

Glutaminolysis and Insulin Secretion

From Bedside to Bench and Back

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Identification of regulatory mutations of glutamate dehydrogenase (GDH) in a form of congenital hyperinsulinism (GDH-HI) is providing a model for basal insulin secretion (IS) and amino acid (AA)-stimulated insulin secretion (AASIS) in which glutaminolysis plays a key role. Leucine and ADP are activators and GTP is an inhibitor of GDH. GDH-HI mutations impair GDH sensitivity to GTP inhibition, leading to fasting hypoglycemia, leucine hypersensitivity, and protein-induced hypoglycemia, indicating the importance of GDH in basal secretion and AASIS. The proposed model for glutaminolysis in IS is based on GDH providing NADH and α -ketoglutarate (α -KG) to the Krebs cycle, hence increasing the β -cell ATP-to-ADP ratio to effect insulin release. The process operates with 1) sufficient lowering of β -cell phosphate potential (i.e., fasting) and when 2) AAs provide leucine for allosteric activation and glutamate from transaminations. To test this hypothesis, IS studies were performed in rat and GDH-HI mouse models. In the rat study, rat islets were isolated, cultured, and then perfused in Krebs-Ringer bicarbonate buffer with 2 mmol/l glutamine using 10 mmol/l 2-aminobicyclo[2,2,1]-heptane-2-carboxylic acid (BCH) or a BCH ramp after 50 or 120 min of glucose deprivation. In the GDH-HI mouse study, the H454Y GDH-HI mutation driven by the rat insulin promoter was created for H454Y β -cell-specific expression. Cultured, isolated islets were perfused in leucine 0–10 mmol/l with 2 mmol/l glutamine 0–25 mmol/l, AA 0–10 mmol/l, or glucose 0–25 mmol/l. Rat islets displayed enhanced BCH-stimulated IS after 120 min of glucose deprivation, but not when energized by fuel. H454Y and control islets had similar glucose-stimulated IS, but H454Y mice had lower random blood glucose. Leucine-stimulated IS and AASIS occurred at lower thresholds and were greater in H454Y versus control islets. Glutamine stimulated IS in H454Y but not control islets. The clinical manifestations of

GDH-HI and related animal studies suggest that GDH regulates basal IS and AASIS. Energy deprivation enhanced GDH-mediated IS, and H454Y mice were hypoglycemic, substantiating roles for GDH and its regulation by the phosphate potential in basal IS. Excessive IS from H454Y islets upon exposure to GDH substrates or stimuli indicate that regulation of GDH by the β -cell phosphate potential plays a critical role in AASIS. These findings provide a foundation for defining pathways of basal secretion and AASIS, augmenting our understanding of β -cell function. *Diabetes* 51 (Suppl. 3):S421–S426, 2002

Despite great advances in the study of insulin secretion (IS) stimulated by glucose, the pathways for maintenance of basal IS and for amino acid (AA)-stimulated IS (AASIS) remain poorly defined. The recent finding of regulatory mutations of the enzyme glutamate dehydrogenase (GDH) causing a form of autosomal dominantly inherited congenital hyperinsulinism (GDH-HI) (1,2–4) is providing new insights into the mechanisms of AASIS and its regulation. The mitochondrial matrix enzyme GDH plays a critical step in glutaminolysis by catalyzing the oxidative deamination of glutamate to α -ketoglutarate (α -KG) with NADP as a cofactor. Positive allosteric effectors of GDH include leucine, 2-aminobicyclo[2,2,1]-heptane-2-carboxylic acid (BCH; a nonmetabolizable analog of leucine), and ADP, whereas GTP is a potent allosteric inhibitor (5–11). Allosteric activation of glutaminolysis is one mechanism by which the AA leucine stimulates IS (6–10,12). GDH involvement in leucine-stimulated IS (LSIS) and its regulation by the cellular phosphate potential, with ADP and GTP as messenger molecules, suggest that in the β -cell, whose primary task is to respond appropriately to energy states of the organism, this enzyme might assume a critical role in IS.

