In vitro insulin secretion by pancreatic tissue from infants with diazoxide-resistant congenital hyperinsulinism deviates from model predictions

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Congenital hyperinsulinism (CHI) is the major cause of persistent neonatal hypoglycemia. CHI most often occurs due to mutations in the ABCC8 (which encodes sulfonylurea receptor 1) or KCNJ11 (which encodes the potassium channel Kir6.2) gene, which result in a lack of functional KATP channels in pancreatic β cells. Diffuse forms of CHI (DiCHI), in which all β cells are abnormal, often require subtotal pancreatectomy, whereas focal forms (FoCHI), which are characterized by localized hyperplasia of abnormal β cells, can be cured by resection of the lesion. Here, we characterized the in vitro kinetics of insulin secretion by pancreatic fragments from 6 DiCHI patients and by focal lesion and normal adjacent pancreas from 18 FoCHI patients. Responses of normal pancreas were similar to those reported for islets from adult organ donors. Compared with normal pancreas, basal insulin secretion was elevated in both FoCHI and DiCHI tissue. Affected tissues were heterogeneous in their secretory responses, with increased glucose levels often producing a rapid increase in insulin secretion that could be followed by a paradoxical decrease below prestimulatory levels. The KATP channel blocker tolbutamide was consistently ineffective in stimulating insulin secretion; conversely, the KATP channel activator diazoxide often caused an unanticipated increase in insulin secretion. These observed alterations in secretory behavior were similar in focal lesion and DiCHI tissue, and independent of the specific mutation in ABCC8 or KCNJ11. They cannot be explained by classic models of β cell function. Our results provide insight into the excessive and sometimes paradoxical changes in insulin secretion observed in CHI patients with inactivating mutations of KATP channels.

Introduction

Congenital hyperinsulinism (CHI) is the major cause of persistent hypoglycemia in newborns and infants (1, 2). The underlying genetic etiology and biochemical mechanisms are multiple, resulting in heterogeneous clinical presentation from relatively mild, medically responsive forms to severe forms requiring near total pancreatectomy. The most common and severe cases of CHI are caused by inactivating mutations in the ABCC8 (90%) and KCNJ11 (10%) genes, which are both located on chromosome 11p15 and respectively encode sulfonylurea receptor 1 (SUR1) and Kir6.2, the regulatory and pore-forming subunits of ATP-sensitive K (KATP) channels with membrane hyperpolarization, lowering of [Ca<sup>2+</sup>]<sub>i</sub> and cessation of insulin secretion.

Drugs that increase or lower β cell [Ca<sup>2+</sup>], independently of metabolism reproduce the secretory changes mediated by the triggering pathway (9). Antidiabetic sulfonylureas augment insulin secretion by closing K<sub>ATP</sub> channels, whereas diazoxide inhibits insulin secretion by opening the channels. This explains the use of diazoxide as primary medical treatment of CHI (1). This approach is successful except in most patients with a mutation of K<sub>ATP</sub> channels, who therefore require other medical and dietary treatments and eventually surgical pancreatic resection in the most severe cases. Histologically, 2 forms of diazoxide-resistant hyperinsulinism can be distinguished (10, 11). In diffuse forms of CHI (DiCHI), all β cells in all islets are affected. The genetic cause is a recessive (more rarely dominant) mutation in ABCC8 or KCNJ11 (2, 6, 7, 10, 11). In contrast, focal hyperinsulinism (FoCHI) is characterized by localized hyperplasia of abnormal β cells. In FoCHI, the genetic cause is a dominant mutation in either ABCC8 or KCNJ11 (2, 6, 7). A decrease in Mg-ADP concentrations in the cytosol, which close K<sub>ATP</sub> channels. As a consequence, the membrane depolarizes and voltage-gated Ca<sup>2+</sup> channels open, allowing Ca<sup>2+</sup> influx and leading to a rise in the cytosolic free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) that triggers exocytosis of insulin granules. Simultaneously, glucose activates an amplifying pathway that does not elevate [Ca<sup>2+</sup>]<sub>i</sub>, further but augments the secretory response to high [Ca<sup>2+</sup>]<sub>i</sub>. Conversely, a fall in blood glucose is normally followed by opening of K<sub>ATP</sub> channels with membrane hyperpolarization, lowering of [Ca<sup>2+</sup>]<sub>i</sub>, and cessation of insulin secretion.

Glucose control of insulin secretion is normally achieved through the interaction of triggering and amplifying pathways in β cells (reviewed in ref. 8). K<sub>ATP</sub> channels play an essential role in the triggering pathway. When β cells sense a rise in blood glucose, their metabolism accelerates, causing an increase in ATP and

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ATP

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of heterozygosity (with isodisomy or duplication of the paternal ing maternal segment of chromosome 11p15, which leads to loss

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12, 13). In focal forms (FoCHI), a localized hyperplasia of abnor

maling maternal segment of chromosome 11p15, which leads to loss of heterozygosity (with isodisomy or duplication of the paternal mutated allele) (14, 15). The same 11p15 region also contains several imprinted genes involved in cell replication: the maternally expressed CDKN1C (p57) and H19, and the paternally expressed IGF-II, which respectively inhibit and promote cell proliferation. The local β cell hyperplasia is attributed to the imbalance between these genes (16, 17). Distinction between these 2 forms has major therapeutic implications: whereas medically unresponsive DiCHI usually requires near total pancreatectomy, FoCHI can be cured by a selective localized resection of the lesion (18, 19).

