0021-972X/04/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89(2):795–805

doi: 10.1210/jc.2003-031120

Induction of β-Cell Rest by a Kir6.2/SUR1-Selective K_{ATP}-Channel Opener Preserves β-Cell Insulin Stores and Insulin Secretion in Human Islets Cultured at High (11 mM) Glucose

ROBERT A. RITZEL, JOHN B. HANSEN, JOHANNES D. VELDHUIS, AND PETER C. BUTLER

Division of Endocrinology and Diabetes, Keck School of Medicine, University of Southern California (R.A.R., P.C.B.), Los Angeles, California 90033; Medicinal Chemistry, Research and Development, Novo Nordisk A/S (J.B.H.), DK 2760, Måløv, Denmark; and Endocrine Division, Mayo Medical and Graduate Schools of Medicine, Mayo Clinic (J.D.V.), Rochester, Minnesota 55905

In health, most insulin is secreted in pulses. Type 2 diabetes mellitus (TTDM) is characterized by impaired pulsatile insulin secretion with a defect in insulin pulse mass. It has been suggested that this defect is partly due to chronic overstimulation of β-cells imposed by insulin resistance and hyperglycemia, which results in depletion of pancreatic insulin stores. It has been reported that in TTDM overnight inhibition of insulin secretion (induction of β-cell rest) leads to quantitative normalization of pulsatile insulin secretion upon subsequent stimulation. Recently, decreased orderliness of insulin secretion has been recognized as another attribute of impaired insulin secretion in TTDM. In the current studies we sought to address at the level of the isolated islet whether chronic elevated glucose concentrations induce both defects involved in impaired insulin secretion in TTDM: deficiency and decreased orderliness of insulin secretion. We use the concept of β-cell rest, induced by a novel β-cell selective K_{ATP}-channel opener (KCO), NN414 (6-chloro-3-(1-methylcyclopropyl)amino-4*H*-thieno[3,2-*e*]-1,2,4-thiadiazine 1,1-dioxide), to test whether preservation of insulin stores leads to normalization of both processes in response to glucose stimulation. Human islets were isolated from three cadaveric organ donors and studied in perifusion experiments and static incubation. Acute activation of K_{ATP}-channels suppressed insulin secretion from perifused human islets by ~90% ($P < 0.0001$). This KCO also inhibited glucagon secretion in a dose-dependent manner ($P = 0.01$). Static incubation at 11 and 16 *vs.* 4 mM glucose for 96 h decreased islet insulin stores by approximately 80% and 85% ($P < 0.0001$, respectively). In subsequent perifusion experiments, total insulin secretion (~30%; $P < 0.01$) from these islets and insulin pulse mass (~40%; $P < 0.05$) were both decreased (11 *vs.* 4 mM). The inhibition of insulin secretion during static incubation with KCO reduced the loss of islet insulin stores in a dose-dependent manner ($P < 0.0001$) and resulted in increased total insulin secretion (2.6-fold; $P < 0.01$) and insulin pulse mass (2.5-fold; $P < 0.05$) during subsequent perifusion. The orderliness of insulin secretion was significantly reduced after chronic incubation of human islets at 11 mM glucose ($P = 0.04$), but induction of β-cell rest at 11 mM failed to normalize the regularity of insulin secretion during subsequent perifusion. We conclude that physiological increased glucose concentrations (11 mM), which are frequently observed in diabetes, lead to a loss of islet insulin stores and defective pulsatile insulin secretion as well as reduced orderliness of insulin secretion. Induction of β-cell rest by selective activation of β-cell K_{ATP}-channels preserves insulin stores and pulsatile insulin secretion without restoring the orderliness of insulin secretion. Therefore, the concept of β-cell rest may provide a strategy to protect β-cells from chronic overstimulation and to improve islet function. Impaired glucose-regulated insulin secretion in TTDM may, however, partially involve mechanisms that are distinct from insulin stores and insulin secretion rates. (*J Clin Endocrinol Metab* 89: 795–805, 2004)

TYPE 2 DIABETES (TTDM) is characterized by impaired insulin secretion due to islet dysfunction. There is increasing evidence from animal models and *in vitro* experiments with human islets that islet dysfunction in diabetes may be due at least partly to reduced insulin stores (1–7). A partial loss of β-cell insulin stores occurs when insulin secretion rates exceed the capacity for insulin synthesis. This might occur as a consequence of chronic hyperglycemia (8, 9) and/or a deficit of β-cell mass (1) leading to chronically increased demands for insulin secretion per β-cell. An increased proinsulin/insulin ratio, present in animals (10) and humans after partial pancreatectomy (11) or with a β-cell deficit as in TTDM (12), supports the idea of an imbalance between secretory demand and supply leading to the secretion of immature insulin granules. Islets chronically exposed to high glucose have a comparable increased proinsulin/insulin secretion ratio (6) that is prevented by concurrent exposure of islets to high glucose and diazoxide, which inhibits insulin secretion (7).

Insulin is almost exclusively secreted in discrete secretory bursts, which occur at approximately 5-min intervals (pulsatile insulin secretion) (13–16). The regulation of insulin secretion is achieved by modulation of the mass of these secretory bursts. Thus insulin secretion following meal ingestion is accomplished by a prompt approximately 600% amplification of insulin burst mass (17). Glucose-stimulated insulin secretory burst mass is deficient in patients with

Abbreviations: ApEn, Approximate entropy; KCO, K_{ATP}-channel opener; KRB, Krebs-Ringer-bicarbonate; TTDM, type 2 diabetes mellitus.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

TTDM (18), who are characterized by an approximately 60% deficit in β-cell mass (19). Pigs with a comparable deficit in β-cell mass similarly exhibit impaired glucose-stimulated insulin secretory burst mass, leading to defective insulin secretion (4). These data imply that β-cell insulin stores, which are required for immediate secretion to generate an insulin secretory burst or first phase insulin secretion, become deficient under conditions of an approximately 50% or greater loss of β-cell mass and/or with chronic hyperglycemia. The concept of deficient insulin stores as a contributing factor to β-cell dysfunction in TTDM was appreciated 25 yr ago when the K_{ATP}-channel opener diazoxide was used to inhibit insulin secretion (and induce β-cell rest) in patients with TTDM, leading to restoration of subsequent first phase insulin secretion (20). Similarly, induction of overnight β-cell rest with somatostatin restored first phase and pulsatile insulin secretion as well as the proinsulin/insulin ratio in TTDM (18).

Impaired insulin secretion in TTDM is due not only to deficient hormone release, but also to disturbed regularity or reduced orderliness of the insulin release process (21–24). The orderliness of hormone secretion is an integrative measure of the factors that contribute in neuroendocrine cells to the sensing of stimuli, to positive or negative feedback, and to the discharge of hormone to elicit a secretory response. Thus, assessment of the orderliness of hormone secretion, for example by approximate entropy (ApEn), provides a more complete analysis of insulin secretion than each independent measure. Application of this method has been found to discriminate pathological from physiological patterns of neuroendocrine secretion (25). Recently, we reported that acute glucose elevations act as a coordinating trigger to enhance the orderliness of insulin secretion from isolated nondiabetic human islets (16).

In the present studies we sought to address at the level of the isolated islet whether chronic elevated glucose concentrations induce both defects involved in impaired insulin secretion in TTDM: deficiency and decreased orderliness of insulin secretion. We use the concept of β-cell rest, induced by a novel β-cell-selective K_{ATP}-channel opener (KCO), NN414, to test whether preservation of insulin stores leads to normalization of both processes. We hypothesize 1) that a β-cell selective KCO inhibits glucose-mediated insulin secretion from human islets (β-cell rest); 2) that human islets cultured at glucose concentrations typical of TTDM (11 mM) have diminished insulin stores and defective insulin secretion due to a decrease in insulin pulse mass; 3) that the induction of β-cell rest with KCO in human islets cultured under these conditions leads to a relative retention of insulin stores and subsequent insulin secretion and insulin pulse mass with glucose stimulation; 4) that chronic exposure of human islets to 11 mM glucose disrupts the orderly secretion of insulin consequent upon an increment in glucose concentration; and 5) that this disrupted orderliness of insulin secretion can be restored by prior β-cell rest.

Materials and Methods

Islet culture

Human pancreatic islets were isolated from the whole pancreas retrieved from three heart-beating organ donors by the Diabetes Institute for Immunology and Transplantation, University of Minnesota (Minneapolis, MN; Bernhard J. Hering), and by the Northwest Tissue Center (Seattle, WA; R. Paul Robertson). The donors did not have a history of diabetes. After isolation, islets were maintained in RPMI culture medium (5 mM glucose) supplemented with 10% fetal bovine serum at 37 C in humidified air containing 5% CO_2. Experiments were performed after islets had been maintained for 1–7 wk in culture. We have seen no loss of islet function from human islets with prolonged (6- to 7-wk) culture.

KCO compound

β-Cell rest was induced with the β-cell selective KCO, NN414 (6-chloro-3-(1-methylcyclopropyl)amino-4*H*-thieno[3,2-*e*]-1,2,4-thiadiazine 1,1-dioxide) (26). This compound is a diazoxide analog and selectively opens K_{ATP}-channels with the subunits Kir6.2 and SUR1 (K_{ATP}-channels on β-cells), without activating K_{ATP}-channels with the subunits Kir6.2/SUR2A (cardiac muscle) or Kir6.2/SUR2B (smooth muscle) (27). The KCO compound was dissolved in dimethylsulfoxide to prepare a stock solution of 100 mM. The stock solution was further diluted with RPMI culture medium or Krebs-Ringer-bicarbonate (KRB) buffer for preparation of final concentrations of 0, 0.3, 3, and 30 μM. Final dimethylsulfoxide concentrations were always <0.04%.

