Extremes of clinical and enzymatic phenotypes in children with hyperinsulinism due to glucokinase activating mutations.

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Objective. Heterozygous activating mutations of glucokinase have been reported to cause hypoglycemia due to hyperinsulinism (GK-HI) in a limited number of families. We report three children with de novo GK-HI mutations who displayed a spectrum of clinical phenotypes corresponding with marked differences in enzyme kinetics.

Research Design and Methods. Mutations were directly sequenced and mutants were expressed as GST-GK fusion proteins. Kinetic analysis of the enzymes included determinations of stability, activity index, the response to glucokinase activator drug and the affect of glucokinase regulatory protein (GKRP).

Results. Child 1 had an ins454A mutation, child 2 a W99L mutation and child 3 an M197I mutation. Diazoxide treatment was effective in child 3 but ineffective in child 1 and only partially effective in child 2. Expression of the mutant glucokinase ins454A, W99L and M197I enzymes revealed a continuum of high relative activity indexes in the three children (26, 8.9 and 3.1 respectively; WT = 1.0). Allosteric responses to inhibition by GKRP and activation by the drug RO0281675 were impaired by the ins454A but unaffected by the M197I mutation. Estimated thresholds for glucose stimulated insulin release were more severely reduced by the ins454A than the M197I mutation and intermediate in the W99L mutation (1.1, 3.5 and 2.2 mmol/l respectively; WT = 5.0 mmol/l).

Conclusions. These results confirm the potency of glucokinase as the pancreatic β-cell glucose sensor and demonstrate that responsiveness to diazoxide varies with genotype in GK-HI resulting in hypoglycemia that can be more difficult to control than previously believed.

Non-standard Abbreviations:
HI, hyperinsulinism; GK-HI, glucokinase-hyperinsulinism; GDH, glutamate dehydrogenase; K\textsubscript{ATP}, ATP-dependent potassium channel; GIR, glucose infusion rate; GKRP, glucokinase regulatory protein; MODY2, maturity onset diabetes of youth; GST, glutathione S-transferase; GST-GK, glutathione S-transferase-gluokinase fusion protein; S6P, sorbitol-6-phosphate; GKA, glucokinase activator
Hypoglycemia in infants with congenital hyperinsulinism has been associated with mutations that affect the regulation of insulin secretion by all three major classes of metabolic fuels: glucose, amino acids, and fatty acids (1-6). The most common of these disorders is due to recessive mutations of the \( \beta \)-cell ATP-dependent potassium channel \( (K_{\text{ATP}}) \); these mutations cause severe neonatal hypoglycemia that does not respond to medical therapy with diazoxide, a \( K_{\text{ATP}} \) channel agonist, and often requires near-total pancreatectomy (7, 8). Other genetic forms of congenital hyperinsulinism, such as dominant mutations of glutamate dehydrogenase, cause less severe disease, with hypoglycemia that may not be recognized until childhood or even adult life and responds well to diazoxide therapy (4, 9-11). In 1998, the first case of hyperinsulinism due to a dominant, gain of function mutation of glucokinase was reported (12). This remains one of the rarest forms of hyperinsulinism and information on its clinical and biochemical manifestations is limited since only a few cases have been reported subsequently (13-19). Most of these cases have been identified because of family histories of hypoglycemia with dominant patterns of transmission and most affected individuals were reported to have relatively mild disease that could be managed medically with diazoxide.

Glucokinase catalyzes the first step in glucose metabolism in pancreatic beta-cells and liver (20). It exists as a monomer in three conformations that control catalytic function: a closed form, an open form, and a super open form (21). Transitions between these conformations are controlled by glucose concentration, giving a sigmoidal enzyme activity curve, as well as by allosteric modulators. Binding of novel glucokinase activator (GKA) molecules, such as RO0281675, to the allosteric site increases glucokinase activity resulting in both augmented hepatic glucose uptake and lowering of the beta-cell threshold for glucose-stimulated insulin release (22). In the liver, glucokinase enzyme activity is inhibited by binding of glucokinase regulatory protein (GKRP), which also leads to nuclear sequestration of the enzyme (23).

Glucokinase serves a critical physiologic function as the beta-cell glucose sensor. It determines the glucose threshold for insulin release because of the low affinity of the enzyme for its substrate, glucose (half maximal activity, \( S_0.5 \), occurs at 7.5 mmol/l glucose). Heterozygous mutations that reduce enzyme activity cause a subtype of maturity onset diabetes of youth (MODY2) while, as noted above, heterozygous activating mutations cause hypoglycemia. Expression of these activating mutations shows increased affinity for glucose with elevations of calculated enzyme activity indexes and lower calculated glucose thresholds for insulin release (24).

