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ARTICLE

Insulin secretion from human beta cells is heterogeneous and dependent on cell-to-cell contacts

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Abstract

Aims/hypothesis We assessed the heterogeneity of insulin secretion from human isolated beta cells and its regulation by cell-to-cell contacts.

Methods Insulin secretion from single and paired cells was assessed by a reverse haemolytic plaque assay. The percentage of plaque-forming cells, the mean plaque area and the total plaque development were evaluated after 1 h of stimulation with different secretagogues.

Results Not all beta cells were surrounded by a haemolytic plaque under all conditions tested. A small fraction of the beta cell population (20%) secreted more than 90% and 70% of total insulin at 2.2 and 22.2 mmol/l glucose, respectively. Plaque-forming cells, mean plaque area and total plaque development were increased at 12.2 and 22.2 compared with 2.2 mmol/l glucose. Insulin secretion of single beta cells was similar at 12.2 and 22.2 mmol/l glucose. Insulin secretion of beta cell pairs was increased compared with that of single beta cells and was higher at 22.2 than at 12.2 mmol/l glucose. Insulin secretion of beta cells in contact with alpha cells was also increased compared with single beta cells, but was similar at 22.2 compared with 12.2 mmol/l glucose. Delta and other non-beta cells did not

increase insulin secretion of contacting beta cells compared with that of single beta cells. Differences in insulin secretion between 22.2 and 12.2 mmol/l glucose were observed in murine but not in human islets.

Conclusions/interpretation Human beta cells are highly heterogeneous in terms of insulin secretion so that a small fraction of beta cells contributes to the majority of insulin secreted. Homologous and heterologous intercellular contacts have a significant impact on insulin secretion and this could be related to the particular architecture of human islets.

Keywords Beta cells · Heterogeneity · Human islets · Insulin secretion · Reverse haemolytic plaque assay

Abbreviations

CMRL	Connaught Medical Research Laboratory
IBMX	3-isobutyl-1-methylxanthine
IEQ	islet equivalent
KRB 2.2	KRB Hepes buffer supplemented with 0.1% BSA and 2.2 mmol/l glucose
MPA	mean plaque area
PFC	plaque-forming cells or cell pairs
PMA	phorbol-12-myristate-13-acetate
RHPA	reverse haemolytic plaque assay
TPD	total plaque development

Introduction

Endocrine cells are not randomly distributed within pancreatic islets. In most laboratory animals, islets consist of a core of insulin-secreting beta cells surrounded by alpha, delta and pancreatic polypeptide cells, which secrete glucagon, somatostatin and pancreatic polypeptide respec-

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tively [1]. This complex architecture, which favours homologous cell-to-cell contacts, has been shown to be crucial for the normal function of the islet [2–5]. In animal models of diabetes, the well-defined islet architecture is disturbed [6, 7] so that glucagon- and somatostatin-containing cells are intermingled with beta cells. The same altered cellular organisation was reproduced in vitro by exposing islet cells to cytokines known to be secreted by leucocytes that infiltrate islets during diabetes onset [8].

The importance of homologous contacts between beta cells is further supported by the observation in rodents that the functioning of isolated beta cells is heterogeneous [9–13]. For instance, beta cells respond unequally to glucose stimulation in terms of insulin secretion and biosynthetic activity [12, 14, 15]. It has been suggested that intercellular contacts coordinate the functioning of beta cells by homogenising and synchronising their activities. This coordination is mediated at least in part by junctional structures and cell adhesion molecules. Specifically, beta cells are interconnected by gap junctions, which form channels across the extracellular space and allow direct exchanges of small cytoplasmic molecules, ensuring electrical coupling and promoting insulin secretion [16]. Adhesion molecules such as integrins, cadherins and neural cell adhesion molecule have been described in islets and they somehow influence insulin secretion and are also involved in maintaining correct islet architecture [17–19].

The cytoarchitecture of human islets has been examined in detail only recently [5, 20]. Compared with animal models, the pattern of distribution of islet cells is unique in that the different endocrine cell types are distributed throughout the islet and not sorted into central and peripheral compartments. In addition, this structural dissimilitude has been associated with functional differences. Indeed, in contrast to mouse or rat islets, human islets displayed oscillations in intracellular calcium concentration that were not synchronised between beta cells. It has been suggested that this discontinuous activity of beta cells originates from the particular layout of beta cells in human islets [5].

Considering these above observations, we analysed whether heterogeneity in terms of insulin secretion also exists between human beta cells and how insulin secretion is affected by intercellular contacts. To this end, human islets isolated from different pancreases were dissociated and insulin secretion from single beta cells and aggregated cell pairs comprising beta and non-beta cells were studied by a reverse haemolytic plaque assay (RHPA) [21].

Methods

Islet isolation Human islets were isolated from human pancreases removed from brain-dead heart-beating donors.

The use of human tissue for research was approved by our local institutional ethical committee, and our centre is a member of the European Consortium for Islet Transplantation for the islet research distribution programme, under the supervision of the Juvenile Diabetes Research Foundation. Islets were isolated and purified using Ricordi's automated method [22] with local modifications [23]. After isolation, islets were suspended in Connaught Medical Research Laboratory (CMRL) medium containing 5.6 mmol/l glucose and 10% FCS, and incubated for 24 h at 37°C and at 24°C thereafter.

Adult male DBA/1 mice were purchased from Janvier laboratories (Le Genest-Saint-Isle, France). All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee, Department of Territory, State of Geneva. Mouse islets were isolated and purified using techniques previously described [17, 24]. Viability, as assessed by propidium iodide/fluorescein diacetate staining, was always higher than 80%. Isolated islets were then kept for 24 h and at 37°C in RPMI medium containing 10% FCS before static incubation tests were done.