Design

Selective activation of β-cell K_{ATP}-channels inhibits glucose-mediated insulin secretion from human islets. Groups of 6–10 human islets were placed in a perifusion apparatus and perifused at 37 C with oxygenated and prewarmed KRB buffer at 11 mM glucose (n = 12 runs). After an equilibration period of 40 min, the effluent was collected at 1-min intervals for 160 min. From 40–100 min the KCO compound was infused at a concentration of 3 μM to activate K_{ATP}-channels. Samples were kept frozen at −80 C.

Impact of chronic elevated glucose concentrations and β-cell rest on human islet insulin stores and islet function. In static incubation, human pancreatic islets from each donor (60–70 islets/dish) were cultured at 4, 11, and 16 mM glucose without or with KCO (0.3, 3, or 30 μM) for 96 h. This concentration range of KCO was selected to encompass the concentration range currently under investigation in clinical studies (~1–3 μM). At 0 and 96 h, samples of culture medium were obtained to determine insulin secretion during static incubation. Samples were kept frozen at −80 C. At the end of the 96-h culture period, the islets were sonicated for the determination of islet insulin content.

After completion of the 96-h static incubation period, aliquots of human islets incubated at 11 mM (0 and 3 μM KCO; n = 9 runs each) and 4 mM (0 μM KCO; n = 12 runs) were placed in the perifusion apparatus and perifused at 37 C with oxygenated and prewarmed Krebs-Ringer-bicarbonate buffer at 16 mM glucose. Human islets from each donor were used for 3 or 4 perifusion runs, respectively. After an equilibration period of 40 min the effluent was collected in 1-min intervals for 55 min. Samples were kept frozen at −80 C.

Islet perifusion

The details of the islet perifusion system have been described previously (28). Briefly, human islets were suspended in Bio-Gel P-2 beads (Bio-Rad, Hercules, CA) and placed in perifusion chambers in aliquots of four to eight islets. The perifusion system (ACUSYST-S, Cellex Biosciences, Inc., Minneapolis, MN) consisted of a multichannel peristaltic pump that delivered perifusate through maximally six parallel tubing sets via a heat exchanger and six perfusion chambers at a constant rate of 0.3 ml/min. The perifusion buffers (Krebs-Ringer-bicarbonate buffer: 115 mmol/liter NaCl, 4.7 mmol/liter KCl, 2.5 mmol/liter $CaCl_2$, 1.2 mmol/liter $MgCl_2$, and 5 mmol/liter NaOH, pH 7.4) were supplemented with 0.2% human serum albumin, preheated to 37 C, and oxygenized with 95% O_2 and 5% CO_2. The perifusate was delivered to the perifusion chambers containing the human islets, and the effluent was collected in 1-min intervals for determination of insulin concentrations.

Laboratory determinations

Glucose concentrations in the perifusion buffers and in the culture medium were measured with the glucose oxidase method (Glucose

Analyzer 2, Beckman Instruments, Brea, CA). Insulin was measured in duplicate with a two-site immunospecific ELISA as previously described (29). There is no cross-reactivity with proinsulin and split 32,33 and 31,32 proinsulins. The lower detection limit is about 4 pmol/liter, and the assay range is 4–2000 pmol/liter. The intraassay coefficient of variation ranged from 1.7–3.2%. The interassay coefficient of variation ranged from 3.5–4.5%. Glucagon was measured in duplicate with a specific RIA (Glucagon DA, ICN Pharmaceuticals, Inc., Orangeburg, NY).

Calculations and statistical analysis

Insulin secretion during islet perifusion (protocols 1 and 2) is expressed in mass units per islet per minute. Insulin and glucagon secretion during the 96-h static incubation period (protocol 2) are expressed in mass units per islet per 96 h. The insulin concentration-time series were analyzed by deconvolution to quantify insulin secretion dynamics and approximate entropy (ApEn) to assess the orderliness of pulsatile insulin secretion. Deconvolution analysis is a multiparameter technique to detect and quantify insulin secretory bursts as described previously (30, 31). Deconvolution analysis has been specifically validated for this perifusion system (28). Briefly, deconvolution analysis computes the insulin secretion rate from the insulin concentration-time profile, while identifying the position, duration, mass, and amplitude of insulin secretory bursts within this secretion profile. These calculations are possible because of the known half-life for the hormone in this perifusion system (0.63 min).

ApEn is a model-independent and scale-invariant statistic designed to quantify the regularity or orderliness of (hormone) time series (32). Technically, ApEn measures the logarithmic likelihood that runs of patterns that are close (within r) for m contiguous observations remain close (within the tolerance width r) on subsequent incremental comparisons. This regularity metric is validated for parameter choices of r = 0.2 × SD in the individual time series and m = 1, as used here (23, 33, 34). Insulin concentration data from islet perifusion experiments were analyzed by calculating normalized ApEn as the number of SDs was removed from mean random ApEn (defined by shuffling the cognate series 300 times). Lower values of the SD indicate a higher degree of irregularity (process randomness). A precise mathematical definition is given by Pincus (32).

ANOVA and unpaired *t* test were used to test insulin concentration, insulin secretion, parameters of insulin pulsatility as determined by deconvolution analysis, and orderliness of insulin secretion as assessed by ApEn for statistical significance. Linear regression analysis was used to analyze the relationship between the islet insulin content and total insulin secretion *vs.* ApEn SD derived from human islet perifusion after static incubation. $P < 0.05$ was considered to denote significant differences.

Results

Does the novel β-cell selective K_{ATP}-channel opener (NN414, KCO) inhibit glucose-mediated insulin secretion from human islets?

To address this question human islets were perifused at 11 mM glucose for 160 min (0–160 min), and KCO was added to the perifusate from 40–100 min. Before exposure to the KCO compound (0–40 min) insulin secretion from perifused human islets was pulsatile, with a mean pulse interval of 4.0 ± 0.2 min/pulse (Fig. 1). KCO at a concentration of 3 μM rapidly inhibited insulin secretion, so that in 8 of 12 experiments the insulin levels in the effluent temporarily dropped below the detection limit of the assay (~4 pmol/liter). On average, 3 μM KCO suppressed insulin secretion from isolated human islets perifused at 11 mM glucose by 89.6 ± 2.3% ($P < 0.0001$; Figs. 1 and 2). In the four experiments in which the perifusate insulin concentrations were still detectable during KCO, we were able to document that insulin secretion was still characterized by secretory bursts (Fig. 3). These data imply that the generation of pulsatile insulin secretion may not be dependent on oscillations of the membrane potential, because activation of K_{ATP}-channels, which induces hyperpolarization of β-cells, does not disrupt the pulsatile nature of insulin secretion. After discontinuation of exogenous β-cell K_{ATP}-channel activation, there was a prompt recovery of insulin secretion through the mechanism of restoration of insulin pulse mass to the pre-KCO baseline plateau (Fig. 1). The regularity of insulin secretion as determined by ApEn before and directly after acute inhibition with KCO was not changed (P = NS), implying that the KCO had no action on orderliness that outlasted its presence in the perfusate.

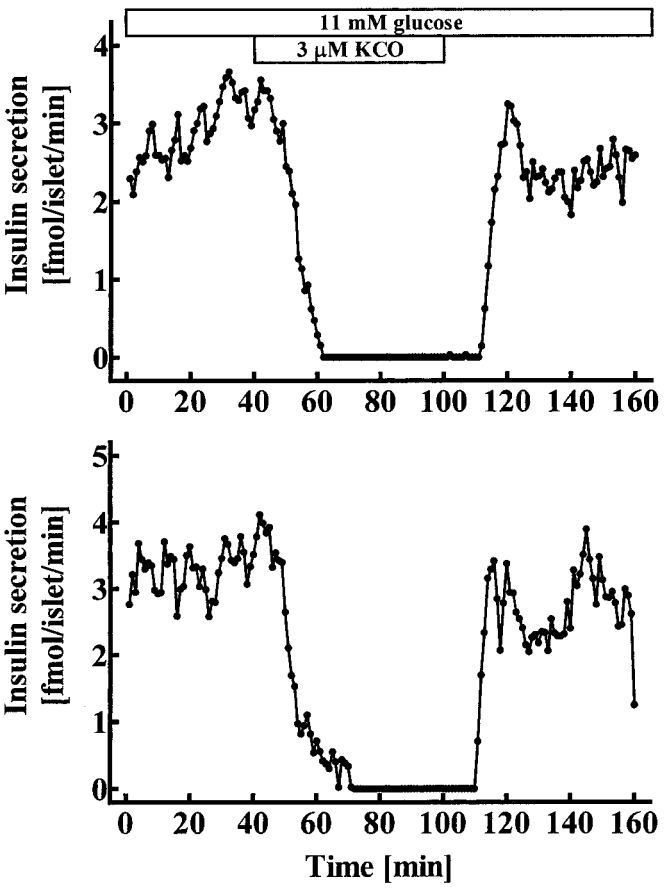

FIG. 1. Insulin secretion profiles of two representative human islet perifusion experiments with acute activation of K_{ATP}-channels. Islets were studied at 11 mM glucose. During the interval from 40–100 min, KCO was infused at a concentration of 3 μM. Inspection of the figure shows an approximately 20-min delay between the onset of KCO perifusion and the accomplishment of steady-state inhibition of insulin secretion. There is a 10-min delay between the delivery of perifusate into this system and its entry into the perifusion chambers containing the islets. Half of the 20 min in delayed onset can therefore be accounted for by the perifusion apparatus, indicating that it takes approximately 10 min for the actions of the KCO to accomplish steady-state inhibition of insulin secretion at the level of the islet. Interestingly, the offset of KCO action at the islet also takes approximately 10 min.

Do human islets cultured over 96 h at elevated glucose concentrations have deficient insulin secretion, and if so is this associated with a concurrent decline in islet stores?

