Hypothalamic AMP-activated protein kinase activation with AICAR amplifies counterregulatory responses to hypoglycemia in a rodent model of type 1 diabetes


Department of Internal Medicine and Endocrinology, Yale University, New Haven, Connecticut

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HYPOGLYCEMIA REMAINS THE MAJOR limiting factor to intensive insulin therapy in type 1 diabetes (T1DM) (9). Individuals with T1DM are particularly prone to hypoglycemia both because of their need for exogenous and unregulated insulin therapy and because of defects in the normal physiological counterregulatory response to acute hypoglycemia (29). Developing therapies to reduce the frequency or severity of hypoglycemia will require a greater understanding of the physiological mechanisms underlying hypoglycemia detection and the impact of T1DM on these mechanisms.

Falling glucose levels are detected within discrete regions of the brain (3–5, 14, 40), and periphery (11). Of these, the ventromedial hypothalamus (VMH) is thought to play a key role in the detection and integration of hypoglycemia signals and subsequent triggering of a counterregulatory defense response (3–5, 13, 30, 31, 33, 42, 47). Emerging evidence supports a key role for the serine/threonine kinase AMP-activated protein kinase (AMPK) in the sensing of hypoglycemia within the VMH. Local activation of AMPK in the VMH with 5-aminoimidazole-4-carboxamide (AICAR) amplified the glucose counterregulatory response to hypoglycemia in normal Sprague-Dawley rats (31) and reversed impaired hormonal counterregulatory responses in normal rats exposed to recurrent hypoglycemia (RH) (30). Moreover, selective downregulation of AMPK in the VMH using a locally delivered short hairpin RNA significantly suppressed the counterregulatory response to subsequent hypoglycemia (32). This work is supported by others who have shown hypothalamic AMPK is activated in response to fasting or central glucoprivation (24, 26, 34), and, moreover, that intracerebroventricular preadministration of the AMPK inhibitor, compound C, or hypothalamic overexpression of a dominant-negative AMPK suppresses the counterregulatory response to acute hypoglycemia (17).

However, AMPK can also be regulated by chronic exposure to high glucose. Acute hyperglycemia (up to 5 h) can reduce AMPK activation in muscle and liver (25), kidney (27), and heart (23), as well as in incubated muscle (21). AMPK may therefore act bidirectionally, potentially functioning as a feedback mechanism to limit glucose uptake into tissues under conditions of hyperglycemia. This raises the possibility that the fluctuating metabolic state of T1DM, namely intermittent hypo- and hyperglycemia may have a variety of effects on AMPK in the brain, the net effect of which may be that AMPK activation in the VMH in T1DM has little or no effect on counterregulatory responses to acute hypoglycemia. We, therefore, sought to determine whether VMH AMPK activation would amplify counterregulatory responses to hypoglycemia in diabetic BB rats. The diabetic BB rat, a rodent model of autoimmune diabetes, was chosen because, like its human counterpart, it has an absolute requirement for exogenous insulin therapy, develops defective glucagon responses to acute hypoglycemia shortly after disease development (22), and develops defective epinephrine responses following RH (38). These are all hallmarks of human T1DM, which makes this rodent model particularly relevant to individuals with T1DM.

MATERIALS AND METHODS

Animals. Male diabetic BB/Wor rats (Biomedical Research Models, Worcester, MA) with established insulin-requiring diabetes (n = 30; 14–28 days disease duration) and male Sprague-Dawley rats (n =...
were housed in the Yale Animal Resource Center on a 12:12-h day-night cycle, fed a standard pellet diet (22% protein, 5% fat, and 51% carbohydrate; cat. no. 2018; Harlan, Boston, MA) and maintained on once-daily PZI insulin (BCP Veterinary Pharmacy, Houston, TX). The animal care and experimental protocols were reviewed and approved by the Yale Animal Care and Use Committee.

Rodent surgery. Ten days prior to each study the rats were anaesthetized with an intraperitoneal injection (1 ml/kg) of a mixture of xylazine (20 mg/ml AnaSed; Lloyd Laboratories, Shenandoah, IA) and ketamine (100 mg/ml Ketaset; Aveco, Fort Dodge, IA) in a ratio of 1:2 (vol:vol). The rats initially underwent vascular surgery for the implantation of chronic vascular catheters, as described previously (41). The catheters [PE-50 tubing with a tip made from Silastic laboratory tubing (0.51 mm ID)] are inserted via a neck incision into the internal jugular vein and carotid artery and extended to the level of the right atrium and aortic arch, respectively. They are then tunneled subcutaneously and externalized at the nape of the neck where the catheter ends are left free. Catheter patency is maintained by filling them with a heparin/polyvinylpyrrolidone solution. After catheter insertion, VMH (anterior-posterior, −2.6 mm; medial-lateral ± 3.8 mm; and dorsalventral, 8.3 mm; at an angle of 20 degrees) microinjection guide cannulas were inserted stereotaxically as described previously (4, 5). The coordinates chosen leave the guide cannula tip 1 mm from the VMH and minimizes tissue damage and gliosis in the area of interest. Previous studies have shown that microinjection to the VMH results in relatively little spreadout with the immediate microinjection site (5).

