glucagon) or markedly blunted (i.e., the sympathoadrenal response) (6,7).

While hypoglycemia is caused by absolute or relative insulin excess, the role of insulin in regulating the counterregulatory response is unclear. Studies have demonstrated that increased insulin levels may augment (8–11), diminish (12), or not change (13–16) the sympathoadrenal response to hypoglycemia. Given recent evidence indicating that insulin acts in the brain (17), some studies have investigated whether insulin’s putative actions in regulating the counterregulatory response might be mediated via actions in the central nervous system. Again, conflicting reports suggest that insulin may act centrally to enhance (18–20), reduce (21), or not alter (22) the sympathoadrenal response to hypoglycemia. If insulin acts in the brain, its likely site of action is glucose-sensing neurons located in the ventromedial hypothalamus (VMH) (21,23–26). These glucose-sensing neurons share metabolic similarities to other well-characterized glucose-sensing cells (i.e., pancreatic β-cells), especially with regard to glucose transport and metabolism (27–29). On the basis of the expression of insulin receptors in the majority of glucose-sensing neurons in the VMH (30), it is postulated that brain insulin action may mediate its effects on central glucose sensing by regulating expression of GLUTs and/or glucokinase.

In this study, the neuronal specific insulin-receptor knockout (NIRKO) mouse model, which chronically lacks central nervous system (CNS) insulin signaling (17,31), was used to investigate the role and mechanism by which brain insulin action regulates central glucose sensing and the counterregulatory response to hypoglycemia.
RESEARCH DESIGN AND METHODS

Mice homozygous for the floxed insulin receptor allele (IRlox-lox) were bred with transgenic mice that express Cre recombinase cDNA from the rat neitin promoter to generate (IRlox-lox;nestin-Cre”) NIKRO mice (17). Genotypes were determined by PCR of tail DNA. Unless otherwise indicated, 2- to 4-month-old NIKRO (IRlox-lox;nestin-Cre”) and littermate control (IRlox-lox;nestin-Cre”) mice were used for these experiments. All mice were housed on a 12:12-h light/dark cycle and fed a standard rodent chow (Mouse Diet 9F, PMI Nutrition International, St. Louis, MO) ad libitum. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Animal Studies Committee of Washington University.

Hypoglycemic-hyperinsulinemic glucose clamps. Mice anesthetized with ketamine/xylazine (87 and 13.4 mg/kg i.p.) were implanted with catheters (MIRE 025, Braintree Scientific Inc., Braintree, MA) into both the right internal jugular and the left carotid or femoral artery. After a 5- to 7-day recovery period, hyperinsulinemic (20 mU/kg/min) hypoglycemic clamps were performed in 5-h fasted, awake, unrestrained, NIKRO and control mice (n = 6–9 per group). To create different degrees of hypoglycemic stress, arterial blood glucose was measured at 10-min intervals and the rate of intravenous 50% dextrose infusion was carefully adjusted to achieve equivalent levels of mild (70 mg/dl), moderate (50 mg/dl), and severe hypoglycemia (30 mg/dl) as well as a euglycemic (110 mg/dl) control. High-performance liquid chromatography– purification methods were used for the determination of plasma insulin as previously described (30,34). The following primary antibodies were used: GLUT1 and GLUT3; 1:100 (Abcam, Cambridge, MA), glucokinase (1:1,000, Calbiochem). The blots were subjected to transfer. The following primary antibodies were used: GLUT1 (1:5,000, Chemicon), GLUT3 (1:1,000, Chemicon), GLUT4 (1:1,000, kindly supplied by Dr. M. Mueckler), glucokinase (1:1,000, Calbiochem). The blots were developed using a horseradish peroxidase-conjugated secondary antibody (1:5,000). Primary antibody binding was detected by enhanced chemiluminescence reagents (Perkin Elmer, Wellesley, MA) on ISO-MAX films and quantified by ImageQuant software analysis (Amersham Pharmacia, Piscataway, NJ). An antibody against β-actin (1:2,000, Sigma, St. Louis, MO) served as a loading control.

Immunohistochemistry. Cryoprotected brains were processed for DAB 3,3′-diaminobenzidine peroxidase substrate immunohistochemistry or immunofluorescence. Briefly, free-floating hypoglycemic sections (20–30 μm) throughout the VMH/arcuate nucleus (ARC) were taken from 1.46 to 1.82 mm caudal to bregma, blocked, and incubated overnight at 4°C in primary antibodies. The following antibody dilutions were used: GLUT1 (1:1,000), c-fos (1:2,000, Ab-5, Calbiochem). For immunofluorescence, goat anti-rabbit Texas Red (1:200, Molecular Probes) was used as the secondary antibody. Subsequently, the sections were mounted on slides using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). For DAB immunohistochemistry, immunoreactivity was performed with biotinylated goat anti-rabbit immunoglobulin G (1:200) using the Elite ABC kit (Vector Laboratories). As a negative control, alternative sections were incubated without primary antibodies. Regions of interest were identified using anatomical landmarks (36), and positively stained cells were counted by a blinded investigator. Four to six brain sections per mouse were quantified for statistical purposes.