Human islets were cultured for 96 h at glucose concentrations of 4, 11, and 16 mM. After this treatment islets were

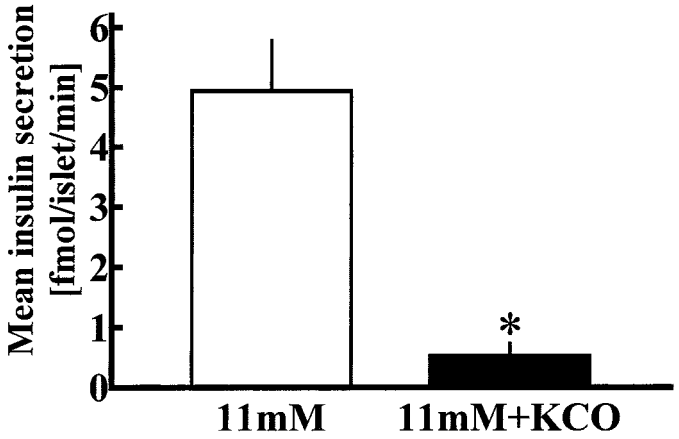

FIG. 2. Mean insulin secretion from human islets in perifusion experiments (n = 12 runs) with acute activation of K_{ATP}-channels. □, Experimental period 0–40 min; ■, experimental period 60–110 min (in Fig. 1). Data are the mean ± SEM. *, $P < 0.05$ *vs.* 11 mM.

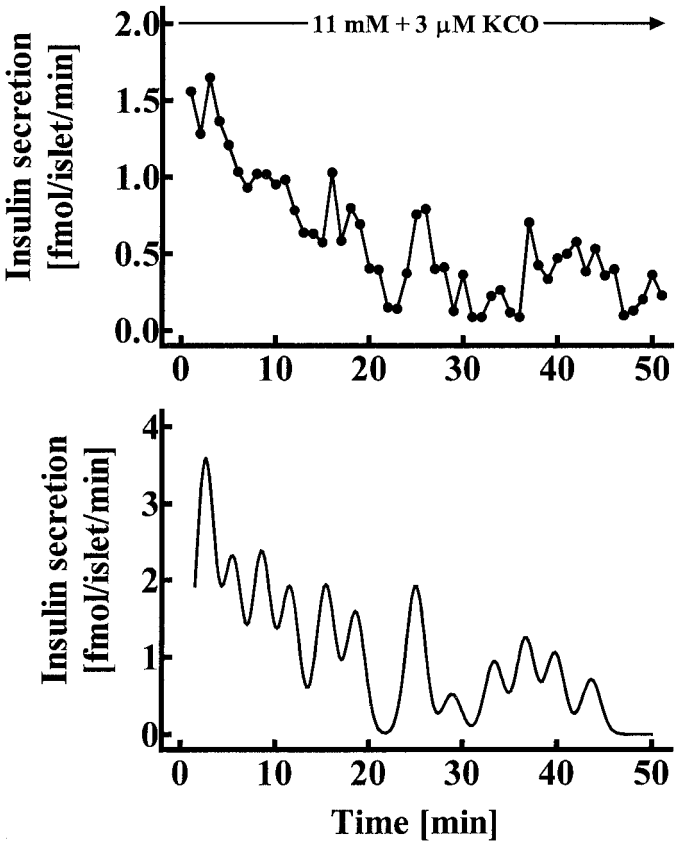

FIG. 3. Raw insulin secretion profile (*top panel*) and insulin secretion derived by deconvolution analysis (*bottom panel*) during islet perifusion at 11 mM glucose and acute activation of β-cell K_{ATP}-channels with 3 μM KCO, corresponding to experimental period from 60–110 min (Fig. 1). In eight of 12 runs insulin secretion during this period was suppressed below the detection limit of the assay. Depicted is one of the experiments with detectable insulin secretion, which occurred in a pulsatile fashion.

perifused at 16 mM glucose, and the dynamics of insulin secretion were quantified. During the 96-h static incubation of islets at glucose concentrations of 11 or 16 mM, there were 23- and 21-fold increases in the insulin secretion rate, respectively, compared with that in islets cultured at 4 mM glucose ($P < 0.0001$; Fig. 4). This sustained glucose-induced augmentation of insulin secretion was associated with 77 ± 8% and 84 ± 7% depletions of islet insulin stores, respectively ($P < 0.001$), by the end of the 96-h static incubation (Fig. 4). When these islets were subsequently perifused, they secreted insulin in a pulsatile fashion (Fig. 5) with a stable pulse interval of 4.0 ± 0.2 min/pulse. There was an approximately 30% deficit in insulin secretion in the islets previously cultured at a glucose concentration of 11 *vs.* 4 mM ($P < 0.01$; Fig. 6). The mechanism subserving this glucose-stimulated decline in insulin secretion was an approximately 40% decrease in insulin pulse mass ($P < 0.05$) with no change in insulin pulse frequency ($P = 0.95$).

These data confirm that islets cultured for 96 h at glucose concentrations typically present in diabetes have impaired glucose-mediated insulin secretion through the mechanism of attenuated insulin secretory burst mass. This deficit is associated with a decline in islet insulin stores.

Does the novel KCO compound prevent loss of insulin stores and deficient glucose-mediated insulin secretion in islets exposed to high glucose concentrations?

To establish the optimal concentration of KCO for inhibition of glucose-mediated insulin secretion, islets were

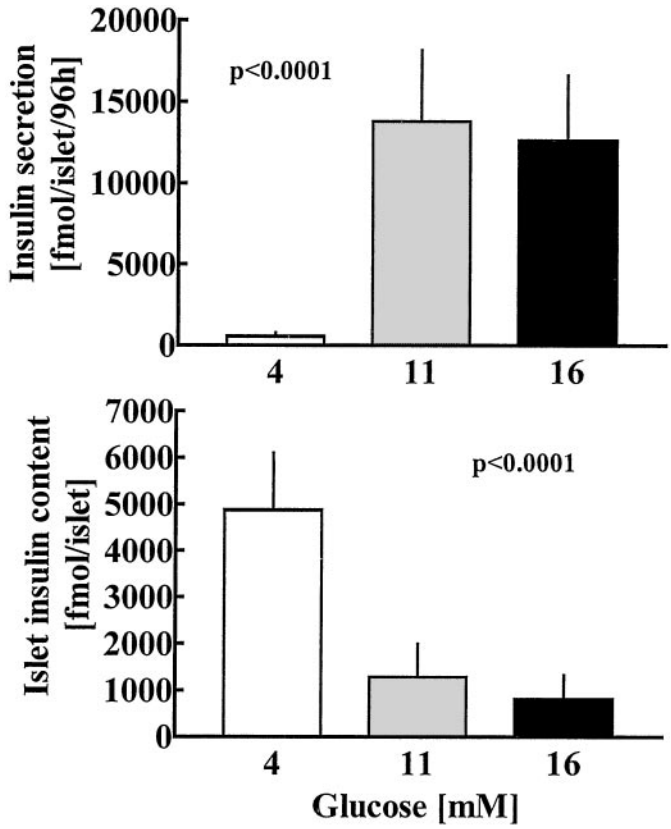

FIG. 4. Insulin secretion (*top panel*) and islet insulin content (*bottom panel*) of human islets (60–70 islets/dish; n = 3 donors) in static incubation for 96 h at 4, 11, and 16 mM glucose. Data are the mean ± SEM. Statistics were determined by ANOVA.

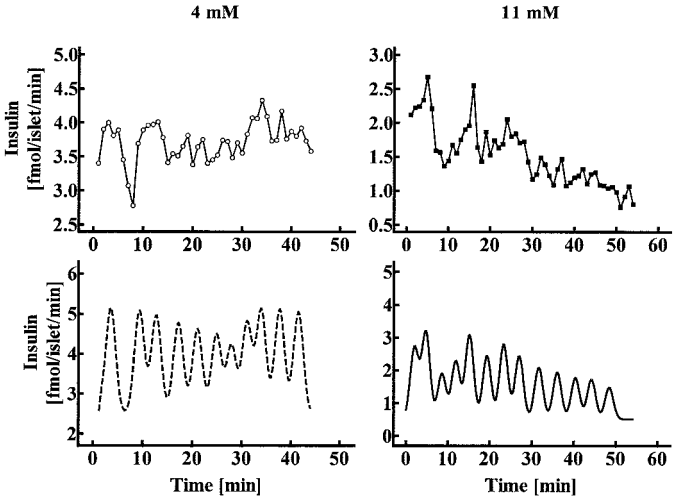

FIG. 5. Effect of elevated glucose on raw insulin secretion (*top panels*) and insulin secretion rates derived by deconvolution analysis (*bottom panels*) in two representative human islet perifusion experiments (6–10 islets each) at a stimulatory glucose concentration of 16 mM. The islets were previously incubated for 96 h at 4 mM (*left panels*) and 11 mM (*right panels*). Note the difference in y-scales to accommodate the range of insulin secretion observed.

cultured at glucose concentrations of 4, 11, and 16 mM as described above, but also with or without KCO at concentrations of 0, 0.3, 3, and 30 μM. KCO dose-dependently decreased insulin secretion during static incubation at all three glucose concentrations (Fig. 7; $P < 0.0001$). At the highest KCO concentration (30 μM), there was a tendency toward a paradoxical loss of the inhibitory effect on insulin secretion ($P = 0.08$; 3 *vs.* 30 μM; n = 9 from all three glucose concentrations). This concentration far exceeds those that are being examined at present in clinical studies (1–3 μM). The inhibition of insulin secretion reduced the loss of islet insulin stores in a dose-dependent manner (Fig. 7; $P < 0.0001$). The 50% effective concentration of KCO to inhibit insulin secretion and prevent depletion of human islet insulin stores during static incubation was in the range of 0.2–0.7 μM. Therefore, we used a concentration of 3 μM KCO for subsequent studies to establish the impact of induction of β-cell rest by KCO in islets cultured at high glucose on subsequent glucose-mediated insulin secretion during perifusion. During static incubation of human islets, KCO also inhibited glucagon secretion in a dose-dependent manner (Fig. 8).

