Congenital Hyperinsulinism Caused by Hexokinase I Expression or Glucokinase-Activating Mutation in a Subset of β-Cells

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Congenital hyperinsulinism causes persistent hypoglycemia in neonates and infants. Most often, uncontrolled insulin secretion (IS) results from a lack of functional KATP channels in all β-cells or only in β-cells within a resectable focal lesion. In more rare cases, without KATP channel mutations, hyperfunctional islets are confined within few lobules, whereas hypofunctional islets are present throughout the pancreas. They also can be cured by selective partial pancreatectomy; however, unlike those with a KATP focal lesion, they show clinical sensitivity to diazoxide. Here, we characterized in vitro IS by fragments of pathological and adjacent normal pancreas from six such cases. Responses of normal pancreas were unremarkable. In pathological region, IS was elevated at 1 mmol/L and was further increased by 15 mmol/L glucose. Diazoxide suppressed IS and tolbutamide antagonized the inhibition. The most conspicuous anomaly was a large stimulation of IS by 1 mmol/L glucose. In five of six cases, immunohistochemistry revealed undue presence of low-Km hexokinase-I in β-cells of hyperfunctional islets only. In one case, an activating mutation of glucokinase (I211F) was found in pathological islets only. Both abnormalities, attributed to somatic genetic events, may account for inappropriate IS at low glucose levels by a subset of β-cells. They represent a novel cause of focal congenital hyperinsulinism. Diabetes 62:1689–1696, 2013

Congenital hyperinsulinism (CHI) is the major cause of severe and persistent hypoglycemia in neonates and infants and is a brain-damaging and potentially life-threatening condition (1–3). Excessive secretion of insulin by pancreatic β-cells has been linked to mutations in several genes. Most cases are caused by inactivating mutations in ABCC8 (encoding sulfonylurea receptor 1) or KCNJ11 (encoding Kir6.2) (4,5), the two subunits of ATP-sensitive K( KATP) channels that mediate the effects of glucose on β-cell membrane potential (6,7). Histologically, two forms of the KCNJ11 channel–related CHI have been identified (8,9). In diffuse forms inherited in an autosomal-recessive (rarely dominant) manner, all β-cells in all islets are affected, and subtotal pancreatectomy may be necessary. In focal forms, a localized adenomatous hyperplasia of abnormal β-cells is present in an otherwise normal pancreas, and its selective resection cures the patient (10,11). One important clinical feature of KATP channel–related CHI patients is their usual resistance to medical treatment with diazoxide (1–3). In vitro studies of islets or pancreatic fragments from operated patients have verified the following predictable consequences of this lack of functional KATP channels in β-cells: membrane depolarization; uncontrolled influx of Ca2+; increase in the cytosolic concentration of free Ca2+; and high rate of insulin secretion at low glucose levels (12–14). They further showed that drugs acting on KATP channels are unable to produce their normal stimulatory (tolbutamide) or inhibitory (diazoxide) effects on insulin secretion (14).

Less often CHI is caused by activating mutations in GCK (encoding glucokinase), GLUD1 (encoding glutamate dehydrogenase), or SLC16A1 (encoding monocarboxylate transporter 1) or by inactivating mutations in HADH (encoding 3-hydroxyacyl-CoA dehydrogenase), UCP2 (encoding mitochondrial uncoupling protein 2), HNF4A (encoding HNF4a), or HNF1A (encoding HNF1a) (15–21). It is unclear how mutations in HNF4A and HNF1A alter β-cell function. All the others affect distinct metabolic pathways in β-cells in such a way that too many otherwise normal KATP channels are closed at any glucose concentration. In these cases of accelerated β-cell metabolism, diazoxide retains its ability to open the channels, which explains the sensitivity of the patients to medical treatment with the drug (1–3). The islet features in KATP channel–unrelated, diazoxide-treatable CHI cases are not well-known because of the rarity and only exceptional surgical treatment of these cases.

