OBJECTIVE—Impaired glucose counterregulation during hypoglycemia is well documented in patients with type 1 diabetes; however, the molecular mechanisms underlying this defect remain uncertain. We reported that the inhibitory neurotransmitter γ-aminobutyric acid (GABA), in a crucial glucose-sensing region within the brain, the ventromedial hypothalamus (VMH), plays an important role in modulating the magnitude of the glucagon and epinephrine responses to hypoglycemia and investigated whether VMH GABAergic tone is altered in diabetes and therefore might contribute to defective counterregulatory responses.

RESEARCH DESIGN AND METHODS—We used immunobots to measure GAD65 protein (a rate-limiting enzyme in GABA synthesis) and microdialysis to measure extracellular GABA levels in the VMH of two diabetic rat models, the diabetic BB rat and the streptozotocin (STZ)-induced diabetic rat, and compared them with nondiabetic controls.

RESULTS—Both diabetic rat models exhibited an ~50% increase in GAD65 protein as well as a twofold increase in VMH GABA levels compared with controls under baseline conditions. Moreover, during hypoglycemia, VMH GABA levels did not change in the diabetic animals, whereas they significantly declined in nondiabetic animals. As expected, glucagon responses were absent and epinephrine responses were attenuated in diabetic rats compared with their nondiabetic control counterparts. The defective counterregulatory response in STZ-diabetic animals was restored to normal with either local blockade of GABAA receptors or knockdown of GAD65 in the VMH.

CONCLUSIONS—These data suggest that increased VMH GABAergic inhibition is an important contributor to the absent glucagon response to hypoglycemia and the development of counterregulatory failure in type 1 diabetes.

Increased GABAergic Output in the Ventromedial Hypothalamus Contributes to Impaired Hypoglycemic Counterregulation in Diabetic Rats

Owen Chan, Sachin Paranjape, Daniel Czyzyk, Adam Horblitt, Wanling Zhu, Yuyan Ding, Xiaoming Fan, Margretta Seashore, and Robert Sherwin

Iatrogenic severe hypoglycemia is the most serious acute complication in insulin-treated diabetes, and it remains the limiting factor in maintaining proper glycemic control (1,2). The brain, and particularly the ventromedial hypothalamus (VMH), plays a crucial role in sensing hypoglycemia and initiating the physiological counterregulatory responses that rapidly correct it (3–6), namely the release of glucagon and epinephrine (7). In longstanding type 1 diabetes, however, these mechanisms are either lost or become impaired, making these individuals more susceptible to the threat of hypoglycemia (8). Although the mechanism(s) underlying these defects have not been identified, it has been postulated that impaired glucose counterregulation in type 1 diabetes stems from a number of factors. The first of which is the simultaneous loss of endogenous insulin secretion and, in association, the capacity to release glucagon in response to hypoglycemia (9). The latter is thought to be the result of a number of intra- and extrapancreatic factors, including the loss of β-cells and thus the capacity to suppress the local release of insulin (10–12), zinc (13,14), and the neurotransmitter γ-aminobutyric acid (GABA) (15) during hypoglycemia. This, when coupled with excessive administration of exogenous insulin, acts to suppress glucagon release. Second, adaptations that occur within the central and peripheral nervous system have been implicated in the impaired glucagon as well as epinephrine responses as well, including alterations in brain glucose and monocarboxylic acid transport and metabolism, and changes in neural innervation of the islet (16–23). The precise mechanisms and the relative contributions of the many disturbances in peripheral and central signals to counterregulatory failure in type 1 diabetes still are unclear.

