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# Mitochondrial GTP Insensitivity Contributes to Hypoglycemia in Hyperinsulinemia Hyperammonemia by Inhibiting Glucagon Release

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**Mitochondrial GTP (mtGTP)-insensitive mutations in glutamate dehydrogenase (GDH<sup>H454Y</sup>) result in fasting and amino acid-induced hypoglycemia in hyperinsulinemia hyperammonemia (HI/HA). Surprisingly, hypoglycemia may occur in this disorder despite appropriately suppressed insulin. To better understand the islet-specific contribution, transgenic mice expressing the human activating mutation in  $\beta$ -cells (H454Y mice) were characterized in vivo. As in the humans with HI/HA, H454Y mice had fasting hypoglycemia, but plasma insulin concentrations were similar to the controls. Paradoxically, both glucose- and glutamine-stimulated insulin secretion were severely impaired in H454Y mice. Instead, lack of a glucagon response during hypoglycemic clamps identified impaired counterregulation. Moreover, both insulin and glucagon secretion were impaired in perfused islets. Acute pharmacologic inhibition of GDH restored both insulin and glucagon secretion and normalized glucose tolerance in vivo. These studies support the presence of an mtGTP-dependent signal generated via  $\beta$ -cell GDH that inhibits  $\alpha$ -cells. As such, in children with activating GDH mutations of HI/HA, this insulin-independent glucagon suppression may contribute importantly to symptomatic hypoglycemia. The identification of a human mutation causing congenital hypoglucagonemic hypoglycemia**

**highlights a central role of the mtGTP–GDH–glucagon axis in glucose homeostasis.**

Insulin potently lowers glucose, so it is not surprising it has received the lion's share of attention in syndromes of hypoglycemia. Rare human conditions with dramatic phenotypes offer unique clues to less obvious contributing mechanisms and can provide unexpected physiological insights. Hyperinsulinemia hyperammonemia (HI/HA) syndrome is an autosomal-dominant childhood disorder characterized by hypoglycemia following a protein-rich but carbohydrate-poor meal (1–3). While the actual prevalence is unknown (estimated ~1:200,000), these patients have a noteworthy but asymptomatic elevated plasma ammonia level from excessive oxidative deamination of amino acids (4). The condition arises from activating mutations at a key allosteric inhibitory site of the mitochondrial enzyme glutamate dehydrogenase (GDH; E.C. 1.4.1.2) (5). L-leucine is a potent endogenous allosteric activator of GDH, and acute physiologic changes in plasma leucine are primarily influenced by the absorption of dietary protein (6). Mitochondrial GTP (mtGTP) is a metabolic switch that allosterically inhibits GDH to prevent excessive glutamate deamination and preserves nitrogen balance (7). Synthesis

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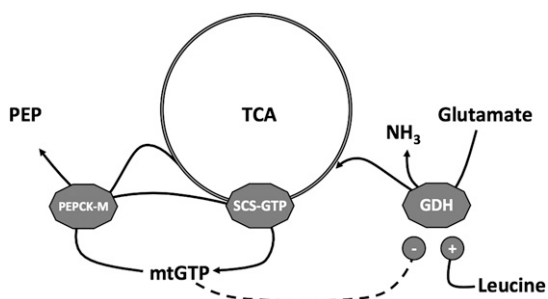
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See accompanying article, p. 4008.

of mtGTP occurs by the tricarboxylic acid (TCA) cycle enzyme succinyl-CoA synthetase (SCS-GTP; E.C. 6.2.1.4), and increased TCA cycle flux will proportionately increase mtGTP to restrict GDH activity. Such a metabolic feedback loop prevents inappropriate amino acid catabolism when SCS flux is sufficient (Fig. 1) (8). mtGTP contributes to glucose-stimulated insulin secretion (GSIS) via the production of phosphoenolpyruvate (PEP) by mitochondrial isoform of PEPCK (PEPCK-M) (9,10). In this sense,  $\beta$ -cell GDH integrates the relative carbohydrate and protein content of a meal by balancing mtGTP inhibition with leucine activation. Recently, this same mechanism of mtGTP and PEPCK-M-dependent synthesis of PEP was also shown to be crucial for endogenous glucose production (EGP), supporting a broad role for this mechanism in the overall maintenance of glucose homeostasis (11).

At least 14 HI/HA-causing mutations in GDH have been identified with loss of the mtGTP inhibition of GDH correlating with phenotypic severity (12–15). The consequent unregulated oxidative deamination of glutamate is the putative cause of hyperinsulinemic hypoglycemia. In vitro data support a model in which elevated plasma leucine concentrations following a protein-rich meal inappropriately floods  $\beta$ -cell mitochondria with  $\alpha$ -ketoglutarate (16). Subsequently, anaplerotic  $\alpha$ -ketoglutarate metabolism then augments both NADH and mtGTP synthesis to generate ATP and mitochondrial PEP to stimulate insulin secretion.

Leucine-induced hyperinsulinemic hypoglycemia is an undisputed and prominent diagnostic feature of HI/HA. Leucine-stimulated hypoglycemia is not limited to HI/HA but can also be observed in other congenital disorders of hypoglycemia as well as in normal control subjects (17–22). The biochemical diagnosis of HI/HA is aided by dramatic hyperinsulinemia following intravenous leucine infusion (2,20). Nevertheless, in the more physiologically relevant scenario of a protein-rich meal, an unexplained



**Figure 1**—mtGTP regulation of anaplerosis and cataplerosis. mtGTP is made from GDP plus inorganic phosphate by the TCA cycle enzyme SCS-GTP during the conversion of succinyl CoA to succinate. GTP is metabolized back to GDP by the action of mitochondrial PEPCK that converts anaplerotic oxaloacetic acid into cataplerotic PEP. mtGTP allosterically inhibits GDH preventing excessive anaplerotic entry of glutamate carbons into the TCA cycle when energy levels are high. Leucine activates GDH to deaminate glutamate to balance mtGTP inhibition.

observation in HI/HA is that hypoglycemia can develop despite the same plasma insulin response as control subjects (23,24).

Another unexplained observation is that fasting hypoglycemia is also frequent in HI/HA and occurs in the absence of elevated plasma leucine concentrations (3). Furthermore, during the hypoglycemia following a controlled fast, plasma insulin concentrations were often appropriately suppressed. These indicate that insulin per se is not the sole driver of hypoglycemia, and another uncharacterized insulin-independent hypoglycemic factor must be involved (3,21,22,24).

Since in addition to  $\beta$ -cells, GDH expression is high in liver, kidney, and brain, it is possible that dysregulated GDH in other tissues contributes to hypoglycemia. To investigate the islet-specific role in hypoglycemia,  $\beta$ -cell-specific mice transgenic for human GDH (hGDH) with the pathogenic mtGTP-insensitive H454Y mutation were generated (16). Initial studies in these humanized mice phenocopy HI/HA with lower fasting plasma glucose concentrations as well as having islets that are hyperresponsive to amino acids. The in vivo characterization of H454Y mice reveals  $\beta$ -cell mtGTP insensitivity impairs the  $\alpha$ -cell independent of insulin. This is the first identification of congenital hypoglycemia associated with impaired  $\alpha$ -cell function that is independent of hyperinsulinemia.