A novel anatomopathological form of CHI was described recently in 16 patients (22). Its hallmark is a mosaicism of the islets. Morphologically hyperfunctional islets containing β-cells with large nuclei, abundant cytoplasm, and signs of intense proinsulin synthesis coexist with resting islets containing β-cells with small nuclei, shrunken cytoplasm, and signs of low proinsulin synthesis. Whereas hypoactive islets are present in the whole pancreas, hyperactive islets are located in one or in a few adjacent lobules. This concentration in a limited region of the gland explains why selective partial pancreatectomy often was curative (22). Despite some similarities, this pathological entity differs from KATP channel–related focal CHI by a lack of germinal mutation in ABCC8 or KCNJ11 and a clinical responsiveness of the cases to treatment with diazoxide (22).

Insulin secretion by pancreatic fragments from six of these patients could be investigated in vitro using the same methods as in our recent study of KATP channel–related CHI pancreas (14). We show that β-cells from the hyperactive region have functionally normal KATP channels, and
that the inappropriate secretion of insulin can be attributed to an increased responsiveness to glucose that, on the basis of immunohistochemical or genetic analyses of the tissues, we attribute to undue expression of low-K_{m} hexokinase I (HK-I) or to an activating mutation in GCK in hyperfunctional islets only.

**RESULTS**

Morphological aspect and sampling of tissue. Intraoperative diagnosis was based on examination of frozen pancreatic biopsy specimens stained with toluidine blue (Fig. 1). The characteristic feature is the coexistence of two types of islets (mosaicism). In small hypoactive islets, β-cells are poor in cytoplasm and have small nuclei (Fig. 1A), whereas hyperactive islets contain cytoplasm-rich β-cells with some enlarged nuclei (Fig. 1B). As previously described, hyperactive islets are concentrated in a few lobules of the gland (22). This pathological region was resected with a rim of normal tissue, and fragments from both regions were saved for functional studies. Postoperative examination of fixed specimens confirmed the segregation of the two types of islets, but separation of pathological and normal regions was not always complete. In particular, samples labeled “normal” still contained some hyperfunctional islets in cases 3 and 5. A small proportion of hypofunctional islets also were often present in pathological samples.

**Insulin content.** The initial insulin content of the tissue was not directly measured but was estimated as previously described (14). The pathological region did not contain more insulin (22.5 ng/mg; range, 6.5–47.5) than the normal region (23.1 ng/mg; range, 10.9–43.1).

**Effects of glucose, diazoxide, and tolbutamide on insulin secretion.** All experiments were performed in the presence of 1 μmol/L forskolin to increase β-cell cAMP levels. In normal pancreas (Fig. 2), an increase in the glucose concentration from 1 to 15 mmol/L stimulated insulin secretion several-fold. This stimulation was abolished by 100 μmol/L diazoxide and reversibly restored by 100 μmol/L tolbutamide. Except for a less pronounced biphasic pattern, this response is similar to that observed in normal adult islets (23) and in fragments of normal pancreas from infants (14). Figure 2 also shows responses of the pathological region from the same pancreas. In five of five cases (one comparison was not possible), insulin secretion rate in 1 mmol/L glucose was two-fold to many-fold higher than in adjacent normal pancreas. An increase in secretion was induced by 15 mmol/L glucose in five of six cases, and it was sustained in four cases. Strikingly, diazoxide inhibited insulin secretion to similar low levels as in normal pancreas, and tolbutamide consistently increased it in a reversible manner (Fig. 2). Altogether, these results indicate that K_{ATP} channels are functional in hyperactive islets.

