Human insulinomas show distinct patterns of insulin secretion in vitro

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ABSTRACT
Insulinomas are β-cell tumors that cause hypoglycemia through inappropriate secretion of insulin. Characterization of the in vitro dynamics of insulin secretion by perifused fragments of ten human insulinomas permitted their subdivision in three functional groups with similar insulin content. Group A (4 cases with fasting and/or postprandial hypoglycemas) showed qualitatively normal responses to glucose, leucine, diazoxide, tolbutamide and extracellular CaCl$_2$ omission or excess. The effect of glucose was concentration-dependent but, compared to normal islets, insulin secretion was excessive in both low and high glucose. Group B (3 cases with fasting hypoglycemas) was mainly characterized by large insulin responses to 1mmol/l glucose, resulting in very high basal secretion rates that were inhibited by diazoxide and restored by tolbutamide, but not further augmented by other agents except high CaCl$_2$. Group C (3 cases with fasting hypoglycemas) displayed very low rates of insulin secretion and virtually no response to stimuli (including high CaCl$_2$) and inhibitors (CaCl$_2$ omission being paradoxically stimulatory). In group B, the presence of low-$K_m$ hexokinase-I in insulinoma β-cells (not in adjacent islets) was revealed by immunochemistry. Human insulinomas thus show distinct, though not completely heterogeneous, defects in insulin secretion that are attributed to undue expression of hexokinase-I in 3/10 cases.
Insulinomas are uncommon, usually benign, tumors of pancreatic β-cells, which cause hyperinsulinemic hypoglycemia (1-4). Early morphological studies have led to distinct classifications of insulinomas on the basis of their histological and structural organization (5-6). They also suggested correlations between ultrastructural appearance of the tumors and either their insulin content (5) or the efficacy of diazoxide to inhibit excessive insulin secretion in the patient (6). However, a study based on a large series of 76 cases reported that the two main morphological groups of insulinomas, trabecular and solid (or medullary), are not homogeneous but display highly variable insulin immunostaining patterns (7). These findings have been confirmed (8), leading to the conclusion that the histological structure of insulinomas is not a satisfactory marker of distinct functional properties.

The characteristics of insulin secretion by human insulinoma cells are poorly known. Several in vitro studies have addressed the question, but no clear picture of the differences with normal β-cells has emerged for a number of reasons. First, the techniques were disparate. Minced pieces of the tumor were incubated either immediately (9,10) or after culture for several days or weeks (11-13). In other studies, pieces of the tumor were digested with collagenase before measurement of insulin secretion either immediately (14,15) or after 1-8 weeks of culture (16-20). Second, only few, dissimilar protocols of stimulation or inhibition were usually tested in static incubations. Third, with a few exceptions (12,13), these functional studies were limited to single cases.

Over a period of 10 years we obtained a fragment from 10 insulinomas, of which we then characterized the dynamics of insulin secretion in vitro, using the same methods as in our recent studies of the pancreas of infants suffering from congenital hyperinsulinism (21,22). The results show heterogeneity in the responses, but sufficient similarities to permit a tentative subdivision in 3 functional groups. In one of these, abnormal insulin secretion is attributed to undue expression of hexokinase-I (HK-I) in tumoral β-cells.
RESEARCH DESIGN AND METHODS

Subjects. Between 2001 and 2010, we obtained a fragment of fresh insulinoma tissue from 9 patients operated at the University Clinics Saint-Luc in Brussels and 1 patient operated at the Hôtel-Dieu in Paris. The clinical diagnosis of insulinoma had been established on the basis of classic medical, biological and imaging criteria (3,4,23,24) that led to surgical ablation of a tumor, which resulted in cessation of hypoglycemic episodes in all patients. The study was conducted with the requested approvals and according to the regulations of the Commission d'Ethique Biomédicale of the Faculty of Medicine of the University of Louvain.