## RESEARCH DESIGN AND METHODS

### Animals and Diets

The generation and initial characterization of the  $\beta$ -cell-specific wild-type hGDH and mutant hGDH mice carrying the HI/HA causing H454Y mutation (hGDH<sup>H454Y</sup>) have been described (16). Sixteen- to 18-week-old male transgenic mice were cohoused with littermates, backcrossed at least seven generations onto C57Bl6 (The Jackson Laboratory, Bar Harbor, ME), and maintained on a regular chow diet (TD2018; Harlan, Teklad, Madison, WI) at the Yale University School of Medicine in accordance with the Institutional Animal Care and Use Committee guidelines.

### In Vivo Studies

Mice were studied following an overnight fast by 3 mIU/kg/min euglycemic hyperinsulinemic clamp, 300 mg/dL hyperglycemic clamp, and 1 g/kg intraperitoneal glucose tolerance tests (IPGTT) by Yale's Mouse Metabolic Phenotyping Center as previously described (25,26). A 90-min primed continuous intravenous infusion of 25  $\mu$ mol/kg/min L-glutamine was used to determine the glutamine-driven insulin secretory response. During the hypoglycemic clamps, mice were initially clamped following a 9-mIU/kg/min insulin infusion at 110 mg/dL by variable glucose infusion for an hour before reducing the glucose infusion rate to clamp at 50 mg/dL. Saline or 10 mg/kg epigallocatechin gallate (EGCG) was delivered by intraperitoneal injection 2 h prior to performing the IPGTT. Plasma glutamine was determined by liquid chromatography-tandem mass spectrometry as previously described (11).

### Islet Studies

Pancreatic islets were isolated by the collagenase method, hand picked, and then perfused on an eight-channel BioRep device (Miami, FL) in Krebs Ringer bicarbonate with 0.2% fatty acid free BSA with the indicated stimuli as previously described (25). Insulin, glucagon, and amylin were measured in the perfusate using LINCplex (Millipore). Whole islets were stained for insulin, glucagon, and somatostatin as previously described (27).

### Statistical Analysis

Data are reported as the mean  $\pm$  SEM. Comparisons between groups were made using unpaired, two-tailed Student *t* tests. For the clamp data, *t* tests and one- and two-way ANOVA were used where appropriate as indicated. A value of  $P < 0.05$  was considered significant. Statistical analyses were performed using GraphPad Prism 6 software.

## RESULTS

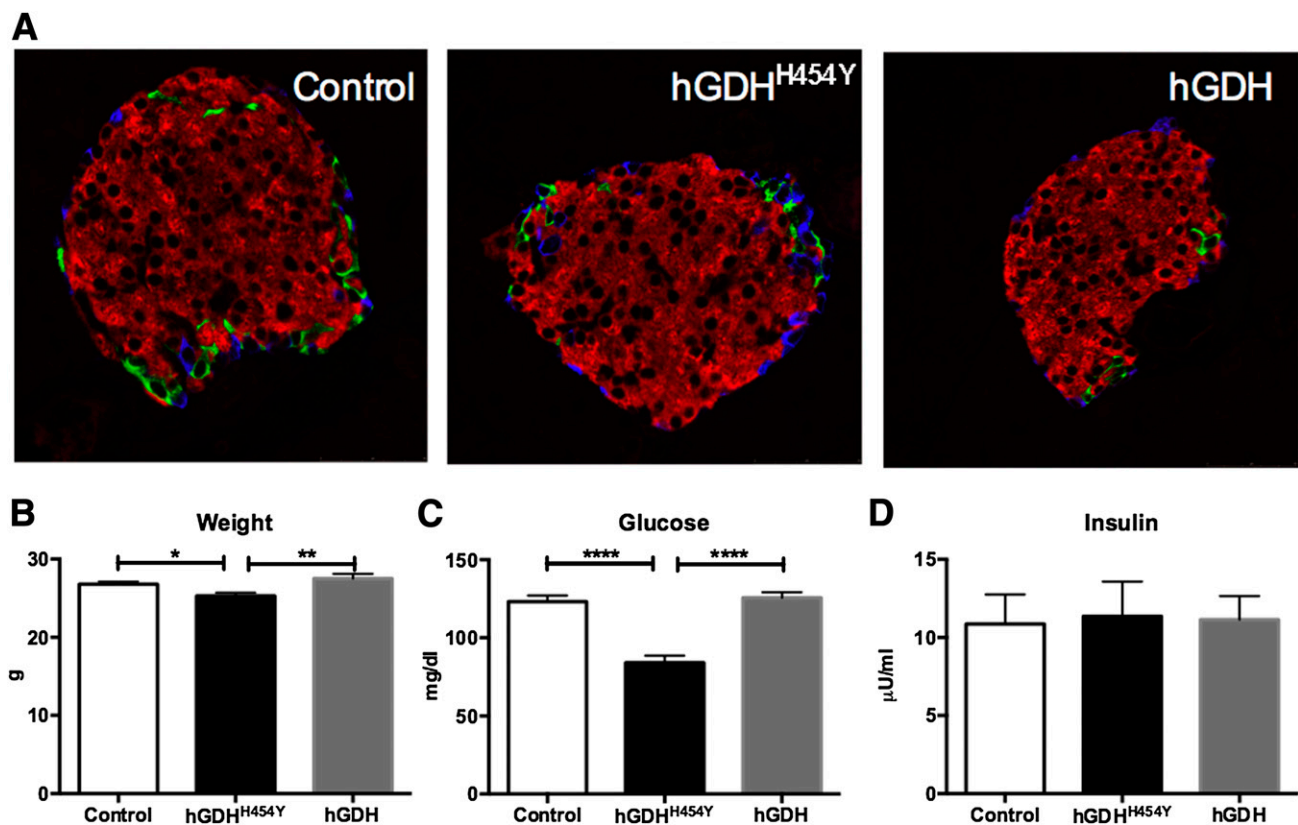
### Hyperglycemic Clamp Studies

In contrast to the severely disrupted islet architecture observed in mice with germline deletion of GDH (28), both hGDH and hGDH<sup>H454Y</sup> transgenic mice had normal  $\alpha$ -,  $\beta$ -, and  $\delta$ -cell distribution (Fig. 2A). The total islet

