# Chronic Hypoglycemia and Diabetes Impair Counterregulation Induced by Localized 2-Deoxy-Glucose Perfusion of the Ventromedial Hypothalamus in Rats

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**Previous studies have demonstrated that the ventromedial hypothalamus (VMH) plays a critical role in sensing and responding to systemic hypoglycemia. To evaluate the mechanisms of defective counterregulation caused by iatrogenic hypoglycemia and diabetes per se, we delivered 2-deoxy-glucose (2-DG) via microdialysis into the VMH to produce localized cellular glucopenia in the absence of systemic hypoglycemia. Three groups of awake chronically catheterized rats were studied: *1*) nondiabetic (with a mean daily glucose [MDG] of 6.9 mmol/l) BB control rats ($n$ = 5); *2*) chronically hypoglycemic nondiabetic (3–4 weeks, with an MDG of 2.7 mmol/l) BB rats ($n$ = 5); and *3*) moderately hyperglycemic insulin-treated diabetic (with an MDG of 12.4 mmol/l) BB rats ($n$ = 8). In hypoglycemic rats, both glucagon and catecholamine responses to VMH glucopenia were markedly (77–93%) suppressed. In diabetic rats, VMH 2-DG perfusion was totally ineffective in stimulating glucagon release. The epinephrine response, but not the norepinephrine response, was also diminished by 38% in the diabetic group. We conclude that impaired counterregulation after chronic hypoglycemia may result from alterations of the VMH or its efferent pathways. In diabetes, the capacity of VMH glucopenia to activate the sympathoadrenal system is only modestly diminished; however, the communication between the VMH and the α-cell is totally interrupted.**
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Severe hypoglycemia is a major risk of insulin therapy, especially in patients with type 1 diabetes who are aggressively attempting to achieve near-normoglycemia (1). Patients with diabetes are more vulnerable to low blood glucose levels, not only because they are unable to synchronize insulin delivery with meal ingestion and activity but also because the counterregulatory responses that normally protect against hypoglycemia are defective. In the early stages of type 1 diabetes, the capacity to release glucagon during hypoglycemia is lost in the early stages of the disease (2) and subsequently because epinephrine responses may also diminish (3). In addition, intensive insulin therapy for diabetes causes reversible suppression in sympathoadrenal responses to hypoglycemia (4,5). This phenomenon is thought to be a consequence of iatrogenic hypoglycemia, because even brief periods of mild hypoglycemia in nondiabetic and diabetic subjects have been shown to reduce counterregulatory hormone responses to subsequent hypoglycemic challenges (6,7). It has been hypothesized that such defects might be related to adaptive changes in glucose transport or glucose metabolism that serve to make the central nervous system (CNS) less vulnerable to neuroglycopenia (8–12). Paradoxically, if the CNS glucose sensors also become resistant to neuroglycopenia, this adaptation might delay and impair activation of protective counterregulatory responses to falling plasma glucose levels.

Recent studies from our laboratory indicate that the ventromedial hypothalamus (VMH) is a dominant center within the CNS for sensing of glucopenia (13–15). In rats, focal lesioning of the VMH abolishes hormonal response to systemic hypoglycemia (13), as does selective prevention of glucopenia in the VMH by glucose perfusion via stereotaxically placed microdialysis probes (14). Conversely, production of local neuroglycopenia by perfusion of 2-deoxy-glucose (2-DG) directly into the VMH of awake rats triggers the release of counterregulatory hormones in the absence of systemic hypoglycemia (15).

In the present study, we used the microdialysis technique to perfuse the VMH with 2-DG in awake, unrestrained, and chronically hypoglycemic nondiabetic rats and spontaneously diabetic BB rats. These animal models were chosen because they express similar defects in counterregulatory hormone secretion (16), as is seen in patients with insulinomas (17) and type 1 diabetes (3) during hypoglycemia. The aim was to directly examine whether chronic hypoglycemia or diabetes alters the capacity of glucose sensors in the VMH to recognize and respond to local neuroglycopenia.

