Original Article

Activation of AMP-Activated Protein Kinase Within the Ventromedial Hypothalamus Amplifies Counterregulatory Hormone Responses in Rats With Defective Counterregulation

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Defective counterregulatory responses (CRRs) to hypoglycemia are associated with a marked increase in the risk of severe hypoglycemia. The mechanisms leading to the development of defective CRRs remain largely unknown, although they are associated with antecedent hypoglycemia. Activation of AMP-activated protein kinase (AMPK) in the ventromedial hypothalamus (VMH) amplifies the counterregulatory increase in glucose production during acute hypoglycemia. To examine whether activation of AMPK in the VMH restores defective CRR, controlled hypoglycemia (~2.8 mmol/l) was induced in a group of 24 Sprague-Dawley rats, all of which had undergone a 3-day model of recurrent hypoglycemia before the clamp study. Before the acute study, rats were microinjected to the VMH with either 5-aminoimidazole-4-carboxamide (AICAR; $n = 12$), to activate AMPK, or saline ($n = 12$). In a subset of rats, an infusion of H^3-glucose was additionally started to calculate glucose turnover. Stimulation of AMPK within the VMH was found to amplify hormonal CRR and increase endogenous glucose production. In addition, analysis of tissue from both whole hypothalamus and VMH showed that recurrent hypoglycemia induces an increase in the gene expression of AMPK α_1 and α_2. These findings suggest that the development of novel drugs designed to selectively activate AMPK in the VMH offer a future therapeutic potential for individuals with type 1 diabetes who have defective CRRs to hypoglycemia. *Diabetes* 55:1755–1760, 2006

Single or recurrent episodes of acute hypoglycemia in nondiabetic or type 1 diabetic (1–3) individuals are known to impair hormonal counterregulatory responses (CRRs) to a subsequent episode of hypoglycemia. Defective hormonal counterregulation to hypoglycemia is closely associated with both altered glucose thresholds for activation of the CRR and reduced symptomatic awareness of hypoglycemia, a combination of clinical syndromes that collectively have been termed hypoglycemia-associated autonomic failure (4). Defective hormonal CRR is in itself associated with a markedly increased risk of severe hypoglycemia (5).

The mechanism through which recurrent hypoglycemia per se induces defective hormonal CRR remains largely unknown. Potential candidate mechanisms include alterations in key steps in the glucose-sensing pathway in the brain (6,7), increased glucose and/or alternate fuel uptake by the brain (8–11), increases in the brain glycogen pool (12), and an effect of hypothalamopituitary axis activation (13,14). In addition, we have recently demonstrated that pharmacological activation of the serine/threonine kinase AMP-activated protein kinase (AMPK) in the ventromedial hypothalamus (VMH), a key central glucose-sensing region (15), amplifies the glucose CRR to acute hypoglycemia. In that study AMPK activation in the VMH during acute hypoglycemia resulted in a marked increase in endogenous glucose production (R_a), with a corresponding reduction in the requirement for exogenous glucose, in the absence of a change in the hormonal CRR (15).

Activation of AMPK follows a rise in AMP-to-ATP ratio, as well as phosphorylation by a kinase kinase (16). AMPK then in turn phosphorylates a number of metabolic enzymes and transcription factors involved in the regulation of cellular metabolism, culminating in the suppression of energy-depleting anabolic pathways and activation of energy-repleting catabolic pathways (17). AMPK can therefore be seen as a metabolic master switch, responding to alterations in cellular energy charge (18). AMPK is widely expressed in brain (19), showing a mainly neuronal distribution, although the α_2 catalytic subunit is also found in activated astrocytes (19), and its expression is greatest in those brain regions with the highest rates of glucose utilization (19). Within the hypothalamus, AMPK expression and activity have been shown to change in response to glucose (20–23), insulin (22,24), leptin (22–25), and refeeding (21,22,24). Glucose given peripherally or directly to the brain via the lateral ventricle decreases AMPK activity within the VMH, among other hypothalamic nuclei (22), and intraperitoneal injections of 2-deoxyglucose (an analog of glucose that is taken up by cells but not metabolized) increases hypothalamic AMPK expression and activity as well as increasing food intake (21). More recently, it was shown in studies in two neuronal cell lines

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AICAR, 5-aminoimidazole-4-carboxamide; AMPK, AMP-activated protein kinase; CRR, counterregulatory reponse; VMH, ventromedial hypothalamus.