In vitro studies of insulin secretion. The fragment of insulinoma used for in vitro studies of insulin secretion was macroscopically sampled from the tumor, avoiding contamination with normal pancreatic tissue when present. Upon arrival in the laboratory, the tissue was minced and collagenase-digested like pancreatic fragments from infants undergoing surgery for congenital hyperinsulinism (21). After ~20 h of culture in RPMI medium containing 5mmol/l glucose, portions of digested tissue were distributed into perifusion chambers for measurement of insulin secretion (21) and, at the end of experiments, of insulin content (25). Because the amount of tissue placed in each chamber was variable, insulin secretion rate was expressed relative to the insulin content of the tissue (fractional insulin secretion as percentage of insulin content/min). Details on the system, techniques and solutions (containing 1μmol/l forskolin during perifusions) can be found elsewhere (26). The number of tests performed with each preparation was determined by the amount of available tissue. Two protocols evaluating acute and concentration-dependent insulin responses to glucose and testing the effects of diazoxide and tolbutamide could be done in all cases. Insulin secretion in response to high extracellular CaCl₂ was tested in 9/10 cases. Two other protocols were done in a number of cases indicated in text and legends. The initial insulin content of the tumor was not directly measured but estimated by adding insulin secreted during culture and experiments.
to the insulin content measured at the end of perifusions (21). In two cases, attempts to measure insulin secretion from pancreatic tissue outside the tumor were unsuccessful because insulin was not measurable in perifusion medium of the small available fragments.

**Morphological studies.** Fragments of insulinomas, with sometimes small adjacent regions of normal tissue, were fixed in formalin and/or Bouin solution for conventional microscopy and immunohistochemical identification of insulin, glucagon, and somatostatin (27) or of the proliferation marker Ki-67 (28). The volume density of β-cells in insulinomas was determined by point-counting on slices immuno-stained for insulin (27). Staining with Congo red was used to identify amyloid deposits. Immunodetection of HK-I was performed as described (22).

**RESULTS**

Subdivision of the studied insulinomas in three functional groups was done retrospectively, on the basis of the pattern of insulin secretion observed in vitro and on the presence or absence of HK-I in insulinoma β-cells (see below). The major characteristics of the 10 cases, all negative for mutations in the *MEN-1* gene, are given in Table 1. The first symptoms of hypoglycemia occurred 8-50 months before operation and were clearly documented postprandially in 3 cases of group A.

**Morphological aspect of insulinomas.** The diagnosis of insulinoma was histologically confirmed in all cases. They were classified as “solid” or “trabecular” according to established criteria (6,7) (Table 1). Typical appearance of the two forms can be seen in Figure 1. As reported previously (6-8), insulin immunostaining was often polarized in β-cells of
trabecular cases (Fig 1, case 8) and more diffuse and heterogeneous in β-cells of solid cases (Fig 1, case 2). Notably, both histological types were observed in the three functional groups (Table 1). All were well-differentiated, with a low proliferation index (2 to 10%) (Supplemental Fig 1). The size of the tumor ranged between 0.4 and 1.9 ml in 9 cases and was very large (16 ml) in case 4. The volume density of β-cells in the tumors averaged 44%, but was highly variable (13-73%) owing to sometimes high proportions of mesenchymal tissue, but not of non-β endocrine cells (Table 1). After staining with Congo red, amyloid deposits were detected in 5/9 cases, a similar proportion as in a larger study (29) (Supplemental Fig 1). They were abundant in only 2 cases of group A and rare or absent in cases from groups B and C (Table 1).

**Insulin concentration in insulinomas.** The tumor insulin concentration was highly variable, ranging from 40 to 395ng/mg (mean = 132ng/mg). This variability persisted after normalization for differences in β-cell proportions (Table 1). None of the 3 groups was characterized by consistently high or low insulin concentrations (Table 1).