Patch-clamp studies (reviewed in ref. 5) have shown that β cells, obtained after near total pancreatectomy of diazoxide-resistant DiCHI patients, have no or very few functional \( K_{\text{ATP}} \) channels in their plasma membranes. They are depolarized, with variably elevated [Ca\( ^{2+} \)], in the presence of low glucose concentrations, and do not display changes in [Ca\( ^{2+} \)], upon application of high glucose concentrations, tolbutamide, or diazoxide (20, 21). Studies of 3 FoCHI cases showed that functional \( K_{\text{ATP}} \) channels are absent from β cells within the focal lesion and present and normally regulated in the adjacent pancreas (22). In vitro data on insulin secretion by CHI pancreas are scanty and sometimes controversial. Three studies were performed before the pathogenesis of the disease started to be understood. Islets isolated from 1 diazoxide-resistant CHI patient doubled insulin secretion in response to glucose (23). Islet cell clusters from 4 diazoxide-resistant DiCHI patients poorly responded to glucose but were stimulated by cAMP (24). Islet cell clusters from 4 DiCHI patients were stimulated by cAMP, were insensitive to high glucose, and variably responded to other fuels while maintained in culture for 1–4 weeks (25). More recently, insulin secretion was studied in islets from 3 DiCHI patients lacking functional \( K_{\text{ATP}} \) channels in their β cells because of a mutation in \( ABCC8 \). Tolbutamide and KCl were ineffective in low glucose conditions, but high glucose conditions were stimulatory. The effect

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Table 1

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Months at surgery</th>
<th>Post-op</th>
<th>Mutation Gene</th>
<th>Nucleotide level</th>
<th>Protein level</th>
<th>Impact on channel function (ref.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FoCH11</td>
<td>5</td>
<td>Cured</td>
<td>( KCNJ11 ) Exon 1( ^{a} )</td>
<td>c.244_247dupAGCT</td>
<td>p.Trp38X</td>
<td>Intra cellular retention (58, 59)</td>
</tr>
<tr>
<td>FoCH12</td>
<td>7.5</td>
<td>Cured</td>
<td>( ABCC8 ) Exon 4</td>
<td>c.560T&gt;A</td>
<td>p.Val187Asp</td>
<td>No ( K_{\text{ATP}} ) current (57)</td>
</tr>
<tr>
<td>FoCH13</td>
<td>2</td>
<td>Cured</td>
<td>( ABCC8 ) Exon 4</td>
<td>c.560T&gt;A</td>
<td>p.Val187Asp</td>
<td>Intra cellular retention (58, 59)</td>
</tr>
<tr>
<td>FoCH14</td>
<td>2</td>
<td>Cured</td>
<td>( ABCC8 ) Exon 4</td>
<td>c.560T&gt;A</td>
<td>p.Val187Asp</td>
<td>No ( K_{\text{ATP}} ) current (57)</td>
</tr>
<tr>
<td>FoCH15</td>
<td>14</td>
<td>Cured: 2 op</td>
<td>( ABCC8 ) Intron 4</td>
<td>c.580-16&gt;G&gt;C</td>
<td>p.?</td>
<td>Intra cellular retention (60)</td>
</tr>
<tr>
<td>FoCH16</td>
<td>7</td>
<td>Cured</td>
<td>( ABCC8 ) Exon 5</td>
<td>c.683G&gt;A</td>
<td>p.Gly228Asp</td>
<td>No ( K_{\text{ATP}} ) current (66)</td>
</tr>
<tr>
<td>FoCH17</td>
<td>11</td>
<td>Cured</td>
<td>( ABCC8 ) Intron 7</td>
<td>c.1176+2T&gt;C</td>
<td>p.?</td>
<td>Intra cellular retention (58, 59)</td>
</tr>
<tr>
<td>FoCH18</td>
<td>9</td>
<td>Cured: 3 op</td>
<td>( ABCC8 ) Exon 12</td>
<td>c.1792C&gt;T</td>
<td>p.Arg598X</td>
<td>No ( K_{\text{ATP}} ) current (57)</td>
</tr>
<tr>
<td>FoCH19</td>
<td>3.5</td>
<td>Cured</td>
<td>( ABCC8 ) Exon 12( ^{a} )</td>
<td>c.1817G&gt;A</td>
<td>p.?</td>
<td>Intra cellular retention (58, 59)</td>
</tr>
<tr>
<td>FoCH20</td>
<td>6</td>
<td>Cured</td>
<td>( ABCC8 ) Exon 15</td>
<td>c.2064G&gt;A</td>
<td>p.Trp688X</td>
<td>No ( K_{\text{ATP}} ) current (57)</td>
</tr>
<tr>
<td>FoCH11</td>
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<td>Cured</td>
<td>( ABCC8 ) Exon 22( ^{a} )</td>
<td>c.2588_2591delATCT</td>
<td>p.His863fs</td>
<td></td>
</tr>
<tr>
<td>FoCH12</td>
<td>2</td>
<td>Cured: 2 op</td>
<td>( ABCC8 ) Intron 32</td>
<td>c.3991+2_3991+15del14</td>
<td>p.?</td>
<td>Intra cellular retention (58, 59)</td>
</tr>
<tr>
<td>FoCH13</td>
<td>2.5</td>
<td>Cured</td>
<td>( ABCC8 ) Exon 36</td>
<td>c.4325delC</td>
<td>p.Pro1442fs</td>
<td>Intra cellular retention (58, 59)</td>
</tr>
<tr>
<td>FoCH14</td>
<td>8</td>
<td>Cured</td>
<td>( ABCC8 ) Exon 36( ^{a} )</td>
<td>c.4354T&gt;C</td>
<td>p.Trp1452Arg</td>
<td></td>
</tr>
<tr>
<td>FoCH15</td>
<td>10</td>
<td>Cured: 2 op</td>
<td>( ABCC8 ) Exon 36( ^{a} )</td>
<td>c.4356G&gt;T</td>
<td>p.Glu1453Asp</td>
<td></td>
</tr>
<tr>
<td>FoCH16</td>
<td>10.5</td>
<td>Cured</td>
<td>( ABCC8 ) Exon 36</td>
<td>c.4406G&gt;T</td>
<td>p.Gly1470Val</td>
<td></td>
</tr>
<tr>
<td>FoCH17</td>
<td>5</td>
<td>Cured</td>
<td>( ABCC8 ) Intron 36</td>
<td>c.4415-13G&gt;A</td>
<td>p.?</td>
<td></td>
</tr>
<tr>
<td>FoCH18</td>
<td>6</td>
<td>Cured</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>DiCH1</td>
<td>3</td>
<td>Diabetes</td>
<td>( ABCC8 ) Exon 5</td>
<td>c.683G&gt;A; Homoz</td>
<td>p.Gly228Asp</td>
<td></td>
</tr>
<tr>
<td>DiCH2</td>
<td>5</td>
<td>Diabetes</td>
<td>( ABCC8 ) Exon 9</td>
<td>c.1420C&gt;T; Homoz</td>
<td>p.Gin474X</td>
<td></td>
</tr>
<tr>
<td>DiCH3</td>
<td>2.5</td>
<td>Glucose intoler.</td>
<td>( ABCC8 ) Intron 28( ^{a} )</td>
<td>c.3561-3C&gt;G; Homoz</td>
<td>p.?</td>
<td>No ( K_{\text{ATP}} ) current, but rescue</td>
</tr>
<tr>
<td>DiCH4</td>
<td>6</td>
<td>Diabetes: 2 op</td>
<td>( ABCC8 ) Exon 34</td>
<td>c.4160C&gt;T</td>
<td>p.Ser1387Phe</td>
<td></td>
</tr>
<tr>
<td>DiCH5</td>
<td>7</td>
<td>Hyposis</td>
<td>( ABCC8 ) Exon 37</td>
<td>c.4480C&gt;T; Homoz</td>
<td>p.Arg1494Trp</td>
<td></td>
</tr>
<tr>
<td>DiCH6</td>
<td>18</td>
<td>Diabetes</td>
<td>( ABCC8 ) Exons 28( ^{a} ) and 37</td>
<td>c.3559A&gt;G; c.4481G&gt;A</td>
<td>p.Arg1187Gly; p.Arg1494Gln</td>
<td></td>
</tr>
</tbody>
</table>

