Hypothalamic Leucine Metabolism Regulates Liver Glucose Production

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Amino acids profoundly affect insulin action and glucose metabolism in mammals. Here, we investigated the role of the mediobasal hypothalamus (MBH), a key center involved in nutrient-dependent metabolic regulation. Specifically, we tested the novel hypothesis that the metabolism of leucine within the MBH couples the central sensing of leucine with the control of glucose production by the liver. We performed either central (MBH) or systemic infusions of leucine in Sprague-Dawley male rats during basal pancreatic insulin clamps in combination with various pharmacological and molecular interventions designed to modulate leucine metabolism in the MBH. We also examined the role of hypothalamic ATP-sensitive K^+ channels (K_{ATP} channels) in the effects of leucine. Enhancing the metabolism of leucine acutely in the MBH lowered blood glucose through a biochemical network that was insensitive to rapamycin but strictly dependent on the hypothalamic metabolism of leucine to α-ketoisocaproic acid and, further, insensitive to acetyl- and malonyl-CoA. Functional K_{ATP} channels were also required. Importantly, molecular attenuation of this central sensing mechanism in rats conferred susceptibility to developing hyperglycemia. We postulate that the metabolic sensing of leucine in the MBH is a previously unrecognized mechanism for the regulation of hepatic glucose production required to maintain glucose homeostasis. *Diabetes* 61:85–93, 2012

Ample evidence has established that protein and amino acids exert profound effects on insulin action, glucose metabolism, and food intake (1–8); however, the mechanisms behind these effects are not yet fully understood. Early work established that postprandial elevations of plasma amino acids stimulate endogenous secretion of both insulin and glucagon (9,10) and thus might modulate hepatic glucose metabolism by changing the portal insulin-to-glucagon ratio (11). Furthermore, amino acids act as substrates for gluconeogenesis (12), a pathway that contributes to glucose production. These direct and indirect effects of amino acids on hepatic glucose metabolism have been studied in various models, mainly using amino acid mixtures. Although these studies have provided a great deal of information, the use of mixtures did not allow for a direct examination of the unique actions of specific amino acids on glucose metabolism. Moreover, the majority of these studies emphasized the actions of amino acids in liver and skeletal muscle. The potential role of the mediobasal hypothalamus (MBH) in the mechanism(s) by which amino acids influence glucose metabolism has not been investigated. The arcuate nucleus of the MBH has emerged as a major center where signals involved in the determination of nutrient availability and energy balance converge. Nutrients engage biochemical sensors in the MBH that in turn exert a negative feedback on food intake and liver glucose production (13–16). Amino acids gain access to the central nervous system through a facilitative transport system (17), and their concentration in the hypothalamus reflects their circulating levels (18). This work focuses on leucine, a branched-chain ketogenic amino acid that plays a critical role in cellular signaling (19–21) via activation of the mammalian target of rapamycin (mTOR) pathway. In fact, leucine has been shown to modulate food intake by activating mTOR in the hypothalamus (6). Leucine metabolism generates acetyl-CoA, which can be oxidized to carbon dioxide or used in de novo lipid synthesis (Fig. 1A). The metabolism of leucine is initiated by its transamination to α-ketoisocaproic acid (KIC), catalyzed by the branched-chain amino acid aminotransferase (BCAT) (22). BCAT is highly expressed in the muscle and brain of humans and rodents (22,23). KIC undergoes oxidative decarboxylation catalyzed by the branched-chain ketoacid dehydrogenase (BCKDH) (22,23), yielding isovaleryl-CoA, which is further oxidized to acetyl-CoA (Fig. 1A), which is subsequently carboxylated to malonyl-CoA by acetyl-CoA carboxylase (ACC). Here, we tested the hypothesis that the metabolism of leucine to acetyl- and malonyl-CoA within the MBH couples the central sensing of leucine with the control of glucose production by the liver and that this regulatory mechanism contributes to the maintenance of euglycemia.

RESEARCH DESIGN AND METHODS

Animal preparation

Rats. We performed the studies in 10- to 12-week-old male Sprague-Dawley rats (24), which first underwent stereotaxic surgery to have inserted either a single cannula into the third cerebral ventricle (intracerebroventricular) or bilateral cannulae into the MBH. The correct placement of the cannulae was verified as previously described (24). A week later, we placed indwelling vascular catheters for pancreatic clamp studies. Satisfactory postoperative recovery was monitored by daily measurements of food intake and body weight gain for 7 days.