**Effects of glucose, diazoxide and tolbutamide on insulin secretion.** In group A, “basal” insulin secretion rate, measured in 1mmol/l glucose, ranged from 0.02 to 0.12%/min. Increasing the glucose concentration to 15mmol/l stimulated insulin secretion several-fold in the four cases (Fig 2A). This stimulation was partially (case 1) or completely inhibited by 100µmol/l diazoxide and reversibly restored by 100µmol/l tolbutamide. These responses are thus qualitatively similar to those observed in isolated islets from normal subjects (26) and fragments of normal pancreas from infants (21). The major difference is a sometimes more sluggish onset and less pronounced biphasic pattern of the response to glucose.

In group B, cases 5 and 6 were characterized by a very high insulin secretion rate in 1mmol/l glucose (0.40 and 0.19%/min) and, except for a short-lived initial increase, no
further stimulation by 15mmol/l glucose (Fig 2B). In case 7, a slower but more sustained response to 15mmol/l glucose occurred above a less markedly elevated “basal” rate in 1mmol/l glucose (0.09%/min) (Fig 2B, lower panel). The three insulinomas were sensitive to inhibition by diazoxide, which lowered insulin secretion well below values measured in 1mmol/l glucose, and to tolbutamide, which reversibly reversed this inhibition (Fig 2B).

In cases from group C, the “basal” rate of insulin secretion in 1mmol/l glucose was low, ranging from 0.01 to 0.02%/min. Stimulation with 15mmol/l glucose only transiently and minimally increased insulin secretion (case 8) or was ineffective (case 9); diazoxide and tolbutamide were also without action (Fig 2C, upper panel). It was only in case 10 that qualitatively normal but quantitatively small responses to glucose, diazoxide and tolbutamide were observed (Fig 2C, lower panel).

**Effects of stepwise increases in glucose concentration on insulin secretion.** In group A, stepwise stimulation with glucose augmented insulin secretion in a concentration-dependent manner (Fig 3A). In cases 1, 2 and 3, 1mmol/l glucose approximately doubled (1.5, 2.2 and 2.4-fold) insulin secretion rate, which then kept increasing up to 5-10mmol/l glucose. In case 4, in which the experiment was started in 1mmol/l glucose, stimulation of secretion occurred only at 5 mmol/l glucose and above (Fig 3A, lower panel).

In group B, the concentration-dependency of the response to glucose was markedly altered (Fig 3B). In cases 5 and 6, the insulin secretion rate displayed a rapid and large peak in response to 1mmol/l glucose (40-fold and 6-fold increases), and then regularly declined in face of stepwise increases in glucose concentration. The response of case 7 was slightly different (Fig 3B, lower panel). Insulin secretion was also induced by 1mmol/l glucose (4.5-fold increase), but the response was of slower onset and remained sustained when the glucose concentration was increased further. In no case was the switch from 10 to 1mmol/l glucose
followed by an obvious decrease in insulin secretion, whereas return to a glucose-free medium was inhibitory in cases 5 and 7.

In group C, no stimulation of insulin secretion occurred in response to stepwise increases in glucose (Fig 3C). In cases 8 and 9, a paradoxical increase in secretion was seen when the glucose concentration was lowered from 10 to 1mmol/l (Fig 3C, upper panel).

**Effects of various stimulatory and inhibitory conditions on insulin secretion.** In group A, addition of leucine and glutamine (5mmol/l each) to a medium containing 3mmol/l glucose induced biphasic insulin secretion in 4/4 cases. This is illustrated for cases 1 and 2 in Figure 4A. Omission of extracellular CaCl$_2$ reversibly abolished secretion, which was also strongly inhibited by activation of α$_2$-adrenoceptors with clonidine. In group B, only case 5 was tested. The two amino acids were apparently ineffective, but the high rate of insulin secretion induced by 3mmol/l glucose was completely and reversibly abolished by omission of CaCl$_2$, and partially inhibited by clonidine (Fig 4B). In group C, the combination of leucine and glutamine was ineffective and omission of CaCl$_2$ was followed by a paradoxical, reversible increase in secretion (Fig 4C).