Based on the initial cases reported, glucokinase hyperinsulinism has been assumed to be a mild form of hypoglycemia that can easily be managed medically. However, one reported case with a more severe clinical phenotype of uncontrollable hypoglycemia suggests that the range of manifestations of glucokinase hyperinsulinism may be greater than has been appreciated (14). The purpose of this report is to describe three children with hyperinsulinism due to de novo glucokinase mutations who exhibit marked differences in responsiveness to medical therapy that correlate with differences in enzyme activity indexes.

**RESEARCH DESIGN AND METHODS**

All clinical studies were approved by the IRB of the Children’s Hospital of Philadelphia. Patient tests were performed in
the Clinical Translational Research Center of the Children's Hospital of Philadelphia. Protein sensitivity and acute insulin response tests to calcium, leucine, and glucose were carried out as previously described (25, 26). Insulin assays were performed using an ELISA kit (Mercodia, ALPCO Diagnostics, Salem, NH).

**Identification of Glucokinase Gene Mutations:** Genomic DNA from peripheral lymphocytes was amplified by PCR using published primer sequences for glucokinase (GK) (Genbank accession no.: M88011) (27). Products were sequenced using the BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and analyzed using Sequencher 3.1 (Gene Codes Corp., Ann Arbor, MI).

**Biochemical Characterization of ins454A, W99L and M197I mutants:** Recombinant human islet wild-type GK, the three patient mutants ins454A, W99L and ins454A, known instability mutant E300K, and a series of designed mutations (M197A, D, E, F, K, L, T and V) were generated using methods previously described (28). Briefly, the mutations were cloned for expression as glucokinase fusion proteins containing a C-terminal glutathionyl S-transferase (GST). GST-GK was produced in E. coli, then purified from crude extracts to near homogeneity by affinity-chromatography using GSH-agarose (Sigma, St. Louis, MO, U.S.A). Characterization of GST cleaved enzymes was also performed by cleavage of GST with Factor Xa. The product was then purified again using glutathione agarose and Factor Xa with benzamidine sepharose 6B to yield the cleaved proteins.

Kinetic analysis of the expressed forms of glucokinase was performed using the protocols developed previously (13, 16) both in the presence and absence of the glucokinase activator compound RO0281675. The activity index, an expression of the proposed enzyme's *in situ* phosphorylation capacity, and the theoretical threshold for glucose stimulated insulin release were calculated as previously described (13, 20). Kinetic analysis was also performed in the presence of recombinant human glucokinase regulatory protein (GKRP), a competitive inhibitor of glucokinase, with and without sorbitol-6-phosphate (S6P), a structural analog of fructose-6-phosphate that enhances GKRP inhibition (16). For this purpose the assay was performed with glucose at 3 mmol/l for wild-type and the glucose concentration was adjusted based on *S₀.₅* of the different mutants both in the presence and the absence of 10 μM S6P.

The stability of mutant and wild-type GST-GK to low glucose and high temperature was tested as previously described by spectrophotometric enzyme assay (16, 29). The stability of W99L was measured using tryptophan fluorescence by comparing the quantum (photonic) yield of wild-type glucokinase to the mutant glucokinase protein solution (30).

**Structural Analysis:** Structural modeling of M197I, W99L, ins454A and published activating mutations was performed using the structure of the super-open form of apo-glucokinase and closed form determined by Kamata et al (21). Modeling was performed using SwissPdb Viewer (31).

**Patients:** Child 1 is a 17 year old male who had a large for gestational age birth weight of 4.8 Kg at term. Hypoglycemia was detected in the first hour after delivery; high rates of intravenous glucose infusion were required to control blood glucose levels (18 mg/kg/min; normal < 5-6 mg/kg/min). Plasma insulin levels at times of hypoglycemia were found to be elevated (8-15 μU/ml; normal < 3). He was not fully responsive to diazoxide, defined as being able to fast greater than 12 hours with blood glucose of more than 70 mg/dl. However, treatment with a high dose of diazoxide, 20 mg/kg/day and feedings every 4 hours
prevented symptoms of hypoglycemia, although plasma glucose levels remained largely between 2.8 and 3.3 mmol/l (50-60 mg/dl). There were no family members with hypoglycemia.