Glucose and glucose deprivation have been shown to alter GABA levels within the brain (24–27), but the evidence for its role in regulating glucose counterregulation remains somewhat controversial. Our laboratory reported that GABA acts within the VMH to modulate the magnitude of both the glucagon and epinephrine responses to hypoglycemia in nondiabetic rats (28). Subsequently, we demonstrated that increased GABAergic tone in the VMH was an important contributor to counterregulatory failure in nondiabetic rats exposed to recurrent antecedent hypoglycemia (29). Studies in nondiabetic humans also have shown that activation of GABA receptors with systemic delivery of the benzodiazepine analog alprazolam, reduces counterregulatory and neuroendocrine responses to hypoglycemia in primates and healthy human subjects (30–32). Conversely, administration of modafinil to healthy human subjects to lower brain GABA concentrations was reported to improve adrenergic sensitivity and some aspects of cognitive function during hypoglycemia but did not significantly affect counterregulatory hormone release (33).

The current study was undertaken to investigate whether GABA inhibitory tone is increased in the VMH in two rodent models of type 1 diabetes and whether GABA contributes to defective counterregulation during hypoglycemia in diabetes. Our data suggest, for the first time,
that increased hypothalamic GABAergic neurotransmission plays a significant role in the loss of the glucagon response as well as the impairment of epinephrine release seen in rats with type 1 diabetes during acute hypoglycemia and that these counterregulatory defects are reversed by specifically reducing excessive GABA tone in the VMH.

RESEARCH DESIGN AND METHODS

All rats started with a body weight of ~300 g and were individually housed in the Yale Animal Resources Center in temperature-controlled (22–23°C) and humidity-controlled rooms. The animals were fed rat standard diet (Agway Prolab 3000, Syracuse, NY) and water ad libitum and were acclimated to handling and a 12-h light cycle (lights on between 0700 h and 1900 h) before experimental manipulation. Principles of laboratory animal care were followed, and experimental protocols were approved by the institutional animal care and use committee at Yale University.

BB diabetic rats. Diabetic BB/Wor rats (*n = 6) were obtained from the University of Massachusetts (Worcester, MA). Blood glucose levels were monitored twice daily using a glucometer (Bayer Contour), and a single subcutaneous injection of protamine zinc insulin was administered to maintain plasma glucose concentrations between ~350 and 450 mg/dL. Once blood glucose levels were stable within this range, the BB rats were maintained at this time for at least 2 weeks before being studied. The insulin dose was reduced on the day prior to the study to prevent unexpected hypoglycemia during the fasting period.

Streptozotocin-induced diabetic rats. Diabetes was induced in male SD rats (~300 g; Charles River) using a single intraperitoneal injection of streptozotocin (STZ, 65 mg/kg in citrate buffer). Nondiabetic control animals received a single intraperitoneal injection of an equivalent volume of citrate buffer. A 10% glucose solution was provided in the drinking water for the first 24 h to prevent hypoglycemia. STZ-induced diabetic animals were not insulin treated, and the condition was maintained for a total of 2 weeks before the animals were studied. Blood glucose was monitored twice daily with a glucometer to ensure adequate hyperglycemia (>250 mg/dL) in our diabetic animals. Nondiabetic SD rats served as controls.

Surgery. Ten days prior to the experiment, the animals underwent aseptic surgery to have vascular catheters implanted into the left carotid artery for blood sampling and right jugular vein for infusion. These catheters were tunneled subcutaneously and exteriorized at the back of the neck. Subsequently, the animals were placed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA) and bilateral stainless steel guide cannulas (Kopf, Kyoto, Japan) for microdialysis and/or microinjection (from bregma: VMH: 2.6 mm posterior, 3.8 mm lateral, and 8.0 mm ventral at an angle of 16°; paraventricular nucleus [PVN]: 1.5 mm posterior, 3.8 mm lateral, and 7.9 mm ventral at an angle of 22°), which were bilaterally inserted into the brain and secured in place with screws and dental acrylic. These coordinates position the 1-mm microdialysis probes into the ventrolateral portion of the VMH. The animals were then allowed to recover for ~1 week in their home cages before being used for the experiment.