In normal β-cells, glucose controls insulin secretion through changes in the cytosolic Ca$^{2+}$ concentration (triggering pathway) and amplification of Ca$^{2+}$ effects on exocytosis of insulin granules (metabolic amplifying pathway) (30). Metabolic amplification can be studied when all β-cell K$_{ATP}$ channels are closed by a high concentration of sulfonylurea. In cases 3 and 4 from group A, 500µmol/l tolbutamide triggered a large peak of insulin secretion followed by stabilization of the secretion rate above pre-stimulatory values. Subsequent augmentation of the glucose concentration to 15mmol/l, in the continuous presence of tolbutamide, increased insulin secretion in a reversible manner (Fig 4D). In group B, high tolbutamide was hardly (case 6) or transiently (case 7) effective in 1mmol/l glucose, and the
subsequent addition of 15mmol/l glucose had no obvious effect (Fig 4E). In group C, high tolbutamide was ineffective in 1mmol/l glucose and the subsequent increase of glucose to 15mmol/l was slightly but reversibly inhibitory (Fig 4F).

**Effects of high extracellular CaCl\(_2\).** A sudden increase in the concentration of extracellular CaCl\(_2\) from 1.25 to 10mmol/l in a medium containing 5mmol/l glucose evoked a large reversible peak of insulin secretion in all tested cases from groups A and B (Figs 5A and 5B). In group A, the relative stimulation ranged from 9.7 to 14.7-fold. It was smaller in group B, ranging from 4.9 to 7.6-fold. In contrast with all other cases, insulinomas from group C did not increase insulin secretion in response to high extracellular CaCl\(_2\) (Fig 5C).

**Expression of hexokinase-I in some insulinoma cells.** Stimulation of insulin secretion by as little as 1mmol/l glucose in certain cases led us to search for the presence of a low-Km hexokinase in insulinoma cells. Immunocytochemistry for HK-I was negative in the 3 cases of group A that could be tested (Table 1). As illustrated for case 2, β-cells within the tumor (and islets outside the tumor) were not labeled (Fig 6A), in contrast to neural, centro-acinar and vascular cells (Fig 6A, inset). In the 3 cases of group B, insulinoma cells were positive for HK-I (Fig 6B), whereas β-cells in islets outside the tumor were negative (Fig 6B, inset). In cases 5 and 6, all insulinoma cells were labeled, though with a variable intensity, whereas areas of negative and positive cells coexisted in case 7. The 3 cases of group C displayed a punctated positivity for HK-I in many insulinoma cells intermingled with negative cells (Fig 6C), whereas β-cells of the islets were negative (Fig 6C, inset).

**DISCUSSION**

Although our 10 cases constitute the largest series of insulinomas functionally studied in vitro, its limited size, inherent to the rarity of the pathology, remains a handicap in our
attempt to classify these endocrine tumors according to functional features. Our subdivision in three groups has limitations and would have been strengthened by genetic analysis of the tumors. It may well be an oversimplification of the heterogeneous behavior of these tumoral β-cells, but enough similarities appear to justify their grouping rather than multiplication of smaller categories.

No consistent difference in symptom duration, tumor size, histological type or insulin concentration was found between the three functional groups of insulinomas. For the 10 cases, the average tumor insulin concentration (132ng/mg) was similar to that measured in normal autopsy pancreas (125ng/mg) (31), but the inter-individual variability was larger (10 versus 4-fold). Higher average insulin concentrations and even greater variability (100-200-fold) were found in larger series (5,32). Insulinomas contain variable, sometimes high, proportions of mesenchymal tissue (vessels, amyloid deposits and connective tissue) that decreases the apparent insulin concentration when expressed per weight. Nevertheless, because the β-cell fraction (13-73%) largely exceeded that in a normal pancreas (~1.25%), it is clear that the concentration of insulin per β-cell is much lower in insulinomas than in normal islets, as also concluded by others (5,32). Finally, from tumor size and β-cell volume density, we can calculate that the mass of insulinoma β-cells ranged between 155 and 2080mg in our 10 cases, as compared with 300 to 1500mg (average of ~900mg) β-cells in the pancreas of healthy subjects (31). It is therefore unlikely that this additional number of β-cells would cause profound hypoglycemia if these insulinoma cells were functionally normal.