The mutation nomenclature is based on the reference sequences \( KCNJ11 \) NM_000525.3 and \( ABCC8 \) NM_000382.3 corresponding to the L72808 isoform (1,582 amino acids), which incorporates the extra serine residue in exon 17 (8). All mutations identified at heterozygous state with the exception of DiCH1, -2, -3, and -5 (homozygous), and DiCH6 (compound heterozygous). Mutations believed to be novel. References to the others in refs. 6, 7. Post-op, post-operative; glucose intoler.; glucose intolerant; homoz, homozygous; op, operations.
of glucose was Ca\(^{2+}\) dependent, but it is not known whether it was sensitive to diazoxide (21). Unfortunately, in none of the above studies was appropriate control tissue available for comparison.

In the present study, we used a perifusion system to characterize in vitro the kinetics of insulin secretion by the pancreas of 24 infants suffering from diazoxide-resistant FoCHI or DiCHI because of inactivating mutations of K\(_{ATP}\) channels in their β cells. In all cases, intraoperative microscopic examination of frozen sections established the differential diagnosis (19) and dictated the localization and extent of pancreatic resection. This also made it possible to select fragments of normal and pathologic tissue from the pancreas of patients with FoCHI and to compare their secretory behavior. Such a comparison is crucial for identification and correct interpretation of abnormalities in the focal lesion and in the whole pancreas from DiCHI patients.

**Results**

**Morphological aspect of DiCHI and FoCHI pancreas.** The 6 DiCHI and 18 FoCHI studied subjects (Table 1) underwent pancreatic surgery because of their clinical resistance to diazoxide treatment. In DiCHI pancreas, the number, size, and general appearance of the islets (Figure 1A) are similar to those in the pancreas from control infants (11). Intraoperative diagnosis, based on the presence of β cells with an abnormally large nucleus and abundant cytoplasm in frozen specimens (Figure 1B), was subsequently confirmed on fixed specimens. The pattern is different in FoCHI pancreas. As shown in Figure 1D, focal lesions consist of an agglomerate of large islets. The size of the lesion is variable (usually < 10 mm in diameter), and its limits are not always as regular as in the figure. Outside the focal lesion, islets contain β cells with a small nucleus and relatively little cytoplasm, which give the impression of packed cells (Figure 1E and ref. 11). In DiCHI, CDKN1C is normally expressed, so that all islets contain cells positive for the CDKN1C (p57) protein (Figure 1C). In FoCHI pancreas, CDKN1C immunostaining is positive in islets outside the lesion (Figure 1F) but negative in the focal lesion (Figure 1G) because of the loss of maternal 11p15 (16, 17). These characteristics were present in all studied cases.