Islet dissociation Aliquots of 1,000 islet equivalents (IEQ; average islet diameter 150 µm) were rinsed twice with 10 ml PBS, resuspended in 1 ml Accutase (Innovative Cell Technologies, San Diego, CA, USA) and incubated at 37°C with gentle pipetting every 30 s. From 5 min onwards, complete cell dissociation was checked by microscopic observation of 10 µl aliquots sampled every min. When dissociation was considered to be complete (usually between 7 and 10 min), cells were diluted with 10 ml cold CMRL containing 10% FCS (vol./vol.). Before analysis by RHPA, cells were rinsed and incubated in this medium for either 1 to 2 h (in a first set of experiments) or for 24 h (in a second set of experiments) in order to increase cell-to-cell aggregation.

Insulin secretion by RHPA Cunningham glass chambers (60 µl) were coated with 0.1 mg/ml poly-L-lysine (molecular mass 150,000–300,000; Sigma-Aldrich, Buchs, Switzerland). Islet cells were then rinsed with KRB Hepes buffer (125 mmol/l NaCl, 4.74 mmol/l KCl, 1 mmol/l CaCl₂, 1.2 mmol/l KH₂PO₄, 1.2 mmol/l MgSO₄, 5 mmol/l NaHCO₃, 25 mmol/l HEPES, pH 7.4) supplemented with 0.1% BSA (wt/vol.) and 2.2 mmol/l glucose (KRB 2.2). Then, islets cells were diluted in KRB 2.2 to a density of 25×10^5 per ml and erythrocytes (sheep blood; Dade Behring, Servion, Switzerland) previously coated with Protein A (Amersham Bioscience, Dübendorf, Switzerland) were added to the cell suspension at a concentration of 5% (vol./vol.). This final preparation (60 µl) was introduced into a Cunningham

chamber and placed in a damp box at 37°C. After 1 h, chambers were rinsed three times with 200 µl KRB 2.2, once with 200 µl testing buffer and once again with 100 µl testing buffer supplemented with a guinea pig polyclonal anti-insulin antibody prepared as described by Wright et al. [25] (dilution 1:300). Testing buffers were KRB 2.2 and KRB supplemented (1) with 12.2 mmol/l glucose, (2) with 22.2 mmol/l glucose, (3) with 2.2 mmol/l glucose + 25 mmol/l KCl, (4) with 22.2 mmol/l glucose + 25 mmol/l KCl and (5) with 22.2 mmol/l glucose + 25 mmol/l KCl + 0.5 mmol/l 3-isobutyl-1-methylxanthine (IBMX) + 100 nmol/l phorbol-12-myristate-13-acetate (PMA) + 10 µmol/l forskolin. After 1 h at 37°C, chambers were rinsed with KRB 2.2, then with this same buffer supplemented with guinea pig complement (dilution 1:40; Dade Behring). After 1 h at 37°C, chambers were rinsed with 0.04% Trypan Blue solution and incubated 5 min at room temperature, rinsed once with KRB 2.2 and finally filled with Bouin's fixation solution.

Immunocytochemistry To identify endocrine islet cells in RHPA, the Cunningham chambers were un-mounted and slides with fixed cells were rinsed in PBS, passed through a series of graded ethanols (30, 50, 70 and 90% [vol./vol.]), incubated for 15 min in PBS supplemented with 0.1% BSA and exposed for 1 h at room temperature to primary antibodies, diluted in PBS containing 0.1% BSA (wt/vol.). For simple staining, we used a guinea pig anti-insulin antibody (1:500 prepared as described by Wright et al. [25]); for double staining, we used this same antibody in combination with a mouse anti glucagon (1:400; Sigma) or a rabbit anti somatostatin antibody (1:3,000; Dako, Glostrup, Denmark). After rinsing with PBS, slides were incubated for 1 h at room temperature with secondary antibodies, diluted in PBS. For simple staining we used an FITC-conjugated goat anti-guinea pig antibody (1:400; Jackson ImmunoResearch Laboratories, West Grove, PA, USA); for double staining we used this same antibody in combination with a tetramethylrhodamine-conjugated donkey anti-mouse or a tetramethylrhodamine-conjugated donkey anti-rabbit antibody (1:200 and 1:900 respectively; Jackson ImmunoResearch Laboratories). After a last rinsing with PBS, slides were coverslipped with a solution of 0.02% of paraphenylenediamine in PBS-glycerol (1:2, vol./vol.) and sealed.

RHPA analysis Slides were examined under a microscope (Zeiss Axiophot, Göttingen, Germany) with both phase-contrast and fluorescence illumination. Analysis was restricted to Trypan Blue-negative cells. Beta cells were identified by their fluorescent labelling. When simple staining was performed, single beta cells, beta cell pairs and pairs comprising one beta and one non-beta cell were analysed. Altogether, these cells represented $81 \pm 13\%$

(mean \pm SD, $n=14$) of the total beta cell population. Single beta cells represented $50 \pm 13\%$ (mean \pm SD, $n=14$) of the total beta cell population. When double staining was performed, single beta cells in direct contact with alpha and delta cells were analysed additionally. With phase-contrast illumination and using a calibrated grid eyepiece, diameters of haemolytic plaques formed around secreting single beta cells and cell pairs were measured. The percentage of plaque-forming cells or cell pairs (PFC) and the corresponding mean plaque area (MPA) were calculated. The total plaque development (TPD) reflecting the total amount of insulin secreted by 100 cells or cell pairs was calculated by multiplying the PFC by the MPA.