In previous in vitro investigations of human insulinoma cells, insulin secretion was unaffected by high glucose in 5 cases from 3 studies (11,12,16) and variably augmented in 7 single cases (9,10,14,17-20). Other agents were rarely tested: insulin secretion was increased by a sulfonylurea in 2/2 cases (18,19) and by high CaCl$_2$ in 1/1 case (13), whereas leucine was ineffective in 1/1 case (11). Inhibition of insulin secretion was observed upon CaCl$_2$
omission in 1/1 case (11), whereas diazoxide addition was ineffective in 2/2 cases (9,12). In our experience, basal insulin secretion by isolated human islets perfused with 1mmol/l glucose was about 0.01% of their insulin content per min (26). Much higher (5-50 x) “basal” rates of secretion were measured here in insulinomas from groups A (3/4 cases) and B (3/3 cases). Distinct procedures for tissue preparation may contribute to the difference but are not the sole explanation. Thus, compared with normal islets, the difference is only 1.5 to 2-fold in insulinomas from group C, and only 2 to 3-fold in collagenase-digested fragments of normal pancreas from infants (21,22).

In group A, the four insulinomas shared several features that make them qualitatively similar to normal islets (26) or fragments of normal pancreas (21). These included sustained insulin secretion in response to 15mmol/l glucose, normal inhibitory and stimulatory effects of diazoxide and tolbutamide, respectively, normal stimulation by amino acids and inhibition by omission of CaCl$_2$ or addition of clonidine, and functioning of the metabolic amplifying pathway. All these characteristics suggest qualitatively normal stimulus-secretion coupling with operative K$_{ATP}$ channels. However, insulin secretion was quantitatively excessive. Thus, cases 1, 2 and 3 displayed abnormally high secretion rates in low and high glucose, and a left-shift of the glucose-dependency of the response. Whereas the normal threshold is at 3mmol/l glucose (21,26), doubling of insulin secretion was already observed at 1mmol/l glucose, which could explain occurrence of fasting hypoglycemias in the patients. This threshold lowering was not correlated with HK-I detection in insulinoma cells, as in group B (see below), perhaps because expression of the enzyme was weaker, also explaining the smaller response to 1mmol/l glucose than in group B. The insulin secretion rate induced by high glucose (0.3-0.4%/min) was also ~2-fold higher than in normal islets or fragments of normal pancreas tested under similar conditions (~0.15%/min) (21,26). We have no mechanistic explanation for this excessive insulin response, which probably explains why hypoglycemias
also occurred after meals as documented in cases 2 and 3. Unlike the other 3 cases of group A, case 4 did not show an increase in glucose sensitivity with elevation of the secretion rate at low glucose, but the response to high glucose was also exceedingly large, which might explain why hypoglycemias occurred only after meals. Notably, this case was also exceptional by the large size of the tumor that was filled with amyloid deposits.