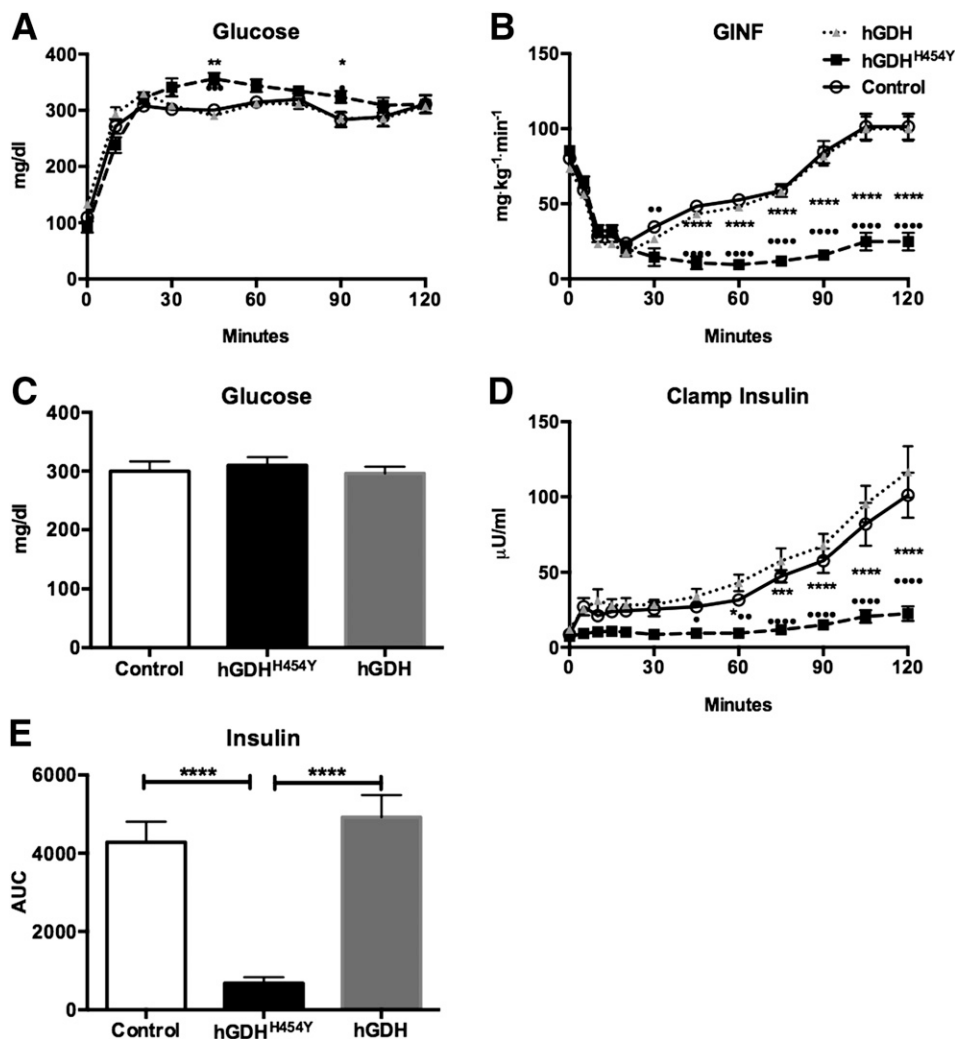
content of insulin, amylin, and glucagon was not different from controls (not shown). Generally, hyperinsulinemia favors weight gain, but the hGDH<sup>H454Y</sup> mice were slightly but significantly lighter than littermate controls (Fig. 2B). Overnight-fasted plasma glucose was 40 mg/dL lower in the hGDH<sup>H454Y</sup> mice (Fig. 2C). Despite differences in glucose, plasma insulin levels were the same (Fig. 2D). While a high insulin to glucose ratio is consistent with hyperinsulinemia, low glucose could also be explained by differences in insulin sensitivity. A hyperglycemic clamp assessed the  $\beta$ -cell-specific effect of the mutant transgene in vivo. No difference in clamped plasma glucose was observed, though glucose infusion ( $G_{INF}$ ) rates were slower with H454Y (Fig. 3A–C). hGDH<sup>H454Y</sup> mice had virtually no insulin secretion in contrast to the  $\sim$ 12-fold increase in controls (Fig. 3D). The area under the curve (AUC) secretion was 85% lower for the H454Y mice (Fig. 3E), indicating a profound defect in the ability to respond to glucose in vivo.

### Glutamine Infusion Studies

$\beta$ -Cell glutaminase deaminates exogenous glutamine to make glutamate, the substrate for GDH. Though glutamine stimulates glucagon release from normal islets, without GDH activation (by agonists such as leucine), it



**Figure 2**—A: Confocal images of pancreatic sections from control, hGDH<sup>H454Y</sup>, and hGDH mice stained for insulin (red), glucagon (green), and somatostatin (blue). B–D: Body weights and plasma glucose and insulin following an overnight fast ( $n = 24/21/20$  mice, respectively). Data are presented as mean  $\pm$  SEM. Significance calculated by ANOVA. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ .



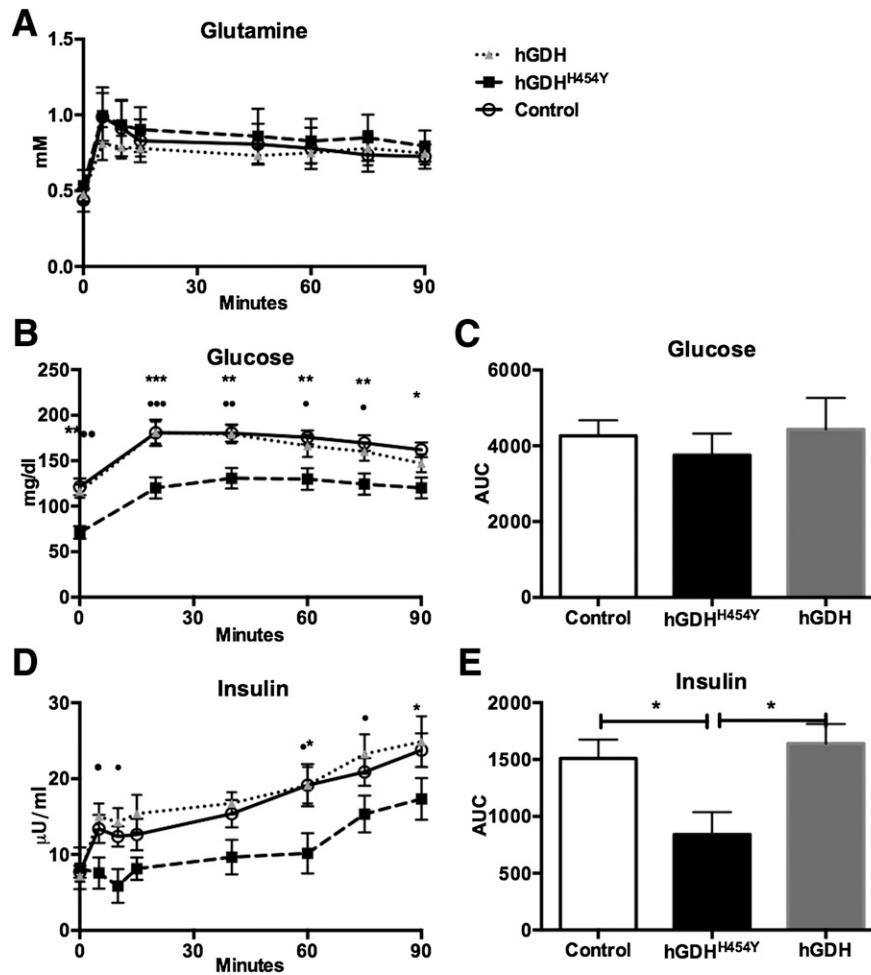
**Figure 3**—Hyperglycemic clamp study. A primed/variable infusion of dextrose clamped plasma glucose at 300 mg/dL in awake, overnight-fasted mice. *A*, *B*, and *D*: Shown for control (open circle), hGDH<sup>H454Y</sup> (black square), and hGDH (gray triangle) mice are plasma glucose, the glucose infusion rate (GINF), and plasma insulin at the indicated times. *C* and *E*: Average plasma glucose values during the clamp (90–120 min) and AUC insulin for the duration of the clamp are shown. Data are presented as mean  $\pm$  SEM ( $n = 9/9/9$  mice). Significance calculated by ANOVA vs. control (\*) or vs. hGDH (•). \*, • $P < 0.05$ ; \*\*, •• $P < 0.01$ ; \*\*\*, ••• $P < 0.001$ ; \*\*\*\*, •••• $P < 0.0001$ .