When intraoperative diagnosis of DiCHI was established, subtotal pancreatectomy was performed and an unselected fragment of the whole organ was saved for functional studies. In FoCHI, the resection was limited to the lesion and a zone of normal tissue at the periphery. The pieces of normal and pathologic tissue destined for functional studies were selected by morphological examination of adjacent frozen samples, with a preference for the inconvenience of small size over the risk of overlapping zones.

**Insulin content and insulin secretion during culture.** The initial insulin content of fresh pancreas was not directly measured but estimated by adding the total amount of insulin secreted during the experiments for functional studies was appropriate control tissue available for comparison.
and the tissue content at the end of the experiments (see Methods). The normal region of FoCHI pancreas contained 46 ng insulin/mg on average and secreted 21.5% of this content during the approximately 19 hours of culture in the presence of 5 mM glucose (Table 2). In the focal lesion, the insulin content was 14-fold greater than in the normal region, but fractional insulin secretion during culture (29.5%) was not consistently larger (on average 1.4-fold, NS by paired t test). In DiCHI pancreas, insulin content (39 ng/mg) and fractional insulin secretion during culture (24.7%) were not different from those in the normal region of FoCHI pancreas (Table 2). The surprising similarity between fractional insulin secretion rates in FoCHI and DiCHI tissue and in normal pancreas is attributed to the stimulation of normal islets by the concentration of 5 mM glucose in culture medium (see below).

**Acute effects of glucose on insulin secretion.** Stimulation of the normal pancreas of FoCHI subjects by a rapid increase in glucose from 1 to 15 mM induced biphasic insulin secretion (Figure 2A). Second phase was suppressed by diazoxide and restored by tolbutamide. At the end of the experiments, addition of forskolin to increase cAMP in β cells doubled insulin secretion (Figure 2A). The presence of forskolin during the whole experiment augmented the amplitude of both first and second phases of glucose-induced secretion, but did not alter the inhibitory and stimulatory effects of diazoxide and tolbutamide (compare Figure 2C with Figure 2A and note the different scales). These changes, here measured with partially digested pancreas, are superimposable on those observed with isolated islets from adult organ donors (26). At least one of the protocols shown in Figure 2, A and C, was performed with normal pancreas of each FoCHI patient and gave qualitatively similar results, attesting to the quality of the tested tissues. Supplemental Figures 1 and 2 (supplemental material available online with this article; doi:10.1172/JCI58400DS1) show all individual responses in the absence and presence of forskolin, respectively.
The focal lesion of the same subjects was characterized by an elevated basal insulin secretion rate in 1 mM glucose (2.8-fold higher than in paired control pancreas; \( P < 0.001 \)) (Figure 2A). Stimulation by 15 mM glucose induced a modest first phase of insulin secretion in 6 of 10 focal lesions (\( P < 0.01 \) for all responses), and a small second phase increase above the high prestimulatory values was detected in only 3 of 10 subjects. Diazoxide never inhibited insulin secretion but unexpectedly increased it in 3 of 10 subjects (Supplemental Figure 1), although the mean change was not significant (Figure 2A). Tolbutamide was always ineffective, and forskolin, added at the end of the experiments, approximately doubled the secretion rate (Figure 2A).

When forskolin was present from the start of the experiments, basal insulin secretion was augmented and the difference with paired control pancreas now averaged 4.1-fold (\( P < 0.001 \)) (Figure 2A). Stimulation with 15 mM glucose induced a small first peak of secretion in 5 of 11 preparations (\( P < 0.05 \) for all responses). This small peak was followed by a paradoxical decrease (\( P < 0.01 \)) during second phase. We acknowledge that, in the absence of paired experiments in low glucose throughout, it is debatable whether the average decrease in insulin secretion rate (Figure 2C) was induced by high glucose or was spontaneous. However, examination of the individual responses (Supplemental Figure 2) suggests that an inhibition by glucose was real in 6 of 11 cases. Equally paradoxical was the increase in insulin secretion produced by diazoxide in 6 of 11 cases (Supplemental Figure 2) (\( P < 0.05 \) for all responses). In contrast, tolbutamide was always without effect (Figure 2B). In 3 other experiments, omission of CaCl\(_2\) from the perifusion medium markedly inhibited the high rate of insulin secretion in FoCHI pancreas (not shown).

Mean insulin secretory responses of DiCHI pancreas were similar to those of focal lesions (Figure 2, B and D). Responses of individual DiCHI cases tested without and with forskolin are shown in Supplemental Figures 3 and 4. In the presence of forskolin throughout, 15 mM glucose induced an initial peak of insulin secretion in 4 of
6 subjects and a paradoxical decrease below initial values during second phase in 5 of 6 subjects ($P < 0.01$). The stimulatory effect of diazoxide was consistently observed (6 of 6) ($P < 0.01$). Pinacidil, a structurally unrelated $K_{ATP}$ channel opener with greater affinity for SUR2 than SUR1, inhibits glucose-induced insulin secretion in normal islets (27) and, like diazoxide, is inactive in Sur1-knockout islets (28). Pinacidil also paradoxically increased insulin secretion in FoCHI lesion and DiCHI pancreas perifused with 3 mM glucose ($P=0.01$), while having no effect in normal pancreas (Supplemental Figure 5).