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and ex vivo hypothalamic tissue that reduction of intracellular ATP triggers a cascade of events via AMPK that lead to the increased expression of the orexigenic neuropeptide AgRP, steps that might be expected to lead to changes in feeding behavior designed to restore the energy supply (21). Taken together, these studies imply a significant role for AMPK within the brain, and in particular the hypothalamus, in coordinating central and peripheral adaptations to changes in an individual's nutritional status.

Here we report that in vivo pharmacological activation of AMPK within the VMH amplifies hormonal CRR to acute hypoglycemia in rats with hypoglycemia-induced defective CRR to hypoglycemia. Moreover, we show that recurrent hypoglycemia is associated with an increase in mRNA for both AMPK α_1 and α_2 isoforms in whole hypothalamus and in the VMH.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley (weight 250–350 g) were housed in the Yale Animal Resource Center, fed a standard pellet diet (Agway Prolab 3000), and maintained on a 12/12-h day/night cycle. The animal care and experimental protocols were reviewed and approved by the Yale Animal Care and Use Committee.

One week before each study, the rats ($n = 24$) were anesthetized with an intraperitoneal injection (1 ml/kg) of a mixture of Xylazine (AnaSed 20 mg/ml; Lloyd Laboratories, Shenandoah, IA) and Ketamine (Ketaset 100 mg/ml; 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, followed by the stereotaxic insertion of VMH (anterior-posterior −2.6 mm, mediolateral ±3.8 mm, and dorsoventral −8.3 mm at an angle of 20°) microinjection guide cannulas, as described previously (15). The rats were then allowed to recover and subsequently studied after they had undergone the recurrent hypoglycemia protocol described below. Rats were studied in the overnight-fasted state, awake and unrestrained.

A further group of animals ($n = 26$) underwent surgery as described above for the insertion of vascular catheters only. On days 4–6 postsurgery, rats were microinjected with insulin ($n = 13$) or saline ($n = 13$; as described below) to induce consecutive episodes of hypoglycemia (insulin) or euglycemia (saline). On day 7, overnight-fed rats were killed, their brains removed rapidly, and either the whole hypothalamus ($n = 4$ each group) was dissected, or the whole brain ($n = 9$ in each group) was rapidly removed and frozen. In this latter group, VMH micropunches were obtained from 600-μm sections taken through the hypothalamus.

Recurrent hypoglycemia protocol. At 9:00 A.M. on the 3 consecutive days before study, overnight-nonfasted rats were injected intraperitoneally with human regular insulin (Eli Lilly, Indianapolis, IN) at a dose of 10 units/kg, as described previously (14). Food was then withheld for 3 h to allow for moderate sustained hypoglycemia (tail vein glucose ~30–40 mg/dl). At the end of this period, the rats were given free access to food. Control rats received 0.9% saline as an intraperitoneal injection under the same conditions.

Microinjection. On the morning of the study, 22-gauge microinjection needles, designed to extend 1 mm beyond the tip of the guide cannula (Plastics One, Roanoke, VA), were inserted through the guide cannula bilaterally into each VMH. The study rat was then microinjected over 5 min (0.1 μl/min) with either 5-aminoimidazole-4-carboxamide (AICAR) (total dose 16 ng, $n = 12$; Sigma-Aldrich) dissolved in 0.9% saline or 0.9% saline as a control ($n = 12$), using a CMA-102 infusion pump (CMA Microdialysis, North Chelmsford, MA). Following microinjection, the needles were left in place for 5 min before being removed. At the end of the study, the rats were killed and probe position confirmed in all rats histologically.