## RESEARCH DESIGN AND METHODS

**Animals.** Male nondiabetic and spontaneously diabetic BB rats were purchased from BB/Wor Laboratories (University of Massachusetts, Worcester, MA). Nondiabetic BB rats were members of the diabetes- resistant strain of BB rats, of which <1% develop diabetes. Animals were housed in an environmentally controlled room

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CNS, central nervous system; 2-DG, 2-deoxy-glucose; MDG, mean daily glucose; PZI, protamine zinc insulin; VMH, ventromedial hypothalamus.

with a 12-h light/dark cycle, and they were maintained on a standard ad libitum rat diet (Agway Prolab 3000, Waverly, NY) comprised of 22% protein, 5% fat, and 51% carbohydrate (the remaining 22% consists of ash, crude fiber, and moisture). Three groups of rats (4–6 months old, weighing 330–370 g) were studied. For each group, the mean plasma glucose was determined in the fed state by measuring plasma glucose from the tail vein at least three times between 0900 and 2400, and often more frequently, every 2–3 days to adequately determine the overall glycemic response to each insulin treatment, including the times of the greatest probability of hypoglycemia, normoglycemia, or hyperglycemia.

Group 1 consisted of untreated nondiabetic rats, aged 4–5 months, with a mean plasma glucose level of 6.9 ± 0.5 mmol/l (mean ± SE), that served as the control group ($n$ = 5).

Group 2 consisted of nondiabetic rats that at 3–4 months of age were made chronically hypoglycemic using gradually increased doses of protamine zinc insulin (PZI) (Lilly, Indianapolis, IN) over 1 week, followed by 3–4 weeks of treatment with twice-daily injections of 9–10 U/kg PZI insulin, resulting in mean plasma glucose values of 2.7 ± 0.6 mmol/l ($n$ = 5).

Group 3 consisted of diabetic rats, aged 5–6 months, that were in moderate glycemic control (mean plasma glucose of 12.4 ± 1.0 mmol/l) achieved by daily injections of PZI insulin in doses of ~11–12 U/kg. The doses of insulin were adjusted during treatment to avoid hypoglycemia by frequent monitoring throughout the 24-h diurnal cycle ($n$ = 8).

**Surgical procedures.** Rats were anesthetized as previously described (14) and placed on a stereotaxic frame. The skull was exposed, and holes were drilled bilaterally in chosen coordinates (14,15) through which the guide cannulas were lowered slowly into the brain and then secured with stainless steel screws and dental acrylic. Immediately after stereotaxic surgery, animals underwent an additional aseptic surgical procedure for placement of internal jugular vein and carotid artery catheters. At the end of the procedure, both catheters were filled with heparin (42 U/ml) and polyvinylpyrrolidone (1.7 g/ml) solution, plugged, tunneled subcutaneously around the side of the neck, and externalized behind the head through a skin incision. Animals were then allowed to recover for 5–7 days. Only those animals that appeared healthy and were able to maintain their weight were used. The evening before the experiment, animals received their last dose of PZI insulin, and the microdialysis probes (10.5 mm in length) of side-by-side design were inserted into the guide cannulas, as previously described (18). The exposed microdialysis membrane was 1.0–1.5 mm (approximately the size of the VMH), so that we could selectively perfuse this brain region. At 1 h before the experiment, the VMH perfusion medium was loaded into 1-ml syringes and delivered at a flow rate of 2.5 µl/min using a Harvard perfusion pump (model 22; Harvard Apparatus, Holliston, MA).

At the end of each experiment, the accuracy of probe placement was confirmed histologically by cresyl violet staining. Only those animals that showed bilateral probe placement into the desired brain region were included.