Immunohistology Mouse pancreases and human pancreas samples were fixed in 4% paraformaldehyde, then embedded in paraffin and sectioned. Sections were incubated for 15 min in PBS supplemented with 0.1% BSA and exposed for 2 h to an anti-glucagon antibody (1:100; Dako) and subsequently for 1 h to FITC-conjugated goat anti-rabbit antibody (1:500; Jackson ImmunoResearch).

Static incubation assay to assess insulin secretion from intact islets Islets cultured for 24 h were rinsed three times with KRB 2.2 and aliquots of 50 IEQ were pre-incubated for 1 h in the same buffer at 37°C. Islets were then successively incubated for 1 h in KRB 2.2 (basal condition), then in KRB 12.2 or 22.2 mmol/l glucose (stimulated conditions) and once again in KRB 2.2 (re-basal condition). Incubation buffers were collected and frozen for further insulin measurements. After the last incubation, insulin was extracted from islets with acid-ethanol. Insulin was measured by ELISA using an ultra-sensitive human or mouse insulin detection kit (Mercodia, Uppsala, Sweden).

Statistical analysis Data are expressed as means \pm SEM. Statistica, version 6.0 software (Statsoft, Tulsa, OK, USA) was used for statistical analysis. Student's *t* test and one-way ANOVA followed by least significant difference or Tukey's post hoc tests were used for comparison of data. Values of $p < 0.05$ were considered as significant.

Results

Insulin secretion of single human beta cell is heterogeneous In RHPA, the reaction between insulin, anti-insulin antibodies and complement induces the lysis of protein A-bearing erythrocytes localised around secreting beta cells; beta cells are identified by immunofluorescence (Fig. 1). Under basal and stimulated conditions, we identified subpopulations of beta cells surrounded or not by a haemolytic plaque

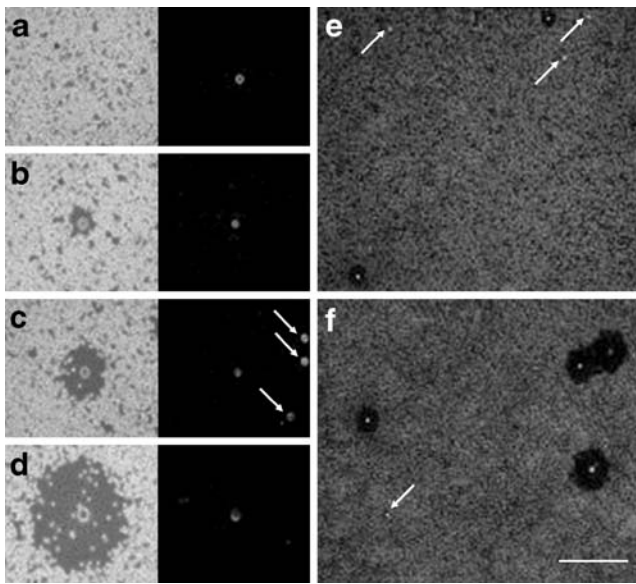


Fig. 1 Heterogeneous formation of haemolytic plaques around single beta cells. Cells were photomicrographed at the end of the RHPA after being stained by immunofluorescence for insulin. **a–d** Phase-contrast views showing single cells surrounded (**b–d**) or not (**a**) by a haemolytic plaque. The corresponding immunofluorescence views indicate that cells produced insulin. After 1 h stimulation with 22.2 mmol/l glucose, beta cells surrounded by intact refringent erythrocytes (**a**) coexisted in the same preparation with cells surrounded by small (**b**), intermediate (**c**) or large (**d**) haemolytic plaques, which consisted of complement-lysed erythrocytes. When photomicrographed at a lower magnification using dark-field illumination (**e, f**), the plaques appear as round dark areas centred around beta cells. At 2.2 mmol/l glucose (**e**), few beta cells were surrounded by small haemolytic plaques, whereas at 22.2 mmol/l glucose (**f**) the number of plaques and their size were higher. Beta cells not surrounded by haemolytic plaque were identified by immunofluorescence for insulin (arrows). Scale bar (**a–d**) 50 μm , (**e, f**) 200 μm

(Fig. 1). Beta cells not surrounded by a haemolytic plaque were observed with all secretagogues used (see below). In addition, as illustrated in Fig. 1b–d, dimensions of haemolytic plaques formed around beta cells were very

variable. The diameters of haemolytic plaques formed around single beta cells in the same preparation frequently ranged from 25 to 300 μm . As plaque area is proportional to insulin secreted, this means that the ratio of insulin secreted by cells with the greatest secretion to insulin secreted by cells with the lowest secretion was ~ 140 .

Insulin secretion from single human beta cells in response to glucose and other secretagogues After 1 h at 2.2 mmol/l glucose, we found few cells surrounded by a haemolytic plaque (Fig. 1e). After 1 h at 22.2 mmol/l glucose the number of PFC increased, although some beta cells not surrounded by haemolytic plaque were still observed (Fig. 1f). PFC, MPA and TPD were evaluated for single beta cells after 1 h of incubation at 2.2, 12.2 and 22.2 mmol/l glucose (Fig. 2). At 2.2 mmol/l glucose, about 30% of beta cells were surrounded by a haemolytic plaque. This value significantly increased to about 70% for beta cells incubated at 12.2 mmol/l glucose (Fig. 2a). At 2.2 mmol/l glucose, the MPA was around 2500 μm^2 and increased to around 4000 μm^2 for cells incubated at 12.2 mmol/l glucose (Fig. 2b). Consequently, TPD was three- to fourfold higher at 12.2 than at 2.2 mmol/l glucose. No difference in PFC, MPA and TPD was observed in cells stimulated with 12.2 or 22.2 mmol/l glucose. Values were similar in cells isolated from islets cultured for 1 or 5 days (Fig. 2).

