Dynamics of glucose-induced insulin secretion in normal human islets

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ABSTRACT

The biphasic pattern of glucose-induced insulin secretion is altered in type 2 diabetes. Impairment of the first phase is an early sign of β-cell dysfunction, but the underlying mechanisms are still unknown. Their identification through in vitro comparisons of islets from diabetic and control subjects requires characterization and quantification of the dynamics of insulin secretion by normal islets. When perifused normal human islets were stimulated with 15mmol/l glucose (G15), the proinsulin/insulin ratio in secretory products rapidly and reversibly decreased (~50%) and did not re-augment with time. Switching from pre-stimulatory G3 to G6-G30 induced biphasic insulin secretion with flat but sustained (2h) second phases. Stimulation index reached 6.7 and 3.6-fold for first and second phases induced by G10. Concentration-dependency was similar for both phases, with half-maximal and maximal responses at G6.5 and G15, respectively. First phase response to G15-G30 was diminished by short (30-60min) pre-stimulation in G6 (versus G3) and abolished by pre-stimulation in G8, whereas second phase was unaffected. After 1-2 days of culture in G8 (instead of G5), islets were virtually unresponsive to G15. In both settings, brief return to G3-G5 or transient omission of CaCl₂ restored biphasic insulin secretion. Strikingly, tolbutamide and arginine evoked immediate insulin secretion in islets refractory to glucose. In conclusion, we quantitatively characterized the dynamics of glucose-induced insulin secretion in normal human islets and showed that slight elevation of pre-stimulatory glucose reversibly impairs the first phase, which supports the view that the similar impairment in type 2 diabetic patients might partially be a secondary phenomenon.
INTRODUCTION

Glucose homeostasis depends on a precise quantitative and temporal regulation of insulin secretion by pancreatic β-cells. When the concentration of glucose is rapidly and steadily increased, insulin secretion displays a biphasic time-course. This peculiar dynamics was suspected from measurements of insulin in peripheral and portal blood in humans (4,10), and convincingly established by in vitro experiments using the perfused rat pancreas and perifused islets (12,41). Experimental studies in rodents have variably attributed the biphasic pattern to the release of distinct pools of insulin granules or to the time course of intracellular signals, in particular free cytosolic Ca^{2+}, in β-cells (reviews in 28,51,60,62,63).

Although blood glucose concentrations never increase rapidly and markedly enough to induce biphasic insulin secretion in daily life, the pattern is a most sensitive expression of adequate β-cell functioning (9). Pioneer studies measuring changes in plasma insulin after intravenous bolus administration of glucose, and more recent ones calculating the insulin secretion rate by deconvolution of C-peptide concentrations during hyperglycemic clamps, have established that impairment of the first phase of glucose-induced insulin secretion is an early marker of β-cell dysfunction during development of type2 diabetes (reviews in 15,21,23,55,56). The underlying cellular mechanisms are still unclear and their elucidation will require in vitro comparisons of islets from patients and healthy controls. In the present study, we used a high resolution perifusion system to precisely characterize and quantify the dynamics of glucose-induced insulin secretion by normal human islets in vitro, and to evaluate the impact of variations in the pre-stimulatory glucose concentration on the biphasic response.
MATERIALS AND METHODS

Islet isolation and culture. This study was performed with islets isolated from 22 human pancreas obtained from multiorgan donors. Isolations were performed in transplantation units of the Medical Faculties of the University of Louvain in Brussels (14/22) and the University of Lille (8/22), using described procedures of pancreas digestion and islet purification (18,40). The cold ischemia time averaged 5.8h (range 1.1-12.7h; >10h for 2 pancreas only). In accordance with the study design, only islet preparations of at least 70% purity were used. The average purity (assessed by dithizone staining) and viability (assessed by trypan blue exclusion) of the 22 preparations were 83% (70-95) and 93% (87-98), respectively. Approval of the experimental use of the islets had been granted by ethical committees of both institutions and consent was given by the donors’ families. None of the donors was known to have diabetes. Their sex ratio was 13M/9F, their mean age 46 years (range 16-63) and their mean BMI 24.8 Kg/m² (range 18-31).

Islets isolated in Lille were initially cultured for 24h in CMRL1066 medium containing 5.6 mmol/l glucose, 0.6% fatty acid-free BSA, penicillin (100IU/ml), and streptomycin (100μg/ml). They were then transferred into RPMI medium containing 5 mmol/l glucose and 10% heat-inactivated FCS, penicillin and streptomycin, and, within 36h of isolation, rapidly (in less than 3h) transported to the laboratory in Brussels, where they were cultured in RPMI medium until their use in experiments performed 1-3 days after arrival. Islets isolated in Brussels were cultured in RPMI medium throughout. No differences were observed between the two groups of islets.