Microdialysis. Animals were fasted overnight in the microdialysis cages to allow sufficient time for acclimation. The next day, the animals were connected to infusion pumps and bilateral microdialysis/microinjection probes were inserted. Artificial extracellular fluid (aECF) was perfused through the microdialysis probes at a constant rate of 1.5 μL/min for 2.5–3 h to allow GABA levels to stabilize prior to the start of the microdialysis and baseline blood sample collection. Microdialysate samples were then collected at 10-min intervals for the duration of the study.

Glucose clamp. Following a baseline collection period of 35 min, a constant insulin (50 mU/kg/min) and variable 50% dextrose infusion was used to induce baseline euglycemia (115 ± 10 mg/dL) for at least 30 min. Once plasma glucose levels were stable, blood samples were taken at this time to assess plasma hormone concentrations during hyperinsulinemic-euglycemia, and, subsequently, the rats were microinjected with 0.1 μL of aECF vehicle over the course of 1 min using a CMA 402 syringe pump (CMA Microdialysis, North Chelmsford, MA). This microinjection protocol minimizes the spread of the compound and has been shown to localize the injection to the ventromedial nucleus (33). To determine whether the counterregulatory defect could be reversed by diazepam, the GABAergic receptor antagonist, bicuculline methiodide (BIC; 12.5 μmol/L, per side), into the VMH of one subgroup of STZ-induced diabetic animals just prior to the induction of hypoglycemia. The dose of BIC used here was a subconvulsive dose determined in a previously described pilot study (28). Specificity of the response to VMH BIC injection was assessed in a subgroup of animals that received a similar injection of BIC into the PVN of the hypothalamus. Following microinjection, the glucose infusion rate was decreased and plasma glucose levels were lowered and maintained at ~50 ± 5 mg/dL for 90 min. Blood samples were collected at 30-min intervals throughout the hypoglycemic clamp portion of the study for measurement of plasma glucagon and catecholamine responses and at the end of the study for plasma hormone concentrations. Following each sample collection, the euthyroid rats were resuspended in an equivalent volume of artificial plasma (34) and reinfused back into the animal to prevent volume depletion and anemia. At the end of the study, following collection of the final blood and microdialysate samples, the animals were killed with an overdose of sodium pentobarbital, and the brains were removed and frozen on dry ice. Subsequently, accuracy of probe placement was determined histologically by visual inspection of coronal brain sections. Only data obtained from those animals with correctly positioned microdialysis probes were analyzed.

GAD65 knockdown. To address the issue of pharmacological specificity with the use of bicuculline, we used a gene knockdown approach to locally decrease the expression of the rate-limiting enzyme in GABA synthesis, GAD65, in the VMH of STZ-induced diabetic rats. In two subgroups of STZ-induced diabetic rats, an adeno-associated virus (AAV) that expressed either a short-hairpin RNA (shRNA) against GAD65 or a scrambled RNA sequence that does not adhere to any known sequences listed in Genbank were microinjected into the VMH 1 week after the induction of diabetes at a rate of 0.1 μL/min for 15 min. This method of delivery limits the spread of the virus to the VMH (35). Non-diabetic control animals received an injection of the AAV with the scrambled RNA sequence. One week after injection of the AAV, animals were subjected to a hyperinsulinemic-euglycemic-hypoglycemic clamp, as described above, to assess counterregulatory hormone profiles. The shRNA sequence, which has 100% homology with both rat and mouse GAD65, is 5′-TCTGGTTTTGTACCTCCTAGTT-3′ and that for the scrambled sequence is 5′-GGAATCTCATTGGATCATAAC-3′.

Hormone and microdialysate analysis. Plasma catecholamine concentrations were analyzed by high-performance liquid chromatography, using electrochemical detection, whereas plasma hormone concentrations were determined using commercially available radioimmunoassay kits. VMH GABA concentrations from microdialysate samples were determined using liquid chromatography–tandem mass spectrometry after butylation, as previously described (36).