RESULTS

Brain insulin action is necessary for full sympathoadrenal response to hypoglycemia. To characterize the counterregulatory response to hypoglycemia, a series of hyperinsulinemic glucose clamps were performed. Blood glucose was clamped at 110, 70, 50, and 30 mg/dl in control and NIKRO mice to induce different degrees of hypoglycemia (mild = 70 mg/dl, moderate = 50 mg/dl, and severe hypoglycemia = 30 mg/dl) or no hypoglycemia (euglycemic clamp = 110 mg/dl) (Fig. 1). In response to insulin infusion, plasma insulin levels were similarly elevated in NIKRO and control mice (Fig. 2A). Severe hypoglycemia (30 mg/dl) resulted in a sixfold increase in glucagon levels and ~60% increase in corticosterone levels, but these increases were similar in both groups (Fig. 2B and C).

BRAIN INSULIN ACTION REGULATES GLUCOSE SENSING

Heat stress. Awake control and NIKRO mice (n = 6 per group) were exposed to an ambient temperature of 42°C for 90 min to induce heat stress. Blood samples were taken at the end of the heat stress period to measure catecholamines. Subsequently, cryoprotected brains were analyzed for heat stress–induced c-fos immunoreactivity.

Western blots. The medial basal hypothalamus, defined anatomically as posterior to the optic chiasm, anterior to the mamillary body, inferior to the thalamus, and ±1 mm lateral to the midline, was dissected and frozen for analysis. Homogenized hypothalamic protein extracts (20 μg for GLUT1 and GLUT3, 100 μg for GLUT4 and glucokinase) were fractionated by electrophoresis on a 10% Bis-Tris Criterion XT (Biorad, Hercules, CA) gel and subjected to transfer. The following primary antibodies were used: GLUT1 (1:5,000, Chemicon), GLUT3 (1:1,000, Chemicon), GLUT4 (1:1,000, kindly supplied by Dr. M. Mueckler), glucokinase (1:1,000, Calbiochem). The blots were developed using a horseradish peroxidase-conjugated secondary antibody (1:5,000). Primary antibody binding was detected by enhanced chemiluminescence reagents (Perkin Elmer, Wellesley, MA) on ISO-MAX films and quantified by ImageQuant software analysis (Amersham Pharmacia, Piscataway, NJ). An antibody against β-actin (1:2,000, Sigma, St. Louis, MO) served as a loading control.

Immunohistochemistry. Cryoprotected brains were processed for DAB 3,3′-diaminobenzidine peroxidase substrate immunohistochemistry or immunofluorescence. Briefly, free-floating hypoglycemic sections (20–30 μm) throughout the VMH/arcuate nucleus (ARC) were taken from 1.46 to 1.82 mm caudal to bregma, blocked, and incubated overnight at 4°C in primary antibodies. The following antibody dilutions were used: GLUT1 (1:1,000), c-fos (1:2,000, Ab-5, Calbiochem). For immunofluorescence, goat anti-rabbit Texas Red (1:200, Molecular Probes) was used as the secondary antibody. Subsequently, the sections were mounted on slides using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). For DAB immunohistochemistry, immunoreactivity was performed with biotinylated goat anti-rabbit immunoglobulin G (1:200) using the Elite ABC kit (Vector Laboratories). As a negative control, alternative sections were incubated without primary antibodies. Regions of interest were identified using anatomical landmarks (36), and positively stained cells were counted by a blinded investigator. Four to six brain sections per mouse were quantified for statistical purposes.

RT-PCR. Sections (400 μm) were taken from brain sections 1.46–1.86 mm caudal to bregma. Bilateral punch biopsy samples (0.5 mm) from the VMH and ARC (0.75 mm from the piniform cortex) were collected from NIKRO mice and littermate controls (n = 7–8 per group). The mRNA extracted with Trizol (Invitrogen Corporation, Carlsbad, CA) was subject to quantitative two-step RT-PCR performed in triplicate in a fluorescent temperature cycler (GeneAmp 7,700 Sequence Detector, Applied Biosystems) with glucokinase primers (glucokinase probe 5′-5′-5′-5′-ACC GCC AAT GTG ATG TCG GCA G3′-5′; glucokinase reverse 5′-5′-5′-5′-CAG TGG TCA CCA AAC TCA-3′; and glucokinase forward 5′-5′-5′-5′-CCA TGA TCT CCT GCT ACT ATG X-3′). The results were quantified after normalizing to rRNA L22 mRNA.

Plasma assays. Blood glucose was measured by a glucometer (Becton, Dickinson and Company, Franklin Lakes, NJ), while plasma glucose was assayed by the glucose oxidase method and a spectrophotometer (BioTek Instruments, Inc., Winooski, VT). Radioimmunoassays were performed for glucocorticoids (ICN Biomedicals, Inc., Costa Mesa, CA). Insulin was assayed by ELISA (Chystal Chem. Inc., Downers Grove, IL). Plasma epinephrine and norepinephrine were measured with a single isothe derivative (radioenzymatic) method (37).