**Effects of stepwise increases in glucose on insulin secretion.** In normal pancreas, glucose-induced insulin secretion was concentration-dependent, with a maximum already reached at 7 mmol/L (Fig. 3). The lack of a response in case 6 is considered unreliable because of low amounts of insulin in the sample. This concentration dependency was markedly altered in pathological pancreas. When the test was started in the absence of glucose (five of six cases), addition of only 1 mmol/L glucose consistently induced a large peak of secretion, whereas subsequent increases had no or little further effect except in case 6 (Fig. 3). At the end of the experiments, switching from 1 to 0 mmol/L glucose (in four of six cases) was followed by a further decrease in secretion rate. Although the threshold for glucose-induced insulin secretion in normal adult
islets or fragments of infant pancreas is at 3 mmol/L (14,23), an increase was observed in response to 1 mmol/L glucose in normal pancreas of cases 3 and 5. We attribute this premature response to the presence of a small proportion of abnormal β-cells in the sample. Another puzzling observation is that in three of four cases, insulin secretion rate was higher in pathological than in normal pancreas in absence of glucose.

**Effects of various agents on insulin secretion.**

Enough tissue for additional tests was available in only three cases. In two of these, basal insulin secretion (no glucose) was again slightly higher in pathological than in normal tissue (Fig. 4). Tolbutamide (25 μmol/L) consistently increased secretion, with a similar or greater efficacy in pathological than in normal pancreas. In mouse islet cells, inosine is split to hypoxanthine, which has no effect on insulin secretion, and ribose-1-phosphate, the subsequent metabolism of which increases ATP levels (24). Inosine mimics most effects of glucose in mouse islets (25) and induces insulin secretion in human adult islets (23). It was effective in pathological and in normal pancreas (Fig. 4). Finally, stimulation with 1 mmol/L glucose increased insulin secretion in pathological pancreas of the three cases, had no effect in normal pancreas of two cases, and was slightly effective in normal pancreas of case 5. These observations back-up those shown in Fig. 3.

In a last series performed with 1 mmol/L glucose throughout, lactate and pyruvate were without effect on insulin secretion by pathological and normal pancreas (three of three) (Fig. 5), which indicates that the pathology is not underlain by abnormal transport of monocarboxylic acids into β-cells (17,26). In contrast, membrane-permeant phenylpyruvate increased secretion two-fold to three-fold in both pathological and normal pancreas. A similar stimulation occurs in rodent islets through acceleration of β-cell mitochondrial metabolism and direct inhibition of K<sub>ATP</sub> channels (27).

**Expression of HK-I in hyperfunctional β-cells.**

The stimulation of insulin secretion by as little as 1 mmol/L glucose in pathological fragments led us to search for the presence of a low-K<sub>m</sub> hexokinase in β-cells. Immunocytochemistry for HK-I was positive in morphologically hyperfunctional islets from five cases as follows: strongly in cases 1, 3, 4, and 5, and weakly in case 2. However, it was consistently negative in their hypofunctional islets. Figure 6 illustrates this striking difference in case 3, in which all islets were labeled in the pathological region in contrast to islets in a normal adjacent lobule. Figure 6 also shows insulin immunolabeling in hypofunctional (Fig. 6B) and hyperfunctional islets (Fig. 6C) from case 1. HK-I was clearly negative in β-cells of hypofunctional islets (Fig. 6D) and positive in β-cells from hyperfunctional islets (Fig. 6E). Glucokinase was present (immunodetection) in β-cells positive for HK-I (Supplementary Fig. 1A and B).

**Glucokinase mutation in hyperfunctional islets.**

Because hyperfunctional islets from case 6 were negative for HK-I, we looked for a mutation in GCK after RNA extraction from sections of pathological and normal islets. As shown in Fig. 7, a mutation (I211F) was identified in the pathological region that also contained wild-type RNA. The presence of both abnormal and normal transcripts suggests that only one allele was mutated. No mutation was found in the normal region, which, together with the lack of GCK mutation in blood DNA of the patient (22),
indicates a somatic mutation in hyperfunctional islets. No mutation in GCK was found in islets from two cases showing weak (case 2) or strong (case 3) HK-I labeling in their hyperfunctional β-cells.