does not stimulate insulin secretion (29). Previously, glutamine-stimulated insulin secretion of hGDH<sup>H454Y</sup> islets occurred at concentrations 5–10-fold higher than found in vivo (16,30). Therefore, a primed-continuous infusion of glutamine was performed to determine whether a substrate-driven increase in  $\beta$ -cell GDH activity itself was sufficient to provoke hyperinsulinemic hypoglycemia. Overnight-fasted plasma glutamine concentrations were the same and doubled during the glutamine infusion in all mice (Fig. 4A). Glutamine infusion also increased plasma glucose levels  $\sim 60$  mg/dL from baseline in all three groups (Fig. 4B and C). Control and hGDH mice both exhibited a strong insulin response to the infusion, while hGDH<sup>H454Y</sup> mice lacked early insulin secretion and had a severely blunted late phase (Fig. 4D). The total AUC insulin secretion was 45% lower than controls (Fig. 4E). While glucose levels were lower in the GDH<sup>H454Y</sup> mice and

may have impacted insulin secretion, the mice still displayed no glutamine-stimulated secretion even though glucose levels had moved into the normal range. Thus, hGDH<sup>H454Y</sup> was sufficiently inhibited during a fast to prevent glutamine stimulation and not able to cause hyperinsulinemic hypoglycemia by itself.

#### Euglycemic Hyperinsulinemic Clamp

During the hyperglycemic clamps shown above,  $G_{\text{INF}}$  was considerably lower for the H454Y mice (Fig. 3B). Possible explanations included diminished insulin secretion and/or increased insulin resistance. Therefore, basal and euglycemic hyperinsulinemic glucose turnover measurements were made (Fig. 5). Basal EGP was 30% lower than littermate controls, consistent with enhanced hepatic insulin sensitivity (Fig. 5A). Clamped glucose was not different during the euglycemic hyperinsulinemic clamp (Fig. 5B). The  $G_{\text{INF}}$



**Figure 4**—Glutamine infusion study. A primed/continuous infusion of L-glutamine was performed in awake, overnight-fasted control (open circle), hGDH<sup>H454Y</sup> (black square), and hGDH (gray triangle) mice. A, B, and D: Plasma glutamine, glucose, and insulin are shown at the indicated times. The AUC plasma glucose above baseline (C) and the total AUC insulin (E) are shown as indicated for the duration of the study. Data are presented as mean  $\pm$  SEM ( $n = 8/7/8$  mice). Significance calculated by ANOVA vs. control (\*) or vs. hGDH (●). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

and  $R_d$  were 30% higher in the transgenic mice, confirming increased insulin sensitivity (Fig. 5C–E). Both groups had similar drops in EGP during the clamp, although it was completely suppressed in the hGDH<sup>H454Y</sup> mice (Fig. 5F). Thus, the H454Y mice unexpectedly had increased insulin sensitivity compared with littermate controls, suggesting that hGDH<sup>H454Y</sup> exerts significant influence outside of the  $\beta$ -cell.

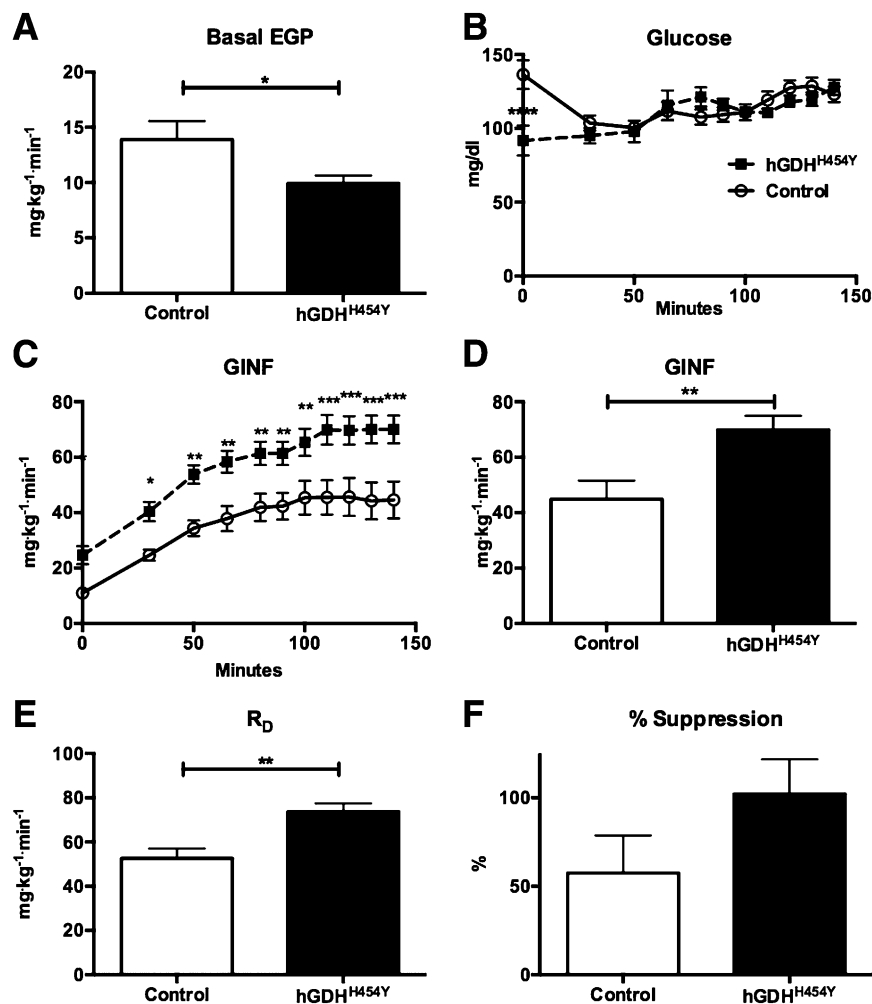
#### Hypoglycemic Clamp Studies

Dysfunctional  $\alpha$ -cell regulation could potentially explain the reduced EGP in the setting of decreased  $\beta$ -cell function. Mice were clamped at euglycemia for 1 h, this time with higher-dose insulin (9 mIU/kg/min), to ensure that islets from both groups of mice experienced similar plasma glucose and insulin concentrations before acutely decreasing the glucose infusion rate to promote hypoglycemia (Fig. 6A). There was no difference in  $G_{INF}$  at either phase of the clamp (Fig. 6B). Peripheral basal glucagon was inappropriately low for hGDH<sup>H454Y</sup> and did not change during the euglycemic phase of the clamp (Fig.

6A and C). Clamped glucose and insulin were the same during hypoglycemia (Fig. 6D and E), but hGDH<sup>H454Y</sup> plasma glucagon was only 40% of control mice (Fig. 6F). Thus, independent of circulating and, by inference, intraislet insulin, a deficient  $\alpha$ -cell response identifies impaired counterregulation contributing to hypoglycemia.