Infusion protocol. All animals were fasted overnight. On the morning of the study, the vascular catheters were opened, and the animals were allowed to settle and recover from any stress of handling for at least 90 min. Thirty minutes ($t = -30$ min) before the commencement of the hyperinsulinemic glucose clamp, each animal was microinjected with either AICAR or control as described above. Thereafter, a hyperinsulinemic-hypoglycemic clamp technique as adapted for the rat (26) was used to provide a standardized hypoglycemic stimulus. At $t = 0$, a 2-h 10 mU · kg^{-1} · min^{-1} infusion of human regular insulin (Eli Lilly) was begun. The plasma glucose was allowed to fall to ~2.8 mmol/l (aiming to achieve target glucose levels within 30–40 min) and was then maintained at this level for 120 min using a variable rate 10% dextrose infusion based on frequent plasma glucose determinations. Samples for glucose, insulin, epinephrine, and glucagon were obtained at regular intervals during the baseline and hypoglycemic states.

In addition, in a subset of rats ($n = 5$ in each group), a primed infusion of H^3-glucose was started at $t = -120$ min and continued throughout the hypoglycemia studies to compare the effects of VMH-AICAR versus VMH-control on rates of endogenous glucose production (R_a) and peripheral glucose utilization (R_d) during insulin-induced hypoglycemia. Glucose turnover was calculated according to the method of Wall et al. (27). Endogenous production was calculated by subtracting the exogenous glucose infusion rate from total R_a.

Analytical procedures. mRNA levels were evaluated with a Brilliant SYBR Green QRT-PCR master mix kit (QIAGEN). Total cellular RNA from rats' whole hypothalamus was obtained using TRIzol reagent (Invitrogen) and cleaned up with Rneasy Mini kit (QIAGEN) according to the manufacturer's instructions. VMH micropunches were obtained using 18-gauge needles. Subsequently, total cellular RNA from punches was extracted with PicoPure RNA isolation kit (Arcturus, CA). The forward primer for rat AMPK α_1 consisted of 5′-ATCCGCAGAGAGATCCAGAA-3′, and the reverse primer consisted of 5′-CGTCGACTCTCCTTTTCGTC-3′. The forward primer for rat AMPK α_2 consisted of 5′-GCTGTGGATCGCCAAATTAT-3′, and the reverse primer consisted of 5′-gcatcagcagagtggcaata-3′. Quantitative RT-PCR kits were made by combining 12.5 μl of 2× SYBR RT-PCR Master Mix, 0.5 μl of upstream primer (5 μmol/l), 0.5 μl of downstream primer (5 μmol/l), 0.0625 μl of StrataScript RT/Rnase block enzyme mixture, 1.4375 μl of RNase-free water, and 10 μl (20 ng/μl) of RNA template. No-template and no-RT controls were introduced in each run. The quantitative RT-PCR was performed using DNA Engine Opticon 2 (MJ Research), in which the mixture was heated to 50°C for 30 min for reverse transcription, heated to 95°C for 10 min, and then cycled 40 times at 95°C for 30 s, 56°C for 1 min, and 72°C for 50 s. To verify the specificity of the amplification reaction, melting-curve analysis was performed. The size of the amplified product was confirmed by electrophoresis. The level of rat β-actin mRNA was determined in all the samples, and the expression of AMPK was normalized to rat β-actin. The threshold cycle (C_T) value is taken as the fractional cycle number at which the emitted fluorescence of the sample passes a fixed threshold above the baseline. The $2C_T$ method was used to calculate the relative differences between experimental and control groups as fold change in gene expression (28). All the reactions were run in a minimum of four independent assays.