DISCUSSION
Histologically, the pancreas from CHI patients can display two major aspects. In diffuse forms, all islets look alike because all β-cells are affected through inheritance of a recessive or dominant germinal mutation in various genes involved in the control of insulin secretion. In focal forms, only a subset of islets contains abnormal β-cells. These islets are concentrated in a restricted zone of the pancreas, referred to as the focal lesion. Thus, focal CHI is a topographical, not a mechanistic, characteristic of the disease. At least two types exist.

The most frequent and best-defined focal lesions result from two genetic events, inheritance of a recessive paternal mutation in ABCC8 or KCNJ11 and somatic deletion of the corresponding maternal segment of chromosome 11p15 in a clone of β-cells. This causes loss of heterozygosity in these β-cells (28,29) and their hyperplasia because of the lack of maternally expressed tumor-suppressor

**FIG. 3.** Effects of stepwise increases and decreases in glucose concentration (G in mmol/L) on insulin secretion by normal (closed circles) and pathological pancreas (open circles) from each of the six studied cases. There was no period in G0 at the start or the end of the experiment with tissue from cases 1 and 2. Normal tissue was not available for case 4. The low insulin content of normal fragments from case 6 makes the results uncertain. Forskolin (Fk, 1 μmol/L) was present throughout.

**FIG. 4.** Effects of 25 μmol/L tolbutamide (Tolb) and 5 mmol/L inosine on insulin secretion by normal (closed circles) and pathological pancreas (open circles) from three cases. The experiments were started in the absence of glucose (G0), which was added at 1 mmol/L (G1) at 60 min. Forskolin (Fk, 1 μmol/L) was present throughout.
**CDKN1C** and **H19** associated with the unrestrained expression of paternal **IGF2** (28,30,31). The inactivating nature of the paternal mutation makes β-cells of these adenomatous focal lesions resistant to diazoxide that cannot inhibit insulin secretion in vivo (1–3) or in vitro (14). The second type of focal lesion is less sharply defined. It is characterized by a mosaicism of the islets, with hyperfunctional islets (without β-cell hyperplasia) preferentially localized in just a few lobules and resting islets in the rest of the pancreas. These patients have no mutation in **KATP** channel genes, have no loss of 11p15 heterozygosity in hyperfunctional β-cells, and are at least transiently sensitive to diazoxide treatment (22). The focal nature of the lesion is compatible with a somatic genetic event, but none has been identified so far.

The present in vitro study of the pancreas of six patients with a clinically diazoxide-sensitive focal pathology identified major differences with focal lesions linked to a mutation in **ABCC8** or **KCNJ11** genes. First, whereas the insulin concentration is ~14-fold higher in **KATP** channel-related focal lesions than in the adjacent normal pancreas (14), there is no difference between pathological and normal regions in this series. This may seem surprising because of the larger size of islets and β-cells in the lesion. However, immunodensitometry of insulin labeling showed that β-cells in small islets within the normal region contain more insulin than large islets within the pathological region (22). The second difference is the presence of functional **KATP** channels in β-cells from both pathological and normal regions. Thus, diazoxide produced a similar inhibition of insulin secretion, and tolbutamide was able to reverse this inhibition and to stimulate secretion in the absence of glucose and diazoxide. A third difference is that glucose increased insulin secretion in the lesion, whereas it did so inconsistently in **KATP** channel-related focal lesions (14). However, the concentration dependency of the response to glucose was strikingly abnormal. When we studied our first case (case 1), the experiments were started with 1 mmol/L glucose and revealed a much higher insulin secretion rate in the lesion than in normal tissue, despite evidence of functional **KATP** channels. This intriguing observation led us to begin subsequent studies in the absence of glucose, which uncovered that 1 mmol/L glucose caused a rapid and large peak of insulin secretion in the five other cases. Further stepwise increases in glucose produced no or little additional stimulation, but an increase from 1 to 15 mmol/L was often effective.