Measurements of insulin secretion. To measure the dynamics of glucose-induced insulin secretion, cultured islets were perifused (1ml/min) as described in our previous studies of
human or mouse islets (27,29). The perifusion medium was a bicarbonate-buffered solution continuously gassed with a mixture of O$_2$:CO$_2$ and supplemented with 1mg/ml BSA. An initial equilibration period of 60min was followed by stimulation with one or several agents as indicated at the top of each figure. Effluent fractions were collected every 2-3min, or every 0.7-1min to characterize the first phase of insulin secretion with great resolution. For each experiment, a portion of the cultured islet preparation was directly transferred into each perifusion chamber without hand-picking of individual islets. To normalize results, islets were recovered from each chamber at the end of experiments and transferred into acid-ethanol for insulin extraction (16). Insulin in effluent fractions and in diluted extracts was measured by a double-antibody radioimmunoassay using human insulin as a standard. Fractional insulin secretion rate was calculated as the percentage of insulin content that was secreted per min (27,29). At selected times of some experiments, proinsulin was measured (in two consecutive fractions) using a kit (HPI-15K) from Millipore, which does not recognize intact insulin but cross-reacts (95%) with 31,32, des-split proinsulin. In the insulin assay, the cross-reactivity with human proinsulin is approximately 85%. The percentage of proinsulin in total insulin-like material was then calculated.

Data presentation and analysis. Results are presented as fractional insulin secretion rate (means ± SEM) for the indicated number of islet preparations. Pre-stimulatory (“basal”) insulin secretion rate was computed over the last 10min preceding stimulation. First phase of glucose-induced insulin secretion was quantified by peak (average of the two highest values) and mean secretion rate (average over 7min) between 2 and 9min of glucose stimulation. Second phase was computed as mean insulin secretion rate over periods of variable durations (depending on protocols), and starting 11min after application of glucose stimulation. Values are given in absolute terms (% of content per min) or used to calculate a stimulation index.
(fold-increase above basal secretion). All related protocols (e.g. comparison of different glucose concentrations) were performed on the same day and with the same islet preparation, thereby permitting pairing of results in subsequent analysis. Exceptions are mentioned in figure legends. The statistical significance of differences was assessed by t-test or Anova followed by Newman-Keuls test, as appropriate.

RESULTS

Basic characteristics of the islets. The quality of islet preparations was established by assessing the following hallmark features of insulin secretion (27): biphasic increase upon stimulation with 15mmol/l glucose (G15), suppression of this stimulation by opening $K_{ATP}$ channels with diazoxide, reversal of this inhibition by closure of these channels with tolbutamide, amplification of secretion by raising cAMP with forskolin and reversibility of the stimulation. This control protocol was used in 20/22 preparations, which displayed the individual responses shown in Figure 1A-D. Although the amplitude of the responses was variable, the pattern was consistent between preparations, which resulted in the mean response shown in Figure 1E. The control protocol was slightly different in 2/22 preparations (Fig 1F).

Islets were not hand-picked (see Methods) but, from the amount of provided islets and the volume of material transferred into chambers, we can estimate that approximately 370 islets (range 250-470) were used for each perifusion. In these 22 control experiments, total islet insulin content, measured at the end of the perifusions, averaged 6500 ± 660 ng per chamber (range 2430 -13580), which would correspond to 17.8 ± 1.7 ng/islet (range 7.3 – 32.1), a value within the range of contents published by many laboratories (1,3,11,19,47).
Proinsulin secretion by perifused islets. In 7 of these experiments (Fig 2), proinsulin was also measured at selected times of the perifusions. The changes in proinsulin secretion were qualitatively, but not quantitatively, similar to those of insulin, which resulted in variations in the proinsulin/insulin ratio (Fig 2, inset). During the pre-stimulatory period in G1, the proinsulin/insulin ratio in the effluent averaged 3.9 ± 0.7%. This ratio decreased to 2.1 ± 0.3% (P<0.01) at the peak of first phase, remained low during second phase, increased about 2-fold during inhibition with diazoxide, and decreased again when stimulation was restored by tolbutamide. The proportion of secreted proinsulin was thus higher under basal conditions than during stimulation of β-cells.