Plasma levels of glucose were measured by the glucose oxidase method (Beckman, Fullerton, CA). Catecholamine analysis was performed by high-performance liquid chromatography using electrochemical detection (ESA, Acton, MA); plasma insulin and glucagon were measured by radioimmunoassay (Linco, St. Charles, MO). All data are expressed as means ± SE and analyzed statistically using either Student's t test or repeated-measures ANOVA followed by post hoc testing to localize significant effects as indicated (SPSS 11.0 for Windows; SPSS).

RESULTS

Hypoglycemia studies. Mean ± SE plasma glucose achieved in each group (60–120 min) was 2.8 ± 0.1 mmol/l for the AICAR group and 2.7 ± 0.1 mmol/l for controls; these levels did not differ significantly (P = NS). Glucose infusion rates (for whole group; $n = 12$ in each), however, differed markedly between groups, with the VMH-AICAR–injected rats requiring significantly less exogenous glucose to maintain the hypoglycemic plateau. Over the last 60 min of the hypoglycemic clamp, the mean glucose infusion rate was 1.8 ± 0.3 vs. 10.3 ± 2.1 mg · kg^{-1} · min^{-1} in the AICAR-injected versus the control rats ($P < 0.001$).

The tracer studies showed that this resulted primarily from a marked and significant increase in the rate of endogenous glucose production (R_a), with no significant overall increase in whole-body glucose uptake (R_d) in the AICAR-injected rats (Fig. 1*A* and *C* shows calculated R_a and R_d for the rats, $n = 5$ in each subgroup, in which tracer studies were additionally performed). Although small differences in basal rates of R_a and R_d were apparent before microinjection, these were not statistically different, and, moreover, no significant effect of VMH microinjection was seen on basal rates of R_a and R_d in either the AICAR or control group. When the mean of glucose turnover mea-