At one year of age he was referred to the Children’s Hospital of Philadelphia because of persistently low plasma glucose values, 2.2 to 3.3 mmol/l (40-60 mg/dl). A trial of octreotide gave only transient improvement in control of hypoglycemia (32). Due to persistent hypoglycemia at 23 months of age a 95% pancreatectomy was performed, however, this failed to control hypoglycemia. Diazoxide treatment was re-tried, but plasma glucose fell to 3.3 mmol/l (60 mg/dl) after only 5 hours of fasting. Treatment consisted of feedings given frequently, day and night. Despite the low levels of plasma glucose, hypoglycemic symptoms rarely occurred, mainly triggered by exercise or high carbohydrate feedings. His parents report that his hypoglycemia appeared to improve after age 11 years and night-time feedings were stopped. However, they also report that he now has occasional hyperglycemia after meals, suggestive of mild glucose intolerance. Currently, child 1 has normal school performance.

Child 2 is a boy with a normal term birth weight of 3.2 Kg. Hypoglycemia was not recognized until age 6 years when he became unarousable a few hours after eating and was found to have a blood glucose of 48 mg/dL (2.7 mM). There was no family history of hypoglycemia. Treatment with 10 mg/Kg/day of diazoxide failed to control hypoglycemia. He was suspected to have an insulinoma due to the apparent new onset of hypoglycemia. Endoscopic ultrasound suggested a lesion in the head of the pancreas that was found to be a lymph node at surgery; biopsies of the neck and body of the pancreas were normal. Octreotide failed to control hypoglycemia. Successful control of hypoglycemia has required a combination of diazoxide (15 mg/Kg/day) plus a low rate of continuous dextrose overnight by gastrostomy-tube (2 mg/Kg/min). School performance was considered to be normal.

Child 3 is a 16 year old boy with a large for dates birth weight of 4.9 Kg at 41 weeks of gestation. Hypoglycemia was detected in the first hours after birth requiring treatment with intravenous dextrose at 12 mg/kg/min. There was no history of hypoglycemia in the parents or in two older siblings. Hyperinsulinemic hypoglycemia was documented but was considered to have resolved after two months of treatment with prednisone. There was no further suspicion of hypoglycemia until 14 years of age when he suffered two brief seizures, each occurring three hours after breakfast. Plasma glucose was found to be 2.2 mmol/l (40 mg/dl). Hyperinsulinemic hypoglycemia was diagnosed. A high dose of diazoxide at 900 mg (15 mg/kg/day) was required to maintain fasting plasma glucose levels above 3.9 mmol/l (70 mg/dl). School performance was considered normal, although not on a par with his siblings.

RESULTS

Figure 1 shows the diurnal profile of plasma glucose levels in child 1 and child 3 (at ages 4 and 15 years, respectively). Glucose values ranged between narrow limits, rarely rising into the normal range even after meals, but also rarely falling very low, even during 12 hours of overnight fasting (child 3). Child 1, whose hypoglycemia was more difficult to manage, had significantly lower mean plasma glucose levels than child 3 (50 ± 2 vs. 63 ± 1 mg/dl respectively, m ± SEM, p < 0.0001). As shown in Figure 2, child 2 fasted for 24 hours with a stable plasma glucose ranging from 3 to 4 mM (54-72 mg/dL) before dropping to 2.8 mM (50 mg/dL). During the fast he developed a significant ketonemic response, albeit lower than
normally seen at this level of blood glucose (BOB > 2.5 mM).

Table 1 shows the responses in child 1, and child 3 to stimulation tests of insulin secretion. Both children showed exaggerated acute insulin responses (AIR) to intravenous glucose. In child 3, acute insulin responses to calcium and leucine were normal, in contrast to the hyper-responsiveness to calcium and leucine seen in patients with K\textsubscript{ATP} channel or GDH mutations respectively. In further contrast to patients with K\textsubscript{ATP} or GDH hyperinsulinism, hypoglycemia was not provoked by oral protein tolerance tests in either child 1 or child 3. All three children had normal serum concentrations of cholesterol and triglyceride.

Review of the surgical specimen of resected pancreas from child 1 showed a normal islet distribution, shape and size from head to tail. There were occasional large endocrine cell nuclei that occupied an area at least three times as large as the surrounding endocrine cell nuclei, similar to the histologic features seen in recessive K\textsubscript{ATP}-HI. The surgical biopsies from child 2 showed normal pancreas.