Immunoblot analysis. Frozen tissue micropunches from the VMH (~12 to 16 mg of tissue [bregma]) were homogenized in 30 μL of lysis buffer (20 mMol/L HEPES, 50 mMol/L glycerol-2-phosphate, 2 mMol/L EGTA, 1 mMol/L dithiothreitol, 10 mMol/L NaF, 1 mMol/L sodium orthovanadate, 1% Triton X-100, and 10% glycerol), using a plastic pestle and ultrasonicator. Protein content was assessed with the Bradford protein assay (BioRad). Equal amounts of protein (15 μg) were resolved under reducing conditions in a 4–20% SDS-polyacrylamide gradient gel. Protein samples were then transferred onto nitrocellulose membranes. Success of the protein transfer was assessed by exposure of the nitrocellulose membranes to Ponceau S solution. Subsequently, the membranes were thoroughly washed prior to being soaked in 5% blocking solution for 1 h at room temperature. The membranes were then washed with 0.01 mol/L PBS, incubated with primary antibodies directed against GAD and β-actin, washed with PBS, and incubated with an appropriate secondary antibody conjugated to peroxidase. Following incubation with the secondary antibody, the membranes were washed with PBS and immersed into chemiluminescent reagent. The membranes were then exposed to Kodak X-OMAT film. Film analysis was undertaken using a computarized image analysis system running Kodak Digital Science One D software. Protein levels are expressed as a ratio of GAD and β-actin in order to correct for differences in loading and transfer.

Statistical analysis. Treatment effects were analyzed using one- or two-way ANOVA for independent or repeated measures, as appropriate, followed by post hoc analysis using the Statistica suite of analytical software for personal computers by StatSoft. P < 0.05 was set as the criterion for statistical significance.

RESULTS

Table 1 summarizes baseline levels of glucose and glucocorticoid hormones. Diabetic rats exhibited marked elevations in plasma glucose levels. Despite the presence of hyperglycemia in the diabetic animals, fasting plasma insulin concentrations were inappropriately reduced. In stressed rats, plasma glucagon levels were more than twofold higher in BB diabetic rats but not significantly increased in the STZ-induced diabetic animals compared with nondiabetic
controls. Epinephrine concentrations did not differ significantly between control and diabetic rats.

Under basal conditions, VMH GAD65 protein levels were elevated by ~50% in both of our diabetic animal models (Fig. 1A). No differences were observed in GAD67 protein levels between control and diabetic animals (Fig. 1B).

Figure 2A shows a schematic of our experimental protocol. Plasma glucose concentrations of diabetic animals were significantly higher than their nondiabetic counterparts (Fig. 2B) at the beginning of the study. After the start of the insulin infusion, plasma glucose levels of nondiabetic animals were maintained at euglycemia, whereas those of the diabetic animals were gradually lowered over the course of 2 h to match the euglycemic levels in nondiabetic controls. Stable euglycemia was then maintained for 30 min in all animals prior to microinjection and the induction of hypoglycemia. Average plasma glucose levels during the hypoglycemic plateau were similar between all of the treatment groups.

The increase in GAD65 corresponded with an approximate doubling of baseline interstitial GABA levels in the VMH of diabetic animals (Fig. 3A). During hyperinsulinemic-euglycemia, extracellular GABA levels did not change from their respective baseline levels. As expected, in response to hypoglycemia, VMH GABA levels decreased by ~50% in nondiabetic animals. This drop was associated with activation of both the glucagon and epinephrine responses (Fig. 3B and C, respectively). In contrast, VMH GABA levels in both diabetic animal models did not fall from their high baseline concentrations in response to

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Control rats</th>
<th>BB rats</th>
<th>STZ rats</th>
<th>STZ + BIC rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td>128 ± 1</td>
<td>589 ± 64*</td>
<td>293 ± 35*</td>
<td>274 ± 56*</td>
</tr>
<tr>
<td>Glucagon (pg/mL)</td>
<td>38 ± 6</td>
<td>89 ± 8†</td>
<td>59 ± 14</td>
<td>41 ± 7</td>
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<tr>
<td>Insulin (ng/mL)</td>
<td>14 ± 2</td>
<td>1 ± 1</td>
<td>8 ± 3</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>Epinephrine (pg/mL)</td>
<td>64 ± 20</td>
<td>51 ± 31</td>
<td>45 ± 12</td>
<td>83 ± 25</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. *P < 0.03 vs. control. †P < 0.01 vs. control. ‡P < 0.003 vs. control.