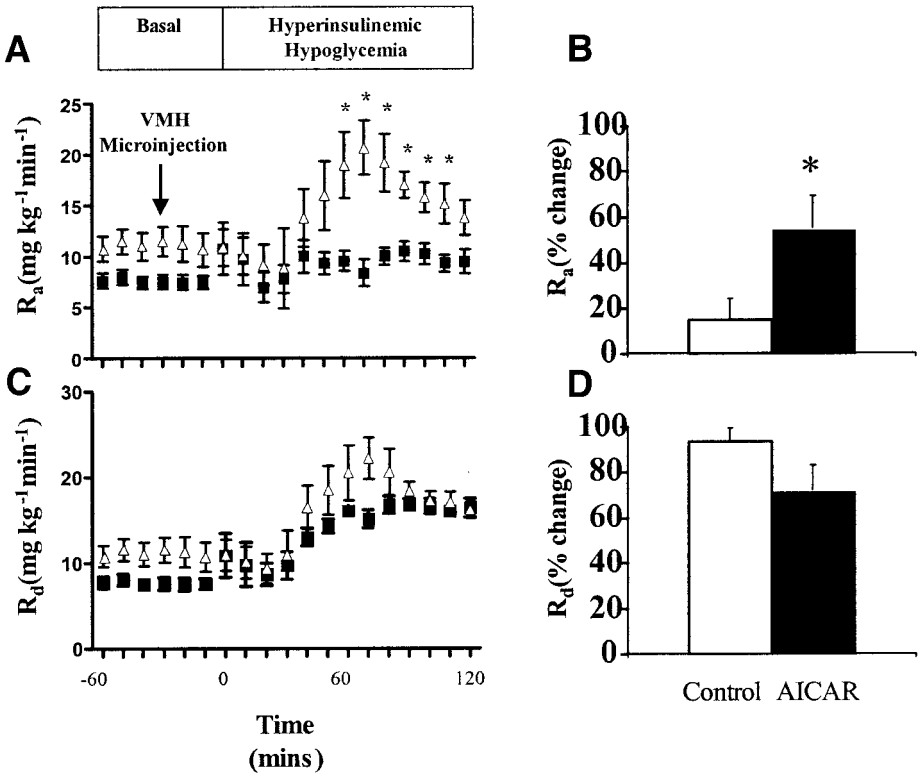

FIG. 1. In a subgroup of rats (both $n = 5$), an infusion of H^3-glucose was started at $t = -120$ min and continued throughout the hyperinsulinemic-hypoglycemic clamp studies to allow calculation of glucose kinetics. Rates of endogenous glucose production (R_a) (*A*) and peripheral glucose utilization (*C*) are shown for control (■) and AICAR (▲) groups at each point of measurement during basal and hypoglycemic periods. In addition, percent change in R_a (*B*) and R_d (*D*) from baseline for control (□) and AICAR (■) studies at 60–120 min are shown. Values are shown as means ± SE. *$P < 0.05$ vs. control.

surements from 60 to 120 min of hypoglycemia were compared with basal levels, VMH microinjection of AICAR was found to have increased R_a by 55 ± 14% vs. 15 ± 9% in the control group (Fig. 1*B* overall group effect $F = 7.4$, $P < 0.05$), whereas R_d increased similarly from basal levels in both AICAR (72 ± 11%) and control (94 ± 5%) groups (Fig. 1*D*, P = NS).

The microinjection of control or AICAR had no significant effect on plasma epinephrine (0.22 ± 0.05 to 0.26 ± 0.05 nmol/l vs. 0.24 ± 0.06 to 0.13 ± 0.05 nmol/l, respectively; both P = NS) or plasma glucagon (control 36 ± 3.4 to 43 ± 4.1 ng/l vs. AICAR 37 ± 3.7 to 41 ± 4.1 ng/l; both P = NS) in the period before the induction of hypoglycemia. However, during hypoglycemia there was a significantly greater rise in the hormone CRR following VMH microinjection of AICAR (Fig. 2*A* and *B*). Mean ± SE plasma levels of epinephrine (control 2.60 ± 0.3 and 4.23 ± 0.61 nmol/l vs. AICAR 5.83 ± 0.90 and 8.70 ± 1.18 nmol/l at 60 and 120 min of hypoglycemia, respectively; $F = 14.55$, $P < 0.01$; Fig. 2*A*) and glucagon (control 101 ± 10.1 and 81.9 ± 25.8 ng/l vs. AICAR 326 ± 38.9 and 193 ± 32.3 ng/l at 60 and 120 min of hypoglycemia, respectively; $F = 11.03$, $P < 0.01$; Fig. 2*B*) were markedly raised following activation of AMPK in the VMH during acute hypoglycemia.

The effect of recurrent hypoglycemia on AMPK α_1 and α_2 gene expression in whole hypothalamus of rats was also determined using quantitative real-time PCR. In comparison to saline-injected control rats, recurrent hypoglycemia induced significant increases in both AMPK α_1 and α_2 gene expression (Fig. 3*A* and *B*). To determine whether this increase in AMPK expression following recurrent hypoglycemia was also reflective of similar changes within the VMH, AMPK mRNA from VMH micropunches was also measured using quantitative real-time PCR. Analysis of tissue obtained from VMH micropunches also showed a significant increase in AMPK α_1 and α_2 gene expression following recurrent hypoglycemia.

DISCUSSION

We previously demonstrated a potential role for the AMPK signaling cascade in glucose sensing within the VMH by showing that the in vivo delivery of a pharmacological activator of AMPK to the VMH augmented the response of