Mice. Twelve-week-old wild-type ($n = 8$) and SUR1-null ($Sur1^{-/-}$) ($n = 13$) male mice (27–32 g) underwent stereotaxic surgery under anesthesia for implantation of a single cannula in the third cerebral ventricle. A week later, the animals were catheterized for pancreatic clamp studies. Each animal was monitored daily for food intake and body weight gain to ensure complete postoperative recovery. The animal studies were approved by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine.
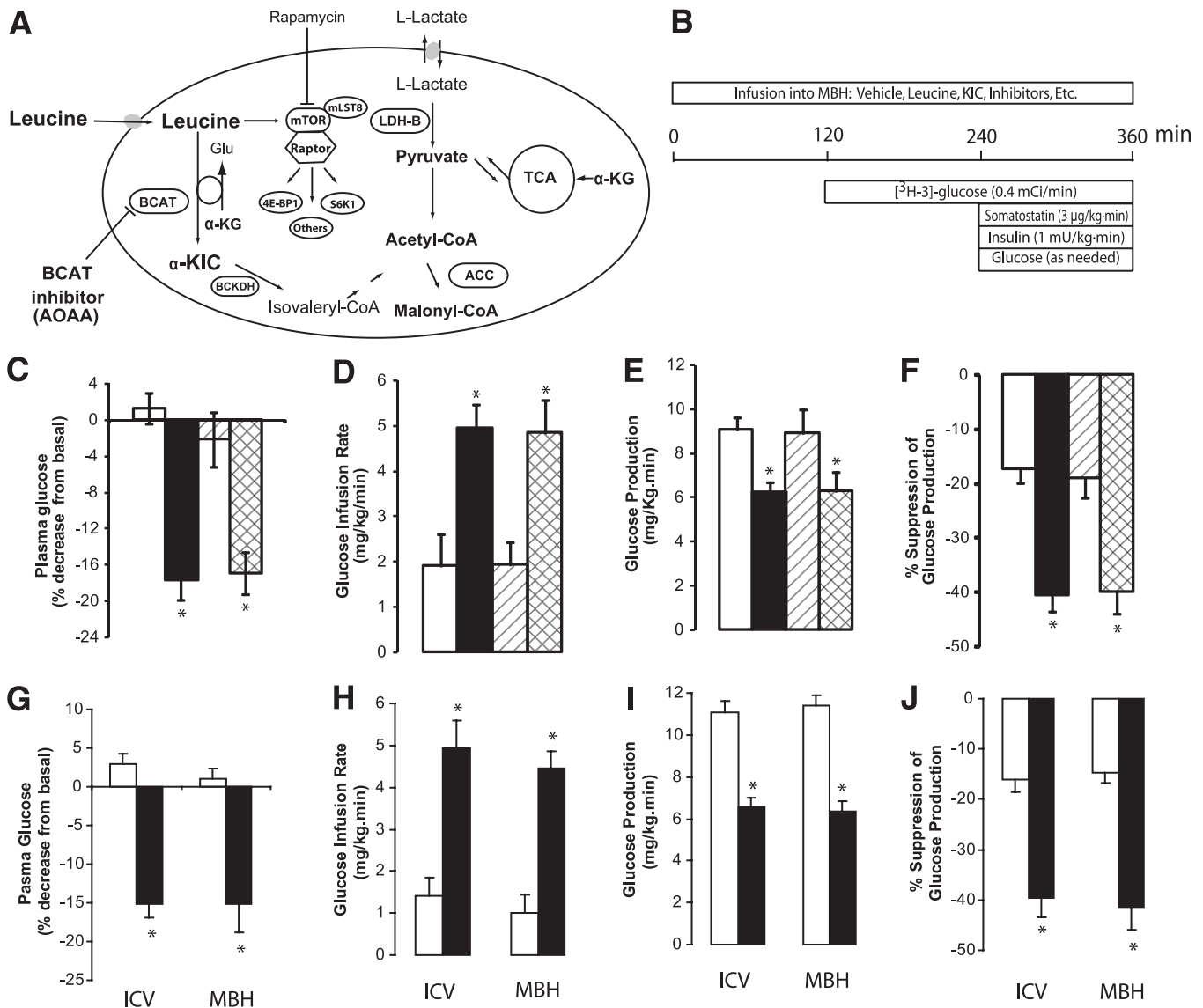
FIG. 1. Central administration of leucine lowers plasma glucose through KIC-dependent inhibition of liver glucose production. *A*: Metabolism of leucine and activation of the mTOR pathway. *B*: Protocol for pancreatic clamp and central infusions (MBH) in rats. *C–F*: Effect of the central administration (MBH) of vehicle (*n* = 6) (□), leucine (*n* = 6) (■), leucine plus BCAT inhibitor (amino-oxyacetic acid [AOAA]; *n* = 5) (▨), and leucine plus mTOR inhibitor (rapamycin; *n* = 4) (▩) on basal plasma glucose (*C*) and whole-body glucose metabolism during pancreatic insulin clamps (*D–F*). *G*: Effect of the administration of vehicle (*n* = 6) (□) or KIC (*n* = 6) (■) into the third cerebral ventricle (intracerebroventricular) or the MBH of rats on plasma glucose. *H–J*: Effect of central infusion of vehicle (*n* = 6) (□) or KIC (*n* = 5) (■) into the third cerebral ventricle (intracerebroventricular) or the MBH on whole-body glucose metabolism during pancreatic insulin clamps (*H–J*). All values are means ± SEM. *$*P < 0.05$ vs. vehicle.

Central or systemic infusions and pancreatic clamp procedure in rats. The studies were carried out in rats fasted for 6 h before the experiments and consisted of central (hypothalamic) or systemic infusions combined with pancreatic clamps (Figs. 1*B*, 5*A*, and 5*F*). The various central infusions were as follows: 5 μl/h i.c.v. and 0.33 μL/h into the MBH. The concentration of inhibitors was chosen based on their half-maximal inhibitory concentration (IC$_{50}$). Infusions were as follows (intracerebroventricular or MBH vehicle [artificial cerebrospinal fluid]): 90 nmol KIC i.c.v., MBH leucine, and 600 pmol KIC; 400 pmol MBH aminooxyacetic acid and 1 pmol MBH rapamycin; 1 nmol MBH α-chloroisocaproate (α-CIC) (pH ~7.4); and 1 μg MBH SHU9119. The central infusions lasted 360 min (Fig. 1*B*). At *t* = 0 min, a primed continuous infusion, either intracerebroventricular or into the MBH, of each of the various solutions described was initiated and maintained for the remainder of the study. At *t* = 120 min, a primed continuous infusion of [3-³H] glucose (40 μCi bolus, 0.4 μCi/min; New England Nuclear) was initiated and maintained for the last 4 h of the study. A basal insulin clamp was initiated at *t* = 240 min and maintained for 2 h (24). Throughout the study, the plasma levels of metabolites were monitored. At the end of the experiments, the rats were killed and tissue samples immediately freeze-clamped in situ and stored at –80°C for subsequent analysis.

Central infusions and pancreatic clamp procedure in mice. The studies lasted a total of 90 min and were carried out in animals fasted for 6 h. The experiments consisted of intracerebroventricular infusions combined with pancreatic insulin clamps (Fig. 4*E*). Groups of animals received central infusions (1 μL/h) as follows: vehicle (artificial cerebrospinal fluid) or 30 pmol KIC. During the infusions, a basal insulin pancreatic clamp was performed as previously described (25,26).

Biochemical analyses and determination of amino acids in plasma. Plasma metabolites and hormones were measured as previously described (25,26). Leucine, isoleucine, and valine were measured in samples of plasma (rats) as previously described (27,28) (Scientific Research Consortium, St. Paul, MN).