Insulin responses to pulse stimulation. Stimulation by 10-min pulses of G10, separated by 10-min intervals in G3, consistently evoked rapid insulin secretion, but the amplitude of the response decreased (P<0.001), mainly between the first and second stimulations (Fig 3A), as also observed in mouse islets (58). Similar changes occurred during stimulation by pulses of 30mmol/l KCl in G3 (Fig 3A). Lengthening the intervals to 30min between glucose pulses did not prevent the decrease in the insulin response (Fig 3B). Quantification of first phase glucose-induced insulin secretion may thus be incorrect when successive stimulations are applied.

Glucose dependency of biphasic insulin secretion. Islets were thus stimulated by increasing glucose from G3 to either G6, G8, G10, G15 or G30 for 2h. Biphasic insulin secretion was consistently observed (Fig 4A and B). The initial acceleration of secretion was similar with the 4 highest glucose concentrations, and the peak of first phase occurred with a similar delay of 5min (after correction for a dead-time of 1min). The increase in insulin secretion was more sluggish in response to G6, with the peak of first phase occurring at least 1min later. Second phase of insulin secretion was stable over the 2h of stimulation with all glucose concentrations (Fig 4A). Upon return to G3 at the end of experiments, the insulin secretion
rate decreased, but this decrease was preceded by a paradoxical transient increase (“off-response”) in some preparations stimulated with the highest glucose concentrations. The glucose-dependency was similar for the first phase (both peak and 7-min integration) and the second phase of insulin secretion (110-min integration), with half-maximal responses at 6.5 to 7mmol/l glucose and maximal responses at 15mmol/l glucose (Fig 4C). A stimulation index (fold-change above baseline) is often used to quantify insulin secretion by human islets. As shown in Figure 4D, the index was different for first and second phases. For example, in response to G15 the stimulation index averaged 8.8 ± 0.12 for the peak of first phase, 6.0 ± 0.8 for integrated first phase and 4.8 ± 0.3 for second phase.

Proinsulin secretion was also measured during prolonged stimulation with G15. The proinsulin/insulin ratio decreased from 4.0 ± 0.6% (n=7) in G3 to 1.9 ± 0.5% (P<0.001) during the first phase, and remained low during the whole second phase (2.1 ± 0.3% after 60 min and 2.2 ± 0.4% after 120 min).

*Acute modulation of biphasic insulin secretion by the pre-stimulatory glucose concentration.* To evaluate how pre-stimulatory glucose influences biphasic insulin secretion, islets were initially perifused with G3, G6, G8 or G10 before being challenged with G15. As expected, initial insulin secretion rate increased with the pre-stimulatory glucose concentration (Fig 5A). The insulin response to G15 remained clearly biphasic when experiments started in G6 instead of G3; the peak of first phase was slightly lower (P<0.05), but integrated first phase was unaffected because of an earlier onset (Fig 5A and B). However, initial perifusion in G8 or G10 virtually suppressed first phase insulin response to G15 without affecting the insulin secretion rate during second phase (Fig 5A and B). The stimulation index, calculated for the peak of first phase, integrated first phase or second phase, decreased with increasing pre-stimulatory glucose concentrations (Fig 5C).
We next investigated whether this alteration of first phase insulin secretion produced by an elevation of pre-stimulatory glucose is a rapidly reversible phenomenon (Fig 6A). Switching from G3 to G8 induced biphasic insulin secretion with a sharp first phase, but a further increase to G15, 40 min later, was clearly less efficient in inducing a rapid response. However, returning to G3 for 10 min before stimulation with G15 was sufficient to restore a clear first phase without impacting the second phase (Fig 6A). Both peak (0.077 ± 0.007 vs 0.054 ± 0.003 %/min, P<0.01) and integrated first phase (0.054 ± 0.03 vs 0.045 ± 0.03 %/min, P<0.05) were larger than in the control group without return to G3. Other combinations of glucose concentrations were also tested. Stimulation with G10 from pre-stimulatory G4 or G6 induced biphasic insulin secretion, with similar second phases but small differences in first phase (Fig 6B). For the peak of first phase, the stimulation index was much smaller when starting in G6 than G4 (3.6 ± 0.5 vs 6.9 ± 1.0, P<0.005) largely because the pre-stimulatory insulin secretion rate was higher. Absolute peak values were also smaller in 7/9 paired experiments, but the difference between mean values (0.057 ± 0.04 vs 0.069 ± 0.07 %/min) did not reach statistical significance. Switching from G10 to G30, 40 min later, was less efficient (P<0.05) in inducing a rapid response than if a 10-min lowering to G6 preceded the final stimulation with G30 (Fig 6B).