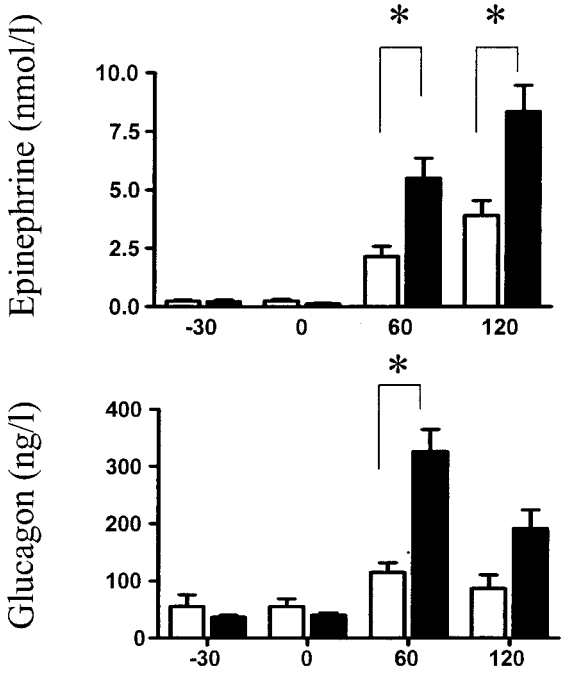

FIG. 2. Counterregulatory hormone response to acute hypoglycemia in recurrently hypoglycemic rats following VMH microinjection of control or AICAR. Samples for plasma epinephrine and glucagon were taken before ($t = -30$ min) and after ($t = 0$ min) microinjection of control solution (□) or 100 μmol AICAR (■) and in response to hyperinsulinemic hypoglycemia (60 and 120 min). Values are shown as means ± SE. *$P < 0.05$ vs. control.

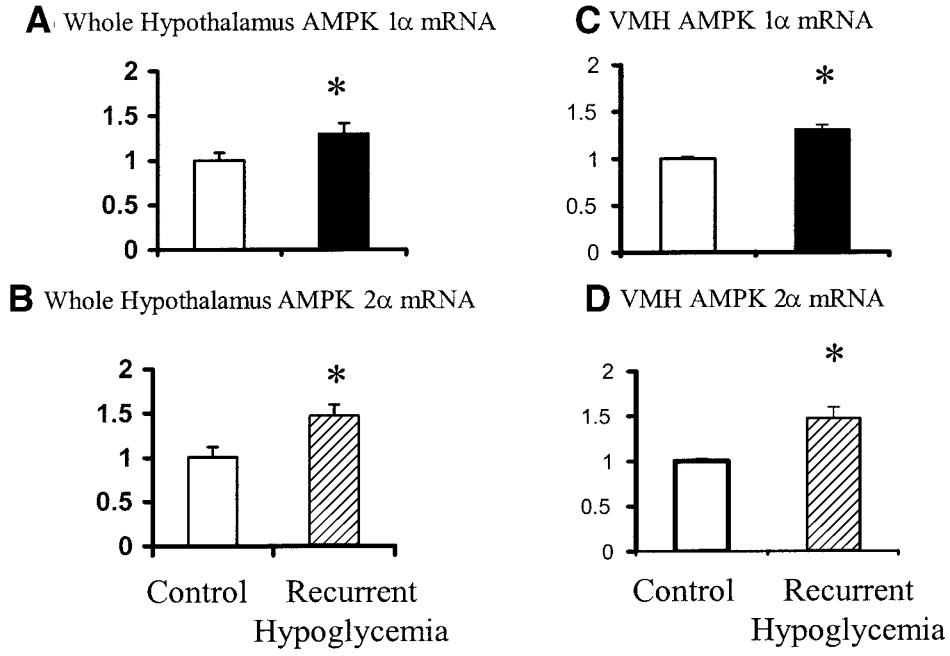

FIG. 3. Effects of recurrent hypoglycemia on AMPK α_1 and α_2 gene expression. The levels of AMPK α_1 (■) (*A* and *C*) and AMPK α_2 (▨) (*B* and *D*) mRNA (real-time quantitative RT-PCR) were assessed following three consecutive, daily episodes of acute hypoglycemia in whole hypothalamus and VMH and compared with saline-injected controls (□). The values are shown as means ± SE in a minimum of four assays. *$P < 0.01$ AMPK α_1 and α_2 gene expression.

hepatic glucose production to insulin-induced hypoglycemia. That study, however, failed to show an effect of AMPK activation on the hormone CRR to hypoglycemia even though it stimulated endogenous glucose production. The present study confirms and extends our original findings by demonstrating that pharmacological activation of AMPK in the VMH can also to a large extent reverse the hormonal counterregulatory defect associated with recurrent antecedent hypoglycemia.