**Genetic mutation analysis:** In child 1, mutation analysis of genomic DNA identified a heterozygous novel missense mutation, ins454A (nt 1363-1364 ins CGG). In child 2, a glucokinase mutation was suspected because of the stability of his hypoglycemia (Figure 2); he was found to have a heterozygous novel W99L missense mutation (nt 296 G>T). In child 3, a mutation in glucokinase was also suspected because of the stability of hypoglycemia; he had a heterozygous novel M197I mutation (nt 591 G>T). The mutations were not detected in any of the children’s parents and were not found in 100 normal chromosomes. No mutations were found in other hyperinsulinism genes, including ABCC8, KCNJ11 and GLUD1.

**Biochemical analysis of expressed ins454A, W99L and M197I glucokinase mutations:** Table 2 shows the enzymatic activities of the purified mutant forms of glucokinase. The ins454A mutation resulted in markedly increased affinity for glucose (decreased glucose S\textsubscript{0.5}). The glucose S\textsubscript{0.5} in child 2 and 3 were reduced to a lesser degree. The k\textsubscript{cat} of the ins454A mutation was similar to wild-type glucokinase; however, k\textsubscript{cat} for the GST-GK fusion M197I mutant was approximately 60% of WT and k\textsubscript{cat} for W99L was increased when compared with WT. Hill numbers for all three mutations were only slightly reduced compared with wild-type. Kinetic variables for the GST-GK fusion proteins were similar to their cleaved counterparts (data not shown). For all mutations, the calculated enzyme activity index was increased compared with wild-type, consistent with a gain of glucokinase enzyme function. This was especially true for child 1 whose ins454A mutation resulted in an activity index greater than 25 times normal, consistent with his poorer response to diazoxide therapy compared to children 2 and 3.

Since enzyme stability can potentially affect glucokinase activity, we examined the effect of incubating ins454A and M197I mutant enzymes under conditions of low glucose and high temperature (Figures 3A and 3B). The stability of enzyme activity of the two mutants after incubation at low glucose concentrations was similar to that of wild-type glucokinase and was much greater than that of the known diabetes-associated instability mutant, E300K (Figure 3A). The thermal stability of the ins454A and M197I mutant proteins was intermediate between that of wild-type and E300K glucokinase (Figure 3B). The thermal stability of W99L was investigated by tryptophan fluorescence under similar glucose conditions (30) and was also intermediate between that of wild-type and the E300K instability mutant (data not shown).
As shown in Table 2, the ins454A GST-GK mutant was unresponsive to the glucokinase activator compound, RO0281675, suggesting that its activity was already maximal. The response of the W99L and M197I GST-GK mutants to the activator was similar to wild-type. The latter finding may be due to the M197I mutation being 19Å away from the activator site and, therefore, unlikely to interfere with activator binding. Another plausible explanation is the moderate degree of enzyme activation leaving room for further activation by the GKA as in the case of W99L.

Figures 3C and 3D show the response of two of the mutant glucokinase enzymes to inhibition by GKRP, the hepatic glucokinase regulatory protein. W99L and M197I GST-GK mutants had similar responses to GKRP as wild-type (W99L results not shown). The ins454A mutant showed little response to inhibition by GKRP.

Biochemical analysis of additional mutants at residue 197: As shown in Table 2, to evaluate the function of the M197 residue, eight additional mutations were designed that substituted a range of amino acids from hydrophobic to hydrophilic at this site. Generally, substitution of hydrophobic residues for the normal methionine resulted in increased affinity for glucose and increased enzyme activity indexes, while changes to hydrophilic or amphipathic residues reduced enzyme activity.

DISCUSSION

The results of these studies demonstrate that congenital hyperinsulinism in these three children was caused by three novel activating mutations of glucokinase, ins454A, W99L and M197I. Expression of the mutations demonstrated changes in glucokinase kinetics consistent with increased enzyme activity. Increases in calculated glucokinase activity indexes ranged from 25 times normal in child 1, with the ins454A mutation, to three times normal in child 3, with the M197I change. These differences in enzyme activation correlated with the more severe, diazoxide-unresponsive hypoglycemia of child 1; the intermediate, partially diazoxide-responsive hypoglycemia of child 2; and the milder, diazoxide-responsive hypoglycemia of child 3.