GDH-HI has a number of distinguishing features that suggest that GDH plays a key role in the hypersecretion of insulin, both basal secretion and that induced by AAs. As with other forms of congenital HI, the dysregulated IS of GDH-HI is associated with fasting hypoglycemia, although in this syndrome the manifestation may be subtle, as shown in Fig. 1A. Symptomatic postprandial hypoglycemia is frequently uncovered in patients with GDH-HI and likely reflects the protein-induced hypoglycemia that is a hallmark of this condition (Fig. 1A) (13). Because LSIS occurs through allosteric activation of GDH, the protein sensitivity of GDH-HI was hypothesized to reflect excessive stimulation of mutant GDH by leucine. Indeed, as shown in Fig.

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AA, amino acid; AASIS, AA-stimulated insulin secretion; BCH, 2-aminobicyclo[2,2,1]-heptane-2-carboxylic acid; GDH, glutamate dehydrogenase; HI, hyperinsulinism; IS, insulin secretion; α -KG, α -ketoglutarate; KIC, ketoisocaproate; Leu-AR, acute insulin response to leucine; LSIS, leucine-stimulated IS; SUR1, sulfonylurea receptor-1.

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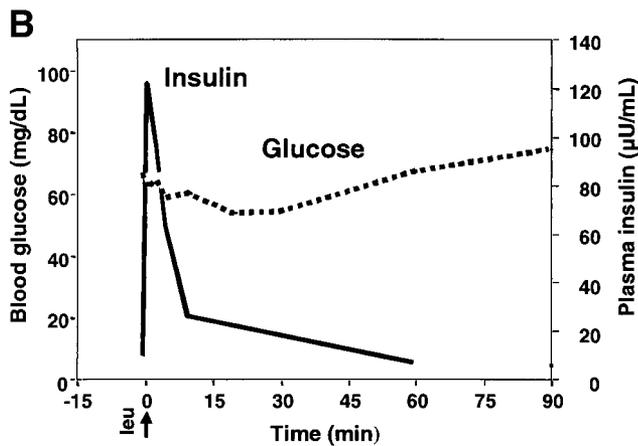
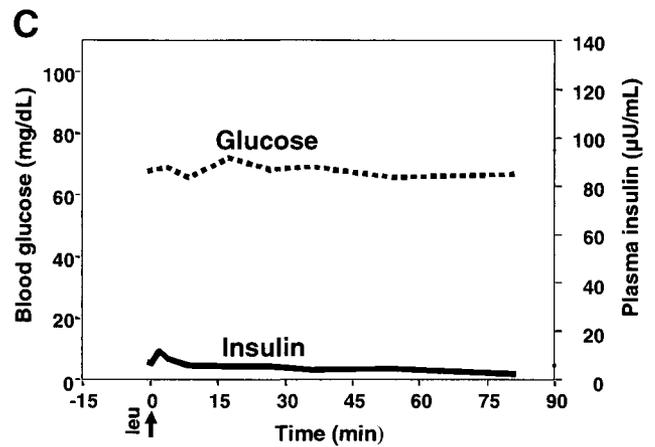
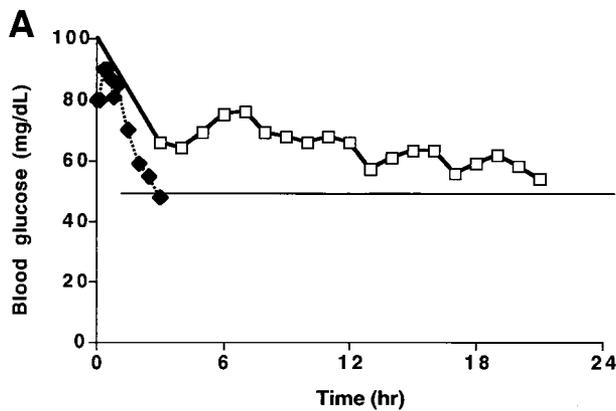