Statistics. All values are presented as the mean ± SEM. Statistical significance was set at P < 0.05, as determined by Student t test.
The epinephrine response was significantly impaired in NIRKO mice during moderate (50 mg/dl) and severe (30 mg/dl) hypoglycemia (Fig. 3A). The epinephrine responses were highly correlated to glycemia levels in both control ($R^2 = 0.76$) and NIRKO ($R^2 = 0.75$) mice but were different between groups as indicated by a shift in the hypoglycemia–epinephrine response curve (Fig. 3A, inset). Norepinephrine levels trended lower in NIRKO mice during moderate (50 mg/dl) and severe (30 mg/dl) hypoglycemia, but the difference did not reach significance (Fig. 3B). In response to the high dose of insulin (20 mU/kg/min), hepatic glucose production was completely inhibited during the hyperinsulinemic clamp at glycemic levels of 100, 70, and 50 mg/dl. During severe hypoglycemia (30 mg/dl), hepatic glucose production rose significantly; however, the rise in hepatic glucose production was significantly blunted in NIRKO mice (Fig. 3C).

**Absent CNS insulin action impairs hypothalamic neuronal activation to hypoglycemia.** To assess the brain’s response to hypoglycemia, c-fos-based functional mapping was used to demonstrate activated neurons and functional circuits that respond to hypoglycemic stress (38). Euglycemic (~110 mg/dl) controls displayed low c-fos expression in the hypothalamus (Fig. 4B). In response to insulin-induced hypoglycemia (31.5 ± 3.1 mg/dl), both NIRKO and control mice markedly increased c-fos expression within the paraventricular nucleus (PVN) of the hypothalamus (Fig. 4A). However, NIRKO animals showed a threefold impairment in c-fos activation as compared with controls (control: 99 ± 16 vs. NIRKO: 31 ± 5, $P < 0.01$) (Fig. 4B).

**Impaired glucose sensing in individual glucose-inhibited neurons.** Whole-cell current-clamp recordings were performed to evaluate the glucose sensitivity of individual glucose-inhibited neurons in the VMH. As expected for VMH glucose-inhibited neurons bathed in sufficient 2.5 mmol/l glucose, action potentials in this basal state were absent in recordings from both control and NIRKO mice. There were also no group differences in membrane potential (MP) or input resistance (IR) in 2.5 mmol/l glucose (control: MP = $-57 ± 4$ mV, IR = $1,209 ± 272$ Ω; NIRKO: MP = $-59 ± 3$ mV, IR = $1,016 ± 162$ Ω). Further, no group differences were observed in glucose-inhibited neurons in response to a maximal glucose decrease from 2.5 to 0.1 mmol/l (not shown). In contrast, glucose-inhibited neurons in NIRKO mice had a significantly impaired change in membrane potential and input resistance (66 and 80% impairment, respectively) in response to a glucose decrease from 2.5 to 0.5 mmol/l (Fig. 4C and D).

**Absent CNS insulin signaling does not influence response to restraint or heat stress.** NIRKO and control mice were subjected to a mild stressor (restraint stress) and a more profound stressor (heat stress) to evaluate sympathoadrenal activation in response to glycemia-independent stress. In response to milder restraint stress, plasma epinephrine levels rose similarly twofold in both littermate controls and NIRKO mice (Fig. 5A). The physiological increased heart rate to restraint stress was also similar in control and NIRKO mice (Fig. 5B). Heat stress induced a more pronounced catecholamine elevation than restraint stress (to levels observed with hypoglycemia), but the rise in both epinephrine and norepinephrine in response to heat stress was again not significantly different between groups (Fig. 6C and D). To determine whether this defect in neuronal activation was unique to hypoglycemia, c-fos expression was also assessed in response to heat stress. Increased c-fos expression was again noted in the PVN in response to heat stress (Fig. 6A), to levels observed with hypoglycemia; however, in response to heat stress, there was no difference in c-fos expression between controls and NIRKO mice.
FIG. 3. Catecholamine and hepatic glucose production levels in a series of hyperinsulinemic glucose clamps. Results are shown for NIRKO (closed bars) and control (open bars) mice ($n = 6–8$ mice per group). A: The epinephrine response was significantly impaired in NIRKO mice ($P < 0.05$) during moderate (50 mg/dL) and severe (30 mg/dL) hypoglycemia. The inset picture demonstrates a shift in the hypoglycemia dose–response curve by the solid (controls) versus dashed (NIRKO) lines. B: Norepinephrine levels in both treatment groups rose significantly higher from the basal period during moderate and severe hypoglycemia, but there was no difference between NIRKO and control responses. C: Hepatic glucose production, in the basal period prior to insulin infusion, was the same in control and NIRKO mice. During the hyperinsulinemic glucose clamps at mild and moderate hypoglycemia, HGP was suppressed. Despite the hyperinsulinemia, during severe hypoglycemia (30 mg/dL), hepatic glucose production rose significantly but remained lower in NIRKO as compared with control mice. $*P < 0.05$. 
groups (Control: 123 ± 4 vs. NIRKO: 129 ± 10, P = NS) (Fig. 6B).