An unexplained phenomenon was observed on stimulating with G15 or G30 from pre-stimulatory G8 or G10 (Fig 6A inset and Fig 6B). A rapid and short-lived acceleration of insulin secretion preceded the larger, sustained increase. It occurred in about 2/3 of the preparations and was not observed when pre-stimulatory glucose was lower (see Fig 4B).

Brief lowering of the glucose concentration was not the only way to restore rapid insulin secretion. Figure 6C shows that chelation of extracellular CaCl₂ with EGTA inhibited insulin secretion during the second phase of the response to G8, and that removal of EGTA,
10 or 20 min later, was followed by a biphasic increase in insulin secretion, although glucose was not changed.

The above approaches of glucose lowering or CaCl$_2$ chelation were accompanied by decreases in insulin secretion, which theoretically could permit refilling of a pool of granules necessary for rapid insulin secretion. This interpretation is not supported by the experiments shown in Figure 6D and E. Thus, islets maintained in G8, G10 or G15 responded by a rapid increase in insulin secretion when challenged by 5mmol/l arginine or 25µmol/l tolbutamide. Granules are thus available for immediate release under these conditions.

**Modulation of biphasic insulin secretion by islet culture in different glucose concentrations.** Experiments described above established that the first phase of glucose-induced insulin secretion is acutely attenuated by increased pre-stimulatory glucose. We next investigated the impact of longer exposure of islets to a moderately elevated glucose concentration. Islets from the same preparations were cultured in parallel, in G5 or G8 for 48h, or in G8 for the last 24h only. They were then perifused for 60min in G5 or in G8 before being stimulated with G15 (Fig 7 A-C).

Let us first consider the impact on first phase response to G15. Confirming results shown in Figure 5A, when islets cultured in G5 were initially perifused in G8, first phase insulin secretion induced by G15 was blunted, both in relative terms (Fig 7A, fold-changes given in inset) and absolute terms (Fig 7C, compare middle columns labeled A). After 48h culture in G8, islets displayed elevated basal insulin secretion rate during initial perifusion with either G8 or G5 (Fig 7B and Fig 7C - left-hand columns). When these islets remained in G8 until stimulation with G15, the first phase was minimal, whereas a large first phase was observed if islets had initially been perifused in G5 for 60min. Similar results were obtained after only 24h of culture in G8, as quantified by columns labeled B in Fig 7C. In this
particular group, the stimulation index was 4.9 in islets pre-perifused in G5 and only 1.3 in islets pre-perifused in G8.

Let us next consider second phase of the response to G15. The insulin secretion rate was higher (P <0.01) in islets cultured in G8 (similar after 24 and 48h) than in those cultured in G5 only, but was unaffected by the glucose concentration (G5 or G8) during the pre-stimulatory period (Fig 7C, right-hand columns). Regardless of culture conditions, the stimulation index for this second phase was larger after initial perifusion in G5 rather than G8 simply because of a lower pre-stimulatory insulin secretion rate (Fig 7A and B, and insets). In summary, islets maintained in G8 for 24-48h have virtually completely lost the ability to respond to G15, but 60 min of pre-stimulation in G5 are sufficient to restore this responsiveness.

In contrast, when stimulated with tolbutamide or arginine, islets cultured for 48h in G8 quickly and markedly increased their insulin secretion rate (Fig 7D). This increase was even larger in G8 than G5, exactly as in mouse islets, in which β-cell depolarization and rise in cytosolic Ca\(^{2+}\) produced by both agents augment with the glucose concentration (30,34). Strikingly, transient chelation of extracellular CaCl\(_2\) by EGTA was followed by a biphasic increase in insulin secretion in islets cultured in G8 for 48h, and either maintained at the same glucose concentration thereafter or stimulated with G15 (Fig 7E).
DISCUSSION

Progress in our understanding of the pathophysiology of the human β-cell can be expected from the increased availability of isolated human islets. Yet, extrapolation of in vitro studies of islet function to the in vivo situation is complicated by the trauma of isolation and the possible influence of culture conditions and duration.

Characteristics of our islet preparations. To compare the responsiveness of over 200 islet preparations received from 15 isolation centers, a recent study (39) evaluated insulin secretion by hand-picked islets, which were perifused (3-min resolution) and subjected to a standardized protocol consisting of two sequential, brief (10-min), stimulations by high glucose without and with isobutyl-methylxanthine. The authors identified several distinct insulin secretion profiles that could not be correlated with differences in donor profiles, isolation center or characteristics of the isolation (39). The main conclusion of the study was that, although most preparations of human islets are well functioning, researchers should beware of including confounding, dysfunctional preparations in their series when they design experimental studies. Pre-experimental functional assessment of islet preparations was thus recommended (39).