Study 1. In this study, the effect of providing an additional pharmacological stimulus to AMPK in the VMH in chronically hyperglycemic or recurrently hypoglycemia diabetic BB rats was examined. Diabetic BB rats require insulin therapy to prevent ketosis and death. The diabetic rats in our facility are treated with once-daily PZI insulin (BCP Veterinary Pharmacy) injected subcutaneously at 1700 with doses based on body weight, tail vein glucose at 0900, and study protocol. Diabetic BB rats in the present study were divided into two groups; Chronic hyperglycemia (CH) and RH. For the CH group, insulin doses were adjusted pre- and postoperatively to avoid exposure to hypoglycemia and to maintain glucose levels in the moderate- to high-range. The average morning tail vein glucose throughout this preclamp phase was 303 ± 14 mg/dL. RH diabetic rats in addition to basal PZI insulin replacement at 1700 received an IP 10 U/kg dose of human regular insulin (Eli Lilly, Indianapolis, IN) at 0900 on the 5 consecutive days prior to surgery. Postoperatively the rats were allowed a 5-day recovery period with moderate glucose control and then underwent a second 5-day period of recurrent once-daily hypoglycemia. During the hypoglycemia period, food was withheld for 3 h to allow for moderate sustained hypoglycemia. At the end of this period the rats were given free access to food. Average tail vein glucose readings for the 5-day preoperative, postoperative, and 5-day prestudy were, respectively, 39 ± 1, 304 ± 18, and 38 ± 2 mg/dL (the readings on the hypoglycemia study days were taken at the end of the 3-h period of hypoglycemia). On the clamp study day, the CH and RH diabetic BB rats were microinjected into the VMH with either AICAR or saline; thus the same pre- and postoperative hypoglycemic period. During the hypoglycemia period, food was withheld for 3 h to allow for moderate sustained hypoglycemia. At the end of this period the rats were given free access to food. Average tail vein glucose readings for the 5-day preoperative, postoperative, and 5-day prestudy were, respectively, 39 ± 1, 304 ± 18, and 38 ± 2 mg/dL (the readings on the hypoglycemia study days were taken at the end of the 3-h period of hypoglycemia). On the clamp study day, the CH and RH diabetic BB rats were microinjected into the VMH with either AICAR or saline; thus the same pre- and postoperative hypoglycemic period. During the hypoglycemia period, food was withheld for 3 h to allow for moderate sustained hypoglycemia. At the end of this period the rats were given free access to food. Average tail vein glucose readings for the 5-day preoperative, postoperative, and 5-day prestudy were, respectively, 39 ± 1, 304 ± 18, and 38 ± 2 mg/dL (the readings on the hypoglycemia study days were taken at the end of the 3-h period of hypoglycemia). On the clamp study day, the CH and RH diabetic BB rats were microinjected into the VMH with either AICAR or saline; thus the same pre- and postoperative hypoglycemic period. During the hypoglycemia period, food was withheld for 3 h to allow for moderate sustained hypoglycemia. At the end of this period the rats were given free access to food. Average tail vein glucose readings for the 5-day preoperative, postoperative, and 5-day prestudy were, respectively, 39 ± 1, 304 ± 18, and 38 ± 2 mg/dL (the readings on the hypoglycemia study days were taken at the end of the 3-h period of hypoglycemia). On the clamp study day, the CH and RH diabetic BB rats were microinjected into the VMH with either AICAR or saline; thus the same pre- and postoperative hypoglycemic period. During the hypoglycemia period, food was withheld for 3 h to allow for moderate sustained hypoglycemia. At the end of this period the rats were given free access to food. Average tail vein glucose readings for the 5-day preoperative, postoperative, and 5-day prestudy were, respectively, 39 ± 1, 304 ± 18, and 38 ± 2 mg/dL (the readings on the hypoglycemia study days were taken at the end of the 3-h period of hypoglycemia). On the clamp study day, the CH and RH diabetic BB rats were microinjected into the VMH with either AICAR or saline; thus the same pre- and postoperative hypoglycemic period. During the hypoglycemia period, food was withheld for 3 h to allow for moderate sustained hypoglycemia. At the end of this period the rats were given free access to food. Average tail vein glucose readings for the 5-day preoperative, postoperative, and 5-day prestudy were, respectively, 39 ± 1, 304 ± 18, and 38 ± 2 mg/dL (the readings on the hypoglycemia study days were taken at the end of the 3-h period of hypoglycemia).
tively. These data show that RH-control rats required markedly more exogenous glucose to maintain the hypoglycemic control than RH-AICAR injected rats.