BCKDH kinase overexpression and assay. A cDNA for full-length rat liver BCKDH kinase (BCKDK) was cloned into the pAAV-CMV-MCS plasmid (Stratagene, La Jolla, CA), and the construct, phospho–adeno-associated virus (pAAV)-cytomegalovirus (CMV)-BCKDK, designed to overexpress BCKDK under control of the CMV promoter, was used for the generation of the adeno-associated virus (AAV, serotype 2) vector AAV-BCKDK. The pAAV-BCKDK plasmid was transfected into human embryonic kidney (HEK)
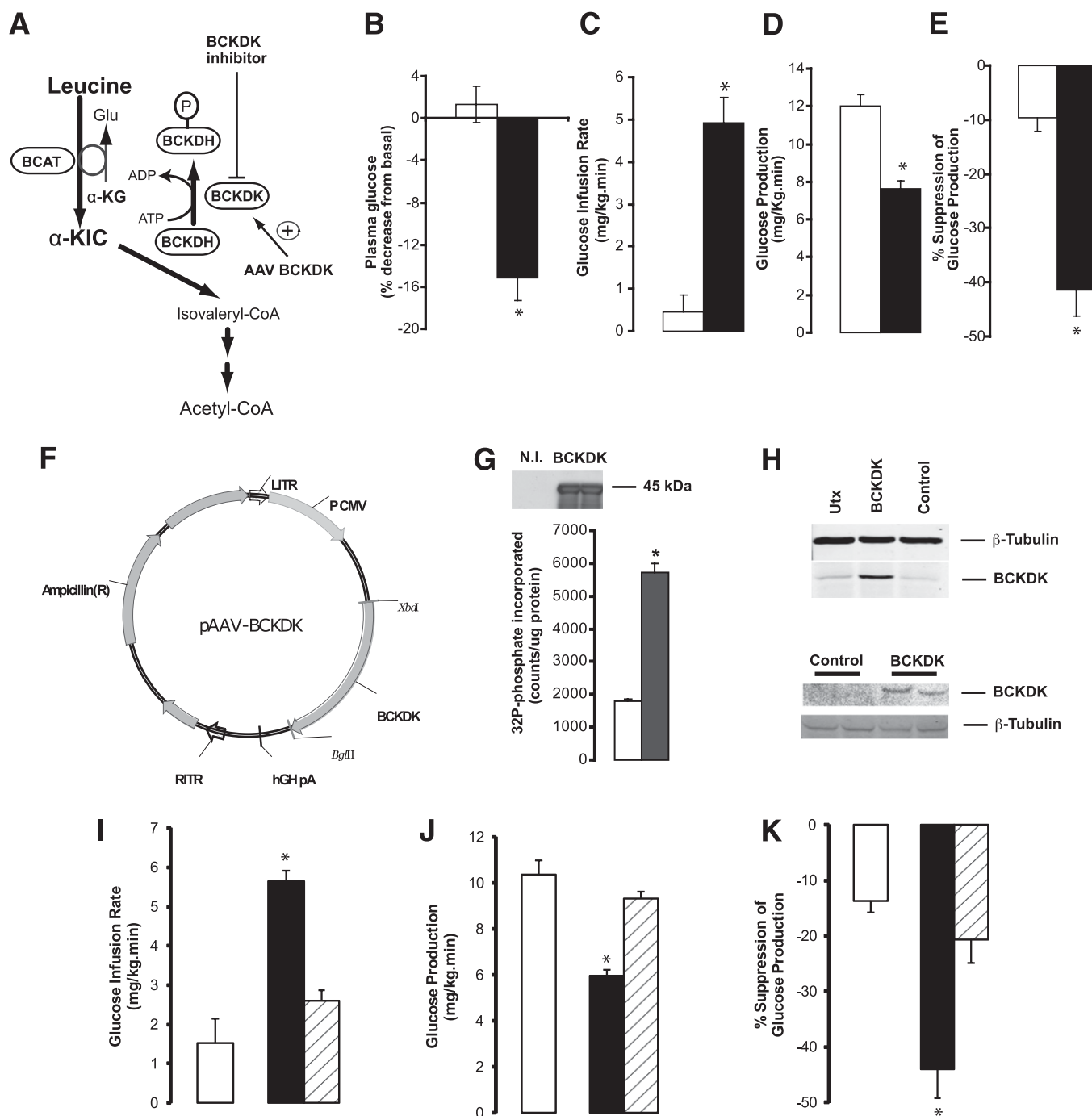
FIG. 2. Central metabolism of leucine/KIC is required for the inhibition of glucose production during physiological hyperleucinemia. *A*: KIC is metabolized to acetyl-CoA by the BCKDH complex, which is inhibited through phosphorylation by BCKDK. BCKDH is indirectly activated by α-CIC. BCKDH activity is inhibited by overexpression of BCKDK. *B–E*: Effect of central infusion (MBH) of vehicle ($n = 6$) (□) or α-CIC ($n = 5$) (■) on basal plasma glucose (*B*) and whole-body glucose metabolism during pancreatic insulin clamps (*C–E*). *F*: Schematic of vector used for the overexpression of BCKDK. LITR, left AAV2 inverted terminal repeat sequence; RITR, right AAV2 inverted terminal repeat sequence; hGH, human growth hormone polyA signal; P CMV, cytomegalovirus promoter. *G*: Western blot analysis (*top panel*; N.I., control (no insert) plasmid.) and activity assay (*bottom panel*; □, control plasmid; ■, BCKDK plasmid.) of overexpression (plasmid) of BCKDK in HEK 293 cells. *H*: Western blot analysis of AAV-driven overexpression of BCKDK in HEK 293 cells (*top panel*) or MBH of rats (*bottom panel*). *I–K*: Effect of the central infusion (MBH) of leucine in rats overexpressing either a control protein (GFP, $n = 4$) (■) or BCKDK ($n = 4$) (▨) in the MBH and vehicle-only ($n = 6$) (□) animals. All values are means ± SEM. *$P < 0.05$ vs. vehicle.

293 cells, which were lysed 48 h later for BCKDK Western blot analysis and activity assays. Aliquots from either an AAV-BCKDK or a control protein (green fluorescent protein [GFP]) vector stock (8×10^{11} genome copies/mL; Applied Viromics, Freemont, CA) were used to transduce HEK 293 cells, which after 5–7 days were analyzed by Western blot. BCKDK activity assays were performed using a commercial kit (Globozymes, Carlsbad, CA). Briefly, aliquots of cell lysates were incubated in the presence of purified BCKDH (substrate) and [^{32}P]-γ-ATP at 37°C for 10 min. Next, the extracts were precipitated with trichloroacetic acid and washed onto filter paper. The filters were dried, placed in scintillation fluid, and counted in a scintillation counter.

Malonyl-CoA decarboxylase overexpression and injection of AAV vector in the MBH of rats. The AAV vector used for the overexpression of malonyl-CoA decarboxylase (MCD) has previously been described (29). Male Sprague-Dawley rats equipped with chronic bilateral cannulae in the MBH were injected with 2 μl per side of the indicated viral preparations. Seven days later,

the animals received intravascular catheters, and upon recovery central infusions combined with euglycemic pancreatic insulin clamps were performed.

Meal challenge test. Groups of rats overexpressing either a control protein (GFP) or BCKDK in the MBH were equipped with jugular catheters. The animals were fed a high-fat, high-protein diet for 7 days. After an 18-h fast, the animals were offered a small meal (8 g) of the same diet (Fig. 5J). Samples of blood were collected right before the meal and postprandially at regular intervals for glucose and insulin determinations.