#### Isolated Islet Studies

Glucose-sensing hypothalamic neurons could contribute to the in vivo phenotype since the hGDH<sup>H454Y</sup> transgene is under the control of the RIP promoter. Islets were perfused at basal glucose (3 mmol/L) followed by hyperglycemia (12 mmol/L) for 15 min before a drop to hypoglycemia (1 mmol/L). After recovery (3 mmol/L), an  $\alpha$ -cell stimulus (100  $\mu$ mol/L glutamate) was followed by KCl depolarization (31). GSIS was deficient in the hGDH<sup>H454Y</sup> mice (Fig. 7A). Interestingly, glucagon secretion in response to hypoglycemia, glutamate, and KCl were all inhibited in the hGDH<sup>H454Y</sup> mice (Fig. 7B). Similar to insulin, amylin release was diminished (Fig. 7C). The green



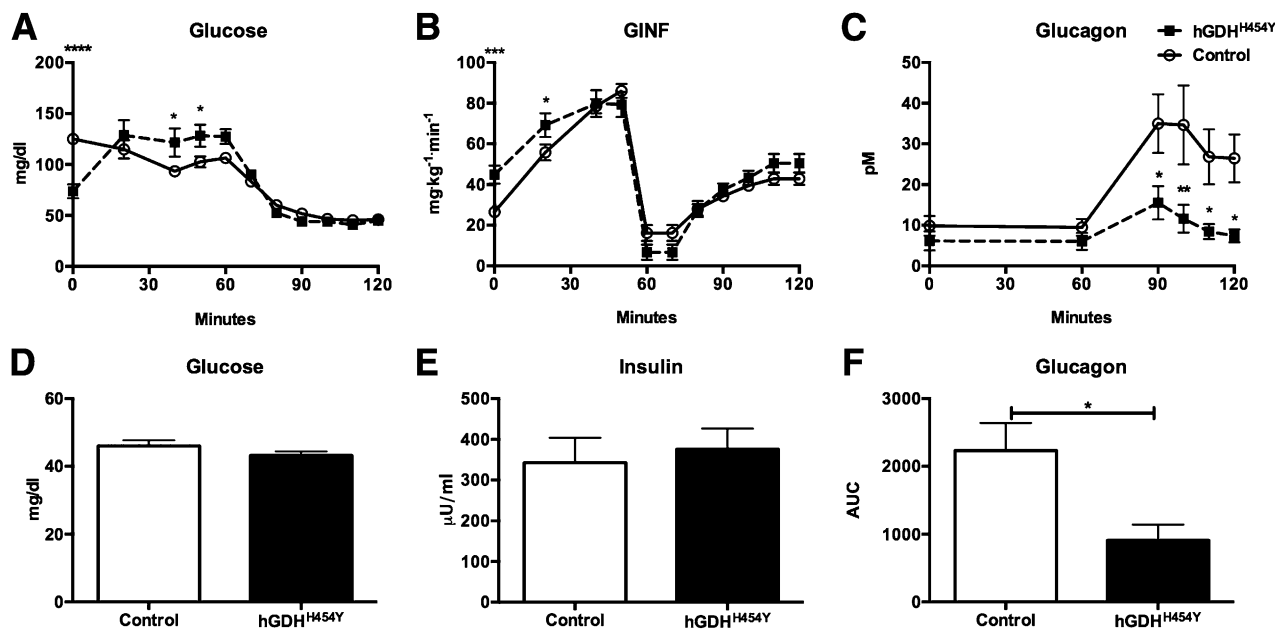
**Figure 5**—Euglycemic hyperinsulinemic clamp study. A primed continuous infusion of insulin with a variable glucose infusion was performed to clamp plasma glucose at 120 mg/dL in awake, overnight-fasted control (open circle) and hGDH<sup>H454Y</sup> (black square) mice. **A**: Basal EGP prior to insulin infusion. **B** and **C**: Plasma glucose and glucose infusion rate (GINF) during the study are shown at the indicated times. **D–F**: Clamp GINF, rate of glucose disposal ( $R_D$ ), and percent suppression of EGP were determined from the clamp. Data are presented as mean  $\pm$  SEM ( $n = 9/8$  mice). Significance calculated by Student  $t$  test. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

tea polyphenol, EGCG, inhibits GDH at 10  $\mu$ mol/L concentrations (30). Acute EGCG normalized glucose- and KCl-stimulated insulin secretion compared with control islets (Fig. 7D–F). Similarly, EGCG restored hypoglycemia-, glutamate-, and KCl-stimulated glucagon release. First-phase insulin secretion and total glucagon secretion (hypoglycemic plus glutamate stimulated) were restored by acute treatment with EGCG (Fig. 7G and H). Notably, since perfused intraislet insulin is estimated to be lower and since EGCG recovered both glucagon and insulin secretion, then  $\alpha$ -cell inhibition is independent of insulin action. Thus, an unidentified GDH-derived metabolic signal in  $\beta$ -cell mitochondria inhibits glucagon secretion.

#### Islet Function Is Improved In Vivo Following EGCG Administration

Chronic EGCG in vivo blunts the glucose drop following a 1.5-g/kg amino acid stimulus or a 24-h fast in

hGDH<sup>H454Y</sup> mice through an undetermined mechanism (30). Because the suppression of both insulin and glucagon secretion was acutely reversible in the isolated islets (Fig. 7), the mechanism was investigated further in vivo. In this cohort of untreated hGDH<sup>H454Y</sup> mice, both basal glucose and insulin were significantly lower, again arguing against hyperinsulinemia alone causing the hypoglycemia (Fig. 8A and B). Following treatment with 10 mg/kg EGCG, the difference between control and hGDH<sup>H454Y</sup> glucose disappeared, but insulin was not lowered in the hGDH<sup>H454Y</sup> mice. Despite significant basal hypoglycemia and hypoinsulinemia, circulating glucagon was not elevated (Fig. 8C). The untreated hGDH<sup>H454Y</sup> mice had greater glucose clearance despite lower insulin during an IPGTT than the controls (Fig. 8D and E). EGCG normalized glucose tolerance without changing insulin secretion (Fig. 8F and G). Contrary to the proposed mechanism (30), EGCG paradoxically normalized glucose tolerance



**Figure 6**—Hypoglycemic clamp study. A primed continuous infusion of insulin (9 mU/kg/min) with variable glucose infusion to clamp glucose at 100 mg/dL was performed for 1 h prior to acutely lowering glucose to clamp at 50 mg/dL in awake, overnight-fasted control (open circle) and hGDH<sup>H454Y</sup> (black square) mice. A–C: Plasma glucose, glucose infusion rate (GINF), and plasma glucagon are presented for the indicated times. D–F: Clamp glucose, clamp insulin, and the AUC glucagon are shown. Data are presented as mean  $\pm$  SEM ( $n = 10/8$  mice). Significance calculated by Student *t* test. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ .

in hGDH<sup>H454Y</sup> mice through a mechanism that does not lower basal or stimulated insulin secretion.