**Abrogated brain insulin action and expression of hypothalamic glucose sensors.** To assess whether CNS insulin action regulates GLUTs and glucokinase in the brain, hypothalamic protein and mRNA expression were assessed. GLUT1 and GLUT3 hypothalamic protein expression were threefold higher than either GLUT4 or glucokinase. GLUT1 protein levels in the hypothalamus were similar in control and NIRKO mice (Fig. 7A and B). Hypothalamic GLUT3 protein levels in NIRKO mice were slightly (80.5 ± 9.8% of control) but not significantly (P = 0.08) reduced (Fig. 7A and B). Glucokinase protein levels were also similar in control and NIRKO mice (Fig. 7A and B). Glucokinase mRNA expression was preferentially expressed in the VMH and arcuate nucleus, but there was no difference in expression levels between experimental groups (Fig. 7C), consistent with the glucokinase protein expression findings. Interestingly, insulin-regulated GLUT, GLUT4, protein levels were significantly reduced (68.5 ± 5.5% of control, P < 0.05) in the hypothalamus of NIRKO mice (Fig. 7A and B). To assess regional localization, immunohistochemistry results demonstrated that GLUT4 protein was highly enriched in the VMH and the ARC of control mice. In NIRKO mice, GLUT4 protein expression was markedly reduced in these regions (Fig. 7D). Despite reductions in GLUT expression in NIRKO mice, regional brain glucose uptake, as assessed during hyperinsulinemic-hypoglycemic clamps, was not different between experimental groups (Fig. 7E).

**DISCUSSION**

Insulin’s role in regulating the counterregulatory response to hypoglycemia is an area of active investigation. Insulin has been shown to increase (8–11,18–20), diminish (12,21), and not alter (13–16,22) the sympathoadrenal response to hypoglycemia. In this study, using a model of chronic brain insulin receptor deficiency, it was demonstrated that insulin action in the brain 1) regulates the glucose sensitivity of glucose-sensing neurons in the VMH, 2) regulates hypothalamic neuronal activation uniquely due to hypoglycemic stress, and 3) modulates the sympathoadrenal response to hypoglycemia by altering the glycemic level required to elicit appropriate sympathoadrenal responses.

In these studies, a ~60% rise in corticosterone was observed in response to severe hypoglycemia in NIRKO and control mice (Fig. 2B). Although not well characterized in mice, this degree of hypothalamic–pituitary–adrenal induced increment in corticosterone is consistent with other groups (39,40). Contrary to the stimulatory effect of
insulin on the cortisol response to hypoglycemia observed in canine models (18,19), these studies in mice demonstrate that the absence of brain insulin action does not impair the hypothalamic–pituitary–adrenal axis response to hypoglycemia.

Reports of insulin action's in the CNS in modulating the glucagon response to hypoglycemia are variable, with studies demonstrating insulin to increase (18,19), decrease (21), or not effect (20) the glucagon response to hypoglycemia. In the current studies, the pancreatic α-cell response to severe hypoglycemia showed a sixfold increase in plasma glucagon levels that was not altered by the absence of CNS insulin receptors in NIRKO mice. Interestingly, although catecholamines stimulate the α-cell, the impaired catecholamine response to hypoglycemia did not diminish the full glucagon response in NIRKO mice (Fig. 2C). These results indicate that factors other than central insulin action and systemic catecholamine responses (perhaps local glycemia, intraislet insulin/zinc, direct innervations, etc.) are more important mediators of the glucagon response to hypoglycemia.

The absence of brain insulin receptors resulted in a significantly impaired epinephrine response in NIRKO mice during moderate (50 mg/dl) and severe (30 mg/dl) hypoglycemia. However, the absence of brain insulin signaling did not result in total deficiency of hypoglycemic counterregulation. An epinephrine response of ~1,500 pg/ml, which was achieved during moderate hypoglycemia (50 mg/dl) in controls, was also elicited at a lower blood glucose level (30 mg/dl) in NIRKO mice (Fig. 3A). Consistently with this finding, the shift in the hypoglycemia–epinephrine response curve (Fig. 3A, inset) indicates that, in the absence of insulin signaling, NIRKO mice needed to reach lower glycemic levels to appropriately activate their adrenomedullary response. While insulin infusion suppressed hepatic glucose production during the clamps, only during severe hypoglycemia (30 mg/dl) was the counterregulatory response of a sufficient magnitude to overcome the suppressive effects of insulin and significantly increase hepatic glucose production. In NIRKO mice, however, the counterregulatory-induced stimulation of hepatic glucose production was significantly blunted during severe hypoglycemia (Fig. 3C), consistent with an impaired sympathoadrenal response. These findings indicate that chronic lack of CNS insulin action alters glucose sensing and/or responsiveness, leading to an impaired sympathoadrenal response and an impaired ability to defend against iatrogenic hypoglycemia.