Stepwise increases of the glucose concentration in the presence of forskolin induced a concentration-dependent increase in insulin secretion in the normal pancreas from FoCHI subjects (Figure 3A). A response was already evoked by 3 mM glucose in 4 of 7 cases ($P < 0.01$ for the whole group), and a maximum was reached at 7 mM glucose. Excellent reversibility was observed on return to 1 mM glucose at the end of the experiments. Whereas the threshold at 3 mM was the same as in adult islets, the maximum effect of glucose was reached at a lower concentration (26). These progressive changes in glucose concentration were without significant effect on insulin secretion by focal lesions in the presence of forskolin (Figure 3A). They were also ineffective in DiCHI pancreas, both in the presence and absence of forskolin (Figure 3B).

In the presence of only 1 mM glucose, tolbutamide, used at the concentration of 500 $\mu$M to close all $K_{ATP}$ channels, consistently stimulated insulin secretion from normal pancreas of FoCHI patients (Figure 3C). An initial peak was followed by a slow decline and stabilization at approximately 1.8-fold above baseline ($P < 0.05$). Subsequently, raising the concentration of glucose to 15 mM in the...
presence of tolbutamide was followed by a slight increase in insulin secretion (4 of 5 subjects) \( (P < 0.05) \), an effect that is attributed to the amplifying pathway \( (8) \). In focal lesion, tolbutamide was ineffective in 1 mM glucose, but the subsequent increase in glucose induced a first peak of insulin secretion \( (4 \text{ of } 5 \text{ subjects}) \ (P < 0.05) \) and a minor and inconsistent \( (2 \text{ of } 5 \text{ subjects}) \) sustained elevation \( (\text{NS for the whole group}) \). A qualitatively similar response was observed in DiCHI pancreas \( (\text{Figure 3D}) \). In focal lesion and DiCHI pancreas, these effects of glucose on top of tolbutamide are not different from those of glucose alone \( (\text{compare with Figure 2B}) \).

**Acute effects of other agents on insulin secretion.** In the presence of 3 mM glucose and forskolin throughout, the combination of leucine and glutamine \( (5 \text{ mM each}) \) induced biphasic insulin secretion from the normal pancreas of FoCHI patients \( (\text{Figure 4A}) \). The effect of the amino acids was reversibly abrogated by omission of extracellular CaCl\(_2\) and markedly inhibited by activation of \( \alpha_2 \)-adrenoceptors with clonidine. In focal lesion, the combination of amino acids evoked a first-phase insulin secretion in 4 of 4 preparations \( (P = 0.05) \) but no secondary elevation. CaCl\(_2\) omission inhibited the high rate of secretion to basal values similar to those in control pancreas, i.e., well below the initial secretion rate in focal lesions. Clonidine caused partial inhibition \( (\text{Figure 4A}) \). In DiCHI pancreas, leucine and glutamine induced a first-phase insulin secretion in only 2 of 4 preparations. Omission of CaCl\(_2\) consistently suppressed secretion, while clonidine was inhibitory in 3 of 4 preparations \( (\text{Figure 4B}) \), as was epinephrine in a previous study \( (25) \). In mouse islets lacking \( K_{\text{ATP}} \) channels because of a knockout of \( Sur1 \), clonidine abrogated insulin secretion by lowering \( [\text{Ca}^{2+}]_c \), and interfering with exocytosis \( (28) \). In a single case \( (\text{DiCHI}) \), glutamine \( (2 \text{ mM}) \) was tested alone and only had a very small effect on insulin secretion, in contrast with the large response induced by a mixture of amino acids \( (\text{Supplemental Figure 6}) \).

In the presence of 15 mM glucose, acetylcholine and KCl reversibly increased insulin secretion in the normal pancreas of FoCHI patients \( (\text{Figure 4C}) \). At the end of the experiments, addition of azide, a mitochondrial poison that lowers ATP levels in islets \( (29) \), abolished insulin secretion. In focal lesions, qualitatively similar but slightly smaller effects were produced by acetylcholine \( (4 \text{ of } 4 \text{ subjects}) \) and KCl \( (3 \text{ of } 4 \text{ subjects}) \). In contrast, azide did not inhibit insulin secretion \( (\text{Figure 4C}) \). The responses of DiCHI pancreas were similar to those of FoCHI pancreas \( (\text{Figure 4D}) \). We tentatively attribute the smaller effects of KCl and acetylcholine to a lesser stimulation of Ca\(^{2+}\) influx in depolarized CHI \( \beta \) cells. The suppression of glucose-induced insulin secretion by azide in control pancreas is attributed to the opening of \( K_{\text{ATP}} \) channels with subsequent lowering of \([\text{Ca}^{2+}]_c\). No such effect can occur in CHI \( \beta \) cells lacking functional \( K_{\text{ATP}} \) channels \( (29) \).

**Acute effects of diagnostic agents on insulin secretion.** Acute stimulation by CaCl\(_2\), tolbutamide, or arginine are commonly used tests in the diagnostic phase of CHI \( (30–33) \). In the presence of 5 mM glucose and forskolin, an increase in extracellular CaCl\(_2\) from 1.25 to 10 mM evoked a large peak of insulin secretion in the normal pancreas of FoCHI patients, and so did 100 \( \mu \text{M} \) tolbutamide and 10 mM arginine \( (\text{Figure 5A}) \). In focal lesions, CaCl\(_2\) and arginine also induced peaks of insulin secretion, whereas tolbutamide had no effect. In DiCHI pancreas, tolbutamide was consistently ineffective in contrast with CaCl\(_2\) and arginine, the effects of which were markedly potentiated by forskolin \( (\text{Figure 5B}) \).