Western blot analysis and quantification and S6 kinase and S6 ribosomal protein phosphorylation analysis. Western blot analysis and quantifications were carried out using the Odyssey Infrared Imaging System (Li-Cor Biotechnology, Lincoln, Nebraska). Hypothalamic wedges were obtained from animals that received central infusions (MBH) of each of the following: 1) vehicle, 2) 1 μmol/L rapamycin (2 μL/side), 3) insulin (4 μU), 4) insulin plus rapamycin, 5) leucine (3 mmol/L), and 6) leucine plus rapamycin. The wedges were processed for Western blot analysis of phosphorylated S6 kinase (S6 K) and S6 ribosomal protein. Additional samples of liver and skeletal muscle from rats that received systemic infusions of either vehicle or leucine during pancreatic clamps were similarly analyzed for phosphorylated S6 K and S6 ribosomal protein.

α-KIC infusions into the paraventricular nucleus of the hypothalamus. Chronic bilateral cannulae were implanted into the paraventricular nucleus of rats followed by vascular catheterization as previously described. Either vehicle or a 3 mmol/L KIC solution was infused into the paraventricular nucleus over a period of 6 h, and pancreatic clamps were performed.

Third cerebral ventricle infusions of KIC and KIC plus SHU9119 and statistical analyses. A single cannula was implanted into the third cerebral ventricle of rats, and upon recovery the animals received vascular catheters. The animals were randomized into three groups to receive 6-h central infusions of the following: vehicle, 3 mmol/L KIC, or 3 mmol/L KIC plus 3 μg SHU9119. Pancreatic clamp studies were performed as described above. Statistical comparisons were assessed by unpaired Student t test or ANOVA.

RESULTS

Brain leucine and glucose homeostasis. To selectively increase the central availability of leucine, we infused this amino acid into the MBH of conscious rats via bilateral cannulae (Fig. 1B). During the first 4 h of the study, we monitored the effects of the intrahypothalamic infusions on plasma glucose and insulin. The infusion of leucine into the MBH lowered plasma glucose and insulin levels (Fig. 1C and Supplementary Table 1, available in Supplementary Data). To investigate the mechanism by which intrahypothalamic leucine lowered glucose levels despite lower insulin levels, we performed a pancreatic basal insulin clamp study (Fig. 1B). During the clamps, we had to infuse more glucose to prevent hypoglycemia when leucine was infused into the MBH compared with vehicle (Fig. 1D). This central effect of leucine was due to a robust suppression of endogenous glucose production (Fig. 1E and F) accompanied by marked decreases in the in vivo rate of glycogenolysis and gluconeogenesis (Supplementary Fig. 1A and B). Conversely, the levels of glucoregulatory hormones and the rate of glucose utilization were unchanged (Supplementary Table 1 and Supplementary Fig. 1C). No significant change in the levels of plasma leucine was observed throughout the experiments (Supplementary Fig. 1D).

In eukaryotic cells, leucine activates a nutrient-sensing mechanism that controls cellular growth and differentiation (20,21,30) through the mTOR pathway (mTOR complex 1), which is inhibited by rapamycin (Fig. 1A). To test whether the central glucoregulatory effect of leucine requires activation of mTOR, we repeated the central infusion of leucine in rats preinfused and then coinfused with rapamycin into the MBH at a dose sufficient to inhibit the stimulatory effect of leucine or insulin on the S6 kinase pathway (Supplementary Fig. 2). Coinfusion of rapamycin failed to alter the effects of central leucine on plasma glucose (Fig. 1C), glucose infusion rate, glucose production (Fig. 1 D–F), and liver glycogenolysis and gluconeogenesis

(Supplementary Fig. 1A and B), indicating that the glucoregulatory action of leucine was mTOR independent.

Leucine metabolism to KIC and glucose homeostasis. The first step in the oxidative metabolism of leucine is its transamination to KIC catalyzed by BCAT (Fig. 1A) (22,23). Amino-oxyacetic acid is a potent BCAT inhibitor (31). To test whether the transamination of leucine to KIC is required for modulation of glucose homeostasis, we examined the effect of intrahypothalamic leucine in the presence of BCAT inhibition. Coinfusion of BCAT inhibitor into the MBH markedly blunted the effect of leucine on plasma glucose (Fig. 1C) and on hepatic glucose metabolism (Fig. 1D–F). Compared with findings in vehicle controls, the central administration of BCAT inhibitor alone did not significantly modify glucose infusion rate (2.3 ± 0.88 vs. 2.4 ± 0.80 mg/kg/min, respectively), glucose utilization (11 ± 0.5 vs. 11 ± 1.4 mg/kg/min), or the ability of insulin to inhibit endogenous glucose production (23 ± 8 vs. 21 ± 8%). Thus, the metabolism of leucine to KIC within the MBH is required for its inhibitory effect on glucose production.

We reasoned that since interfering with the conversion of leucine to KIC obliterated its central effects, a primary increase in the central availability of KIC might be sufficient to regulate glucose homeostasis. The infusion of KIC into the third ventricle of rats lowered plasma glucose levels (Fig. 1G), and during pancreatic basal insulin clamps glucose had to be infused to prevent hypoglycemia (Fig. 1H). This effect of KIC was due to suppression of hepatic glucose production (Fig. 1I and J) and liver glycogenolysis and gluconeogenesis (Supplementary Fig. 3A and B) rather than to increased glucose utilization (Supplementary Fig. 3C).

For localization of its central glucoregulatory effect, KIC was directly infused bilaterally within the MBH. KIC lowered plasma glucose levels (Fig. 1G) and increased the rate of glucose infusion required to maintain euglycemia during pancreatic clamps (Fig. 1H). The latter was due to a robust suppression of liver glucose production (Fig. 1I and J), while glucose utilization was not significantly affected (Supplementary Fig. 3C). Of note, a similar infusion of KIC within the paraventricular nuclei failed to inhibit glucose production (Supplementary Fig. 4A). Thus, the central effects of leucine on glucose metabolism can be entirely replicated by a primary increase of KIC in the MBH. To examine the specificity of BCAT inhibition in blocking the action of leucine, we coinfused KIC with the BCAT inhibitor in the MBH. BCAT inhibition failed to alter the effects of KIC on liver glucose metabolism (Supplementary Fig. 4B).