In group B, the main characteristics of cases 5 and 6 were a large increase in insulin secretion in response to 1mmol/l glucose, followed by a progressive decline in face of subsequent increases in glucose concentration. Strong stimulation already by 1mmol/l glucose may explain why stepping to 15mmol/l glucose was poorly efficient and why diazoxide inhibited insulin secretion well below rates measured at 1mmol/l glucose. Abnormal sensitivity of insulinomas to low glucose with poor further response to higher glucose can result in hypoglycemias in the fasting state and not after meals. Two insulinomas resembling our cases 5 and 6 have previously been studied in vitro (18,20). Compared with a glucose-free medium, 1mmol/l glucose induced a ~10-fold increase in insulin secretion, which was not larger at higher glucose. This excessive sensitivity to low glucose remained unexplained (18) or was tentatively ascribed to strong expression of the high-affinity glucose transporter GLUT-1 in the tumor cells (20). However, it is now established that GLUT-1 and GLUT-3 are the major glucose transporters in normal human β-cells (33-34). Using immunohistochemistry, we detected the presence of the low-\(K_m\) HK-I in tumor β-cells, whereas normal islet β-cells, which do not express HK-I but use the high \(K_m\) glucokinase to phosphorylate glucose (35-36), were negative as expected. We therefore suggest that, in insulinoma cells of group B, glucose metabolism is already markedly accelerated when the glucose concentration is low. The expected consequences are virtually complete closure of \(K_{ATP}\) channels and full activation of the metabolic amplifying pathway (30), which would account for the poor effect of tolbutamide, except when \(K_{ATP}\) channels have been opened by
diazoxide, and for the virtual lack of further stimulation by the combination of leucine and glutamine, which mainly acts through acceleration of metabolism (37-38). In support of our proposal that the activity of HK-I in insulinoma cells is responsible for abnormal insulin secretion at low glucose in cases of group B, we wish to underline the similarities with our previous findings in a group of infants suffering from focal hyperinsulinism (22). The in vitro abnormalities of insulin secretion associated with the presence of HK-I in a subset of their β-cells are quasi superimposable on those of insulinomas from group B. A dominant form of congenital hyperinsulinism has also tentatively been attributed to abnormal HK-I expression in β-cells (39). Case 7 behaved slightly differently from cases 5 and 6. Insulin secretion was also induced by 1mmol/l glucose, but higher concentrations retained some effect, as did tolbutamide even in the absence of diazoxide. A plausible explanation is the weaker and heterogeneous presence of HK-I in the tumor, with some β-cells presumably maintaining a normal control of glucose phosphorylation by glucokinase.

In group C, the major characteristics of the three cases were very low rates of insulin secretion in spite of substantial insulin stores in the tumor, and a virtually complete absence of responses to stimuli and inhibitors active in the other two groups. Poor stimulation by glucose and ineffectiveness of diazoxide and tolbutamide are reminiscent of defects observed in focal lesions of the pancreas causing congenital hyperinsulinism because of a lack of functional \( K_{ATP} \) channels in β-cells (21). However, major differences distinguish the two pathological entities: insulin secretion rates were 5-10 fold lower in these insulinomas than in focal lesions; the decrease in secretion produced by high glucose in the presence of high tolbutamide in insulinomas was never seen in focal lesions; omission of CaCl\(_2\) paradoxically increased insulin secretion in insulinomas whereas it consistently suppressed it in focal lesions; clonidine was ineffective in insulinomas; high extracellular CaCl\(_2\) was ineffective in insulinomas but strongly stimulated insulin secretion in focal lesions (21). Several
morphological features also distinguish focal lesions of infants and insulinomas (27). The functional significance of HK-I detected in insulinomas from group C is unclear in view of the virtually complete absence of effect of glucose on secretion. Intriguingly, HK-I immunostaining was not present in all cells suggesting heterogeneity within the tumor. Poor viability of these 3 insulinomas cannot be formally excluded, but would be expected to cause insulin leakage rather than low baseline secretion, and is not supported by their preserved morphology and similar pattern of HK-I staining. Distal defects in the exocytotic machinery are possible, but how they caused hypoglycemics (in the fasting state only) is unclear.