## DISCUSSION

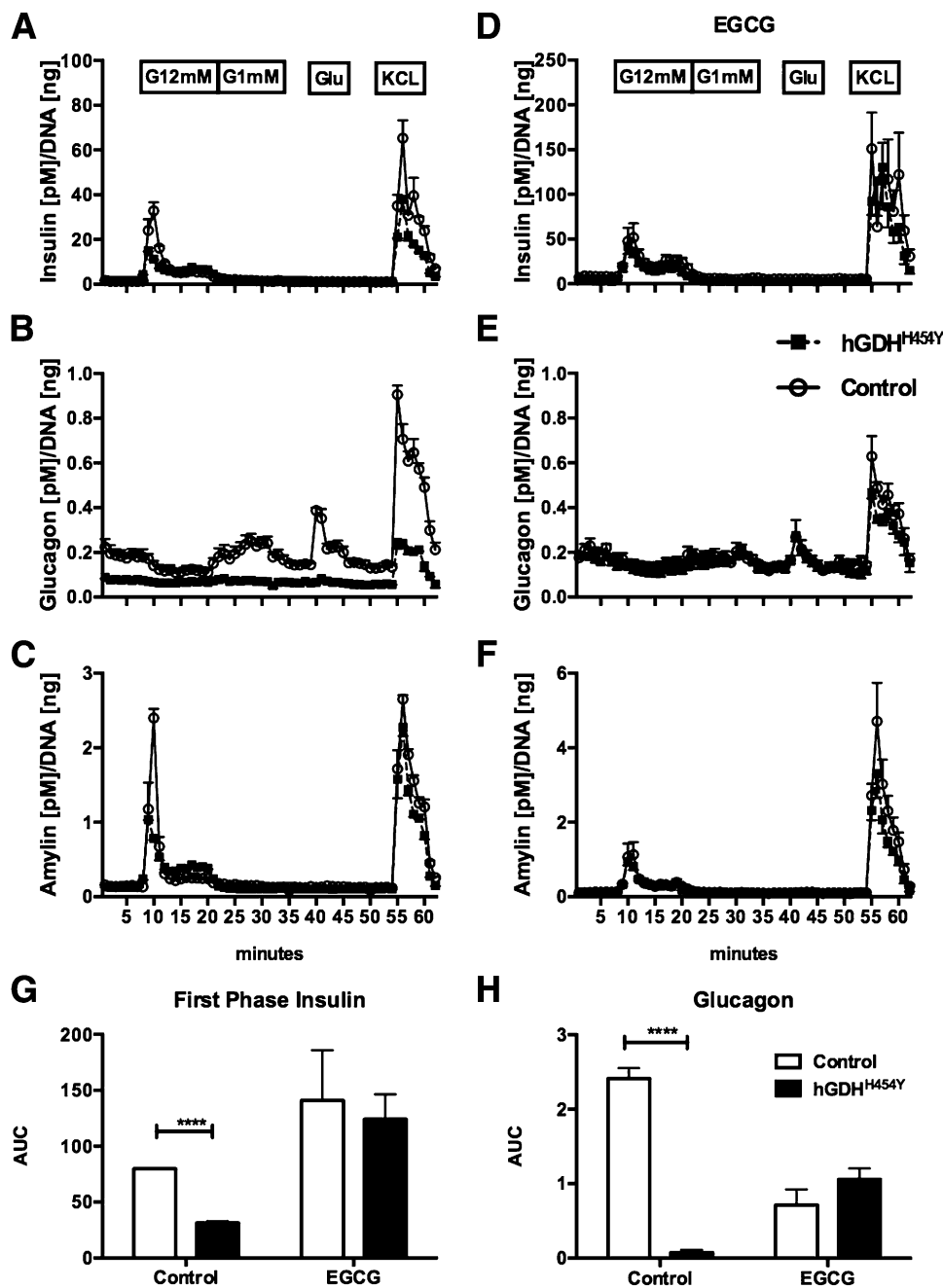
The humanized  $\beta$ -cell-specific GDH<sup>H454Y</sup> mice exhibit the hypoglycemic features of children with HI/HA mutations expressed globally. Like the humans, the mice have fasting hypoglycemia without marked hyperinsulinemia, implicating another element lowering plasma glucose concentrations. Also lacking is either exaggerated hyperinsulinemia or overresponsiveness of pancreatic  $\beta$ -cells to either glucose or glutamine in vivo. Finally, GDH inhibition actually increases GSIS. Clearly, hyperinsulinemia is pathogenic in other focal and diffuse forms of congenital hypoglycemia (23) in which the insulin increases peripheral glucose uptake and suppresses EGP. In contrast, hypoglucagonemia in mice with activating GDH mutations cannot be attributed to elevated insulin. In this study, hypoglycemia is accompanied by reduced EGP and inadequate plasma glucagon concentrations. While these data do not rule out contributory hyperinsulinemia, they do identify an insulin-independent metabolic signal controlled by  $\beta$ -cell GDH that inhibits  $\alpha$ -cells. This is the first report of congenital hypoglycemia where glucagon deficiency is a primary feature (i.e., not just from insulin-mediated suppression of glucagon). Thus, the mtGTP-dependent regulation of GDH is important for both normal insulin and glucagon release.

Defining features of HI/HA include both fasting and protein-induced hypoglycemia (5). Previously, hypoglycemia in HI/HA was explained solely by increased insulin

secretion. Accordingly, unrestrained oxidative flux of glutamate into the TCA cycle in the absence of mtGTP inhibition would provoke insulin secretion via secondary messengers like ATP and PEP (8). Increased GDH activity is the most consistent finding among patients with HI/HA (5,12,13). A more satisfying pathophysiologic explanation of HI/HA comes from considering the mtGTP–GDH–glucagon axis to clinical features of HI/HA such as: 1) fasting hypoglycemia; 2) a hypersensitivity of  $\beta$ -cells to intravenous, enteric, or meal-derived leucine; 3) improvement with diazoxide; and 4) exaggerated glycemic responses to glucagon.

## GDH<sup>H454Y</sup> and Fasting Hypoglycemia

Given the association of hypoglycemia with meal-derived leucine, perhaps it is a little surprising that almost invariably HI/HA patients experience fasting hypoglycemia. Like the mice, humans have lower fasting plasma glucose concentrations without elevated insulin (22). Interestingly, five of the six HI/HA subjects who developed hypoglycemia (<50 mg/dL) during a controlled fast had appropriate (<2  $\mu$ U/mL) plasma insulin levels (21). Similarly, a patient who experienced hypoglycemia during a monitored fast also had suppressed plasma insulin concentrations (24). The fasted humanized hGDH<sup>H454Y</sup> mice had lower plasma glucose concentrations (Figs. 2C, 3A, 4B, 5B, 6A, and 8A), but plasma insulin levels that were either the same as or significantly lower than controls (Figs. 2D, 3D, 4D and E, 6E, and 8B). Notably, these mice also display severely impaired glucose- and glutamine-stimulated insulin secretion in isolated islets (Fig. 7A) as well as in vivo (Figs. 3D and E and 4D and E).