Aliquots of islets that had been incubated for 96 h at 11 mM glucose with or without 3 μM KCO were subsequently studied in perifusion experiments at a stimulatory glucose concentration of 16 mM with no KCO present. During perifusion, insulin secretion from these islets exhibited a pulsatile pattern (Fig. 9). In islets cultured previously at 11 mM glucose with KCO, the mean insulin secretion rate was 2.6-fold increased *vs.* that in islets cultured at 11 mM glucose without KCO ($P < 0.01$; Fig. 10). Interestingly, insulin secretion from islets previously cultured at 11 mM glucose with KCO was not only preserved compared with insulin secretion from islets previously cultured at 4 mM, but actually exceeded it by about 80–90% ($P < 0.05$; Fig. 10). Deconvolution analysis revealed that the restoration of insulin secretion during perifusion by prior culture of islets with KCO and high glucose

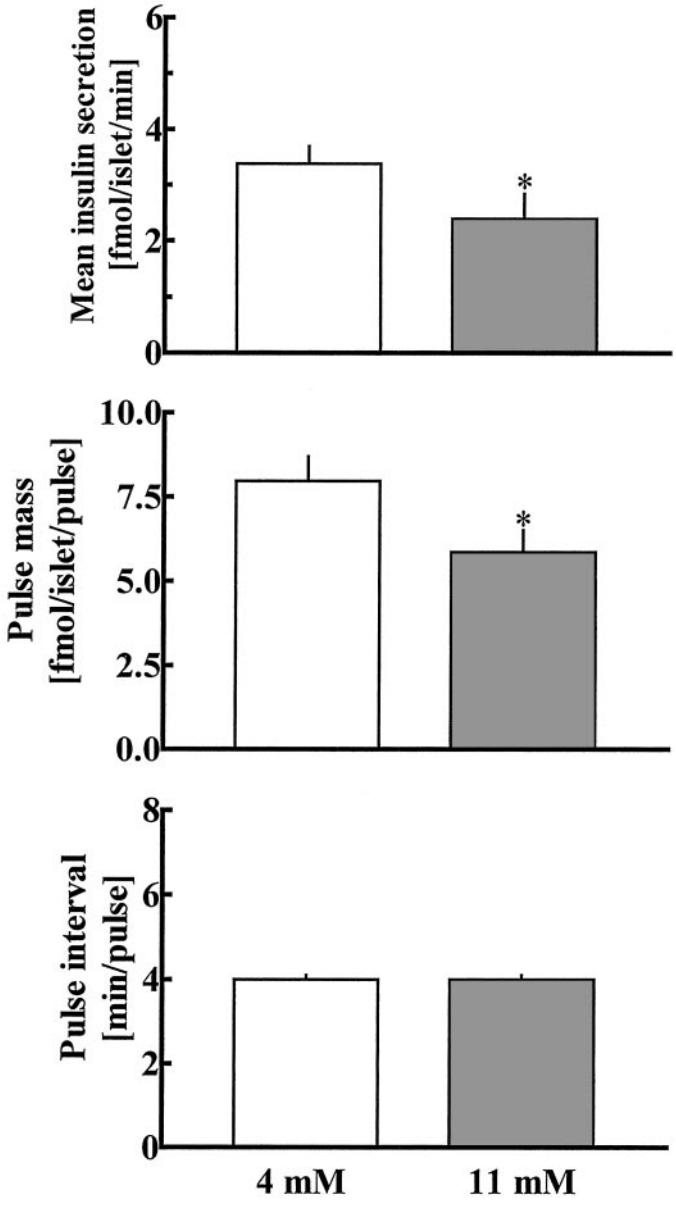

FIG. 6. Mean insulin secretion (*top panel*), pulse mass (*middle panel*), and pulse interval (*bottom panel*) during perifusion of human islets at a stimulatory glucose concentration of 16 mM (n = 9 runs each group, with n = 3 runs per donor). The islets were previously incubated for 96 h at 4 mM (□) or 11 mM glucose (■). Data are the mean ± SEM. *, $P < 0.05$ *vs.* 4 mM.

(*vs.* high glucose alone) was accomplished through the mechanism of amplification of the insulin secretory burst mass (2.5-fold; $P < 0.05$), whereas the interval of pulsatile insulin secretion remained stable ($P = 0.72$; Fig. 10).

These studies support the hypothesis that impaired insulin secretion in islets cultured at glucose concentrations typically present in diabetes can be overcome by concurrent inhibition of insulin secretion (during exposure of the islets to high glucose) by a novel K_{ATP}-channel opener. This observation coupled with the parallel changes in islet insulin stores (and insulin pulse mass) support the idea that the deficit in glucose-mediated insulin secretion in islets induced by chronic

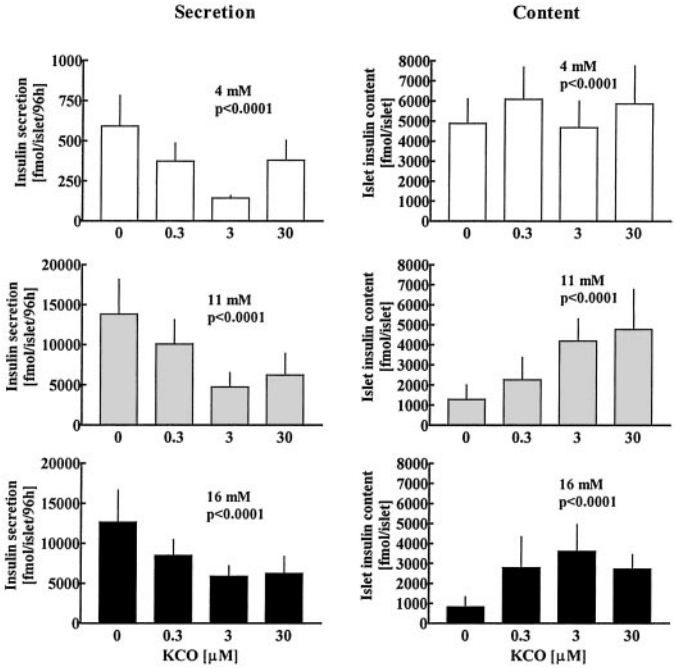

FIG. 7. Effect of KCO concentration on insulin secretion (*left panels*) and islet insulin content (*right panels*) of human islets (60–70 islets/dish; n = three donors) in static incubation for 96 h at 4 (*top panels*), 11 (*middle panels*), and 16 (*bottom panels*) mM glucose. Data are the mean ± SEM. Statistics were determined by ANOVA.

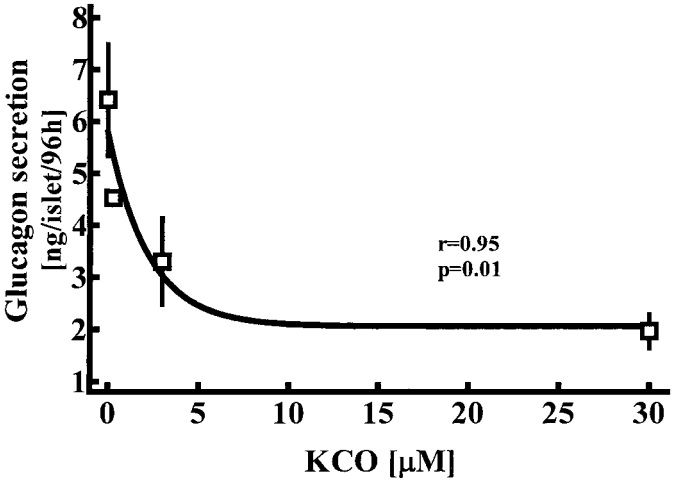

FIG. 8. Relationship between KCO concentration in the culture medium and glucagon secretion from human islets (60–70 islets/dish; n = 3 donors) in static incubation for 96 h at 4 mM glucose. The *solid line* is derived from nonlinear regression analysis. Data are the mean ± SEM. Statistics were determined by ANOVA.

high glucose is mediated through a partial loss of insulin stores, which, in turn, leads to a loss of insulin pulse mass during subsequent glucose stimulation.

Does chronic overstimulation of human islets with glucose (11 mM) disturb the orderliness of stimulated insulin secretion?