The recurrent hypoglycemia protocol used in this study has been previously validated and has been shown to induce defective counterregulation (14,29). Comparison between hormonal CRR to hypoglycemia in the recurrently hypoglycemic rats used in the present study and a group of previously reported nonrecurrently hypoglycemic rats (15) that had undergone exactly the same experimental protocol showed that hormonal responses to acute hypoglycemia were significantly less in those rats who had undergone the recurrent hypoglycemia, showing decrements in both epinephrine (3.9 ± 0.6 vs. 10.5 ± 0.2 nmol/l at 120 min in recurrently hypoglycemic versus normal rats, respectively; $P < 0.05$) and glucagon responses (115 ± 16 vs. 192 ± 24 ng/l at 60 min; $P < 0.05$), confirming the validity of our animal model.

The mechanism through which falling blood glucose is detected by the VMH and translated into an altered firing rate in glucose-sensing neurons is unclear. There is evidence to support significant roles for the ATP-sensitive potassium channel (6,29), glucokinase (7), and now AMPK (15). Glucokinase and the ATP-sensitive potassium channel play key roles in the classical model of glucose sensing, the pancreatic β-cell, but the role of AMPK in this system is unclear. In general, AMPK activation through AICAR or overexpression of constitutively active AMPK in the pancreatic β-cell suppresses glucose-induced insulin secretion (30–32). Moreover, overexpression of AMPK α_1 also reduces tolbutamide- and KCl-induced insulin secretion (33). These findings support the hypothesis that AMPK plays a role in the sensing of low glucose states and suggest that AMPK is integral to this sensing mechanism. Exactly how AMPK integrates with signaling pathways activated by low glucose is unknown. Potential mechanisms in hypothalamic neurons include *1*) a direct action on the ATP-sensitive potassium channel via phosphorylation sites, in both the inward rectifying Kir6.2 and the sulfonylurea receptor subunit (31), or *2*) through activation of nitric oxide synthase (16,34), which could potentially alter γ-aminobutyric acid (35) or norepinephrine release within the VMH (36).

In the present study, and consistent with our previous findings (15), AMPK activation in the VMH was shown to amplify endogenous glucose production during hypoglycemia, although the greater hormone CRR in the VMH-AICAR–injected rats in the present study limits our interpretation of this data. This most probably arose from an increase in the rate of hepatic glucose production, although we cannot exclude potential effects on other organs such as the kidney. It is noticeable that this occurred despite marked physiological hyperinsulinemia, which maintained endogenous glucose production rates at basal levels despite the CRR in control rats. Shimazu (37) first reported in the 1980s that the hypothalamus might play an important role in glucoregulation through direct neural effects on glycogen metabolism in the liver. The VMH and lateral hypothalamus were thought to act reciprocally in regulating intermediary metabolism in peripheral tissues. Electrical stimulation of the VMH was also shown to increase labeled 2-deoxyglucose uptake in peripheral tissues, such as brown adipose tissue (38), and skeletal muscle (38,39). More recently, it was reported that intracerebroventricular infusion of AICAR into mice increased whole-body glucose turnover, muscle glycogen synthesis, and hepatic glucose production (40). It is interesting that both of our studies accord with those of Obici et al. (41), who demonstrated that pharmacological inhibition of, or decreased expression of, carnitine palmityltransferase-1 in the hypothalamus acting via a reduction in fatty acid oxidation served as a signal to suppress hepatic glucose output. They proposed that hypothalamic neurons might have the ability to act as nutrient sensors and could subsequently generate signals that modulate energy homeostasis and hepatic insulin action. AMPK activation in

the hypothalamus, through its known stimulatory effect on fat oxidation (42), would be expected to have the reverse effect as shown in our study. Our findings and those of others provide further support for Shimazu's original contention that the hypothalamus plays a crucial role in the regulation of hepatic glucose production.