The goal of our study was not to compare the responsiveness of different islet preparations but to provide a quantitative characterization of the dynamics of glucose-induced insulin secretion by normal β-cells and to evaluate the impact of variations in the pre-stimulatory glucose concentration on the biphasic response. We obtained islets from only two centers, used preparations with a purity of at least 70%, and rigorously studied them in vitro after only 2-4 days of culture. All these preparations (there was no retrospective selection) exhibited a similar pattern of insulin secretion in our control experiments testing several facets of β-cell function (Fig 1). This greater homogeneity than in the above comparative
study (39) is not obviously linked to any identified feature of the preparations, except perhaps a shorter cold ischemia time (5.8 h versus 10.1 h) and a much shorter transportation time (0.1-3h versus 24h). It is possible that the good reproducibility resides in our use of large numbers of non-selected islets in each experiment (an estimated average of 370). Our procedure of pipetting a portion of the whole cultured material, without individual islet hand-picking, was a reason for not including preparations <70% pure in the design of the study. Although islet hand-picking may be required for valid metabolic or gene expression analyses, a non-selective procedure has the advantage of being more representative of the whole islet population in situ. Small islets, which are not spontaneously hand-picked, have been reported to secrete proportionally more insulin than large islets, with (19) or without (42) a greater stimulation index. Another study using 200 islets per perifusion chamber has also reported excellent consistency between the responses of 12 preparations (2). Anyhow, with our control experiments we have met with the recommendations of the above comparative study (39). Moreover, to optimize resolution of the biphasic pattern of insulin secretion, we collected fractions every 0.7-1 min during the first phase and 2-3 min during the second phase of long stimulations (up to 120 min). To increase the strength of the study, the same protocols were repeated in a paired fashion with many preparations. One weakness was that we only measured insulin secretion, which limits the power of our mechanistic interpretation of some of the observed changes.

**Proinsulin secretion.** During islet stimulation, the proinsulin/insulin ratio in secretory products rapidly and reversibly decreased. Previous in vitro studies of proinsulin secretion by human islets focused on the consequences of long-term stimulation by high glucose. In one of these, a proinsulin/insulin ratio of 4.6% was found in effluent of control β-cells perifused at G2.5, and recalculation of presented data shows a smaller increase of proinsulin than insulin during acute glucose stimulation (33). In our experiments, the proinsulin/insulin ratio was
about 4% in secretory products of non-stimulated islets (G1-G3 or G15+diazoxide) and decreased to about 2% when secretion was stimulated with glucose or restored by tolbutamide in the presence of diazoxide. It is unknown whether these acute changes in the proportion of secreted proinsulin between basal and stimulated conditions reflect exocytosis of granules containing distinct proportions of the prohormone in different islets, in different β-cells of each islet or in every β-cell (different granular pools).

In healthy subjects, the basal proinsulin/insulin ratio in plasma is high (>10%) because of the slower clearance of proinsulin than insulin. Its decrease, in peripheral and portal blood, upon acute stimulation with glucose or arginine, has been attributed to dilution of circulating hormones by proinsulin and insulin secreted in a lower ratio (22,31,36,66). Our results establish that, in addition to mere dilution, a further decrease of this lower ratio also occurs during β-cell stimulation. Our other observation that the secreted proinsulin/insulin ratio did not increase with time over the 2h stimulation with G15 agrees with results of hyperglycemic clamps in normal subjects (50). In fact, this lack of increase is not surprising considering that, even after such a long stimulation, not more than 7% of total insulin reserves have been released.

**Pattern and concentration-dependency of glucose-induced insulin secretion.** Like mouse islets (29,43), human islets display a flat second phase of glucose-induced insulin secretion (13,27). We further show that the same pattern exists from supra-threshold (G6) to maximally effective (G30) glucose concentrations, and is maintained for up to 2h. Static incubations or perifusions with ramp or short (20min) stepwise increases in glucose have established that the concentration-dependency of glucose-induced insulin secretion is shifted to the left in human islets compared with rodent islets, with a threshold at G3 vs G6 (17,25,27). Using stimulations with only one glucose concentration for 2h, the present study determined that concentration-dependency curves are similar for first and second phases, with $K_m$ at G6.5-G7.
in human islets. Although equivalent insulin secretion rates (0.05-0.06% per min) are achieved by G15 in human and mouse islets, they correspond to the maximal response to glucose in human islets and only half-maximal response in mouse islets. Mouse β-cells can thus sustain much higher insulin secretion rates than human β-cells in response to glucose alone (29). Yet, human islets possess a reserve secretory capacity solicited in their high amplitude responses (~0.15%/min) to tolbutamide or arginine in high glucose.