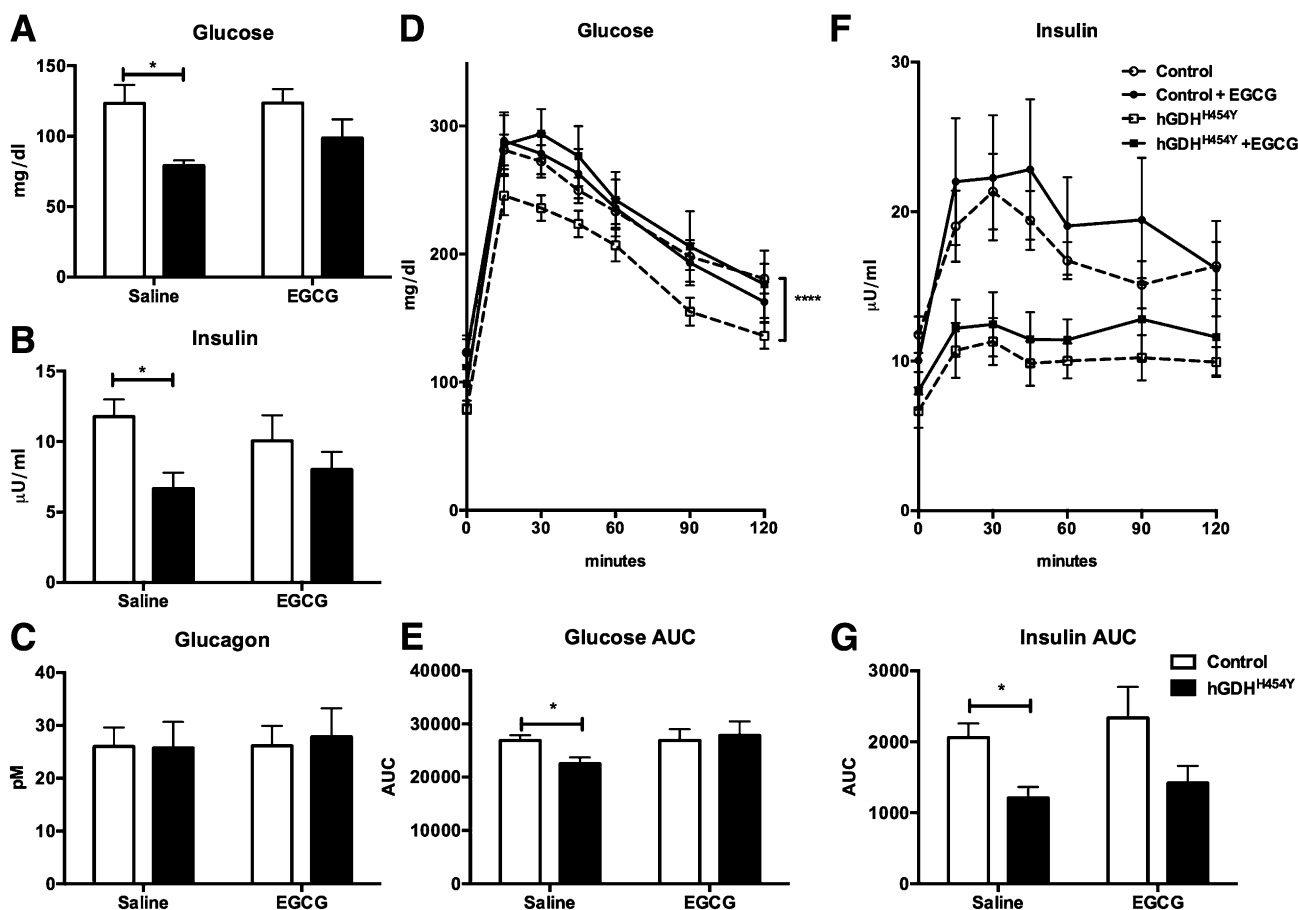
**Figure 7**—Islet perfusion. Islets from control (open circle) and hGDH<sup>H454Y</sup> (black square) mice were perfused in parallel for the indicated conditions (G12, 12 mmol/L glucose; G1, 1 mmol/L glucose; Glu, 100 μmol/L glutamate; and KCL, 30 mmol/L potassium chloride). Perfusate glucose levels were 3 mmol/L unless otherwise indicated. Insulin (A, D), glucagon (B, E), and amylin (C, F) are shown in the absence (A–C) and presence (D–F) of 10 μmol/L EGCG (added at time –60 min). The AUC for first-phase insulin (5–15 min) (G) and glucagon (20–45 min) (H) are shown. Data are presented as mean ± SEM (n = 3/3). Significance calculated by Student *t* test. \*\*\*\**P* < 0.0001.

Thus, it is difficult to explain the hypoglycemia by hyperinsulinemia alone.

An important difference between the mice and humans with HI/HA is the apparently normal GSIS in the latter potentially explained by differences in β-cell mass by effects from other tissues expressing the mutant GDH allele and/or by the presence of elevated ammonia

influencing the direction of GDH metabolism (20). To date, EGP or glucose disposal rates have not been measured in humans with HI/HA. Rather unexpectedly, the β-cell-specific hGDH<sup>H454Y</sup> mice have increased insulin sensitivity. Having lower plasma insulin concentrations concomitant with lower EGP further supports increased hepatic insulin sensitivity from the β-cell-specific





**Figure 8**—In vivo response to GDH inhibition. A–C: Plasma glucose, insulin, and glucagon were measured from control (white bars) or hGDH<sup>H454Y</sup> (black bars) mice 120 min following intraperitoneal injection of either saline ( $n = 8/8$ ) or 10 mg/kg EGCG ( $n = 5/7$ ). D and F: An IPGTT (1 g/kg IPGTT) was performed 2 h following saline (empty) or EGCG (solid) treatment for control (circles) or hGDH<sup>H454Y</sup> (squares) mice. E and G: The AUC from the IPGTT are shown for the indicated groups. Data are presented as mean  $\pm$  SEM. Significance calculated by Student  $t$  test. \* $P < 0.05$ ; \*\*\*\* $P < 0.0001$ .

mutation. While there is no doubt that hyperinsulinemia relative to plasma glucose concentration contributes to HI/HA, nevertheless, it is difficult to reconcile hypoglycemia purely in terms of insulin.

#### GDH<sup>H454Y</sup> and Leucine Hypersensitivity

Enhanced  $\beta$ -cell leucine sensitivity seemingly argues for a primary role of hyperinsulinemia in the hypoglycemia of HI/HA. Leucine activates islet GDH to promote insulin secretion in the presence of glutamine, the precursor for glutamate. Deaminated glutamate then supplies the mitochondria with anaplerotic  $\alpha$ -ketoglutarate. HI/HA causing mutations all have in common a higher  $IC_{50}$  for mtGTP without a change in the  $SC_{50}$  of leucine (13). Leucine-stimulated insulin secretion is highest in the complete absence of glucose (when mtGTP is low) but abrogated in the presence of glucose (when mtGTP is high) (29,32). In fact, insulin secretion is impaired by leucine in normal islets during hyperglycemia (29). Under conditions of hypoglycemia, a slower TCA cycle lowers mtGTP production relaxing GDH inhibition and

promotes glutamate oxidation (Fig. 1). It is counterintuitive, then, why any further loss of mtGTP-inhibition for GDH<sup>H454Y</sup> (during a fast when mtGTP is already low and not inhibitory) would enhance insulin or suppress glucagon release.