Because the adrenomedullary response to hypoglycemia was not impaired during mild hypoglycemia in NIRKO mice, it was speculated that mild (restraint) stress, as noted by modest elevations in epinephrine levels (Fig. 5A), might not have been of sufficient magnitude to detect a differential response between control and NIRKO mice. However, by achieving comparable epinephrine levels during severe stress (heat) and severe hypoglycemia (30 mg/dl) and finding a normal catecholamine response to heat stress in NIRKO mice, these findings indicate that absent CNS insulin signaling does not impair the normal adrenomedullary response even to severe nonhypoglycemic stress (Fig. 3A, Fig. 6A).

Increased c-fos expression in the PVN has been used as a marker of transcriptional activity in stress-related neural circuitry (38,41–43). Expression of c-fos was therefore measured to determine whether the impaired sympathoadrenal response in the NIRKO mice was related to impaired activation of hypothalamic sensing neurons. During hypoglycemia, increased c-fos expression was predominantly observed in the PVN and not seen in the VMH, consistent with other studies (44). Hypoglycemia-induced c-fos activation in the PVN may represent direct activation in response to hypoglycemia or indirect activation in response to afferent input from other areas containing glucose-sensing neurons. Thus, the impaired c-fos activation in the PVN of NIRKO mice in response to hypoglycemia could represent reduced glucose sensing of PVN neurons or, given the abundance of insulin receptors in important VMH glucose-sensing neurons, an indirect reduction in afferent inputs from glucose-sensing neurons in the VMH. Whether this defect indicates impaired direct or indirect glucose sensing, the reduced c-fos activation in NIRKO mice was profound and consistent with other models of impaired glucose sensing and impaired counterregulation (43,45). Further, in response to a nonhypoglycemic stressor, heat stress increased c-fos expression to a similar magnitude as observed during severe hypoglycemia (30 mg/dl); however, no difference in heat-induced c-fos expression was noted between control and NIRKO mice (Figs. 4 and 6C and D). These results indicate that NIRKO mice have an intact neuronal circuitry for sensing and responding to nonhypoglycemic stress; therefore, the impaired responses to hypoglycemia in the NIRKO mice appear to be unique to hypoglycemic stress and/or glucose sensing.

Whole-cell current-clamp recordings of spontaneous electrical activity were made in individual glucose-inhibitory
ited neurons to assess responses of individual glucose-sensing neurons in the VMH. While a direct relationship between glucose-sensing neurons and sympathoadrenal activation has yet to be definitively established, it is noteworthy that the ability of VMH glucose-inhibited neurons to sense a fall in ambient glucose levels is impaired under several conditions where the sympathoadrenal response to hypoglycemia is also impaired (i.e., rats treated with recurrent hypoglycemia or streptozotocin-induced diabetes) (35,46,47). In NIRKO mice, the observed impaired response of VMH glucose-inhibited neurons to reductions in glucose levels (Fig. 4C and D) is entirely consistent with the impaired neuronal (c-fos) activation (Fig. 4A) and the impaired sympathoadrenal activation (Fig. 3A). Further, the electrophysiological findings that NIRKO glucose-inhibited neurons respond normally to maximal glucose deprivation (0.1 mmol/l), but impaired responses at 0.5 mmol/l are consistent with a relative, not absolute, impairment in glucose sensing. These results indicate that insulin acts directly in the brain to regulate the glucose-sensing ability of hypothalamic glucose-inhibited neurons that are critically important and functionally linked in mediating the sympathoadrenal response to hypoglycemia. Of particular interest is that glucose-inhibited neurons of NIRKO mice have an impaired ability to respond to a fall in glucose even in the absence of insulin administration. Combining these in vitro findings to the in vivo findings suggests that it may not solely be a failure of insulin to acutely activate its receptor that leads to impaired glucose sensing and altered neuronal responses; rather, we propose that the chronic lack of insulin signaling in NIRKO mice causes long-term adaptations in gene transcription/transduction (i.e., decrease in GLUT4; see Fig. 7), leading to impaired glucose sensing. Alternatively, because neuronal nitric oxide production is required for glucose-inhibited neurons to sense decreased glucose (48,49) and insulin enhances nitric oxide production in VMH glucose-inhibited neurons (48), the chronic lack of insulin signaling in NIRKO mice may lead to impaired glucose sensing by impairing nitric oxide production. It is entirely plausible that the chronic actions of insulin may be mechanistically very different from the acute actions of insulin in regulating neuronal glucose sensing and the counterregulatory response to hypoglycemia.