**Discussion.**

**Preparation.** Because islet organization is altered in focal adenomatous lesions, we dispersed normal and pathological fragments in exactly the same way. Although morphological \( (\text{Figure 1 and ref. 11}) \) and functional \( (32) \) indices suggest that \( \beta \) cells are quiescent outside focal lesions, secretory responses by fragments of normal pancreas from FoCHI patients were similar to those by islets isolated from healthy adult organ donors \( (26) \). This means that any alterations of \( \beta \) cell function in vivo \( (\text{e.g., by drug treatment or lack of stimulation}) \) have been reversed by the initial 17–22 hours of culture in 5 mM glucose. However, 2 quantitative differences must be pointed out. First, whereas the low threshold of 3 mM for glucose-induced insulin secretion is similar in islets and fragments,
the maximum effect of glucose is reached at a much lower concentration (7 vs. 15 mM) in fragments of infant pancreas. Second, the increase in insulin secretion occurring when the concentration of glucose is raised after closure of K$_{ATP}$ channels by tolbutamide (measure of the amplifying pathway) is of smaller magnitude than in adult islets (26). Because no similar studies with islets from normal young infants are available, it is unclear whether these differences are solely attributable to the age of the subjects. With this reservation, we are confident that our procedure did not distort β cell secretory function. Most importantly, results obtained with focal lesions were included in the study only when healthy pancreas from the same patient was available for comparison and displayed qualitatively normal responses to glucose, diazoxide, and tolbutamide (Supplemental Figures 1 and 2). This control is crucial to ascertain that abnormal responses of the lesion are not simply due to tissue damage. Normal pancreas from FoCHI patients was also used as control for the responses recorded in DiCHI that affects all islets from the patients.

Major abnormalities of insulin secretion in CHI pancreas. We first acknowledge that, in contrast to the good intersubject reproducibility of insulin secretion in normal pancreas, some alterations were inconsistently observed in focal lesion and DiCHI pancreas, which complicates their interpretation. We next emphasize that our study was performed in a subgroup of CHI infants requiring partial or subtotal pancreatectomy because of clinical resistance to diazoxide and other medical treatment. The abnormalities of insulin secretion would be different if the mutations led to expression of partially functional K$_{ATP}$ channels or to defects in other pathways. In both focal lesion and DiCHI pancreas, insulin secretion rate in the presence of 1–3 mM glucose (with or without forskolin) was consistently elevated compared with that in normal pancreas. This is attributable to continuous depolarization of β cells (5) due to the lack of K$_{ATP}$ channels and elevation of [Ca$^{2+}$], even in low glucose concentrations (20). Thus, as previously reported (25), omission of extracellular CaCl$_2$ lowered insulin secretion by CHI pancreas to basal rates similar to those in normal pancreas.

The differences in insulin secretion rates between CHI and normal pancreas disappeared or were reversed at 5 mM glucose and above because the stimulatory action of glucose occurred in normal tissue only. The effects of glucose were indeed peculiar in CHI pancreas. Small stepwise increases in its concentration had no effect, while a larger step (1 to 15 mM) sometimes produced a rapid but small increase in insulin secretion above the already elevated initial level. Most often, the increase was transient and followed by a decrease of insulin secretion (particularly in the presence of forskolin) rather than by a sustained second phase elevation of [Ca$^{2+}$]. Cautious extrapolation to the present observation as are Sur1-knockout islets influenced basal secretion rates in low glucose concentrations and unmasked strong sustained effects of glucose on insulin secretion, which could be attributed to a very efficient amplifying pathway and, more unexpectedly, to a K$_{ATP}$ channel–independent control of Ca$^{2+}$ influx (42, 43, 45). Although our fragments of CHI pancreas were studied after overnight culture in 5 mM glucose, no sustained stimulation of insulin secretion was observed. These discrepancies cannot be attributed to age because islets from 2-week-old Sur1- or Kir6.2-knockout mice behaved like islets from adult mice (43, 45). It seems that a lack of K$_{ATP}$ channels does not lead to the same adaptation mechanisms in mouse and human β cells.

As already alluded to above, the poor efficacy of the amplifying pathway in CHI pancreas is intriguing. This may be one key to the phenotypic differences between the 2 species. In islets from one Sur1-knockout model (39), glutamine potently increased insulin secretion in the presence of low glucose, an effect that was barely augmented by leucine, used to activate glutamate dehydrogenase (40, 51). These and other observations in normal islets prompted the suggestion that glutamine serves as coupling factor in metabolic amplification of exocytotic responses to Ca$^{2+}$ (51, 52). It was also proposed that inhibition of the GABA shunt in Sur1-knockout β cells contributes to their hyposensitivity to glucose and hypersensitivity to amino acids (52). We cannot exclude that simi-
lar alterations underlie the poor responsiveness of CHI pancreas
to glucose but wish to remain very cautious in such extrapolations
between species. Thus, in our own studies using islets from the
other Sur1-knockout model (38), glutamine alone had virtually no
effect on insulin secretion in contrast with activation of glutamate
dehydrogenase (42). We also point out that glutamine alone was
poorly effective in the pancreas of a single DiCHI subject (Supple-
mental Figure 6). Finally, it is puzzling that the amplifying path-
way is less efficient in normal pancreas from FoCHI patients than
in normal adult islets. The question thus remains open.