KIC metabolism to acetyl-CoA and glucose homeostasis. Leucine-derived KIC is further metabolized to acetyl-CoA by the consecutive action of five enzymes (22,23). The rate-limiting step is the oxidative decarboxylation of KIC, catalyzed by BCKDH. This enzyme is inhibited through phosphorylation by BCKDK (Fig. 2A). We reasoned that if the metabolism of KIC to acetyl-CoA is a key signal generated by the central administration of leucine or KIC, then 1) the direct stimulation of KIC flux through BCKDH should recapitulate while 2) the inhibition of KIC flux through BCKDH should blunt the effects of leucine. BCKDH is activated by α-CIC, a potent and specific inhibitor of BCKDK (32). As expected, the infusion of α-CIC into the MBH lowered circulating glucose levels (Fig. 2B) and, during pancreatic clamps, increased the rate of glucose infusion required to maintain euglycemia (Fig. 2C). This effect was due to suppression of liver glucose production (Fig. 2D and E) and liver glycogenolysis and gluconeogenesis (Supplementary Fig. 5A and B) rather than to increased

glucose utilization (Supplementary Fig. 5C). Next, to selectively inhibit KIC flux through BCKDH, we overexpressed BCKDK in the MBH. We constructed the pAAV-BCKDK plasmid, which drives the expression of BCKDK under the control of the CMV promoter (Fig. 2F). Transfection of this plasmid into HEK 293 cells resulted in efficient expression of functional BCKDK (Fig. 2G and H). An AAV BCKDK vector prepared from this plasmid was first tested in HEK 293 cells. Five days after transduction, the cells overexpressed BCKDK (Fig. 2H). AAV BCKDK vectors were injected bilaterally into the MBH of 10- to 12-week-old rats. Ten days later, the expression of BCKDK was demonstrated by Western blot of MBH extracts (Fig. 2H). Importantly, while overexpression of BCKDK in the MBH did not alter glucose metabolism in animals receiving central vehicle (Table 1), it markedly blunted the effect of central leucine on hepatic glucose metabolism (Fig. 2I–K) without modifying glucose utilization (Supplementary Fig. 5D). Taken together, these results clearly indicate that the metabolism of KIC to acetyl-CoA within the MBH is required for the inhibitory effect of leucine on endogenous glucose production.

KIC metabolism to malonyl-CoA and glucose homeostasis. Acetyl-CoA moieties derived from the metabolism of KIC (Fig. 3A) can be further metabolized to malonyl-CoA through the action of ACC, which is in turn inhibited by AMP-activated protein kinase (AMPK). To test whether the conversion of KIC to malonyl-CoA was required for the effects of intrahypothalamic KIC, we coinfused the AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (Fig. 3A) with KIC. The coinfusion of AICAR blunted the effects of KIC on glucose metabolism and hepatic glucose production (Fig. 3B–D and Supplementary Fig. 6A), supporting the idea that formation of malonyl-CoA in the MBH is necessary for the glucoregulatory action of KIC. AICAR alone did not alter glucose fluxes under similar experimental conditions (Table 1). Malonyl-CoA is converted back to acetyl-CoA through the action of MCD (Fig. 3A); thus, an alternative approach to test the role of malonyl-CoA was to enhance the activity of MCD in the MBH (29). We injected AAV MCD (Fig. 3E) into the MBH of rats, and overexpression of MCD was demonstrated by Western blot analysis (Fig. 3F). Importantly, overexpression of MCD in the MBH blunted the effect of central leucine on glucose homeostasis and on hepatic glucose metabolism (Fig. 3G–I and Supplementary Fig. 6B). No significant effect of MCD overexpression per se was observed (29) (Table 1). These results showed that the metabolism of KIC → acetyl-CoA → malonyl-CoA within the MBH is required for the inhibitory effect of leucine on liver glucose production. To further validate the specificity of these effects, we asked

whether the activation of the central melanocortin pathway was required for the action of leucine. The pharmacological antagonism of central melanocortin receptors by SHU9119 failed to alter the effects of central leucine/KIC on glucose production (Supplementary Fig. 4C). To directly test the hypothesis that leucine/KIC-derived hypothalamic malonyl-CoA leads to an increase in the levels of oleyl-CoA (Fig. 3A), we measured the levels of long-chain acyl-CoAs in the MBH. Animals that received central infusions of leucine or KIC displayed a significant increase in the levels of oleyl-CoA (C18:1), while other long-chain acyl-CoAs (e.g., linoeyl-CoA, C18:2) did not change compared with vehicle-infused animals (Fig. 4A).

Amino acid sensing and ATP-sensitive K^+ channels. The activation of hypothalamic ATP-sensitive K^+ channels (K_{ATP} channels) is critical for modulation of blood glucose levels and liver glucose metabolism (26) and for the metabolic effects of other nutrient-dependent signals (15,24,25). To examine the role of central K_{ATP} channels in leucine sensing, we studied the effects of the central infusion of glibenclamide on the modulation of glucose metabolism by KIC. In the presence of the K_{ATP} channel blocker glibenclamide, the infusion of KIC into the MBH failed to modulate the metabolism and production of glucose (Fig. 4B–D and Supplementary Fig. 6C). Glibenclamide alone did not produce significant changes (Table 1). Furthermore, as shown in Fig. 4E–H central administration of KIC also failed to modulate liver glucose metabolism in mice lacking functional K_{ATP} channels in the brain ($Sur1^{-/-}$ mice) (33). Taken together, these findings indicate that the hypothalamic sensing of leucine regulates liver glucose fluxes through a mechanism that requires functional K_{ATP} channels.

Circulating leucine and glucose homeostasis. To examine the physiological relevance of our observations, we performed systemic infusions of leucine to generate increases in its plasma concentration similar to those observed after consumption of diets enriched in protein as well as more moderate increases (34–36) (Fig. 5A). Increasing doses of systemic leucine produced dose-dependent elevations in plasma leucine (Fig. 5B) accompanied by gradual decreases in plasma isoleucine and valine (Supplementary Fig. 7A and B). The latter is a known effect of leucine (36). In the presence of basal insulin and vehicle in the MBH, systemic leucine markedly inhibited glucose production in a dose-independent manner (Fig. 5C–E) without modifying glucose utilization (Supplementary Fig. 7C). More importantly, the infusion of a BCAT inhibitor into the MBH blunted the effect of systemic leucine (Fig. 5F) on glucose production (Fig. 5G–I) without changes in glucose utilization (Supplementary Fig. 7D). Interestingly, the

TABLE 1

Glucose kinetics parameters during euglycemic pancreatic clamps in rats: recombinant viruses and pharmacological modulators control groups

	GIR	Glucose production	Suppression of glucose production	Glucose utilization (R_d)
Recombinant vector control[a]				
AAV BCKDK	1.7 ± 0.4	10.4 ± 0.5	17.0 ± 2.3	12.1 ± 0.6
AAV MCD	1.1 ± 0.4	10.2 ± 0.5	14.1 ± 1.5	11.1 ± 0.7
Pharmacological controls[b]				
AICAR	1.3 ± 0.7	10.0 ± 0.8	14.9 ± 2.4	11.3 ± 0.4
Glibenclamide	2.1 ± 0.7	9.1 ± 0.5	16.8 ± 0.8	11.0 ± 0.8