In addition to glucose, other secretagogues were tested on haemolytic plaque formation from isolated human beta cells (Fig. 3). High concentration of KCl (25 mmol/l), which triggers membrane depolarisation, increased the number of PFC as well as the size of haemolytic plaques at 2.2 and 22.2 mmol/l glucose (Fig. 3c,d). To maximally stimulate insulin secretion, KRB containing 22.2 mmol/l glucose was supplemented with 25 mmol/l KCl, 0.5 mmol/l IBMX, 100 nmol/l PMA and 10 $\mu\text{mol/l}$ forskolin. Under these

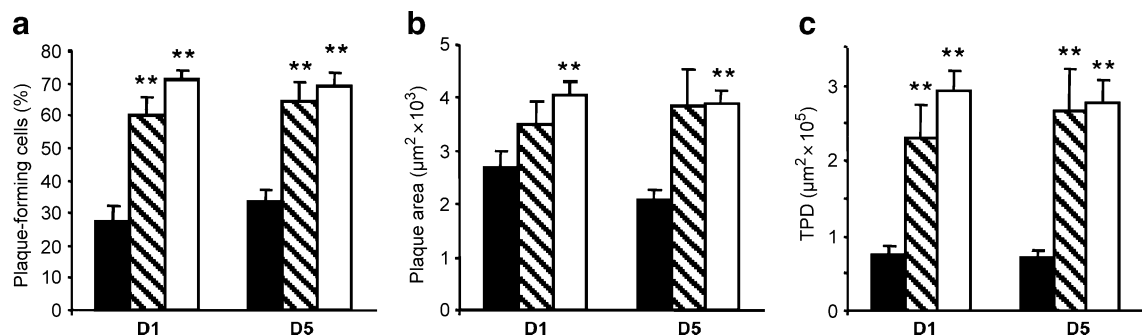


Fig. 2 Effect of glucose on haemolytic plaque formation around single beta cells. One (D1) and five (D5) days after islet isolation, single beta cells were analysed by RHPA. The percentage of PFC (**a**), the MPA (**b**) and the TPD (**c**) were evaluated after 1 h stimulation with 2.2 (black bars), 12.2 (hatched bars) and 22.2 mmol/l glucose (white bars). At D1 and D5, and for all variables analysed, values at

12.2 mmol/l glucose were higher than at 2.2 mmol/l glucose (not significant for plaque area [**b**]) and did not increase any more at 22.2 mmol/l glucose. Results are means \pm SEM of 16 independent experiments for 2.2 and 22.2 mmol/l glucose, and of 11 independent experiments for 12.2 mmol/l glucose. ** $p < 0.01$ compared with 2.2 mmol/l glucose

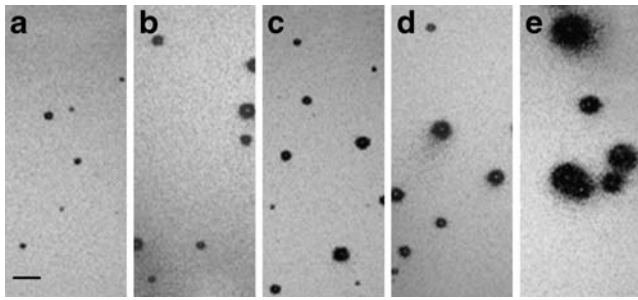


Fig. 3 Effect of different secretagogues on haemolytic plaque formation around beta cells. Cells were photomicrographed at low magnification using dark-field illumination at the end of the RHPA. Haemolytic plaques appear as round dark areas. At 2.2 mmol/l glucose (a), the plaques were rare and small. The number and the size of the plaques gradually increased after stimulation with 22.2 mmol/l glucose (b), 25 mmol/l KCl (c), 22.2 mmol/l glucose plus 25 mmol/l KCl (d) and 22.2 mmol/l glucose plus 25 mmol/l KCl, 0.5 mmol/l IBMX, 100 nmol/l PMA and 10 μmol/l forskolin (e). Scale bar, 200 μm

conditions most beta cells were surrounded by a haemolytic plaque and plaques were larger than those formed under any other conditions (Fig. 3e). However, even under these conditions beta cells not surrounded by a haemolytic plaque were still observed.

Heterogeneity of insulin secretion between donors does not correlate with donor, pancreas and islet cell characteristics In addition to the heterogeneous insulin secretion observed between beta cells isolated from a same pancreas, there is also a great variability of insulin secretion between different pancreases (Table 1). In 15 islet cell preparations from different donors that were stimulated with 22.2 mmol/l glucose, PFC values ranged from 40 to 88%, MPA values from 2,000 to 5,500 μm² and TPD values from 90,000 to 400,000 μm². These values did not correlate with donor characteristics, such as age, BMI and glycaemia, ischaemia time of pancreas, viability and percentage of beta cells (Table 1).

