The mechanisms by which specialized glucose-sensing neurons within the hypothalamus are able to detect a falling blood glucose remain largely unknown but may be linked to some gauge of neuronal energy status. We sought to test the hypothesis that AMP-activated protein kinase (AMPK), an intracellular kinase purported to act as a fuel sensor, plays a role in hypoglycemia sensing in the ventromedial hypothalamus (VMH) of the Sprague-Dawley rat by chemically activating AMPK in vivo through bilateral microinjection, before performing hyperinsulinemic-hypoglycemic or hyperinsulinemic-euglycemic clamp studies. In a subgroup of rats, H3-glucose was infused to determine glucose kinetics. The additional chemical activation by AICAR of AMPK in the VMH during hypoglycemia markedly reduced the amount of exogenous glucose required to maintain plasma glucose during hypoglycemia, an effect that was almost completely accounted for by a three- to fourfold increase in hepatic glucose production in comparison to controls. In contrast, no differences were seen between groups in hypoglycemia-induced rises in the principal counterregulatory hormones. In conclusion, activation of AMPK within the VMH may play an important role in hypoglycemia sensing. The combination of hypoglycemia- and AICAR-induced AMPK activity appears to result in a marked stimulus to hepatic glucose counterregulation. Diabetes 53:1953–1958, 2004

The importance of glucose as a fuel, especially for the brain, ensures that numerous homeostatic mechanisms have evolved that serve to maintain the blood glucose within a relatively narrow physiological range. In type 1 diabetes, supraphysiological insulin replacement therapy and defective glucose counterregulatory mechanisms combine to disrupt normal glucose homeostasis and significantly increase the risk of hypoglycemia (1). As clinicians strive to lower average blood glucose levels further in an attempt to reduce complications related to chronic hyperglycemia, the risk of hypoglycemia increases further (2).

To intervene therapeutically to reduce the risk of hypoglycemia, a greater understanding is required of the mechanisms that have evolved to detect incipient hypoglycemia and to trigger a counterregulatory response. Hypoglycemia detection is thought to occur in specialized glucose-sensing neurons within the portal venous system (3–5) and brain (6–10), with the brain probably playing a predominant role. Within the brain, neurons whose activity appears to be directly linked to fluctuations in the glucose concentration to which they are exposed have been localized to the ventromedial hypothalamus (11) and to the brain stem (12,13). The mechanisms by which these systems are able to detect a falling glucose remain largely unknown. However, given that neurons, with few exceptions, have no significant energy stores, it is likely that neural output within these sensing regions is linked to either metabolism within the neuron or to some gauge of its energy status. To date, most work has focused on the possibility that glucose-sensing neurons use molecular mechanisms similar to those of the pancreatic β-cell (14).

AMP-activated protein kinase (AMPK) is a serine/threonine kinase that has been proposed to function as an intracellular fuel gauge (12,13,15). Activation of AMPK follows a rise in AMP concentration, as well as phosphorylation by a kinase kinase (AMPK kinase) (16). AMPK then in turn phosphorylates a number of proteins involved in the regulation of cellular metabolism, including hydroxymethylglutaryl-CoA reductase (17), acetyl-CoA carboxylase (18), hormone-sensitive lipase (19), and glycogen synthase (20). AMPK is expressed in a wide variety of tissues, including liver, lung, heart, skeletal muscle, and brain (21–25). In the brain, AMPK is widely expressed, with immunostaining revealing a mainly neuronal distribution of all isoforms (24).

To test the hypothesis that AMPK within the ventromedial hypothalamus (VMH) of the rat brain might play a significant role in the detection of hypoglycemia, we have examined in vivo the effect of localized chemical activation of AMPK, using the agent 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), on glucose counterregulation during systemic hypoglycemia.

RESEARCH DESIGN AND METHODS

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

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AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMPK, AMP-activated protein kinase; GIR, glucose infusion rate; HGP, hepatic glucose production; VMH, ventromedial hypothalamus.