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**FIG. 1.** Representative image and densitometric analyses of immunoblots for GAD65 (A) and GAD67 (B) protein in the VMH of control (n = 6), diabetic BB (n = 3), and STZ-induced diabetic (n = 6) rats. Data are presented as relative optical density (R.O.D.) expressed as a ratio over β-actin levels. Results are presented as means ± SE. *P < 0.001 vs. control. (A high-quality color representation of this figure is available in the online issue.)

**FIG. 2.** A: Schematic diagram showing stepped hyperinsulinemic-euglycemic-hypoglycemic clamp protocol. Microdialysate samples were continuously collected throughout the study. Constant insulin (50 mU/kg/min) and variable glucose infusions were started at -150 and continued for the remainder of the study. Plasma glucose concentrations of diabetic animals (dotted line) were lowered to match euglycemic levels (115 ± 10 mg/dL) in nondiabetic animals for 30 min prior to microinjection at 0 and induction of hypoglycemia (50 ± 5 mg/dL). B: Plasma glucose concentrations of nondiabetic controls (Control; n = 6), diabetic BB rats (BB Rat; n = 3), STZ-induced diabetic rats (STZ; n = 6), and STZ-induced diabetic rats microinjected with the GABA_A receptor antagonist (STZ + BIC; n = 6) under baseline conditions (□), during hyperinsulinemic-euglycemia (▲), and during hyperinsulinemic-hypoglycemia (■). Results are presented as means ± SE. *P < 0.000 vs. Control at baseline; †P < 0.001 vs. Control at baseline.
hypoglycemia, and this was accompanied by an absent glucagon and an attenuated epinephrine response.

To assess whether increased inhibitory GABAergic output is responsible for the impairment of counterregulatory hormone release, we microinjected the GABA<sub>A</sub> receptor antagonist, BIC, into the VMH of STZ-induced diabetic animals to block postsynaptic GABA<sub>A</sub> receptors prior to the induction of hypoglycemia. Administration of bicuculline restored both the glucagon and epinephrine responses to normal in diabetic animals, despite the persistence of elevated GABA levels in the VMH (Fig. 3). To establish whether the effects of the bicuculline injection were specific to the VMH, we microinjected bicuculline into another region of the brain that also expresses GABA<sub>A</sub> receptors, the PVN of the hypothalamus, and measured the counterregulatory responses to hypoglycemia. In this study, we noted that blockade of GABA<sub>A</sub> receptors in the PVN did not affect the release of glucagon or epinephrine in either nondiabetic or STZ-induced diabetic animals (Table 2).

Using an alternative and more specific approach to test our hypothesis, we administered an AAV expressing a shRNA against GAD<sub>65</sub> into the VMH of STZ-induced diabetic rats to inhibit GABAergic output.