Similar to its well characterized actions in muscle and fat, insulin-mediated GLUT4 translocation has been demonstrated in neuronal cell lines (50), hippocampus (51), and hypothalamus (52). GLUT4-mediated glucose sensing has been speculated to be important at low glucose concentrations, where insulin-meditated glucose transport
may act to supplement low intracellular glucose levels in hypothalamic glucose-sensing neurons (24,26). Indeed, supporting a glucose-sensing role for insulin receptors and GLUT4 is their coexpression in up to 75% of glucose-responsive neurons in the VMH (30). Further, neuronal GLUT4 has recently been shown to be an important mediator of hypoglycemic counterregulation and glucose sensing, as noted in neuronal GLUT4 knockout mice (53). During hypoglycemia, when glucose transport becomes rate-limiting, it was speculated that decreased GLUT4 expression and/or deficient insulin action would result in reduced glucose uptake in critical glucose-sensing regions.
of NIRKO mice. This study, however, noted equal regional brain glucose uptake during the hyperinsulinemic-hypoglycemic clamp (Fig. 7E), indicating that neither deficient insulin signaling nor the reduced GLUT4 levels altered glucose uptake in these brain areas. Because brain GLUT4 expression is much lower than other glucose transporters, it is likely that glucose uptake was primarily regulated by the more abundant GLUT1 and GLUT3, thus masking any subtle effect caused by decreased GLUT4. While regional brain glucose uptake was not altered in NIRKO mice, an effect of insulin signaling and/or GLUT4 availability on mediating glucose uptake in individual glucose sensing neurons cannot be ruled out.

In summary, it is shown that the chronic lack of insulin receptor signaling in the CNS 1) decreases hypothalamic GLUT4 expression, 2) attenuates individual hypothalamic glucose-inhibited neuronal responses to low glucose, 3) impairs hypothalamic neuronal activation in response to hypoglycemia, and 4) reduces the sympathoadrenal response to hypoglycemia by shifting the glycemic level necessary to elicit appropriate sympathoadrenal responses. These defects are specific for glucose sensing, as the lack of CNS insulin signaling does not restrict neuronal activation or the adrenomedullary response to restraint or heat stress.

It is concluded that insulin acts directly in the brain to regulate both glucose sensing in hypothalamic neurons and the counterregulatory response to hypoglycemia. Because insulin-treated diabetic patients have an impaired ability to sense and appropriately respond to insulin-induced hypoglycemia, the mechanisms by which insulin regulates CNS glucose sensing need to be actively investigated as research scientists endeavor to supplant insulin-induced hypoglycemia as the rate-limiting factor in the glycemic management of diabetes.

ACKNOWLEDGMENTS

We gratefully acknowledge research support from the National Institutes of Health (1F31-DK-084813 [K.A.D.-A.], DK-073683 [S.J.F.], DK-55619 [V.H.R.], DK-081358 [V.H.R.]), Juvenile Diabetes Research Foundation (S.J.F. and V.H.R.), and the core grant support from the Washington University’s Diabetes Research and Training Center (DK-020570 [S.J.F.]) and Nutrition Obesity Research Center (P30-DK-056241 [S.J.F.]). No potential conflicts of interest relevant to this article were reported.

K.A.D.-A. wrote the manuscript, researched data, contributed to the discussion, and reviewed/edited the manuscript. X.Z., Z.S., and D.D.J. researched data. V.H.R. contributed to the discussion and reviewed/edited the manuscript. S.J.F. researched data, contributed to the discussion, and reviewed/edited the manuscript.

Dr. C. R. Kahn graciously supplied the NIRKO mice. Dr. M. Mueckler kindly provided GLUT2 antibodies, and Dr. B. Levin gratefully provided the glucokinase riboprobe. We thank Dr. P. Cryer and his laboratory for performing the catecholamine assay. We also thank Ron Perez for his technical expertise.