Stimulation of insulin secretion by 1 mmol/L glucose suggested an anomaly in glucose metabolism. In rodent and human β-cells, glucose is phosphorylated by the high-Km glucokinase (32,33), whose properties set the threshold for stimulation of insulin secretion at ~3 mmol/L in human islets (23,34). Normal β-cells express no or little low-Km HK. Using immunohistochemistry, we detected the presence of HK-I in islets from five of six of our cases. HK-I was localized in insulin-containing cells of hyperactive islets within the pathological region, whereas hypactive islets were consistently negative for HK-I. We therefore propose that the abnormal insulin secretion in response to 1 mmol/L glucose is attributable to HK-I substituting to glucokinase for phosphorylating glucose. β-cells positive for HK-I also were positive for glucokinase. This may explain why some insulin response persisted on stimulation with glucose concentrations >1 mmol/L. Admittedly, no direct measurements of glucose metabolism are available, but our conclusion is compatible with experimental models. Thus, transgenic mice expressing the low-Km yeast HK-B in β-cells showed lower blood glucose levels with relative hyperinsulinemia. In vitro, their islets displayed increased responsiveness to glucose, with higher insulin secretion rates at basal (3 mmol/L) glucose (36). Forced expression of mammalian HK-I in mouse insulinoma (MIN-6) cells or rat islets also led to increases in glucose metabolism and augmentation of basal insulin secretion (37,38).

Unexpectedly, HK-I was not detected in hyperfunctional islets from case 6, in which no **GCK** mutation had been found in blood cell DNA (22). We therefore searched for a somatic mutation in **GCK** by sequencing RNA extracted from pancreatic sections. One mutation (I211F) was identified together with wild-type RNA in the pathological region but was not detected in the normal region. In vitro experiments have shown that the I211F variant is the most active human glucokinase identified to date (39), but it had not been reported in CHI patients. In our case 6, expression of the hyperactive glucokinase in a subset of β-cells is explainable by a somatic mutation during embryogenesis.

Activating mutations of **GCK** are a rare cause of generally autosomal-dominant CHI. Since the first description (15), 11 mutations have been reported (40). The phenotype ranges from asymptomatic to marked hypoglycemia, with
the most severe cases being caused by the most activating mutations (40). Most cases are responsive to diazoxide, but the needed dose may be high when the activity of glucokinase is strongly increased (40). Interestingly, our case 6 was initially controlled by diazoxide, but the sensitivity to the drug progressively diminished (22), perhaps because of the high activity of the mutated enzyme in a number of β-cells that increased with aging. Patients with an inherited glucokinase mutation have only rarely undergone subtotal pancreatectomy. No islet abnormalities were detected in two patients (40,41). In a third patient, islets were increased in size and contained β-cells with relatively large nuclei (42). These two features also were observed in pathological regions of case 6 with a glucokinase mutation and in pathological regions of the five cases with HK-I expression, which displayed similar features.

Functionally, also, the cases with HK-I expression closely resembled the case with a glucokinase mutation. However, we do not know what caused expression of HK-I in a subset of β-cells segregated from the others. We can only suggest that a somatic genetic event occurred during development of the pancreas, which may have affected the HK-I gene itself or another gene that normally represses expression of the latter. Increased expression of HK-I and excessive insulin secretion at low glucose levels have been observed in islets from mice lacking PKC-α in their β-cells, and both defects were corrected by re-expression of the simultaneously decreased Foxa2 (43). INS-1 cells expressing a dominant-negative Foxa2 also showed upregulation of HK-I, which explained the left shift of the glucose dependency of insulin secretion (44). Deletion of Foxa2 in all β-cells caused severe neonatal hypoglycemia with relative hyperinsulinism (45) that was at least partly attributable to defects in K<sub>ATP</sub> channels (46). K<sub>ATP</sub> channels are functional in pathological islets of our patients, and islets positive for HK-I also stain for Foxa2 (Supplementary Fig. 1C and D). Deletion of Foxa2 in all β-cells caused severe neonatal hypoglycemia with relative hyperinsulinism (45) that was at least partly attributable to defects in K<sub>ATP</sub> channels (46).