Comparison between insulin secretion in vitro and in vivo. Insulin
secretion has been studied in CHI infants before surgery, gener-
ally in an attempt to distinguish between FoCHI and DiCHI. The
tolbutamide stimulation test only poorly distinguishes between
the 2 forms for 2 reasons. First, some mutations do not completely
suppress β cell sensitivity to sulfonylureas, and second, the normal
pancreas in FoCHI does not always strongly respond to tolbuta-
mite, perhaps because of a long-lasting resting state induced by
ambient low glucose or drug treatment (32, 33, 53). This was not
the case in our in vitro studies of FoCHI pancreas, in which tolbuta-
mide was consistently inactive in focal lesion and active in nor-
mal tissue, possibly relieved from any inhibition by the overnight
culture period. In cases of null mutations in ABCC8 or KCNJ11,
the test could still be helpful if performed after elevation of blood
glucose levels to prime the normal pancreas.

In many patients with either form of CHI, an acute increase in
plasma insulin levels was observed after i.v. glucose injection (32,
33, 53). While this increase was anticipated and attributed to the
normal pancreas in FoCHI, it was paradoxical in DiCHI. Our in
vitro results provide an explanation in showing that high glu-
cose acutely stimulates insulin secretion from at least some focal
lesions and DiCHI pancreas. The lack of a sustained second phase
and of any stimulatory effect of a graded increase in glucose con-
centration in vitro is also compatible with in vivo observations in
a single, nonoperated, older DiCHI patient (54).

The acute insulin response to calcium has been used to distinguish
CHI due to K_{ATP} channel mutations from other forms (peripheral i.v.
calcium injection with modest elevation of calcium) and to localize
a focal lesion in the pancreas by selective arterial calcium stimula-
tion of the pancreas with hepatic venous sampling (ASVS) (30, 32, 33, 53).
The test is based on the expectation of a larger calcium influx (hence
of insulin secretion) in β cells that are depolarized because of the
absence of K_{ATP} channels (5). Our in vitro data obtained with a high
CaCl2 concentration are relevant to the ASVS only. We observed that
high CaCl2 induced insulin secretion not only from CHI pancreas
but also from normal pancreas, which is explainable by the fact that
the physiological glucose concentration of 5 mM used in our in vitro
experiments is stimulatory for normal β cells. At best, a quantitative
difference in the responses could be expected in vivo when the nor-
mal pancreas is not stimulated by glucose.

Genotype-phenotype correlations. As already emphasized, insulin
secretory responses of the normal pancreas from FoCHI subjects
were qualitatively similar to those of normal adult islets. This
means that an inactivating mutation of ABCC8 (or KCNJ11) in only
1 allele does not alter β cell function. Our conclusion extends 2 in
vitro studies showing that heterozygous carriers of an ABCC8 muta-
tion (one of which is Val187Asp as in our subjects FoCHI2, -3, and
-4) normally secreted insulin in response to glucose or tolbutamide
(54, 55). Half the number of functional K_{ATP} channels would thus
seem sufficient to ensure normal control of insulin secretion.

The genetic basis of FoCHI and DiCHI is different. Notably,
owing to the loss of maternal CDKN1C (p57) and H19, and unre-
strained expression of paternal IGF-II, focal lesions are character-
ized by an increased proliferation rate, as shown by Ki-67 immu-
nodetection in approximately 8% of β cells within the lesion as
compared with approximately 3% in DiCHI or normal pancreas
(56). In spite of these differences, the alterations of insulin secre-
tion were similar in FoCHI and DiCHI pancreas.

The functional impact of 6 of the ABCC8 mutations found in
our patients has previously been evaluated by expression studies
(Table 1). The mutation identified in subjects FoCHI2, -3, and -4
(57) abolishes K_{ATP} channel expression at the plasma membrane
by causing SUR1 retention in the endoplasmic reticulum (57–59).
In these 3 cases, the paradoxical decrease in insulin secretion in
response to glucose and increase in response to diazoxide were
not observed. However, cases with another mutation also caus-
ing intracellular channel retention (FoCHI6 and DiCHI1, and
DiCHI8) (60, 61) did show the paradoxical response to diazo-
oxide. Except for the fact that high glucose did not decrease insulin
secretion in DiCHI1, the responses of all 6 DiCHI patients were
similar in spite of different mutations. In one case (DiCHI4),
the dominant mutation allows membrane expression (84%) of a
quasi–nucleotide-insensitive K_{ATP} channel (62), but no difference
in insulin secretion was observed with mutations causing com-
plete inhibition of channel trafficking. Finally, no specific altera-
tion of insulin secretion characterized the pancreas of the single
patient with a mutation in KCNJ11 (FoCHI1). With the limita-
tions imposed by the small number of possible comparisons in
our group comprising only diazoxide-resistant CHI patients, this
in vitro study shows that the ultimate impact on insulin secre-
tion is determined by the lack of functional K_{ATP} channels at the
plasma membrane rather than the underlying genetic/cellular
cause of this lack.