The orderliness of insulin secretion was assessed with the regularity statistic ApEn. First, we confirmed that human

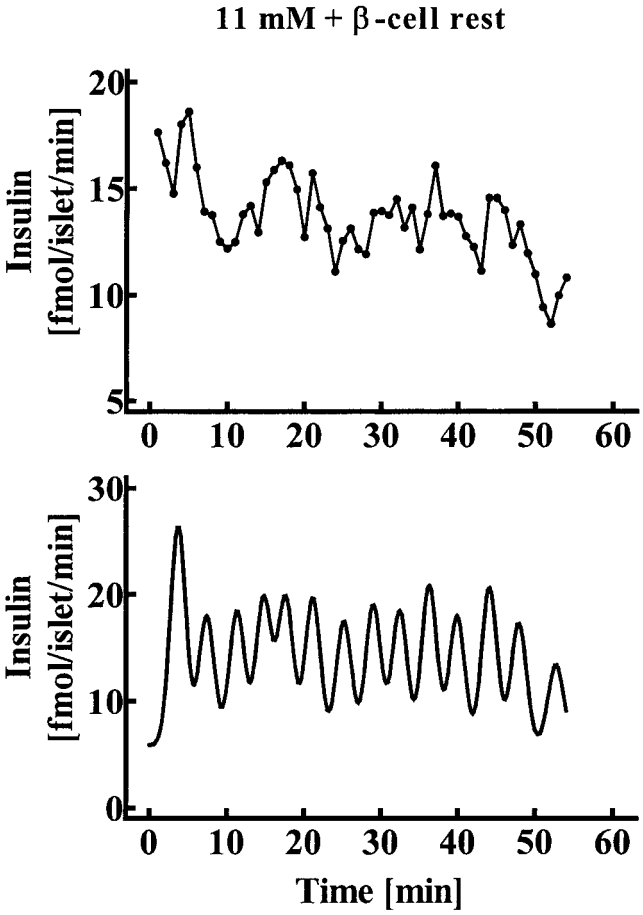

FIG. 9. Raw insulin secretion (*top panel*) and insulin secretion rates derived by deconvolution analysis (*bottom panel*) in a representative human islet perifusion experiment (6–10 islets) at a stimulatory glucose concentration of 16 mM. The islets were previously incubated for 96 h at 11 mM glucose with 3 μM KCO. Note the difference in y-scales compared with Fig. 5 to accommodate the range of insulin secretion observed.

islets previously cultured at normal fasting glucose concentrations (4 mM) and then exposed to an acute increment in the glucose concentration to 16 mM had enhanced orderliness of insulin secretion (Fig. 11). The reference ranges for orderliness of insulin secretion reported in Fig. 11 are derived from previously published data (16). The orderliness of insulin secretion during stimulation with 16 mM glucose was correlated to the islet insulin content in islets previously cultured with 4 or 5 mM glucose (Table 1). In contrast, in human islets previously cultured with 11 mM glucose, the increased orderliness of insulin secretion in response to acute exposure to a glucose concentration of 16 mM was significantly compromised (Fig. 11). In these islets, which had an approximately 80% decrease in islet insulin content (Fig. 4), the orderliness of insulin secretion was not related to the islet insulin content (Table 1). If this defect was primarily due to the loss of islet insulin stores, induction of β-cell rest and preservation of islet insulin content by KCO during culture of islets at 11 mM glucose (Fig. 7) should allow glucose to increase the orderliness of insulin secretion. Interestingly, the induction of β-cell rest in islets cultured at 11 mM glucose did

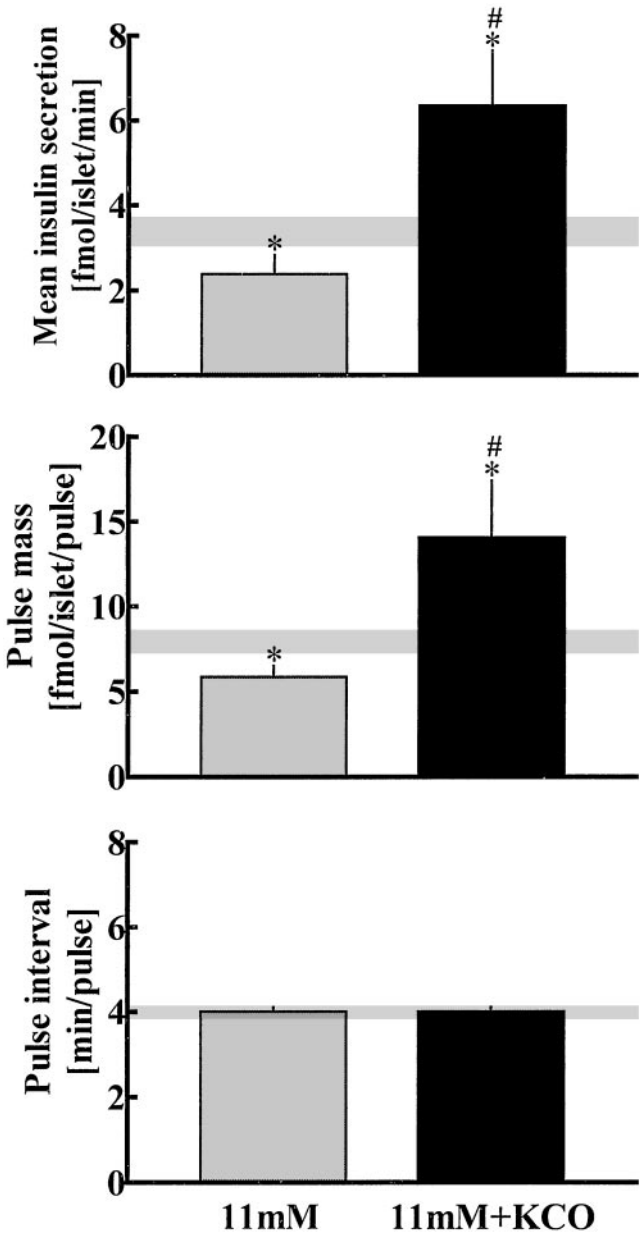

FIG. 10. Mean insulin secretion (*top panel*), pulse mass (*middle panel*), and pulse interval (*bottom panel*) during perifusion of human islets at a stimulatory glucose concentration of 16 mM (n = 9 runs each group, with n = 3 runs/donor). The islets were previously incubated for 96 h at 11 mM glucose (□) or 11 mM glucose with 3 μM KCO (■). The *shaded area* indicates the mean ± SEM data from experiments with islets previously incubated at 4 mM glucose. Data are the mean ± SEM. *, $P < 0.05$ *vs.* 4 mM; #, $P < 0.05$ *vs.* 11 mM.

not lead to normalization of the orderliness of insulin secretion with subsequent glucose stimulation even though this allowed restoration of pulsatile insulin secretion (Fig. 9). Instead, after β-cell rest induced by activation of K_{ATP}-channels, the effect of glucose to coordinate insulin secretion to a more orderly pattern was completely absent (Fig. 11) despite preservation of islet insulin stores (Fig. 7) leading to an enhanced pulse mass of pulsatile insulin secretion. Also, the relationship between islet insulin content and ApEn was not restored (Table 1). The failure of glucose to coordinate insulin secretion from islets with depleted insulin stores was not due to impaired insulin secretion, because in all experimental groups there was no correlation between insulin secretion and ApEn (Table 1).

Discussion

In TTDM, chronic hyperglycemia and overstimulation of β-cells appear to contribute to β-cell dysfunction. Here we confirm that prolonged incubation of pancreatic islets from nondiabetic humans for 96 h at glucose concentrations typically present in diabetes (11 mM) causes depletion of islet insulin stores and impaired insulin secretion by the reduction of insulin pulse mass, recapitulating the disturbances of insulin secretion present in TTDM. We also document that high glucose leads to decreased orderliness of glucose-stimulated insulin secretion. Concurrent culture of islets at a glucose concentration of 11 mM and induction of β-cell rest with a novel β-cell-selective K_{ATP}-channel opener prevented the glucose-induced loss of islet insulin stores and quantitatively preserved subsequent insulin secretion. However, the concurrent β-cell rest during exposure of islets to a glucose concentration of 11 mM for 96 h did not restore subsequent orderliness of glucose-stimulated insulin secretion, implying that measured decreased orderliness of insulin secretion reveals disturbances in glucose-regulated insulin secretion distinct from insulin stores and insulin secretion rate.

The concept of β-cell rest to restore impaired insulin secretion has emerged from both *in vivo* and *in vitro* studies. For example, the insulin response to iv glucagon and tolbutamide was improved in patients with TTDM after intermittent inhibition of insulin secretion with the K_{ATP}-channel opener diazoxide (20). These data were interpreted to support the hypothesis that impaired insulin secretion in humans with TTDM might be due to reduced pancreatic insulin stores induced by chronic overstimulation of the β-cells, and that intermittent inhibition of insulin secretion might avoid depletion of insulin stores and improve subsequent β-cell function. Since then, this concept has been supported in cultured human islets (6, 7) and in rats with chronic stimulation of β-cells caused by continuous glucose infusion or partial pancreatectomy (8, 35) and has been extended to prevent the development of diabetes in Zucker diabetic fatty rats (36) and type 1 diabetes in BB rats (37). Somatostatin induced overnight β-cell rest in humans with TTDM and restored subsequent pulsatile insulin secretion (18). Also, in humans with type 1 diabetes, 3 months of treatment with diazoxide resulted in higher residual insulin secretion 1 yr after discontinuation of diazoxide administration (38). With the present studies we extend the existing data by using a novel β-cell-selective K_{ATP}-channel opener (27) to induce β-cell rest in human pancreatic islets incubated at glucose concentrations typically present in TTDM (11 mM). In the clinical setting, the β-cell-selective action of this compound should provide advantages over diazoxide, which is limited by its nonselective actions on smooth muscle potassium channels and resulting side-effects (hypotension and hirsutism). The present studies show that this novel KCO compound is active in human β-cells. The 50% effective concentration (0.2–0.7 μM) for inhibition of insulin secretion during

static incubation was similar to that determined in rodent islets (26), implying that this KCO might be equally effective in humans as in rats (39). Clinical studies of the novel KCO have been undertaken at plasma levels in the range of approximately 3 μM. Consistent with the present studies in islets, this blood level of the novel KCO leads to an approximately 80–90% reduction in plasma insulin concentrations. We provide evidence that KCO concentrations 1 order of magnitude higher (30 μM) might lead to a paradoxical loss of inhibition of insulin secretion.