FIG. 1. Studies of glucose regulation and IS in patients with GDH-HI. **A:** Blood glucose responses to fasting and protein feeding in GDH-HI. Shown are the concentrations of blood glucose during a fasting test (\square) and after a protein meal (\blacksquare) in a 15-year-old girl with an Arg269His GDH mutation. Unlike this patient, normal individuals do not become hypoglycemic before 36–48 h of fasting and do not develop hypoglycemia after a protein meal. **B and C:** Acute insulin responses to leucine (Leu-AIRs). Intravenous leucine was administered at time 0. Insulin (solid line) and glucose (dashed line) concentrations were obtained at baseline and at 1, 3, and 5 min. GDH-HI patients have hyperresponsive IS in response to leucine (Leu-AIR = 64 μ U/ml), with a baseline serum leucine concentration of 50 μ mol/l and a peak of 630 μ mol/l (**B**), unlike normal control subjects, who have minimal IS in response to leucine, with a baseline serum concentration of 148 μ mol/l and a peak of 750 μ mol/l (**C**). leu, leucine.

1B, individuals with GDH-HI demonstrate exaggerated IS after an intravenous bolus of leucine, unlike control subjects who do not respond to a comparable dose (Fig. 1C) (14). In addition, unlike the severe sulfonylurea receptor-1 (SUR1)/Kir6.2 form of HI, which is typically resistant to medical therapy (15,16), GDH-HI is generally well controlled with diazoxide (17). This agent inhibits IS through its action on SUR1 and hyperpolarization of the β -cell. Its successful use in GDH-HI suggests that the mechanism for GDH-mediated IS to some extent involves the potassium channel-dependent pathway, which responds to changes in the β -cell energy potential, as expressed by the ATP-to-ADP ratio.

The mutations associated with GDH-HI primarily cluster in the region of the GTP binding domain of the enzyme (3,12,17–20). Studies performed by Stanley and colleagues (2,3,21) on lymphoblasts from patients with GDH-HI-associated mutations demonstrated markedly impaired GTP inhibition of GDH activity. These findings suggest that interference with allosteric inhibition of GDH by GTP is responsible for the clinical manifestations of GDH-HI and highlight the significance of strict regulation of GDH.

The finding of diazoxide-responsive HI in the setting of regulatory mutations of GDH provides a model for the role of GDH in AASIS because AA metabolism depends on the normal operation of the process of transdeamination. As shown in the proposed model in Fig. 2, oxidation of glutamate to α -KG by GDH fuels the Krebs cycle and thereby generates ATP. The increase in the ATP-to-ADP ratio leads to closure of the SUR1/Kir6.2 channel complex, depolarization of the β -cell membrane, opening of voltage-

dependent calcium channels, enhanced calcium influx, and augmented IS (22–29). In GDH-HI, loss of GDH sensitivity to inhibition by the β -cell phosphate energy potential is proposed to be the cause of a gain in GDH function, allowing excessive LSIS and protein-induced hypoglycemia because almost all AAs may rely on GDH to stimulate IS. Catabolism of a number of AAs, including leucine, requires the transamination of α -KG to glutamate, thereby supplying not only carbon skeletons to the Krebs cycle but substrate for the GDH reaction. In addition, this model of AASIS implies a possible role for GDH in maintaining basal IS. In the low-energy state, depletion of β -cell GTP would remove inhibition of GDH and maintain basal IS. About half of the daily insulin requirement is supplied by basal release.

GDH-HI is also associated with a peculiar, persistent asymptomatic hyperammonemia that is unaffected by protein intake. The concomitant existence of hyperammonemia with HI corroborates the model of GDH regulatory mutations as causing excessive oxidative deamination of glutamate. In the hepatocyte (Fig. 2), upregulated GDH activity is hypothesized to cause 1) excess ammonia production and 2) depletion of glutamate, which is required for synthesis of N-acetylglutamate, an essential cofactor for carbamoyl-phosphate synthesis, the first committed step in ureagenesis (30). The combination of excess production and impaired disposal of ammonia are thought to account for the elevation of serum ammonium in GDH-HI patients.