Plasma levels of insulin rose significantly in both groups during the hypoglycemia clamp studies to levels of 2,481 ± 458 and 3,212 ± 268 pmol/l for CH-control vs. CH-AICAR, respectively (P = ns). Plasma levels of c-peptide were very low under basal (42 ± 6 vs. 28 ± 5 pmol/l, respectively) and hypoglycemic (37 ± 4 vs. 22 ± 1 pmol/l, respectively) conditions in these CH-control and CH-AICAR diabetic BB rats, and did not differ significantly between groups.

VMH microinjection of AICAR had no significant effect on basal levels (30 to 0 min) of glucagon, epinephrine, and norepinephrine in either CH group, and all hormones rose in response to acute hypoglycemia. VMH AICAR microinjection significantly amplified (180%) glucagon (means ± SE; area under the curve over time (AUC/t) of 144 ± 43 vs. 50 ± 11 ng·l⁻¹·min⁻¹; RH-AICAR vs. RH-control, respectively; P < 0.05) and epinephrine (300%) (4.27 ± 0.96 vs. 1.06 ± 0.26 nmol·l⁻¹·min⁻¹, respectively; P < 0.05), but not norepinephrine (3.07 ± 0.4 vs. 2.06 ± 0.25 nmol·l⁻¹·min⁻¹, respectively; P = 0.85) responses to the hypoglycemia challenge.

Study I (b): VMH AMPK activation in chronically hyperglycemic diabetic BB rats. Plasma glucose profiles (Fig. 3A) in the animal groups were similar and did not differ significantly over the time course of the study (F = 1.3, P = ns). Mean ± SE plasma glucose during the hypoglycemic plateau (60–120 min) was 3.1 ± 0.1 vs. 3.3 ± 0.1 mmol/l for CH-control vs. CH-AICAR, respectively. Mean glucose infusion rates (GIR) required to maintain the hypoglycemia plateau (60–120 min) were slightly, but not significantly reduced in CH-AICAR vs. CH-control BB rats (1.0 ± 0.6 vs. 4.5 ± 2.0 mg·kg⁻¹·min⁻¹; P = 0.1; Fig. 3B).

Plasma levels of insulin rose significantly in both groups during the hypoglycemia clamp studies to levels of 2,481 ± 458 and 3,212 ± 268 pmol/l for CH-control vs. CH-AICAR, respectively (P = ns). Plasma levels of c-peptide were very low under basal (42 ± 6 vs. 28 ± 5 pmol/l, respectively) and hypoglycemic (37 ± 4 vs. 22 ± 1 pmol/l, respectively) conditions in these CH-control and CH-AICAR diabetic BB rats, and did not differ significantly between groups.

Microinjection had no effect on basal levels (−30 to 0 min) of glucagon, epinephrine, and norepinephrine in either CH group, and all hormones rose in response to acute hypoglycemia.
mia (Fig. 4 A–C). VMH AICAR microinjection significantly amplified glucagon (80%) (AUC/t 151 ± 22 vs. 85 ± 22 ng l⁻¹ min⁻¹; CH-AICAR vs. CH-control, respectively; P < 0.05), but not epinephrine (3.25 ± 0.45 vs. 7.00 ± 1.11 nmol l⁻¹ min⁻¹, respectively; P = 0.2) or norepinephrine (2.36 ± 0.9 vs. 2.00 ± 0.50 nmol l⁻¹ min⁻¹, respectively; P = 0.07) responses to the hypoglycemia challenge.