**Comparison of in vitro and in vivo insulin secretion rates.** Our comparison is based on the average insulin content of a human pancreas: 125µg/g x 75g = ~1500 nmoles insulin (57). Using C-peptide deconvolution, different laboratories have calculated insulin secretion rates of about 420 and 850pmol/min at blood glucose concentrations of 8 and 15mmol/l during graded glucose infusions or hyperglycemic clamps (6,35,64). Such insulin secretion rates correspond to 0.028 and 0.055% of the pancreatic insulin content per min. At similar glucose concentrations, we measured insulin secretion rates of 0.033 and 0.058% of islet insulin content per min (Fig 4). The correspondence is thus good for steady-state secretion, as also concluded from calculations based on the granular insulin content (59). Other in vivo studies calculated insulin secretion rates of 1200-1500pmol/min at the peak of the first phase response to acute increases of blood glucose to 12.5-15mmol/l (38,46), which correspond to 0.08-0.10% of pancreatic content per min. Similar insulin secretion rates were measured here in vitro. Finally, we can also compare the amplitude of the response. During hyperglycemic clamps to 8-9mmol/l glucose, insulin secretion rate increased 5.5 and 2.7-fold at the peak of first phase and during second phase, respectively (21), which compares well with our measurements of 5.6 and 3.1-fold changes in perifused islets upon increase from G3 to G8.

**Insulin secretion rate from the β-cell perspective.** Considering that each human β-cell contains ~10000 insulin granules (59), insulin secretion rates measured at the peak of the first phase and during the second phase correspond to about 6 and 3 granules per β-cell/min in
response to G8, and to about 10 and 6 granules per β-cell/min in response to G15. In G15, total first phase (7min) and total second phase (110min) implicate exocytosis of not more than ~50 and ~650 granules, respectively. These rates and proportions of released granules are thus smaller than those estimated through changes of membrane capacitance in single β-cells (59). However, our calculations presuppose that all islets of a preparation and all β-cells within each islet are equally active, which may not be the case owing to the existence of oxygen gradients, particularly within larger islets (8). Furthermore, owing to oscillations of cytosolic Ca²⁺ in glucose-stimulated β-cells (26,48), the insulin secretion rate is pulsatile during the second phase (26,45) and could intermittently be as high as during the first phase.

**Impact of pre-stimulatory glucose on biphasic insulin secretion.** Early human studies, measuring plasma insulin, reported disappearance of the acute insulin response to iv glucose when fasting plasma glucose exceeds 6.5mmol/l (7). These seminal observations were confirmed by studies using deconvolution techniques to calculate insulin secretion rates (24,46), and it was further shown that the first phase already gradually declines in the range of fasting plasma glucose between 4 and 6mmol/l (5,24). This impairment could result from primary defects in β-cell function or be secondary to the elevation of pre-stimulatory glucose. The acute insulin response to high glucose was reported to be unaltered (53) or attenuated (20) after 3-6h of moderate elevation of pre-stimulatory glucose. More recent studies calculating insulin secretion rates showed that, after an initial sustained step to 8mmol/l, a further increase in glucose was no longer able to induce a first phase of insulin secretion in normal lean or obese subjects (44,64). That is exactly what we found in vitro with normal islets: a well-defined first phase response to G15 was observed when starting from between G1 and G6, whereas the insulin secretion rate increased much more sluggishly when stepping from G8-G10 to G15-G30. The negative impact of small increases in pre-stimulatory glucose was not only reflected by lower stimulation index (Fig 5C) but also, and more importantly, by
decreased absolute insulin secretion rates (Fig 5B). Thus, peak first phase to G15 was lower when starting from G6 instead of G3 and peak first phase to G10 tended to be lower when starting from G6 instead of G4. These acute changes indicate that the pre-stimulatory glucose concentration is more important than the amplitude of the change. We next investigated the impact of a longer pre-exposure to elevated glucose. Previous studies, testing the hypothesis of “glucotoxicity”, have reported that glucose regulation of insulin secretion is markedly perturbed in human islets cultured for 2-12 days in G17-G33 (3, 13, 33, 47). We show here that islet culture for 1-2 days in G8, a pathophysiologically more relevant concentration, is sufficient to virtually suppress the insulin response to G15. Our results support the concept that even mild chronic hyperglycemia is detrimental for β-cell function (review in 67).