Further supporting the proposed role of phosphate energy in regulating glutaminolysis and AASIS are studies

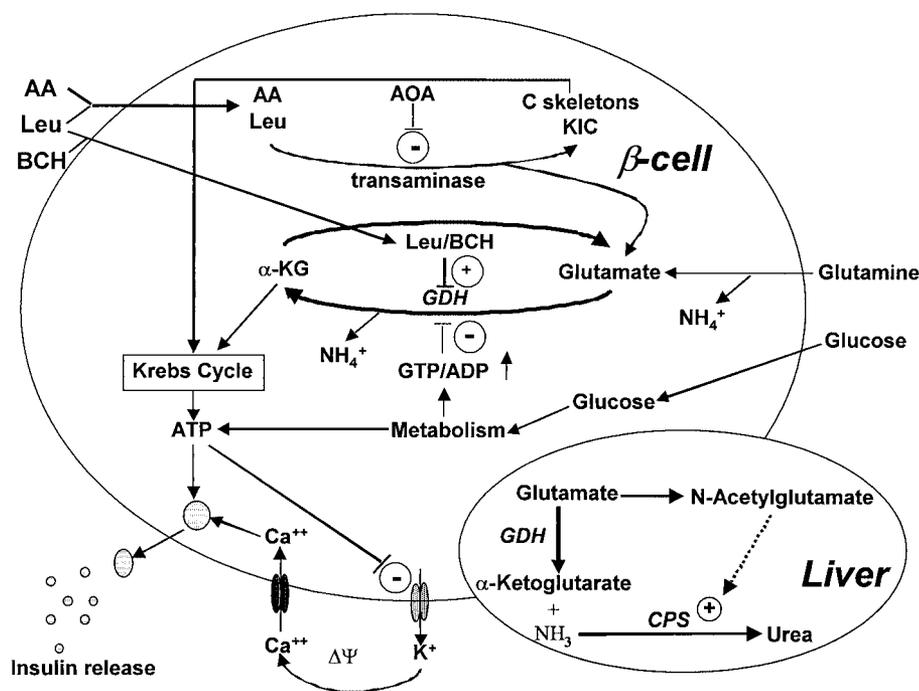


FIG. 2. Model of GDH activity in β -cell IS and hepatic ureagenesis. GDH is responsible for the oxidative deamination of glutamate to α -KG. Oxidation of the α -KG carbon skeleton in the Krebs cycle generates ATP, which then, through the potassium channel complex, activates the same cascade of insulin release as glucose. GDH is allosterically inhibited by GTP. Leucine (Leu) and BCH, a nonmetabolizable analog of leucine, stimulate IS through allosteric activation of GDH. In addition, leucine and other AAs can be transaminated to keto-isocaproate (KIC), in the case of Leu, or other carbon skeletons, and oxidation of KIC and these other carbon skeletons in the Krebs cycle also generate ATP. Moreover, transaminations of Leu and other AAs produce glutamate and hence provide substrate for the GDH reaction. AOA blocks transaminations. In the hepatocyte, GDH activity generates ammonia through oxidation of glutamate. Glutamate is necessary for production of N-acetylglutamate, a necessary allosteric activator of carbamoylphosphate synthetase (CPS), the rate-controlling first step of the urea cycle.

that demonstrated that preincubation of isolated mouse islets in high glucose suppressed LSIS, whereas glucose deprivation enhanced LSIS (31). In isolated rat islets, these studies were correlated with suppression of leucine-stimulated glutaminolysis by glucose and enhancement of leucine-stimulated glutaminolysis by glucose deprivation (31). These findings suggest that the energy state of the β -cell regulates GDH-mediated IS.

These preliminary in vitro studies of GDH-mediated LSIS, and the fasting hypoglycemia, protein hypersensitivity, and exaggerated LSIS of GDH-HI patients, highlight the importance and high precision of GDH regulation by the phosphate potential of the β -cell. To further characterize the regulation of GDH and its role in IS, studies of AASIS and its regulation by glucose are being performed in isolated rat islets and in a transgenic mouse model of GDH-HI.

RESEARCH DESIGN AND METHODS

IS of isolated rat islets. Fasted Wistar rats were anesthetized with an intraperitoneal injection of pentobarbital (100 mg/kg), the abdomen was opened, and the pancreas was removed for preparation of islets. Islets were isolated by collagenase digestion and cultured for 3 days in RPMI-1640 containing 10 mmol/l glucose, 2 mmol/l L-glutamine, and 10% fetal bovine serum (31). Isolated islets were perfused in 2 mmol/l glutamine with a BCH square-wave stimulus of 10 mmol/l or a BCH ramp from 0–25 mmol/l at an incline of $0.5 \text{ mmol} \cdot \text{l}^{-1} \cdot \text{min}^{-1}$. Perfusate was collected frequently and assayed for insulin content to determine the maximal rates of IS and the sensitivity to BCH stimulation of IS. Insulin was measured by radioimmunoassay.