This paradox may be explained, in part, since mtGTP concentration is a function of its production and consumption rates.  $\beta$ -Cells, like liver and proximal tubules, have high mtGTP production rates and so even during hypoglycemia TCA cycle flux may make sufficient mtGTP to inhibit GDH (11). Consumption of mtGTP is dependent on PEPCK-M, and, in the absence of activated pyruvate carboxylase, there may be limited oxaloacetate to consume mtGTP (9) (Fig. 1). Consistent with this, normal islets must first undergo a rundown of mtGTP by perfusion for  $\sim 45$  min with no glucose before glutamine itself stimulates insulin secretion (16,32). As such, mtGTP in the  $\beta$ -cell might be expected to inhibit GDH under all conditions compatible with life. Therefore, an activator (such as prandial leucine) is required to overcome this inhibition. Isolated islets from hGDH<sup>H454Y</sup> mice do

exhibit increased sensitivity to leucine and glutamine. However, leucine only stimulates insulin secretion from the perfused H454Y islets at concentrations one to two orders of magnitude higher than found in vivo, and this secretion is abrogated by glucose (16).

From a diagnostic standpoint, subjects with HI/HA are exquisitely sensitive to intravenous leucine. Nevertheless, fasting plasma leucine is actually lower in HI/HA and reduces the likelihood of leucine-stimulated insulin secretion during episodes of fasting hypoglycemia (2,3,14,33). The doses of leucine used during provocative tests in humans (and with isolated islets) are particularly high and at the lowest doses increase plasma leucine ~10-fold (1–3,20,22,34,35). In comparison, plasma leucine levels are only doubled by a symptom-provoking meal of a hamburger and milkshake (36). Therefore, these provocations, while capable of inducing hyperinsulinemia, may not be indicative of the mechanism of ambulatory hypoglycemia.

While a protein-meal tolerance test may lack the diagnostic sensitivity of the intravenous tests, it arguably is more relevant to the clinical phenotype. Following a protein meal, HI/HA subjects had lower glucose compared with controls but surprisingly no difference in the insulin response (21). Similar results were obtained in a careful study of meal-induced hyperinsulinemia (24). Thus, while there is clearly increased  $\beta$ -cell sensitivity to leucine, the sole linkage of hypoglycemia to increased insulin under physiologic conditions provoking hypoglycemia is more tenuous especially during a fast.

It is interesting that while other amino acids, such as arginine, stimulate insulin secretion, they do not usually cause hypoglycemia (37). Leucine itself has pleotropic effects in vivo that extends beyond the  $\beta$ -cell and has previously been demonstrated to inhibit EGP (17,38–40). In fasted normal humans, L-leucine infusion doubled plasma leucine levels and also reduced blood glucose by ~10 mg/dL without a change in peripheral insulin or glucagon (41). Similarly, there is a direct inhibition of gluconeogenesis by leucine in isolated rodent livers (42). In contrast to the hepatic effect, at physiologic levels (0.2 mmol/L) leucine stimulates glucagon release during basal glucose but not hyperglycemic conditions (29). The opposing roles of leucine on  $\alpha$ -cells (activation) compared with liver (inhibition) can perhaps be better understood when interpreted in relation to the ambient glucose concentration. Amino acid uptake by tissues is insulin dependent; consequently, protein-rich, carbohydrate-poor meals stimulate glucagon in order to counterbalance the insulin needed to dispose amino acids and prevent hypoglycemia. During a combined protein- and carbohydrate-rich meal, the elevated glucose overrides leucine-stimulated glucagon release. In this case, leucine can also directly reduce EGP. With  $\alpha$ -cells inhibited in H454Y mice (and potentially in humans with HI/HA), the normal protein-meal stimulation of glucagon may be absent predisposing to hypoglycemia. Thus, unopposed leucine-stimulated

insulin release likely provides an additional susceptibility to hypoglycemia.

### GDH<sup>H454Y</sup> and K<sub>ATP</sub> Channels

A final argument supporting insulin-induced hypoglycemia with HI/HA is responsiveness to treatment with the K<sub>ATP</sub> channel opener diazoxide. Diazoxide can be an effective treatment for hypoglycemia in HI/HA and could argue for an insulin-only pathogenesis. Indeed, a rise in glucose correlates with insulin lowering in a case of protein-induced hypoglycemia treated with diazoxide (24). The most straightforward interpretation is that hyperpolarization of the  $\beta$ -cell protects from hypoglycemia by reducing insulin secretion. Similar to leucine, diazoxide is promiscuous. K<sub>ATP</sub> channels are found in  $\alpha$ -cells and diazoxide directly stimulates glucagon secretion independent of its effect on insulin (43). Thus, it is possible that a combination of lowering insulin and raising glucagon might explain the beneficial effects of diazoxide therapy.

To date, there are no reports of measured glucagon in patients with HI/HA. Patients with HI/HA like other forms of congenital hypoglycemia have an exaggerated response to glucagon therapy. Other forms of congenital hyperinsulinemic hypoglycemia are known to have relative hypoglucagonemia as well. This has largely been explained by elevated intraislet insulin levels suppressing  $\alpha$ -cell function and is present in patients with focal as well as diffuse disease alike (23). hGDH<sup>H454Y</sup> mice have a blunted response to hypoglycemia in vivo and in vitro as well as impaired glutamate and KCl-induced glucagon that is reversible following acute treatment with an inhibitor of GDH. Interestingly, insulin levels were identical during the hypoglycemic clamps and insulin secretion increased with EGCG treatment strongly arguing against intraislet insulin suppressing glucagon. An important remaining question is how does  $\beta$ -cell GDH regulate  $\alpha$ -cells if not by insulin. Glutamate and several of its metabolites (glutamine, GABA, and, most recently,  $\gamma$ -hydroxybutyrate) modulate glucagon release and suggest that increased catabolism of activators or generation of inhibitors in the absence of mtGTP regulation could be involved (31,44–46). Similarly, increasing basal metabolism by unregulated glutamate metabolism could generate reactive oxygen species or other inhibitory signals (47). Identifying the relevant second messenger(s) will be important for future studies. Taken together, GDH-mediated glucagon deficiency has emerged as a potential contributor to hypoglycemia in HI/HA especially during a fast.

Congenital syndromes of hypoglycemia are associated with key nutrient-sensing mechanisms crucial for GSIS from pancreatic  $\beta$ -cells. In this study, we describe the first congenital form of hypoglucagonemic hypoglycemia associated with the disruption of an important energy-sensing feedback loop. In HI/HA, loss of mtGTP inhibition of GDH leads to hypoglycemia and hyperammonemia. This newly identified association of mtGTP with glucagon, taken together with its roles regulating insulin secretion

and gluconeogenesis, place mtGTP as a metabolic energy sensor central to glucose homeostasis (9–11). The relative contributions of hyperinsulinemia and hypoglycagonemia to hypoglycemia may vary depending on the metabolic condition, but glucagon may have a more predominant role during fasting. These data highlight the importance of the mtGTP–GDH–glucagon axis to glucose homeostasis. By identifying this pathway for  $\alpha$ -cell regulation, this opens up new avenues to control plasma glucose. Whether inhibition of GDH can protect against hypoglycemia or alternatively preventing mtGTP inhibition of GDH to treat hyperglycemia are worth further consideration.

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