Another novel aspect of the present study is the report that KCO dose-dependently inhibits glucagon secretion from human islets. These results suggest that KCO also interacts directly with glucagon-secreting α-cells in human islets, consistent with previous reports showing an inhibitory effect of diazoxide on the electrical activity of clonal α TC cells (40) and isolated rat pancreatic α-cells (41). Given the paracrine inhibitory effect of insulin on glucagon secretion, KCO-induced inhibition of insulin release could have augmented glucagon secretion from human islets, which *in vivo* would have contributed to inadequate suppression of hepatic glucose release. Therefore, the present studies provide evidence that increased glucagon release from the pancreas secondary to inhibition of insulin secretion is not a major limitation in development of the β-cell rest concept for clinical application.

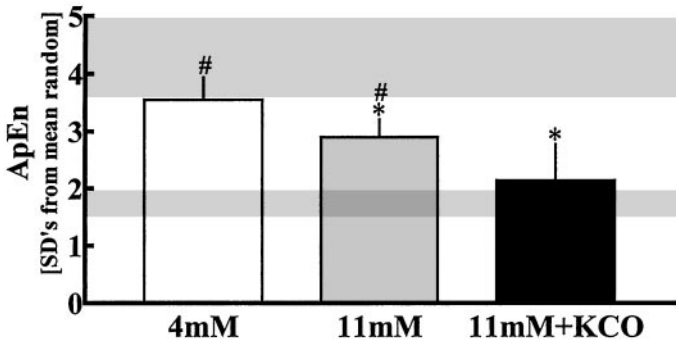

FIG. 11. ApEn defined by the number of SDs removed from mean random ApEn (see *Materials and Methods*) of insulin concentration-time series from perifusion experiments at 16 mM glucose after prior static incubation of human islets for 96 h at 4 mM (n = 12 runs), 11 mM (n = 9 runs), and 11 mM glucose with 3 μM KCO (n = 9 runs). The *shaded areas* indicate the reference ranges for ApEn of insulin secretion at stimulatory glucose concentrations of 4 mM (*bottom*; n = 51 runs) and 16 mM (*top*; n = 14 runs) when human islets were previously kept at 5 mM glucose (16). Higher ApEn SDs from random denote greater secretory regularity or reduced relative randomness of the release process. Data are the mean ± SEM. *, $P < 0.05$ *vs.* 16 mM; #, $P < 0.05$ *vs.* 4 mM.

One of the primary objectives of the current studies was to establish whether prior exposure of human islets to glucose concentrations present in TTDM caused a disturbance of the orderliness of glucose-mediated insulin secretion. We are able to confirm that postulate. These data support the idea that disturbed orderliness of insulin secretion in TTDM may be secondary to hyperglycemia, rather than due to a primary defect in the islet itself. As these studies were performed at the level of the isolated islet, they emphasize the concept that an islet is a functional unit capable of an independent secretory response. *In vivo* pulsatile insulin secretion directs remote target tissues, and feedback and feedforward activities add to the adaptive complexity within this regulatory system. The appeal of a statistic such as ApEn to measure patterns of hormone release is the inclusive *vs.* reductionist nature of this approach. In the current studies we have established that the coordinating action of glucose on insulin secretion (16) is disturbed not only in islets cultured for 96 h with 11 mM glucose, but also in those cultured at the same glucose concentration with KCO, conditions that led to the restoration of pulsatile and total insulin secretion. These data support the evolving concept that the orderliness of insulin secretion is driven partially by underlying pulsatile patterns, but also by subordinate (glucose sensitive) dynamics, which are operative at the level of the islet and are largely independent of the quantity of secretion. In the current circumstances, we might consider the possibility that prior chronic exposure of islets to an elevated glucose concentration (11 mM) leads to stimulation of several of the processes involved in the regulation of insulin secretion at the level of the islet, which are now no longer synchronized by the subsequent increment in glucose concentration (16 mM) during perifusion. An example may be a relative loss of synchrony between the metabolic activity of mitochondria present in each β-cell, which might be manifest in less orderly insulin secretion.

The present studies provide insight into the relationship between glucose-regulated β-cell insulin secretion and synthesis in human islets chronically exposed to glucose concentrations typical of TTDM *vs.* nondiabetics. During static incubation for 96 h with 11 mM glucose, insulin secretion was augmented by 13.8 ± 4.4 pmol/islet/96 h (23-fold) compared with insulin secretion during culture with 4 mM glucose. In consideration of the residual insulin content after static incubation at 11 mM (1.3 ± 0.7 pmol/islet) and the mean insulin content of islets cultured with 4 mM glucose (4.9 ± 1.2 pmol/islet), this reveals that human islets incubated with 11 mM glucose synthesized and secreted approximately 2 times

TABLE 1. Linear regression analysis of the relationship between the islet insulin content (fmol/islet) and total insulin secretion *vs.* ApEn derived from human islet perifusion at five different experimental conditions

	After 5 mM at 4 mM	After 5 mM at 16 mM	After 4 mM at 16 mM	After 11 mM at 16 mM	After 11 mM + KCO at 16 mM
Islet insulin content *vs.* ApEn	r = 0.30	r = 0.56	r = 0.63	r = 0.32	r = 0.02
	$P = 0.03$	$P = 0.04$	$P = 0.03$	$P = 0.40$	$P = 0.96$
Insulin secretion *vs.* ApEn	r = 0.03	r = 0.29	r = 0.27	r = 0.53	r = 0.34
	$P = 0.83$	$P = 0.32$	$P = 0.40$	$P = 0.15$	$P = 0.37$

Stimulation of insulin secretion was performed with 4 or 16 mM glucose after chronic incubation at 5, 4, 11 or 11 mM with 3 μM KCO, as indicated in the *top row*. The results of the first two experimental conditions after chronic incubation at 5 mM glucose derive from data that have been previously published (16).

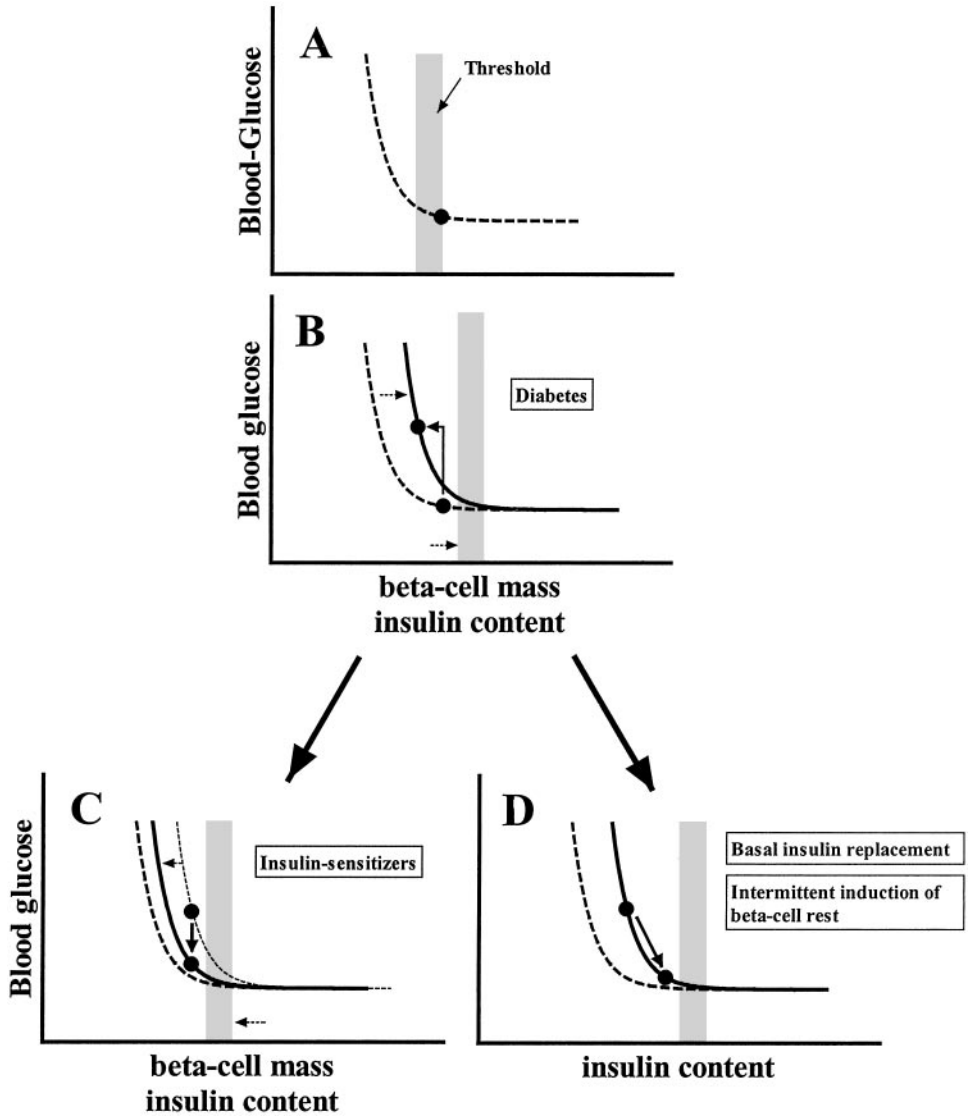

FIG. 12. Model of the hyperbolic relationship between β-cell mass and/or insulin content and blood glucose concentration. The *shaded area* represents the threshold for critical reduction of β-cell mass or insulin content, below which blood glucose concentration is increasing. A, Location of a person on the curve with predisposition for the development of TTDM (●). B, Manifestation of TTDM. Any further decrease in β-cell mass leads to reduced pancreatic insulin content, impaired insulin secretion, and development of hyperglycemia. Treatment with insulin sensitizers shifts the curve to the left (C), whereas basal insulin replacement or intermittent β-cell rest induces a shift along the curve (D).

their insulin content in 96 h, and that this insulin secretion exceeded insulin synthesis, causing an approximately 80% loss of islet insulin content. However, concurrent activation of K_{ATP}-channels and partial inhibition of insulin secretion during exposure to 11 mM glucose largely overcame the imbalance between insulin synthesis and secretion, restricting loss of insulin stores to about 15%. Despite this approximately 15% reduction of insulin stores, islets incubated under these conditions (11 mM glucose with β-cell rest) had an approximately 80% increased rate of total and pulsatile insulin secretion with subsequent glucose stimulation. This may be due to a priming effect of prior elevated glucose on insulin biosynthesis and the secretory machinery (42), which during inhibition of insulin secretion may have enhanced the magnitude of the readily releasable insulin pool. Alternatively opening of ATP-sensitive potassium channels may have enhanced glucokinase activity by inhibiting relocation of glucokinase from the cytoplasm to insulin vesicle membranes, a phenomenon recently recognized in βTC3 cells (43). The combination of enhanced glucokinase activity with a relative preservation of insulin stores, perhaps also released competent stores, may have together contributed to the enhanced glucose-mediated insulin secretion from islets previously cultured with 11 mM glucose and KCO *vs.* islets cultured with 4 or 11 mM glucose without KCO. What are the clinical implications of the present concept to primarily restore pulsatile insulin secretion by prior β-cell rest?