As shown in Figure 4A, the mutations at positions 99, 197 and 454 of glucokinase are located on the reverse side of the enzyme, opposite the catalytic cleft. The ins454A mutation is adjacent to three previously reported glucokinase activating mutations (V455M, A456V and V452L) while the W99L mutation is located at the same position as a previously reported W99R activating mutation (see Table 3 summarizing known GK-HI mutations). Most of the 11 glucokinase activating mutations that have been identified occur in or close to the region of the enzyme that interacts with allosteric activator compounds, such as RO0281675, and with the inhibitory glucokinase regulatory protein. Heredia, et al. have shown that some of these mutations (T65I, A456V) increase glucose binding to the enzyme, whereas the others (W99R, Y214C, V455M) facilitate enzyme isomerization into the active form (33, 34). The two mutations which enhance glucose binding also abrogate interaction with GKRP, suggesting that these mutations cause increased glucokinase activity by favoring maintenance of the closed, active form of the enzyme and preventing opening of the catalytic cleft into the inactive form of the enzyme. The ins454A glucokinase mutation in child 1 appears to fit in this category of mutations, which make the enzyme unresponsive to both inhibition by GKRP and allosteric activation by RO0281675. Loss of inhibition by GKRP does not seem to be a major determinant of clinical phenotype (34), since the Y214C mutation retains sensitivity to GKRP but was reported to have diazoxide
unresponsive hypoglycemia similar to ins454A (Table 3).

The novel M197I amino acid substitution reported here has a unique location which is remote from the other glucokinase activating mutations. As shown in Figures 4B and 4C, the M197I mutation alters a methionine residue that moves in and out of a hydrophobic pocket during the transition between the active and inactive forms of glucokinase. The importance of this hydrophobic lock and key interaction is demonstrated by the series of designed mutations of M197 (Table 2). Substitutions with isoleucine or other hydrophobic amino acids retained or enhanced glucokinase activity. However, substitutions with hydrophilic amino acids essentially inactivated the enzyme. These observations have been confirmed in a recent report by Pal and Miller (35). The M197I mutation indicates that activating mutations of glucokinase need not be restricted to the allosteric domain, where all previous defects have been located, and suggest that additional mutation sites are likely to be identified in patients with GK-HI.

Glucokinase mutations have been found infrequently in mutations analysis of patients with congenital hyperinsulinism, accounting for only 5 of 167 patients in recently reported series (18) and only 3 of 212 cases we have analyzed. Table 3 summarizes the major features of hyperinsulinism due to previously reported activating mutations of glucokinase and the three additional mutations described in this report. Familial cases account for 76% of the 29 known patients. This contrasts with the high proportion of de novo cases in GDH hyperinsulinism and other dominant disorders, suggesting that many cases are not identified in the absence of a family history of hypoglycemia. Only three of the 29 patients had surgery, suggesting a relatively mild hypoglycemia phenotype, especially in the familial cases. Many of the familial cases escaped recognition of their hypoglycemia disorder until beyond the neonatal period or even into adult life. In all of the familial cases, treatment with the β-cell K\textsubscript{ATP} channel agonist, diazoxide, was reported to have been successful in controlling hypoglycemia. Child 3 (M197I mutation) fits this milder, diazoxide-responsive phenotype, although it should be noted that unusually high doses of diazoxide were needed to maintain even low-normal levels of plasma glucose. However, similar to child 2 (W99L), incomplete responsiveness to diazoxide was also apparent in some of the reported cases in the six families with the mild hypoglycemia phenotype (W99R, A456V).

In contrast, children with Y214C and ins454A mutations had a much more severe form of hypoglycemia. Our child 1 with the ins454A mutations could not be controlled on diazoxide, even at a very high dose of 20 mg/Kg/day. In the case of the Y214C mutation, diazoxide responsiveness is unknown since only a low dose was tried. Both of these children required surgery and continued to have poorly-controlled hypoglycemia despite near total pancreatectomy. These cases make it apparent that the clinical manifestations of hyperinsulinism in some glucokinase mutations can be as severe and as unresponsive to diazoxide treatment as in children with hyperinsulinism due to recessive mutations of the K\textsubscript{ATP} channel subunits, SUR1 and Kir6.2.

The observations in the present three cases identify clinical features that may be useful for distinguishing glucokinase hyperinsulinism. A notable feature in the cases presented here was the remarkable stability of their hypoglycemia (Figures 1 and 2), consistent with a resetting of the threshold for insulin release at a value lower than normal. This contrasts with other forms of hyperinsulinism in infants where blood
glucose concentrations can fall without interruption to extremely low values. Insulin responses to secretagogues may also help to distinguish glucokinase hyperinsulinism from other disorders. The glucokinase activating mutations in our three cases are not associated with hyper-responsiveness to calcium (seen in K$_{ATP}$ hyperinsulinism) or leucine (seen in GDH hyperinsulinism), did not predispose to protein-induced hypoglycemia (seen in K$_{ATP}$ and GDH hyperinsulinism), but are accompanied by increased acute insulin response to glucose (blunted in K$_{ATP}$ hyperinsulinism).