The greatest proportion of insulin is secreted by a minority of beta cells To further characterise heterogeneity of insulin secretion, we analysed distribution of beta cells (Fig. 4a) and corresponding TPD (Fig. 4b) according to plaque diameters. At low glucose concentration (2.2 mmol/l), beta cells surrounded by a small-diameter haemolytic plaque (25–37 μm) were most frequent (7–10% of total beta cell population). Then, percentages gradually diminished with increasing plaque diameters. Thus, at low glucose concen-

Table 1 Insulin secretion from single beta cells evaluated per donor by RHPA after stimulation with 22.2 mmol/l glucose, with corresponding pancreas and beta cell characteristics

Donor number	TPD (μm ²)	PFC (%)	MPA (μm ²)	Age (years)	BMI (kg/m ²)	Glycaemia ^a (mmol/l)	Cold ischaemia time ^b (h)	Warm ischaemia time ^c (min)	Beta cell viability ^d (%)	Beta cell content (%)
1	286,913	67.5	4,251	39	21	NA	5.0	133	NA	NA
2	209,644	76.7	2,734	57	20	9.7	8.5	60	95.30	58.6
3	395,678	86.7	4,566	22	28	6.1	12.4	115	90.90	66.1
4	328,735	75.3	4,364	56	28	13.5	6.0	87	94.80	71
5	257,143	72.9	3,530	57	23	13.2	11.3	80	96.10	49.1
6	408,738	79.2	5,138	40	36	5.4	11.0	68	97.30	67.7
7	91,897	40.6	2,262	55	28	13.6	6.4	85	97.60	66.6
8	95,819	47.5	2,017	54	26	8.8	10.5	87	96.80	57
9	408,567	75.6	5,405	42	25	11.1	8.9	36	98.50	40.8
10	387,370	69.2	5,595	43	26	NA	5.1	86	98.60	51.6
11	354,894	88.3	4,018	50	24	5.6	6.5	60	99.20	52.7
12	268,756	68.9	3,903	42	25	6.2	5.1	64	99.20	46.1
13	199,795	58.9	3,391	50	20	5.1	2.4	63	98.50	43.7
14	245,386	64.3	3,817	23	23	7.0	7.0	41	99.30	41.8
15	270,918	64.6	4,196	63	23	9.0	8.8	118	98.40	50.7
Correlation with TPD ^e				-0.43 (0.11)	0.35 (0.21)	-0.08 (0.78)	0.19 (0.51)	-0.04 (0.88)	-0.02 (0.95)	0.01 (0.96)
Correlation with PFC ^e				-0.30 (0.27)	0.12 (0.66)	-0.18 (0.56)	0.30 (0.28)	-0.09 (0.74)	-0.25 (0.39)	0.07 (0.82)
Correlation with MPA ^e				-0.42 (0.12)	0.31 (0.26)	0.07 (0.82)	0.01 (0.96)	-0.01 (0.98)	0.16 (0.57)	-0.11 (0.71)

^aLast glycaemia measured before pancreas removal

^bTime between aortic clamp and start of islet isolation

^cTime between aortic clamp and pancreas removal

^dBeta cells that were Trypan Blue negative

^eCorrelation coefficient (and corresponding *p* value)

NA, not available

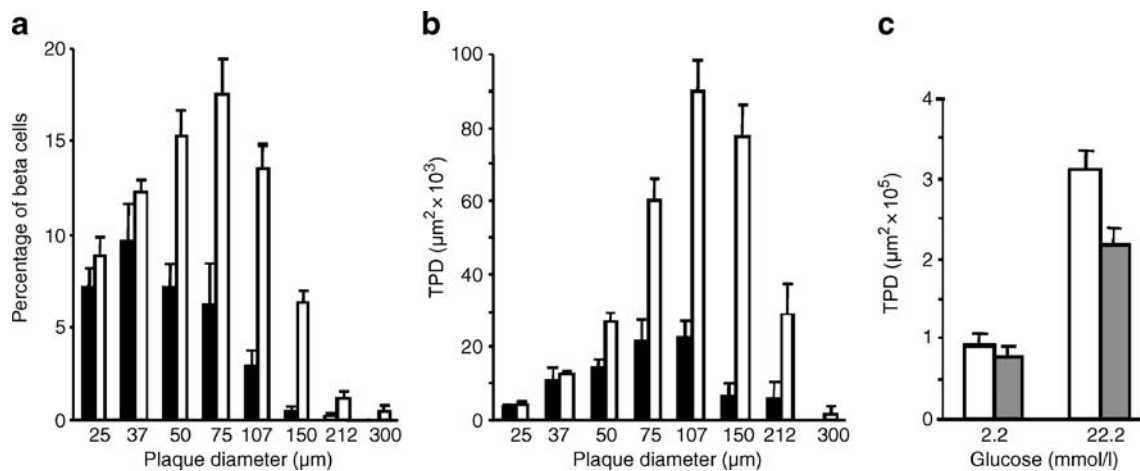


Fig. 4 Secretion pattern in relation to the size of haemolytic plaques. **a** Percentage of secreting beta cells according to the diameter of haemolytic plaques (μm). At 22.2 mmol/l glucose (white bars), percentages of large haemolytic plaques were increased compared with low glucose condition (black bars) where small haemolytic plaques predominated. **b** TPD according to the diameter of haemolytic plaques (μm). At 22.2 mmol/l glucose (white bars), values were higher than at 2.2 mmol/l (black bars), especially for larger haemolytic plaques; the

maximum TPD values were from beta cells with large-diameter haemolytic plaques, as shown by values for 107–150 μm at 22.2 mmol/l glucose, even if the corresponding percentage (**a**) of these cells was low. **c** TPD of the 20% of cells secreting greater levels of insulin (grey bars) compared with global TPD (white bars) after 2.2 and 22.2 mmol/l glucose stimulation. The 20% of cells secreting greater levels of insulin accounted for 90% and 70% of the total insulin secreted after 2.2 and 22.2 mmol/l glucose stimulation, respectively; $n=9$

tration most cells (80%) secreted low amounts of insulin or did not secrete at all. The proportion of cells forming small haemolytic plaques was independent of glucose concentration (9–12% of total beta cell population). In contrast, the frequency of cells forming plaques with a higher diameter gradually increased with glucose concentration, reaching a maximum of 17% for beta cells forming plaques with a diameter of 75 μm . When TPD was expressed as a function of plaque diameter (Fig. 4b), we observed that TPD values were very small for beta cells forming plaques with small diameters, even if, as shown in Fig. 4a, these cells were the most numerous. As shown in Fig. 4b, at both 2.2 and 22.2 mmol/l glucose, haemolytic plaques with 50 μm diameter or below made a low contribution to total insulin secreted. TPD values were considerably higher for beta cells forming plaques with larger diameters (Fig. 4b), even though these cells were less numerous (Fig. 4a). As a consequence of this secretion pattern, we calculated that 20% of the total beta cell population was secreting 90% and 70% of the total amount of insulin released at low and high glucose concentrations, respectively (Fig. 4c).