Transgenic mice. We generated mice that selectively overexpress either the H454Y GDH mutant or the wild-type human GDH in β -cells under the direction of the rat insulin promoter (RIP). The H454Y mutant was chosen because of its severe phenotype in children expressing this mutation. To identify transgenic mice, tail clippings were

performed on 3-week-old pups, DNA was isolated using an overnight digestion with Tris-EDTA proteinase K at 55°C followed by phenol/chloroform extraction and ethanol precipitation, and construct-specific PCR primers were used on isolated genomic DNA. To determine the transgene copy number, Southern blots were performed using construct-specific ^{32}P -labeled probes. To establish islet-specific expression, RNA was isolated using a guanidine isothiocyanate-containing buffer followed by precipitation with ethanol using an RNeasy kit (Qiagen), and RT-PCR was performed using construct-specific primers. Blood samples were obtained via tail clipping at monthly intervals, and random blood glucose concentrations were determined using a blood glucose meter (Freestyle; Therasense, Alameda, CA). Islet isolation was performed as described above. Cultured islets were perfused in Krebs-Ringer bicarbonate buffer using 50-min ramps of either AAs or glucose, as follows: 1) 0–10 mmol/l leucine with 2 mmol/l glutamine, 2) 0–25 mmol/l glutamine, 3) 0–10 mmol/l AA mixture with 2 mmol/l glutamine, and 4) 0–25 mmol/l glucose. GDH activity and sensitivity to GTP inhibition were determined in isolated islets spectrophotometrically, as previously described (3).

These studies were approved by and performed in accordance with the institutional animal care and use committees at the Children's Hospital of Philadelphia and the University of Pennsylvania.

RESULTS

BCH-stimulated IS in isolated rat islets. Studies with isolated mouse islets have previously demonstrated that stimulation of IS by 10 mmol/l leucine or BCH correlates with glutamate oxidation, is enhanced with glucose deprivation, and is suppressed with high glucose. Similarly, in isolated rat islets, BCH stimulates IS (Fig. 3). As shown in Fig. 3, using either a BCH square wave or a ramp, glucose deprivation for 120 min is associated with enhanced BCH-stimulated IS when compared with only 50 min of