Information about islet morphologic abnormalities in patients with glucokinase hyperinsulinism remains limited. In child 1, a small proportion of islet cells showed nucleomegalays similar to, but to a lesser extent than that seen in diffuse K$_{ATP}$ hyperinsulinism. The increase in islet size described by Cuesta (14) in a child with a Y214C glucokinase mutation was not apparent in our child. No abnormalities were noted in the pancreas of the mother of the child with the T65I mutation (16). Although further study may identify specific histologic features, present information suggests that it may not be easy to distinguish GK-HI from K$_{ATP}$ HI or, possibly, from normal pancreas.

Figure 5 shows the effects of various glucokinase activating mutations on predicted glucose threshold for insulin release based on the relative activity indexes of the expressed proteins in vitro. Cases reported to be diazoxide-unresponsive (ins454A and Y214C) have very low glucose thresholds, as does the A456V mutation, which in one patient was not completely responsive to diazoxide. In contrast, the better responses to diazoxide in child 2 and 3 correlate with higher calculated glucose thresholds. It should be noted that the range of plasma glucose levels in our three patients and in the reports of other GK-HI cases (Table 3) tends to be higher than their predicted glucose thresholds (Figure 5). This may partly reflect the effects of counter-regulatory responses to hypoglycemia. An additional potential problem in correlating data on glucokinase kinetics with clinical features in patients is that some of the changes in enzyme properties exert opposing effects. One example of this phenomenon is the V62M mutation which has been associated with MODY2 diabetes (36). When expressed in vitro, this mutation has an increased activity index consistent with causing hypoglycemia, rather than diabetes. The increased instability of this mutant form of glucokinase may counterbalance its enhanced activity and explain why the mutation results in a net loss of function in vivo (36). Similarly, although the ins454A, W99L and M197I mutations give increased activity indexes, they also have slightly reduced stability. Moreover, the impact of the reduced affinity of M197I for ATP in vivo is uncertain. Given these problems, efforts to understand the in vivo and in vitro phenotypes of glucokinase hyperinsulinism mutations will require that accurate data be obtained on the clinical features of affected individuals. For example, these data should especially include careful documentation of the ability of diazoxide treatment to completely normalize plasma glucose levels and accurate estimates of the in vivo glucose “set point”, such as illustrated in Figures 1 and 2.

In summary, these three cases of congenital hyperinsulinism due to activating mutations of glucokinase emphasize the key role that this enzyme plays in setting the glucose threshold of the pancreatic islets. These children had de novo mutations, which made them difficult to recognize. Clues to their diagnosis included persistent, but stable hypoglycemia and exaggerated insulin responses to intravenous glucose stimulation. These cases indicate that the spectrum of hyperinsulinism due to glucokinase activating mutations can range from mild and intermediate cases, which can be managed...
medically with diazoxide, to severe cases that are diazoxide unresponsive and may require additional treatment including near-total pancreatectomy to control hypoglycemia. These cases also illustrate the potentials and limitations of new approaches to develop glucokinase activator drugs for the treatment of type 2 diabetes mellitus.

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Table 1: Acute insulin response (AIR) to insulin secretagogues and plasma lipid concentrations. Reference normals for AIR tests are from [25, 26].

|                     | Child 1 | Child 2 | Child 3 | Normal       |
|---------------------|---------|---------|---------|--------------|
| Glucose AIR (pmol/l)| 483     | 474     | 318 ± 228 |
| Calcium AIR (pmol/l)|         | -24.0   | 0.6 ± 11.4|
| Leucine AIR (pmol/l)|         | 30.0    | 12.0 ± 66 |
| Cholesterol (mmol/l)| 2.9     | 4.3     | 4.3     | 2.8 - 5.8    |
| Triglycerides (mmol/l)| 0.29   | 0.61    | 1.7     | 0.4 - 1.9    |
**Table 2:** Kinetic characteristics of glucokinase ins454A, W99L and M197I. Kinetic data of purified expressed GST-tagged glucokinase with hyperinsulinism-causing ins454A (child 1), W99L (child 2) and M197I (child 3) mutations. Note that the EC_{50} relates to the Activity Index.