Selective arterial calcium stimulation of the pancreas with hepatic venous sampling is used for preoperative localization of insulinomas (23,40-42). We observed strong stimulation of insulin secretion by 10mmol/l CaCl$_2$ in all cases of groups A and B, and no response in group C. In vitro stimulation by high CaCl$_2$ has previously been reported in 3 other cases and attributed to activation of an extracellular Ca$^{2+}$-sensing receptor (13,43), but augmentation of Ca$^{2+}$ influx through the plasma membrane may also contribute. In fragments of normal pancreas from infants, high CaCl$_2$ caused an insulin response qualitatively similar to but quantitatively smaller (3-fold increase) than that in insulinomas from groups A and B (21). The discrepancy with the lack of effect on normal pancreas in vivo (23,40-42) is probably due to higher concentrations of CaCl$_2$ and glucose during in vitro experiments than in vivo tests. Notwithstanding, our results show that false negative results in vivo may be due to unresponsiveness of some insulinomas (group C).

In conclusion, human insulinomas showed distinct, though not completely heterogeneous, defects in insulin secretion in vitro. We subdivided our 10 cases in 3 groups on the following basis. Group A was characterized by a qualitatively normal stimulus-secretion coupling with effective control by glucose. However, increased sensitivity to the sugar caused elevation of baseline secretion at low glucose, and the response to high glucose
was quantitatively excessive, which may explain post-prandial hypoglycemia. No HK-I was detected, but its presence at low levels cannot be excluded. Group B was mainly characterized by undue expression of HK-I in insulinoma β-cells, which resulted in strong stimulation of insulin secretion by as little as 1mmol/glucose and loss of regulation by higher glucose concentrations, two features that may explain fasting hypoglycemias only. Group C showed heterogenous presence of HK-I, very low rates of insulin secretion and a virtually complete absence of responses to stimuli and inhibitors, features that do not readily explain fasting hypoglycemias of these patients. Without investigation of the mechanisms of tumorigenesis and without genetic studies, we cannot determine whether these three groups have distinct origins. However, we can speculate that transitions might exist between groups, as illustrated by case 7 that expressed HK-I in some parts of the tumor only, and was difficult to assign to functional group B rather than group A.

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**Duality of interest.** The authors have no conflict of interest to report.

**Author contributions.** JCH designed research, researched and analyzed data, and wrote the manuscript. MN, YG, JR and CS researched and analyzed data, and reviewed the manuscript. JCH is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
References


**Legends to Figures**

**Figure 1.** Insulin immunostaining in 4 of the studied insulinomas. Cases 2 and 7 show a “solid” organization of β-cells, whereas cases 4 and 8 show the characteristic pattern of “trabecular” insulinomas. In case 4, the abundant yellowish material, visible without staining by Congo red, corresponds to amyloid deposits. Scale bar = 50µm.

**Figure 2.** Effects of glucose and drugs acting on $K_{ATP}$ channels on insulin secretion by insulinoma fragments from the 10 studied cases. All experiments, performed with a medium containing 1µmol/l forskolin to increase cAMP in β-cells, started by a 60-min stabilization period of which only the last 10 min are shown. At time 0, the concentration of glucose was increased from 1mmol/l (G1) to 15mmol/l (G15), before addition of 100µmol/l diazoxide (Dz 100) and 100µmol/l tolbutamide (Tolb 100) as indicated at the top of the panels. Subdivision of the studied cases in 3 groups (A, B and C) was based on similarity of the insulin responses to different stimuli and will thus be consistent throughout. Note the differences in scale between panels, particularly between panels C and the others.

**Figure 3.** Effects of stepwise increases and decreases in glucose concentration (G in mmol/l) on insulin secretion by insulinoma fragments from the 10 studied cases. There was no period in G0 at the start and the end of the experiment with tissue from case 4 (group A). For the sake of clarity, secretion rates were multiplied by 2 in case 7 (group B); actual values are thus twice smaller. Note the differences in scale between panels, particularly between panels C and the others.