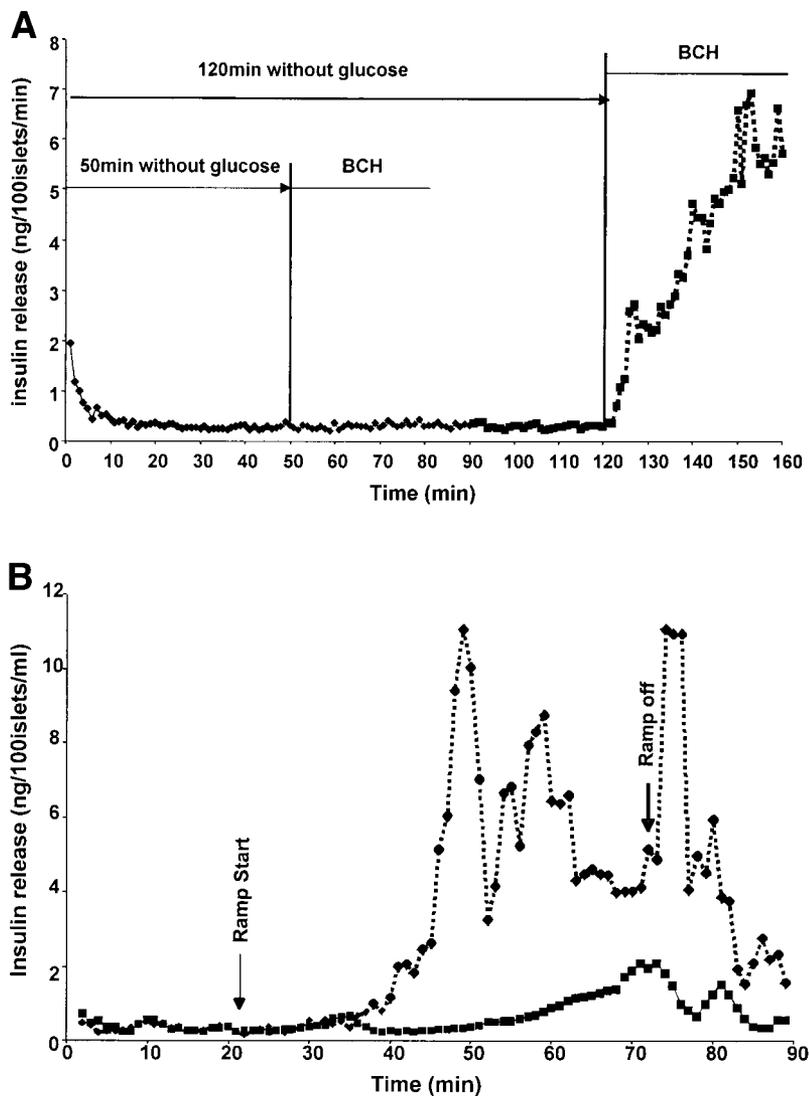


FIG. 3. Effects of “fuel depletion” on BCH-stimulated IS. After brief (50 min, solid line) and prolonged (120 min, dashed line) withdrawal from culture medium, which contained 10 mmol/l glucose, isolated rat islets were perfused with 10 mmol/l BCH (A) or a 0–25 mmol/l BCH ramp at $0.5 \text{ mmol} \cdot \text{l}^{-1} \cdot \text{min}^{-1}$ (B). All studies were performed in the presence of 2 mmol/l glutamine. Islets were not responsive to BCH after a 50-min glucose withdrawal. When the withdrawal time was prolonged to 120 min, BCH stimulated insulin release, which was multiphasic in the presence of a BCH ramp.

glucose deprivation. In addition, islets deprived of glucose for 120 min had a lower threshold for IS in response to a BCH ramp (Fig. 3B).

H454Y-GDH transgenic mice. Random blood glucose concentrations were significantly lower in H454Y-GDH mice ($63 \pm 27 \text{ mg/dl}$) when compared with wild-type human GDH transgenic ($162 \pm 33 \text{ mg/dl}$) and normal control mice ($158 \pm 30 \text{ mg/dl}$). Enzymatic studies revealed that GDH from H454Y mice had impaired inhibition by GTP. In response to perfusion with glucose, H454Y islets had IS that was similar to that of normal islets. However, perfusions of isolated H454Y islets using either a leucine ramp or an AA mixture ramp with 2 mmol/l glutamine revealed lower thresholds for IS and greatly enhanced LSIS and AASIS when compared with normal islets. The addition of glutamine alone was able to stimulate IS in H454Y islets but not in normal control islets.

DISCUSSION

The recent identification of a form of congenital HI that arises from mutations that impair allosteric inhibition of GDH by GTP underscores the significance of a previously underappreciated role of glutaminolysis in IS. The present

studies are being conducted to better define this role and its regulation by the energy state of the β -cell.

GDH is hypothesized to be the key regulator of AASIS, conceptually equivalent to the role of glucokinase as the “glucosensor” of the β -cell. The clinical manifestations of GDH-HI patients support this hypothesis. Impaired sensitivity of GDH to inhibition by GTP leads to protein-induced hypoglycemia, suggesting that GDH-mediated AASIS escapes normal control. Exaggerated IS after leucine stimulation further confirms the role of GDH in mediating specifically LSIS and the indispensability of GTP regulation in restraining this activity. To further explore the implications of studies by Gao et al. (31), which demonstrated suppression of LSIS by 25 mmol/l glucose, the effects of hyperglycemia on LSIS in GDH-HI patients were undertaken. These studies demonstrated suppression of excessive LSIS in GDH-HI patients with blood glucose concentrations raised to 150–180 mg/dl and suggest that impairment in GTP inhibition can be overcome (14). This finding has led to the recommendation that patients with GDH-HI consume carbohydrates before protein in an effort to curb their protein hypersensitivity.