All values represent the mean ± SEM of 5–6 experiments and are expressed as milligrams per kilogram per min. Suppression of GP is expressed as percent of the basal value. [a]Vehicle infused into the MBH. [b]Compound infused alone into the MBH.
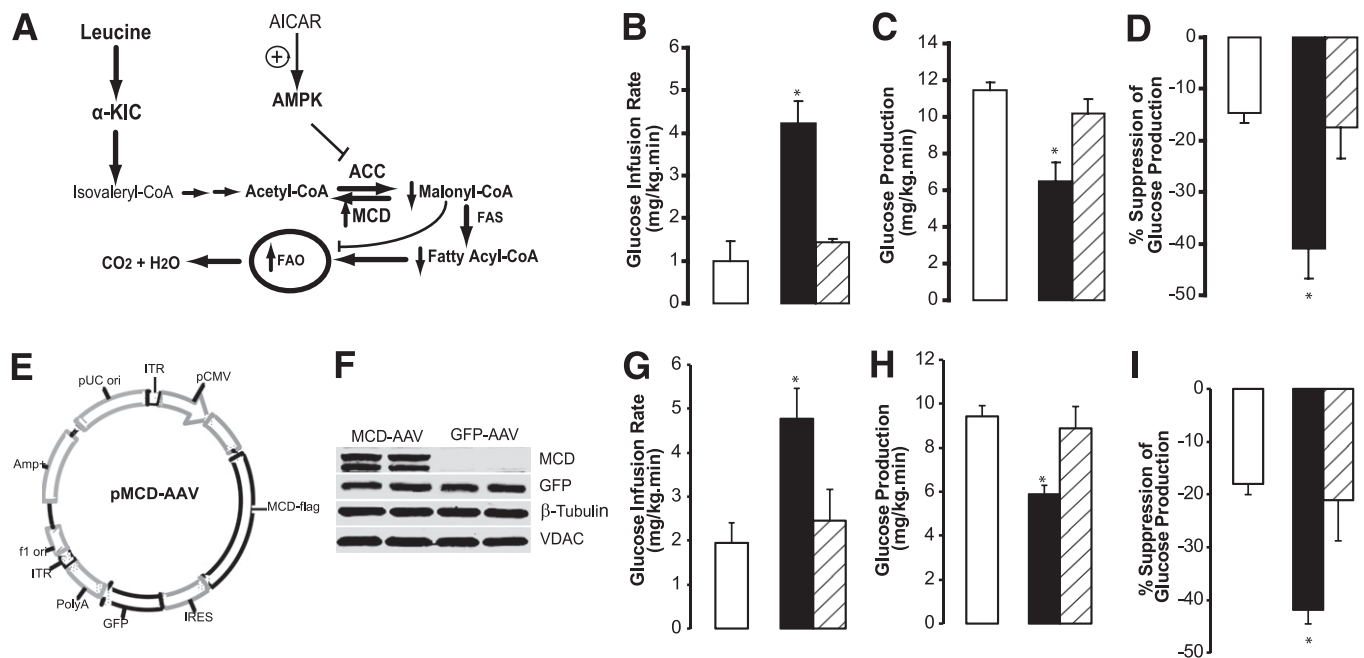
FIG. 3. Hypothalamic metabolism of leucine or KIC to acetyl-CoA and malonyl-CoA is required for the glucoregulatory action of leucine. *A*: Metabolism of leucine. *B–D*: Effect of the central (MBH) infusion of vehicle (□), KIC (■), or KIC plus AICAR (▨) on whole-body glucose metabolism during pancreatic insulin clamps. *E*: pAAV-MCD vector used for the overexpression of MCD in the hypothalamus. ITR, inverted terminal repeat sequence; pUC ori, pUC vector origin of replication; IRES, internal ribosome entry site; Amp, ampicillin resistance gene; ori, origin of replication. *F*: Western blot analysis of hypothalamic wedges from rats injected into the MBH with either control (GFP) (AAV GFP) or AAV MCD. *G–I*: Effect of intrahypothalamic infusion of vehicle (□) or leucine in animals overexpressing a control protein (GFP) (■) in the MBH or of intrahypothalamic leucine in animals overexpressing MCD (▨) in the MBH on whole-body glucose metabolism during pancreatic insulin clamps. All values are means ± SEM. *n* = 4–6. *P < 0.05 vs. vehicle. VDAC, voltage-dependent anion channel; FAS, fatty acid synthase; FAO, fatty acid oxidation.

increase of circulating leucine activated the mTOR pathway in skeletal muscle (Supplementary Fig. 7*E*) and liver (Supplementary Fig. 7*F*). Taken together, these results show that physiological increases of circulating leucine are sufficient to markedly inhibit hepatic glucose production through a mechanism keenly dependent on the metabolism of leucine within the MBH. We next examined the consequences of attenuating the hypothalamic leucine-sensing mechanism on glycemic control. We fed a lard-enriched (10%) diet containing 45% protein (high protein) during 7 days to both control rats and rats with an acquired defect in the metabolism of leucine in the MBH (Fig. 5*J*). This defect was induced by AAV-driven overexpression of BCKDK in the MBH, which impairs leucine sensing (Fig. 2*F–H*). Rats overexpressing BCKDK developed not only higher fasting plasma glucose and insulin but also larger plasma glucose and insulin excursions in response to the meal load compared with control animals (Fig. 5*K*). These results indicate that hypothalamic leucine sensing is required to maintain proper glycemic control.

DISCUSSION

The arcuate nucleus of the hypothalamus has emerged as a critical site for the integration of multiple nutritional cues designed to inform the brain of the nutritional status of the body (37). Here, we have identified a novel circuit coupling the metabolism of leucine in the MBH with the regulation of glucose homeostasis in the liver (Fig. 5*L*). We postulate that in the postabsortive state, the hypothalamic sensing of leucine plays a role in the acute regulation of glucose production in response to elevations of circulating

levels of leucine. The stimulation of this central pathway is sufficient to lower plasma glucose levels through inhibition of liver glucose output through a mechanism keenly dependent on the metabolism of leucine to acetyl-CoA and malonyl-CoA in the MBH. In contrast with recent studies in rats showing that activation of the mTOR pathway is required for the suppressive effect of centrally administered leucine on food intake (6), the effect of leucine on liver glucose production does not require activation of mTOR. These differential mechanisms of leucine sensing potentially allow for selective and independent regulation of food intake and glucose production. The central metabolism of leucine or KIC led to an increase in oleyl-CoA in the MBH, which has been postulated to activate hypothalamic K_{ATP} channels generating the signal(s) that reach the liver via vagal outflow (14,25). In fact, the actions of leucine described here also required functional K_{ATP} channels.