**Figure 4.** Effects of various stimulatory and inhibitory conditions on insulin secretion by insulinoma fragments from several of the studied cases. A-C: The concentration of glucose was 3mmol/l (G3) throughout. Leucine and glutamine (5mmol/l each) were added at time 0 and remained present until the end. Extracellular CaCl$_2$ (normally 2.5mmol/l) was then
omitted (Ca 0, with addition of 100μmol/l EGTA) and 1μmol/l clonidine was added as indicated. The protocol was tested in 4/4, 1/3 and 2/3 cases from groups A, B and C, respectively. D-F: In a medium containing 1mmol/l glucose (G1), 500μmol/l tolbutamide (Tolb 500) was added at 0 min and remained present until the end. The concentration of glucose was then transiently increased to 15mmol/l (G15) as indicated. The protocol was tested in 2/4, 2/3 and 2/3 cases from groups A, B and C, respectively. For the sake of clarity, secretion rates were multiplied by 2 in case 4 (group A); actual values are thus twice smaller. Note the differences in scale between panels, particularly between panels C and F and the others.

**Figure 5.** Effects of high extracellular CaCl$_2$ on insulin secretion by insulinoma fragments from most of the studied cases. In a medium containing 5mmol/l glucose throughout, the concentration of extracellular CaCl$_2$ was increased from 1.25 to 10mmol/l for a period of 10 min as indicated. The protocol was tested in 3/4, 3/3 and 3/3 cases from groups A, B and C, respectively. Note the differences in scale between panels, particularly between panels C and the others.

**Figure 6.** Immuno-histochemical detection of HK-I in certain insulinomas. In group A, β-cells of the insulinoma were negative in contrast to vessel cells shown by arrows (main panel). Outside the tumor (inset), ganglionic neural cells were strongly positive and vessel cells (black arrow) and centro-acinar cells (red arrow) were positive. In group B, β-cells were positive within the tumor (main panel). Outside the tumor (inset), HK-I labelling was clear in vascular and centro-acinar cells, fainter in acinar cells and negative in β-cells of the islets (arrow). In group C, many though not all β-cells of insulinomas showed punctated labelling of HK-I (main panel). Outside the tumor (inset), β-cells of the islets (arrow) were negative whereas centro-acinar cells were positive. Scale bars = 50μm. A blown-up version of the insets is shown in Supplemental Figure 2.
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Legend on next page
Legend to Table 1

Months of symptoms: the onset of symptoms compatible with hypoglycemia was determined according to patients’ self-reports. Case 3: symptoms experienced 120 months before diagnosis of insulinoma occurred during a period of voluntary weight-losing diet.

Symptom timing: defined as post-meal and fasting when symptoms occurred within 4h of meal ingestion and more than 4h after last food intake, respectively. For cases 1 and 6, occurrence of post-meal hypoglycemia could neither be established nor excluded with certainty.

No fixed tissue was available for histological studies in case 3. The percentage of β-cells positive for Ki-67 defines the grade of insulinoma: Grade 1 = 0-2%; Grade 2 = 3-20%. The β-cell volume density (Vv) corresponds to the relative volume (%) of the tumor occupied by β-cells. Rare δ-cells = ~1% at the most. Rare amyloid deposits = ~1-2% of the tumor at the most. The β-cell mass was calculated from β-cell Vv and tumor size (ml=g). The normalized insulin concentration in insulinomas (ng/mg β-cells) was calculated from the global insulin concentration (ng/mg of tumor) and the percentage of β-cells in the tumor (Vv).
Figure 1
Fig 2
Fig 3
Fig 4
Fig 5
Figure 6
Immunodetection of Ki-67 in case 7: the nucleus of a few insulinoma cells is labeled in dark brown. Scale bar = 50 μm.

Staining with Congo Red identifies amyloid deposits that appear in pink. They are abundant in case 1 and rare (arrows) in case 7. Scale bar = 50 μm.

Supplemental Figure 1
Blown-up insets of figure 6. In the normal pancreas outside the tumor, HK-I labeling is strong in ganglionic neural cells, centro-acinar cells and vessel cells, faint in acinar cells, and negative in islet cells. Scale bar = 50 μm.

**Supplemental Figure 2**