Study 2: effect of VMH AICAR during hypoglycemia in normal rats following selective AMPKα downregulation. To control for potential nonspecific effects of AICAR, initial studies were performed in normal Sprague-Dawley rats previously microinjected to the VMH with either a short-hairpin RNA designed to selectively downregulate AMPK (VMH AMPK-siRNA VMH saline or VMH AMPK-siRNA VMH AICAR groups) or a control short-hairpin RNA (VMH control-siRNA VMH saline). Compared with the control siRNA study, and consistent with our previous work (32), VMH AMPK downregulation through local delivery of the AMPK siRNA led to a significant reduction in the glucagon (40%), epinephrine (30%), and norepinephrine (30%) responses to acute hypoglycemia (Fig. 5). This effect was not significantly reversed by microinjection of AICAR to the VMH (Fig. 5; P = ns for all hormones), a finding which supports the use of AICAR in subsequent studies in the BB rat as an activator of AMPK.

DISCUSSION

Perhaps the most noteworthy finding in the present study was the demonstration that VMH AMPK activation in both RH- and CH-diabetic BB rats produced a significant increase in the glucagon response (80–180%) to subsequent hypoglycemia. The inability of individuals with T1DM to produce a substantial rise in plasma glucagon in response to a falling glucose is a major reason for their increased predisposition to severe hypoglycemia (10, 16). The α-cell is subject to both local pancreatic and distal (neural and circulating humoral) influences, because it has proven extremely difficult to determine the principal mechanistic defect(s) that are responsible for impaired glucagon-secretory responses to hypoglycemia in T1DM.

One major factor leading to the development of defective glucagon secretion in T1DM is thought to be the failure of intraislet insulin levels to fall (“switch-off”) during acute hypoglycemia (2, 28, 43). In support of this, restoring the intraislet insulin switch-off signal during hypoglycemia is sufficient to restore glucagon secretion in vitro using perfused islets from streptozotocin-treated Wistar rats (20), and in vivo, albeit in anesthetized rats, using an intrapancreatic artery
infusion of insulin that was abruptly discontinued when streptozotocin-treated Wistar rats (48) and diabetic BB rats (49) became hypoglycemic. However, there remain a number of neural pathways that may also contribute to the development of defective glucagon counterregulation in T1DM and that might, if sufficiently activated, restore glucagon secretory responses to hypoglycemia. The pancreatic α-cell has an extensive and complex autonomic innervation [reviewed in detail in Tabor- sky et al. (44)] with considerable redundancy. All three of the autonomic inputs to the α-cell, epinephrine, sympathetic, and parasympathetic innervation, are capable of stimulating glucagon secretion (44), and autonomic blockade in rodents (18) and primates (19) significantly impairs glucagon secretion during hypoglycemia.

In the present study, we demonstrate that microinjection of the pharmacological AMPK activator, AICAR, increases the magnitude of the glucagon secretory response in both CH- and RH-diabetic BB rats. This finding is consistent with a number of other studies in nondiabetic rodents demonstrating that VMH manipu-
AMPK activation in the VMH. Future studies using neuronal cell type. Thus, it is possible that the effect of AMPK on the counterregulatory response may relate to neuronal and/or glial AMPK activation in the VMH. Future studies using neuronal or glial-specific AMPK knockdowns in rodent models will be needed to address this question.

T1DM individuals exposed to RH develop additional defects in the hormonal counterregulatory response, namely an impaired sympathoadrenal response to subsequent hypoglycemia (9). Recurrent central glucoprivation leads to reduced AMPK activity in the arcuate and ventromedial nuclei in the hypothalamus (1), suggesting that this may contribute to the counterregulatory defect seen. Interestingly, in skeletal muscle, where AMPK is thought to regulate both glucose uptake and fat oxidation during exercise (46), an attenuated increase in AMPK activity is seen following exercise training (where repeated stimulation of AMPK would be anticipated), despite an increase in AMPK expression (12). This is associated with a less marked increase in free AMP in skeletal muscle during subsequent exercise (8). Our finding in the present study that VMH AICAR amplifies the sympathoadrenal response to hypoglycemia in RH-diabetic BB rats, as well as our previous report in nondiabetic RH rats where we also found an increase in VMH AMPK gene expression (30), are consistent with these aforementioned studies and suggest that reduced AMPK activity during hypoglycemia in glucose-sensing neurons such as those in the VMH may contribute to the defective counterregulatory response seen in rodents exposed to recurrent antecedent hypoglycemia.

In summary, we have shown in diabetic BB rats, a rodent model of autoimmune T1DM, that have a severe defect in glucose counterregulation following exposure to RH and CH, that activators of AMPK in the VMH improves glucose counterregulation during subsequent hypoglycemia. Of note, we show for the first time in vivo that a central intervention (VMH AMPK activation) can significantly amplify the glucagon secretory defect to hypoglycemia in a T1DM rodent model, and as such, this may offer a future potential therapeutic target for T1DM patients who suffer recurrent severe hypoglycemia.

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GRANTS

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