Although systemic infusions of leucine, designed to increase circulating leucine to levels similar to those achieved after the consumption of a protein-rich meal (34,35), were sufficient to inhibit liver glucose output, more limited elevations were also effective, supporting the physiological relevance of central leucine sensing. Furthermore, the glucoregulatory effect of systemic leucine is strictly dependent on its metabolism within the MBH, indicating a central mechanism of action. In other studies, leucine-dependent activation of the mTOR pathways in skeletal muscle has been shown to induce insulin resistance as a result of decreased glucose uptake (38). Of interest, in our studies high circulating leucine inhibited liver glucose production despite activation of the mTOR–S6 K pathway in liver and skeletal muscle. Now, it could be argued that
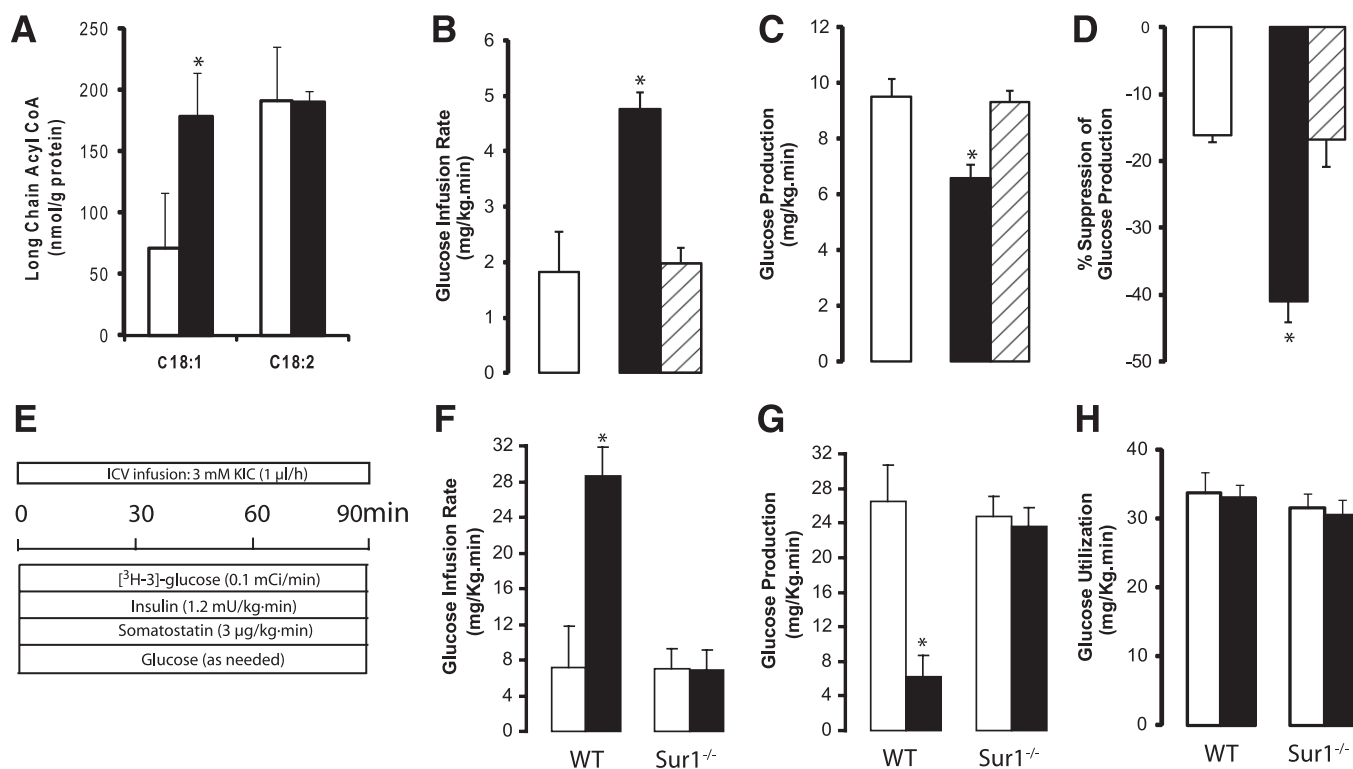
FIG. 4. Central administration of KIC increases hypothalamic oleyl-CoA levels and lowers plasma glucose through a K_{ATP} pathway–dependent inhibition of liver glucose fluxes. *A*: Effect of central administration (MBH) of vehicle (*n* = 6) (□) or KIC (*n* = 6) (■) on hypothalamic long-chain acyl-CoA levels. *B–D*: Effect of central (MBH) vehicle (□), KIC (■), and KIC plus glibenclamide (▨) on whole-body glucose metabolism during pancreatic insulin clamps in rats. *E*: Experimental protocol of central infusions of either KIC or vehicle during pancreatic insulin clamps in mice. *F–H*: Effect of central vehicle (□) or KIC (■) on whole-body glucose metabolism during pancreatic insulin clamps in wild-type (WT) (*n* = 8) and *Sur1*-null (*n* = 13) mice. All values are means ± SEM. *P < 0.05 vs. vehicle.

the decrease in circulating isoleucine and valine associated with hyperleucinemia (36) may have contributed to the glucoregulatory mechanism. However, this possibility is unlikely because *1*) central leucine alone, which does not change the circulating levels of valine or isoleucine, inhibits glucose production to the same extent as systemic leucine and *2*) inhibition of leucine metabolism in the MBH markedly blunted the effect of systemic leucine even in the presence of lower circulating valine and isoleucine. Findings of recent studies in mice unable to metabolize leucine owing to a global deletion of BCAT further support the central leucine-sensing mechanism described here. These BCAT-deficient animals display a lean phenotype with high circulating leucine, enhanced insulin sensitivity, and resistance to diet-induced obesity (39). Interestingly, in these mice only the brain was still capable of metabolizing leucine owing to the presence of an alternative isoform of BCAT unaffected by the targeted disruption. Furthermore, studies of human subjects receiving systemic infusions of leucine have also shown a decrease in glucose production (40,41), although the magnitude of the effect was smaller than in our rodent studies. In contrast to our findings in these human studies, a decrease in glucose utilization was also observed. The reasons for these discrepancies are not immediately clear. Moreover, our findings on the central glucoregulatory effects of leucine are in keeping with recent reports showing that dietary leucine supplementation improved glucose metabolism in both nondiabetic and diabetic mice (42,43). Conceivably, hypothalamic leucine sensing is one of the mechanisms by which high-protein low-fat diets improve insulin action in humans (44–46).