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protocols were reviewed and approved by the Yale Animal Care and Use Committee. One week before each study, all animals were anesthetized 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 animals initially underwent vascular surgery in which chronic vascular catheters were positioned in a carotid artery and jugular vein before being flushed and filled with heparin (942 units/ml) and polyvinylglycolidone (1.7 g/ml). The catheters were then plugged, tunneled subcutaneously around the side of the neck, and externalized behind the head through a skin incision. Following this procedure, and while the animals remained anesthetized, microinjection guide cannulas were bilaterally inserted using stereotactic techniques. The guide cannulas were positioned above the VMH, such that when the microinjectors were inserted, the tip of the microinjector was in the VMH. For stereotaxic surgery, the animals were placed in a stereotactic frame and the skull then exposed through a midline incision. Holes were drilled bilaterally through the skull vault through chosen coordinates and microinjection guide cannulas lowered slowly into the brain. The stereotaxic coordinates from bregma were AP −2.6 mm, ML ±3.8 mm, and DV −8.3 mm at an angle of 20° (26). The coordinates were chosen so that the tip of the guide cannulae lie 1 mm proximal to the target area for microinjection. As a further control, four additional rats had guide cannulas inserted bilaterally into the frontal cortex (coordinates AP 2.7 mm, ML ±2.6 mm, and DV −2.0 mm). All animals were then allowed to recover for 7–10 days and subsequently studied in the overnight-fasted state, awake, and unrestrained.

**Microinjection.** On the morning of the study, a 27-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 with 0.5 μl (0.1 μl/min; total dose 16 ng) of either 100 μmol AICAR (5-aminoimidazole-4-carboxamide; Sigma-Aldrich) dissolved in 0.9% saline or 0.9% saline as a control, using a CMA-102 infusion pump (CMA Microdialysis, N. 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 histologically confirmed in all rats.

**Infusion protocol.** All animals were fasted overnight. On the morning of the study, the vascular catheters were opened and maintained patent by a slow infusion of saline (30 μl/min). During the first 90 min, animals were allowed to settle and recover from any stress of handling. 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 (27), was used to provide a standardized hypoglycemic stimulus. At time zero, a 2-h, 10 mU·kg⁻¹·min⁻¹ infusion of human regular insulin (Eli Lilly, Indianapolis, IN) was begun. The plasma glucose was allowed to fall to −2.8 mmol/l and was then maintained at this level for 120 min using a variable rate 10% dextrose infusion based on frequent plasma glucose determinations. In a separate group of rats following the microinjection of AICAR, a hyperinsulinemic (10 mU·kg⁻¹·min⁻¹)-euglycemic (−6.9 mmol/l) clamp was performed. Samples for glucose, insulin, epinephrine, and glucagon were obtained at regular intervals during the baseline and hypoglycemic states.

The study groups were VMH-AICAR hypoglycemia (n = 11), VMH-control euglycemia (n = 6), VMH-control hypoglycemia (n = 5), and frontal cortex-AICAR hypoglycemia (n = 4). In addition, in a subset of study groups 1 (n = 6) and 2 (n = 5), an infusion of H⁺-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 (Rₑ) and peripheral glucose utilization (Rₒ) during insulin-induced hypoglycemia.

**Analytical procedures.** 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), and plasma insulin and glucagon were measured by radioimmunoassay (Linco, St. Charles, MO). All data are expressed as the mean ± SE and analyzed statistically using repeated-measures ANOVA followed by post hoc testing to localize significant effects as indicated (SPSS version 11.0 for Windows).

**RESULTS**

**Hypoglycemia studies.** Plasma glucose profiles during the hyperinsulinemic-hypoglycemic clamp studies and following microinjection of AICAR or control into the VMH are shown in Fig. 1A. Mean (±SE) plasma glucose achieved in each group (60–120 min) was 2.6 ± 0.1 mg/dl for the AICAR group and 2.7 ± 0.1 mg/dl for controls, levels that did not differ significantly (F = 0.9, P = NS). Glucose infusion rates (GIRs), however, differed markedly between groups, with the VMH-AICAR–injected rats requiring significantly less exogenous glucose to maintain the hypoglycemic plateau (F = 38.9, P < 0.01) (Fig. 1B). Over the last 60 min of the hypoglycemic clamp, the mean GIR was 2.3 ± 0.8 vs. 9.5 ± 1.0 mg · kg⁻¹ · min⁻¹ in the AICAR-injected versus control rats.

The tracer studies revealed that the differences in GIR were completely accounted for by an increased Rₑ (11.0 ± 2.3 vs. 3.1 ± 2.3 mg · kg⁻¹ · min⁻¹; F = 5.6, P < 0.05) (Fig. 2A) during hypoglycemia in VMH-AICAR–injected rats. No significant differences were apparent between VMH-AICAR– and control-injected rats with respect to Rₒ (13.6 ± 2.8 vs. 12.1 ± 2.2 mg · kg⁻¹ · min⁻¹; F = 0.2, P = NS) (Fig. 2B).