Differential secretory response to glucose between single and aggregated beta cells As shown above (Fig. 2), insulin secretion of single beta cells was not increased at 22.2 mmol/l glucose as compared with 12.2 mmol/l glucose. This result was surprising, given that insulin release has often been shown to increase dose-dependently with glucose concentrations from 2.2 to 16.7 or 22.2 mmol/l. We sought to elucidate whether the loss of some characteristics expressed in islets, such as cell-to-cell contacts and

paracrine interactions, could be responsible for the singular secretory pattern of single beta cells. To this end, we studied in a first set of experiments haemolytic plaques developed around: (1) single beta cells; (2) cell pairs formed by two contacting beta cells (beta–beta pairs); and (3) pairs formed by one beta cell contacting one non-beta cell (beta–non-beta pairs) (Fig. 5). As already shown above, TPD of single beta cells was low at 2.2 mmol/l glucose,

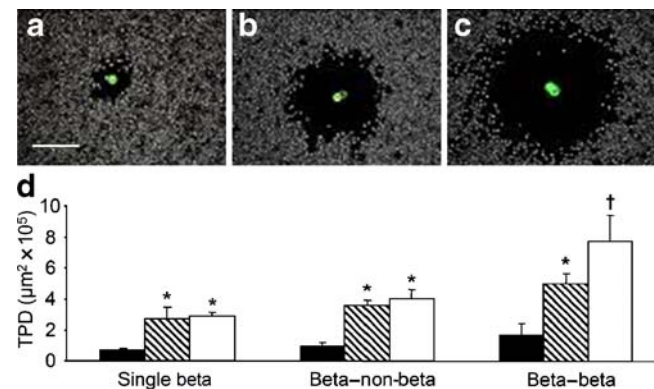


Fig. 5 Insulin secretion of single and paired cells. Photomicrographs of a single beta cell (**a**), a beta cell paired with non-beta cell (**b**) and a beta cell paired with another beta cell (**c**) surrounded by a haemolytic plaque. Scale bar, 50 μm . **d** Corresponding TPD of cells as indicated, evaluated after stimulation with 2.2 (black bars), 12.2 (hatched bars) and 22.2 mmol/l (white bars) glucose. For single beta cells and beta cells paired with non-beta cells (beta–non-beta), TPD increased significantly at 12.2 and 22.2 mmol/l compared with 2.2 mmol/l glucose stimulation, and did not increase further at 22.2 mmol/l compared with 12.2 mmol/l. For beta cell pairs (beta–beta), TPD gradually increased with glucose concentration. $n=5$; $*p<0.05$ compared with 2.2 mmol/l glucose, $^\dagger p<0.05$ compared with 2.2 and 12.2 mmol/l glucose

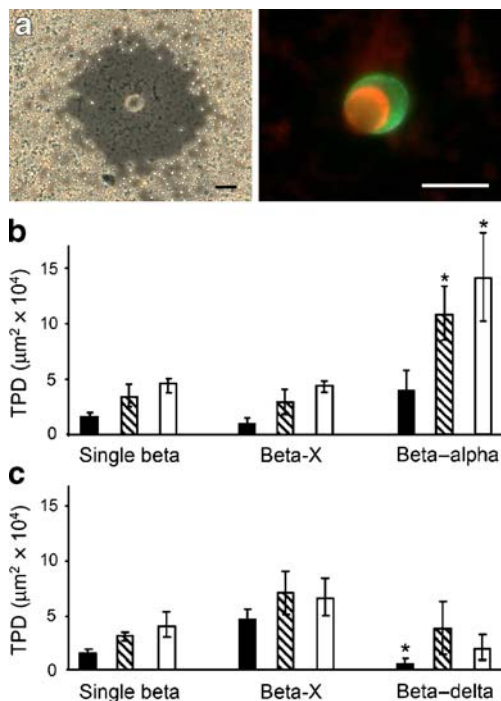


Fig. 6 Insulin secretion of beta cells coupled to alpha and delta cells. **a** A cell pair surrounded by a haemolytic plaque, shown by phase-contrast and fluorescence illumination. Cells were stimulated for 1 h with 22.2 mmol/l glucose and labelled by immunofluorescence for insulin (green) and glucagon (red). Scale bars, 30 µm. **b** At the end of the RHPA, cells were labelled for insulin and glucagon; we analysed single beta cells, beta cells coupled to a non-beta-non-alpha cell (Beta-X) and beta cells coupled to an alpha cell. TPD was evaluated after 1 h stimulation with 2.2 (black bars), 12.2 (hatched bars) and 22.2 mmol/l glucose (white bars). Values are means±SEM of three independent experiments. For each condition, about 350 single beta, 100 Beta-X and 300 beta-alpha cells were analysed. At 12.2 and 22.2 mmol/l glucose, TPD was higher ($p < 0.05$) for beta-alpha than for single beta or Beta-X. **c** At the end of the RHPA, cells were labelled for insulin and somatostatin, and single beta cells, beta cells coupled to a non-beta-non-alpha cell (Beta-X) and beta cells coupled to a delta cell were analysed. TPD was evaluated after 1 h stimulation as above (**b**). Values are means±SEM of three independent experiments. For each condition, about 350 single beta, 300 Beta-X and 40 beta-delta cells were analysed. At 2.2 mmol/l glucose, TPD was lower ($p < 0.05$) for beta-delta than for single beta or Beta-X

increased three- to fourfold at 12.2 mmol/l glucose, but did not increase any more at 22.2 mmol/l glucose. TPD of beta-non-beta pairs was not significantly different from that of single beta cells at any glucose concentrations. By contrast, TPD of beta-beta pairs was increased as compared with single beta cells and beta-non-beta pairs at any glucose concentration ($p < 0.05$). In addition, TPD of beta-beta pairs at 22.2 mmol/l glucose was enhanced up to 1.8-fold compared with 12.2 mmol/l glucose ($p < 0.05$).