REFERENCES

1. Epidemiology of severe hypoglycemia in the diabetes control and complications trial. The DCCT Research Group. Am J Med 1991;90:450–459
2. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. Lancet 1998;352:877–883
3. Laing SP, Swerdlow AJ, Slater SD, Botha JL, Burden AC, Waugh NR, Smith AW, Hill RD, Bingley PJ, Patterson CC, Qiao Z, Keen H. The British Diabetic Association Cohort Study, II. cause-specific mortality in patients with insulin-treated diabetes mellitus. Diabet Med 1999;16:466–471
4. Cryer PE, Davis SN, Shamoon H. Hypoglycemia in diabetes. Diabetes Care 2003;26:1902–1912
5. Jones TW, Davis EA. Hypoglycemia in children with type 1 diabetes: current issues and controversies. Pediatr Diabetes 2003;4:143–150
6. Hirsch BR, Shamoon H. Defective epinephrine and growth hormone responses in type I diabetes are stimulus specific. Diabetes 1987;36:20–26
7. Dagogo-Jack SE, Craft S, Cryer PE. Hypoglycemia-associated autonomic failure in insulin-dependent diabetes mellitus. Recent antecedent hypoglycemia reduces autonomic responses to, symptoms of, and defense to subsequent hypoglycemia. J Clin Invest 1993;91:819–828
8. Davis MR, Mellman M, Shamoon H. Physiologic hyperinsulinemia enhances counterregulatory hormone responses to hypoglycemia in IDDM. J Clin Endocrinol Metab 1993;76:1383–1385
9. Davis SN, Shavers C, Collins L, Cherrington AD, Price L, Hedstrom C. Effects of physiological hyperinsulinemia on counterregulatory response to prolonged hypoglycemia in normal humans. Am J Physiol 1994;267:E402–E410
10. Davis SN, Goldstein RE, Jacobs J, Price L, Wolfe R, Cherrington AD. The effects of differing insulin levels on the hormonal and metabolic response to equivalent hypoglycemia in normal humans. Diabetes 1993;42:263–272
11. Lingenfelder T, Overkamp D, Renn W, Buettner U, Kimmerle K, Schnauffwès A, Hattrup B. Insulin-associated modulation of neuroendocrine counter-regulation, hypoglycemia perception, and cerebral function in insulin-dependent diabetes mellitus: evidence for an intrinsic effect of insulin on the central nervous system. J Clin Endocrinol Metab 1996;81:1197–1205
12. Diamond MP, Hallarmann L, Starick-Zych J, Jones TW, Connolly-Howard M, Tamborlane WV, Sherwin RS. Suppression of counterregulatory hormone response to hypoglycemia by insulin per se. J Clin Endocrinol Metab 1991;72:1388–1390
13. Liu D, Moberg E, Kollind M, Lins PE, Adlson J. A high concentration of circulating insulin suppresses the glucagon response to hypoglycemia in normal man. J Clin Endocrinol Metab 1991;73:1123–1128
14. Mellman MJ, Davis MR, Shamoon H. Effect of physiological hyperinsulinemia on counterregulatory hormone responses during hypoglycemia in humans. J Clin Endocrinol Metab 1992;75:1293–1297
15. Davis SN, Goldstein RE, Price L, Jacobs J, Cherrington AD. The effects of insulin on the counterregulatory response to equivalent hypoglycemia in patients with insulin-dependent diabetes mellitus. J Clin Endocrinol Metab 1993;77:1300–1307
16. Kerr D, Reza M, Smith N, Leatherdale BA. Importance of insulin in subjective, cognitive, and hormonal responses to hypoglycemia in patients with insulin-dependent diabetes mellitus. Dia Metab 1991;16:1075–1079
17. Brinning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, Klein R, Krone W, Muller-Wieland D, Kahn CR. Role of brain insulin receptor in control of body weight and reproduction. Science 2000;289:2122–2125
18. Davis SN, Colburn C, Dobbins R, Nadeau S, Neal D, Williams P, Cherrington AD. Evidence that the brain of the conscious dog is insulin sensitive. J Clin Invest 1995;95:593–592
19. Davis SN, Dunham B, Walnsley K, Shavers C, Neal D, Williams P, Cherrington AD. Brain of the conscious dog is sensitive to physiological changes in circulating insulin. Am J Physiol 1997;272:E567–E575
20. Fisher SJ, Brinning JC, Lannon S, Kahn CR. Insulin signaling in the central nervous system is critical for the normal sympathoadrenal response to hypoglycemia. Diabetes 2005;54:1447–1451
21. Paranjape SA, Chan O, Zhu W, Horbitt AM, McNay EC, Cresswell JA, Bogan JS, McCreinnon RJ, Sherwin RS. Influence of insulin in the ventromedial hypothalamic on pancreatic glucagon secretion in vivo. Diabetes 2010;59:1521–1527
22. Ishihara KK, Haywood SC, Daphna-Iken D, Puente EC, Fisher SJ. Brain insulin infusion does not augment the counterregulatory response to hypoglycemia or glucoprivation. Metabolism 2009;58:812–820
23. Yang XJ, Kow LM, Pfaff DW, Mobbs CV. Metabolic pathways that mediate inhibition of hypothalamic neurons by glucose. Diabetes 2004;53:67–73
24. Levin BE, Routh VH, Kang L, Sanders NM, Dunn-Maynell AA. Neuronal glucosensing: what do we know after 50 years? Diabetes 2004;53:2521–2528
25. Spanswick D, Smith MA, Morishami S, Routh VH, Ashford ML. Insulin activates ATP-sensitive K+ channels in hypothalamic neurons of lean, but not obese mice. Nat Neurosci 2002;5:757–763
26. Cotero VE, Routh VH. Insulin blunts the response of glucose-excited neurons in the ventrolateral-ventromedial hypothalamic nucleus to decreased glucose. Am J Physiol Endocrinol Metab 2009;296:E1101–E1109
27. Yang XJ, Kow LM, Fabunshi T, Mobbs CV. Hypothalamic glucose sensor:
similarities to and differences from pancreatic beta-cell mechanisms. Diabetes 1999;48:1763–1772

28. Schuit, FC, Huypens, P, Heimberg, H, Pipeleers, DG. Glucose sensing in pancreatic beta-cells: a model for the study of other glucose-regulated cells in gut, pancreas, and hypothalamus. Diabetes 2001;50:1–11

29. Pénicaud L, Lelouc C, Lorsignol A, Alquier T, Guillod E. Brain glucose sensing mechanism and glucose homeostasis. Curr Opin Clin Nutr Metab Care 2002;5:539–543

30. Kang L, Routh VH, Kuzhihakandathil EV, Gaspers LD, Levin BE. Physiological and molecular characteristics of rat hypothalamic ventromedial nucleus glucosensing neurons. Diabetes 2004;53:549–559