While AMPK activation also increased endogenous glucose production in hypoglycemia-naïve rats, it did not, as in the present study, amplify hormonal CRR to subsequent hypoglycemia. These findings suggest that recurrent hypoglycemia has resulted in a shift in the activation cascade for AMPK. The AMPK activation cascade is extremely sensitive to changes in intracellular AMP-to-ATP ratio over a small physiological range (43). This arises through the action of adenylate cyclase to convert ADP to ATP and AMP, such that AMP and ATP usually change in reciprocal directions, and the AMP-to-ATP ratio varies as the square of the ADP-to-ATP ratio (43). The findings of our present study would suggest that in hypoglycemia-naïve rats, AMPK activation in the VMH (but not necessarily in all brain regions) is already near maximal at a plasma glucose of 50 mg/dl. Following recurrent hypoglycemia, the shift in the AMPK activation cascade means that AMPK is not fully activated at a glucose of 50 mg/dl, but provision of the extra stimulus to AMPK through AICAR then results in more complete activation of the cascade.

During acute hypoglycemia, there is an increase in AMPK expression and activity (20) in the hypothalamus. In the present study, recurrent hypoglycemia was shown to increase basal (nonfasted) AMPK mRNA in both whole hypothalamus and VMH. In other organs systems, repeated activation of AMPK has also been shown to increase AMPK expression. Repeated exercise increases muscle AMPK protein content (44), and fasting increases AMPK expression in the liver (45,46). However, despite the increase in AMPK, CRRs to acute hypoglycemia are suppressed in recurrently hypoglycemic rats, implying that there is less activation of AMPK. This suggests either an alteration in the upstream activators of AMPK or inhibition of AMPK. The regulation of AMPK is complex (16). For instance, AMP activates AMPK in three different ways (namely, allosteric activation following AMP binding, AMP binding–potentiating activation by phosphorylation at Thr-172 by an upstream kinase, and AMP binding–inhibiting dephosphorylation at Thr-172 by protein phosphatases), all of which are inhibited by ATP. Therefore, it is possible that increased production of ATP during hypoglycemia through increased delivery and/or more efficient oxidation of glucose or lactate might act to inhibit AMPK activation (8–11). Another potential regulator of AMPK that may be affected by recurrent hypoglycemia is the upstream kinase LKB-1, which plays a key role regulating AMPK activation (16). Additionally, it has been shown that regulatory β-subunit of AMPK contains a glycogen binding site. If supercompensation of glycogen does occur following recurrent hypoglycemia (12), this might explain the apparent reduction in AMPK activation during subsequent hypoglycemia despite the increase in AMPK expression.

In conclusion, our data show that it is possible to reverse, to a large extent, the counterregulatory hormonal defect that arises subsequent to recurrent hypoglycemia through additional pharmacological activation of AMPK within the VMH. Recurrent hypoglycemia is associated with an increase in AMPK α_1 and α_2 mRNA in both whole hypothalamus and VMH, and this may represent a compensatory response to recurrent activation of AMPK by the repeated episodes of acute hypoglycemia and could account for the greater sensitivity of the VMH to pharmacological activation. Further studies in animal models of type 1 diabetes will help to clarify the additional impact, if any, on the AMPK activation cascade of the more variable (intermittent hyperglycemia and hypoglycemia) metabolic state of type 1 diabetes. However, these findings suggest that the development of novel drugs designed to activate AMPK in central glucose-sensing regions may offer a future therapeutic potential for individuals with type 1 diabetes who have defective CRRs to hypoglycemia.

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