There is a hyperbolic relationship between β-cell mass and/or insulin content and blood glucose concentrations (4, 5). This relationship is shown diagrammatically in Fig. 12A. The inflection of the hyperbolic curve occurs with an approximately 50% reduction of β-cell mass in humans and large animal models (4, 5, 19). The data from the current studies support the idea that this inflection may indicate the point at which any further decrease in β-cell mass results in chronic demands for insulin secretion per β-cell that exceed the capacity for insulin synthesis, leading to diminished insulin stores and secretion. The available data support the concept that this decompensation of β-cell function might preferentially lead to depletion of the functional readily re-

leasable pool of insulin (44), resulting in deficient first phase insulin secretion and reduced pulse mass of pulsatile insulin secretion. Both of these abnormalities in insulin secretion are characteristic of TTDM and large animal models with decreased β-cell mass (4, 5, 18, 45, 46). If, as the available data suggest, the loss of pulsatile insulin secretion contributes to decreased insulin sensitivity (47–51), the consequence would be a rightward shift of the curve describing the relationship between blood glucose and β-cell mass (Fig. 12B) compounding the relative insulin deficiency, and hyperglycemia. Any strategy that enhanced insulin sensitivity, such as use of pharmacological insulin sensitizers, would be expected to oppose this effect to some degree (Fig. 12C). The current studies introduce another strategy. The use of basal insulin therapy and concurrent inhibition of basal insulin secretion by K_{ATP}-channel activation would allow insulin stores to be replenished (Fig. 12D). As activation of the K_{ATP}-channel, demonstrated by the current islet studies, leads to only partial inhibition of insulin secretion that is overcome by an increased glucose concentration, the combined use of basal insulin replacement and a KCO might be expected to lead to restoration of pulsatile insulin secretion, particularly in relation to meal requirements. This approach would be expected to lead to a shift along the curve to the right (Fig. 12D) and result in the reduction of blood glucose concentrations. Recently, we reported that in human islets the magnitude of the insulin secretory response to a glucose stimulus is a function of the islet insulin content (7, 16). Any increase in the islet insulin stores should therefore result in increased insulin pulse mass and secretion. Consistently, pancreatic insulin content and β-cell responsiveness are increased in rats after 48 h of diazoxide-induced β-cell rest at hyperglycemia (8). Based on data obtained in rodents, it has been suggested that the dynamic movements along the hyperbolic curve (Fig. 12) might be partially governed by differential feedback mechanisms of hyperglycemia on β-cell mass (52). Mild hyperglycemia induces expansion of β-cell mass, whereas more severe hyperglycemia induces β-cell loss. It is not known to what extent these changes are applicable to humans.

The mechanism of glucotoxicity in isolated human islets or in TTDM might be mediated not only by reduced islet insulin stores, but also by functional changes and reduced β-cell survival (53). Functional changes include reduced insulin gene expression and chronic oxidative stress. Prolonged exposure of isolated human islets to elevated glucose concentrations increases β-cell apoptosis (54) consistent with the reported 65% deficit in β-cell mass and increased β-cell apoptosis in TTDM (19). These factors might have contributed to impaired islet function in the present experiments after static incubation of isolated human islets at elevated glucose concentrations. However, recently it has been suggested that activation of K_{ATP}-channels might protect β-cells in human islets from glucose-induced apoptosis (55), and therefore, the positive impact of β-cell rest on human islet function might also be related to increased β-cell survival.

In summary, the novel β-cell-selective KCO, NN414, acutely suppresses insulin secretion from human islets stimulated by elevated glucose. Chronic β-cell stimulation with physiologically increased glucose concentrations (11 mM), which are frequently observed in diabetes, leads to defective insulin secretion (reduced pulse mass and decreased orderliness) that can partly be explained by depletion of islet insulin stores. Induction of β-cell rest prevents the glucose-induced loss of islet insulin stores and preserves insulin pulse mass without restoring the orderliness of insulin secretion. Therefore, the concept of β-cell rest may provide a strategy to protect β-cells from chronic overstimulation and to improve islet function. Also, we provide evidence that impaired glucose-regulated insulin secretion in TTDM partially involves mechanisms distinct from insulin stores and insulin secretion rates.

Acknowledgments

We acknowledge the technical expertise of Lucretia Howard (insulin assays). Human islets were obtained from the Human Islet Distribution Program, University of Minnesota, Diabetes Institute for Immunology and Transplantation (Dr. Bernhard J. Hering), and Northwest Tissue Center (Seattle, WA; Dr. R. Paul Robertson). IRB approval for islet isolation was obtained at each institution.

Received June 30, 2003. Accepted November 5, 2003.

Address all correspondence and requests for reprints to: Peter C. Butler, M.D., Division of Endocrinology and Diabetes, Keck School of Medicine, University of Southern California, 1333 San Pablo Street, BMT-B11, Los Angeles, California 90033. E-mail: pbutler@usc.edu.

This work was supported in part by the Deutsche Forschungsgemeinschaft (Ri 1055), the NIH (DK-61539), and the Hillblom Foundation.

References

1. **Leahy JL, Bonner-Weir S, Weir GC** 1988 Minimal chronic hyperglycemia is a critical determinant of impaired insulin secretion after an incomplete pancreatectomy. J Clin Invest 81:1407–1414
2. **Imamura T, Koffler M, Helderman JH, Prince D, Thirlby R, Inman L, Unger RH** 1988 Severe diabetes induced in subtotally depancreatized dogs by sustained hyperglycemia. Diabetes 37:600–609
3. **Grill V, Bjorklund A** 2001 Overstimulation and β-cell function. Diabetes 50:S122–S124
4. **Kjems LL, Kirby BM, Welsh EM, Veldhuis JD, Straume M, McIntyre SS, Yang D, Lefebvre P, Butler PC** 2001 Decrease in β-cell mass leads to impaired pulsatile insulin secretion, reduced postprandial hepatic insulin clearance, and relative hyperglucagonemia in the minipig. Diabetes 50:2001–2012
5. **Larsen MO, Rolin B, Wilken M, Carr RD, Gotfredsen CF** 2003 Measurements of insulin secretory capacity and glucose tolerance to predict pancreatic β-cell mass in vivo in the nicotinamide/streptozotocin Gottingen minipig, a model of moderate insulin deficiency and diabetes. Diabetes 52:118–123
6. **Bjorklund A, Grill V** 1999 Enhancing effects of long-term elevated glucose and palmitate on stored and secreted proinsulin-to-insulin ratios in human pancreatic islets. Diabetes 48:1409–1414
7. **Song SH, Rhodes CJ, Veldhuis JD, Butler PC** 2003 Diazoxide attenuates glucose-induced defects in first-phase insulin release and pulsatile insulin secretion in human islets. Endocrinology 144:3399–3405
8. **Sako Y, Grill VE** 1990 Coupling of β-cell desensitization by hyperglycemia to excessive stimulation and circulating insulin in glucose-infused rats. Diabetes 39:1580–1583
9. **Leahy JL** 1993 Increased proinsulin/insulin ratio in pancreas extracts of hyperglycemic rats. Diabetes 42:22–27
10. **Leahy JL, Halban PA, Weir GC** 1991 Relative hypersecretion of proinsulin in rat model of NIDDM. Diabetes 40:985–989
11. **Seaquist ER, Kahn SE, Clark PM, Hales CN, Porte Jr D, Robertson RP** 1996 Hyperproinsulinemia is associated with increased β cell demand after hemipancreatectomy in humans. J Clin Invest 97:455–460
12. **Temple RC, Carrington CA, Luzio SD, Owens DR, Schneider AE, Sobey WJ, Hales CN** 1989 Insulin deficiency in non-insulin-dependent diabetes. Lancet 1:293–295
13. **Song SH, McIntyre SS, Shah H, Veldhuis JD, Hayes PC, Butler PC** 2000 Direct measurement of pulsatile insulin secretion from the portal vein in human subjects. J Clin Endocrinol Metab 85:4491–4499
14. **Ritzel R, Schulte M, Porksen N, Nauck MS, Holst JJ, Juhl C, Marz W, Schmitz O, Schmiegel WH, Nauck MA** 2001 Glucagon-like peptide 1 increases secretory burst mass of pulsatile insulin secretion in patients with type 2 diabetes and impaired glucose tolerance. Diabetes 50:776–784
15. **Porksen N, Grofte T, Greisen J, Mengel A, Juhl C, Veldhuis JD, Schmitz O,**