31. Schubert M, Gautam D, Surjo D, Ueki K, Baudler S, Schubert D, Kondo T, Alber J, Gaddis K, Küstermann E, Arndt S, Jacobs AH, Krone W, Kahn CR, Brüning JC. Role for neuronal insulin resistance in neurodegenerative diseases. Proc Natl Acad Sci USA 2004;101:3100–3105

32. Fisher SJ, Kahn CR. Insulin signaling is required for insulin’s direct and indirect action on hepatic glucose production. J Clin Invest 2003;111:463–468

33. Sokoloff L, Reivich M, Kennedy C, Des Rosiers MH, Patlak CS, Pettigrew KD, Sakurada O, Shinohara M. The [14C]deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. J Neurochem 1977;28:907–916

34. Song Z, Routh VH. Differential effects of glucose and lactate on glucosensing neurons in the ventromedial hypothalamic nucleus. Diabetes 2005;54:15–22

35. Song Z, Routh VH. Recurrent hypoglycemia reduces the glucose sensitivity of glucose-inhibited neurons in the ventromedial hypothalamic nucleus. Am J Physiol Regul Integr Comp Physiol 2006;291:R1283–R1287

36. Paxinos G, Franklin K. The mouse brain in stereotaxic coordinates. London, Academic Press, 2001

37. Shah SD, Clutter WE, Cryer PE. External and internal standards in the single-isotope derivative (radioenzymatic) measurement of plasma norepinephrine and epinephrine. J Lab Clin Med 1985;106:624–629

38. Kovacs KJ. c-Fos as a transcription factor: a stressful (re)view from a functional map. Neurochem Res 1988;13:287–297

39. Inouye, K, Shum, K, Chao, O, Mathoo, J, Matthews, SG, Vranic, M. Effects of recurrent hyperinsulinaemia with and without hypoglycaemia on counter-regulation in diabetic rats. Am J Physiol Endocrinol Metab 2002;282:E1369–E1379

40. Chan O, Chan S, Inouye K, Shum K, Matthews SG, Vranic M. Diabetes impairs hypothalamo-pituitary-adrenal (HPA) responses to hypoglycemia, and insulin treatment normalizes HPA but not epinephrine responses. Diabetes 2002;51:1681–1689

41. Tsay HJ, Li HY, Lin CH, Yang YL, Yeh JY, Lin MT. Heatstroke induces c-fos expression in the rat hypothalamus. Neurosci Lett 1999;262:41–44

42. Harikai N, Tomogane K, Sugawara T, Tashiro S. Differences in hypothalamic Fos expressions between two heat stress conditions in conscious mice. Brain Res Bull 2003;61:617–626

43. Paranjape SA, Briski KP. Recurrent insulin-induced hypoglycemia causes site-specific patterns of habituation or amplification of CNS neuronal genomic activation. Neuroscience 2005;130:957–970

44. Niihi M, Sato M, Tamaki M, Wada Y, Takahara J, Kawanishi K. Induction of Fos protein in the rat hypothalamus elicited by insulin-induced hypoglycemia. Neurosci Res 1995;23:361–364

45. Kale, AY, Paranjape SA, Briski KP. I.c.v. administration of the nonsteroidal glucocorticoid receptor antagonist, CP-472555, prevents exacerbated hypoglycemia during repeated insulin administration. Neuroscience 2006;140:555–565

46. Powell AM, Sherwin RS, Shulman GI. Impaired hormonal responses to hypoglycemia in spontaneously diabetic and recurrently hypoglycemic rats. Reversibility and stimulus specificity of the deficits. J Clin Invest 1993;92:2667–2674

47. 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. Physiol Regul Integr Comp Physiol 2007;292:R592–R600

48. 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 2007;292:R1418–R1428

49. Murphy BA, Fakira KA, Song Z, Beuve A, Routh VH. AMP-activated protein kinase and nitric oxide regulate the glucose sensitivity of ventromedial hypothalamic glucose-inhibited neurons. Am J Physiol Cell Physiol 2009;297:C750–C758

50. Benomar Y, Naour N, Aubourg A, Bailleux V, Gertler A, Djiane J, Guerre-Millo M, Taouis M. Insulin and leptin induce Glut4 plasma membrane translocation and glucose uptake in a human neuronal cell line by a phosphatidylinositol 3-kinase-dependent mechanism. Endocrinology 2006;147:2550–2556

51. Piroli G, Grillo CA, Reznikov LR, Adams S, McEwen BS, Charbon MJ, Reigan LP. Corticosterone impairs insulin-stimulated translocation of GLUT4 in the rat hippocampus. Neuroendocrinology 2007;85:71–80

52. Grillo CA, Tamashiro KL, Piroli GC, Milhorn GT, Newson RJ, Reznikov LR, Smith A, Wilson SP, Sakai RR, Reigan LP. Lentivirus-mediated downregulation of hypothalamic insulin receptor expression. Physiol Behav. 2007;92:691–701

53. Fuente E, Daphna-Iken D, Bree A, Suzuki Y, Georgopoulos I, Kahn BB, Fisher S. Impaired counterregulatory response to hypoglycemia and impaired glucose tolerance in brain glucose transporter 4 (GLUT4) knock-out mice (Abstract). Diabetes 2009;58:A13