**TABLE 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>ΔGlucagon (pg/mL)</th>
<th>ΔEpinephrine (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>141 ± 33</td>
<td>4,166 ± 430</td>
</tr>
<tr>
<td>Control + BIC</td>
<td>163 ± 7</td>
<td>3,657 ± 258</td>
</tr>
<tr>
<td>STZ</td>
<td>47 ± 17&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1,520 ± 195†</td>
</tr>
<tr>
<td>STZ + BIC</td>
<td>35 ± 14&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1,186 ± 328†</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. Animals from each treatment group were microinjected with either artificial extracellular fluid or the GABA<sub>A</sub> receptor antagonist, BIC, into the PVN. *P < 0.05 vs. control; †P < 0.001 vs. control.
diabetic rats 1 week prior to the glucose clamp procedure. The GAD65 shRNA lowered GAD65 protein levels in STZ-induced rats with diabetes to normal levels, whereas the scrambled shRNA had no significant effect (Fig. 4). Administration of neither the AAV expressing GAD65 shRNA nor the scrambled shRNA had any significant effect on baseline plasma glucose concentrations compared with control and STZ animals in the above study (Fig. 5). However, the reduction in GAD65 expression resulted in normalization of baseline extracellular GABA levels in STZ-induced diabetic animals and, more importantly, restored the drop in VMH GABA levels during hypoglycemia (Fig. 6A). Knockdown of GAD65 in the VMH of STZ-induced diabetic rats also restored the glucagon and epinephrine responses to hypoglycemia (Fig. 6B and C, respectively).

DISCUSSION

Counterregulatory failure in type 1 diabetes is a well-documented phenomenon. The mechanisms responsible for this defect, however, are not well understood. In previous studies, we showed that inhibitory GABAergic neurotransmission in the VMH is important for modulating the magnitude of glucose counterregulatory responses to an acute bout of hypoglycemia in rats (28). We subsequently reported that marked increases in GABAergic inhibitory neurotransmission within the VMH play an important role in the development of counterregulatory failure following antecedent exposure to hypoglycemia in nondiabetic rodents (29). In the current study, we show that a similar mechanism may be operating in type 1 diabetic rodents and could be a new unrecognized factor contributing to the development of counterregulatory failure, which is a major complication of the disease.

In the first set of studies, we characterized the GABA defect in two different rodent models of type 1 diabetes, the spontaneously diabetic BB rat and the STZ-induced diabetic rat. We observed that under baseline conditions, both diabetic animal models exhibited increased GAD65 protein levels in the VMH, and this was corroborated by a biological marker, the doubling in extracellular GABA levels. The fact that the data are so remarkably similar between these two different animal models of type 1 diabetes suggests that it is likely the diabetic condition itself, and not the potentially toxic side effects of STZ, that is responsible for increasing GABA output within the VMH. Interestingly, no differences were observed with GAD67 protein in the VMH. Our data are consistent with previously published reports showing increased GABA tone in the VMH of STZ-induced diabetic animals. Beverly et al. (37,38) reported increased GABA shunt and GAD activity in the VMH of STZ-induced diabetic rats, whereas Ohtani et al. (39) used microdialysis to show that GABA levels were elevated in the VMH of STZ-induced diabetic rats. Taken together, these data suggest that diabetes raises GABA output in the VMH at least in part by increasing GABA synthesis and release. Although microdialysis provides an assessment of net changes in extracellular GABA levels within the VMH, it does not allow us to specifically identify the source of GABA. Although the majority of GABA is thought to be of neuronal origin, astrocytically derived GABA also has been reported, but its contributions to the VMH interstitial pool and to glucose sensing have not been established (40,41).

In response to a hypoglycemic challenge, VMH GABA levels decrease in our control animals, and this corresponded with activation of both the glucagon and epinephrine responses. In contrast, VMH GABA levels remained elevated during hypoglycemia in both of our diabetic animal models, and this was associated with absent glucagon and attenuated epinephrine responses, establishing for the first time a link between increased inhibitory neurotransmission in the VMH and defective glucose counterregulatory responses in diabetes.

Interestingly, we noted that under hyperinsulinemic-euglycemic conditions, VMH GABA levels in nondiabetic animals did not change significantly from baseline levels. This implies that in normal animals, the release of GABA already may have reached a physiological ceiling under conditions of euglycemia, and the addition of insulin may not provide additional stimulation for GABA release. This is consistent with data from our recent study showing that the administration of increasing doses of glucose into the VMH during peripherally induced hypoglycemia dose-dependently raises extracellular GABA levels. These GABA levels continue to increase until euglycemic levels are reached, at which point they plateau at normal baseline levels (42). Raising VMH glucose concentrations above euglycemic levels does not increase VMH GABA levels further. In the case of the diabetic animals, the fact that GABA levels also remain high under hyperinsulinemic-euglycemic conditions suggests that factors apart from glucose and insulin may be responsible for raising GABAergic output in the VMH.