The classic interpretation of the decrease/loss of first phase is a lack of releasable insulin granules in a critical pool. We do not entirely share this interpretation for several reasons. First, the number of granules released during a full first phase is much smaller than the number of granules located close to the plasma membrane (49). Second, arginine and tolbutamide remained able to induce immediate insulin secretion in islets that were refractory to high glucose. Arginine depolarizes β-cell through its electrogenic transport and tolbutamide depolarizes by closing K\textsubscript{ATP} channels independently of changes in metabolism, and both agents, like glucose, eventually accelerate Ca\textsuperscript{2+} influx (30, 34). There is no evidence that this influx triggers exocytosis from a specific granular pool, distinct from that involved in the rapid response to glucose. Third, lowering the pre-stimulatory glucose concentration for 10-60 min restored the ability of G15 to induce a first phase of insulin secretion even after 48h culture in G8. We rather speculate that, unlike arginine and tolbutamide, acute elevation of glucose from an initial concentration of G8 is unable to induce the rapid, large rise in cytosolic Ca\textsuperscript{2+} that is required to trigger a first phase. Our interpretation is supported by the observation that short-term inhibition of insulin secretion by CaCl\textsubscript{2} chelation, without
decreasing glucose, restored a first phase upon CaCl$_2$ readmission. It is also compatible with the glucose-unresponsive elevation of cytosolic Ca$^{2+}$ found in human islets cultured for 48h in G27 (3), admittedly a much higher glucose concentration than in our study. Although measurements of cytosolic Ca$^{2+}$ under our various conditions would be necessary to prove our hypothesis, substantial support can be found in another study (48). The rapid increase in cytosolic Ca$^{2+}$, which occurred in human islets on stimulation from G3 to G11, was either absent or attenuated and delayed when stepping from G11 to G16.7, whereas tolbutamide addition to G11 caused a marked further increase in cytosolic Ca$^{2+}$ (48).

Our in vitro experiments strikingly reproduced the preserved ability of iv arginine (44,55,64) and iv tolbutamide (14,37) to induce immediate insulin secretion in subjects not responding to iv glucose. Our finding that lowering pre-stimulatory glucose restored the ability of G15 to induce a first phase of insulin secretion is also reminiscent of the partial restoration of first phase observed in type2 diabetic patients after even short-term lowering of blood glucose levels with exogenous insulin (52,65,68) or within 1-3 months of bariatric surgery (54,61). Overall our in vitro results support the idea that the defects in the first phase of glucose-induced insulin secretion are at least partially secondary to the elevation of blood glucose. It is, however, noteworthy that a slight impairment of first phase is detectable in relatives of type2 diabetic patients whose basal plasma glucose concentration is not higher than in controls (5). In contrast, we found that islet pre-exposure to G8 for 1 or 48h did not affect absolute insulin secretion rate during the second phase of stimulation with G15. These experimental maneuvers did not reproduce the in vivo situation in type2 diabetic patients whose second phase of glucose-induced insulin secretion is also blunted (23,32,44). In type2 diabetes, β-cell dysfunction is likely multifactorial, with primary and secondary alterations. In their search, great care must be exerted to design in vitro experiments that neither induce nor erase some of the abnormalities.
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REFERENCES


FIG. 1. Basic characteristics of insulin secretion by the 22 preparations of human islets used in this study. A-E: All perifusions started with a 60-min stabilization period, of which only the last 10 min are shown. Between 0 and 90 min, the concentration of glucose was increased from 1 to 15 mmol/l (G1 – G15). Diazoxide (Dz, 100µmol/l), tolbutamide (Tolb, 100µmol/l) and Forskolin (Fk, 1µmol/l) were then added and withdrawn as indicated at the top of the figure. Panels A, B, C and D show individual responses in 20 preparations, and panel E shows the mean ± SEM. F: In two preparations, the protocol was slightly different, with 1 µmol/l forskolin (Fk) present from the beginning of the perifusion.

FIG. 2. Acute changes in the proportion of Proinsulin and Insulin secreted by perifused human islets. The main panel presents mean insulin secretion in 7 experiments from the group of 20 shown in Figure 1. Proinsulin was measured at selected times of these experiments, and the ratio of proinsulin/insulin concentrations is presented in the inset. *P<0.01 versus G1 and G15 + Dz. Values are means ± SEM for 7 islet preparations.

FIG. 3. Insulin secretion induced by pulses of glucose or KCl in perifused human islets. A: In the first part of the experiment, the concentration of glucose (G) was changed between 3 and 10 mmol/l every 10 min. In the second part, three 10-min pulses of 30 mmol/l KCl (K30) were applied at 10 min intervals in the presence of 3 mmol/l glucose (G3). B: Four pulses of 10 mmol/l glucose were applied at 30-min intervals. Values are means ± SEM for 8 islet preparations. In each experiment, total insulin secretion was computed over each 10-min pulse and expressed as a percentage of secretion measured during the first pulse.