Conclusions. The sole presence of a paternal mutation in ABCC8
or KCNJ11 does not alter in vitro insulin secretion by the pancreas
outside the lesion in FoCHI. Compared with normal pancreas,
insulin content is very high in focal lesion and normal in DiCHI
pancreas, but fractional insulin secretion is not particularly ele-
vated in either pathological tissue at physiological glucose levels.
However, qualitative perturbations are major and similar in the
focal lesion and DiCHI pancreas despite their distinct genetic and
cellular pathology. The in vitro insulin secretion rate is abnormally
elevated in the presence of low glucose and virtually not increased
by high glucose concentrations. Most surprising are the increase
in secretion produced by diazoxide and the decrease sometimes
caused by high glucose concentrations. Neither of these changes
was reported in mouse islets lacking K_{ATP} channels, and neither
can be explained by current models of β cells.

Methods

CHI subjects. The present study was conducted with the approval of and
according to the regulations of the Commission d’Ethique Biomédicale
of the University of Louvain Faculty of Medicine. Informed consent was
obtained from the parents of the children.

The diagnosis of CHI was established on the basis of widely accepted
criteria (1). The patients underwent partial or subtotal pancreactectomy
because diazoxide and other medical measures proved inefficient to pre-
vent hypoglycemia. Between 2000 and 2009, 30 cases of diazoxide-resistant
CHI were included in the study, but only 18 FoCHI and 6 DiCHI cases
could be retained in the final analysis (Table 1).
In FoCHI, mean age at surgery was 6.4 (range 2–11) months. All patients were cured after resection of the focal lesion, sometimes after 2 or 3 operations (Table 1). Four additional subjects were studied but could not be included in the present analysis. In 2 patients, no control tissue was available for comparison with the responses measured in their focal lesion. In the other 2, the fragments of focal lesion were largely contaminated with normal tissue as shown by low insulin content and microscopic evidence that the lesion was poorly delineated.

In DiCHI, mean age at surgery was 4.7 (range 2.5–7) months for 5 patients and 18 months in the sixth patient. Hypoglycemias persisted in 1 patient, and diabetes followed pancreatectomy in the others. Two further subjects could not be included in the study because the insulin content of the pancreatic fragments was too low (<10% of that in other cases) for reliable measurement of insulin secretion.

Genetic studies (authorized in 23/24 subjects) showed that 22 patients had a mutation in ABCC8 (19 different types) and only 1 in KCNJ11 (Table 1). Seven of these mutations are believed to be novel, and the others have previously been described (6, 7). Note that patient DiCHI1 is the sister of FoCHI6 (63) and that patient DiCHI6 is a compound heterozygote.

Postoperative morphological study of the pancreas. Pancreatic samples were fixed in Bouin solution for conventional microscopy and immunohistochemical identification of insulin-containing cells as described elsewhere (17). Other samples were fixed in 4% formalin to perform CDKN1C peptide immunodetection (anti-CDKN1C from NeoMarkers; dilution 1/100).

Sampling and treatment of pancreatic fragments for functional studies. On the basis of intraoperative examinations of frozen samples, the pathologist distinguished between FoCHI and DiCHI, directed the extent of pancreas resection, and sampled fragments for functional studies. Because great care was taken to avoid overlap of normal and pathological regions in FoCHI, available fragments were sometimes very small: average of 102 mg (range 23–295) of whole pancreas. Fragments of tissue were immersed in sterile RPMI culture medium and transported at 4°C to the laboratory in Brussels within 4–8 hours.

On arrival, each fragment was injected with 6 ml of a salt-buffered medium containing 12 mg collagenase, finely minced, and transferred into a tight tube. The suspension of tissue was then shaken by hand for about 3–4 minutes in a water bath at 37°C, gently centrifuged for a few seconds, and rinsed several times at room temperature. Finally, the digested tissue consisting of clusters of cells and debris was resuspended in RPMI culture medium containing 5 mM glucose, 100 IU penicillin/ml, 100 μg streptomycin/ml, and 10% heat-inactivated fetal calf serum, and distributed into 3 cm petri dishes containing a final volume of 2 ml. Insulin secretion was studied the next day, after 17–22 hours of culture at 37°C, in an atmosphere of 5% CO2 in air.

Measurements of insulin secretion. On the day of the experiment, an aliquot of medium culture was saved for insulin determination. Pancreatic tissue was then harvested from culture dishes and distributed into equivalent portions that were transferred into perfusion chambers. Details on the system, technique, and solutions can be found elsewhere (26, 64). At the end of an experiment, pancreatic tissue was recovered from the chambers for measurement of insulin content (65). Insulin in perfusion samples, tissue extracts, and culture medium was measured by radioimmunoassay using human insulin as a standard (26).

Expression of results. Because the amount of tissue placed in perfusion chambers was variable and because this tissue was heterogeneous, insulin secretion rate was expressed relative to the tissue insulin content (fractional insulin secretion as percentage of insulin content/min) (64). Insulin secretion by focal lesion and healthy pancreas of the same patient was directly compared in paired experiments. However, not all protocols could be done in each case because of limited tissue availability. Insulin content of fresh tissue was not measured because this would have reduced even further the amount of tissue for functional studies, but it was estimated as follows: to the insulin content of all tissue fragments from a patient (measured at the end of perfusions), we added the amount of insulin secreted by these fragments during perfusion and culture. From that sum and the weight of the fragment, the initial insulin content of the tissue could be recalculated. Two opposite sources of error are the loss of insulin during tissue mincing and digestion, and insulin biosynthesis during culture.

All values are presented as mean ± SEM for the indicated number of experiments (patients) or with the range in parentheses. Statistical significance (P < 0.05) of changes in secretion was assessed by 2-tailed Student’s paired t test, comparing values before and during application of test agent in the same preparation or values in normal and pathological tissue from the same subject.

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