Although most of GABAergic neuronal cell bodies reside in the perimeter of the ventromedial nucleus, we have shown that a significant number of GAD65-positive nerve terminals exist within the body of the VMH and that a subpopulation of these neurons coexpress ATP-sensitive K+ channels (42). More importantly, this subpopulation of VMH GABAergic neurons responds to local changes in glucose levels. What this suggests is that these neurons in

**FIG. 4.** In vivo validation of GAD65 knockdown in STZ-induced diabetic animals. Representative image showing β-actin and GAD65 protein bands. Graph shows densitometric analyses of immunoblots for GAD65 protein in the VMH of controls with the scrambled shRNA (n = 4), STZ-induced diabetic rats with aECF (n = 4), STZ-induced diabetic rats with the scrambled shRNA (n = 4), and STZ-induced diabetic rats with the GAD65 shRNA (n = 4). 1 week after AAV microinjection. Data are represented as relative optical density (R.O.D.) expressed as a ratio over β-actin levels. Results are presented as means ± SE. *P < 0.001 vs. Control + scrambled shRNA. (A high-quality color representation of this figure is available in the online issue.)
particular are likely either glucose-sensing neurons themselves or that they may form synapses with other glucose-sensing neurons to modulate activity of those neurons as part of the glucose-sensing network that regulates the counterregulatory responses to hypoglycemia. In diabetes, increased activation of GABAergic tone may then prevent this network from initiating an appropriate response.

Having demonstrated that diabetes increases GABA levels in the VMH and that this was associated with an impaired counterregulatory response, we examined whether increased GABAergic neurotransmission in the VMH contributed to suppression of counterregulatory responses to hypoglycemia in diabetic animals using two parallel approaches. In the first approach, we microinjected BIC into the VMH of STZ-induced diabetic rats to locally block postsynaptic GABA\(_A\) receptors prior to the induction of hypoglycemia. This treatment completely restored both the glucagon and epinephrine defects in the diabetic animals, suggesting that increased GABAergic output in the VMH contributes significantly to attenuating the counterregulatory responses to hypoglycemia. The inhibitory effects on glucagon and epinephrine appear to be specific to GABAergic neurotransmission in the VMH because microinjection of BIC into a neighboring nucleus, the PVN, failed to restore the counterregulatory defects in diabetic animals.