FIG. 4. Concentration dependency of glucose-induced insulin secretion in perifused human islets. A: Between 0 and 120 min, the concentration of glucose was increased from 3 mmol/l (G3) to higher values indicated by distinct symbols. B: The same experiments are shown with
an expanded time scale to improve visualization of the first phase of insulin secretion.

Effluent fractions were collected every 42 s during the first 10 min of stimulation, and every 3 min thereafter. C: Concentration dependency curve expressed as a percentage of the responses to 30 mmol/l glucose. D: Concentration dependency curve expressed as a stimulation index (fold-increase above 3 mmol/l glucose). Values are means ± SEM for 8 islet preparations. All glucose concentrations were tested in parallel in the same islet preparations.

FIG. 5. The pre-stimulatory glucose concentration influences biphasic insulin secretion induced by high glucose in human islets. A: Islets were initially perifused for 60 min with 3, 6, 8 or 10 mmol/l glucose (G3, G6, G8, G10) indicated by distinct symbols, before being stimulated with 15 mmol/l glucose. This stimulation lasted 120 min (as in Figure 3), but only the first 40 min are shown. B: Amplitude of the peak of first phase, integrated first phase and integrated second phase responses to 15 mmol/l glucose as a function of the pre-stimulatory glucose concentration. C: Stimulation index (fold-increase above pre-stimulatory glucose concentration). Values are means ± SEM for 9 islet preparations. The 4 pre-stimulatory glucose concentrations were tested in parallel in the same islet preparations.

FIG. 6. Acute alteration and restoration of the first phase of insulin secretion in human islets. A: Islets initially perifused for 60 min with 3 mmol/l glucose (G3) were stimulated by 8 and then 15 mmol/l glucose (G8 and G15). In the test group (open circles), the final stimulation with G15 was preceded by a 10-min decrease from G8 to G3. The inset shows details of the first minutes of stimulation with G15 in the control group. B: Islets initially perifused with 4 or 6 mmol/l glucose (G4, G6) were stimulated by 10 and then 30 mmol/l glucose (G10 and G30). In one group (open circles), the final stimulation with G30 was preceded by a 10-min decrease from G10 to G6. C: Restoration of a “first phase” of insulin secretion by temporary chelation of extracellular CaCl₂ (2.5 mmol/l) with 3 mmol/l EGTA. D: Arginine (Arg, 5 mmol/l) induces immediate insulin secretion in human islets perifused with G8 or G15. E:
Tolbutamide (Tolb, 25µmol/l) induces immediate insulin secretion in human islets perifused with G10. Values are means ± SEM for 9 (A and C), 8 (B) and 4 (D) paired islet preparations, and for 5 preparations in E.

FIG. 7. Culture at different glucose concentrations induces reversible alterations of biphasic insulin secretion in human islets. Islets from the same 8 preparations were cultured for 48h in G5, for 24h in G5 followed by 24h in G8, or for 48h in G8. A: Islets cultured in G5 throughout were perifused for 60 min with G5 or G8 before stimulation with G15. B: Islets cultured in G8 for 48h before perifusions. A-B: Stimulation Index (SI) given in insets correspond to fold-increases of mean first phase (7-min) and mean second phase (110-min) over pre-stimulatory values (last 10 min). C: Comparison of absolute insulin secretion rates during the pre-stimulatory period, at the peak of first phase of the response to G15, and during second phase of the response to G15, in the six groups of islets. Culture conditions are given in the inset. Black columns show islets pre-perifused in G5. Values are means ± SEM for 8 islet preparations. * <0.05 or less versus control islets cultured in G5 throughout (columns A). + <0.05 or less versus corresponding islets pre-perifused in G5 (black columns). D: Islets were cultured in G5 or in G8 for 48h before being perifused at the same glucose concentration and stimulated with tolbutamide (Tolb, 25µmol/l) or arginine (Arg, 5mmol/l). E: Islets cultured for 48h in G8 were initially perifused in G8. Medium CaCl₂ (2.5mmol/l) was temporarily chelated with 3mmol/l EGTA while the glucose concentration remained constant or was eventually increased to G15. In D and E, values are means ± SEM for 5 islet preparations.
Fig 1
Fig 2
Fig 3
Fig 4
Fig 5
Fig 6
Fig 7