Rossle M, Vilstrup H 2002 Human insulin release processes measured by intraportal sampling. Am J Physiol 282:E695–E702
16. **Ritzel RA, Veldhuis JD, Butler PC** 2003 Glucose stimulates pulsatile insulin secretion from human pancreatic islets by increasing secretory burst mass: dose-response relationships. J Clin Endocrinol Metab 88:742–747
17. **Porksen N, Munn S, Steers J, Veldhuis JD, Butler PC** 1996 Effects of glucose ingestion versus infusion on pulsatile insulin secretion. The incretin effect is achieved by amplification of insulin secretory burst mass. Diabetes 45:1317–1323
18. **Laedtke T, Kjems L, Porksen N, Schmitz O, Veldhuis J, Kao PC, Butler PC** 2000 Overnight inhibition of insulin secretion restores pulsatility and proinsulin/insulin ratio in type 2 diabetes. Am J Physiol 279:E520–E528
19. **Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC** 2003 β-Cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. Diabetes 52:102–110
20. **Greenwood RH, Mahler RF, Hales CN** 1976 Improvement in insulin secretion in diabetes after diazoxide. Lancet 1:444–447
21. **Polonsky KS, Given BD, Hirsch LJ, Tillil H, Shapiro ET, Beebe C, Frank BH, Galloway JA, Van Cauter E** 1988 Abnormal patterns of insulin secretion in non-insulin-dependent diabetes mellitus. N Engl J Med 318:1231–1239
22. **Lang DA, Matthews DR, Burnett M, Turner RC** 1981 Brief, irregular oscillations of basal plasma insulin and glucose concentrations in diabetic man. Diabetes 30:435–439
23. **Schmitz O, Porksen N, Nyholm B, Skjaerbaek C, Butler PC, Veldhuis JD, Pincus SM** 1997 Disorderly and nonstationary insulin secretion in relatives of patients with NIDDM. Am J Physiol 272:E218–E226
24. **Schmitz O, Juhl CB, Hollingdal M, Veldhuis JD, Porksen N, Pincus SM** 2001 Irregular circulating insulin concentrations in type 2 diabetes mellitus: an inverse relationship between circulating free fatty acid and the disorderliness of an insulin time series in diabetic and healthy individuals. Metabolism 50:41–46
25. **Veldhuis JD, Johnson ML, Veldhuis OL, Straume M, Pincus SM** 2001 Impact of pulsatility on the ensemble orderliness (approximate entropy) of neurohormone secretion. Am J Physiol 281:R1975–R1985
26. **Nielsen FE, Bodvarsdottir TB, Worsaae A, MacKay P, Stidsen CE, Boonen HC, Pridal L, Arkhammar PO, Wahl P, Ynddal L, Junager F, Dragsted N, Tagmose TM, Mogensen JP, Koch A, Treppendahl SP, Hansen JB** 2002 6-Chloro-3-alkylamino-4H-thieno[3,2-e]-1,2,4-thiadiazine 1,1-dioxide derivatives potently and selectively activate ATP sensitive potassium channels of pancreatic β-cells. J Med Chem 45:4171–4187
27. **Dabrowski M, Ashcroft FM, Ashfield R, Lebrun P, Pirotte B, Egebjerg J, Bondo Hansen J, Wahl P** 2002 The novel diazoxide analog 3-isopropylamino-7-methoxy-4H-1,2,4-benzothiadiazine 1,1-dioxide is a selective Kir6.2/SUR1 channel opener. Diabetes 51:1896–1906
28. **Song SH, Kjems L, Ritzel R, McIntyre SM, Johnson ML, Veldhuis JD, Butler PC** 2002 Pulsatile insulin secretion by human pancreatic islets. J Clin Endocrinol Metab 87:213–221
29. **Andersen L, Dinesen B, Jorgensen PN, Poulsen F, Roder ME** 1993 Enzyme immunoassay for intact human insulin in serum or plasma. Clin Chem 39:578–582
30. **Veldhuis JD, Carlson ML, Johnson ML** 1987 The pituitary gland secretes in bursts: appraising the nature of glandular secretory impulses by simultaneous multiple-parameter deconvolution of plasma hormone concentrations. Proc Natl Acad Sci USA 84:7686–7690
31. **Porksen N, Munn S, Steers J, Veldhuis JD, Butler PC** 1995 Impact of sampling technique on appraisal of pulsatile insulin secretion by deconvolution and cluster analysis. Am J Physiol 269:E1106–E1114
32. **Pincus SM** 1991 Approximate entropy as a measure of system complexity. Proc Natl Acad Sci USA 88:2297–2301
33. **Pincus SM, Hartman ML, Roelfsema F, Thorner MO, Veldhuis JD** 1999 Hormone pulsatility discrimination via coarse and short time sampling. Am J Physiol 277:E948–E957
34. **Veldhuis JD, Pincus SM** 1998 Orderliness of hormone release patterns: a complementary measure to conventional pulsatile and circadian analyses. Eur J Endocrinol 138:358–362
35. **Leahy JL, Bumbalo LM, Chen C** 1994 Diazoxide causes recovery of β-cell glucose responsiveness in 90% pancreatectomized diabetic rats. Diabetes 43:173–179
36. **Aizawa T, Taguchi N, Sato Y, Nakabayashi T, Kobuchi H, Hidaka H, Nagasawa T, Ishihara F, Itoh N, Hashizume K** 1995 Prophylaxis of genetically determined diabetes by diazoxide: a study in a rat model of naturally occurring obese diabetes. J Pharmacol Exp Ther 275:194–199
37. **Vlahos WD, Seemayer TA, Yale JF** 1991 Diabetes prevention in BB rats by inhibition of endogenous insulin secretion. Metabolism 40:825–829
38. **Bjork E, Berne C, Kampe O, Wibell L, Oskarsson P, Karlsson FA** 1996 Diazoxide treatment at onset preserves residual insulin secretion in adults with autoimmune diabetes. Diabetes 45:1427–1430
39. **Carr RD, Brand CL, Bodvarsdottir TB, Hansen JB, Sturis J** 2002 β cell rest, chronically induced by the novel potassium chennel opener NN414, reduces basal hyperglycemia and improves both insulin secretory responsiveness and glucose tolerance in Zucker obese rats [Abstract]. Diabetologia 45:A166
40. **Ronner P, Matschinsky FM, Hang TL, Epstein AJ, Buettger C** 1993 Sulfonylurea-binding sites and ATP-sensitive K^+ channels in α-TC glucagonoma and β-TC insulinoma cells. Diabetes 42:1760–1772
41. **Bokvist K, Olsen HL, Hoy M, Gotfredsen CF, Holmes WF, Buschard K, Rorsman P, Gromada J** 1999 Characterisation of sulphonylurea and ATP-regulated K^+ channels in rat pancreatic A-cells. Pflugers Arch 438:428–436
42. **Nagamatsu S, Nakamichi Y, Katahira H** 1997 Syntaxin, but not soluble NSF attachment protein (SNAP), biosynthesis by rat pancreatic islets is regulated by glucose in parallel with proinsulin biosynthesis. Diabetologia 40:1396–1402
43. **Rizzo MA, Magnuson MA, Drain PF, Piston DW** 2002 A functional link between glucokinase binding to insulin granules and conformational alterations in response to glucose and insulin. J Biol Chem 277:34168–34175
44. **Grodsky GM** 1972 A threshold distribution hypothesis for packet storage of insulin and its mathematical modeling. J Clin Invest 51:2047–2059
45. **Ward WK, Bolgiano DC, McKnight B, Halter JB, Porte Jr D** 1984 Diminished B cell secretory capacity in patients with noninsulin-dependent diabetes mellitus. J Clin Invest 74:1318–1328
46. **Larsen MO, Gotfredsen CF, Wilken M, Carr RD, Porksen N, Rolin B** 2003 Loss of β-cell mass leads to a reduction of pulse mass with normal periodicity, regularity and entrainment of pulsatile insulin secretion in Gottingen minipigs. Diabetologia 46:195–202
47. **Matthews DR, Naylor BA, Jones RG, Ward GM, Turner RC** 1983 Pulsatile insulin has greater hypoglycemic effect than continuous delivery. Diabetes 32:617–621
48. **Bratusch-Marrain PR, Komjati M, Waldhausl WK** 1986 Efficacy of pulsatile versus continuous insulin administration on hepatic glucose production and glucose utilization in type I diabetic humans. Diabetes 35:922–926
49. **Paolisso G, Scheen AJ, Albert A, Lefebvre PJ** 1989 Effects of pulsatile delivery of insulin and glucagon in humans. Am J Physiol 257:E686–E6896
50. **Ward GM, Walters JM, Aitken PM, Best JD, Alford FP** 1990 Effects of prolonged pulsatile hyperinsulinemia in humans. Enhancement of insulin sensitivity. Diabetes 39:501–507
51. **Peiris AN, Stagner JI, Vogel RL, Nakagawa A, Samols E** 1992 Body fat distribution and peripheral insulin sensitivity in healthy men: role of insulin pulsatility. J Clin Endocrinol Metab 75:290–294
52. **Topp B, Promislow K, deVries G, Miura RM, Finegood DT** 2000 A model of β-cell mass, insulin, and glucose kinetics: pathways to diabetes. J Theor Biol 206:605–619
53. **Poitout V, Robertson RP** 2002 Minireview: secondary β-cell failure in type 2 diabetes: a convergence of glucotoxicity and lipotoxicity. Endocrinology 143:339–342
54. **Maedler K, Spinas GA, Lehmann R, Sergeev P, Weber M, Fontana A, Kaiser N, Donath MY** 2001 Glucose induces β-cell apoptosis via upregulation of the Fas receptor in human islets. Diabetes 50:1683–1690
55. **Sturis J, Maedler K, Lehmann R, Spinas GA, Donath MY** 2002 The SUR/Kir6.2 selective potassium chennel opener NN414 prevents glucose-induced apoptosis in human pancreatic islets [Abstract]. Diabetologia 45:A84

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.