| Mutants | Yield (mg/l) | $k_{cat}$ (sec$^{-1}$) | Glucose S_{0.5} (mmol/l) | ATP_{km} (mmol/l) | n_{H} | GI (Activity Index) | Activation by GKA (fold) | EC_{50} for GKA (uM) |
|---------|--------------|-------------------------|--------------------------|-------------------|------|---------------------|-------------------------|-------------------------|
| ins454A | 10.9 ± 3.1   | 53.2 ± 2.9              | 1.1 ± 0.1                | 0.3               | 1.2  | 39.5 ± 1.7          | No activation           | N/A                     |
| W99L    | 19.9 ± 1.7   | 85.6 ± 3.1              | 2.9 ± 0.1                | 0.4               | 1.6  | 13.3 ± 0.8          | 11.8                    | 4.3                     |
| M197I   | 62.1 ± 0.3   | 38.1 ± 4.5              | 2.6 ± 0.2                | 1.5               | 1.6  | 4.8 ± 0.3           | 11.9                    | 4.9                     |
| M197L   | 34.5         | 54.5                    | 5.4                      | 1.0               | 1.4  | 3.8                 | 13.9                    | 6.2                     |
| M197V   | 25.1         | 44.7                    | 2.6                      | 0.5               | 1.7  | 8.0                 | 13.1                    | 3.5                     |
| M197A   | 36.4         | 53.3                    | 3.2                      | 0.3               | 1.7  | 7.0                 | 14.7                    | 3.8                     |
| M197T   | 26.7         | 53.9                    | 4.1                      | 0.5               | 1.6  | 4.4                 | 18.3                    | 5.5                     |
| M197F   | 40.3         | 21.2                    | 54.6                     | 0.2               | 1.3  | 0.1                 | 8.6                     | 6.1                     |
| M197K   | 36.4         | 3.78                    | 86.7                     | 0.2               | 1.1  | 0.03                | 13.6                    | 9.3                     |
| M197D   | 27.7         | 10.3                    | 197.0                    | 0.3               | 1.1  | 0.03                | 6.0                     | 2.8                     |
| M197E   | 6.82         | 21.0                    | 56.2                     | 1.3               | 0.2  | 0.1                 | 9.0                     | 2.7                     |
| WT      | 43.4 ±3.8    | 62.3 ± 4.8              | 7.6 ± 0.2                | 0.4               | 1.7  | 1.5 ± 0.1           | 15.8 ± 0.6              | 6.9 ± 0.4               |
**Table 3:** Comparison of clinical and biochemical features of published glucokinase activating mutants associated with hyperinsulinism.

|                   | V455M | A456V | T65I | W99R | Y214C | G68V | S64Y | V452L | ins454A | W99L | M197I |
|-------------------|-------|-------|------|------|-------|------|------|-------|---------|------|-------|
| **Families**      | 1     | 2     | 1    | 1, de novo | de novo | 1 | de novo | de novo | de novo | de novo | de novo |
| **Number of cases (total=29)** | 5 | 5 | 2 | 2, 1 | 1 | 8 | 1 | 1 | 1 | 1 | 1 |
| **Birth wt (Kg)** | 2.9-4.1 | 2.4-3.8 | 3.1 | 3.1, 4.0 | 4.4 | 1.9-3.7 | 4.3 | 5.9 | 4.9 | 3.2 | 4.9 |
| **Age at diagnosis** |     |       |     |       |       |     |       |       |       |     |       |
| Neonatal (n=8)    | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |     |     |
| Childhood (n=7)   | 2 | 1 |     |     |     | 3 |     |     |     |     |     |
| Adolescence (n=4) | 1 |     |     |     |     | 2 |     |     |     |     |     |
| Adulthood (n=10)  | 2 | 3 | 1 | 1 |     | 3 |     |     |     |     |     |
| **Severity of hypoglycemia** |     |       |     |       |       |     |       |       |       |     |       |
| Mild or untreated (n=12) | 1 | 3 | 1 |     |     | 7 |     |     |     |     |     |
| Diazoxide treated (n=13) | 4 | 2 | 1 | 1, 1 |     | 1 | 1 |     |     | 1 | 1 |
| Diazoxide & Octreotide treated (n=1) |     |     | 1 |     |     |     |     |     |     |     |     |
| Required surgery (n=3) |     | 1 |     |     |     | 1 |     |     |     |     |     |
| Pre-treatment plasma glucose (N > 3.9 mmol/l) | 1.3-2.5 | 2.1-3.5 | 2.2-3.0 | 2.0-3.5, 2.1 | 0.1-2.6 | 1.6-3.3 | 2.0 | 2.6-3.3 | 1.7-2.8 | 2.6-3.7 | 2.6-3.6 |
| Response to diazoxide | Yes | Yes | Yes | Yes, Yes | No | Yes | Yes | Yes | No | Partial | Yes |
| $S_{0.5}$ (normal = 7.55 mmol/l) * | 3.0 | 2.0 | 1.8 | 4.5 | 1.2 | 1.9 | 1.5 | 2.6 | 1.1 | 2.9 | 2.6 |
| **Relative activity index** * | 5.2 | 17 | 3.1 | 4.1 | 130 | 16 | 22 | 11 | 26 | 8.9 | 3.1 |
| Original Clinical Report | Reference | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |

* Values from published reports
FIGURE LEGENDS:

**Figure 1.** Diurnal patterns of plasma glucose concentrations in child 1 and child 3. White circles = child 1; white squares = child 3. Plasma glucose was measured before meals and after overnight fasting conditions. Shaded area indicates the normal range of plasma glucose (4.0 - 5.0 mmol/l, 72 – 90 mg/dl). Child 1 was 4 years old, 2 years after near-total pancreatectomy; child 3 was 15 years old. Neither child was on medical therapy. Mean glucose was 50±2 mg/dl in child 1, 63±1 mg/dl in child 3 (P < 0.0001 between children).

**Figure 2.** Plasma glucose and β-hydroxybutyrate (BOB) responses to fasting in child 2. White triangles = plasma glucose (SureStep bedside meter); black triangles = β-hydroxybutyrate (Precision Xtra bedside meter). Mean plasma glucose was 64±1 mg/dl. β-hydroxybutyrate values increased from 0.1 – 1.8 mmol/l while fasting plasma glucose levels decreased. At the end of fast, plasma c-peptide was suppressed (0.16 nmol/L, normal 0.26 - 1.32 nmol/l). Glucose response to glucagon stimulation was 36 mg/dl. Reference normals when fasting blood glucose < 50 mg/dl: β-hydroxybutyrate: > 2.5 mmol/l; glycemic response to glucagon > 30 mg/dl.

**Figure 3:** Thermal stability of GST-tagged wild-type and mutant glucokinase (Panels A and B). Response of GST-GK mutants to inhibition with glucokinase regulatory protein (GKRP) (Panels C and D). White circles = ins454A; white squares = M197I; white diamonds = E300K; black X = WT. GST-GK levels were 22 nmol/l in all tests.

**Panel A.** Effect of glucose concentration on expressed GST-tagged glucokinase with ins454A and M197I HI mutations and the known E300K instability mutant. For M197I, the studies were not extended below the glucose $S_{0.5}$ of 2.6 mmol/l.

**Panel B.** Effect of temperature on mutant glucokinase enzyme activities. For these assays, enzymes were incubated for 30 min. with glucose levels at their respective $S_{0.5}$.

**Panels C and D.** Inhibition of glucokinase activity by GKRP measured in the presence and absence of sorbitol-6-phosphate respectively.

**Figure 4:**

**Panel A.** Location of glucokinase mutations in the active (closed) form of the enzyme crystallographic structure.

**Panel B.** Location of the M197I residue tucked within a hydrophobic pocket of the active conformation of glucokinase

**Panel C.** Location of the M197I residue projecting into the hydrophilic space of the inactive conformation of the crystal.

**Figure 5:** Calculated thresholds for glucose-stimulated insulin release (GSIR) in activating and inactivating mutations of glucokinase. Black circles = GK-HI mutations; white circles = MODY2 mutations; black X = WT. Thresholds are plotted against the inverse of the mutant enzyme activity index relative to wild-type enzyme. Because relative expression of the glucokinase forms is affected by enzyme affinity for glucose and the ambient glucose concentration, the wild type enzyme dominates the estimated threshold for the heterozygous inactivating defects, but the mutant enzyme dominates the threshold for activating mutations. Thus, the threshold for inactivating mutations plateaus at ~7 mmol/l, whereas the calculated threshold for severe activating mutations approaches zero as the relative activity increases. For
purposes of consistency, all kinetic data in figure are from the Matschinsky lab. Threshold and activity indexes were calculated per reference 24.

Figure 1.

Figure 2.
Figure 3.
Figure 4.

A.

B.

C.
Figure 5. 

![Diagram showing a graph with thresholds for GSIR (mmol/l) on the y-axis and 1/Relative GK Activity Index on the x-axis. Various mutations are marked on the graph, including WT, A53S, M197I, T65I, W99R, V455M, W99L, G68V, S64Y, A456V, Y214C, ins454A, E70K, S263P, E300K, and K414E.]