Dominant mutations in ABCC8 cause a mild form of CHI that can often (47,48), although not always (49,50), be controlled by diazoxide. Expression studies under simulated heterozygous conditions produced channels with variably impaired sensitivity to physiological and pharmacological regulators. In our cases, in vitro sensitivity of pathological islets to diazoxide and tolbutamide thus is theoretically compatible with a somatic dominant mutation in ABCC8 or KCNJ11. Formal exclusion of this possibility faces the major difficulty of sequencing the two genes in tiny amounts of fixed material, and several arguments speak against that explanation. Impairing the function of β-cell K<sub>ATP</sub> channels might increase insulin secretion rate in low glucose levels but is not expected to cause a large response to 1 mmol/L glucose without intervention of an abnormal phosphorylation of the sugar. It is not plausible that mutations in K<sub>ATP</sub> channel subunits and glucokinase occurred simultaneously, and there is no published evidence that HK-I expression occurs secondarily to K<sub>ATP</sub> channel dysfunction. We reinvestigated several cases from our recently studied series of CHI caused by ABCC8 mutations (14) and found that immunostaining for HK-I was negative in islets from patients lacking K<sub>ATP</sub> channels in all their β-cells (diffuse form attributable to homozygous recessive mutations) and in focal lesions (paternal recessive mutation with loss of heterozygosity).

Importantly, no staining for HK-I was detected in islets outside these K<sub>ATP</sub> channel–related focal lesions, where β-cells are heterozygous for the mutation (Supplementary Fig. 2). Functional data also are inconsistent with this explanation. Thus, in some of these K<sub>ATP</sub> channel–related focal or diffuse cases (14), the experimental protocol to test insulin secretion in vitro involved a change from 0 to 1 mmol/L glucose and no increase was observed (Supplementary Fig. 3).

Because of the islet mosaicism that characterizes the pancreas of studied patients, complete separation of normal and pathological pancreatic fragments for subsequent functional studies is virtually impossible. The presence of
some hyperactive islets within “normal” fragments is the most plausible explanation for the small but indisputable response to 1 mmol/L glucose in some cases. However, the possible presence of a minority of normal islets within “pathological” fragments would not invalidate our main conclusions because it could not explain why 1 mmol/L glucose evokes a large peak of insulin secretion and how diazoxide completely inhibits insulin secretion if abnormal β-cells were not also sensitive to the drug. We acknowledge that our study suffers from certain limitations, but we emphasize that the amounts of available tissue are limited, which restricts the number of possible investigations.

In conclusion, we have identified undue presence of HK-I for an unknown cause (five cases) and an activating mutation in GCK (one case) in subsets of β-cells from CHI patients. These molecular abnormalities, which lead to inappropriate secretion of insulin at low glucose levels in vitro, may explain the hyperinsulinemic hypoglycemia in vivo. The pathogeny appears unrelated to KATP channel dysfunction but typical of a metabolic dysfunction that is sensitive to diazoxide treatment. The peculiarity in this group of subjects is that the genetic events are somatic and therefore affect only a fraction of islets concentrated in a focal lesion, the surgical resection of which can cure or markedly improve the patients.

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J.-C.H. and J.R. designed the study, J.-C.H., C.S., M.N., and J.R. analyzed data. J.-C.H. wrote the manuscript. C.S., J.M., S.G., Y.G., and M.N. performed research. C.S., J.M., S.G., Y.G., M.N., and J.R. edited the manuscript. J.-C.H. is the guarantor of this work and, as such, had full access to all the data and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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