The regulation of GDH by the phosphate energy poten-

tial of the β -cell is hypothesized to enable glutaminolysis to also maintain basal IS. Lending credence to this role, fasting provokes the development of hypoglycemia in GDH-HI patients. From this observation, one can extrapolate that GDH is normally turned on in the unfed, "low GTP" state. Regulatory mutations of GDH-HI, however, shift the GDH activity versus GTP curve to the right. Hence, fasting unmasks the effects of GDH regulatory mutations by sufficiently lowering the phosphate potential of the β -cell to remove any GTP inhibition to which mutant GDH may have been sensitive. In the well-characterized potassium channel-dependent model of IS, the β -cell secretes insulin in response to glucose-evoked increases in the ATP-to-ADP ratio. As glucose concentrations fall to <5 mmol/l, glucose loses its stimulant effect on β -cells, but IS does not cease. Removal of GDH inhibition during low-energy states could thus allow glutaminolysis to maintain interprandial IS, a critical task, given basal IS accounts for $\sim 50\%$ of total daily IS.

The finding in isolated rat islets of glucose deprivation enhancing BCH-stimulated IS supports this role of glutaminolysis in maintaining basal IS. In low-energy states, such as fasting, removal of GTP inhibition of GDH permits glutaminolysis to proceed, preventing the development of a diabetic ketoacidotic state. Simple removal of GTP inhibition of glutaminolysis, however, may be insufficient to stimulate IS in "normal" individuals and may require the presence of a secretagogue. As demonstrated by the isolated rat islets, glucose deprivation alone did not stimulate IS but required the presence of the nonmetabolizable analog of leucine, BCH, which stimulates IS through direct allosteric effects on GDH. Hence, the normal increases in serum leucine concentrations that accompany fasting may have teleological significance by serving as the trigger for glutaminolysis-mediated IS.

In addition, the preliminary findings in the H454Y mouse substantiate the role of regulatory mutations of GDH in causing congenital HI and hence provide a transgenic mouse model for GDH-HI. Affected mice have lower blood glucose concentrations than their normal littermates from as early as 3 weeks of age (the first time they are tested). Mice overexpressing wild-type human GDH were not hypoglycemic, suggesting that the regulatory mutation, not overexpression, of GDH is responsible for the HI phenotype in the H454Y transgenic mouse. However, Wollheim and colleagues (32–35), invoking glutamate as the mitochondrial factor directly responsible for IS, have demonstrated in INS-1E cells that overexpressing wild-type human GDH enhanced IS in response to glucose. Studies of the wild-type human GDH transgenic islets have yet to be performed, but isolated islets from the H454Y mutant mice responded similarly to glucose as those of littermate nontransgenic control mice. The role of glutaminolysis in glucose-stimulated IS remains to be fully defined. The opposite conclusions drawn from the work by Wollheim and colleagues clearly requires that the role of GDH in IS should be further explored intensively.

H454Y islets displayed lower thresholds for and overall enhanced IS in response to leucine when compared with control islets. These findings are consistent with the leucine sensitivity of GDH-HI patients and demonstrate a role for glutaminolysis and its regulation by the phosphate

potential of the β -cell in mediating IS. As described earlier, the finding of a reduced threshold for LSIS in H454Y islets allows one to speculate that normal regulation of GDH activity is multilayered. A lowering of the GTP content of the β -cell in combination with increasing serum concentrations of leucine, both of which accompany fasting, may have additive effects on stimulating GDH activity and IS. Enhanced sensitivities of H454Y islets to the stimulant effects of both glutamine and an AA mixture confirm the importance of GDH in stimulating IS. To test our model of AASIS in which AAs supply glutamate for the leucine-activated GDH reaction, aminooxyacetic acid (AOA) will be used to block these transamination reactions.

Identifying the mechanisms of AASIS and basal IS is vital for completing our understanding of β -cell function. The present studies of the GDH-HI syndrome in children and related animal models as well as recent studies suggesting that glutamate, produced through GDH reduction of α -KG (32–35) or by transamination, is a signal per se for IS are a clear expression of the intensified interests in a metabolic pathway long neglected in β -cell research.

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