**Experimental protocol.** Each animal was food deprived for ~4 h before the study. On the morning of the experiment, the vascular catheters were flushed and maintained patent by a slow infusion of saline (20 µl/min) that contained a small amount of heparin (1–2 U/ml), and the VMH microdialysis perfusion was initiated. Rats were conscious and allowed to roam freely in their cages during the experiment. To achieve comparable plasma glucose levels in each group before study, for ~2 h before the experiment, the chronically hypoglycemic animals (group 2) as well as the diabetic animals (group 3) were brought to normoglycemic levels (see RESULTS) by intravenous variable infusion of exogenous 20% glucose or a solution of insulin (20 mU · $kg^{-1}$ · $min^{-1}$), respectively. These infusions were discontinued 20–30 min before initiating the experiment. Thereafter, blood samples were withdrawn for measurement of baseline plasma concentrations of glucose, glucagon, epinephrine, and norepinephrine. Subsequently, for the first 30 min of the experiment, the VMH probes were perfused with a solution that contained 5 mmol/l glucose; this was designated as the control phase. Thereafter, 100 mmol/l 2-DG perfusion was introduced and maintained for 60 min. Arterial blood samples for measurement of plasma glucagon and catecholamines were taken at 15-min intervals during the control phase (15 and 30 min) as well as during VMH glucopenia (45, 60, 75, and 90 min). To avoid dilution by fluid in the dead space of the catheter during blood sampling, 0.5 ml of blood was withdrawn before sample collection. Subsequently, the contents of the initial syringe were reinfused to minimize blood losses. Blood recently obtained from littermates was also transfused during the study to quantitatively replace the blood withdrawn during the experiment. The protocol was reviewed and approved by the Yale Animal Care and Use Committee.

**Analytical methods and calculations.** Plasma glucose was measured in duplicate using a Beckman Glucose Analyzer II (Fullerton, CA). Plasma catecholamines were measured with a radioenzymatic method (Amersham, Arlington Heights, IL), and plasma glucagon was measured using a double antibody radioimmunoassay procedure (Linco Research, St. Charles, MO) as previously described (14).

Data are expressed as means ± SE. Comparison between the experimental groups over time was made by analysis of variance with a repeated measure design, followed by the Student's $t$ test to localize effects.

## RESULTS

**Effect of chronic hypoglycemia.** At the onset of the study, as well as during the baseline period of 5 mmol/l VMH glucose perfusion, mean plasma glucose levels for the chronically hypoglycemic nondiabetic BB rats and their untreated control rats were not significantly different (6.9 ± 0.4 and 7.0 ± 0.5 mmol/l, respectively). However, during perfusion of 2-DG into the VMH, the rise in plasma glucose concentration in the chronically hypoglycemic rats was markedly suppressed compared with the normal control group ($P$ < 0.05, Fig. 1). Similarly, the concentrations of catecholamines and glucagon were stable and not significantly different in the chronically hypoglycemic rats and the control rats during the basal and 5 mmol/l glucose VMH perfusion periods. In the 2-DG phase of the VMH perfusion, the rise in plasma counterregulatory hormones in the chronically hypoglycemic rats were markedly suppressed. The magnitude of the suppression of epinephrine and norepinephrine release was 91 and 93%, respectively. Glucagon responses were also reduced by 77% ($P$ < 0.05), particularly the peak increment at 60 min, which was diminished by 94%.

**Effect of diabetes.** Plasma glucose levels in the insulin-treated diabetic animals in the basal state and during 5 mmol/l glucose perfusion of the VMH were similar to those seen in control animals (6.9 ± 0.3 mmol/l). As shown in Fig. 1, the rise in the peripheral plasma glucose levels in response to VMH glucopenia was slightly, but not significantly, reduced compared with that in control rats. Figure 2 presents the hormonal changes during the 100 mmol/l 2-DG VMH perfusion. There was a 38% reduction ($P$ < 0.05) in the overall plasma epinephrine response in the diabetic rats that was significant at the 60-min time point. However, the reduction in the norepinephrine response in the diabetic group was not significantly different from that in the control group. In contrast, the increase in plasma glucagon that normally followed VMH glucopenia in the nondiabetic animals was totally abolished in the type 1 diabetic rats.