Interestingly, plasma epinephrine (Fig. 3A), glucagon (Fig. 3B), and norepinephrine responses to hypoglycemia did not differ between VMH-AICAR and control groups, showing overall mean differences of 10.277 ± 1.053 vs. 11.134 ± 1.190 pmol/l (F = 1.4, P = NS), 193 ± 36 vs. 228 ± 32 ng/l (F = 0.1, P = NS), and 2.1 ± 0.2 vs. 2.2 ± 0.2 pmol/l (F = 0.3, P = NS), respectively. No significant differences were found when separate analyses of hormone concentrations at individual time points (60, 90, and 120 min) were made. As shown in Table 1, VMH-AICAR and VMH-control animals did not differ significantly during hyperinsuline-
mic euglycemia with respect to GIR (F = 0.6, P = NS), plasma epinephrine (F = 0.1, P = NS), plasma glucagon (F = 0.9, P = NS), or plasma norepinephrine (F = 3.8, P = NS).

To determine whether chemical activation of AMPK in an alternative brain region might effect glucose counterregulation during hypoglycemia, AICAR was injected bilaterally into the frontal cortex before the induction of hypoglycemia. We found no significant differences between these rats and VMH-control rats with respect to GIR (7.2 ± 1.2 mg·kg⁻¹·min⁻¹, F = 2.2, P = NS vs. VMH-control), epinephrine (mean rise 8,711 ± 1,124 pmol/l, F = 3.2, P = NS vs. VMH-control), or glucagon (mean rise 212 ± 44 ng/l, F = 0.1, P = NS vs. VMH-control).

DISCUSSION

The data presented in this study are consistent with the hypothesis that AMPK may play a role in the detection of hypoglycemia by specialized glucose-sensing neurons in the VMH. We have demonstrated that the additional chemical activation of AMPK during hypoglycemia by AICAR resulted in a significant reduction in the amount of exogenous glucose required by the rat to maintain moderate hyperinsulinemic hypoglycemia and that the difference in GIRs was totally accounted for by an increase in endogenous $R_a$. This finding suggests that in the VMH, AMPK activation by hypoglycemia culminates in the generation of a nonhormonally mediated signal that acts to stimulate endogenous glucose production. To the best of our knowledge, our data therefore provide the first in vivo evidence implicating AMPK in the glucose-sensing mechanisms used by the VMH.

AMPK is thought to act as an intracellular fuel gauge that becomes activated by a decrease in the ATP-to-ADP ratio through mechanisms involving phosphorylation by one or more upstream AMPK kinases, allosteric activation, and a decrease in the inhibitory action of phosphatases (15). Increased AMPK activity results in the stimulation of glucose uptake by the muscle, fatty acid oxidation in muscle and liver, and the inhibition of hepatic glucose production (HGP), cholesterol, and triglyceride synthesis, as well as in lipogenesis (28). The role of AMPK within the brain is less well documented. The 1α and 2α catalytic and β and γ noncatalytic subunits of AMPK are widely expressed in brain (21,24). In the mouse, AMPK shows a mainly neuronal distribution, although the 2α catalytic subunit is also found in activated astrocytes (24). Consistent with a potential role for AMPK in regulating cellular metabolism in response to energy depletion is the finding that those brain regions with the highest level of AMPK expression are also those with the highest rates of glucose utilization (24). AICAR is rapidly taken up into cells and phosphorylated intracellularly to form the AMP analog.
ZMP, which activates AMPK usually without changes in AMP or ATP. However, the utility of AICAR as an activator of AMPK is limited by the accumulation of its triphospho-
ylated form (ZTP), which could potentially act as an ATP analog, by ZMP mimicking the effect of AMP on other AMP-sensitive enzymes and by AICAR activating adeno-
sine receptors. Despite these reservations, AICAR has become a widely used and important method for activating AMPK (29). Evidence for its speci-
city for AMPK was demonstrated in those studies, which showed that AICAR could mimic the effect of AMPK activation to stimulate glucose uptake in muscle (30,31) but could not stimulate glucose uptake in mice carrying a kinase-dead AMPK mutant in muscle (32). Overall, we feel it highly likely that VMH microinjection of AICAR has resulted in the chemical activation of AMPK, but we cannot exclude additional effects. There is a need to develop new methods to specifically activate AMPK.