Because of the well-known effect of hormones, such as glucagon and somatostatin, on insulin secretion, we identified in a different set of experiments the non-beta partner in beta-non-beta aggregates by double fluorescence

immunostaining. When insulin and glucagon were stained (Fig. 6a), we observed that aggregates comprising a beta cell coupled to an alpha cell (beta-alpha pairs) secreted significantly more insulin than beta cells alone (Fig. 6b). This effect was attributable to an increase in both PFC and MPA (not shown) and was observed at 12.2 and 22.2 mmol/l glucose (Fig. 6b). When insulin and somatostatin were stained, we observed that TPD of beta-delta pairs was unaffected at 12.2 and 22.2 mmol/l glucose, and decreased ($p < 0.05$) at 2.2 mmol/l glucose compared with single beta cells or beta-non-beta-non-alpha pairs (Fig. 6c). In this set of experiments, beta-beta pairs were also analysed (not shown); their secretion was increased compared with single beta cells and similar to that of beta-alpha pairs.

Different secretory response to glucose in mouse compared with human islets Using static incubation tests, we studied insulin secreted from intact human and mouse islets in response to 1 h of stimulation with 2.2, 12.2 or 22.2 mmol/l glucose (Fig. 7). In human islets, insulin secretion was increased threefold at 12.2 compared with 2.2 mmol/l glucose and did not increase any more at 22.2 mmol/l glucose. These results were similar to those of experiments on single beta cells, beta-non-beta and beta-alpha pairs. By contrast, in mouse islets,

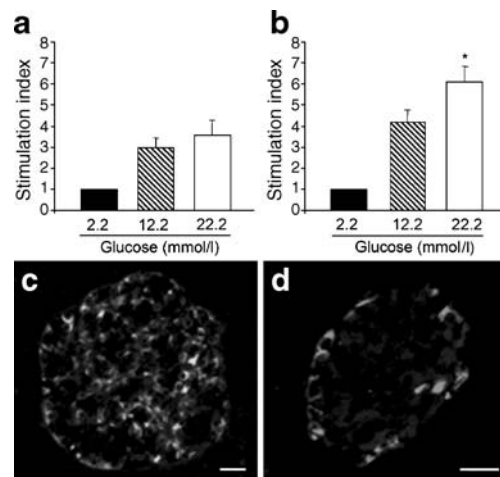


Fig. 7 Insulin secretion from intact islets. **a, b** Insulin secretion from human (**a**) ($n = 7$) and mouse (**b**) ($n = 3$) islets was assessed by static incubation after 1 h incubation at 2.2 (black bars), 12.2 mmol/l (hatched bars) or 22.2 mmol/l (white bars) glucose. Results are expressed relative to 2.2 mmol/l glucose. In human islets (**a**), insulin secretion at 22.2 mmol/l glucose was not significantly increased when compared with 12.2 mmol/l glucose. In mouse islets (**b**), insulin secretion at 22.2 mmol/l glucose was higher ($p < 0.05$) than at 12.2 mmol/l glucose and than the corresponding values of human islets. **c, d** Sections of human and mouse pancreases respectively were labelled for glucagon by immunofluorescence and islets were photomicrographed. Alpha cells are scattered throughout whole human islet (**c**), while in mouse islet (**d**) alpha cells occupy the periphery

insulin release was increased fourfold at 12.2 compared with 2.2 mmol/l glucose, increasing even further by 1.5-fold at 22.2 compared with 12.2 mmol/l glucose. These results were similar to those obtained with beta–beta pairs. Figure 7c,d illustrates the cytoarchitecture of human and mouse islets, respectively. In human islets, alpha cells were scattered throughout the islet (Fig. 7c). By contrast, in mice, alpha cells were mainly localised at the periphery of islets (Fig. 7d). As result of this different organisation, there were fewer homologous intercellular contacts between beta cells in human than in mouse islets. It remains to be shown whether the differences in cytoarchitecture between human and rodent islets is the cause of their different sensitivity to glucose. We are aware that other reasons may be involved, such as dissimilarity in islet handling during and after isolation.

Discussion

This study demonstrates that isolated human beta cells have heterogeneous insulin secretory activity and that cell-to-cell contact affects their insulin secretory activity.