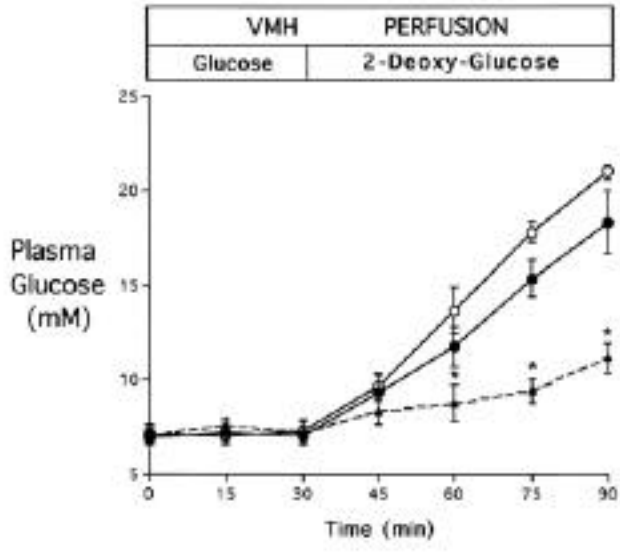


**FIG. 1. Effect of chronic hypoglycemia and type 1 diabetes on peripheral plasma glucose levels during VMH glucopenia. Experimental groups included nondiabetic BB control rats ($n$ = 5) (○), chronically hypoglycemic nondiabetic rats ($n$ = 5) (▲), and moderately hyperglycemic insulin-treated diabetic BB rats ($n$ = 8) (●). *$P$ < 0.05 vs. normal.**

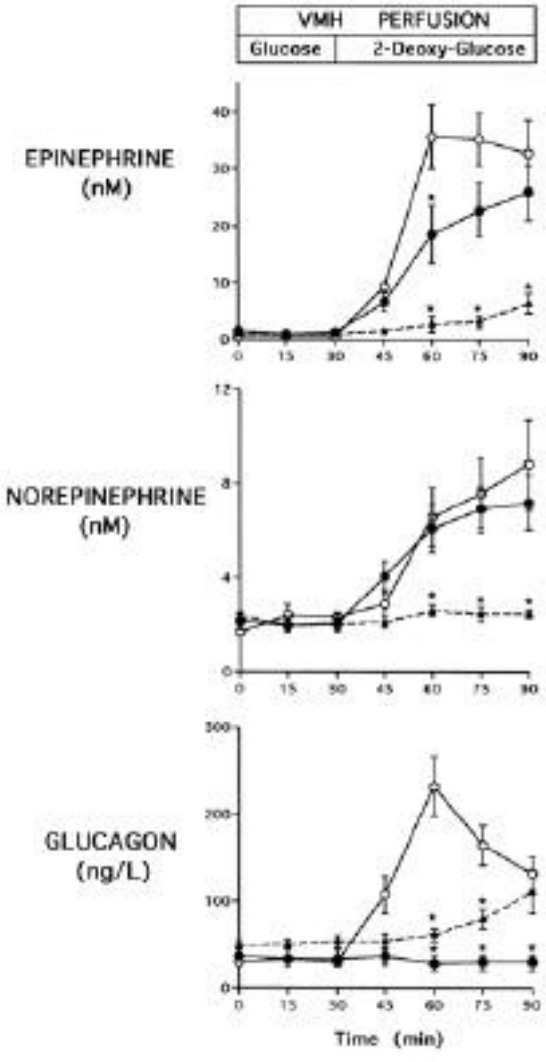


**FIG. 2. Effect of chronic hypoglycemia and type 1 diabetes on plasma counterregulatory hormones during VMH glucopenia. Experimental groups included nondiabetic BB control rats ($n$ = 5) (○), chronically hypoglycemic nondiabetic rats ($n$ = 5) (▲), and moderately hyperglycemic insulin-treated diabetic BB rats ($n$ = 8) (●). *$P$ < 0.05 vs. normal.**

## DISCUSSION

The results of the Diabetes Control and Complications Trial demonstrated the long-term benefits of intensive insulin therapy aimed at near normalization of glucose levels in patients with type 1 diabetes (1). However, severe and frequent hypoglycemia emerged as a serious complication that has limited the application of such regimens (1). Defective counterregulatory responses associated with type 1 diabetes (2,3) as well as suppressed hormonal defense mechanisms induced by iatrogenic hypoglycemia per se (4) contribute to the observed increased risk of severe hypoglycemia. A better understanding of the mechanisms used to sense glucopenia and how they are altered by diabetes and iatrogenic hypoglycemia could therefore offer new therapies aimed at reversing these counterregulatory defects.