To address issues associated with the specificity of BIC, we performed a second series of studies in which we locally administered an AAV that expressed either an shRNA sequence into the VMH of STZ-induced diabetic animals. We microinjected an AAV that expressed either an shRNA sequence into the VMH of STZ-induced diabetic animals. The AAV that expressed the scrambled shRNA sequence into the VMH of STZ-induced diabetic animals did not have any demonstrable effects on VMH GAD\(_{65}\) expression, extracellular GABA levels, plasma glucose, or counterregulatory hormone release. Nondiabetic control animals receiving the scrambled shRNA AAV exhibited the expected fall in baseline VMH GABA levels and activation of the counterregulatory response, whereas STZ-induced diabetic rats injected with the scrambled shRNA AAV showed the expected high VMH GABA levels and impaired counterregulatory responses to hypoglycemia. In stark contrast, when the high VMH GAD\(_{65}\) levels in STZ animals were knocked down into the normal range using a GAD\(_{65}\) shRNA AAV, we not only brought baseline GABA levels back down to normal, but, perhaps more importantly, we also restored the drop in VMH GABA concentrations during hypoglycemia. Knockdown of GAD\(_{65}\) in the VMH restored both the glucagon and epinephrine responses to normal in the STZ-induced diabetic rats. This suggests that 1) a sustained increase in VMH GABAergic inhibition plays a key role in suppressing the glucagon and epinephrine responses to hypoglycemia in diabetes and 2) although the ability to decrease VMH GABAergic tone in response to a hypoglycemic challenge still may be present in diabetes, as evidenced by knockdown of GAD\(_{65}\), it is the abundant overproduction of GABA (most likely stemming from higher GAD\(_{65}\) levels) that prevents GABA levels from dropping below a critical threshold for full activation of counterregulatory responses. This observation underscores the importance of the role of the VMH to counterregulatory failure in type 1 diabetes. Although most studies suggest that the loss of the glucagon response to hypoglycemia in type 1 diabetes is mediated through peripheral mechanisms, including the loss of intraislet factors (10,11,13–15,43) as well as sympathetic innervations to the \(\alpha\)-cell (20), less attention has been given to the role of the brain in this regard. We previously reported that activation of the fuel sensor, AMP kinase, in the VMH of BB rats can improve glucagon responses to hypoglycemia (44), and the current data demonstrate a similar restoration in two different models of \(\beta\)-cell injury (and insulin insufficiency) by modulating GABAergic neurotransmission in the VMH. These observations suggest that alterations in the capacity of the VMH, a key glucose-sensing brain region, to recognize fuel deficit may contribute significantly to the glucagon defect in diabetes as well. Thus, the VMH may play a greater role in counterregulatory failure in diabetes than previously appreciated.

Of particular interest is the fact that the data are remarkably similar to what we observed in animals with antecedent hypoglycemia. In both instances, we saw increases in VMH GABAergic output that was associated with impairments in the counterregulatory responses and improvements in these responses with GABAergic blockade. The fact that these two different conditions can lead to similar changes in the central nervous system and

![FIG. 5. Plasma glucose concentrations of nondiabetic control rats injected with the scrambled shRNA AAV (Control + Scrambled shRNA; n = 6), STZ-induced diabetic rat injected with the scrambled shRNA AAV (STZ + Scrambled shRNA; n = 6), and STZ-induced diabetic rat injected with the GAD\(_{65}\) shRNA AAV (STZ + GAD65 shRNA; n = 6) under baseline conditions (□), during hyperinsulinemic-euglycemia (■), and during hyperinsulinemic-hypoglycemia (▲). Results presented as means ± SE. *P < 0.001 vs. Control at baseline.](diabetes.diabetesjournals.org)
similar pathological outcomes was quite surprising. Although the mechanism responsible is uncertain, this finding suggests that a common adaptation within the central nervous system may occur in both of these conditions that acts to increase GAD65 expression and GABA output in the VMH that in turn contribute to counterregulatory failure.

We conclude that diabetes leads to increased GAD65 expression in the VMH with resulting increases in local GABA concentrations in this critical brain glucose-sensing region. In addition, we have shown, using two parallel approaches, that when GABA tone in the VMH is lowered in STZ-induced diabetic animals, either pharmacologically or genetically, we are able to restore the absent glucagon response and also restore the impaired epinephrine response to normal. Thus, uncontrolled or poorly controlled type 1 diabetes increases GABAergic tone in the VMH.

Although the specific mechanisms responsible for these changes in the synthesis and release of the inhibitory neurotransmitter, GABA, remain to be established, they appear to contribute, at least in part, to defective glucose counterregulation in diabetes and thus be a therapeutic target for diminishing the risk of this serious complication of insulin treatment.

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No potential conflicts of interest relevant to this article were reported.

O.C. conceived and designed the study, researched data, and drafted the manuscript. S.P. researched data and revised the manuscript. D.C. performed mass spectrometry analysis. A.H. researched data and performed mass spectrometry analysis. W.Z., Y.D., and X.F. assisted with the surgeries and studies. M.S. and R.S. revised the manuscript.

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REFERENCES


20. Mundinger TO, Mei Q, Figlewicz DP, Lernmark A, Taborsky GJ Jr. Increased GABAergic OUTPUT AND DIABETIC RATS