Of interest, recent reports in humans (47,48) linked high levels of circulating leucine to the development of insulin resistance and diabetes. However, it is unclear whether the elevated leucine levels are a cause or a consequence of insulin resistance. Our observation that attenuating the hypothalamic sensing of leucine in rats led to hyperglycemia emphasizes the pathophysiological consequences of the faltering of this mechanism. Finally, it is conceivable that neurons of the arcuate nucleus, which are known to play a key role in the regulation of energy balance and glucose metabolism, could be involved in the leucine-sensing mechanism described here. In this regard, the inability of SHU9119 to block the central glucoregulatory action of leucine as well as recent work from several groups (16,49) on the central regulation of glucose metabolism by insulin suggests that agouti-related peptide neurons could be the sensors. Future studies will be required to delineate the cellular anatomy of hypothalamic leucine sensing.

The maintenance of glucose homeostasis is a vital requirement for mammalian survival and a critical priority for the central nervous system. The gathering of accurate biological information is the basic underpinning of any closed-loop homeostatic system. Consistent with a homeostatic loop, the central effects of leucine described herein appear to oppose its peripheral actions on insulin signaling and on gluconeogenesis (2,38,50). In summary, the novel biochemical mechanism for central leucine sensing reported here may be helpful in the development of nutritional and pharmacological interventions for the treatment of diabetes.
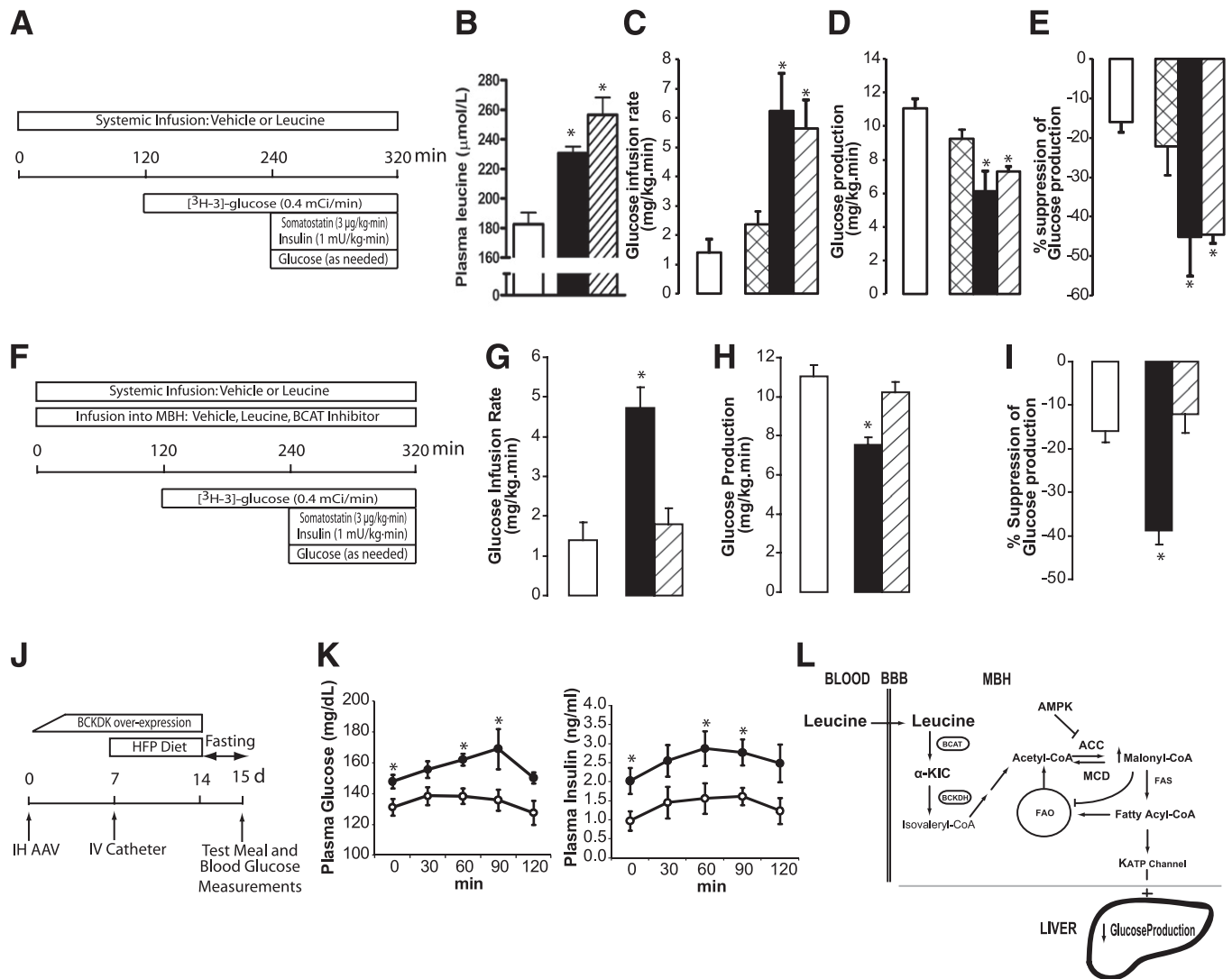
FIG. 5. Central metabolism of leucine is required for the regulation of endogenous glucose production during physiological hyperleucinemia. *A*: Protocol for studies of systemic infusions of leucine. *B*: Plasma leucine: basal (□) or after systemic infusions of leucine at 120 (■) and 240 (▨) μmol/kg/h. *C–E*: Effect of systemic leucine infusion on whole-body glucose metabolism during pancreatic insulin clamps. Rats received vehicle (*n* = 6) (□) or 60 μmol/kg/h (*n* = 5) (▩), 120 μmol/kg/h (*n* = 6) (■), or 240 μmol/kg/h (*n* = 6) (▨) leucine. *F*: Protocol for studies of systemic infusions of leucine during central inhibition of leucine metabolism. *G–I*: Effect of the systemic vehicle (*n* = 6) (□) and leucine (120 μmol/kg/h) in combination with central (MBH) infusion of vehicle (*n* = 6) (■) or BCAT inhibitor (*n* = 6) (▨) on whole-body glucose metabolism during pancreatic insulin clamps. *J*: Protocol for meal challenge test. IH, intrahypothalamic; HFP, high-fat and high-protein diet; IV, intravascular. *K*: Time course of plasma glucose and insulin in rats injected (MBH) with either control AVV (*n* = 7) (○) or BCKDK AAV (*n* = 6) (●). *L*: Proposed mechanism for the regulation of liver glucose production by central leucine sensing. BBB, blood brain barrier; FAO, fatty acid oxidation. All values are means ± SEM. *$P < 0.05$ vs. vehicle except for *K*, where $P < 0.05$ vs. control (GFP) animals.

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