In the current study, we examined how chronic hypoglycemia in nondiabetic rats as well as spontaneous insulin-deficient diabetes in the rat affects the capacity of the VMH to act as a glucose sensor. For this purpose, we measured the hormonal responses to severe local VMH glucopenia produced by 2-DG delivered to the VMH via microdialysis. In previous studies, we also demonstrated that microdialysis probe placement, although invasive, did not interrupt the ability of the VMH to stimulate sympathetic and pancreatic α-cell pathways in experiments in which systemic hypoglycemia was produced (14). The microdialysis probes were perfused with a high concentration (100 mmol/l) of 2-DG, a nonmetabolizable form of glucose, because only a small fraction (~4%) is actually delivered across the dialysis membrane to the extracellular fluid space of the VMH (14). In earlier studies in normal Sprague-Dawley rats, we demonstrated that sufficient 2-DG is delivered by the microdialysis system to produce local neuroglycopenia, and that this, in turn, provokes brisk counterregulatory hormone responses and systemic hyperglycemia (15). Nearly identical changes were observed in the nondiabetic diabetes-resistant strain of BB rats (group 1) used in the current study.

As in humans, we have previously reported that nondiabetic rats made repetitively hypoglycemic with exogenous insulin show delayed and impaired release of catecholamines and glucagon during hypoglycemia, and that these changes are reversible over time (16). We reasoned that if such alterations were mediated by an impairment of VMH glucose-sensing neurons to detect neuroglycopenia, there would be a generalized suppression of the hormonal response to VMH 2-DG perfusion, as was observed in the current study. To the extent that our data can be applied to the clinical situation, it is possible that the suppression of counterregulatory hormone responses induced by iatrogenic hypoglycemia in normal subjects (6) or in intensively treated type 1 diabetic patients (19) may also involve dysfunction of the VMH or any of its efferent pathways. This defect appears to be glucose specific. In our previous studies we showed that glucagon and adrenomedullary responses in chronically hypoglycemic nondiabetic BB rats are preserved to nonspecific stimuli (16). Based on the profound degree of the suppressive effect, one might speculate that it could involve a variety of mechanisms, such as an impairment in VMH signaling pathways or neurotransmitter release and/or action as well as an alteration of glucose transport in this brain area. The exact nature of this defect cannot be directly established from the current experiments.

The spontaneous diabetic BB rat used in this study, an established animal model of type 1 diabetes (20), displays counterregulatory defects remarkably similar to that present in humans with the disease (21,16). In diabetic BB rats, glucagon release during systemic hypoglycemia is totally lost within a week after disease onset, whereas the epinephrine response is initially preserved but tends to diminish over the next 6–8 weeks (22). This pattern is similar to that observed in human type 1 diabetes; however, the time sequence of the changes is much more rapid in the rat model system (3). The discrepancy between the appearance of the glucagon and epinephrine counterregulatory defects in type 1 diabetes may imply that different pathophysiological mechanisms may underlie each of these defects. In the diabetic rats, VMH 2-DG perfusion totally failed to provoke glucagon release. Whereas this effect was complete, catecholamine release was only modestly diminished. Although undetected hypoglycemia induced during insulin treatment of the diabetic rats might have contributed to the mild epinephrine secretory defect, considerable care was undertaken to avoid hypoglycemia for sev-

eral weeks before study, and in no case was hypoglycemia documented by glucose monitoring. Thus, it seems likely that in the diabetic rats, the signal from the VMH was operative, but mildly impaired, at some level in the path between the VMH and the adrenal medulla. In contrast, this VMH signal was totally ineffective in activating the α-cells. Whereas the exact nature of this α-cell defect is uncertain, it is intriguing to speculate that it may be due to changes in neural innervation of the islet, the absence of a β-cell signal, or an intrinsic defect in the α-cell itself. Extrapolation of these observations in the BB rat to the clinical setting should, however, be made with caution. For example, in the clinical setting, type 1 diabetic patients rarely experience persistent hypo- or hyperglycemia.

In summary, our studies demonstrate that chronic hypoglycemia in nondiabetic rats suppresses the ability of the VMH to recognize glucopenia or activate hormonal counterregulation. In diabetic BB rats, the response to VMH glucopenia is only slightly diminished for catecholamines but absent for glucagon. These findings support the hypothesis that the impaired counterregulation after chronic hypoglycemia may result from alterations of the function of the VMH or its efferent pathways, and that in diabetes, there is a distinct independent defect in which the communication between the VMH and the α-cell is interrupted.

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