Beta cell heterogeneity in terms of insulin secretion has been described in rodents [10, 21, 26] and is described here for the first time in humans. In rodents, beta cell heterogeneity was also observed in terms of metabolic, signalling [27, 28] and biosynthetic activities [12, 29]. Mechanisms explaining heterogeneity of beta cells are totally unknown. It cannot be completely ruled out that heterogeneity originates from different levels of beta cell viability in the experimental environment. However, more than 90% beta cells were Trypan Blue-negative and only these were analysed for insulin secretion. In addition, living cells were selected on the basis of the presence of a refringent border corresponding to an intact membrane. It is unlikely that heterogeneity could be related to apoptosis. Indeed, we know from other experiments performed under the same conditions (not shown) that less than 5% beta cells were TUNEL-positive. After maximal stimulation with a mixture of secretagogues comprising glucose, KCl, IBMX, PMA and forskolin, the number of non-secreting beta cells was largely decreased as compared with glucose stimulation. This indicates that most non-secreting cells under glucose stimulation were not damaged, but may need to be stimulated with a secretagogue other than glucose in order to secrete insulin. Whether heterogeneous insulin secretion results from differential cellular insulin contents is unknown and difficult to investigate. Using immunofluorescence, we showed that virtually all beta cells were strongly labelled for insulin and consequently were not degranulated. This indicates that degranulation does not account for the heterogeneity we observed in insulin

secretion. New technologies will be required to explore whether more subtle differences in cellular insulin content could have an effect on insulin secretion.

Even though heterogeneity of insulin secretion was observed in all cell preparations, PFC, MPA and consequently TPD varied considerably between cell preparations. We assessed correlations between RHPA data and donor, pancreas and islet cell characteristics. Characteristics that varied highly between donors were age, BMI and glycaemia. None of these characteristics correlated with RHPA data. Cold and warm ischaemia times are critical pancreas variables that could affect insulin secretion. However, no correlation was observed between ischaemia times and RHPA data. At the cellular level, we analysed viability, beta cell content and percentage of aggregated cells; again no correlation was observed with RHPA data. It remains to be investigated whether other cell characteristics, involving glucose metabolism, cell signalling or insulin biosynthesis, are related to insulin secretion outcome of beta cells.

Studying the repartition of secreting cells in relation to plaque areas, we noticed that the majority of insulin was secreted by very few beta cells. In fact, only 20% of the total beta cell population secreted more than 90% and 70% of the total amount of insulin released at low and high glucose concentration, respectively. There is no evidence in our data that this pattern of secretion exists in whole islets. In rats, heterogeneous secretion of islet beta cells has been demonstrated *in vivo* by systemic perfusion of glucose and glibenclamide [30]. Of course, this kind of experiment is impossible in humans. Based on several reports on the ‘pacemaker’ activity of islet cells [31], we speculate that the cells secreting at high levels could act as ‘leader’ cells that trigger insulin secretion from all beta cells within the same islet. This could occur by cell synchronisation via calcium influx through gap junctions [32, 33], via metabolic [29] or by other extracellular signals [34, 35].

As expected from previous rodent studies [26, 30], homologous contacts between human beta cells potentiated insulin secretion. In addition, and for the first time, we showed that a single intercellular contact between an alpha cell and a beta cell was able to increase insulin secretion. This effect could be explained by a paracrine action of glucagon, meaning that the quantity of glucagon secreted by a single alpha cell, even at high glucose concentration, is sufficient to affect secretion of beta cells. Similar paracrine mechanisms could account for the observed effect of delta cells on insulin secretion. Our results are also consistent with Pipeleers et al. [36], who demonstrated potentiation of first and second insulin secretion phases in coupled rat beta cells and reported that a fraction of beta cells enriched with alpha cells had a secretory response comparable to coupled beta cells [36]. Beside this paracrine hypothesis, it is conceivable that adhesion and junctional molecules also

play a role in the effect of homologous and heterologous intercellular contacts on insulin secretion. Previous work with rat beta cells has shown that intercellular coupling through gap junctions is involved in glucose-regulated insulin secretion [37]. Whether gap junctions or other types of intercellular contact are involved in insulin secretion of human beta cells remains to be investigated.

Intriguingly, insulin secretion in single beta cells was similar at 12.2 and 22.2 mmol/l, a pattern which, just as interestingly, was not found in beta cell pairs. Indeed, when homologous contacts between beta cells were established (as in beta cell pairs), insulin secretion was higher at 22.2 than at 12.2 mmol/l glucose. Therefore these results indicate that homologous contacts between beta cells somehow improve regulated insulin secretion, facilitating insulin secretion when required by extreme metabolic needs. We propose that in human islets, homologous beta cell contacts are reduced by the high number of non-beta cells that are intermingled with beta cells. Consequently in human islets no significant difference could be observed between 22.2 and 12.2 mmol/l glucose. We propose that total insulin secretion from human islets arises from beta cells that are differently regulated according to their neighbouring cells. At moderate glucose concentrations (up to 12.2 mmol/l), insulin secretion from beta cells is potentiated by beta–beta and beta–alpha contacts and not affected or possibly decreased by interactions with other islet cell types. At higher glucose concentrations (22.2 mmol/l), only beta–beta contacts may be able to further potentiate insulin secretion. In rodent islets, homologous contacts between beta cells are predominant and consequently a significant difference is observed in insulin secretion between 22.2 and 12.2 mmol/l glucose.

Differences between species in islet architecture have been described extensively elsewhere [20]. The implications of these findings in islet function have been mentioned in terms of human beta cell de-synchronisation within islets and high sensitivity of human islets to low glucose concentrations [5]. From those findings and our paired cell experiments, it seems that mostly paired cells in the core of mouse islets have a high sensitivity to glucose but poor control in case of rapid variations in the low-glucose state [5], whereas human islets have a more complex system of regulation with both cell-to-cell inhibition/activation of insulin secretion, higher glucagon content and better control in the low-glucose state.

Taken together, our results demonstrate that individual human beta cells are highly heterogeneous with regard to their secretory activity and that both homologous and heterologous intercellular contacts affect regulation of their insulin secretion. We therefore propose that the particular cytoarchitecture of human islets with abundant heterologous cell-to-cell interactions may have a functional relevance in the optimal regulation of insulin secretion.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript

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