The unexpected finding in the present study was that chemical activation of AMPK in the VMH resulted in increased endogenous $R_0$ during hypoglycemia and that this did not appear to occur exclusively through a hormonally mediated mechanism. Given that this effect was not seen with AICAR microinjection in the frontal cortex, it is likely to result from a local hypothalamic effect of AMPK activation. The observed increase in $R_0$ most likely resulted from a stimulation to HGP, although potential contributions from other gluconeogenic organs such as the kidney cannot be excluded. Non-hormonally mediated changes in HGP during hypoglycemia are well recognized (33) and result from both direct neural stimulation and the liver’s ability to self-regulate HGP under conditions of severe hypoglycemia (hepatic autoregulation). It has been shown that hyperglycemia can be induced through electrical stimulation of splanchnic or hepatic nerves in animals that have undergone adrenalectomy and/or pancreatec-
tomy (34,35). In addition, Shimazu, Fukuda, and Ban (36) have shown that stimulation of the VMH specifically results in increased hepatic glycogenolysis and gluconeogenesis. Combined with the current study, these data suggest the possibility of a direct neural link from the hypothalamus to the liver (and less likely the kidney) that is capable of significantly reversing the suppressive effect of hyperinsulinemia on endogenous glucose production. It remains possible that the action of this neural signal could be to increase the sensitivity of the liver to the stimulatory effect of circulating counterregulatory hormones on hepatic glucose output, but the fact that peripheral glucose utilization was higher, albeit not significantly so, in the AICAR-injected rats would argue against an increased sensitivity to catecholamines.

It remains to be explained why we saw no additional effect of AICAR on the counterregulatory hormone response to hypoglycemia. If AMPK is an integral part of the glucose-sensing system, then we might have expected to see additional changes in the counterregulatory hormones (i.e., the additional stimulus to AMPK in the AICAR-injected group emulating severe hypoglycemia centrally). It is conceivable that a separate signaling system exists within the VMH to stimulate counterregulatory hormonal and hepatic responses to hypoglycemia. Alternatively, it is possible that the counterregulatory hormonal response to the specific hypoglycemia stimulus used may already have been near-maximally activated in these rats. The addition of AICAR may have caused hypothalamic glucose sensors to perceive the presence of a more pronounced hypoglycemic stimulus, which has resulted in the activation of other nonhormonal mechanisms, providing a further stimulus to directly increase endogenous glucose production.

There are now numerous reports indicating that the VMH plays a critical role in hypoglycemia sensing (7–9). Specialized glucose-sensing neurons that are stimulated by a rise in glucose (glucose-excited neurons) or that are inhibited by a rise in glucose (glucose-inhibited neurons) have been localized to the VMH (11). The mechanisms used by these specialized neurons remains largely un-
known, although recent evidence suggests that there may be parallels with pancreatic β-cell glucose sensing (14). As in the β-cell, ATP-sensitive K+ channels have been shown in vitro to modulate activity of glucose-excited neurons (14,37). Given that AICAR microinjection to the VMH had no effect on GIRQs, epinephrine, or glucagon during hyper-
insulinemic euglycemia, our data suggest that AMPK is also integral to hypoglycemia sensing. The fact that AICAR had no effect under euglycemic conditions may at first glance appear surprising given that AMPK activity is liable to be low under basal conditions. In vitro cell culture modeling studies have shown that AMPK exhibits an ultrasensitive response to elevation of the activating nu-
cleoside monophosphate (38). This ultrasensitivity arises from at least two factors, namely, that the primary signal AMP acts at more than one step in the cascade and that under basal conditions, the upstream kinase is fully satu-
rated with the downstream kinase (38). As such, the relationship between AMP concentration and AMPK activity is sigmoidal, with a small change in AMP over a critical range having a pronounced effect on AMP activation (38). Under euglycemic conditions, AMP concentrations are
low and the upstream kinase is fully saturated. Moreover, ZMP produced after AICAR administration is ~50-fold less potent than AMP at activating AMPK (38), and under these conditions, we suspect that the intracellular change in AMP/ZMP is insufficient to activate AMPK. However, during hypoglycemia, adenylyl cyclase is activated, converting ADP to ATP and AMP and more markedly increasing intracellular AMP. Under these conditions, we believe that the chemically induced rise in ZMP results in a much more marked activation of AMPK.

It is unknown whether AMPK is acting independently of or plays an integral part in the classical glucose-sensing pathway. Intriguingly, recent data have emerged to suggest that changes in hypothalamic fat oxidation can directly alter endogenous glucose production (39,40). Obici et al. (40) demonstrated that pharmacological inhibition of, or decreased expression of, carnitine palmitoyltransferase-1 (CPT-1) in the hypothalamus, acting via a reduction in fatty acid oxidation or the accumulation of long-chain fatty acid-CoA, 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 (18), would be expected to have the reverse effect if this was true, and, as such, our data are in support of this hypothesis. Moreover, leptin has recently been shown to stimulate fat oxidation by activating AMPK in muscle and to exert additional delayed effects on fat oxidation in muscle through a central hypothalamic-sympathetic effect (41). Taken together with the data from the present study, we hypothesize that AMPK serves as a key downstream signaling enzyme linking both nutrient- and fuel-sensing pathways in the hypothalamus.

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